UNIVERSITI PUTRA MALAYSIA

TRANSFECTION OF HEPG2 CELLS WITH BACTERIOPHAGES T7 AND M13 DISPLAYING REGIONS OF HEPATITIS B SURFACE ANTIGEN

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By

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TRANSFECTION OF HEPG2 CELLS WITH BACTERIOPHAGES T7 AND M13 DISPLAYING REGIONS OF HEPATITIS B SURFACE ANTIGENS

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Dec 2008

Chairman: Associate Professor Dr Tan Wen Siang, PhD

Faculty: Biotechnology and Biomolecular Sciences

Hepatitis B is a major public health problem worldwide that may lead to chronic liver diseases, cirrhosis and hepatocellular carcinoma. It is caused by hepatitis B virus (HBV) which is responsible for 500,000 deaths annually. The preS domain of HBV particularly amino acids 21-47, is believed to be involved in the initial step of HBV infection through attachment to hepatocyte. In order to study virus-cell interactions, both T7 and M13 bacteriophages have been genetically modified to display regions of Hepatitis B surface antigens. In the present study, the recombinant T7 and M13 phages were used to transfect human hepatocarcinoma cell line HepG2. Interestingly, T7-PreS1/2 carrying the second half of preS1 (residues 60-108), but not T7-PreS1/1 which carried the first half of preS1 (residues 1-47), was demonstrated to be the most effective in transfecting HepG2 cells in a dose- and time-dependant manner. The DNA of phage could be recovered from cell lysate and confirmed by PCR whereas infectious form of internalized phage was measured by plaque forming assay. Under fluorescence microscope, internalized phage exhibited the appearance as fluorescent dots. On the other hand, M13-PreS carrying the preS region showed low efficiency of

transfection, thus suggesting that the interaction is also dependant on the valency of targeting ligand. Surprisingly, unmodified phages (MOI ≈ 1.25×10^5) were also capable of transfecting HepG2 at low efficiency and were thought to be taken up by nonspecific uptake. Dynamic light scattering analysis has been carried out to determine the thermostability of recombinant phages. The display of HBV surface antigen did not affect the stability of phages since no significant differences in thermostability were observed between control and recombinant phages. The ability and stability of recombinant phages to transfect HepG2 cells demonstrate the potential of phage displayed system as gene therapy for liver cancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

TRANSFEKSI SEL HEPG2 DENGAN BAKTERIOFAJ T7 DAN M13 YANG MEMPAMERKAN KAWASAN ANTIGEN PERMUKAAN HEPATITIS B

Oleh

TANG KIE HIE

Dis 2008

Pengerusi: Profesor Madya Dr Tan Wen Siang, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Hepatitis B merupakan masalah utama kesihatan awam sedunia yang menyebabkan penyakit hati kronik, sirosis dan barah hati. Penyakit ini disebabkan oleh virus hepatitis B (HBV) dan menjadi punca kematian sebanyak 500,000 setiap tahun. Kawasan liputan preS HBV terutamanya asid amino 21-47, dipercayai terlibat dalam jangkitan awal HBV melalui pelekatan pada hepatosit. Untuk mempelajari interaksi virus-sel, genetik bakteriofaj T7 and M13 telah diubah-suai untuk mempamerkan kawasan-kawasan antigen permukaan Hepatitis B. Dalam kajian ini, faj rekombinan T7 and M13 digunakan untuk menjangkiti sel barah manusia HepG2. Yang menariknya, T7-PreS1/2 yang membawa separuh kedua preS1 (residu 60-108), dan bukannya T7-PreS1/1 yang membawa separuh pertama preS1 (residu 1-47), didapati paling berkesan dalam transfeksi sel-sel HepG2 bergantung kepada dos faj dan masa. DNA faj boleh didapati di dalam sel dan disahkan dengan PCR manakala faj berjangkit dalaman dapat dikira melalui ujian pembentukan plak. Faj dalaman dapat dilihat sebagai bintik-bintik pendarfluor dengan mikroskop pendarfluor. Sementara itu, M13-PreS

yang membawa kawasan preS menunjukkan transfeksi dengan effisiensi yang rendah, mencadangkan bahawa perhubungan tersebut juga bergantung kepada valensi ligan penujuan. Yang menakjubkannya, faj (MOI $\approx 1.25 \times 10^5$) yang tidak diubah-suai juga berupaya menjangkiti HepG2 dengan effisiensi transfeksi yang rendah dan dijangka melalui mekanisma tidak spesifik. Analisis serakan cahaya dinamik telah dijalankan untuk menentukan kestabilan haba faj rekombinan. Pameran antigen permukaan HBV didapati tidak menjejaskan kestabilan faj memandangkan tiada perbezaan ketara diperhatikan antara kestabilan haba faj kawalan dan rekombinan. Oleh itu, kebolehan dan kestabilan faj dalam menjangkiti sel HepG2 telah menunjukkan potensi sistem pameran faj sebagai terapi gen untuk barah hati.

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I certify that a Thesis Examination Committee has met on 1 December 2008 to conduct the final examination of Tang Kie Hie on her thesis entitled "Transfection of HepG2 Cells with Bacteriophages T7 and M13 Displaying Regions of Hepatitis B Surface Antigens" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

I hereby declare that this thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degrees at UPM or other institutions.

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TANG KIE HIE

Date: 16 January 2009

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LIST OF ABBREVIATIONS

α	alfa
β	beta
ε	epsilon
λ	lambda
°C	degree centigrade
μ	micron
AFB_1	aflatoxin B1
AUG	start codon
aa	amino acid
BSA	bovine serum albumin
bp	base pair
CsCl	cesium chloride
cccDNA	covalently closed circular DNA
dsDNA	double-stranded deoxyribonucleic
DHBV	duck HBV
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DR	direct repeat
D_T	translational diffusion coefficient
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay

EN	enhancer
FBA	fluorescent-bacteriophage assay
FBS	fetal bovine serum
GFP	green fluorescence protein
GSHV	ground squirrel hepatitis virus
g	gram
h	hour
HBIG	Hepatitis B Immune Globulin
HBV	hepatitis B virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HCC	hepatocellular carcinoma
HCl	hydrochloride
HepG2	human hepatocellular liver carcinoma cell line
IgE	Immunoglobulin E
kDa	kilodalton
kb	kilobase
L-HBsAg	large surface antigen
M	Molar
MHC	major histocompatibility complex
M-HBsAg	medium surface antigen
mW	miliwatt
mRNA	messenger ribonucleic acid
NTE	sodium-tris-EDTA

NaCl	sodium chloride
NaOH	sodium hydroxide
N-terminal	amino-terminal
nm	nanometer
OD	optical density
ORF	open reading frame
PBS	phosphate-buffered saline
PC	precore
PCR	polymerase chain reaction
PEG	polyethylene glycol
PreS1	N-terminal region of L-HBsAg comprising 108 or 119 amino acid
PreS2	region of M and L-HBsAg comprising 55 amino acid
pH	*Puissance hydrogene*
pHSA	polymerized human serum albumin
pfu	plaque forming unit
pgRNA	pregenomic ribonucleic acid
RF	replicative form
RME	receptor-mediated endocytosis
RNA	ribonucleic acid
RNase	ribonuclease
R_h	hydrodynamic radius
rcDNA	relax-circular deoxyribonucleic acid
SCCA1	squamous cell carcinoma antigen 1
SOC	small outer capsid

S-HBsAg	small surface antigen
ssDNA	single-stranded deoxyribonucleic acid
TLM	translocation motif
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
WHV	woodchucks hepatitis virus
x*g*	centrifugal force
V	volt
v/v	volume/volume
vol	volume
w/v	weight/volume

CHAPTER 1

INTRODUCTION

Hepatitis B virus (HBV) is the prototype of *Hepadnaviridae*, a family which includes related hepatitis viruses which infect woodchucks (WHV), ground squirrels (GSHV) and Pekin ducks (DHBV) (Summers and Mason, 1982). Worldwide, two billion people have been infected with HBV of which 400 million are chronically infected. Chronic infection of HBV causes progressive liver diseases and hepatocellular carcinoma (HCC) that responsible for around 500,000 deaths each year (Perz *et al.*, 2006).

To date, alpha interferon therapy can be used for treatment of chronic HBV infection. Nevertheless, it is ineffective in more than 50% of carriers (Hoofnagle and Di Bisceglie, 1997) and it is associated with several side effects (Torre and Tambini, 1996). Other therapy using nucleoside analogs and inhibitors of virus replication have led to development of drug-resistant mutants due to high rate of viral turn over and error-prone polymerase (Locarnini and Omata, 2006).

A vaccine derived from inactivated hepatitis B surface antigen (HBsAg) purified from human plasma was first licensed in the United States, followed by yeast- and mammalian-derived hepatitis B vaccines which are now widely used (Shouval *et al.*, 1994; Mahoney, 1999). The ultimate goal of hepatitis B vaccination is to control HBV-related chronic liver diseases and hepatocellular

carcinoma. However, HBV with molecular variants in the ‘a’ determinant of HBsAg make it unsusceptible for recognition and neutralisation by anti-HBsAg antibodies.

Gene therapy has been recognized as one of the most promising approaches in curing cancers (Verma and Sonia, 1997). The technology requires a gene delivery vehicle capable of targeting a therapeutic gene to the selected cell type with high efficiency without stimulating significant immune, inflammatory and cytotoxic responses (Michael and Curiel, 1994). Hence, the use of phage display systems for mammalian gene transfer is of great interest due to their stability, direct linkage between genotype and phenotype, inexpensive mass production or purification and excellent safety profile in mammalian cells (Larocca and Baird, 2001). By displaying targeting sequences on the phage coat, bacteriophage vectors which exhibit no natural tropism for mammalian cells may confer mammalian cell-type specific tropism through receptor-mediated endocytosis (RME) (Varga *et al.,* 2000), thus offer an advantage in targeting treatment of cancer.

In the present study, T7 and M13 phages displaying regions of HBsAg which are believed to be involved in infectivity and attachment site for hepatocytes, were used for targeting human hepatocellular carcinoma cell line (HepG2). Therefore, this study was aimed to examine the ability of genetically-targeted T7 and M13 phages in delivering their genes and internalise to HepG2 cells. The thermostability of T7 and M13 particles was also tested with dynamic light scattering (DLS).

CHAPTER 2

LITERATURE REVIEW

2.1 Hepatitis B Virus (HBV)

2.1.1 HBV Morphology

Human HBV is an enveloped DNA virus grouped under the family *Hepadnaviridae* and genus *Orthohepadnavirus* (Ganem and Varmus, 1987). Internal to the envelope is the viral nucleocapsid that comprises of core proteins, a partially double stranded DNA genome of 3.2 kb and a polymerase protein (P) (Ganem, 1991). HBV has three forms of surface antigens (HBsAg): large L-HBsAg, middle M-HBsAg and small S-HBsAg that are embedded in the envelope of the virion. During HBV infection of an individual, HBsAg particles containing only HBsAg are produced in great abundance. These noninfectious particles exist as spherical and filamentous forms of 22 nm in diameter, which is smaller than the 42 nm infectious Dane particles or HBV virion (Dane *et al.*, 1970).

2.1.2 Genomic Organisation and Viral Transcripts

A hepadnavirus virion contains two single-stranded linear DNA molecules of different sizes which paired in an overlapping fashion with breaks at both strands, producing a circular partially double stranded molecule. The

negative (−) strand is about 3200 bases long, which serves as the template for all viral mRNA transcription, whereas the positive (+) strand varies in length, from 1700 to 2800 bases long. A protein, P is covalently attached to 5' end of the (−) strand (Gerlich and Robinson, 1980), while the (+) strand ends in a 19-base-long RNA molecule. At the 5' end of both strands, there is a pair of directly repeated 11-base-long sequences, designated as DR1 and DR2 that play crucial role in the initiation of viral DNA synthesis (Lien *et al.,* 1986; Will *et al.,* 1987).

As shown in Figure 2.1, there are four open reading frames (ORF) present in the DNA, which are C, P, S and X (Wei and Tiollais, 1999). ORF C contains two in-frame start codons, AUGs, which encode the structural protein of the HBV nucleocapsid (Pasek *et al.,* 1979), and a hydrophobic polypeptide bearing hepatitis B e antigen (HBeAg). Instead of incorporating into HBV virions, HBeAg is secreted from cells and accumulating in the serum. The ORF P sequence overlaps the end of ORF C region and continues nearly to the end of the DNA molecule. It encodes the viral polymerase which is utilised in the viral genomic replication as well as the protein primer molecule that is attached to the 5' end of (−) strand DNA (Bosch *et al.,* 1988). Within the ORF P is the ORF S which occurs with three in-frame initiation codons, resulting in the synthesis of three related HBsAg. These proteins of 39 (L-HBsAg), 33 (M-HBsAg) and 24 kDa (S-HBsAg) in size are found on the surface of the virion (Valenzuela *et al.,* 1979; Wong *et al.,* 1985).

Although there are four ORFs in HBV genome, only three mRNAs are synthesized. In addition, the product of ORF X is not well understood. It appears

to encode possibly three *trans* active regulatory proteins that facilitate the transcription from cellular and viral promoter sequences (Seeger and Mason, 2000).

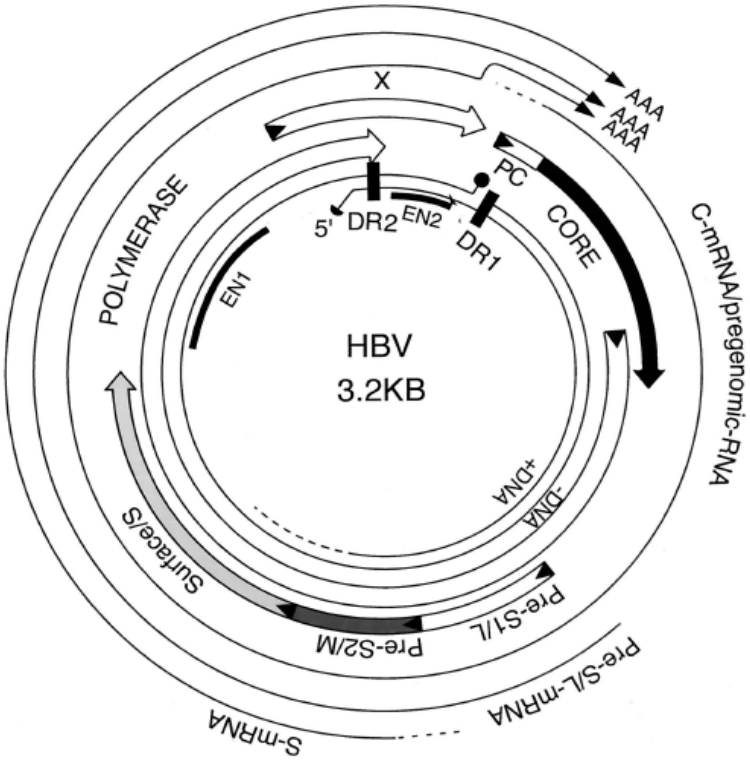

Figure 2.1: Transcriptional and translational map of HBV. The inner circles represent the rcDNA. It comprises of a complete (−) strand DNA and an incomplete (+) strand DNA with the reverse transcriptase and a capped RNA oligomer attached to its 5' end respectively. The position of direct repeat sequences, DR1 and DR2, and the enhancers, EN1 and EN2 are indicated. Three major viral RNAs, the core (C) or pgRNA, preS (L) mRNA an S mRNA are depicted by the outer circle, are controlled by several internal promoters at different sites but are all terminated at a common 3' end, indicated by the letters A. In between the outer and inner circles are the four protein-coding regions which include precore (PC) and core genes, polymerase gene and X gene. The envelope genes, L, M and S overlap with the polymerase ORF (adapted from Seeger and Mason, 2000).

2.1.3 Hepadnavirus Replication

A hepadnavirus particle contains predominantly relaxed-circular DNA molecules (rcDNA) with a complete minus (−) strand and a partially synthesized plus (+) strand. A small amount of linear DNAs as a result of *in situ* priming is also found. The (−) strand is covalently attached to a protein, the viral reverse transcriptase at 5' end while the (+) strand ends in a 19-base-long RNA (Voyles, 2002).

The life cycle of HBV (Figure 2.2) begins by attaching to the host cell membrane via its envelope proteins and the virus release its genome into the nucleoplasm (Lu *et al.,* 1996; Kann *et al.,* 2007). During initiation of infection, the (+) strand is elongated until both strands are of essentially equal length. Both the protein and RNA at the 5' end are removed from the strands and a covalently closed circular DNA (cccDNA) is formed when the viral genome reaches the nucleus. The formation of cccDNA indicates successful initiation of infection as the cccDNA serves as a template for mRNA transcription (Tuttleman *et al.,* 1986).

Replication begins with the synthesis of the pregenomic RNA (pgRNA) molecule which is larger than its genome (Figure 2.2), and has terminal repeats since same set of bases is copied twice. The pgRNA would serve as a template for synthesis of core (C) protein (HBcAg) and preC, viral envelope (S), viral polymerase (P) and X proteins (Summers and Mason, 1982). The P protein binds to the epsilon (ε) stem-loop structure at the 5' end of its own mRNA template and initiates encapsidation into nucleocapsids that are assembled in the cytosol