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Establishment of an Inducible HBV Stable Cell Line that Expresses cccDNA-dependent Epitope-tagged HBeAg for Screening of cccDNA Modulators

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Abstract

Hepatitis B virus (HBV) covalently closed circular (ccc) DNA is essential to the virus life cycle, its elimination during chronic infection is considered critical to a durable therapy but has not been achieved by current antivirals. Despite being essential, cccDNA has not been the major target of high throughput screening (HTS), largely because of the limitations of current HBV tissue culture systems, including the impracticality of detecting cccDNA itself. In response to this need, we have previously developed a proof-of-concept HepDE19 cell line in which the production of wildtype e antigen (HBeAg) is dependent upon cccDNA. However, the existing assay system is not ideal for HTS because the HBeAg ELISA cross reacts with a viral HBeAg homologue, which is the core antigen (HBcAg) expressed largely in a cccDNA-independent fashion in HepDE19 cells. To further improve the assay specificity, we report herein a “second-generation” cccDNA reporter cell line, termed HepBHAe82. In the similar principle of HepDE19 line, an in-frame HA epitope tag was introduced into the precore domain of HBeAg open reading frame in the transgene of HepBHAe82 cells without disrupting any *cis*-element critical for HBV replication and HBeAg secretion. A chemiluminescence ELISA assay (CLIA) for the detection of HA-tagged HBeAg with HA antibody serving as capture antibody and HBeAb serving as detection antibody has been developed to eliminate the confounding signal from HBcAg. The miniaturized HepBHAe82 cell based assay system exhibits high level of cccDNA-dependent HA-HBeAg production and high specific readout signals with low background. We have also established a HepHA-HBe4 cell line

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expressing transgene-dependent HA-HBeAg as a counter screen to identify HBeAg inhibitors. The HepBHAe82 system is amenable to antiviral HTS development, and can be used to identify host factors that regulate cccDNA metabolism and transcription.

Keywords

Hepatitis B Virus; cccDNA; HBeAg; High throughput screening

1. Introduction

Hepatitis B virus (HBV) causes both transient and chronic infections (Block et al., 2007). It has been estimated that HBV has infected about 2 billion people or one third of the world population, resulting in a significant public health burden of approximately 240 million chronic hepatitis B carriers who have an increased risk of liver cirrhosis, hepatocellular carcinoma (HCC), and other severe clinical sequelae (Liang et al., 2015; Trepo, 2014). It is therefore a global health priority to cure HBV infection and prevent its dire consequences (Block et al., 2013; Zoulim and Durantel, 2015).

HBV is a liver tropic, enveloped DNA virus belonging to the *Hepadnaviridae* family (Seeger and Mason, 2000). Upon hepatic sodium taurocholate cotransporting polypeptide (NTCP)-mediated virus entry, HBV relaxed circular (rc) DNA genome is transported into the cell nucleus and converted to a nucleosome-decorated episomal covalently closed circular (ccc) DNA, which serves as template to transcribe viral mRNAs including precore mRNA, pregemonic (pg) mRNA, envelope mRNAs, and X mRNA. In the cytoplasm, viral polymerase (pol) and capsid proteins translated from pgRNA encapsidate their RNA template into the nucleocapsid, inside of which the pol reverse transcribes pgRNA into viral minus strand DNA, followed by asymmetric plus strand DNA synthesis to yield the rcDNA genome. The mature nucleocapsid is either packaged by viral envelope proteins and secreted as virion particle, or recycled to the nucleus where conversion of the rcDNA amplifies and replenishes the cccDNA reservoir. The viral envelope proteins can also secrete independently as subviral particles (HBV surface antigen, HBsAg) (Patient et al., 2009). A viral precore protein is translated from precore mRNA and further processed in ER-Golgi complex by the endoprotease furin and released as e antigen (HBeAg) (Ito et al., 2009; Messageot et al., 2003). Therefore, cccDNA is an essential component of the HBV life cycle, and is responsible for the establishment and maintenance of viral replication and gene expression (Guo and Guo, 2015; Seeger and Mason, 2015a).

To date, although the hepatitis B vaccine is widely used and very effective as prophylaxis against new infections, there is no definitive cure for chronic hepatitis B (Liang et al., 2015). Currently approved drugs for HBV treatment are interferon-α (IFN-α) and 5 nucleos(t)ide analogues. IFN-α only achieves sustained virological response in a minority of hepatitis B patients but with significant adverse effects. The five nucleos(t)ide analogues all act as viral polymerase inhibitors, but rarely cure HBV infection, and the emergence of resistance dramatically limits their long-term efficacy (Zoulim and Locarnini, 2009, 2012). It is generally acknowledged that the major limitation of current drugs is the failure to eliminate

the preexisting cccDNA pool in HBV infected hepatocytes or completely block the *de novo* cccDNA formation from wild-type or drug-resistant virus. Therefore, there is an urgent need for the development of novel therapeutic agents that directly target cccDNA formation, transcription activity, and ideally the stability of cccDNA (Guo and Guo, 2015; Liang et al., 2015; Zeisel et al., 2015; Zoulim and Durantel, 2015).

Even though eradicating cccDNA is the most straightforward approach to a durable cure for hepatitis B, discovery of direct anti-cccDNA agents by high throughput screening (HTS) has not been vigorously pursued, largely because of the paucity of suitable assay systems and practical limitations. In response to this need, we have previously developed the inducible HepDE19 cell culture system in which the production of an easily detectable reporter molecule is dependent upon the presence of cccDNA (Cai et al., 2012). This reporter is the HBeAg, a secreted protein that can be detected by ELISA. In HepDE19 cells, HBeAg mRNA (termed precore (pc) RNA) and protein will only be produced from the cccDNA circular templates because the open reading frame (ORF) of HBeAg and its 5' RNA leader are separated between the opposite ends of the integrated viral genome, and only become contiguous with the formation of cccDNA (Cai et al., 2012; Guo and Guo, 2015). By making use of HepDE19 system, we have previously screened a compound library and identified two inhibitors of cccDNA formation (Cai et al., 2012). However, the existing assay is not ideal because the HBeAg ELISA cross reacts with a viral HBeAg homologue, which is the core antigen (HBcAg) expressed and secreted largely in a non-cccDNA dependent manner from the integrated transgene in HepDE19 cells (Cai et al., 2012; Guo et al., 2007). The genotype D HBeAg shares 154 amino acid (aa) sequence homology with HBcAg (Ito et al., 2009; Messageot et al., 2003), and a highly specific HBeAb or HBeAg ELISA is not available, resulting in a high background noise in the HepDE19 cell-based assay. To further improve the assay specificity and sensitivity, we report herein a "next-generation" cccDNA reporter cell line, named HepBHAe82 cells. In the similar principle of HepDE19 line, we managed to introduce an in-frame human influenza hemagglutinin (HA) epitope tag into the precore domain of HBeAg in the transgene of HepBHAe82 cells without disrupting any *cis*-element critical for HBV replication. The HepBHAe82 cells support the inducible, robust viral DNA replication and cccDNA-dependent HA-tagged HBeAg (HA-HBeAg) expression. In the meantime, we have developed a chemiluminescence ELISA assay (CLIA) for the detection of HA-tagged HBeAg with HA antibody serving as capture antibody and HBeAb serving as detection antibody, which eliminates the background signal from HBcAg. With these improvements, the HA-HBeAg-based cccDNA reporter system is now amenable to the development of a real HTS assay for identification of cccDNA inhibitors.

2. MATERIAL AND METHODS

2.1 Chemicals

Furin inhibitor I was purchased from Calbiochem. Trichostatin A (TSA) was purchased from ApexBio Technology. Bay41-4109 and AT-61 were synthesized by Pharmabridge Inc. according to the published compound structures and synthetic route (Deres et al., 2003;

Perni et al., 2000). CCC-0975 has been described previously (Cai et al., 2012). Lamivudine (3TC) was kindly provided by Dr. William Mason (Fox Chase Cancer Center).

2.2 Plasmids

The tetracycline-inducible HBV replication competent vector pTREHBVDES has been described previously (Guo et al., 2007). To insert an in-frame HA epitope sequence into the coding sequence of precore domain without disrupting the predicted ribonucleotide base pairing in the overlapped coding sequence for epsilon stem-loop region, a DNA fragment which contains HA coding sequence (GTGGACATCTACCCATACGACGTTCCAGATTACGCTGGC, HA sequence is underlined) was inserted into pTREHBVDES at a position immediately upstream of the start codon of core ORF (between HBV nt 1902 and nt 1903, genotype D, subtype ayw), and the resultant plasmid was designated pTREHBV-HAe.

Plasmid pcHBc is a pcDNA3.1-derived HBV core expression vector with HBV core ORF containing sequence fragment (nt 1903–2606/1573–1926) being placed under the control of CMV-IE promoter; whilst plasmid pcHBe is a pcDNA3.1-derived HBV HBeAg expression vector with HBV precore ORF containing sequence (nt 1816–2606/1573–1926) under the control of CMV-IE promoter. The HBV sequence fragment, spanning from nt 1573 to nt 1926, which contains a reported posttranscriptional regulatory element (PRE) (Huang and Liang, 1993), was placed downstream of the core or precore ORF in their respective expression vector to increase the protein expression level. The HA-tagged HBeAg expression vector, namely pcHA-HBe, was constructed by inserting the same aforementioned HA coding sequence-containing DNA fragment (GTGGACATCTACCCATACGACGTTCCAGATTACGCTGGC, HA coding sequence is underlined) into the same position in precore domain in pcHBe as described above.

2.3 Stable cell line establishment and maintenance

HepG2 cells were seeded in collagen-coated plate or flask and maintained in DMEM/F12 medium (Corning) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The tetracycline inducible HBV stable cell line HepDES19 cells were cultured in the same way as HepG2 cells with the addition of 500 μg/ml G418 and 1 μg/ml tetracycline (tet). To induce HBV replication and cccDNA formation in HepDES19 cells, tetracycline was withdrawn from the culture medium and the cells were cultured for the indicated time period (Cai et al., 2012; Guo et al., 2007).

To establish HBeAg constitutive expression cell line, HepG2 cells were stably transfected with plasmid pcHA-HBe by Lipofectamine 2000 (Invitrogen), and antibiotics-resistant single cell colonies were selected by G418 and expanded into cell lines. One cell line with high level of HBeAg expression was designated HepHA-HBe4 cells. HepHA-HBe4 cells were maintained in the same way as HepG2 cells with the addition of 500 μg/ml G418.

To establish tetracycline-inducible HBV stable cell lines expressing cccDNA-dependent HA-HBeAg expression, HepG2 cells were cotransfected by pTREHBV-HAe and pTet-off which expresses tet-responsive transcriptional activator (tTA) (Clontech) with 7:1 molar ratio. The transfected HepG2 cells were selected with 500 μg/ml G418 in the presence of 1

μg/ml tetracycline (tet). G418-resistant colonies were picked and expanded into cell lines. To determine HBV positive cell lines, HBV DNA replication was induced by culturing candidate cell lines in tetracycline-free medium for 6 days, then the cells were lysed in 1% NP40 and the cytoplasmic lysate was denatured by same volume of 1 M NaOH and 1.5 M NaCl for 10 min, followed by neutralization with double volume of 1 M Tris-HCl (pH7.4) and 1.5 M NaCl for 10 min, then the treated lysate was dotted on Hybond-XL membrane by Minifold I dot blotter (GE Healthcare). Next, the air dried membrane was UV crosslinked and subjected to DNA hybridization with α-^{32}P-UTP (800 Ci/mmol, Perkin Elmer) labeled minus strand specific full-length HBV riboprobe as previously described (Cai et al., 2013). The obtained HBV positive cell lines were further assessed for their tetracycline inducibility by confirming the absence of HBV DNA in cells cultured in the presence of tetracycline. A selected tetracycline inducible cell line with high levels of HBV DNA replication and cccDNA-dependent HA-HBeAg expression was designated HepBHAe82 cells. The maintenance and induction of HepBHAe82 cells were performed in the same way as HepDES19 cells.

2.4 HBV RNA and DNA analyses

Total cellular RNA was extracted by TRIzol reagent (Invitrogen). To extract the cytoplasmic encapsidated HBV pgRNA, cells from one well of a 12-well-plate were lysed in 250 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 1% NP-40) in 37°C for 10 min, the cell debris and nuclei were removed by centrifugation at 12,000rpm for 2 min. The supernatant was then incubated with 6 U of micrococcal nuclease (Affymetrix) and 15 μl of 100 mM $CaCl_2$ for 15 min at 37°C to digest unprotected free nucleic acids. Then encapsidated pgRNA was extracted by 750 μl of TRIzol LS reagent (Invitrogen) according to manufacturer's instruction. Total RNA and encapsidated pgRNA samples were subjected to HBV RNA Northern blot hybridization as previously described (Mao et al., 2013).

HBV cytoplasmic core DNA and nuclear cccDNA were extracted from transiently and stably transfected cells and analyzed by Southern blot hybridization according to our previous publications (Cai et al., 2013; Zhang et al., 2014).

For quantitative PCR detection of HBV cccDNA, the extracted cccDNA samples were first heated at 85°C for 5 min to denature the deproteinized HBV rcDNA into single-stranded DNA, followed by Plasmid-safe ATP-dependent DNase (PSAD) (Epicentre) treatment at 37°C for 16 h. The PASD reaction was then stopped by heat inactivation at 70°C for 30 min, and the samples were further purified by DNA clean-up spin column (Zymo Research). Real-time PCR amplification of 2 μl cleaned cccDNA sample was performed with the FastStart Essential DNA Probes Master (Roche) in a 20 μl reaction by mixing with 0.9 μM forward primer (5'-GTCTGTGCCTTCTCATCTGC-3'; nt 1553–1572), 0.9 μM reverse primer (5'-AGTAACTCCACAGTAGCTCCAAATT-3'; nt 1949–1925), and 0.2 μM TaqMan probe (5'-FAM-TTCAAGCCTCCAAGCTGTGCCTTGGGTGGC-TAMRA-3'; nt 1865–1894), followed by annealing and extension steps at 61°C for 50 cycles.

2.5 HBV capsid gel assay

The native agarose gel electrophoresis and enzyme immunoassay (EIA) of cytoplasmic HBV capsid were performed according to previously described (Yan et al., 2015b).

2.6 Immunoprecipitation of HA-tagged HBeAg

One ml of cell culture supernatant sample was incubated with 50 μl of EZview Red Anti-HA affinity gel beads (Sigma-Aldrich) at 4°C overnight with gentle rotation. The beads were washed with TBS buffer (0.15 M NaCl, 0.05 M Tris-HCl [pH 7.4]) for three times at RT. The pelleted beads were dissolved in 1× Laemmli buffer and the immunoprecipitated HA-HBeAg was detected by Western blot using antibodies against HA-tag (Sigma-Aldrich).

2.7 Chemiluminescence ELISA (CLIA) detection of HA-HBeAg

Polystyrene 96-well plate (Cat# 3904, Corning) was coated with 100 μl of anti-HA antibody (cat#: A01244, Genscript; 5 μg/ml in PBS buffer) at 4°C overnight, followed by 5 washes with 150 μl PBS. After removal of the wash buffer, 150 μl of PBS which contains 2% BSA was added into the ELISA wells and incubated at RT for 2 h, followed by wash with 150 μl PBS for 5 times. Then 100 μl of cell culture supernatant samples was added into the wells and incubated at 4°C overnight, followed by 5 washes with 150 μl PBST buffer (0.1% (v/v) Tween 20 in PBS). Then 100 μl of PBST which contains 2% BSA and horseradish peroxidase (HRP)-conjugated anti-HBe antibodies (Cat# 61-H10K, Fitzgerald; 1: 40,000 dilution) was added in the well and incubated at RT for 30 min. After washing with 150 μl PBST for 5 times and 150 μl PBS for 2 times, 100 μl of fresh mixed SuperSignal ELISA Femto Maximum Sensitivity Substrate (Cat# 37074, Thermo Scientific; 1: 1 mixture of luminol enhancer solution and stable peroxide solution) was added and the plate was read on a luminometer.

The detection of HA-HBeAg by the conventional HBeAg ELISA was performed with the commercial HBeAg CLIA kit (Autobio Diagnostics) following the manufacturer’s manual.

3. RESULTS

3.1 Assay design rationale

The design of an HBV cccDNA-dependent HA-tagged HBeAg reporter cell line is according to the similar strategy for previously reported HepDE19 cell line, which expresses the wild type HBeAg in a cccDNA-dependent manner. The strategy is based on the principle that the HBV cccDNA can be made from pgRNA transcript with its translation products (core and pol) *via* the intracellular amplification pathway, the ORF of HBeAg is divided on both termini of pgRNA and is only reconstituted when the pgRNA is retrotranscribed into rcDNA and subsequently circularized into the cccDNA transcription template (Cai et al., 2012; Guo and Guo, 2015). In the new cell line, the transgene contains an integrated HBV genome spanning the entire viral pgRNA and precore ORF region under the control of tetracycline regulated (tet-off) CMV-IE promoter, an in-frame HA epitope sequence is inserted into the precore domain of precore ORF, and the start codon of HA-tagged precore (HA-pC) ORF is mutated to block possible expression of HA-pC from the transgene. Upon tetracycline induction, the transcribed pgRNA will be translated into viral core protein and polymerase

(pol) and retro-transcribed into rcDNA, leading to cccDNA formation. Meanwhile, the start codon of precore ORF at the 3' terminus of pgRNA will be copied into viral DNA sequence, and the ORF of HA-pC will be reconstituted on the cccDNA template. Thus, the authentic precore mRNA can be transcribed only from cccDNA. The precore protein product is further processed and secreted as HBeAg, which serves as a surrogate marker for cccDNA and is conveniently detected by ELISA or other methods (Fig. 1).

3.2 Insertion of an epitope tag into precore ORF of HBV genome

The 5' stem-loop structure (epsilon) of HBV pgRNA is an essential *cis* element for viral replication (Hu and Lin, 2009). It serves as the pgRNA packaging signal and DNA priming site. The epsilon sequence overlaps with the 5' portion of precore ORF and contains the start codon of capsid protein (core, HBcAg) ORF. In order to construct an inducible HBV replicating vector encoding a Human influenza hemagglutinin (HA) fused precore open reading frame without altering the integrity of epsilon structure, an HA-tag-containing DNA sequence was inserted into an in-frame position downstream of the N-terminal signal peptide sequence in precore (pC) ORF and upstream adjacent to the start codon of core ORF in HBV expression vector pTREHBVDES (Fig. 1 and 2A). Plasmid pTREHBVDES was previously used to make the HepDES19 cells, which expresses HBV pgRNA under the control of a tet regulated CMV-IE promoter in a tet-off manner (Guo et al., 2007). After insertion, the 5' 3-aa linker sequence (GTG GAC ATC) replaced the original viral sequence (ATG GAC ATC) of the right arm at the bottom of the epsilon, thereby the base pairing of the epsilon is maintained and the start codon of core ORF was moved to a position downstream of epsilon (Fig. 2B). Between HA-tag sequence and core ATG, the GGC sequence was maintained to keep the authentic Kozak motif of core start codon. The obtained recombinant plasmid was designated pTREHBV-HAe. The plasmid pTREHBV-HAe has a point deletion (ATG to TG) of the 5' end start codon of precore ORF, by which prevents the expression of precore from the HBV genome in the plasmid template. In addition, plasmid pTREHBV-HAe contains two tandem stop codons in the ORF of the small (S) envelope protein (217TTGTTG222 to 217TAGTAG222) to block the production of HBV infectious particles (Fig. 1). Hepadnavirus envelope proteins have been suggested to negatively regulate the intracellular recycling pathway of cccDNA formation (Gao and Hu, 2007; Guo et al., 2007; Lentz and Loeb, 2011; Summers et al., 1990), thus it is expected that blocking envelope protein expression may increase the production of cccDNA.

In a transient transfection assay with cotransfected plasmid pTet-off (which encodes the tetracycline transactivator (tTA) and G418 resistance gene), the recombinant HBV plasmid pTREHBV-HAe was able to replicate HBV rcDNA to a comparable level as achieved by wildtype pTREHBVDES, suggesting that the HA-tag insertion was tolerated by HBV genome replication (Fig. 3). It is worth noting that the size of pgRNA transcribed from pTREHBV-HAe was slightly larger than wildtype pgRNA due to the HA sequence insertion, and the recombinant pgRNA was competent for translation and encapsidation. Since pTREHBV-HAe transient transfection does not produce detectable cccDNA, as expected, no HA-tagged protein was detected by Western blot using HA antibodies (data not shown).

3.3 Expression of HA-tagged HBeAg and ELISA detection

In order to assess the feasibility of HA-tagged HBV precore protein expression and HBeAg secretion, the HA-tag-containing DNA sequence was inserted into the same viral DNA nucleotide position, as described above, in precore expression plasmid pcHBe and the construct was designated pcHA-HBe. Transfection of pcHA-HBe in HepG2 cells led to the intracellular expression of HA-tagged precore (HA-precore) protein and extracellular accumulation of HA-tagged HBeAg (HA-HBeAg) (Fig. 4B), thus confirming that the insertion of HA tag into precore protein does not affect precore expression or posttranslational processing, or HBeAg secretion. Interestingly, because antibodies recognizing the C-terminus of HBV precore/core protein were used in Western blot, the detected intracellular precore and HA-precore should have an uncleaved C-terminus. However, anti-HA antibodies, which would recognize the N-terminus of HA-precore, did not detect the predicted lower band corresponding to the furin-cleaved intracellular HA-HBeAg. This observation indicates that furin processing of precore is transient during HBeAg secretion, without accumulation of intracellular mature HBeAg.

Next, we developed a chemiluminescence ELISA (CLIA) for detecting secreted HA-HBeAg in the supernatant, in which an anti-HA mAb served as the capture antibody, and an HRP-conjugated anti-HBe mAb served as the detection antibody. The HA-HBeAg CLIA assay only detected the HA-HBeAg signal from the supernatant of pcHA-HBe transfected cells, but not from the cells transfected by pcHBe (producing wildtype HBeAg) or pcHBc (expressing HBcAg), demonstrating the specificity of the HA-HBeAg CLIA (Fig. 4C). In accordance with the above, the cell line HepHA-HBe4, which constitutively expresses HA-tagged HBeAg was established by stably transfecting pcHA-HBe into HepG2 cells. HepHA-HBe4 cells secrete high levels of HA-HBeAg as detected by CLIA, and the secretion of HA-HBeAg can be efficiently blocked by furin inhibitor I (Fig. 4D). HepHA-HBe4 cells can serve in a counter screen designed to filter hits that directly target HBeAg metabolism or secretion.

3.4 Establishment of HBV stable cell lines

Plasmid pTREHBV-HAe was stably co-transfected with pTet-off into HepG2 cells to make a tet-inducible HBV cell line. In such a cell line, the HA-precore mRNA can be transcribed only from cccDNA, making secreted HA-tagged HBeAg a surrogate marker for cccDNA (Fig. 1). Through G418 and tet selection, single cell-derived colonies were expanded to clonal cell lines, and the HBV DNA replication in each individual clone was assessed by dot blot hybridization. The dot blot positive clones were subjected to core DNA Southern blot assay. We have obtained 6 cell clones that support high levels of HBV DNA replication in a tet-inducible manner (Fig. 5A), and the cccDNA production in 4 clones with higher DNA replication was analyzed by Southern blot (Fig. 5B). The authenticity of cccDNA bands was confirmed by heat denaturing and EcoR I linearization as previously described (Cai et al., 2013). Among the 4 cccDNA producing cell lines, clone 82 with slightly higher production of cccDNA was named HepBHAe82 cells and used in following studies.

To assess the susceptibility of HBV DNA replication in HepBHAe82 cells to known HBV inhibitors that target different steps in virus replication, the cells were cultured in tet-free

medium and treated with capsid inhibitor Bay41-4109 (Deres et al., 2003), pgRNA encapsidation inhibitor AT-61 (Feld et al., 2007), and nucleoside reverse transcriptase inhibitor 3TC (Xie et al., 1995) (Fig. 6A). As shown in Fig. 6B, all three inhibitors completely blocked HBV DNA replication without reducing the level of pgRNA. In line with their reported mechanism of action, Bay41-4109 blocked capsid assembly and depleted core proteins (lane 2); AT-61 inhibited pgRNA encapsidation but did not block capsid assembly (lane 3); 3TC blocked DNA replication without inhibiting any upstream steps (lane 4). Remarkably, 3TC treatment resulted in large accumulation of the encapsidated pgRNA, which was a consequence of stalled reverse transcription. As expected, all three HBV replication inhibitors completely prevented cccDNA production in HepBHAe82 cells (data not shown). These results suggested that HepBAHe82 cell line is suitable for investigating established and novel antivirals against HBV.

3.5 Validation of cccDNA-dependent HA-HBeAg expression in HepBHAe82 cells

In HepBHAe82 cells, time-dependent kinetics of synthesis and accumulation of viral products, including the core DNA and cccDNA, were observed upon tet withdrawal (Fig. 7A). Next, the HA-HBeAg production in HepBHAe82 cells was assessed. In the culture fluid samples from HepBHAe82 cells, HA-HBeAg was detected at day 6 after the removal of tet and the antigen level gradually increased subsequently (Fig. 7B). The level of HA-HBeAg was proportional to the intracellular level of viral cccDNA, indicating that the cccDNA in HepBHAe82 cells was transcriptionally active.

In order to further validate the cccDNA-dependent HA-HBeAg expression in HepBHAe82 cells, the cells were cultured under three different conditions: 1) in the presence of tet to suppress transgene expression (HBcAg−, core DNA−, cccDNA−); 2) in the absence of tet to induce viral DNA replication (HBcAg+, core DNA+, cccDNA+); 3) in the absence of tet but with 3TC treatment (HBcAg+, core DNA−, cccDNA−). The result showed that the supernatant HA-HBeAg signal appeared at day 6 after tet withdrawal and gradually increased afterward, as a consequence of cccDNA establishment and gene expression. As predicted, HA-HBeAg was barely detected at any time points in the presence of tet or under 3TC treatment (Fig. 8A). At day 10, the average signal: noise (tet- vs tet-/3TC) ratio (S:N) in HepBHAe82 was approximately 40:1, which is superior to the conventional HBeAg ELISA in the HepDE19 system, which exhibited a S:N of ≤5:1 (Cai et al., 2012) (Data not shown). Moreover, when the supernatant samples harvested at day 10 were analyzed by a commercial HBeAg CLIA kit which detects HBeAg with a pair of HBeAbs, a strong background signal was detected from the induced HepBHAe82 cells under 3TC treatment and the S:N was less than 4 (Fig. S1), presumably due to the ELISA cross reaction with supernatant core protein. Furthermore, the cccDNA formation inhibitor CCC-0975 exhibited a dose-dependent inhibition of HA-HBeAg production from HepBHAe82 cells, and the levels of HA-HBeAg were proportional to the intracellular cccDNA levels (Fig. 8B). Therefore, the above data suggest that the production of HA-HBeAg is solely cccDNA-dependent in HepBHAe82 cells, and HA-HBeAg could serve as a specific surrogate marker for cccDNA in HepBHAe82 cells with the HA-HBeAg-specific CLIA assay.

3.5 Development of a condition for analyzing cccDNA stability or transcriptional activity

In our previous screen with the HepDE19 cell system, the testing compound was added immediately after tet withdrawal and the treatment lasted 7 days without medium change to allow for accumulation and easier detection of secreted HBeAg (Cai et al., 2012); therefore the conditions were not ideal for maintaining a compound's activity. In addition, this assay condition increases the hit rate of HBV DNA replication inhibitors and cccDNA formation inhibitors (ie: CCC-0975). In order to develop an assay for specifically identifying direct anti-cccDNA agents, we set out to establish a condition that allows the analysis of HA-HBeAg derived from the preexisting cccDNA pool in a 96-well plate. To this end, tet was removed from HepBHAe82 cells to induce cccDNA formation for certain time period, then tet and 3TC were added to the culture medium to shut down transgene-based pgRNA transcription and viral DNA replication, respectively, hereby preventing the *de novo* replenishment of cccDNA formation. Under this condition, the HA-HBeAg production is linear with cccDNA copy number and transcription activity in HepBHAe82 system, making the assay more cccDNA-specific. As shown in Fig. 9A, induction of cccDNA formation for 14 days followed by tet+3TC treatment exhibited an initial increase of HA-HBeAg in the first 4 days, then the antigen level started to decline, possibly due to the decay of cccDNA and its transcription activity. In addition, the half-life of secreted HA-HBeAg is rather short (12 – 16 hours) in cell cultures (Fig. S2). Therefore for future compound testing, the cultures can be treated for less than 4 days, after cccDNA induction. To validate this assay, we tested a few compounds that may impact cccDNA activity or HBeAg secretion. Because there is no reference compounds available that directly target cccDNA stability, we evaluated an epigenetic regulator trichostatin A (TSA, a histone deacetylase (HDAC) inhibitor) that was expected to affect cccDNA transcriptional activity. We also tested an HBeAg secretion inhibitor (furin inhibitor I). As shown in Fig. 9B, furin inhibitor I dramatically reduced the level of HA-HBeAg, while TSA markedly increased HA-HBeAg expression. As expected, neither compound affected the intracellular cccDNA level. This is consistent with published results that an HDAC inhibitor upregulate HBV cccDNA expression but has no effect on its stability (Pollicino et al., 2006). Our results demonstrated that the productivity of established cccDNA pool in HepBHAe82 cells is susceptible to chemical intervention.

4. Discussion

HBV cccDNA is an undisputed antiviral target for elimination of chronic hepatitis B. However, due to the lack of specific and robust assays for direct measurement of low copy number cccDNA in HBV replicating cells, a cccDNA reporter system is thus needed for identification of cccDNA inhibitors in high throughput screening (Guo and Guo, 2015). Although recombinant HBV reporter viruses have been reported to support cccDNA-dependent infection, replacement or insertion of reporter gene in HBV genome will affect certain step(s) of virus replication and normally require transcomplementation of one or more viral proteins to make the infectious reporter viruses, resulting in decreases in the efficiency of replication or virion formation (Hong et al., 2013; Protzer et al., 1999; Untergasser and Protzer, 2004). In addition, infectious HBV reporter virus systems reproduce only the initial round of infection, and the current HBV *in vitro* infection systems require large amount of virus inoculum, but still exhibit relatively low infectivity and high

variability (Yan et al., 2015a). These disadvantages impede the extensive use of HBV reporter viruses in antiviral drug screen. In contrast, an HBV stable cell line supporting cccDNA-dependent gene expression provides a convenient tool for drug discovery, as represented by HepDE19 cells which express secreted HBeAg in a cccDNA-dependent manner (Cai et al., 2012). Due to its unique ORF organization in the HBV linearized transgene cassette, and its dispensable role in viral DNA replication, HBeAg is the only HBV gene product which could be engineered to be expressed only from the cccDNA template, but not from the integrated transgene, in stably-transfected cells. However, the HBeAg ELISA cross reacts with an HBeAg homologue, which is the core protein expressed predominantly from the transgene and released into the supernatant as naked capsid in HepDE19 cells (Guo et al., 2007). In order to produce a cccDNA surrogate marker with higher specificity for detection, we modified the HBeAg coding sequence in the transgene by inserting an in-frame HA epitope without altering the functions of critical viral *cis* elements and the other viral ORFs. The newly established cell line HepBHAe82, similar to the principle of HepDE19 cells, expresses cccDNA-dependent HA-tagged HBeAg, which completely eliminates the confounding signal from the antigenically related core protein. Therefore, with this improvement, the HepBHAe82 cell system is more suitable for developing into an HTS platform for discovery of HBV cccDNA inhibitors.

To create an epitope tagged HBeAg, the insertion site is limited to the 10 aa precore domain, which is between the N-terminal signal peptide region and the downstream core ORF (Fig. 4A). The precore domain coding sequence is also shared by the upper stem sequence of epsilon in the HBV genome, and the start codon of core ORF is located in the right arm of the lower stem of epsilon (Fig. 2A). Therefore, any exogenous sequence insertion in the precore domain will likely disrupt the base pairing in epsilon structure and result in a reduction of virus replication. In order to maintain the integrity of the epsilon structure, we designed and inserted a segment containing the HA epitope sequence with the following flanking sequences in front of the start codon of the core ORF: 1) A 5’ 9-nt linker was used to maintain the base pairing in the lower stem of epsilon. A single mutation (A1903G) replaced the original core start codon, while maintaining wobble base pairing with the cognate U(T)1855 in the lower stem loop and 2) a 3’ 3-nt linker GGC was used to maintain the Kozak sequence of the core start codon, which, together with the core ORF, was moved to a position downstream of epsilon and the HA-tag insert (Fig. 2B). This strategy minimally alters the sequence of epsilon without affecting HBV pgRNA translation, encapsidation, and DNA replication, and can be used for engineering other epitope tags into HBeAg. Additionally, we have also assessed 6×His tag, Flag tag, and 3×Flag tag in pTREHBVDES vector by using the same cloning strategy, and transient transfection showed that HBV genomic DNA replication was not significantly affected by the tag insertions (data not shown), indicating that the corresponding epitope-tagged HBeAg can be expressed in cccDNA-dependent manner. Among the epitope tags tested, 3×Flag tag, plus the linkers, was the longest insertion (78 nt) tolerated by HBV genome DNA replication. Longer reporter gene sequences, such as luciferase and fluorescence proteins, are currently under testing in our laboratory.

To develop the newly established HepBHAe82 cell system into an HTS platform for screening cccDNA inhibitors, miniaturization is a key consideration for increasing

throughput and reducing the screening cost. With the current HA-HBeAg CLIA, we have miniaturized the HepBHAe82-based assay to 96-well-plate format, in which the detection signals remain sufficiently high under the optimized conditions (Fig. 9). However, the current assay still has weaknesses and needs further improvement. Firstly, the induction of a strong and stable cccDNA-dependent HA-HBeAg signal normally requires more than ten days, which is partly due to the lengthy time required for cccDNA formation and/or the limited sensitivity of CLIA. Secondly, although the HA-HBeAg CLIA assay has good specificity, it is still a traditional ELISA assay that requires supernatant harvest, and multiple washing and incubation steps, which make the use of the assay in an HTS format somewhat cumbersome. Therefore, we have started to develope an AlphaLISA assay format. There are many advantages of AlphaLISA over the conventional ELISA, such as “mix and measure”, no separation or wash steps; low background and wide dynamic range; minimal assay volume; easy to miniaturize; and automation ready; etc (Hertzberg and Pope, 2000). If successful, we expect that the AlphaLISA assay will increase the detection sensitivity of HA-HBeAg, so that the cell induction time could be shortened. More importantly, AlphaLISA will greatly increase the assay robustness and reduce well-to-well variation, thus making the 384-well assay miniaturization possible. In our future HTS development, the performance characteristics of the assay, including variability, reproducibility, and assay dynamic range will be established by Z-factor (Z’), which is the ultimate assessment of the quality and suitability of a HTS assay (Zhang et al., 1999).

In parallel with HepBHAe82 cell line, a stable cell line constitutively expressing HA-HBeAg, namely HepHA-HBe4, has also been generated and can be used as a counter screen. In a future screening paradigm, hits that reduce HA-HBeAg signal in HepBHAe82 cells will be tested in HepHA-HBe4 cells to filter out compounds directly inhibiting HBeAg metabolism or secretion. It is of note that the HBeAg inhibitors may also be potentially valuable for basic and therapeutic research, as it has been reported that HBeAg antagonizes TLR2 innate signaling and HBcAg-specific Th1 cell activation (Lang et al., 2011; Milich et al., 1998), and may induce immunologic tolerance in a fetus from a mother that is HBeAg positive (Milich et al., 1990). As demonstrated in Fig. 9B, this new assay can also pick up HBeAg secretion inhibitors such as the furin inhibitor I.

Since cccDNA persistence involves multiple viral and host factors, there are likely to be many molecular opportunities for intervention (Guo et al., 2012; Levrero et al., 2009; Seeger and Mason, 2015b). It has been demonstrated that the clearance of cccDNA during acute infection can occur through a noncytolytic mechanism that is largely independent of adaptive immune function (Guidotti et al., 1999; Wieland et al., 2004), indicating that cccDNA can be purged by innate cellular defenses that may be activated by small molecule compounds. Furthermore, since cccDNA exists and functions as a minichromosome (Bock et al., 2001), agents that induce cccDNA CpG methylation or alter cccDNA-bound histone modifications (methylation or acetylation) may lead to a reduction of cccDNA transcription (Pollicino et al., 2006; Vivekanandan et al., 2009; Zhang et al., 2014).

Besides potentially serving as a compound screening and testing tool, the HepBHAe82 cccDNA reporter cell line could also aid basic research in the study of host functions that regulate cccDNA formation, stability, and transcription.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Block TM, Gish R, Guo H, Mehta A, Cuconati A, Thomas London W, Guo JT. Chronic hepatitis B: what should be the goal for new therapies? Antiviral research. 2013; 98:27–34. [PubMed: 23391846]

Block TM, Guo H, Guo JT. Molecular virology of hepatitis B virus for clinicians. Clinics in liver disease. 2007; 11:685–706. vii. [PubMed: 17981225]

Bock CT, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, Zentgraf H. Structural organization of the hepatitis B virus minichromosome. Journal of molecular biology. 2001; 307:183–196. [PubMed: 11243813]

Cai D, Mills C, Yu W, Yan R, Aldrich CE, Saputelli JR, Mason WS, Xu X, Guo JT, Block TM, Cuconati A, Guo H. Identification of disubstituted sulfonamide compounds as specific inhibitors of hepatitis B virus covalently closed circular DNA formation. Antimicrobial agents and chemotherapy. 2012; 56:4277–4288. [PubMed: 22644022]

Cai D, Nie H, Yan R, Guo JT, Block TM, Guo H. A southern blot assay for detection of hepatitis B virus covalently closed circular DNA from cell cultures. Methods in molecular biology. 2013; 1030:151–161. [PubMed: 23821267]

Deres K, Schroder CH, Paessens A, Goldmann S, Hacker HJ, Weber O, Kramer T, Niewohner U, Pleiss U, Stoltefuss J, Graef E, Koletzki D, Masantschek RN, Reimann A, Jaeger R, Gross R, Beckermann B, Schlemmer KH, Haebich D, Rubsamen-Waigmann H. Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. Science. 2003; 299:893–896. [PubMed: 12574631]

Feld JJ, Colledge D, Sozzi V, Edwards R, Littlejohn M, Locarnini SA. The phenylpropenamide derivative AT-130 blocks HBV replication at the level of viral RNA packaging. Antiviral research. 2007; 76:168–177. [PubMed: 17709147]

Galibert F, Mandart E, Fitoussi F, Charnay P. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E.coli. Nature. 1979; 281:646–650. [PubMed: 399327]

Gao W, Hu J. Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein. Journal of virology. 2007; 81:6164–6174. [PubMed: 17409153]

Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science. 1999; 284:825–829. [PubMed: 10221919]

Guo H, Jiang D, Zhou T, Cuconati A, Block TM, Guo JT. Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation. Journal of virology. 2007; 81:12472–12484. [PubMed: 17804499]

Guo H, Xu C, Zhou T, Block TM, Guo JT. Characterization of the host factors required for hepadnavirus covalently closed circular (ccc) DNA formation. PloS one. 2012; 7:e43270. [PubMed: 22912842]

Guo JT, Guo H. Metabolism and function of hepatitis B virus cccDNA: Implications for the development of cccDNA-targeting antiviral therapeutics. Antiviral research. 2015; 122:91–100. [PubMed: 26272257]

Hertzberg RP, Pope AJ. High-throughput screening: new technology for the 21st century. Current opinion in chemical biology. 2000; 4:445–451. [PubMed: 10959774]

Hong R, Bai W, Zhai J, Liu W, Li X, Zhang J, Cui X, Zhao X, Ye X, Deng Q, Tiollais P, Wen Y, Liu J, Xie Y. Novel recombinant hepatitis B virus vectors efficiently deliver protein and RNA encoding genes into primary hepatocytes. Journal of virology. 2013; 87:6615–6624. [PubMed: 23552416]

Hu J, Lin L. RNA-protein interactions in hepadnavirus reverse transcription. Frontiers in bioscience. 2009; 14:1606–1618.

Huang J, Liang TJ. A novel hepatitis B virus (HBV) genetic element with Rev response element-like properties that is essential for expression of HBV gene products. Molecular and cellular biology. 1993; 13:7476–7486. [PubMed: 8246965]

Ito K, Kim KH, Lok AS, Tong S. Characterization of genotype-specific carboxyl-terminal cleavage sites of hepatitis B virus e antigen precursor and identification of furin as the candidate enzyme. Journal of virology. 2009; 83:3507–3517. [PubMed: 19193799]

Lang T, Lo C, Skinner N, Locarnini S, Visvanathan K, Mansell A. The hepatitis B e antigen (HBeAg) targets and suppresses activation of the toll-like receptor signaling pathway. Journal of hepatology. 2011; 55:762–769. [PubMed: 21334391]

Lentz TB, Loeb DD. Roles of the envelope proteins in the amplification of covalently closed circular DNA and completion of synthesis of the plus-strand DNA in hepatitis B virus. Journal of virology. 2011; 85:11916–11927. [PubMed: 21900164]

Levrero M, Pollicino T, Petersen J, Belloni L, Raimondo G, Dandri M. Control of cccDNA function in hepatitis B virus infection. Journal of hepatology. 2009; 51:581–592. [PubMed: 19616338]

Liang TJ, Block TM, McMahon BJ, Ghany MG, Urban S, Guo JT, Locarnini S, Zoulim F, Chang KM, Lok AS. Present and future therapies of hepatitis B: From discovery to cure. Hepatology. 2015

Mao R, Nie H, Cai D, Zhang J, Liu H, Yan R, Cuconati A, Block TM, Guo JT, Guo H. Inhibition of hepatitis B virus replication by the host zinc finger antiviral protein. PLoS pathogens. 2013; 9:e1003494. [PubMed: 23853601]

Messageot F, Salhi S, Eon P, Rossignol JM. Proteolytic processing of the hepatitis B virus e antigen precursor. Cleavage at two furin consensus sequences. The Journal of biological chemistry. 2003; 278:891–895. [PubMed: 12417589]

Milich DR, Chen MK, Hughes JL, Jones JE. The secreted hepatitis B precore antigen can modulate the immune response to the nucleocapsid: a mechanism for persistence. Journal of immunology. 1998; 160:2013–2021.

Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? Proceedings of the National Academy of Sciences of the United States of America. 1990; 87:6599–6603. [PubMed: 2395863]

Patient R, Hourioux C, Roingeard P. Morphogenesis of hepatitis B virus and its subviral envelope particles. Cellular microbiology. 2009; 11:1561–1570. [PubMed: 19673892]

Perni RB, Conway SC, Ladner SK, Zaifert K, Otto MJ, King RW. Phenylpropenamide derivatives as inhibitors of hepatitis B virus replication. Bioorganic & medicinal chemistry letters. 2000; 10:2687–2690. [PubMed: 11128652]

Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, Levrero M. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. Gastroenterology. 2006; 130:823–837. [PubMed: 16530522]

Protzer U, Nassal M, Chiang PW, Kirschfink M, Schaller H. Interferon gene transfer by a hepatitis B virus vector efficiently suppresses wild-type virus infection. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96:10818–10823. [PubMed: 10485909]

Seeger C, Mason WS. Hepatitis B virus biology. Microbiology and molecular biology reviews : MMBR. 2000; 64:51–68. [PubMed: 10704474]

Seeger C, Mason WS. Molecular biology of hepatitis B virus infection. Virology. 2015a; 479–480C: 672–686.

Seeger C, Mason WS. Molecular biology of hepatitis B virus infection. Virology. 2015b

Summers J, Smith PM, Horwich AL. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. Journal of virology. 1990; 64:2819–2824. [PubMed: 2335817]

Trepo C. A brief history of hepatitis milestones. Liver international : official journal of the International Association for the Study of the Liver. 2014; 34(Suppl 1):29–37. [PubMed: 24373076]

Untergasser A, Protzer U. Hepatitis B virus-based vectors allow the elimination of viral gene expression and the insertion of foreign promoters. Human gene therapy. 2004; 15:203–210. [PubMed: 14975192]

Vivekanandan P, Thomas D, Torbenson M. Methylation regulates hepatitis B viral protein expression. The Journal of infectious diseases. 2009; 199:1286–1291. [PubMed: 19301974]

Wieland SF, Spangenberg HC, Thimme R, Purcell RH, Chisari FV. Expansion and contraction of the hepatitis B virus transcriptional template in infected chimpanzees. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:2129–2134. [PubMed: 14764900]

Xie H, Voronkov M, Liotta DC, Korba BA, Schinazi RF, Richman DD, Hostetler KY. Phosphatidyl-2', 3'-dideoxy-3'-thiacytidine: synthesis and antiviral activity in hepatitis B-and HIV-1-infected cells. Antiviral research. 1995; 28:113–120. [PubMed: 8585765]

Yan R, Zhang Y, Cai D, Liu Y, Cuconati A, Guo H. Spinoculation Enhances HBV Infection in NTCP-Reconstituted Hepatocytes. PloS one. 2015a; 10:e0129889. [PubMed: 26070202]

Yan R, Zhao X, Cai D, Liu Y, Block TM, Guo JT, Guo H. The Interferon-Inducible Protein Tetherin Inhibits Hepatitis B Virus Virion Secretion. Journal of virology. 2015b; 89:9200–9212. [PubMed: 26109732]

Zeisel MB, Lucifora J, Mason WS, Sureau C, Beck J, Levrero M, Kann M, Knolle PA, Benkirane M, Durantel D, Michel ML, Autran B, Cosset FL, Strick-Marchand H, Trepo C, Kao JH, Carrat F, Lacombe K, Schinazi RF, Barre-Sinoussi F, Delfraissy JF, Zoulim F. Towards an HBV cure: state-of-the-art and unresolved questions--report of the ANRS workshop on HBV cure. Gut. 2015; 64:1314–1326. [PubMed: 25670809]

Zhang JH, Chung TD, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. Journal of biomolecular screening. 1999; 4:67–73. [PubMed: 10838414]

Zhang Y, Mao R, Yan R, Cai D, Zhang Y, Zhu H, Kang Y, Liu H, Wang J, Qin Y, Huang Y, Guo H, Zhang J. Transcription of hepatitis B virus covalently closed circular DNA is regulated by CpG methylation during chronic infection. PloS one. 2014; 9:e110442. [PubMed: 25337821]

Zoulim F, Durantel D. Antiviral therapies and prospects for a cure of chronic hepatitis B. Cold Spring Harbor perspectives in medicine. 2015; 5

Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology. 2009; 137:1593–1608. e1591–e1592. [PubMed: 19737565]

Zoulim F, Locarnini S. Management of treatment failure in chronic hepatitis B. Journal of hepatology. 2012; 56(Suppl 1):S112–S122. [PubMed: 22300461]

Highlights

- A novel HBV cell line HepBHAe82 that expresses HA-tagged HBeAg in a cccDNA-dependent manner.
- HA-tagged HBeAg exhibits superior specificity in ELISA-based assays.
- A counter screen cell line HepHA-HBe4 expressing HA-tagged HBeAg independent of cccDNA.
- HepBHAe82 cell system could be potentially developed into a high throughput screening assay for anti-cccDNA substances.

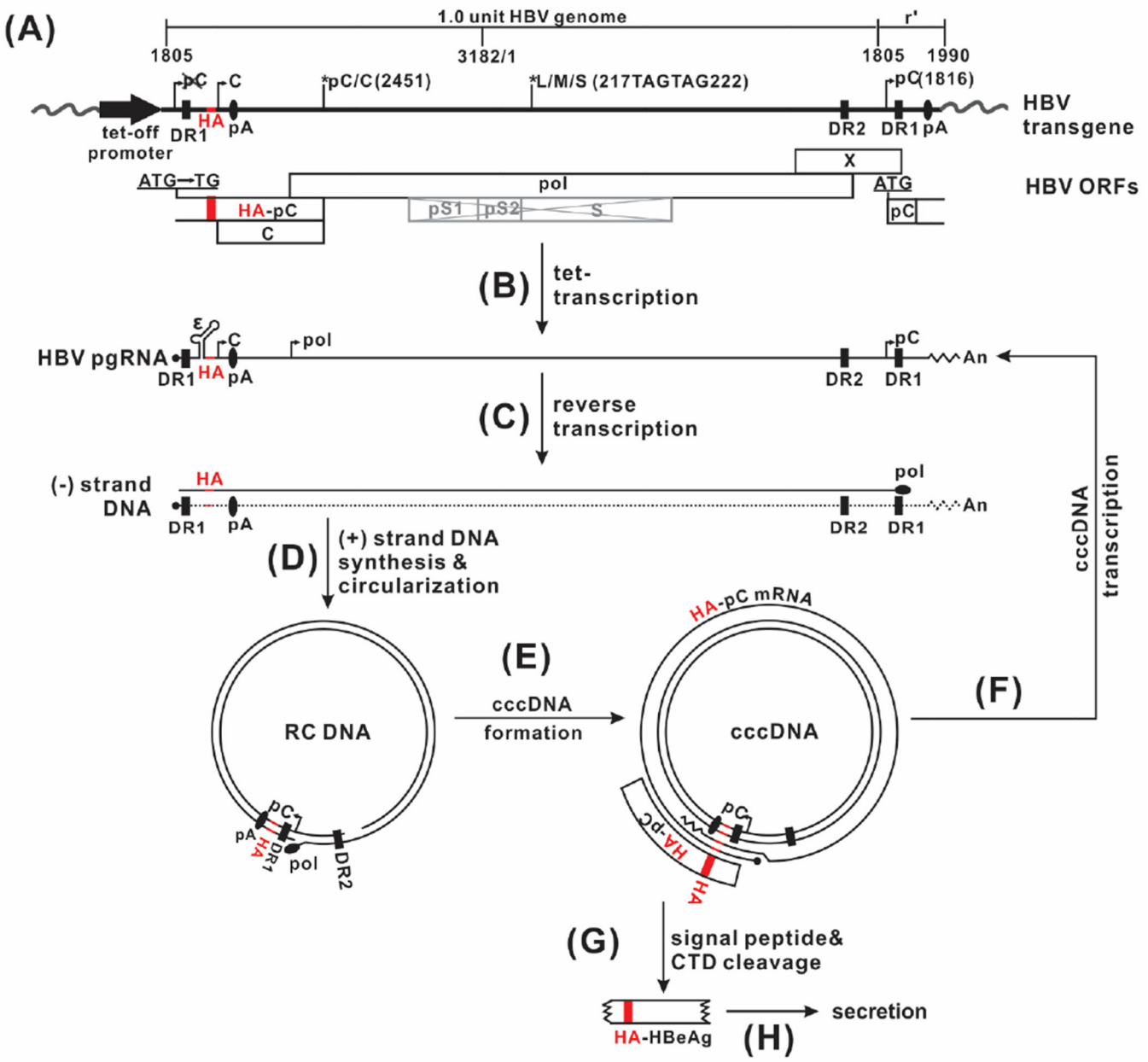

Figure 1. Schematic design of an HBV cccDNA-dependent HA-tagged HBeAg expression cell line

(A) The viral transgene contains a 1.1 overlength HBV genome under the control of tet-CMV promoter. The start codon (ATG) of precore was mutated at the 5' end of HBV DNA, with the second one unchanged at the 3' redundancy. The HA-tag-containing fragment (shown in red) was inserted into the precore domain upstream of the start codon of core ORF. The transgene also contains two tandem stop codons in the small surface (S) ORF to prevent viral envelope protein expression. (B) In the presence of tTA expression, upon removal of Tet from culture medium, pgRNA is transcribed and core and polymerase are produced, resulting in pgRNA packaging and (C) reverse transcription of pgRNA to rcDNA. Host DNA repair mechanisms convert (D) rcDNA into (E) the circular cccDNA template, in which the HA-precore ORF is restored, giving rise to HA-precore mRNA, and (F) pgRNA

for *de novo* viral replication. (G) HA-precore is translated from HA-precore mRNA and processed into secreted HA-HBeAg, which can be detected by ELISA. pC, C, pol, L, M, S and X represent ORF of precore, core, polymerase, large, middle and small s antigen, and X protein, respectively. DR represents direct repeat sequences. CTD represents C-terminal domain.

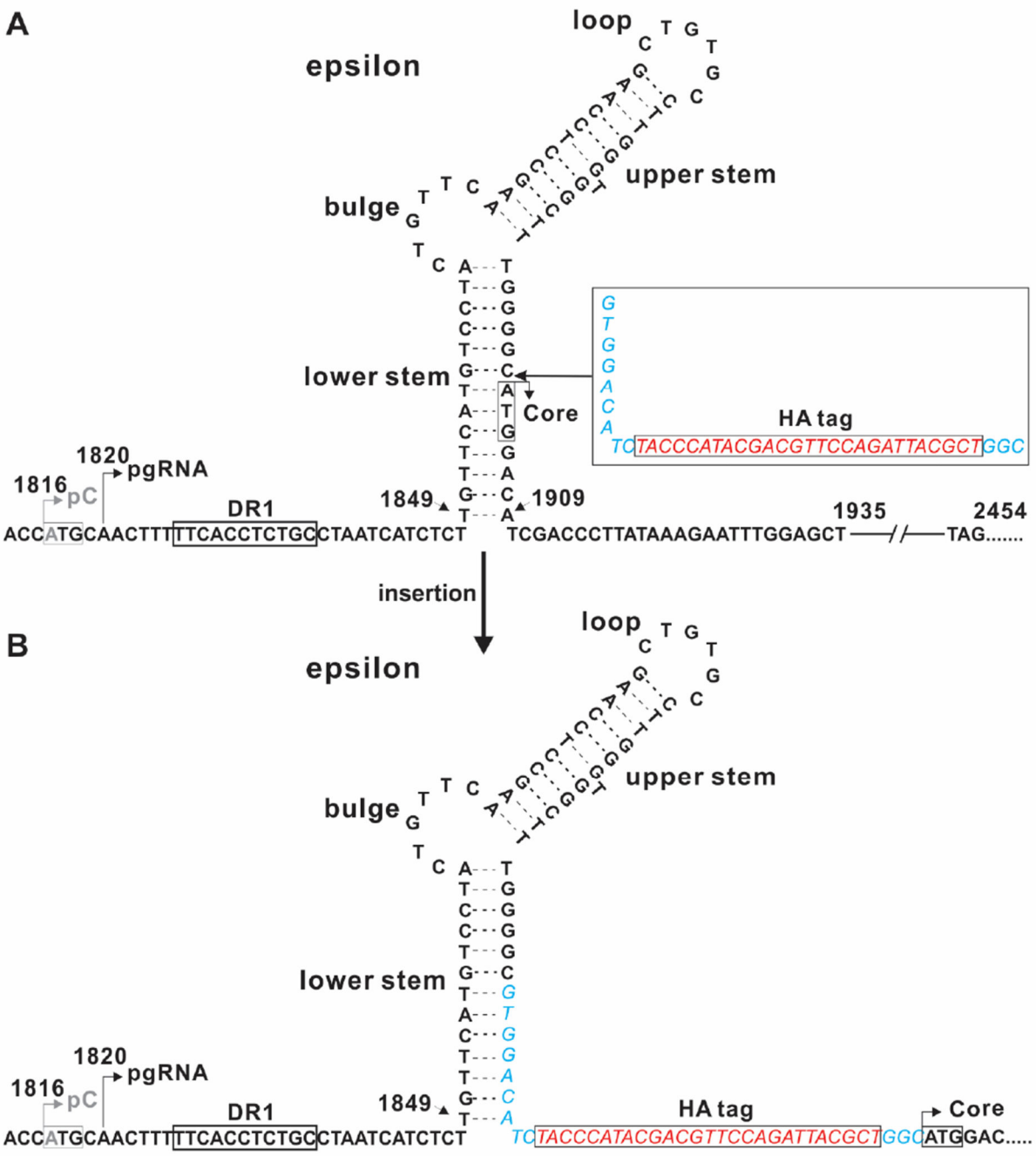

Figure 2. Insertion of the HA-epitope sequence into HBV precore ORF

(A) The precore ORF (genotype D, subtype ayw, nt 1816–2454) located at the 5' end of HBV insertion in plasmid pTREHBVDES is depicted in nucleotide sequence. The sequence between nt 1935 and the stop codon of precore ORF is omitted. The HBV nucleotide position is according to Galibert nomenclature (Galibert et al., 1979). The start codon of precore ORF, direct repeat sequence 1 (DR1), and the in-frame start codon of core ORF are boxed. The start codon precore ORF is mutated (ATG to TG, the deleted nucleotide A is shaded). The authentic pgRNA transcription initiation site (nt 1820) is marked with an

arrow. The overlapped stem-loop structure (epsilon, ε) is illustrated with predicted structures (lower stem, bulge, upper stem, and apical loop). To place an in-frame fused HA-tag sequence into precore ORF without altering the base paring of epsilon, an HA-tag-containing DNA sequence is inserted into an in-frame upstream position adjacent to the start codon of core ORF (see the insert box). The HA-epitope sequence is boxed and shown in red, the linker sequence is shown in blue (B) The sequence modification results in an in-frame fusion of HA-tag plus linker sequences into precore ORF, and the intact ORF of core protein is relocated to a downstream position of epsilon.

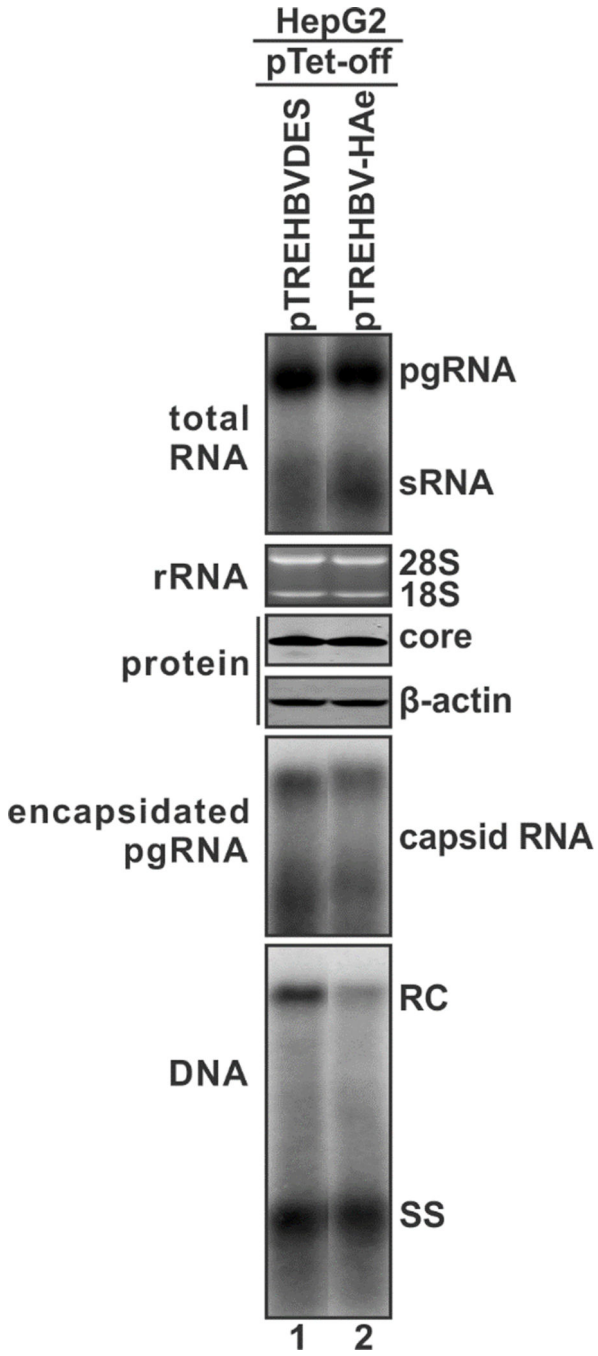

Figure 3. Replication fitness of HA-recombinant HBV genome in transiently transfected cells

HepG2 cells were cotransfected with pTREHBVDES or pTREHBV-HAe and plasmid pTet-off. Cells were harvested 5 days post transfection, and plasmid-based production of HBV RNA, core protein, encapsidated pgRNA, and viral DNA replication were analyzed by Northern blot, Western blot, and Southern blot hybridization, respectively. pgRNA: pregenomic RNA; sRNA: surface RNA; RC: relaxed circular DNA; SS: single stranded DNA. Ribosomal RNA (rRNA) and β-actin served as loading control for Northern and Western blots, respectively.

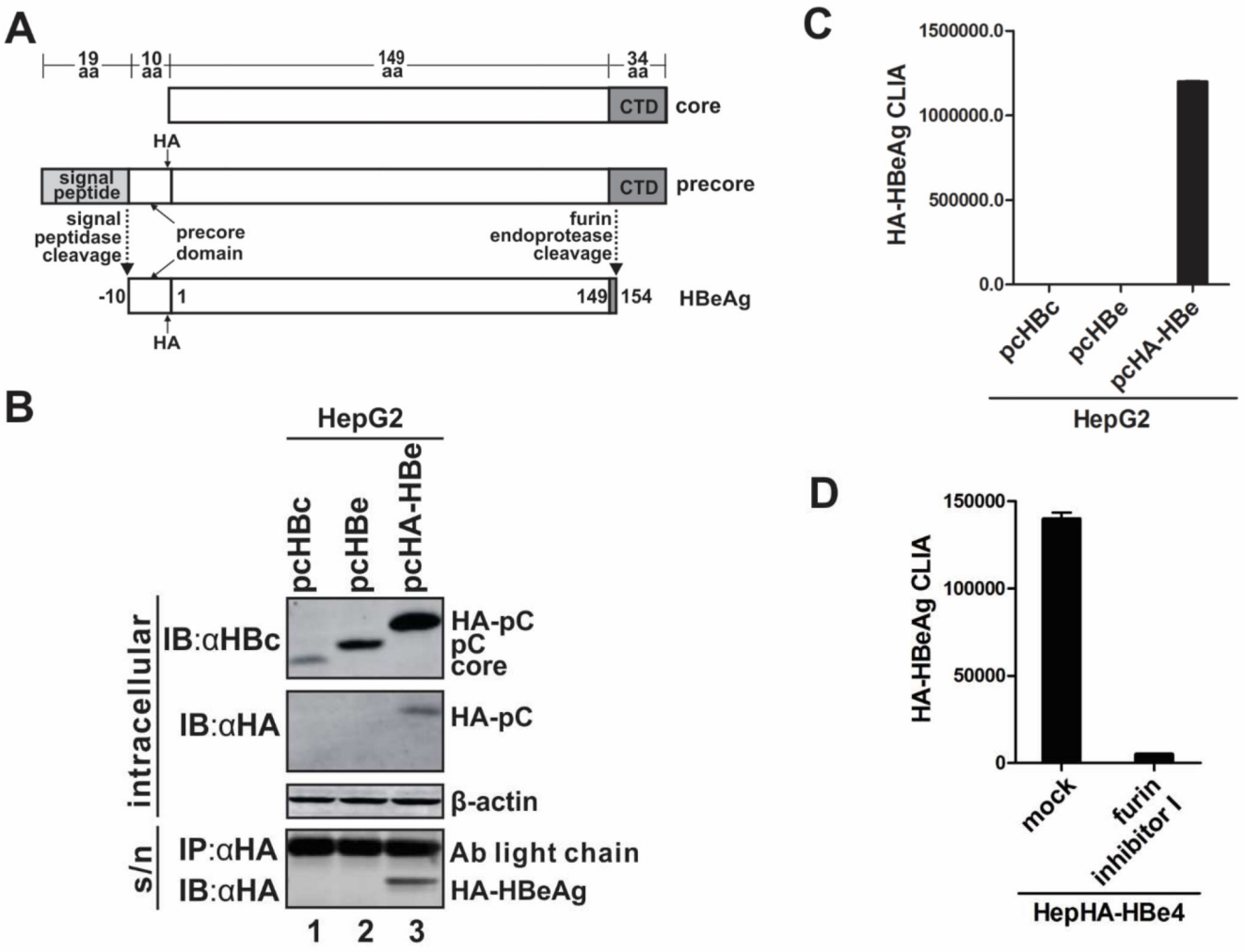

Figure 4. Expression, secretion, and CLIA detection of HA-HBeAg

(A) Schematic illustration of genotype D HBV precore protein, core protein, and HBeAg. Core protein and precore protein share same 183 aa sequence at C-terminus, with the extreme C-terminal 34 aa called C-terminal domain (CTD). Compared to core protein, precore has an N-terminal 29 aa extension, which is comprised of the N-terminal 19 aa signal peptide and the 10 aa precore domain. During precore protein translation and ER-Golgi translocation in cells, the signal peptide is removed by host signal peptidase and the majority of CTD (aa155–183) is removed by the endopeptidase furin, resulting in the secreted HBeAg. The aa position number 1 of HBeAg is assigned to the first methionine of core protein sequence by convention. The insertion position of HA-tag in HA-precore and HA-HBeAg is indicated. (B) HepG2 cells were transfected with plasmid expressing wildtype core (pcHBc), or wildtype precore (pcHBe), or HA-precore (pcHA-HBe). Four days later, the intracellular protein expression was detected by Western using antibodies against core or HA-epitope, the secreted HA-HBeAg in supernatant (s/n) from day 2 to day4 was immunoprecipitated and detected by Western blot. (C) The collected supernatant from (B) was subjected to HA-HBeAg CLIA assay. (D) HepHA-HBe4 cells were left untreated or

treated with furin inhibitor I (1 μM) for 2 days, HA-HBeAg secreted in supernatant was detected by CLIA.

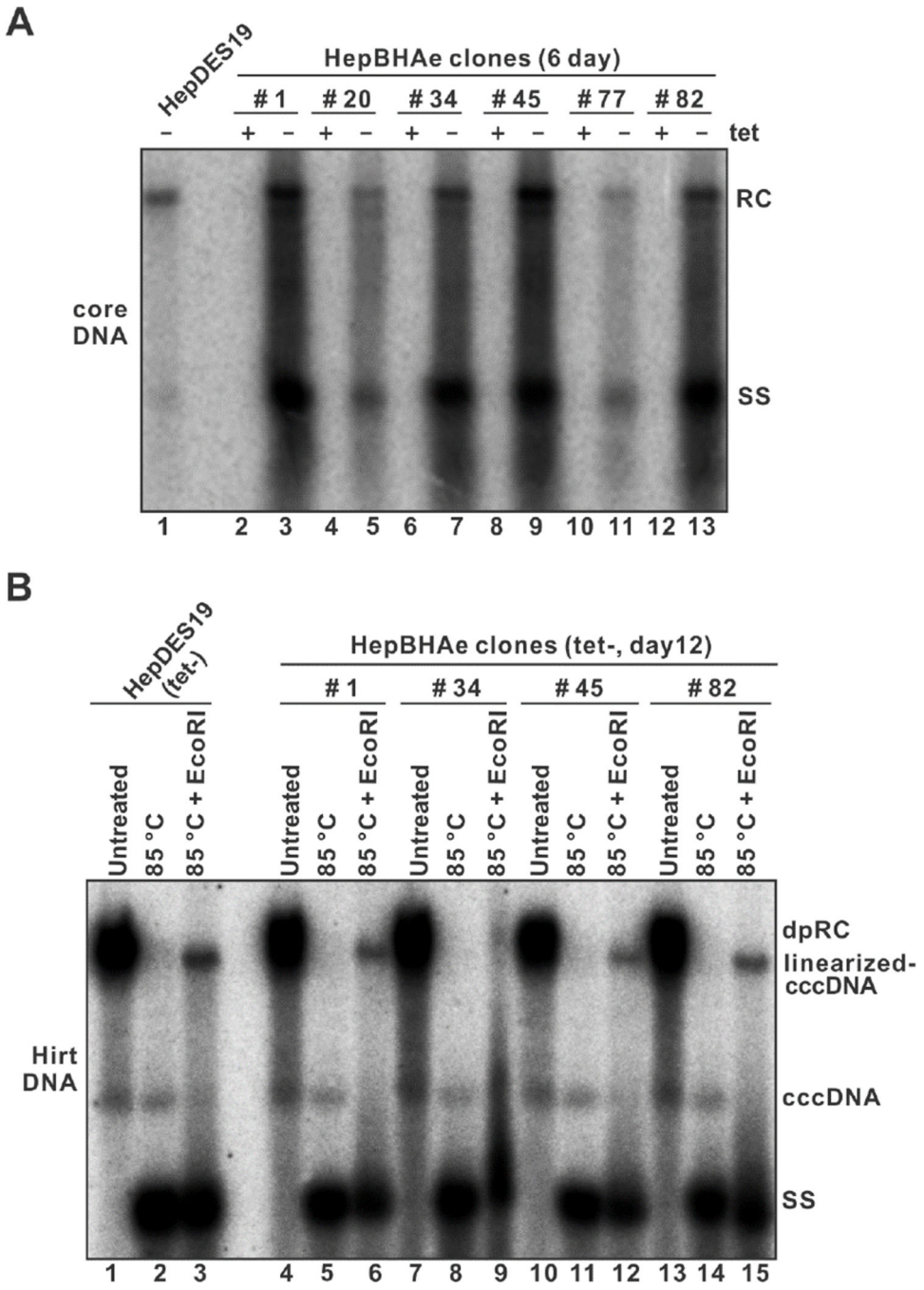

Figure 5. Establishment of inducible cell lines that support HA-recombinant HBV DNA replication

(A) Tet-inducible HBV DNA replication in HepBHAe cells. HepDES19 cells and the newly established HepBHAe cell lines with indicated clone numbers were seeded in 6-well-plates at the same density in the presence of tet. When cells reached confluence, cells were cultured in the presence or absence of tet for another 6 days, followed by viral core DNA Southern blot analysis. (B) cccDNA in HepBHAe cell lines. cccDNA produced in the indicated cell lines was extracted by Hirt extraction and subjected to Southern blot hybridization (lanes 1, 4, 7, 10, 13). To further validate the authenticity of HBV cccDNA,

Hirt DNA samples were heated to 85°C for 5 min before gel loading, a condition that denatures DP-rcDNA into SS DNA, while the cccDNA stays undenatured and its electrophoretic mobility remains unchanged (lanes 2, 5, 8, 11, 14). The heat denatured DNA samples were further digested with EcoR I, by which the cccDNA was linearized to double-stranded DNA (lanes 3, 6, 9, 12, 15).

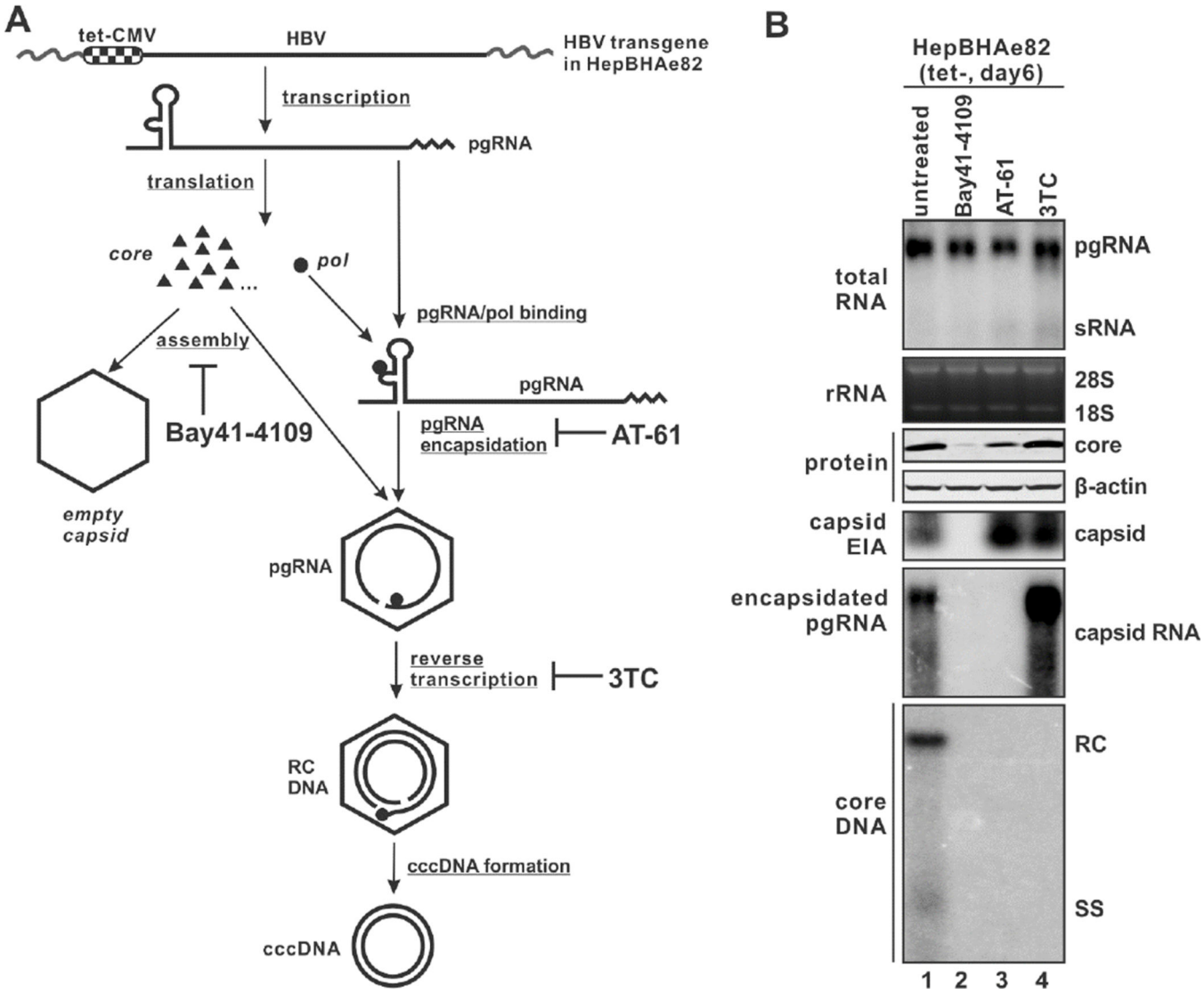

Figure 6. Assessment of antiviral drugs in HepBHAe82 cells

(A) Schematics of HBV replication steps and antiviral targets in HepBHAe82 cells. (B) Upon withdrawal of tet, HepBHAe82 cells were treated with Bay41-4109 (2 μM), AT-61 (25 μM), and 3TC (10 μM) for 6 days. The compound treatment was repeated every two days. Intracellular viral RNA, core protein, capsid particles, encapsidated pgRNA, and core DNA, were analyzed.

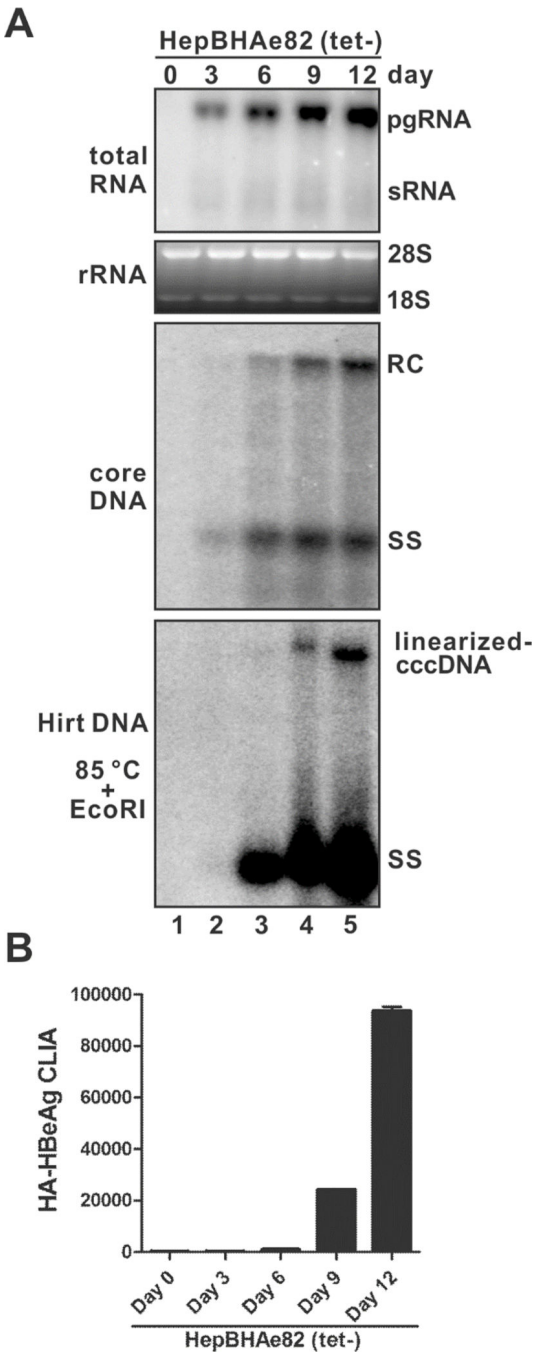

Figure 7. Kinetics of HBV replication and HA-HBeAg production in HepBHAe82 cells

(A) Time course of viral replication and cccDNA synthesis. HepBHAe82 cells cultured in 6-well-plates were harvested at indicated time points after tet withdrawal. Total viral RNA, core DNA, and cccDNA were extracted and analyzed by Northern and Southern blot hybridization, respectively. The cccDNA was heat denatured at 85°C for 5 min and then linearized by EcoR I. (B) Time course of HA-HBeAg secretion. The HA-HBeAg in supernatant samples collected from the experiment described in (A) was detected by CLIA.

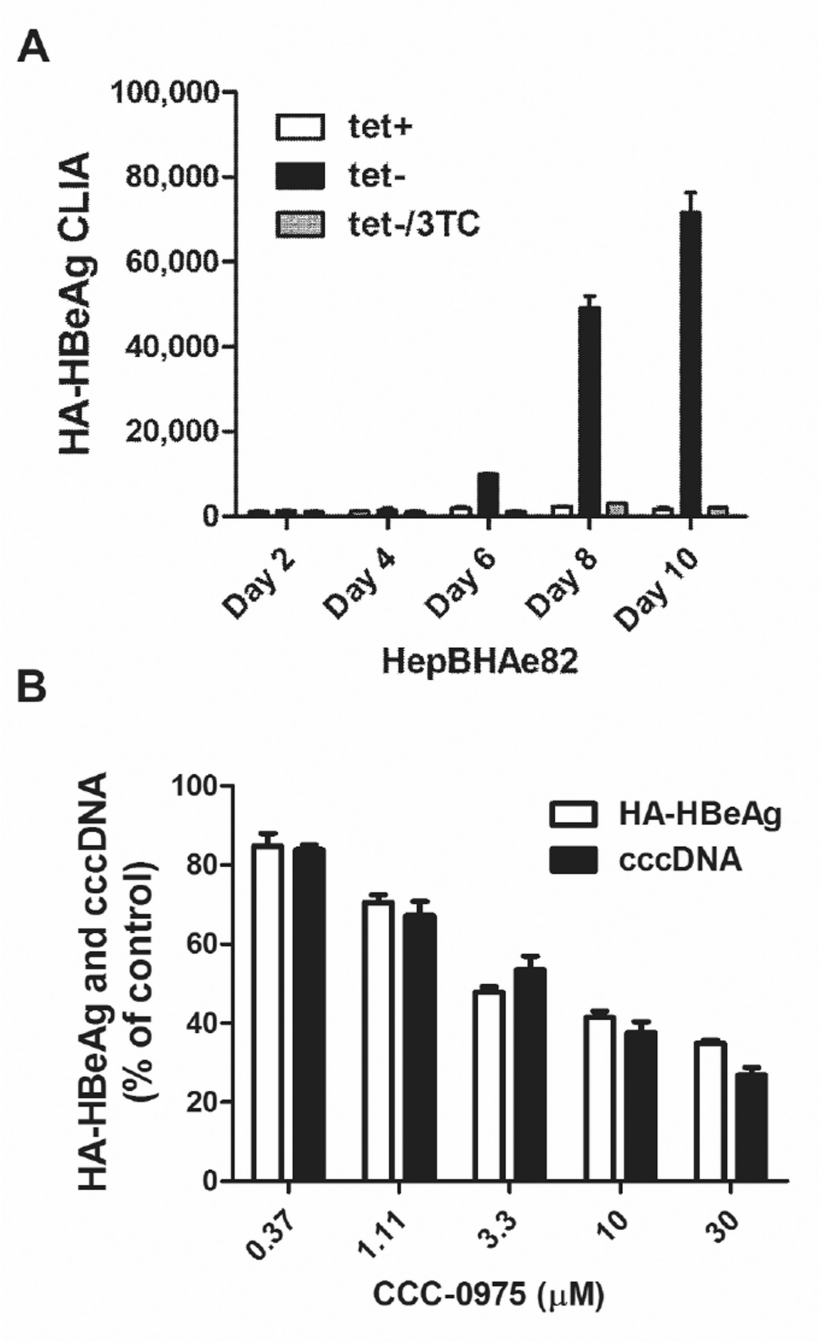

Figure 8. Validation of cccDNA-dependent HA-HBeAg production in HepBHAe82 cells

(A) cccDNA-dependent HA-HBeAg production. HepBHAe82 cells in 12-well-plate were cultured under the indicated conditions, treatment was repeated every other day and the harvested medium was subjected to HA-HBeAg CLIA assay. The CLIA signal readouts were plotted as mean ± SD (n=3). (B) CCC-0975 treatment reduced the production of cccDNA and HA-HBeAg in HepBHAe82 cells. Cells were seeded in a 12-well-plate with tet-free medium to induce HBV replication and cccDNA formation; simultaneously, cells were treated with vehicle (0.1% DMSO) or CCC-0975 at indicated concentrations.

Treatment was repeated every other day for 14 days. Supernatant and cells collected at the treatment end point were analyzed by HA-HBeAg CLIA and cccDNA qPCR, respectively. The HA-HBeAg and cccDNA signals under CCC-0975 treatment were plotted as the percentage of DMSO treatment control (mean ± SD, n=3).

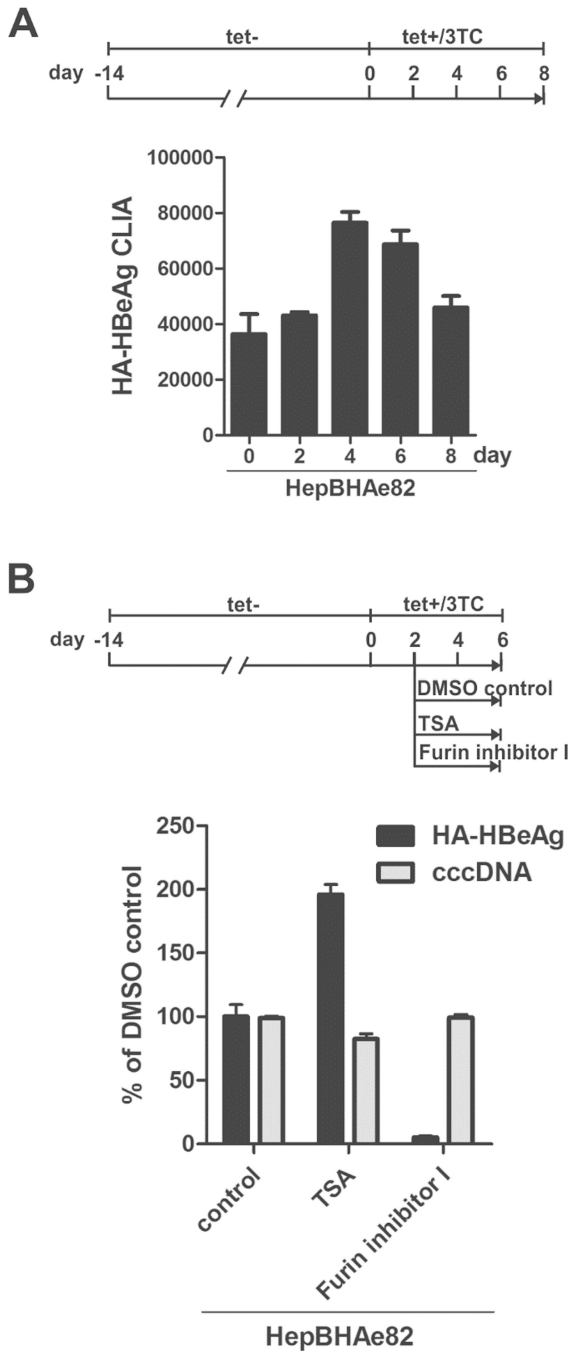

Figure 9. Development of a condition for screening compounds that target cccDNA stability or transcriptional activity

(A) Kinetics of HA-HBeAg expression from the established cccDNA pool. HepBHAe82 cells in 96-well-plate were cultured in tet-free medium for 14 days to accumulate cccDNA, then the cells were treated with tet plus 3TC (10 μM), and medium was harvested every other day and analyzed by HA-HBeAg CLIA. (B) Susceptibility of cccDNA-dependent transcription and HA-HBeAg secretion to compound treatment. HepBHAe82 cells harboring established cccDNA were cultured in medium containing tet and 3TC for 2 days, and then treated with solvent DMSO (0.1%), or furin inhibitor I (1 μM), or TSA (1 μM), tet and 3TC

remained in presence during the treatment. The culture medium was analyzed by HA-HBeAg CLIA after 4 days treatment.