Effect of basal core promoter and pre-core mutations on hepatitis B virus replication

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There are two hypotheses explaining a fulminant outcome after hepatitis B virus (HBV) infection, both of which may be applicable at the same time: (i) basal core promoter (BCP) mutations increase viral replication, allowing rapid spread of the virus through the liver, and (ii) pre-core (pre-C) mutations abrogating hepatitis B e antigen (HBeAg) synthesis remove its tolerogenic effect, leading to a vigorous immune response. This study investigated the effect of these mutations on virus replication efficiency and HBeAg production. Substitutions A1762T/G1764A and T1753C, C1766T and T1768A in the BCP region, and G1896A and G1899A in the pre-C region, were examined either alone or in combination, using a common genetic background. Huh7 cells were transfected with these constructs and real-time PCR was used to quantify released virion-associated and intracellular HBV DNA, pregenomic RNA and pre-C mRNA. In addition, culture supernatants were tested for hepatitis B surface antigen (HBsAg) and HBeAg. The double BCP mutation (A1762T/G1764A) and the pre-C mutations (G1896A, G1899A), either alone or in combination, had no appreciable effect on the replication capacity of the virus. In contrast, clones with mutations at positions 1766/1768, 1762/1764/1766 and 1753/1762/1764 exhibited increased-replication phenotypes. HBeAg was undetectable in all cultures transfected with constructs bearing the G1896A stop-codon mutation, as expected. In contrast, constructs with additional mutations in the BCP region had appreciably lower levels of HBeAg expression than the wild type. Thus, core promoter mutations other than those at 1762/1764 appear to upregulate viral DNA replication and, at the same time, greatly reduce HBeAg production.

INTRODUCTION

Hepatitis B virus (HBV) is the prototype member of the family Hepadnaviridae. Its virions consist of an outer lipoprotein envelope containing the three hepatitis B surface antigens (HBsAg) (Heermann et al., 1984) and an inner core (Robinson & Lutwick, 1976), or nucleocapsid, that encloses the relaxed, circular, partially double-stranded DNA (RC-dsDNA) genome of the virus, and the viral polymerase (Summers et al., 1975). Upon infection of the hepatocytes, the viral genome is delivered to the nucleus, where the incomplete positive strand undergoes completion and ligation, following the removal of the RNA primer and the covalently attached polymerase. In addition, the nick in the negative strand is repaired. The resulting circular, double-stranded molecule is referred to as the covalently closed circular DNA (cccDNA) (Tuttleman et al., 1986), which forms the template for mRNA transcript synthesis. One of the transcripts, termed pre-genomic RNA (pgRNA), is the template for genome replication by reverse transcription (Summers & Mason, 1982), as well as encoding the core and polymerase proteins. The slightly longer pre-core (pre-C) mRNA encodes the protein precursor of HBeAg. Translation of the transcripts occurs in the cytoplasm and encapsidation of the pgRNA into core particles is followed by its reverse transcription to DNA (Pollack & Ganem, 1994). Mature core particles containing DNA genomes are then enveloped and released (Gerelsaikhan et al., 1996) or cycled back to the nucleus to replenish the cccDNA pool, thus perpetuating the chronic infection (Tuttleman et al., 1986).

Infection with HBV causes a wide spectrum of disease manifestations, ranging from asymptomatic infection to acute self-limiting or fulminant hepatitis, or chronic infection with variable disease activity. Chronically infected patients may eventually develop liver failure, cirrhosis or hepatocellular carcinoma. Chronic infection is characterized by well-recognized phases in disease activity, which are separated into the immune-tolerant, the immune-clearance, the non-replicative and finally, in some patients, the reactivation phases (Chu et al., 1985; Hadziyannis & Vassilopoulos, 2001). These phases are characterized by...
distinct serological, biochemical and viral marker patterns, notwithstanding the appearance of well-defined genomic mutations. Such mutations include the double A1762T/G1764A basal core promoter (BCP) mutation (Okamoto et al., 1991) and the G1896A pre-C stop-codon mutation (Carman et al., 1989), the latter often accompanied by a G1899A mutation. Both of these mutations have been associated with the reactivation phase of the disease and fulminant hepatitis when transmitted de novo (Carman et al., 1991; Friedt et al., 1999; Hasegawa et al., 1994; Kaneko et al., 1995; Kosaka et al., 1991; Laskus et al., 1995; Liang et al., 1991; McMillan et al., 1996; Omata et al., 1991; Sato et al., 1995; Sterneck et al., 1996). Moreover, additional mutations in the BCP region that may confer increased replication efficiency to the virus have been described (Baumert et al., 1998; Parekh et al., 2003).

It has been postulated that fulminant disease may be the result of increased viral replication resulting from the BCP mutations, which may upregulate pgRNA, with concurrent downregulation of pre-C mRNA synthesis (Baumert et al., 1996; Buckwald et al., 1996; Moriyama et al., 1996; Sato et al., 1995). However, in vitro experiments addressing these issues have provided conflicting results (Baumert et al., 1998; Gunther et al., 1998; Li et al., 1999; Sterneck et al., 1998). Some studies have reported increased viral replication in conjunction with a decrease in pre-C mRNA synthesis (Baumert et al., 1996; Buckwald et al., 1996; Moriyama et al., 1996), whilst others reported no increase in viral replication, only reduced pre-C mRNA production (Gunther et al., 1998; Sterneck et al., 1998). Such studies have employed reporter-gene constructs containing sub-genomic fragments bearing the relevant mutations in the genetic background that they were found in, or have used infectious clones with the mutations engineered by site-directed mutagenesis.

In the present study, all of the mutations under study were introduced by site-directed mutagenesis in the same genetic background of an infectious clone, either alone or in combination, in order to determine the effect of these mutations per se on replication. In contrast to earlier studies, replication efficiency of the various infectious constructs was documented by quantitative real-time PCR measurements of released virion-associated HBV DNA and of intracellular replicative intermediates, including RC-dsDNA and pgRNA. Finally, promoter analysis was undertaken by using electrophoretic mobility-shift assays (EMSA) employing mutated BCP sequences and nuclear extracts from Huh7 cells.

**METHODS**

**Plasmid constructs and site-directed mutagenesis.** A replication-competent plasmid (p3.8II) containing a 1.2 kb genome length of HBV subtype adr (genotype C) in a pBluescript II KS (+) background (Stratagene) was used as template for the generation of all mutated constructs. This was kindly provided by Professor Yuan Wang, Academia Sinica, Shanghai, China (Fu & Cheng, 1998). The 3.8 kb HBV insert encompassed nt 1400–3213 and 1–1988 of the HBV genome, starting at the 5′ end from enhancer II upstream of the BCP and bearing a 3′-terminal redundant region containing the polyadenylation signal for all viral mRNAs. This plasmid formed the template for use with a QuikChange Multi site-directed mutagenesis kit from Stratagene, as described in the manufacturer’s instructions and using the primers listed in Supplementary Table S1 (available in JGV Online). For combined BCP and pre-C mutations, mutagenesis of the pre-C region was performed first and the resulting constructs were used next as templates for the introduction of the BCP mutations. All constructs were sequenced to ensure that no additional mutations were introduced in the process.

A green fluorescent protein (GFP)-expressing plasmid (pCI-GFP; a gift from Dr M. Moore, Imperial College London, UK) was co-transfected with all mutated and wild-type (WT) constructs to monitor transfection efficiency by fluorescence-activated cell sorting (FACS) analysis, within and between experiments.

**Cell lines and transfections.** The Huh7 and the HBV stably transfected HepG2.2.15 cell lines were grown and maintained in Dulbecco’s minimal essential medium supplemented with 10 % fetal calf serum, 2 mM l-glutamine, 100 IU penicillin ml\(^{-1}\) and 100 μg streptomycin ml\(^{-1}\). All cultures were incubated at 37 °C in a humidified atmosphere and 5 % CO\(_2\).

For the transfection experiments, six-well plates were seeded with 1 × 10\(^5\) cells in 5 ml growth medium per well, followed by a 12–14 h overnight incubation. A plasmid–liposome complex [13 μg recombinant and 2 μg pCI-GFP complexed with 7 μg Lipofectin (Invitrogen)] was then prepared in serum-free OptiMEM as recommended by the supplier (Invitrogen). Briefly, plasmid DNA was diluted in 100 μl OptiMEM, and 7 μl (7 μg) Lipofectin was mixed concurrently with another aliquot of 100 μl OptiMEM. Both were allowed to stand for 45 min at room temperature, after which time they were mixed together and then left to stand for a further 30 min. The 200 μl mixture was then dispensed into a well, having previously washed the cell monolayer with serum-free OptiMEM. After an incubation period of 5 h at 37 °C, the transfection medium was removed and the cell monolayers were washed once again with OptiMEM, followed by the addition of 4 ml fresh growth medium before reincubation for a total of 96 h. All transfections were performed in replicates of seven. The cells from one well were removed by trypsinization and used for determination of transfection efficiency by FACS analysis. The medium from all remaining wells was removed and that from three wells was retained for estimation of the number of released virions. The cells were washed with PBS (pH 7.2) and three wells each were subsequently used to extract total RNA and DNA, as detailed below. The p3.8II construct and the empty pBluescript II KS(+) vector were used as positive and negative controls, respectively. The experiments were repeated on three separate occasions and the results therefore represent the mean of nine replicates. Finally, the cultures were monitored on a daily basis for evidence of cytotoxicity, with no overt signs of such an effect.

**Extraction of HBV DNA from cell-culture media.** Media from transfected cells were first clarified by centrifugation at 3000 r.p.m. for 10 min in order to remove detached cells or cell debris and then aliquots of 200 μl were digested with 1.5 U DNase I (Ambion) and 2 U mung bean nuclease (New England BioLabs) in the presence of 8 mM CaCl\(_2\) and 6 mM MgCl\(_2\) for 20 min at 37 °C (Parekh et al., 2003). This treatment removed non-particle-associated double-stranded DNA, and single-stranded DNA and RNA, respectively. After digestion, the nucleases were heat-inactivated at 75 °C for 5 min and HBV DNA was then extracted from intact virions by proteinase K digestion as described previously (Karayiannis et al., 1995), resuspended in 10 μl ultrapure distilled water and frozen at −20 °C until use. Although cell-culture monitoring during the incubation...
period on a daily basis did not show any overt cytotoxicity, it is acknowledged that some of the HBV DNA may have been derived from released core particles from damaged cells.

**Extraction of intracellular HBV DNA.** Intracellular HBV DNA replicative intermediates, in the form of RC-dsDNA, were extracted from cell lysates following removal of the nuclei. Cells were detached with the help of a rubber policeman and suspended in 400 µl lysis buffer [10 mM Tris/HC1 (pH 8.4) at 4 °C, 1.5 mM MgCl2, 0.14 M NaCl, 2 µl 5% Nonidet P-40] and the nuclei were removed by pelleting as described by Sambrook & Russell (2006). The supernatant containing core particles was removed carefully and DNase I with mung bean nucleas was added as described above, together with adjustment of the MgCl2 to 6 mM and addition of 12 mM CaCl2. After digestion at 37 °C for 20 min and inactivation of the nucleases as described above, the lysates were digested with protease K at 37 °C for 2 h, following addition of 1 % SDS as described previously (Karayiannis et al., 1995). Digests were processed as described above and the HBV DNA pellet was resuspended in 20 µl ultrapure water, followed by storage at −20 °C until use.

**Extraction of HBV transcript RNA.** HBV transcript RNA was prepared by extraction of total cellular RNA using a PUREscript kit according to the manufacturer’s instructions (Flowgen Bioscience). Briefly, 300 µl cell-lysis solution was added to each well and the cell lysate was then transferred to an Eppendorf tube containing 300 µl protein–DNA precipitation solution. The contents of the tube were mixed thoroughly before centrifugation. The aqueous phase was then extracted with phenol/chloroform and the RNA was precipitated by using 2-propanol. After washing with 70% ethanol and drying, the pellet was resuspended in 20 µl ultrapure water and treated with DNase I, followed by its deactivation as described above. The RNA was finally stored at −80 °C.

**cDNA synthesis.** Total RNA (5 µl) and primer BC1 (1 µl; 50 pmol; Carman et al., 1989) were made up to 10 µl with RNase-free water and incubated for 10 min at 65 °C. The mixture was then ice-cooled and the volume was made up to 20 µl by adding the following to final concentrations: 250 mM Tris/HCl, 200 mM KCl, 25 mM MgCl2, 2.5 % Tween 20, 10 mM dithiothreitol, 2.5 mM each dNTP, 20 U RNase inhibitor and 50 U Expand reverse transcriptase (Roche). This mixture was incubated at 42 °C for 90 min in a thermocycler, after which time it was placed immediately on ice.

**Real-time quantitative PCR.** The relative levels of extracellular and intracellular HBV DNA, as well as pgRNA and pre-C mRNA transcript concentrations, were analysed by real-time quantitative PCR with a Quantitech SYBR Green PCR Master-Mix kit (Qiagen). LightCycler capillary tubes contained 10 µl QuantiTech mix, 1 µl each primer at 0.5 mM (F1, 5'-GTAATGTCGCACTTGACTCC-3', positions 436–458; R1, 5'-CCTTGGCGGAGCAGCTA-3', positions 706–689), 7 µl sterile distilled water and 1 µl extracted HBV DNA. Cycling consisted of a holding step at 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. This was followed by a data-acquisition step at 80 °C for 15 s to check the specificity of the PCR product. Further confirmation was obtained by agarose-gel electrophoresis of the PCR products.

For the detection of pgRNA and pre-C mRNA, the cDNA samples were diluted 1:30 and 1 µl diluted sample was used as template in LightCycler reactions set up as described above. The primers used were PCP (5'-TGCTGTACCGACGACCA-3', positions 1797–1814) and BC1 (Carman et al., 1989), which detected pre-C mRNA specifically, and PGP (5'-CACCACCCGATTACATCTCA-3', positions 1826–1846) and BC1, which detected both pre-C mRNA and pgRNA (Laras et al., 2002). Residual HBV DNA contamination of RNA preparations after DNase I digestion was excluded by PCR amplification with primers M3 (which binds upstream of the BCP) and BC1 (Carman et al., 1989).

**Standard curves and quantification.** The copy number of construct p3.8II was determined following spectrophotometric measurement of the A260 of a preparation. This was then used to set up a range of dilutions ranging from 10⁻⁴ to 10⁻² copies ml⁻¹. Transcript copy number was normalized to β-actin levels, which were estimated in each experiment. cDNA synthesis and PCR amplification were run in parallel with HBV DNA tests. β-Actin cDNA synthesis was carried out as described above, using the antisense primer actinAS 5'-CAGTTCAGTGGAATTTGA-3', and for the amplification, sense primer actinS 5'-GAACCTAGGACCATGTG-3' and actinAS. Finally, all results were standardized to 15% transfection efficiency, based on the results obtained by FACs analysis of fluorescing cells co-transfected with pCI-GFP.

**HBV antigen detection.** Culture supernatants were tested for HBsAg by using a Murex HBsAg v. 3 kit (Abbott Laboratories) after a 1:5 dilution. Results were expressed in ng ml⁻¹, using yeast recombinant HbsAg (Recombinax HB3; Merck) to prepare a range of dilutions for a standard curve. HBsAg was detected qualitatively with a VIDAS Hbe/anti-Hbe kit (bioMérieux). Samples giving an index reading of ≥0.1 were deemed positive for HBeAg. Culture supernatants were adjusted based on HBsAg levels and the FACS results, before testing for HBeAg. The index readings were thus taken to reflect semiquantitatively the levels of HBeAg expression in each culture tested.

**EMSA.** Sense and antisense oligonucleotides of the sequence 5'-ATTAGGTTAAAGGTCCTT-3' (positions 1752–1769), bearing the mutations listed in bold in Supplementary Table S1 (available in IGV Online) and representing the BCP mutated regions, were mixed in equimolar quantities in annealing buffer [10 mM Tris/HCl (pH 7.5), 20 mM NaCl] to a final volume of 100 µl (4.8 pmol µl⁻¹). The mixture was heated to 95 °C and then allowed to cool slowly to room temperature. Annealed oligonucleotides were end-labelled by using T4 polynucleotide kinase and [γ-³²P]ATP. Removal of non-incorporated oligonucleotides was achieved by spinning through a 'clean-up' column (MicroSpin S-200HR; Amersham Biosciences). Huh7 cells cultured to confluence were used for extraction of nuclear transcription factors as described by Jiang et al. (1995).

A standard DNA–protein binding reaction comprised 7 µl ultrapure water, 2 µl 5 × Gel Shift binding buffer [20 % glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 250 mM NaCl, 50 mM Tris/HC1 (pH 7.5)], 1 µg poly(dI):poly(dC) µl⁻¹ (Amersham Biosciences) and 5 µg nuclear extract in a final volume of 10 µl. The preparation of the nuclear extract was as described by Jiang et al. (1995). After standing on ice for 10 min, 1 µl appropriate end-labelled double-stranded oligonucleotide was added, followed by incubation on ice for 30 min. In competition experiments, a 50-fold molar excess of unlabelled competitor was added to the mixture prior to the addition of nuclear extracts. Products were electrophoresed on a 4 % non-denaturing polyacrylamide gel in 0.5 × TBE buffer. An oligonucleotide binding in the polymerase region and its complementary sequence were used as a negative control (5'-TTTGCTACGTATAGTG-3', positions 670–687).

**Statistics.** Mean variation between two samples was compared with a Mann–Whitney U test, and Kruskal–Wallis analysis of variance was used in group comparisons. Correlations (two-tailed) were tested with Spearman’s correlation. The spss v. 10 package (SPSS Inc.) was used to perform all of these analyses. A P value of <0.05 was deemed significant.
RESULTS

Replication capacity of pre-C and BCP constructs based on numbers of virions released

The replication capacity of each construct, measured in terms of virion numbers released in the culture medium by 96 h post-transfection, is shown in Fig. 1. There was no significant difference in virion yield between the WT and constructs bearing the G1896A or G1899A substitutions in the pre-C region alone, together or in combination with the double BCP mutation (A1762T/G1764A) (P > 0.05; Fig. 1a). Virion yield ranged between 0.95× and 1.10× that obtained with the WT construct. Similar measurements in culture medium following transfection with constructs bearing each of the BCP mutations alone, together or in combination with additional mutations, as shown in Fig. 1(b), showed that constructs bearing an A1762T or G1764A substitution, or the two together, replicated with efficiency equal to that of the WT. On the other hand, three constructs, with the substitutions C1766T/T1768A, A1762T/G1764A/C1766T and T1753C/A1762T/G1764A/C1766T, released significantly fewer (56-fold; P < 0.001).

Replication capacity of pre-C and BCP constructs based on RC-dsDNA

Measurement of RC-dsDNA levels followed the same trend as that observed for released virions, without exception. Fig. 2(a, b) shows the results obtained with the various constructs containing mutations in the pre-C and BCP regions, respectively. The results were expressed in copies per cell based on cell numbers expressing GFP, as estimated by FACS analysis. These ranged between 38 and 45 for the WT and for other constructs with equal replication capacity, whilst they rose to 61, 63 and 85 for the A1762T/G1764A/
C1766T, T1753C/A1762T/G1764A and C1766T/T1768A constructs, respectively ($P<0.05$), and dropped to 10 for T1753C/A1762T/G1764A/C1766T ($P<0.01$).

**Replication capacity of pre-C and BCP constructs based on HBV RNA transcript detection**

Fig. 3 shows the results obtained for pre-C mRNA and pgRNA transcript measurements. Whether using the constructs with pre-C or BCP mutations, pre-C mRNA ratios were significantly lower than those for pgRNA for the respective cultures. For the WT, the pgRNA:pre-C mRNA ratio was 3.7:1. Looking at pgRNA levels first, there was no difference in the levels detected between the WT and constructs carrying mutations in the pre-C region, when each one was present alone, together or in combination with the A1762T/G1764A double BCP mutation (Fig. 3a). These results were in full agreement with those obtained for virion release and intracellular HBV DNA measurements. In the case of the BCP mutants (Fig. 3b), higher levels of pgRNA transcripts were evident with the constructs that had released more virions into the medium and had higher levels of intracellular HBV DNA. Mutants A1762T/G1764A/}

C1766T, T1753C/A1762T/G1764A and C1766T/T1768A produced 2.1-, 2.2- and 4.3-fold higher levels for this transcript than the WT ($P<0.02$, $<0.02$ and $<0.01$, respectively). On the other hand, the T1753C/A1762T/G1764A/C1766T mutant produced 3.4-fold less pgRNA transcript than the WT ($P<0.01$).

Pre-C mRNA levels were reduced further from those of the WT in cultures of constructs carrying the G1986A stop-codon mutation, and therefore having an HBeAg-negative phenotype. Thus, 4.3-, 3.4- and 3.4-fold lower levels of pre-C mRNA were detected with the G1986A, G1986A/G1899A and A1762T/G1765A/G1896A constructs, respectively ($P<0.03$; Fig. 3a). No significant change was recorded for the other constructs in comparison with the WT. Similarly, there was no significant change in pre-C transcript levels between WT and BCP mutants A1762T and G1764A. In contrast, pre-C mRNA levels were 0.7-fold lower in cultures transfected with mutants A1762T/G1764A and A1762T/G1764A/C1766T than in those transfected with the WT ($P<0.02$). Lower levels were also recorded for the T1753C/A1762T/G1764A construct, but the difference was not significant ($P<0.46$). The exception was the C1766T/T1768A construct, with 1.9-fold higher levels of pre-C mRNA than the WT ($P<0.02$). Finally, a 14-fold decrease was recorded with the T1753C/A1762T/G1764A/C1766T construct compared with the WT ($P<0.001$).

**Detection of HBV antigens**

Cell-culture supernatants 96 h post-transfection were first tested for HBsAg and the results are shown in Fig. 4. The readings, expressed in ng ml$^{-1}$, reflected the trend obtained with all other measurements, with the exception that increases were not as dramatic. HBeAg was undetectable in culture media from all pre-C stop codon-containing variant constructs, as well as in those from the T1753C/A1762T/G1764A/C1766T construct. Table 1 shows the index values (test $A_{450}$ reading divided by that of the standard) following HBeAg testing of supernatants. Reduced HBeAg levels were recorded with BCP mutants T1753C/A1762T/G1764A, A1762T/G1764A and A1762T/G1764A/C1766T in descending order, whilst construct C1766T/T1768A produced higher levels of HBeAg than the WT, reflecting the results obtained for pre-C mRNA measurements.

**Promoter analysis**

Fig. 5 shows the results obtained by EMSA, following interaction of the radiolabelled double-stranded oligonucleotides representing mutated BCP sequences and Huh7 nuclear extracts. At least two bands were visible on the gel, indicating interaction with transcription factors present in the nuclear extract. Nuclear extracts from an osteosarcoma cell line (143-TK$^+$) and an oligonucleotide from the polymerase region produced no such bands (data not shown for the latter). The intensity of the bands was
dependent on the mutations present, and infectious clones that exhibited increased replicative capacity in transfected cells also appeared to bind transcription factors more strongly (C1766T/T1768A, A1762T/G1764A/C1766T and T1753C/A1762T/G1764A). On the contrary, the T1753C/A1762T/G1764A/C1766T oligonucleotide bound the lowest amount of transcription factor(s), reflecting the impaired replication capacity recorded after transfection. Finally, double-stranded oligonucleotides bearing the A1762T and G1764A mutations, either alone or in combination, had reduced binding capacity in comparison to the WT, which, however, did not appear to have a significant reduction in replication capacity.

**DISCUSSION**

In this study, quantitative measurements of released virions and of intracellular replicative intermediates (RC-dsDNA and pgRNA) have been undertaken by using real-time PCR following transfection of HBV constructs bearing mutations in the BCP and pre-C regions. In an attempt to eliminate possible interference from additional mutations elsewhere in the genome, these mutations were engineered into the same genetic background, so that any recorded changes in replicative capacity, if any, would be the result of the specific mutation(s) per se. In addition, we addressed the issue of combined mutations in the BCP and pre-C region and their impact on replication, a topic of some controversy.

Both the double BCP and the pre-C stop-codon mutations can be detected at low frequency during the late HBeAg-positive phase of chronic HBV infection, and become the predominant quasispecies following seroconversion to anti-HBe (Okamoto et al., 1990). These mutations down-regulate or altogether abrogate HBeAg production (Buckwold et al., 1996; Moriyama et al., 1996; Scaglioni et al., 1997) and may therefore contribute to seroconversion to anti-HBe and immune clearance of the WT virus. Reduced or absent HBeAg production may be one of the factors contributing to fulminant hepatitis, as both of

**Table 1.** Index values of HBeAg measurements in supernatants from Huh7 cells transfected with the constructs shown

<table>
<thead>
<tr>
<th>Construct</th>
<th>Index</th>
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<tbody>
<tr>
<td>WT</td>
<td>0.40 (±0.04)</td>
</tr>
<tr>
<td>A1762T/G1764A</td>
<td>0.25 (±0.02)</td>
</tr>
<tr>
<td>A1762T/G1764A/C1766T</td>
<td>0.17 (±0.03)</td>
</tr>
<tr>
<td>T1753C/A1762T/G1764A</td>
<td>0.32 (±0.03)</td>
</tr>
<tr>
<td>T1753C/A1762T/G1764A/C1766T</td>
<td>0.02</td>
</tr>
<tr>
<td>C1766T/T1768A</td>
<td>0.45 (±0.05)</td>
</tr>
<tr>
<td>G1896A</td>
<td>0.01</td>
</tr>
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</table>

Values are from pooled supernatants of three replicates. An index of ≥0.1 denotes a positive result.
the above mutations have been associated with a severe outcome when virus bearing these mutations is transmitted \textit{ab initio} (Carman et al., 1991; Friedt et al., 1999; Hasegawa et al., 1994; Kaneko et al., 1995; Kosaka et al., 1991; Laskus et al., 1995; Liang et al., 1991; McMillan et al., 1996; Omata et al., 1991; Sato et al., 1995; Sterneck et al., 1996). Moreover, a high-replication phenotype, resulting from the presence of core promoter mutations, would be consistent with the suggestion that rapid replication of HBV may be a contributor to fulminant hepatitis (Buckwold et al., 1996; Hasegawa et al., 1994; Liang et al., 1991; Moriyama et al., 1996). Two such genomes associated with fulminant hepatitis B (G1896A/G1899A and C1766T/T1768A) were found to replicate to a very high level (Baumert et al., 1996; Scaglioni et al., 1997). Moreover, additional mutations, such as the T1753C and C1766T substitutions, that also appear to have a high-replication phenotype have recently been described, each in association with the classic A1762T/G1764A double mutation or all together (Parekh et al., 2003).

Previously, the effect of the double BCP mutant (A1762T/ G1764A) on HBV RNA transcription has been assessed by reporter-gene expression, whilst more recent studies of replication capacity have employed Southern blot analysis. These studies have produced conflicting findings, with some reporting increases in viral replication in conjunction with a decrease in pre-C mRNA synthesis (Baumert et al., 1996; Buckwold et al., 1996; Moriyama et al., 1996). A variety of hypotheses have been proposed explaining the increase in viral replication, attributing it to increased encapsidation (Buckwold et al., 1997; Hasegawa et al., 1994; Scaglioni et al., 1997), increased pgRNA transcription (Moriyama et al., 1996; Moriyama, 1997) and enhanced core particle production (Buckwold et al., 1996) and/or downregulation of synthesis of pre-C mRNA, which, as already mentioned, encodes the pre-C protein precursor of HBeAg (Buckwold et al., 1996; Gunther et al., 1998; Moriyama et al., 1996; Moriyama, 1997). In contrast, others have reported no increase in viral replication, only reduced pre-C mRNA production or decreased HBV DNA titres in the presence of BCP mutations, suggesting no increases in viral replication (Gunther et al., 1998; Sterneck et al., 1998).

The single A1762T, G1764A, G1896A and G1899A substitutions had no significant effect on replication capacity in comparison to the WT (Gunther et al., 1998; Parekh et al., 2002; Sterneck et al., 1998); nor did the A1762T/G1764A mutant, as documented by virion release and intracellular RC-dsDNA and pgRNA levels. In contrast, enhanced replication relative to the WT was seen in all core promoter mutants bearing additional mutations in this region (Parekh et al., 2003). A notable exception was the T1753C/A1762T/ G1764A/T1766A mutant, which was severely impaired, this being at variance with the results reported by Parekh et al. (2003). This may relate to the different genotype background used in this study (genotype C) from that in which the mutations were first described (genotype A), an issue discussed further below. No differences in replication capacity were demonstrated by the combined BCP and pre-C recombinants. The effect of these mutations in different genotypic backgrounds on virus replication is an issue warranting further comparative studies.

The levels of pre-C mRNA detected were significantly lower than those of pgRNA in all recombinant constructs (Laras et al., 2002). These were even lower in constructs with core promoter mutations, this being in agreement with readily detectable HBeAg in WT-transfected cells, whilst in most of the BCP recombinants, this marker was detectable at reduced levels (Parekh et al., 2003). The only exception was the C1766T/T1768A construct, in which HBeAg expression was higher than that of WT. Interestingly, constructs bearing the pre-C stop-codon mutation also had pre-C mRNA levels lower than those of the WT. In this case, it would appear that this reduction may be the result of mRNA degradation in the absence of full pre-C protein translation, rather than being due to downregulated synthesis of this transcript. In contrast, the pgRNA levels were raised significantly in all cultures of recombinants exhibiting increased viral replication. HBeAg shares epitopes with hepatitis B core antigen (HBcAg) and has been postulated to induce immune tolerance against itself or HBcAg, or both (Milich et al., 1990). In the presence of reduced or absent serum HBeAg, HBcAg may be targeted directly by both the cellular and humoral immune systems, leading to necrosis of hepatocytes and liver damage, which may be severe enough to cause fulminant disease.

In order to explain the increased viral replication seen with some of the BCP constructs, promoter-activity analysis was undertaken by using EMSA and reporter-gene expression. Increased binding of factors from Huh7 nuclear extracts was evident with oligonucleotides representative of BCP sequences with increased replication capacity. Two main bands were obvious on the gels; the most plausible explanation for this is the binding of two alternative transcription factors to this region. It is well known that the EnhII/CP region, which includes the BCP, binds several transcription factors, some of which have overlapping binding sites (Raney et al., 1997; Yu & Mertz, 1997; Zheng et al., 2004). It is conceivable, therefore, that nucleotide substitutions affecting some of these binding sites may in fact prevent the binding of a particular transcription factor, whilst allowing that of another. Indeed, it is postulated that the double BCP mutation abolishes the binding of chicken ovalbumin upstream promoter transcription factors (COUP-TFs) PPARα-RXRα and TR4, but retains the binding of HNF4 (Moriyama, 1997; Tang et al., 2001; Yu & Mertz, 2001). In addition, this double mutation creates an HNF1-binding site (Li et al., 1999; Tang et al., 2001). The binding of different transcription factors is thought to regulate the synthesis of the pgRNA and pre-C mRNA differentially. Our findings, however, did not show differences in the migration pattern of the bands, which would have been suggestive of alternative transcription factors binding to these sites. Rather, there was a change in band intensity depending on the mutations present, indicating
either a more effective or reduced binding of the same factor(s). Such a suggestion is supported by recent findings showing differential binding of COUP-TF1 between different HBV genotypes (Fischer et al., 2006). Nevertheless, further experiments involving the use of nuclear cell extracts from other cell lines of hepatic origin (to strengthen the above results), as well as experiments to identify the transcription factor(s) involved in the binding, are warranted.

Taken together, the results reported here suggest that a high HBV replication phenotype is associated with additional mutations in the BCP region, other than the well-known double mutation. In addition, it appears that such mutations may allow for a more efficient binding of transcription factors that may, in turn, regulate HBV transcript synthesis differentially.

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