Molecular Characterization and Functional Analysis of Occult Hepatitis B Virus Infection in Chinese Patients Infected With Genotype C

Yong Fang, Xu Teng, Wei-Zhen Xu, Di Li, Hong-Wei Zhao, Li-Juan Fu, Feng-Min Zhang, and Hong-Xi Gu

Department of Microbiology, Harbin Medical University, Harbin, China
Department of Infectious Diseases, Heilongjiang Provincial Hospital, Harbin, China

Occult HBV infection is defined as the persistence of HBV DNA in individuals negative for HBV surface antigen (HBsAg), and many different mechanisms have been reported in different countries. However, in China, one of the endemic areas for HBV infection, no reports have been published on occult HBV infection. The present study investigated the virological features and the mechanism of occult HBV infection in China. Full-length HBV DNA from eight patients with occult HBV infection (S1–S8) and three HBsAg-positive cases (SWT1–SWT3) was cloned and sequenced. Additionally, four entire linear HBV genomes from occult cases were transfected transiently into HepG2 cells. The sequencing results showed two major mutations in patients with occult HBV infection as follows: deletions in the pre-S1 (S3, S4, and S7) and X (S1, S2, and S5) regions. Such deletions covered the S promoter and the basal core promoter (BCP), and function analysis of these variants also showed a decrease in DNA replication and antigen expression. Two patients with occult infection (S6 and S8) had no mutations capable of interfering with viral replication and gene expression in the major viral population. Thus, the deletions in the S promoter and the BCP regions that disable the regulatory elements may be the reason for the absence of HBsAg, and multiple mechanisms may be responsible for occult HBV infection.

INTRODUCTION

Occult HBV infection is defined as the presence of HBV DNA in blood or liver, in the absence of detectable serum HBV surface antigen (HBsAg), and has been classified into seropositive and seronegative infection depending on the presence for antibody to the HBV core antigen (anti-HBc) and/or HBsAg (anti-HBs) [Brechot et al., 2001; Conjeevaram and Lok, 2001]. Occult HBV infection has its own risks of disease transmission and may contribute to acute exacerbation and development of HBV-associated disease such as hepatic inflammation, cirrhosis, and hepatocellular carcinoma (HCC) [Pollicino et al., 2004; Chevrier et al., 2007; Kannangai et al., 2007]. However, the virological features and the mechanism of occult HBV infection have not been elucidated. Many earlier studies have supported the view that rearrangements in the S gene lead to synthesis of an antigenically modified S protein undetectable by the commercially available HBsAg assays [Hou et al., 1995; Minuk et al., 2005], but research on HBV variability has generated conflicting results. Recently, a few studies have extended sequence analysis to the full-length viral genome, to detect mutations outside the S gene capable of interfering with its expression and/or with viral replication, but with differing results [Chaudhuri et al., 2004; Pollicino et al., 2007; Zahn et al., 2008], and regional differences were identified. In China there have been no related reports. The present study attempted to amplify the full-length HBV genome isolated from Chinese patients, and examined the viral genomic variability of isolates, and then evaluated...
replication capacity and antigen expression of typical occult HBV isolates in vitro.

**MATERIALS AND METHODS**

**Patients and Clinical Data**

Between January 2006 and December 2008, the prevalence of occult HBV infection in a group of patients with chronic liver disease without any known cause (such as alcohol abuse, hepatotoxic drug, Wilson’s disease, biliary tract disease, venous outflow obstruction, iron overload, or autoimmune disease) was investigated. None of the patients had been treated with antiviral or immunosuppressive drugs and none were co-infected with HIV, CMV, HCV, HDV, HAV, or HEV. The diagnosis of chronic liver disease was made by conventional clinical, biochemical, imaging, and endoscopic criteria. Cryptogenic chronic liver disease was diagnosed when all known identifiable causes were excluded by relevant investigations.

Occult HBV infection in patients with cryptogenic liver disease was diagnosed when PCR detected HBV DNA in sera for at least two different regions in the absence of detectable HBsAg [Conjeevaram and Lok, 2001]. Patients with occult HBV infection were included in the study. Serum samples were selected at random from the patients with occult HBV infection, for complete genome analysis. The three HBsAg-positive patients that served as a control group were diagnosed with chronic hepatitis B. This study was approved by the Harbin Medical University Committee on Clinical Investigation and informed consent was obtained from all the patients.

**Serological Assays**

Blood samples were centrifuged at 700 g and serum samples were used for serological tests within 4 hr, and the remainder of the same serum samples were stored at −80 °C. HBsAg, anti-HBs, HBV e antigen (HBeAg), anti-HBe, anti-HBc and anti-HCV were measured by ELISA using a diagnostic kit (Kehua Biotechnology, Shanghai, China). According to the instructions, the sensitivity of the assay for HBsAg was 0.05 ng/ml.

Sera from patients negative for all the above tests were investigated for the presence of HBV DNA by PCR. Patients who were negative for HBsAg and other causative factors implicated in the cause of chronic liver disease, but positive for HBV DNA by PCR, were labeled as occult HBV infection as per the diagnostic criteria described earlier [Conjeevaram and Lok, 2001]. Serum samples from these patients were retested for the presence of HBsAg by another commercial kit (Wantai Biotechnology, Beijing, China) to confirm the absence of HBsAg in the serum.

**Isolation and Detection of HBV DNA From Serum**

Viral DNA was extracted from 250 μl of each serum sample by the AxyPrep kit (Axygen Biosciences, Hangzhou, China) according to the manufacturer’s instructions.

For the HBsAg-negative cases, HBV genome detection was performed by nested PCR using three primer sets, each specific for pre-S, precore-core, and X viral genomic regions, respectively [Goshi et al., 2000]. With this approach, only the cases in whom HBV DNA was detected using at least two different sets of primers were diagnosed as occult HBV infection and considered for amplification of the full-length genomes.

In order to prevent carryover contamination during PCR, each step of the procedure was performed in a separate room with dedicated equipment and directional flow from the beginning of the procedure to the end. All samples were tested in duplicate. Control samples included normal sera, HBV-positive sera, and negative controls. Positive and negative controls were included in the extraction steps and in both rounds of amplification.

**Amplification of Full-Length HBV Genomes**

HBV genomes were full-length amplified using different PCR methods. HBV sequences from HBsAg-positive patients were obtained by one-step full-length genome amplification using a single set of primers: sense primer P1 (5’-CCGGAAAGCTTGAGCTCTTCTTTTTCACTC-TGCTTAATCA-3’ 1821–1841) and the antisense primer P2 (5’-CCGGAAAGCTTGAGCTCTTCTTTTTCACTC-TGCTTAATCA-3’ 1806–1825), as in the method used by Günther et al. [1995]. On the other hand, HBV DNA from patients with occult HBV infection was full-length amplified by a two-step PCR approach. The first-round PCR yield was very faint on 0.7% agarose gel after electrophoresis using the primers P1/P2 to amplify the entire genome from occult HBV cases. Therefore, the second-round PCR was performed with different primer pairs: sense primer P3 (5’-GAGCTCTTCTTTTTTCACTC-TGCTTAATCA-3’ 1821–1841) and the antisense primer P4 (5’-GAGCTCTTCTTTTTTCACTC-TGCTTAATCA-3’ 1806–1825). The nucleotide sequences of primers P3 and P4 differed from those of primers P1 and P2 because of the absence of the heterologous nucleotides that contained the HindIII restriction enzyme site. The first-round PCR contained 5 μl 10 × LA PCR buffer, 400 μM each dNTP, 10 μM each primer, 2.5 U LA Taq polymerase (Takara Biotechnology, Dalian, China) and 10 μl template DNA preparation per 50 μl reaction. Amplification was performed for 40 cycles with denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 68 °C for 3 min, with an increment of 1 min after 10 cycles; cycles were followed by an extra incubation at 72 °C for 10 min to ensure full extension of the products. Subsequently, 5 μl (10%) of the first PCR product was re-amplified for 30 cycles in 50-μl assay mixtures with primer set P3 and P4. The second-round PCR conditions were similar to those of the first-round PCR, except annealing was done at 65 °C.

Cloning and Sequencing of the Amplified HBV Genome

The amplicons were recovered from agarose gel with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) and cloned into the pMD18-T Vector (Takara Biotechnology), following the manufacturers' instructions. For each sample, three to four clones were sequenced by an automated DNA sequencer (ABI 3100-Avant Genetic Analyze; Applied Biosystems, Carlsbad, CA) with a set of primers that encompassed the whole HBV genome plus universal M13 primers. HBV primers are described in detail in Supplementary Table SI.

Nucleotide and Amino Acid Analysis

Nucleotide and amino acid sequences of HBV DNA isolates from patients with occult HBV infection and HBsAg-positive controls were aligned with 50 HBV sequences from GenBank using the CLUSTAL W program. The conventional EcoRI position was taken as nucleotide position 1. Genotyping of HBV DNA sequences was performed by phylogenetic comparison with the representative genotypes (HBVADW2 X02763, genotype A) [Valenzuela et al., 1980], HPBADW1 (D00329, genotype B), HPBADRA (M12906, genotype C), XXHEPAV (X02496, genotype D), HHVBE4 (X75664, genotype E), and HHVBF (X75663, genotype F) over the entire genome.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [Kumar et al., 2004] by the neighbor-joining algorithm based on Kimura two-parameter distance estimation.

Cell Culture and Transfection

HepG2 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified incubator with 5% CO2. Four different constructs (S1.3, S3.3, S4.1, and S7.2) that contained full-length HBV genomes isolated from occult HBV patients S1, S3, S4, and S7, respectively, were used to perform transient transfection. Each isolate represented the dominant strain of the HBV population that infected the liver of each patient. Linear HBV monomers were released from S1.3, S3.3, S4.1, and S7.2 constructs and from the wild-type (SWT3.3) control plasmid by cleavage with SspI restriction enzyme (New England Biolabs, Ipswich, MA). Full-length HBV genome DNA (4 μg) from each clone was used to transfect HepG2 cells in six-well culture plates using Lipofection 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s guidelines. Triple plates were used for all samples, and 1 μg reporter plasmid expressing enhanced green fluorescence protein was co-transfected into each culture as an internal control to normalize the transfection efficiency between plates. Cells were collected 72 hr after transfection.

Real-Time PCR Analysis of HBV DNA

HBV DNA was extracted from the intracellular and extracellular core particles using Axygen Mini kit (Axygen Biosciences) at 72 hr post-transfection, and then assayed by real-time PCR using a Diagnostics LightCycler (Roche, Mannheim, Germany), following the manufacturer's guidelines. According to the instructions, an HBV DNA level ≥5.0 × 10^5 copies/ml (2.70 log copies/ml) was considered as a positive response.

HBsAg and HBeAg Assays

At 72 hr post-transfection, the culture supernatant was collected, centrifuged at 1,000g for 5 min to remove cellular debris, and transferred to a clean tube for further analysis. The expression levels of HBsAg and HBeAg were separately assayed using an enzyme immunoassay kit (Kehua Biology, Shanghai, China). According to the instructions, a ratio of sample/negative (S/N) ≥2.1 was considered as a positive response to HBsAg or HBeAg.

Statistical Analysis

Data were expressed as mean ± SD. Data were compared using the F test and one-way ANOVA. All statistical analysis was performed using SPSS 12.0 software (SPSS, Inc., Chicago, IL). P < 0.05 was considered statistically significant.

RESULTS

Patients

During the period 2006 to 2008, 159 patients were diagnosed with cryptogenic chronic liver disease, all of whom had chronic hepatitis. HBV DNA was detected in 45 (28.3%) of these patients. HCV RNA was negative in all of the patients. Seventy-five of the 159 patients, including the 45 HBV-DNA-positive cryptogenic chronic liver disease patients, were positive for anti-HBC IgG. Anti-HBe was not detected in any of these patients. The 45 patients positive for anti-HBc IgG and HBV DNA were categorized as having occult HBV infection. Sera from eight of patients with occult HBV infection were selected randomly for analysis of complete HBV genome (S1–S8). The sera of these eight patients were tested for the presence of anti-HBsAg by using a commercial ELISA kit (Kehua Biotechnology) and were found to be negative for anti-HBsAg. Sera from three HBsAg-positive patients with confirmed chronic hepatitis were used as controls (SWT1–SWT3). Complete genomic analysis of the HBV DNA was also taken in the control group for comparison with any mutation. The clinical data of these patients are shown in Table I. The “infection history” means the length of HBV infection, and the beginning of infection was defined as the first time for finding abnormal liver function in patients with cryptogenic chronic liver disease, or HBsAg in chronic HBV infection patients.

The HBV full-length genomes from eight patients with occult HBV infection were amplified and cloned successfully, and three or four clones from each patient were sequenced. For the HBsAg-positive patients, three clones of each were sequenced. All 11 patients were infected with HBV genotype C strains, which is the predominant genotype in China (Genebank Accession-Numbers: S1–S8 EU916204–EU916232; SWT1–SWT3 EU916233–EU916241) (Fig. 1).

Analysis of the Pre-S–S Genomic Region

In the pre-S1 region, long-stretch deletions of aa 58–118 (nt 3019–3201) were observed in most isolates from three patients (S3, S4, and S7) with occult HBV infection (Fig. 2). In addition, patients S4.4 and S7.4 presented with deletions of aa 1–6, which abolished the pre-S1 start codon. The B-cell antigenic epitope in pre-S1 protein (aa 19–26) [Maeng et al., 2000] was conserved.

Within the pre-S2 region, the start codon was abolished in three isolates (S2.3, S7.1, and S7.2) by a point mutation (ATG!ATA), and the mutation of codon 3 introducing a stop signal terminated the pre-S2 gene in all of the isolates from patient S3 (Fig. 2).

The analysis of the S region showed that three variants (S4.2, S4.3, and S4.4) of patient S4 had three amino acid changes within the putative human leukocyte antigen class I-restricted cytotoxic T lymphocyte (CTL) epitope (aa 28–51) [Tai et al., 1997]. Patients S1, S2, S5, S7, and S8 only had one variant with one or two amino acid changes, and the other patients had no mutations (Table II). Substitutions within the major hydrophilic region of HBsAg (aa 104–172) were found in most patients (one or two amino acids per patient), except patients S4 and S8 (Table II). The immunodominant a determinant (aa 124–147) [Feitelson et al., 1981] of HBsAg had no variation in any of the isolates, except variants S2.2 (A128V), S2.3 (I126T), and S4.4 (I126T), all the substitutions were within the first hydrophilic loop of the a determinant (Table II). The stop codon mutations in variant S1.1, S7.4, and S8.1 were observed in the coding sequence of HBsAg.

Analysis of the Pre-C–C Genomic Region

Within the pre-core region, the nucleotide mutation G1896A, introducing a stop signal at codon 28 and preventing HBeAg synthesis, was found in five isolates (S3.1, S3.3, S3.4, S4.2, and S7.1), and the rest of the isolates had a wild-type pre-core region.

Within the core region, amino acid changes were distributed at random, and different immunogenic antigenic epitopes were observed. Three different amino acid changes (A58V, I59S, and L60V) were detected within the CD4+ T-cell epitope (aa 48–69) [Ferrari et al., 1991], but only a single amino acid substitution (L60V) was present in most of the HBV isolates. Concerning the core protein CD8+ CTL epitopes, only variant S7.3 had three amino acid substitutions (P20A, F23L, and I27V) within the epitope 18–27. CTL epitope 141–151 was conserved in all isolates, except variants


TABLE I. Clinical Manifestations and Laboratory Data of Occult Infection Patients (S1–S8) and HBsAg-Positive Cases (SWT1–SWT3)

<table>
<thead>
<tr>
<th>Patients (IU/L)a</th>
<th>Age (years)</th>
<th>Sex (F/M)</th>
<th>Clinical Manifestations</th>
<th>Viral DNA load (copies/ml)</th>
<th>Alanine transaminase (IU/L)a</th>
<th>Aspartate transaminase (IU/L)a</th>
<th>HBsAg</th>
<th>Anti-HBe</th>
<th>Anti-HBs</th>
<th>HBeAg</th>
<th>Anti-HBs</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>59</td>
<td>M</td>
<td>CH</td>
<td>6.415×10⁴</td>
<td>115</td>
<td>233</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>S2</td>
<td>63</td>
<td>M</td>
<td>CH</td>
<td>2.972×10⁵</td>
<td>105</td>
<td>210</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>S3</td>
<td>52</td>
<td>M</td>
<td>CH</td>
<td>9.347×10⁴</td>
<td>105</td>
<td>170</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>S4</td>
<td>56</td>
<td>F</td>
<td>CH</td>
<td>2.538×10⁵</td>
<td>104</td>
<td>153</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>S5</td>
<td>65</td>
<td>F</td>
<td>CH</td>
<td>3.762×10⁵</td>
<td>104</td>
<td>203</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>S6</td>
<td>65</td>
<td>M</td>
<td>CH</td>
<td>3.689×10⁵</td>
<td>107</td>
<td>169</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>S7</td>
<td>66</td>
<td>F</td>
<td>CH</td>
<td>6.895×10⁵</td>
<td>107</td>
<td>201</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>S8</td>
<td>72</td>
<td>M</td>
<td>CH</td>
<td>7.870×10⁵</td>
<td>175</td>
<td>198</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>SWT1</td>
<td>58</td>
<td>M</td>
<td>CH</td>
<td>6.308×10⁵</td>
<td>115</td>
<td>233</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>SWT2</td>
<td>49</td>
<td>M</td>
<td>CH</td>
<td>7.956×10⁵</td>
<td>104</td>
<td>203</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
<tr>
<td>SWT3</td>
<td>63</td>
<td>M</td>
<td>CH</td>
<td>7.807×10⁵</td>
<td>107</td>
<td>198</td>
<td>C0</td>
<td>C2</td>
<td>C0/C0þ</td>
<td>C0/C0</td>
<td>C0/C0þ</td>
</tr>
</tbody>
</table>

CH, chronic hepatitis. aAlanine transaminase (ALT) and aspartate transaminase (AST) normal values: 0–40 IU/L; –, negative; +, positive.
S4.1 (E145G) and S5.2 (V148I). Two point mutations were found in the arginine-rich motif of the C-terminal domain of the core protein of variants S5.4 (R164C) and S7.2 (R167W). In addition, long-fragment deletion mutations were found in patients with occult infection, S1 (S1.1, aa 81–109; and S1.2, aa 78–106) and S5 (S5.1, aa 65–104; S5.3 and S5.4, aa 84–116), which resulted in a truncated core protein.

Analysis of the Pol Genomic Region

In the terminal protein domain of the polymerase, no typical mutation that may induce occult infection was found. The internal deletions of the pre-S1 region coincided with the spacer region of the polymerase gene, which was tolerant to deletions and substitutions. The L180M substitution inside the conserved YMDD motif of

the reverse transcriptase domain of the Pol gene was found only in variant S7.3. This mutation is usually selected during lamivudine treatment, but none of the patients had been treated previously with antivirals. The RNase H domain was conserved in all isolates.

Analysis of the X Genomic Region

The typical change in the X region was a deletion mutation, the deletion of aa 126–133 observed in patient S2, and deletion of aa 127–133 was observed in patients S1 and S5. Double nucleotide mutations at positions 1762/1764 that resulted in amino acid changes K130M/V131I were found in variants S5.1 and S5.4.

Analysis of Transcription Regulatory Regions

The main regulatory elements were also analyzed in all viral isolates from the 11 studied patients to detect mutations potentially implicated in the inhibition of viral replication and/or gene expression. The pre-S1 promoter had a wild-type sequence in all but two patients (S1 and S8), whereas the same 183 nt deletions (nt 3019–3201) were detected in the S promoter region.

### Table II. Amino Acid Changes in HBV S Protein in Isolates S1–S8

<table>
<thead>
<tr>
<th>Patient isolate</th>
<th>Upstream changes</th>
<th>α determinant</th>
<th>Downstream changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mutant at this position exists as a majority population in all the clones sequenced of the particular isolate.
in three occult infection patients (S3, S4, and S7) (Fig. 2). The deletion covered the CCAAT element, which is known to bind nuclear factor-\(\gamma\) transcription factor and is necessary for S promoter activity. The analysis of enhancer IX promoter indicated only several point mutations, but three occult infection patients (S3, S4, and S6) showed the same mutation (A951T/G) located within the C/EBP binding site of enhancer I. Enhancer II analysis showed only one unique point mutation G1661A (located in the HNF4-binding site) was found in patient S1 (Fig. 3). Analysis of the basal core promoter (BCP) showed the presence of deletion mutations in occult infection patients S1, S2, and S5 (Fig. 3). The double A1762T and G1764A nucleotide exchanges, which have been reported to cause a decrease in HBeAg expression and enhanced viral replication, were found in isolates S5.1 and S5.4.

**Functional Analysis of Occult HBV Genomes**

Frequent deletions were observed in the BCP region (patients S1, S2, and S5) and in the S promoter region (patients S3, S4, and S7), compared with wild-type isolates. To investigate the possibility of altered gene regulation caused by these altered promoter regions, functional assays of these mutants were performed, and wild-type isolate (SWT3.3) was used as control. S1, S2, and S5 had a similar deletion, and therefore, a functional assay of S1.3 isolate was performed. S4.1 only had a deletion mutation in the S promoter, and S7.2 carried the preS2 start codon mutation and deletion in the S promoter. S3.3 had both the pre-core stop codon mutation and the stop codon mutation in the pre-S2 region, and the deletion in the S promoter region. At 72 hr post-transfection, HBV DNA was isolated from cell supernatants and cell lysates, and real-time PCR was performed. As shown in Table III and Figure 4A, except variant S1.3 \((P < 0.05)\), HBV-DNA expression levels of the other three HBVs were similar to those of wild-type strains SWT3.3 in HepG2 cells. In the culture supernatants, HBV DNA was not found in mutant HBV with S promoter deletions, and in variant S1.3, it was significantly lower than that in wild-type strain SWT3.3 (Table III and Fig. 4B). Cells transfected with S-promoter-deleted variant genomes (S4.1, S7.2, and S3.3) were negative for HBsAg secretion, and HBsAg secretion from S1.3 was significantly lower than that from wild-type HBV-transfected cells \((P < 0.05)\) (Table III and Fig. 4C). HBeAg expression levels for all variant genomes were lower than those of the controls \((P < 0.05)\), and HBeAg was negative in the supernatant of S3.3-transfected cells because S3.3 carried the pre-core stop codon (Table III and Fig. 4D).

**DISCUSSION**

Occult HBV infection is a problem in many parts of the world and affects blood banks, the diagnosis of chronic hepatitis, and immunization. In the present study, multiple clones of full-length HBV genomes were obtained from eight patients with occult HBV infection with genotype C and compared with the wild-type HBV genome prevalent in China. The patients were not treated with antiviral drugs or co-infected with other viruses such as HCV and HIV, which can be a feature of HBV infection. The patients were not treated with antiviral drugs or co-infected with other viruses such as HCV and HIV, which can be a feature of HBV infection. There were two possible explanations for the HBsAg-negative HBV infection: the virus produced an antigenically modified S protein undetectable by routine tests [Yamamoto et al., 1994; Chiu et al., 1997; Jeantet et al., 2004]; or the virus carried mutations capable of inhibiting S gene expression and/or viral replication [Blum et al., 1991; Pollicino et al., 2007]. In the present study, entire viral genomes from eight occult HBV carriers were examined and compared with isolates from HBsAg-positive individuals. Then, the capacity of replication and antigen expression of some occult isolates were verified in vitro.

Concerning the genomic HBsAg coding region, amino acid changes were distributed at random. The stop codon mutations in variants S1.1, S7.4, and S8.1 were observed in the coding sequence of HBsAg, which could have abolished HBsAg synthesis. In the α determinant, three point mutations were found in variants S2.2 (A128V), S2.3 (I126T), and S4.4 (I126T). These mutations were not reported previously, and it is unknown whether the mutations I126T and A128V had a role in occult infection. Each of these variants that carried mutations in HBsAg was not the dominant strain in the HBV population infecting the liver of each patient. The results demonstrated the absence in all cases of relevant mutations at the level of the genomic HBsAg coding region, thus indicating an impaired synthesis rather than a failure in antigen detection.

For the main regulatory elements, two major changes (deletion in the S promoter region and BCP region) were observed in six of the eight patients in the present study, which made them the most common observations in occult HBV infection.

In the pre-S1 region, 183-nt deletions were observed in three patients (S3, S4, and S7) with occult infection, which overlapped the surface promoters. The same mutation has been reported in a previous study of occult HBV infection in Spain [Cabrerizo et al., 2000]. Researchers in India have also found deletion mutations in the pre-S1 region, but different from those in the present study [Chaudhuri et al., 2004]. In previous studies, it has also been shown that this deletion mutation in the pre-S1 region is replication-competent by transfecting hepatoma cell lines with this deleted HBV DNA, but the secretion of viral particles into the culture medium did not occur [Melegari et al., 1997]. In the present study, functional analysis also showed the cells transfected with 183-nt deletion variants did not produce HBsAg or secrete viral particles. This is because this deletion removes a region in the S gene promoter which made them the most common observations in occult HBV infection.

### TABLE III. Real-Time PCR Detection of HBV-DNA and Antigen Assay by ELISA

<table>
<thead>
<tr>
<th>Clone</th>
<th>HBV-DNA (log copies/ml)</th>
<th>HBsAg (S/N)</th>
<th>HBeAg (S/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell</td>
<td>Supematant</td>
<td></td>
</tr>
<tr>
<td>S1.3</td>
<td>5.83 ± 0.556e</td>
<td>6.71 ± 0.109e</td>
<td>6.33 ± 0.221e</td>
</tr>
<tr>
<td>S4.1</td>
<td>8.68 ± 0.195</td>
<td>2.63 ± 0.107b</td>
<td>1.92 ± 0.140e</td>
</tr>
<tr>
<td>S7.2</td>
<td>8.26 ± 0.673</td>
<td>2.61 ± 0.066a</td>
<td>1.85 ± 0.174e</td>
</tr>
<tr>
<td>S3.3</td>
<td>8.43 ± 0.500</td>
<td>2.63 ± 0.106b</td>
<td>1.90 ± 0.171a</td>
</tr>
<tr>
<td>SWT3.3</td>
<td>8.53 ± 0.478</td>
<td>8.03 ± 0.448</td>
<td>8.18 ± 0.350</td>
</tr>
</tbody>
</table>

n, negative.

HBV DNA level $\geq 2.70$ log copies/ml was considered as positive HBV DNA response; The ratio of sample/negative (S/N) $\geq 2.1$ was considered as positive HBsAg and HBeAg response.

$^aP<0.05$ versus SWT3.3.
that is a binding site for the transcription factor, SP1, thus causing a drastic reduction in HBsAg synthesis. For assembly of the envelope particles, a specific ratio of major (small) HBsAg and large HBsAg is required. If this ratio is altered, it may alter the HBsAg assembly and secretion [Bock et al., 1997; Gerner et al., 2003] in comparison with that of the wild phenotype, which leads to a decrease in HBsAg and HBV in the serum. If the variants are obtained from serum, this indicates that the HBsAg must be provided in trans by a wild-type virus for the secretion of viral particles that contain mutant genomes. In this sense, the deletion mutant may be accompanied by the wild-type virus in the pre-S1 region.

Further deletion mutations were found in the BCP region in three patients with occult infection, and functional analysis indicated that the cells transfected with the BCP deletion variant showed a decrease in DNA replication and antigen expression. The BCP was sufficient for accurate initiation of both pre-core mRNA and pregenomic (pg) RNA transcription. It contains four AT-rich regions and the initiators for pre-core mRNA and pgRNA transcription. The AT-rich regions can bind TATA-binding protein, which is important for pre-core mRNA and pgRNA transcription. In the present survey, there were four deletion patterns of 18 or 20 bp, which encompassed TA1, TA2, and TA3. One deletion pattern (nt 1753–1772) has been found in occult HBV in Japan [Saito et al., 1999]. These deletion mutations that result in decreased HBV replication caused by a decrease in pgRNA may lead to a decrease in HBV load and HBsAg expression in serum. At another level, all deletions within the BCP result in a frameshift mutation and truncation of the X protein at its C-terminal end, which influence the expression and function of the X protein. This usually affects the transactivating function of the X protein, and loss of this activity may be an additional reason for the decreased viral replication and reduced antigen production seen in patients with occult infection.

HBsAg detection by immunoassay is the mainstay of diagnosis of HBV infection. This is dependent on excess HBsAg in the blood. The regulatory region has much to contribute towards this excess production, as does the relative quantity of the major and large HBsAg. The analysis of mutated promoters observed in the present study showed a decrease in HBsAg expression and viral replication. However, there were occult infection patients who had no mutations capable of interfering with viral replication and gene expression. Thus, it appears that the non-detectability of HBsAg in serum may arise from several mechanisms, caused by alterations in the structural, functional and regulatory regions of the HBV genome. The host immune system is likely to play a critical role, and future research should be carried out.

REFERENCES


Molecular Characterization of Occult HBV