University of Alberta

Using designed zinc finger proteins to inhibit hepatitis B virus transcription in tissue culture

by

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Abstract

Upon infection with hepatitis B virus (HBV) the result will be either a chronic infection or clearance of the virus. For the chronic carriers of HBV, therapy is primarily limited to nucleoside analogs, which inhibits viral replication and prevents production of progeny virus, however the nucleoside analogs have little affect on the reservoir of the virus in the nucleus, the covalently closed circular DNA (cccDNA). Our lab previously designed zinc finger proteins (ZFPs) to target sequences within the duck hepatitis B virus (DHBV) and HBV cccDNAs to inihibit hepadnaviral replication. This is a novel approach to treatment of HBV infection because the reservoir of the virus is directly targeted. The DHBV-specific ZFPs have been shown to inhibit DHBV replication in tissue culture. I have assessed the ability of HBV-specific ZFPs, delivered by lentiviral vectors, to inhibit HBV transcription in two HBV-infected human hepatoma cell lines. The currently designed ZFPs failed to suppress HBV replication.

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List of Abbreviations

3TC	2'-deoxy-3'-thiacytidine (-) enantiomer, lamivudine
ADV	Adefovir dipivoxil
Adv-HBV	Adenovirus expressing HBV
AFP	Alpha-fetoprotein
APS	Ammonium persulfate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
cccDNA	covalently closed circular DNA
cDNA	Complimentary deoxyribonucleic acid
CMV	Cytomegalovirus
CO_2	Carbon dioxide
DCP	Des-gamma-carboxyprothrombin
DHBV	Duck hepatitis B virus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DR	Direct repeat
Ds	Double stranded
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*

ECL	Enhanced chemiluminescence
ECV	Extracellular virus
EDTA	Ethylenediaminetatraacetic acid
EGFP	Enhanced GFP
EMCV	Encephalomyocarditis virus
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
F-12	Ham's F-12 nutrient mix
FAM	6-carboxyfluorescein
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
HAV	Hepatitis A virus
HBcAg	HBV core protein
HBeAg	Precore protein, HBV e antigen
HBIG	Hepatitis B immunoglobulins
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HBx	HBV X protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HE	Homing endonuclease

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	Human immunodeficiency virus 1
HNFs	Hepatocyte nuclear factors
HPRT1	Hypoxanthine phosphoribosyl-transferase 1
HRP	Horseradish peroxidase
HSV-1	Herpes simplex virus 1
ICV	Intracellular virus
IE	Immediate early
IFN-α	Interferon-alpha
IRES	Internal ribosome entry site
Kd	Dissociation constant
L	HBV large surface protein
LAM	Lamivudine, 3TC
LB	Luria Broth
LB/amp	LB plates with 100 μg/mL of ampicillin
LMH	Longhorn male hepatoma
M	HBV medium surface protein
MBP	Maltose binding protein
MCS	Multiple cloning site
miRNA	MicroRNA
MMLV	Moloney Murine Leukemia Virus
MOI	Multiplicity of infection
mRNA	messenger RNA

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Nucleoside analog
NEB	New England Biolabs
NLS	Nuclear localization signal
nt	Nucleotide
ORF	Open reading frame
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with tween 20
PCR	Polymerase chain reaction
pcRNA	precore RNA
Peg	Polyethylene glycol
PEG	Polyethylene glycol
Peg-IFN	Polyethylene glycol conjugated to IFN-α
pgRNA	pregenomic RNA
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
rcDNA	Relaxed circular DNA
RFP	Red fluorescent protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RU	Resonance units
S	HBV small surface protein, HBsAg
SDS	Sodium dodecyl sulfate

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
SV40	Simian virus 40
SVPs	Subviral envelope particles
T	Tween 20
TAMRA	Tetramethylrhodamine
TBS	Tris buffered saline
TBS/T	Tris buffered saline with tween 20
TEMED	N,N,N,N'-tetramethyl-ethylenediamine
TF	Transcription factor
TNF	Tenofovir disoproxil fumarate
TP	Terminal protein
UV	Ultraviolet
VEGF-A	Vascular endothelial growth factor A
VSVG	Vesicular stomatitis virus G protein
X-gal	Bromo-chloro-indolyl-galactopyranoside
ZFP	Zinc finger protein
ε	Epsilon stem loop structure of pgRNA

Chapter 1 – Introduction

1.1 – Global Health Burden from Hepatitis B Virus

1.1.1 – Worldwide Prevalence

Worldwide, approximately 2 billion people have been infected with the hepatitis B virus (HBV), resulting in about 350 million chronic carriers who are at high risk for developing serious, potentially fatal, liver diseases such as cirrhosis and hepatocellular carcinoma. These diseases account for approximately 1 million deaths globally each year [1]. The development of chronic infection after the acute infection is inversely proportional to age, whereby upon exposure 90% of infants, 30 – 50 % of 1-5 year olds and 5% of adults will fail to clear the virus and become chronic carriers [2]. The endemicity of this virus is highly variable throughout the world. There are areas of very high prevalence, for example China with nearly 10% of the population being hepatitis B virus surface antigen positive (HBsAg) [3], to areas of low prevalence with less than 1% of the population being HBsAg positive [4]. In areas of high HBV prevalence (8% or more) the lifetime risk of infection is 60% and typically is in the younger population. These areas include China, the Amazon Basin, parts of the Middle East, most of the Pacific Islands, Southeast Asia and Africa (excluding Japan and South Africa) [2, 4, 5]. In areas of intermediate prevalence (2 -7%) the lifetime risk is 20 – 60% and typically spans all ages [2]. These intermediate areas include South and

Central America, eastern and southern Europe, south-central and southwest Asia and Russia [2]. In areas of low prevalence (less than 2%) the lifetime risk is less than 20% and typically occurs in adults [2]. Areas of low HBV prevalence include the United States, UK, France, Italy, Australia and Canada [2, 4]. In Canada and the United States, HBV prevalence is higher among the Northern communities and the immigrant populations (7% carrier rate for both populations in Canada), predominantly as a result of vertical and horizontal childhood transmission [2]. HBV is transmitted through blood and infected body fluids, including saliva, tears, semen and vaginal secretions [6]. In areas of high endemicity the most common route of infection is perinatally and horizontally among young children, while in low and intermediate areas of prevalence, the most common mode is from sexual contact with infected persons and intravenous drug use [6].

HBV is highly infectious; an estimated 100 times more infectious than the human immunodeficiency virus 1 (HIV-1) and 10 times more infectious than the hepatitis C virus (HCV)[7]. The ease of transmission within a household contributes to the high endemicity of this infection. Worldwide, it is estimated that at least 50% of infections are acquired perinatally or in early childhood [8]. Considering the high prevalence, the infectivity of HBV and the ease of transmission, it is easy to comprehend the global burden of this virus.

1.1.2 – Therapy and Limitations

1.1.2.1 – *Interferon*

The first licensed treatment for treatment of chronic HBV infection was interferon-alpha (IFN-α), an immunomodulatory therapy with natural antiviral properties [9]. This treatment was found to improve liver histology, lower or seroconvert HBV-serum markers (HBeAg and HBsAg) and reduce HBV-DNA [5]. An improvement to interferon treatment was made by the introduction polyethylene glycol (Peg) conjugated to IFN-α (Peg-IFN). This modification increases the half-life of the IFN prolonging its action and improving its pharmacokinetics [10]. The Peg moiety slows the drug's absorption, resulting in a drug level profile that, instead of peaks and troughs seen with interferon therapy, has smooth curves with little difference between maximum and minimum blood concentrations. Therapeutic levels are maintained over the course of 7 days between doses [10] in contrast to IFN-α which is typically given to patients three times weekly. In general, higher doses of IFN-α or peg-IFN for longer durations are associated with better outcomes [5, 11]. However, higher doses result in more severe side effects, including fever, chills, fatigue, myalgia, headaches, flares of autoimmune disease, hepatitis flare, hepatic decompensation, irritability, depression and even suicidal tendency [12]. The severity of these side effects can be difficult to tolerate and patients frequently cease therapy. Furthermore, approximately only 30% of people respond to treatment [13]. However, the benefits of IFN-based therapy over nucleoside analog-based

therapy (next section) include the finite treatment length, higher HBsAg and HBeAg seroconversion, and the lack of viral drug resistance [5].

1.1.2.2 – *Nucleoside Analogs*

The main treatment option for chronic hepatitis B patients, aside from IFN, are nucleoside analogs (NAs). These antiviral drugs are derivatives of natural nucleosides which, after phosphorylation, can be incorporated into a DNA molecule, causing chain termination and irreversible inhibition of the reverse transcription reaction.

Pioneering work in the field of NAs as antiviral agents for the *Hepadnaviridae* family of viruses came out of the University of Alberta in the late 1980's. Lee *et al.* (1989) [14] revealed that there was a significant difference between purine and pyrimidine 2',3'-dideoxynucleosides in the inhibition of replication of duck hepatitis B virus (DHBV). They found that purine 2',3'-dideoxynucleosides were highly active antiviral agents, in fact, *in vivo* experiments with the most active agent resulted in elimination of DHBV DNA in the serum after just two weeks. Howe *et. al.* (1996) demonstrated that the dideoxyguanine (ddGTP) bound to the protein primer of hepadnavirus polymerase during replication, competing with dGTP which is always the first nucleotide to be incorporated [15]. Dr. Tyrrell and his colleagues licensed these compounds to Glaxo (now Glaxo Smith Kline). Through the work with Glaxo, Dr. Tyrrell's laboratory was the first to show lamivudine (2'-deoxy-3'-thiacytidine (3TC) (-) enantiomer) was a potent inhibitor of HBV replication in cell culture and in animal models [14, 16]. The

first clinical study of lamivudine treatment of chronic HBV carriers was reported in 1993 [17]. Drawing on information from clinical studies of HIV-1, Fischer *et al.* in 1996 [18] predicted that lamivudine resistance mutants would arise at the same catalytic site in the hepadnavirus polymerase as the HIV-1 polymerase, and demonstrated *in vitro* that such mutants did indeed confer lamivudine resistance. The conclusion of this paper stated "This report of a genetically engineered lamivudine-resistant hepadnavirus serves as a cautionary note of what could occur when lamivudine is used in the prolonged treatment of chronic HBV infections…". This proved to be accurate when clinical samples of HBV resistant to lamivudine were sequenced [18].

Lamivudine was the first NA approved for treatment of chronic HBV infection in 1998, resulting in significant improvements in patient biochemical and histological data, as well as seroconversion. In fact, after treatment for 48 months, 50% of Chinese patients seroconverted [19]. However, viral resistance mutations are a concern; for example, after lamivudine treatment for five years the resistance rate is reported to be as high as 70% [20, 21]. Nonetheless, lamivudine has a good safety profile and is inexpensive, and as such it is still used as the first oral antiviral in many countries [12, 20].

There are five NAs licensed for use; lamivudine, adefovir dipivoxil (ADV), entecavir, tenofovir disoproxil fumarate (TNF) and telbivudine, and they all inhibit viral DNA synthesis to interrupt the HBV replication cycle. They have been very successful in suppression of HBV replication, however

in some more than others, viral resistance mutations occur rendering the drug ineffective, and in many instances, cross-resistance occurs. For example, mutations that result in resistance to lamivudine generally do not affect sensitivity to ADV but do reduce the sensitivity to entecavir and telbivudine. In general, multiple mutations are required to gain resistance to entecavir, which has very low resistance rates of 1% in HBV treatment-naïve patients after 5 years of therapy [21, 22]. TNF has an even more impressive ability to suppress HBV replication with virtually no resistance. NAs have been shown to slow disease progression, lessen HBV-related liver complications and improve survival [23].

An unfortunate feature of these NAs is that despite their ability to suppress viral replication, they do not directly target the viral reservoir in infected cells; the viral covalently closed circular DNA (cccDNA). Thus, upon cessation of therapy, viral replication simply resumes because of the cccDNA reservoir in the nucleus of an infected hepatocyte.

1.1.2.3 *- Liver Transplantation*

Liver transplantation is a "last resort" for those with end-stage liver disease due to HBV. It is the most common reason for liver transplant in Asia and accounts for 5-10% of liver transplants in the US [24]. If a patient undergoing transplantation does not have antiviral prophylaxis, there is an 80% recurrence rate following transplant [25]. However, if NAs are given pre- and post-transplant along with hepatitis B immunoglobulins (HBIG) post-transplant, the rate of recurrence after 3 years is only 8% [26]. For

example, in a pilot study published in 1996 by Bain *et al.* [27] five HBV-DNA positive liver transplant patients were enrolled in a study to test the efficacy of lamivudine used pre- and post-transplant. They found that lamivudine could effectively inhibit HBV reinfection, with three out of four patients clearing HBeAg and HBsAg post-transplant, all four negative for HBV-DNA in the serum, and three out of four liver biopsies negative for HBV DNA. After follow-up up to 26 months later, two patients had recurrent hepatitis B, and two have normal liver enzymes and liver histology. In addition, the issue of resistant virus arose in those patients with recurrent hepatitis B [27]. Today, liver transplant patients receive a NA plus HBIG routinely following liver transplantation for HBV-induced disease.

1.1.3 – Vaccine

The availability of safe and effective vaccines to prevent HBV has resulted in a decrease in incidence in recent years. A vaccine has been available since 1982 and over 1 billion doses have been given worldwide. By 1992 there were 31 countries with national immunization programs, however, as of December 2006 this increased to 164. In Canada, there are two marketed monovalent vaccines, Engerix-B and Recombivax HB, both of which are based on hepatitis B virus surface antigen (HBsAg) produced and purified from yeast and given intra-muscularly. Additionally, there is a vaccine available for hepatitis A virus (HAV) and HBV, Twinrix, which contains the HBsAg from Recombivax and inactivated HAV. The HBV vaccines

are used for both pre-exposure and post-exposure prophylaxis, and have a variety of administration schedules and dosages depending on various patient factors. These factors may include constraints of delivery, costs associated with delivery or the vaccine itself, or special needs of patients such as atypical immune responses [1, 28].

Though there are multiple subtypes of HBV, the vaccines provide protection to them all. The efficacy of HBV immunization at 5 years post-administration is very good; in a Quebec study, more than 99% of school children had sustained protective responses. On average, after vaccination people aged 20-29 have protective response rates at 95%, 30-39 year olds have 90%, 40-49 year olds have 86%, 50-59 year olds have 71% and those aged 60 or older have between 50 – 70%. The duration of protection has not yet been fully determined, but it is known to persist for at least 15 years in most people who receive the vaccine [28].

1.1.4 – Complications of Chronic Infection

Chronic infection with HBV may result in serious liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC). In fact, an estimated 1 million people die each year from these HBV-associated liver diseases [1, 23, 29]. Other complications including portal hypertension, hepatic decompensation and other extrahepatic manifestations may be present in these patients as well [23]. After several decades 20-25% of chronic carriers will develop cirrhosis and 5-6% will go on to develop HCC [30, 31]. Cirrhosis

is the process of liver scarring and decreasing liver function, and is the result of long-term liver damage. Chronic viral hepatitis, for example from Hepatitis C or Hepatitis B, or from other sources of repeated liver injury such as alcohol abuse, nonalcoholic fatty liver disease, certain medications, among others, may result in cirrhosis of the liver. The gradual scarring of the liver decreases liver functions and reduces blood flow through the liver, and this will result in an array of very serious complications. HCC is a very aggressive form of cancer with a low 5-year survival rate of just 9% in the United States and the incidence is very highly correlated to HBV infection [32-34]. Worldwide, there are more than 700,000 diagnoses and approximately 600,000 deaths due to HCC [35]. A major limitation to the diagnosis of HCC is in its location; within the liver it is hard to detect a small mass, so until it is quite large it may go unnoticed. However, there are serum markers of HCC that are useful in detection, like des-gamma-carboxyprothrombin (DCP), alpha-fetoprotein (AFP) and a variant of AFP, AFP-L3 [36]. In addition, high risk patients undergo ultrasound of the liver every 6-12 months to detect HCC early. For patients that qualify, ie. the tumor is detected early, a liver transplant is an option and recurrent infection in the transplanted liver can be prevented as discussed above.

1.2 – The Hepatitis B Virus

1.2.1 – Prelude

HBV belongs to the family *Hepadnaviridae* containing two genera, the *Orthohepadnaviruses* and the *Avihepadnaviruses*. The prototype viruses of these genera are the HBV and the duck hepatitis B virus (DHBV), respectively. Their partially double-stranded circular DNA genomes, lipoprotein envelope and pararetroviral replication cycle characterize this family of viruses.

HBV is a non-cytopathic virus with primary tropism for hepatocytes, but has been suggested to have alternative replication reservoirs in chronically infected humans, for example in the pancreas, kidney, skin and mononuclear blood cells [37, 38].

Upon infection with HBV, the disease may take one of four courses: acute, occult, chronic, or much more rarely, fulminant hepatitis. Acute infection ranges from self-limiting hepatitis to asymptomatic. The symptomatic hepatitis occurs in less than one percent of neonates and about 10% of 1-5 year old children, in contrast to the adult cohort where one third of those infected experience symptoms [39]. Chronic infection is characterized by two or three stages, depending on the age of the patient at infection. In those who are infected at a young age (perinatally or infant) there are three phases: immune tolerant, immune clearance, and inactive residual, while infection acquired as an adult often lack the immune tolerant

phase [40]. Reactivation may occur in the inactive phase and patients then enter another immune clearance phase [41]. Occult infection is defined by HBsAg clearance but with HBV DNA still detectable at low levels in the serum or in mononuclear cells. This type of infection is a major concern with regards to organ transplantation or immunosuppressive therapy, since reactivation may occur in these situations [42]. Technically defined as an acute infection, fulminant hepatitis occurs in less than one percent of acute adult infections and much more rarely in pediatric patients. It is characterized by rapid, intense immune clearance of the virus, with a mortality rate of approximately 70% [30].

1.2.2 – Genome Characteristics

The 3.2 kilobase (kb) genome of HBV is partially double-stranded, with a complete negative sense DNA strand and a partially complete positive DNA strand, which is approximately two thirds complete [43]. The genome contains four unidirectional, overlapping and frame-shifted open reading frames (ORFs): the precore/core gene, polymerase gene, surface antigen proteins gene and the X protein gene. Five different RNA species are produced, two terminally redundant transcripts at 3.5 kb (precore and pregenomic) and one each of 2.4, 2.1 and 0.7 kb (subgenomic), producing seven gene products: Precore protein, core protein, polymerase, three surface proteins and the X protein (Figure 1.1).

The genome of HBV is very small and entirely coding, the regulatory elements are embedded with the protein coding sequences. All the mRNAs produced share the same polyadenylation signal and receive a 5' cap. There are 4 promoters with the HBV genome: preC/pregenomic, preS1, preS2 and X. Additionally, there are two enhancer elements (I and II) and other negative regulatory elements. The regulatory elements are known to interact with common as well as liver-specific transcription factors [44]. The preS1 controls the transcription of the 2.4 kb RNA transcript, the preS2 controls the transcription of the 2.1 kb RNA transcript, the X promoter controls the transcription of the 0.7 kb RNA transcript, and the preC/pregenomic promoter controls the transcription of the two 3.5 kb RNA transcripts. Enhancer I has been shown to significantly up-regulate the transcription from the preC/pregenomic and X promoters and has a small effect on the preS1 and preS2 promoters [44]. Enhancer II has been shown to significantly up-regulate transcription from preS1, preS2 and X promoters [44].

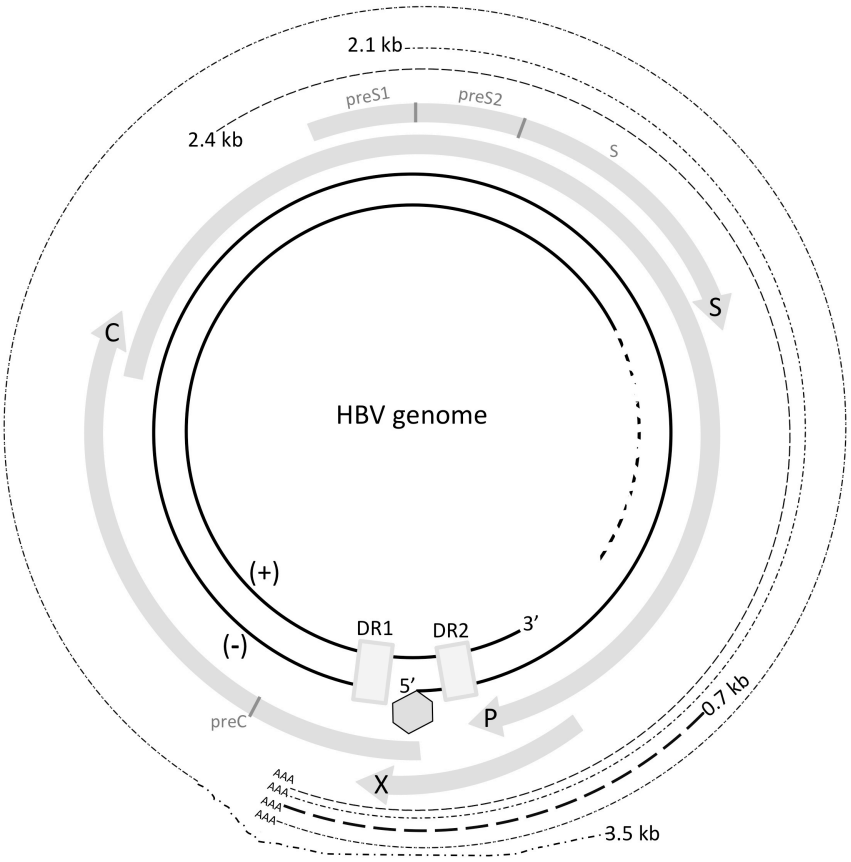

Figure 1.1 - HBV genome organization. Open reading frames (ORFs) are shown in solid grey arrows, DNA is in solid black lines, and RNA transcripts are in dashed lines. Attached viral polymerase depicted with the grey hexagon. Modified from [45].

1.2.3 – Protein Profile

i. *Precore protein.* Produced from the precore RNA (pcRNA), a 3.5 kb transcript which is slightly longer than the pregenomic RNA (pgRNA) [46]. Commonly referred to as HBeAg, this 16 kDa secreted protein has an unknown role in the viral replication cycle but has become a useful diagnostic

marker. Seroconversion from HBeAg-positive to HBeAg-negative (anti-HBe) is associated with approximately 2-log reduction in HBV titers [47]. The precore protein shares antigenic epitopes with the core protein, suggesting a possible role as a buffer to the anti-core immune response that develops soon after infection [47]. It is not required for HBV replication, but it is required for a chronic infection to be established [48, 49].

ii. *Core protein.* Translated from the pgRNA, the second 3.5 kb transcript. This 21 kDa protein also referred to as HBcAg, forms dimers and 120 dimer units self-assemble to form the nucleocapsid (see Figure 1.2). HBcAg has nucleic acid binding domains, which interact with the pgRNA during nucleocapsid assembly and its attached viral polymerase [50, 51].

iii. *Viral polymerase.* This 90 kDa DNA- and RNA-dependent DNA polymerase is translated from the pgRNA. The protein consists of 3 domains: terminal protein at the N-terminus, followed by a spacer region, followed by the polymerase domain and finally at the C-terminus is the RNaseH domain [46]. A tyrosine residue within the terminal protein is the primer for first strand DNA synthesis and it remains linked to the 5' end of the (-) DNA strand (see Figure 1.2) [43]. The

spacer region has been shown to be non-essential for polymerase functions of the protein, acting simply as a tether between flanking domains [52]. The polymerase domain is responsible for the DNA- and RNA- dependent polymerase activities. It has a high error rate of about 10^{-4}-10^{-5} substitutions/base/cycle [46]. Finally, the RNaseH domain is responsible for degradation of the pgRNA as the (-) DNA strand is formed [43].

iv. *Surface proteins.* HBV encodes three surface proteins: small "S", medium "M" or Pre-S2, and large "L" or Pre-S1 (see Figure 1.2), which decorate the viral envelope. The 24 kDa S protein is also known as HBsAg, the most abundant surface protein. Like S, the 30 kDa M protein is translated from the 2.1 kb RNA transcript. The 39 kDa L protein is translated from the 2.4 kb transcript [46, 53]. All three of the surface proteins share a common 24 kDa C-terminus but have independent translation start sites [53]. All three surface proteins are cotranslationally inserted into the endoplasmic reticulum (ER) and subsequently modified by glycosylation or myristoylation [53, 54]. The differential levels of expression of the different surface antigens can be explained by differential transcriptional control [44].

v. *X protein.* The 17 kDa X protein, also called HBx, is translated from the 0.7 kb RNA transcript and is essential for replication [55]. The role of HBx in the viral replication cycle is not clear, but has been implicated in a number of processes, including activation of cellular and viral promoters and the development of HCC [56-58]. HBx has been described as a "promiscuous transactivator of transcription" and has created a conundrum for HBV researchers with regards to its role in HBV replication and pathogenesis [46].

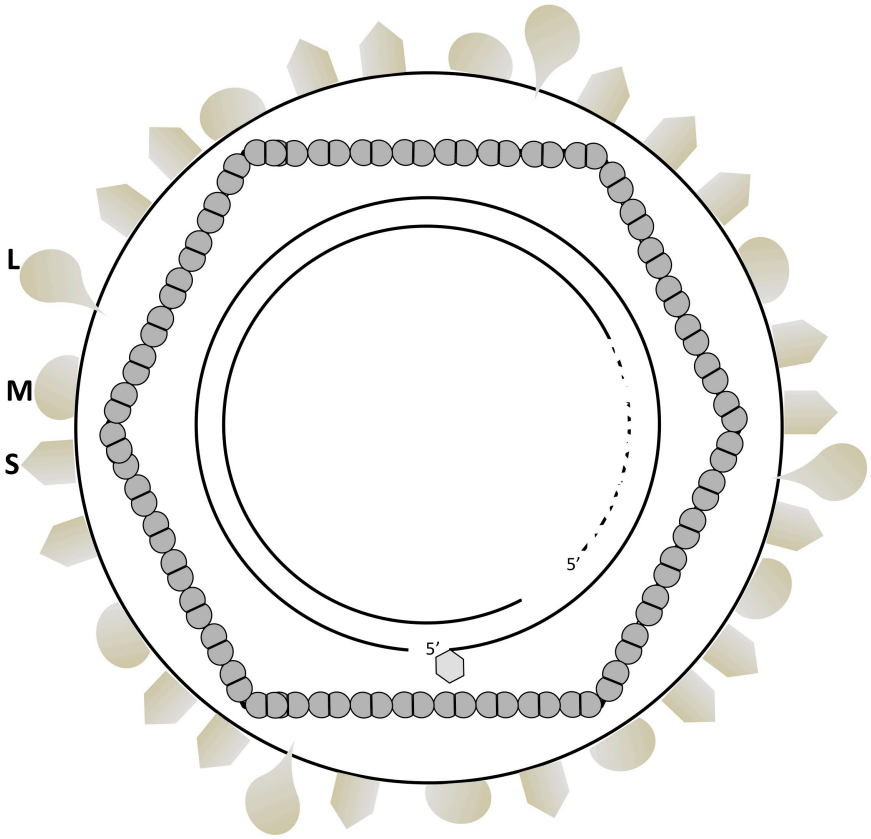

Figure 1.2 - General structure of the HBV Dane particle. The relaxed circular DNA with the attached viral polymerase is shown within the viral nucleocapsid, composed of core dimer units. The surface proteins, L, M and S, are shown studded around the lipid envelope.

1.2.4 – Replication Cycle

The initiation of infection begins with reversible attachment to heparin sulfate proteoglycans on the surface of the hepatocyte. The second interaction is between the viral preS1 protein and an unknown cellular receptor. It is as yet unclear whether entry is by endocytosis or fusion of the viral envelope to the cellular membrane. The nucleocapsid is released into the cytoplasm where it then travels along microtubules to the nuclear pore

complex. Interactions with adaptor proteins of the pore complex facilitate the deposition of the viral relaxed circular DNA (rcDNA) inside the nucleus, while the details of what happens to the capsid structure is not yet clear. The rcDNA, which is covalently linked to the viral polymerase, is converted to fully double-stranded DNA by the accompanying polymerase. Host enzymes remove the polymerase, the short RNA primer that was used for the positive strand DNA synthesis, and ligate the DNA ends to form cccDNA. The cccDNA becomes decorated with histones and non-histone proteins and exists within the nucleus as a stable 'minichromosome', from which all viral transcription occurs. Host and viral factors control regulation of gene transcription, including host CCAAT/enhancer-binding protein (C/EBP) and hepatocyte nuclear factors (HNFs), and the viral core and X protein [51].

From the cccDNA two different types of RNA transcripts are produced with which the HBV lifecycle is dependent on: subgenomic RNAs and pgRNA. The subgenomic RNAs are translated to produce the surface antigen proteins and the X protein. The pgRNA has 5' and 3' terminal repeats, as it is greater than genome length, and is the template for translating the polymerase and core proteins. The interaction between the epsilon stem-loop structure of the pgRNA and the viral polymerase functions to prime the reverse transcription reaction, whereby a short DNA oligonucleotide, starting with a deoxyguanosine nucleotide, is synthesized from a template in the bulged epsilon region and the DNA primer becomes covalently attached to a tyrosine residue in the terminal protein domain of the polymerase [55]. This

oligonucleotide attached to the polymerase is then translocated to the 3' terminal direct repeat (DR), DR1*, and is used for (-) strand DNA synthesis. The terminal protein remains covalently attached to the 5' end of the (-) DNA strand for the duration of the reverse transcription reaction (Figure 1.4). The pgRNA and viral polymerase interaction results in the association with core protein. Subsequently, self-assembly of the RNA-containing nucleocapsid occurs and once it is complete the reverse transcription reaction will be completed. During the (-) DNA synthesis, almost the entire pgRNA template is degraded by the RNaseH activity of the polymerase, until 11-16 nt from the 5' end. This results in an RNA oligonucleotide that is the compliment of DR1. At this point, one of two events can happen: *in situ* priming or primer translocation and third template switch. *In situ* priming is where the RNA oligonucleotide remains at the 3' end of the (-) DNA strand and primes (+) strand DNA, which results in double stranded linear DNA and non-productive virus replication. The other, more frequent and favorable option, for viral replication is primer translocation and a third template switch, whereby elements within the remaining RNA primer sequence mediate DR1 and DR2 being brought closer together, facilitating RNA primer translocation to DR2 at the 5' end of the (-) strand DNA template and circularization. As (+) strand DNA synthesis begins it will reach the physical 5' end of the (-) DNA strand, a template switch occurs by virtue of a terminal redundancy region in the (-) DNA (see Figure 1.4). Positive strand DNA synthesis resulting in rcDNA signals maturation of the viral particle. Mature capsids will either egress

through the ER lumen, which has had the envelope proteins co-translationally inserted into it and be secreted by the cell (infectious Dane particles) or recycle back to the nucleus to build up a cccDNA reservoir. Between 5 and 50 cccDNA molecules will accumulate in the nucleus of an infected hepatocyte, however the details of this regulation are poorly understood. The recycling back to the nucleus is dependent on levels of surface proteins. If surface proteins are low the recycling predominates; if they are abundant the nucleocapsids will bud into the ER and egress via the constitutive secretory pathway (see Figure 1.3). Additionally, subviral envelope particles (SVPs) will be secreted from the cell with envelope proteins but without a nucleocapsid. These SVPs are secreted in huge excess compared to Dane particles in the blood of infected patients. [43, 51, 59, 60].

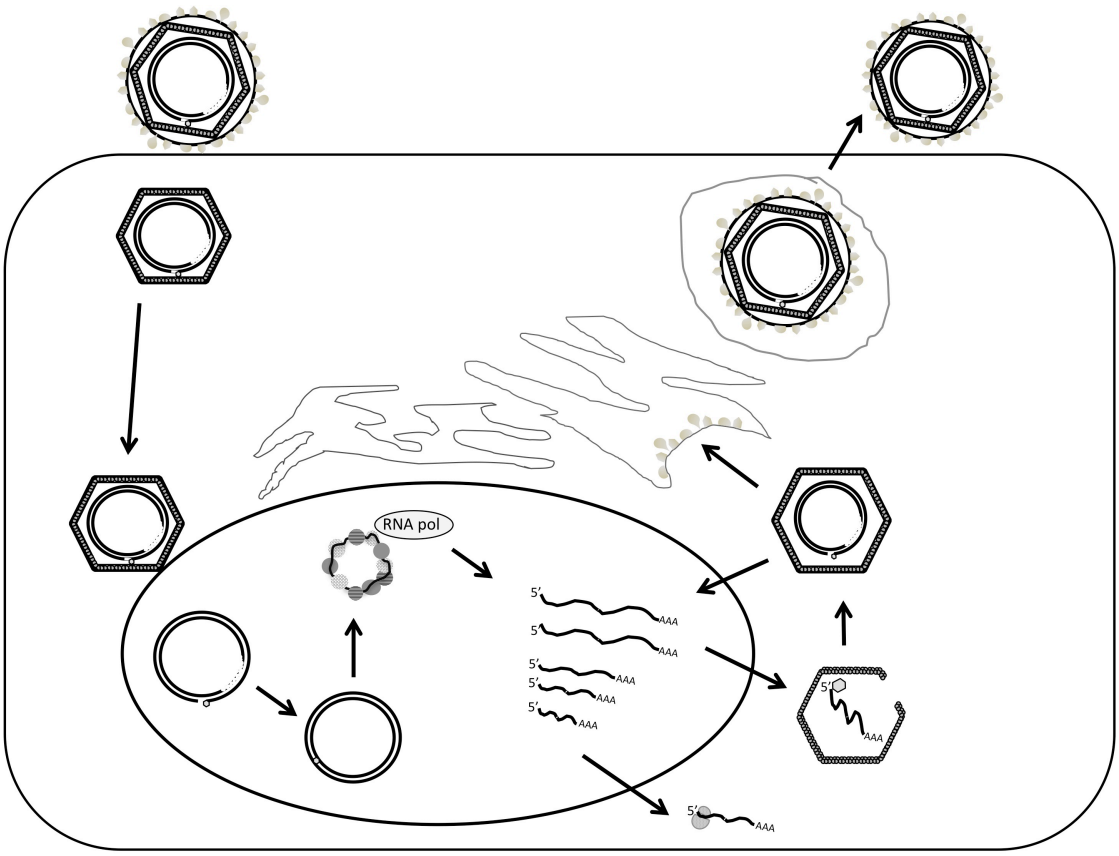

Figure 1.3 - General replication cycle of HBV. The virus attaches to its unknown cellular receptor and then the nucleocapsid is released into the cytoplasm where it traffics to the nucleus. The viral relaxed circular DNA (rcDNA) is deposited in the nucleus where it then becomes fully double stranded. Via host cell enzymes, the viral DNA becomes decorated with histone and non-histone proteins and is converted into covalently closed circular DNA, cccDNA. This stable episome of viral replication is transcribed by host RNA polymerase II, producing 5 transcripts, a 2.4, 2.1 and 0.7 kb and two at 3.5 kb. All transcripts are used for translation, however the pgRNA (3.5 kb) is also used for progeny viral genomes and becomes encapsulated with the nucleocapsid with a viral polymerase. Inside, the pgRNA will be reverse transcribed into rcDNA and either bud out of the cell, through the ER

where it acquires its envelope, or recycles back to nucleus to build up a cccDNA reservoir.

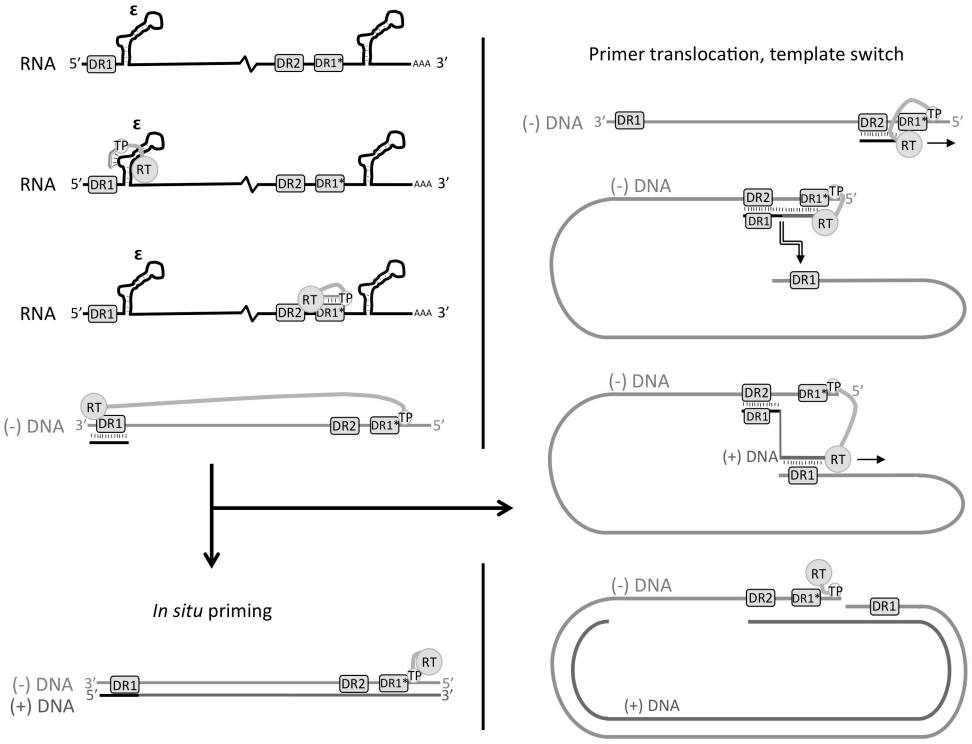

Figure 1.4 - Reverse transcription reaction of HBV. The pregenomic RNA (pgRNA) is shown in black, the (-) strand DNA is shown as light grey lines and the (+) strand DNA is shown as darker grey lines. The terminal protein (TP) domain of the viral polymerase becomes covalently attached to the epsilon stem loop structure (ε) of the pgRNA where a 3-4 nucleotide primer is synthesized, which then translocates to the direct repeat near the 3' end for (-) DNA synthesis. As (-) DNA is made, the pgRNA template is degraded by the RNaseH activity of the polymerase, until 11-16 nucleotides remain, which serve the next priming step. The TP will remain bound to the (-) DNA for the rest of the reaction. (+) DNA synthesis will take place after *in situ* priming resulting in a linear genome (not favorable) or after primer translocation and a template switch resulting in circularization.

1.2.5 – Duck Hepatitis B Virus – A Model Virus

The duck hepatitis B virus (DHBV) has been the primary model system for the study of HBV. DHBV is a member of the *Hepadnaviridae* family, in the *Avihepadnavirus* genera. Most of the key features that we know about HBV were first discovered in DHBV and then confirmed in HBV, such as the dependence on an RNA intermediate in the replication cycle, the role of the cccDNA and the mechanisms of the reverse transcription reaction [61-64]. The similarities between DHBV and HBV are vast, which is why it is such a great model for the study of HBV. The viruses share a similar genomic structure; both have partially double stranded DNA genomes that require an RNA intermediate for replication and have extensive overlap of the ORFs [61]. There are strong similarities in the replication cycles, the structure of the viruses and the host cell-type tropism [61].

Despite these similarities, there are some differences. First, there is no detectable X protein produced by DHBV, however there does appear to be a cryptic ORF [65, 66]. Secondly, there are only 2 surface proteins in DHBV, compared to 3 in HBV. Lastly, the outcome of infection is different between the two viruses. DHBV can be passed to the egg from the infected mother, resulting in chronic infection but this infection has no apparent phenotype and the duck will live a healthy life. When an adult duck is exposed to DHBV, the infection is usually self-limiting and subsequently cleared [61].

1.3 – Zinc Finger Proteins

1.3.1 – Zinc Finger Proteins as tools: Structure and Design

Zinc finger proteins (ZFPs) exist naturally and abundantly in eukaryotic cells, commonly as transcription factors. ZFPs are also known to bind RNA and proteins but are primarily known for interacting with DNA [67-69]. A ZFP consists of multiple finger motifs that can each recognize 3 base pairs, so when finger motifs are arranged in tandem they recognize longer DNA sequences, in multiples of 3 base pairs (see Figures 1.5 and 1.6). Each motif consists of one α-helix and two β-sheets, coordinated by a zinc ion. They have the ability to recognize a specific sequence of DNA by making base-specific contacts with their amino acid side-chains. These base-specific side chains extend from the α-helix of a single finger motif, which lies in the major groove of the DNA making the base-specific contacts [70].

Each finger motif is approximately 30 amino acids, where each motif has a characterized consensus sequence: (F/Y)-X-C-X_{2-5}-C-X_3-(F/Y)-X_5-ψ-X_2-H-X_{3-5}-H, where X is any amino acid and ψ is a hydrophobic amino acid [68]. This sequence will fold into the typical ββα structure when the two cysteines and two histidines coordinate a zinc ion, with the cysteines at the end of one β sheet and the histidines in the α-helix [68]. There are other variations on this sequence that result in functional ZFPs because the majority of the stability of the ββα structure is due to the coordinating zinc ion [68]. Connecting the finger motifs are linker regions; the exact length of these

regions are important because there needs to be appropriate spacing between the finger domains so that they can make their specific DNA contacts. Hydrogen bonding interactions are the main and specific sources of stabilization of the ZFP-DNA complex. However, there are other interactions that help to stabilize the complex as well: hydrogen bonding between the β-sheets and α-helix with the phosphodiester backbone, and intra- and inter-finger interactions [68, 70, 71].

There are a number of ways to design ZFPs [72, 73]. One method screens libraries of ZFPs and sequentially selects for finger motifs that have high affinity. Alternatively, there are simpler yet effective methods, such as using the Zinc Finger Tools Program. This is an online database that designs ZFPs based on a "recognition code" that was developed by Dr. Carlos Barbas III and his colleagues [74-77]. This method is based on a modular approach, with the main factor considered for a binding interaction being those residues in the α-helix that make the base-specific contacts. Inter- and intra-finger interactions are not considered. High affinity, functional ZFPs have been designed using this process [68, 72].

Usually ZFPs function in the context of a larger protein with additional domains having other roles such as mediating protein-protein interactions, transcriptional activation or repression domains, enzymatic activities and so forth [68]. This broad versatility in potential functions and the ease of design of these proteins makes them a useful tool for gene therapy, antiviral therapeutics and biomedical research in general.

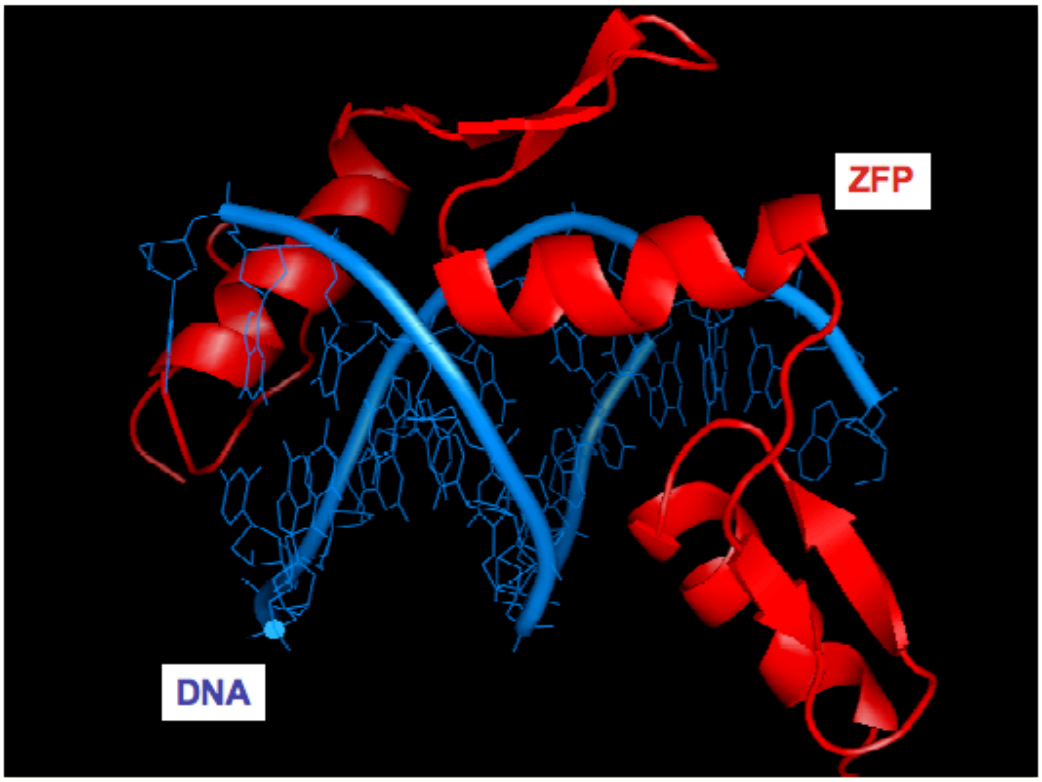

Figure 1.5 – Structure of a ZFP bound to DNA. The protein is in red and the DNA is in blue. The α-helix can be seen lying in the major groove of the DNA. Three finger motifs can be seen. Image from K. Hoeksema [78].

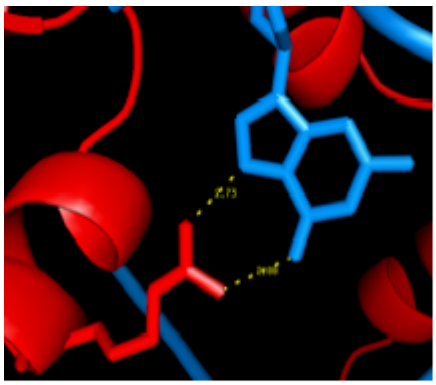

Figure 1.6 – ZFP and DNA interaction. The nucleotide base of the DNA (blue) is hydrogen bonding with a specific residue (red) of the ZFP. Image from K. Hoeksema.

1.3.2 – Zinc Finger Proteins as Therapeutics

Designer ZFPs have been used in a number of ways, including as artificial transcription factors (TFs) to activate or repress transcription, gene correction mediators, and antivirals. The first *in vivo* and *in vitro* demonstration of the ability of designed ZFPs to target specific DNA sequences was achieved by Choo *et al.* [79, 80]. They successfully inhibited transcription of an oncogene with high specificity using three-finger ZFPs in stably transformed murine cells [79]. Liu *et al.* [81] and Rebar *et al.* [82] have used this technology to stimulate new vasculature by promotion of vascular endothelial growth factor A (VEGF-A) gene transcription, *in vitro* and *in vivo*, respectively. The ZFP used in these studies was fused to an activation domain that up-regulated the VEG-F promoter and resulted in angiogenesis in the ear of a mouse model [82]. Attempting gene correction for monogenetic disorders, some researchers have made ZFP-nuclease fusion proteins to introduce site-specific double-strand DNA breaks in host chromosomes carrying the defect making them subject to homologous recombination with wild-type donor DNA [83-85]. Researchers have developed a system whereby designer ZFPs are linked to modified steroid hormone receptor ligand binding domains and transcriptional activation domains, generating novel ligand-dependent transcriptional regulators [86].

Antivirals based on designer ZFPs are a promising approach to treat viral infections. Viral replication has been shown to be inhibited by ZFPs

specific for certain plant viruses, human papilloma virus and notably for HBV (more below) [87-92]. By fusion of a ZFP targeting the long terminal repeat region of the virus with a repression domain, significant viral inhibition of HIV-1 has been observed [93-95].

Recently, researchers have been able to induce targeted viral DNA mutagenesis in certain viruses by using DNA binding proteins (not just ZFPs) fused to DNA cleavage enzymes. For example, the HIV-1 co-receptor molecule CCR5 has been targeted in primary human CD4(+) T cells [96] and in human hematopoietic stem cells [97] with ZFP-nuclease proteins. When engrafted in a humanized mouse model they resulted in lower viremia and slower CD4(+) T cell depletion after HIV-1 challenge. Wilen *et al.* [98] showed that CXCR4, the other co-receptor for HIV-1, could be targeted in primary human CD4(+) T cells by ZFP-nucleases and protect these cells from CXCR4-tropic HIV-1. Furthermore, they showed that when CD4(+) T cells from humans that have a homozygous mutation in the CCR5 gene are treated with ZFP-nucleases targeting the CXCR4 gene, they were resistant to all HIV-1 strains tested [98]. Homing endonucleases (HEs) are enzymes capable of creating double-strand DNA breaks at highly specific target sequences. Aubert *et al.* [99] used an *in vitro* model of HIV-1 latency (lentiviral reporter system) and HEs targeting the provirus, and found that HEs were able to cause mutations that lead to decreased reporter gene expression. Research has been done, although to much lesser extent than with HIV-1, in the field of herpes simplex virus 1 (HSV-1) genome targeting. Grosse *et al.* [100] found that when HSV-1 specific

HEs are expressed in certain tissue culture cells, the cells become more resistant to infection at low to moderate multiplicities of infection (MOIs). They also found that in the remaining viral genomes, up to 16% had mutations at the cleavage site [100].

Recently, HBV has been effectively targeted by ZFPs and ZFP-nucleases [87, 101]. Chen *et al.* [87] designed ZFPs against the core promoter of HBV and tested the antiviral effect in HepG2.2.15 (human hepatoma cells that stably express HBV). After transfection of the ZFP-expression plasmid, they found that HBeAg and HBV DNA in the culture supernatant as well as HBV mRNA were all significantly reduced. Cradick *et al.* [101] designed ZFP-nucleases and co-transfected these expression plasmids along with a plasmid containing the HBV genome and found that 26% of target plasmid remained linear and 10% had been cleaved and were misjoined by cellular repair mechanisms. Lastly, work completed in the Tyrrell laboratory showed that DHBV could be inhibited in a similar tissue culture system [102]. This work is the focus of the next section.

1.3.3 – Previous Research as Proof of Principle

A previous graduate student in the Tyrrell Laboratory, Kimberley Hoeksema (née Zimmerman) completed her PhD in 2010. Her thesis was entitled "Designed zinc finger proteins as novel therapeutics inhibiting the transcription of hepatitis B and duck hepatitis B viruses" and was the basis for this body of work. A publication in the Journal of Virology (2008) [102]

also resulted from her work, which will be reviewed below. I began my research on ZFPs having worked with Kimberley Hoeksema and moving from the duck virus to the human virus. I will briefly review the work done on the human virus before I took over the project, as it is important background information.

In Zimmerman *et al.* [102], DHBV was inhibited by the presence of DHBV-specific ZFPs, as viral RNA, viral proteins and progeny virus were all significantly decreased. The ZFPs were designed with an SV40 nuclear localization signal so that they could interact with the cccDNA driving viral transcription in the nucleus. The ZFPs were targeted to the enhancer region of the DHBV genome, known to be accessible to transcription factors and controlling the core and small surface protein promoters. ZFPs were designed to be able to discriminate between DHBV and chicken or duck genomic sequences. Three 6-finger (18-bp recognition) ZFPs were studied: two DHBV-specific ZFPs were used, as well as a control scrambled ZFP that has no specific binding target. The binding affinity of the ZFPs for their target was assessed by electrophoretic mobility shift assay (EMSA). Dissociation constants (K_d) were in the nM range, indicating high affinity. To confirm the nM K_d values obtained from EMSA, surface plasmon resonance (SPR) was used. This technique measures real-time interactions between ligands that are anchored to a detection surface, while analyte flows over it. Using this technique, nM range K_d was observed for both ZFPs [102]. The specificity of the ZFPs to their target sequence was assessed by competition EMSA, where

competition was observed with specific unlabeled oligonucleotides containing the target sequence but not with unspecific unlabeled oligonucleotides, indicating a specific interaction.

Longhorn chicken male hepatoma (LMH) cells were co-transfected with a plasmid that supports the DHBV replication cycle and with DHBV-specific ZFP-expression vectors. At 24 hours post-transfection, total RNA and lysates were harvested, and at 48 hours, intracellular viral DNA was collected for analysis. DHBV pregenomic RNA was quantitated by quantitiative polymerase chain reaction (qPCR). In the presence of the specific-ZFPs there was a significant reduction observed of this transcript (Figure 1.7 A). Northern blots were performed to see the effect on all the DHBV transcripts, and a trend for decreased transcript production was seen in the presence of the specific ZFPs (Figure 1.7 B & C). Viral core and surface protein expression was measured by Western blot and in the presence of the specific ZFPs, decreased levels of the viral proteins were observed (Figure 1.7 E & F). Intracellular viral DNA was isolated and then analyzed by Southern blot and quantified, and decreased levels were detected in the presence of the specific ZFPs (Figure 1.7 G). Lastly, to measure the toxicity of ZFPs in the LMH cells, MTT assay was performed, and no significant difference was observed between the scrambled control and the DHBV-specific ZFPs (Figure 1.7 D) [102]. In summary, it has been shown that designed ZFPs targeting the enhancer region of DHBV cccDNA are able to inhibit viral transcription and replication with no observed toxicity (Figure 1.7).

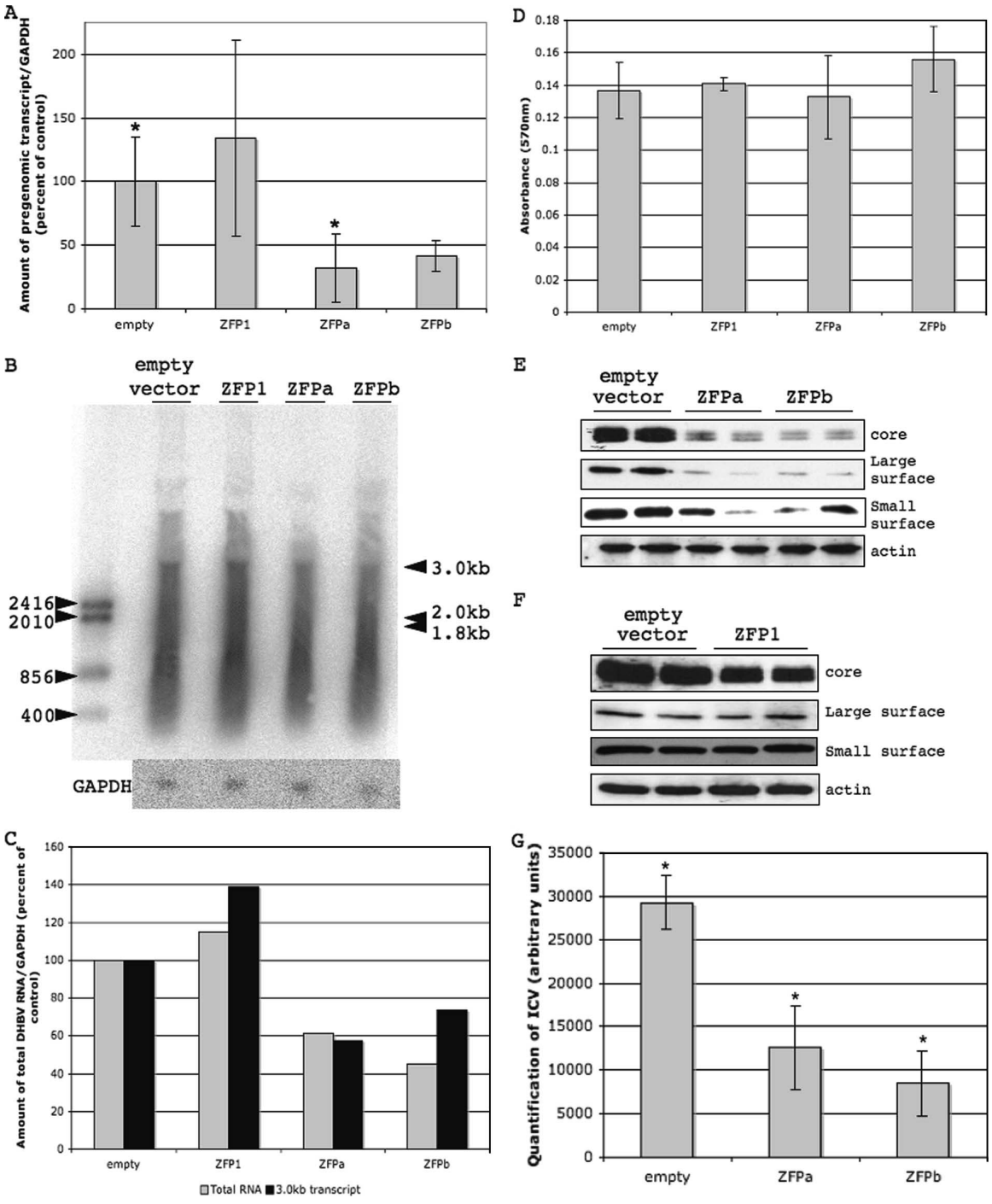

Figure 1.7 - Effects of DHBV-specific ZFPs in LMH cells on DHBV replication, from Zimmerman *et al*. (2008). Data collected from LMH co-transfected with plasmid replicating the DHBV lifecycle and ZFPs or empty vector. (A) cDNA from total RNA harvested at 24 hours, subjected to

quantitative PCR with DHBV-specific and GAPDH-specific primers for normalization. (B) Northern blot with DHBV-specific and GAPDH-specific probes and the DHBV transcripts are indicated with black arrows. (C) Total RNA or 3 kb transcript quantitated from (B) and normalized to GAPDH, and plotted as percent of empty vector control. (D) MTT assay to measure the toxicity of the ZFPs compared to empty vector in LMH cells after 24 hours. (E and F) Western blots with LMH lysates harvested after 24 hours and probed with viral antibodies or actin as loading control. (G) Intracellular virus DNA isolated after 48 hours from LMH cells, ran on a Southern blot and quantified on a phosphorimager.

1.3.4 – Preliminary Data: ZFPk & ZFPn

Kimberley Hoeksema's thesis describes the design and characterization of several HBV-specific ZFPs. The design strategy is very similar to that of the DHBV-specific ZFPs, except the HBV-specific ZFPs are designed to be able to discriminate between HBV and human genomic sequences, and target a different region of the viral genome. Her preliminary work is described here.

The HBV-specific ZFPs are targeted to the preS2 promoter region of HBV strain *ayw* (U95551). This region is known to be accessed by numerous DNA-binding proteins, so it should be accessible to the designed ZFPs [103, 104]. Aside from potentially interfering with the preS2 promoter activity, this region overlaps the preS1 and polymerase ORFs, potentially interrupting these transcripts as well (Figure 1.8).

In addition to the 18 bp recognizing ZFPs, several other ZFPs were designed that recognize smaller target regions (9 bp) that were intended to

function as heterodimers. These heterodimers were intended to be made into ZFP-nuclease fusion proteins, but we experienced much difficulty in the cloning process. Therefore, ZFPk and ZFPn, and the scrambled control ZFPcA, 6-finger (18 bp recognition) ZFPs, are the focus of this thesis.

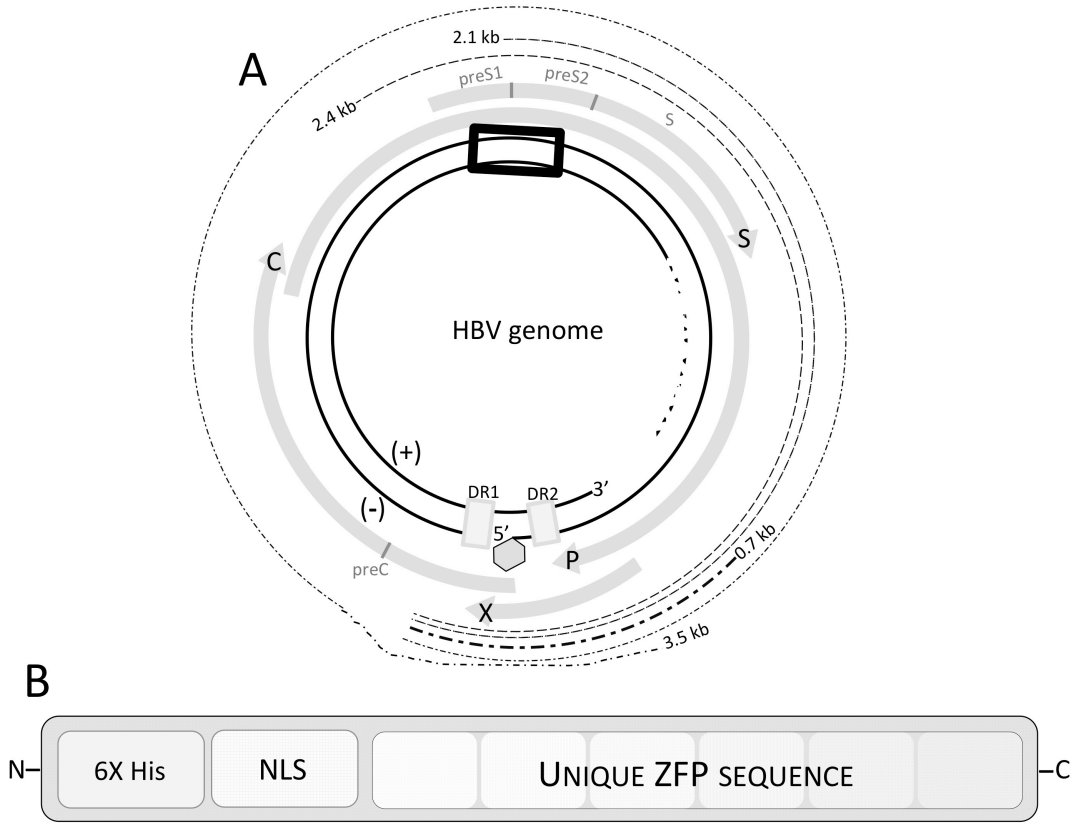

Figure 1.8 - ZFPk or ZFPn target region and outline of their structure. (A) The HBV genome as depicted in Figure 1.1, but the HBV-specific ZFP target site area highlighted with a black box, the preS2 promoter region. (B) A schematic of the structure of the 6-finger HBV-specific ZFPs. There is a 6X histidine tag nearest the amino terminus, followed by a SV40 nuclear localization signal (NLS), and then the unique ZFP coding sequence with 6 finger domains represented as grey boxes.

1.3.4.1 – *Determination of binding affinity*

To determine the binding affinities of ZFPk and ZFPn, EMSA and SPR was performed.

EMSA. Briefly and as described in [102], dsDNA oligonucleotides (with 6 nt flanking each end) were prepared and 1 μM was present in gel-shift buffer with serial dilutions of ZFP at 150 nM to 9.5 nM. Reactions were incubated for 1 hour and then run on 7% non-denaturing polyacrylamide gels for 1 hour. DNA in the gels were then stained with SYBR-Green, imaged, then proteins stained with SYPRO-Ruby, destained and imaged. Band intensity was quantified using Fujifilm ImageGauge v4.22 (2003) software and then non-linear regression plots were produced to calculate dissociation constants. Dissociation constants calculated by EMSA are 44.0 nM for ZFPk and 85 nM for ZFPn (Figure 1.9) (non-linear regression plots not shown) [78, 102].

SPR. Briefly and as described in [102], using BIAcore technology, ZFPs were dialyzed with 1X Hank's Balanced Salts plus 3 mM EDTA and 0.005% surfactant P20, which was also used as the running and sample buffers. Oligonucleotides were biotinylated on the 5' end of the top strand only and then coupled to a sensor chip coated in streptavidin on the BIAcore 3000. ZFPs flowed over the detection surface for 3 minutes followed by 15 minutes for dissociation. A range of concentrations for the ZFPs was used, and between the ZFP injections, the detection surface was regenerated with 0.5% SDS to remove previously bound ZFP. The amount of ZFP binding the

oligonucleotide ligand on the detection surface is measured by the change in refractive index, and this change is calculated as response difference in resonance units (RU). Each line on the graph represents an average for each concentration of ZFP. Kinetic analysis was done on a BIAeval software program. The dissociation constants calculated from SPR are 5.1 nM for ZFPk and 69.4 nM for ZFPn (Figure 1.10) [78].

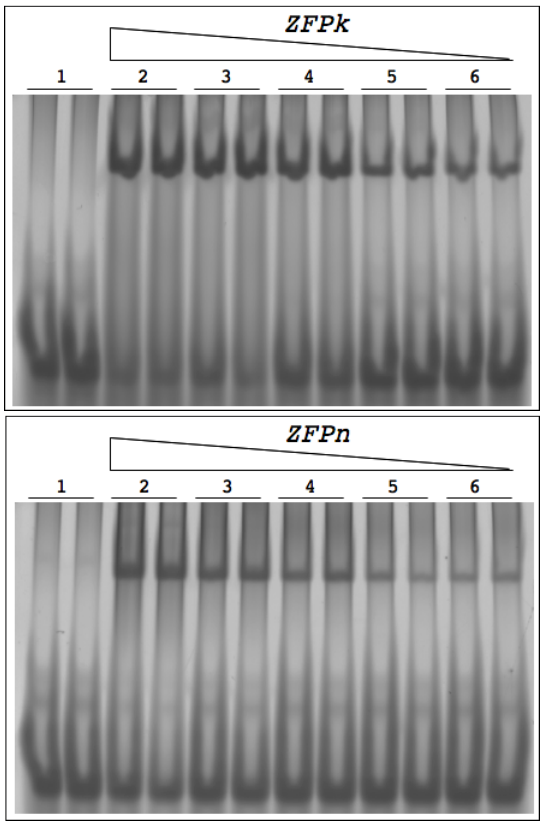

Figure 1.9 – EMSA for ZFPk and ZFPn. Each sample is run in duplicate. ZFP-specific ds oligonucleotides incubated alone (lane 1), or with ZFP-MBP (MBP-maltose binding protein) at 150 nM (lane 2), with serially dilutions of ZFP-MBP down to 9.5 nM (lanes 3-6). Gels stained for DNA with SYBR-Green. Protein-DNA complexes are found nearer the top of the gel in lanes 2-6.

These bands were quantified and used to determine the dissociation constant. Image from K. Hoeksema [78].

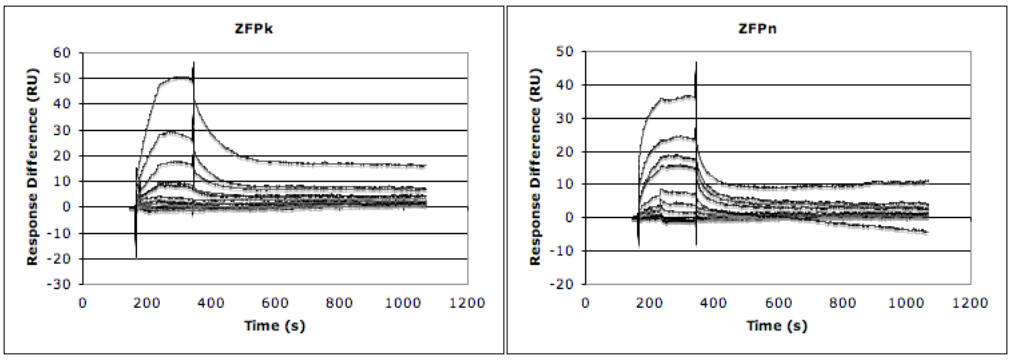

Figure 1.10 – Surface plasmon resonance for ZFPk and ZFPn. On a BIAcore 3000, biotinylated ds oligonucleotides were coupled to streptavidin on a Sensor Chip SA. ZFP-MBP at a range of concentrations were flowed over the detection surface for 3 minutes followed by 15 minutes of dissociation with surface regeneration to remove unbound ZFP between rounds. Each line represents an average for one concentration of ZFP. The response difference in resonance units (RU) reflects the change in refractive index on the sensor chip detection surface caused by ZFP binding the bound oligonucleotides. Image from K. Hoeksema [78].

1.3.4.2 – *Determination of specificity*

To assess the specificities of ZFPk and ZFPn for their target sequences, competition EMSA was performed. Briefly and as described in [102], specific and non-specific dsDNA oligonucleotides (with 6 nt flanking each end) were prepared as unlabeled and ^{32}P-labeled. Specific labeled probes were incubated alone or with 150 nM ZFP, and specific unlabeled oligonucleotides to act as competitors were added at 5, 10 and 50 μM, or unlabeled non-specific oligonucleotides at 50 μM. Also, non-specific labeled probe was

incubated with 150 nM ZFP. Reactions were incubated for 1 hour and run on 7% non-denaturing polyacrylamide gels for 1 hour. Gels were exposed to an image plate overnight and scanned using the Fujifilm FLA-5100 phosphorimager [102]. ZFPk and ZFPn are specific for their target sequences as seen by binding in lane 2 and when unlabeled specific oligonucleotides are added gradually, the probe can be competed off the ZFP (lanes 3-5). The ZFPs bind their respective probes in the presence of unlabeled nonspecific oligonucleotides (lane 6) to the same extent as lane 2 indicating a preference for their target sequence. As expected, the ZFPs do not bind non-specific probe (Figure 1.11) (lane 7) [78].

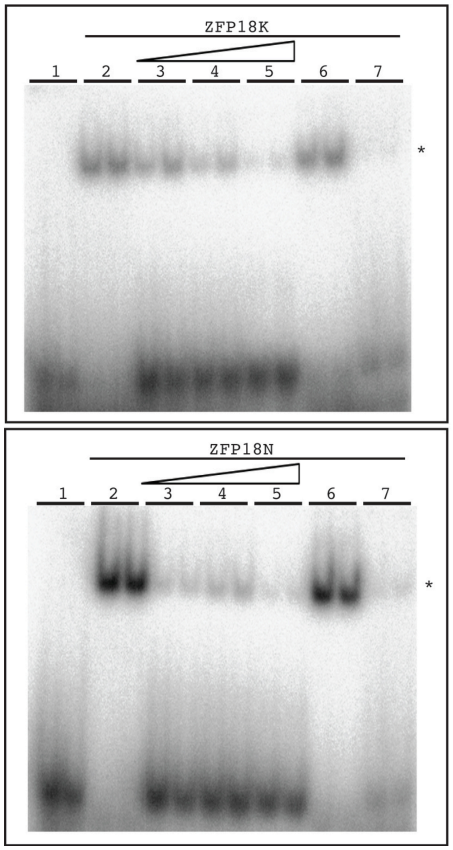

Figure 1.11 - Competition EMSA for ZFPk and ZFPn. Radiolabeled specific ds oligonucleotides were incubated alone (lane 1) or with 150 nM ZFP-MBP (lanes 2-7). Unlabeled specific oligonucleotide was added at 5 μM, 10 μM and 50 μM as competitor DNA (lanes 3-5, respectively) and unlabeled nonspecific competitor DNA at 50 μM (lane 6). Radiolabeled nonspecific oligonucleotides were incubated with 150 nM ZFP-MBP (lane 7). The asterisk (*) shows the ZFP-DNA complexes. Image from K. Hoeksema [78].

1.3.4.3 – *Determination of nucleotide mutation tolerance*

To assess the tolerance of ZFPk for single or double nucleotide substitutions in its target sequence, EMSA was performed. Briefly, oligonucleotides (1 μM) with one or two nucleotide substitutions at various areas in the target sequence were incubated with 75 nM ZFPk for 1 hour.

Samples were then electrophoresed and stained as in section 1.3.4.1. A high tolerance for nucleotide changes is observed by the fact that very few mutations have resulted in a decreased amount of ZFP-DNA binding. However, in panel B lane 4, an A→T mutation in the target of finger 5 seems to have a decreased affinity and a slightly decreased affinity is seen in panel A lane 6, which is a G→C mutation in the target of finger 3. Surprisingly, a C→G mutation in the finger 1 target (panel A lane 4) seemed to exhibit higher binding than the target sequence for which it was designed (lane 2) (Figure 1.12). The design process may explain this off-target binding – the Zinc Finger Tools Program does not consider the secondary interactions that can take place that can affect binding affinities.

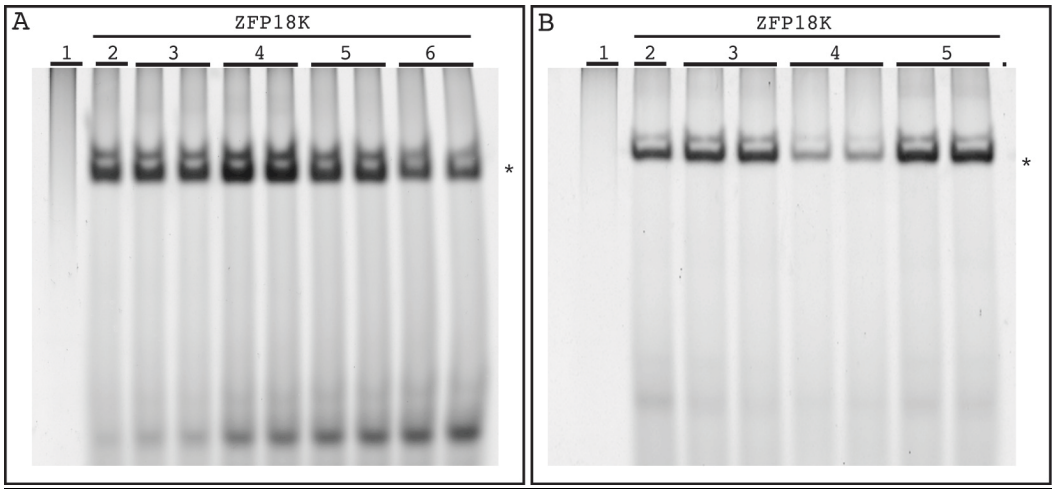

Figure 1.12 – EMSA to determine the tolerance of ZFPk for nucleotide mutations. Panel (A): ds oligonucleotides incubated alone (lane 1) or with 150 nM ZFP-MBP (lanes 2-6). Ds oligonucleotide with the exact target sequence (lane 2), C→T mutation in finger 1 (lane 3), C→G mutation in finger 1 (lane 4), G→A mutation in finger 3 (lane 5) and G→C mutation in finger 3

(lane 6). Panel (B): ds oligonucleotides incubated alone (lane 1) or with 150 nM ZFP-MBP (lanes 2-6). Ds-oligonucleotide with the exact target sequence (lane 2), A→G mutation in finger 5 (lane 3), A→T mutation in finger 5 (lane 4), and double nucleotide mutation in fingers 1 (C→T) and 5 (A→G) (lane 5). Gels stained for DNA with SYBR-Green, the asterisk (*) shows the ZFP-DNA complexes.

1.4 – Hypothesis and Objectives

1.4.1 – Hypothesis

Our hypothesis is that when HBV-specific zinc finger proteins (ZFPs) are expressed in an HBV-infected cell, they will bind the target sequence in the viral cccDNA and inhibit transcription, therefore decreasing the amount of viral RNA and proteins and decrease the levels of virus secreted.

1.4.2 – Objectives

1. To test a ZFP specific for human HBV in tissue culture and 2. If successful in tissue culture, demonstrate activity of a ZFP in HBV infected SCID/beige/Alb-uPA mice [105].

Specific Steps to Achieve the Objectives

1. Design of ZFPs specific for HBV DNA – specific, accessible sites in the cccDNA.

   - HBV-specific ZFPs were designed by Kimberley Hoeksema targeting the SPII promoter region of HBV strain *ayw*. They were designed

using the program "Zinc finger tools", codon optimized for *Homo sapiens* and synthesized by Blue Heron Biotechnology (Bothell, WA).

2. Develop a system for delivery of ZFPs – Lipid-based, baculoviral, and lentiviral delivery systems.
3. Test the effect of the ZFP constructs on the levels of extracellular virus in the supernatant and intracellular virus in tissue culture.
4. Examine the effect of specific ZFPs on viral proteins expressed in HBV infected tissue culture.
5. Examine the effect of specific ZFPs on viral RNA in HBV infected cells.
6. Ensuring the appropriate control ZFP does not have an effect on HBV expression.
7. If successful in tissue culture, attempt to treat the SCID/beige/Alb-uPA mouse.

1.5 – References

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Chapter 2 – Transfections, Adenovirus and Baculovirus Assessments

2.1 – Introduction

Our hypothesis is that when HBV-specific ZFPs are expressed in an HBV-infected cell, they will bind the target sequence in the viral cccDNA and inhibit transcription, therefore decreasing the amount of viral RNA and proteins and decrease the levels of virus secreted. To test the hypothesis, high levels of HBV expression are desirable to be able to see appreciable decreases in HBV virus, proteins or transcripts, in response to HBV-specific ZFPs to be easily detected. It is also important to achieve widespread ZFP expression because any *HBV+/ZFP-* cells will continuously produce HBV and mask any effect of the ZFPs in *HBV+/ZFP+* cells. The data presented in this chapter was produced in our attempt to develop a functional experimental system to test the hypothesis: achieving high levels of HBV and/or ZFP expression in human hepatoma cells.

An HBV-expressing adenovirus vector that is able to reproduce the HBV replication cycle was obtained with the intent of transducing a hepatoma cell line, Huh7, which can also be transfected with ZFP-expression plasmids with reasonable efficiency. This vector supports the full viral replication cycle and has been previously used to transduce primary hepatocytes from mice, ducks and humans, as well as Huh7 and HepG2 tissue culture cells with high efficiency [1, 2]. However, even with a wide range of MOI's tested, this vector did not yield high levels of HBV core protein

expression and the levels of ECV DNA in the supernatant were very low. Therefore, this vector was excluded from further experiments.

Most human hepatoma cell lines are difficult to transfect, including HepAD38 and HepG2.2.15 cells. However, the hepatoma cell line, Huh7, can be transfected with fair efficiency, so these cells were transfected with an HBV-expression plasmid to assess HBV levels post-transfection, with the ultimate goal of a co-transfection with ZFP-expression plasmids. This HBV-expression vector resulted in low HBV expression (data not shown), again undesirable for testing the hypothesis of this thesis. With the availability of hepatoma cell lines that stably express the full HBV replication cycle, HepAD38 and HepG2.2.15, simple transfection of ZFP-expression plasmids into these cells would be ideal. Accordingly, transfection with a variety of reagents, including Lipofectamine 2000, DharmaFECT Duo, DMRIE-C and FuGENE 6, were tried with these cells with unsatisfactory results (see [3] for HepAD38 transfections, HepG2.2.15 transfections presented here).

Baculovirus (Autographa californica nucleopolyhedrovirus) is an insect virus that is widely used for protein production and transduction. They are unable to replicate in vertebrate cells and have been shown to be able to transduce many different cell types, including primary human hepatocytes and hepatocyte-derived cell lines, with high levels of gene expression [4-6]. Baculovirus vectors capable of HBV-specific ZFP expression were previously constructed by Kimberley Hoeksema, a graduate student that preceded me in Dr. Tyrrell's laboratory [3]. The ZFP-expressing baculoviruses were tested in

the HBV-infected hepatoma cell lines and were able to express the ZFPs (Figure 2.5-2.6). However, a beta-galactosidase-expressing baculovirus, provided by Karl Fischer of the Tyrrell laboratory, which is based on the same backbone as the ZFP-expressing baculoviruses, revealed poor transduction efficiency and as such were discontinued from further experiments.

2.2 – Materials and Methods

2.2.1 – Adenovirus expressing HBV (Adv-HBV)

2.2.1.1 - *Cell lines and culture conditions*

i. Huh7.

These cells are a human hepatoma cell line. Huh7 cells were normally cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Cat.# 11965-092) supplemented with 10% fetal bovine serum (FBS) and 50 IU/mL penicillin and 10 μg/mL streptomycin and incubated at 37°C in 5% CO_2.

ii. 293A.

These are human embryonic kidney cells that express the adenovirus E1 gene to facilitate recombinant adenovirus production. These cells were normally cultured in DMEM supplemented with 10%

FBS and 50 IU/mL penicillin and 10 µg/mL streptomycin and incubated at 37°C in 5% CO_2.

2.2.1.2 - *Production and titration of Adv-HBV*

Production. Adv-HBV was provided to us by T. Jake Liang at $1x10^8$ pfu/mL (see Figure 2.1 for a schematic diagram of the Adv-HBV construct). A T-25cm^2 flask of 293A cells was seeded with $2x10^6$ cells, which results in approximately 60% confluent cells one day following seeding. Twenty-five µL containing $2.5x10^6$ pfu of Adv-HBV were used to infect the T-25cm^2 flask of 293A cells. After the 4 days, when the cells were starting to detach, the cells were scraped off the plastic surface with a rubber policeman and transferred into a sterile tube and spun at 100xg for five minutes. The cell pellet was resuspended in 2 mL sterile PBS and then 4 cycles of freeze/thaw in liquid nitrogen and a 37°C water bath, with vigorous vortexing between cycles and without letting the supernatant warm. The solution was spun briefly and the supernatant placed at -20°C for storage. Next, a 50-70% confluent T-175cm^2 flask of 293A cells was infected with 1 mL of Adv-HBV from the first isolation (untitrated virus). Infection proceeded for 3 days and then Adv-HBV was harvested as previously described, with the exception of being initially resuspended in 6 mL of PBS. Finally, five 50-70% confluent T-175cm^2 flasks of 293A cells were infected with 1 mL per flask of Adv-HBV isolated from the previous amplification (untitrated) and harvested after 4 days. The isolation

was the same as above, except all five flasks of infected cells were resuspended in 8 mL of PBS.

Titration. 293A cells were plated in 6-well plates at $1.5x10^5$ cells per well. The following day, 50 µL of Adv-HBV was prepared in eight-log serial dilutions in normal cell culture media and 2 mL of each dilution was applied to cell monolayers and incubated for 2 hours under normal culture conditions. After the 2 hours, the supernatant was removed, the cell monolayers were washed twice with PBS and fresh media was added. The following day, green fluorescent protein (GFP)-positive cells were counted from duplicate wells, averaged and used to calculate the titer of Adv-HBV.

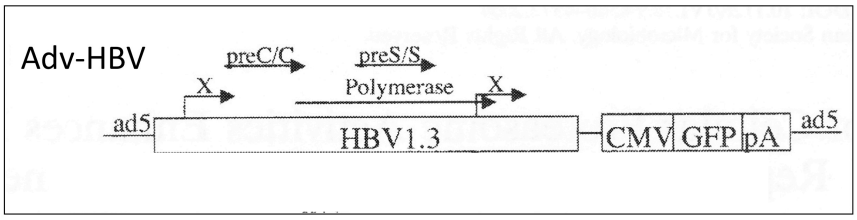

Figure 2.1 – Schematic of Adv-HBV, taken from Zhang *et. al.* [1]. The HBV1.3 is a 1.3-fold-overlength of the HBV subtype *ayw*. A CMV promoter drives the GFP, and natural promoters within the HBV genome, depicted by black arrows drive HBV production.

2.2.1.3 – *Adv-HBV infections*

Adv-HBV was diluted in the appropriate amount of media to yield the desired MOI (0.1 to 50). Cell culture media was removed and replaced with the new media containing Adv-HBV at various MOIs and returned to the

incubator. After 2 hours, the virus containing supernatant was removed and the cells were washed twice with PBS and fresh media was added.

2.2.1.4 – *Cell Culture assessment of HBV*

i. Extracellular virus (ECV) DNA isolation. One mL of cell culture supernatant was collected and incubated at 37°C for 30 minutes with 6 mM $MgCl_2$, 100 µg/mL DNase I, 10 µg/mL RNase A to digest unprotected nucleic acids. After centrifugation at 17,500xg for ten minutes the supernatant was collected and incubated at 4°C overnight in 0.3 volumes of 26% PEG 8000, 1.4M NaCl and 10 mM EDTA to precipitate virus. The samples were centrifuged at 17,500xg for ten minutes and the supernatant was discarded, followed by pellet resuspension in 100 µL TSE buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl and 10 mM EDTA) and the addition of Proteinase K to 800 µg/mL and SDS to 0.1% to digest the viral nucleocapsids. This solution was incubated at 42°C overnight and the following day the viral DNA was phenol chloroform extracted and precipitated with the addition of 10 µg yeast tRNA, two volumes of ice-cold 95% ethanol and 1/10 volume of 3 M sodium acetate at -20°C overnight. The samples were centrifuged at 17,500xg for fifteen minutes and DNA pellets dried and resuspended in 20 µL of water.

ii. Quantitative Real-time PCR. Real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System (ABI) in a 96-well optical plate format. HBV-specific probe (HBV-P3) at 250 nM and primers (HBV-F3 and HBV-R3M3) at 900 nM (amplicon located in the X gene) were used to

quantitate the ECV DNA (Oligonucleotides in Table 2.1). The standard curve, ranging from 10^1 genome equivalents (ge)/μL to 10^6 ge/μL, negative controls and samples were all tested in duplicate. The ABI standard program consisted of 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute to amplify HBV DNA.

Table 2.1 – Oligonucleotides used for quantitative PCR detecting HBV DNA. HBV-F3 is the forward primer, HBV-R3M3 is the reverse primer (containing a 5-nitroindole (i5NitInd), a universal base analog for detecting multiple HBV genotypes) and HBV-P3 is the probe, which is labeled with 6-carboxyfluorescein (FAM) and tetramethylrhodamine (TAMRA) fluorophores [7].

Oligonucleotide name	Sequence (5'-3')	Reference
HBV-F3	ggccatcagcgcatgc	[7]
HBV-R3M3	c/i5NitInd/gctgcgagcaaaaca	[7]
HBV-P3	FAM-ctctgccgatccatactgcggaactc-TAMRA	[7]

iii. SDS-PAGE and Western blot. Cell monolayers were lysed 48 hours post-transduction using radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholic acid in water). The protein concentrations in whole cell lysates were determined using the bicinchoninic acid (BCA) protein assay reagent according to manufacturers specifications (Pierce, Cat.# 23227). The 10%

SDS-polyacrylamide gel was prepared in two steps. First the separating gel was cast (3.75 mL of 2 M Tris-HCl pH 8.8, 200 µL of 10% SDS, 5 mL of 40% acrylamide/bis-acrylamide (29:1), 200 µL of 10% APS, 10.8 mL of water and 20 µL TEMED) and allowed to polymerize for 30 minutes. Second, the stacking gel was prepared and cast (1.25 mL of 1 M Tris-HCl pH 6.8, 100 µL of 10% SDS, 974 µL of 40% acrylamide/bis-acrylamide (29:1), 100 µL of 10% APS, 6.3 mL of water and 10 µL of TEMED) with 1.5 mm well spacers and allowed to polymerize for approximately 30 minutes. SDS-PAGE loading buffer was prepared using 125 mM Tris-HCl pH 6.8, 5% SDS, 10% 2-mercaptoethanol, 15% glycerol and 0.1% bromophenol blue. Samples were prepared using 40 µg of protein per well with 5 µL SDS-PAGE loading buffer and boiled for 5 minutes. The gel apparatus was assembled and the chambers were filled with SDS-PAGE running buffer (30 g/L of Tris, 144 g/L of glycine and 10 g/L of SDS) and then the samples loaded into the wells. Samples were electrophoresed through the gel at 90 V for 15 minutes until the pre-stained SDS-PAGE standards (Rio-Rad, Cat.# 161-0305) began to separate and then the voltage increased to 160 V until the bromophenol blue dye front was approximately 1 cm from the bottom of the gel.

A semi-dry transfer apparatus (Fischer Brand, Cat.# FB-SDB-2020) was used to transfer the electrophoresed proteins onto a nitrocellulose membrane (Amersham Hybond-ECL, GE, Cat.# RPN303D). Specifically, the stacking gel portion of the polyacrylamide gel was removed with a clean scalpel and the separating gel was soaked for approximately 5 minutes in

semi-dry transfer buffer (14.27 g/L of glycine, 3 g/L of Tris and 20% methanol in water). Two pieces of Whatman 3MM filter paper (bigger than the gel) and a piece of the nitrocellulose (same size as gel) were also soaked in semi-dry transfer buffer for approximately 5 minutes. The transfer was set up with the two filter papers nearest the plates of the apparatus with the gel and the nitrocellulose sandwiched in between, with the membrane closest to the positive plate. Transfer was carried out with a limit of 500 mA and voltage maximum at 27 V for 1 hour.

After the transfer, the membrane was blocked with 5% skim milk blocking buffer (5% skim milk powder, 1x Tris buffered saline (TBS) and 0.1% Tween 20 (T)) for one hour. (10x TBS consists of 15.76 g/L of Tris, 87.66 g/L of NaCl). The membrane was washed three times; each wash was five minutes with 1x TBS/T (1x TBS and 0.1% Tween 20). The membrane was incubated with primary antibody for one hour with anti-HBV core protein (1/2,000 rabbit anti-HBV core, DAKO, Cat.# B0586). Anti-actin (1/5000 mouse anti-actin, Millipore, Cat.# MAB1501) was used as a loading control. After washing three times, the membrane was treated with secondary antibodies for 45 minutes (1/5000 goat anti-rabbit Horseradish peroxidase (HRP), Cappel, Cat.# 21974 and 1/5000 goat anti-mouse HRP, Cedarlane, Cat.# CLCC 30007) diluted in 1% skim/TBS/T. Between antibody incubations and afterwards there were 3x washes in TBS/T. Secondary antibodies conjugated to HRP were detected by exposure to Supersignal West Dura Extended Duration Substrate (Pierce, Cat.# 34076) and exposure

to x-ray film (Kodak, Cat.# XAR-5) for 10 minutes to develop and visualize protein bands.

2.2.2 – Transfections

2.2.2.1 – *Cell lines and culture conditions*

i. Huh7.

These cells were previously described (section 2.2.1.1.i).

ii. HepG2.

HepG2 cells are a human hepatoma cell line. These cells are uninfected and were a useful negative control of HBV expression. These cells were normally maintained in a 1:1 mixture of DMEM and Ham's F-12 Nutrient Mix (Life Technologies, Cat.# 11765-054) supplemented with 10% FBS and 50 IU/mL penicillin and 10 µg/mL streptomycin and incubated at 37°C in 5% CO_2.

iii. HepG2.2.15.

These human hepatoma cells are derived from HepG2 cells. To create HepG2.2.15 cells, HepG2 cells were transfected with a plasmid carrying two head-to-tail copies of HBV genome (*ayw*) and the *neo* gene. In these cells stable HBV expression is driven from natural, endogenous promoters of HBV, and these cells support the full replication cycle of HBV and produce infectious virus [8, 9]. These cells were normally maintained in DMEM/F-12 (1:1) supplemented

with 10% FBS and 50 IU/mL penicillin and 10 µg/mL streptomycin and incubated at 37°C in 5% CO_2.

iv. HepAD38.

These human hepatoma cells are derived from HepG2 cells. To create HepAD38 cells, HepG2 cells were co-transfected with a cDNA of the pregenomic (pgRNA) from HBV (strain *ayw*) under the control of the tetracycline-responsive promoter, CMVtet, and a selectable marker. Stable HBV expression is driven from a CMV-IE promoter [10]. These cells support the full replication cycle of HBV and produce infectious virus. These cells were normally maintained in DMEM/F-12 (1:1) supplemented with 10% FBS and 50 IU/mL penicillin and 10 µg/mL streptomycin and incubated at 37°C in 5% CO_2.

2.2.2.2 – *Lipid-based transfections*

Lipofectamine 2000. This lipid-based transfection reagent is composed of a proprietary formulation for transfecting DNA and RNA into eukaryotic cells (Invitrogen, Cat.# 11668-019). This reagent was tested on HepG2.2.15 cells in suspension or as adherent cultures, and with Huh7 cells using a range of DNA:reagent ratios. Aside from varying the DNA:reagent ratios, methods were performed as specified by the manufacturers instructions.

Briefly, for adherent HepG2.2.15 cells in a 48-well plate, the DNA:reagent ratio of 1:2 was used. For each well, 0.42 µg of pd1-EGFPn1

plasmid DNA was diluted in a total volume of 100 µL Opti-MEM reduced serum media (Life Technologies, Cat.# 31985-070) and 0.85 µL of Lipofectamine 2000 was mixed with Opti-MEM to a total volume of 100 µL and incubated for 5 minutes at room temperature. The DNA/Opti-MEM mixture was added drop-wise into the Lipofectamine/Opti-MEM mixture and incubated for 20 minutes. The transfection media was added drop-wise to cell monolayers that contain normal media with no antibiotics and the cells returned to the incubator for 6 hours, followed by a media change. At 24, 48, and 72 hours post-transfection the EGFP-positivity (enhanced green fluorescent protein) was assessed by fluorescent microscopy (Zeiss NLO510 multi-photon microscope) at emission and excitation wavelengths of 488 nm and 509 nm, respectively. For transfection of cell suspensions, methods are the same as for adherent cells except for the following modifications. The DNA/reagent/Opti-MEM mixtures were made in the wells of a 48-well plate which was either poly-L-lysine coated or uncoated. Transfection media was prepared as described above and after the 20-minute incubation, $7.8x10^4$ cells were added drop-wise to each well in transfection media and incubated for 20 hours when the media was changed to normal cell culture media.

For transfection of adherent Huh7 cells, the cells were plated at $2x10^5$ cells/well in 6-well plates. Four µg of DNA (pd1-EGFPn1) was used with varying amounts of Lipofectamine 2000 to give DNA:reagent ratios of 2:1, 1:1, 1:2 and 1:5. The DNA was mixed with Opti-MEM for a total volume of 250 µL. In a separate tube, the Lipofectamine 2000 was mixed with the same

volume of Opti-MEM and incubated for 5 minutes at room temperature. The DNA/Opti-MEM solution and Lipofectamine 2000/Opti-MEM solution were mixed together drop-wise and incubated for 20 minutes at room temperature. This resulting transfection media was added drop-wise to the cell monolayers grown in normal media containing no antibiotics. Six hours later the media was changed to normal media containing antibiotics and at 24 and 48 hours post-transfection the EGFP-positivity was assessed by fluorescent microscopy (Zeiss NLO510 multi-photon microscope) at emission and excitation wavelengths of 488 nm and 509 nm, respectively.

DharmaFECT Duo. This transfection reagent from Dharmacon RNAi Technologies (Part of Thermo Scientific) is a proprietary blend of lipids that is optimized for delivery of both siRNA and plasmid (Thermo Scientific, Cat.# T-2010-01) This reagent was reported to have high transfection efficiency with HepG2.2.15 cells (personal communication with Bill Addison). Even though I am not using siRNAs, the reagent will still deliver plasmid DNA. I tested this reagent on HepG2.2.15 cells in suspension or on adherent cultures, with or without poly-L-lysine coated plates with varying cell densities and the transfection assessed over 48 hours. Methods were performed as specified by the manufacturers instructions. Briefly, for adherent cell transfection, HepG2.2.15 cells were plated at $2x10^5$ cells/well, $2.6x10^5$ cells/well and $3x10^5$ cells/well in 12-well plates. For each well, one µg of pd1-EGFPn1 plasmid was diluted in 100 µL of Opti-MEM, and in a

separate tube, 99-97 µL of Opti-MEM was mixed with either 1 µL, 2 µL, or 3 µL of DharmaFECT Duo to total volume of 100 µL and incubated at room temperature for five minutes. One hundred µL of the Opti-MEM/reagent mixture was added drop-wise into the respective DNA/Opti-MEM mixture and incubated at room temperature for 20 minutes. The culture media was removed from the cells and 800 µL of antibiotic-free normal culture media was added to the DNA/reagent/Opti-MEM and this mixture was applied to the cell monolayers and the cells were returned to the incubator. After 24 hours the media was changed to normal cell culture media and at 24 hours and 48 hours post-transfection the EGFP-positivity was assessed by fluorescent microscopy (Zeiss NLO510 multi-photon microscope) at emission and excitation wavelengths of 488 nm and 509 nm, respectively. For transfection of cell suspensions, the methods were similar as for adherent cells except for the following modifications. The DNA/reagent/Opti-MEM mixtures were made in each well of a 24-well plate which was either poly-L-lysine coated or uncoated. Transfection media was prepared as above except 1.5 µL of reagent was used (1:1.5 DNA:reagent) and after the 20-minute incubation $2.7x10^5$ cells were added drop-wise to the well containing the DNA/reagent transfection media and returned to the incubator for 24 hours. After 24 hours the media was changed to normal cell culture media and at 24 hours and 48 hours post-transfection the EGFP-positivity was assessed by fluorescent microscopy.

DMRIE-C. This transfection reagent from Invitrogen is a liposome formulation consisting of 1:1 (M/M) cationic lipid 1,2-dimyristyloxy-propyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE) and cholesterol (C) (DMRIE-C) in membrane filtered water (Invitrogen, Cat.# 10459-014). This reagent was tested on HepG2.2.15 cells in suspension or as adherent cultures, at high or low reagent:DNA ratios, with or without poly-L-lysine coated plates and assessed over 72 hours. Methods were performed as specified by the manufacturers instructions. Briefly, for adherent cells at $7.8x10^4$ cells/well in a 48-well plate, 0.42 µL reagent for "low" and 1.3 µL reagent for "high" was mixed with 100 µL Opti-MEM, then 0.42 µg DNA (pd1-EGFPn1) was added with Opti-MEM for a total volume of 100 µL and then the DNA/Opti-MEM mixture added to the reagent/Opti-MEM mixture. After a 15-minute incubation, cell monolayers were washed once with Opti-MEM and the 200 µL of transfection media added drop-wise onto the cells in each well. The cells were returned to the incubator for 5 hours and then normal culture media containing 15% FBS was added to the cells. At 24, 48, and 72 hours post-transfection the EGFP-positivity was assessed by fluorescent microscopy (Zeiss NLO510 multi-photon microscope) at emission and excitation wavelengths of 488 nm and 509 nm, respectively. For suspension cell transfection, methods are the same as for adherent cells except for the following modifications. The DNA/reagent/Opti-MEM mixtures were made in the wells of a 48-well plate which was either poly-L-lysine coated or uncoated. Transfection media was prepared as above and after the 15-minute

incubation $7.8x10^4$ cells were added drop-wise into the well/transfection media, returned to the incubator and after 20 hours normal culture media containing 15% FBS was added.

FuGENE 6. This transfection reagent from Roche is a proprietary blend of lipids and other components in 80% ethanol (Roche, Cat.# 11815091001). This reagent was tested on HepG2.2.15 cells in suspension or as adherent cultures, at three DNA:reagent ratios, with or without poly-L-lysine coated plates and assessed over 48 hours. Methods were performed as specified by the manufacturers instructions. Briefly, for adherent cell transfections, the three different reagent:DNA ratios tested were 3:1, 3:2 and 6:1, with variations of 1.26 µL or 0.63 µL of reagent and 0.2 µg or 0.42 µg of DNA (pd1-EGFPn1). HepG2.2.15 cells were plated at $2.5x10^5$ cells/well in a 24-well plate with or without poly-L-lysine coating the day before transfection. For each well, FuGENE 6 was diluted in Opti-MEM to a volume of 21 µL and incubated for 5 minutes at room temperature. The reagent/Opti-MEM solution was added drop-wise into a tube containing the DNA (only DNA) and incubated for 20 minutes at room temperature. The reagent/DNA solution was added to the cell monolayer grown in normal media containing no antibiotics. The cells were incubated in normal culture conditions overnight and the media changed the next day to normal cell culture media. At 24 hours and 48 hours post-transfection the EGFP-positivity was assessed by fluorescent microscopy (Zeiss NLO510 multi-

photon microscope) at emission and excitation wavelengths of 488 nm and 509 nm, respectively. For suspension cell transfection, methods are the same as for adherent cells except for the following modifications. The DNA/reagent/Opti-MEM mixtures were made in the well of a 24-well plate which was either poly-L-lysine coated or uncoated. Transfection media was prepared as above and after the 20-minute incubation $2.7x10^5$ cells were added drop-wise into the well/transfection media and then returned to the incubator.

2.2.2.3 – *Electroporation transfections*

BTX Electro Square Porator. The ECM 830 Electro Square Porator (BTX Harvard Apparatus) is a square wave pulse generator that can be used for mammalian cell transfection. This device was used with two different protocols to test transfection of HepG2 cells using an EGFP reporter plasmid. The first protocol is provided by the manufacturer (Protocol 0370) and the second protocol was from de Marco *et. al.* (2002) in which the authors used the BTX Electro Square Porator to transfect HepG2 cells with different settings than the manufacturer recommends [11]. See the following table for settings (Table 2.2).

Electro Square Porator Protocol # 0370: $4x10^6$ HepG2 cells in 400 µL were mixed with 20 µg of pd1-EGFPn1 plasmid DNA and then transferred to the 4 mm gap cuvette. The electric pulse was performed as described in the protocol shown in the table below. The cells were immediately transferred

onto a poly-L-lysine coated 6-well plate at high and low densities and incubated at 37°C. The cells were examined for EGFP fluorescence at 24 and 48 hours post-transfection.

J. Cell Biol. De Marco *et. al.* (2002) [11] Method: $2x10^6$ HepG2 cells in 200 µL was mixed with 20 µg of pd1-EGFPn1 plasmid DNA and transferred to the 4 mm gap cuvette. The electric pulse was performed as described in the protocol in the table below. Immediately after the pulse, 1 mL of media was added to the cuvette. The cells were allowed to sit for ten minutes and then plated at high and low densities in a poly-L-lysine coated 6-well plate. The cells were examined for EGFP fluorescence at 24 and 48 hours post-transfection.

Table 2.2 – Comparison of protocols for transfecting HepG2 cells using the BTX Electro Square Porator.

	Electro Square Porator Protocol # 0370	J. Cell Biol. (2002) Publication Methods [11]
Voltage	150 V	200 V
Mode	LV	HV
Pulse Length	70 msec.	45 msec.
# of Pulses	1	1
Chamber	4 mm gap	4 mm gap

2.2.3 – Baculovirus

2.2.3.1 – *Cell lines and culture conditions*

i. Sf21 & Sf9.

Sf21 are cells that were derived from the ovary of *Spodoptera frugiperda* (fall army worm) and Sf9 are a clone of Sf21 [12]. These cells are commonly used with baculovirus vectors and have small differences with respect to growth rate and susceptibility to baculovirus infection [13]. They can be grown in suspension or adherent cultures and can be quickly adapted to serum-free medium. The cells used for studies in this thesis were normally cultured in Sf-900 SFM (Life Technologies, Cat.# 10902-088) at 25°C in both adherent and suspension cultures.

ii. HepG2.2.15.

These cells were previously described (see section 2.2.2.1.iii).

iii. HepAD38.

These cells were previously described (see section 2.2.2.1.iv).

iv. Human Hepatocytes.

Primary human hepatocytes were isolated from various donors by Jamie Lewis of the Kneteman laboratory as previously described [14]. Samples of these cells were cryopreserved by Michael Joyce of the Tyrrell laboratory. Samples of cryopreserved cells were thawed and cultured in DMEM supplemented with 1.2 µg/mL insulin, 200

mmol glucose, 11 μM hydrocortisone hemisuccinate, 15 mM HEPES, 3% human serum, 50 IU/mL penicillin, 10 μg/mL streptomycin, 1% DMSO and 20 ng/mL human hepatocyte growth factor, and incubated at 37°C in 5% CO_2.

v. Longhorn Male Hepatoma (LMH) cells.

This chicken hepatoma cell line was normally cultured in DMEM/F-12 (1:1) supplemented with 10% FBS and 50 IU/mL penicillin and 10 μg/mL streptomycin and incubated at 37°C in 5% CO_2. This cell line was previously used for successful transduction with baculovirus by Kimberley Hoeksema [3, 15].

2.2.3.2 – *Production and titration of baculovirus*

Production. ZFP-expressing baculoviruses were originally produced by Kim Hoeksema, as described in her PhD thesis [3]. Briefly, Karl Fischer of the Tyrrell laboratory had modified pFastBac such that a CMV-IE promoter was driving the transgene expression (pFB-CMV1, appendix A). HBV-specific ZFPs were cloned into pFastBac and the resulting pFastBac-ZFP plasmids were transformed into *Escherichia coli* (*E. coli*) carrying a bacmid that undergoes recombination with pFastBac, transferring the desired transgene into the bacmid. The bacmid-ZFP DNA was transfected into Sf9 cells and the supernatant was collected after one week to assay for ZFP-expressing baculoviruses. The resulting baculovirus-ZFP stocks were stored in 2 % FBS at both -80°C for long-term storage, as well as at 4°C for present use.

Subsequent production of ZFP-expressing baculoviruses for work in this thesis was carried out as follows. Sf9 cells were seeded at $1x10^6$ cells/mL in 150 mL spinner flask cultures and allowed to adapt to suspension culture for 5 days. The cells were seeded at $1x10^6$ cells/mL in 150 mL spinner flask cultures and infected at an MOI of 0.1 with the baculovirus expressing ZFP and grown in the dark to preserve the infectivity [16]. After nine days, the cultures were harvested and centrifuged at 200xg for 15 minutes at 4°C to pellet the cells. The supernatant was overlaid on a 25% sucrose cushion (25% sucrose, 5 mM NaCl, 10 mM Tris pH 7.5, 10 mM EDTA) and centrifuged at 76,000xg for 90 minutes in a Beckman Optima LE-80K Ultracentrifuge. Virus-pellets were resuspended in PBS and 5% FBS was added for storage (in the dark) and subsequent titration.

Titration. Baculovirus produced by Kim Hoeksema was titrated as described in [3]. Briefly, a 1961 nucleotide region in the baculovirus (GenBank: L22858.1) gp64 gene (an envelope protein) was amplified from ZFPcA-bacmid DNA which was isolated using Roche High Pure Viral Nucleic Acid Kit (Roche, Cat.# 11858874001) according to manufacturer's instructions. Primers were designed to flank two NotI sites separated by 1476 base pairs in the gp64 gene, so that after the polymerase chain reaction (PCR) product was produced the oligonucleotide could be digested with NotI and ligated into a compatible vector. The primers used were FNot110.06.23L and FNot110.06.23R shown in Table 2.3. The PCR reaction conditions were as follows: 1 unit of Phusion High Fidelity DNA polymerase (New England

Biolabs (NEB), Cat.# M0530L), 10 ng of input DNA (ZFPcA bacmid-DNA), 1 µL of dNTPs (200 µM), 2.5 µL of oligonucleotides (0.5 µM), 10 µL of 5x HF Phusion buffer (provided with enzyme) and water up to 50 µL total volume. The PCR thermocycling conditions were as follows: initial denaturing at 98°C for 30 seconds followed by 25 cycles of denaturing at 98°C for 10 seconds, 54°C annealing for 20 seconds, 72°C extension for 30 seconds, and a final extension at 72°C for 5 minutes. PCR products were electrophoresed through 1% agarose gel (1 µg/mL ethidium bromide (EtBr)) in TAE buffer, visualized using ultraviolet (UV) light and the G:box Gel Documentation System (Syngene) and gel extracted using QIAquick Gel Extraction kit (Qiagen, Cat.# 28706). Ten µg of this purified 1961 nucleotide PCR product, and 1.7 µg of pBluescript KS(+) plasmid DNA was digested with 7.5 units of NotI (Invitrogen, 15441-017) with 2 µL of 10x bovine serum albumin (BSA) (at 0.1 mg/mL), 2µ of 10x NEBuffer 3 (at 1x), and water up to 20 µL total reaction volume for 1.5 hours at 37°C. Twenty µL of the heat inactivated pBluescript digest (at 65°C for 20 minutes) was dephosphorylated with 10 units of Antarctic phosphatase (NEB, Cat.# M0289S) for 40 minutes at room temperature according to manufacturer's instructions. NotI digested and dephosphorylated backbone DNA and NotI PCR products were electrophoresed through 1% agarose gel (1 µg/mL EtBr) in TAE buffer, visualized using UV light and the G:box Gel Documentation System (Syngene) and gel extracted using QIAquick gel extraction kit. The gp64 PCR product was ligated to the pBluescript backbone overnight at room temperature with

one unit of T4 DNA ligase (Invitrogen, Cat.# 15224-017), according to manufacturer's specifications. Chemically competent TOP10 *E. coli* was used for transformation of the ligation mixture by incubating the ligation mixture mixed with the bacteria on ice for 30 minutes, a 42°C heat-shock for 45 seconds and plating onto ampicillin (100 μg/mL) selective Luria Broth (LB) plates. Positive clones were confirmed by NotI digestion, as described above. This resulting plasmid (pBluescript-gp64) was used in quantitative PCR to generate a standard curve for quantitation of baculovirus-ZFP stocks.

Baculoviral DNA was isolated using Roche High Pure Viral Nucleic Acid Kit according to manufacturers instructions. The PCR reaction was performed on an ABI Prism 7900HT Sequence Detection System (ABI) in a 96-well optical plate format. PCR was performed using primers and a probe designed within the gp64 gene present in the pBluescript-gp64 and the baculovirus stocks, as previously described [17]. The primers used were Gp64-F109098 Fwd and Gp64-F109026 Rev and the probe used was Gp64-P109075, as shown in Table 2.3. The PCR reaction conditions were 12.5 μL of 2x Taqman Universal Master Mix (ABI, 4304437), 2.25 μL of 10 μM primers (final concentration at 900 nM), 2.5 μL of 2.5 μM probe (final concentration at 250 nM), 2 μL template DNA and 5.75 μL of water to total volume of 25 uL. The ABI standard thermocycling program consisted of 2 minutes at 50°C and 10 minutes at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minutes to amplify DNA.

Table 2.3 – Oligonucleotides used for cloning of pBluescript-gp64 and quantitative PCR for titration of baculovirus stocks. FNot110.06.23L and FNot110.06.23R are the primers used to amplify the region in the gp64 baculovirus gene that flanks two separate NotI sites. Gp64-F109098 Fwd and Gp64-F109026 Rev primers, and the Gp64-P109075 probe (labeled with FAM and TAMRA fluorophores) were used in real-time quantitative PCR to determine the titer of ZFP-expressing baculovirus stocks.

Oligonucleotide name	Sequence (5'-3')	Reference
FNot110.06.23L	gcatgcatcagctcaatgtt	This thesis
FNot110.06.23R	taaacgtggcccaaaagttc	This thesis
Gp64-F109098 Fwd	cggcgtgagtatgattctcaaa	[17]
Gp64-F109026 Rev	atgagcagacacgcagctttt	[17]
Gp64-P109075	FAM-aaaagtctacgttca ccacgcgccaaa-TAMRA	[17]

2.2.3.3 - *Baculovirus transduction and analysis*

i. Transduction

HepG2.2.15 cells were plated at 2.5x105 cells/well in a 12-well plate the day prior to transduction. The appropriate volume of baculovirus stock to give the desired MOI of 50 was diluted in 0.5 mL of normal cell culture media per well in a 12-well plate. The virus containing cell culture media was overlaid on the cells, which were returned to the incubator and rocked every 10 minutes for an hour. The virus was incubated with the cells overnight. The cell monolayer was washed three times with PBS and then normal culture

media was added. To assess for the presence of ZFP immediately after transduction, the virus-containing media was removed immediately it was overlaid and the cells were harvested for SDS-PAGE (see below) (Figure 2.5).

ii. Analysis: SDS-PAGE and Western blot for ZFP

The HepG2.2.15 cells were harvested at 0, 24 or 48 hours post-transduction. The cell monolayers were lysed, processed, subjected to SDS-PAGE and developed as described in section 2.2.1.4.iii, with the following exceptions. The primary antibody that the membranes were incubated with is a custom in-house made rabbit anti-ZFP antibody (section 2.2.24 of reference [3]), used at 1/10,000 for 1.5 hours. The secondary antibody (1/5,000 goat anti-rabbit HRP, (Cappel, Cat.# 21974)) was incubated with the membranes for 45 minutes. Exposures to x-ray film were between 2 and 30 seconds. There were no loading control antibodies used.

2.2.3.4 – *Beta-galactosidase baculovirus transduction and staining*

Infection. 50 μL, 100 μL, 200 μL, 500 μL and 1 mL of unconcentrated and untitrated beta-galactosidase baculovirus, or 1 μL, 2 μL, 5 μL, 10 μL, or 20 μL of 300x concentrated untitrated beta-galactosidase baculovirus was diluted in media to give a volume of 1 mL in 6-well plates, or 0.5 mL in 12-well plates. The first trial consisted of HepAD38 cells plated at $5x10^5$ cells/well in a 6-well plate being transduced with unconcentrated virus. The second trial consisted of HepAD38 cells plated on poly-L-lysine coated plates

at $6x10^5$ cells/well in a 6-well plate being transduced with the concentrated virus. The third trial consisted of HepAD38 cells plated on poly-L-lysine coated plates at $1.5x10^5$ cell/well in a 12-well plate, in the presence or absence of serum during transduction and transduced with 10 μL or 20 μL of concentrated virus. The last trial consisted of primary human hepatocytes plated at $5x10^5$ cells/well in a 12-well plate, transduced with concentrated virus. After virus was added to the culture, the cells were returned to the incubator with rocking every 10 minutes for the first hour. At 6 hours post-transduction or the following morning the monolayer was washed 3x with PBS and then replaced with normal media. The cells were analyzed for beta-galactosidase expression 48 hours post-transduction.

Staining. The monolayers of cells were washed once with PBS, placed on ice, 2 mL of fixative was added (2% fresh paraformaldehyde, 0.2% glutaraldehyde in PBS) and was kept on ice for an additional five minutes. The cells were rinsed three times with PBS for (1-4 minutes each rinse) and 1.5 mL of stain was added (1.25 mL of 20 mg/mL X-gal stock in dimethylformamide, 50 μL of 0.5 M stock of magnesium chloride, 41 mg potassium-ferricyanide, 53 mg potassium ferrocyanide, to 25 mL in PBS) and the cells incubated overnight at 37°C without CO_2. The beta-galactosidase will cleave X-gal and a by-product of this cleavage is a blue dye. The following morning the cells were rinsed once with PBS and then kept in PBS at 4°C until the cells were viewed and counted under light microscopy.

2.3 – Results

2.3.1 - Adenovirus expressing HBV (Adv-HBV)

Huh7 cells were transduced (section 2.2.1.3) with Adv-HBV at an MOI of 10 and ECV DNA was isolated at day 1, day 3, day 5 and day 7 post-transduction (section 2.2.1.4.i). Quantitative PCR was performed to quantify HBV copies per mL of supernatant (Figure 2.2). The titer was highest on day 1 post-transduction, however this value was very low at approximately $1.5x10^3$ copies/mL.

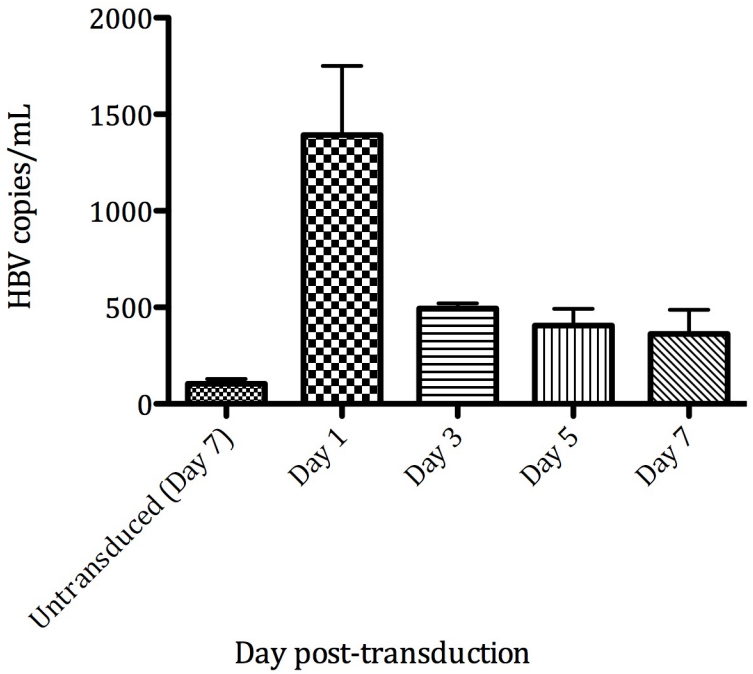

Figure 2.2 – HBV DNA in the supernatant of Huh7 cells after Adv-HBV transduction (MOI 10) at various time points. Huh7 cell monolayers were transduced with Adv-HBV at an MOI of 10 and at 1, 3, 5 and 7 days post-

transduction the supernatant was harvested. The HBV DNA in these supernatants were isolated and quantified by real-time PCR.

Huh7 cells were transduced (section 2.2.1.3) with Adv-HBV at MOIs of 0.1, 1, 10 and 50. After 48 hours ECV DNA was isolated and quantitative PCR was performed to quantify HBV copies per mL of supernatant (Figure 2.3). Cell lysates were harvested for SDS-PAGE and Western blot detection of HBV core protein and actin (Figure 2.4). The supernatants that were quantitated in Figure 2.3 suggest that the best MOI to use is 10, giving titers in range of 10^8 copies/mL. However, the Huh7 cell lysates collected from the corresponding supernatants do not show any HBV core protein at an MOI of 10, but only a small band of protein appearing at an MOI of 50 (Figure 2.4).

At the time of these experiments, SDS-PAGE was the only method I knew to assess HBV core protein levels, and did not know that the DAKO HBV core antibody does not detect HBV core well on Western blots. Since then Bill Addison has developed a protocol that assesses the HBV capsid levels (native capsid Western) instead of HBV core protein monomers and the HBV core antibody that is available works well in this protocol. The ECV DNA data that revealed HBV copies per mL of supernatant in the range of 10^8 could not be reproduced, while from the same cell cultures the Western blot detecting HBV core (granted the assay did not work well) suggests very low levels of HBV. With the knowledge of the capsid Western protocol and if the ECV DNA levels were consistently high, this system may have been a good choice to

test the hypothesis. However, at the time the most logical step was to abandon the system.

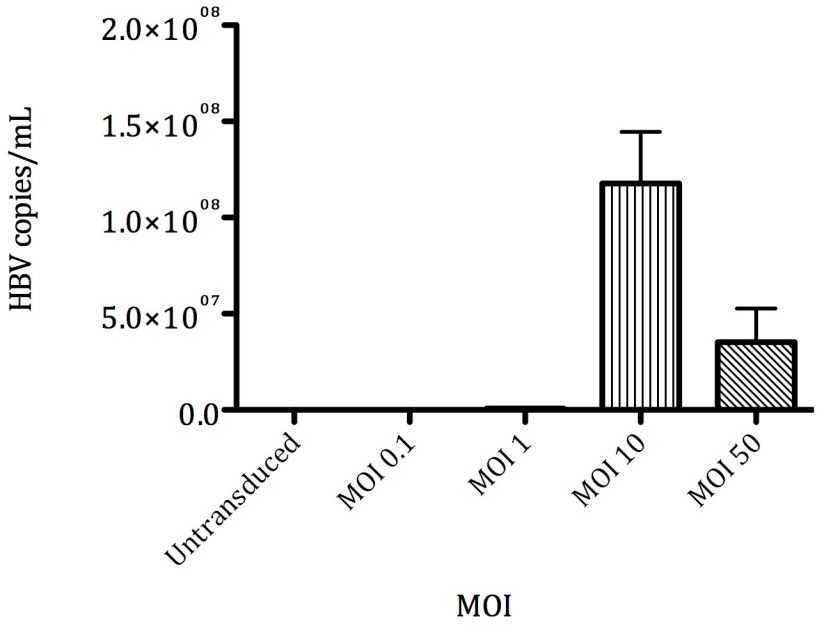

Figure 2.3 – HBV DNA in the supernatant of Huh7 cells 48 hours after Adv-HBV transduction at various MOIs. Huh7 cell monolayers were transduced with Adv-HBV at MOIs of 0.1, 1, 10 and 50, and 48 hours post-transduction the supernatant was harvested. The HBV DNA in these supernatants were quantified by PCR.

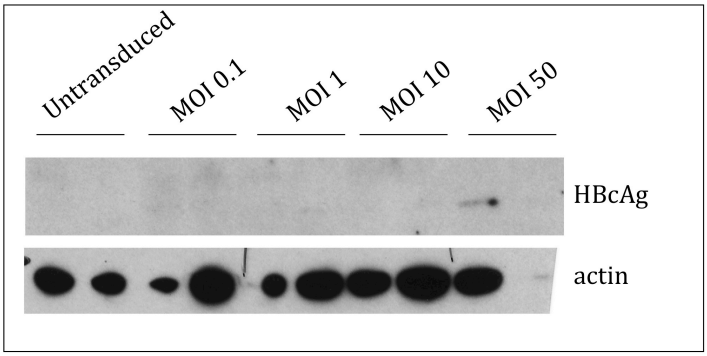

Figure 2.4 – Western blot of Huh7 lysates 48 hours post-transduction with Adv-HBV at indicated MOIs. Huh7 cells were transduced with Adv-HBV at MOIs of 0.1, 1, 10 and 50 and after 48 hours the lysates were harvested and subjected to SDS-PAGE and Western blot detecting HBV core protein (HBcAg) which ran at approximately 21 kDa. Actin was detected as a loading control and ran at approximately 42 kDa. Molecular size markers at 104, 95, 52, 37, 29 and 20 kDa were also run.

2.3.2 – Transfections

2.3.2.1 – *Lipid-based transfections*

i. **Lipofectamine 2000**.

Hepatoma cell lines stably expressing HBV are useful tools for studying HBV, but are very difficult to transfect. If appreciable transfection efficiency could be obtained, we could simply transfect HBV-specific ZFP-expression plasmids into these cells to test our hypothesis. The ability of HepG2.2.15 cells, as adherent or suspension cultures, to be transfected with

Lipofectamine 2000 was optimized and assessed by visual examination of EGFP-transfected cells over 72 hours. Poly-L-lysine coated and uncoated tissue culture plates were compared because these hepatoma cells adhere better and look much healthier when plated on lysine-coated plates. The efficiency was found to be very low and I concluded that this would not be a reasonable system to use to test our hypothesis that expression of HBV-specific ZFPs would inhibit HBV (Table 2.4.1). The efficiencies were low with non-coated, lysine-coated, and with adherent and suspension cultures.

Table 2.4.1 – Lipofectamine 2000 transfections of HepG2.2.15 cells.

HepG2.2.15 cells Lipofectamine 2000	% EGFP positive cells	
adherent cells	Lysine (+) plates	Lysine (-) plates
24 hours	< 1%	< 1%
48 hours	< 1%	< 1%
72 hours	< 1%	< 1%
suspension cells	Lysine (+) plates	Lysine (-) plates
24 hours	0	0
48 hours	1%	< 4%
72 hours	< 1%	1%

The ability of Huh7 cells to be transfected with Lipofectamine 2000 at various DNA:reagent ratios was optimized and assessed by visual examination of EGFP-transfected cell monolayers over 48 hours. The results were reasonable with the highest efficiency of 70% at 48 hours post-

transfection using a DNA:reagent ratio of 2:1 (Table 2.4.2). To test our hypothesis, these cells would have to be efficiently transduced with the Adv-HBV. However, the HBV levels upon transduction with Adv-HBV were low and for this reason this approach was abandoned. I also attempted to co-transfect an HBV-expression plasmid into these cells. The level of HBV produced by the HBV-expression plasmid was assessed as previously described by Western blot detecting HBV core and ECV DNA quantitative PCR, but the results showed very low HBV levels (data not shown).

Table 2.4.2 – Lipofectamine 2000 transfections of Huh7 cells.

Huh 7 cells Lipofectamine 2000	Ratio of DNA:reagent			
adherent cells	2:1	1:1	1:2	1:5
24 hours	45%	40%	40%	25%
48 hours	70%	45%	45%	10%

ii. DharmaFECT Duo.

I attempted to transfect EGFP-expression plasmids into HepG2.2.15 cells using DharmaFECT Duo. The ability of HepG2.2.15 cells to be transfected with DharmaFECT Duo was optimized and assessed by visual examination of EGFP-transfected cell monolayers at 24 and 48 hours. A range of DNA:reagent ratios were compared on cells in suspension or as adherent cultures, as well as cultures being plated on lysine-coated plates or not, and adherent cells at various cell densities. The best result was approximately 10% EGFP-positive cells with limited enhancement seen with cells

transfected in suspension (Table 2.5). However, the transfection levels in cell systems with DharmaFECT Duo were insufficient to test our hypothesis.

Table 2.5 – DharmaFECT Duo transfections of HepG2.2.15 cells.

HepG2.2.15 cells DharmaFECT Duo	% EGFP positive cells			
adherent cells		Ratio of DNA:reagent		
	cell density	1:1	1:2	1:3
24 hours	low	< 1%	5%	10%
	medium	< 1%	7%	10%
	high	< 1%	3%	10%
48 hours	low	< 1%	5%	10%
	medium	< 1%	5%	10%
	high	< 1%	1%	10%
suspension cells	DNA:reagent ratio 1:1.5			
	Lysine (+)	Lysine (-)		
24 hours	8%	5%		
48 hours	10%	5%		

iii. DMRIE-C.

I attempted to transfect EGFP-expression plasmids into HepG2.2.15 cells using DMRIE-C. The ability of HepG2.2.15 cells to be transfected with DMRIE-C was optimized and assessed by visual examination of EGFP-transfected cell monolayers at 24, 48 and 72 hours. High or low reagent:DNA ratios were compared on cells in suspension, adherent cells, and with or without the use of lysine coated plates. Very low transfection efficiencies were found using this reagent in all cell culture systems (Table 2.6). These efficiencies are not adequate to test our hypothesis.

Table 2.6 – DMRIE-C transfections of HepG2.2.15 cells.

HepG2.2.15 cells DMRIE-C		% EGFP positive cells	
adherent cells	Reagent:DNA ratio	Lysine (+)	Lysine (-)
24 hours	low	< 1%	< 1%
	high	0	< 1%
48 hours	low	< 1%	< 1%
	high	< 1%	< 1%
72 hours	low	< 1%	< 1%
	high	0	< 1%
suspension cells	Reagent:DNA ratio	Lysine (+)	Lysine (-)
24 hours	low	< 1%	< 1%
	high	0	< 5%
48 hours	low	< 1%	1%
	high	< 1%	< 5%
72 hours	low	< 1%	< 1%
	high	< 1%	< 3%

iv. FuGENE 6.

I attempted to transfect EGFP-expression plasmids into HepG2.2.15 cells using FuGENE 6. The ability to transfect HepG2.2.15 cells using FuGENE 6 was optimized and assessed by visual examination of EGFP-transfected cell monolayers at 24 and 48 hours. A range of reagent:DNA ratios were compared for cells in suspension or adherent cultures with or without lysine coated plates. Very low transfection efficiencies were found using this reagent, with the highest being 10% EGFP-positive (Table 2.7). This transfection rate was insufficient to test our hypothesis.

Table 2.7 – FuGENE 6 transfections of HepG2.2.15 cells.

HepG2.2.15 cells FuGENE 6	% EGFP positive cells Ratio of reagent:DNA					
	3:1		3:2		6:1	
adherent cells	Lys (+)	Lys (-)	Lys (+)	Lys (-)	Lys (+)	Lys (-)
24 hours	8%	1%	8%	2%	10%	3%
48 hours	10%	1%	8%	1%	10%	2%
	3:1		3:2		6:1	
suspension cells	Lys (+)	Lys (-)	Lys (+)	Lys (-)	Lys (+)	Lys (-)
24 hours	1%	1%	1%	1%	3%	3%
48 hours	5%	3%	5%	2%	5%	5%

2.3.2.2 – *Electroporation transfections*

BTX Electro Square Porator.

The ability of HepG2 cells (the parental cell line of HepG2.2.15) to be transfected with the BTX Electro Square Porator was tested using two different protocols previously published for use with HepG2 cells [11]. Transfection efficiency was assessed by visual examination of EGFP-transfected cells after 24 and 48 hours. However, the efficiency of BTX Electro Square Porator was very low with less than one percent of the cells EGFP-positive (Table 2.8). The protocol published in de Marco *et. al.* (2002) reports a transfection efficiency of 95-99%, however, in my hands the efficiency was much lower at less than 1%. I followed the protocols very closely but in my hands was not able to see transfection efficiency even close to what the authors report. The results are surprising to me but perhaps it

can be explained by cell line differences between research groups that develop over time. Regardless, this transfection efficiency was insufficient to test our hypothesis.

Table 2.8 – BTX Electro Square Porator transfection of HepG2.2.15 cells.

HepG2 cells BTX Electro Square Porator	% EGFP positive cells **24 hours**	 **48 hours**
Electro Square Porator Protocol	< 1%	< 1%
J. Cell Biol. Protocol (2002) (ref. 11)	< 1%	< 1%

2.3.3 - Baculovirus

2.3.3.1 – *Assessment of baculovirus transduction*

With the availability of hepatoma cell lines that stably express HBV, and ZFP-expressing baculovirus vectors; we evaluated this system as a possibility for testing our hypothesis. The experiments using ZFP-expressing baculoviruses were performed using HepG2.2.15 cells and the experiments using the beta-galactosidase baculovirus were performed using HepAD38 cells and primary human hepatocytes.

i. Assessment over 48 hours.

The ability of baculovirus-ZFPk to transduce HepG2.2.15 cells was assessed. HepG2.2.15 cells were transduced with baculovirus-ZFPk at an MOI of 50 and after 0, 24 and 48 hours the cell lysates were harvested and

assessed for the presence of ZFP by Western blot (Figure 2.5). ZFPk could not be detected at time=0 (control), but was clearly visible by Western blotting at 24 and 48 hours post-transduction.

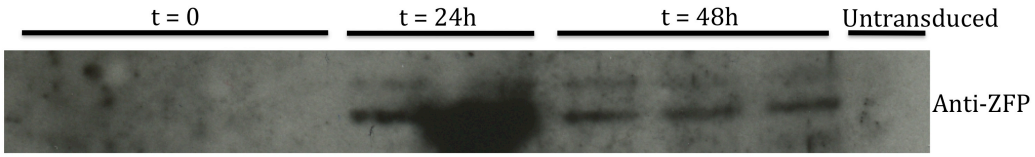

Figure 2.5 – Assessment of ZFP-expressing baculoviruses over 48 hours. HepG2.2.15 cells were transduced with baculovirus-ZFPk at an MOI of 50 and immediately afterwards, after 24 hours and after 48 hours cell lysates were harvested and subjected to SDS-PAGE and Western blot detecting ZFP, which ran at approximately 21 kDa. The multiple lanes represent duplicate experimental samples within the experiment.

ii. Assessment of baculoviruses expressing ZFPca, ZFPk and ZFPn in transduction of HepG2.2.15 cells.

The ability of baculovirus-ZFP to transduce HepG2.2.15 cells was assessed as a possible method to test our hypothesis. HepG2.2.15 cells were transduced with baculovirus-ZFP at an MOI of 50 and after 48 hours the cell lysates were harvested and assessed for the presence of ZFP by Western blot (Figure 2.6). ZFPn and ZFPk are clearly visible, however ZFPcA does not appear to be present. However, in subsequent experiments ZFPcA was confirmed to be produced.

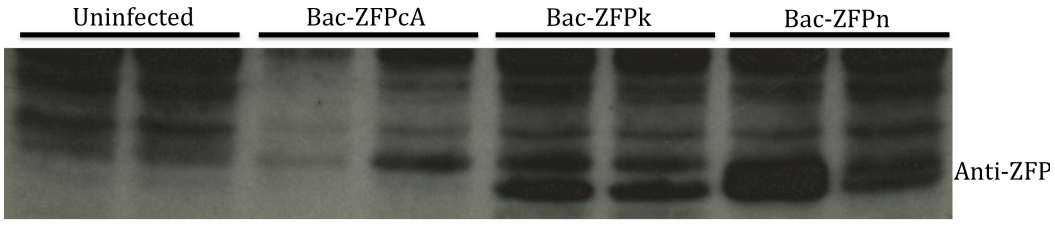

Figure 2.6 – Assessment of ZFP-expressing baculoviruses in HepG2.2.15 cells. Lysates of HepG2.2.15 cells were assessed for ZFP expression by Western blotting 48 hours post-transduction with the three baculoviruses indicated. Protein lysates of transduced cells were subjected to SDS-PAGE and Western blot to detect ZFP, which ran at approximately 21 kDa.

iii. Beta-galactosidase baculovirus assessment.

Since I did not have a way to assess transduction efficiency of the ZFP-expressing baculoviruses, I used a beta-galactosidase-expressing baculovirus from our laboratory. This baculovirus expressing beta-galactosidase uses the same baculovirus used to express the ZFPs and therefore was an ideal surrogate to estimate transduction efficiency. HepAD38 cells and primary human hepatocytes (with the intention of testing in the chimeric mouse model) were used to test the transduction efficiency under various growth and transduction conditions (Table 2.9). Several variables were altered to optimize transduction including using concentrated and unconcentrated virus, serum-free and serum-containing media and growing the cells on uncoated or poly-L-lysine coated plates. Forty-eight hours after transduction the cells were analyzed for beta-galactosidase expression by X-gal staining.

Blue staining cells were counted under light microscopy. The best transduction results were seen with the HepAD38 cells in serum-free media when 20 μL of 300x concentrated virus was applied to the cells (virus stock was not titrated). However, a transduction rate of approximately 2% is inadequate for testing our hypothesis.

Table 2.9 – Experiments with beta-galactosidase-expressing baculovirus. Trials 1 – 3 used HepAD38 cells and trial 4 used primary human hepatocytes. Trial 1 used unconcentrated virus stock with HepAD38 cells plated on uncoated tissue culture plates ($5x10^5$ cells/well). Trial 2 used concentrated virus on HepAD38 cells plated on poly-L-lysine coated plates ($6x10^5$ cells/well). Trial 3 used poly-L-lysine coated plates, concentrated virus and media with or without serum ($1.5x10^5$ cells/well). Trial 4 tested primary human hepatocytes with concentrated virus ($5x10^5$ cells/well). Positive cells appear blue after the X-gal staining procedure (see methods) and these were counted to quantitate the transduction efficiency. NC denotes "not counted".

Trial 1 - HepAD38		
Volume of unconcentrated baculovirus	# of positive cells	estimated % of cells
50 µL	NC	NC
100 µL	NC	NC
200 µL	38	< 1%
500 µL	103	< 1%
1 mL	213	< 1%

Trial 2 - HepAD38, lysine (+) plates		
Volume of concentrated baculovirus	# of positive cells	estimated % of cells
1 µL	13	< 1%
5 µL	NC	NC
10 µL	NC	NC
20 µL	967	< 1%

Trial 3 - HepAD38, lysine (+) plates, +/- serum		
	estimated % of positive cells	
Volume of concentrated baculovirus	serum-free media	serum-containing media
10 µL	< 1%	< 1%

20 µL	1-2%	< 1%

Trial 4 - Primary Human Hepatocytes		
Volume of concentrated baculovirus	# of positive cells	estimated % of cells
1 µL	0	0
2 µL	0	0
5 µL	0	0
10 µL	0	0
20 µL	0	0

2.4 – Discussion

Two ZFPs were previously designed to target the SPII promoter region of HBV cccDNA. We hypothesized that when these ZFPs were expressed in an HBV-infected cell, they will bind their 18-base pair target region in the cccDNA and inhibit viral transcription, therefore decreasing viral protein products and progeny virus. The ZFPs possess a nuclear localization signal (NLS) such that they can be imported into the nucleus where the cccDNA is located. DHBV-specific ZFPs were previously shown to be able to decrease DHBV replication in LMH cells that were co-transfected with a plasmid that reproduces the DHBV replication cycle. These LMH cells have the ability to be easily transfected and thus a co-transfection expressing the DHBV-specific ZFPs and the DHBV-replication plasmid in LMH cells was feasible [15]. However, a system to study the expression of HBV in primary hepatocytes or hepatoma cell lines and subsequently transfecting with ZFP-expressing plasmids was not readily available. The objective of this chapter

was to examine several cell culture systems and different transfection systems to test the basic hypothesis.

The possibility of transducing a hepatoma cell line, Huh7, with an adenovirus vector that is able to express the HBV replication cycle (Adv-HBV), followed by transfection with ZFP-expression plasmids was explored. The expression of HBV from the adenovirus vector was driven by endogenous promoters, while a CMV promoter drives a GFP gene product. While the transducing ability of this vector was very good and resulted in the majority of cells expressing GFP 24 hours post-transduction, the amount of HBV-expression was found to be low. To examine when the HBV level is the best, day 1, 3, 5 and 7 post-transduction supernatants were tested for amount of HBV DNA. The data suggested the HBV DNA was highest in the supernatant at day 1 post-transduction. A range of MOIs was tested, from 0.1 to 50, where the supernatant was harvested at 48 hours post-transduction, as well as the cell lysates for SDS-PAGE and Western blot. The supernatant containing HBV DNA, which was quantitated by real-time PCR, produced data suggesting that an MOI of 10 gives titers in the range of 10^8. However, the lysates from the same cell cultures that were subjected to SDS-PAGE and then Western blot detecting HBV core protein and actin as a loading control reveal that only a faint amount of HBV core protein is produced at an MOI of 50. This inconsistency can be explained by the use of SDS-PAGE and Western blot, which I now know is not a good way to assess HBV core levels. Using the capsid Western protocol would have been useful to determine whether the

amount of HBV in that experiment was truly high or not. This high level of HBV DNA in the supernatant (Figure 2.3) could not be reproduced, so whether or not this system would have been a useful system is unknown, however, at the time it did not seem to be a viable option.

There are a number of potential systems to test the hypothesis involving transfection, co-transfections or transfection and transduction. The most desirable approach would have been to transfect hepatoma cell lines that stably express HBV with ZFP-expression plasmids. However, these cell lines, HepAD38 and HepG2.2.15, do not transfect well. Kimberley Hoeksema exhausted methods to attempt to transfect HepAD38 cells, which is described in her thesis (reference [3]). In my studies I have attempted to transfect HepG2.2.15 cells using a variety of methods, including four different lipid-based transfection reagents and an electroporation device. The protocols for transfection using the electroporation device were followed very closely and so I am surprised by the very low transfection efficiency. The most probable explanation may be that the cells used in the publication and in the development of the manufacturer's protocol are different than the HepG2 cells we have in our laboratory. The history of the cells including factors such as passage number or culture conditions may have an effect on the ability of these cells to be transfected. The cells tested negative for *Mycoplasma sp.* contamination. For each lipid-based reagent a variety of variables were altered in order to optimize the transfection, including poly-L-lysine coating the culture dishes, cell density, adherent versus suspension cultures and

DNA:reagent ratios, all examined over at least 48 hours of culture (Tables 2.4.1, 2.5-2.8). The best result was 10% transfection efficiency, which is too low to test our hypothesis. In order to see an appreciable decrease in HBV levels, we need a high proportion of cells expressing the ZFPs. The results of transfections with Huh7 cells (Table 2.4.2) revealed transfection efficiency of 70%, which is high. A potential system would be to co-transfect these cells with ZFP-expression plasmids and a plasmid that results in HBV replication. Tests were performed using an available HBV-expression plasmid but the results were very similar to those seen with Adv-HBV: very low HBV expression.

The ZFP-expressing baculovirus vectors were originally produced by Kimberley Hoeksema. The ability of these vectors to transduce HepG2.2.15 and HepAD38 cells was assessed in my studies. Initially, the assessment of transduction using these vectors was determined by collecting the cell lysates and subjecting them to SDS-PAGE and Western blot. These data suggested strong ZFP expression, and since there was no marker to assess the transduction efficiency, it was originally thought to be high (Figures 2.5-2.6). However, a beta-galactosidase-expressing baculovirus was provided, which shared a common backbone with the ZFP-expressing baculoviruses. This was a very reliable way to estimate the transduction efficiency of the ZFP-baculoviruses. For optimization, certain variables were adjusted and after examination of transduction efficiency, this vector revealed very low efficiency for HepAD38 and no transducing ability with primary human

hepatocytes (Table 2.9). Consequently, this approach to test the hypothesis was also abandoned.

In summary, in this chapter I assessed various experimental systems to test our hypothesis that ZFP expression in HBV-infected cells will inhibit viral transcription and result in decreased viral proteins, transcripts and progeny. The fundamental objective of this chapter is to achieve high transgene expression in target cells (ZFPs), and the cells must have HBV present. None of the methods presented in this chapter provided a feasible option for testing my hypothesis.

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Chapter 3 – Lentiviral Experiments

3.1 – Introduction

Our hypothesis is that HBV-specific ZFPs expressed in an HBV-infected cell will bind the target sequence in the viral cccDNA and inhibit transcription, thereby decreasing the amount of viral RNA and proteins and decrease the levels of virus secreted. To test the hypothesis, high levels of HBV expression and ZFP expression are required to be able to detect this antiviral effect. We obtained lentivirus expressing GFP under the human ubiquitin C promoter from Dr. Troy Baldwin to test the transduction efficiency of hepatoma cell lines and found this to be successful. Even cell lines that were difficult to transfect, such as HepAD38, could be transduced at an efficiency of approximately 95% at an MOI of 15. This transduction efficiency would provide high ZFP expression in HBV-infected hepatoma cell lines and therefore provided a suitable system to test our hypothesis

Lentiviral vectors are derived from HIV-1 and are able to transduce a wide variety of dividing and non-dividing cells at very high efficiencies [1-3]. These non-replicative vectors integrate into the host cell genome, which results in stable transgene expression. Lentiviral vectors have been used in hepatoma cell lines and maintain transgene expression up to 45 days. Additionally, these vectors have been used for efficient and stable transgene expression in primary human hepatocytes [4-8].

There are two generations of lentiviral vector systems commonly used; the second and third generation. The second-generation system is based on three plasmids: for virus packaging, envelope production and transgene expression. The third-generation system is a four plasmid system that differs in that the packaging plasmid is divided into two plasmids so that certain accessory proteins are separated, ensuring even less chance of replication-competent lentiviruses being produced. Both of the lentiviral systems used in this thesis are based on the second-generation system. The second and third generation systems have increased safety profiles because most of the accessory genes of HIV-1 are removed from the packaging vector.

Optimizing the transfection of 293T cells was performed using Lipofectamine 2000. Obtaining high transfection efficiency results in improved titer of lentivirus. Experiments to test the hypothesis were completed in two HBV-infected hepatoma cells lines, HepAD38 and HepG2.2.15. To assess the impact of the HBV-specific ZFPs in these cells, ECV DNA, ICV DNA and cell lysates were analyzed for the amount of HBV DNA or capsid proteins, and RNA was also analyzed in HepG2.2.15 cells.

To ensure that the ZFPs have a functional nuclear localization signal and are being translocated to the nuclei of infected cells, cell fractions were collected from transduced cells and the nuclear and cytoplasmic compartments were assessed for the presence of ZFP.

Based on visual inspection of transduced cells compared to untransduced controls, from day five post-transduction onwards the cells

have an unhealthy phenotype. To assess the cytotoxicity of transduction to express ZFPs, MTT assay was performed on HepG2.2.15 cells at day 1, 3, 5 and 7 post-transduction.

After experiments testing the hypothesis revealed no antiviral effect of the ZFPs, an alternative experiment was performed to see if the ZFPs are able to bind their target DNA sequence using a dual-luciferase reporter plasmid (aside from the EMSA and SPR performed by Kimberley Hoeksema, section 1.3.4 of this thesis).

3.2 – Materials and Methods

3.2.1 – Cell lines and culture conditions

i. HepAD38.

This cell line has been previously described (section 2.2.2.1.iv).

ii. HepG2.2.15.

This cell line has been previously described (section 2.2.2.1.iii).

iii. 293T.

293T cells are human embryonic kidney cells that have been engineered to express the SV40 large T antigen. Therefore, when a plasmid contains the SV40 origin of replication, the plasmid can be amplified to result in high expression of the transgene. These cells are very easily transfected and widely used for lentiviral vector production. These cells were normally cultured in DMEM

supplemented with 10% FBS and 50 IU/mL penicillin and 10 µg/mL streptomycin and incubated at 37°C in 5% CO_2.

3.2.2 – Cloning of lentiviral expression vectors

Given the success observed with the GFP-expressing lentivirus from Dr. Troy Baldwin, we cloned our ZFPs into the same vector backbone (pLOVE) and used the same packaging and envelope plasmids to attempt to generate lentiviral vectors. Cloning methodology was based on Invitrogen's Gateway Cloning Technology, whereby the transgene is cloned by traditional methods into an entry vector (pENTR) and then, using the proprietary Clonase enzymes, the transgene fragment can be transferred easily into a desirable "destination" expression vector (pLOVE). After cloning, the structures of pLOVE-ZFP expression plasmids were confirmed by transfection of 293T cells to produce the ZFPs and detected by Western blotting. Despite much time trying to generate lentiviral vectors based on the pLOVE/packaging/envelope system suggested by the Baldwin laboratory, I was unsuccessful. I attempted to use multiple protocols including calcium phosphate transfection and various lipid-based transfection reagents to obtain good transfection to generate lentivirus.

A new lentiviral expression vector was obtained from John Law from Dr. Houghton's laboratory. This vector, pTRIP, produces a bi-cistronic message driven by a CMV promoter with the first half of the message encoding the transgene, followed by an Encephalomyocarditis virus (EMCV)

internal ribosome entry site (IRES), which initiates the translation of the second message, a red fluorescent protein (RFP). The pTRIP destination vector possesses the necessary recombination sites to make use of the Clonase enzyme (Gateway system) and the pENTR-ZFP entry vectors that were previously cloned. I tried the Gateway Clonase system without success. Eventually, traditional cloning methods were used to add restriction sites to the flanks of the ZFPs to move them into the pTRIP vector. However, this lentiviral expression vector had a tendency to undergo recombination within its backbone when grown at 37°C, although these recombination events leave the transgene region intact. The recombination events lead to delays in obtaining the full-length expression plasmid, however, growing the bacteria at 30°C solved this problem.

PCR primers were designed to add SpeI and XhoI restriction endonuclease sites to flank the ZFPs to permit cloning of the PCR products into the MCS of the pCR4 and pTRIP vectors. It was necessary to first clone into the pCR4 vector because attempts to clone directly into the pTRIP vector were unsuccessful, possibly due to inefficient digestion to create the sticky ends. The PCR thermocycling parameters to add SpeI and XhoI sites to flank the ZFPs were as follows: 95°C for two minutes, 35 cycles of 95°C for 30 seconds, 61°C for 30 seconds and 72°C for 1 minute, and a final 2 minutes at 72°C. The PCR reaction contained 18 ng of input template plasmid pcDNA3.1(+)ZFPcA, pcDNA3.1(+)ZFPk or pcDNA3.1(+)ZFPn, and two negative controls, pcDNA3.1(+) and no template DNA. To each mixture 1 µL

of 10 mM dNTPs, 12.5 units of recombinant *Taq* DNA polymerase (Invitrogen, Cat.# 10342-053), 0.25 μM of each primer, $MgCl_2$ at 1.5 mM, 5 μL of 10x PCR buffer (Invitrogen, Cat.# 10342-053) and water was added for a final volume of 50 μL. Primer sequences can be found in Table 3.1. The forward primer included a two-nucleotide tail for improved restriction endonuclease digestion (personal communication with John Law), the SpeI cut site and a ribosome binding site sequence, followed by the ATG start codon of the ZFP (the same forward primer was used for each ZFP). The reverse primers have a two-nucleotide tail for improved restriction endonuclease digestion and the XhoI cut site. The PCR products were electrophoresed through 1% agarose gel containing 1 μg/mL EtBr in TAE buffer. DNA bands were visualized using UV light and the G:box Gel Documentation System (Syngene). PCR products were excised with a clean scalpel and extracted using QIAquick Gel Extraction Kit (Qiagen, Cat.# 28706) according to the manufacturer's instructions and eluted in 30 μL of water. To facilitate transfer of the ZFP PCR fragments into the pTRIP vector backbone, they were cloned into pCR4-TOPO TA vectors with the TOPO TA Cloning Kit for Sequencing (Life technologies, Cat.# K4575-J10). The reaction was performed as follows: 0.5 μL salt solution (provided in the kit), 2 μL PCR product and lastly, 0.5 μL pCR4-TOPO vector. The reaction was incubated at room temperature for one hour. The entire ligation mixture was transformed into 100 μL of chemically competent TOP10 *E. coli* and plated on ampicillin-selective (100 μg/mL) LB plates (LB/amp) and incubated at 37°C overnight. Single colony clones were

selected and grown in 2 mL broth cultures of LB/amp for approximately 10 hours at 37°C with agitation. The pCR4-TOPO-ZFP plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Cat.# 27106) according to the manufacturer's instructions and eluted in 50 µL of water. Positive clones were confirmed by double digestion with SpeI and XhoI with the reaction set up as follows: 5 µL input DNA, 15 units of SpeI (Invitrogen, Cat.# 15443-013), 15 units of XhoI (Invitrogen, Cat.# 15231-012), 2 µL of 10x BSA (1x final, or 0.1 mg/mL), 2 µL of NEBuffer 4 and 9 µL water and was incubated at 37°C for two hours. Digests were electrophoresed and visualized as described above. Two positive clones for each ZFP were chosen for further cloning into the pTRIP vector.

The pTRIP plasmid was linearized by digestion with SpeI and XhoI (as described above). The products were electrophoresed, visualized and extracted as described above, except the final elution was with 50 µL of water. To dephosphorylate the pTRIP backbone, 25 µL of the linearized DNA was incubated with 10 units of antarctic phosphatase (NEB, Cat. # M0289S) and 1x reaction buffer (provided with enzyme), according to manufacturer's instructions, for 30 minutes at 37°C and heat inactivated at 70°C for 15 minutes. To prepare the insert DNA, pCR4-TOPO-ZFPcA, pCR4-TOPO-ZFPk or pCR4-TOPO-ZFPn were doubly digested with SpeI and XhoI, electrophoresed and extracted as described above. Ligation reactions to generate pTRIP-ZFPcA, pTRIP-ZFPk and pTRIP-ZFPn were performed as follows: one unit of T4 DNA ligase (1 µL) (Invitrogen, Cat.# 15224-017), 2 µL of 5x ligase buffer

(1x final, provided with enzyme), 1µL pTRIP backbone DNA and 6 µL ZFP insert DNA. Reactions were incubated for 4 hours at room temperature. The entire ligation reaction was transformed into chemically competent TOP10 *E. coli* and plated on LB/amp plates and incubated at 37°C overnight. Single colony clones were selected and grown in 2 mL cultures of LB/amp for approximately 10 hours at 37°C with agitation and the pTRIP-ZFP plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Cat.# 27106) according to the manufacturer's instructions and eluted in 50 µL of water. Positive pTRIP-ZFP clones were confirmed by double digestion with SpeI and XhoI, as described above. The ZFPs were also confirmed by sequencing with a primer designed in the downstream coding sequence of the EMCV IRES, reading the reverse towards the ZFPs.

Table 3.1 – Primers for adding SpeI and XhoI restriction endonuclease cut sites to flank ZFPs for cloning into pTRIP for lentiviral expression plasmids. *Italics* – nucleotide tail, **bold** – cut site, <u>underlined</u> – ribosome binding site, normal text – ZFP coding sequence.

Oligonucleotide name	Sequence (5'-3')	Description
ZFP.fwd	*aa***actagt**<u>gccacc</u>atgcatcatcaccatcaccatcc	SpeI cut site, same primer for all ZFPs
ZFPcA.rv	*tt***ctcgag**ttaggaggtctttttccggtgtgg	XhoI cut site
ZFPk.rv	*tt***ctcgag**ttaaccgatatcagacgtcttcttacc	XhoI cut site
ZFPn.rv	*tt***ctcgag**ttaaccgatatcggacgtcttctttcc	XhoI cut site

3.2.3 - Optimization of 293T cell transfection

In order to obtain high transfection efficiency in 293T cells for lentiviral vector production, Lipofectamine 2000 was used for optimization of transfection for adherent and suspension cell cultures. Transfections were performed using different DNA:reagent ratios. For adherent 293T cells plated at $3.5x10^5$ cells/well in 12-well plates, 4 µL of Lipofectamine 2000 was used with varying amounts of red fluorescent protein (RFP)-expressing DNA (pTRIP-RFP) to give DNA:reagent ratios of 1:2, 1:2.5 and 1:3. The DNA was mixed with Opti-MEM for a total volume of 100 µL. In a separate tube, the Lipofectamine 2000 was mixed with the same volume of Opti-MEM and

incubated for 5 minutes at room temperature. The DNA/Opti-MEM solution and Lipofectamine 2000/Opti-MEM solution were mixed together drop-wise and incubated for 20 minutes at room temperature. This resulting transfection media was added drop-wise to the cell monolayers grown in normal media containing no antibiotics. Six hours later the media was changed to normal media containing antibiotics and at 24 and 48 hours post-transfection the RFP-positivity was assessed by fluorescent microscopy (Zeiss NLO510 multi-photon microscope) at emission and excitation wavelengths of 546 nm and 608 nm, respectively.

For 293T cells in suspension in 12-well plate format, 4 μL of Lipofectamine 2000 was used with varying amounts of plasmid DNA (pTRIP-RFP) to give DNA:reagent ratios of 1:2, 1:2.5 and 1:3. In a sterile tube the DNA was mixed with Opti-MEM for a total volume of 100 μL. In the well of the 12-well plate the Lipofectamine 2000 was mixed with 100 μL of Opti-MEM and incubated for 5 minutes at room temperature. The DNA/Opti-MEM solution was added dropwise to the well containing the Lipofectamine 2000/Opti-MEM solution and incubated for 20 minutes at room temperature. The cell solution containing $4.25x10^5$ cells/well in media without antibiotics was added drop-wise to the well containing the transfection media. After 20 hours the media was changed to normal media containing antibiotics and at 24 and 48 hours post-transfection the RFP-positivity was assessed by fluorescent microscopy (Zeiss NLO510 multi-photon microscope) at emission and excitation wavelengths of 546 nm and 608 nm, respectively.

3.2.4 - Lentivirus production and titration

Production. 293T cells were plated T150cm^2 flasks at 1.3x10^7 cells per flask in normal cell culture media. The following day, cell monolayers were transfected using Lipofectamine 2000, as previously described in section 3.2.3 (adherent 293T cells) and with a DNA:reagent ratio of 1:2.5, scaled-up as follows: 55 µg of total DNA per flask consisting of 27.5 µg of pTRIP-ZFP expression plasmid, 22 µg of pGagpol (packaging vector) and 5.5 µg of pVSVG (envelope vector) [9]. A plasmid map of pTRIP can be found in Appendix A, packaging and envelope vector information can be found from Evans, *et. al*. (2007) [9]. Transfection complexes were left on the cells for 6 hours at 37°C in 5% CO_2, followed by a media change to DMEM with 3% FBS and antibiotics. After 48 hours the culture supernatant was removed, pooled in 50 mL conical tubes and centrifuged at 500xg for 5 minutes to pellet any floating cells. The clarified supernatant was filter-sterilized through a 0.45 µm polyvinylidene fluoride (PVDF) membrane syringe-filter unit. Polybrene was added to 4 µg/mL and HEPES to 20 mM. Virus stocks were aliquoted and stored at -80°C.

Titration. 293T cells were plated in 96-well plates at 2.5x10^4 cells per well. The following day, 25 µL of lentivirus stock was prepared in eight-log serial dilutions in cell culture media consisting of DMEM, 3% FBS, 4 µg/mL polybrene and 20 mM HEPES. Each dilution was done in duplicate and 100

μL of each dilution was applied to cell monolayers. The culture plates were centrifuged at 242xg for 30 minutes at room temperature, then incubated at 37°C overnight. The following morning the supernatant was removed and normal cell culture media was added. After 72 hours, RFP positive cells were counted from duplicate wells, averaged and used to calculate the titer of the lentivirus.

Once ZFP-expressing lentiviral vectors were generated, attempts to generate high-titer lentivirus stocks were difficult. Ellis et. al. (2011) reported that using caffeine at 2-4 mM for 17-41 hours post-transfection resulted in 3- to 8-fold higher lentivirus titers [10]. This was tested in my system, but it did not result in significantly higher titers.

3.2.5 –Lentivirus infections

293T cells were plated at $2.5x10^5$ cells/well in 12-well plates, HepAD38 cells were plated at $5x10^5$ cells/well in 6-well plates, and HepG2.2.15 cells were plated at $5x10^5$ cells/well. The following day the appropriate amount of virus (MOI of 15) was diluted in DMEM cell culture media containing 3% FBS, 4 μg/mL polybrene and 20 mM HEPES. The virus containing media was applied to cell monolayers and the culture plates were centrifuged at 242xg for 30 minutes at room temperature and returned to the cell culture incubator. The following morning the media was changed and 72 hours post-transduction the cells were assessed by visual evaluation of RFP-

positivity for transduction efficiency under fluorescent microscopy. Using an MOI of 15 consistently produced transduction efficiencies of 90-95%.

3.2.6 - Confirmation of ZFP production by transduction

To confirm the ZFPs were being produced upon transduction, Western blot for the detection of ZFP was performed with whole cell lysates of 293T and HepAD38 cells transduced with ZFP-expressing lentiviruses. Fluorescent microscopy was also used to observe productive transduction produced by the RFP encoded by the lentiviral vectors.

293T cells. In this pilot transduction experiment, 293T cells were plated at $2.5x10^5$ cells/well in a 12-well plate. The following day the cells were transduced with untitrated lentiviral vectors diluted 1 in 3 with DMEM cell culture media containing 3% FBS, 4 μg/mL polybrene and 20 mM HEPES. The infection proceeded as described in section 3.2.5. At 72 hours post-transduction the RFP-positivity was assessed by fluorescent microscopy (Zeiss NLO510 multi-photon microscope) at emission and excitation wavelengths of 546 nm and 608 nm, respectively. Whole cell lysates were harvested using 160 μL of RIPA buffer and 20 μL of the cell lysate was prepared with 5 μL of SDS-PAGE loading buffer and boiled for 10 minutes. A 10% polyacrylamide gel was prepared, samples electrophoresed through the gel and semi-dry transferred, as described in section 2.2.1.4.iii with the following exceptions. The membrane was blocked with 2.5% skim milk blocking buffer for two hours. The membrane was washed three times for 5

minutes with 1x TBS/T. The primary antibody was incubated on the membrane for 1.5 hours using rabbit anti-ZFP antibody diluted in 2.5% skim/TBS/T (1/10,000 rabbit anti-ZFP, produced in our laboratory, see [11]) followed by 3x five minute washes with 1x TBS/T. The membrane was incubated with 1/5,000 goat anti-rabbit HRP secondary antibody diluted in 2.5% skim/TBS/T for 45 minutes (Cappel, Cat.# 21974) followed by 3x five minute washes with 1x TBS/T. The HRP-conjugated secondary antibody was detected by exposure to Supersignal West Dura Extended Duration Substrate (Pierce, Cat.# 34076) and exposure to x-ray film (Kodak, Cat.# XAR-5) for 1 second to develop and visualize protein bands.

HepAD38 cells. The HepAD38 cells were plated at $5x10^5$ cells/well in 6-well plates. The cells were transduced with lentiviral vectors expressing ZFPs, harvested and processed as described above for lentivirus expression of ZFPs in 293T cells. The exception is the time for exposure to the x-ray film was 5 seconds.

3.2.7 – Extracellular virus (ECV) DNA isolation

To quantitate the extracellular HBV produced in cell cultures, the virus in the culture media were quantitated by PCR as described in section 2.2.1.4.i. The virus was quantified in the media of HepAD38 cells and HepG2.2.15 cells after 72, 120 or 192 hours post-transduction (indicated in each experiment).

3.2.8 – Intracellular virus (ICV) DNA isolation

Cell monolayers were rinsed with PBS and lysed in ICV Solution #1 (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.3% Triton X-100 and 8% sucrose) and incubated at 37°C for five minutes. Lysates were transferred into a new tube and the nuclei were pelleted by centrifugation at 17,500xg for 10 minutes. The supernatant was transferred into a new tube and $MgCl_2$ was added to 6 mM, DNase I to 100 µg/mL and RNase A to 10 µg/mL and the supernatant incubated at 37°C for 30 minutes. The samples were centrifuged at 17,500xg for 10 minutes and the supernatants were transferred into a new tube. Virus was precipitated overnight at 4°C by the addition of 0.3 volumes of 26% PEG 8000, 1.4 M NaCl and 10 mM EDTA. The samples were centrifuged at 17,500xg for ten minutes to pellet the virus, the supernatant was discarded and the viral pellet was resuspended in 100 µL of TSE buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA). Proteinase K was added to 800 µg/mL and SDS to 0.1% and the solution was incubated overnight at 42°C to digest the nucleocapsids. Phenol and chloroform (100 µL of each) was used to extract the viral DNA and the top aqueous phase containing the DNA was transferred into a new tube. Ten µg of yeast total RNA was added as a carrier to help precipitate DNA, along with two volumes of ice-cold 95% ethanol and 1/10 volume of 3 M sodium acetate. The samples were incubated overnight at -20°C, centrifuged at 17,500xg for 15 minutes and the DNA pellet was resuspended in 20 µL of water.

3.2.9 – RNA isolations

RNA was extracted from cell culture monolayers using TRIzol Reagent (Life Technologies, Cat.# 15596-026) by removing the cell culture supernatant, rinsing once with PBS and adding 1 mL of TRIzol per 10 cm^2 of cells (or per well of a 6-well plate). The cell lysate was passed through the pipette several times and collected into a microfuge tube. After 5 minutes at room temperature, 200 µL of chloroform was added and the tubes were capped and vigorously shaken for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged at 12,000xg for ten minutes at 4°C and the top aqueous phase (RNA remains exclusively in the aqueous phase) was transferred into a new tube and the RNA was precipitated by adding 0.5 mL of isopropanol per mL of TRIzol and incubated at room temperature for ten minutes. The samples were centrifuged at 12,000xg for ten minutes at 4°C to pellet the RNA, the supernatant was removed, and the RNA pellet was washed in 75% ethanol (RNase-free) by vortex and repelleted by centrifugation at 7,500xg for five minutes at 4°C. The supernatant was removed and the RNA pellet was resuspended in RNase-free water.

3.2.10 – cDNA synthesis

The RNA isolated above was used for quantitative real-time PCR. To generate the cDNA, 2 µg of RNA was diluted into a total volume of 9.85 µL with RNase/DNase free water. A master mix was created containing 0.5 µL of

10 mM dNTPs, 1.5 µL of RNase/DNase free water, 0.2 µL of BSA (10 mg/mL), 0.8 µL of RNase OUT (Invitrogen, Cat.# 10777-019), 0.15 µL of 3 µg/µL random primers (Invitrogen, Cat.# 48190-011), 1 µL (200 U) of recombinant Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Cat.# 28025-013), 4 µL 5x first strand buffer (provided with MMLV enzyme) and 2 µL of DTT at 0.1 M (provided with MMLV enzyme). For each reaction, 10.15 µL of the master mix was added to the 9.85 µL of RNA sample. The samples were placed at 22°C for 5 minutes, 37°C for one hour, and 95°C for 10 minutes. Finally, 180 µL of water was added to each sample to yield a final concentration of 10 ng of DNA/µL.

3.2.11 – Quantitative PCR analysis

Real-time quantitative PCR was performed on the cDNA produced from the samples to measure HBV and hypoxanthine phosphoribosyl-transferase 1 (HPRT1), a housekeeping gene used to normalize the HBV measurement/cell. Real-time quantitative PCR for HBV quantitation was performed as described in 2.2.1.4.ii. Real-time quantitative PCR for HPRT1 was performed using the same equipment and reaction chemistry as described in section 2.2.1.4.ii, with the exception that HPRT1-specific primers and probe used were obtained from ABI (Life Technologies, Cat.# 4326321E). The negative controls and samples were all tested in duplicate. The ABI standard program for PCR was 2 minutes at 50°C and 10 minutes at

95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute to amplify the cDNA.

3.2.12 – Capsid Western for HBV

The protein lysates were analyzed using the capsid Western for HBV protocol instead of SDS-PAGE and Western blot because the antibodies available against HBV do not work well in those conditions. However, in the native gel electrophoresis conditions in the capsid Western protocol, the anti-core antibody works well and is a reliable way to analyze HBV capsid levels in the lysate and therefore HBV core levels (personal communication with Bill Addison).

Cell monolayers were washed with two mL of PBS and lysed using one mL of lysis buffer per well of a 6-well plate (50 mM Tris HCl pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100 and 1% Sigma protease inhibitor cocktail (added fresh each use) (Sigma, Cat.# P8340)). After ten minutes at room temperature the cell sheet detaches and the solution was pipetted up and down approximately five times before being transferred into a microfuge tube. The samples were centrifuged for five minutes at 17,500xg at 4°C and 0.9 mL of the supernatant was transferred into a fresh tube. Five µL of RNase A (10 mg/mL) and 2.5 µL DNase I (20 mg/mL) were added to the supernatant and incubated at 37°C for one hour. The samples were centrifuged as above and 0.8 mL of the supernatant was transferred into a new microfuge tube and the total protein content was assessed by BCA

protein assay according to manufacturers specifications (Pierce, Cat.# 23227).

A one percent agarose gel was prepared in running buffer (80 mM Tris H_3PO_4 pH 7.5, 1 mM $Mg(OAc)_2$, 0.1 mM EDTA) and lysates samples were prepared by addition of Orange G loading dye to 1x (6x: 0.35% Orange G, 15% Ficoll 400) and loaded into the gel. The gel was electrophoresed at 4 V/cm for 2 hours. The capsids were transferred onto a nitrocellulose membrane (Amersham Hybond-ECL, GE Cat.# RPN303D) with 1x PBS as the transfer buffer. The transfer was set up using a 13 x 9 inch glass dish filled with approximately 1 liter of 1x PBS and with a glass plate resting across the top. Whatman 3MM filter paper was draped over the glass plate with both ends submerged in the PBS in the dish to wick transfer buffer up to the surface of the glass plate. The gel was placed on the filter paper and the rest of the filter paper surface was covered with pieces of used x-ray film to prevent buffer wicking through any area other than the gel. A piece of nitrocellulose was cut to a size to just cover the gel and floated into a dish of 1x PBS and incubated for a few minutes. The nitrocellulose was laid over the gel and air bubbles between the filter paper, gel and nitrocellulose were removed. A piece of filter paper was placed on top of the nitrocellulose, followed by a stack of paper towels and lastly a book weighing approximately 3 pounds was placed on top of the paper towels. The transfer took place overnight and the following morning the membrane was blocked for 30 minutes in 5% skim milk block buffer made with PBS and 0.1% Tween 20

(5% PBS/T). The membrane was incubated with the primary antibody, anti-HBV core at 1/10,000 (DAKO, Cat.# B0586) diluted in 1% PBS/T for three hours at room temperature with gentle shaking. The membrane was washed three times with 50 mL 1% PBS/T and the secondary antibody was added, goat anti-rabbit HRP, at 1/15,000 (Cappel, Cat.# 21974) for 45 minutes at room temperature with gentle shaking followed by three washes with 50 mL of 1% PBS/T. The membrane was exposed to GE ECL reagent (GE, Cat.# RPN2106) and to x-ray film to visualize HBV capsids (Fuji, Medical x-ray film, Cat.# 47410-08399).

To detect the GAPDH as a loading control, after developing the membrane for HBV capsids it was incubated with 4 mM azide in 1% PBS/T for 30 minutes to inactivate the HRP enzyme conjugated to the goat anti-rabbit antibody. The membrane was washed 3 times with 1% PBS/T to remove any residual azide. With the same procedures as described above, the membrane was exposed to mouse anti-GAPDH antibodies (Millipore, Cat.# H86504M) at 1/3,000 for two hours, washed as above, exposed to goat anti-mouse HRP antibodies at 1/5,000 for 40 minutes, washed and developed as described above.

3.2.13 – Nuclear ZFP enrichment

HepG2.2.15 cells were plated and transduced with lentiviruses as described in 3.2.5. Seventy-two hours post-transduction the cell monolayers were rinsed with PBS and lysed in ICV Solution #1 (10 mM Tris-HCl pH 7.5,

50 mM NaCl, 1 mM EDTA, 0.3% Triton X-100 and 8% sucrose) for five minutes at 37°C. Lysates were transferred into a new tube and the nuclei were pelleted by centrifugation at 17,500xg for 10 minutes. The supernatant was transferred into a new tube and is referred to as the cytoplasmic compartment. The pellets of nuclei were resuspended and lysed in 60 µL of RIPA buffer. After BCA protein assay was performed to determine the total protein content, equal amounts of cytoplasmic or nuclear protein were loaded run on a 12% polyacrylamide gel for SDS-PAGE and then semi-dry transfer, as described in section 2.2.1.4.iii with the following exceptions. Western blot was performed on the membranes, first probing for ZFP using anti-ZFP antibodies at 1/10,000 for 2 hours followed by three washes with TBS/T. Secondary antibody, goat anti-rabbit HRP, was added at 1/7,000 (Cappel, Cat.# 21974) for 45 minutes, followed by three washes with TBS/T, developed with Supersignal West Dura Extended Duration Substrate (Pierce, Cat.# 34076) and exposed to x-ray film for 12 seconds to allow the visualization of the ZFPs. The same membranes were probed for lamin B1 (a nuclear marker) with anti-lamin B1 antibody (Zymed, Cat.# 33-2000) at 1/5,000 for 2 hours, followed by three washes with TBS/T. Secondary antibody, goat anti-mouse HRP, was added at 1/5,000 (Cedarlane, Cat.# CLCC30007) for 45 minutes, followed by three washes with TBS/T. Membranes were exposed to Supersignal West Dura Extended Duration Substrate (Pierce, Cat.# 34076) and exposure to x-ray film for 1 minute allowed for the visualization of Lamin B1 and ZFP.

3.2.14 – MTT assay

HepG2.2.15 cells were plated at $2.1x10^4$ cells/well in 96 cell plates and cultured as described in 3.2.1.iii and transduced as described in 3.2.5. Cell culture media was changed every second day, and cells were harvested at day 1, day 3, day 5 and day 7 post-transduction. Prior to cell harvest, 10 µL of 5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in PBS) was added to culture supernatant and incubated at 37°C, 5% CO_2 for 2 hours. Culture supernatant was aspirated off and 100 µL of DMSO was added to each well. After 10 minutes at room temperature the lysate was transferred into a new 96 plate, sealed and stored at -20°C until all the samples over the 7 days were harvested. To quantitate cell viability, the samples were thawed and the absorbance was measured at 570 nm.

3.2.15 – Dual-luciferase reporter preparation and assay

To investigate whether the ZFPs are able to bind their target DNA sequence in the context of plasmid DNA, another reporter system, a dual-luciferase reporter plasmid, was used. The reporter plasmid, pEZX-MT01 (see Appendix A), which was provided by Rakesh Bhat of Dr. Houghton's laboratory, contains two luciferase genes, Renilla and firefly, where one of the luciferase genes is un-interrupted (Renilla), but the second gene (firefly) has a multiple cloning site (MCS) in the 3' end of the coding region, but before the poly-A signal site. Shende *et. al.* (2011) and Lam *et. al.* (2010) have used

this system to test for miRNA target interactions; instead, I have cloned the HBV target region of the ZFPs into this multiple cloning site [12, 13]. In theory, after co-transfection of ZFP-expression plasmids and pEZX-TS (ZFP target sites), if the ZFPs are expressed and able to bind their target site in the plasmid it should prevent the transcription of the poly-A tail of firefly luciferase and destabilize this transcript. The level of the Renilla luciferase signal will remain unchanged and thus the ratio of Renilla to firefly could be used as a measure of ZFP binding to the specific target.

i. Cloning of ZFP target sites into pEXZ-MT01.

Primers were designed to add XbaI and XhoI restriction endonuclease sites to flank the ZFP-target region within the HBV genome. Primer sequences can be found in table 3.2. The template DNA for target region amplification was 240 ng of puc119HBV1.3. The PCR reaction conditions were as follows: 0.5 μM of each primer, 1 μL of 10 mM dNTPs, 12.5 units of recombinant *Taq* DNA polymerase, $MgCl_2$ at 1.5 mM, 5 μL of 10x PCR buffer and water up to 50 μL. The thermocycling parameters were as follows: 95°C for two minutes, 35 cycles of 95°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 minute, and a final 72°C for 2 minutes. The PCR products were electrophoresed through 1% agarose gel (1 μg/mL EtBr) in TAE buffer and visualized using UV light and the G:box Gel Documentation System (Syngene). PCR products were then excised with a clean scalpel and extracted using QIAquick Gel Extraction Kit (Qiagen, Cat.# 28706) according to the

manufacturer's instructions and eluted in 30 µL of water. To create XhoI and XbaI sticky ends on the PCR products and the pEZX-MT01 plasmid, double digestions with XbaI and XhoI were performed as follows: 5 µL input DNA, 15 units of XbaI (Invitrogen, Cat.# 15226-038), 15 units of XhoI (Invitrogen, Cat.# 15231-012), 2 µL of 10x BSA (1x final, or 0.1 mg/mL), 2 µL of NEBuffer 4 and 9 µL water and was incubated at 37°C for 3.5 hours. Reactions were heat inactivated at 65°C for twenty minutes. Digested PCR products and pEZX-MT01 were electrophoresed, viewed and extracted as previously described. Ligation reactions to generate pEZX-TS (target sites) were performed with one unit of T4 DNA ligase, 1x ligase buffer (provided with enzyme), 6 µL insert DNA, 1 µL of linearized backbone and incubated for 4 hours at room temperature. Ligation reactions were transformed into chemically competent TOP10 *E. coli* and plated on kanamycin (50 µg/mL) selective LB plates. A positive pEZX-TS clone was confirmed by double digestion with XbaI and XhoI, as described above.

Table 3.2 – Primers for adding XbaI and XhoI restriction endonuclease cut sites to flank ZFP-target region in the HBV genome for cloning into pEZX-MT01 for dual-luciferase reporter assays. *Italics* – nucleotide tail, **bold** – cut site, normal text – HBV coding sequence.

Primer name	Sequence	Reference
Targetsites.fwd	*aaaaa***tctaga**atttacacactctatggaaggc	This thesis
Targetsites.rv	*aaaaa***ctcgag**ttctcaaaggtcgagacagc	This thesis

ii. Assays.

293T cells were co-transfected, or transfected and transduced, as described below. The assay methods to read the dual luciferase activity is the same for all experimental outlines and will be described first, followed by the different experimental outlines. To measure dual luciferase activity in cell monolayers, the cells were rinsed with 1x PBS and then lysed in 250 µL of 1x passive lysis buffer in water (Promega, Cat.# E1910). After passing the lysate up and down in the pipette, 10 µL of lysate was transferred into the well of a white 96-well plate (Costar, Cat.# 3912). Using a Perkin Elmer EnSpire 2300 Multilabel Reader, dual pumps were prepared for use and then loaded with LARII substrate in pump 1 and Stop & Glo reagent in pump 2. The reagents were prepared as specified by the manufacturer's instructions (Promega, Cat.# E1910). A program on the plate reader was created with the following parameters ("Dual-Luc"): dispense 50 µL of LARII at 200 µL/s, shake for 1 second at a speed of 100 rpm, delay for 2 seconds, read luminescence of firefly luciferase activity, dispense 50 µL of Stop & Glo at 200 µL/s, shake for 1 second at a speed of 60 rpm, delay for 2 seconds, read luminescence of Renilla luciferase activity.

Co-transfection at 1:1. The dual luciferase reporter plasmid, pEZX-TS, and the ZFP-expression plasmids, pcDNA3.1(+)ZFPcA, pcDNA3.1(+)ZFPk, pcDNA3.1(+)ZFPn or empty vector, pcDNA3.1(+), were co-transfected at a ratio of 1:1. 293T cells were plated at $5x10^5$ cells/well in a 12-well plate and the following day the cells were transfected using Lipofectamine 2000 at a

reagent:DNA ratio of 2:1, with a total amount of 3.2 μL of reagent and 1.6 μg of DNA per well. The 1.6 μg of DNA was equally split between the pEZX-TS and ZFP-expression plasmids for a ratio of 1:1 (0.8 μg of each plasmid). The DNA was mixed with Opti-MEM for a total volume of 100 μL and the Lipofectamine 2000 was mixed with Opti-MEM for a total volume of 100 μL and incubated for 5 minutes at room temperature. The DNA solution and the reagent solution were mixed together drop-wise, incubated for 20 minutes at room temperature and added drop-wise to cell monolayers with normal cell culture media containing no antibiotics. Six hours later the media was changed to normal cell culture media. After 48 hours the cells were lysed and analyzed as described above.

Co-transfection at 1:10. The dual luciferase reporter plasmid, pEZX-TS, and the ZFP-expression plasmids, pcDNA3.1(+)ZFPcA, pcDNA3.1(+)ZFPk, pcDNA3.1(+)ZFPn or pcDNA3.1(+) were co-transfected at a ratio of 1:10, so the ZFP-expression plasmid is in excess. 293T cells were plated at $5x10^5$ cells/well in a 12-well plate and the following day they were transfected using Lipofectamine 2000 at a reagent:DNA ratio of 2:1, with a total amount of DNA at 1.6 μg and 3.2 μL of reagent per well. The 1.6 μg of total DNA consisted of 0.16 μg of pEZX-TS and 1.44 μg of ZFP-expression plasmids for a ratio of 1:10. Transfection, harvest and analysis was performed as described above.

Transfection and transduction. The 293T cells were first transfected in suspension using Lipofectamine 2000 with the pEZX-TS

reporter plasmid and the following morning the cells were transduced with lentiviral vectors to introduce the ZFPs. In the wells of the 12-well plate the transfection complexes were produced with a reagent:DNA ratio of 2:1 using 3.2 µL of reagent and 1.6 µg of plasmid per well. The DNA was mixed with Opti-MEM for a total volume of 100 µL in a separate microfuge tube and the Lipofectamine 2000 was mixed with Opti-MEM for a total volume of 100 µL per well in a 12-well plate. After a 5-minute incubation at room temperature, the DNA solution was added drop-wise into the well, incubated for 20 minutes at room temperature and $5x10^5$ 293T cells were added to the well. The cells were incubated at 37°C and 5% CO_2 overnight and the following morning the cells were transduced with lentivirus-ZFPcA, -ZFPk, and -ZFPn, as described in section 3.2.5. In the incubator, the cells were rocked once every hour for 4 hours, followed by a 7-hour incubation before the media was changed to normal cell culture media. After 48 hours, the cells were lysed and analyzed for luciferase activity as described above.

3.3 – Results

3.3.1 – Optimization of 293T transfection

293T cell transfection with Lipofectamine 2000 was optimized and assessed using a lentiviral expression vector that expresses RFP (pTRIP, see Appendix A). The lentiviral production process requires a triple-plasmid transfection and with the desire to have the highest titers possible,

optimizing of transfection was done. Different DNA:reagent ratios and adherent cell culture (at 24 and 48 hours) or suspension cell culture (at 24, 48 and 72 hours) were assessed. After 48 hours, cells transfected in suspension and at a DNA:reagent ratio of 1:2.5 were found to have an efficiency of 50% (Table 3.3). It was also found that cells transfected in suspension had a higher efficiency than cells transfected as a monolayer. However, during lentiviral production both methods were tried and there was no significant enhancement in titer when the cells were transfected in suspension.

Table 3.3 – Lipofectamine 2000 transfections of 293T cells.

293T cells Lipofectamine 2000	% RFP positive cells Ratio of DNA:reagent		
adherent cells	1:2	1:2.5	1:3
24 hours	5%	5%	5%
48 hours	30%	40%	30%
	Ratio of DNA:reagent		
suspension cells	1:2	1:2.5	1:3
24 hours	20%	25%	25%
48 hours	40%	50%	40%
72 hours	30%	40%	35%

3.3.2 – Confirmation of ZFP production

Transduced 293T cells.

To ensure that the virus-containing supernatant possessed viral vectors capable of producing the ZFPs upon transduction was assessed in the

easily transduced 293T cell line. These cells were transduced with ZFP-expressing lentiviruses diluted 1:3 and the cell lysates were harvested 72 hours later. After SDS-PAGE and Western blotting to detect ZFP, lysates of the cells transduced with ZFPcA, ZFPk and ZFPn, as well as the positive control (previously harvested lysate of cells transfected with ZFP-expression vector) all had bands. The non-transduced control did not have a band for ZFP, as expected (Figure 3.1).

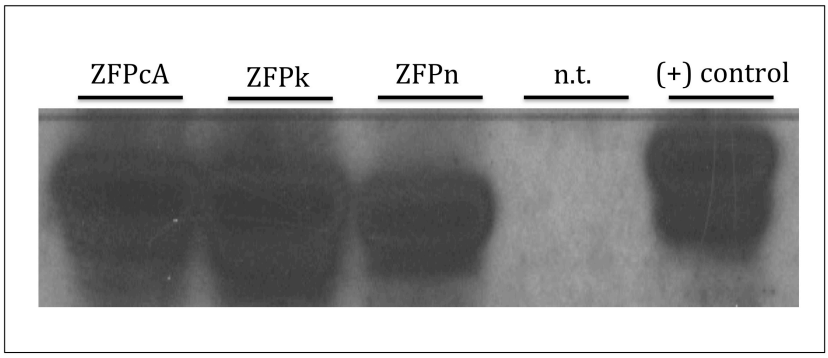

Figure 3.1 – ZFP production in transduced 293T cells. Lysates of 293T cells 72 hours post-transduction with indicated lentiviruses subjected to SDS-PAGE (10% gel) and Western blot to detect ZFP, which ran at approximately 21 kDa. Untreated (untransduced) cell lysates are denoted by "n.t.", and the (+) control is cell lysate from cells transfected with ZFP-expression plasmids.

Transduced HepAD38 cells.

To ensure that the virus-containing supernatant possessed viral vectors capable of producing the ZFPs upon transduction of hepatoma cell

lines such as HepAD38, the ZFP expressing vectors were tested in HepAD38 cells. These cells were transduced with ZFP-expressing lentiviruses diluted 1:3 and the cell lysates were harvested after 72 hours. SDS-PAGE and Western blot for the detection ZFP was performed and the lysates of the cells transduced with ZFPcA, ZFPk and ZFPn, as well as the positive control (previously harvested lysate of cells transfected with ZFP-expression vector) all had bands of ZFP. The non-transduced control did not have a band for ZFP, as expected (Figure 3.2).

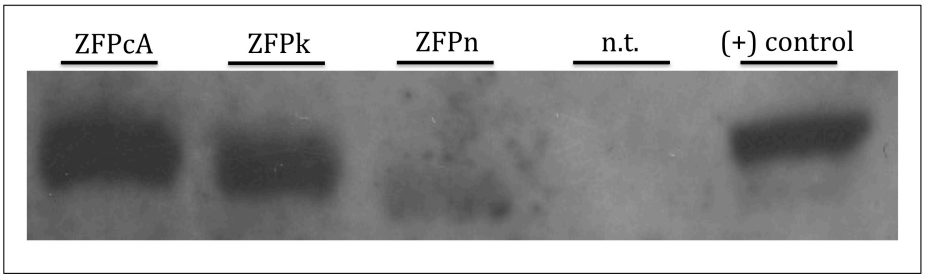

Figure 3.2 – ZFP production in transduced HepAD38 cells. Lysates of HepAD38 cells 72 hours post-transduction with indicated lentiviruses subjected to SDS-PAGE (10% gel) and Western blot detecting ZFP, which ran at approximately 21 kDa. N.t. is untreated (untransduced) cell lysates. The (+) control is cell lysate from cells transfected with ZFP-expression plasmids.

3.3.3 – Evaluation of the hypothesis in HepAD38 cells

HepAD38 cells were plated and transduced (MOI 15) as described in section 3.2.5 with lentivirus-ZFPcA, lentivirus-ZFPk, lentivirus-ZFPn and an

untransduced control. Seventy-two hours post-transduction, the RFP-positivity (transduction efficiency) was determined visually to be approximately 90-95% for each lentivirus expressing ZFPs. ECV DNA was isolated from cell culture supernatant as described in section 2.2.1.4.i and ICV DNA isolated as described in section 3.2.6 and subjected to quantitative PCR for HBV as described in section 2.2.1.4.ii. Cell lysates were harvested and analyzed for HBV capsid levels as described in 3.2.10. This study was repeated two times.

The data presented in Figure 3.3 shows the levels of ECV DNA was not inhibited by the expression of ZFPk and ZFPn, suggesting that virus production was not affected by the presence of HBV-specific ZFPs. Similarly, from the ICV DNA quantification presented in Figure 3.4 shows no effect of ZFPs on the levels of intracellular-capsids. The levels of HBV capsids were not suppressed by the expression of ZFPs, consistent with their lack of effect on viral replication (Figure 3.5). There was no statistical difference between any of the experimental groups, as determined by statistical analysis which used two-way ANOVA with Bonferroni's post-test performed using GraphPad Prism (version 5.00 for Mac OS X).

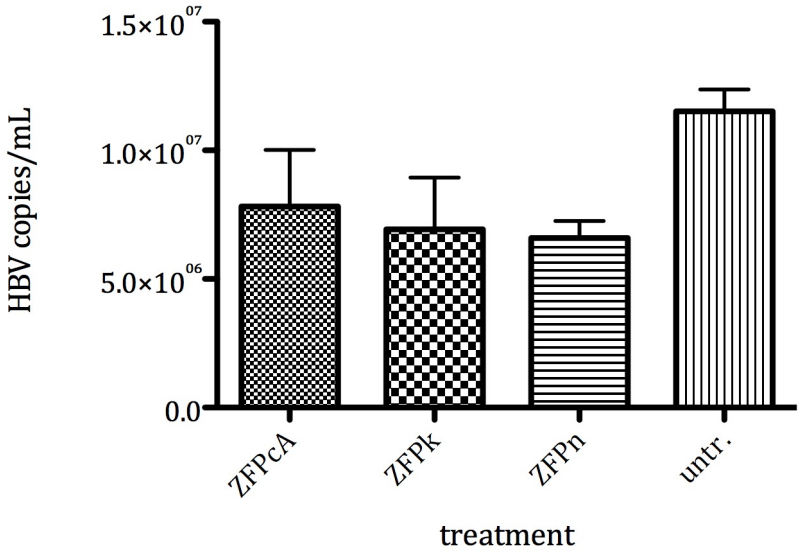

Figure 3.3 – Assessment of the effect of ZFPs on ECV DNA in HepAD38 cells. HepAD38 cells were transduced with indicated lentiviruses and at 72 hours post-transduction the supernatant was collected, HBV DNA was isolated and quantified by real-time PCR. This experiment was repeated twice.

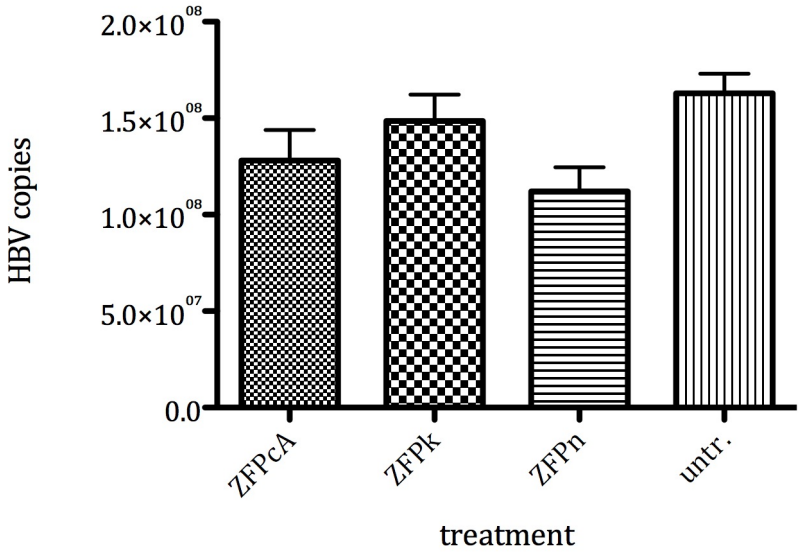

Figure 3.4 – Assessment of the effects of ZFPs on ICV DNA in HepAD38 cells. HepAD38 cells were transduced with indicated lentiviruses and 72 hours post-transduction the cells were harvested for HBV ICV DNA and quantified by real-time PCR. This experiment was repeated twice.

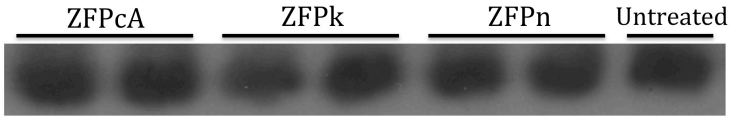

Figure 3.5 – Assessment of the effects of ZFPs on HBV capsid levels in HepAD38 cells. HepAD38 cells were transduced with indicated lentiviruses and 72 hours later the cells were harvested for lysates and analyzed for HBV capsid levels by capsid Western. This experiment was repeated twice.

3.3.4 – Evaluation of the hypothesis in HepG2.2.15 cells

3.3.4.1 – *Day 3 post-transduction*

HepG2.2.15 cells were plated and transduced (MOI 15) as described in section 3.2.5 with lentivirus-ZFPcA, lentivirus-ZFPk, lentivirus-ZFPn, treated with lamivudine (LAM) (10 μM) and an untransduced control. Seventy-two hours post-transduction, the RFP-positivity (transduction efficiency) was determined visually to be approximately 90-95% for each virus. ECV DNA was isolated from cell culture supernatant as described in section 2.2.1.4.i and ICV DNA isolated as described in section 3.2.6 and subjected to quantitative PCR for the detection of HBV as described in section 2.2.1.4.ii. Total RNA was isolated and used to generate cDNA as described in sections 3.2.7 and 3.2.8. The cDNA was used for real-time PCR using HBV-specific probe and primers and HPRT1-specific probe and primers for relative HBV levels normalized to the house-keeping gene, HPRT1. The statistical analysis used was two-way ANOVA with Bonferroni's post-test performed using GraphPad Prism (version 5.00 for Mac OS X). This study was repeated four times.

The data presented in Figure 3.6 shows that levels of ECV DNA did not decrease, showing no antiviral effect with ZFPk and ZFPn, suggesting that viral synthesis in the cells was not affected by the expression of HBV-specific ZFPs. Similarly, the ICV DNA quantification presented in Figure 3.7 shows there is no demonstrable antiviral effect of the ZFPs on the levels of HBV DNA in intracellular-capsids. In fact, the data reveals higher levels of HBV in the

ZFPn treated group, and slightly increased levels of HBV in cells expressing ZFPcA and ZFPk. As expected, the positive-control LAM treated cells have a reduction in HBV DNA in the supernatant and in the cells. The data presented in Figure 3.8, where the levels of HBV RNA have been quantitated, demonstrate that there was virtually no difference between any of the experimental groups and control groups. The LAM treated cells (the positive control) were not expected to have a decreased amount of HBV RNA because the antiviral inhibits DNA synthesis, not RNA synthesis. Notably, there was no decrease seen with the expression of ZFPk and ZFPn in HepG2.2.15 cells.

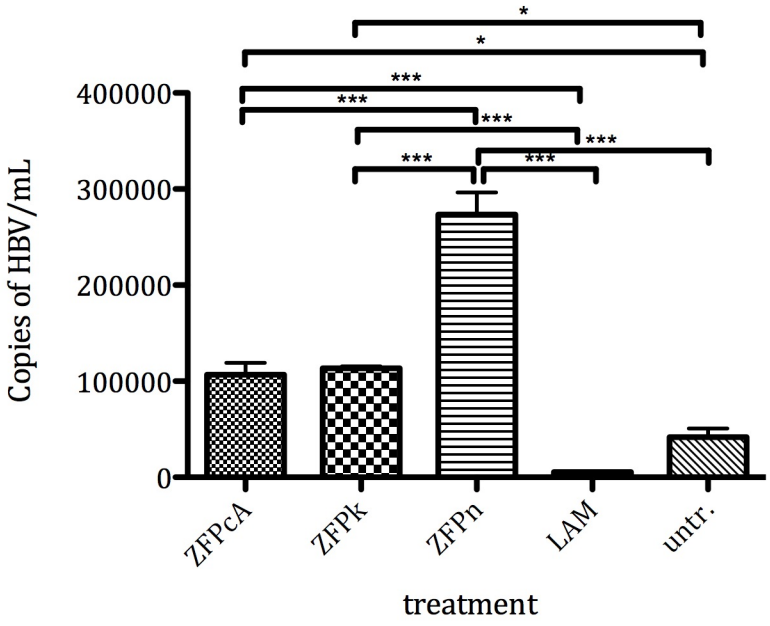

Figure 3.6 – Assessment of the effects of ZFPs on ECV DNA in HepG2.2.15 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and 72 hours post-transduction the supernatant was collected, HBV DNA was isolated and

quantified by real-time PCR. This experiment was repeated four times. * indicates statistical significance ($p<0.05$) between the groups, ** indicates statistical significance ($p<0.01$) between the groups, *** indicates statistical significance ($p<0.001$) between the groups.

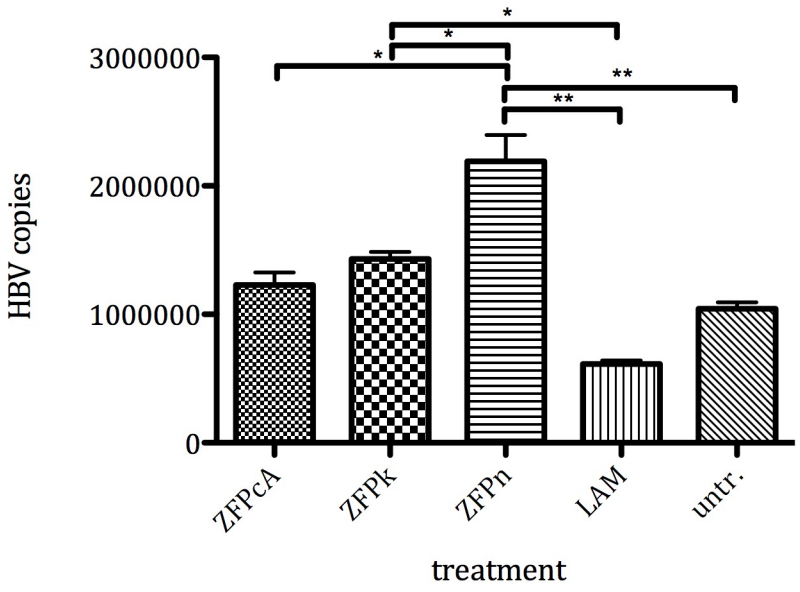

Figure 3.7 – Assessment of the effects of ZFPs on ICV DNA in HepG2.2.1.5 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and 72 hours post-transduction HBV ICV DNA was isolated and quantified by real-time PCR. This experiment was repeated four times. * indicates statistical significance ($p<0.05$) between the groups, ** indicates statistical significance ($p<0.01$) between the groups, *** indicates statistical significance ($p<0.001$) between the groups.

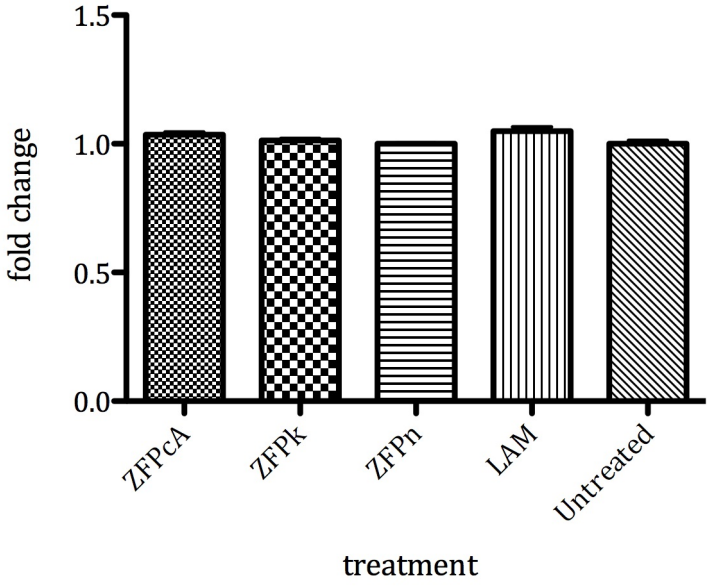

Figure 3.8 – Assessment of the effects of ZFPs on HBV RNA levels in HepG2.2.15 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and 72 hours later total RNA was isolated and used to generate cDNA. HPRT1 and HBV cDNA were quantified by real-time PCR and the data was presented as fold change relative to HPRT1. This experiment was repeated four times.

3.3.4.2 – *Day 5 post-transduction*

HepG2.2.15 cells were plated and transduced (MOI 15) as described in section 3.2.5 with lentivirus-ZFPcA, lentivirus-ZFPk, lentivirus-ZFPn, treated with LAM (10 μM) and an untransduced control. Media was changed every second day, with the addition of fresh LAM. Seventy-two hours post-transduction, the RFP-positivity (transduction efficiency) was determined visually to be approximately 90-95% for each virus. At five days post-

transduction ECV DNA was isolated from cell culture supernatant as described in section 2.2.1.4.i and ICV DNA isolated as described in section 3.2.6 and subjected to quantitative PCR detecting HBV as described in section 2.2.1.4.ii. Cell lysates were harvested and analyzed for HBV capsid levels as described in 3.2.10. Total RNA was isolated and used to generate cDNA as described in sections 3.2.7 and 3.2.8. The cDNA was used for real-time PCR using HBV-specific probe and primers and HPRT1-specific probe and primers, to which the HBV values were normalized. The statistical analysis used was two-way ANOVA with Bonferroni's post-test performed using GraphPad Prism (version 5.00 for Mac OS X). This study was repeated twice.

The ECV DNA quantification data presented in Figure 3.9 reveals elevated HBV DNA in the supernatant of all the transduced experimental groups, but especially in ZFPn treated cells. This trend is consistent with the ICV DNA data in Figure 3.10, where the ZFPn and ZFPk treated groups have higher HBV DNA, however the ZFPcA ICV DNA is lower. In the capsid Western blotting data presented in Figure 3.11, all of the transduced cells have higher HBV capsid levels while the LAM treated and untreated cells appear to have similar levels. A GAPDH loading control was tested and although the loading is not equal in all samples, the trend of higher HBV levels in transduced cells is seen. The capsid Western blotting data was consistent with the ECV DNA data. The HBV RNA quantitation data presented in Figure 3.12 reveal virtually no difference in HBV RNA between experimental groups, and the

LAM control is not expected to have a decrease in HBV RNA due to the activity of the antiviral inhibiting only the reverse transcription of viral DNA.

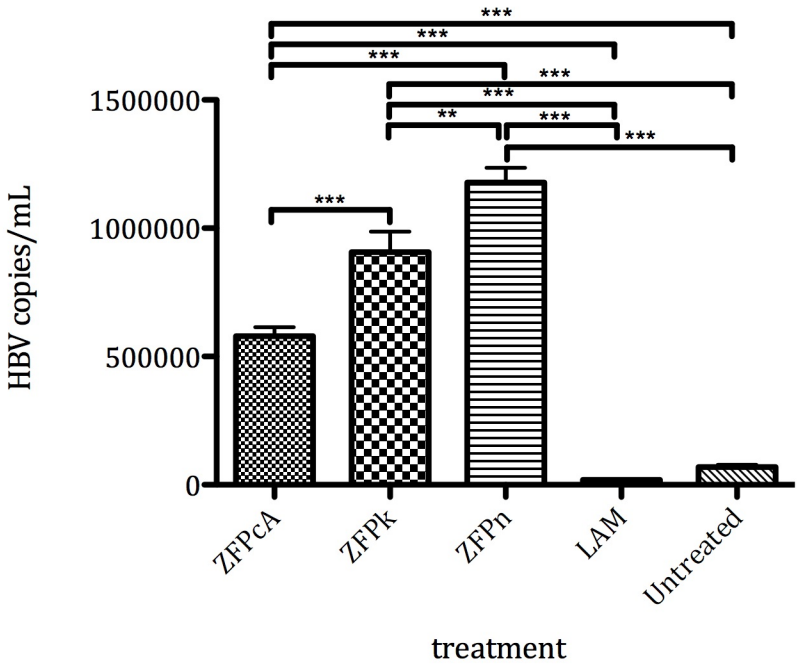

Figure 3.9 - Assessment of the effects of ZFPs on ECV DNA in HepG2.2.15 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and five days post-transduction the supernatant was collected, HBV DNA was isolated and quantified by real-time PCR. This experiment was repeated twice. * indicates statistical significance ($p<0.05$) between the groups, ** indicates statistical significance ($p<0.01$) between the groups, *** indicates statistical significance ($p<0.001$) between the groups.

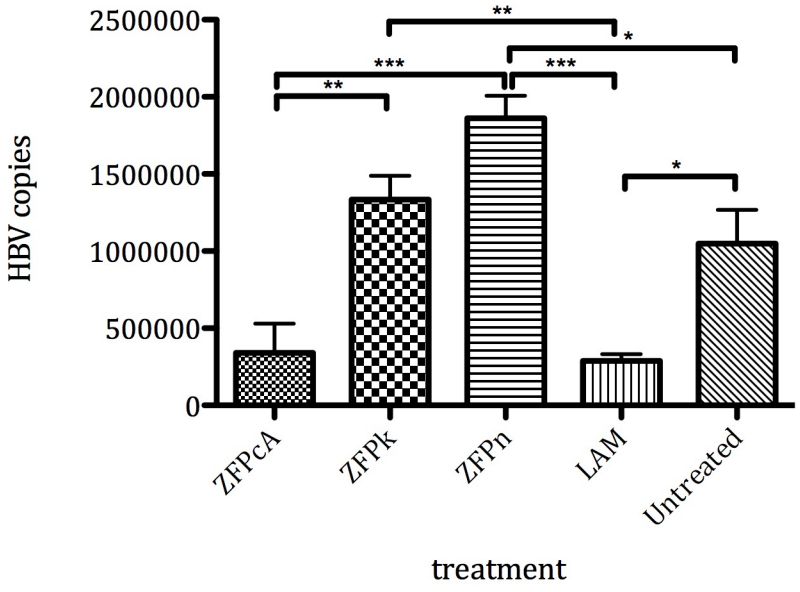

Figure 3.10 - Assessment of the effects of ZFPs on ICV DNA in HepG2.2.1.5 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and five days post-transduction HBV ICV DNA was isolated and quantified by real-time PCR. This experiment was repeated twice. * indicates statistical significance ($p<0.05$) between the groups, ** indicates statistical significance ($p<0.01$) between the groups, *** indicates statistical significance ($p<0.001$) between the groups.

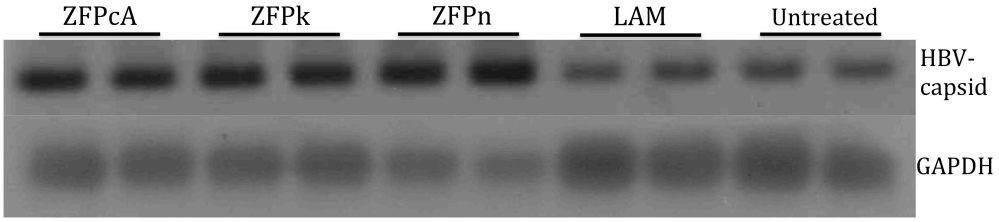

Figure 3.11 - Assessment of the effects of ZFPs on capsid levels in HepG2.2.15 cells. HepG2.2.15 cells were transduced with indicated

lentiviruses and five days later the cells were harvested for lysates and analyzed for HBV capsid levels by capsid Western blotting and GAPDH loading control. This experiment was repeated twice.

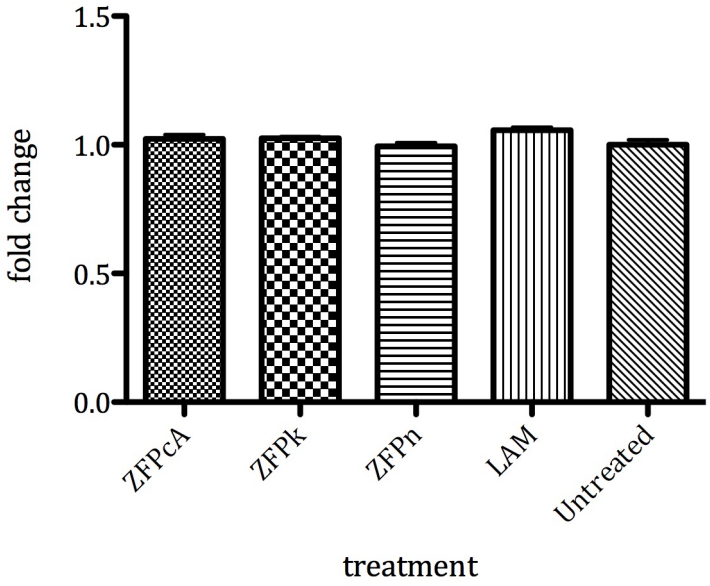

Figure 3.12 - Assessment of the effects of ZFPs on HBV RNA levels in HepG2.2.15 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and five days later total RNA was isolated and used to generate cDNA. HPRT1 and HBV cDNA were quantified by real-time PCR and the data is represented as fold change of HPRT1. This experiment was repeated twice.

3.3.4.3 – *Day 8 post-transduction*

HepG2.2.15 cells were plated and transduced (MOI 15) as described in section 3.2.5 with lentivirus-ZFPcA, lentivirus-ZFPk, lentivirus-ZFPn, treated with LAM (10 μM) and an untransduced control. Media was changed every

second day, with the addition of fresh LAM. 72 hours post-transduction, the RFP-positivity (transduction efficiency) was determined visually to be approximately 90-95% for each virus. At eight days post-transduction ECV DNA was isolated from cell culture supernatant as described in section 2.2.1.4.i and ICV DNA isolated as described in section 3.2.6 and subjected to quantitative PCR detecting HBV as described in section 2.2.1.4.ii. Cell lysates were harvested and analyzed for HBV capsid levels as described in 3.2.10. Total RNA was isolated and used to generate cDNA as described in sections 3.2.7 and 3.2.8. The cDNA was used for real-time PCR using HBV-specific probe and primers and HPRT1-specific probe and primers. The statistical analysis used was two-way ANOVA with Bonferroni's post-test performed using GraphPad Prism (version 5.00 for Mac OS X). This study was performed once.

The data presented in Figure 3.13 showing the ECV DNA quantitation reveal that cells treated with ZFPk have elevated HBV DNA in the supernatant and the other transduced cells have HBV DNA levels comparable to that of untreated cells, and the LAM treated cells are much lower. This trend is consistent with the ICV DNA data presented in Figure 3.14. In contrast, the capsid Western blotting data presented in Figure 3.15 demonstrated a decrease in HBV capsids with all transduced cells; even the LAM treated cells had higher HBV capsid levels. This was not consistent with the ECV and ICV DNA data from the same experiment. The HBV RNA quantitation data presented in Figure 3.16 reveal virtually no difference in

HBV RNA between experimental groups, and the LAM treated cells are not expected to have decreased HBV RNA.

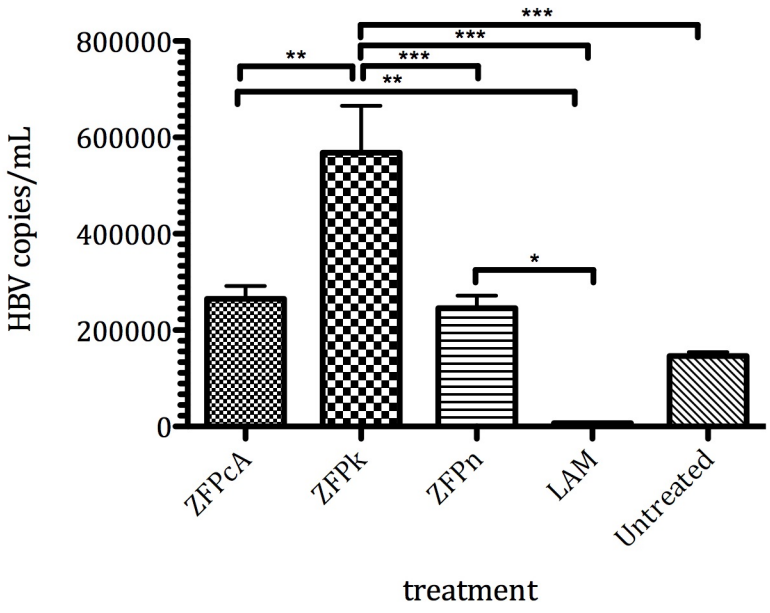

Figure 3.13 - Assessment of the effects of ZFPs on ECV DNA in HepG2.2.15 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and eight days post-transduction the supernatant was collected, HBV DNA was isolated and quantified by real-time PCR. This experiment was performed once. * indicates statistical significance ($p<0.05$) between the groups, ** indicates statistical significance ($p<0.01$) between the groups, *** indicates statistical significance ($p<0.001$) between the groups.

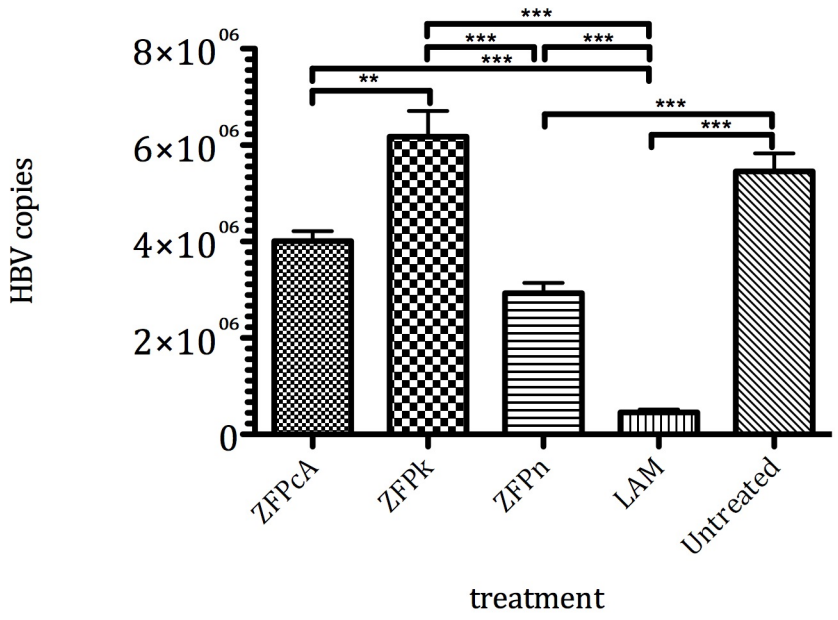

Figure 3.14 - Assessment of the effects of ZFPs on ICV DNA in HepG2.2.1.5 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and eight days post-transduction HBV ICV DNA was isolated and quantified by real-time PCR. This experiment was performed once. * indicates statistical significance ($p<0.05$) between the groups, ** indicates statistical significance ($p<0.01$) between the groups, *** indicates statistical significance ($p<0.001$) between the groups.

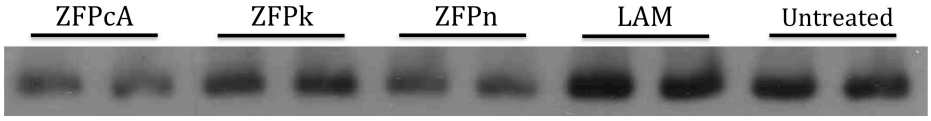

Figure 3.15 - Assessment of the effects of ZFPs on capsid levels in HepG2.2.15 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and eight days later the cells were harvested for lysates and analyzed for HBV capsid levels by capsid Western blotting. This experiment was performed once.

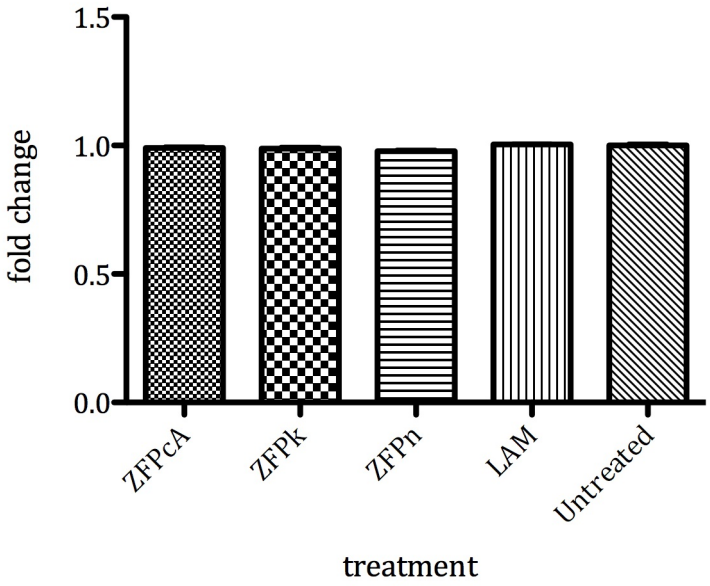

Figure 3.16 - Assessment of the effects of ZFPs on HBV RNA levels in HepG2.2.15 cells. HepG2.2.15 cells were transduced with indicated lentiviruses and eight days later total RNA was isolated and used to generate cDNA. HPRT1 and HBV cDNA were quantified by real-time PCR and the data is represented as fold change of HPRT1. This experiment was performed once.

3.3.4.4 – *Extracellular and intracellular virus DNA levels over time*

The data presented above in sections 3.3.4.1–3.3.4.3 for ECV and ICV DNA levels is combined in Figure 3.17 and 3.18, respectively, showing the effect of the treatment (ZFPs, lamivudine or untreated) over time (Day 3, 5 and 8). The levels of ECV HBV DNA over time remain relatively constant for untreated (Untr) and lamivudine (LAM) treated groups. The levels of ECV

HBV DNA are dramatically heightened at days 5 and 8 post-transduction for all of the transduced groups, including the scrambled control, ZFPcA, although to a slightly lesser extent (Figure 3.17). The levels of ICV HBV DNA remain relatively constant from day 3 to day 5, but at day 8 all groups except LAM treated cells have a dramatic increase, although to a lesser extent, the lentivirus-ZFPn transduced cells (Figure 3.18).

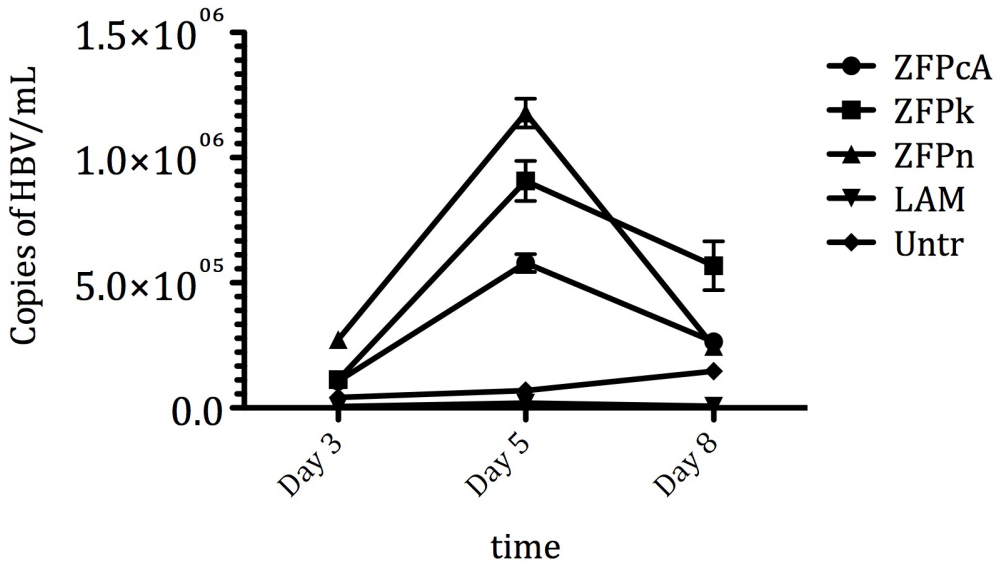

Figure 3.17 – Effect of ZFPs on ECV HBV DNA over time. HepG2.2.15 cells were untreated (Untr), treated with lamivudine (LAM) or transduced with indicated lentiviruses and 3, 5 and 8 days post-transduction the supernatant was collected, HBV DNA was isolated and quantified by real-time PCR.

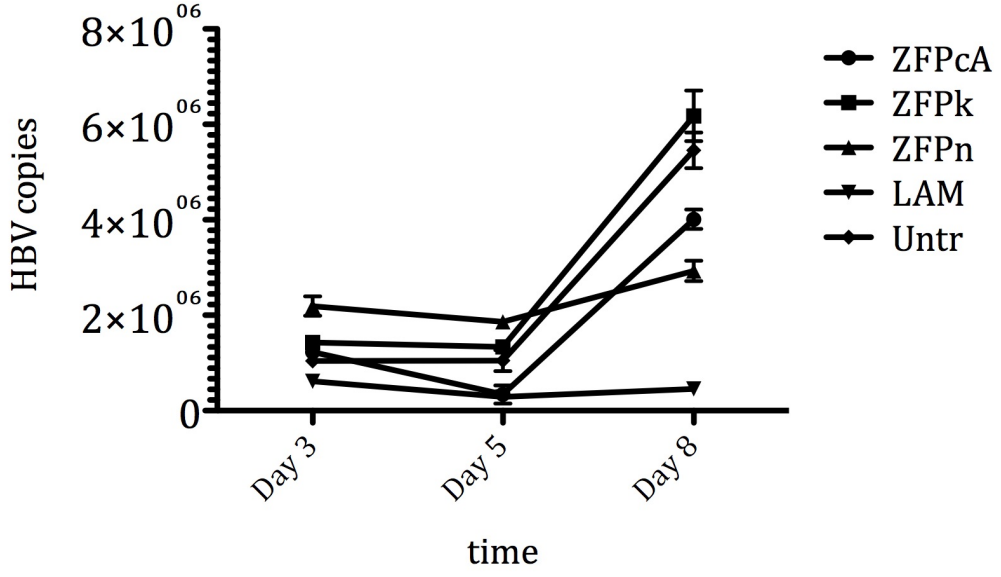

Figure 3.18 – Effect of ZFPs on ICV HBV DNA over time. HepG2.2.15 cells were untreated (Untr), treated with lamivudine (LAM) or transduced with indicated lentiviruses and 3, 5 and 8 days post-transduction HBV ICV DNA was isolated and quantified by real-time PCR.

3.3.5 – Assessment of ZFP nuclear localization

I was surprised not to see suppression of viral DNA synthesis in cells transduced with ZFPs designed to bind the target region of cccDNA of HBV. To ensure that the ZFPs have a functional NLS, it was important to study nuclear localization of ZFPs produced in cells. The nuclear and cytoplasmic compartments of ZFP-expressing cells were compared (described in 3.2.12). HepG2.2.15 cells transduced with lentiviral vectors delivering ZFPs, or a lentivirus not encoding ZFP (no ZFP) (MOI 15) and were harvested 72 hours post-transduction. The cells were lysed, the intact nuclei were pelleted, the cytoplasmic fraction was transferred into a fresh tube and then the nuclei

were lysed with RIPA buffer. Equal amounts of protein from the cytoplasmic and nuclear fractions were run on SDS-PAGE and Western blotting was performed to detect ZFP (21 kDa) and Lamin-BI, a nuclear matrix protein (67 kDa) in each fraction. The Western blot in Figure 3.17 shows that Lamin B1 was exclusively in the nuclear fraction and that the ZFP was present in the cytoplasm but enriched in the nucleus. These results support the idea that there was nuclear localization of the ZFPs expressed in HepG2.2.15 cells. However, a cytoplasmic marker would be necessary to remove the possibility that there was no cytoplasmic lysate contamination of the nuclear fraction. This study was repeated twice.

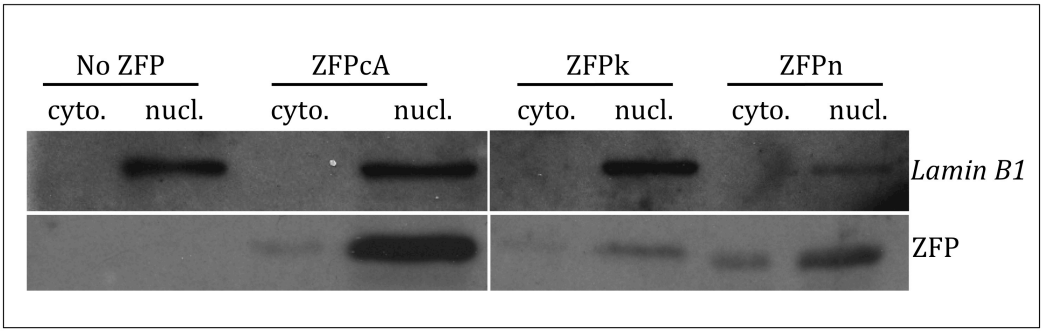

Figure 3.19 – ZFPs are enriched in the nucleus. HepG2.2.15 cell lysates, 72 hours post-transduction with indicated lentiviruses, harvested for nuclear and cytoplasmic compartments and subjected to SDS-PAGE and Western blot for the detection of ZFP and Lamin B1, which ran at 21 kDa and 67 kDa, respectively. This experiment was repeated twice.

3.3.6 – Assessment of toxicity after transduction

Based on visual assessment of HepG2.2.15 cells at various days post-transduction, the cells appeared to be unhealthy and not attaching well, while untreated cells appeared healthy and attached. The viability of transduced cells was assessed at day 1, 3, 5 and 7 post-transduction and compared to untreated cells using an MTT assay. This assay is based on the fact that metabolically active cells will convert the dye (MTT) into a different color dye that can be quantitated by absorbance at 570 nm. A decrease in absorbance (570 nm) correlates with a decrease in the viability of the cells. The data presented in Figure 3.20 shows the relative cell viability after transduction with virus expressing the various ZFPs and compared to untreated controls. The statistical analysis used was two-way ANOVA with Bonferroni's post-test performed using GraphPad Prism (version 5.00 for Mac OS X). The absorbance for the cells at day 1 are lower than the following days because there are fewer cells at that time point. At day 1 the only significant difference in the means of untreated and the different ZFPs is with ZFPk ($p<0.001$). At day 3 there are significant differences between untreated and ZFPcA ($p<0.01$) and ZFPk ($p<0.05$). At day 5 there are significant differences between untreated and ZFPcA ($p<0.001$) and ZFPk ($p<0.001$). There was no statistical difference between untreated and ZFPn at any time point, which was surprising because based on visual assessment cells transduced with lentivirus expressing ZFPn appeared to be the least healthy. Day 1 absorbances are lower than the rest of the time points because there were

fewer cells and therefore less dye was generated. This study was performed once.

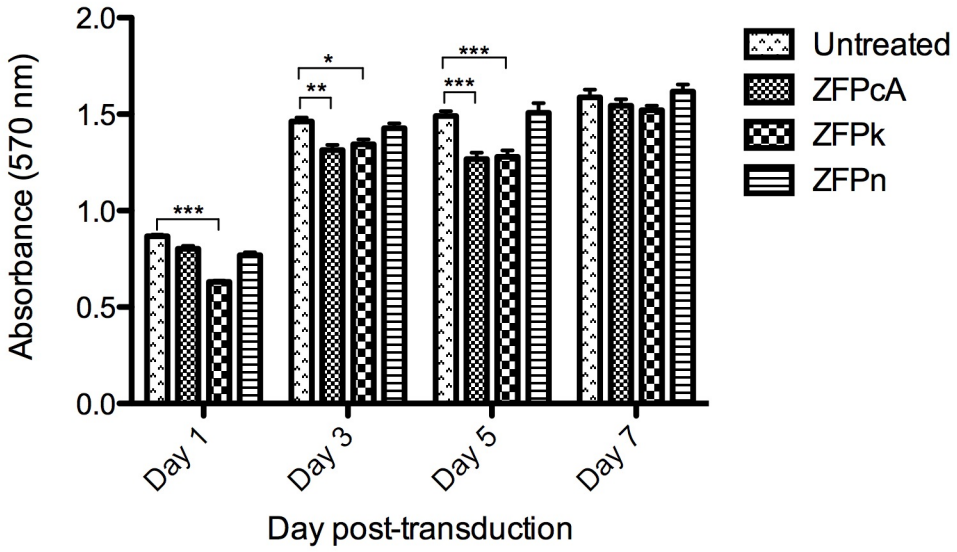

Figure 3.20 – Assessment of the effects of ZFPs on cell viability at various days post-transduction. HepG2.2.15 cells were transduced with indicated lentiviruses and at given time points subjected to MTT assay. A decrease in cell viability is indicated by a decrease in absorbance at 570 nm. The means are representative of 6 wells. * indicates statistical significance ($p<0.05$) between the groups, ** indicates statistical significance ($p<0.01$) between the groups, *** indicates statistical significance ($p<0.001$) between the groups.

3.3.7 – Dual-luciferase reporter assay

An alternative approach to determine if the ZFPs are able to bind their target sequence is to use a dual-luciferase reporter plasmid. Statistical analysis by one-way ANOVA with Bonferroni's post-test was performed using

GraphPad Prism (version 5.00 for Mac OS X). The comparison was between empty vector (or untransduced, Figure 3.23) versus the various ZFPs.

Three ways to perform this test were used. The first is with co-transfection of the reporter plasmid pEZX-TS and ZFP-expression plasmids at a ratio of 1:1. Second, co-transfection of the pEZX-TS and ZFP-expression plasmids at a ratio of 1:10, to reduce the background of reporter plasmid with an excess of ZFPs. Third, transfection (of suspension cells, when the cells were plated) with the pEZX-TS plasmid and transduction with lentiviral vectors to deliver the ZFPs. All tests were performed using 293T cells, are analyzed at 48 hours post-transfection/post-transduction and the data are averages of 6 wells.

Co-transfection at 1:1. The data presented in Figure 3.21 demonstrated that when the ZFP-expression plasmids and the reporter plasmid were present at a ratio of 1:1 there is no significant effect on the luciferase activity with any of the ZFPs present compared to the empty vector control.

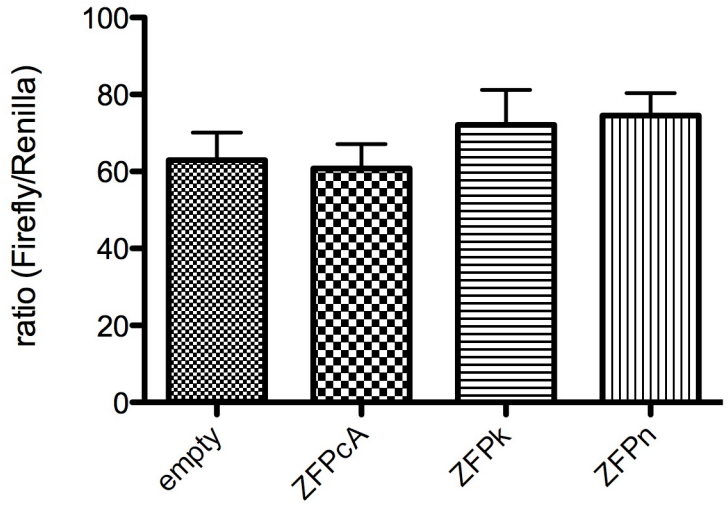

Figure 3.21 – Assessment of the effects of ZFPs on the pEZX-TS reporter plasmid at a DNA ratio of 1:1. 293T cells were co-transfected with dual luciferase reporter plasmid and ZFP-expression plasmids at a ratio of 1:1 and 48 hours later the ratio of firefly luciferase (interrupted) and Renilla luciferase activity was determined. Data is representative of averages of 6 wells. There is no statistical significance between any groups.

Co-transfection at 1:10. The data presented in Figure 3.22 demonstrate that when the ZFP-expression plasmids were in excess compared to the reporter plasmid (1:10) there is a statistically significant reduction in the ratio of firefly to Renilla luciferase activity comparing empty vector to each of the cells transfected or transduced with each ZFP, including the non-specific ZFP, ZFPcA. With the reduction in the ratio seen with ZFPcA, the target site of ZFPcA (a known, random sequence) was searched for in the

sequences of firefly and Renilla luciferase genes. No hits were found, but the possibility of off-target binding is possible, based on the high tolerance of the ZFPs for nucleotide substitutions. This experiment was performed twice.

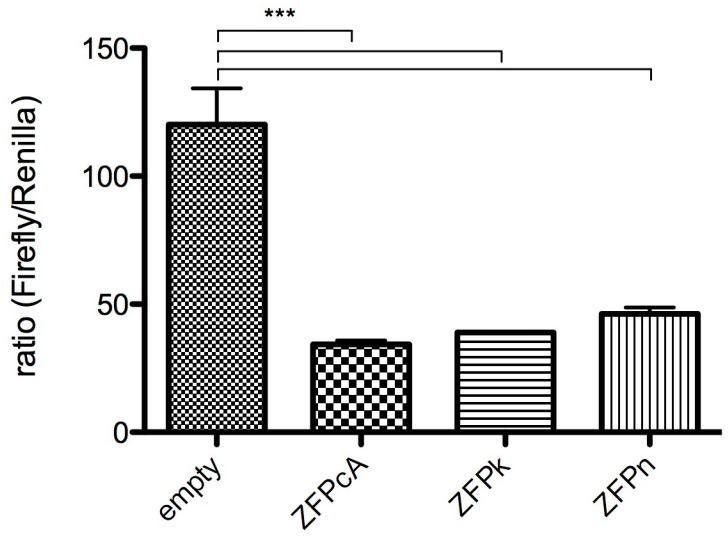

Figure 3.22 - Assessment of the effects of ZFPs on the pEZX-TS reporter plasmid with ZFPs in excess (1:10). 293T cells were co-transfected with dual luciferase reporter plasmid and ZFP-expression plasmids at a ratio of 1:10 and 48 hours later the ratio of firefly luciferase (interrupted) and Renilla luciferase activity was determined. Data is representative of averages of 6 wells. *** indicates statistical significance ($p<0.0001$) between the groups. This experiment was performed twice.

Transfection and transduction. The data presented in Figure 3.23 demonstrate that when the ZFP-expression is introduced with lentiviral

vectors and the reporter plasmid is present there is no significant effect with any of the ZFPs compared to the untransduced control.

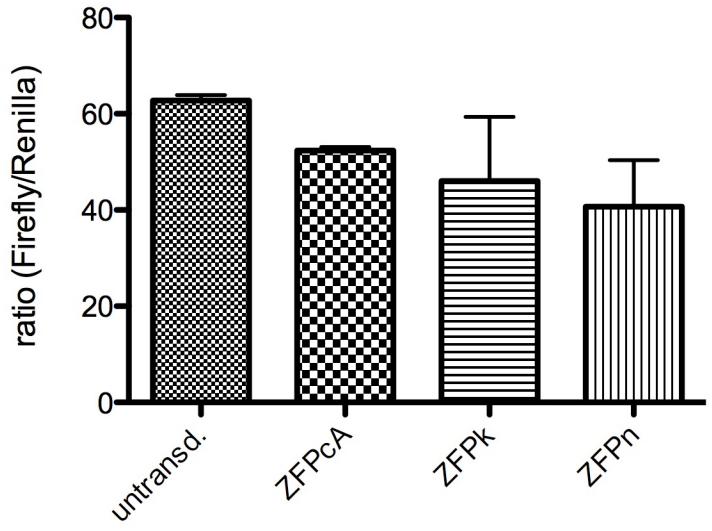

Figure 3.23 - Assessment of the effects of ZFPs when delivered by lentiviral vectors on the reporter plasmid pEZX-TS. 293T cells were transfected in suspension with the dual luciferase reporter plasmid and the following morning transduced with the indicated lentiviruses at MOI of 15. 48 hours later the ratio of firefly luciferase (interrupted) and Renilla luciferase activity was determined. Data is representative of averages of 6 wells. There is no statistical significance between any groups.

3.4 – Discussion

Two ZFPs were previously designed to target the SPII promoter region of HBV cccDNA. We hypothesized that when these ZFPs are present in

an HBV-infected cell, they will bind their 18-base pair target region in the cccDNA and inhibit viral transcription, therefore decreasing viral products. The ZFPs possess an NLS, which permitted them to be imported into the nucleus where the cccDNA is located. DHBV-specific ZFPs were previously shown to be able to decrease DHBV replication in LMH cells that were co-transfected with a plasmid that reproduces the DHBV replication cycle [14]. The intention of the work presented in this thesis was to first develop a system to test the hypothesis in human hepatoma tissue culture.

Success was finally achieved in developing a system to transduce with lentiviral vectors to express the ZFPs in over 90% of transduced HepAD38 cells, which stably express HBV. A number of experiments were done to examine the effects of the ZFPs on HBV expression at 72 hours post-transduction. A study was also performed to examine the levels of ECV 5 days post-transduction (data not shown). There was no inhibitory effect on HBV in any of the experiments (intracellular or extracellular). An important feature of the HepAD38 cell line is that the integrated copy of HBV is driven from a strong CMV promoter leading to high levels of expression. This was desirable because we wanted to be able to see a significant decrease upon exposure to the ZFPs and starting with a high level of HBV would facilitate this observation. However, it is possible that the promoter is too strong and overpowered any effect the ZFPs may have had on HBV transcription. It must be considered that there are two sources of HBV-expression in these cells: the CMV-driven integrated copy and transcription from the cccDNA reservoir

that arises in the natural course of the infection. The latter will have endogenous promoters driving the HBV expression. An alternative approach was to use the HepG2.2.15 cell line, in which the endogenous promoters of HBV drive the expression of HBV in the integrated copy as well as the cccDNA. The levels of HBV in this cell line are not as high as HepAD38, but would work as well if transduced with lentivirus expressing ZFPs. Therefore, the next sets of experiments were performed with the HepG2.2.15 cell line.

In the HepG2.2.15 cells three time points were analyzed for the ZFP effect on HBV expression, day 3 (four repeats), day 5 (two repeats), and day 8 (one repeat). Unfortunately, there was no antiviral effect seen with HBV-specific ZFPk and ZFPn when compared to the control ZFPcA. In fact, the transduced cells expressing HBV-specific ZFPs frequently had levels of HBV higher than that of the untreated control or ZFPcA control.

The Western blotting data for HBV capsids was generally consistent with the ECV/ICV DNA data with no suppression of the viral capsid in cells expressing HBV-specific ZFPs on day 3 or 5 post-transduction.

It was hypothesized that the transduced cells experienced toxicity due to the lentiviral transduction and based on visual inspection, performing an MTT assay seemed logical. When transduced cells were analyzed at day 1, 3, 5, and 7 post-transduction, there was a significant decrease in cell viability with the expression of some ZFPs. When comparing untreated control cells to cells transduced with lentivirus expressing ZFPcA and ZFPk, there was decreased cell viability at more than one time point, while the expression of

ZFPn in HepG2.2.15 cells did not have decreased viability. This was unexpected because based on visual inspection of the cells by day 5 of the time course; the ZFPn transduced cells appeared unhealthy compared to cells transduced with other ZFPs. By day 8, all of the transduced cells appeared unhealthy.

The possibility that the ZFP target region in the virus genome in either cell line had undergone mutations was considered. The cell lines have been passaged for an undetermined number of times, so it seemed possible that over time there could be mutations in the ZFP target sites. ICV DNA from both HepAD38 and HepG2.2.15 was isolated sequenced over the target region, and the results showed that the HepG2.2.15 virus target site was 100% intact and in the HepAD38 virus target site, there was a single nucleotide change (C→A) at the 5' end in the ZFPn target site (data not shown). However, previous work completed by Kimberley Hoeksema determined the tolerance for single and double nucleotide substitutions was quite high (this thesis, section 1.3.4.3, Figure 1.12) [11]. Thus, this is not likely the reason that there was no anti-HBV effect seen in either HepAD38 or HepG2.2.15 cell lines. We also considered the possibility that the ZFP coding sequence in the lentiviral vectors had undergone a mutation(s) that would result in a non-functional, visible ZFP in transduced cells. Therefore, the ZFP-coding region of the lentiviral expression plasmid (pTRIP) was sequenced. The sequences remained 100% intact.

Another possible reason to explain the lack of the anti-HBV effect by transduction with ZFPs could be loss of a functional NLS so that ZFPs could not localize to the nucleus for interaction with the cccDNA. In section 3.3.4 (Figure 3.19) the data presented demonstrates that the ZFPs are enriched in the nuclei of the transduced cells and that the nuclear versus cytoplasmic compartments are clean (no Lamin B1 in the cytoplasm) however I cannot exclude the possibility that there was cytoplasmic lysate contamination of the nuclear fraction. These results are as expected and therefore eliminate the possibility that the ZFPs are not getting into the nucleus.

Given the lack of an anti-HBV effect seen in the infected tissue culture system, it was considered that maybe the ZFPs ability to bind their target and inhibit transcription could be shown with a reporter plasmid. A dual-luciferase reporter plasmid was kindly provided by Rakesh Bhat (Houghton laboratory) and the ZFP target region was cloned into the multiple cloning site. When the two plasmids, the reporter plasmid and ZFP-expression plasmid, were co-transfected at 1:1, or when the ZFPs were delivered by transduction, there was no significant difference between the cells with expression of ZFPs specific for HBV and the non-transduced/empty vector transfected cells or cells transduced/transfected with scrambled ZFP (Figures 3.21 and 3.23). However, when the ZFPs were provided in excess relative to reporter plasmid (10:1) to reduce background luciferase activity from the reporter plasmid not in the presence of ZFPs, there was a significant reduction in the luciferase ratio with the transfections with each ZFP. As the

scrambled control, ZFPcA, should not be able to bind but there was indeed a reduction when it was present, the luciferase genes were analyzed for a possible target site for the ZFPcA (a random sequence). There was no homology found and therefore it is unlikely that ZFPcA was binding elsewhere in the luciferase genes within the reporter plasmid. Therefore, it is difficult to explain the significant reduction in the firefly luciferase (reporter) by ZFPcA. One possible explanation may be that although ZFPs are best known as DNA binding proteins, they also can bind to other proteins. Perhaps the ZFPcA is able to recognize and bind a functionally important motif within one of the luciferase proteins, reduce its activity, and result in reduced signal. However, since the important negative control gave a positive result, we cannot conclude that the effect of ZFPk and ZFPn are specific. It is worth noting that this system is similar to that used by Kimberley Hoeksema with the DHBV-specific ZFPs in the LMH cell line where the target DNA existed within a plasmid (this thesis, section 1.3.3). In contrast to the HBV system where the target exists within the cccDNA species and within the integrated copy in the genome, access to sequences in a plasmid are not hindered by the DNA-binding proteins that are found on the cccDNA and the host cell genome such as histones. Of note, the ZFPs were designed to a region, SPII, of HBV the cccDNA that is known to be an accessible promoter site that interacts with numerous DNA-binding proteins [15, 16]. Perhaps the interactions of the DNA-binding proteins with the cccDNA are competing

with the ZFP binding directly or causing steric hindrance to prevent the ZFP from producing their desired effect.

In summary, in this chapter I attempted to test our hypothesis that a lentiviral vector expressing cccDNA-specific ZFPs in hepatoma cell lines expressing HBV would result in decreased HBV production. I analyzed the ICV DNA, ECV DNA, HBV RNA and HBV capsid levels in these cells. I have addressed the possible causes including changes in the ZFP target site, changes in the sequences of the ZFPs and problems with nuclear localization. The negative results were not attributable to any of these possible explanations. Although the DHBV-specific ZFPs worked in the original testing with DHBV-expression plasmids, the HBV-specific ZFPs failed to have any effect in subsequent cell culture systems tested. Had they worked in cell culture systems, I planned to test their effect in HBV-infected SCID/beige/Alb-uPA mice transplanted with human hepatocytes. These last experiments were not pursued in the absence of positive results in cell cultures. I have concluded that suppression of HBV with the currently designed ZFPs was not possible.

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Chapter 4 – Discussion and Future Directions

The treatment of chronic HBV has evolved rapidly over the last 15 years. Current therapy consisting of nucleoside analogs or interferon suppresses the virus replication but neither directly targets the viral reservoir, cccDNA in the nucleus of infected hepatocytes. The principle of targeting the viral cccDNA as a method of inhibition of viral production is promising. Two groups have recently reported success in inhibiting human HBV replication with ZFP designed to bind cccDNA and our laboratory previously reported similar results in the DHBV system [1, 2].

The main objective of this thesis was to investigate the ability of novel HBV-specific ZFPs to inhibit HBV replication in infected hepatoma cells and if successful, in the HBV-infected chimeric mouse model. The hypothesis was that HBV-specific ZFPs would bind their target sequence in the cccDNA and interfere with the transcription of viral RNA to inhibit HBV replication. The ZFPs must be specific and possess a high binding affinity. Previous work from the Tyrrell laboratory showed that DHBV-specific ZFPs could inhibit the production of DHBV transcripts and viral protein production in the LMH cell line when a plasmid that reproduces the DHBV replication cycle and the ZFP expression plasmids were co-transfected in these cells [3]. In addition, Kimberley Hoeksema, who completed her PhD in Dr. Tyrrell's laboratory, designed HBV-specific ZFPs and determined their binding affinities when I assumed responsibility for this project [4]. The work presented in this thesis

examined a number of systems to transfect or transduce human hepatoma cells with ZFPs. None of these systems worked until lentiviral vectors were tested and were able to transduce hepatoma cell lines with the expression of HBV-specific ZFPs in greater than 90% of cells. Unfortunately, the use of lentivirus vectors for delivery of ZFPs in two HBV-producing hepatoma cell lines failed to show any inhibition, but in fact, an enhancement of the levels of HBV. To ensure that the lack of effectiveness of the ZFP was not due to mutational changes leading to loss-of-function, sequence analysis and the ZFPs indicated they remained unchanged from the original sequence design. The viral target sites in HepAD38 and HepG2.2.15 cells were sequenced and the targets were intact and I concluded that this was not an explanation for the lack of an antiviral effect. To ensure that the NLS was functional and capable of delivering the ZFPs to the nucleus, the functionality of the NLS's in the ZFP constructs was studied and there was good evidence of the ZFP localization to the nucleus. A dual-luciferase reporter plasmid system was used, which is functionally similar to the previously tested DHBV system in the sense that the ZFPs and their DNA target are co-transfected into cells, and the DNA target is in the form of plasmid DNA. This assay resulted in activity with all of the ZFPs, including the negative control ZFPcA, which did not have a predicted binding site within the target site or within the luciferase genes. These results suggest that the ZFPs could not access their target sites in the context of cccDNA or integrated HBV (in the tissue culture cells) possibly because of limited access to the targets – possible interference by histones or

other DNA binding proteins. However, they could bind their target sequence within the dual-luciferase reporter plasmid, but how specific this binding was is difficult to conclude because the negative control ZFP also demonstrated activity.

This potentially cryptic activity of the ZFPs could be from aberrant protein-protein binding or DNA-binding activities. ZFPs are known to interact with substrates other than DNA, including RNA and proteins [5-7]. Interactions between ZFPs and other proteins are possible with ZFPs consisting of as little as a single finger domain, because the surface of proteins are more heterogeneous than a strand of DNA [7]. Therefore, the possibility of interactions between ZFPs and other proteins has to be considered. With our 6-finger ZFPs it is possible that one or two domains on the carboxy- or amino-terminus (or even the interior fingers) is able to interact with other proteins present in the cell. Alternatively, the possibility of aberrant DNA binding activity is a possibility because our EMSA studies demonstrated ZFP binding studies tolerated single or double nucleotide changes (Figure 1.12). This non-specific binding could likely be decreased with improved design or better selection of ZFPs. The methods used for design of our ZFPs was based on a modular approach, where each finger was considered independently and the amino acid residues were chosen based on a "recognition code" but other interactions such as inter-finger interactions or other interactions between the finger and nearby bases, were not considered [8-11]. More recently the methods to design ZFPs have evolved to

obtain more highly specific ZFPs. These methods involve large screening systems which are time consuming and beyond the scope of this thesis. This is a critical issue to be considered if the hypothesis of ZFPs as a potential therapeutic is to be further developed. Highly specific ZFPs will eliminate potential off-target binding and increase the safety profile for this approach to treatment. Additionally, "highly specific" ZFPs versus "specific" ZFPs could have a significant difference on the effect seen; we know that the specific ZFPs are able to bind sequences with at least two nucleotide substitutions, so the potential stimulation effect on other DNA regions is quite possible.

To investigate the potential of aberrant DNA-binding activity, chromatin immunoprecipitation could be performed, although considerable optimization is required to make this assay functional. This assay would be beneficial to determine if the ZFPs were binding at non-target sites and effecting transcription of other host cell genes. The potential of protein-protein binding activity could be investigated by pull-down experiments using amylose (for MBP-conjugated ZFPs) or nickel resin (for 6xHis tagged ZFPs) followed by silver staining and mass spectrometry to identify the potential protein-binding partners. This also would require significant optimization and was beyond the scope of my thesis.

The most likely explanation for the lack of an anti-HBV effect by ZFPs may be the inability of the ZFPs to access their target sequence in chromatinized DNA. Both of the hepatoma cell lines tested possess an integrated copy of HBV in the genomic DNA and the cccDNA species in the

nuclei, which are associated with histones and HBV core protein [12-14]. The ZFPs were designed to target the preS2 promoter region because it is accessible to mulitple transcription factors and therefore was considered as a possible target for the designed ZFPs [15, 16]. There was no antiviral effect observed in the hepatoma cell lines, where the target DNA was highly proteinized. There was an effect with the dual-luciferase assay (albeit the negative control also had an effect) and in previously published work with DHBV [3]. In both cases the DNA target was in plasmid form and less proteinized. It is most probable that the specific ZFPs were unable to access the target sequence in highly proteinized DNA in the cell lines. If this concept was to be further developed, valuable information could be gathered from experiments looking at all the different regions of the cccDNA for availability to binding by a designed ZFP. The preS2 promoter region chosen for the ZFP used in this thesis was chosen because it was predicted to be accessible, but it would be of value to examine other regions within the HBV cccDNA because other regions may be more accessible and therefore potentially allow an antiviral effect to take place. As mentioned above, two research groups have had success that both targeted the core-coding region of the cccDNA [1, 2].

The enhancement of HBV observed in the cells transduced with the different ZFPs, including the scrambled control was not expected but is indeed interesting. It is known that binding of a ZFP to strand of DNA will induce some unwinding of the strand resulting in a wider, deeper major

groove and a compression of the minor groove [17-19]. This slight conformational change may effect the determination of the binding affinity of the ZFP and influence the binding site preferences. Importantly, the unwinding and conformational change induced by the binding of the ZFPs may permit the binding of other DNA-binding proteins to that region of the DNA and make that region more accessible in general. The opening of the DNA from the ZFPs therefore may have enhanced the transcription of HBV by permitting interaction with HBV-specific transcription factors or other non-predicted transcription factors. This could potentially explain the enhancement of HBV that was observed in the data in this thesis.

In summary, this thesis continued the proof-of-concept work performed previously by Kimberley Hoeksema in the laboratory of Dr. Tyrrell, which had shown that ZFPs designed for cccDNA of DHBV had an effect of decreasing DHBV specific RNA and viral proteins. The hypothesis of this thesis was that designed HBV-specific ZFPs could inhibit HBV transcription in HBV-infected human tissue culture. Although the results presented in this thesis did not support the hypothesis using the designed ZFPs for human HBV, the results and the methods developed in this thesis will hopefully provide guidance for future work on the use of ZFPs and inhibition of cccDNA transcription. Inhibiting cccDNA transcription or eliminating cccDNA remain important goals for the potential “cure” of chronic carriers of HBV.

4.1 – References

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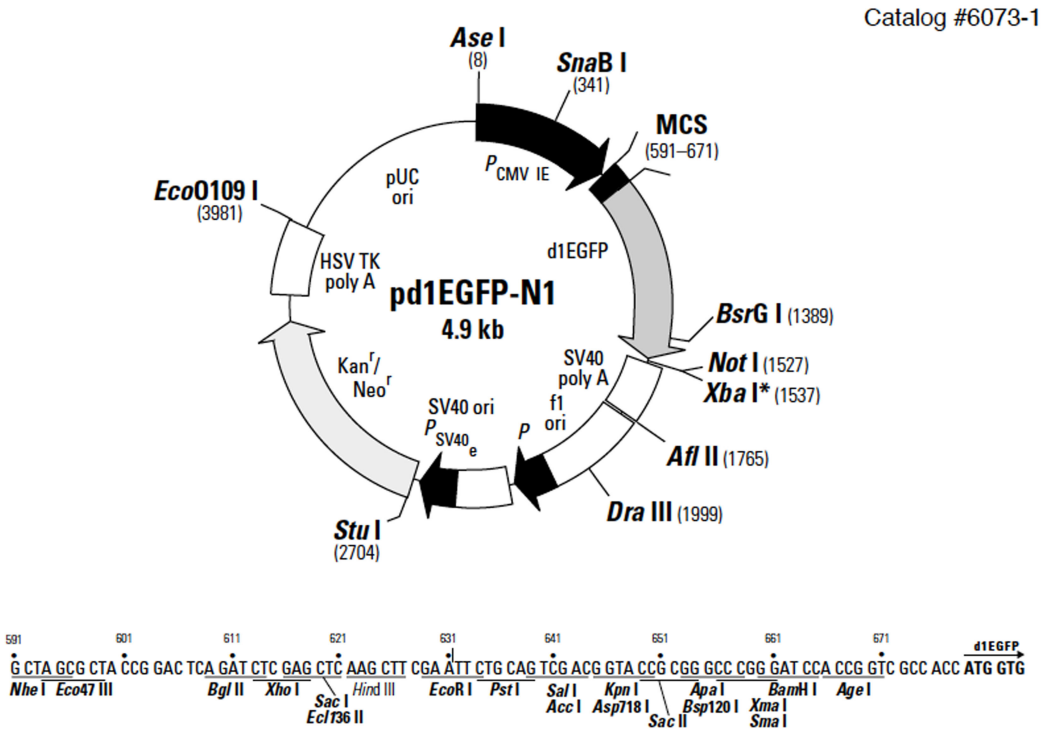
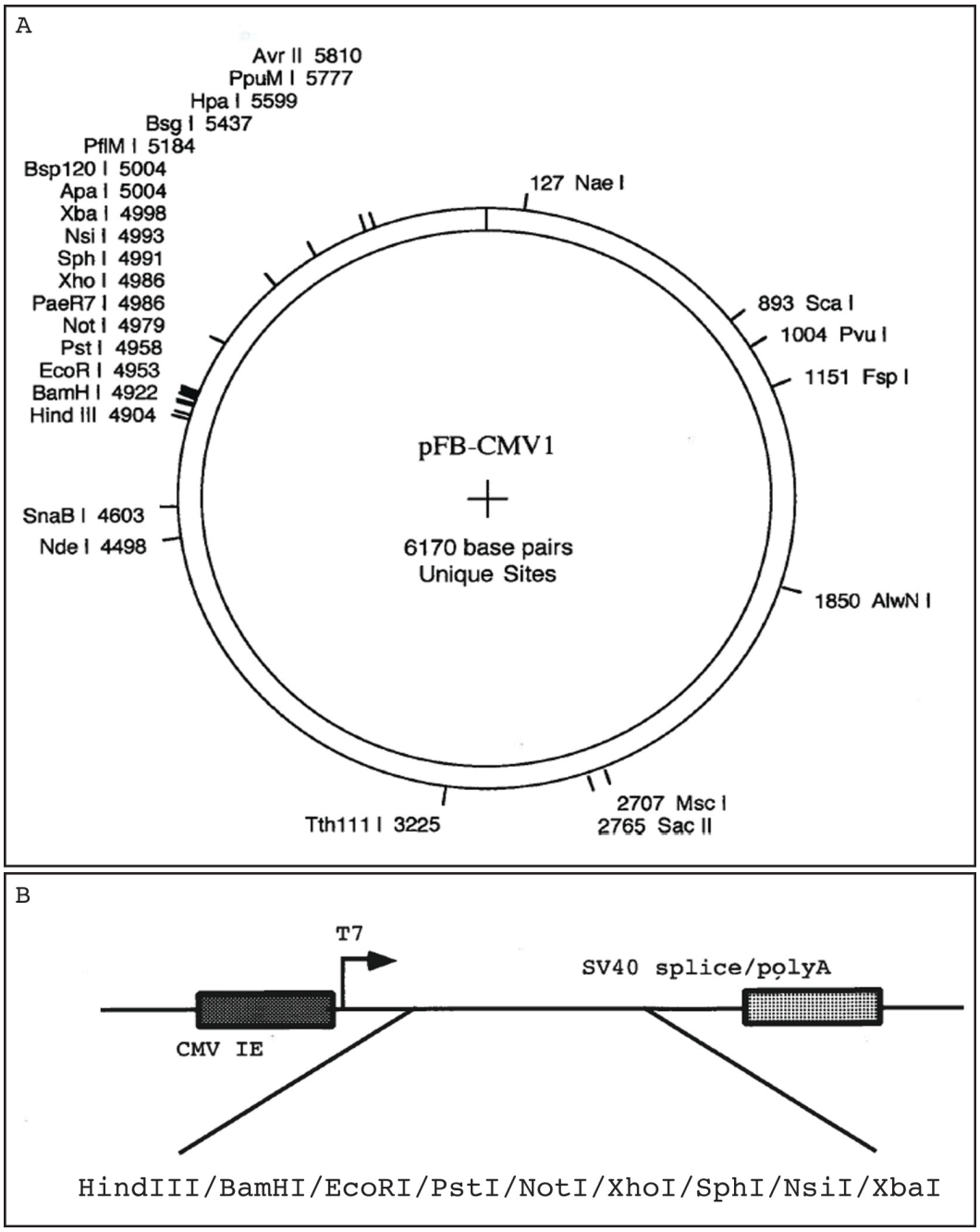
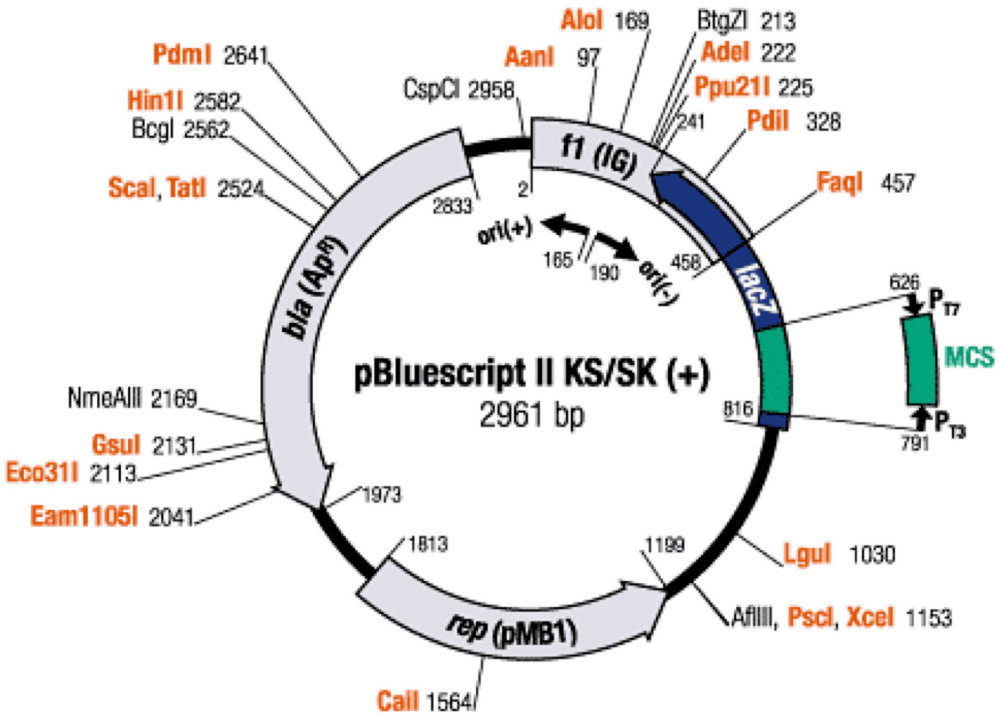
Appendix A – Plasmid Maps



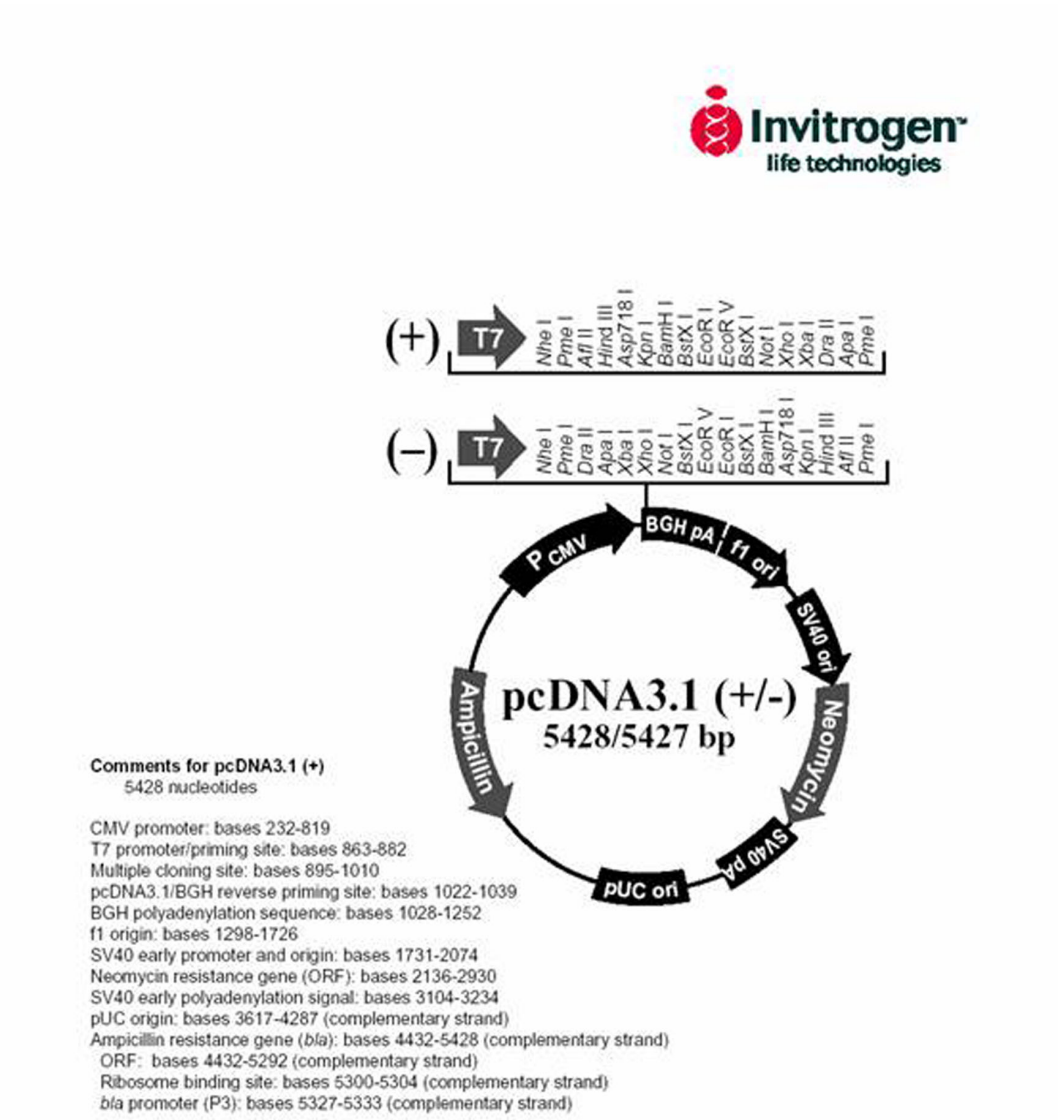
From Clontech Laboratories, Inc.



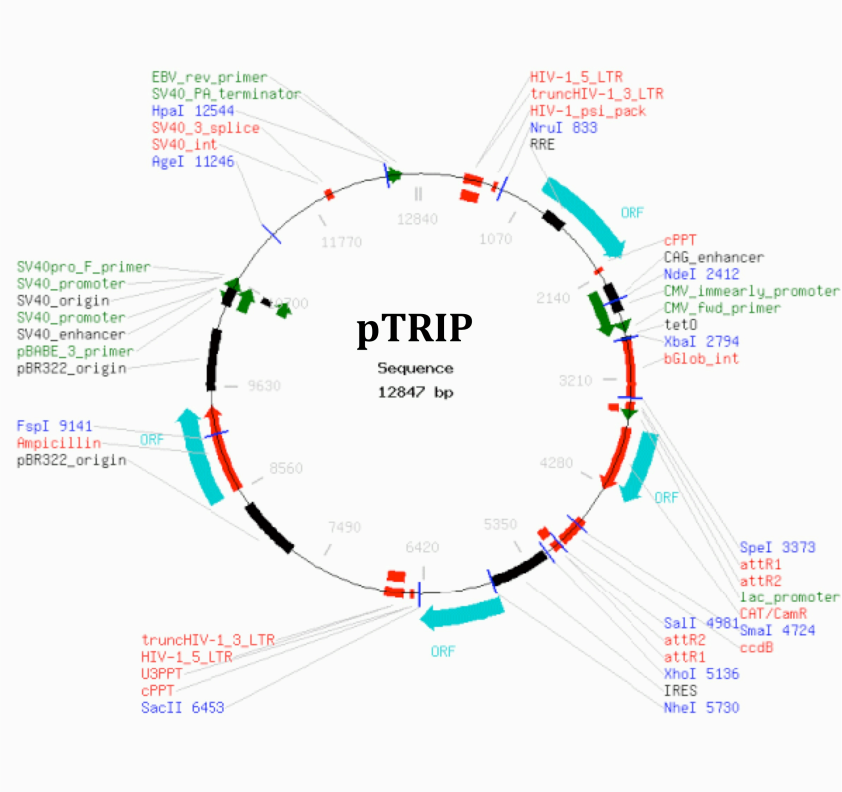
pFastBac (pFB) From Invitrogen. Modified by K. Fischer.



From Thermo Fisher Scientific Inc.

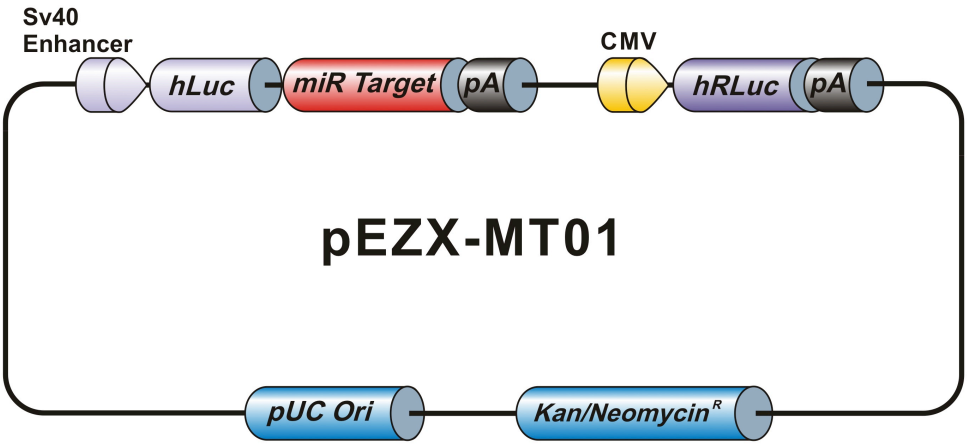


From Invitrogen.



pTRIP

From Schoggins *et al.* (2011) [1].



* miR Target replaced with ZFP target site region

From Genecopoeia

Appendix B – ZFP Information

ZFPk

Nucleotide sequence (5'-3'):
CTCGAGGGTGATATCCTGGAGCCCGGCGAGAAACCGTATAAATGCCC
CGAGTGCGGCAAGTCCTTTAGCCAGAGGGCGCACCTGGAACGGCACC
AAAGAACACATACTGGGGAAAAGCCATACAAGTGCCCTGAGTGCGGC
AAGTCATTCTCTTCACCCGCCGACCTGACAAGGCACCAGAGAACTCAC
ACTGGCGAAAAGCCATACAAGTGCCCTGAATGCGGGAAATCCTTTTCC
CGGGCTGACAATCTGACCGAGCATCAGCGCACCCACACAGGCGAGAA
GCCTTACAAGTGCCCGGAGTGTGGCAAGAGCTTTTCACACACGGGGCA
CCTGTTGGAACATCAAAGGACTCACACTGGCGAAAAGCCCTATAAATG
TCCGGAGTGTGGGAAGAGTTTTAGCACCACCGGGAATCTGACCGTACA
CCAACGGACACACACAGGCGAGAAACCCTACAAGTGCCCCGAATGTG
GCAAATCTTTCAGCGATAAGAAAGATTTGACAAGGCATCAGAGAACA
CACACTGGTAAGAAGACGTCTGATATCGGTACTAGT

Amino acid sequence (N-C):
LEPGEKPYKCPECGKSFSQRAHLERHQRTHTGEKPYKCPECGKSFSSPAD
LTRHQRTHTGEKPYKCPECGKSFSRADNLTEHQRTHTGEKPYKCPECGKS
FSHTGHLLEHQRTHTGEKPYKCPECGKSFSTTGNLTVHQRTHTGEKPYKC
PECGKSFSDKKDLTRHQRTHTGKKTS

DNA binding site: 5'-ACCAATCGCCAGACAGGA-3' on the minus strand at 3105-3121 of HBV subtype *ayw* (U95551)

Finger #	Triplet	Helix
1	GGA	QRAHLER
2	ACA	SPADLTR
3	CAG	RADNLTE
4	CGC	HTGHLLE
5	AAT	TTGNLTV
6	ACC	DKKDLTR

ZFPn

Nucleotide sequence (5'-3'):
CTCGAGGGTGATATCCTCGAACCCGGTGAGAAACCTTATAAGTGTCCC
GAATGTGGGAAGAGTTTCTCCCGCAGCGACGATCTTGTGCGCCACCAA
AGGACACACACAGGGGAGAAACCTTATAAGTGCCCCGAGTGTGGGAA
GAGCTTCAGTCGGTCTGATAACCTGGTGAGGCACCAGAGGACACACA
CCGGCGAAAAACCTTATAAATGTCCCGAGTGCGGCAAAAGTTTTTCAC
GAGCCGATAACCTCACTGAGCATCAACGAACCCATACAGGGGAAAAA
CCATACAAGTGCCCTGAGTGCGGTAAGAGTTTTTCAAGAAGCGACCAC

CTGACTAATCACCAGCGCACCCACACTGGCGAGAAGCCCTACAAGTG
CCCAGAATGCGGTAAATCTTTTTCTCGGTCTGATCACCTTACTACACAC
CAGAGAACGCATACTGGAGAGAAGCCGTACAAATGTCCCGAGTGCGG
AAAGAGCTTTAGCCGCAGTGATCATCTGACCACTCACCAGCGAACCCA
TACCGGAAAGAAGACGTCCGATATCGGTACTAGT

Amino acid sequence (N-C):
LEPGEKPYKCPECGKSFSRSDDLVRHQRTHTGEKPYKCPECGKSFSRSDN
LVRHQRTHTGEKPYKCPECGKSFSRADNLTEHQRTHTGEKPYKCPECGK
SFSRSDHLTNHQRTHTGEKPYKCPECGKSFSRSDHLTTHQRTHTGEKPYK
CPECGKSFSRSDHLTTHQRTHTGKKTS

DNA binding site: 5'-TGGTGGAGGCAGGAGGCG-3' on the plus strand at 3091-3108 of HBV subtype *ayw* (U95551)

Finger #	Triplet	Helix
1	GCG	RSDDLVR
2	GAG	RSDNLVR
3	CAG	RADNLTE
4	AGG	RSDHLTN
5	TGG	RSDHLTT
6	TGG	RSDHLTT

ZFPcA

Nucleotide sequence (5'-3'):
CTCGAGCTGGAACCCGGCGAGAAACCCTATAAGTGCCCCGAGTGCGGGAAATCAT
TTTCTCACAAAAACGCACTGCAAAACCACCAGAGAACGCACACAGGGGAAAAACC
ATACAAGTGTCCTGAATGTGGCAAGTCTTTTTCTGACTGTCGTGATCTGGCCCGG
CACCAGAGAACTCATACCGGCGAAAAGCCATACAAGTGCCCTGAATGCGGAAAAT
CCTTCAGTAGGAAAGACAATCTTAAGAATCACCAGCGCACGCATACCGGAGAAA
AGCCATATAAATGTCCCGAGTGCGGAAAGAGCTTCTCCAGAAGAGATGAGCTGA
ATGTGCACCAAAGGACACATACAGGAGAAAAGCCCTACAAGTGCCCTGAGTGCGG
AAAATCCTTCTCACAGCGGGCTAACCTGCGTGCACATCAAAGAACCCATACCGGG
GAGAAGCCATACAAGTGCCCCGAGTGCGGTAAATCTTTCTCTCAGAAATCAAGCC
TGATTGCTCACCAACGAACCCACACCGGAAAAAAGACCTCCACTAGT

Amino acid sequence (N-C):
LELEPGEKPYKCPECGKSFSQKSSLIAHQRTHTGEKPYKCPECGKSFSQRANLRAH
QRTHTGEKPYKCPECGKSFSRRDELNVHQRTHTGEKPYKCPEC
GKSFSRKDNLKNHQRTHTGEKPYKCPECGKSFSDCRDLARHQRTHTGEKPYKCP
ECGKSFSHKNALQNHQRTHTGKKTSTS

DNA binding site: 5'-ATAAAAATGAAGGCCATT-3' - This is a scrambled, non-specific sequence consisting of scrambled domains of the DHBV-specific ZFPA [2].

Finger #	Triplet	Helix
1	ATA	QKSSLIA
2	AAA	QRANLRA
3	ATG	RRDELNV
4	AAG	RKDNLKN
5	GCC	DCRDLAR
6	ATT	HKNALQN

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