Genetic dissection of naturally occurring basal core promoter mutations of hepatitis B virus reveals a silent phenotype in the overlapping X gene

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During chronic hepatitis B virus (HBV) infection, double substitution mutations in the basal core promoter (BCP) region frequently emerge that include A1762T/G1764A and the neighbouring C1766T/T1768A mutations, here termed BCP1 and BCP2, respectively. Due to a compact viral genome organization, BCP1 and BCP2 mutations result in amino acids changes in the overlapping X gene: K130M/V131I and F132Y, respectively. It has been shown that both BCP mutations lead to a modest increase in viral genome replication. However, the question of whether the alteration that occurs in the overlapping X gene might contribute to the increased viral genome replication has not been properly addressed. This study genetically separated the core promoter from the overlapping X gene using 1.3mer overlength HBV constructs and examined the impact of the X gene mutations on viral genome replication in HepG2 cells. Each BCP mutation resulted in modestly enhanced viral genome replication that occurred via augmented viral transcription. Therefore, it was concluded that these BCP mutations do not affect expression of the overlapping X gene or impair its stimulatory effect on viral genome replication.

INTRODUCTION

Persistent infection with hepatitis B virus (HBV) represents a major health problem worldwide, with more than 350 million chronically infected patients at risk of developing liver cirrhosis or hepatocellular carcinoma (HCC) (Seeger et al., 2007). HBV has a small (3.2 kb) circular DNA genome that encodes the envelope (S), core (pre-C/C) and polymerase (P) genes in a compact, overlapping arrangement that utilizes all three reading frames of the DNA. Two species of overgenome-length 3.5 kb RNAs are transcribed, called C RNA (pre-genomic RNA or pgRNA) and pre-C RNA (Fig. 1a). The C RNA encodes the core protein (HBcAg), whilst the pre-C RNA encodes the pre-core protein, which contains an N-terminal extension of 29 aa (the pre-C region) (Fig. 1a). The pre-C region contains a signal peptide that leads to expression of the e antigen (HBeAg) following truncation of both N-terminal and C-terminal segments (Fig. 1a).

In addition, mammalian hepadnaviruses encode a fourth major open reading frame (ORF), the X gene (encoding the HBx protein) (Seeger et al., 2007). It was thought that the X gene might contribute to viral pathogenicity, as three pathogenic mammalian hepadnaviruses – human hepatitis B virus, woodchuck hepatitis virus and ground squirrel hepatitis virus – contain the X ORF, whilst the less pathogenic avian viruses do not (Bouchard & Schneider, 2004). The biological importance of HBx has been elusive for some time as it appears to be dispensable for viral genome replication in Huh7 cells, a human hepatoma cell line (Blum et al., 1992; Melegari et al., 1998). The requirement of HBx for the establishment of persistent viral infection was first demonstrated in the woodchuck model (Zoulim et al., 1994). Subsequently, it was shown that HBx indeed stimulates viral genome replication in HepG2 cells (but not in Huh7 cells) by about five- to tenfold (Melegari et al., 1998) at both the transcriptional and post-transcriptional levels (Bouchard et al., 2001; Tang et al., 2005). Thus, the HepG2 cell line was used to investigate the impact of HBxs on viral genome replication.

Chronic infection frequently results in the accumulation of core gene region variants (Gunther et al., 1999). In particular, core promoter mutants emerge as the dominant viral population at the late stages of chronic infection (Parekh et al., 2003). The appearance of core promoter mutants is often associated with a reduced HBeAg level but moderately enhanced viral replication (Baumert et al., 1998; Buckwold et al., 1996; Moriyama et al., 1996; Scaglioni et al., 1997). Reduced HBeAg levels and augmented viral replication have been associated with enhanced development of liver diseases. One of the most prevalent mutations includes the double A1762T/G1764A basal core promoter (BCP) mutation (here termed BCP1) (Fig. 1b) (Okamoto et al., 1994). In addition, a C1766T/T1768A double mutation in the BCP region (here termed...
BCP2) has been isolated from a patient with fulminant hepatitis (Baumert et al., 1996, 1998). It has been shown that both BCP1 and BCP2 mutants displayed significantly increased viral genome replication (Baumert et al., 1996, 1998; Buckwold et al., 1996, 1997; Scaglioni et al., 1997), although the underlying mechanisms for this increase differed. However, due to the compact nature of the genomic structure of HBV, both BCP1 and BCP2 mutations result in amino acid alterations in the overlapping X gene, K130M/V131I and F132Y, respectively (Fig. 1b). The impact of the BCP mutations on the overlapping X gene has not been examined directly via genetic dissection of the X gene in previous studies (Baumert et al., 1996; Li et al., 1999). More importantly, all aforementioned studies on the BCP mutations were performed in Huh7 cells, where the stimulation of viral genome replication by HBx has not been observed (Blum et al., 1992; Melegari et al., 1998).

In this report, we genetically dissected the core promoter from the overlapping X gene using a 1.3mer overlength HBV genomic construct. We then introduced the double-substitution mutations into either the core promoter region or the X gene region separately and then tested the effect of these mutations on viral replication in HepG2 cells. The data indicated that both BCP1 and BCP2 mutations led to approximately twofold enhancement of viral genome replication in comparison with the wild type (WT). In addition, enhanced genome replication could be fully accounted for by the increased level of viral transcripts (both pre-C and C RNA). Furthermore, the enhanced viral genome replication was due to changes in the core promoter rather than in the overlapping X gene, as HBx alleles from both BCP1 and BCP2 mutants stimulated viral transcription similarly to WT. Based on these results, we concluded that changes in the BCP, but not in the overlapping X gene, are responsible for the enhanced viral genome replication observed for the BCP mutants.

**METHODS**

**Cell culture and transfection.** HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (Gibco-BRL) and 10 μg gentamicin ml⁻¹ at 37 °C in 5 % CO₂ and passed every third day. Cells were transfected using polyethylenimine (PEI; 25 kDa; Sigma-Aldrich) as described previously (Ryu et al., 2008). Briefly, cells were plated at 40–50 % confluency. Equal amounts of plasmid DNA and PEI were mixed and incubated for 10 min at room temperature. Cells were washed with DMEM and overlaid with the DNA–PEI complex solution. After 20 h, cells were rinsed and fed with fresh medium. Transfection efficiencies of 10–20 % routinely were obtained.

**Plasmid construction.** All substitution and deletions mutants were generated by overlap extension PCR protocols as described previously (Lee et al., 2004). All mutants were sequenced to confirm the base change(s). The details of the molecular cloning of any plasmid construct described in this report will be provided upon request. The 1.3mer overlength WT HBV replicon construct (containing 1.3 units of
the HBV genome spanning nt 1234–1978 of HBV ayw subtype) was prepared as described previously (Cha et al., 2009) and encoded two copies of the X ORF at both the 5’ and 3’ ends of the HBV genome. To preclude HBx expression from the overlapping X ORF, the 5’ end of the X ORF was inactivated by introduction of a stop codon immediately following the first and second AUG codon, as described previously (Cha et al., 2009). The HBx-null control plasmid was constructed as described previously (Cha et al., 2009). Subsequently, substitutions corresponding to the BCP1 and BCP2 mutants were introduced into the BCP region of the WT X-null construct (Fig. 2). The HBx expression plasmid pCMV-HBx, in which three tandem copies of the FLAG epitope were inserted at the C terminus of HBx, has been described previously (Cha et al., 2009). The two mutant HBx expression constructs, HBx/BCP1 and HBx/BCP2, were prepared in parallel.

**Extraction of viral DNA.** Viral DNA from cytoplasmic capsids was isolated from HepG2 cells at 4 days post-transfection, as described previously (Pugh et al., 1988). Briefly, cells were resuspended in lysis buffer [50 mM Tris/ HCl (pH 8.0), 1 mM EDTA, 1% NP-40] and incubated at 37 °C for 15 min. Nuclei and cell debris were removed by centrifugation. Magnesium acetate (final concentration 10 mM) and DNase I (final concentration 500 U ml⁻¹) were added to the supernatant and the mixture was incubated at 37 °C for 45 min to digest the transfected DNA. Polyethylene glycol (6.5%, w/v, PEG8000; Sigma) was added to precipitate the capsids and the mixture was incubated at 37 °C for 45 min. Viral capsids were resuspended in proteinase K buffer [50 mM Tris/ HCl (pH 8.0), 1 mM EDTA, 0.5% SDS] with 300 µg proteinase K ml⁻¹ and incubated for 30 min at 4 °C followed by centrifugation for 4 min. Viral capsid DNA was extracted from the digested capsid with an equal volume of phenol, precipitated with ethanol and resuspended in Tris/EDTA buffer.

**Southern blot analysis.** Southern blot analysis was performed as described previously (Shin et al., 2004). Briefly, the extracted viral DNA was separated by electrophoresis through a 1.3% agarose gel in 0.5 × Tris/acetate/EDTA buffer and transferred onto a nylon membrane. The membrane was pre-hybridized and then hybridized with a 32P-labelled full-length HBV DNA probe in hybridization solution for 16 h at 65 °C. Images were obtained using a PhosphorImager (BAS-2500; Fujifilm).

**Primer-extension analysis.** Primer-extension analysis was performed as described previously (Li et al., 1999). Briefly, primer extension using a primer comprising nt 2051–2024 of the HBV ayw subtype was used to map the 5’ end of the viral transcripts. Total cellular RNA was isolated from HepG2 cells by an acid guanidinium isothiocyanate/phenol/chloroform extraction method (Chomczynski & Sacchi, 1987) and selected by poly(A). The primer was end-labelled with [γ-32P]ATP and hybridized to the poly(A)-selected RNA. Primer extension was carried out using Moloney murine leukemia virus reverse transcriptase (New England Biolabs) according to the manufacturer’s protocol. The primer extension products were separated on a 6% polyacrylamide/urea gel, which was dried and imaged using a PhosphorImager.

**RNase protection assay (RPA).** RNA extraction was performed as described above in the primer-extension assay (Chomczynski & Sacchi, 1987) and an RPA was performed essentially as described previously (Jeong et al., 2000). The riboprobe was derived from the core region (nt 1903–2140) of the promoter as described previously (Cha et al., 2009). Briefly, samples of total RNA or core-associated RNA (30 µg) were hybridized for 16 h at 42 °C with two probes labelled with [x-32P]UTP (3000 Ci mmol⁻¹; Amersham). RNase digestions were carried out with a mixture of RNase A and RNase T1 (Ambion) at 37 °C for 30 min. The digested products were separated on a 6% acrylamide/8% urea gel, which was dried and imaged using a PhosphorImager.

**RESULTS**

**Experimental strategy**

To genetically separate the core promoter region from the X gene, we generated a set of 1.3mer overlength HBV constructs (Fig. 2) that were capable of initiating viral genome replication, as described previously (Cha et al., 2009). To preclude HBx expression from the overlapping X ORF, the 5’ end of the X ORF was inactivated by the introduction of a stop codon immediately following the first and second AUG codons (Fig. 1b), as detailed in Methods. To examine the impact of HBx expression on viral genome replication, the 3’ end of the X ORF was similarly inactivated in the three constructs designated X-null (Fig. 2). Subsequently, BCP1 and BCP2 substitution mutations were introduced into the BCP region, designated BCP1 X-null and BCP2 X-null, respectively (Fig. 2). Previously, it was reported that the BCP1 mutation resulted in approximately twofold enhancement of viral genome replication without affecting the level of C RNA (Buckwold et al., 1996; Li et al., 1999). However, in these previous studies, the impact of HBx may not have been observed because (i) Huh7 cells were employed, in which the stimulation of viral genome replication by HBx does not occur (Blum et al., 1992; Melegari et al., 1998), and (ii) the X gene was not separated genetically.

**Complementation of the BCP1 X-null construct by WT HBx**

To gain more insight into the effect of the BCP1 mutation, we examined the impact of WT HBx on viral genome replication in HepG2 cells, in which stimulation of viral
genome replication by HBx has been reproducibly observed (Bouchard et al., 2001; Cha et al., 2009; Melegari et al., 1998). Cells were transfected with 18 μg of the WT X-null construct or BCP1 X-null construct, along with 1.8 μg of WT HBx expression plasmid per 100 mm plate as indicated in Fig. 3(a). We previously established that HBx expressed under these conditions does not have a saturating effect on genome replication (Cha et al., 2009). Four days after transfection, the viral replication intermediates isolated from capsids were analysed by Southern blot analysis, as detailed in Methods. When only the WT X-null construct was transfected, viral DNA was barely detectable (Fig. 3, lane 1). By contrast, when the WT X-null construct was complemented by the HBx expression plasmid, the level of viral DNA was significantly increased (Fig. 3a, lanes 1 and 2, and Fig. 3b). Consistent with previous reports (Bouchard et al., 2001; Cha et al., 2009; Melegari et al., 1998), these data indicated that WT HBx stimulated viral genome replication by about fivefold (Fig. 3a, lanes 1 and 2, and Fig. 3b). Notably, when the BCP1 X-null construct was complemented by HBx, the level of viral DNA was significantly higher than that obtained with the WT X-null construct (Fig. 3a, lanes 2 and 4, and Fig. 3b). Nonetheless, the magnitude of stimulation by HBx was estimated to be about fivefold (Fig. 3a, lanes 3 and 4, and Fig. 3b), as enhancement of DNA synthesis by the BCP1 X-null construct was approximately twofold higher than that by the WT X-null construct (Fig. 3a, lanes 1 and 3). Collectively, approximately twofold enhancement of viral genome replication was observed when the BCP1 X-null construct was used compared with the WT X-null construct (Fig. 3a, lanes 2 and 4, and Fig. 3b). These data suggested that enhanced genome replication of the BCP1 mutant was more likely attributable to changes in the core promoter than in HBx, as the BCP1 X-null construct alone led to enhanced genome replication relative to the WT X-null construct (Fig. 3a, lanes 1 and 3).

**Complementation of the BCP2 X-null construct by WT HBx**

Next, we examined the impact of WT HBx on the BCP2 X-null construct (Fig. 4). HepG2 cells were transfected as indicated in Methods and viral replication intermediates were measured by Southern blot analysis. These data indicated that the BCP2 X-null construct had an approximately twofold higher level of viral genome replication compared with the WT X-null construct (Fig. 4a, lanes 1 and 3, and Fig. 4b). In addition, the data showed that both WT X-null and BCP2 X-null replicons were similarly stimulated (by approximately fivefold) by HBx.

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**Fig. 3.** The BCP1 core promoter results in enhanced viral DNA synthesis. (a) Southern blot analysis of viral replication intermediates following transfection with the WT X-null and BCP1 X-null constructs. HepG2 cells were transfected as indicated with 18 μg of the 1.3mer overlength HBx-null construct, along with 1.8 μg of the HBx expression plasmid per 100 mm plate. Viral DNA was extracted at 4 days post-transfection and Southern blotting was performed as described in Methods. The replicative DNA intermediates are: RC, relaxed circular DNA; DL, duplex linear DNA; SS, single-stranded DNA. A restriction fragment representing one HBV genomic unit (3.2 kb) served as a size marker (SM; lane 5). (b) Quantification analysis was performed with data obtained from four independent transfections. The value for lane 2 (WT X-null + HBx/WT) was set as 100. Results are shown as means ± SD.

**Fig. 4.** Mutations in the BCP2 core promoter result in enhanced viral DNA synthesis. (a) Southern blot analysis of viral replication intermediates following transfection with the WT X-null and the BCP2 X-null constructs. Transfection and Southern blotting were performed as described in Fig. 3. (b) Quantification analysis was performed with data obtained from four independent transfections. The value for lane 2 (WT X-null + HBx/WT) was set as 100. Results are shown as means ± SD.
WT HBx (Fig. 4b). Notably, this result was indistinguishable from the results for the BCP1 mutant (Fig. 3). Likewise, the enhanced genome replication in this case was most likely attributable to changes in the core promoter rather than in HBx, as only WT HBx was complemented.

Complementation of BCP X-null constructs by cognate HBx mutants

Next, we examined the stimulatory effect of HBx variants on the level of viral genome replication (Fig. 5). To

![Graphs and images showing replication and Western blot analysis results for HBx variants with BCP1 and BCP2 X-null templates.](image)

**Fig. 5.** Neither HBx/BCP1 nor HBx/BCP2 exhibits an altered ability to stimulate viral genome replication. (a) Stimulatory effect of HBx variants on replication of the WT X-null template. Transfection and Southern blotting were performed as described in Fig. 3. The enhancement of viral DNA synthesis by HBx/BCP1 was not significantly different from that of HBx/WT (P>0.05), whilst enhancement by HBx/BCP2 was significantly lower than that by WT (P<0.05). (b) Western blot analysis of HBx variants. Western blot analysis was performed in parallel with the experiment in (a) using anti-Flag antibody (diluted 1:5000; Sigma), as described previously (Cha et al., 2009). Actin detection was used as a loading control. (c, d) The stimulatory effect of HBx variants on the BCP1 (c) and BCP2 (d) X-null template. Transfection and Southern blotting were performed as described in Fig. 3.
measure the impact of each of the three HBx alleles on WT replication, the WT X-null construct was complemented by each HBx mutant individually, as well as by WT HBx, as indicated (Fig. 5a). The data showed fivefold stimulation of viral genome replication by WT HBx, as anticipated (Fig. 5a, lanes 1 and 2). When complemented by BCP1 HBx or BCP2 HBx, the extent of stimulation by BCP1 HBx was comparable to that of WT, whilst the level of stimulation by BCP2 HBx appeared to be slightly reduced (Fig. 5a, lanes 3 and 4). These data suggested that the ability of BCP2 HBx, but not BCP1 HBx, to stimulate viral genome replication was modestly diminished. This result was further substantiated by Western blot analysis performed in parallel, showing that all three HBx proteins were expressed comparably (Fig. 5b) and therefore not affected by the BCP mutations. More importantly, when the BCP1 X-null construct harbouring the BCP1 core promoter was complemented by WT HBx and BCP1 HBx, approximately fivefold stimulation of viral genome replication was observed by both WT HBx and BCP1 HBx (Fig. 5c). Likewise, when the BCP2 X-null construct containing the BCP2 core promoter was complemented by WT HBx and BCP2 HBx, approximately fivefold stimulation of viral genome replication was observed by both WT HBx and BCP2 HBx (Fig. 5d). Overall, regardless of the HBx mutation, all HBx proteins stimulated viral genome replication of the cognate core promoter mutants similarly. Based on these results, we concluded that enhanced genome replication associated with the BCP1 and BCP2 mutants was not a consequence of mutation of the overlapping X gene.

Detection of pre-C and C RNA level

Previously, it has been reported that HBx in the BCP1 mutant (here termed HBx/BCP1) suppresses viral transcription, in particular, of the pre-C RNA (Li et al., 1999). To clarify this issue and to substantiate our above findings, we measured the viral genomic transcripts using primer-extension analysis. We examined both pre-C and C RNA to see whether the BCP mutation differentially affected transcription of these two viral RNAs. The three X-null constructs were complemented by their respective HBx expression plasmids: (i) the WT X-null construct by the WT HBx expression plasmid, (ii) the BCP1 X-null construct by the BCP1 HBx expression plasmid, and (iii) the BCP2 X-null construct by the BCP2 HBx expression plasmid (Fig. 6a). Three days after transfection, whole-cell RNA was extracted and analysed by primer-extension analysis as detailed in Methods. When cells were co-transfected with the WT X-null construct and HBx/WT, both pre-C and C RNAs were detectable, whilst both RNAs were barely detectable when only the WT X-null construct was transfected (Fig. 6a, lanes 1 and 2), indicating that HBx significantly stimulated transcription of both viral RNAs. Importantly, the data indicated that HBx stimulated transcription of pre-C and C RNA to the

**Fig. 6.** Both pre-C and C RNAs are stimulated by HBx in a similar manner. (a) Primer-extension analysis to measure pre-C and C RNA. HepG2 cells were transfected as indicated and primer-extension analysis was performed as described in Methods. Two primer-extension products derived from the pre-C (260 nt) and C-RNAs (236 nt) are indicated. Three PCR fragments, serving as size markers, are shown in the lefthand lane. The magnitude of stimulation (fold increase) of pre-C and C RNA by HBx is shown below the blots. A representative of four experiments is shown. The stimulation of pre-C and C RNA transcription by HBx/BCP1 and HBx/BCP2 was not significantly different from that of HBx/WT (P>0.05). (b) HBeAg levels. The amount of HBeAg secreted into the culture supernatant was measured in parallel with (a) using a commercial enzyme immunoassay kit according to the manufacturer’s instructions (Daisorin). The absorbance was measured at 450 nm. Data are presented as means ± SD of four independent transfections.
same extent (i.e. three- to fourfold). When cells were co-transfected with the BCP1 X-null construct and HBx/BCP1, similar levels of stimulation of pre-C and C RNA transcription by HBx/BCP1 were observed (i.e. three- to fourfold; Fig. 6a, lanes 3 and 4). In contrast with a previous report (Li et al., 1999), suppression of pre-C RNA relative to C RNA was not observed with the BCP1 mutant (Fig. 6a, lane 4) (see Discussion). When cells were co-transfected with the BCP2 X-null construct and HBx/BCP2, stimulation of both pre-C and C RNAs by HBx/BCP2 was similarly observed (Fig. 6a, lanes 5 and 6). Again, in contrast with a previous report (Baumert et al., 1998), the data implied that the sequence alteration in the BCP was probably responsible for the enhanced genome replication of the BCP2 mutant (see Discussion).

To corroborate the above finding, HBeAg secreted into the culture medium of cells transfected in parallel was measured by using a commercial ELISA kit (Fig. 6b). Consistent with the above RNA analysis, the data indicated that HBeAg levels detected in the culture medium were increased approximately threefold by HBx (Fig. 6b). Importantly, the data were in agreement with the results of the primer-extension analysis, indicating that HBx stimulates pre-C RNA transcription to a similar extent, regardless of HBx mutation. Furthermore, HBeAg levels were not reduced in either the BCP1 or BCP2 mutant in comparison with WT (Fig. 6b). Based on the data shown in Fig. 6, we concluded that the enhanced genome replication manifested by both BCP1 and BCP2 mutants was not attributable to either the suppression of pre-C RNA expression or the reduced HBeAg level.

**Encapsidation efficiency of BCP mutants**

In the case of the BCP2 mutant, the enhanced viral replication has been ascribed to elevated capsid assembly (Baumert et al., 1998). Furthermore, it has been reported that the stimulation of viral genome replication by HBx is caused by enhanced production of replication-competent nucleocapsids (Melegari et al., 2005). Thus, it remains possible that changes in the HBx polypeptide contribute to the enhanced capsid assembly observed for the BCP2 mutant (Baumert et al., 1998). To address this issue, encapsidation efficiency was measured by an RPA, as detailed in Methods. The three X-null constructs were complemented with their respective HBx expression plasmids (Fig. 7). The RPA data indicated that the encapsidation efficiency of WT X-null, as measured by the amount of RNA associated with the capsids versus that in the cytoplasm, was approximately 40%, whether or not HBx was complemented, indicating that HBx does not

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<th>WT X-null</th>
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**Fig. 7.** HBx/BCP1 and HBx/BCP2 do not result in enhanced encapsidation. HepG2 cells were transfected as in Fig. 6 and an RPA was performed as described in Methods. RNA extracted from the capsid (C) and total cytoplasmic (T) fractions was analysed by RPA. Encapsidation efficiency (%) was estimated by comparing the amount of RNA detected in the C fraction with that detected in the T fraction. Representative RPA results from six experiments are shown. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured in parallel to serve as a control for both RNA extraction and encapsidation. The encapsidation efficiencies for the BCP1 and BCP2 X-null constructs were not significantly different from that of the WT X-null construct, regardless of the presence of HBx (P>0.05). The positions of riboprobes for the pgRNA and GAPDH mRNAs are indicated by open arrowheads. The protected fragments that were derived from the pgRNA and GAPDH mRNAs are indicated by closed arrowheads.
Impact of HBV core promoter mutations on the X gene

The augmented viral genome replication of the BCP1 mutant has been ascribed to reduced pre-C RNA levels (Buckwold et al., 1996). Subsequent studies indicated that the creation of a hepatocyte nuclear factor 1-binding site in the BCP region resulted in specific suppression of pre-C RNA transcription in the BCP1 mutant (Li et al., 1999; Zheng et al., 2004). In an earlier report, the authors speculated that the two amino acid changes in the HBx polypeptide led to suppressed transcription of pre-C RNAs (Li et al., 1999). In contrast with the previous observation made in Huh7 cells (Li et al., 1999), we did not observe suppression of pre-C RNA by HBx derived from the BCP1 allele in HepG2 cells (Fig. 6a, lane 4). Instead, we observed similar three- to fourfold increases of both pre-C and C RNA levels by BCP1 HBx and WT HBx (Fig. 6a). As the earlier work was performed using Huh7 cells, it is possible that the suppression of pre-C RNA occurs only in Huh7 cells, and not in HepG2 cells.

In the case of the BCP2 mutant, an approximately tenfold augmentation of viral DNA replication was observed in Huh7 cells (Baumert et al., 1996). This augmentation was ascribed to enhanced capsid assembly resulting from enhanced synthesis of core proteins at the post-transcriptional level (Baumert et al., 1998). In contrast, the data presented here showed that (i) the magnitude of augmentation of viral DNA replication by the BCP2 mutant was only about twofold in HepG2 cells (Fig. 4), as opposed to tenfold in Huh7 cells (Baumert et al., 1996), and (ii) the augmentation of viral DNA replication occurred at the transcriptional level, as opposed to the post-transcriptional level. This discrepancy could result from differences in genotype (i.e. genotype D versus genotype A), as a similar twofold increase in viral genome replication of the BCP2 mutant was observed previously with genotype D in Huh7 cells (Scaglioni et al., 1997).

There was also a discrepancy regarding the HBeAg levels detected in Huh7 cells transfected with the BCP mutants. In the BCP1 mutant, earlier work reported markedly reduced HBeAg levels in Huh7 cells (Buckwold et al., 1996; Scaglioni et al., 1997), whereas only a modest reduction (by 20%) was detected by others (Parekh et al., 2003). In the BCP2 mutant, previous work reported no reduction in HBeAg levels (Baumert et al., 1996), whereas a marked reduction in HBeAg was reported by others (Scaglioni et al., 1997). Although the reasons for these disparate results are not clear, they could be attributable to the differences in the constructs used. Our data showed that HBeAg levels were not reduced in HepG2 cells transfected with either the BCP1 or BCP2 mutant when compared with the WT (Fig. 6b). Contrary to the general belief, our results imply that the reduced HBeAg levels in the late phase of chronic infection cannot be ascribed to the BCP mutations. Relevantly, a recent clinical observation revealed a significant increase in viral diversity following HBeAg seroconversion (Lim et al., 2007). Based on our in vitro data in conjunction with the above clinical observation, we speculate that the reduced level of HBeAg might be the cause, rather than the consequence, of BCP mutations.

It was thought that higher viral replication levels associated with variants that accumulate at the late stage of chronic HBV infection are associated with enhanced development of liver diseases (Yim & Lok, 2006). However, our in vitro data showed that both BCP1 and BCP2 mutants were associated with only a modest (twofold) augmentation of viral genome replication, a phenotype that does not appear to be fully responsible for enhanced disease progression. Thus, we speculate instead that the one or two amino acid changes in HBx in the BCP mutants are not merely ‘bystander’ effects for two reasons. First, it is possible that the BCP mutation in the X gene might contribute to the selection of viral variants. Analysis of two individual single point mutants of BCP1 indicated that the A1762T mutation, but not the G1764A mutation, is fully responsible for the enhanced viral genome replication (Buckwold et al., 1997; Moriyama et al., 1996). Furthermore, a single point mutation in the doubly substituted BCP1 mutant has not been found, implicating the selective pressure imposed on the viral genome favouring the double mutation. For
instance, the T-cell response to HBx-derived epitopes, including K130 and V131, could play a role in the selection of the double mutants (Malmassari et al., 2007). Secondly, it is possible that the BCP mutations in the X gene might contribute to the progression of liver diseases, as both BCP mutations were found to be strongly associated with severe liver diseases, in particular, HCC (Baptista et al., 1999; Baumert et al., 1996; Guo et al., 2008; Kao et al., 2003). Importantly, recent retrospective analysis of patients with chronic HBV infection has indicated that the BCP1 mutation is strongly associated with HCC with an odds ratio of 30 (Liu et al., 2006). Therefore, the question of whether the mutations in the overlapping X gene might contribute to the progression of liver diseases such as HCC merits further investigation.

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involving its interaction with 5′ stem–loop structure. *Virology* 373, 112–123.


