AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

Unusual Features of Sodium Taurocholate Cotransporting Polypeptide as a Hepatitis B Virus Receptor

Jisu Li,[a] Li Zong,[a,b] Camille Sureau,[c] Luke Barker,[a] Jack R. Wands,[a] Shuping Tong[a,b]

Liver Research Center, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, Rhode Island, USA[a]; Molecular Virology Laboratory, School of Basic Medical Sciences, Fudan University, Shanghai, China[b]; Laboratoire de Virologie Moleculaire, INTS, Paris, France[c]

ABSTRACT

Cell culture (cc)-derived hepatitis B virus (HBV) can infect differentiated HepaRG cells, but efficient infection requires addition of polyethylene glycol (PEG) during inoculation. Identification of sodium taurocholate cotransporting polypeptide (NTCP) as an HBV receptor enabled ccHBV infection of NTCP reconstituted HepG2 cells, although very little hepatitis B surface antigen (HBsAg) is produced. We found infection by patient serum-derived HBV (sHBV), which required purification of viral particles through ultracentrifugation or PEG precipitation, was PEG independent and much more efficient in HepaRG cells than in HepG2/NTCP cells. In contrast to hepatitis B e antigen (HBeAg), HBsAg was not a reliable marker of productive sHBV infection at early time points. A low HBsAg/HBeAg ratio by ccHBV-infected HepG2/NTCP cells was attributable to dimethyl sulfoxide (DMSO) in culture medium, NTCP overexpression, and HBV genotype D. HepG2/NTCP cells released more viral antigens than HepG2 cells after HBV genome delivery by adeno-associated virus, and stable expression of NTCP in a ccHBV producing cell line increased viral mRNAs, proteins, replicative DNA, and covalently closed circular DNA. NTCP protein expression in HepG2/NTCP cells, despite being driven by the cytomegalovirus promoter, was markedly increased by DMSO treatment. This at least partly explains ability of DMSO to promote ccHBV infection in such cell lines. In conclusion, NTCP appeared inefficient to mediate infection by serum-derived HBV. It could promote HBV RNA transcription while inhibiting HBsAg secretion. Efficient PEG-independent sHBV infection of HepaRG cells permits comparative studies of diverse clinical HBV isolates and will help identify additional factors on virion surface promoting attachment to hepatocytes.

IMPORTANCE

Currently *in vitro* infection with hepatitis B virus (HBV) depends on cell culture-derived HBV inoculated in the presence of polyethylene glycol. We found patient serum-derived HBV could efficiently infect differentiated HepaRG cells independent of polyethylene glycol, which represents a more physiological infection system. Serum-derived HBV has poor infectivity in HepG2 cells reconstituted with sodium taurocholate cotransporting polypeptide (NTCP), the currently accepted HBV receptor. Moreover, HepG2/NTCP cells secreted very little hepatitis B surface antigen after infection with cell culture-derived HBV, which was attributed to NTCP overexpression, genotype D virus, and dimethyl sulfoxide added to culture medium. NTCP could promote HBV RNA transcription, protein expression, and DNA replication in HepG2 cells stably transfected with HBV DNA, while dimethyl sulfoxide could increase NTCP protein level despite transcriptional control by a cytomegalovirus promoter. Therefore, this study revealed several unusual features of NTCP as an HBV receptor and established conditions for efficient serum virus infection *in vitro*.

Hepatitis B virus (HBV) is a major etiological agent for liver cirrhosis and hepatocellular carcinoma (1). The nature of the liver-specific HBV receptor(s) has been a longstanding puzzle in the field (2); this is partly attributable to the paucity of cell lines supporting productive HBV infection. Other than primary human hepatocytes (PHHs) and primary tupaia hepatocytes (PTHs), only the human liver progenitor cell line HepaRG could be infected with HBV after prolonged treatment with dimethyl sulfoxide (DMSO) (3). DMSO promotes the differentiation of HepaRG cell into foci of hepatocytes surrounded by biliary cells. Other human hepatoma cell lines such as HepG2 and Huh7 support HBV DNA replication and virion production upon transfection with cloned HBV genome but not after inoculation with HBV particles. HBV protein expression and genome replication are driven by several coterminal transcripts ranging from 0.7 to 3.5 kb (4). The subgenomic RNAs of 2.4 and 2.1 kb are responsible for the expression of three coterminal envelope proteins termed large (L), middle (M), and small (S), with the M protein having an extra preS2 domain than the S protein and the L protein having an extra preS1 domain than the M protein. In addition to their incorporation into virions, the envelope proteins, especially S and M, are secreted as capsid-free subviral particles that exceed virions by ≥1,000-fold. The large quantity of S protein associated with subviral particles is detected by enzyme-linked immunosorbent assay (ELISA) as hepatitis B surface antigen (HBsAg), which provides a sensitive serological marker of HBV infection. Another serological marker is hepatitis B e antigen (HBeAg), a secreted

Received 14 June 2016 **Accepted** 3 July 2016

Accepted manuscript posted online 6 July 2016

Citation Li J, Zong L, Sureau C, Barker L, Wands JR, Tong S. 2016. Unusual features of sodium taurocholate cotransporting polypeptide as a hepatitis B virus receptor. J Virol 90:8302–8313. doi:10.1128/JVI.01153-16.

Editor: G. McFadden, University of Florida

Address correspondence to Jisu Li, Ji_Su_Li_MD@Brown.edu.

form of capsid (core) protein. HBeAg is translated from a subset of the 3.5-kb RNA called the precore RNA. The other subset, or pregenomic (pg) RNA, serves as mRNA for core protein and DNA polymerase. It is the only RNA to be packaged inside capsid assembled from core protein for conversion to progeny DNA. Since the efficiency of HBV infection *in vitro* remains quite low, measurement of HBeAg and HBsAg from culture supernatant provides simple, sensitive, and quantifiable markers of HBV infection.

According to nucleotide sequence divergence of the entire HBV genome, viral isolates worldwide can be grouped into eight major genotypes (A to H) and two minor genotypes (I and J) (5, 6). Thus far, most *in vitro* infection experiments were based on viral particles concentrated from culture supernatant of HepG2 cells stably transfected with over-length (1.1-copy) HBV genome of genotype D (7–9). Infectivity of such cell culture-derived HBV (ccHBV) particles requires the addition of 4% polyethylene glycol (PEG) during inoculation (10), which has been reported to promote virus attachment to cell surface (11). Independent studies identified heparan sulfate proteoglycans (HSPG) as the low-affinity HBV receptor (11, 12), and a recent work revealed glypican 5 as a major carrier of cell surface HSPG involved in HBV entry (13, 14). The critical HSPG binding sites have been mapped to several basic residues in the "a" determinant of the S domain (15), which could explain the ability of anti-S antibodies to neutralize HBV infectivity. HBV infectivity could also be neutralized by antibodies against the amino terminus of the preS1 domain, which has been implicated in binding to the high-affinity HBV receptor. Recently, Wenhui Li's group identified sodium taurocholate cotransporting polypeptide (NTCP) as a binding partner for myristoylated preS1 peptide 2-48 (nomenclature based on genotype D) (16). NTCP was found by RNA interference to be essential for HBV and hepatitis delta virus (HDV) infection of PHH and HepaRG cells. Conversely, introduction of NTCP cDNA into HepG2 and Huh7 cells conferred susceptibility to infection by HBV and HDV, respectively (16). These seminal findings established NTCP as an HBV and HDV receptor, a demonstration that has been independently confirmed and extended (17–28). Consequently, NTCP substrates or inhibitors such as tauroursodeoxycholic acid (TUDCA), cyclosporine, irbesartan, and ritonavir could suppress ccHBV or HDV infection (18, 20–24). Nevertheless, NTCP-reconstituted HepG2 cells cultured in the presence of DMSO reportedly released up to 100 times more HBeAg than differentiated HepaRG cells after ccHBV infection, but comparable amounts of HBsAg (18). In this regard, the HBsAg/HBeAg ratio seen in differentiated HepaRG cells was closer to, but still lower than that of viremic serum samples derived from chronic HBV carriers (unpublished observations). The greatly distorted HBsAg/HBeAg ratio after NTCP-mediated HBV infection raises questions regarding its role as the physiological HBV receptor *in vivo*. In the present study, we examined factors contributing to reduced HBsAg production in ccHBV infected HepG2/NTCP cells and established conditions for efficient infection of HepaRG cells with serum-derived HBV (sHBV). Furthermore, we found that NTCP expression in HepG2 cells promoted HBV protein expression and DNA replication at a postentry step.

MATERIALS AND METHODS

Chemicals, NTCP expression construct, and antibodies. DMSO and PEG8000 were purchased from Sigma. TUDCA was from Calbiochem. Human NTCP cDNA cloned to pcDNA6 vector under the cytomegalovirus (CMV) promoter was kindly provided by Wenhui Li. The recombinant NTCP protein thus produced contains bovine rhodopsin tag at the carboxyl terminus to enable its detection by a mouse monoclonal antibody (1D4; Santa Cruz), which was used at dilutions of 1:1,000 for Western blotting and 1:100 for immunofluorescence (IF) staining. The other antibodies used included anti-β actin (Sigma), rabbit anti-core (Dako), rabbit anti-preS1 (R271) (29), another rabbit antibody against preS1 residues 12 to 46 of genotypes B/C (MGTNLSVPNPLGFFPDHQLDPAFGA NSNNPDWDFN; GenScript), mouse monoclonal anti-preS2 (S26; Virogen), rabbit anti-S (NB100-62652; Novus), goat anti-S (custom-made), and horse anti-S (anti-Ad/Ay; Abcam).

ccHBV and sHBV. HepDE19 cell line with tetracycline-regulated HBV DNA replication was used as the source of ccHBV (9). It has 1.1 copies of the HBV genome of genotype D stably integrated to the chromosomal DNA of HepG2 cells, with transcription of pgRNA induced by doxycycline removal from the culture medium. Virions and subviral particles were concentrated from culture supernatant by precipitation with 5% PEG (3) or ultracentrifugation at 39,000 rpm for 16 h through 10 to 20% sucrose cushion in a Sorvall SW41 rotor. Particles in the pellet were resuspended in 1/100 the original volume with complete medium or phosphate-buffered saline (PBS) for storage at −80°C, and the genome copy number was determined by dot hybridization using serial dilutions of cloned HBV DNA of known concentration for calibration (30). Similar procedures were used to purify sHBV from viremic serum samples collected from chronic HBV carriers from Shanghai, China, for another study (31). Informed consent was obtained from patients, and the study was approved by Institutional Board and by the Recombinant DNA Committee of Rhode Island Hospital. To determine the HBV genotype of the sHBV isolates, the envelope gene (nucleotides 2836 to 3215 and 1 to 866) was amplified by PCR, followed by direct sequencing and phylogenetic analysis (32). The primers used for PCR and sequencing were sense (5′-GTCACCATATTCTTGGGAAC-3′) and antisense (5′-CATATCCC ATGAAGTTAAGG-3′). All the six sHBV isolates used in the present study belong to genotype C.

HBV-susceptible cell lines, viral inoculation, and assays for infectivity. The HepaRG cell line was kindly provided by Christian Trepo. It was grown in William's E Medium supplemented with hydrocortisone and insulin (3). Confluent cells were further cultured in medium supplemented with 2% DMSO for a minimum of 2 weeks prior to infection. HepG2 cells were transfected with human NTCP cDNA, and stable HepG2/NTCP transfectants were selected by blasticidin (10 μg/ml) as a mixed population. Alternatively, individual clones of stable NTCP transfection were obtained by single cell isolation, followed by expansion. Most infection experiments were performed on confluent cells cultured in 96-well plates without DMSO. Cells were incubated overnight with ccHBV or sHBV, followed by a wash. The medium was changed and collected every 2 or 3 days. HBsAg and HBeAg in cell culture supernatant were measured by commercial ELISA kits (KHB, Shanghai, China). If the optical density values exceeded 2.5, the samples were diluted and measured again to avoid signal saturation. Thus, HBsAg or HBeAg values of >2.5 in some figures were calculated from values measured from diluted samples. The HBsAg/HBeAg ratio refers to HBsAg titer in culture supernatant divided by HBeAg titer from the same volume of culture supernatant. Southern and Northern blot analyses were performed as previously described (29, 31), and for such purposes the cells were seeded in 6- or 12-well plates for infection experiments. To avoid bias in detecting ccHBV or sHBV, the 3.1-kb HBV DNA of genotypes B, C, and D was mixed at 1:1:2 ratio for probe labeling.

HBV DNA replication and protein expression in HepDE19 versus HepDE19/NTCP cells. HepDE19 cells were transfected with NTCP cDNA, followed by growth in blasticidin containing medium to generate HepDE19/NTCP cells as a mixed population. After the removal of doxycycline from confluent HepDE19 and HepDE19/NTCP cells, culture supernatant was collected every 2 to 3 days, and the cells were harvested at

fixed time points. Covalently closed circular DNA (cccDNA) was extracted according to an established method (33). Virions were immunoprecipitated from culture supernatant by a combination of rabbit anti-preS1 antibody (R271) and horse anti-Ad/Ay antibody according to established protocols (29, 31). Virion DNA, intracellular replicative DNA, and cccDNA were detected by Southern blotting, while HBV RNA was detected by Northern blotting. Core, S, and β-actin proteins were detected by Western blotting as described previously (29, 31).

Transient-transfection experiments. HepG2 cells seeded in 12-well plates were cotransfected with secreted alkaline phosphatase (SEAP) cDNA, NTCP cDNA, and tandem dimer of HBV geno1.2 (genotype D) or HBV 6.2 (genotype A) at different ratios, using Lipofectamine 3000 reagents (Invitrogen). The culture supernatant was measured for both HBeAg and HBsAg, whereas the cell lysate was used for HBsAg measurement after dilution with PBS.

AAV-HBV infection. rAAV-HBV1.3, in which 1.3 copies of the HBV genome of ayw subtype (genotype D) or adr subtype (genotype C) was cloned to adeno-associated virus (AAV) 8 vector, was purchased from Beijing Fiveplus Molecular Medicine Institute. The viral stock contained 10^{12} viral genomes/ml. HepG2, HepG2/NTCP, and differentiated HepaRG cells were seeded in 96-well plates, inoculated overnight with rAAV-HBV1.3, and then monitored for HBsAg and HBeAg secretion.

Statistical analysis. Differences between the groups were examined by using a Student *t* test. A *P* value of <0.05 is indicated by an asterisk. All experiments were repeated for ≥ 3 times, and data are presented as means or as means ± the standard deviations (SD).

Accession number(s). Sequences for the six sHBV isolates used in the present study were deposited in GenBank (accession numbers KX300210 to KX300215).

RESULTS

Overview. The present study compared infectivity of two types of HBV inoculum (ccHBV and sHBV) in two human liver cell lines (HepaRG and HepG2/NTCP). The ccHBV isolate was based on genotype D, whereas all of the sHBV isolates used for the present study were of genotype C. Endogenous NTCP expression in differentiated HepaRG cells was driven by its own promoter, while HepG2/NTCP cells overexpressed NTCP under the cytomegalovirus (CMV) promoter. A mixed population of HepG2/NTCP cells was used for most of the infection experiments. The impacts of the method of virus purification, the inoculation size and procedure, and the culture conditions on HBV infectivity were determined. HBV infection was monitored by ELISA detection of HBsAg and HBeAg in the culture supernatant and validated by Northern and Southern blot analyses. Anti-preS and anti-S antibodies were evaluated for their ability to block ccHBV and sHBV infection, which raised questions about the reliability of HBsAg as an early marker of productive sHBV infection. To validate findings from the infection experiments and to establish the postentry effects of NTCP on HBV, HBV genome of genotype D or a non-D genotype was delivered to HepG2, HepG2/NTCP, or HepaRG cells by AAV vector or by transient transfection. Furthermore, NTCP expression construct was transiently cotransfected with the HBV genome into HepG2 cells or stably transfected into HepDE19 cells, the ccHBV producer cell line.

Conditions for efficient ccHBV infection. ccHBV was concentrated from culture supernatant of HepDE19 cell line by ultracentrifugation through sucrose gradient or precipitation with 5% PEG. As expected (10) efficient ccHBV infection of either differentiated HepaRG cells or HepG2/NTCP cells required addition of 4% PEG during inoculation (Fig. 1A). Interestingly, seeding HepG2/NTCP cells at a higher density augmented ccHBV infection (Fig. 1B, upper panel). Culturing HepG2/NTCP cells in medium supplemented with 1% DMSO increased HBeAg production in some experiments (Fig. 1B, compare the upper left and right panels). Surprisingly, a high cell density and especially DMSO treatment markedly increased NTCP protein level in HepG2/NTCP cells (Fig. 1C), despite the fact that NTCP expression was driven by the CMV promoter. We also examined ccHBV infectivity in individual HepG2/NTCP clones. As shown in Fig. 2, a clone with higher NTCP expression (G7) manifested better susceptibility to ccHBV infection than a clone with lower expression (G2), whether according to HBeAg or HBsAg values.

sHBV infection required partial virus purification from viremic serum samples but no PEG during inoculation. Conditions for optimal sHBV infection were tested in differentiated HepaRG cells. Inoculating viremic serum samples directly to HepaRG cells led to low levels of HBeAg and HBsAg, which could be greatly improved by ultracentrifugation of serum samples through sucrose cushion or precipitation of viral/subviral particles with 5% PEG (Fig. 1D). Omitting 10% fetal bovine serum (FBS) in culture medium during inoculation moderately, but consistently, enhanced sHBV infection (Fig. 1E). Therefore, certain soluble factors in human and bovine sera could apparently interfere with sHBV infectivity. Surprisingly, adding 4% PEG during inoculation diminished sHBV infectivity (Fig. 1F), in sharp contrast to its promotion of ccHBV infection (Fig. 1A).

An NTCP substrate failed to block sHBV infectivity if present only during the inoculation step. The findings presented above prompted us to check for additional differences between ccHBV and sHBV infection. In subsequent experiments, ccHBV was concentrated by PEG precipitation while sHBV was purified by ultracentrifugation, with 4% PEG present during ccHBV inoculation. Of several NTCP inhibitors or substrates tested (cyclosporine, ritonavir, ibesartan, and TUDCA), TUDCA added at the inoculation step dose dependently inhibited ccHBV infection of both HepG2/NTCP cells (Fig. 3A) and differentiated HepaRG cells (Fig. 3B). However, it failed to inhibit sHBV infection of HepaRG cells under identical conditions (except for the absence of 4% PEG during inoculation). Rather, adding another aliquot of TUDCA immediately postinoculation was needed to inhibition sHBV infection (Fig. 3C, compare the left and right panels).

HBsAg is not a reliable marker at the early stage of sHBV infection. Although both HBsAg and HBeAg are routinely used as convenient and sensitive markers of HBV infection, they possess different biophysical properties. HBsAg corresponds to subviral particles, which can be copurified with infectious virions by both PEG precipitation and ultracentrifugation. In contrast, HBeAg is a soluble protein that can be removed by such purification procedures. Consequently, subviral particles in the ccHBV preparation could bind to the low-affinity HBV receptor and subsequently shed off, thus contributing to HBsAg signal at 2 or 3 days postinoculation (10). We compared the ability of anti-preS1, anti-preS2, and anti-S antibodies to neutralize ccHBV and sHBV infectivity, which raised questions regarding the reliability of HBsAg as an early marker of productive HBV infection. The rabbit polyclonal anti-preS1 antibody (GenScript) was raised against part of the NTCP binding site, whereas the murine monoclonal anti-preS2 antibody targets preS2 residues 13 to 16 (34). It could partially neutralize sHBV infectivity in PTHs (35). HepG2/NTCP cells or differentiated HepaRG cells were inoculated with ccHBV or sHBV overnight, with or without the presence of antibodies, followed by

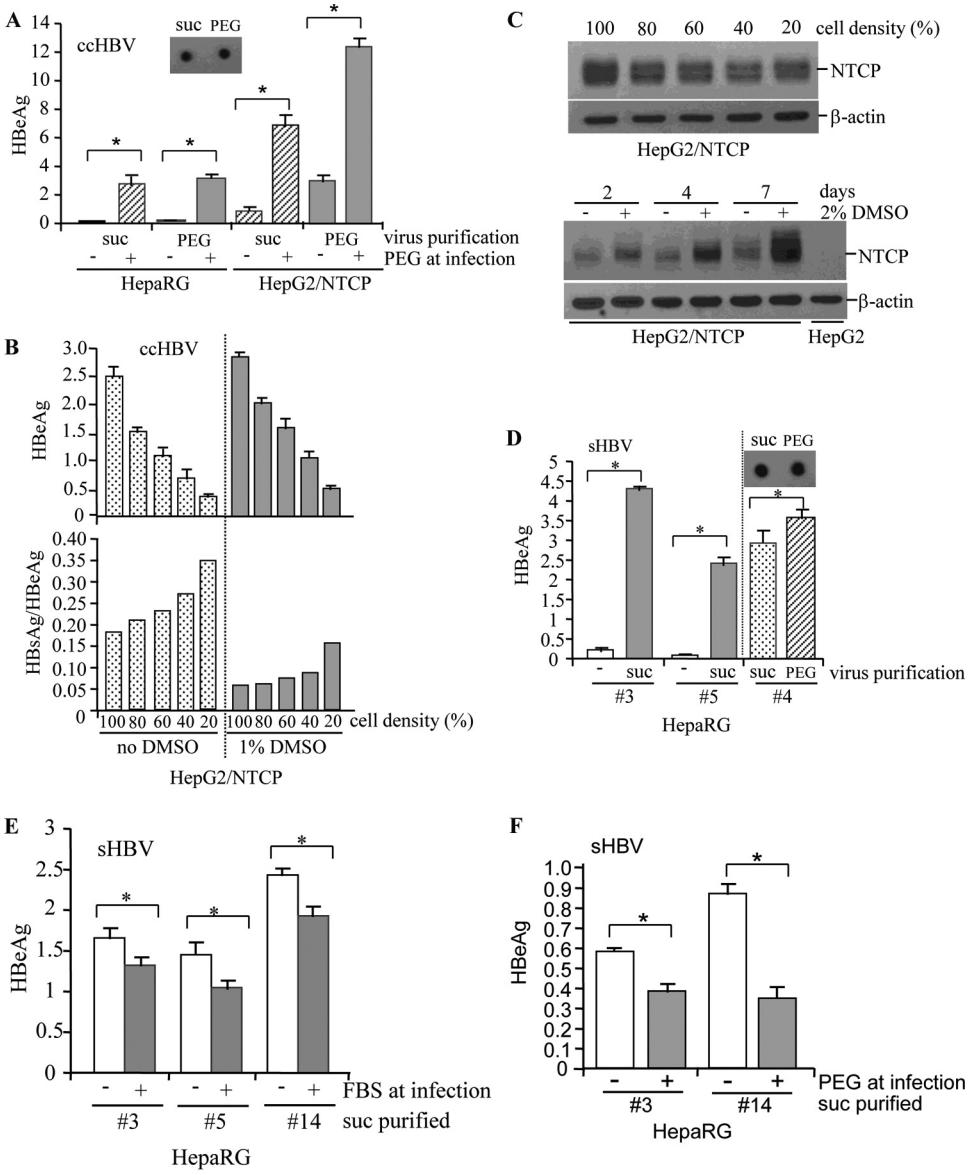

FIG 1 Identification of conditions for efficient ccHBV or sHBV infection. (A) ccHBV particles were concentrated from culture supernatant of HepDE19 cells by ultracentrifugation through sucrose (suc) cushion or PEG precipitation and used to inoculate HepaRG or HepG2/NTCP cells grown in 96-well plates at an MOI of 300, with or without 4% PEG. HBeAg values at day 14 postinfection are shown. (B) HepG2/NTCP cells seeded at the indicated densities were cultured in medium with or without 1% DMSO and then infected with ccHBV at an MOI of 500. Shown are the HBeAg values at day 12 postinfection and the calculated HBsAg/HBeAg ratios. (C) Western blot analysis of NTCP protein level in HepG2/NTCP cells seeded at different densities and harvested at the time of infection (upper panel) or after 2, 4, or 7 days of treatment of confluent cells with 2% DMSO (lower panel). (D) HepaRG cells were infected with serum samples directly or with the same amount of virus after ultracentrifugation (suc) or PEG precipitation (MOI of 800, 400, or 800 for column pairs 3, 5, or 4, respectively). Shown are the HBeAg values at day 16 postinfection. (E) sHBV purified from indicated serum samples by ultracentrifugation was resuspended in PBS and incubated with HepaRG cells in regular medium or medium lacking 10% FBS (MOI of 800, 400, or 800 for column pairs 3, 5, or 14, respectively). HBeAg values at day 7 postinfection are shown. (F) HepaRG cells were infected with sHBV purified by ultracentrifugation, with or without 4% PEG, at an MOI of 800. The finding for HBeAg at day 14 postinfection are presented. The HBeAg values in panels A, C, D, and E correspond to 50 μl of culture supernatant, while those in panel B correspond to 30 μl of culture supernatant. Data are presented as means ± the SD ($n = 3$). *, $P < 0.0001$ to 0.002 (A, D, E, and F) and $P < 0.0001$ (B, top panel). Note that the titers of virus concentrated by ultracentrifugation and PEG precipitation were similar, as revealed by dot blot hybridization using 2-μl portions of virus stocks (A and D).

a wash. The HBsAg and HBeAg titers at different time points postinoculation were measured. Figure 4 presents the results from three independent experiments (panels A to C) using the same ccHBV stock but sHBV from different patients. Based on HBeAg titers at day 7 or 9 postinoculation, ccHBV infectivity in both HepG2/NTCP and HepaRG cells was completely blocked by the anti-preS1 and anti-preS2 antibodies, and also by the goat and rabbit anti-S antibodies [S(G) and S(R)], at the concentrations used (Fig. 4, upper panels). sHBV infectivity, as determined in HepaRG cells, was more difficult to be neutralized, especially by the anti-S antibodies (Fig. 4, second panels). This was probably related to a much higher abundance of sub-

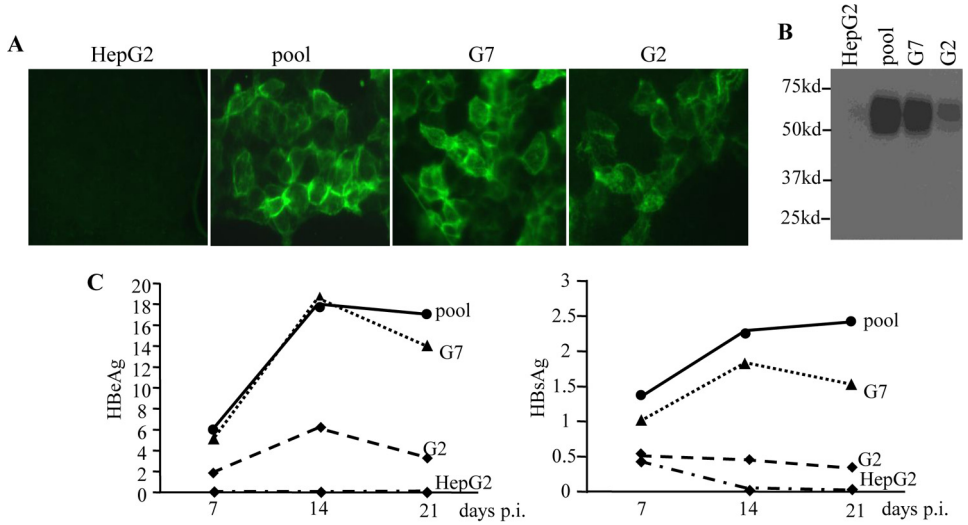

FIG 2 Correlation of ccHBV infectivity with NTCP expression level in pooled HepG2/NTCP cells and individual clones. (A) IF staining of NTCP expression using anti-rhodopsin antibody, with HepG2 cells serving as a negative control. (B) Western blot analysis of NTCP from 20 μg of total cellular proteins. (C) HBeAg and HBsAg titers from 50 μl of culture supernatant at three time points postinfection with ccHBV at an MOI of 200. Pool, pooled HepG2/NTCP cells. G2 and G7 are HepG2/NTCP clones with low and high NTCP expression, respectively.

viral particles in the sHBV preparation relative to the ccHBV preparation, considering that subviral particles will compete with virions for binding to anti-S, but not to anti-preS1, antibodies.

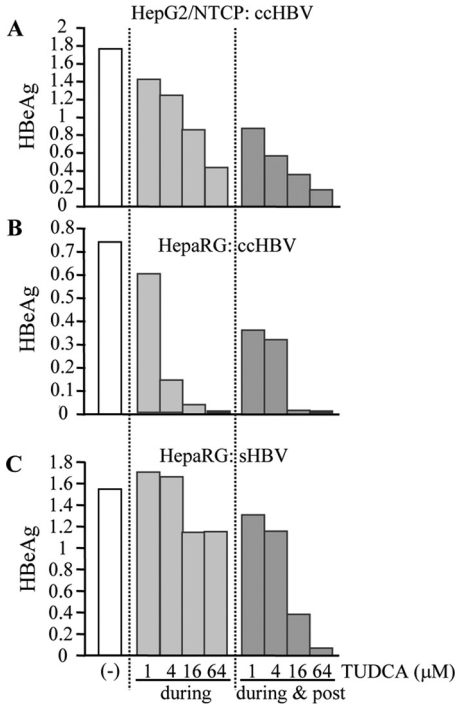

FIG 3 TUDCA added during inoculation alone failed to markedly suppress sHBV infectivity. HepG2/NTCP cells (A) and differentiated HepaRG cells (B and C) were inoculated with ccHBV in the presence of 4% PEG (A and B) or sHBV in the absence of PEG (C). TUDCA at the indicated concentration was added to the inoculum (during), with or without another dose added to the first medium change after inoculation (post). Cells infected in the absence of TUDCA served as controls. HBeAg titers were measured at day 8 (A) or day 7 (B and C) postinoculation from 50 μl of culture supernatant.

Although the anti-preS1 and anti-S antibodies abolished both HBeAg and HBsAg signals from ccHBV-infected cells, they (especially the anti-preS1 antibody) failed to abolish HBsAg in sHBV-infected HepaRG cells at day 7 or 9 postinoculation (Fig. 4, second panels). We interpreted HBsAg positivity unaccompanied by HBeAg as incoming subviral particles, which could be attributed to the high abundance of subviral particles in sHBV preparation, as well as to the inability of anti-preS1 antibodies to prevent subviral particles from attaching to hepatocytes. Consistent with this interpretation, in cells treated with the anti-preS1 antibody the HBsAg titer declined continuously during the 3 weeks of follow-up to a nearly undetectable level (Fig. 4B and C, third panels). Most surprisingly, the anti-preS2 antibody failed to significantly affect HBsAg titer after even ccHBV infection despite complete or nearly complete elimination of HBeAg (Fig. 4, first and second panels). Moreover, the high HBsAg titer persisted during the 3 weeks of follow-up (Fig. 4A and C, third panels).

HepG2/NTCP cells were poorly infectible with sHBV and produced little HBsAg following ccHBV infection. The ccHBV and sHBV infectivity was compared between HepG2/NTCP cells and differentiated HepaRG cells using serial dilutions of the viral stocks. Although infection with either type of virus was dose dependent (Fig. 5A and B), ccHBV-infected HepG2/NTCP cells released more HBeAg but less HBsAg than did HepaRG cells (Fig. 5A and B, left panels). This resulted in a >10-fold difference in the HBsAg/HBeAg ratio between the two cell lines even when HepG2/NTCP cells were cultured in DMSO-free medium (Fig. 5C, right panel). On the other hand, sHBV was much more infectious in HepaRG cells than HepG2/NTCP cells according to both HBeAg and HBsAg (Fig. 5A and B, right panels). Therefore, efficient sHBV infection required additional host factors enriched in HepaRG cells. The HBsAg/HBeAg ratio in sHBV infected HepaRG cells was higher than that of ccHBV-infected HepaRG cells, even at later time points when HBsAg becomes a more reliable marker of sHBV infection (Fig. 5C, right panel).

Patterns of HBV RNA transcription after ccHBV and sHBV infection. HBeAg is translated from the 3.5-kb precore RNA,

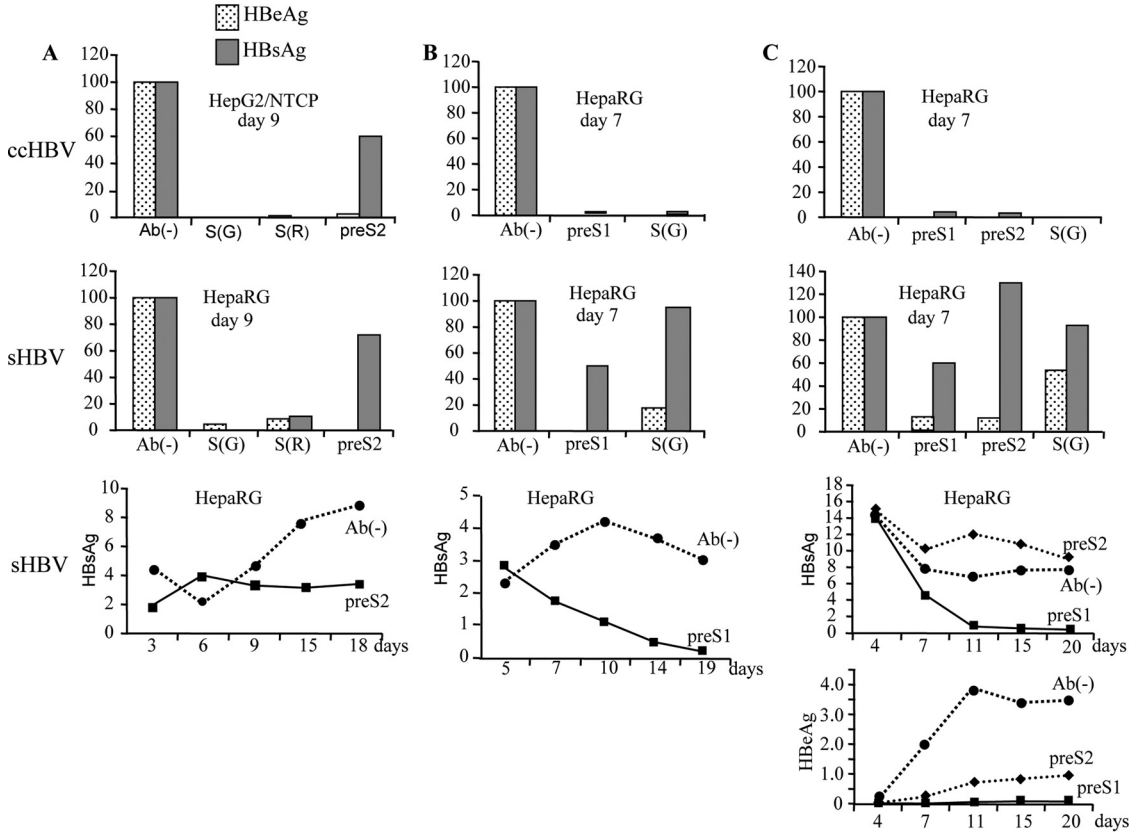

FIG 4 Neutralization of ccHBV and sHBV infectivity by anti-preS1, anti-preS2, and anti-S antibodies: impact on HBeAg and HBsAg titers. Panels A, B, and C represent three separate experiments based on the same ccHBV stock but different sHBV stocks, with the same batch of HepaRG cells infected simultaneously with ccHBV and sHBV (B and C). The top and middle panels show both HBsAg (dotted columns) and HBeAg (gray columns) values at the indicated time point postinoculation with ccHBV (top) and sHBV (middle), respectively, with values from untreated cells set at 100. The third panels show the kinetics of HBsAg from HepaRG cells infected with sHBV in the presence of anti-preS1 or anti-preS2 antibodies, with values from untreated cells shown for comparison. (C) The bottom (fourth) panel indicates the kinetics of HBeAg from HepaRG cells infected with sHBV in the presence of anti-preS1 or anti-preS2 antibodies. The HBsAg and HBeAg values correspond to 50 μl of culture supernatant. The antibody concentrations were 150 μg/ml for goat anti-S [abbreviated as S(G)] in panels A and B but 75 μg/ml in panel C, 10 μg/ml for rabbit anti-S [abbreviated as S(R)], 5 μg/ml for mouse anti-preS2, and 8.9 μg/ml for rabbit anti-preS1.

whereas S protein, the major viral component of HBsAg, is translated from the 2.1-kb subgenomic RNA. To verify whether the abnormal HBsAg/HBeAg ratio seen in ccHBV-infected HepG2/NTCP cells was attributable to distorted ratio of the 3.5-kb RNA over the 2.1-kb RNA, Northern blot analysis was performed. sHBV-infected HepaRG cells showed a typical pattern of HBV RNA transcription, with the 2.4- and 2.1-kb RNAs (which could not be resolved into separate bands) being much more abundant than the 3.5-kb RNAs (Fig. 6A). ccHBV-infected HepaRG cells produced more 3.5-kb RNA but comparable amounts of the 2.4- and 2.1-kb RNAs, which could explain the reduced HBsAg/HBeAg ratio. In ccHBV-infected HepG2/NTCP cells, ratio of the 3.5-kb RNA over the 2.4- and 2.1-kb RNA also increased (Fig. 6A). This was consistent with a recent report that precore/pgRNA were the most prominent HBV RNA species in *de novo*-infected HepG2/NTCP cells (36). Therefore, the differences in HBsAg/HBeAg ratio after sHBV and ccHBV infection of the two cell types were somewhat reflected at the transcription level. The pattern of replicative HBV DNA was similar between ccHBV and sHBV infection and irrespective of the cell type, with the level of HBV DNA in better agreement with HBeAg than with HBsAg titer for both ccHBV and sHBV (Fig. 6B and C). Prolonged differentiation of HepaRG cells led to increased susceptibility to ccHBV infection, as evidenced by higher HBeAg titers and stronger signals of replicative DNA than that observed with HepG2/NTCP cells infected in parallel (Fig. 6C).

Viral genotype, cell type, DMSO treatment, and NTCP overexpression all contributed to distorted HBsAg/HBeAg ratio. As already mentioned, even HepG2/NTCP cells cultured in the absence of DMSO showed >10-fold reduction in HBsAg/HBeAg ratio than ccHBV-infected HepaRG cells cultured with 2% DMSO. Adding 1% DMSO to culture medium of HepG2/NTCP cells further reduced the ratio (Fig. 1B, lower panel). Indeed, Ni and colleagues found an up to 100-fold difference in HBsAg/HBeAg ratio between differentiated HepaRG cells and their batch of HepG2/NTCP cells cultured with 2 or 2.5% DMSO (18). We found that seeding cells at a high density also reduced the HBsAg/HBeAg ratio (Fig. 1B, lower panel). Since both DMSO treatment and high cell density could upregulate NTCP protein level (Fig. 1C), NTCP overexpression might be one of the factors reducing the HBsAg/HBeAg ratio. Considering that ccHBV was genotype D based, and that genotype D isolates release less HBsAg than isolates of genotypes A to C (unpublished observations), the use of genotype D-based

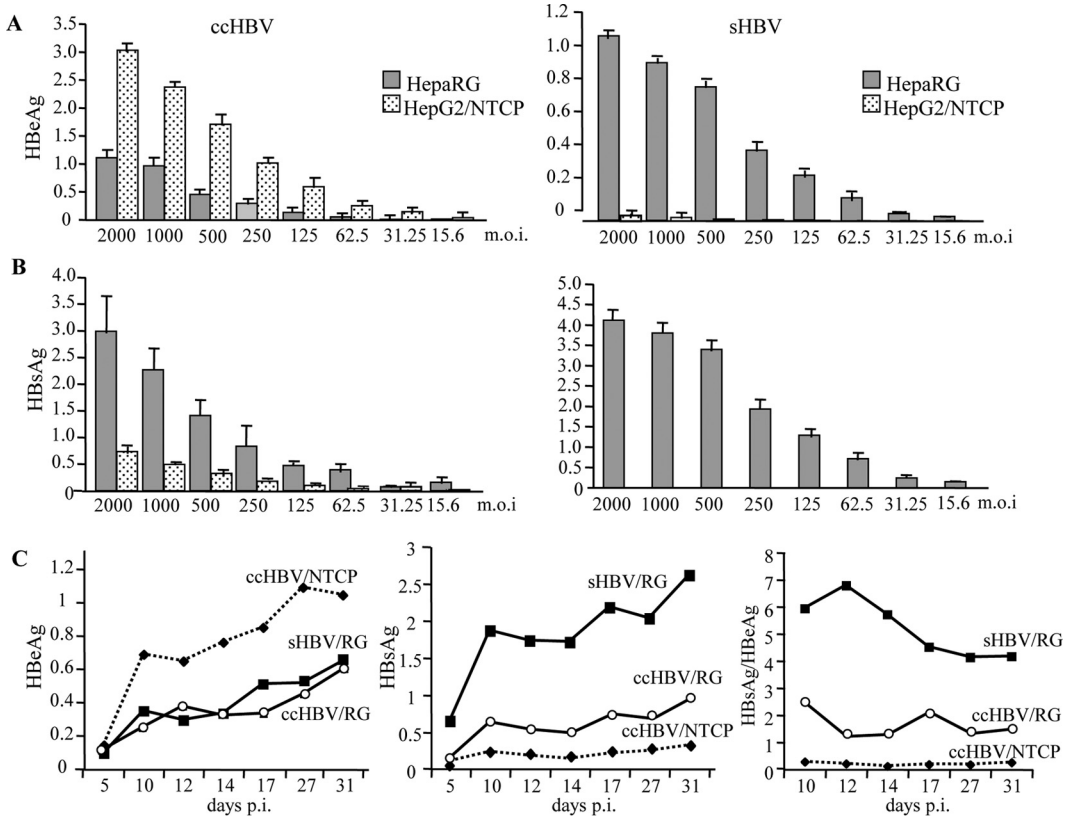

FIG 5 Comparative ccHBV and sHBV infectivity in HepaRG and HepG2/NTCP cells. (A and B) Dose-dependent infection. HepaRG cells (differentiated for 3 weeks) and HepG2/NTCP cells were inoculated with ccHBV or sHBV at the indicated MOI. Shown are the HBeAg (A) and HBsAg (B) titers from 50 μl of culture supernatant at day 7 postinfection. (C) Kinetics of HBeAg, HBsAg, and HBsAg/HBeAg ratio from cells infected at an MOI of 500. NTCP, HepG2/NTCP cells; RG, HepaRG cells. Both HBeAg and HBsAg were measured from 10 μl of culture supernatant.

ccHBV might contribute to the low HBsAg/HBeAg ratio as well.

To test our hypothesis, 1.3 copies of the HBV genome of genotype C or D were delivered by AAV vector (rAAV-HBV1.3) to HepG2, HepG2/NTCP, and differentiated HepaRG cells. HepG2/NTCP cells produced higher levels of HBsAg than did HepG2 cells (Fig. 7A) but even higher levels of HBeAg (Fig. 7B), leading to a reduced HBsAg/HBeAg ratio (Fig. 7C). For these two cell lines, genotype D was associated with a much lower HBsAg/HBeAg ratio than genotype C (Fig. 7C). HepaRG cells inoculated with rAAV-HBV1.3 of genotype C produced more HBsAg and HBeAg than HepG2 or HepG2/NTCP cells, but a comparable HBsAg/HBeAg ratio (Fig. 7D, E, and F). However, they showed an ~10-fold-higher HBsAg/HBeAg ratio than HepG2 cells after infection with rAAV-HBV1.3 of genotype D, primarily due to much higher HBsAg production (Fig. 7D, E, and F). In HepaRG cells, genotype D was rather associated with higher HBsAg/HBeAg ratio than was genotype C (Fig. 7F).

The role of viral genotype and NTCP on the HBsAg/HBeAg ratio was further investigated by cotransfecting HepG2 cells with NTCP expression construct and dimeric HBV DNA of genotype D or A. Such an experimental setting enabled us to alter the NTCP/HBV ratio at will. We cotransfected 0.9 μg of NTCP or SEAP cDNA with 0.1 μg of HBV DNA (9:1 ratio) to mimic NTCP overexpression accompanied by a very low level of HBV infection, as seen in HepG2/NTCP cells. Alternatively, 0.5 μg of NTCP or SEAP cDNA was cotransfected with 0.5 μg of HBV DNA (1:1 ratio) to mimic conditions possibly seen in differentiated HepaRG cells or human liver. Again, genotype D was found to be associated with a lower HBsAg/HBeAg ratio, although increasing the amount of HBV DNA transfected from 0.1 to 0.5 μg diminished the genotypic difference (Fig. 8B, upper panel). The HBsAg/HBeAg ratio was diminished by NTCP, especially at a 9:1 ratio, which could be further reduced by adding 2% DMSO to culture medium (Fig. 8B, compare the upper and lower panels). Quantification of the extracellular/intracellular ratio of HBsAg suggested that both DMSO treatment and NTCP expression could suppress HBsAg secretion (Fig. 8C).

Stable NTCP transfection increased HBV DNA and protein levels. An unexpected finding from rAAV-HBV1.3 infection experiments was higher HBsAg and HBeAg production by HepG2/NTCP cells than that observed with the parental HepG2 cells (Fig. 7A and B). A similar effect was observed after transient transfection of HBV DNA of genotype D or A into the two cell lines, especially at the low DNA dose (data not shown), but not transient cotransfection of NTCP and HBV DNAs (Fig. 8A). These results suggested that NTCP could somehow increase HBV protein expression, probably through a secondary event. To further characterize the postentry effect of NTCP in the HBV life cycle, HepDE19 cells were stably transfected with NTCP expression plasmid to generate the HepDE19/NTCP cell line. After doxycycline removal to induce HBV DNA replication, HepDE19/NTCP

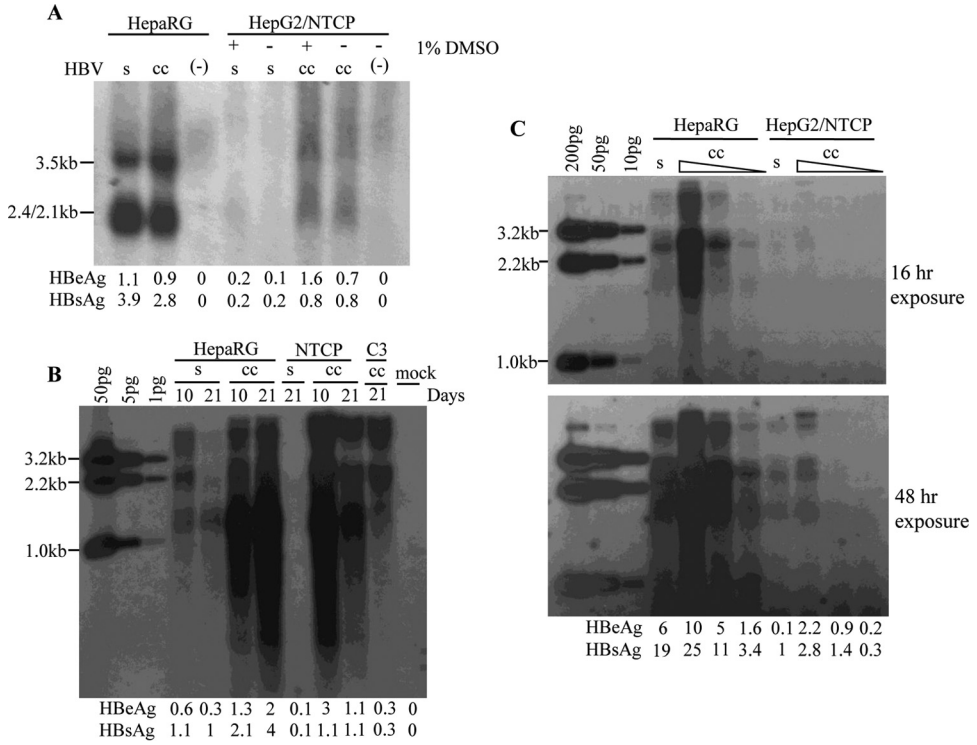

FIG 6 Intracellular HBV RNAs and replicative HBV DNA after ccHBV and sHBV infection. (A) Northern blot analysis at day 14 postinfection (MOI = 300). s, sHBV; cc, ccHBV. (B) Southern blot analysis at day 10 or 21 postinfection (MOI = 500). Restriction enzyme-digested HBV genomes served as size markers. C3, a HepG2/NTCP clone. (C) Southern blot analysis at day 10 postinfection from HepG2/NTCP cells and a batch of HepaRG cells differentiated for 4 months, with an MOI of 500 for sHBV and MOIs of 500, 167, and 50 for ccHBV. Shown are the two different exposures of the same blot. The HBeAg and HBsAg values shown in all panels correspond to 20 μl of culture supernatant.

cells expressed higher levels of HBV core and envelope proteins (Fig. 9B), and secreted more HBeAg and HBsAg, than the parental HepDE19 cells (Fig. 9C). They also showed higher intracellular levels of replicative DNA and cccDNA, as well as extracellular virion DNA (Fig. 9A). Northern blot analysis revealed increased levels of both 3.5-kb and 2.4-/2.1-kb RNAs (Fig. 9A), suggesting upregulation at the transcriptional level. On the other hand, ccHBV particles produced in HepDE19/NTCP cells showed an infectivity comparable to those produced in HepDE19 cells when inoculated at the same multiplicity of infection (MOI; data not shown).

DISCUSSION

A robust, reproducible, and yet authentic *in vitro* system of HBV infection is crucial to characterize the early steps of the HBV life cycle and to identify antiviral agents with the potential to block virus entry *in vivo*. The key components of this system are the target cells, the inoculum, and the culturing conditions/inoculation procedures. PHHs suffer from limited availability, batch-to-batch variability in susceptibility, and inability for serial passage, which was partly overcome with the demonstration that PTHs were, surprisingly, susceptible to HBV infection (37). The establishment of HepaRG cell line provides unlimited supply of HBV-susceptible cells of identical genetic background (3). Still, induction of cellular differentiation essential for HBV infection is time-consuming, and HBV susceptibility differs from batch to batch, partly due to a variable ratio between hepatocytes and biliary cells (38). A role of NTCP in this system of HBV infection was inferred from experiments using NTCP inhibitors, substrates, or shRNAs, although the inhibition was often incomplete. More recently, HepG2 cell line stably transfected with NTCP cDNA offered an alternative *in vitro* system to support HBV infection. Ni et al. reported that adding up to 2.5% DMSO to culture medium could increase HBeAg production from ccHBV infected HepG2/NTCP cells by up to 10-fold, which was about 100 times higher than produced by similarly infected HepaRG cells (18). This, combined with the no need for prolonged differentiation, makes HepG2/NTCP cells a more attractive system of *in vitro* infection (39). However, infection based on NTCP overexpression can be artificial, and the use of an exogenous promoter to drive NTCP expression prevents feedback transcriptional regulation of NTCP expression, which might be operative during the natural course of infection.

As for the inoculum, most investigators used ccHBV produced in HepG2 cells by stable transfection with the HBV genome. The commonly used ccHBV systems are all based on genotype D (7–9). Efficient ccHBV infection requires the addition of 4% PEG during inoculation (3, 10). This practice has greatly facilitated elucidation of the HBV entry process, culminating with the identification of NTCP as an HBV receptor (16). Nevertheless, PEG appears to facilitate HBV virion attachment to cell surface (11), and it may bypass an authentic step of virus-host interaction. Since we performed ccHBV and sHBV infection in the presence and absence of PEG, respectively, different features of ccHBV versus sHBV infection documented here might be partly attributed to PEG. For example, adding TUDCA during inoculation could ef-

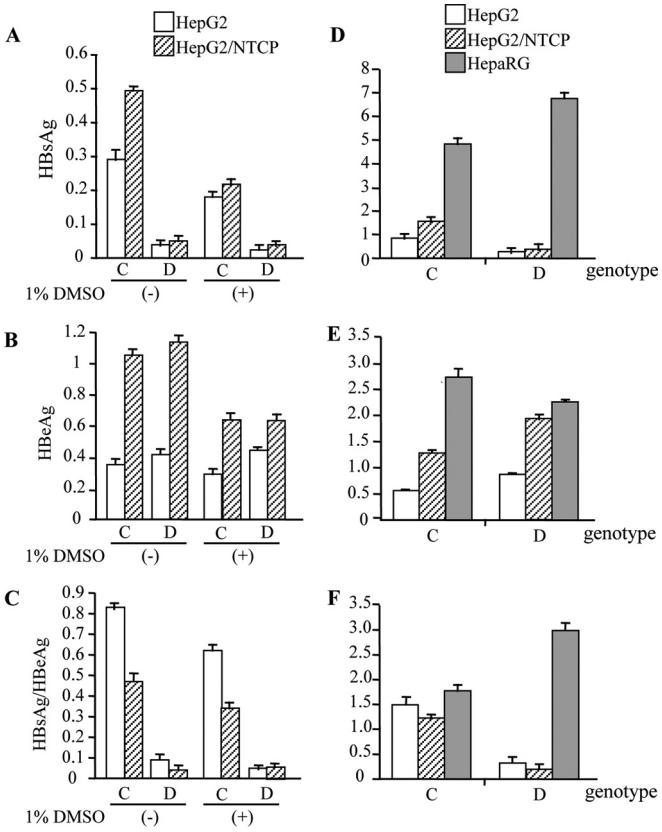

FIG 7 Comparison of rAAV-HBV1.3 infection of HepG2, HepG2/NTCP, and HepaRG cells. (A, B, and C) HepG2 and HepG2/NTCP cells seeded in 96-well plates were infected with rAAV-HBV1.3 of genotype C or genotype D at 3×10^4 genomes/cell and cultured in medium with or without 1% DMSO. Shown are HBsAg (A) and HBeAg (B) titers from 50 μl of culture supernatant at day 10 postinfection, as well as calculated HBsAg/HBeAg ratios (C). (D, E, and F) HepG2, HepG2/NTCP, and differentiated HepaRG cells in 96-well plates were infected with rAAV-HBV1.3 at 1×10^5 genomes/cell. Shown are the HBsAg (D) and HBeAg (E) titers from 5 μl of culture supernatant at day 20 postinfection, as well as the HBsAg/HBeAg ratios (F).

ficiently inhibit ccHBV infection, but its continued presence immediately postinoculation was needed to markedly suppress sHBV infection (Fig. 3). In this regard NTCP, the transporter of TUDCA and the high-affinity receptor, most likely interacts with HBV at a late time point of virus-hepatocyte interaction. PEG probably accelerates this process to enable TUDCA inhibition of ccHBV infection when present at the step of inoculation. PEG may also distort the neutralization efficiency of anti-S antibodies by aggregating viral/subviral particles or facilitating immune complex formation. Indeed, the two polyclonal anti-S antibodies tested here were less efficient at neutralizing sHBV infectivity (Fig. 4), and a 100-fold-higher concentration of a human monoclonal anti-S antibody (40) was needed to inhibit sHBV infection of HepaRG cells (performed in the absence of 4% PEG) than ccHBV infection of the same cell line (in the presence of 4% PEG) (J. Li, unpublished observations). Certainly, the sHBV stock harbors much higher proportion of subviral particles than ccHBV, which could also diminish the neutralizing capacity of anti-S antibody and render HBsAg an unreliable marker of productive sHBV infection, especially at the early time points (Fig. 4).

Compared to differentiated HepaRG cells, HepG2/NTCP cells are highly susceptible to ccHBV infection performed in the presence of 4% PEG but poorly susceptible to sHBV infection, whether in the presence or absence of PEG. This result suggests that host factors other than NTCP are required for efficient sHBV infection. However, since the ccHBV and sHBV used for the present study belong to genotypes D and C, respectively, further studies using ccHBV of genotype C and sHBV of genotype D are warranted to dissect the role of the viral genotype versus the source. Ultracentrifugation or PEG precipitation of viral/subviral particles from human serum samples greatly promoted sHBV infection (Fig. 1), suggesting the presence of soluble inhibitors of sHBV infection. The fact that sHBV infection does not require 4% PEG added during inoculation suggests that certain molecules present on sHBV but not ccHBV mediate initial HBV attachment to cell surface, which is mimicked by PEG. Consistent with our finding, virus purification by ultracentrifugation through discontinuous sucrose density gradient enabled sHBV of genotype D to infect PTH in the absence of PEG (35). PEG-independent sHBV infection of HepaRG cells better reflects natural HBV infection and should help identify additional molecules involved in the initial step of virus-hepatocyte interaction. Moreover, it opens the door to test the infectivity of clinically important HBV genetic variants (5, 6).

As for ccHBV infection of HepG2/NTCP cells, infectivity could be enhanced by a high NTCP protein level (Fig. 2), a high cell density (Fig. 1B), and also DMSO if the issue of cell toxicity could be avoided (18). Surprisingly, a higher cell density and DMSO treatment both increased the NTCP protein level (Fig. 1C). Since NTCP expression was driven by a constitutive CMV promoter and considering that for that particular experiment a blasticidin-resistant HepG2/NTCP pool was used (with the recombinant plasmid integrated randomly into cellular chromosomes), up-regulation of NTCP protein is most likely attributed to a post-translational event such as protein stability. At any rate, this reinforces the correlation between NTCP protein level and the efficiency of ccHBV infection, thus proving a pivotal role of NTCP in the infection process. Interestingly, both high cell density and DMSO treatment also reduced the HBsAg/HBeAg ratio. Experiments using an AAV vector or transient transfection confirmed the role of NTCP overexpression, HBV DNA of genotype D (especially at low level), and DMSO treatment as independent factors reducing the HBsAg/HBeAg ratio. Thus, the drastically reduced HBsAg/HBeAg ratio in HepG2/NTCP cells cultured with 2.5% DMSO and infected with ccHBV of genotype D (18) probably does not challenge NTCP as an HBV receptor *in vivo*, but this rather suggests the artificial nature of the current system of HBV infection based on NTCP overexpression in conjunction with ccHBV of genotype D. It will be interesting to determine whether the HBsAg/HBeAg ratio could be restored by inoculation with ccHBV of non-D genotypes or by switching off NTCP expression postinoculation. Intriguingly, differentiated HepaRG cells produced a high ratio of HBsAg/HBeAg after either ccHBV infection or AAV-mediated delivery of HBV genome of genotype D, despite being cultured in medium supplemented with 2% DMSO. In this regard, differentiated HepaRG cells differ from HepG2/NTCP cells in lacking NTCP overexpression and in the presence of biliary cells surrounding hepatocytes.

Cytosolic L protein could retain the S protein to block HBsAg secretion (41, 42). Considering that NTCP is a binding partner for the amino terminus of preS1 domain, it is possible that cytosolic

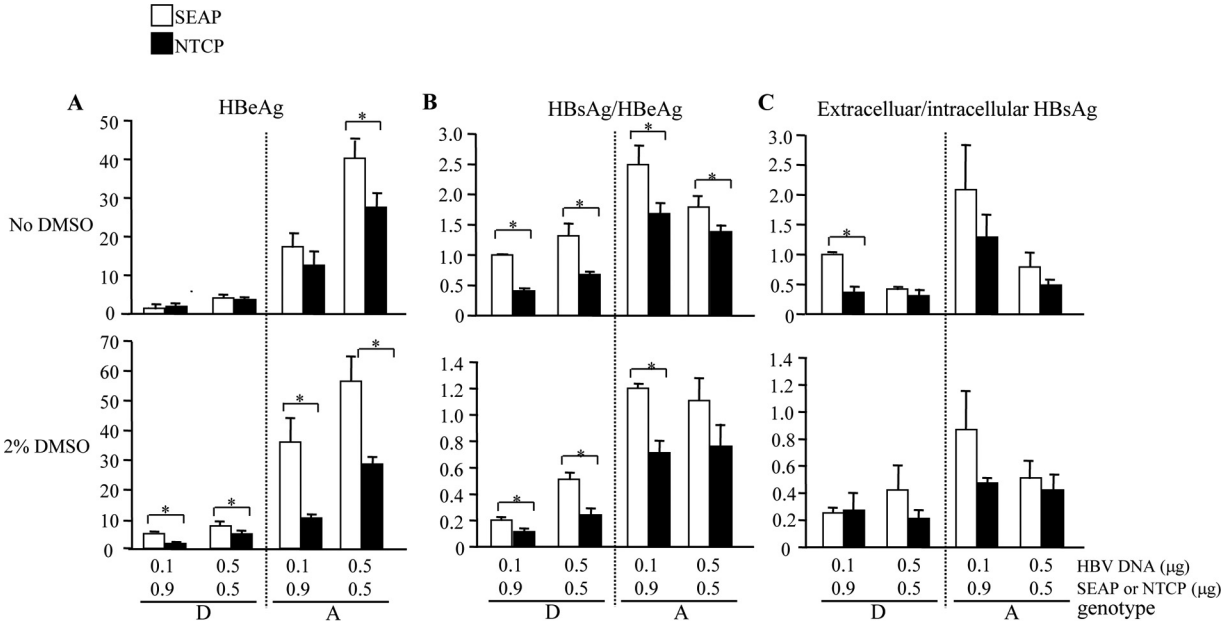

FIG 8 Impact of viral genotype, NTCP, and DMSO on HBsAg/HBeAg ratio in transiently transfected HepG2 cells. HepG2 cells seeded in 12-well plates were cotransfected with a dimeric construct of HBV genotype A (HBV6.2) or genotype D (geno1.2) and SEAP or NTCP cDNA at the indicated amounts. Cells were further cultured in medium with (lower panels) or without 2% DMSO (upper panels), with a medium change at day 3 posttransfection. HBeAg and HBsAg were measured from culture supernatant at day 5 posttransfection. HBsAg was also measured from the corresponding cell lysate. Shown are the HBeAg titers in culture supernatant at day 5 posttransfection (A), the calculated HBsAg/HBeAg ratio in culture supernatant (B), and the extracellular/intracellular HBsAg ratio (C). Data shown are averaged from three independent transfection experiments. Values for the HBsAg/HBeAg ratio and extracellular/intracellular HBsAg ratio were set at 1 for cotransfection between 0.1 μg of HBV DNA of genotype D and 0.9 μg of SEAP cDNA in cells cultured in DMSO-free medium.

NTCP inhibits HBsAg secretion through the L protein. Since ccHBV infected HepG2/NTCP cells are characterized with poor virion secretion (39), a related question is whether overexpressed NTCP suppresses virion secretion as well. Certainly, NTCP did not appear to markedly inhibit HBV virion secretion when HBV DNA replication was maintained at a high level through the strong CMV promoter (Fig. 9A, bottom panel).

The most unexpected finding of this study was that NTCP could augment HBV protein expression, genome replication, and virion secretion independent of its receptor function. This was based on transient transfection of HBV DNA into HepG2 versus HepG2/NTCP cells (data not shown) and rAAV-HBV1.3 infection of these two cell lines (Fig. 7), as well as stable NTCP transfection into HepDE19 cell line (Fig. 9). In the HepDE19 cell line, stable NTCP expression also increased cccDNA formation and the expression of HBeAg, which in this particular cell line serves as a marker of cccDNA-driven transcription (43) even though a fraction of the signal may originate from the cross-reactive core protein. Northern blot analysis suggested that NTCP worked at the step of HBV RNA transcription (Fig. 9A, top panel). However, transient cotransfection of NTCP cDNA with HBV DNA failed to increase HBeAg expression (Fig. 8A, upper panel), suggesting NTCP augments HBV RNA transcription through an indirect mechanism. Whether NTCP distorts the ratio between the 3.5-kb RNA and 2.4-kb/2.1-kb RNAs following ccHBV infection (Fig. 6A) (36) through the same host (transcription) factors requires further investigation, but such transcriptional alteration could partly explain the distorted HBsAg/HBeAg ratio.

One may wonder whether the increased signals of viral RNA, DNA, and proteins in HepDE19/NTCP cells compared to the parental HepDE19 cells (Fig. 9) is due to reinfection of the cells with HBV secreted at earlier time points. Although at present we cannot fully rule out this possibility, it seems unlikely for several reasons. First, ccHBV infection requires concentrated virus inoculated to cells in the presence of 4% PEG, and yet PEG is absent during *in vitro* culture of HepDE19/NTCP cells. Second, the changes in the intracellular levels of HBV RNAs, virion DNA, and core and S proteins can be detected as early as 1 week after induction of HBV pgRNA transcription (Fig. 9A and B). Third, pgRNA transcription, HBV DNA replication, and core protein expression driven by the CMV promoter are much more efficient than those achievable following ccHBV infection. Fourth, ccHBV infection leads to impaired HBsAg secretion, and yet HepDE19/NTCP cells continuously secreted more HBsAg than HepDE19 cells (Fig. 9C, upper panel).

Identification of the nonreceptor function of NTCP in promoting HBV DNA replication and protein expression raises questions about its full-range effect in the HBV replication cycle. Although NTCP is required for HBV infection *in vitro* according to both reconstitution and depletion experiments, and experiments using NTCP inhibitors and substrates suggest a role of NTCP on cell surface, such experiments do not exclude a role at downstream steps of viral life cycle, such as cccDNA formation and RNA transcription. Our findings also have clinical implications. Since most hepatocytes in chronic carriers of HBV are already infected with HBV, targeting an HBV receptor may have limited therapeutic benefit. In con-

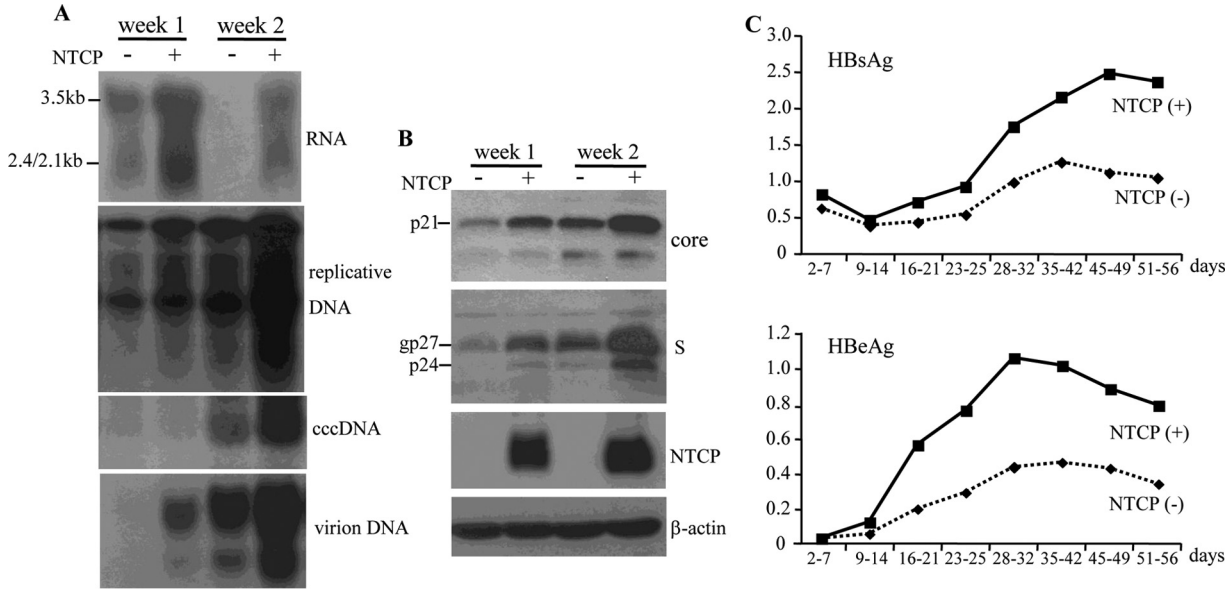

FIG 9 Stable NTCP expression in HepDE19 cell line increased levels of HBV RNAs, DNA, and proteins. (A and B) HepDE19 cells with (+) or without (−) stable NTCP transfection were seeded in six-well plates at 100% density in triplicate. The cells were cultured for 1 or 2 weeks after the removal of doxycycline. One set of cells was lysed for HBV RNA detection, another set for cccDNA extraction, and the third set for a Southern blot of replicative HBV DNA and a Western blot of viral proteins. Culture supernatant was used for the immunoprecipitation of virions. (A) Northern and Southern blot analyses of intracellular viral RNAs, replicative DNA, cccDNA, and extracellular virion DNA. (B) Western blot analysis of intracellular core, S, and NTCP proteins, as well as β actin to serve as a loading control. (C) HepDE19 cells with or without stable NTCP transfection were seeded in 10-cm dishes coated with collagen, and culture supernatant was collected from 2 to 56 days after doxycycline removal. Media from three consecutive time points were combined at an equal ratio, and HBsAg and HBeAg were measured from 15 and 30 μl of 1:10 diluted culture supernatant, respectively.

trast, if NTCP is a major positive regulator of HBV protein expression *in vivo*, then targeting NTCP may help reduce viral antigenemia to promote HBeAg or HBsAg seroconversion.

ACKNOWLEDGMENTS

We are grateful to Christian Trepo for the HepaRG cell line. We thank Wenhui Li for the human NTCP cDNA, Haitao Guo for the HepDE19 cell line, and Yanli Qin for viremic serum samples.

This study was supported by National Institutes of Health grants R21AI103648, R21AI107618, R01AI116639, and R21AI113394.

FUNDING INFORMATION

This work, including the efforts of Jisu Li and Shuping Tong, was funded by HHS | National Institutes of Health (NIH) (R21AI103648, R21AI107618, R01AI116639, and R21AI113394).

REFERENCES

1. **Trepo C, Chan HL, Lok A.** 2014. Hepatitis B virus infection. Lancet **384:**2053–2063. http://dx.doi.org/10.1016/S0140-6736(14)60220-8.
2. **Glebe D, Urban S.** 2007. Viral and cellular determinants involved in hepadnaviral entry. World J Gastroenterol **13:**22–38. http://dx.doi.org/10.3748/wjg.v13.i1.22.
3. **Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C.** 2002. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A **99:**15655–15660. http://dx.doi.org/10.1073/pnas.232137699.
4. **Seeger C, Mason WS.** 2015. Molecular biology of hepatitis B virus infection. Virology **479-480:**672–686. http://dx.doi.org/10.1016/j.virol.2015.02.031.
5. **Tong S, Li J, Wands JR, Wen YM.** 2013. Hepatitis B virus genetic variants: biological properties and clinical implications. Emerg Microbes Infect **2:**e10. http://dx.doi.org/10.1038/emi.2013.10.
6. **Tong S, Revill P.** 2016. Overview of hepatitis B viral replication and genetic variability. J Hepatol **64:**S4–S16. http://dx.doi.org/10.1016/j.jhep.2016.01.027.
7. **Sells MA, Chen ML, Acs G.** 1987. Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci U S A **84:**1005–1009. http://dx.doi.org/10.1073/pnas.84.4.1005.
8. **Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, Seeger C, King RW.** 1997. Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob Agents Chemother **41:**1715–1720.
9. **Guo H, Jiang D, Zhou T, Cuconati A, Block TM, Guo JT.** 2007. Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation. J Virol **81:**12472–12484. http://dx.doi.org/10.1128/JVI.01123-07.
10. **Gripon P, Diot C, Guguen-Guillouzo C.** 1993. Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. Virology **192:**534–540. http://dx.doi.org/10.1006/viro.1993.1069.
11. **Schulze A, Gripon P, Urban S.** 2007. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology **46:**1759–1768. http://dx.doi.org/10.1002/hep.21896.
12. **Leistner CM, Gruen-Bernhard S, Glebe D.** 2008. Role of glycosaminoglycans for binding and infection of hepatitis B virus. Cell Microbiol **10:**122–133.
13. **Verrier ER, Colpitts CC, Bach C, Heydmann L, Weiss A, Renaud M, Durand SC, Habersetzer F, Durantel D, Abou-Jaoude G, Lopez Ledesma MM, Felmlee DJ, Soumillon M, Croonenborghs T, Pochet N, Nassal M, Schuster C, Brino L, Sureau C, Zeisel MB, Baumert TF.** 2016. A targeted functional RNA interference screen uncovers glypican 5 as an entry factor for hepatitis B and D viruses. Hepatology **63:**35–48. http://dx.doi.org/10.1002/hep.28013.
14. **Li J, Wands J.** 2016. Hepatitis B and D viral receptors. Hepatology **63:**11–13. http://dx.doi.org/10.1002/hep.28131.
15. **Sureau C, Salisse J.** 2013. A conformational heparan sulfate binding site essential to infectivity overlaps with the conserved hepatitis B virus a-determinant. Hepatology **57:**985–994. http://dx.doi.org/10.1002/hep.26125.
16. **Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B,**

Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W. 2012. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. eLife **1:**e00049. http://dx.doi.org/10.7554/eLife.00049.

17. **Drexler JF, Geipel A, Konig A, Corman VM, van Riel D, Leijten LM, Bremer CM, Rasche A, Cottontail VM, Maganga GD, Schlegel M, Muller MA, Adam A, Klose SM, Carneiro AJ, Stocker A, Franke CR, Gloza-Rausch F, Geyer J, Annan A, Adu-Sarkodie Y, Oppong S, Binger T, Vallo P, Tschapka M, Ulrich RG, Gerlich WH, Leroy E, Kuiken T, Glebe D, Drosten C.** 2013. Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes. Proc Natl Acad Sci U S A **110:**16151–16156. http://dx.doi.org/10.1073/pnas.1308049110.
18. **Ni Y, Lempp FA, Mehrle S, Nkongolo S, Kaufman C, Falth M, Stindt J, Koniger C, Nassal M, Kubitz R, Sultmann H, Urban S.** 2014. Hepatitis B and D viruses exploit sodium taurocholate cotransporting polypeptide for species-specific entry into hepatocytes. Gastroenterology **146:**1070–1083. http://dx.doi.org/10.1053/j.gastro.2013.12.024.
19. **Tong S, Li J.** 2014. Identification of NTCP as an HBV receptor: the beginning of the end or the end of the beginning? Gastroenterology **146:**902–905. http://dx.doi.org/10.1053/j.gastro.2014.02.024.
20. **Blanchet M, Sureau C, Labonte P.** 2014. Use of FDA approved therapeutics with hNTCP metabolic inhibitory properties to impair the HDV lifecycle. Antiviral Res **106:**111–115. http://dx.doi.org/10.1016/j.antiviral.2014.03.017.
21. **Konig A, Doring B, Mohr C, Geipel A, Geyer J, Glebe D.** 2014. Kinetics of the bile acid transporter and hepatitis B virus receptor Na+/taurocholate cotransporting polypeptide (NTCP) in hepatocytes. J Hepatol **61:**867–875. http://dx.doi.org/10.1016/j.jhep.2014.05.018.
22. **Yan H, Peng B, Liu Y, Xu G, He W, Ren B, Jing Z, Sui J, Li W.** 2014. Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide. J Virol **88:**3273–3284. http://dx.doi.org/10.1128/JVI.03478-13.
23. **Watashi K, Sluder A, Daito T, Matsunaga S, Ryo A, Nagamori S, Iwamoto M, Nakajima S, Tsukuda S, Borroto-Esoda K, Sugiyama M, Tanaka Y, Kanai Y, Kusuhara H, Mizokami M, Wakita T.** 2014. Cyclosporin A and its analogs inhibit hepatitis B virus entry into cultured hepatocytes through targeting a membrane transporter, sodium taurocholate cotransporting polypeptide (NTCP). Hepatology **59:**1726–1737. http://dx.doi.org/10.1002/hep.26982.
24. **Nkongolo S, Ni Y, Lempp FA, Kaufman C, Lindner T, Esser-Nobis K, Lohmann V, Mier W, Mehrle S, Urban S.** 2014. Cyclosporin A inhibits hepatitis B and hepatitis D virus entry by cyclophilin-independent interference with the NTCP receptor. J Hepatol **60:**723–731. http://dx.doi.org/10.1016/j.jhep.2013.11.022.
25. **Peng L, Zhao Q, Li Q, Li M, Li C, Xu T, Jing X, Zhu X, Wang Y, Li F, Liu R, Zhong C, Pan Q, Zeng B, Liao Q, Hu B, Hu ZX, Huang YS, Sham P, Liu J, Xu S, Wang J, Gao ZL.** 2015. The p.Ser267Phe variant in SLC10A1 is associated with resistance to chronic hepatitis B. Hepatology **61:**1251–1260. http://dx.doi.org/10.1002/hep.27608.
26. **Yan R, Zhang Y, Cai D, Liu Y, Cuconati A, Guo H.** 2015. Spinoculation enhances HBV infection in NTCP-reconstituted hepatocytes. PLoS One **10:**e0129889. http://dx.doi.org/10.1371/journal.pone.0129889.
27. **He W, Ren B, Mao F, Jing Z, Li Y, Liu Y, Peng B, Yan H, Qi Y, Sun Y, Guo JT, Sui J, Wang F, Li W.** 2015. Hepatitis D virus infection of mice expressing human sodium taurocholate cotransporting polypeptide. PLoS Pathog **11:**e1004840. http://dx.doi.org/10.1371/journal.ppat.1004840.
28. **Bouezzedine F, Fardel O, Gripon P.** 2015. Interleukin 6 inhibits HBV entry through NTCP downregulation. Virology **481:**34–42. http://dx.doi.org/10.1016/j.virol.2015.02.026.
29. **Garcia T, Li J, Sureau C, Ito K, Qin Y, Wands J, Tong S.** 2009. Drastic reduction in the production of subviral particles does not impair hepatitis B virus virion secretion. J Virol **83:**11152–11165. http://dx.doi.org/10.1128/JVI.00905-09.
30. **Li JS, Cova L, Buckland R, Lambert V, Deleage G, Trepo C.** 1989. Duck hepatitis B virus can tolerate insertion, deletion, and partial frameshift mutation in the distal pre-S region. J Virol **63:**4965–4968.
31. **Qin Y, Tang X, Garcia T, Hussain M, Zhang J, Lok A, Wands J, Li J, Tong S.** 2011. Hepatitis B virus genotype C isolates with wild-type core promoter sequence replicate less efficiently than genotype B isolates but possess higher virion secretion capacity. J Virol **85:**10167–10177. http://dx.doi.org/10.1128/JVI.00819-11.
32. **Qin Y, Zhang J, Mao R, Guo H, Yin Y, Wu X, Weng X, Wands J, Tong S.** 2009. Prevalence of basal core promoter and precore mutations in Chinese chronic hepatitis B patients and correlation with serum HBeAg titers. J Med Virol **81:**807–814. http://dx.doi.org/10.1002/jmv.21439.
33. **Yu M, Summers J.** 1994. Multiple functions of capsid protein phosphorylation in duck hepatitis B virus replication. J Virol **68:**4341–4348.
34. **Sominskaya I, Paulij W, Jansons J, Sobotta D, Dreilina D, Sunnen C, Meisel H, Gerlich WH, Pumpens P.** 2002. Fine-mapping of the B-cell epitope domain at the N terminus of the preS2 region of the hepatitis B surface antigen. J Immunol Methods **260:**251–261. http://dx.doi.org/10.1016/S0022-1759(01)00551-8.
35. **Glebe D, Aliakbari M, Krass P, Knoop EV, Valerius KP, Gerlich WH.** 2003. Pre-s1 antigen-dependent infection of *Tupaia* hepatocyte cultures with human hepatitis B virus. J Virol **77:**9511–9521. http://dx.doi.org/10.1128/JVI.77.17.9511-9521.2003.
36. **Tropberger P, Mercier A, Robinson M, Zhong W, Ganem D, Holdorf M.** 2015. Mapping of histone modifications in episomal HBV cccDNA uncovers an unusual chromatin organization amenable to epigenetic manipulation. Proc Natl Acad Sci U S A **112:**E5715–E5724. http://dx.doi.org/10.1073/pnas.1518090112.
37. **Walter E, Keist R, Niederost B, Pult I, Blum HE.** 1996. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. Hepatology **24:**1–5. http://dx.doi.org/10.1053/jhep.1996.v24.pm0008707245.
38. **Schulze A, Mills K, Weiss TS, Urban S.** 2012. Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. Hepatology **55:**373–383. http://dx.doi.org/10.1002/hep.24707.
39. **Li W.** 2015. The hepatitis B virus receptor. Annu Rev Cell Dev Biol **31:**125–147. http://dx.doi.org/10.1146/annurev-cellbio-100814-125241.
40. **Wang W, Sun L, Li T, Ma Y, Li J, Liu Y, Li M, Wang L, Li C, Xie Y, Wen Y, Liang M, Chen L, Tong S.** 2016. A human monoclonal antibody against small envelope protein of hepatitis B virus with potent neutralization effect. MAbs **8:**468–477. http://dx.doi.org/10.1080/19420862.2015.1134409.
41. **Persing DH, Varmus HE, Ganem D.** 1986. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. Science **234:**1388–1391. http://dx.doi.org/10.1126/science.3787251.
42. **Standring DN, Ou JH, Rutter WJ.** 1986. Assembly of viral particles in Xenopus oocytes: pre-surface-antigens regulate secretion of the hepatitis B viral surface envelope particle. Proc Natl Acad Sci U S A **83:**9338–9342. http://dx.doi.org/10.1073/pnas.83.24.9338.
43. **Cai D, Mills C, Yu W, Yan R, Aldrich CE, Saputelli JR, Mason WS, Xu X, Guo JT, Block TM, Cuconati A, Guo H.** 2012. Identification of disubstituted sulfonamide compounds as specific inhibitors of hepatitis B virus covalently closed circular DNA formation. Antimicrob Agents Chemother **56:**4277–4288. http://dx.doi.org/10.1128/AAC.00473-12.