Kinetics of HBV DNA replication, cccDNA formation and HBsAg production in a "pgRNA launch" HBV replication system and its susceptibility to antiviral agents

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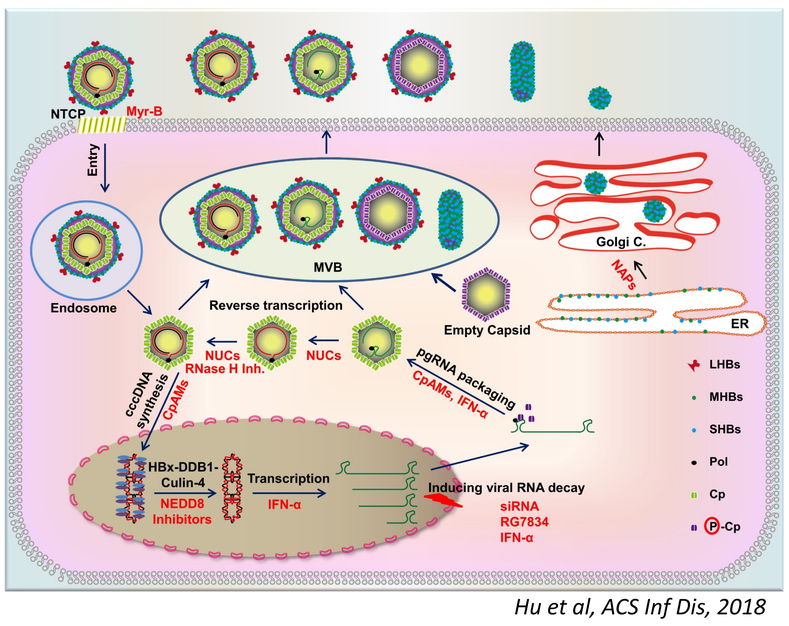
Abstract

Hepatitis B virus (HBV) replicates its genomic DNA by reverse transcription of pregenomic RNA (pgRNA), which also serves as the mRNA for core protein and viral DNA polymerase. Although it had been shown that pgRNA is infectious with DHBV (*Huang and Summers, Journal of Virology, 1991, 65:5435-9*), it was not reported until recently that transfection of *in vitro*-transcribed pgRNA into human hepatoma cell line Huh7.5 initiates HBV replication (*Yu, et al., bioRxiv, Oct. 2, 2019; http://dx.doi.org/10.1101/787630*). Here, we further characterized the kinetics of pgRNA launch HBV replication in Huh7.5 cells and showed that viral capsids, single-stranded DNA, rcDNA and cccDNA became detectable sequentially at 3, 6, 12 and 24 h post-pgRNA transfection, during which the levels of viral DNA replication intermediates and cccDNA peaked at 24 and 48 h post-pgRNA transfection, respectively. HBsAg became detectable in the culture medium at 4 days post-pgRNA transfection, and gradually increased over the following 6 to 8 days. In agreement with the critical role of HBx in the activation of cccDNA transcription, site-directed mutagenesis demonstrated that HBsAg production is strictly HBx-dependent, while the early robust viral DNA replication and cccDNA synthesis does not depend on HBx expression. Taking advantage of the sequential and robust HBV replication within 48 h post-pgRNA transfection, we showed that capsid assembly, pgRNA encapsidation, DNA synthesis and/or cccDNA formation can be specifically targeted by distinct antiviral agents. On the contrary, treatment of the pgRNA-transfected cells starting at 48 h post-pgRNA transfection allows assessment of antiviral agents on mature nucleocapsid uncoating, cccDNA synthesis and transcription, as well as HBV RNA stability. In summary, compared to HBV replicon plasmid transfection, the pgRNA launch system permits accurate dissection of antiviral mechanisms without interference from input plasmid DNA, allowing investigation of cccDNA transcription and metabolism with secreted HBsAg as a quantitative marker.

Introduction

HBV cccDNA can be synthesized from either virion DNA during infection (*de novo* pathway) or progeny cytoplasmic rcDNA (intracellular amplification pathway)



Hu et al, ACS Inf Dis, 2018

- **Due to the lack of cell culture systems without transgenes, transcriptional regulation of cccDNA derived from intracellular amplification pathway, and especially its dependence on HBx, cannot be investigated.**
- **Since dslDNA is minor species of HBV DNA and can be converted into cccDNA, the pgRNA launch HBV replication system also provides an experimental system to investigate the biological property of dslDNA-derived cccDNA.**

Study Objectives

- **To evaluate the cccDNA formation and HBV replication kinetics using the pgRNA launch HBV replication system**
- **To investigate the transcriptional regulation of cccDNA, and to compare the biological properties of cccDNA derived from rcDNA versus dslDNA.**
- **To map the molecular mechanisms and potential drug resistance profiles of antiviral agents.**

1. Establishment of a pgRNA-launch HBV replication system

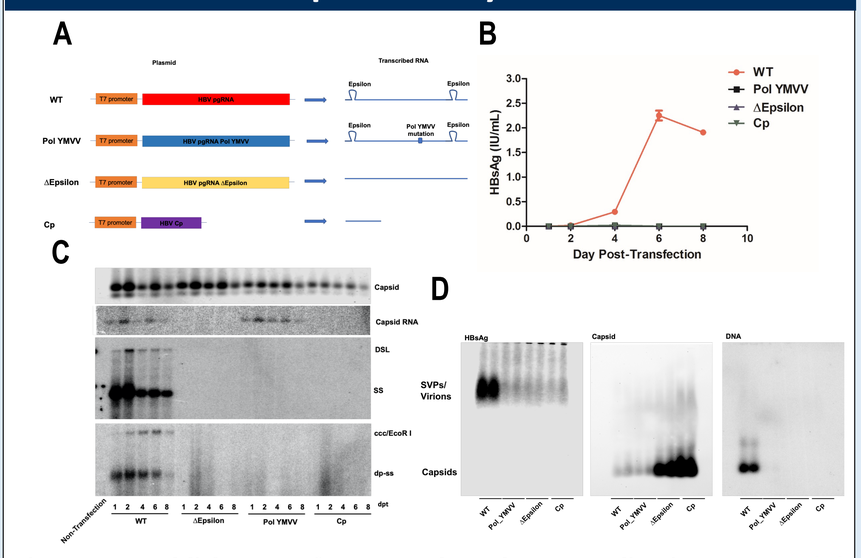


Figure 1. Establishment of a pgRNA-launch HBV replication system. (A) Schematic presentation of plasmid constructs for *in vitro* pgRNA transcription. Huh7.5 cells were transfected with indicated RNA transcript, from which **(B)** cell culture medium was collected at 1, 2, 4, 6 and 8 day-post-transfection (dpt) for HBsAg detection by an ELISA kit, **(C)** cells were harvested at 1, 2, 4, 6 and 8 dpt for the detection of capsids and the associated RNA by a 1.8% agarose gel electrophoresis assay. Core DNA and cccDNA were analyzed by Southern blot hybridization. DNA in Hirt supernatants was denatured at 88°C for 8 min to convert DP-rcDNA into single-stranded DNA, followed by restriction with EcoRI to convert cccDNA into unit-length double stranded linear DNA (3.2 kb). **(D)** Culture media were harvested from 4 to 8 dpt and concentrated by ultracentrifugation. Subviral particles (SVPs)/virions, secreted capsids and their associated DNA were separated by 1.0% agarose gel electrophoresis and detected by immunoassay with indicated antibodies, as well as hybridization with an HBV minus strand-specific riboprobe.

2. Early kinetics of pgRNA-launch HBV replication and its application in antiviral evaluation

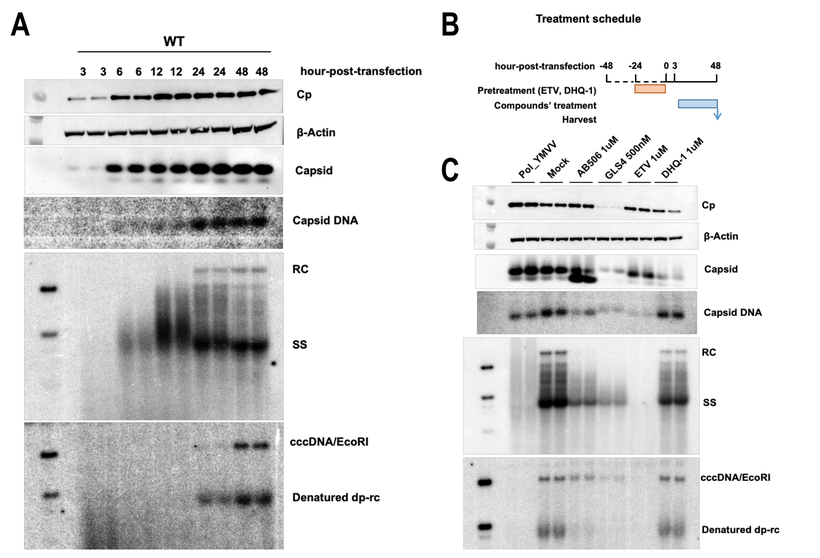


Figure 2. Robust HBV DNA replication and cccDNA synthesis occurs upon transfection of pgRNA in Huh7.5 cells. (A) Huh7.5 cells were transfected with *in vitro*-transcribed pgRNA. Cells were harvested at 3, 6, 12, 24 and 48 h post-transfection. **(B)** Treatment schedule of antiviral agents in pgRNA-launch HBV replication system. **(C)** Huh7.5 cells transfected with *in vitro*-transcribed pgRNA were treated with the indicated antiviral agents and collected at 48 h post-transfection. Intracellular HBV core protein was detected by a Western blot assay with a rabbit polyclonal antibody HBc-170A. β-Actin served as a loading control. The capsids were separated by 1.8% agarose gel electrophoresis, transferred to a nylon membrane and detected by a mouse monoclonal antibody against HBV core protein. HBV DNA replication intermediates and cccDNA were detected by Southern blot hybridization assay.

3. Assessment of anti-HBV agents on mature nucleocapsid uncoating and viral RNA stability

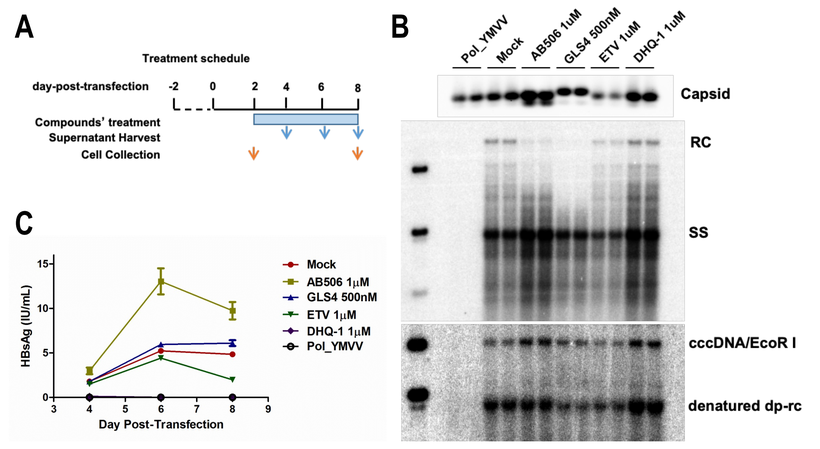


Figure 3. Effects of delayed addition of antiviral agents on HBV replication intermediates. (A) Schedule of antiviral agent treatment. **(B)** Huh7.5 cells were transfected with *in vitro*-transcribed pgRNA and treated with the indicated antiviral agents starting at 48 h post-transfection. Culture media were harvested at 2, 4 and 6 dpt. Cells were harvested at 6 dpt. Intracellular capsids were detected by native agarose gel-based particle gel assay. HBV DNA replication intermediates and cccDNA were detected by Southern blot. **(C)** HBsAg level in the supernatants were measured by ELISA.

4. Expression of HBsAg in pgRNA transfected cells depends on HBx-DDB1 interaction

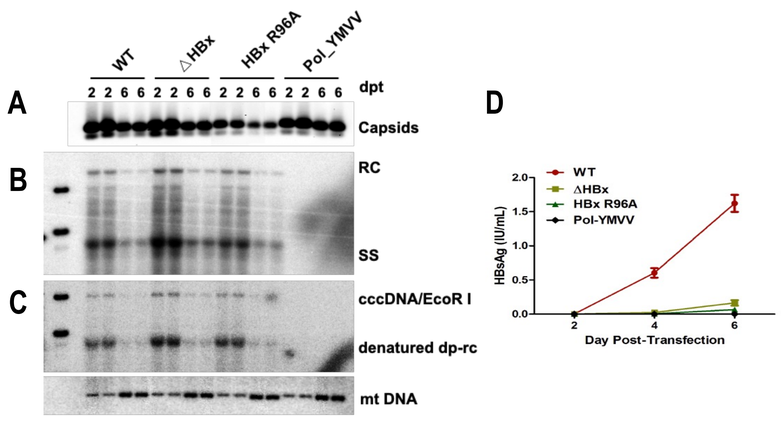


Figure 4. HBsAg expression depends on HBx and its interaction with DDB1. Huh7.5 cells were transfected with *in vitro*-transcribed WT or the indicated mutant pgRNA. Culture media were harvested at 2, 4 and 6 dpt. Cells were harvested at 2 and 6 dpt. **(A)** Intracellular capsids were detected by native agarose gel-based particle gel assay. **(B)** HBV DNA replication intermediates and **(C)** cccDNA were detected by Southern blot hybridization. Mitochondrial DNA served as a loading control for cccDNA. **(D)** HBsAg in culture media was measured by ELISA.

5. Transcription of cccDNA-derived from dslDNA appears to be less dependent on HBx

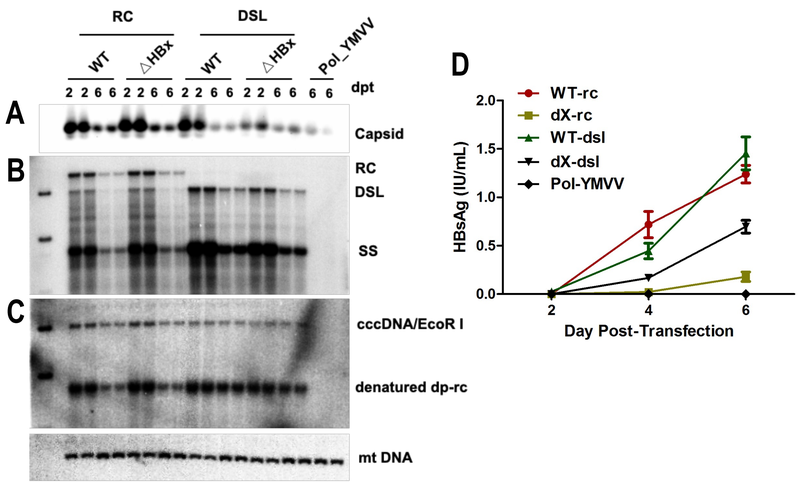


Figure 5. Transcriptional activity of cccDNA-derived from dslDNA appears to be less dependent on HBx. Huh7.5 cells were transfected with *in vitro*-transcribed WT or mutant pgRNA. In pgRNA termed "DSL", the addition of two nucleotides at the 5' terminus prevents the efficient translocation of RNA primer and results in the synthesis of double-stranded linear (dsl) DNA. Culture media were harvested at 2, 4 and 6 dpt. Cells were harvested at 2 and 6 dpt. **(A)** Intracellular capsids were detected by native agarose gel-based particle gel assay. **(B)** HBV DNA replication intermediates and **(C)** cccDNA were detected by Southern blot hybridization. Mitochondria DNA served as a loading control for cccDNA. **(D)** HBsAg in culture media was measured by ELISA.

Conclusions

- **Following transfection of functional pgRNA into Huh7.5 cells, robust levels of HBV DNA replication and cccDNA synthesis occur sequentially. This experimental system permits investigation of HBV cccDNA intracellular amplification and transcription without interference from integrated HBV transgenes.**
- **The robust early cccDNA formation and HBV replication in pgRNA-transfected cells allows for identification of viral replication steps targeted by antiviral agents.**
- **Conversely, delayed treatment of pgRNA-transfected cells allows for investigation of antiviral effects on mature nucleocapsid uncoating, cccDNA transcription and viral RNA stability.**
- **The dependence of HBsAg expression on HBx function allows for monitoring cccDNA transcriptional regulation and HBx function by measuring secreted HBsAg in culture media.**