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Author/s:

Huynh, Thao Thanh

Title:

Identifying factors regulating production and expression of HBV cccDNA and the minichromosome using in vitro and in vivo models of HBV replication

Date:

2021

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Identifying factors regulating production and expression of HBV cccDNA and the minichromosome using *in vitro* and *in vivo* models of HBV replication.

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

by

THAO THANH HUYNH
ORCID ID: 0000-0003-2658-1846

Department of Microbiology and Immunology,
Faculty of Medicine, Dentistry and Health Sciences,
The University of Melbourne
and
Research and Molecular Development,
Victorian Infectious Diseases Reference Laboratory

May 2021

ABSTRACT

Chronic Hepatitis B (CHB) affects more 250 million people globally, causing significant morbidity and mortality from associated sequelae. The etiological agent, hepatitis B virus (HBV) contributes to the majority of cirrhosis and hepatocellular carcinoma (HCC) cases, resulting in nearly a million deaths annually. Carriage of the virus (HBsAg positivity) is associated with elevated risk of developing these liver complications, so while available nucleos(t)ide analogue (NUC/NA) therapy can effectively control viral replication, viral clearance to the point of 'functional cure' is required to significantly lower the risk. Moreover, development of a cure remains paramount as treatment is typically life-long, and cessation of therapy can lead to reactivation of the virus via the nuclear reservoir of a viral genome template known as the covalently closed circular DNA (cccDNA) minichromosome. Unaffected by conventional therapies, cccDNA is the required template for the synthesis of all viral transcripts, including greater-than-genome transcript pregenomic RNA (pgRNA), which in turn, is the template for reverse transcription to form the relaxed circular DNA (rcDNA) genome of progeny virions. Despite immense efforts, much remains to be elucidated about the virus and its replication cycle, largely due to the fact that the HBV research field is restricted by a severe lack of suitable/appropriate models for investigating all stages of the viral cycle, with the cccDNA minichromosome being particularly difficult to interrogate.

This PhD project addressed this knowledge gap by characterising a monomeric/genome-length based transfection system both *in vitro* and *in vivo*, and investigating its utility as a novel model enabling investigation of cccDNA-like molecules. The model was utilised to further explore the impact of different/specific viral-host protein interactions on viral replication. Specifically, this was centred on the viral HBx and cellular DDB1 proteins, as the interaction between these two was recently demonstrated to facilitate viral transcription following viral infection. Finally, a broad proteomics approach involving microarray screening was applied to identify novel host factors/mechanisms associated with HBV replication. HBV interacts with many cellular proteins and factors, redirecting the associated processes to benefit viral replication and propagation. While some of these cellular components have been described previously, likely many more are yet to be discovered, and this project aimed to identify additional potential therapeutic targets that could ultimately contribute to HBV cure.

DECLARATION

I, Thao Huynh, declare that this thesis is a record of my original work towards the degree of Doctor of Philosophy except where indicated in the Preface, and that due acknowledgement has been made in the text to all other material used. This thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Thao Huynh
May 2021

PREFACE

The work presented in this thesis was performed under the joint supervision of Professor Peter Revill (Victorian Infectious Diseases Reference Laboratory, VIDRL), and Professor Hans Netter (VIDRL). I have had additional supervision from Dr Gregor Ebert (Walter & Eliza Hall Institute). The following work presented in this thesis is acknowledged as a contribution/work of others:

Chapter Three: All quantitative serology for detection of HBeAg and HBsAg was performed by Mr Hugh Mason (VIDRL). Viral inoculum for cell culture infection experiments was generated by Ms Vitina Sozzi (VIDRL). Sanger sequencing (capillary separation) was performed by Micromon Genomics (Melbourne, Australia).

- **(in vivo experiments):**

Ms Michelle Clark, Ms Liana Mackiewicz, and Dr Gregor Ebert at the Walter & Eliza Hall Institute were responsible for the study design, performed the hydrodynamic tail vein injections, the serum bleeds, and the harvesting of liver. I extracted the cccDNA, and performed all of the necessary digests and Southern blots, and contributed to the preparation and revision of the manuscript submitted to *Cell Death and Disease* on November 24th, 2020.

Chapter Five: Cell lysates were subjected to antibody microarray analysis by Kinexus Bioinformatics (Vancouver, Canada). Lead candidate selection was performed based on the returned converted and organised data.

ACKNOWLEDGMENTS

Embarking on this PhD journey was not something I had anticipated, but I can honestly say it has been a fulfilling and enjoyable learning experience. Though it has certainly not been a walk in the park, I am fortunate to have been supported by many people along this journey.

To the amazing family and friends who supported me through my academic endeavours: I am forever grateful for your existence. Thank you for keeping me alive and sane, for checking in and boosting my spirits, not only without my prompting but indeed despite my reluctance and many protests. I had always anticipated the thesis writing process to be the greatest struggle personally, but I hadn't accounted for a global pandemic, and resultant uncertainty and changing circumstances in the world around me, as well as the general despair of 2020 to be so affecting. Even before Covid hit, I had made myself scarce. I had promised to be a better and more available friend from 2020...only to have my submission ultimately pushed back more than a year. Through the tough times of social distancing and lockdown, waning motivation and struggling productivity, thank you for assuring me there was indeed a light at the end of the tunnel, even if I couldn't see it.

I am eternally grateful to my supervisor Prof Peter Revill, for giving me the opportunity to complete both an Honours and PhD project, and for generously offering financial support. I truly believe that your unending support and optimism has significantly pushed me to see this PhD through, and not give up - as I have quietly considered on several occasions (or basically every other day, towards the end). Together with Prof Hans Netter, who unwittingly signed on as co-supervisor for my PhD project, you have provided valuable advice, suggestions, direction, and guidance, without which I would not have been able to complete this PhD. I would also give a special mention to Ms Tina Sozzi, who has contributed significantly to my learning experience. Tina has been an absolute mentor, introducing me to the lab working environment and teaching me all the experimental skills I have built over the course of both my Honours and PhD projects. She has provided me with much insight and guidance in the planning, execution and evaluation of my experiments, and I will be forever indebted to her for her generosity in sharing her time and effort. Finally, I would also like to thank Dr Nadia Warner for offering me employment, and for being understanding and supportive even when my submission was continually delayed.

Every other member of the R&MD family I have had the pleasure of coming to know throughout this experience, staff and students, past and present alike, has contributed to my PhD project in some aspect, and I would like to extend my gratitude collectively. Perhaps most importantly, you have all contributed to making my experience an enjoyable and unforgettable one.

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LIST OF ABBREVIATIONS

°C	Degrees centigrade
A	Adenine (nucleotide); Alanine (amino acid)
aa	Amino acid
Ab	Antibody
BCP	Basal core promoter
BSA	Bovine serum albumin
C	Cytosine (nucleotide); Cysteine (amino acid)
cDNA	Complementary DNA
cccDNA	Covalently closed circular DNA
cccDNA-LM	cccDNA-like molecule
C-term	Carboxy (COOH) terminal
CTD	Carboxy terminal domain
DDB1	(UV-)damaged DNA-binding protein 1
DDB2	(UV-)damaged DNA-binding protein 2
DHBV	Duck hepatitis B virus
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleotide triphosphate
dsDNA	Double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
ETV	Entecavir
EV	Empty vector
Exo I/III	Exonuclease I/exonuclease III
FITC	Fluorescein isothiocyanate
x g/rcf	G-force/relative centrifugal force
g	Grams
G	Guanine (nucleotide); Glycine (amino acid)
GSHV	Ground squirrel hepatitis virus
HBcAg	Hepatitis B virus core antigen
HBeAg	Hepatitis B virus e antigen
HBV	(human) Hepatitis B virus
HBx	Hepatitis B virus X protein
HCC	Hepatocellular carcinoma
IFN	Interferon
IU/PE IU	International units/Paul Erlich IU
kDa	KiloDalton
LB	Luria Bertani
M	Molar
MEM	Modified eagle media
-mer	
mg	Milligrams
mins	Minutes
ml	Millilitres
mM	Millimolar
mRNA	Messenger RNA
NA/Nuc	Nucleos(t)ide analogue
N-term	Amino terminal

NaCl	Sodium Chloride
NFW	Nuclease-free water
ng	Nanograms
NP-40	Octylphenoxypolyethoxyethanol (also known as Igepal™)
nt	Nucleotide
NTCP	Sodium taurocholate cotransporting polypeptide
ORF	Open reading frame
p17	17kDa HBV protein, also known as HBe e antigen (HBe/HBeAg)
p21	21kDa HBV protein, also known as HBV core protein (HBc/HBcAg)
p22	22kDa HBV intermediate protein
p25	25kDa HBV protein, also known as HBV precore protein
PBS	Phosphate-buffered solution
pc/pcRNA	Precore/precure RNA
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/streptomycin
PEG	Polyethylene Glycol
PE IU	Paul Erlich international units
pg/pgRNA	Pregenomic RNA
pol	Polymerase
PreS	Pre-surface
rcDNA	Relaxed circular DNA
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecylsulphate
SEAP	Secreted alkaline phosphatase
sec	Seconds
Ser	Serine
SMAC	Second mitochondria-derived activator of caspase
SMC5/6	Structural maintenance of chromosomes 5/6 complex
S.O.C	Super optimal broth with catabolite repression
ssDNA	Single-stranded DNA
T	Thymine (nucleotide); Threonine (amino acid)
Thr	Threonine
T/V	Trypsin/versene
Tris/tris base	tris(hydroxymethyl)aminomethane
µg	Micrograms
µl	Microliters
V	Volts/Voltage
v/v	Volume/volume
VIDRL	Victorian Infectious Diseases Reference Laboratory
w/v	Weight/volume
WHV	Woodchuck hepatitis virus
WT	Wild-type

Chapter One: Introduction

1.1 Hepatitis B virus (HBV)

1.1.1 Epidemiology

Worldwide, over 257 million people, approximately 3.5% of the population, are currently chronically infected with hepatitis B virus (HBV) (WHO Global Hepatitis Report, 2017). Infection is endemic in certain regions of the world, and even in countries where it does not present a significant burden nationally, pockets of endemicity can exist within specific communities (Hahne et al, 2004; Kowdley et al, 2012; MacLachlan et al, 2013; Marschall et al, 2008). HBV is transmitted through contact with bodily fluids, and in the highly prevalent South-East Asian and Sub-Saharan African regions, infection is primarily acquired perinatally from mother-to-child (Beasley et al, 1977; Burk et al, 1994; Okada et al, 1976), or horizontally within the first few years of childhood (Botha et al, 1984; Marinier et al, 1985). In most of these cases, infection results in long-term, often lifelong carriage (Edmunds et al, 1993; McMahon et al, 1985). In contrast, for most immune-competent adults, exposure to the virus is limited to an acute infection that is subsequently cleared within six months. Only a smaller proportion of these exposures, around 5-10%, will progress to chronic infection which is defined by persistent detection of the surface protein for more than six months (Beasley et al, 1983b; McMahon et al, 1985). This has major public health implications as even in the absence of active viral replication, chronic carriers have an intrinsically higher risk of developing associated complications of the liver such as cirrhosis and liver cancer (Beasley & Hwang, 1984; Chen et al, 2010), with chronic hepatitis B infections estimated to contribute to more than 880,000 deaths worldwide in 2015 alone (WHO Global Hepatitis Report, 2017).

While increased research and understanding, medical innovation and intervention has generally aided in lessening the burden of most major communicable diseases (tuberculosis, HIV, malaria etc), viral hepatitis infections and their associated morbidities and mortalities continue to rise (Stanaway et al, 2016), with liver cancers currently the 2nd highest cause of cancer-related deaths (Mortality & Causes of Death, 2016). This is despite the decades-long existence of a successful prophylactic HBV vaccine and effective antiviral treatments. However, these treatments are not completely protective and since chronic HBV infection remains incurable, the risk of developing the associated sequelae persists, even on treatment. Moreover, infections can often manifest asymptotically, and with 257 million people worldwide estimated by the WHO to be affected with viral hepatitis, this represents a significant proportion of the population who are oblivious to their status and potential transmission risk. Taken together, HBV undoubtedly poses a significant global health burden requiring immediate and vigorous attention and concerted global efforts towards its elimination as a substantial health threat (Revill et al, 2019a).

1.1.2 HBV genotypes

Hepatitis B virus exists as nine distinct genotypes (A-I) which are separated by a sequence divergence of more than 8% at the nucleotide level (Arauz-Ruiz et al, 2002; Norder et al, 1992;

Okamoto et al, 1988; Stuyver et al, 2000; Tran et al, 2008). An additional isolate recovered from a Japanese soldier residing in Borneo during World War II was identified to be a recombinant virus and designated as genotype J, though it has not been further observed elsewhere (Tatematsu et al, 2009). HBV genotypes can be further divided into sub-genotypes that differ by greater than 4% in their nucleotide sequence. HBV genotypes largely cluster geographically, meaning they often correlate with a patient's ethnic and migratory/epidemiological background, with marked differences in HBV natural history, disease progression and treatment response to interferon therapy (Croagh et al, 2015; Gunther, 2006; McMahon, 2009). In Australia, there is no dominant genotype but rather all HBV genotypes are present, which is reflective of a highly diverse and multicultural population (Bell et al, 2005). Furthermore, most CHB patients originally hail from countries where HBV infection is comparatively a more significant burden.

In general, in regions carrying the greatest burden of hepatitis infection, the prevailing genotypes are B and C in Asia, and A and E in Africa. In Europe, America, and the Mediterranean regions, patients are predominantly infected with genotype A and D. Multiple studies have described genotype-associated differences in HBV replication (Bannister et al, 2019; Sozzi et al, 2018; Sozzi et al, 2016). The infecting genotypes have implications on an infected individual's health and treatment. For example, adult-acquired genotype A HBV has higher risk of progression to development of CHB compared to other genotypes (Ito et al, 2014; Mayerat et al, 1999). Genotypes C and B are more commonly associated with cirrhosis and hepatocellular carcinoma (HCC), with genotype C in particular identified as an individual risk factor for HCC development (Chan et al, 2004; Chan et al, 2003; Kao et al, 2000a; Yang et al, 2008). High replication phenotypes are often observed with genotypes B and C, as compared to A and D, and the increased production and accumulation of viral products may play a role in inducing liver damage and associated disease progression (Sugiyama et al, 2006). Genotype B and C patients also tend to undergo delayed HBeAg seroconversion so the higher incidence of liver damage associated with these genotypes is developed in the setting of prolonged infection and replication (over decades), and immune-mediated damage associated with persistent but insufficient responses (Chu et al, 2002; Kao et al, 2004). In contrast, genotypes A and D are often associated with earlier HBeAg negativity due to higher prevalence of specific mutations such as the precore G1896A mutation resulting in a premature stop signal and abrogation of HBeAg expression (Kramvis et al, 2008). Some sub-genotypes are also associated with rapid progression to HCC in specific populations, such as A1 in young African males (Kew et al, 2005; Kimbi et al, 2004), or F1 in native Alaskan children and young adults (Gounder et al, 2016; Livingston et al, 2007). Genotype-associated differences are also observed in response to interferon therapy, with genotypes A or B patients typically responding better than patients infected with genotypes C or D (Erhardt et al, 2005; Flink et al, 2006). Despite these general distinctions, it is important to note that these differences may also be contributed to by underlying host genetics, environmental or lifestyle factors associated with the different populations.

In Australia, while overall HBV prevalence is low at an estimated <1%, significantly higher rates are represented within migrant communities where the population largely originate from countries where HBV is endemic (Cowie et al, 2010; MacLachlan et al, 2013). HBV infection has unacceptably significant impacts on indigenous communities in places such as North America,

Alaska and Australia (Livingston et al, 2007; MacLachlan & Cowie, 2015). The Indigenous Australian population of the Northern Territory was exclusively infected with a novel sub-genotype C4 associated with a disproportionately higher burden of liver disease, more than four times that of non-Indigenous Australians (Littlejohn et al, 2014). This is despite a childhood vaccination program being introduced in the early 1990s (Connors et al, 1998) and suggests that available vaccines may not represent an effective strategy for pan-genotypic prevention of HBV infection. Further studies are required to identify the crucial determinants which dictate these discrepancies, in order to find solutions to protect the vulnerable communities.

1.1.3 Natural history

Chronic hepatitis B develops through the progression of four distinct phases, known, according to current European Association for the Study of the Liver (EASL) guidelines (2017) as: HBeAg-positive chronic HBV infection (formerly immune tolerant), HBeAg-positive chronic hepatitis B (formerly immune clearance/active), HBeAg-negative chronic HBV infection (formerly immune control/inactive hepatitis B) and HBeAg-negative chronic hepatitis B (immune escape/reactivation) phases (Figure 1.1). The first phase, HBeAg chronic HBV infection, is absent from the majority of infections acquired later in life, being more frequent and prolonged in perinatal infections from HBeAg-positive mothers, as the immature immune systems allow for viral replication in the context of tolerance and immune evasion. Patients in this first phase are commonly HBeAg-positive, with elevated HBV DNA and normal ALT levels and do not usually receive antiviral therapy (Chan et al, 2010; Jaroszewicz et al, 2010; Liaw & Chu, 2009; Nguyen et al, 2010). This phase can last decades, during which time there is seemingly minimal liver inflammation and damage (Jaroszewicz et al, 2010). The recent debate around its former designation “immune tolerance” arises from questions regarding whether it accurately represents the immune status, with evidence suggesting there was low level immune activity (detection of integration and liver damage) which is not reflective of true tolerance (Bertoletti & Kennedy, 2015). Furthermore, integration was observed even in immune tolerant patients (Mason et al, 2016), which could contribute to transformation and progression to liver cancer (Kim et al, 2018), further raising questions surrounding the benefits of treating patients in the early phases of chronic infection to prevent or lower risk of potential future/late complications.

‘HBeAg-positive chronic hepatitis B’ marks the phase where antiviral responses are actively mounted as the immune system works to control the infection. Intermittent hepatitis flares are characteristic of this phase, during which elevated ALT and reduced DNA levels indicate robust immune responses mounted in attempt to clear infection (Liaw & Chu, 2009). However, this repeated “immune attack” is often insufficient to eradicate the infection and collateral inflammatory damage to the liver is sustained, leading to fibrosis and cirrhosis. HBeAg seroconversion and anti-HBeAg antibody (anti-HBe) production commonly occurs during this phase, and transition to the HBeAg-negative chronic HBV infection (phase 3) is marked by progressively decreasing ALT and HBV DNA levels as the viral infection is effectively controlled, often by therapeutic intervention (Chan et al, 2010; Nguyen et al, 2010).

Also known as the “inactive carrier” state, the third phase (HBeAg-negative chronic HBV infection) is generally characterised by the presence of anti-HBe, normal ALT levels and relatively low (often undetectable) HBV DNA levels, and improvement in liver fibrosis (Chan et al, 2010; Liaw & Chu, 2009; Nguyen et al, 2010). In some cases, infections are not effectively controlled or cleared and viral replication is renewed, a sign of the immune escape or reactivation phase (Phase 4: HBeAg-negative chronic hepatitis B) that is often punctuated by the emergence of immune escape HBV variants (Lim et al, 2007). Commonly observed are the G1896A precore mutant which abrogates HBeAg expression through introduction of a premature stop codon (Tong et al, 1990), and the double A1762T/G1764A basal core promoter (BCP) mutant which reduces transcription of the precore mRNA (Li et al, 1999).

Beyond these major chronic states, the fifth phase, Occult HBV infection is characterised by detectable cccDNA in the liver, low or undetectable serum HBV DNA, but undetectable HBsAg (Raimondo et al, 2019). In some cases, CHB subjects enter a sixth phase (‘resolved HBV infection’), defined by undetectable HBV DNA and HBsAg, which is the current definition of a functional cure (Cornberg et al, 2020; Revill et al, 2019a). In contrast, a complete cure requires permanent inhibition or elimination of the viral cccDNA reservoir within the hepatocyte nucleus. This is not achievable at present, due to the many gaps existing in our understanding of HBV infection, replication and pathogenesis and there is an urgent need for new approaches to study the biology of HBV cccDNA.

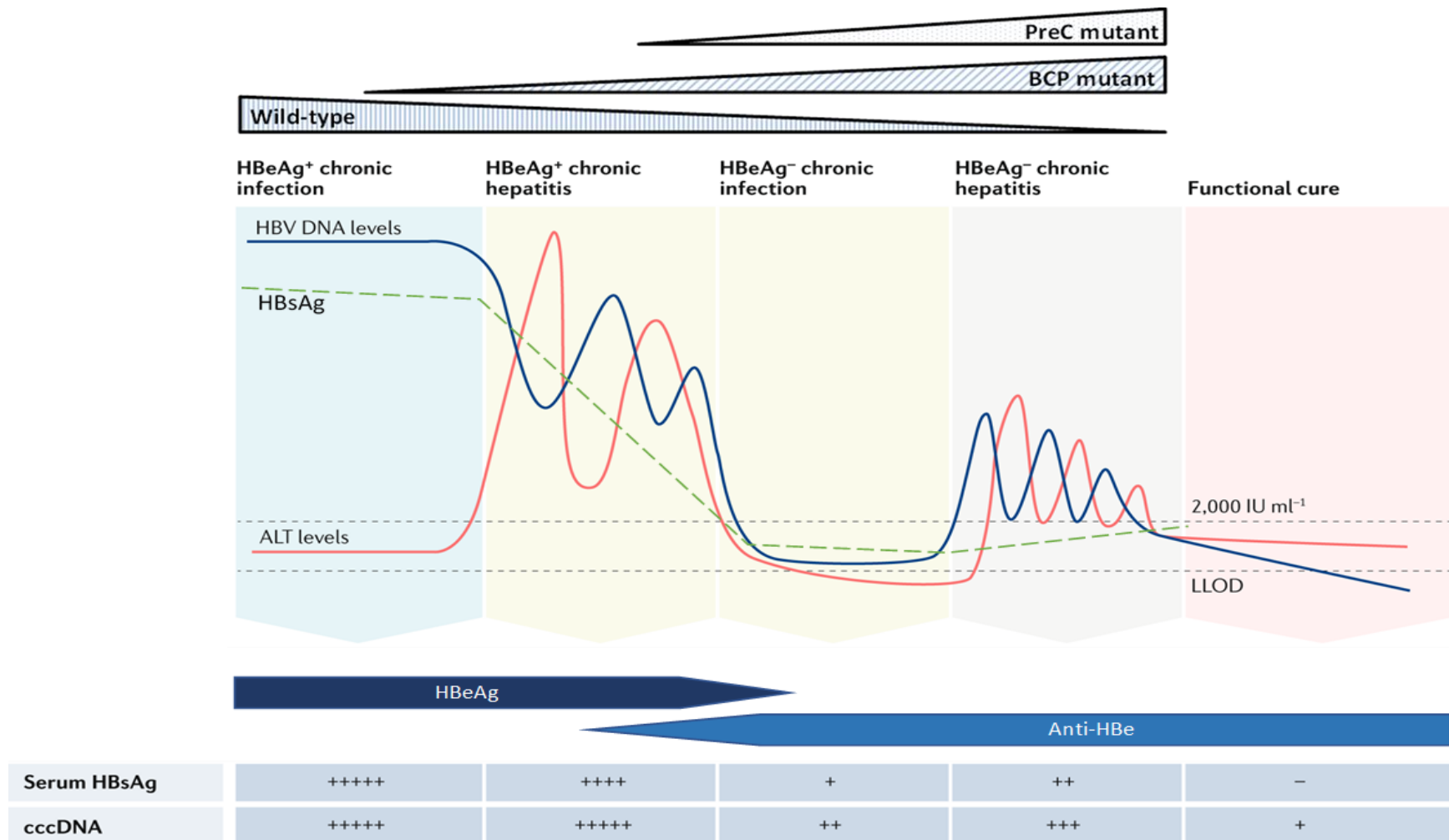


Figure 1.1: Natural history of chronic hepatitis B , depicted through four phases and ending with functional cure. HBeAg, hepatitis B e antigen; HBsAg, hepatitis B S antigen; cccDNA, covalently closed circular DNA; ALT, alanine transaminase; PreC, precore mutant; BCP, basal core promoter mutant; -, not detected; +, detected; LLOD, lower limit of detection. Adapted from (Fanning et al, 2019)

1.1.4 Hepatocarcinogenesis

For the majority (90-95%) of adult individuals, infection with HBV results in a self-limiting, acute infection (McMahon et al, 1985). However, for the remaining proportion who progress to life-long chronic infection (and including those acquiring CHB from mother-to-baby transmission), development of primary liver cancer (hepatocellular carcinoma, HCC) will directly attribute to about half of total HCC cases (WHO Global Hepatitis Report, 2017). Despite substantial epidemiological evidence pointing to HBV being the biggest contributor to HCC incidence/development, the specific mechanisms by which the virus induces transformation and tumorigenesis remain largely undefined. Specific HBV genotypes, mutations, splice variants, HBV integration and partial gene deletions have all been identified as risk factors for HCC development (Liu et al, 2014a; Pan et al, 2018; Sumi et al, 2003; Yotsuyanagi et al, 2002), although the underlying molecular/virological mechanisms for many of these associations is unclear at present (Lin et al, 2007; Sugauchi et al, 2003; Yang et al, 2008).

Integration of sub-genomic HBV fragments into host chromosomes, leading to dysregulation of tumour-suppressor genes and tumorigenic oncogenes is postulated to be a major contributor to hepatocarcinogenesis (Feitelson & Lee, 2007; Jiang et al, 2012; Tu et al, 2001). Moreover, while the HBV genome does not integrate into the host in its entirety, integrated segments result in enhanced expression of some HBV proteins, and resultant over-active immune responses and prolonged inflammation. HBV infection and replication in general induces modulation of cellular physiology and homeostasis, and the dysregulation of cellular processes such as apoptosis, cell proliferation and migration/invasion likely also contribute to cellular transformation. In hepatic tumour cells, aberrant activation of protein kinases including p38 and c-Jun N-terminal kinase (JNK) has been suggested as drivers of tumour initiation and progression (Chen et al, 2009a; Hsieh et al, 2007; Neuveut et al, 2010), and HBV may influence some of these mechanisms.

The hepatitis B X (HBx) protein is considered a major potentiator of carcinogenesis; its multi-regulatory functions potentially leading to aberrant modulation of multiple transcription and signal transduction pathways (Arbuthnot et al, 2000; Benhenda et al, 2009; Ito et al, 2001; Klein & Schneider, 1997). In HCC models and clinical samples, HBx expression and transformative ability has been correlated to facilitation and acceleration of tumour development and HCC progression (Kim et al, 1991; Neuveut et al, 2010; Slagle et al, 1996). Furthermore, the HBx gene or truncated HBx fragments are among those commonly/frequently found integrated into host genomic DNA within HCC tissues (Liu et al, 2008; Unsal et al, 1994). It is possible that genotype-specific differences in the propensity for HCC development are partly attributable to inherently differential capability of HBx variants to mediate HBV replication and other functions (Ali et al, 2014; Elizalde et al, 2019; Liu et al, 2014c). Conclusions drawn from studies are often complicated by usage of overexpression systems where protein expression is artificially enhanced. Investigations conducted in more physiologically relevant systems in the setting of HBV replication are required to provide meaningful insight into elucidating the mechanisms underlying HBV-mediated HCC.

1.2 HBV “life” cycle

1.2.1 HBV replication

HBV infection commences with attachment of the HBV envelope proteins to heparin sulphate peptidoglycans (HSPG) on the hepatocyte membranes (Schulze et al, 2007; Verrier et al, 2016) (Figure 1.2). This bridges the virus into a specific engagement of the myristoylated preS1 region of its large surface protein (LHBs) with the *bona fide* receptor sodium taurocholate cotransporting polypeptide (NTCP), upon which viral entry is mediated by endocytosis (Yan et al, 2012). NTCP is a bile acid transporter expressed on the basolateral membrane of hepatocytes, partially explaining the liver tropism of the virus, though additional factors must be present as conferring expression of NTCP alone is not sufficient to enable infection of mouse hepatocytes post-entry (Li et al, 2014). To facilitate internalization, it was recently discovered that epidermal growth factor receptor (EGFR) is an additional co-factor required for uptake of the virus (Iwamoto et al, 2019).

Once inside the cell, uncoating of the viral envelope and dissociation of the nucleocapsid results in release of the partially double-stranded relaxed circular DNA (rcDNA) genome. This is translocated to the nucleus where it is subsequently converted into covalently closed circular DNA (cccDNA) (Kann et al, 1997; Kann et al, 2007; Kock & Schlicht, 1993; Rabe et al, 2003), which can persist within the nucleus as a minichromosome through association with histone and non-histone proteins (Newbold et al, 1995). The cccDNA, which is untargeted by currently-available therapies, forms the template for RNA transcription and viral genome synthesis thus, the minichromosomes is the viral reservoir largely responsible for viral persistence and reactivation during chronic and latent infections (Chen et al, 2004). Beyond the requirement of the presence of specific entry receptors, HBV replication is also restricted to hepatic cells by the requirement of transcription factors that are enriched in the liver. Nuclear hormone receptors including HNF4, RXR α , and PPAR α have been demonstrated to be essential for RNA transcription and viral replication of HBV (Raney et al, 1997; Tang & McLachlan, 2001). These factors bind to the HBV enhancers and stimulate synthesis of pregenomic RNA (pgRNA), which is the crucial RNA template required for viral replication (Doitsh & Shaul, 2004; Yang et al, 2020).

Viral transcripts derived from the cccDNA template are exported to the cytoplasm, where translation to viral proteins occurs (Huang & Liang, 1993; Huang & Yen, 1995). The major 3.5kb pregenomic RNA (pgRNA) transcript is encapsidated following recognition of its 5' epsilon sequence by the viral polymerase (Bartenschlager et al, 1990; Pollack & Ganem, 1993). Reverse transcription to minus sense, followed by plus sense DNA, producing the rcDNA genome, is mediated by the covalently-linked viral polymerase within the immature core particles (Summers & Mason, 1982). The naked viral core particles can then proceed via two pathways, the first of which is migration back to the nucleus and import of the rcDNA molecules to be converted to cccDNA, thereby adding to the nuclear cccDNA pool (intracellular amplification or recycling pathway) (Tuttleman et al, 1986a). Alternatively, they can proceed through the canonical viral egress pathway via multivesicular bodies (MVB), undergoing envelopment and ESCRT-mediated budding to ultimately be released from the cell as infectious progeny virions (Jiang et al, 2016; Patient et al, 2009; Prange, 2012). Subviral

particles consisting of HBV surface proteins and lipid and which lack the viral genome, are also produced and secreted in excess over the mature infectious particles, with presumable roles in immune evasion and distraction (Chai et al, 2008)(Figure 1.2).

Many of the details surrounding the specific processes involved in viral infection and replication have yet to be realised, which is due in large part, to the viral tropism and absence of appropriate investigative models and systems. As such, this is an important barrier to overcome in the search for a HBV cure.

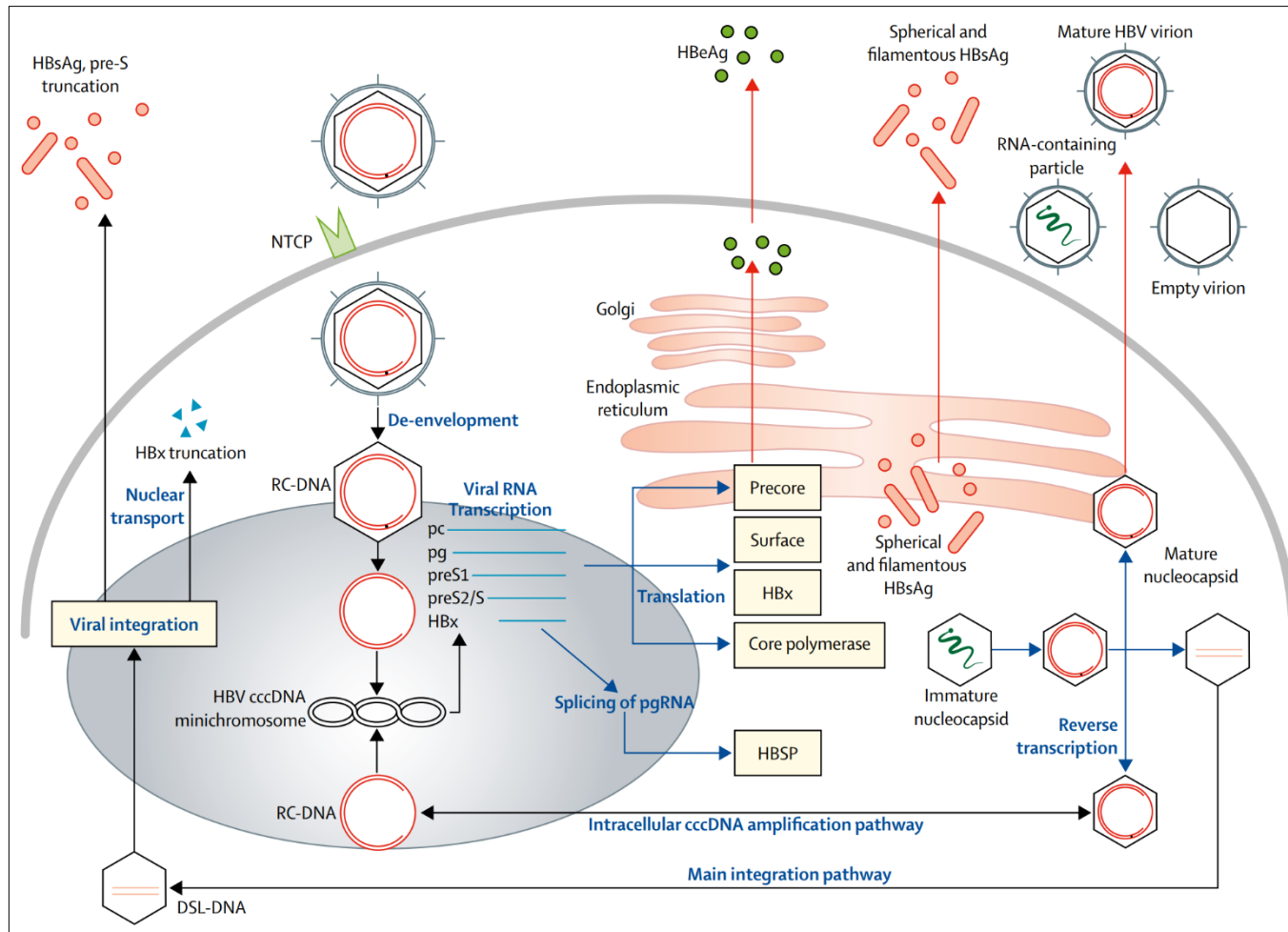


Figure 1.2: Hepatitis B virus replication cycle.

Schematic representation outlining the steps involved in HBV infection and replication; from entry via NTCP receptor, to release of mature progeny virions. NTCP; sodium taurocholate cotransporting polypeptide, HBSP; HBV splice protein, DSL; double-stranded linear, RC; relaxed circular, ccc; covalently closed circular. Adapted from (Revell et al, 2019a)

1.2.2 HBV genome

The 3.2kb HBV rcDNA genome consists of a minus strand covalently-linked to a terminal protein at the 5' end (Gerlich & Robinson, 1980), and a plus strand with a variable length 3' terminus (Figure 1.3)(Landers et al, 1977). The genome encodes four overlapping reading frames, such that all of the genome encodes either a gene or regulatory element, and in some cases both (Galibert et al, 1979; Valenzuela et al, 1980). Four promoters, core, X, preS1, and preS2, regulate production of five viral RNA transcripts: 3.5kb pregenomic (pgRNA) and precore RNA (pcRNA), 2.4kb preS1 mRNA, 2.1kb preS2 mRNA, and 0.7kb HBx mRNA.

Pregenomic RNA encodes the viral RNA-dependent DNA polymerase (Pol), and the core protein (Ou et al, 1990). Pol, a reverse transcriptase with RNase H activity facilitates synthesis of the rcDNA genome of progeny virions. This process occurs following encapsidation of the pgRNA by the capsid shell which consists of 120 assembled dimers of the core protein (Wynne et al, 1999; Zhou & Standring, 1992). The core proteins can also assemble in the absence of RNA to form empty particles (Alberti et al, 1978; Gerin et al, 1975; Kaplan et al, 1976). The COOH terminal domains (CTD) undergo phosphorylation modifications which regulates their activities (Kann & Gerlich, 1994; Lan et al, 1999; Liao & Ou, 1995)

The other 3.5 kb transcript, pcRNA, encodes for the intracellular 25 kDa precore protein (p25). Cleavage of the p25 N-terminal signal sequence produces a 22kDa intermediate cytosolic polypeptide (p22), which undergoes further post-translational modification at the C-terminal end to ultimately yield production of HBeAg (p17), a non-structural protein with sequence identity to the core protein (HBc/p21) but is secreted from the cell and often used as a surrogate marker of active viral replication (Bruss & Gerlich, 1988; Messageot et al, 2003; Ou et al, 1986; Ou et al, 1989). Additionally, HBeAg acts as a tolerogen due to its sequence overlap with the core protein, suppressing immune responses and likely facilitating persistent infection (Chen et al, 2005; Milich, 1999). Although translated from separate mRNA transcripts, HBe- and HBc antigens share a central core domain and differ at their termini (Ou et al, 1986; Standring et al, 1988). These differences preclude HBeAg, a non-essential product for viral replication, from being assembled into viral capsids and distinguishes it from the essential structural component HBc (DiMattia et al, 2013; Nassal & Rieger, 1993; Wasenauer et al, 1993).

The 2.4kb preS1 transcripts encode the large (L) surface antigen (LHBs), while alternative translation start sites dictate synthesis of middle (M) and small (S) surface antigens (MHBs, SHBs, respectively) from the preS2 (2.1 kb) transcripts. LHBs and MHBs proteins contain the SHBs-specific sequence, with additional N-terminal extensions. The three surface proteins form the envelope of 42nm infectious virions (Dane particles)(Bruss & Ganem, 1991; Dane et al, 1970; Ueda et al, 1991), empty 22nm subviral particles (SVPs), and longer filamentous particles (Almeida et al, 1969; Bayer et al, 1968). SHBs proteins contain the major serotype-defining immunodominant epitope (and S domain, with LHBs and MHBs-specific N-terminal extensions), and account for the majority of the viral particles, while the M form appears to be largely dispensable for virion formation(Bruss & Ganem, 1991; Stibbe & Gerlich, 1982). LHBs proteins are the second most abundant and are important for infectivity of the virus as their N-terminal region contains the domain responsible for recognition of HBV by the entry receptor (Le Seyec et al, 1999). Aside from mature genome-containing virions, surface proteins,

predominantly SHBs, also assemble into empty particles (Heermann et al, 1984). These SVPs are produced in excess over the infectious virions and are believed to act as decoys which mask the virus from immune-mediated clearance and therefore facilitate persistence. Interestingly, findings from Pfefferkorn et al (2020) suggest that assessing the different HBsAg forms may help predict the likelihood of HBsAg loss in response to treatment.

The final 0.7kb transcript encodes for the smallest viral protein, HBx, which represents a non-structural protein that is not directly involved in HBV replicative processes (Blum et al, 1992). HBx is a major focus of this thesis and its functions are described in depth in the following section.

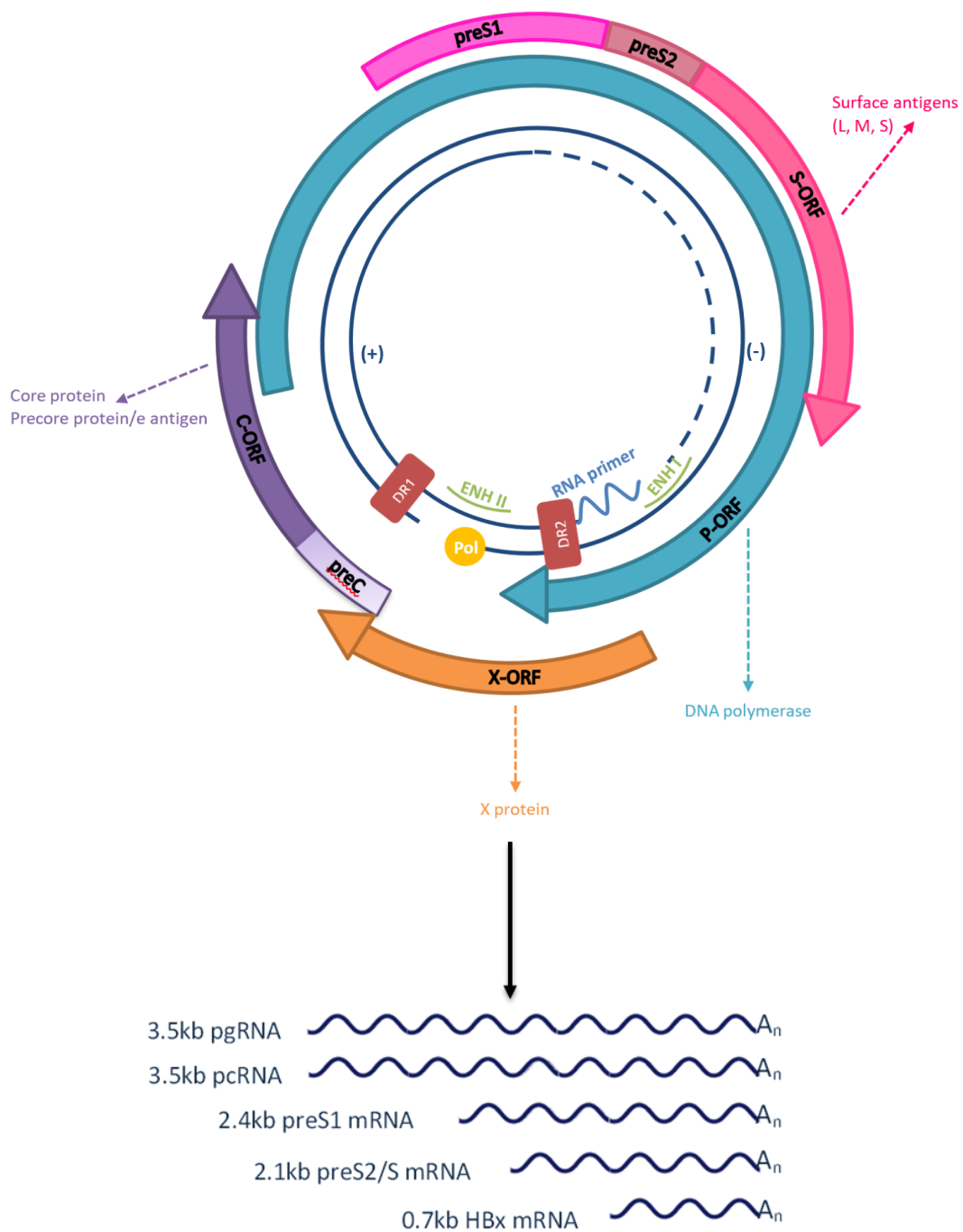


Figure 1.3: HBV genome organisation

Schematic representation of the HBV genome showing the 3.2kb negative (antisense, -) DNA strand and the variable-length positive (sense, +) strand, with the four major overlapping reading frames (ORFs). The 5' ends of the antisense and sense strands are linked to a polymerase and RNA primer, respectively. Translated products of the ORFs are denoted by dashed arrows. The relative positions of the basal core promoter (A1762T/G1764A) mutations are depicted in green.

Adapted from Human Virus Guides – Hepatitis B Virus, Lai, CL. & Locarnini, S. (2008)

1.3 Hepatitis B X protein

1.3.1 HBx gene and protein

The shortest of the four HBV open reading frames encodes for the smallest and most enigmatic HBV protein, HBx (Siddiqui et al, 1987). HBx does not have a direct role in the HBV replication cycle, but interacts with a number of host proteins and HBV cccDNA to impact on HBV replication in a multitude of ways. *In vitro*, HBx has been shown to have a short half-life and plays roles in various pathways including transactivation, cell survival/apoptosis and DNA repair (Becker et al, 1998; Bouchard & Schneider, 2004; Elmore et al, 1997; Jia et al, 1999; Rawat & Bouchard, 2015; Rawat et al, 2012). The multiple functions are thought to be conferred by its dual localization; in the nucleus, where it binds to transcription factors and recruits transcription machinery to confer its transactivation activity, and in the cytoplasm where it interacts with a variety of host proteins to activate signal transduction pathways (Doria et al, 1995). Though not strictly essential to the viral replication cycle, HBx is important for establishing efficient infection and transcription (Lucifora et al, 2011; Tang et al, 2005)

The HBx gene encodes for a translated protein of approximately 17kDa, consisting of 154 amino acids (Figure 1.4). Broadly, HBx can be separated into two major domains consisting of ~50 N-terminal residues, and the remaining C-terminal region (Tang et al, 2005). Two downstream in-frame translation start (AUG) signals exist, for expression of putative 8kDa and 6.6kDa proteins that lack the N-terminal domain but which retain some transactivating activity (Figure 1.4)(Kwee et al, 1992). Predicted to be largely unstructured, the N-terminal domain comprising the first third of the gene (i.e. roughly fifty amino acids) provides negative regulation of protein expression and activity (Murakami et al, 1994). Jiang & Tang (2010) found that this region confers deubiquitinating enzyme activity which ultimately inhibited interferon induction. This region was shown to be largely refractive to proteasomal degradation (Kim et al, 2008), which is in contrast to the remaining carboxy terminal two-thirds responsible for the majority of HBx's transactivation activity (Kumar et al, 1996; Tang et al, 2005). Transcription factors such as AP-1, NF- κ B, CREB have been identified to interact with this C-terminal region (Benn et al, 1996; Cross et al, 1993; Kumar et al, 1996; Seto et al, 1990; Su & Schneider, 1996; Williams & Andrisani, 1995). Interaction with such host factors facilitates transcriptional activation properties of HBx which is not known to directly bind double stranded DNA, and the transactivating properties in turn augment HBV replication (Tang et al, 2005). HBx upregulates expression and transcriptional activity of the transcription factor HIF-1 α (Yoo et al 2003) and the BCP (A1762T/G1764A) double mutant which confers changes in the overlapping HBx protein at K130/V131 further enhanced HIF-1 α expression and transcriptional activity (Liu et al, 2014a). In contrast, C-terminal truncated HBx exhibited weakened ability to promote nuclear localization of HIF-1 α , thus abrogating antiproliferative and transactivation effects (Ma et al 2008).

HBx appears to be a short-lived and unstable protein (Bergametti et al, 2002b; Korniyeyev et al, 2019), being subjected to proteasomal degradation which can be mediated by, or independent of ubiquitylation (Kim et al, 2008). Its stability is positively and negatively regulated by interactions with other proteins which facilitate its post translational modification with small molecules such as ubiquitin and NEDD8 (Hu et al, 1999; Kim et al, 2008; Liu et al, 2017a).

Intracellular localization of HBx is dependent on the level of expression, with a predominantly nuclear localization associated with lower HBx expression, whereas high expression results in a largely cytoplasmic distribution (Cha et al, 2009; Henkler et al, 2001). Furthermore, interaction with host proteins can also affect its subcellular localization (Korniyev et al, 2019; Nag et al, 2001; Saeed et al, 2019). At high levels, a portion of cytoplasmic HBx can also be identified in mitochondria (Takada et al, 1999), and HBx has been implicated to impair immune responses through interaction with mitochondrial antiviral signalling (MAVS) protein (Wei et al, 2010). HBx promoted degradation of MAVS, preventing downstream IFN- β induction and subsequent interferon-stimulated gene (ISG) production, providing a mechanism by which HBV is able to dampen or evade host antiviral immune responses. Mitochondrial HBx is also implicated in calcium homeostasis and modulation of signal transduction cascades (Bouchard et al, 2003; Bouchard et al, 2001b; Geng et al, 2012; McClain et al, 2007). Moreover the mitochondrial association itself can cause aggregation of the mitochondrial structures ultimately leading to cell death (Takada et al, 1999).

Although not essential for HBV infection or morphogenesis *in vitro*, HBx expression has been shown to augment and maintain HBV replication following establishment. For example, through interaction with Spindlin1 and Parvulin 14/17 to facilitate cccDNA transcription (Ducroux et al, 2014; Saeed et al, 2019), or regulating Akt signalling and interacting with anti-apoptotic proteins Bcl-2/Bcl-xL to influence viral replication via modulation of cell death (Geng et al, 2012; Rawat & Bouchard, 2015). HBx interaction with UV-damaged DNA-binding protein 1 (DDB1) was also demonstrated to affect HBV replication, and will be discussed further in more detail as this association is a major focus of this thesis. Changes in HBx sequence mediated by HBV genotypes or specific mutations can affect HBx function and potentially modulate viral replication. A brief summary of some of these discoveries is provided in Table 1.1.

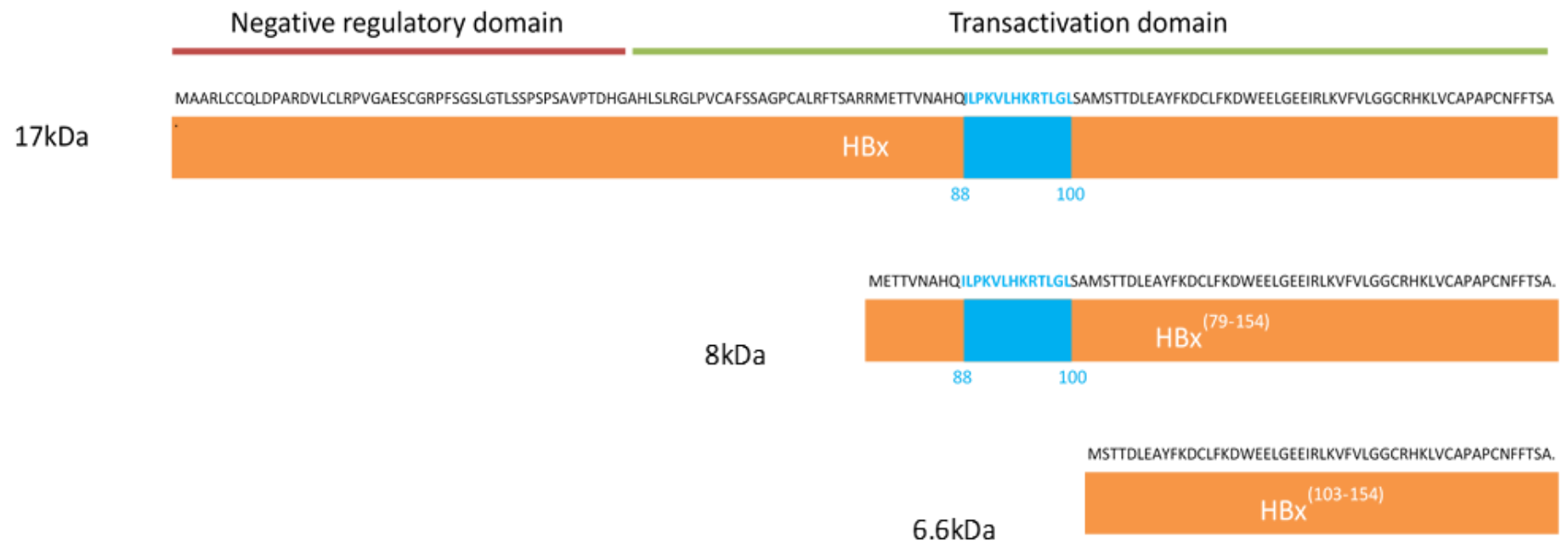


Figure 1.4: Translated and putative truncated products of the HBx gene

Schematic representation of the full length (17kDa) HBx polypeptide, as well as the putative truncated proteins (8kDa and 6.6kDa) resulting from translation initiation at the downstream AUG codons. Major domains are denoted above the full-length HBV by red and green lines. DDB1-binding motif (H-Box) is highlighted in blue.

Table 1.1 Putative HBx point mutations and their functional significance

Position	Function	Mutated residue	Effect	Reference
W120	Bcl-xL-binding α-helix BH3-like domain	A120	Impaired binding affinity to Bcl-xL	(Kusunoki et al, 2019)
W120, L123		A120, A123		(Zhang et al, 2019a)
			Reduced HBV replication	
G124, I127		L124, A127	Disrupted Bcl-xL, Bcl-2 interaction, impaired cytosolic calcium elevation, HBV DNA replication	(Geng et al, 2012)
L98	DDB1-binding	F98	Markedly decreased DDB1-binding activity	(Lin-Marq et al, 2001)
K95, R96		A95, A96	Strongly reduced DDB1-binding activity	
R96		E96	Abolished DDB1-binding activity	
			Unable to rescue HBx-null replication	
R96		A96	Abolished DDB1-binding Able to rescue HBx-null replication	(Kim et al, 2016)
L98		A98		
G99		A99		
C61		Zinc-binding motif	A61	Unable to rescue HBx-null replication Does not induce SMC6 degradation
C69	A69			
C137	A137			
H139	H139			
S31	Akt1 phosphorylation motif	A31	Increased oncogenic potential of HBx	(Khattar et al, 2012)
		L31	Inhibition of Akt activation and HBx-mediated apoptosis	(Lee et al, 2012)
S41, P42	Pin1 binding (phosphorylated S41) HBx phosphorylation motif SPSP(39-42)	A41/A42	(Interaction) Enhanced transactivation activity of HBx	(Pang et al, 2007)
C7	DNA repair/DDB1-binding	S7	Did not affect DDB1-binding	(Becker et al, 1998)
			Inhibited DNA repair	
C61		L61	Abolished DDB1-binding	
C69			Abolished DDB1-binding Did not alter DNA repair	
P90, K91		V90, L91		

Table 1.1 (Cont.) HBx point mutations and their effects on HBx function

Position	Function	Mutated residue	Effect	Reference
K118, D119 (gt A/C/D)	MAVS interaction, impairs aggregation of MAVS	N118, E119 (gt B)	Potent inhibition of RIG-I-MAVS-mediated IFN induction	(Wang et al, 2020)
A10, S144	p53 binding	R10, R144	Impaired binding to p53, upregulated p21 expression, increased cells at G1 phase, impaired inhibition of apoptosis (decreased Bax expression?)	(Shi et al, 2016)
R19	Par14/17 binding	A19	Failed to rescue HBx-null replication	(Saeed et al, 2019)
P20		A20		
R28		A28		
R29		A29		
R19, P20		A19, A20	Impaired Par14/17 interaction, failed to rescue HBx-null replication	
R28, R29		A28, A29		
R19, P20, R28, R29		A19, A20, A28, A29	Abolished interaction, failed to rescue HBx-null replication	
C61	Transactivation activity	T61	Drastic loss of transactivation function, partially overcome by transfecting more	(Kumar et al, 1996)
C69		T69		
C137		T137	Drastic loss of transactivation function	
F116 (??)	Enh II activity?	C116 (??)	Inhibited EnhII transcription through impaired HNF3β-binding, leading to reduced viral replication in vitro,	(Jiao et al, 2019)
L98, L100	Nuclear export signal	A98, A100	Redistributes cytoplasmic HBx to predominantly localise to nucleus	(Forgues et al, 2001)

1.3.2 HBx – host protein interactions

1.3.2.1 HBx and DDB1 interaction

Interaction between the viral HBx and host endogenous DDB1 was first recognised in the 1990s (Lee et al, 1995). HBx binds directly to DDB1, and numerous investigators have used mutagenesis studies to attempt to identify the critical DDB1-interacting region on HBx (Becker et al, 1998; Bergametti et al, 2002a; Lin-Marq et al, 2001; Sitterlin et al, 1997) the results of which are summarised in Figure 1.5. It should be however noted that many of these were a result of *in vitro* yeast-2-hybrid/transcription-based assays and do not necessarily reflect protein binding in mammalian cells, which would be complicated by additional factors. X-ray crystallography showed that HBx occupied a clam-like pocket of the larger DDB1 protein, forming multiple contacts with one of its three β -propeller units (Li et al, 2010a). Studies by various groups have demonstrated effects of the HBx-DDB1 interaction on cellular transactivation, apoptosis/cell survival (Bontron et al, 2002; Lin-Marq et al, 2001), DNA repair (Becker et al, 1998; Jia et al, 1999) and even viral replication (Leupin et al, 2005; Sitterlin et al, 2000a), though it can be noted that many of the early studies were conducted using the equivalent protein of the related woodchuck hepatitis virus (WHV) (Sitterlin et al, 2000a; Sitterlin et al, 2000b). Knockdown of DDB1 further enhanced inhibition of replication by HBx siRNA, in an *in vitro* model (Tang et al, 2008). This dependency on DDB1 for HBx-mediated enhancement of replication supported earlier work, in which a DDB1-binding-impaired HBx mutant was unable to rescue replication of HBx-deficient HBV (Leupin et al, 2005). DDB1 is part of a heterodimeric complex involved in repair of UV-damaged DNA. Together with the smaller subunit DDB2, the (UV-)DDB complex repairs DNA lesions by the nucleotide excision repair (NER) pathway. DDB2 was demonstrated to be critical for nuclear translocation of DDB1, and this association appeared to be mutually exclusive of DDB1-HBx binding, suggesting that both DDB2 and HBx appeared to bind to the same region of DDB1. It was also demonstrated that DDB2 could also bind HBx independently of DDB1, resulting in nuclear accumulation of HBx (Nag et al, 2001).

Beyond its role in DNA repair, another main function of DDB1 involves association with E3 ligase complexes which facilitate degradation of protein substrates. DDB1 binds to Cullin4 (CUL4) protein of the CRL4 E3 ligase complex via one of its three β -propeller units (Angers et al, 2006), while simultaneously accommodating binding of substrate receptors referred to as DDB1-CUL4-associated factors (DCAFs) within a pocket formed by the remaining β -propeller units (He et al, 2006; Lee & Zhou, 2007). In this way, DDB1 acts as an adaptor protein, bridging targeted protein substrates into proximity with the E3 ligase complex to facilitate their ubiquitylation and ultimate proteasomal degradation (Angers et al, 2006; Hu et al, 2004b). It was proposed that HBx could act as a viral DCAF, utilising its interaction with DDB1 to subvert the activity of the E3 ligase complex to its own advantage and targeting an unknown restriction factor. This was later confirmed to be the case, with structural maintenance of chromosomes 5/6 (Smc5/6) complex being identified as the targeted restriction factor (Decorsière et al, 2016; Murphy et al, 2016).

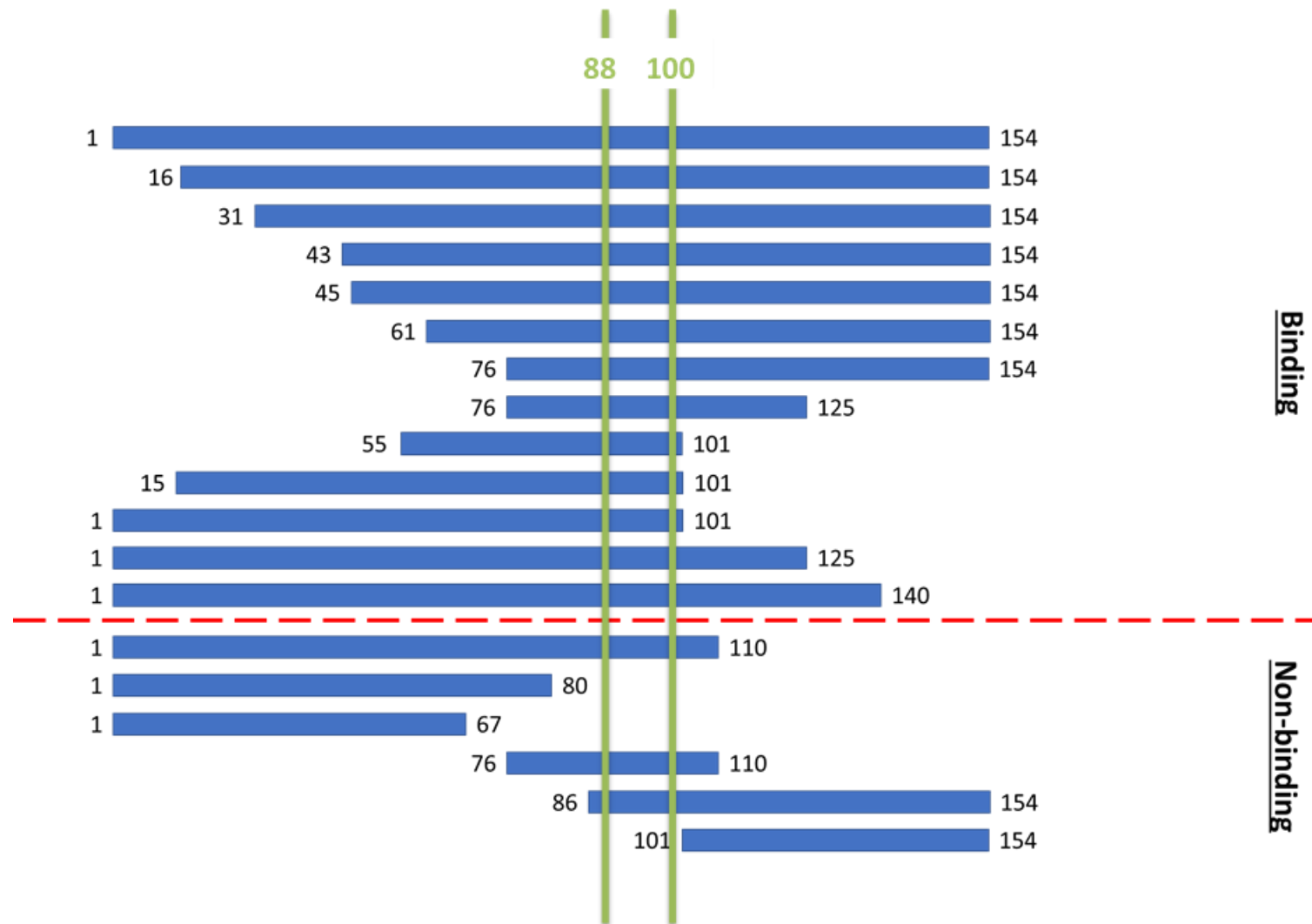


Figure 1.5: Summary of proposed minimal DDB1-binding region of HBx

Schematic of truncated-HBx ability to bind to DDB1, as summarised from mutational analyses by Becker et al (1998); Hodgson et al (2012); Lin-Marq et al (2001); Sitterlin et al (1997); Wentz et al (2000)

Vertical green lines mark the HBx fragment bound to DDB1 (so-called “H-Box”) in co-crystallization analysis by Li et al (2011).

1.3.2.2 HBx-DDB1 facilitation of viral replication

Structural maintenance of chromosomes (SMC) complexes play essential roles in chromatin maintenance and DNA replication and repair. The complexes consist of a heterodimer of SMC proteins with other non-structural proteins, and the SMC5/6 complex is the least well-characterised of the three complexes. In 2016 a link to HBV replication was described for this complex (Decorsière et al, 2016; Murphy et al, 2016). It was demonstrated that in the absence of HBx, the Smc5/6 complex associated with cccDNA, tethering it to ND10 bodies to restrict cccDNA transcription (Niu et al, 2017), and that this restriction also involved interaction of Praja1 (PJA1) (Xu et al, 2018). During HBV infection, the SMC5/6 is targeted for degradation by the DDB1-CRL4 E3 ligase complex (Decorsière et al, 2016; Murphy et al, 2016). This is mediated by its interaction with HBx, which in turn is bound to DDB1, facilitating ubiquitylation of SMC5/6 (Figure 1.6). Thus, this presents a viral mechanism of redirecting cellular machinery and processes to overcome a host restriction factor. These findings raised interesting questions regarding whether the HBx protein or mRNA is part of the virion, as implicated by the suggested requirement of HBx protein expression for the initiation of transcription and facilitation of HBV replication following viral entry. Although this has yet to be proven experimentally, Niu et al (2017) have used RNAseq to demonstrate detection of small HBx-like transcripts present in virus preparations from cell culture and patient plasma, suggesting these transcripts may be packaged into the virion. These could be translated soon after viral entry, and facilitate initiation of infection, wherein more mRNA could be transcribed from the cccDNA, and more HBx proteins produced to propagate the effects. In an earlier study, Doitsh & Shaul (2004) demonstrated that HBV transcripts could be categorised into early and late groups. They showed that EnhI-regulated HBx mRNA was detected earliest around 16 hours following transfection and largely undetectable 5 days later. The remaining EnhII-regulated HBV transcripts were expressed later and could be stimulated by ectopic expression of liver-enriched nuclear receptors. Activation of EnhII required functional EnhI, and postulated expressed HBx protein may act as an early-late switch, repressing EnhI activity at high levels to permit EnhII activation.

The HBx and Smc5/6 interaction indicates that the precursory HBx-DDB1 interaction is crucial for HBV replication. Although differences exist between HBx sequences of different HBV genotypes (Figure 1.7), even within the previously characterised DDB1-binding region (Figure 1.8), it is unclear whether these differences may affect HBx interaction with DDB1 and the ability to relieve transcriptional repression imposed by Smc5/6, therefore contributing to the observed differences in replicative efficiency for different HBV genotypes (Bannister et al, 2019; Bannister et al, 2018; Sozzi et al, 2018; Sozzi et al, 2016).

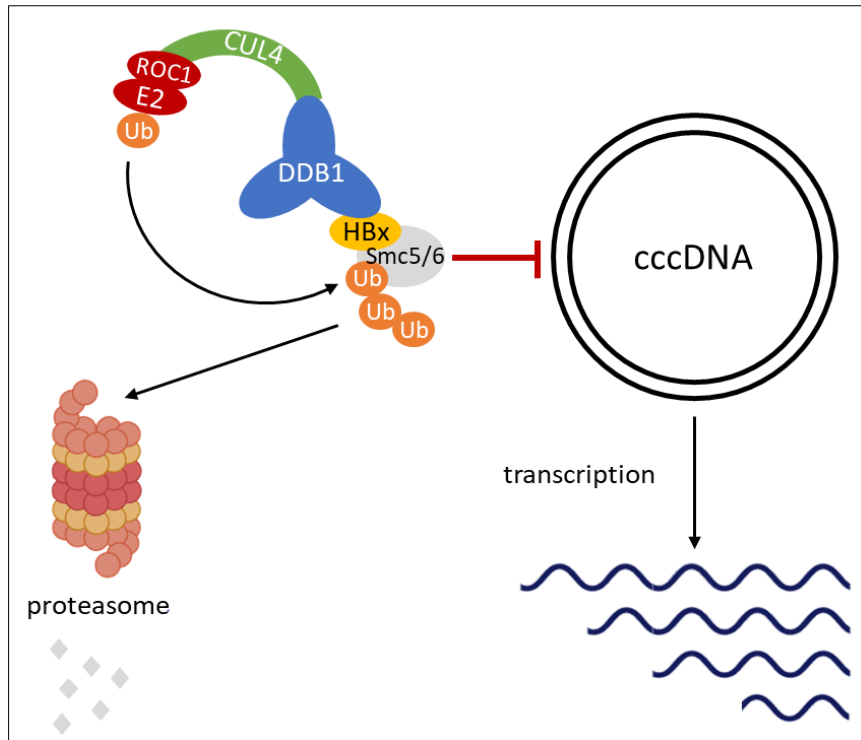


Figure 1.6: HBx- and DDB1-mediated relief of viral transcriptional repression
 Structural maintenance of chromosomes 5/6 (Smc5/6) complex inhibits transcription of HBV cccDNA in the absence of HBx expression. Interaction between HBx and DDB1 facilitates CUL4-E3 ligase complex-mediated ubiquitylation and degradation of Smc5/6 to enable transcription.
 Adapted from Kim et al. (2016).

Majority	M	A	A	R	L	C	C	Q	L	D	P	A	R	D	V	L	C	L	R	P	V	G	A	E	S	X	G	R	P	F	
Position										10										20											30
A1						Y					S	S																		F	
A2																										R			L		
C2					V																									V	
D3	*M	A	A	R			C	Q	L	D	P	A	R	D	V	L	C	L	R	P	V	G	A	E	S		G	R	P		
BCP						C						A														C				F	
R96E																															
Majority	S	G	X	L	G	T	L	S	S	P	S	P	S	A	V	P	T	D	H	G	A	H	L	S	L	R	G	L	P	V	
Position										40										50											60
A1	A	R					V	S									S														
A2			P	L					S				P																		
C2				F	G	T		P	S	P	S	S		A	V	P	A	D	H	G	A	H	L	S	L	R	G	L	P	V	
D3		F					L																								
BCP	S			S	L			S				P					T														
R96E																															
Majority	C	A	F	S	S	A	G	P	C	A	L	R	F	T	S	A	R	R	M	E	T	T	V	N	A	H	Q	I	L	P	
Position										70										80											90
A1																		C										I			
A2																															
C2																											V				
D3	C	A	F	S	S	A	G	P	C	A	L	R	F	T	S	A	R		*M	E	T	T	V	N	A	H	Q		L	P	
BCP																		R										I			
R96E																															

Figure 1.7: HBx sequence alignment of selected HBV genotypes/variants.

Alignment of sequences for wild-type HBx (genotype A1, A2, C2, D3) and two mutant HBx (BCP130M/131I, R96E) in D3 background, with key sites highlighted. Sequences based on replication-competent greater-than-genome length HBV constructs.

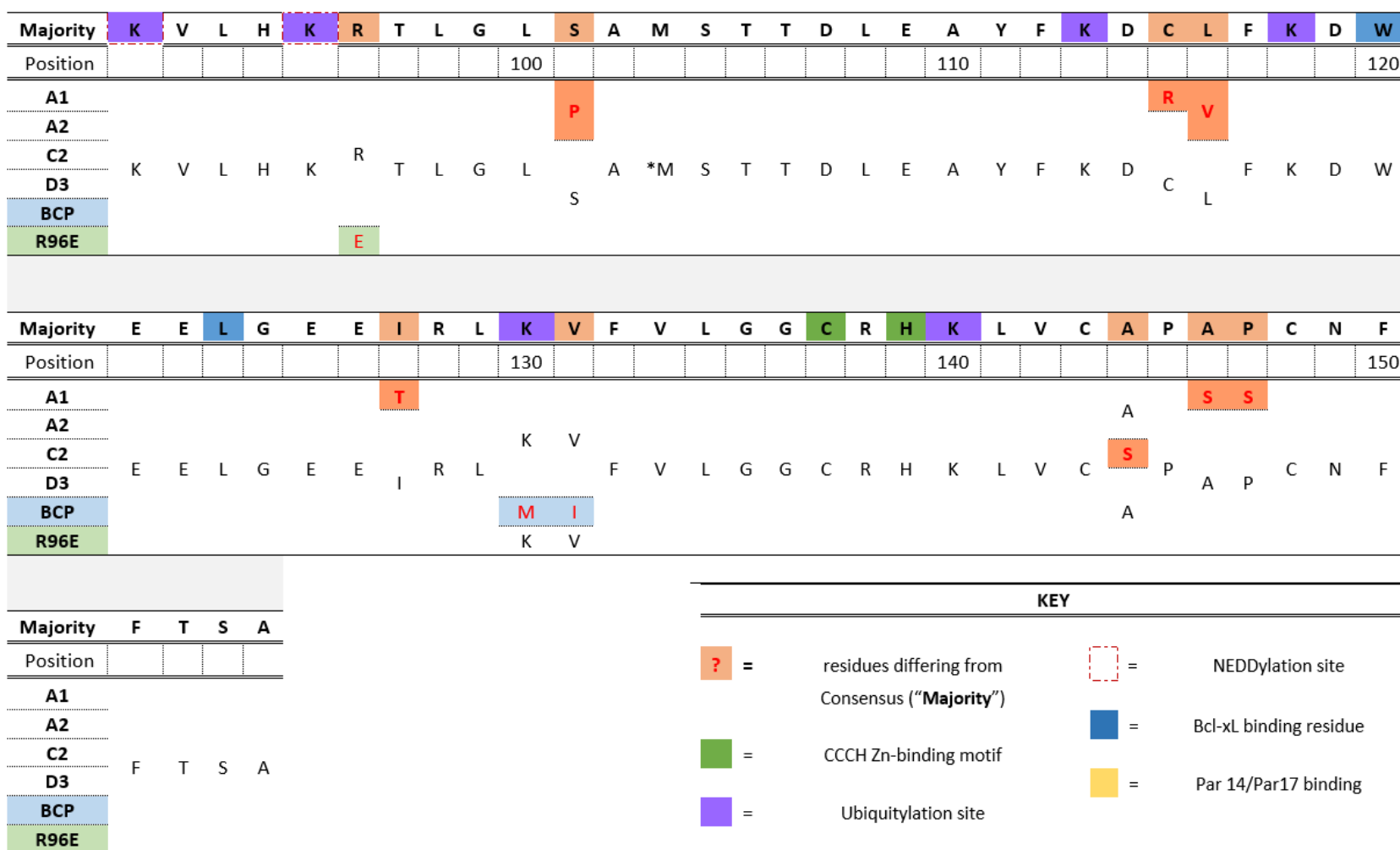


Figure 1.7 (continued): HBx sequence alignment.

Alignment of sequences for wild-type HBx (genotype A1, A2, C2, D3) and two mutant HBx (BCP130M/131I, R96E) in D3 background, with key sites highlighted. Sequences based on replication-competent greater-than-genome length HBV constructs.

	90										100					
	.	.		.	.	.	.		.	.	.	.		.	.	
A2	I	L	P	K	V	L	H	K	R	T	L	G	L	P	A	
B2	N	.	.	.	.	.	.	.	.	.	.	.	.	S	.	
C2	V	.	.	.	.	.	.	.	.	.	.	.	.	S	.	
D3	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	
J	N	.	.	R	.	.	.	.	.	.	.	.	.	S	I	

Figure 1.8: Alignment of a major DDB1-binding region of HBx, across select genotypes.
Alignment of HBx sequences (genotypes A2, B2, C2, D3, J) between residues 88 and 102, encompasses the DDB1-binding “H-box” motif.

In addition to its role in HBx-induced enhancement of HBV replication, DDB1 was recently demonstrated to stimulate HBV replication in an HBx-independent manner (Kim et al, 2016). In contrast to previous findings (Leupin et al, 2005) an HBx-DDB1-binding-impaired mutant restored replication of HBx-deficient HBV, to almost the same extent as the wild-type HBx. The authors proposed that DDB1 could directly stimulate cccDNA transcription through association with the HBV cccDNA minichromosome (Kim et al, 2016). Notable differences between the two studies showing HBx-dependent or HBx-independent, DDB1-mediated enhancement of HBV replication, include the use of HBV constructs harbouring varying copy numbers of the HBx gene, and different specific DDB1-binding-impaired HBx mutants (Kim et al, 2010; Leupin et al, 2005). The different experimental designs may have facilitated the different outcomes.

1.4 cccDNA

During HBV infection, formation of the cccDNA minichromosome is required for the subsequent generation of pregenomic RNA, the major replicative intermediate. Conversion of rcDNA to cccDNA is largely a host-dependent process requiring several steps, and is postulated to be recognised and mediated by multiple cellular DNA repair pathways (Guo et al, 2007a; Schreiner & Nassal, 2017; Sohn et al, 2009). Several of the host factors contributing to these processes have been identified, though due to lack of appropriate models their requirement *in vivo* has been contested. Firstly, the polymerase covalently linked to the 5' end of the minus strand can be removed by tyrosyl DNA phosphodiesterase-2 (TDP2) *in vitro* (Cui et al, 2015; Koniger et al, 2014), though the requirement of TDP2 remains somewhat controversial in HBV-replicating cells since knockdown of TDP2 reportedly enhanced transcription and replication in an inducible stable cell line (Guo & Guo, 2015). Synthesis of the plus strand gap was demonstrated to involve polymerase κ (POLK) as one key factor and potentially additional polymerases as well, such as POLH and POLL (Qi et al, 2016), and Pol α for the intracellular amplification pathway of cccDNA (Tang et al, 2019). Other proteins predicted to be involved in rcDNA repair/cccDNA formation include endonucleases, topoisomerases, ligases (Ji & Hu, 2017; Kitamura et al, 2018; Long et al, 2017; Schreiner & Nassal, 2017; Sheraz et al, 2019). cccDNA formation is also influenced by other viral factors, such as expression of large HBsAg. Summers and colleagues showed that in the presence of an excess of HBV large surface proteins (LHBs), nucleocapsid particles are preferentially enveloped and secreted, whereas nuclear import and cccDNA amplification is favoured when LHBs expression is restricted (Summers et al, 1990).

cccDNA is further arranged into chromatin-like structures by interactions with the viral core and HBx proteins, and histone proteins (Bock et al, 2001; Newbold et al, 1995). Transcription of the cccDNA minichromosome is subject to epigenetic regulation by modifications such as histone acetylation and DNA methylation. Recruitment of histone acetyltransferases (HATs) CBP/p300 and PCAF and hyperacetylation of cccDNA-bound histone H3 and H4 histones was shown to correlate to viral replication (Belloni et al, 2009; Pollicino et al, 2006) by promoting a more permissive chromatin state. Conversely, recruitment of histone deacetylases HDAC1 and Sirt1, and histone hypoacetylation, correlated with transcriptional silencing. DNA methylation suppresses cccDNA transcription (Kim et al, 2011b; Vivekanandan et al, 2009; Zhang et al, 2014) as this process generally interferes with recognition and binding of transcription factors at promoter regions, either through direct interaction or through attachment of methyl binding proteins (MBPs). MBPs in turn recruit histone-modifying/chromatin remodelling complexes such as HDACs which lead to chromatin condensation and transcription repression, and histone methyltransferases (HMTs) which can activate or repress transcription depending on the target sites (Koumbi & Karayiannis, 2015; Levrero et al, 2009).

The HBx protein regulates HBV replication by recruiting chromatin-modifying factors to the cccDNA, changing the chromatin architecture and epigenetically regulating cccDNA transcription (Belloni et al, 2009). Its presence correlated with recruitment of factors to favour a transcriptionally active chromatin state, while also counteracting /reducing recruitment of factors that transcriptionally repress the cccDNA (Figure 1.9), such as Spindlin1 (Ducroux et al, 2014), SETDB1 (Riviere et al, 2015) and PRMT1 (Benhenda et al, 2013).

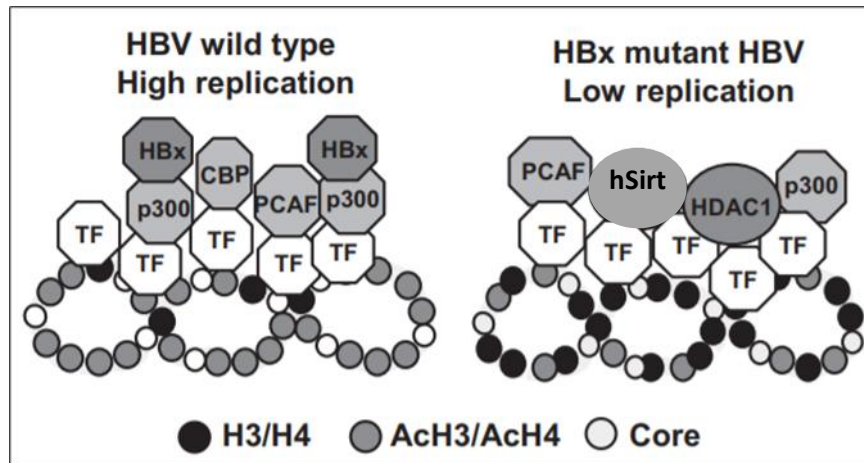


Figure 1.9: HBV minichromosome structural organisation.

Schematic representation cccDNA-bound histones acetylation status (H3/H4; AcH3/AcH4) and the recruitment of chromatin modifying enzymes onto the viral minichromosome in relation to viral replication and HBx status. In cells expressing mutant HBV and replicating at low levels (right panel), cccDNA-bound histones are hypoacetylated, the recruitment of acetyltransferases (PCAF, p300) is severely impaired, whereas the recruitment of the histone deacetylases (hSirt and HDAC1 is increased). Adapted from Belloni et al. (2009). Core, HBV core protein; TF, transcription factor).

The cccDNA minichromosome is generally believed to be stable within the nucleus, with reported copy number varying among hepatocytes and the overall disease/infection stage (Hantz et al, 2009; Spangenberg et al, 2004; Zhang et al, 2003). Studies using other hepadnaviruses indicated copy numbers ranging anywhere from 1 up to 50 (Kajino et al, 1994; Li et al, 2018b; Moraleda et al, 1997; Zhang et al, 2003) but analyses in chronic hepatitis B patients indicated more modest estimates, with decreased levels following HBeAg seroconversion (Laras et al, 2006; Wursthorn et al, 2006). Lacking an origin of replication, cccDNA maintenance is considered to be linked to the longevity of slowly dividing hepatocytes. No evidence of chromosomal tethering has been demonstrated and indeed, studies have observed a diluting effect correlating with (partitioning during) cell division as cccDNA is unequally distributed into daughter cells (Allweiss et al, 2018; Li et al, 2018b).

Although not actively replicated, cccDNA minichromosomes are transcriptionally active, facilitating synthesis of pgRNA which can be encapsidated and reverse transcribed and potentially replenish/amplifying the cccDNA pool by nuclear recycling of rcDNA-containing nucleocapsids. Studies by Lenhoff, Summers and colleagues using the related duck hepatitis B virus (DHBV) uncovered a regulatory function of large HBsAg on intracellular nucleocapsid transport; during early infection, HBsAg expression is low and nuclear import of rcDNA is favoured to amplify the established cccDNA pool. As cccDNA levels increases, transcription and

subsequent expression of HBsAg also increases, directing nucleocapsids towards the maturation pathway to secrete virions (Lenhoff & Summers, 1994; Summers et al, 1990). This regulatory effect was modestly observed in human hepatoma cells (Gao & Hu, 2007; Guo et al, 2007b; Lentz & Loeb, 2011) indicating other factors are involved in its regulation. Viral propagation enables spreading of infection to naïve hepatocytes and establishment of new cccDNA molecules, highlighting the necessity of silencing or eliminating this viral episome in order to achieve complete cure of the infection.

1.5 HBV therapeutic interventions

1.5.1 Prophylactic vaccines

An effective prophylactic vaccine against HBV has been available since 1982 and childhood vaccination programs have been successfully implemented with impressive results (Chang et al, 1997; Chen et al, 1996; Fortuin et al, 1993). Vaccination of babies within one week of birth prevented perinatal transmission from HBeAg-positive mothers in two-thirds of cases, and transmissions were further reduced with additional administration of hepatitis B immunoglobulin HBIG in the case of active replication (Beasley et al, 1983a). In a Taiwanese cohort, 20 years following its introduction, vaccination was estimated to have reduced HBsAg prevalence by up to 87%, childhood mortality from fulminant hepatitis by 68%, and HCC in children ages 6-9 by 75% (Chien et al, 2006; Ni et al, 2007). However, preventative vaccination is of no benefit for the 257 million people already chronically infected. Accordingly, other therapeutic strategies are required to target the virus, with functional cure as the goal, as loss of HBsAg is associated with sustained virologic responses and substantially reduced risk of cirrhosis and HCC development (Beasley et al, 1981).

1.5.2 Direct-acting antivirals

Complete HBV elimination requires that the nuclear cccDNA reservoir be cleared or at least permanently inhibited or silenced. Mechanistically and logistically, this is not achievable at present so most strategies instead aim for a functional cure as an endpoint, defined by persistently undetectable serum HBV DNA, and loss of HBsAg with or without the production of anti-HBs antibodies (Revill et al, 2019a). Decades of research has led to the development of therapeutic strategies that target nearly every stage of the HBV replication cycle (Figure 1.10). Some of these are approved therapies, while others are undergoing clinical evaluation, and yet others are still in preclinical or early developmental stages with promising potential. The following is a brief discussion of available therapies, and new approaches under investigation.

- Reverse transcription inhibitors

Nucleos(t)ide analogues (NAs) such as Tenofovir (pro-drugs tenofovir disoproxil fumarate, TDF, and tenofovir alafenamide, TAF) and Entecavir (ETV) are the current standard of care for treatment of chronic hepatitis B (Buti et al, 2016; Honkoop & De Man, 2003; Murakami et al, 2015). These molecules target the viral polymerase at the reverse transcription stage of viral replication, inhibiting DNA extension and inducing chain termination upon their incorporation during genome replication (Clark & Hu, 2015). Inhibition can also be non-competitive and without incorporation into the viral DNA, as was demonstrated for Clevudine (Lu et al, 2015). NA therapy successfully controls viral replication in nearly all patients, thereby lowering the risk of transmission as well as development of sequelae (Heathcote et al, 2011; Marcellin et al, 2013). Side effects are minimal though with potential risk of long-term toxicity, resistance is currently low particularly with the newer generation drugs, and treatment can even improve liver condition (fibrosis regression)(Marcellin et al, 2013; Murakami et al, 2015; Zoulim & Locarnini, 2009). However, as these therapies only suppress viral replication with no impact on the cccDNA reservoir, treatment is life-long as viral rebound often occurs upon cessation (Fung et al, 2009; Honer Zu Siederdissen et al, 2016; Wong et al, 2013) and furthermore, they reduce

but do not eliminate the risk of developing HCC. The lack of durable response combined with low rate of functional cure (i.e. HBsAg loss) means NAs are not curative and cannot be considered a definitive viral elimination method, and development of alternative therapeutics is required.

- Entry inhibitors

The discovery of the *bona fide* HBV receptor (NTCP) on the hepatocyte membrane was in part validated based on the ability of entry inhibitor peptide Myrcludex B to effectively block both HBV and HDV infection (Gripon et al, 2005; Li & Urban, 2016; Yan et al, 2012). Myrcludex B mimics the preS1 region of the large HBs protein which engages with the NTCP receptor to mediate viral entry (Lutgehetmann et al, 2012; Volz et al, 2013). The strategy is based on competitive inhibition or blocking of the virus-receptor and has been recently approved for use in patients co-infected with hepatitis D virus (HDV) (Blank et al, 2016), the satellite virus which requires the HBV surface proteins for its propagation (Rizzetto, 1989). Entry inhibition with Myrcludex B alone is not useful as a curative approach for chronic hepatitis B, in which cells are already infected, but may be beneficial in combination with other therapies, by protecting naïve hepatocytes from infection in the setting of suppressed replication (Volz et al, 2013).

- Capsid assembly modulators (CAMs)/Core protein allosteric modulators (CpAM)

The HBV core protein is required for formation of the viral capsid which encapsidates pgRNA. Formation of these nucleocapsid particles is a prerequisite for rcDNA synthesis and virion genesis, and knowing the structure of this particle enables rational drug design to develop and improve molecules that can interfere with this process (Nijampatnam & Liotta, 2019; Wynne et al, 1999). Several classes of allosteric modulators have been developed which inhibit HBV replication (Campagna et al, 2013; King et al, 1998; Qiu et al, 2017; Stray et al, 2005; Wu et al, 2017). These small molecule derivatives act by aggregating and degrading core proteins or facilitating the formation of empty particles, thereby disrupting pgRNA encapsidation (Campagna et al, 2013; Deres et al, 2003; Stray et al, 2005; Wu et al, 2017). Current evidence suggests core inhibitors can also impact cccDNA establishment, presumably by inducing disassembly and consequently suppression of re-imported rcDNA-containing nucleocapsids (Guo et al, 2017; Lahlali et al, 2018). Capsid assembly modulators (CpAMs) are generating great interest within the field, with data suggesting good tolerability and viral suppression, though their effectiveness in treating chronically-infected patients is undetermined.

- Surface inhibitors

Reducing viral antigenic burden may be required for reconstitution of HBV-specific immune responses ultimately necessary for viral clearance. Nucleic acid polymers are a class of oligonucleotide-based compounds gaining attention in recent years as a new therapeutic approach (Guillot et al, 2017; Noordeen et al, 2013). These NAPs reduce secretion of the hepatitis B surface antigen to durably reduce or clear HBsAg from the serum and subsequently achieve seroconversion (Vaillant et al, 2018), possibly by targeting a host factor involved in the assembly/secretion of these proteins (Boulon et al, 2020). Combining these with another therapy that controls viral replication such as PEG-IFN or NA demonstrated suppression of HDV RNA and HBsAg in co-infected patients (Bazinet et al, 2020; Vaillant, 2016). Monoclonal antibodies against the HBV surface proteins have also been evaluated for treatment of CHB

(Eren et al, 2000; Galun et al, 2002; Tsuge et al, 2016; van Nunen et al, 2001). Collectively the limited data suggests that these antibodies exhibit some neutralising activity and could reduce serum HBsAg and HBV DNA, though responses were not sustained upon treatment cessation. However, there may be merit in their combined usage with other antiviral therapies, since targeting the HBV envelope protein and reducing the burden of circulating antigen may ultimately be beneficial to the clearance of infection.

- Gene silencing

Small interfering RNAs (siRNA) are an alternative approach being investigated for reducing viral antigen load and overcoming immune exhaustion in CHB (Gish et al, 2015). RNA interference involves use of short oligonucleotides (siRNA) to ultimately trigger degradation of a target mRNA of complementary sequence (Carthew & Sontheimer, 2009; Wilson & Doudna, 2013). Gene silencing by siRNA is a promising strategy to target HBV since its small genome and overlapping reading frames presents an ideal context to potentially simultaneously target multiple viral genes with a single RNA trigger. This in turn enables targeting multiple steps of the viral replication cycle. siRNAs for HBV treatment are currently under evaluation with promising results, reporting reductions in HBsAg as well as other clinical markers (Lee et al, 2019; Schluep et al, 2017; Wooddell et al, 2013; Yuen et al, 2019). An unexpected benefit of this treatment strategy in addition to reduced viral load, was induction of HBsAg loss (Lee et al, 2018; Sepp-Lorenzino et al, 2015; Wooddell et al, 2017; Yuen et al, 2018b; Yuen et al, 2019). A major challenge associated with RNAi technology is the efficiency of delivery to target sites, and can be improved by encapsulation or conjugation of the RNAi trigger to other molecules in order to enhance endocytic uptake (Li & Szoka, 2007; Nair et al, 2014). Another important consideration in design of the molecules is the target sequence, which should ideally also target cccDNA and integrated HBV sequences (Wooddell et al, 2017).

- Gene editing

Gene editing strategies allow inactivation of genes via exploitation of bacterial editing systems such as Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9 RNA-guided nucleases, which have proven efficacy in treatment of patients with the likes of HIV and haemophilia (Sharma et al, 2015; Tebas et al, 2014). Briefly, these systems use guide RNAs to target complementary DNA sequences for degradation by specific nucleases, followed by induction of DNA repair that often introduces changes to the underlying open reading frame (Mali et al, 2013; Ran et al, 2013). Several pre-clinical studies have investigated the use of TALEN or CRISPR/Cas9-mediated degradation of HBV cccDNA, in various experimental systems and with differing target regions of the HBV genome (Bloom et al, 2018; Dreyer et al, 2016; Karimova et al, 2015; Kennedy et al, 2015a; Lin et al, 2014). Gene editing suppressed HBV replication with reductions in total DNA, cccDNA and antigens (Bloom et al, 2013; Bloom et al, 2018; Kennedy et al, 2015b; Moyo et al, 2018), however it is unknown what the long-term effects of such therapy in the clinical setting are, specifically regarding the defective cccDNA formed by non-homologous end-joining (NHEJ) following nuclease cleavage. Furthermore, the ability of the machinery to accommodate for a few mismatches and cleave DNA of imperfect complementarity introduces risk of potentially devastating off-target effects. Finally, efficient and specific delivery methods to the infected hepatocyte will be required in order to target all copies of the cccDNA reservoir and achieve HBV cure (Ely et al, 2020).

- Epigenetic modulators

Transcriptional activity of the HBV cccDNA minichromosome is regulated by modifications of the DNA-bound histone tails, similar to host chromosomes (Koumbi & Karayiannis, 2015). This presents opportunities to regulate viral expression using compounds that modulate the enzymes which regulate transcriptional activity of the viral minichromosome (Hong et al, 2017). The use of histone deacetylase inhibitors (HDACi) to functionally inhibit the removal acetyl groups from histone tails has demonstrated efficacy for treatment of T-cell lymphomas (Duvic et al, 2007; Olsen et al, 2007) and in one particular trial, treatment resulted in reactivation of HBV replication in a presumably latently-infected patient (Ritchie et al, 2009). This could have been caused by minichromosome hyperacetylation as a consequence of HDAC inhibition, suggesting that therapeutics which induce epigenetic suppression might conversely also be effective in silencing cccDNA transcription. Indeed, *in vitro* investigations demonstrated proof of concept that HAT inhibitors reduced HBV replication, while HDAC activators inhibited HBV replication and cccDNA transcription induce active epigenetic suppression (Palumbo et al, 2013). Such epigenetic therapies are already well recognised in the activation of latent HIV reservoirs as part of the “shock and kill” treatment approach (Kumar et al, 2015), although this is yet to result in HIV cure.

1.5.3 Immunomodulatory therapies

Hepatitis B virus is a non-cytopathic virus and it is well understood that development of chronic infection is attributed to immune evasion. Whereas immune responses effectively lead to resolution of acute infection in most adult cases (Guidotti & Chisari, 2001; Guidotti et al, 1999; Hoofnagle et al, 1973), chronic infection is characterised by a notable lack or impairment of such defence mechanisms (Nakamura et al, 2001; Publicover et al, 2013; Publicover et al, 2011). Exacerbation of inflammation by sustained immune responses that are ultimately insufficient to clear viral establishment contribute to liver damage. It has become increasingly evident that direct-acting antivirals alone will likely not be sufficient to eradicate HBV infection, and that alternative or supplementary strategies involving reducing the viral burden in the setting of reconstitution/induction of the immune system would most likely be required for effective curative approaches. Different strategies to target both the innate and adaptive arms of the immune system are currently undergoing investigation.

1.5.3.1 Innate Immunomodulators

Although innate immunity is poorly activated during CHB, responses are sufficient to suppress replication during acute infection (Cheng et al, 2017; Guidotti et al, 1999). Activation of RIG-I, APOBEC, NK cells (Ebert et al, 2011; Kakimi et al, 2000; Nguyen et al, 2007; Noguchi et al, 2005) and induction of cytokines such as IFN, and TNF (Belloni et al, 2012; Xia & Protzer, 2017; Xia et al, 2016) can efficiently control or even clear infection. Therapies targeting innate immunity aim to stimulate/enhance those actions, mimicking an effective/productive antiviral response (Xia & Protzer, 2017).

- Interferon alpha/PEG-IFN α

Early investigations established that virus-specific cytotoxic T lymphocytes (CTLs) can kill a small proportion of infected cells, as well as secrete cytokines (IFN α / β / γ , TNF α) that (non-cytolytically) abolish HBV replication without killing hepatocytes (Cavanaugh et al, 1998; Guidotti et al, 1996; Wieland et al, 2000). Consequently, administration of interferon alpha (IFN- α) to stimulate proinflammatory responses including induction of cytokines, was commonly used to treat HBV infection before the advent of more safe and effective therapies largely superseded its use, such as the use of antivirals. Unlike the oral NAs, IFNs are less convenient as they are typically injected subcutaneously. Treatment course is more finite (usually 12 months, sometimes longer) and virologic responses often sustained after cessation, with some studies even demonstrating impacts on the nuclear reservoir (Belloni et al, 2012; Liu et al, 2013). IFN may also induce cccDNA clearance, although this finding remains controversial (Chisari et al, 2014; Lucifora et al, 2014; Xia et al, 2014). However functional cure rate remains low and tolerability is poor compared to NA therapy (Buster et al, 2008; Lok et al, 2017). Moreover, IFN treatment is more effective for certain genotypes, particularly the subset of patients infected with genotype A HBV who exhibit minimal liver damage (Kao et al, 2000b; Marcellin et al, 2016; Zhang et al, 1996) although the mechanisms are unknown. A recent *in vitro* study by Shen et al (2018) suggested that cell type, rather than HBV genotype, may be contributing to observed *in vitro* differences in IFN response.

- Toll-like receptor agonists

Toll-like receptors constitute a class of pattern recognition receptors (PRR) which are critical for detection of pathogen-association molecular patterns (PAMPs) that are the first step of defence by the innate immune system (Diebold, 2008; Takeda & Akira, 2004). Recognition by PRR activates a cascade of signalling events that ultimately results in production of antiviral genes (Akira et al, 2006; Uematsu & Akira, 2007). HBV may regulate expression of TLRs to inhibit immune responses in CHB (Kondo et al, 2011; Visvanathan et al, 2007; Wu et al, 2009; Zhang & Lu, 2015). Agonists of different TLRs are being investigated as stimulators of IFN, interferon-stimulated gene (ISG) and inflammatory cytokine production for HBV treatment (Zhang et al, 2012). In addition, innate immunity is important for supporting induction of the adaptive immune response and stimulation of TLR7/9 can increase CTL response and produce large amounts of type I and II IFN to suppress viral replication (Guiducci et al, 2009). Agonists of TLR 7 and 8 are among the lead compounds under investigation as stimulators of a suboptimal innate immune response to promote HBV clearance. These reduced viral DNA and antigens and controlled HBV in animal models (Daffis et al, 2020; Lanford et al, 2013; Menne et al, 2015) but efficacy in clinical trials has been lacking to date (Boni et al, 2018; Gane et al, 2015; Janssen et al, 2018). Toxicity concerns arise regarding the lack of specificity and broad induction of cytokines, but increased understanding of PRR distribution in human liver and virus control-correlated cytokine gene expression profile would aid development of better agents.

- RIG-I agonists

While cell surface and endosome-localised TLRs activate immune responses after extracellular stimuli, activation of signal transduction in response to cytosolic PAMPs can be triggered by retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), a family of RNA helicases (Pichlmair

et al, 2006; Samanta et al, 2006; Takahasi et al, 2008; Yoneyama et al, 2004). RIG-I activation induces type III IFN, while the binding of the helicase to pgRNA itself inhibits Pol-epsilon (pgRNA) interaction required for viral replication (Liu et al, 2017b; Sato et al, 2015). As such, RIG-I agonists have been considered anti-HBV treatments through suppression of these activities, in conjunction with the aim to activate innate immune responses. Initial trials demonstrated promising results, exhibiting antiviral activity in infected patients with significant reduction in HBV and RNA, potentially via a mechanism inhibiting a process involved in reverse transcription (Colledge et al, 2018; Suresh et al, 2017; Walsh et al, 2018; Yuen et al, 2018a). However, trials of one such RIG-I agonist – Inarigavir, have recently been suspended due to serious adverse events.

- *Antagonists of cellular apoptosis inhibitors (cIAPs)*

Cell death is an important outcome in many infections, many pathogens subvert or modulate the stimulation of this process to maximise the survival of the host cell. Tumour necrosis factor (TNF) signalling can mediate death of HBV-infected hepatocytes, and cellular inhibitors of apoptosis proteins (cIAPs) were demonstrated to attenuate TNF signalling to restrict the death of these infected cells (Ebert et al, 2015b). This is significant as inadequate immune response and failure or inability to clear HBV infection is a hallmark of chronic hepatitis B. Inhibitors of cIAPs, or Smac mimetics, have been successfully developed as cancer therapeutics by promoting the killing of tumour cells (Bai et al, 2014; Fulda & Vucic, 2012; Oost et al, 2004; Probst et al, 2010). Smac mimetics have also demonstrated promotion of TNF-mediated elimination of HBV-infected hepatocytes in a murine model supporting HBV replication (Ebert et al, 2015a). Establishment of cccDNA has not been reported using this system, and thus the effect of these cIAP antagonists in this context is not known. We sought to address cccDNA presence using the hepatitis B murine model (Ebert et al., 2015). Reduction of the proportion of infected hepatocytes combined with repression of the remaining infected cells for example with an NA could present a viable curative therapeutic approach.

1.5.3.2 Adaptive immunotherapy

Adaptive immune responses are critical for effective control of HBV, as demonstrated by establishment of chronic infection in acutely-infected chimpanzees following CD8⁺ T cell depletion (Thimme et al, 2003). Accordingly, chronic hepatitis B is characterised by quantitative and/or functional deficiency of B and T lymphocytes, the key mediators of adaptive immunity (Burton et al, 2018; Park et al, 2016). Importantly, specific and targeted T cell responses are inefficient and/or defective during CHB, leading to the “T cell exhaustion” phenotype (Boni et al, 2007; Wherry, 2011). Therapeutic strategies targeting adaptive immunity are currently under investigation, largely focus on reconstituting or stimulating/enhancing these HBV-specific T cell activities.

- *Checkpoint blockers*

Checkpoint molecules regulate the immune response to prevent over-activation. Interaction between immunomodulatory molecules such as programmed cell death protein 1 (PD1) and its ligand (PD1-L) protect against autoimmunity by inhibiting T-cell mediated responses and inducing apoptosis in antigen-specific and regulatory T cells. Such inhibitory signals can be

upregulated in CHB (Bengsch et al, 2014; Boni et al, 2007; Park et al, 2016) leading to the dysfunction and the “T cell exhaustion” phenotype characteristic of chronic infections. CHB patients also have atypical B cells, expressing PD-1 which results in functionally reduced antibody production (Gehring & Protzer, 2019). Blockade of this interaction using monoclonal antibodies has proven to promote durable responses as effective cancer immunotherapy (Ansell et al, 2015; Rizvi et al, 2015; Salimzadeh et al, 2018; Zou et al, 2016), and investigators are evaluating this efficacy of this approach in improving specific B- and T-cell responses against HBV (Boni et al, 2007; Burton et al, 2018; Fiscaro et al, 2010; Rizvi et al, 2015). Investigations in woodchuck models demonstrate enhanced efficacy in viral control in combination with the antiviral ETV (Balsitis et al, 2018; Liu et al, 2014b), but further studies are required to determine the best context (predictors) and combination drugs to produce durable responses. There are concerns about the liver toxicity of PD-1 and PD-L1 antibodies, (pathway blockade may lead to severe liver inflammation and result in fulminant hepatitis) however interim results from patient trials did not report severe hepatic toxicity in patients with advanced hepatocellular carcinoma (El-Khoueiry et al, 2017).

- T cell transfer

T cell transfer has been used to overcome inefficient B and T cell responses. Bone marrow transplant from immune donors to chronic patients, was demonstrated to result in immune reconstitution (Ilan et al, 1993; Ilan et al, 2000; Lau et al, 1997). However, this method is limited by the major risks and severe adverse effects associated with graft rejection. Instead, genetically-engineered autologous T cells expressing HBV-specific receptors may be used to treat chronic HBV infection and related complications/HCC (Bertoletti & Ferrari, 2012; Gehring et al, 2011; Koh et al, 2013). The use of designed, antigen-specific TCRs can redirect antigen specificity and increase CTL number and activity to enhance the response against the cognate specific target. Thus, adoptive transfer of these engineered T cells expressing HBV-specific T cell receptors (TCRs, (Qasim et al, 2015)) or chimeric antigen receptors (CARs, (Krebs et al, 2013)) may be an alternative approach to reconstituting function of exhausted T cells (Bohne & Protzer, 2007; Krebs et al, 2013). This approach has been used to specifically recognise viral surface antigens and induce production of IFN- γ and IL-2 and selectively eliminate infected cells (Bohne et al, 2008).

- Therapeutic vaccines

While effective prophylactic vaccines have been successful in reducing significant burden of Hepatitis B globally over the past several decades, they are of no benefit to the already-infected CHB population. Natural resolution of infection is typically characterised by lifelong immunity conferred by specific B and T-cell immune response. Thus, recognising this as a key requirement to curing HBV, therapeutic vaccines are being developed with the aim of boosting HBV immunity and restoring necessary functional anti-HBV activities. Vaccines developed to stimulate specific and multifunctional B- and T-cell responses to HBV envelope and core proteins have shown limited clinical efficacy to date (Heathcote et al, 1999; Jung et al, 2002; Lok et al, 2016; Vandepapeliere et al, 2007) despite promising results in animal studies (Lu et al, 2008; Menne et al, 2002). Vaccine development is complex, with considerations required to be made regarding multiple factors; specific immunogen and presentation, vaccine formulation, delivery and other host determinants can all contribute to optimal vaccine design

and impact the triggering of effective and durable immune responses required to broadly clear HBV infection. This in turn requires better understanding of the viral infection processes and host contributions.

1.5.4 HBV cure

Extensive research into improving currently available, or exploring innovative approaches continually advance our ability to better prevent or control viral infection, improving outcomes and quality of life for those chronically infected with HBV. However, the fact remains that functional cure is only rarely achieved with existing therapies, and new approaches are urgently required. Functional cure of CHB infection would require a finite therapy that suppresses HBV DNA and HBsAg expression, ideally with production of anti-HBs antibodies. For complete (sterilising) cure, all episomal cccDNA templates would need to be eliminated, as well as integrated HBV sequences. Achievement of these goals requires better understanding of both the viral properties and host immune responses, in turn necessitating the need for robust and permissive models. This deeper understanding of HBV replication and pathogenesis in conjunction with improved cell culture and animal models will accelerate development and evaluation of novel therapeutic strategies to cure HBV infection. An integrated approach will likely be required for elimination, a combination of different therapies that strikes the right balance in directly inhibiting different aspects of the viral cycle (Figure 1.10) while stimulating antiviral immune responses to enhance viral clearance (Revill et al, 2019a; Revill et al, 2019b).

Step	Therapeutic Approach
1	Entry Inhibitors
2,3,7	core protein inhibitors
3	cccDNA inhibitors
4	epigenetic inhibitors
5	siRNA
6	translation inhibitors
8	pol/RT inhibitors
9,10	secretion inhibitors

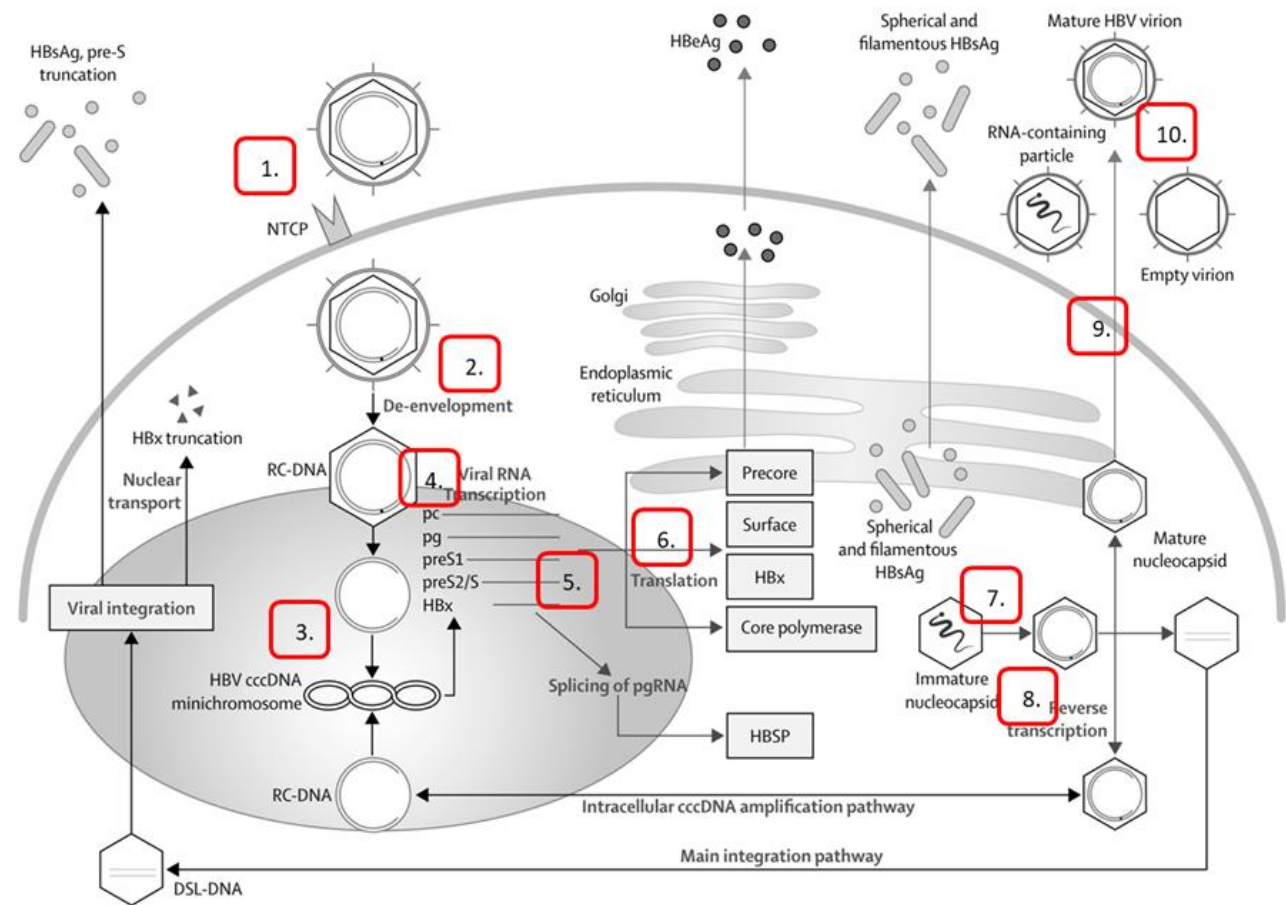


Figure 1.10: Sites of action for different direct-acting antiviral approaches.

Potential therapeutic strategies for treatment of HBV infection, based on steps within the viral replication cycle. 1: Receptor-mediated entry. 2: De-envelopment and nuclear translocation. 3: rcDNA-to-cccDNA conversion. 4: cccDNA-to-mRNA transcription. 5: (splicing &) mRNA export. 6: viral protein translation. 7: pgRNA encapsidation. 8: pgRNA-to-rcDNA reverse transcription. 9: envelopment & maturation. 10: viral secretion.

1.6 *In vitro* models of HBV replication

Recent advancements in understanding HBV molecular biology and infectivity were largely enabled by the discovery of the NTCP entry receptor (Yan et al, 2012), leading to the development of model systems which allow investigations of the complete HBV “life” cycle. Extraordinary species and tissue-specificity has severely hampered the ability to comprehensively study and analyse HBV infection and replication. As such, much of our early understanding of the various viral processes was gained from experiments overexpressing viral proteins or using related hepadnaviruses such as duck hepatitis B virus (DHBV) or woodchuck hepatitis virus (WHV) (Cova & Zoulim, 2004; Mandart et al, 1984; Summers et al, 1978; Tennant & Gerin, 2001). A brief description of available models follows.

1.6.1 Transfection models

- Transient transfection

A common method of analysing HBV replication *in vitro* utilises transfection of tumour cell lines with overlength cDNA constructs encoding the HBV genome. Overlength constructs are necessary for the production of all replicative intermediates, since the linearisation of the circular viral genome cannot facilitate generation of the greater-than-genome RNA transcripts (pgRNA and pcRNA), and inevitably disrupts the reading frames which overlap each other. Furthermore, as viral expression comes directly from the plasmid, there is not conversion of rcDNA genome to form cccDNA templates, as occurs in natural infection. The cell lines most commonly used for HBV transfection-based molecular virology studies are the HepG2 and Huh-7 human hepatoma cell lines, derived from hepatocellular carcinoma (HCC) tissues (Aden et al, 1979; Nakabayashi et al, 1982). Transfection of suitable HBV DNA constructs into either cell line leads to HBV replication, including expression of all HBV DNA and RNA transcripts, generation of infectious viral particles, as well as expression of secreted HBsAg and HBeAg (Acs et al, 1987; Chang et al, 1987; Yaginuma et al, 1987). Combined with the ready ability to manipulate genetic constructs, transient transfection is a convenient model for quickly analysing gene and protein expression as consequences of genetic variation/mutations (Bannister et al, 2019; Sozzi et al, 2016). These rapid transfection-based models have been instrumental in elucidating much of the HBV replication cycle, although their transient nature precludes studying long-term effects of viral expression. This could also be partly attributable to absence of an established stable episome, as per cccDNA minichromosomes of natural infection. This can be overcome with generation of integrated cell lines stably expressing HBV (Ladner et al, 1997; Zhou et al, 2006).

- Stably transfected cell lines

Hepatoma cell lines harbouring integrated HBV genomes of various configurations have been important tools for investigating HBV replication. These cell lines have been transfected with HBV DNA, selected for under specific pressures/conditions, and are able to replicate HBV either constitutively or an inducible manner (Aden et al, 1979; Guo et al, 2007a; Ladner et al, 1997; Sells et al, 1987; Sureau et al, 1986). Among the most popularly utilised stable cell lines are the HepAD38 and HepG2.2.15 lines, both of which are derived from the HepG2 cell line

and have integrated HBV sequences of genotype D HBV. HepAD38 cells contain an integrated cDNA copy of the HBV pgRNA under the control of a tetracycline-responsive CMV promoter (Ladner et al, 1997), while HepG2.15 cells contain two head-to-tail dimers of the HBV genome with retroviral long terminal repeat promoters (Sells et al, 1987). HepAD38 and its derivative cell lines are particularly popular for examining transcriptional regulation of cccDNA, however studies can be complicated due to the continual presence of integrated HBV DNA. Stable cell lines have also been an indispensable source of viral inoculum for *in vitro* infection experiments, with the HepAD38 cell line in particular one of the most productive generators of infectious inoculum and thus one of the most prolifically utilised cell lines. Due to the nature of the integrated DNA, a major caveat for many of these investigations is that they have been restricted to the genotype D HBV. Furthermore, while transiently transfected and stably integrated cell lines have been instrumental to the understanding of the specific aspects of the replication cycle of HBV, induction of replication from introduced transfected templates prevents investigation of early viral processes from entry to cccDNA formation.

- Full-length (monomer) PCR and 1.0mer transfection

In 1995, Günther et al described a PCR strategy that amplified full-length HBV genomes from patient sera (Figure 1.11). They demonstrated that the resulting monomeric PCR products could be transfected into cells where they circularise to form molecules analogous to cccDNA and facilitate HBV replication. Pollicino et al (2006) showed by Southern blot that the cccDNA-like molecules resembled cccDNA from chronic hepatitis B patient liver, and proceeded to use this as a surrogate model to explore the epigenetic regulation of cccDNA. Their findings indicated that the acetylation of cccDNA-bound H3 and H4 histones regulated HBV replication (Pollicino et al, 2006). This monomeric transfection system was a powerful tool for studying replication of variant HBV, building on the conventional cccDNA-deficient overlength HBV transfection-based replication. Since monomeric constructs first require circularisation, these represent a more physiologically relevant model. However, the utility of this system for studying other aspects of HBV replication, and host interactions, had yet to be demonstrated.

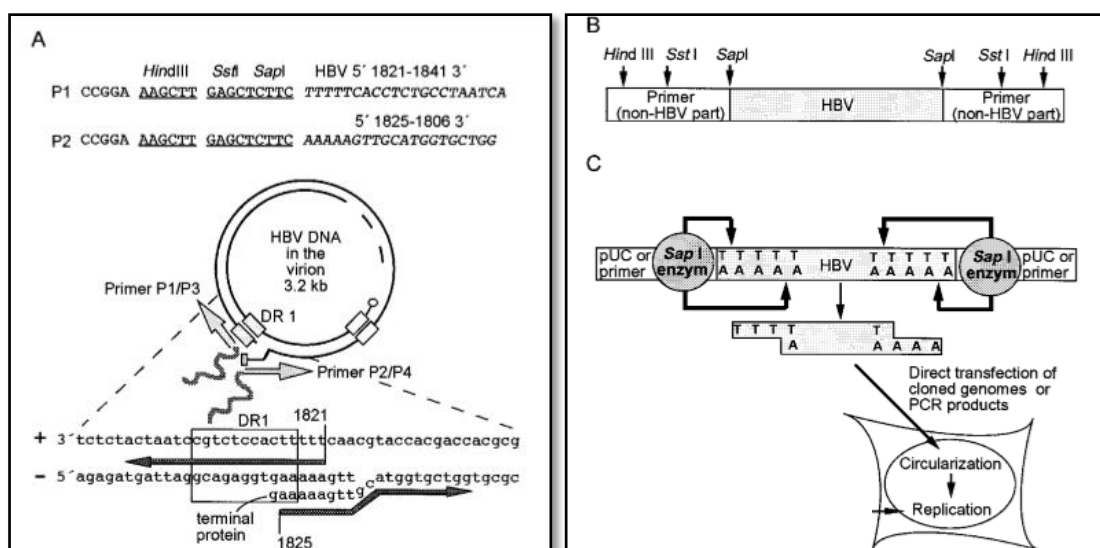


Figure 1.11 Generation and transfection of HBV monomer or "1.0mer" ("Günther PCR")

(A) Primer sequences and binding site for PCR amplification of whole HBV genomes ("1.0mers"). (B) Structure of the Günther PCR product. (C) 1.0mer HBV released by digestion with SapI enzyme can be subsequently transfected into cells to facilitate HBV replication following circularisation. Adapted from Günther et al (1995).

1.6.2 Infection models

- Pre-NTCP infection systems

Permissive and robust infection systems present an undeniably significant gap in HBV research. Lack of a small laboratory animal model for natural HBV infection has directed efforts towards the development of an *in vitro* infection model. Early studies utilised infected patient-derived sera to inoculate primary human hepatocytes (PHH) and was subject to variation from different donors of both hepatocytes and inoculum. However, virus produced by cell lines was then found to be a reproducible source of infectious inoculum (Gripon et al, 1993; Gripon et al, 1988; Rijntjes et al, 1988). Primary hepatocytes undergo a rapid loss of hepatic phenotype and functionality following isolation, presenting a limited window of opportunity during which the cells are susceptible to HBV infection (Heslop et al, 2017; Konig et al, 2014; Yan et al, 2012). These models have significantly improved in recent years due to advanced knowledge and techniques enabling prolonged infection (Winer et al, 2017; Xiang et al, 2019).

Attempts to develop more reproducible *in vitro* infection systems led to the identification of the hepatoma-derived cell line, HepaRG (Gripon et al, 2002; Hantz et al, 2009). These bi-potent cells differentiate into biliary-like or hepatocyte-like cells morphologically and functionally akin to PHHs with susceptibility to HBV infection following differentiation with DMSO and hydrocortisone (Schulze et al, 2012). This development overcame the issue of variability attributed to donor hepatocytes, however lengthy differentiation processes and low infection efficiency have prevented their widespread use.

In a microarray screen Ni et al (2014) NTCP was validated as an HBV entry receptor when upregulated genes in differentiated HepaRG cells were selected for based on plasma membrane association, basolateral sorting, high expression in the liver, low expression in HepG2/NTCP cells, and conservation in primates and rodents.

- NTCP infection systems

The breakthrough discovery of the HBV entry receptor (NTCP), enabled development of an infection model involving genetically-engineered, NTCP-overexpressing HepG2 cells to facilitate HBV infection (Yan et al, 2012). Huh-7 cells have also been engineered to express NTCP with moderate success and have become the preferred system for investigations on HBV-HDV co-infection (Ni et al, 2014; Veloso Alves Pereira et al, 2015). Infection was reported to be more productive in HepG2^{NTCP} cells compared to HepaRG cells, as measured by core protein expression as well as HBsAg and HBeAg secretion (Ni et al, 2014). Although identification of NTCP has produced *in vitro* infection models with comparatively increased robustness and usability, infection rates are low and improvements are required. Furthermore, the restricted HBV replication observed in murine cells expressing hNTCP (Lempp et al, 2016; Li et al, 2014) indicates NTCP may not be the only limiting factor. Furthermore, the issue of acquiring good inoculum remains, as infection experiments are dependent on producing highly concentrated virus inoculum, a highly laborious and often not reproducible process.

- Novel cell culture models

Further advancements in development of *in vitro* models for HBV infection and replication include utilisation of stem cell technology to generate induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (HLCs), shown to maintain prolonged susceptibility and support long-term infection (Shlomaia et al, 2014; Xia et al, 2017). Additionally, modified culturing

conditions has been used to improve existing models. Co-culturing with non-parenchymal stromal cells, three-dimensional cultures using various scaffolds have shown potential in supporting HBV infection and viability of cells *ex vivo* (March et al, 2015; Ortega-Prieto et al, 2018; Zhang et al, 2019b). Organoid systems have quickly gained popularity and traction over recent years owing to their potential applications in the field of personalised medicine and particularly cancer research. These systems provide an attractive model, more closely representing the *in vivo* context, and have already been successfully adapted into infection models for various pathogens including rotavirus and zika virus (Dutta & Clevers, 2017). Work towards establishing liver organoids as more permissive and relevant system for modelling HBV infection is under development with promising results to date (Akbari et al, 2019; Crignis et al, 2019; Nie et al, 2018; Sun & Hui, 2020).

While *in vitro* systems are useful for early investigations, *in vivo* models are still ultimately required for pre-clinical evaluation of new therapies. Importantly, *in vivo* models allow therapeutic studies in the presence of a competent immune system.

1.7 *In vivo* models of HBV replication

1.7.1 *In vivo* hepadnavirus models

Hepadnaviridae are a family of small DNA-containing viruses that preferentially cause infection within the liver of various hosts including humans, higher primates, some birds and rodents. Both the *orthohepadnaviridae* (mammalian virus) and *avihepadnaviridae* (avian virus) replicate via a common reverse transcription strategy involving the production of an RNA intermediate from the DNA genomes. Since HBV has a narrow host range, studies involving various animals and their respective hepatitis B viruses have been instrumental towards our understanding regarding the replication of the prototypic member HBV.

1.7.1.1 Non-human HBV models

In vitro and *in vivo* models enabling the study of related hepadnaviruses, particularly DHBV and WHV, have been instrumental in improving our understanding of HBV (Cova & Zoulim, 2004; Mandart et al, 1984; Mason et al, 1980; Summers et al, 1978; Tennant & Gerin, 2001). Major advantages of the DHBV model included its ease of use under laboratory conditions, with efficient vertical transmission as well as high rates of chronic viremia following intravenous injection of ducklings (Tuttleman et al, 1986b; Urban et al, 1985). This system is extremely efficient, with most cells exhibiting infection (Jilbert et al, 1996; Sprengel et al, 1984; Tuttleman et al, 1986a), however the pathogenesis differs in humans. In ducks, the milder asymptomatic phenotypes rarely resulted in liver disease in these animals (Freiman & Cossart, 1986; Marion et al, 1984). Notably, the DHBV genome also lacks an equivalent HBx open reading frame, and although a vestigial analogous protein was identified, it did not have any apparent functional contribution to infection and replication (Chang et al, 2001). In contrast, the mammalian woodchuck hepatitis virus genome does contain an X open reading frame and pathogenesis of WHV more closely resembles that of HBV. Development of HCC is observed in infected animals, although more frequently and with accelerated progression than typically observed in humans (Popper et al, 1987). Natural history of infection also appears to resemble that of chronic hepatitis B in patients, in that it is largely determined by the age of the animal at the time infection (Frommel et al, 1984; Tennant et al, 1987). Experimental transmission of WHV beyond infancy is more likely to result in acute self-limiting infection, the rate of chronicity decreasing with increasing age (Tyler et al, 1986; Wong et al, 1982).

The related hepadnavirus models have been instrumental in the evaluation of anti-HBV therapies (Balsitis et al, 2018; Daffis et al, 2020; Fourel et al, 1987; Lu et al, 2008; Menne et al, 2002; Noordeen et al, 2013). However, the fact remains that these are distinct viruses that fail to faithfully recapitulate the entire replication cycle and pathogenesis of HBV, prompting the need for robust HBV-specific systems. Beyond infection of non-human higher primates, now rightfully banned on moral and ethical implications, no existing model reproducibly allows for comprehensive analysis of all replicative stages involved in HBV infection.

1.7.1.2 Animal models of HBV infection

HBV only naturally infects humans, but experimentally can also infect chimpanzees. Their close evolutionary/genetic similarity makes them a useful model for studying HBV, and until the advent of more acceptable susceptible small animal models, were the preferred model for

evaluation of vaccine development (Buynak et al, 1976; Purcell & Gerin, 1975). Aside from accessibility constraints pertaining to limited supply and high costs, the routine usage of chimpanzees in research has since been severely restricted due to moral and ethical implications. Other than chimpanzees, the only other animal with known susceptibility to HBV are the Asian tree shrews (*Tupaia belangeri*) that are closely related to primates. These mammals demonstrate low HBV replication efficiency, both *in vitro* and *in vivo* and are also not widely used due to restricted accessibility of the animal model and lack of specific reagents for their investigation (Walter et al, 1996).

Identification of the NTCP receptor for HBV entry has precipitated the advent of new models for HBV infection (Wettengel & Burwitz, 2020). Genetic engineering has enabled the generation of transgenic animals with induced human NTCP expression, to facilitate HBV infection in animals not otherwise susceptible. This has been demonstrated in various animals including, macaques and pigs, (Burwitz et al, 2017; Lempp et al, 2017; Zhou et al, 2019), with the rhesus macaque model showing particular promise, despite only inducing transient expression at present (Wettengel & Burwitz, 2020). However, the generally low efficiency with which infection is established in these systems points to there being additional host restriction factors contributing to the species tropism of human HBV.

1.7.2 Murine models of HBV

Small laboratory animal-based models are preferred for discovery and evaluation of pre-clinical therapeutics. The extraordinary tropism of HBV precludes a natural infection model in mice, however other methods are available for studying hepatitis B in these animals (Table 1.2). Lacking natural susceptibility to HBV infection, murine models representing HBV replication have been limited to two broad models: induction of HBV replication following introduction of HBV DNA, and infection of chimeric mice grafted with human hepatocytes.

- Gene expression models

The former group includes transgenic mice with HBV transgenes for stable expression of HBV proteins (Chisari et al, 1985; Farza et al, 1988). These animals display tolerance to the viral proteins and as such, absence of chronic liver disease, although pathology can develop from liver injury mediated by prolonged exposure to accumulated proteins (Chisari et al, 1987; Dunsford et al, 1990; Kim et al, 1991). Transduction of mice hepatocytes with adenoviruses or adeno-associated viruses carrying HBV genomes is another method used to induce HBV expression in mice. This results in acute self-limiting (Hartman et al, 2007; Sprinzl et al, 2001; von Freyend et al, 2011) or persistent (Huang et al, 2012b; Mingozi et al, 2003; Ye et al, 2015) infection depending on the vector and dose.

An alternative method of inducing gene expression in mice is through the introduction of DNA via the tail vein by hydrodynamic injection (HDI) (Huang et al, 2006; Liu et al, 1999; Yang et al, 2002). HDI allows non-NTCP receptor-mediated entry of the DNA into the hepatocyte. Advantages of this system include induction of HBV replication in immunocompetent mice, but also the ability to influence infection duration by changing the mouse lineage (Ebert et al, 2015a). A popularly utilised method described by Huang et al (2006) involves the delivery of more-than-full-length HBV genomes flanked by inverted terminal repeat (ITR) regions from

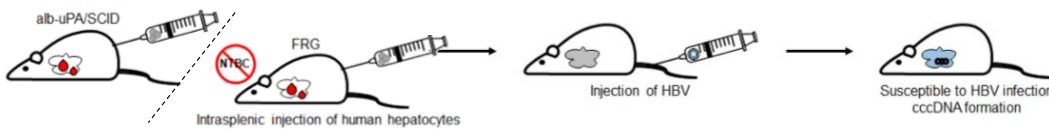
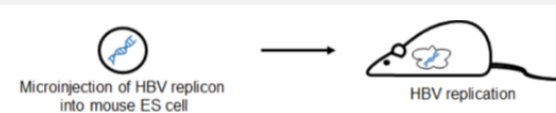
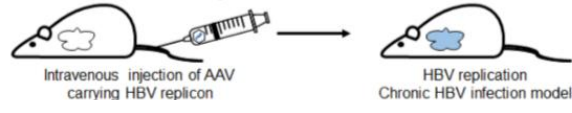
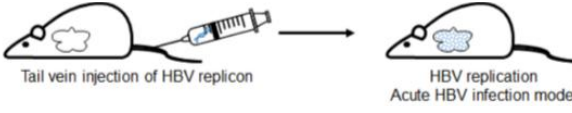
AAV to facilitate integration into host genomes. Analogous to *in vitro* transfection models described earlier, this results in facilitation of near-complete HBV replication. However, instrumental as they are to *in vivo* investigations, these murine models notably still preclude the ability to investigate the early steps of HBV infection and replication since they completely bypass the need for viral entry.

- Infection models

Currently, the only available murine models permissive to HBV infection are chimeric mice with humanised livers (Brown et al, 2000; Lutgehetmann et al, 2012; Ohashi et al, 2000). These mice have been grafted with human hepatocytes and as such are able to support natural infection, and therefore are also the only mice that establish cccDNA. Necessitated by the need to prevent host rejection, these mice are of severely immunocompromised background. To overcome this limitation, trimera mouse have also been generated, which involve additional transplantation of bone marrow/lymphocytes from immunocompetent donors, in a bid to reconstitute the immune response (Eren et al, 1998; Ilan et al, 1999). However, cost and extensive laborious technical expertise required to establish these strains still restricts their wide usage, despite being the only infectable murine model for many decades.

Following discovery of the NTCP entry receptor, conferring susceptibility to HBV infection through engineering a mouse strain that expresses NTCP on the hepatocytes has not been yet successful. In contrast to macaque and pig hepatocytes however (Lempp et al, 2017) despite successful NTCP expression on murine hepatocytes, it was discovered that these cells were unable to fully support HBV replication post-entry (Lempp et al, 2016; Li et al, 2014). This suggests that species restriction of HBV was not due to NTCP alone (Tang & McLachlan, 2002). For this reason, the HBV research field is in desperate need of a suitably permissive small lab animal model that closely resembles HBV infection for investigative and preclinical evaluations.

Table 1.2 Murine models of HBV replication.

	Virological Characteristics				Animal Characteristics	
	Virus	Viral entry	Viral infection /replication	cccDNA formation	Immunocompetent/ Immunodeficient	Class/Order/Family/Genus
Chimpanzee	HBV	Yes	Infection	Yes	Immunocompetent	Mammalia/Primate/Hominidae/Pan
Tupaia	HBV	Yes	Infection	Yes	Immunocompetent	Mammalia/Scandentia/Tupaiaidae/Tupaia
Duck	DHBV	Yes	Infection	Yes	Immunocompetent	Aves/Anseriformes/Anatidae/Anas
Woodchuck	WHV	Yes	Infection	Yes	Immunocompetent	Mammalia/Rodentia/Sciuridae/Marmota
Rhesus Macaque	HBV	Yes				Mammalia/Primate/Cercopithecidae/Macaca
Mouse	HBV					Mammalia/Rodentia/Muridae/Mus
Human chimeric	HBV	Yes	Infection	Yes	Immunodeficient	
						
Transgenic	HBV	Yes	Replication	No	Immunocompetent, tolerant to HBV	
						
Transduced	AAV, HBV	Yes	Replication	Yes	Immunocompetent	
						
Transfected	HBV	Yes	Replication	No	Immunocompetent	
						

Adapted from Hwang & Park (2018) and Guo et al (2018)

1.8 HBV modulation of cell signalling

Since direct antivirals are currently incapable of inducing CHB cure, targeting host components or mechanisms critical for HBV replication may be necessary. Cellular homeostasis is maintained by the specific coordination of various processes, which are in turn regulated by modulation of signal transduction cascades. Generally, stimuli trigger sequential responses that facilitate transcription and expression of genes, which direct functional outcomes. Bacterial and viral pathogens encode proteins that hijack and modulate these pathways/components to their own advantage and benefit, redirecting cellular machinery to facilitate pathogenic replication and propagation. For example, viruses can manipulate cellular structures to evade immune recognition and activation, such as by concealing their genomes in intracellular membranes or the cytoplasm, demonstrated by dengue virus (Uchida et al, 2014) and human immunodeficiency virus 1 respectively (Lahaye et al, 2013). In turn, herpes simplex virus 1 induces degradation of interferon (IFN)-inducing nuclear receptors (Orzalli et al, 2012), and hepatitis C virus (HCV) subverts cellular autophagy leading to enhanced replication (Hansen et al, 2017).

HBV, likewise relies on host cellular proteins and processes in order to replicate and propagate, including engaging cellular receptors to enter cells and utilizing cellular transcription and translation machinery for replication (Cougot et al, 2012; Deng et al, 2017; Iwamoto et al, 2019; Koniger et al, 2014; Seto et al, 1990; Su & Schneider, 1996; Tang et al, 2005; Yan et al, 2012). Besides hijacking cellular proteins to directly facilitate its infectious cycle, HBV replication also modulates general processes such as cellular DNA replication and repair, cell cycle progression, cell survival and proliferation, and induction of apoptosis and autophagy (Arzberger et al, 2010; Becker et al, 1998; Chin et al, 2007; Damania et al, 2014; Guilhot et al, 1996; Lu et al, 2005; Ozer et al, 1996; Rawat et al, 2012; Sasaki et al, 2016; Sir et al, 2010; Wang et al, 2011). Several of these processes are described further below.

- Cell cycle/proliferation:

In vitro studies have identified an inverse correlation between cell proliferation and HBV replication (Ozer et al, 1996). In stable and transiently-transfected cell lines, detection of HBV replicative intermediates was increased when cells were quiescent (G₀/G₁ phase) or in G₁ and G₂ arrest, and expression was inhibited during the proliferating phase (S phase) (Huang et al, 2004; Koike et al, 1994; Wang et al, 2011). HBV-induced G₂ phase arrest correlated with significant upregulation in phosphorylated Erk, Akt (key survival kinase) (Chin et al, 2007), effects now also attributed to HBx (Rawat & Bouchard, 2015; Wang et al, 2014). Furthermore, HBx facilitates HBV replication by inducing quiescent hepatocytes to enter the G₁ phase, while preventing S phase progression, in a calcium-dependent manner (Gearhart & Bouchard, 2010). Conversely, Koike et al (1994) suggested that HBx may contribute to hepatocarcinogenesis by driving deregulated cell cycle progression, describing progression from G₀/G₁ to S phase of most HBx-expressing cells (Koike et al, 1994). These discrepancies could be due to variation in experimental condition, or may indicate that a threshold level of HBx expression may be necessary for induction of DNA synthesis.

- Apoptosis:

Beyond deregulation of cell cycle progression, inhibition of apoptosis is another mechanism by which cancer cells facilitate tumour formation, through increased proliferation. Numerous studies suggest inhibitors of apoptosis proteins (IAPs) are upregulated in cancer cells originating from the pancreas, lung (Ekedahl et al, 2002) and prostate (McEleny et al, 2002). IAPs can also be stimulated by virus infection via modulation of host gene expression, and some viral proteins inhibit apoptosis (Rothe et al, 1995). Lu et al (2005) observed increased expression of cellular IAPs in constitutive HBV-expression HepG2.2.15 cells compared to the parental HepG2 cells. This upregulation was also correlated with HBx protein (Gearhart & Bouchard, 2010), which was described to enhance apoptotic killing by TNF α (Su & Schneider, 1997). Using a HDI mouse model, Ebert et al. (2015) demonstrated that cIAPs permitted viral persistence by attenuating TNF-mediated cell death of HBV-replicating cells (Ebert et al, 2015b), and that antagonising this cIAP function promoted HBV clearance, though the impact on cccDNA reservoir and cure of chronic infection requires clarification.

Other investigations exploring HBx's role in cell death have done so in the context of tumour suppressor protein p53 and transactivation activity; direct interaction leading to apoptosis through cytoplasmic sequestration of p53, which consequently inhibits its transcriptional activation (Elmore et al, 1997; Truant et al, 1995; Wang et al, 1995). In contrast, HBx has also been shown to suppress apoptosis (Kim et al, 2001b; Shih et al, 2000), likely to prevent release of progeny virions following infection (Arzberger et al, 2010). HBx induction or inhibition of apoptosis may depend on the nature of the apoptotic stimulus or specific HBx variants (Lee et al, 2012).

- Kinases and gene expression

HBV replication is implicated in modulation of several mitogen-activated protein kinase (MAPK) signalling pathways, which consist of sequentially phosphorylated kinase cascades. Indeed, activation of these signalling cascades contributes to increased proliferative rates in many human tumours, increased transcriptional activity of factors regulating cytokine gene expression and promote immune responses (Guan, 1994; Kyriakis & Avruch, 2001; Seger & Krebs, 1995), thus leading to their investigation as anti-cancer targets (Arora & Scholar, 2005; Sawyers, 2003; Zhang et al, 2009). HBV replication has been shown to activate a number of cellular kinases; activation of Src tyrosine kinase stimulated viral polymerase activity to contribute to HBV replication (Klein, 1999), and upregulation of phosphorylated ERK, Akt and downstream target GSK3- β was observed in HBV-replicating cells (Chin et al, 2007). Conversely, activation of the PI3K-Akt pathway suppressed HBV replication (Guo et al, 2007b). Activated Akt1 was a consistent feature of HBV-induced HCC, purportedly promoting cell survival in favour of viral replication by inhibiting apoptosis of infected cells (Guo et al, 2007b). Regulation of HBV replication by signal transduction pathways may differ depending on physiological and pathological contexts, since many of these pathways can interact and influence each other.

HBx expression modulates signal transduction pathways via direct protein interaction (Kim et al, 2001a) or augmentation of intracellular Ca²⁺ levels (Bouchard et al, 2001b; Zheng et al, 2003), leading to activation of downstream transcription factors and transactivation of genes (Benn & Schneider, 1994; Benn et al, 1996; Cross et al, 1993; Doria et al, 1995; Henkler et al,

1998; Kim et al, 2001a; Klein & Schneider, 1997; Lee & Yun, 1998). However, the role of HBx protein in the HBV infection and replication cycle remains largely inconclusive/unknown, beyond the recently described relief of transcriptional repression described earlier (Decorsière et al, 2016; Murphy et al, 2016). In addition to HBx, a number of other HBV proteins interact with or modulate host signalling proteins. The HBV core protein contains a C-terminal domain (CTD) which is subjected to phosphorylation, and this state likely determines its subcellular localization, capsid stability and viral function (Ludgate et al, 2011). CTD phosphorylation state was associated with HBV DNA synthesis but not virion secretion (Ning et al, 2017). Phosphorylation of HBc affects its binding to host proteins, which could potentially epigenetically regulate transcription if these HBc proteins are associated with the viral minichromosome.

The Hepatitis B e antigen (HBeAg), which shares sequence identity with HBc, is believed to play a role in immune modulation, down-regulating TLR-2 responses in HBeAg positive individuals (Revill et al, 2015; Visvanathan et al, 2007). Yu et al (2017) reported HBeAg repressed IFN/JAK/STAT signalling to facilitate replication. They found that stimulation of SOCS2 expression ultimately lead to downregulation of IFN receptor expression, in turn inhibiting expression of ISGs.

Cellular proteins play an important role in the HBV replication cycle. The SMC5/6 complex and PRMT1 methyltransferase were identified as restricting factors for cccDNA transcription (Benhenda et al, 2013; Decorsière et al, 2016; Murphy et al, 2016), while Parvulin 14/17 proteins' recruitment to cccDNA via interaction with HBx promotes transcriptional activation and upregulation of HBV replication (Saeed et al, 2019), engagement with surface EGFR facilitated internalization of NTCP-bound virions (Iwamoto et al, 2019), HSP90 facilitated ribonucleoprotein (RNP) complex formation which potentiates reverse transcription (Gyoo Park et al, 2002; Hu & Seeger, 1996). Host polymerases, helicases, and topoisomerases, have also been implicated in the steps involved in rcDNA-to-cccDNA conversion alone (Kitamura et al, 2018; Koniger et al, 2014; Qi et al, 2016; Schreiner & Nassal, 2017; Sheraz et al, 2019; Tang et al, 2019). Although inhibitors of HBV replication have been identified through targeting some of these host-virus interactions, none of these have successfully translated to clinically approved therapies for treatment of chronic hepatitis B.

Systematic analysis of the pathways and factors that are significantly impacted in the context of HBV infection and replication is required to identify all host factors crucial for HBV replication and infectivity, providing mechanistic insights into strategies that could be employed to identify new drug targets leading to a HBV cure. One such approach is antibody microarrays (Zhang & Pelech, 2012), such as the service provided by Kinexus Bioinformatics (Canada), which includes a wide range of antibodies for specific detection of various cellular proteins as well as their phosphosites, that have been recognised as biomarkers for signalling pathway monitoring and disease diagnosis for a range of pathogens. Such broad screening aims to identify regulatory protein changes in response to different experimental conditions, and consequently prompt discovery of targeted inhibitory approaches that might lead to suppression of HBV replication and/or infection. This approach has been used for a range of human pathogens, including malaria, and dengue, zika and HBc. Recently, Haqshenas et al (2017) identified important roles for JAK2 and STAT5 in HCV, and insulin receptor in Dengue

virus infections (Haqshenas et al, 2019), and consequently corresponding inhibitors which could suppress viral replication. Similarly, we propose the application of these techniques could help uncover new insights into virus-host interactions contributing to HBV replication, and this forms the basis of Chapter 5 of this thesis. This knowledge, together with the re-evaluation of known existing inhibitors to host factors, such as kinase inhibitors used under other circumstances, may fast-track novel therapies leading to functional and/or complete cure.

1.9 Hypothesis and Aims

Chronic hepatitis B virus infection (CHB) poses a significant public health burden, affecting more than 250 million people worldwide. Globally, disease progression, severity and even therapeutic outcomes may differ depending on the viral variants causing the underlying infection, including different HBV genotypes. There is an urgent need for an HBV cure underpinned by new *in vitro* and *in vivo* models of HBV replication, permitting preclinical investigation of the complete HBV replication cycle. In particular this must include examination of the HBV covalently closed circular DNA (cccDNA) reservoir, across multiple HBV genotypes and variants.

Transfection of 1.0mer HBV templates, which can be circularised to form cccDNA-like molecules, could enable such investigations. Unlike conventional overlength constructs (which do not allow analysis of cccDNA), these do not contain redundant genome segments, and hence can only enable replication following circularisation of the templates. Furthermore, Hydrodynamic injection (*in vivo*) of monomeric HBV genomes can partially address an important lack of suitably physiologically-relevant *in vivo* models that permit studies of the complete HBV replication cycle post viral entry including generation of a cccDNA-like molecule. Importantly, these models could be used to investigate virus-host interaction of different HBV variants, which in turn could lead to identification of new drug targets to inhibit HBV replication.

The aim of this study was to test the applicability of this 1.0mer replication system in answering pertinent research questions, and were addressed through three specific points:

1. Investigating the utility of the HBV monomeric genome transfection/HDI method as a cccDNA-enabling HBV replication model of variant HBV *in vitro* and *in vivo*.
2. Assessing the DDB1-interacting ability of HBx of different HBV genotypes and evaluating DDB1-specific effect of HBV transcription.
3. Identifying novel host factors involved in HBV replication as potential therapeutic targets.

Chapter Two: Materials and Methods

2.1 Material providers

<u>Provider</u>	<u>Headquarters</u>	
Affymetrix	Santa Clara, CA	USA
Applied Biosystems	Scoresby, VIC	Australia
BDH Prolabo	Radnor, PA	USA
BioRad	Hercules, CA	USA
Biotix	San Diego, CA	USA
BMG Labtech	Ortenberg	Germany
Corning	Corning, NY	USA
Dako	Glostrup	Denmark
Eppendorf	Hamburg	Germany
GE Healthcare Life Sciences	Buckinghamshire	UK
GenScript	Piscataway, NJ	USA
Gibco	Gaithersburg, MD	USA
Greiner Bio-One	West Heidelberg, VIC	Australia
Implen GmbH	Munich	Germany
Invitrogen	Carlsbad, CA	USA
MP Biomedicals	Santa Ana, CA	USA
Merck Millipore	Darmstadt	Germany
Micromon	Melbourne	Australia
MJ Research	St. Bruno (Quebec)	Canada
Olympus	Tokyo	Japan
Perkin Elmer	Waltham, MA	USA
Promega	Madison, WI	USA
Qiagen	Venlo	Netherlands
Roche	Indiana	USA
Santa Cruz Biotechnology	Dallas, TX	USA
Sarstedt	Numbrecht	Germany
Sigma-Aldrich	St. Louis, MO	USA
Thermo Fisher Scientific	Waltham, MA	USA

2.2 General reagents

2.2.1 Infectious cDNA clones

The replication-competent wild-type overlength HBV constructs (1.3mers) for genotypes C2 and D3 (in the pUC57 vector backbone) were previously generated by the lab and utilised in this project. These constructs contain two copies of the viral transcription enhancers I and II as well as the gene encoding HBx. Equivalent genome-length constructs were generated by Genscript for both genotypes, based on the Günther PCR product sequence. Transcription from these plasmids occurred from the native promoters.

Additional variants used in the study (based on the above clones):

- **HBxΔ**, D3 1.3mer, C2 and D3 1.0mer; contains a mutation of the starting AUG of the gene, abrogating the starting Methionine residue (ATG to TTG)
- **BCP^{A1762T/G1764A}**, D3 1.3mer and 1.0mer; contains a double mutation in the basal core promoter region also resulting in changes of HBx gene at residues 130/131
- **HBx^{R96A}**, D3 1.3mer and 1.0mer, contains mutation resulting in a R->A change at residue 96 of HBx gene
- **HBx^{R96E}**, D3 1.3mer and 1.0mer, contains mutation resulting in a R->E change at residue of 96 of HBx gene

2.2.1.1 Preparation of 1.0mer HBV for transfection

HBV 1.0mer constructs must be enzymatically digested prior to transfection, in order to excise the HBV genome from the vector backbone. Approximately 40 µg of plasmid DNA was incubated with 5-7 µl SapI (10 U/µl, NEB) and 10 µl 10x CutSmart Buffer in total volume of 100 µl, for 3 hours at 37°C. The reactions were heat-inactivated by incubation at 65°C for 20 minutes. Two microlitres of the enzyme reaction was used to verify the enzyme digestion by electrophoresis on a 1% agarose gel at 80V for approximately 1 hour. Once confirmed, the reactions were cleaned and concentrated prior to transfection using the Wizard® SV Gel and PCR Clean-Up System (Promega), according to the PCR clean-up protocol. Entire enzyme digest reactions were mixed with an equal volume of Membrane-binding Buffer and loaded onto a single column, DNA was eluted in 20 µl nuclease-free water to ensure a final concentration of at least 1.0 µg/µl. Eluted DNA from multiple columns/digests were pooled together and used to transfect HepG2/Huh7 cells.

2.2.2 Protein expression plasmids

The pCI vector was used for generation of all protein expression plasmids, in which expression of the genes of interest were under control of the CMV promoter. Traditional cloning methods were used to add a single FLAG tag to either terminal of the genotype D3 wild-type HBx construct for immunoprecipitation experiments. DDB1 construct was tagged with c-Myc peptide for immunoprecipitation.

- **HBx^(WT)**, A1, A2, C2 and D3; consisting of the wild-type sequences of the full HBx genes, as extracted from the sequences of the 1.3mer infectious clones for the specified genotypes
- **HBx^{BCP}**; consists of full HBx gene with double BCP A1762T/G1764A mutation which elicits K130M/V131I residue changes in HBx gene, genotype D3 backbone

- **HBx^{R96E/R96A}**, C2 and D3; consists of full HBx gene with mutations that change arginine residue 96 of HBx gene to either alanine or glutamate
- **HBx^{96/8/9A}**, C2 and D3; consists of full HBx gene with mutations that change arginine residues at position 96 and 98, and glycine at 99 of HBx gene to alanine
- **DDB1^{2myc}**, full length DDB1 gene (accession #NM_001923) with a double c-Myc peptide (EQKLISEEDL) attached at the N terminus, separated from the gene by a (GAGA_) linker sequence
- **HBs-myc**, full HBV small surface gene with c-Myc peptide attached at the N-terminus

All plasmids were sequenced and verified against the appropriate reference sequences.

2.2.3 Preparation of media and reagents

All reagents were prepared in Ultrapure water unless otherwise stated.

Table 2.1 Composition of prepared solutions

Solution	Contents
LB agar	1% (w/v) tryptone (BD Biosciences) 0.5% (w/v) yeast extract (BD Biosciences) 1% (w/v) NaCl (Merck Millipore) 1.5% (w/v) agar (Thermo Fisher Scientific) 100 µg/ml ampicillin (Sigma Aldrich)
LB-broth	1% (w/v) tryptone (BD Biosciences) 0.5% (w/v) yeast extract (BD Biosciences) 1% (w/v) NaCl (Merck Millipore) 100 µg/ml ampicillin (Sigma-Aldrich)
1% Agarose gel	1% (w/v) agarose (Promega) 1x TAE buffer (Bio-Rad) 3 µl ethidium bromide (Sigma-Aldrich)/100ml agarose
Cracking Buffer	1 M NaOH 10% SDS 0.5 M EDTA - Bromocresol Green
Laemmli buffer	10% SDS (MP Biomedicals) 40% glycerol (Sigma-Aldrich) 0.05% bromophenol blue (Merck Millipore) 25% β-mercaptoethanol (Sigma-Aldrich) 0.25 M Tris pH 6.8
SDS-PAGE Tris/glycine running buffer	10% (v/v) 10x Tris/glycine/SDS buffer (Bio-Rad)
Transfer buffer	10% (v/v) 10x Tris/glycine buffer (Bio-Rad) 20% (v/v) methanol (Thermo Fisher Scientific)
0.1% PBS-T	10% (v/v) 10x PBS 0.1% Tween-20 (Sigma-Aldrich)
Blocking agent (Western blot)	5% (w/v) skim milk powder - Dissolved in 0.1% PBS-T (above)
HepG2/HEK-293 maintenance media	- MEM (Gibco) 10% (v/v) Foetal Calf Serum (Bovogen) 100 U/ml Penicillin/Streptomycin (Gibco) 2 mM L-glutamine (Gibco)
Huh7 maintenance media	- DMEM (Gibco) 10% (v/v) FCS (Bovogen) 100 U/ml Penicillin/Streptomycin (Gibco)
HepAD38 maintenance media	- DMEM/F12 (Gibco) 10% (v/v) FCS (Bovogen) 100 U/ml Penicillin/Streptomycin (Gibco) - Geneticin
HepG2-hNTCP maintenance media (before experiment)	- Huh7 maintenance media (above) 5 µg/ml Puromycin (Gibco)

HepG2-hNTCP seeding media	• Huh7 maintenance media
40% PEG	• 40 g PEG 8000 • Dissolved in 100 ml PBS
HepG2-NTCP replicative/maintenance media (post-infection)	• Huh7 maintenance media • 2.5% (v/v) DMSO (Sigma-Aldrich)
Cell Lysis Buffer (NP-40 lysis buffer)	• 10 mM Tris, pH 7.5 (Merck Millipore) • 1 mM EDTA, pH 8.0 (Thermo Fisher Scientific) • 50 mM NaCl (Merck Millipore) • 0.5% NP-40 (Sigma Aldrich)
Western lysis buffer	• 150 mM NaCl (Merck Millipore) • 50 mM Tris, pH 7.5 (Merck Millipore) • 1% (v/v) NP-40 (Sigma Aldrich)
Glycerol mounting medium	• 90% glycerol (Sigma-Aldrich) • 20 mM Tris pH 8.0
Western blot stripping buffer	• 200 mM Glycine (BDH Prolabo) • 0.1% SDS (MP Biomedicals) • 1% Tween-20 (Sigma Aldrich) • Adjust to pH 2.2
Blocking agent (immunofluorescence)	• 1% BSA (Sigma-Aldrich) • in PBS
cccDNA Lysis Buffer	• 10 mM Tris-HCl, pH 7.4 • 10 mM EDTA • 0.5% SDS
5x Stop Buffer	• 2.5% (w/v) SDS • 100 mM Tris, pH 7.5 • 125 mM EDTA
Liver homogenization buffer	• 50 mM Tris, pH 7.4 • 10 mM EDTA
Gel Soak 1	• 0.5 M NaOH • 1.5 M NaCl
Gel Soak 2	• 0.5 M Tris, pH 7.5 • 1.5 M NaCl
10x SSC	• 1.5 M NaCl • 0.15 M Sodium citrate
Southern Wash Buffer 1	• 2x SSC • 0.1% SDS
Southern Wash Buffer 2	• 0.1x SSC • 0.1% SDS

2.3 Preparation of plasmid constructs

2.3.1 Preparation of vector and inserts for cloning

2.3.1.1 PCR for insertion

PCR amplification was used to generate epitope-tagged gene fragments (eg HBx-FLAG). Primers were obtained from Integrated DNA Technologies (IDT), and designed such that a single FLAG epitope (DYKDDDDK) was added onto either the N- or C-terminal of the HBx sequence.

Reaction mix (for 50µl reaction):

Reagent	Volume (ul)
10x buffer (containing 15 mM MgCl ₂)	5.0
dNTPs (20 mM)	0.4
Forward primer (20 uM)	0.5
Reverse primer (20 uM)	0.5
HotStar Taq Plus (Qiagen)	0.4
Template DNA	4.0
NFW	39.2

Thermocycling conditions:

Denaturation	95°C	5 min	} 40 cycles
Denaturation	94°C	30 sec	
Annealing	55°C	30 min	
Extension	72°C	40 sec	
Extension	72°C	10 min	
Hold at 10°C			

2.3.1.2 Site-directed mutagenesis

Site-directed mutagenesis using the Quikchange Lightning Site-Directed Mutagenesis Kit (Stratagene) was used to introduce point mutations into existing constructs (eg 1.0mer C2 HBxΔ from WT construct). Primers were designed according to manufacturer's guidelines and obtained from IDT. PCR reactions were prepared according to manufacturer's instructions. Briefly, the protocol involves PCR, digestion with *DpnI* restriction enzyme to digest parental dsDNA, and transformation into XL10-Gold ultracompetent cells. Clones were then checked as described below in Section 2.3.4.

2.3.1.3 Restriction enzyme digestion

Vector and Inserts were digested with the appropriate enzymes (restriction sites for these were incorporated into the inserts by the specifically designed PCR primers) to provide 'sticky ends' for ligation. Double digests were set up when the buffers for both enzymes were compatible, otherwise sequential digests with each individual enzyme was performed. Digests were typically performed at 37°C for 2 hours. Digested DNA was separated by electrophoresis on a 1% agarose gel and the required fragments were obtained for ligation by gel-purification

using the Wizard SV Gel and PCR Clean-Up System (Promega) according to manufacturer's protocol.

2.3.2 Ligation

Ligation reactions were prepared to replace mutated or epitope-tagged genes of interest with the original fragments from the vector backbone. This process utilises a DNA ligase to form bonds between nucleotides at the nick site. Ligation reactions typically used 3 Weiss units of T4 Ligase (Promega), with DNA at a 3:1 insert:vector ratio and were performed 1hr at room temperature or overnight at 4°C.

In some cases, rather than direct cloning into chosen vectors, PCR products were first subcloned into the pGEM-T Easy vector (Promega) with ligations performed according to manufacturer's protocol. Minipreps were prepared from bacteria transformed with the ligated vector, the mutated insert of interest was digested with appropriate enzymes, and swapped out with the original insert in the target (template) plasmid (digested with the same enzymes). Transformation and identification and verification of potential clones is described below.

2.3.3 Bacterial transformation

50µl of competent DH5α E.coli cells (Invitrogen) were transformed using a heat shock method. The entire ligation reaction was added to the cells and the reaction was incubated for 20 minutes on ice. To induce plasmid uptake, cells were heat-shocked for 45 seconds at 42°C, followed by 2 minutes incubation on ice. 200 µl of super optimal broth with catabolite repression (S.O.C) medium (Invitrogen) was added to the transformation reaction and incubated at 37°C for 45-60 minutes in a shaking incubator, and subsequently diluted 1:1000 in S.O.C. medium. 100 µl of this reaction was spread over a Luria Bertani (LB) agar plate containing 100 µg/ml ampicillin (Sigma-Aldrich) and incubated at 37°C overnight. A single bacterial colony was picked and re-inoculated onto a fresh plate the following day and incubated under the same conditions.

2.3.4 Checking potential clones

2.3.4.1 "Pick and patch" plate

To screen for bacteria transformed with successfully ligated constructs, scrapings from single colonies grown on the transformation plate, were used to inoculate small patches on a fresh agar plate. Plates were incubated overnight at 37°C.

2.3.4.2 Cracking gel

30ul of cracking buffer (Table 2.1) was added to 1.5ml tubes. Sterile tips were used to scrape bacteria from a colony of the pick and patch plate and added to the tube with cracking buffer. Tips were swirled/mixed in the buffer, removed and the lids of the tubes were closed. This process was repeated until all patches had been scraped. Tubes were incubated at 65°C for 30 minutes. A 1% agarose gel containing EtBr was prepared and samples were loaded with the gel on the bench. The gel was placed in the gel tank and TAE buffer was added so that it reached the top of the gel without covering the wells. Gel was run for 5 minutes at 50V until the dye front had moved out of the wells, before TAE buffer was topped up and voltage increased to 100V until the dye front had migrated the length of the gel. Gel was imaged on the ChemiDoc

MP instrument and potential positive clones were identified by slower migration compared to the undigested vector only control.

2.3.4.3 Mini cultures

Mini cultures were prepared for further selection of potential clones by inoculating bacteria (taken from pick and patch plates) in 5ml LB broth (Amp+) and incubating on a shaking platform overnight at 37°C. The following day, plasmids were isolated using the Wizard Plus SV Miniprep DNA Purification System according to manufacturer's protocol. 1.5 ml of the mini culture was centrifuged in a 2ml tube for 5 minutes at 13,000 rpm to pellet cells and DNA was eluted in 50ul nuclease-free water. Successful cloning was subsequently confirmed by enzyme digest to check correct sizing, or alternatively by sequencing of the construct.

2.4 Generation of plasmid stocks for transfection

2.4.1 Bacterial transformation

Bacterial transformation were performed as described in Section 2.3.3. For transformations with plasmid DNA, approximately 200 ng of DNA was used to transform 50 µl of competent cells.

2.4.2 Maxi preparation of plasmid DNA

A scraping of a single bacterial colony was inoculated in 3 ml LB broth and incubated at 37°C for 8 hours in a shaking incubator. 1 ml of the starter culture was used to inoculate 150 ml LB broth with 100 µg/ml ampicillin (Sigma-Aldrich) and incubated at 37°C overnight in a shaking incubator. Plasmid DNA was extracted using the Qiafilter Plasmid Maxi Kit (Qiagen) according to the manufacturer's protocol, with the following modifications:

- Bacterial cells were harvested by centrifugation at 4000 rpm for 10 minutes rather than at 6000x g for 15 minutes
- Following precipitation with Buffer P3, the lysate was centrifuged at 4000 rpm for 10 minutes, rather than incubating at room temperature for 10 minutes before transferring to a filter Cartridge
- Purified DNA was resuspended in 200 µl of Endotoxin-free Buffer TE or Nuclease-Free Water (NFW, Promega)

2.4.3 Glycerol stocks

700µl of log-phase maxi-preparation culture was added to 300 µl of 80% glycerol (Sigma-Aldrich), and immediately stored at -70°C for long-term storage of bacterial cultures harbouring plasmids of interest.

2.4.4 Spectrophotometry

2µl of prepared maxi DNA was used to estimate the DNA concentrations by spectrophotometry using the NanoPhotometer (Implen) as per manufacturer's instructions. Maxi DNA can then be digested with restriction enzymes or sequenced to verify the correct clone/sequence.

2.5 PCR and sequencing

PCR and sequencing reactions were performed on the (Bio-Rad PTC-200 DNA Engine and S1000, Eppendorf Mastercycler Gradient thermal cyclers). Sequences were analysed using Lasergene's DNASTAR suite programs including SeqMan, EditSeq and MegAlign.

Table 2.2 Sequences of primers used

Primer	Application	Sequence (5' to 3')
pCISeq (F)	Sequencing	TAATACGACTCACTATAGGC
pCISeq (R)	Sequencing	TCCATTCGCCATTCAGGCTGCGC
M13 (F)	Sequencing	GTAAAACGACGGCCAG?
M13 (R)	Sequencing	CAGGAAACAGCTATGAC?
865	Sequencing	TTCGGAGTGTGGATTGCGAC
1245	Sequencing	GCCTGCTAGGTTCTATCCTAACC
1437	Sequencing	CATGCTGTAGCTCTTGTTC
JM	Sequencing	TTGGGGTGGAGCCCTCAGGCT
2074	Sequencing	CCCTAGAAAATTGAGAGAAGTC
1798	Sequencing	CCACTGCATGGCCTGAGGATG
Seq2	Sequencing	TTGGCCAAAATTCGCAATC
OSX1	Sequencing	TTTTCTTTTGTCTTTGGGTAT
TTA4	Sequencing	GAAAATTGGTAACAGCGG
2254	Sequencing	GAAGATGAGGCATAGCAGCAGG
OSX4	Sequencing	GCTCCAGACCGGCTGCGAGCAA
SeqP2	Sequencing	AAAAAGTTGCATGGTGCTGG
644(F)	Sequencing	AAGCTTCCATGGTGATCGCAGTCCC
1479(F)	Sequencing	CTCTGGTCAGTGAATGGAAGGAGCC
2202(F)	Sequencing	ACCAGGAAGTGTCCTAGTGTTCGG
2810(F)	Sequencing	TCAGTGCTGCTGCTTGCCTACAAGC
948(R)	Sequencing	GTCTCTCCAAGGAGTTCTACACGG
1663(R)	Sequencing	TAATGGGGTGATGTCCAAGCAAGCC
2374(R)	Sequencing	TCCAAAGGAGGTCTCATGAGGAGC
3255(R)	Sequencing	TGAAAGGATCTCCAGAAGGAGTGC
1075	PCR (S ^{thn} probe)	AAGGTGGGAACTTTACTGGGC
PC2	PCR (S/N ^{thn} probe)	GGCAAAAACGAGAGTAACTC
2954	PCR (N ^{thn} probe)	ATATGAACCTTTACCCCGTTGC
C2 HBxR96E (F)	PCR (SDM)	GCCCAAGGTCTTACATAAGGAGACTCTTGGACTCTCAGC
C2 HBxR96E (R)	PCR (SDM)	GCTGAGAGTCCAAGAGTCTCCTTATGTAAGACCTTGGGC
C2 HBx KO (F)	PCR (SDM)	CGGAAGTACACCTCCTTTCTTGGCTGCTAGGGTGTGC
C2 HBx KO (R)	PCR (SDM)	GCACACCCTAGCAGCCAAGGAAAGGAGGTGACTTCCG
D3 HBx N_Flag (F)	PCR (Cloning)	GATCCTCGAGACCATGGACTATAAAGACGACGATGACA AAGCTGTTAGGC
D3 HBx N_Flag (R)	PCR (Cloning)	GATCTCTAGATTAGGCAGAGGTGAAAAAGTTGC
D3 HBx C_Flag (F)	PCR (Cloning)	ATATCTCGAGACCATGGCTGCTAGGC
D3 HBx C_Flag (R)	PCR (Cloning)	ACTGTCTAGATTATTTGTCATCGTCGTCTTTATAGTCGGC AGAGGTG
P1	PCR (GUN PCR)	CCGGAAAGCTTGAGTCTTCTTTTACCTCTGCCTAATCA
P2	PCR (GUN PCR)	CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCATGGTGCTG G

2.5.1 PCR amplification

2.5.1.1 *Günther* PCR

This method has been adapted from Günther et al. (1995) which described amplification, sequencing and analysis of full-length HBV genomes.

Reaction mix (For 50µl reaction):

Reagent	Volume (µL)
Nuclease-free water	39.2 µL
10x buffer (containing 15 mM MgCl ₂)	5.0 µL
P1 primer (20 µM)	0.4 µL
P2 primer (20 µM)	0.5 µL
HotStarTaqPlus (Qiagen)	0.5 µL
DNA template	4 µL

Thermocycling conditions

Denaturation	94°C	15 min	} 40 cycles
(shift to 60°C in 50 sec)	94°C	40 sec	
Annealing	60°C	1 min	
(shift to 72°C in 15 sec)			
Extension	72°C	4 min	
With an increment of 5 sec/cycle for 40cycles			
Hold at 10°C			

2.5.1.2 Sequencing PCR

Automatic sequencing was carried out using the BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) to verify plasmid sequences.

Reaction mix (For 12 µl reaction):

Reagent	Volume (µL)
Nuclease-free water	To 12 µL total volume
10x buffer (containing 15 mM MgCl ₂)	2.0 µL
primer (0.8 µM)	4.0 µL
Big Dye Terminator Ready Reaction Mix	2.0 µL
DNA template	200-400 ng

Thermocycling conditions

Denaturation	96°C	10 sec	} 30 cycles
Annealing	50°C	5 sec	
Extension	60°C	4 min	
Hold at 10°			

Reactions were subsequently precipitated as outlined below

2.5.1.3 Precipitation

Prepared reactions of 8 µl NFW, 2 µl sodium acetate (3 M), 50 µl 96% ethanol (Merck Millipore) and the entire PCR reaction (12 µl) was incubated at room temperature for 20 minutes followed by centrifugation for 20 minutes at full speed. The supernatant was aspirated and washed with 150 µl 70% ethanol (Merck Millipore). The reaction was centrifuged at full speed for 5 minutes, the supernatant removed and the pellet allowed to dry for 10 minutes at 37°C. Reactions were sent as dried pellets to the Micromon DNA Sequencing Facility in the Department of Microbiology, Monash University for gel electrophoresis.

2.5.1.4 1.3mer fragment PCR for sequencing

1.3-mer infectious clones contain two precore regions so in order to confirm the maxi DNA, PCR was required to amplify the two segments for sequencing. Primers used yielded two fragments of 2.6 kb and 1.9 kb in length.

Reaction mix (For 50µl reaction):

Reagent	Volume (µL)
Nuclease-free water	To 50 µL
10x buffer	5.0 µL
dNTPs (20 mM)	0.4 µL
Pc/BacF2 or OSC2 primer (20 µM)	0.5 µL
OS1 or X1 primer (20 µM)	0.5 µL
HotStarTaqPlus (Qiagen)	0.4 µL
Add 100 ng DNA template	

Thermocycling conditions

Denaturation	94°C	3 min	} 30 cycles
	94°C	30 sec	
Annealing	55°C	30 sec	
Extension	72°C	3 min	
	72°C	5 min	
Hold at 4°C			

2.5.2 Agarose gel electrophoresis

PCR amplicons were analysed by gel electrophoresis. 5 µl of PCR product was loaded into a horizontal agarose gel and run in parallel with the GeneRuler 1 kb DNA ladder (Thermo Fisher

Scientific). Gels were electrophoresed in TAE buffer (Bio-Rad) at 100V until the dye-front was half way down the gel and visualised using the ChemiDoc™ MP system (BioRad).

2.5.3 DNA purification and sequencing

2µl of ExoSAP-IT reagent (Affymetrix) was mixed with 5 µl of PCR product, incubated for 15 minutes on a 37°C heat block, followed by inactivation by 30 min incubation at 80°C.

Sequencing reactions were carried out as described Section 2.5.1.2.

2.6 Cell lines

2.6.1 HepG2 cell line and maintenance

HepG2 is an immortalised hepatoma cell line, derived from a hepatoblastoma of a 15-year-old Caucasian male. This cell line was selected for utilisation in transfection experiments. HepG2 cells were maintained in sterile culture vessels in supplemented Modified Eagle Medium (MEM, Gibco), described in Table 2.1. Cells were cultured at 37°C in a humidified incubator (5% CO₂) with media collected and/or replaced every 3-4 days as required.

2.6.2 Huh7 cell line and maintenance

Huh7 is an immortalised hepatoma cell line, derived from a well-differentiated hepatocellular carcinoma of a 57-year old Japanese male. This cell line was selected for utilisation in transfection experiments. Huh7 cells were maintained in sterile culture vessels in supplemented Dulbecco's Modified Eagle Medium as described in Table 2.1. Cells were cultured at 37°C in a humidified incubator (5% CO₂) with media collected and/or replaced every 3-4 days as required.

2.6.3 HepG2-hNTCP cell line and maintenance

The HepG2-NTCP cell line, kindly gifted by Stephan Urban (University Hospital Heidelberg, Heidelberg, Germany), are HepG2 cells stably transfected with human NTCP. The continuous selection of the NTCP required the presence of puromycin (Gibco) in the culture medium (HepG2-hNTCP maintenance media, Table 2.1). Expression of NTCP makes these cells permissive for HBV infection. Cells were culture cultured at 37°C in a humidified incubator (5% CO₂) with media collected and/or replaced every 3-4 days as required.

2.6.4 HepAd38 cell line and maintenance

HepAD38 is a HepG2-derived cell line which has been stably transfected with one cDNA copy of the HBV pgRNA, under the control of a tetracycline-responsive promoter. Addition of tetracycline to the medium suppresses HBV replication. Replicative intermediates and mature virions are produced upon removal of tetracycline. This cell line was used for generation of viral inoculum in infection experiments, as well as replication controls alongside transient transfection of infectious clones. AD38 cells were maintained in sterile culture vessels in supplemented Dulbecco's Modified Eagle Medium/F12 Medium as described in Table 2.1. Cells were cultured at 37°C in a humidified incubator (5% CO₂) with media collected and/or replaced every 3-4 days as required.

2.6.5 Cell passaging and maintenance

One hundred millimetre dishes of cells were washed with 5 ml sterile phosphate buffered saline (PBS) before being trypsinised with 2 ml 0.5% trypsin-EDTA (Gibco) at 37°C for 4 minutes. Eight millilitres of media was added to inactivate trypsin, and cells were resuspended in a volume of fresh media dependent on the passaging ratio. Confluent 100mm dishes of cells were split and re-seeded into smaller culture dishes or plates on the day preceding transfection. Media was replaced prior to transfecting the cells.

2.7 Transient transfection

2.7.1 Transient transfection protocol

Transient transfection of HepG2 and Huh7 cells were performed using the FuGENE 6 Transfection Reagent (Promega), which had already been optimised through prior studies within VIDRL to establish the amount of DNA and FuGENE required per transfection for achieving maximum efficiency.

Table 2.3 (a): Ratios used for transfection of HepG2 cell line

Type of dish	Amount of DNA (μg)	Amount of FuGene (μl)	Amount of OptiMEM (μl)
100 mm	10.8	32.8	200
60 mm	4.0	12	100
6-well plate (per well)	1.8	5.4	50
12-well plate (per well)	0.84	2.5	50

Table 2.3 (b): Ratios used for transfection of Huh7 cell line

Type of dish	Amount of DNA (μg)	Amount of FuGene (μl)	Amount of OptiMEM (μl)
100 mm	10.8	26	200
60 mm	4.0	9.6	100
6-well plate (per well)	1.8	4.3	50
12-well plate (per well)	0.84	2.0	50

Cells were seeded into the appropriate-sized culture vessels as required for the specific experiments conducted. Seeding was performed to ensure 50-80% confluency for transfection the following day. Transfection reactions were prepared in polystyrene tubes. Required volumes of Opti-MEM were aliquoted into a tube, to which the corresponding volume of Fugene was added. These reagents were mixed by gently shaking the tubes, and incubated at room temperature for 5 minutes. The required amount of DNA for transfection was added to a second tube, and the Fugene-Opti mix was transferred to this after the 5 minute incubation. The reaction was mixed by gently shaking the tubes, and incubated at room temperature for 20 minutes. During this time, cells were replenished with fresh media and the transfection reaction was added drop-wise into the culture vessels. Cells were incubated overnight under standard conditions, and media was replaced the following day.

2.7.2 Transfection efficiency

To assess transfection efficiency, transfection experiments were set up in 12-well plates wherein plasmids of interest were co-transfected with 0.1 μg of pRL-TK vector (Promega; Cat no: E224A). Three days post-transfection, cells were lysed and assayed according to the instructions of the Renilla Luciferase Assay System (Promega). This system is designed to provide a fast and sensitive method of detecting sea pansy (*Renilla reniformis*) luciferase, enabling the measurement of Renilla Luciferase activity as a co-reporter for normalisation of experimental variations such as differences in transfection efficiencies. Luminescence was measured using the FLUOstar OPTIMA luminometer (BMG Labtech).

2.8 Protein analysis

2.8.1 Serological testing

Quantitative serological testing was kindly performed on supernatant samples, courtesy of Mr. Hugh Mason (VIDRL) who is trained and experienced on the use of the Cobas e 411 instrument (Roche). Levels of secreted antigens in cell culture supernatant were tested using the commercially available quantitative *Elecsys HBsAg II qant II* and *HBeAg* assays, and results were obtained by calibrating through HBeAg Paul Erlich International reference standard. HBsAg values were converted to international units/ml (IU/ml) by dividing by 18.21, and HBeAg results (PE IU/ml) were obtained by calibrating output values through HBeAg Paul Erlich International reference standard.

2.8.2 Western blot

2.8.2.1 Harvesting protein lysates from transfected cells

Media was removed from transfected cells and cells were washed with ice-cold PBS. Cold western lysis buffer (Table 2.1) supplemented with Pierce Protease/Phosphatase Inhibitor Mini tablets (Thermo Fisher) was added to the cells and incubated at 4°C for 15 minutes on a rocking platform. Cell lysates were collected into pre-chilled 1.5 ml tubes and centrifuged at 13,000xg for 20 minutes at 4°C. The supernatant was transferred to fresh tubes and stored at -20°C until required. Prior to SDS-PAGE, samples were mixed with an appropriate volume of Laemmli buffer and boiled for 5 minutes.

2.8.2.2 Immunoprecipitation

Immunoprecipitation was performed on cell lysates harvested from 2-3 60mm dishes of transfected cells, in a total volume of 500 µl-1ml of western lysis buffer. Antibody was added to lysates according to recommended manufacturer concentrations, and incubated at 4°C for 2 hours on a rotating mixer. 50 µl of Protein G Dynabeads (Thermo Fisher) per sample, was aliquoted into separate tubes and separated using a magnetic rack. The supernatant was aspirated and beads were resuspended in PBST, which was then aspirated before adding the lysate-antibody mix to the beads. The samples were then incubated at 4°C for 1 hr on a rotating mixer. Following separation using the magnetic rack, the lysates were aspirated and beads were washed three times in PBS. Beads were resuspended in 100 µl PBS, before being transferred to fresh tubes. The tubes were placed on the magnetic rack, PBS aspirated and the beads resuspended in 30 µl lysis buffer. An appropriate volume of Laemmli buffer was added to the samples and then boiled for 5 minutes. The beads were separated using the magnetic rack once again and samples were transferred to fresh tubes and stored at -20°C, ready for SDS-PAGE.

2.8.2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Lysates were prepared for SDS-PAGE by being boiled in Laemmli buffer prior to loading. The samples were loaded onto a vertical pre-cast gel (Any kD/4-15% Mini-PROTEAN TGX/Stain-Free Precast Protein Gels, Bio-Rad) and electrophoresed together with the MagicMark XP Western Protein Standard (Invitrogen) and/or Western C Precision Plus Protein Standard (BioRad) to determine band size. The gel was then placed into a tank with SDS-PAGE running buffer (Table 2.1) and electrophoresed at 150V for approximately 45 minutes, until the samples had run the

length of the gel. When required, gels were imaged for total protein using the Stain-Free protocol on the ChemiDoc (Stain-Free gels only). For Protein transfer, the gel was placed on top of a nitrocellulose membrane (BioRad) and sandwiched between extra thick filter paper (BioRad) pre-soaked in transfer buffer (Table 2.1) and transferred using the Trans-Blot Turbo Transfer System (BioRad). Unless otherwise stated, transfers were performed with the pre-programmed standard semi-dry protocol (25V, 30 minutes).

2.8.2.4 Immunoblotting

Following transfer, the membrane was blocked overnight in 5% skim milk powder in 0.1% PBS-Tween (PBS-T) at 4°C on a rocking platform. The membrane was then incubated in a 1:1000 dilution of primary antibody in 1% skim milk diluted in PBS-T for 1.5 hours on a shaking platform or 4°C overnight, then washed 3 x 10 minutes with PBS-T. The process was repeated with a secondary antibody conjugated to horseradish peroxidase (HRP) (Dako) and detection was performed by enhanced chemiluminescence (ECL) using the Western Lightning Chemiluminescence Kit (Perkin Elmer), and visualised using the ChemiDoc MP system and Image Lab software (BioRad).

Table 2.4 Antibodies used in western blot/immunoprecipitation experiments

Antibody	Supplier/Manufacturer	Target
Mouse anti-HBx (X36C)	Santa Cruz	HBx protein
Mouse anti-myc (9B11)	Cell Signalling Technology	c-myc epitope (EQ
Mouse 7e9	-	Precore peptide
Mouse ID8	-	HBc protein
Mouse H166	-	HBs proteins
Mouse anti-B-actin-HRP	Santa Cruz	B-actin
Mouse anti-HBx (gt D)	Gilead (gifted)	HBx protein

2.8.2.5 Loading controls

After ECL detection, membranes were rinsed in PBS-T on a shaking platform for 5 minutes, then incubated in stripping buffer (Table 2.1) twice, for 10 minutes each time. 2x 10 minute washes with PBS, and 2x 5 minute washes with PBS-T then followed, before blocking and immunoblotting as described above. To determine protein loading, blots were probed with a mouse beta-actin HRP antibody, incubated 1hr on a shaking platform.

2.8.3 Kinexus sample preparation

HepG2 were seeded, transfected and maintained as per usual protocol and processed as required for subsequent Kinexus analysis, according to instructions provided, briefly described below.

2.8.3.1 Kinex KAM-900P/1325 Microarray

Following transfection, media was aspirated from cell culture dishes and cells were washed with ice-cold PBS and subsequently lysed in provided Kinexus Lysis Buffer containing DTT and protease and phosphatase inhibitor tablets. Cell lysates collected into 1.5 ml tubes and homogenised by passing through 18- and 26-gauge needles until sample was no longer viscous. Homogenate was incubated in a 45°C water bath for 30 minutes and clarified by

centrifugation at 15,000 rpm for 30 minutes. Resulting supernatant was transferred to new tubes and subsequently stored at 4°C before being shipped at room temperature to Kinexus Bioinformatics (Vancouver, Canada) for analysis.

2.8.3.2 *Potential candidate selection*

For identification of potential candidates representing substantial modulation for follow-up investigation, a series of parameter thresholds were applied to the data to filter out marginal effects or spurious/doubtful results based on evaluation of quality. The four basic criteria that need to be satisfied for further consideration are as follows:

Parameter	Result required for “Potential” designation	Notes
Flag	0,0	Indicates acceptable replicate spots
Signal Intensity	>1000	Indicates stronger signals
%Error	<50	Indicates the replicability of duplicate spots
%Change from Control (CFC)	<-25 or >50	Indicates more substantive changes from the control sample

Below is a brief summary description of each selected Parameter according to Kinexus...

Flag: An indication of quality of the spot, based on its morphology and background. The flagging codes used in the reports are as follows:

0: acceptable spots

1: spots manually flagged for reasons and may not be very reliable

Signal Intensity: for the selection of antibodies for follow on validation studies, it is recommended that antibodies not only demonstrate large changes, but also strong spot intensities. This will improve the prospects for successful detection by western blotting.

%CFC: the percent change of the test sample in Signal Intensity from the specified control (mock)

%Error: a parameter to show how tightly the Signal Intensity for adjacent duplicate spots of the same protein in the sample compare to each other

2.8.3.3 Gene ontology annotation analysis

The open -source web application GOnet (available at <http://tools.dice-database.org/GOnet/>) was used to perform GO term annotation analysis. GOnet provides insight into the functional interconnection of the submitted entries (identified potential candidates). Default parameters were used.

2.9 DNA analysis

2.9.1 DNA extraction

2.9.1.1 Extraction of intracellular core-associated HBV DNA (core prep)

Cells were harvested for intracellular core-associated HBV DNA from 60 mm dishes 5 days post-transfection. Supernatant was collected or aspirated from transfected dishes and cells were washed with 2 ml PBS. 500 µl of Cell Lysis Buffer [Table 2.1] was added and dishes were incubated on a shaking platform for 15 min at room temperature. Lysates were then collected into 1.5 ml tubes and centrifuged at 13,000 rpm (16,000g) for 5 minutes to pellet the nuclei. The lysate (supernatant) was then transferred to a 2 ml safe-lock tube, and incubated with 3 µl (30U) of DNase I and 5 µl of 1 M MgCl₂ for 1 hr at 37°C. The DNase I digest was then repeated once more, as described. Following digest, 110 µl of 5x Stop buffer [Table 2.1] was added, and samples were treated with Proteinase K (final concentration of 0.5 mg/ml) at 37°C overnight. The following day lysates were removed from the heat block, pulsed and subjected to 2x phenol and 1x chloroform extraction. The DNA in the resulting aqueous phase was then precipitated at -20°C overnight with 0.7x volume isopropanol and 5 µl glycogen (2 mg/ml). The following day, the DNA was pelleted by centrifugation for 10 mins and washed with 70% ethanol. After centrifugation for 3 min, the ethanol was aspirated and the pellet allowed to dry at room temperature, at which point they were resuspended in 27 µl nuclease-free water and 3 µl 10x loading dye, ready for southern blot analysis

2.9.1.2 Extraction of cccDNA (Hirt extraction)

Cells were harvested for nuclear, protein-free HBV DNA from 60 mm dishes 5 days post-transfection. Supernatant was collected or aspirated from transfected dishes and cells were washed with 2 ml PBS. 600 µl of cccDNA Lysis Buffer [Table 2.1] was added and a cell lifter was used to scrape cells from dish, lysates being transferred to 1.5 ml tubes. Two-hundred µl of 2.5 M KCl (final concentration of approximately 0.5 M) was added, mixed well by pipetting and incubated at room temperature for 30 minutes. Samples were then centrifuged for 20 min at room temperature to remove insoluble proteins, the supernatant transferred to a 2 ml safe-lock Eppendorf tube and subjected to 2x phenol and 1x chloroform extraction. The DNA in the resulting aqueous phase was then precipitated at room temperature for 30 min with 2x volume room temperature ethanol and 3 µl glycogen (2 mg/ml). Following precipitation, the DNA was pelleted by centrifugation for 10 mins and washed with 70% ethanol. After centrifugation for 3 min, the ethanol was aspirated and the pellet allowed to dry at room temperature, at which point they were resuspended in 20-40 µl nuclease-free water.

2.9.1.3 cccDNA confirmation

Hirt-extracted DNA was treated in multiple ways in order to confirm expression of cccDNA:

- Heat denaturing: to confirm supercoiled nature of cccDNA, which is unaffected by the temperature, unlike other higher MW intermediates
 - DNA was incubated at 85°C in a heat-block for 5 minutes
 - Sometimes followed by enzyme digestion to linearise
- Linearization: to confirm cccDNA consists of circularised monomeric HBV genome
 - DNA was incubated with EcoRI enzyme at 37°C for 1 hr

- Expect to see a size shift from supercoiled cccDNA to linear genome-length HBV (3.2kb)
- Exonuclease digestion: to confirm covalently closed DNA lacking free ends which are substrates for exonuclease digestion
 - DNA was incubated with a combination of Exonuclease I and III for 3 hours at 37°C
 - Expect cccDNA to largely remain unaffected while most other mediates should be digested away

2.9.2 Southern blotting

2.9.2.1 Agarose gel electrophoresis

1% agarose gel in 1x TAE was prepared by melting in the microwave oven with regular swirling to ensure even mixing and check complete melting. After cooling, the melted agarose was poured into a taped gel tray with double combs and allowed to set. Once set, the combs and tape from both ends of the gel tray were removed, the tray placed in the gel tank and the tank filled with 1x TAE. Samples were loaded into the wells along with DIG-labelled DNA molecular weight markers, and run at 100V until the dye front had migrated to the centre of the gel tray (before the second row of wells).

2.9.2.2 Transfer of DNA to a membrane

Following electrophoresis, the gel was soaked in 0.25 M HCl for 15 mins at room temperature on a shaking platform, when cccDNA was to be detected (results in partial depurination of the DNA fragments). The HCl was poured off and after rinsing with water, Gel Soak 1 solution was added and incubated for 20 min at room temperature. This results in denaturation of the DNA. Gel Soak 2 then replaced Gel Soak 1 for a 30 min incubation for neutralisation. To set up the transfer apparatus, a gel tray was overturned and placed within a larger tray. A piece of Whatman 3MM filter paper wider than the width of the agarose gel was prepared, and placed over the length of the upturned gel tray. This filter paper acts as a wick to draw transfer buffer up through the gel to the membrane. 10x SSC buffer was poured over the wick and to fill the large tray to within 1 cm of the bottom of the gel tray. The gel was placed on top of the wick with the DNA side facing down. A piece of positively charged nylon membrane the same size as the gel was wetted in the 10x SSC and placed on top of the gel using forceps. Air bubbles were removed and excess gel trimmed around the edges of the membrane. A piece of Whatman 3MM filter paper the same size as the gel was wetted in 10x SSC and placed on top of the membrane, air bubbles removed. A stack of paper towels approximately 8 cm high and the same size as the membrane, was stacked on top of the Whatman paper. A small weight was placed on top of the stack and the apparatus left at room temperature overnight. The following day, the paper towel stack and Whatman paper were removed, the membrane sandwiched between some pieces of Whatman filter paper and baked at 80°C for 2 hrs.

2.9.2.3 Preparation of DIG-labelled probes

The Roche PCR DIG Probe DNA synthesis kit was used to label probes generated by PCR using 1.3mer WT genotype D3 in pUC57 vector as the template. This kit involves a PCR assay whereby DIG-11-dUTP is incorporated during the PCR process. To generate a DNA probe to be used on Southern blot, primers 1075 and PC2 were used to generate a 2.5 kb probe which covers most of Pol, X and PC but not core. Prior to first use, PCR products are boiled for 5 min

and quenched on ice for 5 min, before being added to 10 ml (1 blot) or 20ml (2 blots) of prehybridization buffer to prepare the probe. Upon re-use, probes were incubated at 65°C for 10 min and quenched on ice for 5 min.

2.9.2.4 Hybridisation

The nylon membrane was placed in a cylindrical hybridization bottle and incubated with 20 ml DIG EasyHyb (Roche) prehybridization buffer at 42°C for 2 hr. The prehybridization buffer was then replaced with the DIG-labelled HBV DNA probe which had been boiled and quenched on ice. This incubation occurred at 42°C overnight.

2.9.2.5 Detection of DNA

Following overnight hybridisation, the membrane was twice incubated with Southern Wash buffer 1 for 5 min at room temperature, followed by twice with Southern Wash buffer 2 for 15 min at 68°C. The DIG Wash and Block Buffer Set (Roche) was subsequently used for the detection of the southern blot. The membrane was briefly rinsed with appropriately diluted Washing Buffer in a plastic container on a shaking platform. The membrane was then blocked for 2hr at room temperature on a shaking platform, with a solution prepared by diluting the Maleic Acid Buffer and Blocking Solution. Twenty millilitres of this solution was set aside and used to dilute the DIG antibody (Anti-Digoxigenin-AP, Fab fragments, Roche), which the membrane was then incubated with for 30 min at room temperature on the shaking platform. The membrane was then washed twice with the Washing Buffer for 15 min each time, incubated with diluted Detection buffer for 3 min. Finally, the membranes were subjected to ECL detection using CPD-Star reagent (Roche) and ChemiDoc MP imager.

2.10 RNA analysis

2.10.1 RNA extraction

Cells were harvested for intracellular total RNA from 60 mm dishes 5 days post-transfection. The Qiagen RNeasy Mini Kit was used, according to the manufacturer's Animal Cell Spin protocol. RNA was eluted in 2x 30 µl Rnase-free water and stored at -70°C until required. Prior to northern blotting, 10 µl sample of RNA was mixed with 10 µl Glyoxal Load Dye and incubated at 50°C for 30 min, then stored at -20°C, ready for northern blotting.

2.10.2 Northern blotting

2.10.2.1 Agarose gel electrophoresis

1% agarose gel in 1x Gel/Running Prep Buffer (Ambion Northern Kit) was prepared by melting in the microwave oven with regular swirling to ensure even mixing and check complete melting. After cooling, the melted agarose was poured into a taped gel tray with combs and allowed to set. Once set, the combs and tape from both ends of the gel tray were removed, the tray placed in the gel tank and the tank filled with 1x Gel/Running Prep Buffer. Samples were loaded into the wells along with DIG-labelled RNA molecular weight markers, and run at 80V until the dye front had migrated the length of the gel.

2.10.2.2 Transfer of RNA to a membrane

Following electrophoresis, gel transfer was set up as described in the Ambion kit protocol. Briefly, a 3 cm high stack of paper towels cut to the size of the gel, was placed on a plastic tray. Three dry pieces of Whatman 3MM filter paper was cut to the size of the gel and placed on top of the paper towel stack. Two more pieces of filter paper were wetted in Transfer Buffer (Ambion) and placed on top of the stack. This was followed by the agarose gel, three more pieces of wet filter paper, and finally, three longer pieces of filter paper "bridges" which act as a wick drawing the transfer buffer up from the reservoir to the transfer apparatus. Transfer occurred for 2hrs at room temperature, after which the membrane was sandwiched between pieces of Whatman filter paper and baked at 80°C for 30 minutes to cross-link the RNA. Membranes were then stored in a Falcon tube at -20°C until ready, or immediately proceeded to hybridisation.

2.10.2.3 Preparation of DIG-labelled probes

The Roche PCR DIG Probe DNA synthesis kit was used to label probes generated by PCR using 1.3mer WT genotype D3 in pUC57 vector as the template. This kit involves a PCR assay whereby DIG-11-dUTP is incorporated during the PCR process. To generate a DNA probe to be used on northern blot, primers 2954 and PC2 were used to generate a 0.7 kb probe covering the end of pol and X but not surface or core. PCR products were verified by agarose gel electrophoresis and stored at -20°C until required. Prior to first use, PCR products are boiled for 5 min and quenched on ice for 5 min, before being added to 10 ml (1 blot) or 20 ml (2 blots) of prehybridization buffer to prepare the probe. Upon re-use, probes were incubated at 65°C for 10 min and quenched on ice for 5 min.

2.10.2.4 Hybridisation

The nylon membrane was placed in a cylindrical hybridization bottle and incubated with 20 ml DIG EasyHyb (Roche) prehybridization buffer at 50°C for 2 hr. The prehybridization buffer was

then replaced with the DIG-labelled HBV DNA probe which had been boiled and quenched on ice. This incubation occurred at 50°C overnight.

2.10.2.5 Detection of RNA

As previously described for DNA in Section 2.9.2.5

2.11 Experimental infections

2.11.1 Transfections to generate infectious viral inoculum

HepG2 cells were seeded into 100mm cell culture dishes and transfected with a 1.3-mer genotype D wild-type clone the following day. Supernatant was collected every 3 days, with media being replaced until day 20. HepAD38 cells were similarly seeded and maintained in the absence of tetracycline to induce viral production. Supernatant was collected every 3 days and replaced until day 20.

2.11.2 PEG purification of HBV virions

100ml of cell culture supernatant was added to 18 ml of 40% PEG 8000 buffer in a tissue culture flask. The mixture was mixed well and incubated at 4°C overnight. The following day, 29.5 ml of the PEG-precipitated supernatant was transferred into 4x JA-20 tubes and centrifuged at 10,000 rpm for 1hr at 4°C and the supernatant was aspirated as much as possible without dislodging the pellets. The pellets were sequentially resuspended in sterile PBS containing 10% FCS in a total volume of 1 ml (i.e. 1/100th of the starting volume was used to resuspend the first tube, then the resuspended sample was used to resuspend remaining pellets). The inoculum was transferred to a 1.5 ml screw-capped tube and placed on a rotating wheel at 4°C for 6 hrs or overnight, before being centrifuged at 8,000 rpm for 5 min and the resulting supernatant transferred to a new screw-capped tube leaving any pellet/debris behind. The inoculum was stored at 4°C, or -70°C for longer storage.

2.11.3 HBV DNA quantification

Concentrated virus stock was diluted 1:10,000 in universal diluent (Roche) and quantified using the Abbott M2000 SP/RT platform (VIDRL). The resulting viral load was used to calculate the volume of stock needed to infect cells at a multiplicity of infection (MOI) of 1000:

Example calculation

Virus (inoculum) titre: 2.1×10^7 copies/ul

Cell count: 1.5×10^6 cells/60mm dish

$$\left(\frac{1.5 \times 10^6}{2.1 \times 10^7} \right) \times 1000 \text{ (MOI)}$$

= 71 ul of inoculum required per 60mm dish

2.11.4 Seeding HepG2-NTCP cells

HepG2-NTCP cells were seeded into collagen-coated 60 mm dishes at a concentration of 1.5×10^6 cells/dish, in supplemented Dulbecco Modified Eagles Medium (DMEM) [Table 2.1].

2.11.5 Infecting HepG2-NTCP cells

Infections were performed the day following seeding, at a MOI of 1000. Purified virus stock was diluted in HepG2-hNTCP seeding medium [Table 2.1] to a final volume of 300 µl per well containing 4% PEG, and used to replace the seeding medium.

2.11.6 Maintenance of infected cells

Day 1 post-infection, media was aspirated, cells were washed 5x with PBS to remove carry-over inoculum, and replaced with replicative media (DMEM containing 2.5% DMSO) and incubated under usual conditions (37°C, 5% CO₂). Process was repeated the following day with 3x PBS washes, and media thereafter replaced every 2-4 days until the end of the experiment.

2.11.7 Analysis

Cell culture media from infected cells were collected at 3-day intervals and detected for HBeAg by Elecsys. Cells were harvested at 13 days post-infection and Hirt DNA was extracted for cccDNA detection by Southern blot (methods outlined in section 2.9).

2.12 In vivo experiments

In vivo work was conducted in collaboration with Marc Pellegrini and Greg Ebert's group at the Walter and Eliza Hall Institute of Medical Research. With the exception of the liver DNA and RNA analysis outlined below in section 2.12.2 and 2.12.3, all mouse experiments were carried out by Michelle Clark, Liana Mackiewicz and technical staff/other members of the Pellegrini/Ebert lab (WEHI).

2.12.1 Hydrodynamic injection (HDI)

C57BL/6 mice were housed and handled at WEHI by the Pellegrini/Ebert lab members. Briefly, mice were injected with 10 μ g of HBV constructs in a total volume of PBS equivalent to 8% of their body weight. The pAAV-HBV1.2 plasmid has been described previously (Huang et al 2006), and HBV 1.0mers were generated by digestion with *SapI* as described in section 2.2.1. The introduction of DNA via the tail vein of mice under immense pressure expands liver fenestrae, allowing the genetic material to enter the hepatocytes. Mice were bled weekly and serum-extracted DNA was used to monitor HBV replication by qPCR assay. Three weeks post-injection, mice were sacrificed, and their livers harvested for later detection of HBV replicative intermediates.

2.12.2 Evaluation of drugs

Experiments and analyses were conducted as described in previous experiments Section 2.12.1, with the additional treatment of ETV or selected SMAC mimetics. Birinapant was administered weekly as 30 mg/kg peritoneal injections, while Entecavir monohydrate (Baraclude) (2 mg/kg) and another SMAC mimetic (100 mg/kg) were given as daily oral gavages. These were dissolved in 10% DMSO or specific solvents ("vehicles") provided by the manufacturers. Treatments began one week post-injection and continued for two weeks.

2.12.3 DNA analysis

50-150 mg of snap-frozen liver tissue was suspended in 1 ml TE buffer (50 mM Tris buffer, pH 7.4, 10 mM EDTA) in a 2 ml round-bottom safe-lock tube (Eppendorf), and homogenised using the Qiagen TissueLyserII (30Hz, 5 min). Samples were allowed to settle for at least 30 min on ice before being transferred into 2 fresh tubes. 120 μ l of 10% SDS and 100 μ l 2.5M KCl were added to each sample and mixed by pipetting. Samples were clarified by centrifugation at 4°C for 10 min at full speed. The resulting supernatant was transferred to fresh tubes and subjected 2x phenol and 1x chloroform extraction. DNA was left to precipitate at -20°C overnight in 0.7x volume of isopropanol and 5 μ l glycogen (2 mg/ml). DNA was pelleted by centrifugation for 10 min at full speed, washed with 70% ethanol, and finally resuspended in a volume of nuclease-free water (30-60 μ l). DNA samples could then be split for multiple confirmatory treatments (e.g. EcoRI digest, Exo I/III digest) as previously described within section 2.9.1.2.

2.12.4 RNA analysis

Approximately half a mouse liver tissue was homogenised in 1 ml Trizol reagent in a 2 ml round-bottom safe-lock tube (Eppendorf) using the Qiagen TissueLyserII before being stored at -70°C. Before extraction, tubes were thawed on ice with frequent vortexing to ensure homogenisation. Once thawed, tubes were incubated at room temperature for 5 minutes

before centrifugation at 4° for 10 min at 12,000xg. The resulting supernatant was transferred to fresh tubes, 200 µl chloroform added, and samples were mixed by vortexing for 15 sec. Samples were incubated at RT for 2-3 mins and centrifuged at 4°C for 15 min at 12,000 xg. The resulting top aqueous phase was transferred to fresh tubes and incubated with 500 µl isopropanol for 10 min at RT. Samples were centrifuged at 4°C for 10 min at 12,000 xg. The supernatant was aspirated and the pellet was washed with 80% ethanol (vortex, centrifuge 5 min at 4°C, 7,500 xg). Ethanol was aspirated and pellet allowed to air-dry for 2-3 min, transferred to a 70°C heat block and incubated for 2-3 min. The pellets were redissolved in 100 µl RNase-free water. RNA was further purified using RNeasy Kit (Qiagen) according to manufacturer's protocol with some modifications. Briefly, 350 µl Buffer RLT (with 10 µl/ml BME) was added to the RNA sample, mixed with 250 µl 100% ethanol and applied to the RNeasy column. Sample was passed through column twice by reapplying the volume to the column following centrifugation at full speed for 1 min. Column was washed twice with 750 µl Buffer RPE (full speed, 1 min), transferred to a new 1.5 ml tube and eluted with RNase-free water. RNA quality and concentration was assessed using the BioAnalyzer and RNA 6000 Nano Kit (Agilent) as per manufacturer's instructions. RNA was prepared and analysed by northern blot as described in Section 2.10.

Chapter Three: Evaluating 1.0mer HBV transfection as an *in vitro* and *in vivo* model for analysis of HBV replication and cccDNA minichromosome biology

3.1 Introduction

It took nearly fifty years following the discovery of HBV before its *bona fide* entry receptor, NTCP, was identified (Yan et al, 2012). This discovery enabled establishment of NTCP-expressing *in vitro* and *in vivo* systems permissive to HBV infection, which up until that point, was largely limited to studies using primary human hepatocytes (Galle et al, 1994; Gripon et al, 1993; Gripon et al, 1988) or HepaRG cells (Gripon et al, 2002) *in vitro*, and chimpanzees (Barker et al, 1975) and tree shrews (Walter et al, 1996; Yan et al, 1996) *in vivo*. The cost and resource restrictions, the subsequent prohibition of research using naturally susceptible chimpanzees, and the limited availability of reagents for tree shrews restricted the wider usage of these animal models. Consequently, the advent of humanised murine models (where human hepatocytes are grown in mice) (Dandri et al, 2001; Hwang & Park, 2018; Lutgehetmann et al, 2012) and stable NTCP-expressing HepG2 cells (Yan et al, 2015; Yan et al, 2012) enabling HBV infection, have transformed the HBV field. However, these approaches also have limitations, affecting the ability to routinely study the complete HBV replication cycle. For example, it is not currently possible to routinely infect HepG2^{NTCP} cells with a wide range of HBV variants; to date only virus derived from patient sera and HepAD38 cells have efficiently infected cells. Limited access to HBV-infected patient sera, means that sources of viral inoculum have largely been limited to stably transfected cell lines, most of which have been integrated with a single strain of HBV – genotype D (Ladner et al, 1997; Sells et al, 1987). Furthermore, infection efficiency in these models is also very low. These constraints are also shared by the humanised mouse studies which additionally are very cost-prohibitive and not widely available.

Consequently, much of our knowledge of HBV replication comes from studies using transient transfection of permissive hepatoma cell lines with overlength HBV cDNA clones (Sureau et al, 1986), or hydrodynamic injection of similar constructs into mice (Huang et al, 2006). Neither approaches represent a true HBV infection as they bypass the critical entry step necessary for generation of cccDNA, the major transcriptional template. Despite their limitations, transient transfection studies using overlength HBV genomes have been instrumental in providing fundamental insights into the molecular basis of HBV replication *in vitro*, in a robust and reproducible manner. To date, this in contrast, has not been reliably achieved using current infection models which are highly technically challenging and/or labour-intensive. In addition, transient transfection more readily permits investigation and comparison of different HBV genotypes and variants.

In 1995, Günther and colleagues described a PCR protocol for amplifying full (unit-length) HBV genomes which they and others subsequently demonstrated resulted in formation of cccDNA-like molecules (cccDNA-LM) upon transfection into hepatoma cells (Gunther et al, 1995; Pollicino et al, 2006). This overcomes some limitations of preceding transfection models, namely the inability to generate cccDNA-like templates. To add, this represents a more physiological replication system, since circularisation of the template (3.2kb) is required for

proper recapitulate replication and production of all viral intermediates, particular the greater-than-genome pgRNA and pcRNA transcript. In contrast, overlength constructs contain repeat genome segments and can transcribe the 3.5kb HBV transcripts (see Appendix A, Figure A-1). Although not a true infection model, various groups have utilised this approach to examine the chromatin organisation of cccDNA-LM and have shown they undergo epigenetic regulation, similar to true HBV cccDNA (Belloni et al, 2009; Koumbi et al, 2016; Pollicino et al, 2006). As such, these cccDNA-LM can act as surrogate models for cccDNA investigation, however extensive evaluations of the model have not been performed.

The aim of this chapter was to further characterise this 1.0mer model (Figure 3.1) and its ability to examine the complete HBV replication cycle (post-entry) using different HBV genotypes. The resulting work was based on the hypothesis that transfecting linear monomeric HBV genomes into cells would permit routine and extensive interrogation of HBV replication using different HBV variants. Furthermore, it would provide the additional and critical advantage over traditional greater-than-genome length HBV DNA transfection of enabling analysis of cccDNA-LM which are not produced in traditional transfection systems.

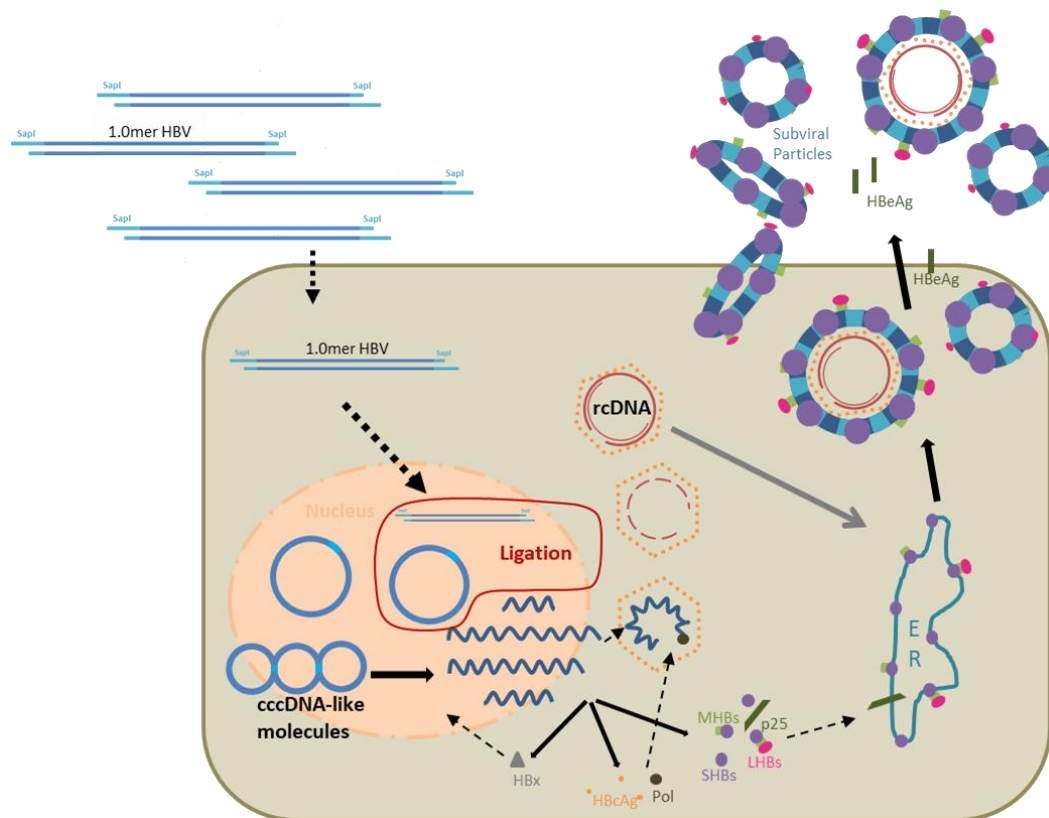


Figure 3.1: Schematic representation of HBV 1.0mer transfection strategy.

Transfection of monomeric HBV genomes results in circularisation and formation of episomes, and induction of HBV replication.

cccDNA can be formed through two major pathways: *de novo* synthesis by conversion of rcDNA molecules from incoming infecting virions, and via the intracellular recycling pathway of naked nucleocapsids transporting back to the nucleus. It is not known how cccDNA-LM originate in this transfection setting, and utilisation of nucleot(s)ide analogues (NUCs) may

assist in providing insight into these mechanisms. In the clinical setting, these NUCs are used to control viral replication in infected patients by inducing chain termination, hence inhibiting reverse transcription of pgRNA to rcDNA. We would expect then, that if 1.0mer cccDNA-LM were mainly a product of the intracellular pathway, then treatment with NUCs such as Entecavir (ETV), a deoxyguanosine analogue, would reduce their expression levels, by lowering the abundance of the template for cccDNA formation. In contrast, if the cccDNA-LMs were mainly a result of the direct circularisation of input transfected linear HBV monomer rather than NUC-targeted rcDNA, then the presence of ETV should not affect the level of resulting cccDNA-LMs.

Furthermore, the utility of this 1.0mer transfection approach in an *in vivo* setting using a murine model, has not been investigated. As mice are not naturally susceptible to HBV infection, the field is in urgent need of a convenient, physiologically relevant, small animal model for investigating HBV. Most currently existing murine models do not permit the establishment of cccDNA, which is particularly pertinent for pre-clinical evaluation of anti-HBV therapies in order to assess their impact on chronic infections and contribution to HBV cure. We addressed this need by establishing a 1.0mer HBV mouse model based on the current hydrodynamic injection model (HDI, (Huang et al, 2006)). Originally, this model involved injection of overlength HBV (genotype A, serotype adw) constructs stabilised by the inverted terminal repeats (ITR) of adeno-associated viral vectors (AAV) (Figure 3.2). The mechanism facilitating HBV replication in this HDI overlength model has not been completely defined, but typically additionally requires the AAV Rep gene. Presumably, the ITR regions flanking the overlength HBV genome constructs facilitate their integration into host genomes, although HBV replication could also result from formation of episomes or extrachromosomal concatemers (Nakai et al, 2001). This pAAV-HBV1.2 HDI model results in HBV replication in approximately 10-20% of hepatocytes with detectable serological markers of chronic infection up to approximately 12 weeks (Ebert et al, 2015b; Huang et al, 2006).

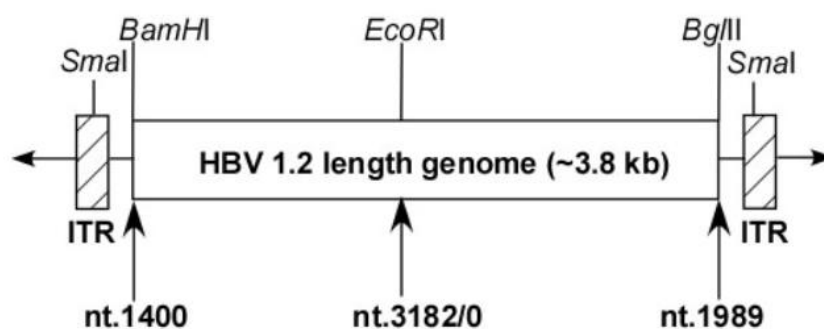


Figure 3.2: Schematic representation of the pAAV-based HBV construct (pAAV-HBV1.2) used for HDI induction of HBV replication in mice. HBV fragment obtained from BamHI/EcoRI-digested fragments of pHBV-48 (genotype A) is flanked by inverted terminal repeats (ITRs) of a rAAV vector. Adapted from Huang et al. (2006).

To evaluate the utility of this putative 1.0mer HDI model, we conducted experiments in collaboration with colleagues at WEHI who had previously shown potential for antagonists of cellular inhibitor of apoptosis (cIAP) to serve as anti-HBV therapy. cIAPs have been shown to

impair clearance of HBV by preventing TNF-mediated death of infected cells and Ebert et al (2015) demonstrated that antagonists of cIAPs could relieve this effect and facilitate clearance of HBV-infected hepatocytes (Ebert et al, 2015a; Ebert et al, 2015b). These molecules are also referred to as SMAC mimetics as they work by mimicking the functional antagonisation of cIAP by endogenous SMAC/DIABLO proteins. In the previous study, HDI mice treated with these SMAC mimetics exhibited reduced viral loads, however their impact on cccDNA was unknown. Given their mechanism of action, it was hypothesised that this treatment would result in reduced cccDNA levels (Figure 3.3). Since cccDNA molecules are not generated in the AAV/HBV1.2 model, the HBV-clearing ability of these SMAC mimetics was re-assessed in the context of 1.0mer HBV-injected mice to determine their capacity for eliminating cccDNA as potential therapy for chronic HBV infection/HBV cure.

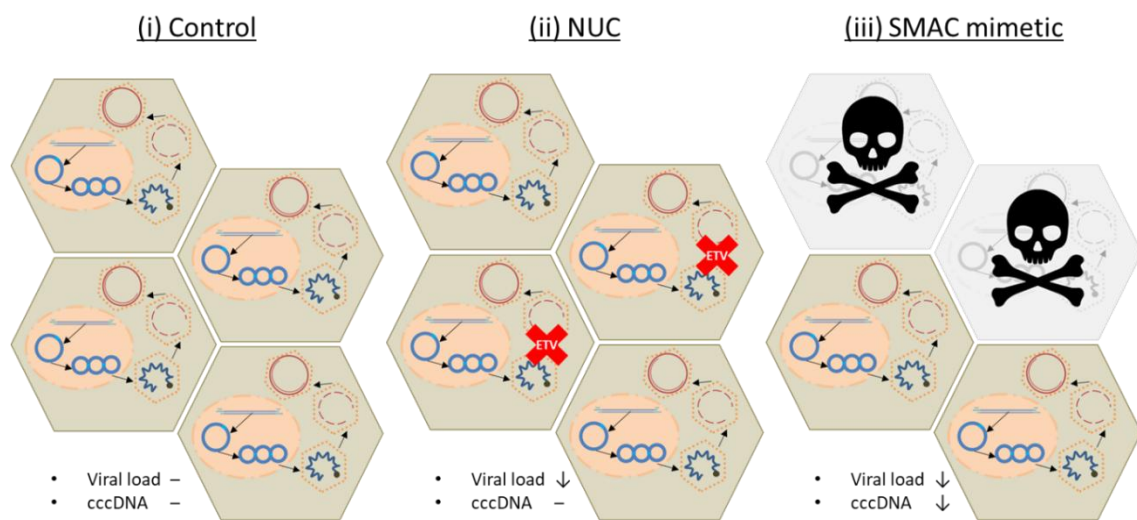


Figure 3.3: Schematic representation of hypothesised change to cccDNA expression following treatment with different therapies. (i) In absence of antiviral therapy, cccDNA is uninhibited and viral expression continues. (ii) Treatment with nucleot(s)ide analogues (e.g. Entecavir, ETV) inhibits reverse transcription; overall replication is suppressed but cccDNA is unaffected and remains constant. (iii) Treatment with cIAP antagonists (e.g. Birinapant) induces cell death; viral replication, including cccDNA is reduced accordingly.

3.2 *In vitro* transient transfection using 1.0mer HBV constructs

Transfection of monomeric HBV genomes demonstrates potential as an alternative transfection system enabling functional investigation of cccDNA. The cccDNA-LM (cccDNA-like molecules) produced using this model were shown to undergo epigenetic modulation and regulation, correlating to HBV replication (Belloni et al, 2009; Koumbi et al, 2016; Pollicino et al, 2006). A comprehensive analysis was conducted to characterise the model in terms of HBV replication *in vitro*, including assessment of cccDNA formation and HBV replicative intermediate expression.

3.2.1 Generation of 1.0mer HBV for transfection

To demonstrate the ability of the HBV “1.0mer” model in enabling comparative replicative analyses of different HBV variants, we generated HBV plasmid constructs containing full-genome PCR products flanked by *SapI* restriction sites within a pUC bacterial vector (~2.7kb) (Günther et al., 1995). Wild-type constructs specific for HBV genotypes C2 and D3 were selected for initial investigations. Prior to transfection, HBV genomes were liberated from vectors by *SapI* as described Section 2.2.2.1. Confirmatory gel agarose electrophoresis revealed bands corresponding to monomeric HBV genomes (3.2kb) with a remaining major vector fragment of approximately 2.4kb (Figure 3.4, lane 3).

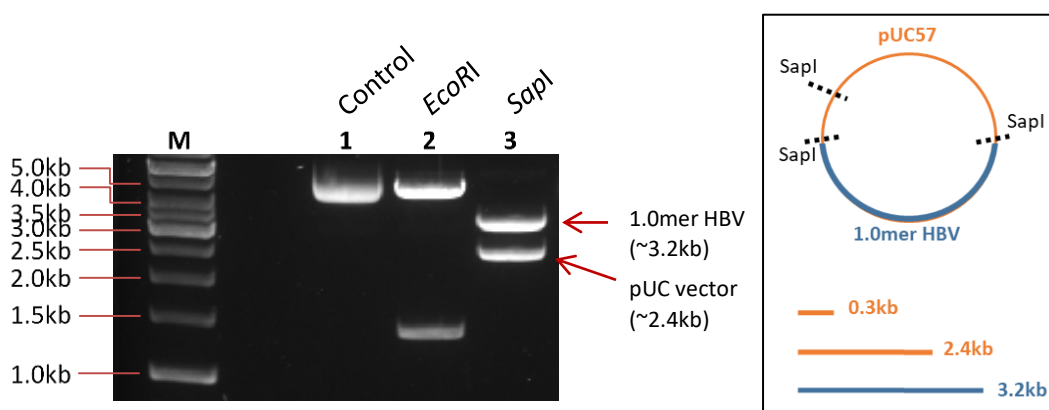


Figure 3.4: Agarose gel electrophoresis analysis of HBV 1.0mer DNA digested by restriction endonucleases.

Digestion of 1.0mer HBV plasmids (lane 1) with the *SapI* enzyme released the HBV genome from the vector backbone to generate “1.0mers” (lane 3).

The 3.2kb HBV fragment is what we refer to as the “1.0mer”. It consists exclusively of the full-length HBV sequence, no vector-derived sequences, and facilitates induction of HBV replication upon transfection and circularisation. Digestion of the plasmid construct with the *EcoRI* restriction enzyme produces two expected bands of approximately 4.5 and 1.4kb (Figure 3.4, lane 2). These bands represent a hybrid combination of viral and vector sequences which is consistent with the *EcoRI* restriction site being present once within the vector, and once within the HBV genome. This result confirms the expected organisation of the 1.0mer HBV plasmid construct, and HBV inserts were also additionally verified by Sanger sequencing (not shown).

3.2.2 *In vitro* replication analysis

To assess the replicative capacity of HBV 1.0mers, the monomeric HBV DNA molecules were transfected into permissive HepG2 or Huh-7 cells as described in (Section 2.7). Transfection of the equivalent 1.3mer constructs was also conducted in parallel for confirmatory and comparative analysis. DNA, RNA and protein extractions were performed and analysed for detection of HBV-specific replicative intermediates using various methods, as outlined in Sections 2.8 – 2.10.

3.2.2.1 *Analysis of HBV DNA*

- Intracellular core-associated DNA expression

To examine HBV DNA replicative intermediates inside cytoplasmic HBV core particles, intracellular core-associated DNA was extracted five days post-transfection, and Southern blot analysis performed. A band of approximately 3.2kb, as well as a slightly larger band, likely corresponding to a double stranded linear (dsIDNA) and relaxed circular DNA (rcDNA) respectively (Figure 3.5) were detected. A lower band around 2.2kb was also present and likely represents a single-stranded DNA (ssDNA) intermediate. These results confirmed that 1.0mer HBV transfection produced replicative HBV DNA intermediates.

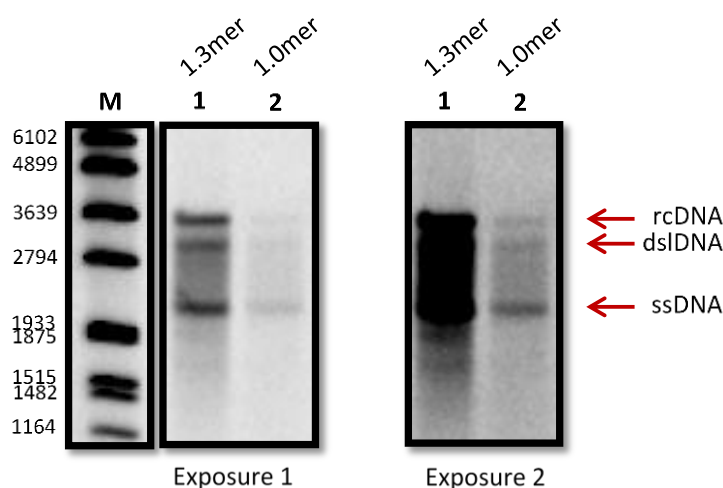


Figure 3.5: Southern blot analysis comparing intracellular core-associated HBV DNA following transfection (1.3- vs 1.0mer).

Intracellular DNA extracted from HepG2 cells 5 days post-transfection with wild-type 1.3 (lane 1) or 1.0mer (lane 2) HBV (Genotype D3).

- HBV cccDNA expression

To assess whether the linear monomeric HBV DNA form cccDNA-like molecules, a Hirt extraction, which specifically selects DNA from the nuclear fraction was performed. Southern blotting of Hirt-extracted DNA from 1.0mer-transfected cells for protein-free, nuclear replicative intermediates revealed bands of approximately 2.0kb, indicative of supercoiled cccDNA-like episomes (cccDNA-LM, Figure 3.6, compare lanes 1&2 with 3&4). This cccDNA-LM band was not produced from 1.3mer-transfected cells, and as shown in Figure 3.7 (lane 4), was indistinguishable from the band present in stably transfected HepAD38 cells which are known to produce cccDNA. Figure 3.6 showed that HBV 1.0mers established cccDNA-LM with similar efficiency, following transfection with different HBV variants (Genotype C2, lanes 2&4; D3 lanes 1&3).

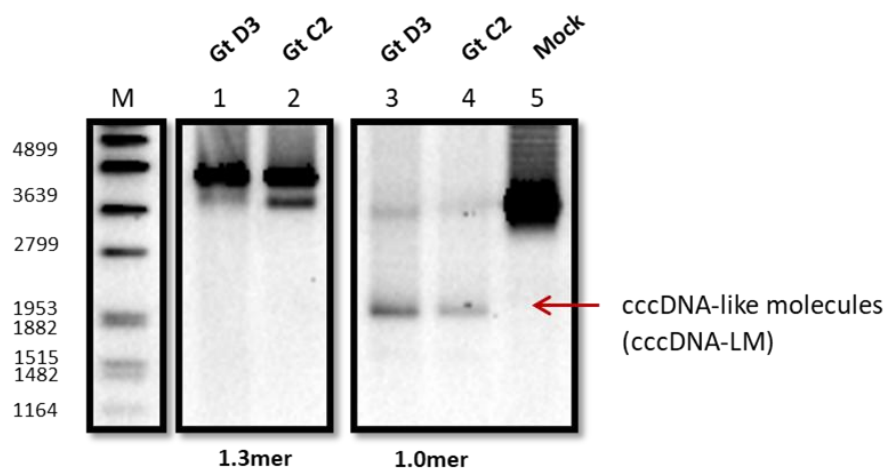


Figure 3.6: Southern blot analysis comparing Hirt-extracted (Exo-digested) HBV DNA following transfection (1.3- vs 1.0mer).

Hirt-extracted DNA from HepG2 cells 5 days post-transfection with wild-type 1.3 (lane 1 and 2) or 1.0mer (lane 3 and 4) HBV, Genotype D3 and C2. Mock control represents the original (not linearised) 1.0mer-containing plasmid (lane 5).

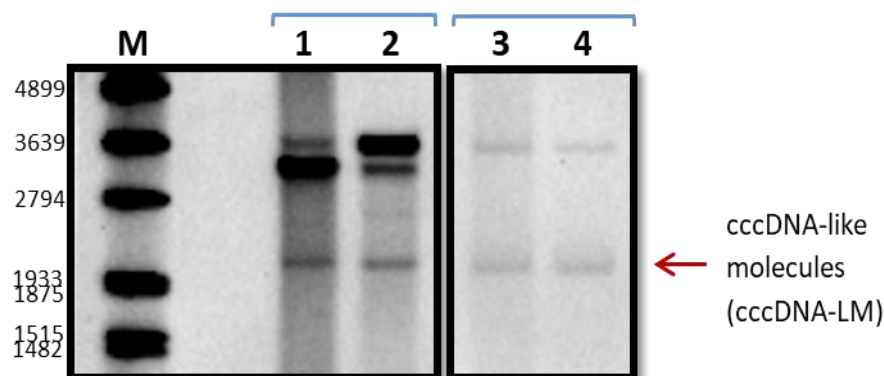


Figure 3.7: Southern blot analysis comparing Hirt-extracted HBV DNA from HepAD38 and 1.0mer-transfected cells..

Hirt-extracted DNA from HepG2 cells 5 days post-transfection with wild-type 1.0mer HBV (Genotype D3, lane 1 and 3), or from replication-induced HepAD38 (lane 2 and 4) cells 7 days post-seeding.

- Verification of cccDNA-like molecules (cccDNA-LM)

To further demonstrate that 1.0mer HBV constructs form cccDNA-LM, comparative studies were performed with stably transfected HBV-producing cells (HepAD38) and cells infected with HBV (HepG2^{NTCP}). Infection of HepG2^{NTCP} cells with HBV inoculum derived from HepAD38 or transiently-transfected 1.3mer (genotype D3, wild-type) cell culture supernatant was conducted as described in Section 2.11. Hirt extracts were prepared and Southern blot analysis performed to compare the cccDNA molecules generated from the different systems. 1.0mer-derived cccDNA-LM produced bands identical in size to those of authentic cccDNA molecules extracted from infected HepG2^{NTCP} (Figure 3.8, lanes 3 and 4, respectively), indicating high similarity in overall topology between the molecules.

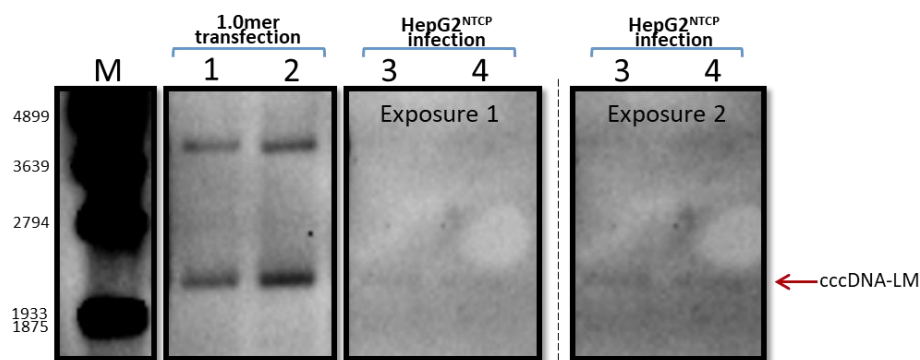


Figure 3.8: Southern blot analysis comparing Hirt-extracted HBV DNA from 1.0mer HBV-transfected HepG2 (lane 1 and 2) and infected HepG2^{NTCP} cells.

Hirt-extracted HBV DNA extracted from HepG2 cells 5 days post-transfection with wild-type 1.0mer HBV (Genotype D3, lane 1 and 2), or 13 days post-infection of HepG2^{NTCP} cells with inoculum derived from induced HepAD38 cells (lane 3) or transfected HepG2 (1.3mer D3 WT HBV, lane 4). DNA was digested with Exo I/III prior to gel electrophoresis and Southern blot.

A time course experiment was conducted to investigate longevity of the cccDNA-LM in our 1.0mer model. Hirt extractions were performed at two, five and seven days post-transfection with 1.0mer HBV, and equivalent amounts of DNA analysed by Southern blot. cccDNA-LM levels visibly decreased with increasing culture period, until day 7 post-infection (Figure 3.9 (a)). A subsequent experiment extended to 12 days post-transfection suggests persistence of these molecules (Figure 3.9 (b)), with a decrease over time possibly caused by dilution due to mitosis of cells, or by cell death.

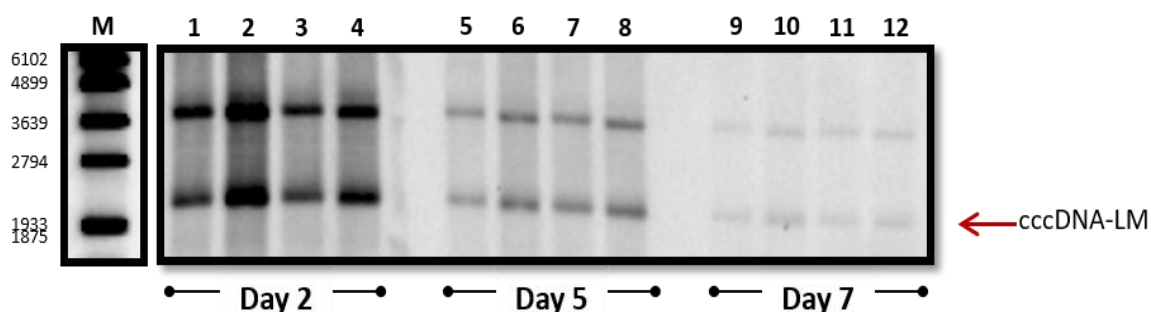


Figure 3.9 (a): Southern blot analysis of HBV cccDNA-like molecules from transfection of different 1.0mer HBV constructs, harvested 2, 5, or 7 days post transfection.

Hirt-extracted HBV DNA extracted from HepG2 cells 2, 5, or 7 days post-transfection with 1.0mer HBV (Genotype D3, odd numbered lanes; C2, even numbered lanes)

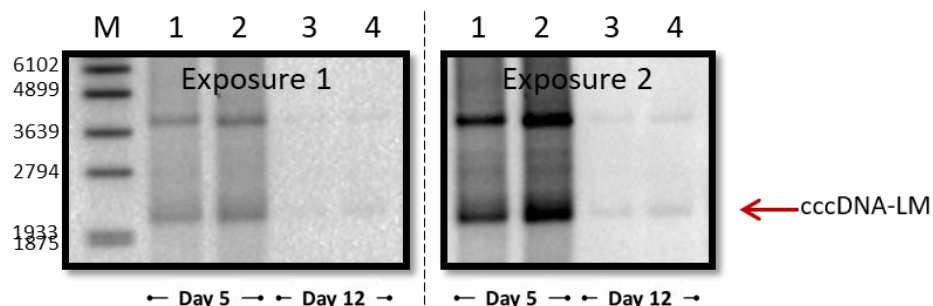


Figure 3.9 (b) Southern blot analysis of HBV cccDNA-like molecules from transfection of different 1.0mer HBV constructs, harvested at 5 and 12 days following transfection.

Hirt-extracted HBV DNA extracted from HepG2 cells 5 or 12 days post-transfection with wild-type 1.0mer HBV (Genotype C2 lane 1 and 3; gt D3, lane 2 and 4)

To further characterise the cccDNA-LM, Hirt-extracted DNA was subjected to enzymatic digestion. Several exonuclease digestion protocols have been described in previous studies as a means of confirming circularised molecules (Luo et al, 2017; Qu et al, 2018). These enzymes select for fully enclosed circular molecules of DNA by degrading linear DNA from free ends, in theory digesting rcDNA (due to incomplete positive strand) and ssDNA intermediates. As seen in Figure 3.6 to Figure 3.9, the presence of the lower molecular weight cccDNA-LM bands (~2.0kb) persisted following treatment with a combination of exonucleases I and III (Exo I/III), confirming resistance which could only be conferred by a covalently closed circular conformation lacking free 3' ends. A higher molecular weight band slightly greater than 3.6kb was often also observed on the blots. The fact that these also remain after combined Exo I/III treatment (Figure 3.6 to Figure 3.9), indicates they are unlikely to represent HBV rcDNA which are characteristically incomplete. Subsequent digestion with the restriction enzyme *EcoRI* which cuts once in the HBV genome, reduced the presence of these molecules with a simultaneous increase in dsDNA intermediates. This suggests these molecules may be an additional form of the circularised 1.0mers representing a more relaxed topological state, contrasting the supercoiled lower cccDNA-LM form (Figure 3.10). *EcoRI* digestion also linearised the supercoiled cccDNA-LM, shifting these lower bands back up to the expected size for linear monomeric HBV genomes (3.2kb), further confirming these cccDNA-LM as 1.0mers which had circularised following transfection (Figure 3.10).

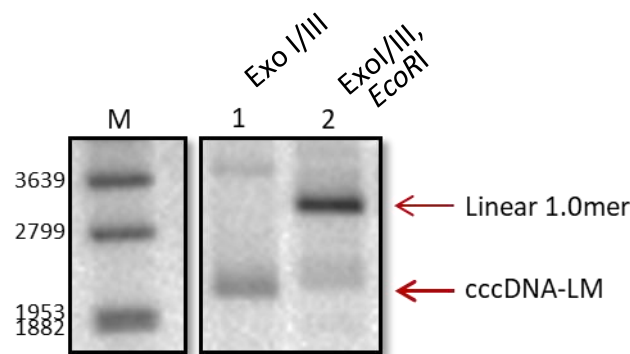


Figure 3.10: Southern blot analysis of *EcoRI*-digested Hirt-extracted HBV DNA following 1.0mer transfection.

HepG2 cells transfected with HBV 1.0mers (genotype D3) were Hirt-extracted 5 days post-transfection. DNA was treated with Exo I/III alone (lane 1) or subsequently digested with *EcoRI* prior to Southern blotting.

- Determining the mechanism of cccDNA-LM generation

To determine whether the observed cccDNA-LM were a result of *de novo* synthesis by circularisation of the transfected linear DNA (input 1.0mers) following entry into the nucleus, or whether they were a product of the intracellular nucleocapsid recycling pathway involving re-import of newly-synthesised rcDNA back into the nucleus and subsequent conversion to cccDNA, we enlisted usage of a NUC. Transfection of 1.0mers were conducted in the presence of Entecavir (ETV) to inhibit reverse transcription. Accordingly, the resulting effect on cccDNA-LM expression would indicate the major pathway by which these molecules were derived. For verification of treatment, extraction and DNA expression, similar experiments using stably transfected HepAD38 and 1.3mer HBV transfection were also conducted in the presence of ETV.

Extraction and Southern blotting of intracellular core-associated DNA revealed decreased levels of HBV DNA in the stably transfected HepAD38 cells, as well as both 1.3mer and 1.0mer-transfected HepG2 cells (Figure 3.11 (a)), confirming the efficacy of the treatment. This effect was inversely correlated with increasing concentrations of ETV, and did not significantly impact the level of secreted HBeAg or HBsAg detected in the cell culture supernatant (Figure 3.11 (b)), which is as expected since these proteins are produced from specific mRNAs.

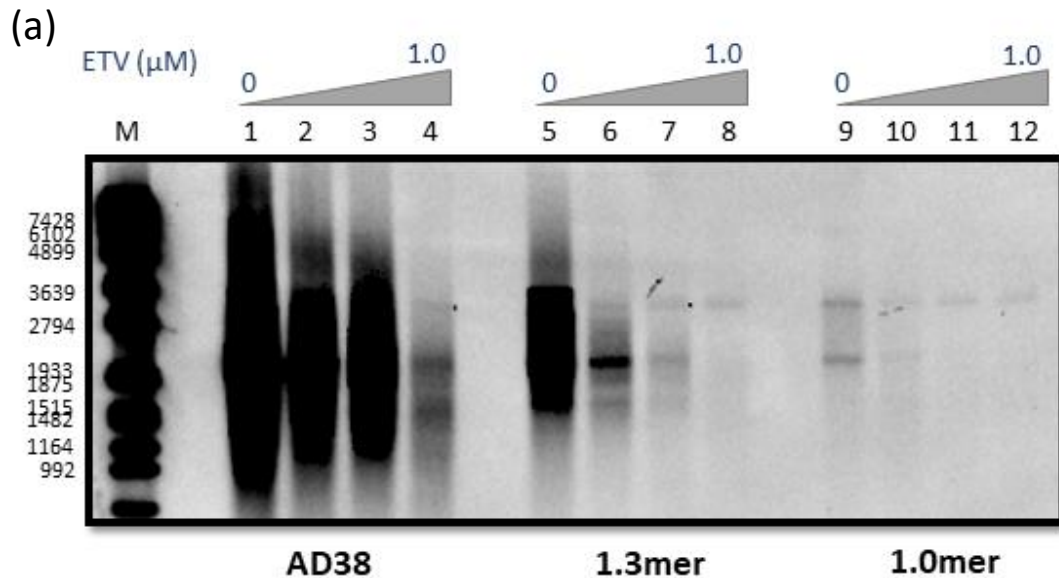


Figure 3.11 (a): Southern blot analysis of intracellular core-associated HBV DNA following Entecavir treatment in stably transfected HepAD38 cells or 1.3/1.0mer HBV-transiently transfected cells. DNA was extracted from AD38 cells at day 7, or transfected HepG2 cells at day 5 post-treatment induction. AD38 cells were seeded and maintained in the presence of increasing concentrations of Entecavir (ETV; 0uM, 0.01uM, 0.1uM, 1.0uM). HepG2 cells were treated with ETV 2hr prior to transfection, then maintained in the presence of ETV.

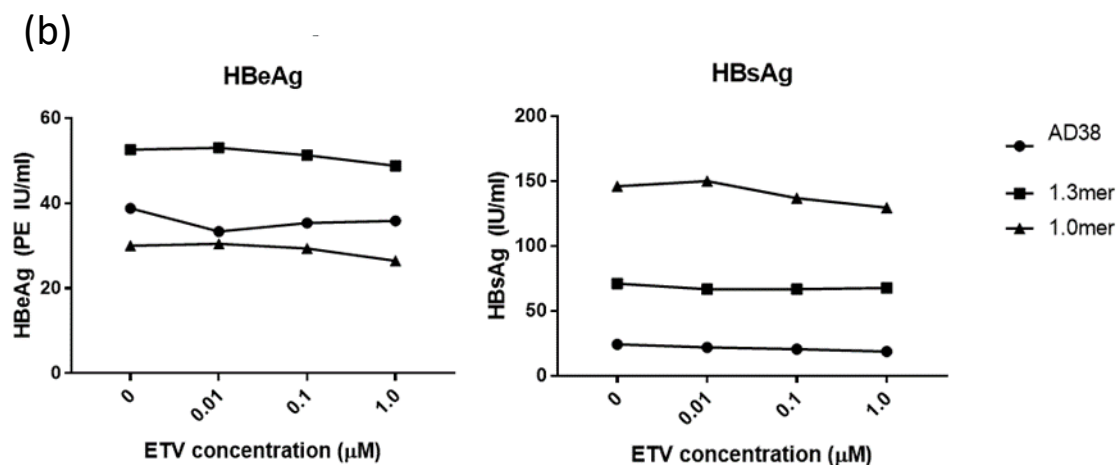


Figure 3.11 (b) Quantitative serology of secreted antigens following Entecavir treatment of stably transfected AD38 cells, or 1.3/1.0mer HBV-transfected cells.

Figure shows results from a single experiment.

Importantly, although ETV reduced intracellular HBV core-associated DNA levels, it had no perceivable impact on the concentration of cccDNA-LMs in the Hirt extracts of 1.0mer-transfected cells (Figure 3.12). This was consistent with a *de novo* method of formation, indicating that the observed cccDNA-LMs in HBV 1.0-mer-transfected cells were predominantly produced from direct circularisation of the linear monomers, rather than through the intracellular recycling of rcDNA-containing core particles.

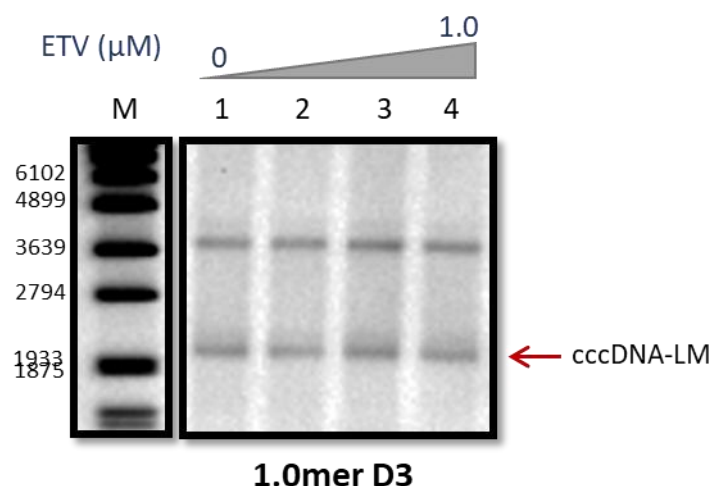


Figure 3.12: Southern blot analysis of HBV cccDNA-like molecules following Entecavir treatment in HepG2 cells transfected with 1.0mer HBV. Cells were maintained on media containing increasing concentrations of ETV 2hr prior to, and following transfection. Hirt DNA was extracted 5 days post-transfection and treated with Exo I/III prior to southern blot

3.2.2.2 Analysis of HBV RNA

HBV RNA expression was next investigated by northern blotting in order to assess transcriptional activity following 1.0mer HBV transfection. Extraction of total cellular RNA following transfection revealed production of major expected HBV transcripts: 3.5kb pregenomic (pgRNA)/precore (pcRNA) RNA, 2.4 preS1 mRNA, 2.1 preS2/S mRNA, 0.7kb HBx mRNA (Figure 3.13). These bands correlated with bands observed from transfection of 1.3mer HBV constructs, though with noticeably lower abundance (Figure 3.13, compare lanes 1-2, 3-4) which was consistent with our DNA results. Similar patterns of RNA transcription were also observed following transfection of HBV 1.0mers of two different genotypes, with higher levels associated with genotype D3 compared to C2 (consistent with 1.3mer). This result confirmed that 1.0mer HBV molecules are transcriptionally active irrespective of specific HBV background.

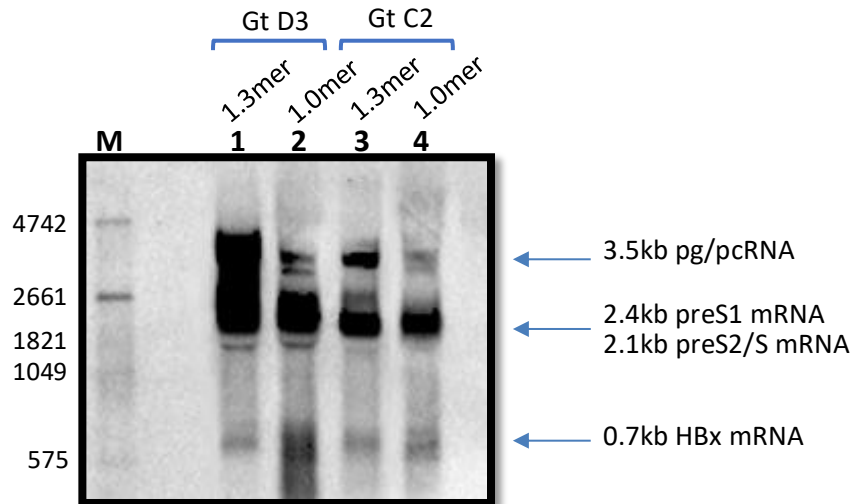


Figure 3.13: Northern blot analysis comparing intracellular HBV RNA from Huh7 cells 5 days post-transfection (1.3-vs 1.0mer). Cells were transfected with wild-type 1.3 (lanes 1&3) or 1.0mer (lanes 2&4) HBV constructs (genotype D3 lanes 1&2, genotype C2 lanes 3&4).

3.2.2.3 Analysis of HBV proteins

To assess gene expression patterns from transfection of 1.0mer HBV, resultant cell culture supernatant and lysates were analysed for detection of HBV-specific proteins.

- Intracellular proteins

Intracellular protein expression was determined by immunoblotting analysis using specific antibodies against HBc, HBx and HBsAg. These results indicated detectable expression of all of these proteins in cell lysates following 1.0mer transfection (Figure 3.14). Small (S) and Large (L) forms of the surface proteins were observed, along with higher bands representing the glycosylated versions (Figure 3.14 (i)). 21kDa core protein was also detected in lysates of cells after transfection with both 1.3 and 1.0mer constructs (ii), although HBx was undetectable in 1.0mer-transfected cells (iii, lane 2).

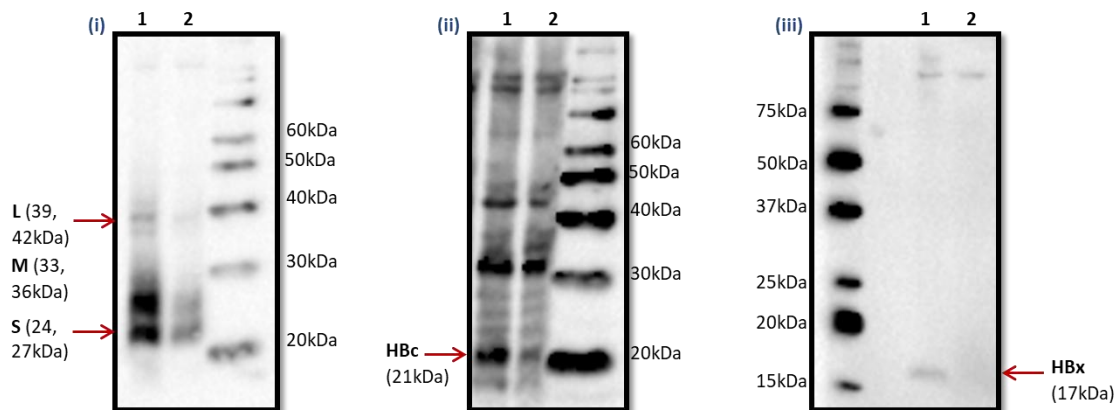


Figure 3.14: Immunoblot detection of HBsAg (i), HBcAg (ii) and HBx (iii) five days post-transfection (1.3-vs 1.0mer). Lysates of cells transfected with 1.3 (lane 1) or 1.0mer (lane 2) wild-type HBV constructs (genotype D3) were probed with anti-HBs/HBc/HBx antibodies.

However, considering that HBx appears to be a generally unstable protein detected at low levels even in 1.3mer-transfected cells, it was unsurprising that it was typically at undetectable levels in 1.0mer-transfected cells (day 5, Figure 3.14, iii). The 1.3mer constructs encode two copies of the HBx gene which include the necessary promoters to drive primary transcripts directly from the plasmid, so it is expected that more HBx protein would be observed from these constructs. This in turn could further contribute to a more favourable expression timeline for detecting X protein (at day 5 post-transfection) compared to the linear 1.0mer constructs, and could possibly be related to protein stability. To confirm that 1.0mer transfection was resulting in the production of the HBx protein, we precipitated the lysates with anti-HBx to concentrate the sample and indeed detected HBx expression (Figure 3.15, lane 3). Enhanced detection was observed in the context of DDB1 co-expression, and will be further explored in upcoming sections.

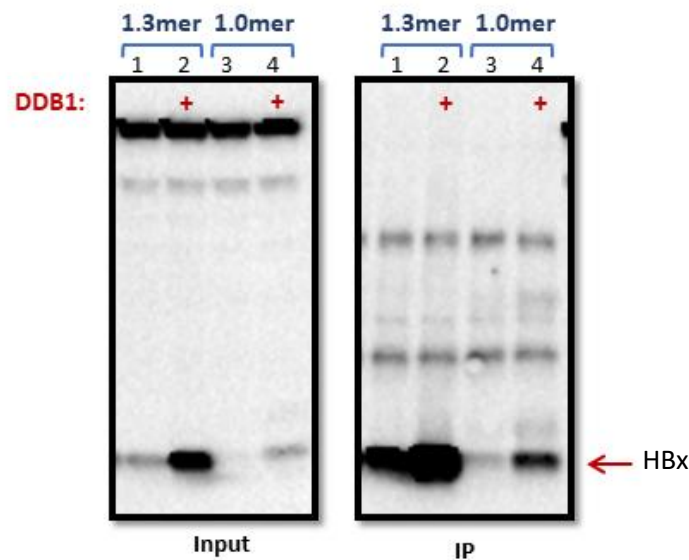


Figure 3.15: Immunoblot detection of HBx in total (Input, left panel) or immunoprecipitated (IP, right panel) lysates of cells transfected with 1.3 (lanes 1-2) or 1.0mer (lanes 3-4) wild-type HBV (genotype D3) in the presence or absence of DDB1 expression plasmid. Lysates were harvested 2 days post-transfection and subjected to immunoprecipitation with anti-HBx antibody before immunoblotting (same antibody).

- Secreted proteins

Secreted HBsAg and HBeAg were detectable in cell culture supernatant using the Elecsys platform (Roche USA), typically with lower expression in the 1.0mer-derived sample compared to 1.3mer transfection (Figure 3.16). This difference appeared to be more pronounced with HBeAg than with HBsAg and may allude to a potential trend toward statistical significance, and is likely due to the extra requirement of circularisation to facilitate proper HBeAg expression following 1.0mer transfection. This is analogous to the natural infection scenario, wherein HBeAg secretion can be considered a surrogate measure of cccDNA formation, due to the requirement of this step for subsequent production of the greater-than-genome-length precore transcripts which are translated into HBeAg.

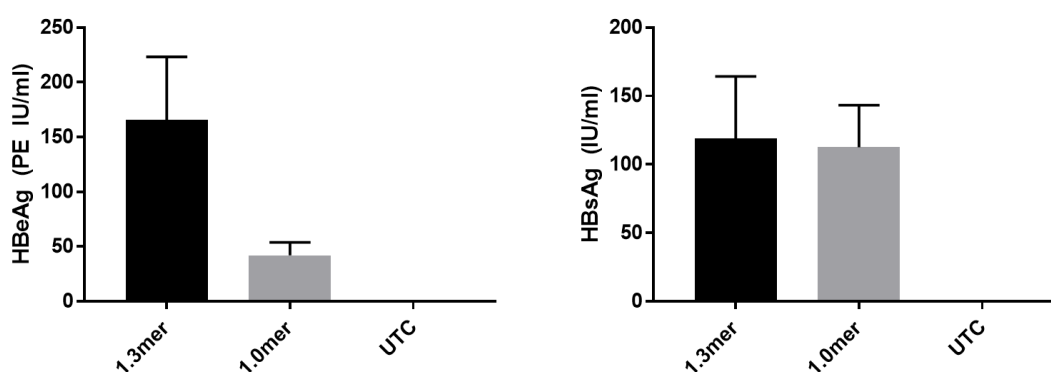


Figure 3.16: Quantitative serology for detection of secreted HBeAg (left) and HBsAg (right) in the cell culture supernatant of transfected HepG2 cells.

Mean ($\pm$ SEM) HBeAg (left) and HBsAg (right) levels detected from supernatant of cells transfected with HBV (genotype D3) 1.3mer (dark bars) or 1.0mer (light bars). N=11 experiments. UTC, untransfected control.

Together, these results indicate that transfection of 1.0mer HBV constructs resulted in production of all expected HBV proteins, similar to that of conventional overlength clones, although at lower expression levels, which was consistent with our analysis of HBV DNA and RNA.

3.3 Characterisation of the 1.0mer model *in vivo*

Mice are not naturally susceptible hosts of HBV, so alternative innovative animal models permissive to comprehensive investigation of replication, are required for enabling pertinent/thorough pre-clinical evaluations of therapeutic interventions. Having confirmed that HBV 1.0mers are completely replication competent *in vitro*, investigations were undertaken to evaluate their adaptation to establish a novel murine model of HBV replication *in vivo*, based on the existing HDI model (Huang et al, 2006). We hypothesised that introducing HBV monomers into mice under the same conditions (HDI) will result in circularisation of the 1.0mers to form cccDNA-LMs which can facilitate HBV transcription and replication, as demonstrated *in vitro*. This would therefore present a non-humanised murine model of HBV replication that would not only enable analysis of cccDNA, but facilitate comparative replication studies of different HBV variants.

3.3.1 Confirmation of HBV replication (WEHI)

Induction of HBV replication following hydrodynamic injection was confirmed by our WEHI colleagues (personal correspondence). The data are not shown here and are currently being prepared for publication. Briefly, mice were bled weekly post-injection, and DNA was extracted from the sera. Subsequently, qPCR of extracted DNA confirmed induction of HBV replication in mice injected with HBV 1.0mers, and this result was demonstrated using three different 1.0mer constructs corresponding to genotypes A2, C2 and D3. Additionally, the presence of HBeAg and HBsAg expression was detected by quantitative serology of mice sera. Finally, HBcAg was detectable by immunohistochemistry within the livers of mice injected with these HBV 1.0mer constructs (unpublished data, WEHI).

3.3.2 Analysis of cccDNA-LM

For the detection of cccDNA-LM, Hirt extractions were performed on snap-frozen livers from 1.0mer-injected mice (Section 2.12.3), and analysed by Southern blot as described in Section 2.9.2 for detection of HBV-specific DNA. cccDNA-like molecules were detected in liver tissue from the 1.0mer HBV-injected mice, but not those injected with the pAAV-HBV constructs or “uninfected” control mice (Figure 3.17 (a)). Similar to results obtained from our *in vitro* cell culture experiments, these cccDNA-LMs were resistant to treatment with Exonucleases I and III (Figure 3.17 (b), lane 3), and the representative supercoiled DNA bands shifted to 3.2kb upon digestion with *EcoRI* to linearise the circularised monomers. These results were consistently observed, irrespective of HBV genotype injected (Figure 3.17).

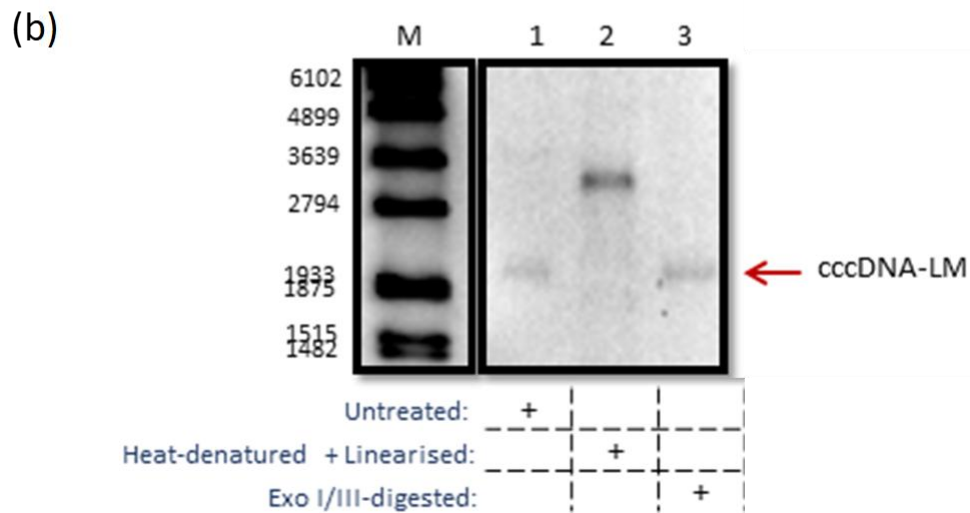
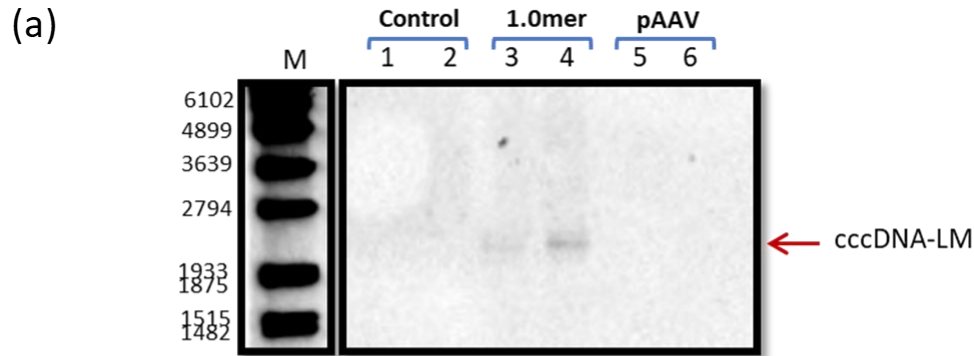


Figure 3.17: Southern blot analysis of Hirt-extracted DNA from snap-frozen liver of C57BL/6 mice.

(a): Hirt-extracted DNA from mice (individual lanes represent separate mice) not injected with HBV DNA (lanes 1-2), injected with wild-type D3 1.0mer HBV (lanes 3-4), or A2 pAAV-HBV1.2 plasmid (lanes 5-6).

(b): Hirt-extracted DNA from 1.0mer HBV (wild-type, genotype D3) injected mice was subjected to heat treatment, linearisation with *EcoRI* or Exo I/III digestion as indicated.

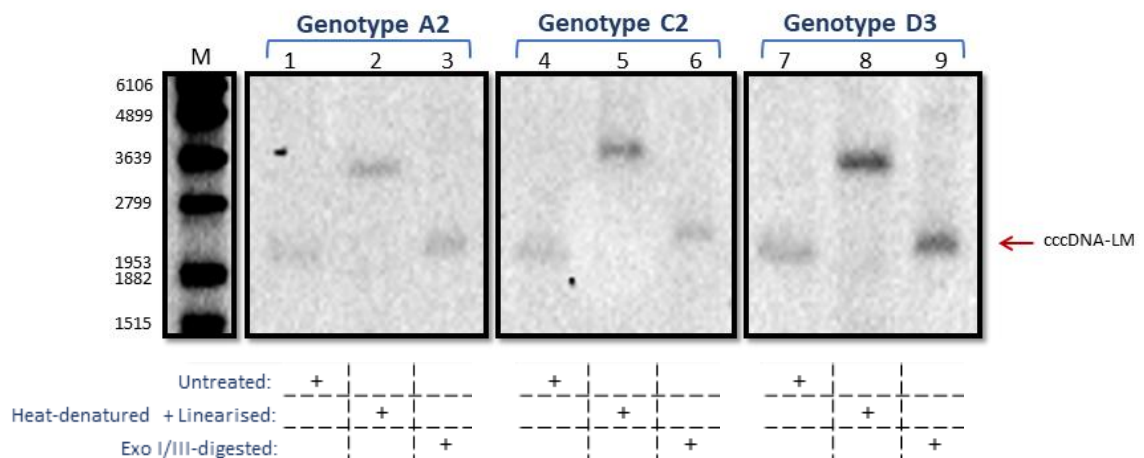


Figure 3.18: Southern blot of Hirt-extracted DNA from snap-frozen liver of C57BL/6 mice injected with wild-type 1.0mer HBV genotypes A2 (lanes 1-3), C2 (lanes 4-6) and D3 (lanes 7-9). Extracted DNA was treated in various manners prior to Southern blot, as indicated below image of blot.

Linearization was performed by *EcoRI* digestion

3.3.3 Analysis of HBV RNA

To examine transcriptional activity of 1.0mer HBV following injection into mice, Trizol-extracted mice liver RNA was analysed for HBV RNA transcripts by northern blot. Detection of 3.5kb pregenomic/precore RNA, 2.4kb and 2.1kb preS1 and preS2 mRNA, as well as 0.7kb HBx mRNA was demonstrated by northern blot for all assessed genotypes (Figure 3.19), indicating that these 1.0mers are transcriptionally active, as they were for cell culture transfection.

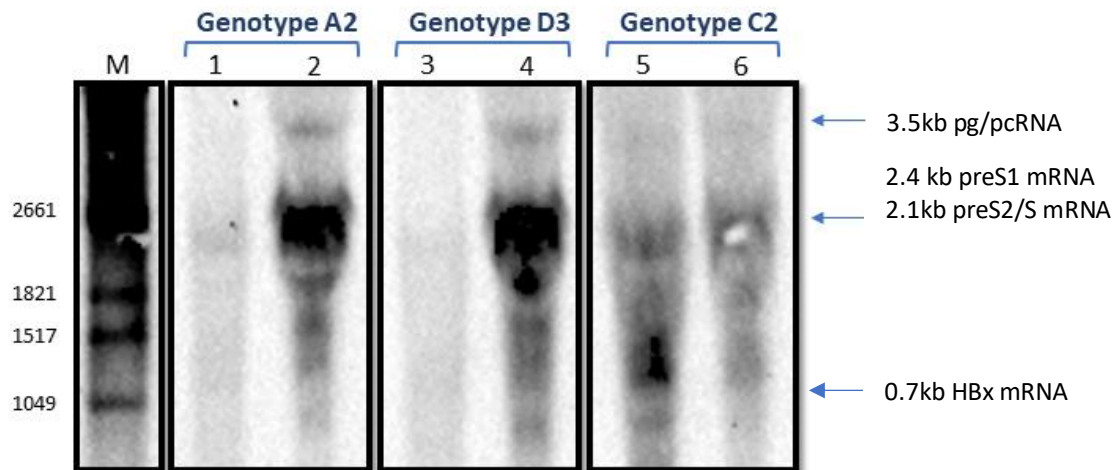


Figure 3.19: Northern blot analysis of HBV RNA from Trizol-extracted RNA liver of C57BL/6 mice injected with wild-type 1.0mer HBV genotypes A2 (lanes 1-2), C2 (lanes 3-4) and D3 (lanes 5-6).

3.4 Determining the utility of the 1.0mer model *in vivo*

Having demonstrated and characterised induction of HBV replication in mice following HBV 1.0mer hydrodynamic injection, including the production of cccDNA-like molecules, we proceeded to evaluate its utility as a model for assessing novel antiviral therapies. In a previous study by our collaborators at WEHI, SMAC mimetics were demonstrated to reduce HBV replication through inducing TNF-mediated death of HBV-infected cells (Ebert et al, 2015a; Ebert et al, 2015b). Reduced viral loads were observed using the AAV/HBV1.2 hydrodynamic injection model, however the impact on cccDNA was not assessable due to the constraints of the utilised model. To continue on from these previous investigations, we re-assessed the HBV-clearing ability of these SMAC mimetics using HBV 1.0mer constructs which demonstrably result in generation of cccDNA-like molecules. Presuming a reduction in the levels of these molecules is observed, this would indicate these SMAC mimetics as potential therapy for chronic infection, potentially contributing to HBV cure.

3.4.1 Assessing SMAC mimetics on cccDNA expression

HBV 1.0mer-injected mice were used to assess the ability of cIAP antagonists to clear cccDNA. Mice injected with HBV (genotype D3) were treated with ETV or selected SMAC mimetics as described in Section 2.12.2, and sacrificed three weeks post-injection. qPCR of extracted serum DNA confirmed that the two different SMAC mimetics (“X”, Birinapant) examined could reduce HBV level, though with less potency than the nucleoside analogue ETV (data not shown, personal correspondence with WEHI collaborators). In livers of 1.0mer-injected mice treated with ETV or the provided control solvent (“vehicle”) in which the mimetic X was prepared, cccDNA-like molecules were detected by Southern blotting (Figure 3.20, lanes 1-3), whereas they were reduced to near-undetectable levels in mice treated with the SMAC mimetics (Figure 3.20, lanes 4&5). This is in line with the mechanism of action of these SMAC mimetics which preferentially eliminate infected cells, in contrast to NUCs which act on an intracellular replication step without impacting cccDNA levels.

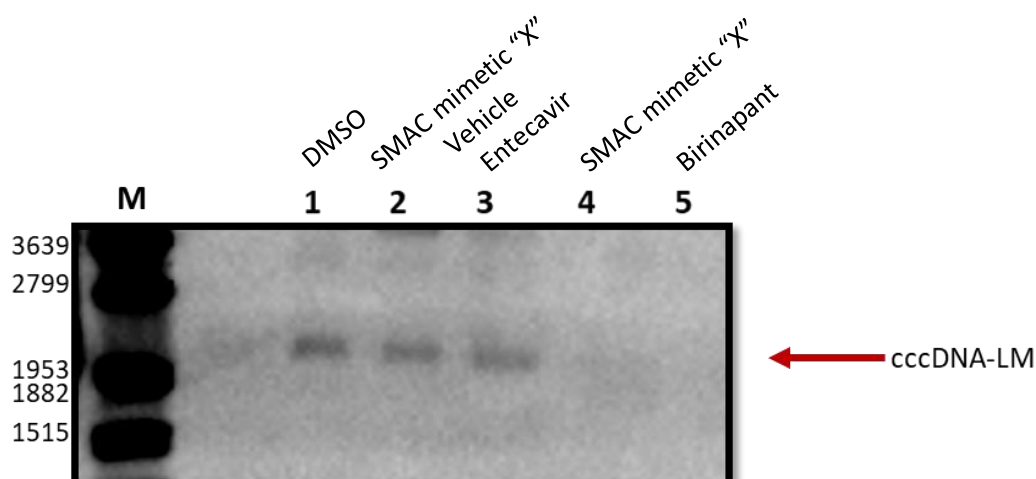


Figure 3.20: Southern blot analysis of Hirt-extracted DNA snap-frozen liver of C57BL/6 mice injected with wild-type 1.0mer HBV genotypes D3 and treated with the NUC Entecavir (lane 3) or two different SMAC mimetics (“X”, Birinapant; lanes 4-5). DMSO was the vehicle for both Entecavir and Birinapant.

These results indicate that SMAC mimetics display effective anti-HBV capability with ability to reduce cccDNA, unlike with conventional NUC therapy. Additionally, they provide supportive evidence that HDI injection of 1.0mer HBV is a viable model for *in vivo* evaluations of cccDNA effect of antiviral therapies. Further investigation may include evaluation of this anti-HBV effect on different HBV genotypes and variants, which could be easily conducted by generating the specific 1.0mer constructs.

3.5 Discussion

The lack of readily available cell culture and animal models of HBV infection and replication that enable investigation of the cccDNA minichromosome is an immense barrier to facilitating curative research (Revill et al, 2019a). As cccDNA formation follows infection of hepatocytes and subsequent translocation of HBV rcDNA to the nucleus, conventional transient transfection methods utilising overlength cDNA constructs of the HBV genome are unsuitable, as they bypass these initial infections steps. In turn, current cell culture infection models do not yet permit routine and reproducible experimentation with different HBV genotypes or variants. We hypothesised that transfection of 1.0mer HBV PCR (Gunther et al, 1995) presents a viable alternative strategy allowing easy comparison of different variants with the additional benefit of producing cccDNA-like molecules (cccDNA-LM). Pollicino and colleagues (2006) had previously shown that this approach produced cccDNA-like molecules in hepatoma cells, detected by Southern blot, which closely resembled cccDNA detected in HBV-infected patient liver tissue. Their study demonstrated by ChIP assays that these 1.0mer cccDNA-LM were associated with histone proteins, and that the acetylation of these histone proteins correlated with HBV replication. Koumbi et al (2016) then used this model to further show that basal core promoter (BCP) mutants exhibited lower replication than wild-type HBV, and that this correlated with reduced acetylation of the cccDNA-bound histones (Koumbi et al, 2016). Though the Pollicino study demonstrated 1-mer transfection resulted in cccDNA production using two HBV genotypes, none of these previous studies (Belloni et al, 2009; Koumbi et al, 2016; Pollicino et al, 2006) included comprehensive analyses of overall HBV replication with different HBV variants using this model.

In this project, we further characterised the model to demonstrate that 1.0mer HBV transfection is a relatively simple and reproducible way of recapitulating almost all stages of the HBV replication cycle, post-entry, for different HBV genotypes or variants. Using the overlength HBV transfection system, our group has previously conducted comparative replicative analysis to demonstrate genotype-associated differences in HBV replication and protein expression (Bannister et al, 2019; Sozzi et al, 2018; Sozzi et al, 2016). We likewise observed similar replication patterns in comparing genotypes C2 and D3 using 1.0mer HBV transfection, which was consistent with the previous 1.3mer study. We confirmed that the 1.0mer transfection system resulted in detection of HBV-specific replicative intermediates including DNA, RNA and proteins using various methods, albeit at lower expression levels compared to transfection with the conventional overlength constructs (Sozzi et al, 2016).

This reduced replication efficiency may be attributed to several reasons, one of which is that transfected 1.0mers must first be circularised once inside the cell in order to form the template for transcription of pgRNA, the critical replicative intermediate. This additional requirement presents a barrier not present with the overlength constructs which contain redundant sequences and therefore are able to directly facilitate transcription of the pgRNA, making the 1.3mer replication more efficient in comparison. Further to this, the 1.3mer constructs contain two copies of the HBx gene, which is a known transcriptional activator of viral and host promoters with demonstrated enhancing effects on HBV replication (Doria et al, 1995; Kwee et al, 1992; Tang et al, 2005; van Breugel et al, 2012), and is therefore also likely to contribute to enhanced replication efficiency of the 1.3mers over the 1.0mers. Finally,

transfection and subsequent analysis was conducted in parallel for direct comparison of the two methods, but the timelines to reach certain replication milestones may well differ between 1.3- and 1.0mers, and it would be pertinent to take this into consideration for future investigation. Interestingly, despite exhibiting lower expression of most other intermediates, 1.0mer transfection often resulted in comparable secretion of HBsAg, which, in addition to cccDNA-LM, could potentially also result from transfected linear 1.0mers or integrated segments, though we have not yet investigated integration in this model.

However, despite lower replication overall under these experimental conditions, 1.0mer transfection has the undeniable advantage over 1.3mer transfection in the formation of detectable cccDNA-like molecules which are not present upon transfection with the conventional overlength clones. Expression of these molecules were not affected when the cells were treated with ETV to inhibit reverse transcription (Figure 3.12), indicating they likely originated predominantly through *de novo* route of circularised linear monomers, rather than through the nucleocapsid recycling/re-import pathway. These molecules are presumably authentic templates for replication of the virus, as there are no other DNA intermediates present that could properly support transcription of pgRNA. We observed in our experiments that 1.0mer transfection-derived cccDNA molecules were consistently much more readily detected by Southern blot, compared to those naturally derived from infected HepG2^{NTCP} cells (Figure 3.8). While this could indicate that the produced 1.0mer cccDNA-LM may be intrinsically more efficiently formed compared to the authentic infected-HepG2^{NTCP} cccDNA, we acknowledge the probability that the cccDNA level could likely be enhanced with improved infection efficiency and optimised harvest time to correlate with the peak of replication. However, this further serves to highlight the difficulty of infection systems which require laborious generation of inoculum that is ultimately unpredictable in infectivity, let alone resultant detectable cccDNA generation. In contrast, 1.0mer transfection reliably induces cccDNA-LM expression much more efficiently than HepG2^{NTCP} infection.

Expression of 1.0mer-derived cccDNA-LM progressively declined over a week following transfection (Figure 3.9), perhaps due to immature, incomplete or irregularly-formed molecules which are susceptible to degradation by endogenous cellular mechanisms, as well as our introduced exonuclease treatments following extraction. These treatments are designed to degrade linear, un-ligated and nicked DNA ends. Therefore, we speculate that the remaining cccDNA-LM molecules at day 7 would represent more resistant, matured forms and anticipate that cccDNA-LM expression would thereafter stabilise and reach a plateau rather than continue to taper off completely. Indeed, in subsequent experiments, expression level appeared to stabilise with no perceivable difference at 12 days post-transfection. Dezhbord et al (2019) reported in a recent study that two distinct populations of protein free-rcDNA were detectable upon infection of HepG2^{NTCP} cells: one that peaked earlier in infection derived from the incoming virions, which thereafter declined and was superseded by a later population (from ~15 dpi) arising by *de novo* synthesis following establishment of cccDNA. Thus cccDNA is initially formed from the incoming rcDNA molecules shortly after infection, while the stable levels of cccDNA observed late after infection (~30 dpi) was believed to be maintained by continual replenishment of cccDNA derived from the later population of rcDNA. Similarly, we could postulate that that transfected input 1.0mers are critical for initial cccDNA-LM establishment following transfection, after which rcDNA-containing nucleocapsids are formed

and can contribute to further formation and maintenance of cccDNA molecules in the nucleus via the intracellular pathway, at later time points. This would suggest that cccDNA levels would remain quite stable (and maintain replication) for extended lengths of time, reliant on the intrinsic intracellular amplification pathway. Assessing the production of virions resulting from this pool of transcriptional templates may yield interesting insights into HBV infectivity and further utility of this transfection model.

We have yet to analyse the HBV virions resulting from 1.0mer transfection and we will be following this up in future to see how these compare to current methods of generating inoculum using transient transfection or stable cell lines. At present, inoculum derived from transient transfection experiments with overlength clones is poorly infective, however inoculum derived from 1.0mer transfections is yet to be tested. HBV envelopes are comprised of a combination of the three forms of the surface protein, the exact proportions and level of glycosylation of which can vary within the virion. As such, it would not be unreasonable to imagine that, at least in the transient transfection context, HBV replication could produce a heterogeneous population of viral particles which range in their composition/morphology and hence susceptibility to infection, possibly explaining the great variability and difficulty in establishing *in vitro* infections. An intriguing proposition is that fully mature virions are more efficiently produced at later time points from the stable cccDNA-like molecules established following transient 1.0mer transfection. That is, although replication tends to peak earlier (~day 5) according to standard measurements of replicative intermediates, it may later recover following a presumed decline, and the virus produced in this stage could represent more mature and infectious virions. However, this may be balanced by the reduced levels of replication, resulting in less secreted virus. These hypotheses are yet to be tested. Another factor to consider, is that to more easily obtain sufficient amounts of template, we opted not to gel-purify our 1.0mer HBV fragments prior to their transfection. This could be considered to further optimise the model, as delivery of more specific HBV DNA in the absence of vector backbone DNA may well demonstrate enhanced, more productive replication than presently observed due to template. However, this approach is highly labour- and time-intensive.

The other advantage of the 1.0mer method over infection is that it enables extensive investigation of HBV variants with relative ease. Generation of 1.0mers removes the requirement of difficult cloning and reconstruction, and can directly utilise DNA extracted from patient sera as a template, as demonstrated in the original study by Günther et al (1995). With the advent of modern gene synthesis services (eg Genscript), generation of these HBV 1.0mers has been significantly simplified and fast-tracked. This enables investigation of replication across an unrestricted range of HBV genotypic variants or specific mutants, with the added advantage of production of a cccDNA-like molecule. Additionally, it presents a potential alternative method for generating infectious inoculum of selected HBV variants, broadening the scope of investigations using infections that are currently largely restricted to genotype D, particularly that derived from stable HepAD38 and HepG2.2.15 cell lines. Further work needs to be conducted to characterise the inoculum generated by 1.0mer transfection to determine whether this is in fact a viable and practical solution.

The chimpanzee infection model remains the best representation of natural HBV infection, but has been severely restricted on moral and ethical grounds and is now largely banned. Recently,

a macaque model of infection has been established by transducing hNTCP expression *in vivo* (Burwitz et al, 2017). While non-human primate models may be particularly pertinent in specific cases such as pre-clinical evaluations, their sentient nature and the associated moral and ethical implications, as well as the technical complexity and economic burden limit their common usage and restrict the justification of use for early and smaller exploratory and proof of concept studies (i.e. more basic research). Accordingly, small lab-based animal models are required for the advancement of HBV research, however, since mice are not naturally susceptible to infection, alternative methods are applied to enable *in vivo* analysis.

Commonly used, currently available murine models include those involving natural infection of mice with humanised liver, and expression of HBV genomes/genes from integrated transgenes or transient viral vectors delivered by various methods. These models differ in their availability, usability and capability of translational research, but similarly restrict comprehensive molecular investigation of HBV replication. Excitingly, we have demonstrated for the first time that introduction of HBV 1.0mers in C57BL/6 mice via hydrodynamic injection is a relatively simple and flexible method of comprehensively investigating different HBV variants *in vivo*. This directly addresses an unmet need in the field of HBV research as suitable animal models permitting such analyses are currently unavailable. Hydrodynamic injection of 1.0mer HBV induced replication in immunocompetent mice, with demonstrated production of anticipated intracellular DNA, RNA, proteins as well as extracellular DNA and secreted antigens. Importantly this represented a non-humanised murine model enabling establishment of detectable of cccDNA-like molecule, a first for the HBV field, laborious chimeric infectious model notwithstanding (Dandri et al, 2001). The original HDI method described by (Huang et al, 2006) using the pAAV-HBV1.2 construct, upon which this model was based, does not produce detectable cccDNA. Minicircle technology has also been used in recent years to establish recombinant cccDNA in cells and mice, and although similar to our described Günther-based 1.0mer method, generation of these constructs is complex and results in cccDNA-LM which are less authentic as they retain residual recombination sites (Guo et al, 2016; Li et al, 2016; Wu et al, 2018) (Li et al, 2018a; Yan et al, 2017). In our system, HBV 1.0mers are authentic HBV replicons, which are simply excised from their vectors by enzyme digestion, and injected into mice without further manipulation. Lucifora et al (2017) also detected cccDNA in C57BL/6 mice transduced with adenovirus carrying AAV-HBV vectors, but the inclusion of the AAV8 virus as a delivery system may complicate interpretation of results and induce host responses (Martino & Markusic, 2020) that could potentially impact HBV replication. Accordingly, caution should be exercised when using this model so as to differentiate between AAV8- or HBV-specific viral and host/immune responses, which may be difficult to discriminate.

The pAAV-HBV1.2 hydrodynamic injection model currently represents an acute/borderline chronic model of HBV replication, with viral clearance observed in mice after approximately 12 weeks post-injection (Ebert et al, 2015b). In our own 1.0mer study, experiments were terminated by 4 weeks in order to capture the highest replicative stage and increase the likelihood of detecting intrahepatic DNA, particular HBV cccDNA-LM. However, there is potential to extend this model for longer-term analyses by using different mouse strains. In support of this, Ebert et al have shown that hydrodynamic injection of C3H mice results in a longer-term infection using the pAAV-HBV1.2 constructs, with sustained viremia for at least 20

weeks (Ebert et al, 2015b), and we predict this chronic effect to be similarly observed using our 1.0mers. Different animal genetic backgrounds influence experimental outcomes and should be carefully considered and adapted to best represent the investigational context when designing an experiment.

As further validation of the utility of this model, we provided an example of how it may be applied to pre-clinical evaluation of antiviral treatments to assess impact on the nuclear reservoir of cccDNA. This is an important consideration in the real-world, in the context of treating chronically infected patients with the aim to cure infection, eradication of cccDNA being the gold standard treatment endpoint. Our investigation compared two different HBV therapeutic strategies; the first, a direct antiviral agent targeting viral replication (ETV), and the second an immunotherapy targeting infected cells (Ebert et al, 2015b). Treatment of 1.0mer HDI mice with either drugs reduced serum HBV DNA through different modes of actions, however only the SMAC mimetics which induce death of infected cells (Ebert et al, 2015a), resulted in concomitant reduction of intrahepatic 1.0mer-derived cccDNA-like molecules. Nucleoside therapy inhibited reverse transcription of pgRNA and would not be predicted to significantly impact cccDNA levels unless their establishment was predominantly reliant on the intracellular nucleocapsid recycling pathway, a notion both our *in vitro* and *in vivo* data suggested was not the major pathway in the 1.0mer model, at least in the initial instance. Our experiments confirmed that targeting SMAC to induce death of HBV-infected cells is a feasible option, and could potentially result in cure. However, much like gene-editing approaches such as the use of epigenetic inhibitors, CRISPR, Talens (Bloom et al, 2018; Moyo et al, 2018) potential side and off-target effects need to be carefully considered. For example, if all HBV-infected cells were targeted and a significant portion of the patient's liver was infected, this may present a danger to the patient through increased hepatic damage. Nevertheless, we have demonstrated by proof of principle that this treatment approach can be used to reduce levels of surrogate cccDNA-LM in a 1.0mer HBV *in vivo* model.

In conclusion, 1.0mer HBV is suitable for investigation of all aspects of the HBV replication cycle, post-development of HBV cccDNA. Though replication is generally at lower levels compared to transfection with overlength constructs, the formation of cccDNA-like molecules provides an undeniable advantage that will overcome some of these limitations, and further optimisation such as gel-extracting the specific 1.0mer band may enhance this. Likewise, further characterisation of the mechanisms behind 1.0mer circularisation following transfection (other cellular enzymes/exonucleases may be involved in modifying overhangs to form intact junctions) may also have implications for replication efficiency.

The relative ease in which experiments can be set up and conducted makes 1.0mer transfection an attractive alternative method to flexibly induce cccDNA-LM formation and HBV replication of different variants for extensive analyses. We have also shown it is a suitable model in the context of *in vivo* drug evaluation to assess impact on chronic infection and cccDNA, which also offers the potential for developing high throughput assays for drug testing *in vitro*, although acknowledging the limitation that despite similarities, cccDNA-LM may have differences to authentic cccDNA. Further characterisation of the established cccDNA-like molecules in regards to their repair, chromatin organisation and epigenetic regulation could provide important molecular insights into authentic cccDNA and its regulation, and hopefully,

may facilitate discovery of novel therapeutic strategies toward silencing or eliminating cccDNA, which is currently a major barrier for HBV cure. Such discoveries may be preceded by investigating the specific interactions between virus and host proteins, and their impacts on the cccDNA minichromosome biology and function.

Chapter Four: Investigating the importance of HBx – host factor interactions for HBV replication

4.1 Introduction

Hepatitis B Virus X protein (HBx) plays a critical role in the HBV life cycle, particularly for the establishment of productive viral transcription and replication following viral infection (Lucifora et al, 2011). Without a clearly defined role, it is likely that HBx contributes to and mediates viral processes through multiple mechanisms (Cha et al, 2009; Doria et al, 1995). Located intracellularly in the nucleus and various cytoplasmic compartments, HBx interacts with a multitude of cellular proteins and factors to regulate many processes such as cell cycle progression, proliferation, apoptosis, DNA repair (Bagga et al, 2016; Becker et al, 1998; Cho et al, 2015; Lin-Marq et al, 2001; Rawat & Bouchard, 2015). Generally, cytosolic HBx largely exerts its effects through interactions with various proteins to modulate signal transduction pathways, while nuclear localization enables recruitment and/or interaction with transcription factors and basal transcription machinery to augment gene expression (Cheong et al, 1995; Doria et al, 1995; Lin et al, 1997). Furthermore, transcription from HBV minichromosomal templates has been correlated to epigenetic regulation by histone-modifying enzymes recruited through HBx, which itself has no known DNA-binding properties (Belloni et al, 2009; Mohd-Ismail et al, 2019)). Such enzymes identified to modify cccDNA-bound histones and impact HBV transcription include hSirt1 (Deng et al, 2017), HDAC1 (Levrero et al, 2009; Pollicino et al, 2006) , SETDB1 (Riviere et al, 2015) PRMT1 (Benhenda et al, 2013).

To date, the relationship between HBx and the cellular UV-Damaged DNA Binding (UV-DDB) subunit DDB1 is one of the most well-characterised of its identified interactions. Co-expression of HBx and DDB1 was reported to be mutually stabilising for both proteins, potentially contributing to the observed impacts on HBx-linked effects (Becker et al, 1998; Sitterlin et al, 2000a; Sitterlin et al, 2000b). Notably, DDB1 was demonstrated to enhance HBx-mediated stimulation of HBV replication (Hodgson et al, 2012; Tang et al, 2008), and in 2016, this effect was mechanistically linked to a HBx-DDB1 specific interaction (Decorsière et al, 2016; Murphy et al, 2016). These studies demonstrated that the HBx-DDB1 interaction facilitated degradation of a host factor (structural maintenance of chromosomes complex, SMC5/6) otherwise restricting cccDNA transcription. In this context, HBx acted as a substrate receptor, recruiting the SMC5/6 complex and subjecting it to the actions of an E3 ubiquitin ligase complex via its interaction with DDB1. Hence, DDB1 acted as an adaptor protein which bridged the two components together, initiating a series of reactions which ultimately relieved transcriptional repression of HBV. This illustrated the importance of DDB1 and describes one mechanism for HBx-mediated enhancement of HBV replication. Li et. al. (2010) had previously described an alpha helical motif between amino acids (aa) 88-100 of HBx, and identified this “H-Box” region as a major DDB1-binding motif (Figure 4.1).

HBV genotypes and subgenotypes affect the severity and course of chronic infection and disease (Kim et al, 2011a; Kramvis, 2014), and the response to specific treatment interventions (Erhardt et al, 2005; Kao et al, 2000b). These differences may be partly attributed to various HBx functions, since differential replication capacity and inhibition of IFN induction have been described for proteins of varying genotypes and subgenotypes.(Elizalde et al, 2019), (Wang et al, 2020). Specific HBx mutations have also been described to affect various activities, including inhibition of Akt activation by S31L substitution (Lee et al, 2012), impaired p53 binding with

A10R/S144R substitutions (Shi et al, 2016), and enhanced transactivation by S41A/P42A substitutions (Pang et al, 2007). Furthermore, HBV viruses with double basal core promoter (BCP) mutations A1762T/G1764A characterised by reduced HBeAg expression (Buckwold et al, 1996), also encode mutant HBx proteins (K130M/V131I) which are strongly associated with increased likelihood of HCC progression (Yang et al, 2008; Yin et al, 2011). These mutations have been shown to impact on HNF1 binding (Li et al, 1999), upregulate HIF-1a expression and transcriptional activity (Liu et al, 2014c), and increase p53 binding capability (Iyer & Groopman, 2011)

At present it is not known whether, different genotype-specific HBx sequences interact differently with DDB1. Even within more defined interacting region such as H-Box domain, the impact of sequence changes is unclear, due to contentious results observed in separate studies. For example, one investigation showed that the substitution of the original arginine residue to a glutamic acid (R96E) completely abrogated HBx ability to rescue replication of an HBx-deficient HBV (Leupin et al, 2005). However, another study showed that R96A substitution had seemingly no effect and that this mutant restored HBV replication just as effectively as wild-type HBx (Kim et al, 2016).

Genotypic variation was identified to induce amino acid changes at certain positions within this (“H-Box”) region between different HBV genotypes (Figure 4.1) and it was hypothesised that these, and additional proximal genotype-associated amino acid sequence differences may affect its specific interaction with DDB1, and consequently its ability to facilitate HBV replication. This in turn may contribute to differences in HBV replication identified across different genotypes (Sozzi et al, 2018; Sozzi et al, 2016).

	90								100							
	.	.		.	.	.	.		.	.	.	.		.	.	.
A2	I	L	P	K	V	L	H	K	R	T	L	G	L	P	A	.
B2	N	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.
C2	V	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.
D3	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.
J	N	.	.	R	.	.	.	.	.	.	.	.	.	S	I	.

Figure 4.1: Sequence variation in DDB1-binding region of HBx across genotypes.
Alignment of HBV genotypes, within a central HBx domain which encompasses the major DDB1-binding motif (H-Box, aa 88-100).

In this chapter, the impact of genotype-associated changes in the HBx sequence on the interaction with DDB1 was examined. This was achieved by expressing the different variants in the presence or absence of ectopic DDB1, and subsequent immunoprecipitation experiments to assess interacting abilities. A mammalian cell expression system was then used to explore protein expression and DDB1-interacting ability of select HBx variants (different genotypes and H-Box mutants) to assess their implications on HBV replication efficiency.

Complimentary to its synergistic effects, it has also been reported that DDB1 itself can stimulate replication and enhance replication, without the requirement or assistance of HBx (Kim et al, 2016). DDB1 was shown to precipitate with HBV cccDNA in a HepG2-NTCP infection model, suggesting it associates with cccDNA to directly stimulate its transcription (Figure 4.2).

Based on this premise, our characterised 1.0mer *in vitro* transfection model (Chapter 3) was utilised to further explore this mechanism. It was postulated that DDB1 would similarly enhance HBV transcription of 1.0mer cccDNA-LM in an HBx-independent manner, and that the 1.0mer model would permit the assessment of this association in greater detail, due to its production of a cccDNA-like molecule.

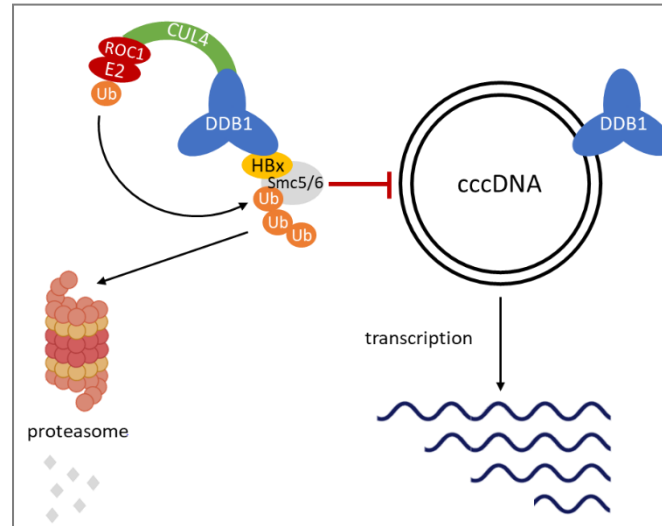


Figure 4.2: HBx-dependent (left) and –independent (right) mechanism of transcriptional stimulation by DDB1.

Left: DDB1-mediated relief of transcriptional repression involving direct interaction with HBx and subsequent degradation of host restriction factor Smc5/6. Right: DDB1 association with cccDNA leading to HBx-independent stimulation of transcription. From Kim et. al. (2016)

4.2 Assessing the effect of DDB1 expression on HBV replication

DDB1 has been proposed to directly stimulate HBV transcription via association with cccDNA (Kim et al, 2016). This was demonstrated using HepG2^{NTCP} infection model, however has not been assessed using the 1.0mer HBV replication system which generates cccDNA-LM. If this effect was observed to be consistent, it would allow comparative studies with mutant viruses, enabling identification of sequences involved in the DDB1 interaction, and their impact on regulation of HBV replication.

4.2.1 Assessment of HBx expression levels in the presence of ectopic DDB1 expression

Two independent experiments were performed to determine the impact on HBx protein expression levels in the presence of the DDB1-encoding plasmid.

4.2.1.1. *Confirmation of HBx expression from transfected plasmids*

Constructs for expression of HBx and DDB1 proteins were generated to study their interactions and impact on HBV replication. The human DDB1 gene (Accession number NM_001923), was cloned into the CMV-driven pCI expression vector (Stratagene) by Genscript (USA). An equivalent construct had previously been generated at VIDRL for the expression of the HBx protein (genotype D3). At the commencement of this project, suitable antibodies facilitating HBx detection by immunoblotting were not available, and this limitation was overcome by using traditional PCR and cloning methods to generate HBx constructs encoding a single FLAG epitope tag (DYKDDDDK) (Material and Methods, Section 2.3). HBx expression was confirmed by immunoblotting using an anti-FLAG antibody in transfected cell lysates (Figure 4.3, panel 1, lanes 1-3). These data demonstrate that N- and C-terminally-tagged HBx is expressed at higher levels in the presence of DDB1 (Figure 4.3, panel 1, lanes 4, 5) compared to samples in the absence of DDB1 (Figure 4.3, panel 1, lanes 2, 3). Wild-type HBx was not tagged and hence not detected by anti-FLAG (lane 3).

Subsequently, two HBx-specific antibodies were obtained, one commercially available (Santa Cruz, X36C), the other a kind gift from Gilead Sciences (USA). The X36C antibody was determined to efficiently detect HBx expression, to similar extent as the FLAG antibody (Figure 4.3, third panel). The anti-HBx monoclonal antibody provided by Gilead is specific for the HBx protein encoded by HBV genotype D (Gilead Sciences, personal communication) and was subsequently used for experiments involving genotype D due to its superior sensitivity compared to the X36C mAb (not shown). These results indicate that specific monoclonal antibodies can be used to confirm expression of HBx protein within mammalian cells, following transfection of the corresponding generated expression plasmids. A more detailed follow-up study was performed to assess HBx expression at different time points post-transfection.

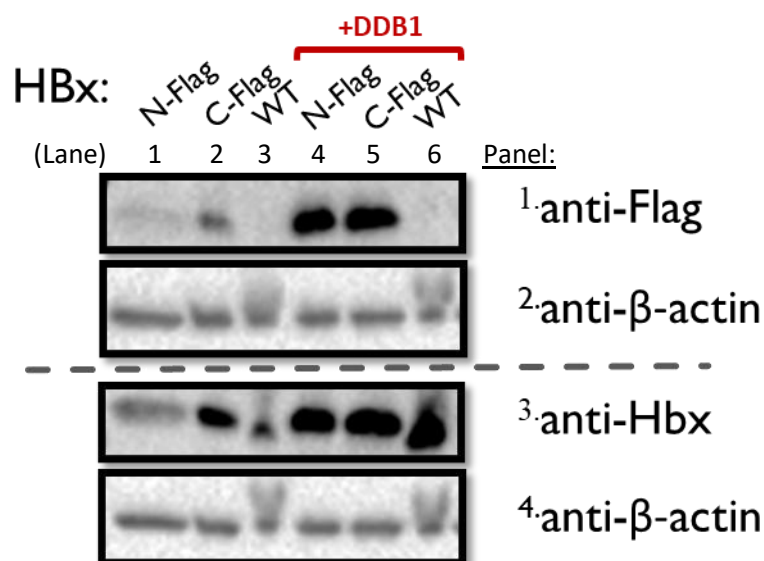


Figure 4.3: Detectable HBx expression from transiently-transfected expression constructs.

Immunoblot showing HBx expression from HepG2 cells transfected wild-type (WT), or N-terminal/C-terminal-tagged HBx proteins (N-Flag/C-Flag) alone (lanes 1-3), or with the DDB1 expression plasmid (lanes 4-6). Cells were lysed 2 days post-transfection and detected using monoclonal anti-FLAG (panel 1) or anti-HBx (panel 3) antibodies (Santa Cruz).

4.2.1.2. Detection of stabilised HBx expression when expressed with DDB1

To investigate in more detail whether the presence of ectopic DDB1 influences the level of HBx, HBx and DDB1 were co-expressed. Co-transfection experiments using HBx and DDB1 expression plasmids in hepatoma cells showed that detection of HBx was generally low when expressed in the absence of DDB1 (Figure 4.4, lanes 1-3). Expression appeared to diminish over time, with highest expression at day 2 post-transfection reducing greatly by day 5 post-transfection (Figure 4.4, compare lanes 1, 4, 7). These results confirmed the results of the prior experiment showing enhanced HBx levels in the presence of DDB1-expressing plasmid (Figure 4.3). This effect was observed for both the HepG2 and Huh-7 cell lines, with HBx expression generally higher in Huh-7 cells (not shown). Co-expression of exogenous DDB1 appeared to stabilise HBx protein, resulting in increased detection at day 5 post-transfection (compare lanes 7 and 8). This effect was consistently observed using wild-type HBx expression constructs, FLAG-tagged HBx constructs (Figure 4.3, lanes 4-6), and replication competent 1.3mer and 1.0mer HBV genome constructs (Figure 4.5). Collectively, these experiments indicated more stable HBx protein expression levels from specific plasmids, in the presence of a DDB1-encoding plasmid. Failure to detect DDB1 in immunoblots using anti-DDB1 antibody (#5428, CST), prompted the generation of myc-tagged DDB1 protein to confirm correlating DDB1 expression with HBx stabilisation (Section 4.2.1.3).

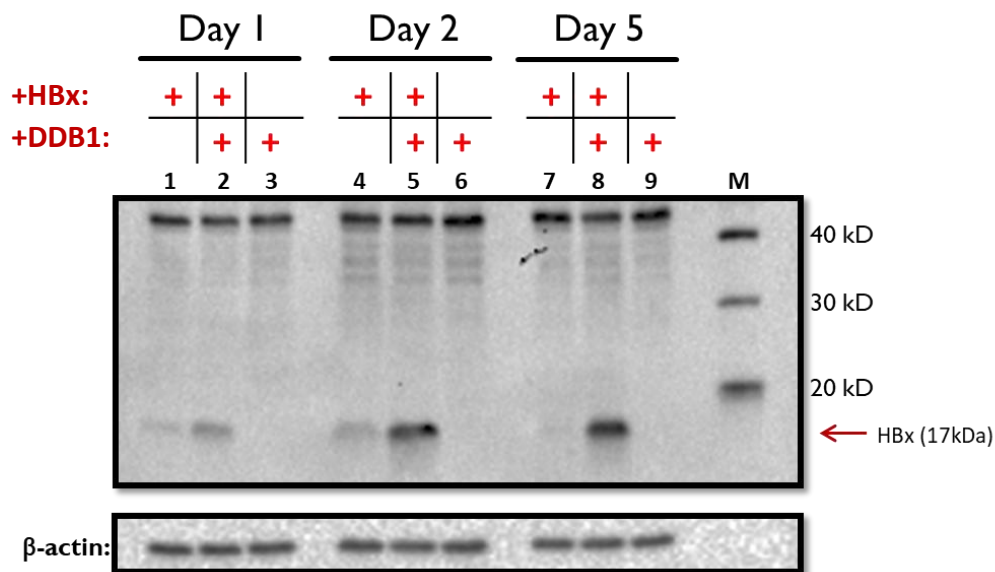


Figure 4.4: Immunoblot showing HBx expression up to 5 days post-transfection. HBx construct (D3, wild-type) was transfected alone, or with the DDB1 expression plasmid into HepG2 cells and harvested at the indicated days post-transfection for immunoblot analysis using an HBx-specific monoclonal antibody (Gilead Sciences).

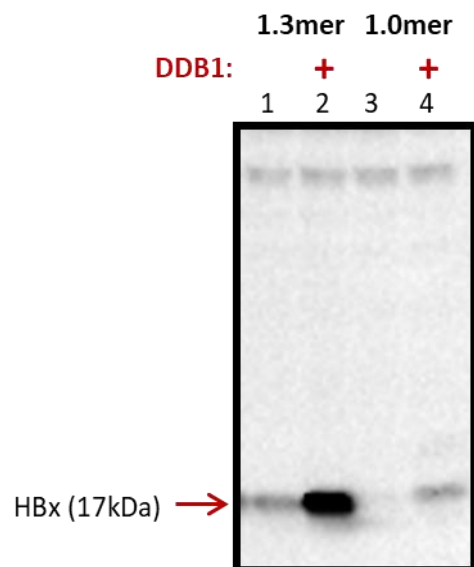


Figure 4.5: HBx expression from replication-competent HBV constructs. WT 1.3mer (lanes 1, 2) or 1.0mer (lanes 3, 4) HBV clones were transfected into Huh7 cells alone, or with DDB1 expression plasmid (lanes 2, 4). Lysates were harvested 2 days post-transfection for immunoblot analysis using specific monoclonal HBx antibody (Gilead Sciences, GS-).

4.2.1.3. Confirmation of DDB1 overexpression from transfected plasmids

Although enhanced HBx expression was detected following over-expression of DDB1 (Fig 4.3-4.5), immunoblot experiments using an anti-DDB1 antibody (Cell Signaling Technology, #5428S) failed to unambiguously confirm DDB1 expression (not shown). Consequently, c-myc epitope tags (EQKLISEEDL) were added to the termini of DDB1 sequence by PCR and cloning (Section 2.3). Comparative analyses were performed to detect DDB1 with the myc-tag fused to the N- or C-terminus (Figure 4.6, lanes 3, 4, 6). The presence of the double myc-tag at the N-terminus (DDB1-N^{2myc}) correlated with stronger bands detected using anti c-myc mAb (#2276, CST), relative to the background levels observed with a construct containing a single myc-tag at the C-terminus (DDB1-C^{myc}) (Figure 4.6, lanes 4, 6), as well as the slightly lower molecular weight background bands (untransfected: lane1, untagged DDB1: lane 3, HBx only: lane 5).

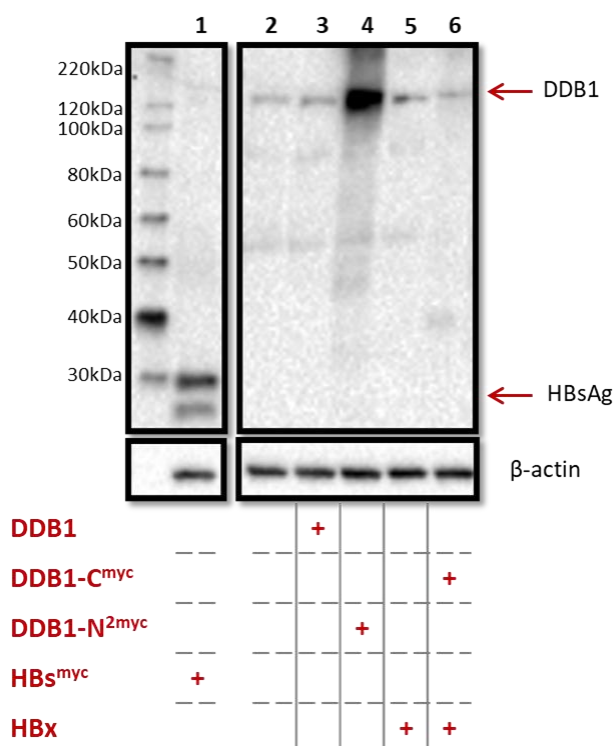


Figure 4.6: DDB1 expression from various expression constructs, indicated below blot

Huh-7 cells were transfected with various DDB1 expression plasmids (lanes 3, 4, 6) and harvested 2 days post-transfection for immunoblot detection using monoclonal anti-myc antibody (CST). Myc-tagged HBs was included as a positive control for antibody specificity. Two bands are visible, representing glycosylated and non-glycosylated forms of HBsAg (lane 1). Lane 2 represents untransfected cells, cells were transfected with HBx construct only in lane 5.

The absence of these bands in samples immunoprecipitated with anti-HBx antibody (Figure 4.7, middle panel) suggest these might represent unrelated endogenous proteins not directly interacting with HBx. Similar to the native DDB1 expression construct, expression of DDB1-N^{2myc} was confirmed to also enhance HBx expression (not shown) and was selected as the DDB1 expression plasmid utilised in subsequent investigations.

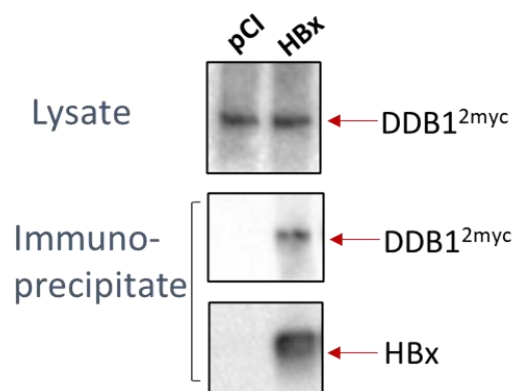


Figure 4.7: Direct interaction between expressed HBx and DDB1-N^{2myc} proteins.

Huh-7 cells were transfected with myc-tagged DDB1 expression plasmids, with mock (pCI) or HBx co-transfection. Lysates were harvested 2 days post-transfection and immunoprecipitated using HBx-specific antibody. Immunoblots show DDB1 (top and middle panel) and HBx (bottom panel) expression detected using antibodies to c-myc epitope and HBx, respectively.

4.2.2 HBV replication analysis

4.2.2.1. Verification of HBxΔ variant viability

To assess the effect of DDB1 on HBV replication, independent of HBx, comparative studies were performed using 1.3mer and 1.0mer HBV WT genomes, and equivalent constructs deficient in HBx expression (HBxΔ). 1.3mer HBxΔ constructs containing mutations in both copies of the HBx gene to completely abrogate expression of the HBx protein, was previously generated by Mrs Sally Rodgers and Dr. Nadia Warner at VIDRL. Equivalent 1.0mer constructs were generated by site-directed mutagenesis (SDM) of the WT 1.0mer construct to mutate the initiating codon of HBx, abrogating its expression. Transfection into HepG2 and Huh-7 cells was performed, and analysed for HBV replicative intermediates, in order to evaluate the viability and replication competency of these HBxΔ constructs, as was previously confirmed for the WT constructs (Chapter 3).

4.2.2.2. Confirmation of HBx (expression) abrogation in HBx-deficient (HBxΔ) constructs

HBx detection was confirmed for both 1.3mer and 1.0mer WT constructs from transfected cells, but not in cell lysates derived from cells transfected with the HBxΔ constructs (Figure 4.8, lane 1 and 3, compared to 2 and 4, respectively). Accordingly, DDB1 co-expression enhanced HBx detection from WT (lanes 7 and 9) and as expected had no effect on the HBxΔ variants (lanes 8 and 10).

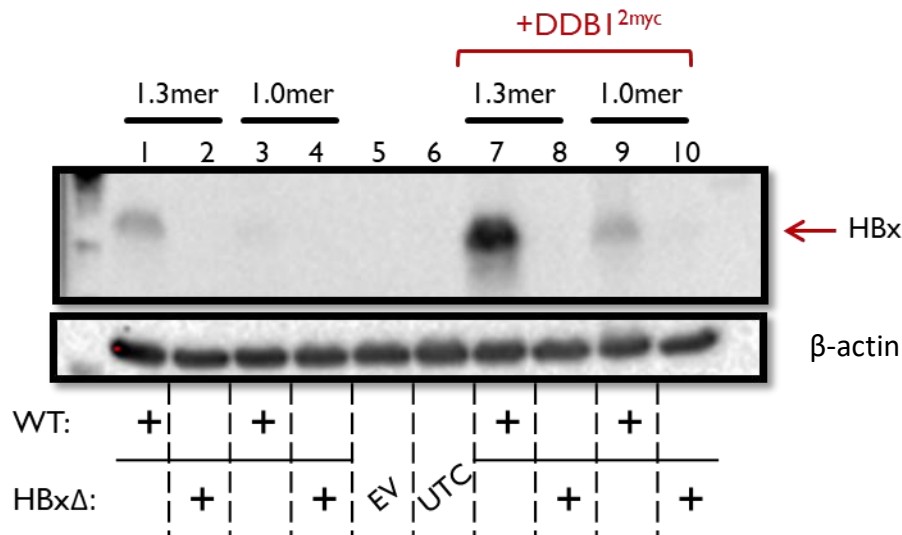


Figure 4.8: Immunoblot showing HBx expression from replication competent HBV constructs. WT or HBx-deficient (HBxΔ) constructs were transfected into Huh7 cells and analysed by immunoblotting with monoclonal HBx antibody (Gilead Sciences), 5 days post-transfection.

4.2.2.3. Confirmation of HBV DNA expression

All constructs were confirmed to be replication-competent, as determined by analysis of HBV core-associated and nuclear DNA by Southern blotting. In the previous chapter, a higher replication level was demonstrated with 1.3mer HBV compared to 1.0mer HBV. Likewise, higher HBx expression was observed following transfection with 1.3mer HBV compared to 1.0mer constructs (Figure 4.8, lane 1 vs 3, 7 vs 9). Southern blot analysis confirmed detection of expected bands HBV from core particle-associated DNA (rcDNA, dsIDNA, ssDNA, Figure 4.9), with HBxΔ constructs exhibiting lower levels than the WT counterpart (compare lanes 1 and 2). In contrast, this difference was not apparent for the equivalent 1.0mer constructs (compare lanes 3 and 4). Hirt extractions following transfection of these variant 1.0mer HBxΔ constructs also confirmed production of cccDNA-LM (Figure 4.10), as had been previously demonstrated for the WT HBV constructs in Chapter 3 (Figure 3.6). These results confirmed that HBx-deficient 1.0mer HBV constructs were not impaired in the formation of cccDNA-LM following transfection, and were replication-competent.

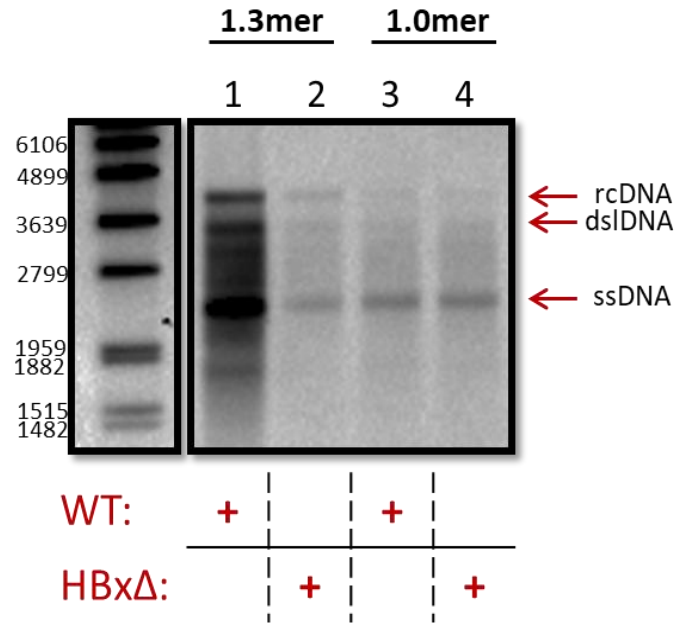


Figure 4.9: Detection of core-associated, HBV DNA from replication competent 1.3mer and 1.0mer HBV (Genotype D3) construct by Southern blot. HepG2 cells were transfected with wild-type (WT, lane 1 and 3) and HBx-deficient (HBxΔ) mutants (lanes 2 and 4), intracellular core-associated DNA isolated and analysed with a DIG-labelled probe specific for HBV DNA. rcDNA: relaxed circle DNA, dsl: double-stranded linear DNA, ssDNA: single-stranded DNA

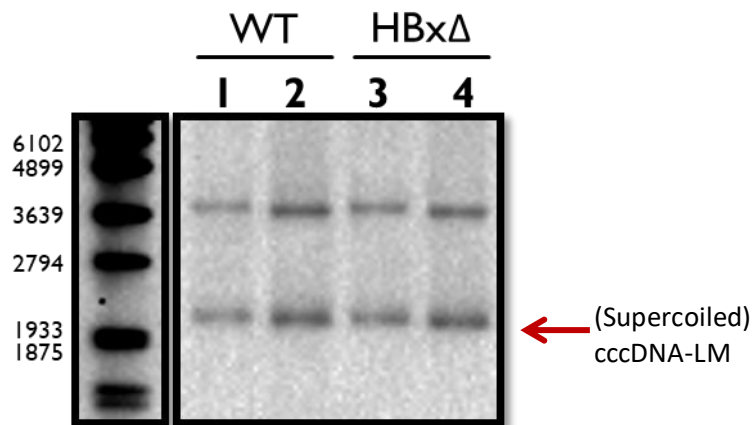


Figure 4.10: Detection of nuclear HBV DNA from replication competent 1.0mer HBV (genotype D3) constructs by Southern blot. HepG2 cells were transfected with WT (lanes 1 and 2) and HBxΔ mutants (lanes 3 and 4), nuclear DNA isolated and analysed with a DIG-labelled probe specific for HBV DNA. cccDNA-LM: cccDNA-like molecule

4.2.2.4. Confirmation of HBV RNA synthesis

To confirm the ability of the 1.0mer HBV constructs to serve as templates for mRNA synthesis, total mRNA was isolated and analysed according to Section 2.10. Northern blotting of total RNA extracted from cell lysates using HBV-specific probes, confirmed that all HBV constructs were transcriptionally active, with detection of 3.5kb, 2.4kb/2.1kb, and 0.7kb HBV-specific transcripts (Figure 4.11). The identification of greater-than-full length transcripts strongly indicates that the linear monomeric molecule circularises to form a cccDNA-like molecule. As observed for HBV DNA, lower levels of HBV RNA were observed following transfection with 1.0mer (lanes 4 and 5) constructs than with 1.3mer (lanes 2 and 3). Also consistent with the DNA results (Figure 4.9, lane 1 and 2), HBxΔ variants similarly produced lower levels when compared to the WT construct, the difference being more pronounced between the respective 1.3mer constructs (Figure 4.11, compare lanes 2 and 3), while expression appeared comparable between the 1.0mer HBV variants (compare lanes 4 and 5) and was consistent with only marginal differences in DNA expression between both variants (Figure 4.9, lane 3 and 4).

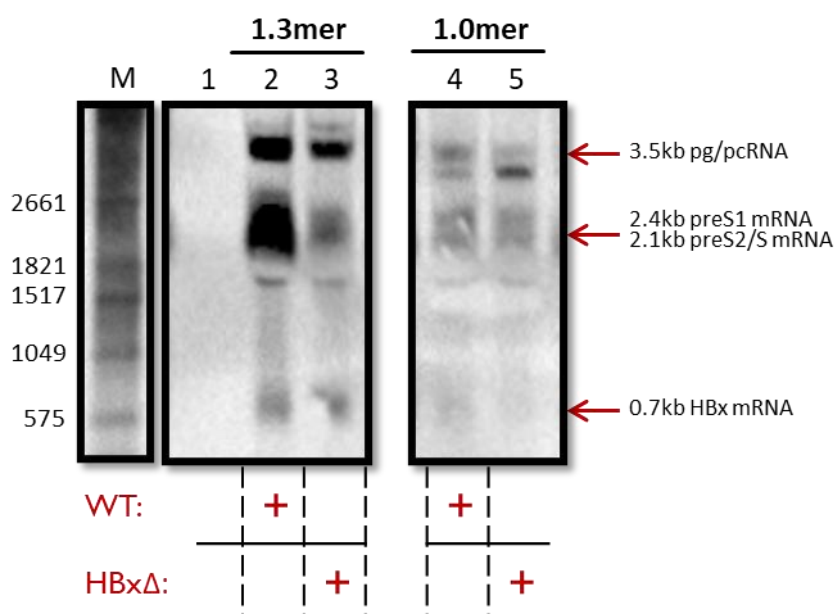


Figure 4.11: Detection of HBV RNA following transfection of infectious HBV constructs, by northern blot.

Huh-7 cells were transfected with genotype D3 WT or HBxΔ HBV constructs (1.3mer: lanes 2, 3; and 1.0mer: lanes 4, 5). Total intracellular RNA was isolated 5 days post-transfection and analysed with a DNA-labelled probe specific for HBV RNA. Lane 1: untransfected cells. pgRNA: pregenomic RNA, pcRNA: precore mRNA, preS/S: pre-surface/surface

4.2.2.5. Confirmation of HBV protein secretion

To demonstrate HBV protein expression, secreted antigens in the supernatant of transfected cells were measured using the Elecsys platform (Roche). These results confirmed expression and secretion of both HBsAg (Figure 4.12, left panel) and HBeAg (Figure 4.12, right panel) following transfection with 1.3mer and 1.0mer HBxΔ constructs. Lower levels of HBeAg were observed from 1.0mer than 1.3mer constructs, while HBsAg levels were more comparable between the two genome configurations.

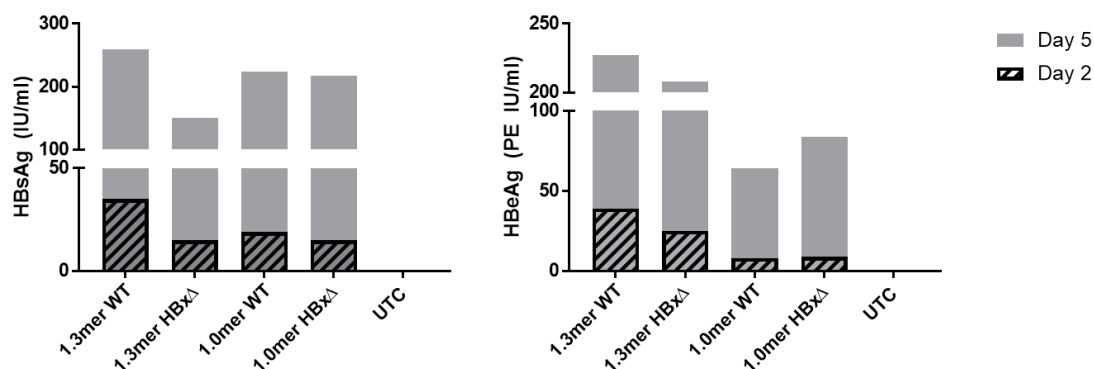


Figure 4.12: Quantitative HBsAg (left panel) and HBeAg (right panel) analyses of cell culture supernatant following transfection with various HBV constructs.

WT or HBxΔ HBV constructs of HBV genotype D3, were transfected into HepG2/Huh7 cells. Cell culture supernatant was collected 5 days post-transfection and analysed for secreted antigens on the Elecsys platform (Roche). Results represent a single experiment.

Although investigations are focused on HBV genotype D3 in this particular study, equivalent genotype C2 1.0mer constructs have also been generated. Transfection of these constructs likewise produced detectable HBsAg and HBeAg in the cell culture supernatant (Figure 4.13, left and right panel, respectively). As observed with the 1.3mer constructs, HBxΔ constructs produced lower levels of secreted HBeAg and HBsAg than the WT counterparts, and this was observed with both genotypes investigated. These results further demonstrate the utility of this cell culture transfection model in facilitating comparative investigations involving different HBV genotypes and potentially other interesting HBV variants. Together, these results suggest that 1.3mer HBV constructs produce more efficient replication in cells than do 1.0mers, and that the effect of HBx protein on replication levels may be greater in the 1.3mer system compared to 1.0mers.

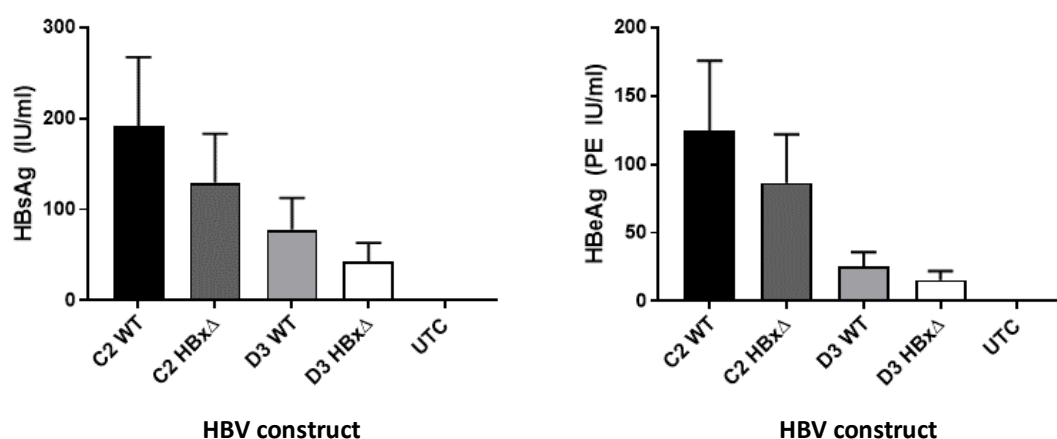


Figure 4.13: Quantitative HBsAg (left panel) and HBeAg (right panel) analyses of cell culture supernatant following transfection with various 1.0mer HBV constructs.

WT or HBxΔ HBV constructs specific for genotype C2 or D3, were transfected into HepG2 or Huh-7 cells. Cell culture supernatant was collected 5 days post-transfection and analysed for secreted antigens on the Elecsys platform (Roche). Bars represent averaged HBeAg/HBsAg from 3 independent experiments.

4.2.2.6. *DDB1-mediated enhancement of HBV replication*

Having confirmed that the HBxΔ HBV constructs were replication-competent, these were then used to investigate the impact of DDB1 expression on HBV replication. The 1.0mer transfection model was specifically used to investigate DDB1 stimulation of transcription from episomal templates in the presence or absence of HBx expression. Replication initiated from 1.3mer HBV constructs is reportedly enhanced in the presence of ectopic DDB1 protein expression (Kim et al, 2016), and this finding was confirmed in our initial experiments. Follow-up studies using 1.0mer constructs also replicated the outcome achieved with the 1.3mer constructs, demonstrating elevated replication in the presence of DDB1. These experiments confirmed increased replication from 1.3mer WT HBV when co-transfected with DDB1 expression plasmids, as demonstrated by Southern (Figure 4.14 (left) lane 1 vs 2) and northern blotting (Figure 4.14 (right) lane 1 vs 2) for the detection of intracellular HBV DNA and RNA, respectively. Increased replication was also observed from transfected 1.0mer HBV constructs following DDB1 co expression (Figure 4.14, lanes 3 vs 4).

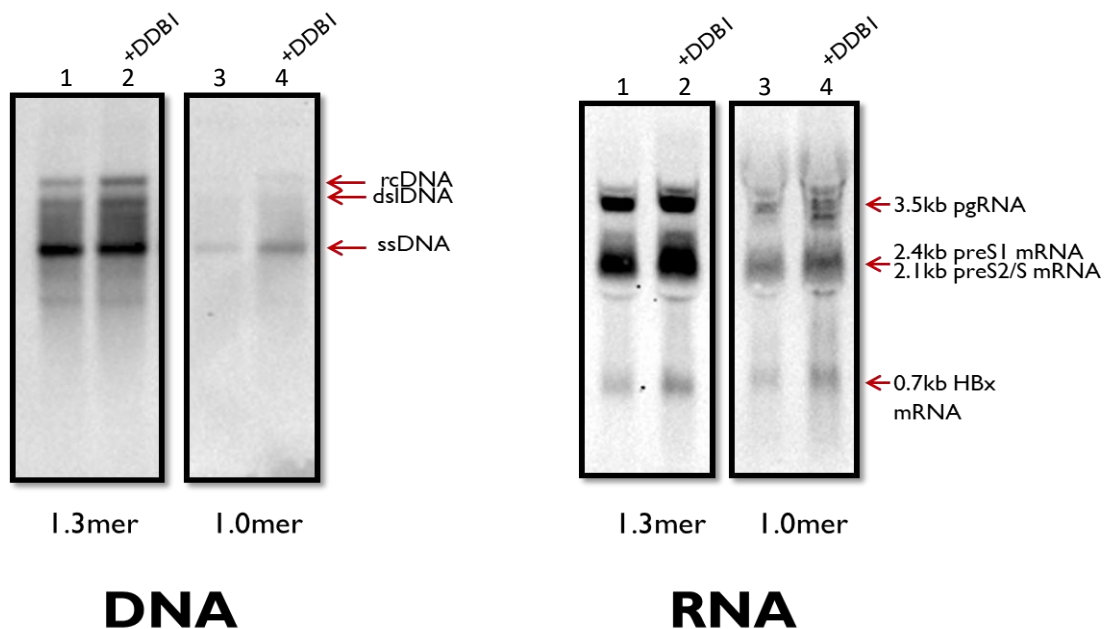


Figure 4.14: Southern and northern blot of intracellular core-associated HBV DNA and intracellular HBV RNA 5 days following transfection.

HepG2 and Huh-7 cells were transfected with 1.3mer and 1.0mer WT HBV constructs alone, or co-transfected with DDB1 expression plasmids. Cells were harvested 5 days post-transfection and extracted for HBV DNA or RNA and detected using HBV-specific probes. rcDNA: relaxed circle DNA, dsI: double-stranded linear DNA, ssDNA: single-stranded DNA, pgRNA: pregenomic RNA, pcRNA: precore mRNA, preS/S: pre-surface/surface

To evaluate the reported HBx-independent effect of DDB1 on replication, experiments were repeated using the HBx-deficient 1.0mer HBV constructs, since these form cccDNA-LM. Previous reports using 1.3mer transfection and HepG2^{NTCP} infection models suggested that DDB1 enhanced HBV replication independently of HBx expression, by associating with and stimulating transcription of cccDNA. Indeed, replication following transfection with 1.0mer HBxΔ constructs was observed to slightly increase following co-transfection with the DDB1 expression plasmid (Figure 4.15). Southern and northern blotting of intracellular replicative

intermediates of HBV, showed increased expression when HBx or DDB1 were provided in *trans* (compare lane 1 to lanes 2 and 3).

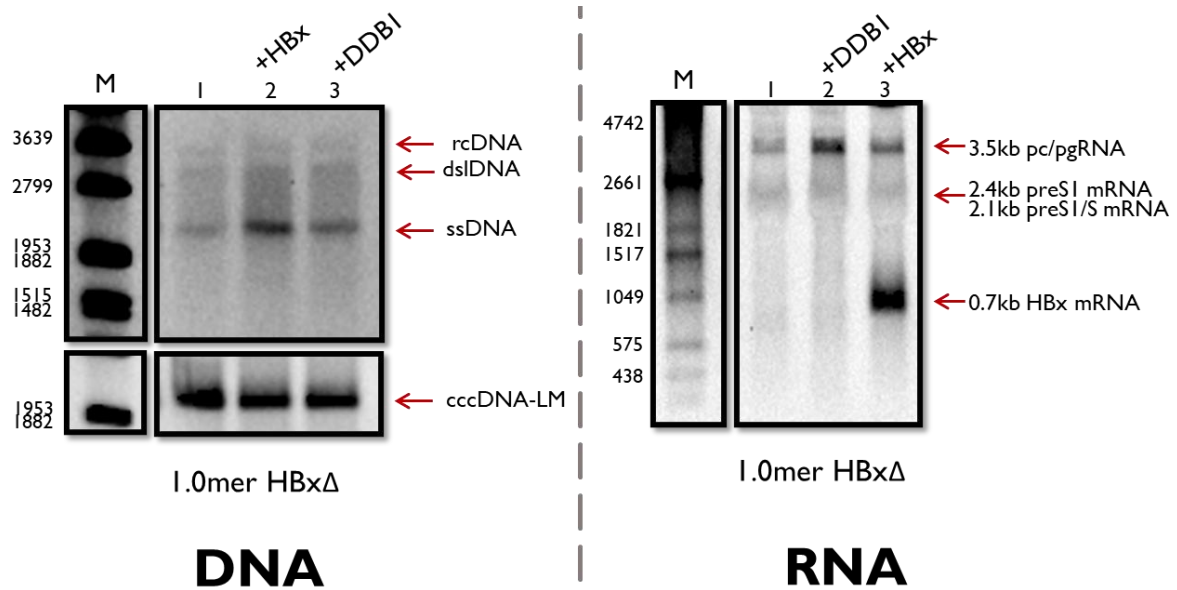


Figure 4.15: Southern and northern blot of intracellular core-associated HBV DNA and intracellular HBV RNA 5 days following transfection.

Cells were transfected with 1.0mer HBx-deficient HBV constructs alone (lane 1), or co-transfected with HBx (left, lane 2; right, lane 3) or DDB1 (left, lane 3; right, lane 2) expression plasmids. Core-associated DNA (left, top panel) and nuclear DNA (left, lower panel) were extracted using in-house methods and detected by Southern blotting with HBV-specific probes. Total RNA was extracted using Qiagen RNEasy Mini Kit and detected by northern blotting with HBV-specific probes.

In addition, quantitative serology of secreted HBeAg and HBsAg in the cell culture supernatant also exhibited slightly increased levels (Figure 4.16, grey bars). In contrast, DDB1 expression had no observable effect on the expression level of the cccDNA-LM itself (Figure 4.15, left, lower panel). However this finding is from one preliminary experiment and further experiments are necessary to confirm if the observed differences are statistically significant.

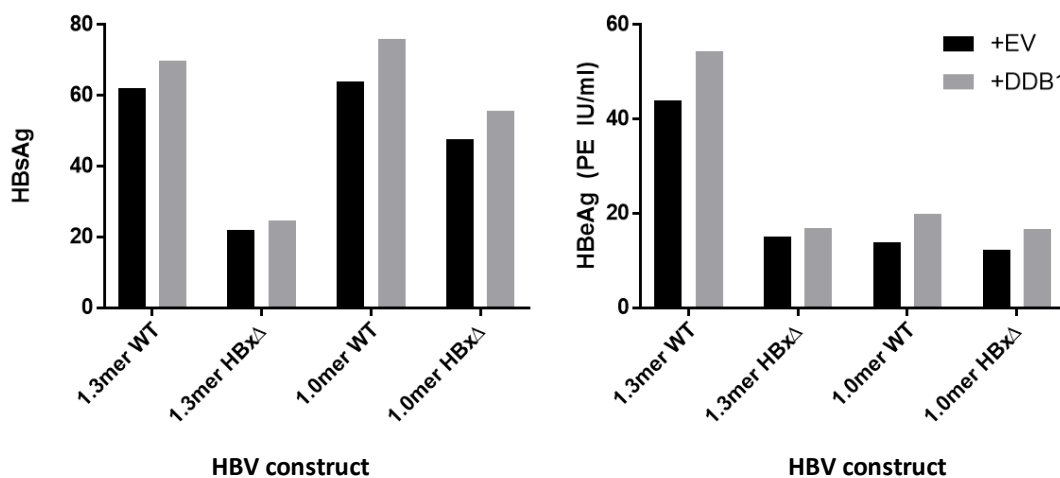


Figure 4.16: Quantitative detection of HBeAg and HBsAg in supernatant of cells transfected with various HBV constructs. Cells were transfected with WT/HBxΔ HBV constructs in conjunction with DDB1-expressing plasmid or empty vector (EV). Cell culture supernatant was collected and tested for levels of secreted HBsAg (left) and HBeAg (right) using the Elecsys platform (Roche). Results from a single experiment.

The outcomes indicate a moderate enhancement of HBV replication markers in the absence of HBx (1.0mer HBxΔ construct) and in the presence of DDB1, indicating that DDB1 may act on HBV replication in a HBx-independent manner. These findings lend further support to the previous findings of Kim et. al., (2016) in which it was demonstrated that DDB1 could enhance HBV replication in a HBx-independent manner. They proposed that this was due to association of DDB1 with HBV cccDNA to directly stimulate transcription, although specific mechanisms were not proposed.

Moreover, our findings show that the use of the 1.0mer construct provides a suitable model for functional studies of interactions between HBx, host proteins and HBV cccDNA, to investigate their impact on HBV replication *in vitro*. The 1.0mer system is likely more physiologically relevant than the 1.3mer transfection model, due to the production of the episomal cccDNA-like template. Future studies should utilise the 1.0mer transfection model and chromatin immunoprecipitation-based assays to further interrogate interactions between DDB1 and cccDNA, and elucidate the mechanisms behind the proposed transcriptional regulation.

4.3 The impact of DDB1 on HBx expression across genotypes

Previously, the HBx-DDB1 interaction was thought to be indispensable for facilitating HBV transcription, and thought to be the major mechanism by which HBx mediates its enhancing effects on HBV replication. Having identified sequence variation between different HBV genotype HBx genes, within the DDB1-binding H-Box motif, we hypothesised these changes may impact on the HBx-DDB1 interaction and by implication, replication of different HBV genotypes. To investigate this, a series of variant HBx expression plasmids were generated and co-transfected into hepatoma cells along with the DDB1 expression plasmid, and their impact on HBx and DDB1 expression investigated by immunoprecipitation and immunoblotting. (Methods described in Section 2.8.2)

4.3.1 Analysis of HBx-DDB1 interaction

4.3.1.2. Sequence variability in the HBx gene did not impair DDB1-mediated enhancement of HBx expression

To determine the impact of genotype-associated sequence changes in the HBx gene on its interaction with DDB1, CMV-driven expression plasmids (Genscript) of wild-type HBx protein for selected HBV genotypes (A1, A2, C2, D3), and a selection of HBx variants were generated (Table 4.1). Variants included the clinically relevant BCP A1762T/G1764A double mutant which corresponds to HBx amino acid K130M/V131I mutations, as well as previously described DDB1-binding-impaired HBx mutants R96E (Lin-Marq et al, 2001). Permissive hepatoma cell lines were either singly or doubly transfected with the different HBx constructs and the DDB1 expression plasmid, and were harvested for analysis at multiple time points post-transfection, as per usual protocol (Section 2.8.2).

Table 4.1 Protein Expression Plasmids (in pCI backbone)

Construct	Description
Wild-type HBx - A1 - A2 - C2 - D3	Wild-type HBx sequences within pCI vectors (Stratagene, USA) for listed HBV genotype
Tagged HBx - N-Flag - C-Flag	Wild-type HBx (D3) sequences with a single FLAG (DYKDDDDK) epitope sequence added to either amino (N-Flag) or carboxy (C-Flag) terminus, within pCI vectors
Mutant HBx - Basal core promoter (BCP) - R96E - R96A - 96/8/9A	Genotype D3 HBx sequence with introduced mutations by site-directed mutagenesis of the wild-type sequence - BCP: A→T/G→A substitutions; resulting in K130M/V131I amino acid changes [BCP: A1762T/G1764A] - R96E: A→G/G→A substitutions; resulting in R96E amino acid change - R96A: A→G/G→C/ G→T substitutions; resulting in R96A amino acid change - 96/8/9A: : ○ A→G/G→C/ G→T substitutions; resulting in R96A amino acid change ○ C→G/T→C substitutions; resulting in L98A amino acid change ○ G→C/ A→T substitutions; resulting in G99A amino acid change
Wild-type DDB1	Human DDB1 sequence within pCI vector
Tagged DDB1 - C^{myc} - N^{2myc}	- Human DDB1 sequence within pCI vector with single c-myc (EQKLISEEDL) epitope sequence added to the carboxy terminus (C^{myc}), or double tag added to amino terminus (N^{2myc})

Expression of all HBx variants was confirmed by immunoblotting (Figure 4.17). Subtle differences were observed in HBx expression levels between different constructs, such as higher expression with the genotype A1 construct, and lower expression of the BCP mutant construct (genotype D3) compared to its WT counterpart. One-way ANOVA analysis of combined densitometric analysis from multiple experiments showed no significant difference between mean expression of any of the genotype-specific or mutant HBx (BCP/R96E) constructs (Tukey's multiple comparison test). Exogenous DDB1 expression consistently upregulated HBx expression as detected on immunoblots, for all wild-type constructs for genotypes A, C and D, as well as the HBx D3^{BCP} variant. Analysis of densitometry data (Holm-Sidak) again showed this difference in expression when DDB1 was added to be statistically insignificant for any of the constructs investigated. Moreover, no discernible differences in HBx expression were observed across genotypes following addition of DDB1 (Figure 4.17, lanes 7-11), indicating that genotype-mediated sequence variability in the HBx sequence did not adversely impact the HBx-DDB1 interaction, despite introducing amino acid changes in the DDB1-binding H-Box motif (Figure 4.1). Similar findings were observed for the HBV BCP variant which specifically encoded altered HBx residues at positions 130 and 131 (lane 10 vs 11), indicating these mutations did not negatively affect interaction with DDB1. This finding is

consistent with these residues being located outside of the major DDB1-interacting H-Box region of HBx. In contrast, enhancement was significantly impaired with an H-Box mutant in which the arginine residue at position 96 was changed to glutamate (R96E, Figure 4.17, lane 12). This mutant has been previously characterised as a DDB1-binding-impaired variant (Leupin et al, 2005; Lin-Marq et al, 2001), and was included in all experiments as a negative control.

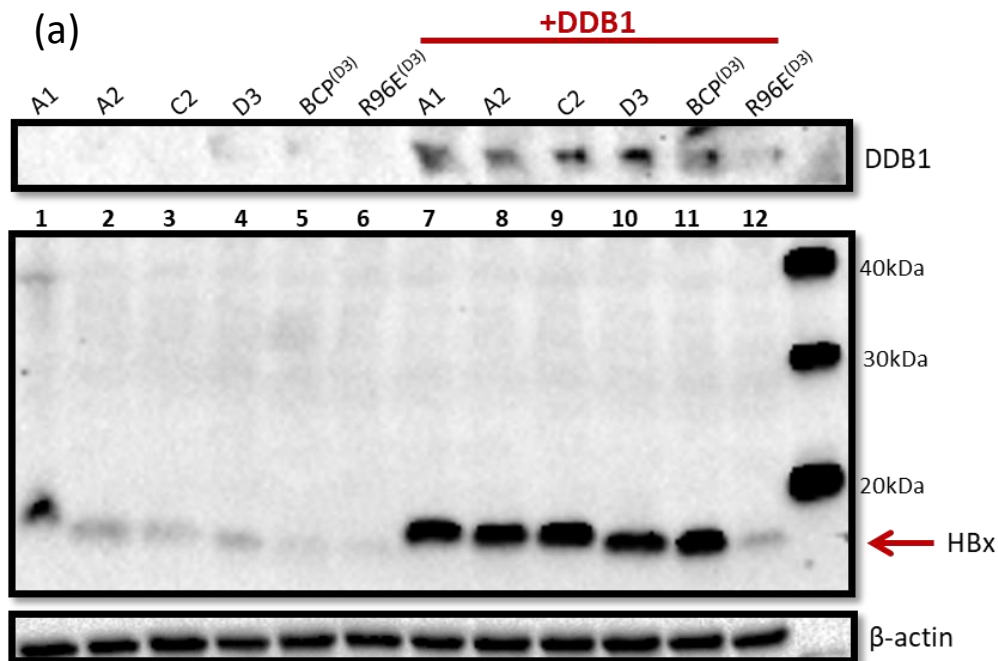


Figure 4.17 (a): Immunoblot showing variant HBx expression (middle panel) +/-DDB1 co-expression (top panel). WT HBx expression plasmids (genotype A1, A2, C2, D3) and mutant HBx (gt D3 background-BCP, K130M/V131I mutant; DDB1-binding impaired R96E mutant)) were transfected alone, or with DDB1 expression plasmid and harvested 2 days post-transfection. Western blotting was performed to detect expression of HBx and DDB1 using specific antibodies.

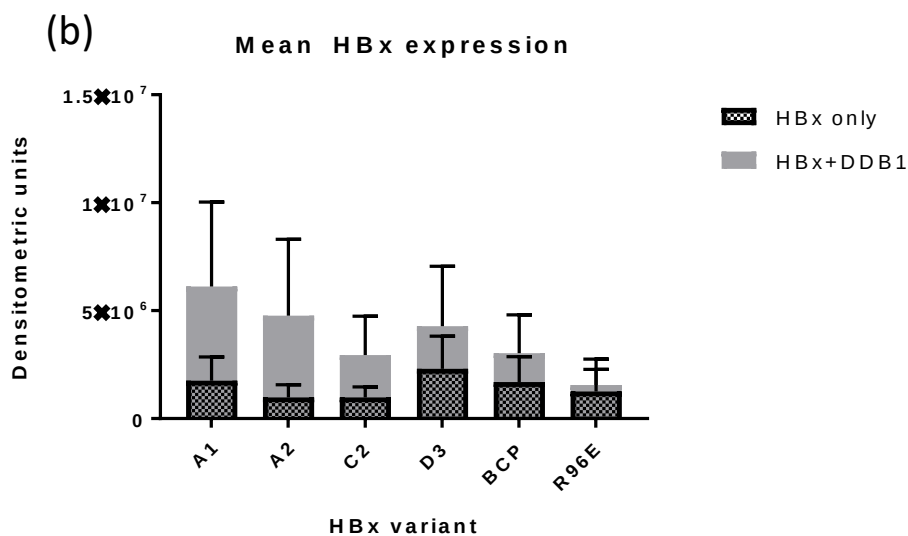


Figure 4.17 (b): HBx expression following transfection with HBx expression plasmid alone, or in conjunction with DDB1 expression plasmid. Bars represent averaged results from densitometric analysis of multiple independent immunoblotting experiments.

4.3.1.3. *DDB1 dependence in HBx-mediated enhancement of HBV replication/DDB1-binding* *HBx mutant expression*

Early evidence established a requirement for DDB1 HBx-mediated enhancement of HBV transcription and replication, supported by experiments using a R96E HBx mutant which severely impacted its ability to interact with DDB1 (Leupin et al, 2005), and consequently was incapable of rescuing HBV replication from a HBx-deficient construct. In contrast, Kim et al., (2016) demonstrated that the R96A HBx mutant was able to rescue replication of HBx-deficient HBV, to almost the same level as that of WT HBx. Protein expression and interaction experiments were conducted to explore these conflicting results and directly compare the different DDB1-binding-impaired HBx mutants. Both mutants (R96A and R96E) were included in these investigations. In addition, a mutant HBx protein was investigated which combines different mutations analysed by Kim et al (2016). The mutant protein 96/8/9A contains three alanine residues at amino acid 96, 98, and 99 which replace R, L, and G, respectively. These various constructs were transfected into HepG2 and/or Huh7 cells for subsequent immunoblot analysis.

These transfection experiments showed that WT and mutant HBx proteins are expressed at similar levels and exhibit similar patterns, with relatively high expression at 2 days post-transfection which decline by day 5 (Figure 4.18). This reduced expression was not apparent when HBx was co-transfected with DDB1 (lane 2 in each panel). Interestingly, expression of HBx at day 5 appeared to be higher in Huh7 cells than HepG2 cells, for reasons that were unclear, but may be a contribution of higher level of endogenous DDB1 expression in Huh7 cells compared to HepG2 as noted by Leupin et al (2005).

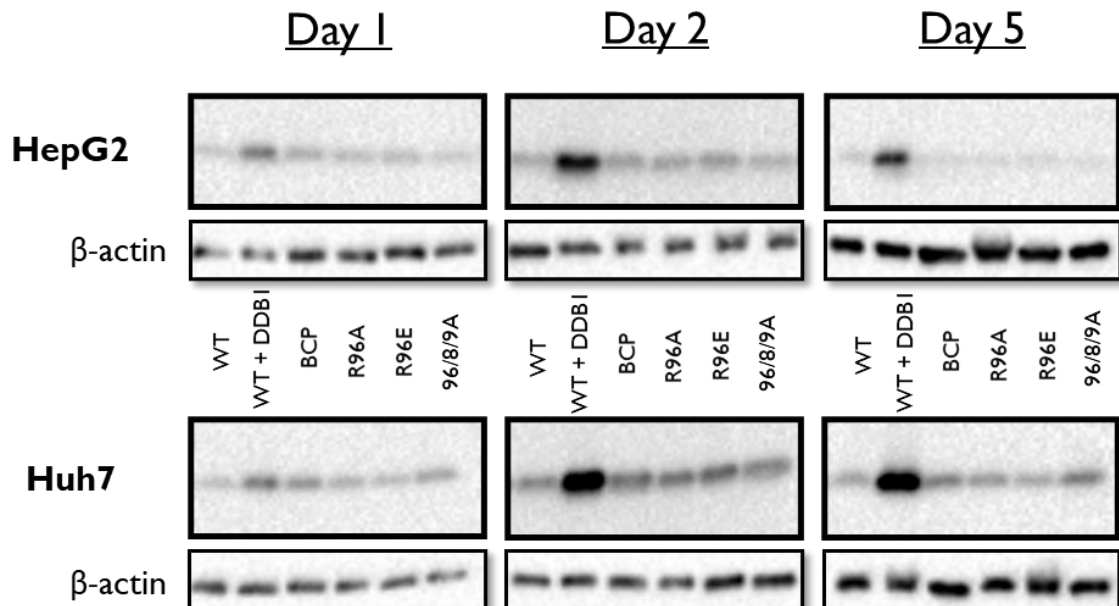


Figure 4.18: Immunoblot detection of mutant HBx expression in a time-course experiment, in HepG2 and Huh7 cells.

WT or mutant HBx (genotype D3) expression plasmids were transfected into HepG2 or Huh7 cells and harvested at the indicated days post-transfection for western blot analysis using an HBx-specific monoclonal antibody (Gilead Sciences).

All H-Box mutants examined (R96A, R96E and 96/8/9A, Table 4.1) impaired DDB1-mediated enhancement of HBx expression as compared to the WT HBx (Figure 4.19), consistent with the outcome achieved for the R96E HBx mutant described earlier (Figure 4.17). Curiously, impairment appeared more substantial for the R96E mutant in Huh7 cells compared to HepG2 cells, though the reason was unclear.

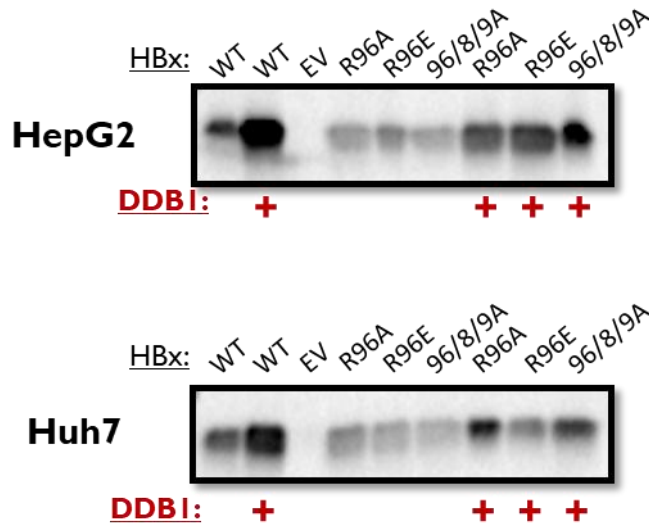


Figure 4.19: Immunoblot showing mutant HBx expression in HepG2 and Huh7 cells. WT or mutant HBx (genotype D3) expression plasmids were transfected alone, or with DDB1 expression plasmid into HepG2 or Huh7 cells and harvested at 48 days post-transfection for western blot analysis using an HBx-specific monoclonal antibody.

Immunoprecipitation experiments were performed to further investigate the interaction between DDB1 and these HBx mutants. Because preceding results showed there was no difference in DDB1-mediated augmentation of HBx expression across HBV genotypes (Figure 4.17), studies were limited to HBV genotype D HBx. Mutant HBx and DDB1 expression constructs were co-transfected as described previously, and lysates were immunoprecipitated using HBx- or c-Myc-specific (for DDB1-N^{2myc}) antibodies prior to immunoblotting. A non-interacting HBs-myc expression plasmid was also transfected as a control.

Immunoprecipitation with anti-HBx antibody confirmed expression of all HBx constructs, with slightly lower expression levels observed for DDB1-binding mutants (Figure 4.20). Furthermore, only marginal differences were observed in DDB1 expression following HBx pull-down, indicating possible interaction between DDB1 and these HBx mutants. These results were surprising as we had previously observed that DDB1-binding had markedly reduced ability to enhance HBx levels (Figure 4.17), possibly compromising the interaction. This suggests that regions outside of the HBx H-Box motif may contribute to DDB1-binding but may not contribute to HBx expression or stability of the HBx protein, although we have confirmed that the BCP/HBx 130/131 mutation does not contribute to this association.

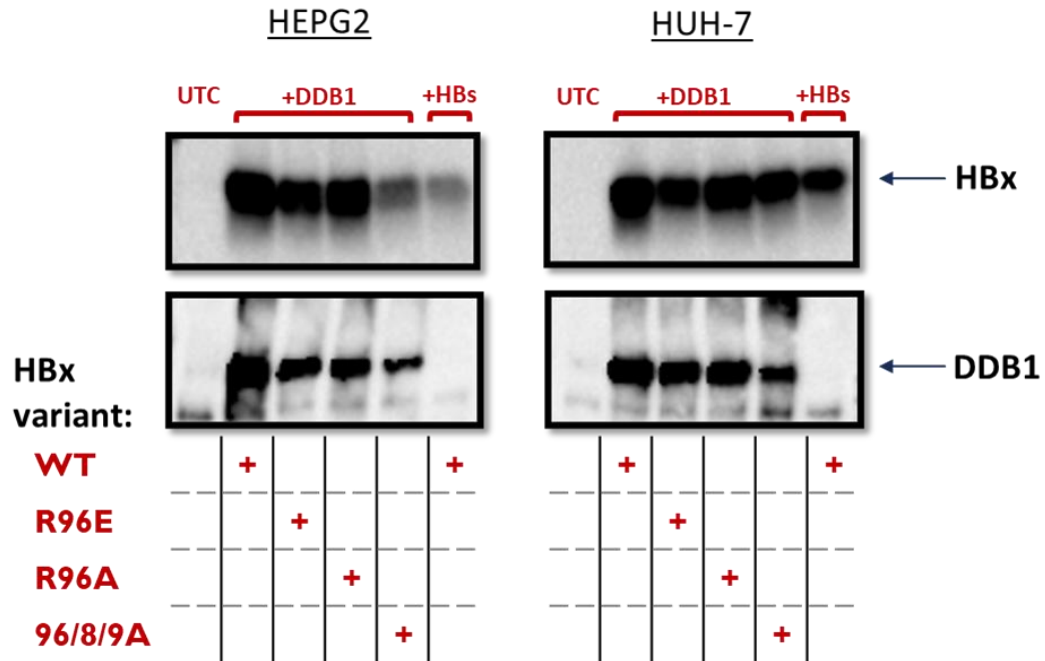


Figure 4.20: Immunoblot showing interaction between HBx mutants and DDB1
HepG2 (left) and Huh7 (right) cells were co-transfected with wild-type and DDB1-binding-impaired HBx mutants, and myc-tagged DDB1 expression plasmids (or HBs-myc as control). Lysates were harvested 2 days post-transfection and subjected to immunoprecipitation using anti-HBx antibody. HBx and DDB1 expression in these precipitated samples were detected using monoclonal antibodies to HBx or c-myc (DDB1). UTC, ut-transfected control.

4.4 Discussion

The role and contribution of the enigmatic viral HBx protein in the HBV replication cycle has proved difficult to elucidate. Many early studies utilised protein overexpression systems due to low physiological expression and lack of specific antibodies, and found that HBx interacted with a multitude of proteins within various signalling pathways. Such pathways include those involving Ras-Raf, Akt, JAK/JNK MAP Kinases (Doria et al, 1995; Kim et al, 2001a; Lee et al, 2012; Lee & Yun, 1998; Rawat & Bouchard, 2015; Wang et al, 2014). The overall significance of many of these findings however, remain unclear in the context of natural HBV infection and replication, due to the underlying experimental conditions used.

Most recently, renewed interest in HBx biology has directed research towards HBx interaction with host protein DDB1, for which early studies had indicated effects on apoptosis, transactivation and DNA repair (Becker et al, 1998; Leupin et al, 2005; Lin-Marq et al, 2001; Sitterlin et al, 2000b). DDB1, as a component of the heterodimeric UV-DDB1 complex, works in conjunction with partner protein DDB2, to identify and repair UV-damaged DNA strands via the nucleotide excision repair (NER) pathway (Li et al, 2006). In later studies, DDB1 was recognised as an adapter protein for the CRL4-E3 Ubiquitin Ligase complex, wherein it indirectly (via other substrate receptors) bridges proteins into association with the complex, ultimately targeting them for ubiquitination and subsequent proteasome-mediated degradation (Angers et al, 2006; He et al, 2006). Only recently has this particular function been linked to HBV replication, with the HBx-DDB1 interaction demonstrated as a critical facilitator of HBV cccDNA transcription, through degradation of a host restriction factor, SMC5/6 (Mitra & Guo, 2016).

DDB1 expression stabilised HBx expression across HBV genotypes

The absence of proof-reading ability from the HBV reverse transcriptase means that the HBV replication strategy generates a multitude of variants or quasispecies. Consequently, HBV genotypes, which differ in their overall sequences by >8%, exhibit markedly divergent replication phenotypes (Sozzi et al, 2018; Sozzi et al, 2016), disease outcome/severity and therapeutic response (Kramvis, 2014). In turn, minor changes in the HBV sequences can have major impacts on virus replication and protein expression. Notable examples include the G1896A precore mutation resulting in abrogation of HBeAg and is often associated with HBeAg-negative HBV infection (Carman et al, 1989). The double A1762T/G1764A BCP mutation also resulting in reduced HBeAg expression, is considered as a risk factor of HCC development (Buckwold et al, 1996; Yang et al, 2008; Yin et al, 2011) and could involve HBx-related mechanisms due to its effect on the overlapping HBx amino acid sequence at residues 130/131.

Since reports demonstrated a critical role for HBx-DDB1 interaction in HBV replication (Decorsière et al, 2016; Leupin et al, 2005; Murphy et al, 2016; Sitterlin et al, 2000a), we were interested in determining the impact of sequence variability in the HBx gene on DDB1 interaction. Li et al (2010a) identified a central domain within the HBx amino acid sequence termed the “H-Box” motif, which forms an alpha helix structure directly contacting DDB1 residues within a clam-like pocket. We identified amino acid differences within this H-Box domain between different HBV genotypes (Figure 4.1), and hypothesised that these changes

may impact HBx DDB1-binding ability, in turn explaining some of the variation in replication efficiency observed between different HBV genotypes and variants. Following transfection of protein expression plasmids for HBx and DDB1 in hepatoma cell lines, lower HBx levels were observed in the absence of DDB1, rapidly decreasing to near-undetectable levels by 5 days post-transfection. It has been previously suggested that expression of DDB1 stabilises HBx expression (Bergametti et al, 2002b), and concordantly, we observed that co-transfection with a DDB1 expression plasmid led to seemingly increased stability of HBx protein, with protein more readily detectable at later time points (Figure 4.4).

DDB1-mediated enhancement of HBx expression levels was observed for various HBx constructs derived from HBV genotypes A, C and D, following co-transfection. No difference was observed, suggesting the HBx-DDB1 interaction was not severely impaired for the major common HBV subgenotypes A2, C2 or D3 (Figure 4.17). This suggests that the genotype-specific sequence differences we observed in the H-Box likely do not contribute to known differences in HBV replication across different genotypes (Bannister et al, 2019; Bannister et al, 2018; Sozzi et al, 2018; Sozzi et al, 2016). While it is possible that HBx-independent activity of DDB1 may contribute to genotype-mediated differences in HBV replication, further studies are required to investigate this possibility. Expression of the BCP HBx variant, derived from the clinically relevant BCP A1762T/G1764A (HBx K130M/V131I) double mutant which is often associated with reduced HBeAg levels, similarly showed these mutations had no impact on DDB1-mediated enhancement of HBx expression. Koumbi et al (2016) showed that reduced replication of this variant was correlated with less acetylation of cccDNA-bound histones, and our results suggest this effect is likely unrelated to interactions between HBx and DDB1.

H-Box mutant HBx retained DDB1-interacting ability

In contrast to naturally-occurring mutations, studies have shown that substitutions of specific HBx residues within the H-Box domain can adversely impact on DDB1 interaction and HBV replication (Leupin et al, 2005). In particular, although both the R96A and R96E mutants have been described to exhibit impaired DDB1-binding ability, the consequence of these mutations on HBV replication have been contradictory. Leupin et al (2005) and Li et al (2010) demonstrated that the R96E mutant was unable to rescue replication of HBx-deficient HBV replicon when expressed in *trans* in HepG2 cells. In contrast, Kim et al (2016), utilised a R96A mutant which although exhibiting reduced association with DDB1, was still able to rescue replication of HBx-deficient HBV to almost the same level as the wild-type protein. In light of these conflicting results, investigations were conducted with the inclusion of several of these previously-used HBx mutants. Expectedly, HBx mutants harbouring substitutions that reportedly impair its ability to interact with DDB1 (including R96A/E, 96/8/9A) displayed an attenuated increase of protein levels compared to their wild-type counterparts (Figure 4.19), which is consistent with impaired DDB1-mediated stability. However, immunoprecipitation experiments did not reflect the same profound effects on the HBx-DDB1 interaction. These results in fact showed similar levels of DDB1 co-precipitating with the HBx mutants, as was observed for wild-type HBx (Figure 4.20). Put together, this may suggest that these mutants could in fact still efficiently interact with DDB1, but that the interaction does not confer stability, hence observed impairment in DDB1-mediated enhancement of HBx expression. Additionally, these mutations may also impact on other functions of HBx that potentially contribute to this effect through unidentified mechanisms.

Regardless of underlying mechanism, these findings contrast with similar experiments performed by Kim et al (2016), who showed that HBx H-Box mutants essentially abrogated the interaction with DDB1. This effect is unlikely related to the cell system used, as our findings were consistent in two hepatoma cell lines (Figure 4.19) and HEK293 cells (not shown). Alternatively, additional explanations could include differences in experimental procedures or epitope tags used potentially affecting intrinsic properties or its off-target interactions. It may be worth further pursuing these peculiar results and assess the impact downstream of the interaction.

Crystallography analysis revealed R96 forms two hydrogen bonds with DDB1 (Li et al, 2010a), so the effect of mutating this residue to Glu or Ala may produce different effects with regards to binding to DDB1 and/or other proteins, protein localization, and other downstream functional effects including SMC5/6 degradation. There may be an intermediate protein involved, linking both HBx and DDB1 together and able to compensate for interrupted HBx-DDB1 association. Since all three proteins are interacting within the same complex, immunoprecipitation experiments would yield similar results (HBx and DDB1 indirectly linked by third protein), while impaired enhancement of detected HBx expression could be attributed to reduced DDB1-mediated stability. Hypothetically, substitution to a glutamic acid at that specific position may adversely affect its interaction with this putative intermediate protein, while an alanine residue would leave this association unimpaired and therefore compensatory to ultimate downstream effects. Alternatively, the different mutations could induce alternative compensatory interactions with DDB1 to mediate HBx function. Future directions could include further comprehensive analyses into direct HBx variant interaction with DDB1 (IP) and functional consequence on downstream effects, such as HBx stability or localization, and SMC5/6 degradation.

DDB1 enhances HBV replication in both HBx-dependent and -independent manners

Studies led by Murphy et al (2016) and Decorsiere et al (2016) showed that the HBx and DDB1 interaction was crucial for HBV cccDNA transcription and replication. Their investigations identified the SMC5/6 complex as a host factor that associated with HBV cccDNA to restrict its transcription. However, in the presence of HBx, interaction with DDB1 led to hijacking of the CRL4 E3 ubiquitin ligase activity, targeting SMC5/6 for ubiquitination and proteasomal degradation. This ultimately relieved transcriptional repression and facilitated HBV propagation. On the other hand, a subsequent study by Kim et al (2016) found that DDB1 could stimulate HBV transcription via an HBx-independent mechanism. The authors therefore speculated that DDB1 might contribute to HBV transcription via two distinct mechanisms: one requiring the DDB1-HBx interaction and another alternatively, independent of a direct HBx-DDB1 interaction. The former, as described by the Murphy/Decorsiere reports (2016), results in targeted degradation of the SMC5/6 restriction factor to facilitate cccDNA transcription, while the latter involves an unidentified mechanism wherein DDB1 associates with the cccDNA to stimulate its transcription. They proposed that both mechanisms exist, with the predominant pathway dictated by the specific experimental systems utilised. This suggests that host restriction between cells may play a role; for example, in a natural infection setting using primary human hepatocytes, SMC5/6 restriction is predominant, and subsequent HBx-DDB1 interaction becomes paramount. In contrast, SMC5/6 restriction in HepG2 cells could be

comparatively negligible, in turn making the HBx-DDB1 interaction largely dispensable, and explaining the HBx-independent transcriptional stimulation effect of DDB1.

Since both mechanisms impact HBV cccDNA transcription, this thesis utilised the 1.0mer HBV *in vitro* transfection model, which produced a transcriptionally active cccDNA-like molecule (Chapter 3). A greater-than-genome-length construct was also used for comparison, as overlength constructs were used in previous studies (Kim et al, 2016; Leupin et al, 2005). HBx-deficient HBV constructs (HBxΔ) were also included to distinguish from HBx-independent effects. Analyses of HBV replication confirmed that the HBxΔ constructs were replication competent, though replicated less efficiently compared to the WT HBV (Figure 4.9, Figure 4.11) which was consistent with HBx requirement for efficient replication. This effect was more apparent with the 1.3mer construct, likely due to encoding two copies of the HBx gene, as opposed a single copy in the 1.0mer constructs. Introduction of ectopic DDB1 into this system resulted in increased HBV DNA, RNA and secreted proteins from wild-type 1.3mer HBV constructs, and also showed to be true with 1.0mer HBV (Figure 4.14). Additionally, in support of the findings from Kim et al. (2016) demonstrating that DDB1 expression stimulates cccDNA transcription in an HBx-independent manner within hepatoma cell lines, we also observed increased HBV replication with the addition of DDB1, from HBx-deficient (HBxΔ) HBV 1.0mer. These results suggest DDB1 can enhance HBV replication via both HBx-dependent and – independent mechanisms. Whether DDB1 associates with cccDNA remains to be determined and is subject of future work. The 1.0mer system provides opportunities to further investigate this association for multiple HBV variants and genotypes, enabling confirmatory chromatin immunoprecipitation-based experiments.

A potential role for DDB2 in DDB1-mediated enhancement of HBV replication?

As part of the UV-DDB complex, DDB1 and DDB2 aid the repair of UV-damaged DNA via the NER pathway (Li et al, 2006; Moser et al, 2005; Yeh et al, 2012). This process also involves ubiquitination and degradation of various factors (Groisman et al, 2003; Matsuda et al, 2005; Sugawara, 2009) and we hypothesise that through facilitating cccDNA formation, these repair factors and processes may contribute to an HBx-independent mechanism of enhanced transcription (Figure 4.21). Supporting evidence to this effect was reported at a recent international HBV conference (Marchetti, et. al., 2019, October. *Host DNA damage binding protein 2 is involved in cccDNA formation*. Abstract presented at HBV International Meeting – The Molecular Biology of Hepatitis B Viruses, Melbourne, Australia) Kim et. al., (2016) used ChIP-Seq to demonstrate that DDB1 associated with cccDNA in a HepG2^{NTCP} infection system, and other components of the UV-DDB complex may also be involved. For example, the inclusion of the DDB2 protein in this process is a likely possibility, and we speculate that DDB2 protein could act as the linker between cccDNA and DDB1, which is not known to have direct DNA-sensing/binding properties (Li et al, 2006, Luijsterburg et al, 2007). In this way, DDB1 would associate with cccDNA-bound DDB2 to catalyse the repair and efficient expression of the minichromosomal template (Figure 4.21).

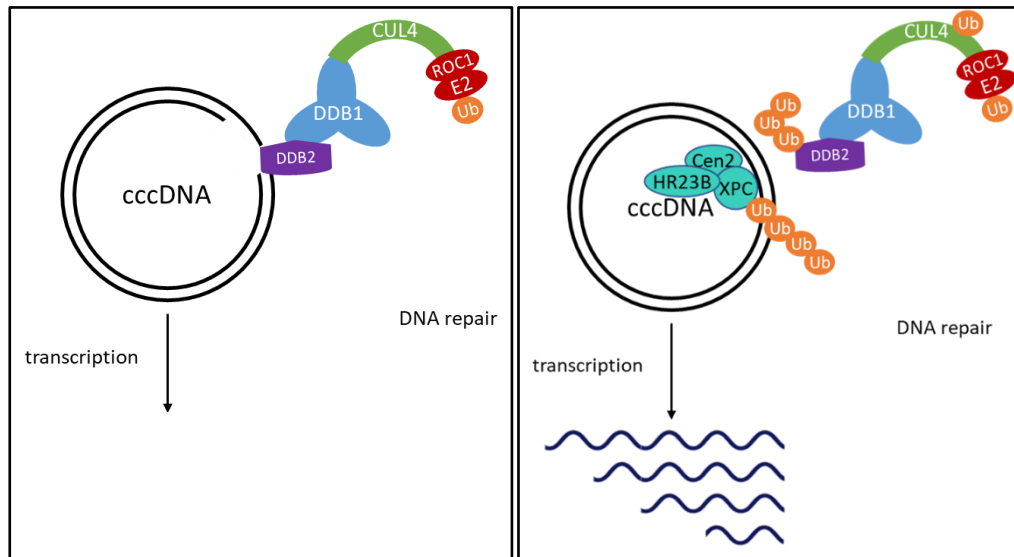


Figure 4.21: UV-DDB complex-mediated repair of the transcriptional template (cccDNA) involving both DDB1 and DDB2 components. Schematic diagram describing proposed mechanism of HBx-independent, DDB1 transcriptional enhancement. In the absence of HBx involvement, DDB1 and DDB2 could facilitate the repair of cccDNA minichromosome. These processes typically involve ubiquitination of additional recruited repair factors. Adapted from Kim et al., (2016)

Alternatively, DDB2 could also be involved in the HBx-DDB1 degradation pathway facilitating removal of restriction factors. In this scenario, HBx could act as the linking protein, binding both HBx and DDB1 targeting SMC5/6 and/or other (unidentified) host restriction factors for degradation via recruitment of the CUL4 E3 ubiquitin ligase complex (Figure 4.22, left). This would allow HBx mutants that have lost the ability to efficiently interact with DDB1, to still facilitate cccDNA transcription. Another possible scenario could involve DDB2 displacing other factors and/or targeting them for degradation while directly bound to cccDNA (right).

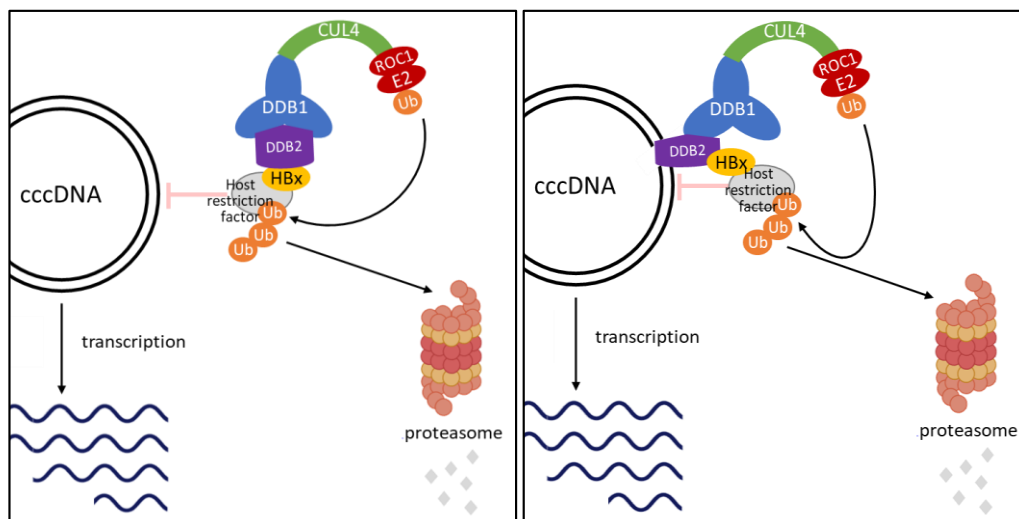


Figure 4.22 Alternative configurations proposed for HBx and UV-DDB complex-mediated degradation of cccDNA-restricting host factors. Schematic diagram of proposed mechanism of HBx-dependent, DDB1 transcriptional enhancement by degradation of host restriction factor and subsequent relief transcriptional repression. Adapted from Kim et. al. (2016)

Finally, this mechanism may occur in the absence of HBx expression altogether, interacting with other unknown proteins to target, as yet unidentified restriction factors in direct association with UV-DDB complex components (Figure 4.23). These investigations should be prioritised in future studies.

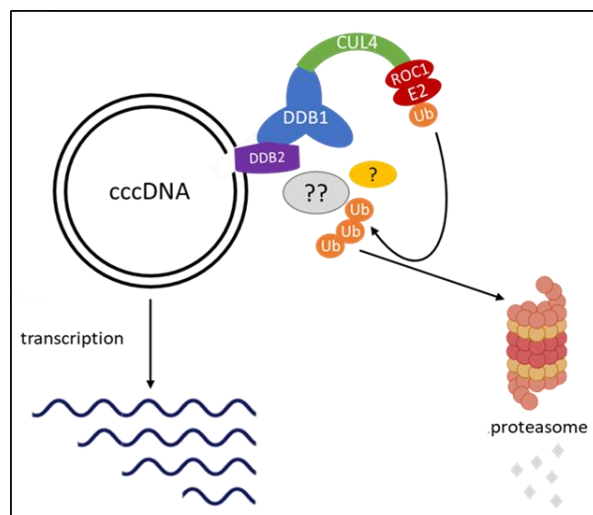


Figure 4.23: Proposed UV-DDB complex-induced, HBx-independent transcriptional enhancement.

Schematic diagram of HBx-independent relief of transcriptional repression wherein degradation of unidentified cccDNA-restricting host factors is mediated via direct interaction with DDB1 and/or DDB2.

HBx is undeniably a complex protein with many interacting factors reported to influence its expression and/or function (Iyer & Groopman, 2011; Park et al, 2009; Xian et al, 2010; Zhao et al, 2011), and many more likely remain to be uncovered. Conflicting reports on its activities and contribution to HBV replication may be associated with cellular contexts and other external factors therein. It has been previously noted that HBx did not enhance HBV replication in Huh-7 cells, as it did in HepG2 cells (Leupin et al, 2005), so cellular systems utilised may need to be taken into consideration when comparing results. Our experiments showed generally consistent results between different mammalian cell lines, albeit with higher HBx levels typically observed in association with Huh7 cells over HepG2 cells. The reason for this is unclear, but could be related to intrinsic properties of the cell, such as p53 expression, and the associated implications of that on HBx expression (Iyer & Groopman, 2011; Leupin et al, 2005; Xian et al, 2010). Although use of expression plasmids are good starting points for analysing specific protein/protein interactions, focus should always be brought back to the physiological context. Interesting effects observed in standalone expression systems should be validated with replication models to verify relevance in the broader context of HBV replication/infection. This is especially important when considering how different viral proteins can interact and regulate individual activities. While infection systems continue to be optimised and improved, transfection models such as 1.0mer HBV that readily permit investigation of different HBV genotypes and variants, may be viable, and physiologically-closer alternative models to facilitate these studies in the foreseeable future.

When we assessed the HBx-DDB1 interaction across genotypes by co-expressing DDB1 with HBx of the different HBV genotypes, we observed that this upregulating effect of HBx expression imposed by DDB1, was consistent across the tested variants. This included wild-type HBx of genotypes A2, C2 and D3. We included an additional sub-genotype of A, as A1 is curiously attributed to significant progression to liver cancer within a specific sub-population, and HBx is traditionally believed to have transformative/hepatocarcinogenic properties. Genotype A1 and A2 HBx proteins also vary quite a bit in their N-terminal domain. We also included two other HBx variants in the background of sub-genotype D3 HBx. The BCP variant is derived from the clinically relevant BCP A1762T/G1764A double mutant which is often associated with reduced HBeAg levels. The presence of these mutations have also been correlated with higher replication and identified as risk factors towards HCC development. The overlap of the core promoter and HBx genes is such that this double mutation also alters residues 130 and 131 of HBx to Methionine and Isoleucine respectively.

We observed comparable levels of HBx expression across the different variants when co-expressed with DDB1 by western blot, even despite slight variations in baseline expression in the absence of DDB1 sometimes observed (Figure 4.17). This could suggest that while genotypic variation or specific mutations did not appear to adversely impact interaction with DDB1, some variants may mediate stronger interactions. Alternatively, the distribution of HBx proteins localised within different cellular compartments could vary across genotypes and therefore affect the proportion of proteins available for interaction with DDB1. Our results appear to be consistent with predominantly cytoplasmic localization at high expression levels as described in the literature, although comprehensive confocal microscopy studies would help elucidate this further and quantitate the different proportions. Although we saw no obvious difference in HBx expression level across genotypes when co-transfected with DDB1 and detected by western blot, we still cannot completely rule out the possibility that these sequence changes could affect the interaction between the proteins. We would need to perform co-immunoprecipitation experiments with each of these HBx variants with DDB1 to confirm either alternative conclusion, especially considering the inconsistencies of results later observed with the DDB1-binding mutants. Of course we also cannot rule out simple experimental variation such as transfection or translation efficiency between the different conditions within or between experiments, such as differential expression of HBx and/or DDB1.

Enhanced HBx detection in the presence of DDB1 has been described since (Lee et al, 1995) though early studies typically utilised yeast 2-hybrid/other system (Becker et al, 1998; Lin-Marq et al, 2001) or analysed the related WHx protein as a surrogate of HBx (Bergametti et al, 2002b; Sitterlin et al, 2000a; Sitterlin et al, 2000b), which may complicate direct comparisons. Collectively, general consensus appears to be that the HBx-DDB1 interaction results in protein stability. The most popular theory for this effect seems to be that DDB1 interaction protects HBx from ubiquitylation and proteasome degradation, and previous reports have accordingly demonstrated reduced Ub-HBx/WHx and increased HBx/WHx expression with DDB1 (Bergametti et al, 2002b; Hu et al, 1999). This effect was similarly achieved by blocking degradation using proteasome inhibitors (MG-132), and was not significantly further enhanced with addition of DDB1. In contrast, Kim et al (2016) observed an increase in detection of DDB1-binding impaired HBx mutants (and HBx mRNA) with MG-132 treatment while DDB1 was

knocked down, rather proposing DDB1 regulated HBx expression at the transcriptional level instead of protein stability.

To further investigate reported HBx-independent DDB1 enhancement of HBV replication, we once again utilised cell culture transfection systems. HBx expression was detectably lower in these HBV replication-competent constructs (1.3mer or 1.0mer) compared to previous HBx expression plasmids. This could reflect an intrinsically more efficient system in which HBx overexpression is driven by the CMV promoter, unlike the full length HBV constructs which are under the control of native HBV promoters. The induction of HBV replication with the inclusion of other HBV proteins could also result in induction of host antiviral responses to ultimately restrict replication. For example, Kim et al (2003) demonstrated that co-expression of HBc stimulated proteasomal degradation of HBx to significantly reduce its expression level. We constructed HBx-deficient HBV constructs (HBx Δ) to better separate the specific effects. These constructs were replication competent, though less efficiently replicating compared to the WT HBV. This effect was very apparent with the 1.3mer construct, but less so with our 1.0mers. This greater dependence on HBx in the 1.3mer system may simply be an attribute to its two copies of the gene, as opposed to the single copy in 1.0mers.

Consistent with previous reports, with the addition of ectopic DDB1 expression, we observed increased HBV DNA, RNA and secreted proteins from WT HBV 1.3mer constructs. This effect was reproduced with HBV 1.0mers which can produce cccDNA-LM, albeit the effect was subtler. Subsequent experiments showing similar results using an HBx Δ 1.0mer lends further support to the notion Kim et al (2016) suggestion that DDB1 can stimulate cccDNA transcription in an HBx-independent manner. Whether DDB1 associates with cccDNA remains to be seen is subject to future work, and the 1.0mer system certainly presents opportunities to further investigate, likely with confirmatory chromatin immunoprecipitation experiments we did not have the capacity or expertise to perform within the scope of this project. In this project we have investigated the specific interaction and HBx and DDB1 proteins as these have demonstrated effects on HBV replication in past studies. Identification of specific interactions between other host proteins that play unidentified roles in HBV replication, of which there are bound to be many, will open up doors to new therapeutic avenues to follow in pursuit of a cure for chronic HBV infection.

Chapter Five: Identifying key cell signalling components regulating HBV infection and replication

5.1 Introduction

Pathogens manipulate a range of cellular components and processes to overcome host defence mechanisms, in order to establish and maintain infection. Strategies utilised by viral or bacterial pathogens include interference with cellular pattern recognition receptor (PRR) activity and/or subsequent modulation of signalling transduction pathways, as demonstrated by bacteria such as enteropathogenic *E.coli*, *Salmonella enterica* and *Staphylococcus aureus* which express numerous effector proteins that modulate receptor signalling and dampen immune responses (Askarian et al, 2014; Baruch et al, 2011; Cirl et al, 2008; Newman et al, 2006). Viruses similarly manipulate and subvert host mechanisms to facilitate their replication and propagation. Examples include manipulation of cellular structures to evade immune recognition and activation by both hepatitis C virus (HCV) and Dengue viruses (Neufeldt et al, 2016; Uchida et al, 2014), and degradation of nuclear receptor IFI16 leading to loss of interferon (IFN) induction by Herpes Simplex virus-1 (Orzalli et al, 2012). Evasion of cell death is another common mechanism utilised by various pathogens in order to prolong host viability and facilitate their survival and dissemination (Byrne & Ojcius, 2004; Carpenter et al, 2015; Knodler et al, 2005), as demonstrated with subversion of autophagy for viral replication by HCV (Hansen et al, 2017).

Cell viability is maintained by critical processes such as transcription, translation, proliferation, and DNA repair, that are tightly regulated by signalling cascades and pathways often involving multiple mitogen-activated protein kinases (MAPKs) (de Nadal et al, 2011). As major components of signal transduction pathways, MAPKs induce phosphorylation and activation of various substrates in response to stimuli (transcription factors, cytoskeletal proteins, other signalling proteins) to ultimately augment biological responses pertaining to cell growth, differentiation and apoptosis (Krishna & Narang, 2008; Seger & Krebs, 1995; Yang et al, 2013b). Precise control of these activating signals is required as even subtle changes may have dramatic effects on the outcome, and many pathogens are known to encode proteins which directly modulate MAPK activation by varying mechanisms (reviewed by .

For a number of key cellular pathways, including several MAPK signalling pathways, modulation was induced by HBV and presumed to facilitate viral replication and persistence. HBV replication upregulates phosphorylation and activation of kinases (Chin et al, 2007), which can in turn suppress HBV replication (Guo et al, 2007b; Zheng et al, 2003), indicating that regulation of cell signalling must be finely balanced to facilitate viral replication and persistence. Several HBV proteins have also been directly attributed to modulation of cell signalling. HBx modulation of cellular proliferation and cell cycle progression through activation of MAPK signalling may be one mechanism by which the protein contributes to supporting HBV replication (Bouchard et al, 2001a; Gearhart & Bouchard, 2010; Klein et al, 1999; Klein & Schneider, 1997; Lee & Yun, 1998). The HBV core protein critical for viral replication and assembly is itself subject to phosphorylation by host kinases, influencing its subcellular localization, capsid stability and viral function (Ludgate et al, 2011; Ning et al,

2017). Repression of IFN/JAK/STAT signalling by HBeAg was shown to facilitate HBV replication, through stimulation of SOCS2 expression which downregulated expression of the IFN receptor, and consequently of IFNR-stimulated genes (ISG) (Yu et al, 2017). The HBeAg, which is critical for the establishment of chronic infection and is a known immune tolerogen also suppresses TLR2 expression and TNF α production, believed to facilitate persistence (Visvanathan et al, 2007). This was shown to be via interaction with adaptor proteins TRAM and Mal, and was not observed for the related HBV core antigen (HBc)(Lang et al, 2011). However the complete repertoire of host cell signaling pathways and molecules modulated by HBV replication is yet to be described.

Identifying the components and signalling pathways critical for viral infection and replication may uncover new drug targets and ultimately guide the development of novel therapeutic strategies. There is global consensus that curative strategies for chronic HBV infection will likely require a combination of direct acting antivirals, as well as host targets that restrict viral infection and replication (Alter et al, 2018; Revill et al, 2016; Revill et al, 2019a; Revill et al, 2019b). Microarrays, such as the antibody microarray service provided by Kinexus Bioinformatics (Vancouver, Canada), provide a broad snapshot of gene expression and/or phosphorylation status of a cell in response to infection. Kinexus microarrays include more than 400 pan-specific antibodies to determine protein expression level, and over 800 phospho-specific antibodies indicating phosphorylation status. Target proteins include protein-serine/threonine kinases, protein-tyrosine kinases, transcription factors, adaptor/scaffold proteins, and protein/lipid phosphatases among others. This approach permits a broad assessment of cell protein expression and phosphorylation status that may lead to identification of novel drug targets impacting viral replication and/or infectivity, and has been used to identify pathways modulated by pathogens including the malaria parasite (Solyakov et al, 2011) and HCV (Haqshenas et al, 2017). Using a cell culture model, JAK2 and STAT5 were identified as key molecules for promotion of HCV replication, and accordingly, selective inhibition of MAP4K2 markedly reduced HCV replication (Haqshenas et al, 2017). These microarray screens have also been utilised for Zika virus, and Dengue virus, with insulin receptor activity identified as a limiting factor for dengue replication in Wolbachia-infected cells (Haqshenas et al, 2019). This demonstrates the utility of antibody microarrays as a viable approach for both identifying key proteins and pathways for replication and potential drug targets.

Deregulated kinase activity is implicated in many human afflictions including Alzheimer's disease (Chang et al, 2012; Shukla et al, 2012) and cancers (Blume-Jensen & Hunter, 2001; Dhillon et al, 2007; Sun & Xiao, 2003). Kinases are heavily implicated in cell proliferation and survival processes leading to tumour development, and have become attractive cancer therapeutic targets, such as for the treatment of chronic myeloid leukaemia (Druker et al, 2006; O'Brien et al, 2003). Our understanding of the specific host components and processes involved and required for HBV replication is incomplete, largely hindered by the lack of appropriate model systems. Viral dependence on host cell mechanisms for replication suggests host components interacting with/are involved in viral processes or factors represent attractive alternative antiviral targets. This is supported by preclinical studies with the PLK-1 (Polo-like kinase 1) inhibitor BI-2536 demonstrating inhibition of HBV infection using a humanised liver murine model (Diab et al, 2017). The mechanism of action is likely mediated

through the HBV core protein, as elevated PLK1 expression and activity leads to HBc phosphorylation, facilitating capsid formation and DNA synthesis following HBV infection. PLK1 has also previously been suggested as a therapeutic target for HCC, since its depletion led to G2/M arrest, inhibition of cell proliferation and promotion of apoptosis (He et al, 2011)

For this chapter, Kinexus antibody microarrays were used to screen and identify key cell signalling proteins and pathways modulated by HBV replication and/or protein expression, with the intent to identify potential novel therapeutic targets. Given the considerable evidence for HBx-mediated modulation of numerous signalling pathways, and the recent identification of a link between HBV replication and its interaction with host DDB1 , the initial screen investigated the effect of expressing these proteins either in isolation or together. Experiments were also performed in the setting of HBV replication, using wild-type and HBx-deficient variants (HBx Δ) to verify the cellular factors identified by the screen with HBx and to identify other potential novel targets involved in HBV replication. This was achieved using the HBV 1.0mer cell culture transfection model validated in Chapter 3.

5.2 Protein expression screen – HBx and DDB1

The initial screen was performed in the context of HBx and DDB1 overexpression, the interaction of which relieves transcriptional repression of HBV cccDNA following infection (Decorsière et al, 2016; Murphy et al, 2016). Independent contributions to HBV replication have also been described for both proteins (Hodgson et al, 2012; Kim et al, 2016) which is suggestive of additional roles that we were interested to explore. Investigation of cell signalling pathways modulated by both proteins was achieved by performing an antibody microarray screen on HepG2 cell lysates following transfection with either pCI-HBx, pCI-DDB1, or both constructs expressing HBx and DDB1. Transfections with empty plasmid vectors were included as negative controls. Cells were harvested 2 days post-transfection, processed according to the provided guidelines (briefly described in Section 2.8.3), and sent to Kinexus (Canada) for analysis using the KAM-900P or KAM-1325 antibody microarray services, for assessing the protein expression levels and for determining the phosphorylation status. Data was mined for outstanding ‘hits’ and tagged as “Potential” subject to satisfaction of defined criteria (intended to highlight most promising candidates), primarily upregulation by more than 50% or downregulation by more than 25% (Table 5.1).

Table 5.1 Definition for “Potential” candidate designation (i.e. for further consideration)

Set Parameters for “Potential”	Value
Flag	0,0
Signal	>1000
%Error	<50
%Change from Control (CFC)	<-25 or >50

After applying the above criteria, modified expression levels or changes of the phosphorylation status was identified for 170 targets following transfection with either HBx, DDB1, or the combination of HBx+DDB1. Sixty targets showed differences in protein expression (“Pan”-specific) and 110 represented phosphorylation status (“Phospho”-specific). Results were independently evaluated in the context of HBx expression alone, HBx+DDB1 co-expression and DDB1 alone, for satisfaction of set parameters (Table 5.1) and consequent “Potential” designation. Figure 5.1 depicts the distribution of targets satisfying the set parameters among the groups, with DDB1 expression showing the most modulated candidates of the 3 groups. The potential candidates were entered into the web-based application GOnet (Pomaznoy et al, 2018) to perform gene ontology annotation analysis to facilitate a biological classification of the identified candidates. This resulted in a ranked list of various biological processes (GO terms) and the number of genes within the data set that were associated with each of those terms. In comparing the lists for each of the HBx and DDB1 potential candidates, it was noted that the top 5 entries were consistent between the two groups, albeit in a slightly different order (Table 5.2). These were: cellular protein modification process (GO:0006464), signal transduction (GO:0007165), anatomical structure development (GO:0048856), response to stress (GO:0006950), and cell differentiation (GO:0030154). The ranking varied thereafter between the two groups, indicating that different processes were modulated by HBx expression compared to DDB1 expression.

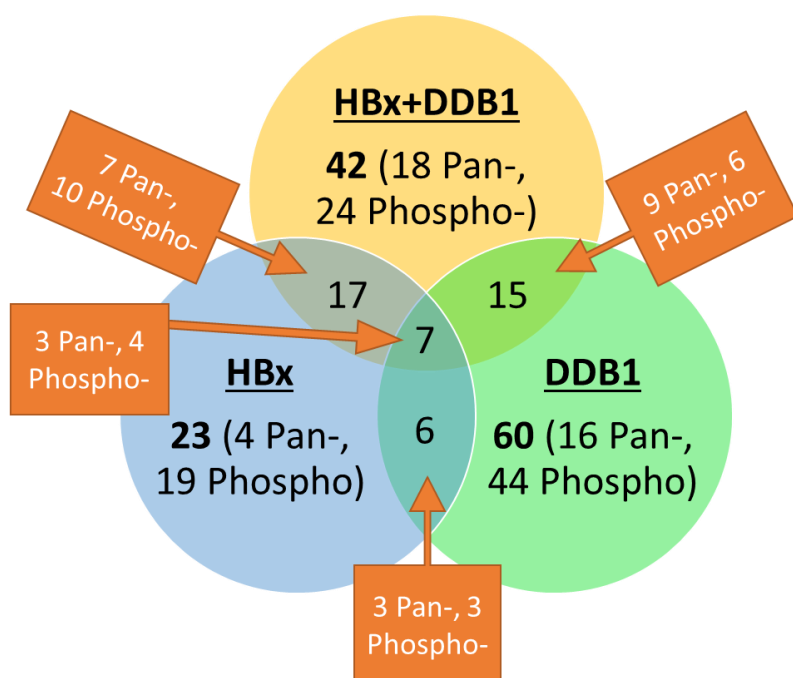


Figure 5.1 Separation of Potential candidates that were modulated following HBx, DDB1, or HBx+DDB1 over-expression.

Table 5.2 Comparison of the top ranking biological processes significantly modulated by overexpression of HBx or DDB1 (as determined by the number of associated potential candidates)

HBx		GO_term_ID	GO_term_def	DDB1	
Rank	No. of Genes			Rank	No. of Genes
1	19	GO:0006464	cellular protein modification process	3	34
2	18	GO:0007165	signal transduction	1	42
3	16	GO:0048856	anatomical structure development	4	31
4	14	GO:0006950	response to stress	2	34
5	12	GO:0030154	cell differentiation	5	26
6	10	GO:0006810	transport	10	16
7	9	GO:0009058	biosynthetic process	12	14
8	8	GO:0007049	cell cycle	7	18
9	8	GO:0044403	symbiotic process	15	13
10	7	GO:0040011	locomotion	14	13
11	7	GO:0022607	cellular component assembly	13	14
12	7	GO:0034641	cellular nitrogen compound metabolic process	8	17
13	7	GO:0008219	cell death	11	15
14	6	GO:0016192	vesicle-mediated transport	26	8
15	6	GO:0048870	cell motility	18	10
16	6	GO:0002376	immune system process	6	23
17	6	GO:0000003	reproduction	21	10
18	5	GO:0015031	protein transport	29	7
19	5	GO:0000278	mitotic cell cycle	17	11
20	5	GO:0000902	cell morphogenesis	20	10

From the list of 170 potential targets, a short list of six priority candidates were selected for further investigation (Table 5.3, Figure 5.2), with prioritisation based on previously demonstrated associations with HBV, or with cellular processes known to be important for HBV replication. Of the six unique targets selected, two candidates were modulated by HBx over-expression (eIF4E, mTOR, MST1), four candidates by DDB1 over-expression (eIF4E, eIF4G, HDAC4, HSP90) and two candidates by combined HBx and DDB1 expression (mTOR, MST1). An associated shift in gene expression/activity of these candidates in the HBx or DDB1-expressing samples therefore may indicate a role for these proteins in HBV replication (via modulation of these activities). Further descriptions of these results and potential relevance of these candidates can be found in Appendix B. Several results were deemed spurious despite seemingly significant modulation, as they did not satisfy all criteria required for priority hits (Table 5.3, asterisked).

Table 5.3 Short-listed potential candidates from HBx and/or DDB1 protein expression screen

Candidate	Phospho- or Pan-specific antibody	%CFC			
		HBx	HBx+ DDB1	DDB1	Priority in which sample?
eIF4E ^a	Phospho (S209)	-26	6	2389*	HBx
eIF4E ^b	Phospho (S209)	65*	-3	-27	DDB1
eIF4G	Phospho (S1106)	-10	-75*	-59	DDB1
HDAC4	Pan	-1	26	82	DDB1
HSP90a/b	Pan	20	49	301	DDB1
mTOR	Pan	184	136	38	HBx, HBx+DDB1
MST1 ^a	Pan	31	67	40	HBx+DDB1
MST1 ^b	Pan	83	82	21	HBx, HBx+DDB1

*considered Potential candidate according to %CFC value, but excluded based on other set parameters (Table 5.1);

^a and ^b represent independent antibodies

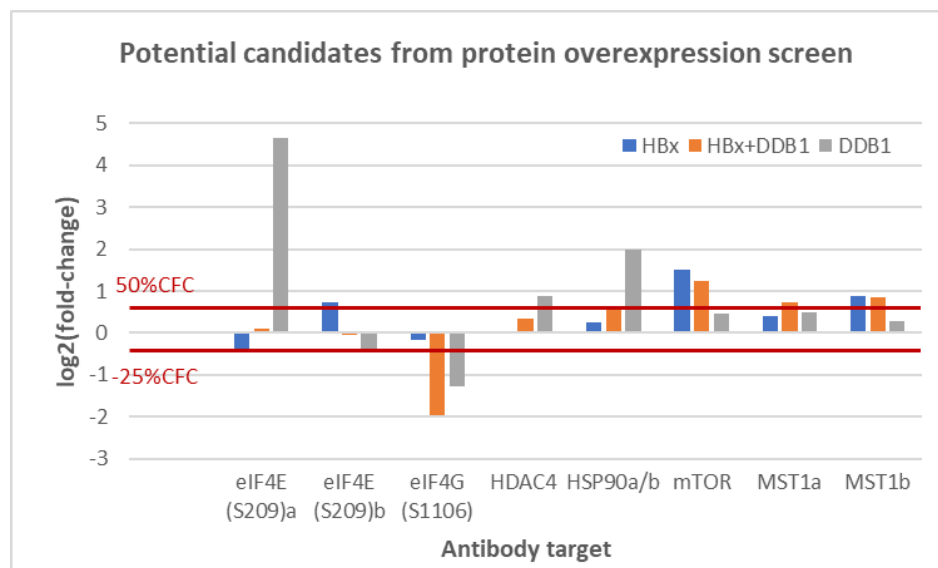


Figure 5.2 Graphic representation of selected candidates of targets significantly modulated by protein overexpression, in relation to control cells (empty vector transfection). CFC: change from control

5.3 HBV replication screen

Having identified several HBx and/or DDB1-modulated candidates potentially implicated in HBV replication, our next step was to assess cell modulation in a broader replication context. As HBx is not strictly required for HBV infection or establishment of replication, analysis in the context of HBV replication could potentially elucidate additional as-yet-unidentified factors or mechanisms which contribute to HBV replication and which would not be identified by studying HBx-mediated effects alone. To facilitate this, we employed the HBV 1.0mer cell culture model optimised in Chapter 3, which generates cccDNA-LMs and encodes single copy of the HBx gene. This more faithfully represents natural replication than replication driven by plasmids with overlength HBV cDNA inserts. An HBx-deficient 1.0mer variant (HBxΔ) was included for comparison with the WT HBV 1.0mer construct, which enabled examination of differentially modulated signals by HBV replication, in the presence or absence of HBx expression. In addition to the protein expression constructs in our prior screen (Section 5.2), we had also included a conventional overlength HBV replication-competent construct (1.3mer genotype D3; Section 2.2.1) and compared these results with those generated from the 1.0mer analysis as further validation.

5.3.1 Host factor modulations induced by 1.0mer HBV replication

Host-virus interactions were studied in the context of 1.0mer HBV transfection, since these constructs more closely represent authentic templates and viral replication occurs following formation of cccDNA-LM comparable to HBV cccDNA. Transfection of 1.3mer HBV (wildtype) cDNA which encodes an extra copy of the HBx gene, was used for comparative purposes only. This revealed a total of 118 substantially modulated subjects (not shown), while modulated signals for 198 potential candidates were observed following transfection with 1.0mer HBV (Figure 5.3). Forty-nine of these signals represented modulated protein expression, while 149 indicated changes in phosphorylation status. Wild-type HBV was attributed to 73 of these results (73/198), 65 were associated with HBx-deficient HBV, and the remaining 60 candidates were modulated by both HBV variants. Of note, all 60 (52 unique candidates) of these signals exhibited similar changes for WT and HBxΔ, in both direction and magnitude (Figure 5.4). Candidate factors are summarised in Table 5.4.

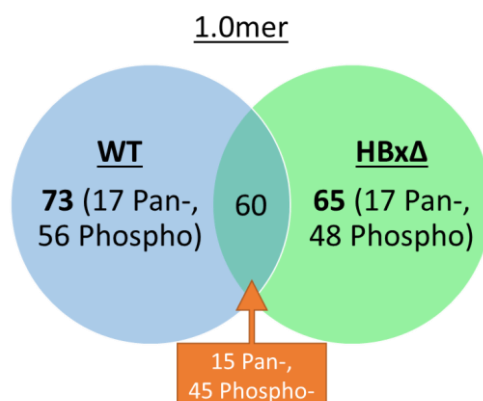


Figure 5.3 Summary of observed signals modulated in response to 1.0mer WT and HBxΔ HBV transfection

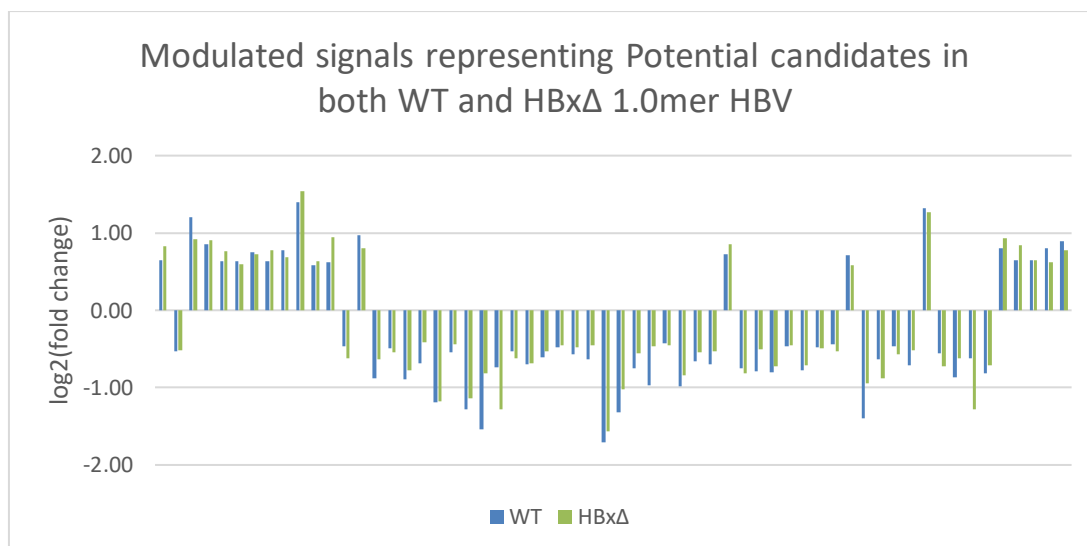


Figure 5.4 Modulated signals (represented by each pair of bars along the x-axis) marked as Potential (according to previously defined criteria) in both WT (blue) and HBxΔ (green) 1.0mer HBV groups.

Table 5.4 Summary of the Potential candidates showing modulation (Figure 5.4) by both WT and HBxΔ 1.0mer HBV constructs, sorted by observed Up- or Down-regulation, relative to the mock Control.

Highlighted entries represent candidates that also showed substantial modulation (i.e. met the criteria for Potential candidates) by 1.3mer WT HBV replication.

Up-regulated targets	Down-regulated targets	
AMPKa1 (PRKAA1)	APC	PFTAIRE2 (ALS2CR7)
AurKA (Aurora A, AIK, STK15)	HRAS	PKCg (PRKCG)
ErbB2 (HER2, Neu)	HSP90AB1 (HSP90B; HSP90-beta)	PKD1 (PRKCM, PKCm, PRKD1)
ErbB2 (HER2, Neu)	IRAK1	PLCG1
FGFR3	ITK	PTPN1 (PTP1B)
Frk	Ksr1	PTPN14
Fyn	LATS2 (KPM)	Raf-B (BRaf)
GFAP	LOK	Raf1 (RafC)
GluR1	MAFG	RIPK2 (RICK; RIP2; CARD3)
GSK3b	MEK1 (MKK1, MAP2K1)	SIK (SNF1LK)
H3F3A	MPL	SLK
HSP90AB1 (HSP90; HSP84; HSP90B; HSPC2; HSPCB) - Pan	MST1 (STK4, Krs2)	SPT5
PLCB3	NFKB p65 (Rel A)	STAT3
RSK1 (RPS6KA1, p90RSK)	p38b MAPK (MAPK11)	TAK1 (MAP3K7)
STAT5	p38d MAPK (MAPK13)	TAO1 (TAOK1)
TRRAP	p53 (TP53)	TBK1
VEGFR1 (Flt1)	p70S6K (S6Ka, RPS6KB1)	TEC
WNK1	PAK1 (PAKa)	
YAP1		

To assess the consistency of modulated signals between HBV constructs of different composition (1.0mer vs 1.3mer) we further examined the 60 candidates that were modulated by 1.0mer transfection (Table 5.4) and compared their signals against those following 1.3mer transfection. Taking into account only the direction of observed modulation (up or down, relative to mock control) of these sixty signals revealed that eighteen of these were similarly up- or downregulated in the 1.3mer replication-competent array (Figure 5.5, blue), while a further eighteen were modulated in the opposite direction (Figure 5.5, yellow). For the remaining 24 candidates it was not possible to compare, as these targets were not included in the 1.3mer-based array conducted previously. As for meeting the criteria for “Potential” candidate designation, only six targets were shared between 1.3mer WT and 1.0mer HBV (both WT and HBxΔ, Table 5.4, highlighted entries), three with modulation in the same direction (p70S6K, PKCg, TEC) and three in the opposite direction (STAT3, STAT5, H3F3A). Overall, this indicates that although similarities were observed between cellular effects mediated by HBV replication from unit- or –overlength constructs, the extent of the modulation seen in these targets was substantially different, with only six candidates consistently identified to be potential targets as defined by the set parameters outlined in Table 5.1 (highlighted, Table 5.4). These host factors are involved in a extensive variety of biological processes, including signal transduction (GO:0007165), cell-cell signalling (GO:0007267), response to stress (GO:0006950), immune system process (GO:0002376), anatomical structure development (GO:0048856) (Appendix C, Figure C-1).

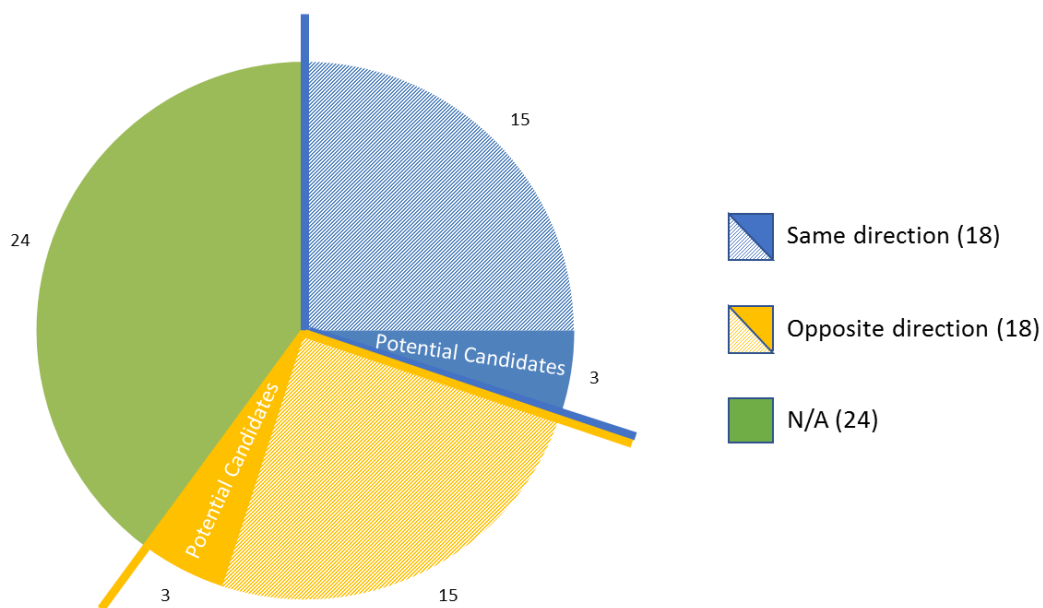


Figure 5.5 Proportion of Potential 1.0mer HBV-modulated candidates (60) that were also modulated by 1.3mer WT HBV transfection

To identify host factors generally contributing to HBV replication that may potentially represent targets for HBV therapy, we examined the signals which were consistently modulated by both WT and HBxΔ replication-competent 1.0mer HBV constructs. Fifty-two of the sixty modulated signals identified by both 1.0mer HBV variants represented unique targets in terms of protein or antibody recognition site (Table 5.4). Targets chosen for potential further investigation were selected based on existing evidence in literature, that may indicate a role for these factors in HBV replication, as shown either by a previous association to HBV, or more indirectly via a link to cellular process that is relevant to HBV replication.

From this analysis, seven unique candidates were ultimately shortlisted for potential follow-up, based on interesting evidence (of their functions and/or associations) that could suggest a role in HBV replication. These were YAP1, VEGFR1, TRRAP, TAO1, MST1, HSP90, and GluR1 (Figure 5.6). These host factors are commonly involved in biological processes pertaining to signal transduction (GO:0007165), homeostatic process (GO:0042592), protein-containing complex assembly (GO:0065003), cell differentiation (GO:0030154), response to stress (GO:0006950), cellular protein modification process (GO:0006464) (Appendix C, Figure C-2). The two signals representing modulated protein expression (TRRAP and HSP90) were both upregulated compared to controls, while the remaining phosphorylation signals were evenly split, with four showing increase from control, and four being reduced. Intriguingly, HSP90 showed upregulated expression, but downregulated phosphorylation at Y484. Although these effects were observed in both the 1.0mer WT and HBxΔ variants, arguing against HBx protein being a major determinant, it nonetheless suggests that these responses may be important for HBV replication, as they were modulated in response to both replication-competent constructs.

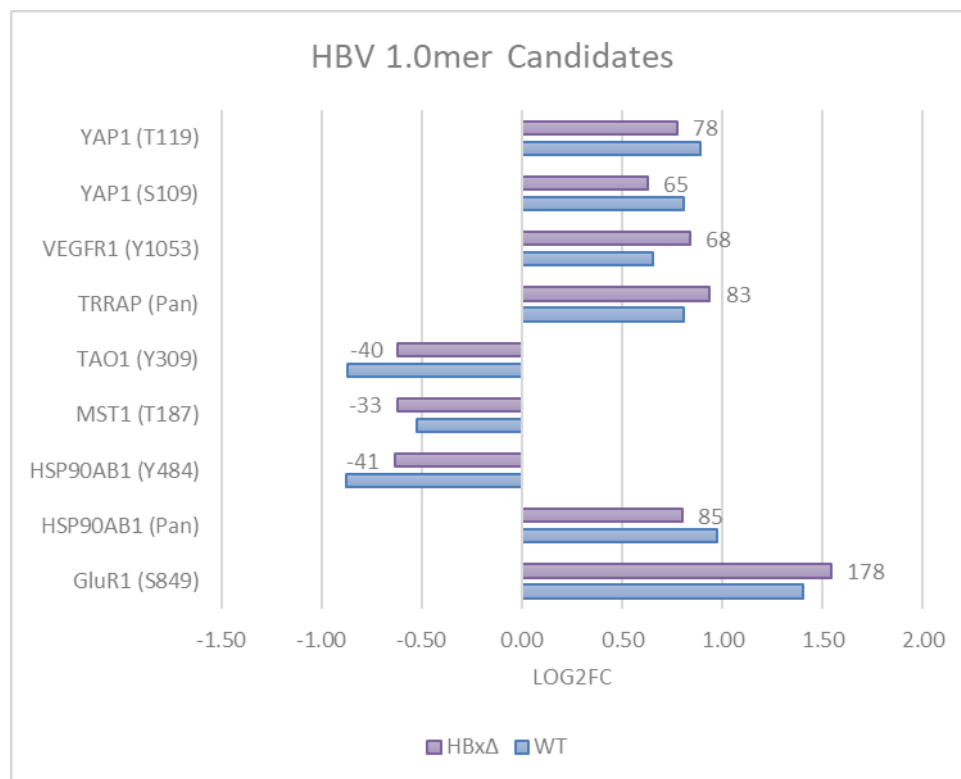


Figure 5.6 Targets upregulated/downregulated by 1.0mer HBV. The values shown beside the bars correspond to averaged %CFC (from both 1.0mer results). CFC, change from control (compared to mock control)

5.3.2 Host factor modulation specifically induced by WT or HBxΔ 1.0mer HBV

The results of the screen were also examined for targets that were differentially modulated by either WT or HBxΔ HBV replication, in order to determine whether there were any major differences in the cellular changes elicited by HBV induction either in the presence or absence of HBx. In this regard, more potential candidates were identified in the context of WT HBV replication than there were for HBxΔ HBV (Figure 5.7). Most of the identified candidates were phosphorylation specific targets, and the lack of overlapping targets suggests (discussed in the following section; Table 5.5, Table 5.6) that the cellular effects of WT and HBxΔ HBV replication largely appear to be independent of each other, and that the absence of HBx may have changed modulation of virus-host interactions .

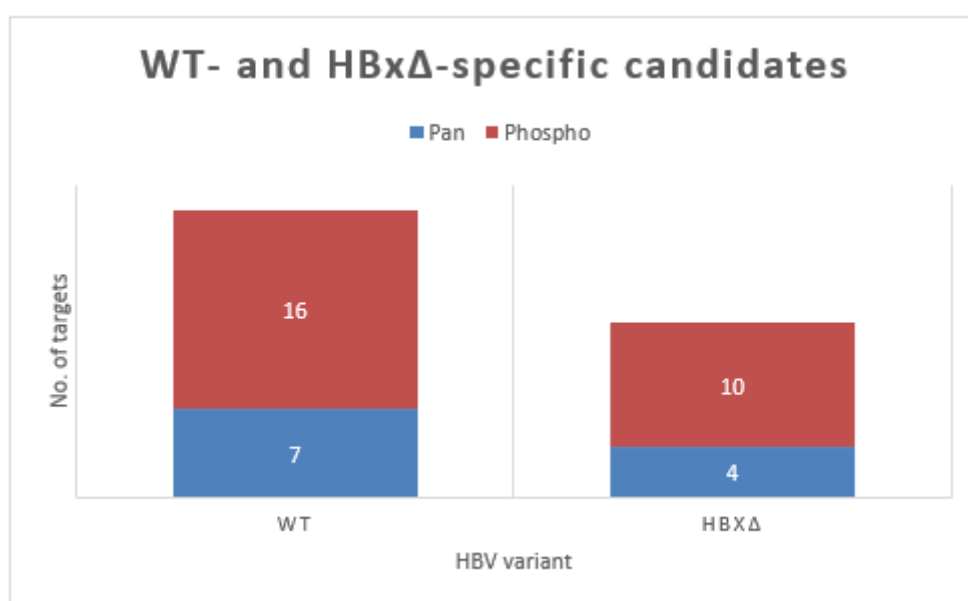


Figure 5.7 Summary of Priority candidates specific to either 1.0mer WT, or HBxΔ only.

Twenty-three of the 73 identified by WT HBV replication (Figure 5.3) were specifically augmented by this construct, based on only marginally modulated signals in the reciprocal HBxΔ sample (increased by <20% or decreased by up to 10%) (Table 5.5). Five of these signals were similarly augmented in the same direction (up/downregulation) by pCI-HBx expression (Table 5.5, green cells). Although these were not considered significant candidates in the HBx context, that they were modulated in the same direction arguably still lends support to a role for HBx in mediating these effects. Conversely, 14 targets were identified to be augmented by HBxΔ but not WT HBV (Table 5.6). Six of these exhibited modulation in the opposite direction to that observed with HBx overexpression (Table 5.6, green cells) and may also represent HBx-mediated effects.

Table 5.5 Potential candidates specifically modulated by 1.0mer wild-type (WT) HBV (and not HBxΔ). Highlighted targets (green cells) are those that also elicited changes of similar direction (up/down-regulation) in pCI-HBx-transfected samples.

Target Name	P-Site
Abl (Abl1)	Pan-specific
ATM	Pan-specific
eIF4G (eIF4G1)	S1231
Flt3 (STK1)	Pan-specific
FOXO1	T611
GSK3b	Pan-specific
H3F3A	T3
HMGCR	S872
JNK2 (MAPK9)	Pan-specific
MEK1 (MKK1, MAP2K1)	S222
Met	T1241
MSK2 (RPS6KA4)	T194+S196

NOS2	S745
PFKFB3	S461
PKCa (PRKCA)	Y195
PKCt (PRKCQ)	Y545
PKD2 (PRKD2)	S197+S198
PKG1a (PRKG1A)	T515+T517
Ron (RONa)	Y1238
RSK1 (RPS6KA1, p90RSK)	S221
Sgk269 (PEAK1)	Y635
SHIP1 (INPP5D)	Pan-specific
TYK2	Pan-specific

Table 5.6 Potential candidates specifically modulated by 1.0mer HBx-deficient (HBxΔ) HBV (and not WT). Highlighted targets (green cells) are those that were modulated in the opposite direction (up/down-regulation) in pCI-HBx-transfected samples.

Target Name	P-Site
CaMK4 (CaMPK4)	Pan-specific
CDC2L5 (CHED, CDK13)	Pan-specific
GSK3a	S278+Y279
GTF2F1	S385+T389
IκBa	Pan-specific
IKKγ (NEMO)	S377
LMR2 (LMTK2, KPI-2)	S1450

MAPKAPK2 (RPS6KC1)	Y225+T226
MEK1 (MKK1, MAP2K1)	Pan-specific
MKK3 (MAP2K3, MEK3)	S218
PKCb (PRKCB1)	S661
RARA	S77
Sgk223	Y413
STAT1	S727

In addition to the shortlisted 1.0mer HBV-associated targets identified in Figure 5.6, we also included a further two candidates from the analyses of specific HBV constructs (WT vs HBxΔ). Inclusion of these targets was based on their observation that PKCt/PRKCQ and PKD2/PRKD2 both showed substantial down-regulated phosphorylation signals in the WT 1.0mer (but not HBxΔ) and were also down-regulated by 1.3mer HBV and HBx over-expression. As such, they represent prime/priority candidates for further consideration/investigation as potential host factors involved in HBV replication processes, via HBx-mediated mechanisms.

Interestingly, there was little overlap observed between specific candidate host factors identified by WT (Table 5.5) and HBxΔ (Table 5.6) replication, and GO annotation was performed to further investigate pathways potentially affected by these modulated signals. This revealed strong concordance between the most relevant/populated/dominant biological processes represented by the targets of each group, with the same four processes (signal transduction, cellular protein modification, response to stress, anatomical structure development) occupying the top ranks (Table 5.7). This suggests that although there were some differences in specific targets modulated by WT and HBx-deficient HBV, the biological processes most affected were similar.

Table 5.7 Highest ranked processes relating to candidates identified by WT or HBxΔ HBV-specific modulation

GO_term_ID	GO_term_def	WT		HBxΔ	
		Rank	No of genes	Rank	No of genes
GO:0007165	signal transduction	1	18	1	12
GO:0006464	cellular protein modification process	2	16	2	12
GO:0006950	response to stress	4	15	3	9
GO:0048856	anatomical structure development	3	16	4	8
GO:0002376	immune system process	6	10	5	8
GO:0006810	transport	18	4	6	7
GO:0030154	cell differentiation	5	13	7	6
GO:0008219	cell death	7	7	8	4
GO:0044403	symbiotic process	26	3	9	4
GO:0040011	locomotion	8	7	10	3

Overall our analyses of HBV replication screen identified ten host factors (Figure 5.6, and PKCt, PKD2) that could have unidentified roles in HBV replication processes as suggested by their modulation in the context of HBx/1.0mer HBV transfection, and/or previously unknown link to HBx/DDB1 proteins, based on observed augmentation by these proteins in the previous screen (Table 5.3) as well as in the 1.0mer HBV context. Of these ten candidates, two (PKCt, PKD2) can be further prioritised for follow-up investigation, based on their consistent down regulation by 1.3mer HBV replication, 1.0mer HBV replication (WT, but not HBxΔ), and HBx overexpression, which potentially implicates HBx as an important factor in the regulation of these signals.

5.4 Discussion

HBV modulates the cellular landscape, facilitating its survival and propagation. There is an urgent need to develop new drugs to cure chronic HBV infection, and hence host factor-virus interactions that are critical for HBV replication represent an attractive therapeutic target. In this study, an antibody microarray approach was used to screen for prospective proteins, and by extension processes/mechanisms that contribute to HBV replication, thereby identifying potential new therapeutic targets for resolution of HBV infection. This approach had been used previously to identify targets for other pathogens, notably including the liver-tropic virus HCV (Haqshenas et al, 2017). As far as we know, the same method has not been utilised for HBV, for which many questions still remain regarding the specific mechanisms involved in its infection and replication.

HBx is necessary for efficient establishment of transcription and replication following HBV infection (Lucifora et al, 2011). One mechanism through which this is achieved involves interaction with host protein DDB1, and results in the relief of transcriptional repression on cccDNA. Since both HBx and DDB1 have also independently demonstrated to be involved in positive transcriptional regulation (Hodgson et al, 2012; Kim et al, 2001a; Kim et al, 2016), antibody arrays were performed to gain further insights into factors contributing to HBx and/or DDB1-mediated regulation of HBV replication. We also investigated mechanisms critical for HBV replication, in the setting of WT and HBx-deficient viruses, using the 1.0mer model optimised in Chapter 3.

5.4.1 Host factors modulated by HBx and DDB1 over-expression

Antibody microarray screens identified 170 potential targets substantially modulated following overexpression of either HBx or DDB1 alone, or following co-expression of both proteins. Of these, 23 were preferentially significantly modulated by HBx expression alone, 60 by DDB1 expression alone, and 42 only when both proteins were expressed (Figure 5.1). Reassuringly, the top ranking biological processes represented by the targets identified by HBx overexpression reflected those which HBx has previously been attributed to, namely cellular protein modification (Chung et al, 2003; Jung et al, 2007; Srisuttee et al, 2012), signal transduction (Bouchard et al, 2003; Doria et al, 1995), cell differentiation (Cho et al, 2015; Gearhart & Bouchard, 2010; Huang et al, 2012a), and cell cycle regulation (Bouchard et al, 2001a; Wang et al, 2019). Beyond the first few ranks shared with HBx, highest modulation by DDB1 expression was observed for processes likely specific to its function. For example, cellular nitrogen compound metabolic process (GO:003541) describes nucleobase-, pyridine-, and pyrimidine-containing compound metabolic processes, and may be indicative of DDB1's known role in DNA repair (Dualan et al, 1995; Li et al, 2006). In turn, regulating immune detection and response to damaged/defective DNA might contribute to maintenance of cell homeostasis, influencing their high rankings (immune system process (GO:0002376) ranked 6; homeostatic process (GO:0042592) ranked 9). Symbiotic process (GO:0044403) ranked higher for HBx overexpression compared to DDB1. This GO term is defined as *“process carried out by gene products in an organism that enable the organism to engage in a symbiotic*

relationship...with another organism", which is consistent with HBx and its role in the HBV replication cycle (Murakami, 2001; Tang et al, 2006).

Ultimately, 6 unique targets (8 signals in total) whose expression or activity (as suggested by phosphorylation) was significantly modulated by expression of viral HBx and/or host DDB1 proteins, were selected for further investigation/consideration. These were translation elongation initiation factors 4E and 4G (eIF4E, eIF4G), histone deacetylase 4 (HDAC4), heat shock protein 90 (HSP90), mammalian target of Rapamycin (mTOR) and mammalian sterile 20-like kinase 1 (MST1). Interestingly, the two proteins with changed phosphorylation status (eIF4E and eIF4G) were both down-regulated, while the remaining four proteins had up-regulated signals for expression (Table 5.8). Organising these results based on the groups in which they exhibited these effects showed HBx overexpression led to upregulation of mTOR and MST1 expression, and downregulation of eIF4E^{S209} phosphorylation. Co-expression with DDB1 also upregulated mTOR and MST1 protein expression, while DDB1 expression alone downregulated eIF4E^{S209} and eIF4G^{S1106} phosphorylation, and upregulated expression of HDAC4 and HSP90. Modulation of these targets could represent unidentified contributions to HBV replication, or more specifically facilitation of such effects by HBx and/or DDB1 interactions. More detailed summaries of these candidates can be found in Appendix B.

Table 5.8 Summary of the effect on selected targets modulated in response to expression of HBx and/or DDB1:

HBx	HBx+DDB1	DDB1
eIF4E ^a - S209 (↓)	mTOR – Pan (↑)	eIF4E ^b – S209 (↓)
mTOR – Pan (↑)	MST1 ^a – Pan (↑)	eIF4G – S1106 (↓)
MST1 ^b – Pan (↑)	MST1 ^b – Pan (↑)	HDAC4 – Pan (↑)
		HSP90a/b – Pan (↑)

In summary, analyses of this antibody microarray screen has identified several host candidates that may be playing previously unidentified roles in facilitating HBV replication and/or transcription, based on their modulation in the context of HBx and/or DDB1 expression. These host factors appear to be largely involved in processes pertaining to transcription and translation, and these biological effects will next need to be investigated in an HBV replication/infection system. Verification of these effects in this context (such as by utilising siRNA, evaluation in clinical samples etc.) could present new mechanisms of HBx and/or DDB1-mediated regulation of HBV replication, potentially leading to novel therapeutic interventions involving drugs against specific candidates.

5.4.2 Host factors modulated by HBV Replication

Having identified targets modulated in the context of HBx and/or DDB1 over-expression, the 1.0mer HBV cell culture model was subsequently used to investigate targets modulated by HBV replication. This model has the advantage of producing cccDNA-like molecules, mimicking the natural HBV replication cycle, in contrast to the traditional overlength HBV transfection models. Importantly, cells were transfected with wild-type or HBx-deficient 1.0mer HBV constructs to investigate the impact on cell signalling in the presence and absence of HBx protein. From this experiment, a total of 198 modulated signals were observed in response to either WT or HBxΔ 1.0mer HBV transfection, relative to mock transfection (Figure 5.3). Twenty-three targets were observed to be specifically substantially modulated by WT, but not HBxΔ HBV (Table 5.5), while reciprocal analysis highlighted effects for 14 targets that were modulated by the HBxΔ and not by WT HBV (Table 5.6). Comparing each set of targets against the results of the HBx overexpression screen, revealed five (modulated in the same direction), and six candidates (modulated in the opposite direction) from each respective group, that may represent HBx-mediated effects. Since the lists of candidates for each construct did not overlap, it could be hypothesised that the presence/absence of HBx augmented the modulation of these targets, possibly by masking/revealing specific sites of interaction or modification. GO analysis was performed to explore the functional interconnections of selected targets, and comparing the 23 WT-specific and 14 HBxΔ-specific targets showed that although the individual targets differed between the two groups, the biological processes they are associated with were largely in accordance (Table 5.7). Overall, this could mean that the presence or absence of HBx protein during HBV replication induces differential modulation of varying sets of host factors, but that ultimately HBV replication and the identified modifications impact on the same cellular processes.

Besides modulated signals specifically associated with either WT or HBxΔ HBV, sixty targets (52 unique host factors) were identified to be substantially, though not differentially, augmented by both constructs (Figure 5.4, Table 5.4). These may represent additional factors more broadly/generally involved in HBV replication and are also interesting targets for consideration since HBx is not strictly necessary for establishment of infection. Comparison of the 60 targets modulated by Wt and HBxΔ 1.0 mer variants with targets modulated by 1.3mer HBV, identified 18 signals each that were modulated in the same or opposite direction to the results observed from 1.0mer HBV transfection (Figure 5.5). Furthermore, only three targets in each group were considered to also be Potential candidates in the 1.3mer WT HBV analysis (not shown). The overall low concordance (of specific target modulation) between the 1.0mer HBV and 1.3mer HBV results though interesting, were not considered to be particularly concerning. Instead, this is believed to be representative of the inherent differences between the HBV constructs and their replication mechanism and capacities (see Chapter 3).

Encouragingly, analysis for HBV 1.3mer and 1.0mer samples showed that the biological processes affected were very similar (not shown), even though specific targets modulated in each group were not identical. This may indicate that although HBV replication generally affects the same cellular processes or pathways, 1.3mer HBV replication mediated these effects differently to 1.0mer HBV. These differences could have been due to difference in the constructs themselves, as 1.3mer derived HBV replicates at higher efficiency than 1.0mer HBV

(see Chapter 3) and encodes 2 copies of the HBx gene, compared to the more biologically relevant 1.0mer which encodes 1 copy of HBx. It is also important to note that the 1.3mer and 1.0mer microarray experiments were not conducted at the same time, making direct comparisons between these constructs difficult. Furthermore, as the 1.3mer HBV is presented in the context of a vector, transcripts can be driven from cryptic promoters present in the plasmid backbone, and therefore affect the expression of gene products (Lemp et al, 2012; Rosfjord et al, 1994). For these reasons, our findings have focused on the more authentic system represented by 1.0mer HBV replication-competent HBV (genome-length construct, formation of cccDNA-LM). Further selection of candidates for investigation was based on previously described functions in processes relevant to HBV replication, or, where links had already been established in relation to HBV, based on some potential new mechanism in the context of HBx and/or DDB1. Nine unique targets were ultimately shortlisted for potential follow-up, two of which (MST1, HSP90) overlapped with targets identified in the previous protein overexpression screen. The remaining candidates included YAP1, VegFR1, TRRAP, TAO1, and GluR1 (Table 5.9), as shown in Figure 5.6 and summarised in Table 5.9. Further summary discussion of these shortlisted candidates and their functions can be found in Appendix D.

Table 5.9 Summary of the effect on selected targets modulated in response to 1.0mer HBV replication (both WT and HBxΔ:

Up-regulated targets	Down-regulated targets
GluR1 – S849	HSP90a/b – Y484
HSP90a/b – Pan	MST1 – T187
TRRAP – Pan	SLK – S189
VEGFR1 – Y1053	TAO1 – Y309
YAP – S109	
YAP – T119	

5.4.2.1 Role/brief summary of priority candidates modulated by HBV replication and HBx over-expression

To identify potential HBx-specific targets, results were compared for all constructs excluding that of the 1.0mer HBV construct lacking wild-type HBx expression (Table 5.6). Of the twenty-three targets specifically associated with replication of 1.0mer WT, but not HBxΔ HBV (Table 5.5), five also exhibited shifts in the same direction (increase or decrease) following transfection with the pCI-HBx construct (Table 5.5, green), and two of these signals (PKCt, PKD2 phosphorylation) were additionally decreased by WT 1.3mer HBV construct which encodes two copies of the HBx gene. This could suggest that HBV-induced, decreased phosphorylation of PKCt (Y545) and PKD2 (S197+S198) could somehow regulate/contribute to HBV replication, and that these effects may be mediated by HBx protein. Based on these results, we propose that these host factors present prime candidates for prioritised further investigation as potential therapeutic targets, requiring extensive work to first validate both a biological effect, and involvement of HBx.

Role of the prime candidates

- PKCt

Protein Kinase Cs are a family of serine-threonine kinases that play major roles in signal transduction. Protein Kinase Cs (PKCs) are thought to be involved in cancer biology through major roles in signal transduction and cell regulation, and the decreased PKCt expression in HCC tissue was found to be well-correlated with poor cancer grade (Lu et al, 2010). PKC isoform theta (PKCt/PKCθ) belongs to the novel class of PKCs which are activated by diacylglycerol, and is a critical regulator of biology and function of T cells, in which it is reported to be highly expressed (Hayashi & Altman, 2007). Mutation of PKCt results in poor T cell activation and survival, aberrant expression of apoptosis-related proteins (Hayashi & Altman, 2007), and in HIV infection, it was suggested Nef-induced loss of PKCt could contribute to impairment of T cell function (Smith et al, 1996). Although previous reports suggest effective innate signals following viral infections are sufficient to overcome the requirement for PKCt in mounting antiviral responses, (Berg-Brown et al, 2004; Giannoni et al, 2005; Marsland et al, 2005), immune responses in the context of CHB infection are known to be generally poor (Nakamura et al, 2001; Publicover et al, 2013; Publicover et al, 2011), indicating PKCt dysregulation could be a potential contributor to establishment of persistent infection. Although it is mainly reported to be expressed in T cells, low level expression was previously reported in liver as well as HCC tissue (Chang et al, 1993; Lu et al, 2010). Nevertheless, this finding likely warrants considering of closely related, more ubiquitous isoforms of PKC known to be expressed in hepatocytes such as the delta isoform, with which it shares the highest sequence similarity (Osada et al, 1992).

PKCt residue Y545 falls within the kinase domain conserved across PKC isoforms. These domains contain an activation loop, phosphorylation of which is required for PKC catalytic activity (Liu et al, 2002; Newton, 2003), and although the effect of Y545 phosphorylation has not been directly described, it is possibly associated with PKCt activity since phosphorylation of one site on PKC can reportedly influence phosphorylation of another site (Liu et al, 2002).

Extensive cross-talk also exists between different isoenzymes and determines the overall cellular response. HBV capsids indiscriminately encapsidate the alpha isoform of PKCs, which were proposed to compete with RNA for binding of HBc (Wittkop et al, 2010). Previously, release of RNA from HBc phosphosite enabled core phosphorylation, reduced capsid stability, RNA encapsidation capacity, resulting in impaired virion secretion (Kann & Gerlich, 1994). However Wittkop et al (2010) reported that inhibition of PKCa also led to reduced virion secretion (Wittkop et al, 2010), suggestively attributing these contrasting effects to the existence of other specific isoforms or kinases (other than PKCa) within the lumen that differentially influence HBc phosphorylation. Furthermore, it has been suggested that phosphorylation of HBc at different sites by various protein kinases mediates distinct functions on the HBV life cycle.

Since decreased phosphorylation of PKC ϵ^{Y545} was consistently observed for WT 1.0mer HBV, 1.3mer HBV, and HBx over-expression, it is suggested that modulation of this target may be involved in a mechanism relating to HBV replication, and that this effect may be at least partially mediated by HBx.

- PKD2

PKD2 (serine/threonine protein kinase D) is expressed in endothelial cells of various tissues and is known to play an important role in angiogenesis through modulation of key growth factors involved in endothelial cell biology (Hao et al, 2009). It may also regulate expression of other genes involved in angiogenesis including cell cycle regulators, and was identified as a potential HSP90 client (Moulick et al, 2011)- which was another potential candidate identified in the microarray screen. HSP90-mediated stabilization of PRKD2 can contribute to tumor growth and angiogenesis (Azoitei et al, 2014; Azoitei et al, 2015). In HCC PKD2 is the predominant isoform, with enhanced expression compared to non-tumour tissue. High phosphorylation and expression of PKD2 is associated with a high predicted metastasis risk signature, high invasive potential, since PKD2 stimulates EMT and invasion of HCC (Zhu et al, 2016). Silencing of PKD2 markedly inhibited endothelial cell proliferation and migration, as well as upregulating expression of ICAM-1 which has known roles in inflammation and immunity (Hao et al, 2009), and PKD2 has also been implicated in immune responses. IFN- α induces PKD2 catalytic activity and subsequent interaction with IFNAR1. PKD2 attenuates type I IFN signalling by degradation of IFNAR1, and inhibition/knockdown of PKD2 augments IFN- α induced signalling and enhances antiviral effects (Zheng et al, 2011). PKD2 catalytic activity is also required for efficient antigen receptor-induced cytokine production in T cells, as well as T-cell dependent antibody responses (Matthews et al, 2010).

Expression of PKD is predominantly cytoplasmic, with activation inducing transient translocation to the plasma membrane. Unlike other isoforms, rather than translocating to the nucleus, PKD2 remains in the cytosol upon membrane dissociation where it can mediate various effects, presumably through activation of pathways including NF- κ B, MAPK, and CREB (Ozgen et al, 2008; Zou et al, 2012). This activation and dissociation requires PKC activity (Rey et al, 2003). PKC-mediated serine phosphorylation in the catalytic domain is important for PKD activation and downstream signalling, and PKC ϵ has been implicated in regulation of PKD activation, and loop phosphorylation and activation in response to antigen receptor ligation.

Thus during HBV replication, modulation of PKC ϵ may consequentially modulate PKD2, impacting various downstream effects to potentially facilitate or contribute to persistence and progression to HCC. Moreover, these mechanisms may involve HBx, since over-expression of this viral protein also similarly augmented these signals. Furthermore, GO analysis of these two specific candidates (PRKD2 = PKD2; PRKCQ = PKC ϵ) revealed associations in multiple varying biological pathways (Figure 5.8), which is also reminiscent of the multi-functional nature described of HBx.

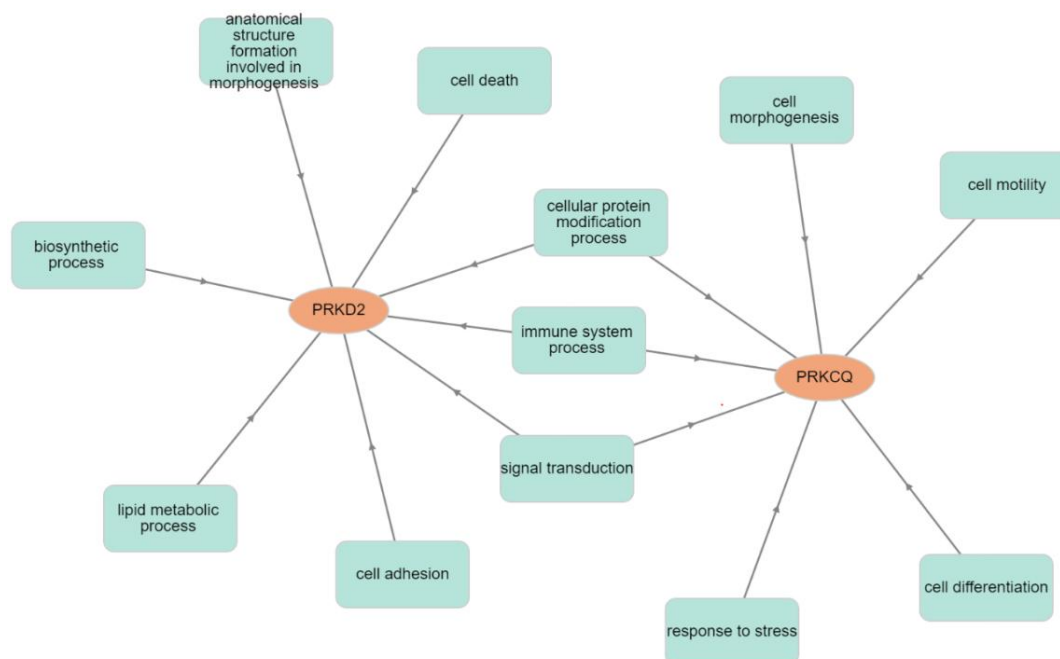


Figure 5.8 GO analysis showing biological processes associated with PKCt(PRKCQ) and PKD2 (PRKD2).

This study has identified a number of host signalling pathways modulated by HBV replication and/or HBx expression which may represent novel targets for therapeutic intervention. The importance of these targets in HBV replication needs to be verified, using inhibitory RNAs (siRNA) or by gene editing systems (CRISPR) in a HBV replication model. Ideally this would be done using the newly developed NTCP-HBV infection system or in primary human hepatocytes (Shen et al, 2018; Yan et al, 2012) with inclusion of multiple viral variants for a broader, more complete picture/analysis of potential broader efficacy in varied infected populations. Following validation, a range of existing inhibitors that have already been tested in human studies could be investigated for efficacy in suppressing or clearing infection/replication. Examples of inhibitors which have demonstrated efficacy against the identified host targets candidates, (as summarised from Selleckchem.com) and which could be readily investigated for inhibitory effects on HBV infection, are shown in .

Table 5.10 Summary of selected host targets and their potential inhibitors (listed on Selleckchem.com)

Target	Inhibitor
PKCt	• Sotrastaurin • PKC-theta inhibitor
PKD2	• CID 2011756 • 1-Naphthyl PP1(1-NA-PP1)
eIF4E	• 4EGI-1 • 4E1RCat
eIF4G	• SBI-0640756
HDAC4	• Tasquinimod • CUDC-101 • Quisinostat • BML-210 (CAY10433) • TMP269
HSP90	• Tanespimycin (17-AAG) • Luminespib (NVP-AUY922) • Ganetsepib • BIIB021 • Onalespib • KW-2478
mTOR	• Rapamycin • Everolimus (RAD001) • AZD8055 • Ridaforolimus • Sapanisertib (MLN0128) • Gedatolisib (PKI-587) • Torin 2 • WYE-125132 (WYE-132) • ETP-46464 • Dactolisib
MST1	• XMU-MP-1
YAP1	• CA3 (CIL56) • YAP-TEAD Inhibitor 1 (Peptide 17) • Super-TDU • TED-347 • Verteporfin
VEGFR1	• MGCD-265 • Motesanib (AMG-706) • Axitinib • Motesanib Diphosphate (AMG-706) • Linifanib (ABT-869) • Cediranib • Pazopanib • Tivozanib
TRRAP	• ?WM-1119
TAO1	• Compound 43 TAO Kinase Inhibitor
GluR1	• IEM 1754 2HBr

Chapter Six: Concluding remarks

Chronic hepatitis B affects more than 257 million people globally, causing significant morbidity and mortality from related cases of liver disease (Beasley & Hwang, 1984; Chen et al, 2010). Accordingly, the etiological agent, HBV, is a major public health burden, contributing close to one million deaths annually (WHO Global Hepatitis Report, 2017). A unique and complex viral replication strategy presents challenges to development of therapeutic strategies that will result in functional or complete sterilizing CHB cure. Functional cure is rarely achieved, and at best, patients achieve persistent viral suppression and reduced (but not eliminated) risk of liver sequelae on conventional anti-HBV treatments/interventions (Beasley & Hwang, 1984; Chen et al, 2010; Cornberg et al, 2020). Gaps in current understanding are attributed to lack of appropriate models that enable comprehensive investigation of all stages of the HBV replication cycle, particularly in relation to the nuclear reservoir of cccDNA which is the template for transcription of all viral RNAs. Moreover, most non-clinical investigations on HBV are restricted to HBV genotype D, as routine models for comparison of different HBV genotypes/subgenotypes permitting investigation of the complete HBV replication cycle are not yet available. In this thesis, we described an alternative *in vitro* cell culture transfection system that addresses several of these limitations.

We demonstrated that a 1.0mer HBV transfection model is a viable approach to comprehensively investigate HBV replication across HBV genotypes. We have characterised the system and shown that this model permits expression of all expected HBV DNA, RNA, and proteins both *in vitro* in hepatoma cell lines, and *in vivo* in immunocompetent mice. Importantly, a major advantage of this system over the current conventional transfection systems is the production of cccDNA-like molecules, which have been shown to act as transcriptional templates akin to authentic cccDNA molecules produced following natural HBV infection. The system is amenable to studying different HBV variants, being relatively easy to manipulate and generate different genotype or variants, which allows for exploration and comparison of different HBV viruses. Moreover, application of the model to an *in vivo* context using a murine system enables investigations of cccDNA and HBV variants, following therapeutic interventions. To this effect, we have used this model to demonstrate SMAC mimetics decreased expression of cccDNA-like molecules in contrast to standard nucleoside treatment, consistent with its identified mechanism of action. A manuscript including these murine studies has been submitted.

We have also used the 1.0mer model to investigate the interactions of host DDB1 and HBx in HBV replication. Previous studies had suggested that DDB1 could enhance HBV replication in both HBx-dependent and –independent manners (Decorsière et al, 2016; Kim et al, 2016; Murphy et al, 2016). In support of the latter, we have also shown that DDB1 could increase HBV replication in an HBx-independent manner. The mechanism for this is unclear, however, we suggest that DDB2, a critical partner in the DNA-repairing activity of DDB1, could be involved in both HBx-dependent and –independent mechanisms for DDB1-mediated transcriptional enhancement, via degradation of various host factors (unidentified or otherwise) and/or contribution to repair of cccDNA templates. This model facilitates future follow-up studies wherein these DDB1- and DDB2-mediated transcriptional effects can be

closely examined, to potentially identify new therapeutic targets for intervention, although off-target host effects will need to be carefully monitored.

Previous reports identified a link between HBx-DDB1 interaction and relief of cccDNA transcriptional silencing otherwise imposed by SMC5/6 complex. We hypothesised that this mechanism may contribute to the differences in replication capacity observed between different HBV genotypes (Bannister et al, 2019; Sozzi et al, 2018; Sozzi et al, 2016), based on sequence differences in a major DDB1-binding region of HBx. Utilising over-expression constructs for viral HBx and host DDB1 proteins, we showed that genotype-associated differences in the H-Box motif did not impair DDB1's ability to stabilise HBx expression, suggesting this was not a major contributing factor to genotype-associated differences in HBV replication. We observed that DDB1 could efficiently and consistently stabilise WT HBx protein expression from various genotypes, with no perceived impairment. In contrast, enhanced stability/expression of HBx mutants known to be impaired in DDB1-binding was expectedly suppressed.

To further investigate novel host factors or processes that may be involved in HBV replication, and may represent new antiviral targets, we performed comprehensive antibody microarray screens to identify host proteins modulated by HBV replication and/or HBx and DDB1 expression. Investigating cells over-expressing HBx and/or DDB1 proteins, suggested potential contributing roles in HBx- and/or DDB1-mediated HBV replication for eIF4E, eIF4G, mTOR, MST1, HDAC4 and HSP90. Additionally, we also identified potential roles in HBV replication for GluR1, HSP90, MST1, PKC ϵ , PKD2, TRRAP, VEGFR1, and YAP1. These were selected based on consistent modulation observed by both WT and HBx Δ 1.0mer HBV transfection. We have briefly described each of these targets, and suggested how they may interact with each other and HBV within Appendices A and C. Many have also been linked to tumour development and thus may also be implicated in HBV-associated HCC. One limitation of the current study is that all experiments were performed in cancer-derived cell lines, and though these have played indispensable roles in scientific discovery and development, undeniably still display distinct profiles to primary cells. Since cell signalling pathways are often altered in tumour cell lines, for example in relation to cell growth and protein synthesis, future studies using primary cells would thus be illuminating in validating the roles of these identified candidates in HBV replication. These results need to be further and comprehensively validated, but if HBV-associated effects are verified, there are existing pre-validated inhibitor compounds that could be tested for anti-HBV efficacy, presenting novel therapeutic strategies that could potentially lead one step closer to achieving HBV cure.

In conclusion, this study has shown that the 1.0mer HBV model readily permits cell culture and animal-based studies for evaluation of the persistence of cccDNA-like molecules. We have demonstrated the utility of this system for evaluation of novel therapeutic approaches, particularly those that target HBV-infected cells. Further refinement of the model utilising methods such as ligation of monomers prior to HDI or transfection should be further investigated. We have also demonstrated the utility of the 1.0mer system for screening host factors involved in HBV replication. This in turn led to identification of host components that are targets for further investigations; as viral replication-associated host determinants, and consequently potential drug targets. In the absence of more convenient and easy-to-use NTCP-

based HBV infection models, the 1.0mer HBV transfection model should prove to be an important tool that will advance our understanding of HBV replication across genotypes, and facilitate development of new treatments benefitting the 257 million people worldwide currently living with life-long chronic HBV infection.

Appendix A: Genome arrangement of HBV 1.3mer vs 1.0mer template

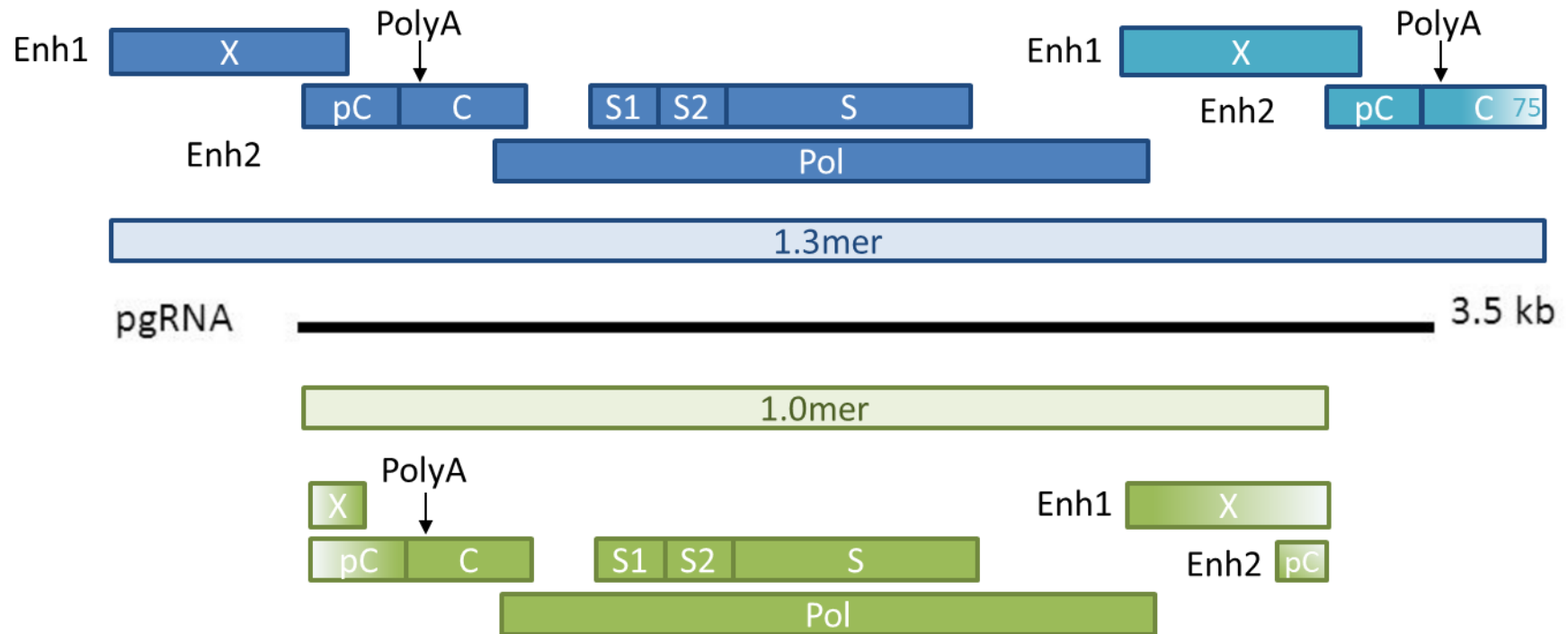


Figure A-1 Schematic depicting the approximate genome arrangement of 1.3mer and 1.0mer HBV constructs relative to each other. Note that 1.0mer templates (3.2kb) are less than the length of the 3.kb pg/pcRNA, hence the requirement of circularisation for replication.

Appendix B: HBx and/or DDB1 overexpression Kinexus analysis shortlisted candidates

Results

In transfected cells expressing either HBx or DDB1 alone, or in conjunction, six host proteins showing modulated signals in response to HBx and/or DDB1 expression were shortlisted as being potentially interesting targets for further investigation. The following is a brief summary of the observed effects, followed by a short discussion of potential implications.

EIF4E

The first selected candidate was the eukaryotic initiation factor 4 (eIF4E), which is an mRNA cap-binding proteins important in protein translation initiation. HBx and DDB1 over-expression reduced phosphorylation of eIF4E at S209 (Figure A-1). Phosphorylation was reduced by 26% following HBx over-expression (antibody A), and 27% following DDB1 over-expression (Antibody B). Spurious results such as the 2389% increase with DDB1 overexpression (antibody A), and a 65% increase with HBx expression (antibody B), were ignored due to their questionable Flag and higher error values, respectively. These results suggest that independent expression of either HBx or DDB1, but not both, reduces eIF4E phosphorylation at S209.

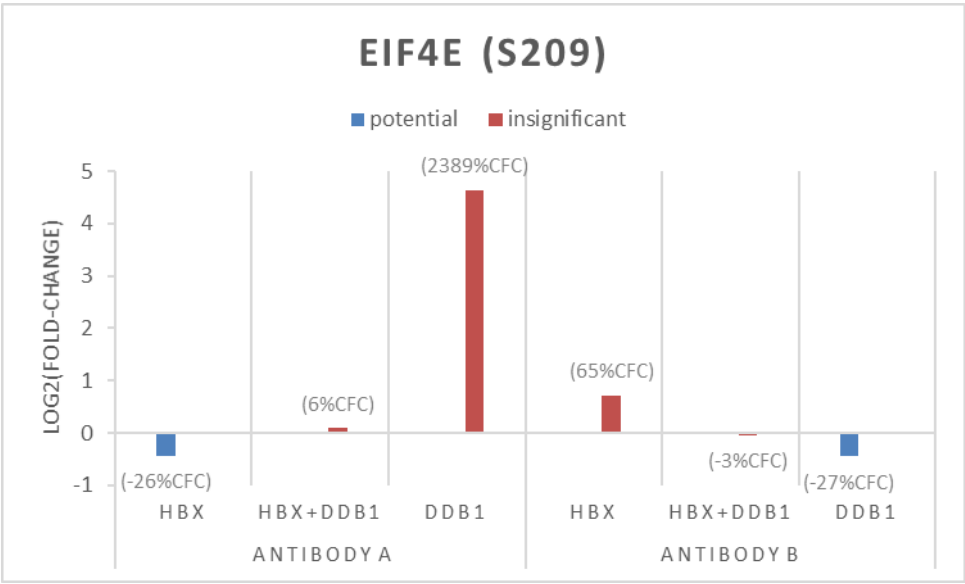


Figure B-1 Phosphorylation of eIF4E at S209 in HepG2 cells transfected with HBx, HBx+DDB1, or DDB1.

EIF4G

The multi-protein translation initiation complex (eIF4F) which involves eIF4E, also includes a larger protein eIF4G. Phosphorylation of eIF4G at S1106 was significantly reduced by DDB1 expression alone, and only marginally by HBx expression alone (Figure A-2). Although this reduction was further enhanced when both proteins were expressed together, this result was not considered a potential hit as it did not satisfy the required criteria. This suggests that DDB1 expression, but not HBx expression (alone or with DDB1) substantially decreases EIF4G phosphorylation at S1106.

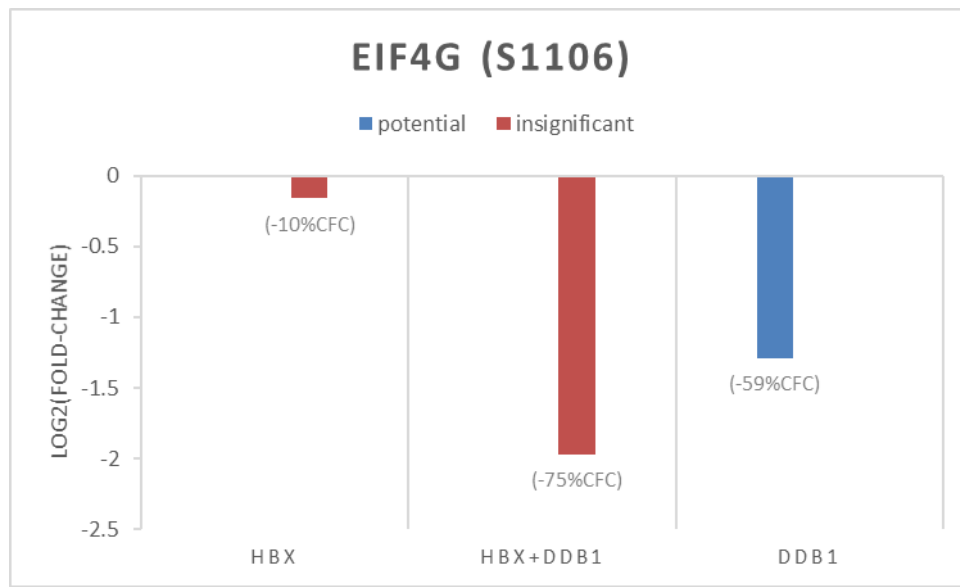


Figure B-2 Phosphorylation of eIF4G at S1106 in HepG2 cells transfected with HBx, HBx+DDB1, and DDB1.

HDAC4

Histone deacetylases regulate transcription by modifying chromatin architecture via modification of the associated histones. In this screen HDAC4 expression was substantially increased by DDB1 overexpression alone, and to a lesser extent when HBx and DDB1 were co-expressed (Figure A-3). However HBx over-expression itself had no perceivable impact on HDAC4 expression. Interestingly, DDB1 expression also significantly increased phosphorylation of HDAC5 at S498, however the error value was too high (>50%) for the result to be considered a potential and was consequently not included in the final shortlist (not shown). This data indicates that HBx expression alone or with DDB1 does not substantially modulate expression of HDAC4, while DDB1 expression alone significantly increases expression.

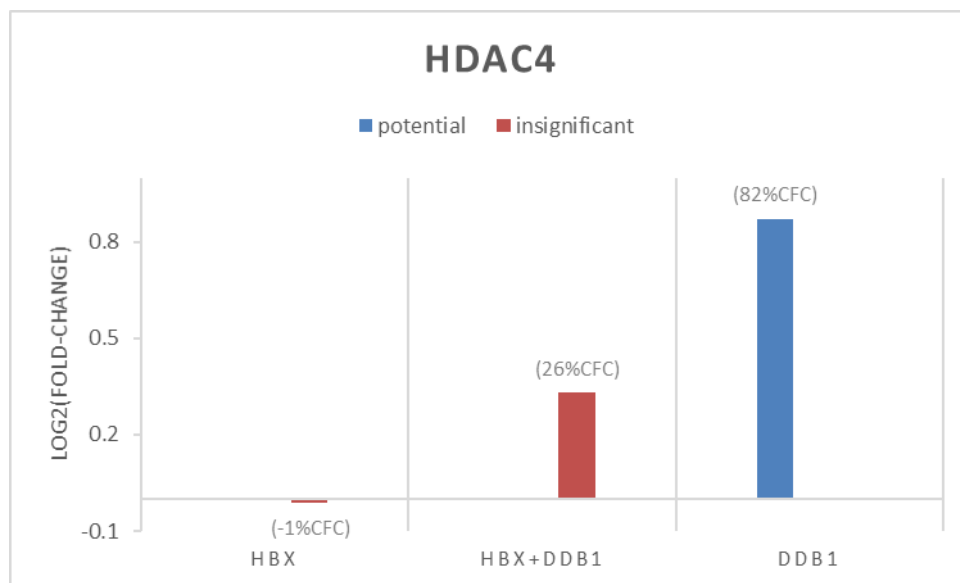


Figure B-3 Expression of HDAC4 in HepG2 cells transfected with HBx, HBx+DDB1, and DDB1.

HSP90

Molecular chaperones, such as heat shock proteins, play important roles in protein folding and assembly, and thus prevention of aggregation to maintain cellular homeostasis (Taipale et al, 2010; Tsutsumi & Neckers, 2007). DDB1 over-expression resulted in increased HSP90 expression (Figure A-4). Although increased HSP90 expression was also observed for HBx and the HBx+DDB1 co-expression, these did not meet the threshold criteria to qualify as Potential candidates. This data indicates HBx expression alone or with DDB1 marginally increases HSP90 expression, while independent DDB1 expression significantly upregulates HSP90 expression.

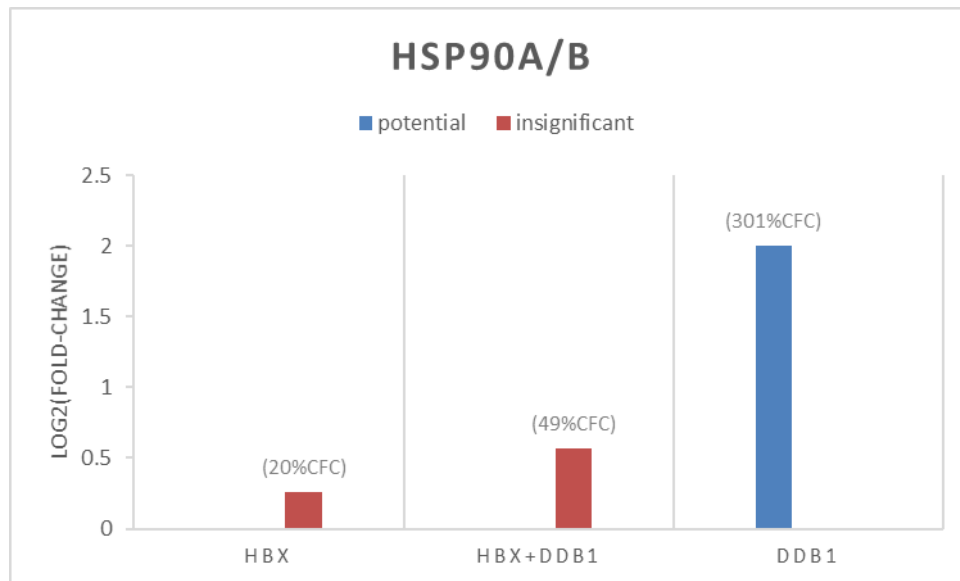


Figure B-4 Expression of HSP90a/b in HepG2 cells transfected with HBx, HBx+DDB1, and DDB1.

mTOR

Mammalian target of rapamycin (mTOR) is a member of the PI3K-related protein kinases (PIKK), whose signalling are important for cell growth and survival (Laplane & Sabatini, 2012; Sabatini, 2017). mTOR protein expression was increased when HBx was transfected alone or in junction with DDB1 expression plasmid (A-5). DDB1 expression alone also induced increase in signal, although this did not meet the defined threshold for a Potential candidate. This result indicates that DDB1 expression only marginally modulate mTOR expression, but independent HBx expression or HBx-DDB1 co-expression both significantly increase expression of mTOR.

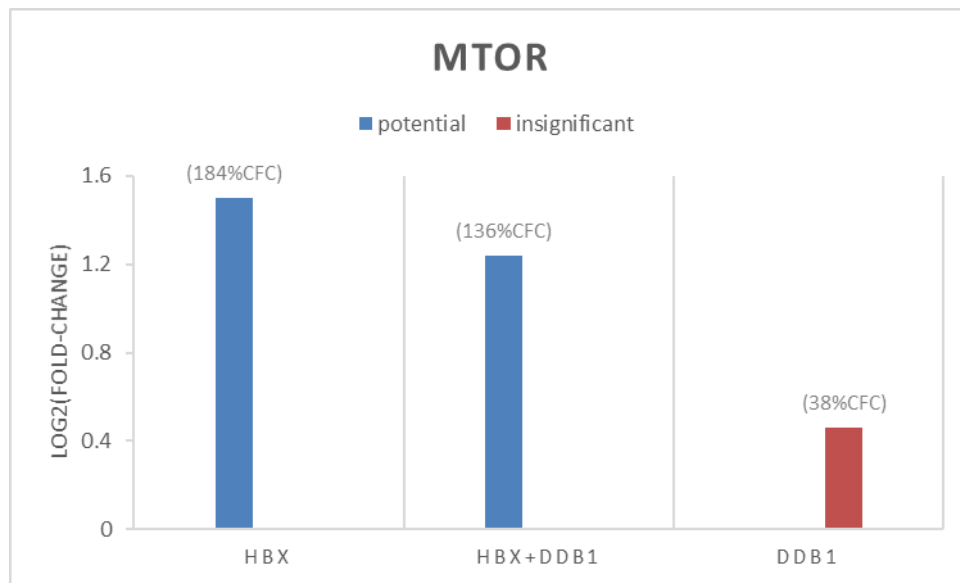


Figure B-5 Expression of mTOR in HepG2 cells transfected with HBx, HBx+DDB1, and DDB1.

MST1

Mammalian Sterile 20-like kinase 1 (MST1) is a serine/threonine kinase with pro-apoptotic activities which make it a putative tumor suppressor. Two different MST1 pan-specific antibodies (Kinexus antibody codes NK113-1 and NK113-2) showed MST1 upregulation in all samples to varying degrees (A-6). For the first antibody, this change was significant in the HBx+DDB1 sample, while the second antibody additionally exhibited significant changes in HBx expression alone. This data suggests that while HBx+DDB1 co-expression, and possibly independent HBx expression upregulates MST1 expression, DDB1 expression alone only modestly modulates expression.

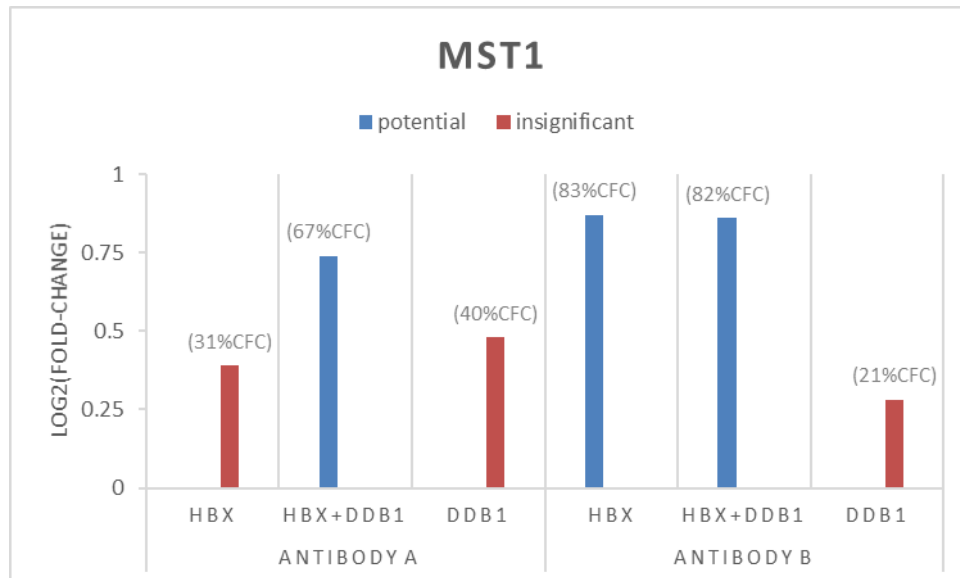


Figure B-6 Expression of MST1 in HepG2 cells transfected with HBx, HBx+DDB1, DDB1 and HBV (1.3-mer D3 HBV). Asterisks represent "Priority"-flagged results.

Discussion

eIF4E, eIF4G, mTOR:

Many viruses positively or negatively regulate eIF4F complex formation to inhibit cellular protein synthesis and selectively regulate viral protein translation (Connor & Lyles, 2002; Cuesta et al, 2000; Montero et al, 2015; Yanguéz et al, 2012). A significant decrease in eIF4E^{S209} phosphorylation was observed following HBx expression alone and DDB1 co-expression, by 2 different antibodies (Figure A-1). Phosphorylation of eIF4E at S209 was described to reduce its binding affinity to mRNA cap structures by electrostatic repulsion (Scheper et al, 2002; Zuberek et al, 2004; Zuberek et al, 2003), consequently leading to the dissociation of the eIF4F translation initiation complex from the capped mRNA. In turn, this release of eIF4F may serve to enhance unwinding/ribosome migration and translation initiation of the mRNA, or it may facilitate translation initiation of alternate RNAs, thus “reprogramming” the translational machinery in response to certain stimuli. eIF4E has previously been shown to bind HBV pol (Kim et al, 2010; Ryu et al, 2010), so interrupting these interactions via modulation of eIF4E may be a mechanism for promoting viral replication. In this regard, Duncan et al showed dephosphorylation of eIF4E correlated with inhibition of cellular protein synthesis following heat shock (Duncan et al, 1987), while increased expression and phosphorylation of eIF4E has been observed in several human cancers (Coleman et al, 2009; De Benedetti & Graff, 2004; DeFatta et al, 1999; Fan et al, 2009), often associated with poor prognosis (Graff et al, 2009; Nathan et al, 1997; O'Reilly et al, 2009). Therefore, HBx-mediated modulation of eIF4E and presumably protein synthesis, may be one mechanism by which HBx facilitates optimal HBV-permissive cell conditions and contributes to HBV-mediated carcinogenesis.

Phosphorylation of eIF4E is mediated through its interaction with Mnk1/Mnk2 kinase-bound eIF4G (Pyronnet et al, 1999; Waskiewicz et al, 1999). Modulation of eIF4G, possibly via dephosphorylation by DDB1 (and HBx to a lesser extent) (Figure A-2), could interrupt its associations with Mnk1/2 or other kinases (Diaz-Meco et al, 1994; Whalen et al, 1996), thereby resulting in reduced phosphorylation of eIF4E. Phosphorylation of eIF4G also correlated with reduced translation in a study by (Ling et al, 2005). Therefore it may be speculated that in the setting of HBV replication, the individual effects of HBx and DDB1 on eIF4E and eIF4G could coordinately regulate translation to the ultimate benefit of viral replication. In addition, mTOR may also be contributing to this effect. Expression of the mammalian target of Rapamycin (mTOR) was substantially increased by HBx expression or HBx+DDB1 co-expression (and less significantly by DDB1 expression alone). Activated mTOR leads to hyperphosphorylation of translation-inhibiting eIF4E-binding proteins (4E-BP). This effectively inhibits the 4E-BPs, resulting in their release from eIF4E, and thereby promoting eIF4E-eIF4G association and ultimately protein translation (Gingras et al, 1999). Deregulation of the PI3k/Akt/mTOR (a survival pathway) is increasingly implicated in HCC carcinogenesis (Chen et al, 2009b; Gedaly et al, 2012; Zhou et al, 2010), suggesting another mechanism by which HBx and DDB1 could contribute to promotion of HBV-associated HCC.

Collectively, our microarray results showing modulated expression/activity of eIF4E, eIF4G and mTOR suggest that expression of HBx and DDB1 may coordinate multiple pathways/actions to augment cellular protein synthesis, ultimately creating a cellular environment conducive to enhanced HBV replication. Translation may be accordingly promoted or inhibited in a temporally-regulated manner so that inhibition of global cellular protein synthesis is halted in favour of viral protein synthesis at critical points to promote virus replication. This may be

involve additional interactions coordinated by other viral proteins during infection and replication processes, and furthermore may relate to promotion of hepatocarcinogenesis

HDAC4:

Increased expression of HDAC4 was observed following ectopic DDB1 overexpression (A-3). Chromatin-modifying proteins play important roles in the transcriptional regulation of chromosomal DNA. Modification of tails of DNA-bound histones by histone acetyltransferases (HAT) induces a relaxed, transcriptionally active chromatin state (Ausio & van Holde, 1986; O'Neill & Turner, 1995), whereas removal of these acetylation marks by histone deacetylases (HDAC) induces chromatin condensation which reduces access to transcriptional machinery and results in a transcriptional silencing phenotype (Hassig et al, 1998; Kadosh & Struhl, 1998). In cell culture models and chronic infected patient liver, increased HDAC1 recruitment correlated with low HBV viremia, and treatment with HDAC inhibitors increased acetylation of cccDNA-bound histones paralleled by increased replication (Pollicino et al, 2006). HBx is also known to interact with numerous histone-modifying proteins to recruit them onto cccDNA and promote transcription (Belloni et al, 2009), and furthermore has been shown to induce expression of HDAC1 (Yoo et al, 2008). Similar to HDAC1-3, recruitment of HDAC4 to promoters regulated transcription by targeting transcription factors (MEF2C, recruits HDAC to repress transcription) (Wang et al, 1999). Although it was demonstrated that HDAC4 is able to suppress HBV replication (Zhang et al, 2011), the specific mechanism behind this is unclear. Downregulation of HDAC4 expression has been described among the actions of miR-1 leading to enhanced HBV replication. Although we did not observe direct modulation of HDAC4 by HBx overexpression, our findings suggest that HDAC4 modulation may be one of the mechanisms by which DDB1 contributes to the epigenetic restriction of HBV cccDNA.

HSP90:

Overexpression of DDB1 led to increased expression of HSP90 (A-4). Heat shock proteins are molecular chaperones critical for ensuring proper folding and assembly of protein/protein complexes (Yonehara et al, 1996) (Buchner, 1996; Jakob & Buchner, 1994). HSP90 has been found to associate and regulate proteins including steroid receptors and protein kinases (Pratt, 1993; Stancato et al, 1993; Xu & Lindquist, 1993). This ensures their proper function and is essential for cellular homeostasis and viability. In the context of HBV, HSP90 binds core protein dimers, stabilizing them and facilitating capsid formation (Shim et al, 2011). It also binds to TP and RT domains of human HBV Pol (Cho et al, 2000) and induces conformational changes required for *in vitro* priming activity of Pol (Gyoo Park et al, 2002). Interaction between polymerase and pgRNA essential for initiation of reverse transcription (Hu et al, 2004a; Hu & Seeger, 1996) and these experimental results suggest DDB1 could be facilitating this through regulation of HSP90. Additionally, HSP90 may also be contributing to replication through chaperone activities involving recruitment and assembly of DDB1-containing complexes that regulate cccDNA transcription. Therefore, we speculate that HSP90 may be an important factor in DDB1-mediated regulation of HBV transcription.

MST1:

Mammalian Sterile 20-like kinase 1 (MST1) is a serine/threonine kinase with pro-apoptotic activities indicative of tumour suppressor activity that is particularly potent in the liver (Zhou et al, 2009) (Song et al, 2010). MST1 controls phosphorylation of effectors within the Hippo signaling pathways involved in monitoring organ size and oncogenesis (Camargo et al, 2007; Pan, 2007).(Qin et al, 2013) MST1 has also been demonstrated to promote growth of hepatocellular carcinoma cells suggesting a role in promoting liver cancer (Ng et al 2013). In this experiment, MST1 expression was substantially upregulated in the context of HBx+DDB1 expression, suggesting that modulation of MST1 may be another mechanism by which HBx contributes to HCC progression in chronic HBV infection, and that this mechanism may also involve DDB1.

Appendix C: 1.0mer and 1.3mer HBV Kinexus analysis

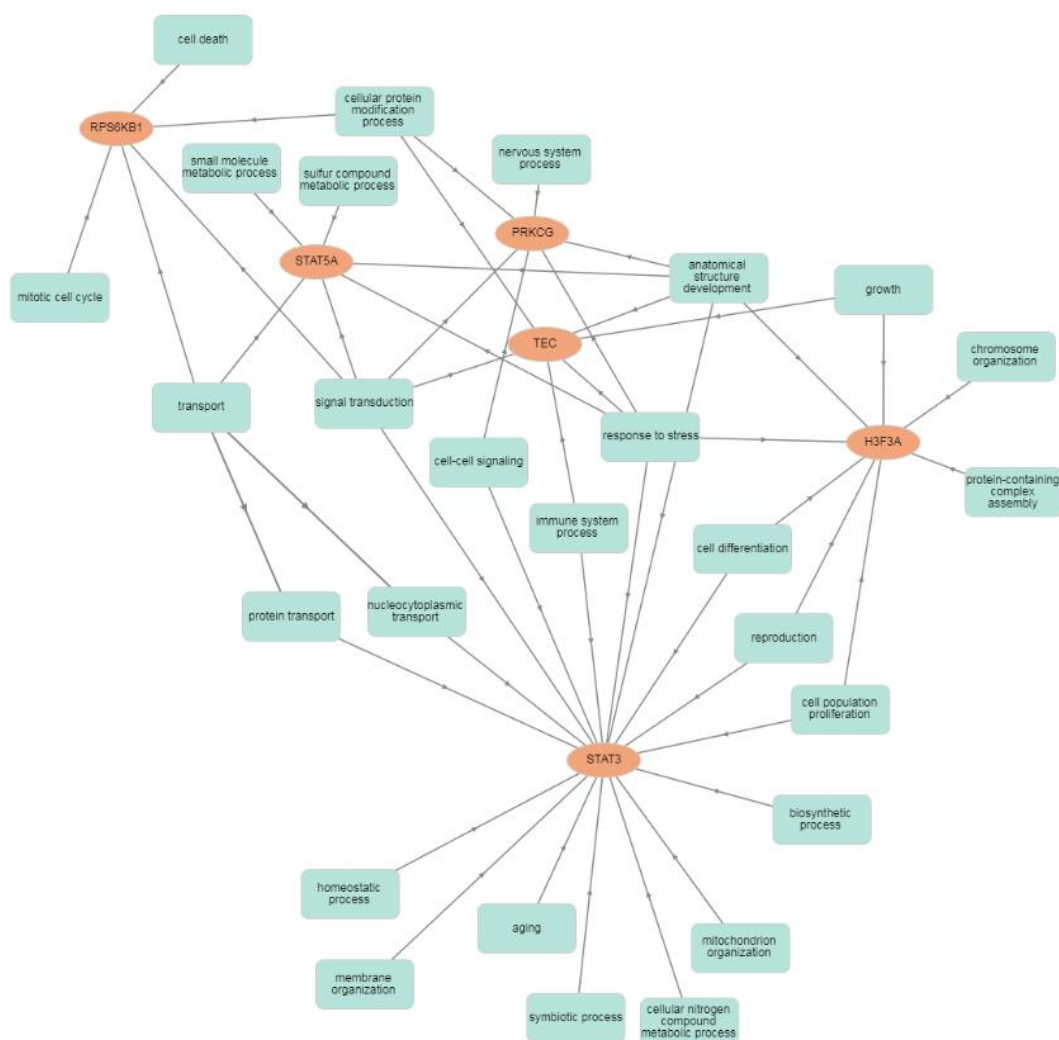


Figure C-1 Biological processes associated with host factors modulated by both 1.0mer and 1.3mer HBV. RPS6KB1, Ribosomal protein S6 kinase beta-1; STAT5A, Signal transducer and activator of transcription 5A ; PRKCG, Protein kinase C gamma type; TEC, Tyrosine-protein kinase Tec ; H3F3A, Histone H3.1; STAT3, Signal transducer and activator of transcription 3

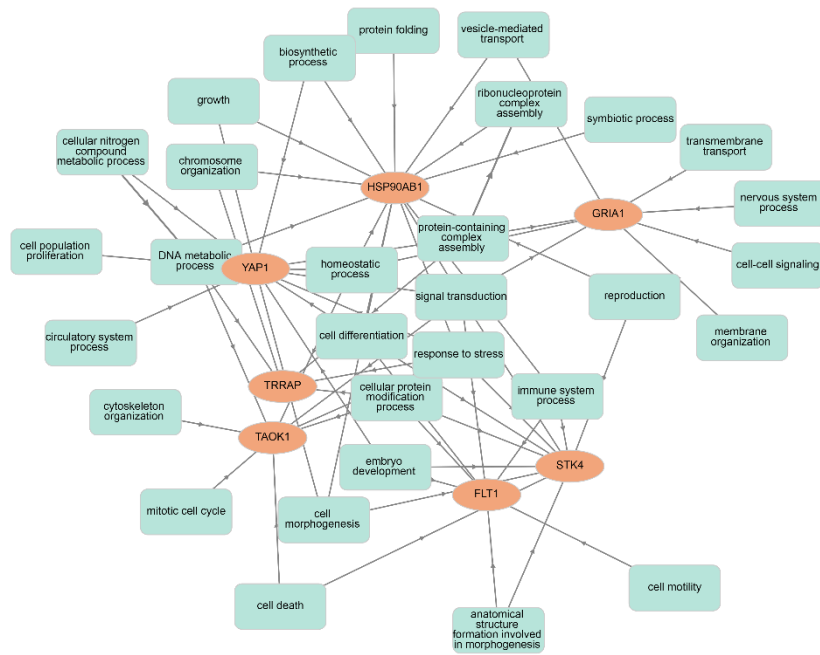


Figure C-2 Biological processes associated with candidate host factors modulated by both WT and HBxΔ 1.0mer HBV. HSP90AB1, Heat shock protein HSP 90-beta; GRIA1, Glutamate receptor 1; YAP1, Transcriptional coactivator Yes-associated protein 1; TRRAP, Transformation/transcription domain-associated protein; FLT1, Vascular endothelial growth factor receptor 1; STK4, Serine/threonine-protein kinase 4

Appendix D: 1.0mer HBV Kinexus analysis shortlisted candidates

Discussion

HSP90/MST1/YAP1

Induction of HBV replication from transfected 1.0mers modulated expression of HSP90 and MST1, both of which we previously showed were modulated by HBx and/or DDB1 overexpression. HBV replication increased HSP90 expression but decreased its phosphorylation at Y484. This induced modulation of HSP90 may be contributing to HBV replication directly, via enhanced facilitation of reverse transcription (Hu et al, 2004a; Hu & Seeger, 1996), or indirectly through broader regulation of cellular processes in order to create a permissive cell state (Geller et al, 2012). That expression was also enhanced by DDB1 overexpression might suggest a role for DDB1 in HBV replication via HSP90. Perhaps the chaperone is required for the recruitment and assembly of proteins/complex involved in conversion of rcDNA to cccDNA. Furthermore, HSP90a is abundantly expressed in HCC (Tang et al, 2020), potentially promoting tumor progression by maintaining active conformation of oncoproteins. Thus HSP90a may be implicated in HBx-mediated promotion of tumorigenesis and HCC, since HSP90a expression was enhanced following HBx activation of Ras/Raf/ERK1/2 signal cascades and proto-oncogenes (Li et al, 2010b).

HBV replication also decreased phosphorylation of MST1 at T187, although our previous screen saw increased expression by HBx over-expression. Phosphorylation of activation loop residues Thr¹⁷⁷ and Thr¹⁸⁷ are critical for kinase activity (Glantschnig et al, 2002). This could indicate that although HBx expression may upregulate expression of MST, in the context of replication, this does not necessarily correlate to increased activity. Murine Mst1 is most abundant in lymphoid tissues and functions as an important T cell regulator (Dong et al, 2009; Katagiri et al, 2009; Zhou et al, 2008). Mst1 deficiency has been described to cause immunodeficiency syndrome primarily characterised by loss of naive T cells and resulting in recurrent infections (Abdollahpour et al, 2012; Nehme et al, 2012). It could be possible that HBV-mediated reduction in phospho-activation results in a phenotype akin to that described for Mst1-deficiency, albeit in a more muted manner that could relate to the insufficient immune responses characterizing chronic HBV infections.

Furthermore, cleavage of MST1 by caspases during apoptosis results in its translocation into the nucleus, where it can induce chromatin condensation (Ura et al, 2001). In mouse liver it was demonstrated that MST1 is cleaved and constitutively activated, leading to proliferation and HCC development. This was mediated by loss of inhibitory phosphorylation of YAP1^{S127} which functions in tumour suppression (Zhou et al, 2009). Since YAP1^{T187} is critical for kinase activity (Glantschnig et al, 2002), augmenting its phosphorylation may be one mechanism by which HBV modulates its function to regulate HBx-mediated development/progression of HBV-associated HCC via MST1-related mechanisms.

Phosphorylation of transcriptional activator Yes-associated protein (YAP1) at T119 and S109 was increased following 1.0mer HBV transfection. YAP1^{T119} is phosphorylated during normal mitosis and G2/M phase arrest by CDK1 (Yang et al, 2013a), and also by JNK1 and JNK2 (Tomlinson et al, 2010) regulating UV-induced apoptosis. YAP1^{S109} is a LATS/ NDR/Warts (Zhang et al, 2015) phosphorylation site. YAP1 phosphorylation can lead to cytoplasmic retention,

inhibiting transcription activation and growth promoting functions of YAP1. Enhanced nuclear abundance and proliferative function was observed by suppression of YAP1 phosphorylation (MST1/2 deficiency) in colon cancer (Zhou et al, 2011). MST1/2 also phosphorylates YAP in liver (Zhou et al, 2009).

As part of the Hippo signalling pathway, YAP is an important regulator of liver development, repair, cell fate determination and tumorigenesis (Nguyen et al, 2015; Patel et al, 2017). YAP1 activates transcription of pro-apoptotic genes to induce apoptosis in response to DNA damage (Levy et al, 2008), but can also influence gene expression through protein-protein interactions (Nguyen et al, 2015). YAP promotes cell proliferation and chromosomal instability, and dysregulation is associated with development and poor prognosis in liver cancer (Weiler et al, 2017) (Liu-Chittenden et al, 2012). Murine studies show YAP1 over-expression increases the likelihood of developing liver cancer (Kowalik et al, 2011; Liu et al, 2010), and with HBx expression upregulating YAP in HCC cells (Wu et al, 2016), suggests a potential role in contributing to the development of HBx-mediated hepatocarcinogenesis. Thus increased phosphorylation may contribute to creating permissive cellular state for optimal viral replication and propagation and ultimately leading to promotion of HCC.

GluR1

Phosphorylation at S849 of GluR1 was also upregulated by HBV. Glutamate receptor 1 is a G-protein coupled receptor involved in signalling, whose upregulated expression was observed in HBV-infected tumorous liver. In mouse cell lines, stimulation of Grm1 activated ERK1/2 (Marin et al, 2006) and Akt (Shin et al, 2010) a mechanism potentially contributing to their transformation, as ectopic GluR1 expression was shown to be sufficient for melanoma development *in vivo* (Pollock et al, 2003). Regulation of glutamate receptor function is mediated by phosphorylation by protein kinases such as PKC and CaM-KII (Raymond et al, 1994) (Roche et al, 1996; Tan et al, 1994). PKC phosphorylates GluR1^{S849} and is important for activation, intracellular localisation and processing (Ghafari et al, 2015; Tavalin, 2008). Therefore, increased phosphorylation of S849 may be a mechanism induced by HBV leading to HCC development. Intriguingly, GluR1 modulation and potentially impaired glutamergic synaptic transmission is suggested to be a mechanism for cognitive impairment in liver disease patients, as suggested by altered phosphorylation, expression and activity of GluR1/AMPA receptors in rat liver failure models (Monfort et al, 2007).

TAO1

TAO1 (Thousand-and-one amino acid kinase 1) also functions in the Hippo signalling pathway regulating phosphorylation of MST kinases to restrict cell proliferation and tissue growth during development (Tao-1 directly phosphorylates T195 in Hippo activation loop) (Boggiano et al, 2011). TAO1 can suppress activity of YAP, and phosphorylation and activation MST2 at undefined residues (Poon et al, 2011). Given their high similarity, especially within catalytic domains (Creasy et al, 1996; Creasy & Chernoff, 1995), TAO1 might also phosphorylate the homolog MST1. Decreased TAO1 phosphorylation may result in increased activation of pro-growth YAP, a phenotype that has been observed with TAO1 deficiency, and which suggests TAO1 may be required for activation of MST1/2 (Poon et al, 2011). TAO1 is a Ste20-like kinase that regulates microtubule dynamics and is an important regulator of apoptosis and cell cycle progression (Draviam et al, 2007; Sato et al, 2007). Cells undergoing mitosis exhibit increased protein expression and kinase activity compared to those in S phase, and depletion

of TAO1 reduces the density of interphase and mitotic microtubules in human cells (Shrestha et al, 2014). Decreased phosphorylation at Y309 was observed with HBV replication, which has been known to increase in G1 and G2 phases (Ozer et al, 1996; Wang et al, 2011), so inhibiting TAO1 activity could provide a replication and survival advantage by inducing cell cycle arrest.

TRRAP

Transformation/transcription domain-associated protein, a large protein and member of ATM superfamily with homology to the PI3K family members. Found in association with HAT complexes, appears to be responsible for the recruitment of these complexes to chromatin during transcription, replication and DNA repair (Ikura et al, 2000) (Allard et al, 1999; Murr et al, 2006). Recruitment of these complexes to promoters induces histone acetylation and activates transcription of target genes, though it can also contribute to these processes in through participation in non-HAT complexes.

Recent evidence suggests HAT complexes play important roles in DNA repair, using similar mechanisms to their role in transcriptional activation (Brand et al, 2001) (Martinez et al, 2001), relaxing compacted chromatin and enabling access by DNA repair factors. 1.0mer HBV replication increased TRRAP expression, which suggests that it might be involved in repair and transcription of cccDNA, though mechanism and regulation of this would be important to distinguish as TRRAP has also been implicated in suppression of HBV. Nishitsuji and colleagues found that TIP60 complex bound to HBV promoter on cccDNA to suppress transcription, through recruitment of Brd4 to acetylated of histone H4. As a component of the complex, silencing of TRRAP resulted in increased transcription (Nishitsuji et al, 2018).

TRRAP also interacts with B-catenin. Deregulation of B-catenin degradation and the associated Wnt pathway may contribute to tumour development (Murr et al, 2007). It has been reported that expression of TRRAP was higher in HCC compared to matched non-tumour liver, and that high expression levels was associated with poor prognosis (Sheel et al, 2019) (Kwan et al, 2020). Kwan et al demonstrated CRISPR depletion of TRRAP induced senescence resulting in decreased cell growth (G2/M arrest) and colony formation in various HCC cell lines. Both TRRAP and its binding co-factor KAT5/Tip60 (Ikura et al, 2000) were required for transcriptional activation of mitotic genes (including TOP2A) and contribute to HCC cell proliferation by promoting G2/M progression. Targeting these protein interactions could functionally inhibit the HAT complex and suppress HBV replication (Gao et al, 2014; Wang et al, 2018).

VEGFR1

Vascular endothelial growth factors and their tyrosine kinase receptors are potent mediators of angiogenesis (Hoeben et al, 2004) and as such are implicated in promotion of cancer and other pathologies (Kaplan et al, 2005). VEGF has been suggested to be predictive of HCC metastatic recurrence and clinical outcomes (Qin & Tang, 2004). VEGFR1 is a tyrosine kinase expressed in vascular endothelial cells and in non-endothelial cells including hematopoietic stem cells and monocytes/macrophages (Sawano et al, 2001). In liver, VEGFR1 stimulation mediates release of growth factors, which mediates regeneration following liver injury (LeCouter et al, 2003). Serum VEGFR1 level in HCC patients was significantly higher than control (healthy) group, strong association between VEGFR1 level and advancement of cancer stage, presence of metastases and poor tumour differentiation (Kemik et al, 2010) VEGFR1 in

bone marrow was also used as a predictor of HCC recurrence (Kamiyama et al, 2012). HBx was reported to upregulate VEGFR3 (Lian et al, 2007) and VEGF (via upregulated mTOR signalling) in HCC (Yen et al, 2012). Transfection of 1.0mer HBV upregulated VEGFR1-Y1053 phosphorylation. Phosphorylation of this site and others in the VEGFR1 tyrosine kinase domain mediates its effects (Ito et al, 1998), and autophosphorylation at Y1053 (and Y1048) has been implicated in VEGFR1-VEGFR2 cross-talk for induction of VEGFR2-induced proliferative signals (Mokhdomi et al, 2016). Increase of VEGFR1 phosphorylation may dysregulate its signalling, activating and enhancing its effects to contribute to HBV-associated HCC in similar manners.

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