Functional Characterization of Novel HBV Subgenotypes/Mutations Associated with Increased Risk for Hepatocellular Carcinoma (HCC)

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Chronic infection of hepatitis B virus (HBV) increases the risk of hepatocellular carcinoma (HCC) by more than 100-fold. However, the underlying molecular mechanism of this process is not fully understood. Several recent studies have shown that A1762T and G1764A mutations of HBV were associated with the aggressiveness of liver disease, in which inactive carriers would develop active hepatitis, and eventually liver cirrhosis and HCC. In Asia, genotypes B and C are the predominant genotypes of HBV infections. Our longitudinal five-year follow-up study of 426 chronic hepatitis B patients in Hong Kong found that the genotype C HBV (normally with A1762T/G1764A mutations) was closely associated with higher risk of HCC than genotype B HBV (non-frequent mutations with A1762T/G1764A).

After alignment of 300 HBV sequences randomly downloaded from GenBank, we found that the frequency of A1762T and G1764A mutations in genotype C was found as high as 64%, while 34% was found for other genotypes (A, B, D to H). Besides, recent clinical studies have also shown that A1762T/G1764A mutations occur frequently in HCC patients with genotype B infection (81%, 30 of 37 patients), but were relatively lower in asymptomatic carriers (43%, 22 of 51 patients). These indicate that the contribution of A1762T/ G1764A mutations to liver cancer might not

be limited to genotype C. As the double mutations are present within the region of HBV Enhancer II/Basal core promoter (BCP) and cause residue substitution of HBx (Lys130Met and Val131Ile); therefore, their effects on the promoter and HBx activities were examined.

In this study, systemic site-directed mutagenesis studies, promoter assays, replication capacity assays and overexpression of HBx assays were carried out to demonstrate the molecular mechanisms of these mutations for the increases risk of HCC. Three conclusions were drawn from this study. (1) A1762T and/or G1764A mutations of HBV could reduce BCP activities in a synergistic manner with 1764A contributing more. Reversed T1762A and/or A1764G mutations increase the BCP activities also in a synergistic manner with 1764G contributing more; (2) HBx could increase HBV BCP activity, HBV replication and HBsAg expression. The Lys130Met and Val131Ile mutations of HBx could further increase the above abilities while the A1762T/G1764A double mutations in the BCP region could not affect the interaction of HBx and HBV BCP; (3) The G1677T/A1679C and T1706C mutations could increase the BCP activity; The ectopic expression of HBx could further increase the BCP activity while the mutated HBx (^{130}Met and ^{131}Ile) has less effect on these mutated promoters.

摘要:

由乙型肝炎病毒（HBV）引起的慢性感染可以使肝细胞癌（HCC）发生的危险性增加超过100倍，但是其中的分子机理目前仍然不是十分清楚。有研究证实HBV A1762T/G1764A突变与肝脏疾病的进展密切相关，它可以使隐匿性携带者发展为活动性肝炎并最终导致肝硬化甚至肝癌。在亚洲，HBV感染主要以B型和C型为主。我们在香港的一项426例慢性肝炎的5年随访研究发现C型HBV（通常伴有A1762T/G1764A突变）相对于B型HBV（通常不伴有A1762T/G1764G突变）与肝细胞癌的发生相关性更高。

通过对从GenBank下载的300个HBV全序的比对，我们发现在C型HBV中A1762T/G1764A的发生率高达64%，而在其他基因型（A，B，D到H）中只有34%。此外，最近的临床研究也表明A1762T/G1764A突变在B型HBV引起的肝细胞癌中也经常发生（81%，30/37），但是在携带者中突变率较低（43%，22/51）。这表明A1762T/G1764A突变对肝细胞癌的影响并非局限于C亚型HBV。由于此双突变位于HBV增强子II/基本核心启动子（BCP）区而且会导致HBx基因发生氨基酸突变（Lys130Met和Val131Ile）；因此我们对双突变对基本核心启动子以及HBx的影响进行了研究。

本研究中我们通过系统的定点突变研究，启动子活性研究，HBV复制能力研究以及HBx过表达研究来阐明此双突变对肝细胞癌发生的分子机理。我们得到3个结论。（1）HBV A1762T和/或G1764A突变可以协同降低BCP的活性，而1764A贡献相对较大。反之，T1762A和/或A1764G突变会协同增强BCP活性，而1764G贡献较大；（2）HBx会增强HBV BCP活性，促进HBV的复制，并增加HBsAg的表达。Lys130Met和Val131Ile突变会增强HBx的上述功能。

但是BCP区的A1762T/G1764A双突变并不会影响HBx对HBV BCP的作用；（3）G1677T/A1679C 和T1706C突变会增强BCP的活性，HBx的外源性表达会增强突变BCP的活性，但是带有^{130}Met 和 ^{131}Ile突变的HBx对带有以上突变的BCP相对影响较小。

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DONG Qingming

Table of Contents **page**

List of figures

Symbols and Abbreviations used

Symbols

α	Alpha
β	Beta
γ	Gamma
κ	Kappa

Abbreviations

aa	amino acid
ADV	adefovir dipivoxil
AFP	alpha fetoprotein
ALT	alanine aminotransferase
APC	adenomatous polyposis coli
AP-1	activating protein-1
AsC	asymptomatic carrier
ATF	activating transcription factor
BCP	basal core promoter
bZip	basic region/leucine zipper
C/EBP	CAAT-enhancer binding protein
cccDNA	covalently closed circular DNA
CDK	cyclin-dependent kinase
CMV-IE	cytomegalovirus immediate early gene
COUP-TF1	chicken ovalbumin upstream promoter transcription factor 1
CPE	cytopathic effect
CRE	cAMP response element
CREB	cAMP response element-binding protein

CT	computed tomography
CURS	core upstream regulatory sequence
DDB	damaged DNA binding protein
DHBV	duck hepatitis B virus
DIP	defective interfering particle
DMEM	dulbecco's modified minimum essential medium
DR1	direct repeat 1
DR2	direct repeat 2
dsDNA	double-stranded DNA
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
Enh I	enhancer I
Enh II	enhancer II
ER	endoplasmic reticulum
EtBr	ethidium bromide
FBS	fetal bovine serum
FTF	fetoprotein transcription factor
GSHV	ground squirrel hepatitis virus
HAP	heteroaryldihydropyrimidine
HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B e antigen
HBIG	hepatitis B immune globulin
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HBx	hepatitis B virus X protein
HBXIP	hepatitis B X-interacting protein
HCC	hepatocellular carcinoma

HIF	hypoxia-inducible factor
HIV	human immunodeficiency virus
HLF	hepatic leukemia factor
HNF	hepatocyte nuclear factor
HSP	heat shock protein
hTERT	human telomerase reverse transcriptase
IL	interleukin
ITR	inverted terminal repeat
Lamivudine	(−) 2'3'-dideoxy-3'-thiacytadine (3TC)
LTR	long terminal repeat
MOI	multiplicity of infection
MRI	magnetic resonance imaging
NA	nucleotide analogue
NF-1	nuclear factor 1
NFκB	nuclear factor κ B
NRE	negative regulatory element
NRs	nuclear receptors
Oct-1	octamer binding protein 1
OD	optical density
ORF	open reading frame
PFU	plaque-forming unit
pgRNA	pregenomic RNA
PPAR	peroxisome proliferator-activated receptor
PSMA7	proteasome (prosome, macropain) subunit, alpha type, 7
PSMC1	proteasome (prosome, macropain) 26S subunit, ATPase, 1
PTH	primary tupaia hepatocyte
RARE	retinoic acid response element

RFX1	regulatory factor X gene 1
RPB5	RNA polymerase II (RNAPII) subunit 5
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP1	specificity protein 1
ssDNA	single-stranded DN
STAT	signal transducer and activator of transcription
TBP	TATA box-binding protein
TFIIB	transcription factor IIB
TFIIH	transcription factor IIH
TGF-β	transforming growth factor-β
TNFα	tumor necrosis factor α
TR2	the testicular receptor 2
TRE	transcription response element
WHO	World Health Organization
WHV	woodchuck hepatitis virus
WMHBV	woolly monkey HBV
XAP	hepatitis B virus X-associated protein
XAPC7	proteasome alpha subunit type 7
XIP	hepatitis B virus x interacting protein
XPB	xeroderma pigmentosum B
XPD	xeroderma pigmentosum group D

Chapter 1

General Introduction

Hepatitis is a general term which means inflammation of the liver. Such inflammation can be induced by different viruses such as hepatitis A, B, C, D and E. Since the development of jaundice is the only landmark of liver disease, a correct diagnosis could only be made by testing the patients' sera for the presence of specific anti-viral antigens or antibodies (Hollinger *et al.*, 2001; Mahoney *et al.*, 1999; Robinson *et al.*, 1995). Among all viral causes of human hepatitis, hepatitis B virus (HBV) bears most global importance than the others. Hepatitis B is a common cause of serious liver disease which affects millions of people worldwide. Infection occurs mostly in early childhood yet is asymptomatic. Thus, it often leads to chronic liver cirrhosis and hepatocellular carcinoma (HCC) (Hollinger *et al.*, 2001; Mahoney *et al.*, 1999; Robinson *et al.*, 1995; Ganem *et al.*, 2001).

1.1 Etiology

1.1.1 Morphology of HBV

HBV belongs to the family of hepadnaviruses (Orito *et al.*, 1989; Bollyky *et al.*, 1999; Mason *et al.*, 2005). It is a 42nm partially double stranded DNA virus (Dane *et al.*, 1970; Ganem 1991), composed of a 27nm nucleocapsid core (HBcAg) (Robinson *et al.*, 1976), surrounded by an outer lipoprotein envelope containing the surface antigen (HBsAg) (Hollinger *et al.*, 2001; Mahoney *et al.*, 1999; Robinson *et al.*, 1995; Ganem *et al.*, 2001). In addition to virions, HBV-infected cells produce two distinct subviral lipoprotein particles: 20-nm spherical

and filamentous forms of similar diameter (Ganem *et al.*, 2004). These HBsAg particles contain solely enveloped glycoproteins and host-derived lipids, and also typical outnumber virions from 1000:1 to 10,000:1 (Ganem *et al.*, 2004). An electron microscopic view and a schematic diagram of HBV and related particles are shown in Figs.1-1 and 1-2.

1.1.2 Genome and transcription of HBV

The genome of HBV is a relaxed-circular, partially duplex DNA of 3.2kb (Summers *et al.*, 1975). The compact genome encodes four overlapping open reading frames (ORF) including pre S/S, pre C/C, X and P; and 5 RNA species-3.9kb, 3.5kb, 2.4kb, 2.1kb and 0.7kb. The 5 RNA species encode for 7 viral proteins: 3 envelope proteins-large (pre S1, pre S2, and S), middle (pre S2 and S), and small (S); the nucleocapsid core protein (HBcAg); the secretory hepatitis B e antigen (HBeAg); the viral reverse transcriptase/polymerase, and the X protein (Chisari *et al.*, 1997; Mahoney *et al.*, 1999; Robinson *et al.*, 1995; Ganem *et al.*, 2001). HBV sequences are transcribed under the control of four promoters, the pre C/pregenomic, S1, S2 and X promoters (Moolla *et al.*, 2002). The presence of two enhancers, Enhancer I (EnhI) and Enhancer II (EnhII), and negative regulatory elements (NRE) in the HBV genome further augment the controlled synthesis of HBV genome (Huan *et al.*, 1993). HBV transcription control elements also display a preference for liver-specific or liver-enriched trans-factors, which contributes to the liver tropism of the virus (Moolla *et al.*, 2002; Schaller *et al.*, 1991; Shaul, 1992). The genome and transcription map of HBV is shown in Fig.1-3.

1.1.3 The HBV life cycle

The liver is composed of many different types of cells; much of the functional activity resides in hepatocytes, bile ductule epithelium, and Kupffer cells (Gartner *et al.*, 1997). The hepatocytes are the major liver cell type (which constitute 70% of the liver) and are the major target of infection by HBV which is known as a liver-tropic virus. Bile ductule epithelial cells, cells in the pancreas, kidney and lymphoid system may also be the targets of infection (Blum *et al.*, 1984; Hadchouel *et al.*, 1988; Jilbert *et al.*, 1987; Korba *et al.*, 1989; Lieberman *et al.*, 1987; Nicoll *et al.*, 1997; Shimoda *et al.*, 1981; Yoffe *et al.*, 1986). However, the evidences for replication of HBV in bile ductules and at extrahepatic sites are controversial or incomplete, thus they are not usually considered when discussing the issues of viral reproduction and pathogenesis.

The virus enters the hepatocyte via an unknown route, and the viral genome is transported to the nucleus in which a covalently closed circular DNA (cccDNA) is generated by repairing the partially double-stranded genome, whereupon viral transcription occurs (Tuttleman *et al.*, 1986; Ghany *et al.*, 2007). Encapsidation of the pregenomic RNA (pgRNA) occurs in the cytoplasm via a complex interaction among epsilon, core particles, HBV polymerase, and various chaperone proteins. Reverse transcription which leads to negative and then positive strand synthesis occurs inside the viral nucleocapsid (Pollack *et al.*, 1994). Viral assembly then takes place in the endoplasmic reticulum (ER). Mature nucleocapsids finally undergo assembly and coating with envelope proteins followed by budding and virion secretion into the blood. The details of replication process are shown in Fig. 1-4.

1.1.4 Cellular immune response to HBV

The HBV replication is not genuinely cytotoxic to cells. This is in accordance with the phenomenon that many HBV carriers are asymptomatic and have mild liver injury, despite extensive and ongoing intrahepatic replication of the virus (de Franchis *et al.*, 1993). It is now believed that host immune responses displayed on infected hepatocytes to viral antigens are the principal determinants of hepatocellular injury. This notion is consistent with the clinical observation that patients with immune defects who are infected with HBV often have mild yet acute liver injury but high rates of chronic carriage (Stevens *et al.*, 1975). HBV replicates in hepatocytes to produce HBsAg particles and virions. Both types of particle can be taken up by antigen presenting cells, which degrade the viral proteins to peptides that are then presented on the cell surface bound to MHC class I or II molecules. These peptide antigens can be recognized by $CD8^+$ or $CD4^+$ T cells, respectively, which are thereby sensitized. Virus-specific $CD8^+$ cytotoxic T cells (with help from $CD4^+$ T cells) can recognize viral antigens presented on MHC class I chains on infected hepatocytes. This recognition process leads to either direct lysis of the infected hepatocytes or the release of interferons and tumor necrosis factor α (TNFα), which could combat viral replication in surrounding hepatocytes without killing the cells directly (Fig. 1-5).

1.1.5 HBV serotype

The genetic variability of HBV is very high. Before the establishment of the genotypes, HBV strains were distinguished by serological analysis according to ten HBsAg subtypes designated ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw3, adw4q $^-$, adrq+, and adrq $^-$ (Norder *et al.*, 2004; Kay *et al.*, 2007). Further details are summarized in Table 1-1.

1.1.6 HBV genotype

Based on the definition of divergence of the whole HBV genome sequence ≥8% or variance of the S gene sequence ≥4%, HBV is classified into 8 genotypes named from A to H (Chinese Society of Hepatology *et al.*, 2007; Okamoto *et al.*, 1988; Orito *et al.*, 1989; Norder *et al.*, 1992, 2004; Kidd-Ljunggren *et al.*, 2002; Norder *et al.*, 1994; Vieth *et al.*, 2002; Arauz-Ruiz *et al.*, 2002), in which both B and C genotypes are prevalent in Southeast Asia including China (Ding, 2001). Due to the genetic diversity of HBV, most genotypes are now divided into subgenotypes with distinct virological and epidemiological properties, and specific geographic distribution (Sumi *et al.*, 2003; Chan *et al.*, 2005). To date, HBV subgenotypes are known to differ by at least 4% in their genome sequences (Norder *et al.*, 1993, 2004; Lindh *et al.*, 1997). In addition, recombination among HBV genotypes also increases the variability of HBV. Differences between genotypes affect the disease severity, course and likelihood of complications, and therefore treatment outcome and possible vaccination used. So far, little is known about the driving forces behind the divergence of HBV into genotypes, the reason for their specific geographic distribution, and the timing of the evolutionary events. The geographic distributions of HBV genotypes and subgenotypes are shown in Fig. 1-6 and summarized in Table 1-3. The fundamental properties of genomes and differences between HBV genotypes are shown in Table 1-2.

1.1.7 HBV mutant

Mutation of HBV genome is a common event occurred in the process of HBV infection. Due to the high replication capacity of HBV with a release rate up to 10^{13} viral particles per day

and the high error rate of the viral polymerase, HBV genomes are susceptible to all possible mutations in every nucleotide (Nowak *et al.*, 1996; Brechtbuehl *et al.*, 2001). By analyzing the HBV DNA isolated from the plasma of four symptom-free carriers, the mutation rate of HBV was estimated to be approximately 1.4 to 3.2 $\times$ 10^{-5} substitutions/site/year (Okamoto, *et al.*, 1994). HBV is evolving gradually with time, resulting in a large amount of genetic diversity, despite the constraints imposed by the complex genetic organization of the viral genome (Kay *et al.*, 2007). This diversity is partly due to an infidelity of the viral replication machinery (Summers *et al.*, 1982), virus/host interactions and partly due to parallel evolution in geographically distinct areas. Recombination also appears to be an important element in HBV evolution. Human intervention, in form of mass vaccination and antiviral treatment, will reduce the burden of HBV-related liver diseases but may also accelerate the viral evolution (Kay *et al.*, 2007). These mechanisms provide a basis for both evolutionary virus drift and selection of specific variants in a patient.

During specific phases of chronic infection, the major virus population could be completely replaced by specific variants within weeks or months. These variants include HBV strains with a defect in pre-core protein expression, with amino-acid changes in core protein and hepatitis B e antigen, with mutations in pregenomic/core promoter, with deletions in C gene, pre S1 or pre S2 region, and with amino-acid changes in a-determinant of hepatitis B surface antigen (Günther, 2006). Different mutations also lead to different characteristics and results. For example, A1762T/G1764A mutations in the basal core promoter and X protein region enhance HBV replication, reduce the HBeAg expression and are found related to the function of X protein (Scaglioni *et al.*, 1997; Buckwold *et al.*, 1996, 1997; Li *et al.*, 2002). G1896A

mutation in the Pre-C region leads to a premature stop codon in the ORF of HBeAg protein, causing e antigen negative as well as differential distribution among the viral genotypes (Carman *et al.*, 1989; Omata *et al.*, 1991; Imamura *et al.*, 2003; Laskus *et al.*, 1993; Okamoto *et al.*, 1990; Rodriguez-Frias *et al.*, 1995).

As for the drug-resistant mutants, many mutation sites have been found in the polymerase region of HBV, following the anti-virus therapy with the use of Lamivudine (Seta *et al.*, 2000; Stuyver *et al.*, 2001; Yuen *et al.*, 2001), Telbivudine (Lai *et al.*, 2005), Adefovir (Angus *et al.*, 2003; Schildgen *et al.*, 2006; Locarnini *et al.*, 2005; Borroto-Esoda *et al.*, 2006), Tenofovir (Delaney *et al.*, 2006; Sheldon *et al.*, 2005), Entecavir (Tenney *et al.*, 2003, 2004; Warner *et al.*, 2003). Summary of all these drug-resistant mutations is shown in Fig. 1-7.

1.2 Epidemiology of hepatitis B

HBV infection is a global health problem, yet the infection rates are quite different in different areas. The world could generally be divided into three areas where the prevalence of chronic HBV infection is high (>8%), intermediate (2-8%), and low (<2%) (WHO, 2001). Fig. 1-8 illustrated the HBV infection rate in various global regions. It is known that its prevalence is highest in Asia, Africa, southern Europe, and Latin America, where the rate of HBsAg carriage in the general population ranges from 2% to 20% (Margolis *et al.*, 1998; Kane *et al.*, 1995).

In China, the prevalence of HBV is high. The HBsAg carrier rate among the general population is 9.09%; the rates among the vaccinated and unvaccinated population are 4.51% and 9.51% respectively (Liang *et al.*, 2005). The prevalent serotypes of HBV in China are

adrq+ and adw2+; while the rare serotype, ayw3, is found mainly in Xinjiang, Tibet and Inner Mongolia autonomous regions. The prevalent genotypes in China are C and B (Fan *et al.*, 1998). The HBV infection could cause acute and chronic liver diseases (Chen *et al.*, 1993). Chronic carriage of HBV, as defined by the presence of HBsAg and persistent increase of serum aminotransferase concentrations for more than 6 months after infection, is a serious public health problem because the carriers are reservoirs of the infectious agent, and are themselves prone to develop liver diseases (Kao *et al.*, 2002; Evans *et al.*, 1998). Following acute HBV infection, the risk of developing chronic infection varies inversely with ages. Chronic HBV infection occurs in about 90% of infants whom are HBV infected at birth, 25-50% of children infected at 1-5 years of age and about 1-5% of persons infected in later childhood or adulthood (Ganem *et al.*, 2001; Hollinger *et al.*, 2001).

According to a recent report, there are more than 2 billion people who have been infected with HBV. Among all, about 350 million remain chronically infected and later became virus carriers (WHO, 2001; Viral Hepatitis Prevention Board, 1996; Margolis *et al.*, 1995; Kao *et al.*, 2002; Parkin *et al.*, 2005). Besides, three quarters of the world's population live in areas with high levels of infection. Every year, there are over 4 million acute clinical cases of HBV, and around 1 million of them die annually from chronic active hepatitis, hepatic decompensation, cirrhosis or HCC (WHO, 2001; Evans *et al.*, 1998; Parkin *et al.*, 1999). HBV is regarded as the major cause in up to 80% of all cases of HCC worldwide, second to tobacco among all human carcinogens (Hollinger *et al.*, 2001; WHO, 2001; Viral Hepatitis Prevention Board, 1996).

An effective vaccine has been employed for preventing HBV infection for nearly 20 years, and there is also attempt to try universal vaccination in some developing countries including

China, where there are around 120 million carriers (Parkin *et al.*, 1999). Although vaccination is not an option for established infections, its effectiveness in preventing blood-borne transmission from an infected mother to her newborn is as high as 90% (Stevens *et al.*, 1985). However, the high cost of the vaccination programs has impeded its introduction in many poor countries where HBV infections are prevalent, afflicting 5 to 20% of the population (Seeger *et al.*, 2000). Table 1-4 summarize the milestones and future prospects in global control of HBV infection since the virus was discovered.

1.3 Clinical features of hepatitis B

1.3.1 Clinical symptoms and phases

HBV infection may either be self-limiting or long-standing. Self-limited infection is defined as the disappearance of the viral antigens and the appearance of anti-HBs antibody (Prince *et al.*, 2001). Persons with self-limiting infection clear the infection spontaneously within weeks to months and such situation happens mostly in adults (Wright *et al.*, 1993; Hoofnagle *et al.*, 1981). Acute infection with HBV is associated with acute viral hepatitis - an illness that begins with general ill-health, loss of appetite, nausea, vomiting, body aches, mild fever, dark urine, and then progresses to the development of jaundice. The illness lasts for few weeks and then gradually improves in most infected people. However, some patients may develop more severe liver illness such as fulminant hepatic failure, and die over it. The infection could be entirely asymptomatic and completely unrecognized.

Chronic hepatitis B infection is defined as the presence of the HBV for more than 6 months after the initial infection. There are three phases best describe the invasion action of

HBV: the immunotolerant phase, the immune clearance phase, and the inactive or low/non-replication phase (Lok *et al.*, 2001; Kao *et al.*, 2002). During the first phase, patients have no symptoms, serum ALT is normal, HBeAg is positive and the HBV DNA is high. In the second phase, some HBV carriers start to have symptoms and acute hepatitis. In the last, the patients are symptom-free and liver disease becomes inactive. The age at the time of HBV infection is the primary risk factor relating to the progression of chronic infection; 90% of those perinatally exposed patients would develop chronic infection, whereas 25%-30% of children who have been exposed to HBV would develop chronic infection (Chinese Society of Hepatology, *et al.*, 2007). Chronic infection with HBV may be either asymptomatic or may be associated with a chronic inflammation in the liver (chronic hepatitis), leading to cirrhosis over a period of several years. This type of infection dramatically increases the incidence of HCC which is predominantly immune-mediated. Chronic carriers are encouraged to avoid consuming alcohol as it increases their risk for cirrhosis and liver cancer.

1.3.2 Laboratory test

The assays for detecting HBV infection involve serum or blood tests, which could detect either viral antigens or antibodies produced by the host (Bonino *et al.*, 1987).

The HBsAg is the most frequently used candidate in screening the presence of HBV, since it is the first detectable viral antigen to appear upon infection. However, it is still possible that such candidate marker remains undetectable when the testing is carried out too early, or too later after the antigen has been cleared up by the host. The infectious virion contains an inner "core particle" which encloses the viral genome. The icosahedral core particle is made of 180 or

240 copies of core protein, alternatively known as hepatitis B core antigen, or HBcAg. During the 'window' phase in which the host remains infected but is successfully clearing the virus, the presence of IgM antibodies targeting the hepatitis B core antigen (anti-HBc IgM) may be the only serological evidence of infection.

Shortly after the appearance of HBsAg, another antigen named the HBeAg will appear. Traditionally, the presence of HBeAg in host's serum is associated with higher rate of viral replication and infectivity; however, certain variants of HBV do not produce the 'e' antigen, so this rule does not always hold true. During the natural course of infection, the HBeAg may be cleared, and antibodies to the 'e' antigen (anti-HBe) will start to generate immediately right after. This conversion is usually associated with a dramatic decline in viral replication.

If the host is able to clear the infection, eventually the HBsAg will become undetectable and IgG antibodies to the HBsAg and core antigen (anti-HBs and anti HBc IgG) will appear. With this in mind, a person negative for HBsAg but positive for anti-HBs may indicate either a complete clearance of HBV or a previous vaccination.

Individuals who remain HBsAg positive for at least six months are considered to be hepatitis B carriers. Carriers of the virus may have chronic hepatitis B, which would be reflected by elevated serum alanine aminotransferase (ALT) levels and inflammation of the liver, as revealed by biopsy. Carriers who have seroconverted to HBeAg negative status, particularly those who acquired the infection as adults, have lower viral multiplication and hence may be at lower risk of long-term complications or transmitting infection to others.

The patterns of serologic and molecular markers in acute self-limited and chronic HBV infection are different. Figs. 1-9 and 1-10 show the levels of hepatitis B viral antigens and

antibodies in the blood following acute and chronic HBV infection, respectively.

More recently, polymerase chain reaction (PCR) and real-time fluorescent quantitative PCR (FQ-PCR) have been employed to measure the quantity of viral nucleic acid in clinical specimens (Weinberger *et al.*, 2000; Tedder *et al.*, 2002; Zoulim, 2006). These tests are called viral loads and are used to assess the infectious status of patient as well as to monitor the treatment outcome.

1.3.3 Diagnostic imaging

Ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) technologies have been employed in previous studies (Chinese Society of Hepatology, *et al.*, 2007) in screening the HCC and diagnosing/monitoring the progress of chronic hepatitis B.

1.3.4 Possible ways of transmission

HBV can be transmitted via the exposure to infectious blood or body fluids. Possible forms of transmission include unprotected sexual contact, blood transfusions, re-use of contaminated needles & syringes, and vertical transmission from mother to child during childbirth (Zuckerman, 2007; Liu *et al.*, 2006). HBV is not a virus that spreads through food or water, sharing eating utensils, breastfeeding, hand holding, coughing, or sneezing.

1.3.5 Treatment

Acute hepatitis B infection might not usually require treatment because most adults could combat the infection spontaneously. Early antiviral treatment may only be required in less than

1% of patients, whose infection takes a very aggressive course ("fulminant hepatitis") or who are immunocompromised. On the other hand, treatment of chronic infection may be necessary to reduce the risk of cirrhosis and HCC. Chronically infected individuals with persistently elevated serum ALT and HBV DNA levels are suggested for therapy immediately.

Although none of the commercially available drugs could completely clear the infection, these drugs can halt viral replication, and therefore prevent the liver from further damages such as cirrhosis and HCC. The commonly used antiviral drugs are lamivudine (Puoti *et al.*, 2002), adefovir (Marcellin *et al.*, 2003), tenofovir (Nelson *et al.*, 2003; Ristig *et al.*, 2002), entecavir (Lai *et al.*, 2002), emtricitabine (Chin *et al.*, 2001), and clevudine (Chin *et al.*, 2001); and immune system modulators such as interferon alpha (Wong *et al.*, 1993; Ghany *et al.*, 2007; Chinese Society of Hepatology *et al.*, 2007) and peginterferon alfa-2a (Fried *et al.*, 2008; Marcellin *et al.*, 2004; Hui *et al.*, 2005). However, some individuals respond better to certain kinds of drugs and this might be due to different genotypes or patient's heredity. Infants of mothers with known hepatitis B carrying clinical record must be injected with hepatitis B immune globulin (HBIG). This injection should be done within the first 12 hours after birth, so as to reduce the risk of acquiring hepatitis B by 95%. This treatment also allows the mother to breastfeed her child safely.

Nucleotide analogues (NA) inhibit the production of HBV DNA by competitively inactivating the HBV polymerase and/or acting as chain terminators (not all NAs are chain terminators). Phenolpropenamide inhibits the encapsidation process, and heteroaryldihydropyrimidines (HAPs) acts by inhibiting the encapsidation step and degrading the nucleocapsid. Peptidomimetic compounds act further downstream by inhibiting the

assembly of HBV. Glucosidase inhibitors work by inhibiting the glycosylation of HBsAg and thereby inhibit the assembly of virus (Ghany *et al.*, 2007). The molecular targets of drugs which repress HBV replication is shown in Fig. 1-4. The flow chart summarizing the antiviral treatment for chronic hepatitis B is shown in Fig. 1-11.

1.3.6 Vaccination

Hepatitis B is a vaccine-preventable disease. WHO recommends that hepatitis B vaccine should be included in routine immunization services in all countries. Vaccines are composed of the HBsAg and produced from plasma or recombinant DNA (Zuckerman, 2006). When administered properly, hepatitis B vaccine induces protection in about 95% of recipients (Hollinger *et al.*, 2001; Robinson, 1995).

1.4 HBV and HCC

HCC is the third leading cause of cancer death in China. Liver cancer causes more than 500, 000 deaths annually throughout the world, with a male to female ratio of 4:1 (El-Serag *et at.*, 2004; Llovet *et al.*, 2003; Parkin *et al.*, 2001; Shibuya *et al.*, 2002). The high mortality associated with HCC is due to its unresponsiveness to treatment and late diagnosis among HCC patients (Blum, 2005). The incidence of HCC varies with geography, race, age and sex. HCC is responsible for 90% of the primary malignant liver tumors found in adults (Wilson, 2005; El-Serag *et al.*, 2007). Although the major viral and environmental risk factors for HCC have been identified, the understanding on the pathways of HCC development is still incomplete. Genes most frequently affected in HCC can be divided in four main regulatory pathways: p53

pathway, $p16^{INK4A}$/Rb pathway, Wnt/adenomatous polyposis coli (APC)/β-catenin pathway and transforming growth factor-β (TGF-β) pathway (Tannapfel *et al.*, 2001; Staib *et al.*, 2003; Tommasi *et al.*, 2007).

The frequency of HCC follows the same general geographic distribution pattern as that of persistent HBV infection. Recently, it has been estimated that about 53% of HCC cases worldwide are related to HBV (Parkin *et al.*, 2001). The age distribution of patients with clinic ally recognized tumors suggests that these tumors appear after a mean duration of about 35 years of HBV infection.

Chronically infected subjects of HBV have a risk of HCC that is 100 times more than that of non-carriers (Beasley, 1988). Persons with higher risk of developing HCC are male adults and chronic hepatitis B patients with cirrhosis who contracted HBV at their early childhood. Only 5% of the patients with cirrhosis develop HCC. On the other hand, between 60% and 90% of HCC patients have underlying cirrhosis. Although the role of HBV in provoking HCC is undisputed, the underlying cellular and molecular mechanisms remain not fully understood (Fourel *et al.*, 1990; Matsubara *et al.*, 1990; Popper *et al.*, 1987; Lauer *et al.*, 1992; Kim *et al.*, 2007). Given these facts, a twice-a-year screening of chronically infected patients with measurements of serum alpha fetoprotein (AFP) or hepatic ultrasonography, or both, is warranted (Lok *et al.*, 2001).

To date, HBV infection could promote carcinogenesis via two pathways. First, the viral DNA can integrate in the host genome, and then induces chromosome instability. Integration is not essential for the viral replication but it allows persistence of the viral genome. Almost all of HBV-associated HCCs harbor chromosomally integrated HBV DNA (Beasley *et al.*, 1981;

Brechot *et al.*, 1980; Paterlini-Bréchot *et al.*, 2003). The specific parts of HBV genome enter the chromosome of the liver cells and may then disrupt their normal genetic structure, thereby causing the liver cells to become cancerous (Aoki *et al.*, 1996). The second pathway involves the integration of HBV genome at specific sites of human chromosome which activates endogenous genes such as retinoic acid β-receptor (Dejean *et al.*, 1986), cyclin A (Wang *et al.*, 1990), mevalonate kinase (Graef *et al.*, 1994), c-myc or N-myc (Bruni *et al.*, 2004; Jacob *et al.*, 2004), sarco/endoplasmic reticulum calcium ATPase 1 (Brechot *et al.*, 2000) and human telomerase reverse transcriptase (hTERT) (Horikawa *et al.*, 2001, 2003; Hytiroglou *et al.*, 2006). The genetic material of HBV is frequently found in the host cancer cells. This suggests that viral integration in the vicinity of genes that controls cell proliferation, viability and differentiation is a mechanism frequently involved in HBV-associated hepatocarcinogenesis (Lupberger *et al.*, 2007). More recently, 15 genes were found to be altered at protein level by HBV integration in tumors, suggesting that viral integration in the host genome alter the activity of these candidate genes, thus induce hepatocarcinogenesis (Ferber *et al.*, 2003; Horikawa *et al.*, 2003; Paterlini-Brechot *et al.*, 2003). Apart from the integration of HBV DNA mentioned above, there are other HBV viral factors that were thought to relate to HCC. They include Pre S1/Pre S2/S, HBeAg, HBV DNA virus load, HBV genotype, A1762T/G1764A mutations and HBx, etc. (Toshkov *et al.*, 1994; Iloeje *et al.*, 2006; Kuang *et al.*, 2004; Feitelson, 1999).

In recent studies, it was revealed that HBsAg carriers have 25-37 times higher risk of developing HCC when compared with non-infected people (Hassan *et al.*, 2002; Yang *et al.*, 2002). An accumulation of the large Pre S1/S2/S HBV envelope protein induces a direct toxic

effect on hepatocytes, independent of the immune response to the viral protein (Toshkov *et al.*, 1994; Wang *et al.*, 2003). Apart from triggering the liver cell proliferation, an accumulation of viral proteins, for example HBsAg in so-called 'ground glass' hepatocytes, may modify detoxification pathways such as those involving cytochrome P450 enzymes; this effect may enhance the metabolism of chemical carcinogens (Kremsdorf *et al.*, 2006).

There are conflicting data about the influence of HBV genotypes on HCC development (Kao, 2002; Orito *et al.*, 2003; Sugauchi *et al.*, 2004). Recent studies from Taiwan provided profound evidence for HBeAg-positive patients that HBV genotype C causes a more aggressive disease course as compared with genotype B (Chen *et al.*, 2005; Kao *et al.*, 2004; Yu *et al.*, 2005). On the other hand, there are reports from Taiwan manifesting that more than 50% of the HBV-related HCC patients are infected with genotype B. Another 15 years follow-up study on Taiwanese pediatric patients with chronic HBV infection showed that genotype B is identified in 74% of the children with HBV-associated HCC (Ni *et al.*, 2004).

A recent study revealed that the relative risk of HCC is 6-fold higher among patients who were positive for both HBsAg and HBeAg, as compared with those who were positive for HBsAg alone (Yang *et al.*, 2002). Based on this finding, it was suggested that that HBeAg might be a useful marker for estimating the risk of a patient in developing HCC, since HBeAg reflects productive HBV replication. The risk of HCC seems to be elevated with increasing HBV viral load (Iloeje *et al.*, 2006).

Another interesting observation is that the prevalence of the A1762T/G1764A mutation in the basal core promoter region increases with the progression of liver diseases. Since this mutation seems to be associated with HCC development, it might represent a helpful prognostic

biomarker (Kuang *et al.*, 2004; Liu *et al.*, 2006).

Hepatitis B virus X protein (HBx) has also been long suspected to be involved in hepatocarcinogenesis, although its oncogenic role remains controversial (Rossner, 1992; Feitelson, 1999). HBx activates a broad variety of different promoter elements which trigger activation of transcription factors like activator protein-1 (AP-1), nuclear factor kappa B (NF-κB), specificity protein 1 (SP1) and octamer binding protein 1 (Oct-1) (Zhang et al., 2006; Waris et al., 2003). HBx is a multifunctional regulator that modulates transcription, signal transduction, cell cycle progress, protein degradation, apoptosis, and genetic stability by direct or indirect interaction with host factors (Caselmann *et al.*, 1998; Elmore *et al.*, 1997; Gottlob *et al.*, 1998; Kim *et al.*, 1991; Yu *et al.*, 1999).

1.5 Objectives and significance of the present study

As mentioned earlier, chronic HBV infection is a major risk factor for the development of HCC; however, the pathogenesis of HBV-induced HCC is poorly understood. Our clinical studies have shown that some subgenotypes/hot spot mutations are associated with increased risk of HCC. This study aimed at extending our clinical observations to exploring fundamental mechanisms by characterizing the biological and virological functions of novel HBV mutations associated with increased risk of HCC. The specific aims were as follows:

- To investigate the replication capacity and promoter activity of HBV strains isolated from asymptomatic HBV carriers aged over 60 and HCC patients aged about 30;
- To construct novel HBV mutations as observed in HCC patients by site-directed mutagenesis and determine their promoter activity;

- To investigate whether substitution mutations at X protein enhance or impair its functions on HBV replication and promoter activity.

This study will translate clinical findings to exploratory work on the basic mechanisms of HBV-associated HCC. The outcome of this study will provide new knowledge of HBV-associated HCC. Furthermore, experiments of HBx on the promoter activity of HBV could shed new light on the mechanism by which HBx increases HBV replication and the related preventive strategy.

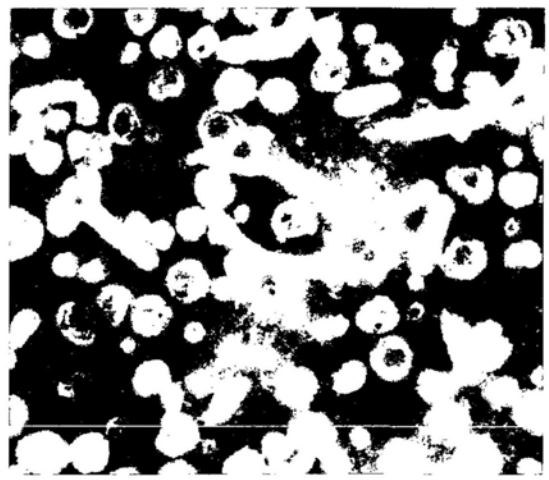

Fig. 1-1. Electron Microscopy (EM) image of HBV related particles (Ganem *et al.*, 2004).

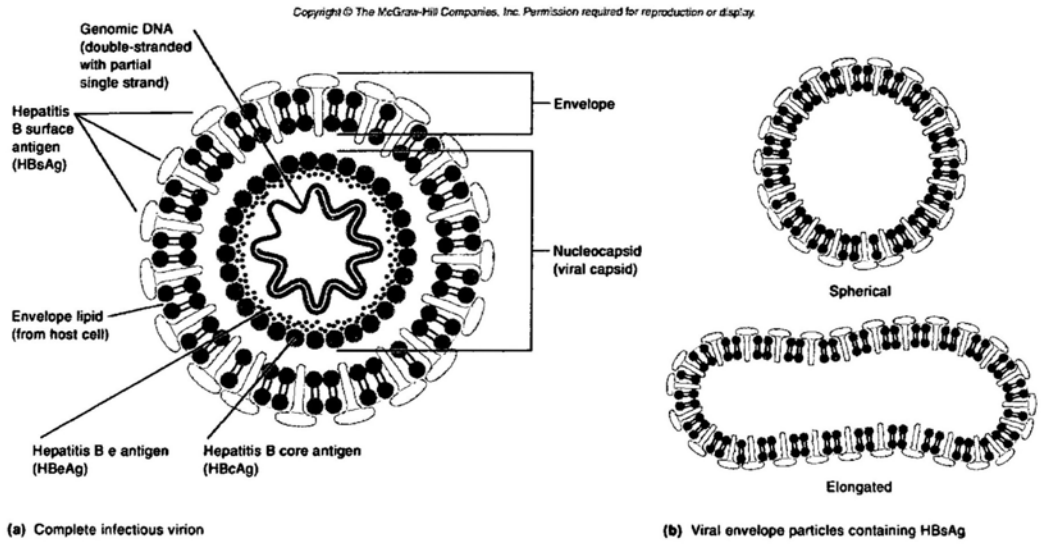

Fig. 1-2. Schematic representation of the HBsAg-associated particles. Panel a shows HBV virions, Panel b shows 20-nm HBsAg particles (http://www.mcgraw-hill.com/).

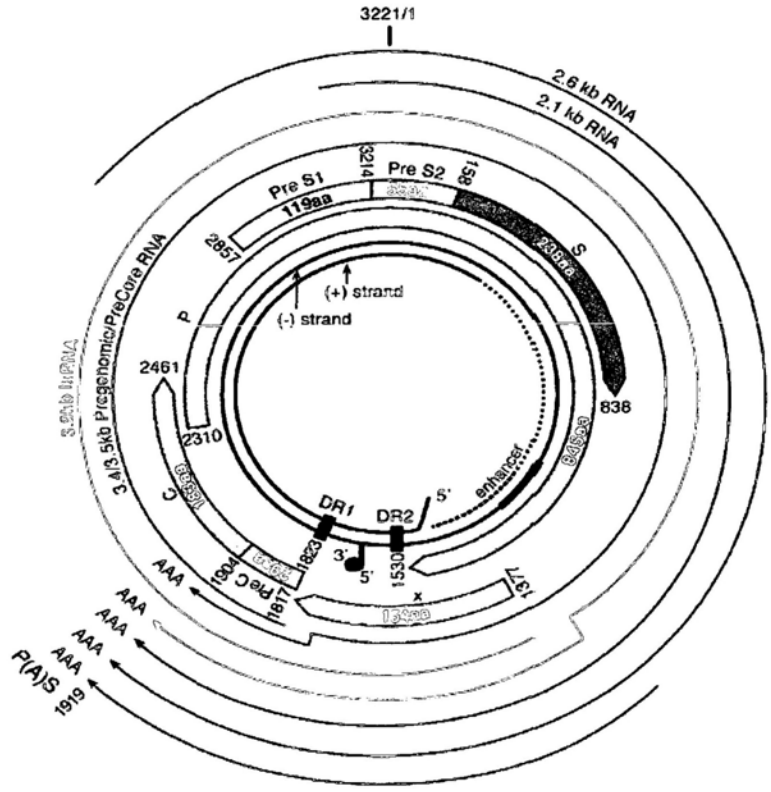

Fig.1-3. Genome and transcription map of HBV (adw). Inner circles represent the viral partial dsDNA, wide arrows represent the overlapping viral ORFs, and the outer thin arrows represent the different viral transcripts, including the 3.9kb RNA, initiating from the X promoter. Nucleotides are numbered according to the unique EcoR I site=1. Transcription polyadenylation site is located within the Core gene coding sequence, at position 1919. P, RT-polymerase; DR, direct repeats (Doitsh *et al.*, 2003).

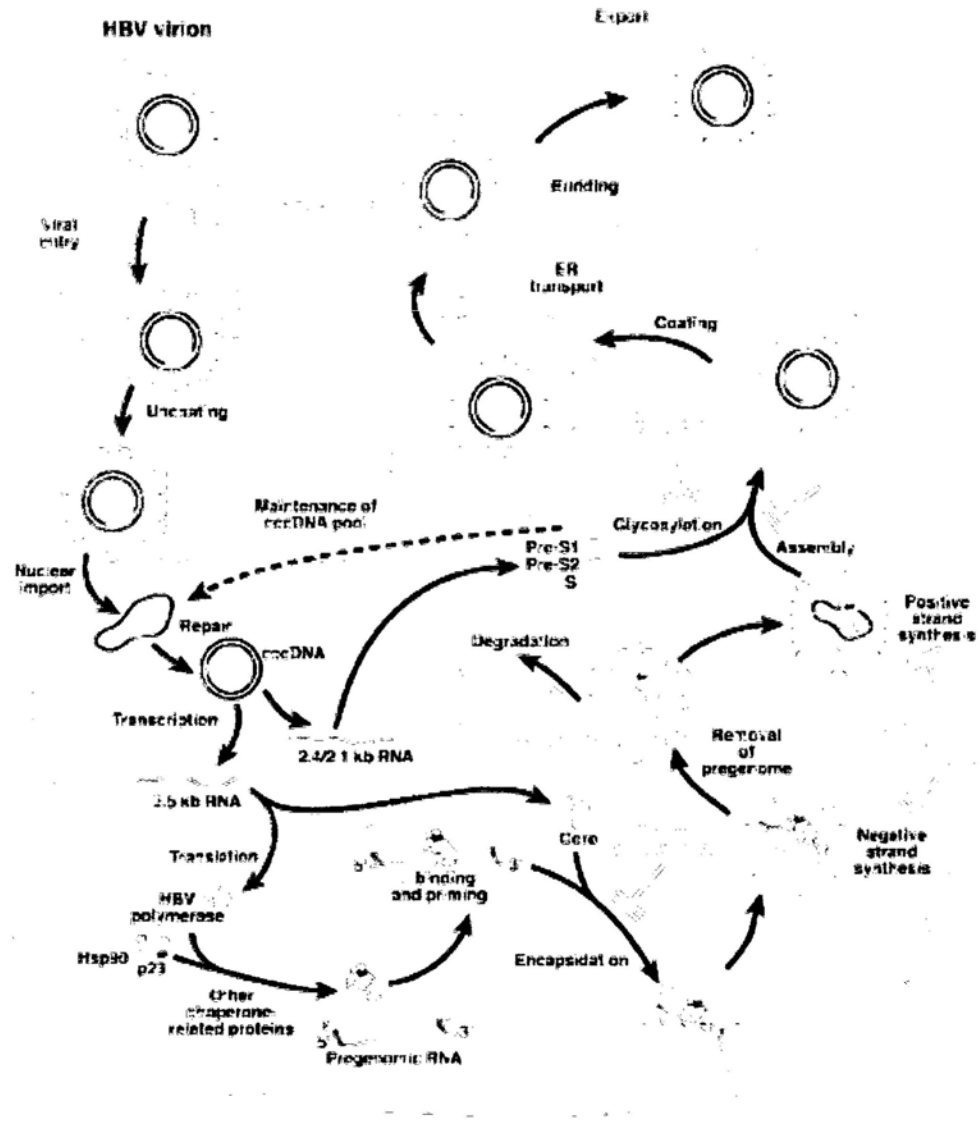

Fig. 1-4. Replication cycle of HBV and molecular targets of HBV replication (Ghany *et al.*, 2007).

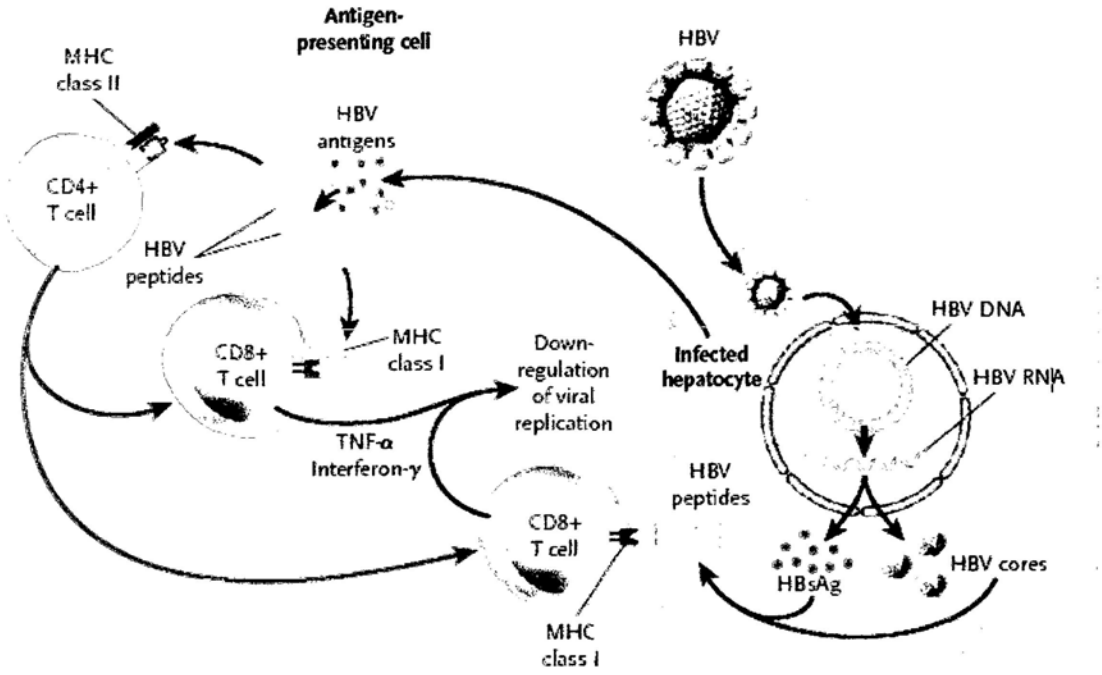

Fig. 1-5. Cellular immune responses to HBV (Ganem *et al.*, 2004).

Table 1-1. Distinctive feature of HBV serotypes (Kay *et al.*, 2007).

Serotype	Amino acid signature on HBsAg sequence
ayw1	122R + 160K + 127P + (134F and/or 159A)
ayw2	122R + 160K + 127P
ayw3	122R + 160K + 127T
ayw4	122R + 160K + 127L
ayr	122R + 160R
adw2	122K + 160K + 127P
adw3	122K + 160K + 127T
adw4q−	122K + 160K + 127L + 178Q
adrq+	122K + 160R + 177V + 178P
adrq−	122K + 160R + 177A

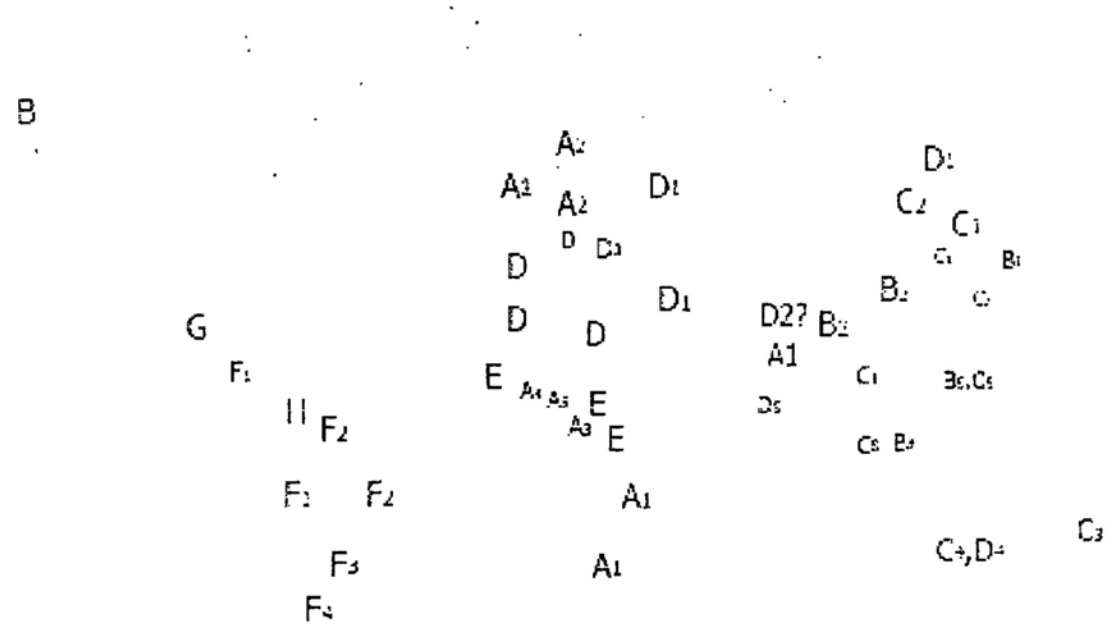

Fig. 1-6. Geographic distribution of HBV genotypes and subgenotypes (Schaefer, 2007).

Table 1-2. Fundamental properties of genomes and differences between HBV genotypes (Schaefer, 2007).

Genotype	Genome length (bp)	ORF differences
A	3221	Insertion of aa 153 and 154 in HBc
B	3215	
C	3215	
D	3182	Deletion of aa 1-11 in pre S1
E	3212	Deletion of aa 11 in pre S1
F	3215	
G	3248	Insertion of 12 aa in HBc Deletion of aa 11 in pre S1
H	3215	

Table 1-3. HBV subgenotypes and geographic prevalence (Schaefer, 2007; Kay *et al.*, 2007).

Genotype	Subgenotype	Serotype[A]	Geographic origin
A	A1 (Aa, A')	**adw2**, ayw1	Africa, Asia, South America
	A2 (Ae, A-A')	**adw2**, ayw1	Northern Europe, North America
	A3 (Ac)		Gabon, Cameroon
	A4		Mali
	A5		Nigeria
B	B1 (Bj)	**adw2**	Japan
	B2 (Ba)	**adw2**, adw3	Asia without Japan
	B3	**adw2**, ayw1	Indonesia, Philippines, China
	B4	**ayw1**, adw2	Vietnam, Cambodia
	B5		Philippines
C	C1 (Cs)	**adrq$^+$**, ayr, adw2, ayw1	South East Asia
	C2 (Ce)	**adrq$^+$**, ayr	Far East (Korea, Japan, Northern China)
	C3	adrq$^+$, adrq$^-$	Micronesia, Pacific Islands
	C4		Australia
	C5		Philippines, Vietnam
D	D1	**ayw2**, adw1, ayw1	Mongolia, Belarus, Europe, Egypt, Asia
	D2	**ayw3**, ayw1	India, Europe, Japan
	D3	**ayw3**, ayw2, ayw4	South Africa, East India, Serbia, USA
	D4	**ayw2**,ayw3	Australia, Japan
	D5		East India
E		**ayw4**, ayw2	West Africa, UK, France
F	F1	**adw4**, ayw4	South and Central America
	F2	**adw4**	South America
	F3	**adw4**	Bolivia, Columbia
	F4	**adw4**	Argentina, Bolivia, France
G		**adw2**	USA, France , Germany and Mexico
H		**adw4**	Central and South America

[A]: The main serotypes are shown in bold.

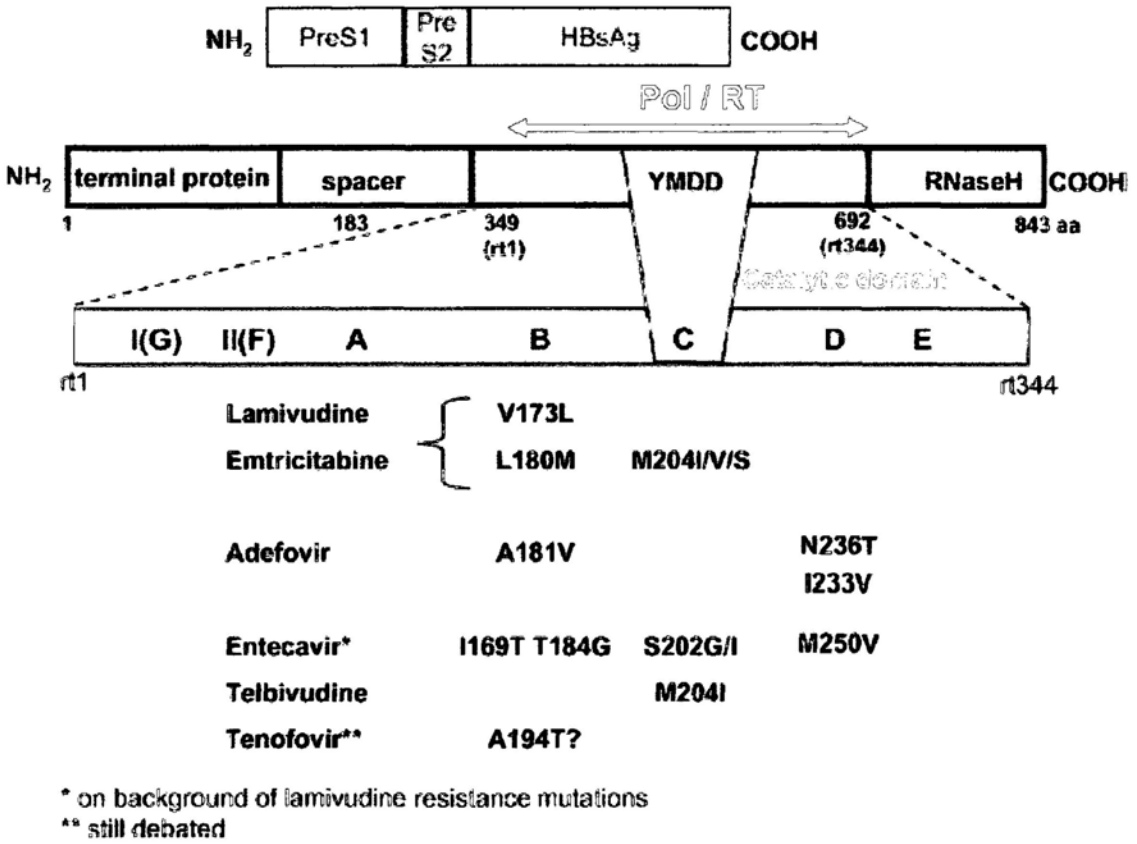

Fig. 1-7. HBV polymerase drug-resistant mutants. Schematic representation of the HBV polymerase and the envelope proteins whose open reading frame overlaps the polymerase gene. The four domains of the polymerase are indicated, in which the Pol/RT domain is magnified to show the well-conserved sub-domains. The numbering system used to identify drug-resistant mutants follows the recommendations of literature (Stuyver *et al.* 2001; Kay *et al.*, 2007).

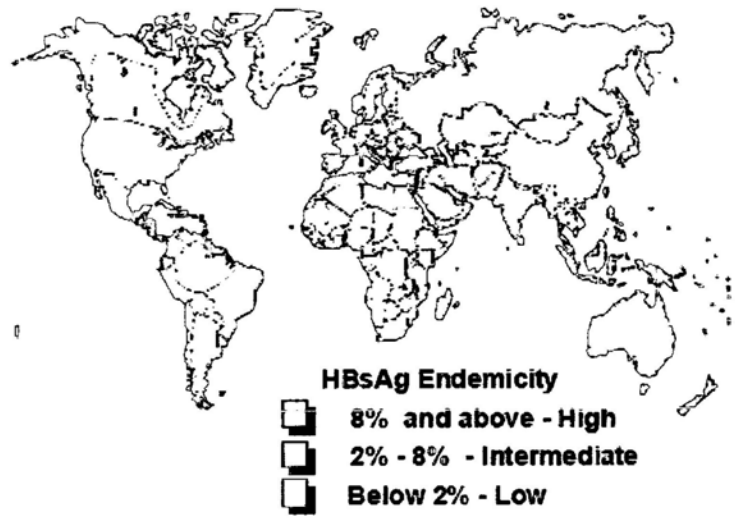

Fig. 1-8. Geographical distribution of chronic hepatitis B virus infection (World Health Organization. Introduction of hepatitis B vaccine into childhood immunization services, Geneva, WHO, 2001).

Table 1-4. Milestones and future prospects in global control of HBV infection (Kao *et al.*, 2002)

Year	Milestone and perspective
1960s	Discovery of Australia antigen
1970s	Serological assays for HBV markers
1980s	Hepatitis B vaccine available Interferon approved for treatment of chronic hepatitis B Quantitative assay for HBV DNA
1990s	PCR assay for detection of HBV DNA Lamivudine approved for treatment of chronic hepatitis B
2000s	Novel antiviral agents (pegylated interferon, nucleoside analogues or therapeutic vaccine)
2010s	Combination therapy for chronic hepatitis B
2020s	Cure for chronic hepatitis B
2050s	Global control of hepatitis B virus infection

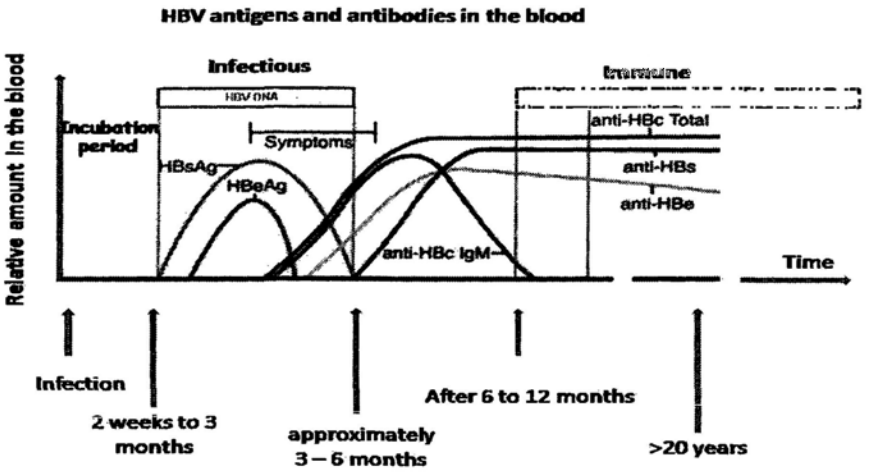

Fig. 1-9. Hepatitis B viral antigens and antibodies detectable in the blood following acute infection (http://en.wikipedia.org/wiki/Hepatitis_B_virus).

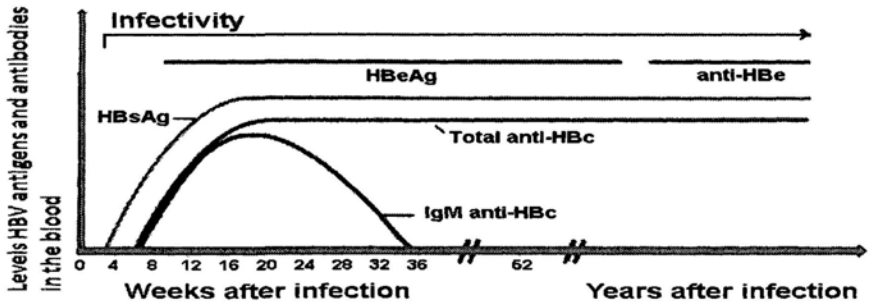

Fig. 1-10. Hepatitis B viral antigens and antibodies detectable in the blood of a chronically infected person (http://en.wikipedia.org/wiki/Hepatitis_B_virus).

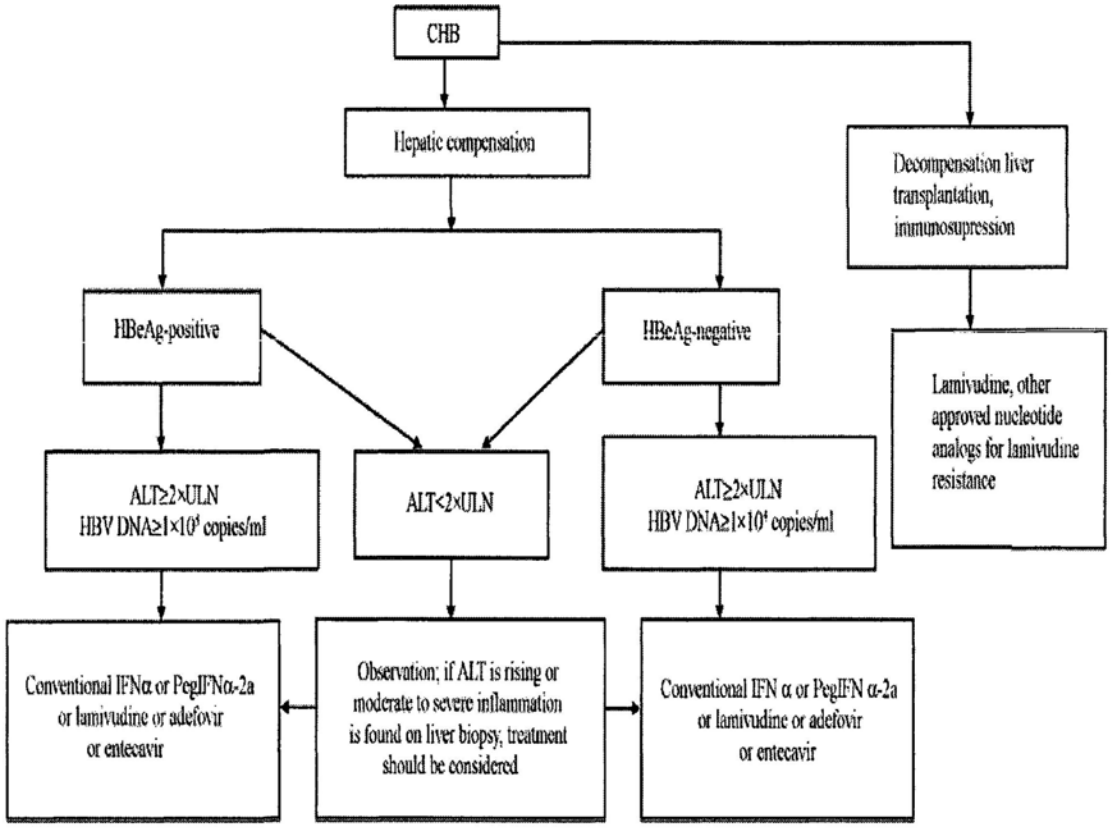

Fig. 1-11. Flow chart for chronic hepatitis B antiviral treatment (Chinese Society of Hepatology *et al.*, 2005).

Chapter 2

Full sequence analyses of four HBV strains in which two from HCC and two from AsC

2.1 Introduction

Hepatitis B virus is a member of hepadnaviruises family (Orito *et al.*, 1989; Bollyky *et al.*, 1999; Mason *et al.*, 2005). Its genome contains a relaxed-circular, partially double-stranded DNA of 3.2 kb (Summers *et al.*, 1975). It encodes four overlapping open-reading frames including (ORF)-pre S/S, pre C/C, X and P (Chisari *et al.*, 1997; Mahoney *et al.*, 1999; Robinson *et al.*, 1995; Ganem *et al.*, 2001). It has been suggested that HBsAg and HBx protein are oncogenic. Based on the definition of divergence of the whole HBV genome sequence ≥8% or variance of the S gene sequence ≥4%, HBV is classified into 8 genotypes from A to H (Chinese Society of Hepatology *et al.*, 2007; Okamoto *et al.*, 1988; Orito *et al.*, 1989; Norder *et al.*, 1992, 1994, 2004; Kidd-Ljunggren *et al.*, 2002; Vieth *et al.*, 2002; Arauz-Ruiz *et al.*, 2002). Differences between genotypes affect the disease severity, course and likelihood of complications, and response to treatment and possibly vaccination.

HBV is one of the major causative agents of HCC in Asia, including China, where HBV genotype B and C are prevalent. It has been proposed that HBV genotypes may influence the course of disease and relevant biological functions. A case control study from Taiwan suggested that HBV genotype C is more closely associated with cirrhosis and HCC in those who are older than 50 years; whereas genotype B is more common in patients with HCC aged

less than 50 years (Kao *et al.*, 2003). Recently studies reported that basal core promoter mutations (A1762T and G1764A) are associated with more aggressive progression of liver disease from inactive carrier to active hepatitis, and eventually to liver cirrhosis and HCC (Kao *et al.*, 2000; Hsieh *et al.*, 2004; Tralhao *et al.*, 2002). Several HBV genes, including truncated pre S2/S and X gene have been found in hepatoma tissue (Chami *et al.*, 2003; Brechot *et al.*, 2004; Wang *et al.*, 2004). Another hot spot mutation in the core promoter region is the G1896A mutation (Tralhao *et al.*, 2002; Murakami *et al.*, 2001). However, the relationships among HBV genotypes, hot spot mutations, biological and virological consequences, and hepatocarcinogenesis remain largely unclear. In this part of study, the characteristics of full sequences of four strains of HBV in which two from HCC patients and two from AsCs were analyzed. Genotype, subgenotype, hot spot mutations and other genome variations were examined and discussed.

2.2 Materials and methods

2.2.1 Patients

One hundred HBV-related HCC patients and one hundred age-matched HBV-infected controls were recruited at Prince of Wales Hospital in Hong Kong. Informed consent was obtained from patients in prior, and this study was approved in advance by the Ethics Committee of the Chinese University of Hong Kong. Our studies were based on the findings that A1762T/G1764A mutations occurred frequently in HCC patients with genotype B infection (81%, 30 of 37 patients), but were relatively lower in AsCs (43%, 22 of 51 patients) (Sung *et al.*, 2008). So our criteria of selecting patients in our studies include two points. First, double

mutations were observed in HBV strains isolated from HCC patients but not from carriers. Second, representative young HCC patients and old HBV carriers would be selected. To meet the criteria, four typical HBV strains (genotype B) from four patients (two HCC patients and two HBV carriers) were selected for this study. Those samples from HCC patients were named H60 and H293, whilst the two from HBV AsCs were named C265 and C1691. The subjects were all male of 31, 26, 66, and 71 years old, respectively. All patients recruited had full-length HBV genomes sequenced. Serum samples from all patients were stored at -80°C immediately after collection.

2.2.2 Extraction of DNA from sera

HBV DNA was extracted from sera using QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions.

2.2.3 Amplification of complete virion-encapsidated HBV genomes

HBV full sequences were amplified by PCR using primer HBV-P1 and HBV-P2 (Table 2-1) in the gap region of minus strand of HBV genome. Thermocylcing was performed using the high fidelity HotStar Taq polymerase (Qiagen, Hilden, Germany). The PCR conditions were carried out as follows: 94°C for 3 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 6 minutes; then a final extension phase was performed at 72°C for 10 minutes. The PCR products were separated by electrophoresis on 1.0% agarose gel and stained with 0.5 μg EtBr/ml gel. The results were visualized using a UV transilluminator (Uvitec, Cambridge, UK) and photo-documented.

2.2.4 Cloning of PCR products and verification

The PCR products of complete HBV genomes were purified from agarose electrophoresis using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pCR2.1-TOPO (Invitrogen, CA, USA) according to the manufacturer's instructions. For primary verification of the right clones, EcoR I (NEB, MA, USA) restriction endonuclease was used.

2.2.5 Sequencing of HBV full genomes

Five primers were used for sequencing the HBV full genomes: HBV-P3, HBV-P4, HBV-P5, M13-F and M13-R (Table 2-1). The former three sequences were HBV specific and the latter two were included in the vector pCR2.1-TOPO.

2.2.6 Sequence assembling and comparison

Four full sequences of HBV for each genotype (A, C, D, E, F, G and H) and eight sequences for genotype B (four for genotype Ba and four for genotype Bj) were randomly extracted from GenBank database for genotype and subgenotype analysis together with the four complete HBV sequences employed in this study. The sequence assembling and alignment was carried out using Vector NTI Suite 9, GeneDoc 2.6 and SimPlot 2.5. The substitution model used for amino acid sequence alignment and for defining nucleotide similarity scores is the Dayhoff model. The phylogenetic trees were constructed with TreeView 1.5 based on neighbor-joining method (Saitou and Nei, 1987). The reliability of phylogenetic tree analysis was assessed by bootstrapping resampling method (Felsenstein, 1985).

2.3 Results

2.3.1 Full genome PCR of HBV

The PCR products of the whole genome of HBV are shown in Fig. 2-1. The figure shows a band about 3.2kb of length is present for all samples. Another band appears at around 2.5kb for sample H60. This 2.5kb band might be a spliced variant of HBV. Therefore, all of the five bands were cut, purified and cloned into T-A vector pCR2.1-TOPO. After PCR screening and plasmid purification, the restriction endonuclease EcoR I was used to screen for the right clones. The inserts at around 3.2kb and 2.5kb appeared in the expected positions on the agarose gel (Fig. 2-2).

2.3.2 Sequencing of full genome of HBV

The five clones inserted with HBV full genomes were sequenced using five primers. After five fragments for every sample were assembled, the right HBV genomes were produced. They were named H60, H293, C265, C1691 and H60-2.5kb, respectively. All HBV genomes sequenced in this study were deposited in GenBank with the following accession numbers: DQ995801, DQ995802, DQ995803, DQ995804 and DQ995805.

2.3.3 Alignment of five HBV full sequences

The alignment result is shown in Fig. 2-3. The genome is 3215bp in length for H60, H293, C265 and C1691, whilst 2494bp for H60-2.5kb. Double mutations in the core promoter (A1762T/G1764A) were observed in H60 and H293 sequences, while point mutation in the precore (G1896A) was found in C265 and C1691. HBx was truncated at the 3'-end and short of around 105 nucleotides for H60. It was found constituted by 119 amino acids in H60 whilst

HBx proteins in the other three stains were constituted by 154aa.

2.3.4 Genotype and subgenotype analysis of HBV full sequences

Through comparing the full sequences of the four HBV strains with reference sequences of genotype A to H of HBV, all our sample sequences (i.e. H60, H293, C265 and C1691) were found to belong to genotype B of HBV with the nucleotide identity more than 92% (Table 2-2). When compared with subgenotye Ba and Bj, the four strains of HBV fit nearer to Ba subgenotype with a similarity as high as 95-97% to Ba and 94-95% to Bj. Fig. 2-4 shows the phylogenetic tree which constructed based on the complete genome of the four isolates and HBV genotypes (i.e. A to H strains). Notably, HBV isolates of subtype Ba possess the recombination with genotype C over the precore region plus core gene, while those of subtype Bj do not (Sugauchi *et al.*, 2002). To verify the same issue in our samples, software SimPlot 2.5 was used to compare the entire sequences of our sample strains and Bj strain (AB014366) with genotype C strain (AB195956) nucleotide by nucleotide. Fig. 2-5 shows the results, which indicate a high similarity between the four samples and genotype C strain (AB195956) but comparatively lower similarity when compared with Bj strain (AB014366) and genotype C strain (AB195956) in nucleotide region nt1840 to nt2380. This may be the recombinant site for genotype B with genotype C which produces subgenotype Ba. Then, the sequences between nt1840 and nt2380 were compared between all of the four sample strains in this study and genotype A to H reference strains of HBV. As seen in Table 2-3, the nucleotide identity is very high (92-96%) between our sample strains and genotype C. Phylogenetic tree constructed based on nt1840 to nt2380 of Pre C and C region of HBV genomes is also shown in Fig. 2-6. The

phylogenetic tree shows that the four strains employed in this study are evolutionarily closer to genotype C than Bj subgenotype in this region. All of the above indicate that the four samples in our study are all belonged to genotype B and subgenotype Ba of HBV with recombination with genotype C from nt1840 to nt2380 in the Pre C and C region of HBV.

2.3.5 Features of the spliced variant of HBV

The sequence alignment of the spliced variant of HBV from H60 with the other four full sequences in this study is shown in Fig. 2-3. The deletion region is shown in hyphen. The spliced variant is 2494bp in length. It has three deletion regions: a 487bp deletion from nt2447 to nt2935, a deletion of 183bp from nt3019 to nt3203 and a deletion of 53bp from nt3215/nt1 to nt54. However, it has two more nucleotides "AG" in the position nt3215. It has complete Pre C, C, S and X genes but incomplete Pre S1, Pre S2 and the corresponding Terminal protein (TP), Spacer, DNA polymerase/Reverse transcriptase (Pol/RT) of Polymerase gene. Moreover, it has no S1 promoter and short S2 promoter, yet both the Enhancer I/X promoter and Enhancer II/Basal core promoters are complete. Furthermore, it has a direct repeat sequence of 5'-TTCACCTCTGC-3' for DR1 (nt1590-nt1600) and DR2 (nt1824-nt1834) and a complete 5'-end encapsidation signal (nt1853-nt1982). The schematic structure of the spliced variant is shown in Fig. 2-7.

2.4 Discussion

2.4.1 The relationship between HBV genotype and liver disease

As mentioned earlier, HBV is classified into 8 genotypes designated by the letters from A

to H (Okamoto *et al.*, 1988; Norder *et al.*, 1994; Stuyver *et al.*, 2000; Arauz-Ruiz *et al.*, 2002). Genotypes of HBV have distinct geographic distribution and they can influence the clinical course of hepatitis B. Genotype A is prevalent in Europe, Africa and South-East Asia, as well as the Philippines (Sugauchi *et al.*, 2002). Genotype B and C are predominant in Asia. Genotype D is common in the Mediterranean area, the Middle East and India. Genotype E is localized in sub-Saharan Africa. Genotype F and H are restricted to Central and South America. Moreover, Genotype G has been found in France, Germany and the United States (Chu *et al.*, 2003; Stuyver *et al.*, 2000; Vieth *et al.*, 2002). Since genotypes A and D frequently occur in Western countries, while genotypes B and C are common in Asia (Kao *et al.*, 2000; Orito *et al.*, 2001; Ding *et al.*, 2001) including China, Taiwan and Japan, clinical differences between genotype A and D, as well as B and C, have been studied extensively. To date, clinical courses could differ, even among the groups infected with the same genotypes. Emerging evidence has linked the more severe liver disease with longer duration of HBeAg (i.e. particularly genotype C HBV) in serum, accompanied by the delayed seroconversion of antibody to HBeAg (anti-HBe) (Lindh *et al.*, 1999; Kao *et al.*, 2002). Furthermore, HCC was also found to develop more frequent in patients infected with HBV genotype C than B (Kao *et al.*, 2000). In Taiwan, infection with HBV genotype B induces HCC at a much higher rate than that in Japan in adults younger than 50 years (Kao *et al.*, 2000; Orito *et al.*, 2001). This also happened in our study as our four sample strains also show very different clinical outcome despite the fact that they all belong to genotype B of HBV. In our study, the patients of younger age (i.e. 31 and 29 years old) all developed HCC, while the older subjects (i.e. 66 and 71 years old) were still AsCs. Other clinical differences between genotype B and C of HBV are also summarized in Table 2-4.

For genotype B, it was further classified into subgenotype namely from B1 to B5. Subgenotype B1 (Bj) is restricted to Japan with suffix 'j' standing for Japan; B2 (Ba) with the widest distribution in most Asian countries with suffix 'a' representing Asia; B3 is restricted to Indonesia; B4 is restricted to Vietnam; and B5 is restricted to Philippines (Liu *et al.*, 2007; Sugauchi *et al.*, 2002). Recombination between different genotypes occurs frequently in retroviruses, represented by human immunodeficiency virus type 1 (HIV-1) (Lole *et al.*, 1999). For RNA viruses, homologous recombination and template switching have been proposed as the mechanisms for intragenic recombination (Lole *et al.*, 1999). Recombination between HBV isolates of distinct genotypes has also been reported (Bollyky *et al.*, 1996; Morozov *et al.*, 2000), which may endow recombinants with a phenotype for virological characteristics or disease-inducing capacity distinct from those of parental genotypes. Examples for recombination events between HBV genotypes are listed in Table 2-5. The recombination between HBV isolates of distinct genotypes might be relevant to HBV replication, which involves the reverse transcription of an RNA intermediate (Summers *et al.*, 1982). Notably, HBV isolates of subtype Ba possess the recombination with genotype C over the precore region plus core gene, while those of subtype Bj do not (Sugauchi *et al.*, 2002). In this study, the four sample strains from Hong Kong are belonged to subgenotype Ba, yet with quite different pathological outcome. How does the recombination between genotype B and C occur, thus affect the clinical outcome, and why it is geographically distributing so differently are yet to be elucidated.

In previous years, researchers have found a higher frequency of HBeAg in serum (Kobayashi *et al.*, 2005; Sugauchi *et al.*, 2003) and the double mutations in the core promoter

(A1762T/G1764A) in the patients infected with subtype Ba than Bj (Sugauchi *et al.*, 2003). In view of the response to lamivudine, it is also poorer in patients infected with subtype Ba than Bj (Akuta *et al.*, 2003).

Mutations in the core promoter and precore region were known to down-regulate and abolish the synthesis of HBeAg, respectively, under the influence of HBV genotypes. The double mutations in the core promoter (A1762T/G1764A) are detected more frequent in the patients infected with genotype C than B and the point mutation in the precore (G1896A) is less frequent (Lindh *et al.*, 1999; Orito *et al.*, 2001). So far, if the core promoter region of subtype Ba is replaced by that of genotype C, it would be more prone to the mutation for A1762T/G1764A than that of subtype Bj. Since the A1762T/G1764A mutations are found to implicate hepatocarcinogenesis in HBV patients (Kao *et al.*, 2003), a high frequency of this mutation in subgenotype Ba infection would be responsible, at least in part, for Taiwanese HBV patients to develop HCC during early adulthood (Kao *et al.*, 2000; Sugauchi *et al.*, 2003). Another explanation is that putative cytotoxic-T-cell epitopes are presumed on the product of the HBV core gene (Chisari *et al.*, 1995). Since the recombination with genotype C in HBV isolates of genotype Ba was restricted to the pre C region as well as the core gene, cytotoxic-T cell epitopes of subgenotype Ba would be more efficiently expressed in them than in the HBV of the genuine genotype B (Bj) for enhancing the disease-inducing capacity.

2.4.2 Spliced variant of HBV

"Incomplete particles" were discovered during successive undiluted passages of the influenza viruses in 1940s (von Magnus, 1947). In general, the incomplete particles contain less

than one full-length genome and are replication defective. They can be rescued by, and interfere with, the replication of homologous helper viruses. Another important characteristic of incomplete particles is their ability to enrich their proportion in the total viral yield in cells infected with wild-type and incomplete viruses (Crick *et al.*, 1966; Holland *et al.*, 1987; Roux *et al.*, 1991). Based on these properties, Huang and Baltimore defined these biologically active incomplete particles as defective interfering particles (DIP) and the replication-competent homologous virions as standard viruses (Huang and Baltimore, 1970). DIPs are widespread among many DNA and RNA viruses in bacteria, plants, and animals. In tissue culture, DI viruses are capable of establishing persistent viral infections (Holland *et al.*, 1987). In animal models, some DI viruses have been shown to modulate the course of disease by attenuating the virulence of standard viruses (Barret and Dimmoch, 1986). Coinfection with DI RNA of tomato bushy stunt virus could modify the course of disease induced by the wild-type tomato bushy stunt virus infection (Hillman *et al.*, 1987). In humans, it has been proposed that DI particles might be responsible for the chronic recurrence of viral diseases. Perturbation of the balance between DI and standard viruses could trigger a new episode of disease manifestation (Huang and Baltimore, 1970).

For HBV, the DI particle is known as spliced variant. HBV has unspliced and spliced mRNAs. The spliced mRNAs may become spliced variant of HBV virus. Five unspliced messenger (mRNA) transcripts of 3.5kb (short and long), 2.4kb, 2.1kb, and 0.8 kb are synthesized from the HBV genome and encode for the capsid, envelope, polymerase, and transactivator X viral proteins (Seeger *et al.*, 2000). All these transcripts are unspliced, have different 5'-ends, and share the same 3'-ends located at the unique poly (A) site of the HBV

genome. Only the pregenomic RNA which contains a functional encapsidation signal at its 5'-end is encapsidated into the core particles and reverse transcribed into progeny HBV DNA (Ganem, 1996). In addition to unspliced HBV mRNA, singly and doubly spliced 2.2-kb mRNAs arised from 3.5-kb pregenomic mRNA have been identified in HBV DNA–transfected cell lines and in HBV-infected liver (Wu *et al.*, 1991; Chen *et al.*, 1989; Suzuki *et al.*, 1989, 1990; Su *et al.*, 1989; Günther *et al.*, 1997; Terré *et al.*, 1991; Huang *et al.*, 2000). On the other hand, Günther *et al.* also found 11 types of spliced genomes with heterogeneity and common features from sera of chronic carriers (Günther *et al.*, 1997) and 1.5kb to 2.2kb spliced genome variants of HBV with deletions and insertions in the C gene and Pre S region in immunosuppressed kidney transplant patients, who also have end-stage liver cirrhosis that was believed to be associated with an accumulation of complex variants (Günther *et al.*, 1995; Märschenz *et al.*, 2006). Similar C gene deletions have been found mainly in patients with chronic active hepatitis (Ackrill *et al.*, 1993; Takayanagi *et al.*, 1993; Wakita *et al.*, 1991). It was therefore postulated that core proteins with deletions may alter the immune recognition and might be important for T-cell-mediated liver damage. Suzuki *et al.* found spliced RNA of 0.7kb, 1.1kb, 1.7kb, 2.0kb, 2.1kb and 2.3kb from HepG2 cell line transfected with HBV genome (Suzuki *et al.*, 1989). Wu *et al.* found a singly spliced transcript in hepatoma cell lines transfected with cloned viral DNA and a doubly spliced one in naturally infected human liver tissue, yet those spliced transcripts were suggested unnecessary for HBV replication (Wu *et al.*, 1991). Sommer *et al.* found a new kind of HBV genomes with a 60 to 100 internal poly (A) sequence. The 5'-ends of the internal poly (A) sequences are located at the authentic poly (A) sites of the RNA pregenome, whilst the positions of the 3'-ends vary due to different sizes of

vicinal deletions (Sommer *et al.*, 1997). Similar spliced variants were also found in HIV genomes (Vartanian *et al.*, 1997).

Splicing of the pregenomic RNA is not unique for HBV but was also observed for the related woodchuck (WHBV) and duck (DHBV) hepatitis viruses (Hantz *et al.*, 1992; Ogston *et al.*, 1996). Moreover, a spliced version of the DHBV pregenomic RNA was shown to be essential for productive infection of ducks but dispensable for virus replication when DHBV DNA was introduced into hepatoma cell lines by transfection (Obert *et al.*, 1996). Similarly, the major types of spliced pregenomic HBV RNAs are dispensable for virus replication when tested by transfection of cells with HBV DNA (Su *et al.*, 1989; Wu *et al.*, 1991), but an *in vivo* function is not excluded. In fact, it was suggested that defective genomes derived from the major singly spliced RNA (2447/489) might play crucial roles in virus persistence, as they were found predominantly in the sera of patients with chronic infection and less frequently in those with acute self-limited course (Rosmorduc *et al.*, 1995). In addition, these genomes were shown to express with a much higher levels of HBc, HBe and HBx proteins than the wild-type genome in transfection experiments. These proteins are crucial components for immune recognition of the virus, viral persistence, and pathogenicity (Rosmorduc *et al.*, 1995). Recently, Marschenz *et al.* carried out a co-replication analysis of naturally occurred defective HBV variants with wild-type HBV. His team found that the variants shows enhanced replication and enrichment in different transfected variant-wt HBV mixtures both in Huh 7 and HepG2 cells (Märschenz *et al.*, 2008). Günther *et al.* have also shown that spliced mRNA is packaged, reverse transcribed, and thus leads to the secretion of defective viral particles (Günther *et al.*, 1995, 1997; Terré *et al.*, 1991; Rosmorduc *et al.*, 1995; Sommer *et al.*, 2000; Soussan *et al.*, 2008). Furthermore, an

elevated dHBV (defective HBV)/HBV ratio was found associated with liver necroinflammation and fibrosis disease, suggesting that the dHBV expression was accompanied by severer state of liver disease (Soussan *et al.*, 2008).

In this study, a new type of spliced variant of HBV, which is 2494bp in length, was found in the HCC patient, H60. Although three deletion regions were found in the sample strain, it has complete Pre C, C, S and X genes. The Enhancer I/X promoter, Enhancer II/Basal core promoter, DR1, DR2 and the 5'-end encapsidation signal are all complete. Therefore, there is a possibility that it could encapsidate into the functional HBV particle, and then induces certain extent of liver disease including cirrhosis and HCC. However, further investigations will be necessary to clarify this postulation.

Table 2-1. Primers for HBV full sequence PCR and sequencing

Primer	Nucleotide sequence (5'-3')	Position (nt)	Polarity
HBV-P1	CACCTCTGCCTAATCATCTCWTGTTCATG	1826-1854	Sense
HBV-P2	AAAAAGTTGCATGGTGCTGGTGAACRCAC	1825-1797	Antisense
HBV-P3	AGAAAGGCCTTGTAAGTTGGC	1121-1101	Antisense
HBV-P4	GGGTCACCATATTCTTGGGAACAAGA	2814-2839	Sense
HBV-P5	AGTGTGGATTCGCACTCCT	2269-2287	Sense
M13F	TGTAAAACGACGGCCAGT	in the vector	Antisense
M13R	CAGGAAACAGCTATGACC	in the vector	Sense

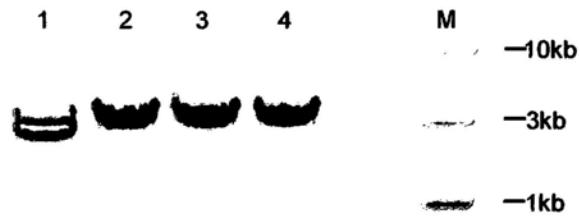

Fig.2-1. Agarose gel analysis of HBV genome PCR products. 1. H60. 2. H293. 3. C265. 4. C1691. M. Promega 1kb DNA Ladder.

Fig.2-2. Agarose gel analysis of pCR2.1-TOPO-HBV by EcoR I digestion.1. H60. 2. H293. 3. C265. 4. C1691. 5, H60-2.5kb. M. Promega 1kb DNA Ladder.

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                  *        20        *        40        *        60        *        80
C265       : CTCCACCACTTTCCACCAAACTCTTCAGGATCCCAGAGTCAGGGCCCTGTACTTTCCTGCTGGTGGCTCCAGTTCAGAAA :   80
C1691      : ........AG.................A.........................A.......................G.. :   80
H60        : ........................C..A.........................C.......................G.. :   80
H293       : ............................A.....................................................G.. :   80
H60-2.5kb  : --------------------------------------------------.....................G.. :   27

                  *       100        *       120        *       140        *       160
C265       : CAGTGAGCCCTGCTCAGAATACTGTCTCTGCCATATCGTCAATCTTATCGAAGACTGGGGACCCTGTACCGAACATGGAG :  160
C1691      : ....A.....................................................................C..... :  160
H60        : ..............................................C..................................... :  160
H293       : ................................................................................ :  160
H60-2.5kb  : ..............................................C..................................... :  107

                  *       180        *       200        *       220        *       240
C265       : AGCATCGCATCAGGACTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAAAATCCTCACAATACC :  240
C1691      : .A.....................................................C.......................... :  240
H60        : .A............................................................................. :  240
H293       : .A....T.................................G.............C.......................... :  240
H60-2.5kb  : .A.................................G.....................................................  187

                  *       260        *       280        *       300        *       320
C265       : ACAGAGTCTAGACTCGTGGTGGACTTCTCTCAGTTTTCTAGGGGGAACACCCGTGTGTCTTGGCCAAAATTCGCAGTCCC :  320
C1691      : ..............................G......C......G.................................... :  320
H60        : ................................................................................ :  320
H293       : ..............................A...........A..T.................................... :  320
H60-2.5kb  : ..............................................A..................................... :  267

                  *       340        *       360        *       380        *       400
C265       : AAATCTCCAGTCACTCACCAACCTGTTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTTTCTGCGGCGTTTTATCATC :  400
C1691      : .....................................................G.......................... :  400
H60        : ..................................C.....................C..................... :  400
H293       : C.....................................................C......G..................... :  400
H60-2.5kb  : ..................................C.....................A..................... :  347

                  *       420        *       440        *       460        *       480
C265       : TTCCTCTGCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCT :  480
C1691      : .....................................................C.......................... :  480
H60        : ................................................................C.....          :  480
H293       : ................................................................................ :  480
H60-2.5kb  : ................................................................................ :  427

                  *       500        *       520        *       540        *       560
C265       : AATTCCAGGATCATCAACAACCAGCACCGGACCATGCAAAACCTGCACGACTCCTGCTCAAGGAACCTCTATGTTTCCCT :  560
C1691      : ................................................................................ :  560
H60        : ..................................G........AG.............................. :  560
H293       : ..............................................AG..............T............... :  560
H60-2.5kb  : ..................................G........A.............................. :  507

                  *       580        *       600        *       620        *       640
C265       : CATGTTGCTGTACAAAACCTACGGACGGAAACTGCACCTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTA :  640
C1691      : ..............................................................................T.... :  640
H60        : ...........G.................................................................... :  640
H293       : ................................................................................ :  640
H60-2.5kb  : ..............................................T..................................... :  587

                  *       660        *       680        *       700        *       720
C265       : TGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGATTCGTAGGGCTTTCCCCCAC :  720
C1691      : ..............................................G...........G..................... :  720
H60        : ..............................................................G..................... :  720
H293       : ..........................................T..............G..................... :  720
H60-2.5kb  : ..................................C.....................G..................... :  667

                  *       740        *       760        *       780        *       800
C265       : TGTCTGGCTTTTAGTTATATGGATGATGTGGTTTTGGGGGCCAAATCTGTACAACATCTTGAGTCCCTTTATGCCGCTGT :  800
C1691      : ...........C.....................A...........GA.................................. :  800
H60        : ...........C.................................G.........................A....... :  800
H293       : ...........C.................................G.........................A....... :  800
H60-2.5kb  : ...........C.................................GA........................A....... :  747
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                  *       820         *       840         *       860         *       880
C265       : TACCAATTTTCTTTTGTCTTTGGGTATACATTTAAACCCTCACAAAACAAAAAAGATGGGGATATTCCCTTAACTTCATGG :  880
C1691      : ...TC...C.................................................................... :  880
H60        : ................................................................................ :  880
H293       : ................................................................................ :  880
H60-2.5kb  : ................................................................................ :  827

                  *       900         *       920         *       940         *       960
C265       : GATATGTAATTGGGAGTTGGGGCACATTGCCACAGGAACATATTGTACAAAAAATCAAAATGTGTTTTAGGAAACTTCCT :  960
C1691      : ..........................................................C..................... :  960
H60        : ..................................................C.............G...... :  960
H293       : ..........................................................C..................... :  960
H60-2.5kb  : ..................................................C.............G...... :  907

                  *       980         *      1000         *      1020         *      1040
C265       : GTAAACAGGCCTATTGATTGGAAAGTATGTCAACGAATTGCGGGTCTTTTGGGGTTCGCCGCCCCTTTCACGCAATGTGG : 1040
C1691      : .....................................T.........A.....T.............A........ : 1040
H60        : .....................................T......G........T.................... : 1040
H293       : .....................................T.............T.....A.................. : 1040
H60-2.5kb  : .....................................T.............T.....................  :  987

                  *      1060         *      1080         *      1100         *      1120
C265       : CTATCCCGCTTTAATGCCTTTATATGCATGTATACAAGCAAAACAGGCTTTTACTTTCTCGCCAACTTACAAGGCCTTTC : 1120
C1691      : A.....T.............................T.................T...........T.......... : 1120
H60        : A.....TA..................................................................... : 1120
H293       : A.....T.................................G.........C.......................... : 1120
H60-2.5kb  : A.....T..................................................................... : 1067

                  *      1140         *      1160         *      1180         *      1200
C265       : TAAGTAAACAGTATCTGAACCTTACCCCCGTTGCTCGGCAACGGCCTGGTCTGTGCCAAGTGTTTGCTGACGCAACCCCC : 1200
C1691      : .G.....................TA.................................................... : 1200
H60        : .......................TA.................................................... : 1200
H293       : .CC..G........T........TA........T.......................................... : 1200
H60-2.5kb  : .......................TA.................................................... : 1147

                  *      1220         *      1240         *      1260         *      1280
C265       : ACTGGTTGGGGCTTGGCCATAGGCCATCAGCGCATGCGTGGAACCTTTGTGTCTCCTCTGCCGATCCATACTGCGGAACT : 1280
C1691      : ....................T..................................................... : 1280
H60        : ................................................................................ : 1280
H293       : ................................................................................ : 1280
H60-2.5kb  : .....................................G.......................................... : 1227

                  *      1300         *      1320         *      1340         *      1360
C265       : CCTAGCCGCTTGTTTTGCTCGCAGCAGGTCTGGGGCAAAACTCATCGGGACTGACAATTCTGTCGTGCTCTCCCGCAAGT : 1360
C1691      : .T....A.................C.......A.............................................A. : 1360
H60        : ........................C....................................................... : 1360
H293       : ...................................G............................................ : 1360
H60-2.5kb  : ........................C....................................................... : 1307

                  *      1380         *      1400         *      1420         *      1440
C265       : ATACATCATTTCCATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTGCGCGGGACGTCCTTTGTTTACGTCCCGTCGGCG : 1440
C1691      : ....G.......................................................................... : 1440
H60        : ................................................................................ : 1440
H293       : .C............................................................................. : 1440
H60-2.5kb  : ..........................................A..................................... : 1387

                  *      1460         *      1480         *      1500         *      1520
C265       : CTGAATCCCGCGGACGACCCCTCTCGGGGCCGCTTGGGGCTCTACCGCCCGCTTCTCCGCCTGTTGTGCCGACCGACCAC : 1520
C1691      : ......................C.....................................C.A.............. : 1520
H60        : ..........................................................A.............. : 1520
H293       : ......................C.................C.....................A.............. : 1520
H60-2.5kb  : ..........................................................A.............. : 1467

                  *      1540         *      1560         *      1580         *      1600
C265       : GGGGCGCACCTCTCTTTACGCGGACTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGC : 1600
C1691      : ................................................................................ : 1600
H60        : ...A.....................................................................T..... : 1600
H293       : ................................................................................ : 1600
H60-2.5kb  : ................................................................................ : 1547
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                  *      1620         *      1640         *      1660         *      1680
C265       : ACGTCGCATGGAGACCACCGTGAACGCCCACGGGAACCTGCCCAAGGTCTTGCATAAGAGAACTCTTGGACTTTCATCCA : 1680
C1691      : ..............................C.....T.....................G..............GTA. : 1680
H60        : ..............................................................G..............G.A. : 1680
H293       : ............A..............C..............................G..............G.A. : 1680
H60-2.5kb  : ..............................................................G..............G.A. : 1627

                  *      1700         *      1720         *      1740         *      1760
C265       : TGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGTGTGTTTACTGAGTGGGAGGAGTTGGGGGTGGAGGTTAGGTTA : 1760
C1691      : ..................................................................A.......A....A..... : 1760
H60        : ........................T.........................A.............A.............. : 1760
H293       : ..................................................A.....................A..... : 1760
H60-2.5kb  : ........................T.....................................A.............. : 1707

                  *      1780         *      1800         *      1820         *      1840
C265       : AAGGTCTTTGTACTAGGAGGCTGTAGGCATAAATTGGTGCGTTCACCAGCACCATGCAACTTTTTCACCTCTGCCTAATC : 1840
C1691      : ........................................T..................................... : 1840
H60        : .T.A..................................................................... : 1840
H293       : .T.A.....................................T..................................... : 1840
H60-2.5kb  : .T.A..................................................................... : 1787

                  *      1860         *      1880         *      1900         *      1920
C265       : ATCTCATGTTCATGTCCTACTATTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTAGGGCATGGACATCGACCCGTATAA : 1920
C1691      : ......................G...........G.................................T.......... : 1920
H60        : ......................G.C.............................G............T.......... : 1920
H293       : ......................G.............................G..A.........T...A.C..... : 1920
H60-2.5kb  : ......................G.............................G..A.........T.......... : 1867

                  *      1940         *      1960         *      1980         *      2000
C265       : AGAATTTGGAGCTTCTGTGGAGTTACTCTCTTTTTTGCCTTCTGACTCCTTTCCTTCTATTCGAGATCTCCTCGACACCG : 2000
C1691      : ................C...A..GA..............G......T..........G.............T....... : 2000
H60        : ..........................................T................................. : 2000
H293       : ..........................................TT................................ : 2000
H60-2.5kb  : ..........................................T......G.............C........... : 1947

                  *      2020         *      2040         *      2060         *      2080
C265       : CCTCTGCTCTGTATCGGGAGGCCTTAGAGTCTCCGGAACATTGTTCACCTCACCATACGGCAATCAGGCAAGCTATTCTG : 2080
C1691      : ................................A.........A.............A..................... : 2080
H60        : ........T.................................................C.................. : 2080
H293       : ........................................A...................C..............G.. : 2080
H60-2.5kb  : ........T.....................................................C.................. : 2027

                  *      2100         *      2120         *      2140         *      2160
C265       : TGTTGGGGTGAGTTGATGAATCTAGCCACCTGGGTGGGAAGTAATTTGGAACATCCAGCATCCAGGGAATTAGTAGTCAG : 2160
C1691      : ....................................TG..........CG..........A..A............G. : 2160
H60        : ..............A.....................................G.......................... : 2160
H293       : ..............A.....................................G.........................T.. : 2160
H60-2.5kb  : ..C...........A.....................................G.......................... : 2107

                  *      2180         *      2200         *      2220         *      2240
C265       : CTATGTCAACGTTAATATGGGCTTAAAATTCAGACAACTATTGTGGTTTCACATTTCCTGTCTTACTTTTGGAAGAGAAA : 2240
C1691      : ......................C..............A.T.................A.....C........G....CC. : 2240
H60        : ......................C.....A.....................................G....... : 2240
H293       : ....................AC.....A.....................................G...C... : 2240
H60-2.5kb  : ......................C.....A.....................................G....... : 2187

                  *      2260         *      2280         *      2300         *      2320
C265       : CTGTTCTTGAATATTTGGTGTCTTTTGGAGTGTGGATTCGCACTCCTCCTGCATATAGACCACAAAATGCCCCTATCTTA : 2320
C1691      : ..................................................................G.............. : 2320
H60        : ..........................................................................GC.. : 2320
H293       : T.........................................................................C.. : 2320
H60-2.5kb  : .C...AC.....................................................................GC.. : 2267

                  *      2340         *      2360         *      2380         *      2400
C265       : TCAACACTTCCGGAAACTACTGTTGTTAGACCAAGAGGCAGGTCCCTTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG : 2400
C1691      : ................................T........C.................................... : 2400
H60        : ............G.............G.........A...C.................................... : 2400
H293       : ............................A.............C.................................... : 2400
H60-2.5kb  : ............G............TGC.........A...C.................................... : 2347
```

```
                  *      2420         *      2440         *      2460         *      2480
C265       : GTCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAACCCCAATGTTAGTATTCCTTGGACACATAAGGTGGGAAAC : 2480
C1691      : ......................C...................T............C........T.............. : 2480
H60        : ........................................T.T..................................... : 2480
H293       : ............................A..........CT.T..................................... : 2480
H60-2.5kb  : ........................................T.T....------------------------------- : 2394

                  *      2500         *      2520         *      2540         *      2560
C265       : TTTACTGGGCTTTATTCTTCTACGGTACCTTGCTTTAATCCTAACTGGCAAACTCCTTCTTTTCCTGACATTCATTTGCA : 2560
C1691      : .....G.....................................T.....................A.............. : 2560
H60        : .....G.....................................................................A. : 2560
H293       : .....G..............G.....................T......C............T..............G : 2560
H60-2.5kb  : ------------------------------------------------------------------------------ :    -

                  *      2580         *      2600         *      2620         *      2640
C265       : GGAGGACATTGTTGATAGATGTAAGCAATTTGTGGGACCCCTTACAGTCAATGAAAACAGGAGACTAAAATTAATTATGC : 2640
C1691      : ......................G.............G.....CG....A........AG..................... : 2640
H60        : ........................................G............A............A.................. : 2640
H293       : ........................................G.........T.A.......C....................... : 2640
H60-2.5kb  : ------------------------------------------------------------------------------ :    -

                  *      2660         *      2680         *      2700         *      2720
C265       : CTGCTGGGTTTTATCCCAATGGTACTAAATATTTTCCCTTAGATAAAGGGATCAAACCGCATTATCCAGAGTATGTAGTT : 2720
C1691      : .....A..............T...C........G.......................TT..........AC........ : 2720
H60        : .....A..............T............G.........A..............T..................... : 2720
H293       : .C...A..............TC...........G.......................T...........A........ : 2720
H60-2.5kb  : ------------------------------------------------------------------------------ :    -

                  *      2740         *      2760         *      2780         *      2800
C265       : AATCATTACTTCCAGACGCGACATTATTTACACACTCTTTGGAAGGCGGGGATCTTATATAAAAGAGAATCCACACGTAG : 2800
C1691      : ..................A.................................T.........C.........G.......... : 2800
H60        : ..................................................................G.......... : 2800
H293       : ..............................A.T.....................................G.......... : 2800
H60-2.5kb  : ------------------------------------------------------------------------------ :    -

                  *      2820         *      2840         *      2860         *      2880
C265       : CGCCTCATTTTGCGGGTCACCATATTCTTGGGAACAAGATCTACAGCATGGGAGGTTGGTCTTCCAAACCTCGAAAAGGC : 2880
C1691      : ..............................................................................  : 2880
H60        : ..................................................C........................... : 2880
H293       : ..............................................................................  : 2880
H60-2.5kb  : ------------------------------------------------------------------------------ :    -

                  *      2900         *      2920         *      2940         *      2960
C265       : ATGGGGACAAATCTTTCTGTCCCCAATCCCCTGGGATTCTTCCCCGATCATCAGTTGGACCCTGCATTCAAAGCCAACTC : 2960
C1691      : ..............................................................................  : 2960
H60        : ..............................................................................  : 2960
H293       : ..............................................................................  : 2960
H60-2.5kb  : ----------------------------------------------------------..................... : 2420

                  *      2980         *      3000         *      3020         *      3040
C265       : AGAAAATCCAGATTGGGACCTCAACCCGCACAAGGACAACTGGCCGGACGCCAACAAGGTGGGAGCGGGAGCATTCGGGC : 3040
C1691      : ...................T.....................................................T.............. : 3040
H60        : ..................................................T..............T.............. : 3040
H293       : ...................T.....A.....................A..............T.........T.... : 3040
H60-2.5kb  : ..........................................A..............-------------------- : 2479

                  *      3060         *      3080         *      3100         *      3120
C265       : CAGGGGTCACTCCTCCCCATGGGGGACTGTTGGGGTGGAGCCCTCAGGCTCAGGGCCTACTCACAACTGTGCCAGCAGCT : 3120
C1691      : .....T.............................G.............C......A..T....C.............. : 3120
H60        : .....T....C.................................................................. : 3120
H293       : .....T....C....TT............................................................. : 3120
H60-2.5kb  : ------------------------------------------------------------------------------ :    -

                  *      3140         *      3160         *      3180         *      3200
C265       : CCTCCTCCTGCCTCCACCAATCGGCAGTCAGGAGGGCAGCCTACTCCCTTATCTCCACCTCTAAGGGACACTCATCCTCA : 3200
C1691      : ................................A............................................... : 3200
H60        : ................................A.......A....................................... : 3200
H293       : ................................A............................................... : 3200
H60-2.5kb  : ------------------------------------------------------------------------------ :    -
```

```
C265       : GGCCATGCAGTGGAA-- : 3215
C1691      : .....C.........-- : 3215
H60        : ...............-- : 3215
H293       : ...............-- : 3215
H60-2.5kb  : --.............AG : 2494
```

Fig.2-3. Sequence alignment of four HBV full genome sequences and one 2.5kb spliced variant from H60. Dot represents the identical nucleotide as above and hyphen for deletion.

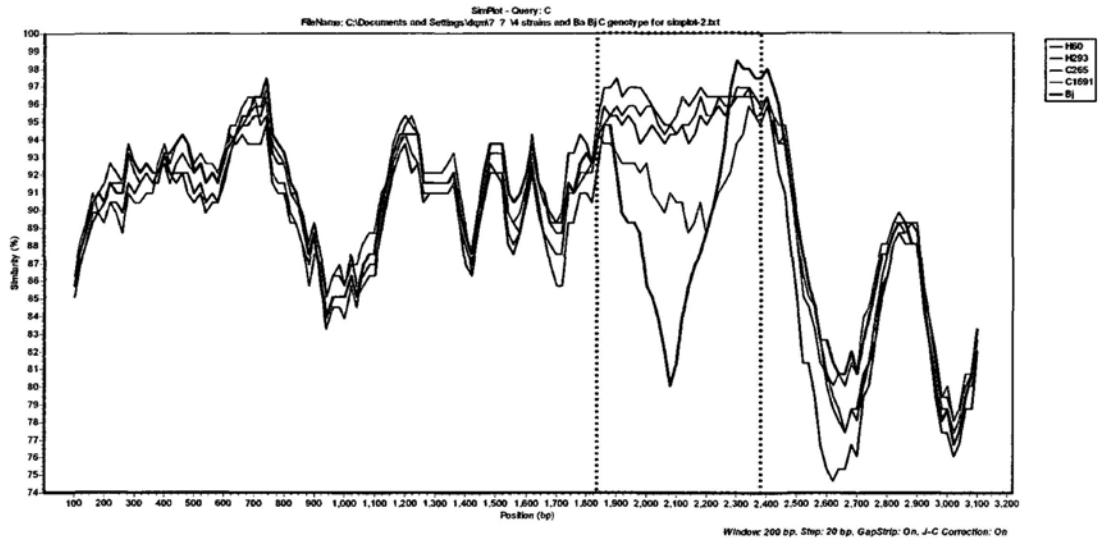


Fig.2-5. Comparison of nucleotide similarity for full genomes of four HBV isolates in this study and genotype Bj strain (AB014366) with reference genotype C strain (AB195956) conducted with SimPlot program and bootscanning procedure.

Table 2-2. Similarity of nucleotide over the complete genome among the four isolates in this study with different HBV genotypes

	A	*Ba*	*Bj*	C	D	E	F	G	H
H60	90	***96-97***	***94-95***	90-91	87-88	87-88	83-86	86	85
H293	89	***96-97***	***94***	90	86-87	87	83-85	85-86	85
C265	89-90	***96-97***	***94***	90	87	87-88	83-85	85	85
C1691	89	***95-96***	***94***	90	87-88	87	83-85	85	84-85

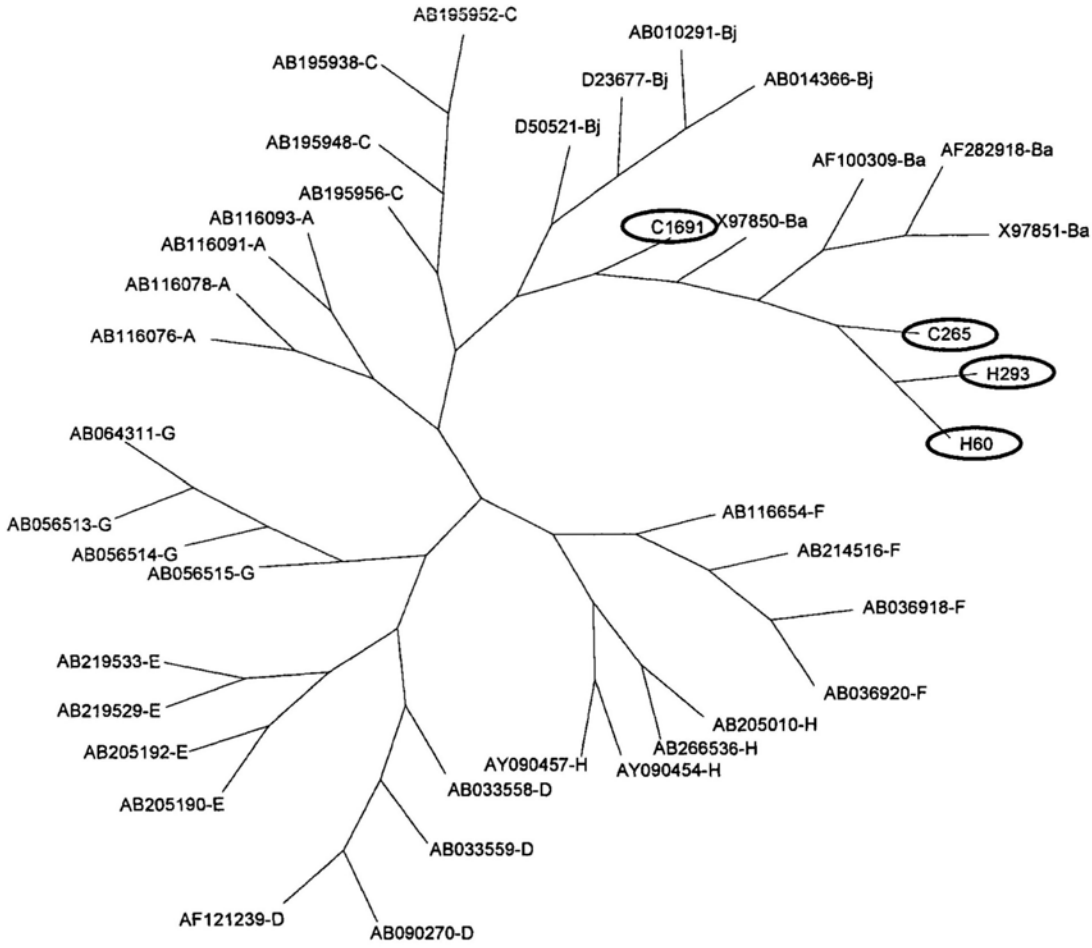


Fig. 2-4. Phylogenetic tree constructed on the complete genome of the four isolates in this study and HBV genotype A to H strains.

Table 2-3. Similarity of nucleotide over nt1840 to nt2380 of PreC and C region of HBV genome among the four isolates in this study with different HBV genotypes

	A	*Ba*	*Bj*	C	D	E	F	G	H
H60	87	***97-98***	***89-90***	94-96	88-90	87-90	74-87	84	86-87
H293	86	***95-97***	***88-89***	94-95	97-89	87-88	73-87	83	85-86
C265	86-87	***96-97***	***88-89***	94-95	88-89	87-89	73-86	83	85
C1691	84-85	***93-94***	***87***	92	86-88	86-87	72-85	81-82	84

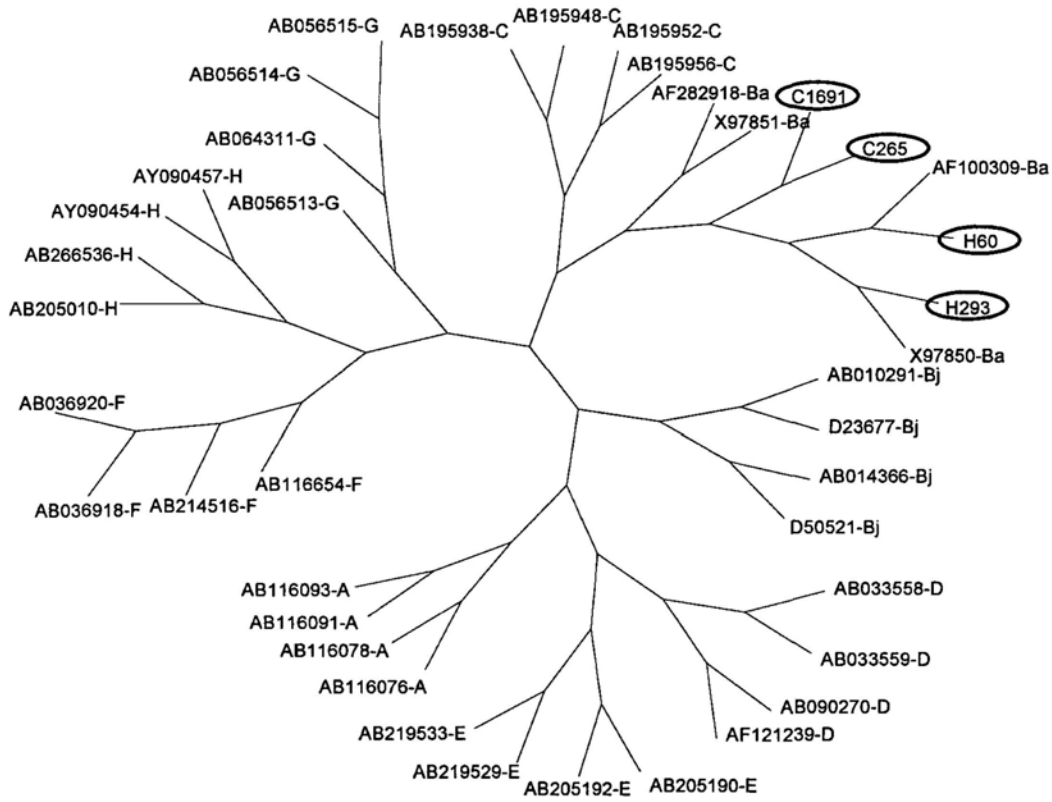


Fig. 2-6. Phylogenetic tree constructed on nt1840 to nt2380 of Pre C and C region of HBV genome of the four isolates in this study and HBV genotype A to H strains.

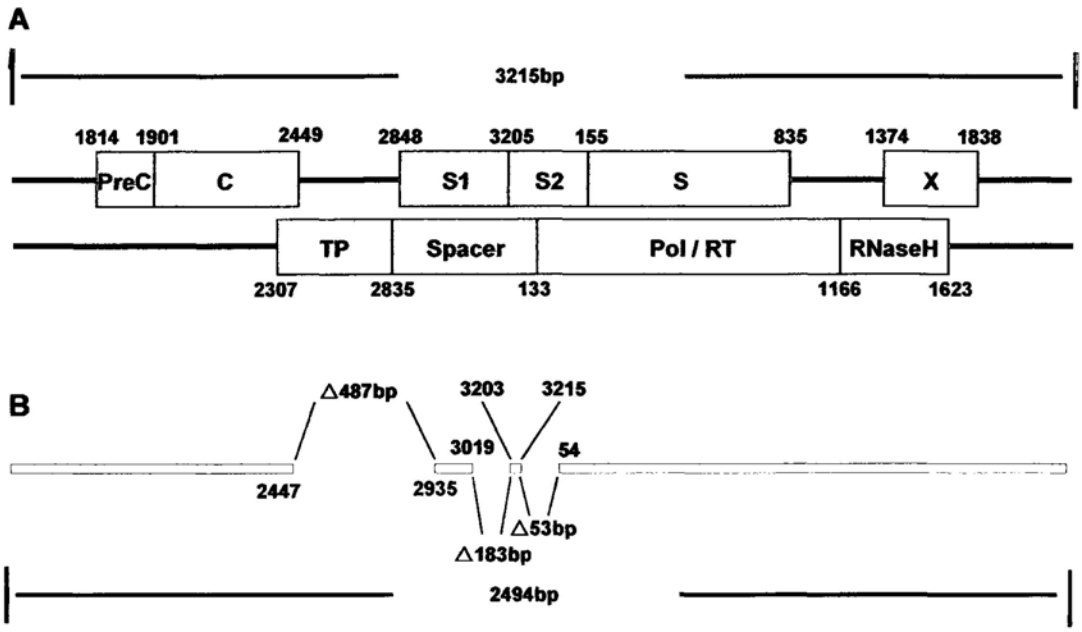


Fig. 2-7. Gene organization of full-length and 2.5kb spliced variant of HBV DNA genome. A: Full-length HBV DNA. The numbers show the nucleotide position bordering certain ORF of HBV. P gene overlaps with the C, S and X genes and consists of four functional regions, namely Terminal protein (TP), Spacer, DNA polymerase/Reverse transcriptase (Pol/RT) and RNaseH. B: 2.5kb spliced HBV DNA from H60. △: Indicates the deleted region. The ORF and the nucleotide position is the same as panel A.

Table 2-4. Clinical and virological differences between genotype B and genotype C (Kao *et al.*, 2002)

Items	Genotype C	Genotype B
Positivity of HBeAg	More	Less
Immune clearance phase	Longer	Shorter
Severity of acute exacerbations	More	Less
Histological activity	Higher	Lower
Serum HBV DNA level	Higher	Lower
Frequency of precore stop codon mutation	Less	More
Frequency of basal core promoter mutation	More	Less
Response to antiviral treatment	Worse	Better
Clinical outcome (cirrhosis and liver cancer)	Worse	Better (Japan, China), variable (Taiwan)

Table 2-5. Examples for recombination events between of HBV genotypes (Schaefer, 2007)

Genotype of		Recombination Breakpoint	
Backbone	Insert	5'	3'
A	C	1801	2865
A	D	2895	327
		2820	386-586
		?	670
B	C	1740-1838	2443- 2485
B	C	3120	3171
		3060	3191
		2910	2950
C	B	1731-1838	2437- 2479
D	A	129	2339
		495	780
		822	1775
G	C	1860	2460
A	E	882	1060

Chapter 3

Construction of HBV-1.5X for four HBV strains

3.1 Introduction

The genome of HBV is a relaxed-circular, partially duplex DNA of 3.2kb (Summers *et al.*, 1975), which encodes four overlapping ORFs (pre S/S, pre C/C, X and P) and 5 RNA of 3.9kb, 3.5kb, 2.4kb, 2.1kb and 0.7kb, thus finally generate 7 viral proteins. The transcript responsible for virus replication is the pregenomic RNA (pgRNA), either 3.9kb or 3.5kb in length, encompassing the entire genome length plus a terminal redundancy of about 120 nt that contains a second copy of the direct repeat 1 (DR1) and the ε signal, plus the poly (A) tail. Its primary essential role is to act as mRNA for the core protein and the reverse transcriptase. In addition, pgRNA is the template for generating new DNA genomes by reverse transcription (Beck *et al.*, 2007). During the life cycle of HBV, the virus enters the hepatocytes and the viral genome is transported into the nucleus in which the cccDNA is generated by repairing the partially double-stranded genome, whereupon viral transcription occurs (Tuttleman *et al.*, 1986; Ghany *et al.*, 2007). Then, encapsidation of the pgRNA occurs in the cytoplasm via a complex interaction between epsilon, core particles, HBV polymerase, and various chaperone proteins. At last, negative and positive strand synthesis occurs within the viral nucleocapsid through reverse transcription (Pollack *et al.*, 1994).

Previous studies showed that HBV enters and replicates in primary fetal human hepatocytes (Ochiya *et al.*, 1989) as well as in normal human adult hepatocytes in the presence

of DMSO (Gripon *et al.*, 1988). However, no mammalian hepadnaviruses including HBV have ever been found possible to propagate *in vitro* (i.e. cell culture). Furthermore, the underlying mechanism of how the HBV start the invasion is not yet elucidated. So the development of improved antiviral drugs has been considerably limited. In fact, chimpanzee is the only animal species that is fully permissive and well tested for HBV infection. However, chimpanzee experiments are limited by cost, availability, and ethical considerations. Then enormous progresses in HBV research have been made using avian and mammalian HBV-related viruses, which offer ample opportunities for studies in naturally occurring hosts. But the HBV-related viruses are also considered to possess some kind of differences from HBV. Therefore, some human hepatoma cell lines were generated which hopefully would support the replication of HBV DNA and virion formation when they were transfected with constructs with a redundant HBV genome (Ganem *et al.*, 1987). These constructs include HBV 1.1, 1.2 and 1.3, etc. which have 1.1, 1.2 and 1.3 copies of HBV genome (Guidotti *et al.*, 1995). The structures of these constructs are shown in Fig. 3-1. The HBV 1.1, 1.2 and 1.3 genome constructs shared a common 3′ terminus at nt1982, just downstream of the unique poly (A) site in the HBV genome, while they differed in their overall length by positioning their 5′ termini either at the upstream of the nucleocapsid promoter and enhancer II (1.1 genome), in the middle of the X gene (1.2 genome), or upstream to the X promoter and enhancer I (1.3 genome) (Guidotti *et al.*, 1995). Guidotti *et al.* reported that a high level of HBV replication has been achieved in the liver and kidney tissues with three independent lineages containing the 1.3 genome construct while only low-level, kidney-preferential replication occurred with the shorter constructs (Guidotti *et al.*, 1995; Araki *et al.*, 1989). Yang *et al.* carried out hydrodynamic injection of viral DNA HBV

1.3 and constructed a mouse model of acute HBV infection (Yang *et al.*, 2002). The HBV 1.3 is regarded differently from 1.1 or 1.2, as it has an extra copy of the entire HBV enhancer I at the 5' end of the integrated transgene. This factor might explain for the higher level of HBV gene expression in the liver of mouse model.

We have four strains of HBV isolated from two HCC patients and two AsCs. To study the characteristics of the four viruses and the interaction between these viruses and hepatocelluar cells, the first and most important step is to construct an *in vitro* system which can support virus replication and produce virions to mimic the HBV life cycle. In this study, a construct termed as HBV 1.5X which is longer than the HBV 1.3 redundant HBV genomes was generated in order to give a higher level of HBV production. This was helpful as it facilitated the surveillance of the pathogenesis of different HBV strains, which might also be useful for other HBV studies in the near future.

3.2 Materials and methods

3.2.1 HBV full genome cloning

Please refer to Chapter 2, section 2.2.4, on page 32.

3.2.2 Construction of HBV-1.5X

Fig. 3-2 shows the workflow of the present study. All the primers used in this study are listed in Table 3-1. All the restriction endonucleases were from NEB (MA, USA).

3.2.2.1 T-A cloning of HBV full sequence into pCR 2.1-TOPO

Please refer to Chapter 2, sections 2.2.2, 2.2.3 and 2.2.4 for details on page 32. The Primer P1 was the same as HBV-P1 and the Primer P2 was the same as HBV-P2.

3.2.2.2 Self-ligation of HBV full sequence

Two 5'-end phosphated primers 5-P-HBV-1826S and 5-P-HBV-1825A were used for full sequence amplification. The condition was the same as that mentioned in Chapter 2, section 2.2.3, on page 32. The PCR products were then self-ligated to form HBV cccDNA using T4 DNA ligase (NEB, MA, USA).

3.2.2.3 Cloning of two fragments from HBV cccDNA

By the use of the same sense primer HBV 715S but different antisense primers HBV 2325A and HBV 2844A, two fragments of 1.6kb and 2.1kb, respectively, were amplified. The conditions were the same as that mentioned in Chapter 2, section 2.2.3, on page 32, except that the extension time was a little shorter that was 4 minutes. The two fragments were then cloned into pCR 2.1-TOPO vector for later construction.

3.2.2.4 Site-directed mutation to delete Kpn I site in pCR 2.1-TOPO vector

Since there is a unique Kpn I site at the position nt2509 in all of our full HBV sequences and we would use this unique site in the later step, the Kpn I site was chosen as the deletion target in the multiple cloning site of pCR 2.1-TOPO vector using primers Kpn I Mutation F and Kpn I Mutation R. Site-directed mutagenesis was performed using a one step muatagenesis kit (GeneTailor Site-Directed Mutagenesis System, Invitrogen, CA, USA) according to

manufacturer's instructions.

3.2.2.5 Ligation of HBV full sequence with nt715 to nt2325 fragment

The HBV full sequences were ligated with fragments nt715 to nt2325, thus formed a 3.7kb sequence of HBV at the sites of Aat II (position nt1419 in the HBV sequence) and Sac I (in the multiple cloning site of pCR 2.1-TOPO).

3.2.2.6 Ligation of HBV 3.7kb sequence with nt715 to nt2844 fragment

By acting on the unique Kpn I (position nt2509 in the HBV sequence) and Xho I (in the multiple cloning site of pCR 2.1-TOPO) sites, the HBV 3.7kb sequences were ligated with fragments nt715 to nt2844 which formed a 4.8kb sequence of HBV that was named HBV-1.5X. HBV-1.5X meant one and a half times of HBV genome.

3.2.2.7 Restriction endonuclease digestion of HBV-1.5X

For the verification of the right clones of HBV-1.5X, single digestion with Xho I and double digestion with Xho I and KpnI restriction endonucleases were performed.

3.2.3 Cell culture and transfection

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum under 5% CO_2 incubation at 37°C. DNA was transfected into the HepG2 cells using Lipofectamine 2000 (Invitrogen, CA, USA) and the cells were harvested after 24h and 48h post transfection. The final amount of plasmid DNA was 4 μg per

transfection in each well of a 6-well plate. The cells were treated with 200 µl cell lysis buffer and stored at -80°C for later use.

3.2.4 HBsAg, HBeAg and HBV-DNA detection

The ELISA kit used for the detection of HBsAg was Murex HBsAg version 3 while the one for HBeAg detection was Murex HBeAg/anti-HBe (Abbott, IL, USA). The experiment was performed according to the manufacturer's instructions. The optical density (OD) values were detected at 450nm. For the cell supernatant, 75µl of each was added to the ELISA kit well for detection. For the cell lysates, they were diluted by ten times to a final volume of 75µl and then used for two antigen detection. HBV DNA was extracted from the supernatant and cell lysates using QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. The samples were treated with Dnase I (NEB, MA, USA) to digest the DNA outside the HBV particles in prior. For HBV-DNA quantification, real-time PCR was carried out using Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) and primers HBC-F and HBC-R. The thermal cycling was carried out using an ABI 7500 Real Time PCR System with the following conditions: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 40 sec. The results were analyzed using ABI 7500 Real Time SDS software.

3.2.5 Statistics

All statistical analyses, t-test and one way ANOVA, were done using SPSS 13.0 for Windows. A p-value less than 0.05 was considered as statistically significant.

3.3 Results

3.3.1 Restriction endonuclease digestion of HBV-1.5X

For the verification of the right clones of HBV-1.5X, single digestion of Xho I (Fig. 3-3) and double digestion of Xho I and Kpn I (Fig. 3-4) were performed. For Xho I digestion, a band of about 8.7kb (3.9kb for pCR2.1-TOPO vector and 4.8kb for HBV-1.5X insert) appeared. For Xho I and Kpn I double digestion, two bands (i.e. one around 1.8kb and the other around 7kb), appeared also at the expected positions.

3.3.2 HBsAg, HBeAg and HBV-DNA detection

3.3.2.1 HBsAg

The HBsAg in the supernatant and cells after 24 h and 48 h of transfection are shown in Figs. 3-5A and Fig. 3-5B. The OD values determined at 48 h after transfection were all higher than those detected 24 h for the four samples of HBV-1.5Xs ($p<0.05$). In the supernatant, the H60-1.5X sample produced the highest level of HBsAg with an OD value as high as 3.5 ($p<0.05$). For the cell lysates, both H60-1.5X and C265-1.5X produced much higher HBsAg level with a high OD value of 3.5 after 48 h ($p<0.05$).

3.3.2.2 HBeAg

The level of HBeAg in the supernatant and cells after 24 h and 48 h of transfection are shown in Figs. 3-5C and Figs. 3-5D. The HBeAg was positive for H60-1.5X and H293-1.5X, yet negative for C265-1.5X and C1691-1.5X after 24 h and 48 h of transfection. Although the HBeAg was positive for H60-1.5X and H293-1.5X, the OD values were very low (< 0.6) at

both time points.

3.3.2.3 HBV DNA quantification

The HBV virus load in the supernatant and cells after 24 h and 48 h of transfection are shown in Figs. 3-5E and Fig. 3-5F. The virus loads were all higher at 48 h than 24 h post transfection ($p<0.05$) with log10 of virus loads about 6 to 7 per milliliter and 10 to 11 per milliliter for the supernatant and cell lysates respectively.

3.4 Discussion

3.4.1 Animal models for the study of HBV

3.4.1.1 The chimpanzee and the tupaia model

After the tests for HBsAg was developed in the late 1960s, chimpanzee was the first animal found to be susceptible to HBV infection (Barker *et al.*, 1973). Though chimpanzees do not develop chronic liver disease, they are the only primates known to develop a cellular immune response similar to that observed in humans during acute infection by HBV (Bertoni *et al.*, 1998). Therefore, they were regarded as a reliable HBV infection model in which immunological effects and the pathogenesis of acute HBV infection could be evaluated (Will *et al.*, 1982). Like humans, HBV-infected chimpanzees could become asymptomatic carriers; therefore, they have also been used to investigate the underlying mechanisms of viral persistence (Shikata *et al.*, 1980; Shouvat *et al.*, 1980). Furthermore, chimpanzees have also been employed in the development of safe vaccines (Berthetot *et al.*, 1984; Ohmura *et al.*, 1987; Fujisawa *et al.*, 1990; Ogata *et al.*, 1999) and in the evaluation of therapeutic efficiency of

anti-HBs and anti-HBc antibodies (Iwarson *et al.*, 1985; Ogata *et al.*, 1999).

Although experimental and naturally occurring infections of HBV have been reported in gibbons, orangutans and rhesus monkeys (Pittot, 1990; Shouval, 1994; Warren *et al.*, 1999; Lanford *et al.*, 2000; Takahashi *et al.*, 2000), the development of hepatic lesions and elevation of hepatic enzymes in these animals has never been reported. Nonetheless, the large size, the strong ethical constraints and the high costs of monkeys severely restrict their use for research purposes.

Based on the close phylogenetic relationship between tree shrews and primates, the tree shrew species *Tupaia belangeri* has been analyzed for the study of HBV infection both *in vitro* and *in vivo*, taking advantage of the adaptability of these animals to the laboratory environment (von Weizsacker *et al.*, 2004; Baumert *et al.*, 2005). Interestingly, Yan *et al.* reported an induction of HCC in tree shrews after they committed HBV and/or aflatoxin B1 (Yan *et al.*, 1996a, b).

3.4.1.2 HBV-related animal models: The hepadnaviruses family

Due to the limited host range of HBV and its inability to grow *in vitro*, much of the studies about the HBV life cycle have come from studies of other members of the hepadnaviruses family, especially the woodchuck hepatitis virus (WHV) and the duck HBV (DHBV) (Tuttleman *et al.*, 1986b). WHV was the first nonhuman hepadnaviruses being discovered and was found to carry high incidence of chronic hepatitis and HCC in the colony of Eastern woodchucks (*Marmota monax*) which were maintained in the Philadelphia Zoo (Summers *et al.*, 1978). Many studies have been done on the WHV for the studying of HBV (Cote *et al.*, 1990,

1991, 1992, 2000). For example, Korba *et al.* found enhanced antiviral benefit of combination therapy with lamivudine and famciclovir over corresponding monotherapies against WHV replication in chronic WHV carrier woodchucks (Korba *et al.*, 2000). A similar enhancement of antiviral effectiveness was also been reported against DHBV replication in cultures of primary duck hepatocytes (Colledge *et al.*, 1997). Suppression of lamivudine-resistant B-domain mutants by adefovir dipivoxil in the woodchuck hepatitis virus model has also been observed (Jacob *et al.*, 2004). Besides, a series of similar viruses have been isolated from ground squirrels (ground squirrel hepatitis virus, GSHV) (Marion *et al.*, 1980; Ganem *et al.*, 1982a, b), arctic squirrels (Testut *et al.*, 1996), tree squirrels (Feitetson *et al.*, 1986), as well as from woolly monkey (Lanford *et al.*, 1998), three avian species, domestic duck (Mason *et al.*, 1980), grey heron (Sprenget *et al.*, 1988) and snow goose (Chang *et al.*, 1999).

In general, there are known crucial distinctions between different members of the hepadnaviruses family. So far, the avian viruses are the most divergent members from HBV. However, all of these viruses share a number of common characteristics: (1) enveloped virions containing a relaxed circular, partially double stranded DNA of 3 to 3.3 kb; (2) a virion-associated polymerase with reverse transcriptase activity; (3) the production of excess subviral particles composed of envelope proteins and lipids only; (4) narrow host range; and (5) production of persistent infection displaying predominant hepatotropism (Ganem, 1996). Despite the common characteristics among the viruses family, the woodchuck is still the most commonly employed model in the research field of HBV. This is because the WHV is more similar to HBV in terms of genomic organization than the avian hepadnaviruses which allows us to investigate the entire HBV life cycle in natural host hepatocytes both *in vitro* and *in vivo*

more easily.

3.4.1.3 HBV-replicating mouse models

3.4.1.3.1 HBV-transgenic mice

Like every other models used, none of the above mentioned models is ideal for HBV investigation. For example, DHBV and WHV are genetically divergent from HBV, and immunological studies in ducks and woodchucks are difficult. Chimpanzee experiments are limited by cost, availability, and ethical considerations.

However, the limitation of the host range of viruses can actually be overcome, partly if not all, by genetically engineered animals which could possess viral genomes as demonstrated using HIV (Leonard *et al.*, 1988; Morrey *et al.*, 1991), papilloma virus (Lacey *et al.*, 1986), and human HBV (Chisari, 1995a, b, 1996; Guidotti *et al.*, 1995). Enormous progress has also been achieved through the development of various mouse strains that carry HBV transgenes. These offer the opportunity to investigate some of the pathogenetic mechanisms responsible for HBV-associated liver diseases, as well as to test specific viral genes for their potential oncogenic function *in vivo* (Dunsford *et al.*, 1990; Koike *et al.*, 1994; Araki *et al.*, 1989; Farza *et al.*, 1988). Many transgenic mice have been produced thus to express portions of the HBV genome coding for the envelope (Farza *et al.*, 1987; DeLoia *et al.*, 1989; Gilles *et al.*, 1992a, b; Ando *et al.*, 1993), core (Guidotti *et al.*, 1994; Milich *et al.*, 1994), precore (Milich *et al.*, 1990; Guidotti *et al.*, 1996), and X (Lee *et al.*, 1990; Perfumo *et al.*, 1992; Koike *et al.*, 1994) proteins. The use of these transgenic mice yielded invaluable knowledge on the viral induction, protein transport, assembly, secretion, disease pathology and HBV-specific immune responses.

However, they may not be considered suitable for antiviral studies on viral replication, as they only express portions of the viral genome. In previous years, many attempts have been made to generate transgenic mice that carry the complete linear viral genome (Araki *et al.*, 1989; Gilles *et al.*, 1992a; Nagahata *et al.*, 1992; Perfumo *et al.*, 1992; Guidotti *et al.*, 1994a, b; Tsui *et al.*, 1995). So far, the most valuable information from transgenic mice models has been focused on the inflammatory processes and responses during HBV infection (Guidotti *et al.*, 1994,1996) and the role of MHC class I restricted CTL response (Ando *et al.*, 1993). HBV-transgenic mice are immunologically tolerant to HBV antigens, and therefore, they do not develop chronic liver disease. Experiments with transgenic mice expressing HBV have demonstrated the model's utility for evaluating potential anti-HBV compounds such as interleukin-12 (Cavanaugh *et al.*, 1997), (−)2'3'- dideoxy-3'-thiacytadine (lamivudine, 3TC) (Morrey *et al.*, 1999a; Li *et al.*, 2005), interferon-α (Morrey *et al.*, 1999b; Li *et al.*, 2005), adefovir dipivoxil (ADV) (Julander *et al.*, 2002) and bis-POM-PMEA (Kajino *et al.*, 1997). Nevertheless, the mouse model is less expensive to employ than other hepatitis animal models which allows for more convenient antiviral testing due to the relatively simple manipulation skills.

3.4.1.3.2 HBV-transfected mice

To initiate viral replication in mouse livers, adenovirus vectors containing hepadnaviral genomes have been used. Secretion of infectious virions was demonstrated (Sprinzl *et al.*, 2001) and dynamics of the CTL response to HBsAg were studied in mice after intramuscular DNA immunization and infection by a HBV-recombinant adenovirus (Isogawa *et al.*, 2005).

As with the other models, there are some disadvantages in using HBV-transgenic or

-transfected mice. Although infectious virus can be produced in mice, their hepatocytes are not permissive for infection. Therefore, the early steps in viral infection including virus receptor recognition and mechanism of entry can not be addressed in these systems. Furthermore, all viral RNAs are synthesized in HBV-transgenic mice from a chromosomally integrated copy of the virus, whereas no cccDNA, the natural template of viral transcription *in vivo*, could be produced. Therefore, the study on the complete abrogation of virus production by antiviral drugs, which requires elimination of cccDNA and knowledge of its half life, would not be carried out in the transgenic mice.

3.4.1.3.3 Human-mouse chimera models of HBV infection

In 1999, Ilan and colleagues developed a human mouse radiation chimera system, the HBV Trimera mouse (Ilan *et al.*, 1999). Normal mice, preconditioned by lethal total body irradiation and reconstituted with SCID mouse bone marrow cells, were transplanted with *ex vivo* HBV-infected human liver fragments. Though the number of engrafted cells and viraemia were low, the presence of HBV DNA and cccDNA in the liver of these animals was promising. Since the follow-up was limited to 15 weeks, it was impossible to assess if those infected mice developed chronic HBV infection (Wu *et al.*, 2001).

3.4.2 Cell line for HBV study

In the last decades, many efforts have been made to establish cell system that allows replication of HBV. Some researchers have used HCC-derived cell lines in which the production of HBsAg and HBcAg was successfully observed under limited circumstances

(Macnab *et al.*, 1976; Yoakum *et al.*, 1983; Marquardt *et al.*, 1984). Hirschman *et al.* described a HeLa cell line which could transiently produce HBV-like particles when it was transfected by recircularized HBV DNA (Hirschman *et al.*, 1980). Elfassi *et al.* reported a lymphoblast cell type, which was obtained from a bone marrow aspirate of an acute HBV patient with viral DNA maintained in an extrachromosomal form, could also produce virus-like particles (Elfassi *et al.*, 1984).

3.4.2.1 HepG2.2.15

The HepG2.2.15 came basically from the hepatoblastoma cell line HepG2 transfected with pDolTHBV-1, a vector that contains two head-to-tail dimers of HBV in a tail-to-tail orientation (Sells *et al.*, 1987; Glebe *et al.*, 2001). The HepG2.2.15 produces high levels of HBeAg and HBsAg. HBV DNA is carried along by these cells as chromosomally integrated sequences and episomally as relaxed circular, covalently closed, yet incomplete copies of the HBV genome. Viral DNA was detected also in conditioned growth medium at the buoyant densities characteristic for infectious Dane and immature core particles. Morphologically, the cell produced 22-nm spherical and filamentous HBsAg particles and 42-nm Dane particles visualized by immune electron microscope. As with virus isolated from infected patients, the virus produced by HepG2.2.15 cells has thus less capable to infect HepG2 cells directly. Consequently, it is assumed that the HepG2 cells might not have receptor(s) for viral attachment and penetration, and that episomal HBV DNA and virus production was maintained for longer than 9 months via a constant replenishment of circular episomal DNA from the chromosomally integrated HBV DNA sequences. However, HBV produced by HepG2.2.15

cells has been reported to cause hepatitis in chimpanzees (Acs *et al.*, 1987). Therefore, the HepG2.2.15 cell line can support the assembly and secretion of both the replicative intermediates of HBV DNA and also Dane-like particles. This *in vitro* system can now also be used to study the life cycle of HBV, antiviral research, the interaction of specific cytotoxic T lymphocytes and cytolytic antibodies with infected liver cells (Sells *et al.*, 1987; Acs *et al.*, 1987). Despite the fact the HBV expression level is quite difficult to control, HepG2.2.15 is still the most widely used HBV producing cell line in antiviral research (Acs *et al.*, 1987; Sells *et al.*, 1987, 1988). Some researchers used this cell line to show the enhanced effectiveness of combination therapies for treating chronic HBV infection (Korba, 1996; Zembower *et al.*, 1998; Perigaud *et al.*, 1999).

3.4.2.2 HepAD38

Ladner *et al.* reported the development and isolation of a cell line, termed HepAD38 that replicates human HBV under conditions that can be regulated with tetracycline (Ladner *et al.*, 1997). In the presence of the antibiotic, this cell line is free of virus due to the repression of pgRNA synthesis. Upon removal of tetracycline from the culture medium, the cells express viral pgRNA, accumulate subviral particles in the cytoplasm that contain DNA intermediates characteristic of viral replication, and secrete virus-like particles into the supernatant. Since the HepAD38 cell line can produce high levels of HBV DNA, it might be useful for analyzing the viral replication cycle that depends upon viral DNA synthesis in a synchronized manner. In addition, this cell line has been formatted into a high-throughput, cell-based assay that permits the large-scale screening of diverse compound libraries for new classes of inhibitors of HBV

replication (Ladner *et al.*, 1997). The above advantages make the HepAD38 assay economically feasible to use for large-scale screening of diverse chemical libraries for identifying new classes of inhibitors for HBV replication. The new regulable HBV producing cell lines would be invaluable tools to address both scientifically and pharmaceutically relevant queries regarding HBV.

3.4.2.3 Hep 3B and PLC/PRF/5

Hep 3B cell lines were initially derived from liver biopsies of two children with primary hepatoblastoma and HCC (Knowles *et al.*, 1980; Aden *et al.*, 1979; Martin *et al.*, 1975). The Hep 3B and PLC/PRF/5 cell lines are known to synthesize HBsAg (MacNab *et al.*, 1976; Aden *et al.*, 1979; Knowles *et al.*, 1980). Other Hep 3B cell lines such as HEp-3B 217, HEp-3B 14, HEp-3B Fl contained two, one and one HBV genome DNA equivalents per cell, respectively. Southern blot hybridization analysis demonstrated that HBV DNA was integrated into the chromosomes of these cell lines (Twist *et al.*, 1981), so they could produce HBV particles.

3.4.2.4 Other HBV models

Except for chimpanzees, experimental HBV infection is mainly restricted to primary hepatocytes of humans. Until recently, the primary tupaia hepatocyte (PTH) is regarded as one of the feasible alternatives which are contractable by serum-derived HBV (Kock *et al.*, 2001; Glebe *et al.*, 2003; Glebe *et al.*, 2005; von Weizsacker *et al.*, 2004) and Woolly Monkey HBV (WMHBV) (Kock *et al.*, 2001; Lanford *et al.*, 1998). However, no net amplification of the virus is achieved in the cell line (Gripon *et al.*, 2002).

In 2006, Sun *et al.* constructed two stable HepG2- and Huh7-based human hepatoma cell lines for efficient regulated expression of infectious HBV (Sun *et al.*, 2006). The yields of secreted HBV particles, when compared to HepG2.2.15 cells, were modestly higher in the Huh7.93 and at least 5-fold higher in the HepG2.117 cells. Other cell lines including HB611 and HepG2-BV3 have also been reported to produce HBV (Tsurimoto *et al.*, 1987; Mabit *et al.*, 1994).

3.4.3 HBV-1.5X

In this study, four HBV-1.5Xs were constructed which possessed a 5'-end redundant sequence of 1.1kb to nt715 with another copy of Enhancer I/X promoter and Enhancer II/Basal core promoter, and 3'-end redundant sequence of 500bp to nt2325 after the poly (A) sequence. They are longer than both the pgRNA and the reported HBV 1.1, 1.2 and 1.3 constructs which generated promising results. In our study, HBV-related antigens, including HBsAg and HBeAg, were detected in both the supernatant and cell lysates. In the supernatant with H60-1.5X, the OD values of HBsAg were very high at both 24 h and 48 h post-transfection. In the cells even after 10 times dilution of the cell lysates, the OD values for HBsAg are still high, especially for H60-1.5X and C265-1.5X. These results indicated that our HBV 1.5X system produced very high level of HBsAg which is the most important antigen for HBV infection. For HBeAg, the values are comparatively lower than HBsAg for all the HBV-1.5Xs both in the supernatant and in the cells, 24 h or 48 h. For low or negative HBeAg detection during HBV infection, the most possible reason is mutagenesis including A1762T/G1764A and G1896A mutations. This is also the case for this study. From the sequence alignment in Chapter 2 (page 47), the double

mutations in the basal core promoter (A1762T/G1764A) were noted to appear in H60 and H293 strains while the point mutation (G1896A) was happened in the precore region in both the C265 and C1691 strains. As we know that the A1762T/G1764A mutations could reduce the HBeAg production whilst the G1896A mutation could terminate HBeAg synthesis, these might be the reasons why the level of the HBeAg is lower than that of HBsAg in this study. Our results are generally consistent with the sequence alignment.

For HBV-DNA FQ-PCR, the virus loads are very high at 24 h and 48 h post-transfection with log10 of virus loads of about 6 to 7 per milliliter in the supernatant and 10 to 11 per milliliter in the cell lysates for all the four strains. This fact indicates that the HBV-1.5X system has produced HBV virions in the cell and then secreted outside the cell.

Based on the levels of HBsAg, HBeAg and HBV DNA detected in our study, the HBV-1.5X system was actually producing very high level of HBV virus. This might because it has another copy of complete Enhancer I/X promoter, Enhancer II/Basal core promoter and HBx gene, which can all increase the replication of HBV. This system might already provide the most important step in clone construction which might also be useful for future study either in constructing cell lines or transgenetic mice for studying the four viral strains and other related characteristics.

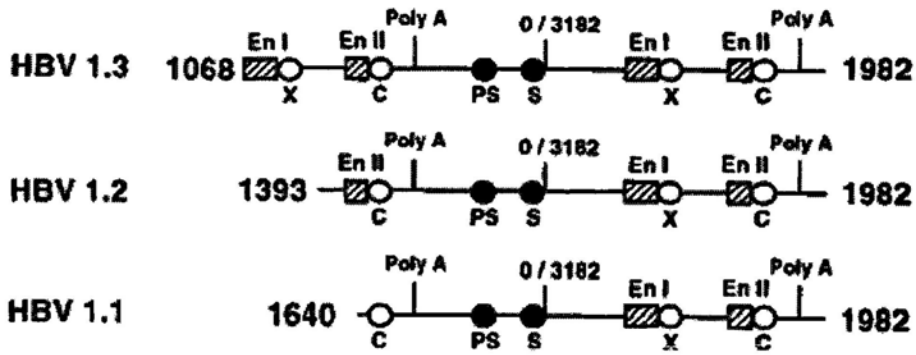
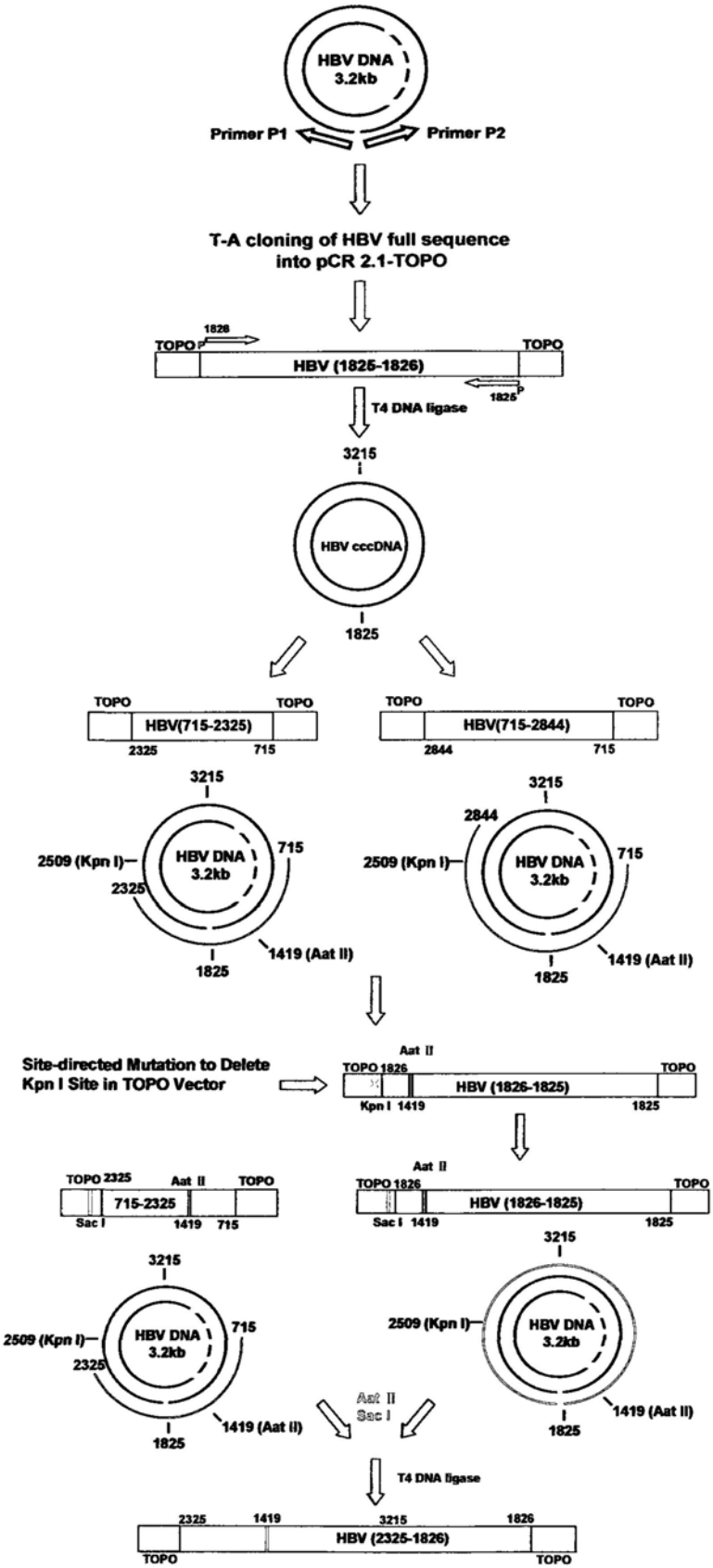


Fig. 3-1. Structures of HBV 1.1, 1.2 and 1.3. Schematic representations of the HBV-derived constructs that contain terminally redundant, greater-than-genome length (1.1, 1.2 and 1.3) copies of the complete HBV genome are shown. En, enhancer; Poly A, polyadenylation signal; X, C, PS, and S indicate the X, core, pre-S, and S promoter, respectively. (Guidotti *et al.*, 1995)

Table 3-1. Primers for the HBV-1.5X construction

Primer	Nucleotide sequence (5'-3')	Position (nt)
5-P-HBV-1826S	P-CACCTCTGCCTAATCATCTCWTGTTCATG	1826-1854
5-P-HBV-1825A	P-AAAAAGTTGCATGGTGCTGGTGAACRCAC	1825-1797
HBV715S	CCCCACTGTCTGGCTTTCAG	715-734
HBV2325A	GTTGATAAGATAGGGGCATTT	2305-2325
HBV2844A	AAGACCAACCTCCCGTGCTG	2863-2844
Kpn I Mutation F	CATGATTACGCCAAGCTTCATACCGAGCTC	In the vector
Kpn I Mutation R	AAGCTTGGCGTAATCATGGTCATAGCTG	In the vector
HBC-F	AGTGTGGATTCGCACTCCT	2269-2287
HBC-R	GAGTTCTTCTTCTAGGGGACCTG	2381-2359


HBV DNA
3.2kb
Primer P1
Primer P2
T-A cloning of HBV full sequence
into pCR 2.1-TOPO
1826
TOPO
HBV (1825-1826)
1825
T4 DNA ligase
3215
HBV cccDNA
1825
TOPO
HBV(715-2325)
2325
715
HBV(715-2844)
2844
2509 (Kpn I)
2325
715
1419 (Aat II)
Site-directed Mutation to Delete
Kpn I Site in TOPO Vector
Aat II
HBV (1826-1825)
Kpn I 1419
715-2325
Sac I
1419
Aat II
Sac I
T4 DNA ligase
2325
1419
3215
1826
HBV (2325-1826)

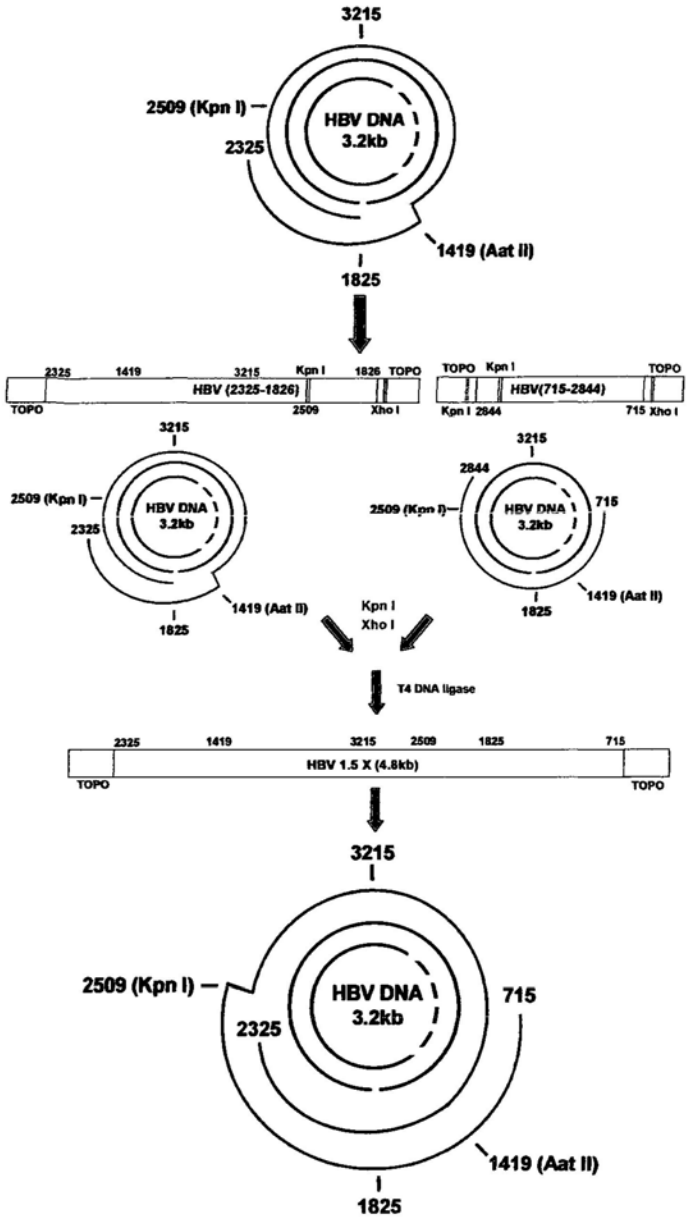


Fig. 3-2. Workflow for the construction of HBV-1.5X.

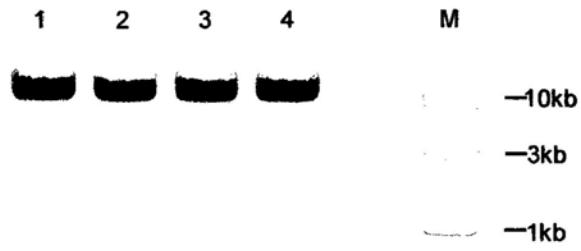


Fig.3-3. Agarose gel analysis of pCR2.1-TOPO-HBV-1.5X by Xho I digestion. 1. H60-1.5X. 2. H293-1.5X. 3. C265-1.5X. 4. C1691-1.5X. M. Promega 1kb DNA Ladder.

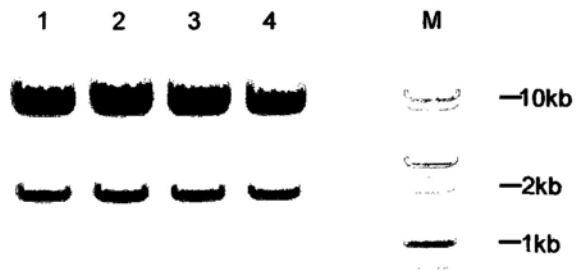


Fig.3-4. Agarose gel analysis of pCR2.1-TOPO-HBV-1.5X by Xho I and Kpn I double digestion. 1. H60-1.5X. 2. H293-1.5X. 3. C265-1.5X. 4. C1691-1.5X. M. Promega 1kb DNA Ladder.

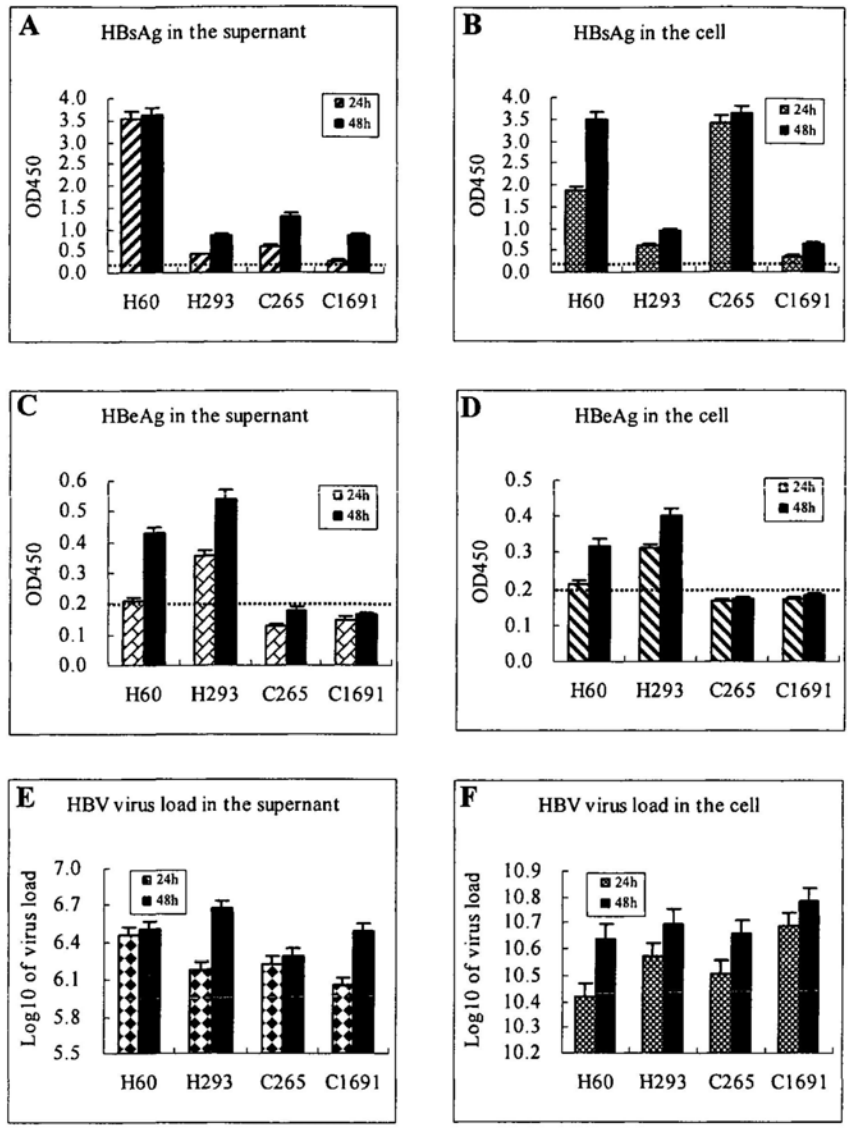


Fig. 3-5. HBsAg, HBeAg and HBV virus load detection in the supernatant and cell of HBV-1.5X transfected HepG2 cell. The experiment was repeated three times in triplicate. The OD values were determined for the cell lysates after they were diluted for 10 times. A. HBsAg in the supernatant; B. HBsAg in the cell; C. HBeAg in the supernatant; D. HBeAg in the cell; E. HBV virus load in the supernatant; F. HBV virus load in the cell. The broken line represents the cut-off value.

Chapter 4

The association between A1762T/G1764A mutations of HBV and HCC – the reduction of basal core promoter activities

4.1 Introduction

HBV sequences are transcribed under the control of four promoters: the preC/pregenomic, S1, S2 and X promoters (Gunther *et al.*, 1998). Except for S1, all the other HBV promoters lack the TATA box motifs which were essential in the formation of transcription initiation complex, and so they represent a unique model of transcription. Two enhancers and negative regulatory element in the HBV genome further increase the controlling of HBV-RNA synthesis. All these transcription elements are embedded within the ORFs of HBV genome. This feature shows the remarkable capacity of HBV virus to maximize the function of its small genome. HBV transcription control elements also display a preference for liver-specific trans-factors, which contributes to the liver tropism of the virus.

Enh I has a primary ability in up-regulating transcription in an orientation independent manner (Guo *et al.*, 1991). It can increase the transcription of pregenomic/preC and HBx mRNA dramatically. However, it has a modest effect on the surface antigen transcription. Detailed analysis of enhI showed three distinct domains: a modulator element, a core domain and a domain that overlaps with the HBx ORF (Fukai *et al.*, 1997). The structure of Enh I/X promoter complex is shown in Fig. 4-1.

Enh II is located immediately upstream of the BCP on the HBV genome and overlaps with the CURS sequence of the core promoter. Enh II stimulates transcriptional activity of S1, S2 and the X promoter in an orientation independent manner (Yuh *et al.*, 1990). The structure of Enh II /Basal core promoter complex is shown in Fig. 4-2.

Chronic infection by HBV was known to increase the risk of HCC by more than 100-fold (Beasley *et al.*, 1981). However, the molecular mechanism of the inducing process is still unclear. In Asia, genotypes B and C are the predominant genotypes of HBV infections. In a longitudinal follow-up study of 426 chronic HBV infections for up to 5 years in Hong Kong, genotype C HBV was found to be associated with higher risk of HCC than genotype B HBV (Chan *et al.*, 2004). Several recent studies also showed that A1762T and G1764A mutations were closely associated with the aggressiveness of liver disease, in which inactive carriers have developed active hepatitis, and eventually liver cirrhosis and HCC (Kao *et al.*, 2003; Hsieh *et al.*, 2004; Tralhao *et al.*, 2002). In this study, 100 genotype C sequences and 200 other genotype sequences were randomly selected from the GenBank database. After alignment, the frequency of A1762T and G1764A mutations in genotype C was found around 64%, while for other genotypes (A, B, D to H) was generally lower (34%). These findings suggest an important role of A1762T/G1764A mutations in hepatocarcinogenesis in genotype C patient. Recent clinical studies have shown that A1762T/G1764A mutations also occur frequently in HCC patients with genotype B infection (81%, 30 of 37 patients), but are relatively lower in AsCs (43%, 22 of 51 patients) (Sung *et al.*, 2008). This indicates that the contribution of A1762T/ G1764A mutations to liver

cancer is not limited to genotype C HBV. In this study, the effect of A1762T and G1764A mutations on the Enhancer II/Basal core promoter activity of HBV was demonstrated for the first time. Our findings deduce a mechanistic explanation on viral evasion that may provoke or facilitate the progression of HCC.

4.2 Materials and methods

4.2.1 Patients, workflow and primers

For patients information please refer to Chapter 2, section 2.2.1, on page 31. A diagram of basal core promoter activity workflow is shown in Fig. 4-3. All the primers used in this study are listed in Table 4-1.

4.2.2 HBV sequence alignment

Three-hundred complete genome sequences of HBV genotypes B, C, and others were randomly downloaded from GenBank database (http://www.ncbi.nlm.nih.gov/Genbank). Enhancer I/X promoter sequences from our 4 samples and basal core promoter sequences from 12 HCC patients and 12 AsCs controls in our study were analyzed. The sequence alignment was carried out using Vector NTI Suite 9, GeneDoc 2.6 and SimPlot 2.5. The substitution model used for defining nucleotide similarity scores is the Dayhoff model. The phylogenetic trees were constructed with TreeView 1.5 and constructed by the neighbor-joining method (Saitou and Nei, 1987). The reliability of phylogenetic tree analysis was assessed by bootstrapping resampling method (Felsenstein, 1985).

4.2.3 Extraction of DNA from sera and plasmid construction

Please refer to Chapter 2, section 2.2.2, on page 32 for DNA extraction method.

Enhancer I/X promoters were amplified by PCR using primer HBV F-902-925 and HBV R-1358-1339. The Enhancer II/Basal core promoters were amplified by PCR using primer HBV F-1601-1615 and HBV R-1815-1801 (spanning nt1601 to nt1815). The PCR products were resolved by 1.5% agarose gel electrophoresis and purified using a DNA purification kit (Qiagen, Hilden, Germany). The thermocycling conditions were set as follows: 94°C for 3 min, 35 cycles of 94°C for 30 s, 55 °C for 30 s and 72°C for 30 s, followed by a final extension of 72°C for 5 min. The PCR products for Enhancer I/X promoter were digested with Kpn I and Bgl II, whilst the products for Enhancer II/Basal core promoters were digested with XhoI and HindIII. These fragments were then inserted into the multiple cloning sites of pGL3-Basic vector (Promega, WI, USA). Sequence correction of the constructs was confirmed by DNA sequencing using primer pGL3-for and pGL3-rev. A sketch map of constructs is shown in Fig. 4-4.

4.2.4 Site-directed mutagenesis

Site-directed mutagenesis was performed using a one step mutagenesis kit (GeneTailor Site-Directed Mutagenesis System, Invitrogen, CA, USA) according to the manufacturer's instructions. Site-directed mutagenesis primers in position nt1762, nt1764, nt1677/nt1679, nt1706 and nt1733 were listed in Table 4-1. All the constructs used in this study were confirmed by sequencing using primer pGL3-for and

pGL3-rev. The procedure of site-directed mutagenesis is described in Fig. 4-5.

4.2.5 Cell culture, transfection, and luciferase assays

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum under 5% CO2 incubation at 37°C. DNA was transfected into the HepG2 cells using Lipofectamine 2000 (Invitrogen, CA, USA) and the cells were harvested at 24 h post-transfection. Transfection efficiency was routinely monitored by cotransfecting 100 ng of the pRL-SV40 vector as the internal control. To verify the results, all transfections were performed in triplicate. The final amount of plasmid DNA was 1 μg per transfection in 12-well plate. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega, WI, USA). Firefly luciferase activity of the reporter plasmid was normalized to renilla-luciferase activity and expressed as fold induction of control.

4.2.6 Statistics

All statistical analyses, t-test and one way ANOVA, were done using SPSS 13.0 for Windows. A p-value less than 0.05 was considered as statistically significant.

4.3 Results

4.3.1 Sequence alignment for Enhancer I/X promoter

The sequence alignment of the four HBV strains in this study for Enhancer I/X promoter is shown in Fig. 4-6.

4.3.2 Enhancer I/X promoter activity

The promoter activities of the Enhancer I/X promoters of the four HBV sample strains are shown in Fig. 4-7. There are no difference among the four strains ($p>0.05$).

4.3.3 Enhancer II/basal core promoter activity

Fig. 4-8 shows the promoter activities of the Enhancer II/Basal core promoters of our strain samples. There are significant difference among the four strains ($p<0.001$) with H60 revealed the lowest activity whilst C265 got the highest. The promoter activities of strains H293, C265 and C1691 were about 10, 33 and 12 times to that of H60, respectively.

4.3.4 High frequency of A1762T/G1764A mutations in genotype C HBVs and genotype B HBVs with HCC

It has been reported that genotype C HBV is associated with more aggressive development of liver cirrhosis and HCC (Chan *et al.*, 2004; Kao *et al.*, 2000). Interestingly, the mutation rate of A1762T/G1764A of genotype C was also reported to be much higher than that of genotype B. The high frequency of A1762T/G1764A mutations in genotype C was found to correlate with an increased risk of HCC (Kao *et al.*, 2003). To determine whether A1762T/G1764A mutations occur commonly in aggressive genotype C globally, 300 full genome sequences of genotype B (100), genotype C (100) and other genotypes (100) of HBV were randomly downloaded from GenBank. These sequences were from different groups in Asia, Europe, Africa

and North America. After alignment, the mutation rate of A1762T/G1764A was 40%, 64% and 35% for genotype B, C and the other genotypes, respectively. A recent study has suggested a close relationship between the A1762T/G1764A mutations on genotype B and higher risk of HCC development (Sung *et al.*, 2008). These results further support the fact that A1762T/G1764A mutations play an important role in the progression of HBV-associated diseases, including HCC.

4.3.5 Reduction of the basal core promoter activities of HBVs by A1762T and G1764A mutations in AsC

In this study, genotype B strains were chosen to demonstrate the effects of various mutations on basal core promoter activities. After alignment with the promoter region, the enhancer II / Basal core promoter regions of the genotype B HBVs were found highly conserved among carriers and HCC patients except nt1762 and nt1764 (Figs. 4-9 and 4-10). The nucleotides 1762T and 1764A were observed in the HCC samples, while 1762A and 1764G were observed in the AsC samples. Therefore, the promoter activities were tested for the isolates which were taken from two representatives in each group (i.e. HCC patients: H60 and H293; AsCs: C265 and C1691) with the GenBank Accession Nos. DQ995801, DQ995802, DQ995803, and DQ995804, respectively. In order to determine the effect of nt1762/nt1764 mutations on the HBV Enhancer II/Basal core promoter activities, the luciferase reporter vectors containing HBV Enhancer II/Basal core promoter were constructed from strains isolated from these typical patients (Fig. 4-11).

In the case of C265, basal core promoter activity was found decreased by 15% and 25% by 1762A to 1762T and 1764G to 1764A mutations, respectively. Simultaneous mutation of 1762A to 1762T and 1764G to 1764A synergistically reduced the promoter activity by about 50% (Fig. 4-11.). In the case of carrier C1691, promoter activity was decreased by about 30% and 35%, when 1762A was mutated into 1764T and 1764G into 1764A, respectively. Promoter activity was further reduced to 55% by simultaneous mutations of 1762A into 1762T and 1764G into 1764A. In combination, A1762T mutation reduced the overall promoter activity by around 23% while G1764A mutation reduced it by 30%, suggesting that the 1762G might contribute in a larger proportion in regulating the promoter activity than that of 1762A. In both cases, A1762T/G1764A double mutations reduced promoter activities ($p<0.001$) significantly by about 50%. These results indicate that HCC-associated A1762T/G1764A mutations could decrease the basal core promoter activity of HBV in a statistically significant manner.

4.3.6 Enhancement of the basal core promoter activities of HBVs from HCC patients by reverse T1762A and A1764G mutations

If our observations of A1762T/G1764A mutations with an increased risk of HCC were correct, the basal promoter activities of HBVs isolated from HCC patients ought to be increased by reverse mutagenesis. In the case of HCC patient H60, the promoter activity of HBV has increased by 48% and 68% when 1762T and 1764A were reversely mutated into 1762A and 1764G, respectively (Fig. 4-12). In the case of

patient H293, the promoter activity of HBV has increased by 50% and 140% when 1762T and 1764A were reversely mutated into 1762A and 1764G, respectively. In both cases, the reversed T1762A/A1764G double mutations synergistically increased the promoter activities by at least two fold ($p<0.001$). These results provide evidences that the A1762T and G1764A mutations of HBVs in HCC patients could decrease the basal core promoter activities, with 1764G playing a more important role than 1764A.

4.3.7 The promoter activity of sample strain from HCC patient H60 and some mutants

From the results of Enhancer II/Basal core promoter sequences alignment for all the strains from HCC patients and AsCs (Fig. 4-9), we observed the main differences between H60 and C265 are situated in positions nt1706, nt1733 and nt1677/nt1679 except nt1762/nt1764. Therefore, site-directed mutagenesis for H60 was carried out as follows; Mutation 1 (M1): T1706C; M2: A1733G and M3: G1677T/A1679C. In this order of mutations, the promoter activity was increased by 1.31, 15.62 and 2.26 times, respectively (Fig. 4-13; $p<0.001$).

4.4 Discussion

Previous studies of HBV transcription revealed the requirement of two enhancer elements. EnhI is located upstream of the X promoter and is targeted by multiple activators, including basic leucine zipper proteins. EnhII is located upstream to the PreCore promoter and is targeted mainly by nuclear receptors (NRs) (Doitsh *et al.*, 2004). Both enhancers show high activity in liver cell lines, and they also function

together with different promoters (Ben-Levy *et al.*, 1989; Garcia *et al.*, 1993; Honigwachs *et al.*, 1989; Huan *et al.*, 1995; Tang *et al.*, 2001; Yu *et al.*, 2001; Zhang *et al.*, 1992). EnhI regulates not only the X promoter but also all the other viral promoters (Fukai *et al.*, 1997; Antonucci *et al.*, 1989; Doitsh *et al.*, 1999; Honigwachs *et al.*, 1989; Hu *et al.*, 1991). EnhI was known as an essential element for HBV transcription and it can also be partially replaced by the simian virus 40 enhancer (Hu *et al.*, 1991). HBV-transgenic mice lacking EnhI at the 5' end of the inserted DNA were found defective in virion production and poor liver-specific HBV expression was also noted (Guidotti *et al.*, 1995). A region within EnhI was known to bind multiple transcription activators of the basic leucine zipper family, including C/EBP (Dikstein *et al.*, 1992), the AP-1 complex (Faktor *et al.*, 1990), and ATFs (Maguire *et al.*, 1991). This region possesses an intrinsic enhancer activity in a variety of liver cell lines (Faktor *et al.*, 1990; Kosovsky *et al.*, 2000; Vannice *et al.*, 1998). In addition, cellular factors involved in cell cycle control and apoptosis, including the tumor suppressor protein p53 (Ori *et al.*, 1998), its homologue p73 (Doitsh *et al.*, 1999), the proto-oncoprotein c-Abl (Dikstein *et al.*, 1992), and RFX-1 (Katan *et al.*, 1997; Siegrist *et al.*, 1993), could specifically bind and regulate EnhI activity. Recently, *in vivo* footprinting analysis has showed that the particular EnhI region is occupied by the aforementioned cell cycle control proteins (Shamay *et al.*, 2001). On the other hand, EnhII was situated immediately upstream to the pgRNA/PreC promoter and has been found related to the transcription regulation of pre S2/S promoters (Kramvis *et al.*, 1999; Yuh *et al.*, 1990). The mode of interplay between these enhancers and their

unique contributions in regulating HBV transcription remained obscure. In this study, no significant difference was found in the activities of Enhancer I/X promoter of all of our HBV strains. This might be explained by the fact that the Enhancer I/X promoter is so essential and is predominant for HBV replication and gene expression (Doitsh *et al.*, 2004). So it is comparatively conserved to increase the chance of viral survival. For Enhancer II/Basal core promoter, there was significant difference among the four sample strains. Through sequence alignment, specific nucleotide positions were found and the difference was noted between H60 and C265. The activities of Enhancer II/Basal core promoter were found up-regulated in all strains by T1762A, A1764G, T 1706C, A1733G, G1677T/A1679C mutations. These changes may therefore be useful to attract different transcriptional factors or stronger substrates to bind to their nucleotide regions, thus enhance the activity of Enhancer II/Basal core promoter.

The contribution of HBV to the pathogenesis of liver cancer is multifactorial and complicated by the identification of mutant variants of HBV that modulate the carcinogenesis process (Locarnini *et al.*, 2003). Clinical studies have shown that HBV-infected patients with A1762T/G1764A mutations have low levels of HBeAg and are often associated with HCC. This mutation pattern also occurred in the genome of HBV isolated from the HCC patients (e.g., H60 and H293) in this study. Therefore, our study provided for the first time some laboratory direct evidences that could support the clinical observations. In this study, we showed that the promoter activities of HBVs isolated from AsCs were significantly reduced when wild-type 1762A and 1764G were mutated into 1762T and 1764A (C265 and C1691). Conversely, reversed

mutations from 1762T/1764A to 1762A/1764G were shown to increase the basal core promoter activities of HBVs isolated from HCC patients. Although the nucleotide context of the promoters of HBV strains exerts some influence on 1762T or 1764G, the overall consequence of A1762T/G1764A double mutations reduced basal core promoter activity by about 50%. Therefore, we postulated that such mutations might closely associate with the diminished precore mRNA (PreC mRNA) transcription. This might be the consequence of the changed hepatocyte nuclear factor 4 (HNF-4) binding site 2 and the TATA box-like sequence of the core promoter. Other evidence (Gunther *et al.*, 1998) also suggested that the double mutations resulted in an introduction of an additional hepatocyte nuclear factor 1 (HNF-1) binding site in the core promoter and specifically repressed preC RNA synthesis. The double mutations also changed 2 codons of HBx, with the mutant HBx appeared to have superior HNF-1 trans-activation properties (Gunther *et al.*, 1998). Moreover, other transcriptional factors might also contribute to the Enhancer II/Basal core promoter activity variance related to A1762T/G1764A mutations. These include retinoid x receptor a (RXRa), peroxisome proliferator activated receptor a (PPARa), chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) and human testicular receptor 2 (TR2) (Li *et al.*, 1998; Gunther *et al.*, 1998; Raney *et al.*, 1997). The transcription factors which could bind around nt1762/nt1764 are shown in Fig. 4-14. Our studies suggest for the first time that both 1762A and 1764G are important for binding those factors, and that 1764G plays a very significant role in determining the binding affinity.

Although the mechanism of A1762T/G1764A mutations in inducing HCC remains elusive, ours and some of the previous studies might provide several clues for later references. Briefly, the core promoter region of HBV directs the transcription of both species of 3.5 kb pgRNA and PreC mRNA (Kramvis *et al.*, 1999). The pgRNA is translated into core protein and polymerase protein (reverse transcriptase), serving as a template for reverse transcription of HBV after being packaged into core particles. The PreC mRNA is translated into a PreC/core fusion protein that is post-translationally modified to yield HBeAg. The A1762T/G1764A mutations might then reduce the HBeAg level, (i.e. to induce immune tolerance to itself), the HBcAg or both antigens. This finally increases the viral replication and viral load, and eventually to HCC. In addition, the HBV A1762T/G1764A double mutations also affect the amino acid sequence of the HBx gene as they reside in codons 130 and 131, result in the substitutions of lysine to methionine and valine to isoleucine, respectively (Li *et al.*, 1999). The X gene protein was found to display various types of biological activity (Arbuthnot *et al.*, 2000; Murakami *et al.*, 2001), but its specific role and that of this mutant protein in the pathogenesis of liver cancer have yet to be elucidated.

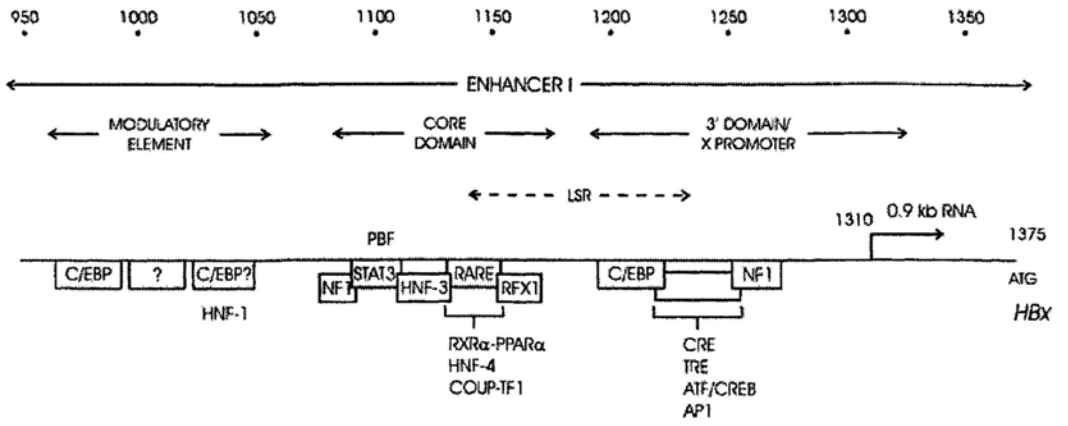


Fig. 4-1. Enhancer I/X promoter complex. Nucleotide coordinates for the HBV ayw subtype are given. Functional domains of the enhancer I element are shown. Sites for interaction with several transcription factors are also shown (Moolla *et al.*, 2002).

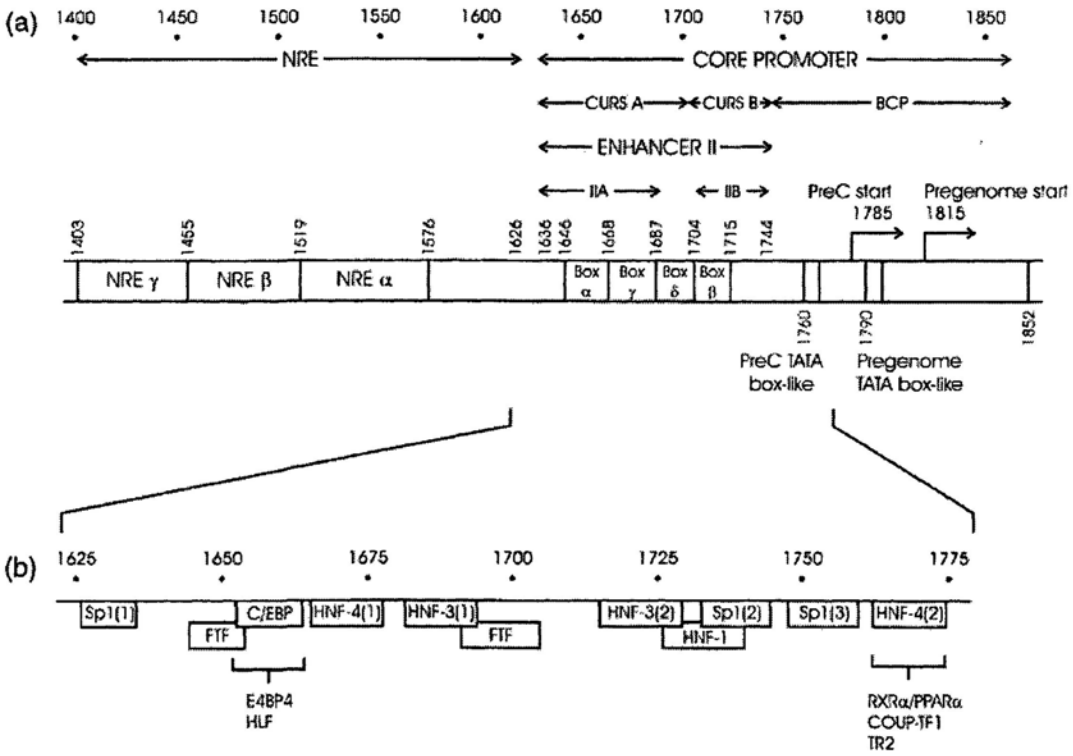


Fig. 4-2. Enhancer II / Basal core promoter complex. (a) Schematic representation of the structural domains of the basic core promoter (BCP), its upstream regulatory regions-core upstream regulatory sequence (CURS) and negative regulatory element

(NRE) and the enhancer II element. The TATA box-like sequences and transcription initiation sites of the precore and pregenomic RNA are also indicated. (b) Sites of interaction of several ubiquitous and liver-specific transcription factors that regulate core promoter and enhancer II transcription activity. Transcription factor binding sites between HBV nucleotide coordinates 1625 and 1775 are indicated as a schematic footprint (Moolla *et al.*, 2002).

Table 4-1. Primers for cloning and site-directed mutagenesis of Enhancer I/X promoter and Enhancer II / Basal core promoter of four HBV strains in this study

Primer	Nucleotide sequence (5'-3')	Direction
HBV F-902-925	CGGGGTACCGCACATTGCCACAGGAACATATTG	Sense
HBV R-1358-1339	GGAAGATCTTTGCGGGAGAGCACGACAGA	Antisense
HBV F-1601-1615	CCGCTCGAGACGTCGCATGGAGAC	Sense
HBV R-1815-1801	CCCAAGCTTATGGTGCTGGTGAAC	Antisense
HBV-1762-1764F	TCTTTGTACTAGGAGGCTGTAGGCATAAA	Sense
HBV 1762A-1764A-R	AGCCTCCTAGTACAAAGATCTTTAACCTAA	Antisense
HBV 1762T-1764G-R	AGCCTCCTAGTACAAAGACCATTAACCTAA	Antisense
HBV 1762A-1764G-R	AGCCTCCTAGTACAAAGACCTTTAACCTAACC	Antisense
HBV 1762T-1764A-R	AGCCTCCTAGTACAAAGATCATTAACCTAA	Antisense
HBV-BCP-G1677T A1679C-F	GACTCTTGGACTTTCATCCATGTCAACGAC	Sense
HBV-BCP-G1677T A1679C-R	TGCTGAAAGTCCAAGAGTCCTCTTATGCAA	Antisense
HBV-BCP-T1706C-F	GACCGACCTTGAGGCATACTTCAAAGACTG	Sense
HBV-BCP-T1706C-R	TATGCCTCAAGGTCGGTCGTTGACATTGCT	Antisense
HBV-BCP-A1733G-F	AGAGGAGTTGGGGGAGGAGGTTAGGTTAAT	Sense
HBV-BCP-A1733G-R	CTCCTCCCCCAACTCCTCCCACTCAGTAAA	Antisense
pGL3-for	CTAGCAAAATAGGCTGTCCC	Sense
pGL3-rev	TATGTTTTTGGCGTCTTCC	Antisense

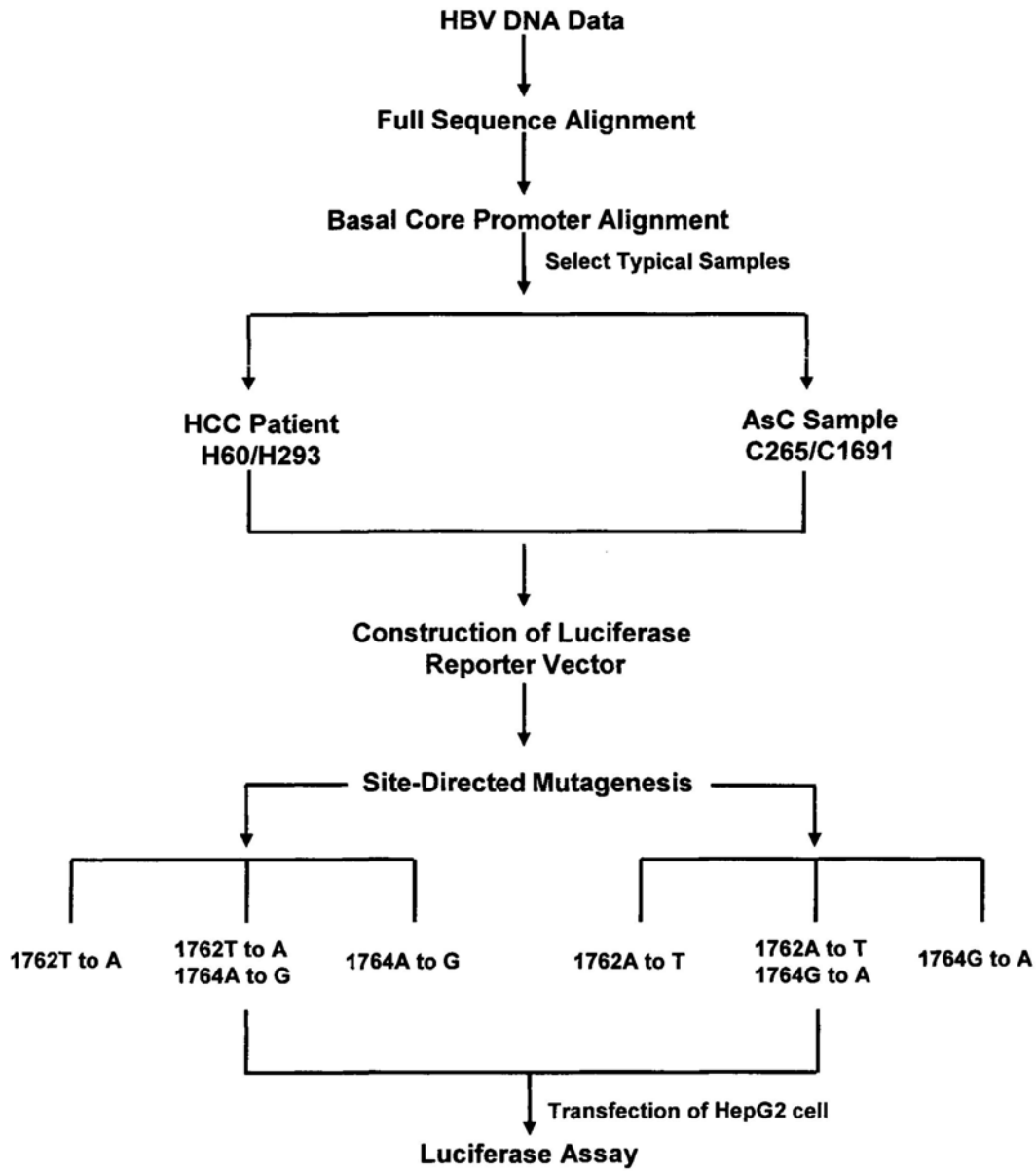


Fig. 4-3. A flow chart for the basal core promoter studies

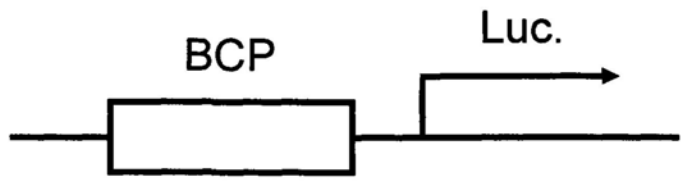


Fig.4-4. Schematic diagram of construct for basal core promoter assay.

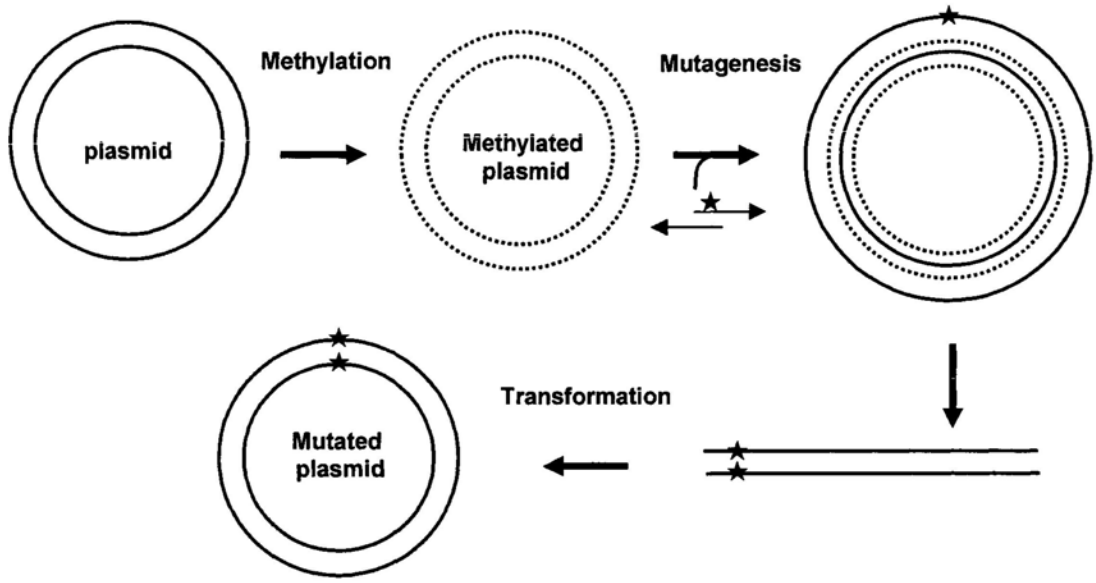


Fig. 4-5. Workflow of site-directed mutagenesis.

1. Methylate plasmid DNA (isolated from any source) with DNA methylase at 37°C for 1 hour.
2. Amplify the plasmid in a mutagenesis reaction with two overlapping primers, one of which contains the target mutation. The product is linear, double-stranded DNA containing the mutation.
3. Transform the mutagenesis mixture into wild type *E. coli*. The host cell circularizes the linear mutated DNA, and *Mcr*BC endonuclease in the host cell digests the methylated template DNA, leaving only unmethylated, mutated product.

```
                *       920         *       940         *       960         *       980
H60    : GCACATTGCCACAGGAACATATTGTACAAAAAATCAAACTGTGTTTTAGGAAGCTTCCTGTAAACAGGCCTATTGATTGG :   80
H293   : ....................................A........C....A.......................... :   80
C265   : ....................................A............A.......................... :   80
C1691  : ....................................A........C....A.......................... :   80

                *      1000         *      1020         *      1040         *      1060
H60    : AAAGTATGTCAACGAATTGTGGGTCTGTTGGGGTTTGCCGCCCCTTTCACGCAATGTGGATATCCTACTTTAATGCCTTT :  160
H293   : ........................T..............A.......................G.............. :  160
C265   : ..................C......T........C.......................C.....CG.............. :  160
C1691  : ........................T..A...................A..............G.............. :  160

                *      1080         *      1100         *      1120         *      1140
H60    : ATATGCATGTATACAAGCAAAACAGGCTTTTACTTTCTCGCCAACTTACAAGGCCTTTCTAAGTAAACAGTATCTGAACC :  240
H293   : ...................G.........C.............................CC..G........T....... :  240
C265   : ..................................................................................... :  240
C1691  : .................T.................T...........T...........G..................... :  240

                *      1160         *      1180         *      1200         *      1220
H60    : TTTACCCCGTTGCTCGGCAACGGCCTGGTCTGTGCCAAGTGTTTGCTGACGCAACCCCCACTGGTTGGGGCTTGGCCATA :  320
H293   : ............T.................................................................... :  320
C265   : ..AC.......................................................................... :  320
C1691  : .....................................................................................T :  320

                *      1240         *      1260         *      1280         *      1300
H60    : GGCCATCAGCGCATGCGTGGAACCTTTGTGTCTCCTCTGCCGATCCATACTGCGGAACTCCTAGCCGCTTGTTTTGCTCG :  400
H293   : ..................................................................................... :  400
C265   : ..................................................................................... :  400
C1691  : ...........................................................T....A.................. :  400

                *      1320         *      1340         *
H60    : CAGCCGGTCTGGGGCAAAACTCATCGGGACTGACAATTCTGTCGTGCTCTCCCGCAA :  457
H293   : ....A..........G.................................................. :  457
C265   : ....A.......................................................... :  457
C1691  : ............A.......................................................... :  457
```

Fig.4-6. The sequence alignment of Enhancer I/X promoters of the four HBV strains in this study.

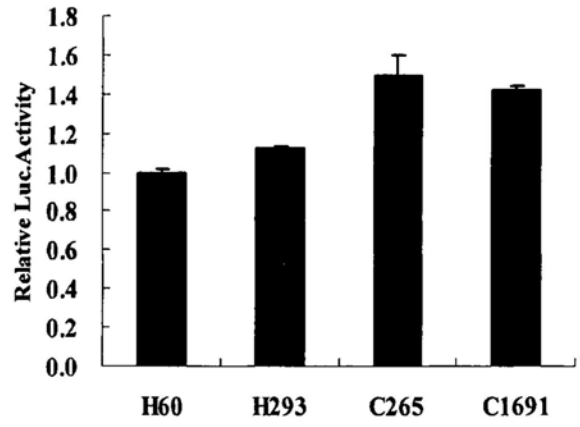


Fig. 4-7. The promoter activities of Enhancer I/X promoters of the four HBV strains in this study.

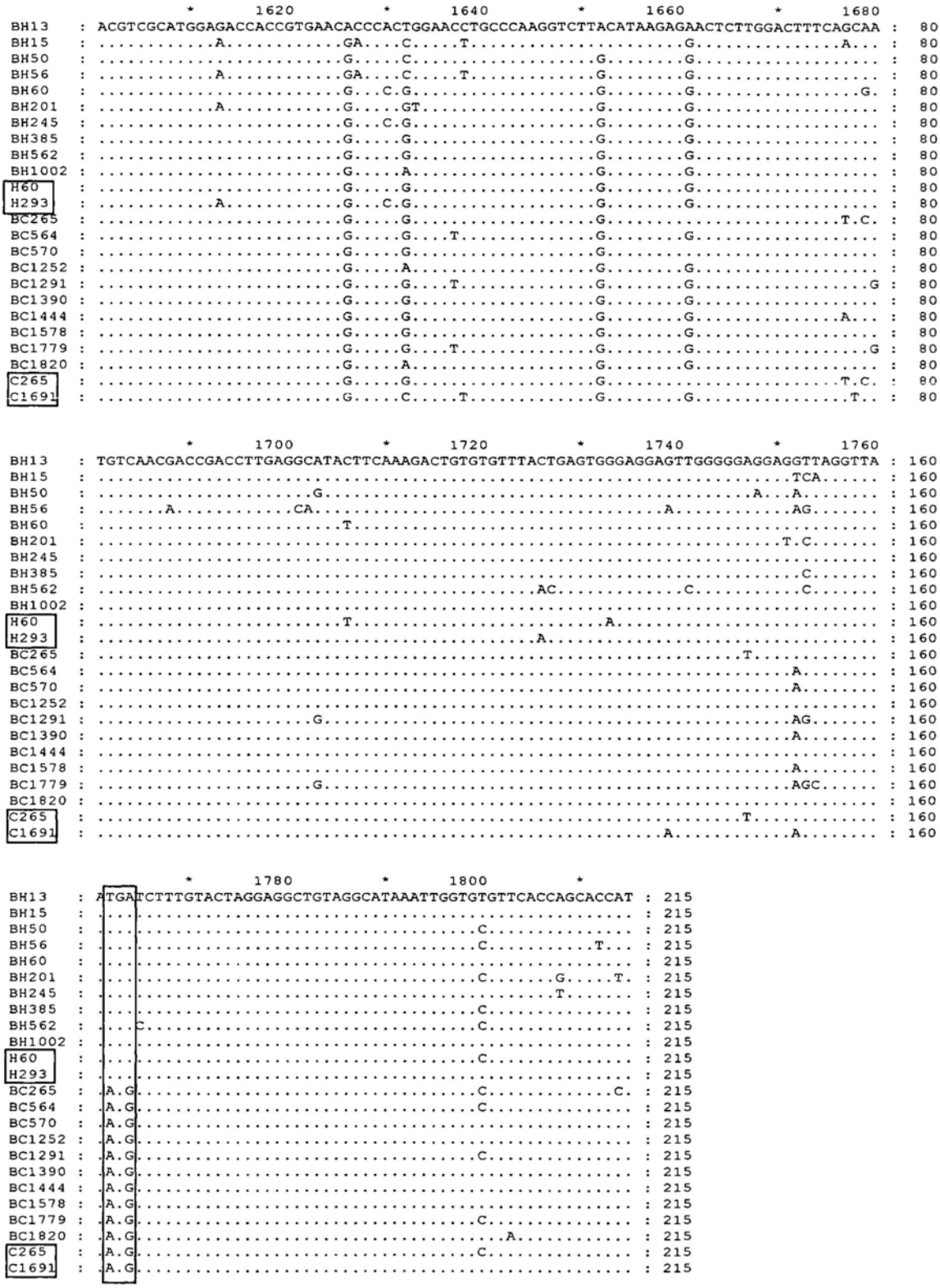


Fig.4-9. The sequence alignment of basal core promoters of genotype B HBVs. The

sequences numbered with H are from HCC patients, and those numbered with C are from asymptomatic carriers.

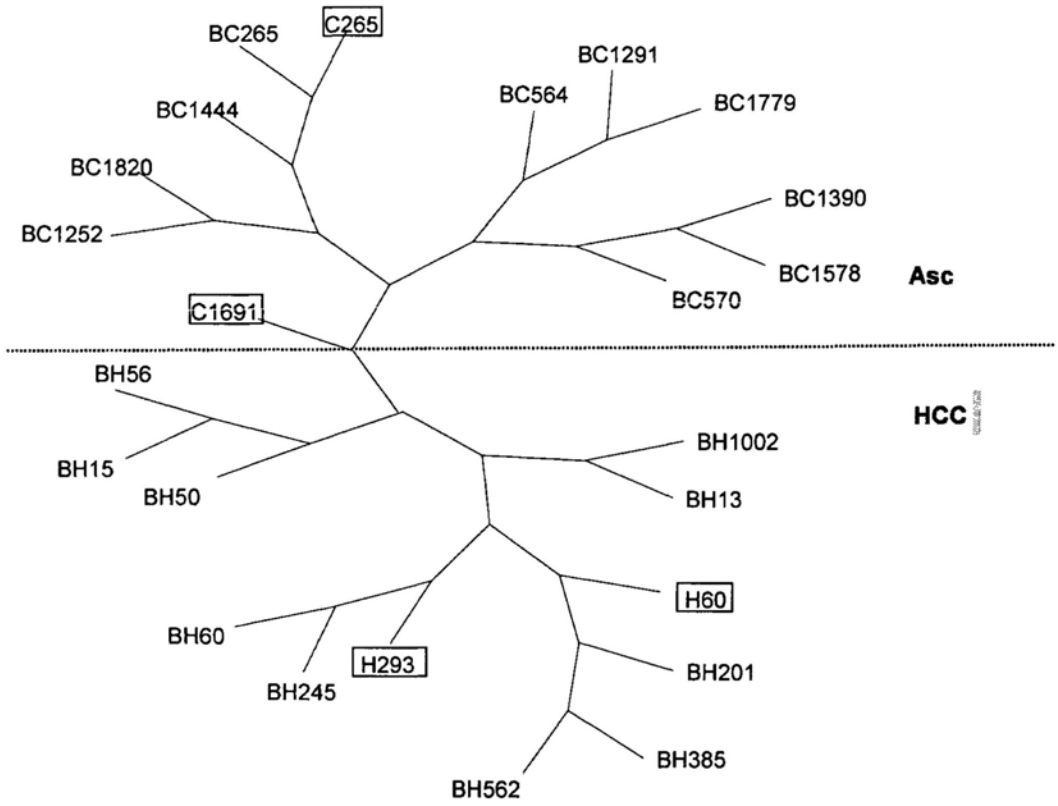


Fig. 4-10. The phylogenetic tree based on the basal core promoter sequences.

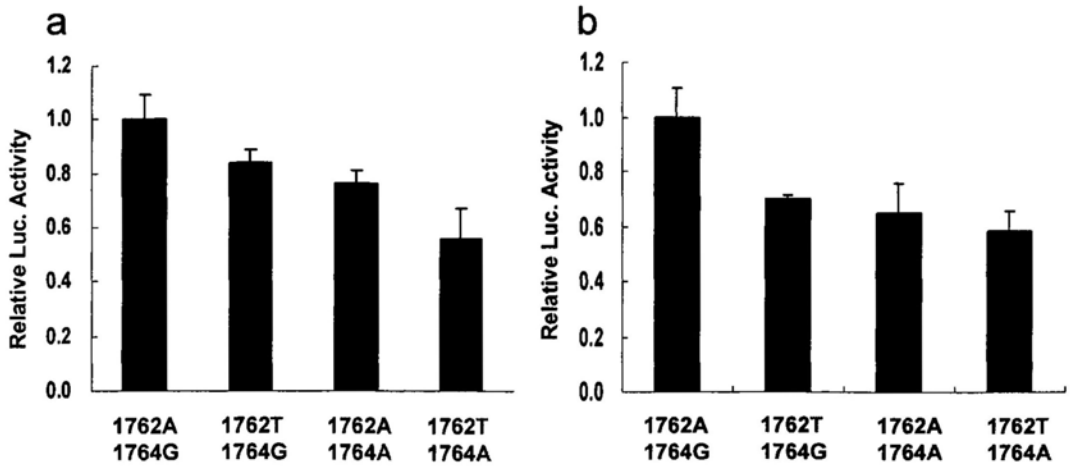


Fig. 4-11. The promoter activities of HBVs isolated from asymptomatic carrier C265

(a) and C1691 (b) before and after site-directed mutagenesis. The original promoter activity of HBV from C265 and C1691 was defined as 1. The experiments were repeated at least 3 times.

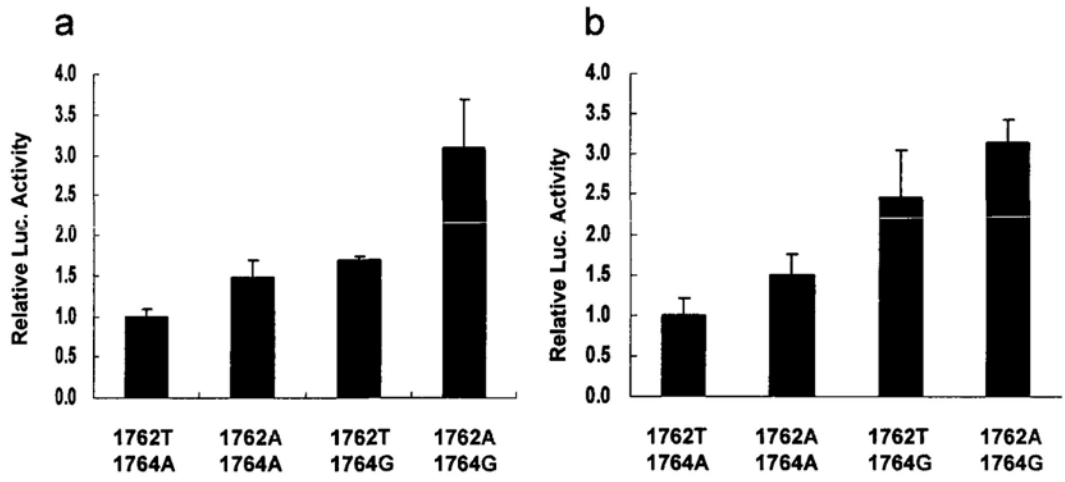


Fig. 4-12. The promoter activities of HBVs isolated from HCC patients H60 (a) and H293 (b) before and after reverse mutagenesis. The original promoter activity of HBV from H60 and H293 was defined as 1.

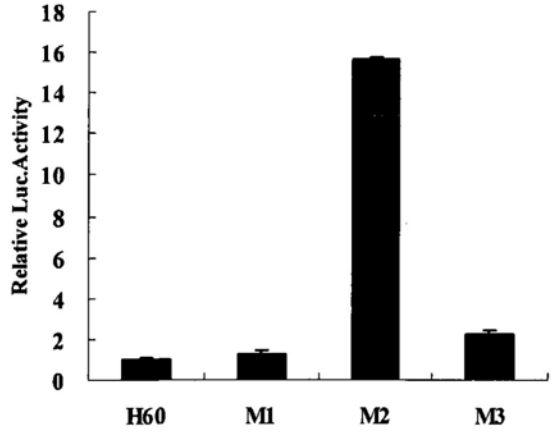


Fig. 4-13. The promoter activity of the strain isolated from HCC patient H60 and some mutation ones. The original promoter activity of H60 was defined as 1. M1:T1706C. M2:A1733G. M3:G1677T/A1679C.

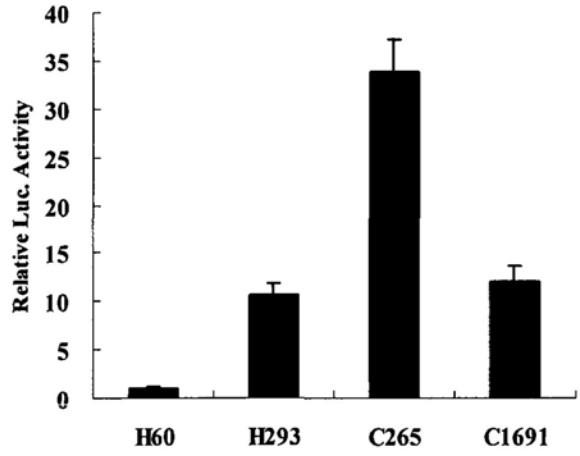


Fig. 4-8. The promoter activities of Enhancer II/Basal core promoters of the four HBV strains in this study.

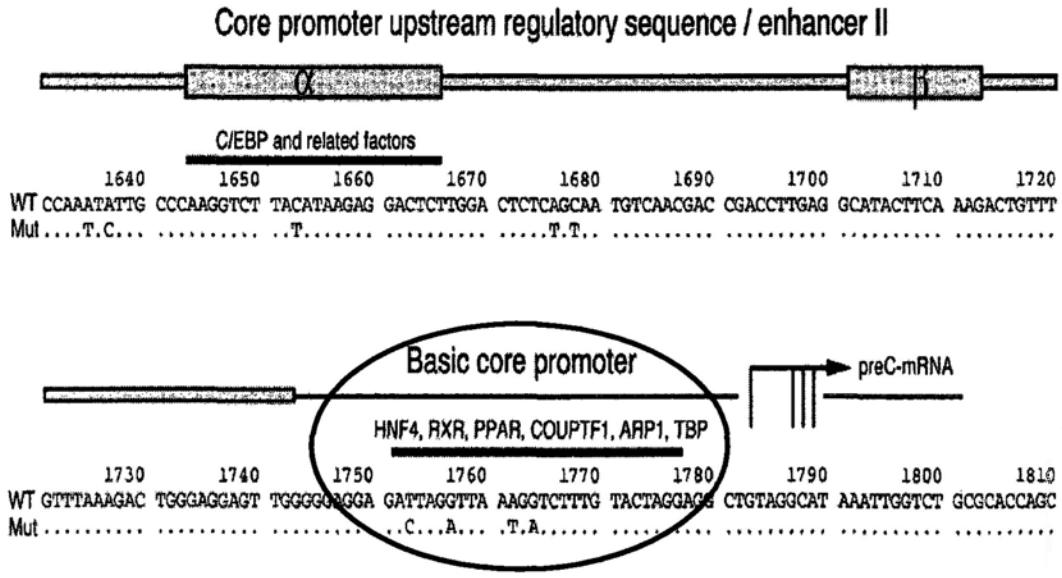


Fig. 4-14. Nucleotide sequences of the mutant and wild-type of enhancer II/Basal core promoter of HBV. The transcription factors which bind around nt1762/nt1764 are shown (Gunther *et al.*, 1998).

Chapter 5

The influence of HBx natural variants on HBV replication and Enhancer II/Basal core promoter activity

5.1 Introduction

Mammalian hepadnaviruses encode a small regulatory protein termed X protein, for which no obvious counterpart exists in the nononcogenic avian hepatitis viruses (Duflot *et al.*, 1995). The X protein is well conserved among all mammalian hepadnaviruses which also shown expression during infection both in human (Su *et al.*, 1998) and in woodchuck (Dandri *et al.*, 1996; Jacob *et al.*, 1997). In addition, it was known to be required for the viral life cycle in the woodchuck (Chen *et al.*, 1993; Sitterlin *et al.*, 2000). HBV is a causative agent of acute and chronic hepatitis also found implicated in HCC. HBV genome encodes a single regulatory protein termed HBx which contains 154 amino acids and has a molecular weight of 17 kDa. The HBx protein localizes in both the cytoplasm and nucleus (Sitterlin *et al.*, 2000; Wang *et al.*, 1991). Its sequence is divided into six regions ranged from A to F and encodes two separate functional domains, the regulatory domain in the N-terminal (Chen *et al.*, 1993) and the transacting domain in the C-terminal (Fig. 5-1). The transacting domain is related to the main transcriptional function of HBx together with other factors and proteins. The regulatory domain of HBx was suggested as a crucial factor for the negative regulation that avoids overexpression of HBx (Murakami *et al.*, 1994; Faktor *et al.*, 1990). Expression of HBx is controlled by Enhancer 1 and X promoter (Colgrove *et al.*, 1989; Spandau *et al.*, 1988; Zhang, 1992, Dikstein

et al., 1990; Faktor *et al.*, 1990), with the X expression believed to be autoregulated (Murakami *et al.*, 1994; Hidaka *et al.*, 1988; Greene, 1991).

HBx has been associated with hepatocarcinogenesis. It does not directly bind to DNA but it can bind to transcription factors to enhance their DNA binding activities and modulate biological processes such as viral replication, transcription, signal transduction, cell cycle progress, protein degradation, cell proliferation, apoptosis, genotoxic stress response and genetic stability (Murakami, 1999, 2001; Bouchard *et al.*, 2004; Arbuthnot *et al.*, 2000; Qadri *et al.*, 1995; Maguire *et al.*, 1991; Lin *et al.*, 1997; Shamay *et al.*, 2002; Andrisani *et al.*, 1999). These transcription factors include AP-1 (Natoli *et al.*,1994), NF-κB (Su *et al.*, 1996), ATF/CREB (Choi *et al.*, 1997; Williams *et al.*, 1995), acidic activators (Haviv *et al.*, 1995), p53 (Elmore *et al.*, 1997), Smad4 (Lee *et al.*, 2001), hypoxia-inducible factor-1α (HIF-1α) (Yoo *et al.*, 2004) RXR (Kong *et al.*, 2000), PPARγ (Choi *et al.*, 2004), TFIIB (Lin *et al.*, 1997) and TFIIH (Qadri *et al.*, 1996), etc. Other functions of HBx also involved the interactions with UV-damaged DNA-binding protein (Sitterlin *et al.*, 1997), proteasome complexes, such as PSMA7 and PSMC1 (Huang *et al.*, 1996), and protease tryptase TL2 (Rakotomahanina *et al.*, 1994), Hsp60 and Hsp70 (Zhang *et al.*, 2005).

On the other hand, HBx also activates several signal transduction pathways including NF-κB, mitogen-activated protein kinase, protein kinase C, and JAK/STAT pathways (Pan *et al.*, 2001; Kekule *et al.*, 1993; Luber *et al.*, 1993; Benn *et al.*, 1994; Lee *et al.*, 1998; Waris *et al.*, 2001). It modulates mitochondrial calcium release (Bouchard *et al.*, 2002, 2003; Chami *et al.*, 2003; Oh *et al.*, 2003), thus leads to the activation of the Src family of tyrosine kinases (Klein *et al.*, 1997, 1999), as well as Ras and Jun N-terminal protein kinase pathways (Benn *et*

al., 1996; Natoli *et al.*, 1994). HBx has been shown to promote tumor cell invasion (Lara-Pezzi *et al.*, 2002) whilst several studies have showed its crucial role in regulating apoptosis (Huo *et al.*, 2001; Takada *et al.*, 1999; Su *et al.*, 1997; Bergametti *et al.*, 1999; Shirakata *et al.*, 2003). Taken together, these studies indicate that HBx plays a prominent role in HBV-induced hepatocarcinogenesis.

Although many steps of the HBV replication have been elucidated, there is little information about the regulation of viral replication. Several lines of evidence suggest that HBx can enhance HBV replication in both cell culture and the transgenic mice model (Zoulim *et al.*, 1994; Bouchard *et al.*, 2001; Reifenberg *et al.*, 2002; Xu *et al.*, 2002; Zhang *et al.*, 2001; Melegari *et al.*, 1998, 2005; Singh *et al.*, 2003). This was done via the activation of viral transcription (Colgrove *et al.*, 1989; Melegari *et al.*, 1998; Zhang *et al.*, 2001) or reverse transcription activity of the viral polymerase (Bouchard *et al.*, 2001; Klein *et al.*, 1999). In previous study, HBx has been demonstrated to trans-activate a variety of viral and cellular promoters including the HBV promoters (Murakami, 1999, 2001).

In this study, we have four natural variant HBx genes that were isolated from two HCC patients and two AsCs. The HBx gene from HCC patient H60 was found truncated, while the one from another patient H293 has A1762T/G1764A double mutations. For the HBx gene from AsCs, C265 and C1691, the nt1762/nt1764 site was found similar to the wild type. The A1762T/G1764A double mutations were also known to affect the Enhancer II/Basal core promoter sequence. However, whether these four natural variant HBx proteins can influence the HBV Enhancer II/Basal core promoters harboring 1762A/1764G, 1762T/1764A or other mutations is still unknown. In this study we have investigated the HBx influence on the

Enhancer II/Basal core promoter activity, the HBV replication and antigen expression.

5.2 Materials and methods

5.2.1 Patients and workflow

For patients information please refer to Chapter 2, section 2.2.1, on page 31. Process of constructing recombinant adenovirus with the Adeno-X™ Tet-Off Expression Systems 1 is shown in Fig. 5-2.

5.2.2 Plasmid construction

5.2.2.1 Amplification of HBx

HBx gene was amplified by PCR using primer HBVX-1362-1385-KpnI-F and HBVX-1845-1820-Xba I-R (Table 5-1) from the self ligated HBV genome. The thermocycling conditions were set as follows: 94°C for 3 min, then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min followed by a final extension of 72°C for 10 min. The high fidelity pfu Taq polymerase (Promega, WI, USA) was used. The PCR products were separated by electrophoresis on 1.0% agarose gel and detected by EtBr staining with a UV transilluminator.

5.2.2.2 Cloning of PCR products of HBx into pcDNA4/HisMax-B and sequence alignment

The PCR products of HBx were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and digested with Kpn I and Xba I (NEB, MA, USA). After purification, the fragments were cloned into pcDNA4/HisMax-B (Invitrogen, CA, USA) through the Kpn I and Xba I sites. The positive clones were sequenced using primer BGHrev (pcDNA4) (Table 5-1) by Techdragon (Hong Kong). The sequence alignment was carried out

using Vector NTI Suite 9 and GeneDoc 2.6.

5.2.2.3 Cloning of HBx into pAdeno-X vector

PCR was performed using primer HBVX-PCDNA4-NotI-F and HBVX-1845-1820-Xba I-R (Table 5-1). The condition employed has been described earlier in 5.2.2.1. The PCR products were digested with Not I and Xba I (NEB, MA, USA), then purified and cloned into pTRE-Shuttle2 vector (BD, NJ, USA). Thereafter, the HBx gene was cloned from pTRE-Shuttle2 vector through PI-Sce I and I-Ceu I sites into pAdeno-X vector (BD, NJ, USA). All the work was done according to the instructions of the Adeno-X™ Tet-Off® Expression System 1 (BD, NJ, USA). The Adeno-X-HBx plasmids were then transformed into DH5α. The transformants for recombinant Adeno-X-HBx DNA were screened using Adeno-X forward PCR primer and Adeno-X reverse PCR primer/HBVX-1845-1820-Xba I-R (Reverse primer 2). For the recombinant clones, PCR yielded a 287bp segment or 1.5kb fragment (Fig. 5-3). The recombinant pAdeno-X-HBx Viral DNA plasmids were analyzed by restriction digestion with PI-Sce I and I-Ceu I. The insert was about 2kb in length.

5.2.3 Production of recombinant adenovirus

Adeno-X contains two Pac I restriction sites, which are located at both ends of the viral genome, 3' and 5' to the inverted terminal repeats (ITR). The ITRs contain the origins of adenovirus DNA replication and must be positioned at the termini of the linear Ad DNA molecule to correctly support the formation of the replication complex. Therefore, plasmid was digested with Pac I before the packaging the recombinant Adeno-X-HBx.

5.2.3.1 Transfection of HEK 293A cells with Pac I-digested Adeno-X-HBx

HEK 293A cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum under 5% CO_2 incubation at 37°C. First of all, HEK 293A cells were seeded at a density of 1-2 x 10^6 cells per 60-mm culture plate 12-24 h before transfection. The cell line was a gift from Dr. Chen Xinchun of Shenzhen Donghu Hospital. Plated samples were then left to incubate at 37°C in a humidified atmosphere under 5% CO_2. For best results, the cells should be 50-70% confluent at the time of transfection. Prior to transfection, the cells were checked for flat morphology and strong adherence to the plate. Each 60-mm culture plate was transfected with 10 µl of Pac I-digested Adeno-X-HBx using Lipofectamine 2000 (Invitrogen, CA, USA). One day later and periodically thereafter, the samples were checked for cytopathic effect (CPE) under microscope. One to two week later, cells were transferred to a sterile 15-ml conical centrifuge tube. Infected cells that still adhered to the bottom or sides of the culture plate were dislodged into the medium by gentle agitation. Then sample suspension was left to centrifuge at 1,500x g for 5 min at room temperature. Supernatant was discarded whilst pellet was resuspended in 500 µl sterile PBS. Lysed cells were put to undergo three consecutive freeze-thaw cycles with each cycle conditioned as follows: cells were freeze at -80°C; then thawed by placing the sample containing tube in 37°C water bath, yet the tube was taken out before it reached 37°C. The samples were vortex mixed thoroughly after each freeze/thaw cycle. The samples were left to centrifugation to remove pellet debris. Supernatant was stored at -20°C or used immediately.

Fresh 60-mm culture was infected by adding 250 µl (50%) of the cell lysate that we prepared earlier. The lysates were directly added to the medium, then left to incubate as normal.

CPE should be observed within one week. When more than 50% of the cells were detached from the plate, viral stock was prepared as described earlier in this session. Those stocks were named "primary amplification" and stored at -80°C. Finally, the adenoviral titer was determined by FQ-PCR using primer Ade-X-F and Ade-X-R (Table 5-1) while End-Point Dilution Assay was employed for titering the adenovirus in the end.

5.2.3.2 Amplifying recombinant adenovirus: preparing high-titer stocks

The following protocol was used to amplify recombinant adenovirus and to produce additional stocks of the Adeno-X Tet-Off® regulatory and Adeno-X TRE-βgal control viruses. First of all, the HEK 293A cells were plated in a T75 flask approximately 24 hours before infection. For best results, the monolayer should be 50-70% confluent at the time of infection on the following day. Then the cells were incubated overnight at 37°C in a humidified atmosphere at 5% CO_2. On the following day, the medium was replaced with 5 ml of fresh growth medium that contains adenovirus with a multiplicity of 1-5 pfu/cell. After that the cells were incubated for 90 min at 37°C in a humidified atmosphere at 5% CO_2. Then the cells were incubated for 3-4 days at 37°C after replacing the old medium with 10 ml of fresh growth medium. When approximately 50% of the cells had detached, the suspension was transfered to a sterile, 15-ml conical centrifuge tube. The virus was at last isolated using the freeze-thaw method and adenoviral titer was determined as described earlier in this session.

5.2.3.3 Infecting HepG2 cell with adenovirus

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum under 5% CO_2 incubation at 37°C. First of all, the HepG2 cell was

seeded in 6-well plate 12–24 hrs before infection. The cell was ≥90% viable at the time of infection. Then the growth medium was removed from cell cultures, and the regulatory virus Adeno-X Tet-Off and response virus (Adeno-X-HBx-H60, -H293, -C265 and -C1691 and Adeno-X TRE-βgal) were added to the center of each plate the next day. The multiplicity of infection (MOI) was 1, 2, 5 and 10. The ratio of regulatory virus to the response virus was 1:1. The plate was tipped to spread the viruses evenly over the cells. After that the plate was covered and incubated in a humidified 5% CO_2 incubator at 37°C for 4 hours to allow the virus to infect the cells. Then fresh complete growth medium without doxycycline was added and cells were incubated in a humidified 5% CO_2 incubator at 37°C. At last, HBx expression at 24 hrs and 48 hrs following viral infection was analyzed.

5.2.4 Total protein extraction for Western blot analysis

HepG2 cells were lysed for 30 min on ice with lysis buffer. They were then centrifuged at 16,000×g for 30 min at 4°C. Protein concentration of the samples was measured using the Bradford assay (BioRad, CA, USA) according to the manufacturer's instructions. Equal amounts of protein were loaded on a 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel. SeeBlue Plus2 Pre-Stained Standard markers (Invitrogen, CA, USA) were used as the standards. After electrophoresis, the separated proteins were transferred onto a 0.45 μm nitrocellulose membrane. The membranes were blocked with 5% fat-free milk for 1 h at room temperature. After washing three times with phosphate buffered saline containing 0.1% Triton X-100 (PBST), the membranes were incubated with anti-HBx (Abcam, MA, USA) in 5% fat-free milk O/N at 4°C. On the next day, the membranes were washed thrice, each for 10 min

in PBST and then incubated with secondary antibody in 5% fat-free milk for 1 h at room temperature. Signals were developed using an enhanced chemiluminescence (ECL+) kit (GE Healthcare Bio-Sciences Corp, NJ, USA).

5.2.5 Luciferase assays

DNA was transfected into the HepG2 cells using Lipofectamine 2000 (Invitrogen, CA, USA). Transfection efficiency was routinely checked by cotransfecting 100 ng of the pRL-SV40 vector as an internal control. The HBV BCP plasmids include BCP-H60-1762A/1764G, BCP-H60-1762T/1764A, BCP-C265-1762A/1764G, BCP-C265-1762T/1764A, H60-BCP-1677T/1679C, H60-BCP-1706C and H60-BCP-1733G. In order to verify the results, all transfections were routinely performed at least in triplicate. The final amount of plasmid DNA was 1 μg per transfection in 12-well plate. After 4h of transfection, regulatory virus Adeno-X Tet-Off as well as response virus (Adeno-X-HBx-H60, -H293, -C265 and -C1691 and Adeno-X TRE-β gal) were added to the center of each plate. The MOI noted was 10. The ratio of regulatory virus to the response virus was 1:1. Cells were harvested after 24 h of virus infection. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega, WI, USA). Firefly luciferase activity of the reporter plasmid was normalized to renilla luciferase activity and expressed as fold induction of control.

5.2.6 Adeno-X-HBx and HBV-1.5X co-transfection

The transfection and virus infection method was the same as that described in last session

(5.2.5) except that the HBV BCP plasmids were changed to C265-HBV-1.5X. The HBV DNA quantification and HBsAg detection methods were the same as mentioned in Chapter 3, section 3.2.4, on page 59.

5.2.7 *Statistics*

All statistical analyses, t-test and one way ANOVA, were done using SPSS 13.0 for Windows. A p-value less than 0.05 was considered as statistically significant.

5.3 Results

5.3.1 HBx sequencing

The HBx gene on the vector pcDNA4/HisMax-B was sequenced and analyzed. The amino acid alignment of the HBx proteins from the four sample strains is shown in Fig. 5-4. For C265, C1691 and H293, the HBx protein is 154aa in length, while the one for H60 was clearly truncated at the C terminal, with only 119aa in length. The HBx protein from H293 has also noted to possess Lys130Met and Val131Ile mutations.

5.3.2 Adeno-X-HBx plasmid confirmation

After transformation and selection on LB/Amp+ plates, *E. coli* containing recombinant Adeno-X-HBx DNA could be rapidly identified by PCR using the BD Adeno-X Forward PCR Primer and Reverse PCR Primer, and HBx specific reverse primer. For all the positive recombinant constructs, PCR yielded a 287bp segment or 1.5kb fragment.

Restriction analysis of recombinant Adeno-X-HBx viral DNA with PI-Sce I and I-Ceu I

was shown in Fig. 5-5. As seen in the figure, a band about 2kb appeared at the expected position. Pac I digestion result for the transfection and package of Adeno-X-HBx was shown in Fig. 5-6. The digestion results were both as expected.

5.3.3 HBx expression

The Western blot results for HBx protein expression (i.e. after 48 h of Adeno-X-HBx virus infecting HepG2 cells with MOI from 1 to 10) is shown in Fig. 5-7. The expression level of HBx was clearly very low for H60. For the other three samples, HBx expression levels were almost the same. Since the HBx protein sequences from the strain C265 and C1691 were highly homologous, HBx proteins from H293 (with Lys130Met and Val131Ile mutations) and C265 were selected for the following studies.

5.3.4 HBx influence on the HBV BCP activity

The influence of HBx protein on the HBV BCP activity is shown in Fig. 5-8. The HBx protein from strain C265 increased the BCP (with 1762A/1764G) activity of both C265 and H60 by 80% ($p<0.01$) and 52% ($p<0.01$), respectively, whilst it increased the BCP (with 1762T/1764A) activity for both C265 and H60 by 58% ($p<0.05$) and 21% ($p<0.05$), respectively. The HBx protein from strain H293 increased the BCP (with 1762A/1764G) activity of both C265 and H60 by 110% and 75%, respectively ($p<0.01$). It also increased the C265 and H60 BCP (with 1762T/1764A) activity respectively by 94% and 31% ($p<0.01$). These results suggest that the HBx proteins from both H293 (with Lys130Met and Val131Ile mutations) and C265 could increase the HBV BCP activity no matter the BCP harbors

1762A/1764G or 1762T/1764A. Besides, HBx protein from strain H293 has higher augment capacity on the HBV BCP activity no matter the BCP harbors 1762A/1764G or 1762T/1764A (Fig. 5-9; $p<0.05$).

For both HBx proteins, the activity of BCPs from strain C265 or its mutant has been increased to a higher level than those from strain H60 and its mutant (Fig. 5-8). This indicates that, apart from the nt1762/nt1764 site, the BCP of C265 has other nucleotide mutations that can increase its activity compared with the BCP from H60. These point mutations have been discussed in Chapter 4, section 4.3.7, on page 85. For the BCP from strain C265, it possesses G1677T/A1679C and T1706C mutations when it is compared with H60 BCP. However, whether these mutations could genuinely affect the interaction between HBx and HBV BCP is yet to be elucidated. So far, results from our studies have indicate that the HBx protein from strain C265 could increase the H60-BCP with 1677T/1679C activity by 35% ($p<0.05$), and the H60-BCP with 1706C activity by 58% ($p<0.05$). The HBx protein from strain H293 could increase the H60-BCP with 1677T/1679C and 1706C by 10% ($p<0.05$) and 50% ($p<0.05$), respectively. All of these results are shown in Fig. 5-10. Comparison made between HBx proteins from strain C265 and H293 on HBV BCP mutants (with 1677T/1679C and 1706C) activity also showed that for those mutations the HBx of C265 could increase the BCP activity to a higher level than the HBx from H293 (Fig. 5-11; $p<0.05$). For 1733G mutation of BCP, the HBx influence pattern on the BCP is the same as that for nt1762/nt1764 mutations (data not shown).

5.3.5 HBx influence on the HBV replication and HBsAg expression

The HBx protein from strain C265 was found to increase the C265 HBV virus replication by 2.2 times (log10 copies/ml, 5.8 vs. 5.45, $p<0.05$), and the HBsAg expression by 1.73 times (OD450, 1.018 vs. 0.589, $p<0.05$). The HBx protein from strain H293 increased the C265 HBV virus replication by 3.8 times (log10 copies/ml, 6.03 vs. 5.45, $P<0.05$) whilst it could also increase its HBsAg expression by 4.48 times (OD450, 2.641 vs. 0.589, $p<0.01$). Our results also showed that the HBx protein from H293 (with Lys130Met and Val131Ile mutations) has higher capacity in increasing HBV replication ($p<0.05$) and HBsAg expression ($p<0.01$) than the one from C265 (Fig. 5-12).

5.4 Discussion

5.4.1 HBx protein natural variants

In humans, HBx has been shown to be expressed in HCC tissues and is the most preferential sequence retained in the integrated form of HBV (Paterlini *et al.*, 1995; Zhu *et al.*, 1993). Several mutants have been identified from sera and/or liver tissues from patients with HCC. HBx sequences isolated from integrated HBV genomes in tumor tissues have frequently been reported to have a deletion at the 3'-end (Wei *et al.*, 1995; Hsia *et al.*, 1997; Poussin *et al.*, 1999; Yamamoto *et al.*, 1993; Schluter *et al.*, 1994; Tsuei *et al.*, 1994). In this study, the HBx sequence from H60, sample from a patient of 31 years old, was truncated with only 119 amino acids. Similar HBx truncation has also been reported before in chronic hepatitis B patients, but at a lower incidence than that seen in HCC (Koike, 1998). As a matter of fact, truncated HBx was believed to enhance the transforming ability of Ras and Myc (Tu *et al.*, 2001), which suggested that such truncation might play a role at the early stage of liver carcinogenesis and be

selected during the development of HCC.

Recently, a Ser31Ala point mutation (Yeh *et al.*, 2000) and a linked-point mutation, Lys130Met and Val131Ile (Takahashi *et al.*, 1998; Baptista *et al.*, 1999), in HBx were shown to be prevalent in HCC patients. In this study, HBx protein with Lys130Met and Val131Ile mutations was also observed in HCC patient (i.e. H293, who was aged 26 when the sample was collected). However, the biological functions of these mutants have not been explored so far.

5.4.2 HBx expression in HepG2 cell line

In the present study, the HBx proteins were all expressed in HepG2 cell line for all the four samples. However, the expression level of HBx was extremely low in H60 when it was compared with the other three HBx proteins. This might be explained by the lost of the transcription activity domain, which genuinely listed at the truncated C-terminal of HBx derived from H60. This truncation might cause the lost of auto-regulation and decrease its transcription activity, therefore, just a minimal level of HBx could be found in H60.

5.4.3 HBx influence on the Enhancer II/Basal core promoter activity

Based on our study, both the HBx proteins from strain H293 (with Lys130Met and Val131Ile mutations) and C265 could increase the BCP activity of strain C265, H60 and their nt1762/nt1764 mutated variants. For HBx protein from H293, it has higher capacity in increasing the HBV BCP activity when it was compared with that from C265. These results indicate that the Lys130Met and Val131Ile mutations of HBx could increase its ability in enhancing the HBV BCP activity no matter the BCP harbors 1762A/1764G or 1762T/1764A.

However, it was also suggested that the nt1762/nt1764 double mutations in the BCP region had no effect on the interaction of HBx and HBV BCP as revealed in this study. Previous studies have highlighted some explanation for the enhancing ability of HBx on the Enhancer II/Basal core promoter activity. Since HBx protein has transcriptional activity but no DNA biding domain, its influence on the promoter activity may be mediated through the interaction of HBx with other transcription factors. For example, Choi *et al.* reported that the direct interaction of HBx and C/EBPα could strongly activate the Enhancer II/BCP of HBV in a synergistic manner (Choi *et al.*, 1999). They did serial deletion analysis of the enhancer II/BCP and had identified the responsible region (nucleotides 1639-1679), in which two C/EBP-binding sites were located. Domain analysis of HBx showed that the central region (amino acids 78-103) was necessary for direct interaction with C/EBPα. However, the complete form of HBx was necessary for the synergistic activation of the HBV BCP. In this study, the 1677T/1679C and 1706C mutations of HBV BCP have also been found to increase the interaction of HBx protein from C265 (without Lys130Met and Val131Ile mutations) with HBV BCP. The mutation region from nt1677 to nt1706 of BCP might actually prefer the binding of the HBx protein (without Lys130Met and Val131Ile mutations) and related transcriptional factors complexes, thus to increase the corresponding promoter activity.

5.4.4 HBx influence on the HBV replication and antigen expression

In vivo HBV replication requires the expression of its regulatory protein, which is known as the HBx (Bouchard *et al.*, 2004; Keasler *et al.*, 2007). Indeed, viral infection cannot be established in woodchucks by injecting HBx-deficient woodchuck HBV DNA (Chen *et al.*,

1993; Zoulim *et al.*, 1994). In the HepG2 human hepatoma cell line and mouse model, an HBx-deficient virus replicates at a much lower rate than that of HBx^+ virus, yet such replication could be completely restored by adding a complementing plasmid encoding HBx (Bouchard *et al.*, 2001, 2003; Melegari *et al.*, 1998, 2005; Keasler *et al.*, 2007). Again, this finding supports an important role of HBx in stimulating HBV replication. In our study, HBx was found to up-regulate HBV replication and HBsAg expression. The mutated HBx with Lys130Met and Val131Ile mutations from H293 was found to increase HBV replication at a much higher rate than that of HBx without the mutations from C265. This might be one of the reasons that HBx could induce HCC by up-regulating HBV replication as well as HBsAg expression, with the HBsAg also known as one of oncoproteins.

Apart from increasing HBV replication, HBx could also influence other viruses. For example, it could induce ongoing HIV-1 replication and HIV-1 long terminal repeat (LTR) transcription by synergizing with Tat protein and with T-cell activation signals. These indicate that the presence of HBx would cause a faster progression of AIDS in HBV-HIV co-infected individuals (Gómez-Gonzalo *et al.*, 2001). Besides, Assogba *et al.* also showed that the HBx protein could cause transcriptional activation of the promoter of human cytomegalovirus immediate early gene (CMV-IE) via the NF-κB site (Assogba *et al.*, 2002).

But the mechanism for HBx to increase HBV replication remains still unknown. Tang *et al.* showed that the transcriptional transactivation function of HBx protein is crucial in its augmentation role during HBV replication. The C-terminal two-thirds (amino acids 51 to 154) of HBx, which contains the transactivation domain, was known to be crucial for the augmentation function yet not the N-terminal one-third (aa 1 to 50). Furthermore, the regions

between aa 52 to 65 and aa 88 to 154 were believed to be pivotal also for the augmentation function of HBx in HBV replication (Tang *et al.*, 2005). From previous studies, the underlying mechanism which might fit for the augmentation function of HBx was deduced as the following: 1) The alteration of cytosolic calcium by HBx. Bouchard *et al.* showed that the alteration of cytosolic calcium was a fundamental requirement for HBV replication and was mediated by HBx protein (Bouchard *et al.*, 2001). The regulation of intracellular calcium is crucial for controlling cellular metabolism, cell cycle, signal transduction, protein synthesis, transcription, and apoptosis (Clapham *et al.*, 2007). It is therefore not surprising that a number of viruses, including retroviruses such as HIV, rotovirus, adenovirus, and Rubella virus, encode regulatory proteins that alter normal calcium homeostasis to benefit one or more aspects of viral replication (Matthews *et al.*, 1998; Beatch *et al.*, 2000; Brunet *et al.*, 2000; Schubert *et al.*, 1996). 2) Increasing core protein serine phosphorylation by HBx. HBV core protein forms the capsid of viral particles and is essential for viral genome DNA replication and maturation. The C terminus of core protein contains three serines at positions 155, 162, and 170, phosphorylation of which is important for viral DNA replication. Melegari *et al.* demonstrate that the phosphorylation of these serines is stimulated by HBx protein (Melegari *et al.*, 2005). So HBx might therefore stimulate HBV replication by increasing core serine phosphorylation. 3) The interaction of HBx with DDB1. The interaction between Hbx and cellular protein DDB1 could mediate cell death (Leupin *et al.*, 2003; Lin-Marq *et al.*, 2001; Sitterlin *et al.*, 2000). However, Leupin *et al.* has showed that such interaction is critical for HBx to promote HBV genome replication in human hepatoma cells (Leupin *et al.*, 2005). 4) The influence of HBx on Enhancer II/Basal core promoter activity as demonstrated in our study. Faktor *et al.* reported

that transactivation of HBV enhancers and promoters could be done by HBx (Faktor *et al.*, 1990; Nakatake *et al.*, 1993). Based on our findings, an increase of the Enhancer II/Basal core promoter activity might induce a corresponding increase in the replication of HBV.

In conclusion, this study has shown that HBx protein could actually increase the Enhancer II/Basal core promoter activity, HBV replication and HBsAg expression. The Lys130Met and Val131Ile mutations of HBx could further increase these abilities. At the same time, the A1762T/G1764A double mutations in the BCP region might not affect the interaction between HBx and HBV BCP. However, the 1677T/1679C and 1706C mutations of HBV BCP could increase the interaction of wild type HBx protein (without Lys130Met and Val131Ile mutations) with HBV BCP.

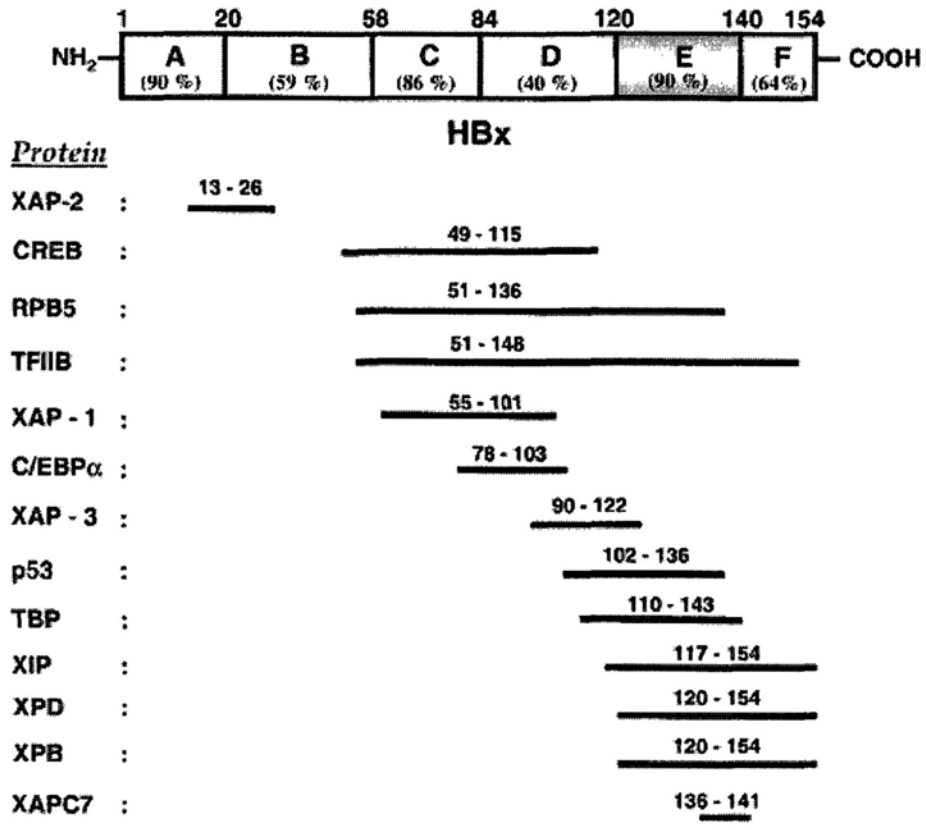


Fig. 5-1. The HBx regions and HBx-binding proteins. The HBx sequence has been divided into six regions (A-F) based on its homology to the X protein of ground squirrel hepatitis and woodchuck hepatitis virus. The regions of homology along with the percentage of homology are depicted on the top panel. The X binding proteins are shown below this panel. The binding co-ordinates of these proteins are positioned along the regions of interaction on HBx (Reddi *et al.*, 2003).

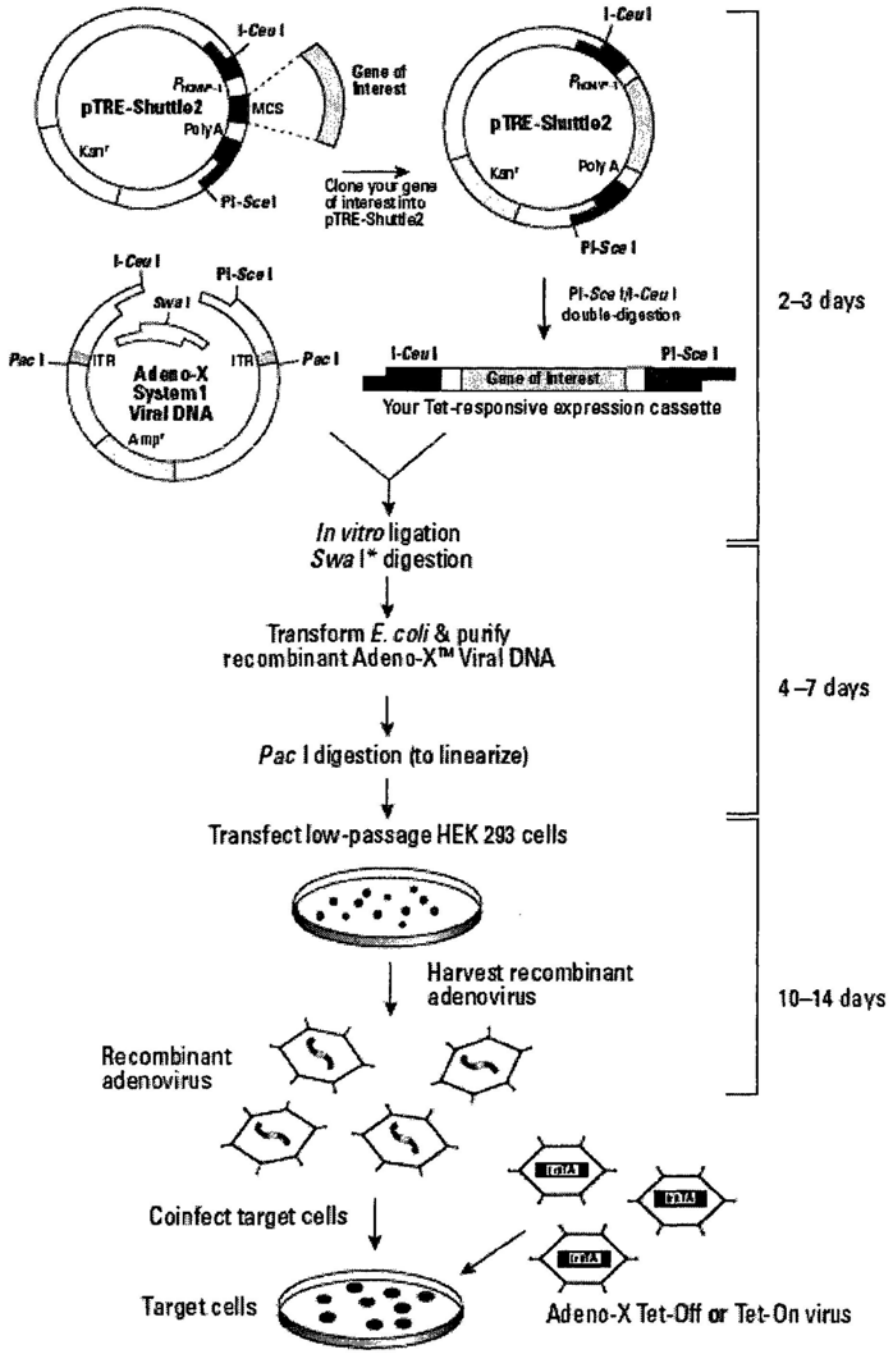


Fig. 5-2. Process of constructing recombinant adenovirus with the Adeno-X™ Tet-Off Expression Systems 1.

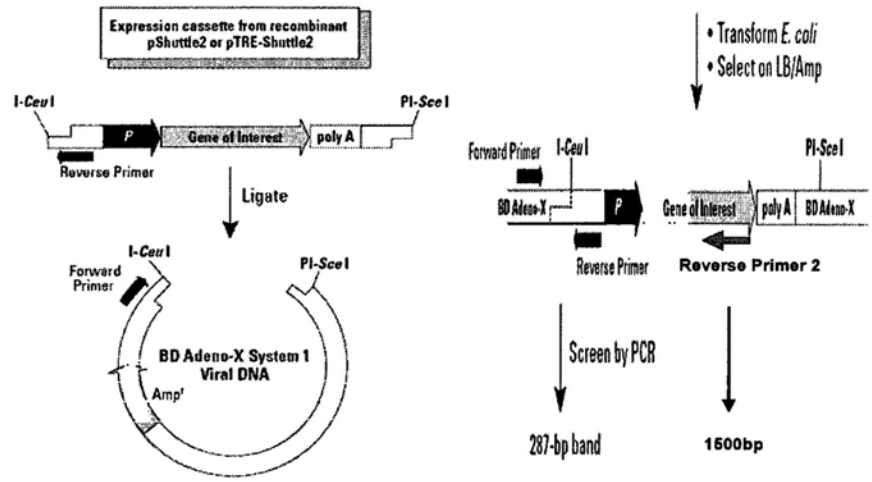


Fig. 5-3. Screening transformants for recombinant pAdeno-X-HBx DNA using the BD Adeno-X™ System 1 PCR Screening Primer Set and HBx specific primer.

```
                *         20         *         40         *         60         *
C265  : MAARLCCQLDPARDVLCLRPVGAESRGRPLSGPLGALPPASPPVVPTDHGAHLSLRGLPVCAFSSAGPCALRFTSARR :  78
C1691 : ............................P.............L................................. :  78
H293  : ............................P.....P..................................... :  78
H60   : ..................................................T.......................... :  78

        80         *        100         *        120         *        140         *
C265  : METTVNAHGNLPKVLHKRTLGLSSMSTTDLEAYFKDCVFTEWEELGVEVRLKVFVLGGCRHKLVRSPAPCNFFTSA : 154
C1691 : ........R.............V.....................E.I.....................C.......... : 154
H293  : .......P.............A.............N......E....MI..........C.......... : 154
H60   : ....................A.............----------------------------------------- : 119
```

Fig. 5-4. Amino acids alignment of the four HBx proteins in this study. For C265, C1691 and H293, the HBx protein is 154aa in length; for H60, it has only 119aa. The HBx from H293 has Lys130Met and Val131Ile mutations. The point indicates the amino acid is the same as the first line. The broken line indicates the deletion region for the HBx protein from H60.

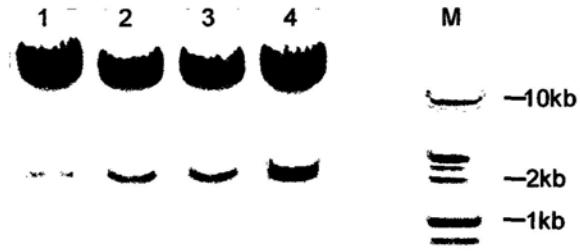


Fig. 5-5. Restriction analysis of recombinant Adeno-X-HBx viral DNA with PI-Sce I and I-Ceu I. The digests were resolved on a 0.8% agarose gel. The insert is about 2kb in length. 1. H60. 2. H293. 3. C265. 4. C1691. M. Promega 1kb DNA Ladder.

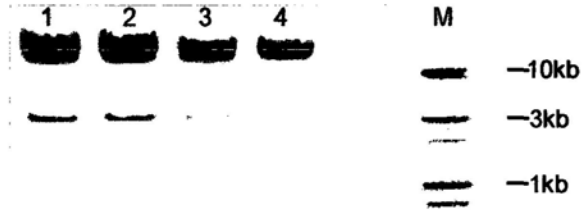


Fig. 5-6. Pac I digestion result for the transfection and package of Adeno-X-HBx. The digests were resolved on a 0.8% agarose gel. 1. H60. 2. H293. 3. C265. 4. C1691. M. Promega 1kb DNA Ladder.

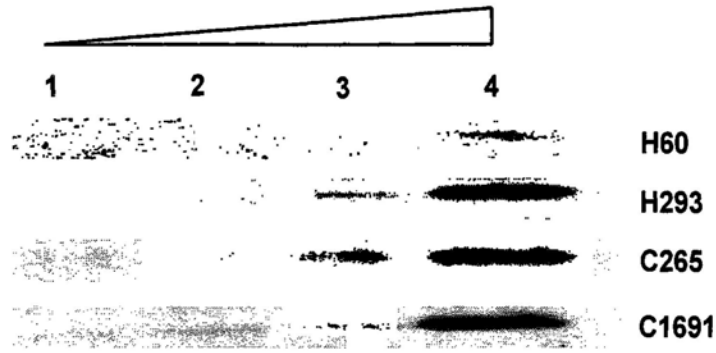


Fig. 5-7. Western Blot result for HBx protein after Adeno-X-HBx virus infected HepG2 cells 48h. 1. MOI=1. 2. MOI=2. 3. MOI=5. 4. MOI=10.

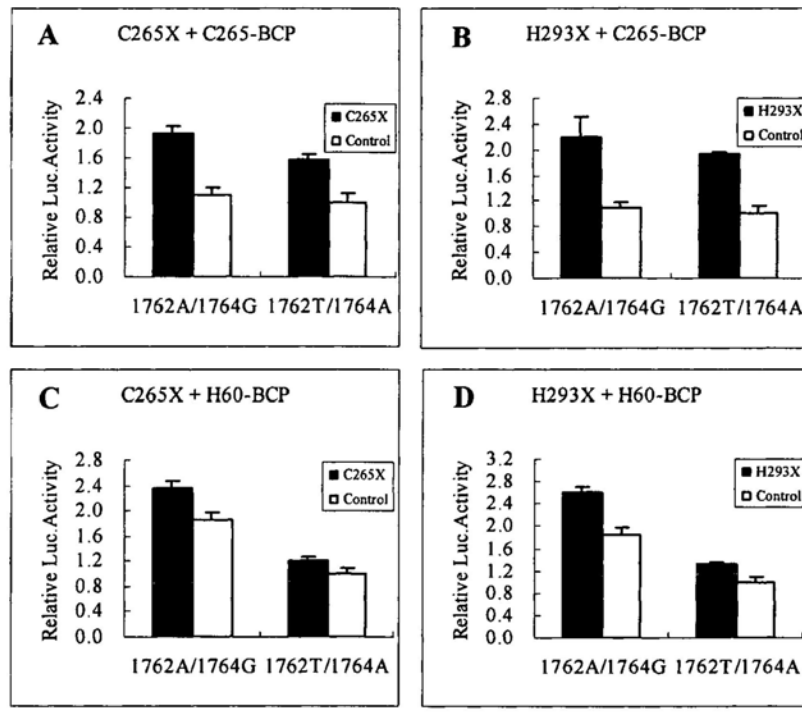


Fig. 5-8. HBx protein influence on the HBV BCP (with 1762A/1764G and 1762T/1764A). A. Effect of HBx protein from strain C265 on the BCP (with 1762A/1764G) activity from C265 and its mutant (with 1762T/1764A). B. Effect of HBx protein from strain H293 on the BCP activity from C265 and its mutant. C. Effect of HBx protein from strain C265 on the BCP activity from H60 (1762T/1764A) and its mutant (with 1762A/1764G). D. Effect of HBx

protein from strain H293 on the BCP activity from H60 and its mutant.

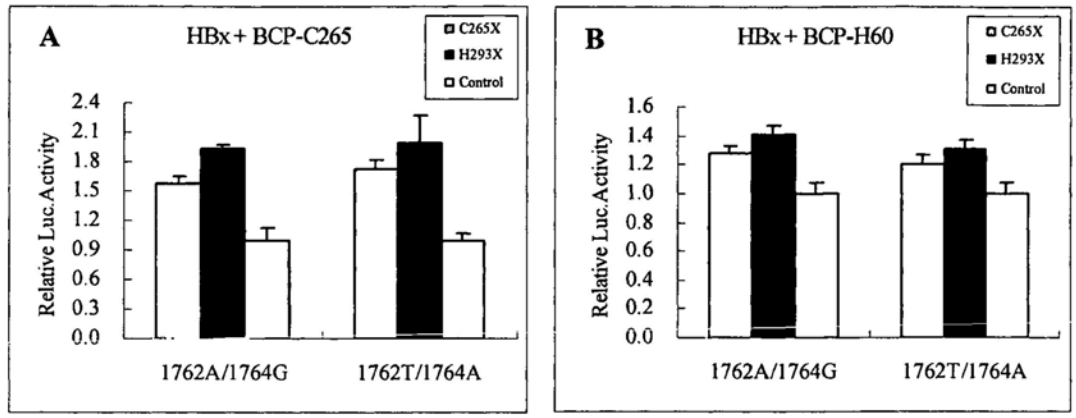


Fig. 5-9. Comparison of influence between HBx proteins from strain C265 and H293 on HBV BCP (with 1762A/1764G and 1762T/1764A) activity. A. Influence of HBx on the BCP from strain C265 and its mutant. B. Influence of HBx on the BCP from strain H60 and its mutant.

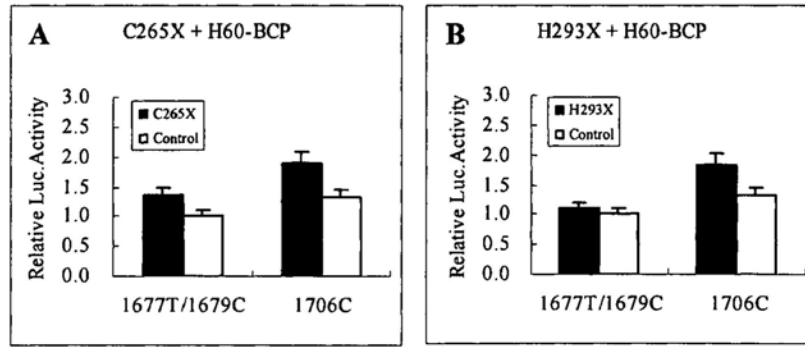


Fig. 5-10. Influence of HBx proteins from strain C265 and H293 on the H60 BCP mutants (with 1677T/1679C and 1706C). A. Effect of HBx protein from strain C265. B. Effect of HBx protein from strain H293.

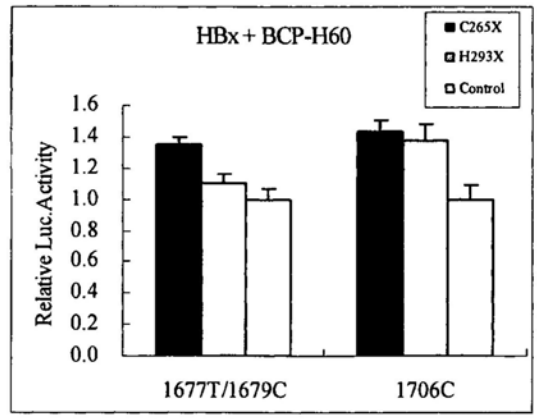


Fig. 5-11. Comparisons of influence between HBx proteins from strain C265 and H293 on HBV BCP mutants (with 1677T/1679C and 1706C) activity.

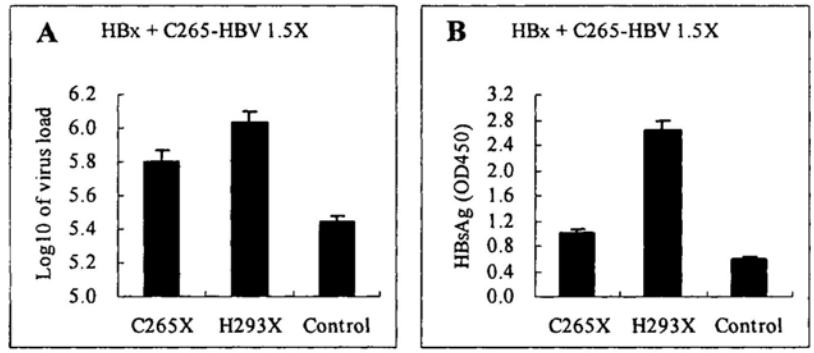


Fig. 5-12. Comparisons of influence of HBx proteins from strain C265 and H293 on the replication and HBsAg expression of HBV strain C265 virus. A. HBV replication. B. HBsAg expression.

Table 5-1. Primers for HBx studies

Primer	Nucleotide sequence (5'-3')	Direction
HBVX-1362-1385-KpnI-F	CGGGGTACCTTACATCATTTCCATGGCTGCTAGG	Sense
HBVX-PCDNA4-NotI-F	AATGCGGCCGCTCCGAAACCATGGGGGGTTCT	Sense
HBVX-1845-1820-Xba I-R	TGCTCTAGAGAGATGATTAGGCAGAGGTGAAAAAG	Antisense
BGHrev (pcDNA4)	TAGAAGGCACAGTCGAGG	Antisense
Adeno-X Forward PCR Primer	TAGTGTGGCGGAAGTGTGATGTTGC	Sense
Adeno-X Reverse PCR Primer	AGATCTGAGCTTTCGCTACC	Antisense
Ade-X-F	TAGTGTGGCGGAAGTGTGATGTTGC	Sense
Ade-X-R	AGATCTGAGCTTTCGCTACC	Antisense

Chapter 6

General Discussion

HBV is a major causative agent of HCC especially in China, where genotype B and C are prevalent. Many characteristics possessed by the virus itself have complicated the pathogenesis of HBV. Firstly, each genotype of HBV has specific and different geographic distribution, which affects the clinical outcomes of HBV infection. Secondly, a number of spliced variant of HBV have been discovered, which made the prognosis of both liver cirrhosis and HCC patients more complicated. Thirdly, the HBV replication is mediated through a reverse transcription step and the RNA polymerase has no proofreading function. These characteristics have introduced lots of mutant strains of HBV. For example, the A1762T/G1764A double mutations have been suggested to be related to HCC. The nt1762/nt1764 sites are found within the Enhancer II/Basal core promoter and HBx gene regions. Therefore, the double mutations may change their respective and interactional functions because the HBx protein is a transcriptional factor. Fourthly, HBsAg and HBx protein are oncoproteins, but many of their functions remain still unknown. Taken together, the characteristics of HBV its own are of great importance in the pathogenesis of chronic hepatitis and HCC, etc. All these issues will be discussed below for the four strains of HBV, two from HCC patients aged about 30 and two from HBV carriers aged more than 60.

6.1 The relationship between HBV genotype and liver disease

Genotype B HBV infection has been reported to induce HCC more frequently in younger individuals in Taiwan than those in Japan (Kao *et al.*, 2000; Orito *et al.*, 2001). Similar observation has been noted in our study. The four strains in our study all belong to genotype B

of HBV but of different aged patients as well as different clinical outcomes. In the cases of H60 and H293, they belonged to two younger adults of age 31 and 26 years old, respectively. By the time the sample collection was carried out, both of them have developed HCC already. In the cases of C265 and C1691, both of them belonged to older patients of 66 and 71 years old, respectively. By the time of sample collection, they were just HBV carriers without any signs of cancer development.

For genotype B, it was further classified to subgenotype namely from B1 to B5. Subgenotype B1 (Bj) is restricted to Japan while B2 (Ba) has the widest distribution in Asia. Notably, HBV isolates of subtype Ba possessed the recombination with genotype C over the precore region plus core gene, while those of subtype Bj did not (Sugauchi *et al.*, 2002). In this study, sequence alignment showed that the two HBV strains from those younger patients with HCC belonged to Ba subgenotype, which is a recombinant type of genotype B and C. Therefore, we postulated that recombination between genotypes B and C might partly, if not all, responsible for the occurrence of HCC in the younger HBV patient group. However, further investigations will be necessary to clarify the mist.

6.2 Spliced variant of HBV

. HBV has unspliced and spliced mRNA with the spliced ones may become spliced variants of HBV. Singly and doubly spliced 2.2-kb mRNAs arised from 3.5-kb pregenomic mRNA had been identified in HBV DNA-transfected cell lines and in HBV-infected liver (Wu *et al.*, 1991; Chen *et al.*, 1989; Suzuki *et al.*, 1989, 1990; Su *et al.*, 1989; Günther *et al.*, 1997; Terré *et al.*, 1991; Huang *et al.*, 2000). Günther *et al.* also found 11 types of spliced genomes with heterogeneity and common features from sera of chronic carriers by full-length PCR (Günther *et al.*, 1997), whilst 1.5 to 2.2 kb spliced genome variants were found with deletions and

insertions in the C gene and Pre S region in immunosuppressed kidney transplant patients. Those patients were also suffered from end-stage liver cirrhosis; therefore, it was believed that the development of cirrhosis was associated with the accumulation of the complex HBV variants (Günther *et al.*, 1995; Märschenz *et al.*, 2006). In a recent study, Soussan *et al.* found an elevated dHBV (defective HBV)/HBV ratio, which indicated an association between liver necroinflammation and fibrosis disease, and suggested a regulatory mechanism on dHBV expression corresponding to the severity of the liver disease (Soussan *et al.*, 2008).

In this study, a new type of spliced variant of HBV with 2494bp in length from HCC patient H60 was revealed. It has three deletion regions, yet it also possesses complete Pre C, C, S and X genes. In addition, the Enhancer I/X promoter, Enhancer II/Basal core promoter, DR1, DR2 and the 5'-end encapsidation signal are also all complete in this variant. Therefore, it may be encapsidated into functional HBV particle, thus induce some kind of liver diseases including cirrhosis and HCC.

6.3 Construction of HBV-1.5X

In this study, four HBV-1.5Xs were constructed which all have a 5'-end redundant sequence (1.1kb) to nt715 with another copy of Enhancer I/X promoter and Enhancer II/Basal core promoter and a 3'-end redundant sequence of 500bp after the poly (A) sequence. All of them were longer than the pgRNA and the previously reported HBV 1.1, 1.2 and 1.3 constructs (Guidotti *et al.*, 1995). In the present study, the HBV-1.5X system produced very high level of HBV virus replication (HBsAg, and HBV DNA) which might because it has another copy of complete Enhancer I/X promoter, Enhancer II/Basal core promoter and HBx gene. This system might be useful for further studies on stable cell line selection and transgenetic mice research in

order to get a comprehensive understanding on the characteristics of the four viruses.

6.4 A1762T/G1764A mutations of hepatitis B virus, associated with the increased risk of hepatocellular carcinoma, reduce basal core promoter activities

The contribution of HBV to the pathogenesis of liver cancer is multifactorial and complicated by the emergence of natural HBV variants that modulate the carcinogenesis process (Locarnini *et al.*, 2003). Clinical studies have shown that HBV patients with A1762T/G1764A mutations have low levels of HBeAg and were often associated with HCC. This mutation pattern also occurred in the genome of HBVs isolated from the HCC patients (e.g., H60 and H293). For the first time, the findings of our study provided some insights to explain these clinical observations. In our study, the promoter activity of HBVs isolated from carriers (C265 and C1691) was significantly reduced when wild-type 1762A and 1764G were mutated into 1762T and 1764A without changing any other nucleotides. Conversely, we also showed that reversed mutation from 1762T/1764A to 1762A/1764G increased the basal core promoter activities of HBVs isolated from HCC patients. Although the nucleotide context of the HBV promoters exerts some influence on 1762T or 1764G, the overall consequence of A1762T/G1764A double mutations still reduced the basal core promoter activity by about 50%. Therefore, we suggest that these mutations might be associated with the diminished PreC mRNA transcription that was likely a consequence of the altered HNF-4 binding site 2 and the TATA box-like sequence of the core promoter. Our results suggest that the double mutations could result in the introduction of an additional HNF-1 binding site in the core promoter and could specifically repress preC RNA synthesis. It is postulated that both 1762A and 1764G are

important for the binding of these factors, and that 1764G plays a very significant role in determining the binding affinity.

Although the mechanism of A1762T/G1764A mutations for HCC remains elusive, several indications were noted in our study. The core promoter region of HBV was reported to direct the transcription of both species of 3.5 kb messenger RNA: pgRNA and PreC mRNA (Kramvis *et al.*, 1999). The pgRNA could be translated into core protein and polymerase protein, serving as a template for reverse transcription of HBV after being packaged into core particles. The Pre C mRNA could also translate into a Pre C/core fusion protein that was post-translationally modified to yield HBeAg. The A1762T/G1764A mutations were found to reduce the HBeAg level (postulated to induce immune tolerance against itself), the HBcAg or both antigens, which might increase the viral replication and viral load. Finally, this leads to the development of HCC. Furthermore, the HBV A1762T/G1764A double mutations also affect the amino acid sequence of the HBx gene as they reside in codons 130 and 131, results in an alternation of lysine to methionine and valine to isoleucine, respectively (Li *et al.*, 1999). The HBx protein was found to display various types of biological activity (Arbuthnot *et al.*, 2000; Murakami *et al.*, 2001), but its role or the primary function of this mutant protein in the pathogenesis of HCC is yet to be elucidated.

6.5 The HBx protein natural variant

In this study we found a C terminal truncated HBx which had only 119 amino acids from a HCC patient H60 aged 31 and the expression level of it was comparatively very low *in vitro*. The HBx truncation may be implicated in the carcinogenesis of HCC. Actually several mutants

have been identified from sera and liver tissues from patients with HCC. HBx sequences isolated from integrated HBV genomes in tumor tissues have frequently been reported to have a deletion at the 3'-end (Wei *et al.*, 1995; Hsia *et al.*, 1997; Poussin *et al.*, 1999; Yamamoto *et al.*, 1993; Schluter *et al.*, 1994; Tsuei *et al.*, 1994). HBx truncation may also be found in chronic hepatitis B patients, but at a lower incidence than that seen in HCC (Koike, 1998). Based on the fact that truncated HBx can enhance the transforming ability of Ras and Myc (Tu *et al.*, 2001), it was suggested that HBx truncation might play a role at an early stage of liver carcinogenesis and be selected during the development of HCC. Furthermore, another linked-point mutation, Lys130Met and Val131Ile (Takahashi *et al.*, 1998; Baptista *et al.*, 1999), in HBx were shown to be prevalent in HCC patients. In this study, an HBx protein with Lys130Met and Val131Ile mutations was also found from a HCC patient H293 aged 26. This mutated protein may be related to young HCC patients under age 50.

6.6 The influence of natural HBx variants on HBV replication and Enhancer II/Basal core promoter activity

As discussed earlier, our results indicate that the Lys130Met and Val131Ile mutations of HBx can increase the HBV BCP activity no matter the BCP contains 1762A/1764G or 1762T/1764A. On the other hand, the nt1762/nt1764 double mutations in the BCP region have no effect on the interaction between HBx and HBV BCP. However, it is still true that HBx can actually increase the Enhancer II/Basal core promoter activity. Another interesting finding is that 1677T/1679C and 1706C mutations of HBV BCP could increase the interaction of HBx protein from C265 (without Lys130Met and Val131Ile mutations) with HBV BCP. This might because of the mutation region from nt1677 to nt1706 of BCP is actually fonder to bind the complexes of wild type HBx protein (without Lys130Met and Val131Ile mutations) and related

transcriptional factors, which thus induced an increase of BCP activity. However, more investigations are needed to study the underling mechanism of natural HBx variants' influence on the BCP activity. Electrophoretic mobility shift assay (EMSA) is a good choice for further studies in this aspect.

On the other hand, the HBx protein was found to increase both the HBV replication and HBsAg expression. Such ability was noted significant higher in the mutated one with Lys130Met and Val131Ile mutations from H293, but not at the absence of Lys130Met and Val131Ile mutations (i.e. sample from C265). This might be one of the reasons why the HBx could induce HCC by increasing the HBV virus replication and HBsAg expression as HBsAg is also an oncoprotein. The augmentation of HBV replication by HBx may be carried out via the regulation of intracellular calcium, increasing core serine phosphorylation, interaction of HBx with DDB1 and influence on Enhancer II/Basal core promoter activity as discussed earlier.

6.7 Conclusion

In this study, many A1762T/G1764A double mutations related issues, such as HBV BCP activities, the interactions of HBx with HBV BCP as well as HBV replication, were all investigated. It was found that: (1) A1762T and/or G1764A mutations of HBV could reduce BCP activities in a synergistic manner with 1764A contributing more. Reversed T1762A and/or A1764G mutations increase the BCP activities also in a synergistic manner with 1764G contributing more; (2) HBx could increase HBV BCP activity, HBV replication and HBsAg expression. The Lys130Met and Val131Ile mutations of HBx could further increase the above abilities while the A1762T/G1764A double mutations in the BCP region could not affect the interaction of HBx and HBV BCP; (3) The G1677T/A1679C and T1706C mutations could increase the BCP activity; The ectopic expression of HBx could further increase the BCP

activity while the mutated HBx (^{130}Met and ^{131}Ile) has less effect on these mutated promoters.

6.8 Future prospect

In future studies, it will be worthwhile to study the influence of natural HBx variants on the Pre S1 and Pre S2 promoters' activities, since our present studies show that HBx can increase the expression of HBsAg. More investigations can be carried out to establish transgenic mice models using the HBV-1.5X system. Therefore, the pathogenesis of HBV *in vivo* can be examined. Besides, it can be invaluable to determine the function of H60-2.5kb spliced variant in both *in vitro* and *in vivo* conditions as it might be implicated in HCC occurrence. Also, there may be a need to study the functions of truncated HBx (from the sample H60) on the pathways that related to HBx-induced carcinogenesis or apoptosis. Lastly, the influence of hot spot mutations on viral replication, antigen expression and cellular changes using HBV-1.5X may be worth for investigation, since the direct evidences about the hot spot mutations' influence are still needed to be found out.

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Publication

1. **Dong Q**, Liu Z, Chan DP, Zhang B, Kung HF, Chan HL, Sung JJ, He ML. A1762T and G1764A mutations of hepatitis B virus, associated with increased risk of hepatocellular carcinoma, reduce the enhancer II/basal core promoter activities. Biochem Biophys Res Commun, 2008, 374: 773-776.
2. **Dong Q,** Xu B, Tan Y, Liu Z, Tian L, Zhang B, Lin CK, Kung HF, Sung JJ, He ML. The genetic variability of MDR1 C3435T polymorphisms in four Southern Chinese populations. Biomed Pharmacother, 2008, PMID:18926661.
3. Li Z, Yao H, Ma Y, **Dong Q**, Chen Y, Peng Y, Zheng BJ, Huang JD, Chan CY, Lin MC, Sung JJ, Yuen KY, Kung HF, He ML. Inhibition of HBV gene expression and replication by stably expressed interferon-alpha1 via adeno-associated viral vectors. J Gene Med, 2008, 10(6):619-27.
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