Molecular characterization of occult hepatitis B virus in genotype E-infected subjects

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Occult hepatitis B virus (HBV) infection (OBI), defined as the presence of HBV DNA without detectable HBV surface antigen (HBsAg), is frequent in west Africa, where genotype E is prevalent. The prevalence of OBI in 804 blood donors and 1368 pregnant women was 1.7 and 1.5 %, respectively. Nine of 32 OBI carriers were evaluated with HBV serology, viral load and complete HBV genome sequence of two to five clones. All samples except one were anti-HBV core antigen-positive and three contained antibodies against HBsAg (anti-HBs). All strains were of genotype E and formed quasispecies with 0.20–1.28 % intra-sample sequence variation. Few uncommon mutations (absent in 23 genotype E reference sequences) were found across the entire genome. Two mutations in the core region encoded truncated or abnormal capsid protein, potentially affecting viral production, but were probably rescued by non-mutated variants, as found in one clone. No evidence of escape mutants was found in anti-HBs-carrying samples, as the ‘a’ region was consistently wild type. OBI carriers constitute approximately 10 % of all HBV DNA-viraemic adult Ghanaians. OBI carriers appear as a disparate group, with a very low viral load in common, but multiple origins reflecting decades of natural evolution in an area essentially devoid of human intervention.

INTRODUCTION

Hepatitis B virus (HBV) chronically infects >15 % of the Ghanaian population; by age 40, virtually 100 % of the population has been in contact with HBV and carries antibodies against the HBV core antigen (anti-HBc) (Allain et al., 2003). By age 16, 75 % of the population is anti-HBc-positive and the majority of the 15 % of chronic carriers have already developed anti-HBe [antibodies against the HBV ‘e’ antigen (HBeAg)] and carry a relatively low viral load (Candotti et al., 2006). It is therefore unsurprising that approximately one in 60 Ghanaian blood donors have been identified as carriers of ‘occult’ HBV infection (OBI), defined as the presence of HBV DNA without detectable HBV surface antigen (HBsAg) (Owusu-Ofori et al., 2005).

Previous reports have shown that, during the late stages of the natural history of the infection, OBI in Ghana is characterized by a very low viral load and the presence either of both anti-HBc and antibodies against HBsAg (anti-HBs), or of anti-HBc without anti-HBs, although anti-HBe may or may not be detectable in either of these two categories of infected individual (Candotti et al., 2006). The presence of anti-HBs suggests recovery from an acute infection, although the simultaneous presence of HBV DNA suggests persistence of the virus, as described previously (Rehermann et al., 1996; Yotsuyanagi et al., 1998). In contrast, individuals carrying anti-HBc only can be either chronic carriers at the tail end of carriage, where HBsAg levels are below the limit of sensitivity of screening assays, or individuals who have recovered from the infection, but no longer carry detectable anti-HBs, an antibody with considerable shorter longevity than anti-HBc (Allain, 2004). In this article, full-genome sequences...
of nine strains of occult genotype E HBV from Ghana have been studied in order to assess whether specific viral mutations could characterize OBI and explain the low levels of viral replication. The disparity and variety of mutations found suggest multiple origins and mechanisms responsible for this condition.

METHODS

Samples. Human immunodeficiency virus (HIV)- and hepatitis C virus (HCV)-negative plasma samples from 1368 pregnant women (labelled PW) and 804 blood donors (labelled BD) were collected and screened for HBsAg by a rapid test (Determine HBsAg/AgHBs; Abbott Laboratories) at the Komfo Anokye Teaching Hospital in Kumasi, Ghana. Samples that tested negative for HBsAg were stored at −20 °C or below until used. Sample G2451, from an HBsAg-positive blood donor with high HBV DNA load (5 × 10⁸ IU ml⁻¹), was used as a control. The study was approved by the University of Science and Technology Ethics Committee, Kumasi, Ghana. Informed consent was obtained from PW and BD, who presented with no clinical symptoms suggestive of HBV-related illness.

Serological testing. The presence of HBsAg was tested further by using Murex HBsAg version 3 (Murex Biotech) or Hepanostika HBsAg Ultra (bioMérieux). HBeAg and anti-HBe were detected with Murex HBeAg-anti-HBe (Murex Biotech). Anti-Hbc and anti-HBs were detected with Wellcozyme anti-HBe and Murex anti-HBs (Murex Biotech) enzyme immunoassays (ELAs), respectively. Both assays were performed according to the manufacturers’ instructions.

HBV DNA purification and quantification. HBV DNA was isolated from 200 μl plasma by using a High Pure Viral Nucleic Acid kit (Roche Diagnostics), according to the manufacturer’s instructions. HBV DNA was quantified by using the Mx3000P Multiplex Quantitative PCR system (Stratagene). The probe BS-1 and the primers HBV-Taq1 and HBV-Taq2 were designed from the sequences of the conserved regions of the HBV surface gene (Weinberger et al., 2000). The fluorogenic probe was 5’-labelled with Cy5 dye and 3’-labelled with Black Hole Quencher 2 (Biosearch Technologies). Amplification was performed with a Brilliant Quantitative PCR Core Kit (Stratagene). A quantitative PCR contained 1 × Core PCR buffer, 5 mM MgCl₂, 0.8 mM each dNTP, 1.8 μM each primer, 0.2 μM fluorogenic probe, 3 × 10⁻⁴ mM reference dye (carboxy-X-rhodamine), 2.5 U SureStart Taq polymerase (Stratagene) and 10 μl template DNA preparation per 50 μl reaction. After an initial incubation at 95 °C for 10 min, 50 two-step cycles of 1 min at 60 °C and 30 s at 95 °C were carried out. For each run, duplicates of a tenfold serial dilution of WHO International Standard for HBV DNA for NAT assays 97/746 (National Institute for Biological Standards and Control, Potters Bar, UK), containing 0.16–1600 IU HBV genome per reaction, were analysed. Based on the mean number of threshold cycles (Ct) for each dilution, a linear regression was constructed, with Ct as a function of the log of the number of template molecules per reaction. By using this regression analysis, the number of template molecules per reaction was calculated from the mean of duplicates for each plasma sample (Allain et al., 2003).

Amplification of nearly full-length HBV genome. The full-length HBV genome was amplified with a nested PCR using the Expand High Fidelity PCR system (Roche). Outer primers were P1WRS (5’-TTTTTCACCTCTGCCTAATCA-3’; forward) and P5W (5’-AAAAAGTTGGCATGRTMTGG-3’; reverse), and inner primers were P3WRS (5’-CTACTGTTCAGCCCTGAAAGC-3’; forward) and P4WRS (5’-CGCAGACCATTTATGCGCTAC-3’; reverse). First-round amplification was performed in a volume of 50 μl, containing 1 × Expand High Fidelity buffer, 2.25 mM MgCl₂, 0.2 mM dNTPs, 0.2 μM each primer, 5.25 U Expand High Fidelity Enzyme mix and 10 μl DNA. After an initial denaturation step at 95 °C for 3 min, 40 amplification cycles were performed with denaturation at 94 °C for 40 s, annealing at 50 °C for 35 s and elongation at 68 °C for 3.5 min, with an increment of 1.5 min after each 10 cycles. The second PCR round was performed by using the same conditions, except that 2.5 U enzyme mix was used, annealing was done at 55 °C and 6 μl of the first-round product was loaded in the second round. When the HBV DNA load was ≤20 IU ml⁻¹ (sample BD2), amplification was performed by using 10 μl DNA obtained after ultracentrifugation of 10 ml plasma using an Optima L-90K ultracentrifuge and an SW41Ti rotor (Beckman Instruments) (Roseman et al., 2005). Plasma was centrifuged at 39,000 r.p.m. for 3 h, pellets were resuspended in 200 μl of water and nucleic acid was purified as described above.

The 276 bp basic promoter/pre-core (BCP/PC) region, including 50 bp separating the binding site of the primers used in the full-length PCR, was amplified by using hemi-nested PCR as described previously (Candotti et al., 2006).

Cloning and sequencing of the amplified HBV genome. Full-length and BCP/PC amplicons were gel-purified by using a QIAEX II Gel Extraction kit (Qiagen) and cloned by using a TOPO TA Cloning kit (Invitrogen), following the manufacturers’ instructions. Plasmid DNA was purified by using a QIAprep spin miniprep kit (Qiagen), and full-length HBV DNA inserts were identified by restriction-enzyme digestion with EcoRI. For each sample, two to five clones were sequenced by using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit, version 1.0, and an ABI Prism 3100 Genetic Analyzer (both from Applied Biosystems), according to the manufacturer’s instructions, with a set of primers encompassing the whole HBV genome plus universal M13 primers. HBV primers are detailed in Supplementary Table S1, available in JGV Online.

Sequence analysis. Electrophoregrams were analysed with the SeqMan II program from the Lasergene package (DNASTAR Inc.) and checked manually to confirm base assignment. The consensus sequences of the full-length and BCP/PC regions were obtained from an alignment of the sequences of two to five clones by using the MacVector program version 7.2, with the HBV EcoRI site taken as nucleotide position 1.

OBI sample sequences were aligned with 24 full-length HBV genotype E sequences obtained previously from HBsAg-positive infected individuals and from the control G2451 included in this study (GenBank accession numbers: AB032431, AB091255, AB194947, AB194948, AB091256, AB106564, AB205188–AB205192, AJ739675, DQ060822–DQ060830, X75657 and X75664). Amino acid substitutions in OBI sample sequences that were not present in any of the reference isolates were designated uncommon mutations. The BCP/PC sequences were compared with 96 previously characterized partial sequences from HBsAg-positive Ghanaian blood donors infected with HBV genotype E (Candotti et al., 2006).

Phylogenetic analysis was performed with PAUP* by the neighbour-joining algorithm based on Kimura two-parameter distance estimation (Swofford & Sullivan, 2003). Diversity was defined as the mean value for pairwise distance between sequences within the same group, calculated as the number of nucleotide differences between two individual sequences, corrected for sequence length. The ratio (dₙ/dₛ) of synonymous (dₙ) to non-synonymous (dₛ) substitutions was estimated by using the Nei–Gojobori method (http://www.hiv.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html).
RESULTS

Virological and serological data

Individual plasma samples collected from 1368 PW and 804 BD in Kumasi, Ghana, were screened for HBsAg and HBV DNA (Allain et al., 2003; Owusu-Ofori et al., 2005). Twenty (1.5%) and 12 (1.7%) of these PW and BD samples, respectively, were HBsAg non-reactive/confirmed HBV DNA-positive, and were categorized as carrying OBI. A 3160 bp region, including the nearly full-length HBV genome and the corresponding BCP/PC region, was amplified successfully and cloned from seven PW and two BD samples. HBV DNA load ranged from <20 to 2030 IU ml\(^{-1}\) (Table 1). For sample BD2 (<20 IU ml\(^{-1}\)), full-length amplicons were obtained after ultracentrifugation of 10 ml plasma. Full-length HBV genome amplification failure in 23 OBI samples was essentially related to extremely low viral load (<20 IU ml\(^{-1}\)) and lack of volume to perform ultracentrifugation.

Serological investigations were performed on the fully sequenced OBI samples (Table 1). All were confirmed as being HBsAg-negative by EIA. Anti-HBc was detectable in all samples except for PW7. Three samples (PW1, PW2 and PW4) contained anti-HBs, suggesting recovery from infection. The other five samples suggested either chronic or recovered infection, with no-longer-detectable HBsAg or anti-HBs, respectively. PW1, PW2 and BD2 were HBeAg-reactive, PW4 and PW5 contained anti-HBe, and PW3 was negative for both markers. In sample PW7, HBV DNA was the only detectable marker of HBV infection. None of the subjects had been vaccinated against HBV or had received antiviral treatment, and all were clinically asymptomatic.

Sequence analysis

The efficiency of full-genome amplicon cloning was limited by the size of the amplicons, and most clones contained a truncated insert. Only two, four and five plasmids containing the intact 3160 bp HBV insert were obtained for samples PW7 and BD1, samples PW1 and BD2, and samples PW2, PW3, PW4 and PW5, respectively (Table 2). All OBI sample sequences were of genotype E (100% bootstrap value over 1000 replicates) (Fig. 1). Neither nucleotide nor amino acid OBI sample sequences for each open reading frame (ORF) showed any evidence of clustering; they were distributed among sequences derived from HBsAg-positive subjects infected with HBV genotype E (Fig. 1; data not shown). Clone sequences from sample PW7 were identical, in contrast to the other eight samples, which showed quasispecies variation. Variants of each quasispecies were clearly related to each other and formed separate, distinct clusters (Fig. 1). In addition, two distinct subclusters were observed among PW5 clones and were supported by bootstrap values of 100% (PW5-cl4 and PW5-cl7) and 69% (PW5-cl1, PW5-cl2 and PW5-cl11) (Fig. 1). This suggested that, following initial infection, two related HBV lineages diverged and evolved separately over time in carrier PW5. This hypothesis is supported by the fact that this woman was considerably older (44 years) than the other individuals with OBI (Table 1) and did not carry anti-HBs.

The intra-quasispecies diversity over the entire genome except for the BCP/PC region ranged from 0.2 to 1.28%, and consisted of rare single nucleotide polymorphisms (Table 2). Overall data for the core, polymerase, pre-S/S and X regions mainly showed \(d_S/d_Q\) ratios >1, indicative of sequence stability or negative selection. The lowest and highest \(d_S/d_Q\) ratios were generally observed within the polymerase and the core coding regions, respectively. Ratios <1, indicating positive selection, were observed for the core (0.84) and polymerase (0.94) coding regions from BD2 and PW3.

Intra-sample amino acid diversity was statistically significantly higher in the polymerase than in the core protein (\(P=0.046\)) and the S antigen (\(P=0.03\)) (Spearman rank

Table 1. HBV serology and viral load of subjects with OBI

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex/age (years)</th>
<th>Viral load (IU ml(^{-1}))</th>
<th>Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HBsAg</td>
</tr>
<tr>
<td>PW1</td>
<td>F/25</td>
<td>2030</td>
<td>−</td>
</tr>
<tr>
<td>PW2</td>
<td>F/31</td>
<td>21</td>
<td>−</td>
</tr>
<tr>
<td>PW3</td>
<td>F/29</td>
<td>32</td>
<td>−</td>
</tr>
<tr>
<td>PW4</td>
<td>F/26</td>
<td>48</td>
<td>−</td>
</tr>
<tr>
<td>PW5</td>
<td>F/44</td>
<td>32</td>
<td>−</td>
</tr>
<tr>
<td>PW6</td>
<td>F/20</td>
<td>94</td>
<td>−</td>
</tr>
<tr>
<td>PW7</td>
<td>F/18</td>
<td>78</td>
<td>−</td>
</tr>
<tr>
<td>BD1</td>
<td>M/18</td>
<td>117</td>
<td>−</td>
</tr>
<tr>
<td>BD2</td>
<td>M/35</td>
<td>15–20</td>
<td>−</td>
</tr>
</tbody>
</table>
correlation test). No significant difference was observed between core and S antigens (P=0.17) (Table 2). In addition, several clones from the majority of the infected individuals showed major mutations, resulting in potentially defective viruses (not shown). PW3-cl4 showed the insertion of a single guanosine (position 578 in the polymerase ORF), introducing a frame shift that abolishes translation of the polymerase and the long S antigen. PW4-cl10 showed one nucleotide deletion in the core ORF (position 287) and a stop codon mutation in the N-terminal part of the long S antigen (aa 5). The insertion of 1 nt in the overlapping Pol/S genes of PW5-cl1 (position 452 in the polymerase ORF) abolished translation of the polymerase and the long S antigen. PW6-cl6 showed a stop-codon mutation (position 102) in the core antigen. The two clones from PW7 had one adenosine insertion (position 225), disrupting the core ORF, and a 3 nt deletion at positions 53–55 (core ORF). Finally, BD1-cl21 and BD2-cl13 showed deletion of a single cytosine (position 616) and guanosine (position 222) in the Pol gene, respectively.

A nucleotide consensus sequence was deduced for each sample by aligning the corresponding clone sequences to be representative of the most frequent nucleotide found at each position. Each consensus sequence was combined with the corresponding BCP/PC sequence obtained by direct sequencing in order to generate a 3212 bp consensus HBV genome sequence for each OBI sample. The consensus nucleotide sequences and derived amino acid sequences from the nine OBI samples were compared with 24 full-genome reference sequences of HBV genotype E strains obtained from HBsAg-positive individuals.

**Table 2.** Intra-species genetic diversity and \(d_{\text{NS}}/d_S\) ratio for coding regions of the main genes

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. clones sequenced</th>
<th>Mean (range) genetic diversity* (%)</th>
<th>Mean (d_{\text{NS}}/d_S) ratio</th>
<th>Mean (range) amino acid diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Core</td>
<td>Pol</td>
<td>PreS/S</td>
</tr>
<tr>
<td>PW1</td>
<td>4</td>
<td>0.58 (0.41–0.76)</td>
<td>2.71</td>
<td>2.17</td>
</tr>
<tr>
<td>PW2</td>
<td>5</td>
<td>0.27 (0.13–0.48)</td>
<td>4.21</td>
<td>1.36</td>
</tr>
<tr>
<td>PW3</td>
<td>5</td>
<td>0.38 (0.25–0.54)</td>
<td>4.12</td>
<td>0.94</td>
</tr>
<tr>
<td>PW4</td>
<td>5</td>
<td>0.37 (0.0–0.63)</td>
<td>4.31</td>
<td>1.45</td>
</tr>
<tr>
<td>PW5</td>
<td>5</td>
<td>1.28 (0.0–2.28)</td>
<td>3.09</td>
<td>1.36</td>
</tr>
<tr>
<td>PW6</td>
<td>2</td>
<td>0.03</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PW7</td>
<td>2</td>
<td>0.00</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>BD1</td>
<td>2</td>
<td>0.40</td>
<td>NA</td>
<td>3.82</td>
</tr>
<tr>
<td>BD2</td>
<td>4</td>
<td>0.32 (0.16–0.47)</td>
<td>0.84</td>
<td>5.41</td>
</tr>
</tbody>
</table>

*Intra-species genetic diversity was calculated as the number of nucleotide substitution differences between sequences corrected for sequence length (3160 bp).
†NA, Not available. \(d_{\text{NS}}/d_S\) was not calculated if either \(d_S\) or \(d_{\text{NS}}\) equalled zero.

Mutations in viral regulatory elements

Mutations observed in the regulatory elements of OBI consensus sequences are summarized in Table 3. There were no deletions or insertions in promoter or enhancer regions. Only a few uncommon scattered point mutations were observed and were generally simultaneously present in two or three strains (Table 3). All identified important regulatory elements were wild type. Samples PW2, PW4 and PW7 showed the \(\text{A}^{1762\text{T}}\text{/}^{1764\text{A}}\) BCP double mutation (Table 3) in all sequenced clones. Mutation \(\text{A}^{1757\text{G}}\) was observed in samples PW2, PW5, PW7 and BD1. The Kozak initiation sequence of pre-core was consistently wild type (AGCAAC).

Analysis of the pre-core and core regions

The pre-core region of HBV strains from OBI or HBsAg-positive samples presented similar features. Pre-core stop codon 28 mutant (\(\text{G}^{1896\text{A}}\)) was observed in samples PW2, PW5 and BD1 (Table 3) and was associated with the \(\text{A}^{1762\text{T}}\text{/}^{1764\text{A}}\) double mutation in samples PW2 and PW5. However, \(\text{A}^{1762\text{T}}\text{/}^{1764\text{A}}\) and \(\text{G}^{1896\text{A}}\) were associated with detectable HBeAg and anti-HBe in samples PW2 and PW5, respectively (Tables 2 and 3). In addition, the wild-type pre-core sequence was not associated with detectable HBeAg in samples PW3 and PW4, but was detected in samples PW1 and BD2.

The core proteins of samples PW1 and PW3 were wild type, whilst samples PW2, PW4 and BD1 showed a few uncommon mutations (Fig. 2). Anti-HBc-positive sample PW5 had more mutations. By using different predictive methods, these changes did not appear to affect the hydrophobicity or antigenicity profile of the PW5 protein significantly (data not shown). No mutations were found in the arginine-rich motif (Hatton et al., 1992; Nassal, 1992) or in any of the SPRRR motifs (Liao & Ou, 1995) of the C-terminal domain of the core protein of OBI strains. In the case of sample PW6, one clone was wild type for the core protein, but the second one contained a stop codon at position 102, resulting in an 82 aa-truncated core protein.
(Fig. 2). Finally, anti-HBc-negative sample PW7 showed a single-base insertion (adenosine) at position 2125, causing a frame shift that completely altered the 18 aa after position 74, with a stop codon appearing at position 92 (Fig. 2). This single-base insertion was present in both sequenced clones.

**Analysis of the predicted surface antigens and polymerase**

Between none and three uncommon mutations per OBI strain were observed in the pre-S1 unique region (Fig. 2), although four of them $^{R34K}$ (PW4 and PW3), $^{R38K}$ (PW4) and $^{H44N}$ (PW5) were located in the aa 21–47 region, which is involved in hepatocyte attachment (Neurath et al., 1986; Petit et al., 1991; Pontisso et al., 1989). Within the pre-S2 unique protein, sample PW5 had a mutated initiation codon in all clones, affecting the synthesis of the middle surface protein.

Regarding the S protein, all OBI strains were genotypically of subtype ayw4 (Huy et al., 2006). Only a few mutations were observed when comparing consensus OBI strain sequences with other reported genotype E non-OBI strain sequences (Fig. 2). The major hydrophilic loop (aa 100–
Table 3. Uncommon mutations in HBV regulatory elements of OBI strains

Mutations shared by two or more OBI strains are shown in bold. AT-1, AT region 1 inside BCP (basic core promoter); URR, upper regulatory region; NRE, negative regulatory element; CURS, core upstream regulatory sequence; BEM, basal enhancer module; AEM, accessory enhancer module.

<table>
<thead>
<tr>
<th>OBI subject</th>
<th>Promoters</th>
<th>Core</th>
<th></th>
<th>BCP</th>
<th>URR</th>
<th>Rest of promoter</th>
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<tr>
<td></td>
<td>PreS1†</td>
<td>PreS2/S‡</td>
<td>X§$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PW1</td>
<td>G3074C</td>
<td>A1319C</td>
<td>C1287A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PW2</td>
<td>A1762T,</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>G1764A</td>
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<tr>
<td>PW3</td>
<td>A1762T,</td>
<td></td>
<td></td>
<td></td>
<td>A1757G</td>
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</tr>
<tr>
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<td>G1764A</td>
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<td></td>
<td></td>
<td>G1809A</td>
<td></td>
</tr>
<tr>
<td>PW4</td>
<td>C1632A</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>A1757G</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>A1128T</td>
<td>A1128T</td>
</tr>
<tr>
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<td>C1221C</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

†Position according to Fujiwara *et al.* (2005). HNF1, SP1 site 2 and HNF3 site 2 are totally conserved.
‡HNF1, SP1 (sites 1–3) and NF-Y according to Lu & Yen (1996), Raney *et al.* (1992) and Shaul *et al.* (1986) are totally conserved.
||Positions according to Erhardt *et al.* (2000) and Kramvis & Kew (1999). HNF1, SP1, sites 2 and 3 and HNF3-binding sites (Ha-Lee *et al.*, 2001) are totally conserved.
¶Positions according to GB/RANTES and EP sites are totally conserved.
GB/RANTES and EP sites are totally conserved.

160) was essentially wild type, except for substitution P105R in sample PW1, S140T in three of five PW5 clones and S140L in BD1 (Fig. 2). Other substitutions affected the transmembrane domains.

The OBI sequences showed a few uncommon polymerase substitutions. In the terminal protein domain of the polymerase, substitution P105R was found in both samples PW3 and PW4. In the reverse transcriptase (RT) region, the YMDD motif was conserved in all strains. In sample PW5, three mutations were detected in the catalytic domains of the RT (Poch *et al.*, 1989): N421H (domain A), S575A (domain D) and V598I (domain E), the first two mutations being non-conservative (NC). Uncommon mutations shared by at least two strains were at positions K678T (NC) in samples BD1, PW3 and PW4; S468D (NC) in samples BD2 and PW2; S468D (NC) and PW5; and K663R (conservative) in samples PW3 and PW4 (Fig. 2).

In the X protein, except for the substitutions directed by the double nucleotide mutations at positions 1762/1764 resulting in amino acid changes K130M/V131I in samples PW2, PW4 and PW5, the sequence was wild type.

**DISCUSSION**

Only a few studies have reported the analysis of complete HBV genomes from a limited number of individuals (one to nine) with occult HBV (Chaudhuri *et al.*, 2004; Jeanet *et al.*, 2002; Kremsdorf *et al.*, 1993; Preisler-Adams *et al.*, 1993; Schories *et al.*, 2000; Uchida *et al.*, 1994, 1995). In the present study, multiple clones of full-length HBV genomes
were obtained from Ghanaian adults with genotype E OBI. The features of the nine OBI strains from the present study represent a facet of HBV natural history that is undisturbed by human interventions, such as passive or active immunization or antiviral drugs, or by co-infections with either HIV or HCV. In Ghana, systematic HBV vaccination of newborns started in 2003, and only a minority of adults have been immunized.

OBI is frequent in Ghana, as reported previously in BD (Owusu-Ofori et al., 2005) and confirmed here in PW. The prevalence is around 1.5%, or nearly 10% of total viraemic individuals. HBV infection was confirmed in eight OBI cases by the detection of anti-HBc. Anti-HBc-negative sample PW7 can probably be explained by an HBV strain containing a core gene single-base insertion interrupting the normal core protein at aa 74 (Table 1). This 93 aa-long truncated core protein appears incompatible with capsid formation, but virion assembly may have been rescued by a minor, undetected wild-type variant for core expression (Günther et al., 2000) and may have affected virion formation significantly; this may constitute an explanation for the low viral load and absence of detectable HBsAg and anti-HBc. Another example of this potential mechanism http://vir.sgmjournals.org 415

Fig. 2. Schematic representation of the uncommon mutations found in HBV proteins from Ghanaian OBI strains. Consensus deduced amino acid sequences for the complete genome of the nine OBI individuals studied are represented by horizontal lines. The positions of the 'a' determinant in HBsAg and the catalytic domains (A–E) of the polymerase RT are indicated by shading. Uncommon mutations (for definition, see Methods) are represented by lollipops; mutations found in the pre-core, preS and X proteins are shown facing up and mutations in the polymerase protein are shown facing down. For the sake of simplicity, uncommon mutations in the polymerase spacer domain are not shown, because this region has been described to be tolerant to deletions and substitutions (Radziwill et al., 1990). As the BCP/PC region of BD2 was not amplified, information on the pre-core region is missing for this sample. As only two clones were obtained for sample BD1, six mutations that were wild type in one clone and mutated in the second are not represented (i.e. aa 49 and 140 in the S protein, aa 29 in X and aa 16, 495 and 627 in the polymerase protein). Mutations shared by two or more OBI samples are highlighted, indicating their position numbers. NC (non-conservative) and C (conservative) substitutions are given according to Mirny & Shakhnovich (1999). A pre-core mutation at aa 28 (grey-filled square lollipop) and mutation at AUG of the S2 protein (empty square lollipop) are indicated.
for OBI is provided by sample PW6, where the dominant variant did not encode the full-length core protein (102/184 aa), but contained a variant potentially able to rescue virion formation. The production of anti-HBc antibodies in PW6, but not PW7, could be explained by the location of the three identified B-cell epitopes in the core protein at aa 75, 107–118 and 128–135 (Carman et al., 1997).

Another element for the classification and potential mechanism of OBI is provided by the simultaneous presence of HBV DNA and anti-HBs, suggesting viral persistence after recovery. In the present study, anti-HBs detected in three of eight samples (PW1, PW2, and PW4; Table 1) was consistent with another study conducted in areas where genotypes A and D were prevalent, showing that 50% of OBI cases were detected in anti-HBs carriers (Brojer et al., 2006). One suggested mechanism of OBI in this situation of persistent, presumably low-replicating virus was related to escape mutants hidden from recognition in sanctuaries such as the liver (Allain, 2004; Rehermann et al., 1996; Yotsuyanagi et al., 1998). This does not seem to be applicable to our three cases, as the S protein ‘a’ region was wild type. An alternative hypothesis might be that the detected anti-HBs antibodies are poorly neutralizing and have lost the ability to control the level of circulating virus.

Among the four samples with anti-HBc, but no detectable anti-HBs, sample PW5 is interesting, as it has a higher level of genetic diversity resulting in two distinct clusters of clones. This feature is compatible with a relatively high level and prolonged replication in this 44-year-old woman, who was probably infected chronically during childhood (Table 1; Fig. 1).

The detection of HBeAg without detectable HBsAg in three samples (PW1, PW2 and BD2) was unusual, as previous data showed a clear association between low viral load and anti-HBe (Allain et al., 2003). Surprisingly, sample PW2 showed a combination of mutations G1896A and A1762T/G1764A, which are known to affect HBeAg production negatively (Table 3). This suggested the presence of a minority wild-type variant that escaped identification in the limited number of clones sequenced. Two other samples (PW4 and PW5) contained anti-HBe antibodies, in association with either the A1762T/G1764A double mutation alone or combined with G1896A. This seromolecular diversity of OBI strains is not significantly different from that in HBsAg-positive genotype E chronic infections (Allain et al., 2003; Candotti et al., 2006).

At the genomic level, each OBI strain was composed of variants with limited diversity and, although genetically distinct, the strains did not differ more from each other than they differed from the available full-genome sequences of HBsAg-positive genotype E consensus sequences (Fig. 1). These findings were consistent with the previously recognized limited diversity of genotype E strain sequences (Mulders et al., 2004) and suggest that OBI strains do not constitute a specific cluster. Quasispecies diversity within OBI samples was limited (0.20–0.58%), except for sample PW5 (1.28%) as indicated above, and possibly reflected the low level of replication. Within each sample, fewer non-synonymous than synonymous changes were generally observed in all regions of the viral genome (Table 2). The positive selection in the polymerase gene, suggested by a low dN/dS ratio, might tend to reduce the viral enzyme activity rather than suppress it. In contrast, analysis of the core region suggested relatively negative selection, despite this region being an important immune target. It suggests that the anti-core response might lack efficiency in individuals with OBI compared with chronic carriers (Osiowy et al., 2006).

Alignment of genomic sequences showed the presence of rare and evenly distributed nucleotide substitutions throughout the entire genome, suggesting the lack of preferential immune targets or natural selection. By examining each HBV gene separately, although a number of mutations not described previously were identified, none was common to all or to more than three OBI sequences. Whether by phylogenetic analysis or by examination of specific gene sequences or deduced amino acid sequences, these nine OBI consensus sequences were indistinguishable from those of HBsAg-positive strains with a higher viral load that were available for genetic comparison (Fig. 1). Many regions of the HBV genome are involved in replication, and significant mutations in any of these regions may have an impact on viral production. In none of the nine OBI sequences studied here were significant mutations found that could be predicted to affect viral replication (Fig. 2). Short of being able to assess the replicating capacity of these strains individually in vitro, there was no predictable evidence of specific defects in the replicative capacity of these strains.

Taken together, the data obtained suggest that genotype E OBI strains are a disparate group of HBV strains unified by their mode of detection: highly sensitive genomic amplification. For five of the nine strains examined, a mechanism leading to a very low viral load can be suggested. In two cases, the low level of virion production may be related to the dominance of variants carrying a lethal core mutation in the quasispecies, partly rescued by the presence of minority core wild-type variants. In three more cases, viral production is probably limited by the presence of anti-HBs and the existence of equilibrium between viral production and a moderately effective host immune system. The last group of four samples falls into the category of ‘anti-HBc only’, which constitutes the lower end of a continuum in chronic infection, ranging from high-level production to nearly complete immune control. The inability to detect HBsAg in the apparently chronic occult cases represents only the difference in sensitivity between DNA amplification and HBsAg capture assays in detecting the presence of HBV virions.

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