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Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays

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Abstract

Currently available antiviral nucleoside analogs for the treatment of chronic hepatitis B virus (HBV) infections profoundly reduce virus load, but rarely cure the virus infection. This is due, at least in part, to their failure to eliminate viral covalently closed circular (ccc) DNA from the nuclei of infected hepatocytes. To screen compound libraries for antiviral drugs targeting cccDNA, we set out to develop a cell-based assay suitable for high throughput screening. Since cccDNA is time-consuming to assay, it was desirable to use a viral gene product that could serve as a reporter for intracellular cccDNA level. We predicted that the secretion of HBV e antigen (HBeAg) by HepAD38 cells, a tetracycline inducible HBV expression cell line, would be cccDNA-dependent. This is because a large portion of pre-core mRNA leader sequence in the 5′ terminus of integrated viral genome was deleted, preventing HBeAg expression from transgene, but could be restored from the 3' terminal redundancy of pre-genomic RNA during viral DNA replication and subsequent cccDNA formation. Our experimental results showed that following induction, HepAD38 produced and accumulated cccDNA, which became detectable between 7 and 8 days. HBeAg synthesis and secretion into culture fluid were dependent upon and proportional to the level of cccDNA detected. Therefore, the secretion of HBeAg by HepAD38 cells could potentially serve as a convenient reporter for the high throughput screening of novel antiviral drugs targeting HBV cccDNA.

Keywords: Hepatitis B virus; cccDNA; Antiviral assay

Despite the fact that most adulthood HBV infections are transient, approximately 10% of infected adults and over 90% of infected neonates fail to mount a sufficient immune response to clear the virus and develop a life-long chronic infection which could progress to chronic active hepatitis, cirrhosis and primary hepatocellular carcinoma (McMahon, 2005). It is estimated that there are more than 400 million chronic HBV carriers worldwide (Lee, 1997). Currently available antiviral medications for the management of chronic HBV infections include alpha interferon (IFN-α) and three nucleoside analogs (lamivudine, adefovir and entecavir) that inhibit viral nucleocapsid formation and block viral DNA synthesis by premature chain termination, respectively (Perrillo, 2005; Wieland et al., 2005). Despite the potent inhibition of viral replication, even prolonged nucleoside analogue treatments rarely cure HBV infection and virological relapse is common, following the discontinuation of therapy (Lai et al., 2002; Liaw et al., 2000; Marcellin et al., 2003). In addition, the development of drug-resistance to the polymerase inhibitors by the virus will most likely limit their long-term efficacy (Locarnini, 2005). IFN-α has been used to treat HBeAg-positive chronic hepatitis B and its therapeutic efficacy has been improved by the recent introduction of pegylated interferon, but 48-week treatment of peginterferon alpha 2a only results in HBV e antigen (HBeAg) seroconversion and reduction of viral load in 30–40% of patients (Janssen et al., 2005; Lau et al., 2005). It is therefore a public health priority to develop novel therapeutic agents to cure chronic HBV infection and prevent its severe clinical sequelae.

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HBV is a small DNA virus that contains a relaxed circular (rc) partially double stranded DNA genome(Summers et al., 1975). Unlike other mammalian DNA viruses, HBV replicates via reverse transcription of its pre-genomic (pg) RNA (Summers and Mason, 1982). In marked contrast to retroviruses, HBV genomic DNA integration into host cellular chromosomes is not an essential step in its life cycle. Instead, upon infection, incoming viral rcDNA is transported into the nucleus and converted into episomal covalently closed circular (ccc) DNA, which serves as the template for the transcription of viral RNAs. The viral pgRNA is translated to produce both the core protein and the reverse transcriptase (RT) (Summers and Mason, 1982). The RT protein binds to the epsilon sequence within the pgRNA to prime viral DNA synthesis and initiate nucleocapsid assembly (Wang and Seeger, 1992; Wang and Seeger, 1993). Subsequently the viral polymerase converts the pgRNA into rcDNA. The nucleocapsids mature as rcDNA is formed and can either be enveloped and secreted out of cells or deliver its rcDNA into the nucleus to amplify nuclear cccDNA (Ganem and Varmus, 1987; Seeger and Mason, 2000; Wu et al., 1990). HBV infections are maintained by the presence of a small and regulated number of cccDNA in the nuclei of infected cells (Summers et al., 1990; Tuttleman et al., 1986). The copy number of cccDNA in a hepatocyte infected by duck hepatitis B virus (DHBV) ranges from 1 to 50 (Zhang et al., 2003). In spite of potent inhibition of viral DNA synthesis, the current viral RT inhibitor-based antiviral therapy has little direct effect on cccDNA. Long-term therapy of adefovir or entecavir mediates significant reduction in cccDNA, but still fails to eliminate chronic HBV infections (Sung et al., 2005; Zoulim, 2004).

It is therefore conceivable a therapy that inhibits cccDNA formation and/or eliminates established cccDNA from infected hepatocytes would be an important compliment to the current management of the infection. Based on the current knowledge, the formation of cccDNA requires capsid disassembly, import of viral DNA into nucleus and conversion of rcDNA to cccDNA (Seeger and Mason, 2000). The molecular details for all those steps are still elusive, but many viral and cellular proteins should be involved and could serve as potential targets for therapeutic intervention. Once cccDNA is made, it has been shown to be stably maintained in non-dividing cells (Moraleda et al., 1997). The previous assumption is that cccDNA can only be eliminated due to the killing of infected cells by cytotoxic T lymphocytes or be diluted out by cell division (Guo et al., 2000; Summers et al., 2003; Zhang et al., 2003). However, recent studies indicated that cccDNA might be subject to turnover, and interferon and/or other cytokines induced antiviral programs might be able to noncytolytically purge cccDNA from infected hepatocytes (Anderson et al., 2005; Wieland et al., 2004). Thus, therapeutic activation of this intracellular innate immune response by small molecules might promote the decay of cccDNA.

To find small molecules that could eliminate cccDNA from infected cells, we developed a cell-based assay suitable for high throughput screening. In particularly, we found in this study that the secretion of HBeAg by HepAD38 cells (Ladner et al., 1997), a tetracycline inducible HBV expression cell line, is cccDNA-dependent. The level of HBeAg in culture fluid could serve as a convenient reporter for the amount of intracellular cccDNA.

1. Materials and methods

1.1. Cell culture

HepAD38 cell (a gift from Dr. Christoph Seeger, Fox Chase Cancer Center) was cultured in 60 mm dishes with DMEM/F12 medium (invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Ladner et al., 1997). Where needed, tetracycline was routinely added at 1 μg/ml to suppress HBV pgRNA transcription. Lamivudine was obtained from Glaxo Smith Kline. IFN-α was obtained from PBL, Inc. Cells and culture media were harvested at indicated time points. Media were clarified by centrifugation at 1000 × *g* for 10 min and cells were washed with cold PBS and stored at −70 °C.

1.2. Nucleic acid analysis

Intracellular viral core DNA was extracted as described previously (Guo et al., 2005). Briefly, cells from one 60 mm dish were lysed with 1 ml of lysis buffer containing 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% NP40 and 8% sucrose at 37 °C for 10 min. Cell debris and nuclei were removed by centrifugation and the supernatant was mixed with 250 μl of 35% PEG-8000 containing 1.5 M NaCl. After 1 h incubation in ice, viral nucleocapsids were pelleted by centrifugation at 12,000 × *g* for 10 min at 4 °C, followed by 1 h digestion at 37 °C in 400 μl of digestion buffer containing 0.5 mg/ml pronase (Calbiochem), 0.5% SDS, 150 mM NaCl, 25 mM Tris–HCl (pH 8.0) and 10 mM EDTA. The digestion mixture was extracted twice with phenol and DNA was precipitated with ethanol, dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). One sixth of the DNA sample from each plate was resolved by electrophoresis into a 1.5% agarose gel. Gel was then subjected to denaturalization in a solution containing 0.5 M NaOH and 1.5 M NaCl, followed by neutralization in a buffer containing 1 M Tris–HCl (pH7.4) and 1.5 M NaCl. DNA was then blotted onto Hybond-XL membrane (GE Health care) in 20× SSC buffer.

Total cellular RNA was extracted with TRIzol reagents (invitrogen), by following the instructions of the manufacturer. Ten microgram of total RNA was resolved in 1.2% agarose gel containing 2.2 M formadelhyde and transferred onto Hybond-XL membrane in 20× SSC buffer.

cccDNA extraction was carried out by using a modified Hirt extraction procedure (Hirt, 1967). Briefly, cells from one 60 mm dish was lysed in 3 ml of 10 mM Tris–HCl (pH 7.5), 10 mM EDTA and 0.7% SDS. After 30 min incubation at room temperature, the lysate was transferred into a 15 ml tube followed by addition of 0.8 ml of 5 M NaCl and incubation at 4 °C overnight. The lysate was then clarified by centrifugation at 12,000 × *g* for 30 min at 4 °C and extracted twice with phenol and once with phenol:chloroform. DNA was precipitated with two volume of

ethanol overnight at room temperature and dissolved in 60 μl TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). One third of the cccDNA sample was then denatured at 85 °C for 5 min and separated in a 1.2% agarose gel and transferred onto Hybond-XL membrane.

For the detection of HBV DNA and RNA, membranes were probed with either an α-^{32}P-UTP (800 Ci/mmol, Perkin-Elmer) labeled minus or plus strand specific full-length HBV riboprobe. Hybridization was carried out in 5 ml EKONO hybridization buffer (Genotech) with 1 h pre-hybridization at 65 °C and overnight hybridization at 65 °C followed by a 1 h wash with 0.1× SSC and 0.1% SDS at 65 °C. The membrane was exposed to a phosphoimager screen and hybridization signals were quantified with Quantity One software (Bio-Rad).

1.3. RT-PCR detection of pre-core mRNA

Five microgram total RNA extracted with TRIzol reagents was digested with 5 units RQ1 RNase free DNase I (Promega) and further purified with RNAeasy mini-prep kit (Qiagen). One microgram purified total RNA was mixed with 100 ng Oligo(dT)$_{20}$, and cDNA was synthesized using SuperScript II (Invitrogen), based on the manufacturer's instruction. Primers used for specific amplification of pre-core RNA were 5′ AGGCATAAAT TCTGCGCAC3′ (corresponding to nt.1787–1808) and 5′ TCTCATTAACTG TGAGTGGGCCTA3′ (complementary to nt. 2618–2595). PCR conditions were set as 30 cycles of 20 s at 94 °C, 30 s at 57 °C and 1 min at 72 °C. The 832 bp PCR fragment was cloned into pGEM-T Easy vector (Promega) and sequenced by automatic DNA sequencer.

1.4. Viral particle assay

Viral particles (including virions, subviral particles and nucleocapsids) in culture medium were precipitated by adding polyethyleneglycol (PEG) 8000 to a final concentration of 10% and incubated on ice for 1 h and followed by centrifugation at 6000 × *g*, 4 °C, for 10 min. Pellets were dissolved in TNE buffer containing 10 mM Tris–HCl, pH8.0, 100 mM NaCl and 1 mM EDTA. Viral particles were resolved in a 1% agarose gel and transferred onto nitrocellulose membrane as described above. Viral DNA was detected by hybridization with a α-^{32}P-UTP (800 Ci/mmol, Perkin-Elmer) labeled minus strand specific full-length HBV riboprobe.

1.5. Elisa

Culture medium was diluted 1:50 in DMEM/F12 and the level of HBeAg was measured by using the EKB-PLUS ELISA kit (Diasorin) according to the manufacturer's direction.

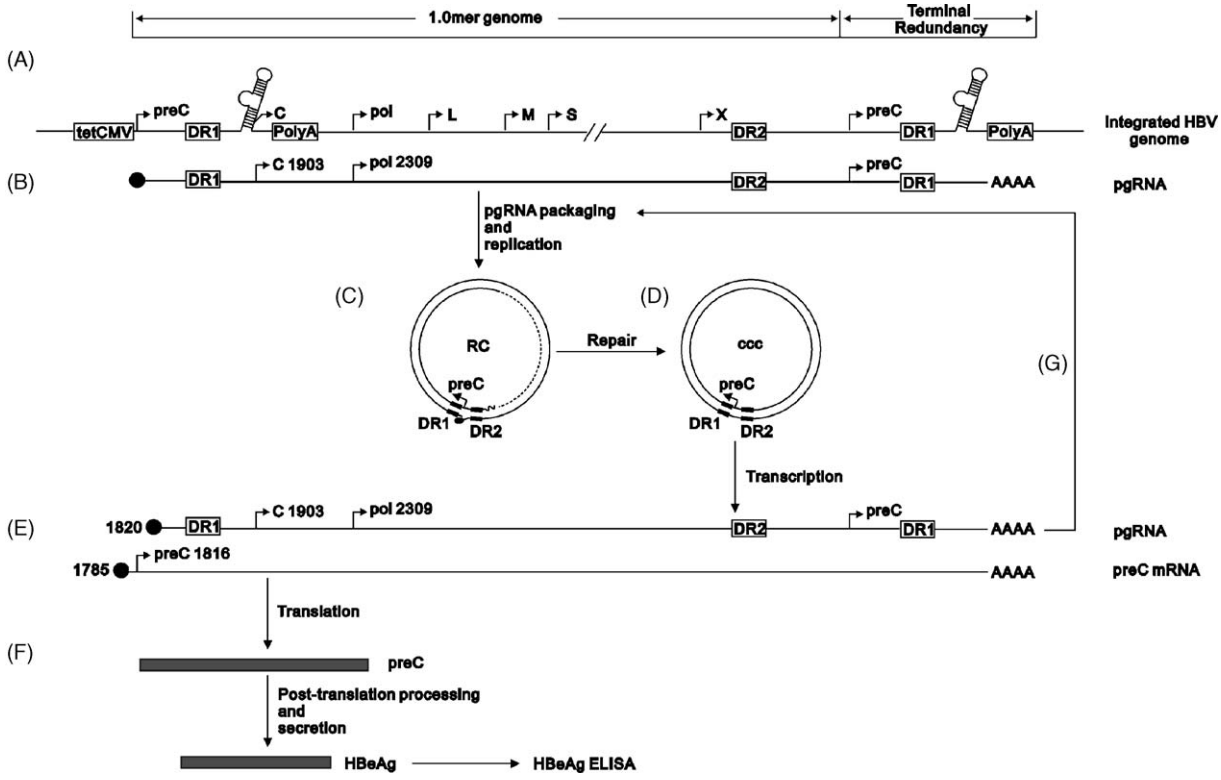

Fig. 1. Schematical representation of experimental strategies. The integrated HBV DNA in HepAD38 cells is composed of a 1.1 overlength HBV genome, beginning from nt 1807, placed downstream of a tetCMV promotor (A). Upon removal of tetracycline from the cultures, viral pgRNA transcription begins (B), followed by DNA replication (C) and cccDNA formation (D). The deleted pre-core mRNA leader sequence is restored during DNA replication and cccDNA formation. The transcription from a cccDNA template gives rise to both pre-core mRNA (E), which is translated into HBeAg (F), and pgRNA, which serves as templates for new-rounds viral DNA synthesis (G).

2. Results

2.1. Experimental strategies

HepAD38 is a stably transfected HepG2 cell line in which HBV replication occurs in a tetracycline inducible manner (Ladner et al., 1997). It contains a 1.1 unit length HBV genome under the control of a tetracycline regulatory element and minimum CMV promoter (tetCMV). Briefly, HBV sequences, beginning at the Fsp I site (nt 1807), were placed fifteen nucleotides (nt) downstream of the TATA box of the tetCMV promoter (Fig. 1). Transcription of HBV pgRNA is induced upon tetracycline removal from culture medium, leading to capsid assembly and DNA synthesis. A small portion of the de novo synthesized rcDNA is shuttled into the nucleus and converted to cccDNA, which, in turn, serves as a transcriptional template for viral mRNAs.

Using the tetCMV promoter, pgRNA would be synthesized which, unlike authentic pgRNA, would contain the AUG of the pre-core open reading frame (ORF). However, since this pgRNA would begin just 2 nt upstream of the pre-core start codon, we hypothesized that this RNA would not be able to translate HBeAg because of a very short leader sequence. Once cccDNA is formed, the missing leader sequence of pre-core mRNA would be restored from the 3′ terminal redundancy of pgRNA during viral DNA replication. Consequently both authentic pre-core mRNA and pgRNA could be transcribed under the control of viral pre-core/core promoter from the cccDNA template. We therefore predicted that HBeAg would be only produced from cccDNA, but not from the integrated viral genome. If this is the case, the amount of HBeAg in culture medium could serve as a reporter for intracellular cccDNA level. This would provide us a convenient cell-based assay system for high throughput screening of novel antiviral compounds targeting HBV cccDNA.

2.2. Kinetics of HBV DNA replication and cccDNA accumulation upon tetracycline removal

Previous work indicated that HBV cccDNA is not efficiently formed in HepG2 cells transiently transfected with HBV genome (Scaglioni et al., 1997). We therefore initially characterized the kinetics of HBV RNA transcription and DNA synthesis in HepAD38 cells upon tetracycline removal and determined if cccDNA could be detected. As shown in Fig. 2A, 3.5 kb HBV viral RNA transcripts were induced at 2 days after tetracycline removal and continued increasing over next 12 days (Fig. 2A). Consistent with previous reports, the transcription of 2.4 and 2.1 kb HBV envelope protein mRNAs also appeared to be induced upon tetracycline removal, indicating that the tetCMV enhancer could operate beyond a certain distance, since it is more than 500 nt away from the envelope gene transcriptional starting sites (Fig. 2A) (Guo et al., 2003).

HBV DNA replication intermediates (core DNA) became detectable 2 days after 3.5 kb viral RNA appearance and increased over the next 10 days (Fig. 2B) to reach approximately 2000 copies per cell. With an improved Hirt DNA extraction method (see Section 1), cccDNA could be detected at day 8 after

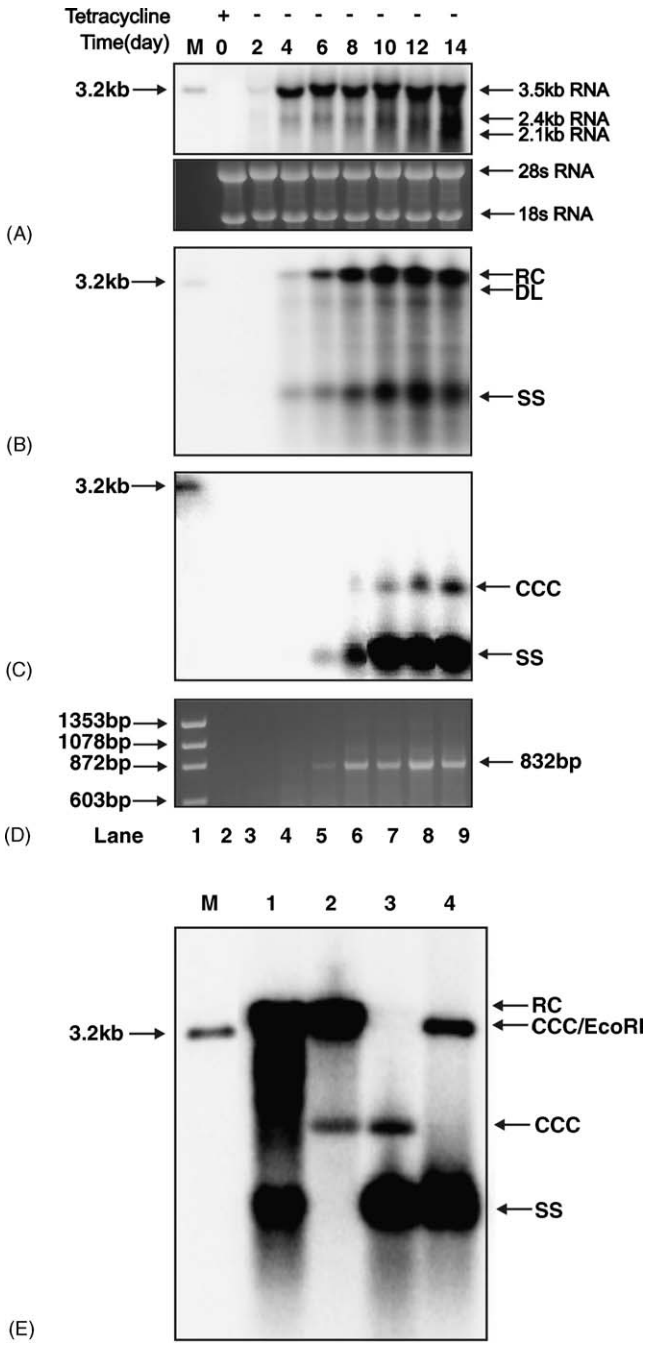

Fig. 2. Kinetics of HBV replication after tetracycline removal. HepAD38 cells were cultured in 60 mm dishes until confluent and tetracycline was then removed from culture medium to induce HBV replication. Cells were harvested at the indicated times before and after tetracycline removal. The levels of intracellular viral RNA (A), core DNA (B), cccDNA (C) and pre-core mRNA (D) were determined by Northern blot, Southern blot hybridization and RT PCR assay as described in materials and methods. (E) To confirm the identity of cccDNA, Hirt DNA preparations made from HepAD38 cells cultured in the absence of tetracycline for 8 days were separated on an agarose gel without treatment (lane 2), after denaturalization at 85 °C for 5 min (lane 3) and digestion with EcoRI after denaturalization at 85 °C for 5 min (lane 4). Core DNA prepared from same cultures (lane 1) and 50 pg of unit length HBV DNA (lane M) served as controls. RC, relaxed circular DNA. DL, double-stranded linear DNA. ccc, covalently closed circular DNA. ss, single-stranded DNA.

the removal of tetracycline and slowly increased from approximately 4 copies per cell at day 8 to 20 copies per cell at day 14 (Fig. 2C). The identity of the observed cccDNA was confirmed by heat denaturing and further EcoRI digestion, which converted cccDNA into a unit-length linear DNA (Fig. 2E).

In summary, we observed that upon the removal of tetracycline, HBV mRNA transcription, core DNA synthesis and cccDNA formation sequentially occurred in HepAD38 cells, which is consistent with the current model of HBV replication cycle. The copy numbers of cccDNA in HepAD38 cells are at levels similar to those observed in HBV infected human hepatocytes (Maynard et al., 2005; Werle-Lapostolle et al., 2004).

2.3. Transcription of pre-core mRNA is cccDNA-dependent

Because pre-core mRNA is only 35 nt longer than pgRNA, it is difficult to distinguish them by Northern blot hybridization. To test our hypothesis that, in these cells, pre-core mRNA could only be transcribed from cccDNA template, but not the integrated genome, we designed a pre-core mRNA specific RT-PCR assay. Briefly, one PCR primer was designed to anneal with the 5′ sequence of pre-core mRNA (nt.1787–1808) and a second, the downstream, primer to a sequence (nt. 2618–2595) common to both pre-core and pgRNA. Therefore, an 832 bp long DNA fragment could only be amplified from pre-core mRNA.

Using the same RNA samples from the kinetics studies shown in Fig. 2A, we found that the predicted RT PCR products from pre-core mRNA could be detected at day 6 after tetracycline removal and increased in parallel with cccDNA in following 8 days (Fig. 2D). PCR performed with the RNA samples without reverse transcription did not yield any amplified product, indicating that the observed RT PCR products were amplified from pre-core mRNA, not from possible viral DNA contamination. The RT-PCR products were cloned into pGEM-T Easy vector and authentic sequences were confirmed by sequence analysis. Using the RT-PCR assay, the pre-core transcript was detected 2 days prior to the time cccDNA was detected by Southern blot hybridization (compare lanes 5–7 in Fig. 2C and D). This is most likely because the RT-PCR assay for pre-core mRNA was more sensitive than Southern blot hybridization assay for cccDNA.

To further determine the relationship between cccDNA formation and pre-core mRNA transcription, we compared the cccDNA and pre-core mRNA accumulation kinetics in HepAD38 cells replicating viral DNA and cells in which viral DNA replication had been arrested by lamivudine treatment. The drug treatment procedures are depicted in Fig. 3A. Briefly, HepAD38 cells were cultured in the absence of tetracycline and at same time, left untreated (Group 1) or treated with lamivudine to completely arrest HBV DNA synthesis (Group 2) for 7 days. The tetracycline was then added back to the cultures to prevent viral RNA transcription from integrated DNA and the cultures were maintained without or with lamivudine treatment for another 7 days. Analysis of viral RNA transcription, core DNA synthesis, cccDNA accumulation and pre-core mRNA expression revealed the following results: First, viral pgRNA were induced upon tetracycline removal in both untreated and lamivudine treated cells and reached similar levels at day 7 post

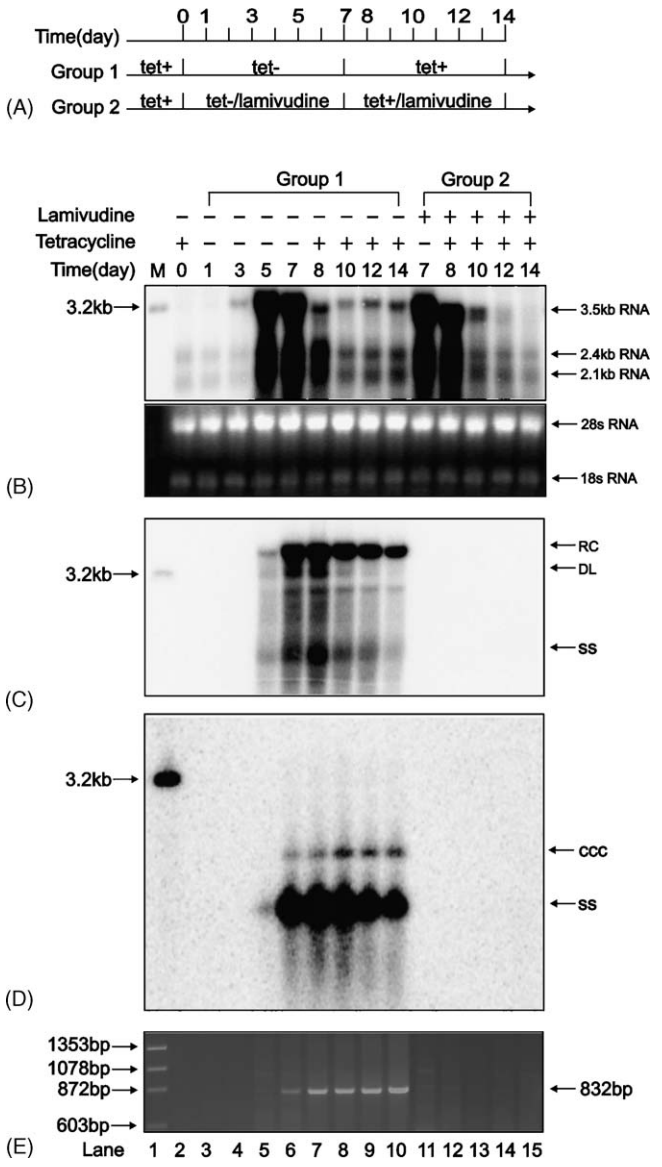

Fig. 3. Relationship of HBV cccDNA formation and pre-core mRNA transcription. (A) Experimental procedures. HepAD38 cells were cultured in 60 mm dishes until confluent and tetracycline was then removed from cultures medium and the cells were left untreated (Group 1) or treated with 50 μM lamivudine (Group 2) for 7 days (day 0–7). The tetracycline was then added back to cultures of both untreated and lamivudine treated cells and incubation continued without or with lamivudine for another 7 days (day 7–14). Cells were harvested at the indicated time points. The levels of viral RNA (B), core DNA (C), cccDNA (D) and pre-core mRNA (E) were determined by Northern blot, Southern blot and RT-PCR assays as described in materials and methods. Fifty picograms of unit length HBV DNA (lane 1) served as hybridization control.

tetracycline removal (Fig. 3B). Second, as expected, core DNA synthesis and cccDNA formation occurred only in untreated, but not lamivudine treated cells (Fig. 3C and D). Third, without de novo transcription from integrated viral genome, viral core DNA declined four-fold in the next 7 days, while cccDNA levels increased about two-fold during the same period of time, indicating a continuous conversion of pre-existed rcDNA into cccDNA (Fig. 3C and D). Fourth, after the addition of tetracycline back to culture medium, viral RNA levels declined quickly in both

untreated and lamivudine treated cells, but appeared to increase 3 days later in untreated cells. This increase is in parallel with the increase of cccDNA levels (Fig. 3B and D). However, in spite of approximately 10 copies of cccDNA per cell at day 14, viral RNA levels reached less than 10% of the levels obtained from a single copy integrated viral genome (comparing lanes 6–10 in Fig. 3B). Hence, either only a small fraction of cccDNA served as transcription templates or transcription from cccDNA is not as efficient as from the tetCMV promoter in integrated viral DNA. Fifth, the initial drop of viral pgRNA and envelope mRNAs after the addition of tetracycline in lamivudine treated cells delayed about 24 h in comparison with untreated cells (compare lanes 7 and 12 in Fig. 3B). The reason for this observation is currently not clear. Finally, despite the fact that similar amounts of 3.5 kb viral RNA were induced in both untreated and lamivudine treated cells, pre-core mRNA could only be detected coincidentally with cccDNA in untreated, but not lamivudine treated cells (Fig. 3E). This implies that pre-core mRNA was indeed transcribed only from cccDNA template, but not the integrated viral DNA.

2.4. Correlation of HBeAg secretion and intracellular cccDNA levels

To explore the possibility that HBeAg could serve as a reporter for nuclear cccDNA, we measured the levels of HBeAg by ELISA assay in the supernatants from HepAD38 cell cultures for the kinetics studies described above (Fig. 2). The results showed that HBeAg became positive at day 4 after tetracycline removal and increased during the next 10 days. There was generally a good correlation between levels of nuclear cccDNA and HBeAg in the culture medium (Fig. 4A).

We further compared the levels of HBeAg in the culture media harvested from cells harboring cccDNA (Group 1) and cells in which viral DNA replication were arrested by lamivudine treatment since the removal of tetracycline (Group 2) as described in Fig. 3. The results showed that the levels of HBeAg detected by the ELISA assay in the medium of untreated cells at day 7 was only slightly higher than that in the medium from lamivudine treated cells. After the addition of tetracycline back to culture media, HBeAg levels in the media of untreated cells increased in parallel with the increases of cccDNA and pre-core mRNA, but slightly decreased in the supernatants from lamivudine treated cells (Figs. 3D, E and 4B). The differences of HBeAg levels between the samples from untreated and lamivudine treated cells could reach about Seven- to eight-fold after day 10. These results implied that HBeAg was only translated from pre-core mRNA, while most pgRNA is transcribed from integrated viral DNA.

The low level HBeAg signals still detected in the supernatants harvested from day 3 to 7 after tetracycline removal and lamivudine treated cells might be due to cross reaction between anti-HBe antibody and free core protein and/or core particles released from dead cells. Results from a sucrose gradient fractionation analysis indicated that anti-HBe antibody used in this study could not react with core particles, but the possibility that the antibody could react with unassembled or disassembled core proteins could not be ruled out (results not shown). Neverthe-

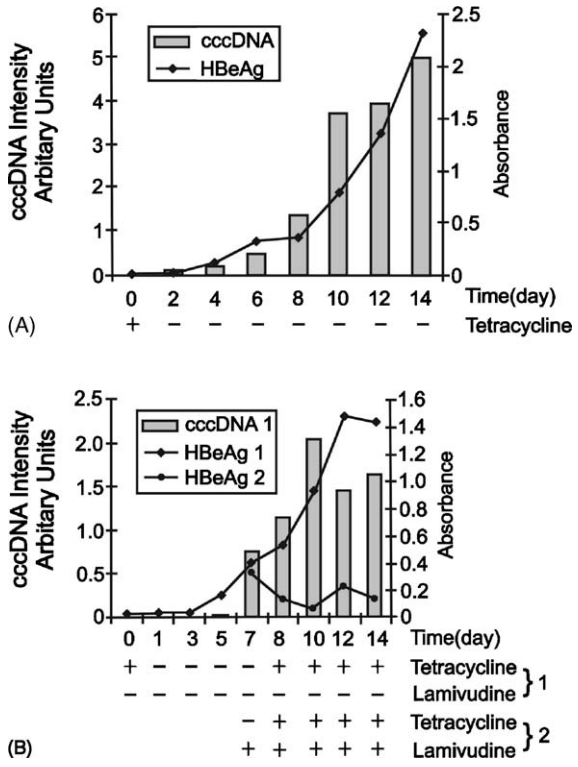

Fig. 4. Correlation of HBV cccDNA formation and HBeAg secretion. HBeAg levels in the culture media harvested from (A) the kinetics studies described in Fig. 2 and (B) the experiments described in Fig. 3 were determined by ELISA assay and plotted as a function of time (days). The levels of viral cccDNA were quantified using Bio-Rad QuantityOne program from the Southern blots shown in Figs. 2C and 3D, and superimposed onto graph (A) and (B), respectively.

less, the differential power of HBeAg assay between cell culture media from cells with and without cccDNA was approximately eight-fold, which would be sufficient to screen for effects of compounds on HBV cccDNA.

2.5. Stability of cccDNA in confluent HepAD38 cells

In order to use HepAD38 cells to evaluate the effects of compounds on cccDNA formation and stability, it was desirable to know the stability of the cccDNA pool in confluent HepAD38 cells. The results shown in Fig. 3C indicated that cccDNA pools in HepAD38 cells were quite stable over a short time period after the cessation of viral pgRNA transcription from integrated viral genome. But the observed stability could be due to either a dynamic balance between cccDNA decay and cccDNA formation from newly synthesized viral DNA, or the inherent stability of cccDNA molecules.

To distinguish these possibilities, we took the advantage of the HBV reverse transcriptase inhibitor lamivudine to arrest viral DNA synthesis and measured the rates of decay of both core DNA and cccDNA in confluent HepAD38 cells. HepAD38 cells were at first cultured in the absence of tetracycline for 8 days to induce DNA synthesis and cccDNA formation. The tetracycline was then added back to the cultures to inhibit the transcription of viral RNA from the integrated genome. One set of cultures was left untreated (Fig. 5, Lanes 7–13) and another set was treated

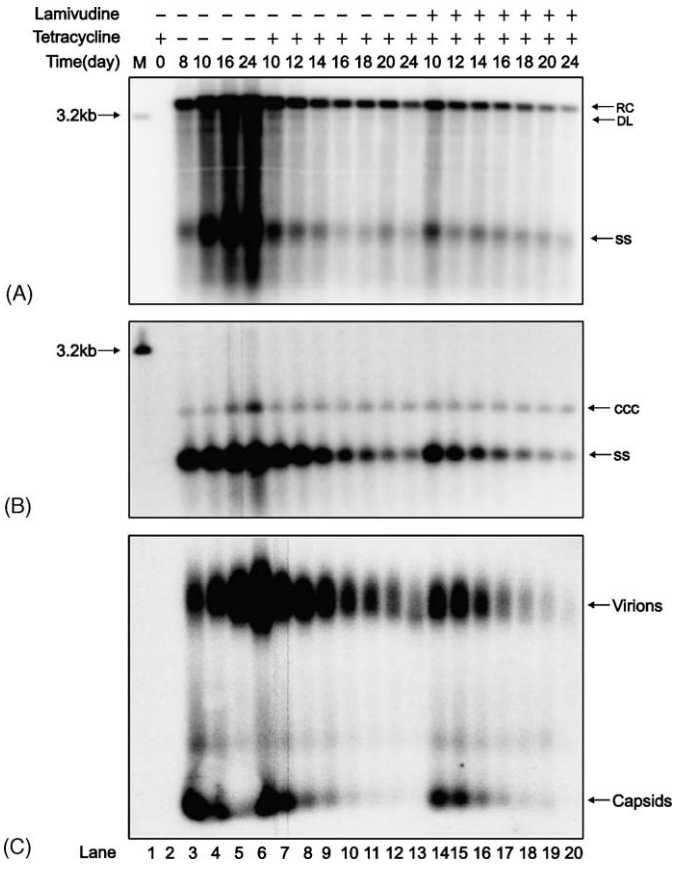

Fig. 5. Stability of HBV capsids and cccDNA in HepAD38 cells. HepAD38 cells were cultured in the absence of tetracycline for 8 days (day 0–8) to induce viral DNA replication and cccDNA formation. One set of cells was continued to be cultured in the absence of tetracycline for another 16 days (day 8–24). Cells were harvested at day 0, 8, 10, 16 and 24 (lanes 2–6). The tetracycline was added back to the remaining cultures at day 8, which were left untreated or treated with 10 μM lamivudine for 16 days (day 8–24). Untreated (lanes 7–13) and lamivudine treated cells (lanes 14–20) were harvested every other day and the levels of intracellular viral core DNA (A) and cccDNA (B) were determined by Southern blot hybridization and quantified by using Quantity One (Bio-Rad). (C) Enveloped viral particles (virions) and "naked" nucleocapsids (cores) in culture medium were determined by particle assay as described in Section 1. Fifty picograms of unit length HBV DNA (lane 1) served as hybridization control.

with lamivudine (Fig. 5, lanes 14–20) for 16 days. Lamivudine was intended to arrest ongoing DNA synthesis that might result from pgRNA transcribed from cccDNA.

The analysis of viral core DNA and cccDNA revealed that in the absence of de novo pgRNA synthesis from integrated viral genome, the levels of viral core DNA was decreased four-fold and eight-fold in 16 days in untreated and lamivudine treated cells, respectively. Those results indicated that cccDNA dependent viral RNA transcription continued to support new rounds of DNA synthesis, albeit at a much lower level, and this synthesis could be inhibited by lamivudine.

The levels of cccDNA in both untreated and lamivudine treated cells remained at constant levels over the 16 days for which it was measured (Fig. 5A and B). As expected, the levels of HBeAg in culture medium did not change after the addition of tetracycline and lamivudine (results not shown). Those results implied that cccDNA in confluent HepAD38 cells were either inherently stable or its decay could be compensated by the conversion from pre-existed rcDNA (Moraleda et al., 1997).

The observed decline of core DNA after the addition of tetracycline and treatment with RT inhibitor could be explained by core particle degradation and/or secretion. The results from viral particle assay indicated that HBV virion secretion gradually decreased, but still continued after the secession of viral RNA transcription from integrated viral genome and arresting of ongoing viral DNA synthesis by lamivudine (Fig. 5C). Those results suggested that the nucleocapsids in which DNA synthesis was arrested could still be enveloped and secreted. This is consistent with the observation made in acyclovir treated woodchucks (Tencza and Newbold, 1997).

3. Discussion

Cell-based assays for the discovery of antiviral drugs against HBV mainly utilize HepG 2.2.15 and other stable cell lines (Acs et al., 1987; Fu and Cheng, 2000). HBV surface antigen (HBsAg) and HBeAg are constitutively produced from integrated viral DNA and viral transcripts from cccDNA templates comprise less than 10% of total viral RNA in those cells (Chou et al., 2005). Hence, the levels of HBsAg and HBeAg in the culture media of those cells cannot be used as reporters for the inhibition of viral DNA replication and cccDNA formation by drugs.

HepAD38 is a HepG2 derived stable cell line supporting tetracycline inducible HBV replication (Ladner et al., 1997). A unique property of this cell line is that viral pgRNA transcription from integrated viral DNA can be turned on or off at will by removal or addition of tetracycline in culture medium, respectively. Therefore, it is possible to specifically study viral gene transcription from cccDNA by turning off the transcription from integrated viral genome. Here we show that HBV precore mRNA transcription and secretion of its translation product HBeAg in HepAD38 cells were strictly cccDNA-dependent (Figs. 2 and 3). Furthermore, we demonstrated that the levels of HBeAg in culture fluids are quantitatively correlated with the amounts of cccDNA in nuclei (Fig. 4).

Taking advantage of those two properties of the HepAD38 cell line, we demonstrated that without de novo viral RNA transcription from integrated viral DNA, nuclear HBV cccDNA could be stably maintained for at least 2 weeks in confluent HepAD38 cells (Fig. 5B). Moreover, under those conditions, the difference between the signal and background in the HBeAg reporter assay was greater than seven-fold (Fig. 4B), which should be sufficient for determination of the effects of compounds on HBV cccDNA. In comparison to the currently available cccDNA assays based on membrane hybridization and PCR technologies (Guo et al., 2003; Werle-Lapostolle et al., 2004), the HBeAg reporter assay is more convenient, cost effective and most importantly, suitable for high throughput screening of compounds that could prevent cccDNA formation and/or purge existing cccDNA from the nuclei of HBV infected hepatocytes.

The observed stability of cccDNA indicated that it could be either very stable in confluent HepAD38 cells as observed in primary woodchuck hepatocytes (Moraleda et al., 1997) or replenished by the conversion of the pre-existing rcDNA to cccDNA,

a process that may not require viral reverse transcriptase activity (Kock and Schlicht, 1993; Moraleda et al., 1997). This observation corroborates the long half-life estimates of hepadnavirus cccDNA, observed in HBV infected chimpanzees (Wieland et al., 2004), woodchuck hepatitis virus infected woodchucks (Zhu et al., 2001) and duck hepatitis B virus infected ducks (Addison et al., 2002) receiving viral RT inhibitor therapies, which were 9–14, 33–50 and 35–57 days, respectively. However, it is in contradiction to the relatively short half-life (3 days) of cccDNA in HBV recombinant baculovirus infected HepG2 cells (Delaney et al., 1999). The reason for this discrepancy is currently not clear.

In spite of the obvious advantages, using an HBeAg reporter assay to screen anti-HBV compounds might have some inherent caveats. First, any compounds that inhibit viral gene transcription, translation, HBeAg post-translational processing and secretion could reduce HBeAg levels in culture medium and would be scored as positive hits. An example is ellagic acid, a flavonoid purified from herbal medicine Phyllanthus *urinaria*, which was reported to inhibit HBeAg secretion, but do not affect viral DNA replication and HBsAg secretion (Shin et al., 2005). A simple resolution to this problem is the simultaneous measurement of HBsAg and some cellular secreted proteins, such as alpha-fetal protein and alpha-antitrypsin, in culture media. The information obtained from those assays should not only help to exclude the above confounding factors, but also provide information on cytotoxicity of the compounds. Secondly, compounds that interfere with the HBeAg assay could reduce the HBeAg signals reported by ELISA assays. Therefore, it is essential to perform a second round screening by directly applying the compounds identified in primary screening in ELISA assays to rule out the false positives. Finally, due to the current absence of drugs that directly reduce intracellular cccDNA levels, we could not evaluate the performance of the assay. Nevertheless, the HBeAg reporter assay described here provides a potentially applicable cell based high throughput platform for the discovery of antivirals specifically targeting cccDNA.

It is worthy of note that the improved cccDNA assay described in this work would allow the further studies of the fate of cccDNA during cell division. Moreover, by adding tetracycline back to culture medium after a prolonged induction of viral replication in HepAD38 cells, the continuation of HBV replication under this condition would absolutely rely on cccDNA, which mimics the situation of HBV infected hepatocytes. It is, therefore, a useful cell culture model to evaluate potential therapeutic agents and devise the optimal conditions to cure the HBV infected cells. In combination with currently available human siRNA library, HepAD38 cells would also be a suitable cell system to identify the host cellular factors essential for cccDNA synthesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2006.05.001.

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