Video Article

Development of a Hepatitis B Virus Reporter System to Monitor the Early Stages of the Replication Cycle

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Abstract

Currently, it is possible to construct recombinant forms of various viruses, such as human immunodeficiency virus 1 (HIV-1) and hepatitis C virus (HCV), that carry foreign genes such as a reporter or marker protein in their genomes. These recombinant viruses usually faithfully mimic the life cycle of the original virus in infected cells and exhibit the same host range dependence. The development of a recombinant virus enables the efficient screening of inhibitors and the identification of specific host factors. However, to date the construction of recombinant hepatitis B virus (HBV) has been difficult because of various experimental limitations. The main limitation is the compact genome size of HBV, and a fairly strict genome size that does not exceed 1.3 genome sizes, that must be packaged into virions. Thus, the size of a foreign gene to be inserted should be smaller than 0.4 kb if no deletion of the genome DNA is to be performed. Therefore, to overcome this size limitation, the deletion of some HBV DNA is required. Here, we report the construction of recombinant HBV encoding a reporter gene to monitor the early stage of the HBV replication cycle by replacing part of the HBV core-coding region with the reporter gene by deleting part of the HBV pol coding region. Detection of recombinant HBV infection, monitored by the reporter activity, was highly sensitive and less expensive than detection using the currently available conventional methods to evaluate HBV infection. This system will be useful for a number of applications including high-throughput screening for the identification of anti-HBV inhibitors, host factors and virus-susceptible cells.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54849/>

Introduction

Chronic infection with hepatitis B virus (HBV) is a major risk factor for chronic liver diseases¹. Although current therapeutic strategies are based on nucleotide analogs that inhibit HBV pol function and/or the administration of type I interferon that activates immune responses in infected individuals as well as indirectly suppressing HBV proliferation through interferon-stimulated gene functions², these treatments cannot eliminate HBV DNA completely³. Moreover, the emergence of HBVs that are resistant to anti-pol agents is of concern⁴. The administration of combined anti-viral agents directly targeting the different steps of human immunodeficiency virus (HIV) or hepatitis C virus (HCV) life cycle was shown to successfully suppress or eradicate the virus(es). Similar to this idea, the development of anti-HBV agent(s) that act directly on the different stages of the HBV life cycle is important for establishing future HBV therapies.

In general, the establishment of a simple *in vitro* culture system of the target virus facilitates the development of anti-virus agents. However, there are at least two barriers to the development of *in vitro* culture systems to screen anti-HBV agents. The first is the lack of a convenient *in vitro* cell culture system for HBV infection/proliferation. Unlike other viruses, such as HIV and HCV, which are propagated in established cell lines, it is difficult to cultivate HBV *in vitro* because of experimental limitations including a narrow host range. The use of specific cell culture systems such as the human hepatoma cell line HepaRG, which is susceptible to HBV infection,^{5,6,7} have been developed to overcome these problems. Moreover, PXB cells, isolated from urokinase-type plasminogen activator transgenic/SCID mice inoculated with primary human hepatocyte (PHH), were shown to be susceptible to HBV infection and replication⁸. However, HBV replication levels in HepaRG are dependent on the cellular differentiation state after culture, which can cause inconsistent and irreproducible results of HBV infection/replication levels. PXB is commonly used for HBV infection experiments but is limited by its availability. A tetracycline inducible HBV expression cell line, HepAD38, has also been widely used to study HBV replication, but this system only allows evaluation after transcription and not at the entry step of HBV infection⁹. Recently, the identification of sodium taurocholate cotransporting polypeptide (NTCP) as a functional receptor for HBV has allowed the development of a variable HBV culture system¹⁰. Indeed, NTCP expression in non-susceptible hepatocarcinoma cells such as Huh7 and HepG2 enables HBV infection¹⁰ and thus, the choice of HBV susceptible cell lines has been expanded, resolving many of the experimental limitations. The second problem is the lack of a simple assay system to evaluate HBV infection and replication. Evaluation of HBV infection is usually conducted by analyzing HBV DNA, RNA and proteins. However, quantification of these virus markers is time consuming, often costly and not always simple. Therefore, the development of a simple assay system, such as using a reporter gene, might overcome problems associated with HBV assay systems.

However, because the genome size that can be packaged into an HBV capsid is limited — less than 3.7 kb¹¹ — the size of a reporter gene should be as short as possible. Furthermore, the presence of multiple cis elements scattered throughout the genome, which are essential for viral replication, limits the positions available for insertion of the reporter gene into the genome. Several reports have attempted to insert foreign genes, including HIV-1 Tat, green fluorescent protein, and DsRed, into the HBV genome^{11,12,13}. However, these recombinant HBVs are not useful for screening HBV infection/replication, or for the high-throughput screening of factors affecting HBV infection/replication. This is mainly because of the low productivity of recombinant viruses and the reduced intensity of reporter gene expression caused by inefficient virus production.

To overcome these issues, we constructed a reporter HBV with a high yield of virus production. This virus is highly sensitive for monitoring the early stages of the HBV replication cycle, from entry to transcription. To achieve this, NanoLuc (NL) was chosen as a marker gene because it is a small (171 amino acids) engineered luminescent reporter¹⁴. Moreover, NL is approximately 150-fold brighter than firefly or Renilla luciferase, and the luminescent reaction is ATP-independent, suggesting that the false hit rate will be low for high-throughput screening. The production efficiency of the recombinant HBV is approximately 1/5 of the parent HBV, and similar to levels reported for previous HBV recombinant viruses; however, the brightness of NL overcomes virus productivity issues so it can be used for the mass screening of anti-HBV agents.

Screening of anti-HBV agents using primary hepatocytes, HepaRG, HepAD38 and NTCP-transduced hepatocytes might be useful for the screening of anti-HBV agents by conventional method(s). However, the system described here has various advantages such as simple handling, high sensitivity, and low cost for screening. These advantages are suitable for high throughput assays to develop and identify new HBV agents for therapeutic purposes.

Protocol

1. Production of Recombinant HBV Encoding the Reporter Protein

1. Preparation of HepG2 cells

1. Prepare cell culture medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 U/ml nonessential amino acids).
2. Plate 4×10^6 HepG2 cells in a 10-cm collagen-coated dish in 10 ml of culture medium the day before transfection. Incubate HepG2 cells at 37 °C in a humidified 5% CO₂ incubator.
NOTE: When approximately 4×10^6 HepG2 cells are plated in a 10 cm collagen-coated dish in 10 ml of culture medium, they will be 70-90% confluent the next day.

2. Transfection

1. Transfect HepG2 cells with 5 µg of pUC1.2HBV delta epsilon¹⁵ and 5 µg of pUC1.2HBV/NL¹⁵ using a transfection reagent as per manufacturer's instructions.
2. The next day, remove the culture medium and add 10 ml of fresh culture medium.
3. One week after transfection, transfer the culture medium containing the recombinant HBV to a 50 ml-tube and proceed to step 1.3.1. Add 10 ml of fresh culture medium to the plate containing the transfected cells.
NOTE: Production of the recombinant HBV is maintained for 4 weeks. The culture medium containing recombinant HBV can be stored at 4 °C for 1 month.

3. Purification of recombinant HBV

1. Remove cell debris from the culture medium containing recombinant HBV by centrifugation (2,300 x g for 5 min).
2. Pass the supernatant through a 0.45 µm membrane filter.
3. Add an equal volume of 26% PEG/1.5 M NaCl (polyethylene glycol 6000: 130 g, NaCl, 49 g, 1 ml of 0.5 M EDTA pH 8.0 and 5 ml of 1 M HEPES pH 7.6 in 500 ml) to the culture medium containing recombinant HBV and mix gently. Incubate overnight at 4 °C.
4. Centrifuge at 2,300 x g for 20 min at 4 °C.
5. Discard the supernatant and dissolve the pellet in 0.5 ml of TNE (10 mM Tris, 50 mM NaCl, 1 mM EDTA).
6. Remove the debris by centrifugation at 2,300 x g for 5 min.
7. Load 0.5 ml of TNE containing recombinant HBV onto 0.8 ml of 20% sucrose in TNE.
8. Centrifuge at 100,000 x g for 3 hr at 15 °C.
9. Discard as much of the supernatant as possible, and save the pellet. Resuspend it in 1 ml of serum free DMEM per 40 ml of starting culture medium.
10. Incubate overnight at 4 °C.
11. Filter through a 0.45 µm filter. Prepare 0.5 ml aliquots and store at -80 °C.
NOTE: If reporter protein contamination of the original virus sample is observed, purify the virus by density gradient ultra-centrifugation of 5-30% sucrose in TNE at 100,000 x g for 2 hr, or CsCl density equilibrated centrifugation from 1.1-1.6 g/ml at 150,000 x g for 50 hr.

2. Infection of Recombinant HBV

1. Culture HepG2 cells stably expressing NTCP (HepG2/NTCP)¹⁵ that are susceptible to HBV infection in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 µg/ml G-418 and 100 U/ml nonessential amino acids at 37 °C in a humidified 5% CO₂ incubator.
2. One day before infection, plate approximately 5×10^4 HepG2/NTCP cells¹⁴ into a 96-well collagen coated plate in 0.1 ml of culture medium.
3. Thaw recombinant HBV in a 37 °C water bath until there is a small bit of ice remaining in the vial.
4. Prepare the medium for infection by combining the following: 10 µl of 40% PEG8000 in 1x phosphate buffered saline (PBS), 2 µl of dimethyl sulfoxide (DMSO), 10 µl of recombinant HBV and 78 µl of fresh culture medium per well of a 96-well plate.
5. Add 100 µl of recombinant HBV solution to one well of the 96-well plate.

- One day after infection, wash the infected cells 3 times with 300 μ l PBS per well to remove the contaminating reporter protein from the virus fraction.
- Incubate infected cells in 200 μ l of culture medium containing 2% DMSO for one week or less.

3. Analysis

- One week after infection, wash the infected cells 3 times with PBS.
- Add 50 μ l of lysis buffer to the infected cells.
- Rock the culture plate for 5 min, and then centrifuge at 2,000 x g for 5 min.
- Add 50 μ l of the reporter substrate into the luminometer plate.
- Add 20 μ l of cell lysate to a luminometer plate containing the reporter substrate. Mix by vortexing briefly.
- Place the plate in the luminometer and initiate reading¹⁵.

Representative Results

Figure 1 shows a schematic of the HBV genome, transcribed RNAs, virus proteins and their coding region on the genome. The reporter gene and its location in the genome are also indicated. **Figure 2** shows the HBV reporter plasmid and helper plasmid containing the reporter gene. The pUC1.2HBV/NL reporter was constructed by deleting nucleotide positions 223-811 from the transcription initiation site of the preCore mRNA of pUC1.2HBV¹⁶ and then inserting the reporter gene. The pUC1.2HBVdelta with point mutations in the encapsidation signal sequence was generated by site-directed mutagenesis PCR. Reporter plasmid (pUC1.2HBV/NL) and helper plasmid (pUC1.2HBVdelta) were digested with *HindIII* and *EcoRI*, and then subjected to gel electrophoresis. The expected bands, which are 3.0 kb for the pUC plasmid and 3.5 kb for 1.2HBV/NL or 1.2HBVdelta, are shown in **Figure 2B**. **Figure 3** shows HepG2 cells expressing NTCP infected with recombinant HBV. To establish HepG2 cells stably expressing NTCP, HepG2 cells were transfected with pCAN-NTCP-myc encoding NTCP-myc and a neomycin resistant gene. G418-resistant cell clones were selected and expanded. The HepG2-NTCP-myc-clone22 is susceptible to HBV infection. The lysates of HepG2 or HepG2-NTCP-myc-clone22 were subjected to western blotting. NTCP is a glycoprotein of 349 amino acids, with an extracellular N-terminus containing two N-linked glycosylation sites (Asn5 and Asn11). The 43 kDa band of unglycosylated NTCP and the 65 kDa band of glycosylated¹⁰ are shown in **Figure 3A**. HepG2-NTCP-myc-clone22 cells expressing NTCP, but not HepG2 cells, were susceptible to recombinant HBV infection. **Figure 4** shows the kinetics of the reporter gene (A and B), virus RNA and DNA (A) levels in recombinant HBV-infected HepG2-NTCP-myc-clone22 (A) or human primary hepatocyte (PHH) cells (B). The levels of HBV RNA and reporter activity were elevated at 3 days after infection in HepG2-NTCP-myc-clone22 or PHH cells. In contrast, there was no change in DNA levels at 9 days after infection because recombinant HBV is deficient for functional core and pol that have an important role in the intracellular DNA replication pathway. **Figure 5** shows the inhibition of recombinant HBV by hepatitis B immune globulin (HBIG), heparin and IFN- β . Entry inhibitors such as HBIG and heparin strongly suppressed the reporter activity by less than 10% at 75 U/ml and 150 U/ml, respectively, while IFN- β suppressed the reporter activity by less than 50% at 1,000 U/ml. The inhibitory effect of these inhibitors was dose dependent. **Figure 6** shows the life cycle of HBV.

Figure 1: Schematic of the HBV genome and relative location of virus RNA and proteins on the genome. HBV DNA is shown by an arc in black. The location of the enhancer and promoters for the transcription of virus RNAs on the arc is shown in yellow. The location of viral RNAs and proteins on the genome are represented by arrows with dotted blue lines (RNA) and with colored arrows (proteins), respectively. Virus RNAs are discriminated by size: 3.5 kb and 3.4 kb mRNAs indicate PreCore/C and pregenomic RNA/Core, respectively, 2.5 and 2.1 kb RNAs represent preS1 and preS2/S, respectively, and the 0.8 kb RNA indicates X gene¹⁷. A reporter gene is substituted by the relevant size of core coding sequence. The reporter gene is driven by a core promoter containing enhancer II. Pro: Promoter, EnhI/pro: Enhancer I/promoter, EnhII/Pro: Enhancer II/promoter, Pol: Polymerase, S: Surface antigen. [Please click here to view a larger version of this figure.](#)

A

B

HindIII/EcoRI digestion

Figure 2: Schematic for the generation of recombinant HBV. (A) Blue lines indicate the pregenome RNA. The "A" stretch represents poly A at the 3' terminus. Blue boxes indicate the coding region for each virus protein. The red box indicates a reporter gene translated from its own initiation codon. The reporter plasmid cannot produce PreCore and Pol, and the helper plasmid containing 2 mutations in the encapsidation signal (CTGTGCC to CTATGTC) expresses all HBV proteins. E: Encapsidation signal. (B) The reporter plasmid, helper plasmid and pUC plasmid were digested with *EcoRI* and *HindIII*. These digested plasmids were subjected to gel electrophoresis. [Please click here to view a larger version of this figure.](#)

Figure 3: Recombinant HBV infects HepG2 expressing NTCP. (A) A stable cell line expressing NTCP was established by the transfection of HepG2 cells with a plasmid encoding Myc-tagged NTCP followed by selection with 500 μg/ml of G418 for 3 weeks. The level of NTCP-Myc in HepG2 cells stably expressing NTCP (HepG2-NTCP-Myc-clone22) cells was determined by western blotting using anti-Myc antibodies (1:1,000 dilution). (B) HepG2-NTCP-Myc-clone22 cells were infected with recombinant HBV in the presence of 2% DMSO and 4% PEG8000. At 72 hr after infection, the level of reporter activity was determined by reporter assay. Results are representative of three independent experiments, and error bars show the standard deviations of the means. [Please click here to view a larger version of this figure.](#)

Figure 4: Time course of recombinant HBV infection. (A) HepG2-NTCP-Myc-clone22 cells were infected with recombinant HBV in the presence of 2% DMSO and 4% PEG8000. The level of HBV infection was determined by reporter activity (black line) at 3, 6 or 9 days after infection. Levels of HBV RNA (blue line) and DNA (orange line) were simultaneously measured by RT-PCR and PCR¹⁵, respectively, at each infection time point. (B) Primary human hepatocytes (PHH), isolated from urokinase-type plasminogen activator transgenic/SCID mice inoculated with PHH, were infected with recombinant HBV in the presence of 2% DMSO and 4% PEG8000. The level of HBV infection was determined by reporter activity at 3, 6, or 9 days after infection. [Please click here to view a larger version of this figure.](#)

Figure 5: Effect of known anti-HBV agents on recombinant HBV infection. HepG2-NTCP-Myc-22 cells were infected with recombinant HBV in the presence of HBIG, heparin and IFN-β at the doses indicated, as well as 2% DMSO and 4% PEG8000. The level of HBV replication (**A**) and cell viability (**B**) was determined by reporter activity 6 days after infection. HBIG is an antibody that neutralizes HBV infection and heparin is an inhibitor for enveloped viruses. Results are representative of three independent experiments, and error bars show the standard deviations of the means. [Please click here to view a larger version of this figure.](#)

Figure 6: Schematic of the HBV life cycle. HBV enters into cells through virus receptors including NTCP. Capsid-associated relaxed circular DNA (rcDNA) is uncoated and converted to covalently closed circular DNA (cccDNA) in the nucleus. cccDNA functions as a template for mRNA transcription. The genomic RNA is encapsidated to the virus capsid by assembling with proteins for core and pol. Before further assembling with HBS proteins, the capsid is involved in amplifying HBV DNA by a process called the cccDNA replication process. Virus particles assembled with HBS proteins eventually are released outside the cell. [Please click here to view a larger version of this figure.](#)

Discussion

The HBV genome has four primary open reading frames (ORFs) including the core, polymerase, surface and X ORFs (**Figure 1**). Transcription of these four HBV ORFs is tightly regulated by four promoters: the precore/core promoter containing enhancer II, S1 promoter, S2 promoter and X promoter containing enhancer I¹⁸. The 3.5 kb and 3.4 kb mRNAs are translated into PreCore and Core proteins, respectively. The large envelop protein (L) is produced from the largest subgenomic mRNA (2.5 kb), and the middle (M) and small surface proteins (S) are translated from the shorter transcript (2.1 kb). The 0.8 kb transcript is the template for translation of the X protein^{19,20}. The 5' and 3' ends of pregenomic RNA contain multiple functional cis elements that include direct repeat 1 (DR1), DR2 and a packaging signal^{21,22}. These functional elements limit the insertion of reporter or marker genes into the pregenomic RNA. We constructed recombinant HBV by using trans-complementation of two vectors, a transfer vector containing the reporter gene and a helper vector. The transfer vector was constructed by replacement of the core and pol regions with the reporter gene (**Figure 2**).

NTCP was identified as a cellular receptor for HBV entry¹⁰. Because the expression of NTCP mRNA is very low in HepG2 cells, which are not susceptible to HBV infection, we established a stable HepG2 cell line expressing NTCP-myc (**Figure 3A**). Whereas recombinant HBV infection of HepG2 cells showed no reporter activity, NTCP expression in HepG2 cells conferred susceptibility to recombinant HBV infection (**Figure 3B**). The time course analysis of recombinant HBV infection demonstrated that both reporter activity and HBV RNA were detectable within 3 days of recombinant HBV infection and peaked at 9 days postinfection; however, increased HBV DNA levels were not observed (**Figure 4**). Entry

inhibitors, such as HBIG or heparin, inhibited recombinant HBV infection (**Figure 5**). These results indicated this system can be used to monitor early stage HBV replication from virus entry to transcription, but not DNA replication and the re-infection of virus released from infected cells (**Figure 6**).

Since the reporter protein often contaminates the recombinant HBV fraction, washing the virus infected cells with PBS less than three times may result in a high background. Therefore, it is important to check the background level by measuring reporter activity in medium from HBV-unsusceptible HepG2 treated with the recombinant virus. If the background is high, add a washing step (3 times washing with 300 μ l PBS per well in a 96-well plate) the day before step 3.1.

If the production of the recombinant HBV is low, check the transfection efficiency with a control plasmid expressing a fluorescent protein, such as GFP or DsRed. In general, the efficiency of transfection of HepG2 cells should be greater than 35%. If the transfection efficiency is low, optimize specific transfection conditions to achieve high transfection efficiencies. If the transfection efficiency is high, check whether infectious recombinant HBV is produced by using it to infect primary human hepatocytes. Infectivity of HBV to primary human hepatocytes is usually higher than to hepatoma cell lines expressing NTCP. If the efficient infection of recombinant HBV is achieved in primary human hepatocytes, the problem is not the recombinant HBV but the cells to be infected. Establish a number of stable HepG2 clones expressing NTCP and select highly infectious cell clones. Low cell confluency in culture at the time of infection may result in poor infectivity. Increasing the number of cells in the culture improves the infection efficiency. For HBV infection, 80-95% confluency for HepG2 cells expressing NTCP at the time of infection provides a high infection rate.

The development of several reporter HBVs has been reported by many groups^{11,12,13}, but most of these reporter HBVs show poor productivity. Moreover, the reporter activity in infected cells is not as high, and would be difficult to use for screening anti-HBV agents using a high-throughput assay. In contrast, our reporter HBV system produces a strong reporter signal in the infected cells that makes it easy to conduct mass screening for anti-HBV agents using a relatively small amount of the virus compared to other reporter viruses reported thus far.

Here, we generated a novel HBV reporter system to monitor the early stages of the HBV replication cycle and this was validated by measuring the reporter protein activity after infection. The described method is a simple, rapid, and cost-effective HBV vector system and will be useful for the identification of HBV host factors as well as anti-HBV compounds by using high-throughput screening methods. Indeed, we identified HBV host factors and anti-HBV genes by whole-genome RNAi technology¹⁵. Of note, this system is not suitable for the evaluation of the late stages of HBV replication such as the DNA replication step, capsid, and virus assembly, and budding, because recombinant HBV is deficient for the production of a functional core and pol. The further development of recombinant HBV is required to evaluate the entire HBV life cycle.

In summary, this system can be used to evaluate HBV infection by measuring reporter activity. Therefore, there is no need to conduct complicated assay techniques such as PCR, RT-PCR, HBV Core or S antigen ELISA to evaluate HBV infection/replication. Finally, because this reporter HBV is replication incompetent, there is no risk of infection.

Disclosures

The authors declare that they have no competing financial interests.

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