Replicative Competence of the T131I, K141E, and G145R Surface Variants of Hepatitis B Virus

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Variants of hepatitis B surface antigen have been described in different clinical settings, but their replicative capacity in vitro has remained unexplored. Point mutations leading to sT131I, sK141E, and sG145R amino-acid substitutions were engineered by site-directed mutagenesis into an infectious plasmid clone of the virus. The mutated constructs were transfected into Huh7 cells, and their replication capacity was documented by LightCycler (Roche Diagnostics) measurements of virion-associated hepatitis B virus (HBV) DNA, intracellular relaxed circular double-stranded DNA, and pre-genomic RNA. The sT131I and sG145R variants replicated with efficiency equal to that of the wild type, whereas the sK141E variant was replication impaired.

Hepatitis B virus (HBV) particles have an outer lipid bilayer envelope into which are inserted the large, middle, and small hepatitis B surface antigen (HBsAg) proteins. All 3 proteins share the group-specific “a” antigenic determinant, a hydrophilic region with a conformational structure that constitutes the main neutralization epitope of the virus [1]. Variants of the virus that have amino acid substitutions in this region have been described in different epidemiological and clinical settings. These variants exhibit altered antigenicity, which causes assays either to fail to detect them or to have greatly reduced sensitivity. Such variants have been described in vaccinees, liver-transplant patients who have received monoclonal or polyclonal anti-HBs treatment after transplant, cases of occult infection, and cases of disease reactivation after immunosuppression or cytotoxic drug treatment for malignancies [2].

The coexistence of these variants with the presence of anti-HBs, which may lead to their partial suppression, raises questions relating to their replicative fitness. Because the HBsAg open reading frame overlaps with that of the polymerase, substitutions in the “a” determinant region may have a detrimental effect on polymerase function. The present study aimed to investigate the replication capacity of the following 3 HBsAg variants: the sG145R variant, most often detected in vaccinated children worldwide [3]; the sK141E variant, described in a vaccinated child in Gambia [4]; and the sT131I variant, found in patients with occult infection [5, 6]. Two of these substitutions also lead to amino acid changes in the polymerase region. Replicative fitness was assessed by LightCycler (Roche Diagnostics) measurements of HBV-DNA levels in cell culture supernatants and of intracellular levels of replicative intermediates—such as relaxed circular double-stranded (RC-ds) HBV DNA and pre-genomic (preG) RNA, the template for negative strand DNA synthesis—and these levels were compared with those found in the wild type (WT). In addition, the levels of preG RNA were compared with those of precore (preC) mRNA, which encodes for the hepatitis B e antigen (HBeAg).

**Methods.** The desired mutations were engineered using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) in accordance with the manufacturer’s instructions. A plasmid (p3.8II; Prof. Yuan Wang, Academia Sinica, Shanghai) containing a 1.2-times-genome-length fragment of HBV (Genotype C, subtype adr) in a pBluescript II KS (+) background was used as template. Sense primer 5′-CTGCTCAAGGATCTCTTA-TGTTCCTCC, was used for the sT131I substitution, 5′-TGTGCCTCAGAAACCTTCG was used for the sK141E substitution, and 5′-TGTTTGCG-TGTACAGAACCTTCG was used for the sG145R substitution (point mutations are underlined); the complementary, antisense primers were used for mutagenesis. The entire HBV insert was then sequenced to ensure that no additional mutations had been introduced.

Huh7 hepatoma cells were transfected with 3 μg of the appropriate construct complexed with Lipofectin (Invitrogen), as recommended by the supplier. The complex also contained 2 μg of a green-fluorescence protein (GFP)-expressing plasmid (pCI; Promega) to monitor transfection efficiency by use of fluorescence-activated cell-sorter analysis. Six-well plates were seeded with 1 × 10⁶ cells in 5 mL of Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum, 2 mmol/L l-glutamine, 100 IU/mL penicillin, and 100 μg/mL.
streptomycin. Transfections were performed 16 h after seeding, and cultures were processed 96 h later. All incubations were performed at 37°C in a humidified atmosphere containing 5% CO₂. Each construct was tested in triplicate on 3 different occasions.

Virion-associated HBV DNA was extracted from cell-culture supernatants after clarification by low-speed centrifugation. The clarified supernatants were first treated with 2 U of DNaseI (Ambion) to remove extraneous DNA, followed by heat inactivation of the enzyme. HBV DNA was then extracted from intact virions by proteinase K digestion as described elsewhere [7], resuspended in 10 μL of ultrapure distilled water, and frozen at −20°C until used.

For preparation of intracellular HBV replicative intermediates, cells were detached by use of a flexible rubber scraper and were resuspended in 400 μL of phosphate-buffered saline (pH 7.4), which was then divided into two aliquots. The first was recentrifuged and the cell pellet was resuspended in lysis buffer (10 mmol/L Tris HCl [pH 8.4] at 4°C, 1.5 mmol/L MgCl₂, 0.14 mol/L NaCl, and 2 μL of 5% Nonidet P-40) for the removal of nuclei by pelleting, as described by Sambrook and Russell [8]. RC-ds DNA was then extracted from the supernatant by proteinase K digestion as described elsewhere [7]. The HBV-DNA pellet was resuspended in 20 μL of ultrapure water and was stored at −20°C until used. Total cellular RNA, including HBV transcript RNA, was extracted from the second cell aliquot by use of a Purescript kit (Gentra Systems) used in accordance with the manufacturer’s instructions. The RNA pellet was resuspended in 20 μL of ultrapure water and treated with DNaseI as described above, before being stored at −80°C.

For cDNA synthesis, sufficient RNase-free water was added to 5 μL of total RNA and 1 μL (50 pmol) of primer BC1 [9] to produce 10 μL, which was incubated for 10 min at 65°C and then was ice-cooled. The volume was then increased to 20 μL by adding the following to create final concentrations: 250 mmol/L Tris-HCl, 200 mmol/L KCl, 25 mmol/L MgCl₂, 2.5% Tween, 10 mM dithiothreitol, and 2.5 mmol/L of each dNTP, 20 U of RNase Inhibitor, and 50 U of Expand Reverse Transcriptase (Roche Diagnostics). This mixture was incubated for 90 min at 42°C and then was ice-cooled.

Real-time quantitative polymerase chain reaction (PCR) was performed by use of a QuantiTech SYBR Green PCR Master-Mix Kit (Qiagen), with 1 μL of extracted HBV DNA in a total solution of 20 μL, which included 10 μL of the mix described above, 1 μL of 0.5 mmol/L of each primer (F1 [5′-GTATGGTTGC-CCTTGTGTTTCC, positions 436–458], and R1 [5′-CCCTGCGG-AACCATGAA, positions 706–689]), and 7 μL of sterile distilled water. Cycling consisted of a holding step for 15 min at 95°C followed by 45 cycles of denaturation for 15 sec at 94°C, annealing for 30 sec at 60°C, and extension for 30 at 72°C sec. This was followed by a data-acquisition step for 15 sec at 80°C to check on the specificity of the PCR product. Moreover, PCR products were run on a 2% agarose gel to further confirm the presence of specific bands of the correct size.

For the detection of preG RNA and preC mRNA, the cDNA samples were diluted 1:30 and 1 μL of the dilution was used as template in LightCycler reactions set up as described above. The primers used were PCP (5′-GTCTGTCACCAGCAGCA, positions 1797–1814) and BC1, which specifically detected pre-C mRNA, and PGP (5′-CACCTCCTGCTAATCATCTCA, positions 1826–1846) and BC1, which detected both preC mRNA and preG RNA [10]. preG RNA levels were estimated by subtraction of preC mRNA from the total. Residual HBV-DNA contamination of RNA preparations after DNaseI digestion was excluded by PCR amplification with primers M3 and BC1 [9].

Quantitative values were obtained from a standard curve of 10-fold dilutions (range, 10⁸–10¹⁰ copies/mL) of p3.8II, the concentration of which was determined spectrophotometrically. Transcript copy number was normalized to those of β-actin, amplified concurrently with primers 5′-CAGTCTCATGATGGATTGTAATTG (antisense) and 5′-GAACTCCAGAGCAACTGTG (sense). Finally, all results were standardized to 15% transfection efficiency, which was based on the results obtained by fluorescent-activated cell-sorter analysis of fluorescent cells co-transfected with pCI-GFP.

Finally, tissue-culture supernatants were tested by use of the Murex (version 3; Abbott Laboratories) HBsAg detection kit using a monoclonal antibody on the solid phase. The mean variation between 2 samples was compared by use of the Mann-Whitney U test in SPSS (version 10; SPSS), and significance was set at P < .05.

Results. The real-time PCR results for virion and RC-ds HBV DNA, as well as for preG RNA, were validated using the HBV stably transfected HepG2.2.15 cell line (data not shown). (figure 1A) shows the results obtained for HBV-DNA levels in culture supernatants. HBV plasmid constructs of variants sT131I and sG145R replicated as efficiently as did the WT virus, whereas the sK141E variant replicated with reduced efficiency. There was a 5-log₁₀ difference between the level of HBV DNA recorded for the WT virus and that recorded for the sK141E variant (P < .001). These results were further substantiated by testing for RC-ds HBV DNA, a replicative intermediate extracted from intracellular core particles. Without exception, the RC-ds HBV DNA levels followed the same trend as that for released virions with a significant decrease being apparent once again for the sK141E variant (P < .001; figure 1B).

preG RNA constitutes the template for negative-strand DNA synthesis, and its detection is an additional measure of viral replicative capacity. The preG-RNA levels were compared with those of preC mRNA (figure 1C). Both transcripts showed an obvious reduction in their levels in cultures of the sK141E
Figure 1. Replication efficiency of hepatitis B virus (HBV) constructs bearing mutations in the “a” determinant region, as demonstrated by real-time polymerase chain reaction of virion-associated HBV DNA (A), relaxed circular double-stranded (RC-ds) HBV-DNA levels (B), and transcript levels of pregenomic (preG) RNA and precore (preC) mRNA (C). Transfection efficiency was estimated by use of fluorescence-activated cell-sorter analysis of green-fluorescence protein–expressing cells. All results were normalized to 15% transfection efficiency, and in addition, transcript levels were normalized to those of \( \beta \)-actin.

variant. In all cultures, the preC-mRNA levels recorded were lower than the corresponding preG-RNA levels.

Finally, the Murex-kit tests for HBsAg in culture supernatants from cells transfected with the WT and surface variant constructs showed significantly reduced levels for the variants, as depicted in figure 2 (\( P < .02, .01, \) and .001 for sG145R, sT131I, and sK141E respectively).

Discussion. After the introduction of the HBV vaccine for infant immunization and the use of hepatitis B immune globulin to prevent reinfection of transplanted liver graft in chronic HBV carriers, it became apparent that some individuals harbored HBsAg variants despite the concurrent detection of anti-HBs. What is more, although antibodies were detectable by the relevant immunoassays, HBsAg was undetectable or detected with reduced sensitivity, depending to a large extent on the format of the assay used. The sG145R variant was the first to be described in a vaccinated child [3], and it represents by far the most common variant in this setting [2]. Antibody-binding studies using monoclonal anti-HBs have shown that amino acid substitutions in the “a” determinant region of HBsAg result in altered antigenicity and reduced sensitivity in immunoassays [11, 12].

Although HBV DNA from HBsAg variants is detectable by PCR, there have been no systematic studies of sequential monitoring of HBV DNA levels in patients with these variants. The replication phenotype of these viruses therefore remains unexplored. Because the open reading frame of HBsAg is overlapped in its entirety by that of the polymerase, it follows that any amino acid substitutions in the “a” determinant region may alter the structure and functional activity of the enzyme. Indeed the “a” determinant overlaps the fingers subdomain of the polymerase, and, as has been shown recently, some of the substitutions identified in the polymerase region that confer resistance to lamivudine (a nucleoside analogue used in the treatment of chronic HBV infection) lead to amino acid substitutions in HBsAg, substitutions that altered its antigenicity and detection sensitivity [13].

In the present study we have investigated the replication phenotype of 3 HBsAg variants, after transfection of Huh7 cells with plasmid constructs bearing the sT131I, sK141E, and sG145R coding sequences. The findings indicate that there was no detrimental effect on polymerase function in the sT131I and...
sG145R variants, which replicated with an efficiency equal to that of the WT virus, as documented by quantitative measurements of released virion HBV DNA, RC-ds HBV DNA, and preG RNA. It follows that these mutations also did not have any negative effect on virus morphogenesis. The findings for sG145R agree with those reported by Torresi et al. [14], whereas the sT131I with no concurrent change in the polymerase behaved as the WT did. In contrast, by all 3 parameters measured, the sK141E variant exhibited reduced replication efficiency. Of note are the greatly reduced levels of both preG RNA and preC mRNA, in comparison with those of the WT. This is unlikely to be due to reduced transcriptional capacity in the mutant, because the relevant promoter was intact; more likely, it relates to subsequent steps in the virus life cycle and requires further investigation.

Of the 3 amino acid substitutions in the surface protein, only 2 resulted in amino acid substitutions in the reverse transcriptase (rt) domain of the polymerase. The sT131I change maintained the N (asparagine) residue at position rt139, whereas the sK141E and sG145R changes resulted in changes to rtK149R and rtR153Q, respectively, in the overlapping polymerase region. Therefore, the rtK139R substitution appears to have a negative impact on polymerase function.

Further study of the replication capacity of variants with substitutions in the “a” determinant region is warranted, to include those with multiple mutations. This is particularly important in the light of recent findings showing that substitutions in the “a” determinant region may act to compensate for any replication-capacity deficit due to amino acid changes conferring resistance to lamivudine [14] that occur in functional domains of the reverse transcriptase. Torresi et al.[14] demonstrated that 2 surface variants, sP120T and sG145R, which cause amino acid changes rtT128N and rtW153Q, respectively, in the polymerase region, were able to restore the replication phenotype of a construct containing the rtL180M/M204V lamivudine-resistant mutations.

References