

## A CELL-BASED SYSTEM FOR HEPATITIS B VIRUS REPLICATION: SIGNIFICANCE OF CLINICALLY ENHANCED VIRAL REPLICATION IN RELATION TO DELETIONS IN VIRAL CORE PROMOTER

Hong Pan<sup>1</sup>, Lisa FP Ng<sup>2</sup>, Ee Chee Ren<sup>2</sup> and Wei Ning Chen<sup>1</sup>

<sup>1</sup> Hepatitis Viruses and Liver Cancer Research Laboratory, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive 05N-10, Singapore 637551 and <sup>2</sup> Genome Institute of Singapore, 60, Biopolis Street, Genome, #02-01, Singapore 138672

### TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
  - 3.1. Construction of Replicative HBV Genome with Deletion in Core Promoter
  - 3.2. Transfection of HepG2 Cells and Measurement of Secreted HBsAg
4. Results and discussion
5. Acknowledgments
6. References

### 1. ABSTRACT

The core promoter of hepatitis B virus (HBV) is crucial in viral replication. Our previous investigation in clinical serum samples indicated that deletions in the viral core promoter (from nucleotide 1758 to 1777 and 1749 to 1768 respectively) may lead to increase in viral replication. We propose to characterize these deletion mutants in a cell-based system that supports HBV replication. Replicative HBV genome developed in our laboratory was used as template to generate deletions in viral core promoter corresponding to those found in clinical samples. These deletion constructs were used to transfect HepG2 cells, which then support the subsequent infection of cells from these deleted but replicative genomes. The effect of deletion on the viral replication was measured by comparing the amount of hepatitis B virus surface antigen (HBsAg) secreted in the culture medium with that from cells transfected with the wild type HBV genome. The amount of secreted HBsAg in cells transfected with deletion mutants was significantly lower than those transfected with the wild type genome. Our results provide a new system for molecular characterization of mechanism of HBV replication. Contrary to the increased replication observed in clinical samples, the reduced viral replication in genomes carrying deletions in the core promoter suggests a complex regulatory mechanism of viral replication which may involve other elements in the viral core promoter.

### 2. INTRODUCTION

Hepatitis B is one of the world's most common and serious infectious diseases. Approximately 350 million

people are chronic carriers of hepatitis B virus (HBV) and are at risk of developing a wide range of liver diseases including hepatocellular carcinoma (HCC) (1)

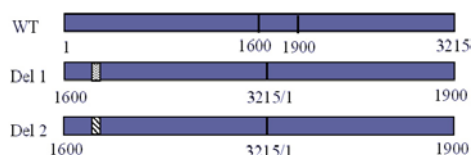
HBV is a DNA virus that replicates through an RNA intermediate, the 3.5-kb pregenomic mRNA. The core promoter (1591-1822 of the HBV genome, with the G of the EcoRI restriction site as nucleotide 1) plays an important role in viral replication, particularly in the production of 3.5 kb pregenomic RNA (2, 3).

Naturally occurring mutations have been observed during HBV infection due to its replication through a low fidelity reverse transcriptase. These mutants are either capable of escaping neutralization by HB vaccines or are resistant to antiviral agents (4, 5). Some newly discovered variants are located within functional domains of viral proteins and are now linked to various stages of liver disease, including the acute hepatitis and the development of HCC (6).

Mutations in the core promoter—notably an A to T change at position 1762 together with a G to A change at position 1764—are frequently detected in patients with fulminant liver diseases but were recently also found in anti-HBe positive asymptomatic carriers (7, 8). These mutations are reported to also affect the viral replication in various ways (9, 10). Other mutations reportedly implicated in the seroconversion from HBeAg positive to anti-HBe positive status include the precore stop codon mutation, which converts the codon 28 (W) to a stop codon thereby abolishing the translation of HBeAg

**Table 1.** List of Oligonucleotide Primers for Deletion PCR

<b>Deletion 1 (1758-1777)</b>	
Fragment A	5'-GCGGCCGCACGTCGCATGGAGACCACCG-3'
Fragment B	5'- CCAATTTATGCCTACAGCCTCCTAACCTCTCTCCCCAACT-3'
Fragment C	5'-AGTTGGGGGCGGAGGTTAGGAGGCTGTAGGCATAAATTGG-3'
Fragment D	5'-GCGGCCGCGAATTCCACTGCATGGCCTGAGG-3'
<b>Deletion 2 (1749-1768)</b>	
Fragment A	5'-ACGTCGCATGGAGACCACCG-3',
Fragment B	5'-GCCTACAGCCTCCTAGTACAACCTCCCCAACTCCTCCCACT-3',
Fragment C	5'-AGTGGGAGGAGTTGGGGGAGTTGTACTAGGAGGCTGTAGGC-3',
Fragment D	5'-TCGGCCGCGAATTCCACTGCATGGCCTGAGG-3'.

**Figure 1.** Generation of Replicative HBV Genomes.

Wild type HBV genome in pBR325 was used as template. The region 1600-1900 contained the core promoter as well as the overlapping transcription termination region (11). Deletions were generated by a sequential PCR method (see Fig. 2). Replicative genomes were cloned by a two-step PCR strategy (12).

(13). Similar to the mutations in the core promoter, the precore stop codon mutation has been found in patients with fulminant hepatitis or chronic active hepatitis (14).

We have recently reported a cross-sectional analysis of mutation pattern in the core promoter from Singapore HBV carriers (15). High HBV replication level as measured by serum HBV DNA level (around 228.16 pg ml<sup>-1</sup>) as well as HBsAg level (around 140 µg/ml) was found in three patients. Interestingly, deletions within the same region (nucleotide 1758-1777) of the viral core promoter were found in all three samples. As other mutations occurring concurrently on these viral genomes may impact on the observed viral load (16), the relationship between the viral DNA level with the deletions in the core promoter would need to be further assessed.

We report here our molecular analysis of such a correlation using a cell-based HBV replication system. Our results indicated that replicative HBV genomes containing deletions in the viral core promoter, corresponding to those found in the clinical samples displayed decreased viral replication level as measured by their respective HBsAg level in culture medium. Nevertheless, such a cell-based HBV replication system should be helpful in future molecular characterization of other naturally occurring mutations/deletions in HBV genome.

### 3. MATERIALS AND METHODS

#### 3.1. Construction of Replicative HBV Genome with Deletion in Core Promoter

The HBV genome-containing pBR325 plasmid (ATCC, USA) was used as template. The HBV genome

was cloned in pBR325 at EcoRI site and starting nucleotide of the viral genome was defined as 1 and the ending nucleotide as 3215 (WT, Figure 1).

To generate deletion mutant constructs, two pairs of primer were designed for two separate rounds of PCR. The primer sequences are shown in table 1.

HBV genomes carrying such deletions in pBR325 were used as template to generate by PCR the first fragment (1600-3215/1), with deletions in viral core promoter (1749-1768 and 1758-1777 respectively). The wild type HBV genome in pBR325 was in turn used as template to generate by PCR the second fragment (3215/1-1900), with a wild type transcription termination region (1600-1900). In-frame ligation of each of the first fragments (carrying respective deletion in viral core promoter) with the common wild type second fragment into mammalian expression vector pcDNA3.1 (Invitrogen, USA) resulted in respective replicative HBV genomes (Figure 1).

#### 3.2. Transfection of HepG2 Cells and Measurement of Secreted HBsAg

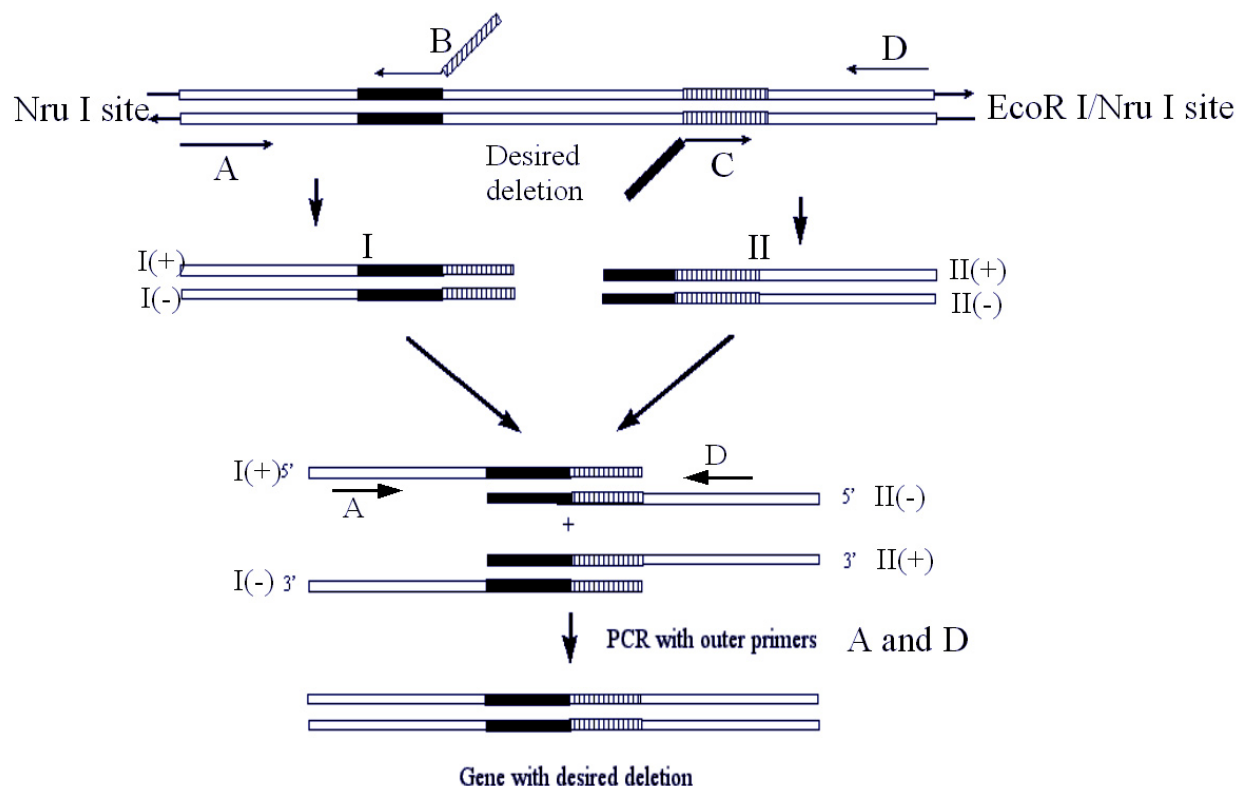
The HepG2 cells (ATCC, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) from Gibco BRL (Life Technologies, USA) containing 10% fetal calf serum (FCS) from Pierce (USA) at 37°C in 5% CO<sub>2</sub>/95% air at 95% relative humidity. 2 µg of each of the constructs with deletions in the viral core promoter, as well as the wild type HBV genome in pcDNA3.1 vector were transfected separately into HepG2 cells using Effectene transfection reagent (Gibco BRL, Life Technologies, USA). Each transfection was carried out in two independent experiments. Three days after transfection, culture medium was collected and 200 µl were used for the measurement of HBsAg (Auszyme kit, Abbott Laboratories, USA).

### 4. RESULTS AND DISCUSSION

Deletions in the viral core promoter have been identified in HBV carriers with high viral DNA levels in our recent study (15). To assess the significance of such a finding, a cell-based system which supports HBV replication was used in this study. The system was based on transfecting a replicative HBV genome into HepG2 cells prior to the onset of subsequent infection of other cells. The successful infection of HepG2 cells has been shown by the large amount of secreted

**Table 2.** Semi-Quantitative Measurement of Secreted HBsAg

Type of HBV Construct	Percentage of Secreted HBsAg
Wild type HBV genome	100
HBV genome with deletion 1749-1768	3.8
HBV genome with deletion 1758-1777	5.5



**Figure 2. Generation of deletion in viral core promoter by sequential PCR.** Oligonucleotide primers were designed for each deletion mutant. Specifically, oligonucleotide primers B and C being perfectly complementary to each other, allowing the elimination of the region between these two primers in subsequent PCR. The first round of PCR involved pairing of primers A and B, C and D separately. The pairing of primers A and D in the second round of PCR, using the mixture of first round PCR product as template, resulted in the generation of desired deletions.

virions in the culture medium, as examined under electron microscope (17). Such replicative genome has been successfully used to analyze various HBV mutants (12). In the case of vaccine escape mutants, replicative genome carrying such mutation created by site-directed mutagenesis has been shown to display altered viral antigenicity after transfecting HepG2 cells (12).

Deletions in the viral core promoter, corresponding to those found in the clinical samples (1749-1768 and 1758-1777 respectively), were generated by a sequential PCR strategy (Figure 2). This was followed by a two-step PCR and ligation into mammalian expression vector pcDNA3.1 to generate replicative HBV genomes with deletions in their respective viral core promoter (Figure 1). Sequencing of these constructs revealed no other mutations in other part of the viral genomes (*data not shown*).

To assess the role of the deletions in core promoter on the viral replication, each of these replicative

HBV genome constructs (wild type, deletion 1 and deletion 2) was used to transfect HepG2 cells. Transfection for each construct was carried out in two independent experiments. The respective culture medium was collected for comparative measurement of the viral replication. Based on the reported correlation between the secreted HBsAg and HBV DNA level in HBV-producing 2.2.15 cells and other clinical studies (18, 19), the culture medium was semi-quantitatively assessed for the amount of HBsAg by ELISA assay (Auszyme, Abbott Laboratories, USA).

Results shown in Table 2 indicated a significant decrease of secreted HBsAg for both deletion 1 and deletion 2 HBV genomes, as compared with the wild type HBV genome.

While in sharp contrast to our previous observation (15), our molecular analysis suggested that the deletions in viral core promoter were not involved in the high viral load found in three clinical samples. As our

cross-sectional analysis focused mainly on the mutation pattern at the precore residue 28 and 1762/1764, it remains possible that mutations in other part of the core promoter in these three samples have contributed to the enhanced viral load.

Taken together, our data have provided molecular information on the role of core promoter deletions in HBV replication. While our results could be validated by including other measurements such as HBV DNA level, The cell-based system for HBV replication should be a valuable tool for molecular characterization of other deletion/mutations not only in the core promoter, but also in other regions of the HBV genome.

## 5. ACKNOWLEDGMENTS

This work was supported by grant 03/1/22/18/229 (WN Chen) from the Biomedical Research Council, Agency for Science, Technology and Research, Singapore. H Pan is a recipient of a graduate research scholarship from Nanyang Technological University, Singapore. L Ng is supported by a Postdoctoral Fellowship Award from the Singapore Millennium Foundation (Singapore Technologies).

## 6. REFERENCES

1. R. B. Birrer, D. Birrer & J. V. Klavins: Hepatocellular carcinoma and hepatitis virus. *Ann Clin Lab Sci* 33, 39-54 (2003).
2. Chen WN & C. J. Oon: Human hepatitis B virus mutants: significance of molecular changes. *FEBS Lett* 453, 237-242 (1999)
3. Kramvis A & Kew MC: The core promoter of hepatitis B virus. *J Viral Hepatitis* 6, 415-427 (1999)
4. Locarnini S: Molecular virology of hepatitis B virus. *Semin Liver Dis* 24, 3-10 (2004)
5. Zuckerman AJ: Effect of hepatitis B virus mutants on efficacy of vaccination. *Lancet* 355, 1382-1384 (2000)
6. Gunther S, L. Fischer, I. Pult, M. Sterneck & H. Will: Naturally occurring variants of hepatitis B virus. *Adv Virus Res* 52, 25-137 (1999)
7. Honda A, O. Yokosuka, T. Ehata, M. Tagawa, F. Imazeki & H. Saisho: Detection of mutations in the enhancer 2/core promoter region of hepatitis B virus in patients with chronic hepatitis B virus infection: comparison with mutations in precore and core regions in relation to clinical status. *J Med Virol* 57, 337-344 (1999)
8. Kurosaki M, N. Enomoto, Y. Asahina, I. Sakuma, T. Ikeda, S. Tozuka *et al.*: Mutations in the core promoter region of hepatitis B virus in patients with chronic hepatitis B. *J Med Virol* 49, 115-123 (1996)
9. Moriyama K: Reduced antigen production by hepatitis B virus harboring nucleotide deletions in the overlapping X gene and precore-core promoter. *J Gen Virol* 78, 1479-1486 (1997)
10. Scaglioni PP, M. Melegari & J. R. Wands: Biologic properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame. *Virology* 233, 374-381 (1997)
11. Weiss L, A. S. Kekule, U. Jakubowski, E. Burgelt & P. H. Hofschneider: The HBV-producing cell line HepG2-4A5: a new in vitro system for studying the regulation of HBV replication and for screening anti-hepatitis B virus drugs. *Virology* 216, 214-218 (1996)
12. Chen WN, C. J. Oon & I. Toh: Altered antigenicities of hepatitis B virus surface antigen carrying mutations outside the common "a" determinant. *Am J Gastroenterol* 95, 1098-1099 (2000b)
13. Carman WF, M. R. Jacyna, S. Hadziyannis, P. Karayiannis, M. J. McGarvey, A. Makris & H. C. Thomas: Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 2, 588-591 (1989)
14. Omata M, T. Ehata, O. Yokosuka, K. Hosoda & M. Ohto: Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *New Engl J Med* 324, 1699-1704 (1991)
15. Chen WN & C. J. Oon: Mutations and deletions in core promoter and precore stop codon in relation to viral replication and liver damage in Singaporean hepatitis B virus carriers. *Eur J Clin Invest* 30, 787-792 (2000a)
16. Friedt M, P. Gerner, P. Wintermeyer & S. Wirth S: Complete hepatitis B virus genome analysis in HBsAg positive mothers and their infants with fulminant hepatitis B. *BMC Gastroenterol* 4, 11 (2004)
17. Oon CJ, W. N. Chen, K. T. Goh, S. Mesenas, H. S. Ng, G. Chiang, C. Tan, S. Koh, S. W. Teng, I. Toh, M. C. Moh, K. S. Goo, K. Tan, A. L. Leong & G. S. Tan: Molecular characterization of hepatitis B virus surface antigen mutants in Singapore patients with hepatocellular carcinoma and hepatitis B virus carriers negative for HBsAg but positive for anti-HBs and anti-HBc. *J Gastroenterol Hepatol* 17, S491-496 (2002).
18. Janssen HL, C. J. Kerhof-Los, R. A. Heijtkink & S. W. Schalm: Measurement of HBsAg to monitor hepatitis B viral replication in patients on alpha-interferon therapy. *Antiviral Res* 23, 251-257 (1994)
19. Liu MC, M. Yu, N. L. Zhang, W. B. Gong, Y. Wang, W. H. Piao, Q. H. Wang & G. Q. Wang: Dynamic analysis of hepatitis B virus DNA and its antigens in 2.2.15 cells. *J Viral Hepat.* 11, 124-129 (2004)

**Key Words:** Hepatitis B Virus, HBV, Core Promoter, Deletions, Replication

**Send correspondence to:** Prof. Wei Ning Chen, Hepatitis Viruses and Liver Cancer Research Laboratory, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive 05N-10, Singapore 637551, Tel: 65-63162870, Fax: 65-62259865, E-mail: WNChen@ntu.edu.sg

<http://www.bioscience.org/current/vol10.htm>