Novel Recombinant Hepatitis B Virus Vectors Efficiently Deliver Protein and RNA Encoding Genes into Primary Hepatocytes

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Hepatitis B virus (HBV) has extremely restricted host and hepatocyte tropism. HBV-based vectors could form the basis of novel therapies for chronic hepatitis B and other liver diseases and would also be invaluable for the study of HBV infection. Previous attempts at developing HBV-based vectors encountered low yields of recombinant viruses and/or lack of sufficient infectivity/cargo gene expression in primary hepatocytes, which hampered follow-up applications. In this work, we constructed a novel vector based on a naturally occurring, highly replicative HBV mutant with a 207-bp deletion in the preS1/polymerase spacer region. By applying a novel insertion strategy that preserves the continuity of the polymerase open reading frame (ORF), recombinant HBV (rHBV) carrying protein or small interfering RNA (siRNA) genes were obtained that replicated and were packaged efficiently in cultured hepatocytes. We demonstrated that rHBV expressing a fluorescent reporter (DsRed) is highly infective in primary tree shrew hepatocytes, and rHBV expressing HBV-targeting siRNA successfully inhibited antigen expression from coinfecting wild-type HBV. This novel HBV vector will be a powerful tool for hepatocyte-targeting gene delivery, as well as the study of HBV infection.

Humans are the only natural hosts of hepatitis B virus (HBV), and hepatocytes are the only recognized cells that support productive HBV infection *in vivo* (1). Viral gene transcription and replication in hepatic cells have been extensively studied (1), and high levels of infectious HBV virions can be produced in cultured cells with relative ease (2). The extremely restricted host and hepatocyte tropism of HBV infection, as well as the possibility of obtaining large amounts of virus *in vitro*, makes HBV an ideal candidate for the development of hepatocyte-targeting delivery vectors. HBV-based vectors are also invaluable for the study of HBV infection mechanisms.

The highly compact HBV genome contains four overlapping open reading frames (ORFs) (preC/C, P, preS1/preS2/S, and X) (Fig. 1A). Multiple essential *cis* elements overlap these ORFs and function at the DNA or RNA level during different stages of the viral life cycle. Mature virions contain partially double-stranded, relaxed circular DNA (rcDNA) genomes. Upon infection of hepatocytes, rcDNA genomes are converted into covalently closed circular DNA (cccDNA), which serves as a transcription template for viral RNA species. Viral pregenomic RNA (pgRNA), which also functions as mRNA for polymerase, is bound by newly translated polymerase, preferentially in *cis*. The pgRNA-polymerase complex is then packaged by viral core proteins, also translated using pgRNA as mRNA. Reverse transcription and synthesis of rcDNA take place within the capsids. Mature capsids are subsequently enveloped by membranes containing viral large/middle/small (L/M/S) surface proteins, encoded by the preS1/preS2/S ORF, to produce progeny virions that bud into the endoplasmic reticulum (ER) lumen to be secreted (1).

The compact nature of HBV genome organization makes adapting it into a viral vector complicated. Several previous attempts focused on searching for sites in the HBV genome that tolerate insertions of foreign genes and support their expression

(3–6). These efforts met with very limited success: either small numbers of recombinant HBV (rHBV) virions were obtained due to weak replication and/or poor packaging, or the recombinant virions failed to achieve efficient infectivity/cargo gene expression. The causes of this lack of success are manifold. First, there is a strict limit on the genome size that can be packaged by HBV capsids (4, 7). Second, multiple *cis* elements in the genome essential for viral replication limit the possible choices of dispensable HBV genome segments to be replaced by cargo sequences. Third, and perhaps most important, polymerase translated from pgRNA acting in *cis* is much more favorable for initiation of reverse transcription and subsequent capsid packaging than *trans*-complemented polymerase (8). Consequently, rHBV vectors dependent on polymerase expressed by helper plasmids for replication often suffered from low production yield in cultures, which made their characterization and application difficult.

In this work, we addressed the above-mentioned problems by taking a different approach to rHBV vector design. Instead of experimentally testing insertion sites within the wild-type HBV genome, we screened clinical HBV strains for replicative deletion mutants. A naturally occurring mutant strain that possesses a

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FIG 1 rHBV vector based on the highly replicative deletion mutant 6898. (A) Schematic representation of plasmid constructs. ORFs and promoters are represented by open boxes and arrows, respectively. Stop codons in the S ORF are indicated by asterisks. (B) Southern blot analysis of viral genome replication and virion production of 6898-derived constructs. Huh7 cells transfected with the specified plasmids were analyzed for HBV DNA associated with intracellular capsids (top), secreted capsids and/or virions in the supernatant (middle), and secreted virions immunoprecipitated from the supernatants by anti-preS1 MAb (bottom). HBV-specific probes (left) and Tat-specific probes (right) were used sequentially on the same blots. The difference in transfection efficiency was normalized using cotransfected enhanced green fluorescent protein (EGFP) reporter. (C) pCMV1.1-6898Tat expressed functional Tat protein. Huh7 cells were cotransfected with the specified plasmids, LTR firefly luciferase reporter, and a *Renilla* luciferase control. Relative luciferase activities were measured using a dual-luciferase reporter system (Promega). Each transfection was performed in duplicate, and data from three independent repeats are shown. The error bars indicate standard deviations.

fairly large (207-bp) deletion in the preS1/polymerase spacer region but replicates efficiently in cultured hepatocytes was identified. By applying a novel insertion strategy that preserves the continuity of the polymerase ORF and, consequently, a functional polymerase working in *cis* on recombinant pgRNA, the mutant

HBV was engineered into an rHBV vector. rHBV harboring foreign protein or small interfering RNA (siRNA) cassettes replicated efficiently in cultured hepatocytes and could produce and secrete virions when viral envelope proteins were *trans*-complemented. Furthermore, we verified the infectivity of recombinant virions on

primary tree shrew (*Tupaia belangeri*) hepatocytes (PTH). Finally, rHBV expressing HBV-targeting siRNA inhibited antigen expression in PTH from coinfecting wild-type HBV.

MATERIALS AND METHODS

Plasmids. An HBV mutant designated 6898 (genotype C) was isolated from an adefovir-treated hepatitis B patient (GenBank accession number [FJ518810](#)). The 6898 genome was amplified and cloned into the pHY106 vector downstream of the cytomegalovirus (CMV) promoter to create pCMV1.1-6898, as described previously (9). p1.2-HBV containing a 1.2× terminally redundant copy of the wild-type HBV genome has been described previously (10). The wild-type HBV genome was amplified from p1.2-HBV and cloned into pYHY106 to create pCMV1.1-HBV. The helper plasmid pLMS encoding wild-type envelope proteins was constructed by deleting sequences upstream of the Sp1 promoter from p1.2-HBV. Further engineering of pCMV1.1-6898 to create the vector designated 5c3c was conducted using standard mutagenesis methods. Cargo sequences to be inserted into the 6898 or 5c3c vector were either amplified by PCR from corresponding plasmids or chemically synthesized when necessary to obtain sequences with desired properties.

RNA interference (RNAi) plasmids targeting wild-type HBc (ci, 5'-G ATCTCAATCTCGGAATCTCA-3') and HBx (xi, 5'-CCAGGTCTTGC CCAAGGTCTTACAT-3') coding sequences were created using pSuper vector as described previously (11). Flag-tagged HBc and HBx expression plasmids were constructed on a pcDNA3 (Invitrogen) backbone. RNAi-resistant target sequence mutations were introduced into HBc (cm, 5'-G ATCTCAATCTCCAGCTTCTCA-3') and HBx (xm, 5'-CCAGGTGCTC CCGAAGGTCTTACAT-3') coding sequences (mutations are underlined) using site-directed-mutagenesis methods. For insertion into the 5c3c vector, H1 promoter plus short hairpin RNA (shRNA) sequences were amplified by PCR from pSuper-based plasmids. The sequences of all recombinant plasmids were confirmed by automatic sequencing, and the relevant sequences of 5c3c and 5c3c-based constructs are presented in Fig. S1 in the supplemental material.

Cell culture, transfection, and HBV nucleic acid analysis. Huh7 and HepG2 cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 2 mM L-glutamine, 50 U/ml penicillin, and 10% fetal bovine serum (Invitrogen). Transfections were performed at 80 to 90% cell confluence using plasmid DNA and polyethylenimine (Sigma) at a 1:2 ratio.

Extraction of intracellular capsid-associated DNA and extracellular virion-associated DNA was performed as described previously (12). To immunoprecipitate Dane particles, culture supernatants were concentrated using Amicon Ultra-15, and 2 µg anti-preS1 monoclonal antibody (MAb) (125E11; kindly provided by Alpha Inc., Shanghai, China) (13), 30 µl protein G agarose (Roche) beads, and 2% bovine serum albumin (BSA) (Sigma) were added. After incubation at 4°C overnight with gentle shaking, the protein G agarose beads were precipitated by centrifuging and washed 5 times with 0.5% Tween 20 in phosphate-buffered saline (PBS) and once with 0.5% NP-40 in Tris-EDTA buffer. HBV DNA was then extracted using the same protocol as for extracellular HBV DNA extraction. The HBV DNA was analyzed using agarose gel electrophoresis and Southern blotting as described previously (12). HBV-specific probes labeled with ³²P were prepared from a DNA fragment containing nucleotides (nt) 96 to 1509 of the wild-type HBV genome.

For analysis of viral transcripts derived from wild-type HBV, total RNA was extracted from Huh7 cells using an RNeasy minikit (Qiagen) 3 days after transfection. Twenty micrograms of total RNA from each transfection was separated on a 1% agarose gel containing formaldehyde. Northern blotting was carried out according to standard protocols using the ³²P-labeled probes prepared using DNA fragments corresponding to the deleted region in 5c3c as the template.

CsCl density gradient centrifugation. Transfection supernatants, as well as HBV-infected patient serum, were concentrated using 6% polyethylene glycol (PEG)-8000 (Sigma) precipitation and subjected to CsCl

density gradient (1.1 to 1.4 g/cm³) centrifugation at 38,000 rpm for 20 h using a SW41 rotor on a Beckman L80. Fractions were assayed for HBsAg using the Abbott Architect assay, and HBV DNA was extracted and analyzed using Southern blotting as described above. To visualize virus particles, concentrated supernatants were counterstained with tungstophosphoric acid and examined with a transmission electron microscope (Philips model CM-120).

Infection of primary tree shrew hepatocytes. PTH were prepared by following a two-step perfusion protocol, as previously described (14). Freshly prepared PTH were seeded into collagen-coated 6-well plates (BD Biosciences) at 5 × 10⁵ cells per well and maintained in Hepato-Stim Hepatocyte Defined medium (BD Biosciences). For infection assays, PEG-concentrated transfection supernatants containing about 1 × 10⁸ HBV genome equivalents (geq) quantitated using a real-time PCR HBV DNA detection kit (Qiagen) were added to each well at 24 h postseeding. After incubation at 37°C overnight, the cells were washed 5 times with PBS and cultured with a daily change of fresh medium. In coinfection experiments, wild-type HBV virions prepared from patient sera or recombinant HBV particles prepared from transfection supernatants were used at 1 × 10⁸ geq each (1:1 ratio) per well. PTH infected with recombinant virus expressing a fluorescent reporter (DsRed) were visualized under fluorescence microscopy, and percentages of infected cells were calculated by manually counting DsRed-positive versus total cells in 10 randomly selected views. Protocols for animal handling were approved by the Animal Ethics Committee of Shanghai Medical College.

Immunofluorescence. Ten days after infection, PTH were washed 3 times with ice-cold PBS, fixed in 3.7% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature, and washed twice with ice-cold PBS. The cells were then incubated for 15 min in PBS containing 0.5% Triton X-100, washed with PBS, blocked in 1% BSA in PBST (PBS plus 0.5% Tween 20) for 30 min, and incubated with polyclonal rabbit anti-HBcAg (1:700; Dako) or monoclonal mouse anti-HBsAg (1:1,000; Dako) in 1% BSA in PBST in a humidified chamber for 1 h at room temperature. Subsequently, the cells were washed with PBST, incubated with Alexa Fluor 488 goat anti-rabbit IgG(H+L) (1:1,000; Life Technologies) or Alexa Fluor 488 donkey anti-mouse IgG(H+L) (1:1,000; Life Technologies) in 1% BSA in PBST for 1 h at room temperature in the dark, and washed 3 times with PBST in the dark. Finally, the cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (Sangon) for 5 min at room temperature and examined under fluorescence microscopy (AMG EVOS Fluorescence Microscope).

RESULTS

Identification and characterization of a highly replicative HBV deletion mutant. In an attempt to create an rHBV vector system, we screened clinical HBV strains for naturally occurring deletion mutants that retain high replication competence. One mutant, designated 6898, that harbors a 207-bp deletion affecting amino acids (aa) 42 to 110 of preS1 and the overlapping polymerase spacer region was isolated from an adefovir-treated hepatitis B patient (Fig. 1A). The deletion also obliterates most of the Sp2 promoter, which, coupled with two nonsense mutations in the downstream S-coding region, renders the mutant incapable of producing envelope proteins.

Despite the deletion, the 6898 genome under the control of the CMV promoter (pCMV1.1-6898) produced more intracellular capsid-associated replication intermediate DNA than wild-type HBV (pCMV1.1-HBV) in transfected Huh7 cells (Fig. 1B). Due to the lack of expression of envelope proteins, no DNA-containing virions could be immunoprecipitated by anti-preS1 MAb from pCMV1.1-6898 transfection supernatants (Fig. 1B). However, cotransfection with a helper plasmid expressing wild-type L/M/S envelope proteins (pLMS) resulted in secretion of enveloped virions to a level higher than that of the wild-type control (Fig. 1B).

FIG 2 Characterization of HBV virions derived from the naturally occurring deletion mutant 6898. Concentrated supernatants from Huh7 cells transfected with the specified plasmids were subjected to CsCl density gradient ultracentrifugation. The collected fractions were analyzed for buoyant density using a refractometer, for HBsAg using the Abbott Architect assay, and for HBV DNA using real-time PCR. HBV DNA was also analyzed in Southern blotting using HBV-specific probes. Particles in concentrated supernatants were visualized by electron microscopy (insets). Sera from HBV-infected patients were used as positive controls.

Virions produced by pCMV1.1-6898/pLMS cotransfection were then subjected to CsCl density gradient ultracentrifuge for comparison with wild-type HBV virions. Two particle populations, both containing HBV DNA, were observed at ~ 1.25 -g/ml and ~ 1.3 - to 1.35 -g/ml buoyant densities, respectively (Fig. 2). Particles sedimenting at ~ 1.25 g/ml were also observed in HBV-infected patient serum and pCMV1.1-HBV transfection superna-

tants, but not in pCMV1.1-6898 transfection supernatants. The ~ 1.3 - to 1.35 -g/ml particles, on the other hand, were observed in both pCMV1.1-HBV and pCMV1.1-6898 transfection supernatants, but not in patient serum. Fractions containing the ~ 1.25 -g/ml particles were positive for HBsAg in enzyme-linked immunosorbent assay (ELISA), and examination under electron microscopy identified mature HBV virions (42-nm

Dane particles) in supernatants containing ~ 1.25 -g/ml particles (Fig. 2). Furthermore, virions produced by pCMV1.1-6898/pLMS cotransfection displayed the same morphology as virions in patient serum, as well as virions in wild-type pCMV1.1-HBV transfection supernatants (Fig. 2). The ~ 1.3 - to 1.35 -g/ml fractions were negative for HBsAg, and the DNA-containing particles therein were likely immature, unenveloped core particles produced by transfected cells, as previously reported (2, 15). Southern blot analysis confirmed that these particles contained more fast-migrating immature DNA than ~ 1.25 -g/ml Dane particles (Fig. 2).

These results demonstrated the high replication competence of 6898 in spite of the deletion in preS1/polymerase. In addition, mature HBV virions indistinguishable from wild-type HBV virions could be efficiently produced by 6898 in the presence of *trans*-complemented L/M/S.

6898 can be engineered into a recombinant HBV vector. To test the possibility of engineering 6898 into an rHBV vector, we inserted a 207-bp fragment encoding the N-terminal 68 residues of HIV Tat plus a stop codon into the deletion site in the 6898 genome, using the Sp1 promoter to drive transcription. To ensure functional polymerase acting in *cis* on recombinant pgRNA, *tat* sequence was inserted in frame with the preS1 ORF so that Tat and polymerase would not affect each other, instead of with the P ORF, as Chaisomchit et al. did in a previous attempt at creating an rHBV vector (3). Importantly, several silent mutations were introduced in the *tat* sequence to avoid creating stop codons in the overlapping polymerase ORF so that the polymerase is not prematurely terminated. The resultant recombinant 6898Tat genome encoded a polymerase of the same length as the wild type but with 69 residues in its spacer region replaced by unrelated sequence. In reporter assays, pCMV1.1-6898Tat activated the HIV long terminal repeat (LTR) substantially (Fig. 1C), indicating that functional Tat was expressed, as designed.

In transfected Huh7 cells, pCMV1.1-6898Tat produced less replication intermediate DNA than pCMV1.1-6898 but comparable to pCMV1.1-HBV (Fig. 1B). Cotransfection with pLMS resulted in secretion of enveloped virions, also at a level lower than that of pCMV1.1-6898/pLMS but comparable to the wild type (Fig. 1B). Apparently, introduction of unrelated amino acid sequence into the spacer of 6898 polymerase negatively affected viral replication, but the recombinant 6898Tat still retained relatively high replication and packaging competence.

These data demonstrated that, by applying a novel insertion strategy to preserve the polymerase ORF, the deletion mutant 6898 could carry foreign genes without suffering a major loss in replication competence and virion production efficiency. In other words, 6898 was a suitable starting point for designing rHBV vectors.

Creation of an optimized 5c3c vector based on 6898. Previous work had shown that rHBV genomes could only exceed the wild-type genome size (3,215 bp for genotype C) by a very limited amount before packaging efficiency started to decrease dramatically (4, 7). Therefore, to maximize the capacity for carrying cargo sequences, we incrementally expanded the deletion in 6898 in both directions and tested the constructs obtained for replication competence in cultured cells. The results indicated that additional deletions in both directions of no more than 90 bp were well tolerated, with minimal decrease in replication (Fig. 3A). Based on this finding, we constructed the pCMV1.1-6898-5c3c (abbrevi-

FIG 3 Generation of the 5c3c vector from 6898. (A) Serial deletions to maximize 6898's capacity for cargo sequences. The natural deletion in pCMV1.1-6898 was expanded incrementally in either direction (top), and replication efficiency in transfected Huh7 cells was measured by Southern blotting of intracellular capsid-associated HBV DNA using HBV-specific probes (bottom). The maximal deletions that were tolerated in either direction were combined to create the 5c3c vector, which contained a synthetic 15-bp linker within the 384-bp deletion site (not depicted). The start codon of the preS1 ORF was also mutated to ACG in 5c3c. 5c3c-Red was created by inserting DsRed sequence with start and stop codons into the synthetic linker in 5c3c in frame with an ATG-less preS1 ORF (see Fig. S1 in the supplemental material). (B) Replication of 5c3c-Red in transfected Huh7 cells compared to parental 5c3c. Southern blot analysis of intracellular capsid-associated HBV DNA was performed as described above.

ated as 5c3c) vector, which has a total of 384 bp (128 aa) of the preS1/polymerase spacer region deleted compared to wild-type genotype C HBV. A 15-bp synthetic fragment containing two restriction enzyme recognition sites was inserted to ease cargo gene insertion while keeping the polymerase ORF uninterrupted. In

FIG 4 Infection of primary tree shrew hepatocytes with rHBV vector carrying the DsRed reporter gene. Concentrated supernatants from 5c3c-Red/pLMS-cotransfected Huh7 cells were used, with or without preincubation with anti-preS1 MAb or normal mouse IgG control, to infect freshly prepared PTH cultures. Concentrated supernatants from mock transfection and HepG2 cells were used as negative controls. Concentrated supernatant from 5c3c-Red-only transfection, which contains only naked capsids, was used to rule out the possibility of capsid-mediated transfection of PTH. (A) Cells were subjected to DAPI staining and visualized using fluorescence microscopy. Scale bars, 100 μ m. (B) HBcAg was detected using immunofluorescence. Scale bars, 200 μ m.

addition, the upstream preS1 start codon was mutated to ACG, which does not affect the polymerase amino acid sequence (see Fig. S1 in the supplemental material). 5c3c replicated nearly as efficiently as the parental 6898 in transfected cells (Fig. 3A).

rHBV expressing DsRed reporter efficiently infects primary tree shrew hepatocytes. rHBV expressing a fluorescent reporter enables monitoring of HBV infection and replication nondisruptively and in real time, which will have broad applications in both virological and pharmaceutical studies. To produce such an rHBV, we inserted the coding sequence of fluorescent DsRed into 5c3c in frame with ATG-less preS1 to create 5c3c-Red (Fig. 3A). The choice of DsRed was based on its strong fluorescence, small size (225 aa), and absence of stop codons in the overlapping P ORF after insertion. The insertion resulted in a polymerase 104 aa longer than the wild type and increased the genome size to 3,527 bp, nearly 10% longer than the wild type. In transfected Huh7 cells, 5c3c-Red replicated less efficiently than 5c3c but nearly comparably to wild-type HBV (Fig. 3B).

To assess the infectivity and cargo gene expression of 5c3c-Red virions, concentrated transfection supernatants were used to infect PTH, which are susceptible to HBV infection (14, 16). Incubation of PTH with 5c3c-Red/pLMS cotransfection supernatants resulted in marked DsRed expression in infected cells (Fig. 4A), whereas incubation of PTH with concentrated supernatant from 5c3c-Red-only transfection did not lead to appreciable DsRed expression compared to a mock-transfected control (Fig. 4A). The

same pattern was observed when HBcAg was stained by immunofluorescence as an indicator of HBV infection (Fig. 4B).

To further prove that delivery of DsRed expression into PTH was achieved through rHBV infection, concentrated supernatants from 5c3c-Red/pLMS cotransfection were preincubated with anti-preS1 MAb or mouse IgG control before being used for PTH infection. Preincubation with anti-preS1 MAb markedly reduced the number of DsRed-positive cells, whereas no appreciable reduction was observed after preincubation with mouse IgG control (Fig. 4A). In addition, HBcAg staining gave similar results (Fig. 4B). These data reconfirmed the infectivity of rHBV virions produced by 5c3c-Red in the presence of *trans*-complemented envelope proteins, as well as the ability of these virions to initiate efficient cargo gene expression upon infection of hepatocytes.

rHBV delivers siRNA expression into primary tree shrew hepatocytes. Due to their smaller sizes, small functional RNA-like ribozyme, siRNA, and microRNA (miRNA) genes are especially ideal for delivery vectors with strict limits on insertion size, including rHBV vectors.

Sequences for two shRNA precursors of siRNA that target HBc (ci) and HBx (xi) coding sequences, respectively, were placed under the control of the H1 promoter and demonstrated to be capable of knocking down expression of cotransfected Flag-tagged HBc and HBx (Fig. 5A), as well as inhibiting wild-type HBV replication (Fig. 5C). The shRNA expression cassettes, including the H1 promoter were then inserted into the 5c3c vector to create

FIG 5 Engineering rHBV vectors to deliver RNA interference. (A) Validation of HBV-targeting siRNA function and specificity. Flag-tagged HBc and HBx expression plasmids were used as targets and cotransfected with the specified plasmids carrying siRNA precursors targeting HBc (ci) or HBx (xi) sequence. Target sequences in HBc and HBx were also mutated (Flag-cm and Flag-xm, respectively) and tested for escape of RNAi targeting. Flag-tagged target protein expression was detected using anti-Flag MAb, and beta-actin was used as a loading control. (B) Replication of 5c3c vectors carrying siRNA precursor expression cassettes. Intracellular capsid-associated HBV replication intermediate DNA in transfected Huh7 cells was measured by Southern blotting using HBV-specific probes. Target sequences of ci and xi were mutated in 5c3c-ci and 5c3c-xi to create 5c3c-cm-ci and 5c3c-xm-xi, respectively. (C) Inhibition of p1.2-HBV replication and transcription by siRNA expressed from pSuper and 5c3c vectors. Intracellular capsid-associated HBV DNA (top) and cytoplasmic HBV RNA (middle) in transfected Huh7 cells were analyzed in Southern and Northern blots using probes specific for sequences deleted in 5c3c. (D) Knockdown of DsRed expression in PTH by coinfection with 5c3c vectors carrying HBV-targeting siRNA expression cassettes. Concentrated supernatants from Huh7 cells cotransfected with specified 5c3c vectors and pLMS were used to infect freshly prepared PTH cultures. DAPI-stained cells were visualized using fluorescence microscopy (left), and DsRed-positive and total cells in 10 randomly selected views were manually counted to calculate the percentage of DsRed-expressing cells (right). Scale bars, 100 μ m.

5c3c-ci and 5c3c-xi, following the insertion strategy described above. In transfected Huh7 cells, 5c3c-ci and 5c3c-xi displayed much lower replication (<10%) than 5c3c (Fig. 5B). However, when target sequences of ci and xi on the respective vectors were silently mutated to sequences that escape interference (cm and xm, respectively) to create 5c3c-cm-ci and 5c3c-xm-xi (Fig. 5A), replication efficiency was restored to about 30% that of 5c3c

(Fig. 5B). Upon cotransfection with wild-type p1.2-HBV into Huh7 cells, both 5c3c-cm-ci and 5c3c-xm-xi displayed marked inhibition of wild-type HBV transcription and replication (Fig. 5C).

To test whether virions derived from the 5c3c-cm-ci and 5c3c-xm-xi vectors could engender effective RNA interference in infected cells, we first used the 5c3c-Red reporter infection system

established as described above and coinfecting PTH with supernatants from 5c3c-Red/pLMS, along with 5c3c-cm-ci/pLMS or 5c3c-xm-xi/pLMS cotransfections, respectively, at a 1:1 qeq ratio. Coinfection with 5c3c-xm-xi virions reduced the percentage of DsRed-positive cells from about 19% following 5c3c-Red infection to about 5%. Coinfection with 5c3c-cm-ci viruses also resulted in fewer DsRed-expressing cells postinfection, but only reduced to 9.5% (Fig. 5D). A similar difference in inhibition efficiency between ci and xi was observed in transfection assays (Fig. 5C), which might be partially explained by the fact that xi target sequences exist on all HBV RNA species, whereas ci target sequences exist only on preC/pgRNA (Fig. 1A). Such a difference in inhibition potency between the recombinant viruses also suggests that intracellular interference, rather than extracellular competition for membrane receptors, is the underlying mechanism.

We went on to probe the effectiveness of the more potent 5c3c-xm-xi against wild-type HBV in a PTH infection assay. Wild-type HBV virions were prepared from patient sera, and the existence of xi target sequence was verified by sequencing. Infection of PTH by wild-type HBV resulted in production of HBsAg and its secretion into the culture supernatant after day 7 postinfection (Fig. 6A), which was also confirmed by detecting intracellular HBsAg in an immunofluorescence assay (Fig. 6B). Due to the lack of functional envelope protein ORFs, infection by 5c3c-xm-xi virions did not lead to any HBsAg expression postinfection (Fig. 6). When 5c3c-xm-xi virions were used to coinfect PTH with wild-type HBV, marked reduction of both secreted and intracellular HBsAg production could be observed in comparison to wild-type infection alone (Fig. 6). Similar but more potent inhibition of HBsAg production was observed when wild-type HBV was preincubated with anti-preS1 antibody (Fig. 6). No apparent reduction in postinfection HBsAg expression was observed when 5c3c-Red virions were used to coinfect with wild-type HBV (Fig. 6). This observation once again indicates that intracellular RNA interference, rather than extracellular competition for receptors, is responsible for causing the inhibition.

Taken together, these results demonstrated that, in addition to protein-encoding genes, the 5c3c vector is also capable of delivering functional RNA genes into infected hepatocytes.

DISCUSSION

In this work, we engineered the naturally occurring, highly replicative HBV mutant 6898, which harbors a 207-bp deletion in the preS1/polymerase spacer region, into an efficient rHBV vector, 5c3c. A novel cargo gene insertion strategy was developed that avoids disruption of the overlapping polymerase ORF in order to preserve the replicative competence of rHBV. The 5c3c vector was demonstrated to be capable of tolerating insertions of at least 675 bp of cargo sequences while retaining replication efficiency comparable to that of wild-type HBV. When supplemented with HBV envelope proteins *in trans*, recombinant 5c3c vectors produced mature enveloped virions from cultured hepatocytes, which efficiently infected primary hepatocytes and mediated high-level expression of cargo genes. Furthermore, the 5c3c vector could also carry shRNA genes and deliver its expression in infected hepatocytes. The availability of such a novel rHBV vector system will enable innovative applications in both basic and applied research regarding HBV and the human liver.

Naturally occurring deletion mutants of HBV are not uncommon, but large deletions are relatively rare, because replication

and/or packaging is often affected. The 207-bp deletion in 6898 is located within the coding sequence of the polymerase spacer region, which overlaps the Sp2 promoter and the preS1/preS2 ORF (Fig. 1A). The spacer presumably assumes no particular higher structure and functions as a physical “tether” between the N-terminal terminal-protein (TP) domain and the C-terminal reverse transcriptase/RNase H (RT/RH) domains (17). Separately expressed TP and RT/RH domains of duck hepatitis B virus (DHBV) and HBV could reconstitute, at least partially, polymerase functions *in vitro* (18–20). Previous work has also shown that the spacer region is more variable in amino acid sequence than the rest of the polymerase and can tolerate moderate deletions or insertions without major loss of polymerase activity (17). It is therefore not very surprising that replication competence could be retained even with fairly large deletions (6898 and 5c3c) or extensive amino acid changes (6898Tat, 5c3c-Red, etc.) in the spacer region, as long as the continuity of the polymerase ORF is preserved.

Interestingly, there have been a few independent reports of clinically isolated HBV mutants harboring 183-nt (61-aa) “in-frame” deletions at nearly identical places near the C terminus of preS1 (21–24), highly similar to the deletion in 6898. Two of the reported mutant strains belonging to genotype D were demonstrated to be nearly as replication competent as the corresponding wild type (23, 24), and one was shown to produce enveloped virions when *trans*-complemented with wild-type envelope proteins (23). These results suggest that high replication competence of 6898 is not restricted to this particular mutant strain or this particular genotype. It is therefore very likely that highly replicative vectors like 5c3c could also be constructed from strains of other genotypes in a similar fashion.

Insertion of protein-encoding sequences into the polymerase spacer region can use three possible reading frames. Chaisomchit et al. inserted coding sequences for the N-terminal 89 aa of HIV Tat in frame with polymerase into the spacer region preceding the preS1 ORF of the wild-type HBV genome (3). Tat expressed as a polymerase fusion protein retained the ability to activate the HIV LTR, whereas polymerase with embedded Tat displayed much lower activity than the wild-type control. In this work, protein-encoding sequences were inserted into 6898 or 5c3c using the preS1 reading frame instead. Such a scheme avoids embedding the protein of interest into the HBV polymerase, so that their folding, trafficking, and functions would be independent of each other. The requirement to keep the polymerase ORF uninterrupted in recombinant 5c3c vectors constitutes a restriction on cargo gene insertions. It is possible that introduction of certain amino acid sequences may interfere with the protein folding and function of upstream TP and/or downstream RT/RH domains. This could have partially contributed to the apparent reduction in replication efficiency of the 5c3c-cm-ci and 5c3c-xm-xi vectors. Nevertheless, in addition to Tat and DsRed, we have successfully inserted modified human alpha, beta, gamma, and lambda interferon coding sequences into 5c3c and achieved interferon expression without abolishing polymerase activity (data not shown).

Two previously reported rHBV vectors used *trans*-complemented polymerase for replication in production cultures (4, 5). Although recombinant virions could be obtained, infectivity in primary human hepatocytes appeared to be very low, as demonstrated by reporter expression (4, 5). The low infectivity could be a result of lack of infection, low transcription and translation postinfection, or both. The recombinant 5c3c vectors described in

FIG 6 rHBV expressing HBV-targeting siRNA reduced wild-type HBV antigen expression in coinfecting primary tree shrew hepatocytes. Wild-type HBV virions prepared from patient sera and supernatants from Huh7 cells cotransfected with the specified 5c3c vectors and pLMS were used to infect or coinfect freshly prepared PTH cultures. (A) Measurement of secreted HBsAg in culture media using ELISA. The error bars indicate standard deviations. (B) Intracellular HBsAg in infected or coinfecting PTH was detected by immunofluorescence. DAPI-stained cells were visualized using fluorescence microscopy. The error bars indicate standard deviations. Scale bars, 200 μ m.

this work contain all the elements required for viral replication and can be expected to initiate amplification of recombinant genomes upon infection, which might have contributed to the high-level expression of DsRed observed in PTH infected by 5c3c-Red virions (Fig. 4). This property is desirable when efficient expression of delivered genes is crucial. In addition, the 5c3c-Red infection assay enables nondisruptive and continuous monitoring of

HBV infection and makes identification of infected cells much easier. Such a system will be a powerful tool for the study of HBV infection and will facilitate the identification of essential molecules involved in HBV entry. The availability of assays like this will also greatly improve efficiency in pharmaceutical applications, such as screening and characterization of HBV entry inhibitors (25, 26).

Due to its large cell mass and easy access to the bloodstream, the liver has long been regarded as an attractive target of gene therapy, not only for liver-related diseases, but also for non-liver-related diseases that could be remedied by expression of functional proteins, including diabetes and hemophilia (27). Other viral vectors, like adenovirus, adeno-associated virus, retrovirus, and lentivirus, have all been used for liver-targeting gene delivery, in addition to nonviral vectors. These delivery methods all suffer from low hepatocyte specificity, even when liver-homing molecules are used for packaging and hepatocyte-specific promoter/enhancer elements are used for driving transcription. Intrinsically hepatotropic rHBV vectors like 5c3c provide the means for highly specific liver targeting and efficient expression of protein and RNA genes in hepatocytes.

Using 5c3c-derived vectors expressing HBV-specific siRNA precursors or human interferons for treatment of chronic HBV infection represents a most interesting and promising application of rHBV vectors. Chronically infected patients are generally tolerant of HBV antigens, and the common problem of anti-vector immune responses facing viral vectors for gene therapy is likely nonexistent. More importantly, recombinant 5c3c vectors would be able to produce progeny recombinant virions in hepatocytes coinfecting by wild-type HBV, which provides *trans*-complementation of envelope proteins. Such *in vivo* production and expansion of recombinant virions carrying therapeutic cargo genes would greatly enhance the scale and duration of cargo gene expression, contributing to a better therapeutic outcome. Nevertheless, modifications and improvements to the 5c3c vector system are needed before any *in vivo* experiments could be considered. For instance, the helper plasmid expressing envelope proteins needs to be modified to avoid the possibility of creating wild-type HBV in production cultures through homologous recombination. Further work is warranted to address potential issues and to probe the clinical applicability of this novel HBV-based vector system.

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