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Formation of covalently closed circular DNA in Hep38.7-Tet cells, a tetracycline inducible hepatitis B virus expression cell line

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ABSTRACT

Hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) plays a central role in chronic HBV infection. However, analysis of the molecular mechanism of cccDNA formation is difficult because of the low efficiency in tissue cultured cells. In this study, we developed a more efficient cccDNA expression cell, Hep38.7-Tet, by subcloning from a tetracycline inducible HBV expression cell, HepAD38. Higher levels of cccDNA were produced in Hep38.7-Tet cells compared to HepAD38 cells. In Hep38.7-Tet cells, the cccDNA was detectable at six days after HBV induction. HBV e antigen (HBeAg) secretion was dependent upon cccDNA production. We screened chemical compounds using Hep38.7-Tet cells and HBeAg secretion as a marker. Most of the hit compounds have already been reported as anti-HBV compounds. These data suggested that Hep38.7-Tet cells will be powerful tools for analysis of the molecular mechanism of cccDNA formation/maintenance and development of novel therapeutic agents to control HBV infection.

1. Introduction

It is estimated that two billion people worldwide have been infected with hepatitis B virus (HBV) [1]. Chronic HBV infection is currently a major public health burden, affecting approximately 240 million individuals worldwide [2]. These patients have an elevated risk of chronic active hepatitis, cirrhosis or primary hepatocellular carcinoma [3–5].

Following HBV infection, the viral genomic relaxed circular DNA (rcDNA) is translocated into the cell nucleus and converted into episomal covalently closed circular DNA (cccDNA), which serves as a transcription template for viral mRNA. After transcription and nuclear export, cytoplasmic viral pregenomic RNA (pgRNA) is assembled by HBV polymerase and capsid proteins to form the nucleocapsid. Polymerase-catalyzed reverse transcription in the nucleocapsid yields minus-strand DNA, which is subsequently copied into plus-strand DNA to form the progeny rcDNA genome. Mature nucleocapsids are then either packaged with viral envelope proteins to egress as virion particles or shuttled back to the nucleus to amplify the cccDNA reservoir through the intracellular cccDNA amplification pathway [6–8].

Establishment of infection and viral persistence are both dependent on the formation of cccDNA during the HBV replication cycle [9–13]. The half-life of cccDNA is longer than other viral nucleic acids ranging from days to months in animal and tissue culture models [14–16]. Thus, there is an urgent need for the development of novel therapeutic agents that directly target cccDNA formation/maintenance. Formation of cccDNA in HepG2 cells transiently transfected with HBV genome is not efficient [17]. In HepAD38 cells, a tetracycline inducible HBV expression cell line, production of secreted HBV e antigen (HBeAg) is predominantly cccDNA dependent and thus might be useful as a surrogate marker of cccDNA formation [18–20]. To identify small molecules that inhibit cccDNA formation, we developed a more efficient cccDNA expression cell system. In the present study, we used Hep38.7-Tet cells subcloned from HepAD38 cells to investigate the levels of cccDNA formation, mRNA transcription, replication, viral particle secretion and HBeAg secretion.

2. Materials and methods

2.1. Cell culture

HepG2.2.15 [21] and HepG2.2.15.7 cells (unpublished data, M. Iwamoto and K. Watashi) were maintained in DMEM/F12 medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/mL Penicillin, 100 μg/mL Streptomycin,

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400 μg/mL Geneticin and 5 μg/mL Insulin. HepAD38 (a gift from Dr. Christoph Seeger at Fox Chase Cancer Center) and Hep38.7-Tet cells were maintained in the same way as HepG2.2.15 cells but with the addition of 400 ng/mL tetracycline.

The cells were seeded onto 60 mm dishes at a density of 8.0×10^5 cells/well with tetracycline-containing medium. After 24 h incubation, tetracycline was removed from the medium to induce HBV replication. The plate was incubated for 6 days. The tetracycline was then added back to the medium to prevent HBV pgRNA transcription from integrated DNA [22]. HBV replication, cccDNA accumulation, pre-core mRNA transcription and HBeAg secretion were only induced from cccDNA formed in the 6 days without tetracycline. The plate was incubated for another 6 days. The cells and culture medium were harvested at indicated time points.

2.2. Compound sources

Entecavir was purchased from Wako Pure Chemical Industries, Ltd. CCC-0975 was synthesized at Japan Tobacco Inc. FDA approved drug screening library (Selleck Chemicals, Houston, TX) which consisted of 414 compounds in total. Compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM.

2.3. Compound screening

Hep38.7-Tet cells were seeded into 96-well plates at a density of 3.0×10^4 cells/well with tetracycline-containing medium. After 24 h incubation, tetracycline was removed from the medium and then compound-containing medium was added to screening plates at a final concentration of 10 μM in 0.1% DMSO. Screening plates were incubated for 6 days. Tetracycline was then added back to the medium and incubation continued for another 6 days. The activity of compounds was evaluated by measurement of secreted HBeAg in the medium. The cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Japan).

2.4. Nucleic acid analysis

Intracellular core DNA was extracted as described previously [23,24]. Fifteen micrograms of extracted DNA was resolved by electrophoresis with a 1.2% agarose gel and transferred onto Hybond-XL membrane (GE Healthcare, Piscataway, NJ) in 20× SSC buffer. Total cellular RNA was extracted with TRIzol reagents (Life Technologies). Ten micrograms of total RNA were resolved in a 1.2% agarose gel containing 2.2 M formaldehyde and transferred onto Hybond-XL membrane. Extraction of cccDNA was carried out using a modified Hirt extraction procedure [25–28]. DNA (15 μg) was resolved and separated in a 1.2% agarose gel and transferred onto Hybond-XL membrane. For the detection of viral DNA and RNA, membranes were probed with full-length HBV DNA labeled with AlkPhos direct labeling reagents (GE Healthcare). After incubation with hybridization buffer for 6 h at 65 °C, the membrane was quantified by digital imaging with a LAS-4000 (GE Healthcare).

2.5. Viral particle assay

Viral particles (including virions, subviral particles and nucleocapsids) were extracted as described previously [29]. Viral particles in culture medium were precipitated by adding PEG8000 to a final concentration of 10% and incubated on ice for 1 h, followed by centrifugation at 8000 rpm at 4 °C for 10 min. Pellets were dissolved in TNE buffer.

2.6. Real-time PCR assay

Viral DNA was quantified using EXPRESS SYBR GreenER qPCR Supermix (Life Technologies). Core DNA and DNA containing particles selective primers were 5′-CTCGTGGTGGACTTCTCTC-3′ (Forward) and 5′-AAGATGAGGCATAGCAGCA-3′ (Reverse). Primers selective for cccDNA were 5′-CGTCTGTGCCTTCTCATCTGC-3′ (Forward) and 5′-GCACAGCTTGGAGGCTTGAA-3′ (Reverse). The cycling parameters were as follows: 50 °C for 2 min, 95 °C for 2 min, then 45 cycles of 95 °C for 15 s and 60 °C for 1 min with an Applied Biosystems 7500 sequence detection system (Life Technologies). The HBV plasmid was diluted over a range of 10^7–10^2 copies and used as a standard.

2.7. Real-time reverse transcription-PCR assay

Total RNA was extracted as described previously [20]. Five micrograms of total RNA extracted with TRIzol reagents were digested with 5 units RQ1 RNase-free DNase (Promega) and further purified with RNeasy mini kit (QIAGEN, Hilden, Germany). Synthesis of cDNA was from 1 μL purified total RNA using SuperScript III First-Strand Synthesis System (Life Technologies), based on the manufacturer's instruction. The selective primers used to transcribe cDNA from HBV RNA were 5′-GACCACCAAATGCCCCTATC-3′ (Forward) and 5′-GATTGAGATCTTCTGCGACGC-3′ (Reverse). The cycling parameters as described above.

2.8. Reverse transcription-PCR assay

The cDNA transcribed from pre-core RNA were quantified using PrimeSTAR Max DNA Polymerase (Takara, Japan). The primers were 5′-TAGGCATAAATTGGTCTG-3′ (Forward) and 5′-GATTGAGATCTTCTGCGACGC-3′ (Reverse). The cycling parameters were as follows: 94 °C for 1 min, then 45 cycles of 98 °C for 10 s, 55 °C for 5 s and 72 °C for 1 min. DNA was resolved and separated in a 1% agarose gel.

2.9. Indirect immunofluorescence analysis

Indirect immunofluorescence analysis was performed essentially as described previously [30,31]. Briefly, after fixation with 4% paraformaldehyde and permeabilization with 0.3% Triton-X-100, an anti-HBV core antibody (DAKO) was used as the primary antibody.

3. ELISA

The level of HBeAg in culture medium was measured using the HBe monoclonal ELISA kit (SIEMENS, Munich, Germany) according to the manufacturer's instructions.

4. Results

4.1. Intracellular cccDNA formation in HBV expression cell lines

We subcloned from HepAD38 cells and selected Hep38.7-Tet cells that showed the highest replication levels among the established subclones (unpublished data, M. Iwamoto and K. Watashi). We also subcloned HepG2.2.15.7 cells from HepG2.2.15 cells (unpublished data, M. Iwamoto and K. Watashi). To validate cccDNA levels in HBV expression cell lines, we compared cccDNA formation in tetracycline inducible Hep38.7-Tet, HepAD38 cells and non-inducible HepG2.2.15, HepG2.2.15.7 cells. As shown in Fig. 1, cccDNA could be detected after day 6 in all four cell lines. Hep38.7-Tet cells showed the highest levels of cccDNA

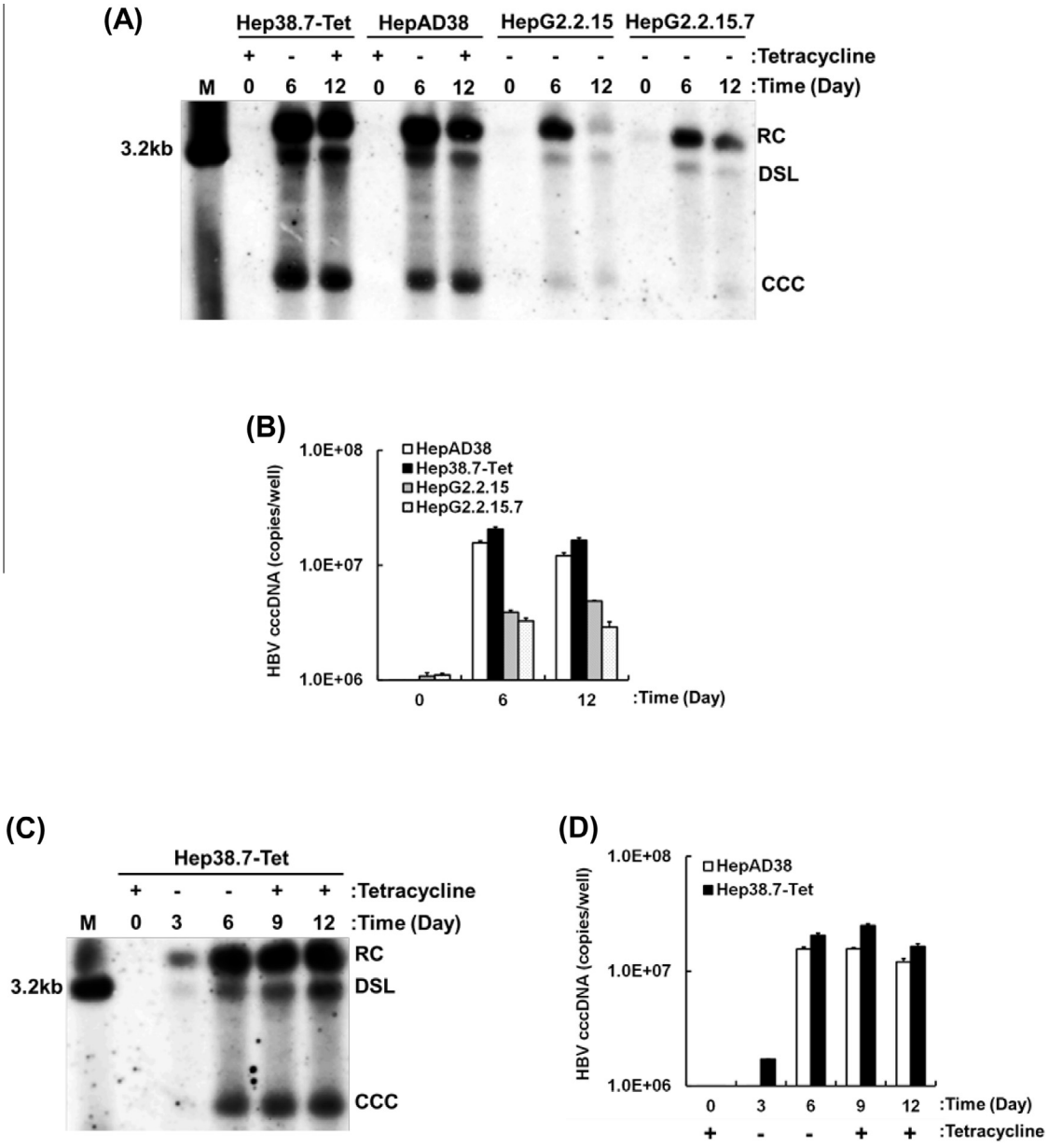

Fig. 1. Intracellular HBV cccDNA formation in Hep38.7-Tet cells. Hep38.7-Tet, HepAD38, HepG2.2.15 and HepG2.2.15.7 cells were harvested on days 0, 6 and 12. HBV cccDNA was extracted from the cells and analyzed by Southern blot (A) and Real-time PCR assay (B). Hep38.7-Tet and HepAD38 cells were harvested on days 0, 3, 6, 9 and 12. HBV cccDNA was extracted from the cells and analyzed by Southern blot (C) and Real-time PCR assay (D). Full length HBV DNA (3.2 kb) served as a control (lane M). The positions of relaxed circular DNA (RC), double stranded linear DNA (DSL) and covalently closed circular DNA (CCC) are indicated.

accumulation among the cell lines tested by Southern blot analysis and Real-time PCR assay (Fig. 1A and B). Higher levels of cccDNA were formed in tetracycline inducible cell lines compared to non-inducible cell lines.

4.2. Kinetics of intracellular cccDNA formation

We further investigated the kinetics of cccDNA levels after HBV induction in Hep38.7-Tet cells. The cells were seeded into 60 mm dishes with tetracycline-containing medium. After 24 h incubation, tetracycline was removed from the medium and the cells were cultured for 6 days. The tetracycline was then added back to the medium and incubation was continued for another 6 days. Southern blotting indicated that HBV cccDNA could be detected at day 6 and slowly increased from day 6 to day 12 (Fig. 1C). The accumulation of cccDNA in Hep38.7-Tet cells was higher than in HepAD38 cells at all timepoints examined (Fig. 1D). We calculated the copy number of cccDNA in Hep38.7-Tet cells from the results of the Real-time PCR assay. At day 3 cccDNA was present at 2 copies per cell and increased to 16 copies per cell at day 12. Liver biopsies have been shown to contain 1–50 copies of cccDNA per cell by Real-time PCR assay [32–35]. The copy number of cccDNA in Hep38.7-Tet cells was similar to that observed in HBV infected human hepatocytes.

4.3. Kinetics of intracellular HBV DNA synthesis

To determine HBV DNA replication in Hep38.7-Tet cells, we analyzed intracellular core DNA synthesis. As shown in Fig. 2, HBV core DNA could be detected by Southern blot analysis at day 3 and increased until day 12 (Fig. 2A). Using a Real-time PCR assay, core DNA synthesis in Hep38.7-Tet cells was found to be 2–4 times higher than in HepAD38 cells under similar conditions (Fig. 2B).

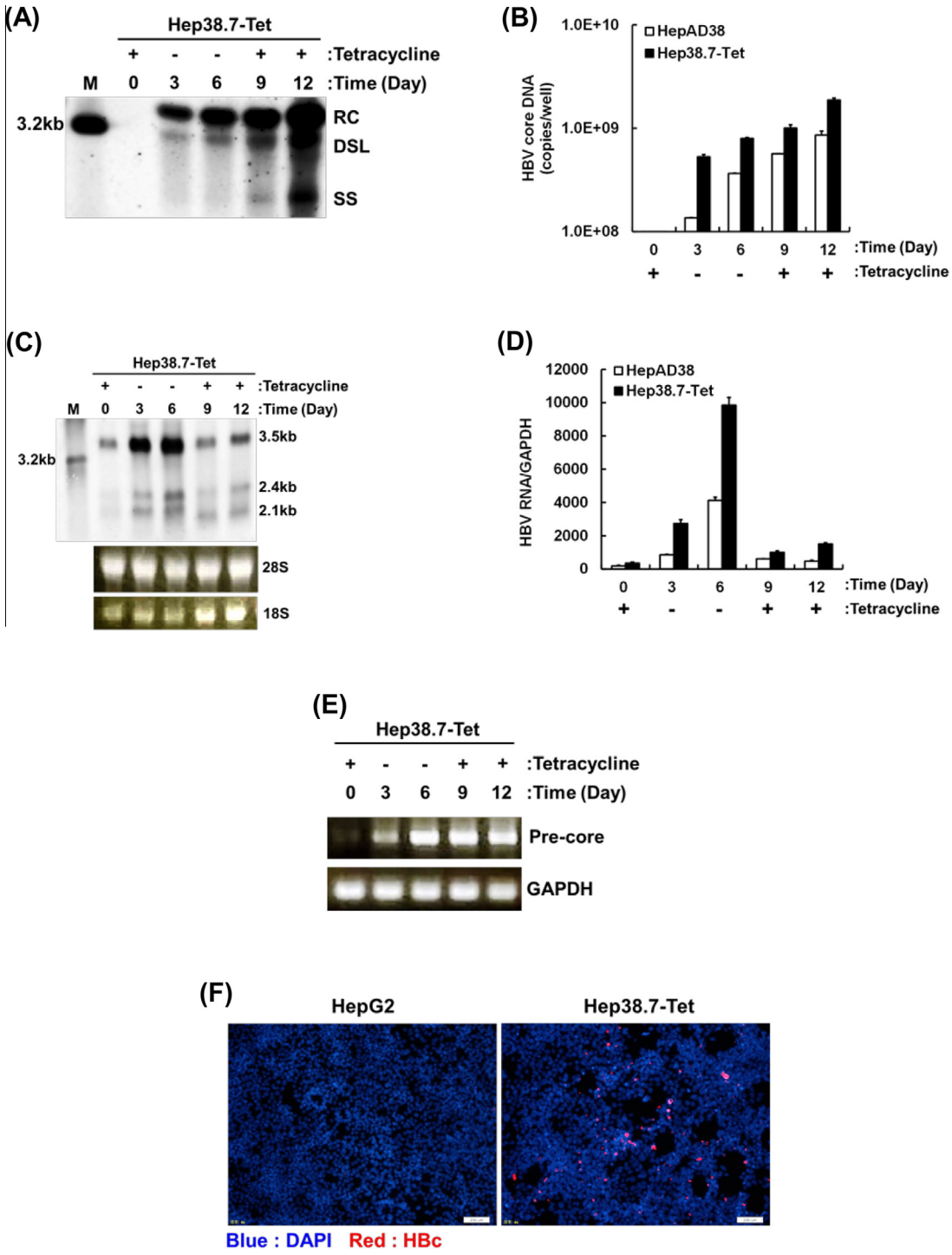

Fig. 2. Intracellular HBV core DNA synthesis, HBV mRNA transcription and HBc protein expression in Hep38.7-Tet cells. Hep38.7-Tet and HepAD38 cells were harvested on days 0, 3, 6, 9 and 12. HBV core DNA was extracted from the cells and analyzed by Southern blot (A) and Real-time PCR assay (B). Full length HBV DNA (3.2 kb) served as a control (lane M). The positions of relaxed circular DNA (RC), double stranded linear DNA (DSL) and single stranded DNA (SS) are indicated. Total cellular RNA was extracted and HBV RNA was detected by Northern blot (C) and Real-time reverse transcription-PCR assay (D). rRNA (28S and 18S) served as a loading control. The positions of HBV pgRNA (3.5 kb) and surface mRNAs (2.4 kb and 2.1 kb) are indicated. Pre-core mRNA was detected by Reverse transcription-PCR assay (E). GAPDH served as a loading control. By indirect immunofluorescence analysis, Hep38.7-Tet and HepG2 cells were stained for HBcAg on day 12 (F).

4.4. Pre-core mRNA transcription is cccDNA dependent

We next evaluated the synthesis of HBV RNA in Hep38.7-Tet cells. Northern blotting analysis showed that 3.5 kb HBV mRNA transcription increased until day 6 and then decreased following the addition of tetracycline (Fig. 2C). Using a Real-time reverse transcription-PCR assay, the transcription of HBV mRNA in Hep38.7-Tet cells was 2–3 times higher than in HepAD38 cells (Fig. 2D). These results implied that mRNA transcription, core DNA synthesis and cccDNA formation sequentially occurred during the HBV replication cycle in Hep38.7-Tet cells.

Because pre-core mRNA is only 35 nt longer than pgRNA, it is difficult to analyze by Northern blot and we therefore designed a pre-core mRNA specific reverse transcription-PCR assay. Using the same RNA samples shown in Fig. 2C, we found that the predicted reverse transcription-PCR product from pre-core mRNA

could be detected at day 3 and increased in parallel with cccDNA in the following 6 days (Fig. 2E). After the addition of tetracycline back into the medium, viral pgRNA and envelope mRNA, but not pre-core mRNA transcription declined from integrated viral DNA. This result suggested that pre-core mRNA was transcribed from cccDNA but not integrated viral DNA.

4.5. Intracellular HBc protein expression

To analyze the intracellular HBV protein expression, we evaluated HBc protein in the cell using a specific HBc antibody. The intracellular HBc protein was detected in Hep38.7-Tet cells but not HepG2 cells by indirect immunofluorescence analysis (Fig. 2F).

4.6. Kinetics of viral particle formation

To analyze viral particle formation, we evaluated the extracellular viral particles including virions and naked capsids. As shown in Fig. 3, extracellular HBV DNA in Hep38.7-Tet cells was higher than in HepAD38 cells indicating that virions and nucleocapsids were secreted into the medium (Fig. 3A).

4.7. Correlation of HBeAg secretion and cccDNA formation

To explore the possibility that HBeAg could serve as a reporter for formation of cccDNA, we measured the levels of its secretion in Hep38.7-Tet cells. ELISA results indicated that the levels of HBeAg increased until day 12. HBeAg secretion in Hep38.7-Tet cells was approximately twice that in HepAD38 cells (Fig. 3B). There was a good correlation between cccDNA formation and HBeAg secretion (Fig. 1C).

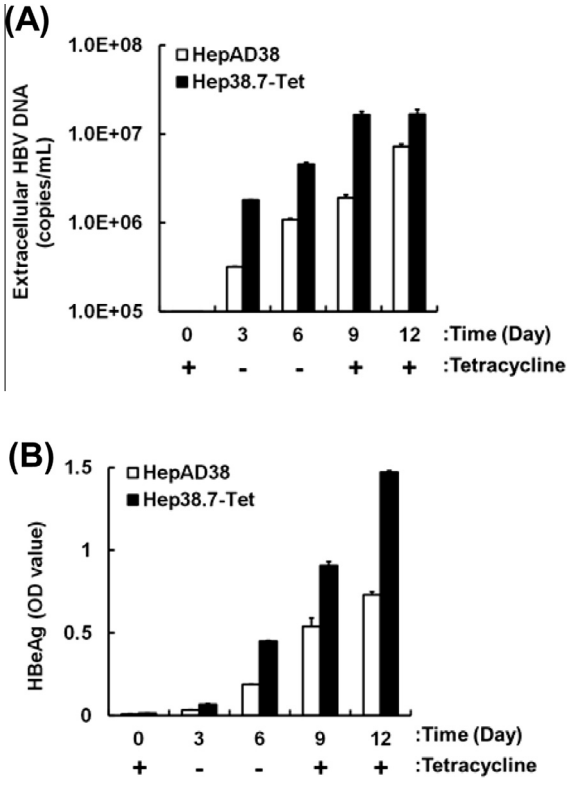

Fig. 3. Extracellular HBV DNA and HBeAg secretion in Hep38.7-Tet cells. Culture media of Hep38.7-Tet and HepAD38 cells were collected on days 0, 3, 6, 9 and 12. HBV particles (including virions and naked capsids) were precipitated by adding PEG8000 from the medium. HBV DNA was extracted from the HBV particles and analyzed by qPCR assay (A). HBeAg in culture medium was determined by ELISA (B).

4.8. Identification of anti-HBV compounds from Hep38.7-Tet cell-based assay

We evaluated the inhibitory activities of anti-HBV compounds, reverse transcriptase inhibitor (Entecavir) and cccDNA formation inhibitor (CCC-0975). Hep38.7-Tet cells were seeded into 96-well plates with tetracycline-containing medium. After 24 h incubation, tetracycline was removed from medium and then compound-containing medium was added to the screening plates. Screening plates were incubated for 6 days. Tetracycline was then added back to the medium and incubation continued for another 6 days. The activity of compounds was evaluated by measurement of secreted HBeAg in the medium at day 12. As shown in Fig. 4, these compounds had dose dependent inhibitions against cccDNA formation and showed similar inhibitory activities as previous reports [36,37]. The inhibition of cccDNA was proportional to the HBeAg reduction (Fig. 4A). Southern blot analysis showed that these compounds caused a dose dependent reduction of cccDNA (Fig. 4B). These results support the use of Hep38.7-Tet cells for screening to identify compounds that affect the HBV life cycle, including cccDNA formation.

Next, to identify compounds that affect cccDNA formation and maintenance, we screened chemical compound library at a final concentration of 10 μM. As shown in Table 1, CCC-0975 caused a 78% inhibition of HBeAg levels compared with control. Twelve compounds caused more than 50% inhibition of HBeAg levels without cytotoxicity for primary hits. These compounds included reverse transcriptase inhibitors, HMG-CoA reductase inhibitor, a steroid hormone, immunosuppressant agents and tetracycline.

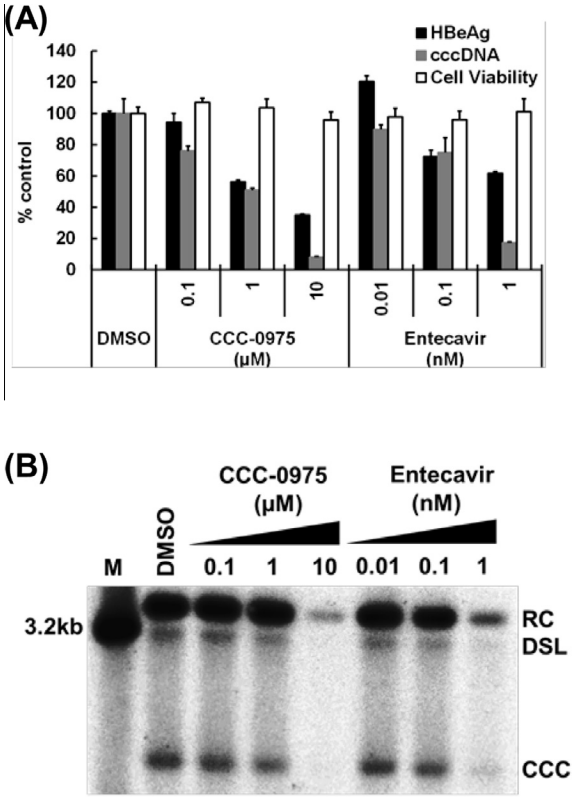

Fig. 4. Inhibitory activities of anti-HBV inhibitors in Hep38.7-Tet cells. Cells and culture medium were collected on day 12. HBV cccDNA was extracted from the cells and analyzed by Real-Time PCR assay. HBeAg in culture medium was determined by ELISA (A). HBV cccDNA was extracted from the cells and analyzed by Southern blotting (B). Full length HBV DNA (3.2 kb) served as a control (lane M). The positions of relaxed circular DNA (RC), double stranded linear DNA (DSL) and covalently closed circular DNA (CCC) are indicated.

Table 1
Antiviral activities of hit compounds.

Compound		HBeAg inhibition (% control)
Telbivudine	Reverse transcriptase inhibitor	24
Entecavir	Reverse transcriptase inhibitor	26
Tenofovir	Reverse transcriptase inhibitor	23
Emtricitabine	Reverse transcriptase inhibitor	29
Zalcitabine	Reverse transcriptase inhibitor	32
Nelarabine	Reverse transcriptase inhibitor	28
Pitavastatin	HMG-CoA reductase inhibitor	41
Progesterone	Steroid hormone	31
Mycophenolic	Immunosuppressant agent	32
Leflunomide	Immunosuppressant agent	24
Oxytetracycline	Tetracycline antibiotic	12
Methacycline	Tetracycline antibiotic	11
CCC-0975	cccDNA formation Inhibitor	22

Tenofovir and Entecavir are clinically used for HBV treatment [38]. Immunosuppressant agents have been reported to have anti-HBV activity [39]. Tetracycline antibiotics stopped HBV induction. These results indicate that Hep38.7-Tet cells are a suitable system to identify potential therapeutic agents.

5. Discussion

We subclones Hep38.7-Tet cells from HepAD38 cells which is a HepG2 derived cell line supporting tetracycline inducible HBV replication. Upon tetracycline withdrawal, the transcribed pgRNA will express viral core protein and polymerase and initiate reverse transcription to generate rcDNA. The start codon of the C-terminally truncated pre-core open reading frame (ORF) at the 3′ end of the pgRNA is copied into the viral DNA sequence and the pre-core ORF is restored during rcDNA conversion into cccDNA. Thus, the authentic pre-core mRNA will be transcribed only from cccDNA, with the translated pre-core protein being further processed into HBeAg, which is secreted into the culture medium and serves as a marker for cccDNA formation.

Hep38.7-Tet cells exhibited higher levels of HBV mRNA transcription, replication, cccDNA formation, virion secretion and HBeAg secretion than parental HepAD38 cells. These results may be due to the earlier transcription initiation of HBV pgRNA from integrated HBV after removal of tetracycline in Hep38.7-Tet cells than that in HepAD38 cells. We also confirmed that pre-core mRNA transcription was dependent on cccDNA formation and HBeAg secretion was quantitatively correlated with cccDNA formation. Moreover, the secreted HBeAg levels were sufficient to discover antiviral compounds in Hep38.7-Tet cells. In fact, we found some hits from the small-molecular compound library that significantly reduced the HBeAg levels. Many of these hit compounds had been identified as anti-HBV compounds previously. In this cell-based assay, any compounds that inhibit viral gene transcription, translation, HBeAg post-translational processing and secretion would be selected as positive hits. For example, CCC-0975 was discovered as an inhibitor of cccDNA production from a cccDNA-dependent HBeAg-producing cell line, HepDE19 [37]. This compound reduced the HBeAg levels in primary screening and reduced the levels of cccDNA and its putative precursor, deproteinized relaxed circular DNA (DP-rcDNA) in further mechanistic studies. Therefore, it is essential to evaluate the intracellular cccDNA levels in order to find a cccDNA inhibitor. Nevertheless, Hep38.7-Tet cells serve as a high throughput cell-based assay to identify cccDNA formation inhibitors. In conclusion, Hep38.7-Tet cells will be a powerful tool to analyze the molecular mechanism of HBV cccDNA formation and will facilitate the development of novel therapeutic agents for HBV infection.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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