Functional analysis of ‘a’ determinant mutations associated with occult HBV in HIV-positive South Africans

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Occult hepatitis B is defined by the presence of hepatitis B virus (HBV) DNA in the absence of hepatitis B surface antigen (HBsAg). Occult HBV is associated with the development of hepatocellular carcinoma, reactivation during immune suppression, and virus transmission. Viral mutations contribute significantly to the occult HBV phenotype. Mutations in the ‘a’ determinant of HBsAg are of particular interest, as these mutations are associated with immune escape, vaccine escape and diagnostic failure. We examined the effects of selected occult HBV-associated mutations identified in a population of HIV-positive South Africans on HBsAg production in vitro. Mutations were inserted into two different chronic HBV backbones and transfected into a hepatocyte-derived cell line. HBsAg levels were quantified by enzyme-linked immunosorbent assay (ELISA), while the detectability of mutant HBsAg was determined using an HA-tagged HBsAg expression system. Of the seven mutations analysed, four (S132P, C138Y, N146D and C147Y) resulted in decreased HBsAg expression in one viral background but not in the second viral background. One mutation (N146D) led to a decrease in HBsAg detected as compared to HA-tag, indicating that this mutation compromises the ability of the ELISA to detect HBsAg. The contribution of occult-associated mutations to the HBsAg-negative phenotype of occult HBV cannot be determined adequately by testing the effect of the mutation in a single viral background, and rigorous analysis of these mutations is required.

INTRODUCTION

Hepatitis B surface antigen (HBsAg) is commonly used as a diagnostic marker for hepatitis B virus (HBV) infection. Patients with detectable HBsAg in their serum have an active HBV infection, and other serologic markers are used to further categorize the infection. However, a subset of patients are infected with HBV but do not have detectable HBsAg in their serum and are said to have occult HBV – defined by the presence of HBV DNA in patient serum in the absence of HBsAg (Said, 2011). Anti-HBc and/or anti-HBs antibodies are not reliable for distinguishing occult from chronic infection, as 20–46% of people with occult HBV have no detectable serological markers of HBV (Hollinger & Sood, 2010; Powell et al., 2014). Because of these factors, occult HBV infection often remains undiagnosed and is rarely treated.

Occult HBV is linked to several negative clinical outcomes, including the development of hepatocellular carcinoma (HCC) in patients with cryptogenic liver disease (Chen et al., 2009; Ikeda et al., 2009). In southern Africa, a region of high HBV prevalence, HBV DNA was detected in 75% of black Africans with HCC that were positive for any anti-HBV antibody but negative for HBsAg (Kew et al., 2008). Additionally, during immune suppression, occult HBV can reactivate to chronic HBV. When the immune system is suppressed, viral replication increases and viral loads may increase by 100-fold before serological markers appear and the infection is detected (Coppola et al., 2011; Hui et al., 2006). Immune
response to the increased viral load, either during immunosuppression or following immune reconstitution, can lead to acute hepatitis and even fulminant hepatic failure. Finally, occult HBV can be transmitted to others via blood transfusion or liver transplantation, despite low HBV viral loads in the donor (Chazouilleres et al., 1994; Levicknik-Stezinar et al., 2008; Yuen et al., 2011).

Many factors may play a role in the HBsAg-negative phenotype of occult HBV. Differences in host immune response have been implicated (Ahmadabadi et al., 2012; Arababadi et al., 2010; Hassanshahi et al., 2010; Martin et al., 2009). Occult HBV also occurs more frequently in people with hepatitis C virus (HCV) and/or human immunodeficiency virus (HIV), suggesting that viral co-infections play a role (Cacciola et al., 1999; Kao et al., 2002; Mphahele et al., 2006; Shire et al., 2004). Multiple studies have identified viral mutations that are associated with occult HBV and alter the ability of HBsAg to be produced, secreted from the cell or detected by commercial assays (Araujo et al., 2008; Candotti et al., 2006; Chaudhuri et al., 2004; Martin et al., 2010; Powell et al., 2014; Svicher et al., 2012). Though many occult HBV-associated mutations have been identified throughout the viral genome, functional analysis of these mutations is rare. Importantly, mutations were generally introduced into one wild-type HBV genome, and background-specific effects were not examined.

We previously identified 235 occult-associated mutations in HIV-positive South Africans (Powell et al., 2014). This population has an extremely high rate of occult HBV – 8–23 % compared to 0.8–2.3 % of HIV-negative South Africans – though rates of chronic HBV do not differ greatly by HIV status (Bell et al., 2012; Finrather et al., 2011; Lukhwareni et al., 2009; Mphahele et al., 2006; Powell et al., 2014). The aim of this study was to determine the effect of the occult-associated mutations identified in this population that occur in the ‘a’ determinant, the region of HBsAg where antibody binding occurs. Mutations in this region have been shown to HBsAg expression and alter both HBsAg detection; thus, they may play a key role in the phenotype of occult HBV (Huang et al., 2012).

RESULTS

Characterization of full-length chronic HBV backgrounds

Both amplified chronic HBV backgrounds were genotype A1 (Fig. 1) and were 3 % different from one another based on nucleotide comparison. No previously reported drug resistance, vaccine escape, immune escape or occult-associated mutations were found in these wild-type HBV sequences. Additionally, no mutations were found in the surface or pre-core/core promoter (Bruni et al., 2011; Carman, 1997; Carman et al., 1990; Chen et al., 2010; Chong-Jin et al., 1999; Coleman et al., 1999; Cui et al., 2015; Ghaziasadi et al., 2013; He et al., 2001; Hsu et al., 1999; Ireland et al., 2000; Ishigami et al., 2007; Kew et al., 2001; Lee et al., 2001, 2015; Ngui et al., 1997; Oon et al., 1996; Pivert et al., 2015; Seddigh-Tonekaboni et al., 2001; Tseng et al., 2015; Wiseman et al., 2009; Yoshida et al., 2000; Zuckerman & Zuckerman, 2003). When transfected into the HuH7 cell line, both wild-type viruses resulted in high-level HBsAg expression, with higher HBsAg levels in viral background 2 compared to background 1 (Fig. 2) demonstrating that 1) wild-type viruses produce robust levels of HBsAg in this system, and 2) there may be differences in HBsAg expression based on the wild-type vector into which the occult-associated mutation is placed. Thus, data in subsequent experiments were normalized to the HBsAg levels produced within the corresponding wild-type HBV background.

Effect of occult-associated mutations on HBsAg expression in full-length HBV system

Levels of HBsAg were measured in the cell supernatant and lysate following transfection with WT HBV or HBV in which a single occult-associated mutation was inserted (Fig. 3). All HBsAg concentrations were normalized to the HBsAg concentration of the sample transfected with WT, and mutations were inserted into two different viral backgrounds to evaluate background-specific effects. In viral background 1, occult-associated mutations S132P, C138Y, N146D and C147Y led to significantly decreased supernatant HBsAg concentrations compared to WT (Fig. 3a). In background 2, no mutations led to a significant reduction in supernatant HBsAg, though N146D did result in a decrease that trended towards significance (p = 0.0525). No occult-associated mutations led to a significantly different lysate concentration of HBsAg in either chronic HBV background (Fig. 3b). In contrast, N146S resulted in a significant increase in lysate HBsAg in both viral backgrounds. Transfection efficiency was not altered by the presence of occult-associated mutations, as shown in Fig. S1 (available in the online Supplementary Material).

Effect of occult HBV-associated mutations on HBsAg ELISA efficacy

To evaluate the effect of the ‘a’ determinant mutants on HBsAg ELISA efficacy, HA-tagged small HBsAg was synthesized using an expression vector system. Absolute levels of HBsAg are less relevant in this system than in the full-length HBV system, since an exogenous promoter drives HBsAg expression. Instead, comparison of the amount of HBsAg detected by ELISA and the amount of HA-tag detected by Western blot allows for the determination of the detectability of each mutant HBsAg without relying on HBsAg/anti-HBs interactions (Fig. S2). Mutants with ELISA/Western blot ratios of 1 are as detectable as WT HBsAg of the same viral background. Mutants with ELISA/Western blot ratios of <1 are less detectable than the corresponding WT HBsAg utilizing the HBsAg ELISA from this study. No mutations resulted in decreased detectability within viral background 1. However, one
Fig. 1. Genotype of full-length chronic HBV viral backgrounds. Full-length chronic HBV sequences from HIV-positive South Africans were aligned with HBV sequences of known genotype downloaded from GenBank. The two chronic HBV viral backgrounds generated in this study are highlighted in red. Significant posterior probabilities are shown.
mutation (N146D) resulted in significantly reduced detection of HBsAg in viral background 2. This difference was observed both in the lack of detectable HBsAg via ELISA (Fig. 4a) versus the robust detection of HA via Western blot (Fig. 4b) and the very low ELISA/Western blot ratio (Fig. 4c).

**DISCUSSION**

Occult HBV-associated mutations have been identified in many studies, but few have characterized their effect on HBsAg production, secretion and detection. Several studies have demonstrated that occult-associated mutations can lead to decreased detection of HBsAg, though the mechanisms vary (Hass et al., 2005; Huang et al., 2012; Jammeh et al., 2007; Martin et al., 2012; Slicher et al., 2012). For example, one study found that one occult-associated mutation altered the splicing of preS2/S mRNA and prevented post-transcriptional production of HBsAg but did not alter viral replication (Hass et al., 2005). Another study suggested that occult-associated mutations may introduce novel glycosylation sites or alter capsid/surface interactions (Slicher et al., 2012). Occult-associated mutations may exhibit increased HBsAg retention in cells, and fluorescence microscopy has shown localization to the endoplasmic reticulum (Biswas et al., 2013; Martin et al., 2012). Only one of these previous studies examined occult-associated mutations within more than one viral background (Martin et al., 2012). That study utilized an HBsAg expression system exclusively, and no background specific effects were observed.

In the current study, in chronic HBV background 1, four of the seven 'a' determinant mutations resulted in a decreased HBsAg in the supernatant. In chronic HBV background 2, none of the mutations resulted in altered supernatant HBsAg levels, although one mutation did result in a decrease that trended towards significance ($p=0.0525$). In the cell lysate, no mutations resulted in decreased HBsAg, and one mutation resulted in an increase in HBsAg as compared to wild-type. Taken together, these data indicate that five of the seven 'a' determinant mutations result in increased retention of HBsAg in a viral background-specific manner.

The data presented in the current study indicate that the impact of an occult-associated mutation in one viral
background may not be replicated in other backgrounds. Alternatively, occult-associated mutations can be reverted to the wild-type amino acid. If the reversion results in increased HBsAg, the occult-associated mutation can be said to decrease HBsAg. Taken together, these data indicate that occult-associated mutations – both in the ‘a’ determinant and throughout the S ORF – can decrease extracellular HBsAg by increasing intracellular retention of HBsAg, though this likely requires interaction with other amino acids, resulting in the background specific effects observed. Covariance analysis to determine which amino acids evolve together may provide further insight as to which amino acids contribute to the action of the ‘a’ determinant mutants (Donlin et al., 2012).

Previous studies have found association between mutations in the ‘a’ determinant of HBsAg and failure of diagnostic assays to detect HBsAg, even when it is known to be present in patient serum (Huang et al., 2012). The ‘a’ determinant mutations examined here may prevent or decrease the detection of HBsAg by ELISA in cell supernatant and lysate. These findings are applicable only to the HBsAg ELISA utilized in this study and not universally or to clinical assays, as each HBsAg assay utilizes distinct – and typically proprietary – antibodies to detect HBsAg. Using the HA-tagged HBsAg system, one mutation (N146D) decreased HBsAg detectability in one viral background. HBsAg was undetectable using HBsAg ELISA, but robust levels of HA-tag were detected using Western blot. Despite very low detectability of mutant N146D HBsAg expression in background 2,

Fig. 3. Effect of occult-associated mutations on HBsAg expression. Results are shown for cell supernatant (a) and cell lysate (b). Fold change was determined by dividing the HBsAg concentration of each sample by the average HBsAg concentration of the samples transfected with WT HBV in each experiment. The inserted mutation is indicated on the y-axis. Negative control indicates transfection with an equivalent amount of pGEM instead of HBV. Black bars represent the results from inserting mutants into viral background 1, while white bars represent viral background 2. Experiments included duplicates of each sample, and the experiments were performed four times each. Error bars represent standard error. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
neither supernatant nor lysate levels of HBsAg were decreased in the full-length HBV system. This may indicate that small HBsAg is not detected by the ELISA, while medium and large HBsAg are detectable. The full-length system can produce all three sizes of HBsAg, while the expression vector system produces only small HBsAg. Additionally, while the HA-tag may interfere with detection of this mutant HBsAg, this is unlikely, as all other mutants were similarly tagged and no interference was observed. Because no differences in HBsAg detectability were observed for any other mutation in either viral background, differences in ELISA detectability of HBsAg are unlikely to be a major factor in the lower levels of supernatant HBsAg produced by several mutants in the full-length HBV system.

The current study has several potential limitations. First, this study reveals the importance of viral background in determining the effects of occult-associated mutations, but mutations were only analysed in two backgrounds. To more fully elucidate the role these occult-associated mutations may have in decreasing HBsAg detected in infected patients, mutations should be inserted into additional chronic HBV backgrounds. This would also allow more careful study of the influence of other amino acids on the effect of the occult-associated mutation. Additionally, some of the 'a' determinant mutations were identified in genotype A2 occult HBV. However, these mutations were tested in genotype A1 chronic HBV. This was necessary as we were unable to amplify full-length chronic HBV from any patients with genotype A2. While this prevents exploration of sub-

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**Fig. 4.** Detectability of HBsAg with occult-associated mutations. HA-tagged HBsAg was measured using HBsAg ELISA (a) and Western blot with anti-HA antibodies following anti-HA immunoprecipitation (b). Both methods were normalized to the average of the WT samples. The detectability of HBsAg (c) was calculated by dividing the HBsAg fold change by HA Western blot for each sample. Black bars represent the results from inserting mutants into viral background 1, while white bars represent viral background 2. Experiments included duplicates of each sample, and the experiments were performed twice each. Error bars represent standard error. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
genotype-specific differences, separation of samples by sub-genotype is rare in occult HBV studies and there are very limited data to suggest that sub-genotype is relevant. Previous studies have tested the effect of occult-associated mutations within genotypes instead of sub-genotypes (Jammeh et al., 2007; Martin et al., 2012; Svicher et al., 2012). Moreover, the full-length HBV system utilized here permits HBsAg production from endogenous viral promoters, but levels of viral genome replication are likely low (Cavallone et al., 2013). This system requires the transfection of viral genomes into hepatocytes and thus bypasses the initial steps of infectious life cycle. If occult-associated mutations alter the binding of HBsAg to cell receptors or viral entry into cells, this cannot be determined using this system. Finally, in the detectability experiments, HBsAg levels were compared to HA-tag levels in the same sample. This detectability value, which is internally normalized, was then compared between samples. Because of this, absolute HBsAg quantities cannot be compared among mutants for the HBsAg expression vector system.

In summary, seven ‘a’ determinant occult-associated mutations identified in HIV-positive South Africans were evaluated for their effect on HBsAg production and detection. Four of the seven mutations resulted in decreased HBsAg in cell supernatant when mutant viruses of one viral background – but not a second viral background – were transduced into HuH7 cells. An additional mutation resulted in increased HBsAg in cell lysate and the same amount in cell supernatant. Taken together, five of the seven mutations studied resulted in increased intracellular retention of HBsAg as compared to wild-type virus in at least one background. This study highlights the importance of viral background in assessing the effect of occult-associated mutations, and future studies should involve inserting the mutations to be studied into multiple HBV backgrounds.

**METHODS**

**Site-directed mutagenesis and plasmid preparation.** Occult-associated mutations were identified as described previously (Powell et al., 2014). Briefly, the surface and polymerase open reading frames were sequenced from 30 patients with occult HBV and 19 patients with chronic HBV from HIV-positive South Africans. Three clones per patient were sequenced to identify dominant/common mutations. Occult-associated mutations were identified as those that appear exclusively in sequences of occult HBV but do not appear in chronic HBV sequences from the same population or genotype-matched chronic HBV references from GenBank.

Full-length HBV was amplified as previously described from two patients with chronic HBV from the same population (Günther et al., 1995). To analyse genotype, the full-length chronic HBV sequences from HIV-positive South Africans were aligned with HBV sequences of known genotype downloaded from GenBank. Alignment was performed with Clustal X. Phylogenetic inference was performed with a Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the Bayesian Evolutionary Analysis by Sampling Trees v1.8.2 program under an uncorrelated log-normal relaxed molecular clock, and the Hasegawa, Kishino, and Yano model with a nucleotide site heterogeneity estimated using a gamma distribution. The BEAST MCMC analysis included a chain length of 10 000 000 with sampling every 1000th generation. Results were visualized in Tracer v1.5 to confirm chain convergence, and the effective sample size (ESS) was calculated for each parameter. All ESS values were >500, indicating sufficient sampling. The maximum clade credibility tree (Fig. 1) was selected from the posterior tree distribution after a 10% burn-in using TreeAnnotator v1.7.3. Posterior probabilities >90% were considered statistically significant.

Full-length HBV amplification was attempted from samples from all 19 patients with chronic HBV. However, due to limitations of the procedures (low viral load, primer mismatch, etc.), full-length HBV was obtained only from two patient samples. As described previously, these full-length chronic HBV clones were screened for recombination, and no recombination was found (Powell et al., 2014). The full-length chronic HBV (also referred to as wild-type or WT) was cloned into the pGEM-T Easy vector system (Promega, Madison, WI, USA). Mutations within the ‘a’ determinant (amino acids 124–147 of small HBsAg or sHBsAg) were inserted individually into the two full-length chronic hepatitis B viruses using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) and appropriate primers (Table 1). All mutations were confirmed via sequencing.

WT and mutant plasmids were transformed into JM109-competent cells (Protein Express, Cincinnati, OH, USA). Following bacterial growth, plasmids were prepared via plasmid maxiprep kits (Qiagen, Valencia, CA, USA). HBV was liberated from the plasmid via Sap I digestion (New England Biolabs, Ipswich, MA, USA). Digestion products were purified and concentrated using the QiAquick PCR Purification kit (Qiagen). HA-tagged HBsAg expression vectors were created.

**Table 1. Primers used for site-directed mutagenesis**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Amino acids at site*</th>
<th>Mutagenesis primer (5'-→3')</th>
</tr>
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<tbody>
<tr>
<td>S132P</td>
<td>S</td>
<td>CTCCTGCTCAAAGGCACCCCTATGTGTTGCCCTCATATGT</td>
</tr>
<tr>
<td>F134L</td>
<td>F</td>
<td>TCAAGGCAAATCTATGCTTCCCTCATATGTGTTGCT</td>
</tr>
<tr>
<td>C137Y</td>
<td>C</td>
<td>TCAAGGCAAATCTATGCTTCCCTCATATGTGTTGCT</td>
</tr>
<tr>
<td>C138Y</td>
<td>C</td>
<td>CTCATGTTTCCCTCATATGTGTTGCTAAACACCTACGGG</td>
</tr>
<tr>
<td>N146D</td>
<td>N</td>
<td>CAAAACCTACGGATGGAGATTGCACCTGATATTCCC</td>
</tr>
<tr>
<td>N146S</td>
<td>N</td>
<td>ATCACAAGGTGCAACTCTCCATCCCGTAGGTTTTGTTGACACG</td>
</tr>
<tr>
<td>C147Y</td>
<td>G/G</td>
<td>TGTTACAAAAACCTACGGATGGAGTTTACACGTTCTATTCCC</td>
</tr>
</tbody>
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*Indicates any amino acid found at indicated site in any of 235 sequences of Genotype A chronic HBV downloaded from GenBank.
Cell culture and full-length HBV transfection. Huh7 cells were grown in DMEM media ( Gibco, Grand Island, NY, USA) supplemented with 10% bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO₂. Cells were seeded at a density of 1 x 10⁵ per well in a 12-well plate 24 h before transfection. Two micrograms total DNA (1 μg HBV and 1 μg pGEM for full-length HBV) were transfected per well using 3 μL of Lipofectamine 3000 (Life Technologies, Grand Island, NY, USA). To measure transfection efficiency, 50 ng of pcDNA3.1His/LacZ plasmid (Life Technologies, Grand Island, NY, USA) was co-transfected. Cells were washed with warmed phosphate buffered saline 24 h post-transfection, and media was replaced. On day 3 post-transfection, supernatant and cells were collected because previous work showed robust expression of HBsAg at this time point (data not shown). Cells were counted using the Scepter cell counter (Millipore, Billerica, MA, USA), and two wells were stained per mutation. Supernatant was clarified by centrifugation at 14 000 g for 5 min. Cell lysate was prepared by mixing 180 μL of cell suspension with 20 μL of 10x RIPA buffer (Abcam, Cambridge, MA, USA), incubating on ice for 5 min, and clarifying by centrifugation at 14 000 g for 15 min. HBsAg in supernatant and lysate was measured using HBsAg ELISA (Biochain, Newark, CA, USA). Samples were run in duplicate, and the experiment was repeated a total of four times for each viral background. To determine transfection efficiency, cells were stained using a β-galactosidase staining kit (Life Technologies). Following staining, five high-powered fields were counted per well, and two wells were stained per mutation.

HA-tagged HBsAg transfection and measurement. Cells were cultured as described above. Cells were seeded at a density of 1.5 x 10⁵ cells per well in a 12-well plate 24 h before transfection. Two micrograms of plasmid DNA were transfected per well using 3 μL of Lipofectamine 3000 (Life Technologies). Cells were washed with warmed phosphate buffered saline 24 h post-transfection, and media were replaced. On day 3 post-transfection, supernatant and cells were collected because previous work showed robust expression of HBsAg at this time point (data not shown). Cells were counted using the Scepter cell count (Millipore, Billerica, MA, USA), and all samples were adjusted to the same cell count. Supernatant was clarified by centrifugation at 14 000 g for 5 min. Cell lysate was prepared by mixing 180 μL of cell suspension with 20 μL of 10x RIPA buffer (Abcam, Cambridge, MA, USA), incubating on ice for 5 min, and clarifying by centrifugation at 14 000 g for 15 min. HBsAg in supernatant and lysate was measured using HBsAg ELISA (Biochain, Newark, CA, USA). Samples were run in duplicate, and the experiment was repeated a total of four times for each viral background. To determine transfection efficiency, cells were stained using a β-galactosidase staining kit (Life Technologies). Following staining, five high-powered fields were counted per well, and two wells were stained per mutation.

STATISTICAL ANALYSIS All experiments were run at least three times independently. For Figs 3 and 4 in which data from multiple independent experiments are shown, a one-way ANOVA was utilized to analyze differences in mean fold change. For all analyses, p-values of less than 0.05 were considered statistically significant. Fig. 2, Figs S1 and S2 are representative experiments.

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