Enhanced hepatitis B virus (HBV) pre-genomic RNA levels and higher transcription efficiency of defective HBV genomes

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Defective hepatitis B virus (dHBV) particles contain genomes corresponding to singly spliced HBV RNA. A limited number of studies show that dHBV is present in all chronically HBV-infected patients. Clinical studies have linked dHBV and dHBV gene products to high virus loads and liver damage. The replication characteristics of dHBV genomes remain poorly understood. We found that the splice donor/acceptor sites critical for the formation of dHBV genomes are conserved across HBV genotypes. We report a novel method to create dHBV constructs from corresponding wild-type (WT) HBV constructs. We assessed the transcriptional characteristics of the dHBV constructs with those of the corresponding WT HBV constructs. Interestingly, dHBV constructs had higher pre-genomic RNA levels, transcription efficiency, HBV e antigen levels and intracellular HBV core antigen levels compared with the corresponding WT HBV constructs. Our findings highlight previously unrecognized fundamental molecular characteristics of dHBV genomes and their potential role in the pathogenesis of HBV infection.

INTRODUCTION

Hepatitis B virus (HBV) proteins are encoded by unspliced mRNAs (Liang, 2009). Subgenomic HBV fragments are produced as a result of HBV RNA splicing (Su et al., 1989; Terré et al., 1991). The 2.2 kb singly spliced HBV RNA is the predominant form of spliced HBV RNAs (Soussan et al., 2008), produced as a result of splicing between nt 2447 and 489 in the pre-genomic RNA (pgRNA). Splice donor/acceptor sites (GT/AG) have been identified at the splice junction; a point mutation at these sites inhibited the production of singly spliced pgRNA (Su et al., 1989). The singly spliced HBV RNA encodes the HBV splice-generated protein (HBSP), a novel protein linked to the frameshift caused by splicing (Soussan et al., 2000). HBSP has been detected in HBV-infected livers (Soussan et al., 2000). Importantly, the singly spliced HBV RNA is packaged, reverse transcribed and secreted as defective HBV (dHBV) particles with the help of the polymerase from the WT (Rosmorduc et al., 1995; Terré et al., 1991). The presence of dHBV particles containing subgenomic HBV fragments corresponding to the singly spliced HBV RNA has been reported in patients with acute/chronic HBV infection and also in cell culture models (Su et al., 1989; Terré et al., 1991). Defective HBV is detected in all individuals with chronic HBV infection and the proportion of dHBV : WT HBV can vary substantially; dHBV may contribute up to 69 % of total HBV DNA (Soussan et al., 2008). Higher dHBV : HBV ratios in patients with chronic HBV infection have been associated with severe liver necrosis and fibrosis (Soussan et al., 2008). In addition, the expression of HBSP has been linked to higher HBV DNA levels (Preiss et al., 2008; Soussan et al., 2003). Recent clinical studies linking dHBV to virus loads and liver disease have renewed the interest in dHBV.

While the splice acceptor and donor sites essential for HBV RNA splicing resulting in dHBV genomes are known, it remains unknown whether the splice acceptor (AG) and splice donor (GT) residues critical for splicing are conserved across HBV genotypes. Mutations leading to the disruption of the splice junction sites (GT/AG) render the WT genome incapable of forming singly spliced HBV RNA (Su et al., 1989), which is essential for the subsequent formation of dHBV particles. It remains unknown whether all the HBV genotypes have the potential to form dHBV genomes.

The packaging of dHBV RNA (singly spliced RNA) and the subsequent synthesis of HBV DNA replicative intermediates and secretion of dHBV particles (virions) require help from the WT HBV-encoded HBV polymerase.
Nonetheless, transcription and translation from dHBV genomes is done using the host machinery and is independent of the presence of viral polymerase. Therefore, dHBV genomes can produce HBV RNA and HBV proteins without help from WT HBV.

Replication activity is an important characteristic of the HBV life cycle and is measured as the ratio of HBV pgRNA : HBV covalently closed circular DNA (cccDNA) or the number of HBV pgRNAs produced per HBV cccDNA (Laras et al., 2006). Transcription efficiency of HBV is determined by the amount of HBV pre-core transcripts per cccDNA (HBV pre-core RNA : cccDNA ratio) (Laras et al., 2006). The pgRNA levels (normalized to cccDNA) and transcription efficiency of dHBV genomes have not been investigated.

Here, we investigate the nucleotide sequence conservation of splice acceptor (AG) and splice donor (GT) residues critical for HBV RNA splicing across HBV genotypes: if these critical residues are conserved across genotypes, this may reflect their compatibility with the splicing event leading to the formation of dHBV genomes. We have also developed a novel sequence-independent restriction digestion-based method to create dHBV constructs from the WT HBV genome. We then used these constructs to study the pgRNA levels, transcription efficiency and HBV proteins in cell culture.

RESULTS AND DISCUSSION

Critical residues at the splice junction site are conserved across HBV genotypes

The critical nucleotide residues at the splice junction sites (GT/AG) are conserved in over 98% (157/160) of the sequences analysed (Fig. 1). The splice junction sites (GT/AG) were disrupted in a total of three sequences: one each from genotypes A, E and H. The nucleotides residues critical for splicing were highly conserved compared with 20 nt flanking the sites (splice donor site: 98.8 ± 7.8 vs 89.3 ± 8.2; P < 0.001; splice acceptor site: 99.4 ± 5.5 vs 87.5 ± 6.9; P < 0.001). Our results clearly indicated that the nucleotide residues critical for HBV RNA splicing are present and are highly conserved across all HBV genotypes. Interestingly, all three sequences (GenBank accession nos AJ344115, AB219534 and GU565217; Fig. 1) with disrupted splice donor/acceptor sites were reported from individuals without detectable HBV surface antigen (HBsAg) (Fujiwara et al., 2005; Jeantet et al., 2002; Zaaijer et al., 2011). While the conservation of nucleotide residues at the splice junction site represents an interesting finding, it is not possible to determine whether this conservation is a result of these nucleotides (GT/AG) encoding a critical amino acid.

A novel method for creation of dHBV constructs from corresponding WT constructs

To the best of our knowledge, there has been only one attempt to create dHBV constructs (Rosmorduc et al., 1995), where dHBV constructs derived from the liver of a chronically HBV-infected patient were compared with WT HBV constructs derived from a different source. We believe that this method is not ideal for assessing the replication characteristics of dHBV, as major differences in replication do exist among different HBV strains (Qin et al., 2011a) and also among quasi-species circulating in the same individual (Parekh et al., 2003). We developed a novel method to create dHBV constructs from the corresponding WT construct (Fig. 2). Importantly, the dHBV and WT constructs created using this method had identical sequences (baring the deletion) and hence these constructs are appropriate for studies that investigate the replication properties of dHBV genomes. We successfully demonstrated the functionality of our unit-length dHBV constructs by transfection into liver cells (described below).

Defective HBV has enhanced pgRNA levels and higher transcription efficiency

Replication activity is measured as the ratio of HBV pgRNA : HBV cccDNA (Laras et al., 2006). The levels of cccDNA (normalized to cellular DNA) were found to be comparable between WT HBV and dHBV (Fig. 3a). Interestingly, dHBV constructs had almost twofold higher pgRNA levels (normalized to cccDNA, i.e. pgRNA : cccDNA) compared with the corresponding WT constructs (P < 0.05; Fig. 3b). To the best of our knowledge, this is the first report describing increased pgRNA levels for dHBV. Higher pgRNA levels of dHBV genomes may have important implications for our current understanding of HBV replication. dHBV genomes do not encode a functional polymerase (Su et al., 1989). dHBV genomes are packaged and secreted as dHBV particles (Rosmorduc et al., 1995) from hepatocytes co-infected with WT HBV and dHBV genomes. However, when dHBV particles infect a hepatocyte, they will be able to produce dHBV transcripts and most HBV proteins (except the surface and polymerase proteins) without help from the WT virus. The epsilon encapsidation signal required for packaging is intact in dHBV pgRNA and therefore higher pgRNA levels of dHBV in co-infected hepatocytes implies that dHBV pgRNA may have an edge over WT pgRNA to be packaged and secreted. This raises the important question of how the virus benefits from making more dHBV pgRNA and secreting dHBV particles. It is possible that dHBV particles may serve as a decoy to mop up neutralizing antibodies produced against HBV, thereby allowing immune escape of the WT virus. Our finding of higher pgRNA levels in dHBV genomes may be explained, at least in part, by clinical studies linking HBSP to higher HBV DNA levels (Soussan et al., 2003).

Transcription efficiencies (HBV pre-core RNA : cccDNA) for dHBV constructs were about twofold higher than those for the corresponding WT constructs (P < 0.05; Fig. 3c). While the precise reason for increased pgRNA
levels and transcription efficiencies of dHBV genomes remain unclear, plausible explanations include the following: (i) efficient transcription of shorter genomes: transcription of HBV cccDNA takes place in the nucleus with the help of the host RNA polymerase; however, the steady-state mRNA levels for mammalian genes ranging from 2 to 20 nt are substantially lower. 

![Fig. 1. Nucleotide sequence alignment of the HBV genome at the splice junction site (nt 2447/489). The nucleotide residues critical for the splicing event (GT/AG) are highlighted in yellow; 20 nt flanking the splice junction sites across HBV genotypes A–H are also shown. A total of 160 sequences were aligned (20 sequences from each genotype) using CLUSTAL Omega. The accession numbers of three sequences with disrupted donor/acceptor sites (GT/AG) are highlighted in grey.](http://jgv.microbiologyresearch.org)
**Fig. 2.** (a) Schematic diagram describing the creation of dHBV constructs. PCR amplification of HBV DNA (step 1) was followed by cloning into the pSc-A cloning vector (step 2). Restriction digestion using BspQI (step 3) released unit-length WT HBV DNA with sticky ends complementary to each other. dHBV DNA amplification was then carried out after in vitro ligation (step 4). PCR amplification of ligated full-length WT HBV DNA with primers D1 and D2 (see b) deleted the region between nt 2447 and 489 (step 5); this 2 kb amplicon corresponding to dHBV was then cloned into pSc-A (step 6). The dHBV clone was digested using BspQI to release unit-length dHBV DNA of ~2 kb with sticky ends complementary to each other (step 7). In vitro ligation of dHBV DNA (step 8) and PCR amplification using primers P1 and P2 (step 9) were then performed to obtain the dHBV clone with a 3.2 kb insert (step 10).
carried out. The amplicon corresponding to dHBV was cloned into pSC-A (step 10) and digested using BspQI to release the dHBV DNA (step 11) for transfection. 

(b) Sequence of primers D1 and D2 used for the amplification of dHBV DNA using WT HBV DNA as the template. 

(c) Schematic showing dHBV amplified using primers D1 and D2 cloned into pSC-A vector.

(d) Restriction digestion using BspQI released dHBV DNA with complementary ends [see step 7 in (a)]. Note that \( \bullet \) represents the BspQI recognition sequence included as the primer overhang.

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4 kb are comparable (Chiaromonte et al., 2003), suggesting that differences in length between the WT and dHBV constructs are unlikely to affect their transcription efficiencies; (ii) a potential role of the dHBV-encoded HBSP in enhancing the replication and transcription of dHBV genomes, as clinical studies have linked HBSP expression to higher virus loads (Preiss et al., 2008; Soussan et al., 2003); and (iii) the possibility of as yet unknown negative regulatory elements in the deleted region (nt 2447–489) of the HBV genome.

**Defective HBV produces more HBV e antigen (HBeAg) than the WT**

As expected, the WT HBV constructs produced HBsAg and the dHBV constructs did not produce HBsAg, as the gene encoding HBsAg is partially deleted in the dHBV construct (Fig. 4a). Of note, dHBV constructs produced significantly higher levels of HBeAg compared with the WT constructs (Fig. 4b); this finding is in keeping with the higher transcription efficiency of the dHBV genome compared with
that of the WT HBV. The presence of HBeAg has been linked to aggressive liver disease (Hsu et al., 2002) and a high risk of hepatocellular carcinoma (Yang et al., 2002). The association between dHBV genomes and higher levels of HBeAg in our study is particularly interesting, as this could potentially represent one of the mechanisms by which dHBV contributes to the pathogenesis of chronic HBV infection. In addition, epidemiological studies have linked the presence of HBeAg to higher virus loads (Chu et al., 2003). One may also argue that the higher levels of HBeAg produced by dHBV constructs could potentially play a role in increased pgRNA levels of dHBV genomes. Our finding that dHBV genomes produced more HBeAg than the WT HBV suggested that the proportion of dHBV in an infected individual could potentially influence seroconversion to anti-HBe, an important therapeutic end point for chronic HBV patients on antiviral therapy (Liaw, 2009).

**Defective HBV is linked to intracellular accumulation of hepatitis B core antigen (HBCAg)**

Cells transfected with dHBV constructs were seen to accumulate higher levels of HBCag (more than twofold; \( P < 0.05 \); Fig. 4c) compared with those transfected with WT constructs. This may be explained in part by the inability of defective particles to be packaged and secreted outside the cell (unless WT HBV co-inficts the same hepatocyte). Alternatively, higher levels of HBCag accumulation in cells transfected with dHBV constructs compared with those transfected with WT constructs may vindicate the higher transcription efficiency of dHBV constructs. Higher levels of HBCag have also been reported in dHBV constructs derived from the liver of a chronically HBV-infected patient compared with WT HBV constructs derived from a different source (Rosmorduc et al., 1995). The presence of higher levels of HBCag in cells transfected with the dHBV construct was particularly interesting as it...
may potentially influence the dynamics of HBV packaging and secretion.

In summary, we demonstrate that the critical nucleotide residues at the splice junction sites (GT/AG) are highly conserved across HBV genotypes. We have also reported a novel method to create dHBV constructs from corresponding WT HBV constructs for cell culture studies. Our findings clearly demonstrate that dHBV genomes have higher pgRNA levels and transcription efficiency compared with the corresponding WT genomes. Regardless of the underlying mechanism, the ability of dHBV genomes to produce HBV pgRNA and transcribe more efficiently than the WT HBV genomes has interesting and noteworthy implications. First, despite being present in small proportions compared with the WT, dHBV genomes could potentially compete with the packaging of the WT genome. However, the dynamics of packaging dHBV pgRNA with the help of the WT polymerase warrants further studies as Bartenschlager et al. (1990) have demonstrated that mutations in HBV pgRNA can adversely affect their packaging efficiency. Secondly, enhanced production of HBeAg from dHBV constructs suggests that dHBV genomes could potentially influence HBV replication and contribute to the pathogenesis of chronic HBV infection. Taken together, our findings suggest that the dynamics between dHBV and WT HBV could potentially contribute to long-term outcomes in chronic HBV infection. Studies investigating the mechanisms underlying higher pgRNA levels in dHBV genomes and co-transfection of WT and dHBV genomes at clinically relevant ratios may shed more light on the role of dHBV in the pathogenesis of chronic HBV infection.

**METHODS**

**Analysis of splice junction site conservation across HBV genotypes.** A total of 160 full-length sequences, comprising 20 sequences from each HBV genotype (A–H) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank) to assess the presence and conservation of the splice donor/acceptor sites. The mean nucleotide conservation within the donor/acceptor sites and 20 nt flanking the sites was calculated using CLUSTAL Omega (Goujon et al., 2010; Sievers et al., 2011).

**Creation of WT and dHBV constructs.** The limitations of splicing by overlap extension, especially for manipulation of DNA segments greater than 1 kb, are well documented (Vallejo et al., 2008). We therefore developed a novel sequence-independent restriction digestion-based method to create dHBV constructs from the WT HBV constructs. The serum samples of two HBeAg- and HBsAg-positive blood donors (samples 1 and 2) infected with genotype D were used for DNA isolation. HBV DNA levels measured using real-time PCR with β-globin DNA levels measured using real-time PCR with β-globin DNA

**Quantification of HBV cccDNA, pre-core RNA and pgRNA.** A QIAamp DNA mini kit (Qiagen) was used to extract DNA, and Trizol reagent (Invitrogen) was used to extract RNA. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad) from 1 μg DNase I-treated RNA. Primers specifically targeting pre-core transcripts and pgRNA (Laras et al., 2006) were used for real-time PCR quantification of the respective targets using Sso Fast Evagreen Supermix (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase mRNA levels were used to normalize HBV RNA levels (Li et al., 2012). The extracted DNA was subjected to plasmid-safe DNase (Epicentre) digestion following the manufacturer’s instructions. Plasmid-safe DNase digests all linear forms of DNA; HBV cccDNA will remain unaffected by plasmid-safe DNase digestion (Laras et al., 2006). The column was purified and used as a template for the quantification of HBV cccDNA by real-time PCR using primers 5’-GTGCC-CTITCTCATCTGCGCGG-3’ (nt 1555–1573) (Laras et al., 2006) and 5’-GAACATTAGGCCCATATTGAG-3’ (nt 2191–2170) and a Taqman probe, 5’ FAM-TTCAGCTCAGCCATGCTGGTGGGG-TGGC-TAMRA-3’ (nt 1863–1892) (Hennig et al., 2002) by using Premix Ex Taq (Takara). This assay is highly specific for HBV cccDNA and does not amplify linear transfected HBV DNA (WT or dHBV constructs). The HBV cccDNA real-time PCR did not amplify high concentrations (×10^9 copies) of the WT or dHBV constructs, confirming the specificity of this assay to distinguish transfected linear DNA and HBV cccDNA. A schematic detailing the HBV cccDNA primers and probe binding region in the HBV genome is shown in Fig. 5. The HBV cccDNA levels were then normalized to cellular DNA using β-globin DNA levels measured using real-time PCR with appropriate primers (Lever et al., 2009); normalized cccDNA levels were used for all subsequent analyses. Standard curves were generated for all real-time PCR assays using serial dilutions of the appropriate clones to facilitate absolute quantification. The lower limit of detection for all HBV real-time PCR assays (pgRNA, pre-core RNA, HBsAg and HBV cccDNA) was <10 copies per reaction.

**Estimation of HBV proteins.** Commercially available HBsAg (MONOLISA; Bio-Rad) and HBeAg (DiaSorin) ELISAs were used for

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estimation of secreted HBV proteins following the manufacturer’s instructions. Samples were diluted appropriately to ensure that the absorbance values were within the linear range of the assay. Intracellular HBcAg was estimated from cell lysates using a commercially available quantitative HBcAg ELISA kit (Cell Biolabs). The HBcAg levels were normalized to the total intracellular protein levels estimated using a Bradford assay.

dHBV genomes are incapable of synthesizing relaxed circular HBV DNA replicative intermediates (as they do not have a functional HBV polymerase to reverse transcribe HBV pgRNA) and secreting virions without help from WT HBV; we therefore did not assess these parameters.

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**Fig. 5.** Schematic diagram showing the primer and probe binding regions for the HBV cccDNA assay. The shaded regions indicate complementary sticky ends. (a) Primer and probe binding regions in BspQI-digested WT HBV or dHBV constructs used for transfection. The filled area in the middle of the genome (nt 2447–489) shows the region that is spliced (and hence deleted) from the dHBV construct. (b) Primer and probe binding regions in WT or dHBV constructs following circularization after transfection into Huh7 cells to form HBV cccDNA.
REFERENCES


