Identification of cellular transcription factors binding to HBV promoter with naturally occurring deletions

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Identification of Cellular Transcription Factors Binding to HBV Promoter with Naturally Occurring Deletions

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of the requirement for the degree of Doctor of Philosophy

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Abstract

Identification of Cellular Transcription Factors Binding to HBV Promoter with Naturally Occurring Deletions

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submitted for the degree of PhD

Hepatitis B virus (HBV) is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Suppressing viral replication in chronic HBV carriers is an effective approach to controlling disease progression. Even though antiviral compounds are available, the host factors that have a significant effect on viral replication efficiency need to be better characterized. Our group previously reported a significant association between patient serum viral load with a naturally occurring deletion within the HBV viral promoter. The purpose of our study was to analyze the impact of the deletion in the HBV viral promoter on viral replication efficacy, and characterize cellular transcription factor(s) that may be involved in the altered HBV replication. Our data showed that replication-competent HBV genome with deletion in the viral promoter had decreased replication level as compared with the wild type HBV genome in our cell-based replication system, supported by the decrease in HBsAg secretion and extracellular HBV viron. Concurrently, our promoter activity assay shows that the activity of the deleted promoter is lower than that of wild type. In addition, a new transcription factor recognizing the viral promoter with deletion has been revealed in the gel shift assay using nuclear extracts. This new transcription factor was subsequently identified as SMARCE 1 through DNA-protein array assay. The ability of SMARCE 1 in modulating the replication efficiency of HBV was further demonstrated in cell transfection studies, in particular an overexpression of SMARCE 1 led to a decreased HBV replication. Our findings therefore suggest a wider role of SMARCE 1 beyond maintaining host cellular functions and more importantly the SMACRCE 1 may represent a novel target for pharmacologic intervention of HBV replication.

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Abbreviations

2D	2-demension
ARP1	apoAI regulatory protein
bp	base pair (s)
BSA	bovine serum albumin
BCP	basic core promoter
cDNA	complementary DNA
COUP-TF	chicken ovalbumin upstream promoter- transcription factor
C/EBP	CCAAT/enhancer binding protein
CP	core promoter
CURS	core upstream regulatory sequence
dNTP	3'-deoxynucleotide triphosphate
dATP	3'-deoxyadenosine triphosphate
dCTP	3'-deoxycytidine triphosphate
dTTP	3'-deoxythimidine triphosphate
dGTP	3'-deoxyguanian triphosphate
Del 1	deletion 1
Del 2	deletion 2
DMEM	Dulbecco's Modified Eagle medium
DMSO	dimethyl sulphoxide
DNA	3'-deoxyribonucleic acid
DR-1	direct repeat minus 1

		Abbreviation
DTT	dithiothreitol	
ECL	enhanced chemiluminescence	
EDTA	ethylene-diamine-tetraacetic acid	
EMSA	electrophoretic mobility shift assay	
FBS	fetal bovine serum	
Enh II	enhancer II	
hr	hour(s)	
HBV	hepatitis B virus	
HEPES	N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid]	
HPLC	high pressure liquid chromatography	
HRE	hormone response element	
HNF1	Hepatocyte nuclear factor 1	
HNF4	Hepatocyte nuclear factor 4	
IEF	isoelectric focusing	
IPG	immobilized pH gradient	
kbp	kilobase pair(s)	
kD	kilodaltons	
LEF	liver-enriched factor	
LB	Luria-Bertani	
mAb	monoclonal antibody	
NRE	negative regulatory sequence	
NEB	New England Biolabs	
nt	nucleotide	

Abbreviation	
MOPS	3-[N-morpholino]propanesulphonic acid
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
pgRNA	pregenomic RNA
PMSF	phenylmethsulphonyl fluoride
PPAR	peroxisome proliferator-activated receptor
Pre-C	precore
rpm	revolutions per minute
RNLE	rat liver nuclear extract
RT	room temperature
RXR	retinoid X receptor
RE	restriction enzyme
SD	standard deviation
SDS	sodium dodecyl sulphate
Sec	second(s)
TBP	TATA binding protein
TEMED	N,N,N',N'-tetramethylethlenediamine
TF	transcription factor
T _m	melting temperature
TR	testicular receptor

Abbreviation	
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
URR	upper regulatory region
UV	ultraviolet
WT	Wild type
v/v	volume per volume
w/v	weight per volume

The single letter and triplet codes for amino acid residues are used. Restriction enzymes are referred to their three letter names, other abbreviations are defined in the text when first used.

1. Introduction

1.1 Hepatitis B Virus’s Epidemiology

Hepatitis B is one of the serious and common infectious diseases in the world. In some developed country, the rate of hepatitis B carrier is not very high. However, in some developing region such as Asia, Africa, the Middle East and the Pacific Islands, hepatitis B infection is still a major heath problem with the carrier rate ranging between 10 to 20% (Hollinger *et al.* 2001). Some carriers can develop severe liver disease including liver cirrhosis and hepatocellular carcinoma (Zuckerman *et al.* 1996; Birrer *et al.* 2003). Studies have also shown that chronic hepatitis B infection increases the risk of HCC by roughly 100 fold (Mahoney *et al.* 1999).

FIG. 1-1. Transcripts and promoters. (obtained from webpage of Laboratory of Alan McLachlan)

The numerical annotation on the HBV genome [0-3, 221 base pairs (bp)] are referred to the HBV subtype adw₂. S, C, X and P represent region for surface antigen, core/e antigen, HBx and viral polymerase. PreS1, preS2, Xp and Cp

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represent the promoter regions, Enhancer (Enh) I and II refer to the enhancer elements. The HBV transcripts are shown by incomplete circles.

1.2 Molecular Biology and Structure of HBV

HBV belongs to the hepadnaviridae family which specifically attacks liver cells. It is an enveloped DNA virus with a partially double stranded DNA genome. The size of the genome is approximately 3200 base pairs in length (Fig 1-1).

HBV has a peculiar genomic organization in that the open reading frames (ORFs) of four HBV genes are overlapping with each other. These ORFs include core gene, x gene, polymerase gene, and surface gene. The transcription of these four gene was regulated by two enhancer element and at least five promoters. They are referred to as enhancer I (Enh I) enhancer II (Enh II), and Pre S1, Pre S2 promoter, PreC/C promoter, X promoter respectively.

Specific information of each promoter and the regulation of the viral gene are listed below:

1. The core promoter is a key promoter that controls the transcription of the 3.5kb pregenomic mRNA(pgRNA) and the precore mRNA. This pgRNA is the template for the translation of core protein and polymerase, while serving as template for reverse transcription of HBV. This makes pgRNA a key component of the HBV replication process. On the other hand, the core protein is the sole component of nucleocapsid enveloping the HBV genome (Bruss *et al.* 1998).
2. The precore mRNA is expressed as the precore protein which is the precursor of the circulating HBeAg. The secreted HBeAg is found in HBV-infected individuals.

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3. The transcription of the smallest viral protein HBx is regulated by the X promoter (Schek *et al.* 1991). HBx is a versatile viral protein that is involved in many cellular processes such as transcription, signal transduction, cell cycle, apoptosis, and in addition, studies also show that increasingly HBx is related with hepatocarcinoma (Hong *et al.* 2006).
4. The pre-S1 promoter regulates the transcription of viral RNAs that encode three related envelope proteins known as pre-S1, pre-S2, and major S proteins. All these surface antigen proteins are encoded by the same coding region but initiated at three different in-frame start sites. The termination of translation is common for these surface antigen proteins (Fig 1-1). As a result, there are three surface antigen sharing the common S region. The large hepatitis B surface antigen (LHBsAg) is the largest of the 3 surface proteins. It contains the Pre-S1 domain, Pre-S2 and S domain (Fig 1-1). The middle hepatitis B surface antigen is the medium HBV surface protein which contains an extra 55 amino-acid domain (pre-S2), but without the preS1 portion. The major S promoter regulates the expression of the smallest hepatitis B surface protein (HBsAg), which only has the major S region. This additional level of transcription for the major S has resulted in higher proportion of HBsAg compared with large and medium viral surface protein.

1.3 Replication of HBV

1.3.1 HBV Replication

HBV infect the host liver cells which can support its replication. The entry of HBV in liver cells is through unknown mechanism, probably via receptor-mediated endocytosis. After

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decoating the surface antigen at plasma membrane, the nucleocapsid containing the HBV partially double-stranded DNA is translocated into nucleus. Inside of the nucleus, the partially double strand DNA is repaired by the viral polymerase, and is then converted as covalently closed circular DNA (cccDNA). The reverse transcription process is subsequently initiated using the cccDNA as template. Four different mRNA transcripts are generated using the host RNA polymerase II, they are 3.5kb pgRNA, 2.4kb Mrna, 2.1kb mRNA, 0.7kb mRNA. Synthesis of four viral RNA transcripts is controlled by the respective viral promoters as above-mentioned. Each transcript terminates at a common polyadenylation site which is located within the coding region of the core protein. (Fig 1-1, Fig1-2).

Fig.1-2. life cycle of HBV (obtained from Robert's HBV page)

Legend is the same as paragraph 1.3.1 in the text.

1.3.2 HBV DNA synthesis

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Once synthesized in the nucleus, the pgRNA is released in the cytoplasm. This is followed by the binding of the viral polymerase to the stem-loop structure on the pgRNA. This binding will trigger the recruitment of core protein to form the nucleocapsid with the pgRNA-polymerase complex inside. The reverse transcription will then start inside the nucleocapsid.

Fig. 1-3 A diagrammatic representation of HBV's replication process. (This diagram was obtained from www.stanford.edu)

DR1 and DR2, also named for direct repeat sequences, consist of 12 bp each. pgRNA is synthesized by host polymerase II using cccDNA as template. Once synthesized, this pgRNA becomes longer than cccDNA due to the formation of the repeated terminal ends. The pgRNA then exits the nucleus and is translated into the core protein and HBV DNA polymerase. The core protein envelops the

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pgRNA-Polymerase complex after the binding of the latter to the former. The (-) strand DNA is then transcribed inside the nucleocapsid by reverse transcription. The C-terminal RNaseH of the viral polymerase then digests the pgRNA away, leaving a small stretch of RNA oligoribonucleotide. The oligomer is then annealed to the DR2 portion of the DNA at the 5' end. The HBV polymerase then generates complementary (+)DNA using this oligomer as primer.

The mechanism of conversion from pgRNA to dsDNA has been well reported. One significant characteristic is the generation of two direct repeat sequences (DR1 and DR2) as well as a stem-loop structure which is also called the encapsidation signal for the viral polymerase (Fig. 1-3). Once the polymerase bound to the stem-loop structure of pgRNA, it synthesizes a primer of 3 to 4 bases and the reverse transcription is initiated. Firstly, the minus (-) strand of DNA will be synthesized using this primer from the 5'-end of the RNA pregenome (Fig.1-3). As the (-) DNA strand continue to be elongated, the copied pgRNA template is degraded by RNase H activity of the polymerase protein except a stretch of 15 to 18 oligonucleotides at the 5' end of the pgRNA (Miller *et al.* 1984; Summers *et al.* 1982). This oligonucleotide then serves as the primer for the synthesis of the (+) DNA strand after moving to the 5' DR2 region on the (-) DNA strand (Lien *et al.* 1986).

Once the oligonucleotide is translocated to the 5' DR2 site, the synthesis of the (+) DNA starts and continues towards the end of the 5' end of the (-) DNA strand. In general, the (+) DNA strand is not completed before the re-entry of the virion into another host cell. This leads to the typical partially double-stranded genome in nucleocapsid. One possibility is that there may not

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be sufficient amount of deoxyribonucleic acids inside the nucleocapsids which prevents further genome replication.

1.3.3 Budding and Secretion of Mature Virions and Non-Infectious Particles

One direct result of individual promoter controlling the synthesis of mRNA coding for the major surface antigen (HBsAg) is that a much higher amount of HBsAg is produced, as compared with middle hepatitis B surface proteins (MHBs) and large hepatitis B surface protein (LHBs). HBsAg is translated into the endoplasmic reticulum (ER), to form aggregates in specific regions of the Golgi complex. The process of aggregation also excludes host proteins from joining the aggregates. The aggregates then bud into the lumen of the ER to produce the secreted 22nm particles with no HBV genome inside (Cheng *et al* 1986; Chisari *et al.* 1986; Persing *et al.* 1986; Standring *et al.* 1986).

On the other hand, the newly formed nucleocapsid containing HBV genome are associated with areas of the Golgi that are high in HBsAg. LHBs interact with the nucleocapsid to form the 42 nm enveloped particles which then exit from the infected cells via the process of exocytosis.

1.4 Structure and Function of the Core Promoter

The core promoter (CP) of HBV plays a key role in HBV replication. It directs the transcription of 3.5kb pregenomic RNA (pgRNA). The pgRNA serves as the template for reverse transcription of HBV once it is bound to the viral polymerase and enveloped into the

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nucleocapsid. The pgRNA also codes for the core protein and the viral polymerase. The sequence of the CP is believed to be contained between nt.1591 and 1851 as shown in Fig. 1-4.

The CP region overlaps part of two HBV genes: the 3' end of the HBx open reading frame and the 5' end of the pre-C/C coding region. The CP region can be divided into two regions, the upper regulatory region (URR), and the basic core promoter region (BCP). The BCP is involved in the initiation of both pre-C mRNA and pgRNA transcription (Yuh *et al.* 1992), and the different initiation sites of pre-C mRNA and pgRNA are shown on Fig. 1-5. The URR (nt.1631-1742) is mainly involved for regulation of BCP. It contains two regulatory elements, core upstream regulatory sequence (CURS nucleotide 1636-1742) and a negative regulatory element (NRE nt.1613-1636) (Fig1-4).

FIG. 1-4 Organization of the CP of HBV (from Ref. [Kramvis A *et al.* 1999])

Legend is identical to text in paragraph 1.4

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The enhancer II (Enh II) is located upstream of the HBV core promoter (Fig. 1-4). The critical sequence required for the full enhancer activity is found to cover nucleotide 1687–1805. (Yee *et al.* 1989). As shown in Fig. 1-4, Enh II can be separated into two smaller sequence regions (box α and β). These two regions appear to work together for the full enhancer activity. It is reported that Enh II may function as CURS through activating the CP in a position- and orientation-dependent way. In addition, Enh II also can stimulate the S and X promoters (Yuh *et al.* 1991; Yuh *et al.* 1993; Lo *et al.* 1994).

1.4.1 The Basic Core Promoter

The Basic Core Promoter (BCP) is defined within the region covering nts1742-1849 [Yu *et al.* 1992] (Fig. 1-5). This region contains DR1 region which plays important role in HBV DNA reverse transcription. BCP also includes the initiation site of pre-C and pgRNA (Wang *et al.* 1990).

Fig. 1-5 Initiation sites of precore (pre-C) mRNA and pregenomic RNA transcription map within the BCP, they are shown as horizontal arrows. (Kramvis A *et al.* 1999)

The BCP does not have conventional TATA box (TATAAA) but has four TATA-like boxes (AT-rich regions) that can be recognized by the TATA-binding protein (TBP) (Figure 1-5).

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One of these TATA-like boxes, TA4 (nt1788-1795), is both a TBP-binding site for initiation of pgRNA transcription and the starting site for the full transcription of precore mRNA. Overall, the transcription of pre-C mRNA and pg RNA is regulated in a synchronized way (Chen *et al.* 1995).

1.5 Transcription Regulation

The HBV genome contains four genes including the core, the polymerase, HBx, and the surface gene respectively. The transcription of all these 4 genes is regulated by individual viral promoter as well as two enhancer regions. These two enhancer regions are shown on Fig. 1-6 (Kramvis, *et al.* 1999).

Fig. 1-6. Two enhancers of HBV. The enhancers are indicated as arrow, ENI and ENII.

(Kramvis *et al.* 1999)

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1.5.1 Core Promoter Binding Proteins

Like many other viruses, HBV has its own host range and tissue specificity towards liver cells (Guidotti *et al.* 1995). As we mentioned before, the two enhancers may bind to liver-specific host factors. These factors may also bind to other regions of BCP. Therefore, the liver cell specificity is determined by the interaction of the host factors and the *cis*- acting elements of virus (Honigwachs *et al.* 1989).

According to literature review, there are many transcription factors that bind to various regulatory sequence elements. The binding of transcription factors in turn plays either positive or negative roles on HBV promoters and enhancers. The core promoter plays a key role in HBV replication. Therefore, those nuclear receptors which can bind to the core promoter region may have very important regulatory function (Kramis *et al.*1999). The transcription factors which have been shown to bind to the core promoter region are shown as Fig.1-7. Some of them increase the activity of transcription, while others repress the transcription. These different functions subsequently affect the viral replication (Gunther *et al.* 1996).

Fig. 1-7 Map of transcription factor binding sites on the core promoter (CP) of HBV (Kramvis *et al.* 1999). Binding sites shown rat nuclear liver extract (RNLE).

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CCAAT/enhancer binding protein (C/EBP), hepatocyte nuclear factor 1 (HNF1), HNF3, HNF4, liver enriched factor (LEF), Sp1 protein, and TATA-binding protein (TBP)

As shown in Fig 1-7, transcription factors that bind to the BCP region include RNLE, C/EBP, SP1, TBP, hepatocyte nuclear factor 4 (HNF4), HNF1 and LEF. LEF is also known as the Liver Enriched Factor. It includes some members of nuclear receptor superfamily such as retinoid X receptor (RXR), chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1), peroxisome proliferators-activated receptor (PPAR) and apolipoprotein AI regulatory protein 1 (ARP1). LEF is shown to differentially regulate the synthesis of the precore mRNA and pgRNA. In addition, the RXR α -PPAR α activates the viral transcription from the nucleocapsid promoter by interacting with HNF4 site 2, and COUP-TF1 and ARP1 (Yu *et al.* 1997). It is reported that HNF4 stimulates the transcription of the precore RNA and core RNA from the wild-type (WT) core promoter. The two binding sites of HNF4 are from nt1652-1684, nt1751-1780 (Fig 1-8). On the other hand, HNF 1 activates some of liver specific genes. CCAAT/enhancer binding protein (C/EBP), a liver-specific nuclear factor, has been shown to activate the CP activity at low concentrations but represses the activity at high concentrations. Finally, the Sp1 protein is one of the best known transcription factors. For HBV replication, it directs synthesis of pgRNA by binding to three binding sites (Zhang *et al.* 1993). The binding site of Sp1 is also shown on Fig 1-8. There are four TATA binding boxes in this region, which can be bound by TATA-binding protein (TBP). The ubiquitous TBP also plays an important role in the regulation of general transcription (Chen *et al.* 1995). Thus the synthesis of pre-C mRNA and pg RNA occurs from separate promoters and these promoters are differentially regulated (Yu *et al.* 1996, Raney *et al.* 1997).

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It is reported that female individuals are prone to develop HCC in HBV-infected patients compared to male individuals (Beasley *et al.* 1988). It is believed that hormones may play some roles in hepatocarcinogenesis, by regulating the expression of HBV genes. And recently, several hormone response elements (HRE) have also been identified in HBV genome (Fig 1-9) (Zhang *et al.* 1993; Yu *et al.* 1996; Raney *et al.* 1997). By way of binding to different nuclear receptor super families, HRE should influence the regulation of viral gene expression at the transcriptional level.

Fig. 1-9 Schematic diagram of HRE. The HREs and glucocorticoid response element (GRE) are indicated by small shaded squares (Yu *et al.* 1997)

Recently it has been shown that nuclear hormone receptors play an important role in regulating HBV RNA synthesis and viral replication. Among these transcription factors, the most important one is the transcription factor which binds the proximal nuclear hormone binding in the nucleocapsid promoter (Tang *et al.* 2001).

The site of DR1 HRE is from nts 1751-1775. The HNF4 binding site 2 can bind to this region, it spans the TATA box-like sequence of the pre-C promoter. Chicken ovalbumin upstream

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promoter transcription factor 1 (COUP-TF1), human testicular receptor 2 (TR2), and peroxisome proliferator-activated receptors (PPARs) as heterodimers with retinoid X receptors (RXRs), can also specifically bind this DR1 HRE. It is well reported that different nuclear receptors can compete for binding to the same HRE (Tsai *et al.* 1994). Thus, the activities of viral promoters are likely to be modulated by different HRE binding proteins.

1.6 Significance of Core Promoter Mutation in HBV Genome

During HBV infection, both humoral and cellular host-immune response and antiviral therapy exert selection pressure on HBV. Mutations in HBV have emerged to enhance their survival rate under such pressures. In addition, the HBV DNA polymerase is devoid of proof reading ability and consequently mutations are introduced into the genome throughout viral replication. There are therefore many naturally occurring mutations in HBV genome, either resistant to antiviral agents or capable of escaping neutralization by HB vaccines (Locarnini *et al.* 2004; Zuckerman *et al.* 2000). Some of 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 (Gunther *et al.* 1999).

For chronic HBV infection, it is reported that the high prevalence of PreC and BCP mutants are found in most viral strains (Chu *et al.* 2003). Considering the key role that core promoter plays in the viral life cycle, any mutation found in this region may theoretically have consequences on the subsequent viral replication (Honda *et al.* 1999; Kurosaki *et al.* 1996). Some of the mutations detected in this region have been linked to hepatocarcinogenesis, including the frequently found nt A1762T/G1764A substitution (also referred to as mutational hotspot)

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(Frank *et al.* 2004). Furthermore, these double core promoter mutations can moderately enhance viral genome replication, and meanwhile, while reducing the expression of HBeAg.

It has separately been reported that high replication rate is associated with naturally occurring core promoter mutants in outbreaks of fulminant hepatitis (Parekh *et al.* 2003). These mutations are able to affect the viral replication in various ways (Scagkioni *et al.* 1993). Another quite common mutation is the precore stop codon mutation. Viral strains carrying such a mutation are characterized by the absence of HBeAg. This mutation has been found in patients with fulminant hepatitis or chronic active hepatitis (Omata *et al.* 1991). Other studies have reported specific point mutations in the BCP in HBV from patients with fulminant hepatitis (Shih *et al.* 2003; Hasegawa *et al.* 1994; Horikita *et al.* 1994; Kaneko *et al.* 1995). In addition to point mutations in the core promoter, short deletions have also been reported in patients with chronic hepatitis B (Sato *et al.* 1995; Okamoto *et al.* 1994). Taken together, it is very likely that a particular clinical phenotype is a result of specific mutations in the core promoter (Takahashi *et al.* 1995).

At the viral replication level, it has been shown that HBeAg may not be critical for viral replication. However, it is essential to establish persistent infection possibly by decreasing the immune response against the core protein. Both the precore gene and the core gene code for the expression of HBeAg. Interestingly, some of core promoter mutants express less HBeAg due to its down regulation (Bang *et al.* 2005). As HBeAg shares common immune epitopes with the core protein, it can buffer the immune response caused by core protein. This explains why many core promoter mutations have correlation with severe clinical symptoms or high viraemia (high viral load). Another important issue is the fact that mutations in the core promoter may

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affect viral replication through their mutational effects on the overlapping viral genes, in particular HBx.

1.7 Naturally Occurring Deletions and Their Effect on HBV Replication

Deletions within the core promoter have been found in patients with various HBV related diseases such as chronic hepatitis, HCC, liver cirrhosis, active hepatitis. Of great interest is the fact that most of the deletions reported are found within the basic core promoter region. To date, at least 20 types of naturally occurring deletions have been reported in the basic core promoter. The size of deletions varies from 8 to 21 bp (Fig. 1-10). *In vitro* assay have shown a reduced production of pre-C mRNA and enhanced production of pgRNA in patients carrying viruses with deletions in the core promoter region (Moriama *et al.* 1997).

Fig. 1-10 Deletions in the basic core promoter (BCP) of HBV (20 deletion patterns in the BCP, overlapping the X gene, have been detected (Kramvis *et al.* 1999).

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Similarly to point mutations mentioned earlier, the deletion will cause either deletion or frameshift of the X gene as a result of the overlapping core promoter with HBx gene. These various factors contribute to the clinical outcome of HBV infection in association with deletions in the core promoter.

1.8 Research Scope and Objectives of This Project

1.8.1 Research Scope

The core promoter of hepatitis B virus (HBV) is crucial in viral replication. Previous studies showed that some deletions in the core promoter result in suppression of viral replication and HBV DNA (Uchida *et al.* 1995; Brunetto *et al.* 1990). However, our previous investigation in clinical serum samples indicated that deletions in the viral core promoter may lead to increases in viral replication. Unusually high HBV replication levels as indicated by serum HBV DNA level (around 228.16 pg/ml) as well as HBsAg level (around 140 µg/ml) were detected in three patients. Interestingly, deletions within the same region (nts 1758-1777, 1749-1768) of the viral core promoter were identified in these three samples. Their average serum HBV DNA level at 228.16 pg/ml is significantly higher than that of other patients (at 36.39 pg/ml). (Table 1, see Appendix) (Chen *et al.* 2000). Since the deletions in the core promoter seem to have direct impact on viral replication, it will therefore be of interest to characterize the underlying mechanisms at molecular level.

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Based on published data, the deletion 1 (from nts 1749-1768) spans the region of DR1 HRE, TA1, TA2 (Fig. 1-5), enhancer II (Fig. 1-4), and DR1 HRE (Fig. 1-6). Several known transcription factors are known to bind to this region including HNF4, COUP, RXR, SP1, CEBP, TBP. On the other hand, the deletion 2 (from nts1758-1777) spans the region of DR1 HRE, enhancer II, TA2, and TA3. Known transcription factors which bind to this region include HNF4 and RXR/PPAR. It is also known that the core promoter is transactivated in the presence of RXR and PPAR. Over-expression of the HNF4 protein leads to an increase in the level of transcription by three fold, whereas over-expression of RXR α -PPAR α increases the level of transcription by four fold [Raney *et al.* 1997].

As for the well known Sp1 protein, it has been reported that its binding to the core promoter (three binding sites) is critical for the transcription of the core RNA and the precore RNA. Its role can however be positive or negative depending on the specific location of the binding sites. For example, binding to the downstream site (nts1745-1754) has no apparent effect on the transcription of the S gene and the X gene. On the other hand, binding of Sp1 to the upstream site (nts1733-1742) has been found to negatively regulate the transcription of the S gene and the X gene. Sp1 binding to the site in the Enh II (nts1624-1633) has been found to positively regulate the expression of all of the HBV genes (Li *et al.* 2001).

Another transcription factor, C/EBP, binds to at least five sites on the CP with two of them overlapping Enh II. C/EBP modestly activates the activity of the CP at a low concentration of the protein, but represses the activity when its concentration is higher (Lopez *et al.* 1990; Lopez *et al.* 1991).

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It has been shown that the recombinant TBP binds to the four TA rich sequences in the core promoter region. While binding of TBP protein to TA3 positively regulates the transcription, its binding to TA1 and TA2 have not been found to influence the transcription level. Interestingly, binding of TBP to TA4 has been shown to play a role in the initiation of expression of pgRNA (Chen *et al*, 1995).

The HBV core promoter contains some binding sites for nuclear receptors. A natural deletion in this binding site, which deleted the nucleotides (nts 1749 -1768 and nts1758-1777) as reported in our lab. These deletions selectively abolish the binding sites of several nuclear receptors. In addition, these deletions also result in deletions and frame shift changes of HBx protein.

The deletion 1 affects binding sites for Sp1, HNF4, RXR which implies possible changes in the viral replication. It also affects the binding sites TA1 and TA2 for TBP although these two sites have no obvious influence on the viral replication. As for deletion 2, the binding site TA3 for TBP is affected which may imply that to increase the transcription of core promoter. In addition, the HNF4 binding site 2 was also deleted in deletion 2 mutant. Although the clinical data suggest an increase in HBV replication in patients carrying viral strains with deletion 1 and deletion 2 in the core promoter, the exact mechanism leading to the increase remains unclear. While both deletions remove binding sites for known transcription factors, it is also possible that the same deletions lead to rearranged sequences thus generating new binding sites for other transcription factors. This possibility is supported by the fact that the double mutation of nt 1765 from A to T and nt 1767 from G to A not only removes a nuclear receptor binding site but also creates a new binding site, with an overall suppression of transcription and enhancement of viral replication (Li *et al*. 1999).

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Previous studies have shown that the dominant HBV population in immunosuppressed patients with severe liver disease contains genomic changes such as deletions, insertions and/or base changes in the core promoter region. Most of the mutations create binding sites for hepatocyte nuclear factor 3 (HNF3) or HNF1. Recent report also indicates that a single mutation at 1752 (from A to G) generates the binding site for hnRNPK (Ng *et al.* 2005). Therefore, it is likely that these two deletion sites result in variation in the transcription level, either due to the loss of binding of transcription factors or due to the binding of novel TF to the rearranged region. Despite numerous reports on deletions in the core promoter, no molecular mechanism has been analyzed to reveal the association between the core promoter deletion and the HBV replication rate.

1.8.2 Significance of This Project

Current antiviral therapies for HBV carriers include alpha-interferon or lamivudine, but the long-term recovery from disease is not optimal due to the development of drug-resistant viral mutants and low seroconversion rates (Hoofnagle *et al.* 1997). The antiviral therapy also induces various mutations in the viral genome. These mutations not only affect the treatment efficiency, but also influence the replication capacity of the mutant viruses. Some carriers infected with HBV with mutations in the core promoter exclusively have high HBV DNA titers compared with the wild type virus (Okamoto *et al.* 1994). It has been reported that a 21 bp deletion in the basic core promoter (BCP) in patients displayed different clinical rate of HBV replication. Detailed investigation on HBV mutants will provide us with better understanding of HBV-related pathogenesis. To date, direct relationship between host-virus interactions and chronically infected patients remains to be characterized, including the relationship between the core promoter deletion and the HBV replication rate.

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Based on our previous clinical study, we postulate that deletions in the core promoter may generate new binding sites for cellular transcription factor, which in turn leads to the increase in HBV replication. Comparative transfection experiments are to be conducted for 2 naturally occurring core promoter deletions with wild-type clones and analyzed viral replication, virion secretion, and expression of HBsAg. The present study aims to first analyse the replication potency of the mutant viruses (with deletions in their core promoter) and secondly to identify and characterize such transcription factors.

1.8.3 The Objectives of the Project

The objective of this study was to analyze the impact of the different deletions in the BCP on viral replication efficacy. Characterization of these deletion mutants is to be carried out in a cell based system that supports HBV replication with the following objectives:

- Analyse the HBV replication for mutants with deletions in their core promoter
- Identify and characterize cellular transcriptional factor involved in the increased HBV replication due to deletions in core promoter

2. Materials and Methods

2.1 HBV Infection in a Cell Culture System

A new cell culture system has been developed to analyze various HBV mutants. This is based on transfecting a replicative HBV genome into HepG2 cells. As shown in Fig. 2-1 and Fig 2-2, the promoter and transcription termination of the 3.5kb pregenomic RNA are located in the same region. A linear version of a replicative HBV genome should therefore contain this region at its 5' end (serving as promoter) and also at its 3' end (serving as transcription termination region) (Fig. 2-1). This can explain why promoter/termination regions were put on both ends of HBV genome.

Fig. 2-1 Schematic representation of linear HBV viral genome

The region of nts 1600-1900 has been marked, because this region contained the core promoter as well as the overlapping transcription termination region. And later will be used in the replicative genome construction as the tandem region.

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Fig. 2-2 Genomic organization of plasmid pcDNA3.1-1.1x HBV

This Fig shows the genomic organization of plasmid pcDNA3.1-1.1x HBV, the sequence actually include two fragments: the first fragment, from nts1600-3221 and the second fragment: 1-1900. It shows the HBV ORFs and the most important restriction sites along the HBV genome, together with their nucleotide positions, starting at the unique *EcoRI* (3221/1) site within the HBV genome. Because the X-ORF was truncated, it is therefore delineated in dashed lines. The transcription mRNA products include 3.5kb mRNA, 2.1kb mRNA, 2.4kb mRNA, 0.7kb mRNA,

In our laboratory, the promoter/termination regions were located in the central part (nt 1600-1900) of the HBV genome obtained from ATCC. Therefore, the replicative genome has been constructed. The replicative DNA constructs was composed of two fragment: the first fragment (from nts 1600-3215) and the second fragment (from nts 1-1900) (1 and 3215 are in fact immediately adjacent positions on a circular HBV genome or when the genome is appropriately linearized). The two fragments would be ligated into mammalian expression vector pcDNA3.1+ sequentially (Fig. 2-4).

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Such replicative genome has been successfully used to analyze various HBV mutants. In the case of vaccine escape mutants, point mutations have been found at antigenic region. Replicative genome carrying such mutation created by site-directed mutagenesis has been shown to display altered viral antigenicity after transfecting HepG2 cells. Such cell culture system was also evidenced to be useful for analyzing other types of HBV mutants, including those with deletion in core promoter.

2.1.1 Construction of Plasmids Containing the Core Promoter Deletions within the Context of the Reference HBV Genome

The wild type HBV replicative genome was the kindly gift from Prof William Chen's previous laboratory. It was used as the template for construction of plasmids containing the core promoter deletions. When the deleted replicative genome constructed, EcoR I site on mammalian cell expression vector was destructed firstly, then 2 PCR fragments, the first Fragment: nts 1-1900bp, the second Fragment: nts 1600-3215bp, were amplified out respectively, actually the second fragment which include the deletions was obtained by assembly PCR. The principle of assembly PCR was schematically described as shown in Fig. 2-3.

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Fig. 2-3 Generation of deletion in viral core promoter by assembly 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.

2. Materials and Methods

Two steps cloning strategy was employed in this study, which is listed as below (Fig. 2-4),

- 1) The second fragment was cloned into pcDNA3.1+ (Nru I site).

- 2) The first fragment was cloned into EcoR I site which was located at the end of the second fragment.

Fig. 2-4 Construction of the replicative plasmid which includes mutated core promoter.

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2.1.2 Plasmid Construction for Replication Model (Deletion 1, Deletion 2)

2.1.2.1 Amplification of the Vector

The mammalian cell’s expression plasmids pcDNA3.1+ was purchased from Invitrogen. The plasmids listed in Table 2-1 include the cloning vectors and the clones constructed in this study. Plasmid pcDNA3.1+ map is shown in Appendix C.

Table 2-1 List of the bacterial and plasmids for cloning

Bacterial strain /plasmids	Relevant characteristics	Source or reference
E coli strain DH5 α		Purchase from Invitrogen
pcDNA3.1+vector	This vector contains an CMV promoter, size: 5.428bp	Purchase from Invitrogen
pcDNA3.1+HBV WT	This is a vector with HBV replicative genome A wild type inserted upstream of the CMV promoter	This study
pcDNA3.1+HBV Del1	This is a vector with HBV replicative genome A -deletion 1 inserted upstream of the CMV promoter	This study
pcDNA3.1+HBV Del 2	This is a vector with HBV replicative genome A -deletion 2 inserted upstream of the CMV promoter	This study
pBIND	This is a vector which can encode Renilla luciferase	Purchase from Promega

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2.1.2.1.1 Bacterial Strain

The bacterial strains and plasmids used in this study have been listed in Table 2-1. *Escherichia coli* (*E. coli*) strain DH5 α was used as a host of the mammalian expression plasmid pcDNA3.1+ for molecular cloning. It was grown on Luria-Bertani (LB, Appendix-D) medium at 37°C. *E. coli* cells containing recombinant plasmids, which encode resistance to ampicillin, was maintained on LB agar (Appendix D) supplemented with the antibiotic used at a final concentration of 100 μ g/ml (Appendix D). The bacterial strains were kept on LB agar stored at 4°C for short-term storage (around 1 month). Bacterial cultures were also kept in LB broth with 15% glycerol and frozen at -80°C for more than one year.

2.1.2.1.2 Preparation of Competent Cells

The cell used in this study is DH5 α (purchased from Invitrogen). A single freshly isolated colony of *E.coli* strain DH5 α was inoculated into 5ml of LB broth and grown at 37°C overnight with shaking. 1ml of the cell culture was inoculated into 100ml of LB broth and incubated at 37°C with shaking for several hours (normally 2 hr) when OD₆₀₀ of culture was around 0.3-0.4. The cell culture was then centrifuged at 5,000 rpm for 5min at 4°C. The supernatant was discarded and the cell pellet was gently resuspended in 30ml of 0.1M ice cold MgCl₂ (appendix D). The mixture was then centrifuged at 5,000 rpm for 5min at 4°C. The supernatant was discarded and the cell pellet was gently resuspended in 20ml of 0.1M ice cold CaCl₂ (appendix D). The reaction mixture was incubated on ice for 30min. Centrifugation of the reaction mixture was subsequently conducted for 5min with 5,000 rpm at 4°C. Then the supernatant was removed, after that the cell pellet was resuspended in 1.5ml of ice cold 0.1M CaCl₂/15% glycerol (appendix D) , following that, it was aliquoted to 150 μ l each eppendorf tube and kept in -80°C freezer until required.

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2.1.2.1.3 Transformation of *E.coli* Strain DH5 α

75 μ l of freshly prepared competent cells of *E.coli* strain DH5 α was each mixed with the respective plasmid DNA and incubated for 5min on ice, the reaction mixture was subsequently heat-shocked at 42°C water for 1 min. Prior to the addition of 1ml of LB broth, the reaction mixture was incubated on ice for 5min again. Then the reaction mixture was centrifuged at 14000rpm for 2min, after which the supernatant was discarded. The cell pellet was resuspended in 100 μ l of LB broth and diluted accordingly. The cell culture was then spread onto LB agar plates with ampicillin (100 μ g/ml) and incubated overnight upside down at 37°C.

2.1.2.1.4 Growth of *E.coli* Strain

A single colony of *E.coli* strain DH5 α - pcDNA3.1+ was each picked up from the agar plates and inoculated into 5ml of LB broth containing ampicillin (100 μ g/ml). The cell culture was then incubated at 37°C, with shaking at 250rpm, for 8 hrs, after that, 0.5ml of cell culture was inoculated into 200 ml of fresh LB broth containing ampicillin (100 μ g/ml) and the resultant cell culture was incubated at 37°C overnight with shaking. After culture for 16-18 hr, the cell pellet was accumulated through centrifugation (Sigma centrifuge) at 5,500 rpm at room temperature, and the cell pellet was kept in -20°C until use.

2.1.2.1.5 Preparation of Plasmid DNA

Large scale preparation of plasmid DNA from *E.coli*

Vector pcDNA3.1+ was isolated by using the Qiagen MIDI kit (Appendix C) according to the manufacturer's instructions. 1ml of overnight culture was subcultured in 100 ml of fresh LB/amp medium, after shaking for 8 hours at 300rpm. The culture was harvested by centrifugation at 5500rpm for 15min at 4°C (Sigma centrifuge). The cell pellet was resuspended

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thoroughly in 4ml of ice-cold buffer P1, 4ml buffer P2 was added and the sample was mixed thoroughly by inverting for 4-6 times and incubated at room temperature for 5 min. Subsequently, 4ml of chilled buffer P3 was added, after inverted for 4-6 times, it was incubated on ice for 20min, after that, the cell debris was removed by centrifugation at 20,000 g for 30min at 4°C. After the QIAGEN-tip 100 has been equilibrated by 4ml of buffer QBT, the supernatant sample was allowed to enter the resin by gravity flow. After the QIAGEN-tip was washed for two times by buffer QC, the DNA was eluted with 5ml of buffer QF, in order to precipitate the DNA, 3.5ml of room temperature isopropanol was added to the eluted DNA, after centrifuged at 20,000 g for 30min at 4°C, the DNA pellet was washed with 2ml of 70% ethanol, and centrifuged at 20,000g for 10min, the pellet was air-dried, and redissolved in 100ul of buffer EB (Appendix D).

2.1.2.1.6 Mini-preparation of Plasmid DNA

This was carried out using the Qiagen miniprep kit (appendix D) according to the manufacturer's instructions. 5ml of overnight culture was centrifuged at 5,500 g for 5min, the cell pellet was resuspended in 250µl buffer P1 and transferred to a micorcentrifuge tube, then 250µl of buffer P2 was added to the reaction mixture to lyse the cells, then buffer N3 was added and the sample was mixed immediately and thoroughly by inverting the tube for 4-6 times. After centrifugation for 10min at 13,000 rpm in a table-top micro centrifuge, the supernatant was applied to the QIAprep spin column by decanting, then the column was centrifuged at maximum speed for 30-60 sec. After washed with 0.75ml of buffer PE, the DNA was eluted out with 30 µl of buffer EB.

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2.1.2.2 Construction of Replicative HBV Genome with Deletion in Core Promoter

All the replicative genome now constructed harbor more than one HBV genome, eg: the HepG2-2.2.15, contains two head-to-tail copies of the RNAs. HB611 HBV which was established by transfecting Huh6- c15 cells with plasmid p3HB-neo carries three tandemly arranged HBV genomes. (Weiss *et al.* 1996) The replicative genome constructed by our group contains a merely terminally redundant 1.1× HBV genome. The terminal redundancy of the recombinant viral genome is necessary because HBV replicates via reverse transcription of a 3.5-kb pregenomic RNA, resulting in a 3.2-kb partially double-stranded DNA genome, which is part of the virion (outlined in Fig. 2-2). Plasmid pcDNA3.1-HBV contains each of the four major open reading frames only once and exclusively under the control of their autologous regulatory elements (Fig. 2-2).

The HBV genome containing pBR325 plasmid (ATCC, USA) was used as a template, The HBV genome was cloned in pBR325 the sequence was from 1600-3221/1-1900, which was call 1.1 fold HBV genome. Because The region 1600-1900 contained the core promoter as well as the overlapping transcription termination region. (Weiss et al 1996) This genome was the kindly gift from Prof William Chen's previous Lab. To generate deletion mutant constructs, two pairs of primer were designed for two separate rounds of PCR. The primer sequences are shown in Table 2-2. HBV genomes in pBR325 were used as a template to generate by assembly PCR the first fragment (nts1600-3221), with deletions in viral core promoter (nts1749-1768 and nts1758-1777 respectively). The wild type HBV genome in pBR325 was in turn used as a template to generate by normal PCR the second fragment (nts1- 1900), with a wild type transcription termination region (nts1600-1900). In-frame ligation of each of the first fragments (carrying respective deletion in viral core promoter) with the common wild type

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second fragment into mammalian expression vector pcDNA3.1 (Invitrogen, USA) resulted in respective replicative HBV genomes (Fig. 2-4(2)). So the replicative genome sequence was shown schematically on Fig 2-5.

Table 2-2 List of Oligonucleotide Primers for Deletion PCR

Primer for 1 st fragment (nts1600-3221)	
Deletion 1 (nts1749-1768)	
primer A	5'-GCAT <u>TCGCGA</u> CACGTTGCATGAGACCACC -3'
primer B	5'-GCCTACAGCCTCCTAATACTCCCCAGCTCCTCCCAG-3'
primer C	5'-CTGGGAGGAGCTGGGGGAGTGTATTAGGAGGCTGTAGGC-3'
Primer D	5'-GCT <u>TCGCGA</u> <u>GAATTC</u> TCCACTGCATGGCCTGAGG-3'
Deletion 2 (nts1758-1777)	
primer A	5'-GCAT <u>TCGCGA</u> CACGTTGCATGAGACCACC -3'
Primer B	5'- CCAATTTATGCCTACAGCCTCCTAATCTCCTCCCCAGCT- 3'
primer C	5'-AGCTGGGGGAGGAGATTAGGAGGCTGTAGGCATAAATTGG - 3'
primer D	5'-GCT <u>TCGCGA</u> <u>GAATTC</u> TCCACTGCATGGCCTGAGG-3'
Primer for 2 nd fragment (nts 1-1900)	
2 nd Fragment forward	5'-GCGAATTCCACCACTTTCCACCAAA-3'(nts1-20)
2 nd Fragment reverse	5'-GCGAATTCAAAGCCACCCAAGGCACAGCGC-3' (nts1880- 1900)

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Fig. 2-5 Generation of replicative HBV genomes

The sequence for HBV replicative (WT, Del1, Del2)genome are all from nts 1600-3221/1-1900.

Cloning procedure

The deleted core promoter fragment was amplified from primers bearing the appropriate restriction enzyme sites. The region of interest is flanked by the Nru I (5') restriction site and EcoR I (3') restriction site (Table 2-2). Hence, the pcDNA3.1+ vector was digested with Nru I. The enzyme digestion reactions were carried out in a volume of 20µl containing 1× restriction enzyme (RE) buffer (from NEB), 5µl of DNA vector and 15µl of insert, 1µl of each of the appropriate REs (NEB) and were topped up to 20 µl with ddH₂O. The reaction was carried out at 37°C for 4 hrs. The digestion result was interpreted by running an agarose gel to ensure complete digestion prior to subsequent manipulation. The products of these cleavage reactions were then extracted from agarose gel using QIAquick Gel Extraction Kit (APPENDIX D, Qiagen, Germany).

2.1.2.2.1 Preparation of the first Fragment (from nts 1600-1900)

The HBV-deletion (include Del 1 and Del 2) full-length replicative clone (genotype A) was constructed by using a HBV genome-containing pBR325 plasmid as a template, primers were

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designed to amplify two fragments: the first fragment: nts 1600-3221, and the second fragment: nts 1-1900. The region nts 1600-1900 contained the core promoter and the overlapping transcription termination region (Fig. 2-2). In frame ligation of the two fragments using the internal EcoR I (1/3221) ensures continuous viral open reading frames. The whole genome was cloned into the Nru I site in pcDNA3.1+, resulting in the replicative construct. Viral transcription was under its own promoter control as the viral insert was cloned at the Nru I site, located upstream of the plasmid's CMV promoter (Fig. 2-4).

First round PCR (to get fragment I and II, Fig. 2-3)

The first fragment, nts1600-3221, was generated as a two step PCR product from the HBV-pBR325 plasmid and cloned into pcDNA3.1+. The PCR reactions were carried out in a volume of 50µl containing 5×*pfu* buffer (Promega), 1µl of template (PBR325 hbv-wt) DNA vectors and 1µl of primer A(C), 1µl of primer B(D), 1µl of 10mM dNTP and 1µl of *pfu* polymerase (purchase from Promega) and the reaction mixture were topped up to 50 µl with ddH₂O. The reaction was carried out by using the protocol as follow: heated to 95°C and kept at this point for 5min, followed by 35 cycles of 95°C for 1min and 50°C for 2min; then heated to 72°C and kept for 5min, and lastly followed by 72°C for 10min. The PCR products were assigned as del1 fragment I, del1 fragment II, del2 fragment I, and del2 fragment II, respectively.

Second round PCR (to get the first fragment)

(by using fragments I and II as templates to generate the fragment including the deleted region)
The PCR reactions were carried out in a volume of 50µl containing 5×*pfu* buffer (from Promega), 1µl of each fragment I and II of Del 1 or Del2 served as template and 1µl of primer A, 1µl of D, 1µl of 10mM dNTP and 1µl *pfu* polymerase (from Promega) was

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added into the reaction mixture and lastly topped up to 50 μ l with ddH₂O. The PCR protocol was the same as first round PCR. The result was interpreted by 1% agarose gel.

Sequencing was done for verification of the constructs.

Preparation of the second fragment (from nts 1-1900)

The second fragment, comprised of nts 1-1900 of the HBV insert, was generated by PCR from pBR325-HBV and was cloned downstream of the first fragment in pcDNA3.1+. The PCR reactions were carried out in a volume of 50 μ l containing 5 μ l of 10 $\times$ *pfu* buffer (from Promega), 1 μ l of template (PBR325 HBV-wt) DNA and 1 μ l of the second fragment forward primer, 1 μ l of second fragment reverse primer, 1 μ l of 10mM dNTP and 1 μ l *pfu* polymerase (from Promega), lastly, the reaction mixture were topped up to 50 μ l with ddH₂O. The PCR protocol was the same as the one above.

2.1.2.2.2 Pretreatment of Vector pcDNA3.1+

As the first step, the EcoR I site in the MCS was removed. The pcDNA3.1+ was digested by EcoR1 enzyme, after the digestion was finished by confirmation with agarose gel, the DNA was purified by column. As the subsequent step, 1 μ l of 10mM dNTP and 1 μ l T4 kinase was added into the reaction system, after incubated at room temperature for 1 hour, the reaction mixture was undergone purification again to remove those enzyme and dNTP. Then 1 μ l of T4 ligase (from NEB) and 2 μ l of ligase buffer were added to perform self ligation. After colony screening, one of the correct colony which has pcDNA3.1+ vector without EcoR1 site in MCS was acquired.

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2.1.2.2.3 Restriction Enzymes (RE) Digestion of DNA Vectors and Inserts

Enzymes (Nru 1 or EcoR 1) were obtained from New England Biolabs Inc. (U.S.A), and were used in the buffers and conditions specified by the manufacturer. In general, an appropriate amount of DNA and the corresponding 10× restriction buffer were dispensed into eppendorf tubes and made up to the correct volume with distilled water. A ratio of 0.5-1 units of restriction endonuclease per µg of substrate DNA was employed; the contents were mixed and incubated at a specific temperature characteristic of the enzyme. The total volume of enzyme added must not exceed one tenth of the total volume, as the enzyme solutions used in this study contained 50% glycerol which, in excess, affects the activities of the enzyme.

For double restriction digestion, the best NEB buffer to provide reaction conditions that are amenable to both restriction endonucleases was selected according to the NEB buffer chart. Depending on an enzyme's activity, rating in a non-optimal NEB buffer, the number of units of incubation time of the reaction may be adjusted to compensate for slower rate of cleavage.

If no single NEB buffer can be found to satisfy the buffer requirements of both enzymes, the reaction can be done sequentially. First, cleavage was done with the enzyme that requires the lower salt reaction conditions, subsequently, the salt concentration of the reaction was adjusted (using a small volume of concentrated salt solution) to approximate the reaction conditions of the second restriction endonuclease. The second enzyme was added and incubated to complete the second reaction.

Another way of doing sequential double digests was after the first enzyme digestion, the DNA was purified through Qiagen quick DNA purification kit, after the DNA was digested by the second enzyme.

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2.1.2.2.5 Dephosphorylation of Vector DNA

To prevent self-ligation of digested vectors with compatible or different ends, 5µl of linearised DNA vector was dephosphorylated using 1µl of calf intestinal phosphatase (CIP) (NEB) in a 20µl reaction with the manufacturer's recommended buffer. After incubation at 37°C for 1 hr, the DNA was purified after gel extraction. For both the vector and inserts, those fragments were all gel extracted by using the Qiagen gel extraction kit.

2.1.2.2.6 Construction of the New Plasmid Including Deletion Part

By using the DNA which acquired from mini preparation of pcDNA3.1+ without EcoRI, the deletion fragment I (from nts 1600-3215 bp which included the deleted core promoter) of HBV was cloned into the constructed plasmid at Nru I site, and fragment II was cloned into the EcoRI site sequentially.

2.1.2.2.7 Ligation of DNA Vectors with the Desired Inserts

The ligation reactions were carried out in 20µl of reaction mixtures containing 1× T4 ligation buffer (NEB), 1 unit of T4 ligase (NEB), purified DNA vectors, inserts and ddH₂O. For the vector, insert ratio of the ligation of pcDNA3.1+ to the first HBV fragment was kept at 1:3. In general, 50ng of vectors and 150ng of the first fragment were used in the ligation reactions, ligations were conducted at 4°C overnight, or at RT for 4hrs. The products of reactions were subsequently used to transform competent *E.coli* strain DH5α cells.

2.1.2.2.8 Transformation

The same procedure as described in 2.1.2.1.3 (Transformation of *E.coli* strain DH5α) was carried out.

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2.1.2.3 Screening of the Positive Clone

Single colonies observed on the overnight cultures were transferred to fresh LB agar plates with ampicillin (100µg/ml) and numbered accordingly. Prior to another round of overnight incubation at 37°C, these colonies were inoculated into 5ml each of LB broth with ampicillin (100µg/ml) and grown at 37°C, with shaking, for 16 to 18hrs. Plasmids from these potential candidates of recombinant clones were extracted using a Qiagen mini preparation kit (Germany) according to the manufacture's instruction.

The extracted plasmids were used as DNA templates in subsequent rounds of PCR screenings for the desired recombinant clones. In other words, if the desired band (i.e. 1900bp) was observed after a round of PCR, its DNA template would be considered as a recombinant plasmid (vector carrying the desired insert) and its origin, a recombinant clone. The concentration of the recombinant plasmids were quantified at 260nm and stored at -20°C until required for transfection assays. The positive clone was sequenced to identify the deletion.

2.1.2.4 Determination of Plasmid DNA Concentration

DNA samples were diluted 125 times with sterile water and placed in quartz cuvettes. The absorbances at wavelengths of 260 and 280 nm were determined on a spectrophotometer (Biospec-mini DNA/RNA/Protein Analyser). Taking an absorbance of 1 unit at 260nm to be equivalent to 50µg/ml double-stranded DNA, the concentration of each DNA sample was calculated accordingly, taking into account the dilution factor. The ratio of the absorbance at 260nm to that at 280nm gives an indication of the purity of the DNA sample. A ratio of 1.8 suggests that the sample contains pure double-stranded DNA. A value significantly greater than

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1.8 indicates RNA contamination. While a ratio less than 1.8 indicates protein contamination. Then the sample was kept in the -20°C freezer until use.

2.2 Cell Culture and Transfection of HBV DNA

2.2.1 Cell Manipulation

2.2.1.1 Origin of Cell Lines

HepG₂ cell line was used in this study. Table 2-6 has shown the origin and characteristics of each cell line. The cell line was purchased from the American Type Culture (ATCC) and stored frozen in liquid nitrogen in our lab.

2.2.1.2 Culture of Cell Lines

The typical procedure for cell culture and uses of cells at different parts of this study is the same. Cell lines stored frozen in liquid nitrogen were thawed in a 37°C water bath and resuspended in 12ml of cold modified eagle medium (Appendix D) under sterile conditions. Cells were pelleted by centrifugation at 2,000rpm for 2min and subsequently resuspended gently in 2ml of DMEM which was supplemented with 10% fetal bovin serum (FBS) and antimycotics (Appendix D). All cells were cultured initially in 75cm² sterile canted-neck tissue culture flask (NUNC) in an incubator with humidified atmosphere of 5% CO₂ and 37°C. When the cells reached 80% confluence, they were trypsinized for sub culturing or harvesting.

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2.2.1.3 Trypsinization of Cell Lines

Upon achieving growth of 80% confluence, the cells were trypsinized. Culture medium was first removed and the cells were washed once with 10ml of sterile Phosphate Buffer Saline (PBS, Appendix D). Prior to use, trypsin (Appendix D) was warmed to 37°C and approximately 4ml of trypsin was added to each 75cm² culture flask, after the addition of trypsin, the culture flask was shaken gently for about 1 minute to ensure that the trypsin covered all the cells. After being incubated at 37°C for cells to detach, the flask was then gently patted to detach the cells from the matrix. The effect of trypsin was subsequently neutralized by the addition of 8ml of fresh DMEM medium (pre-warmed to 37°C).

2.2.1.4 Subculture and Harvesting of Cell Lines

Following trypsinization as mentioned previously in section 2.2.1.3. The cell suspension was then centrifuged at 2,000rpm for 2 min and the supernatant discarded. Subsequently, cells were treated according to the procedures they were required. The cells were resuspended in 12ml of DMEM medium and distributed into 3 new culture flasks to be incubated. Subculturing of cells was repeated until sufficient number of cells for harvesting was obtained.

2.2.1.5 Freezing of Cell Lines

Cells cultured in the 75cm² flask were trypsinized once 90% confluence was achieved. The cell suspension was centrifuged at 2,000rpm for 5 min and the supernatant was discarded. Cell pellet was then resuspended in 1.5 ml ice-cold freezing medium (Appendix D).

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2.2.1.6 Cell Culture

The adherent cell lines used in this study are HepG₂ cells. The cells were cultured and seeded for transfection studies according to the protocol that was stated above. Cells were plated onto 24 well culture plates (Iwaki, Japan) 24 hr before transfection at an average seeding density of 8×10^4 cells per well with 1.5 ml per well of DMEM. The cells were incubated in CO₂ incubator at 37°C for 24hrs to achieve confluence of 80% before transfection.

2.2.2 Transfection study

2.2.2.1 Transfection Reagent

Effectene transfection reagent (Appendix D) was chosen in this study.

2.2.2.2 DNA

Plasmid DNA for transfection should be free of protein, RNA and chemical contamination (e.g. an A₂₆₀/A₂₈₀ ratio of 1.8-1.9). DNA was suspended in sterile water to a final concentration of 0.2-1mg/ml. The optimal amount of the DNA to be used in the transfection would vary widely depending upon the type of DNA and the target cell line. In this study, the cells have been optimally transfected with 200ng of the different construction of pcDNA3.1+ vectors using the effectene reagent in a 24 well plate format for adherent cells. Increasing the amount of transfected DNA did not necessarily result in a higher transfection efficiency.

2.2.2.3 Time

The optimal transfection time is dependent upon the cell line and DNA used. For optimization experiments, transfection time was attempted from 2 hr to overnight; cell morphology was

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monitored during the transfection interval. It was evidenced that for the HepG₂ cells, 4 hr is the optimal transfection time, if transfection lasted for overnight, most of the cells would die.

2.2.2.4 Transfection of HepG₂ Cells

Transfection of cells with wild type HBV replicative genome and two recombinant plasmids of constructs [pcDNA3.1-HBV, pcDNA3.1-HBV(del1), pcDNA3.1-HBV(del2)]. 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% CO₂/95% air with 95% relative humidity. The HepG₂ cells grown in T₂₅ flask were used, when the cells came to 50-60% confluence, 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 the HepG₂ cells using Effectene transfection reagent (Qiagen, Effectene transfection reagent) (the transfection procedure is shown in Fig. 2-6). Briefly, plasmids were mixed with 8µl enhancer and 150µl buffer EC, the mixture was incubated at room temperature for 5 min, then 25µl Effecten reagent was added into the mixture, and incubated for 10 min, then the mixture was dissolved in 1ml of culture medium and the transfection mixture was overlaid on the cells drop wisely. After incubation at 37°C for 4 hr, the culture medium was replaced with 12ml of fresh DMEM containing 10% FCS after washing three times with phosphate-buffered saline (PBS), and the flasks were further incubated for 48hr. 200µl of culture medium was examined for the production of HBsAg. Day 1, cells were seeded at 80% confluence, and Day 3 cells or medium were collected for

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subsequent analysis. Culture medium was collected for the secreted HBsAg analysis. The cells were collected for real-time PCR analysis.

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Fig. 2-6 Overview of transfection with adherent cells (Adapt from Qiagen Effecten reagent kit).

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2.3 Measurement of HBsAg in Culture Medium

Auszyme kit (Abbott Laboratories, USA) was used to measure the secreted HBsAg. 160 µl of each constructs was transferred into the machine's assigned sample well. The reagent pack was gently inverted at least 5 times to ensure the microparticles in bottle #1 were resuspended. After carousel was placed on the center post and the reagent pack was put in the heater block in correct order, the door was closed and then the machine began to run. 1hr later, the result of the secreted HBsAg was collected.

2.4 Quantification of HBV DNA from Supernatant.

2.4.1 Isolation of Culture Medium Viral Particles

QIAamp DNA Blood Mini Kit (Appendix C) was used in this experiment. 20µl of QIAGEN protease was added into 200µl of culture medium sample, and 200µl of buffer AL was added to the reaction mixture. The mixture was incubated at 56°C for 10min, and then it was applied to the QIAamp spin column with inclusive of 200µl, while the flow through was spun and discarded. After the column has been washed by AW1 and AW2, it was eluted in 200µl of AE buffer.

2.4.2 Amplification of the HBV-Specific Fragments

HBV DNA isolated from the cultured medium was amplified by PCR in 50µl reaction buffer. The primer designation is tabulated in Table 2-3.

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Table 2-3 The primers designed for partial HBV core protein

HBV Core forward:	5'-atggacattgacccttataaag-3'
HBV Core reverse:	5'-agactctaaggcttctcgat-3'

Plasmid pcDNA3.1+ (HBV) was amplified with primers (as shown on table above). (94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 50°C for 2 min, 72°C for 2min, and lastly followed by 72°C for 10min) and with *Taq* DNA polymerase (NEB). The PCR product was resolved in 1% agarose gel to identify the size.

2.4.3 Quantification of HBV DNA from Supernatant

Real-time analysis of PCR enables truly quantitative analysis of starting template copy number with accuracy and high sensitivity over a wide dynamic range. Specialized thermal cyclers equipped with fluorescence detection modules were used to monitor the fluorescence as amplification occurs. The iQ™5 Multicolor Real Time PCR Detection System employed in this study builds on the strengths of the iCycler® Thermal Cycler (Bio-Rad), which provides the optimum performance for PCR. 0.15µl of HBV DNA product, was used as the real-time PCR template, GAPDH was used as the internal control.

The protocol employed is as follows, 1µl of diluted forward primer (1:98) and 1µl of diluted reverse primer (1:98), 1.05µl of cDNA sample and 7.5µl of Sybr green reagent were mixed together in an PCR tube by vortex; for negative control, 1.05µl of ddH₂O was served as the template, then the PCR with the following protocol was run: 50°C for 2 min, 95°C for 10 min,

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followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, and lastly followed by dissociation at 60°C for 10min. The PCR product was resolved in 1% agarose gels.

2.5 Quantification of pgRNA Product in the Cells Transfected with Different Constructs

2.5.1 Collection of Cells

The cells were trypsinized for collection. 5ml of trypsin was pipetted into the T₂₅ flask, after incubated in CO₂ incubator for 5 min; the cells were collected into the 1.5 eppendorf tube each. After centrifuged at 2,000 rpm for 2min, the cell pellet was kept in -80°C freezer until use.

2.5.2 Total RNA Isolation

Total RNA was isolated with RNeasy kit (Appendix D) according to the manufacturer's instructions. The eppendorf tube with cell pellet was taken out from -80°C freezer, 350µl of buffer RLT with 3.5µl of β-mecaptoethanol was added into the cells and it was sonicated to make lysis of the cells. After 1 volume of 70% ethanol was added, the mixture was applied into the column, and 700µl of RW1 was added into the column followed by centrifugation. Once the column was washed by buffer RPE for two times, the sample was eluted by 50µl of RNase free water.

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2.5.3 mRNA Isolation

In order to omit the contamination of transfected DNA, mRNA isolation was performed with Oligotex mRNA Mini Kit (Appendix D). The appropriate amount of buffer OBB (binding buffer) was added into the total RNA sample and mixed well, then it was incubated at 70°C for 3 min. After the sample was put on the bench for 10 min, the mixture was palleted with the addition of appropriate volume of oligotex. Washing was subsequently conducted with buffer OW2 (wash buffer) for two times, mRNA was then eluted with 20µl of buffer OEB (elution buffer).

2.5.4 RT-PCR

RT reaction

Before performing the RT reaction, mRNA acquired from last step was heated in a 10µl volume at 65°C for 5 to 10 min and then quenched on ice. Then the RT-PCR reaction mixture was prepared as follows: 5µg of heat denatured RNA, 3.0 µl of 10 x PCR buffer, 2.5 µl of 10mM dNTP, 6.0 µl of 25mM MgCl₂, 1.0 µl of random primers (1.8 mg/ml), 0.5 µl of reverse transcriptase (from Clontech), and 17.0 µl of ddH₂O were mixed together, and this reaction mixture was left at 25°C for 10 minutes, later the sample was incubated at 42°C for 1 hr, and after that, the cDNA sample was denatured at 95°C and placed on ice. Subsequently, a normal PCR was conducted to confirm whether the template can produce some PCR product. The reaction mixture was prepared as follow: 6.0 µl of cDNA product, 1.5 µl of 10 x PCR buffer, 0.2 µl of *Taq* polymerase (NEB), 0.5 µl of primer 1 (1.0 mg/ml), 0.5 µl of primer 2 (1.0 mg/ml), and 10.3 µl of ddH₂O was mixed together in a 0.5ml PCR tube. The PCR reaction was as

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follows: 95°C for 2 min, 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 45 sec, 72°C for 60 sec and lastly followed by 72°C for 10 min.

2.5.5 Real-time PCR assay

0.15µl of cDNA product was used as the template for real-time PCR, and the procedure is the same as that described in 2.4.3.

2.6 Promoter Activity Assay

2.6.1 Transfection procedure

2.6.1.1 Plasmids

The plasmids pGL3-Control (a luciferase plasmid with a simian virus 40 enhancer and promoter) and pGL3-promoter (an enhancerless luciferase plasmid with a simian virus 40 promoter upstream from the luciferase gene) were purchased from Promega Corp (USA). The plasmids listed in Table 2-4 include the cloning vectors and clones constructed in this study. The plasmid pGL3-promoter was used as the cloning vector for the transfection and luciferase expression studies. Restriction maps of the two vector plasmids, pGL3-Control and pGL3-promoter, are shown in Fig. 3 (Appendix C). The complete sequence of the multiple cloning sites of pGL3-promoter was used as the cloning vector (Fig. 4, Appendix B). Vector pGL3-promoter was used as the reporter vector to study the region of interest, HBV Enhancer II (EnhII-Luc) (131bp) was used as the insert to be studied. Vector pBIND was used to normalize the transfection efficiency.

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2.6.1.2 Plasmid Constructs

Plasmid pGL3-promo/EnhII-wt was constructed by ligation of the EnhII-Luc (131bp) MluI-BglII fragment (nt 1686-1801) with reporter plasmid pGL3 Promo/wt. The fragment containing the basic functional unit of the Enhancer II was amplified by PCR using the primers Luc F and Luc R (HBV position nts 1686 to 1705 and nts 1779 to 1801, respectively). MluI and BglII sites are underlined and cloned into the MluI /BglII restriction site of the luciferase reporter vector (pGL3-promoter). pcDNA3.1+vector cloning with the first fragment (nts1600-3215) was used as the template for pGL3-promoter-del1/del2 construction, and the pBR325-(HBV wild type) as the template for cloning of pGL3-promoter-wt (Table 2-4). The competent cell which was used in transformation experiment was E coli strain DH5 α from Invitrogen.

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Table 2- 4 List of the plasmids for cloning

plasmids	Relevant characteristics	Source of reference
pGL3 control vector	This vector contains an SV40 enhancer, size: 5256bp	promega
pGL3-promoter vector	This vector does not contain SV40 enhancer, size: 5010 bp promoter	promega
pGL3-promoter-wt vector	This is a vector with HBV wild type core promoter inserted in the MCS of pGL3-promoter vector	This study
pGL3-promoter-del1 vector	This is a vector with HBV deletion 1 core promoter inserted in the MCS of pGL3-promoter vector	This study
pGL3-promoter-del1 vector	This is a vector with HBV deletion 2 core promoter inserted in the MCS of pGL3-promoter vector	This study

2.6.1.3 Primer Designation

The primer was designed as listed in Table 2-5. The expected PCR product included the whole length of the Enhancer II element.

Table 2-5 Primer for luciferase assay (from nts1684-1798)

LucF	5'-GCACGCGTCAACGACCGACCTTGAGG-3'
LucR	5'-GCAGATCTACCAATTTATGCCTACAGCCTC-3'

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As a functional assay to study the effect of base pairs deletion on the Enhancer II element, the various clones of pGL3-promoter vector were transfected into various cell lines (Table 2-6). Constructed plasmid DNA together with control/promoter luciferase plasmid DNA (3:1) were used for each transfection mixture.

Table 2-6 Cell lines used for transfection studies (all these cell lines are purchased from ATCC)

Cell line	Tissue	HBV Status	Cell origin reference
HepG ₂	Hepatoma	HBV-	Aden et al(1979) [Nature 282: 615-616] (1980) [Science 209:497-499] (1987) [in vitro cell Dev. Biol. 23:349-354] (1990) [J.Biol.Chem.265:22,474-479]
Chang	liver	HBV-	Yang.M.H et al (2006) [oncogene Aug 28] Kang-Park S et al; (2006) [virus research July 24]
293T	kidney	HBV-	Lopez-Contreras et al (2006) [J. Biol.Chem Aug 17]

2.6.2 Luciferase Assay

After 48 hr post transfection, the culture medium was carefully removed from each well and the cells were washed with 2 ml of prewarmed PBS, 500 µl of 1×Glo-lysis buffer which was provided in the Bright-Glo luciferase assay system (Appendix D) was added to each well and incubated for 5 min at room temperature (the Glo lysis buffer was equilibrate to 22°C beforehand). Then the lysate was transferred to luminometer tube and a volume of Bright-Glo™ Assay Reagent equal to the volume of Glo Lysis Buffer was added. They were shaken

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slightly to make sure that they were mix very well. And luciferase activity measured as relative light units (RLU) was determined with a Turner 20/20 luminometer (Promega, USA).

The results of the luciferase activity were expressed as a percentage of the internal positive control (pBIND). To control variations in transfection efficiency, experiments were performed in triplicates and repeated a minimum of 3 times, pBIND (Appendix C) which encoded the renilla luciferase was used to monitor transfection efficiencies. After cell lysis, the stop-and-glo reagent (Appendix D) was added to measure the Renilla luciferase activity. The results of the luciferase activity were normalized by the transfection efficiency.

2.7 Isolation of a Novel Transcription Factor

2.7.1 In Silico Analysis of the Possible Transcription Factor of the Rearranged Core Promoter

TRANSFAC (The Transcription Factor Database) was utilized to seek possible transcription factor of the rearranged core promoter. The rearranged core promoters were used as the target sequences for the searching. The target sequences are as follows, deletion 1 (nts 1749-1768): (10 bps before the deletion and 10 bps after the deletion) **gctgggggag tgtattagga**. Deletion 2 (nts 1758-1777): (10 bps before the deletion and 10 bps after the deletion) **ggagattagg aggctgtagg**.

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2.7.2 2-Dimension (2D) Gel

There should be some difference in protein level of the cells transfected with different constructs. The 2D gel was therefore performed to elucidate the difference.

2.7.2.1 Transfection

Three different constructs were transfected into 3 of 80% confluence T₇₅ flasks of HepG2 cells.

2.7.2.2 Harvesting of Cells

48 hr after the transfection, the cells was harvested, after the cells was trypsinized, the cells was pelleted by centrifugation, then the cell pellet were washed with Phosphate Buffered Saline (PBS) for 3 times, and before last centrifugation, the cells were counted. At last, the cells pellet was kept in -80°C freezer.

2.7.2.3 Protein Extraction by Use of Lysis Buffer

Firstly, the volume of lysis buffer which need to be added into cell pellet was determined, the general rule-of-thumb was 200µl of Lysis Buffer per 1×10^6 cells; then, Proteinase Inhibitor (to prevent break-down of proteins, ie. 40µl per 1ml extraction), and Nuclease Mix (remove nucleic acids, ie. 10µl per 1ml extraction) were added to the buffer before use. After the appropriate amount of Lysis Buffer was added to sample, the reaction mixture was vortex for 1 min and was centrifuged at 14,000 rpm (4°C) for 1 hr. The supernatant containing proteins were then transferred to a new tube and aliquoted into tubes of 100 µl, as last step, the quantity of protein was checked by using Amersham 2D-Quant Kit and stored in -80°C before usage.

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2.7.2.4 Rehydration of IEF (Isoelectric Focusing) Gel Strip

Before use, DTT (20mM) and IPG buffer (0.5%) was added to the rehydration buffer. And around 350 μ l of rehydration buffer was used on a strip holder per gel strip, then the plastic backing was removed from the strip and gel face was laid down on the rehydration buffer in an even manner, as last step, approximate 2.5ml of cover fluid (mineral oil) was put on the gel and surrounding area, and the gel strip was placed on a flat surface overnight (minimum 10 hours).

2.7.2.5 Running of IEF

Next morning (or after 10 hours), the strip was removed from the rehydration strip holder and was washed briefly with milliQ water. Then the gel was dab dried and placed on a universal strip holder with the gel facing up this time round, positive end facing the longer side of the electrode strip. The electrode pad was cut and wetted with distilled water, then the water was removed by pressing the gel against filter paper so that the electrode pad was left moist but not wet. Then it was placed onto both ends of the strip and electrodes were placed on it, and then, the cup was placed near the positive end of the strip (near barcode). Before sample loading, 20mM DTT and 0.5% IPG (immobilized pH gradient) buffer were added in the sample, and approximately 0.2 ml of cover fluid was applied on the sample. Then the entire area was covered with cover fluid (~4ml total), and sample was placed on ETTAN IPGhor II. After these steps, IEF started to run. Once the running has completed, the strip was removed immediately and stored in equilibration tube at -80°C until 2nd dimension run on SDS-PAGE.

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For the preparation of the 10% PAGE gel, 10% PAGE gel was casted and left standing overnight for proper polymerization, and a layer of water-saturated butanol was placed at the top layer to level the gel.

2.7.2.6 IEF Program for HepG2 Cells

The protocol for IEF was divided into 5 phases. At phase I, the voltage was set at 300 volt (V). The voltage was increased to 500 V at phase II. And 1,000 V of the voltage was set for the phase III. Phase IV was a gradient phase, which had a voltage of 8,000 V. At the last phase, phase V, the voltage was set at 8,000 V as well, but the constant was 24,000 Vhr.

2.7.2.7 Equilibration of IEF Gel Strip for 2nd Dimension SDS-PAGE

Firstly, the equilibration buffer should be prepared. 0.1g of DTT and 0.25g of IAA was added into two tubes of equilibration buffer respectively (2 tubes of 10ml equilibration buffer per gel strip), then the gel strip was taken out from -80°C to thaw for a while. After the gel strip was equilibrated with DTT for 15min, and followed by equilibration with IAA for 15min, the IAA-equilibration buffer was decanted, and gel strip was washed with milliQ water, the electrode pad area was removed and gel strip was cut to the appropriate size.

2.7.2.8. Running of 2nd Dimension SDS-PAGE Gel

As the first step, the rainbow marker was prepared, 9.5µl of marker and 0.5µl of 2-mercaptoethanol were mixed together and heated at 95°C for 5 min and placed on ice for use. 2µl of processed marker was pipetted on cut filter paper, and it was allowed to air-drying before

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application onto SDS-PAGE gel beside gel strip. Subsequently, the PAGE gel was covered by melt molten Agarose Sealing Solution (melt at 65°C), after that, the equilibrated gel strip was carefully placed on top of PAGE gel, gel facing front, and the gel was posited by spatula to make sure that the gel strip has touched PAGE gel completely. As the next step, the air-dried molecular weight marker was added beside the gel strip for estimation of molecular weight. After the sealing solution has solidified, the entire setup was brought to the cold room for electrophoresis. The gel was run at 30mA (15mA per gel), for the initial 15 min, the voltage was adjust to the highest value, following that, it was run at 60mA (30mA per gel) for the subsequent 5.5 hours or so (until dye front reaches about from the base). Silver staining was then proceeded using PlusOne Silver Staining kit after. The gel was first fixed overnight if the process was done late.

2.7.3 Gel Shift Assay

2.7.3.1 Gel Retardation Assay

Gel retardation assay is also known as protein mobility electrophoresis, gel electrophoresis DNA-binding assay and mobility-shift DNA-binding assay. This assay is based on the principle that DNA-protein complexes have different mobilities to uncomplexed DNA during polyacrylamide gel electrophoresis (Spence, 1991). Hence, by investigating the changed mobilities of restriction fragments in a gel when complexed to DNA-binding proteins, the localization of the binding regions is correlated with the restriction fragments. This assay is also used to determine if there is more than one protein involved in binding which may be different fragments or to the same fragments, and requires assay of the protein itself. Once the binding is localized to a defined DNA fragment, then the method can be used as an assay to

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monitor purification of the DNA-binding protein (Spence, 1991). In this study, gel retardation assay was used to confirm the binding of the deletion region and the novel TF. The LightShift® Chemiluminescent EMSA Kit (PIERCE) (Appendix C) was used to perform the gel shift assay in this study; it used a nonisotopic method to detect DNA-protein interactions. Biotin end-labeled DNA containing the binding site of interest was incubated with a nuclear extract or purified factor. This reaction was then subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled DNA was detected using the Streptavidin-Horseradish Peroxidase Conjugate and the Chemiluminescent Substrate.

2.7.3.2 Preparation of Crude Nuclear Protein Extracts

Nuclear extraction from cells (NE-PER Extraction, Appendix D) was conducted according to the following protocol, $\sim 2 \times 10^7$ cells were obtained from one T₇₅ flask of 100% confluent cells (HepG₂). The cell pellet was washed with ice-cold 1× PBS and stored at -80°C till ready for use. When nuclear protein extraction started, the cell pellet was resuspended in 400µl of cold CER1 which complete inhibitors and 0.5mM DTT were included (18 µl of 25×complete +432 µl CER1 +0.2 µl 1M DTT). Then, the cell mixture was vortexed vigorously for 15 sec, and incubated on ice for 10 min, next, 22µl of ice-cold CERII was added into reaction mixture. And cell mixture was vortex again vigorously for another 5 sec and incubated on ice for 1 min, then the cell mixture was vortex once again for 5 sec and spun at maximum speed for 5 min at 4°C. Next step, the supernatant (cytoplasmic extract) was transferred to another tube (keep or discard) and the cell pellet (nuclear extract) was resuspended in 200µl of cold NER (with complete inhibitors and 0.5 mM DTT). Then the nuclei mixture was vortex vigorously for 15

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sec and placed on ice for 10 min, and during this period of time, the sample was vortex again vigorously for 15 sec every 5-10 min. as last step, the sample was spun at maximum speed for 10 min at 4°C and the supernatant was collected and dialyzed with mini dialysis units (Pierce #69550) in 100 µl aliquots in 1 liter cold dialysis buffer at 4°C for 2hr. then, the nuclei extract (~100–120µl) was collected and the concentration of nuclear protein was measured, and sample was stored at –80°C.

2.7.3.3 Quantification of Protein Concentration

The protein concentration was quantified using the Bio-Rad Protein Assay kit (Bio-Rad, USA) based on the Bradford Dye Assay. IgG was used as the standard protein in the range of 2 to 40µg with 1 ml of diluted dye. Samples were incubated for 5 min before the absorbance at 595 nm on a spectrophotometer was read. Typical yield was about 1-2 mg/ml of nuclear protein isolated from 1×10^7 HepG₂ cells.

2.7.3.4 Probe Preparation for Membrane Hybridization

Probe (P) (Table 2-7) used were synthesized as complementary single-strand (forward [F] and reverse [R]). Oligonucleotides were synthesized (Sigma Proligo Corp, USA) and purified by high-pressure liquid chromatography (HPLC). These single-stranded oligonucleotides were then annealed to form the double stranded oligonucleotides probes.

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Table 2-7 Probes used for membrane hybridization assay

probe	sequence
WT forward	5'-(biotin) gggaggagct gggggaggag attaggtaa aggtctttgt attaggagc tgta-3'
WT reverse	5'-taca gcctcctaata acaaagacct ttaacctaata ctctccccc agctcctccc-3'
DEL1 forward	5'-(biotin) gggaggagctggggggag tgtattaggaggctgta-3'
DEL1 reverse	5'-tacagcctcctaataca ctccccagctcctccc -3'
WT compete forward	5'-gggaggagct gggggaggag attaggtaa aggtctttgt attaggagc tgta-3'
DEL1 competitive forward	5'- gggaggagctggggggag tgtattaggaggctgta-3'

2.7.3.4.1 Annealing of the Probe

The single-stranded oligonucleotides were annealed based on the method as described by Vinson et al. (1988) with some modifications, 5μg of each complimentary oligonucleotide were mixed with 10×T4 polynucleotide kinase buffer (New England Biolabs) in a 100μl reaction, and annealed using the following profile: 65°C for 15min, 37°C for 15min, 24°C for 15min, and 4°C for 15min. After the annealing has finished, 5μl sample was interpreted on 3% agarose gel, and the annealed sample left was stored in -20°C until use.

2. Materials and Methods

2.7.3.4.2 Gel Purification of the Probe

The band of the annealing product was excised from the agarose gel, and the gel was mixed with resuspended QIAEX II by vortexing. Then the gel mixture was incubated on 50°C for 10min to solubilize the gel and the DNA will bind with the suspension as well. After washing the suspension with buffer QX1 and PE, the double strand DNA was eluted in 20µl of ddH₂O. Then the sample was aliquoted and stored in -20°C until use.

2.7.3.5 Preparation of Native Polyacrylamide Gel

Gel retardation assay was performed using the vertical slab unit from *bio-rad*. For the gel preparation, 6% polyacrylamide gel was first prepared, 4ml of acrylamide:Bis (29:1) was added into a 50ml Falcon tube, and 2ml of 50% glycerol was added, followed by 2ml of 5×TBE (Tris-Borate) and 12ml of ultrapure water. 60µl of 10% APS and 60µl of TEMED was added. The gel was allowed to set for 1 hr at room temperature. Once the gel was set, the comb was removed and the wells were flushed with ddH₂O to remove unpolymerized material.

2.7.3.6 Preparation of Protein-DNA Binding Reaction

Binding reaction procedures were performed (Pierce Lightshift Chemiluminescent EMSA Kit, Appendix D). For specific competition, the binding reaction was pre-incubated with 200-fold excess of unlabeled probes. For the sample preparation, 3 tubes of reactions were included in each set of sample, #1 was negative sample which only include labeled probe; #2 was testing sample which include labeled probe and nuclear extract; # 3 was the sample with competent probe in which 200- fold excess unlabeled probe was added except for the labeled probe and nuclear extracts.

2. Materials and Methods

First of all, the stock reaction mixture for each tube was prepared, the formula was as follows: 2 μ l of 10 $\times$ binding buffer, 1 μ l of 1 μ g/ μ l poly (di/dc), 1 μ l of 50% glycerol, 1 μ l of 1% NP-40, 1 μ l of 1M KCL, 1 μ l of 100mM MgCl₂, and 1 μ l of 200mM EDTA were added, and for negative control, 10 μ l of ultra pure water was added, for testing tube, 7 μ l of ultra pure water was added, and for sample with compete probe, 5 μ l of ultra pure water was added. Then, these reaction mixtures were vortex vigorously to mix well and were incubated on ice for at least 10 min.

As next step, the nuclear extracts and unlabeled probe were added. For tube #2 and tube #3, 3 μ l of nuclear extracts was added into each tube, and for tube #3, 2 μ l of 2pm unlabeled probe was also added. Then the sample was mixed gently by pipetting up and down and incubated on ice for 20 min. Lastly, biotin-labeled probe (10 fmol) was added in each tube. And the sample was mixed gently by pipetting up and down and incubated on room temperature for 20 min. then, 5 μ l of loading buffer was added to each reaction and was mixed gently. Then, the whole reaction was left on ice until loading. At the same time, when polyacrilamide gel has solidified, the gel was pre-run in 0.5 $\times$ TBE buffer in cold room for 40 min. Then the prepared samples were loaded onto the gel and the gel was run at 100V for 2hr. When the gel was running, Nylon membranes (Pierce Biodyne B, #77016) and thick filter papers (2 pieces per gel) were presoaked in cold 0.5 $\times$ TBE for at least 10min. After gel running has finished, the gel was moved to a piece of thin filter paper and was transferred to membrane using Semi-Dry Method (BioRad) at 21 V for 45min. After transfer was completed, the membrane was placed on a dry filter paper with bromophenol blue side up. Then the membrane was cross-linked by UV on the transilluminator for 15 min with DNA side face the machine.

2. Materials and Methods

Detection of biotin-labeled DNA by chemiluminescence (Pierce#89880) was performed according to the following protocol:

The blocking buffer and the 4× washing buffer were warmed up to 37°C in a water bath until all particulate is dissolved. Then the membrane was blocked with 20ml of blocking buffer for 15min with gentle shaking. At the same time, the conjugate/blocking solution was prepared by adding 66.7ul stabilized streptavidin-HRP conjugate to 20ml blocking buffer (1:3,000). Then, the blocking buffer was removed from the membrane and conjugate/blocking buffer was added, and membrane was incubated for 15min with gentle shaking. When shaking was performing, the 1×wash buffer was prepared by adding 40ml of 4×wash buffer to 120ml ultra pure water. Then, the membrane was transferred to a new container and was rinsed briefly with 20ml of 1×wash buffer. After the membrane was washed for 4 times (the membrane was shaken in 20ml of 1×wash buffer for 5 min each time), the membrane was transferred to a new container incubated with 30ml of substrate equilibration buffer with shaking for 5min, meanwhile, the substrate working solution was prepared by adding 6ml of luminal/enhancer solution to 6ml of stable peroxide solution. Then the membrane was removed from substrate equilibration buffer and blotted on filter paper to remove excess buffer. After that, the membrane was placed in a clean container and substrate working solution were pour onto membrane, then it was incubated for 5 min without shaking with DNA side face down, after that, the membrane was removed from working solution and blotted on a piece of filter paper for a few second to remove excess buffer. Then, membrane was wrapped in a piece of plastic wrap. The last step was exposure; the membrane was exposed by using CL-Xposure film (Pierce #34090) for 1 to 5 min.

2. Materials and Methods

2.7.4 Membrane Hybridization

The TranSignal™ TF Protein Array (Appendix C) provides a method by which researchers can very quickly survey direct interactions between TF proteins and their complimentary cis-binding element. This means that by simply producing a biotinylated version of promoter region of interest, people can assess which transcription factors arrayed on the membrane have the potential to bind to this region. Once these have been identified it starts to give clues about which transcription factors, and their associated signaling pathways, might be regulating the expression of your gene of interest. The flow chart of the TranSignal™ TF Protein Array DNA is shown in Fig. 2-7.

Fig. 2-7 Flow chart of the TranSignal™ TF protein DNA array. (From Panomics TranSignal™ TF protein DNA array handbook)

2. Materials and Methods

Two membranes were placed into two separated containers which containing 4ml 1× binding buffer each, and the containers were put on a shaker and incubated for 2 hr at room temperature. Then 100ng (2-3μl) oligo biotinylated probe was diluted in 4ml of binding buffer. After the membrane with probes was incubated at room temperature for 30 min with gentle shaking (49 rpm), it was washed 2 times with 4ml of 1×washing buffer for 3min at room temperature. Then the streptavidin-HRP (1:1000 folds) was diluted in 1×wash buffer and the membrane was incubated with the diluted streptavidin-HRP for 30 min at room temperature. After the membrane was washed for 3 times with 4ml of 1×washing buffer for 5min at room temperature, it was detected by chemiluminescent way. The membrane was placed onto one sheet of clean wrap, the detection buffer was pipetted onto the membrane and the buffer mixture was evenly distributed over the membrane without bubble. After incubated for 5 min at room temperature, the film went through exposure procedure. Unipro protein data base was used to search for the tissue specificity of the TF on each membrane.

2.7.5 Confirmation of Interaction

2.7.5.1 Over-expression of the Potential TF in Mammalian Cells

Isolation of interesting potential TF from HepG₂ cells total RNA was achieved by two steps:

1. to get the total RNA from HepG₂ cells using the same method as mentioned above, and
2. to isolate the potential TF from the HepG₂ cells total RNA by using RT-PCR.

2. Materials and Methods

2.7.5.1.1 RT-PCR

Titanium one-step RT-PCR kit (Appendix C) was used in this experiment. Firstly, master mix was made which includes: 5µl of 10× one-step buffer, 1µl of 50× dNTP mix, 0.5µl of recombinant RNase inhibitor (40 unit/µl), 25µl of thermo stabilizing reagent, 10µl of GC-melt PCR solution, 1µl of oligo (dT) primer, 1µl of 50× RT-TITANIUM Tag enzyme mix; secondly, and 2µl of total RNA, 4µl of primer (20µM 5'primer 2µl, 20µM 3'primer µl)(table 2-8), and 0.5µl of RNeas free water were added into the reaction mixture.

Table 2-8 PCR primers for potential TF isolation

Name of primers	sequence
pXJ40- SMARCE1 forward primer (BamH1)	5'-atcggatcctcaaaaagaccatcttatgc-3'
pXJ40- SMARCE1 reverse primer (Not 1)	5'-ccgagcggccgccttattctttttctcatctt ctg-3'

The PCR protocol used was as follows: 50°C for 1hr, 94°C for 5min, followed by 35 cycles of 94°C for 30sec, 55°C for 30 sec, and 68°C for 3 min, lastly followed by 68°C for 2 min After the PCR procedure has finished, 5µl of PCR product was interpreted on 1% agarose gel. As the next step, the PCR product was cloned into pXJ-40.

2. Materials and Methods

2.7.5.1.2 Cloning of the RT-PCR Product into pXJ40 Vector

Cloning of the potential TF into pXJ-40 vector was conducted with the primer designation being designed as described in Table 2-8. Cloning of the 2 PCR products into pXJ-40 vector was carried out afterwards. Table 2-9 lists the information about pXJ-40.

Table 2-9. The vectors used in this study

plasmids	Relevant characteristics	Source of reference
pXJ-40- vector	This vector is an mammalian cell's expression vector with HA tag at 5'end, size is 5310bp	The kindly gift from Dr Koh Cheng Gee (SBS)

The new TF construct was transfected into 293T cells in order to check whether it can be over expressed or not. Western blotting was conducted to detect the over expressed protein by HA tag.

2.7.5.1.3 Transfection of the Construct into Mammalian Cells

4×10⁵ 293T cells were seeded in a 6-well plate one day before transfection, when the cells were 60% confluence, 400ng of plasmid DNA was transfected into each well according to the manual of Qiaeffecten transfection kit (Appendix D).

2.7.5.1.4 Western Blot Analysis

Preparation of 7.5% SDS PAGE gel was made. For separating gel, 4ml of Acrylamide/Bisacrylamide mix, 2.5ml of 1M Tris/HCl (pH=8.8), 3.3ml of distilled H₂O, 100μl of 10%SDS, and 100μl of ammonium persulfate were added into a 50ml falcon tube, followed by addition of 5μl of TEMED. A total of 10ml gel solution was prepared which was enough for 1 mini gel. For stacking gel, 0.83ml of Acrylamide/Bisacrylamide Mix, 0.63ml of

2. Materials and Methods

1M Tris/HCl (pH=6.8), 3.4ml of distilled H₂O, 50 μ l of 10% SDS, 50 μ l of 10% ammonium persulfate were added in a 15ml falcon tube, and lastly 5 μ l of TEMED was added, consequently 10ml gel solution was prepared which was enough for 1 mini gel. Sample was then prepared. Cells were collected 48 hrs after the transfection. After the cells were pelleted, the pellet was mixed with 2 $\times$ sample buffer, resuspended for several times, and heated on 100°C heat block for 5-10 min to denature the protein.

After the sample was loaded onto the well, the gel was run at 100 volts for 100min until the blue dye came to the end of the electrophoresis tank. After the electrophoresis has finished, the gel was moved to a thin filter paper and transferred to membrane using Semi-Dry Method (BioRad) at 21 V for 45min.

Detection of Protein was done by Chemiluminescent. The membrane was put in the container, and shaken in blocking buffer for 30min/overnight, after that, it was shaken again in blocking buffer with appropriate concentration of 1st antibody after 3 times wash in washing buffer for 10 min each time, then the membrane was shaken in blocking buffer with appropriate concentration of 2nd antibody for 1 hr. After washed for another 3 times in washing buffer, the signal was detected by chemiluminescent. The method is the same as that mentioned in section 2.6.3:

2.7.6 Confirmation Experiment of the Binding Assay

The nuclear extracts of the 293T cells which were transfected with pXJ-40 empty vector and pXJ-40 SMARCE 1 respectively were prepared by using the method previously mentioned.

2. Materials and Methods

The gel shift assay was performed by using the nuclear extracts from cells which over express potential TF protein.

2.7.7 Correlation between Potential TFs Binding Protein and HBV Replication

2.7.7.1 Promoter Activity Assay

4 reaction mixtures were prepared: 1) pGL3promoter-WT and pXJ40 empty vector cotransfected into 293T cells, 2) pGL3promoter-WT and pXJ40-SMARCE 1 vector cotransfected into 293T cells, 3) pGL3promoter-DEL1 and pXJ40 empty vector cotransfected into 293T cells, and 4) pGL3promoter-DEL1 and pXJ40-SMARCE 1 vector cotransfected into 293T cells. After 48 hrs transfection, the cells' lysate were collected to measure the value of promoter activity. The luciferase assay method is the same as that mentioned previously (2.5.2).

2.7.7.2 HBsAg Detection

4 reaction mixtures were also prepared: 1) pcDNA3.1+(HBV-WT) and pXJ40 empty vector cotransfected into 293T cells, 2) pcDNA3.1+(HBV-WT) and pXJ40-SMARCE 1 vector cotransfected into 293T cells, 3) pcDNA3.1+(HBV-DEL1) and pXJ40 empty vector cotransfected into 293T cells, and 4) pcDNA3.1+(HBV-DEL1) and pXJ40-SMARCE 1 vector cotransfected into 293T cells. After 48 hrs of transfection, the culture media were collected to measure the value of HBsAg. Surface antigen detection is the same as that mentioned previously (section 2.2.3).

2.8 Statistical Analysis

The statistical method which was used in this experiment was T test.

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3.1 Identification of Novel HBV Variants

Our laboratory's previous clinical result shows that three serum samples had deletions in the core promoter region (nucleotides 1758±1777 for CHBV06 and CHBV17, and 1749±1768 for CIR09, Table 1), and Intriguingly, high levels of HBV DNA were seen in all three deletion samples identified in this study (the copy number of HBV DNA are 212.0 pg/ml, 280.4pg/ml, 192.1pg/ml respectively, which is much higher than that of average level. (the average value of HBV DNA of other patient was 31.51pg/ml.

I examined 839 HBV strains with CURS/BCP nucleotide sequences which were deposited in the hepatitis virus database (<http://s2as02.genes.nig.ac.jp/>), however, no similar mutations were found. In addition, we searched 530 complete genomes for this deletion mutation in the HBV database, however, no similar deletions were found in any part of the complete genome sequences. This is the first report of characterization of this kind of deletions (*data not shown*). From this result we know that, these two deletions are different from those mutation which has been identified before, so it will be interesting to investigate these two promoter mutations.

3.2 In Vitro Study Using Replication Model (Replicon)

In order to test the effect of the BCP mutations on HBV replication, either the wild type sequence or the deletions were introduced into the HBV genome. Replication-competent plasmid vectors with the 1.09-fold HBV genome were generated, and sequences of all clones were checked for the correct occurrence of deletions in the BCP and PC region. The constructs were tested by transient transfection in the human hepatoma cell line HepG₂. Each panel of constructs was tested in at least three repeated experiments, and similar results were

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obtained. Transfection efficiency was controlled in all experiments by cotransfection of pBIND plasmid and measurement of *Renilla* luciferase activity.

3.2.1 Construction of Replicative HBV Genome with Deletions in Core

Promoter

In order to further verify whether the deleted HBV (including two deletion fragments in the core promoter) has a high replication rate, an experiment was designed and conducted. The strategy of the experiment is as follows: firstly, PBR325 (HBV) was used as template to get PCR fragment, secondly, the HBV including deletions was constructed. In order to get rid of the effect of the strong CMV promoter on the vector, Nru I site was chosen as the cloning site.

3.2.1.1 Preparation of the Vector

As the first step, pcDNA3.1+ was digested with EcoR I (Fig. 3-1). Klenown enzyme was used to destruct the EcoR I site on the vector. Self ligation was performed, followed by mini preparation to check the self ligation product (Fig. 3-2). Further digestion was carried out again using EcoR I to confirm the effect of the site destruction (Fig. 3-3). From the result, we used the lane 3 sample as the vector. The vector was then digested with Nru I. After purification, the treated vector was used to ligate with the insert.

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Fig. 3-1 EcoR I digested pcDNA3.1+ (size: 5.4kb).

Fig. 3-2 Miniprep result of pcDNA3.1+ self ligation

Fig. 3-3 Result of pcDNA3.1+, which has been digested by EcoR I overnight, lane 5 is 1kb marker, lane 3 will be used subsequently.

3.2.1.2 Preparation of the Inserts

PCR statagy was described as (Fig 3-4).The PBR325 (HBV) was used as the template (Fig. 3-5). First round PCR was used to get fragment I (Fig. 3-6) and II (Fig. 3-7) for deletion 1 and deletion 2. And then Fragment I and II was used as the template, and the fragment including the deletion part (first fragment from nts1600-1900) was obtained (Fig. 3-8). The sequence of the1st fragment cloning product was confirmed by sequencing result. After confirmation, the gel extraction was conducted. After the processing, the PCR product is ready for the ligation.

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Fig. 3-4 Generation of deletion in viral core promoter by sequential PCR.

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Fig. 3-5 Template pBR325 (HBV).
Lane 1: pBR325 (5.9kb) HBV (3.2kb)
Lane 2: 1kb marker

Fig. 3-6 First round PCR result,
lane1: fragment I for deletion 1; lane 2:
fragment I for deletion 2; lane 3: 1kb marker.

Fig. 3-7 First round PCR result
lane 1: fragment II for deletion 1 ; lane 2:
fragment II for deletion 2. lane3: 100bp
marker;

Fig. 3-8 Second round PCR product
1st fragment: from nts (1600-1900, lane 1: 100bp
marker; lane2: fragment include deletion1; lane3:
fragment include deletion 2; lane4: Nru I digested
pcDNA3.1+ vector; lane 5: 1kb marker.

3.2.1.3 Ligation of the Two Inserts

The 1st and 2nd fragments of two deletions have been successfully ligated sequentially. The 1st fragment of deletion 1 has been successfully ligated and confirmed by Nru I digestion. The 2nd fragment of deletion 1 was ligated and confirmed by EcoR 1 digestion. In addition, it was confirmed by PCR that the 2nd fragment was cloned in the correct direction. The deletion region in the core promoter region was identified by sequencing. The sequencing result is as follows:

Alignment of pcDNA3.1+HBVWT and HBV genome of ours:

		1693		1727
pcDNA3.1+HBVWT	(67)	ACCTTGAGGCCTACTTCAAAGACTGTGTGTTTAAG		
HBV Genome OURS	(1693)	ACCTTGAGGCCTACTTCAAAGACTGTGTGTTTAAG		
Consensus	(1693)	ACCTTGAGGCCTACTTCAAAGACTGTGTGTTTAAG		
		1728		1762
pcDNA3.1+HBVWT	(102)	GACTGGGAGGAGCTGGGGGAGGAGATTAGGTTAAA		
HBV Genome OURS	(1728)	GACTGGGAGGAGCTGGGGGAGGAGATTAGGTTAAA		
Consensus	(1728)	GACTGGGAGGAGCTGGGGGAGGAGATTAGGTTAAA		
		1763		1797
pcDNA3.1+HBVWT	(137)	GGTCTTTGTATTAGGAGGCTGTAGGCATAAATTGG		
HBV Genome OURS	(1763)	GGTCTTTGTATTAGGAGGCTGTAGGCATAAATTGG		
Consensus	(1763)	GGTCTTTGTATTAGGAGGCTGTAGGCATAAATTGG		
		1798		
pcDNA3.1+HBVWT	(172)	T		
HBV Genome OURS	(1798)	T		
Consensus	(1798)	T		

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Alignment of pcDNA3.1+HBVDEL1 and HBV genome of ours:

	1678		1712
pcDNA3.1+HBVDEL1	(1678)	CAATGTCAACGACCGACCTTGAGGCCTACTTCAAA	
HBV Genome OURS	(53)	CAATGTCAACGACCGACCTTGAGGCCTACTTCAAA	
Consensus	(1678)	CAATGTCAACGACCGACCTTGAGGCCTACTTCAAA	
	1713		1747
pcDNA3.1+HBVDEL1	(1713)	GACTGTGTGTTTAAGGACTGGGAGGAGCTGGGGGA	
HBV Genome OURS	(85)	GACTGTGTGTTTAAGGACTGGGAGGAGCTGGGGGA	
Consensus	(1713)	GACTGTGTGTTTAAGGACTGGGAGGAG TGGGGGA	
	1748		1782
pcDNA3.1+HBVDEL1	(1748)	G-----TGTATTAGGAGGCT	
HBV Genome OURS	(120)	GGAGATTAGGTTAAAGGTCTTTGTATTAGGAGGCT	
Consensus	(1748)	G TGTA TAGGAGGCT	
	1783		
pcDNA3.1+HBVDEL1	(1783)	GTAGGCATAAATTGGT	
HBV Genome OURS	(135)	GTAGGCATAAATTGGT	
Consensus	(1783)	GTAGGCATAAATTGGT	

Alignment of pcDNA3.1+HBVDEL2 and HBV Genome of ours:

	1696		1735
pcDNA3.1+HBVDEL2	(69)	TTGAGGCCTACTTCAAAGACTGTGTGTTTAAGGACTGGGA	
HBV Genome OURS	(1696)	TTGAGGCCTACTTCAAAGACTGTGTGTTTAAGGACTGGGA	
Consensus	(1696)	TTGAGGCCTACTTCAAAGACTGTGTGTTTAAGGACTGGGA	
	1736		1775
pcDNA3.1+HBVDEL2	(109)	GGAGCTGGGGGAGGAGATTAGG-----	
HBV Genome OURS	(1736)	GGAGCTGGGGGAGGAGATTAGGTTAAAGGTCTTTGTATTA	
Consensus	(1736)	GGAGCTGGGGGAGGAGATTAGG	
	1776		1798
pcDNA3.1+HBVDEL2	(134)	--AGGCTGTAGGCATAAATTGGT	
HBV Genome OURS	(1776)	GGAGGCTGTAGGCATAAATTGGT	
Consensus	(1776)	AGGCTGTAGGCATAAATTGGT	

Taken together, deletions in the viral core promoter, corresponding to those found in the clinical samples (nts1749-1768 and nts1758-1777 respectively), were generated by a sequential PCR strategy. 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. Sequencing of these constructs revealed no other mutations in other part of the viral genomes. These clones can be used in next step *in vitro* experiment.

3.2.2 Influence of HBV Deletions on HBsAg Secretion and Extracellular HBV Virion

3.2.2.1 Influence of HBV Deletions on HBsAg Secretion

To assess the role of the deletions in core promoter on the viral replication, we cloned replication-competent HBV vectors (genotype A, adw2) with deletions in the core promoter region. Each of these replicative HBV genome constructs (wild type, deletion 1 and deletion 2) was used to transfect HepG₂ cells. The transfection efficiency was controlled by the luciferase assay. Cotransfection of cells with pBIND plasmid to control the transfection efficiency was done, the ratio of the plasmid used in this experiment was 1:1. The luciferase assay results were shown in Fig.3-9. From this result we can see that the transfection efficiency is almost equal for these 3 constructs. Therefore, the result of surface antigen is comparable.

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Fig. 3-9 Transfection efficiency comparison
HepG2 cells were co-transfected with full-length replicative HBV clones (indicated by wt,del1, del2), pcDNA3.1+ serve as a control. Water (H₂O) was serve as a negative control. The ratio of co-transfected vector was 1:1. Renilla Luciferase activity were measured in HepG2 cells 48hr after transfection Transfections were performed in triplicate, and the same experiment was repeated for 3 times. Standard deviations are shown.

Transfection for each construct was carried out in three 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 (Janssen *et al.* Kerhof *et al.* 1994 ; Liu *et al* 2004), the culture medium was semiquantitatively assessed for the amount of HBsAg by ELISA assay (Auszyme, Abbott Laboratories, USA). Results shown in Fig. 3-10 indicates a significant decrease of secreted HBsAg for both deletion 1 and deletion 2 HBV genomes, as compared with the wild type HBV genome ($p<0.001$), while in sharp contrast to our previous observation (Chen *et al.* 2000).

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Fig. 3- 10 Semi-quantitative masurement of screted HBsAg
400ng of different replicative construct DNA was transfected into 4×10^5 HepG2 cells, 48 hr after transfection, 160 μ l of culture medium of each sample was collected, and HBsAg concentration was examine by using of IMX machine. Mean values of HBsAg were obtained from three independent experiments measured in the supernatant, Values are normalized to the HBsAg Means and standard deviations are based on more than three independent experiments; values are given relative to wild type. (preset the value of wild type as 100).

HBsAg concentrations in the supernatant decrease significantly between the HBV deletions and wild type (Fig. 3-10).It shows that the cells which was transfected with wild type will secret out more surface antigen. This experiment was performed in triplicates and repeated a minimum of 3 times, and got the same result, standard deviation was marked on the bar chart. (T test was used as the statistical method).

Our data have provided molecular information on the role of core promoter deletions in HBV replication. 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; the surface antigen value is much lower in cells

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transfected with core promoter deletion construct. Which means, compared to wild type, deletion type replicative genome can push less HBsAg to be secreted out.

3.2.2.2 Influence of HBV Deletions on Extracellular HBV Virion

In order to further validate the influence of core promoter deletions on the HBV replication capacity, the extracellular HBV DNA level was measured. HBV DNA replication is asymmetric. The negative strand is synthesized first from the pregenomic RNA template and, after degradation of the RNA template, positive-strand DNA is synthesized by using the negative-strand DNA as a template (Ganem *et al.* 2001, Summers *et al.* 1982). Only core particles containing such double-stranded viral genome are enveloped and secreted into serum (Ganem *et al.* 2001), although human hepatoma cell lines transfected with HBV DNA may release naked core particles containing single-stranded genome (Gerelsaikhan *et al.* 1996; Wei *et al.* 1996; Yuan *et al.* 1999). Parallel to the detection of replicating viral DNA within transfected cells, we extracted extracellular virion's DNA.

Newly synthesized HBV virions released into supernatant were collected 2 days after transfection and measured using a quantitative real-time PCR approach (1ul virus DNA as template). Each of these replicative HBV genome constructs (wild type, deletion 1 and deletion 2) was used to transfect HepG2 cells. The transfection efficiency was controlled by the luciferase assay, cotransfection of pBIND plasmid to control the transfection efficiency was done, the result of the luciferase assay shows transfection results are comparable (*data not shown*). The result shows deleted mutants displayed decreased levels of released HBV virions in the supernatant 2 days after transfection (Fig. 3-11). In contrast, BCP deletions can produce HBV virion numbers less than those of the wt HBV construct (difference got great significance, $p < 0.001$). We observed decreased accumulation of HBV virions in the

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supernatant. That means the replication capacity of these three constructs is also different, and wild type one seems has stronger replicative capacity.

Fig. 3-11 Quantification of HBV copy number from supernatant by quantitative real-time PCR

400ng of HBV replicative DNA constructs (wild type, del 1, and del2) were transfected into HepG2 cells, 48 hr after transfection, the medium was collected, and HBV DNA was extracted from medium by using Qiagen Blood Mini Kit, then 1 μ l of DNA sample was used as the template for Real-Time PCR reaction, this bar chart showed the relative HBV copy number of del1 and del2. Mean values and standard deviations from three independent experiments are shown. Values are normalized to HBV copy number of the wt construct (100%). There are significant differences compared with wt expression. Average values and standard deviations are based on more than three independent experiments; values are given relative to wt. (preset the value of wild type as 100).

The results showed that cells transfected with deleted replicative genome can produce less virions in the culture medium, which means, the core promoter region deleted replicative genome has less replication capacity. This experiment was performed in triplicates and repeated a minimum of 3 times, and got the same result, standard deviation was marked on the bar chart. (T test was used as the statistical method).

3.2.3 Influence of HBV Deletions on Intracellular Replication Efficacy

Since we know that there is difference between wild type and mutated construct on the protein level, the question is whether it is caused by the difference in the mRNA level, so the real time PCR in mRNA level was carried out.

After HepG2 cells were transiently transfected with different constructs which include pcDNA3.1+(HBV-WT), pcDNA3.1+(HBV-DEL1), and pcDNA3.1+(HBV- DEL2) for 48hrs, the total RNA was extracted from HepG₂ cells. In order to exclude the template contamination, the mRNA extraction also was done, 5µg of mRNA of each sample was used as the template to do the reverse transcription, after the dsDNA was achieved, normal PCR was performed by using of 1µl dsDNA of each sample as the template, 5µl of PCR product was loaded on the 1% agarose gel, as shown on Fig. 3-12. It is clear that there is no band for the negative control (template which was not gone through the reverse transcription), which means there is no internal contamination for the Real time PCR.

Fig. 3-12 Agarose gel result of RT-PCR

Lane 1: negative control, normal PCR by using the template of 1 μ g mRNA(extracted from cells transfected with wild type replicative genome), Lane 2: RT –PCR product (partial c gene) by using the template of 1 μ l cDNA (synthesis by the template of mRNA which extracted from cells transfected with wild type replicative genome), Lane 3: RT –PCR product (partial c gene) by using the template of 1 μ l cDNA(synthesis by the template of mRNA which extracted from cells transfected with deletion 1 replicative genome), Lane 4: RT –PCR product (partial c gene) by using the template of 1 μ l cDNA(synthesis by the template of mRNA which extracted from cells transfected with deletion 2 replicative genome), Lane 5: 1kb marker.

I also applied an RT-PCR, by using the primer pairs covering the HBV-C for amplification. GAPDH as a cellular housekeeping gene was included as an internal control. Quantification of the PCR products in the calculated linear area of amplification showed significantly decreased C protein in the deleted mutants (Fig. 3-12). It can be seen that compared to the wild type, the gene expression level in the cells transfected with deleted replicative genome is much lower, the difference is of great significance. This also indicates that the pregenomic RNA levels are reduced in deleted mutants.

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Fig. 3-13 Quantification of the C product relative to GAPDH expression

1g of different replicative constructs DNA was transfected into 1 T₇₅ flask (70% confluence) of HepG2 cells, 48 hr after transfection, cells were collected, mRNA from each sample was extracted by using Oligotex mRNA extraction kit, then, 10µl of mRNA sample was used as template, and an RT-PCR performed to get cDNA for each sample, lastly, 1.05µl of cDNA was used as template for real-time PCR, GAPDH was served as internal control for this reaction, different sample was indicated as (wt, del 1 and del 2), pcDNA3.1+ was used as a negative control for core gene expression. Quantification of the C protein expression of cells transfected with deletion replicon relative to that of cells transfected with wild type replicon. Means and standard deviations are based on more than three independent experiments; each PCR sample was triplicated. values are given relative to wild type. Significant differences compared with wt expression are marked.

In vitro study using replication model (replicon) was also conducted. RT-PCR analysis from total RNA isolated from HepG2 cells 48 hr after transfection revealed that the pcDNA3.1+HBV replicon with the deletion in core promoter region replicated much less than the pcDNA3.1+HBV wild-type replicon.

The above results are consistent with the influence of HBV deletions on extracellular replication efficacy. Transcriptional activity of the different HBV deletions was analyzed by

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Real-time PCR. Pregenomic RNA levels were significantly reduced on the core promoter deletion construct. This experiment was performed in triplicates and repeated a minimum of 3 times, and got the same result, standard deviation was marked on the bar chart. (T test was used as the statistical method).

3.2.4 Promoter Activity Assay

Because the deletions were occurred in the core promoter region, and previous result showed that there are great difference on the pgRNA level and from gene analysis, it was reported that the enhancer II region was from nts 1687-1805. As this deletion just resided in the viral enhancer element (Fig. 3-14a). Because of the high sensitivity and the easy way to carry out compared to the CAT or GFP and ALP, the luciferase assay was chosen for the promoter activity analysis. We proceeded to study the effects of these base deletions on transcriptional efficiency by cloning a 131- base-pair Enh II fragment bearing nts 1749-1768, nts1758-1777 bp deletions upstream of a SV40 promoter–luciferase reporter gene vector (Fig. 3-14b). Transient transfections were carried out on the hepatic cell line HepG₂ and subsequently assayed for luciferase activity.

(a)

(b)

Fig. 3-14 Construction of the luciferase assay plasmid

(a) The Enh II is located just upstream of the core promoter. The minimum sequence for enhancer activity has been previously defined at nucleotide 1687–1805 as shown. And two deletion regions are boxed. (b) Amplified Enh II fragments including the deleted region were inserted upstream of the SV40 promoter in an enhancerless luciferase reporter vector---pGL3-Promoter.

3.2.4.1 Construction of the luciferase Assay Plasmids

Plasmids pGL3-Control (a Luciferase plasmid with a simian virus 40 enhancer and promoter) and pGL3-Promoter (an enhancerless Luciferase plasmid with a simian virus 40 promoter upstream from the Luciferase gene) were obtained from Promega. Plasmid pGL3-Promo/wt

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was constructed by amplifying the basic functional unit of Enh II by PCR using primers LucF and LucR comprising HBV nucleotide positions 1686–1801. These fragments were isolated by PCR from the different template. For wild type, the template is pcDNA3.1+ HBV-WT, for del1 and del2, the templates are nest PCR products of the first fragment (Fig. 3-8). The enhancer II PCR products are shown as Fig. 3-15. These three different fragments were MluI/BglII-digested and ligated with MluI/BglII digested pGL3-Promoter (Fig. 3-16). The ligation product was confirmed by enzyme digestion (Fig. 3-17). The sequence of each constructs was identified by sequencing result (shown on page 89).

Fig. 3-15 PCR product of enhancer II region lanes 1,2: wild type promoters (size 131bp); lanes 3,4: deletion 1 promoter (111bp), and lanes 5,6: deletion 2 promoter (111bp). Lane7: 1kb marker

Fig. 3-16 Vector of pGL3-promoter, it was digested by both Bgl II and MluI, its size is 5,010kb.

Fig. 3-17 Enzyme digestion confirmation of the 3 clones, lanes 1,2: pGLpromoter -del2; lanes 3,4: pGLpromoter-del2; lanes 5,6: pGLpromoter-wt; lane 7: 1kb marker,.

Sequencing result of pGL3-promoter-wt/del1/del2

The alignment result of pGL-promoter-wt and pcDNA-HBV-WT

		1688	1722
pGL-promoter-wt	(56)	TCAACGACCGACCTTGAGGCCTACTTCAAAGACTG	
HBV Genome OURS	(1683)	TCAACGACCGACCTTGAGGCCTACTTCAAAGACTG	
Consensus	(1688)	TCAACGACCGACCTTGAGGCCTACTTCAAAGACTG	
		1723	1757
pGL-promoter-wt	(91)	TGTGTTTAAGGACTGGGAGGAGCTGGGGGAGGAGA	
HBV Genome OURS	(1718)	TGTGTTTAAGGACTGGGAGGAGCTGGGGGAGGAGA	
Consensus	(1723)	TGTGTTTAAGGACTGGGAGGAGCTGGGGGAGGAGA	
		1758	1792
pGL-promoter-wt	(126)	TTAGGTTAAAGGTCTTTGTATTAGGAGGCTGTAGG	
HBV Genome OURS	(1753)	TTAGGTTAAAGGTCTTTGTATTAGGAGGCTGTAGG	
Consensus	(1758)	TTAGGTTAAAGGTCTTTGTATTAGGAGGCTGTAGG	
		1793	
pGL-promoter-wt	(161)	CATAAATTGGT	
HBV Genome OURS	(1788)	CATAAATTGGT	
Consensus	(1793)	CATAAATTGGT	

3. Results

The alignment result of pGL3-promoter-del1 and pcDNA-HBV-WT

		1678	1712
HBV Genome OURS	(1678)	CAATGTCAACGACCGACCTTGAGGCCTACTTCAAA	
pGL3-promoter-del1	(53)	C---GTCAACGACCGACCTTGAGGCCTACTTCAAA	
Consensus	(1678)	C GTCAACGACCGACCTTGAGGCCTACTTCAAA	
		1713	1747
HBV Genome OURS	(1713)	GACTGTGTGTTTAAAGGACTGGGAGGAGGTGGGGGA	
pGL3-promoter-del1	(85)	GACTGTGTGTTTAAAGGACTGGGAGGAGGTGGGGGA	
Consensus	(1713)	GACTGTGTGTTTAAAGGACTGGGAGGAGGTGGGGGA	
		1748	1782
HBV Genome OURS	(1748)	GGAGATTAGGTTAAAGGTCTTTGTATTAGGAGGCT	
pGL3-promoter-del1	(120)	G-----TGTATTAGGAGGCT	
Consensus	(1748)	G TGTATTAGGAGGCT	
		1783	
HBV Genome OURS	(1783)	GTAGGCATAAATTGGT	
pGL3-promoter-del1	(135)	GTAGGCATAAATTGGT	
Consensus	(1783)	GTAGGCATAAATTGGT	

The alignment result of pGL3-promoter-del2 and pcDNA-HBV-WT

		1689	1723
HBV Genome OURS	(1689)	ACCGACCTTGAGGCCTACTTCAAAGACTGTGTGTT	
pGL3-promoter-del2	(62)	ACCGACCTTGAGGCCTACTTCAAAGACTGTGTGTT	
Consensus	(1689)	ACCGACCTTGAGGCCTACTTCAAAGACTGTGTGTT	
		1724	1758
HBV Genome OURS	(1724)	TAAGGACTGGGAGGAGCTGGGGGAGGAGATTAGGT	
pGL3-promoter-del2	(97)	TAAGGACTGGGAGGAGCTGGGGGAGGAGATTAGG-	
Consensus	(1724)	TAAGGACTGGGAGGAGCTGGGGGAGGAGATTAGG	
		1759	1793
HBV Genome OURS	(1759)	TAAAGGTCTTTGTATTAGGAGGCTGTAGGCATAAA	
pGL3-promoter-del2	(131)	-----AGGCTGTAGGCATAAA	
Consensus	(1759)	AGGCTGTAGGCATAAA	
		1794	
HBV Genome OURS	(1794)	TTGGT	
pGL3-promoter-del2	(147)	TTGGT	
Consensus	(1794)	TTGGT	

3.2.4.2 Luciferase Assay

Transient transfection of HepG₂ cells was carried out with an equal amount of the two plasmids, the pGL3 control vector (or pGL3-promoter constructed vector which encodes the firefly luciferase) and pBIND vector which encodes the Renilla luciferase (served as an internal control efficiency of transfection). Three different constructs (pGL3-promoter-wt. pGL3-promoter-del1. pGL3-promoter-del2) as well as positive control and negative control vectors were cotransfected with pBIND vector, the ratio of firefly luciferase activity to Rennilla luciferase activity was normalized comparing to positive control supplied by the

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system. Results from the independent transfections for each combination are shown in Fig. 3-18(a).

The results show that the constructs with these 2 deletions both strongly decreased the SV40 promoter when linked to it in cis orientation, resulting in lower levels of luciferase expression as compared to wild type (Fig. 3-19(a)). As a positive control, the vector containing both the SV40 promoter and enhancer sequences resulting in optimal luciferase expression was used. (the enhancer for the positive control is different with enhancer of HBV) (“positive” in Column 1 in Fig. 3-19a). In addition, the cloning vector itself containing only the SV40 promoter–luciferase gene but without the enhancer element (“negative” in Column 2 in Fig. 3-18) served as the negative control (normalized by luciferase activity of pBIND). It can be seen that the luciferase activity of del1 and del2 construct is lower than that of wild type. To determine if these results were specific to only the host HepG₂ cell line, we repeated the transfections in two other cell lines: Chang liver cell line and 293T. The results were consistent with the initial results being observed with HepG₂ (Figs. 3-18(b),(c)), which further substantiates that the 20 base pair deletions in nts1749-1768 and nts1758-1777 have a significant effect on the transcriptional efficiency of Enh II. These *in vitro* assays provide strong support for the effect of these two 20 bps deletions on lower HBV viral load, but it is also possible that there may be additional contributing factors (This value was normalized by the value of PBIND luciferase activity which served as the transfection efficiency control).

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(a)

(b)

(c)

Fig. 3-18 Promoter activity results of 3 different constructs on different cell lines

(a) HepG₂, (b) Chang liver cell, and (c) 293T. 400ng of pGL3 -wt/del1/del2 were trasfected into 3 different cell lines, 48 hr after transfection, the cells were collected and lysed, the luciferase activity was measured by luminometer, pBIND vector was also cotrasfected with each clone to normalize the transfection efficiency. For each cell type, the first column represents the ratio of the luciferase activity of internal positive control (promoter and enhancer) to pBIND. The second column (“negative”) represents the ratio of activity of the internal negative control (promoter only) to that of pBIND. The other columns of each cell line represent the the ratio of activity between vectors with the promoter and the Enh II (wt, del1, del2) to that of pBIND. Results of the luciferase assay were normalized to the level of the internal positive control (arbitrarily set at 100%). This experiment was performed for 3 time independently, and each sample was done triplicated.

From Fig. 3-18(a), the positive control gave out a normalized ratio and was set as 100%. The negative control between pGL3control and pBIND showed 2.59% activity comparing to the positive control. It showed that deletion 1 construct has a less than 1/3 activity of that of a wild type activity, and deletion 2 also has less than ½ activity compared with that of wild type. The result on the other cell lines got the same tendency. The decreased luciferase activity strongly supports the hypothesis that the decrease of HBV viral replication was occurred on

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the transcription level. This experiment was performed in triplicates and repeated a minimum of 3 times, and got the same result, standard deviation was marked on the bar chart. (T test was used as the statistical method).

In summary, the results showed that the construct with these 2 deletions all strongly decreased the SV40 promoter when linked to it in cis, resulting in lower levels of luciferase expression as compared to wild type, which means deleted promoter has less activity than that of wild type.

3.3 Identification of a Novel Transcription Factor

Because the deletions occurred in the core promoter region, most probably, the difference in the surface antigen secretion was caused by the different transcription efficiency, for deleted core promoter region will affect the binding of transcription factor (TF) directly. We therefore hypothesized that there is a novel TF that can bind to del1 promoter region. And from previous *in vitro* cell system analysis, compared to the deletion2, deletion 1's difference was of greater significance, so deletion 1 was transiently chosen for further study.

3.3.1 In Silico Analysis Results Obtained from Website

The *in vitro* experiment results as above can be explained in several ways. First, the binding of new cellular transcription factor leads to decreased viral replication. Second, removal of a positive regulator element leads to the decreased viral replication. In other words, However, the fact that the negative regulation region is located in a different region (nts1613-1636) suggests that new cellular transcription factor binding to the core promoter with deletion may be responsible for the observed increase in viral replication. There is a possibility that positive

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TF cannot bind to the deleted promoter. Although there are some factors like HNF4, SP1 and COUP etc can bind to the deleted region, but the combined function of all those TF are still not clear, from paper literature, it is also quite possible that there is a novel TF can bind to the rearranged region. Through the Transfac database searching (Table 3-1), it was found that there are some transcription factors binding with DNA sequence just at the junction site. For deletion 1, LyF-1 (lymphocyte-specific DNA binding protein) can bind with DNA sequence gttaggagg, and Sp1 (an activator of numerous TATA-containing and TATA less promoters) can bind to DNA sequence aggagg. For deletion 2, ADR1 (the yeast transcriptional activator) can bind to DNA sequence gggagt and GAmYb (MYB transcription factor) can bind to DNA sequence gggagttg. The results showed that, although they were detected from different species, there are several proteins that can bind to the rearranged promoters. It is obvious that, for wild type, the present searched results are consistent with those reported by other researchers. Hopefully, it is possible to find a novel transcription factor in HBV.

Table 3-1 Database searching results: target element, factor, and binding sequence of the three promoters

	Target element	Factor	Binding sequence
Wild type	Tgggggaggagattagggttaaaggtcttt (1741-1769)	SP1	gggagg
		C/EBP	taggttaa
Del1	ggggag ttgtactaggagg (1742-1748, 1769-1781) deletion:1749-1768	ADR1	gggagt
		GAmYb	gggagttg
Del2	cggaggttagg aggctgtagg (1747-1757, 1778-1787) deletion: 1758-1777	LyF-1	gttaggagg
		SP1	aggagg

There are several proteins that can bind to the rearranged promoters. The results showed that, although they were detected from different species, It is obvious that, for wild type, the

present searched results are consistent with those reported by other researchers. Hopefully, it is possible to find a novel transcription factor in HBV.

3.3.2 2 Dimensional (2D) Gel Result

3.3.2.1 A 2D Gel Analysis of Cellular Proteins Associated with Different Replicative Genome

To identify cellular proteins associated with the different replicative genome and could therefore be used as a reference for next study. These two different constructs (pcDNA 3.1+(HBV-wt) and pcDNA 3.1+(HBV-del1) were transfected into HepG₂ cells. The cells were maintained for 2 days after transfection, and protein extracts from 3×10^6 transfected cells were prepared as described (in chapter 2). One hundred and fifty micrograms of protein lysate, quantified using Amersham Pharmacia 2D Quant Kit, were subjected to 2D electrophoresis using Amersham Pharmacia IPGphor IEF Unit according to established conditions (see chapter 2). Silver staining of the gels was then performed and the gels were scanned using a Molecular Dynamics Personal Densitometer SI as described (in chapter 2). The 2D gels were run three times for the same lysate of HepG₂ cells transfected with each type of construct (wild type replicative genome, deletion 1 replicative genome separately) to minimize gel to gel variation.

3.3.2.2 Preliminary Identification of Cellular Proteins Associated with Different Replicative Genome

Results shown in Fig. 3-19 revealed differences among the two gels. The different proteins spots revealed in Fig. 3-19 were further analyzed based on their molecular weight and

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isoelectric point (*pI*) value. The *pI* value and molecular weight of these different proteins were assessed and summarized in Table 3-2. Results indicate three main categories of differences:

- (I) Proteins which were not present in the cells transfected with deletion 1 replicative genome, but present in cells transfected with wild type replicative genome. These include spots group B,C;
- (II) Proteins which were not present in the cells transfected with wild type replicative genome, but present in cells transfected with deletion 1 replicative genome. These include spots group D;
- (III) Protein which was present in both cells transfected with two different replicative genome, but the intensity of spots is obviously decreased. These included spots group A.

Table 3-2 Isoelectric points (*pI*) and molecular weight (MW) values of cellular proteins associated with two different replicative genomes (wild type and deletion1)

Spot group	Approximate <i>pI</i> (pH values)	Approximate molecular weight (MW) (kDa)	Wild type replicative genome	Deletion 1 replicative genome
A	5.6	69	+	+(down)
	5.7	69	+	+(down)
B	7.4	35	+	-
C	7.1	17	+	-
	7.2	23	+	-
	7.3	23	+	-
	7.5	19	+	-
D	6.5	24	-	+

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Fig. 3-19 2D gel result

2D SDS-PAGE gel analysis. Total proteins were extracted from HepG2 cells which was transfected with wild type replicative genome; (b) deletion1 replicative genome.

2g of different replicative genomes (wild type and deletion 1 respectively) were transfected into HepG2 cells (T_{75} flask, 70% confluence), 48 hr after transfection, the cells were collected, and total protein was extracted by using the lysis buffer, the 2 D gel was run as instructed (method please refer to 2.7.2)

Differences among the two gels were boxed. Identification of differences (grouped into A-D). Differences in intensity or presence of protein spots between the two graphs were identified using a Molecular Dynamics Personal Densitometer SI. These spots were boxed and numbered. This experiment was repeated for 3 times independently.

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In summary, our results revealed that specific cellular proteins were associated with exposure to a particular replicative genome. the difference in 2D-gels between wild type-transfected cells and deleted genome-transfected ones can be different as physiological responses in two different HBV genomes-transfected cells are changed by differential effects of each genome. Cells which were transfected with deletion 1 replicative genome really got some new proteins which can not be found in cells transfected with wild type. In addition, our data also indicated that the proteomics approach could be used to identify cellular proteins associated different replicative genomes.

3.4 Isolation of a Novel Transcription Factor

From the 2D result we can see that compared to the cells transfected with wild type and deletion 1 replicative genome, it is true that there are some new proteins appeared, we hypothesized that maybe there is a novel binding protein that can bind to the rearranged core promoter sequence. In order to validate this suspicion, we performed the gel shift assay experiment, the total nuclear extracts from HepG2 cells were used initially.

3.4.1 Gel Shift Assay

3.4.1.1 Identification of the Optimal Concentration of the Probe

In order to perform the gel shift assay, the optimal concentration of the probe which can be detected needed to be identified firstly (Fig. 3-19), while because after gel extraction, the concentration of the probe is very low, and it was quite difficult to be quantified by the spectrophotometer. Therefore dot blot assay was performed firstly to confirm the lowest concentration of the probe.

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After gel purification, wild type and deleted probe were dissolved in 20μl of distilled water. And 1 μl of labeled probes was diluted by five fold, which means, 20μl labeled probe was diluted in 100μl water respectively and stored in -20°C. While the 20μl of unlabeled probe was stored in -20°C without dilution. Then, the labeled probe was undergone a serials dilution which was described in Table 3-3.

Table 3-3 Serials dilution of biotin labeled probe

No.	lane 1	Lane 2	Lane3	Lane4	Lane 5
Sample name	Positive control	Labeled probe(wt/del1)	Labeled probe(wt/del1)	Labeled probe(wt/del1)	Labeled probe(wt/del1)
Dilution fold	no dilution	1:10000	1:1000	1:100	1:10

Fig. 3-20 Identification of the optimal concentration of the probe.

Probe was diluted serially, dot blot experiment was performed to see the optimal concentration can be used.

Lane1: 2μl of positive control probe from kit, Lane2A(B): 1μl of 1:10000 dilution of deletion 1 (wild type) probe, Lane3A(B): 1μl of 1:1000 dilution of deletion 1 (wild type) probe, Lane4A(B): 1μl of 1:100 dilution of deletion 1 (wild type)probe, Lane5A(B): 1μl of 1:10 dilution of deletion 1 (wild type) probe.

It shows (Fig. 3–20) that there is an obvious gradient decrease of the intensity of probe on each dilution fold respectively, and that compared to the intensity of positive control, the dilution of 1:10,000, and 1:1,000 are too low, so that they cannot be detected. However, till the dilution of 1:100, 1μl of each probe seems to be comparable with 2 μl of positive control,

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therefore 1:100 dilution of labeled probe of both wild type and deletion 1 was used in the next step gel shift assay.

3.4.1.2 Gel Shift Assay

In order to further establish and confirm the biological significance of this deletion observed in the Enh II region, I wanted to demonstrate the presence of any direct physical HBV DNA–host interaction at the new generated nucleotide site. To this end, 54-mer oligonucleotide probes were designed to contain 17bp before deletion 1 and 17bp after deletion 1 nucleotide, with wild type probes taken from the whole 54bp sequence and also cover 20bp deletion 1 region. An electrophoretic mobility shift assay (EMSA) was performed to test the binding affinity difference between wild type probe and deletion 1 probe with the nuclear extract from HepG2 cells. Protein concentration in nuclear extracts was quantified by Bradford protein assay kit (Biorad). 1: 100 dilution of biotin labeled probe was used for the shift assay. 200 fold of cold probe of each type was used in competent experiment. And probes were incubated together with 5 μ g of HepG2 nuclear extracts. Later, the reaction mixture was electrophoresed on a 6% nondenaturing polyacrylamide gel, then the gel was transferred on the membrane, after blocking, washing and incubating with streptavidine, the signal was detected by ECL. The results are shown in Fig. 3-21. This gel shift assay result indicates that three complexes can be detected, they are I, II and III, while, complexes I and II can be seen both on the wild type (wt) probe lane and deletion 1 probe lane, and they cannot be competed by the cold probe. This is probably because complexes I and II were only unspecific DNA–protein binding complexes. For complex IV, wild type also got the band shift. However, the formation of complex III was entirely lost for the wild type probe. And this one can also be clearly competed by adding of excess cold probe. Densitometric analysis of the bands indicated that the protein detected by deletion 1 was very clear, which means the binding affinity is strong.

Fig. 3-21 Analysis of deletion effects of nts 1749-1768 on the binding of protein factors from HepG2 nuclear extract.

Electrophoretic mobility shift assays were performed using HepG2 nuclear extracts with two different probes: the wild type and deletion 1 sequence (sequence described in chapter 2) was used as a probe, the DNA fragment was end labeled with biotin, incubated with 5 µg of HepG2 nuclear extract, and electrophoresed on a 6% nondenaturing polyacrylamide gel. The experiment was electrophoresed as described as the penal above the Fig.3-21, Lanes: 1, labeled wild type probe alone without nuclear extract; 2, labeled and unlabeled wild type probe with HepG2 nuclear extract; 3, labeled wild type probe with HepG2 nuclear extract; 4, Labeled deletion 1 probe alone without nuclear extract; 5, labeled and unlabeled deletion 1 probe with HepG2 nuclear extract; 6, labeled deletion 1 probe with HepG2 nuclear extract probe. I, II, III, and IV mark the locations of complexes I, II, III and IV respectively.

This result suggests that: there is an unknown protein which can bind with rearranged deletion sequence while can not bind with wild type sequence. Specific protein interaction with biotin-labeled probe should be decreased when the unlabeled probe was added; it seems to me that complex III has specific interaction with promoter. It indicates that there might be a novel TF

which can bind to the rearranged core promoter region. These data lend support to the possibility that host DNA-binding proteins interact directly with the HBV viral sequence.

3.4.2 Membrane Hybridization Assay Result

3.4.2.1 Selection of Appropriate Membrane

To study the interaction between the deletion probe and potential TFs, we employed a newly developed protein-DNA array technology. This array is a high throughput, DNA-based system that facilitates profiling of the activities of multiple TFs in one assay. There are four commercial arrays available: Array I, II, III, IV. After searching on Uniprot databank for the tissue specificity of each TF, array III was chosen for this experiment, because it has more liver enriched transcription factors (Fig. 3-22).

TF Protein Array Version III																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	PFDN4		PCU2AF1		PPAR α		PPAR β		PPAR γ 1		PPAR γ 2		PSMC5		PTGER2		PTTG		PTTG2		PURA		pos	
B	PXR-2		REL		ETS1		REVERB		RFXANK		RXR1B		RXRG		RYBP		SCML1		SHP		SIX2		pos	
C	SMARCB1		SMARCE1		MEF2A		SP1		SP3		SP4		STAT1		STAT3		STAT4		STAT6		SURB7		pos	
D	TCEA1		TCF7L2		PXR-2		TF2D		TFE3		TFEC		TLX		TRb1		TRCP		TRIP13		v-Myc		pos	
E	YY1																						pos	
	pos		pos		pos		pos		pos		pos		pos		pos		pos		pos		pos		pos	

Fig. 3-22 TF protein array version III

3.4.2.2 Membrane Hybridization Assay

To identify TFs which can bind to this specific deletion 1 probe, membrane hybridization assay was carried out by using the deletion 1 and wild type probe respectively as well as the

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cold probes. 3ul of 1:100 diluted biotin labeled probe and 10 fold of cold probe was used in this experiment, 5µg of HepG₂ nuclear extracts was used as well, after incubation membrane with blocking buffer, washing buffer, and streptavidin, the signal was detected by ECL. To confirm the result, this experiment was performed 2 times separately.

Array analysis result (Fig. 3-23) shows that when Fig 3-23 A and Fig 3-23 B was compared, the most striking changes noted were increased binding of PTTG1, PTTG2, SIX2, PXR2 and TFE3, which means those above TFs can bind with the deletion 1 probe, and the binding can be competed by adding the cold probe. From this result, it can be seen that for PXR2, SIX2, the intensity of the dot indicated a 2-fold increase compared with the compete result, and for PTTG1, PTTG2 and TFE3 the intensity of the dot indicated a 1-fold increase compared with the compete result, another TF named SMARCE1 can weakly bind to deletion 1 probe and this binding can be completely competed by adding the excess cold probe. When Fig 3-23 C and Fig 3-23 D compared, The most striking changes noted were increased binding of REVRB, SP3, which means those above TFs can bind with the wild type probe, and the binding can be competed by adding the excess cold probe. Another dot which can be seen on each membrane named RYBP. Compare with Fig A and C, the signal of PTTG2, SIX2, TFE2 are strong on Fig A, whereas those protein's signal is weak on Fig C, which means those proteins can strongly bind with deletion 1, while weakly bind with wild type; however PTTG1, SMARCE1, PXR2 can only be seen on the membrane incubated with deletion 1 probe, and cannot be seen on the membrane incubated with wild type probe.

Deletion 1

Wild type

Fig. 3-22 DNA-protein array result of different probes. The response elements on the array are spotted in duplicate. The right and bottom sides of the array indicate where biotinylated DNA has been spotted. Most TFs are named according to standard nomenclature. A,B: results of the arrays when screening protein- deletion 1 interaction. The TF protein array III was incubated with the biotinylated deletion1 Probe (A) and both biotinylated and unlabeled deletion1 probe (B). Specific Protein/DNA interactions (boxes) were competed out with unlabeled probe, whereas non-specific binding of the probe was not. C,D: results of the array when screening protein- wild type interaction. The TF protein array III was incubated with the biotinylated wild type Probe (C) and both biotinylated and unlabeled wild type probe (D). Specific Protein/DNA interactions (boxes) were competed out with unlabeled probe, whereas non-specific binding of the probe was not. The rectangle box showed (method as 2.7.4) TFs which can be competed. (This experiment has been repeat twice, and got the same result).

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The data suggest that, PTTG2, SIX2, and TFE3 have stronger binding activity with deletion 1 probe than with wild type, and PTTG1, SMARCE1, PXR2 can bind with deletion 1 only. The dot of RYBP which can be seen on each membrane were ignored, it was thought to be just the unspecific binding of the DNA probe. The full name of the potential TF is listed in Table 3-4.

Table 3-4 The interesting proteins which were obtained from DNA-protein array analysis

Accession number	Name of TF	Full name
NM_004219	PTTG 1	pituitary tumor-transforming 1
NM_006607	PTTG 2	pituitary tumor-transforming 2
NM_000965	PXR-2	retinoic acid receptor, beta
NM_016932	SIX 2	sine oculis homeobox homolog 2 (Drosophila)
NM_003079	SMARCE 1	SMARCE1: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1
NM_006521	TFE 3	transcription factor binding to IGHM enhancer 3

3.5 Gel Shift Confirmation

Because the protein-DNA array is a high throughput method, the results require verification by a secondary assay. For the next step EMSA will be performed with deletion 1 and wild type probe (probes that were identical in sequence to the probes used in the protein-DNA array assay).

3.6 Confirmation of DNA-Protein Interaction

To search for the deletion 1 binding protein(s), we screened a TF array which include 45 differentiated TFs with labeled deletion sequence. A TF which was named SMARCE1 was obtained. SMARCE1 contains 411 amino acids (46649 Da).

3. Result

3.6.1 Cloning of the SMARCE1 into Mammalian Expression Vector pXJ-40**3.6.1.1 Total RNA extraction.**

Commercial RNeasy® Mini Kit was used to extract ~100 µg RNA (50 µl) from A 90% confluence Hep G₂ cells. Fig. 3-24 shows the extracted total RNA electrophoresed on a denaturing formaldehyde/agarose gel, alongside an RNA marker. Two bands, corresponding to 18s and 28s ribosomal RNA, were observed.

Fig. 3-24 Total RNA extracted from HepG2 cells:

2×10⁶ of HepG2 cells was collected, and total RNA was extracted by using Qiagen RNeasy Mini Kit 2µl of RNA was loaded on the DEPC denaturation gel. Lane 1, RNA sample , Lane 2: RNA ladder

3.6.1.2 Isolation of Potential TF from Total RNA.

Titanium one-step RT-PCR kit was used in this experiment, 2µl of total RNA was used as template, and primer sequence design is copied from NCBI SMARCE1 (accession No. NM_003079) the full length of the SMARCE1 gene was fished out from the HepG₂ cDNA library. The size of this gene is 1235bp (Fig. 3-25).

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Fig. 3-25 Vector and insert for the cloning of SMARCE1 fusion protein. Lane 1: smarce 1 PCR product isolated for HepG₂ cDNA library size: 1235bp, Lane 2: mammalian expression vector pXJ40 size 4.5kb; lane 3: 1kb marker.

Fig. 3-26 Confirmation of cloning of smarce 1. Lane 1: enzyme digestion result of pXJ40-SMARCE1, Lane 2 : 1kb marker.

3.6.1.3 Cloning of the Smarce1 into Mammalian Expression Vector pXJ40.

To examine the binding specificity of SMARCE1, recombinant SMARCE1 protein was obtained by fusion of the SMARCE1 open reading frame with that of the HA tag. Plasmids pXJ40 (a mammalian cell expression vector) was used as the vector in this construction. The insert of SMARCE1 is the PCR product which achieved from the last step, the full length of SMARCE1 gene was BamH I/Not I -digested and ligated with BamH I/Not I -digested pXJ40. The cloning result was confirmed by double enzyme digestion as shown in Fig. 3-26, and the sequence of the insert was identified by sequencing result (*data not shown*).

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3.6.2 Western Blot Analysis

To do the overexpression study for this potential TF, Western blot analysis was carried out in HepG₂ cells transfected with pXJ40-SMARCE1 constructs using anti-HA antibody. However, no protein band was observed. This could be due to the low transfection efficiency for HepG₂ cells. Therefore next step we compare the cellular transfection efficiency of different cell lines. The transfection experiment shows that the transfection efficiency of 293 T cells is much higher than that of HepG₂ cells (*data not shown*), and previous promoter assay experiment result also shows the tendency of the promoter activity to be the same for replicative genome in different cell lines. Furthermore, previous result in our laboratory also indicated that when replicative genome was transfected into 293T cells, HBsAg can also be detected in the cultured medium, which means virion of HBV can be packaged and secreted out normally in this cell line (*data not shown*). So the same pXJ40-SMARCE1 construct was transfected into the epithelial cell line 293 T which generally yields a higher protein level compared with HepG₂ cells. 400ng of pXJ40-SMARCE1 was transfected into 293 T cells, and 400ng of pXJ40 vector was also transfected as the negative control, 48hrs post transfection. The cells were collected and lysed in 2×SDS sample buffer, after gel running and membrane transfer, 1:1000 anti-HA monoclonal antibody was used as 1st antibody, after ECL development, the western result is shown in Fig. 3-27 which indicates the SMARCE1 protein can be successfully overexpressed in 293 T cells.

3. Result

Fig. 3-27 Analysis of SMARCE1 Protein Level.

Western blot analysis was carried out using HA tag monoclonal antibody. A 46kD protein corresponding to SMARCE1 was detected in lane 1 (293T cells transfected with pXJ40-SMARCE1). No protein was detected in lane 2 (293T cells transfected with pXJ40 plasmid).

3.6.3 Confirmation of the Protein Binding to the Rearranged Core Promoter Region

We then examined the binding specificity of overexpressed SMARCE1 protein in cells. An experiment was designed to confirm that overexpressed SMARCE1 indeed bound to deletion 1 region while did not bind to wild type region. A 1.2-kb RT-PCR fragment coding for the full-length SMARCE1 gene from total RNA extracted from HepG₂ cells was cloned into the mammalian expression vector pXJ40. Transient-transfection assays were then used to prepare nuclear extracts. 293T cells were transfected with pXJ40-SMARCE1 or empty vector without the SMARCE1. Total cell lysates from these transfected cells were obtained for gel shift experiments. As shown in Fig. 3-27, SMARCE1 were detected with anti-HA monoclonal antibody. The apparent molecular mass of this protein is 46kD. Then, 5µg of nuclear extracts from each cell lysate were obtained for incubation with probes of deletion 1 and probes of wild type separately. The nuclear extracts were incubated with 1:1000 diluted labeled double-stranded oligonucleotides corresponding to the wild type and deletion 1 promoter sequence, 200 fold Oligonucleotides containing unlabeled sequences corresponding to the wild type and

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deletion 1 sequence were added in molar excess in separate mixture for gel shift assay. After electrophoresis and membrane transfer, both complexes and probes were identified with streptavidin- horseradish peroxidase and substrate (Fig. 3-28). Fig. 3-28(a) represents the gel shift assay result by using nuclear extracts from cells transfected with empty vector. Fig. 3-28(b) represents the gel shift assay result by using nuclear extracts from cells transfected with pXJ40-SMARCE1 vector. The reagents added in each lane are tabulated in Table 3-5.

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Table 3-5 Description of reagents added in each lane.

Lane No.	1	2	3	4	5	6
Cells Nuclear extracts	+	+	--	+	+	--
Labeled wt probe	--	--	--	+	+	+
Unlabeled wt probe	--	--	--	+	--	--
Labeled del1 probe	+	+	+	--	--	--
Unlabeled del1 probe	+	--	--	--	--	--

Fig. 3-28 DNA binding specificity of overexpressed smarce1

(a) Binding of probe to the nuclear extracts without SMARCE1 overexpression. 293 T cells at density of 2.0×10^7 cells per T_{75} flask were transfected with $2\mu\text{g}$ of pXJ40 empty vector as indicated. Nuclear extracts were prepared as described in Materials and Methods. Five micrograms of mini-nuclear extracts was incubated with $1\mu\text{l}$ of 1:1000 dilution biotin labeled probe (both wt and del1) in gel shift experiments. 200 fold oligonucleotides containing unlabeled sequences were added in molar excess as competitors in gel shift assays. I, II, III, IV and V mark the location of the complexes. (b) Binding of probe to the nuclear extracts with SMARCE1 overexpression. 293 T cells at of density of 2.0×10^7 cells per T_{75} flask were transfected with $2\mu\text{g}$ of pXJ40empty vector as indicated, Nuclear extracts were prepared as described in Materials and Methods. Five micrograms of mini-nuclear extracts was incubated with $1\mu\text{l}$ of 1:1000 dilution biotin labeled probe (both wt and del1) in gel shift experiments. 200 fold Oligonucleotides containing unlabeled sequences were added in molar excess as competitors in gel shift assays. I, II, III, IV and V mark the location of the complexes.

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By comparing the two results in Fig 3-28, figure 3-28 (b) showed that there is one band (complex II) that indicated an TF has bound with deletion 1 probe (lane 2) and cannot bind with wild type probe (lane 5), and this binding can be completely competed by adding of excess unlabeled deletion 1 (lane 1). Although in Fig 3-28 (a) this binding activity was also presented in cells transfected with empty vector only, but the intensity is much lower and it also can be competed. These results indicated that SMARCE1 could indeed bind to the deleted sequence. It is known that even in the 293T cell itself, there are also some endogenous SMARCE1 which explained why Fig 3-28 (a) showed slight traces of binding. Beside the complex II, there are the presences of 4 complexes which can also be seen on lane of wild type probe too, which indicated nonspecific binding for the deletion 1 probe.

EMSA verified that nuclear extracts from which SMARCE1 was over expressed from the 293 T cells has one more stronger binding protein with deletion 1 probe (complex II) compared to that with wild type probe. Adding cold DNA to the EMSA reaction abolished the band shifts, confirming that the detection was specific.

3.7 Influence of SMARCE1 on HBV Replication

3.7.1 Influence of SMARCE1 on HBsAg Secretion

This section seeks to demonstrate the functional connection of SMARCE1 to the regulation of HBV viral load. The SMARCE1 expression constructs were co-transfected into 293T, together with a full-length nts1749-1768 deleted replicative clone of HBV and wild type replicative clone of HBV, respectively to determine the effects of SMARCE1 on the replicative efficiency of the HBV construct. The ratio of the two vector is one to one, After 48hr, the respective

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culture medium was collected for comparative measurement of the viral replication. Then the culture medium was semiquantitatively assessed for the amount of HBsAg by ELISA assay. The whole experiment was repeated at least three times to obtain the following results. Results shown in Fig. 3-29 suggested that: 1) for the wild type replicative genome, no matter whether overexpress SMARCE1 or not, there is no great difference on HBsAg secretion ($p>0.05$); 2) but for the deletion 1 replicative genome, the HBsAg value is lower than that without overexpression of SMARCE1, the difference is of significance ($p<0.05$), which is almost $\frac{1}{4}$ or 25%.

Fig. 3-29 Semi-quantitative measurement of secreted HBsAg
2 days after transient cotransfection with HBV replicative genome and SMARCE1 overexpression vector and empty vector, the culture medium was collected and HBsAg was measured by IMX machine. Mean HBsAg values were obtained from three independent experiments measured in the supernatant. Standard deviation was marked on the bar chart, this experiment was repeated for 3 times independently.

3. Result

The cells transfected with deletion 1 replicative genome with overexpression of SMARCE1 were shown to secrete out less HBsAg. This experiment were performed in triplicates and repeated a minimum of 3 times, and got the same result, standard deviation was marked on the bar chart. (T test was used as the statistical method). This in turn suggests that SMARCE1 may play a role in lowering the HBV replication capacity on cells transfected with deletion 1 replication genome. Another possible reason is that there is a negative effect on the HBsAg promoter or surface transcripts, however in our cell based replicative system, the only difference between wild type strain and del1 strain is the deletion in core promoter region, while this sequence difference did not overlap with promoter of surface gene. So, it is quite possible that this decrease is occurred at transcription level because of SMARCE1.

3.7.2 SMARCE1 Is an Essential Modulator of Virus Promoter Activity

In order to further validate the SMARCE1 function on promoter activity, the SMARCE1 expression constructs were co-transfected into 293T, together with a pGL3promoter-del1 vector and pGL3promoter-wt vector to determine the effects of SMARCE1 on the different promoter's activity. The same amount of pBIND vector was also transfected to serve as the transfection efficiency control. The ratio of the three vector was one to one. Transfection for each construct was carried out in three repeated experiments. And each sample was triplicated each time. 48hr later, cells was lysed and luciferase reagent was added to measure the luciferase activity of each sample. Results from the independent transfections for each combination are shown in Fig. 3-30. It shows that: 1) it does not affect much on the wild type promoter activity, no matter whether it overexpresses SMARCE1 or not ($p>0.05$), and 2) the deletion 1 promoter activity value was lower when samrce1 was overexpressed, the difference is of significance

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($p < 0.05$). However, for wild type promoter activity it seems that overexpression of SMARCE1 did not have any effects on HBsAg secretion. This experiment were performed in triplicates and repeated a minimum of 3 times, and got the same result, standard deviation was marked on the bar chart (T test was used as the statistical method).

Fig. 3-30 Luciferase result of different constructs with or without overexpression of SMARCE1 HepG2 were transfected with the respective Enh II clones (wt, del1). For each cell type, the first column represents the ratio of the luciferase activity of internal positive control (promoter and enhancer) to pBIND. The last column (“negative”) represents the ratio of activity of the internal negative control (promoter only). The other columns represent the the ratio of activity between pGL3-Enh II (wt, del1) vectors to that of pBIND .Those cotransfected with SMARCE1 overexpress vector was in shaded column. Results of the luciferase assay were normalized to the level of the internal positive control (arbitrarily set at 100%). (RLU stands for relative luminescence)

In summary, the results showed that when SMARCE1 was overexpressed on the cells transfected with deleted HBV core promoter construct, the luciferase expression level was

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decreased. It therefore states that SMARCE1 may play an important role in decreasing the viral replicative capacity on transcription level.

4. Discussion

4.1 Identification of Deletions in Core Promoter

In this project, I have identified HBV-genomes with deletions in the core promoter region. I examined the CURS/BCP nucleotide sequences of 839 HBV strains in the hepatitis virus database (<http://s2as02.genes.nig.ac.jp/>). However, no similar deletions were found. Therefore, our study represented the first report of characterization of this kind of deletions. The investigation has provided us with better understanding on the association between the deletions and the HBV replication.

Our previous clinical result has shown that, in three patients, a high HBV replication level has been detected as measured by serum HBV DNA level (around 228.16 pg/ml) and HBsAg level (around 140 µg/ml). Interestingly, the same deletion has been found in viral core promoter region in these patients. The deletions cover two regions nts1758-1777 and nts1749-1768. The average serum HBV DNA level of these three samples is 228.16 pg/ml, which is significantly higher than that of other patients, 36.39 pg/ml (Appendix A).

4.2 Effect of Core Promoter Deletions on HBsAg Secretion

A high HBV DNA level in serum attracted our interest. Is there any correlation between the high HBV replication rate and HBV genotype? In order to assess the significance of such a finding and also to determine the impact of the BCP deletions on viral replication efficiency in different HBV constructs, we cloned the deleted core promoter region into replication-

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competent HBV vectors successfully and through transient transfection into human hepatoma cells, these constructs were tested *in vitro* in a cell –based system. The system was based on transfecting a replicative HBV genome into HepG2 cells. Some studies show it can support HBV virus replication with a large amount of secreted virions in the culture medium (Oon *et al.* 2002). Such replicative genome has been successfully used to analyze various HBV mutants (Chen *et al.* 2000b).

In this thesis, we reported the molecular analysis of the correlation between HBV replication rate and different HBV replicative genome using of this cell-based system. The results indicate that replicative HBV genomes containing deletions in the viral core promoter displayed decreased viral replication level. This in turn indicated that, even with the truncated core promoter region, the construct can still produce HBsAg albeit at a lower level.

The result of our HBsAg measurement showed that the amount of secreted HBsAg in the cells transfected with deletion mutants was significantly lower than that of those transfected with the wild type genome. This was opposite to the situation with high level of HBV DNA in clinical samples (Chen and Oon *et al.* 2000). This paradox could be explained by the fact that the clinical samples may be heterogeneous with mutations or insertions or deletions in other parts of the genome. However, cross-sectional analysis focused mainly on the mutation pattern at the precore residue 28 and nt 1762/1764, therefore, the mutation in other part in these three samples may have contributed to the viral load enhancement. In contrast, only the deletions in the core promoter were analysed in our cell-based system. Nevertheless, the *in vitro* analysis reported in our study shows that it was a valuable platform to study the potential mechanism of regulation in a homogeneous condition.

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The result of our HBsAg measurement showed the amount of secreted HBsAg in the cells transfected with deletion mutants was significantly lower than that of those transfected with the wild type genome. The possible explanations were as follow, 1) the decrease occurred at transcription level, this hypothesis can be tested by promoter activity assay; 2) the transcription products of deletion constructs were the same as or even higher than that of wild type, but the decrease occurred at translation level. Because the HBsAg measurement was a combination of both the DANE particle and empty HBsAg, HBV DNA detection could be used to see whether the decrease is triggered by true lower replication or only because of the secretion of low empty surface antigen. Because the difference of our constructs lies in core promoter region instead of S promoter region, these two promoter sequence were also not overlapped. So as the next step, the relatively number of DNA copy of different constructs could be tested.

My results provide a new system for investigation of the molecular characterization of mechanism of HBV replication. Although our *in vitro* assay result was contrary to the clinical result, they suggested that there may be a complex regulatory mechanism in the viral replication which means some other elements in the viral core promoter may be involved. My results could be validated by further experiment such as the measurements of HBV DNA level. Nevertheless, such a cell-based HBV replication system should be helpful for further molecular characterization of other naturally occurring mutation and deletions of HBV genome.

4.3 Effect of Deletions in Core Promoter on Viral Replication

In order to further validate the replicative capacity of mutated HBV constructs, HBV DNA in culture medium was extracted and relatively compared with one another by real time PCR. The

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result showed that the constructs with the deletion mutations displayed decreased replication capacity compare to that of wild type, as indicated by the secreted HBV virus. The detection of HBV DNA in the culture medium also showed that even with an incomplete core promoter region, there was the existence of the “pregomic-like” molecules. Importantly, this mRNA can be packaged, reverse transcribed and the corresponding cDNA also can be secreted in viral particle encapsidated form.

Our *in vitro* results indicated that HBV mutants with naturally occurring deletions in the core promoter had lower viral replication level compare to that of wild type. This is opposite to the clinical observation which shows higher HBV DNA levels were detected for those core promoter partially deleted samples. One possible explanation is that these deleted strains are actually defective in viral replication. The high viral DNA levels which we observed may be a result of co-existing small amount of wild-type strains with the deleted strains, it is reported that the wild type strains can complement partly the replication deficiency of the mutant strains. And there might be some interaction between the wild type a deleted strain which is still unknown, the final consequence of the interaction showed the lower HBV replication capacity. The other possibility is that there are other mutations on the genome of these clinical mutant viruses which contribute to the observed HBV DNA levels in the clinical setting. Further investigation including sequencing of full genomes of the clinical strains should shed lights on the detailed mechanism of the discrepancies between the clinical and cell culture data.

The result of trasfection with deletion mutants alone was characterized by low-level of viraemia. One possible reason might be the loss of some functions because of the deletion. The deletion lies in the core promoter region which contains binding sites for regulators of

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transcription. I hypothesize that BCP deletion may have decreased HBV replication rate as a result of combined factors: altered transcription factor binding to the promoter, deletion in the overlapping HBx protein, and changes in the ratio of PreCore to pregenomic RNA. Possible explanations are listed as follows,

- 1) Loss of some of transcription binding sites. It was found in this study that part of CURS/BCP was deleted, which covers the HNF4, SP1, C/EBP, TA binding protein site. For HNF4 site, nucleocapsid promoters were transactivated in the presence of RXR and PPAR. If it has been removed, the transcription level would be decreased. For SP1 site, it can bind to the region of nts1745-1754 in the core promoter. It has been reported that this binding did not affect the transcription of the S gene and the X gene, SP1 site abolished therefore may not affect the transcription. The C/EBP also covers this region. The function of C/EBP is contradict, the *in vitro* experiment shows that C/EBP modestly activates expression from the CP at a low concentration, but represses expression at high concentrations, so what is the true effect if this binding site was abolished is still not clear. TATA box binding protein also plays an important role in the transcription and initiation of pgRNA. It is not clear so far yet what the combined function of these TF is. Another possibility is that there is one or more novel TFs that can bind to the rearranged core promoter region, and play negative regulatory role which decrease the transcription.
- 2) Changes in the ratio of precore to pregenomic RNA. The core promoter regulates the transcription of the core gene and precore gene (Yen *et al.* 1993). The coding sequence for precore gene cover the whole core gene coding sequence as well as the additional 29

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amino acids amino-terminus. This extra amino-terminus consists of a signal peptide, the function of this signal peptide is to send the precore protein to the endoplasmic reticulum (ER) (Ou *et al* 1997). Then, this signal peptide was cleaved in the ER lumen, after that, the shortened precore protein transferred out of the ER lumen, and is cleaved once again at its carboxy terminus, lastly, it was secreted out of the cell. (Ou *et al* 1997) The secreted final product is called hepatitis B e antigen. Our result shows the DNA replication capacity is low with the naturally occurring deletion constructs, there are some factors may be involved in the decreased HBV DNA, 1) studies show that precore protein can inhibit HBV DNA synthesis, those deletions of course will affect proper precore protein expression, 2) the stem-loop structure play an important role in viral packaging and starting of the reverse transcription, previous reports shows, with the mutants in precore region, the stem-loop structure was also affected, therefore it will affect packaging efficiency of the polymerase-pregenomic RNA complex. From this angle, It is possible that HBeAg increases the HBV replication, and in these two deletion cases, e antigen expression obviously was affected. We only know that the final result is the decrease of HBV replication, the concrete role it plays in this procedure still needs to be further studied. There might be some other more complicated regulatory mechanism involved in the replication procedure afterwards

- 3) The role of HBx protein in the decreased replication. HBx is believed to be essential for HBV infection. It can interact and activate the Src and related signaling pathway, this function is very important for HBV DNA replication. For example, it is reported that HBx can activate the Src kinase activator: the cytosolic calcium-dependent proline-rich tyrosine kinase (Pyk2). And by inhibiting Pyk2, the activation function of HBx

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on HBV DNA replication can be blocked (Michael *et al* 2001). Obviously, because the overlapping region of core promoter and X gene, X protein expression was also affected therefore, truncation of X protein as a result of the deletions in the core promoter investigated in our study could be another reason that accounts for the lower replicative capacity of the deleted constructs.

4.4 Effect of Deletions in Core Promoter on Viral Replication

In order to further identify intracellular virus replication, pgRNA product of 3 different constructs was compared, and the result has been shown in Fig. 3-12. The constructs with the deletion mutations decreased replication capacity. It is indicated as the reduced pregenomic-RNA level. Theoretically, core promoter mutation affects pregenomic RNA transcription. The result was expected in that the pgRNA of the cells transfected with deletion1 was lower than that of the cells transfected with wild type.

Along with the reduced secretion of progeny virus and surface antigen caused by the deletion in 1749-1768 and 1758-1777, there was a consistency decrease in the production of pgRNA (Fig. 3-12). Therefore, I hypothesize that the decrease in progeny virus production most likely occurred at or immediately prior to the pregenome encapsidation step of viral assembly. Further experiments are required to confirm the mechanism by which this decrease of viral production occurs.

Our RT-PCR result showed that the construct produced less amount of mRNA than wild type produced. The possible explanations were as follows: 1) the deletion resides in the nuclear

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receptor binding site of the core promoter and abolishes the binding of COUP-TFs, the heterodimer of PPAR α -RXR α and TR4. Without these TF's binding, the transcription decreased; 2) there are some repressors that can bind to these regions, or in other words, there are some other transcription factors that can bind to the region. This region generated by deletion would decrease the transcription level. Our next step was to isolate this possible "repressor" by using panomics membrane hybridization kit.

Taken together, we transfected these different construct into HepG2 cells and tested the deleted core promoter sequence in the context of the wild type genome. The result shows low level of pgRNA synthesis in the recombinant HBV genome with deleted promoter. We can still draw the conclusion that the recombinant HBV genome with the truncated promoter can produce pregenomic RNA-like transcripts, and these transcripts can also be encapsidated and reverse transcribed.

My data have provided helpful molecular information regarding the role of core promoter deletions in HBV replication. For these two core promoter deletions, our data shows that the viral replication was decreased and HBsAg expression was lower compared with wild type constructs. I hypothesize that, most probably, the BCP deletion mutations may decrease HBV replication via altered transcription factor binding to the promoter.

Our data showed that the wild type produced more pgRNA than viruses with deletion 1 and deletion 2. This result indicated that the decrease in progeny virus production likely occurred at or immediately before the pregenome encapsidation step. Because the deletion region is just on the Enh II region, Enh II is a critical *cis*-element for the liver-specific transcriptional regulation

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of HBV gene expression. Is it possible that at the transcription level, the decrease occurred already? To understand whether the natural deletions at nts 1749-1768 and nts 1758-1777 had some difference transcription level, I cloned 3 reporter constructs and tested the ability to drive reporter gene transcription of all these 3 constructs. It was evident that wild type had higher activity than the deletion 1 and deletion 2 constructs (Fig. 3-17). To investigate the potential mechanism for this decreased transcriptional activity, we searched for the possible interaction of DNA sequence and there specific binding protein. Using an initial gel shift assay followed by a membrane hybridization analysis, the effects of the BCP deletion of 1749-1768 on HBV gene expression and replication were further elucidated.

4.5 Isolation of a Novel Transcription Factor

Since promoter assay showed lower truncated core promoter activity, there may be a “repressor” binding to the rearranged core promoter region.

4.5.1 Sequence Analysis of Rearranged Core Promoter - Possible Cellular Binding Protein

We proposed that the rearranged promoter created several new possible binding sites for the putative transcription factor. Through the database searching (Table 3-1), it was found that there were some transcription factors binding with DNA sequence just at the junction site. For deletion 1, ADR1 (the yeast transcriptional activator) binds to DNA sequence gggagt, GAmYb (MYB transcription factor) binds to DNA sequence gggagttg. For deletion 2, LyF-1 (lymphocyte-specific DNA binding protein) can bind with DNA sequence gttaggagg, Sp1 (an activator of numerous TATA-containing and TATA less promoters) can bind to DNA sequence

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aggagg. The results indicated that, although they were detected from different species, theoretically, there may be several proteins that can bind to the rearranged promoters. It is obvious that, for wild type, the present searched results were consistent with those reported by other researchers.

4.5.2 2-Dimensional Gel Electrophoresis

In order to further confirm the existence of the novel transcription factor, the two different constructs were transfected into Hep G2 cells, and 2D gel experiment was performed to elucidate the cellular protein differences of two constructs. In all the *in vitro* experiments conducted, the difference between the deletion 1 result and the wild type result is shown to be core drastic as such, they were chosen for the further study.

The different protein spots revealed in Fig. 3-18 A and B were further analyzed based on their molecular weight and isoelectric point (pI) values. Results shown in Fig. 3-18 revealed differences among the two gels. Some of the proteins were present in the cells transfected with wild type, but not present in the cells transfected with deletion 1. Some of the proteins were present in the cells transfected with deletion 1, but not present in the cells transfected with wild type. The results therefore suggested that, with exposure to a different replicative genome there were specific cellular proteins associated with the replication of distinct viruses.

4.5.3 Gel Shift Result

Using the deletion1 and wild type sequences as probes and HepG2 nuclear extracts, the EMSA experiment showed the presence of an unknown TF binding to the deletion 1 probe, but not to the wild type probe (Fig. 3-20).

4.6 Interpretation of Membrane Hybridization Result

From the previous gel shift result, it was clear that there was an unknown TF which can bind to deletion 1 probe, but not to the wild type probe. The next step was therefore to identify this protein. DNA-array assay was performed to screen out the possible TF. Result (Fig. 3-22) showed that 5 TFs specifically bound to deletion 1 probe (Table 3-4).

PTTG1

Pituitary tumor transforming gene (PTTG) has been recently identified as a new oncogene. It can be used as a marker of malignancy for several cancers, including the endocrine malignancies such as pituitary adenomas. The location of it is in cytoplasm and nucleus, its N-terminal destruction box (D-box) can serve as a recognition signal for degradation via the ubiquitin-proteasome pathway. Its tissue distribution is ubiquitous, however, in pituitary adenomas it is expressed in high level, while in other tissue, it is relatively expressed at low level. (Kakar *et al.* 1999). The main functions of the PTTG1 are listed as follows:

- 1) By inhibit the separase activity, this protein can inhibit the premature chromosome separation which was called as an anaphase inhibitor, thus to initiate anaphase, it should be degraded. As a result, PTTG is involved in several critical cellular processes such as mitosis, cell cycle progression, DNA repair and apoptosis.
- 2) It is found that in pituitary adenomas, the expression of this protein is very high, so it was used as a marker for pituitary adenomas. Except for this, PTTG 1 is also take part

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in the pathogenesis of other tumors. It can induce aneuploidy and up-regulate FGF-2, a potent mitogenic and angiogenic protein (Tfelt *et al.* 2006).

- 3) It is also reported that it can inhibit cellular growth via up-regulation of the cell cycle protein p21. this conclusion was drawn from the experiment done on human A549 lung cancer cells (Mu *et al.* 2003).

PTTG2

Pituitary tumor-transforming 2 (PTTG2) which was isolated from human testis has also regarded as an oncogene. Because in various human tumors and tumor cell lines its RNA detection shows high levels. It has been reported that, similarly to PTTG1, *in vitro* assay shows that over-expression of hPTTG2 in mouse fibroblast cells can cause an increase in cell proliferation, and promote tumor formation in nude mice. Like PTTG1, PTTG2 is located in both cytosol and nucleus. It also has a lot of functions. It plays the role in chromosome organization and biogenesis and it was detected in pituitary adenoma (Prezant *et al.* 1999).

SIX2

Sine oculis homeobox homolog 2 (SIX2) belongs to the SIX/Sine oculis homeobox family.. Associated with its DNA-binding property, it is found in the nucleus. It shows high expression level in skeletal muscle and may be involved in limb tendon and ligament development (Ikeda *et al.* 2002).

TFE3

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TFE3 is a transcription factor that binds to IGHM enhancer 3. it has RNA RNA polymerase II transcription factor activity, and it is an ATP binding protein, The molecular function lies in its phenylalanine-tRNA ligase activity. (Chung *et al.* 2001).

RXP2

Retinoic acid is a morphogen and has profound effects on vertebrate development. RXP2 is a receptor for retinoic acid and also known as RAR-beta (retinoic acid receptor, beta) and RAR-epsilon. It has been shown to be activated by HBV infection. This receptor regulates cell processes by directly modulating the gene expression machinery. It is located in placenta, mammary tumor and liver. Three functional domains have been identified: a modulating N-terminal domain, a DNA-binding domain and a C-terminal steroid-binding domain. Interestingly, it has been identified in hepato cellular carcinoma (Benbrook *et al.* 1988).

Sum in all, all these proteins obtained from the DNA-array assay are transcription factors, two of them triggered our interests, 1) from the membrane hybridization result, only SMARCE 1 can be completely competed when adding the excess quantity of unlabeled probe, and 2) RXP2 belongs to nuclear receptor, and it also correlates much with liver, therefore I decided to choose these two proteins for the further study. However when I tried to use the RXP-2 primer to get the RT- PCR product from the HepG2 cells, it always failed. Nevertheless, SMARCE 1 was chosen for the further confirmation experiment.

4.7 Role of SMARCE 1 in HBV Replication

Using an initial electrophoretic mobility shift assay followed by a membrane hybridization analysis, a novel oligonucleotide region with deletion 1 included was able to enrich a host binding factor. The binding factor turned out to be SMARCE 1, a known protein that has been shown to be involved in a number of cellular functions. SMARCE 1 has been identified by different investigators and has been known under various names, including SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1-related (SMARCE 1-related protein) (HMG domain protein HMG20B) (Structural DNA-binding protein BRAF35) (BRCA2- associated factor 35) (Sox-like transcriptional factor). It has been found in both nuclear and cytosol. Members of the Swi/Snf family of chromatin-remodeling complexes have shown to be involved in transcriptional control. Based on the similarities of their ATPase subunits, these complexes can be divided into three classes: Swi2/Snf2, Isw1, and Mi-2 proteins (Martens JA 2003).

Earlier investigations have suggested important roles of the SWI/SNF complexes in fundamental cellular processes such as transcription, replication, and the repair of chromatin (martens JA, *et al*, 2003, Peterson CL 2004). These in turn have wide implications in diverse physiological and pathological processes, including cell proliferation and differentiation, retrovirus infection, and carcinogenesis (Chen J *et al* 2005). Interestingly, it has been shown that the whole complex can bind directly to double stranded DNA (Wang W *et al* 1998).

In this study, I was able to show that a host protein—SMARCE 1 can be isolated by direct binding to a viral fragment derived from the HBV variant of the infected patient. The cotransfection experiment and gel shift assay further confirmed the binding affinity of

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SMARCE 1 and deletion1 probe. An important next step was to show if SMARCE 1 acts on a full-length HBV genome. To this end, I constructed two full-length replicative HBV clones, which were identical except for core promoter deletion in the region 1749-1768. The wild type and Deletion 1 replicative clone variants were co-transfected with SMARCE 1 expression constructs respectively. The results once again confirmed that the wild type replication was more efficient than the deletion1, while there was no significant difference for wild type between with or without overexpression of SMARCE 1 in enhancing or decreasing viral load. However, with overexpression of SMARCE 1, the HBsAg value of the deletion 1 variants decreased. This suggests that SMARCE 1 plays some roles on decreasing HBsAg secretion. It can be seen from the cotransfection experiment that in the cells with overexpression of SMARCE 1, the secreted HBsAg value is lower (Fig. 3-28). Furthermore, the promoter activity is also lower in the cells with SMARCE 1 overexpression (Fig. 3-29). Does this TF repress promoter activity at the transcription level? From literature review, it seems that there are possibilities that SMARCE 1 down regulates the transcription. Recent chromatin immunoprecipitation (ChIP) experiments showed that Swi/Snf components were detected at repressed promoters. It seems that repression by Swi/Snf need to recruit other regulatory proteins. For example, the first step of tumor suppressor by repression of E2F activation is shown to recruit Swi/Snf to particular E2F-dependent promoter where they inhibit E2F-mediated transcription (Wang S, *et al* 2002).

Perhaps other cellular protein factors may also act in to support the replication activity of HBV in the host. Indeed, a deeper understanding of how its work will provide insights into viral replication. The mechanistic aspect of SMARCE 1 on HBV replication needs to be further explored.

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Our study shows that the overall viral replication efficiency is determined by a combination of both viral sequence and interaction with specific host proteins. As the researchers final aim is to find the antiviral drugs. To find the interaction between host cells and virus is one of the channel for us to reach the target. Further investigation is required.

This study shows that the host protein—SMARCE 1 can be isolated by direct binding to a viral fragment derived from the HBV variant of the infected patient. The binding affinity of SMARCE 1 and deletion1 probe was confirmed. SMARCE 1 binds to and modulates the replicative efficiency of HBV at a precise location at the Enh II regulatory region. Overexpression studies show the dependence of HBV on a host factor to modulate its replication efficiency, and give us promise as a new class of targets for the intervention of chronic hepatitis B infection.

SMARCE1 has first been isolated in 1999. It is known as BAF57, a subunit of the Swi/Snf complex. The complex is a 2-MDs multisubunit ATP-dependent chromatin remodeling complex, consisting of a core ATPase subunit (called BRG) and associated proteins (BAF=Brg-Associated Factors). The activity of the ATPase ensure the remodeling activity while BAF is involved in interacting with other cellular proteins, mostly transcription regulators. Such transcription regulators are divided into two classes: 1) histone modification factors and 2) chromatin remodeling proteins (Swi/Snf complex).

Multiple functions of SMARCE1 have been reported. These include 1) the transcription activation by recruiting co-activator of gene expression in estrogen-induced response (Belandia

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et al., 2002); 2) the transcription repression by recruiting repressor protein involved in neuronal specific expression (Battaglioli *et al.*, 2002); and 3) direct DNA binding of tumor suppressor gene and leading to the activation of its expression (Wang *et al.*, 2005). Our results were therefore similar to the function of SMARCE1 in light of its direct binding to HBV core promoter.

Further investigation of SMARCE1 in chromatin remodeling in the context of HBV genome will be interesting. One peculiar characteristic of HBV genome is its conversion into ccc DNA once translocated into the host nucleus. It is then organized into mini chromosomes by complexing with histone and non-histone proteins (Teresa *et al.*, 2006). As a result, the number of nucleosomes formed by HBV cccDNA is estimated to be around 18 (Bock *et al.*, 1994).

In addition to its direct binding to HBV core promoter, SMARCE1 could also interact with other proteins to exert its repressor role on HBV replication. For example, it could recruit other repressor proteins to the core promoter which in turn occupy the binding site for transcription activators. As a result, transcription of pgRNA is repressed and HBV replication reduced.

More work can be done in the future. First, SMARCE 1 has been identified out by membrane hybridization assay, the Far Western could be done to further confirm the binding of this TF with specific promoter region. In addition, the chromatin immunoprecipitation could also be carried out to testify the binding quantitatively. Since it is reported that HBx is able to enhance the HBV replication (Hong *et al* 2006), the truncated HBx would decrease the HBV replication. This can be verified by cotransfection experiments. In addition, another interesting question would be which one of the TFs plays the major role. These experiments can be done in

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the future to further explore those factors which affect HBV replication. The results can be further taken as a reference for drug development for HBV.

Taken together, our investigation provides a general cell-based platform to study the regulation of gene expression from various aspects. Although it is applied to HBV promoter in this study, this cell-based replication assay provides the functional validation of the transcription regulation, while the direct identification of transcription factors via membrane hybridization provides a general and effective approach toward the analysis of DNA-protein interactions.

Summary

Hepatitis B virus (HBV) infection is a serious public health problem in many parts of the world that results in complications such as cirrhosis and hepatocellular carcinoma. Suppression of HBV replication, a causative agent for chronic hepatitis, is an effective approach to controlling disease progression. Host factors have a significant effect on viral replication efficiency and need to be better characterized.

Our group has reported the association between clinical virus load and deletions in HBV viral promoter previously. The interesting observation that chronic HBV carriers have different serum viral loads prompted me to investigate host proteins involved. To understand whether the natural deletions at 1749-1768 and 1758-1777 had any functional impact, reporter constructs were developed to test the effects of such deletions on their ability to drive reporter gene transcription. It is evident that both deletions had lower activity than the wild type constructs. Cellular transcription regulator recognizing the promoter with the deletion was revealed in gel shift assay and subsequently identified as SMARCE 1 through DNA-protein array assay. Furthermore, SMARCE1 was found to bind to and repress the replicative efficiency of HBV genome containing the same deletion in viral promoter.

The approach used in this thesis has opened a new way to study DNA-protein interaction and more significantly holds promise in identifying a new class of targets for the intervention of chronic hepatitis B infection. This research also suggests that SMACRCE 1 may represent a novel target for pharmacologic intervention of HBV replication.

References

- Arii M, Takada S, Koike K. Identification of three essential regions of hepatitis B virus X protein for *trans*-activation function. *Oncogene* 1992; 7: 397–403.
- Battaglioli E, Andres ME, Rose DW, Chenoweth JG, Rosenfeld MG, Anderson ME, Mandel G. REST repression of neuronal genes requires components of the hSWI/SNF complex. *J Biol Chem* 2002; Oct 25; 277(43): 41038-41045.
- Beasley, R. P. Hepatitis B virus: the major etiology of hepatocellular carcinoma. *Cancer* 1988; May 15; 61(10): 1942-1956.
- Belandia B, Orford RL, Hurst HC, Parker MG. Targeting of SWI/SNF chromatin remodeling complexes to estrogen responsive genes. *EMBO J* 2002; 21(15): 4094-4103.
- Birrer RB, Birrer D and Klavins JV. Hepatocellular carcinoma and hepatitis virus. *Ann Clin Lab Sci* 2003; 33: 39-54.
- Bock CT, Schranz P, Schroder CH, Zentgraf H. Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell. *Virus Genes* 1994; 8(3): 215-229.
- Brunetto MR, Stemler M, Bonino F, Schodel F, Oliveri F et al. A new hepatitis B virus strain in patients with severe anti-HBe positive chronic hepatitis B. *J hepatol* 1990; 10: 258-261
- Brunetto MR, Rodríguez UA, Bonino F. Hepatitis B Virus Mutants. *Intervirology* 1999; 42: 69-80.
- Bruss, V. and Gerlich, W.H. Formation of Transmembranous Hepatitis B e-antigen by Cotranslational in vitro Processing of the Viral Precore Protein. *Virology*; 1988;.163: 268-275.

References

- Buckwold VE, Zhichang X, Yen TSB, Ou J-H. Effects of a frequent double-nucleotide basal core promoter mutation and its putative single-nucleotide precursor mutations on hepatitis B virus gene expression and replication. *J Gen Virol* 1997; 78: 2055–2065.
- Carman WF, Jacyna MR, Hadziyannis SP, Karayiannis MJ, McGarvey A, Makris & Thomas HC, Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 2 1989; 588- 591.
- Chen IH, Huang CJ, Ting LP. Overlapping initiator and TATA box functions in the basal core promoter of hepatitis B virus. *J Virol* 1995; 69: 3647–3657.
- Chen WN, Oon CJ, 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* 2000; 30: 787-792.
- Chen WN, Oon CJ and Toh I. Altered antigenicities of hepatitis B virus surface antigen carrying mutations outside the common 'a' determinant. *Am J Gastroenterol* 2000; 95: 1098-1099.
- Chen J, Archer TK. Regulating SW1/SNF subunit levels via protein-protein interactions and proteasomal degradation: BAF155 and BAF 170 limit expression of BAF57. *Mol Cell Biol* 2005; Oct; 25(20): 9016-9027.
- Chen LL, Rashmi P, Elliot J. Lefkowitz and Sham S. Kakar. Identification of the human pituitary tumor transforming gene (hPTTG) family: molecular structure, expression, and chromosomal localization. *Gene* 2000; 248: 41-50.
- Cheng, C. et al., Hepatitis B large surface protein is not secreted but is immunogenic when selectively expressed by recombinant vaccinia virus. *J Virol* 1986; 60: 337-344.

References

- Chisari, F. et al. Expression of hepatitis B large envelope polypeptide inhibits hepatitis B surface antigen secretion in transgenic mice. *J Virol* 1986; 60: 880-887.
- Chung MC, Kim HK, Kawamoto STFEC can function as a transcriptional activator of the nonmuscle myosin II heavy chain-A gene in transfected cells. *Biochemistry* 2001; Jul 40(30): 8887-897.
- Chu, CJ, Keefe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, Carey W, Brown RS, Luketic JVA, Terrault N, and Lok AS. Prevalence of HBV precore/core promoter variants in the United States. *Hepatology* 2003; 38: 619-628.
- Dominguez A, Ramos-Morales F, Romero F, Rios RM, Dreyfus F, Tortolero M, Pintor-Toro JA. HPTTG, a human homologue of rat PTTG, is overexpressed in hematopoietic neoplasms. Evidence for a transcriptional activation function of hPTTG. *Oncogene* 1998; 17: 2187-2193.
- Feitelson MA, Duan LX, Guo J et al, Precore and X region mutants in hepatitis B virus infections among renal dialysis patients. *J Viral Hep* 1995; 2: 19-31.
- Feitelson MA, Duan LX, Guo J et al, X region deletion mutants associated with surface antigen-positive hepatitis B virus infections, *Gastroenterology* 1995; Jun; 108: 1810-1819.
- Frank Tacke, Christina Gehrke, Tom Luedde, Albert Heim, Michael P. Manns, and Christian Trautwein . Basal Core Promoter and Precore Mutations in the Hepatitis B Virus Genome Enhance Replication Efficacy of Lamivudine-Resistant Mutants. *J Virol* 2004; August; 78(16): 8524–8535.
- Ganem D, Varmus HE. The molecular biology of the hepatitis B viruses. *Annu Rev Biochem* 1987; 56: 651–693.
- Ganem D et al. The X files—One step closer to closure. *Science* 2001; Dec 14; 294: 2299–2300.

References

- Garcia AD, Ostapchuk P and Hearing P. Functional interaction of nuclear factors EF-C, HNF-4, and RXRa with hepatitis B virus enhancer I. *J Virol* 1993; 67: 3940–3950.
- Ganem D and Schneider R.. Hepadnaviridae: the viruses and their replication. In D. Knipe and P. Howley (ed.), *Fields virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa. 2001; p2923–2970.
- Ganem, D., and H. E. Varmus. The molecular biology of the hepatitis B viruses. *Annu Rev Biochem* 1987; 56: 651-693.
- Genie B, Kim KH, Guarnieri M et al. Effect of mutating the two cysteines required for HBc antigenicity on hepatitis B virus DNA replication and virion secretion. *Virology* 2005; 332: 216-224.
- Gerelsaikhan T, Tavis J and Bruss V. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. *J Virol* 1996; 70: 4269–4274.
- Guidotti LG, Matzke B, Schaller H and Chisari FV. High-level hepatitis B virus replication in transgenic mice. *J Virol* 1995; 69 (10): 6158-6169.
- Guo WM, Chen TS, Yen B and Ou JH. Hepatocyte-specific expression of the hepatitis B virus core promoter depends on both positive and negative regulation. *Mol Cell Biol* 1993; 13: 443–448.
- Gunther SL, Fischer I, Sterneck PM and Will H. Naturally occurring variants of hepatitis B virus. *Adv Virus Res* 1999; 25-137.
- Gunther S, Fischer L, Irmgard P et al. Naturally occurring variants of Hepatitis B virus. *Adv Virus Res*. 1997; 52: 25-50.
- Gunther S, Nils P, Alicja I et al. Type, prevalence, and significance of core promoter/enhancerII mutations in hepatitis B viruses from immunosuppressed patients with severe liver disease. *J Virol* 1996; Dec: 8318-8331.

References

- Hasegawa K, Huang J, Rogers SA, Blum HE and Liang TJ. Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *J Virol* 1994; 68: 1651–1659.
- Hollinger FB, Liang TJ, Knipe DM et al. Hepatitis B Virus. *Fields Virology* 4th ed. Philadelphia, Lippincott Williams & Wilkins, 2001; 2971–3036.
- Honda AO, Yokosuka T, Ehata M, Tagawa F, Imazeki and Saisho H. 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* 1999; 57: 337–344.
- Honigwachs J, Faktor O, Dikstein R, Shaul Y, Laub O. Liver-specific expression of hepatitis B virus is determined by the combined action of the core gene promoter and the enhancer. *J Virol* 1989; 63: 919–924.
- Hoofnagle JH, DiBisceglie AM et al. The treatment of chronic viral hepatitis. *N Engl J Med* 1997; 336: 347–356.
- Hong T, Naoki O, Shuichi K, Seishi M. Molecular functions and biological roles of hepatitis B virus x protein. *Cancer Sci* 2006; Oct; 97: 977–983.
- Horikita M, Itoh S, Yamamoto K, Shibayama T, Tsuda F, and Okamoto H.. Differences in the entire nucleotide sequence between hepatitis B virus genomes from carriers positive for antibody to hepatitis B e antigen with and without active disease. *J Med Virol* 1994; 44: 96–103.
- Huan B, and Siddiqui A. Retinoid X receptor RXR α binds to and trans-activates the hepatitis B virus enhancer. *Proc Natl Acad Sci USA* 1992; Oct 89: 9059–9063.

References

- Huan B, Kosovsky MJ and Siddiqui A. Retinoid X receptor a transactivates the hepatitis B virus enhancer 1 element by forming a heterodimeric complex with the peroxisome proliferator-activated receptor. *J Virol* 1995; 69: 547–551.
- Ikeda K, Watanabe Y, Ohto H and Kawakami K. Molecular interaction and synergistic activation of a promoter by Six, Eya, and Dach proteins mediated through CREB binding protein. *J Mol Cell Biol* 2002; 22: 6759-6766.
- Ishida H, Ueda K, Ohkawa K, Kanazawa Y et al. Identification of multiple transcription factors, HLF, FTF, and E4BP4, controlling hepatitis B virus enhancer II. *J virol* 2000; 74: 1241-1251.
- Janssen HL, Kerhof-Los CJ, Heijtkink RA and Schalm SW. Measurement of HBsAg to monitor hepatitis B viral replication in patients on alpha-interferon therapy, *Antiviral Res* 1994; 23: 251-257.
- Johnson JL, Raney AK, McLachlan A. Characterization of a functional hepatocyte nuclear factor 3 binding site in the hepatitis B virus nucleocapsid promoter. *Virology* 1995; 208: 147–158.
- Junker M, Galle P, Schaller H. Expression and replication of the hepatitis B virus genome under foreign promoter control. *Nucl Acids Res* 1987; 15: 10117–10132.
- Kakar SS, Jennes L, Zhang X, Horwitz GA, Prezant TR, Valentini A, Nakashima M, Melmed S. Structure, expression, and function of human pituitary tumor-transforming gene (PTTG). *Mol Endocrinol* 1999; 13: 156-166.
- Kanai K, Kako M, Aikawa T et al. Core promoter mutations of hepatitis B virus for the response to interferon in e-antigen-positive chronic hepatitis B. *Am J Gastroenterol* 1996; 91: 2150-2156.

References

- Kaneko M, Uchida T, Moriyama M, Arakawa Y, Shikata T, Gotoh K., and Mima S. Probable implication of mutations of the X open reading frame in the onset of fulminant hepatitis B. *J Med Virol* 1995; 47: 204–208.
- Kidd LK, Öberg M, Kidd AH. Hepatitis B virus X gene 1751–1764 mutations: implications for HBeAg status and disease. *J Gen Virol* 1997; 78: 1469–1478.
- Kramvis A and Kew MC, The core promoter of hepatitis B virus. *J Viral Hepat.* 1999 Nov; 6(6): 415-427.
- Kramvis A, Kew MC, Bukofzer S. Hepatitis B virus precore mutants in serum and liver of Southern Africa Blacks with hepatocellular carcinoma. *J Hepatol* 1998; 28: 132-141.
- Kurosaki M, Enomoto N, Asahina Y, Sakuma I, Ikeda T, Tozuka S. et al. Mutations in the core promoter region of hepatitis B virus in patients with chronic hepatitis B. *J Med Virol* 1996; 49: 115-123.
- Li J, Victor E, Buckwold et al. Mechanism of suppression of Hepatitis B virus precore RNA transcription by a frequent double mutation. *J Virol* 1999; Feb: 1239-1244.
- Li M, Xie Y, Wu X, Kong Y, Wang Y. HNF3 binds and activates the second enhancer, ENII, of hepatitis B virus. *Virology* 1995; 214: 371–378.
- Li M, Xie YH et al. Cloning and characterization of a novel human hepatocyte transcription factor, hB1F, which binds and activates enhancer II of hepatitis B virus *J Biol Chem* 1998; 273(44): 29022–29031.
- Lien, J.M. et al., Evidence that a capped oligoribonucleotide is the primer for duck hepatitis B plus-strand DNA synthesis. *J Virol* 1986; 57: 229-236.
- Loeb, D.D. et al., Sequence-independent RNA cleavages generate the primers for plus strand DNA synthesis in hepatitis B viruses: implications for other reverse transcribing elements. *EMBO J* 1991; 10: 3533-3540.

References

- Li J, Ou JH. Differential regulation of hepatitis B virus gene expression by the Sp1 transcription factor. *J Virol* 2001; Sep;75(18): 8400-8406.
- Li M, Xie YH, Kong YY, Wu X, Zhu L, Wang Y. Cloning and characterization of a novel human hepatocyte transcription factor, hB1F, which binds and activates enhancer II of hepatitis B virus. *J Biol Chem* 1998; 273: 29022-29031.
- Liu MC, Yu M, Zhang NL, Gong WB, Wang Y, Piao WH, Wang QH and Wang GQ. Dynamic analysis of hepatitis B virus DNA and its antigens in 2.2.15 cells. *J Viral Hepat* 2004; 11: 124-129.
- Locarnini S et al. Molecular virology of hepatitis B virus. *Semin Liver Dis* 2004; 4: 3-10.
- López-Cabrera M, Letovsky J, Hu KQ, Siddiqui A. Transcriptional factor C/EBP binds to and transactivates the enhancer element II of the hepatitis B virus. *Virology* 1991; 183: 825-829.
- Lopez-Cabrera M. Letovsky J. Hu KQ, Siddiqui A. Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: trans-activation and repression by CCAAT/enhancer binding protein. *Proc Natl Acad Sci USA* 1990; 87: 5069-5073.
- Lo WY, Ting LP. Repression of enhancer II activity by a negative regulatory element in the hepatitis B virus genome. *J Virol* 1994; 68: 1758-1764.
- Mack, D.H., Bloch, W., Nath, N. and Sninsky, J.J. Hepatitis B Virus Particles Contain a Polypeptide Encoded by the Largest Open Reading Frame: A Putative Reverse Transcriptase. *J Virol* 1988; 62: 4786-4790.
- Mahoney FJ, Kane M. Hepatitis B vaccine. In: Plotkin SA, Orenstein WA (Eds). *Vaccines*. 3rd Edition. W.B. Saunders, Philadelphia, 1999.
- Mangelsdorf DJ and Evans RM. The RXR heterodimers and orphan receptors. *Cell* 1995; 83: 841-850.

References

- Moriyama K et al. Reduced antigen production by hepatitis B virus harbouring nucleotide deletions in the overlapping X gene and precore-core promoter. *J Gen Virol* 1997; 78: 1479–1486.
- Mehdi, H., Kaplan, M.J., Anlar, F.Y., Yang, X., Bayer, R., Sutherland, K. and Peeples, M.E. Hepatitis B Virus Surface Antigen Binds to Apolipoprotein H. *J Virol* 1994; 68: 2415-2424.
- Miller, R.H. et al., Hepatitis B DNA-RNA hybrid molecules in particles from infected liver are converted to viral DNA molecules during an endogenous DNA polymerase reaction. *Virology* 1984; 139: 64-72.
- Miyakawa Y, Mayumi M. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen, *J Virol* 1994; 68: 8102–8110.
- Nassal M, Hepatitis B virus replication: Novel roles for virus-host interactions. *Intervirology* 1998; 42: 100–116.
- Ng LP, Chan M, Chan SH, Cheng PCP, Leung HC, Chen WN, Ren EC. Host Heterogeneous ribonucleoprotein K (hnRNP K) as a Potential Target to Suppress Hepatitis B Virus Replication. *PLoS Med* 2005; Jul; 2(7): 673-683.
- Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Joseph A Martens and Fred. Recent advances in understanding chromatin remodeling by Swi/Snf complexes. *Curr Opin Genet* 2003; 13: 136–142.
- 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* 1991; 324: 1699-1704.
- Oon CJ, Chen WN, Goh KT, Mesenas S, Ng HS, Chiang G, Tan C, Koh S, Teng SW, I. Toh., Moh MC, Goo KS, Tan K, Leong AL and Tan GS. Molecular characterization of hepatitis B

References

- 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* 2002; 17: S491-496.
- Ou JH. Molecular biology of hepatitis B virus e antigen. *J Gastroenterol Hepatol* 1997; 12: S178-S187.
- Paran Nir, Assaf Ori, Izhak Haviv, and Yosef Shaul. A composite polyadenylation signal with TATA Box Function. *Mol Cell Biol* 2000; Feb: 834-841.
- Parekh S, Zoulim F, Ahn SH, Tsai A, Li J, Kawai S, Nasser K, Christian T, Jack W, and Tong S. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. *J Virol* 2003; June: 6601-6612.
- Persing, D. et al.. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. *Science* 1986; 234: 1388-1392.
- Peterson CL, and Cote J. Cellular machineries for chromosomal DNA repair. *Genes Dev* 2004; 18: 602-616.
- Prezant TR, Kadioglu P, Melmed S. An intronless homolog of human proto-oncogene hPTTG is expressed in pituitary tumours: evidence for hPTTG family. *J Clin Endocrinol Metab* 1999; Mar; 84(3): 1149-1152.
- Raney AK, Johnson JL, Palmer CNA, McLachlan A. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. *J Virol* 1997; 71: 1058-1071.
- Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, Tsuda F, Tanaka T, Okamoto H, Miyakawa Y, and Mayumi M. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. *Ann Intern Med* 1995; 122: 241-248.

References

- Scaglioni PP, Melegari M. and. Wands JR. Biologic properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame. *Virology* 1997; 233: 374-381.
- Seeger C, Mason W. Hepatitis B virus biology. *Microbiol Mol Biol Rev.* 2000; 64: 51–68.
- Shih, C. Functional significance of naturally occurring hepatitis B virus variants, In S. Locarnini and C. L. Lai (ed.), *Human virus guides—human hepatitis B viruses*. International Medical Press, London, United Kingdom. 2003; 23-41.
- Standring, D.N., Assembly of viral particles in *Xenopus* oocytes: pre-surface-antigens regulate secretion of the hepatitis B viral surface envelope particle. *Proc Natl Acad Sci USA* 1986; 83: 9338-9342.
- Summers J and Mason W. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* 1982; 29: 403– 415.
- Staprans, S. et al., 1991. Mutations affecting hepadnavirus plus-strand DNA synthesis dissociate primer cleavage from translocation and reveal the origin of linear viral DNA. *J Virol* 50: 563-571.
- Schek, N., Bartenschlager, R., Kuhn, C. and Schaller, H. Phosphorylation and Rapid Turnover of Hepatitis B Virus X-Protein Expressed in HepG2 Cells from a Recombinant Vaccinia Virus. *Oncogene* 1991; 6: 1735-1744.
- Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, Yoshizawa H, and Mishiro S, The precore/core promoter mutant (T1762 A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J Gen Virol* 1995; 76: 3159–3164.
- Tang H, Banks KE, Anderson AL, McLachlan A. Hepatitis B virus transcription and replication. *Drug News Perspect* 2001; 14: 325–334.

References

- Tang H, Anneke k, and Raney et al. Replication of the wild type and a natural hepatitis B virus nucleocapsid promoter variant is differentially regulated by nuclear hormone receptors in cell culture. *J Virol* 2001; Oct: 8937-8948.
- Tavis, J.E. et al. Expression of functional hepatitis B virus polymerase in yeast reveals it to be the sole viral protein required for correct initiation of reverse transcription. *Proc Natl Acad Sci USA* 1993; 90: 4107-4111.
- Tavis, J.E. et al. Hepadnaviral reverse transcription initiates within the RNA stem-loop of the viral encapsidation signal and employs a novel strand transfer. *J Virol* 1994; 68: 3536-3543.
- Teresa Pollicino, Laura Belloni, Giuseppina Raffa, Natalia Pediconi, Giovannin Squadrito, Giovanni Raimondo, Massimo Levrero. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-Bound H3 and H4 histones. *Gastroenterology* 2006; 130: 823-837.
- Tfelt-Hansen J, Kanuparthi D, Chattopadhyay N. The emerging role of pituitary tumor transforming gene in tumorigenesis. *Clin Med Res* 4, (2): 130-137.
- Tsai MJ and O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor super family members. *Annu Rev Biochem* 1994; 63: 451-486.
- Toh, H., Hayashida, H. and Miyata, T.. Sequency Homology Between Retroviral Reverse Transcriptase and Putative Polymerases of Hepatitis B Virus and Cauliflower Mosaic Virus. *Nature* 1983; 305: 827-829.
- Uchida T, Gotoh K, Shikata T. Complete nucleotide sequences and the characteristics of two hepatitis B virus mutations causing serologically negative acute or chronic hepatic B. *J Med Virol* 1995; 45: 247-252.

References

- Victor E, Buckwold, Xu ZC, Chen M, Benedicat TS et al. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol* 1996; 70: 5845–5851.
- Wang S, Zhang B, Faller DV. Prohibitin requires Brg-1 and Brm for the repression of E2F and cell growth. *EMBO J* 2002; 21: 3019-302.
- Wang W, Chi T, Yutong et al. Architectural DNA binding by a high-mobility-group/kinesin-like subunit in mammalian SWI/SNF-related complexes (BRG1-associated factory chromatin transcription) *Biochemistry* 1998; 95: 492–498.
- Wang WX, Li M, Wang Y, Li ZP. HNF1 is critical for the liver-specific function of HBV enhancer II. *Res Virol* 1998; 149: 99–108.
- Wang W, Chi T, Xue Y, Zhou S, Kuo A, Crabtree GR. Architectural DNA binding by a high-mobility-group/kinesin-like subunit in mammalian SWI/SNF-related complexes. *Proc Natl Acad Sci U S A* 1998; 95(2): 492-498.
- Wang Y, Tao Q. Function of direct repeat sequences in DNA replication of hepatitis B virus. *Zhonghua Yi Xue Za Zhi* 1990; 70(11): 604-742.
- Wang, G-H et al, The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. *Cell* 1992; 71: 663-670.
- Wang, G-H, et al. Novel mechanism for reverse transcription in hepatitis B viruses. *J Virol* 1993; 67: 6507-6512.
- Wang, G-H et al., The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. *Cell* 1992; 71: 663-670.
- Wang Li, Rober A. Baiocchi, Sharmistha Pal, George Mosialos, Michael Caligiuri, Said Sif. The BRG1- and Hbrm-associated factor BAF57 induces apoptosis by stimulating expression of the cylindromatosis tumor suppressor gene. *Mol Cell Biol* 2005; 25(18): 7953-7965

References

- Wei Y, Tavis J and Ganem D. Relationship between viral DNA synthesis and virion envelopment in hepatitis B viruses. *J Virol* 1996; 70: 6455– 6458.
- Yee JK et al. A liver-specific enhancer in the core promoter region of human hepatitis B. *Science* 1989; 24: 658–661.
- Yen TSB et al. Regulation of hepatitis B virus gene expression. *Semin Virol* 1993; 4: 33–42
- Yuh CH, Chang YL, Ting LP. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. *J Virol* 1992; 66: 4073–4084.
- Yuh CH, Ting LP et al. C/EBP-like proteins binding to the functional box-alpha, and beta of the second enhancer of hepatitis B virus. *Mol Cell Biol* 1991; 11: 5044–5052.
- Yuh CH, Ting LP et al. Differentiated liver cell specificity of the second enhancer of hepatitis B virus. *J Virol* 1993; 67: 142–149.
- Yu X, Mertz JE. Differential regulation of the pre-C and pregenomic promoters of human hepatitis B virus by members of the nuclear receptor superfamily. *J Virol* 1997; 71: 9366–9374.
- Yu X, Mertz JE. Promoters for synthesis of the pre-C and pregenomic mRNAs of human hepatitis B virus are genetically distinct and differentially regulated. *J Virol* 1996; 70: 8719–8726.
- Yuan, T, G. Sahu, W. Whitehead, R. Greenberg, and C. Shih. The mechanism of an immature secretion phenotype of a highly frequent naturally occurring missance mutation at codon 97 of human hepatitis B virus core antigen. *J Virol* 1999; 73: 5731–5740.
- Zhang, P. and McLachlan A. Differentiation-specific transcriptional regulation of the hepatitis B virus nucleocapsid gene in human hepatoma cell lines. *Virology* 1994; 202: 430–440.
- Zhang P, Raney AK, McLachlan A. Characterization of functional Sp1 transcription factor binding sites in the hepatitis B virus nucleocapsid promoter. *J Virol* 1993; 67: 1472–1481.

References

Zheng YY, Li J and Ou JH. Regulation of Hepatitis B virus core promoter by transcription factor HNF1 and HNF4 and the viral X protein. *J Virol* 2004; 6908-6914.

Zuckerman AJ et al. Progress towards the comprehensive control of hepatitis B in Africa: a view from South Africa. *Gut* 1996; 38 (suppl): S31-6

Zuckerman AJ. Effect of hepatitis B virus mutants on efficacy of vaccination. *Lancet* 2000; 355: 1382-1384

Appendix A

Table 1 Mutation pattern of patients showing two deletion mutations

Patient	Age (years)	Sex	HBsAg ($\mu\text{g mL}^{-1}$)	HBVDNA (pg mL^{-1})	HBe/Ag/anti-HBe	PIIIP (U mL^{-1})	ALT (IUL^{-1})	PreCore (residue 28)	1762-1764 (core promoter)
<i>Patients with chronic HBV infection</i>									
CHBV01	27	F	100.0	58.2	+/-	0.72	nd	W	A.G
CHBV02	36	M	6.5	31.0	-/+	1.48	285	W/stop	A/T.G/A
CHBV03	43	M	130.0	29.6	+/-	1.32	1191	W/stop	A.G
CHBV04	51	M	2.5	3.2	-/+	0.48	46	Stop	A.G
CHBV05	38	M	109.0	49.6	+/-	0.56	48	W/stop	A.G
CHBV06	33	M	140.0	212.0	+/-	0.76	96	W	Deletion (1758-1777)
CHBV07	53	F	320.0	139.5	+/-	0.52	65	W	A.G
CHBV08	46	M	6.3	8.4	-/+	0.96	510	Stop	A.G
CHBV09	26	F	110.0	53.3	+/-	0.74	24	W	A.G
CHBV10	62	M	110.0	92.7	+/-	0.49	65	W	A.G
CHBV11	18	M	71.0	38.3	+/-	0.74	24	W	A.G
CHBV12	30	F	140.0	60.6	+/-	0.3	45	W	A.G
CHBV13	30	F	79.5	95.2	+/-	0.56	57	W	A.G
CHBV14	46	M	7.5	5.3	+/-	0.76	71	W	T.A
CHBV15	26	F	100.0	74.7	+/-	0.54	19	W	A.G
CHBV16	37	F	25.5	39.7	+/-	0.66	91	W	A.G
CHBV17	45	M	147.5	280.4	+/-	0.40	26	W	Deletion (1749-1768)
<i>Patients with cirrhotic liver</i>									
CIRR01	46	F	2.1	25.0	+/-	1.58	97	W	T.A
CIRR02	45	M	14.0	13.4	+/-	1.38	95	W	T.A
CIRR03	59	M	6.4	26.0	-/+	1.04	373	Stop	T.A
CIRR04	50	M	5.3	3.7	-/+	1.58	75	W/stop	T.A
CIRR05	46	M	2.2	3.8	-/+	1.46	129	W/stop	T.A
CIRR06	44	M	0.49	7.6	-/+	1.16	1546	Stop	A.G
CIRR07	40	M	2.8	5.1	+/-	1.12	426	W	T.A
CIRR08	47	M	3.5	6.3	-/+	1.44	73	Stop	A.G
CIRR09	38	M	55.0	192.1	+/-	1.15	718	W	Deletion (1758-1777)
CIRR10	75	F	4.0	3.2	-/+	1.53	16	Stop	T.A
<i>Patients with hepatocellular carcinoma</i>									
HCC01	51	M	23.0	1.6	-/+	0.95	84	Stop	T.A
HCC02	66	M	2.6	2.7	-/+	1.58	97	Stop	A.G
HCC03	72	M	2.4	13.8	-/+	1.08	41	Stop	T.A
HCC04	44	M	4.6	36.8	+/-	1.22	375	W	T.A
HCC05	64	F	8.1	11.6	-/+	1.08	196	Stop	A.G
HCC06	56	M	12.0	5.5	+/-	1.13	68	W	T.A

Appendix B: HBV Genome — wild type ayw

HBV genome (our lab's)

1 t tccactgcct tccaccaaac tctgcaggat
 32 cccagagtca ggggtctgta tcttctgct ggtggtcca gttcaggaac agtaaaccct
 92 gctccgaata ttgccttca catctgtca atctccgca ggactgggga ccctgtgacg
 152 aacatggaga acatcacatc aggattccta ggaccctgc tegtgttaca ggcgggggtt
 212 ttctgttga caagaatcct cacaataccg cagagtctag actcgtggtg gacttctctc
 272 aattttctag ggggatctcc cgtgtgtctt ggccaaaatt cgcagtcctc aacctccaat
 332 cactcaccaa cctctgtcc tccaattgt cctggttacc gctggatgtg tctgcggcgt
 392 ttatcatat tctcttcat cctgtgcta tgcctcatct tcttattggt tcttctggat
 452 tatcaaggta tgttgccctt ttgtcctcta attccaggat caacaacaac cagtacggga
 512 ccatgcaaaa cctgcacgac tctgtctcaa ggcaactcta tgtttccctc atgttctgt
 572 acaaaacctt cggatggaaa ttgcacctgt attcccatcc catctctctg ggctttcgca
 632 aaatacctat gggagtgggc ctcatctgt ttctcttggc tcagtttact agtgccattt
 692 gttcagtgtg tcttagggct tccccact gtttggttt cagctatatg gatgatgtgg
 752 tattgggggc caagtctgta cagcatctg agtcccttta taccgtgtt accaattttc
 812 tttgtctct gggatatacat taaacctt acaaaacaaa aagatggggt tattccctaa
 872 acttcatggg ctacataatt ggaagttggg gaactttgcc acaggatcat attgtacaaa
 932 agatcaaaca ctgttttaa aaacttctg ttaacaggcc tattgattgg aaagtatgtc
 992 aaaaaattgt gggctttttg ggctttgtg ctccatttac acaatgggga tctctgcct
 1052 taatgccttt gtatgatgt atacaagcta aacaggcttt cactttctcg ccaactfaca
 1112 aggcctttct aagaaaacag cacatgaacc ttccccctg tgcctggaaa cggcctggtc
 1172 tgtgccaagt gtttctgac gcaaccccca ctggtgggg cttggccata ggccatcagc
 1232 gcattcgtgg aacctttgtg gctcctctgc cgatccatac tgcggaactc ctacccgtt

Appendix

1292 gttttgctcg cagccggtct ggagcaaagc tcatcggaac tgacaattct gtcgtcctct
1352 cgcggaaata tacatcgttt ccatggctgc taggctgtac tgccaactgg atccttcgcg
1412 ggacgtcett tgtttacgtc ccgtcggcgc tgaatcccgc ggacgacccc tcgcggggcc
1472 gcttgggact ctctcgtccc cttctcgtc tgcggtcca gccgaccacg gggcgcacct
1532 ctctttaegc ggtcctccc tctgtgcctt ctcatctgcc ggtcctgtg cacttcgctt
1592 cacctctgca cgttgcattg agaccaccgt gaacgcccac cagatcctgc ccaaggtctt
1652 acataagagg actcttggac tcccagcaat gtcaacgacc gaccttgagg cctactcaa
1712 agactgtgtg ttaaggact gggaggagct gggggaggag attaggttaa aggtctttgt
1772 attaggagcg ttaggcata aattggtctg cgcaccagca c
1813 catgcaactt ttacacctct gcctaactat ctctgtaca tgcccactg ttcaagctc
1873 caagctgtgc cttgggtggc ttggggcat ggacattgac cttataaag aatttgagc
1933 tactgtggag ttactctctg tttgccttc tgactcttt ccttcctga gagatctct
1993 agacaccgcc tcagctctgt atcgagaagc cttagagtct cctgagcatt gtcacctca
2053 ccatactgca ctacggcaag ccattctctg ctggggggaa ttgatgactc tagctacctg
2113 ggtgggtaat aatttggaag atccagcatc cagggatcta gtagtcaatt atgttaatac
2173 taacatgggt taaagatca ggcaactatt gtggttcat atatcttgc ttacttttg
2233 aagagagact gtacttgaat atttggctc ttcggagtg tggattcgca ctctccagc
2293 ctatagacca ccaaagccc ctatctatc aacacttcg gaaactactg ttgttagcg
2353 acgggaccga ggcaggtccc ctagaagaag aactccctc cctcgcagac gcagatctca
2413 atgccgcgt cgcagaagat ctcaatctc ggaatctca tggtagtatt ccttgactc
2473 ataaggtggg aaactttacg gggctttatt cctctacagt acctatctt aatcctgaat
2533 ggcaaactcc ttctttctt aagattcatt tacaagagga cattattaat aggtgtcaac
2593 aatttgggg cctctcact gtaaatgaaa agagaagatt gaaattaatt atgcctgcta
2653 gattctatcc taccacact aaatattgc ccttagacaa aggaattaaa ccttattatc

2713 cagatcaggt agttaatcat tacttccaaa ccagacatta ttacatact ctttgaagg
2773 ctggtattct atataagagg gaaaccacac gtagcgcac atttgcggg tcaccatatt
2833 cttgggaaca agagctacag catgggaggt tggatcaaa aacctcgcaa aggcatgggg
2893 acgaatcttt ctgttccaa ccctctggga ttcttcccg atcatcagtt ggaccctgca
2953 ttgggagcca actcaaaaa tccagattgg gacttcaacc ccatcaagga ccactggcca
3013 gcagccaacc aggtaggagt gggagcattc gggccagggc tcaccctcc acacggcgt
3073 atttggggt ggagccctca ggctcagggc atatggacca cagtgtcaac aattctcct
3133 cctgctcca ccaatcggca gtcaggaagg cagctactc ccatctctcc acctctaaga
3193 gacagtcac ctcaggccat gcagtggaa

Appendix C: Vector Map

Fig. 1 pcDNA3.1+ vector map

pcDNA3.1 Vectors

Map of
pcDNA3.1(+) and
pcDNA3.1(-)

The figure below summarizes the features of the pcDNA3.1(+) and pcDNA3.1(-) vectors. The complete sequences for pcDNA3.1(+) and pcDNA3.1(-) are available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 13). Details of the multiple cloning sites are shown on page 3 for pcDNA3.1(+) and page 4 for pcDNA3.1(-).

Fig. 2 pBIND vector map

pBIND Vector multiple cloning region. Bases listed in triplets correspond to codons in the open reading frame of the GAL4 fusion protein. Stop codons (underlined) in three different reading frames are located downstream of the multiple cloning region and before the T3 promoter sequences.

pBIND Vector circle map and sequence reference points.

pBIND Vector sequence reference points:

CMV immediate-early enhancer	1-659
CMV immediate-early promoter	669-750
chimeric intron	890-1022
T7 RNA polymerase promoter	1067-1085
GAL4 fusion protein	1116-1556
multiple cloning region	1570-1619
T3 RNA polymerase promoter	1639-1657
SV40 late polyadenylation signal	1666-1887
phage f1 origin of replication	1982-2437
SV40 early enhancer/promoter	2502-2872
SV40 minimum origin of replication	2768-2833
hGH intron for <i>Renilla</i> gene	2924-3183
<i>Renilla</i> luciferase gene coding region	3208-4143
synthetic polyadenylation signal	4201-4249
β -lactamase (<i>Amp^r</i>) coding region	4668-5528

Fig. 3 pGL3-Control vector map

pGL3-Control Vector circle map. Additional description: *luc+*, cDNA encoding the modified firefly luciferase; *Ampr*, gene conferring ampicillin resistance in *E. coli*; *f1 ori*, origin of replication derived from filamentous phage; *ori*, origin of replication in *E. coli*. Arrows within *luc+* and the *Ampr* gene indicate the direction of transcription; the arrow in *f1 ori* indicates the direction of ssDNA strand synthesis.

pGL3-Control Vector Sequence Reference Points:

Multiple cloning region	1-41
SV40 Promoter	48-250
Luciferase gene (<i>luc+</i>)	280-1932
GLprimer2 binding site	281-303
SV40 late poly(A) signal	1964-2185
SV40 Enhancer	2205-2441
RVprimer4 binding site	2499-2518
ColE 1-derived plasmid replication origin	2756
β -lactamase gene (<i>Amp^r</i>)	3518-4378
f1 origin	4510-4965
Synthetic poly(A) signal	5096-5249
RVprimer3 binding site	5198-5217

Fig. 4 pGL3-promoter vector map

The pGL3-Promoter Vector circle map. Additional description: *luc+*, cDNA encoding the modified firefly luciferase; *Amp^r*, gene conferring ampicillin resistance in *E. coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in *E. coli*. Arrows within *luc+* and the *Amp^r* gene indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis.

pGL3-Promoter Vector Sequence Reference Points:

Multiple cloning region	1–41
SV40 Promoter	48–250
Luciferase gene (<i>luc+</i>)	280–1932
GLprimer2 binding site	281–303
SV40 Enhancer	(none)
SV40 late poly(A) signal	1964–2185
RVprimer4 binding site	2253–2272
ColE 1-derived plasmid replication origin	2510
β -lactamase gene (<i>Amp^r</i>)	3272–4132
f1 origin	4264–4719
Synthetic poly(A) signal	4850–5003
RVprimer3 binding site	4952–4971

Appendix D: Reagents and solutions

General reagents

General reagents and sovents were obtained from Amersham Pharmacia Biotech, Amersham International plc, BD clontech, BDH Chemicals Ltd. Bio-Rad Laboratories, Cole-Parmer, Fluka Chemie Gmbh, FMC Bioproducts Gibco-BRL Ltd, ICN Biomedicals, Invitrogen Corporation, Merck KGaA, PIERCE, Promega Corporation, and Sigma-Aldrich Chemie Gmbh. Other reagents and their sources are listed below. Solutions were sterilized when required by autoclaving at 120°C for 20 minutes or by passing through a 0.22µm filter (Millipore, Sartorius)

Enzymes

Restriction endonucleases, Taq DNA polymerase, Pfu DNA polymerase, T4 DNA ligase, Proteinase and other enzymes were purchased from Amersham International Plc., Amersham Pharmacia Biotech, Bethesda Research Laboratories, New England Biolabs Inc., and Promega Corporation, unless otherwise stated, and were used according to the supplier’s recommendations.

Commercially available kits

QIAprep Spin Miniprep Kit	QIAGEN Ltd.
QIAprep Spin Midiprep Kit	QIAGEN Ltd.
QIAquick Gel extraction Kit	QIAGEN Ltd.
QIAquick PCR purification Kit	QIAGEN Ltd.
RNeasy Mini Kit	QIAGEN Ltd.
Qiagen Effectene transfection Kit	QIAGEN Ltd

Appendix

QIAamp DNA Blood Mini Kit	QIAGEN Ltd.
Oligotex mRNA Mini Kit (12)	QIAGEN Ltd.
QIAEX II Gel Extraction Kit (150)	QIAGEN Ltd.
Sybr Green Real Time PCR Kit	Bio-Rad Corp.
The LightShift® Chemiluminescent EMSA Kit	PIERCE
ECLTM Western Blotting Analysis System	Amersham Pharmacia Biotech
Titanium One-Step RT-PCR Kit	BD-Bioscience Clontech
Bright-Glo Luciferase Assay System	Promega
NE-PER Extraction,	Pierce
Transignal TF Protein Array Kit	Panomics
Stop-and-Glo Reagent	Promega
B-per Bacterial Protein Extraction Reagent	PIERCE

DNA Vectors

pcDNA3.1(+)	Invitrogen Corp.
pbind	Promega Corp
pGL3 –control	Promega Corp
pGL3-promoter	Promega Corp
pxj-40	From Dr. Cheng Gee Koh (SBS, NTU)

cell lines

<i>E.coli</i> strain DH-5 α	Stratagene
<i>E.coli</i> strain BL21	Stratagene
HepG(2) cells	From ATCC
293 T cells	From ATCC

Appendix

Chang liver cells From ATCC

Antibodies

HA-Probe Anti-HA From Santa Cruz Biotechnology

Goat Anti-Mouse From PIERCE

SOLUTIONS, BUFFERS AND MEDIA

Laboratory stocks

20mg/ml Ampicillin filtered (0.22 μ M) and stored at -20°C

10m/ml BSA Stored at -20°C

0.1M DTT Stored at -20°C

1M DTT Stored at -20°C

0.5M EDTA, pH 7.5 Stored at RT

0.1M EDTA, pH 7.5 Stored at RT

0.1% ethidiom bromide Stored at 4°C

glycerol loading dyes 30% glycerol, 5mM EDTA pH 7.5, 0.1%
bromophenol blue

1M HEPES, Ph 7.4 Stored at 4°C

1M MgCl₂ Stored at RT

3M NaCl Stored at RT

2× sample buffer 0.16 M Tris Ph 8.0, 8M Urea, 1.6%
SDS, 0.08% bromophenol blue

10%(w/v) SDS Stored at RT

100mM spermidine Stored at -20°C

Appendix

10×TBE	0.89 M Tris pH 8.0, 0.89M Boric acid, 25mM EDTA
TE buffer	10mM Tris pH 8.0, 0.1Mm EDTA
1M Tris-HCl, pH 8.0	Stored at RT
1.5M Tris-HCl, pH 8.8	Stored at RT
1M Tris-HCl, pH 6.8.	Stored at RT
Iso-propanol	Stored at RT
1×PBS	Stored at RT
Commassie Blue Staining Buffer	50% methanol, 10% glacial acid 40% H ₂ O, 0.25g Brilliant Blue in 1 Liter, Stored at RT
Commassie Blue Destaining Buffer	30% methanol, 10% glacial acid 60% H ₂ O, Stored at RT

Media for Bacterial Culture

LB medium	1%(w/v) Bacto-tryptone (BD), 0.5% (w/v) Yeast (BD), 1%(w/v) NaCl (Merck)
LB agar	LB medium plus 1.5% (w/v) Bacto-agar(BD)
LB plates(amp ^r)	LB agar plates of 100µg/ml ampicillin
LB plates(kana ^r)	LB agar plates of 50µg/ml kanamycin

Solutions for making competent bacterial cells

Appendix

CaCl₂ 1M, 4°C

MgCl₂ 1M, 4°C

Solutions for Tissue culture

Minimum Essential Medium (MEM) Gibco, Invitrogen Corp. 10%FBS
100µg/ml antibiotics/antimycotics
phosphate Buffered Saline, pH 7.2
Gibco, Invitrogen Corp.

Cell freezing mixture 10% DMSO in FBS

Trypsin-EDTA Gibco, Invitrogen Corp.(0.25%
Trypsin, EDTA Na

RPMI/HEPES RPMI 1640, FBS, HEPES 10mM,
pH 7.4

Solutions for cell lysates and protein extraction

B-per lysis buffer

100Mm PMSF Prepared fresh in ethanol

Buffers for SDS-PAGE

10× Running buffer 0.25 M Tris, 1.9M Glycine, 1%(w/v)
SDS

4×Separating gel buffer 1.5M Tris, 0.4%(w/v) SDS, pH8.8

4×Stacking gel buffer 0.5M Tris, 0.4%(w/v) SDS, pH8.8

Appendix

sample loading buffer	0.01%(w/v) bromophenol blue, 2%(w/v) SDS, 8M urea, 0.2M Tris pH 8.0
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Protein Blotting Buffers

Blotting buffer	12.5mM Tris Ph 8.0, 96mM glycine, 10%(w/v)ethanol
Blocking buffer	3% skim milk, 0.05% Tween 20 in PBS
Washing buffer	0.1%(v/v)Tween 20 in PBS

Appendix E: Published Papers

1. Identification of Cellular Transcription Factors Binding to HBV Promoter with Naturally Occurring Deletions. Hong Pan, Wei Ning Chen, paper submitted.
2. A Cell-Based System for Hepatitis B Virus Replication : Significance of Clinical Enhanced Viral Replication in Relation to Deletions in Viral Core Promoter. Hong Pan, Lisa FP Ng, Ee Chee Ren and Wei Ning Chen. Frontiers in Bioscience 10, 2001-2004, September 1, 2005.