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# 建立肝臟去氧核醣核酸病毒中共價閉合環狀去氧核醣核酸的報告基因系統

# Construction of a Reporter System for the formation of hepadnavirus covalently closed circular DNA

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本論文係 莊淳喬 君（學號 R00445105 ）在國立臺灣大學微生物學所完成之碩（博）士學位論文，於民國 101 年 7 月 18 日承下列考試委員審查通過及口試及格，特此證明

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論文題目：建立肝臟去氧核醣核酸病毒中共價閉合環狀去氧核醣 核酸的報告基因系統
指導教授：楊宏志

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# 中文摘要

Hepadnavirus 科包含 B 型肝炎病毒（HBV），擁有一局部雙股去氧核糖核酸基因組，稱為 RC-DNA。RC-DNA 進入肝細胞後會轉換成病毒生活史中必要的模板，稱為共價閉合環狀去氧核醣核酸 （cccDNA）。然而對於 RC-DNA 如何轉換成 cccDNA 的機轉並不了解。南方墨點法為目前偵測 cccDNA 的方法，但此方法非常的耗時及耗人力。因此有必要發展出一套有效率的 cccDNA 偵測系統。而目前 B 型肝炎病毒並沒有一個可以有效率被感染及產生 cccDNA 的活體內外系統。相對而言，鴨子的 B 型肝炎病毒（DHBV）在前基因體核糖核酸（pgRNA）有產生下可以有效率地產生 cccDNA。本研究用 DHBV 的系統建造偵測 cccDNA 產生與否的系統。首先，我們結果顯示在破壞 DHBV 基因使其無法產生核蛋白（core）或聚合酶（polymerase）情況下可以外加 core 及 polymerase 將之補回來。接著我們在 core 的轉譯起點到 polymerase 起點區域間建造一系列去除片段序列的突變進行研究。在外加 core 的情況下發現唯有在區域多腺苷酸化 (poly A) 到 polymerase 的轉譯起點可被補回來且 cccDNA 的產生量沒有明顯變少。因此我們在此區域插入偵測基因，分別為抗 Zeocin 抗生素的基因及螢光蛋白質。在插入後，我們利用南方墨點法偵測他們生活史不會受到影響且會產生 RC-DNA 及 cccDNA。這證實了此偵測系統的可行性。此一偵測系統還需要進一步的實驗來證實它在偵測 cccDNA 的真實性。

**關鍵字：**B 型肝炎病毒、鴨子 B 型肝炎病毒、共價閉合環狀去氧核醣核酸、偵測基因、南方墨點法

# ABSTRACT

Hepadnavirus, including hepatitis B virus (HBV), possesses a partial double-strand DNA genome, also known as relaxed circular DNA (RC-DNA), which is converted to covalently closed circular DNA (cccDNA), a critical template for hepadnavirus replication, after entry to the hepatocytes. However, little is known about the detailed mechanisms regulating the conversion from RC-DNA to cccDNA. Detection of cccDNA usually requires Southern blotting, which is quite labor-intensive. Therefore, a convenient *in vitro* cccDNA reporter system should facilitate the research of cccDNA. However, in the present there is still no *in vivo* and *in vitro* system that can support efficient HBV infection and cccDNA formation. In contrast, duck hepatitis B virus (DHBV) can efficiently form cccDNA in human cell lines as long as its pregenomic DNA is made. In this study, we utilized DHBV system to construct the cccDNA reporter for ready detection of cccDNA formation. We first showed that trans-complementation of core and polymerase could rescue the replication cycle of core- and polymerase-deficient DHBV mutants. We then generated an array of deletion mutations that spanning the region between the start codons of core and polymerase genes. Interestingly, we found that the region between the poly A signal and the start codon of polymerase gene could be

deleted without significant reduction of cccDNA formation. Thus, we inserted two reporter genes, the zeocin-resistant gene and a fluorescent protein into this region. Using Southern blotting, we demonstrated that they both maintain the ability to produce cccDNA and RC-DNA, indicating the feasibility of our reporter system. Further experiments are required to prove its utility in serving as a reporter of hepadnavirus cccDNA.

Keywords: Hepatitis B virus (HBV), Duck hepatits B virus (DHBV), cccDNA, reporter genes, Southern blot, complementation,.

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# 1. INTRODUCTION

## 1.1 The history of hepatitis B virus

In 1960s, Dr. Baruch Blumberg and colleagues discovered human hepatitis B virus (HBV)[1,2,3], when they were searching through the blood of Australian aborigine for polymorphic serum proteins and found a previous unknown protein[4]. Initially, this protein was named Australia (Au) antigen, and then it became clear that it was related to type B hepatitis. Later, Au antigen was only found in patients infected with HBV by other investigators in 1968[5]. Furthermore, in 1970, D.S. Dane visualized virus-like particles under the electron microscope in serum from patients suffering type B hepatitis. Thus, Au antigen was identified as HBV surface protein (HBsAg) and this particle was infectious and transmissible agent[6].

## 1.2 The classification of hepatitis B virus

HBV belongs to the family Hepadnaviridae. The Hepadnaviridae family contains two genera, *Avihepadnaviruses* and *Orthohepadnaviruses*. *Orthohepadnaviruses* include the prototype human HBV and others that infect other

mammalian hosts such as woodchucks, ground squirrels, arctic squirrels, woolly monkeys, etc. *Avihepadnaviruses* , including duck hepatitis B virus (DHBV), heron hepatitis B virus (HHBV) and snow goose hepatitis B virus (SGHBV), infect avian hosts [7]. The two genera of the Hepadnaviridae family, *Avihepadnaviruses* and *Orthohepadnaviruses*, share only 40% of nucleic acid homology[8]. However, all members of the Hepadnaviridae family have similar orientation of open reading frames (ORFs) and replication strategies for infections that cause either acute or persistent diseases[7].

Based on more than 8% difference, human HBV can be grouped into eight genotypes, A through H. Extensive phylogenetic analysis subdivides human HBV into subgenotypes, which differ by at least 4%[8]. The distribution of genotypes also varies geographically. For example, Eastern Africa and Western Asia have genotypes A and D respectively [9].

**1.3 Epidemiology and the natural history of HBV infection**

HBV affects more than 2 billion people worldwide. The number of chronic carriers is more than 350 millions, even after introduction of vaccine against HBV since 1982. The chronic HBV carriers have higher risk of hepatic decompensation, cirrhosis,

and hepatocellular carcinoma, which can eventually lead to mortality. The rate of mortality associated with the chronic hepatitis B is about 0.5 to 1.2 million each year[10].

HBV can be transmitted perinatally and horizontally. Perinatal transmission of HBV from the mother to the child is the main route in the endemic areas, especially in Asian countries. In Africa, horizontal transmission is the main route during early childhood. Horizontal transmission includes infection through the non-sterile syringe and needle from blood transfusion[11].

The infection of HBV can be acute or last for more than 6 months and become chronic infection. In acute infection, the patients can be symptomatic or asymptomatic. Symptomatic patients initially have malaise, anorexia, nausea, vomiting, fever and flu-like symptoms, and some of them may have jaundice. The patients with complete recovery from acute infection develop a long-term protection for HBV infection[11].

The definition for chronic hepatitis B is the persistence of serum HBsAg for more than 6 months. According to the serum HBeAg and HBV DNA status, the chronological course of the three phases of chronic hepatitis B are immune tolerance, immune clearance and low or non-replication of HBV. Patients with vertical transmission of HBV infection are initially in the immune tolerance phase, which is characterized by

seropositive HBeAg and high HBV replication, and then enter the HBV clearance phase with elevation of ALT and decline of HBV DNA. After the immune clearance phase, they will enter the low or non-replicative phase with HBeAg seroconversion, normal ATL and low HBV DNA[11].

## 1.4 The current challenges in treatment of chronic HBV infection

Currently available treatment for chronic hepatitis B consist of interferon-alfa and nucleoside analogues (NAs). Interferon-alfa enhances the immune response to control HBV infection[12]. NAs, such as entecavir and tenofovir, directly inhibit the HBV reverse transcriptase[13]. However, HBV relapses once antiviral treatment is discontinued. Thus, it often requires life-long treatment with NAs[12]. It has been known that the relapse of HBV is caused by the persistence of the HBV covalently closed circular DNA (cccDNA) in the nuclei of the infected hepatocytes. Since the NA treatment cannot eradicate the persistent cccDNA, HBV resumes the replication cycle once the treatment stops. Therefore, eradication of cccDNA is the challenging issue for current HBV treatment.

**1.5 Virion structure and genome organization**

HBV has a spherical particle with a diameter of 42nm and 28 nm electron-dense cores[14]. They consist of inner nucleocapsid (core), surrounded by lipid envelopes, and virally encoded surface proteins[15]. The nucleocapsids have two sizes of particles, which are approximately 34 nm and 30 nm in diameter. Large particles form the majority, and the remaining 15 percent of nucleocapsids are small particles. The nucleocapsids have a basic protamine-like C-terminal region involved in packaging pregenomic RNA[16].

The genomes of hepadnaviruses are approximately 3 to 3.3 kilobases, and exist as partially double-stranded DNA (dsDNA). The genome of HBV has four ORFs, including polymerase, core (and precore), surface, and HBx. The longest ORF encodes polymerase, which has three catalytic subdomains that are the terminal protein priming domain, reverse transcriptase (with DNA-polymerase function), and RNase H. Surface proteins' ORFs overlap with the ORF of polymerase. It encodes three different surface proteins – large (LHBs), middle (MHBs), and small (SHBs). They can be distinguished by different glycosylation status. Core and precore proteins are encoded by precore/core ORFs. The precore sequence contains a signal peptide for translocation into the

endoplasmic reticulum (ER). Last but not least, HBx proteins interact with many cellular partners and are involved in HBV-associated carcinogenesis[17].

## 1.6 The replication cycle of human HBV

### 1.6.1 HBV replication cycle

When HBV enters the target cells, its relaxed circular DNA (RC-DNA) genome is released from nucleocapsids and transported to the nucleus, where it is converted into cccDNA[18]. This cccDNA is an important intermediate for hepadnavirus replication. It serves as the transcriptional template for the pregenomic RNA (pgRNA) and various subgenomic RNAs. The longer precore messenger RNA (mRNA) are translated and processed into HBeAg, which serves as a serological marker for HBV replication. The shorter pgRNA serves as not only mRNAs for capsid and polymerase proteins, but also the template for reverse transcription. Polymerase protein then binds to the pgRNA from which it is translated[19]. When binding to pgRNA, polymerase initiates encapsidation of the complex into a RNA-containing nucleocapsids[20]. The RNA inside the nucleocapsids are reversely transcribed by the polymerase into RC-DNA. The newly formed

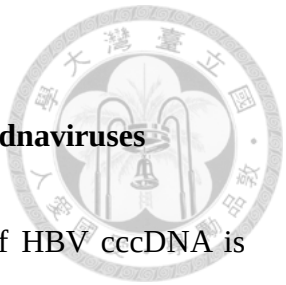
RC-DNA-containing nucleocapsids can either re-enter nuclei to continue another round of replication cycle or can interact with the envelope proteins and be secreted as new infectious virions[21,22,23,24].

### 1.6.2 From pgRNA to RC-DNA (Reverse transcription)

Polymerase binds to the epsilon (ε) of pgRNA and starts DNA synthesis. The bulge region of epsilon is used as the template for polymerase to synthesize the 5'end 4 nucleotides [24], which are covalently linked to the tyrosine residue of the TP domain of polymerase. After the template switch to the proximal direct repeat 1 (DR1) in the 3'end, this oligonucleotide initiates the synthesis of the full-length negative DNA strand [25], along with which the pgRNA is digested by RNase H domain. Upon the completion of negative strand synthesis, there remains short 5'-end RNA of the pgRNA containing the DR1 sequence, which serves as the RNA primer for initiation of the positive strand synthesis by transfer of the RNA primer to DR2 close to the 5' end of the negative strand. Through the 5' and 3' ends negative strand redundancy (about 8 nt), the growing end of the positive DNA strand can switch to the 3' end of the negative strand and proceed to complete the positive strand synthesis and form RC-DNA[24,26].

### 1.6.3 From RC-DNA to cccDNA

The synthesis of cccDNA from RC-DNA requires several steps. First of all, it needs to fill the gap of plus-strand DNA. Second, the 5'-capped RNA primer at the 5' terminus of plus-strand needs to be removed. Third, it has to remove viral polymerase that is attached to the 5' end of minus-strand DNA. Forth, removal of the terminal redundancies of the negative-strand DNA is required. Last, both strands of the DNA should be ligated[24,27]. However, the detailed molecular mechanisms and the involved host factors from RC-DNA to cccDNA remain largely unknown. Recently, it has been suggested that RC-DNA lacking polymerase at the 5'-end of the negative strand might be the precursor of cccDNA[28]. Furthermore, it has been predicted that cellular DNA endonuclease cleaves the negative-stranded DNA downstream of the 5' end. Therefore, the cellular DNA repair system is suggested to involve the process, such as DNA2, FEN1 and ligase[29].



### 1.7 DHBV, a good model for studying cccDNA formation of hepadnaviruses

The molecular pathway of formation and amplification of HBV cccDNA is poorly understood, for the reason that it produces very little cccDNA in human hepatoma cell lines transfected with the HBV-expressing plasmid. A previous study suggested that the major block for cccDNA formation is the conversion of HBV RC-DNA into cccDNA. In contrast, in DHBV such block does not exist[30].

Both DHBV and HBV belong to the family Hepadnaviridae. They have the common virion structure and genome organization and share a similar replication strategy. The DHBV genome has only three ORFs, surface protein, core protein, and polymerase protein[31]. Therefore, DHBV serves a good model for studying replication of hepadnavirus. Actually, most features of hepadnaviral infection are first discovered in the DHBV model system and then confirmed in HBV[32].

### 1.8 DHBV cis-acting sequences that contribute to the replication cycle

#### 1.8.1 DHBV cis-acting sequences involved in encapsidation

In hepadnavirus, ε sequence is located at the 5’ end of the pgRNA. It forms a

phylogenetically conserved secondary structure that the polymerase can bind for encapsidation. In avian hepadnavirus, a sequence about 900 nucleotides downstream of ε called region II is required for encapsidation. Deletion of these sequences causes defect for encapsidation[33,34]. Deletion of sequences between ε and region II (the intervening sequence) affect the encapsidation efficiency by the deletion size. Complete removal affects encapsidation, but smaller deletions have no effect[35].

**1.8.2 DHBV cis-acting sequence that contribute to negative-strand DNA synthesis.**

Synthesis of the negative-strand DNA is initiated by the viral polymerase, which serves as a primer and a reverse transcriptase binding to ε of pgRNA. The first four nucleotides of the negative-strand DNA are then synthesized and transferred by base-pairing with a UUAC sequence at the DR1 (12 nucleotides direct sequence) near the 3' end of the pgRNA[36,37]. The region between nucleotides 251 and 450 (near 5' of region II) and between nucleotides 2058 and 2153 are most required for the synthesis of the negative-strand DNA. However, how the sequence between nucleotides 2058 and 2153 is involved in synthesis of the negative-strand DNA is not yet known[38].

### 1.8.3 DHBV cis-acting sequence that contribute to RC-DNA positive-strand synthesis and cccDNA formation.

At the end of negative strand synthesis, the primer is then translocated to the DR2 position near the 5' end of the minus-strand DNA template and initiate positive-strand synthesis[39]. Thus, DR2 is indeed required for positive-strand synthesis. Furthermore, the terminal redundancy on the negative-strand DNA template for template switch is necessary for the synthesis of the plus-strand DNA[40]. Nucleotides between 2549 and 2561 (named 3E), 723 and 833 (named M), and 2205 and 2642 (named 5E) are required for positive-strand synthesis which involve template switch or priming[41,42,43,44].

In spite of the discovery of the cis-acting sequence that is necessary for the hepadnavirus encapsidation and DNA synthesis, little is known about the cis-acting sequence that is required for the synthesis of cccDNA.

## 1.9 The effect of the size of the inserted DNA on DHBV replication

The DHBV pgRNA with additional 1000 nucleotides failed to form RC-DNA due to the large DNA size that could not be packaged. However, if the pgRNA was added with 180 nucleotides, RC-DNA could be detected[45]. These results indicate that larger size

of the genome affects the packaging efficiency. Thus, a large insertion size can potentially block the replication cycle.

# 2. SPECIFIC AIM

In this study, we aim to create a cccDNA reporter system for ready detection of cccDNA formation. Two reporters, the drug resistant gene and fluorescent protein, will be utilized. We will also validate the ability of these cccDNA reporters in formation of cccDNA *in vitro* by using Southern blotting.

# 3. MATERIAL AND METHODS

## 3.1 Plasmids

The following plasmids are used: DHBV (WT), DHBV1S, ΔCore, ΔPol, Δ400, Δ200-1, Δ200-2, Δ100-1, Δ100-2, D1SVC155, D1SZeocin, CMV-Core, CMV-pol, DHBV1S dimer.

Both DHBV and DHBV1S plasmids contain pgRNA sequences (about 1.2 mer) including full length DHBV genome (3021bp) and extra 490 nucleotide s(from transcription start site to *EcoRI* site) driven by the CMV promoter. The only difference between DHBV and DHBV1S is that DHBV1S has surface premature stop codons (1327 T to A, 1345 T to A, 1348 T to A), so that surface proteins are deficient, Thisenhances cccDNA formation by promoting intracellular recycling of rcDNA[46,47]. These two plasmids are gifts from Dr. Ju-Tao Guo at Drexel Institute for Biotechnology and Virology Research.

The followings are all derived from DHBV1S using site-direct mutagenesis. ΔCore was generated by placing a premature stop codon at 2672. ΔPol was generated by deleting the nucleotides 719 to 722 (Figure 4a). Δ400 has nucleotides deleted between 2646 (core

ATG) and 2771 and between 2771 and 20 (2771-3021, 1-20). Δ200-1 has nucleotides deleted between 2646 and 2771. Δ200-2 has nucleotides deleted between 2771 and 3021, 1-20. Δ100-1 has nucleotides deleted between 2646 and 2746. Δ100-2 has nucleotides deleted between 2672 and 2771 (Figure 5a).

The whole core or polymerase genes are added to pcDNA3.1 backbone to generate CMV-Core and CMV-pol. Therefore, core or pol is expressed under the CMV promoter when transfected into the cells.

D1SZeocin and D1SVC155 are both generated by adding the Zeocin-resistance gene (D1SZeocin) and Venus (fluorescence protein) C-terminal 155 amino acid sequences (D1SVC155), respectively, into Δ200-2 at the nucleotides that were deleted from DHBV1S. T2A sequence is added before Zeocin and Venus sequence (Figure 7a and figure 8a).

DHBV1S dimer was generated by cutting with *EcoRI* on DHBV1S template and annealing to the TA vector without a CMV promoter (Figure 9a).

**3.2 Cell lines and cell culture system**

Human embryonic kidney 293T (HEK293T) and hepatocyte-derived

hepatocellular carcinoma (Huh7) cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% bovine fetal serum (BSA) , 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamate. They were cultured in 5 % carbon dioxide at 37 ˚C.

**3.3 DNA transfection**

DNA transfection was performed by calcium phosphate transfection and lipofectamine protocol. In calcium phosphate transfection, $1.5x10^6$ cells were cultured on 6cm dish one day before transfection, 2.5 M $CaCl_2$, 2X HBS buffer and 0.1X TE buffer were pre-warmed until reaching 37 ˚C. 10 μg DNA was first diluted with 450 μl 0.1X TE buffer and then 50 μl 2.5 M $CaCl_2$ was added drop by drop. DNA: $CaCl_2$ mixture was then added into the plastic tube containing 500 μl 2X HBS drop by drop and vortexed simultaneously. The mixture was incubated in 37 ˚C for 30 min. The transfection medium was then added into cells. For complementation, the DNAs were added at the ratio of 1:1. In lipofectamin transfection, $5x10^6$ cells were cultured on 6mm dish one day before transfection, DNAs were mixed with Opti-MEM reduced serum medium. Lipofectamin was mixed with Opti-MEM reduced serum medium. After 10 minutes incubation at room

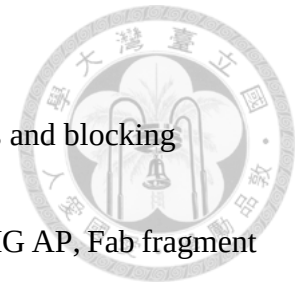
temperature, DNAs with Opti-MEM reduced serum was mixed with lipofectamin with Opti-MEM reduced serum medium and incubated for 20 mins at room temperature before being added to the cells for transfection.

### 3.4 Modified Hirts' extraction method

After 48hr of transfection, cells from 6 cm-diameter dish were transferred to 15ml centrifuge tube. Hirts lysis buffer (10 mM Tris-HCl pH 7.6, 10 mM EDTA pH 8.0, 0.7 % SDS) was then added (3ml) and mixed at room temperature for 30 mins. Cell samples were then treated with 0.8 ml 5 M NaCl overnight at 4 ˚C. The supernatant was collected by 10,000 rpm (RA-410M3) centrifugation for 30 mins at 4 ◦C. After transferred to new 15ml centrifuge tube, they were treated with 3 μl (final concentration 150 μl/ml) of protease K (Promega) for 2 hr at 37 ˚C. The DNA was then extracted by saturated phenol twice and phenol:chloroform once. After extraction, the DNA was precipitated with twice volume of 100 % ethanol overnight. The DNA was then precipitated by 10,000 rpm centrifugation for 10 mins at 4 ˚C. The DNA pellet was transferred and washed with 1 ml 70 % ethanol in the eppendorf. After centrifugation 10,000 rpm for 10 mins at room temperature, the DNA pellet was dried and then resolved in 30 μl dd$H_20$.

**3.5 Southern blot analysis**

DNA samples were first digested with *DpnI* for 2hr and Plasmid-safe DNase (PsD) for 1hr in a volume of 30 μl. After loading into 1% agarose gel (0.5X TAE), they were run at 40 volt for 17 hr. Agarose gel was then treated with 0.25 N HCl 15 mins once, 0.5N NaOH 15 mins twice, 0.5X TAE buffer 10 mins once and 0.25X TAE for 10 min. The DNA samples were then transferred to the positively charged Nylon membrane (Roche) by electrophoresis for 4 hr. Transferring construction from the negative to positive are: Sponge, 3 M paper, agarose gel, positively charged Nylon membrane, 3 M paper, sponge. After transferring, the nylon membrane was exposed to UV light thrice by UV crosslinker box (UV stratalinker, Stratagen) with Auto-crosslink program. The membrane was then pre-hybridized at 42 ◦C for 30 min with 7 ml DIG Easy Hyb Granules (Roche) and hybridized at 42 ◦C overnight with 7 ml DIG Easy Hyb Granule containing DIG-labeled hybridization probe, which was synthesized by Roche PCR DIG probe synthesis kit. At the next day, the membrane was washed with low stringency buffer twice at room temperature and high stringency buffer twice at 55 ◦C. After that, it was treated with 1X washing buffer for 5 mins. The membrane was subsequently blocked with blocking



solution 1 (1x blocking solution, 1x Malic acid solution) for 30 mins and blocking solution 2 (1x blocking solution) containing 1:10,000 dilution anti-DIG AP, Fab fragment (Roche) for 30 mins. After blocking, the membrane was washed with 1x washing buffer (1x Malic acid and 0.3-0.5 % Tween 20) twice for 15 mins and rinsed with 1x detection buffer (0.1 M Tris-HCl, pH 9.5 and 0.1 M NaCl) for 5 mins for detection. Last, the chemiluminescence signal was detected by UVP Image system (UVP AutoChemiTM System) and X-ray films.

**3.6 DIG-labeled DNA probe synthesis**

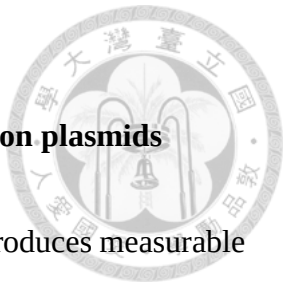
The DIG- labeled DNA detection probes were synthesized by Roche PCR DIG Probe Synthesis Kit. They were used for nonradioactive DIG system for Southern blotting analysis. The two pairs of primers that were used for synthesis of the DIG-labeled DNA probe are: Forward 5'-AAA GTC CTC TGT ACC TTT GC-3' , Reverse 5'-GCA AGA ATC ATA AAC AAT GG-3'. PCR with the primer pair generated a probe with around 500 bp in length. The PCR was performed with above primer pairs in the following condition: 94 ˚C for 5 mins, [94 ˚C x 30s - 55 ˚C x 30 s - 72 ˚C x 40 s] x 35 cycles, 72 ˚C x 40 s. And the single reaction contain 10 μM forward primer 1μl, 10 μM

primer reverse primer 1 μl, Vial 2 (dNTP contained DIG-11-dUTP) 5 μl, Vial 3 (PCR buffer) 5 μl, Vial 1 (enzyme mix) 0.8 μl, 50 ng DHBV-1S plasmid 1 μl and $ddH_2O$ 34.2μl.

# 4. RESULTS

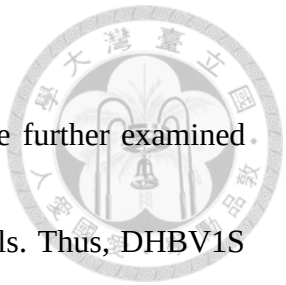
## 4.1 The strategies to construct a DHBV cccDNA reporter

An ideal cccDNA reporter system should allow the expression of the reporter gene only when the cccDNA is generated. Actually, e antigen of DHBV is a good candidate for this purpose. In the replication cycle of DHBV, the pgRNA does not generate e antigen, which can only be produced by the precore mRNA transcribed from cccDNA. Taking advantage of this unique property of e antigen during DHBV replication, we design a cccDNA reporter by cloning the reporter gene to the region of the core gene in frame with the e antigen. To make the expression of the reporter gene being controlled by the production of e antigen, we destroyed the start codon (ATG) of the core gene. Therefore, in our cccDNA reporter system, the pgRNA is generated under the control of the CMV promoter and produces no reporter proteins. However, once cccDNA is formed, the precore transcript can be made, and allow for the expression of the reporter gene (Figure 2).



**4.2 Production of RC-DNA and cccDNA from the DHBV expression plasmids**

To investigate cccDNA, we need a system that efficiently produces measurable cccDNA. Since HBV, compared to DHBV, poorly produces cccDNA, we used the DHBV system to study cccDNA. First of all, we need to verify whether cccDNA and RC-DNA can be detected *in vitro*. HEK293T cells were transfected with either the wild type (WT) DHBV or the surface-deficient DHBV1S. After 48 hours of transfections, DNAs were collected using the modified Hirt's extraction protocol and analyzed by Southern blotting. The results showed that, in DNA samples from DHBV1S-transfected HEK293T cells, two major bands were detected at the size between 3.0 to 4.0 kb (RC-DNA) and between 2.0 to 2.5 kb (ccc-DNA) (Figure 3). In comparison, WT DHBV produced much less cccDNA (Figure 3). These results are consistent with the previous report. The increase of cccDNA formation by DHBV1S was probably due to the promotion of the intracellular recycling pathway[46,47]. To confirm whether these two bands were indeed RC-DNA and cccDNA, we treated the DNA with the restriction enzyme *XhoI* (Figure 3), which only has a single restriction site within the DHBV genome. The results demonstrated that, following *XhoI* treatment, both bands disappeared and were shifted to a single band with the size of around 3 kb, compatible with the genome size of the DHBV (3021 bp).



Since hepadnavirus replicates primarily in hepatocytes, we further examined whether DHBV replicates in Huh7 cells as it does in HEK293T cells. Thus, DHBV1S was transfected into Huh7 cells. After 48 hours of transfection, DNAs were collected using the modified Hirt's extraction protocol and analyzed by Southern blotting. The results showed the same pattern as that in HEK293T cells (Figure 3). We also treated the extracted DNA with *XhoI* to confirm the RC-DNA and cccDNA identity of these bands (Figure 3).

To summarize, DHBV and DHBV1S-expressing plasmids can generate RC-DNA and cccDNA in both HEK293T and Huh7 cells. Furthermore, DHBV1S produces more cccDNA than DHBV.

**4.3 Complementation of the core- and polymerase-deficient DHBV by the core- and polymerase-expressing vectors**

In order to generate a DHBV cccDNA reporter system, the reporter gene is planned to be inserted into the region of core or polymerase genes. This strategy unavoidably destructs the expression of core and polymerase genes. Therefore, complementation of the cccDNA reporters with the core- or polymerase-expressing

vectors is necessary for production of cccDNA. Therefore, we first examined the efficiency of complementation. Four plasmids are generated for this purpose: the core-deficient (ΔCore), the polymerase-deficient (ΔPol) DHBV1S expression vectors, the core-expressing (CMV-Core), and the polymerase-expressing (CMV-pol) vectors. ΔCore has a premature stop codon in the core gene of DHBV1S genome. ΔPol has four nucleotides deletion in the polymerase gene of the DHBV1S genome. The CMV-Core and CMV-Pol have core or polymerase genes cloned to the pcDNA3.1 backbone and under the regulation of the CMV promoter.

When HEK293T cells were transfected with ΔCore or ΔPol plasmid alone, no production of RC-DNA and cccDNA were found (Figure 4b). However, RC-DNA and cccDNA could be readily detected when 293T cells were co-transfected with ΔCore and ΔPol (Figure 4b), indicating that these two DHBV mutants could complement each other. Furthermore, generation of RC-DNA and cccDNA were found by co-transfection of ΔCore plus CMV-Core (Figure 4b) or ΔPol plus CMV-Pol (Figure 4b). The results also showed that polymerase complementation was less efficient. To further confirm the identity of RC-DNA and cccDNA, the samples were cut with *XhoI*. After the digestion with *XhoI*, the two bands of RC-DNA and cccDNA disappeared, but a band with the size

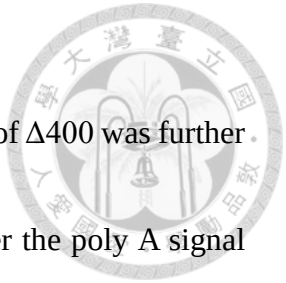
of about 3kb appeared (Figure 4c). The latter is compatible with the size of linear DHBV genome (3021 bp).

In summary, replication of the core-deficient and polymerase-deficient DHBV can be achieved by trans-complementation.

**4.4 Construct of a series of core-deletion DHBV mutants**

To generate the cccDNA reporter, we plan to place the reporter gene in the region of the core gene and in frame with the precore gene. Because the size of the DHBV genome affects the replication efficiency, we searched for the regions of the core gene that could be deleted and allow for insertion of the reporter gene without dramatic change of the genome size. Thus, we generated a series of core-deletion DHBV mutants to discover which region of the core gene can be deleted without affecting the replication cycle.

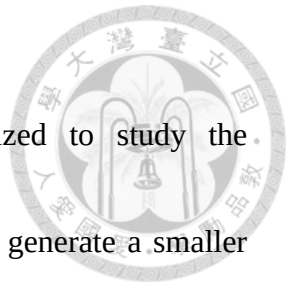
Constructs with the serial deletion of the core gene were generated using site-directed mutagenesis. Five core-deletion mutants, Δ400, Δ200-1, Δ200-2, Δ100-1, and Δ100-2, were created (Figure 5a). The regions of deletions were described below: The deletion of Δ400 spanned from the start codon (ATG) of core to the start codon of



polymerase, sparing the poly A signal sequence. The deletion region of Δ400 was further separated to two regions: before the poly A signal (Δ200-1) and after the poly A signal (Δ200-2). The deletion regions of Δ100-1, and Δ100-2 were generated by separating the deletion region of Δ200-1 into two halves. We found that, none of these 5 core-deletion mutants produced RC-DNA and cccDNA, but only Δ200-2 could produce RC-DNA and cccDNA by complementation with core (Figure 5c). Actually, these two bands were smaller than the WT RC-DNA and cccDNA, compatible with the deletion of 264 bps. We also validated the identity of the bands of RC-DNA and cccDNA by digestion with *XhoI* (Figure 5d).

**4.5 Construct of the cccDNA reporters D1SVC155 and D1SZeocin**

To generate the cccDNA reporter, we chose two forms of reporters, the fluorescent protein and the drug-resistant gene. As mentioned above, the size of the hepadnavirus cccDNA affects the replication efficiency, so we managed to reduce the size of the reporter gene. Bimolecular fluorescence complementation (BiFc) is a new technique that two separate parts of an individual fluorescent protein, N-terminal and C-terminal parts, can still produce fluorescence as long as two parts are co-expressed and



interact with each other. The BiFc technique is usually utilized to study the protein-protein interaction. Here, we take the advantage of BiFc to generate a smaller fluorescent reporter. In our study, we used Venus as the fluorescence gene[48], which is separated into two parts, VN173 (N-terminal of Venus protein to 174 amino acids) and VC155 (C-terminal of Venus protein from 155 amino acids). When co-transfected in HEK293T cells, these two parts could produce the fluorescence (Figure 6d).

D1SVC155 was constructed by adding VC155 sequence into the deletion part of DHBV1S in Δ200-2. T2A sequence was added before the VC155 sequence (Figure 7a). We demonstrated that when HEK293T cells were co-transfected with D1SVC155 with CMV-Core, RC-DNA and cccDNA could be detected (Figure 7b). The bands of RC-DNA and cccDNA were a little up-shift compared to that of DHBV1S, consistent with that the size of the added sequence is larger than that of the deleted sequence. We also digested the DNAs with *BamHI* (single cut site) to examine the identity of these two bands of RC-DNA and cccDNA (Figure 7c). Taken together, our data indicate that our reporter construct could still produce both RC-DNA and cccDNA.

Another gene we used as the reporter is the zeocin-resistant gene, with a small size of 378bp. D1SZeocin is generated in a strategy very similar to that used for

constructing D1SVC155. We ligated zeocin with the T2A sequence and inserted this complex sequence to the deletion part of DHBV1S in Δ200-2 (Figure 8a). Formation of RC-DNA and cccDNA by D1SZeocin could be rescued by complementation with core (Figure 8b). To summarize, we constructed two cccDNA reporters, both of which could still produce RC-DNA and cccDNA validated by Southern blotting.

**4.6 Construct of the DHBV1S dimer for analysis the activity of the DHBV promoter in Huh7 cells**

In our cccDNA reporter design, following the formation of cccDNA, the transcription of the reporter genes that uses precore ATG for translation can only be activated by the promoter located in the DHBV genome for production of the precore mRNA. In order to verify the activation of DHBV promoter in Huh7 cells, we generated a DHBV1S dimer construct (Figure 9a) that has the duplicate sequences of DHBV. The results show that when Huh7 cells were transfected with DHBV1S dimer, RC-DNA could be detected (Figure 9b). Although the signal of the bands was relatively lighter than that of samples transfected with the CMV-driven DHBV gene. As a result, the reporter genes can be transcribed.

# 5. DISCUSSION

The results showed that trans-complementation of core and polymerase could rescue the replication cycle of core- and polymerase-deficient DHBV mutants. Thus, using this system we can scan an array of mutants that spanning the region between the start codons of core and polymerase genes. Interestingly, we found that the region between the poly A signal and the start codon of polymerase gene (nucleotide 2777-3021, 1-20) could be deleted without significant reduction of cccDNA formation. Thus, we inserted two reporter genes, the zeocin resistant gene and fluorescent protein (VC155) into this region. Using Southern blotting, we demonstrated that they both maintain the ability to produce cccDNA and RC-DNA, indicating the feasibility of our reporter system.

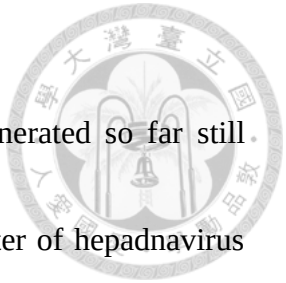
Due to the peculiarities of hepadnavirus genome organization, replication strategies, and the defined inner volume of the icosahedral capsid, the generation of reporter system of hepadnavirus has been difficult. Recently, the generation of hepadnavirus reporter systems has focused on the infection process and immunity[49,50]. One reporter system for cccDNA that is quite related to our system has been generated by

using HBeAg as a marker, which has to be screened by ELISA. However, using ELISA for the purpose of screening is still quite labor-intensive and costly. Our system takes advantage of drug-resistant and fluorescence genes and should be more convenient and cost-effective for the screening purpose.

In the past, deletion of the genome of hepadnavirus was considered infeasible. However, several recent studies have identified the cis-acting elements critical for encapsidation and reverse transcription (negative and positive strands synthesis). For examples, DR1, DR2, ε, region II, 3E, 5E, and M are known to be required for the replication cycle[33,36,41]. However, very few studies focus on the step involved in conversion of RC-DNA to cccDNA. Our cccDNA reporter system places the reporter genes, which can only be translated in parallel with the precore gene. Interestingly, the genome region at 2777-3021, 1-20 can be deleted without significantly affecting the formation of RC-DNA and cccDNA. We also deleted other regions (2646 to 2771, 2646-2746, and 2672-2771), but none of them could produce RC-DNA and cccDNA by complementation with core. Since the size of the genome affects the replication efficiency, the ability in deletion of a larger region theoretically can accommodate a larger reporter gene. We will further examine whether we can actually delete the core region other than

2777-3021, 1-20. The previous studies have demonstrated that the insertion of foreign DNA sequence into DHBV affects the replication cycle by disabilizing encapsidation[45]. The replicative intermediate decreased sharply when the insertion genome increased from 399bp to 720bp[49]. In our results, it is surprising that addition of T2A-Zeocin (461bp) and T2A-VC155 (341bp), RC-DNA and cccDNA can still be produced. Considering the deletion is only 264bp, the inserted sequence is about 100 or 200 bp longer. However, D1SZeoncin produces more cccDNA than D1SVC155 although the former is larger than the latter. The reason for this observation is unknown. One plausible explanation is that the sequence of VC155 may interfere more with DHBV replication compared to that of zeocin resistance gene.

The low signal of RC-DNA in Huh7 cells is considered as the reason of the large construct of DHBV1S dimer that might affect the transfection efficiency. The previous study has shown that, when transfected DHBV dimer into Huh7 cells, significantly amount of RC-DNA and cccDNA could be still detected[45], indicating that there should not be a problem in using the DHBV promoter in Huh7 cell lines. Furthermore, it was mentioned in the previous study that even in much reduced replication capacity, it can still yield a useful reporter readout[49].



Although the cccDNA reporter systems that we have generated so far still require further experiments to prove its utility in serving as a reporter of hepadnavirus cccDNA, we have shown a critical step that both reporter systems can produce RC-DNA and cccDNA. In the future, we have to demonstrate that these two reporters can authentically reflect the production of cccDNA in hepatoma-derived cell lines, like HepG2 and Huh7. For example, we will select D1SZeocin-transduced stable cells with zeocin and then examine whether or not the survived cells indeed produce cccDNA. Similarly, D1SVC155 has to be examined for its ability in production of cccDNA and fluorescence. After verification, these reporter systems will be utilized to identify the host cellular factors that are involved in cccDNA synthesis by screening with the human shRNA library.

In conclusion, we have created two DHBV cccDNA reporter systems and validated their ability in production of RC-DNA and cccDNA. Further experiments are still required to prove its application. We believe that this tool should be able to facilitate the research of cccDNA. In addition, these systems may serve the purpose in screening for the host factors and small molecules that regulate hepadnavirus cccDNA formation.

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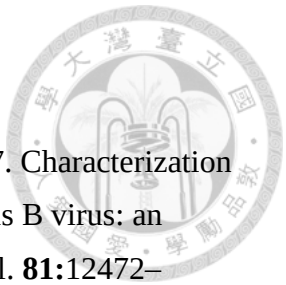

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# 7. FIGURE

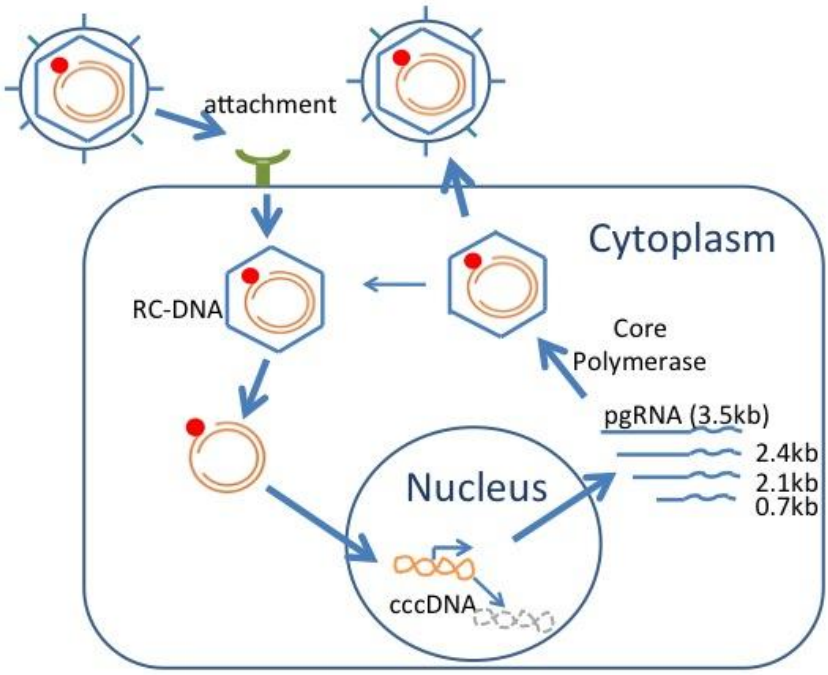



**Figure 1. The life cycle of HBV.**

Enveloped virions infect hepatocytes. Following entry, the nucleocapsid is released into the cytoplasm and the RC-DNA genome is transported to the nucleus and converted into cccDNA. The genome is transcribed into subgenomic RNAs and pgRNA from which core and polymerase protein are translated. After pgRNA encapsidation, reverse transcription is initiated inside the capsid. Mature, RC-DNA containing nucleocapsids can re-deliver the RC-DNA to nucleus for cccDNA amplification, or be secreted via interaction with the envelope proteins as progeny virions.

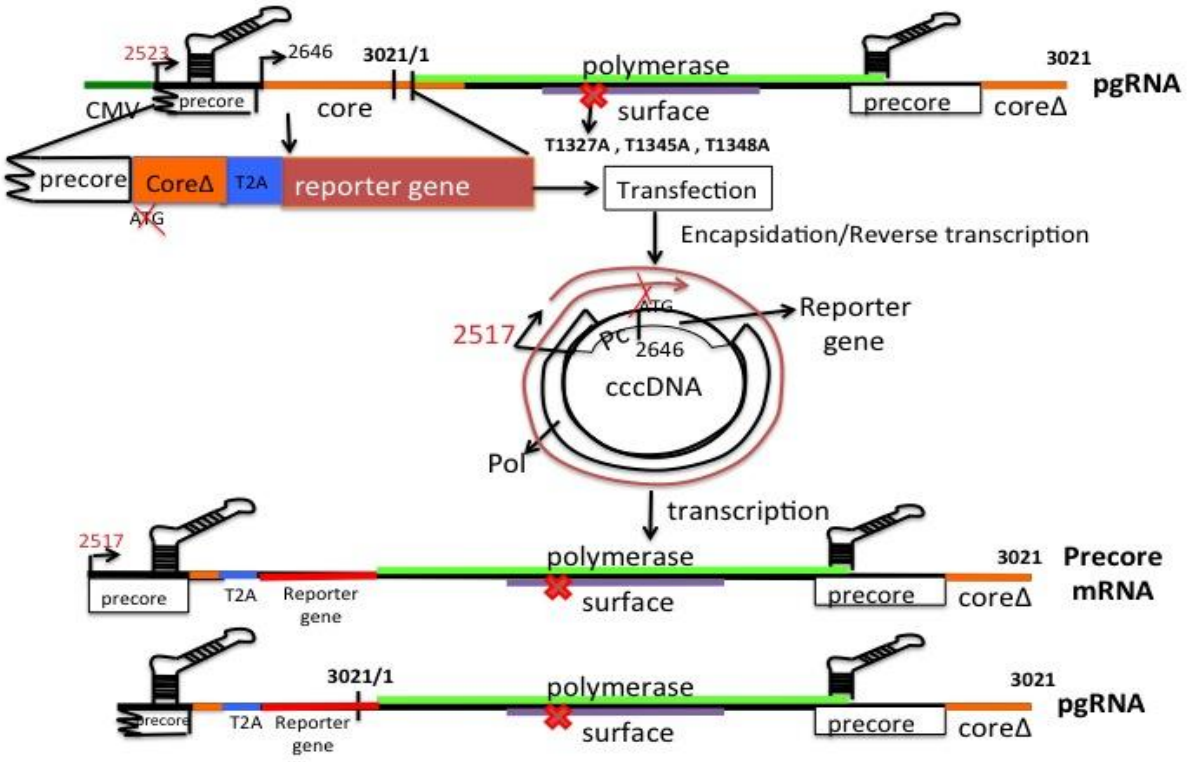



**Figure 2. Establishment of a cccDNA reporter system.**

The upper panel illustrates the genome organization of the DHBV-expressing plasmid. The red cross mark represents the nonsense mutations (T1327A, T1345A, T1348A) of the surface gene. The numbers above the boxed regions are based on the numerical system of the DHBV genome. The middle and lower panels demonstrate the strategy and structure of the DHBV cccDNA reporter system. Mutation of the start codon ATG of the core gene is indicated. The reporter gene is ligated with a T2A sequence at its 5'end and then inserted in-frame with the precore sequence T2A. T2A can process self-cleaved to prohibit leading peptide interfere with reporter protein or secrete out of the cells.

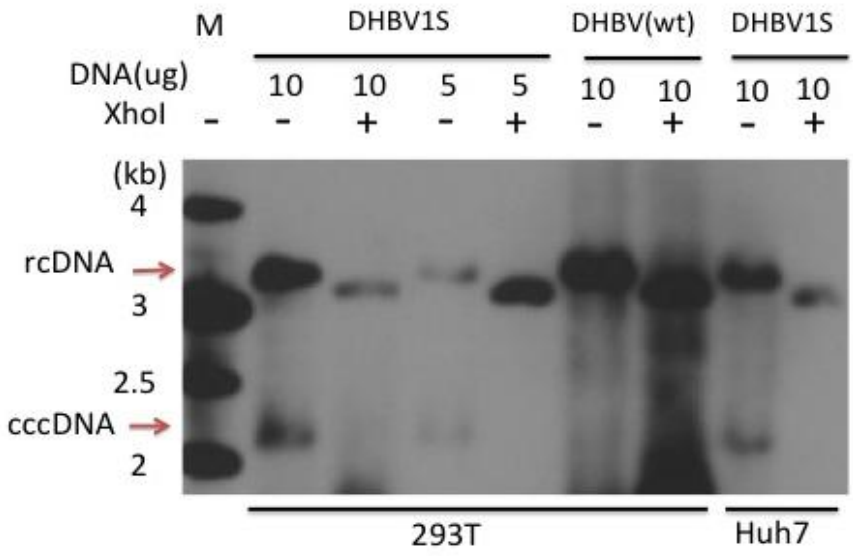



**Figure 3. Formation of RC-DNA and cccDNA in 293T and Huh7 cells transfected with DHBV and DHBV1S plasmids.**

DHBV and DHBV1S were transfected into HEK293T/Huh7 cells for 48 hr. Extracted DNAs were analyzed by Southern blot. The amount of transfected plasmids is indicated above. RC-DNA and cccDNA are marked with the red arrows.

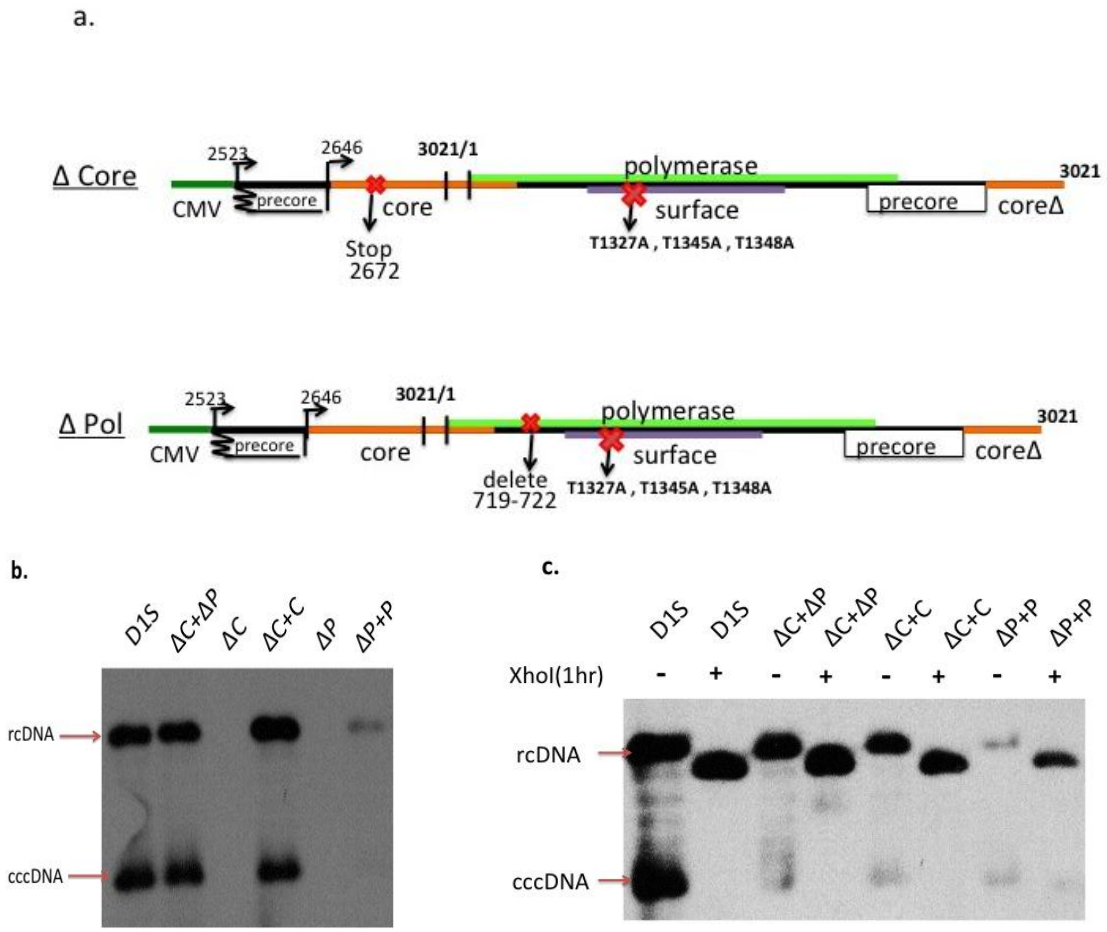
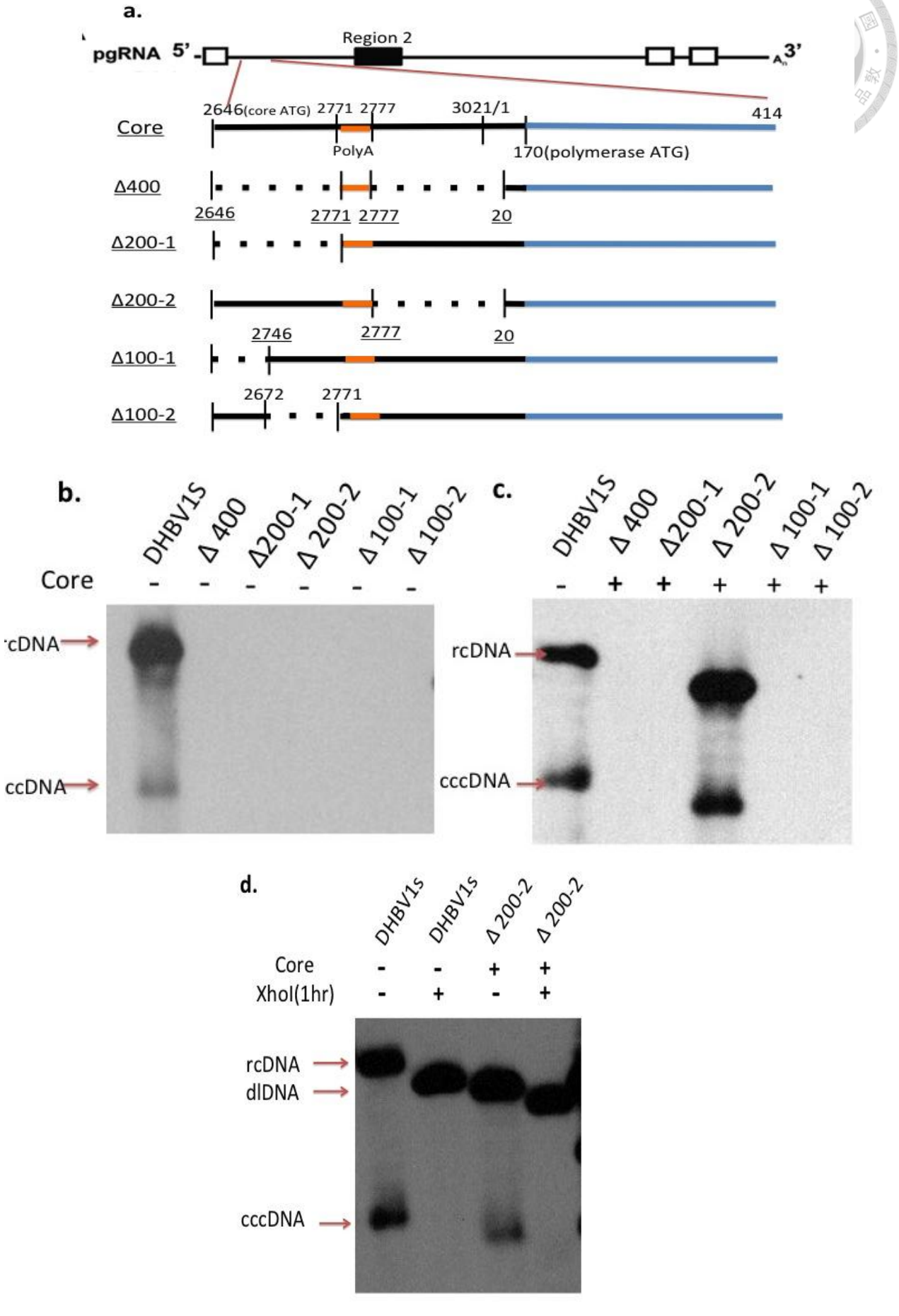



**Figure 4. Complementation of core-deficient and polymerase-deficient DHBV mutants.**

(a) Generation of core- and polymerase-deficient DHBV1S mutants-expressing plasmids. The nonsense mutation of the core gene and a 4-nucleotide deletion of the polymerase gene are indicated with the red cross marks. ΔCore was generated by substituting a premature stop codon at 2672 on DHBV1S plasmid. ΔPol was generated by deleting nucleotide 719 to 722 on DHBV1S plasmid. (b) Different combination of ΔCore (ΔC), ΔPol (ΔP), CMV-Core (C), and CMV-pol (P) were transfected into HEK293T cells for 48 hr. Extracted DNAs were analyzed by Southern blot . The ratio of transfected plasmids in

complementation experiments is 1:1. (c) Extracted DNAs were treated with *XhoI*.


a.
pgRNA 5'
Region 2
3'
Core
2646(core ATG) 2771 2777
3021/1
414
PolyA
170(polymerase ATG)
Δ400
2646
2771 2777
20
Δ200-1
Δ200-2
2746
2777
20
Δ100-1
2672
2771
Δ100-2
b.
DHBV1S
Δ 400
Δ200-1
Δ 200-2
Δ 100-1
Δ 100-2
Core
- - - - - -
cDNA
ccDNA
c.
DHBV1S
Δ 400
Δ200-1
Δ 200-2
Δ 100-1
Δ 100-2
- + + + + +
rcDNA
cccDNA
d.
DHBV1s
DHBV1s
Δ 200-2
Δ 200-2
Core - - + +
XhoI(1hr) - + - +
rcDNA
dlDNA
cccDNA

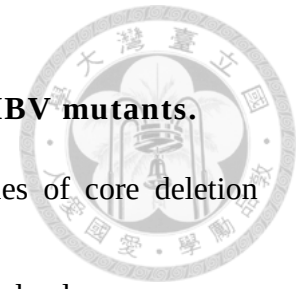


**Figure 5. Complementation of series of core-deletion DHBV mutants.**

(a) The construct of the DHBV1S core deletion mutants. A series of core deletion mutations spanning the region between the start codons of the core and polymerase genes are illustrated. Δ400: deletion in nucleotides (nt) 2646 (core ATG) -2771 and 27713021, 1-20. Δ200-1: deletion in 2646-2771. Δ200-2: deletion in 2771-3021, 1-20. Δ100-1: deletion in 2646-2746. Δ100-2: deletion in 2672-2771. Δ400, Δ200-1, Δ200-2, Δ100-1, and Δ100-2 were transfected alone (b) or co-transfected with CMV-Core at the ratio of 1:1 (c). After 48hr of transfection, DNAs were extracted and analyzed by Southern blot. (d) Extracted DNAs were treated with *XhoI*. Region II : encapsidation signal that could not be deleted for replication cycle.

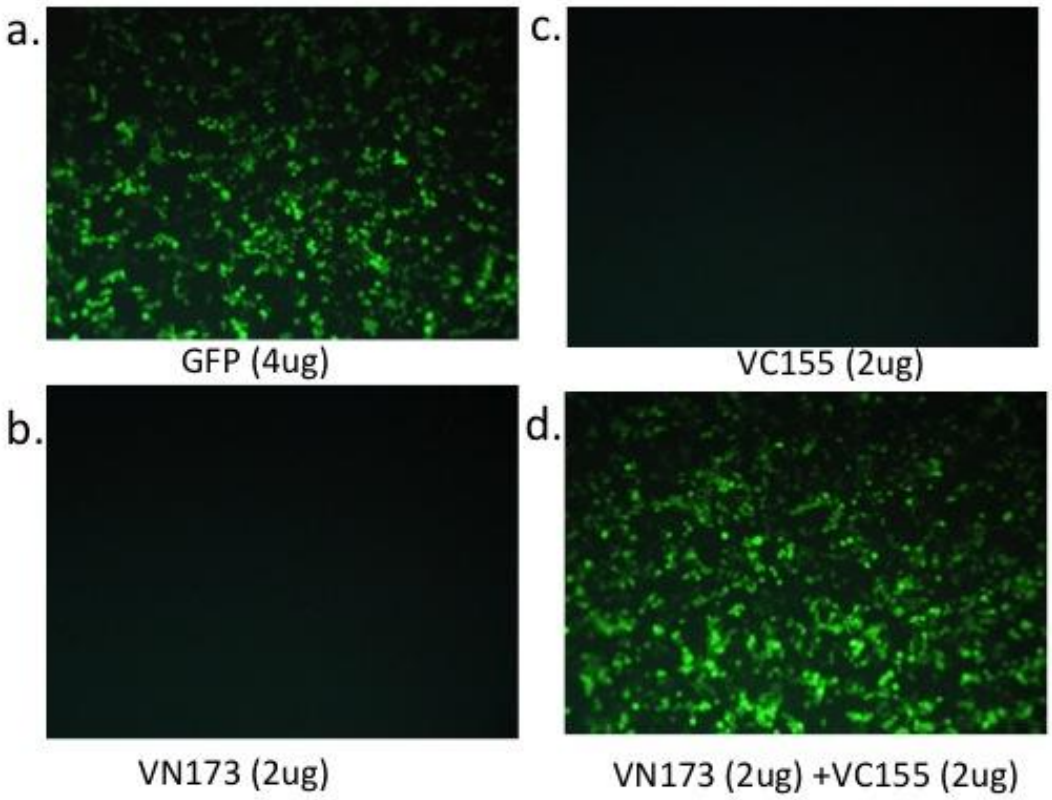



**Figure 6. Bimolecular fluorescence complementation of Venus fluorescence genes.**

VN173 and VC155 were transfected alone (b,c) or co-transfected with the ratio of 1:1 (d). The pictures were captured after 48hr of transfection. VN173 = N-terminal of venus protein to 174 amino acids. VC155 = C-terminal of venus protein from 155 amino acids. Ocular lens = 1X. Objective lens = 10X.

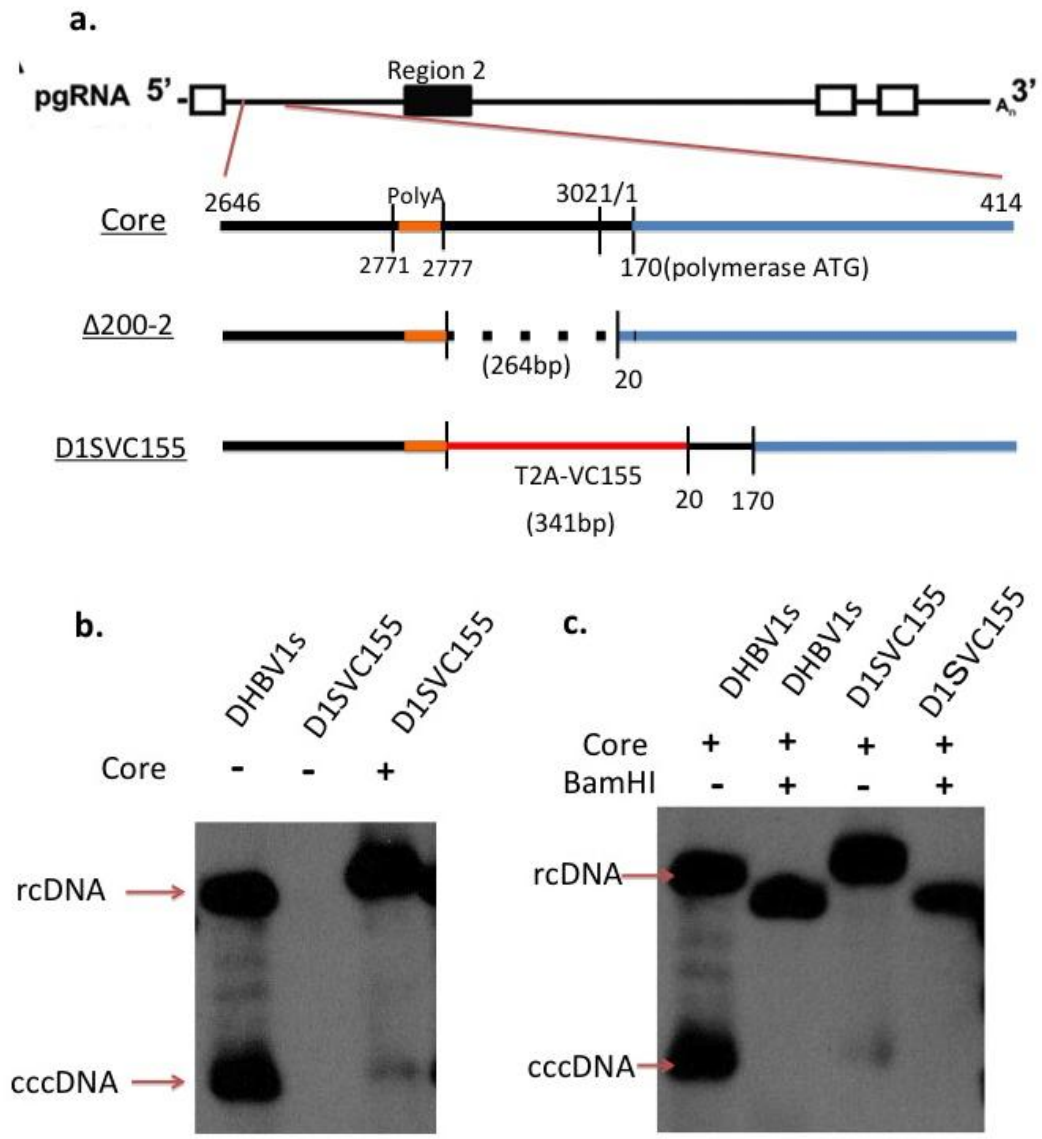



**Figure 7. Complementation of D1SVC155.**

(a) D1SVC155 is generated from the DHBV1S core deletion mutant Δ200-2 by adding gene segment encoding the C-terminal 155 amino acid sequences of Venus (fluorescence protein). T2A sequence is added at the upstream of the Venus sequences. D1SVC155 were co-transfected with CMV-Core at the ratio of 1:1 (b). After 48hr of transfection, DNAs were extracted and analyzed by Southern blot. (c) Extracted DNAs were treated with *BamHI*

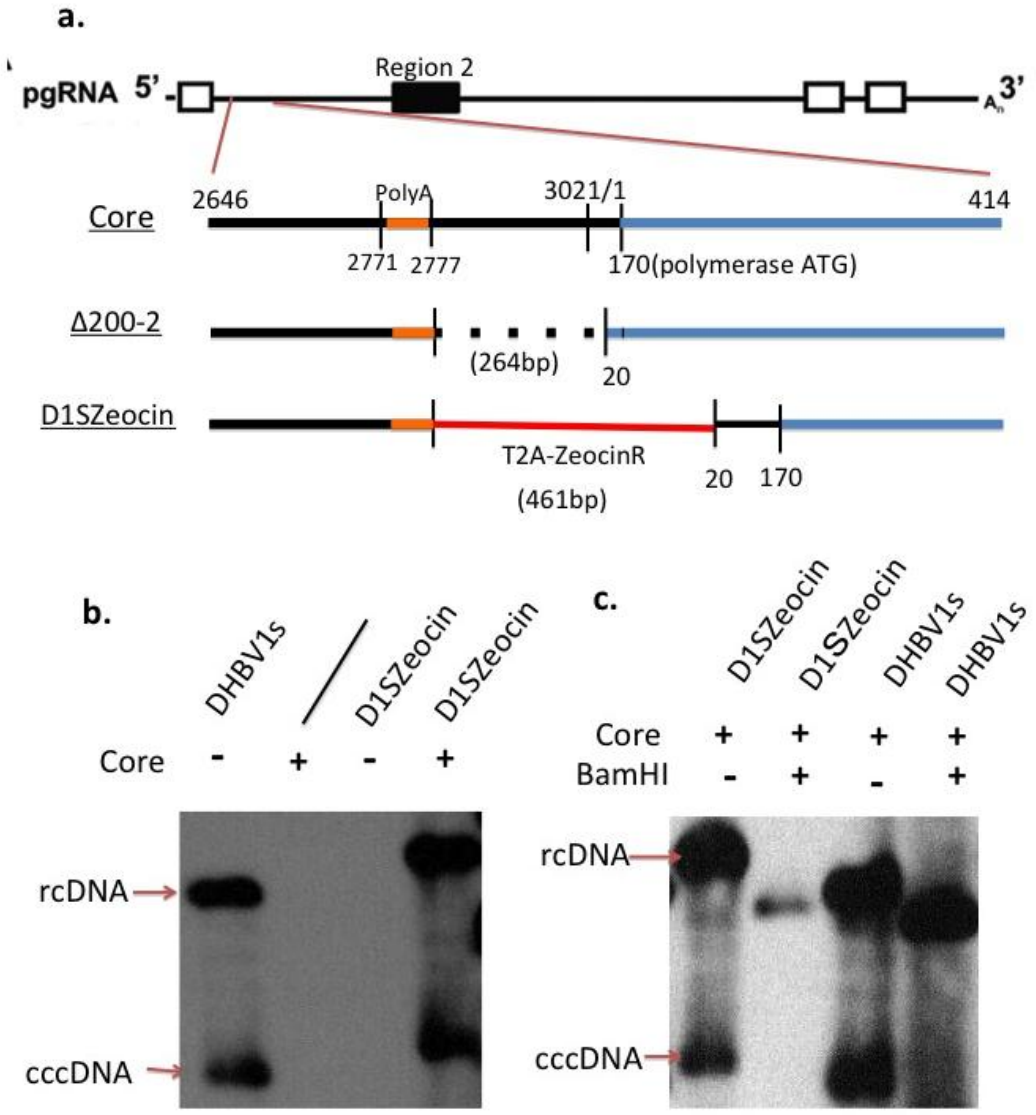



**Figure 8. Complementation of D1SZeocin.**

(a) D1SZeocin is generated from the DHBV1S core deletion mutant Δ200-2 by adding zeocin resistance gene. T2A sequence is added at the upstream of the Zeocin sequences. D1SZeocin were co-transfected with CMV-Core at the ratio of 1:1 (b). After 48hr of transfection, DNAs were extracted and analyzed by Southern blot. (c) Extracted DNAs were treated with *BamHI*.

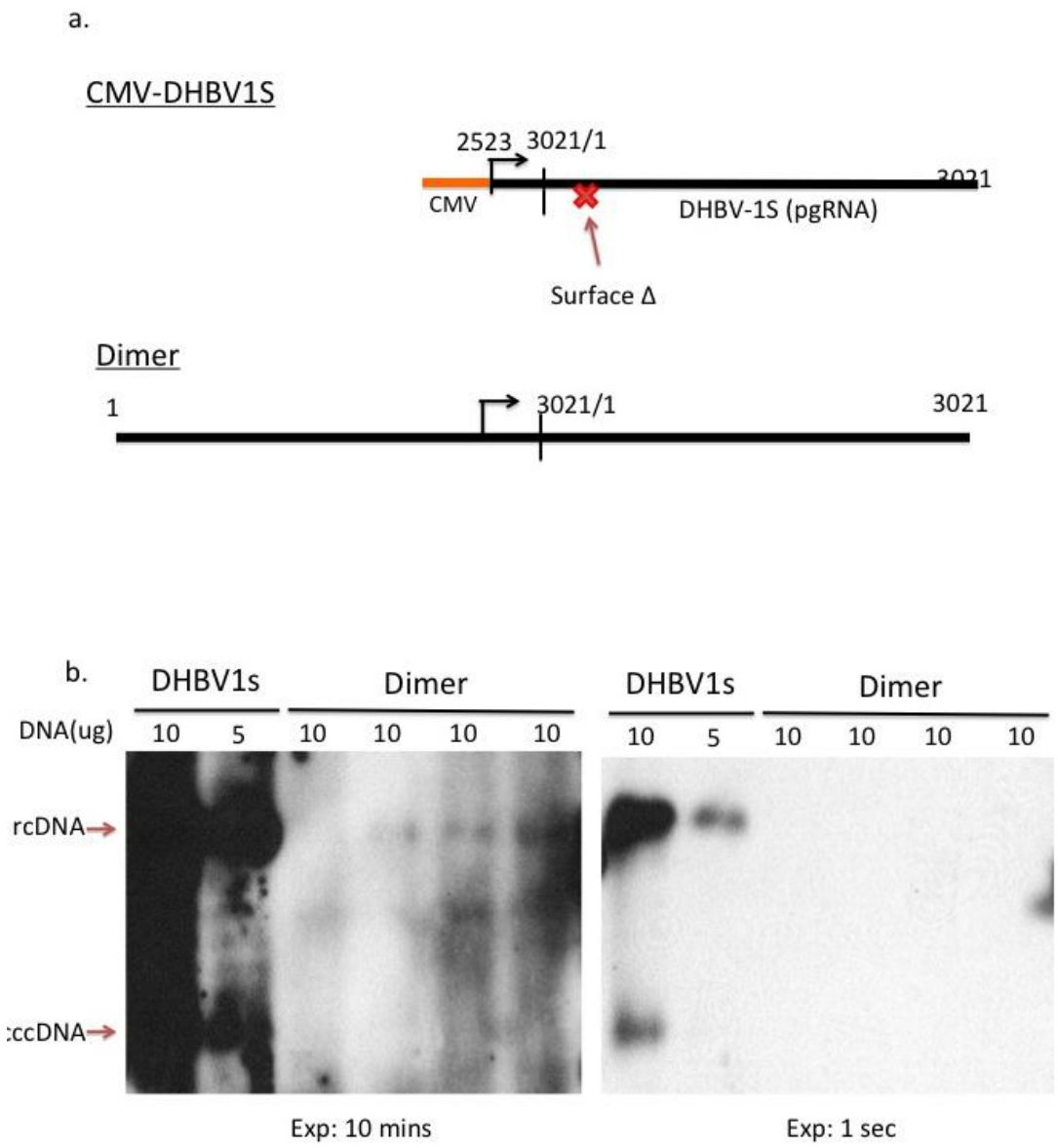



**Figure 9. Formation of RC-DNA and cccDNA in Huh7 cells transfected with DHBV1S dimer plasmid.**

(a) The DHBV1S dimer is generated from the DHBV1S plasmids by duplicating DHBV1S sequence and is added to the TA vector. (b) After 72hr of transfection, DNAs were extracted and analyzed by Southern blot. Exp: exposure time.