Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A

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Complete nucleotide sequences of 19 hepatitis B virus (HBV) isolates of genotype A (HBV/A) were determined and analysed along with those of 20 previously reported HBV/A isolates. Of the 19 HBV/A isolates, six including three from Japan and three from the USA clustered with the 14 HBV/A isolates from Western countries. The remaining 13 isolates including four from The Philippines, two from India, three from Nepal and four from Bangladesh clustered with the six HBV/A isolates reported from The Philippines, South Africa and Malawi. Due to distinct epidemiological distributions, genotype A in the 20 HBV isolates was classified into subtype Ae (e for Europe), and that in the other 19 into subtype Aa (a for Asia and Africa) provisionally. The 19 HBV/Aa isolates had a sequence variation significantly greater than that of the 20 HBV/Ae isolates (2·5 ± 0·3 % vs 1·1 ± 0·6 %, $P < 0·0001$); they differed by 5·0 ± 0·4 % (4·1–6·4 %). The double mutation (T1762/A1764) in the core promoter was significantly more frequent in HBV/Aa isolates than in HBV/Ae isolates (11/19 or 58 % vs 5/20 or 25 %, $P < 0·01$). In the pregenome encapsidation (ε) signal, a point mutation from G to A or T at nt 1862 was detected in 16 of the 19 (84 %) HBV/Aa isolates but not in any of the 20 HBV/Ae isolates, which may affect virus replication and translation of hepatitis B e antigen. Subtypes Aa and Ae of genotype A deserve evaluation for any clinical differences between them, with a special reference to hepatocellular carcinoma prevalent in Africa.

INTRODUCTION

Hepatitis B virus (HBV) has been classified into seven genotypes based on a sequence divergence over the entire genome exceeding 8 % (Norder *et al.*, 1994; Okamoto *et al.*, 1988; Stuyver *et al.*, 2000), and they are designated by upper-case letters from A to G. A possible eighth genotype is proposed with a tentative designation of H that is closely related to genotype F phylogenetically (Arauz-Ruiz *et al.*, 2002). The six major HBV genotypes (A–F) have distinct geographical distributions (Lindh *et al.*, 1997; Magnius & Norder, 1995). HBV genotypes A and D are predominant in Europe, North America and Africa, while genotypes B and C are prevalent in east and south Asia. On clinical fronts, there have been increasing lines of evidence to indicate influences of HBV genotypes on the outcome of liver diseases in hosts and the

The sequences reported in this article have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB116076–AB116094.

response to antiviral therapies, especially between genotypes A and D prevalent in Western countries as well as B and C common in Asia (Chu *et al.*, 2002; Kao *et al.*, 2002; Kobayashi *et al.*, 2002; Mayerat *et al.*, 1999; Orito *et al.*, 2001; Sugauchi *et al.*, 2002a; Wai *et al.*, 2002). Information is limited for geographical distribution or clinical relevance of genotypes G and H which were discovered recently (Arauz-Ruiz *et al.*, 2002; Kato *et al.*, 2002a, b; Stuyver *et al.*, 2000).

Virological characteristics and clinical manifestations may differ, however, even amongst HBV isolates of the same genotype. We have reported two subtypes of genotype B, designated Ba (a for Asia) and Bj (j for Japan), of which Ba has the recombination with genotype C over the precore region plus core gene, while Bj does not (Sugauchi *et al.*, 2002b). Response to antiviral therapies and the prevalence of hepatitis B e antigen (HBeAg) differed amongst patients with chronic liver diseases who were infected with HBV/Ba and HBV/Bj (Akuta *et al.*, 2003; Sugauchi *et al.*, 2003b). Likewise, amongst isolates of HBV genotype A (HBV/A), two subtypes have been reported, one of which distributes widely in European countries and the USA, while the other prevails in sub-Saharan Africa (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Sugauchi *et al.*, 2003a). The subtype of genotype A, designated A′ by Bowyer *et al.* (1997), seems to be virologically distinct from the original genotype A and associated with reduced serum levels of HBV DNA and a low prevalence of HBeAg in serum (Kramvis *et al.*, 1997, 1998). In addition, subtype A′ may have an association with hepatocellular carcinoma prevalent in Africa (Attia, 1998; Edman *et al.*, 1980; Olweny, 1984).

Complete nucleotide sequences were determined for 19 HBV/A isolates recovered from the USA and Asian countries. Including the sequences of 20 HBV/A isolates retrieved from the DNA databases, 20 isolates of the original genotype A and 19 isolates of subtype A′ were compared phylogenetically and for unique mutations in their nucleotide sequences. Due to distinct epidemiological distributions, together with marked virological differences, we would like to propose the classification of the original genotype A prevalent in European countries into subtype Ae (e for Europe) and A′ common in African and Asian countries into subtype Aa (a for Africa/Asia).

METHODS

Serum samples. Nineteen serum samples containing HBV/A were collected from native HBV carriers in various countries [Bangladesh, $n=4$; India, $n=2$; Japan, $n=3$; Nepal, $n=3$; The Philippines $n=4$; USA, $n=3$ (none of African ethnicity)]. Samples from India, Nepal and The Philippines were submitted by doctors who attended the Second Workshop on Hepatocellular Carcinoma in Asia held on 21 February 2002 in Tokyo by the Miyakawa Memorial Research Foundation. HBV genotypes were determined by ELISA with a commercial kit (HBV GENOTYPE EIA, Institute of Immunology Co., Ltd) involving monoclonal antibodies to type-specific epitopes in the preS2 region product (Kato *et al.*, 2002b; Usuda *et al.*, 1999, 2000) as well as by RFLP on the small-S gene sequence amplified by PCR with nested primers (Mizokami *et al.*, 1999). The entire nucleotide sequences of the HBV/A isolates in the 19 serum samples, which had been stored at −20 °C, were determined. The study protocol was approved by the Ethics Committees of the institutions, in accordance with the 1975 Declaration of Helsinki, and an informed consent was obtained from each HBV carrier.

Determination of the full-length sequence of HBV. Nucleic acids were extracted from serum (100 µl) using a DNA extractor kit (Genome Science Laboratory). HBV DNA fragments covering the entire genome sequence in 19 samples were amplified by the method reported previously (Sugauchi *et al.*, 2001). Amplified HBV DNA fragments were sequenced directly by the dideoxy method using a *Taq* Dye Deoxy Terminator cycle sequencing kit and a fluorescent 3100 DNA sequencer (Applied Biosystems).

Phylogenetic analysis. Complete genome sequences of 46 HBV isolates were aligned using the CLUSTAL W software program (Thompson *et al.*, 1994), and the alignment was confirmed by visual inspection. The genetic distances were calculated with the 6-parameter method (Saitou & Nei, 1987), and the phylogenetic tree was constructed by the neighbour-joining method using the ODEN program of the National Institutes of Genetics (Mishima, Japan) (Ina, 1994). To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times.

Statistical analyses. Frequencies between groups were compared by the chi-square test or by Fisher's exact test. Differences were considered significant for P values less than 0·05.

RESULTS

Phylogenetic relatedness and genetic diversity of the two subtypes of HBV/A

Complete nucleotide sequences of 19 HBV/A isolates were determined. Of the 19 HBV/A genomes, 18 had a genome length of 3221 bp and one (HBV-NEP40) possessed a deletion of 21 nt in the preS1 region, as did three HBV/A isolates retrieved from the DDBJ/EMBL/GenBank databases (accession nos AF297623, AF297625 and V00866). The insertion of 6 nt characteristic of genotype A was present in the core region in all 19 HBV/A isolates. Together with the 20 complete genome sequences of HBV/A isolates retrieved from the databases, the 19 determined in the present study were subjected to phylogenetic analysis along with seven HBV isolates representative of genotypes B, C, D, E, F, G and H (Fig. 1). Three recombinant HBV strains of genotypes A and D [AF297621 (Kramvis *et al.*, 2002); AF418674 and AF418682 (unpublished)], as well as a single recombinant strain of genotypes A and A′ (Bowyer *et al.*, 1997), were excluded from the phylogenetic analysis.

Of the 19 HBV/A isolates for which full-length sequences were determined in the present study, 13 including four from The Philippines, two from India, three from Nepal and four from Bangladesh were classified into subtype Aa and clustered with the HBV isolates from The Philippines, South Africa and Malawi retrieved from the databases; they differed from one another in $2{\cdot}5 \pm 0{\cdot}3$ % (range 1·1–4·6 %) of the entire genome sequence by pairwise comparison. The remaining six HBV/A isolates including three from the USA and three from Japan were classified into subtype Ae, and clustered with the 14 HBV isolates from Western countries

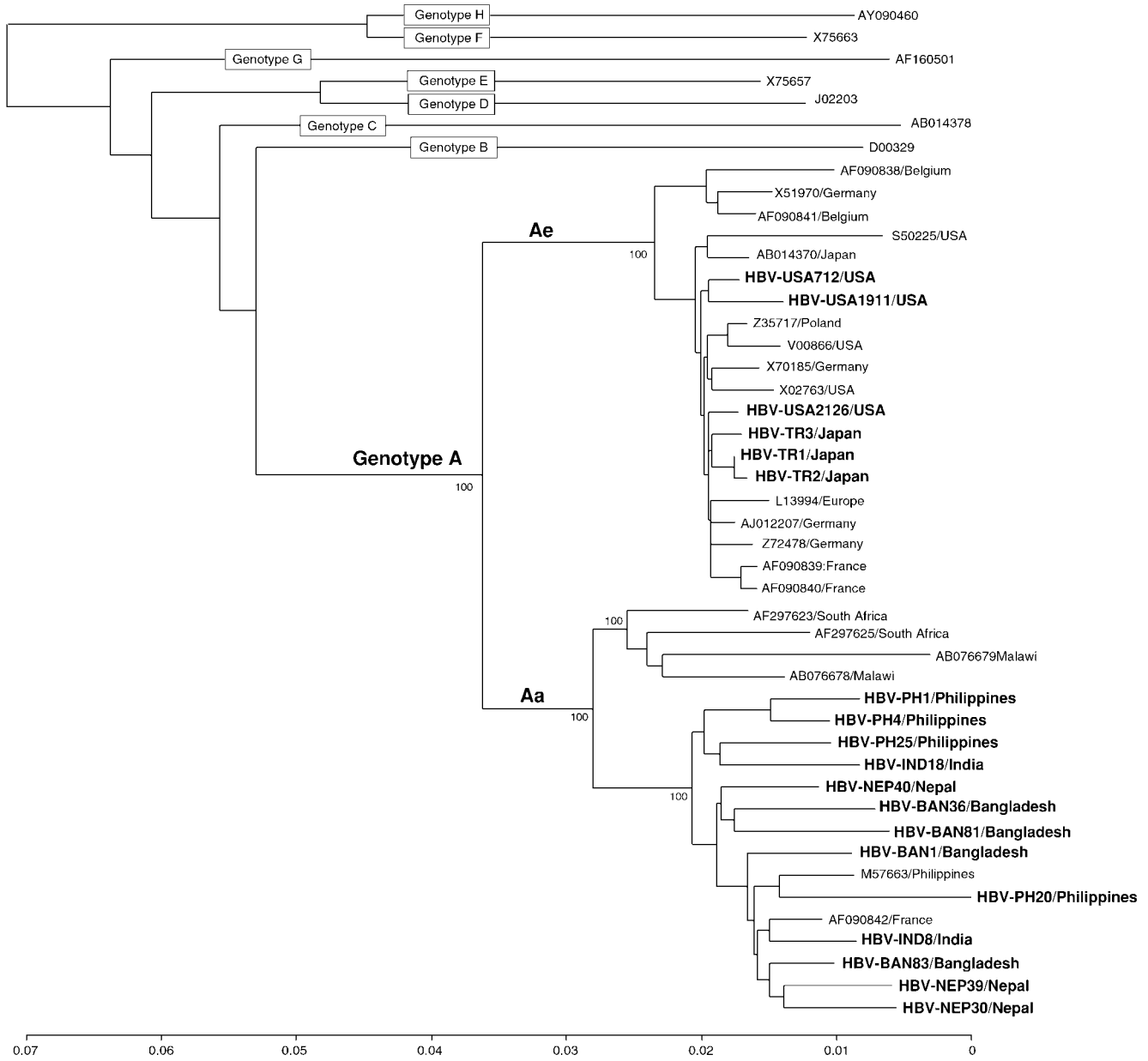

Fig. 1. Phylogenetic tree constructed using the entire nucleotide sequences of 46 HBV isolates. The 39 HBV/A isolates were compared with seven HBV isolates representing genotypes B–H. HBV/A isolates clustered on two branches, Ae (the original European genotype A) and Aa (the new African/Asian genotype A corresponding to A′ proposed by Bowyer *et al*., 1997). The 19 HBV/A isolates, the sequences of which were determined in this study, are shown in boldface; accession numbers are given for sequences of the other 27 HBV isolates. The country of origin is indicated after a solidus for each HBV/A isolate. Genetic distance is indicated below the tree. Bootstrap values are shown at the nodes of the main branches.

and Japan whose sequences were retrieved from the databases. These 20 HBV/Ae isolates had a sequence variation of $1{\cdot}1 \pm 0{\cdot}6$ %, which was significantly smaller than the $2{\cdot}5 \pm 0{\cdot}3$ % in the 19 HBV/Aa isolates ($P < 0{\cdot}0001$) (Table 1). The inter-group sequence divergence between the 20 and 19 isolates of genotypes Ae and Aa, respectively, was $5{\cdot}0 \pm 0{\cdot}4$ % (4·1–6·4 %) by pairwise comparison.

Phylogenetic analyses were performed on 19 HBV/Aa isolates and six HBV/Ae isolates, the sequences of which were determined in the present study and retrieved from DNA databases, within four reading frames, i.e. the preS1/preS2 region, the S gene, the X gene and the precore/core region (Fig. 2). A clear separation of subtype Ae from Aa is seen in the tree topology for the preS1/preS2 region, the X gene and

Table 1. Mean number of differences in nucleotide sequences of the entire genome and its reading frames within 20 HBV/Ae and 19 HBV/Aa isolates as well as between them

The mean ± SD values are shown with the ranges in parentheses.

Reading frames	Differences within Ae or Aa isolates (%)		Differences between Ae and Aa isolates (%)
	Ae	Aa	
Entire genome	1·1 ± 0·6 (0·1–3·6)	2·5 ± 0·3 (1·1–4·6)	5·0 ± 0·4 (4·1–6·4)
preS1/preS2	1·4 ± 0·2 (0–4·6)	3·3 ± 0·4 (0·8–6·9)	6·6 ± 0·9 (5·0–8·6)
S gene	0·5 ± 0·4 (0–2·1)	1·4 ± 0·6 (0·3–2·9)	1·9 ± 0·5 (0·9–3·7)
X gene	0·9 ± 0·2 (0–1·9)	2·4 ± 0·4 (0·4–4·1)	4·1 ± 0·7 (2·2–5·6)
Precore/core	1·3 ± 0·2 (0–5·4)	3·0 ± 0·3 (0·6–6·4)	4·6 ± 0·6 (2·8–7·9)
Polymerase gene	1·0 ± 0·1 (0·1–2·4)	2·5 ± 0·2 (1·2–4·3)	5·0 ± 0·4 (4·1–6·0)

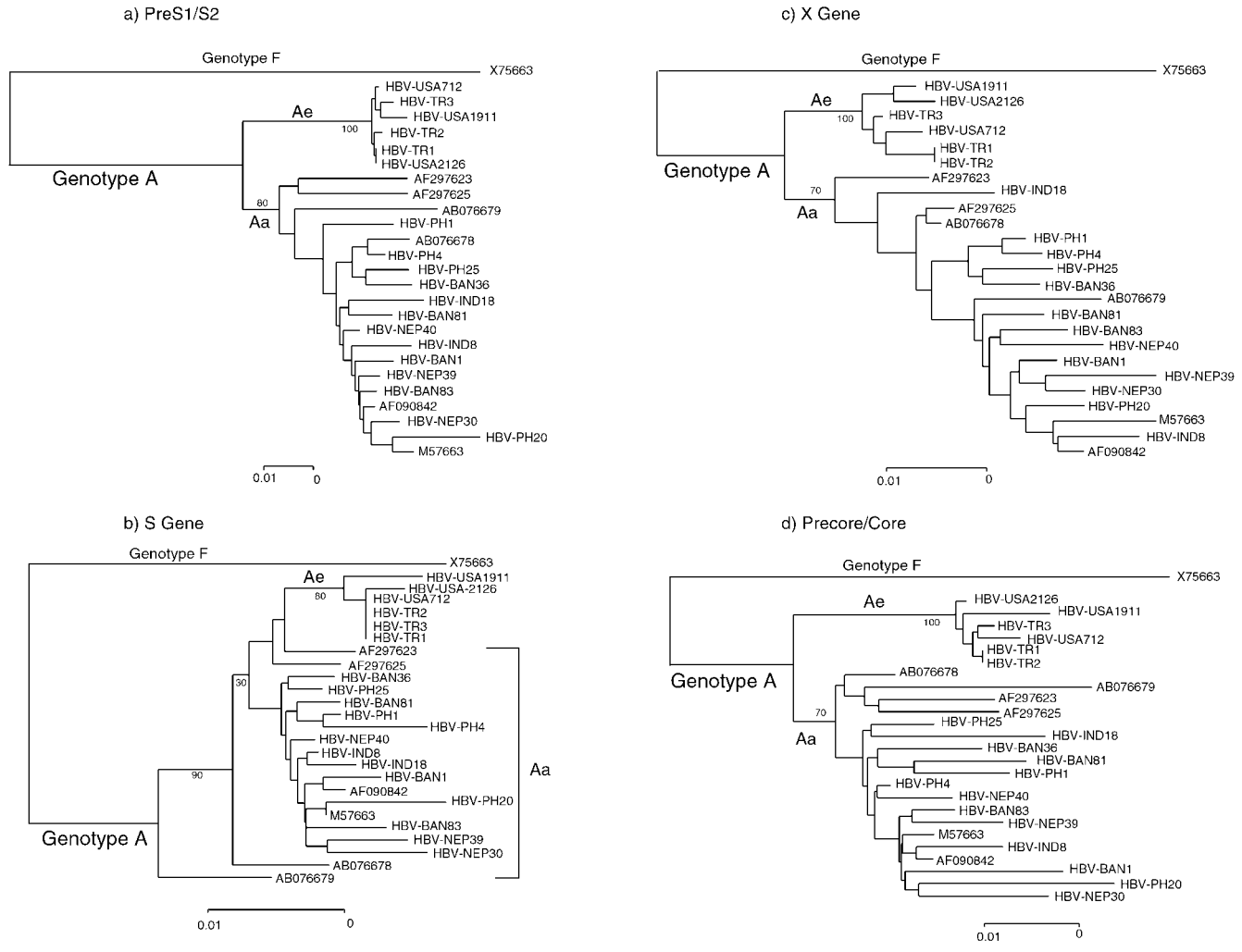

Fig. 2. Phylogenetic trees constructed using partial genome sequences of 25 HBV/A isolates. Nineteen full-length sequences were determined in the present study. Four trees representing (a) the preS1/preS2 region, (b) the S gene, (c) the X gene and (d) the precore region plus the core gene are shown with the sequence of genotype F serving as an outgroup. Genetic distance is indicated below each tree. Bootstrap values are shown at the nodes of the main branches.

the precore/core region. Phylogenetic trees constructed from S gene sequences, however, revealed no significant bootstrap values at the bifurcation of Ae and Aa.

With the availability of many sequences of the preS2 region and the S gene for genotype A isolates, 49 preS2/S sequences of genotype A were retrieved from the DNA databases, and a phylogenetic tree was constructed from them along with those of the 19 genotype A isolates sequenced in the present study (Fig. 3). Genotype A isolates from African countries (South Africa, Malawi and Zimbabwe) clustered with those from Asian countries (Bangladesh, India, The Philippines and Nepal) that were classified into subtype Aa, and they were separated from subtype Ae isolates from Western countries.

Pairwise genetic distances within 20 HBV/Ae and 19 HBV/Aa isolates, as well as between them, in the complete genome and each reading frame are shown in Table 1. The genetic divergence between HBV subtypes Ae and Aa was largest in the preS1/preS2 region amongst all the reading frames compared. Furthermore, HBV/Aa isolates had greater genetic divergence than HBV/Ae isolates in the complete genome as well as in all reading frames.

The serotype of hepatitis B surface antigen (HBsAg) was *adw* in all 20 HBV/Ae isolates and in 16 of the 19 HBV/Aa isolates. It was *ayw* in three HBV/Aa isolates including two (HBV-PH1 and HBV-PH4) from the present study and AB076678 from Malawi. Serotypes were deduced by codons 122 and 160 for either lysine or arginine (Okamoto *et al.*, 1987).

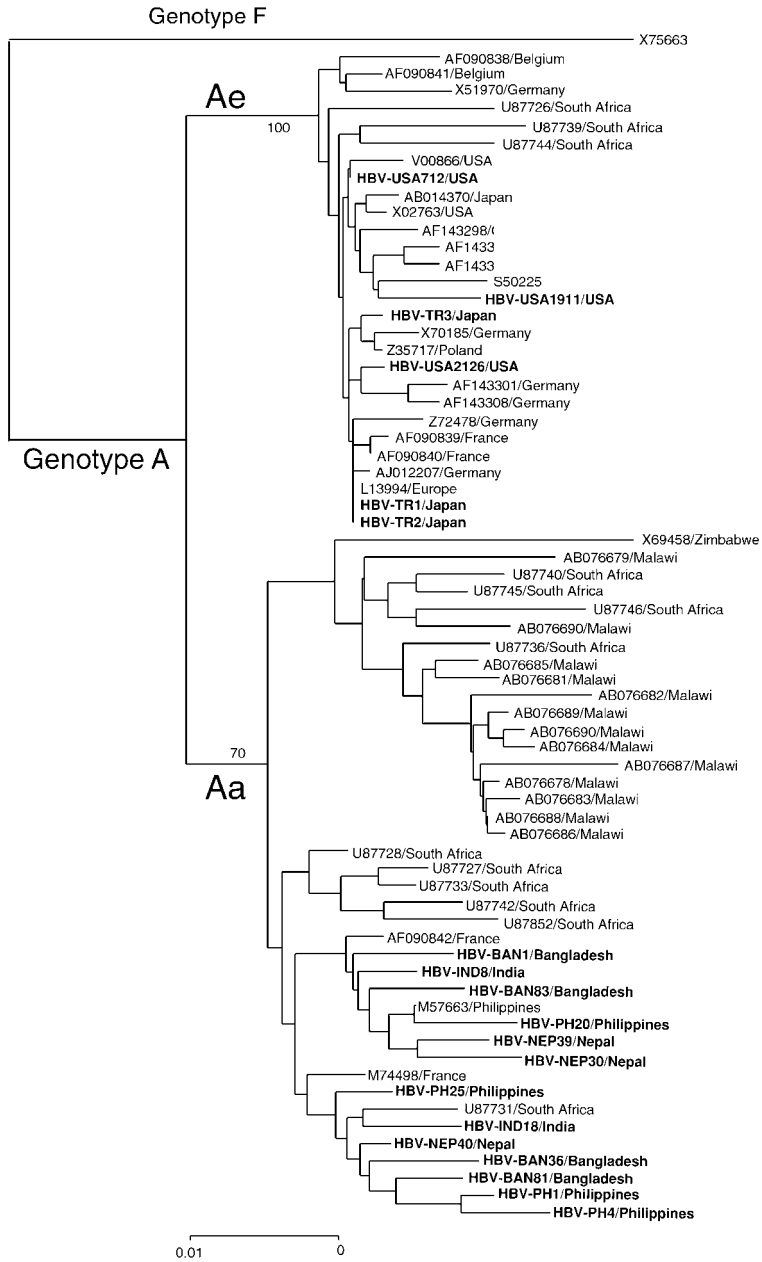

Fig. 3. Phylogenetic tree constructed using the preS2 and S gene sequences of 68 HBV/A isolates. They clustered on two separate branches, Ae (European genotype A) and Aa (African/Asian genotype A corresponding to A′ proposed by Bowyer *et al.*, 1997). The 19 HBV/A isolates, the sequences of which were determined in this study, are shown in boldface; accession numbers are given for sequences of the other 49 HBV isolates. The country of origin is indicated after a solidus for each HBV/A isolate. Genetic distance is indicated below the tree. Bootstrap values are shown at the nodes of the main branches.

Comparison of nucleotide sequences in the basic core promoter and precore region between HBV/Ae and HBV/Aa isolates

The double mutation (T1762/A1764) was significantly more frequent in HBV/Aa than in HBV/Ae isolates (11/19 or 58 % vs 5/20 or 25 %, $P<0{\cdot}01$). Point mutations for T1809 and T1812, which were not known in HBV isolates of genotypes other than A, were found frequently in HBV/Aa isolates (18/19 or 95 % and 16/19 or 84 %, respectively). Sequences of the pregenome encapsidation (ε) signal in the precore region are compared between HBV/Ae and HBV/Aa isolates in Fig. 4. Remarkably, the point mutation from G to A or T at nt 1862 and that from G to A, C or T at nt 1888 occurred frequently in HBV/Aa isolates (16/19 or 84 % and 17/19 or 89 %, respectively); these point mutations were seen only in HBV/Aa isolates. The precore stop mutation (A1896), accompanied by a C-to-T mutation at nt 1858 making a pair with it, was found in a single HBV/Ae isolate from Europe (accession no. AF090838).

DISCUSSION

Genotype A is different from the other genotypes of HBV in that it has a C at nt 1858 that prohibits the G-to-A point mutation at nt 1896 for creating a stop codon in the precore region (Li *et al.*, 1993); C1858 and T1896 make a pair in the lower stem of the pregenome encapsidation (ε) signal. The G1896A mutation prohibits the translation of the HBeAg precursor (Carman *et al.*, 1989; Okamoto *et al.*, 1990) and, by doing so, prevents the seroconversion from HBeAg to the corresponding antibody. Despite the presence of C1858, individuals infected with HBV/A in Africa seroconvert to anti-HBe very frequently, and only 5 % of them possess HBeAg in their serum when they reach adulthood (Dusheiko *et al.*, 1985). Furthermore, HBV/A strains in Africa seem to be different from those in Western countries in that they induce hepatocellular carcinoma very often (Attia, 1998; Olweny, 1984). HBV accounts for most cases of hepatocellular carcinoma in Africa, but only for 15–20 % of cases in the USA where HBV/A is prevalent (Di Bisceglie *et al.*, 1998).

Bowyer *et al.* (1997) reported a subgroup of HBV/A isolates from Africa which clustered on a branch separate from that harbouring isolates from Europe and the USA, based on a phylogenetic analysis of preS2/S sequences. They designated genotype A prevalent in Africa as A′ to distinguish it from the original genotype A. Differences between A and A′ have been corroborated by comparison of the entire genomic sequences (Kramvis *et al.*, 2002). These two subgroups of genotype A are also serologically different. The African genotype A′ encodes HBsAg of serotype *adw* or *ayw*, unlike the original genotype A that encodes HBsAg of serotype *adw* (Bowyer *et al.*, 1997; Sugauchi *et al.*, 2003b). Although A and A′ belong to the same genotype, they seem to be very different epidemiologically and in their capacity to encode HBeAg and induce hepatocellular carcinoma.

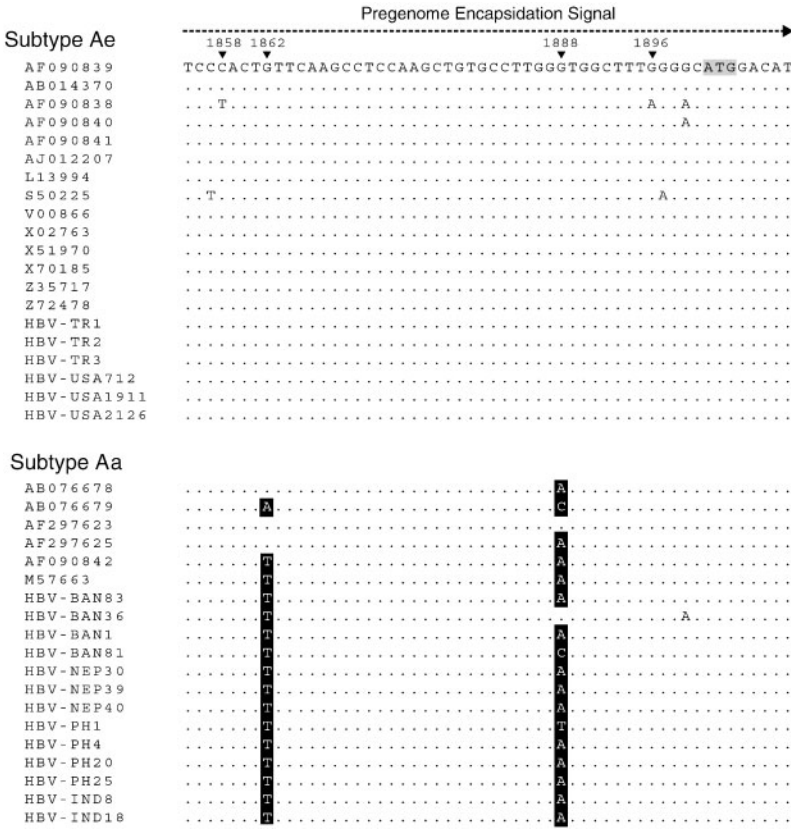

Fig. 4. Nucleotide sequences of the pregenome encapsidation (ε) signal in HBV/Ae and HBV/Aa isolates. Positions of C1858 making a pair with G1896 and those of the subtype-specific mutations at nt 1862 and nt 1888 are indicated by ▼. The start codon in the C gene is highlighted.

In the present study, 19 HBV/A isolates from various countries in Asia and from the USA were sequenced in full-length and, along with 20 sequences retrieved from the DDBJ/EMBL/GenBank databases, were examined phylogenetically. The 39 HBV/A isolates clustered on two separate branches for A and A′, as Bowyer *et al.* (1997) observed in their analysis of partial genomic sequences. It came as a surprise that the three HBV/A isolates from Japan were of subtype Ae, confirming previous reports. Hence, most HBV/Ae infections in Japan would have been imported from Western countries. They are definitely different, however, from HBV/Aa infections prevailing in other Asian countries, albeit subtype Aa was found in a minor population of HBV/A isolates from Japan in this study and is reported (accession no. AB014370) (Takahashi *et al.*, 1998).

Amino acids specific to subtype A′ isolates from South Africa clustering in the preS region and the P gene have been reported (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002). In the present study, also, there were amino acids in the preS region and the P gene that were unique to subtype Aa isolates from Asian countries. They were not found in other genotypes of HBV (Ae and B–H), while some of them were shared by subtype Aa isolates from African countries (54Q, 74V, 86A and 91V in the preS1 region; 32L in the preS2 region; 91A, 236T, 256C and 268G in the P gene).

Recombination was not responsible for differences between subtypes Ae and Aa, because recombinants of genotype A with another genotype (Owiredu *et al.*, 2001) were excluded from our phylogenetic analyses of the 39 HBV/A isolates. The comparison of subtype Aa with Ae revealed many differences, some of which have been described previously, while others have not. Sequence variation greater in HBV/Aa than HBV/Ae isolates reported in the preS/S gene (Bowyer *et al.*, 1997) was confirmed and extended to the entire genomic sequence as well as to the other reading frames (Table 1). The divergence in the preS1/preS2 sequence was found to be greatest between HBV/Aa and HBV/Ae isolates.

Probably of the most important virological relevance, nt 1862 was invariably G in the 20 HBV/Ae isolates, while it was frequently found to be T in the HBV/Aa isolates and was detected in 15/19 (79 %) of them; A at this position was found in a single (5 %) HBV/Aa isolate. Furthermore, nt 1888 was exclusively G in HBV/Ae isolates, but it was replaced by A ($n=14$), C ($n=2$) or T ($n=1$) in 17/19 (89 %) HBV/Aa isolates. These two nucleotides are positioned in the 6 nt bulge and upper stem, respectively, that make essential elements in the pregenome encapsidation (ε) signal (Fig. 5). Nt 1862 is a G in wild-type HBV and occupies the third position in the 6 nt bulge. The conversion of G1862 to any of the other three nucleotides does not interfere with the encapsidation of pregenomic RNA (Rieger & Nassal, 1995), but it does seem to affect the replication of HBV (Nassal & Rieger, 1996). How G1862 in HBV/Aa isolates is involved in hepatocarcinogenesis in Africa, where these isolates are prevalent, is a matter of clinical concern (Kramvis *et al.*, 1998).

It has been proposed that the G-to-T missense mutation at nt 1862 would interfere with the processing of the HBeAg precursor by its position close to the cleavage site of signal peptidase (Kramvis *et al.*, 1997). It creates phenylalanine two positions upstream of the signalase cleavage site in the amino acid sequence of the precore region, which makes it difficult for signalases to act properly (Kramvis *et al.*, 1997), and has been shown to reduce the production of HBeAg by *in vitro* transfection studies (Hou *et al.*, 2002). This could be a reason for suppressed production of HBeAg in African individuals infected with HBV/Aa (Dusheiko *et al.*, 1985), and might be implicated in fulminant hepatitis B in Chinese patients who were infected with HBV/B with G1862T in the absence of any mutations that abrogate or down-regulate the production of HBeAg (Hou *et al.*, 2002). Fulminant hepatitis B is caused by HBV variants with mutations in the precore region or core promoter that abort or reduce the synthesis of HBeAg (Kosaka *et al.*, 1991; Liang *et al.*, 1991; Omata *et al.*, 1991; Sato *et al.*, 1995).

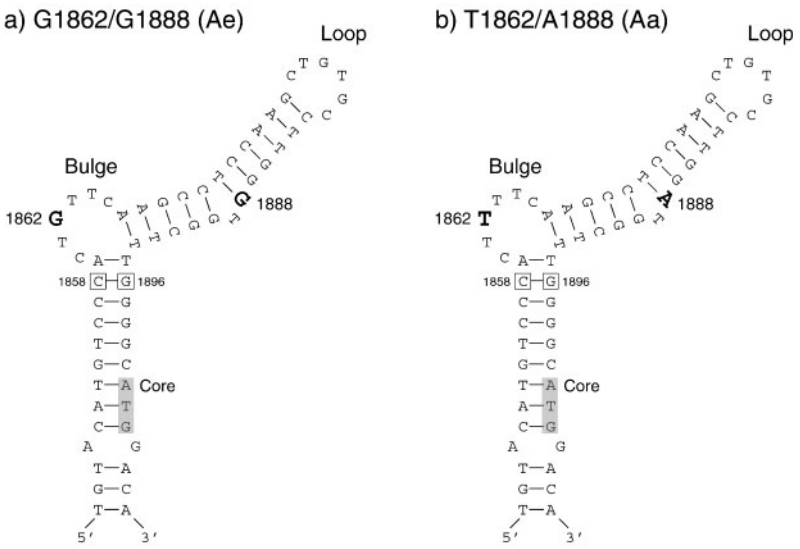

Fig. 5. Conformation of the pregenome encapsidation (ε) signal for HBV genomes of subtypes Ae and Aa. All 19 isolates of subtype Ae possess G1862 and G1888 (a), in contrast to T1862 and A1888 in 15 (79 %) and 14 (74 %), respectively, of the 19 HBV/Aa isolates (b). A Watson–Crick pair between C1858 and G1896, which is characteristic of genotype A (Li *et al.*, 1993), is boxed and the initiation codon of the C gene is shaded.

Although the G1862T mutation was prevalent and detected in 15/19 (79 %) HBV/Aa isolates for which the full-length sequences were known, in remarkable contrast to 0/20 HBV/Ae isolates, it was not exclusive to subtype Aa of genotype A. T1862 is present in 7/27 (26 %) HBV/B isolates from patients with fulminant hepatitis in China (Hou *et al.*, 2002), as well as in two isolates of HBV genotype C [accession nos D23683 (Horikita *et al.*, 1994) and X85262 from Italy]. Moreover, it was detected in the full-length sequences of HBV isolates from gibbons [accession nos AJ131574 (Grethe *et al.*, 2000) and AY077735 (Noppornpanth *et al.*, 2003)]. In addition, G1862 has been documented in many HBV isolates of unspecified genotypes from patients with chronic hepatitis (Carman *et al.*, 1995; Horikita *et al.*, 1994; Kramvis *et al.*, 1997; Loriot *et al.*, 1995; Santantonio *et al.*, 1991; Tran *et al.*, 1991; Valliammai *et al.*, 1995), fulminant hepatitis (Hou *et al.*, 2002; Laskus *et al.*, 1993) and hepatocellular carcinoma (Kramvis *et al.*, 1998). The prevalence of G1862T in African HBV/Aa isolates needs to be surveyed on a large scale; however, in the four African HBV/Aa isolates whose full-length sequences are available, nt 1862 is G in three and A in one (accession no. AB076679).

In conclusion, a comparison of 20 HBV/Ae and 19 HBV/Aa isolates over their entire genomic sequences has disclosed many previously reported and unknown differences between them. Inasmuch as these differences may affect the replication of HBV as well as the translation of HBeAg, and can modify the clinical courses of acute and chronic infections, the prevalence of HBV/Ae and HBV/Aa would need to be determined in a number of epidemiological and clinical settings. The classification of genotype A into Ae and Aa subtypes would be more appropriate than the A/A′ grouping in which A′ tends to sound subordinate to A. It may turn out that isolates of A′ are more frequent than those of A on a worldwide basis and that they are also much older phylogenetically. This view would be supported by sequence variation in the entire genome significantly wider in HBV/Aa than HBV/Ae isolates.

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