STUDY OF THE FUNCTIONAL SIGNIFICANCE OF MUTATIONS IN THE HEPATITIS B VIRUS GENOME, FOLLOWING TRANSFECTION OF LIVER CELL LINES WITH CLONED INFECTIOUS DNA

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"I was always looking outside myself for strength and confidence, but it comes from within. It is there all the time".

Anna Freud

"Give the world the best you have, and it may never be enough; Give the world the best you've got anyway".

Agnes Gonxha Bojaxhiu

"Our lives are shaped by those who love us and by those who refuse to love us".

John Powell, S.J

For her unconditional love, care, and compassion, the work herein is a dedication to my great-grandmother, Hajjah Oumie Sanyang "Yayoe" (1899-2004).

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Abstract

There are conflicting reports regarding the replication efficiency of hepatitis B virus (HBV) *in vitro*, as related to fulminant hepatitis (FH) disease outcome, when using infectious DNA clones bearing core promoter (CP) or precore (preC) mutations. In most of these studies, the mutations had been cloned in the genetic context they were found in, which may explain partly the varied findings. Here, we have introduced these mutations into a common HBV genetic background by Site-directed mutagenesis, either alone (CP, preC), or in combination (CP/preC), and analysed their possible influence on replication efficiency following transfection of hepatoma cell lines (Huh7 and HepG2). Evidence for viral replication was based on immunological identification and/or quantification of viral proteins and — using the LightCycler technology to quantify — HBV DNA of released virions, and RNA transcript levels (preC and pregenome). The results show significant ($P < 0.05$) replication and transcription differences between some of the mutant- and the wildtype-constructs. Furthermore, correlations existed between virion release and HBsAg or total transcription levels ($\rho = 0.665$, and $\rho = 0.621$, respectively, significant at $P < 0.05$). For comparative purposes, the replication capacities of these mutants were assessed not only relative to wildtype, but also to documented mutants bearing mutations in the reverse transcriptase (RT)/polymerase (P) and "a" determinant/surface (S), genes. Using the parameters listed above, the effect of lamivudine (LMV) on viral transcription and replication of mutations in the "a" determinant/S gene alone were first tested, followed by mutations in the RT/P gene alone, and then the two combined, in the presence and absence of LMV. The results show that the S gene mutations can restore the replication phenotype of mutants bearing P gene mutations.

In order to explain the underlying basis for the augmented replication seen in the above experiments, promoter-analysis studies were undertaken using bandshift and luciferase assays. The data show that mutations in regulatory regions can indeed affect viral replication and thus antigen expression, as a result of varied binding of nuclear factors. This in turn affected EnhII/CP activity which drives RNA transcription, the basis for DNA replication. In conclusion, FHB disease may be the result of increased viral replication controlled by the CP region, but may also be influenced by a combination of viral host factors.

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Abbreviations

aa.	amino acid(s)
A-INF	alpha interferon
Aa	Australia antigen
β-actin	beta-actin
bp	base pairs
BCP	basic core promoter
BSA	bovine serum albumin
C	core (gene or protein)
Ci	Curie
C-terminal	carboxyl-terminal
ccc	covalently closed circular
C/EBP	CCAAT enhancer binding protein
cDNA	complementary DNA
CP	core promoter
Cpm	counts per minute
COUP-TF1	chicken ovalbumin upstream promoter transcription factor 1
CURS	core upstream regulatory sequence
DDT	dithiothreitol
DEPC	diethyl pyrocarbonate
DHBV	duck hepatitis B virus
DMEM	Dulbecco-modified eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
dNTP	deoxynucleotide 5' triphosphate
DS	double-strand
DR	direct repeat
ε	epsilon
E. coli	*escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EMSA	electro mobility shift assay
ELISA	enzyme-linked immunosorbent assay
Enh	enhancer
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
fg	fentogram
GFP	green fluorescence protein
FITC	fluorescein isothiocyanate
FHB	fulminant hepatitis B
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B *e* antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HIV	human immunodeficiency virus
HKG	housekeeping gene
HNF4	hepatocyte nuclear factor

hr	hour
Ig	immunoglobulin
kb	kilobase pairs
LB	Lauria berterni broth
LMV	lamivudine
MHC	major histocompatibility complex
MHR	major hydrophilic region
mRNA	messenger RNA
M.Wt	molecular weight
N-terminal	amino-terminal
NRE	negative regulatory element
nt.	nucleotide
OD	optical density
ORF	open reading frame
P	polymerase (gene or protein)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
preC	precore
preG	pregenome
RC	relaxed circular
RNase	ribonuclease
rRNA	ribosomal RNA
RPII	reverse polymerase II
RT	reverse transcriptase/transcription
RT-PCR	reverse transcriptase PCR

S	surface (gene or protein)
SDM	Site-directed mutagenesis
SS	single-stranded
TBE	tris borate buffer
TE	tris EDTA buffer
TEMED	N N N' N' tetramethylethylenediamine
Tris	tris[hydroxymethl]aminoethane
UV	ultraviolet
V	volts
WHsAg	woodchuck hepatitis B virus
WT	Wildtype p3.8II
w/v	weight to volume
X GAL	5 bromo 4 chloro 3 indoyl β D galactopyranoside

Nucleic Acids

Adenosine	A
Cytidine	C
Guanosine	G
Thymidine	T
Uridine	U

Amino Acids

Amino Acid	Abbreviations		Triplet Codon					
Alanine	A	Ala	GCT	GCC	GCA	GCG		
Arginine	R	Arg	CGT	CGC	CGA	CGG	AGA	AGG
Asparagine	N	Asn	AAT	AAC				
Aspartic acid	D	Asp	GAT	GAC				
Cysteine	C	Cys	TGT	TGC				
Glutamic acid	E	Glu	GAA	GAG				
Glutamine	Q	Gln	CAA	CAG				
Glycine	G	Gly	GGT	GGC	GGA	GGG		
Histidine	H	His	CAT	CAC				
Isoleucine	I	Ile	ATT	ATC	ATA			
Leucine	L	Leu	TTA	TTG	CTT	CTC	CTA	CTG
Lysine	K	Lys	AAA	AAG				
Methionine (start)	M	Met	ATG					
Phenylalanine	F	Phe	TTT	TTC				
Proline	P	Pro	CCT	CCC	CCA	CCG		
Serine	S	Ser	TCT	TCC	TCA	TCG	AGT	AGC
Threonine	T	Thr	ACT	ACC	ACA	ACG		
Tryptophan	W	Trp	TGG					
Tyrosine	Y	Tyr	TAT	TAC				
Valine	V	Val	GTT	GTC	GTA	GTG		
Termination Codons			TAA	TAG	TGA			

Chapter 1

Introduction

CHAPTER 1. INTRODUCTION

1.1 The Discovery of The Hepatitis B Virus

It has been known for centuries that some form of hepatitis exists that afflicts humans and harms lives. However, before World-War II, physicians neither knew that it was virus-inflicted, nor that several types of viral hepatitis existed. It was assumed to be contagious because epidemics of hepatitis often occurred in crowded, unsanitary conditions, but how the contagion was transmitted from person to person was a mystery. Headway into solving this mystery was made in 1947 when a British physician who specialised in liver disorders, Dr. F.O. MacCallum, identified the Hepatitis B Virus (HBV) when he was researching yellow fever. MacCallum suspected that a virus carried in human blood could cause hepatitis and classified viral hepatitis into two types, infectious hepatitis, now known as hepatitis A, and serum hepatitis or hepatitis B (The Hepatitis B Story – Hepatitis B: A Debilitating Disease, www.beyonddiscovery.org). A series of observations in volunteers by MacCallum and others shortly afterwards strengthened this hypothesis and established that the illness could be transmitted through the faecal-oral route for Hepatitis A Virus (HAV) (Feinstone *et al.* 1973), and through exposure to contaminated blood, for HBV (Blumberg *et al.* 1967a).

During the next decade and a half, researchers at various laboratories attempted in vain to isolate the infectious agents that caused the two types of hepatitis. The only evidence that suggested that the culprits were viruses was the finding that they were small enough to pass through some of the smallest-pore filters used in experiments. In spite of these initial results, hepatitis research had reached a deadlock by the mid-1960s. A remarkable advance was made with the discovery of a mysterious protein, whilst researching leukaemia (Blumberg *et al.* 1967b). Blumberg subsequently named this unknown protein *Australia antigen* (*Aa*) after the homeland of the aborigine whose blood led to its discovery. Encouraged by Blumberg's findings, another virologist confirmed the link between *Aa* and hepatitis (Prince 1968). At about the same time, other scientists centred on the molecular details of *Aa*. With the aid of electron microscopy, the consistent presence of virus-resembling particles in the sera and liver cells of patients who tested positive for *Aa* confirmed that these particles were indeed those of HBV (Dane *et al.* 1970b).

Since the discovery of the virus more than three decades ago, advances have been made in understanding its molecular and cellular biology, natural history, and the mechanisms underlying its pathogenesis and disease progression. These results gave rise to promising developments in blood screening and vaccines, and led to the belief that viral hepatitis could be controlled and, need no longer be a threat to human health, as it had been for thousands of years. However, other areas of HBV infection that continue to draw attention are the prevalence of occult HBV infection, the changing epidemiology and clinical significance of HBV variants (mutants), in particular precore (preC) and core promoter (CP) mutants, and the mechanisms of immune clearance and pathogenesis of virus related liver diseases.

1.2 Transmission and Epidemiology

More than two billion people worldwide have evidence of past or current HBV infection and 350 million are chronic HBV carriers. Three quarters of the world's population live in areas where there are high levels of infection (Update of a WHO Consultation, organised with the Expanded Programme on Immunisation committee, Geneva 1996).

The clinical expression of hepatitis B in different parts of the world depends not only on the prevalent genotypes but also on their modes of transmission. In fact, host and epidemiologic factors influence the clinical expression of hepatitis B dramatically. Figure 1 compares areas of low endemicity, such as the U.S.A; with a hepatitis B surface antigen (HBsAg) prevalence of 0.1% to 0.2%, *vs.* areas of high endemicity such as Asia and sub-Saharan Africa, with prevalences of 10% to 15%, or higher (Kew 1992; Parkin 1998). In Asia, for example, the high prevalence of hepatitis B in the general population is reflected by its high prevalence among women of childbearing age. Many infections are transmitted perinatally from mother to offspring. Neonatal infection is associated with a high level of immunological tolerance, which is very difficult to overcome. As a result, the likelihood of chronicity exceeds 90% (McMahon *et al.* 1985e).

In contrast, the U.S.A has relatively few hepatitis B carriers and, because the prevalence of HBV infection in women of childbearing age is comparably low, perinatal infections are rare. Most infections, instead, are acquired in early adulthood via occupational exposure (for example, in healthcare workers), or injection drug use or, most commonly, sexual activity (that is, via human behaviours that foster the

spread of bloodborne viruses). The adult immune system mounts a robust response to the presence of the virus in liver cells, resulting in immunologic destruction of virus-infected cells, and in acute hepatitis B which resolves with containment of the virus. The likelihood of chronicity in such clinically apparent acute hepatitis B cases is low (probably $\leq$ 1%) (Tassopoulos *et al.* 1987a). Moreover, the clinical expression of disease is reflected by another dichotomy between areas of high and low HBV prevalence. In highly endemic areas, the lifetime risk of succumbing to complications (cirrhosis and liver cancer) has been estimated to be as high as 40%, whereas in low prevalence areas the risk of HCC in those with adult-acquired chronic hepatitis B is very low; as shown (Figure 1.1).

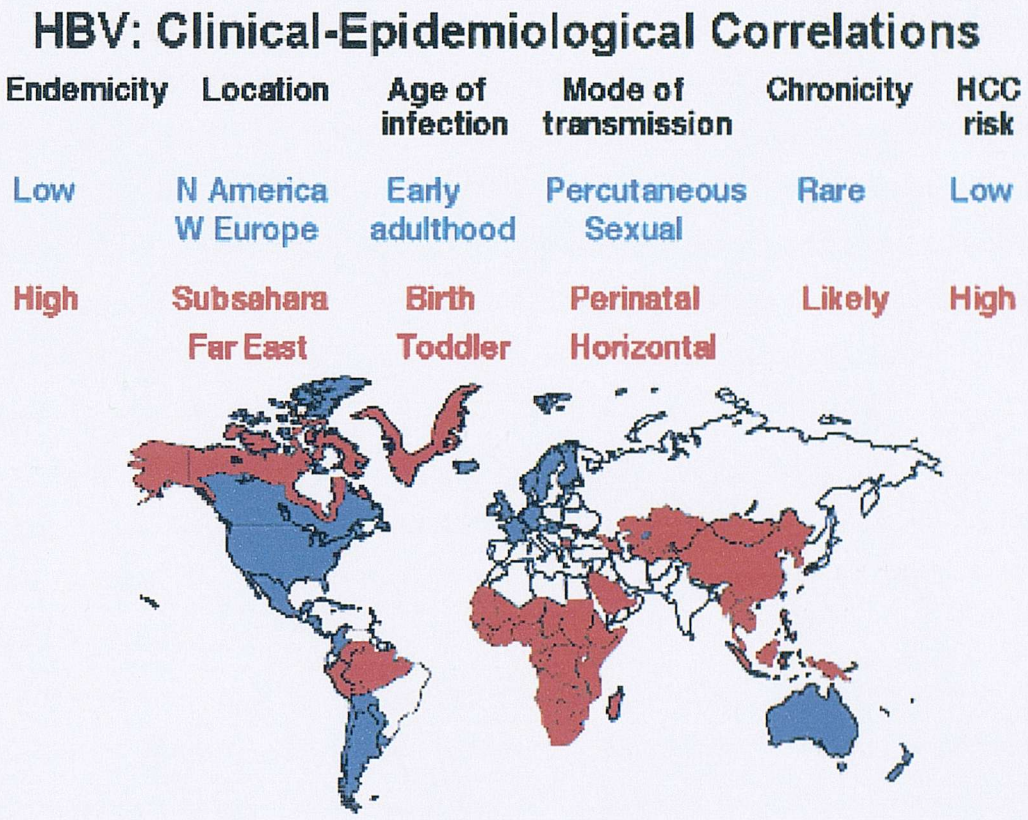


Figure 1.1 Worldwide distribution of HBV prevalence: a comparison of low versus high endemicities.
The geographical variation of HBV infection is significant and ranged from high endemic regions such as Africa and South East Asia (in red) to those of low endemicity such as the USA, Australia and Western Europe (in blue). Mediterranean countries and Russia form part of a range of countries with intermediate infection (colourless). Adapted from www.cdc.gov/ncidod/disease/hepatitis

1.2.1 Sequence diversity

HBV has sequence diversity which has led to its classification into genotypes and serological subtypes. The former is generally defined as the genetic constitution of an organism or cell. If this genetic composition — or genetic sequence, in viral terms — is serologically characterised, then subtypes or serotypes can be defined (Francois *et al.* 2001a). Genotypes have been recognised following analysis of nucleotide sequences, more recently employing phylogenetic tree construction. There are eight genotypes of the virus labelled A–H. There exist distinct geographic variations in frequencies of the various genotypes (Gan *et al.* 1984; Okamoto *et al.* 1986b). Thus, geographical clustering of these genotypes has been observed: A and D prevail in European countries (Okada *et al.* 1976), while B and C are predominantly found in South East Asia, including Japan (Kao *et al.* 2000). Genotypes A, D and E have been reportedly found in Africa (Lindh *et al.* 1997b; Norder *et al.* 1993b; Norder *et al.* 1993a), whilst F is almost exclusively found in South America.

To-date, apart from the eight genotypes, nine serological subtypes have also been defined. Serological subtypes were originally defined by antibodies against the common "a" determinant of HBsAg and the occurrence of at least two mutually exclusive subdeterminants, *d/y* depending on a lysine/arginine at position 126, and *w/r* on a lysine/arginine at position 160. This gives rise to four serological subtypes, *adw*, *ayw*, *adr*, and *ayr* (Okamoto *et al.* 1988). The *adw* and *ayw* serological subtypes are more commonly found in Europe, North America and Africa, whilst the *adr* subtype predominates in the Far East (Odemuyiwa *et al.* 2001; Sugauchi *et al.* 2003). Genetic analysis has, revealed that the four stated serological subtypes do not correspond to single genotypes (Grob 1998b; Ohba *et al.* 1995).

Whether the nature of genotypes and serological subtypes does play any role in influencing the clinical course of hepatitis B infection still remains a subject of controversy: whilst the correlation of HBV genotype to disease and treatment outcome has been suggested (Janssen *et al.* 2005; Mayerat *et al.* 1999), others remain sceptical (Grob 1998a).

1.3 Hepadnaviruses

Although this thesis focuses only on human HBV, a brief description of other members of the *Hepadnaviridae* family of which HBV is the prototype, may be relevant. Their relevance lies in the fact that research into understanding HBV

infection has also centred around other family members, with the aim of unravelling similarity in DNA sequence and/or genome organisation, for the eventual purpose of exploiting such similarities or differences to develop antiviral agents and therapies.

The *Hepadnaviridae* family is a group of small, enveloped DNA-viruses found in mammals and birds. Those most closely related to HBV have been found in woodchucks and ground squirrels (Seeger & Mason 2000c; Tennant *et al.* 1991; Testut *et al.* 1996). Such viruses have about 70% sequence homology to HBV, but are not known to infect humans or other primates; in contrast, HBV is infectious for the great apes. Because of the similarities in DNA sequence and genome organisation, the viruses infecting mammals are grouped within the genus *Orthohepadnavirus.* More distantly related viruses, with a similar genome organisation but almost no sequence homology, found in ducks and geese, form the *Avihepadnavirus* genus (Mason *et al.* 1998; Sprengel *et al.* 1988).

1.4 Hepatitis B Virus

1.4.1 Genome structure and virion morphology

Very early studies of the HBV virion, also known as the *Dane* particle, described it as a doubled-shelled particle 42–47nm in diameter (Bayer *et al.* 1968c; Dane *et al.* 1970a; Robinson & Lutwick 1976). Using electron microscopy, these scientists further described the HBV virion as being surrounded by an outer envelope which contains high amounts of HB surface antigen (HBsAg). In turn, this envelope surrounds an inner nucleocapsid consisting of core protein arranged as an icosahedron. As well as the HB polymerase, the nucleocapsid also contains the HBV genome.

Dane also described two other subviral particles that can be found in serum from an infected individual, namely the HB filaments and spheres. Both particles had a diameter half the size of a *Dane* particle, and were composed solely of HBsAg. The spheres contain small and middle HBsAg proteins whereas filaments also contain the large HBsAg protein. High levels of these non-infectious particles can be found during the acute phase of infection (Huang & Liang 1993), the presence of which may allow the infectious viral particles to remain in the blood stream undetected by neutralising antibodies (Rehermann *et al.* 1995a). Robinson and Summers showed that purified virions possess a nucleocapsid which consists of hepatitis B core antigen

(HBcAg) (Robinson *et al.* 1974a; Summers *et al.* 1975). The formation of the nucleocapsid leads at the same time to the encapsidation of the viral genome and polymerase. In the mature virion, this core particle is enveloped by HBsAg proteins.

1.4.2 Genome organisation

The HBV genome is a circular DNA molecule of 3200 base pairs (bp) and appears to be (among mammalian viruses) the smallest fully replication-competent genome (Seeger and Mason, 2000). Every nucleotide forms part of an Open Reading Frame (ORF) (Figure 1.2), encoding one or more viral protein(s). The virus is remarkable for its highly condensed genomic organisation. As a consequence, all of the many viral essential *cis*-acting elements (direct repeats DR1 and DR2, encapsidation signal, promoter, and enhancer elements) lie within a coding region. A striking feature of the genome is its partially double-stranded (DS) structure. The plus strand of the virion is incomplete and variable in length at its 3′–end, while the 5′–end is fixed. The minus strand has well-defined 5′– and 3′–ends and possesses a terminal redundancy of nine bases that creates a triple-stranded overlap in this region of the genome, (Figure 1.3).

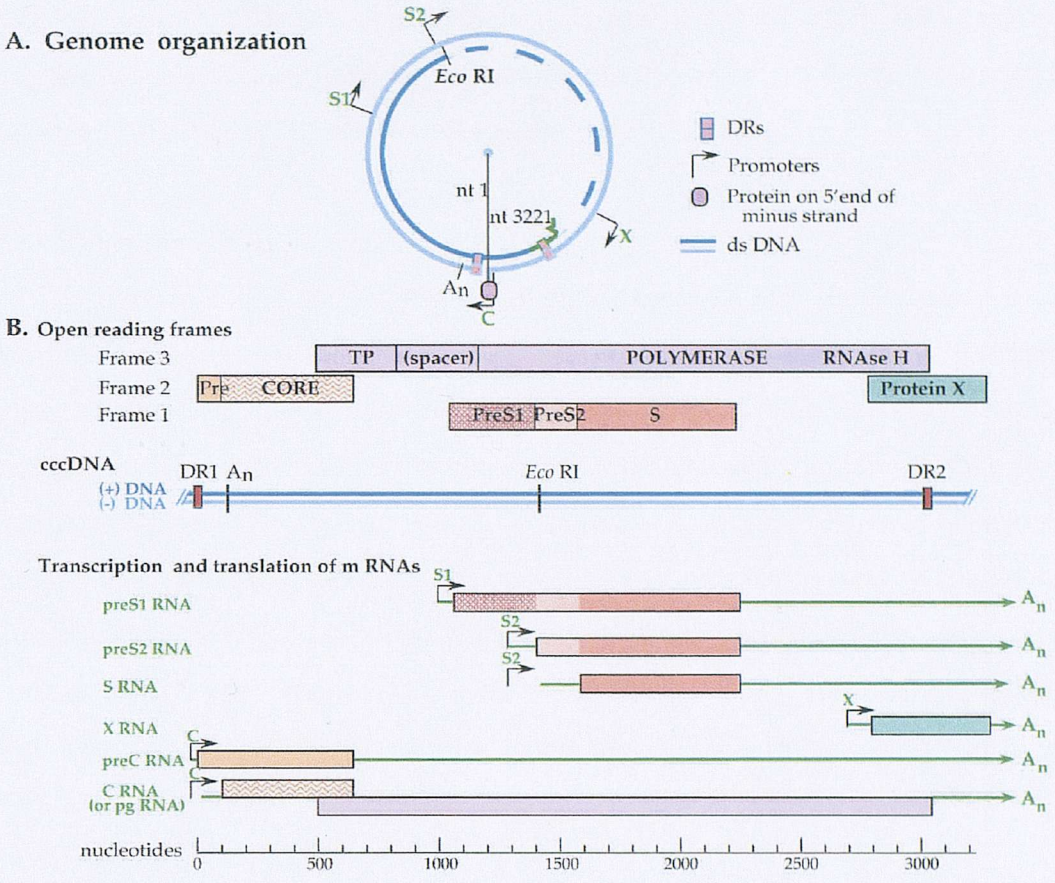


Figure 1.2 Schematic representation of the HBV genome, ORFs and transcripts.
A. Circular map of the HBV genome showing the locations of the repeated elements (DR1 and DR2), the four known promoters (black arrows), and the gap of variable length in the plus strand of the DNA. This map is numbered from the beginning of the preG RNA. Most authors number the nucleotides from the unique *EcoRI* site (here 1407).
B. Linearsised map of cccDNA showing the open reading frames. Below this, are shown the six mRNAs and the proteins (coloured blocks) translated from them. Note that the preC mRNA and preG RNA are of more than genome-length, and that all mRNAs end at the polyadenylation site at nucleotide 121 (but have been shown extended to the right for clarity). All transcripts are made from the (−) DNA (light blue) as template, in a clockwise direction on the circular map, or left in the linearised map. Figure was adapted (Strauss & Strauss 2002).

An overview of the HBV genome organisation is provided here since this is the area that was exploited in this investigation to design primers for the amplification of replicative intermediates: covalently closed circular (ccc) DNA, pregenomic (preG) and preC RNA transcripts were differentially amplified from total intracellular DNA and RNA transcripts, respectively, using techniques which will be explained in later sections of this chapter. At this stage, in view of the relevance of HBV viral intermediates in this study, it is important to describe how they come about during

viral replication. Understanding their role, and those of other HBV replication intermediates, is a prerequisite to this study. First, however, a detailed description of the proteins/antigens that make up the *Dane* particle is given:

1.5 Hepatitis B Virus Proteins

There are four partially or totally overlapping ORFs in the genome which altogether encodes seven different HBV proteins, through the use of different in-frame start codons: the polymerase (P) ORF occupies the majority of the genome and encodes the viral polymerase; the surface (S) ORF encodes the three surface proteins (small, middle and large); the core (C) ORF encodes both the HBe and core proteins; ORF X encodes the X protein. The genome also contains genetic elements which regulate levels of transcription, polyadenylation, encapsidation and replication (Nassal & Schaller 1996; Robinson *et al.* 1974b). HBsAg, HBeAg and core antibodies are used when testing for HBV markers in the blood. Of relevance is the level of the virus in the blood (HBV DNA) used to track the natural history of HBV infection in an individual.

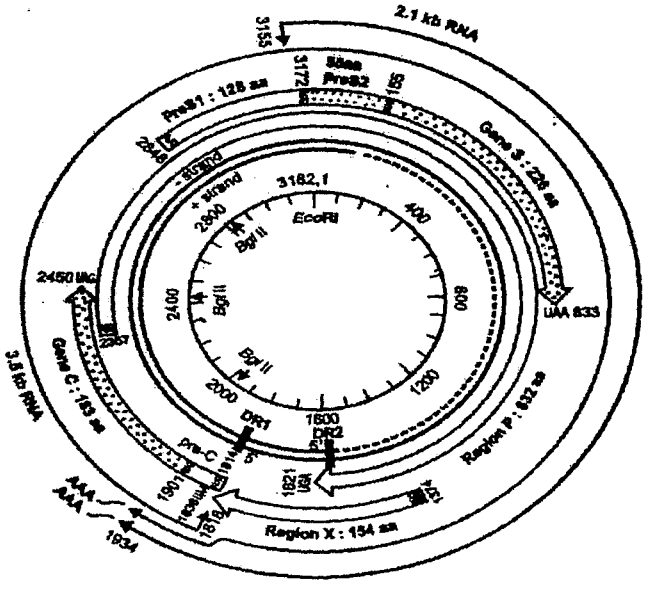


Figure 1.3 Fine structure of the HBV genome organisation.
Inner circles represent the partially DS virion DNA; dashes signify the incomplete positive strand. The locations of DR1 and DR2 are also indicated. Boxed-arrows surrounding the genome indicate the four ORFs in the direction of translation. The outer arrows represent the two major mRNA transcripts, 3.5 and 2.1kb in length. Image adapted (Kramvis & Kew 1998a).

1.5.1 HBV surface protein

The S ORF contains three in-frame start sites that direct synthesis of three distinct surface proteins, all of which share a common termination codon (Figure 1.2B). A shared region known as the S-domain relates all these HBV surface proteins to each other. Two promoter regions, namely the preS1 and preS2, control expression of these proteins. The preS1 promoter controls transcription of a single 2.4kb RNA molecule which includes the entire S ORF region, whereas, the preS2 promoter controls transcription of a family of transcripts, 2.1kb in length. The preS2 promoters have been shown to be stronger than the preS1 promoter (Heermann *et al.* 1984c; Robinson *et al.* 1974c; Wong *et al.* 1985a). This results in more middle and small surface proteins being expressed than large surface protein. Mutation(s) in the specific "shared" region of these proteins known as the "a" determinant, has been associated with HB immunoglobulin treatment failure and the escape from vaccine-induced antibodies to HBsAg (anti-HBs) which neutralise HBV (Alexopoulou *et al.* 2004; Carman *et al.* 1990b; Fortuin *et al.* 1994b; Karthigesu *et al.* 1994c).

In an effort to understand the biology of mutations known to affect the "a" determinant, a generally accepted model of this region is used (Figure 1.4). This model is described as a two-looped structure with a tight loop between amino acids 121 and 124. The tertiary structure of the whole Major Hydrophilic Region (MHR), which includes the "a" determinant, has been shown to be important for antigenicity. It has been divided into five antigenic regions, named HBs1 (up to position 120), HBs2 (120–123), HBs3 (124–137), HBs4 (139–147), and HBs5 (148–169) (Carman 1997). (In a later section, surface mutants, their effect and reasons for evading vaccine therapy or the natural immune system, will be highlighted).

1.5.1.a. Small Hepatitis B Surface Antigen (HBsAg or SHBsAg)

This protein is the smallest of the surface proteins, containing solely the S-domain. Historically, it also has been referred to as *Aa* (Bayer *et al.* 1968b; Ishikawa & Ganem 1995c; Wong *et al.* 1985b; Yuasa *et al.* 1991a). It is highly hydrophobic, containing four-transmembrane spanning regions. The protein is the prime constituent of all HB particle forms and as such, it appears to be expressed by the virus in high quantities. It also contains a highly antigenic epitope, analyses of which allow for the subtyping and genotyping of HBV carriers (Ashton-Rickardt & Murray 1989a; Bayer *et al.* 1968a; Courouce-Pauty *et al.* 1983; Okamoto *et al.* 1986a).

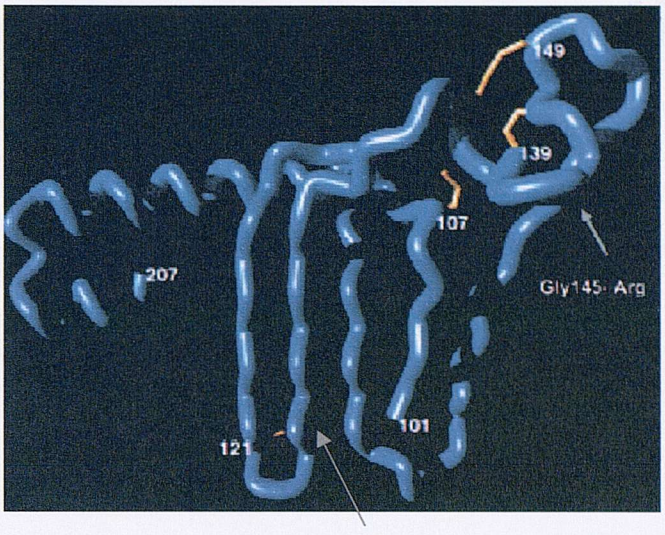


Figure 1.4 Model of the "a" determinant.
The position of the Gly → Arg (G → R) substitution (145) in the projected 139–147 amino acid region is shown. This mutant produces false-negative results in some commercial assays. Image adapted from (Chen *et al.* 1996).

1.5.1.b. Middle Hepatitis B Surface Antigen (MHBsAg)

Initiation at the second AUG of the S ORF produces the intermediate or middle-sized HBV surface protein that contains an additional 55 amino-acid (aa.) domain known as preS2 (Heermann *et al.* 1984b; Wong *et al.* 1985c). Together with preS1 and parts of S, this domain is hydrophilic and appears to reside extracellularly. This envelope protein (MHBsAg) has been shown to be translocated into the lumen of the endoplasmic reticulum (ER) during biosynthesis, explaining why it (M protein or preS2) is glycosylated, as opposed to another surface antigen polypeptide, LHBsAg (that additionally contains preS1) whose sequences are predominantly or exclusively cytoplasmic (Ostapchuk *et al.* 1994; Peng *et al.* 2005a; Prange & Streeck 1995) has not got a preS1 glycosylation site. The function of the MHBsAg is not well understood; the preS2 domain is apparently not essential for virus infection of hepatocyte cultures (Fernholz *et al.* 1993a). Since work on Site-directed mutagenesis (SDM) suggests that virion secretion requires the glycosylation in the preS2 domain of MHBsAg (Lu *et al.* 1995; Mehta *et al.* 1997), the MHBsAg oligosaccharide may have a role as a potential therapeutic target.

1.5.1.c. Large Hepatitis B Surface Antigen (LHBsAg)

This, the largest of the surface proteins, contains the PreS1 domain as well as the PreS2 and S domains. The PreS1 between aa. 21–47, is the ligand involved in virus binding to the hepatocyte receptor (Neurath *et al.* 1990; Pontisso *et al.* 1989; Xu *et al.* 1997a). Further work has linked ER retention of the protein as giving rise to the "ground-glass" hepatocytes being due to its overexpression, (Xu *et al.* 1997). However, it was first suggested that ER retention was due to a cytosolic factor that bound the LHBsAg as a transmembrane protein (Eble *et al.* 1986; Eble *et al.* 1990). More recent evidence has shown formation of intracellular particles of LHBsAg in the lumen of the ER (Franco *et al.* 1992a; Guo & Pugh 1997a). These authors suggest that retention appears to be due to the binding of LHBsAg to calnexin, a protein believed by most, as the one responsible for mediating viral attachment onto host cells. The isolation of this vital receptor has however proved elusive to molecular virologists involved in HBV research.

Several studies have shown that when this virus replicates in liver cells, S proteins are produced in much larger quantities than any other component of HBV (Alka *et al.* 2000b; Guidotti *et al.* 1999e; Heermann *et al.* 1984a; Pawlotsky 2002; Rehermann *et al.* 1995b; Robinson *et al.* 1974d; Wong *et al.* 1985d).

Like the *Dane* particles, the excess spheres and rods of the surface antigen can also enter the bloodstream in large numbers and, when present, they potentially indicate an active HBV infection that can be either acute or chronic (Alka *et al.* 2000a; Guidotti *et al.* 1999d; Tassopoulos *et al.* 1987b; Wong *et al.* 1985e). Therefore, only a portion of HBsAg is involved in *Dane* particle formation and individuals with this antigen should be considered infectious to others.

Laboratory tests can usually detect HBsAg about fourty to fourty-five days after infection, although detection may range from one to twelve weeks. The protein may precede the onset of symptoms such as jaundice and elevation of liver enzymes (which indicates injury of liver cells) by one to seven weeks (Guidotti *et al.* 1999c). Although some studies have demonstrated that anti-HBs alone is sufficient to provide protection against HBV infection, there is good reason to believe that the preS1 domain may be, at least in part, the substrate for the still elusive viral receptor. Epitopes in preS1 can also elicit virus-neutralising antibodies (Lambert *et al.* 1990; Lambert *et al.* 1991; Yuasa *et al.* 1991b) and alter the host range of the virus upon

genetic recombination (Ishikawa & Ganem 1995b). Besides its role in infection, the preS1 domain provides the ligand for core particles during the assembly of the viral envelope. This requires display of preS1 epitopes on the cytosolic side of the viral membrane (Guo & Pugh 1997b; Ishikawa & Ganem 1995a).

1.5.2 HBV C and *e* proteins

The C ORF is divided into preC and the core regions. The names used for these two different proteins are HBcAg and HB *e* antigen (HBeAg). The HBcAg is the major component of the nucleocapsid shell that packages the HBV genome. This 185 amino acid-long protein is expressed in the cytoplasm of infected cells during viral replication (McMahon *et al.* 1985d). The HBcAg forms dimers which are linked to form the nucleocapsid, which in turn encapsidates HBV DNA and the polymerase. *In vivo* studies have revealed that HBcAg is not found in the bloodstream at any time, but is readily detectable in hepatocytes following a liver biopsy (Guidotti *et al.* 1999b; Hadziyannis & Vassilopoulos 2001a; McMahon *et al.* 1985c; Ning & Shih 2004b).

Sequence analysis indicates that HBcAg is predominantly composed of hydrophilic and charged amino acids. The region of the antigen associated with nucleocapsid assembly, appear to be involve in nucleic acid packaging (Shafritz & Lieberman 1984; Strauss & Strauss 2002; Zoulim 2005b). HBcAg is able to form particles with outward pointing spikes even if a foreign peptide sequence is spliced into the HBc coding region. As such, HBcAg can be used for displaying foreign protein domains on the surface of HBV nucleocapsids (Aiba *et al.* 1997a; Bock *et al.* 1996; McMahon *et al.* 1985b; Ning & Shih 2004a).

Unlike the core protein, HBeAg has a different fate. The *e* antigen is so-named due to its "early" appearance during an acute HBV infection. HBeAg is expressed by the translation of sequences upstream of the HBcAg initiation codon in the 3.5kb preC mRNA. The preC sequence which contains the first initiation codon at position 1814 leads to the synthesis of a protein encoded by both the preC (containing a signal sequence) and C ORFs. A specific sequence encodes a hydrophobic transmembrane domain that results in the translocation of the preC/C protein into the lumen of the ER following synthesis. Cleavage of the signal sequence in the ER-lumen is followed by further proteolytic processing by a host protease at aa. 149, immediately upstream of the arginine-rich C-terminus, and by secretion of the resultant soluble 17kd polypeptide (*e* antigen) which is soluble into circulation

(Blackberg & Kidd-Ljunggren 2000b; Hultgren *et al.* 1997c; Milich *et al.* 1990d; Peng *et al.* 2005b). The described fundamental change in the location of protein expression is thought to alter its antigenicity such that it does not share antigenic homology with HBcAg, despite having nearly identical amino acid sequences. The purpose of producing this secretory form of HBc is little-understood, but is not thought to be involved in viral replication, but instead to be secreted from infected cells (Garcia *et al.* 1988; Standring *et al.* 1988c). It is detectable in serum of highly viraemic HBV-infected carriers where there are antibodies against it. Notwithstanding the clinical importance attached to the antigen in detecting the virus in patients, HBeAg has been shown to be irrelevant for replication either *in vivo* or *in vitro* (Chang *et al.* 1987a; Chen *et al.* 1992; Schlicht *et al.* 1987; Standring *et al.* 1988b). One hypothesis is that high levels of HBeAg may prevent the immune system from eliminating HBV-producing cells and thus act as a tolerogen within neonates (Milich *et al.* 1990c; Standring *et al.* 1988a), promoting the establishment of persistent infection following vertical transmission of the virus. Similarly, HBeAg translated from the core gene, may be responsible for diverting the immune response, (Hultgren *et al.* 1997b; Milich *et al.* 1997a).

During natural infection, HBeAg-negative mutants arise and are positively-selected over wildtype virus (Santantonio *et al.* 1991; Tran *et al.* 1991). In support of this, Chen *et al.* (1992) have found that woodchucks do not undergo persistent WHV infection if an HBe-minus variant is used for infection. In humans, however, these variants are infectious causing FHB (Chan *et al.* 1999c).

A large number of mutations in the core protein affect T and B-cell epitopes. A cluster of mutations overlap with the dominant T helper-cell epitope between positions 50 and 69, which is recognised by virtually all patients irrespective of MHC class II background (Ferrari *et al.* 1991). Whether these mutations reduce the Th cell response currently remains unknown. The overlapping HBe1 and HBc B-cell epitopes (Salfeld *et al.* 1989) are often affected also by one or more amino acid substitutions during the course of chronic hepatitis (Gunther *et al.* 1998g). However, what has still not been clarified is how mutations in HBc/*e* B-cell epitopes interfere with clearance of the virus, because HBeAg and its precursors are not an integral part of the virion and the nucleocapsid is surrounded by the lipid envelope of the virion. Mutations minimising or even hindering the CTL response *in vitro* have been

observed in a chronic carrier, whose CTL response was narrowly focussed on the corresponding epitope (Penna *et al.* 1991). Another study indicated that this type of response as well as the escape of HBV from CTL recognition, is a rare event (Rehermann *et al.* 1995d).

1.5.3 HBV X protein

The presence of a fourth gene, termed X, is unique to mammal-infecting *hepadnaviridae*. The product of the X ORF has transactivating properties (Rossner 1992). Its function during natural viral infection is, however, not known. It is essential for *in vivo* virus replication, but unnecessary for viral DNA synthesis in transfected tissue culture cells (Chen *et al.* 1993a; Zoulim *et al.* 1994). Evidence that the gene is expressed *in vivo* stems from the detection of antibodies present in sera of HBV-infected humans and naturally infected animals (Chen *et al.* 1993b; Vitvitski-Trepo *et al.* 1990), and from protein analysis of liver samples obtained from WHV-infected woodchucks (Dandri *et al.* 1996).

Investigators have shown the X gene as the part of the HBV genome that is most often included among HBV DNA integrants (Unsal *et al.* 1994; Wang *et al.* 1994b). Moreover, there is evidence that the X protein binds p53 and abrogates its normal functions (Truant *et al.* 1995; Ueda *et al.* 1995). Ueda and co-workers used a transgenic mouse model expressing the X protein to show that this protein formed a complex with p53 protein in the cytoplasm of Hepatocellular Carcinoma (HCC) cells, and that the latter was completely absent from nuclei, its normal site of action (Ueda *et al.* 1995).

Additionally, experiments using an *in vitro* interaction and electrophoretic mobility shift assay showed that HBx augmented the DNA binding activity of C/EBPα by direct interaction with it; its basic leucine zipper domain was responsible for the interaction with HBx (Choi *et al.* 1999b). Domain analysis of HBx showed that the central region of the protein (aa. 78–103) was necessary for the interaction with C/EBPα. However, the complete form of HBx was necessary for the synergistic activation of the HBV preG promoter (Choi *et al.* 1999a). The results of this study suggest that the interaction of HBx and C/EBPα enhances HBV transcript synthesis directed by the preG promoter.

1.5.4 HBV polymerase

The largest ORF in the HBV genome encodes for the viral polymerase (P). Its protein is 90kD in size and has both RNA and DNA dependent polymerase activities; it plays a key role in HBV genome generation as well as preG RNA encapsidation (Tang & McLachlan 2002b; Tavis *et al.* 1994) (Section 1.6.3). The C-terminal part of polymerase proteins has been found to be versatile and to share amino acid homology with RNase H domain, and reverse transcriptases. The data also suggested that a conservative N-terminal region of polymerase proteins functions as protein primer for DNA synthesis in hepadnaviruses (Khudyakov & Makhov 1989; Radziwill *et al.* 1990; Toh *et al.* 1983). The polymerase protein has been divided into four characteristic domains starting from its amino-terminus end containing, namely: a terminal protein, a spacer region, the Reverse Transcriptase (RT)/P and the RNase H domain which lies at the carboxyl end (Figure 1.2, panel B). The terminal protein plays a critical role in the packaging of preG RNA and in the priming of minus strand DNA synthesis (Section 1.6). The normally highly conserved RT/P domain has been defined as the central enzymatic activity of replication (Bosch *et al.* 1988) and is a target for drug therapy. Studies have described drug-resistant HBV mutants in patients, later found predominantly to harbour virus with amino acid substitutions in this domain (Dienstag *et al.* 1999; Lau *et al.* 2000; Liaw *et al.* 1999a; Ling & Harrison 1999a; Tipples *et al.* 1996a). (These mutants and their effect on drug therapy will be discussed in more detail in a later section, 1.8).

1.6 HBV Viral Replication

1.6.1 Hepatocyte specificity, virion attachment, and transport

Since hepatocytes are the primary site of viral DNA replication, it is tempting to conclude that tissue specificity is controlled mainly by a hepatocyte-specific receptor. However, earlier work has found that hepatotropism is also evident at the level of viral gene expression. Various studies have indicated virus selectivity for productive infection in only very specific cell line (Halpern *et al.* 1984; Jilbert *et al.* 1987; Tagawa *et al.* 1987).

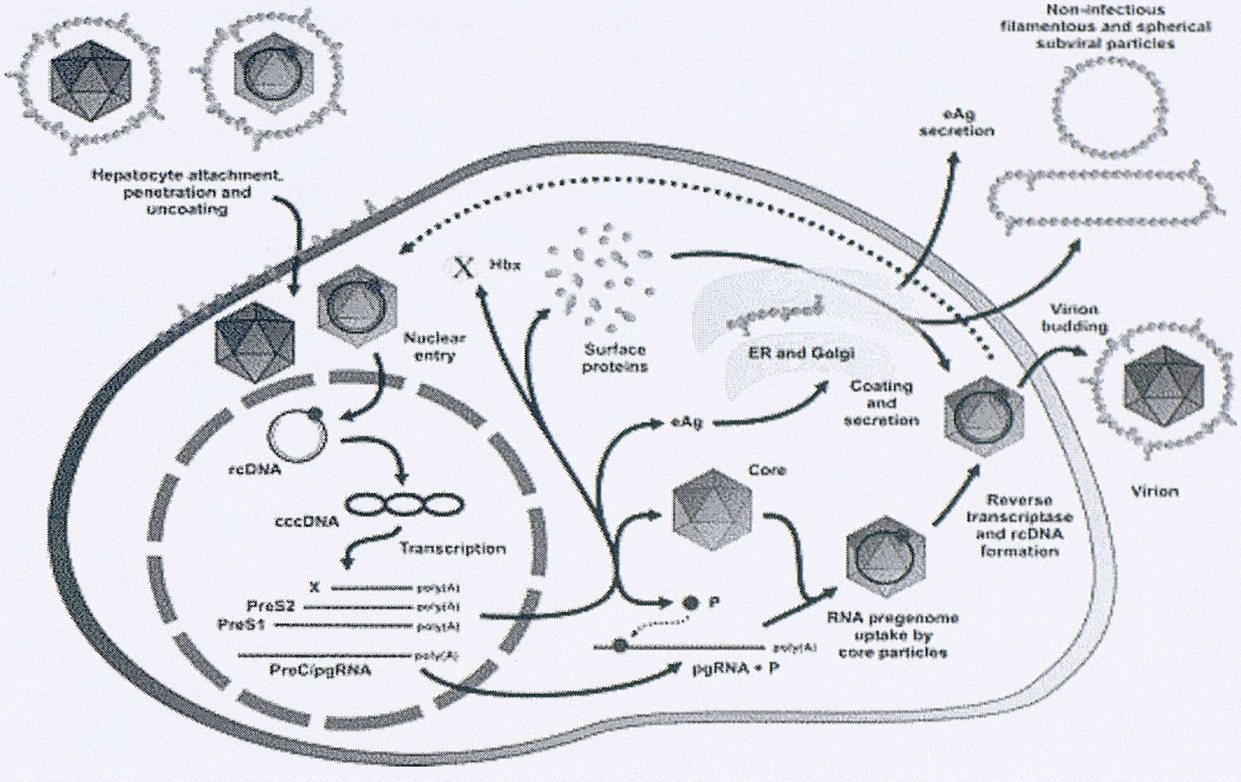
Summarised in Figure 1.5, the initial steps following HBV entry are not clearly defined although it is known that the virion initially attaches to a susceptible

hepatocyte through recognition of a cell surface receptor(s) whose identification still remains a challenge to virologists. The small DNA viruses, such as hepadnaviruses, are thought to maintain their genome in an encapsidated state until they arrive in the nucleus (Greber & Fassati 2003; Shafritz & Lieberman 1984). In the nucleus, their DNA genomes are repaired (see later section: 1.6.1) (Tuttleman *et al.* 1986f) to form cccDNA which subsequently serves as template for transcription, using host RNA polymerase III. Once formed, these transcripts are transported to the cytoplasm for translation to form the different HB viral proteins. Nucleocapsids are then assembled, at the same time, incorporating a single preG RNA into the assembled cores. Encapsidation of this RNA triggers reverse transcription to synthesise double-stranded (DS) circular DNA, whose sequential synthesis has been described. Firstly, the negative DNA strand is synthesised from the encapsidated preG RNA template (described above). For this to occur, the polymerase initially binds to the epsilon encapsidation signal, the side bulge of which is used to synthesise a short (3 nt.), DNA primer. This is used to prime positive DNA synthesis by binding to the DR1 sequence at the 3′–end of the preG RNA. Concurrently with DNA synthesis, the RNA template is degraded by the RNase H activity of the polymerase, except for this extreme 5′–end, which is used in turn as primer for the synthesis of the positive DNA strand and using the newly made negative DNA strand as a template (Summers & Mason 1982a; Will *et al.* 1987c). Some core particles bearing the mature genome are transported back to the nucleus, where their DNA genomes can be converted to cccDNA thus maintaining a stable intranuclear pool of transcriptional templates (Tuttleman *et al.* 1986e).

The replication of HBV has been shown to occur almost exclusively in the liver. However, using Duck Hepatitis B Virus (DHBV) as a model to characterise hepadnaviral replication, Halpern and colleagues (1984) have documented replication in a subset of exocrine cells, in the endocrine islets of pancreas, and in the proximal tubular epithelium of the kidney in ducks. Nevertheless, the liver remains the primary site of replication and the liver cell, or hepatocyte, is the cell that supports viral replication in full.

Figure 1.5 Schematic model for the Hepadnavirus life cycle

Arrows indicate direction of replication. Reference: biology.kenyon.edu/slonc/bio38/scuderi/parti.html



Summary of the main stages in the HBV life cycle, *in vivo*

1. **Attachment**: HBV virions bind to surface receptors and are internalised.
2. **Uncoating and Nuclear Transport**: The viral coat is removed and the nucleocapsid is transported to the nuclear pore.
3. **HBV Genome Repair**: The released genome is repaired to cccDNA, the template for viral messenger RNA (mRNA) transcription within the nucleus.
4. **Transcription**: The resulting viral mRNA, after transport, is translated in the cytoplasm to produce the viral surface, core, polymerase, and X proteins.
5. **Nucleocapsid Assembly**: There, progeny viral capsids assembled encapsidating the viral RNA (RNA packaging) and a copy of the polymerase. This RNA is reverse-transcribed into viral DNA.
6. **Virion release**: The resulting cores can either bud into the ER to be enveloped and exported from the cell, or recycle their genomes into the nucleus for conversion to cccDNA.

1.6.2 HBV genome repair and transcription

During initiation of infection, the viral RT/P attached to the 5′–end of the plus strand, and a short RNA attached to the 5′–end of the minus strand, are removed from the viral RC-DNA genome. The incomplete positive strand is "filled-in" and the nick in the negative strand is ligated, to form a double-stranded (DS) circular DNA molecule, referred to as cccDNA. This "repair" mechanism is only known to occur in the nucleus after the virus had entered the hepatocyte (Ganem 1982b; Ganem *et al.* 1994; Gripon *et al.* 2002; Parekh *et al.* 2003j; Seeger & Mason 2000b; Gerlich *et al.* 1993). The cccDNA now serves as the template for transcription of viral mRNAs, (Figures 1.2 and 1.5). One of these, the preG RNA, serves as the mRNA for the synthesis of core protein (nucleocapsid subunit) and viral RT/P. The latter subsequently binds to the 5′–end of its own mRNA template, forming a complex which is ultimately packaged into nucleocapsids, where viral DNA synthesis occurs. Translation of the polymerase protein and preG RNA packaging into viral nucleocapsids are tightly coupled events (Bartenschlager *et al.* 1990b; Hirsch *et al.* 1990; Junker-Niepmann *et al.* 1990a). The RT/P binds to an RNA stem-loop structure termed epsilon, at the 5′–end of the preG RNA, triggering encapsidation into the core particles (Pollack & Ganem 1993a; Wang *et al.* 1994a). Epsilon (ε) therefore constitutes the viral encapsidation signal.

1.6.3 Encapsidation signal

Numerous authors have described the structure of ε, transcribed from the distal preC region and proximal C gene. This is formed by inverted repeat sequences consisting of 70 nucleotides (positions 1846–1916, numbering from the *EcoRI* site; (Chiang *et al.* 1992; Galibert *et al.* 1979; Kawamoto *et al.* 1994) (Figure 1.6). These inverted repeat sequences are a prerequisite to the formation of a secondary, or possibly tertiary structure, that is recognisable as a packaging signal (Junker-Niepmann *et al.* 1990b). The consensus amongst investigators is that despite the fact that both genomic HBV transcripts contain the encapsidation signal ε at their 5′–ends, only the shorter (preG RNA) transcript is encapsidated (Bartenschlager *et al.* 1990a; Enders *et al.* 1987; Junker-Niepmann *et al.* 1990c; Will *et al.* 1987b).

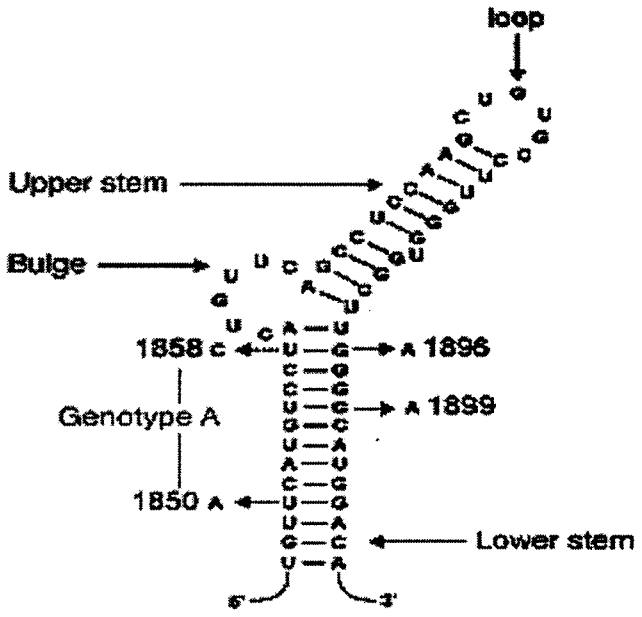


Figure 1.6 Proposed secondary structure of the ε encapsidation signal
The secondary structure of ε of Genotype A, based on computer predictions, chemical and enzymic probing. Image adapted from (Kramvis & Kew 1998b).

Lok *et al.* (1994) have suggested that only 3-nt. segment (1856-1858 and 1896-1898) and probably the adjacent nucleotides (1855 or 1899) are crucial for the function of ε, possibly due to proximity to the start codon of the core gene. Besides encapsidation, ε is involved in activation of the RT/P, in template restriction and in DNA synthesis initiation by reverse transcription. In addition, ε is a constituent of the DNA template that gets translated into the preC/C fusion protein which ultimately undergoes post-translational modification to produce HBeAg, as already described (Shafritz & Lieberman 1984), (Section 1.5.2).

1.6.4 HBV virion packaging and release

Once partially DS-DNA has been produced, nucleocapsids can follow one of two routes:

1. They may migrate to the nucleus to increase the copy number of cccDNA. Since cccDNA does not undergo semi-conservative replication, all cccDNA is derived from viral DNA made in the cytoplasm via the reverse transcription pathway (Tuttleman *et al.* 1986d). Accumulation of viral envelope proteins prevents excessive cccDNA formation, which can be toxic to hepatocytes (Summers *et al.* 1990b; Summers *et al.* 2003).

2. They may undergo a maturation event that facilitates their acquiring an outer envelope via budding into the ER. For envelope acquisition, the core of mature virions binds to the N-terminal domain of the LHBsAg which results in translocation (budding) of the core particles across the ER membrane. Enveloped particles containing all three surface proteins (S, M and L) are thought to be transported through the ER into the Golgi complex. Completion of the envelopment process triggers the secretion of mature virions into the bloodstream (Huovila *et al.* 1992).

1.7 HBV Replicative Intermediates

1.7.1 cccDNA detection

As alluded to, cccDNA appears to be the requisite intermediate in HBV viral replication, and a key aspect to monitoring viral replication is its detection. A main determinant to achieving this aim successfully involves designing primer-pairs that can only bind cccDNA, e.g., one of the primers either binds before DR1, or DR2, whilst the complementary primer binds sequences that are common to all HBV DNA molecular species (Maillard & Pillot 1996). A primer-pair that would include sequences in both DR1 and DR2 would provide additional stringency and binding specificity for the intended cccDNA sequence target, thus possibly eliminating false positive results. Furthermore, to characterise the significant role of cccDNA in HBV persistence and/or mutation, replication intermediates not only need to be detected, but also quantified. Previous studies for detecting them are found in Millard and Phillot (1996), and more recently (Yang *et al.* 2004). In this thesis, the highly sensitive Real-time PCR method was employed rather than a semi-quantitative approach as in previous studies. Described in detail later (Section 1.16), this technique works by both detecting and quantifying at the same time. This assay has also been used in this thesis to measure two other replicative intermediates, the preG and preC mRNA transcripts. The reason for both detecting and quantifying these replicative intermediates was based on the hypothesis that the results would provide not only evidence of active viral replication but also enable the relative comparison in replication capacity terms to be examined among different recombinants. Since a vital aspect of this investigation includes the detection of RNA intermediates, an overview of how their production is regulated is given.

1.7.2 Transcriptional regulation of preC and preG mRNA

The HBV transcripts encoding the viral proteins described so far are synthesised by the host RNA polymerase II, and all of them terminate at a common polyadenylation signal, located within the core protein coding region (Yuh & Ting 1990b; Yuh *et al.* 1992c) (Figure 1.7). As described earlier, transcription of cccDNA produces two longer than genome-length RNAs (3.5kb), and subgenomic surface and X mRNAs. Studies have shown that each of the two 3.5kb RNAs varies in length: whilst the initiation site for the shorter transcript (preG mRNA) is situated at nucleotide (nt.) 1815 ± 5, that of the longer transcript (preC RNA), is situated at positions 15 to 35 upstream (Figure 7.1).

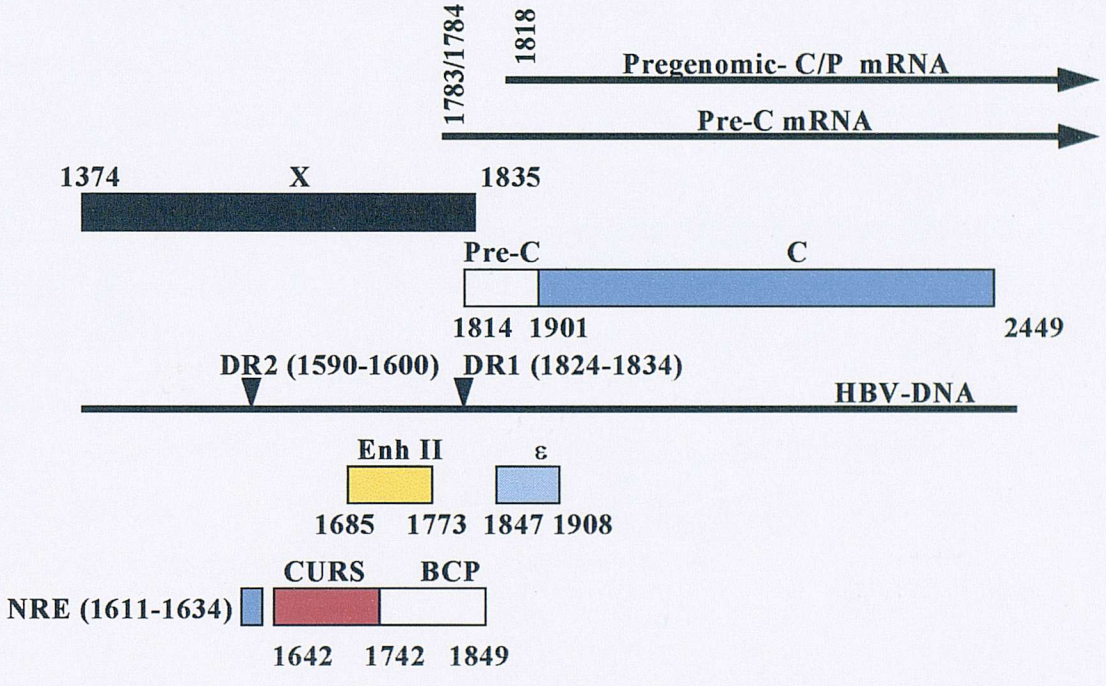


Figure 1.7 Schematic diagrams of the HBV preC and preG transcripts in relation to the CP region.
EnhII, CP, preC and X ORF are indicated. Horizontal arrows indicate the two viral transcripts and the direction of transcription. A non-arrowed line represents the full-length HBV genome on which the position of DR1 and DR2 are indicated. Basic Core Promoter (BCP), Core Upstream Regulatory Sequences (CURS), ε and the negative regulatory element (NRE) are also shown. Image adapted from (Okamoto *et al.* 1994a).

The preC RNA encodes preC/C protein, the precursor of HBeAg. The preG RNA, forms the replicative intermediate, but in addition, encodes the viral core and

polymerase proteins (Moriyama *et al.* 2003; Yu & Mertz 1996e; Yu & Mertz 1997d). Mutations introduced in the C and P genes of a replication-competent HBV construct will be employed in this investigation in order to measure the change in replication efficiency, using a wildtype construction as reference.

The production of the preG RNA and preC mRNA are controlled by the liver cell-specific BCP (Shaul *et al.* 1985d). Two enhancer regions with liver specific characteristics have been identified in the HBV genome (Figure 1.5): Enhancer I (EnhI) is positioned between nts. 1080–1234, and lies between the S and X ORFs. EnhII is situated at positions 1685–1773 and lies upstream of the CP region, overlapping the X ORF (Chen *et al.* 1995a; Gunther *et al.* 1996a). CP comprises nts. 1744–1751 and forms part of the BCP region of the virus (nt. 1742–1849, as shown). Sequences (1643–1742) upstream of the Basic Core Promoter (BCP), which incorporate Enhancer II (EnhII), are referred to as the Core Upstream Regulatory Sequences (CURS) and have a positive effect on promoter function (Chen *et al.* 1995b; Gunther *et al.* 1996b; Laras *et al.* 2002a; Liu *et al.* 2004d; Tang & McLachlan 2002a; Xie *et al.* 2001c; Chen *et al.* 1995c; Gunther *et al.* 1996c; Laras *et al.* 2002b; Liu *et al.* 1994c). In addition, a Negative Regulatory Element (NRE) has been identified which has the opposite effect as CURS. Studies have also shown that the BCP, CURS and NRE regions bind a number of transcription factors including Hepatocyte Nuclear Factor 4 (HNF4), and CCAAT Enhancer Binding Protein (C/EBP) (Chen *et al.* 1995d; Liu *et al.* 1994b). Mutations in the transcription binding sites have also been reported in a number of studies, but their effect on the actual replication fitness of the virus has not been ascertained.

1.8 HBV Mutants and Variants

It is surprising that for such a small DNA virus, HBV exhibits such a high mutation rate (2×10^{-4} base substitutions per site per year) (Mimms 1995b). There is evidence to suggest that such mutations may lead to the synthesis of altered, inactive proteins, or to the loss of their ability to produce the original functional proteins (Carman *et al.* 1990a; Hess *et al.* 1997; Kramvis *et al.* 1997a; Schormann *et al.* 2006; Guarnieri *et al.* 2006c). In genetic terms, a mutant (from *mutare*, to change) is defined as a gene, chromosome, organism, or population differing from the corresponding wildtype by one or more nucleotide alterations. A mutation in turn is a

relatively permanent, transmissible change in the genetic material, involving either a biochemical change in a codon, a physical change in interchromosomal relations or a protein sequence alterations. In the context of this study, point mutations resulting from nucleotide substitution were created. These mutations are classified as either missense (a codon for a particular amino acid is altered to one specifying a different amino acid) or nonsense mutations (a base change which produces a nonsense codon resulting in premature termination of protein synthesis), for example the A1762T/G1764A and G1896A, respectively, which converts A → G/G → A, and G → A, respectively.

Differentiation between "variants" and "mutants" can be made using the following criteria: the former, classified as naturally-occurring, give rise to well-recognised serological subtypes and genotypes; the latter are sequence changes selected by host influences, such as immune pressure (Francois *et al.* 2001b). [However, the two meanings (variants and mutants) are often used interchangeably]. The global variations of HBV genome sequence heterogeneity, or genotypes, have been correlated to the prevalence of HBV mutants in various geographic regions with disease outcome and response to antiviral treatment (Li *et al.* 1993b).

Although many of these mutations would be lethal to the virus, a number of studies have shown that some of these mutations may actually be advantageous, offering either a replication advantage, facilitating immune escape, or enabling resistance to nucleoside analogue treatment. These are represented by the preC and/or CP mutants, the "a" determinant/S and the RT/P mutants, respectively (Brunetto *et al.* 1989a; Carman *et al.* 1989d; Chayama *et al.* 1998; Chuang *et al.* 1993; Waters *et al.* 1992e).

1.8.1 PreC gene mutants

The result of a G → A substitution at position 1896 of the preC region, which converts codon 28 for tryptophan (TGA) to a translational stop-codon (TAG), leads to premature termination of synthesis of the preC/C protein, (Carman *et al.* 1989c). Less frequent preC region mutations have also been reported. These involve initiation codon mutations (at positions 1814 or 1815), a nonsense mutation at 1874, a missense mutation at 1862, and frameshift mutations (Hou *et al.* 2002g; Kramvis *et al.* 1997b). The preC mutant, common in anti-HBe-positive patients, causes active liver disease

and, when transmitted to naïve individuals, infants or adults may develop in Fulminant Hepatitis B (FHB) (Aritomi *et al.* 1998; Hasegawa *et al.* 1994a; Komatsu *et al.* 2001b; Omata *et al.* 1991b; Sato *et al.* 1995a).

As stated earlier, HBeAg is a soluble protein that has been found to have a tolerogenic effect on the immune response of the host, thus favouring chronic infection. This finding is further strengthened by reports that the protein acts negatively to regulate viral replication by reducing encapsidation of preG RNA. Its abrogation or reduced synthesis may increase HBV replication efficiency, heightening the host immune response to the virus (Baumert *et al.* 1996f; Buckwold *et al.* 1996a; Liang *et al.* 1991a). An aggressive host immune response to HBV antigens may thus result in FHB, possibly due to over-expression of (reportedly) its core protein, the main target for cytotoxic T cells, as a result of its efficient presentation to the immune system (Karayiannis et al., 1995).

Earlier investigations have linked preC mutations to different regions of ε. Analysis of these mutants has permitted the identification of the various segments of ε that function in RNA packaging, in the activation of the polymerase protein, and in reverse transcription (reviewed in Kramvis and Kew, 1998). However, of relevance to this thesis, are the naturally-occurring mutations in the lower stem of ε (Figure 1.6). Nonetheless, it is worth briefly highlighting the effect of mutations in other regions of ε, such as mutations in the upper stem that do not significantly affect the general functioning of ε, suggesting that sequence-specific determinants in this region may be irrelevant (Beck *et al.* 1997). Moreover, a specific mutation in the bulge of ε was not critical for encapsidation (Fallows & Goff 1995; Knaus & Nassal 1993b; Pollack & Ganem 1993b) although it is essential to encapsidation and viral replication. Studies have also described mutations in the loop of ε where deletion and major changes in the size and sequence in this region impaired encapsidation and HBV replication (Knaus & Nassal 1993a; Tong *et al.* 1992). Using heron and duck HB viruses, gross mutations within this region prevented efficient physical binding of ε to the polymerase protein and established the importance of the loop in binding host factors for encapsidation activation (Beck & Nassal 1997; Pollack & Ganem 1993c).

In the ascending limb of the lower stem, position 1858 — which pairs with 1896 (Figure 1.6) — can either be a thymidine (*T*) or a cytosine (*C*), both coding for proline in codon 15 (1856-1858). A T1858 (U1858 in RNA) would destabilise the

Watson-Crick base-pairing with G1896 in the descending limb, whereas normal base-pairing would exist with a C1858. Studies have therefore linked the effects of G1896 mutations to specific HBV genotypes, and to *C* or *T* at position 1858 (Li *et al.* 1993a; Lindh *et al.* 1997a; Rodriguez-Frias *et al.* 1995). C1858 predominates amongst A and F viral genotypes, whilst T1858 has been reported to occur mostly in genotypes B, C, D and E. Studies have documented that the G1896A mutation may be accompanied by an A → T transversion (in genotypes A and F) at position 1858, the joint effect of which further stabilises ε, leading subsequently to increased viral replication (Owiredu *et al.* 2001). Also reported is the association of the G1896A with the G1899A. This association jointly acts to stabilise ε (Kramvis *et al.* 1997c; Ogata *et al.* 1993b).

1.8.2 Core promoter mutants

Although the G1896A stop codon mutation, as noted, has been detected in a large number of FHB patients and was initially thought to play a causal role in the disease (Carman *et al.* 1993c; Fujiwara *et al.* 1998; Maruyama *et al.* 1998; Zheng *et al.* 2003d), this mutant is not always isolated from FHB patients (Aye *et al.* 1994; Laskus *et al.* 1994; Zheng *et al.* 2003c). Other mutations may be implicated and there is mounting evidence that mutations occurring in the CP may have a role in FHB (Baumert *et al.* 1996e; Buckwold *et al.* 1997h; Carman *et al.* 1989b; Chan *et al.* 1999b; Dumpis *et al.* 2001; Hou *et al.* 2002f; Omata & Ebata 1991). This region consists of the BCP which contains binding sites for a variety of transcription factors, including HNF4 and Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF) (Raney *et al.* 1997e; Yu & Mertz 2003c; Zheng *et al.* 2003b). (These will be described in more detail in a later section).

CP mutants are the dominant viral species not only at the anti-HBe-positive stage, but also earlier at the late HBeAg-positive stage of infection (Blackberg & Kidd-Ljunggren 2000a; Bozdayi *et al.* 2001; Scaglioni *et al.* 1997a). Most of the promoter mutations affect the transcription factor binding sites of BCP. The most frequent and extensively studied change is in the common binding site involving an A → T nucleotide exchange at position 1762 combined with a G → A nucleotide exchange at position 1764. This double mutation has been linked to FHB (Buckwold *et al.* 1996b; Moriyama *et al.* 1996a; Scaglioni *et al.* 1997b). These and other

investigators agree that changes in transcription factor binding possibly account for the downregulation of preC mRNA and HBeAg production seen in *in vitro* studies with the double mutant. What remains controversial are the results generated in terms of the effect of this double mutation on viral replication in transfection studies. Although Moriyama *et al.* (1996) and Buckwold *et al.* (1997) showed it to enhance viral replication, the investigators explained their results differently: one group interpreted the increased viral replication as being due to enhanced encapsidation (Buckwold *et al.* 1997g), whilst the other ascribed it to increased preG RNA transcription (Moriyama 1997a). On the other hand, Gunther and colleagues (1998) found downregulated preC mRNA transcription only, and no increased viral replication in Huh7 cells. The fact that these mutants have been detected in viral isolates from asymptomatic carriers, would argue against a role in upregulating viral replication (Baptista *et al.* 1999b; Kurosaki *et al.* 1996).

Other naturally occurring BCP mutants have also been described *in vitro*. Similar to the A1762/G1764T mutant, mutations at positions 1766, 1768 and 1753 have been causally associated with FHB (Baumert *et al.* 1998f; Parekh *et al.* 2003i). The mutations at these positions have not yet been found to occur individually, but in combinations of two, three or even four. Like A1762/G1764T, these mutants have been described by Baumert *et al.* (1998) and Parekh *et al.* (2003) as having a similar HBeAg phenotype and also upregulating viral replication, generally. These findings are supported by recent data from patients (Sendi *et al.* 2005).

What still remains to be reported are results of a comprehensive *in vitro* investigation looking at the replication effects of various preC and CP mutations, when they occur alone or in combination, compared to the wildtype genotype. It could be of particular relevance to observe effects of the mutations *in vitro*, and compare them to published *in vivo* results (Peng *et al.* 2005c; Yoo *et al.* 2003).

1.8.3 Surface gene mutants

Point mutations in various parts of the S gene have been widely described (Fernholz *et al.* 1993b; Melegari *et al.* 1994; Melegari *et al.* 1997; Xu *et al.* 1997b; Carman *et al.* 1995b). Mutations in the preS1 region dysregulate S protein expression which in turn causes direct or immune-mediated liver cell injury in HBV transgenic mouse models (Ando *et al.* 1993; Chisari *et al.* 1987a). Fernholz's group (1993) have

also described PreS2 deletion variants, and observed *in vitro* that these variants can still replicate independently, concluding therefore, that this region is neither essential for HBV replication nor for infectivity.

Of interest to this study (reported in this thesis), however, is the "a" determinant region, common to all three surface proteins, and the major immunogenic site against which neutralising antibodies are raised. Mutations in this area can emerge during chronic infection and are most prevalent in patients that tested negative for HBsAg or positive for anti-HBs. Vaccinated chimpanzees challenged with a specific "a" determinant mutant were protected from developing infection (Ogata *et al.* 1999), but this has not proved to be the case in humans.

Mounting interest in the investigation of S gene mutants began with the identification of an HBV vaccine escape mutant in an infant born to an HBeAg-positive mother who developed breakthrough infection despite having undergone full passive–active immunoprophylaxis (Carman *et al.* 1990f). Both HBsAg and anti-HBs were detected concurrently in the circulation in this child. The most frequent escape mutation is due to a glycine → arginine (G → R) substitution at amino acid position 145 in the HBs4 region (G145R), reflecting a guanosine → adenosine change at nt. position 587 (Fujii *et al.* 1992a; Waters *et al.* 1992d). This mutant, found predominantly in S.E Asia and the Far East (Fujii *et al.* 1992b; Hsu *et al.* 1997; Zuckerman *et al.* 1994) persists for many years and is quite stable (Fujii *et al.* 1992c; Zuckerman & Zuckerman 1999). Since the discovery of the G145R mutant, several other vaccine-associated mutants have been described, including L141E (Carman 1997; Karthigesu *et al.* 1994a) and T131I (Carman *et al.* 1995a; Carman *et al.* 1997; Grethe *et al.* 1998; Kato *et al.* 1996; Yamamoto *et al.* 1994a).

Monoclonal antibodies that bind to epitopes within the common "a" determinant of HBV have been shown to bind to two cyclical peptide analogues, containing aa. 124-137 and 139-147, respectively (Torresi *et al.* 2002f; Waters *et al.* 1992b). For the studies given in this thesis, therefore it was important to use such antibodies to characterise S gene mutants, prior to analysing their effects in subsequent transfection experiments.

1.8.4 Polymerase mutants

Mutations in the P gene have been causally linked with resistance to the therapeutic effects of nucleoside analogues used in the treatment of chronic infection

(Allen *et al.* 1998a; Liaw *et al.* 1999b; Tipples *et al.* 1996b). Lamivudine (2′, 3-dideoxy-3′-thiacytidine, LMV) is a potent inhibitor of HBV RNA-dependent DNA polymerase, irreversibly blocking reverse transcription and inhibiting viral replication. It thus effectively reduces the viral burden in chronic HBV carriers (Francois *et al.* 2001c; Fu *et al.* 1999). The drug inhibits HBV replication and improves liver function in chronically-ill patients (Ling & Harrison 1999b). LMV-resistant HBV mutants are characterised by the change in the viral RT/P gene of methionine → isoleucine (M → I) (M204I) or valine (V) (M204V) in the YMDD motif (Stuyver *et al.* 2001) of the catalytic center of the enzyme (Allen *et al.* 1998; Ling and Harrison, 1999; Ling *et al.*1996; Tipples *et al.* 1996). The latter mutation is also accompanied by a compensatory leucine (L) → M change at codon 180 (L180M/M204V) (Allen *et al.*, 1998; Ling and Harrison, 1999; Ling *et al.*, 1996; Tipples *et al.*, 1996). The positions of these mutations are based on the recently revised nomenclature for the HBV polymerase (Stuyver *et al.* 2001). These mutants are resistant to LMV both *in vitro* and *in vivo* (Ling and Harrison, 1999; Ling *et al.*, 1996; Ono-Nita *et al.*, 1999a; Tipples *et al.*, 1996). Resistance of HBV to LMV is explained by steric hindrance between the oxathiolane ring of LMV-triphosphate and the β-branched chain of *I* and *V* (Das *et al.* 2001).

In a recent comprehensive review highlighting the effects of some of the currently available therapies for chronic HBV infection (Karayiannis 2004), about 18% of HBeAg positive patients were stated to seroconvert to anti-HBe following LMV treatment, resistance being linked to viral replication. LMV-resistant mutants appear to be less replication competent than the wildtype strain, an effect associated with mutations within the catalytic domain of the P gene (Karayiannis 2004; Ling & Harrison 1999c; Ono-Nita *et al.* 1999b; Torresi *et al.* 2002e; Marrone *et al.* 2005a; Buckwold *et al.* 1997f; Shaul *et al.* 1985c; Wang *et al.* 1998d; Wolters *et al.* 2001a; Marrone *et al.* 2005b; Shaul *et al.* 1985b). Of particular interest to the work reported herein are those classified as occurring within the YMDD motif of the core sub-domain of the P gene. The amino acid substitutions involved in these mutational changes are M → I and M → V, at position 204 generating YIDD and YVDD sequences, respectively.

1.8.5 Regulatory element mutations

Mutations in *cis*-acting transcriptional regulatory elements (CP) of the HBV genome are of particular functional relevance. Published studies have suggested that whilst mutations in EnhI were rather uniformly distributed, those of EnhII were focussed within the BCP region (nts. 1742-1849), and possibly contributed to development of FHB (Fang *et al.* 1998; Sterneck *et al.* 1996b; Torresi *et al.* 2002d). The major effect of such mutations may be a reduction in preC mRNA synthesis and consequently of HBeAg production, as observed in transfection studies (Buckwold *et al.* 1997e; Moriyama *et al.* 1996b; Scaglioni *et al.* 1997c). Sequences located 5′–end of the BCP, involve the upstream regulatory region that is divided into two regions, a Negative Regulatory Element (NRE) and a Core Upstream Regulatory Sequence (CURS). While the former region has a negative regulatory effect on BCP, the latter, although not a promoter in itself (and does not mediate its activity via EnhII), is known to regulate positively BCP in a liver-specific manner via *cis*-acting elements at positions 1636-1672 (Chen *et al.* 1995e; Crescenzo-Chaigne *et al.* 1995b; Gunther *et al.* 1996d; Ishida *et al.* 2000a; McNair *et al.* 2000a).

The role of CP in HBV has been extensively studied. Without the support of EnhII for added activity, it is described as a weak promoter. CP, made up of EnhII and BCP, has been shown to act preferentially in well-differentiated hepatoma cell lines (Yu & Mertz 1996d; Zheng *et al.* 2004e). EnhII has been described as multifunctional. Whilst maintaining its role as stimulant of the S and X promoters from a downstream location, it also functions as CURS in a position-and-orientation-dependent manner (Gunther *et al.* 1996e; Scafe *et al.* 1990; Xie *et al.* 2001b; Xu *et al.* 2006; Yu & Mertz 1996c). The CP *cis*-acting elements are known to interact with transactivating factors present in various cell types, during liver cell differentiation (Yu & Mertz 1996b; Yuh *et al.* 1992b). This interaction is necessary for the liver specific expression role of the CP (Yu & Mertz 1996a; Yuh & Ting 1990a).

The BCP region is known to be a binding site for a number of transcription factors, including HNF4, HNF1, and C/EBP. Buckwold *et al.* (1996 and 1997) ascribed the alteration of preC mRNA transcription to changes in transcription factor binding. Over-expression of HNF4 has been reported to repress the activity of the CP and to downregulate production of the preC transcript. These investigators (Buckwold *et al.*) implied that mutations in the BCP may alter the position and

orientation of binding sites, which in turn may affect binding of certain liver-specific transcription factors, and create novel sites for other transcription factors, probably accounting for reduction of the preC mRNA. Mutations in the transcription binding sites have been reported (Buckwold *et al.* 1997d; Shaul *et al.* 1985a; Wang *et al.* 1998c), but to date their effect on virus replication has not been fully evaluated.

1.9 Hepatitis B Pathology

Viral hepatitis B leads to liver cell destruction. A typical histological section of a chronically HBV infected liver is characterised by a mononuclear inflammatory cell infiltrate that extends from portal areas to disrupt the limiting plate of hepatocytes, which undergo necrosis. This interface of chronic active hepatitis can clinically become "fulminant", i.e., characterised by extensive necrosis termed "ballooning degeneration" (Demetris *et al.* 1986b; McMahon 2004). The inflammatory cells have been identified as consisting mostly of lymphocytes sometimes forming lymphoid follicles, including plasma cells containing immunoglobulins mostly of IgG type and macrophages containing iron granules (Koziel MJ. 1998). The histopathological pattern of injury is further described as showing lobular disarray, Kupffer cell hypertrophy, lymphohistiocytic lobular inflammation, spotty acidophilic necrosis of hepatocytes, and variable portal inflammation. The presence of inclusion bodies, or "ground glass" cells, can be used to distinguish HBV from hepatitis caused by the Herpes virus group. Ground glass cells are uncommon during the initial presentation (Demetris *et al.* 1986a; Demetris *et al.* 1990), therefore, microorganism identification is usually made with the aid of laboratory studies to detect viral antigens and/or nucleic acids in blood or tissues.

1.10 Hepatitis B Pathogenesis

Detection of HBcAg by immunostaining in hepatocytes is a marker of active viral replication. Core particles formed by copies of the HBc protein are assembled in the cytoplasm of cells. They encapsidate preG RNA and a copy of the polymerase protein. These then bud off the cisternae of the ER where the surface proteins are inserted. It appears that the virus is not cytotoxic, and cytolysis that occurs is apparently produced by cytotoxic lymphocytes sensitive to one or more viral antigens (Dudley *et al.* 1972). In 1981 it was discovered that HBV DNA in hepatocytes may

exist as an integrated (DS, intranuclear) form or in an episomal (free, circular, low-molecular weight, single-stranded, intracytoplasmic) form. Both forms may exist in the same hepatocyte (Rehermann *et al.* 1995c; Shafritz & Hadziyannis 1987). Active viral replication is associated with predominantly episomal viral DNA, while low level replication is associated with integrated viral DNA. Cases of chronic hepatitis of short duration (one-two years) exhibit predominantly episomal (cytoplasmic) viral DNA while cases of longer duration (6–8 years) exhibit integrated, intranuclear viral DNA. Cytotoxic T-lymphocytes sensitive to viral antigens mediate the lysis of infected hepatocytes (Montano *et al.* 1983; Thomas *et al.* 1982b).

1.10.1 HBV persistence

HBV is not cytotoxic, but destroys liver cells indirectly by provoking an immunologic response. Kupffer cells endocytose viral antigens and present them bound to MHC class II molecules, to T-helper cells (Ghavami *et al.* 2005; Zoulim 2004c). These CD4+ cells recognise the antigens and release cytokines that direct B-cell and cytolytic T-cell (CTL) activity. Stimulated B cells produce specific antibodies, including neutralising antibodies (Ellett 1989; Fagan *et al.* 1986; Zoulim 2004b). CTLs recognise viral peptides bound to MHC class I molecules on hepatocyte surfaces, leading to destruction of infected hepatocytes. In persons who fail to mount a sufficiently vigorous immune response to HBV during acute infection, chronic infection develops, and persists, resulting in progressive liver damage and fibrosis (Koziel MJ. 1998; Zoulim 2004a).

1.11 Clinical Course of Hepatitis B

The disease outcome of HBV infection can be summarised by the following highlights of the natural history of infection with HBV: it is determined by the interactions existing between viral replication and host immune response. Broadly speaking, there are four types of host-virus relationship, which account for the various outcomes:

1. The immune response of the host is strong, resulting in elimination of the virus and of the infected liver cells: this, results in acute hepatitis that resolves. The reaction may be superacute and accompanied by massive hepatocellular necrosis, as in fulminant hepatitis.

2. The immune response of the host is weak but sufficient. The infection remains asymptomatic and progresses towards cure. This is more common in young children.
3. The immune response of the host is weak and inadequate. Partial tolerability occurs, associating prolonged replication of HBV (persistent HBsAg) and low-grade destruction of hepatic tissue. This situation of chronic rejection (chronic hepatitis) may last for several months or several years and progress to post-hepatic cirrhosis and/or primary liver cancer.
4. The immune response is nil. This situation is seen in chronic asymptomatic carriers occasionally tolerating massive viral replication.
The persistence of viral replication, whether or not symptomatic, constitutes a danger in terms of viral transmission.

1.11.1 Acute hepatitis B

Acute hepatitis B can range from subclinical disease to fulminant hepatic failure in about 2% of cases. Many acutely infected individuals develop clinically apparent acute hepatitis with loss of appetite, nausea, vomiting, fever and abdominal pain. In a minor number of cases, there may follow an icteric phase, with jaundice and worsening symptoms (Howard & Balfour, Jr. 1975). Occasionally there is a window in time when neither HBsAg nor anti-HBs are detected and anti-HBc is the only detectable marker. This is referred to as the pre-HBs phase of the "window period" (White D.O & Fenner F 1994). There are a number of possible outcomes. Less than 1% of icteric cases die of acute FHB; the majority of patients recover fully with liver cell regeneration within two to three months, but a proportion progress to chronic infection. In cases of fulminant hepatic failure from acute HBV infection, orthotopic liver transplantation can be life-saving. About 90% to 95% of acutely infected adults recover without sequelae. About 5% to 10% of acutely infected adults become chronically infected (Shafritz & Hadziyannis 1987). Diagnosis of acute HBV infection is made either serologically or after testing for HBV DNA. Positive results with these tests are diagnostic of HBV infection, but do not discriminate between acute and chronic infections (McMahon *et al.* 1985a).

1.11.2 Chronic hepatitis B

Patients who do not recover from acute infection progress to chronic infection, defined as longer than six months persistence of HBs antigenaemia. Chronic hepatitis B infection can be asymptomatic, but may cause progressive liver damage and eventually lead to cirrhosis and/or primary hepatocellular carcinoma (HCC) (Chu *et al.* 1999b; Ganem 1982a; McMahon 2004). It can either be "replicative" or "non-replicative". In non-replicative infection, the rate of viral replication in the liver is low and serum HBV DNA concentration generally low; HBeAg is not detectable (the patient may be anti-HBe-positive). In "replicative" infection, the patient usually has a relatively high serum concentration of viral DNA and detectable HBeAg. Patients with chronic hepatitis B and "replicative" infection have a generally worse prognosis and a greater chance of developing cirrhosis and/or HCC than those without HBeAg. In rare strains of HBV, with mutations in the preC gene, "replicative" infection can occur in the absence of detectable serum HBeAg (Chu *et al.* 1999a; McMahon 2004; Tong & Tu 2004; Wands *et al.* 1992; Wolters *et al* 2001b).

1.12 Diagnosis

1.12.1 Serological markers

Diagnosis of HBV infection is generally made on the basis of serology. Virtually all individuals infected with HBV, either acutely or chronically, will have detectable serum HBsAg. In acute infections, HBsAg is detectable several weeks after infection and its appearance coincides with the onset of clinical symptoms (Worman 1997a). The most widely used test for HBsAg detection is Enzyme Linked Immunosorbent Assay (ELISA). This assay is typically quantitative (technical manual, ELISA kit, Abbott Diagnostics) and provides an absorbance reading correlating with the antigen concentration within a specific range. A cut-off value provides the level below which readings are negative. More than 98% of patients with chronic HBV infection test positive in anti-HBV ELISAs. Negative tests are occasionally seen in immunocompromised patients with HBV.

Apart from HBsAg, HBeAg is also detectable in acute infections characterised by high rates of viral replication. At around the same time, IgM antibodies against HBcAg are detectable in serum. Subsequently, IgG antibodies against core are produced. As acute infection resolves, IgG antibodies against core antigen persist and

IgM antibodies and HBsAg become undetectable. Subjects who develop an immune response against HBV develop antibodies against HBsAg (anti-HBs); such antibodies are also produced by vaccination (Fortuin *et al.* 1994a). Most people who have had acute infection that has resolved continue to have IgG antibodies against HBcAg for life. Some, but not all, maintain anti-HBs levels. It is thought, that such "seronegative" patients are still protected from future infection.

1.12.2 Virological markers

Viral genomic sequences can be amplified directly by Polymerase Chain Reaction (PCR) techniques. Because viral load may be predictive of response to treatment and prognosis, it may sometimes be useful to use semi-quantitative PCR to estimate serum viral concentrations. Equally sensitive is the COBAS AMPLICOR™ Analyser. This is a benchtop system PCR that fully automates amplification and detection steps in a single instrument. It combines five instruments into one (thermal cycler, automatic pipettor, incubator, washer and reader). Of growing significance in diagnosis however, is quantitative "Real-time" PCR using a LightCycler, as described in Section 1.18.

Less sensitive assays, such as the Abbott liquid hybridisation or membrane assays, can detect HBV DNA in the serum of approximately half of the patients with HBeAg-negative HBV infection (Zoulim *et al.* 1992). Assays utilising branched-DNA analysis can also be used to quantify HBV DNA in serum (Urdea MS 1993); although not as sensitive as RT-PCR, this is a simple and rapid detection and quantification method that uses branched oligonucleotides consisting of unique HBV specific sequence, which hybridises to the respective target sequence. Multiple copies of small oligonucleotides labelled with alkaline phosphatase then bind the branched oligonucleotide enabling detection.

Sequencing of HBV DNA amplified by PCR can be useful in identifying mutant viruses with clinical significance. For example, (Carman *et al.* 1989a; Ulrich *et al.* 1990a) mutations in the HBV preC region of the genome were identified, these abrogate production of HBeAg but allowed the continued assembly of infectious virus. These preC mutants were associated with severe liver disease in the absence of HBeAg (Carman *et al.* 1989e). A relatively recent report has also shown that clinicians infected with preC mutant strains, without detectable serum HBeAg, can transmit HBV to patients (The Incident Investigation Teams and others 1993). In

addition, to achieve a safe blood supply for transfusion purposes, other rapid tests for viral serological markers and nucleic acid testing have been described (Allain & Lee 2005).

1.13 HBV Prophylaxis and treatment

Inadequate screening of pre-transfused blood still poses some risk of HBV infection in developing countries (Allain *et al.* 2003). In the Western world, where screening is routine, major routes of transmission among adults are intravenous drug use and sexual contact, with the risk of HBV infection being notably high in promiscuous homosexual men. Health-care workers and patients receiving haemodialysis are also at increased risk of infection. Effective vaccines are available for the prevention of HBV infection and all individuals at risk for infection should be vaccinated. Post-exposure prophylaxis with hepatitis B immune globulin is also effective for non-immune individuals after a known exposure (for example, needle stick) (Worman 1997b). Despite all these methods of prophylaxis, infection is probably best prevented by individuals (especially promiscuous homosexual) taking the necessary lifestyle precautions to reduce risk factors associated with HBV transmission. In endemic areas such as The Gambia, where child-to-child horizontal transmission is known to be a mode of transmission, vaccination programmes have achieved remarkable decreases in the incidence of acute and chronic infection, (Fortuin *et al.* 1993; VanDamme *et al.* 1997; Viviani *et al.* 1999; Whittle *et al.* 1991).

There are currently four licensed drugs for the treatment of chronic HBV infection (reviewed, Karayiannis 2004). These are recombinant interferon (INF)-α (normal and pegylated), nucleoside analogues LMV (Epivir) and entecavir as well as the nucleotide analogue, adefovir dipivoxil (Hepsera). The efficacy of these drugs in achieving the main goal of arresting HBV replication, or permanently suppressing it (to at least 1000 copies/ml) varies according to the drug and the group of patients under treatment.

α-interferons were the first drugs approved in the United States for the treatment of chronic hepatitis B. Interferon treatment is recommended for individuals who are HBeAg-positive. About 40% of such individuals will loose serum HBeAg sixteen weeks after the start of treatment with INF-α and few treated patients (less than 10%) may even be cured as assessed by the loss of HBsAg (Westland *et al.* 2003b). Loss of HBeAg is correlated with an improved prognosis.

The nucleoside analogue, LMV, has a clear role in the treatment of patients with HBV-related cirrhosis before and after receiving a liver transplant, and for treating patients with HBeAg-negative viraemia (i.e. patients with a preC mutant virus) against which interferon has little long-term efficacy (Karabay *et al.* 2005). The main problem associated with LMV therapy however, is the development of resistance due to the YMDD mutations (positions 180 and 204 of RT/P) which after three years of therapy, occurs in half of all treated patients (Wolters *et al.* 2001c; Wright 2004; Yeh *et al.* 2000). It was initially thought wiser to keep patients with the YMDD mutants on therapy as they replicate less efficiently than the wildtype virus. Subsequent studies have, however, indicated that incomplete suppression of HBV replication reduces the degree of benefit. Drug resistant viruses favours the selection of increasingly fit and equally pathogenic viruses by viral adaptation, and given the complex immune response to hepatitis B, such mutants may indeed be more pathogenic over time (Bock *et al.* 2002a; Dienstag *et al.* 2003a; Dienstag *et al.* 2003c; Wiegand *et al.* 2004). Recent findings from a study using large cohorts of Asian patients on LMV monotherapy for up to five years support this conclusion, and show continuing liver damage in the presence of the YMDD mutant viruses (Dusheiko 1999). LMV in combination with interferon has not proved more effective than either treatment alone. There is therefore an obvious need for other antiviral agents.

In September 2002, the United States Food and Drug Administration (FDA) approved adevofir dipivoxil, also effective against HIV, for the treatment of hepatitis B. The drug has been shown in clinical trials to inhibit viral replication effectively and to overcome LMV resistance. In patients that were treated with the drug, serum HBV DNA was found to have been decreased significantly (Westland *et al.* 2003a).

Loss of HBeAg and associated suppression of viral replication have also been reported with other nucleotide analogues, such as tenofovir, telbuvidine, and emtricitabine. These, undergoing trials at the moment, lead to biochemical remission, histological improvement, and, in a small percentage of individuals, loss of HBsAg (Dusheiko GM 2003; Keeffe *et al.* 2004; Lok *et al.* 2001).

1.14 Experimental Background to Work Leading to This Thesis

The last twenty-five years or so have witnessed a revolution in our understanding of cell and infectious diseases. This explosion of knowledge has been credited to the many technical and experimental advances that are now central to modern molecular biology, a number of which were employed in this investigation. The principles underlying these techniques are described below.

1.14.1 Site-directed mutagenesis

In vitro Site-directed mutagenesis (SDM) is an invaluable technique for studying protein-structure function relationships, and gene expression and control. Many of the used methods require single-stranded (SS) DNA as a template for achieving high levels of efficient mutagenesis (Kunkel *et al.* 1987). The reason for this, historically, has been the need for separating the complementary strands of DNA to prevent re-annealing. Use of PCR in SDM (mainly on low molecular weight DNA) accomplishes strand separation by using a denaturing step to separate the complementing strands and allowing efficient polymerisation of PCR primers. PCR site-directed methods thus allow site-specific mutations to be incorporated in virtually any double-stranded (DS) plasmid, eliminating the need for SS requiring vectors or SS rescue.

Normally, PCR-based approaches require the synthesis of two complementary oligonucleotides that contain the desired mutation(s), and their use to prime a plasmid DNA template. Subsequent digestion of the reaction mixture with a restriction enzyme such as *Dpn*I removes the template DNA, leaving intact the newly synthesised DS mutant PCR product, which can then be used to transform competent Escherichia coli (*E. coli*). This method allows for high-efficiency mutagenesis in a fairly short period of time (Li & Wilkinson 1997). *Dpn*I restriction endonuclease has a recognition target sequence, 5′–Gm6ATC–3′, where the *A* residue is methylated, allowing newly synthesised to be selected from parental DNA. DNA isolated from almost all common strains of *E. coli* is Dam-methylated at the sequence 5′–GATC–3′.

For PCR-based SDM, it is often desirable to reduce the number of copying cycles to prevent clonal expansion of any (undesired) second-site mutations. Limited cycling, which would result in reduced product yield can be offset by increasing the starting template concentration. Secondly, selection may be required to reduce the number of parental molecules coming through the reaction. And thirdly, because of

the "extendase" activity of some thermostable polymerases, it is often necessary to incorporate an "end-polishing" step into the procedure to remove one or more extraneous nucleotides prior to end-to-end ligation of a PCR-generated product containing incorporated mutations into vectors for subsequent studies.

A standard method for SDM (Stratagene), accomplishes the above desired features by incorporation of the following steps: (i) increasing template concentration approximately 1000–fold over conventional PCR conditions; (ii) reducing the number of cycles from 25–30 to 15–18; (iii) adding *DpnI*; (iv) using *Taq* Extender in the PCR mix for increased reliability for PCR on up to 10 kb substrates; (v) using *Pfu* DNA polymerase to polish the ends of the PCR product, and (vi) efficient intramolecular ligation in the presence of T4 DNA ligase (in the case of the multi-site kit). [Details of the specific mutagenesis kit used to introduce specific mutations are given in Section 2.2.8].

1.14.2 Transcript-specific PCR

Transcription of RNA is an important event in the HBV replication process. Under the direction of the HBV CP, the synthesis of the two overlapping 3.5kb viral transcripts which only differ at their 5′ initiation sites (Will *et al.* 1987a; Yaginuma *et al.* 1987) are produced. A major obstacle in analysing and monitoring the synthesis of RNA transcripts from the HBV CP *in vivo* and *in vitro* has been the lack of a sensitive technique that would permit the differential detection of preC mRNA and preG RNA transcripts, especially when using the small amount of total RNA extracted from minute biopsy samples.

A rapid differentiation of the 3.5kb-related transcripts with distinct, but known or predicted 3′–ends, was previously achieved via RT (reverse transcriptase)-PCR (Edwalds-Gilbert *et al.* 1997; Schutz *et al.* 2000; Wahle & Kuhn 1997). Laras *et al.* (2002) extended this method to quantify HBV RNA transcripts. They utilised the unique structure of the HBV genome and transcripts, and developed a Transcript-specific RT-PCR assay that allows for the specific detection of preC mRNA molecules in as little as 330ng of total liver RNA. This technique simultaneously monitors the level of total HBV RNA transcription (preC mRNA plus preG RNA). Using specifically synthesised primers that differentially recognise the 5′–ends of *in vitro* synthesised transcripts, they increased sensitivity to allow them to detect preC mRNA with preC 5′–end binding primer in 10^3 target copies; synthetic preG RNA

transcripts were undetectable over the various concentrations tested (10^2–10^5). A preG 5′–end binding primer, on the other hand, detected both transcripts with the same sensitivity. This technique is adaptable to the study of the effect of mutations on the synthesis of preC mRNA and/or preG RNA in the liver of patients with chronic HBV infection, and also for *in vitro* studies.

1.14.3 Gene delivery into cells (transfection)

Under appropriate conditions, eukaryotic cells can accept exogenous DNA, and a limited portion of this DNA localises in the nucleus. The phenomenon has reportedly been exploited to obtain both transient and stable expression of various genes (Felgner *et al.* 1987a). However, due in part to the size and charge of DNA and to the multitude of enzymic and membrane barriers imposed by the cell, the spontaneous entry of intact DNA into the cell and its subsequent expression in the nucleus is a very inefficient process. For this reason, a wide variety of methods have been developed to facilitate this process.

At present, viruses are still the most efficient gene transfer tools available but, due to their inherent limitations, the development of more flexible and adaptable systems has become an attractive goal. The transfer of genetic material using non-viral systems preceded the development of viral-based vectors. Non-viral gene transfer can in fact be traced back to the early work of Avery and others who showed that genes were transferred into bacteria by nucleic acids (Avery *et al.* 1944). Several non-viral gene transfer methods (protoplasts, polycations, calcium-phosphate, liposome fusion, microinjection and electroporation) were reportedly employed in the 1960s. However, all of these methods suffer from one or more problems related to either cellular toxicity, poor reproducibility, inconvenience, or inefficiency of DNA delivery. Only in 1987 was the potential of cationic lipids as an efficient means to transfer genes into cells realised (Felgner *et al.* 1987b).

Felgner *et al.* (1987) synthesised a cationic lipid that forms liposomes that can interact spontaneously with DNA, fuse with cells, and facilitate the delivery of functional DNA into them. The technique is simple, highly reproducible, and more efficient than some other, commonly used, procedures. Since then, mounting evidence has accumulated in support of their effectiveness in transferring nucleic acids into a variety of cell types (Bebok *et al.* 1996a; Friend *et al.* 1996; Gao & Huang 1995; Jarnagin *et al.* 1992; Watanabe *et al.* 1994; Weber *et al.* 1995). All cell

populations appear transfectable, although lipid requirements varied for cell types and, for cultured human hepatocytes, with the age of the culture. Using a reporter gene to monitor transfection efficiency, Jarnagin *et al.* (1992) found transfection efficiency at 48hrs to be as low as 10% with a doubling percent efficiency seen only seven days following transfection. Watanabe *et al.* (1994) recorded a maximum transfection efficiency of 60% between 6 and 48hrs post transfection. In non-hepatocyte cells, >95% of transfection efficiency was reportedly observed in a cystic fibrosis respiratory epithelial cell line (2CFSME0-1), within the first hour of transfection (Bebok *et al.* 1996b). Other studies have also shown variations in transfection efficiencies. However, methods of determining transfection efficiencies have varied. And, in general, targeting the nucleus (stable transfection) remains a low efficient process. (In a later section, the methods used in this study for determining transfection are described).

1.14.3.a. Cell lines

Before the 1980s, very little was known about the HBV genome or its full life cycle. This was largely due to the lack of an *in vitro* tissue culture system for HBV propagation. In the quest of a solution for this situation, several mammalian cell lines were used to transfect cloned HBV DNA (Dubois *et al.* 1980; Moriarty *et al.* 1981; Siddiqui 1983; Wang *et al.* 1983; Will *et al.* 1984). However, although these experiments generated sizable amounts of HBsAg and HBeAg, none was capable of producing neither DNA replicative intermediates — similar to those identified in the livers of animals infected with other hepadnaviruses (Enders *et al.* 1985; Glebe *et al.* 2001; Moroy *et al.* 1985; Summers & Mason 1982b) — nor *Dane*-like viral particles. A breakthrough experiment came in 1987 when an *in vitro* system was successfully set up to study the HBV life cycle and the reaction of immunocompetent cells with cells carrying HBV (Sells *et al.* 1987b). Since then, although other similar cell lines containing stably integrated HBV genomes have been described (Chang *et al.* 1987b; Tsurimoto *et al.* 1987a; Weiss *et al.* 1996), the HepG 2.2.15 cell line described by Sells *et al.* (1987) appears to be the more widely used one. Derived from the HepG2 human hepatoma cell line, and transfected with a replication-competent construct containing two head-to-tail dimers of HBV in a tail-to-tail orientation, it maintains a substantial number of liver-specific functions and supports many phases of HBV

replication. The system has also served as an *in vitro* model system for analysis of the immune response against disease and recovery from disease (Muller *et al.* 1983).

Although the 2.2.15 cell line contains multiple copies of integrated HBV DNA, which unlike retroviruses integrate viral DNA into the host genome, hepadnavirus genomes are not normally integrated but, instead, are maintained in the nucleus of infected cells *in vivo* as a pool of episomal cccDNA molecules (Tuttleman *et al.* 1986c). Despite the fact that the integration of HBV DNA in human hepatocytes can occur (Brechot *et al.* 1980), it is not an obligatory part of the HBV lifecycle. HepG2 cells can thus, and have been, widely used in transient transfection studies (Delaney & Isom 1998e; Delaney *et al.* 1999b; Doitsh & Shaul 2003; Li *et al.* 1999b; Mimms 1995a).

Other human cell lines of different origins have also been used to transfect infectious HBV DNA for the purpose of comparing HBV replication in several cell lines, including Huh7, another hepatoma cell line (Tsurimoto *et al.* 1987b), 293T cells of kidney origin (Lambert & Prange 2004), and murine fibroblasts, for example LTK^- cells (Seifer *et al.* 1990). Cells of osteosarcoma origin, for example, $143\text{-}TK^-$ have also been used in transfection studies (Li *et al.* 1993c).

1.14.3.b. Monitoring of transfection efficiency *in vitro*

Transfection efficiencies can vary with biological entities, cell lines, cell density, cell passage number, or with changes in transfection reagent and general transfection conditions (Pollard *et al.* 1998b). The many possible combinations of cell types and transfection conditions can result in unpredictable cellular responses to transfection. Transfection reagents alone have been shown to produce inconsistent results based on cell passage number, reagent shelf life, or to have synthetic batch-to-batch variation (Bonetta 2004). To control for this variability, determination of transfection efficiency for every experiment is recommended. This becomes a requirement in studies where the results of transfections of different constructs are being compared with each other, and/or to a wildtype entity.

Various methods of determining transfection efficiency in cells have therefore been used. Some of these methods are "direct", others are "indirect". Similarly, some of the techniques are more versatile than others. One method involves the use of reporter genes where transfection efficiency is usually determined by co-transfection of the desired substrate with a reference gene under the control of a constitutively

strong active promoter. Determination of the reference enzyme activity, e.g., for reporter genes, such as *firefly* luciferase or beta-galactosidase, generally linked to a viral promoter, is generally assumed to represent the transfection efficiency in the experiment. The direct determination of an introduced plasmid concentration in lysates of transfected cells is suitable for monitoring the transfection efficiency in reporter gene assays even if different cell lines are compared. This method has been simultaneously used to study the promoter and/or enhancer activities of genes (Park 2001; Wu & Wiltbank 1999). (This protocol will be elaborated on in a later section). Another way of using a reporter gene to determine transfection efficiency in *in vitro* studies involves co-transfecting with a second plasmid that expresses a fluorescence protein, such as the widely used Green Fluorescence Protein (GFP) (Buhlmann *et al.* 2003; Gouskos *et al.* 2004a; Moradpour *et al.* 2004). However, determination of the number of cells that express this protein, and hence transfection efficiency, is an indirect method since the transfection of the fluorescent plasmid into a certain number of cells does not necessarily reflect transfection of the test construct into the same number of cells, even when restricting transfection conditions to a mole:mole relationship. It is important to calculate and standardise all transfection efficiencies to produce unbias results. When fluorescent reporter constructs are used for transfection efficiency determination, percent transfection efficiency is either determined by fluorescence-activated cell sorting (FACS) or by fluorescent microscopy. However, the latter has a major disadvantage in that, obtaining statistically relevant results by manual counting is tedious and time consuming. On the other hand, cell-autofluorescence can interfere with accurate FACS analysis. FACS analysis, followed up by visual confirmation of transfection efficiency using conventional fluorescent microscopy, provides a relatively adequate "two-in-one" methodology for determining transfection efficiency in cells.

FACS Analysis

FACS allows the counting and analysis of physical and molecular attributes of cells. One of the most widely used applications in the analysis of cell populations that expresses a particular type of marker on a single cell level, involves the use of green-fluorescing cells that are tagged with a GFP-expressing plasmid. The cell sorter, an instrument with sophisticated optics, lasers and electronic processors, automates the task of identifying and quantitatively analysing individual cells, and of separating and

rapidly sorting closely related cell populations. By measuring the physical and chemical properties of cells, such as fluorescence, then by physically separating cells while still alive, the cell sorter has become an important tool for biomedical research and clinical medicine.

1.14.4 Imunocytochemistry:

Cell antigen localisation

Immunocytochemistry is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions, the site of antibody binding being identified either by direct labelling of the antibody, or by use of a secondary labelling method. As *in situ* hybridisation, a related technique allows for the demonstration of specific nucleic acid sequences (genes) in their cellular environment. Using these techniques, the relationship between the presence of HBV antigens, their localisation, and HBV replication has been described (Dienstag *et al.* 2003b; Jolivet-Reynaud *et al.* 2001a; Roingeard *et al.* 1990; Glebe & Gerlich 2004). In such studies, either HBcAg or HBsAg was identified by staining, following transfection of cloned HBV DNA into hepatoma cells. Intracellular HBV antigen expression was detected by immunofluorescence.

This ability to detect HBV antigen in cells using antibodies has been exploited for research and clinical purposes (Muller *et al.* 1983). Although immunocytochemistry has been more extensively used to study intracellular localisation of HBV antigens and/or their structures, when used with FACS and fluorescence microscopy, it can also be used as a marker for transfection in *in vitro* studies (Schaefer & Gerlich 1995).

Using monoclonal and polyclonal primary antibodies, Roingeard *et al.* (1990) and Aiba *et al.* (1997) reported two clear-cut types of HBcAg staining in transfected HepG2 and 143-TK$^-$ cells, and identified antigen localisation as dependent on the sequence of the transfected C gene, rather than cell type used: whereas transfection with wildtype C genes into cells showed strong nuclear staining, staining for the antigen was more pronounced in the cytoplasm using C gene mutants.

Diagnosis

Apart from its use in cellular localisation, monoclonal antibodies have been used to diagnose diseases. Although several assays for the quantitative measurement of HBV DNA have been developed, some of which have earlier been described, for clinical diagnosis, these methods tend to generate highly divergent results (Pawlotsky *et al.* 1997; Pawlotsky *et al.* 2000; Quint *et al.* 1995). Further, they require cumbersome procedures and expensive equipment, which in turn demands considerable operator skill and results in high costs. For such needs, therefore, the easy and inexpensive ELISA is a favourite. The technique combines the specificity of antibodies with the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme with a high turnover number. ELISAs can provide a useful measurement of antigen or antibody concentration. One of the most useful of the immunoassays is the two antibody "sandwich" ELISA. This assay is used to determine the antigen concentration in unknown samples. The assay is fast and accurate, and if a purified antigen standard is available, it can determine the absolute amount of antigen in an unknown sample. The sandwich ELISA requires two antibodies that bind to epitopes that do not overlap on the antigen. This can be accomplished using either two monoclonal antibodies that recognise mutually exclusive discrete sites, or a batch of affinity-purified polyclonal antibodies.

1.14.5 Real-time PCR:

A simultaneous qualitative and quantitative technique

Gene expression analysis plays an increasingly important role in many fields of biological research and the use of PCR to achieve this task has increased to the point where it is now accepted as the "gold standard" for detecting nucleic acids from a number of sources. It has become an essential tool in research and diagnostic laboratories. The recently developed Real-time PCR quantification method has gained wide acceptance due to its many advantages over the conventional quantifications in terms of accuracy, sensitivity, dynamic range, high-throughput capacity, and lack of requirements for post-PCR manipulations (Heid *et al.* 1996; Higuchi *et al.* 1993).

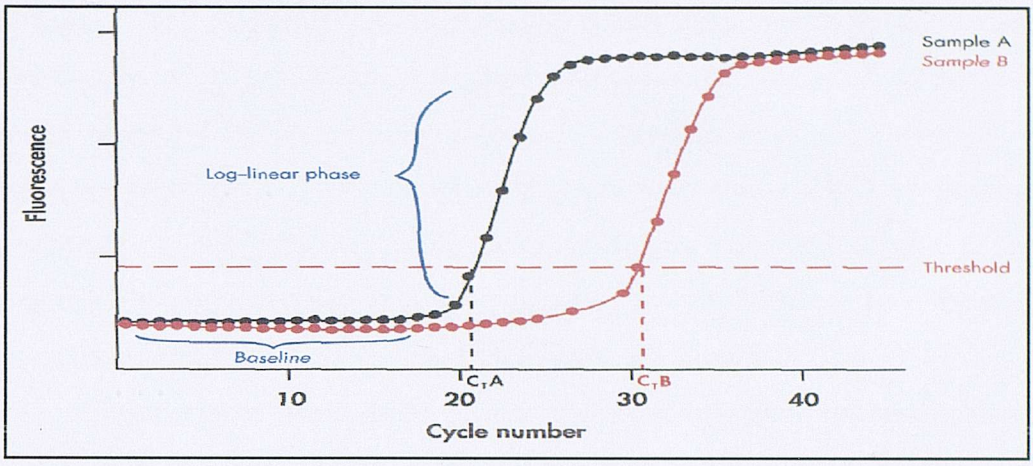
There are currently five main chemistry protocols used for the detection of PCR products during Real-time PCR. Of these, sequence-specific fluorescence-labelled probes (for example, TaqMan) have been adopted as a standard detection format for

many applications (Bustin 2000c; Lie & Petropoulos 1998). They are disadvantageous in that they are not well suited for quantification of a large number of different sequences since a new, and relatively expensive, probe may be required for each amplicon under investigation.

Elsewhere, optimised and validated self-fluorescing amplicon assays have been adopted, using the DS DNA-binding dye, SYBR® green. This provides a much more economical alternative for quantifying any given DNA or transcript in a reaction (Lekanne Deprez *et al.* 2002; Vandesompele *et al.* 2002a; Wang *et al.* 2002; Al-Robaiy *et al.* 2001b; Al-Robaiy *et al.* 2001a). Using the SYBR® Green assay, product denaturation, annealing and extension can be effected within each cycle. Substantial product-to-product annealing, however, occurs during later amplification cycles; it can become a major hazard, producing a "plateau" effect (Figure 1.8, I) (Wittwer *et al.* 1997a). Continuous within-cycle monitoring, however, allows rapid optimisation of amplification conditions and should be particularly useful in developing new, standardised clinical assays. Using such a generic dye, Vendesomepele *et al.* (2002), and other investigators (Ririe *et al.* 1997), have accurately differentiated between target amplicons from non-specific, and cross-priming amplification products, by the generation of so-called DNA melting-curves and first-derivative melting-peaks (Figure 1.8, II). With the inclusion of a serially diluted DNA of known concentrations to generate an external standard curve, these investigators and others have demonstrated the technique as specific and sensitive in accurately detecting and quantifying genes of interest with unknown starting concentrations.

Since its inception and optimisation, other studies have extended the system's versatility beyond detection and quantification of genes (Abe *et al.* 1999; Addison *et al.* 2000; Chen *et al.* 2001; Jun-Bin *et al.* 2003; Paraskevis *et al.* 2002; Sum *et al.* 2004; Wong *et al.* 2004), to distinguishing between mutant and wildtype sequences (Aleem *et al.* 2001; Loeb *et al.* 2000; Whalley *et al.* 2001; Wittwer *et al.* 1997b; Zhang *et al.* 2002). A limiting factor in using this technique lies in the initial selection of primer-pairs. For example, Tichopad *et al.* (2002) have shown variability in performance of the technique when primer sets of varying lengths were used to amplify the same gene.

(I)



(II)

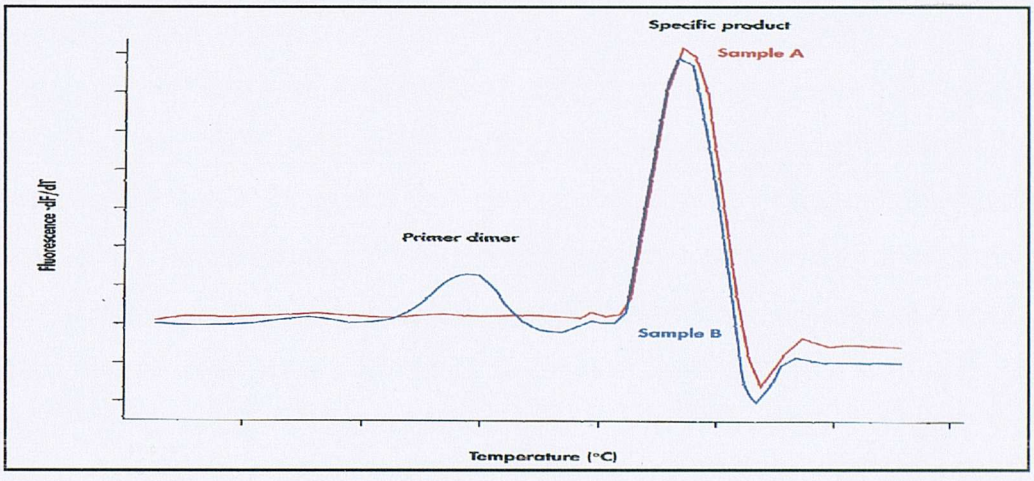


Figure 1.8 A typical Amplification Plot (I) and Melting Curve Analysis of PCR products (II).
(I). Shows increases in fluorescence from two samples, A and B. Sample A contains a higher amount of starting template than sample B. The first detectable increase in fluorescence (C_T) is indicated for each of the two samples. **(II).** Indicate the melting curve analysis of the products resulting from the amplification.
The Figures have been re-produced from technical bulletin of the QuantiTect SYBR® Green PCR Kit.

The use of housekeeping genes in gene quantification

In spite of problems, quantitative Real-time PCR has established itself as the method of choice for rapid and reliable quantification of mRNA transcription, its problems being offset by the benefits of highly accurate "Real-time" quantification. To overcome major problems related to exact gene transcription analysis, amplification of the mRNA of a second "housekeeping" gene (HKG) can be used as a reference, for calibrating measurements of gene expression (Eisenberg & Levanon

2003a). Ideally the HKG should not be regulated or influenced by the experimental procedure. The accurate quantification of a true reference gene allows for the normalisation of differences in the amount of amplifiable RNA or cDNA in individual samples generated by: (i) different amounts of starting material, (ii) the quality of the starting material; and (iii) differences in RNA preparation and cDNA synthesis, since the reference gene is exposed to the same preparation steps as the gene of interest. For gene transcription studies in different tissues, the investigation of a reference gene exhibiting constant RNA transcription in all tissues is important.

Housekeeping Genes have been categorised into four different groups: (i) structure-related genes such as beta (β)-actin, and Tubulin; (ii) metabolism-related genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and glucose-6-phosphate dehydrogenase (G6PDH) (iii) transcription-related genes such as TATA-binding protein (TBP) and Reverse Polymerase II (RPII), and (iv) genes which cannot clearly be categorised into one of these groupings, including albumin (Alb), and Peptidylprolyl isomerase A (PPIA) (Radonic *et al.* 2004b). Although these genes are constitutively expressed in most tissues, studies have shown that the expression of some of them varies under certain experimental conditions which may lead to a serious distortion of results (Eisenberg & Levanon 2003b; Thellin *et al.* 1999). The Radonic *et al.* (2004) study showed that RPII is constitutively expressed in most tissues, including liver. It encodes a main enzyme of mRNA transcription; its transcript is part of a self-regulating cycle and its protein is involved in the mRNA synthesis of several cellular mRNAs. The limiting factor to RPII is the fact that it is one of the lowest of the expressed HKGs and it therefore can be difficult to amplify (Radonic *et al.* 2004a). In the Radonic *et al.* (2004) study, β-actin was found to be expressed in relatively high concentrations across many tissues. Other studies have shown that genes such as β-actin (Glare *et al.* 2002), GAPDH (Deindl *et al.* 2002; Hamalainen *et al.* 2001a; Zhong & Simons 1999) and 18S RNA (Raaijmakers *et al.* 2002; Solanas *et al.* 2001) may be stimulated under certain mitogenic conditions in various cell types. In conclusion, these studies have provided enough evidence to previous suggestions that control genes in Real-time RT-PCR experiments analysing transcript abundances have to be evaluated for each individual cell type. Therefore, careful choice of the HKG to fit a given situation is essential for quantitative RT-PCR analysis.

1.14.6 Analysis of promoter function

As already described, gene expression is controlled at several steps, including transcription. Because the promoter region of a gene provides the key to transcriptional regulation, techniques that monitor regulation of promoters, particularly if they encode transcription factors, can be valuable. Different methods that fulfil this task include Electrophoretic Mobility Shift Assays (EMSA), and/or the use of reporter systems.

1.14.6.a. Electrophoretic mobility shift assay

The EMSA technique is based on the observation that protein:DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide gel electrophoresis (Ceglarek & Revzin 1989; Revzin 1989). Because the rate of DNA migration is shifted or retarded by protein binding, the assay is also referred to as a gel shift or gel retardation assay. Until the development of the EMSA (Fried & Crothers 1984a; Garner & Revzin 1981), protein:DNA interactions were studied primarily by nitrocellulose filter-binding assays (Stockley 1994). An advantage of studying DNA:protein interactions electrophoretically is its ability to resolve complexes of different stoichiometry or conformation. Another major advantage for many applications is the fact that the source of the DNA-binding proteins may be a crude nuclear or whole cell extract rather than a purified preparation. Gel shift assays have been used qualitatively to identify HBV transcription factors in crude lysates and, in conjunction with mutagenesis, to identify important binding sequences within promoter region of genes (Buckwold *et al.* 1997c; Gunther *et al.* 1998f; Liu *et al.* 1994a; Lo & Ting 1994; McNair *et al.* 2000b; Spandau & Lee 1992a; Xie *et al.* 2001a; Xu *et al.* 2004a; Yu & Mertz 1997c).

The ability to resolve protein:DNA complexes depends largely upon the stability of the complex during the brief time (approximately 1min) it is migrating into a gel. Sequence-specific interactions are transient and are stabilised by the relatively low ionic strength of an electrophoresis buffer which can be important to stabilising such interactions. Upon gel entry, protein complexes are quickly resolved from free DNA, in effect, freezing the equilibrium between bound and free DNA. In a gel, the complex may be stabilised by "caging" effects of the gel matrix, meaning that if the complex dissociates, its localised concentration remains high, promoting

prompt re-association (Fried & Crothers 1981; Fried & Crothers 1984b). Therefore, even unstable complexes can often be resolved by this method.

Parameters critical to EMSA reactions have been determined, and include the length of target DNA, the labeling and detection processes, and the results obtained with a nonspecific competitor. Typically, short (20-50bp) linear DNA fragments containing the binding sequence(s) of interest are used in EMSAs. If circular DNA is used in the reaction, the protein:DNA complex may actually migrate faster than the free DNA. Traditionally, DNA is 5′–radiolabeled with ^{32}P using [$\gamma^{32}P$]ATP and T4 polynucleotide kinase. Nonspecific competitor DNA, such as poly(dI•dC) or poly(dA•dT), can be included in the reaction to minimise the binding of nonspecific proteins to the labelled target DNA. These repetitive polymers provide an excess of nonspecific sites to absorb proteins in crude lysates that will bind to non-specific DNA sequences.

While there are characteristic shifts caused by the binding of specific protein(s) to the target DNA, the change in relative mobility does not identify the bound protein. Identification of the DNA-bound protein may be accomplished by including an antibody, specific for the DNA-binding protein in question, to the binding reaction. If the protein of interest binds to the target DNA, the antibody may bind to that protein:DNA complex, further decreasing its mobility relative to unbound DNA. This is called a "supershift" assay. In some cases, however, the antibody may disrupt the protein:DNA interaction and result in loss of the characteristic shift, without producing a supershift. However, supported by the proper controls, even this "negative" result can support the identification of the protein of interest, as shown elsewhere (Viollet *et al.* 1997a).

1.14.6.b. Reporter vectors: role in the analysis of promoter sites

The most common method of analysis of promoter regulation involves the use of promoter-reporter fusions, wherein a viral promoter and/or enhancer is fused to a reporter gene so that by assaying the reporter gene product, one can study the promoter activity. The basic requirements for the assay are: an easily quantified reporter protein that does not affect any physiologic process inside cells, an efficient transfection method, and a suitable cell line. Reporter assays can be carried out both *in vitro* and *in vivo*. *In-vitro* assays refer to procedures in which the reporter protein is quantified using the cell lysate or the cultured medium for secreted reporter proteins.

Several kinds of reporter vectors have been developed to study the promoter and/or enhancer activities of genes. These include a luciferase vector (deWet *et al.* 1987), which has been most favoured due to the fact that it is a rapid, sensitive, and reproducible assay (Wood 1991) . It has been used in HBV promoter analysis studies (Cai *et al.* 2003; Nishizono *et al.* 1995). In the luciferase assay, *luciferin* and other components are added to cell extracts, and the production of light from extracts expressing the luciferase gene is measured conveniently by a luminometer or scintillation counter (Seliger & McElroy 1960). Some of the disadvantages are that luciferase lacks "reproducibility" between samples due to rapid emission of light. The half-life of luciferase is very short, and the assay requires a luminometer or scintillation counter, which can also be a limiting factor.

1.15 Hypothesis and Aims of investigation

1.15.1 Hypothesis

In view of previous findings indicating that HBV isolates bearing the CP and preC mutations are associated with fulminant disease, we have hypothesised that such isolates may have had the capacity to replicate more efficiently than wildtype virus.

HBV is not known to be directly cytopathic to cells (Ando *et al.* 1994a; Ando *et al.* 1994b; Chisari 1996; Edgington & Chisari 1975; Guidotti *et al.* 1994). This fact agrees well with the observation that many HBV carriers are asymptomatic and have minimal liver injury, despite extensive and ongoing intrahepatic replication of the virus (de Franchis *et al.* 1993). It is now thought that host immune responses to viral antigens displayed on infected hepatocytes are the principal determinants of hepatocellular injury (Franco *et al.* 1992b; Franco *et al.* 1997; Nayersina *et al.* 1993). This notion is consistent with the clinical observation that patients with immune system defects who are infected with HBV often have mild liver injury but high rates of chronic carriage (Stevens *et al.* 1975). Therefore, although the immune may be a contributory factor in liver injury during fulminant disease, the experiments reported here were solely concerned with the role that the virus plays in this outcome.

Previous studies comparing the effect of mutations on viral replication have used various isolates from different patients and geographical regions, and variant reporter systems. To avoid heterogeneity in viral sequences, this study concentrated on the introduction of mutations using a common HBV genetic background.

Therefore, any differences recorded in the replication efficiency of such constructs would be due to the mutations *per se*.

1.15.2 Aims

The overall aim in this work was to investigate the effects of mutations in HBV genes on their biological and pathological properties. This was achieved by:

➢ Developing and optimising an SDM approach for the construction of recombinant clones bearing the mutations under study, and then,

➢ Introducing the following mutations into such constructs:

- CP mutations (*e.g.*, A1762G/G1764T),

- preC mutations (*e.g.*, G1896A)
- Combined CP/preC mutations (*e.g.*, A1762G/G1764T/G1896A)
- "a" determinant/S gene mutations (*e.g.*, L141E)
- RT/P gene mutations (*e.g.*, M204V)
- Combined "a" determinant/RT gene mutations (*e.g.*, G145R/M204V)

➢ To assess their replication capacity in hepatoma cell lines *e.g.* HepG2 and Huh7, by detecting and measuring relative levels of the following parameters, compared to wildtype sequences:

- Virion release
- Intracellular HBV DNA:
  - cccDNA, and RC-DS DNA
- RNA transcripts:
  - preC mRNA and preG RNA
- Viral proteins:
  - HBsAg (quantitative)
  - HBeAg (qualitative)

➢ To evaluate the effect of mutations in the S gene on the replication competence of the virus, and possible effects on the functioning of the overlapping P gene.

➢ To study the effect of the CP mutations in the regulatory elements on viral replication and the binding of transcription factors, and document this by promoter activity analysis using a reporter gene.

Chapter 2

Materials & Methods

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffers and solutions

Reagents used for the preparation of buffers and solutions were purchased from MERCK (UK) or Sigma (UK), except where stated. All solutions were prepared with Milli Q deionised water, unless otherwise stated. For pH calibration, a 211 Microprocessor pH Meter (Hanna Instruments, RI, U.S.A) was used.

Glycogen Stock	20mg/ml (Roche Diagnostics, Sussex, UK)
EDTA solution	0.5M EDTA in Milli Q water [pH 8.0].
Ethidium bromide stock	10mg/ml
Milli Q water	Purified and de-ionized water prepared using an Elga Option 3 water purifier and Milli Q Organex-Q system
Miniprep solution I	25 mM Tris-HCl pH 8.0, 50mM glucose, 10 mM EDTA, autoclaved prior to use and stored at 4°C
Miniprep solution II	0.2 M NaOH, 1% SDS, prepared fresh immediately prior to use
Nuclease free water	HiPersolve water from VWR (Poole, UK).
Phosphate buffered saline (PBS)	137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4 \cdot 7H_2O$, 1.4mM KH_2PO_4. (pH 7.1), or prepared by dissolving 1 PBS tablet per 200ml of water. Autoclaved to sterilise.
PCR dNTPs (10X stock)	10mM each of dATP, dTTP, dCTP, dGTP (Promega, UK), stored at -20°C
Phenol	Purchased ready saturated with water from Appligene and stored at +4°C
Phenol: chloroform	Phenol:Chloroform:Isoamyl alcohol 25:24:1, pH 4.7
Sodium Acetate	3M Sodium Acetate, pH 5.2, autoclaved to sterilise

TBE Buffer (10x)	0.9M Tris base, 0.9M Boric acid, 25mM EDTA

2.1.2 E. *coli* strains

One Shot® TOP10 Chemically Competent *E. coli*	Invitrogen, Paisley, UK
MAX Efficiency® DH5α ™ Competent *E. coli*	Invitrogen

2.1.3 *E. coli* growth media

Agar	Bacto-agar added to 2% (W/V) Luria Bertani (LB) broth
Luria broth (LB)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, autoclaved to sterilise
SOC broth	Invitrogen, stored at -20°C

2.1.4 Cell culture media

Dulbecco-modified Eagle's medium (DMEM), supplied by Invitrogen, was supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, penicillin (100 International Units/ml) and streptomycin (100 μg/ml). Lipofectin® and opti-MEM® (Invitrogen) were used for transfections.

2.1.5 Human cell lines

Cell lines used in this study were of human origin and provided by Dr. Jenny Waters, Department of Medicine A, Imperial College, London. They were:
Huh7, HepG2, hepatocellular carcinoma, (Nakabayashi *et al.* 1982),
293T, kidney carcinoma, (Yang *et al.* 2003), and
143-Thymidine kinase-negative (TK^-) osteosarcoma, (Aiba *et al.* 1997).

2.1.6 Plasmids

p3.8II and m8 (Wu *et al.* 2001)	Provided by Professor Yuan Wang, Institute of Biochemistry, Academia Sinica, Shanghai.
pCI-GFP (Moore *et al.* 2005)	Provided by Dr. M.D. Moore, Department of Infectious Diseases, Imperial College, London.

2.1.7 Vectors

pBluescript® II KS (+)	Stratagene (Cambridge, UK)
pGEM®-T-Easy	Promega (Southampton, UK)
pGL2®: Basic and Control	Provided by Dr. R. Watson, Department of Virology, Imperial College, London

2.1.8 Oligonucleotides

All oligonucleotides were purchased from Sigma-Genosys. Oligonucleotides used in these studies are classified according to purpose and shown in Table 2.1. Some primers used in assays are not specified with regard to specific use, for example, the Real-time/Transcript-specific oligonucleotides also served in the RT-PCR reactions. Also, some of those used in the SDM PCRs were also used in EMSA experiments (Results, Chapter 8).

Polarity	Name	Sequence	Tm°C
Oligonucleotides Utilised in Site-directed mutagenesis PCR			
+ −	A1762T--S A1762T--AS	5'-GAGGAGATTAGGTTAATGGTCTTTGTACTAGG-3' 5'-CCTAGTACAAAGACCATTAACCTAATCTCCTC-3'	66
+ −	G1764A--S G1764A--AS	5'-GAGGAGATTAGGTTAAAGATCTTTGTACTAGG-3' 5'-CCTAGTACAAAGATCTTTAACCTAATCTCCTC-3'	64 67
+ −	A1762T/ G1764A --S A1762T/ G1764A --AS	5'-GAGGAGATTAGGTTAATGATCTTTGTACTAGG-3' 5'-CCTAGTACAAAGATCATTAACCTAATCTCCTC-3'	65
+ −	C1766T/ T1768A --S C1766T/ T1768A --AS	5'-GGTTAAAGGTTTTTGTACTAGG-3' 5'-CCTAGTACATAAACCTTTAACC-3'	66
+ −	A1762T/G1764A/C1766T --S A1762T/G1764A/C1766T --AS	5'-GAGGAGATTAGGTTAATGATTTTTGTACTGGAG-3' 5'-C TCCAGTACAAAAATCATTAACCTAATCTCCTC-3'	64 67
+ −	T1753C/A1762T/G1764A --S T1753C/A1762T/G1764A --AS	5'-GGAGGAGACTAGGTTAATGATCTTTGTACTA-3' 5'-TAGTACAAAGATCATTAACCTAGTCTCCTCC-3'	66
+ −	T1753G/A1762T/ G1764/C1766T --S T1753G/A1762T/G1764A/C1766T --AS	5'-GGAGGAGACTAGGTTAATGATTTTTGTACTA-3' 5'-TAGTACAAAAATCATTAACCTAGTCTCCTCC-3'	62
+ −	G1896A--S G1896A--AS	5'-GGGTGGCTTTAGGGCATGGACATTGAC-3' 5'-GTCAATGTCCATGCCCTAAAGCCACCC-3'	76
+ −	G1899A--S G1899A--AS	5'-GGGTGGCTTTAGGACATGGACATTGAC-3' 5'-GTCAATGTCCATGTCCCAAAGCCACCC-3'	77
+ −	G1896A/G1899A --S G1896A/G1899A --AS	5'-GGGTGGCTTTAGGACATGGACATTGAC-3' 5'-GTCAATGTCCATGTCCTAAAGCCACCC-3'	73

Polarity	Name	Sequence	Tm°C
	Cont. *Oligonucleotides Utilised in Site-directed mutagenesis PCR*		
+ −	HBV 141--S HBV 141--AS	5'-TGTTGCTGTACAGAACCTTCG-3' 5'-CGAAGGTTCTGTACAGCAACA-3'	63
+ −	HBV 131--S HBV 131--AS	5'-CTGCTCAAGGAATCTCTATGTTTCC-3' 5'-GGAAACATAGAGATTCCTTGAGCAG-3'	63
+ −	L180M--S L180M --AS	5'-CCTCAGTCCGTTTCTCATGGCTCAGTTTACTAG -3' 5'-CTAGTAAACTGAGCCATGAGAAACGGACTGG-3'	73
+ −	M204I –S ‡ M204I –AS	5'-GGCTTTCAGTTATATCGATGATGTGGTATTGGGGG-3' 5'- CCCCCAATACCACATCATCGATATAACTGAAAGCC-3'	77
+ −	M204V --S M204V --AS	5'-GGCTTTCAGTTATGTGGATGATGTGGTATTGGGGG-3' 5'- CCCCCAATACCACATCATCCACATAACTGAAAGCC-3'	76
	Oligonucleotides used for sequencing		
−	S1	5'-ATGGAGAACATCACATCAGGA-3'	50
+	S2	5'-GGCCAAGACACACGGGTGAT-3'	56
−	Pol2	5'-ACGGGGTAAAGGTTC-3'	50
−	Pol3	5'-AAGATCCAGTTGGC-3'	51
+	M3	5'-CTGGGAGGAGTTGGGGGA-3'	55
−	BC1	5'-GGAAAGAAGTCAGAAGGCAA-3'	57
−	3C	5'-CTAACATTGAGATTCCCGAGA-3'	50
−	Pol7	5'-AAGATTCGTCCCCATGCC- 3'	66
+	PO5'	5'-TGCGGGTCACCATAT-3'	42
+	T7	5'-ATTATGCTGAGTGATATCCC-3'	55
+	GLprimer1	5'-TGTATCTTATGGTACTGTAACT-3'	53
	Oligonucleotides used in Real-time PCR		
+ −	F1: 436-458 R1: 706-689	5'-GTATGTTGCCCGTTTGTCC-3' 5'-CCCTGCGAACCACTGAA -3'	63 64
+ −	cccDNA—S+: 1547-1568 cccDNA—C-: 1877-1896	5'-CCGTCTGTGCCTTCTCATCTGC-3' 5'-CAAAGCCACCCAAGGCACAG-3'	70 70
+	**1** PCP: 1797-1812 *	5'-GTCTGTTCACCAGCACCA -3'	61
+	**1** PGP: 1826-1846 *	5'-CACCTCTGCCTAATCATCTCA-3'	62
−	**1** BC1: 1974-1955 *	5'-GGAAAGAAGTCAGAAGGCAA-3'	61
+	M3: 1730-1754 *	5'-GAGTTGGGGGAGGAGATTAG-3'	61

[1] Numbering of primer positions starts at the *EcoRI* site. The binding sites for these primers are duplicated: occuring in both X genes within the p3.8II construct (Figure 3.1). The two binding sites for each of the primers when the construct numbering is used are: 398–415 and 3608–3625 (for PCP), 426–446 and 3644–3664 (for PGP), and 576–556 and 3792–3773 (for BC1). This numbering takes the first *BamHI* site (vector/insert junction) as the start of the numbering.

Oligonucleotides used to amplify Housekeeping genes			
+	β-actin--S	5'-GAACTCCAGCAGGACCATGTG-3'	67
−	β-actin -- AS	5'-CACTTCATGATGGAATTGA -3'	57
+	RPII--S	5'-GCACCACGTCCACTGACAT-3'	57
−	RPII--AS	5'-GTGCGGCTGCTTCCATAA-3''	65
β-globin oligonucleotides used in PCR			
+	β-globin--S	5'-ACACAACTGTGTTCACTAGC-3'	55
−	β-globin--AS	5'-CAACTTCATCCACGTTCACC-3'	63
Additional oligonucleotides utilised in Promoter-Analysis studies			
+	A1752G/T1754G/A1772T/ G1774A --S	5'-GAGGAGGTGAGGTTAAAGGTCTTTGTTCAAGG-3'	
−	A1752G/T1754C/A1772T/G1774A --AS	5'-CCTTGAACAAAGACCTTTAACCTCACCTCCTC-3'	68
+	T1753C/T1754G/A1762T/ C1766T --S	5'-GGAGACGAGATTAATGGTTTTTG -3'	
−	T1753C/T1754C/A1762T/C1766T --AS	5'-CAAAAACCATTAATCTCGTCTCC-3'	62
+	G1764A/C1766T --S	5'-GGAGATTAGATTAAAGTTTTTTG -3'	
−	G1764A/C1766T --AS	5'-CAAAAAACTTAATCTAATCTCC-3'	55
+	T1752G/T1754G --S	5'-GGAGATGAGATTAAAGGTCTTTG-3'	
−	T1752G/T1754TG--AS	5'-CAAAGACCTTAATCTCATCTCC-3'	62
+	HBV-Mlu I 1604-22	5'-gattacgcgtTCGCATGGAGACCACCGTG-3'	78
−	HBV--Bgl II 1792-1810	5'-agatctGGTGCTGGTGAACAGACC-3'	70

Table 2.1 Oligonucleotides used in this study

All the above listed oligonucleotides were HBV-binding, except those of T7 and GLprimer1. These two oligonucleotides had binding sites within specific sites in the pBluescript® II KS(+), pGEM®-T-Easy, and pGL2-basic® vectors, respectively, and were used in the Promoter-Analysis section of this thesis.

SDM oligonucleotides were named according to the positions of the nucleotide(s) that are mutated. Mutated positions are shown in bold. Letters preceding numbers given denote WT sequence at that position, whilst those following, denote the substitution. Nucleotide numbering is from the *EcoRI* site (position) for all mutagenic oligonucleotides except for the "a" determinant/S and RT/P mutant recombinants. For these two, numbering begins at the start of the small S gene and the RT domain of the P gene, respectively.

Key:

+, sense polarity (*S*)

−, antisense polarity (*AS*)

Tm, melting temperature

Primers marked with * were also used for reverse transcription.

Primers marked with ‡ were also used as non-specific competitors in the electromobility shift assay reactions.

Bold, lower case nucleotides designate engineered restriction sites.

2.1.9 Enzymes

All enzymes were purchased from New England Biolabs, Promega or Roche.

2.1.10 Radiochemical

[γ^{32}P]ATP (> 45,000 Ci/ mmol 165.5 TBq/mmol); (MP Biochemicals, Geneva, Switzerland).

2.1.11 Photography and autoradiography

Agarose gels were photographed with Type 667 Polaroid film (Polaroid, St. Albans, UK). Radioactive gels were exposed to BioMax MS or EKTAScan B/RA films (IBI-Kodak, Herts, UK). X-ray films were developed using an M35-MX-OMAT processor and developer (Kodak).

2.1.12 Commercial kits

Rapid DNA Ligation (Roche Diagnostics, Sussex, UK)

Luciferase Assay System kit (Promega, Southampton, UK)

QIAEX® II Gel Extraction (QIAGEN, West Sussex, UK)

QIAquick PCR Purification Kit (QIAGEN, West Sussex, UK)

pGEM®-T-Easy Vector System (Promega, Southampton, UK)

GenElute™ Plasmid Miniprep Kit (Sigma, Poole, UK)

QuantiTect SYBR® Green PCR (QIAGEN, West Sussex, UK)

QuickChange® Site-Directed Mutagenesis (Stratagene, Netherlands)

TNT® Coupled Reticulocte Lysate System (Promega, Southampton, UK)

PURESCRIPT® RNA Isolation Kit, (Gentra Systems, Nottingham, USA)

Murex HBsAg Version 3 (ELISA) kit (Abbott Diagnostics, Dartford, UK)

2.1.13 Molecular weight standards

25bp, 100bp and 1kb double-stranded, DNA markers used to estimate the size of DNA fragments on agarose gels were purchased from Bioline (London, UK), except where stated otherwise.

2.1.14 Antibiotics

Ampicillin for bacterial cell culture was purchased from Sigma. It was made as a stock solution (100 mg/ml) 1,000X the working concentration, filter sterilised, and stored in aliquots at –20°C. Autoclaved media were cooled to 45°C or below, prior to addition of antibiotic to avoid its degradation.

2.1.15 Immunological reagents

Primary monoclonal antibodies raised in mice [anti-HBe (RFHBe) and anti-HBs antigens (RFHBs 1, 2, 4, 6, 7, 18; Waters *et al* 1992, Seddigh-Tonekaboni *et al* 2000)] were used in this study for purposes of *in situ* detection of viral proteins within cells. They were provided by Dr. Jenny Waters. Polyclonal antibodies against transcription factors such as Chicken Ovalbumin Upstream Promoter Transcription Factor (Coup-TF), Hepatocyte Nuclear Factors, 1, and 4 (HNF1, and HNF4) (Viollet *et al.* 1997) used in the DNA-protein binding studies were provided by Dr. Mireille Vasseur-Cognet, Institute Cochin, Inserm, France. The CY3-labelled secondary (goat anti-mouse) antibody was purchased from Chemicon International (Hampshire, UK).

2.1.16 Biological sample

Liver biopsy tissue (for optimisation of cccDNA extraction) came from a man with histologically proven chronic hepatitis B (HBsAg-positive) and was stored at -70° C. Biological sample was collected following ethics approval by the Ethics Committee at St. Mary's Hospital.

2.1.17 Chemicals and plasticware

Most standard laboratory reagents were supplied by VWR International (Butterworth, UK) or Sigma. Bacto-tryptone, Yeast extract and Bacto-agar were purchased from Difco Labs Ltd (Surrey, UK). Tissue culture plastics were purchased from Becton & Dickinson (Oxford, UK).

2.1.18 Statistical analyses

Statistical interpretations of the results obtained in the study were based on values obtained by non-parametric methods: Mean-variation between two samples

was compared with the *Mann-Whitney U* test. *Kruskal-Wallis analysis of variance,* used in group comparisons, was replaced by the *Wilcoxon's matched pairs* test in the LMV study. Correlations (2-tailed) were tested with Spearman's correlation; rho (ρ). SPSS version 10 (SPSS Inc., USA) was used to perform these analyses. Values that fell between 0.50 and 0.99 are said to have a strong positive correlation (manual, SPSS).

2.1.19 Project permission

This project was approved by the Safety Committee of the Imperial College School of Medicine (project permit number: GMIC-01105) and by the Health and Safety Executive (UK). *In vitro* transfections of replication-competent recombinants were undertaken in the Department's Category III Laboratory, in accordance with the recommendations and guidelines of the appropriate College committee on genetic manipulation.

2.2 – 2.8 Methods

2.2 Nucleic Acid Manipulation

General molecular biology techniques employed during this work were modeled on those found in "Molecular Cloning, A Laboratory Manual 2 (Sambrook *et al.* 1989). However, these techniques were optimised at various stages, as described, where appropriate.

Enzyme reactions were carried out in 1.5ml or 0.4ml microcentrifuge tubes. Reactants were mixed prior to incubation by gently flicking the base of the tube. The contents were next brought to the bottom of the tube with a brief spin in a microfuge.

2.2.1 Plasmid DNA preparation from bacterial cells

2.2.1.a. Alkaline lysis method (Miniprep)

Small scale preparation of plasmid DNA was carried out by the alkaline lysis method (Birnboim and Doly 1979; Ish-Horowicz and Burke 1981; Sambrook et al. 1989). Single colonies of *E.coli* were individually, carefully picked from an agar plate into 1.5ml of LB broth within a 20ml universal tube and grown overnight at 37°C with shaking at 190–200g. Bacteria from this overnight culture were pelleted in

a 1.5ml microcentrifuge tube (10,000g, 30sec) and the supernatant aspirated and discarded. The bacterial pellet was then resuspended in 140µl of solution I (section 2.1.1). This was incubated at room temperature (r.t) for 5min, then, 280µl of solution II were added, mixed (by inversion) and ice-incubated for 5min. Finally, 200µl of ice-cold miniprep solution III were added, mixed and left on ice for 10min. The colourless precipitate was then pelleted (10,000g, 10min) and the supernatant transferred to a fresh tube. 0.5µl *RNaseA* (10mg/ml) was next added and the mix was incubated for 15min at 37°C. 500µl of a mixture of phenol:chloroform (1:1) was then added to the sample and the products vortexed. The phases were separated by centrifugation for 3min at 13,000g and the upper aqueous layer transferred to a new tube. This was re-extracted with an equal volume of chloroform and the phases separated again by centrifugation. The aqueous phase product was then precipitated with 100% ethanol and the pellet washed with 500µl 70% ethanol, then re-centrifuged for 3min at 13,000g. The supernatant was carefully aspirated, the pellet allowed to air-dry and the DNA was then resuspended in 30µl nuclease-free water and stored at –20°C until use.

2.2.1.b. Plasmid miniprep purification kit

A commercially produced miniprep purification kit was also used to prepare DNA, according to the manufacturer's instructions. In brief, through the sequential addition of 200µl of buffers I–III to pelleted bacteria from 1.5ml of overnight culture, bacterial protein and DNA were precipitated and removed by centrifugation (5min, 13,000g). DNA was further purified by centrifugation through a spin column, washed twice with 700µl washing solution and then eluted in 40µl commercial Ultrapure water.

2.2.2 Restriction enzyme digestion of DNA

As a general protocol, for the production of DNA fragments for subsequent cloning, 3µg of DNA was digested with 25 units (U) of a restriction enzyme in 15µl of 1X buffer (from a 10X stock). Digestions were carried out at 37°C for at least 1.5hrs. For double-digestions with the two enzymes that were active in the same buffer, the process followed the single-digest protocol. With enzymes requiring non-compatible buffers, the product of the first digestion was purified using the QIAquick PCR Purification Kit before use in the second digestion. Enzymic digestion was

monitored by comparing an aliquot of the digestion mixture with a similar amount of uncut DNA, by gel electrophoresis.

For analytical studies, such as for the restriction analysis of potential recombinant constructs, the reaction conditions were modified. In a 10µl final volume, 2–5µl of miniprep DNA (Section 2.2.1) was digested with 5U of restriction enzyme in 1X reaction buffer. The mixture was either incubated for 1–2hrs or overnight.

2.2.3 Purification of DNA fragments from agarose gels

DNA fragments from a PCR reaction or restriction enzyme digest (Section 2.2.2) were resolved by agarose gel electrophoresis (Section 2.5.1) and visualised on an ultraviolet (UV) transilluminator following ethidium bromide staining. Appropriate bands were excised using a clean scalpel blade and transferred to a microcentrifuge tube. DNA was then extracted from the agarose using Qiaex resin (QIAEX II gel extraction Kit). In this method, DNA binds to silica gel particles in the presence of chaotropic salts, after solubilisation of the agarose. Wash steps were performed under high salt conditions to eliminate remnants of the agarose and subsequent ethanol-based washes were carried out to remove residual salts and dyes. DNA was eluted from the silica gel particles using 20–30µl of Ultrapure water.

2.2.4 Capture cloning of PCR products

The pGEM®-T Easy vector system was employed during this study to facilitate the cloning of small (211bp) DNA promoter fragments produced by PCR. This kit takes advantage of the template independent activity of some thermostable polymerases, which add a deoxyadenosine residue to the 3′–ends of PCR products (for example, the Expand™ High Fidelity enzyme). The PCR product can then be ligated into linearised pGEM®-T Easy vector DNA, which contains single 3′ thymidine overhangs at the insertion site (Appendix # 4, vector 2). The thymidine residues prevent re-circularisation of the plasmid DNA and, in providing a compatible overhang for PCR products with an added 3′ deoxyadenosine residue, greatly improve the efficiency of ligation.

The pGEM®-T Easy vector carries both an ampicillin resistance gene (β-lactamase) and a gene encoding the β-galactosidase α-peptide (*Lac*). Insertion of a

fragment into the multiple cloning site of this plasmid ablates the β-galactosidase function, which catalyses the cleavage of X-gal to produce a blue colour. Bacterial colonies containing a recombinant plasmid with an insert appear white when plated onto agar containing ampicillin and X-gal compared to non-recombinants which are blue.

Ligations were carried out using 1μl pGEM®-T Easy vector, 1μl T4 DNA ligase and 3μl gel-purified PCR product in 5μl 2X ligation buffer (supplied), and the mixture was incubated overnight at 4°C. Chemically competent *E.coli* (MAX Efficiency® DH5α or One Shot® Top10) were transformed (Section 2.6.2) with 2–4μl of ligation mix and plasmid minipreps were made from overnight cultures seeded with a single white colony (Section 2.2.1). The presence of the desired insert was confirmed by restriction analysis (Section 2.2.2) and DNA sequencing (Section 2.2.9).

Restriction sites for *MluI* and *BglII* in the multiple cloning sites of the pGL2®-Basic luciferase vector (without any promoter), were engineered into the two PCR primers [Sense and Antisense (Table 2.1)], and the corresponding restriction enzymes were used to double-digest plasmids from the different colonies (obtained by SDM, Section 2.2.2), for subsequent re-cloning of the insert into the pGL2®-Basic luciferase expression vector. This involved double-digestion and agarose gel-purification of both the vector (Appendix #3, vector 3) and the individual plasmids. 50ng of the purified vector was then ligated to the HBV core promoter (CP) insert (nt. 1604–1815), using a 1:3 vector-to-insert ratio (Section 2.2.6).

2.2.5 Determination of concentration and purity of DNA

DNA concentrations were determined by measuring UV absorbance. Absorbance was determined at 260nm and 280nm in a quartz cuvette with a 10mm path length, using an Ultraspec 2000 spectrophotometer (Pharmacia). DNA samples were diluted so that an A_{260} reading fell in the linear range between 0.1 and 1.0. The DNA concentration was then calculated from the standard estimation that 1.0 A_{260} absorbance unit corresponds to 50μg/ml double-stranded DNA. The purity of the sample was assessed by calculating the A_{260}/A_{280} ratio where a ratio of 1.7–1.9 indicates sufficiently pure DNA for most molecular biology purposes. To a large extent, this nucleic acid quality control test was also used for RNA extracted from cells prior to its use in RT-PCR.

2.2.6 Ligation of DNA

DNA molecules possessing compatible terminals and a 5′–phosphate group may be joined using T4 DNA ligase. Approximately 50ng of linearised, vector was added to insert DNA at molar ratios of between 1:1 and 10:1. For T-A ligations, gel purified PCR fragments (which have *A* over-hangs) were directly ligated to 50ng of pGEM®-T-Easy vector (which bore *T* over-hangs). Appropriate amounts of inserts were used to achieve the same vector:insert molar ratios as described above. 1µl of T4 DNA ligase (400U) and 1µl of 10X, or 5µl of 2X T4 DNA ligase buffers was added and the volume made up to 10µl with Ultrapure water. Mixtures were gently mixed and incubated overnight either at 16°C (water bath) or 4°C (refridgerator). The Rapid DNA Ligation system (Roche) works similarly except that it requires only an incubation time of 5min, and incubation is at r.t.

2.2.7 Primer design

With minimal modifications, all the primers (Table 2.1) utilised in the mutagenesis and RT-PCR reactions, as well as the β-actin and β-globin primers, were taken from published work. Primers utilised in sequencing reactions were designed by others in the Department, whilst the remaining primers (ccc F/R, F1/R1 and, RPII primer-pairs) were designed by the investigator. All primers were generated by reference to published HBV sequences and were applicable to all genotypes since restricting primer-design to the specific genotype and subtype of wildtype virus strain was irrelevant for the purposes of this study. Primers that were used for sequencing purposes and, in RT-PCR, have been used in other studies in the Department or in studies from the literature (Laras *et al.* 2002). covalently closed circular (ccc)DNA primers were designed to bind only to ccc HBV DNA, having taken into account the nature of the HBV genome (Chapter 1, Sections 1.4).

Primer length ranged from 18 to 32 nucleotides. The Netprimer program (PREMIER Biosoft International, Palo Alto, U.S.A) was used to analyse primers for secondary structure, particularly hairpins and cross-priming in primer pairs. In Real-time PCR in particular, formation of secondary structures can generate false copy number values since the software takes all amplifications into account when providing amplification results (Chapter 1, Sections 1.16). Nonetheless, secondary structures

between some primer-pairs were inevitable due to constrain, relating to the amplification of the intended regions.

Netprimer was also used to predict the melting temperature (Tm) of primers. Great effort was made to design primers with Tms of at least 50°C, but not exceeding 80°C. Notwithstanding the use of Netprimer, primers were modified on several occasions to optimise PCR. Primers used in the Promoter-Analysis section of this investigation contained nucleotides added to the 5′–end to create unique restriction sites. Engineering of restriction sites to the 5′–end of PCR products was undertaken to facilitate ligation of the amplified 211bp promoter region into the pGL2®-Basic vector (Section 2.4.1).

Primers designed for the purpose of SDM took Stratagene suggestions into account with regard to, Tm, primer length and G–C content (SDM Technical bulletin). Using Netprimer as above, primers were designed for the amplification (Real-time PCR) of released HBV virions post transfection in hepatoma cell lines. All primers were synthesised by Sigma-Genosys (Cambridge, UK) and purified by de-salting.

2.2.7.a. Annealing temperature (Ta)

The Ta for each PCR reaction was initially set at 3–5°C below the Tm of the primer with the lower Tm in the primer-pair. On occasion, this was adjusted for PCR optimisation.

2.2.8 Site-directed mutagenesis:

Principle of QuikChange® II Site-Directed Mutagenesis Kit

The basic procedure of the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) utilises a supercoiled double-stranded (DS) DNA plasmid with an insert of interest and two synthetic oligonucleotide primers, both containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuUltra*™ DNA polymerase, without primer displacement. Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *DpnI*. The *DpnI* endonuclease (target sequence: 5′–Gm6ATC–3′) is specific for methylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesised DNA (refer to

section on Site-directed mutagenesis: 1.14.1). The circular, nicked DS-DNA containing the desired mutation(s) is then transformed into XL1-Blue supercompetent cells (supplied with kit). After transformation, the XL1-Blue supercompetent cells repair the nicks in the mutated plasmid.

Protocol of Site-directed mutagenesis

A replication-competent plasmid (p3.8II) containing a 1.2-times genome-length of HBV subtype *adr* was initially used as template in a full-length Site-directed mutagenesis (SDM) reaction. This plasmid was based on pBluescript® II KS(+), and the HBV fragment was cloned into the vector's unique *BamHI* and *SmaI* restriction sites. The insert was 3,808 base pairs (bp) long and consisted of bp 1400 to 3215 plus 1 to 1986. When full-length SDM failed (as described in Results, Section 3.2), this plasmid was then first rendered incompetent by removing a 900bp fragment (which was comprised of the 600bp repeat, plus a 3000bp fragment from the P gene) (Results, Section 3.0). The new plasmid, p3.8IIΔ, was subsequently used as template in SDM using standard PCR reactions. SDM was performed using the QuikChange® Site-Directed Mutagenesis Kit according to instructions and recommendations of the manufacturer (see principle below).

A number of precautions were taken to limit the risk of PCR-contamination. Gloves were worn at all times and frequently changed. Ultrapure water was used in all PCR-related procedures and all reagents were aliquoted into working volumes so that the stock supply was infrequently accessed. The PCR reactions were prepared with aerosol-free filter tips. The PCR products were opened for further analysis in a separate laboratory with separate tips and pipettes. All pipettes were frequently exposed to UV light to degrade potential contaminating DNA templates.

In brief, the SDM protocol involved the initial generation of two complementary oligonucleotides in which the specifically desired mutations were introduced (Table 2.1). All primers were designed with the aid of the Netprimer program (Section 2.2.7) and all reagents were kit-provided except where stated otherwise. Each mutagenesis reaction was composed of 5µl of 10X reaction buffer, 50ng of p3.8IIΔ, 1.25µl each of the two mutation-specific complementary primers and 1µl of dNTP mix in a PCR tube. This mixture was subsequently diluted with Ultrapure water to a volume of 49µl. 1µl of 2.5U/µl *PfuUltra*™ DNA polymerase was finally added to the PCR-mix to bring it to a final volume of 50µl. Samples were then

placed in a thermocycler for 12–14 cycles of 1min denaturation at 95°C, 1min of annealing at the appropriate Ta and 13min of extension. The Ta varied according to the Tm of the primers used. Prior to cycling, samples were initially denatured at 95°C for 1min.

Following temperature cycling, reactions were placed on ice for 2min. Once cooled, 1µl of 10U/µl *DpnI* restriction enzyme was added to the reaction and the contents mixed by pipeting up and down several times. The reaction mixtures were briefly centrifuged in a microcentrifuge and immediately incubated at 37°C for 1hr to digest the non-mutated residual p3.8IIΔ template.

Following the *DpnI*-parental plasmid digestion, 1µl was transformed (Section 2.6.2) into XL1-Blue supercompetent cells (provided with the mutagenesis kit), and plasmid DNA was isolated from selected colonies (Sections 2.4.2 and 2.2.1, respectively). The size of the resulting plasmid was checked by *BamH1*-digestion (Section 2.2.2), followed by sequencing to confirm the introduction of the desired mutation(s) and to check for mutations introduced in error (Sections 2.2.9 and 2.3.2).

2.2.9 DNA sequencing

DNA was sequenced by the Clinical Science Centre Genetics Core Facility at the Hammersmith Hospital Campus of Imperial College, London. 500ng of miniprep DNA was mixed with 3.2pmol of sequencing primer and made up to 10µl with Ultrapure water. The samples were processed using reagents and instruments from Applied Biosciences, and the sequences were provided in electronic form.

2.2.10 Extraction of extracellular viral particles

HBV virions were extracted from culture media based on modified previous protocols (Karayiannis *et al.* 1995, Parekh *et al.* 2003). For cell-debris removal, 200µl of supernatant was prespun at 4,000rpm for 10min and the resulting supernatant transferred into a fresh microcentrifuge tube. This supernatant potentially contained both Dane and naked core particles. Transfected HBV DNA was digested away at 37°C for 30min by 2U of *DNaseI* (Ambion's Kit) and 1.5U of mung bean nuclease (New England Biolabs) in the presence of 8mM $CaCl_2$ and 6mM $MgCl_2$. [Mung bean nuclease degrades single-stranded (SS) DNA and RNA with exonuclease activity to yield 5'–phosphate terminated products]. Then, 100µl of Proteinase K, digestion buffer was added to give final concentrations of 25mM/L NaAc, 2.5mM/L

ethylenediaminetetra-acetic acid (EDTA), 1% Sodium Dodecyl Sulfate (SDS), 2mg/ml Proteinase K and 10μg/ml of yeast transfer RNA as carrier. Digestion at 65°C was for 2hrs and DNA was phenol-extracted and ethanol-precipitated (as described in Section 2.2.1.a) after which the resulting pellet was suspended in 10μl of Ultrapure water. Samples were stored at –20°C.

2.2.11 Extraction of HBV replicative intermediates

HBV DNA intracellular replicative intermediates; relaxed-circular double-stranded (RC-DS) and covalently closed circular (ccc) DNA were extracted from transfected cells as described below. Total HBV viral RNA was isolated for the differential detection of preC and preG transcripts, following amplification (Methods, Sections 2.2.11.a and Results, Figures 4.3 and 4.4).

2.2.11.a. Viral RNA extraction

A number of precautions were taken to avoid viral RNA degradation by exogenous RNAses. All glassware was treated with 0.1% diethyl pyrocarbonate (DEPC), a strong inhibitor of RNAses. Glassware was soaked for 2hrs in DEPC and then rinsed in sterile water. Glassware and plasticware were autoclaved for 30min at 120°C at 38 lb/in^2. Work surfaces were decontaminated with RNasezap® (Austin, TX, USA). Disposable gloves were worn at all times and frequently changed. The preparation of RNA was performed in a designated laboratory following the cell-lysis stage in a Category III Laboratory. RNA extraction was carried out either on freshly lysed or stored cells. Cells destined for storage were first flash-frozen in liquid nitrogen and subsequently kept at –80°C.

RNA extraction employed the PURESCRIPT® RNA Isolation Kit, which provided all solutions used in the extraction process. The manufacturer's instructions were adhered to. In brief, individual wells (6-well plate) of transfected cells were first rinsed with PBS and 300μl of Cell-Lysis Solution (provided with the kit) were subsequently added. Plates were rocked from side to side, followed by pipetting of lysed cells into a 1.5ml microcentrifuge tube. Wells were rinsed with an additional 100μl of the Cell-Lysis Solution. Following this cell-lysis step, an aliquot of 300μl of the cell lysate was then used in the actual extraction protocol. 100μl of protein-DNA precipitation solution was added to the cell lysate to precipitate proteins and DNA. RNA contained in the final supernatant was recovered with Isopropanol precipitation

followed by washing with 70% ethanol and air-drying of the pellet. This pellet was resuspended in 20µl RNase-free water. DNase treatment (2.2.10.a) either followed directly, or the RNA was stored at –80 °C until later use.

2.2.11.a.i. DNAse treatment of RNA

Extracted RNA (as above) was DNase-treated to remove potential DNA contaminants. *DNaseI* is an endonuclease enzyme that acts by cleaving DS or SS circular DNA. The treatment was carried out with the use of Ambion's DNaseI Kit[1] (RNase-free). 1X (of a supplied 10X) DNaseI Buffer and 4U of enzyme were added to the total RNA, mixed gently, and incubated at 37°C for 20–30min. A 0.1 volume of DNase Inactivation Reagent was added to the mixture, briefly vortexed, and incubated for 2min at r.t. The mixture was centrifuged at 10,000g for 1min to pellet the DNase Inactivation Reagent and the supernatant containing the RNA was then transferred to a new DNase-free tube and stored at –80°C.

2.2.11.b. Extraction of total intracellular DNA and ccc DNA

In the initial phase of the study, optimisation of the specificity and sensitivity to detect ccc– and RC-DS– HBV DNA necessitated the differential isolation of each one of them. RC-DS DNA was extracted (from cells and from a liver biopsy sample) as total intracellular HBV DNA (RC-DS plus ccc). cccDNA was differentially isolated from 2.2.15 cells, as described below.

Extraction of total intracellular HBV DNA (RC-DS plus ccc) from cells

A modified version of a previously described protocol (Abdelhamed *et. al* 2001) was used for the extraction of intracellular (RC-DS plus ccc) HBV DNA from transfected cells. Cells were first rinsed with PBS, subsequently trypsinised and resuspended in DMEM, this was then subjected to centrifugation for 10min at 6,000g, washed in PBS and repelleted at 6,000g for 10min. The resulting pellet was resuspended in 50µl of magnesium acetate (final concentration, 10mM) and DNase-treated (initially) as explained above (Section 2.2.11.a.i) to remove non-encapsidated DNA.

[1] The use of this kit excludes heat inactivation of the enzyme.

Furthermore, in the subsequently transfected assays, the presence of non-encapsidated DNA was later found to be either unlikely at day 4 post transfection (Abdelhamed *et. al* 2001), or negligible (Results, Section 5.2.3)]. This preliminary precautionary step of DNAse-treatment was therefore excluded from further extraction processes, and the pellet was directly lysed and DNA extracted following the second 10min centrifugation.

400µl[1]of Cell-Lysis Solution (provided in the PURESCRIPT® RNA Isolation Kit) was added to three individual wells to release intracellular HBV DNA. This was then transfered to a clean 1.5ml microcentrifuge tube, and 700µl of Neutralization Solution (S3)[2] was subsequently added. This was thoroughly mixed by repeated inversion, and cell debris pelleted by a 20min centrifugation at maximum speed (12,000–14,000g). The supernatant was dispensed into a fresh 1.5ml microcentrifuge tube, and DNA was extracted with phenol-chloroform and precipitated with isopropanol (as described in Section 2.2.1.a). After washing with 70% ethanol, and air-drying, the pellet was suspended in 10µl of Ultrapure water and stored at –20°C, or the pellet was immediately treated with the appropriate nuclease, as described below.

30U of Plasmid-Safe ATP-dependent DNase (Biolab, Cambridge, UK) was added to the 10µl of HBV-DNA from above in the presence of 1X (final concentration) of 10X buffer (supplied with enzyme). Digestion was at 37°C for 3hrs. Plasmid-Safe ATP-dependent DNase acts selectively to digest linear DS- or circular SS-DNA but has no activity on nicked, ccc or supercoiled DNA (Technical Manual, Biolab).

Following the above digestion, total intracellular HBV DNA (RC-DS plus ccc HBV DNA) was extracted as described above. The resulting pellet was resuspended in 10µl of Ultrapure water and stored at –20°C.

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[1] In the optimisation stage, half of this volume (200µl) was used for the isolation of RC-DS DNA and the remainder was used for cccDNA extraction. When total intracellular DNA was extracted, the entire 400µl was used for that purpose.

[2] The Neutralization Solution (S3) acts to precipitate cell debris, proteins, lipids, and SDS, following centrifugation for 20min at ≥ 12, 000g (technical bulletin, GenElute™ Plasmid Miniprep Kit). [This solution is a component of the GenElute™ Plasmid Miniprep Kit (Sigma, Poole, UK)].

Extraction of cccDNA from cells[1]

The protocol used for the extraction of non-protein-associated circular HBV DNA from transfected cells was a modified version of a previously described method (Delaney *et al.* 1999). Nucleic acids were essentially isolated as described above (Extraction of total intracellular HBV DNA from cells). The Plasmid-Safe ATP-dependent DNase was replaced by mung bean nuclease. The pellet was resuspended in 10µl of Ultrapure water and stored at –20°C.

Extraction of total intracellular HBV DNA from tissue sample[1]

Total HBV DNA extraction from liver sample was carried out in a derogated Category III laboratory. An autoclaved and DEPC-treated tissue grinder was used to homogenise 75mg of the frozen specimen. The rest of the procedure was as described above for the extraction of total intracellular HBV DNA from cells.

2.2.12 Reverse transcription polymerase chain reaction (RT-PCR)

The Polymerase Chain Reaction (PCR) is an *in vitro* method for the amplification of a specific DNA sequence. Two primers are annealed to the complementary strands of a DNA molecule at positions which flank the region of interest and this region of DNA then amplified through multiple cycles of denaturing, annealing and elongation using a DNA polymerase. In order to use this method to detect and quantify HBV viral transcripts, reverse transcription (RT) of the viral RNA to complementary DNA (cDNA) was required to provide a DNA template for PCR (including Real-time). In general, cDNA could be used directly as template in Real-time PCR. The integrity and success of the RT process was first tested by performing standard PCR with the Real-time primers (Section 2.2.12.b).

2..2.12.a. cDNA synthesis

HBV RNA was reverse-transcribed using Expand™ Reverse Transcriptase (Roche Diagnostics, Essex, UK), which is a genetically engineered version of the Moloney Leukaemia Murine Virus reverse transcriptase. The primers used are indicated with an asterisk in Table 2.1.

[1] This protocol was used only in the optimisation phase of extraction and detection, see Results; Section 4.2.

5µl of total RNA and 1µl of the primer (50 pmoles) were made up to 10µl with RNase-free water, incubated for 10min at 65°C in a thermocycler and immediately ice-cooled. To this mixture, 4µl buffer, 2µl of 100mM dithiothreitol (DTT), 2µl of 10 mM dNTP mix, 0.5µl RNase Inhibitor (20U), 0.5µl sterile water and 1µl Expand™ Reverse Transcriptase (50U) were then added to give a final concentration of 250mM Tris-HCl, 200mM KCl, 25mM $MgCl_2$, 2.5% Tween®, 10mM DTT and 2.5mM of each dNTP. This solution was incubated at 42°C for 90min in a thermocycler, after which it was immediately placed on ice.

2..2.12.b. Polymerase chain reaction

Three different DNA polymerases were used for PCR amplification during the course of these studies: *Taq*, Expand™ High Fidelity PCR System (Roche), and *PfuUltra*™ polymerases. Stringent PCR conditions (Section 2.2.8) were taken to minimise contamination of PCR with extraneous DNA. Negative controls were included at the cDNA synthesis stage and during PCR.

Taq DNA polymerase

Taq polymerase is a highly processive 3′-5′ DNA polymerase that lacks 3′-5′ exonuclease (proofreading) activity (Chien *et al.* 1976). It was originally isolated from the thermophilic eubacterium *Thermus aquaticus.* PCR using *Taq* polymerase generates high yields of DNA, but is prone to misincoporation of bases, as a result of the absence of a proof-reading capacity. In this study, *Taq* polymerase was used in initial PCRs to verify the presence of HBV DNA and during the optimisation of techniques. Reactions were carried out in 1X polymerase buffer (supplied with the enzyme) supplemented with 2.0mM $MgCl_2$ in a 0.2ml PCR tube. To this, 2µl of cDNA or extracted HBV DNA were added together with 1µl 10mM dNTP mix, 0.5µl of each of the sense and antisense primers (30 pmoles) and 0.6–1µl (3–5U) *Taq* polymerase, and the volume was adjusted to 50µl with Ultrapure water. The PCR tubes were then placed in a thermal cycler. The DNA template was denatured at 95°C for 3min, and this was followed by 30 cycles of denaturing at 95°C for 30sec, annealing at the appropriate Ta for 30sec and elongation at 72°C for 1min. After the final annealing step, the polymerisation was allowed to proceed for 7min to minimise the formation of single strands at the ends of the amplified DNA molecules. PCR

products were stored at 4°C and a portion (at least half) of the reaction analysed by agarose gel electrophoresis.

PfuUltra™ DNA polymerase

The enzyme was either provided with the QuikChange® II Site-Directed Mutagenesis Kit, or separately purchased from the same company (Stratagene). *PfuUltra™* DNA polymerase is a thermostable enzyme that replicates DNA and exhibits 3'–5' exonuclease activity. It was originally isolated from *Pyrococcus furiosus*. It is highly suitable for use in applications where high polymerase fidelity is important, such as in SDM. The high-fidelity of the polymerase (18–fold higher fidelity in DNA synthesis than *Taq*, Stratagene Technical Bulletin) eliminates second-site errors and prevents propagation of amplified errors in mutagenic reactions (Section 2.2.8).

Expand™ High Fidelity PCR System

This system consists of a mixture of *Taq* and *Pwo* DNA polymerases, designed to give PCR products high yields in addition to high fidelity and specificity, as a result of its proofreading capability. This system was used in generating all the final mutated promoter fragments for cloning into the PGL2 luciferase basic® vector, required in the Promoter-Analysis study of this investigation (Section 6.8). PCR reactions were carried out as above (for *Taq* Polymerase).

2.2.13 Real-time quantitative PCR

The relative HBV DNA and transcript concentrations were analysed by Real-time quantitative PCR with the use of the QuantiTech™ SYBR® Green PCR Master-Mix Kit. Whilst the underlying principle of the method, as well as the sensitivity, efficiency and reproducibility of the kit has already been described, below is a summary of the methodology, taken from the manufacturer's instructions. Real-time assays were carried out in a specially allocated laboratory and sterile techniques (as in standard PCR Section 2.2.8) were stringently applied to avoid cross-contamination between samples and/or from other sources of nucleic acid. However, despite all these precautions, low-level contamination was sometimes detected.

HBV DNA samples (Section 2.2.10 and sub-Section 2.2.11.b) did not require any further preparation and were used neat in quantitative reactions. cDNA samples were diluted between 1:10 and 1:30 and the dilution factor taken into account in the final normalisation (to β-actin) and standardistion (to 15% transfection efficiency) calculations (see Appendix #3). Samples for standard-curve generation were constructed as described below (Section 2.2.13.a).

QuantiTech™ SYBR® Green PCR Master Mix Kit

This ready-made master mix contains the fluorescent dye SYBR® Green I which enables rapid analysis of many different targets. High specificity and sensitivity in PCR is achieved by the use of the hot-start enzyme, HotStartTaq DNA polymerase, together with a specialised buffer. The enzyme is provided in an inactive state and has no enzymic activity at ambient temperatures. This prevents the formation of misprimed products and primer-dimers during the reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification (technical handbook, QuantiTech™ SYBR® Green PCR). The enzyme is activated by a 15min incubation step at 95°C.

QuantiTech™ SYBR® Green PCR mix contains a balanced combination of KCl and $(NH_4)_2SO_4$, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This serves to create stringent primer annealing conditions, leading to increased specificity. It is normally not necessary to optimise by titration of Mg^{2+}, since the $MgCl_2$ concentration only marginally influences primer annealing when using this buffer (Qiagen Technical Bulletin).

2.2.13.a. Preparation of standard-curve samples

A requirement for analysing samples by quantitative Real-time is the inclusion of serially-diluted samples of known concentrations, for the generation of a standard-curve which enables the machine's software to calculate the precise starting concentration (in copy number) in test samples.

Although it may have been ideal to use the WHO-approved International Reference standard, it was thought convenient to create a Real-time PCR standard for use in the generation of a standard curve by way of cloning a PCR product into a plasmid vector (Section 2.2.4) preferably using the same specific primer-pair that was to be used in the actual Real-time amplification reaction. Subsequent to cloning, the

resulting plasmid was linearised at the vector's Multiple-Cloning Site, by restriction digestion, and the linearised product was gel-extracted (Sections 2.2.2 and 2.2.3) for use in the generation of the standard curve. For example, the fragment amplified using the F1-RI HBV DNA primer-pair (272bp) was subjected to gel-electrophoresis (Section 2.5.1), purified and then ligated into pGEM®-T-Easy (Sections 2.2.3 and 2.2.6). This was sequentially followed by transformation, selection of recombinant clones, digestion with *Pst1* for plasmid linearisation and gel-extraction (Sections 2.2.2 and 2.2.3, respectively). The concentration of the linearised product was determined spectrophotometrically (Section 2.2.5), then serially diluted for generation of a standard-curve (Appendix #1).

This process was repeated in constructing standard plasmids with the other primer-pairs that were used in the Real-time PCR quantification of cccDNA, preC and preG transcripts, and β-actin and RPII HKGs.

2.2.13.b. Preparation of samples for Real-time PCR

A Real-time PCR master-mix for one sample was prepared as follows: 10μl of the QuantiTech™ SYBR® Green, 1μl of 0.5μM of each of the sense and anti-sense primers, and 7μl of sterile water were gently vortexed, subjected briefly to microcentrifugation and subsequently dispensed into a LightCycler capillary tube (Roche). 1μl of the DNA sample was added and the capillary was re-centrifuged for 45–60sec then placed in the LightCycler. The software was programmed to run the following cycling conditions: initial polymerase activation step at 95°C for 15min, followed by 45 cycles of denaturation at 94°C for 15sec, annealing at an appropriate Ta for 30sec and extension at 72°C for 30sec. Precautions to eliminate potential primer-dimers (Section 4.3.a) being quantified involved, an additional data acquisition step at 80°C for 15sec into the amplification stage of the above cycling protocol. This acquisition temperature was determined in a preliminary experiment to establish the Tm of each target product and that of any dimers that may have resulted from the reaction. In general, it was found that primer-dimers (for example as formed between PCP and BC1 or, M3 and BC1 primer-pairs: Results, Section 4.3) had a Tm of between 72 and 75°C, whereas the Tms for the desired products were at least 80°C.

For the RNA transcript analysis, each cDNA sample was used separately to quantify both the targeted HBV species, as well as the β-actin (and RPII, initially) gene. The latter was included for normalisation purposes: copy number of the target

gene was first divided by that of the β-actin, the result is therefore a ratio of the two, and has no unit. Then, each result was standardised to 15% transfection efficiency (Appendix #3) for all Real-time PCR results presented in the Results sections.

2.3 Protein Analyses

In the initial phase of the study, transfection efficiency determination was by directly staining for HBcAg in transfected cells. Transfected cells were also stained with a panel of monoclonal antibodies that recognised different HBsAg epitopes. The same protocol was used in both procedures. Qualitative detection of HBeAg and quantitative testing of HBsAg were also undertaken, (see Section 2.3.2).

2.3.1 Immunocytochemistry: *In situ* stainings for HBV antigens

Hepatoma cells (Huh7) were maintained as monolayer cultures on 22 x 22 mm coverglass slips (Thermo Electron Corporation, San Hose, CA, USA) in 6-well plates. Following transfection by a HBV replication-competent recombinant, and incubation (see Section 2.6.5) for 3–4hrs, cells were rinsed with PBS, and subsequently transferred into a fresh 6-well plate for staining.

The coverglasses were first covered with an undiluted preparation of the mouse monoclonal antibody; RFHBs18, and allowed to stand at r.t for 30–45min. Cells were then washed 3 times with PBS and then covered with a 1:500 dilution of rabbit anti-mouse before incubating in the dark for 30–45min at r.t, followed by repeated washing with PBS. The antibody was diluted in a "blocker" solution of 10% FCS (in PBS), incubated and washing with PBS, as above. Excess PBS was absorbed onto a laboratory paper-towel and the coverglass was mounted face-down on a microscope slide (Thermo Electron Corporation) using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Inc., CA, USA). Samples were stored in the dark at 4°C and analysed by fluorescence microscopy, using an appropriate filter, at least 45min later. DAPI is a DNA-specific dye (4′, 6, diamidino-2-phenylindole) which produces a blue fluorescence with excitation at 360nm and emission at 460nm when bound to DNA, essentially staining nuclei blue.

2.3.2 Analysis of secreted HBV antigens

2.3.2.a. HBsAg: quantitative analysis

The relative HBsAg levels secreted into the culture supernatant was determined using the HBsAg Monoclonal II ELISA kit (Murex HBsAg Version 3, Abbott Laboratories). [Except were stated, all reagents were kit-supplied]. Samples were first centrifuged at 6,000g for 30sec to remove cellular debris, transferred to a clean tube and stored at –20°C for later use, or assayed for the antigen immediately. To avoid signal saturation, supernatants collected from transfected cells were diluted with serum-free medium before assaying: dilution range of 1:2 to1:5 were used. Sample of the diluted medium (75µl) was made up to 100µl with sample diluent (supplied), and added to a microtitre plate pre-coated with murine monoclonal anti-HBsAg. Following incubation at 37°C for 1hr, 50µl of goat anti-HBsAg conjugated to horseradish peroxidase (HRP) was added and further incubated at 37°C for 30min. Wells were washed five times with Borate Wash Buffer to remove unbound conjugate. The HRP substrate, 3,3′,5,5′-tetramethyl- benzidine (TMB), was added and the reaction allowed to continue incubating at 37°C for 30min. The reaction was terminated by the addition of 50µl of 1M H_2SO_4 and the concentration of HBsAg in each sample read at a wavelength of A_{450} using a microtitre plate reader (Dynatech Immunoassay Systems, Dynatech Laboratories, Chantilly, VA, USA). Positive and negative control sera provided by the manufacturer were included in each run. The value of this negative control was subtracted from all sample values which were compared to an internal control (sample medium from cells transfected with empty vector).

To enable the conversion of the final absorbance values to µg/ml (using the formula below), a series of standard dilutions were also included. This involved serially diluting a WHO-approved yeast recombinant HBsAg [Recombivax HB® (Merck & Co INC, NJ, USA)] to achieve a five-point concentration range between 250pg/ml and 50ng/ml. Concentrations were estimated using the formula:

Concentration (µg/ml) = A_{450} value × dilution factor × A_{260} unit for DS-DNA[1]

[1] The standard for A_{260} unit value for double-stranded DNA = 50µg/ml.

2.3.2.b. HBeAg: qualitative analysis

In the absence of a sensitive quantitative HBeAg ELISA kit, only qualitative testing was undertaken for purposes of detecting the antigen in the conditioned media of transfected cells. Selected samples were sent to the routine Virology Laboratory, St. Mary's Hospital, and VIDAS HBe/Anti-HBe kits were used (Biomerieux, Marcy L'Etoile, France). A reading above 0.015 PEI U/ml was deemed a positive result (PEI, Paul Elrich Institute).

2.4 Electrophoretic Techniques

2.4.1 Agarose gel elctrophoresis of DNA

Agarose gels were formed using a variety of formatted gel electrophoresis tanks. 0.7 to 2.0% (w/v) agarose in 1X TBE buffer, was heated in a microwave oven, allowed to cool to at least 65°C before adding 15μg ethidium bromide, and the mixture then poured into the assembled apparatus taking care to avoid air bubbles. Once the gel had set, it was covered with 5mm of 1X TBE buffer and the template combs removed from the wells. Generally, 0.7% to 1.0% (w/v) agarose was used for separation of larger fragments (> 5kb) and 2 to 2.5% (w/v) for smaller fragments (< 1kb).

An appropriate amount of loading buffer was added to the sample which was then carefully pipetted into individual wells. The gel was run at a constant current of 65mA, until the bromophenol blue tracking dye had migrated a requisite distance along the gel. DNA bands were visualised on a UV transilluminator and photographed when appropriate. Due to the dilution of ethidium bromide during electrophoresis, staining was not always evident. To remedy this, the gel was soaked in 1.5μg/ml ethidium bromide for 15min, excess ethidium bromide was washed-off in distilled water for 8–10min by shaking on an orbital shaker, and the gel then viewed again. For preparative gels, UV exposure was kept to a minimum to prevent DNA damage.

2.4.2 Acrylamide gel elctrophoresis of proteins

A 4% non-denaturing acrylamide gel (similarly prepared and set up as above) was used to resolve DNA-protein complexes (Section 6.8). Gels consisted of 1.0ml 10X TBE buffer, 1.25ml 40% (w/v) acrylamide/bisacrylamide mix (Biorad, London,

UK), 0.75ml 40% (w/v) acrylamide, 0.625ml 80% glycerol, 20µl TEMED, and150µl 10% ammonium persulphate (APS, freshly made). This mixture was pipetted into the pre-assembled glass plates in a mini gel apparatus and polymerisation allowed to proceed at r.t for 45–60min. Gels were run in 0.5X TBE buffer at 4°C and a constant current of 30mA, until the dye reached the bottom of the plate. They were then fixed in a solution of acetic acid (5%), methanol (45%) and water (50%). Fixing was on an orbital shaker, for 30min. Gels were transferred onto two layers of Whatman 3MM paper and covered with a thin plastic membrane for drying. Dried gels were exposed to an X-ray film in an autoradiography cassette, and stored in a –70°C freezer, for at least 16hrs, subsequent to which the cassette (containing the exposed film) was allowed to warm-up to r.t, the film was then developed, and the results visually analysed.

2.5 Bacterial Techniques

2.5.1 Maintenance of *E.coli* strains

E.coli strains were maintained on LB agar plates containing ampicillin at 4°C, for up to two weeks. For long term storage, 0.75ml of 50% glycerol was added to 0.75ml of an overnight culture of *E.coli* in a 2ml screw cap microcentrifuge tube, and the tubes stored at –80°C.

2.5.2 Transformation of $CaCl_2$ competent *E.coli* cells with plasmid DNA

For the transformation of ligation reactions, commercially obtained DH5α (Invitrogen) and TOP10 (Invitrogen) supercompetent cells (35–50µl) were removed from storage and thawed on ice. The recombinant plasmid was then introduced into the microcentrifuge tube containing the cells, mixed gently and left on ice for 30min. 2µl of a sub-cloning ligation reaction (pGEM®-T Easy) and 5–7µl of other ligation reactions were used for this purpose. Cells were then heat-shocked for 30–45sec in a 42°C water bath, then immediately placed on ice for 2min. 0.5ml of SOC medium was dispensed into tubes and these were incubated at 37°C with gentle shaking for 60min, before plating on LB agar plates containing ampicillin (50µg/ml). Blue/white selection was achieved by the addition of 20µl of X-gal (40mg/ml in dimethylformamide) to the agar plates 30min prior to use. Bacteria were grown in inverted plates overnight at 37°C.

2.5.2.a. Selection of recombinant clones

White colonies, containing recombinant plasmid DNA, were picked from the agar plates using autoclaved plastic pipette tips and inoculated into 5mls of LB broth (containing 50μg/ml ampicillin), in 30ml sterile universal bottles. These were incubated for at least 16hrs at 37°C with shaking to allow bacterial growth prior to DNA preparation (Section 2.2.1).

2.6 Cell Culture Techniques

2.6.1 General handling considerations

All cell lines used in this study were maintained as horizontal monolayer cultures in plastic culture vessels (T75 canted-neck flasks). Cultures were grown at 37°C in the presence of CO_2, and were split 1:3 or 1:4 every 3–5 days.

2.6.2 Splitting of Confluent Cells

Medium was first aspirated and the cells were washed with PBS. 0.5ml of 10X trypsin was added and the flask was incubated at 37°C for 2–3min to allow cells to detach. To complete the detachment process, the flask was briefly shaken and 2ml of DMEM growth medium added (with supplements as described in Section 2.1.4). This was then aspirated and transferred to a sterile 20ml universal container and the volume made up to 10ml with DMEM growth medium. Cells were centrifuged for 5min at 1,200g and the resulting pellet resuspended in 1ml of growth medium. 200-300μl of this was then added to T75 flasks, supplemented with 20ml of growth medium, before being placed in the incubator.

2.6.3 Cell counting and viability estimation

The number of cells that were plated out for transfection experiments was standardised. For this, cells were assayed using Trypan Blue (Invitrogen) prior to seeding, a dye taken up by only non-viable cells. A drop of a 10X stock solution was added to an aliquot of cells (at ratios of 1:4) and allowed to stand for 5min. A sample of the mixture was then analysed using a haemocytometer (Sigma) under an inverse-phase microscope (Kodak). The number of viable cells was then expressed as a percentage of the total number of cells in the etched squares of the haemocytometer.

To determine the number of cells in a suspension for seeding purposes, the following formula was used:

Number of cells/ml = N × (1 x 10^{-4}) × D, where:
N = number of cells in the etched squares.
1x10^{-4} = volume of liquid covering the etched squares, and
D = dilution factor

2.6.4 Freezing and thawing of cell stocks

Cells for freezing were pelleted by centrifugation and their supernatant discarded. Pellets were resuspended in DMEM and 0.5ml added to an equal volume of freezing mixture in a cryovial. The freezing mixture was prepared as follows: 10ml DMEM was added to an equal volume of 10% FCS and mixed well. 5ml sterile dimethyl sulphoxide (DMSO) were next added and cooled to 4°C. The cryovials were slowly frozen (1°C per min for 3hrs) in the vapour phase of liquid nitrogen prior to storage in liquid nitrogen.

Frozen stocks were thawed in water at 37°C, then transferred to a universal container containing 20ml of pre-warmed DMEM and the cells were centrifuged for 5min at 1,000g then resuspended in fresh growth medium.

2.6.5 Transfection of cells

1mg/ml of Lipofectin (stock solution), a liposome preparation formulated from cationic lipids, was used for transfection of cells with plasmid DNA according to Invitrogen instructions: a 6-well plate was seeded with 1 x 10^5 cells in 3-5ml of medium per well and left to incubate for at least 12–14hrs. Where appropriate, 2hrs before transfection, cells were treated with 4.0μM of LMV. A relatively high concentration of LMV was selected (corresponding to the IC_{90} of LMV for the inhibition of HBV replication; Delaney *et al.* 2001) in order to achieve almost complete inhibition of LMV-sensitive HBV. This protocol was adapted (Torresi *et al.* 2002c).

A plasmid-liposome complex (3μg of recombinant and 2μg of the pCI-GFP green fluorescence-expressing plasmid complexed with 7μg of Lipofectin®) was prepared in serum-free medium (OptiMEM®, Invitrogen), in a final volume of 100μl. This was allowed to incubate for 45min at r.t. 100μl of OptiMEM® was similarly

incubated, then added to the above mix. Transfections were performed in replicates of seven so the above mixture was scaled up by a factor of 7.5 (to allow for any loss). Following a further incubation of the mixture for 30min, cells were washed and 200µl of the mixture was dispensed into each well, before incubating for 5hrs at 37°C. Following incubation, the transfection mixtures were replaced by 4ml of DMEM growth medium and incubation was continued for another 96hrs, after which time cells were harvested (Sections 2.2.10 and 2.2.11). Wells included for negative control purposes, had the pBluescript® II KS(+) empty vector instead of the HBV plasmid in the above mix.

A single well (the seventh well in the above protocol) was used for the purpose of transfection efficiency determination by FACS.

2.6.5.a. Transfection of cells for replication efficiency determination

Co-transfection with a plasmid carrying a reporter gene was used in all experiments. The latter plasmid was for the purpose of determining transfection efficiency. A mole:mole ratio, rather than amount in µg, was used for these co-transfections to avoid discrepancies due to the different sizes of the recombinant plasmids (6.8kb) and the reporter plasmid (4.6kb) (Bigger *et al.* 2001).

2.6.5.b. Transfection of cells for promoter-analysis using luciferase as reporter

2 x 10^4 cells were seeded in 96-well plates, as described above. Co-transfected samples were incubated for 24hrs, after which cell extracts were assayed as described in Section 2.8.1. As with the replication capacity experiments, normalisation was done exactly as was for the replication efficiency, that is by co-transfecting with the green fluorescence expressing protein (pCI-GFP) on a mole:mole ratio [500ng:396ng of promoter (5.8kb), and fluorescence plasmids (4.6kb), respectively]. Transfection efficiency was determined by FACS analyses as described below (Section 2.6.2). The data summarised in Results, Figure 9.9, was following statistical standardisation to 15% transfection efficiency.

2.6.6 Transfection efficiency determination

Cells destined for transfection efficiency determination by FACS were harvested 48hrs following co-transfection (Section 2.6.5), then washed with unsupplemented DMEM, re-suspended in a fresh medium (unsupplemented) and

pelleted by centrifugation at 1,200g for 5min. Resuspension and pelleting was repeated and the pellet was finally resuspended in 1ml PBS containing 1% of formaldehyde fixative, before incubation at r.t for 20–30min. Fixed cells were re-centrifuged and the pellet resuspended in 1ml PBS before being analysed for expression of the green fluorescence protein using a FACS Calibur machine (Becton Dickinson, UK).

Transfection efficiency results obtained by FACS were verified by rough estimation using fluorescence microscopy. For this, 300µl of the remaining FACS cell-suspension was first pelleted, and the pellet was resuspended in a small volume of the overlying liquid by pipetting. The resulting suspension was mounted in DAPI for viewing (Section 2.3.1).

2.7 Electrophoretic Mobility Shift Assays (EMSA)

2.7.1 Extraction of DNA-binding proteins from Huh7 cell nuclei

Huh7 cells were cultured to confluency (Section 2.6) and subsequently used for extraction of nuclear transcription factors for use in the protein-DNA binding assay (below). The extraction procedure was that described previously (Jiang *et al.* 1995a). During the extraction procedure, tubes were kept on ice, and all centrifugations were carried out at 4°C.

Briefly, adherent cells were rinsed with cold PBS, and then detached by adding 1ml of a 1:1 ratio of cold PBS to trpysin. The suspension was transferred to a microfuge tube and the cells centrifuged for 10sec to wash-off the trypsin. Cells were then re-suspended in 2ml cold PBS and centrifuged again for 10sec. The pellet was finally resuspended in 400µl of cold buffer A [10mM HEPES-KOH pH 7.9, 10mM KCL, 1.5mM $MgCl_2$, 0.5mM dithiothreitol (DTT)], allowed to swell on ice for 10min then vortexed for 10sec and centrifuged for 10sec. The resulting pellet was resuspended in 40µl cold buffer C (20mM HEPES-KOH, pH 7.9, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT), then incubated on ice for another 20min for high salt content extraction. Cellular debris were removed by centrifugation for 2min and an aliquot of the supernatant fraction containing the DNA binding proteins taken for determination of protein concentration. The remainder was stored at –70°C.

2.7.2 Determination of protein concentration

Protein concentrations were generally determined using a Bradford assay (Bradford, 1976): 5µl of protein solution was diluted with 95µl of Ultrapure water to give a 100µl final volume. Similarly, BSA (stock 2mg/ml) was serially diluted (0-100µg) and used to generate a standard curve. Diluted samples were dispensed in plastic cuvettes and 2ml of the assay reagent added. The contents of the tubes were mixed by inversion and left to stand at r.t for 2min, after which absorption at A_{595} was measured in an Ultraspec 2000 spectrophotometer (Pharmacia). The absorbance readings were subsequently extrapolated from the standard curve for conversion to concentration values.

2.7.3 Preparation of ^{32}P-labelled DNA sequences

In the analysis of promoter-binding, two complementary oligonucleotides (Section 2.7.4.a) were ^{32}P-labelled (Section 2.7.3) and tested for binding with Huh7-extracted nuclear transcription factors (Section 2.7.1).

2.7.3.a. Annealing of oligonucleotides

Equimolar quantities of the two complementary oligonucleotides (480pmol/µl) were diluted with annealing buffer (10mM Tris-HCl pH 7.5, 20mM NaCl) to a final volume of 100µl (4.8pmol/µl). The solution was heated to 95°C in a heating block, then, allowed to cool slowly on the workbench to r.t. Annealed oligonucleotides were directly radiolabeled (as below) or stored at –20°C.

2.7.3.b. Labeling of annealed oligonucleotides

Annealed oligonucleotides were labeled in a phosphorylation reaction using fresh [γ^{32}P]ATP. The labelling reaction was undertaken in a designated area for radioactive work and all College rules and guidelines, adopted from those laid down by the National Radiological Protection Board (1991) for handling radioactive material, strictly adhered to.

The labeling reaction comprised of the following, added consecutively: 2µl of annealed oligonuclotides, 1µl T4 Polynucleotide Kinase (PNK) buffer, 4µl of Ultrapure water, 1µl (5U) T4 PNK enzyme (New England Biolab) and 1µl [γ^{32}P]ATP (3,000Ci/mmol at 10mCi/ml). The mixture was subsequently incubated at 37°C for 30min. At the end of the incubation, the reaction was diluted with 40µl of Ultrapure

water. Removal of non-incorporated oligonucleotides present in the reaction mixture was achieved using a "clean-up" column (MicroSpin S-200HR, Amersham): the reaction was directly dispensed onto the column and ^{32}P-labelled annealed oligonucleotides were eluted by centrifugation (3,000g) in a microfuge for 1min. Non-labelled oligonucleotides, together with most of the free ^{32}P, were retained by the resin. Such "Hot" probes were stored on a short-term basis at –20°C.

2.7.4 Binding Reactions

2.7.4.a. Bandshift EMSA

A standard DNA-binding reaction was comprised of 7µl Ultrapure water, 2µl 5X Gel Shift Binding Buffer (20% glycerol, 5mM $MgCl_2$, 2.5mM EDTA, 2.5mM EDTA, 250mM NaCl, 50mM Tris HCl pH 7.5), 1µg/µl poly(dI).poly(dC) (Amersham) and 5µg nuclear extract (Section 2.6.2) in a final volume of 10µl. The sample was incubated on ice for 10min, then 1µl of the above synthesised radioactive probe (Section 2.7.5.b.) containing approximately 50–90fmol of [γ^{32}P]ATP, corresponding to 30,000 to 50,000cpm, was added (Section 2.7.3). In competition experiments, a 50-fold molar excess of unlabelled competitor was added to the mixture prior to the addition of nuclear extracts. This mixture was allowed to incubate 30min on ice. Then, 1µl 10X gel loading buffer (250mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 40% glycerol) was added to each sample. Products were electrophoresed on a 4% non-denaturing polyacrylamide gel in 0.5X TBE buffer (Section 2.4.2). Primers that recognised a region in the polymerase gene were also annealed and used in non-specific competitor reactions (Table 2.1).

2.7.4.b. Supershift EMSA

The above protocol was essentially used for supershift assays; Ultrapure water (4µl instead of 6µl) was added to allow for the addition of the 2µl of a specific antibody: Coup-TF, HNF1 and HNF4. The reactions were further incubated on ice for 20min after adding specific antibodies to the appropriate tubes. The protocol was adapted as previously described (Buckwold *et al.* 1997b; Viollet *et al.* 1997b; Yu & Mertz 1997b).

2.8 Luciferase Assays

An extension of the DNA-protein binding studies (described above) involved transfection of constructed reporter gene-promoter plasmids (Section 2.2.4), and assaying for luciferase expression.

2.8.1 Extracting luciferase from cultured cells

The "active cell-lysis" protocol (www.stratagene.com) was used to extract luciferase from Huh7 cells transfected (Section 2.6.5) with a luciferase-expressing promoter/pGL2-basic plasmid. In brief, 24hrs following transfection, cells were washed with 1X PBS buffer. 25µl 1X Cell-Lysis Reagent (Promega, kit-supplied as 5X diluted with Ultrapure water) was used to lyse cells and the lysate was then used for analysis, as below.

2.8.2 Luciferase activity assay

50µl of luciferase-substrate buffer mixture (kit-supplied, Promega) was added to 20µl cell lysate and the mixture was loaded into an opaque 96-well plate designed for use with a luminometer (Berthold Technologies, Hertfordshire, UK). The instrument was programmed to measure luminescence for a period of 10sec, commencing 2sec after the automated injection of the supernatant.

Chapter 3

Results

CHAPTER 3. RESULTS

3.0 Creation of Recombinant Mutants

Parent plasmid p3.8II (Wildtype) has been previously described (Wu *et al.* 1999, 2001). It contained a "more than genome-length" copy of HBV *adr* subtype, genotype C. Using molecular cloning and Site-directed mutagenesis (SDM), this plasmid was used as the common genetic backbone to create a panel of constructs bearing well-characterised mutations in the precore (preC), core promoter (CP), or in both regions within a single construct. The positions mutated were chosen based on well known, or new, mutations reported in the literature and are shown in Tables 3.1 –3.3.

3.1 p3.8II construct analysis

The HBV insert and flanking regions of p3.8II were sequenced in order to determine its primary structure and gene organisation (Sections 3.1.1, 3.1.2 and Figure 3.1), using primers given in the Methods section.

3.1.1 Construct organisation

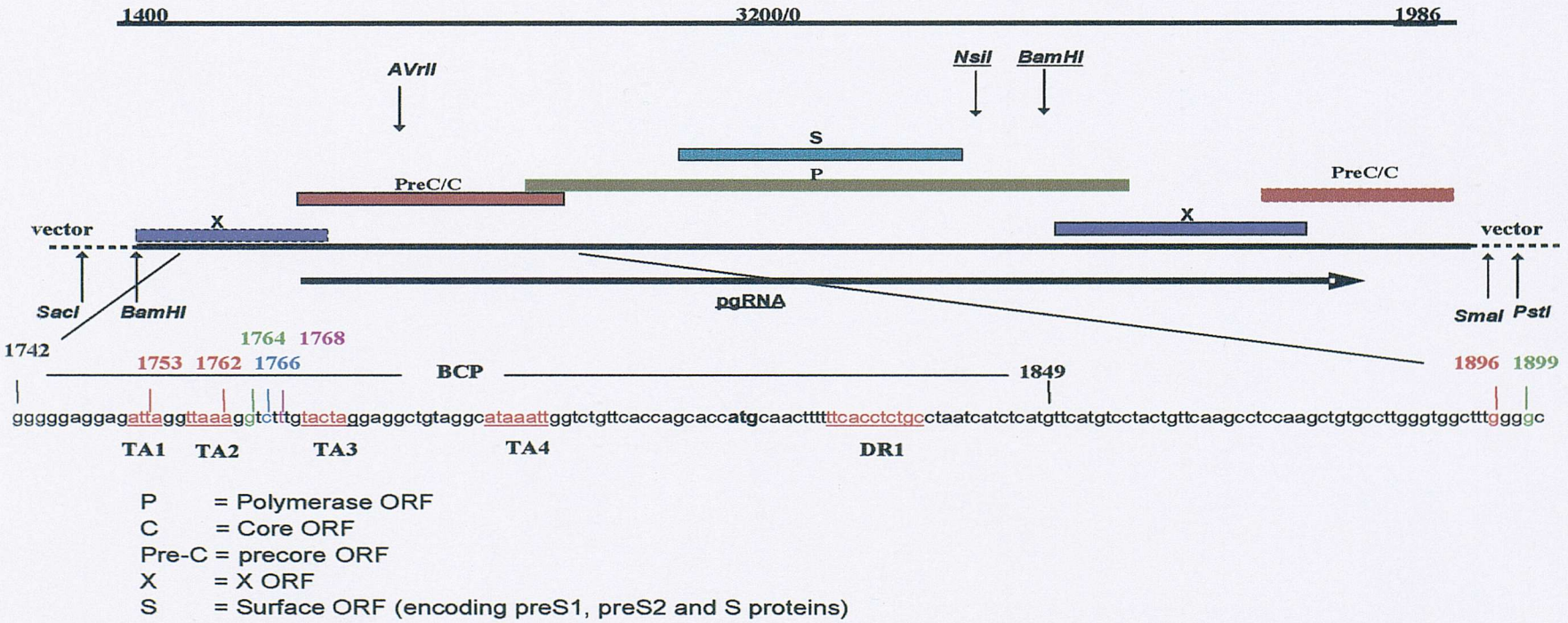
A schematic representation of the construct components that contained the full HBV insert was generated, after sequence editing (See Figure 3.1). Vector-to-insert ligation junctions and vector restriction sites adjacent to these junctions were also mapped (see full vector map in Appendix #4).

Sequencing revealed that the construct contained an HBV fragment of 1.2 times genome-length, with a 5′ terminal redundancy encompassing the entire X Open Reading Frame (ORF), that is, the Enhancer (Enh)II, CP, and Origin of Replication [direct repeat (DR)1, and 2)] regions, as well as the preC ORF and part of the C ORF encompassing its polyadenylation site (Figure 3.1). This recombinant has been shown to be replication competent in transfection experiments (Wu *et al.* 1999). In this study, a panel of mutant recombinants as created by orientation-cloning and PCR-mediated SDM, as shown in Tables 3.2-3.4. They included: preC (for example G1896A and G1899A), CP (for examples C1766T/T1768A, and T1753G/A1762T/G1764A), combined preC/CP (for example A1762T/G1764A/G1896A), "a" determinant/surface (S) (for examples G145R and T131I), and reverse transcriptase (RT)/polymerase (P) (for examples L180M and M204I). The m8 mutant recombinant (also from Professor

Jinlin Hou), based on p3.8II, had the characteristic G1896A and G1899A substitutions in the preC region.

3.1.2 Analysis of p3.8II

Adr – AB026815 showed a 97.9% sequence homology with other *adr* subtypes from the *Genbank.* Nucleotides within the Negative Regulatory Element (NRE) (1611–1634), the Core Upstream Regulatory Sequence (CURS) (1643–1742) and preC (1814–1901) regions were conserved (Table 3.2). Conservative substitutions were seen in two nucleotide positions of the BCP (1719 and 1725) in the WT sequence, which was carried over to all recombinants, but did not lead to an amino acid change. PCR errors also noted on occasion, did not lead to amino acid substitutions and did not affect regulatory regions of the virus. These recombinants were therefore retained for study.



P = Polymerase ORF
C = Core ORF
Pre-C = precore ORF
X = X ORF
S = Surface ORF (encoding preS1, preS2 and S proteins)

Figure 3.1 Schematic representation of the p3.8II construct and the positions of the mutated sites of the core promoter and precore regions. Nucleotide positions that were mutated are indicated with matching colour numbers, letters, and lines. A solid black line represents the 1.2 copy of the HBV genome in pBluescript II KS(+) cloned into *BamHI* and *SmaI* sites. The different ORFs are indicated with filled coloured boxes; those that are truncated are highlighted with dashed-line bordering. Truncation of the ORFs are at the position of insertion into the vector at 1400 (X) and 1986 (preC/C). Nucleotide numbering is from the *EcoRI* site. Lower case letters indicate the BCP and precore region sequences. The TA-rich and DR1 regions are underlined in red, and the preC initiation codon is highlighted in bold.

Tables 3.1–3.3 show nucleotide and amino acid sequence alignment and edition, using the DNASIS program (Hitachi software).

Positions of site-specific mutagenesis in the CP and preC regions	Nucleotide position
	1713 1723 1733 1743 1753
Reference(AB026815)	TACTTCAAAG ACTGTTTATT TAAAGACTGG GAGGAGTTGG GGGAGGAGAT
Wildtype (adr)	TACTTCAAAG ACTGTTTGTT TAAGGACTGG GAGGAGTTGG GGGAGGAGAT
A1762T	---------- -------G-- ---G------ ---------- ----------
G1764A	---------- -------G-- ---G------ ---------- ----------
A1762T/G1764A	---------- -------G-- ---G------ ---------- ----------
C1766T/T1768A	---------- -------G-- ---G------ ---------- ----------
A1762T/G1764A/C1766T	---------- -------G-- ---G------ ---------- ----------
T1753C/A1762T/G1764A	---------- -------G-- ---G------ ---------- ---------C
T1753C/A1762T/G1764A/C1766T	---------- -------G-- ---G------ ---------- ---------C
G1896A	---------- -------G-- ---G------ ---------- ----------
G1899A	---------- -------G-- ---G------ ---------- ----------
G1896A/G1899A	---------- -------G-- ---G------ ---------- ----------
A1762T/G1764A/G1896A	---------- -------G-- ---G------ ---------- ----------
A1762T/G1764A/G1899A	---------- -------G-- ---G------ ---------- ----------
	1763 1773 1783 1793 1803
Reference(AB026815)	TAGGTTAAAG GTCTTTGTAC TAGGAGGCTG TAGGCATAAA TTGGTCTGTT
Wildtype (adr)	TAGGTTAAAG GTCTTTGTAC TAGGAGGCTG TAGGCATAAA TTGGTCTGTT
A1762T	--------T- ---------- ---------- ---------- ----------
G1764A	---------- A--------- ---------- ---------- ----------
A1762T/G1764A	--------T- A--------- ---------- ---------- ----------
C1766T/T1768A	---------- --T-A----- ---------- ---------- ----------
A1762T/G1764A/C1766T	--------T- A-T------- ---------- ---------- ----------
T1753C/A1762T/G1764A	--------T- A--------- ---------- ---------- ----------
T1753C/A1762T/G1764A/C1766T	--------T- A-T------- ---------- ---------- ----------
G1896A	---------- ---------- ---------- ---------- ----------
G1899A	--a------- ---------- ---------- ---------- ----------
G1896A/G1899A	---------- ---------- ---------- ---------- ----------
A1762T/G1764A/G1896A	--------T- A--------- ---------- ---------- ----------
A1762T/G1764A/G1899A	--------T- A--------- ---------- ---------- ----------
	1963 1973 1983 1993 2003
Reference(AB026815)	GTCCTACTGT CAAGCCTCC AAGCTGTGCC TTGGGTGGCT TTGGGGCATG
Wildtype (adr)	GTCCTACTGT CAAGCCTCC AAGCTGTGCC TTGGGTGGCT TTGGGGCATG
A1762T	---------- ---------- ---------- ---------- ----------
G1764A	---------- ---------- ---------- ---------- ----------
A1762T/G1764A	---------- ---------- ---------- ---------- ----------
C1766T/T1768A	---------- ---------- ---------- ---------- ----------
A1762T/G1764A/C1766T	---------- ---------- ---------- ---------- ----------
T1753C/A1762T/G1764A	---------- ---------- ---------- ---------- ----------
T1753C/A1762T/G1764A/C1766T	---------- ---------- ---------- ---------- ----------
G1896A	---------- ---------- ---------- ---------- --A-------
G1899A	---------- ---------- ---------- ---------- -----A----
G1896A/G1899A	---------- ---------- ---------- ---------- --A--A----
A1762T/G1764A/G1896A	---------- ---------- ---------- ---------- --A-------
A1762T/G1764A/G1899A	---------- ---------- ---------- ---------- -----A----

Table 3.1 Nucleotide alignments of parts of the EnhII and C ORF of recombinants bearing mutations in the CP, preC or both regions combined.
The sequence of the reference *adr* subtype (accession number AB026815), and of the WT (p3.8II) are shown at the top from positions 1738–1803, and from 1963–2003. The recombinants generated and the positions of the specific mutations are given in the first column. Nucleotides that were specifically mutated are underlined; non-mutated sequences are noted by short dashes. Bold-marked nucleotides indicate base changes in the reference that differ from the WT sequences. Lower case letters indicate base changes that were sporadically introduced during the mutagenesis PCR. Nucleotide numbering starts at the *EcoRI* site.

Positions of site-specific mutagenesis in the "a" determinant/S, and RT/P regions	Nucleotide position
	35 45 55 65 75
Consensus (AB026815)	CATGGAGAAC ACAACATCAG GATTCCTAGG ACCCCTGCTC GTGTTACAGG
Wildtype (adr)	CATGGAGAGC ACAACATCAG GATTCCTAGG ACCCCTGCTC GTGTTACAGG
L180M	--------G- ---------- ---------- ---------- ----------
M204I	--------G- ---------- ---------- ---------- ----------
L180M/M204V	--------G- ---------- ---------- ---------- ----------
G145R/M204I	--------G- ---------- ---------- ---------- ----------
M204V	--------G- ---------- ---------- ---------- ----------
G145R/L180M/M204V	--------G- ---------- ---------- ---------- ----------
T131I	-------G- ---------- ---------- ---------- ----------
L141E	-------G- ---------- ---------- ---------- ----------
G145R	-------G- ---------- ---------- ---------- ----------
	85 95 105 115 125
Consensus (AB026815)	CGGGGTTTTT CTTGTTGACA AAAATCCTCA CAATACCACA GAGTCTAGAC
Wildtype (adr)	CGGGGTTTTT CTTGTTGACA AGAATCCTCA CAATACCACA GAGTCTAGAC
L180M	---------- ---------- -G-------- ---------- ----------
M204I	---------- ---------- -G-------- ---------- ----------
L180M/M204V	---------- ---------- -G-------- ---------- ----------
G145R/M204I	---------- ---------- -G-------- ---------- ----------
M204V	---------- ---------- -G-------- ---------- ----------
G145R/L180M/M204V	---------- ---------- -G-------- ---------- ----------
T131I	---------- ---------- -G-------- ---------- ----------
L141E	---------- ---------- -G-------- ---------- ----------
G145R	---------- ---------- -G-------- ---------- ----------
	135 145 155 165 175
Consensus (AB026815)	CCAAAATTCG CAGTCCCCAA CCTCCAATCA CTCACCAACC TCTTGTCCTC
Wildtype (adr)	CCCAAATCGG CAGTCCCCAA CCTCCAATCA CTCACCAACC TCTTGTCCTC
L180M	--C----CG- ---------- ---------- ---------- ----------
M204I	--C----CG- ---------- ---------- ---------- ----------
L180M/M204V	--C----CG- ---------- ---------- ---------- ----------
G145R/M204I	--C----CG- ---------- ---------- ---------- ----------
M204V	--C----CG- ---------- ---------- ---------- ----------
G145R/L180M/M204V	--C----CG- ---------- ---------- ---------- ----------
T131I	--C----CG- ---------- ---------- ---------- ----------
L141E	--C----CG- ---------- ---------- ---------- ----------
G145R	--C----CG- ---------- ---------- ---------- ----------
	235 245 255 265 275
Consensus (AB026815)	CAATTTGTCC TGGCTATCGC TGGATGTGTC TGCGGCGTTT TATCATATTC
Wildtype (adr)	CAATTTGTCC TGGTTATCGT TGGATGTGTC TGCGGCGTTT TATCATATTC
L180M	---------- ---T-----T ---------- ---------- ----------
M204I	---------- ---T-----T ---------- ---------- ----------
L180M/M204V	---------- ---T-----T ---------- ---------- ----------
G145R/M204I	---------- ---T-----T ---------- ---------- ----------
M204V	---------- ---T-----T ---------- ---------- ----------
G145R/L180M/M204V	---------- ---T-----T ---------- ---------- ----------
T131I	---------- ---T-----T ---------- ---------- ----------
L141E	---------- ---T-----T ---------- ---------- ----------
G145R	---------- ---T-----T ---------- ---------- ----------
	285 295 305 315 325
Consensus (AB026815)	CTCTTCATCC TGCTGCTATG CCTCATCTTC TTGTTGGTTC TTCTGGACTA
Wildtype (adr)	--TC------ -----G---- -G-------- ---------- -----A----
L180M	--TC------ -----G---- -G-------- ---------- -----A----
M204I	--TC------ -----G---- -G-------- ---------- -----A----
L180M/M204V	--TC------ -----G---- -G-------- ---------- -----A----
G145R/M204I	--TC------ -----G---- -G-------- ---------- -----A----
M204V	--TC------ -----G---- -G-------- ---------- -----A----
G145R/L180M/M204V	--TC------ -----G---- -G-------- ---------- -----A----
T131I	--TC------ -----G---- -G-------- ---------- -----A----
L141E	--TC------ -----G---- -G-------- ---------- -----A----
G145R	--TC------ -----G---- -G-------- ---------- -----A----
	385 395 405 415 425
Consensus (AB026815)	GCACGGGACC ATGCAAGACC TGCACGATTC CTGCTCAAGG AACCTCTATG
Wildtype (adr)	GCACGGGACC ATGCAAGACC TGCACGATTC CTGCTCAAGG AACCTCTATG
L180M	---------- ---------- ---------- ---------- ----------
M204I	---------- ---------- ---------- ---------- ----------
L180M/M204V	---------- ---------- ---------- ---------- ----------
G145R/M204I	---------- ---------- ---------- ---------- ----------
M204V	---------- ---------- ---------- ---------- ----------
G145R/L180M/M204V	---------- ---------- ---------- ---------- ----------
T131I	---------- ---------- ---------- ---------- --T-------
L141E	---------- ---------- ---------- ---------- ----------
G145R	---------- ---------- ---------- ---------- ----------
	435 445 455 465 475
Reference (AB026815)	TTTCCCTCTT GTTGCTGTAC AAAACCTTCG GACGGAAACT GCACTTGTAT
Wildtype (adr)	TTTCCCTCCT GTTGCTGTAC AAAACCTTCG GACGGAAACT GCACTTGTAT
L180M	--------C- ---------- ---------- ---------- ----------
M204I	--------C- ---------- ---------- ---------- ----------
L180M/M204V	--------C- ---------- ---------- ---------- ----------
G145R/M204I	--------C- ---------- ---------- ---A------ ----------
M204V	--------C- ---------- ---------- ---------- ----------
G145R/L180M/M204V	--------C- ---------- ---------- ---A------ ----------
T131I	--------C- ---------- ---------- ---------- ----------
L141E	--------C- ---------- -G-------- ---------- ----------
G145R	--------C- ---------- ---------- ---A------ ----------
	535 545 555 565 575
Reference (AB026815)	CAGTCCGTTT CTCCTGGCTC AGTTTACTAG TGCCATTTGT TCAGTGGTTC
Wildtype (adr)	CAGTCCGTTT CTCCTGGCTC AGTTTACTAG TGCCATTTGT TCAGTGGTTC
L180M	---------- ---A------ ---------- ---------- ----------
M204I	---------- ---------- ---------- ---------- ----------
L180M/M204V	---------- ---A------ ---------- ---------- ----------
G145R/M204I	---------- ---------- ---------- ---------- ----------
M204V	---------- ---------- ---------- ---------- ----------
G145R/L180M/M204V	---------- ---A------ ---------- ---------- ----------
T131I	---------- ---------- ---------- -
L141E	---------- ---------- ---------- -
G145R	---------- ---------- ---------- -
	585 595 605 615 625
Reference(AB026815)	GCAGGGCTTT CCCCCACTGT TTGGCTTTCA GTTATATGGA TGATGTGGTA
Wildtype (adr)	GTAGGGCTTT CCCCCACTGT TTGGCTTTCA GTTATATGGA TGATGTGGTA
L180M	-T-------- ---------- ---------- ---------- ----------
M204I	-T-------- ---------- ---------- --------C- ----------
L180M/M204V	-T-------- ---------- ---------- -----G---- ----------
G145R/M204I	-T-------- ---------- ---------- --------C- ----------
M204V	-T-------- ---------- ---------- -----G---- ----------
G145R/L180M/M204V	-T-------- ---------- ---------- -----G---- ----------

YMDD motif

itions of site-specific mutagenesis in the "a" determinant/S, and RT/P regions	Nucleotide position
	685 695 705 715 725
Reference(AB026815)	CAATTTTCTT TTGTCTTTGG GTATACATTT GAACCCTAAT AAAACCAAAC
Wildtype (adr)	CAATTTTCTT TTGTCTTTGG GTATACATTT GAACCCCAAT AAAACCAAAC
L180M	---------- ---------- ---------- ------C--- ----------
M204I	---------- ---------- ---------- ------C--- ----------
L180M/M204V	---------- ---------- ---------- ------C--- ----------
G145R/M204I	---------- ---------- ---------- ------C--- ----------
M204V	---------- ---------- ---------- ------C--- ----------
G145R/L180M/M204V	---------- ---------- ---------- ------C--- ----------
	735 745 755 765 775
Reference(AB026815)	GTTGGGGCTA CTCCCTTAAC TTCATGGGGT ATGTAATTGG AAGGTGGGGT
Wildtype (adr)	GTTGGGGCTA TTCCCTTAAT TTCATGGGAT ATGTAATTGG ATGTTGGGGT
L180M	---------- T--------T --------A- ---------- -T-T------
M204I	---------- T--------T --------A- ---------- -T-T------
L180M/M204V	---------- T--------T --------A- ---------- -T-T------
G145R/M204I	---------- T--------T --------A- ---------- -T-T------
M204V	---------- T--------T --------A- ---------- -T-T------
G145R/L180M/M204V	---------- T--------T --------A- ---------- -T-T------

Table 3.2 Alignment of nucleotide sequences from the RT/P domain (includes, 9–775) and the overlapping S gene (includes, 1–530) regions.
Sequences for parts of the overlapping S and RT domains A–E are presented. Nucleotide numbering is from the start of the RT domain; nucleotide 26 coincided with the first base of the initiation codon (bold and underlined ATG) of the small S gene. Specifically substituted nucleotides are underlined; non-mutated sequences are noted by short dashes. Bold-marked nucleotides indicate base changes in the reference that differ from the WT sequences. The area of the YMDD motif, are from nucleotides 607 to 619.

(A)

Positions of site-specific mutagenesis in the "a" determinant/S region	Amino acid position
	10 20 30 40 50
Reference (AB026815)	MENTTSGFLG PLLVLQAGFF LLTKILTIPQ SLDSWWTSLN FLGGAPTCPG
Wildtype (adr)	MESTTSGFLG PLLVLQAGFF LLTRILTIPQ SLDSWWTSLN FLGGAPTCPG
T131I	--S------- ---------- ---R------ ---------- ----------
L141E	--S------- ---------- ---R------ ---------- ----------
G145R	--S------- ---------- ---R------ ---------- ----------
	60 70 80 90 100
ReferenceAB026815)	QNSQSPTSNH SPTSCPPICP GYRWMCLRRF IIFLFILLLC LIFLLVLLDY
Wildtype (adr)	PNRQSPTSNH SPTSCPPICP GYRWMCLRRF IIFLLILLVC VIFLLVLLNY
T131I	P-R------- ---------- ---------- ----L---V- V-------N-
L141E	P-R------- ---------- ---------- ----L---V- V-------N-
G145R	P-R------- ---------- ---------- ----L---V- V-------N-
	110 120 130 140 150
Reference (AB026815)	QGMLPVCPLL PGTSTTSTGP CKTCTIPAQG TSMFPSCCCT KPSDGNCTCI
Wildtype (adr)	QGMLPVCPLL PGTSTTSTGP CKTCTIPAQG TSMFPSCCCT KPSDGNCTCI
T131I	---------- ---------- ---------- I--------- ----------
K141E	---------- ---------- ---------- ---------- E---------
G145R	---------- ---------- ---------- ---------- ----R-----

(B)

Positions of site-specific mutagenesis in the RT/P region	Amino acid position
	69 79 89 99 109
Reference (AB026815)	PKFAVPNLQS LTNLLSSNLS WLSLDVSAAF YHIPLHPAAM PHLLVGSSGL
Wildtype (adr)	PKSAVPNLQS LTNLLSSNLS WLSLDVSAAF YHIPSHPAAM RHLLVGSSEL
L180M	--S------- ---------- ---------- ----S---G- R-------E-
M204I	--S------- ---------- ---------- ----S---G R-------E-
L180M/M204V	--S------- ---------- ---------- ----S---G- R-------E-
G145R/M204I	--S------- ---------- ---------- ----S---G- R-------E-
M204V	--S------- ---------- ---------- ----S---G- R-------E-
G145R/L180M/M204V	--S------- ---------- ---------- ----S---G- R-------E-
	119 129 139 149 159
Reference (AB026815)	PRYVARLSST SRNINYQHGT MQDLHDSCSR NLYVSLLLLY KTFGRKLHLY
Wildtype (adr)	PRYVARLSST SRNINYQHGT MQDLHDSCSR NLYVSLLLLY KTFGRKLHLY
L180M	---------- ---------- ---------- ---------- ----------
M204I	---------- ---------- ---------- ---------- ----------
L180M/M204V	---------- ---------- ---------- ---------- ----------
G145R/M204I	---------- ---------- ---------- ---------- ----Q-----
M204V	---------- ---------- ---------- ---------- ----------
G145R/L180M/M204V	---------- ---------- ---------- ---------- ----Q-----
	169 179 189 199 209
Reference (AB026815)	SHPIILGFRK IPMGVGLSPF LLAQFTSAIC SVVRRAFPHC LAFSymddVV
Wildtype (adr)	SHPIILGFRK IPMGVGLSPF LLAQFTSAIC SVVRRAFPHC LAFSymddVV
L180M	---------- ---------- -M-------- ---------- ----------
M204I	---------- ---------- ---------- ---------- -----I----
L180M/M204V	---------- ---------- -M-------- ---------- -----V----
G145R/M204I	---------- ---------- ---------- ---------- -----I----
M204V	---------- ---------- ---------- ---------- -----V----
G145R/L180M/M204V	---------- ---------- -M-------- ---------- -----V----
	219 229 239 249 259
Reference (AB026815)	LGAKSVQHLE SLFTSITNFL LSLGIHLNPN KTKRWGYSLN FMGYVIGSWG
Wildtype (adr)	LGAKSVQHLE SLFTSITNFL LSLGIHLNPN KTKRWGYSLN FMGYVIGCWG
L180M	---------- ---------- ---------- ---------- -------C--
M204I	---------- ---------- ---------- ---------- -------C--
L180M/M204V	---------- ---------- ---------- ---------- -------C--
G145R/M204I	---------- ---------- ---------- ---------- -------C--
M204V	---------- ---------- ---------- ---------- -------C--
G145R/L180M/M204V	---------- ---------- ---------- ---------- -------C--

Table 3.3 Alignment of the deduced amino acid sequences of parts of the HBV small S (1–150 amino acids) and P (09–349 amino acids) genes.

(A). Amino acid alignment of a region of the S (small) gene. Numbering shown takes the ATG at the start of the gene as 001. **(B)**. Amino acid alignment of domains A–E of the RT/P gene. Indicated numbering takes the GAG (Glu) at the start of the RT region as 001 (Stuyver *et al*, 2001). Specifically-introduced nucleotides are underlined; non-mutated sequences are noted by short dashes. Bold-marked nucleotides indicate base changes in the reference that differ from the WT sequence. The area of YMDD motif is from amino acids 205 to 208, and indicated with lower case letters.

3.2 Full-length Site-Directed Mutagenesis

Full-length SDM was initially tested in order to engineer CP and preC mutations, using p3.8II as template. At this stage, problems were encountered in generating these constructs.

3.2.1 Strategy 1. Site-directed mutagenesis by the QuikChange® Multi Site system

Introduction of specific mutations into the p3.8II was first attempted using the QuickChange® Multi Site-Directed Mutagenesis Kit (Stratagene, LaJolla, California), a technology engineered to introduce specific mutations in primers at multiple sites (up to five) in a single round using almost any template up to 8kb (www.stratagene.com).

3.2.1.a. Primer design

Mutagenic primers were designed based on Stratagene's "Mutagenic Primer Design" recommendations suitable for the procedure:

1. All primers used were designed to anneal to the sense strand.
2. Primers were selected to bind to separate regions on the same strand of the template plasmid (for example, CP and preC regions).
3. Primers contained between 25 and 45 nucleotides, with a Tm of $\geq$ 75 °C and a G-C content of 40%. Primer working concentration: 100ng/primer.
4. The specific mutations were placed close to the middle of a primer, which terminate in one or more "*C*" or "*G*" nucleotides at the 3′–end.

Despite adherence to the recommendations for mutagenic primer design, the results of the mutagenesis experiments showed problems that were later discovered to be caused by the presence of the 600bp repeat in the p3.8II parental template. On restriction digestion of plasmid DNA from mutagenic colonies and subsequent sequencing, it was noted that most of the resulting PCR product were truncated. Although some of the "looping-out" can be explained as being due to the release of a 2.9kb HBV-DNA fragment at the region that is located between the two preC/core (C) regions of the p3.8II construct (Figures 3.1), truncation seen in most of the remaining colonies following *BamHI* digestion (as Figure 3.2, lanes 4–6, 8, 11–13 and 15) were hard to explain. The looping-out of the 2.9kb fragment, stated above is detailed below:

Subsequent to *BamHI* digestion of the mutated constructs generated, sizes were estimated as app. 3.9kb; that is, 3.0kb of vector + 0.9kb fragment from the remaining HBV insert (Figure 3.2, lane 7). These single bands obtained with mutants after digestion with *BamHI* was at variance with the expected two bands. This was confirmed in lane 14 (Figure 3.2) which was loaded with a *BamHI*-digestion product of the parent plasmid; 3.6 and 3.2kb fragments are clearly seen. This indicated that part of the sequence containing a *BamHI* site was missing.

When a representative of each of the two types of truncated clones (lanes 4 and 7) described above were used as templates in an *AvrII/NsiI* double-digestion (see parent plasmid map, Figure 3.1), an 890bp (app. 0.9kb) HBV fragment was released (lanes 2 and 9: digestion was duplicated) for the lane 7 digestion, but no fragment release was seen when the undigested clone in lane 4 was *AvrII/NsiI* double-digested (lanes 1 and 10: digestion was duplicated). At least for lanes 2 and 9, the results seen confirm that the *BamHI* site at the insert-vector junction may still be intact.

Several attempts were made to rectify this problem, incorporating Stratagene suggestions. These included increasing the volume (thus the U/μl) of the supplied QuikChange® Multi enzyme blend (of unspecified concentration), in the hope of providing optimal conditions for ligation of the PCR strands during the PCR.

Notwithstanding the above alterations, ligation efficiency was only marginally improved.



Figure 3.2 Restriction enzymic digestion of plasmid DNA following full-length SDM of the p3.8II template.
Lanes 4–6, 7, 8, 11–14, and 15 were loaded with mutant colonies digested with *BamHI,* giving a linearised fragment (> 5.00kb) consisting of vector + HBV fragment. Lanes 1 and 10 contain mutant colony (lane 4) double-digested with *AvrII* and *NsiI* giving an 890bp fragment. Lanes 2 and 9 (in duplicate) contain mutant colony (lane 7) double-digested with *AvrII* and *NsiI* giving an 890bp fragment. Lane 3 was loaded with undigested plasmid DNA.
Lane 14: *BamHI*-digestion of the p3.8II parent construct.
Lane M, 1kb DNA markers (Promega). Resolution of bands was on a 1.5% (w/v) agarose gel.

The following restriction endonucleases have digestion sites in the p3.8II construct at nt. positions: 1400 and 3217 (*BamHI)*, 1995 (*AvrII*), and 2885 (*NsiI*). (See p3.8II map: Figure 3.1).

3.2.1.b. Sequence fidelity of PCR products

The "loop-out" problem was not encountered with constructs that involved mutagenesis of the RT and "a" determinant regions. However, on sequencing such constructs, sporadic nucleotide substitutions were discovered (Figure 3.3, panel A) in some of the generated full-length mutant clones. These erroneous point mutations occurred despite the use of the high fidelity polymerase; they would have defeated the purpose of using a common genetic background to create various mutants. Furthermore, some of these mutations affected important regulatory elements, such as polyadenylation sites, where an insertion of a *T* was found in some of the analysed constructs (3.3, panel B).

To reduce mutations caused by erroneous DNA synthesis, the concentration of magnesium ions, dNTPs, and pH found to affect sequence fidelity were altered

(Ling and Robinson 1997). For the same reason, the PCR cycle number was also reduced (from the recommended 30 cycles, down to 15). However, the result was an enormous decrease in the number of colonies to < 10%, and, in spite of improved fidelity, a mutation rate of 1–2% *vs.* 4–7% was observed. The fact that isolating full-length mutant colonies were rare following optimisations, made obtaining a desired mutant with low sporadic mutations a difficult, possibly, unsurmountable challenge. Using a larger amount of the p3.8II methylated template (150ng *vs.* 100ng) which was readily removed by digestion with *DpnI*, led only to a marginal improvement. These protocol optimisations continued to create non-specifically introduced mutations at conserved HBV regions in some of the recombinants. Therefore, a strategy similar to that adopted for use with the CP and preC mutants was explored for creating some of the RT/P and "a" determinant/S mutant recombinants.

3.3 New strategy for Site-directed mutagenesis

To circumvent the above described SDM experimental difficulties, enzymic digestion and sub-cloning protocols were employed. The position of restriction enzyme sites in the construct were first determined and mapped using the HBV-insert sequencing results and pBluescript® II KS(+) nucleotide details (www.stratagene.com, and Appendix 4): p3.8II was rendered replication-incompetent by removing a 0.9kb fragment which contained the 0.6kb repeat segment to generate a construct designated p3.8IIΔ. Following SDM, the repeat fragment that had been removed was re-introduced into each mutant for replication-competency. Figure 3.4 is a summary of the new strategy used for this purpose.

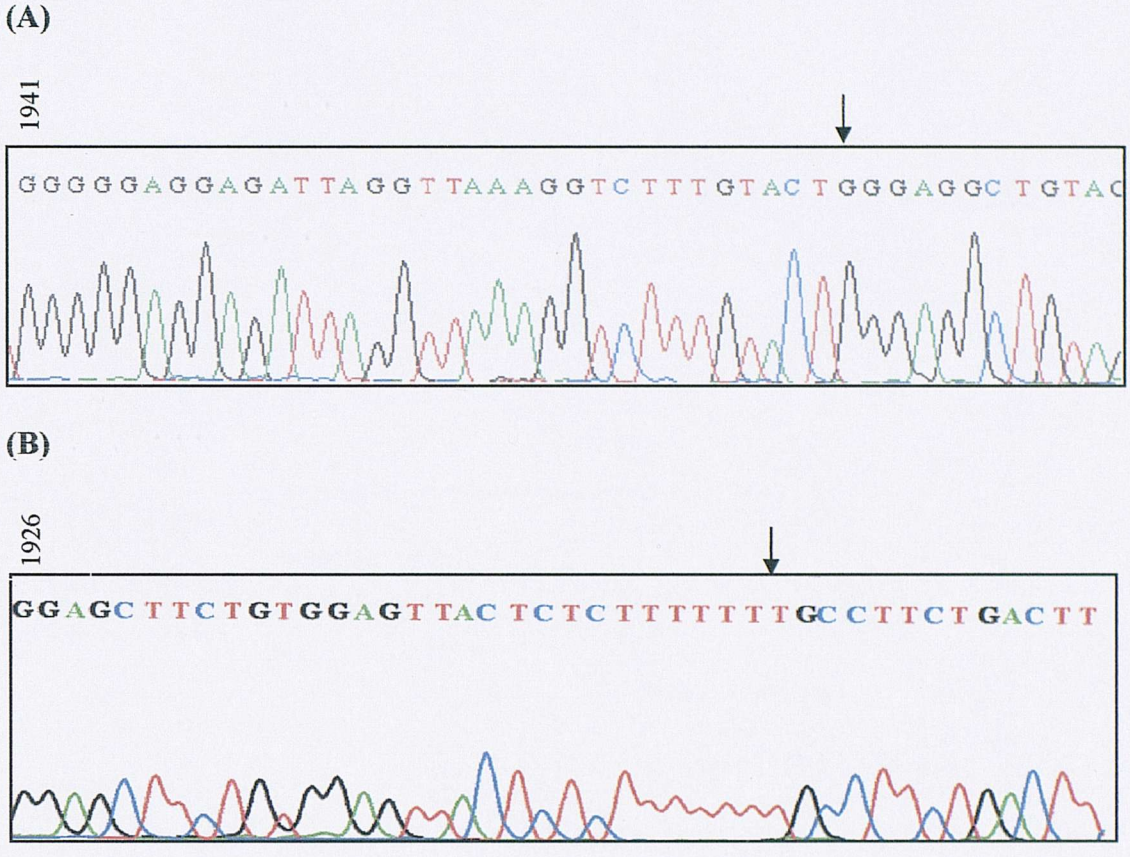


Figure 3.3 Electrophoregrams showing random base substitutions in the p3.8II CP and preC regions during full-length SDM.
Sequences were obtained using an automated sequencer and T7 primer binding to the RNA polymerase promoter in pBluescript® II SK(+). Mutagenesis was carried out with the QuikChange® Multi Site-Directed Mutagenesis Kit, using primers that bore specific nucleotide substitution(s). **(A)**. nucleotide substitution of A → G at CP position 1775, as indicated. **(B)**. Insertion of an extra *T* at position 1956 in the core-encoding region.
The start of nucleotide numberings, are shown. Nucleotide numbering starts at the *EcoRI* site.

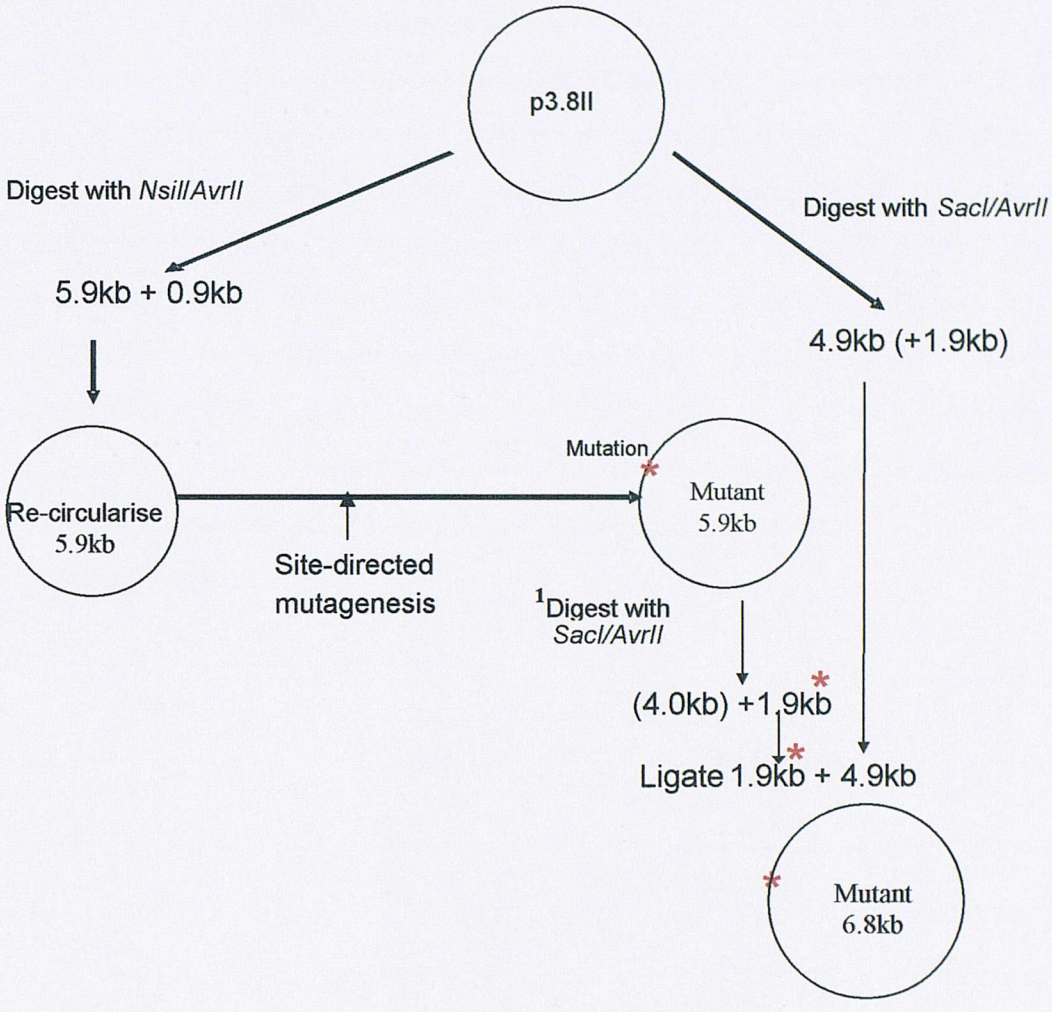


Figure 3.4 Strategy adopted for the introduction of mutations by SDM into the common genetic background (p3.8IIΔ).
Following *PstI/NsiI* double-digestion of the parent plasmid, the larger linearised 5.9kb fragment was re-circularised and used as template in all SDM experiments. Subsequent to mutagenesis, plasmid DNA from mutant colonies were double-digested with *SacI/AvrII* to release a 1.9kb fragment containing the desired mutation (*), or mutations (not shown). This fragment was then ligated to a 4.9kb fragment generated by double-digestion of the p3.8II parent plasmid with *SacI/AvrII*. The final re-ligated product was a 6.8kb construct, (as shown), containing the desired mutation (*), or mutations (not shown).

[1]This stage proved unnecessary for the synthesis of S and P gene mutant recombinants. Instead, following successful SDM, the plasmid was double-digested with *PstI/NsiI* enzymes, and the previously removed 0.6kb repeat then re-ligated to generate full-length mutant recombinants.

3.3.1 Template preparation for mutagenesis

In preparation for SDM, parent plasmid, p3.8II, was digested with *NsiI* and *PstI* restriction endonucleases. Two fragments resulted from this digestion: (1) a smaller 0.9kb fragment containing the 0.6kb repeat plus a 0.3kb fragment from the P gene and (2) a larger 5.9kb fragment with the entire 3.0kb vector plus 2.9kb of the insert (Figure 3.5). Following gel purification, the larger 5.9kb fragment, designated p3.8IIΔ, was re-circularised and used as template for subsequent SDM attempts. Its compatible "sticky ends" obtained by *NsiI* and *PstI* double-digestion facilitated re-circularisation of this fragment. Recombination deficient (*recA*) cells transformed with p3.8IIΔ produced colonies which were analysed for potential use as templates for mutagenesis.

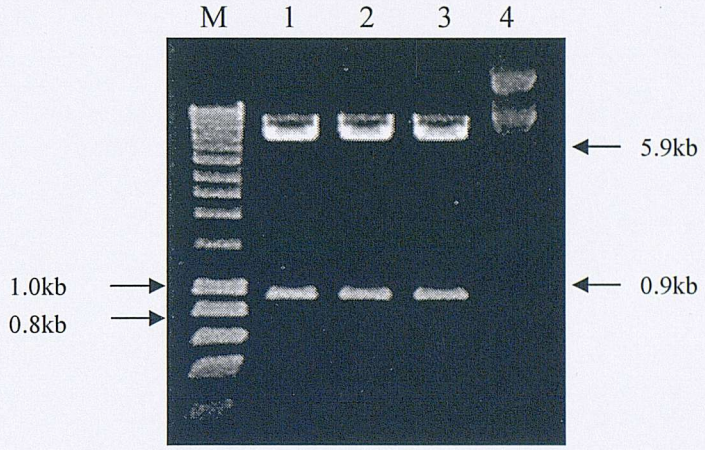


Figure 3.5 *NsiI/PstI* double-digestion of p3.8II.
Lanes 1, 2 and 3 show the small (0.9kb) and large (5.9kb) fragments produced from three colonies isolated. Lane 4 contains undigested plasmid (from colony 3). Lane M contains 1kb DNA markers. Resolution of bands was on a 1% (w/v) agarose gel.

3.3.2 Analysis of template plasmids

In the initial stages following transformation of p3.8IIΔ, DNA isolated from transformants were *BamHI*-digested to eliminate possible contamination with colonies from the original 6.8kb construct which may have been carried over during gel extraction of the 5.9kb fragment. DNA from such contaminating colonies when digested with *BamHI* would have released fragments of 3.2kb and 3.6 kb, as shown (Figure 3.6, lane 3). The pBluescript® II KS(+) vector into which the full-length HBV insert was ligated has no other *BamHI* site other than the one present at the vector-insert junction (See Figure 3.1).

DNA preparations yielding linearised 5.9kb fragments with *BamHI* digestion (Figure 3.6, lanes 1, 2 and 4) indicated successful generation of the desired p3.8IIΔ plasmid template. These were subsequently sequenced to identify any plasmids with sporadic mutations in critical positions produced during the mutagenesis stage.

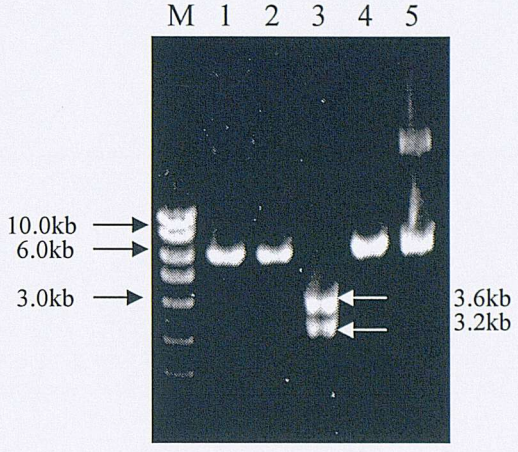


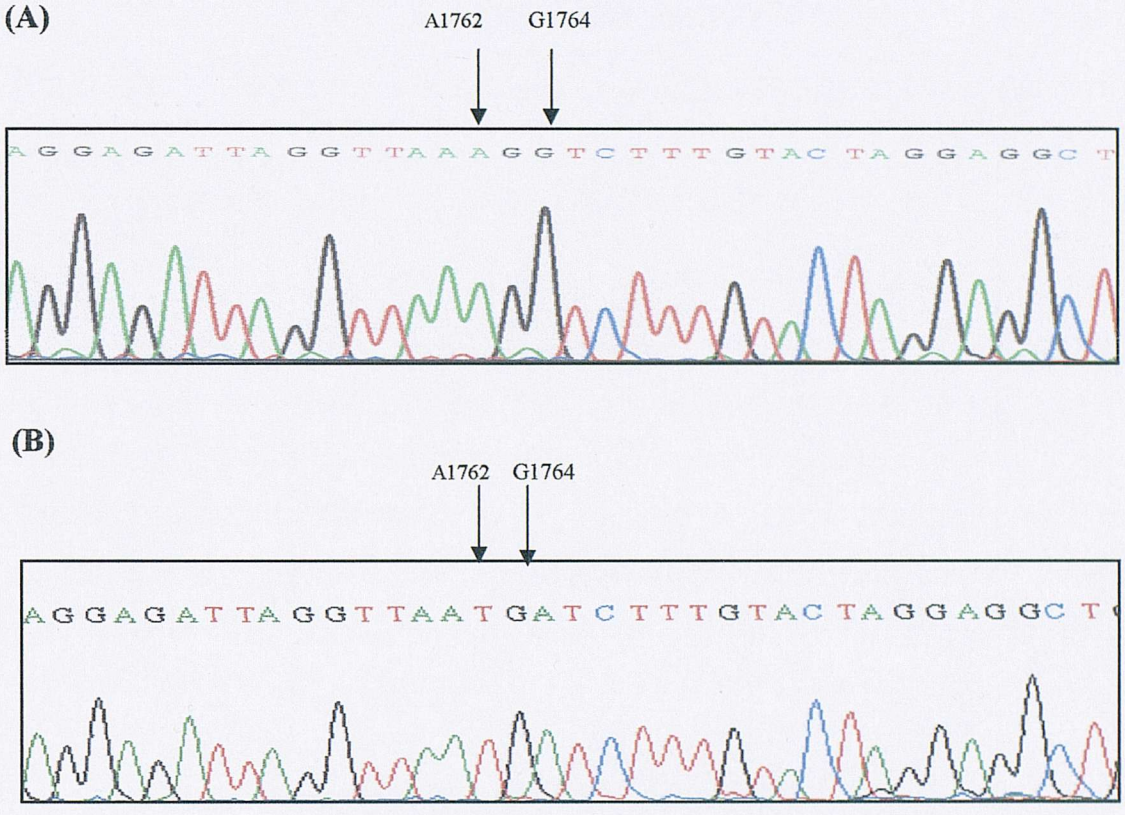
Figure 3.6 *BamHI* restriction digestion of p3.8IIΔ, and p3.8II (as control). Lanes 1, 2 and 4, linearised p3.8IIΔ (5.9kb) from different colonies. Lane 3, p3.8II digestion yielding expected 3.2kb and 3.6kb fragments. Lane 5, undigested p3.8IIΔ showing linear, and super-coiled DNA. Lane M, 1kb DNA markers. Band resolution was on a 1% (w/v) agarose gel.

3.3.3 Mutant sequence confirmation

Since DNA isolated from most *E.coli* strains is methylated, PCR mutagenesis products were first *DpnI*-treated prior to transformation. Methylation of *A* bases in *DpnI* restriction sites renders the p3.8IIΔ template susceptible to digestion whilst leaving intact newly synthesised mutated unmethylated DNA. DNA extracted from potential mutant transformants was digested with *BamHI* and sequenced to eliminate potential "false" positive colonies from the p3.8IIΔ template and colonies with PCR "error" corresponding to nucleotide substitutions, deletions or insertions.

To confirm the presence of desired mutations as, for example, the A1762T/G1764A double mutations (Figure 3.7) in the CP or, the G1896A mutation in the preC region, constructs were sequenced using a T7 primer with complementary homology to the promoter of the pBluescript® II KS(+) plasmid. This primer generally produced sequences of 600-800bp corresponding to regions between the *BamHI* ligation site in pBluescript® II KS(+) and the HBV insert (position 1400 onwards, Figure 3.1). Recombinants that contained the desired mutation(s) were

further sequenced to check the integrity of the remainder of the HBV insert; only DNA colonies with no mutations in conserved areas were chosen for further analysis. Overall, a number of sequencing experiments were carried out aimed at identifying appropriate mutations for further studies.



Figures 3.7 Electrophoregrams indicating WT and mutant sequences within the BCP region of parent and recombinant plasmids.
(A). WT sequence with *A* and *G* at positions 1762 and 1764, respectively.
(B). CP recombinant with A1762T and G1764A substitutions.

3.3.4 Generation of the 6.8kb mutant recombinants

Fully sequenced 5.9kb mutant plasmids selected for further study on the basis of mutant integrity and location were double-digested with *SacI* and *AvrII* to release a 1.9kb fragment which bore the mutation(s) of interest, and a 4.9kb fragment (Figure 3.8, lanes 1–3).

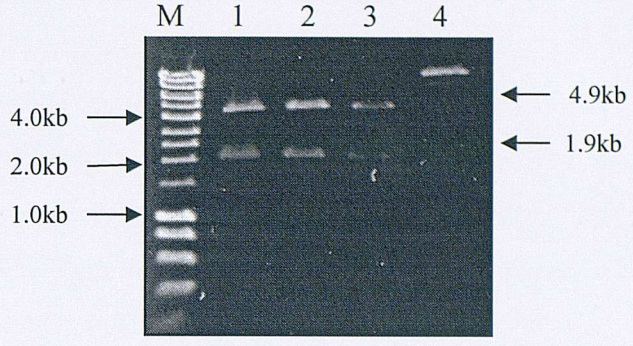


Figure 3.8 ***Sac I/AvrII*** **double-digestion of mutated p3.8IIΔ constructs.**
Lanes 1–3, double-digestion of mutated p3.8IIΔ plasmid (from the same colony[1]). Products of the correct lengths, 1.9kb and 4.9kb were observed by electrophoresis on a 1.0% (w/v) agarose gel. Lane 4, undigested plasmid and Lane M, 1kb DNA markers.

(The above stage would be excluded in constructing the "a" determinant/S and RT/P gene mutant recombinants). The 1.9kb mutant fragment was gel extracted for subsequent sub-cloning. Concurrently, the original 6.8kb plasmid was double-digested with the above two enzymes, to produce 4.9kb (backbone) and 1.9kb fragment (Figure 3.9, 1–3). The 4.9kb backbone had ends compatible with those in the 1.9kb mutated fragment, facilitating the ligation of the two. Full 6.8kb recombinants were generated in this way as determined, in Figure 3.11

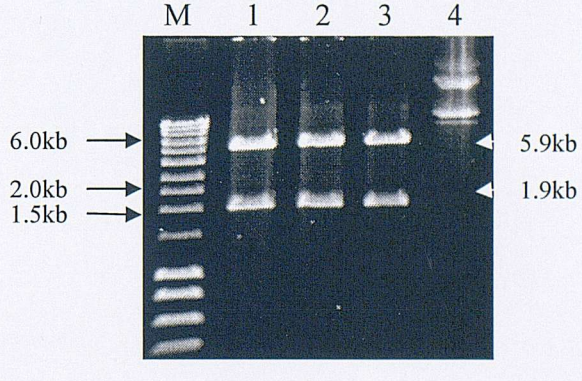


Figure 3.9 ***SacI/AvrII*** **double-digestion of the p3.8II parent plasmid.**
Lanes 1–3, p3.8II, (from the same colony[1]). Lane 4, undigested plasmid. Lane M, 1.0kb DNA markers. Electrophoresis was on 1% agarose gel (w/v).

[1] Gel-extracted DNA from the different wells, loaded with digestion product of the same colony (Figures 3.8, and 3.9), were pooled to increase concentration of final product.

3.3.5 Confirmation of full-length mutant recombinants

The ligation of the 1.9kb mutated fragment to the 4.9kb WT backbone was followed after bacterial transformation. Two control transformations were also set up, one with un-ligated linearised backbone plasmid DNA (4.9kb) and the other with the un-ligated mutant inserts (1.9kb). When colonies from control transformations corresponded to more than a third of the total number of colonies found on test plates, the experiment, apparently due to incomplete *DpnI* digestion, was repeated. To circumvent this problem, enzyme concentration was increased, and longer periods of time allowed for complete digestion of the template. Where digestion efficiency was sufficiently adequate, colonies were picked, and plasmid DNA extracted from transformants was digested with *BamHI* (Figure 3.10) to confirm the re-constitution of the original 6.8kb plasmid length. The region that spanned the two *AvrII/SacI* re-ligated junctions was sequenced to confirm identity and purity of the mutant. At this stage, amino acid sequencing showed a single sporadic substitution only (Figure 3.1), which was of a conservative nature and was therefore not considered significant. The above described procedure was adopted for the construction of each recombinant mutant generated for eventual transfection into hepatoma cell lines.

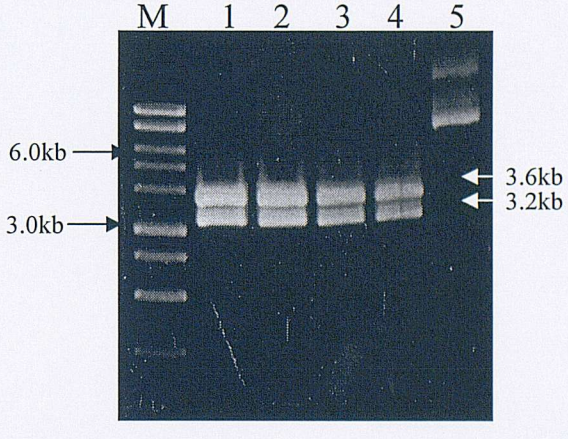


Figure 3.10 *BamHI*-digestion of re-constituted 6.8kb mutant recombinants. Lanes 1–4, *BamHI* digests of mutated recombinant 6.8kb plasmids (from different colonies). Expected products, of 3.2kb and 3.6kb in size, reflected successful ligation. Lane 5, undigested plasmid. Lane M, 1.0kb DNA markers (Promega). Resolution of bands by electrophoresis was on a 1% (w/v) agarose gel.

4.0 Validation of Assays

To validate the sensitivity and specificity of the techniques subsequently used in this study, the kinetics of HBV replication in the 2.2.15 hepatoma cell line were examined.

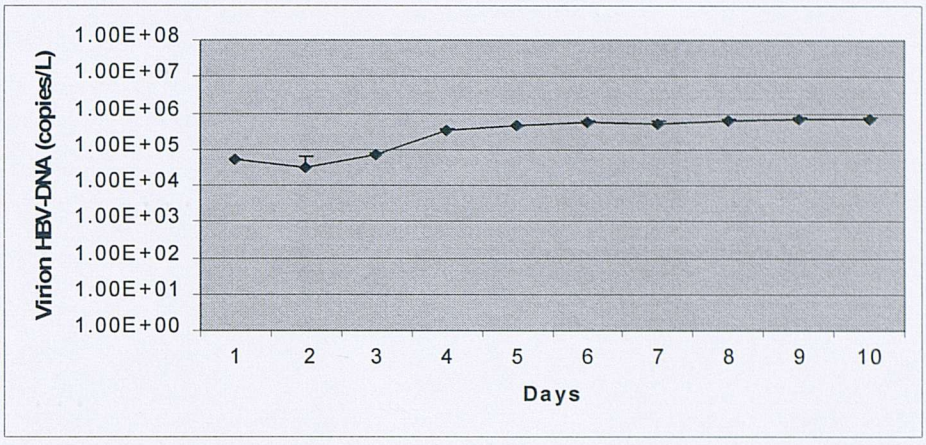
4.1 HBV kinetics over time in 2.2.15 cells

The rate of release of HBV particles from the 2.2.15 cell line was determined over a 10-day period. Real-time PCR monitoring (Figure 4.1, panel A) of HBV DNA demonstrated a gradual and constant increase in virion release over time, as shown. A zero time point was not included since 2.2.15 is a stably transfected cell line, with continuous replicating HBV. Control experiments included HepG2 cells (parent cell line) transfected with empty pBluescript® II KS(+) vector.

In this experiment (Figure 4.1, panel B), HBsAg was detectable from the first day post seeding, and increased steadily over time.

At present, there is no sensitive quantitative HBeAg ELISA kit, therefore only qualitative testing was undertaken for the antigen marker, at days 1, 2, 4, 6, 8 and 10. Antigen was not detectable on days 1 and 2 but at all other time points. Positive results were values above 0.015 PEI Units/ml, taken as the cut-off value. The data are qualitative (providing only a negative or positive result) and therefore not represented as a graph.

(A)



(B)

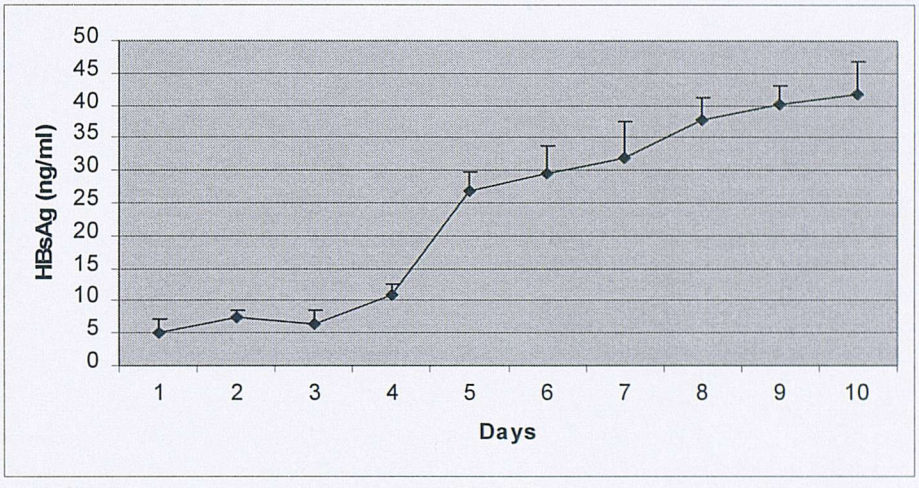


Figure 4.1 Quantitation of HBV DNA and HBsAg in 2.2.15 cell culture media. Conditioned media of 2.2.15 cells seeded at a starting density of 70% in 25-cm^2 flasks were sampled from days 1–10, post seeding. Virion HBV DNA was extracted and analysed by Real-time PCR.
(A). A logarithmic scale used to indicate released virion copy number. Values are given per litre due to low copy numbers.
(B). HBsAg quantified with a standard curve set up using yeast recombinant HBsAg. All results shown are the average of triplicates from the same experiment and shown as the mean ±1 SD. The assay was repeated on two further occasions. The trend identified in the results was repeatable.

4.2 Intracellular HBV DNA replicative intermediates in 2.2.15 cells

To validate the extraction and detection techniques for total HBV DNA for differential detection of HBV DNA replicative intermediates, total cellular DNA was extracted from a liver biopsy specimen from an HBsAg-positive patient ("T"). This extracted DNA served as positive control for the detection of both types of HBV replicative intermediates: RC-DS DNA and cccDNA. Similarly, total DNA was

extracted from 2.2.15 cells. cccDNA was also extracted exclusively from 2.2.15 cells in the presence of mung bean nuclease, as described in Methods, Section 2.2.11.b.

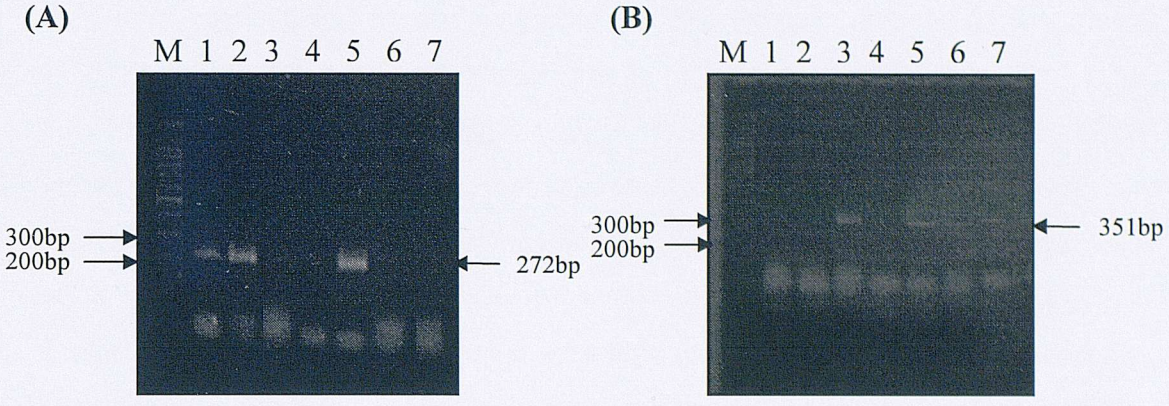


Figure 4.2 Analysis of intracellular HBV DNA replicative intermediates extracted from 2.2.15 cells.
Total DNA extracted from a pooled duplicate of cells 10 days post seeding in 25-cm^2 flasks. HepG2 cells transfected with empty pBluescript® II KS(+), were used as negative control. Expected amplicon sizes were: **(A)** 272bp with the F1-R1 primer-pair, and **(B)** 351bp with the cccDNA primer-pair. Electrophoresis was on a 2.0% (w/v) agarose gel. Lanes M, 100bp DNA markers (Promega).

(A): Lanes 1 and 2 (duplicated), total HBV amplicons from 2.2.15 cell extracts obtained with the F1-R1 primer-pair. Lane 3 and 4 (duplicated), amplification of DNA released in the supernatant of 2.2.15 cells, using the cccDNA primer-pair. Lane 5, PCR amplification of total intracellular DNA from Patient T liver biopsy. Lane 6, F1-R1 primer-pair used to amplify total intracellular DNA extracted from negative control cells. Lane 7, PCR negative control (water) with the F1-R1 primer-pair. **(B):** Lane 1 and 2 (duplicated), total extracellular DNA from 2.2.15 culture media amplified with the cccDNA primer-pair. Lane 3, total DNA extracted from Patient "T" liver biopsy, amplified with the cccDNA primer-pair. Lane 4, F1-R1 primer-pair used to amplify total intracellular DNA extracted from negative control cells: HepG2. Lanes 5 and 6 (duplicated), total DNA extracted from 2.2.15 and amplified with the cccDNA primer-pair. Lane 7, cccDNA extracted from 2.2.15 cells and amplified with cccDNA primer-pair.

Using the total DNA extract from the patient biopsy as template, separate PCR analyses were used to detect cccDNA and total intracellular HBV DNA (RC-DS plus ccc) differentially. Figure 4.2, panel A, shows the PCR product obtained with F1-R1 primer pair (lanes 1, 2, and 5) used for total replicative intermediates, and the product obtained with the cccDNA primers (Panel B, lanes 3, 5–7).

The same template and primer concentrations were used in the separate PCR amplification processes. In Figure 4.2, it is obvious that the products generated with

the F1-R1 primer-pair were more concentrated than those obtained with the cccDNA primers (panels A and B, respectively). This result, which did not use a fully quantitative technique, may reflect binding of the F1-R1 primer-pair to both types of HBV DNA present, as opposed to a situation with primers that should selectively amplify cccDNA (the cccF-cccR primer set).

This suggestion was strengthened by testing the specificity of the cccDNA primers. That is, using cccDNA extracted from 2.2.15 cells as a template with the cccDNA primer-pair, an amplicon was obtained (Panel B, lane 7). In contrast, with the same primer set, no amplicons were obtained when total extracellular DNA extracts from the 2.2.15 culture media were used as template, as shown in lanes 3 and 4 (duplicates), panel A. cccDNA amplicons were detectable only when cccDNA extract was used as template and amplified with cccDNA primers. All 2.2.15 cell-extracts were from cells harvested ten days post seeding.

4.3 Monitoring of mRNA transcription in the 2.2.15 cell line

The synthesis of RNA transcripts directed by the HBV EnhII/CP sequences in transfected cells was investigated by Transcript-specific PCR. This technique, described previously (Chapter 1), enables the differential and specific detection of both total mRNA (preC mRNA plus preG RNA), and preC mRNA. The preC mRNA-specific 5′–primer, PCP, allowed for specific detection of the same transcripts without interference from preG RNA (Figures 4.3 and 4.4). The 5′–primer PGP could detect both preC and preG transcripts (Figure 4.4, lanes 3 and 4). The possible presence of residual contaminant DNA, notwithstanding treatment with *DNaseI*, was checked using the 5′–primer M3, which binds to sequences upstream from both preC mRNA and preG RNA initiation sites. Primer-dimers (PD) were evident with the PCP-BC1 (lane 1) and PGP-BC1 primer-pairs (PD) (lane 4). No primer dimer was seen with the M3-BC1 primer-pair, lane 5.

In the subsequent Huh7 and HepG2 transfection studies (Sections 5 and 6), it is important to note that the Transcript-specific primers will potentially detect two types of transcripts: the longer than genome-length transcripts (3.5kb) which the primers were intended to target, but also any shorter than genome-length transcript which may have been generated from the repeat fragment. This shorter repeat fragment which also has the promoters to transcribe RNA (see Figure 3.1) is necessary for replication-competency of parent plasmid.

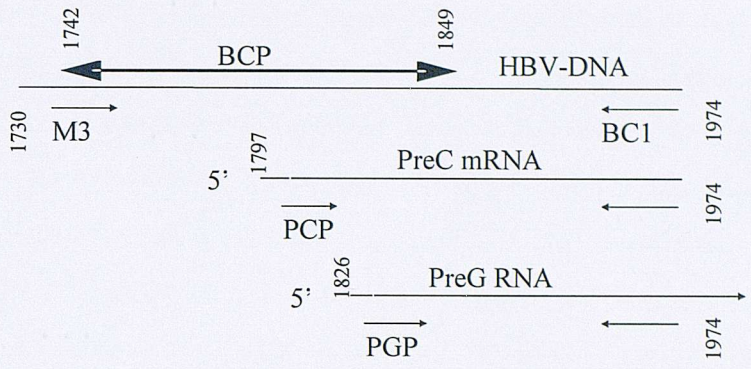


Figure 4.3 Schematic representation of the HBV BCP region showing the orientation of primers employed in the detection of preC and preG transcripts. The 5' ends of the 3.5kb preC mRNA and preG RNA transcripts are indicated. Primers utilised for the Transcript-specific RT-PCR assays (PCP, PGP and BC1) are designated by short horizontal arrows. The binding position for the first nucleotide of each primer is given. The start and end of BCP is indicated (double-headed arrow). Sense primers, PCP and PGP, were used to detect and quantify the two viral transcripts. Primer M3 was used to detect possible contamination with HBV DNA. A common antisense primer, BC1, was used throughout.
Nucleotide numbering starts at the *EcoRI* site.

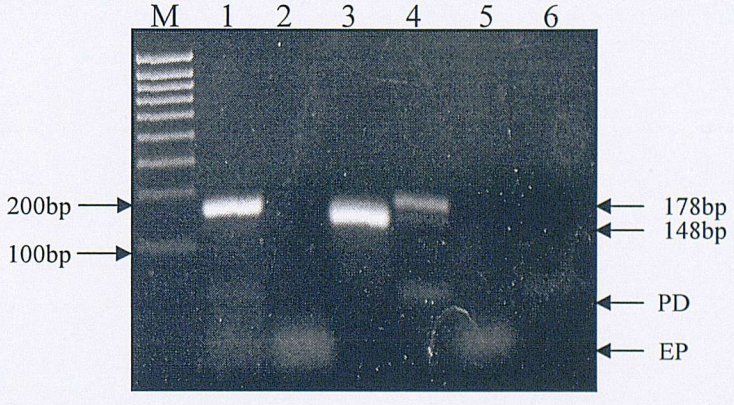


Figure 4.4 Transcript-specific PCR using RNA extracted from the 2.2.15 cells. A 2% (w/v) agarose gel used to detect amplicons of HBV preC mRNA and preG RNA following RT-PCR. Total RNA was extracted from the 2.2.15 cell line 10 days post seeding. Lanes 1 and 2, cDNA amplified with primers PCP and BC1 (148bp). Lanes 3 and 4, cDNA amplified with primers PGP and BC1 (178bp). Lane 5, PCR product generated with M3 and BC1 primers to detect contaminating DNA from cells. Lane 6, PCR negative control with the PGP-BC1 primer-pair. Primer-dimers (PD) are seen in Lanes 1, 4 and 6 (just visible). Excess primer(s) (EP) are also obvious in Lanes 1, 2 and 5. Lane M, 100bp marker.

4.3.a.Secondary structure of primer-pairs

Although the programme Netprimer was used to check for any potential inter- or intra-homology in all primers or primer-pairs that were used for the detection of both transcripts (PreC mRNA, and PreG RNA), the Transcript-specific technique revealed some secondary structure. Due to the short distance (Figure 4.3) between the two 5′–ends of the transcripts, Netprimer was unable to eliminate all potential secondary structures. Nonetheless, the 5′–, and 3′–primers that were ultimately selected (PCP and PGP), in spite of their potential dimerisation with BC1 (Figure 4.5, panel A), lacked additional secondary structures (hairpins and self-dimerisation) generated with other primers tested initially.

(A)

I. Secondary structure of primer-pair

Most stable cross dimer:
ΔG = -5.13 kcal/mol

```
5' GTCTGTTCACCAGCACCA 3'
      ||||    |   |
3' AACGGAAGACTGAAGAAAGG 5'
```

II. Secondary structure of primer-pair

Most stable cross dimer:
ΔG = -10.76 kcal/mol (3' Cross Dimer)

```
5' CACCTCTGCCTAATCATCTCA 3'
        |||||  |  |  ||
     3' AACGGAAGACTGAAGAAAGG 5'
```

(B)

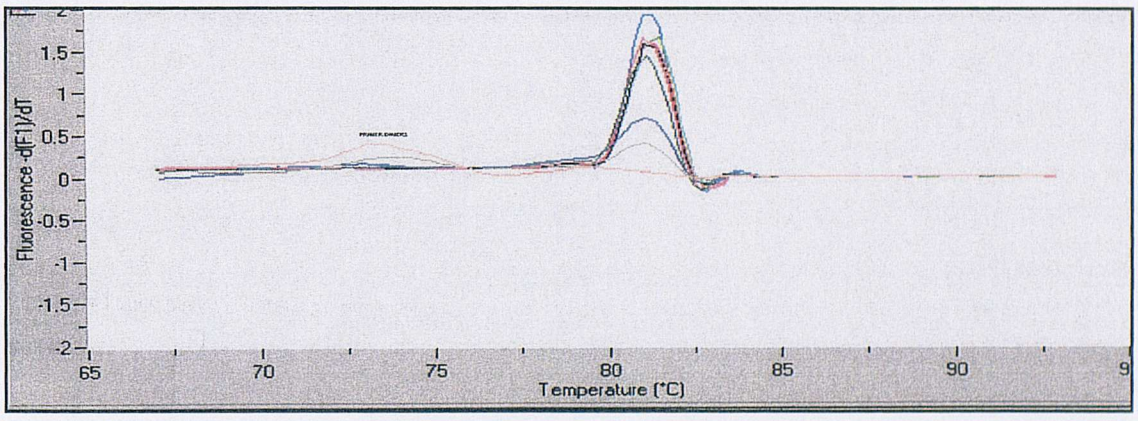


Figure 4.5 Primer-dimerisation in Transcript-specific PCR.
(A). Cross dimerisation between the 5′–ends of sense primers PCP (I) and PGP (II), with the common antisense BC1 primer.
(B). Melting Curve analysis showing the presence of dimerisation products (low peaks) with a lower Tm, compared to the higher melting peaks of the specific products. The PGP-BC1 primer-pair was used here.

Since these same Transcript-specific primers were to be used in the Real-time quantification of the transcripts, it was particularly important that a preliminary run be performed to characterise further both the desired PCR products and any primer-dimers by their characteristic melting behaviour. The data for this are shown in Figure 4.5, panel B, where pure PCR product produce single, sharply defined melting curves with narrow peaks at between 80.5 and 81.0°C. In contrast, cross-dimers melted at relatively lower temperatures with generally broader peaks. After this finding, an extra step was added in the amplification process to quantify products selectively that melted only beyond 80°C. During PCR, primer-pairs that produce dimers compete with the formation of specific PCR products, leading to reduced amplification efficiency and potentially less successful PCR. To compensate for this, primer concentrations were increased. On gels, excess unbound primer, migrate faster than dimers. Free, excess primer (EP), were mostly seen in lanes 1, 2 and, 5 (Figure 4.4), where no amplicons were obtained.

4.4 Detection of internal control genes in 2.2.15 cell line

The purpose of the following experiment was to detect control, or so-called "housekeeping" genes (HKG) expressed by the 2.2.15 cell line, ten days post seeding. As described before (Introduction, Section 1.14.5), quantitative gene expression data are often normalised to the expression levels of HKGs. The principle supporting their use as internal controls in quantitative Real-time RT-PCR was based on the fact that the ratio between HBV RNA and HKG copy numbers allows for a correction against potential errors during stages of RNA manipulation. To do this, two HKGs were selected: β-actin and RPII. Using standard semi-quantitative PCR, both HKGs were detectable from RNA extracts of 2.2.15 cells (Figure 4.6). Primers specific for the β-globin gene were included to control for cell-specificity contamination; no amplicon was observed with these primers (Figure 4.6, lane 3).

The β-actin transcripts proved more abundant than those of RPII (Figure 4.6: compare lanes 1 and 2) although comparable amplification protocols were used for both: template was taken from the same cDNA preparation, and equimolar concentrations of starting template and primers were used in the same run for the amplification of the three genes (including β-globin). Therefore, β-actin transcripts were chosen for RNA transcript normalisation in subsequent experiments. Although

Radonic *et al.* (2003) have shown that RPII is expressed in most cells and cell lines, including hepatoma ones, its low abundance would have made detection difficult. Whereas 4µl of a corresponding cDNA template amplified with β-actin primer-pair gave the results in lane 1, similar amounts of the same template, amplified with RPII primer-pair gave a weaker band (lane 2), ten days post seeding. (The cDNA was amplified from RNA extracted from 2.2.15 cells pooled from two 25-cm^2 flasks).

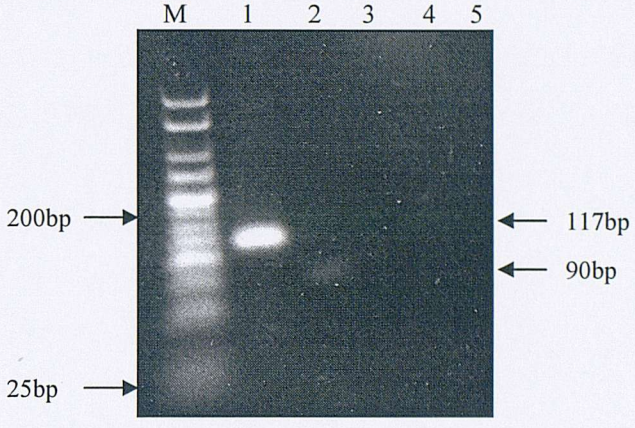


Figure 4.6 PCR amplification of ß-actin and RPII HKGs, as expressed in 2.2.15 cells.
2% (w/v) agarose gel electrophoresis of HKGs amplified from cDNA synthesised with RNA extracted from 2.2.15 cells, ten days post seeding. Lane A: β-actin amplicon (117bp). Lane 2, RPII amplicon (90bp). Lane 3, loaded with PCR product using the β-globin primer-pair (110bp). Lanes 4 and 5: PCR negative controls for both β-actin and RPII, respectively. Lane M: 25bp DNA marker.

In the subsequent quantitative RT-PCR assay results (Sections 5.4.5, 6.2.2 and, 7.3), the resulting copy number of each transcript (preG or preC mRNA) were normalised against those obtained for β-actin. The resulting value represents the ratio of the two, that is:

$$\frac{\text{Copy number of target gene (HBV transcript)}}{\text{Copy number of control/HK gene (β-actin)}}$$

The above is based on the inherent assumption that expression of the HK/control gene remains constant in the cells under investigation, despite the fact that exceptions to this assumption are well documented, as previously described (Section 1.14.5).

5.0 *In vitro* Analysis of the Effect of Mutations in preC and CP

Following construction of mutant recombinants and validation of techniques using the 2.2.15 cell line, the effect of the preC and CP mutations on the replication capacity of the virus was next fully analysed by Real-time PCR. For this, recombinants were first transiently co-transfected into Huh7 cells. Supernatant was collected 96hrs post transfection and media HBV DNA was subjected to quantitative Real-time PCR. At the same time, intracellular replicative intermediates of total HBV DNA, cccDNA and total RNA were similarly quantified. Real-time Transcript-specific PCR was used to quantify the levels of preC mRNA and preG RNA. Assay results with mutant recombinants were always compared to those of the p3.8II WT construct.

Standard curves were constructed (Appendix #1) for use in all the Real-time PCR assays, and transcript quantification results were normalised to β-actin. Using standard PCR conditions, each cDNA sample was first tested for the β-globin gene to confirm the hepatic origin of extracted RNA. Real-time PCR was carried out only if this test gave negative results.

5.1 Preparation of standards for Real-time PCR

In order for the Real-time software to calculate and assign values corresponding to the concentration of the starting template automatically, a standard curve was concurrently generated, as described in Section 2.2.13.a., and Appendix #1.

5.2 Determination of WT transfection efficiency in Huh7 cells

The efficiency of transfection of the WT construct in Huh7 cells was initially determined by immunocytochemistry. However, due to lost of cells encountered during the staining process, the method was deemed unreliable and was replaced by an indirect method involving co-transfection with an expressing green fluorescent protein plasmid. FACS analysis, combined with fluorescence microscopy, was used to determine transfection efficiency.

5.2.1 Transfection efficiency by immunocytochemistry

Determination of transfection efficiency in Huh7 cells involved *in situ* detection of HBcAg following transfection with the WT recombinant. Figure 5.1 (panels B and C, white-arrows) shows that the antigen was predominantly localised in

the cytoplasm, with a diffused distribution surrounding the blue (Dapi)-stained nuclei (panel C). There does, however, appear some nuclear staining also, as shown in panel B (arrow). The mock control transfection with the empty vector [pBluescript® II KS(+)] showed no such staining (panel D). These results were deemed adequate for the purpose of this task. However, although this technique was successful, cell densities were consistently reduced probably due to cell detachment during the staining procedure. Sparsely distributed cells as a result of cell loss (see panels A and C) created gaps on previously cell-confluent coverslips.

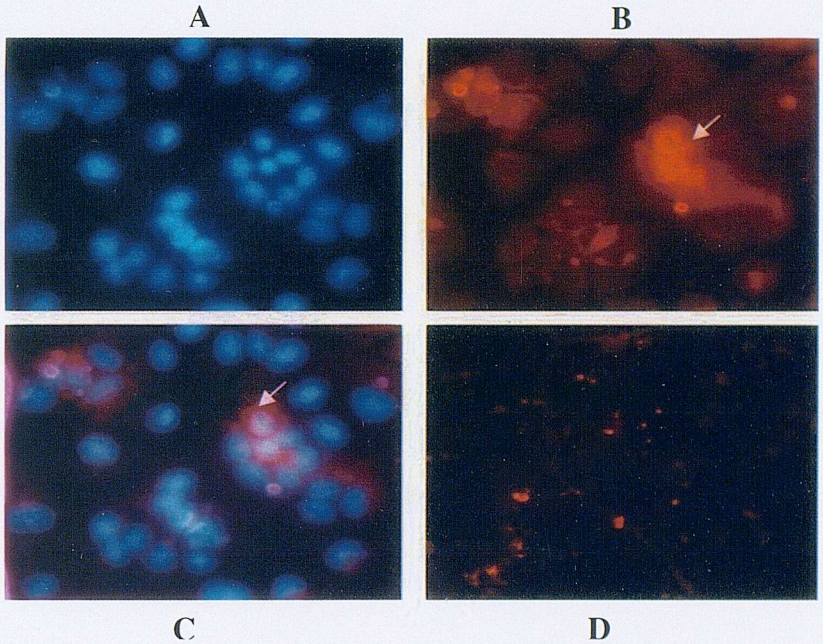


Figure 5.1 HBcAg staining of Huh7 cells transfected with the WT recombinant: transfection efficiency determination.
Confluent layers of cells were stained for HBcAg 48hrs following transfection (x400 magnification). **A.** Blue (Dapi)-stained nuclei. **B.** HBcAg staining with anti-HBe polyclonal antibody. **C.** Merged field (A and B) showing blue (DAPI)-stained nuclei surrounded by the red-stained HBcAg. **D.** Mock control, of transfection.

5.2.2 Determination of optimum transfection concentration into a hepatoma cell line

A 4.6kb plasmid (pCI-GFP) containing the reporter gene for green fluorescent protein (GFP) was used for transfection efficiency determination at a given time (48hrs). Using a concentration range of 0.5–10μg, pCI-GFP was transfected into Huh7 cells. An average peak (26% maximum), transfection efficiency was seen with 2μg pCI-GFP DNA (Figure 5.2). Concentration increases beyond 2.0μg did not

improve the overall transfection efficiency markedly, as determined by FACS. 2μg of the plasmid DNA was thus used in subsequent co-transfection experiments. Between wells (of a triplicate transfection) variations of 2–5% in transfection efficiency (increases or decreases) were seen in two further repeats of the experiment, ($P < 0.05$).

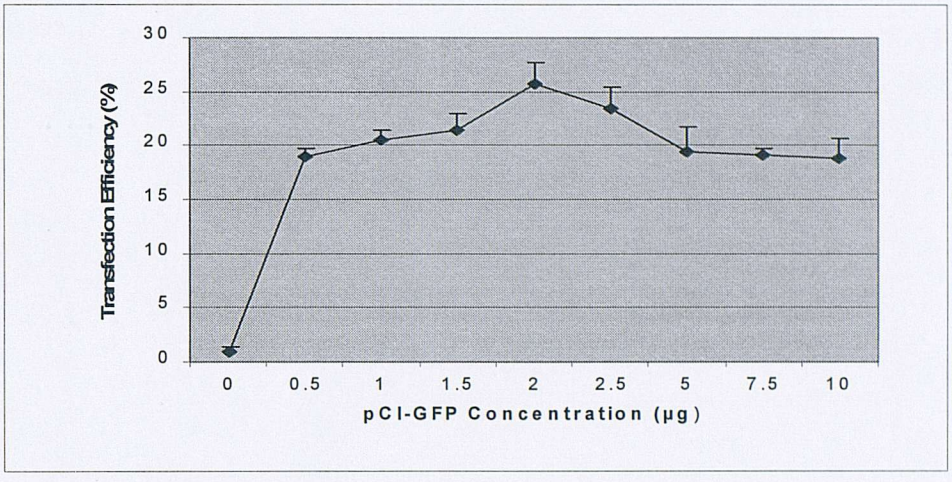


Figure 5.2 Transfection efficiency determination.
pCI-GFP concentrations ranging from 0–10μg were used to transfect Huh7 cells, which were analysed by FACS 48hrs later. The percentage of positively transfected cells was worked out having excluded dead or autofluorescing cells (R1, Figure 5.4). Results are the average of triplicate experiments and are shown as the mean ±1 SD.

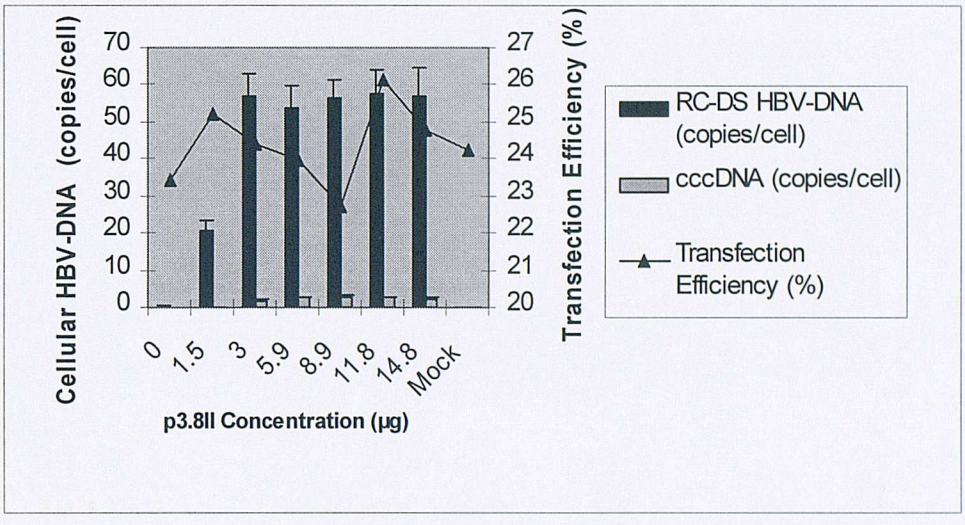
5.2.3 Optimum co-transfection concentration for pCI-GFP and p3.8II in hepatoma cells

Following on from experiment 5.2.2, the optimum concentration for co-transfection with p3.8II (WT) was subsequently determined. In brief, 3μg of "pCI-GFP mix" [2μg of pCI-GFP plus 1μg of pBluescript® II KS(+) vector] were co-transfected with p3.8II at concentrations equimolar to the 1.0μg-10μg pCI-GFP range that is, from 1.5μg to 14.5μg. (See Appendix # 2 for mole:mole sample calculation). 1.5μg of p3.8II DNA failed to produce detectable cccDNA over the time period of the experiment (Figure 5.3). Notwithstanding the fact that cccDNA was subsequently detected when 3.0μg of "pCI-GFP mix" was co-transfected with 2.9μg of p3.8II, this replicative intermediate, was not consistently detected in any triplicate from the same assay. The replicative intermediate was only detected in cell-lysate from only one well (of a triplicate-well assay), when 3μg of "pCI-GFP mix" was co-transfected with 11μg of the WT plasmid (Figure 5.3, panel A). In two

subsequent attempts (results not shown), detection of cccDNA was only consistent in one experiment (triplicate-transfected wells), whilst in the other, it was detectable in cell-extract from only two wells of the three. The replicative intermediate was not detected at sufficient sensitivity (per cell) to allow meaningful conclusions. On the other hand, both types of RNA transcripts (preC and preG) were readily detectable at all concentrations (Figure 5.3, pane B), confirming the replication-competency of the input plasmid, as well as the results in panel A.

[See Appendix #3.a.ii for sample calculation of intracellular HBV DNA per cell].

(A)



(B)

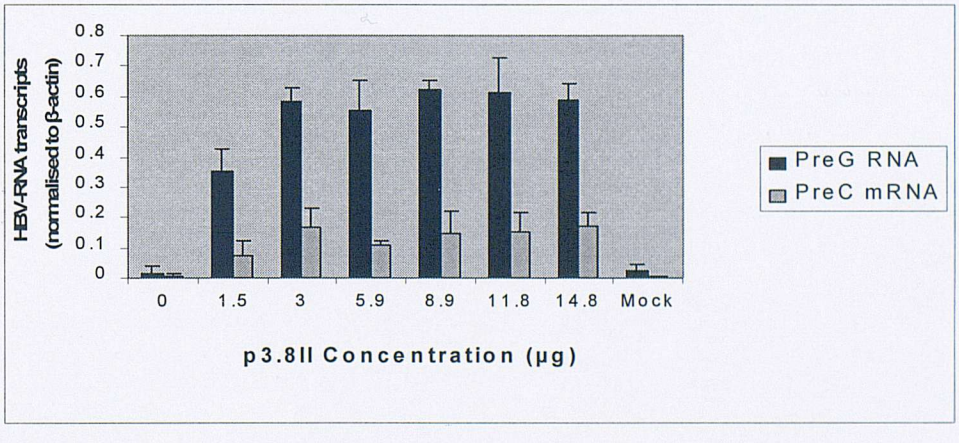


Figure 5.3 Copy numbers of cellular HBV DNA and RNA transcripts as indicated, following co-transfection of Huh7 cells.

Cells destined for transfection efficiency determination, by FACS, were harvested and assayed 48hrs after co-transfection. **(A).** HBV DNA replicative intermediates (total and ccc) were quantified by Real-time PCR 96hrs post-transfection.

(B). HBV RNA transcript (preG RNA and preC mRNA) copy numbers determined by RT Real-time PCR and normalised to β-actin.

It was noted that the preC mRNA transcript was consistently less abundant than that of preG RNA. As stated earlier (Section 4.3), what cannot be prevented in this assay — as it specifically applies here — is the additional and, possible detection of transcripts shorter than genome-length. These transcripts are potentially amplified from the 600bp repeat fragment. It is encouraging to note that the data summarised in the two panels did correlate, suggesting that in this experiment with WT plasmid, the amount of transcribed RNA depended on the amount of input plasmid that was eventually transcribed. This preliminary experiment also served to test the replication-competency of the WT, at the intracellular level.

5.2.4 Determination of optimum time for transfection

To determine the optimal expression time of pCI-GFP, transfected cells were examined at 24, 48 and 72hrs, post transfection, by FACS (Figure 5.4) and fluorescent microscopy (Figure 5.5). A clearer (and more reproducible) signal was obtained at 48hrs or 72hrs, than at 24hrs; the 48hrs time-point was used in all subsequent estimations. This allowed assessment of transfection efficiency at an early stage, and permitted repeat of the experiment earlier if the transfection was unsuccessful.

Cells from one of four wells normally co-transfected, and a mock control, were subjected to FACS analysis 48hrs post transfection (Figure 5.4). The strategy of using a single well for transfection efficiency estimation was initially validated by harvesting three individual wells after co-transfection with the p3.8II recombinant. Transfection efficiencies of 21.81%, 23.03% and 23.96% were obtained. Harvesting product from a single well from triplicate transfections was thus deemed to be a near representative of the transfection efficiency in the remaining two. Final FACS analysis excluded non-viable cells fluorescing in the range of mock co-transfected cells (scattered around the circle and labelled R1, Figure 5.4, panel A,).

To provide support for the FACS results, a fixed amount of the residual FACS preparation was visually inspected by fluorescence microscopy. The findings suggested that FACS, seconded by visual microscopic analysis (shown in Figure 5.5), provided a reliable method for determining transfection efficiency. Similar results were obtained following co-transfection of HepG2 cells, also of hepatic origin.

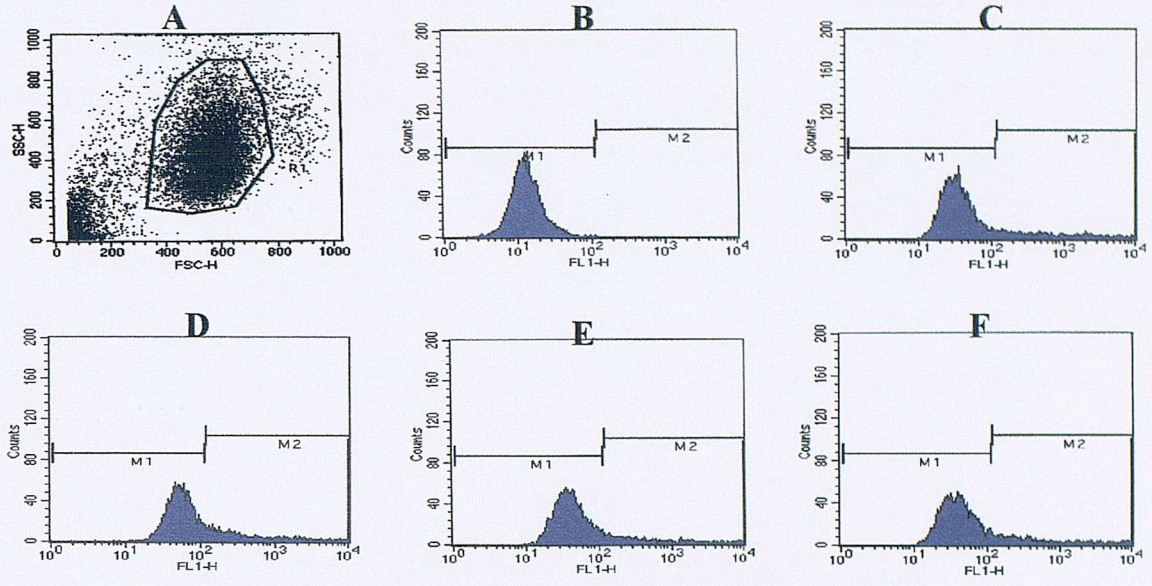


Figure 5.4 Transfection efficiency determination by FACS using varying amounts of p3.8II and a constant amount of pCI-GFP.
A. Mock co-transfection control. Auto-fluorescing, dead or dying cells, scattered outside the circle [labelled R1 (hardly visible)], were excluded from the analyses. **B**, **C**, **D**, **E** and **F**. Varying p3.8II concentrations from 0.0, 0.5, 2.0, 2.5 and 7.5µg, respectively. Cells were deemed transfected if they fell in the M2 zone of the FL1-H range of the mock transfection. Cells in the M1 zone are negative controls, transfected with the empty pBluescript® II KS(+).

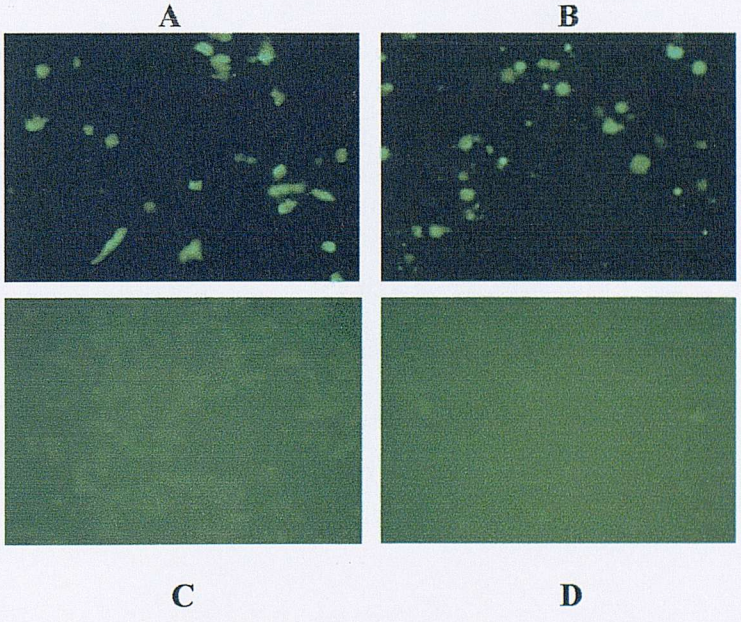


Figure 5.5 Expression of the green fluorescence protein by pCI-GFP in cells, as given, after co-transfection with p3.8II.
A. HepG2 cells. **B.** Huh7 cells. **C.** HepG2 "mock" transfected cells. A, B and C were visualised 48hrs post transfection.
D. Huh7 cells. Visualisation was 24hrs post transfection.
Fields of view at x400 magnification showing pCI-GFP expression is representative.

5.2.5 Expression of the green fluorescence protein in non-hepatic cell lines

Non-hepatoma cell lines such as 293T (human kidney origin) and 143-TK$^-$ (human osteosarcoma origin) were co-transfected with p3.8II and pCI-GFP (Figure 5.6) to compare transfection efficiencies with those obtained in Huh7 and HepG2 cells using similar co-transfection protocol and conditions. Transfection efficiencies for 143-TK$^-$ and 293T cells were 0.9–14% and 57–63%, respectively. The high 293T cell line transfection efficiency results were generally consistent with those previously obtained (M.D. Moore, Ph.D Thesis, 2005, Department of Infectious Diseases, Imperial College, London).

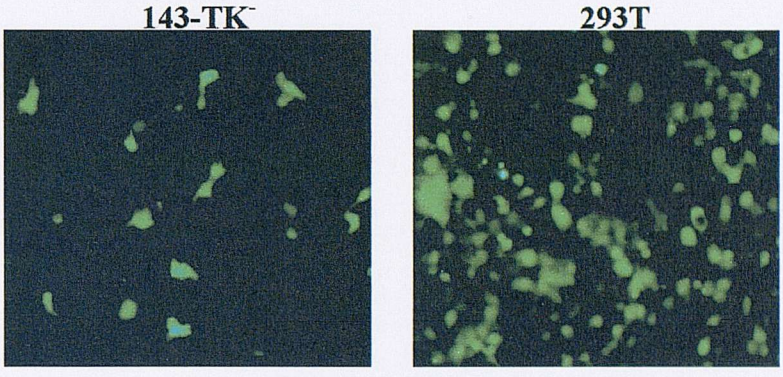


Figure 5.6 Expression of green fluorescence protein in non-hepatoma cell lines following co-transfection of pCI-GFP and p3.8II.
Fields of view at x400 magnification showing pCI-GFP expression is representative.

5.3 Control validations for experimental work

To validate the techniques that have been tested using the 2.2.15 cells (Section 4.1), transient transfection of the WT construct was performed in Huh7 cells and various parameters, as described, measured to determine the replication capacity of the construct. Data (Figures 5.7–5.9) presented are the mean of each assay in triplicate; results of two further repeats are also presented. All results were first standardised to 15% transfection efficiency prior to averaging (Appendix #3 for sample calculation of standardisation).

5.3.1 Quantification of released virions by Huh7 cells co-transfected with p3.8II and pCI-GFP

Real-time PCR was used to detect and quantify virion DNA extracted from the media of co-transfected Huh7 cells. In view of the greatly increased detection of HBV DNA, the results (summarised in Figure 5.7) show that the p3.8II construct was

replication-competent. These observations were confirmed in the two repeat experiments, which exhibited no significant difference ($P > 0.05$). Residual input plasmid was detected at time point 0 (insignificant at $P < 0.001$).

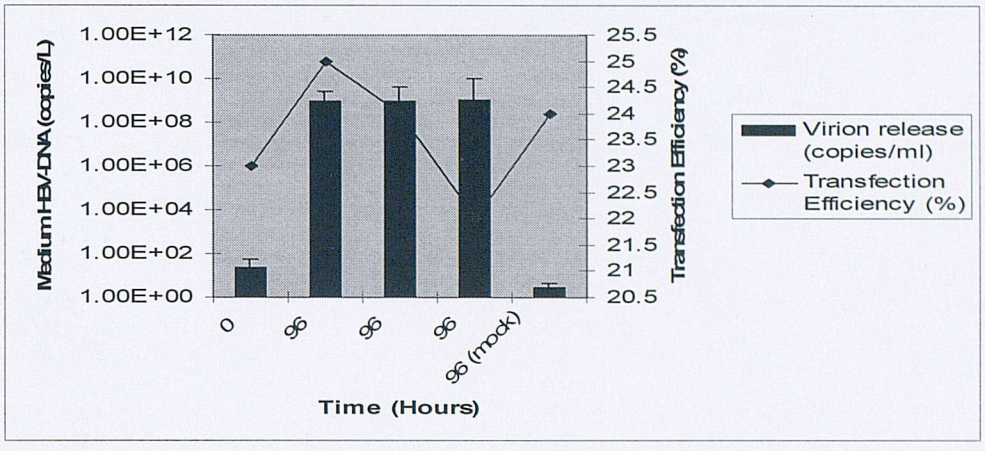


Figure 5.7 Transfection efficiency and quantification of released HBV DNA, following co-transfection of Huh7 cells with WT p3.8II and pCI-GFP.
Black bars indicate the HBV DNA values in copies per ml at 96hrs following transfection and a line indicates transfection efficiency.
[All designations are meant to represent each bar it appears under, and not at intervals].

5.3.2 Quantification of HBV DNA replicative intermediates in Huh7 cells co-transfected with WT p3.8II and pCI-GFP

RC-DS DNA levels at 96hrs were readily detectable, as shown in Figure 5.8.

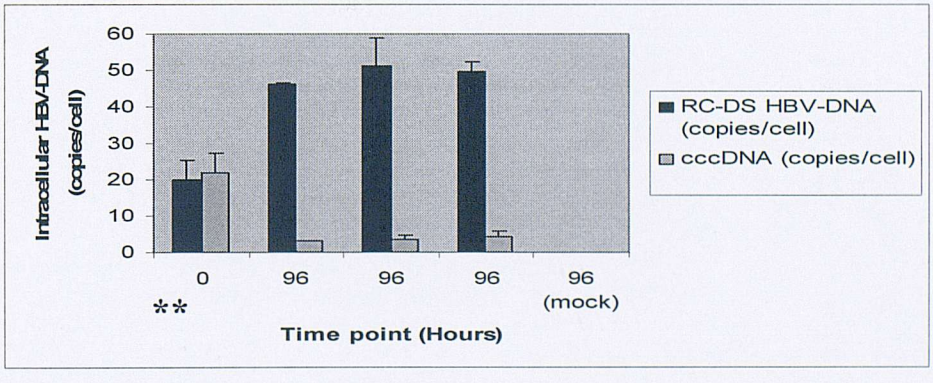


Figure 5.8 Quantification of HBV DNA replicative intermediates following p3.8II transfection into Huh7 cells.
Each of the bars represents the average of triplicates from the same experiment, shown as ±1 SD. Results of three experiments at different times are shown for the 96-hr assays only; the mock and time 0 bars are taken from one of three triplicate assays.

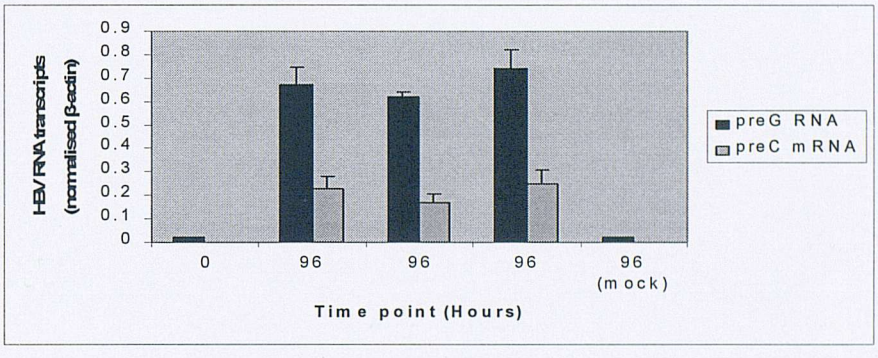
** Represent total transfected plasmid amplified with the two sets of primer-pairs.

In contrast, nuclear cccDNA levels were inconsistently detectable between replicates. At time 0, the two sets of primers (F1-R1, and cccF-cccR) were binding to a target DNA sequence (highly likely to be input plasmid at this stage) with high specificity and demonstrated increased amplification compared to values obtained 96hrs post transfection. In relation to the levels of RC-DS DNA, cccDNA levels were very low (ranging from 12:1 to 15:1; significant at $P < 0.001$), when consistently detected. A similar trend in results has been observed previously (Delaney *et al.* 1999a). HBV amplification was not observed in the mock control.

5.3.3 Assessment of WT BCP in directing HBV transcription in Huh7 cells

The levels of HBV transcripts synthesised by the WT construct (p3.8II) at 96 hrs post transfection in Huh7 cells were determined using the Real-time Transcript-specific PCR. Results obtained from these assays are presented in Figure 5.9.

(A) β-actin normalisation



(B) RPII normalisation

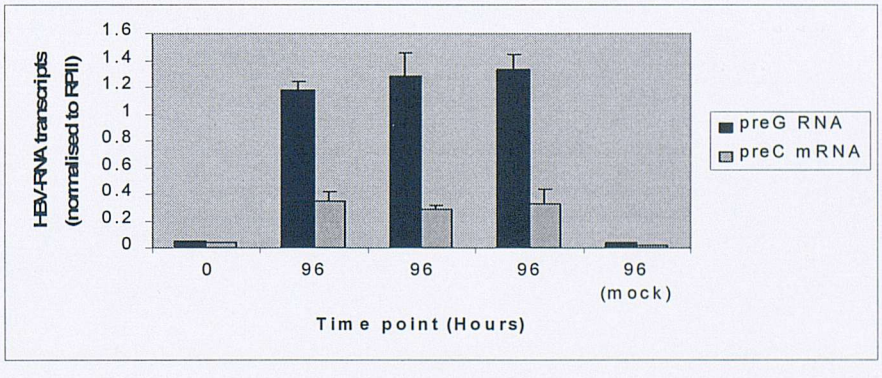


Figure 5.9 Determination of the transcription levels in Huh7 cells transfected with p3.8II.
(A). β-actin, and **(B).** RPII, HBV transcript normalisations
Each bar (preG RNA and corresponding preC mRNA) represents the average of triplicates from the same experiment, shown as ±1 SD. The results of two further repeat experiments are also shown. Standardised data of a single triplicate assay are shown for time 0 and for the mock control.

The difference between the values for total transcript and preC mRNA provided values for preG RNA. These preliminary WT transcript quantification results were normalised to both β-actin and RPII transcripts. The ratio of preG RNA to preC mRNA was at least 3:1 (Figure 5.9). Controls using sterile water and "DNA" extracted from the mock transfection served to monitor for contamination at the PCR and transfection stages, respectively.

5.3.4 Assessment of HBV antigens in Huh7 cells transfected with WT

The ability of p3.8II to express HBV gene products 96hrs post transfection in Huh7 cells was tested by monitoring conditioned media for the presence of HBsAg and HBeAg. (HBeAg tests were carried out in the Routine Virology Laboratory, St. Mary's Hospital; the antigen was found to be consistently detectable in media). The antigens were not detected at 0 time point, or in the mock control samples.

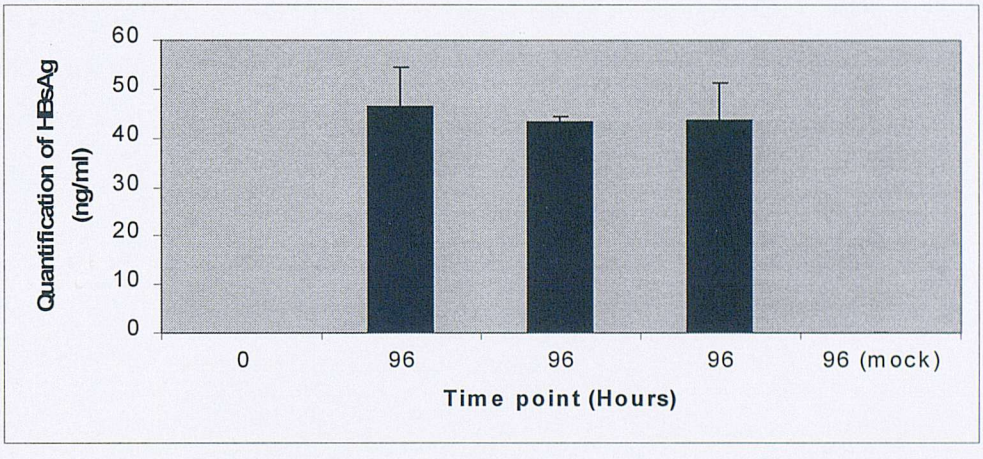


Figure 5.10 HBsAg detection by quantitative ELISA following transfection of WT.
ELISA samples were read at A_{450} and quantified (ng/ml) from an HBsAg (yeast recombinant) standard curve. Each bar represents the average of a triplicate assay from the same experiment, shown as ±1 SD following 15% transfection efficiency standardisation.

5.4 Mutant evaluation: *In vitro* assessment of the replication capacity of the CP and preC recombinants

To define the role of the CP and preC mutant sequences in the regulation of HBV replication, mutations associated with these regions were introduced into the p3.8II genetic background either alone or in combination, and their replication competency tested by transfection into Huh7 cells. As shown (Figures 5.7–5.10), the

residual plasmid DNA in the media (time 0) is insignificant and/or not replication-competent. This time point was therefore excluded from further experiments. Each mutant recombinant was, however, compared to those of the p3.8II parent plasmid, and to negative control mock transfections.

5.4.1 Quantification of virions released in culture media following co-transfection of CP mutant recombinants with pCI-GFP

FACS results indicated a mean transfection efficiency of 22.5%, and a range of 21–24%. All recombinants were capable of releasing HBV particles into media, indicating that they were replication-competent (Figure 5.11). Differences in virion concentrations were noted between different recombinants: A1762T/G1764A/C1766T and T1753C/A1762T/G1764A showed significant increases in relation to WT (4.4–, and 3.9–fold, respectively) ($P < 0.05$). On the other hand, a significant decrease in the replication capacity of T1753C/A1762T/G1764A/C1766T was observed in comparison to the WT; minus(−)56.5-fold, (significant at $P < 0.001$). When compared to WT, the C1766T/T1768A recombinant exhibited the largest increase (11.8–fold, $P < 0.001$). There was no statistically significant difference between WT and each of the single CP mutant recombinants, A1762T and G1764A ($P = 0.69$ and 0.9, respectively).

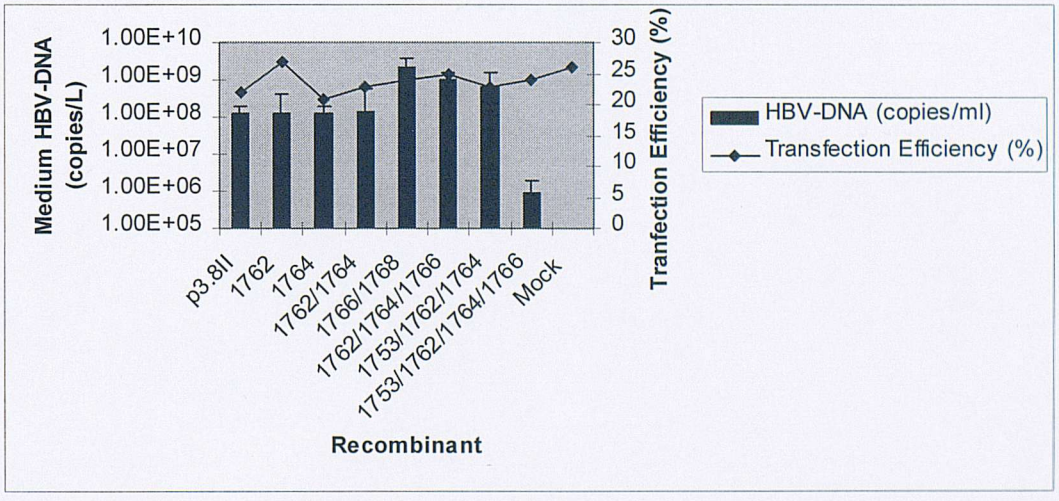


Figure 5.11 Virion HBV DNA concentrations in culture media following co-transfection of CP recombinants with pCI-GFP in Huh7 cells
The solid line gives the transfection efficiencies obtained with the various constructs (right y-axis), as determined by FACS. Vertical bars indicate copy numbers as means ±1 SD, standardised to 15% transfection efficiency, they represent the average of triplicate analyses from the same experiment.

Notably, the different recombinants that bore the A1762T/G1764A double mutations in addition to other CP mutations (Figure 5.11) showed varying significant differences in relation to parental A1762T/G1764A-only recombinant, which showed no significant difference in virion release relative to WT ($P < 0.05$). Similar findings for some of the recombinants have been reported (Baumert *et al.* 1998e; Gunther *et al.* 1998e; Parekh *et al.* 2003h).

The differences in the observed replication efficiency among the various recombinants, compared to the WT, cannot be explained by differences in transfection efficiency, since all data were statistically standardised at a fixed transfection efficiency of 15%.

5.4.2 Quantification of virion release into culture media following co-transfection of preC or combined CP mutations, with pCI-GFP

To examine the effect of the G → A substitution at positions 1896 and 1899, constructs bearing these mutations either individually, or in combination, were tested for replication efficiency, in Huh7 cells.

Neither of the two preC recombinants with single mutations had any significant effect on replication as compared with WT ($P > 0.05$). The third base of codon 15 in the WT is normally a *T* (genotype non-A) (Table 3.1), which makes the base-pairing of T1858 with an A1896 much stronger (reviewed in Kramvis and Kew, 1998).

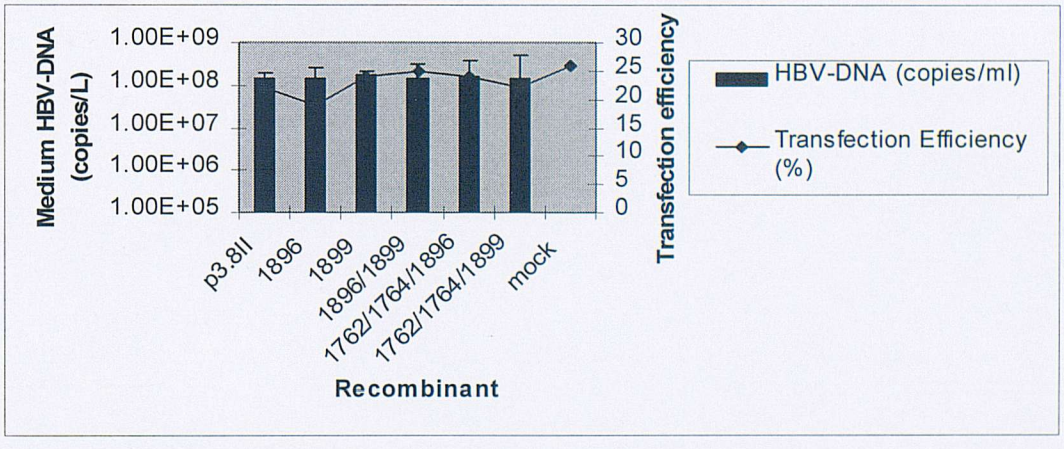


Figure 5.12 Virion release following co-transfection of Huh7 cells with pCI-GFP and recombinants harbouring mutations in preC, in combination with the CP mutations.
The presented results are averages of triplicate assays, from the same experiment, with ±1 SD values.

In our hands, this mutation, however, had no significant ($P > 0.05$) effect on replication. Furthermore, the introduction of both preC mutations, G1896A/G1899A, did not significantly alter replication under the conditions used in this investigation. This is notwithstanding the fact that the second U-G wobble-pair (codons 14 and 29, respectively) in the lower stem of ε now achieved a more stable base-pairing between *U* and *A* as a result of the 1899 mutation.

Moreover, when each of the single preC mutations was introduced into the A1762T/G1764A mutant recombinant, to give A1762T/G1764A/G1896A and A1762T/G1764A/G1899A, no significant replication alteration was obtained (Figure 5.12) in relation to the WT. The *Mann-Whitney U Test*, relevant in this case, did not indicate any difference in viral replication capacity (insignificant at $P > 0.05$) in any of these constructs (see Section 5.4.1).

5.4.3 Analysis of intracellular HBV DNA replicative intermediates in Huh7 cells transfected with the CP recombinants

Total intracellular HBV DNA (RC-DS plus ccc) was extracted from Huh7 cells as previously described, and the DNA concentration in each sample determined by Real-time PCR.

Figure 5.13 indicates the comparison between RC-DS DNA and cccDNA levels 96hrs after transfection.

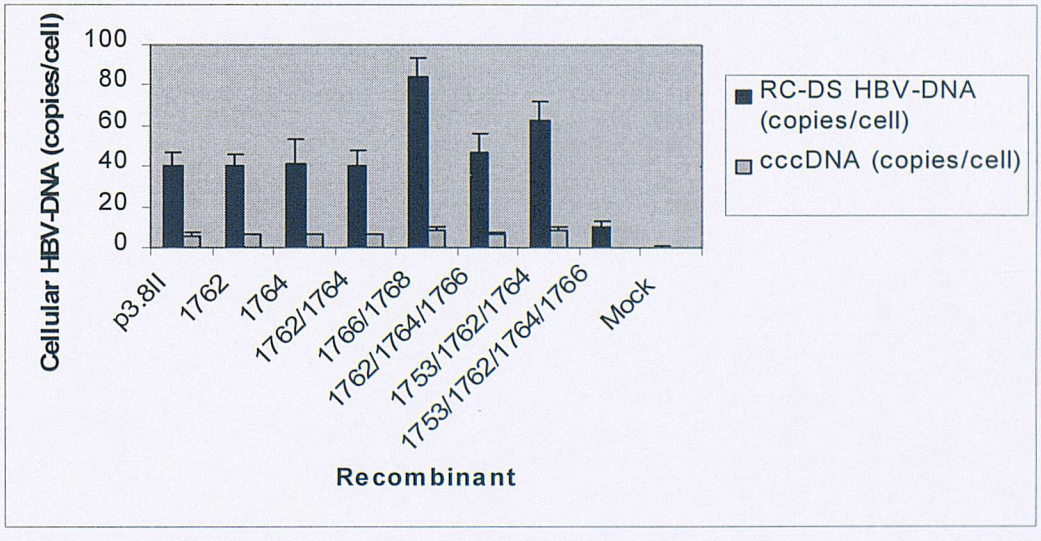


Figure 5.13 RC-DS HBV DNA and cccDNA levels in Huh7 cells transfected with various CP constructs bearing the mutations shown.
Results are the average of triplicate assays from the same experiment, with ±1 SD values.

Precise values for intracellular RC-DS DNA were obtained by subtracting the low cccDNA levels from the total HBV DNA values. For all samples, the data showed that total HBV DNA levels were significantly higher ($P < 0.001$) than those of cccDNA (Figure 4.2). However, because cccDNA was not consistently detected (per cell) in three (out of eight) recombinants, of the set of triplicate assays from the same transfection experiment, precise ratios between the two could not be determined in general. For the recombinants in which cccDNA was consistently detected; WT, C1766T/T1768A, A1762T/G1764A/C1766T, and T1753C/A1762T/G1764A, however, the ratio of RC-DS DNA to cccDNA ranged from 7:1 to 8:1, respectively. The cccDNA values for the three readings of these recombinants showed negligible deviation to each other ($P > 0.05$). However, in two further repeats the pattern of cccDNA detection was not wholly reproducible in cells transfected with the same mutant recombinants, casting some doubt on the reliability of the cccDNA results. Notably, on a per cell basis, cccDNA was not detected in the T1753C/A1762T/G1764A/T1766A recombinant. However, in comparison to the WT, a decrease of 4.2–fold total intracellular DNA was observed for this mutant ($P < 0.05$).

Figure 5.13 shows that there were differences in total intracellular DNA replicative intermediates between the various constructs, with the exception of the recombinants that harboured single CP mutations, and A1762T/G1764A. Moreover, with the exception of the mutant recombinants (C1766T/T1768A, A1762T/G1764A/C1766T, and T1753C/A1762T/G1764A) in which cccDNA was consistently detected in the triplicates from the same assay, the difference (of total intracellular DNA) relative to WT, was significant ($P < 0.05$) (2.2– and 1.5–fold, respectively). For the remaining recombinants, minor differences in copies per cell were not significant.

Despite the high-replicating phenotype of the C1766T/T1768A recombinant as determined from the virion release data, intracellular HBV DNA levels (2.2– *vs.* 10.8–fold) did not exhibit a corresponding increase. Notwithstanding this, in general, virion release data correlated with that of total intracellular HBV DNA: compared to WT, significantly ($P < 0.05$) more DNA was observed in the cells transfected with the C1766T/T1768A (2–fold) and T1753C/A1762T/G1764A (2.4–fold). On the other hand, a decrease of 4.2–fold ($P < 0.001$) was seen for the T1753C/A1762T/G1764A/C1766T mutant.

Two further repeat experiments confirmed the RC-DS DNA results, but not those of cccDNA.

5.4.4 Total intracellular HBV DNA in Huh7 cells transfected with preC mutant recombinants alone and combined with the CP mutations

As with the CP mutant recombinants, lysates from Huh7 cells transfected with recombinants bearing mutations in the preC region only, and those with combined CP and preC mutations, were assessed *in vitro* for their replication capacities. Again, similar to the CP mutant recombinants, whilst the RC-DS HBV DNA replicative intermediate was consistently detected in all triplicates from assays at any one time — cccDNA was not — except for the G1896A, A1762T/G1764A/G1896A, and A1762T/G1764A/G1899A recombinants (Figure 5.13). In these recombinants, a ratio of 10:1, 13:1, and 17:1, respectively, of RC-DS DNA *vs.* cccDNA was calculated. However, little weight should be put on the above figures since this replicative intermediate (cccDNA) was not consistently detected in all triplicates, from the same assay. Moreover, the cccDNA data that is presented in Figure 5.14 was not reproducible on repeat experiments.

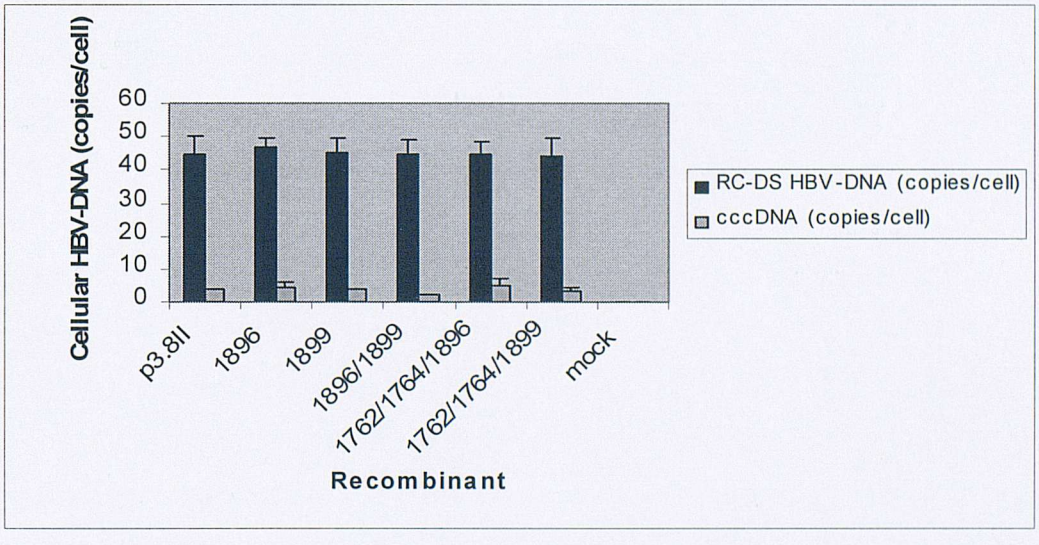


Figure 5.14 HBV DNA replicative intermediates following transfection of Huh7 cells with recombinants harbouring mutations in preC combined CP, and preC mutations.
Results are the average of triplicate assays from the same experiment, with ±1 SD values.

In relation to WT, the data did not show any significant variation in the levels of total intracellular (RC-DS) HBV DNA by the A1762T/G1764A/G1896A and A1762T/G1764A/G1899A recombinants ($P < 0.05$) (Figure 5.14), and no differences between the three preC recombinants ($P > 0.05$). As with the CP recombinants, two repeats (2X) of these transfections did not show any deviation from this trend (Figure 5.14).

5.4.5 HBV RNA transcripts in Huh7 cells transfected by recombinants carrying mutations in either CP, preC, or in both

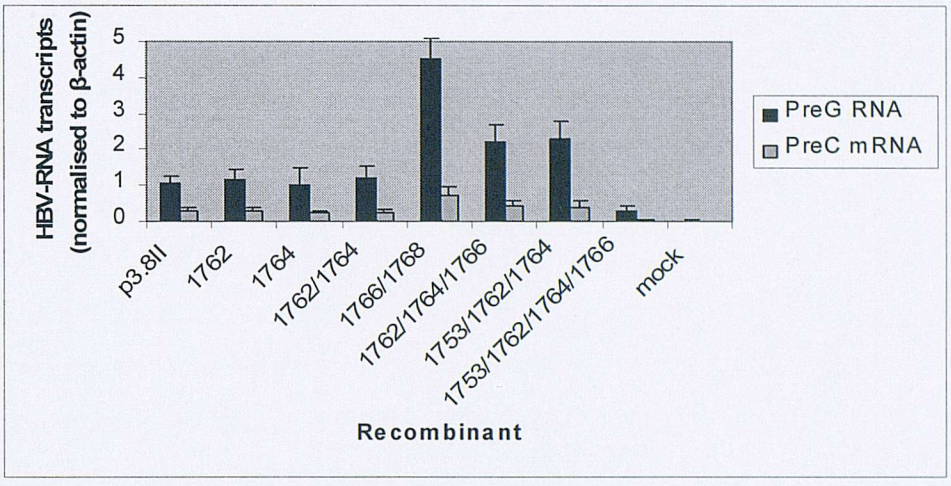
To document further the replication capacity of the mutated constructs, and to obtain further evidence in support of the HBV DNA findings, the levels of HBV RNA transcripts were investigated by Transcript-specific Real-time PCR, 96hrs after transfection of Huh7 cells (Figure 5.15, panels A, and B). The results for the WT show consistently 3.6:1 and 3.8:1 more preG RNA than preC mRNA.

Figure 5.15, panel A, showing transcript levels with the CP recombinants, demonstrates that the A1762T/G1764A recombinant did not exhibit significant preG RNA alterations (1.1–fold, $P > 0.05$) over those found in WT. Likewise, the mutant's preC mRNA was not altered compared with the WT. These results mirrored intracellular HBV DNA levels of the same mutant, which showed no significant difference, relative to WT (Figure 5.13). Similarly, this mutant showed insignificant upregulation in virion release, compared with the WT (Figure 5.11). Mutating the WT at either position A1762T, G1764A or the two combined, did not appear to affect viral replication at the transcription level, as monitored by the capacities of these constructs to release virions (Figure 5.11). The phenotype associated with the remaining CP recombinants, was in general, mirrored by their preG RNA transcription levels (Figure 5.15), where C1766T/T1768A, A1762T/G1764A/C1766T, and T1753C/A1762T/G1764A exhibited 4.3–, 2.1–, and 2.2–fold more preG transcripts than the WT, respectively ($P < 0.05$). On the other hand, compared to WT, preG RNA transcription of T1753C/A1762T/G1764A/C1766T was significantly downregulated (3.4-fold; $P < 0.01$). With regard to preC mRNA transcript levels, whilst differences between WT and each of the single CP, as well as the A1762T/G1764A mutants were not significant ($P > 0.05$), this appeared not to be the case with the remaining CP mutants that demonstrated significant preG transcription levels: A1762T/G1764A/C1766T (1.5–fold), T1753C/A1762T/G1764A (1.3–fold).

For T1753C/A1762T/G1764A/C1766T, the transcript was undetectable (Figure 5.15) whilst there was a 2.5–fold increase with C1766T/T1768A ($P < 0.05$). Similar findings have been documented by others (Buckwold *et al.* 1996c; Gunther *et al.* 1998d; Okamoto *et al.* 1994b).

Figure 5.15, panel B indicates the level of transcripts in Huh7 cells transfected by preC mutant recombinants or, recombinants bearing preC and CP mutations.

(A)



(B)

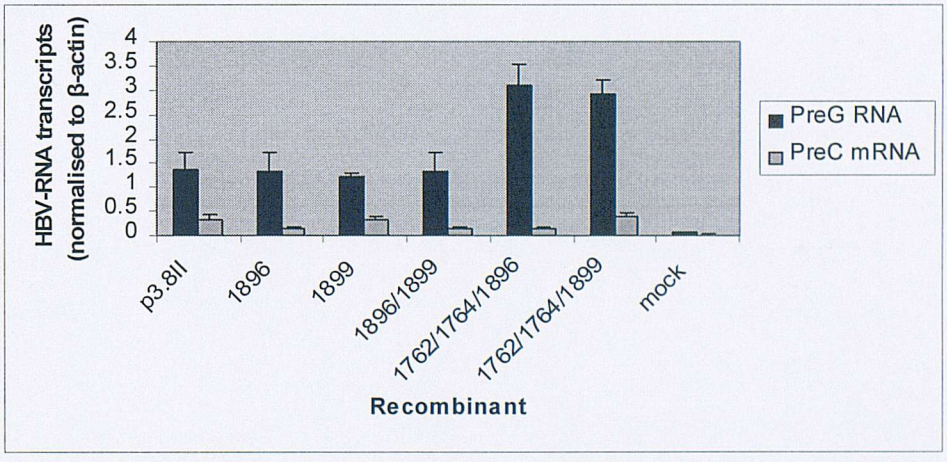


Figure 5.15 Differential detection of HBV transcripts in Huh7 cells transfected with recombinants bearing mutations in preC, CP, or in both.
HBV-RNA was extracted from Huh7 cells at 96hrs. Data were standardised to 15% transfection efficiency and normalised to β-actin. Results are the average of triplicate assays, showing ±1 SD values.
(A). PreC recombinants. **(B)**. Combined preC/CP recombinants.

Similar to the data for the CP mutant recombinants (Figure 5.15, panel A), both RNA transcripts (in Figure 5.15, Panel B) were expressed at ratios similar to

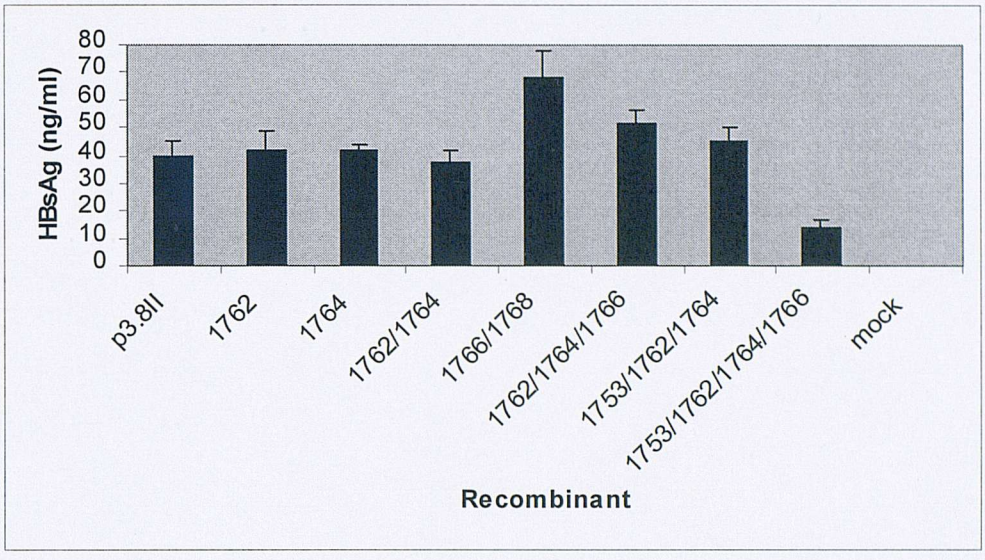
those seen with the CP mutants, with regard to WT. Only two mutant recombinants showed similar transcription capacity as the WT: G1899A, and A1762T/G1764A/G1899A. The recombinants that harboured the stop-codon mutation (G1896A), had significantly ($P < 0.05$) reduced preC transcription, when compared to WT. These data corroborates previous findings (Moriyama *et al.* 1996c). No differences were demonstrated in their preG RNA levels when compared to WT ($P > 0.05$), as also seen by Gunther *et al.* (1998), although not by others (Moriyama 1997).

5.4.6 Detection of HBV antigens in Huh7 cells following transfection with recombinants bearing mutations in either CP, preC or both

To characterise further the replication capacity of the various mutant recombinants, HBsAg released in culture media was quantitatively detected by ELISA. With the exception of the two recombinants bearing single mutations in the CP region, these were further also qualitatively assayed for the presence of HBeAg. Since mutations in the preC region had been shown earlier to affect HBeAg production (Brunetto *et al.* 1989b; Carman *et al.* 1989f; Kramvis *et al.* 1998; Lok *et al.* 1994; Tong *et al.* 1993b), all the recombinants harbouring a preC mutation were assayed for the presence of this antigen. The results were negative for all the tested recombinants except for the A1762T/G1764A, T1753C/A1762T/G1764A and G1899A recombinants. The results for the triple and quadruple mutant-recombinants correlated with their significantly reduced preC mRNA transcripts level (Figure 5.5, panel A). (The single CP mutants were not tested for the antigen). The positive results obtained with these mutants were not however completely reproducible: two samples transfected at different times were tested: one was negative and one positive for the T1753C/A1762T/G1764A and A1762T/G1764A mutants; whilst both tests were positive for the G1899A mutant. A negative result either indicated downregulation, or inhibition of HBeAg production. Thus, HBeAg production was abrogated in cultures transfected with constructs bearing the preC stop-codon mutation in agreement with Peng *et al.* (2005). In addition, Peng *et al.* (2005) demonstrated significant reduction in HBeAg production as a result of mutations in the CP region, regardless of whether or not they were accompanied by mutations in the preC region. However, due to the non-quantitative nature of the assay, reliable comparisons cannot be made. Although, the HBeAg qualitative assay used in this

study was optimised for use with patients' sera, the negative results suggest the truncation of HBeAg production, HBeAg downregulation, or if present, it may have been below the detection limit of the assay.

(A)



(B)

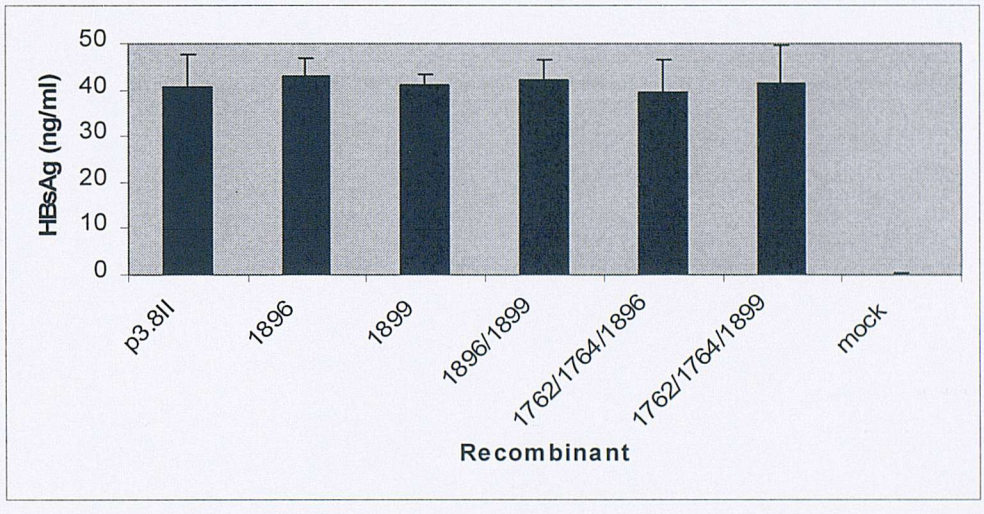


Figure 5.16 HBsAg detection in culture media from Huh7 cells transfected by recombinants harbouring mutations in the CP, preC or in both.
(A). CP mutants. **(B).** PreC, or combined CP and preC region mutants.
Results represent the average of triplicate assays from the same experiment, giving ±1 SD values.

Results obtained for HBsAg showed that all the recombinants expressed and secreted this antigen in cell culture media (Figure 5.16). Levels of HBsAg produced by the different recombinants in Huh7 cells reflected the levels of virion release recorded for each construct (Figures 5.11 and 5.12). For the CP recombinants, a

significant upregulation ($P < 0.05$) in the release of surface antigen in relation to WT was seen for each of the recombinants. In general, although, there was a correlation between virion release and antigen concentration, consistently higher levels of HBsAg were observed compared with virion release values. In comparison with the WT, whilst significant HBsAg increases were detected with three recombinants: T1766T/T1768A, A1762T/G1764A/C1766T, and T1753C/A1762T/G1764A (alterations were at least $P < 0.05$); the antigen was downregulated in T1753C/A1762T/G1764A/C1766T (−3.0–fold: significant at $P < 0.01$). There was no evidence of significant differences between HBsAg binding, or its expression by the two single CP, or A1762T/G1764A recombinants ($P < 0.05$). Some of the data are consistent with the fact that this antigen is produced in excess in the form of HBs "spheres" and "filaments", the production of which has been shown to be independent of virion production and release (Chisari *et al.* 1986). An example of this is the fact that T1753C/A1762T/G1764A showed a higher replication activity, compared to A1762T/G1764A/C1766T (Figures 5.11–5.13). However, this situation was reversed for HBsAg data.

Similar results were obtained with the combined CP and preC mutant constructs (Figure 5.16, panel B). A1762T/G1764A/G1899A and A1762T/G1764A/G1896A did not alter the antigenicity, or its expression, compared to WT.

6.0 Replication Capacity of the CP, preC and CP/preC Combined Mutant Constructs in HepG2 Cells Following Co-transfection with pCI-GFP

The replication capacity of all recombinant constructs was also determined using HepG2 cells. As with Huh7 cells, the HepG2 cell line is a differentiated hepatoma cell line routinely used for *in vitro* transfection of replication-competent HBV constructs. Functional *in vitro* studies have demonstrated that some HBV mutations did exhibit enhanced replication capacity in HepG2 than Huh7 cell lines (Buckwold *et al.* 1996d; Moriyama *et al.* 1996d; Zheng *et al.* 2003a). However, in these published studies, strains with varied genetic background, variable quality and concentration of plasmid preparations, or mutations with unknown relevance, could account for the differences observed. In this investigation the genetic background is the same for all the recombinants and, if performed with the same protocol and experimental conditions, any significant findings with regard to replication capacity should be attributable to the specifically introduced mutation(s), or to the cell line itself (Suk *et al.* 2002). To confirm that the data obtained (and given in this thesis) in a study with Huh7 cells is a reflection of the phenotype of the mutant recombinants, the same constructs were used to transfect HepG2 cells.

6.1 Determination of virion release in HepG2 cells co-transfected with various mutant constructs and pCI-GFP

To eliminate transfection efficiency differences as a potential determinant for variations in results obtained by different investigators, the method of standardisation to a fixed transfection efficiency value (15%) was implemented for HepG2 cells as it was for Huh7 cells. In general, transfection efficiencies in HepG2 cells proved lower than found in Huh7 cells as shown in Figure 6.1, panel A. Furthermore, compared to the latter (Figures 5.11 and 5.12), virion release from HepG2 cells (Figure 6.1, panel B) was marginally raised, but not significantly so ($P > 0.05$).

Notably, however, property alterations relative to WT were only manifested by the same mutations that showed variation in virion release in Huh7 cells. Interestingly, the pattern of virion release by all mutant recombinants in the two cell lines, were very similar, with minimal fluctuations between experiments and cell lines: relative to WT, virion release was upregulated in different recombinants by various orders of magnitude. C1766T/T1768A (12.2–fold), A1762T/G1764A/C1766T (5.1–fold), T1753C/A1762T/G1764A (5.9–fold); a massive downregulation by 87.4–fold was

seen for the T1753C/A1762T/G1764A/C1766T recombinant (significant at least at $P < 0.01$). Similarly, in comparison to WT, no replication alteration was evidenced in each of the single CP mutant recombinants, nor in the two combined (A1762T/G1764A) (insignificant at $P < 0.05$). Furthermore, this trend was repeated for the recombinants in panel B, including A1762T/G1764A/G1899A and A1762T/G1764A/G1896A.

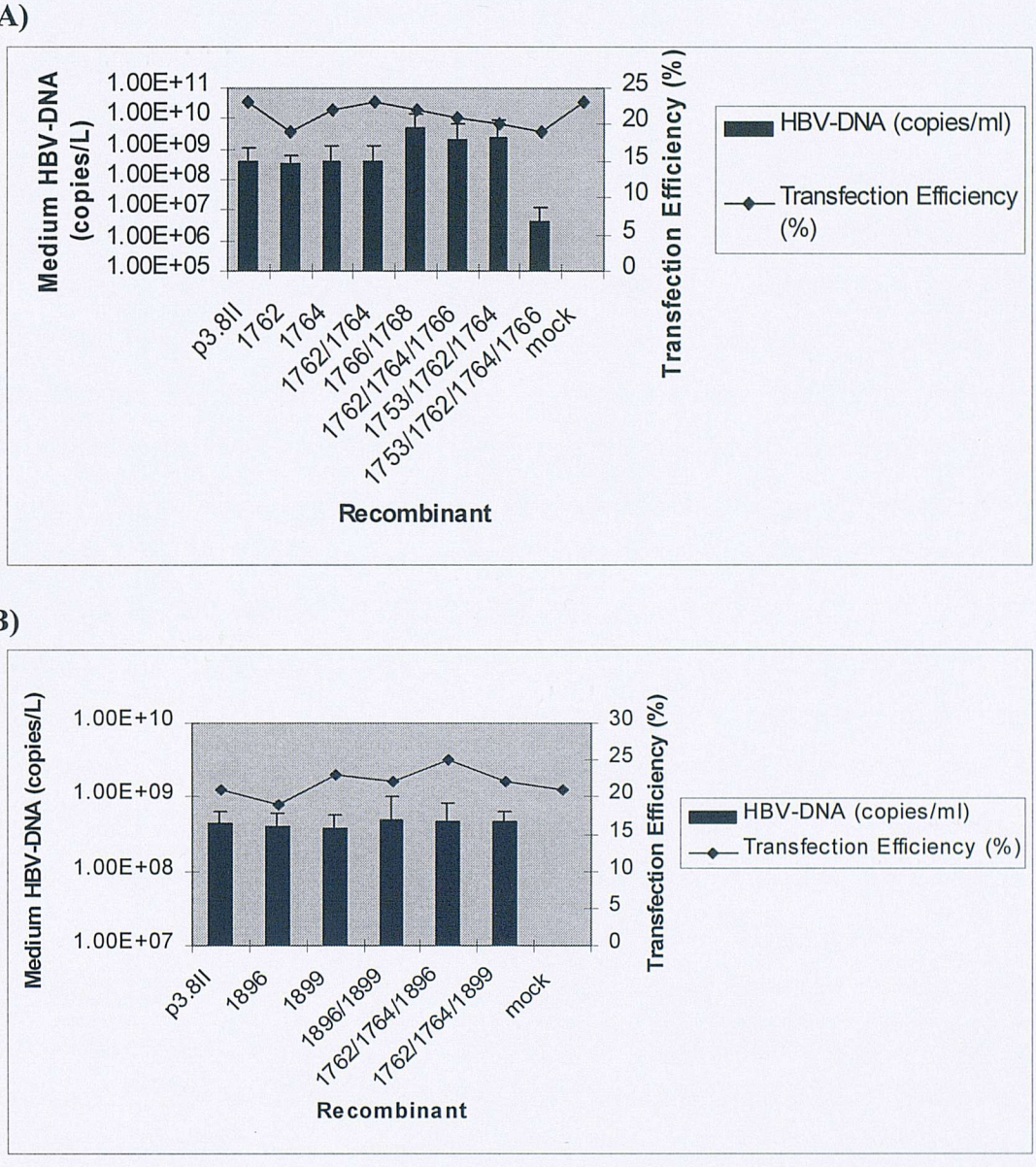


Figure 6.1 Transfection efficiency and virion release from HepG2 cells co-transfected with mutant constructs and pCI-GFP.
(A). CP mutants. **(B)**. Combined preC and CP mutants. All results are averages of triplicate analyses from the same experiment and showed ±1 SD values.

6.2 Analysis of intracellular replicative intermediates of recombinants in transfected HepG2 cells

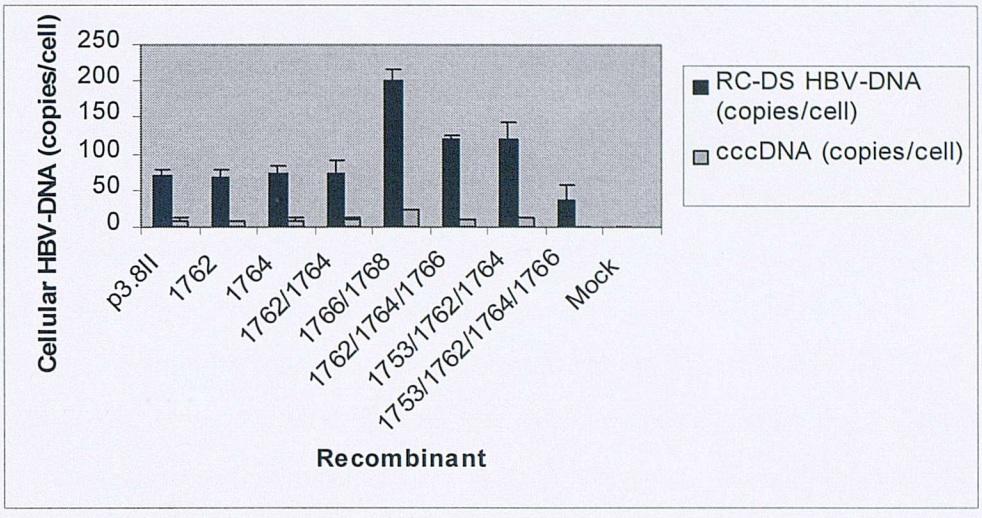
The differences in HBV DNA replication amongst WT, and the recombinants which showed virion release augmentation in Huh7 cells, appeared to originate from the altered ability of mutants to transcribe RNA from cccDNA and to synthesise HBV DNA. To confirm that virion release increases seen with some of the mutant recombinants co-transfected with pCI-GFP into HepG2 cells (Figure 6.1) correlated with similar increases in replicative intermediates, the latter were assessed by measuring RC-DS DNA and cccDNA levels, as described for Huh7 cells.

6.2.1 Detection and quantification of HBV DNA replicative intermediates

The general pattern of results obtained with HepG2 cells (Figure 6.2), mirrored that of the Huh7 cells (Figures 5.13 and 5.14). Although RC-DS HBV DNA was readily detected in triplicate assays from the same transfection for every recombinant, this was not the case with regards to cccDNA, perhaps for the reasons given earlier for Huh7 transfections. However, in spite of its significantly low levels, contrary to the Huh7 results, here, it was detectable consistently in all but one recombinant: A1762T. In further repeats, however, results were not reproducible for WT, the single CP, and the A1762T/G1764A mutant recombinants. In those mutants where it was consistently detected (Panel A), varying significant ($P < 0.05$) differences in the ratio of RC-DS DNA to cccDNA were noted: WT:C1766T/T1768A = (9:1); WT:G1764A or A1762T/G1764A = (8:1); WT:A1762T/G1764A/C1766T = (12:1), and WT:T1753C/A1762T/G1764A = (10:1). cccDNA was not detected for the T1753C/A1762T/G1764A/C1766T recombinant.

Similarly, C1766T/T1768A, A1762T/G1764A/C1766T, and T1753C/A1762T/G1764A constructs showed significantly ($P < 0.05$) more intracellular HBV DNA than WT giving values of 3–, 1.6–, and 1.8–fold. By contrast, T1753C/A1762T/G1764A/C1766T downregulated WT replication by 3.3–fold ($P < 0.05$). With regard to panel B, compared to WT, changes in intracellular HBV DNA levels were not noted as significant ($P < 0.05$), neither for each of the single CP, nor the two combined.

(A)



(B)

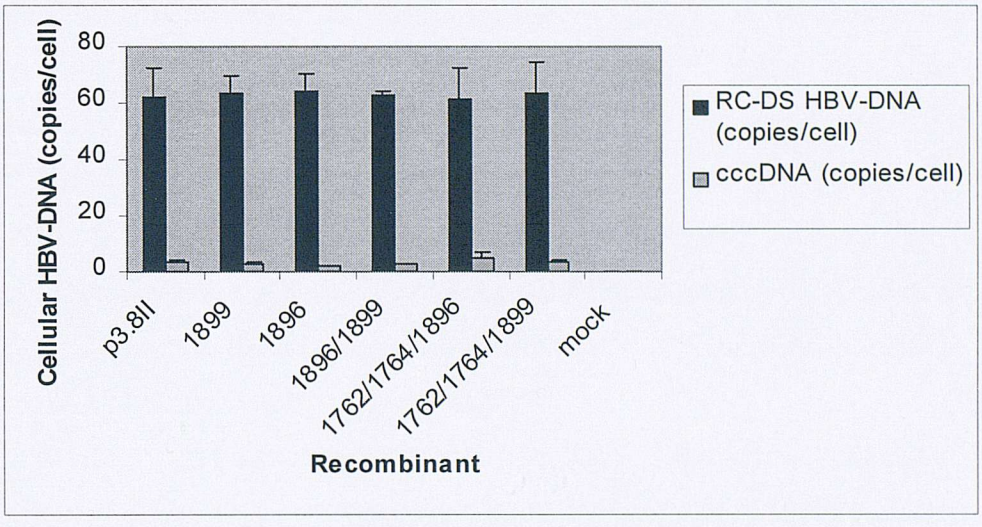


Figure 6.2 Quantification of intracellular HBV DNA in HepG2 cells transfected with various mutant HBV recombinants.
(A). CP mutant recombinants. **(B)**. Combined preC and CP mutants.
Results are the average of triplicate assays from the same experiment, giving ±1 SD values.

Correlations between extracellular and intracellular HBV DNA was seen for each of the single preC mutant recombinants, and for the two combined CP/preC recombinants (A1762T/G1764A/G1896A, and A1762T/G1764A/G1899A), relative to WT. Further, cccDNA was not consistently detected (per cell) in only two recombinants: G1896A, G1896A/G1896A. This contrasted with the Huh7 cccDNA data (where cccDNA was not consistently detectable in half the recombinants, Figure 5.2). However, as seen with the Huh7 data, cccDNA results were not reproducible in repeats. Statistically, the cccDNA results (where it was detected consistently, in both panels) did not show any significant variations relative to WT.

6.2.2 Assessment of transcription in HepG2 cells transfected by CP and preC mutant recombinants

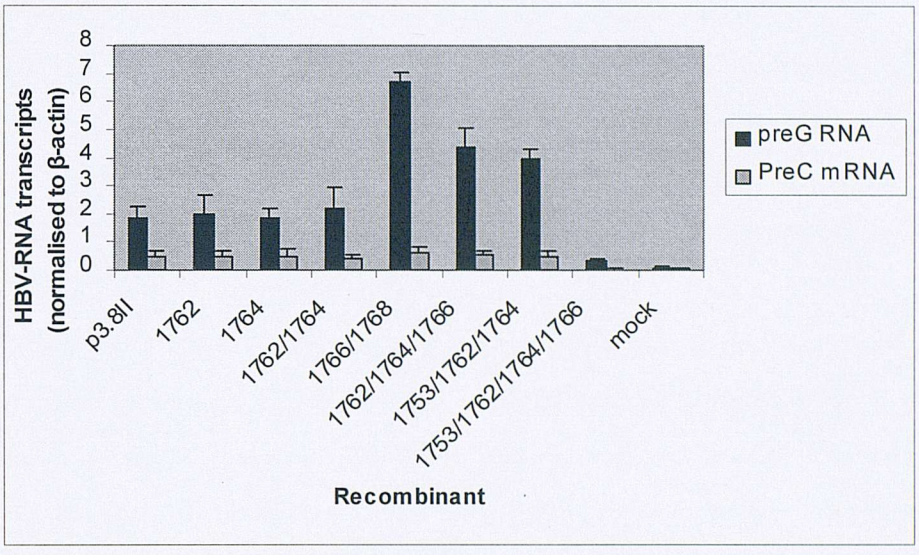
HBV transcript synthesis was next examined (Figure 6.3, panels A, and B). Based on the Real-time Transcript-specific PCR technique that was used to detect transcripts in Huh7 cells, preG RNA and preC mRNA transcripts were assayed for, in total cellular-derived RNA: A similar pattern of CP activity was apparent in both cell lines (Figure 6.3). However, the ratio of preG RNA to preC mRNA compared with WT was different; whereas in the Huh7 it ranged between 3.6:1 and 3.8:1, here it was 3.7:1 to 5.4:1. Notwithstanding this, as seen in HepG2 cells, for virion release (Figure 6.1) and total intracellular HBV DNA (Figure 6.2), the levels of HBV transcripts were higher than observed for Huh7 cells. Compared to WT, the same recombinants that showed a significant increase ($P < 0.05$) in replication activity in Huh7 cells also showed upregulated replication in HepG2 cells. Values indicating significant ($P < 0.05$) total transcript level changes were: C1766T/T1768A (3.1–fold), A1762T/G1764A/C1766T (2.2–fold), and T1753C/A1762T/G1764A (1.7–fold); T1753C/A1762T/G1764A/C1766T exhibited massive downregulation (5.8–fold) of transcription. In terms of trend, no significant differences ($P > 0.05$) were observed when total transcript levels were compared between the two cell lines; WT values were taken as reference in these comparison.

In panel B, significant variations in preG RNA were not demonstrated by any of the mutant recombinants. These data agree with the results of the parameters that were already assayed for — virion release and intracellular HBV DNA — for these recombinants. In addition, the transcript results for A1762T/G1764A/G1896A and A1762T/G1764A/G1899A were not significantly different from that obtained for the A1762T/G1764A construct, transfected in Huh7 cells (1.1–fold upregulation) — suggesting that the introduction of the single preC mutation in the A1762T/G1764A recombinant did not affect its replication capacity, in either cell line.

With regard to preC mRNA, transcript levels were not significantly altered in the CP mutant recombinants, except for the T1753C/A1762T/G1764A/C1766T construct, which indicated suppression of the transcript to undetectable levels. Transcription differences between the two cell lines were significant ($P < 0.05$). P ranged between 0.5 and 1.0, for the three recombinants that upregulated preG transcript levels (as stated above): C1766T/T1768A, A1762T/G1764A/C1766T, and T1753C/A1762T/G1764A.

In support of its extracellular and intracellular HBV DNA data (Figures 6.1 and 6.2), no variation in preC mRNA levels was obtained between WT and the G1899A and A1762T/G1764A/G1899A mutant recombinants; ($P > 0.05$) (Figure 6.3, panel B). However, as already seen with the Huh7 data (Section 5.4.5), significant transcript downregulation was observed with the stop-codon mutants.

(A)



(B)

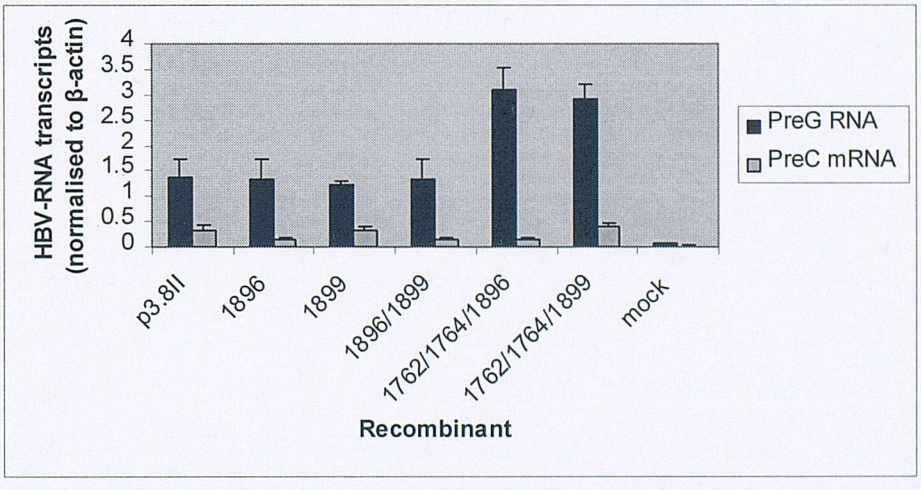


Figure 6.3 Differential detection of HBV transcripts in HepG2 cells with mutant recombinants of CP, preC or both.
(A). CP mutants. **(B).** Combined preC and CP mutants.
Results are means of triplicate assays from the same experiment, giving ±1 SD values.

In general, however, significant upregulation of transcription levels was seen in HepG2 *vs.* Huh7 cells. This ranged from 1.5– to 1.9–fold more tanscript, for the above listed CP recombinants. Taken together, it appears that under the experimental

conditions used here, the various mutant recombinants and WT behaved in a different manner in the two cell lines, with Huh7 cells yielding lower values. Notwithstanding this, the patterns of replication showed a similar trend in the two cell types. These potentially important conclusions were confirmed in two further repeat experiments.

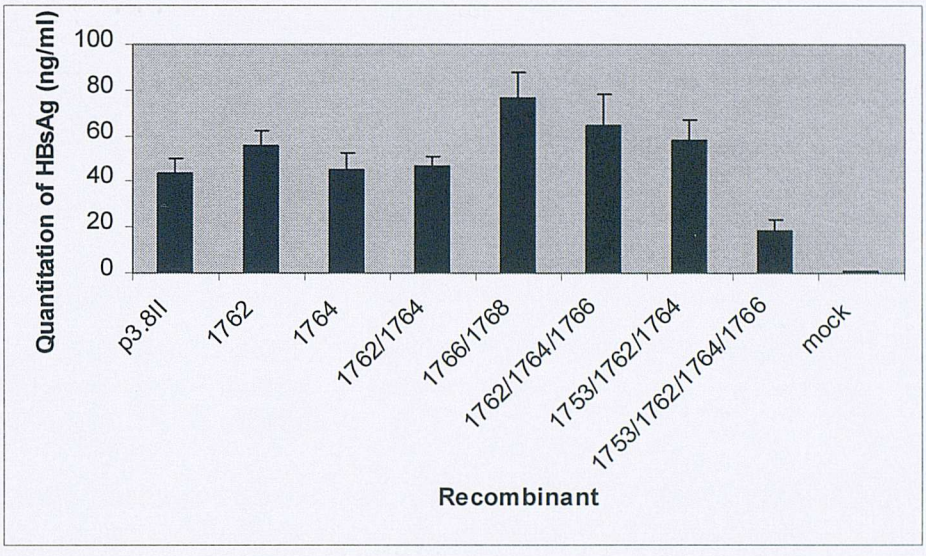
6.3 Analyses of antigens release in media from HepG2 cells transfected with mutant recombinants

HepG2 cell culture media were analysed for HBeAg production 96hrs post transfection, to assess the activity of the CP in these cells. The results obtained were similar to those found in Huh7 cells, with the apparent exception of the A1762T/G1764A/G1899A construct; this result was, however, not reproducible in the two further repeats.

Next, the amount of HBsAg secreted in the culture media was determined by quantitative ELISA in HepG2 cells. All recombinants were found to secrete HBsAg in detectable amounts (Figure 6.4). When the relative amounts of the antigen were compared, an upward shift was found amongst all recombinants compared to the Huh7 HBsAg results (Figure 5.16). What was interesting moreover was the fact that the difference between each recombinant and WT was lower than observed for the Huh7 cells. G1764A and A1762T/G1764A CP mutant recombinants showed negligible differences compared to WT. Using WT as reference however, antigen upregulations (at least significant at $P < 0.05$) were observed with C1766T/T1768A, A1762T/G1764A/C1766T, and T1753C/A1762T/G1764A. On the other hand, a decrease (significant at $P < 0.01$) in antigen release was seen with the T1753C/A1762T/G1764A/C1766T recombinant. Notwithstanding this downregulation, there was a correlation ($\rho = 0.667$) between concentrations of antigen and virion, in the supernatant. The differences observed between the two cell lines used were significant ($P < 0.05$). Surprisingly, a significant ($P < 0.05$) increase in HBsAg expression was seen for the G1762A mutant recombinant, relative to WT (1.3–fold), unlike the data obtained for the two other recombinants (G1764A and A1762T/G1764A); contrary to general conventional evidence, this particular data did not correlate with corresponding virion release data (Figure 6.1, panel A), which saw no significant difference between virion release of this particular recombinant, compared to WT. No other investigation (*in vitro* or *in vivo*) has yet shown this

mutant as having a high HBsAg-releasing phenotype. What other investigators have also observed however, is the discrepancy between levels of virion release and HBsAg for CP, and/or preC recombinants (Chisari *et al.* 1987b). This was associated with excess surface antigen in cells, in the form of spheres and filaments, as previously explained. However these specific data were only reproducible once, but not for the second time as was the case with the others.

(A)



(B)

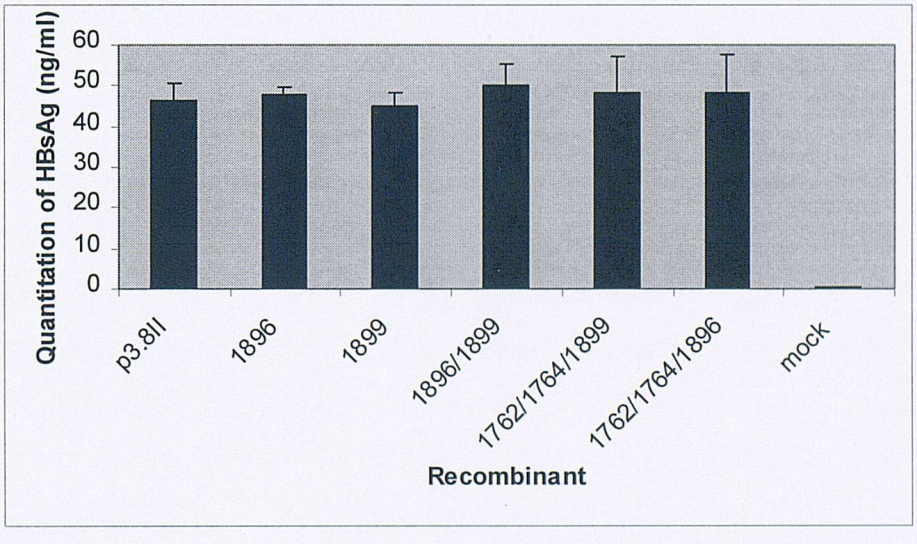


Figure 6.4 Quantification of HBsAg released into the media of HepG2 cells transfected with the various mutant recombinants and WT
(A). CP mutants. **(B)**. Combined preC and CP mutants.
Results are average of triplicate assays from the same experiment, given as ±1 SD.

For the two combined CP and preC mutant recombinants, neither HBsAg synthesis nor its binding capacity, as assayed for by ELISA, were affected. These results bore no evidence of alteration to that of G1764A, and A1762T/G1764A mutant recombinants (panel A). Generally, the trend was similar to that noted with the Huh7 transfection results.

All the stated HepG2 transfection results are summarised in Table 6.1.

Position of CP mutations	DNA replication	DS intracellular DNA	preG RNA	preC mRNA	HBsAg
WT	+++	+++	+++	+	+++
1762	+++	+++	+++	+	+++
1764	+++	+++	+++	+	+++
1762/1764	+++	+++	+++	+/–	+++
1766/1768	++++++	++++++	++++++	+/–	++++++
1762/1764/1766	++++	++++	+++++	+/–	+++++
1753/1762/1764	++++	++++	++++	+/–	+++++
1753/1762/1764/1766	+	+	–	–	+

Table 6.1 Summary of the parameters measured after transfection of HepG2 cells with constructs bearing CP mutations.
All comparisons were referenced to the WT.

[The results obtained following transfection of the mutant recombinants bearing mutations in the preC region show no significant differences from those obtained with the WT. Additionally, similar findings applied to comparisons between constructs with combined preC and CP mutations and the 1762/1764 construct alone. These observations also applied to transfections in HepG2 cells].

6.4 Correlation between HBV DNA, HBV RNA and HBs antigen (HBsAg)

Since this study has shown that the pattern of replication of the various recombinants is generally similar in the two hepatoma cell lines, the *Spearman's* correlation coefficient (ρ) was calculated using only the HepG2 cell line standardised data. Further, since results for cccDNA were either very low, or inconsistently detectable, only data for RC-DS HBV DNA was utilised in calculations to represent intracellular HBV DNA. Total HBV transcript levels were used.

There proved to be a strong correlation between the concentration of released HBV DNA (virions) and HBsAg ($\rho = 0.523$; $P < 0.05$), as well as between released HBV DNA and total transcribed HBV RNA ($\rho = 0.621$; $P < 0.05$).
[See Section 2.1.18 for statistical description].

7.0 The Effect of HBV Surface Gene Mutations on Polymerase Function

In order to study the effect that mutations in the HBV "a" determinant region of the S gene might have on the function of the RT region of the overlapping P gene, preliminary experiments included detection and localisation of the surface protein in Huh7 cells. The binding characteristics of the protein were assessed using a panel of monoclonal antibodies to HBsAg (anti-HBs) directed against the "a" determinant. Subsequently, different naturally occurring S and P gene mutant recombinants generated in the common genetic backbone (p3.8II) by SDM were co-transfected (with pCI-GFP) into Huh7 cell line in the presence or absence of the nucleoside analogue, lamivudine (LMV). Replication levels were assessed by Real-time PCR and HBsAg binding specificity determined by ELISA, and the results are given below.

7.1 Localisation of HBsAg

In situ staining for HBsAg following transfection of the WT recombinant in Huh7 cells showed that there was both sub-membrane (double-headed arrow, Figure 7.1, panel A), and cytoplasmic staining (single arrow, Figure 7.1, panel B), surrounding the blue DAPI-stained nuclei. By contrast, there was evidence (panels C and D) of poor binding specificity to the RFHBs18 primary antibody by the G145R mutant recombinant. Unlike the HBsAg staining characteristics of the WT (panels A and B) which demonstrated an "even" antigen distribution, the G145R variant surface antigen either did not stain, or showed very weak punctate staining (Figure 7.1). These findings are further represented by Table 7.1. Similar to the results of the HBcAg staining (Section 5.2), cell lost were encountered following the staining procedure (Figure 7.1)

To prove the binding specificity of the primary antibody for human WT-HBsAg, WT transfected Huh7 cells were also stained with a primary antibody specific for woodchuck hepatitis surface antigen (anti-WHsAg). Poor binding only was observed (Figure 7.1, panels E, and F).

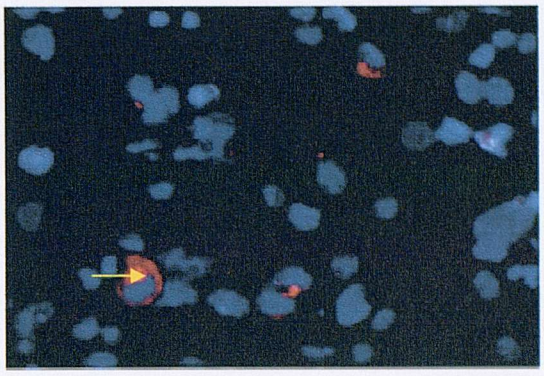
Mock transfection with the empty vector, did not show any HBsAg binding signal (Figure 7.1, panels G and H).

Figure 7.1 Immunofluorescence detection of HBsAg in Huh7 cells transfected with the WT or G145R recombinants using the RFHBs18 as primary antibody.
A. CY3-labelled antibody staining of WT HBsAg under a red filter. Double-headed arrow indicates sub-membrane HBsAg localisation.
B. DAPI-stained nuclei merged with **A** (blue filter). Arrow points to HBsAg cytoplasmic localisation in the same cell as in **A**.
C. CY3-labelled antibody staining of the G145R mutant HBsAg;
D. DAPI-stained nuclei merged with **C**;
E. CY3-labelled anti-WHs staining of WT HBsAg;
F. DAPI-stained nuclei merged with **E**;
G. CY3-labelled antibody staining of pBluescript® II KS(+) vector;
H. Green-fluorescence protein detection under a green filter, expressed by pCI-GFP.

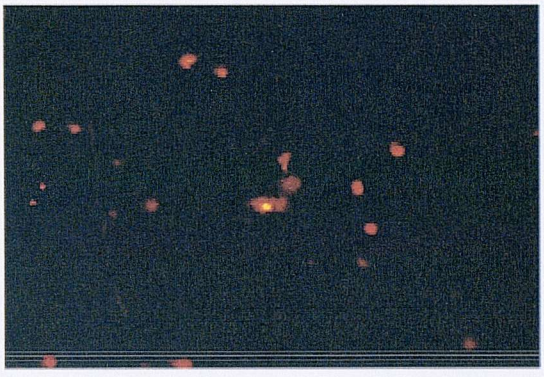
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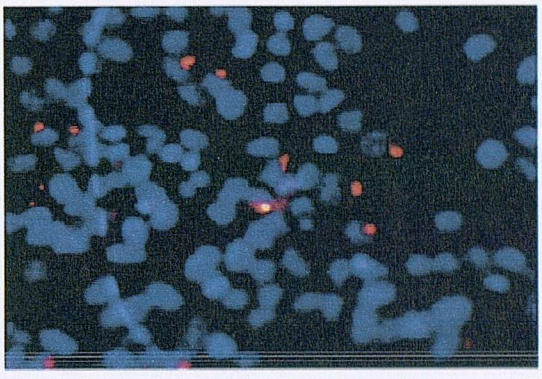
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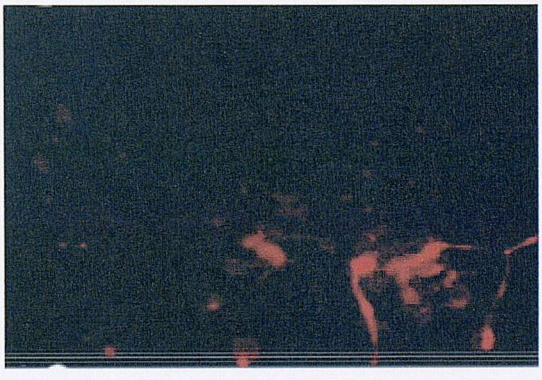
C



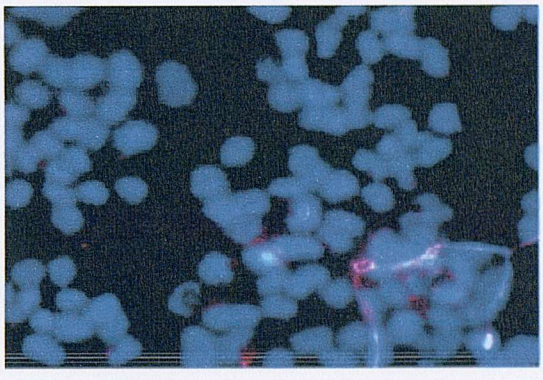
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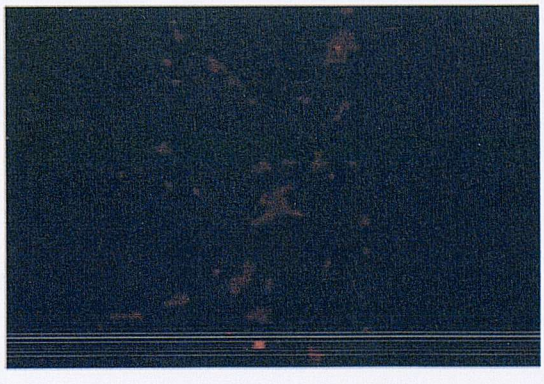
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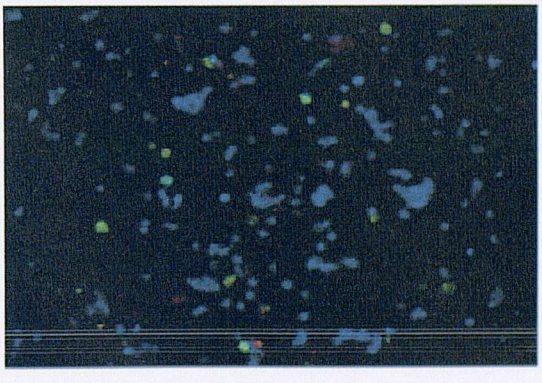
F



G



H



7.2 HBsAg detection in Huh7 cells transfected with constructs bearing mutations in the "a" determinant

To determine the effect of mutations introduced in the "a" determinant region of the S gene on the antigenicity of HBsAg, the aforementioned panel of anti-HBs monoclonals were used. Wells containing coverglasses were seeded with the same number of Huh7 cells and transfected with the various constructs on the same day. These cells (on the coverglasses) were then used for detection of HBsAg. No other method of standardisation was employed here.

The three single vaccine-associated S gene mutations that were identified and subsequently introduced into the p3.8II background by SDM (Methods and Results Sections 2.2.8 and 3.3, respectively) are shown in Table 7.1. The binding capacity of mutant recombinant antigens was compared to that of the WT. No binding was seen between neither RFHBs1, 2, and 6, and the mutant recombinants. Unlike WT where RFHBs4, 7 and 18 bound strongly, the mutant recombinants gave a very weak signal with the same antibodies. As control, cells transfected with WT were reacted with an anti-Woodchuck (WHs) monoclonal. This bound poorly to the HBsAg, indicating a lack of specificity of the antibody for the antigen, as shown previously in Figure 7.1, panel E.

Anti-HBs monoclonal	Cyclical peptide binding site	T131I	K141E	G145R	Wildtype	G145R/L180M/ M204V
RFHBs 1	124-137	-	-	-	+	-
RFHBs 2	124-137	-	-	-	+	-
RFHBs 4	124-137	+	+	+	++	+
RFHBs 7	139-147	+	-	+	+++	+
RFHBs 6	Not defined	-	-	-	+	-
RFHBs18	Not defined	+	+	+	+++	+

Table 7.1 Binding characteristics of a panel of human anti-HBs monoclonals directed against the group specific "a" determinant, in cells transfected with the WT and mutant recombinants

7.3 Molecular analysis of changes in P function as a result of S gene mutations

With the aid of the techniques previously described, that is, Real-time PCR, Transcript-specific PCR and quantitative ELISA, and building on the results obtained in the previous two (7.1 and 7.2) sections, the effect of mutations in the S gene on the function of the overlapping P encoding region were examined.

7.3.1 Assessment of the replication fitness of S gene mutant recombinants

The effect of the S mutations on virus replication was analysed. 96hrs after transfection of Huh7 cells, virion release was quantified by Real-time PCR. Figure 7.2 shows an insignificant difference in the replication capacity of the G145R mutant recombinant (or T131I) relative to WT ($P > 0.05$). By contrast, recombinant K141E replicated less efficiently than WT or mutants G145R and T131I ($P < 0.05$). The pattern of replication of these S mutant recombinants suggested that at least one of them (K141E) was defective in viral replication.

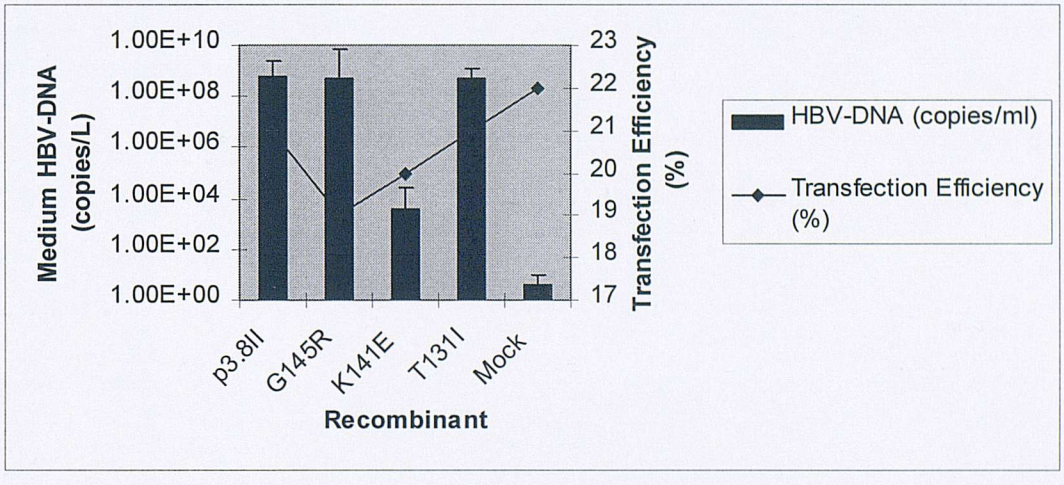


Figure 7.2 Virion release in culture media from Huh7 cells co-transfected by the WT or "a" determinant/S gene mutant recombinants, and pCI-GFP.
Results are shown as an average of triplicate assays from the same experiment, giving ±1 SD values.

The levels of total cellular HBV DNA and the mRNA transcription capacity of the BCP were also measured. Figure 7.3A shows that the surface recombinant K141E had significantly less cellular RC-DS HBV DNA relative to WT (or other mutants, G145R and T131I) indicating that the impairment in the replication efficiency of the construct (K141E) reflected an intracellular defect ($P < 0.05$).

cccDNA was consistently detected among triplicate assays, but not at all in K141E transfected cells.

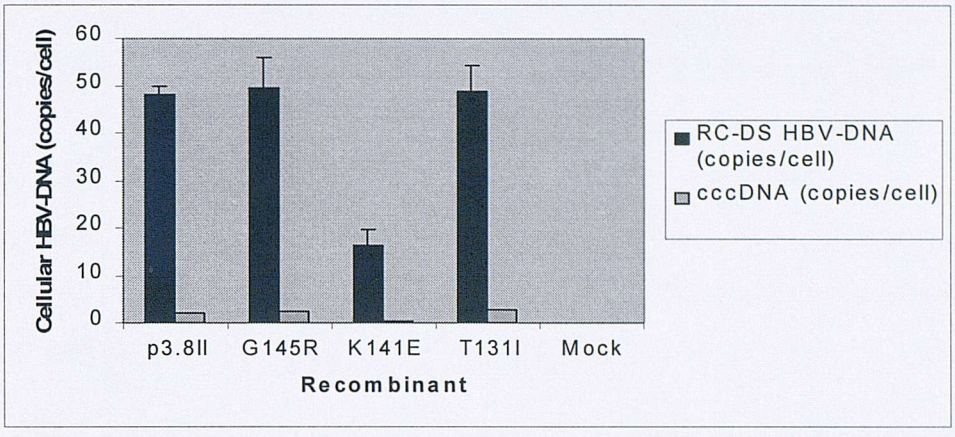


Figure 7.3A Quantification of intracellular HBV DNA replicative intermediates of "a" determinant/S gene mutant recombinants transfected into Huh7 cells.
Results indicate average of triplicate assays from the same experiment, giving ±1 SD values.

Examination of transcription levels again showed downregulation of both transcripts (preG RNA and preC mRNA) as compared with either WT or mutants (Figure 7.3B) in Huh7 cell lysates of K141E transfections. The difference was statistically significant ($P < 0.05$). The data were in agreement with the virion release results (Figure 7.3A) and, taken together, demonstrate that the K141E mutant did not replicate efficiently (see discussion).

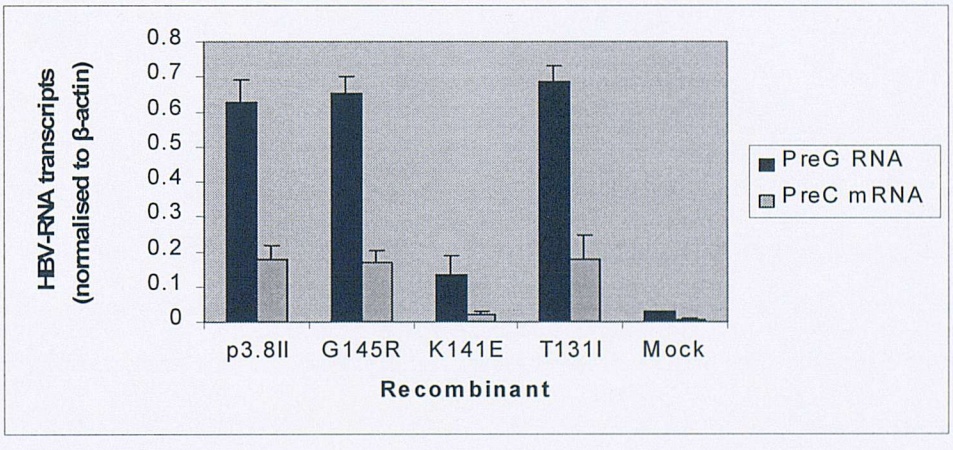


Figure 7.3B HBV transcript detection in Huh7 cells transfected with WT or S gene mutant recombinants.
Results are shown as an average of a triplicate from the same experiment, giving ±1 SD values.

Quantitative ELISA allowed the binding capacity determination of HBsAg released in the media of cells transfected by the HBsAg mutant recombinants to be assessed. Compared to WT, highly reduced HBsAg binding capacity was observed in the mutants (Figure 7.4). Unlike WT, it is evident that there is no correlation between HBsAg detection (Figure 7.4) and virion release (Figure 7.2) in the G145R and T131I mutant recombinants. Due to the low absorbance readings that were obtained here, it was thought prudent not to convert the A_{450} HBsAg absorbance values into concentration values (ng/ml) — as was the case in the two previous sections. Nevertheless, standardisation to 15% transfection efficiency was employed here, as in previous experiments. (See Appendix #3b, sample 2).

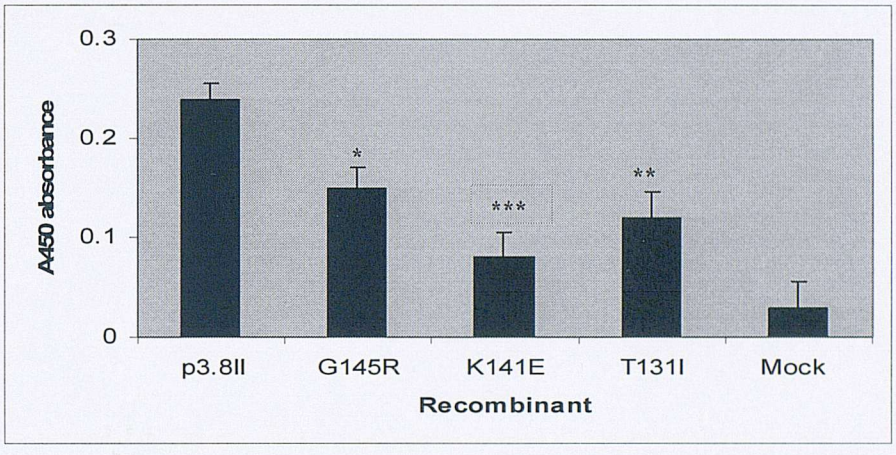


Figure 7.4 Kinetic detection of HBsAg in culture media of Huh7 cells transfected by WT or S gene mutant recombinants.
Results are shown as an average of triplicate assays from the same experiment, giving ±1 SD values.

*, **, and ***: $P < 0.05$, 0.01, and 0.001, respectively.

7.3.2. *In vitro* analysis of recombinants bearing mutation(s) in the RT region of the P gene, the "a" determinant of the S gene, or both

Together with other well-recognised P (RT) gene mutations (conferring LMV-resistance), the G145R S gene mutation was also introduced into the p3.8II genetic background and transfected into Huh7 cells in the presence or absence of LMV. The data (Figure 7.5) support the enhanced replication phenotype of G145R/L180M/M204V compared to L180M/M204V, as observed by others ($P < 0.05$) (Delaney *et al.* 2003a; Ono-Nita *et al.* 1999a). Despite this upregulation by the triple-mutation recombinant compared to WT, virion release remained

significantly less ($P < 0.05$), implying that the replication capacity of the L180M/M204V was only partially restored by the introduction of the G145R mutation. However, up to 96hrs post transfection in LMV-treated cells, the reduction in extracellular HBV DNA in G145R/L180M/M204V cultures was not significantly different from that in the non-treated G145R/L180M/M204V cultures ($P > 0.05$), indicating its resistance to the drug. The opposite effect was observed with WT which responded to the drug with a nearly 75% reduction in virion release over the same period. Following LMV-treatment, a similar reduction was seen with the two single RT/P gene mutant recombinants, L180M and M204V, both of which replicated at an efficiency similar to WT (Figure 7.5).

Whilst no difference in replication was seen between the G145R recombinant and WT, when G145R was introduced into a recombinant that bore the single M204I substitution, the resulting recombinant showed similar replication capacity as the M204I recombinant (Figure 7.5). Replication efficiencies given in Figure 7.5 represent results of transfections with LMV and without LMV for each recombinant. One transfected well was reserved for FACS analysis. All results shown were subsequent to standardisation to 15% transfection efficiency, using a similar method of calculation as before. [Refer to Appendix #3, for sample calculations of transfection efficiency standardisation].

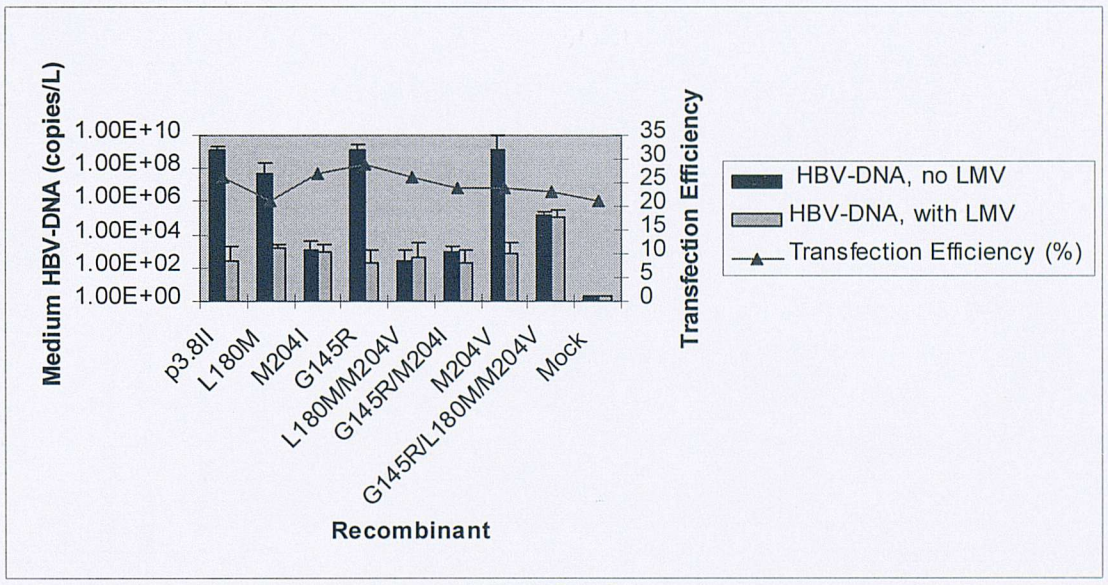
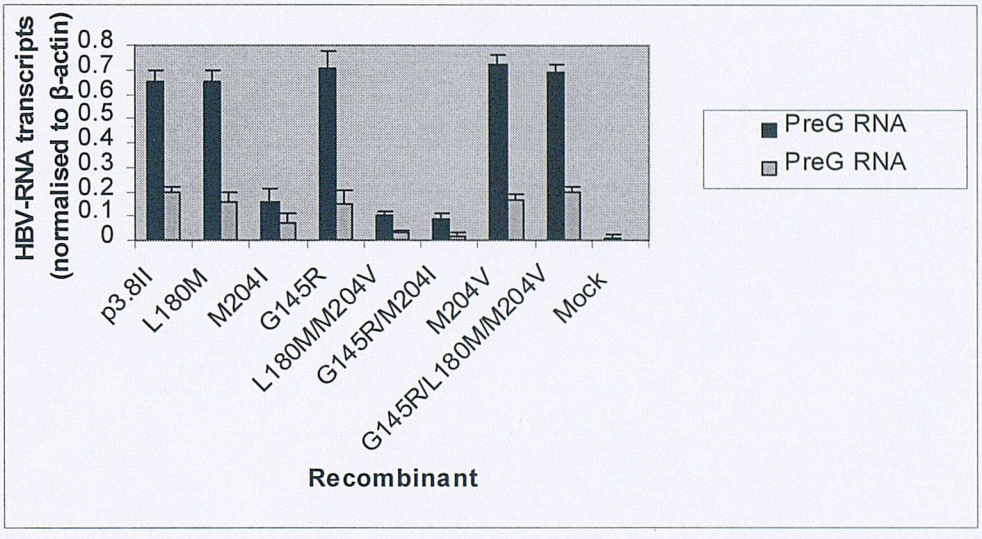


Figure 7.5 HBV DNA from virions released in culture media of Huh7 cells co-transfected with the constructs and pCI-GFP, shown in the presence and absence of LMV.
Results are shown as an average of a triplicated assay from the same experiment, giving ±1 SD values.

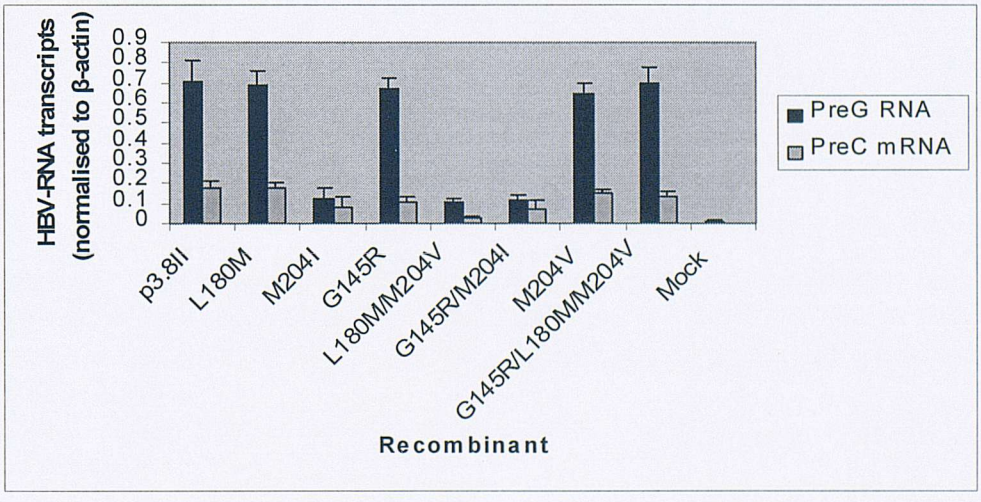
7.3.3 Replicative intermediates in Huh7 cells transfected with "a" determinant/ S, and RT/P mutant recombinants

To determine whether the increased level in virion production conferred by the G145R/L180M/M204V recombinant showed a parallel increase in intracellular HBV DNA and transcription, cells were assayed 96hrs post transfection, as previously described. Figure 7.6; panel A, shows transcription elevation by the recombinant harbouring the above triple-mutation, mirroring the increases in virion release seen in Figure 7.5. This increase correlated with the observed increase of intracellular total HBV DNA (RC-DS plus ccc) (Figure 7.6, panel B), as well as with enhanced levels of replicative intermediates ($P < 0.05$).

(A) HBV RNA transcript, in the absence of LMV

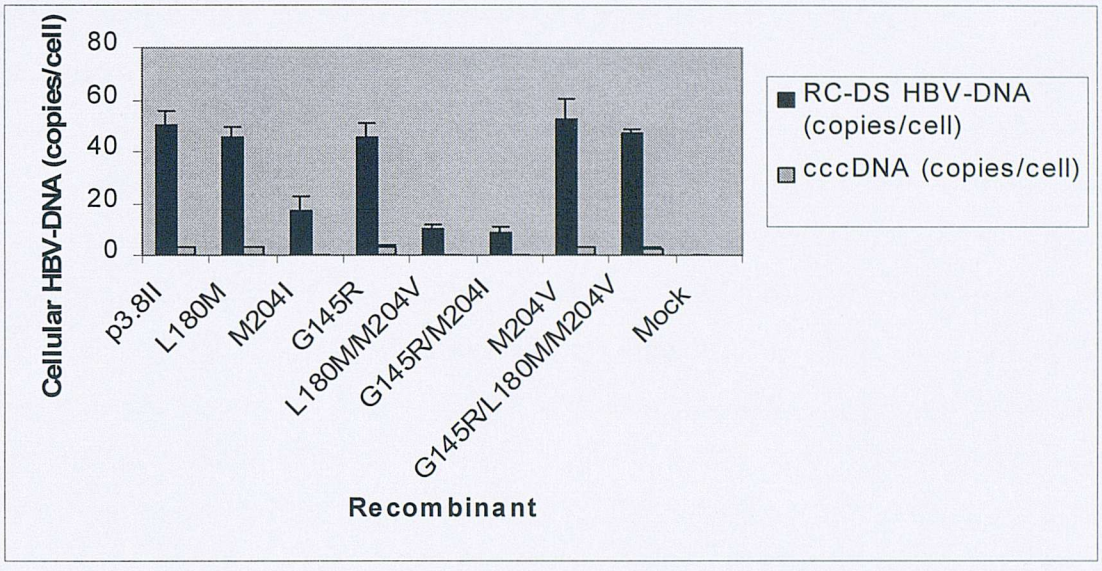


(B) HBV RNA transcript, with LMV



For some recombinant lysates, the two forms of intracellular HBV DNA assayed for were either not consistently detected, or not detected at all in the case of ccDNA (Figure 7.6, panels C and D) independent of the presence or absence of LMV. This is likely due to low copy numbers.

(C) Intracellular HBV DNA, in the absence of LMV



(D) Intracellular HBV DNA, with LMV

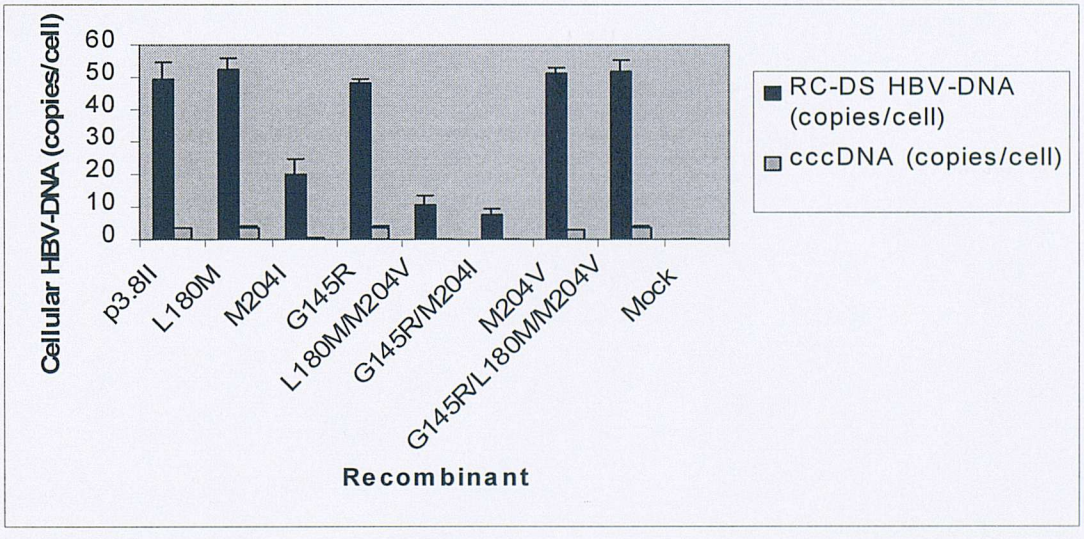


Figure 7.6 Detection of replicative intermediates extracted from Huh7 cells transfected with recombinants harbouring mutations in the RT/P gene alone or in combination with those in the "a" determinant/S gene, in the presence or absence of LMV.

Results are shown as an average of triplicate assays from the same experiment, giving ±1 SD values.

7.3.4 Detection of HBeAg and HBsAg synthesised by recombinants in transfected Huh7 cells

The fact that the ratio of preC mRNA to preG RNA was unaffected by LMV would suggest no change in the capacity of these recombinants to produce HBeAg. This was tested using G145R and G145R/L180M/M204V. The assay indicated that the antigen was undetectable in the former, whilst positive (≥ 0.015PEI/ml) in the latter. However, as previously explained for preC and CP recombinants, the HBeAg assay used, optimised for detecting the antigen in patient samples, was qualitative only.

Binding of HBsAg produced by the recombinants was also assessed (Figure 7.7). Compared to both WT, and the double mutant recombinant, L180M/M204V, there was a reduced binding capacity to HBsAg from recombinants harbouring the G145R substitution (G145R/L180M/M204V, G145R/204I) ($P < 0.05$). Both this assay and the corresponding virion release assay (Figure 7.5) suggest the mutant (L180M/M204V) to be partly defective in replicative capacity. Although the results of virion release for the G145R implied similar replication efficiency to WT, the mutant (G145R) had a reduced capacity for antigen binding to antibodies, as expected. Overall, the findings showed replication upregulation as a result of introducing the G145R substitution into an RT/P mutant recombinant, confirmed by data given in Figures 7.5 and 7.6.

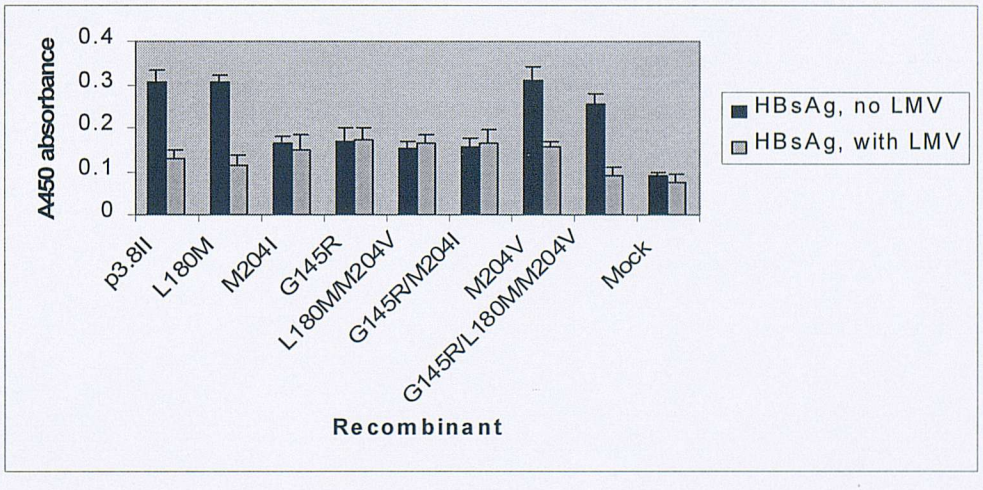


Figure 7.7 Detection of HBsAg in culture media of Huh7 cells transfected with recombinants harbouring mutations in the RT/P gene or in combination with "a" determinant/S gene mutations, in the presence and absence of LMV. Results are shown as an average of triplicate assays from the same experiment, given as ±1 SD values.

When media containing LMV were tested for HBsAg binding, the results (Fig 7.7) for WT showed a lower antigen concentration (2–fold) ($P < 0.05$) compared to that of the non-LMV treated cells. This effect was similarly seen with the L180M, M204V, and L180M/M204V recombinants. However, the remaining mutant recombinants either failed to demonstrate any differences in binding capacity, or showed marginal alterations ($P > 0.05$) when their transfected media with or without LMV are compared (Figure 7.7).

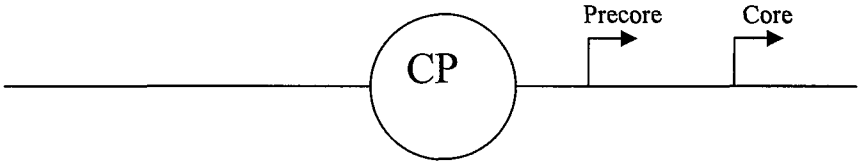
In general, the results demonstrated that P gene mutations in the RT domain, when not accompanied by the G145R substitution, may have no effect on HBs antigenicity, as implied by the ELISA assay data.

8.0 Promoter-Analysis of CP mutations:

8.1 The effect of CP mutations on transcription factor binding

To further define the HBV sequences responsible for enhanced replication of FHB strains, the same CP mutations investigated in this study were assessed and compared to WT in *in vitro* EMSA, using oligonucleotides representing transcription factor binding sites in HBV CP region (Figure 8.1, B: CP1–CP7). Other FHB isolates (Figure 8.1, B: CP8–CP11) were also studied for binding to hepatoma cell nuclear transcription factors.

(A)



(B)

WT	ATT AGG TTA AAG GTC TTT **I R L K V F**	**WT**
CP1	ATT AGG TTA **A**TG GTC TTT **I R L M V F**	A1762T
CP2	ATT AGG TTA AAG **A**TC TTT **I R L K I F**	G1764A
CP3	ATT AGG TTA **A**TG **A**TC TTT **I R L M I F**	A1762T/G1764A
CP4	ATT AGG TTA AAG G**T**T **T**AT **I R L K V Y**	C1766T/T1768A
CP5	ATT AGG TTA **A**TG **GTT** TTT **I R L M V F**	A1762T/G1764A/C1766T
CP6	**A**CT AGG TTA **A**TG **A**TC TTT **T R L M V F**	T1753C/A1762T/G1764A
CP7	**A**CT AGG TTA **A**TG **ATT** TTT **T R L M V F**	T1753C/A1762T/G1764A/C1766T
CP8	**GTC** AGG TTA AAG GTC TTT **G**TA **C**TA **V R L K V F V L**	A1752G/1754C/A1772T/T1774A

CP9	GTC AGG TTA AAG GTT TAT **V R L K V Y**	A1752G/T1754C/T1768A
CP10	ATT AGG TTA AAG ATT TTT **I R L K V F**	G1764A/C1766T
CP11	GTC AGG TTA AAG GTT TTT **V R L K V F**	A1752G/T1754C

Figure 8.1 Binding sites of transcription factors to HBV CP sequences from various mutants
(A). The CP and transcription initiation sites of the preC mRNA and preG RNA are illustrated. **(B).** The CP sequence between positions 1752 and 1769 (1752–1775 for CP8) for the WT compared with those from a number of mutants. The boldface letters in the gene highlight the locations of nucleotide mutations, and the alterations produced in amino acid sequences are given. [See Figure 3.1 for detail positions of substituted nucleotides].

8.2 Competition assay for transcription factor binding to the WT CP sequence

Preliminary experiments were concerned with the determination of specific and non-specific binding of the oligonucleotides to transcription factors. In this assay, the binding of transcription factors from Huh7 cell lysates was conducted in the presence of increasing molar ratios of non-labelled WT DS-annealed oligonucleotide. As shown in Figure 8.2, lane 3, two major shifted bands (labelled I and II), and two minor bands (III and IV) — which migrated slower on the gel — were detected when WT sequence was used in the binding reaction. When the same molar concentration of a specific competitor was used (lane 4), the pattern and intensity of the shifted bands were indistinguishable from WT (lane 3). However, although the intensity of the major bands was reduced compared to the WT when the concentration of the competitor was increased to 3.0–fold (lane 5), even this concentration was insufficient to prevent binding completely. Increasing the competitor to 5.0–fold (lane 6), still did not block WT binding. When binding of transcription factors to un-related sequences was assayed for, that is, cold oligonucleotides that bound to an unrelated region (P gene) also using concentrations designated 1.0–, 3.0– and 5.0–fold (lanes 7–9), there was no reduction in the intensity of the shifted bands compared to WT.

Lane 1 shows that factors that bind WT sequence are specific to hepatoma cells since there was no binding when proteins extracted from Huh7 cells were replaced by those of 143-TK$^-$ (osteosarcoma cells). As expected, no bandshift was seen in lane 2, (a negative control) since WT oligonucleotide end-labelled with [γ-^{32}P]ATP was incubated without nuclear extract. The drawn conclusion allowed by the observed binding results is that WT sequence, as used, binds strongly and specifically to transcription factors present in the hepatoma cell extract.

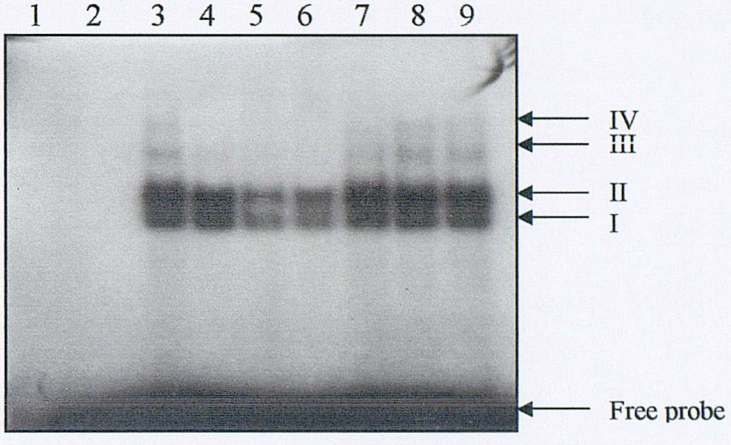


Figure 8.2 EMSA for WT
Annealed oligonucleotides were end-labelled with [γ-^{32}P]ATP and incubated with 6µg of Huh7 protein extract. A competition assay was performed by using the WT DNA represented by lane 3, as probe in the presence of protein extract from 143-TK$^-$ cells (lane 1). Huh7 cell extract was not added to the negative control (lane 2). The probe was specifically competed with 1.0–, 3.0–, or 6.0–fold of un-labelled CP10 sequence (lanes 4, 5 and 6, respectively). 1.0–, 3.0–, or 6.0–fold "cold" un-related competitor, as control, was added to lanes 7, 8 and 9, respectively.

8.3 Role of transcription factor binding in the replication efficiency of CP mutants

Since deletion mutant analyses showed that the region 1752 to1775, forming part of the EnhII/BCP region, contain *cis*-acting elements (Ishida *et al.* 2000b; Raney *et al.* 1997d; Viollet *et al.* 1997c), it follows that some transcription factors must be associated with this region. Using as probes various DS-annealed oligonucleotide that bear CP nucleotide substitutions within this region (between 1752 and 1769) further assays for transcription factor binding were undertaken in gel retardation analyses, similar to those outlined in Figure 8.2.

Figure 8.3, lane WT contains two specific (I and II) gel retarded bands and supports the presence of transcription factors in Huh7 cells which bind to the WT CP sequence. This binding appears to be specific since there was competition for the signal when 20.0–fold, "cold" competitor was used (lane SI). Similarly, under as near as possible the same reaction conditions, different mutations examined (lanes CP1–CP7) exhibited binding to these nuclear factors. Relative to the WT, binding was strongest with CP4 and weakest with CP7 (see Figure 8.1 for nucleotide compositions). These mutations corresponded to C1766T/T1768A (CP4) and T1753C/A1762T/G1764A/C1766T (CP7), respectively. Consistent with previous results (Figure 8.2), two major bands (labelled I and II) were shifted. A third band (labelled III) is barely visible in each of lanes CP4 and CP6. The production of at least two bands suggests that more than one factor present in the Huh7 nuclear extract may bind to nt. 1752 to 1769, to regulate EnhII/CP activity. An alternative explanation may be that, dimers (or other forms) of the same transcription factor may bind, with apparent equal affinity.

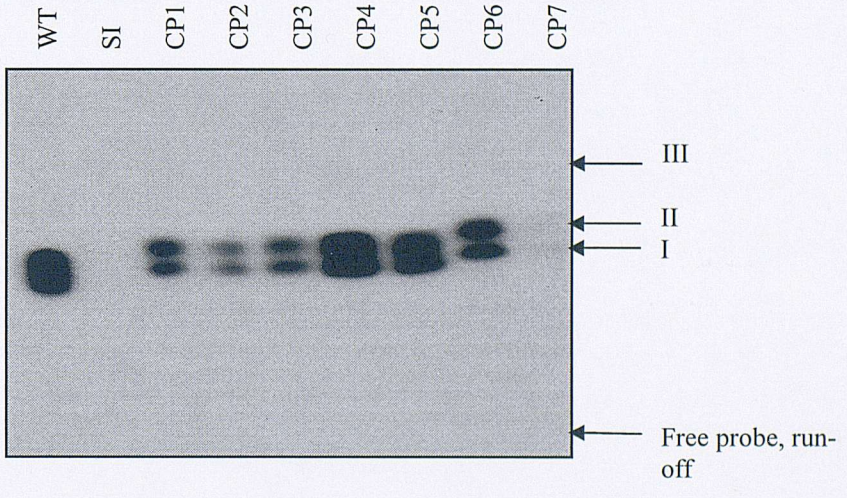


Figure 8.3 Gel retardation analysis of HBV WT oligonucleotides in nt. 1752 to 1769 region and mutations.
[γ-^{32}P]ATP-end-labelled DS-annealed oligonucleotide containing the sequence from nt. 1752 to 1769 (WT and CP1–CP7, as designated) incubated with Huh7 nuclear extract and products resolved on polyacrylamide gel. Two major bands (I and II) are observed for WT and CP1–CP7. A 20.0–fold unlabelled specific competitor abolished the specific band-shifts (lane SI).

Other FHB isolates that were available were similarly used in gel retardation assays for comparison purposes (Figure 8.4). Interestingly, they all showed the doublet transcription factor binding pattern, seen in Figure 8.3. However, they differed in intensity from WT, and appeared to demonstrate earlier weak patterns, as observed with CP7 (Figure 8.3). Despite their weak binding intensities, the residual signals were strong enough to conclude that at least two major shifted bands of similar intensity (I and II) were observed for each reaction. A very weak minor band (III) was also evident in some cases. The intensity of this band appears to correlate with those of the two major bands, being more pronounced in the WT, and least so in CP9 and CP10. The specific inhibition reaction (SI) was almost completely out-competed by 50.0–fold unlabelled WT competitor.

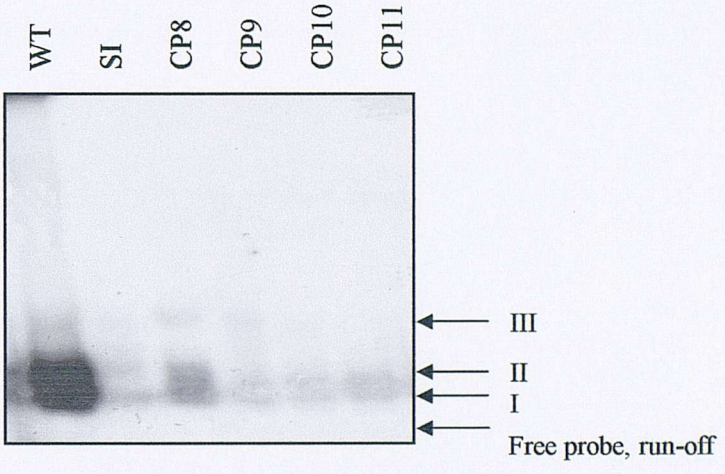


Figure 8.4 Gel retardation analysis of HBV bearing mutations in the nt. 1752 to 1775 region.
[γ-^{32}P]ATP-end-labelled DS-annealed oligonucleotides containing sequences between nt. 1752 to 1775 (WT and CP8–CP11, as designated) incubated with Huh7 nuclear extracts, and products resolved on a polyacrylamide gel. Two major bands (I and II) are seen for all the reactions. A 20.0–fold unlabelled specific Wildtype competitor (lane SI) removed the specific band-shifts.

The above data support the assumption that the highly reduced shifts seen with most mutants must be due to the effect of the introduced mutations. The latter are possibly the same in the Wildtype and mutants, since the bandshift pattern is similar (or identical).

8.4 Identification of transcription factors that bind to the nt. 1752 to 1775 region

The role of members of the nuclear receptor superfamily of transcription factors in regulating HBV transcription was next investigated. Previous analyses had demonstrated that transcription from the EnhII/CP is driven by nuclear factors in differentiated hepatoma cell lines (Raney *et al.* 1997c; Spandau & Lee 1992b; Viollet *et al.* 1997d; Wang *et al.* 1998b; Zheng *et al.* 2004d). Detailed analysis of this promoter-site identified multiple binding sites for liver-enriched and ubiquitous transcription factors located between 1752 and 1775. This region include the start site for the preC RNA (Nishizono *et al.* 1995). The EnhII/CP region was shown to include binding sites for protein factors mainly expressed in the liver: HNF4, COUP-TF, and HNF1 (Crescenzo-Chaigne *et al.* 1995a; Raney *et al.* 1997b). Each of these three nuclear factors are examples of proteins not present in all tissues but present at high concentrations in liver extracts (Lopez-Cabrera *et al.* 1990; Spandau & Lee 1992c; Xanthopoulos *et al.* 1991b).

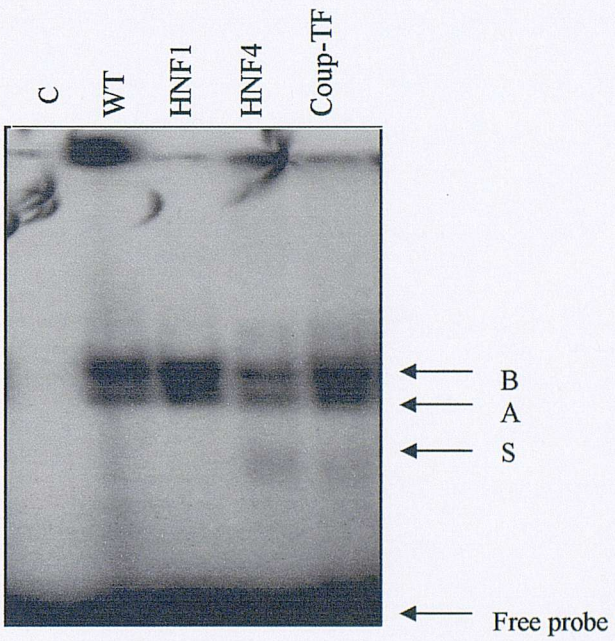


Figure 8.5 EMSA using antibodies (supershift) directed against various steroid hormone receptor superfamily members.
EMSA performed using [γ-^{32}P]ATP-end-labelled DS-annealed oligonucleotide from WT (1752 to 1769), and electrophoresed on polyacrylamide gel. The lanes contain probe without nuclear extract (C), Huh7 extract without antibody, (WT), and with various antibodies (HNF1, HNF4, COUP-TF) as shown. A and B marks the locations of specific bands.

Figure 8.5 shows the result of reactions carried out in an attempt to identify the transcription factor(s) causing the bandshifts seen (Figures 8.3 and 8.4) and involves supershift assays using the WT probe and polyclonal antibodies directed against the three named factors.

`The supersift experiment shown in Figure 8.5 yielded two major bands with the probe (A and B), for both the positive control (WT), and those reacted with the various antibodies. Additionally, a minor band is seen in each of the reactions with HNF4 and Coup-TF (labelled S). This faint lower band had not been detected in previous WT reactions (Figures 8.2 and 8.3). The reaction in lane C served as negative control. It contained DS-annealed WT oligonucleotide, end-labelled with [γ-^{32}P]ATP and incubated without nuclear extract. As similarly seen with the bandshift assays, it was apparent that factor(s) present in the nuclear extract bound to the WT probe and produced a bandshift in every case. However, none of the antibodies chosen caused any obvious supershift — all the antibody test reactions appeared to exhibit bands of similar size as the positive control (lane WT) — therefore, the role that any of these three factor(s) play in modulating transcription in the WT (or presumably mutant recombinants) (Sections 5.4.5 and 6.2.2) following transfection in Huh7 cells, remains unproved. Specificities of these antibodies directed against the tested nuclear factor binding sites have been shown (Viollet *et al.* 1997e).

8.5 Functional analysis of mutations within the EnhII/CP sequences *in vitro*

In order to determine if the binding heterogeneity exhibited by bands I and II (and possibly band III) (Figures 8.3 and 8.4) was reflected by their capacity to direct expression in a liver cell environment, promoter activity assays were undertaken.

The sizes of the HBV insert in CP/pGL2-basic constructs were checked by restriction digestion, and sequence fidelity was confirmed by sequencing (Figures 8.7, panel A, and, 8.8, respectively). [Each recombinant plasmid contained a 211bp fragment (nt. 1604–1815; Figure 8.6, 8.7) which bears the binding site for several transcription factors in the WT (Xanthopoulos *et al.* 1991a)].

The constructs, were generated using the transcription factor binding sites corresponding to the full-length mutant recombinants that were previously transfected in the hepatoma cell lines (see Results, Sections 3.3.1–3.3.5) to determine their

biological effects. Therefore, they also bore the same mutations that were present in the oligonucleotides (Figure 8.1) used in the EMSAs. Once generated, these CP/EnhII FHB constructs (CP1–CP7), containing luciferase as the reporter gene, were transiently transfected into Huh7 cells, then assayed for luciferase activity, and hence measurement of promoter activity.

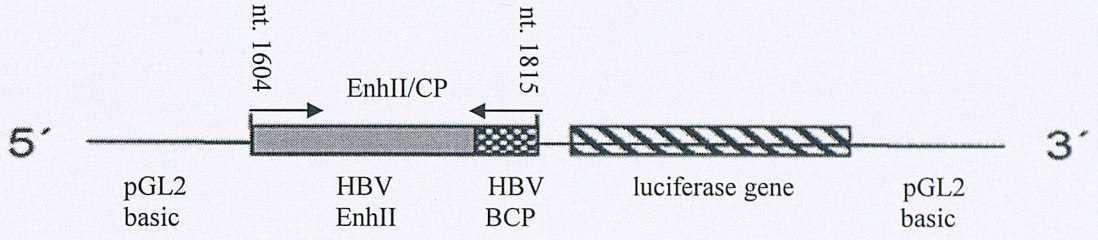


Figure 8.6 Structure of reporter constructs.
Restriction sites *MluI* and *BglII* were engineered in the sense and antisense primers, respectively. In a position-orientation dependent manner, the resulting EnhI/CP amplicons (nt. 1604–1815) were each cloned into the pGL2-basic® vector, immediately upstream of the luciferase gene.

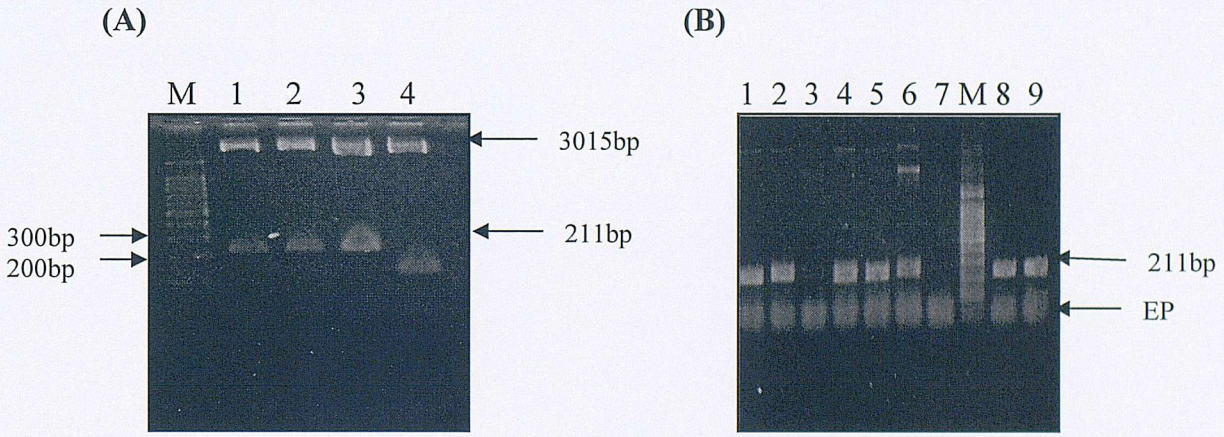


Figure 8.7 2% agarose gel electrophoresis of pGL2-basic/ EnhII/CP clones.
(A). Restriction digestion of clones, with MluI and BglII. A fragment of 211bp is released in all lanes (except 4), leaving behind the larger (3015bp) and empty cloning vector (pGEM®-T-Easy). Lane 4, undigested plasmid (WT). The lanes contain digestion products of various recombinant constructs (lanes 1, 2, and 3: WT, CP1, and CP2, respectively).
(B). Prior to digestion of clones (as in A), PCR was carried out to check for correct insert sizes using HBV-BglII 1792–1810 and HBV-MluI 1604–22 primers. The expected 211bp size fragment was seen in all clones, except 3, and 7. EP, excess primer. The lanes contain the PCR products of various recombinant constructs (lanes 1–7, 8, and 9: WT, CP1–CP6, CP7, and CP8).
M, 100bp markers.

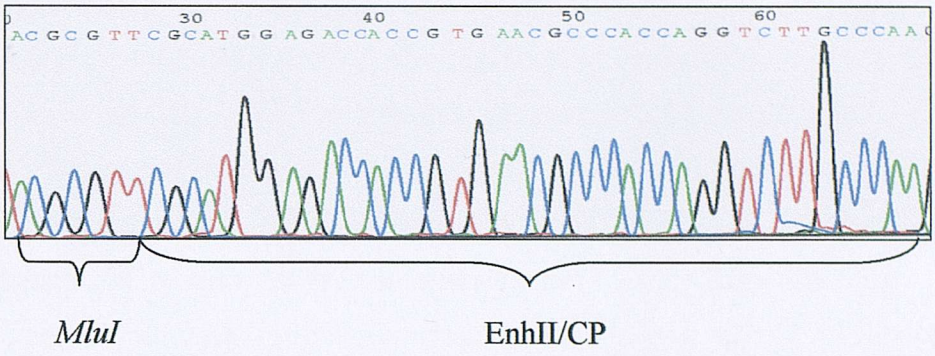


Figure 8.8 Electrophoregram of the sequence of WT EnhII/CP sequence. Nucleotides 1604 to 1646 are indicated, incorporating the 3′–end of the 211bp fragment and the engineered *MluI* site sequence. [Full sequence, Appendix 4].

The functional significance of the observations obtained thus far was investigated by transient transfection analysis in Huh7 cells. It has previously been demonstrated that the region from 1604–1815 is an independent sequence which is sufficient to drive transcription (Nishizono *et al.* 1995). The results show increased activity (Figure 8.9) in each of the recombinants that additionally harbour the double CP mutations at positions 1762/1764, but not the double mutant in isolation, which has luciferase expression levels comparable to WT.

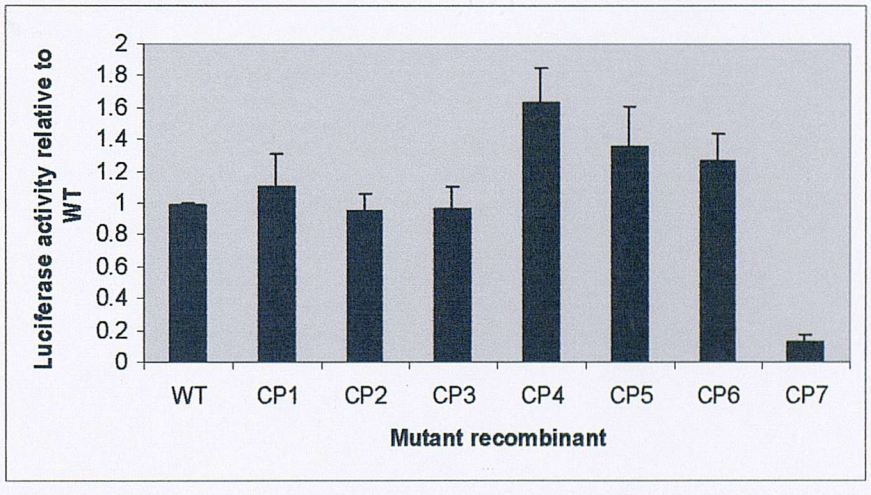


Figure 8.9 Luciferase activity of pGL2-basic/EnhII/CP constructs. Huh7 cells co-transfected with pCI-GFP on a mole:mole ratio and incubated. The activity of luciferase, were measured for each mutant, as indicated, and results are expressed as a ratio of WT (assigned a value of 1). Each result represented the average of triplicate experiments from the same assay, showing ±1 SD. Note the low CP7 activity and high activities of CP5, CP6, and (especially) CP4.

These data support those relating to the replication capacity of the mutants. In support of the replication efficiency data (Sections 5, and 6), the single CP constructs did not significantly differ in their activities to drive replication from WT. Likewise, the luciferase activity of CP7 transfected in hepatoma cells agreed with its replication capacity, suggesting that its reduced replicative capacity (Sections 5 and 6) may have been as a result of its weak promoter activity (2.3–fold less, compared to WT). Furthermore, in relation to WT, significantly ($P < 0.05$) more luciferase activities were observed with the transfection of the different constructs: CP4 (1.6–fold), CP5 (1.4–fold), and CP6 (1.3–fold). Consistent with the transfection capacity results (Sections 5.3.4–6.3), CP7 vastly downregulated luciferase activity (8.3–fold, $P < 0.001$).

Following FACS analyses, the average efficiency of transfection was 22%, with a range of 21–24%.

Chapter 4

Discussion

CHAPTER 9. DISCUSSION

Here, an attempt is made to analyse critically and discuss the findings reported in the Results, subheadings 3 – 8, in order. In so doing, parallels have been drawn between these findings and those in the literature, and efforts have been devoted to explain in scientific terms, the reasons for discrepant findings, whilst acknowledging results in agreement. Areas where problems were encountered have been highlighted and attempts will be made to discuss them. Finally, I review the relevance of the scientific findings documented in this thesis.

Generation of replication competent mutant recombinants

Currently, the best model systems available for the *in vitro* study of HBV replication include stably transfected hepatocyte-derived cell lines, such as the HepG2-derived 2.2.15 cells (Sells *et al.* 1987a; Sells *et al.* 1988), and HB611, from the hepatoma cell line Huh-6 (Tsurimoto *et al.* 1987c; Tuttleman *et al.* 1986b). Alternatively, plasmid DNA containing the HBV genome can be transiently transfected into cell lines that support HBV replication, including HepG2 and Huh7. For a comprehensive comparative *in vitro* study of viral replication of specific HBV mutants, such as conducted in this thesis, replication-competent clones carrying full-length HBV genomes with each of the four intact HBV ORFs are deemed here a prerequisite. As the genome is circular, a set of intact ORFs can be obtained by using longer than genome-length fragments (e.g 1.2–fold); this entails the duplication of part of the genome.

For introducing mutations in long targets such as p3.8II (6.8kb), Stratagene's QuikChange® SDM kits have become the preferred choice. However, as experienced with the multi-choice kit (section 3.2), the kit becomes less efficient when the specific sequence targetted for mutagenesis is repetitive in the template (Figure 3.1). This was the problem in the initial attempts to generate core promoter (CP) and precore (preC) mutants using the full-length p3.8II construct as template. This approach to introduce multiple mutations in a single polymerase chain reaction (PCR) was subsequently abandoned due to looping-out of DNA segments, as indicated in Section 3.2, with the subsequent creation of deletion "variants" that are replication-incompetent.

Although there is no perfect method for generating full-length mutant constructs, with such a long construct as template, Stratagene's QuikChange® Multi Site-Directed mutagenesis kit was thought to be ideal, since up to five individual primers can be used at once thereby reducing the introduction of sporadic mutations. With a proof-reading enzyme, *Pfu-Ultra*™, non-specific mutations can be further restricted in number, although not completely prevented. After transformation, clones with non-specific mutations can be identified by sequencing and removed from further studies. The main advantage of this multi-kit therefore lies in its versatility, allowing introduction of several mutations at a time, saving effort and time.

An alternative kit, also supplied by Strategene (QuikChange® Site-Directed Mutagenesis Kit) required the use of a pair of complementary mutagenised primers designed to introduce specific mutation(s) in a single-site DNA target. However, the same problems that were encountered with use the multi-site kit were also experienced with this kit.

As a way forward to the proposed study, p3.8II parent construct was rendered replication-incompetent by removing the duplicated sequences (p3.8IIΔ). Then, with the use of the QuikChange® Site-Directed Mutagenesis Kit, followed by sequencing, clones with the desired mutations, as presented in Section 3.3, were generated. Although this new strategy of molecular cloning and sequencing was time-consuming, and laborious, it circumvented sensitivity and specificity issues associated with the full-length approach. However, not all the mutant constructs were constructed using this new strategy. Few of the surface (S) and polymerase (P) gene mutant recombinants were generated using the full-length, p3.8II as template, since none of these areas were duplicated in the Wildtype (WT) p3.8II construct. Due to the long length of the template, nevertheless sporadic base substitutions occasionally occurred in some mutant recombinants, some of them in regulatory regions [such as seen when an extra *T* was inserted in the polyadenylation site (Figure 3.4)]. Some clones that bore mutations assumed to be either silent or missense, and therefore not thought to alter protein function; for example, the mutant that retained Pro at nt. 178, or the Phe → Leu change at nt. 97, were also used in subsequent analysis. When sporadic PCR-introduced mutations repeatedly occurred in conserved regions — i.e using the full-length HBV construct was used as template to generate the remaining S and P mutant recombinants — a similar, but less laborious strategy as used to create the

preC and CP mutants was used (review Resuslts, sub-Section 3.2.1.b and legend of Figure 3.4).

Kinetics of 2.2.15 cells replication

Since establishment of the 2.2.15 cell line by Acs *et al.* (1987), a number of studies have benefitted from its use in studying the molecular mechanisms of HBV replication. The availability of the cell line has also allowed the effects of antivirals and cytokines on HBV production to be explored (Doong *et al.* 1991; Heijtink *et al.* 1994; Hostetler *et al.* 1997; Innaimo *et al.* 1997; Korba & Gerin 1992; Lee *et al.* 1996; Schinazi *et al.* 1994). In this thesis, 2.2.15 cells were used for the purpose of optimising the techniques that were ultimately utilised to evaluate the replication capacity of the various mutants. As with other findings, this investigation found that HBV expression and replication was generally not high (Delaney & Isom 1998d; Liu *et al.* 2004c). Despite this, the 2.2.15 cell line was the ideal choice for use in optimisations in this study because, without further manipulatons to synchronise the population, each cell contained virus at all phases of the replication cycle. This was particularly important in the initial time-course analysis, since any differences in replication would be solely the result of the viral kinetics and not dependent on possible variation in the cell number or concentration of transfected plasmid. To prove this, transfection quantitation protocols was the same for all mutants, additionally, all results were standardised by calculation.

Notwithstanding its low replication efficiency, as determined from virion release (Figure 4.1), it was possible to validate the extraction protocols that were used in this study. Although the covalently closed circular (ccc)DNA isolated from 2.2.15 cells was of relatively low concentration, as seen earlier (Figure 4.2, panel B, lane 7), this was probably due to the fact that the replicative intermediate is generally present in cell nuclei in very low quantities, as was seen with transiently transfected cells (discussed later). In spite of this problem, it could be confirmed that cccDNA-specific primers were not randomly binding to other forms of DNA, since no amplicon resulted when they were used in amplification studies on HBV DNA released into supernatant (Figure 4.2, panel A, lanes 3 and 4). The method of extraction, and/or the non-specific binding of cccDNA primers may well be the reason for previously reported disparities in cccDNA detection (Chen *et al.* 2004; Delaney & Isom 1998c; Moraleda *et al.* 1997a; Summers *et al.* 1990a; Tuttleman *et*

al. 1986a; Zhang *et al.* 2003; Zoulim 2005a). In this study, mung bean nuclease was used in isolating cccDNA from other DNA replicative intermediates. This is a single-strand (SS) specific DNA and RNA endonuclease that catalyses the degradation of single-stranded DNA and RNA to yield blunt, ligatable ends. In this instance, both the positive (nicked) and negative strands of the HBV genome [reviewed, Section 1.4.2 (genome organisation)] will be degraded. After confirmation that the cccDNA primers bound specifically to the target when using total DNA as template (section 4.2), it was concluded that cccDNA should be amplified from total DNA preparations, instead of performing separate isolations. This conclusion was based on the finding that there appeared to be negligible differences in the concentration of cccDNA amplified using a cccDNA template, and those from total DNA (Figure 4.2, Panel B, Lanes 5–7). In designing the cccDNA-specific Real-time PCR detection assay, the primers were positioned at conserved regions flanking the direct repeats (DR)1 and 2, and the nicks in both negative and positive strands. The cccDNA copy number was subsequently obtained after subtraction from the total DNA copy number obtained by the F1-R1 primer-pair: the latter was designed to target both cccDNA and relaxed circular (RC) double-stranded (DS) DNA. The difference after subtraction represents the copy number of the latter DNA.

Apart from intracellular DNA, 2.2.15 cells were also used to test the specificity and sensitivity of the Transcript-specific assay. Described in detail in the experimental background section (1.14), the specific amplification of pregenomic (preG) RNA (total transcripts) and preC mRNA were achieved, by utilising the sequences of their common polyadenylation site (3′–end), and taking advantage of the variation of the unique sequences present at their 5′–ends, which allow distinction between the two different transcripts. Results in this thesis confirmed the specificity of the 5′ PGP primer for use in determining total CP-directed HBV transcription, whilst the 5′ PCP primer specifically targets the longer transcript, PreC, only (Figure Figure 4.4).

As explained earlier (Sections 4.3 and 5.2.3), it is worth emphasising that the results representing the Transcript-specific assays may not represent only those of the longer than genome-length tanscripts, but also those of much shorter lengths (of under 250bp). Whilst the former are transcribed by the promoters and enhancers present at the 3′–end of the cloned HBV-DNA insert, those of the latter are transcribed from promoters present in the repeat fragment and located at the 5′–end

(Figure 3.1). Unfortunately this "double amplification" is inevitable because of the nature of the construct that ensure its replication capacity. The use of oligo(dT) primers — as opposed to the common BC1 reverse primer used in the Real-time HBV RNA quantification would not have solved this problem, since these shorter transcripts extended beyond the polyadenylation site (to which an oligo(dT) primer may bind, by at least 30bp (see Figure 3.1).

It was encouraging to note that data obtained in the optimisation of this assay — using WT — did indicate correlation between mRNA normalised for β-actin, to those for RPII. Moreover, a good correlation coefficient ($\rho = 0.523$; $P < 0.05$) was obtained between extracellular HBV DNA and, total mRNA, as previously observed (Liu *et al.* 2004b). This is possibly indicative of any of the following speculations: (1) for unknown reason, the shorter-than-genome length transcripts may be of much less occurrence in the pool of transcripts, (2) the fact that its length is much shorter than the normal transcripts (250bp *vs.* 3500bp), may render it susceptible for degradation, since it will not be competent for translation and remains in the cell-cytoplasm for a longer period than the 3.5kb transcripts, which are used as template for translation of viral proteins, (3) during the final stages of the RNA extraction process, these shorter transcripts may not be heavy enough to precipitate and would therefore remain floating in the supernatant which is removed, leaving behind the longer and heavier 3.5kb transcripts.

The use of the 2.2.15 cell line for optimisation was also extended to the detection of the housekeeping genes (HKGs), beta (β)-actin and reverse polymerase II (RPII). Expression levels of target genes measured by Real-time RT-PCR are usually normalised by comparison with transcript abundances of reference genes. However, evaluation of expression stability of well established and frequently used control genes such as β-actin and GAPDH revealed that expression levels of control genes may vary considerably in the samples of interest, (Bustin 2000b; Hamalainen *et al.* 2001b; Schmittgen & Zakrajsek 2000; Suzuki *et al.* 2000; Vandesompele *et al.* 2002c) which may interfere with result accuracy. This illustrates the necessity for validation of control genes for every single cell type. Accordingly, evaluation of expression stability of control genes for each individual experiment has been claimed in a number of previous publications (Bustin 2000a; Hamalainen *et al.* 2001c; Livak & Schmittgen 2001; Vandesompele *et al.* 2002b).

For the purposes of the studies reported in this thesis, although it initially seemed advantageous to use RPII as a HKG, due to its constant expression in cells, its non-vulnerability to mitogenic stimulation (as described in Section 1.14), and most importantly due to its role in mRNA expression, its very low abundance (Section 4.4) in cells did not favour its use. These adverse effects could have hampered the Transcript-specific Real-time assay, since it was possible that it might not be expressed to a level sufficiently high for comparison purposes. On the other hand, although it has been shown that β-actin may be regulated under conditions of mitogenic stimulation by ionomycin treatment, β-actin has been found to be constitutively expressed in cells at relatively high concentrations (Radonic *et al.* 2004c). Such stimulation may have been problematic in experiments using lamivudine (LMV)-treated cells as the drug is potentially mitogenic. However, LMV now has been used for years, without such problems. Furthermore, there was no disparity in the transcript detection results of the LMV studies that could be attributed to LMV side-effects. After consideration, it was felt that β-actin would be appropriate for use in this investigation.

Reproducible transient transfections for the study of hepatitis B virus replication based on co-transfection with GFP, and standardisation

To facilitate studies on genotypic variation, pathogenesis, and the role of emerging anti-viral resistant mutations, it is essential to have available *in vitro* and *in vivo* recombinant HBV systems that can expedite such analyses accurately and efficiently. The method of analysis that was used in this study has distinct advantages over some of those used in other transient transfection studies to examine HBV replication (Allen *et al.* 1998b; Delaney *et al.* 2003b). Central to making meaningful interpretation of data produced in a transient transfection system, is the capacity to consistently reproduce results. In the context of mammalian cell culture transfection, the effectiveness of this strategy has been undermined by several factors: (a) the difficulty associated with accurately determining the concentration of DNA successfully transfected, into mammalian cells, (b) ensuring comparable levels of transfection for all samples being transfected, and (c) limiting intra-experimental variation of duplicate or triplicate results. To date, many reporter plasmids, including beta (β)-galactosidase, luciferase, and chloramphenicol acetyl transferase (CAT) have facilitated the standardisation of intra-experimental transfection efficiencies.

Their use in co-transfection experiments has relied on the assumption that the level of expression of the reporter gene correlates with transfection efficiency of the plasmid of interest.

The process of co-transfection cannot control for errors in the quantity of individual and different DNA plasmids that are successfully transfected into discrete populations of cells. Attempts to address this problem have included standardising transfections according to intracellular HBeAg expression or secreted HBsAg in culture supernatants (Delaney *et al.* 2003c; Torresi *et al.* 2002g). However, these systems are limited to the study of mutations of HBV that do not influence the expression of these antigens. They are not suitable therefore for the study of preC mutants that are HBeAg-negative, or vaccine escape mutants which produce HBsAg that may not be adequately recognised in commercial immunoassays. This problem is further confounded when one considers the emergence of drug resistance mutations. Given that the P gene of HBV totally overlaps the S gene, and that all of the drug resistant mutations reported thus far occur within this region of overlap, the use of this antigen (HBs) cannot be deemed a reliable marker of transfection efficiency without first ensuring that the secretion of the antigen is unchanged. It has also been demonstrated that mutations within various regions of the P gene result in changes in the overlapping envelope gene and impair viral secretion, causing the intracellular retention of HBsAg (Kalinina *et al.* 2001a). Some of the above findings were also supported by those reported in this thesis. In this instance, the determination of secreted HBsAg is no longer a reliable measure of transfection efficiency. Furthermore, the results documented herein have demonstrated that LMV resistant mutations in the polymerase, with their corresponding changes in the S gene, result in HBsAg with reduced binding affinity to anti-HBs. Therefore, the correction of transfection efficiency of LMV resistant HBV mutants using immunoassays to determine HBsAg in culture supernatants is also unreliable for some HBV mutants and makes any comparison of WT and LMV resistant HBV transfections of doubtful meaning.

Although for the purposes of this investigation it would have been best to determine transfection efficiency by directly staining for a viral protein whose expression was deemed unaltered by the mutations under study, for example HBcAg, this method was undermined by problems encountered during the washing stages of the staining protocol. Cells were repeatedly lost from coverglasses, which translated

into an underestimation of the actual number of cells that were successfully transfected. In the process however, what was determined with success was the localisation of the HBcAg in Huh7 cells. In agreement with other investigators, it was found that the core protein was present in the WT cytoplasm (and possibly in the nuclei) (Aiba *et al.* 1997b). For purposes of transfection efficiency determination, it was sufficient that cells stained for the core could be clearly distinguished.

As explained above, although the method of co-transfecting a fluorescent plasmid in association with the plasmid of interest is not in itself ideal, this investigation (documented in this thesis) has allowed optimisation in such a way that circumvents many of the above problems. First, the rationale for the use of equimolar concentrations of HBV DNA (mutant) to that of the fluorescent-expressing (pCI-GFP) plasmid is based on the theoretical basis that the same number of plasmid copies will be delivered to cells in each case and would allow an unbiased comparison of any differential effect observed as a consequence of mutations. Weight:weight based transfection would have compared equal weights of the two plasmids (pCI-GFP and HBV), but not allow for molar ratios due to their varying sizes; 4.6kb and 6.8kb, respectively. Use of the mole:mole ratio with adjustment of the pCI-GFP (being the smaller of the two plasmids) weight to that of the HBV plasmid allows for the same chance of transfection of both plasmids into cells (Bigger *et al.* 2001). Noteworthy are the summary results reported in this thesis and given in Figure 5.3, suggesting that co-transfecting an excess of 3μg of the HBV plasmid with the fluorescent plasmid leads to saturation of the cell. This can be counterproductive since high DNA concentration may be toxic to the cells (Gouskos *et al.* 2004b).

Although the above approach may have been accurate enough in terms of reproducibility, it was concluded that the results should be standardised to a fixed percentage that was consistently surpassed during transfection efficiency optimisations (15%). Whilst other systems, including baculovirus and adenovirus expression systems, have been developed to study HBV replication, stable HBV expressing cell lines and transgenic mice have also been used to address questions related to problems associated with non-reproducibility of transfection efficiency. However, they are all limited by the laborious nature of each system (Delaney & Isom 1998b; Feitelson *et al.* 1988; Guidotti *et al.* 1999a; Ren & Nassal 2001; Sprinzl *et al.* 2001). Furthermore, the use of promoter expression plasmids was not suitable in this study since it will defeat the purpose of driving viral replication solely using

the HBV endogenous promoters. The optimised transfection strategy described in this study provided a convenient and reliable transient transfection system that could be utilised to "fast track" the examination of virtually all co-transfection experiments.

An overall conclusion that can be drawn from the transfection results is that whilst Huh7 and HepG2 cells are more resistant to transfection than 293T, the osteosarcoma cells (143-TK$^-$) used in this study was however seen to have lower transfection efficiency than the above three cell lines.

PreC and CP mutants can enhance viral replication in transfected Huh7 and HepG2 cells

The mechanisms determining the emergence of viral mutants in response to immune selection may be important in considering possible therapeutic interventions designed to prevent their pathophysiological consequences. Several HBV mutants with altered amino acid sequences in immunodominant epitopes of the major surface antigen have been identified. These presumably escape elimination because they are not recognised by the normal antibody responses to the viral envelope proteins (Gunther *et al.* 1999). Similarly, viral mutants that fail to express HBeAg may not be efficiently cleared from patients in whom the immune response is primarily directed against nucleocapsid antigens (Akahane *et al.* 1990b; Gunther *et al.* 1998c; Naoumov *et al.* 1992b; Okamoto *et al.* 1994c). Consistent with this suggestion, various viral mutants have been obtained from patients who have seroconverted from HBeAg-positive to an anti-HBe status. The most common mutations observed in these mutants contain a stop codon in the preC coding region that prevents the synthesis of HBeAg (Akahane *et al.* 1990a; Gunther *et al.* 1998b; Naoumov *et al.* 1992a). Apart from such mutations, and the well-known double CP mutation (A1762T/G1764A), recent studies have identified *in vivo*, another cluster of mutations in the viral CP region, which are associated with FHB or severe hepatitis (Komatsu *et al.* 2001a; Omata *et al.* 1991a; Sato *et al.* 1995b; Scaglioni *et al.* 1997d; Ulrich *et al.* 1990b). Although functional studies have demonstrated that these mutations exhibited a high replication phenotype, none have compared a variety of such mutations with the wildtype, by looking at various replication parameters. Furthermore, it seems important that all the recombinants should be produced in the same genetic background, to prevent skewing of results as a result of potential occurrence of additional mutations elsewhere in the genome which may or may not affect

replication of the virus. However, what may be considered as the advantage of producing various muatations in the same genetic background could well be a rate-limiting step of this study, as will be discussed further, in this chapter (including studies by Parekh *et al.* 2003 and Gunther *et al.* 1998). To examine these issues, without interventions from the immune system, this study set out to evaluate individual and combined effects of mutations in a tissue culture system. The rare occurrence of certain apparently *e*-defective mutations is suggested to be likely due to the fact that they do not seem to show any replication advantage over the WT (Yuan *et al.* 1995). Results given in this thesis has demonstrated that the single A1762T, G1764A, G1896A, and G1899A substitution have no significant ($P > 0.05$) replication advantage over the WT, strengthening this finding.

The paradox that high replicating viral strains were isolated from patients with high viraemia titres, while sera with low viral titres contained viral strains with limited replication capacity, has been refuted using hepatoma cell lines (Parekh *et al.* 2003g). Nevertheless, a high replication capacity of CP mutants is consistent with their frequent association with FHB (Friedt *et al.* 1999; Kalinina *et al.* 2001b; Liang *et al.* 1991b; McMillan *et al.* 1996; Ogata *et al.* 1993a; Sato *et al.* 1995c; Sterneck *et al.* 1996a; Stuyver *et al.* 1999), and two such genomes associated with FHB (G1896A/G1899A and C1766T/T1768A) were found to replicate at a very high level (Baumert *et al.* 1996d; Hasegawa *et al.* 1994b). However, each of these studies used constructs on different genetic backgrounds, which could influence the results. The results in this thesis suggest, moreover, that high replicating capacity is a feature shared by naturally occurring CP mutants. What cannot be concluded from the findings given herein is that such enhanced replicating capacity is limited to CP mutants implicated in FHB.

It was important during the optimisation stage of this work, that the presence of any input plasmid was not present at the time of extracting released HBV DNA. To ensure that this was the case, 96hrs following transfection, cells were first *DNaseI*-treated prior to being lysed for extraction of HBV DNA. It has been suggested that transfected (or input) plasmid DNA is initially reduced following incubation before possibly increasing again (Delaney *et al.* 2001). This preliminary decrease may be due to its removal either by export from cell or by degradation of input plasmid (Abdelhamed *et al.* 2002b). In addition, although results of Real-time PCR for quantification of extracellular HBV DNA did not distinguish between the

different forms of the virus, it is important to note that whilst single-stranded (SS) HBV DNA molecules predominate during the first 24hrs after transfection, starting from 48hrs onwards the predominant form of DNA in cytoplasmic nucleocapsids is the mature genomic form which would ultimately be released into the medium (Delaney and Isom, 1998; Abdelhamed *et al.* 2002). These authors showed that HBV DNA present in medium of transfected cells three days post transfection with a replication-competent HBV plasmid is predominantly present in the form of RC-DS HBV DNA. In comparison to other studies that used a blotting method to detect intracellular replicative intermediates, the use of an assay as sensitive as Real-time PCR avoids false results which may have been the source of discrepancies in previous *in vitro* studies (Gerelsaikhan *et al.* 1996; Parekh *et al.* 2003f; Yuan *et al.* 1999a). The studies that suggested an increased replication in some of the CP and preC mutant recombinants may have been due to the added detection of release immature virions containing SS HBV DNA (Abdelhamed *et al.* 2002a). These studies not only did not isolate intracellular RC-DS HBV DNA only [as was the purpose for using Plasmid-Safe ATP-dependent DNase here (Section 2.2.11.b.)], but also, the DNA non-descrimating blotting (Southern) technique was used for detection. This would have potentially detected all forms of DNA that was present each time. *In vivo*, only core particles containing the DS viral genome are enveloped and secreted into serum (Ganem & Schneider 2001), although human hepatoma cell lines transfected with HBV DNA may release naked core particles containing a SS genome. Since the release of naked core particles may therefore be an artefact of hepatoma cells, such results demonstrate that measuring release particles only, may not provide wholly accurate information for phenotyping mutants.

Although the same protocols and similar experimental conditions were utilised in the *in vitro* assays with the two cell lines, ideally, the two experiments should have been conducted in parallel, to minimise any errors between such experiments. The difficulty in using large numbers of samples at once was however considered a limiting-factor in this approach since the handling of many samples and in replicates, may have introduced further errors. Notwithstanding the fact that the two sets of experiments were carried out at various times, it was encouraging to observe the near consistent pattern of replication efficiency in the two cell lines used. The slightly higher replicating capacity of the tested mutants in HepG2 cell line may reflect the different metabolic activities of the two cell types, or differences in factors

needed for efficient replication (Bonetta 2004; Pollard *et al.* 1998a). In particular, the HepG2 cell line is known to be more closely related to human liver cells than Huh7 cells, by virtue of the former's biological and physiological capability to synthesise and secrete albumin (Strous & Van 1989). The differences in replication capacity noted between the different mutants in the two cell lines may also be attributed to the introduced mutations as opposed to the cell line used. Challenged by the difficulty to consistently and perhaps accurately represent levels of cccDNA on a per cell basis, in both cell lines, and especially Huh7, it is thought that this problem most likely results from the low concentration of the replicative intermediate in cells, rather than from experimental variations. It is hoped that another DNA replicative intermediate (RC-DS) could be useful in the mutant studies (reported in this thesis) and would be adequately represented by the data summary, as obtained from the two cell lines.

The results obtained in this investigation did demonstrate that the enhanced replication capacity observed by some of the mutants was as a consequence of the upregulation of intracellular replicative intermediates. Enhanced replication relative to WT was seen in all CP mutants, except those with single lesion, and the A1762T/G1764A mutant. A significant, reproducible, downregulation was noted with the T1753C/A1762T/G1764A/T1766A mutant. In addition, no differences in replication capacity were demonstrated by the combined CP and preC recombinant mutants. The fact that there was no apparent increase in extracellular, intracellular, and promoter activity (discussed later) in each of the preC gene mutations, or when combined with the A1762T/G1764A CP mutations, may suggest that the combined effect of preC and CP mutations in a single construct may not necessarily mean a more aggressive immune response is not invoked, when these HBeAg-negative mutants are present in FHB patients. Indeed, it must be emphasised that the replication phenotype of the various introduced mutants are being tested, only. Similar studies have concluded that the accumulation of mutations in other regions of the genome, host immune response, *ab initio* infection with the mutants may significantly affect and determine HBV production and course of liver disease in such anti-HBe-positive patients (Chan *et al.* 1999a; Laras *et al.* 2006a). Interestingly, however, the G1899A mutation has been found to restore the low replicating capacity of another HBeAg-negative, FHB CP mutant, G1862T (Guarnieri *et al.* 2006b). This is discussed below.

With regards to mutants harbouring substitutions in the preC region only, the mutation most often reported in patients with FHB is G1896A. The results in this study found no significant difference in replication capacity between any of the three preC mutants tested, compared to WT. The results for the single G1896A substitution may be explained by the fact that the G → A substitution at position 1896 was accompanied by a C → T transversion at nucleotide 1858 in the other strand of the stem of ε, opposite to nucleotide 1896. This resulted in an A-T instead of an A-C base pair at this site, which would have otherwise occurred, destabilising ε and precluding viral replication (Kramvis & Kew 1998c; Owiredu *et al.* 2001; Tong *et al.* 1993a). It remains unclear whether the G → A mutation at 1899 has any immunological significance, but studies have found its occurrence could be higher than previously thought since its presence is not limited to the *e*-antigen-negative patients (Gunther *et al.* 1992). On the other hand, the predominance of the double preC mutant could be due to a growth advantage of these viruses over other non-predominant preC mutations (Yuan *et al.* 1999b). However, in a recent report, it was shown that the replication defect of G1862T (Hou *et al.* 2002e), was largely relieved by the co-occurrence of the G1899A mutation (Guarnieri *et al.* 2006a). The G1862T mutant of genotype B has been implicated in FHB (Hou *et al.* 2002d). Interestingly, a number of isolates in that study (Hou *et al.* 2002c) of Chinese patients harboured the G1899A mutation but not the G1896A stop-codon mutation or CP mutations. Since enhanced replication of the HBV genome is considered critical to the induction of FHB (Baumert *et al.* 1996c), the presence of the G1899A mutation in such strains may not be a mere coincidence. In summary, however, due to the marginal difference in replication capacity compared with that of the WT, it is concluded that the preC mutants produce no replication advantages over WT, under the experimental conditions used herein.

In general, results of virion release correlated with HBsAg expression, as expected. However, there were instances when a lack of correlation was observed. Such correlation discrepancies may be due to the production of excess HBsAg in the form of spheres and filaments, of unknown explanation, as shown by *in vitro* and *in vivo* studies (Delaney & Isom 1998a; Liu *et al.* 2004a; Prange *et al.* 1995) and (Hantz *et al.* 1980). From data presented in this thesis, the concentration of excess surface antigens may not necessarily correlate with virions in the media, but notably, ELISA assays make no distinction between excess HBsAg and Dane particles.

As for the HBeAg test results, it is concluded that they must remain inconclusive due to the fact that the kit that was used for the test was optimised for patients' sera, rather than for *in vitro* studies. Furthermore, the test was purely qualitative. However, in spite of these reservations, it may be relevant that the antigen was detected in WT-transfected cells, whilst it was undetectable in most of the CP recombinants that evidenced significant decrease in preC mRNA transcription, including the C1766T/T1768A mutant (Baumert *et al.* 1998d; Parekh *et al.* 2003e). It is known that the CP mutations downregulate HBeAg production, but do not abolish it altogether. The origin of this *e*-antigenic reduction was seen at the level of preC mRNA transcripts: with the exception of the single CP mutants, all other recombinants harbouring mutations in their CP regions significantly downregulated preC mRNA transcription. This transcript is the precursor of HBeAg (Buckwold *et al.* 1996e; Gunther *et al.* 1998a; Moriyama *et al.* 1996e; Moriyama 1997b). On the HBeAg data alone, however, this study (documented in this thesis) cannot allow detailed comparisons to be made between the different recombinants, nor draw firm conclusions, since the *e*-antigen results were only qualitative, and the assay was not optimised for transfection studies, as highlighted above.

The results obtained with the T1753C/A1762T/G1764A/T1766A mutant construct were significantly different, both compared to WT and to the other recombinants. Results documented here were partly supported by previous *in vivo* findings (Parekh *et al.* 2003d). However, these investigators compared two different strains, and obtained varying results for the same type of mutant; one clone was defective in virion secretion, whilst another was not. In addition, they did not amplify the full-length sequences from the two patients using, rather, only the CP region that was under study. In other words, there could have been additional mutations elsewhere in the genome, which may have been missed. If this was the case, the added mutation(s) may potentially have had a role in upregulating replication, as reported for one of the clones studied (Parekh *et al.* 2003c). The findings given in this thesis demonstrated that the specifically introduced mutations downregulated WT replication activity in the tested parameters (extracellularly and intracellularly). Its phenotype must have an intracellular origin. This was of interest, given the fact that mutants associated with FHB have often a high-replicating phenotype. Therefore, there must be an additional factor — virologically- or immunologically-related — that accounts for the FHB status reported (Parekh *et al.*

2003b) in the FHB patient that harboured this mutant. What is a concern, however, with Parekh's findings is that they can be seen as inconclusive: using a FHB patient serum, these investigators targeted only the CP mutation "hotspot" area, and created a replication-competent recombinant, potentially implying that the remainder of the HBV sequence must be different from that of the patient. When such an infectious clone was analysed *in vitro,* the phenotype was low replicating. However, when a full-length construct with the same hotspot mutations was generated using the whole genome from another FHB patient, an upregulation in replication was observed. Nothing was stated as to the presence, or absence of mutations occurring elsewhere in the genome, which could alter viral replication.

Further, using clones with different genetic backgrounds (quasispecies), investigators found either significantly reduced HBeAg expression with the C1766T/T1768A recombinant (Baumert *et al.* 1998), or no expression (Parekh *et al.* 2003). The latter study also found that CP mutant recombinants that additionally harbour a substitution at position 1753 showed reduced capacity to express HBeAg and either a 3-fold increase in replication, or no difference in comparison to the WT. Additionally, these authors found that some high replicating CP mutants expressed as much HBeAg as the WT, contradicting conventional concepts of mutants that predominate in FHB patients. However one cannot directly compare the results which were obtained with constructs of varied genetic background, to those using the same genetic make-up, as was the case in our study. The conclusion that can be drawn from these findings is that HBeAg-negativity may be a feature of FHB. Indeed, as described earlier in the Introductory Chapter, this antigen normally exerts an inhibitory effect on the immune response of the host (Baumert *et al.* 1998c; Milich *et al.* 1997b; Uchida *et al.* 1993). Furthermore, the HBeAg precursor protein acts as a negative regulator of viral replication by decreasing encapsidation of preG RNA (Baumert *et al.* 1996b; Buckwold *et al.* 1996f). In this direct or indirect way, absent or reduced HBeAg synthesis appears to increase the replication efficiency of the virus, which will in turn heighten the host immune response to the virus. Indeed, these investigators, amongst others have implicated a heightened host immune response as a contributing factor in FHB infection. However, these findings excluded HBeAg-positive situations, as a correlation between levels of preG RNA and HBeAg has been shown in these patients(Laras *et al.* 2006b).

Much controversy still surrounds the replication capacity of the A1762T/G1764A mutant, and its role in disease outcome: this study, and those of others (Gunther *et al.* 1998), found no alteration in preG RNA levels in the A1762T/G1764A recombinant, but saw a significant reduction (WT-compared) in preC mRNA transcription. Although an RNA quality control test of samples had been conducted — an A_{260}/A_{280} purity test must be >1.7 — it can be, reasonably, argued that small amounts of RNA degradation, might have contributed in sample-to-sample variability of measured expression values. However, RNA transcript copy numbers were normalised to an internal control (β-actin), suggesting that possibly inconsistencies due to RNA integrity were very unlikely, thus not contributing in sample-to-sample variation in mRNA levels.

Hence, the decrease in viral transcription seen as a consequence of the A1762T/G1764A mutations, may partly explain the presence of this recombinant in FHB patients. HBeAg shares epitopes with HBcAg (Milich *et al.* 1988d) and this (HBeAg) has been postulated to induce immune tolerance against itself or HBcAg or both (Carman *et al.* 1993b; Milich *et al.* 1990b). In the presence of reduced or absent serum HBeAg, HBcAg may be targeted directly by both the cellular and humoral immune systems (Milich *et al.* 1988c) leading to necrosis of hepatocytes and liver damage (Brunetto *et al.* 1991; Kaneko *et al.* 1995). HBcAg elicits a significantly more vigorous antibody response *in vivo* than HBeAg (Milich *et al.* 1988b). Results given in this thesis are contradicted by work from other laboratories that reported viral upregulation with the same type of mutant (Buckwold *et al.* 1997a; Moriyama *et al.* 1996f). Indeed, the fact that these mutants have been detected in viral isolates from sera of assymptomatic-carrier patients, which have low HBV DNA titers (based on the fact that HBV DNA could be detected only by using nested PCR), argues against these mutations causing increased viral replication (Baptista *et al.* 1999a). Therefore, the prevalence of this double mutant in FHB patients appears not to reflect increased viral replication, but may be related to decrease HBeAg expression.

Mutations in the "a" determinant/S gene can restore the replication phenotype of P gene mutants

Most naturally occurring antibodies to HBsAg are found to bind to the "a" determinant between codons 124 and 147. This region is essentially conserved, although sequence alignments demonstrate a greater degree of genetic variation lying

between codons 124 and 137 than between codons 138 and 147. This variation seen among genotypes may be functionally significant as the surface (S) and polymerase (P) genes do overlap and one would expect significant genetic constraint in this part of the gene.

It is well-documented that amino acid substitutions and insertions in the *a* determinant result in antigenic and immunogenic changes in HBsAg (Carman *et al.* 1990c; Harrison *et al.* 1991; Hou *et al.* 1995; Karthigesu *et al.* 1994b; Waters *et al.* 1992a; Yamamoto *et al.* 1994b). Additionally, Site-directed mutagenesis (SDM) and amino acid replacement studies (Bruce & Murray 1995; Ashton-Rickardt & Murray 1989b; Steward *et al.* 1993) by studying the effect of their substitutions on HBsAg antigenicity have identified important HBsAg residues. For example, the major vaccine and immune escape variant G145R abrogates the binding of many murine monoclonal antibodies directed against the second loop and "a" conformational determinants (Waters *et al.* 1992). How this radical amino acid (aa.) change brings this about, whether by altering the folding of the polypeptide loops or by removing part of the backbone from the accessible structure, it is not known. Because the RFHBs18 primary antibody has no defined cyclical peptide binding site in the "a" determinant (Waters *et al.* 1992), and the ultrastuctural details of the protein are not available, which of the two cyclical regions (124 and 137, or 138 and 147) are externalised, cannot be concluded. Notwithstanding, the observations (documented in this thesis; Table 7.1) confirm that the first loop forms a suitable diagnostic and perhaps immunological target as was shown in the context of the genetic make-up of the WT using fluorescence microscopy. Immunofluorescence appears to be a reliable method, with regard to its sensitivity for the detection of HBsAg or surface coat antigen, in hepatoma cell lines. Results in this thesis showed strong fluorescence for the HBsAg localised in Huh7 cell membranes (Figure 7.1). In general, this corresponds to published reports (Hadziyannis *et al.* 1972; Ijaz *et al.* 2003; Jolivet-Reynaud *et al.* 2001b; Shikata *et al.* 1974), although there also appears to be some nuclei staining. Until electron microscopy has been carried out, it cannot be a certainty however, that what appears as nucleic staining may in fact be cytoplasmic protein lying over the nucleus (Figure 7.1, panel B).

The antibodies used in this study were selected for their ability to differentially bind to the two cyclical peptides of HBsAg. RFHBs1 and 4 bind to peptide 124–137, and RFHBs7 to 139–147 although with reduced affinity compared

to their binding to solubilised native molecules (Waters *et al.* 1991). Using epitope mapping, it has been shown that RFHBs18 partially cross-competes with RFHBs7 (Seddigh-Tonekaboni *et al.* 2000a). These investigators further demonstrated that the antibodies recognised different epitopes within the "a" determinant; they did not completely inhibit the binding of each other. Contrary to previous findings (Waters et al. 1992), which indicated a very high binding of *adr* WT to RFHBs1, results given in Table 7.1 showed poor binding of the antibody to the *adr* WT. This was also observed by Seddigh-Tonekaboni *et al.* (2001). RFHBs1 has been shown to neutralise HBV infectivity in chimpanzee challenge studies using both *ad* and *ay* standard HBV inocula (Iwarson *et al.* 1985) and therefore is also active in the *ayw* background.

Overall, the findings reported in this thesis demonstrate that amino acid changes within the two above mentioned "a" determinant cyclical peptides can alter the antigenic structure, and ultimately affect binding to antibody.

With regards to the replication kinetics of the three "a" determinant/S mutants, this study has demonstrated that low binding of anti-HBs to virus does not necessarily indicate lack of binding capacity, as was the case with the G145R and T131I mutants, but may in fact be due to the replication-incompetency of the virus resulting from specific mutation(s), as was seen with the K141E mutant. These findings were summarised by Figures 7.2-7.4. Figures 7.2 and 7.3 (panels A, and B) showed that G145R and T131I mutants replicate similarly to the WT, whereas the K141E mutant replicated significantly less efficiently. These results support previous findings (Seddigh-Tonekaboni *et al.* 2000b; Torresi *et al.* 2002h; Waters *et al.* 1992c). The HBsAg assay results, however, indicated that compared to WT, all three "a" determinant mutants have a significantly reduced binding capacity to anti-HBs, as seen by immunoassays. The findings reported in this thesis are interesting, as since the specific mutations in the K141I and G145R recombinants lies in the same conserved epitope (aa. 139–147) it would therefore be tempting to hypothesise that they would have a similar replication capacity. All three S gene mutations are located in an important region of the "a" determinant; aa. 124 to 147. A mutant virus, which has a point mutation in the genome that results in an amino acid substitution at position 145 of the HBsAg, has been described in a child who despite an adequate anti-HBs response became HBV infected (Carman *et al.* 1990d). Arg, which is substituted for Gly, is a much larger residue and is (positively) charged. As a result,

the hydrophobicity profiles of this region of the two antigens are quite different (Carman *et al.* 1990e), and would be expected to affect the secondary and tertiary structures of the antigen. This amino acid substitution lies within an important region of the "a" determinant and so potentially alters the epitopes recognised by the protective immune response.

The P gene completely overlaps the envelope gene, and as a consequence, mutations selected in the envelope gene may produce important mutations in the polymerase protein. The vaccine and HBIg selected changes in the "a" determinant of the HBsAg protein, produce changes in the overlaping polymerase protein that could alter the structure and functional activity of the RT/P enzyme. Mutations of the tyrosine, methionine, aspartate, aspartate (YMDD) motif [methionine either substituted by isoleucine (M204I) or valine (M204V)] in the C domain and leucine substituted by methonine (L180M) in the B domain of the HBV DNA P gene were investigated in LMV and non-LMV-treated Huh7 cells by Real-time PCR and ELISA.

It was found that the G145R mutation in the "a" determinant, selected as a consequence of vaccine escape or HBIg treatment, can partially restore the replication of some LMV-resistant HBV mutants carrying changes in the YMDD motif. The occurrence of unique LMV-resistant HBV mutants associated with the development of severe hepatitis and liver failure following recurrence of hepatitis B infection in liver transplant recipients has been reported (Tillmann *et al.* 1999). *In vitro* studies demonstrated that these patients were infected with HBV mutants carrying combinations of mutations in both the S and polymerase genes (Bock *et al.* 2002b). The changes in the S gene overlapped with the RT region of the polymerase protein. These combination mutants demonstrated an increased replication phenotype in the presence of LMV compared to WT virus (Figure 7.6).

The combination of HBV mutations examined in this study produced levels of total intracellular HBV replication intermediates that were comparable to WT levels, while remaining resistant to LMV. When the G145R mutation was added to the LMV-resistant mutants M204I, or L180M/M204V, the levels of RNA transcripts were greater than those of the latter two alone, reinforcing the compensatory nature of this change on the function of the HBV polymerase. The HBsAg results correlated with those of virion release and generation of replicative intermediates, except in the case of the G145R mutant which responded poorly to the assay, as already described.

The findings in this study were similar to those reported by others (Torresi *et al.* 2002b).

It is also important to note that the mutants L180M, and M204V did not show any replication difference compared to the WT, possibly indicating that these mutations on their own do not affect viral replication, hence rarely found in isolation.

Viral clearance may occur in infected animals without significant liver damage (Guo *et al.* 2000). The exact sequence of molecular events during this process remains unclear. Viral clearane provides the basis for using a combination of nucleoside analogs and cytokines to hasten the process. The complex host immune response interaction with infected cells accounts for three phases of the natural history of chronic HBV infection. The fact that HBV replication does not induce a cytopathic effect, and that viral cccDNA may have a long-half life in infected hepatocytes, are key determinants in viral persistence and selection of resistant mutants. In other words, since cccDNA does not undergo semiconservative replication (Seeger & Mason 2000a; Yuen *et al.* 2005), all the cccDNA inside the hepatocytes is derived from viral DNA produced in the cytoplasm. Therefore, containment of HBV infection not only relies on the inhibition of active viral replication that will prevent infection of new hepatocytes, but also requires a total elimination of cccDNA in order to prevent the synthesis of new HBV. Other investigators using cell cultures with non-dividing hepatocytes, also on studies in duck HBV, have shown the high stability of cccDNA *in vivo* (Addison *et al.* 2002; Moraleda *et al.* 1997b).

Effect of EnhII/CP mutations on protein factor binding and transcription activity

With the exception of the single CP mutants, all the other naturally-occurring HBV sequences have been previously isolated from patients with FHB (Baumert *et al.* 1996a; Baumert *et al.* 1998b; Parekh *et al.* 2003a; Hou *et al.* 2002b). Amongst others, these investigators generally demonstrated, or implied, that the mutants expressed reduced levels of the preC mRNA, and had upregulated viral replication. The increased replication seen with some of the mutants in this investigation may provide an explanation as to why HBV carrying these mutations often becomes the dominant quasispecies during FHB. The increase in virus progeny production likely occurs at, or immediately prior, to the pregenome encapsidation step of the virus life-cycle,

since the packaging efficiency of the preG RNA was found to be upregulated by double CP mutants (Buckwold *et al.* 1997i). This effect was shown to be partially reversed if a preC expression plasmid was used in a *trans*-complementation experiment, suggesting a negative role for the preC gene in HBV replication. This suggestion was supported by a study which indicated that the preC gene suppressed HBV replication in transgenic mice (Guidotti *et al.* 1996).

In spite of the likelihood that 1762 A →T, and 1764 G → A single-nucleotide mutations are intermediates that lead to the eventual appearance of the double mutant (1762 A →T plus 1764 G → A), each of these single mutants are rarely detected in HBV patients. In order to understand the mechanism that underlies the upregulated viral replication seen with these CP mutations (described in Results, Sections 3, 5 and 6), a detailed analysis of promoter activity — beginning with an electromobility shift assay (EMSA) — was carried out. For a detailed comparison, other substitution mutations implicated in FHB (Hou *et al.* 2002a) were included in the EMSA study.

The results obtained showed the binding activity of at least one transcription factor with varying strength, to the different mutant promoter-sites, when compared to the WT. In spite of the weak binding of factor(s) to CD7–CD11, it was obvious that the results implied that the factor(s) that was (were) responsible for the bandshift may be similar in all the mutants, at least under the conditions used here. Other studies have also reported multiple bands using EMSA (Yu & Mertz 1997a; Zheng *et al.* 2004c), where major band(s) that often appeared were stronger than the remaining shifted non-specific bands, it can be concluded from the data presented in the Results chapter, Sections 3.3 and 3.4, that either one factor is binding as a heterodimer, or two factors bound at once, and with similar strength, which is possible since the binding site common to all the recombinants used here is known to have the capacity to bind several factors (Raney *et al.* 1997a; Xanthopoulos *et al.* 1991b). The possibility for the latter case has not been confirmed and may be questioned as there are two major shifted bands and they appear to act coordinately. Other EMSA studies have shown that only one factor dominates binding at this site (McNair *et al.* 2000c; Xanthopoulos *et al.* 1991b).

In an effort to identify the protein factor(s) responsible for this binding activity, three of the (at least) five factors that are known to bind in these sites were tested for binding. The gel retardation analyses (supershift), however, failed to identify the putative transcription factor(s) in the Huh7 nuclear extract which mainly

caused similar bandshift results with each factor, as seen in Figures 8.3, and 8.4. What is repeatedly obvious is that whatever nuclear factor(s) is responsible for the bandshifts, it must be enriched in hepatocytes as seen (Figure 8.5), since no bandshift was observed when the Huh7 (of hepatoma origin) nuclear extract was replaced with 143-TK$^-$ (of osteosarcoma origin) (Figure 8.2, lane 1). The supershift results appear chiefly inconclusive for one main reason: that is, they showed a similar binding pattern in each antibody assay, compared to WT. However, what appears as a residual shift of similar size and intensity were noted in each of two reactions: HNF4 and Coup-TF. Since these cannot be seen as condiderable shifts due to the fact that the two major bandshifts (A and B) remains, the general results may be suggestive that perhaps only one factor, which was not tested for in this experiment, dominated the binding site, and once bound, inhibited the binding of the other factors tested for in this study. Alternatively, it is possible that transcription factors HNF4 and Coup-TF were indeed binding but at such low levels, as reflected on the residual shift results described above, and demonstrated (Figure 8.5). Indeed, although different studies have implicated different nuclear factors in the binding of WT EnhII/CP, most of these studies implicated HNF4 (Cooney *et al.* 1992a; Li *et al.* 2000a; Viollet *et al.* 1997f; Xanthopoulos *et al.* 1991b; Xu *et al.* 2004b; Yu & Mertz 2003b; Zheng *et al.* 2004b). Other studies have also shown significant binding of C/EBP, and HNF3 (Li *et al.* 1995b; Viollet *et al.* 1997g; Xanthopoulos *et al.* 1991b), and retinoid X receptor alpha (RXRα; another member of the nuclear superfamily similar to HNF4) (Jiang *et al.* 1995b). Other lesser known transcription factors have also been reported to interact with this region, and were adequate for EnhII/CP activity (Ishida *et al.* 2000c; Lai & Ting 1999a; Li *et al.* 1998). There is a possibility that the cell-cycle stage at the time of nuclear protein extraction may also play a determining role with regard to factors that bind, at a significant level. With a sequence that responds to different factors, this is a distinct possibility.

Finally, EnII/CP activity may result from the cooperative action of various factors, which can be both of ubiquitous (for example SP1), and hepatoma origins (such as HNF1, HNF4, C/EBP, COUP-TF) (Ishida *et al.* 2000d). The factors which stimulate transcription of HBV gene must be expressed in hepatocytes; the absence of even one of them may result in a great decrease in transcription, as may be reflected in the case of T1753C/A1762T/G1764A/C1766T (CP7). The EnhII sequence is believed to have two roles when acting as a *cis* element; one as an enhancer of all

promoters of HBV and another as a "helper" in driving CP transcription. The CURS and EnhII regions overlap, but (EnhII) applicable only to CP and functions in a position- and orientation-dependent manner (Yuh *et al.* 1992a). For the luciferase activity study in this thesis, EnhII was probably taking the latter role, that of a transcription "helper".

Since some of the above transcription factors were demonstrated to be involved in HBV gene expression, (Harish *et al.* 2001; Ishida *et al.* 2000e; Ktistaki & Talianidis 1997) — though their function in our study was not characterised — HBV replication can be explained by the combined action of hepatocyte-enriched factors leading to hepatotropism of HBV. We suggest that controlling the functions of these factors may contribute to the suppression of HBV replication. If so, this could account for the difference between the high replicating recombinant, T1766T/T1768A (CP4), and the low replicating one T1753C/A1762T/G1764A/C1766T (CP7). Despite the fact that there was a general correlation in these mutants between their levels of luciferase activity, extracellular HBV DNA and, intracellular replicative intermediates, there were several fold-differences amongst them. It can be speculated that the reason for the lack of correlation between the luciferase, and bandshift data for some of the recombinants may be the fact that various protein factors could be binding to the different promoter sites (Cooney *et al.* 1992b; Li *et al.* 2000b; Viollet *et al.* 1997h; Xanthopoulos *et al.* 1991b; Xu *et al.* 2004c; Yu & Mertz 2003a; Zheng *et al.* 2004a), notwithstanding their similarity in the pattern of bandshift results. Indeed studies have shown that HNF1 may only dominate binding to this promoter site when the A1762T/G1764A substitution mutations are present (Li *et al.* 1999a; McNair *et al.* 2000d; Wang *et al.* 1998a). This factor may exhibit weaker binding in other situations. In spite of this, a low binding capacity may nonetheless translate to no significant difference in replication capacity (Li *et al.* 1999), as seen in the work documented in this thesis. Apart from this, what is yet to be proved is whether there are any specific nuclear factors that dominate binding of this region, as suggested by the increase promoter activity seen in some of the FHB sequences used in this investigation.

Concluding Remarks

These studies have examined the possibility that mutations in the CP region of the HBV genome upregulate viral replication in FHB patients. As a comparison,

low-replicating clone known to affect the S gene was included — leading the engagement further in a comprehensive study encompassing mutations in the overlapping P gene, and at the effect of the former mutations (S gene) on polymerase function. The conclusions drawn from the results of Sections 3, 5 6, and 8, largely support the concept of viral replicative activity enhancement as a potential immune system trigger for the onset of FHB, as suggested in other studies (Carman *et al.* 1993a; Hadziyannis & Vassilopoulos 2001b; Hultgren *et al.* 1997a; Milich *et al.* 1988a; Milich *et al.* 1990a; Thomas *et al.* 1982a). Similarly, from this study (reported in this thesis) it is concluded that S gene mutations can influence P gene function, and ultimately its response to drug therapy. However, this work also raises other questions, which can only be addressed by further research.

A number of confounding factors, such as the identification of an adequate SDM approach, and a reproducible method for transfection efficiency determination, were identified early in the investigation, and measures were taken to resolve them. As a result, the data obtained in this study were neither skewed by the potential influences of sporadic mutations in recombinants, nor, it is thought, by differences in transfection efficiencies. Thus, the findings should be directly attributable to the specifically-introduced mutations *per se*. Although it is postulated that FHB mutants may be exclusively high-replicating, data documented here did not totally prove that, although the replication capacity studies did show a clear increase by all the recombinants that harboured at least two mutations in the CP region. The exceptions were A1762T/G1764A, and T1753C/A1762T/G1764A/C1766T mutants. Other data supporting these observations have been published (Baumert *et al.* 1998a; Parekh *et al.* 2003k). However, the role of the T1753C/A1762T/G1764A/C1766T mutant in contributing to FHB disease outcome needs further elucidation. In the current context, the fact that the low replicating capacity of this mutant was rooted in its weak promoter have been corroborated through the Promoter-Analysis study, which showed reduced binding of nuclear factors to the CP region. Additionally, the luciferase results supported the apparently weak promoter activity of this mutant, as compared to those seen with the WT and/or the remaining mutant recombinants. Although the data demonstrate clearly that nuclear factor(s) present in hepatoma cells bound to the WT EnhII/CP site, and initiated transcription, further work, using for example, additional antibodies that are specific to other nuclear factors, needs to be carried out. Generating similar constructs using the additional FHB sequences

(CP8-CP11, Figure 8.1), followed by luciferase activity measurements should provide further data to enable a thorough comparison of mutant behaviours. Indeed, there is evidence that nuclear factors other than the three used in this study (HNF1, HNF4, and COUP-TF), may bind the specific CP sequences tested here, which have adequate capability to regulate transcription (Ishida *et al.* 2000f; Lai & Ting 1999b; Li *et al.* 1995a; Xanthopoulos *et al.* 1991b). What can be concluded based on the available data is that there are nuclear factor(s) as yet to be identified which must be binding to the promoters to affect the levels of transcription, and ultimately replication. Understanding the possible role that nuclear receptors, particularly ligand-dependent nuclear receptors, might play in transcription start site selection might suggest approaches to modulate the HBeAg status of chronically HBV-infected individuals, which may have useful therapeutic implications. For example, one ligand which acts to suppress preG RNA may be useful in the treatment of FHB, whilst another which activates synthesis of preC mRNA may be helpful in curtailing HBV virion production and eliciting an earlier immune response in acutely infected patients. Further bandshift assays are needed to address this issue.

Finally, it must be re-emphasised that this work, attempted to unravel the role of mutants in triggering FHB. This is by no means the only contributing factor. In children in particular, in addition to the host immune status, age at infection and maternal HBsAg/HBeAg status, are major contributors in determining the onset of FHB. Furthermore, the clinico-pathological observations made in other patients with FHB suggest that, in the absence of a functioning immune system as it occurs in SCID mice and severely immunosuppressed patients, HBV can replicate and express its proteins in an unrestrained fashion. This leads to excessive accumulation of viral proteins, cell damage, and sometimes fatal hepatic disease (Meuleman *et al.* 2006).

The role of mutations in the S gene region, and how these may affect the functioning of the P gene with which it overlaps, have been the subject of speculation for a long time. However, emerging evidence suggests that the replication efficiency of low replicating LMV-resistant P gene mutant have been partially restored by an S gene mutations (Torresi *et al.* 2002a). In this thesis, studies identified some phenotypic restoration of one LMV-resistant mutant, L180M/M204V. The possibility that recombinant viruses — for example containing the G145R/180M/M204V mutation in "a" determinant of HBsAg that may potentially decrease the ability of anti-HBs to neutralise it — may also have the potential to

behave as vaccine escape mutants, is challenging. Further virological and immunological studies could resolve this question.

Chapter 10

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Appendices

APPENDICES

#1. Example of standard curve calculations and construction.

#2. Example of mole:mole calculation for the co-transfection of pCI-GFP and HBV recombinant.

#3. Sample calculations for standardisation to 15% transfection efficiency:

3.a. of Real-time PCR results.

3.b. for quantification of HBsAg (by ELISA) in transfection media.

#4. Sample electrophoregram of EnhII/CP fragment in pGL2-Basic® vector

#5. Vector maps and DNA markers.

Appendix # 1

Example of standard curve calculations and construction:

To generate a standard curve using a known template, the copy number of the starting plasmid was first determined spectrophotometrically. Subsequently, a series of 10-fold dilutions from 10^3-10^9 were generated. Per microlitre, the various dilutions were 30ng, 3ng, 300pg, 30pg, 3pg, 400fg, 4fg. During the Real-time PCR assay, each dilution was assigned a value indicating the calculated number of molecules in relation to the concentration of the plasmid present in each capillary. The assigned values were obtained from the following sample calculation where, 30ng is used:

Molecular Weight (M.Wt) of unknown = M.Wt of 1 base-pair x S = 660g/bp x S
(S = number of base-pairs in the vector + insert)
The molecular weight in grams contains 1 mole of molecules, which is equal to 6.02×10^{23} molecules, therefore:

Number of molecules per gram = $(6.02 \times 10^{23}) \div$ M.Wt of plasmid

For example pGEM-T-Easy vector + preC mRNA = 3015 + 148 = 3163bp
Therefore the Molecular Weight of preC mRNA = 3163bp × 660g/bp = 2,087,580g
Number of molecules per gram of pGEM-T-preC mRNA = $(6.02 \times 10^{23}) \div (2.08758 \times 10^6)$
Number of molecules per ng = $(6.02 \times 10^{14}) \div (2.0889 \times 10^6) = 2.88 \times 10^8$
Number of molecules per 30ng = $(2.884 \times 10^8) \times 30 = 8.65 \times 10^9$
The starting concentration of each unknown sample was automatically extrapolated from the standard curve generated from the same run.

Appendix # 2

Example of mole:mole calculation for the co-transfection of pCI-GFP and HBV recombinant:

Information:

Length of pCI-GFP = 4.6kb

Length of HBV recombinant (final transfected construct) = 6.8kb

Approximate weight of 1base = 330g, and 1 base pair (1bp) is 660g/bp.

Question:

What concentration of HBV recombinant (for example p3.8II) is eqimolar to 2.0μg of pCI-GFP?

Calculation:

M.Wt of pCI-GFP = 4,600 x 660 = 3.036×10^6 g/mol

1 mole = weight (g) ÷ molecular weight (g/mol)

Thus: 1 mole = 0.002g ÷ 3.036×10^6g/mol = 6.588×10^{10} mol of pCI-GFP

M.Wt of 6.8kb recombinant (p3.8II) = 6,800 × 660 = 4.488×10^6 g/mol.

A p3.8II equimolar concentration of 6.588×10^{10} mol of pCI-GFP is:

$$6.588 \times 10^{10} \text{ mol} = \frac{\text{Weight of p3.8II (g)}}{4.488 \times 10^6 \text{ g/mol}}$$

Weight of p3.8II = (6.588×10^{10}) mol × (4.488×10^6) g/mol = 0.002956g (2.956μg)

Therefore, 3.0μg of a 6.8kb recombinant was an equimolar concentration for co-transfecting with 2.0μg of the pCI-GFP.

Appendix # 3

#3.a. Calculation example for standardisation to 15% transfection efficiency of Real-time PCR results:

#3.a.i. Information:

Cells were seeded at a density of 1.5×10^5 in 0.003L of medium, then transfected 12hrs later. 96hrs following transfection, 300µl of supernatant were removed for HBV DNA (or replicative intermediate) extraction. Extracted DNA was diluted in a final volume of 10µl distilled water. 1µl of neat DNA extract was used in Real-time assays.

Question:

Given the above information, if the Real-time PCR result was 1.35×10^8 copies/µl of extracted product, what was the total viral copy number/ml in the 3ml supernatant of 20% transfection efficiency?

Calculation:

100% transfection efficiency would have transfected 1.5×10^5 cells (that is all cells that were plated out).

Therefore, if transfection efficiency was in 20%, 3.0×10^4 cells would have been transfected.

Since released DNA was extracted from 300µl supernatant and eluted in 10µl of distilled water, the total extracted DNA contained:

$10\mu l \times (1.35 \times 10^8)$ copies/µl = 1.35×10^9 copies of HBV DNA

Therefore, if 300µl of supernatant contained 1.35×10^9 copies, then a total of 3000µl (3.0ml) supernatant will contain 1.35×10^{10} copies in other words. In other words, 20% transfection efficiency would produce a total of 1.35×10^{11} copies of HBV virions in the original 0.003L of medium.

Cont. #3.a.i

For standardisation to 15%:

15% × (1.35×10^{10}) = 2.03×10^{11} divided by the original transfection efficiency of 20% = 1.02×10^{10} copies/3000µl at 15% transfection efficiency.

In a 0.001L (or 1000ml = 1×10^{6}µl) of medium contained 3.4×10^{9} virus particles in the above example.

EXCEPTION: Results of HBV RNA transcripts (preG and preC) were similarly standardised except that cDNAs were first diluted at various dilution factors ranging from 1:10 to 1:30, following which 1µl of the diluent was used as template for detecting transcript and control gene (HKG) copy numbers. Transcripts copy nuymbers were normalised to the HKG by dividing the former by the latter. [Refer to Section 4.4]. The result representing a ratio of the two, were next multiplied by the cDNA dilution factor to revert to the undiluted state. This was then standardised to 15% transfection efficiency, as in the above example.

#3.a.ii. Information:

Following on from the information given above (in #3.a.i), if intracellular HBV DNA (RC– or ccc–DNA) was detected from a triplicated assay, and the average copy number in 1µl (from an original neat stock of 10µl) was 1.35×10^{5}, how many HBV particles were present per cell? Transfection efficiency was 20%. 1.5×10^{5} cells were originally transfected.

Calculation:

100% transfection efficiency would have transfected 1.5×10^{5} cells (i.e the total number of cells that were plated out). However, if transfection efficiency was 20%, 3.0×10^{4} cells would have been transfected.

Therefore, how many of the 1.35×10^{5} HBV RC-DS (or ccc) DNA copies were present in each cell in the 3.0×10^{4} transfected cells.

$$\frac{1.35 \times 10^{5}}{3.0 \times 10^{4}} = 4.5 \text{ copies/cell in1µl}$$

Since the total HBV DNA was present in an eluant of 10µl, then:

4.5 copies/cell/1µl of extract × 10µl = 45 copies/cell (for 20% transfection efficiency).

15% standardisation: $\frac{15\% \times 45}{20\%}$ = 34 copies/cell.

#3b. Calculation examples for standardisation to 15% transfection efficiency for quantification of HBsAg (by ELISA) in transfection media:

A standard curve was constructed using a yeast recombinant HBsAg (see Methods, Section 2.2.13.a). Samples, in triplicate, were initially diluted 1:5 and the mean (in ng/µl) determined from the standard curve. The final result was subsequently standardised to 15% transfection efficiency.

Information provided:

A mean absorbance (A_{450}) of 1.2 corresponded to 8.5ng/ml from the plotted standard curve. How much of this concentration will represent 15% transfection efficiency, down from an initial transfection efficiency of 20%?

Calculation:

Sample I:

Original contained 8.5ng/ml × dilution factor (5) = 42.5ng/ml.

42.5ng/ml → 20%

Thus for 15% transfection efficiency: HBsAg amount (ng/ml) = $\frac{15\% \times 42.5\text{ng/ml}}{20\%}$

15% transfection efficiency will yield 31.88ng/ml of HBsAg.

Cont. #3b

Sample II:

Original contained absorbance (A_{450}) of 1.2 × dilution factor (5) = A_{450} of 6.0.

A_{450} of 6.0 → 20%

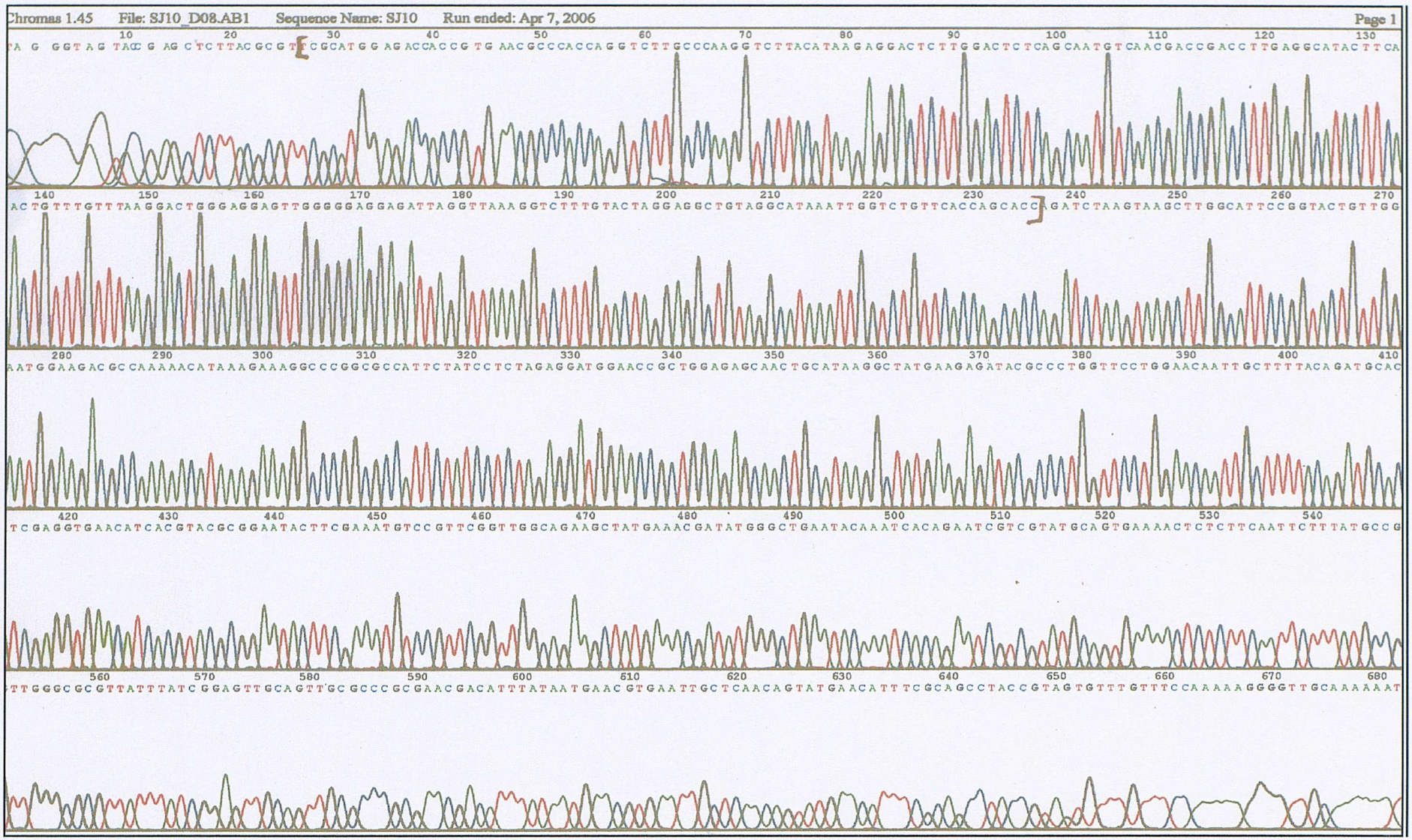
Thus for 15% transfection efficiency: HBsAg absorbance (A_{450}) = $\frac{15\% \times 6.0\ (A_{450})}{20\%}$

15% transfection efficiency will theoretically result to an A_{450} of 4.5.

Appendix # 4.

Sample electropherogram of WT EnhII/CP fragment in pGL2-Basic® vector.

The sequences were derived using an automated sequencer and a sense sequencing primer (GL1primer1), which binds immediately upstream the start of the vector multiple cloning sites at nucleotide positions 5565-5587. Bars indicate the limits of the cloned HBV insert. [For vector map, see appendix #5, Vector 5].


Chromas 1.45 File: SJ10_D08.AB1 Sequence Name: SJ10 Run ended: Apr 7, 2006 Page 1

Appendix # 5

#5a. Vectors used in the project:

Vector 1: pBluescript® II KS(+) (Stratagene):

(a) Used to clone the parent 1.2X (3.8kb) HBV DNA insert. It was the "backbone" for all recombinants.

(b) Served as a mock control in all transfection studies.

Vector 2: pGEM-T-Easy (Promega) was used to:

(a) clone specific HBV inserts for generating the standard curves used in the quantitative Real-time PCR, and to

(b) sub-clone the various HBV promoter genes in the promoter-analysis studies.

Vector 3: pGL2-Basic (Promega), used to clone the promoter sequences for the Promoter-Analysis studies.

Vector 4: pGL2-control (Promega), the control vector.

#5b. DNA markers used in this project

DNA markers were supplied by Bioline London (hyperladders™), or Promega (100bp):

Marker 1: Hyperladder™ I

Marker 2: Hyperladder™ IV

Marker 3: Hyperladder™ V

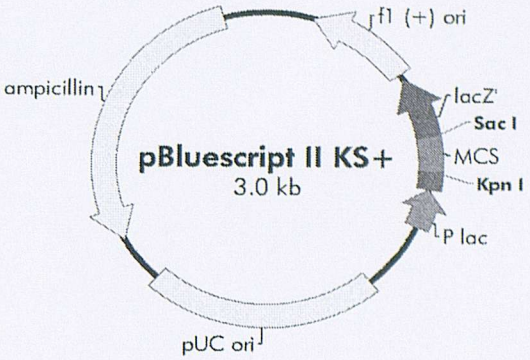
Marker 4: 100bp ladder

All vector and marker diagrams were reproduced from the supplier technical manuals.

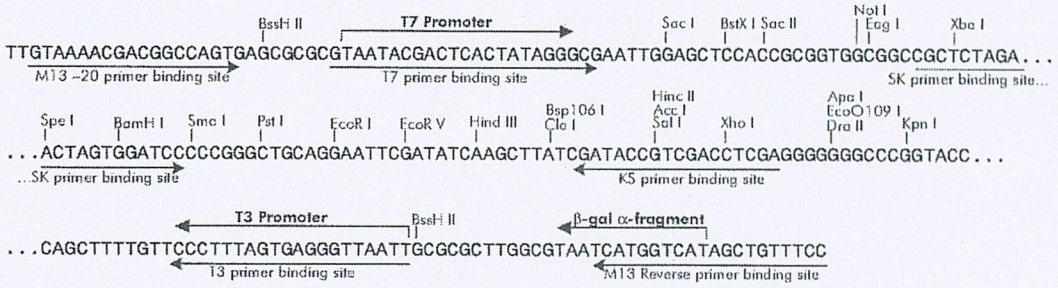
#5a. Vectors

Vector 1: pBluescript® II KS(+)

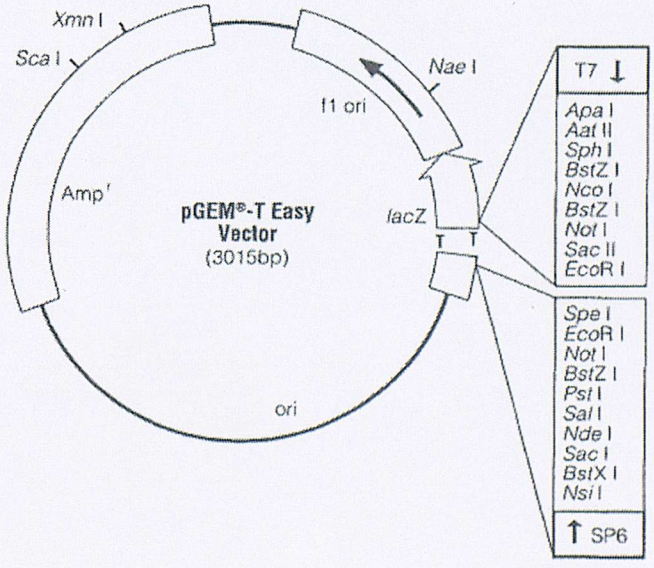
f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



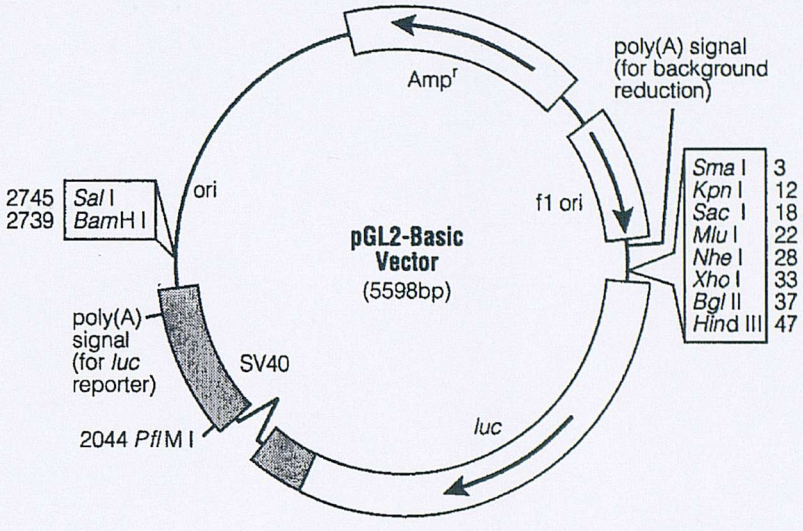
pBluescript II KS (+/–) Multiple Cloning Site Region (sequence shown 598–826)



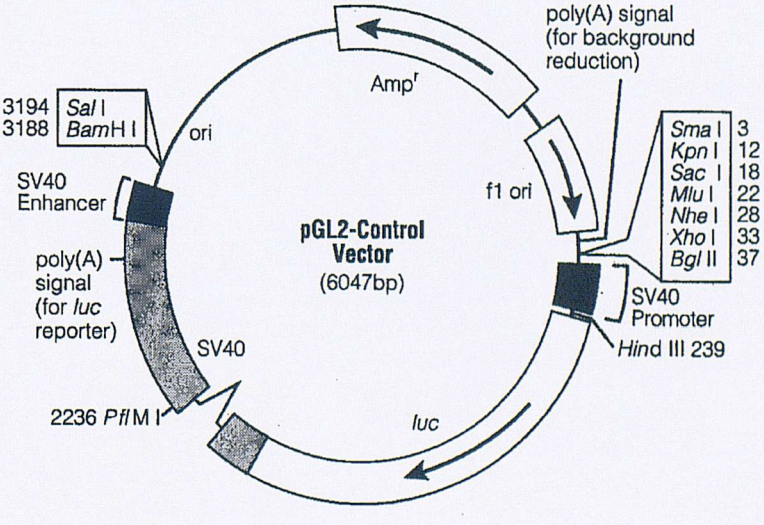
Vector 2: pGEM-T-Easy ®



Vector 3: pGL2-Basic®

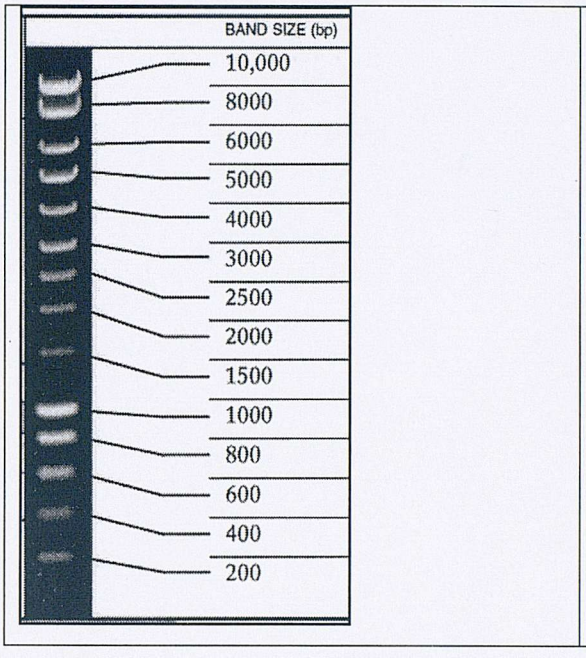


Vector 4: pGL2-Control®

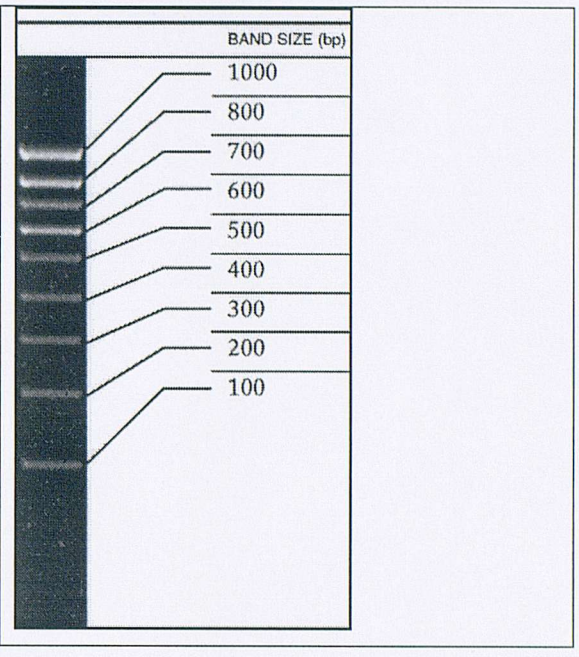


#5b. DNA markers

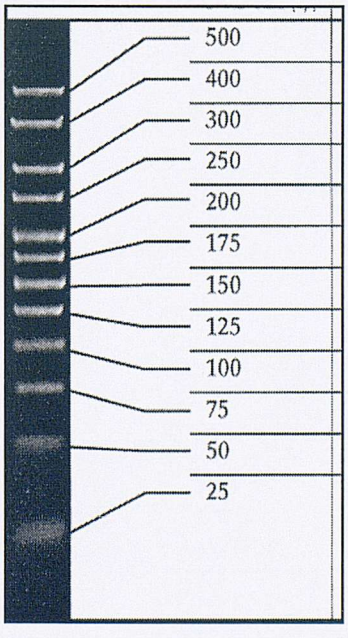
Marker 1: Hyperladder™ I



Marker 2: Hyperladder™ IV



Marker 3: Hyperladder™ V



Marker 4: 100bp ladder

