Development of an mRNA Vaccination Strategy for the Prevention and Treatment of HBV Infection

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Johannesburg, 2017

DECLARATION

I, Camilla Lamb, hereby declare that this dissertation is my own work. This dissertation and the work included have not been submitted for any other degree, or for examination at this or any other University.

..... (Signature)

.....Day of ....., Johannesburg, 2017

RESEARCH OUTPUT RELATED TO DISSERTATION

Publications

1. Lamb, C & Arbuthnot, PA. (2016). Activating the innate immune response to counter HBV infection. *Expert Opinion on Biological Therapy*. 16:12 pp 1517-1527

International Training

1. Masters Student Internship – Centre for Chronic Immunodeficiency (CCI) at the Universitätsklinikum Freiburg, Germany. Length of internship – 1 month. Supervisors: Dr Tatjana Cornu & Marianna Romito (PhD Candidate). Skills learned: *In vitro* mRNA Production, Nucleofection, culture of primary cells.

Intellectual Property

1. The results of this study form part of a larger research effort conducted by Prof. Patrick Arbuthnot into the use of mRNA vaccines targeted against HBV. Currently, these results are being formulated into a preliminary patent application for the generation of intellectual property involving the use of mRNA in an anti-HBV vaccine formulation.

ABSTRACT

Persistent HBV infection carries an elevated risk of developing cirrhosis and hepatocellular carcinoma (HCC). Infection is preventable by immunisation with a recombinant protein vaccine encompassing the major surface antigen (HBsAg) of Hepatitis B virus (HBV). The vaccine is globally administered resulting in a notable decrease in global carrier rates. However, some recipients (5-10%) remain as hypo- or non-responders associated with minimal to no protective antibody levels. HBV-specific DNA-vaccines have demonstrated potential for induction of potent antibody responses and target-specific activation of cellular immunity with purported application in chronic HBV infection (CHB) treatment. Furthermore, *in situ* production of HBsAg allows for inclusion of neutralising and cross-specific preS epitopes absent from the current vaccine. Immunisation with mRNA accompanies a superior safety profile and offers several other advantages over DNA, but no such vaccine targeting HBV exists. To develop an anti-HBV mRNA-based vaccine, vectors engineered for mRNA production were constructed. From the production vectors, *LHBs* (large HBsAg) and *SHBs* (small or major HBsAg) mRNA was synthesised by *in vitro* transcription using phage T7 polymerase, and translated in transfected cells to produce detectable LHBs and SHBs. SHBs was readily secreted while LHBs was retained intracellularly. This investigation constitutes a preliminary step towards the preclinical development of an anti-HBV mRNA-based prophylactic and therapeutic vaccine formulation and has provided proof-of-principle that LHBs encompassing preS epitopes is expressible *in situ* from synthetic mRNA transcripts. Further developments will include modification of the mRNA synthesis protocol to comply with GMP regulations and functional testing of the vaccine candidate in appropriate animal models.

Keywords: *HBV, HCC, HBsAg, preS, DNA-vaccines, CHB, mRNA-based vaccine*

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LIST OF ABBREVIATIONS

aa	Amino acid
AGL	Antigenic loop
APC	Antigen presenting cell
BSA	Bovine serum albumin
cAg	Core antigen
cccDNA	Covalently closed circular DNA
CHB	Chronic HBV infection
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMXAA	5,6-dimethylxanthenone-4-acetic acid
DNA	Deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein
eIF2α	α-Complex of elongation initiation factor 2
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Fetal calf serum
GMP	Good manufacturing practice
HBsAg	Hepatitis B surface antigen
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B 'e' antigen

HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IFN-α	Interferon-alpha
IL	Interleukin
IP-10	IFN-gamma-induced protein 10
IRF3	Interferon regulatory factor 3
ISG	Interferon stimulated gene
kb	Kilobases
kDa	Kilodaltons
LB	Luria Bertani
LHBs	Large surface antigen
LTβR	Lymphotoxin β receptor
MCS	Multiple cloning site
MDA5	Melanoma differentiation-associated protein 5
MHBs	Middle surface antigen
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NA	Nucleic acid
NF-$_K$B	Nuclear factor $_K$-light-chain-enhancer
NIC	No-insert control
NK	Natural killer (cells)
NKT	Natural killer T (cells)

nm	Nanometre
NPC	Non-parenchymal cell
NTCP	Sodium-taurocholate co-transporting peptide
OVN	Overnight
ORF	Open reading frame
P	Polymerase
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PD-1	Programed death protein 1
pDC	Plasmacytoid dendritic cell
pDNA	Plasmid DNA
PFA	Paraformaldehyde
PHH	Primary human hepatocyte
PKR	RNA-dependent protein kinase
PM	Plasma membrane
PRR	Pattern recognition receptor
pgRNA	Pregenomic RNA
rcDNA	Relaxed circular DNA
RIG-I	Retinoic-acid inducible gene-I
rNTP	Ribonucleotide triphosphate
RT	Room temperature
sAg	Surface antigen
SHBs	Small surface antigen

SMC	Structural maintenance of chromosomes
SVP	Subviral particle
T	Temperature
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TM	Transmembrane
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
UTR	Untranslated region
VLPs	Virus-like particles
VLVs	Virus-like vesicles
WHV	Woodchuck Hepatitis Virus
ZIKV	Zika virus

1. INTRODUCTION

1.1 Molecular Virology of HBV

HBV is a relatively small, enveloped, non-cytopathic virus with a partially double-stranded, relaxed circular DNA (rcDNA) genome of approximately 3.2 kilobases (kb) (Summers *et. al.*, 1975). HBV was first visualised as a 42-nm viral particle by electron microscopy, sometimes referred to as the Dane particle (Dane *et. al.*, 1970). Dane particles consist of a nucleocapsid core, housing the genomic material and viral polymerase, enveloped in a host-derived lipid envelope embedded with surface glycoproteins. The genome is packaged into an icosahedral nucleocapsid (Seeger & Mason, 2000) with a triangulation number T=4 or T=3 (reviewed in: Steven *et. al.*, 2005). The rcDNA genome has a negative strand, the coding strand, and a non-coding positive strand of incomplete length. The HBV genome is produced from a pregenomic RNA (pgRNA) replicative intermediate by reverse transcription during capsid assembly and maturation. Reverse transcription is the function of a viral-derived polymerase (P) co-encapsidated with the pgRNA template during virion assembly. Within infected cells, the rcDNA is translocated to the nucleus where it is converted to a highly stable, episomal covalently closed circular DNA (cccDNA) minichromosome (reviewed in: Seeger & Mason, 2000).

The cccDNA serves as the transcriptional template of HBV and encodes the structural and non-structural proteins essential for the completion of the replication-cycle. Viral proteins are produced from four overlapping open reading frames (ORFs) that complete the HBV genome (Figure 1). Unidirectional transcription of the cccDNA template yields four viral mRNAs which are subsequently translated into seven viral proteins (Moolla *et. al.*, 2002). All mRNA transcripts

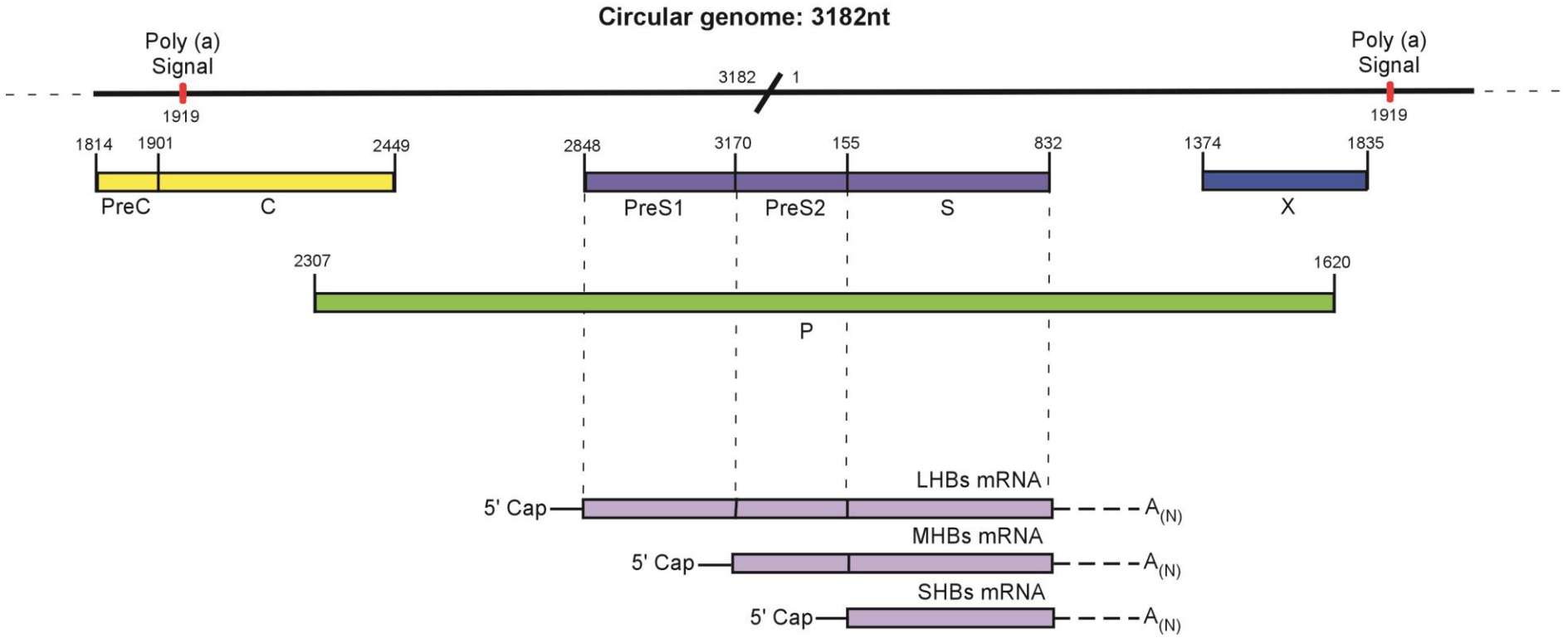


Figure 1: Organisation of the HBV genome and transcription products of the HBV surface antigen (*PreS1/PreS2/S*) gene. Schematic of the linearised genome, the genome is normally circular. The four ORFs of the HBV genome are the *Core*, *Surface*, *X* and *P*. All four mRNA transcripts share a polyadenylation sequence at the 3' end (indicated in red). The *S* ORF is transcribed into the 2.1kb and 2.4kb transcripts encoding the major (S) HBs and large (L) HBs proteins respectively. Differential processing of the 2.4kb transcript during mRNA maturation forms the mRNA for the middle (M) HBs protein.

terminate at a polyadenylation signal located at the shared 3' end (Figure 1). A recombination event in the nucleus can sometimes result in the random integration of HBV genomic DNA into host chromosomes (Wang & Rogler, 1991; Sung *et. al.*, 2012). Episomal persistence of cccDNA or integrated HBV genomic elements within an infected liver establishes chronic infection and the consequent continual production of HBV antigens acts to drive the immunotolerant phenotype.

The four mRNA transcripts produced from cccDNA include three subgenomic transcripts of 2.1kb, 2.4kb and 0.9kb, translated into the surface antigen (HBsAg) glycoproteins (2.1kb and 2.4kb) (Figure 2a) and HBx protein (0.9kb). The surface antigen glycoproteins occur in three forms: small surface antigen (SHBs), middle surface antigen (MHBs) or large surface antigen (SHBs). The surface glycoproteins occur at varying frequencies within the HBV envelope and can undergo post-translational glycosylation or myristoylation with effects on protein function (see Section 1.2.1). Spherical and filamentous subviral particles (SVPs) noted alongside the Dane particles in electron micrographs have been determined to be secreted particulate matter of HBsAg (Dane *et. al.*, 1970). The SVPs are non-infectious and form the basis of clinical detection and disease monitoring in HBV-infected patients.

The second class of mRNA transcript produced is the greater-than-genome-length pgRNA transcript which functions as the genomic template during viral replication and is translated into the core (HBcAg), precore (HBeAg) and HBV polymerase (P) proteins (Nassal *et. al.*, 1990). The 25-kDa HBeAg precursor protein or precore protein (Ganem, 1996; Seeger & Mason, 2000) is processed in the endoplasmic reticulum (ER) to give the secreted HBeAg of 17kDa (Ou, 1997). Detection of HBeAg is used as a diagnostic marker for high infectivity (Hollinger, 1996). HBcAg and HBeAg are both variants of the core protein where HBcAg

contributes to nucleocapsid assembly and HBeAg is readily secreted with a proposed function in promoting immune tolerance (Chen *et. al.*, 2004). P initiates reverse transcription via interactions with the pgRNA and covalently associates with the negative strand (Pollack & Ganem, 1993). Once within the nucleocapsid, P reverse transcribes the pgRNA *in situ* to give the rcDNA form of the genome.

The function of the HBx protein in the viral replication-cycle is not well defined and remains a topic of debate. Recently it was determined that HBx binds structural maintenance of chromosome (SMC) complex proteins 5/6 (SMC5/6) targeting them for ubiquitination and subsequent proteosomal degradation (Murphy *et. al.*, 2016). SMC5/6 proteins recognise and bind extrachromosomal DNA with inhibitory effect on gene expression. Degradation of SMC5/6 results in their inability to bind cccDNA and prevent the expression of HBV proteins. HBx may indirectly regulate HBV replication by this mechanism.

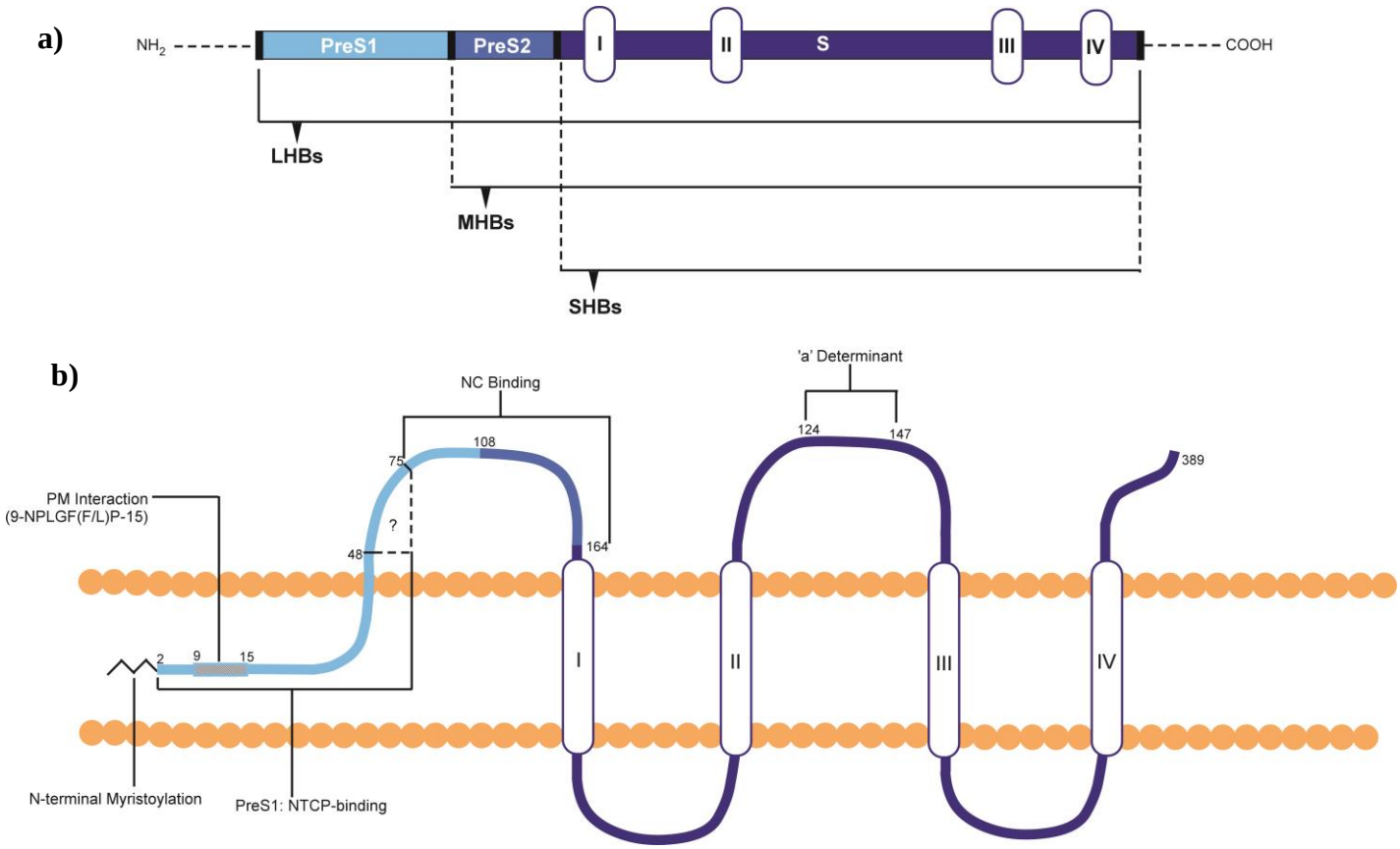


Figure 2: Translation products of the HBV surface antigen (*preS1/preS2/S*) gene. **a)** Domains of the LHBs protein. LHBs protein includes preS1/preS2/S protein domains, MHBs includes only preS2/S and SHBs or major HBsAg encompasses only the S protein region. The roman numerals indicate the placement and numbered identity of the putative transmembrane domains in the shared S domain. **b)** Functional organisation of the LHBs protein. The myristoylated N-terminal domain is required for PM-association. PreS1 (aa 2-48) mediates NTCP-binding with a conserved motif (9-NPLGF(F/L)P-15) playing a crucial role in entry receptor association. The NC-binding region (aa 75-164) of preS1/preS2 functions in envelopment of the virion. TM helix I contains 3 (φ) hydrophobic clusters implicated in membrane fusion. The 'a' determinant within the AGL between TM II and III is a primary determinant of infectivity and is structurally maintained by disulphide bridge formation (not shown).

1.1.1 Surface Antigens of HBV

The LHBs, MHBs and SHBs proteins have distinct and diverse functions in the completion of the HBV replication-cycle and the propagation of infection. All three isoforms have a common C-terminal domain (S domain) which is responsible for embedding the protein into the viral envelope via the four putative transmembrane helical domains (Figure 2b). The preS1 and preS2 domains are N-terminal extensions of the major HBsAg (SHBs). PreS1 and preS2 function in mediating specific interactions with the nucleocapsid during envelopment and virion release (Nassal, 1996) as well as entry receptor-binding (Le Seyec, *et. al.*, 1999; Glebe, *et. al.*, 2003; Barrera *et. al.*, 2005). Only recently, Yan and colleagues identified the sodium taurocholate co-transporting polypeptide (NTCP), a hepatocyte-specific bile salt transporter, as being the entry receptor for HBV and HDV (Yan *et. al.*, 2012). A highly conserved, 75-amino acid (aa) region (aa 157-165; Figure 2b) in the preS1 domain of LHBs mediates virion binding to the NTCP entry receptor. Differing lengths of the preS-binding regions in MHBs and LHBs impart different affinities to NTCP indicating that the entire 75-aa region of preS1 (Figure 2b) may also participate in NTCP association (reviewed in: Urban, *et. al.*, 2014).

Initially, LHBs is internalised within the viral particle and is therefore not available for receptor binding. Myristoylation of the N-terminus of LHBs (Persing *et. al.*, 1987) results in subsequent post-translational translocation (Lambert & Prange, 2001) exposing the preS-binding domains on the external surface of the mature virion. N-terminal myristoylation, therefore, is a crucial determinant of HBV infectivity (Gripon *et. al.*, 1995; Bruss *et. al.*, 1996). Le Seyec and colleagues mapped functionally important sites within the preS domains in HBV subgenotype D (Le Seyec *et. al.*, 1998). They found that mutations within the first 77 amino acids of LHBs

completely blocked infectivity while mutations within the preS2 domain and preS1 (aa 114-163) disrupted capsid assembly and could not be conclusively classified in terms of infectivity (Figure 2b). The generation of broadly neutralizing antibodies against LHBs alone could potentially eliminate binding to surface entry receptors. In this study, LHBs and SHBs proteins were evaluated further.

Other determinants of infectivity include the antigenic loop (AGL) protein region located between TM-I and TM-II of the S domain in all three forms of HBsAg (Figure 2b). An alanine-scanning mutagenesis approach was used by Salisse and Sureau to map a set of highly conserved residues within the AGL that are critical for infection, namely the 'a' determinant (Salisse & Sureau, 2009), the first discovered marker of HBV infection (Blumberg, 2002). The conformation of the AGL, as dictated by disulphide bridge formation between Cys-121 and Cys-149 (Figure 2b), is a determinant of the structure of the 'a' determinant (Abou-Jaoudé & Sureau, 2007). This sequence is currently the most effective neutralisation epitope included in the broadly administered anti-HBV vaccine (Zanetti *et. al.*, 2008). Disruption of the disulphide bridge conformation by membrane-impermeable reducing agents negated infectivity (Abou-Jaoudé & Sureau, 2007).

Upon gaining entry into a cell and becoming exposed to the reducing cytoplasmic environment, dissolution of the disulphide bridges of the 'a' determinant may function in a post-binding stage of infection. A similar mechanism has been proposed for the infection pathway of duck HBV (Grgacic & Schaller, 2000). The structure of the disulphide bridges in duck HBV maintains the HBsAg in a so-called pre-fusion state (Grgacic & Schaller, 2000). A highly hydrophobic protein domain comprising 3 hydrophobic (φ) clusters embedded in the TM-I domain of LHBs in duck HBV is crucial for the fusion of viral and cellular membranes during

infection (Chojnacki *et. al.*, 2005). A similar region has been found in human HBV (Lepère-Douard *et. al.*, 2009) though the exact mechanism of membrane fusion in human infection remains to be completely characterised. The φ clusters role in infectivity, though, appears restricted to the LHBs despite being found in all three isoforms.

In summation, the determinants of HBV infectivity are confined to the surface antigen proteins and include (i) crucial sequence regions in PreS1, (ii) N-terminal myristoylation of LHBs, (iii) φ clusters of LHBs, (iv) the AGL region and (v) the 'a' determinant within the AGL. Compromising the determinants of infectivity could serve a prophylactic and therapeutic purpose. It is reasonable to assume that anti-preS1 antibodies, therefore, would be neutralizing and highly protective. Evidence in support of this approach stems from other studies wherein direct interference of receptor binding by a synthetic preS1-derived lipopeptide effectively blocked HBV infection (reviewed in: Urban *et. al.*, 2014). The synthetic peptide, Myrcludex B, is currently being evaluated in phase 2 clinical trials.

1.2 HBV Infection

Historically, hepatitis has been a recognised clinical condition for many centuries, typically presenting as jaundice. An outbreak of hepatitis in 1883 in Germany following a national smallpox immunization campaign marked the first documented cases of a parenterally transmissible form of the condition (Lurman, 1885). It was only in the 1940's that the viral aetiology of hepatitis was first described identifying two main routes of transmission, faecal-oral or through exposure to contaminated blood products (reviewed in: Van Damme *et. al.*, 2013). Faecal-orally transmitted hepatitis is now universally referred to as Hepatitis A infection while

parenteral infection is now accepted as being caused by Hepatitis B virus (HBV). An important landmark was reached in HBV research when, in 1965, Blumberg and colleagues accidentally isolated the surface antigen, or so-called 'Australian antigen', of HBV (HBsAg) by isoprecipitation (Blumberg, 2002).

HBV is the prototypical member of the family *hepadnaviridae* consisting of hepatotropic viruses with a relatively narrow range of host specificity. HBV causes significant liver injury in primates and humans while other *hepadnaviridae* infect avian or rodent hosts (Seeger and Mason, 2000). Such a select host range often complicates HBV experimentation and the development of novel therapeutics because of limited access to animal models representative of the human disease. There are many questions regarding HBV immunopathology and disease progression that remain to be elaborated. Despite the associated challenges, global efforts have deepened the understanding of natural HBV history, aetiology, epidemiology and molecular biology all with the aim of eventual eradication of the virus from the human population.

1.2.1 Immunopathology of Acute and Chronic HBV Infection

HBV is one of five distinct viral agents known to cause hepatitis in humans and HBV infection is recognised as being responsible for the majority of the global hepatitis disease burden. Usually, acute infections are spontaneously resolved by the host adaptive and innate immune responses. However, approximately 5% of infected adults (~ 390 million people) fail to clear viral replicates resulting in the establishment of chronic infection (Stanaway *et. al.*, 2016). Age is a contributing factor and if exposed to HBV within the first year of life, 90% of infants progress from acute to chronic infection. The risk decreases if exposure occurs between one and

four years of age where approximately 30% of children develop symptoms of chronic disease (Stanaway *et. al.*, 2016).

Chronic HBV infection (CHB) is responsible for around 600 000 deaths annually because of an increased risk of infected individuals developing hepatocellular carcinoma (HCC) and cirrhosis (Stanaway *et. al.*, 2016). An estimated 25% of CHB patients develop HCC which coincides statistically with HBV being responsible for as much as 80% of the worldwide occurrence of liver cancers. HBV, therefore, is attributed as being the world's second most important carcinogen after tobacco (Lok & MacMahon, 2009). CHB sufferers coinfected or superinfected with the satellite virus Hepatitis D virus (HDV) are at risk of an accelerated progression of serious disease consequences because HDV uses HBV envelope proteins for dissemination of viral progeny (Rizzetto, 2013). CHB is considered to be a significant challenge to the global health status and has been the focus of extensive research initiatives aimed at developing control and prevention strategies.

Acute HBV infection is usually spontaneously resolved by the host's immune system and natural elimination of the virus involves both cellular and humoral immune responses (reviewed in: Maini *et. al.*, 2014). HBV infection has an extended incubation period of 6-8 weeks during which the virus is undetectable. Clinically, patients will only develop symptoms 10-12 weeks post-infection, if symptoms develop at all (Rehermann & Nascimbeni, 2005). Thus, a critical phase of initial adaptive and innate immune responses remains incompletely understood because, upon diagnosis, the critical window for studying these responses has been lost. Information regarding the immune component of initial viral replication has had to be derived in hindsight from data generated in chronically-infected patients and animal models of CHB.

HBV is a non-cytopathic virus and innate immune responses appear to be very limited during the initial phases of infection (Schlomai *et. al.*, 2014; Sato *et. al.*, 2015). In a chimpanzee model of acute infection there is no measurable type I IFN response (Wieland *et. al.*, 2004) which corroborates observations made in human patients (Dunn *et. al.*, 2009). Two hypotheses exist to explain the possible means by which critical innate immune function is undetected in HBV hosts: (i) the 'stealth virus' hypothesis and (ii) the virus-derived suppression hypothesis. The 'stealth virus' hypothesis states that HBV can avoid innate immune detection completely. During initial phases of replication, cccDNA is confined to the nucleus and viral genomic material is shielded by the nucleocapsid within the cytosol hypothetically rendering it unavailable for binding to innate pattern recognition receptors (PRRs). Though there is no measurable type I IFN response, there is a detectable peak in natural killer (NK) and natural killer T (NKT) cell populations preceding the logarithmic phase of replication (Fisicaro *et. al.*, 2009). HBV is, therefore, detected by the innate immune system but does not stimulate a typical antiviral response. Hypothesis (ii) seems more plausible and HBV-derived proteins have been shown to interfere directly with type I IFN production and secretion (Bertoletti & Ferrari, 2011).

Adaptive immune effector function specific for HBV is likely a crucial element of self-limiting infection. A population of polyfunctional, HBV-specific T lymphocytes present in the pre-logarithmic phase of replication appears to correlate with resolution of infection (Asabe *et. al.*, 2009). CD-4+ T cells with HBV-antigen specificity prime CD-8+ T cells and promote their survival. CD-8+ T cells can direct non-cytolytic clearance of HBV through the production of tumour necrosis factor-α (TNF-α) and IFN-γ. There is a complex interplay between CD-4+ and CD-8+ T cells that induces robust T_H1 responses. Both cell types play critical roles in affecting viral clearance. CD-4+ T-cell depletion studies in chimpanzee models resulted in CD-8+ cell

dysfunction and failure to clear the infectious HBV (Asabe *et. al.*, 2009). At peak viraemia, depletion of CD-8+ cells in chimpanzee models eliminated the capacity of the host's immune system to resolve the acute infection (Thimme *et. al.*, 2003). HBV-specific CD-4+ cells can also induce humoral immune responses resulting in the production and secretion of anti-HBV antibodies by antigen-specific B cells.

Failure of the host's immune defenses to clear HBV during acute infection ultimately results in viral persistence and the establishment of chronic infection. Chronic HBV infection is progressive and patients are found to undergo five distinct phases of disease as summarized by Spearman and colleagues in 2013 (Spearman *et. al.*, 2013). In the first phase, or phase of immune tolerance, viral replication occurs at high rates, seemingly unhindered by the host immune system. During the phase of immune clearance (second phase), viral replication slows but the disease complications often increase in severity as a response to elevated levels of cytotoxic immune activity within the liver. In patients infected in adulthood, HBeAg seroconversion during the second phase of chronic infection allowing progression to phase three where patients live as inactive carriers of the virus. Instead of HBeAg seroconversion, induced changes in the viral genome may result in limited production of HBeAg in which case the patient appears to be in phase three but is in fact suffering from HBeAg-negative chronicity, with a much higher associated incidence of cirrhosis and HCC. Patients in phase two or phase three are at risk of reverting back to active HBV replication. Finally, patients can suffer from occult HBV infection (phase 5) in which there is no detectable HBsAg in the serum but HBV genomic DNA is still present.

Some evidence suggests that HBV may play an active role in the suppression of innate and adaptive effector functioning to prevent elimination of the virus from the infected host. A

suggested mechanism of HBV-induced immune suppression is the attenuation of Toll-like receptor (TLR) signalling (Wu *et. al.,* 2009). TLRs are a highly conserved family of PRRs that sense pathogen-associated molecular patterns (PAMPs) resulting in a signal transduction cascade to increase production of pro-inflammatory cytokines and type I IFNs, and upregulate co-stimulatory molecules on the surface of innate immune cells (reviewed in: Akira *et. al.*, 2006). Studies performed *ex vivo* in cultured murine hepatocytes of parenchymal and non-parenchymal origin (NPCs) revealed that TLR signalling by NPCs was abrogated in the presence of HBV virions, HBsAg or HBeAg (Wu *et. al.*, 2009). The HBV-derived proteins were found to downregulate nuclear factor $_{K}$-light-chain-enhancer (NF-$_{K}$B) of activated B cells and effector proteins of the MAPK pathway (Wu *et. al.,* 2009), both downstream signalling molecules of TLR activation.

The retinoic-acid inducible gene-I (RIG-I)/melanoma differentiation-associated protein 5 (MDA5) signalling axis permits the cytosolic detection of viral RNA and subsequent innate immune activation. HBsAg and HBeAg (Wu *et. al.*, 2009) as well as HBV polymerase inhibit interferon regulatory factor-3 (IRF3) activation disrupting the RIG-I signal transduction cascade in murine or primary human hepatocytes (PHH) respectively (Yu *et. al.*, 2010). A recent study provided evidence that HBV may have a direct inhibitory effect on the production and function of IFN-α (Lütgehetmann *et. al.*, 2011) which may explain the poor effectivity of IFN-α treatment in many CHB sufferers. The evidence generated to explain the suppression of innate immune function during progression to CHB is compelling; however, the data are derived from artificial models of infection and remain to be validated *in situ*. Lack of availability of an appropriate animal model of HBV immunopathology renders many conclusions made on the progression of

HBV infection to a chronic state theoretical, but a direct inhibitory role of HBV-derived proteins on innate immune function would seem plausible.

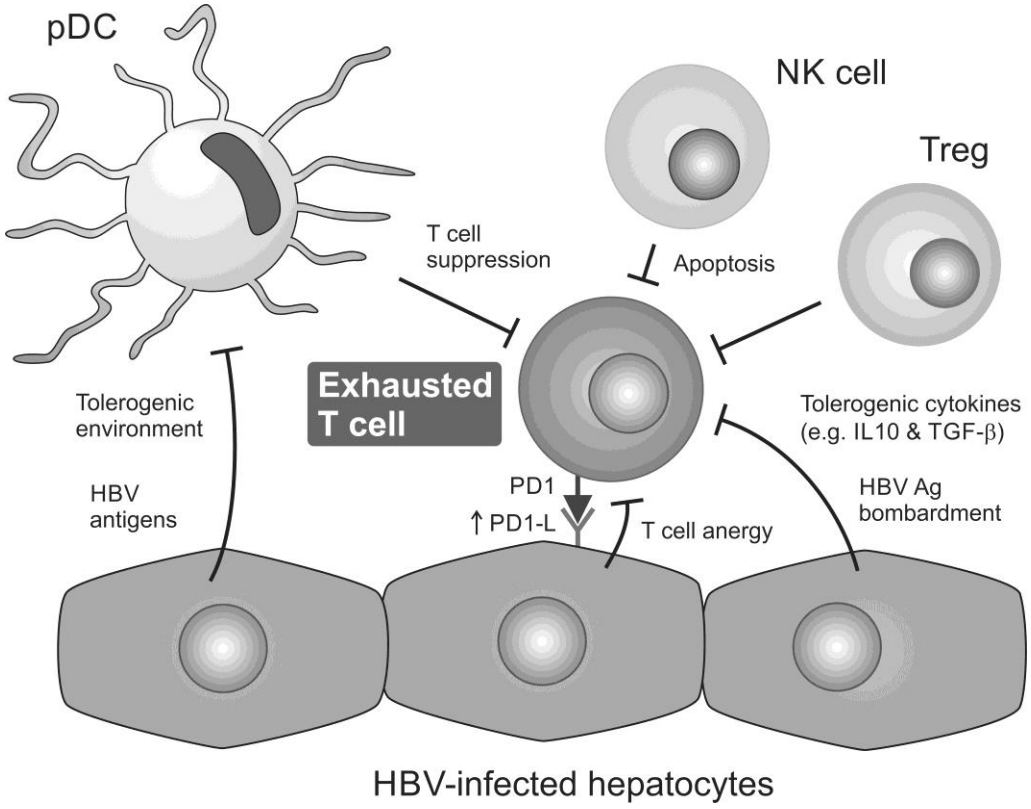


Figure 3: Suppression of HBV-specific immunity in the chronically-infected liver. CHB patients are unable to resolve HBV infection partially because of a tolerogenic hepatic milieu and because of an exhausted T-cell phenotype. Unabated antigen exposure with the absence of appropriate activating secondary signals causes T-cell anergy. NK cells can deplete HBV-specific T-cell populations through activation of apoptotic pathways. HBV can suppress T-cell functioning by upregulating the expression of the co-inhibitory signalling molecules on the cell surface resulting in their suppression rather than activation when encountering MHC-II restricted HBV-derived peptides. Chronic HBV infection results in the upregulated production of

immunosuppressive cytokines IL10 and TGF-β to promote immune tolerance rather than activation. Though incompletely understood, there may be a role for the induction of Tregs in the establishment and maintenance of chronic HBV infection. Image has been adapted with permission from: Lamb, C. and Arbuthnot, P., 2016. Activating the innate immune response to counter chronic hepatitis B virus infection. *Expert Opinion on Biological Therapy*, *16*(12), pp.1517-1527.

1.2.2 Immunological Milieu of the Chronically-Infected Liver

Resolution of acute HBV infection requires robust cellular and humoral immune responses in which CD-4+ and CD-8+ cells play critical roles in the elimination of viral particles and infected hepatocytes (Guidotti *et. al.*, 2015). CHB induces a tolerogenic hepatic microenvironment characteristic of an exhausted T-cell phenotype (Isogawa *et. al.*, 2013). Following resolution of acute infection, CD-8+ T cells form populations of highly functional, memory T cells with properties that distinguish them from their antigen-naïve, memory-like T cell counterparts. Properties of memory CD-8+ T cells include (i) rapid re-activation upon antigen re-encounter, (ii) high proliferative potential upon re-activation, (iii) homing to secondary lymphoid tissues and (iv) prolonged persistence through the homeostatic maintenance of population numbers by interleukin 7 (IL-7)- and IL-15-driven proliferation (Virgin *et. al.*, 2009).

In CHB, high viral replication rates and unabated antigen exposure results in an exhausted CD-8+ T cell phenotype characterised by the loss of IFN-γ production in response to antigenic stimulation (Guidotti *et. al.*, 2015) (Figure 3; Lamb & Arbuthnot, 2016). Exhausted memory CD-8+ T cells no longer produce IL-2, lose their high proliferative capacity and lose their killing

capacity during early stages of the conversion to the exhausted phenotype (Wherry, 2011). As the cells become more dysfunctional they lose the ability to produce TNF-α and severe exhaustion results in complete loss of IFN-γ production and cytotoxic functioning. The terminal stage of T-cell exhaustion is the physical loss of existing antigen-specific T-cell populations. CD-4+ T cell populations are also affected during chronic viral infection (Brooks *et. al.*, 2005) albeit that this process is far less well understood than the effect of chronic infection on CD-8+ cells. It is likely that CD-4 cell dysfunction can result in decreased IL-21R production, a cytokine that helps maintain antiviral CD-8+ populations (Fröhlich *et. al.*, 2009) implying a synergistic loss of adaptive immune control. Elevated IL-10 levels may also be explained by CD-4+ T cell dysfunction (Brooks *et. al.*, 2006). The more exact role of dysfunctional CD-4+ T cells on the mediation of CD-8+ T cell effector function and viral persistence remains an important topic of investigation.

In CHB specifically, several viral and host-derived factors contribute to the establishment of exhausted HBV-specific T cells and diminished anti-HBV immunity. Constant exposure to HBsAg, HBcAg and HBeAg without the appropriate secondary signals for T-cell activation may cause T-cell anergy in CHB patients (Reignat *et. al.*, 2002; Chen *et. al.*, 2005). Elevated levels of IL-10 and transforming growth factor-beta (TGF-β) in the chronically-infected liver promote tolerance rather than immune activation against HBV. Upregulation of the inhibitory programed death protein 1 (PD-1) on hepatocytes, increased production of cytotoxic T-lymphocyte antigen 4 (CTLA-4), T-cell immunoglobulin and mucin domain 3 (TIM-3), all act to supress immune activity and also promote tolerance (Peppa *et. al.*, 2010; Guidotti *et. al.*, 2015). Furthermore, IFN-γ-deficient NK cells and regulatory T cells (Tregs) all contribute to a loss of CD-8+ T-cell responsiveness to HBV antigens (Isogawa *et. al.*, 2013) (Figure 3). Strategies to restore proper

immune function in CHB using immunotherapeutic agents have become a popular avenue of investigation for HBV therapy development. Many advances have been made in the attempt to reactivate innate immune functioning to augment protective and therapeutic adaptive immune responses (reviewed in: Lamb & Arbuthnot, 2016).

1.2.3 Current HBV Immunotherapy

Therapy options available to CHB sufferers are limited in effectiveness and often fail to eliminate HBV-infection. These treatments do not prevent but rather prolong the eventual onset of severe liver disease complications. First line therapies include treatment with one of five clinically approved nucleoside or nucleotide (NUC) analogues which inhibit activity of the HBV polymerase; alternatively, patients receive a 48-week course of PEGylated interferon-alpha (IFN-α) (Pol & Lampertico, 2012). IFN-based therapeutic strategies have been applied in an attempt to reactivate immune effector functions within the microenvironment of the chronically infected liver. These strategies are found to be effective in only a small percentage of patients and flares in liver disease upon cessation of the therapy occur commonly. IFN-based therapies remain relatively ineffective even when administered in combination with NUC analogues and are accompanied by significant cost and risk of severe side-effects (reviewed in: Rehermann & Bertoletti, 2015). First line therapies are able to supress viral load as monitored by anti-HBsAg seroconversion, and in doing so reduce the risk of complicating disease (Hosaka *et. al.*, 2013). However, seroconversion alone is not indicative of curative therapy. Disease resurgence is common and is likely to occur because of the persistence of cccDNA in infected hepatocytes during non-replicative phases of infection (Zoulim & Locarnini, 2009).

The field of anti-HBV therapy development is extensive and covers a broad range of therapeutic strategies including gene therapy. The use of RNA interference technology (RNAi) (reviewed in: Ivacik *et. al.*, 2011) or gene editing technologies such as TALENs (transcription activator-like effector nucleases) (Bloom *et. al.*, 2013) to disrupt viral gene expression and degrade viral genomic material respectively have become popular means of HBV genome silencing. Other strategies involve immunotherapy and the activation of immune responses (innate and adaptive) with the goal of eliminating HBV from an infected patient. The use of exogenous activators of the innate immune system has gained popularity in recent years as well as the possibility for therapeutic vaccination against HBV.

An ideal anti-HBV immunotherapy would ensure the synergistic actions of the innate and adaptive arms of the immune system to eradicate cccDNA and prevent *de novo* infection of hepatocytes by circulating viral particles. Curative therapy would result in (i) seroclearance, (ii) durable antiviral effect eliminating the need for life-long, chronic medication, (iii) restoration of liver health and function as measured by the normalization of liver markers (eg. serum alanine aminotransferase (ALT)) and (iv) no risk of disease resurgence post-treatment. Novel immunotherapeutics, and potentially therapeutic vaccination strategies, are necessary and should be developed with these goals as key focal points.

Delivery of recombinant, exogenous immune-activating cytokines and chemokines would seem to be a relatively easy way to replace those immunogenic molecules absent in the immunological milieu of chronic HBV infection. Though TNF-α and IFN-γ are critical cytokines for non-cytolytic control of HBV replication, the administration of cytokines to patients could result in lethal systemic inflammatory responses severely limiting their clinical application (Guidotti *et. al.*, 1994; McClary *et. al.*, 2000). IFN-α exerts its effects by destabilizing cccDNA

(Belloni *et. al.*, 2012; Lucifora *et. al.*, 2014), mediating the innate immune system to activate adaptive responses and inhibiting viral gene expression (Rang *et. al.*, 1999; Wieland *et. al.*, 2000; Pasquetto *et. al.*, 2002). IFN-α has been approved for human use and has been extensively evaluated as a possible immunotherapeutic. Patient data suggest that IFN-α is curative in only a very small number of patients and the potential for dangerous side-effects from high doses is substantial (Perrillo, 2005).

Despite the challenges faced when developing novel immunotherapeutics for HBV, the idea that activators of the innate arm of the immune system may be curative is encouraging. Artificial agonists of TLR7 and 8 have shown great promise as novel therapies. A small molecule, GS9620, developed by Gilead Sciences is an orally administered TLR7 agonist which stimulates the production of IFN-α by plasmacytoid dendritic cells (pDCs) (Lanford *et. al.*, 2013). By stimulating *in situ* IFN-α production, the cytokine levels are under the control of endogenous regulatory mechanisms (reviewed in: Swiecki & Colonna, 2015) and are therefore unlikely to reach levels that are harmful to the recipient. Activated pDCs provide the nexus for the innate and adaptive arms of the immune system. Activated pDCs can then activate NK cells and CD-8+ cells by antigen cross-presentation (Figure 4a). In mice and cynomolgus monkeys, GS9620 upregulated the expression of interferon stimulated genes (ISGs) and promoted a type I IFN response (Fosdick *et.al.*, 2014). In humans, GS9620 elevated levels of IFN-gamma-induced protein 10 (IP-10) localised to the liver (Gane *et. al.*, 2015). Short term administration to chimpanzees (Lanford *et. al.*, 2013; Fosdick *et. al.*, 2014) and woodchucks (Menne *et. al.*, 2015) correlated with suppression of HBV (or Woodchuck Hepatitis Virus, WHV) replication markers, reduction of cccDNA and viral mRNA. Also noted was a marked decrease in the risk of developing HCC indicating great therapeutic potential in humans. However, chimpanzees and

woodchucks were administered low dosages when compared with those needed for efficacy in human patients, which may result in unforeseen side effects during clinical testing.

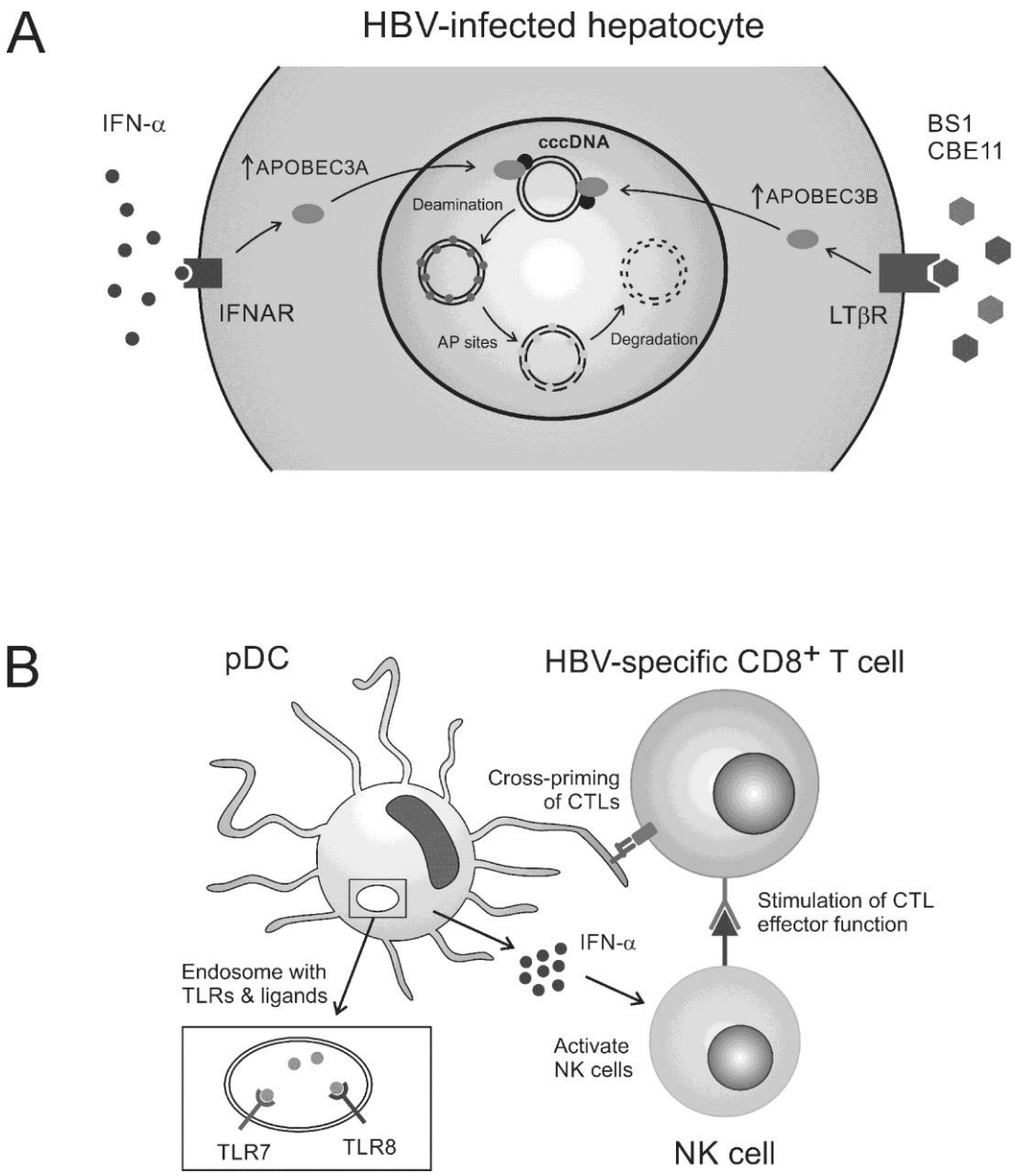


Figure 4: Hypothetical mechanisms of action for HBV immunotherapeutic compounds. **A)** IFN-α and LTβR agonists. IFN-α activates APOBEC3A and BS1 or CBE11 activates APOBEC3A

expression. APOBEC proteins bind HBcAg and are directed to cccDNA where they deaminate cytidine nucleotides resulting in the generation of apyrinic/apyrimidinic (AP) sites. AP nucleases act at AP sites to cause specific, non-cytolytic degradation of the HBV replication template. **B)** TLR7/8 agonists. Agonists of TLR7/8, for example GS9620, activate pDCs and induce localised production of IFN-α. IFN-α activates NK cells which induce CTL effector functioning in CD-8+ T-cells. HBV-specific CTL responses are mediated by antigen cross-presentation to HBV-specific CD8+ T-cells by activated pDCs. The pDCs provide a nexus for the innate and adaptive arms of the immune system to overcome HBV-induced immune tolerance and eradicate cccDNA-containing hepatocytes. Adapted with permission from: Lamb, C. and Arbuthnot, P., 2016. Activating the innate immune response to counter chronic hepatitis B virus infection. *Expert Opinion on Biological Therapy*, *16*(12), pp.1517-1527.

Activation of IFN pathways as an alternative to administering recombinant IFN shows great therapeutic promise as indicated by the results from evaluating efficacy of GS9620. Another activator of innate anti-HBV effect through IFN mechanisms is STING (Burdette & Vance, 2013). STING is activated by cyclic dinucleotides that form an integral part of the bacterial signal transduction cascade. STING acts as an adaptor molecule for PRRs that are activated by DNA detected in the cytosol of infected cells. A STING agonist, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), has been assessed as an HBV therapy (Guo *et. al.*, 2015) because STING activation results in the production of IFN amongst other important cytokines. After intraperitoneal administration in murine models, DMXAA induced a robust type I IFN response

that was effective in reducing HBV replication. The compound remains to be assessed further but thus far the results are promising.

The formation and persistence of cccDNA is critical in the establishment of CHB in humans. Removal of cccDNA either through the destruction of cccDNA itself or through the removal of cccDNA-containing hepatocytes would be considered a curative therapy and is the ultimate goal of all novel therapeutic development. Recently, Lucifora and colleagues were able to demonstrate that stimulation of the IFN pathways could cause direct, nontoxic degradation of cccDNA in infected hepatocytes by activating the lymphotoxin β receptor (LTβR) (Lucifora *et. al.*, 2014). Tetravalent bispecific (BS1) and bivalent (CBE11) agonistic anti-LTβR antibodies stimulated the APOBEC3B-mediated degradation of cccDNA. APOBEC proteins bind HBcAg and target deaminating enzymes to the nuclear cccDNA (Figure 4a). In experimental conditions, the process was limited to cccDNA and was not found to cause cellular DNA damage, but whether or not this would be true if administered *in vivo* or in CHB patients remains to be evaluated. The field of immunotherapy development is very active and there are many examples of novel immunotherapeutics in varying stages of development. Here we draw specific attention to the prospect of combined therapeutic and prophylactic vaccination as a means of initiating protective antibody responses concurrently with re-activating exhausted CD-8+ T cell anti-HBV responsiveness.

1.2.4 Prophylactic and Therapeutic Anti-HBV Vaccination

The fortuitous discovery of HBsAg demarcated the first known infection marker of HBV and its discovery led to the development of a highly sensitive immunoassay, the HBsAg ELISA, used

routinely for diagnosis of infection (reviewed in: Gerlich, 2013). The inherent immunogenicity of HBsAg has since supported the development of the first recombinant antiviral vaccine in human history. HBsAg produced recombinantly in yeast (Barbacid *et. al.*, 1982) was incorporated into a commercially available vaccine in 1982 and currently forms part of globally-implemented vaccination regimen.

To date, anti-HBV vaccination is carried out in over 180 countries worldwide and has been accredited with reducing global chronic carrier rates by 70-90% (reviewed in: Gerlich, 2014). Despite significant success in early childhood and neonate immunization, the effectiveness of the recombinant vaccine in adult populations is suboptimal. Even following multiple administration attempts, between 2.5 and 5% of healthy adult recipients remain as non- or hyporesponders (with no detectable levels or no protective levels of anti-HBsAg antibodies respectively), which increases by 5-10% for every decade past 30 years of age. Non-responsiveness is exacerbated in circumstances of immunosuppression such as in overweight and diabetic individuals, transplant recipients and patients on dialysis as well as people co-infected with HIV (reviewed in Kubba *et. al.*, 2003). A large proportion of the adult population, therefore, remains vulnerable to infection rendering the goal of HBV eradication unlikely within the paradigm of the current vaccination regimen.

First-generation HBV vaccines were derived from the plasma of chronic HBV carriers; Hepatitis B surface antigen (HBsAg) was purified from donated plasma, concentrated and treated to eliminate residual infectivity. Administration of the vaccine was associated with a decrease in the incidence of new infection within high-risk cohorts (Maupus *et. al.*, 1978; Szmuness *et.al.*, 1980). However, significant safety concerns regarding the retention of infectivity despite treatment, or the potential for accidental transmission of other infectious agents (for example

HIV), greatly limited the application. Over the same period of first-generation vaccine development, researchers in the field were avidly exploring a means by which to apply recombinant DNA technology to the production of HBsAg. Successful attempts were made by artificially expressing the *S* gene of the HBV genome in transformed yeast cultures, providing the basis for the second-generation HBV vaccine (Valenzuela *et. al.*, 1982) which was approved for commercial distribution in 1982.

The recombinant HBsAg vaccine currently in global use contains only the SHBs isoform and lacks any pre-S epitopes. PreS-containing HBsAg is difficult to produce recombinantly in yeast as the protein product is cytotoxic and therefore subjected to intracellular proteolytic degradation (Imamura *et. al.*, 1987). Yeast-derived envelope proteins are not processed via the secretory pathway and so, as well as lacking the pre-S epitopes, the HBsAg recombinant proteins do not become glycosylated (Imamura *et. al.*, 1987). Structural differences between the recombinant HBsAg and the native protein have notable effects on the production of conformational anti-HBs antibodies and subsequent immunogenicity.

Evidence suggests that LHBs expressed and processed in transfected mammalian cells confer enhanced immunogenicity when compared with conventional recombinant SHBs (Heerman *et. al.*, 1987). PreS1/preS2/S protein in the native fold constitutes the group- and subtype-specific antigenic determinants. Therefore, anti-preS1 humoral immune responses have the potential to produce cross-specific, broadly neutralizing antibodies. Immunization with preS1/preS2/S recombinant protein was found to restore full responsiveness in hyporesponders or non-responders to the conventional vaccine (Milich *et. al.*, 1985). Sci-B-Vac^{TM}, a mammalian cell-derived recombinant preS1/preS2/S vaccine (reviewed in: Shouval *et. al.*, 2015) has proven efficacy in special risk circumstances and has an enhanced immunogenicity when compared with

the standard SHBs recombinant vaccine. The development of a vaccine containing the preS regions of HBsAg is justifiable as a method by which to further optimise the prophylactic effectiveness of the current vaccine.

Mammalian cell-based production processes are not desirable as they are costly and laborious. This provided a rationale for the development of a third-generation, DNA vaccination strategy for the *in vivo* expression of antigenic gene products. DNA vaccination with synthetic plasmid DNA (pDNA) encoding viral antigens is an attractive antiviral approach as DNA vaccines have been demonstrated to be capable of inducing cell-mediated and humoral immune responses in small animal models, nonhuman primates and human patients (Mallilankaraman *et. al.*, 2011; Belisle *et. al.*, 2011; Bagarazzi *et. al.*, 2012). Endogenous antigens expressed from delivered gene cassettes are capable of being processed by the cells to induce major histocompatibility class I (MHCI) restricted cytotoxic T lymphocyte activation resulting in a T_H1 response and cellular immunity (Figure 5). If targeted to professional antigen presenting cells (APCs), endogenous antigens are processed by the MHCII-restriction pathway to induce robust antibody responses (Figure 5). *In situ* antigen production from nucleic acid vaccine constructs, therefore, has the dual benefit of prophylaxis and immunotherapy.

Anti-HBV DNA vaccination strategies have been in development since the 90's (Davis *et. al.*, 1996) and interestingly, anti-HBV DNA vaccination was the first of its kind to have demonstrable efficacy in humans (Rottinghaus *et. al.*, 2003). In 2013, Obeng-Adjei and colleagues developed a DNA-based multivalent vaccine cocktail from which either LHBs or SHBs was expressed concurrently with HBcAg in mice and rhesus macaques (Obeng-Adjei *et. al.*, 2013). The antigen-coding sequences delivered intramuscularly as naked pDNA elicited anti-HBV antibody production directed against multiple epitopes within the S and preS regions. Also,

antigen-specific CTL responses were observed by IFN-γ ELISPOT assay and intracellular cytokine staining. The results of the 2013 study are compelling evidence in support of DNA vaccination but delivery of naked pDNA in human patients is unfeasible. Naked pDNA will activate PRR-mediated innate immunity directed against the vaccine inoculum rather than the expressed antigens with potentially harmful consequences. Delivery of DNA vaccines is a limiting factor of the technology and DNA therapies are most efficiently delivered by viral vectors, the application of which is accompanied by additional and extensive safety concerns. Another anti-HBV DNA vaccine cocktail developed by Kim and colleagues in 2008 (Kim *et. al.*, 2008), HB-110, is currently in the early stages of clinical trials (phase I) in South Korea.

In vivo expression of multiple HBV gene products stimulated multi-specific Th1 (T-helper one) and Th2 (T-helper two) immune function supporting the observation that DNA vaccines are associated with a cytotoxic T lymphocyte (CTL) response not previously noted for subunit or recombinant vaccines (Davis *et. al.*, 1995). CTL responses contribute to the recognition and elimination of infected hepatocytes in chronic HBV sufferers or carriers with the effect of terminating viral persistence. A nucleic acid vaccination approach, therefore, has potential for both prophylactic and immunotherapeutic benefit. Remarkably, repeated vaccination with DNA encoding preS1/preS2/S proteins, Hepatitis B core antigen (HBcAg), Polymerase (Pol), HB*x* and an interleukin (IL)-12 adjuvant elicited potent anti-HBV humoral and cell-mediated immunity (Yang *et. al.*, 2006). Obtaining maximal effect from *in situ* encoded antigens requires specific delivery to APCs, unachievable by electroporation. Targeting of the DNA vaccine to DC-cells using recombinant protein significantly improves the immunogenic effect of the vaccine (Wang *et. al*., 2016) and reduces the risk of aberrant immune stimulation in non-target tissues.

Therapeutic vaccination would encompass a means by which a vaccination inoculum could induce both cellular and humoral immunity with direct antiviral effect. Proof of this concept derives from repeated (monthly) Sci-B-Vac^{TM} injections co-administered with daily oral lamivudine doses to sufferers of persistent HBV infection (reviewed in: Shouval *et. al.*, 2015). The therapeutic campaign supressed viral replication and prompted HBsAg seroconversion in ~50% of recipients. It is thought that the strategy was sufficient in overcoming T cell exhaustion and breaking HBV immune tolerance but the facts remain to be fully elucidated in experimental settings. Vaccination of patients with chronic hepatitis C virus (HCV) using pseudo-typed chimpanzee adenoviral vectors encoding non-structural HCV proteins successfully induced robust and potent anti-HCV T cell responses (Kelly *et. al.*, 2011). DNA vaccination, therefore, has an established potential for the treatment of hepatotropic viral infections. The prospect of therapeutic DNA-based vaccine technology specific to HBV has gained popularity in recent years though there remains some hesitancy regarding the safety profile of therapeutic DNA administration.

1.2.5 A Novel Approach: mRNA-based Anti-HBV Vaccination

Apart from difficulties involving the optimal and successful delivery of DNA sequences to targeted tissues, DNA vaccine studies often yield conflicting and highly variable efficacy data. Consideration of the variability of results stemming from DNA vaccination studies, combined with pre-existing safety concerns surrounding the application of DNA therapies in human patients, the application of DNA-based vaccination strategies for HBV remains in its infancy. It seems unlikely that DNA vaccines will be immediately favoured over other immunotherapies

currently in development, whether used alone or in combination with classical NUC analogue treatment (Yoon *et. al.*, 2015). mRNA-based vaccination is a method by which to elicit potent antigen-specific humoral and cell-mediated immune responses with a superior safety profile when compared with DNA vaccines.

There are additional, distinct features of mRNA that support its preferential use as a source of immunogen for vaccination: (i) mRNA has a relatively short intracellular half-life and is therefore transiently expressed rendering the bioavailability of the antigenic product controllable and predictable, (ii) mRNA translation is carried out in the cytosol and nuclear targeting is not necessary reducing the complexity of the delivery method when compared with DNA and (iii) there is no possibility for exogenous mRNA to alter the genetic material of the hose cell. The ultimate goal of mRNA vaccination is that the antigen will be taken up, expressed and processed by APCs *in vivo* succeeded by the priming of CD8+ and CD4+ T-cell responses. However, the majority of mRNA constructs will be taken up by non-immune cells and the antigen, therefore, will need to be readily secreted in order to be retaken up and processed by APCs to the effect of eliciting humoral immune responses (Figure 5).

Antigen-encoding mRNA for the induction of CTL immune responses was first evaluated for its application as a cancer immunotherapy or for the potential treatment of HIV (Boudreau *et. al.*, 2011; Vanham & Van Gulck, 2012). Originally, mRNA immunotherapy was performed by isolating DCs from an infected individual and engineering them *ex vivo* with the mRNA construct before reintroduction. Though clinically feasible and safe, engineering DCs *ex vivo* is a complex and costly form of personalised medicine which greatly limits its application within the general population. In an HBV-specific context, the regions in which HBV infection is endemic are often economically challenged, developing countries without the necessary resources for

providing personalised medicine to the afflicted population. Recent advances in non-viral nanotechnology facilitates *in vivo* RNA delivery while at the same time improving mRNA stability and enhancing endolysosomal escape upon cell entry (Schlegel *et.al.*, 2013). Furthermore, particulate matter is actively phagocytosed by APCs and, combined with some chemical modification, could result in better APC-targeting. The application of a lipoplex delivery method will be incorporated into the design of the technology here described at a later stage of development.

1.3 Rational Design of the mRNA Transcripts for Vaccination

Application of mRNA as a therapeutic alternative to DNA was first described by Jirikowski and Sanna in 1992 (Jirikowski & Sanna, 1992). Their approach was largely based on an observation made in a seminal article published by Wolff and colleagues (Wolff *et. al.*, 1990) a few years before wherein injection of exogenous nucleic acid into murine muscle was capable of producing functional protein. Despite a propensity for degradation by abundant nucleases *in vivo,* unprotected pDNA and even mRNA, known to be particularly susceptible to extracellular nucleolytic degradation (Probst *et. al.*, 2006), penetrated the cytosol and nucleus. Exogenous nucleic acid was processed by cellular mechanisms with the final result being the production of the encoded protein localised to the injection site (Wolff *et. al.*, 1990). Specific production of antigenic protein from exogenous DNA (Ulmer *et. al.*, 1993) was closely followed by the first reported application of mRNA in a vaccine-type formulation (Conry *et. al.*, 1993). However, unmodified naked mRNA is highly immunogenic and is capable of inducing undesirable

inflammatory responses in recipients limiting its application for human therapies (Kariko *et. al.*, 2005).

The 5' triphosphate and unmodified bases of exogenous RNA activates a myriad of innate immune receptors including but not limited to TLR-3/7/8 and RIG-I. It is likely that inter- and intrastrand secondary structural elements of synthetic mRNA mediates activation of RNA-dependent protein kinases (PKR) (Bevilacqua *et. al.*, 1998). PKR recognises the double-stranded RNA genomes of infiltrating viruses during natural infection and becomes activated by autophosphorylation. In turn, activated PKR phosphorylates the α-subunit of translation initiation factor 2 (eIF-2α) thereby suppressing translation (Hunt & Ehrenfeld, 1971; Ehrenfeld & Hunt, 1971). Base modifications in endogenous mRNA are common and represent a discriminatory molecular feature of endogenous mRNA versus RNA derived from invading pathogens or viruses. Some viruses, such as the Herpes Simplex virus, will increase the frequency of base modifications as a means of immune evasion (Bokar & Rottman, 1998). The inclusion of chemically-modified nucleosides, most commonly pseudouridine, is found to promote evasion of PKR recognition (Anderson *et. al.*, 2010) and enhance translational capacity of synthetic mRNA (Kariko *et. al.*, 2008). Optimisation of synthetic mRNA with the incorporation of chemically-modified ribonucleotide bases does not affect delivery efficiency (Anderson *et. al.*, 2010) and is indistinguishable from the unmodified counterpart by agarose gel electrophoresis (Kariko *et. al.*, 2005).

Pseudouridine is the most common modified nucleoside in mRNA and is produced from the isomerisation of uridine. Inclusion of pseudouridine promotes base stacking and in doing so stabilises the duplex regions (Davis, 1995). In rabbit reticulocytes transiently transfected with pseudouridine-modified mRNA, protein production from the modified mRNA was 10-fold

greater than for the unmodified counterpart (Kariko *et. al.*, 2008). The improved translational capacity could be accredited to the enhanced stability and decreased immunogenicity of the mRNA which in turn extends the half-life of the synthetic transcripts. Interestingly, it is a topic of debate as to whether base modification is useful for mRNA vaccine design where the intrinsic adjuvanticity of the mRNA could prove beneficial. Inclusion of pseudouridine suppresses the induction of TNF-α and IL12 secretion from monocyte-derived DCs by reducing the production of IFN-γ (Kariko *et. al.*, 2008). TNF-α and IL12 enhance CTL and NK responses, and APCs polarise T-cell responses in an IL12-dependent manner (Koski *et. al.*, 2004). IFN-γ acts as the priming signal to maximise IL12 secretion by maturing DCs and immunogenic RNA substitutes this signal. It is logical, therefore, that pseudouridine-modified mRNA in a vaccine formulation could actually limit vaccine efficacy.

A means of circumventing the inclusion of modified bases without resulting in suboptimal translational capacity of the mRNA is by sequence-engineering the mRNA production constructs. Such an approach is the focus of the current study and has been applied during the design process. Sequence-engineering of mRNA occurs in two ways: (i) sequence optimisation by considering adapted codon usage in different expression organisms and (ii) selection of appropriate regulatory sequences (UTRs) (Thess *et. al.*, 2015). These approaches have proven efficient in attaining protein production levels comparable to the non-engineered, chemically modified transcripts (Thess *et. al.*, 2015). In the current study, the gene of interest was processed using a codon-optimisation algorithm to produce a sequence adapted for optimal translation in humans as the primary and mice as the secondary organisms of expression. The mRNA here produced includes the 5' UTR of the *β-globin* gene and 3' UTR of *α-globin* which encompasses pyrimidine-rich sequences that act to stabilise the mRNA by binding the ubiquitous

α-complex of eIF2 (reviewed in: Pascolo, 2008). Sequence-engineering of the synthetic mRNA is a critical aspect of the current study and has been applied here in an attempt to ensure optimal applicability of the mRNA in a vaccine formulation at a later stage.

Additional aspects are included in the design of the mRNA production process to ensure optimal translation of the transcripts: (i) incorporation of a Kozak sequence (Kozak, 1978) in the 5'UTR that overlaps the start codon to optimise translation, (ii) 5'-capping of the transcripts concurrently with transcription using an anti-reverse cap analogue (ARCA) and (iii) the incorporation of a poly (A) tail mimic by transcription of the poly T:A sequence stretch (~95nt) downstream 3'UTR. By these methods, the synthetic mRNA will structurally mimic endogenous transcripts. ARCA will be incorporated concurrently with nascent bases which will ensure that ~80% of the transcripts will be in the correct orientation for productive translation. ARCA-capped mRNA has a two-fold greater translational efficiency than uncapped mRNA in rabbit reticulocytes (Stepinski *et. al.*, 2001) possibly because it has a longer half-life (Grudzein *et. al.*, 2009) and therefore prolonged protein expression (Zohra *et. al.*, 2007). The ~95nt poly (A) tail along with the globin UTRs should function in stabilising the synthetic mRNA (Mockey *et. al.*, 2006; Holtkamp *et. al.*, 2006).

The current study was aimed at the development of an anti-HBV mRNA vaccine for the purpose of improved prophylactic potency through the inclusion of all critical HBsAg epitopes and as well as exploring the use of mRNA vaccination as a novel immunotherapeutic. This dissertation describes the early development and design stages of the mRNA vaccine complement in which codon-optimised DNA sequences were successfully subcloned into specially engineered mRNA production vectors for downstream *in vitro* transcription of synthetic LHBs and SHBs mRNA. We were able to produce mRNA using this method that was

translated into detectable LHBs and SHBs in cultured hepatoma cells *in vitro*. However, it was found that SHBs was readily secreted into the cytoplasm while LHBs remained trapped inside the cell, an important fact that will require downstream optimisation to ensure full immunogenic benefit of the vaccine when being tested *in vivo*. Overall, the current study has provided the framework for further development of the vaccine and establishment of functionality at a later stage of development.

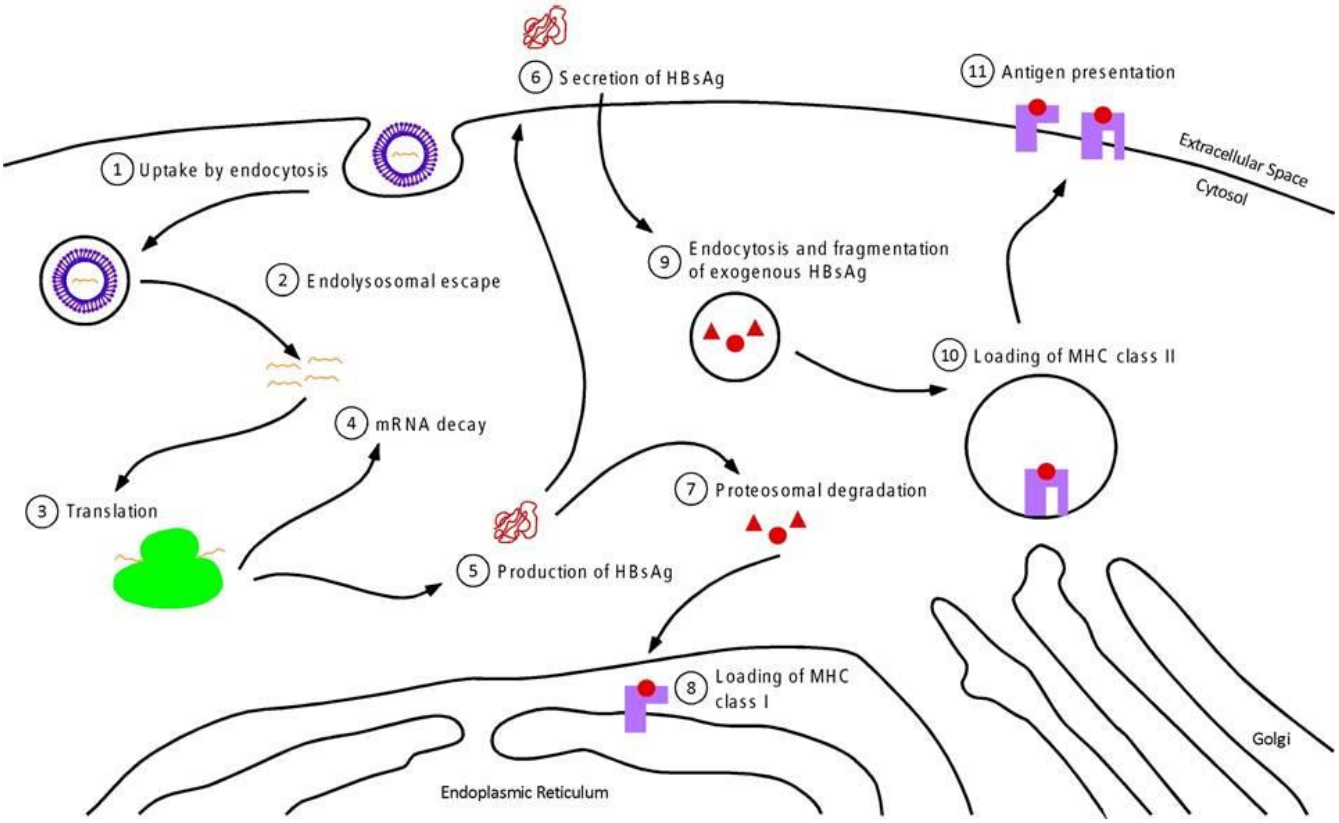


Figure 5: Theoretical initiation of humoral and cellular immunity from mRNA vaccine products *in situ.* Lipoplex-associated mRNA is protected from extracellular endonucleases and (1) nanoparticle uptake from the extracellular environment is mediated by endocytosis. (2) Within the acidic environment of the endolysosome, the acid-base chemistry of the lipoplex delivery vehicles directs an increase in overall positive charge to allow for attachment and fusion to the anionic, endosomal membrane; the mRNA payload escapes degradation and is released into the cytosolic compartment. (3) The mRNA constructs enter the endogenous protein synthesis pathway and are translated to produce S-HBsAg or L-HBsAg. (4) mRNA has a relatively short half-life and is rapidly degraded by intracellular RNAses upon termination of translation. (5) The protein produced is either (6) secreted or (7) degraded in a proteasome-dependent manner; peptides from the degraded protein enter the endoplasmic reticulum and become bound to MHC class I molecules which are transported to the surface for MHC class I-restricted antigen presentation and the induction of CD8+ T-cell responses (9). (8) Re-uptake of the secreted protein by endocytosis is followed by endosomal degradation and peptide binding to MHC class II molecules which are then transported to the cell surface for MHC class II-restricted antigen presentation to CD4+ T-cells (9). Antigenic products of a DNA vaccine would ultimately be processed in much the same way with the exception that the DNA would first need to be translocated to the nucleus for transcription and the delivery method may differ from the lipoplex formation here shown.

2. MATERIALS AND METHODS

2.1 Generation of mRNA production templates and in vitro *expression vectors*

2.1.1 Design of the mRNA production pDNA backbone

To produce synthetic mRNA by T7 transcription *in vitro*, a suitable mRNA pDNA production template was designed to serve as the backbone into which all mRNA-encoding DNA sequences were subcloned. T7 transcription is a method whereby the phage T7 polymerase is used for high fidelity synthesis of mRNA transcripts under the transcriptional control of the promoter specific to the phage protein, the T7 promoter. The mRNA production backbone is referred to as pmRNA-MCS-95A where MCS is the abbreviation for 'multiple cloning site'. The pmRNA-MCS-95A backbone was designed to contain (in a 5' to 3' direction) (i) a M13 forward (M13F) sequencing primer-binding site (not shown), (ii) a T7 promoter, (iii) the 5' untranslated region (UTR) of the rapine *β-globin* gene (iv) followed immediately downstream by the MCS, (v) the 3' UTR of the murine α-globin gene, (vi) a stretch of 95 thymidine residues on the coding strand (poly A:T), followed further downstream by the (vii) M13 reverse (M13R) sequencing primer-binding site (not shown) (Figure 6).

The sequences M13F, T7, α-globin 5'UTR, β-globin 3'UTR and M13R are standard sequences and were obtained from GenBank® NIH genetic sequence database (available at: https://www.ncbi.nlm.nih.gov/genbank/). Within the 5' UTR is the Kozak consensus core sequence, (gcc)gccRccAUGG (Kozak, 1978), included to optimise translation of the mRNA (appendix A1-1). The MCS sequence was pieced together using known, overlapping Type II

restriction endonuclease binding sites (Appendix A1-1). The MCS ensured that the plasmid template is amenable to other subcloning experiments to be performed at a later stage if needed. The stretch of sequences between the M13F and the T7 promoter, as well as from the 3' terminus of the poly (T) sequence to the M13R were abstracted from the standard pUC57 backbone. Downstream of the poly A:T sequence, a *Hind*III restriction site was included for use in the linearization step of the mRNA production protocol (Section 2.3).

The sequences were pieced together in FASTA format and sent to GenScript® for synthesis using the GenPlus™ High-throughput Gene Synthesis next-generation gene synthesis technology platform. The synthesized genetic fragment was subcloned by GenScript® into a pUC57 backbone and returned to us accompanied by a certificate of analysis and confirmation of sequence identity. The plasmid DNA was prepared and purified for downstream application using the Qiagen® Plasmid Purification Maxi Kit (appendix A2-1).

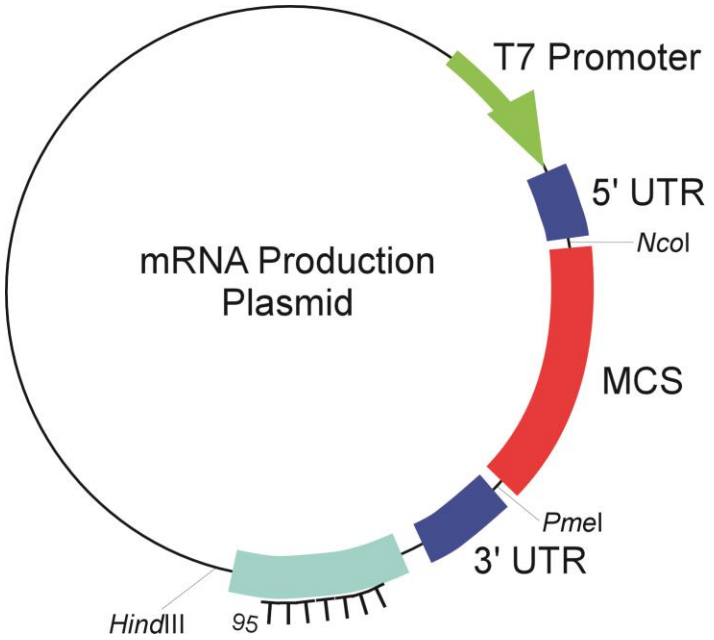


Figure 6: mRNA Production Template. The mRNA production sequences include a T7 promoter which drives *in vitro* transcription by T7 polymerase, followed by the 5' UTR of the rapine β-globin gene and the 3'UTR of the murine α-globin gene punctuated by the MCS. Included in the MCS are *Nco*I and *Pme*I restriction sites used for subcloning of mRNA-coding sequences. Immediately downstream of the 3'UTR is the poly A:T stretch on the coding strand which is transcribed into a poly (A) tail mimic during *in vitro* transcription. Several (~8) nucleotides downstream from the 3' terminus of the poly A:T sequence is a *Hind*III restriction site used for template linearization during mRNA production.

2.1.2 Codon-optimisation of the LHBs coding sequence

For the production of sequence-engineered mRNA, a sequence codon-optimisation approach for the HBsAg-coding sequence was adopted. Different species have adapted codon-usage and codon optimisation is a way to improve translatability of the mRNA. The LHBs sequence of HBV Genotype A1, isolate Mart-B27 (Brichler *et. al.*, 2013) was sourced from GenBank® NIH genetic sequence database (accession number: HE974370.1). The sequence was extracted from the total genomic sequence and saved in FASTA format with a *Nco*I restriction site added at the 5' end and a *Pme*I restriction site at the 3' end, preceded by a guanidine dinucleotide sequence (for reconstruction of the complete 3'UTR). Towards the 3' end of the LHBs gene is the SHBs ORF which was extracted by PCR at a later stage (see section 2.1.3.1).

The FASTA sequence file was sent to GenScript® for synthesis as described above (Section 2.1.1).The synthesised gene was codon-optimised by GenScript® using the patented OptimumGene™ (Liu *et. al.*, 2012; US Patent 8326547B2) optimisation algorithm with humans as the primary, and mice as the secondary organism for expression. The codon-optimised LHBs sequence was processed by Genscript and returned as described in section 2.1.1. The pDNA, referred to as pUC57-preS1/preS2/S (CO) was prepared and purified for downstream application using the Qiagen® Plasmid Purification Maxi Kit (Qiagen, Germany) (appendix A2-1).

2.1.3 Construction of the mRNA production and *in vitro* expression vectors

Three mRNA production plasmids were generated for the purposes of the current study: (i) pmRNA-LHBs-95A, (ii) pmRNA-SHBs-95A and (iii) pmRNA-eGFP-95A. The pmRNA

plasmids were modified to include a CMV promoter/enhancer sequence upstream of the T7 promoter and in frame with the mRNA-coding DNA sequences (Figure 7). A CMV promoter/enhancer controls strong expression of downstream DNA sequences in mammalian cells and is used commonly in eukaryotic expression vectors. The modified plasmids; (i) pmRNA-CMV-LHBs, (ii) pmRNA-CMV-SHBs and (iii) pmRNA-CMV-eGFP (Table 1), could therefore be used to assess protein expression from the cloned sequences *in vitro* prior to mRNA production. The pmRNA-eGFP-95A plasmid was included as a control plasmid for protein expression from the DNA and from the produced mRNA. An additional control plasmid, pmRNA-CMV-MCS, was constructed to be used a mock control plasmid during DNA transfection experiments.

2.1.3.1 *PCR amplification of the required sequences*

The LHBs, SHBs, eGFP and CMV promoter/enhancer sequences were amplified from various donor plasmids by standard polymerase chain reaction (PCR). The eGFP sequence and CMV promoter/enhancer sequences were amplified from pCI-neo-eGFP while both the *LHBs* and *SHBs* sequences were amplified from pUC57-preS1/preS2/S (CO) (Figure 8). All amplification reactions were performed using the KAPA Taq ReadyMix PCR Kit (Kapa Biosystems, MA, USA) according to the manufacturer's instructions. Forward and reverse primers for each amplification reaction were synthesised by standard phosphoramidite chemistry (Integrated DNA Technologies, IA, USA) and contained no additional modifications. Primer sequences as well as specifics of each PCR reaction are described in Table 2. Primers were designed to incorporate a 5' *Nco*I and 3' *Pme*I site to each amplified sequence for subsequent subcloning into pmRNA-MCS-95A. Amplification was performed using a T100^{TM} Thermal

Cycler (Bio Rad Laboratories, RSA) and the thermal cycler profiles for each reaction are shown in appendix A3-1.

Table 1: List of plasmid constructs and their intended use.

Description	Plasmid Construct	Experimental Application
Foundational constructs	pmRNA-MCS-95A	Subcloning
	pUC57-preS1/preS2/S (CO)	Source of codon-optimised *LHBs* and *SHBs*
mRNA production vectors (premature stage of subcloning)	pmRNA-eGFP-95A	mRNA production by *in vitro* transcription using T7 phage polymerase
	pmRNA-LHBs-95A	
	pmRNA-SHBs-95A	
mRNA production and *in vitro* expression vectors	pmRNA-CMV-MCS	Mock control
	pmRNA-CMV-eGFP	Transfection efficiency control
	pmRNA-CMV-LHBs	Expression of *LHBs in vitro* and production of LHBs mRNA
	pmRNA-CMV-SHBs	Expression of *SHBs in vitro* and production of SHBs mRNA

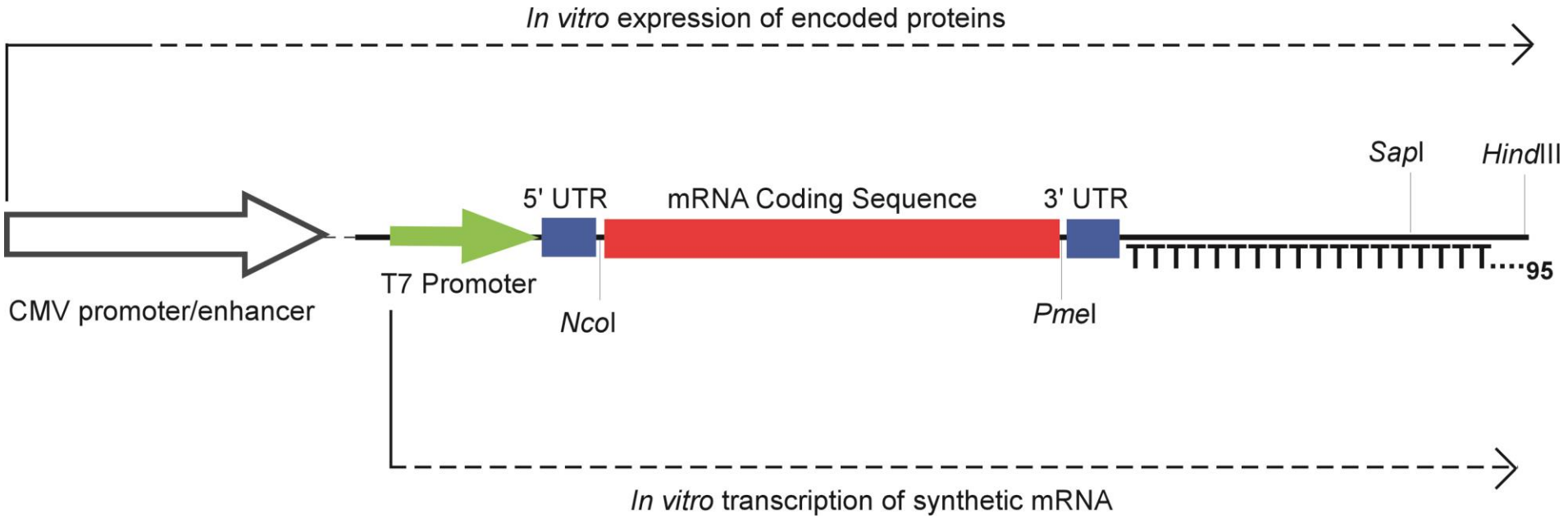


Figure 7: Schematic of critical sequence elements within the pmRNA-CMV plasmid constructs. The plasmids were designed for dual experimental purpose: (i) drive protein expression *in vitro* in cultured cells and (ii) permit *in vitro* transcription for mRNA synthesis. The CMV promoter/enhancer drove transcription of a long mRNA inclusive of all the sequence elements needed for mRNA production. Translation of the encoded protein, which was subsequently expressed from the synthetic mRNA transcripts, was initiated at the translational start site (within the *Nco*I recognition and restriction sequence). The design of this vector allowed for pre-evaluation of protein production before initiating mRNA transcript synthesis from the same plasmid construct. This was particularly important for assessment of HBsAg expression from codon-optimised *LHBs* and *SHBs*.

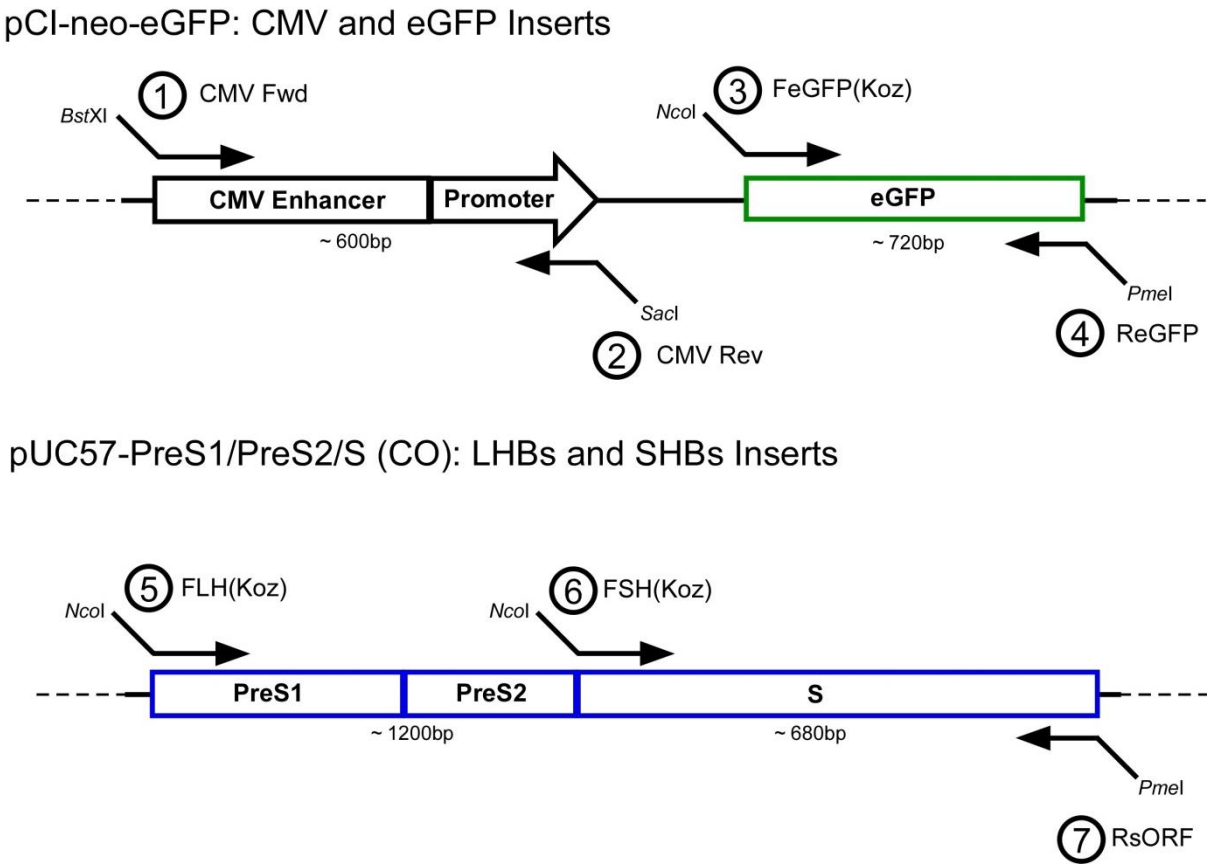


Figure 8: Generation of cloning fragments by PCR. The eGFP cloning fragment was amplified from pCI-neo-eGFP using the FeGFP(Koz) and ReGFP forward and reverse primers respectively. The amplicon was approximately 720bp in length. The CMV enhancer/promoter sequence of approximately 600bp was amplified from the same donor plasmid, pCI-neo-eGFP, using the CMV Fwd and CMV Rev primer set. LHBs (~1200bp) and SHBs (~680bp) were amplified from pUC57-preS1/preS2/S (CO) using the FLH(Koz) and FSH(Koz) forward primers respectively and the same reverse primer, RsORF.

Approximately 1 ng of respective pDNA donor template and 20 pmole each of forward and reverse primer were included in each amplification reaction. The amplification cycles were repeated 35 times in each instance and for each respective PCR, 5 independent reactions were set-up and amplicons of the same identity were then pooled for purification. Immediately post-amplification, 1 µl of each reaction was analysed by gel electrophoresis through a 1% agarose gel (120V, 35 minutes) for visualisation (ethidium bromide-staining) of amplicon quality and purity. High quality amplicons of the same identities were pooled and purified further using the QIAquick PCR Purification Kit (Qiagen, Germany) operated according to the manufacturer's instructions. Purified amplicons were eluted in 30 µl of double-deionised water (ddH_2O) and quantified using the NanoDrop 1000 (Thermo-Scientific, MA, USA) spectrophotometer. An elution check of the purified amplicon was performed by agarose gel electrophoresis (according to the conditions described above) loaded with approximately 250 ng of the amplicon eluent. All eluents were stored at -20^{o}C if not used immediately.

Table 2: Specific primer sequences and PCR conditions (restriction sites are underlined).

Amplicon Identity	Primer Sequences (5'-3')		Annealing T (^{o}C)	Elongation Time (minutes)
eGFP	Fwd	**FeGFP(Koz):** *TAG CCG CCA CCA TGG TGA GCA AGG GCG AG*	50	1:30
	Rev	**ReGFP:** *CAG CGT TTA AAC GGG CCC TTA CTT GTA CAG CTC GTC*		
CMV	Fwd	**CMV Fwd**: *CGG CCA GTG AAT TGG CAT TGA TTA TTG ACT*	50	1:30
	Rev	**CMV Rev**: *CTA AAC GAG CTC TGC TTA TAT AGA CCT CCC*		
LHBs	Fwd	**FLH(Koz):** *TAG CCG CCA CCA TGG GGG GGT GGT CAA GC*	55	2:15
	Rev	**RsORF:** *CAG CGT TTA AAC GGG CCC TCA AAT GTA GAC CCA CAG*		
SHBs	Fwd	**FSH(Koz):** *TAG CCG CCA CCA TGG AGA ATA TTA CTT CC*	50	1:30
	Rev	**RsORF:** *CAG CGT TTA AAC GGG CCC TCA AAT GTA GAC CCA CAG*		

2.1.3.2 Amplicon sequence confirmation

Purified amplicons were subcloned into linearised pTZ57/R using the InsTAclone™ PCR Cloning Kit (Thermo-Scientific, MA, USA). Manufacturer's instructions for ligation reaction set-up were followed exactly using an insert/vector ratio of 1:3. Calculations of vector and insert amounts were performed according to the table provided in the manual of the kit (Table A3-2.1). The final ligation volume was 30 µl and the ligations reactions were incubated overnight (O/N) (~16 hours) at 22°C in a refrigerated water bath. Included in the ligation reaction layout was a positive control provided by the kit as well as a no-insert control (NIC) wherein only the linearised backbone was present in the ligation reaction.

For the transformation reactions, 50 µl of competent XL10 Gold® *E. coli* were transformed with 2.5 µl of each ligation reaction mix (Appendix A2-2) and plated onto ampicillin plates coated with X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and IPTG (isopropyl thiogalactoside) (Appendix A2-1). The plates were left to incubate upside-down O/N at 37°C and then individual white colonies from the plates were used to inoculate 20 ml of Luria Bertani (LB) medium (Appendix A2, table A2 1.2). The pDNA was extracted and purified and included in various restriction digest reactions to screen for positive clones (Table 3). Positive clones were sent for standard Sanger sequencing by Inqaba Biotec, RSA. Sequencing was performed using M13F and M13R sequencing primers.

Sequencing data extracted from the chromatograms provided by Inqaba Biotec were saved in FASTA format (query sequence). A multiple sequence alignment algorithm, BLASTN (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), was used to align the extracted sequence with the original, desired sequence (subject sequence). Sequences

with 100% query identity were considered true positive clones and selected for scaled-up pDNA preparation (Appendix A2, Table A2 1.2).

2.1.3.3 Subcloning of mRNA-coding sequences and CMV promoter/enhancer

The mRNA-coding sequences (eGFP, LHBs and SHBs) contained a 5' *Nco*I and 3' *Pme*I site introduced during PCR amplification. The *Pme*I restriction site in pmRNA-MCS-95A is 2 nucleotides downstream of the start of the 3' UTR but this sequence region was reconstituted by the reverse primers (Table 2). The strategy employed to construct the final *in vitro* expression vectors involved 2 distinct cloning steps because there is an *Nco*I restriction site within the CMV promoter/enhancer sequence. First, the mRNA-coding sequences excised from the positive pTZ clones using *Nco*I-HF® and *Pme*I (NEB, MA, USA) were subcloned into complementary sites within the linearised pmRNA-MCS-95A backbone (Figure 9). Confirmed positive pmRNA clones, and the selected pTZ-CMV, were digested with *Bst*XI and *Sac*I (Thermo-Scientific, MA, USA). The CMV promoter/enhancer insert was then subcloned into the relevant sites within the linearised pmRNA (with coding insert) backbone to construct the dual *in vitro* expression and mRNA production plasmids (Figure 9). The pmRNA-CMV-MCS mock control plasmid was included only in the second stage of the cloning protocol (Table 1). Identical experimental proceedings were applied at all stages of subcloning which are described in detail in Appendix A3-2.

Restriction digestion with *Nco*I-HF® and *Pme*I (NEB, MA, USA) was performed as a double digestion reaction in CutSmart™ buffer (NEB, MA, USA) within a final reaction volume of 50 µl. *Bst*XI and *Sac*I (Thermo-Scientific, MA, USA) have optimal activities in different buffer systems and at different temperatures. Two separate digestion reactions were therefore performed consecutively, on the same DNA sample, to obtain the necessary fragments. The first

stage of the digestion reaction using *Bst*XI was performed in 1× Buffer O (Thermo-Scientific, MA, USA) in a total reaction volume of 20 µl. The reaction was incubated at 55^{o}C in a T100^{TM} Thermal Cycler (Bio Rad Laboratories, RSA) for 60 minutes. The digested DNA was purified from the reaction volume using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the kit manual and eluted in 30 µl of ddH_2O. A 1 µl volume of eluent was used for an elution check (agarose gel electrophoresis). Upon confirmation of successful digestion, the remaining volume of eluent was included in the second stage of the restriction digestion reaction (Figure 9). The *Sac*I digestion reaction was set up in a final reaction volume of 50 µl which included 1× *Sac*I buffer (Thermo-Scientific, MA, USA). The reaction mix was incubated at 37^{o}C for 60 minutes. The protocol outlined in appendix A3-2 was then completed using the extracted inserts and vector backbones.

Screening for positive clones for all the generated plasmids was performed by restriction mapping with several different enzymes chosen specifically for each plasmid type. The restriction enzymes used, as well as the respective reaction conditions for screening final *in vitro* expression and mRNA production plasmids is outlined in Table 3. All restriction enzymes used were FastDigest^{TM} enzymes (Thermo-Scientific, MA, USA) and all reactions were incubated at 37^{o}C for 30 minutes. Digestion reactions were analysed by agarose gel electrophoresis and positive clones were selected based on the band sizes of the DNA fragments resulting from each digestion. A GeneRuler^{TM} DNA Ladder Mix (Thermo-Scientific, MA, USA) was run alongside the samples for size comparison.

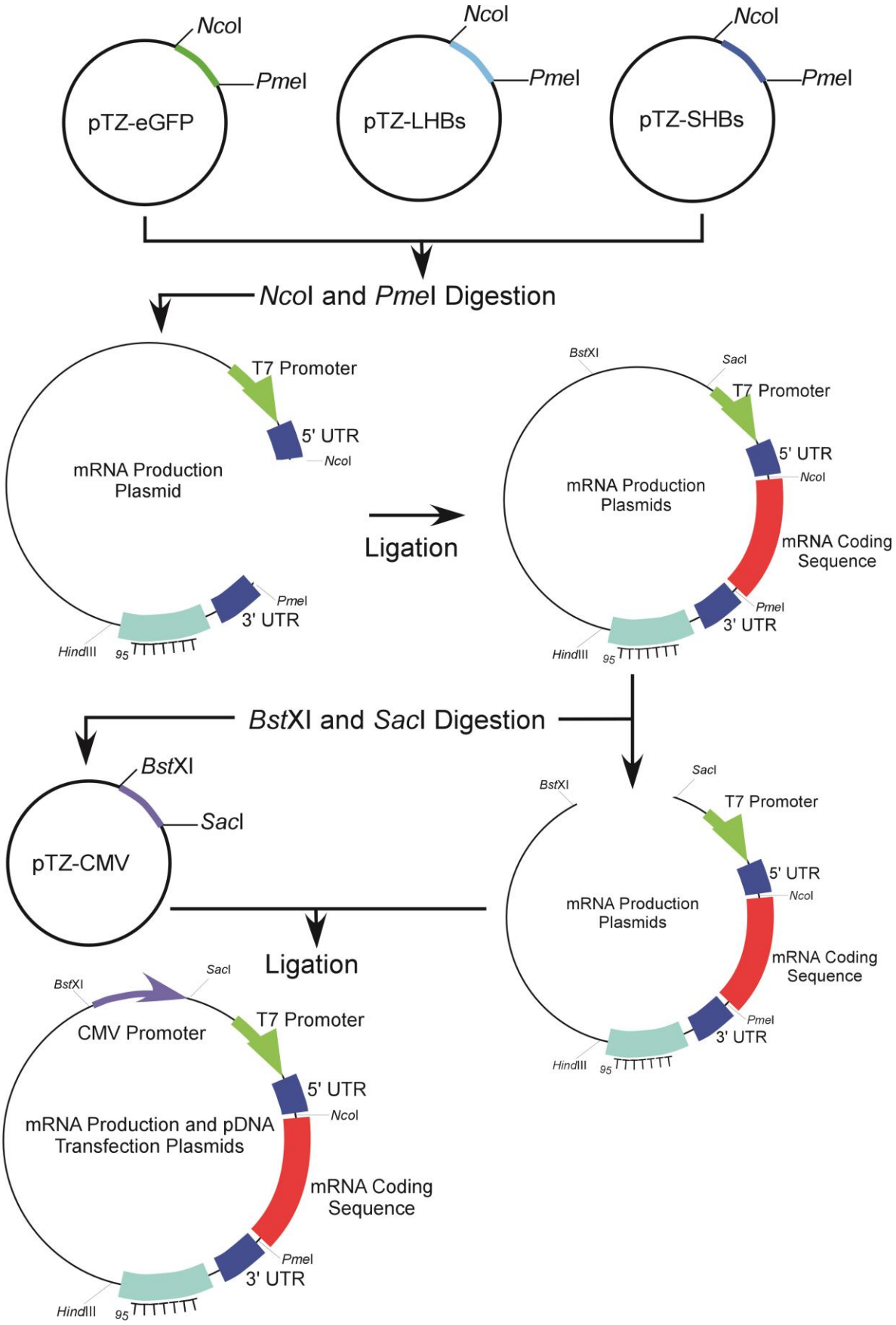

NcoI
PmeI
pTZ-eGFP
NcoI
PmeI
pTZ-LHBs
NcoI
PmeI
pTZ-SHBs
NcoI and PmeI Digestion
T7 Promoter
5' UTR
NcoI
mRNA Production Plasmid
PmeI
3' UTR
HindIII
95
Ligation
BstXI
SacI
T7 Promoter
5' UTR
NcoI
mRNA Production Plasmids
mRNA Coding Sequence
PmeI
3' UTR
HindIII
95
BstXI and SacI Digestion
BstXI
SacI
pTZ-CMV
Ligation
BstXI
SacI
T7 Promoter
5' UTR
NcoI
mRNA Production Plasmids
mRNA Coding Sequence
PmeI
3' UTR
HindIII
95
BstXI
SacI
CMV Promoter
T7 Promoter
5' UTR
NcoI
mRNA Production and pDNA Transfection Plasmids
mRNA Coding Sequence
PmeI
3' UTR
HindIII
95

Figure 9: Overview of the cloning strategy for generating mRNA production and *in vitro* expression vectors. Sequence-checked mRNA-coding inserts were excised from the pTZ clones using *Nco*I and *Pme*I. The excised inserts were then ligated into specific free ends on the pmRNA backbone digested with the same enzymes to create the mRNA production plasmids. These plasmids were then included in an additional subcloning step wherein they were digested with *Bst*XI and *Sac*I. The CMV promoter/enhancer sequence was extracted from pTZ-CMV using *Bst*XI and *Sac*I and ligated into the linearised mRNA production plasmid backbones to give the final plasmids which were used for *in vitro* expression analyses and mRNA production.

Table 3: Restriction digestion: Screening for positive clones. All enzymes shown are FastDigest™ enzymes (Thermo-Scientific, MA, USA). The reactions were made up in a final volume of 20µl and were incubated at 37° C for 30 minutes.

	Enzymes		**Buffer**
	Screen 1	**Screen 2**	
pmRNA-CMV-MCS	*Spe*I + *Sca*I	*Eco*RI + *Not*I	1X FastDigest™ Green Buffer
pmRNA-CMV-EGFP	*Spe*I + *Sca*I	*Eco*RI + *Bam*HI	
pmRNA-CMV-LHBs	*Spe*I + *Sca*I	*Apa*LI	
pmRNA-CMV-SHBs	*Spe*I + *Sca*I	*Apa*LI	

2.2 Optimisation of immunofluorescence protocol for the detection of intracellular HBsAg

2.2.1 Antibodies

For the purposes of the current study, we needed to detect LHBs and SHBs and distinguish between the two protein isoforms using immunofluorescence. Three murine monoclonal primary antibodies were selected, namely anti-S (S14; ab8636, Abcam®, Cambridge, UK), anti-preS1 (AP1; sc-5776, Santa Cruz Biotechnologies, TX, USA) and anti-preS2 (S26; sc-23944, Santa Cruz Biotechnologies, TX, USA). The anti-S antibody detected SHBs, and LHBs while the anti-preS1 and anti-preS2 antibodies were specific for LHBs. A goat anti-mouse IgG polyclonal secondary antibody conjugated to Alexa Fluor® 488 (Thermo-Scientific, MA, USA) was used for detection of bound primary antibody. A standard immunofluorescence protocol was followed (Appendix A2-3); however, it was necessary to optimise primary antibody incubation conditions for optimal detection of intracellular HBsAg. This was because the antibodies have not been broadly applied for detection of *in vitro* intracellular HBsAg protein. Primary antibodies AP1 and S14 were used at a dilution of 1:100 and S14 was used diluted 1:50.

2.2.2 Culture of HepG2.2.15 Cells

HepG2.2.15 cells are G418-resistant derivatives of the HepG2 hepatoblastoma cell line which carry four tandem copies of the HBV genome (5'-3') (Sells *et. al.*, 1987). Typically, HepG2.2.15 cells are used as *in vitro* replication models for HBV and constitutively produce all the HBV-associated proteins including HBsAg. Cultured HepG2.2.15 cells, therefore, allowed for optimisation of the immunofluorescence staining protocol prior to DNA or RNA transfection.

HepG2.2.15 cells were maintained in Gibco® Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, CA, USA) supplemented with penicillin (10 000 units/ml), streptomycin (100µg/ml) and 10% heat-inactivated fetal calf serum (FCS). Cultures were incubated at 37°C and 5% CO_2.

2.2.3 Conditions Assessed for Optimisation

HepG2.2.15 cells were seeded in 96 well culture dishes at ~38 000 cells/well (~80% confluency) and incubated overnight or until the confluency reached ~90%. Once the cells had grown to a sufficient confluency, they were immediately fixed in 4% (w/v) paraformaldehyde (PFA), permeabilized in 0.1% (v/v) Triton™-X100 (Sigma-Aldrich, MS, USA) and either used immediately or stored at 4°C in a humidified chamber (Appendix A2-3). Three incubation conditions for the primary antibodies were tested: (i) incubation in primary antibody for 24 hours at 4°C, (ii) extending the blocking step to 24 hours overnight at 4°C or (iii) an in primary antibody incubation of 1 hour at RT. All incubation conditions were performed in a humidity chamber. The immunofluorescence protocol was completed and the stained cells imaged using the Zeiss Axio Imager inverted fluorescence microscope (Zeiss, Germany) and the AxioVision (V3.8.1) imaging software.

2.3 In vitro *synthesis of mRNA transcripts*

The synthesis of mRNA transcripts was completed in two stages: (i) linearisation and purification of the pmRNA template and (ii) *in vitro* T7 transcription using the linearised

template (Figure 10). Initially, the pmRNA template was designed to be linearised using a Type IIS restriction enzyme, *Sap*I. The recognition site of *Sap*I was just downstream of the poly A:T sequence and the restriction site 5 thymidine residues before the 3' terminus of the poly A:T such that the final nucleotide on the linear coding strand was a thymidine. The restriction activity of Type IIS enzymes relative to Type II enzymes differs in cleavage efficiency. Often, Type IIS enzymes require more than one recognition site to be present to cleave the DNA and the pmRNA backbone harboured two *Sap*I sites ~350 bp apart. Incomplete linearisation of plasmid template was consistently noted (Appendix A4-1) whereby the DNA was cleaved at only 1 of the 2 sites and only a small portion of the DNA was cleaved at both sites. As a result of using pmRNA templates of different lengths, mRNA transcripts of differing lengths were produced as an impure mixture. Instead, a *Hind*III site located ~8nt downstream of the 3' terminus of the poly A:T sequence was used because *Hind*III is a high fidelity, robust restriction enzyme that achieved complete linearisation more efficiently.

Table 4: Reaction set-up for *in vitro* transcription by T7. mMESSAGE mMACHINE® T7 ULTRA Kit Reagents are listed in the order of their addition to the reaction mix

	Positive Control (µl)	**Experimental Sample (µl)**
Ambion® Nuclease-free H_2O	4	(make volume up to 20µl)
rNTPs/ARCA	10	10
10X Reaction Buffer	2	2
Linear DNA Template	2	(up to 1µg)
T7	2	2
V_T	**20**	**20**

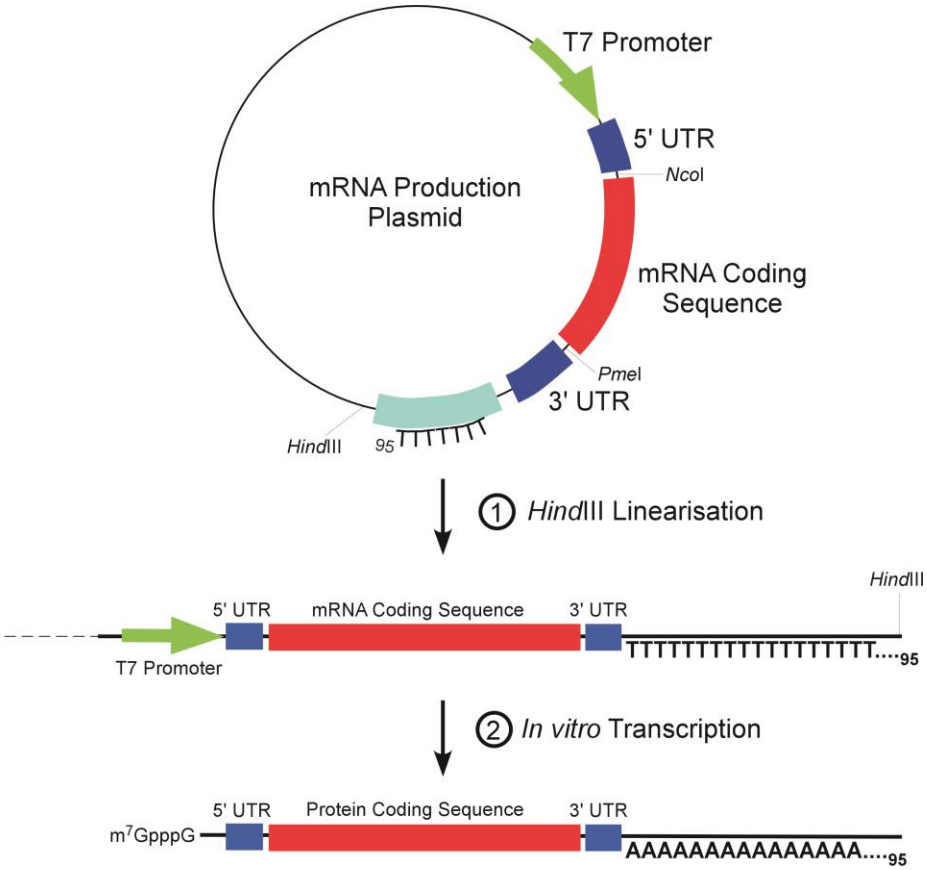


Figure 10: Schematic of mRNA production protocol. Synthesis of mRNA transcripts by *in vitro* T7 transcription was performed in two stages: (i) Linearisation of the DNA template and (ii) production of transcripts by *in vitro* transcription. *Hind*III was used to linearise the plasmid template by cleaving the DNA downstream of the poly (T) sequence on the coding strand. In doing so the mRNA transcripts produced in (ii) are of a predetermined length and all transcripts produced are identical. Included in the T7 transcription reaction set-up was ARCA (m^7GpppG) which functions as a mimic of a 5' cap found in endogenous mRNA. The poly A:T sequence was converted to a poly (A) sequence in the transcripts which functions in mimicking the poly (A) tail found in eukaryotic mRNA.

The linearisation reaction was set-up using 10 µg of high quality, high purity pmRNA plasmid per reaction. Included in the final reaction volume of 50 µl were 10 U *Hind*III-HF® and 1× CutSmart® Buffer (NEB, MA, USA). The reaction volume was made up in ddH_2O and mixed thoroughly before incubation for 60 minutes at 37°C. Immediately post-incubation, 1µl of the restriction digest reaction was analysed using agarose gel electrophoresis to assess complete linearisation. If confirmed to be completely linear, the remainder of the restriction digest reaction (49 µl) was purified using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The linear template was eluted in 30 µl of TE buffer (provided in kit) and quantified using the NanoDrop 1000 spectrophotometer. An elution check by agarose gel electrophoresis was performed using 1 µl of the eluent. Linearisation was completed on the day of, or the day before, the *in vitro* transcription reaction and no template was stored at -20°C for more than 24 hours before use.

T7 *in vitro* transcription was achieved using the mMESSAGE mMACHINE® T7 Ultra Kit (Life Technologies, CA, USA). In brief, the reaction was set up according to table 4 and incubated for exactly 2 hours at 37°C in a thermal cycler. A quantity of 1 µg purified linear DNA was used as the transcription template. The reaction set up included the rNTPs in specific ratios as well as the anti-reverse cap analogue (ARCA) included to mimic the 5' cap of endogenous eukaryotic mRNA. The final reaction volume of 20 µl was made up in Ambion® nuclease-free water (Life Technologies, CA, USA). To eliminate the pmRNA template, 1 µl of TURBO® DNAse was added followed by another 15 minute incubation at 37°C.

After incubation, the reaction (20 µl) was thoroughly mixed with 30 µl of ice-cold 7.5 M lithium chloride (LiCl) solution (containing 50 mM EDTA) (provided in kit) and incubated at -20°C for 60 minutes to allow for precipitation of the mRNA. The solutions were kept on ice from

this point to preserve mRNA integrity. To isolate the mRNA by precipitation the LiCl solutions were centrifuged at 12 000 ×g for 15 minutes (4^{o}C). The supernatant was carefully removed and the mRNA pellet was washed in 1 ml of ice-cold 70% (v/v) ethanol prepared fresh using Ambion® nuclease-free water (Life Technologies, CA, USA). The ethanol wash step required centrifugation at 12 000 ×g for 15 minutes (4^{o}C) following which as much of the ethanol as possible was removed by pipette aspiration without disturbing the mRNA pellet. The pellets were then subjected to additional centrifugation steps of 1 minute at 12 000 ×g (4^{o}C) followed on by aspiration of residual ethanol until all of the ethanol had been removed. The pellets were resuspended in 10 µl of Ambion® nuclease-free water (Life Technologies, CA, USA) and a 1:10 dilution was made for quantification by spectrophotometry using the NanoDrop 1000. Once quantified, the mRNA was assessed by formaldehyde-agarose gel electrophoresis (appendix A2-4). Pure, non-degraded mRNA was aliquoted in 2 µg aliquots and stored at -80^{o}C until needed.

2.4 Transfection of Huh7 cells to assess HBsAg expression

The human hepatoma-derived cell line, Huh7 (Nakabayashi *et. al.*, 1982), was maintained in Gibco® DMEM (Life Technologies, CA, USA) supplemented with 10% heat-inactivated FCS, penicillin (10 000 units/ml) and streptomycin (100 µg/ml). Cells were seeded at ~40% confluency (38 000 cells/well) in a 48 well culture dish in antibiotic-free Gibco® DMEM supplemented with 10% heat-inactivated FCS. The seeded cells were incubated at 37^{o}C and 5% CO_2 O/N (~20-24 hours) or until reaching ~80% confluency before proceeding with transfection. Transfection efficiency was measured by the inclusion of pDNA/mRNA expressed to produce eGFP which was then visually assessed using standard fluorescence microscopy on the Zeiss

Axio Imager inverted fluorescence microscope (Zeiss, Germany) and the AxioVision (V3.8.1) imaging software.

2.4.1 pDNA Transfections

Seeded cells were transfected with 250 ng/well each of pmRNA-CMV-MCS, pmRNA-CMV-eGFP, pmRNA-CMV-LHBs or pmRNA-CMV-SHBs. An HBV replication-competent plasmid, pCH9-3091, was used as a positive control (Nassal, 1992). pmRNA-CMV-MCS was used as a mock control and pmRNA-CMV-eGFP was used for recording transfection efficiency. DNA transfections were performed using Lipofectamine™ 3000 reagent (Invitrogen, CA, USA) where the protocol was modified to accommodate a 48 well culture dish format. The transfection mix contained 250 ng of pDNA, 0.5 µl of P3000™ reagent and 0.4 µl of Lipofectamine™ 3000 in a total volume of 50 µl per well made up in Opti-MEM™ (Thermo-Scientific, MA, USA). The transfection reactions were mixed by vortexing for at least 3 seconds and then left to incubate for 20 minutes at RT before being added drop-wise to the cells. Otherwise, the protocol provided by the manufacturer was followed as instructed.

Cells were left to incubate in the transfection mix for 8 hours at 37^{o}C and 5% CO_2 before the medium was replaced with fresh, pre-warmed Gibco® DMEM (Life Technologies, CA, USA) supplemented with 10% heat-inactivated FCS, penicillin (10 000 units/ml) and streptomycin (100µg/ml). Transfected cells were incubated for a further 48 hours before being processed for intracellular and secreted HBsAg detection.

2.4.2 mRNA Transfections

Seeded cells were transfected with 500 ng/well each of eGFP mRNA or TriLink eGFP mRNA to serve as transfection efficiency controls. TriLink Biotechnologies offers a service in the production of high-quality mRNA transcripts. Here we used their services to produce eGFP mRNA for use as a positive control. The eGFP mRNA produced from pmRNA-CMV-eGFP was also included in a transfection experiment prior to the initiation of *in vitro* mRNA transcript synthesis to establish the produced mRNA is translated using the lipofection method of transfection. An untransfected control was used as a negative control. Experimental cell samples were transfected with 500 ng/well of either LHBs mRNA or SHBs mRNA. As a positive control for immunofluorescence pCH9-3091 (Nassal, 1992) was used at a dilution of 150 ng/well. The mRNA transfections were performed using Lipofectamine^{TM} 3000 (Invitrogen, CA, USA) applying a protocol similar to that used for pDNA transfections (section 2.4.1) in a 48 well culture dish with the exception that P3000^{TM} reagent was excluded from the transfection mix. The transfection reactions were mixed by vortexing for at least 3 seconds and then left to incubate for 20 minutes at RT before being added drop-wise to the cells. Otherwise the protocol provided by the manufacturer was followed as instructed.

Cells were left to incubate in the transfection mix for 8 hours at 37^{o}C and 5% CO_2 before the medium was replaced with fresh pre-warmed Gibco® DMEM (Life Technologies, CA, USA) supplemented with 10% heat-inactivated FCS, penicillin (10 000 units/ml) and streptomycin (100 μg/ml). Transfected cells were incubated for a further 48 hours before processing for detection of intracellular and secreted HBsAg.

2.5 Detection of HBsAg

Intracellular LHBs and SHBs was detected using immunofluorescence performed according to the protocol in appendix A2-3 and modified according to the optimised primary antibody incubation conditions (see section 2.2). Cells were fixed, permeabilized and prepared for immunofluorescence 48 hours post-transfection.

Extracellular HBsAg was detected using an HBsAg-specific sandwich-type enzyme-linked immunosorbent assay (ELISA). The supernatant of transfected cells was harvested 48 hours post-transfection and processed using the Monolisa^{TM} HBsAg ULTRA kit (Bio-Rad, CA, USA) according to the instruction manual provided. Briefly, 100 µl of cell supernatant was added to respective wells on a 96-well microtiter plate coated with murine monoclonal antibodies specific for the S domain of HBsAg present in both LHBs and SHBs. A half-volume (50 µl) of Conjugate Solution was dispensed into each well and left to incubate, covered, in a 37^{o}C incubator. The conjugate solution contains monoclonal mouse and polyclonal goat anti-HBsAg antibodies conjugated to peroxidase. The microtiter plates were then washed a minimum of 5 times using Washing Solution (provided by the kit) diluted to 1 × in dH_2O. A 100 µl volume of development solution (provided by kit) was added to each well and the samples left to incubate a further 30 minutes at RT in the dark After the final incubation period, 100 µl of Stopping solution (1 N H_2SO_4; provided by kit) was added and left to incubate for 5 minutes at RT. The ELISA data were extracted as optical density measurements at 450/690 nm using the iMark Microplate Absorbance Reader (Bio-Rad, CA, USA). Captured data was processed using GraphPad PRISM® software (V5.0).

3. RESULTS

3.1 Generation of transcriptional templates and in vitro *expression vectors*

The pmRNA plasmid backbone designed for the purposes of mRNA synthesis (section 2.1.1) contains *Nco*I and *Pme*I restriction sites within the MCS. The *Nco*I site is located at the 3' terminus of the 5' UTR sequence and the *Pme*I site is located at the 5' terminus of the 3' UTR sequence. The restriction sites are absent from *LHBs, SHBs* and *eGFP* making them suitable choices for subcloning (Figure 9). The *Nco*I and *Pme*I restriction sites were introduced at the 5' and 3' termini of *LHBs, SHBs* and *eGFP* by modification of the forward and reverse primer sequences for PCR amplification (Table 2). Similarly, the CMV promoter/enhancer sequence was amplified from the pCI-neo-eGFP plasmid using PCR primers that introduced a *Bst*XI site at the 5' terminus and a *Sac*I site at the 3' terminus of the sequence (Table 2). High purity, homogenous solutions of the *LHBs, SHBs, eGFP* and *CMV* amplicons were obtained and the PCR conditions were considered optimal (Figure 11a). High fidelity amplification of the target sequences was confirmed by Sanger sequencing of the amplicons subcloned into pTZ57R/T backbone (see representative chromatogram in appendix A4-2). The pTZ clones containing sequences confirmed as being correct were prepared for downstream application. On average, out of 4-6 clones selected for sequencing, at least 2 harboured sequences matching the original sequence.

Initially, *LHBs, SHBs* and *eGFP* sequences were excised from the positive pTZ clones and inserted into the pmRNA-MCS-95A backbone. Subcloning of the coding sequences was performed prior to subcloning of the *CMV* because of an *Nco*I site within the promoter sequence

(Figure 9). The final plasmid constructs are described in Table 1 and shown schematically in Figure 7. The CMV promoter/enhancer was included to drive protein expression from the pDNA in transfected Huh7 cells. T7 polymerase directs mRNA transcript synthesis from the T7 promoter downstream of the CMV promoter/enhancer. Using this rationale, the ability for the *LHBs*, *SHBs* and *eGFP* to produce functional protein could be assessed before those same plasmids were used for *in vitro* transcription. Positive clones containing all the critical sequence elements indicated in Figure 7 were identified by restriction mapping. Restriction enzymes were chosen specifically to be diagnostic for the CMV promoter/enhancer and the protein-coding sequences (Table 5) in the correct orientations. Representative digestion reactions of positive clones are shown in Figure 11b.

*Spe*I cleaves the DNA within the CMV promoter/enhancer sequence and does not have any additional restriction sites within *LHBs*, *SHBs* or *eGFP*. *Spe*I is a unique cutter of the plasmid constructs and the presence of two bands within the *Spe*I and *Sca*I double digestion reaction indicates the presence of the CMV promoter/enhancer (Figure 11b). *Apa*LI digests the plasmid constructs at three different sites generating two bands of identical length for pmRNA-CMV-LHBs and pmRNA-CMV-SHBs. The bands are 1250 bp and 500 bp in length (Figure 11b). The third band is diagnostic of the presence of *LHBs* or *SHBs*. Therefore, pmRNA-CMV-LHBs and pmRNA-CMV-SHBs contain the CMV promoter/enhancer and HBsAg-coding sequences as needed. *Eco*RI is a unique cutter and cleaves the DNA immediately downstream of the M13F. *Not*I is an enzyme of the MCS and therefore only cleaves pmRNA-CMV-MCS (Figure 11b). *Bam*HI cuts within *eGFP* and the presence of a second band indicates the insertion of the *eGFP* sequence (Figure 11b). By this analysis, all the plasmid constructs were confirmed

as being correct and containing all the sequence elements needed for later use in mRNA production and *in vitro* expression experiments.

Table 5: Predicted band sizes of positive clones following restriction digestion with multiple sets of restriction enzymes.

Plasmid	Enzymes	Expected Band Sizes (bp)
pmRNA-CMV-MCS	*Spe*I and *Sca*I	1000
		2800
	*Eco*RI and *Not*I	800
		3000
pmRNA-CMV-eGFP	*Spe*I and *Sca*I	1000
		3500
	*Eco*RI and *Bam*HI	2800
		1100
		700
pmRNA-CMV-LHBs	*Spe*I and *Sca*I	950
		3900
	*Apa*LI	3200
		1250
		500
pmRNA-CMV-SHBs	*Spe*I and *Sca*I	950
		3400
	*Apa*LI	2700
		1250
		500

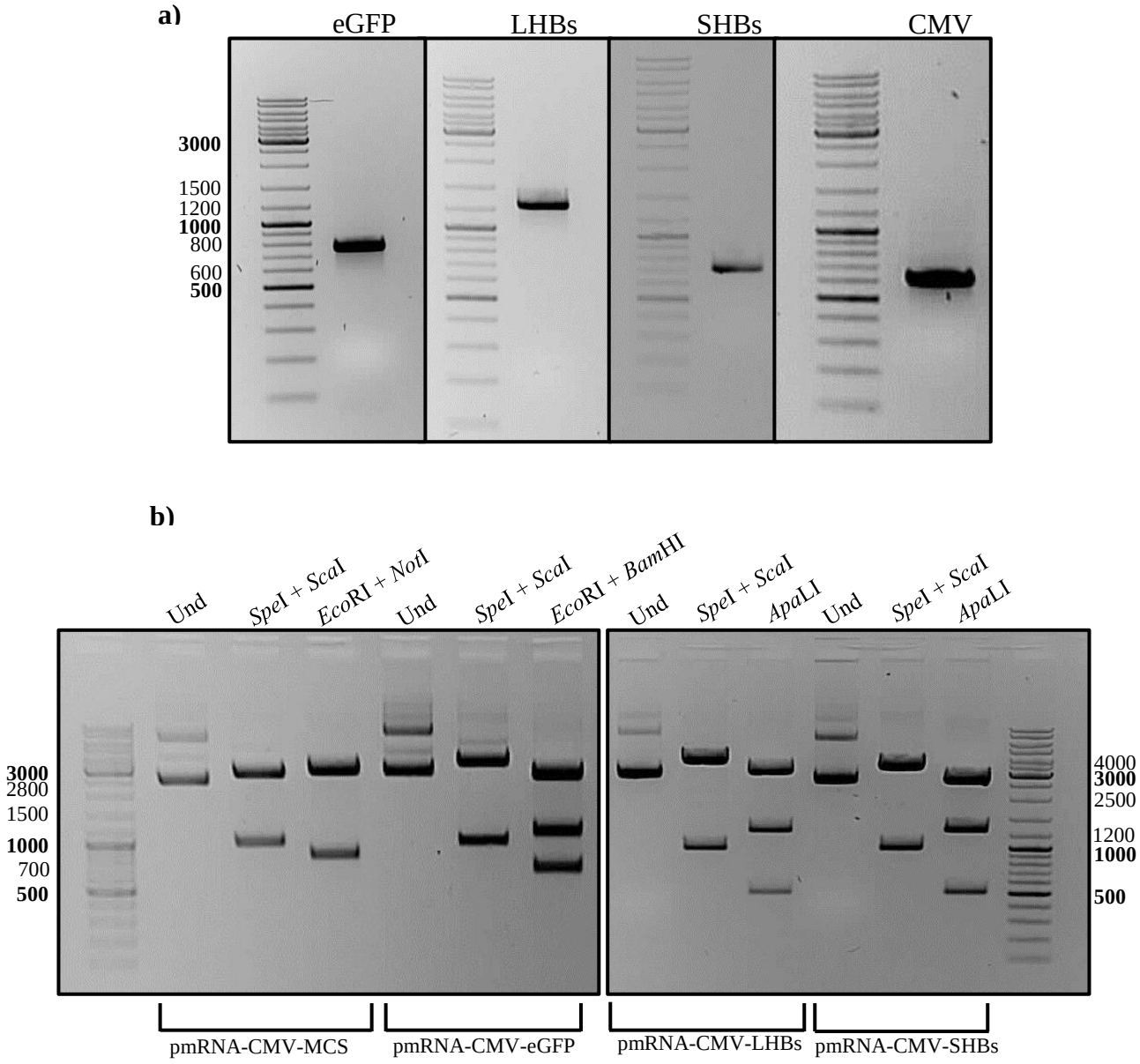


Figure 11: Molecular cloning of mRNA production plasmids and *in vitro* expression vectors. a) Purified PCR amplicons. *LHBs*, *SHBs*, *eGFP* and *CMV* amplicons. b) Restriction mapping of constructed plasmids. Representative restriction digestion reactions of positive clones containing the CMV promoter/enhancer upstream of the T7 promoter and downstream coding sequences are shown. The O'GeneRuler^{TM} DNA ladder mix (Thermo-Scientific, MA, USA) was run alongside the samples and undigested pDNA controls (Und) for size comparison.

3.2 Optimisation of immunodetection of intracellular HBsAg

Immunofluorescent detection of expressed HBsAg present in the cytosol of transfected cells was performed using three primary antibodies: (i) anti-S, (ii) anti-preS1 and (iii) anti-preS2. The use of three antibodies binding to three distinct regions of protein served a dual purpose. Firstly, binding of the antibodies would confirm the presence of intracellular HBsAg expressed from the codon-optimised constructs and secondly, preS-specific antibodies would distinguish between LHBs and SHBs. The antibodies were used at dilutions recommended by the manufacturer but the incubation conditions for each antibody needed to be optimised. Following the application of the standard immunofluorescent imaging protocol (Appendix A2-3) with one hour incubation in primary antibody at RT, no fluorescent signal was detectable above background.

The imaged cells transfected with pmRNA-CMV-LHBs or pmRNA-CMV-SHBs were indistinguishable from the untransfected control and the control excluding primary antibody, indicating that non-specific binding of secondary antibody was not occurring. Instead, we reasoned that the primary antibodies were not binding sufficiently to the protein or that the protein was not being expressed at detectable levels from the pmRNA-CMV-LHBs or pmRNA-CMV-SHBs. For optimisation independent of transfection we used HepG2.2.15 cells which constitutively express LHBs, MHBs and SHBs for optimisation of primary antibody incubation conditions. HepG2.2.15 cells are stably transfected with the complete genome of HBV and therefore constitutively express all three forms of HBsAg (Sells *et. al.*, 1987). Thus, if we were able to detect HBsAg in HepG2.2.15 cells but failed to detect the same protein in transfected

cells using the same protocol, we could conclude that there was a problem with the coding sequences.

Primary antibody-binding can be affected at two steps of the protocol, at the blocking step and at the incubation step. Three conditions were evaluated for each of the three antibodies, namely (i) incubation in primary antibody O/N at 4^{o}C in a humidified chamber, (ii) incubation in blocking buffer, (1% w/v) BSA O/N at 4^{o}C in a humidified chamber followed by an incubation in primary antibody for 1 hour at RT and (iii) incubation in primary antibody for 1 hour at RT. Anti-preS1 and anti-preS2 antibodies were used at a 1:100 dilution while anti-S was used at a 1:50 dilution. Anti-preS1 and anti-S antibodies bind optimally following O/N incubation at 4^{o}C in a humidified container. Anti-preS2 binds optimally when the cells are blocked in 1% BSA O/N at 4^{o}C in a humidified container followed by one hour incubation in primary antibody at RT the following day (Figure 12). These conditions were applied in all later immunodetection protocols wherein the three antibodies were used. The lack of detectable signal above background in the control sample wherein incubation with primary antibody was omitted indicates there is no non-specific binding of secondary antibody within the samples and signal therefore originates from detected protein only.

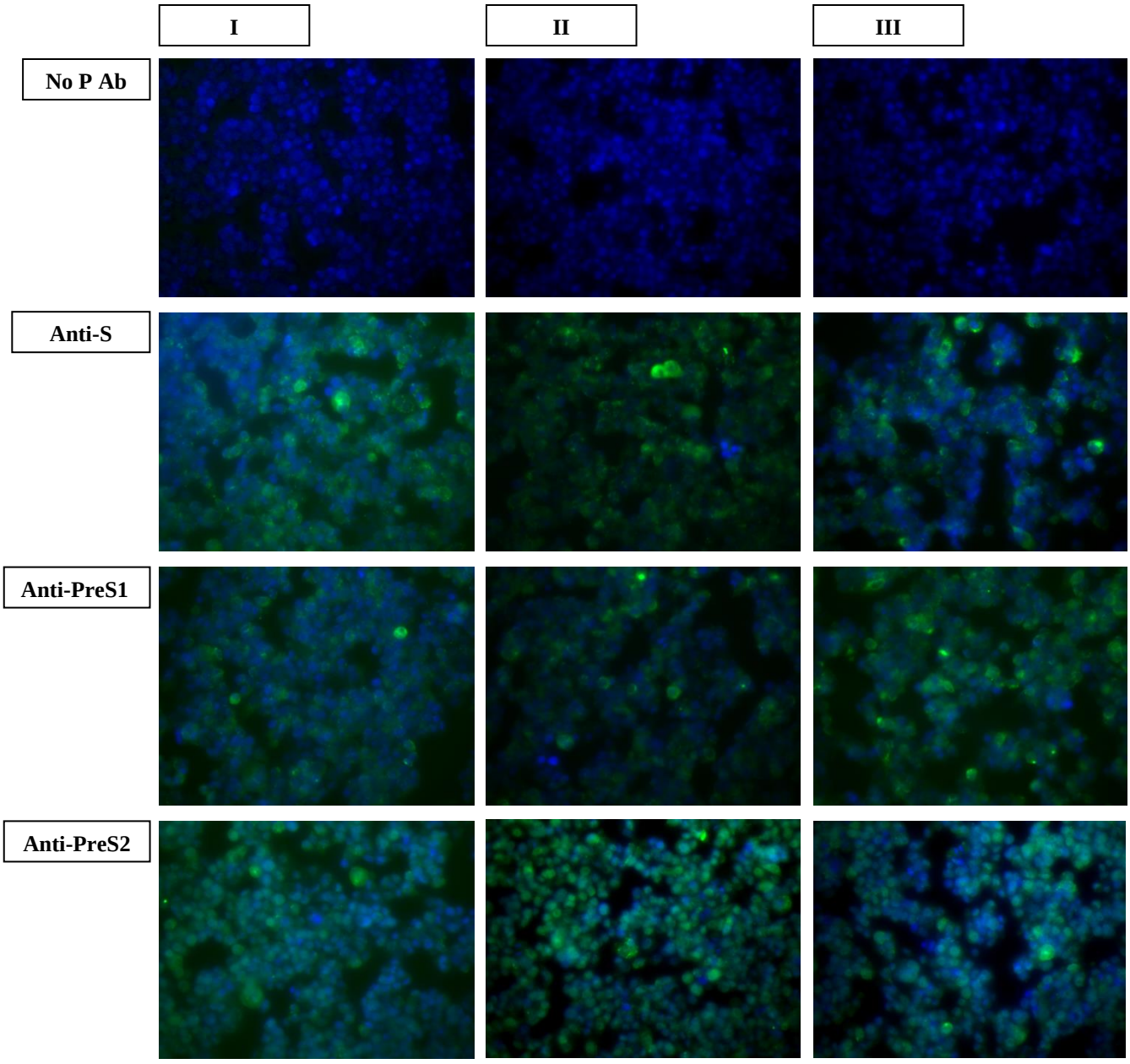


Figure 12: Optimisation of immunofluorescence protocol using HepG2.2.15 cells. Three primary antibodies binding epitopes present within the PreS1, PreS2 and S regions were assessed for optimal binding and specificity using three different incubation or protocol conditions. (I) Incubation in primary antibody O/N at 4^{o}C**.** (II) Incubation in blocking buffer (1% (w/v) BSA in PBS) O/N at 4^{o}C before incubating in primary antibody for 60 minutes at RT. (III) Incubation in

blocking buffer (1% (w/v) BSA in PBS) for 60 minutes at RT and then incubation in primary antibody for 60 minutes at RT. The anti-preS1 and anti-preS2 antibodies were used at a 1:100 dilution; the anti-S antibody was used at a dilution of 1:50. A goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor® 488 was used at a dilution of 1:200. A control sample in which a primary antibody incubation step was omitted (No P Ab) was included to demonstrate specificity of binding. Images were captured using the Zeiss Axio Imager inverted fluorescence microscope (Zeiss, Germany) and the AxioVision (V3.8.1) imaging software.

3.3 CMV-driven production of native protein from subcloned genes

Initially, Huh7 cells were transfected with pmRNA-CMV-eGFP (Figure 13). Fluorescent eGFP was detected indicating that the pmRNA-CMV plasmid construct design (Figure 7) was able to produce native protein despite the additional, unconventional sequence elements included in the mRNA transcripts upstream of the translational start site. The plasmids expressing eGFP also allowed for visual assessment of transfection efficiency using Lipofectamine™ 3000. To assess whether codon-optimised *LHBs* and *SHBs* inserted into the pmRNA-CMV backbones are expressed to produce LHBs and SHBs respectively, cultured Huh7 cells were transfected with pmRNA-CMV-LHBs and pmRNA-CMV-SHBs using the same transient transfection protocol used with pmRNA-CMV-eGFP. As a positive control for HBsAg expression, Huh7 cells were also transfected with pCH9-3091 (Nassal, 1992). Expression of LHBs, MHBs and SHBs from pCH9-3091 in cells of hepatic origin is driven by endogenous HBV promoters and therefore likely to occur at levels comparable to those noted during physiological HBV infection.

Transfection of Huh7 with pmRNA-CMV-MCS as a mock control validated that HBsAg expressed in Huh7 is only detected when HBsAg-coding DNA was present.

Secreted HBsAg (LHBs and SHBs) was detected within the medium of Huh7 cells 48 hours post-transfection by HBsAg-specific ELISA. The HBsAg ELISA detects only S protein region and therefore does not discriminate between LHBs and SHBs. SHBs expressed from pmRNA-CMV-SHBs is secreted at levels comparable to those of the positive control whereas LHBs was not detected in the media of cells transfected with pmRNA-CMV-LHBs (Figure 14). The data shown are mean OD_{490nm} readings representative of experiments performed in triplicate with SEM shown (Figure 14). SHBs is thus readily secreted from transfected cells whereas LHBs is not. However, LHBs is expressed from pmRNA-CMV-LHBs but appears to be retained within the intracellular space (Figure 15).

Huh7 cells transiently transfected with pmRNA-CMV-LHBs exhibited positive immunofluorescent signals when incubated with anti-preS1, anti-preS2 and anti-S antibodies (Figure 15). The same result was obtained for Huh7 transfected with pCH9-3091 (Nassal, 1992). In contrast, transfection with pmRNA-CMV-SHBs resulted in detectable signal only when incubated with anti-S antibodies (Figure 15) consistent with the absence of preS1 and preS2 epitopes. We were able, therefore, to distinguish between LHBs and SHBs using the different primary antibodies. By qualitative assessment, LHBs appeared more abundant in the intracellular space than SHBs but anti-S antibodies detected far less of the S-domain in cells transfected with pmRNA-CMV-LHBs than pmRNA-CMV-SHBs (Figure 15). It cannot be excluded that anti-S binds its target with lower affinity than anti-preS1 or anti-preS2 which may account for the lower than expected levels of intracellular LHBs in the cells transfected with pmRNA-CMV-LHBs. In conclusion, native LHBs and SHBs is expressed from codon-optimised *LHBs* and *SHBs*

respectively and the pmRNA-CMV-LHBs and pmRNA-CMV-SHBs constructs were therefore used for *in vitro* transcription of synthetic LHBs and SHBs mRNA.

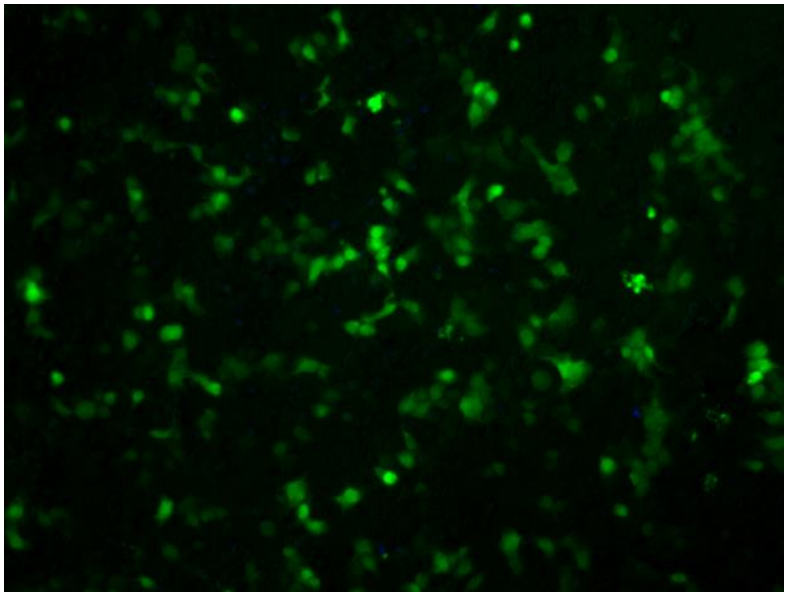


Figure 13: Efficiency of transient transfection protocol measured by eGFP expression. Huh7 cells were transfected with pmRNA-CMV-eGFP and transfection efficiency was visually assessed by the apparent frequency of eGFP-positive cells within the overall population. Green fluorescence was detected using the Zeiss Axio Imager inverted fluorescence microscope (Zeiss, Germany) and the AxioVision (V3.8.1) imaging software. Shown here is a representative image of three separate experiments performed in triplicate. Cells are shown at 200× magnification.

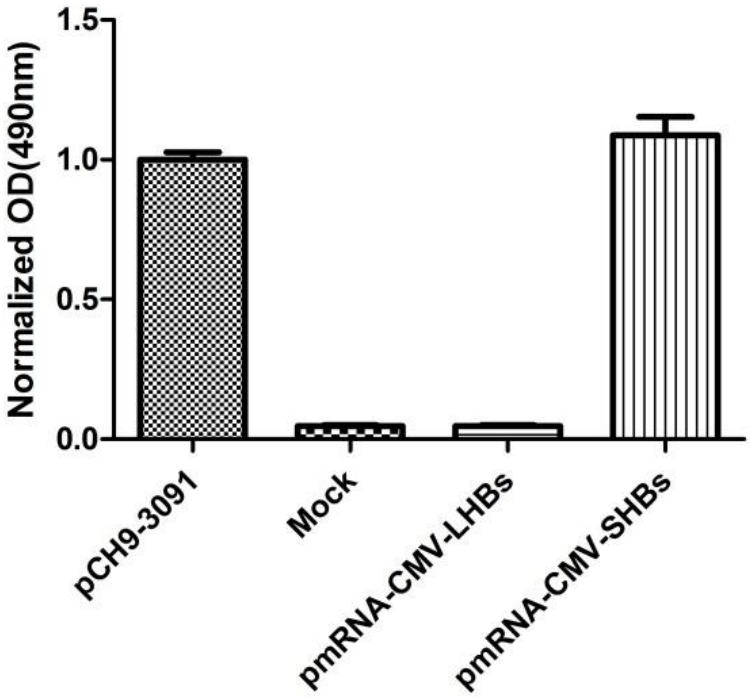


Figure 14: Detection of HBsAg secreted from transfected Huh7. Huh7 cells were transiently transfected with pmRNA-CMV-LHBs, pmRNA-CMV-SHBs, pmRNA-CMV-MCS (mock) and pCH9-3091 (positive control). The media removed from the cultures of transfected cells 48 hours post-transfection was subjected to an HBsAg ELISA for quantitative assessment of secreted HBsAg. Data are represented as mean optical density measurements (at 490nm) normalised to the mean measurement for the positive control (pCH9-3091) (+/- SEM, n=3).

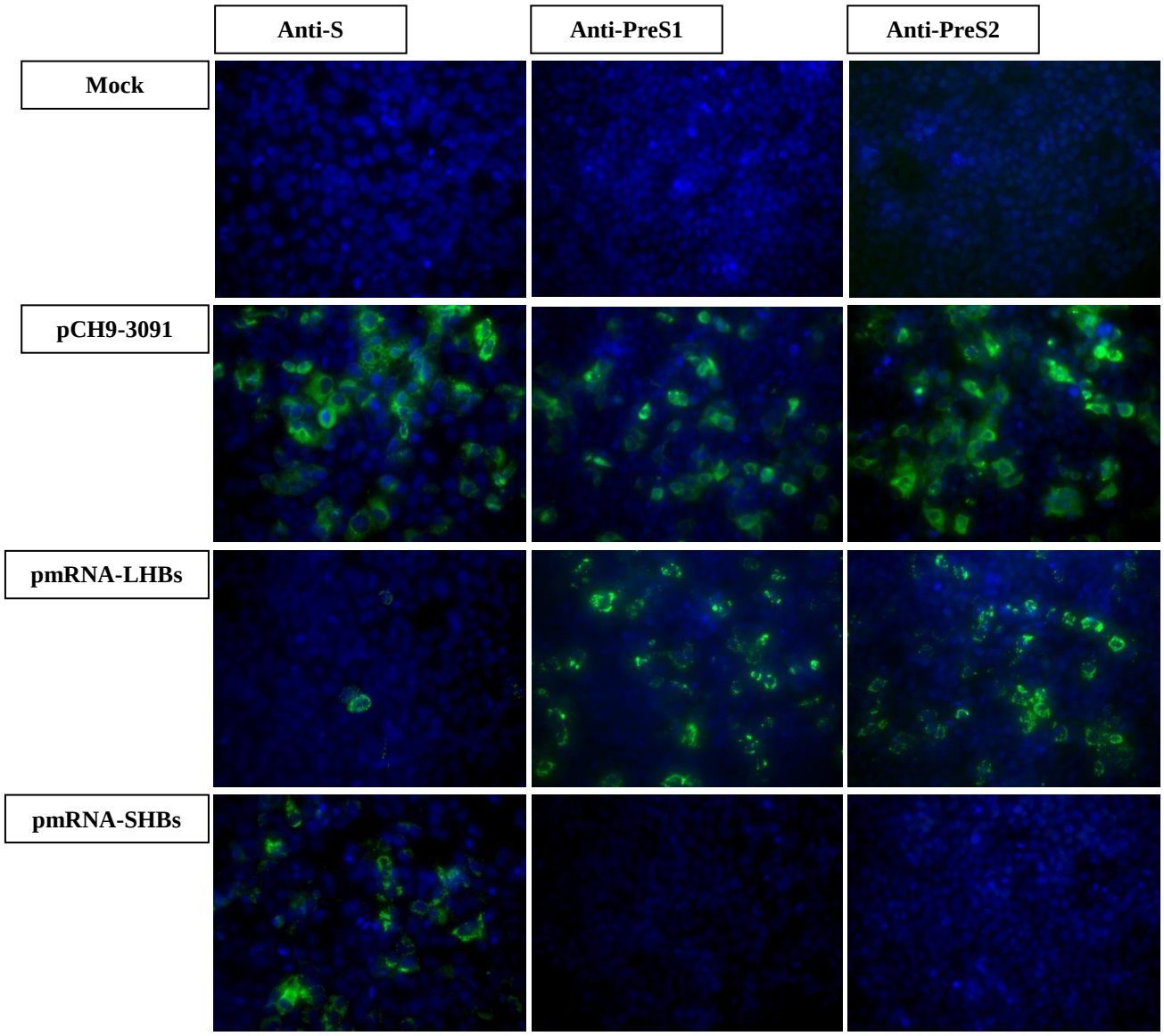


Figure 15: Immunodetection of intracellular HBsAg. Immunodetection of intracellular LHBs and SHBs using anti-preS1, anti-preS2 and anti-S primary antibodies and an Alexa Fluor®-488 conjugated secondary antibody. Green fluorescence was measured using the Zeiss Axio Imager inverted fluorescence microscope (Zeiss, Germany) and the AxioVision (V3.8.1) imaging software. Images shown are representative of experiments repeated in triplicate. Images are shown at 200× magnification and exposure for green fluorescence (FITC Filter) was kept constant at 500 ms. The cells were counterstained with DAPI.

3.4 mRNA Synthesis

Prior to inclusion in the T7 *in vitro* transcription reaction, the pDNA has to be linearised by restriction digestion. The linearisation step is critical to the success of the transcription reaction because incompletely linear DNA would result in a heterogeneous population of mRNA transcripts. The positioning of the linearisation restriction site dictates the universal termination point for all transcripts and ensures that they are all of similar length. The design of our pmRNA backbone includes a poly (A:T) sequence on the coding DNA strand. The stretch of 95 'T' nucleotides is transcribed into a poly (A) tail mimic by *in vitro* transcription (Figure 10).

Ideally, the final rNTP of the mRNA transcript should be rATP because the final dNTP on the linear pDNA is 'T'. An attempt to achieve this was made by utilising *Sap*I, a Type IIS restriction enzyme, for template linearisation. The *Sap*I recognition site is just downstream of the poly (A:T) sequence while its restriction site is 5 nt into the poly (A:T) such that linearisation would result in ~90 thymidine nucleotides on the coding strand. However, there are two *Sap*I recognition sites within the pmRNA constructs spaced approximately ~350 bp apart. Instead of cutting at both sites as expected, *Sap*I usually cleaved the DNA only at one or the other restriction site (Appendix A4-1). The DNA template produced from *Sap*I linearisation was a mixture of two different sizes which proved problematic for mRNA synthesis wherein use of the template mixture produced a heterogeneous mixture of transcripts of varying lengths (Appendix A4-1b). As an alternative to *Sap*I, there is a *Hind*III restriction site ~8 nt downstream of the termination point of the poly (A:T). We opted to use *Hind*III instead because it is a robust, broadly used Type II restriction enzyme. Linearisation using *Hind*III was highly efficient and

only a negligible amount of DNA template is assumed to remain undigested, undetectable by standard agarose gel electrophoresis in a 1% agarose gel (Figure 16a).

When setting up *in vitro* transcription reactions and handling the mRNA product, great care needed to be taken to ensure that the mRNA did not become degraded. The mRNA was far less stable than DNA and is highly susceptible to digestion by ubiquitous RNases, a major contaminant of all laboratory surfaces and equipment. The mRNA production procedures were therefore carried out in strictly controlled environments using equipment and solutions reserved specifically for use with RNA. Formaldehyde-agarose gel electrophoresis was used not only to assess the homogeneity of the mRNA transcript population but also to ensure that the mRNA is intact. By our method, a pure, homogenous solution of mRNA was produced from each pmRNA template that was of high quality and contains no degradation products (Figure 16b). Within the linearised template there was only a single T7 promoter followed on by a single translational start site (Figure 7). It is reasonable to assume that only one type of mRNA transcript would be synthesised and that transcript would contain the sequence encoded for by the DNA template. Thus, no RNA ladder was included for size determination but differences in mRNA transcript length are evident in Figure 16b because of differences in the length of *LHBs, SHBs and eGFP*. Earlier, total RNA extracted from Huh7 showing the 18S and 23S was used to estimate mRNA transcript sizes (data not shown). The mRNA production protocol was successful and is considered optimal within the limitations of the kit used.

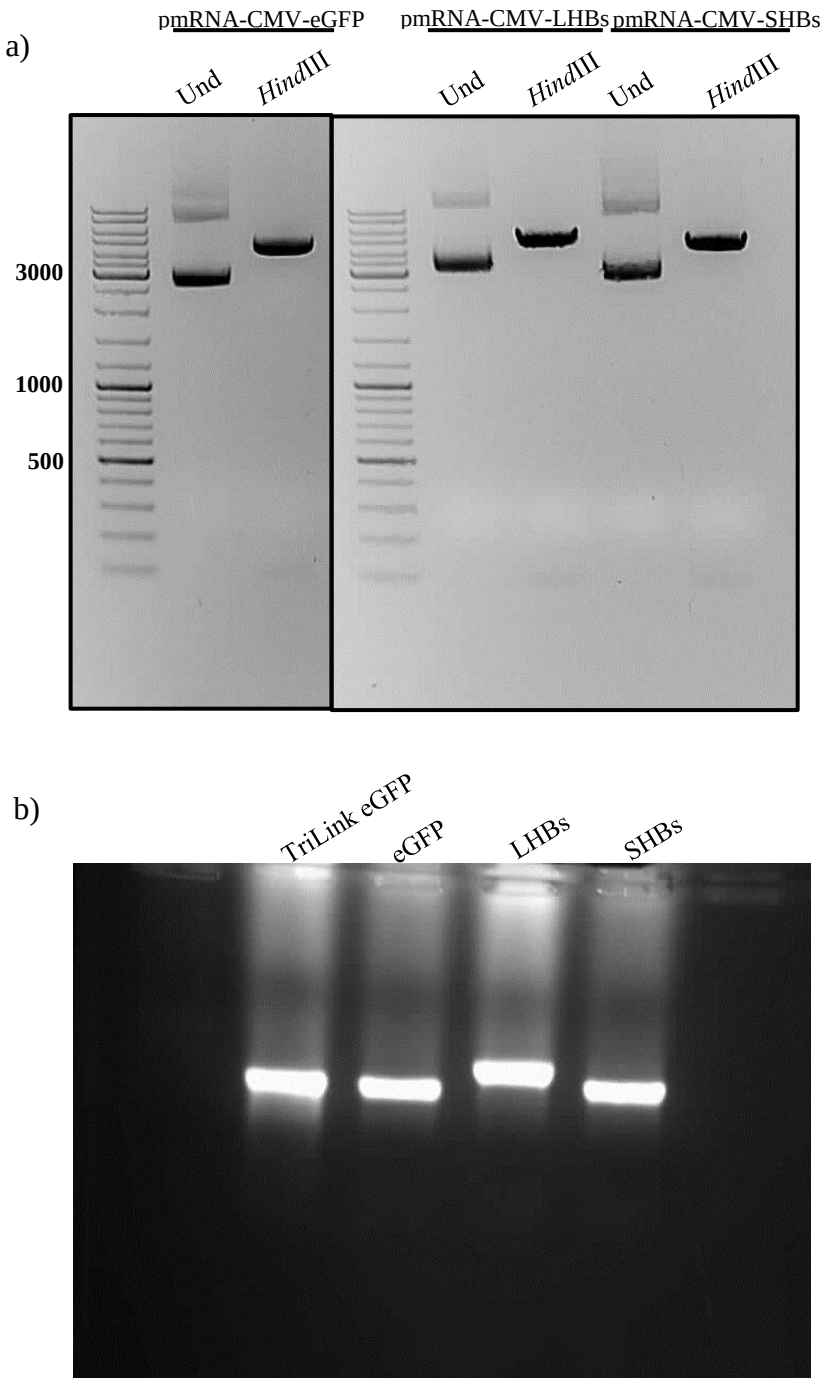


Figure 16: mRNA Synthesis. a) Linearisation of mRNA production plasmids. The mRNA production plasmids pmRNA-CMV-eGFP, pmRNA-CMV-LHBs and pmRNA-CMV-SHBs were linearised by digestion with *Hind*III. An undigested control was included (Und) to demonstrate linearisation. High purity linearised pDNA was included as the template for

synthetic mRNA production by *in vitro* T7 transcription. b) *In vitro* transcribed mRNA. Synthetic mRNA produced from the linearised templates visualised by EtBr-staining in a 1% formaldehyde-agarose gel run at 80 V for 45 minutes. eGFP mRNA Produced by TriLink Biotechnologies, CA, USA (TriLink eGFP) was used as a quantity loading control and mRNA integrity control.

3.5 Translation of the synthetic mRNA transcripts in transfected Huh7 cells

The Huh7 cells were transfected with 0.5 μg of mRNA per well in a 48 well culture dish using Lipofectamine^{TM} 3000. Lipofection is not an ideal method of transfection with mRNA transcripts; it is more common to use Nucleofection or electroporation to minimise the time of incubation before transfection and in doing so minimise the risk of degradation. Using *eGFP* mRNA produced by TriLink Biotechnologies or produced from pmRNA-CMV-eGFP, Huh7 cells were transfected using the Lipofection method and eGFP expression was assessed using fluorescence microscopy (Figure 17). Increased frequency of GFP-positive cells following transfection with TriLink *eGFP* mRNA as opposed to *eGFP* mRNA produced from pmRNA-CMV-eGFP could indicate a greater translational capacity of the TriLink mRNA (Figure 17). The TriLink mRNA contains similar sequence elements to the pmRNA templates: (i) the 5' and 3' UTRs as well as the transcribed poly (A:T) sequence (albeit only ~50 'T' nucleotides are present on the coding strand. However, the synthesis protocol is proprietary and it is not clear which method of production or purification is used. It is likely that a step included in the preparative protocols results in greater translational capacity over the mRNA produced by the method here described. Green fluorescent protein is produced from the eGFP produced by our

means indicating that synthetic transcripts are translatable albeit at lower levels than TriLink *eGFP* mRNA (Figure 17).

HBsAg-ELISA of Huh7 cell medium after transfection with LHBs and SHBs mRNA reveals a similar pattern of HBsAg secretion to that noted for Huh7 transfected with pmRNA-CMV-LHBs and pmRNA-CMV-SHBs (Figure 18). Though not ideal, a lack of an mRNA-based positive control necessitated the use of pCH9-3091 to control for LHBs and SHBs detection intracellularly and within the cell media. SHBs secreted from cells transfected with *SHBs* mRNA is at levels comparable to the positive control whereas the secreted levels of protein resulting from transfection with LHBs mRNA are comparable to the untransfected, negative control (Figure 18). The most robust primary antibody as identified in previous experiments, namely anti-preS2, was used for immunodetection of intracellular LHBs. LHBs protein was detected within the intracellular space of Huh7 transfected with *LHBs* mRNA (Figure 19); however, the frequency of LHBs-positive cells was far lower than that noted after transfection with pmRNA-CMV-LHBs. The LHBs translated from *LHBs* mRNA was also present at far lower levels within the transfected cells than that produced from pCH9-3091 (Nassal, 1992). These results show that both LHBs and SHBs proteins were translated from the synthetic mRNA transcripts. Therefore it was concluded that by our method, native protein is producible from synthesised mRNA *in vitro*. This is important for *in situ* antigen production whereby the native fold of a protein maintains structural epitopes important for the generation of functional antibodies.

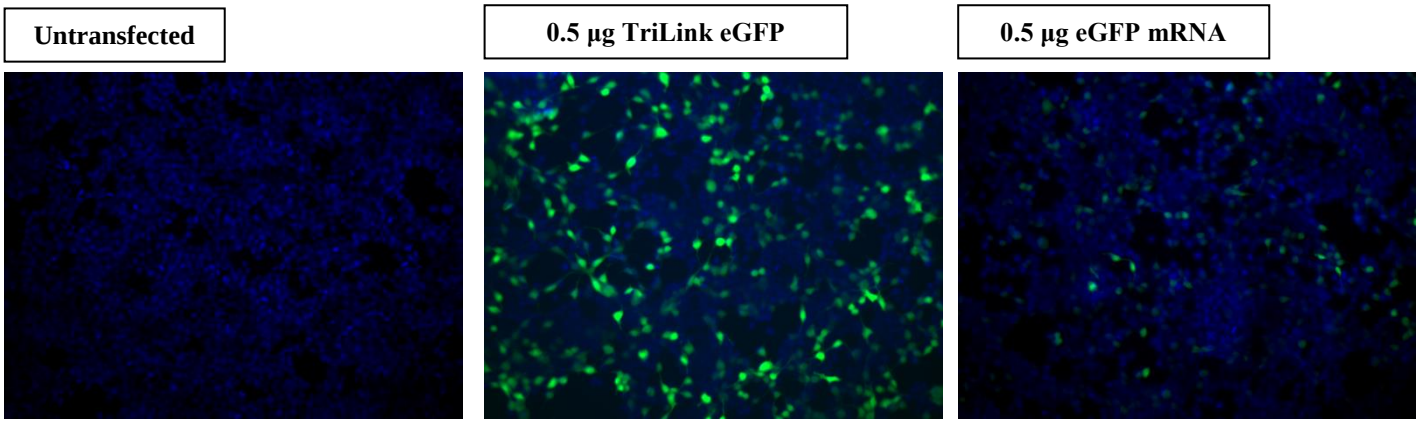


Figure 17: Transfection of Huh7 cells with eGFP mRNA. Huh7 cells were transfected with 0.5 µg TriLink eGFP and eGFP mRNA respectively. Transfection efficiency was visually assessed by determining the number of eGFP-positive cells within the overall transfected population. Green fluorescence was detected using the Zeiss Axio Imager inverted fluorescence microscope (Zeiss, Germany) and the AxioVision (V3.8.1) imaging software. Cells are shown at 100× magnification.

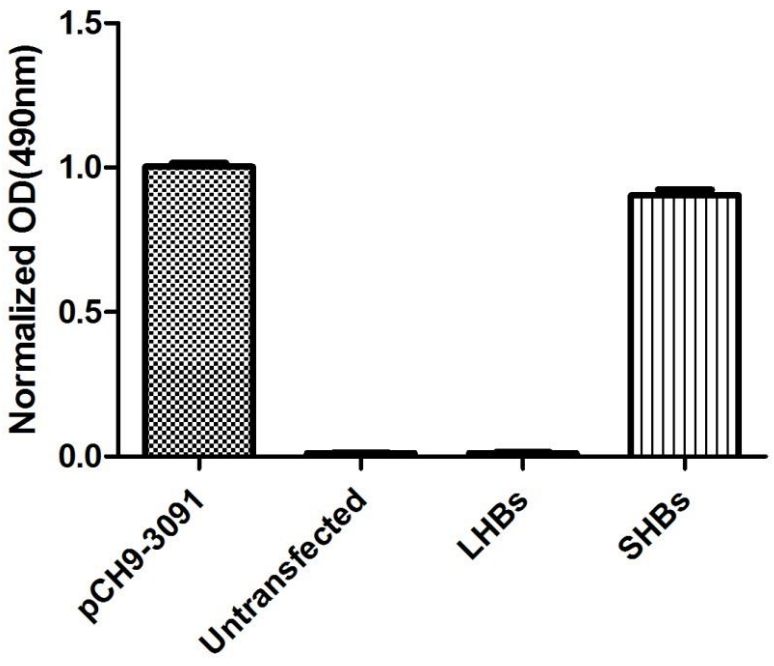


Figure 18: Detection of secreted SHBs from mRNA-transfected Huh7 cells. Huh7 cells were transiently transfected with LHBs mRNA, SHBs mRNA and pCH9-3091 pDNA (positive control). The media removed from the cultures of transfected cells 48 hours post-transfection were subjected to an HBsAg ELISA for quantitative assessment of secreted HBsAg (SHBs). Data are represented as mean optical density measurements normalised to the mean measurement for the positive control (pCH9-3091) (+/- SEM, n=9). Untransfected cells were used as a negative control.

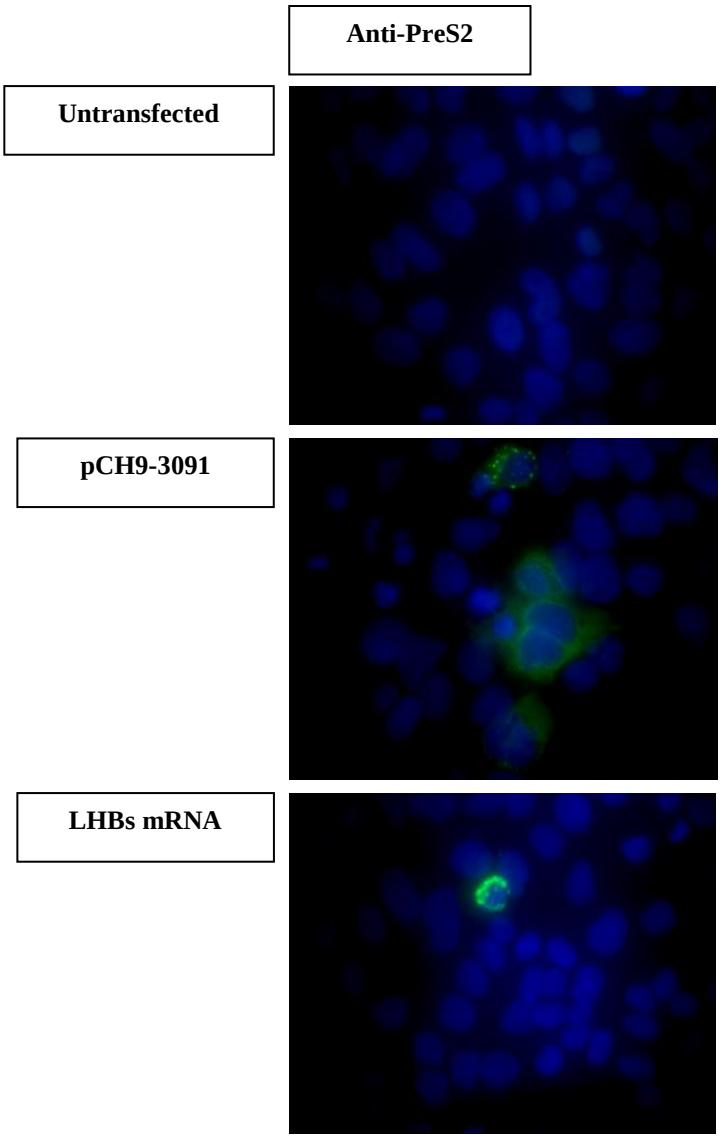


Figure 19: Detection of intracellular LHBs translated from synthetic mRNA in Huh7. Immunodetection of intracellular LHBs using an anti-preS2 primary antibody and an Alexa Fluor®-488 conjugated secondary antibody. Green fluorescence was measured using the Zeiss Axio Imager inverted fluorescence microscope (Zeiss, Germany) and the AxioVision (V3.8.1) imaging software set on the FITC (Green) filter. Images are shown at 400× magnification and exposure for green fluorescence (FITC filter) was kept constant at 1.5 s. Cells are counterstained with DAPI. LHBs is detected in the cytosol of cells transfected with pCH9-3091 (positive control) and *LHBs* synthetic mRNA.

4. DISSCUSSION

4.1 ***Synthesis of LHBs and SHBs mRNA***

Vaccine formulations containing antigen-encoding mRNA have proven antiviral potential and increased research efforts will further strengthen development of this field. Our study relates to the adaptation of antiviral mRNA vaccination strategies for dual application in anti-HBV prophylaxis and therapy for CHB. This study describes the developmental stages of an anti-HBV mRNA-based vaccine wherein we successfully produced high quality LHBs- and SHBs-encoding synthetic mRNA transcripts that were translated in cultured cells to produce detectable LHBs and SHBs protein.

The method of mRNA production by *in vitro* transcription is the most common approach to mRNA synthesis. However, any fault in the design of the template pDNA or in the production process itself can result in the inability to produce mRNA of sufficient quantity and quality, as well as mRNA having compromised translational capacity. There are several factors critical to the rational design of an mRNA production template and process which include: (i) translatability of the encoded mRNA, (ii) choice of linearisation enzyme, (iii) inclusion of application-appropriate regulatory sequences (5' and 3' UTRs) and (iv) structural resemblance of the mRNA transcripts to endogenous mRNA.

Codon-optimisation of the protein-coding sequence was a crucial aspect of the rational design process. The genetic code is degenerate and thus as many as six different codons can encode a single amino acid. The tRNA-gene copy numbers within the genomes of various multicellular eukaryotic organisms including *Homo sapiens* and *Drosophila melanogaster*

revealed that differential codon usage in eukaryotes is significant (Kanaya *et. al.*, 2001). Biased codon usage can diminish interspecies translational efficiency of exogenous DNA or synthetic transcripts with consequences for antigen production from nucleic acids in vaccine formulations. The immunogenicity of a polynucleotide vaccine encoding the L1 and L2 capsid proteins of human papillomavirus was significantly improved after codon optimisation of the protein-coding sequences (Liu *et. al.*, 2001). Heterologous L1 and L2 proteins produced from the modified DNA sequence induced neutralising, protective antibody responses and potent CTL responses in immunized mice. Codon-optimisation is readily applied in the production of sequence-engineered templates for synthesis of mRNA transcripts used as high-expression genetic vectors for antigen expression (Thess *et. al.*, 2015). Here, the coding sequence for LHBs and SHBs were codon-optimised for expression in humans (primary organism) and in mice (secondary organism).

Codon-optimisation for optimal production of heterologous protein relies on a central assumption that the incorporation of frequently used codons to replace rarer codons in a given sequence results in high level protein expression. A seminal study carried out in 2009 (Kudla *et. al.*, 2009) showed that applying randomly selected codon usage to generate a library of 154 *GFP* genetic variants resulted in as much 250-fold variation in functional GFP production when comparing the low-expression and high-expression library constructs. Interestingly, low level production of GFP did not correlate with rare codon usage but rather cell fitness. It appears that mRNA transcripts containing high frequencies of rare codons compromised cell viability resulting in lower fluorescence (GFP expression). Within the context of generating mRNA for optimal antigen production, one cannot rely on codon-optimisation alone. Specific regulatory

sequences as well as inclusion of the Kozak sequence (Kozak, 1978) may act synergistically to promote mRNA stability and optimal translational capacity (Pascolo, 2008).

The translational start codon is included within the Kozak sequence and the translational stop codon concludes the codon-optimised protein-coding sequence at the 3' terminus. Between the T7 promoter and the translation initiation signal, as well as between the translation termination signal and the start of the poly (A), are stabilization and destabilization sequences that contribute to the highly regulated half-life of mRNA transcripts (Ross, 1995). The mRNA is stabilised by pyrimidine-rich sequences in the 3' UTR which are responsible for binding to the 'α-complex' (Holcik & Liebhaber, 1997). Therapeutic mRNA molecules and those used for prophylaxis commonly include the 5' and 3' UTRs of the *β*- and *α*-globin genes respectively because they have been found to promote long-term stability of mRNA in anucleate red blood cells (Malone *et. al.*, 1989). The UTR sequences act to promote stability concurrently with the length of the poly (A). An optimal length of the poly (A) has been determined as being more than 100 nt but no less than 30 nt (Mockey *et. al.*, 2006; Holtkamp *et. al.*, 2006). In the design of our pDNA templates, the 5' UTR of *β*-globin and 3' UTR of *α*-globin were included and a poly (A) tail of approximately 95 nt was transcriptionally introduced to maximise stability of the synthesised mRNA.

The length of the poly (A) tail may have a significant impact on the success of an mRNA vaccination construct. Typical eukaryotic mRNAs have a poly (A) tail between 80 and 200 nt long (Dreyfus & Regnier, 2002), while prokaryotes produce RNA with a poly (A) of between 30 and 60 nt if at all (Sarkar, 1997). The innate immune system uses poly (A) tail length as a distinguishing molecular factor between endogenous and pathogen-associated mRNAs. mRNA lacking a long poly (A) upregulates IL12 secretion by transfected APCs (Koski *et. al.*, 2004).

IL12 acts to polarise T-cells towards a T_H2 response and in doing so promote B-cell expansion, increased secretion of IgM as well as activation of macrophages. The T_H2 response is skewed towards supporting humoral immunity and limiting the spread of extracellular pathogens; induction of high-level IL12 could elicit protective antibody responses in vaccine recipients. Maximisation of IL12 secretion by maturing DCs requires two signals, a priming signal from IFN-γ and a secondary signal from binding to CD40L. Immunogenic mRNA can substitute the priming signal and thus, poly (A) length is an important consideration for mRNA vaccine design (Koski *et. al.*, 2004). Vaccine efficacy would depend on balancing translational efficiency with intrinsic adjuvanticity. In the context of the study here described, the impact of the poly (A) tail length on translatability, and the relevance thereof in the context of vaccine design will be discussed in greater detail in section 4.2.

The synthetic mRNA constructs have been designed to closely mimic endogenous mRNA molecules by transcriptional inclusion of the poly (A) and a 5' cap dinucleotide analogue. Inclusion of the standard Cap analogue in the *in vitro* transcription reaction typically results in around 80% capping efficiency. Standard Cap can be incorporated into the transcript in one of two orientations. Only the orientation whereby the 3' OH of the pentose, which harbours the unmethylated guanine, is used for phosphodiester linkage to the nascent mRNA produces translatable mRNA. Thus, only 40% of transcripts produced would be translated into protein (Stepinski *et. al.*, 2001). The use of an anti-reverse cap analogue (ARCA) overcomes this problem and allows for generation of 80% translatable transcripts with a five-fold increase in protein yield over standard-capped mRNA (Stepinski *et. al.*, 2001). The ARCA here used is included in the mMessage mMachine® T7 ULTRA kit (Thermo-Scientific, MA, USA) and is the subject of proprietary information. Alternatives to ARCA are currently under investigation in an

attempt to avoid the use of previously patented material in the production process and therefore reduce cost without compromising translation.

The choice of restriction enzyme used for template linearisation is a critical stage of the design process. As a result of the RNA-dependent-RNA polymerase activity and processivity of the T7 bacteriophage polymerase (Arnoud-Barbe, 1998), an enzyme that does not produce 3' overhangs must be used to avoid the production of larger-than-desired transcripts. The linearisation enzyme must digest the entire template DNA content of the reaction with high fidelity to avoid transcription of non-target sequences (Schlake, *et. al.*, 2012). Initially we utilized *Sap*I, a Type IIS restriction enzyme, for template linearisation (Appendix A4-1). *Sap*I appeared to cleave the DNA only once in some instances, at one or the other *Sap*I site. As a result, a doublet of linearised template was produced wherein the linear DNA fragments differed by approximately 350 bp (Appendix A4-1).

To improve the efficiency of the linearisation reaction we chose to use *Hind*III as an alternative to *Sap*I. *Hind*III cuts DNA with the characteristics necessary to conform to the requirements of the mRNA production protocol and a cleavage efficiency of as much as 100% was achieved by *Hind*III digestion (Figure 16 a). However, the location of the *Hind*III site within the template DNA is not ideal and linearisation results in an 8 nt extension on the 3' end of the poly (A) tail mimic with as yet unknown consequences on translational efficiency. Future effort would need to modify the template to exclude or minimise the nonessential additional nucleotides. Our method of mRNA production yielded a homogeneous preparation, free of transcripts of irregular length and of sufficient quantity for downstream experimentation (Figure 16 b). The production procedure was highly reproducible and considered optimal for fulfilling the purposes of the current study.

4.2 ***Translation of synthetic mRNA to produce LHBs and SHBs***

Inclusion of a CMV promoter/enhancer upstream of the mRNA production sequences (Figure 7) allowed for assessment of surface antigen expression from the codon-optimised sequence prior to the initiation of mRNA production. Detectable surface antigen was produced from the pDNA harbouring *LHBs* and *SHBs* in cultured Huh7 (Figure 14; Figure 15). Quantification of extracellular or secreted surface antigen by ELISA revealed that the amount of SHBs produced from pmRNA-CMV-SHBs was comparable to that of the positive control (pCH9-3091) (Figure 14). LHBs, however, was detectable only within the intracellular space (Figure 15) and does not appear to be secreted from the cells (Figure 14). The observation that LHBs is not secreted when expressed as a heterologous protein concurs with observations made in previous studies (Chisari *et. al.*, 1986; Cheng *et. al.*, 1986).

Translation of SHBs and LHBs from the synthetic mRNA transcripts was achieved within the cultured cells indicating that the rational design process had been successful. SHBs expressed from SHBs mRNA and subsequently secreted was detected at levels comparable to the positive control by ELISA while LHBs protein was not (Figure 18) confirming that inhibition of secretion occurs post-translationally. LHBs expressed from synthetic mRNA was detected within the cell (Figure 19) albeit at far lower levels than those seen post-transfection with the pDNA equivalent of the LHBs sequence (Figure 15). Furthermore, the eGFP mRNA transcript synthesised by our method as opposed to that synthesised by TriLink Biotechnologies produced eGFP at notably lower levels (Figure 17). We identified four possible explanations for this: (i) the transfection method may be suboptimal (ii) the stability of the mRNA may be compromised, (iii) the additional 8 nt at the 3’ terminus that remain as a product of the location of the *Hind*III

site during linearisation may negatively impact translational capacity or (iv) the length of the poly (A) may be insufficient.

The preferred method of evaluating protein expression from mRNA *in vitro* is by nucleofection or electroporation. Lipofection in this instance did result in detectable protein levels (Figure 17) and the cells expressing eGFP from TriLink eGFP mRNA or our transcripts were transfected using a similar protocol. Lipofection, therefore, cannot account for differences in translational capacity but it may be worthwhile to utilise Nucleofection or electroporation in later experiments to optimise detectable protein output. It is possible that the TriLink mRNA is more stable; however, the UTR sequences for both TriLink transcripts and our transcripts are derived from globin genes. Chromatographic recovery of mRNA as is applied at TriLink may remove destabilising contaminants carried over from the transcription reaction but more thorough investigation is needed to substantiate this. It is most likely that the additional 8 nt proceeding from the 3' terminus of the mRNA or the length of the poly (A) affects translational capacity.

The 5' Cap and the poly (A) tail have mutually dependent functions in regulating the translation of endogenous and synthetic mRNA (Gallie, 1991). Poly (A) binding protein (PAB), a highly conserved eukaryotic protein (Grange *et. al.*, 1987), functions as a regulatory determinant of translation by affecting the functioning and biogenesis of the 60S ribosomal subunit as well as the formation of the ribosomal complex between 60S and 80S ribosomal subunits (Sachs *et. al.*, 1987). The poly (A) tail acts in *trans* with the 5' Cap to bind translational initiation factors and regulate protein production (Gallie, 1991). A poly (A) tail of 95 nt may be insufficient in directing the appropriate molecular binding events for optimal translation efficiency. It may be possible to overcome this by excluding the poly (A:T) sequence from the

pDNA template and instead including the mRNA in a post-transcriptional poly (A)-tailing reaction. By this method, however, the length of the poly (A) is not easily controlled and may exhibit a high degree of variability within a given preparation of mRNA transcript. An unknown poly (A) length is undesirable for GMP-mRNA production and a longer poly (A) may have unforeseen effects on vaccine efficacy. It is recommendable to eliminate all other options to improve translational capacity before altering the method of poly (A) tail addition.

4.3 ***Secretory capacity of expressed LHBs and SHBs***

LHBs is a large ORF containing three translation start sites and terminated by a shared stop codon (Figure 1). Therefore, all three forms of HBsAg share a common C-terminal S sequence (major surface protein) while the MHBs contains an additional preS2 region and the LHBs contains both a preS1 (at the N-terminus) and preS2 region. The ELISA antibodies are directed against the S portion (major surface antigen) of the protein present in both LHBs and SHBs. Therefore, this assay should detect any HBsAg present extracellularly regardless of which form of the protein is present. The lack of detectable secreted LHBs (Figure 14 and Figure 18) is probably not as a result of assay limitations; however, an ELISA microtiter plate coated with anti-preS1 or anti-preS2 antibodies could be used to unequivocally prove this assumption. Bound antigens would be detected using a secondary antibody-conjugate detection system.

The *LHBs* gene in the pDNA and LHBs mRNA contain all three transcriptional and translation start sites needed to produce LHBs, MHBs and SHBs. During a natural replication cycle of HBV, the three forms of the surface antigen are produced at varying levels and actively secreted (Ganem, 1996). There are two *surface* promoter elements and the stronger element

directs translation of SHBs (Ganem, 1996). It would be expected then that SHBs would also be produced from the *LHBs* pDNA and mRNA but this is not confirmed by the ELISA data (Figure 14; Figure 18). The relative distribution of LHBs, MHBs and SHBs remains similar within the Dane particles, 22 nm subviral spheres and filaments (Gerin *et. al.*, 1983; Theilmann *et. al.*, 1986). LHBs is co-secreted with the Dane particle and enriched within the 22 nm subviral filaments relative to SHBs and MHBs, which are more abundant in the 22 nm spheres relative to LHBs (Gerin *et. al.*, 1983; Takahashi *et. al.*, 1986). The 22 nm subviral spheres constitute the dominant form of secreted HBsAg and the relatively low levels of LHBs within these particles may serve to explain low level secretion of LHBs present in patient serum (Theilmann *et. al.*, 1986).

In transgenic mice, increased production of LHBs resulted in a significant decrease in SHBs detected in the serum (Chisari *et. al.*, 1986). Normally, SHBs is readily secreted and present at very low levels within the cytosol. Based on a property of the preS protein regions, and in particular preS1, LHBs in excess supresses the secretion of SHBs and causes it to be retained within the Golgi apparatus (Chisari *et. al.*, 1986). It is likely, therefore, that the heterologous expression of LHBs ultimately inhibits the secretion of the other forms of HBsAg. However, secretion of SHBs produced from pCH9-3091 (Nassal, 1992) is unaffected by the LHBs produced from the same vector (Figure 14). Possibly, the endogenous HBV sequences within pCH9-3091 (Nassal, 1992) limit the production of LHBs, which subsequently permits continual secretion of SHBs as per the normal replication cycle. In terms of *in situ* antigen expression for immunisation purposes, LHBs would need to be secreted in order to taken up by APCs and processed for MHCII-restriction and induction of humoral responses.

Self-assembling protein nanoparticles in the form of virus-like vesicles (VLVs) have been assessed for anti-HBV therapeutic vaccination potential (Reynolds *et. al.*, 2015). Administration of a VLV-vector additionally encoding MHBs in mice resulted in CTL responses detectable by ELISPOT assay. MHBs-specific antibody responses were shown but no direct assay on secreted MHBs was performed. It would be interesting to assess whether MHBs alone is more readily secreted than LHBs. The contribution of preS1 and preS2 to intracellular retention of surface antigen remains unclear (Chisari *et. al.*, 1986). During nucleocapsid assembly and maturation, the preS1 domain is embedded in the envelope of the virion and faces the interior of the viral particle (Figure 2 b). The N-terminus becomes myristoylated (Persing *et. al.*, 1987) and LHBs undergoes complex post-translational translocation (Lambert *et. al.*, 2001) allowing for the PreS1 region to be exposed on the virion surface capable of mediating interactions with NTCP (Yan *et. al.*, 2012). It is possible that a putative feature of the PreS1 region, known to embed the protein in the membrane, does not undergo post-translational modification in the absence of other HBV proteins and as a result accumulates in the Golgi. Exclusion of the PreS1 region may restore secretory capacity but at the expense of including critical epitopes directing potentially-broadly neutralizing antibody responses.

LHBs cannot be produced recombinantly in yeast (Imamura *et. al.*, 1987) and attempts made to produce the protein using mammalian cells (Chisari *et. al.*, 1986) proved that the application of the protocol would be insufficient for large-scale immunogen production. As the protein is not secreted at high levels (Chisari *et. al.*, 1986), recovery and purification of recombinant membrane-bound LHBs from within intracellular organelles would be costly and laborious. Furthermore, induction of robust cell-mediated and humoral immune responses after administration of a nucleic acid-based vaccine depends on high-level secretion of the encoded

immunogen. Possible future work could investigate directing LHBs to the secretory pathway through inclusion of a signal sequence produced in the encoded antigen at the N-terminus. Simultaneous targeting of APCs in preference to non-immune cells could further promote the elicitation of protective and therapeutic immune responses (Boyle *et. al.*, 1997; Hung *et. al.*, 2001).

4.4 ***Synthesised mRNA within the context of a vaccine formulation***

In recent years, the application of mRNA-based vaccination has gained increasing attention in various fields of medical research including anti-tumour vaccination (Boczkowski *et. al.*, 2000), anti-allergy (Weiss *et. al.*, 2010) and antiviral therapeutic vaccination (Martinon *et. al.*, 1993). The mRNA biomolecule is biologically labile, highly programmable and intrinsically immunogenic making it an ideal alternative to DNA for development of novel vaccination strategies. mRNA offers some distinct advantages when compared with DNA: (i) when injected into murine skin, protein produced from naked mRNA exceeds the amount produced from naked pDNA of similar molarity (Probst *et. al.*, 2007), (ii) mRNA is a transient biological macromolecule and the protein product is produced over a limited, controllable time period and (iii) exogenous mRNA can in no way alter the genetic material of a recipient cell and there is little to no risk of associated genotoxicity (reviewed in: Pascolo, 2008).

Vaccination of patients with a repertoire of autologous tumour antigens in the form of total tumour mRNA is a viable option to overcome immune tolerance and promote tumour destruction (Boczkowski *et. al.*, 2000). Anti-tumour mRNA vaccines encompassing mRNA amplified from tumour tissue promote survival in tumour-bearing mice (Ashley *et. al.*, 1997) and

supress the formation of metastases (Boczkowski *et. al.*, 1996). Developmental research into mRNA vaccine formulations produced by virtue of rational design and *in vitro* transcription is still in its infancy. An mRNA vaccine against influenza virus developed in 1993 (Martinon *et. al.*, 1993) is the first example of a prophylactic and therapeutic mRNA vaccine with proven antiviral effect. Immunization of mice with liposomes loaded with mRNA encoding the influenza nucleoprotein induced potent antigen-specific CTL responses. The genomes of the mice carried one of three distinct MHC haplotypes and the induction of CTL responses was similar in all experimental groupings. The results suggest that antigens expressed from minimal genetic vectors such as mRNA circumvent MHC haplotype restrictions allowing for induction of protective antiviral immunity regardless of MHC genetic limitations on peptide presentation.

Influenza viral infections represent a unique health challenge on a global scale because pandemic strains can arise rapidly and unexpectedly. As mRNA is highly programmable, the encoded sequences can be quickly altered to sequence-match changes in immunogenic proteins and produced for large-scale administration without reinitiating clinical testing (Petsch *et. al.*, 2012). In similar circumstances, towards the end of 2016 there was an unexpected outbreak of Zika virus (ZIKV) in South America with devastating neuropathological consequences for infected adults and infants (Pierson & Graham, 2016). Vaccination with nucleoside-modified mRNA encoding the pre-membrane and envelope glycoproteins of ZIKV has been recently proposed as a highly effective, potent immunization strategy for the prevention of future outbreaks (Pardi *et. al.*, 2017).

The ZIKV mRNA vaccine formulation was designed in accordance with many of the principles here discussed. The mRNA was produced by *in vitro* transcription and a 101 nt poly (A) tail mimic was encoded for by the pDNA template (Pardi *et. al.*, 2017). The envelope

protein-coding sequences were also codon-optimised. However, the production process differed from that described here in that the synthesised mRNA transcripts were post-transcriptionally capped with ARCA in a separate reaction and the mRNA was recovered and purified by chromatography. Whereas we chose to rely on sequence-engineering of the template pDNA alone to optimise mRNA production and translation, Pardi and colleagues opted for the use of chemically-modified nucleotides, namely 1-methylpseudouridine-5'-triphosphate. The approach of using chemically-modified bases in an mRNA vaccine was successful in eliciting robust, protective antibody production in animal models and thus this method of mRNA optimisation does not appear to interfere with induction of humoral immunity. However, the vaccine formulation was tested prophylactically and not therapeutically. It is not conclusive from the data as to whether base modification will undermine induction of cellular immune, cytotoxic responses as is needed for the treatment of CHB. It is worth exploring if the inclusion of modified bases will rectify the low level expression noted *in vitro* (Figure 19) without compromising therapeutic vaccine efficacy at a later stage of development.

Recombinant HBsAg anti-HBV vaccines, in contrast to nucleic acid vaccine, do not have potential use for therapy. Vaccines based on recombinant proteins are unable to elicit potent cell-mediated immune responses in human recipients (Shen *et. al.*, 2010; Chen *et. al.*, 2011) which are essential for promoting viral clearance. DNA-based antiviral vaccine platforms have proven ability to activate humoral and cell-mediated antigen-specific immunity in small-animal models, primates and humans (Mallilankaraman *et. al.*, 2011; Belisle *et. al.*, 2011; Bagarazzi *et. al.*, 2012). Upon administration, the majority of the nucleic acid (DNA or mRNA) will most likely be taken up by and translated within non-immune cells. Intracellular proteins degraded within the proteasome are presented to surveiling immune cell by MHC-I restriction (Figure 5) and

abnormal, or viral-derived peptides and immune cells would stimulate T_H1-polarised responses. In contrast, the antigens produced would ostensibly need to be secreted and then taken up by APCs to be processed for MHC-II restriction to elicit a T_H2 antibody response (Figure 5). A means of overcoming the limitation of antigen-secretion would be to reprogram DCs *ex vivo* for antigen-production (Boczkowski *et. al.*, 2000) or attempt to target pDCs directly *in vivo* by using appropriate methods of delivering the mRNA (Wang *et. al.*, 2016). The efficiency and ultimate effectiveness of a prophylactic and therapeutic anti-HBV nucleic acid vaccine, therefore, would depend on the secretion of the encoded HBV immunogen, in this instance LHBs. This would need to be carefully addressed in the further development of the mRNA-vaccine formulation here described. LHBs secretion would need to be promoted or an APC-targeted delivery method implemented using chemically modified lipid nanoparticles (LNPs).

An anti-HBV DNA vaccine formulation designed by Obeng-Adjei and colleagues in 2013 comprised consensus *core* and *surface* antigen sequences, including LHBs, from HBV genotypes A and C administered concurrently as a multivalent vaccine cocktail (Obeng-Adjei *et. al.*, 2013). The DNA vaccine cocktail was able to activate polyfunctional T-cell responses in immunized mice and rhesus macaques with the effect of inducing potent HBV-specific CTL activity and generation of high-titer, cross-reactive protective antibodies (Obeng-Adjei *et. al.*, 2013). No experiments directly measuring secreted LHBs were reported in the 2013 study; however, the data indicate preS1- and preS2-specific CTL responses which corroborate with successful MHC-I and MHC-II peptide restriction.

It is possible that the co-administration of consensus *core* sequences with *surface* antigen promoted the secretion of viral-like particles (VLPs) in which LHBs was incorporated. Interactions between the LHBS within the envelope and the underlying core proteins of the

nucleocapsid function in viral assembly and maturation before non-cytolytic release of the virions into the extracellular space (Hollinger, 1996). Artificially replicating these interactions may permit LHBs secretion as part of a VLP. Such an approach could be applied to improving the secretion profile described here. As a future objective of the current study, co-transfection with pDNA encoding core sequences and pmRNA-CMV-LHBs could be applied to assess the impact of the core protein on promoting LHBs secretion. If such an approach were to be successful, it may be necessary to deliver the mRNA vaccine as a mixture of LHBs and core mRNA for enhanced efficiency.

Highly conserved N-terminal sequences of LHBs including the N-terminal aa 9-NPLGF(F/L)P-15 of the preS1 (Figure 2 b) are crucial for mediating viral entry (Barrera *et. al.*, 2005). Making these sites unavailable for receptor-binding essentially blocks the infection or re-infection cycle. A peptide, Myrcludex B, inhibits HBV entry by blocking the N-terminal aa 9-NPLGF(F/L)P-15 and is currently in phase 2 of clinical trials (reviewed in: Urban *et. al.*, 2014). Ostensibly, anti-preS1 antibodies would occlude receptor-binding and prevent receptor-mediated endocytosis. Inclusion of the preS1 protein domain in a vaccine formulation would prevent *de novo* infection and if administered to CHB patients undergoing liver transplantation surgery, could prevent reinfection of the newly transplanted liver. Also, inclusion of preS1 and preS2 protein domains in a vaccine formulation may establish rapid seroprotection in previous non-responder vaccine recipients (Rendi-Wagner *et. al.*, 2006). It is critical, therefore, that future efforts be made to ensure that LHBs becomes the primary, functional immunogen of the mRNA vaccine here described to fulfil the therapeutic and prophylactic potential. Furthermore, inclusion of a TLR9 agonist as a vaccine adjuvant may support immunogenicity of the vaccine formulation by activating the necessary effector cell populations (Steinhagen *et. al.*, 2011).

The production of mRNA for clinical application is scalable and can be made to adhere to good manufacturing practice (GMP) regulations. Our method of production represents a pre-GMP phase of development and would need to be modified at a later stage in consideration of GMP requirements. For example, the preparation of the pDNA template required culturing of bacterial clones in an ampicillin-containing bouillon (LB) (Appendix A2-1). GMP insists that because of the prevalence of penicillin allergies and the spread of penicillin-resistance, no antibiotic utilised at any stage of the mRNA preparative process should be of the ampicillin family (Pascolo, 2008). The mRNA produced was recovered from the T7 transcription mix by precipitation in lithium chloride precipitation solution (section 2.3). GMP mRNA must be recovered by size exclusion chromatography (SEC) within a buffer system containing only sodium ions. SEC allows for the elimination of aberrant or irregular transcripts as well as trace contaminants introduced during the enzymatic transcription process. Future development of the methodology here described will aim at complying with GMP standards.

5. CONCLUDING REMARKS

The aim of the study here described was to design and implement an mRNA production procedure for the ultimate purpose of being included in an anti-HBV prophylactic and therapeutic vaccine formulation. To the best of our knowledge such an approach has not yet been applied to HBV therapy and the proven efficacy of such a vaccine would have significant impact on the field of HBV immunotherapy development. As noted earlier this year (2017) with the successful development of an anti-ZIKV mRNA vaccine (Pardi *et. al.*, 2017), mRNA vaccination

is a valid and potentially valuable novel approach to antiviral prophylaxis. We hope to demonstrate its application as an effective immunotherapy in the near future.

The most critical aspect of the early stages of mRNA vaccine development is the successful generation of high quality, high purity mRNA transcripts that are translated to give functional protein or antigen. We were able to produce high quality mRNA transcripts that were translated into detectable LHBs and SHBs *in vitro* in transfected Huh7 using a highly reproducible method. However, the LHBs protein was translated at lower than anticipated levels *in vitro* and was not readily secreted as is a requirement for a successful mRNA-base therapeutic vaccine. LHBs is accepted as being a difficult protein to produce by heterologous expression and is retained within membranous organelles in the cytosol rather than entering the secretory pathway. The efficacy of the anti-HBV mRNA vaccine, particularly in the context of a prophylactic vaccine, may depend on the secretion of LHBs. Future efforts will be directed at (i) improving the secretory profile of the LHBs protein, (ii) scaling up the production procedure within the parameters of GMP, (iii) assessment of base modification as a means to optimise antigen expression *in vivo*, (iv) design of a LNP-based delivery method for direct targeting of APC and (v) testing vaccine efficacy *in vivo*.

The results of this study are promising and it is likely that the antigens produced from the mRNA *in vivo* would elicit immune responses in the recipient animal. This study will be expanded to include development of a nanoparticle-based delivery platform inclusive of an appropriate TLR9 adjuvant. Thus, future work will provide an intersection of multiple disciplines within anti-HBV research and its success will contribute to the advancement of anti-HBV prophylaxis as well as the therapeutic outcome of CHB treatment.

APPENDIX

A1 ADDITIONAL SEQUENCE INFORMATION AND PLASMID MAPS

A1-1 pmRNA-MCS-95A Sequence Insert

>pmRNA-MCS-95A Insert: Coding strand (3'-5')

CACGTGGTATACCATTTTGCTGCCGGTCACTTAAAGCTCGAGCCATGGATTATGCTGAGTGATATCCCT

TTATTCTCTCTTTTCTTCTCATTCTTCTTTATATCGGCGGTGGTACCGCGCGGACGTCCGCCGGCGATC

GGGCCCTCTAGAATTAATTCCCGGGCAAATTTGCGACGGAAGACGCCCGAACGGAAGACCGGTACGG

GAAGAAGAGAGGGAACCGTGGACATGGAGAACCAGAAACTTATTTCGGACTCATCCTTCCGATCTTTTTT

TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

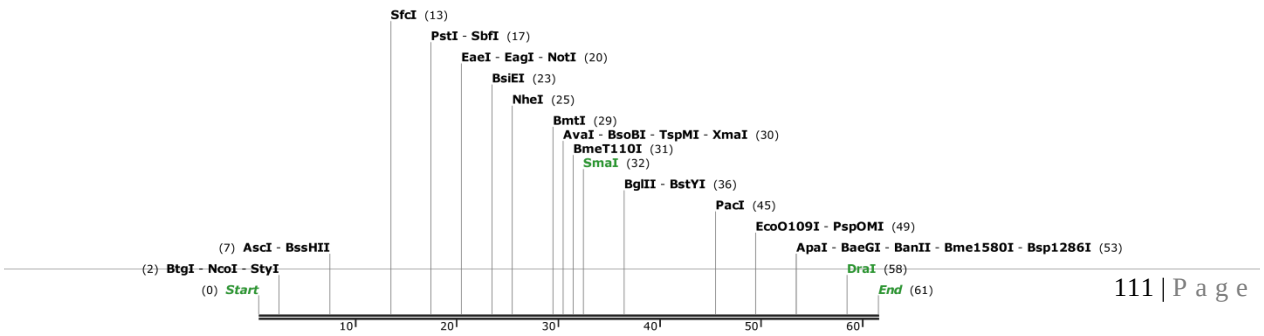
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTTCTCGTTCGAACCGCATTAGTACCAGTATC

GACAAAGGACTGTACACTCGTT

Sequence Information:

- T7 promoter sequence shown in green
- 5' and 3' UTRs are underlined
- Kozak sequence is shown in purple
- MCS is shown in red
- Poly A:T sequence is shown in blue



GENERAL LABORATORY METHODS AND PROTOCOLS

A2-1 Production and Purification of Plasmid DNA

Reagents

Luria Bertani Medium & Agar Plates

Luria Bertani Medium (LB) was prepared by dissolving 10% (w/v) of tryptone (Sigma-Aldrich, MO, USA), 5% (w/v) of NaCl and 5% (w/v) of yeast extract (Sigma-Aldrich, Y1625) in deionized water (dH_2O). LB medium for agar plates was prepared in the same way but 1.5% (w/v) of bacteriological agar (Sigma-Aldrich, MO, USA) was included in the recipe. The solution was sterilized by autoclaving at 121^oC for 20 minutes and left to cool at room temperature (RT); the LB medium was stored at RT. For pouring plates, agar-containing LB solution was allowed to cool to ~60^oC and was then treated with 1000X ampicillin (to a final concentration of 1X), mixed thoroughly and then poured into plastic bacterial petri dishes. The agar plates were left to polymerise at RT and then stored at 4^oC for no longer than 1 month.

1000X Ampicillin

One gram of ampicillin (Sigma-Aldrich, MO, USA) was dissolved in 10ml of 70% (v/v) ethanol and stored at -20^oC.

X-Gal

Twenty milligrams of X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Sigma-Aldrich, MO, USA) was dissolved in 1ml of dimethyl formamide, wrapped in tin foil to protect the solution from light and stored at -20^oC until needed.

1000X IPTG

One hundred milligrams of IPTG (Isopropyl β-D-1-thiogalactopyranoside; Thermo-Scientific, MA, USA) was dissolved in 1ml of dH_2O and filter sterilized through a 0.45µm filter. IPTG solution was stored at -20^oC until needed.

X-gal IPTG Ampicillin Plates

LB agar plates were prepared as described above. Just before use, any excess moisture was removed from the surface of the agar by incubating the plates at 37^oC with the lid slightly opened. To each plate, 40µl of X-Gal solution and 8µl of 1000X IPTG needed to be added. The solutions were premixed in the correct ratio to a final volume that was dependent on the number of plates included in the experiment. The solutions were thoroughly mixed and 48µl was added dropwise to the agar surface before being spread into an even thin layer. The plates were placed back into the incubator at 37^oC until the surface was completely dry. The X-Gal/IPTG plates were prepared fresh just prior to the completion of the relevant transformation reactions.

Procedure:

Scaled-up Purification

Plasmid DNA for mRNA production, subcloning and pDNA transfection was prepared and purified using the Qiagen® Plasmid Purification Maxi Kit according to the manufacturer's instructions and using the buffer system included in the kit. The average yield for a purification run was 2.5-3.5µg/µl in 50µl as measured on the NanoDrop 1000 (Thermo-Scientific, MA, USA) spectrophotometer.

Small-scale Purification

Plasmid DNA purification for screening of positive clones during subcloning experiments was performed using EconoSpin™ Prepacked Silica Membrane Spin Columns (Epoch Life Science Inc., TX, USA). The protocol followed was modified slightly from the manufacturer's instructions but used the suggested buffer system prepared according to the provided recipes (table A2-1.1). The average yield for a purification run was 0.5-1.0 µg/µl in 30µl as measured on the NanoDrop 1000 (Thermo-Scientific, MA, USA) spectrophotometer.

Table A2-1.1: Recipes for the buffer system used with the EconoSpin™ Spin Columns for purification of pDNA

	Description	**Recipe**	**Storage**
Buffer MX1	Suspension Buffer	• 50mM Tris-HCl • 10mM EDTA, pH 8.0 (25°C) • 100µg/ml RNAse A	4°C
Buffer MX2	Lysis Solution	• 0.2M NaOH • 1% (w/v) SLS	RT
Buffer MX3	Neutralization and Binding Solution	• 4M Guanidine hydrochloride • 0.5M Potassium acetate • pH 4.2 (25°C)	4°C
Buffer WS	Wash Buffer	• 10mM Tris-HCl, pH 7.5 (25°C) • 80% (v/v) Ethanol	RT

Protocol:

1. Harvested the bacterial pellet by centrifugation at 10 000rpm for 20 minutes at 4^{o}C
2. Discarded the supernatant and resuspended the pellet in 500µl of MX1 by pipette aspiration
3. Added 500µl of MX2 and vigorously inverted tube until solution was homogenous and viscous
4. Incubated the bacterial suspension for 5 minutes at RT
5. Added 700µl of MX3 (ice-cold) and vigorously inverted tube until the cellular DNA became visibly fragmented and the remaining solution cleared
6. Pelleted the fragmented cellular DNA by centrifugation at 12 000 rpm for 10 minutes at 4^{o}C and transferred the supernatant to an EconoSpin^{TM} column (800µl).
7. Centrifuged the column for 1 minute at 4 000 rpm (at RT) and discarded the flow-through
8. Added 500µl of WS Buffer to the column and centrifuged for 1 minute at 4 000 rpm at RT (repeated twice discarding the flow-through each time)
9. Allowed the column to stand at RT for 5 minutes and then centrifuged at 12 000 rpm for 2 minutes
10. Transferred the column to a clean collection tube and added 25-30µl of dH_2O and allowed to stand at RT for 5 minutes
11. Eluted the pDNA by centrifugation at 12 000 rpm for 1.5 minutes
12. Stored pDNA in aliquots at -20^{o}C

(**Note**: EconoSpin^{TM} Columns were stored between 4-8^{o}C)

Overall Process: pDNA Preparation and Purification

Table A2-1.2: Procedure followed when performing large-scale (Maxi Prep) and small-scale (Mini Prep) pDNA purification

	Step	Description	Maxi Prep	Mini Prep
Day 1	1.	Transformation (see **A2-2**)	• ~10ng of pDNA used to transform 50µl XL10 Gold *Escherichia coli* competent bacteria • Plated full transformation volume (50µl) onto LB-agar plate and incubated overnight (OVN) a 37^{o}C (upside down)	
Day 2	2.	Inoculation – 1 colony per culture	• 100ml LB (100µg/ml ampicillin) • Incubated OVN at 37^{o}C with 160 rpm shaking	• 20ml LB (100µg/ml ampicillin) • Incubated OVN at 37^{o}C with 160 rpm shaking
Day 3	3.	pDNA Preparation and Purification	• Qiagen® Plasmid Purification Maxi Kit	• EconoSpin™ Column Protocol
	4.	Elution	• 50-100µl of dH_2O	• 25-30µl of dH_2O
	5.	Quantification	• Used the NanoDrop 1000 Spectrophotometer • Recorded A260/A280 as a measure of purity (ratio of ~1.8 considered pure)	
	6.	Restriction Mapping	• Confirmed pDNA identity using multiple restriction digest reactions	

A2-2 Transformation Reaction Protocol

Reagents

1. Transformation Buffer I

- 10mM 2-(N-morpholino) ethanesulfonic acid (MES), pH5.8 at RT
- 10mM Rubidium chloride ($RbCl_2$)
- 10mM Calcium chloride dihydrate ($CaCl_2.2H_2O$)
- 50mM Manganese tetrahydrate ($MnCl_2.4H_2O$)

2. Transformation Buffer II

- 10mM Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), pH6.8 at RT
- 75mM $CaCl_2.2H_2O$
- 10mM $RbCl_2$
- 3.75ml of 15% (v/v) Glycerol

(**Note**: Transformation buffers were stored frozen at -20^{o}C in aliquots and defrosted completely before use)

Preparation of Competent Bacterial Cells

1. A small volume of competent XL10 Gold® *E. coli* cells (Agilent Technologies, CA, USA) from frozen stock was used to inoculate 4ml of LB medium (no ampicillin) in a snap-cap plastic bacterial culture tube
2. The inoculation was incubated OVN at 37^{o}C with 160rpm shaking

3. The 4ml inoculation mix was then used to inoculate 100ml of LB (no ampicillin) which was then incubated until the OD_{550} ~ 0.4 (approximately 2 hours)
4. The inoculation mix was then placed immediately on ice and chilled for 5 minutes
5. The cells were pelleted by centrifugation at 4^{o}C for 5 minutes at 2 500 xg
6. The pellet was resuspended in Transformation Buffer I and left to chill on ice for another 5 minutes
7. The cells were pelleted again by centrifugation at 4^{o}C for 5 minutes at 2 500 xg
8. The pellet was resuspended in 2ml of Transformation Buffer II and left on ice for 15 minutes
9. Cells were quickly dispensed in 100µl aliquots and stored immediately at -80^{o}C

Transformation Reaction

1. An aliquot of chemically-competent XL10 Gold® cells was removed from storage and thawed on ice
2. Once the cells had just thawed, a 50µl aliquot of competent cells was removed and incubated with ~10ng of pDNA on ice for 20 minutes
3. The transformation reaction was then incubated at 42^{o}C for 90 seconds and then immediately placed on ice for a further 2 minutes
4. The transformation reaction was then plated on LB-agar plates (with 100µg/ml ampicillin) and incubated upside-down OVN at 37^{o}C

A2-3 Immunofluorescence Imaging Protocol

Reagents

1. 1X Phosphate-buffered Saline (PBS)

- 1 PBS tablet (Thermo-Scientific, MA, USA) was dissolved in 500ml dH_2O
- The solution was autoclaved - 1kPa, 121^{o}C for 20 minutes

2. 4% (w/v) PFA

- 4g of PFA was dissolved in 80ml of 1X PBS in a water bath set at 60^{o}C for approximately 2 hours
- While the solution was mixed by magnetic stirring, 1M NaOH was added dropwise until the solution cleared
- The solution was allowed to cool to RT and then the pH was adjusted to 6.9
- The final volume of 100ml was made up with 1X PBS

3. 1% (w/v) BSA

- Prepared fresh – 1g of BSA (Roche Diagnostics, Switzerland) was dissolved in a final volume of 1ml 1X PBS

4. 0.1% (v/v) (Sigma-Aldrich, MO, USA)

- Prepared fresh - 10µl of Triton^{TM}-X100 was added to 10ml 1X PBS and mixed thoroughly

5. 1000X DAPI

- 1mg of DAPI was dissolved in 1ml of dH_2O
- The 1000X DAPI solution was frozen and stored at -20^{o}C until use

Protocol

1. Removed media from cells and immediately fixed the cells in 50µl of 4% PFA for 15 minutes at RT
2. Cells were washed 2X in phosphate buffered saline (PBS) and permeabilized by adding 50µl of 0.1% (v/v) Triton^{TM}-X100 followed by a 10 minute incubation at RT
3. Cells were again washed 2X in phosphate buffered saline (PBS) and either used immediately or stored in PBS at 4^{o}C until needed

4. The cells were blocked in 1% (w/v) BSA either for 1 hour (condition 1 and condition 3) or overnight at 4^{o}C (condition 2) and then washed 3X
5. This was followed by the primary antibody incubation step – varied according the optimisation condition being tested for
6. A stringent wash-step of 3X PBS washes was followed on by incubation with 30µl secondary antibody (diluted 1:200 in 1% (w/v) BSA) for 40 minutes at RT (in a humidity chamber)
7. The cells were washed 3X in PBS and incubated in 1X DAPI for 5 minutes at RT
8. The cells were washed a final 2X in PBS before imaging on the Zeiss Axio Imager Fluorescence Microscope (Zeiss, Germany) using the AxioVision Software V3.8.1

A2-4 Formaldehyde-agarose gel electrophoresis

Reagents

1. 10X MOPS Buffer

- 0.2M 3-(N-morpholino) propanesulfonic acid (MOPS), pH7.0 at RT
- 20mM Sodium Acetate (NaOAc)
- 10mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0 at RT

2. DEPC-H_2O

- 0.05% (v/v) diethyl pyrocarbonate (DEPC) in dH_2O
    - Stirred for 30 minutes at RT using a magnetic stirrer bar
    - Autoclaved immediately – 1kPa, 121^{o}C for 20 minutes

Making the Formaldehyde-Agarose Gel

1. For preparation of a 100ml gel at 1%, 1g of agarose was dissolved in 72ml of DEPC-H_2O in a microwave oven

2. The melted agarose was allowed to cool to exactly 60^{o}C as measured by a thermometer before the addition of 10ml 10X MOPS Buffer (final concentration of 1X) followed immediately by the addition of 18ml 32% Formaldehyde solution (Sigma-Aldrich, MO, USA).
3. The gel was mixed, poured immediately into a setting tray with a 3mm comb and allowed to polymerise for a minimum of 30 minutes and a maximum of 60 minutes before loading the gel.

Sample Preparation

1. Approximately 500ng of mRNA was used per sample
2. The mRNA was mixed with 8µl of 2X RNA Loading dye (Thermo-Scientific, MA, USA) and 1µl of 200µg/ml EtBr
3. The samples were boiled at 70^{o}C for 10 minutes and then immediately placed on ice until being loaded onto the gel

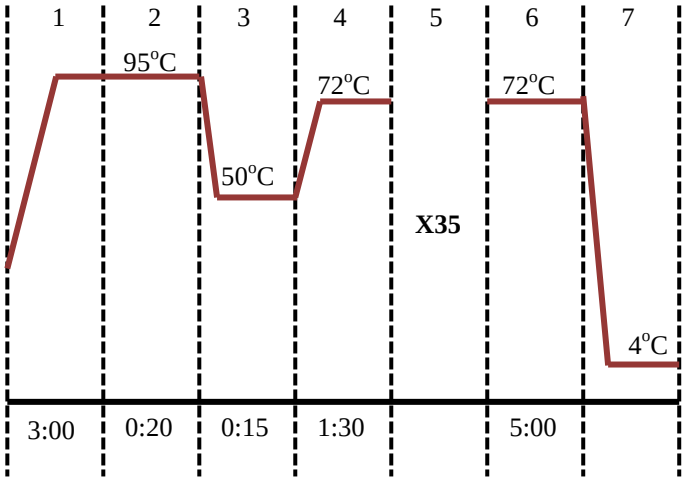
Electrophoresis

1. The gels were placed in an electrophoresis tank connected to a high capacity power pack in the cold room (2-8^{o}C)
2. MOPS Buffer (1X) was poured into the tank such as to just cover the top surface of the gel and fill up the sides
3. The samples were loaded into their respective lanes
4. The gel was electrophoresed at 80V for 45-60 minutes
5. The gel was viewed as per a normal agarose gel

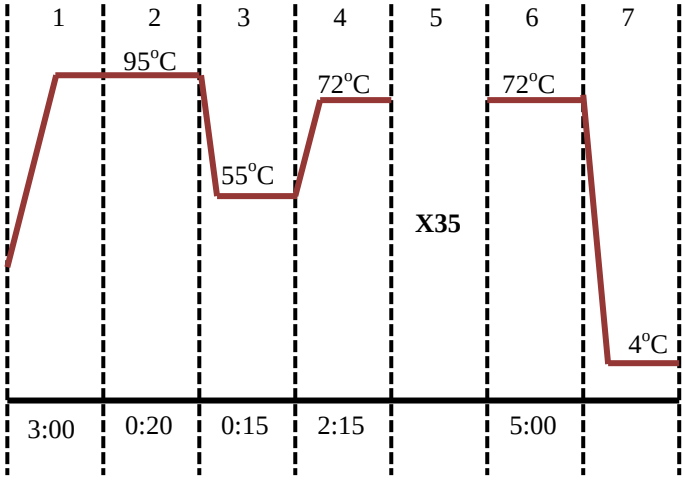
A3 ADDITIONAL EXPERIMENTAL DETAIL

A3-1 PCR Thermal Cycler Profiles

eGFP, CMV and SHBs:



LHBs:

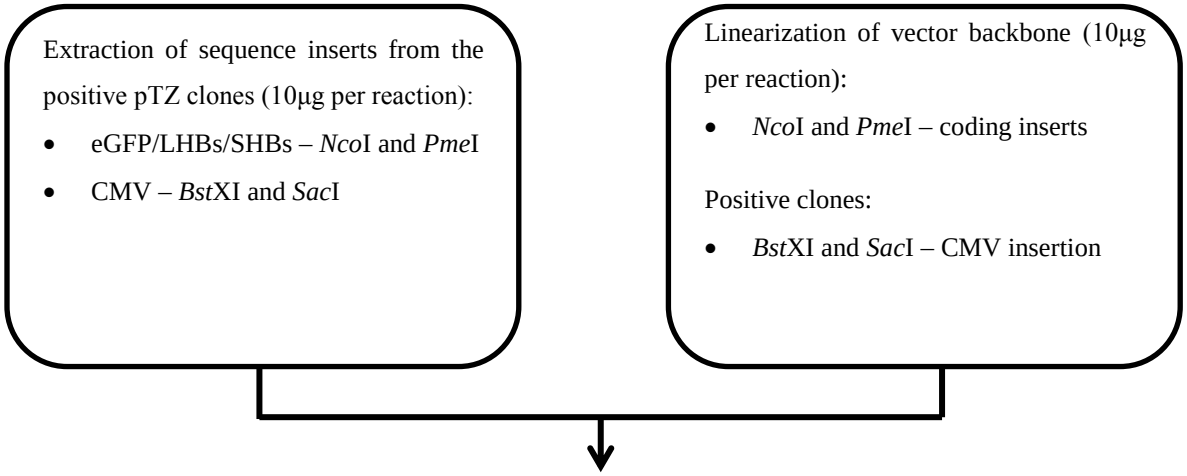


Profile Information:

1. Initial denaturation step
2. Denaturation
3. Annealing
4. Elongation
5. Number of Cycles
6. Final elongation step
7. Baseline T

A3-2 Experimental process of subcloning

1. Restriction digestion reactions:



2. Purification of desired DNA fragments by gel extraction

- The total volume of the restriction digestion reactions were electrophoresed through a 1% agarose gel (120V, 35 minutes)
- The gels were viewed directly by ultra-violet light (ethidium bromide staining of DNA) and the bands of correct sizes (run concurrently with GeneRuler^{TM} DNA Ladder Mix; Thermo-Scientific, MA, USA) were excised using a sharp, clean scalpel.
- The DNA-containing gel slices were weighed and the DNA fragments purified using the MinElute® Gel Extraction Kit (Qiagen, Germany) according to the provided protocol in the kit manual. Fragments were eluted in 20µl of ddH_2O
- The purified fragments were quantified using the NanoDrop 1000 spectrophotometer (Thermo-Scientific, MA, USA).
- An elution check was done by gel electrophoresis (1% agarose, 120V, 35 minutes) loaded with 1µl of purified fragment eluent. Fragment identitiy was confirmed by size comparison with the DNA ladder.

3. Ligation reactions: Used a vector/insert ratio of 1:3 based on molarity (0.172 pmole: 0.52 pmole of available ends)– quantity of each fragment to be included in the reaction were calculated using table A3-3.1

Table A3-2.1: Calculation guideline for DNA fragment quantity included in ligation reactions. Amounts of DNA fragment included calculated according to fragment size and estimated weight. Adapted from the InsTAClone PCR Cloning Kit (Thermo-Scientific, MA, USA) manual

Insert/vector backbone size (bp)	**Optimal quantity for inclusion in ligation reaction (ng)**
100	17
300	51
500	88
1000	172
2000	343
3000	515
4000	686
5000	858

- Ligation was carried out using T4 DNA Ligase (Thermo-Scientific, MA, USA) and set-up as shown below:

		NIC	Experimental Reactions
	Needed	**Volumes (µl)**	
Backbone	According to table A3-2.1		
Insert		NONE	According to table A3-2.1
ddH_2O		To make up V_T = 30µl	
10X T4 DNA Ligase Buffer	1X	3	3
T4 DNA Ligase		1	1
V_T		**30**	**30**

- Ligation reactions were incubated OVN (~16 hours) in a refrigerated water bath set to 14^{o}C

4. Transformation of XL10 Gold® *E. coli*:
    i. Protocol followed according to A2-2: 2.5µl of each ligation reaction mix was used to transform 50µl of competent bacterial cells
    ii. Transformation reactions were plated onto standard LB-agar plates (A2-1)
5. Screening for positive clones:
    i. Individual colonies were used to inoculate separate 20ml LB cultures (A2-1)
    ii. Plasmid DNA was prepared using EconoSpin™ Spin Columns (A2-1)

iii. Restriction mapping was used to identify positive clones

iv. DNA from positive clones was prepared on a large-scale and processed appropriately according to downstream application (A2-1)

v. All plasmid DNA was stored at -20^{o}C

A4 SUPPLEMENTARY DATA

A4-1 mRNA Transcripts produced from template DNA linearised with *Sap*I

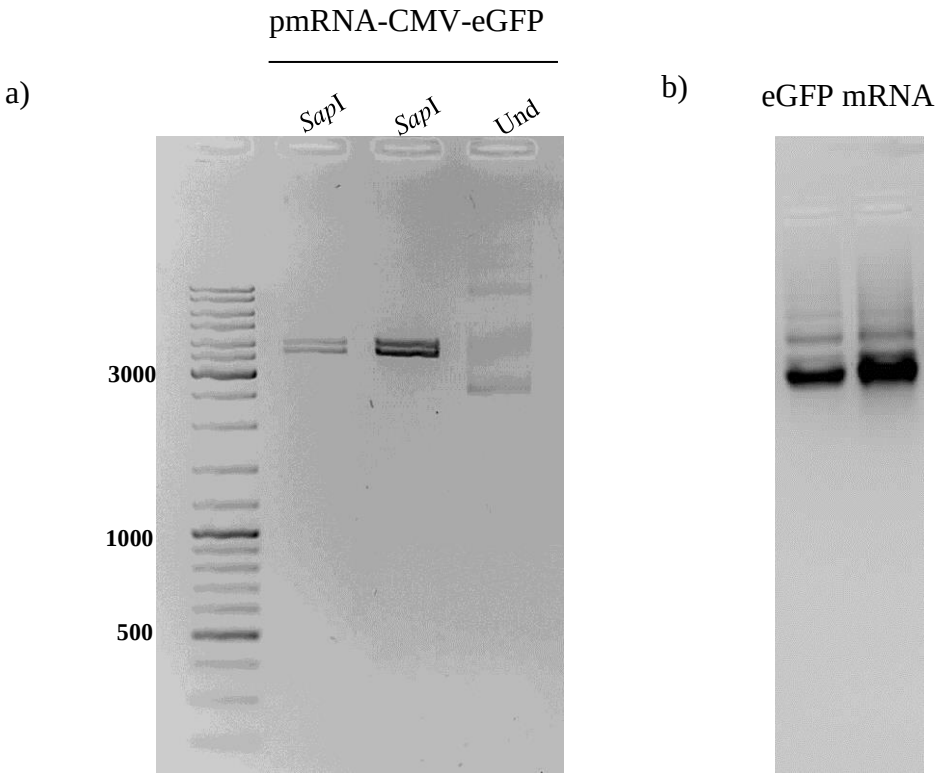
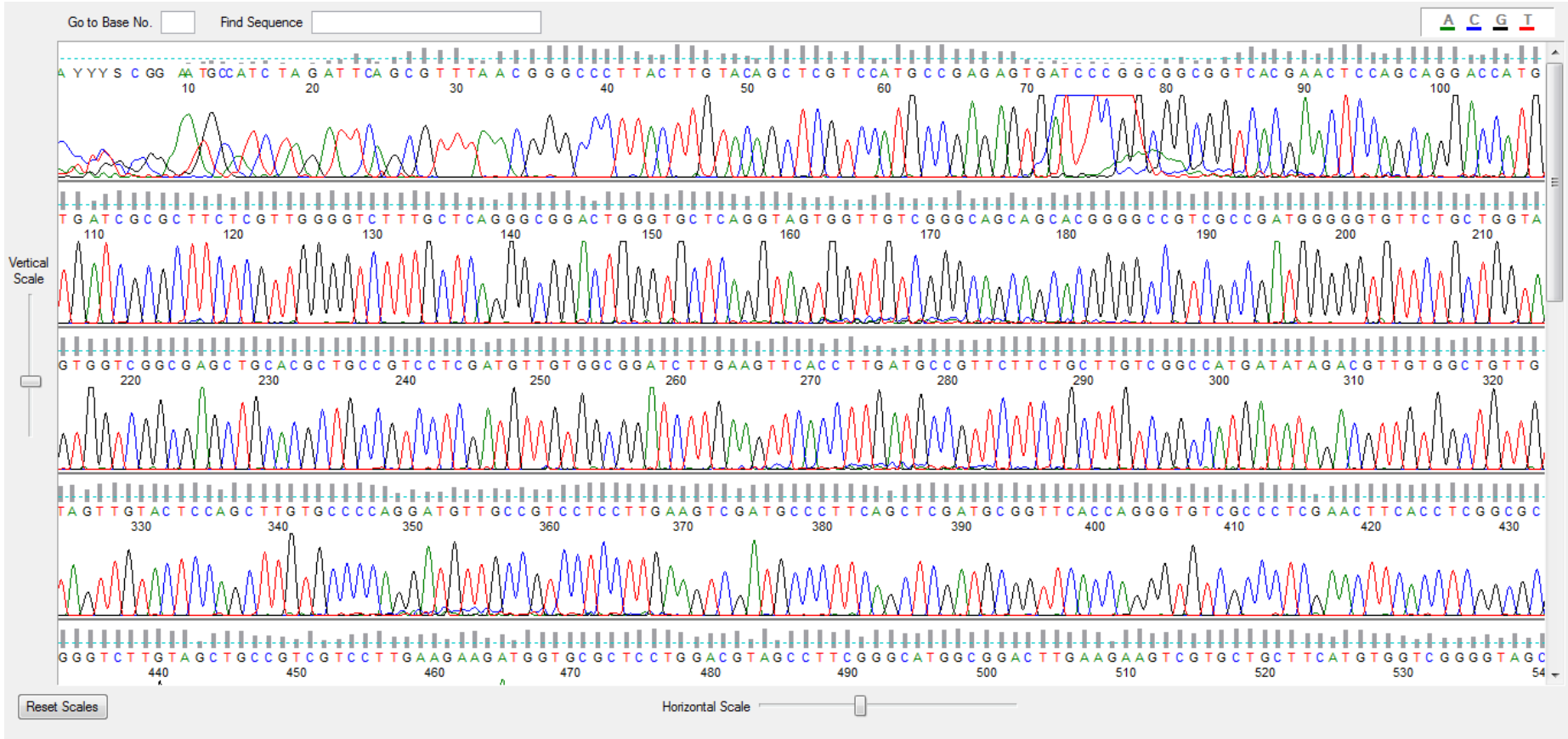


Figure A4-1: <u>*Sap*I linearisation of pmRNA-eGFP</u>. The experiments shown are duplicates. *Sap*I linearisation was performed under multiple conditions altered according to reacting amount of *Sap*I included in the reaction and incubation time. Shown here are representative images of the (a) doublet consistently noted after linearisation with *Sap*I and the (b) consequent heterogeneous mRNA preparation

A4-2 Chromatogram representative of sequencing data



A5 ETHICS CLEARANCE AND INFORMATION

A5-1 Biosafety approval for SA MRC/WITS-Antiviral Gene Therapy Research Unit

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Genetic Resources, Private Bag X973, PRETORIA, 0001
Harvest House Room 165, 30 Hamilton Street, Arcadia, Pretoria, 0002

From: Ms N L Mkhonza
Tel: (012) 319-6165 • **Fax:** (012) 319-6298 • E-**mail:** BathobileM@daff.gov.za
Enquiries: Ms B Mahlangu • **Ref:** 39.2/University of Witwatersrand – 14/003

Prof P B Arbuthnot
University of Witwatersrand
Antiviral Gene Therapy Research Unit
Department of Molecular Medicine and Haematology
Private Bag 3
Wits
2050

+27 (0) 11 717 2365 (Tel)
+27 (0) 11 717 2395/ +27 (0) 86 529 6833 (Fax)
Patrick.Arbuthnot@wits.ac.za (E-mail)

Dear Prof P B Arbuthnot

RE: REGISTRATION OF FACILITY

With reference to the application to register a facility, submitted in terms of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997). Registration number **39.2/University of Witwatersrand-14/041.**

The facility is hereby registered; please find attached your certificate which serves as proof of registration. Please familiarize yourself with the Standard Operating Procedure approved for Regulation 2(2) to determine whether your current activities or any future activities would require an additional contained use permit or not.

Please consult the website of the Department at www.daff.gov.za (Branches/ Agricultural Production, Health & Food Safety/ Genetic Resources/ Biosafety) for the latest application forms and the SOP document referred to in the above paragraph.

If any of the provisions of the Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997), including any condition of any permit issued in terms of the GMO Act, is not complied with at all times, you will be subject to prosecution in terms of Section 21 of the GMO Act, 1997.

Yours sincerely

Ms N L Mkhonza
Registrar: Genetically Modified Organisms Act, 1997 (Act No. 15 of 1997)

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/44/C

APPLICANT: B Moyo

SCHOOL: Molecular Medicine and Haematology

LOCATION: Faculty of Health Sciences

PROJECT TITLE: *Disabling hepatitis B virus replication in transgenic mice using lipoplex formulations to deliver mRNA encoding virus-targeting transcription activator like effector nucleases*

Number and Species

88 Mice

Approval was given for to the use of animals for the project described above at an AESC meeting held on 26 August 2014. This approval remains valid until 25 August 2016.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: _____ (Chairperson, AESC) Date: 14th SEPT 2014

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 11th SEPTEMBER 2014

cc: Supervisor: N/A
Director: CAS

Works 2000/lain0015/AESCCert.wps

A5-3 M&E Document: Student-specific ethics clearance

AESC 2009

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Buhle Moyo

b. Department: Antiviral Gene Therapy Research Unit, Molecular Medicine and Haematology

c. Experiment to be modified / extended **AESC NO:**

Original AESC number	2014	45	C
Other M&E's : N/A			

d. Project Title: Disabling hepatitis B virus replication in transgenic mice using lipoplex formulations to deliver mRNA encoding virus-targeting transcription activator like effector nucleases

e. Number and species of animals **originally approved**:	88	Balb-c HBV transgenic mice
f. Number of additional animals **previously allocated on M&Es**:	N/A	
g. Total number of animals **allocated** to the experiment to date:	18	
h. Number of animals **used** to date:	18	
i. Specific modification / extension requested: We are requesting to add Ms Camilla Lamb (550176) as a co-worker to the study		
j. Motivation for modification / extension: Ms Lamb will be involved in assisting with the maintenance of the mouse colony		

Date: 15 June 2016 Signature: .....

RECOMMENDATIONS: Approved. Inclusion of Ms C Lamb as a co-worker.
Ms Lamb should attend a 'first time user' session facilitated by the CAS before working with the mice.

Date: 15 June 2016 Signature: .....
Chairman, AESC

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