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The pregenome/C RNA of duck hepatitis B virus is not used for translation of core protein during the early phase of infection *in vitro*

Qiang Liu[a,b,1], Juan Huang[a,b,1], Renyong Jia[a,b,c,*,1], Mingshu Wang[a,b,c,1], Dekang Zhu[b,c], Shun Chen[a,b,c], Mafeng Liu[a,b,c], Zhongqiong Yin[c], Yin Wang[c], Anchun Cheng[a,b,c,**]

[a] *Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan 611130, PR China*
[b] *Avian Disease Research Center, Sichuan Agricultural University, 46 Xinkang Road, Ya'an, Sichuan 625014, PR China*
[c] *Key Laboratory of Animal Disease and Human Health of Sichuan Province, Chengdu, Sichuan 611130, PR China)*

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ABSTRACT

Over the course of duck hepatitis B virus (DHBV) replication, one type of RNA (pregenome/C RNA, 3.5 kb) that corresponds to the whole genome of DHBV is generated from the transcription of viral cccDNA. Previous work has proposed three functions for the pregenome/C RNA: it can serve as the pregenome and be packaged into the core protein during the process of replication, and it encodes the mRNA for both the capsid protein and the viral polymerase. However, little is known about the timing of these functions during the different stages of viral infection. In this study, a reverse transcription quantitative real-time PCR assay was developed to analyze the dynamic transcription process of the pregenome/C RNA. The dynamic expression of the core protein was investigated using an indirect immunofluorescence assay (IFA) and by western blot analysis. The generation of pregenome/C RNA began at 12 h post infection and peaked at 20 h post infection; however, the core protein was not detectable until 24 h post infection. These results demonstrate that the core protein appeared approximately 12 h later than the pregenome/C RNA. These results suggest that the DHBV pregenome/C RNA is not used for the translation of the viral core protein during the early stages of infection.

1. Introduction

Duck hepatitis B virus (DHBV), which was discovered in 1980 (Mason et al., 1980), belongs to the Avihepadnavirus genus of the Hepadnaviridae family of viruses. DHBV shares several fundamental features with human hepatitis B virus (HBV) and serves as an important model virus for HBV studies. Most key features of Hepadnaviridae viruses were first discovered in DHBV and subsequently confirmed in HBV; such features include the replication of the viral genome by reverse transcription of a long RNA (Summers and Mason, 1982), details of reverse transcription by the virus (Wang and Seeger, 1992), and mechanisms by which covalently closed circular (ccc) DNA is formed and amplified (Tuttleman et al., 1986). Thus, studies of DHBV are of use in elucidating details of the replication mechanisms used by HBV or pathogenic features of the virus.

The life cycle of DHBV starts with the attachment of viral particles to their receptors on the surface of a target host cell (Stoeckl et al., 2006). After binding, viral particles are taken up into the cell by receptor-mediated endocytosis (Kock et al., 1996). The nucleocapsid is released from the endosomal compartment into the cytosol and transported to the nucleus to initiate productive infection (Funk et al., 2004). After reaching the nuclear membrane, the core protein is presumably phosphorylated, and a nuclear localization signal is exposed; this leads to uptake of the nucleocapsid into the nucleus (Kann et al., 1999). In the nucleus, the relaxed form of the viral genome (relaxed circular DNA or rcDNA) is converted into a plasmid-like, covalently closed circular form (cccDNA). Several genomic and subgenomic RNAs are transcribed from the cccDNA by cellular RNA polymerase II (Rall et al., 1983); of these, the pregenomic RNA (pgRNA) is selectively packaged into progeny capsids and reverse transcribed by the co-packaged P protein into new rcDNA genomes. Mature rcDNA-containing nucleocapsids can be used for intracellular cccDNA amplification or be enveloped and released from the cell in progeny virions.

* Corresponding author at: Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan 611130, PR China. Tel.: +86 13980859871.
** Corresponding author at: Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan 611130, PR China.
E-mail addresses: jiary@sicau.edu.cn (R. Jia), chenganchun@vip.163.com (A. Cheng).
[1] These authors contributed equally to this work.

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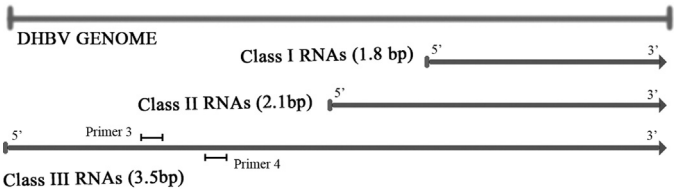

Fig. 1. Schematic representation of viral RNAs and locations of primers 3 and 4. Three different classes of viral RNA are transcribed from DHBV cccDNA. Class I (1.8 kb) viral RNAs encode a small surface protein, class II (2.1 kb) viral RNAs encode large surface proteins and class III (3.5 kb) RNAs encode the polymerase or core protein or serve as the pregenome used to reverse transcribe the DHBV genome. Primers 3 and 4, located in the 5′ end of the longest viral RNA, were used in the qPCR assay to quantify pregenome expression.

Three classes of viral RNA are known to be generated during the process of cccDNA transcription *in vivo* (Fig. 1). RNAs from all three classes are polyadenylated and posses a cap structure (Seeger et al., 1986). They all have the same 3′ end but possess different 5′ ends due to their different transcription initiation sites. All the viral RNAs are transported into the cytoplasm and translated. One class (1.8 kb) of viral RNA encodes the small surface protein, which is the most abundant DHBV protein. A second class of viral RNA (2.1 kb) encodes the large surface protein. The third class and the longest RNA (3.5 kb) is the whole genome, which has three functions: encoding the viral core protein, encoding the viral polymerase and serving as the pregenome (Seeger et al., 1986). The pregenome and its translated proteins are essential for the assembly of nucleocapsids; however, few studies have investigated how the use of the longest RNAs is balanced among their different purposes. Thus, it would be useful to investigate the uses of the longest RNAs over time, to determine when they serve as the pregenome and when they are used for the translation of the core protein or the polymerase.

Translation occurs at different time points during the infectious cycles of hepadnaviruses, and the timing of the translation of a given gene depends primarily on the functions of that gene (Tombacz et al., 2009). The early genes are responsible for functions associated with viral replication, including nucleotide synthesis, the regulation of transcription, and DNA cleavage and packaging, and these genes are usually transcribed and translated early in the infectious cycle. Genes that participate in protein modifications, virion formation, and virion maturation are usually translated at a relatively late stage. Regardless of whether genes are translated early or late during the course of infection, their protein levels always correspond to their transcription levels, as has been demonstrated for pseudorabies virus (Tombacz et al., 2009) and duck enteritis virus (Cai et al., 2010; Lian et al., 2010; Shen et al., 2009). The goal of this study was to determine whether the pregenome/C RNA was used for the translation of viral core proteins during the early phase of virus infection.

2. Materials and methods

2.1. Cells, virus and antibodies

Primary duck hepatocytes (PDHs) were obtained from 7-day-old Pekin ducklings known to be negative for DHBV. The DHBV CHv strain (GenBank no. EU429325) was isolated and stored by our laboratory. DHBV-containing duck serum used for infecting PDHs was collected from experimentally infected ducks. The copy number of DHBV particles in sera was confirmed by quantitative PCR. Polyclonal anti-core protein antibodies were prepared by immunizing rabbits with DHBV recombinant core protein (rDHBc) that had been expressed in a prokaryotic expression system (Liu et al., 2014).

2.2. Development and evaluation of a quantitative PCR assay for the PreC/C gene

DHBV genomic DNA extracted from DHBV-positive sera was used as a template for conventional PCR. The primers P1 (5′-ATGGATATCAATGCTTCTAGA-3′) and P2 (5′-TTTCCTAGGCGA-GGGAGA-3′) were designed to amplify the entire PreC/C gene (786 bp). The PCR products were subsequently cloned into the pMD18-T vector to generate the recombinant plasmid pMD18-T/PreC/C, which was used to produce the qPCR standard curve. Pregenome/C RNA levels were quantified by qPCR using the duck β-actin gene as a reference gene. For this purpose, a pMD18-T/β-actin standard plasmid was constructed as previously described (Wu et al., 2011). Primers P3 (5′-GGATCGGGCTAGGAGATTGC-3′) and P4 (5′-CCTGAGCCACTTGGATTGGT-3′ were used to quantify PreC/C gene expression levels, while the primers P5 (5′-GTATCAGCAGCAGTCTTA-3′) and P6 (5′-GCGAGTAACTTCCTGTAA-3′) were used to quantify β-actin expression levels. Both amplified PCR products were located within the fragments of PreC/C and β-actin that had been cloned into the vector, and the lengths of the PCR products obtained using the primer pairs P3/P4 and P5/P6 were 173 bp and 148 bp, respectively.

After the primers were designed, the annealing temperatures for the PreC/C gene and the β-actin reference gene were optimized for the qPCR assay. Two-step qPCR was performed using the CFX Connect™ real-time PCR detection system (Bio-Rad, California, USA). The qPCR reaction for the PreC/C gene was performed in a 20 μL volume containing 10 μL SsoAdvanced™ SYBR Green Supermix (Bio-Rad, California, USA), 0.6 μL of primers P3 and P4 (10 μM) and 100 ng of pMD18-T/PreC/C. Cycling parameters included pre-denaturation at 95 °C for 30 s and 45 cycles of denaturation at 95 °C for 20 s followed by annealing using a temperature gradient of 56.8–64.8 °C over 30 s. The qPCR assay for β-actin was similarly optimized, with the annealing temperature optimized using a temperature gradient of 47.9–53.9 °C. The optimized annealing temperatures for the PreC/C and β-actin qPCR assays were confirmed based on their amplification curves, and melting curves were established to assess the specificity of the primers. Next, preparations of the pMD18-T/PreC/C and pMD18-T/β-actin standard plasmids were serially diluted 10-fold to generate concentrations of 10^{-2}–10^{-7} relative to the primer stocks. A total of 2 μL of each dilution served as the DNA template for the PreC/C and β-actin assays under the optimized conditions. Standard curves were created using the Bio-Rad CFX Manager. The amplification efficiencies and correlation coefficients for the efficiency curves were calculated and compared.

2.3. Cell culture and virus infection

Primary duck hepatocytes (PDHs) were obtained by collagenase perfusion of 7-day-old Pekin ducklings known to be negative for DHBV as described previously (Rigg and Schaller, 1992). Cells were seeded onto 24-well plates (Corning, USA) at a density of 4×10^5 cells/well and maintained in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, 10 μM hydrocortisone 21-hemisuccinate and 1 μg/mL insulin (Sigma, USA). After the cells were incubated for 48 h, the media containing 10% FBS was replaced with serum-free media, and the cells were incubated for an additional 6 h. To infect DHBV-negative PDHs, DHBV-containing sera were diluted in serum-free medium, and approximately 1×10^8 copies of DHBV were added to each well. Negative control wells were mock-infected with PBS. After 6 h of incubation, the cells were washed