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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.

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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).

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

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

demonstrated significant associations between the HBV genotypes and the severity of liver disease, clinical outcomes and the response to antiviral therapies (Kramvis & Kew, 2005). Moreover, it was also demonstrated that the clinical and virological characteristics may also differ among patients infected with the same genotype (Miyakawa & Mizokami, 2003). The existence of different subtypes (subgenotypes) within same genotype helps to explain this for HBV/B, where one of the subtypes (subgenotypes) (widespread in Asia; Ba) possesses a recombination with genotype HBV/C, while another (indigenous to Japan; Bj) does not (Sugauchi *et al.*, 2003). Similarly, two subtypes (subgenotypes) have been reported for HBV/A: one of them, Aa (A'/A1) prevails in sub-Saharan Africa and South Asia, while the other, Ae (A2), is widely distributed in Europe and the USA (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Sugauchi *et al.*, 2004). The subtypes (subgenotypes) of HBV/A show no evidence of distinguishing recombination; nevertheless, they are associated with differences in replicative activity, and in the mechanisms of HBeAg seroconversion as a result of specific nucleotide substitutions in the core promoter and precore regions (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004).

The characterization of isolates from indigenous populations, especially in Africa where HBV is hyperendemic, may assist in revealing the origin of HBV and clarify the many questions about its evolutionary history (Kramvis *et al.*, 2005). The genetic diversity and distribution of HBV genotypes in Central West Africa, particularly in Cameroon, are poorly documented. No data were available for the HBV strains from Pygmies in this region. The objectives of the present study were to assess the prevalence of HBV and hepatitis C virus (HCV) markers among Bantus and Pygmies, to compare the distribution of HBV genotypes and to analyse the genomic characteristics of the HBV/A strain in Cameroon. Six full genome sequences, including four representing a new subtype (subgenotype) of HBV/A and two HBV/E strains from the Cameroonian Pygmies, were analysed.

METHODS

Blood serum samples. Blood serum samples were collected in 1994 from 544 voluntary donors, including representatives of two relatively isolated populations (Bantu and Pygmies) in Cameroon, Central West Africa. The Pygmies enrolled were from two forest encampments in the East province, and the Bantu were enrolled from five provinces across the country (Central, South, North, West and East). None of the donors had clinical symptoms of liver disease. Written informed consent was obtained from all subjects enrolled. After isolation of the serum fraction from whole blood, the samples were stored at −40 °C until use. The number of subjects studied in each group, their ages and sexes are summarized in Table 1.

Serological assays for hepatitis virus markers and HBV genotyping. HCV (anti-HCV) and HBV serological markers (HBsAg, HBeAg, anti-HBs and anti-HBc) were examined using a chemiluminescent immunoassay (Ortho Clinical Diagnostics).

HBsAg-positive samples were subjected to HBV genotyping using an enzyme-linked immunoassay (EIA) with monoclonal antibodies to type-specific epitopes of the preS2 region (Usuda *et al.*, 1999), using commercial kits (HBV Genotype EIA; Institute of Immunology Co.).

Table 1. The distribution of HBV and HCV serological markers and HBV genotypes among two populations in Cameroon

Population	Bantu ($n=370$)	Pygmies ($n=174$)	P
Male/Female*	177/188	87/82	NS†
Age (years); mean ± SD	34·2 ± 14·5	29·9 ± 9·2	<0·05
Anti-HCV	75 (20·3 %)	4 (2·3 %)	<0·0001
HBsAg	33 (8·9 %)	13 (7·5 %)	NS
HBeAg	5 (15·2 %)	0	NS
Anti-HBs	77 (20·8 %)	17 (9·8 %)	0·001
Anti-HBc	322 (87·0 %)	150 (86·2 %)	NS
HBV/A	15 (45·5 %)	5 (38·5 %)	NS
HBV/E	13 (39·4 %)	7 (53·8 %)	NS
HBV/D	5 (15·1 %)	1 (7·7 %)	NS

*Gender and age data were not available for some of the specimens.
†NS, Not significant.

Amplification, quantification of HBV DNA and nucleotide sequencing. DNA was extracted from 27 serum samples, in which HBV/A and HBV/E had been identified by genotyping EIA: 20 (15 from Bantu and five from Pygmies) and seven (only from Pygmies), respectively. Total DNA was extracted from 100 µl serum using a QIAamp DNA mini kit (Qiagen) and suspended in 100 µl storage buffer (supplied by the kit manufacturer). A real-time PCR assay, allowing detection of up to 100 viral DNA copies ml^{-1} (Abe *et al.*, 1999), with slight modifications (Tanaka *et al.*, 2004), was used for HBV DNA screening.

Two overlapping HBV DNA fragments covering the entire genome sequence were amplified using specific primers and PCR conditions that have been described previously (Sugauchi *et al.*, 2001). Amplified HBV DNA fragments were sequenced directly using a Prism Big Dye v3.0 kit (Applied Biosystems) on an ABI 3100 DNA automated sequencer (Applied Biosystems). All sequences were analysed in both the forward and reverse directions. Complete and partial HBV genomes were assembled using GENETYX v11.0 (Software Development). The nucleotide sequence data reported in this paper appear in the GenBank/EMBL/DDBJ nucleotide sequence databases with the accession numbers AB194947–AB194955.

Sequence analysis. Sequences were aligned using the CLUSTAL W software program (Thompson *et al.*, 1997). Phylogenetic trees were constructed using neighbour-joining (NJ) analysis incorporating the six-parameter distance correction method (Gojobori *et al.*, 1982) with bootstrap test confirmation performed on 1000 resamplings using the Online Hepatitis virus database (http://s2as02.genes.nig.ac.jp/). Preliminary trees were constructed for Cameroonian HBV strains obtained in this study and corresponding data of 632 HBV genome sequences available from the GenBank/DDBJ databases (the trees are available from the authors). The final trees presented herein were constructed for Cameroonian strains together with the selected GenBank/DDBJ references including the HBV/A strains of various geographical origins, and representatives of other known human HBV genotypes.

Nucleotide divergence over complete genomes was calculated using the CLUSTAL method implemented in the MEGALIGN software (Clewley & Arnold, 1997).

Detection of recombination. All Cameroonian strains' complete genome sequences were examined for the presence of recombination with other HBV genotypes, as described previously (Robertson *et al.*, 1995). Bootscan analysis implemented in the SimPlot software program (Lole *et al.*, 1999) was performed for each of the strains.

Statistical analysis. All statistical values were calculated using the Mann–Whitney U test, Fisher's exact test and the χ^2 test with Yate's correction, implemented in the STATA v8.0 software program (Stata). Differences were considered significant for P values less than 0·05.

RESULTS

Hepatitis virus serological markers and HBV genotypes in Cameroon

Table 1 summarizes results of the serological screening and HBV genotyping. The overall anti-HCV seroprevalence was very high (14·5 %), and was significantly higher in Bantus (20·3 %) than in Pygmies (2·3 %, $P<0·0001$), demonstrating that transmission networks of the infection are relatively isolated between two populations (blood transfusion and medical procedures probably contributed to transmission in the Bantus but not in the Pygmies; Kowo *et al.*, 1995). Nevertheless, HBsAg prevalence was equally high in both

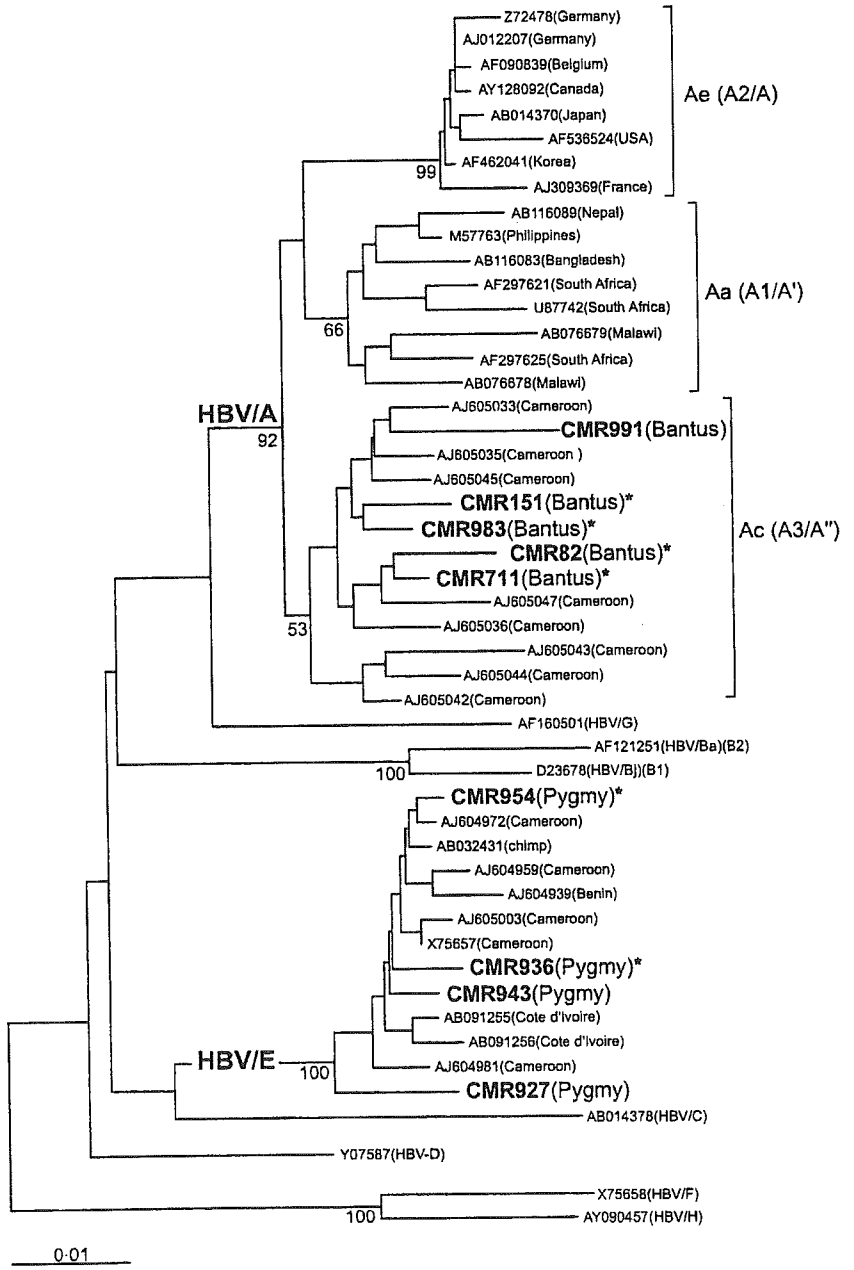


Fig. 1. A phylogenetic NJ tree constructed using the HBV preS2/S nucleotide sequences. Nine strains from Cameroon isolated in this study are indicated in bold. Reference sequences were retrieved from GenBank/EMBL/DDBJ with their accession numbers and origin (in parentheses) indicated. Bootstrap values are indicated in the tree roots. Asterisk (*) marked strains, four HBV/A and two HBV/E, from Cameroon were used for further analyses based on the complete genome sequences.

populations (8·9–7·5 %). HBeAg examined among HBsAg-positive carriers was determined in 10·9 % of cases, all of which were in the Bantus infected with HBV/E (mean age 21·2 years, range 1–30 years). The mean age of HBeAg-negative carriers in the Bantus was 31·6 years, range 17–90 years. All HBsAg-positive carriers among the Pygmies were negative for HBeAg (mean age 29·5 years, range 27–38 years). Thus, the mean age of the HBeAg-negative group was relatively young for HBsAg carriers in both populations in Cameroon, suggesting early HBeAg seroconversion. Anti-HBc seroprevalence was very high in both populations (mean 86·7 %), with no significant difference (86·2 vs 87 %), concordant with a previous report (Ndumbe *et al.*, 1993) and indicating a high incidence of HBV infection in both populations, probably attributable to effective horizontal transmission at a young age, as reported previously in African countries (Kramvis *et al.*, 2005). There was no significant difference in the distribution of the examined viral markers among the Bantu population in different provinces, or among the Pygmies population in the different encampments. A total of 46 serum samples found to be positive for HBsAg were subjected to HBV genotyping using the EIA method. Genotypes A and E identified in 43·5 % of cases were equally predominant in both of the populations, and genotype D was found in a minority (13 %) of cases. No significant difference in distribution of the genotypes was found within the same population in different provinces or between the two populations.

In order to study the molecular genetic characteristics of the prevalent HBV genotypes in Cameroon, 20 HBV/A and seven HBV/E samples, for which sufficient volume was available, were subjected to further investigation. Of the

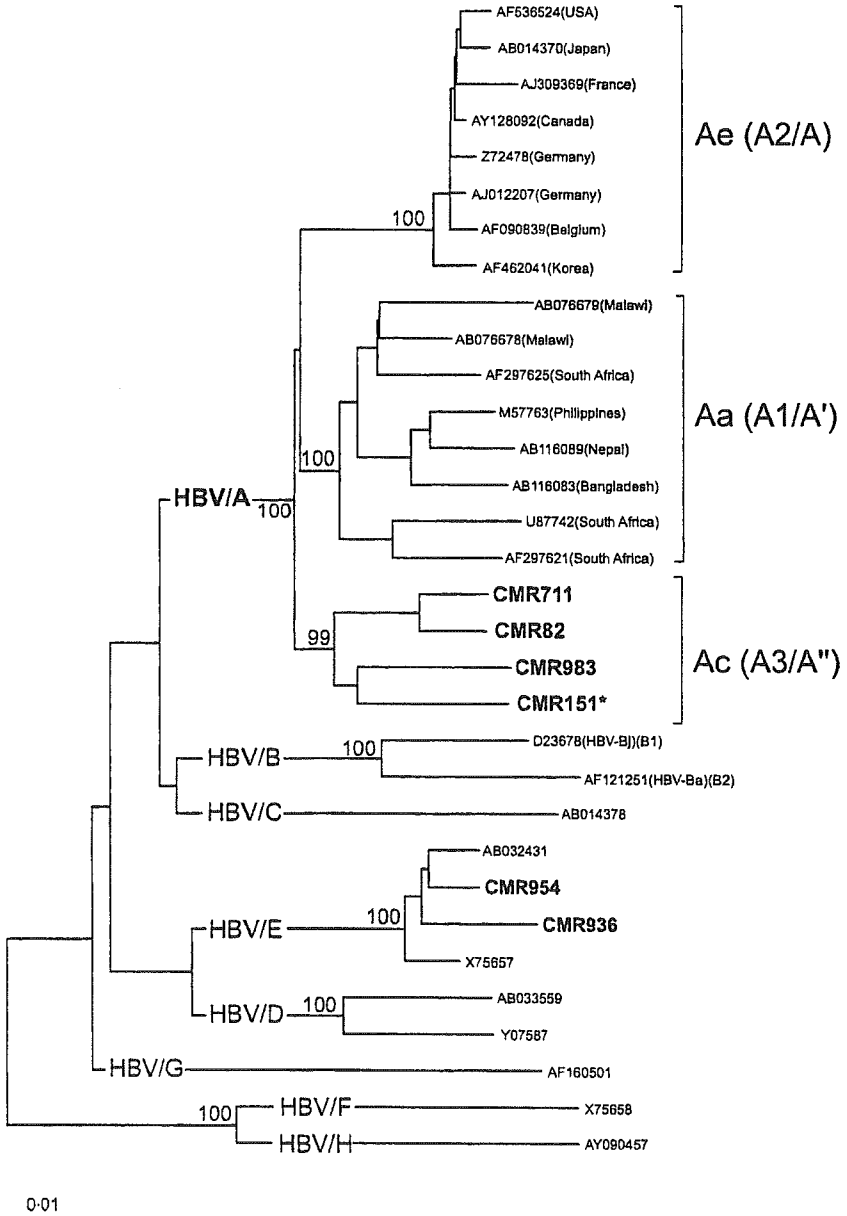
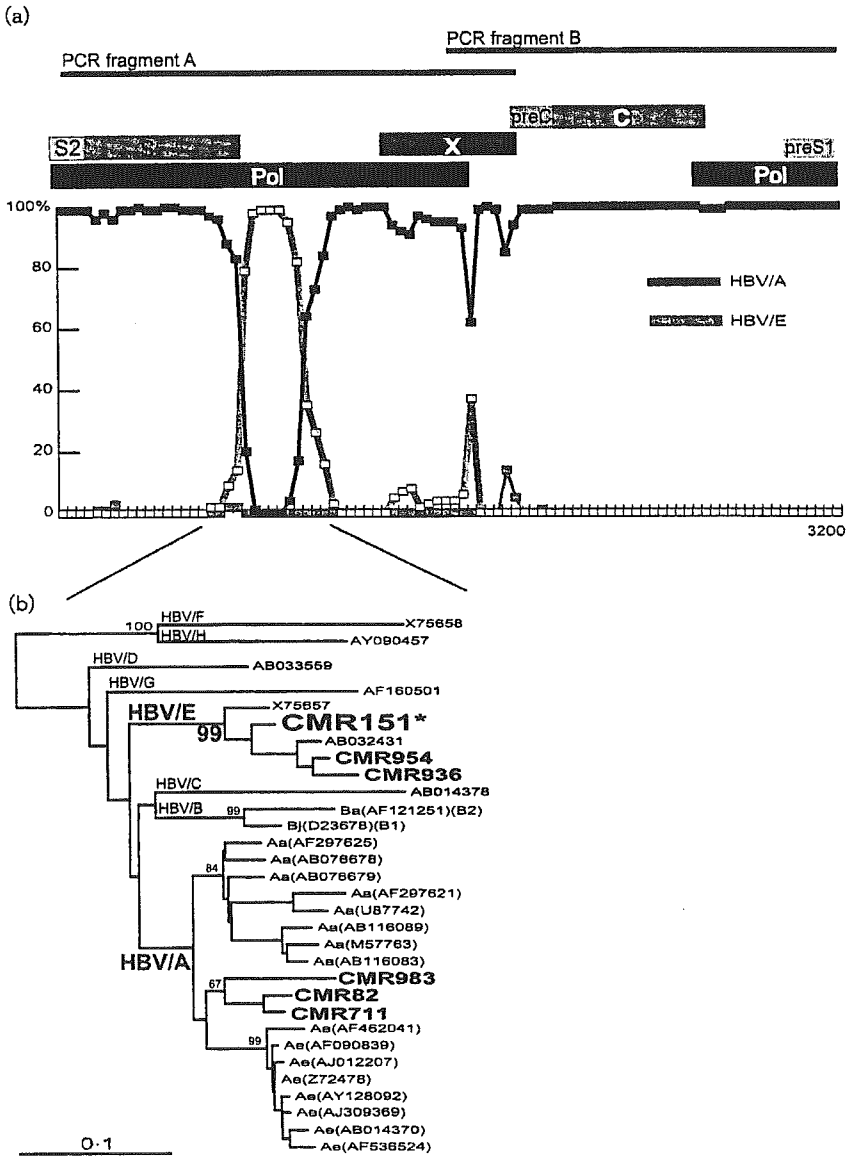


Fig. 2. A phylogenetic NJ tree constructed using the complete HBV genome. Six strains from Cameroon isolated in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. The origins of the previously published HBV/A strains are indicated in parentheses. Bootstrap values are indicated in the tree roots. The strain from Cameroon with the recombination between HBV/A and HBV/E is marked with an asterisk (*).

samples, only 1/27 was HBeAg-positive (HBV/E by EIA), which was obtained from a 1-year-old child, and the rest (26/27) of the HBsAg-positive carriers had undergone HBeAg seroconversion.

HBV DNA quantification, sequencing, phylogenetic relation and genetic diversity of HBV/A subtypes (subgenotypes)

HBV DNA was detected in only 10/27 serum samples: 5/15 Bantus and 5/12 Pygmies. The highest HBV DNA level ($3{\cdot}4 \times 10^{10}$ copies ml^{-1}) was detected in the sample obtained from a 1-year-old child. The rest of the nine positive samples were obtained from (mean) 26-year-old carriers (range 21–30 years), with HBV DNA levels ranging from $1{\cdot}1 \times 10^{3}$ to $7{\cdot}8 \times 10^{5}$ copies ml^{-1}. HBV DNA-negative carriers were (mean) 30·4 years old, range 17–50 years, showing a general tendency of HBV DNA level to decline with age (not statistically significant, probably due to small numbers). HBV large S coding region sequences were successfully amplified from 9/10 samples. The sequences were subjected to a similarity search throughout GenBank/DDBJ using the BLAST search engine, and the most similar strains were used for phylogenetic analysis together with the reference sequences of all known human HBV genotypes. The phylogenetic relationship of the ~800 nt (positions 31–835) preS2/S sequences of the HBV strains is represented in Fig. 1. Within the HBV/A phylogenetic cluster, the HBV/Aa (A1) and HBV/Ae (A2) strains separated out into two clusters and the five Cameroonian strains sequenced in this study together with other Cameroonian strains retrieved from GenBank/DDBJ clustered separately. The Cameroonian strains retrieved from GenBank/DDBJ were previously designated A″ cluster according to partial (Large S) genome sequence (Mulders *et al.*, 2004). The Cameroonian and HBV/Aa (A1) subclusters, however, did not have significant bootstrap indexes.



The complete genome of six strains (four HBV/A and two HBV/E) were sequenced successfully (marked by asterisks in Fig. 1). The lengths of the complete genomes corresponding to HBV/A and HBV/E were 3221 and 3212 nt, respectively. The phylogenetic analysis of the complete genome sequences (Fig. 2) revealed three distinct bootstrap test supported groups within the HBV/A cluster: HBV/Aa (A1), HBV/Ae (A2), and the third group formed by samples from Cameroon. We denoted the third phylogenetic group as 'HBV/Ac', where 'c' stands for Cameroon and Central Africa. The distinctive grouping of HBV/Ac (A3) strains was also confirmed when preS1/S2, preC/C, and complete Pol genes were analysed phylogenetically. S and X genes were phylogenetically related between the HBV/Aa (A1) and HBV/Ac (A3) groups. Estimated inter-group percentage nucleotide divergence over complete genome sequences consisted of [mean ± SD (range)]: 4·9 ± 0·4 (4·2–6·1), Aa (A1) versus Ae (A2); 5·1 ± 0·5 (4·0–6·0), Aa (A1) versus Ac (A3); and 5·2 ± 0·3 (4·7–5·8), Ae (A2) versus Ac (A3). On the other hand, intra-group percentage nucleotide divergence was similar for HBV/Aa (A1) and HBV/Ac (A3) [mean ± SD (range)]: 3·6 ± 0·8 (4·0–4·6) and 3·9 ± 1·1 (1·8–4·8), respectively, and lowest for Ae (A2): 0·9 ± 0·3 (0·4–1·6) ($P < 0·0001$).

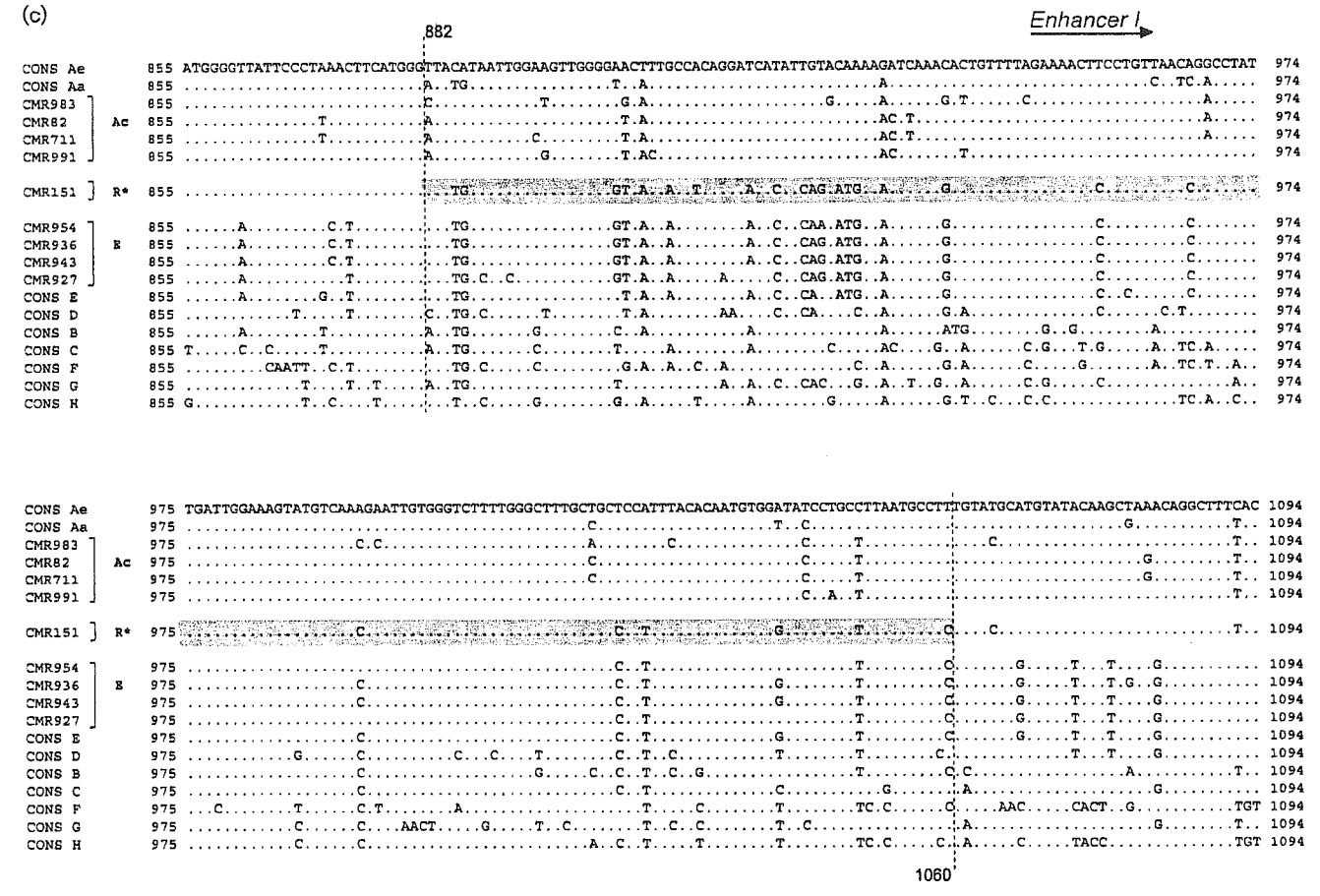


Fig. 3. (a) SimPlot analysis demonstrating the recombination in the non-overlapping part of the polymerase coding region of the CMR151 strain. The strain was subjected to bootscan analysis over the entire genome using the SimPlot program (Lole *et al.*, 1999) with a window size of 300 bp and a step size of 30 bp, under the F84 (ML) model, with bootstrap resampling performed 1000 times. Initially, consensus sequences of each human HBV genotype were used as references; after manual confirmation of the sequence alignment, the final plot was constructed using the consensus of the HBV/A, HBV/E and HBV/D genotypes only. The sequences were obtained from two overlapping PCR fragments, indicated by two lines ('A' and 'B'). HBV genome coding regions are indicated by standard abbreviations (S2, S, Pol, X, preC, C and preS1). (b) The phylogenetic NJ tree constructed using the recombinant segment. Six strains from Cameroon in this study are indicated in bold. Accession numbers are given for reference sequences retrieved from GenBank/EMBL/DDBJ. (c) Alignment of all human HBV genotype genome nucleotide sequences in the region corresponding to the recombination in the Cameroonian strain CMR151 (shaded in grey). Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Dashed lines at 882 and 1060 represent the breakpoints.

Recombination

Evidence of recombination between HBV/A and HBV/E was observed in one of the Cameroonian strains (CMR151, marked by an asterisk in Fig. 2). The result of the bootscan analysis for the complete genome sequence of the strain is presented in Fig. 3(a). The phylogenetic tree constructed using the corresponding sequence segment confirmed the grouping of the CMR151 strain together with the HBV/E strains, with strong bootstrap support (Fig. 3b). The recombinant segment corresponded to a part of the non-overlapping HBV DNA polymerase in the reverse transcriptase (RT) domain and a part of the enhancer I–X promoter. (Fig. 3c). The breakpoints at nucleotide positions 882 and 1060 were estimated by mapping the informative sites and using χ^2 confirmation (Robertson *et al.*, 1995).

Enhancer/promoter elements and amino acid characteristics of the HBV/Ac (A3) strains

A comparison of the nucleotide substitutions within the *cis*-acting elements among the four HBV/Ac (A3) strains and the consensus sequences of the HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes) as well as the other HBV genotypes (including HBV/Ba, Bj, B2, B1) are summarized in Table 2. Nine specific nucleotide substitutions were found in HBV/Ac (A3) strains: $G^{1173}A$ (enhancer I–X promoter), $C^{1473}G$, $G^{1512}A$ and $C^{1703}T$ (enhancer II–core promoter), $A^{2742}G$ (S1–promoter), $C^{3021}T$, $C^{3042}T$,

Table 2. Subtype (subgenotype) specific sites (bold) within enhancers and promoter regions of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Nucleotide positions correspond to the HBV genome reference sequence, GenBank accession no. NC_003977. Consensus sequences were composed according to 60 % or higher incidence at the corresponding nucleotide position.

Region	Position (nt)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
Enhancer I–X promoter (950–1350)	963	**C**	T	T	T	T	T
	1041	T	A	A	A	G	A
	1173	G	–	**A**	**A**	**A**	–
	1320	A	A	A	A	A	G
	1350	**T**	–	–	–	–	C
Enhancer II–core promoter (1400–1850)	1404	**T**	–	–	–	–	C
	1464	**G**	T	T	T	T	T
	1473	C	**G**	**G**	–	**T**	–
	1484	A	–	–	–	–	**C**
	1511	G	–	–	–	–	**A**
	1512	**T**	**A**	**A**	–	**A**	**G**
	1703	C	**T**	**T**	**T**	A	–
	1727	A	–	–	–	–	**G**
	1740	T	–	–	–	–	**C**
	1809–1812	**TCAT**	-TC-	--C-	----	G--C	G--C
Encapsidation signal (1846–1908)	1888	**A**	G	G	G	G	G
S1–Promoter (2716–2806)	2720	**A**	T	T	T	T	**G**
	2742	A	**G**	**G**	**G**	**G**	–
	2744	C	**A**	**A**	–	–	–
	2777	G	C	T	T	T	T
S2–Promoter (2999–3219)	3013–3014	**CA**	--	--	--	--	GC
	3021	C	**T**	**T**	**T**	**T**	–
	3042	C	**T**	**T**	**T**	–	–
	3052	T	–	–	–	–	**C**
	3057/60	T/C	C/T	C/T	C/T	C/T	C/T
	3069	A	–	–	–	–	**C**
	3072–3073	TG	A-	C-	--	--	-A
	3076	T	**C**	**C**	**C**	**C**	–
	3111	T	**T**	**T**	**T**	**T**	**C**
	3118	C	–	–	–	–	**T**
	3121	G	–	–	–	–	**A**
	3124	G	–	–	–	–	**A**

$T^{3076}C$ and $C^{3111}T$ (S2–promoter). Interestingly, three of four strains had substitutions in the Kozak sequence (1809–1812) (Ahn *et al.*, 2003; Tanaka *et al.*, 2004), and two had a basal core promoter double mutation (1762/1764).

Although HBV/Ac (A3) amino acid motifs in general were more similar to HBV/Aa (A1) than to HBV/Ae (A2) motifs, HBV/Ac (A3) strains had some specific sites: Thr^{84} in preS1, Ala^{146} in Pol/terminal protein, Ser^{239}, Trp^{246}, Ser^{257} in Pol/spacer, Asp^{356}, Arg^{501}, Ser^{607} in Pol/RT, and Thr^{47} in X proteins when compared with consensus sequences composed according to 60 % or higher incidence at the corresponding amino acid position (Table 3). Pre-core/core amino acid patterns had no specific substitutions among HBV/A subtypes (subgenotypes).

Table 3. Subtype (subgenotype) specific sites (bold) in amino acid sequences of HBV/Aa (A1), HBV/Ac (A3) and HBV/Ae (A2)

Consensus sequences were composed according to 60 % or higher incidence at the corresponding amino acid position.

ORF	Position (aa)	HBV/Aa (A1)	HBV/Ac (A3)				HBV/Ae (A2)
			CMR711	CMR82	CMR983	CMR151	
PreS1	54	**Q**	–	–	–	–	**A**
	67	**F**	–	–	–	–	**L**
	74	**V**	–	–	–	–	**I**
	84	**I**	**T**	**T**	**T**	–	–
	86	**A**	T	T	T	T	T
	89	**P**	–	–	–	–	**S**
	90	**A**	–	–	–	–	**T**
	91	**V**	–	–	–	–	**I**
PreS2	32	**L**	V	V	V	V	V
	47	**S**	–	–	–	–	**A**
S	209	**L**	–	–	–	–	**V**
Pol/terminal protein	17	**E**	G	G	G	G	**G**
	33	**E**	–	–	A	A	**A**
	74	**P**	**Q**	**Q**	–	–	–
	102	**T**	**N**	**N**	–	–	–
	120	**N**	–	–	–	–	**T**
	146	**T**	**A**	**A**	**A**	**A**	–
Pol/spacer	236	**T**	–	–	–	–	S
	239	**P**	**S**	**S**	**S**	**S**	–
	246	**R**	**W**	**W**	**W**	–	–
	257	**F**	**S**	**S**	**S**	**S**	–
	269	**Y**	–	–	–	–	H
	271	**A**	–	–	–	–	**V**
	273	**S**	–	–	–	–	**N**
	308	**S**	K	K	–	–	**C**
	334	**Q**	–	–	K	K	K
	338	**K**	E	E	E	E	E
	348	**L**	–	–	–	–	**R**
Pol/RT	356	**E**	**D**	–	**D**	**D**	–
	501	**W**	**R**	**R**	**R**	**R**	–
	607	**T**	**S**	**S**	**A**	**S**	–
	617	**I**	**L**	L	–	–	–
	619	**H**	–	–	**D**	**D**	–
	666	**K**	**R**	**R**	–	–	–
X region	11	**S**	–	–	–	–	P
	31	**A**	S	S	S	S	S
	34	**L**	**V**	**V**	–	**F**	–
	47	**S**	**T**	**T**	–	**T**	A
	146	**S**	**F**	–	–	A	A
	147	**S**	–	–	–	P	P

DISCUSSION

A previous study carried out in Cameroon among the Bantus and the Pygmies (Kowo *et al.*, 1995) demonstrated a high (18·6 %) overall seroprevalence of HCV, which was significantly higher in Bantus (31·7 %) than in Pygmies (11·1 %). The results of the present study also indicate the very high HCV seroprevalence (14·5 %), and support the difference between the two populations. However, in our study, HCV seroprevalence among the Pygmies was lower (2·3 %), which might be attributed to the younger age of examined subjects compared with the cohort previously studied (Kowo *et al.*, 1995). The difference in HCV seroprevalence between the two populations might be explained by exposure of the Bantus to transmission routes such as medical procedures and blood transfusion, to which the Pygmies are not exposed. However, HBV seroprevalence (HBsAg and anti-HBc) was equally high among the two populations and different regions of the country, which is concordant with previous data (Ndumbe *et al.*, 1993). Further epidemiological investigation is required to evaluate factors contributing to the difference in HBV and HCV transmission in the Pygmies, in contrast with neighbouring Bantus.

The only data available on HBV genotypes in Cameroon demonstrated the predominant prevalence of HBV/A in human immunodeficiency virus-positive cohort (Mulders *et al.*, 2004). The present study revealed that both HBV/A and HBV/E are distributed equally in both native populations in Cameroon. The phylogenetic analysis revealed a close relationship in the large S coding region among the Cameroonian strains sequenced in this study and those from the same country available from previous reports (Mulders *et al.*, 2004; Norder *et al.*, 1992). Based on phylogenetic analysis of the complete genome, including four sequences in this study, the presence of a third phylogenetic cluster was confirmed within HBV/A in this study. The cluster was distinct from known HBV/Aa (A1) and HBV/Ae (A2) subtypes (subgenotypes), and designated HBV/Ac (A3) (where 'c' stands for Cameroon and Central Africa). The inter-subtype (subgenotype) nucleotide divergence over the complete genome sequences falls within the 4–8 % range that justifies the classification of HBV/Ac (A3) into a distinct subtype (subgenotype) according to the recent proposals on HBV nomenclature (Kato *et al.*, 2005; Kramvis *et al.*, 2005). The high intra-subtype (subgenotype) nucleotide divergence of four HBV/Ac (A3) complete genomes suggests a long natural history of this subtype (subgenotype) within the native population of Cameroon, as has been reported for subtype (subgenotype) HBV/Aa (A1) in southern African Blacks (Kimbi *et al.*, 2004). On the other hand, HBV/E strains obtained from the Pygmies did not group together separately from the strains isolated in different geographical regions, even though the Pygmies represent an isolated population in Africa. The presence of low divergent HBV/E genotype among the Pygmies might not support the hypotheses proposed previously that HBV/E has a very short history in humans (Mulders *et al.*, 2004).

The newly described subtype (subgenotype) HBV/Ac (A3) possesses a combination of the sites specific for either HBV/Aa (A1) or HBV/Ae (A2) within the corresponding enhancer/promoter elements and amino acid motifs (Kimbi *et al.*, 2004; Sugauchi *et al.*, 2004; Tanaka *et al.*, 2004). Moreover, the subtype (subgenotype) also has HBV/Ac (A3) unique substitutions. The recombination affecting a short, non-overlapping segment of the polymerase RT domain found in one of the Cameroonian strains is the first event documented to have occurred between HBV/A and HBV/E. The sequencing data generated in the present study could be used to design assays that can discriminate between HBV/Ac (A3) and the other subtypes (subgenotypes) of HBV/A in order to characterize its clinical–virological features. Cohort studies are required to investigate a possible association of HBV/Ac (A3) infection with early HBeAg/anti-HBe seroconversion and low HBV DNA levels in carriers indicated by the tendencies observed on the small number investigated in present study.

At the present time, investigation of HBV molecular heterogeneity, global distribution of HBV genetic forms, including recombination and mutations as well as efficient implications of the data, is one of the major directions in the field of virus research (Kramvis *et al.*, 2005). In this respect, further standardization of the HBV nomenclature and, an efficient and logical classification should be based on a consensus of the accumulated data including recent studies.

In conclusion, the complete genome of the third subtype (subgenotype) of HBV/A, identified in Cameroon, has been analysed and unique nucleotide/amino acid substitutions have been identified within this subtype (subgenotype). The high intra-group divergence suggests that this subtype (subgenotype) represents an indigenous HBV strain with a long natural history. Recombination between this subtype (subgenotype) and genotype E is described.

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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

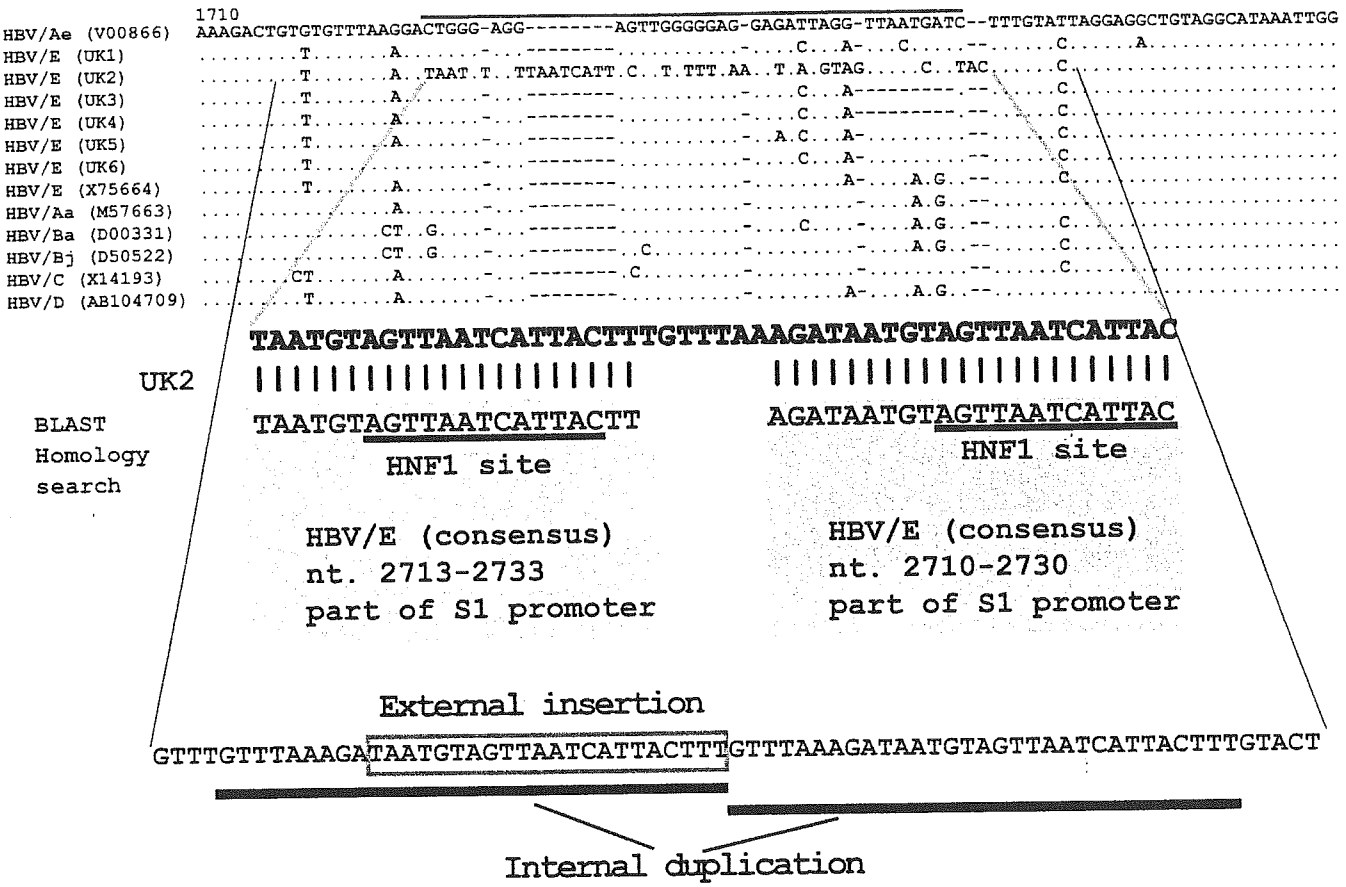

FIG. 1. Nucleotide alignment of the six HBV genotype E sequences of the core upstream regulatory region to the basic core promoter region, with references to other genotypes and subtypes. The sequence enlarged under the alignment shows the UK2-specific 50-nucleotide sequences which were analyzed by BLAST 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

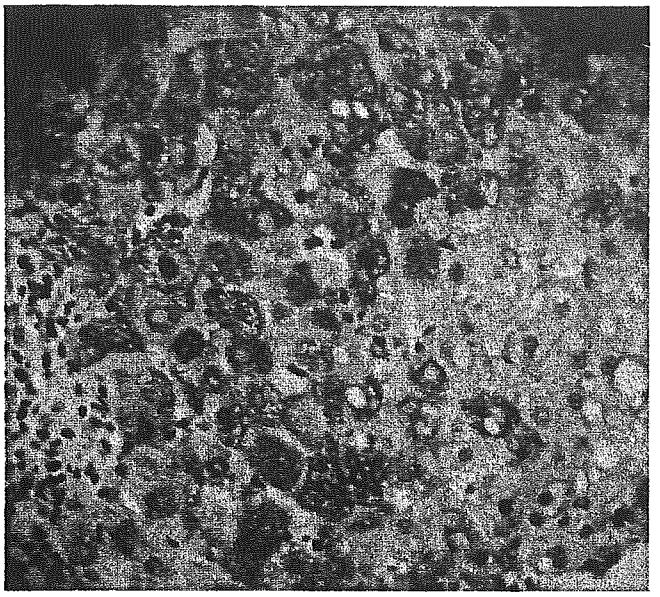

FIG. 2. Immunohistochemical detection of HBcAg in the liver of the patient 2. Strong staining is observed in both nuclei and cytoplasm.

(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.

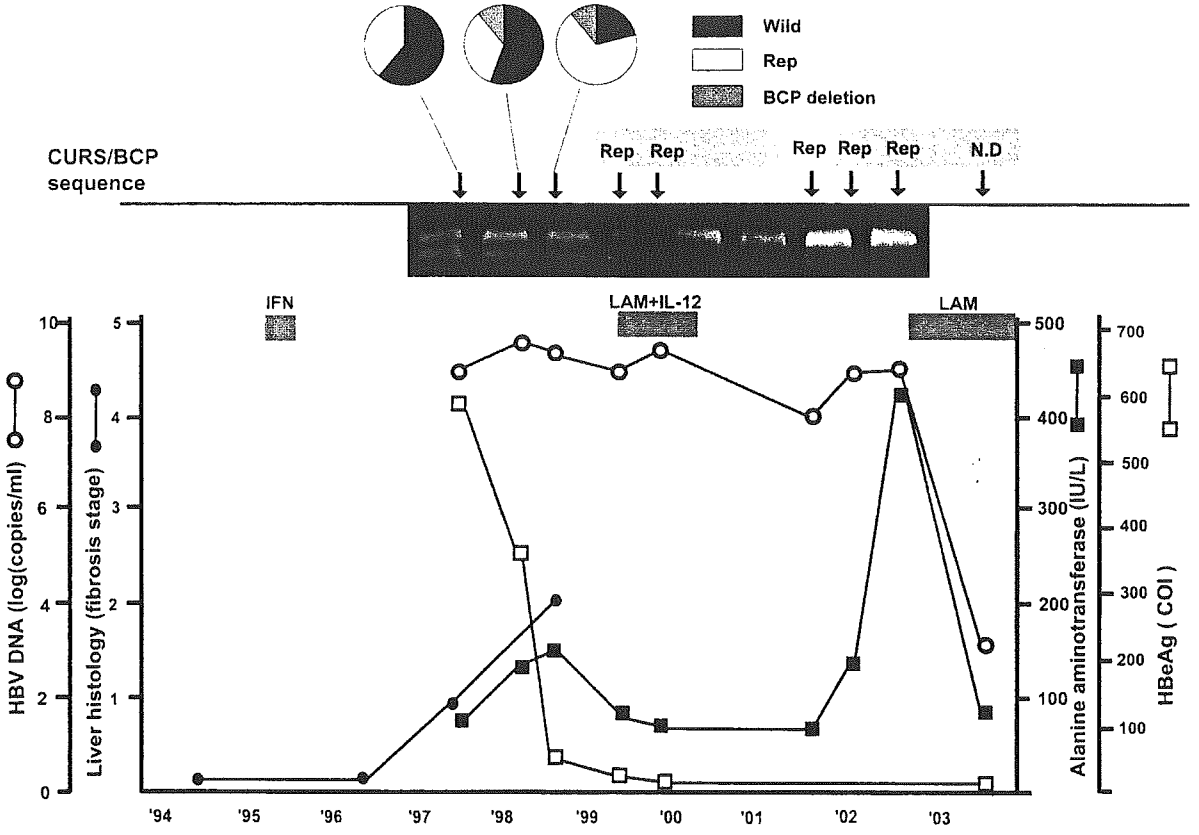

FIG. 3. Clinical course of patient 2 based on HBV DNA levels, fibrosis stage, HBeAg titer, and ALT levels. The three circular charts show how the proportions of wild type, replacement mutation (Rep) type, and BCP deletion type changed during the time course. The electropherogram (5%) shows that mixed 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

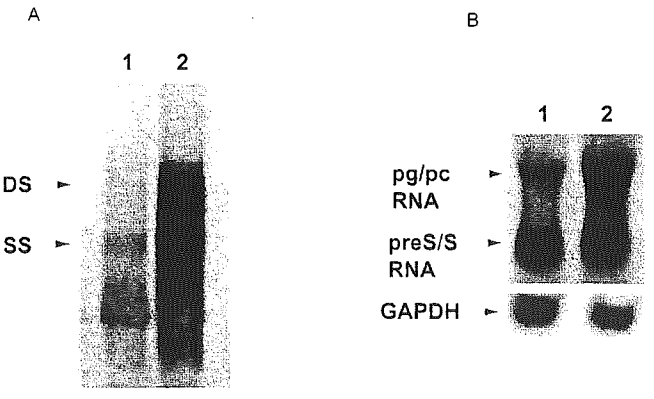

FIG. 4. (A) Southern blot analysis of intracellular replication competence of replacement mutation with HNF1 site. Double-stranded (DS) DNA and single-stranded (SS) DNA are indicated by arrows. The pUC19-HBV/E replacement replicon (lane 2) replicates at a much higher rate than the pUC19-HBV/E wild-type 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.

accumulation of hepatitis B core protein in nuclei and cytoplasm of hepatocytes.

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

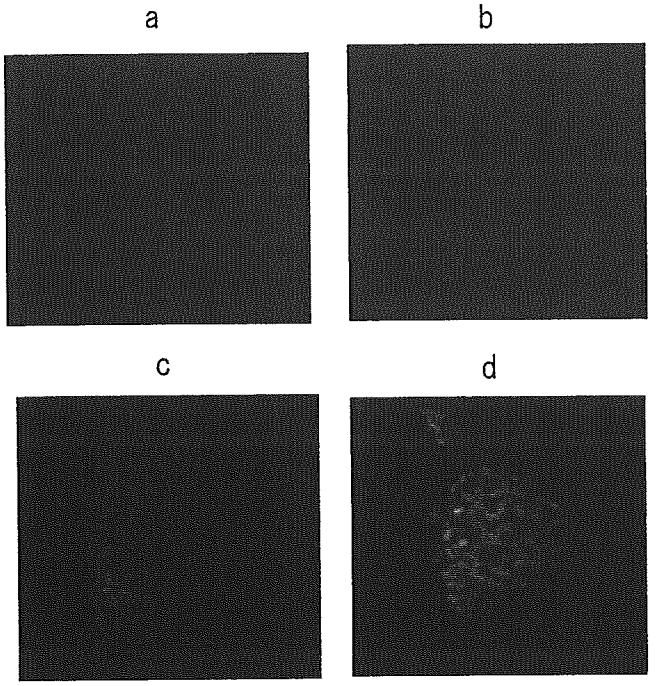

FIG. 5. Intracellular localization of HBcAg (magnification, ×400). Immunofluorescence staining of HBcAg in HuH-7 cells transfected with (a) pUC19-HBV/E wild-type replicon and (c) pUC19-HBV/E replacement replicon. (b and d) Nuclear staining for the same set of cells was performed by use of DNA staining. 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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Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism

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Abstract

Recently hepatitis B virus genotype C (HBV/C) has been classified into geographically typical two subtypes (subgenotypes); HBV/C1 in Southeast Asia (Cs) and HBV/C2 in East Asia (Ce). Our aim is to develop a rapid subtyping assay and to examine the virological features of these two subtypes. Based on 171 HBV/C strains retrieved from the database, 17 single nucleotides polymorphisms (SNPs) were found between two subtypes. Taking advantage of five SNPs in non-overlapping polymerase region, a restriction fragment length polymporphism method with three endonucleases was newly developed for distinguishing between HBV/Cs and HBV/Ce. The method was applied to 49 HBV/C carriers from Japan and Hong Kong. The 24 in Hong Kong were classified into HBV/Cs, and the 25 in Japan were HBV/Ce, confirmed by sequencing. Some specific mutations were detected in the encapsidation signal; precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858, was found in HBV/Ce strains, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found in HBV/Cs. Especially, two closely linked mutations (A1896 and A1899) in HBV/Ce could stabilize the epsilon loop structure more efficiently and influece viral replication. Hence, these virological differences between the two subtypes might influence clinical features.

Keywords: Hepatitis B virus; Single nucleotides polymorphisms; Subgenotypes

1. Introduction

HBV genotypes have a distinct geographical distribution and correlate with severity of liver disease [1,2]. Genotypes B and C are prevalent in Asia, and genotype C causes more serious liver disease than genotype B [3,4]. HBV strains even of the same genotype may differ both virologically and clinically. There are two subtypes (subgenotypes) of genotype B in distinct geographical distributions, designated Ba ("a" standing for Asia) and Bj ("j" for Japan) provisionally [5], and clinical differences between patients infected with HBV/Ba and HBV/Bj are coming to the fore [6,7]. Additionally, there have been some lines of evidence for virological and clinical differences between HBV/Aa in Africa and HBV/Ae in Europe and the US [8,9]. Infection with HBV/Aa is associated with low serum levels of HBV DNA as well as low prevalence of hepatitis B e antigen (HBeAg) in serum, and is implicated in the high incidence of HBV-induced hepatocellular carcinoma (HCC) in Africa [10,11].

Recently, phylogenetic analysis of the pre-S1/pre-S2 genes revealed two major groups within genotype C: one for strains from southeast Asia including Vietnam, Myanmar and Thailand (named HBV/C1) and the other for strains from

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(far) East Asia including Japan, Korea and China (named HBV/C2). This finding was confirmed by phylogenetic analyses based on the complete sequences of 32 HBV/C strains [12], and by a recent independent study in Hong Kong [13]. The latter paper designated the two subtypes (subgenotypes) as HBV/Cs in Southeast Asia and HBV/Ce in the (far) East Asia that have different epidemiological distributions [13]. However, further studies are required to evaluate clinical and virological significance between HBV/C1 (Cs) and HBV/C2 (Ce), and development of a simple and efficient method for classification is essential.

In this study, we investigated single nucleotides polymorphisms (SNPs) between HBV/Cs and HBV/Ce at complete genome levels, and developed a novel polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method in the non-overlapping polymerase region involving five SNPs to distinguish between HBV/Cs and HBV/Ce precisely.

2. Materials and methods

2.1. Subjects

A total 49 sera containing HBV/C determined by the ELISA on preS2-region products [14,15], with the results confirmed by PCR-RFLP of the S gene [16], were obtained from chronic carriers of HBV who visited Nagoya City University hospital in Japan or Queen Mary Hospital in Hong Kong. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and an informed consent was obtained from each HBV carrier. To determine SNPs between HBV/Cs and HBV/Ce, 34 HBV/Cs and 137 HBV/Ce complete sequences were additionally recruited from DDBJ/EMBL/GenBank database.

2.2. PCR-RFLP for distinguishing between subtypes (subgenotypes) Cs and Ce of HBV genotype C

Nucleic acids were extracted from 100 μL of serum using QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany). A novel method for specific determination of HBV/C consisted of two PCR cycles with hemi-nested primers followed by RFLP with the restriction site specific for HBV/Cs or Ce. The first-round PCR was performed with a sense primer (HBV964F: 5′-ATT AGA CCT ATT GAT TGG AAA GT-3′ [nt 964-986]) and an antisense primer (HBV1272R: 5′-AGT ATG GAT CGG CAG AGG AG-3′ [nt 1272-1253]) within non-overlapping polymerase region. The second-round PCR was performed with a sense primer (HBV970F2: 5′-CCT ATT GAT TGG AAA GTA TGT CA-3′ [nt 970-992]) and an antisense primer (HBV1272R). To determine HBV/Cs, a portion (5 μl) of the amplification product of 309 base pairs (bp) in size was digested with 5 U of *Ase*I at 37 °C and *BstE*II at 60 °C for 1 h each. For HBV/Ce digestion, *Nci*I was used at 37 °C for 2 h. Digests with these enzymes were run on electrophoresis in 3.0% (w/v) agarose gel, stained with ethidium bromide and examined for their sizes under the ultraviolet light.

2.3. Amplification and sequencing of the core promoter as well as the precore region plus core gene

To confirm the results by PCR-RFLP, HBV DNA sequences bearing the core promoter and precore/core regions were amplified by PCR with hemi-nested primers by the method described previously [17], with slight modifications. In brief, the first round of PCR was performed with sense primer (HB7F-2: 5′-CAT GGA GAC CAC CGT GAA CGC-3′ [nt 1607–1627]) and antisense primer (HB8R-2: 5′-ATA GGG GCA TTG GTC T-3′ [nt 2314–2299]) for 40 cycles (94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min [6 min in the last cycle]) in a 96-well cycler (GeneAmp 9700, Perkin-Elmer Cetus, Norwalk, CA). The second round of PCR was performed with sense primer (HB7F-2) and antisense primer (HB7R-2: 5′-CCT GAG TGC TGT ATG GTG AGG-3′ [nt 2072–2052]) for 35 cycles, under the same conditions as in the first-round PCR. The standard precautions for avoiding contamination during PCR were exercised carefully, and a negative control serum was included in each run of tests to ensure the specificity. Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

2.4. Molecular evolutionary analyses of HBV

Reference sequences were retrieved from the DDBJ/EMBL/GenBank database and their accession numbers for identification. Nucleotide sequences of HBV were aligned by the program CLUSTAL X, and the genetic distance was estimated with the six-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/). Based on these values, a phylogenetic tree was constructed by the neighbor-joining method with the mid-point rooting option.

3. Results

3.1. SNPs for distinguishing between HBV/Cs and HBV/Ce in complete genomes

When the 171 HBV/C (34 HBV/Cs and 137 HBV/Ce) strains, retrieved from the DDBJ/EMBL/GenBank database were compared over the complete genomes, 17 SNPs were found between two subtypes (subgenotypes) (Table 1). Of them, five SNPs in non-overlapping polymerase region include restricted enzyme sites: *BstE*II site (nt 1041 of T [T1041] and C1044), *Ase*I site (A1050 and A1053) and *Nci*I site (C1155). Interestingly, the 34 HBV/Cs strains possessed *BstE*II site (G/GTNACC [nt 1039–1045]) and/or *Ase*I

Table 1
Subtype-specific mutations in the complete genomes of HBV/Cs and HBV/Ce

SNPs no.	Nucleotide position	Cs ($n = 34$)	Unmatched	Amino acids/region	Ce ($n = 137$)	Unmatched	Amino acids/region	Enzymes
1	166	C	0	Thr/S, **His/P**	A	1	Thr/S, **Asn/P**	
2	312	T	2	**Leu/S**, Phe/P	C	1	**Ser/S**, Phe/P	
3	400	C	3	Ile/S, **Leu/P**	A	0	Ile/S, **Ile/P**	
4	**1041**	**T**	**6**	**Gly/P**	**C**	**10**	**Gly/P**	*BstE*II
5	**1044**	**C**	**0**	**Thy/P**	**T**	**1**	**Thy/P**	*BstE*II
6	1047	A	0	Pro/P	T	3	Pro/P	
7	**1050**	**A**	**2**	**Ala/P**	**C**	**20**	**Ala/P**	*Ase*I
8	**1053**	**A**	**1**	**Leu/P**	**A/G**	**1**	**Leu/P**	*Ase*I
9	**1155**	**T**	**0**	**Ala/P**	**C**	**9**	**Ala/P**	*Nci*I
10	1721	A	1	Val/X	G	0	**Leu/X**	
11	2065	A	0	Leu/C	C	7	Leu/C	
12	2158	A	2	Val/C	C	5	Val/C	
13	2559	A	0	Lys/P	C	3	**Gln/P**	
14	2561	A	1	Lys/P	G	5	**Gln/P**	
15	2633	G	0	Leu/P	A	0	Leu/P	
16	2958	T	2	**Phe/P**, Asn/PreS1	C	4	**Leu/P**, Asn/PreS1	
17	3008	C	1	**Ser/P, Ala/PreS1**	A	5	**Arg/P, Asp/PreS1**	

(AT/TAAT [nt 1050–1055]), while the 137 HBV/Ce strains had neither *BstE*II nor *Ase*I sites. On the other hand, 128 of 137 (93%) HBV/C2 strains possessed *Nci*I site (CC/SGG [nt 1154–1158]) and none of the HBV/C1 strains had *Nci*I site due to T1151. Additionally, according to the SNPs, eight amino acids differences were found between two subtypes (subgenotypes) (Table 1).

3.2. PCR-RFLP for distinguishing between HBV/Cs and HBV/Ce

Geographically, typical genetic representatives for HBV/Cs and HBV/Ce (eight strains each) were selected. The partial genome sequence alignment including restriction sites is shown in Fig. 1. HBV/Cs strains were obtained

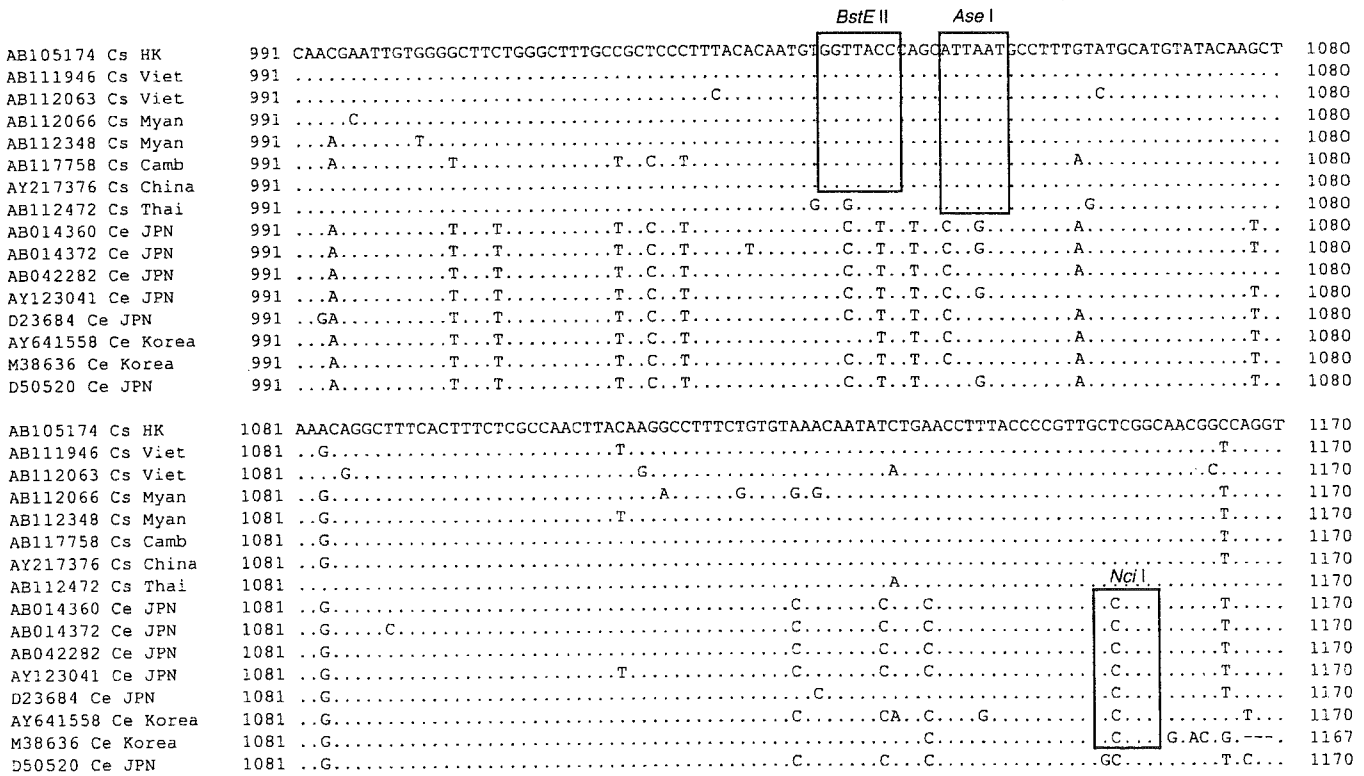

Fig. 1. Alignment of 8 HBV/Cs (C1) and 8 HBV/Ce (C2) sequences in non-overlapping polymerase region. The specific *BstE*II and *Ase*I sites are specific for HBV/Cs strains, while *Nci*I site is found in HBV/Ce strains. All sequences from the database are identified with accession numbers, followed by subtype and the country of origin in abbreviation for Cambodia (Camb), Hong Kong (HK), Japan (JPN), Myanmar (Myan) and Vietnam (Viet).