INHIBITION OF HEPATITIS B VIRUS SUBGENOTYPE A1 REPLICATION USING ACTIVATORS OF RNA INTERFERENCE

Maluta Steven Mufamadi

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree

of

Master of Science in Medicine

Johannesburg, 2008.

Declaration

I, Maluta Steven Mufamadi declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……day of ……..2008

Dedication

To my Mother and my Brothers

Masindi, Peter, Avhatakali, Masala and Mufandilani Mufamadi

PUBLICATION AND PRESENTATIONS

Publication

1. Weinberg MS, Ely A, Barichievy S, Crowther C, Mufamadi MS, Carmona S. and Arbuthnot P. (2007) specific inhibition of HBV replication in vitro and in vivo with expressed long hairpin RNA, Molecular Therapy. 2007 Mar; 15(3):534-41.

2. Weinberg MS, Ely A, Passman M, Mufamadi SM and Arbuthnot P. Effective anti HBV hammerhead ribozymes derived from multimeric precursors, Oligonucleotides. 2007 Spring; 17(1):104-12 3.143).

3. Abdullah Ely, Tanusha Naidoo, Steven Mufamadi, Carol Crowther, Patrick Arbuthnot. Expressed anti HBV primary microRNA shuttles inhibit viral replication efficiently in vitro and in vivo, Molecular Therapy (Manuscript -ID MT-2006-633).

Conference Proceedings

1. Ely A, Carmona S, Crowther C, Mufamadi M S, Weinberg M and Arbuthnot P. Inhibiting HBV gene expression with long hairpin RNA sequences that target

the viral X open reading frame. Advances in RNAi research conference; 2006 March 22-23; Oxford, United Kingdom.

2. Ely A, Carmona S, Crowther C, Mufamadi M S, Barichievy S, Weinberg M and Arbuthnot P. Expression of Long Hairpin RNA sequences inhibit HBV replication *in vivo* without inducing an interferon Response. ASGT; 2006 May 31 – June 4; Baltimore

3. Mufamadi MS, Ely A, Kramvis A, Arbuthnot PB. Generation of a plasmid containing a greater than genome length sequence of the a1 subgenotype of HBV that is replication competent in vivo. SASBMB ^{xx}th 2006; 2006 March 22-23; Pietermaritzburg, South Africa.

4. Ely A, Carmona S, Crowther C, Mufamadi M S, Barichievy S, Weinberg M and Arbuthnot P. Expression of Long Hairpin RNA sequences inhibit HBV replication *in vivo* without inducing an interferon Response. SASBMB ^{xx}th; 2006 March 22-23; Pietermaritzburg, South Africa.

5. Moganavelli Singh, Mario Ariatti, Patrick Arbuthnot, Steven Maluta Mufamadi. Lipoplex Formation by Synthetic Targeted Cationic Liposome-Mediated DNA Carrier Systems. The 16th International Microscopy Congress; 2006 September 3 -8; Sapporo, Japan

6. Abdullah Ely, Tanusha Naidoo, Steven M Mufamadi, Carol Crowther, Patrick B Arbuthnot P. MicroRNA-like Hairpin sequences capable of inhibiting HBV replication efficiently *in vitro* and *in vivo*. ASGT; 2007 May 30 – June 3; Seattle, Washington, USA

ABSTRACT

Infection with the hepatitis B virus (HBV) is still a major global health problem with an estimated 6% of the world's population chronically infected with the virus. Chronic infection with HBV subgenotype A1, which is hyperendemic to southern Africa, is associated with a particularly high incidence of liver cancer and cirrhosis. Understanding HBV replication and developing effective HBV treatment to prevent liver cancer remain important medical priorities. Although there is a preventative vaccine for HBV, efficacy of currently available treatment of established infection is limited. Exploiting the RNA interference (RNAi) pathway through the use of small interfering (siRNA) and short hairpin RNA (shRNA) is an attractive new approach for the development gene therapies against HBV infection. Our laboratory has designed and demonstrated the efficacy both *in vitro* and i*n vivo* of several shRNAs designed to target the *X* open reading frame (ORF) of HBV. Thus, the objective of this study was to construct a replication competent plasmid vector of the A1 subgenotype, a reporter plasmid vector of HBV and to assess the efficacy of RNAi effecters against these vectors both *in vitro* and *in vivo*. The first HBV replication competent vector, pCR-HBVA1 1.3x, containing the sequence of an HBV subgenotype A1 isolate, was successfully constructed by generating a greater than genome length sequence of HBV, that starts just upstream of endogenous HBV basic core promoter (BCP) and ends just downstream of the unique HBV polyadenylation (pA) site. Human hepatoma (Huh7) cells transfected with this plasmid secreted HBV surface antigen (HBsAg) into

culture supernatants. In the murine hydrodynamic injection model of HBV replication, serum HBsAg, hepatitis B e antigen (HBeAg) and viral particle levels as well as relative surface and core mRNA levels were shown to be significantly elevated as compared to mock-injected mice. The second HBV vector, pCH-FLuc, was successfully generated by replacing the surface ORF with the sequence encoding Firefly Luciferase. The ability of pCH-FLuc to express Firefly Luciferase was demonstrated in a liver cell line (Huh7 cells). Co-transfection of the reporter plasmid, pCH-FLuc, with shRNAs targeted to HBV caused a significant reduction in Luciferase expression. Co-transfection/injection of the pCR-HBVa1 1.3x with shRNAs caused significant inhibition in the level of viral antigens (HBsAg, HBeAg and hepatitis B core antigen (HBcAg) as well as relative surface and core mRNA levels. This was observed both *in vitro* and *in vivo*. Our results demonstrate the potential this model allows for the study of HBV replication as well as the assessment of potential therapeutic strategies in a regionally significant subgenotype of HBV.

ACKNOWLEDGEMENTS

1. I would like to express my deepest thanks to my supervisor, Prof. P B Arbuthnot, for invaluable guidance, support, and encouragement.

2. I would like to thank Mr. Abdullah Ely for assistance and patience during generation of the plasmid pCR-HBVA1 1.3x and numerous practical aspects of my project.

3. I would also like to thank my colleagues Mrs. Carol Crowther for assistance with QPCR experiments, Dr. Mark Passman for assistance with generation of the plasmid pCH-FLuc, Ms. Margaret Badenhorst for assistance with immunohistochemical staining and Mrs Gladys Gagliardi for providing technical support.

4. I would also like to acknowledge all the members of the Hepatitis B Virus Research Unit (HBVRU) for offering me help and advice when it was needed.

5. I would like to express my sincerest gratitude and appreciation to Prof. Anna Kramvis (Molecular Hepatology Research Unit) for providing me with the plasmid pCR-XL-TOPO- HBVa 1 (pCR-HBVa1 1x) and for HBV subgenotype A1 sequence alignments.

6. I would also like to thank the serology unit of the National Health Laboratory Service (NHLS) in Johannesburg, for performing the HBV e antigen ELISAs.

7. To my mother and brothers, thank you for your encouragement and support during this degree, and for always being there for me.

8. I would like to extend thanks to the financial assistance provided to me by the University of Witwatersrand (HBVRU), the Poliomyelitis Research Foundation (PRF), and the Medical Research Council (MRC).

9. Finally, I gratefully acknowledge God almighty who gave me strength and courage to carry out this study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Page

LIST OF ABBREVIATIONS

1. Anti-HBc	-	hepatitis B core antibody
2. Anti-HBs	-	hepatitis B surface antibody
3. ASHV	-	Arctic Squirrel hepatitis virus
4. BCP	-	basic core promoter
5. cccDNA	-	covalently closed circular DNA
6. CTLs	-	cytotoxic T-lymphocytes
7. DNA	-	deoxyribonucleic Acid
8. dsRNA	-	double-stranded RNA
9. DHBV	-	duck hepatitis B virus
10. *E. coli*	-	*Escherichia coli*
11. EDTA	-	ethylene diamine-tetra-acetic
12. ELISA	-	enzyme-Linked Immunosorbent Assay
13. FCS	-	foetal calf serum
14. FDA	-	food and drug administration
15. GAPDH	-	glyceraldehyde-3-phosphate dehydrogenase
16. GFP	-	green fluorescent protein
17. GSHV	-	ground squirrel hepatitis virus
18. HBV	-	hepatitis B virus
19. HBcAg	-	hepatitis B core antigen
20. HBeAg	-	hepatitis B e antigen
21. HBsAg	-	hepatitis B surface antigen

22. HBx	-	hepatitis B virus X protein
23. HBVRU	-	Hepatitis B Virus Research Unit
24. HCC	-	hepatocellular carcinoma
25. Huh7	-	human hepatoma cells
26. IFN-α	-	Interferon alpha
27. IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
28. Kb	-	kilobase
29. mRNA	-	messenger RNA
30. miRNA	-	micro RNA
31. nt	-	nucleotides
32. ORF	-	open reading frame
33. pA	-	polyadenylation
34. PCR	-	polymerase chain reaction
35. pgRNA	-	pregenomic RNA
36. PoI III	-	RNA polymerase III
37. PTGS	-	posttranscriptional gene silencing
38. qPCR	-	quantitative polymerase chain reaction
39. RdRP	-	RNA-dependent RNA polymerase
40. RISC	-	RNA-inducible silencing complex
41. RNA	-	ribonucleic acid
42. RNAi	-	RNA interference
43. rpm	-	revolutions per minute
44. RPMI medium	-	Roswell Park Memorial Institute medium

45. RT-PCR	-	reverse transcriptase PCR
46. shRNA	-	short hairpin RNA
47. siRNA	-	small interference RNA
48. TGS	-	transcriptional gene silencing
49. UTR	-	untranslated region
50. YMMD	-	tyrosine-methionine-aspartate-Aspartate
51. X-gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
52. WHV	-	woodchuck hepatitis virus

CHAPTER 1

1 INTRODUCTION

1.1 The hepatitis B virus

1.1.1 Hepatitis B virus biology

The hepatitis B virus (HBV) causes both acute and chronic infection of the liver (1). There are approximately 350 million chronic carriers of HBV (2). Chronic HBV infection is endemic in several areas including sub-Saharan Africa, east and South-East Asia, and the western Pacific islands where between 8-15% of the population are chronic carriers (3). The clinical course of HBV infection is variable and may be influenced by individual viral and host variants (4). For example, chronic infection with HBV subgenotype A1, which is hyperendemic to sub-Saharan Africa, is associated with a particularly high risk of hepatocellular carcinoma (5). An effective vaccine has been available for nearly 20 years and vaccination is included as part of Expanded Programme of Immunization (EPI) in developing countries including areas of endemic infection. It's effectiveness in preventing blood borne transmission from a mother to her newborn child is about 90% (6). However, vaccination does not treat established infections. Licensed treatments which include interferon-α, lamivudine and adefovir dipivoxil are the only available treatments for chronic HBV infection (7). These drugs have only 20 to 30% efficacy in patients with chronic HBV infection (8).

Understanding HBV replication and pathogenesis for the development of effective HBV treatment to prevent liver cancer therefore remain important medical priorities. Exploiting the RNA interference (RNAi) pathway has shown exciting promise for the development of novel anti HBV therapy (9).

1.1.2 Life cycle of HBV

HBV is a small enveloped hepatotropic DNA virus which replicates essentially in parenchymal cells of the liver (10). The HBV life cycles involves attachment of infectious virions to cellular receptor(s), uncoating and releasing nucleocapsids that migrate to the cell nucleus (Figure 1.1). Early events in the viral life cycle are poorly understood due to the lack of knowledge about the mode of receptor mediated infection of the host hepatocytes and absence of cell lines and small animals that are susceptible to HBV infection. Although the mechanisms by which HBV enters the cell is poorly understood, it appears that receptor components are necessary for HBV-mediated infection of hepatocytes (10). After the virus has entered into hepatocytes it is uncoated and releases its nucleocapsid which migrates to the cell nucleus, where the partially double-stranded viral genome is converted into covalently closed circular DNA (cccDNA), which is the template for the transcription of four viral (the pregenomic RNA, the pre-S (L) mRNA, the S mRNA and the X mRNA). Translation occurs following transcript export to the cytoplasm.

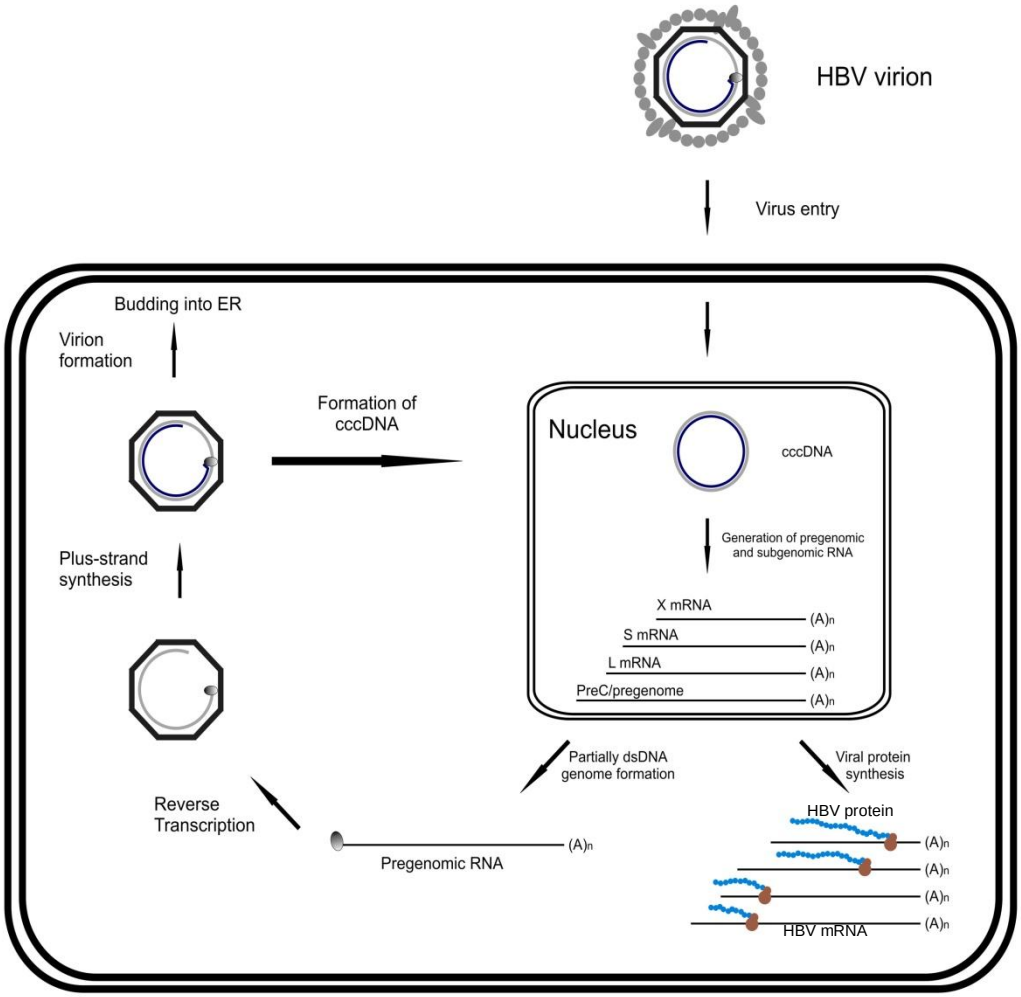


Figure 1.1: Schematic representation of the HBV life cycle. The virus is recognised by cell and binds to cellular receptor(s), uncoating and releasing nucleocapsids that migrate to the cell nucleus, where the partially dsDNA genome is converted into cccDNA. The cccDNA then serves as the template for transcription of functional genome and subgenomic mRNAs. Pregenome RNA is translated into polymerase. The dsDNA HBV can re-enter the nucleus to form more cccDNA or mature virus can assembled with envelope in the ER membranes and virion are released from cells (1-2).

The pregenomic RNA (pgRNA) serves as the mRNA for the synthesis of core protein and reverse transcriptase. HBV replicates its genome by reverse transcription of the pgRNA. The reverse transcriptase binds to the 5' end of its own mRNA template, and the complex is packaged into nucleocapsids, where viral DNA synthesis occurs through reverse transcription. After partially double stranded DNA has been produced, the mature nucleocapsids containing the HBV DNA genome are assembled with envelope polypeptides in the endoplasmic reticulum membranes, and the virions are released from the cell (1, 2, 10).

1.1.3 The viral genome and proteins

The HBV genome is the smallest of all animal viral genomes being 3.2 kilobase (Kb) in size (10) (Figure 1.2). It is composed of relaxed circular, partially double stranded DNA (rcDNA) (2). The long (or minus) strand covers the complete viral genome (3.2 kb) and has the viral polymerase priming protein (reverse transcriptase) bound to its 5' terminus. The short (or plus) strand is incomplete, varies in length and maintains the genome circularity by a cohesive overlap across the 5' and 3' ends of the minus strand. The HBV genome has four primary open reading frames (ORFs) which partially overlap each other and encode the surface, core, polymerase and X proteins. Transcription from the ORFs is initiated by 4 promoters: the Nucleocapsid/Core promoter, X promoter and two Surface promoters and two enhancers (Enhancer I and Enhancer II). In addition, *cis*-acting negative regulatory elements also function in the

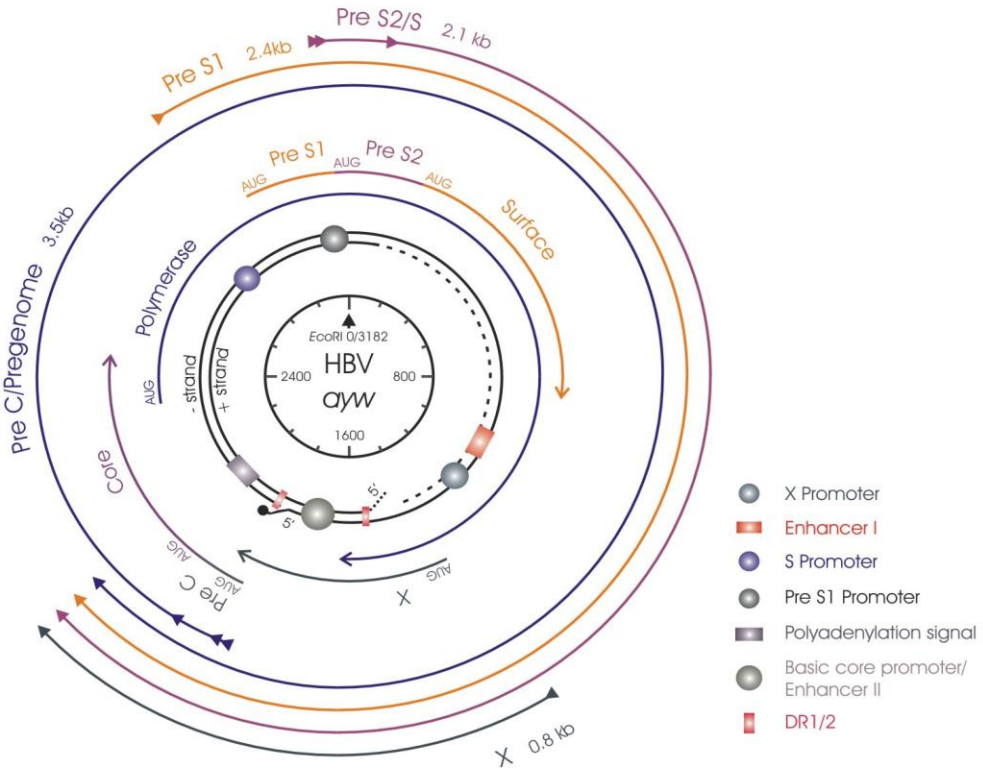


Figure 1.2: Schematic representation of the HBV genome (46). Coordinates of the genome are given relative to the single *EcoR* I restriction site. The partially double stranded HBV DNA genome comprises + and – strands with cohesive complementary 5' ends. The *cis*- elements that regulate HBV transcription are represented by the circular and rectangular symbols. Arrows immediately surrounding the HBV genome indicate the four open reading frames. Four arrows, which give the 5' to 3' polarity, indicate the HBV transcripts.

regulation of viral genes. Transcription from the four ORFs results in formation of the 4 transcripts of 3.5 2.4, 2.1 and 0.9 kb. The single polyadenylation signal terminates transcription at a common 3' end for all transcripts. The 3.5 kb pgRNA serves as template for the core and polymerase proteins. The 2.4 kb RNA encodes the large surface antigen; the 2.1 kb transcript encodes the middle and small surface antigens and the 0.9 mRNA codes for hepatitis B virus X protein (HBx). The three viral surface antigens (HBsAg) are thought to play key roles in the binding of the virus to the host-cell receptors, in the assembly of the virion and its release from cells. The preC/C (precore/core) region encodes the viral proteins, known as HBV core antigen (HBcAg) and HBV e antigen (HBeAg). The HBcAg forms the nucleocapsid; whereas HBeAg is dispensable for infection *in vivo*. The P region is specific for the viral polymerase, DNA synthesis and RNA encapsidation. The X ORF encodes the viral X protein which is required for infection and replication in liver cells *in vivo* (11-13).

The core promoter consists of the basic core promoter (BCP) and an upstream regulatory sequence. The BCP is defined as the region which has *cis*-acting elements that direct the precise initiation of both the preC and pregenomic RNAs. The sequence immediately 5' of the BCP is referred to as the core upstream regulatory sequence (CURS). The CURS region contains several domains, designated boxes α, β, δ and γ. Box α and box β form part of the bipartite structure of the enhancer II element. Several sequence motifs have been shown to interact with ubiquitous and

liver specific transcription factors and are located within the BCP and its upstream regulatory region (12, 13).

1.1.4 Genotypes and subgenotypes

Genetic analysis of full length HBV genomes has led to the virus being classified into eight genotypes (A to H) (4). These genotypes are distinguished by intergenotypic differences of more than 8% in the entire genome, which consists of approximately 3200 base pairs, or at the level of the surface gene by a difference of greater than 4%. All eight genotypes have different geographic distributions, virological characteristics, clinical manifestations and response to antiviral therapies (14, 15).

The dominant genotype in South Africa is genotype A, which consists of two subgenotypes A1 and A2. Subgenotype A1 predominates over subgenotype A2 (16). Subgenotype A1 has unique features that are likely to contribute to mutation, disease progression and response to antiviral therapy. This subgenotype is hyperendemic to southern Africa and is associated with a particularly high incidence of liver cancer and cirrhosis (5). Subgenotype A1 has distinctive sequence characteristics within its open reading frames that may affect both replication of the virus and the expression of its proteins. It is believed that these sequence alterations might account for the significantly higher risk of developing hepatocellular cancer (HCC). Because of greater nucleotide divergence and endemic infection compared to other genotypes,

subgenotype A1 is believed to have a very long natural history within the South African black population (16, 17). The unique features of HBV subgenotype A1 and in particular the high risk of developing HCC associated with this subgenotype make, the understanding of HBV replication and development of effective HBV treatment remain important medical priorities.

1.2 Models of HBV replication

HBV has host- and cell-specific requirements that have made development of cell culture systems difficult. Furthermore, effects in cell culture cannot be used reliably to predict effects in animals and especially in man. A major way to overcome this obstacle to the research is to generate an animal models of HBV replication to investigate further the viral life cycle, the development of liver disease (18) and antiviral drug efficacy (19).

1.2.1 *In vitro* model of HBV replication

Passman and colleagues (20) have generated a reporter plasmid of HBV expressing enhanced green fluorescent protein (EGFP) as a marker of HBV replication by replacing the *preS2/S* ORF with DNA encoding EGFP. Transfection of cultured cells with this reporter plasmid was able to express EGFP instead of HBsAg. In addition,

deletion of *preS2/S* also disrupted the polymerase ORF. The highly conserved *HBx* region which overlaps all viral ORFs was not disrupted.

The two major cell culture models of HBV are primary human hepatocytes and hepatoma-derived cell lines. Primary human hepatocytes for infection studies have the advantage that they retain important cellular characteristics of differentiated hepatocytes allowing natural penetration (i.e. infection), and full replication of HBV (18). However, difficulties arise with this system because within several days they lose the capability of supporting HBV replication and culture supernatants from infected cells do not always give similar results (21). In addition, infection of these cells with HBV has poor viral replication and low viral yields. Although there are some difficulties with this model of HBV infection, it has demonstrated infectivity by the virus and may be useful for studying early stages of the viral life cycle (18).

Hepatoma-derived cell lines (e.g HepG 2.2.15) have been generated by the integration viral cccDNA under the control of constitutive or inducible promoters. The advantages of these cell lines are that integration of the complete HBV genome, allows for the expression of HBV RNAs, viral proteins and secretion of virus-like particles (22). The disadvantage of this cell line is that it cannot be used to study HBV-host cell infection processes such as viral attachment, penetration, and uncoating (21).

1.2.2 *In vivo* models of HBV replication

The lack of convenient animal models of HBV infection has stymied the progress of HBV research. Chimpanzees are the only animals fully permissive and well tested for HBV infection (23). Studies performed in chimpanzees played a critical role in the discovery of HBV and continue to play an essential role in defining the natural history of this important human pathogen (23). The discovery of related hepadnaviruses such as woodchuck hepatitis virus (WHV), ground squirrels hepatitis virus (GSHV), Arctic Squirrel hepatitis virus (ASHV) and duck hepatitis B virus (DHBV) offers opportunities for *in vivo* studies in various animals with naturally occurring hepadnaviruses. Recently HBV research has shown progress with the development of various mouse strains carrying HBV transgenes (24). HBV replicating mouse models offer the opportunity to investigate some of the pathogenic mechanisms responsible for HBV associated liver disease, as well as testing specific viral genes for their potential oncogenic function *in vivo* (25).

1.2.2.1 The chimpanzee model

A chimpanzee model of HBV infection is associated with development of acute liver infection (26). Although these animals do not develop chronic liver infection, they are the only primates known to develop a cellular immune response similar to that observed in humans acutely infected with HBV (27). Like humans, when

chimpanzees are immunized with HBsAg, they develop high titers of antibodies to HBsAg. Chimpanzees have played an important role in investigating the mechanism of viral persistence (28) and the development of safe vaccines (29). Due to the limited availability, ethical constraints, the large size of the animals, high cost and lack of chronic disease of chimpanzees, alternative animal models would be desirable.

1.2.2.2 Surrogate animal models

Several non primate HBV-related hepadnaviridae have been identified and studied in their natural hosts. Summer and colleagues (30) were first to discover non primate hepadnaviruses called WHV, which infects only Eastern woodchucks (*Marmota monax*) in the northeastern part of the USA. WHV was found to be closely related to HBV in its structure, genetic organization and mechanism of replication. Natural infection of this animals model is associated with chronic liver disease and HCC (31). Other viruses that have similar properties to WHV include GSHV (32), ASHV (33) as well as viruses infecting ground squirrels, tree squirrels, and three avian species (34). Mammalian hepadnaviruses (in particular WHV) rather than the avian hepadnaviruses are the more often used animal models of HBV infection due to similarity with HBV in terms of genomic organization and effects of persistent infection (23). WHV models have contributed significantly to the understanding of viral replication, chronic infection, and hepatic carcinogenicity of hepadnaviruses. WHV models are not ideal models of HBV infection for various reasons, e.g. these viruses are genetically divergent from HBV, woodchucks are difficult to breed and there is a

lack of immunological reagents to study lymphocyte subsets. Although these animals are usefully to establish proof of principle, immunotherapeutic agents against WHV will not be effective against HBV, and vaccines against HBV cannot be tested in woodchucks. Furthermore, these animals do not develop cirrhosis (18).

1.2.2.3 Rodent models of HBV replication

Despite the availability of permissive animal models (e.g. Chimpanzees) and a good model of antiviral therapy and chronic carriers (e.g. Woodchuck model) there is need to develop a model of HBV infection that permits researchers to study viral replication, gene expression, and immunopathogenesis (35). Recent studies show the development of two groups of HBV-replicating mouse models (i.e. HBV transgenic mice and hydrodynamically injected mice) with the potential to bridge this gap in HBV studies (35).

Transgenic mouse model: Transgenic mice are manipulated by insertion of a gene, which is subsequently expressed in every host cell. HBV-transgenic mice have been generated expressing HBsAg, preS and X antigens (35). This animal model provides a way to study the function of viral proteins and replication of HBV. Although transgenic mice support viral replication and have high levels of HBsAg in circulation, HBV is not directly cytopathic (35). In one of the transgenic mouse studies, researchers demonstrated that high levels of HBx in the liver led to the development

of HCC (25) and that the X protein impairs the function of p53 (37). Another study with transgenic HBV mice indicated that cytotoxic T-lymphocytes (CTLs) may clear HBV by non-cytolytic mechanisms via cytokines (38).

Hydrodynamic injection model: During hydrodynamic tail vein injection, a large volume of DNA-containing saline is injected via the tail vein of mice and causes widening of hepatic sinusoidal fenestrations and creating pores in the plasma membrane of hepatocytes (39), thereby enabling the efficient entry of DNA into cells (40). Yang and Colleagues (36) demonstrated that the hydrodynamic injection of a HBV replication-competent plasmid lead to gene expression and viral replication in mice. In the case of the hydrodynamic injection model of HBV, after injection of the replication-competent plasmid, viral antigens and replicative intermediates are synthesized and virus is secreted into blood. Viral antigens are detectable in the liver tissues and sera of mice. HBsAg and HBeAg may be measured using Enzyme-Linked Immunosorbent Assay (ELISA) and HBcAg by immunohistochemical staining of liver sections. Viral antigens disappear from blood as early as 7 days after injection, after the appearance of antiviral antibodies. HBV transcripts and replication intermediates also disappear from liver by day 15, after the appearance of antiviral CD8+ T cells. It is also thought that this method may be valuable for the characterisation of newly discovered viruses as long as they have the potential to replicate in the liver. In the case of HBV, this methodology may be useful in the study of generation of HBV variants, new models systems to evaluate anti HBV drugs (36).

Although infectious virus can be produced from HBV transgenic mice or mice hydrodynamically injected, a limitation of the model is that mouse hepatocytes are not permissive for HBV infection. Therefore, the still unknown early steps in viral infection, such as receptor recognition and mechanism of entry, cannot be addressed in these systems (23).

1.3 Treatment of HBV Infection

Despite the availability of an effective vaccine against HBV, the prevalence of chronic infection has not significantly decreased (8). Twenty-five percent of patients chronically infected with HBV are at risk of developing cirrhosis and primary (41). The development of potential antiviral treatment aims to prevent such an undesirable outcome. Currently HBV therapy consists of treatment with interferon alpha (IFN-α), an immunomodulator, the nucleoside analogue lamivudine and the nucleotide analogue adefovir dipivoxil. The standard treatment with these drugs results in a loss of HBeAg with or without seroconversion to anti-HBe, normalisation of serum transaminase levels, loss of HBV DNA and improvement in liver histology (42). IFN-α was approved by the United States of America Food and Drug Administration (FDA) for the treatment of chronic HBV infection in 1992 (43). Although, IFN-α is licensed as an antiviral agent for treatment of chronic HBV infection its use has numerous disadvantages, e.g. expense, poorly toleration (15) and association with several undesirable side effects including: fever, myalgias and depression (44)**.** Also, IFN-α is

only effective in carriers with actively replicating virus (i.e. HBeAg positive carriers) (45). Nucleoside analogues (such as lamivudine) are chemically synthesized molecules that are structurally similar to naturally occurring nucleosides. Lamivudine was approved by the FDA in 1998 for the treatment of chronic HBV infection. Since lamivudine structurally mimics natural nucleosides it can be incorporated into newly reverse transcribed HBV DNA causing chain termination thereby inhibiting viral replication (42). Lamivudine has been shown to be more effective in HBeAg-negative carriers than HBeAg-positive carriers during 12 months treatment (46). The emergence of drug resistant strains of HBV against lamivudine indicates a major drawback of the use of this drug. Resistant HBV strains harbor point mutations in the HBV polymerase gene, predominantly in the well conserved YMDD (Tyrosine-methionine-aspartate-Aspartate) motif, such that the methionine (M) at position 552 is changed either to valine (YVDD) or isoleucine (YIDD) (47). Adefovir dipivoxil (a nucleotide analogue) was approved by the FDA in 2002 for the treatment of chronic HBV infection. However, adefovir dipivoxil acts as a stimulant for natural killer cell activity and induces endogenous interferon production (42).

Only 30 to 40 percent of HBV patients respond to treatment with INF-α, lamivudine, and adefovir dipivoxil (43). Therefore, there is an urgent need to develop novel antiviral therapy for treating chronic HBV infection. Currently, molecular approaches, such as the use of RNA interference (RNAi) have become a major focus of developing new therapeutic strategies against HBV (48). RNAi is a gene silencing

mechanism conserved in all eukaryotes, in which double-stranded RNAs suppress the expression of a cognate gene inducing degradation of mRNAs or blocking mRNA translation (49-51).

1.4 RNA interference

RNAi is a novel regulatory mechanism that limits gene expression by mediating sequence-specific degradation or translational repression of mRNA (posttranscriptional gene silencing or PTGS) or by suppressing transcription (transcriptional gene silencing or TGS) (50).

RNAi was originally discovered in 1998 when Fire and colleagues (52) found that the injection of dsRNA into the nematode worm *Caenorhabditis elegans* led to efficient sequence-specific gene silencing. RNAi has been observed in nematodes, plants, fungi, vertebrates and mammals and appears to be present in almost all eukaryotic organisms.

1.4.1 The RNAi pathway

Exploiting the RNAi pathway has shown exciting promise for the development of novel antiviral therapy. Naturally, the mechanism involves processing of larger dsRNAs to ultimately form RNA duplexes (small interfering RNAs or siRNAs) that are

21-22 nucleotides (nt) in length with symmetric 2-3 nt 3' overhangs and 5'-phosphate and 3'-hydroxyl groups (Figure 1.3) (53). Dicer, which belongs to a family of RNase III enzymes, first cleaves long dsRNAs to produce siRNAs (54)**.** The second step involves incorporation of siRNAs into the multiprotein RNA-inducible silencing complex (RISC). The duplex siRNA is unwound, leaving the antisense strand to guide RISC to its homologous target mRNA for endonucleolytic cleavage (55)**.** Finally, the targeted mRNA is cleaved at a single site in the centre of duplex region between the guide siRNA and the targeted mRNA, approximately 10 nt from the 5' end of the siRNA (56).

Mutations in genes encoding RNA-dependent RNA polymerases (RdRP) have been shown to be deleterious highlighting the role these genes play in the spread of RNAi throughout plants and *C. elegans* as well as between generations in *C. elegans*. The mechanism has been elucidated (57). Using a siRNA as a primer and its target as the template, the RdRP generates dsRNA which is recognised by Dicer and cleaved to form secondary siRNAs (or new siRNAs). The new siRNAs may be incorporated into RISC to function as guides and mediate gene silencing by mRNA degradation or re-enter the so-called random degradative PCR pathway (58).

Recent studies revealed that the natural RNAi pathway employs endogenously encoded small RNAs (microRNAs or miRNAs) to regulate gene expression (59). miRNAs are small RNAs processed from double stranded hairpin structures that are

encoded in the genome. miRNAs have been found to be abundant in plants, humans, *Drosophila melanogaster* and *C. elegans*, and are known to be involved in numerous aspects of gene expression (60, 61). Like siRNAs, miRNAs may mediate gene suppression by mRNA degradation when they are completely complementary to their target site. However, miRNAs are usually only partially complementary to sites within the 3' untranslated region (UTR) of the target mRNA, resulting in repression of translation and inhibition of protein synthesis (62). siRNAs that are designed to be partially complementary to their target sites have also been shown to inhibit gene expression by translational suppression.

1.4.2 Delivery of RNAi-inducing sequences

Inhibition of gene expression using the RNAi pathway is rapidly becoming the method of choice for studying gene function in human hepatoma cell line. However, successful knockdown of the target gene requires efficient delivery of siRNAs. Several technologies have been developed that enable effective delivery of siRNAs to both cells in culture and mouse models of HBV replication (63).

Delivery of siRNA or short hairpin RNA (shRNA): A number of transfection reagents are being employed for transfection of siRNAs or shRNAs into different cell lines. Lipofectamine 2000, liposomes, Oligofectamine and adenoviruses are routinely used

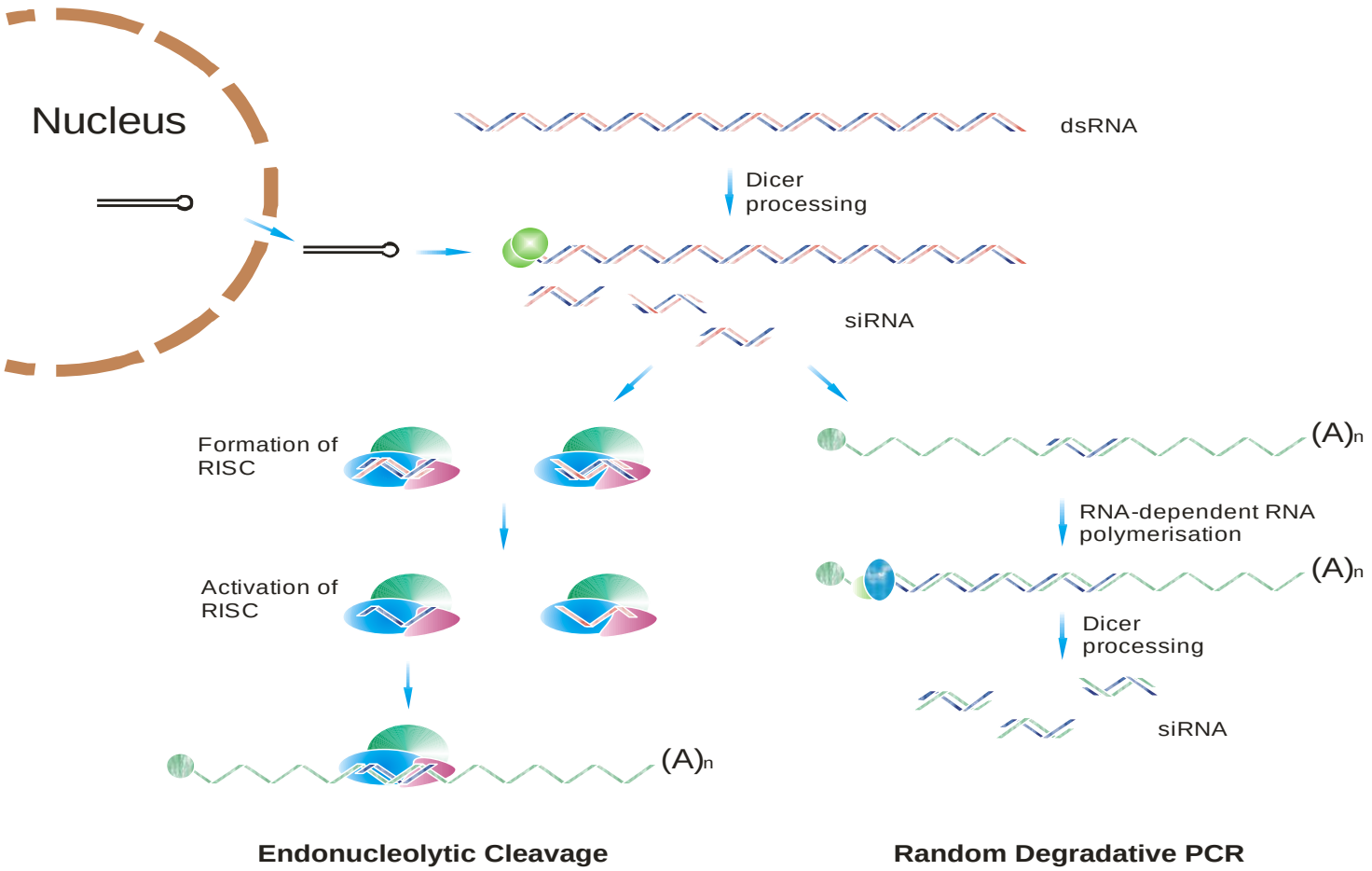


Figure 1.3: Schematic representation of the RNAi pathway. Long dsRNA is recognised and processed by Dicer, an RNase III enzyme. Synthetic siRNA or endodgenous siRNA are incorporated into RISC and guide the protein complex to target mRNA for endonucleolytic cleavage. In plants and worms (*C. elegans*) the siRNA may act as primers for an RdRP to generate nascent dsRNA, which in turn is processed by Dicer to produce secondary siRNA.

for siRNA delivery. Lipoplexes and eletroporation has been used successfully to introduce siRNAs or shRNAs into cell lines as well as into parasites (48, 64, 65).

siRNA expression vector: An alternative to the use of synthesised siRNAs to induce RNAi is to use a vector that expresses siRNAs in the cell. RNA polymerase III (PoI III) promoters are commonly used to drive expression of shRNAs that are processed into siRNAs. Pol III promoters have the ability to initiate and terminate RNA transcription at well defined positions, which allows for the design of precisely defined constructs. The Pol III promoters used most often for shRNA expression are the human and mouse U6 promoters and the human H1 promoter(63).

1.4.3 RNAi-mediated inhibition of HBV

Several studies of siRNA sequences against HBV have shown significant inhibition of gene expression and replication. Efficacy of several different siRNA sequences has been assessed in cultured cells as well as murine models of HBV replication (9).

McCaffrey and colleagues (66) assessed the efficacy of the human U6 promoter cassettes that encoded shRNA sequences. The six U6 shRNA cassettes were targeted to different HBV ORFs (viz. *polymerase*, *core*, *surface* and *HBx* ORFs). Of these RNAi inducers, shRNA sequences targeted to the *surface* and *polymerase* ORF demonstrated impressive inhibition of markers of HBV gene expression *in vitro*

and *in vivo.* Giladi and colleagues (67) assessed the efficacy of five synthetic siRNAs that targeted different sites within the *surface* ORF of HBV. The siRNA that targeted sequence 9-27 nt downstream of the ATG translation start site was found to be most effective. HBsAg, HBeAg and the production of HBV RNA from transiently transfected HepG2 and HepG2.2.15 as well as in the hydrodynamically injected mice was significantly knocked down by this siRNA. In the same study, HBsAg and HBcAg expression in liver sections were strongly decreased by this synthetic siRNA. Carmona and colleagues (48) assessed the efficacy of a panel of 10 Pol III U6 promoter-encoded shRNAs that target conserved sequences of the oncogenic *HBx* ORF. The *HBx* ORF was chosen as the target region as it is common to all HBV transcripts. The two shRNAs which exhibited the highest level of knockdown *in vitro* were shown to be equally effective *in vivo*. Transfection of shRNAs into Huh7 cells markedly diminished the HBsAg secretion in culture supernatants by 80-100%. Approximately 100% of HBsAg, HBcAg, and *in vivo* viral particle equivalents were diminished in mice hydrodynamically injected with an HBVreplication-competent plasmid and a shRNA cassette. In the same study, significant knockdown was observed in transgenic mice of HBV treated with U6 shRNA 5 and U6 shRNA 6 delivered using recombinant adenoviral vectors. Ying and colleagues (64) assessed the effects of three anti HBV 21-bp shRNAs targeted to the *HBsAg*, *HBcAg* and *polymerase* ORFs. HBsAg secretion was reduced by 80% in cell culture and approximately 90% *in vivo*. HBeAg secretion was also significantly inhibited after treatment with RNAi effecters. Analysis of HBsAg and HBcAg expression in liver

sections also revealed substantial reduction in these proteins in mice treated with shRNAs. These shRNAs also showed a significant inhibition in the levels of HBV mRNA, as measured by reverse transcriptase polymerase chain reaction (RT-PCR) and HBV DNA (as detected by southern blot). The shRNA targeted to the surface region was a more effective inhibitor of HBV antigen secretion in these studies (63).

1.5 Aims

This study is aimed at developing target vectors as a tool for studying gene function, and assessing exploitation of the RNAi pathway to inhibit HBV gene expression. These will be achieved by:

(a). Constructing a HBV reporter vector. The vector generated should expresses Firefly luciferase as a marker of HBV replication in order to allow for accurate quantification of HBV viral replication.

(b). Constructing a HBV replication-competent plasmid of subgenotype A1. The vector should a greater than genome length of HBV subgenotype A1.

(c). Assessing the efficacy of RNAi effecters against these vectors both *in vitro* and *in vivo*. The ability of effecters of RNAi to mediate inhibition of HBV replication was to be assessed in the human hepatoma cell line, Huh7 and in a mouse model of HBV replication.

This study used three U6 shRNA (U6 shRNA 5, U6 shRNA 6 and U6 shRNA 10) previously described by Carmona and collogues (48) to target the viral *HBx* ORF. The HBV genome has four primary ORFs which have a common polyadenylation signal immediately downstream of the *HBx* ORF. The *HBx* ORF is thus common to all transcripts and RNAi effecters targeted to the *HBx* ORF would therefore act against all viral transcripts. HBx also plays an important role in the establishment of a successful viral infection and replication *in vivo*. These factors make the *HBx* ORF an ideal therapeutic target.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Target vector and reporter plasmid

2.1.1 Construction of pCH-FLuc

The plasmid pCH-9/3091 (68) contains a greater than genome length sequence of HBV DNA cloned downstream of the CMV promoter, allowing the plasmid to simulate viral replication when introduced into cells. Replacing the *surface* ORF with the sequence encoding Firefly luciferase would generate a plasmid expressing Firefly luciferase as a marker of HBV replication.

The reporter plasmid was generated using a PCR-based approach. The primers used (Inqaba Biotech, SA) were designed such that the amplicons generated contained a *Spe* I restriction site upstream and an inframe *Xho* I restriction site downstream of *Firefly luciferase*. The *Firefly luciferase* sequence was amplified by PCR (PCR Master Mix; Promega, WI, USA) from pCI-neo-Luc (pCI-neo containing the Firefly luciferase sequence) using the primer set in Table 2.1. PCR reaction conditions were as follows: initial denaturation at 95^{o}C for 2 minutes, followed by 30 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds and extension at 72^{o}C for 1 minute; a final extension step was carried out at 72^{o}C for 2 minutes.

The amplicons were subjected to agarose gel electrophoresis on a 0.8% gel, eluted and purified using the MinElute™ Gel Extraction Kit (Qiagen, CA, USA). The purified fragments were ligated into the pTZ57R/T cloning vector according to the manufacturers' instructions (Fermentas, WI, USA) (Figure 2.1A). Briefly, the fragments were ligated at 22^{o}C overnight in a 30 µl reaction volume containing T4 DNA ligase and ligase buffer. Ten microlitres of ligation mix was used to transform 50 µl of chemically competent *Escherichia coli* (*E. coli)* (DH5α, Invitrogen, CA, USA). For preparation and transformation of chemically competent *E.coli* see Appendix 6.1.1. The transformed *E. coli* were plated on Luria Bertani amplicillin-containing agar plates (see appendix 6.1.1) and incubated at 37^{o}C overnight. White colonies were selected and screened after restriction enzyme digestion with *Xho* I and *Spe* I.

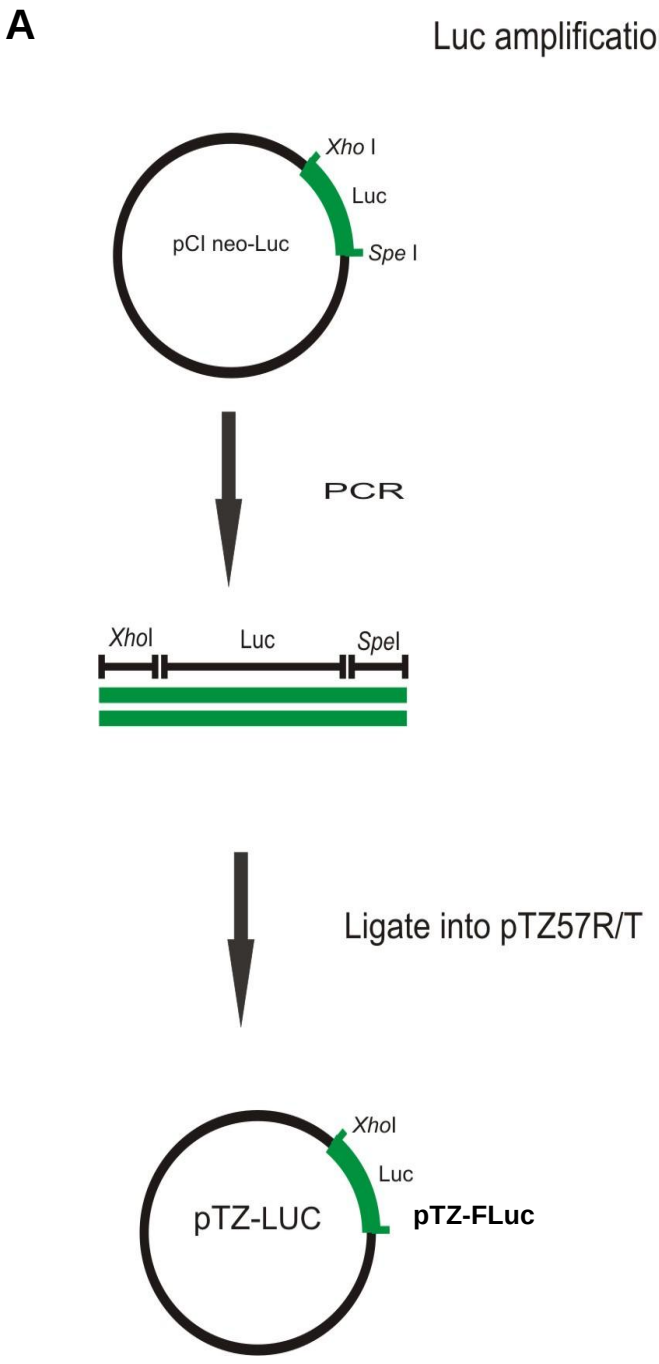
To incorporate the DNA sequence encoding Firefly luciferase into pCH-9/3091, pTZ-FLuc was restricted with *Xho* I and *Spe* I (Figure 2.1B). The digested fragments were subjected to agarose gel electrophoresis and the desired fragments eluted using the MinElute™ Gel Extraction Kit. pCH-9/3091 was digested with *Xho* I resulting linearised product. The linearised pCH-9/3091 was partially digested with *Spe* I in buffer Tango 10x (Fermentas, WI, USA) at 37^{o}C for 30 minutes (Table 2.2). The pCH-9/3091 backbone lacking the *surface* ORF was eluted from 0.8% agarose gel using standard phenol chloroform purification procedures. A ligation reaction was set up with 3 µg of the *Firefly Luciferase* fragment together with 1 µg of the pCH-9/3091 backbone in a 30 µl reaction volume containing T4 DNA ligase (Fermentas, WI, USA)

(Figure 2.1). Purified PCR product was quantified spectrophotometrically (Shimadzu Corporation, Japan) at 260 nm, A_{260} of 1.0 = 50 µg/ml DNA. The ligation mixture was incubated at 22^{o}C overnight. Chemically competent *E. coli* (DH5α) were transformed with 10 µl of the ligation mix, plated on ampicillin positive Luria Bertani agar plates and incubated at 37^{o}C overnight (Appendix 6.1.1). Clones positive for single unit insert (pCH-FLuc) were screened with *Xho* I and *Spe* I.

2.1.2 Construction of pCR-HBV A1 1.3x

The plasmid pCR-XL-TOPO-HBV (pCR-HBVA1 1x), kindly provided by Prof. Anna Kramvis, Molecular Hepatology Research Unit (MHRU), Department of Medicine, University of the Witwatersrand, was generated by cloning the full length genome of a subgenotype A1 isolate from a South African HBV chronic carrier.

The target plasmid pCR-HBVA1 1.3x was also generated using a PCR based approach (Figure 2.2). The primers (Integrated DNA Technologies, IA, USA) were designed such that the amplicons generated contain a *Hind* III site upstream and a *Pci* I site downstream of the Basic Core Promoter (BCP), and an *Fsp* I site upstream and an *Apa* I site downstream of the polyadenylation signal (pA) (Table 2.2). The sequences encoding the BCP and pA were amplified by PCR from pCR-HBVA1 1x.


A
Luc amplification
Xho I
Luc
pCI neo-Luc
Spe I
PCR
XhoI
Luc
SpeI
Ligate into pTZ57R/T
XhoI
Luc
pTZ-LUC
pTZ-FLuc

B

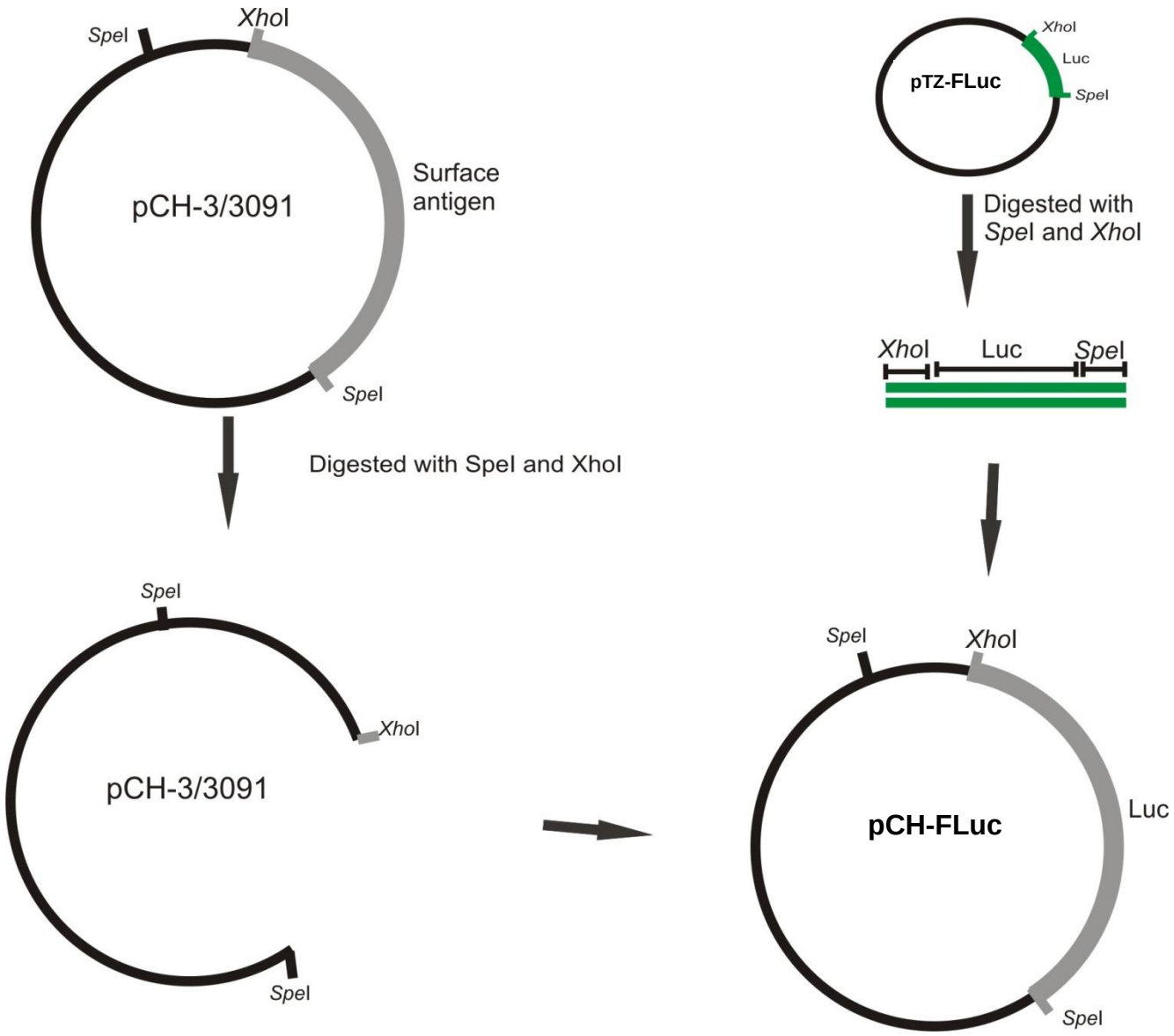


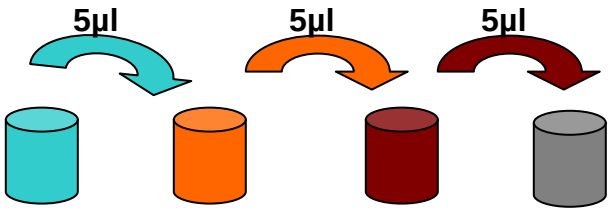
Figure 2.1 Diagrammatic illustration of cloning strategy for the generation of the HBV plasmid expressing Firefly Luciferase (pCH-FLuc) as a marker of HBV replication. (A) Construction of pTZ-FLuc, and (B) Construction of the pCH-FLuc reporter plasmid.

Table 2.1: Oligonucleotide sequences for the generation of Firefly Luciferase fragment.

Luciferase (Forward)	5' - ACTGCTCGAGGATTGGGGACCCTGCGCTGAACATGGAAGACGCCAAAAAC -3'
Luciferase (Reverse)	5'- ACTGACTAGTTTACACGGCATCTTTCC -3'

Restriction sites *Xho* I and *Spe* I in the forward and reverse primers respectively are underlined

Table 2.2: Serial dilution used to perform partial digest with *Spe* I from linearised pCH-9-3091 product (pCH-9/3091 digested with *Xho* I).



	Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
pCH-9/3091 (*Xho* I)	10µl	10 µl	10 µl	5 µl
Buffer Tango 10x	4 µl	2µl	2µl	2µl
Spe I	1µl	-	-	-
Sterile H_20	5µl	3µl	3µl	3µl
Total volume	**15µl**	**15µl**	**15µl**	**15µl**

Table 2.3 Oligonucleotide sequence used to amplify BCP and pA fragments from pCR-HBVa1 1x.

PA(Forward)	5'- GATCTGCGCACCATCATCATGCAACTTTTTCACCTCTGCCTAATC - 3'
PA(Reverse)	5'- GATCGGGCCCGGACTGAAGGAAAGAAGTCAG - 3'
BCP(Forward)	5'- GATCAAGCTTATGTACAAGCGAAACAGGC - 3'
BCP(Reverse)	5'- GATCACATGTACAAGAGATGATTAGGCGAGAGGTGAAAGTTGCAT GATGATGATGGTTGCGCA - 3'

Restriction sites are underlined. *Fsp* I and *Apa* I in forward and reverse pA primers, respectively and *Hind* III and *Pci* I in the forward and reverse BCP primers, respectively.

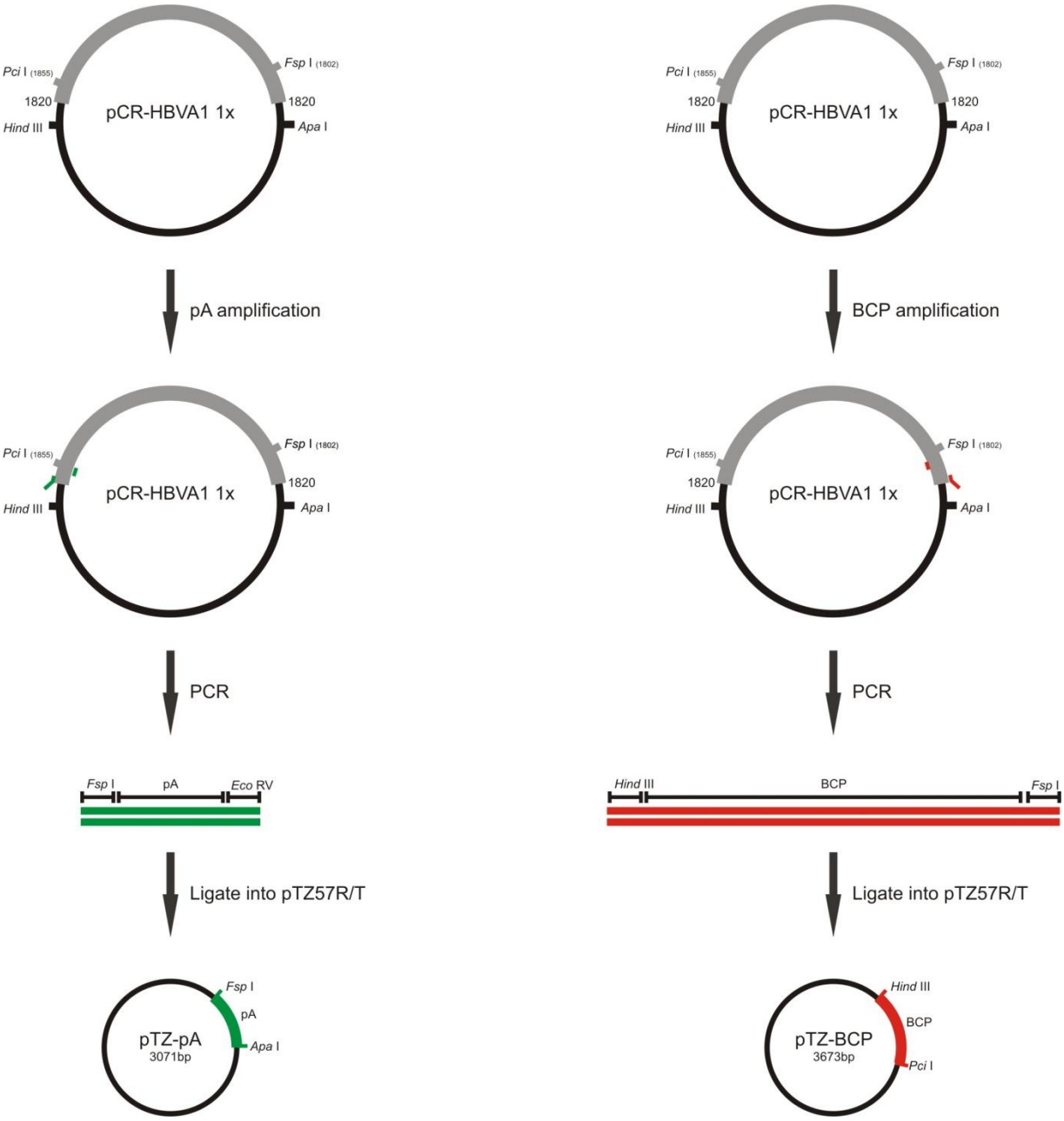
PCR reaction conditions were as follows: initial denaturation at 95^{o}C for 2 minutes, followed by 30 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds and extension at 72^{o}C for 1 minute, final extension step was carried out at 72^{o}C for 2 minutes.

The amplicons were subjected to agarose gel electrophoresis and purified using the MinElute™ gel Extraction Kit. The purified fragments were ligated into the pTZ57R/T cloning vector according to the manufacturer's instructions (Figure 2.2 A). Ten microlitres of ligation mix was used to transform 50 µl of chemically competent *E. coli*. The transformed *E. coli* were plated on Luria Bertani ampicillin containing agar plates. Colonies were selected and screened by restriction enzyme digestion with *Hind* III and *Pci* I, *Fsp* I and *Apa* I for BCP and pA sequences, respectively.

The BCP and pA sequences were cloned upstream and downstream of the full length subgenotype A1 sequence, respectively to generate the replication competent plasmid pCR-HBVA1 1.3x (Figure 2.2 B). The sequence encoding the BCP was removed from pTZ-BCP with *Hind* III and *Pci* I. Similarly the pA signal was also removed from pTZ-pA with *Fsp* I and *Apa* I. pCR-HBVa 1x was digested separately; first with *Hind* III an *Apa* I to yield the pCR-XL-Topo backbone and then with *Pci* I and *Fsp* I to yield the HBV subgenotype A1 fragment. A Four-way ligation reaction was set up with the purified BCP, pA, HBVa1 and pCR-XL-Topo fragments in a 20 µl reaction volume containing T4 DNA ligase. The ligation mixture was incubated at 25^{o}C overnight. Chemically competent bacterial strain (DH5α) were transformed with 10 µl of the ligation mix, then plated on Kanamycin positive Luria Bertani agar plates and incubated at 37^{o}C overnight (Appendix 6.1.1). Clones positive for single unit insert (pCR-HBVA1 1.3x) were screened with *Hind* III and *Pci* I as well as *Fsp* I and *Apa* I restriction enzymes.

2.2 Construction of short hairpin RNA expression cassettes

Construction of the short hairpin (shRNA) expression cassettes has been previously described (48). Briefly, the sequences of local HBV isolates were aligned with the Bioinformatics programme, GeneDoc and the most conserved region of the *HBx* ORF chosen as target sites. shRNA expression cassettes were then designed to target the most conserved regions of the *HBx* ORF. The shRNA were designed with 25 nt stems


Pci I (1855)
Fsp I (1802)
1820
1820
Hind III
Apa I
pCR-HBVA1 1x
pA amplification
PCR
Fsp I
pA
Eco RV
Ligate into pTZ57R/T
pTZ-pA
3071bp
Fsp I
pA
Apa I
BCP amplification
PCR
Hind III
BCP
Fsp I
Ligate into pTZ57R/T
pTZ-BCP
3673bp
Hind III
BCP
Pci I

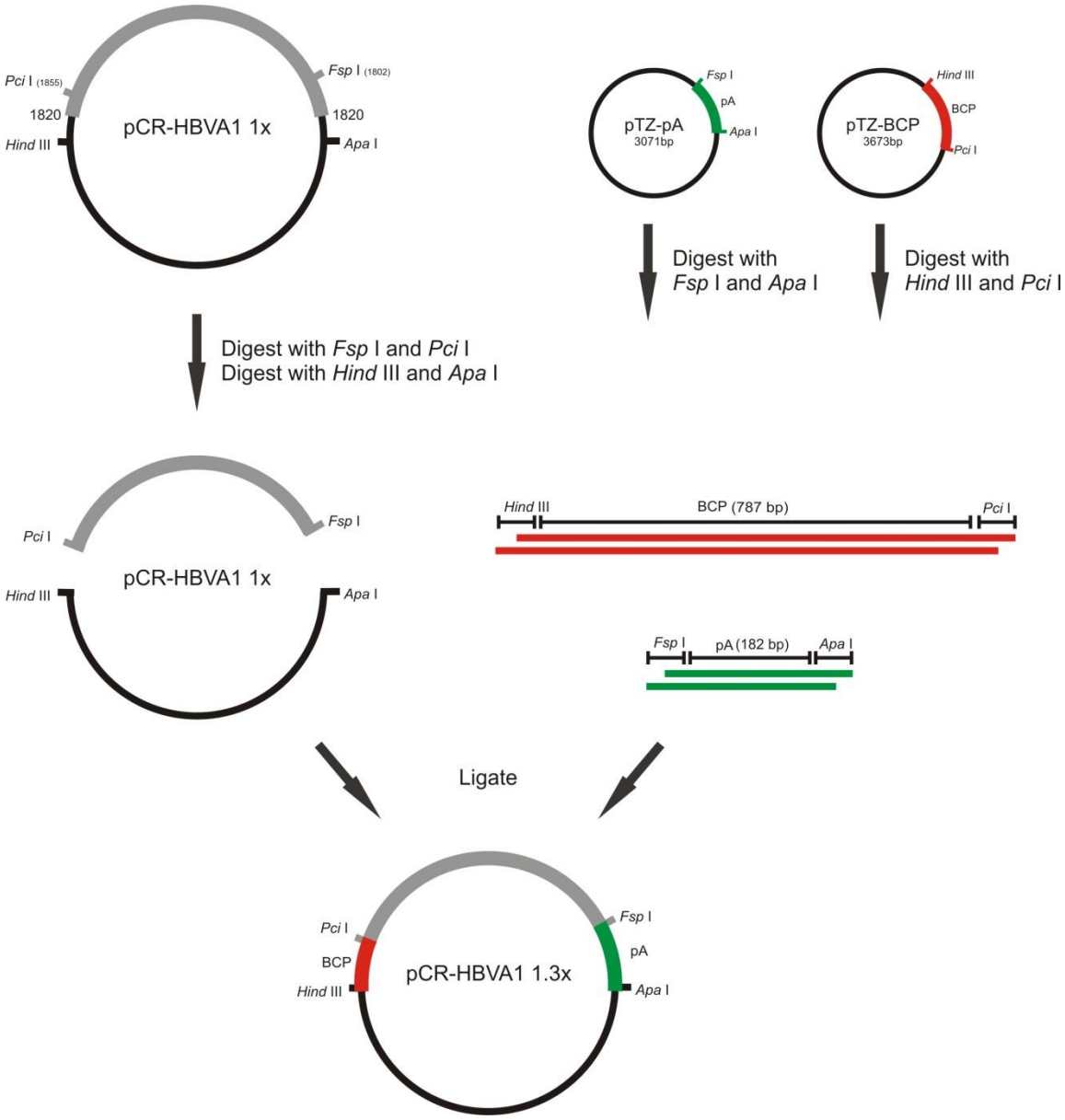


Figure 2.2 Diagrammatic illustration of cloning strategy for the generation of HBV replication competent plasmid (pCR-HBVA1 1.3x) containing a greater than genome length sequence of the viral A1 subgenotype.

containing G:U wobble bases and loop sequence of miRNA-23. The expression cassettes were constructed in accordance with the PCR-based techniques develop by Castanotto and colleagues (69).

2.3 DNA extraction

2.3.1 Isolation of plasmid DNA

All plasmids used in this study are listed in Table 2.4. Plasmid DNA was isolated using EndoFree® Plasmid Maxi kit (Qaigen, GmbH, Germany). Two hundred and fifty milliliters of Luria Bertani medium (Appendix 6.1.1) was inoculated with a single colony containing the plasmid of interest and incubated at 37°C with shaking (100 rpm) overnight. The plasmid DNA was isolated according to the manufacturer's protocol (Appendix 6.1.2). The quality of plasmid DNA was assessed by agarose gel electrophoresis (Appendix 6.1.3). The quantity of plasmid DNA was also determined spectrophotometrically (Shimadzu Corporation, Japan).

2.4 Characterisation of HBV plasmids

2.4.1 Characterisation of HBV vectors in cultured cells

2.4.1.1 Transfections

Human hepatoma cells, Huh7, were maintained in RPMI supplemented with 2.5% foetal calf serum (FCS), penicillin (50 IU/ml) and streptomycin (50 µg/ml) (Gibco BRL, UK). On the day prior to transfection, 150 000 Huh7 cells (counted using haemocytometer) were seeded in wells 1 cm in diameter. To assess the expression from pCH-FLuc and pCR-HBVA1 1.3x plasmids, Huh7 cells were transfected with 1.7 µg of the respective HBV vector. The plasmid DNA was transfected into Huh7 cells as per Lipofectamine 2000 (Invitrogen, CA, USA) protocol (Appendix 6.1.4).

2.4.1.2 Assays for markers of HBV replication

Luciferase activity

To assess the luciferase activity of pCH-FLuc, Huh7 cells were transfected with 1.7 µg of pCH-FLuc and 0.2 µg pCMV-GFP (a vector encoding enhanced green fluorescent protein). Forty eight hours post-transfection cells were analysed for EGFP expression by Confocal Microscopy (Axiovert 100M, Zeiss, Germany) under a FITC filter. Lysates were then prepared from transfected cells as per luciferase protocol

(Appendix 6.1.5) and then analysed by Dual Luciferase® Reporter Assay System (Promega, WI, USA) using the Veritas Microplate luminometer (Appendix 6.1.5).

HBsAg secretion

Forty eight hours post-transfection the medium was collected from cells, to assess the replication competency of pCR-HBVA1 1.3x. The cells were transfected with 1.7 µg of pCH-9/3091 (control replication competent plasmid), or pCR-HBVA1 1x (HBV plasmid that is not replication competent) or pCR-HBVA1 1.3x. An additional 0.2 µg of pCMV-GFP was added for the assessment of transfection efficiency. HBsAg secretion into the culture supernatants was measured using the Monolisa® Ag HBs Plus immunoassay kit (Bio-Rad, CA, USA). Briefly, 100 µl of the cultured supernatants was incubated with an immobilised monoclonal HBsAg specific antibody for an hour at 37^{o}C. After the hour incubation the plate was washed and developing solution added. The plate was allowed to develop at room temperature in the dark for 30 minutes. After the 30 minutes incubation stopping solution was added and the ratio of the optical density at 450nm and 690 nm measured using Bio-Rad's Model 680 Microplate Reader (Bio-Rad, CA, USA).

2.4.2 Characterisation of pCR-HBVA1 1.3x targeted vector *in vivo*

2.4.2.1 Hydrodynamic tail vein injections

In vivo studies were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee (see last page of Appendix). Six to eight week old female mice were used for the experiments. To assess the replication competent plasmid pCR-HBVA1 1.3x, a mixture of 5 µg plasmid and saline comprising 10% of the mouse's body mass was injected via the tail vein over 5 to 10 seconds. Saline solutions containing three plasmid vectors pCR-HBVA1 1.3x, pCH-9/3091 or pCR-HBVA1 1x were injected into mice.

The mice were bled on days 2 and 5 post-injection. Blood was collected by the retro-orbital bleeding method. The blood was centrifuged at 3000 rpm at room temperature for 5 minutes and serum collected. Animals were sacrificed on day 5 post-injection, and the livers harvested for RNA extraction.

2.4.2.2 HBsAg and HBeAg secretion

Serum collected from mice at days 2 and 5 post-injection were analysed for HBsAg and HBeAg secretion. One hundred microlitres were used to quantify the amount of HBsAg and HBeAg secreted into mice sera. HBsAg levels were measured using the Monolisa® Ag HBs Plus immunoassay kit as described in Section 2.2.1.2. HBeAg

levels were measured using Axsym (Elisa) immunoassay Kits. The serology Unit of the National Health Laboratory Service (NHLS), Johannesburg, South Africa performed the HBeAg analysis.

2.4.2.3 Viral particle equivalents

One hundred microlitres of serum collected at days 2 and 5 post-injection were used to detect the HBV DNA levels (viral particle equivalents). Viral particles were extracted from serum on the MagNA Pure LC using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany). PCRs were carried out on the extracted samples using the Roche Lightcycler 2.0 (Roche Diagnostics, Mannheim, Germany). Thermal cycling parameters consisted of a hot start (30 seconds at 95^{o}C) followed by 50 cycles of PCR (denaturation at 95^{o}C for 5 seconds), annealing at 58^{o}C for 10 seconds and extension at 72^{o}C for 10 seconds, and finally melting curve analysis (95^{o}C for 30 seconds, 65^{o}C for 30 seconds, and 95^{o}C at 0.2^{o}C/second ramp rate. Viral particle equivalents (VPEs) were determined using crossing point analysis with Roche software. VPEs were calculated using the WHO International Standards for quantitation of HBV DNA for Nucleic Acid Amplification Technology (NAT) assays (National Institute for Biological Standards and Control, UK).

2.4.2.4 HBV mRNA measurement

To measure the concentrations of HBV mRNA, total RNA was isolated from liver tissues (Appendix 6.1.7) and reverse transcribed using Sensiscript Reverse Transcription Kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. To amplify HBV *core*, *surface* and housekeeping *Glyceraldehyde-3-*

phosphate dehydrogenase (GAPDH) cDNA, the procedures and primers combinations described by Song and colleagues (70) were used (Table 2.4). All real-time PCR was carried out using the Roche Lightcycler 2.0. Controls included water blanks and RNA extracts that were not subjected to reverse transcription. Taq readymix with SYBR green (Sigma, MO, USA) was used to amplify and detect DNA during the reaction. Thermal cycling parameters were the same as for viral particle equivalents (Section 2.2.2.3). Specificity of the PCR products was verified by melting curve analysis. Relative quantitation analysis was performed by determining the ratio of HBV RNA to GAPDH RNA.

Table 2.4 Primer sequences for *surface*, *core* and *GAPDH* mRNA

Surface(Forward)	5’ - TGCACCTGTATTCCCATC - 3’
Surface(Reverse)	5’ - CTGAAAGCCAAACAGTGG - 3’
Core(Forward)	5’ - ACCACCAAATGCCCCTAT- 3’
Core(Reverse)	5’ - TTCTGCGACGCGGCGA - 3’
GAPDH(Forward)	5’ - GAAGGTGAAGGTCGGAGTC -3’
GAPDH(Reverse)	5’ - GAAGATGGTGATGGGATTTC - 3’

2.5 Assessing efficacy of U6 shRNA cassettes against HBV

2.5.1 Assessing efficacy of U6 shRNA in cultured mammalian cells

2.5.1.1 Transfections

Cell culture and transfections of Huh7 cells were carried out as described in Section 2.2.1.1. To assess efficacy of U6 shRNA cassettes against the reporter plasmid, 0.4 µg, 0.2 µg and 0.16 µg of pCH-FLuc was co-transfected with 0.9 µg, 1.1 µg and 1.14 µg of U6 shRNA vectors. Addition of 0.2 µg phRL-CMV (Promega, WI, USA) allowed to correct for transfection efficiency. Efficacy of the shRNA expression cassettes against the replication-competent plasmid (as measured by HBsAg and HBV mRNA quantitation) was assessed by transfecting 0.25 µg of pCR-HBVA1 1.3x with 0.6 µg of U6 shRNA vectors. Addition of 0.2 µg of pCMV-GFP allowed for transfection efficiency to be determined.

2.5.1.2 Assay for markers of viral replication *in vitro*

Luciferase Assays

Forty eight hours post-transfection cell lysate was prepared from cells transfected with pCH-FLuc and shRNA vectors. Luciferase activity was measured using the Dual

Luciferase® Reporter Assay System as described in Section 2.2.1.2 and Appendix 6.1.5.

HBsAg and relative HBV mRNA quantitation

Forty eight hours post-transfection, 100 µl of culture medium was collected from cells cotransfected with pCR-HBVA1 1.3x and shRNA expression vectors and analysed for HBsAg secretion. Levels HBsAg was measured by ELISA as described previously (Section 2.2.1.2). Total RNA (Appendix 6.1.6.1) was isolated from cells to assess the level of HBV *core* and *surface* mRNA as well as *GAPDH* mRNA. The level of HBV mRNAs were analysed using the Roche Lightcycler 2.0 as described in Section 2.4.2.4.

2.5.2 Assessing efficacy of U6 shRNA *in vivo*

2.5.2.1 Murine hydrodynamic injections

To assess efficacy of U6 shRNA cassettes against HBV *in vivo*, six to eight week old female mice were used. A saline solution comprising 10% of the mouse's body mass was injected via the tail vein over a period of 5 to 10 seconds. The saline solution contained a combination of three plasmid vectors: 5 µg target DNA (pCR-HBVA1 1.3x), 5 µg anti HBV sequence (U6 shRNA encoding plasmid) and pCMV-GFP (for

delivery efficacy). The mice were bled on days 3, 5 and 7 post-injection. Serum was collected as described in section 2.2.2.1. Animals were sacrificed on day 7 post-injection and their livers harvested for RNA extraction and immunohistochemical staining. Animal protocols were performed in accordance with the University of Witwatersrand Animals Ethics Screening Committee.

2.5.2.2 Assay for markers of viral replication *in vivo*

HBsAg and HBeAg secretions

Serum collected at days 2, 5, and 7 after hydrodynamic tail vein injection were analysed for HBeAg and HBsAg using Axysym (ELISA) immunoassay kit and Monolisa® (Elisa) immunoassay kit, respectively. The serology unit of NHLS, Johannesburg, South Africa performed the HBeAg analysis.

qPCR

RNA was extracted from the liver of injected mice (Appendix 6.1.7 for extraction of RNA) at time of sacrifice. HBV mRNA expression (*core* and *surface* mRNA) was analysed from total RNA using the Roche Lightcycler 2.0 as described in Section 2.2.2.4.

Immunohistochemistry

Approximately 4 g of liver was harvested and fixed for intrahepatic staining for HBcAg positive cells (Appendix 6.1.8). Cells positive for HBcAg stain brown as observed by Light Microscopy.

Statistical Analysis

Analysis of statistically significant differences was carried out using the student's paired two tailed t-test with the GraphPad Prism 4 software (GraphPad software, Inc., CA, USA)

Table 2.5: Plasmids used in this study for transfection and injection

Plasmid	Description	Reference/source
pCH-FLuc	HBV plasmid surface ORF replaced by Firefly Luciferase encoding sequence	Current study
pCMV-GFP	Plasmid vector encoding Green Florence protein	(20)
pCH-9/3091	Control HBV replication-competent plasmid	(68)
pCR-HBVa1 1x	HBVA1 non replication-competent plasmid	MHRU Lab
pCR-HBVa1 1.3x	HBVA1 non replication-competent plasmid	Current study
phRL-CMV	Plasmid encoding *Renilla* luciferase	Promega, WI, USA
pG-U6 shRNA 5	Plasmid encoding shRNA 5 sequences under transcriptional control of the U6 promoter	(48)
pG-U6 shRNA 6	Plasmid encoding shRNA 6 sequences under transcriptional control of the U6 promoter	(48)
pG-U6 shRNA 10	Plasmid encoding shRNA 10 sequences under transcriptional control of the U6 promoter	(48)

CHAPTER 3

3 RESULTS

3.1 Generation of plasmid vectors of HBV

3.1.1 Generation of a plasmid vector (pCH-FLuc) expressing Firefly luciferase as a marker of HBV replication.

To generate a vector expressing Firefly luciferase as a marker of HBV replication, the preS2/S ORF of pCH-9/3091 was replaced with the sequence encoding Firefly luciferase (Figure 2.1), leaving all other ORFs intact. First, the *Firefly luciferase* sequence (1650 bp) was amplified from pCI-FLuc by PCR (Figure 3.1) to introduce the unique sites *Xho* I and *Spe* I. The amplified sequence was then cloned into the PCR cloning vector pTZ-57R/T. Clones positive for insert were identified by restriction with *Xho* I and *Spe* I. As indicated in Figure 3.2 several clones were positive for inserts of the desired size (i.e. 1650 bp). To replace the preS2/S ORF of pCH-9/3091 with the sequence encoding Firefly luciferase, pTZ-FLuc was restricted with *Xho* I and *Spe* I (Figure 3.3). The preS2/S was removed from pCH-9/3091 by first completely restricting the plasmid with *Xho* I followed partial digestion with *Spe* I (Figure 3.4). Finally the *Xho* I-*Spe* I restricted *Firefly luciferase* fragment was ligated into the *Xho* I-*Spe* I sites of pCH-9/3091. To confirm successful insertion of the *Firefly luciferase* sequence into pCH-9/3091 the resulting clones were screened with *Xho* I and *Spe* I

digestion. A single clone was positive for insertion. This clone was further analysed by restriction enzyme digestion (Figure 3.4). The results from Figure 3.4 confirm successful cloning of the sequence encoding Firefly luciferase into the position previously occupied by the PreS2/S.

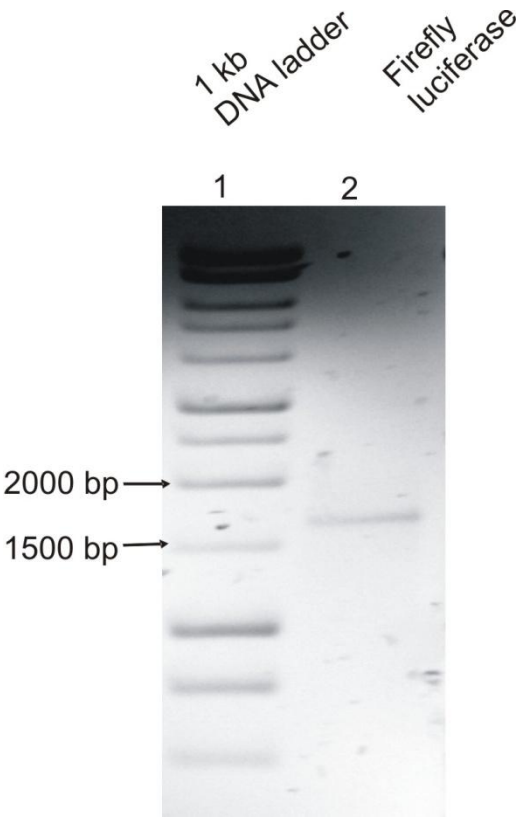


Figure 3.1: Agarose gel electrophoresis of the purified Firefly luciferase PCR product. A 1 kb DNA ladder was used as a molecular weight marker.

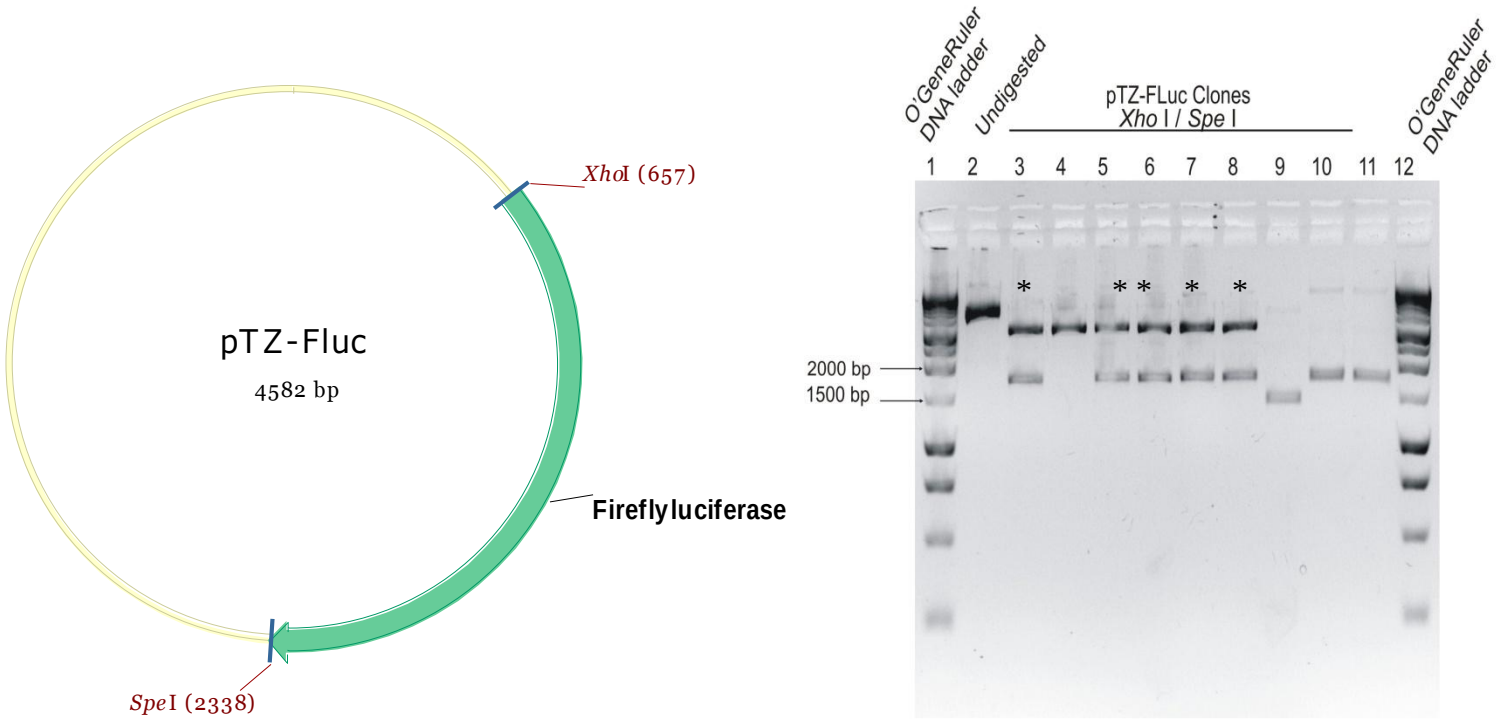


Figure 3.2: Screening for the presence of the *Firefly luciferase* insert. Plasmid DNA from colonies positive for an insert were restricted with *Xho* I and *Spe* I to confirm the presence of the *Firefly luciferase* sequence. Lanes 1 and 12: Molecular weight markers, lane 2: Undigested pTZ-FLuc clone, lanes 3 – 11 DNA from clones 1-10 restricted with *Spe* I and *Xho* I. Asterisk indicates putative positive clones.

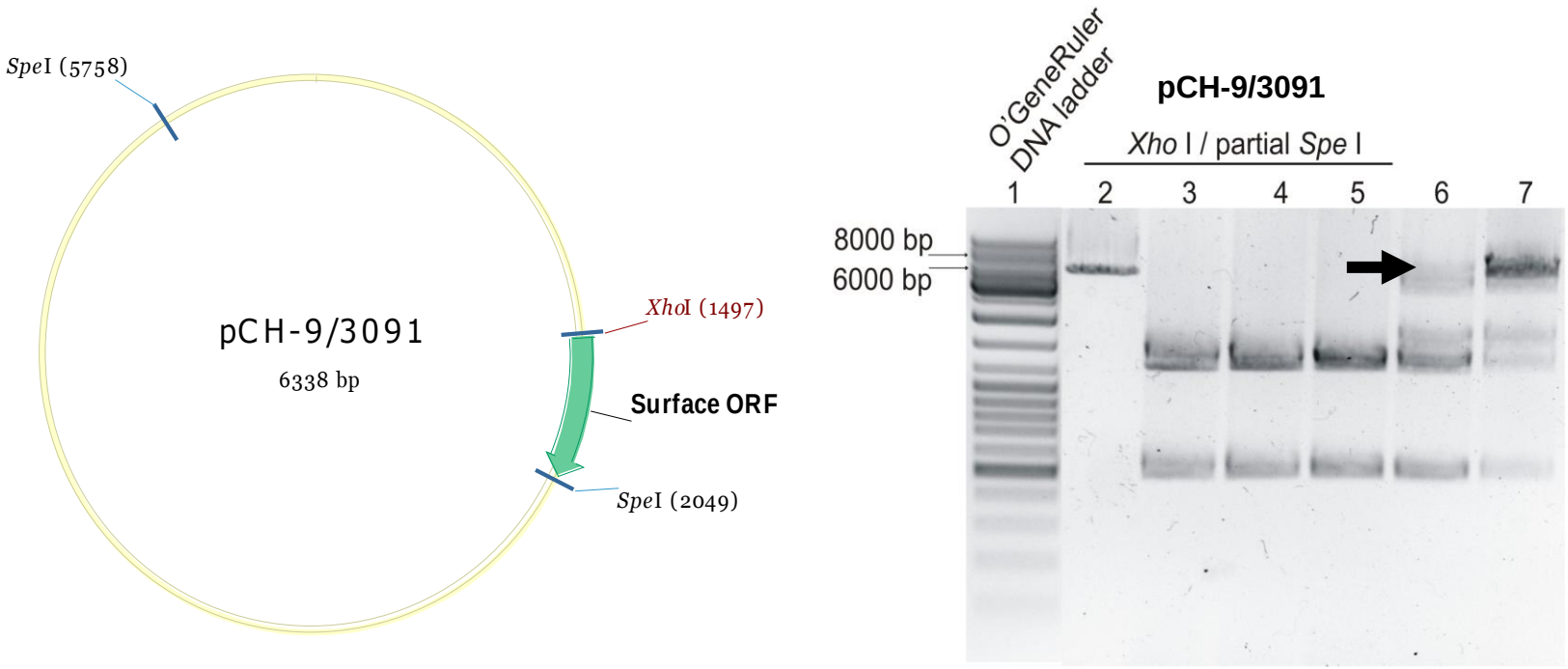


Figure 3.3 Agarose gel electrophoresis of the pCH-9/3091 first completely restricted with *Xho* I followed partial digestion with *Spe* I. Lane 1: O'GeneRuler DNA ladder, Lane 2 pCH-9/3091 completed digested with *Xho* I, lane 3 -7 *Xho* I restricted pCH-9/3091 serially digested with decreasing concentrations of *Spe* I. Arrows indicates the isolated fragment.

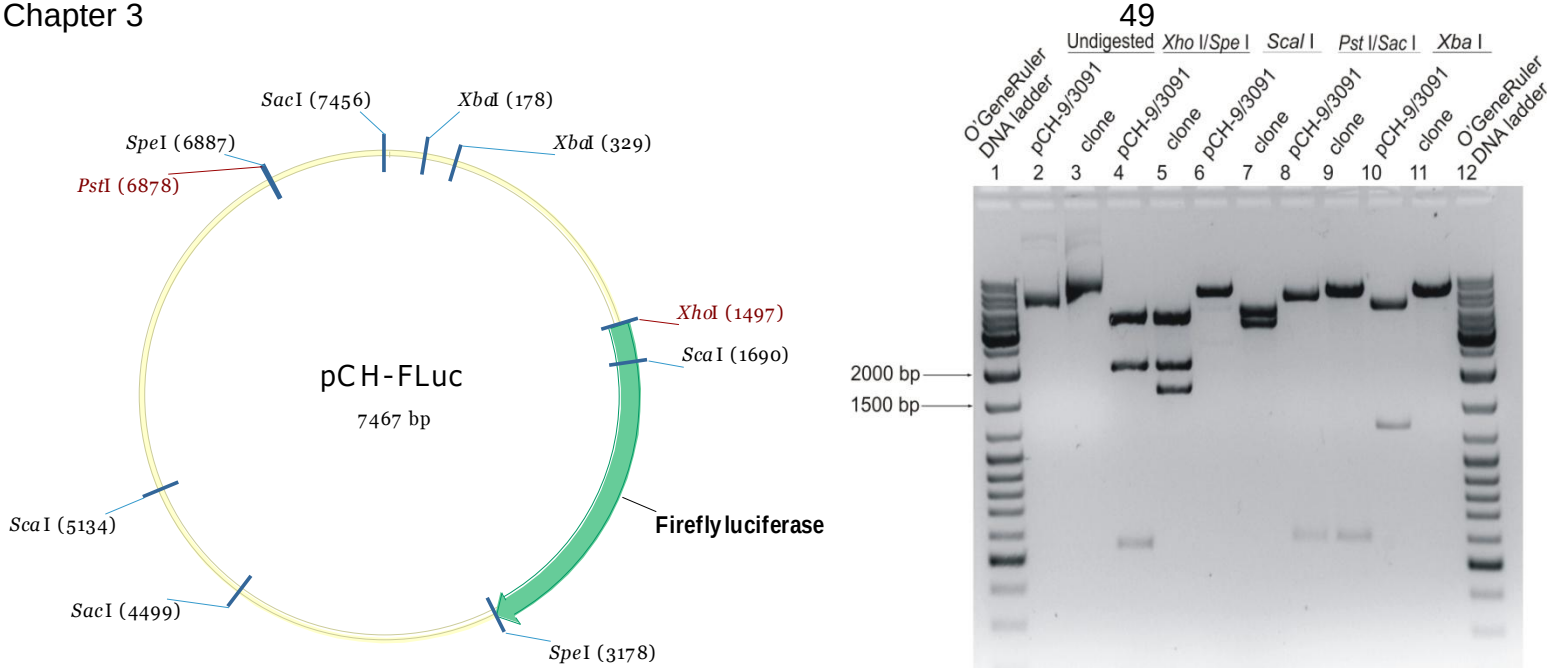


Figure 3.4 Screening for *Firefly luciferase* sequence insertion into *PreS2/S* ORF of pCH-9/3091. Lane 1 and 12: O'GeneRuler DNA ladder, lanes 2 and 3: undigested plasmids, lanes 4 and 5: *Xho* I-*Spe* I digestions, lanes 6 and 7: *Sca* I digestion, lanes 8 and 9: *Pst* I-*Sac* I digestions lans 10 and 11: *Xba* I digestion.

3.1.2 Generation of a replication competent plasmid (pCR-HBVA1 1.3x) containing a greater than genome sequence of the viral A1 subgenotype.

Figure 2.2 shows the strategy for the creation of a vector containing a greater than genome length sequence of HBV subgenotype A1. The BCP (787 bp) and pA (182 bp) sequences were amplified from pCR-HBVA1 1x by PCR (Figure 3.5). Amplification introduced the unique sites *Hind* III and *Pci* I into the BCP sequence and *Fsp* I and *Apa* I into the pA sequence. The amplified sequences were then cloned into pTZ-57R/T to generate pTZ-BCP and pTZ-pA. Clones positive for inserts were screened by restriction with *Hind* III-*Pci* I (pTZ-BCP) and *Fsp* I-*Apa* I (pTZ-pA). The restriction analysis of the DNA isolated from positive clones showed that the BCP and pA sequences had been correctly inserted into the pTZ-57R/T vector and inserts were of desired sizes (i.e. 787 bp and 182 bp) (Figures 3.6 and 3.7). To generate a greater than genome length sequence of HBV subgenotype A1, pCR-HBVA1 1x was restricted with *Hind* III and *Apa* I (full length sequence of HBV fragment) and with *Fsp* I and *Pci* I (pCR-XL-TOPO plasmid backbone fragment) (Figure 3.8). Finally the *Hind* III-*Pci* I restricted BCP fragment and *Fsp* I-*Apa* I restricted pA fragment were ligated together with the *Hind* III-*Apa* I and *Fsp* I-*Pci* I fragments from pCR-HBVA1 1x. To confirm successful insertion of BCP and pA sequences into pCR-HBVA1 1x, clones were restricted with *Hin*d III and *Apa* I (Figure 3.9). These results confirm successful cloning of the BCP sequence and the pA sequence upstream and downstream of full length sequence of HBV subgenotype A1, respectively.

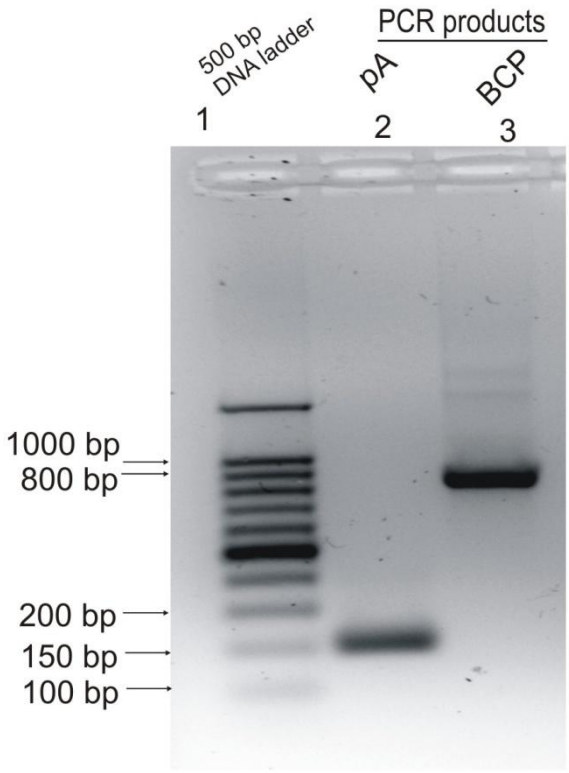


Figure 3.5: Agarose gel electrophoresis of the purified pA and BCP PCR products. Lane 1: 500 bp DNA ladder was used as a molecular weight marker, lane 2: pA amplicon and lane 3: BCP amplicon.

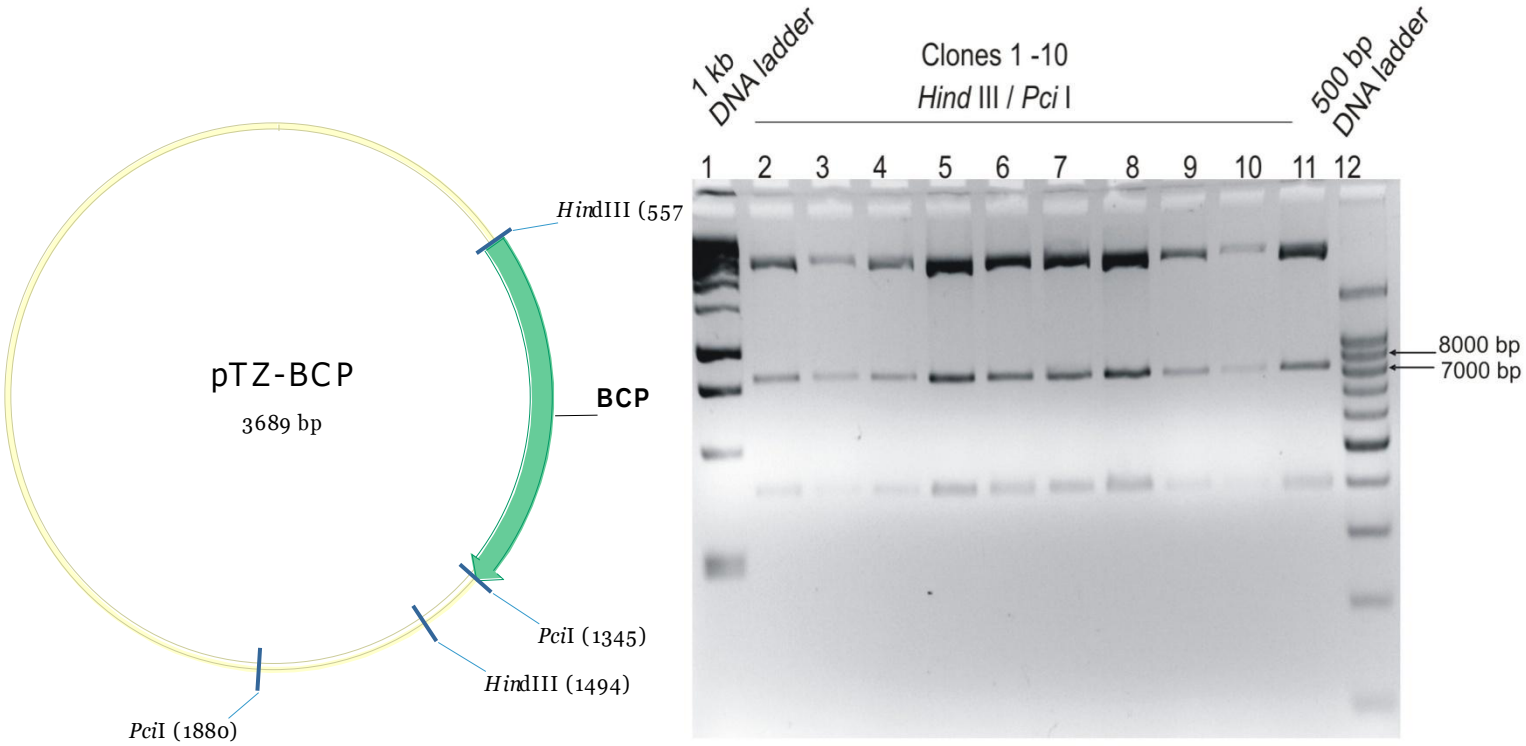


Figure 3.6: Screening for the successful insertion of the BCP fragment into pTZ-57R/T. Lane 1 and 12: 1 kb and 500 bp DNA ladder, lane 2–11 pTZ-BCP clones digested with a combination of *Hind* III and *Pci* I restriction enzymes. All clones are positive clones in figure legend.

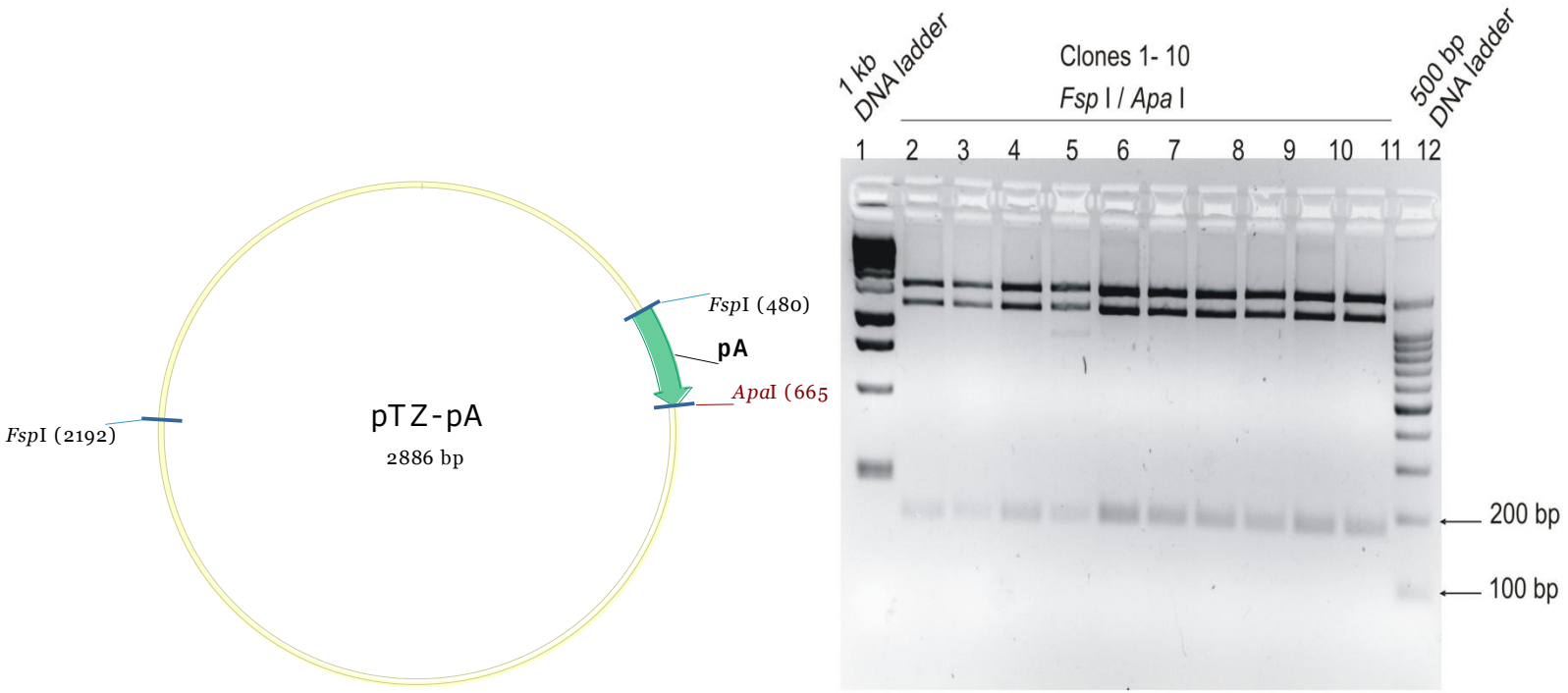


Figure 3.7: Screening for the successful insertion of the pA sequence into pTZ-57R/T. Lane 1 and 12: 1 kb and 500 bp DNA ladder, lane 2–11 pTZ-pA clones digested with a combination of *Apa* I and *Fsp* I restriction enzymes. All clones are positive in figure legend.

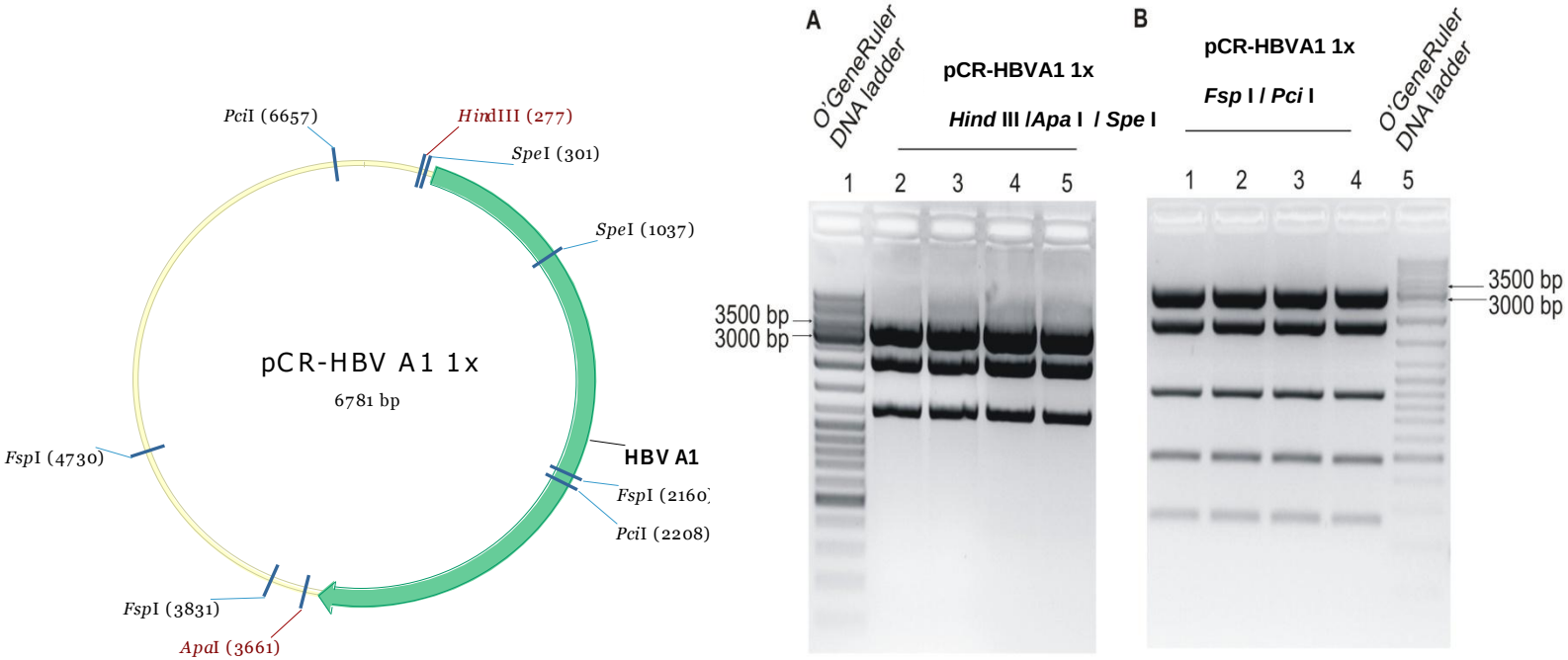


Figure 3.8: Agarose gel electrophoresis of pCR-HBV1 1x restricted for (A) full length sequence of HBV and (B) vector backbone (pCR-XL- TOPO backbone). (A) lane 1: O'GeneRuler DNA ladder, lane 2-5 pCR-HBVA1 1x digestion (*Hind* III-*Apa* I-*Spe* I). (B) Lane 1-4: pCR-HBVa 1x digestion (*Fsp* I and *Pci* I), lane 5 O'GeneRuler DNA ladder. The 3200 bp HBV and 178 bp pA insert can be seen in the restriction analysis from all ten clones.

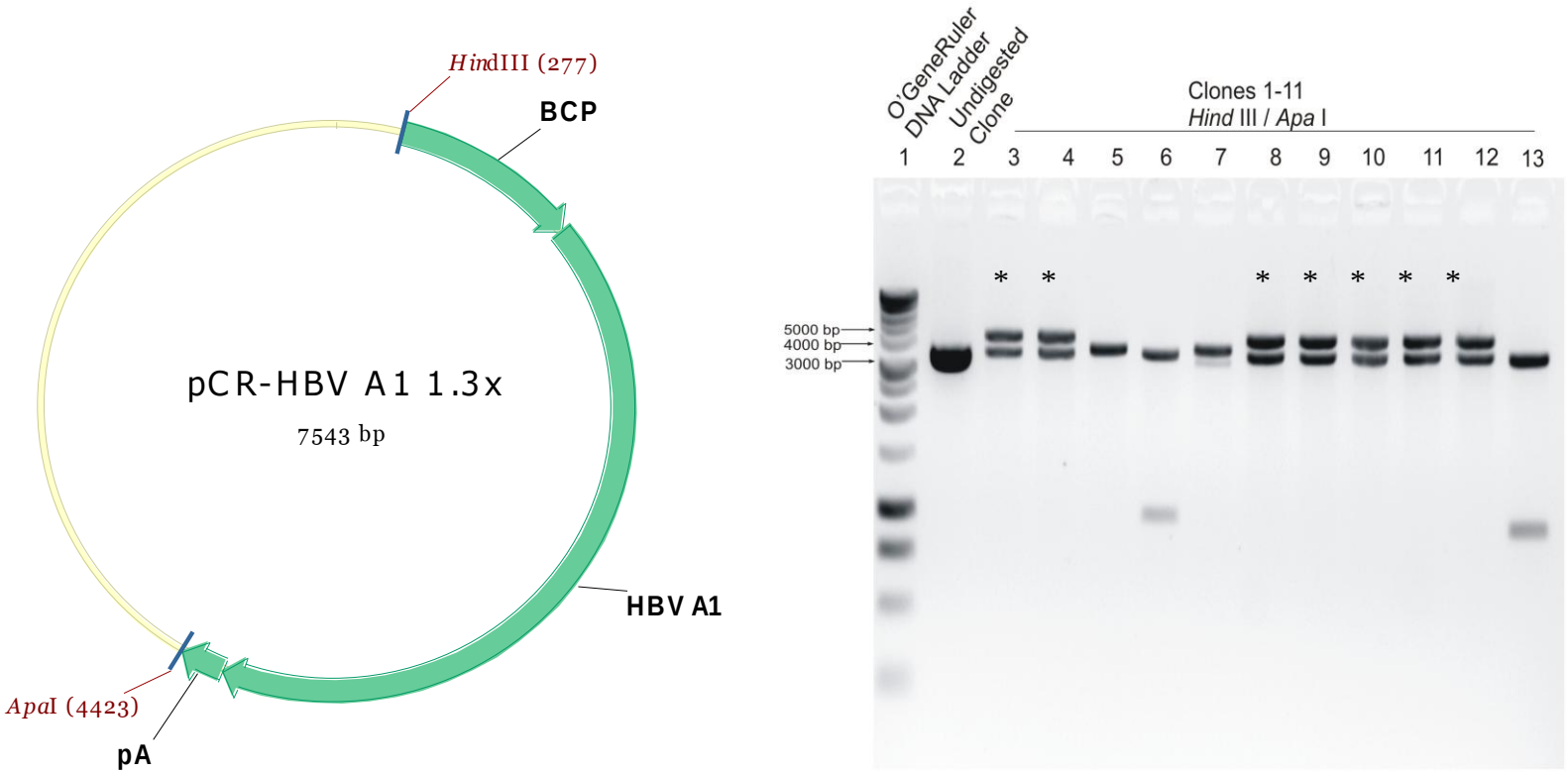


Figure 3.9: Screening for the presence of both BCP and pA inserts. Clones were selected and DNA was digested to confirm the presence of BCP and pA. Lane 1: O'GeneRuler DNA ladder, lane 2 undigested pCR-HBVA1 1.3x clone, lane 3, 4, 8–12 positive clones, lane 5 and 7 looks like undigested clones, lane 6 and 13 erroneous clones. Asterisk indicates putative positive clones.

3.2. Expression of Firefly luciferase as a marker of HBV replication in cultured mammalian cells.

To express luciferase as a marker of HBV replication, the *surface* ORF of pCH-9/3091 was replaced with the sequence encoding Firefly luciferase (Figure 2.1). To assess functionality of this construct (pCH-FLuc), it was co-transfected with a phRL-CMV (plasmid expressing *Renilla* luciferase) in cultured mammalian cells (Huh7). A plasmid expressing EGFP was also included to control for transfection efficiency. As can be seen from Figures 3.10 A and 3.10 B equal expressions of green cells indicate that transfection efficiency was equivalent. Figure 3.10 C shows Firefly luciferase activity in transfected cells. From the figure it is evident that cells transfected with pCH-FLuc expressed Firefly luciferase whereas the mock transfected cells did not (Figure3.10). The results demonstrate that Firefly luciferase is expressed within the HBV genome construct of the pCH-FLuc vector.

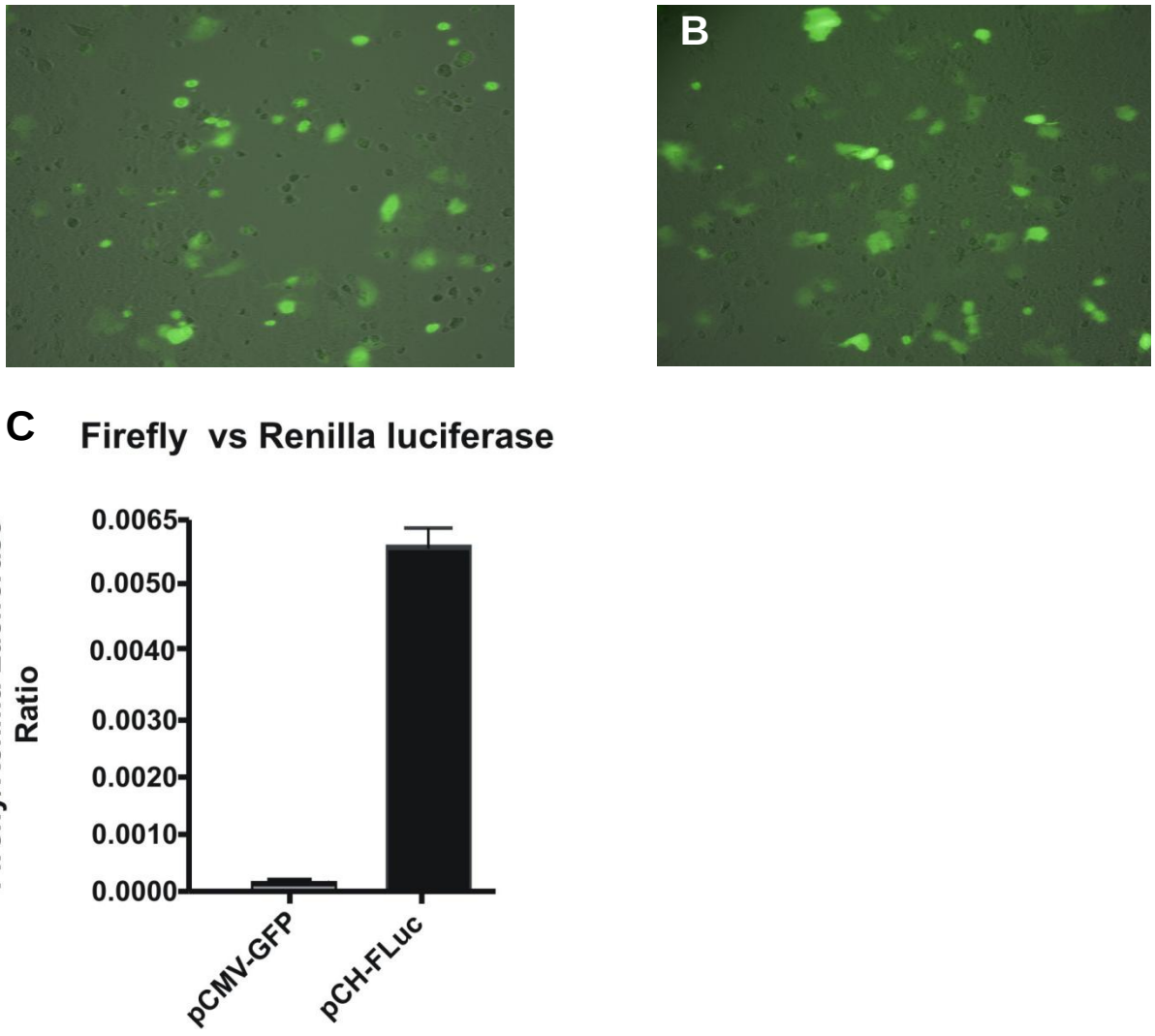


Figure 3.10: Luciferase expression in Huh7 cells. The transfection was performed in triplicate and the quantities of luciferase were analysed by the Dual Luciferase Reporter Assay System. Fluorescence microscopy fields of Huh7 cells transfected with (A) pCH-FLuc and pCMV-GFP (B), pCMV-GFP only (C), Luciferase activity in Huh7 cells transfected with pCH-Fluc.

3.3 Assessing functionality of the replication competent plasmid derived from HBV subgenotype A1 (pCR-HBVA1 1.3x)

3.3.1 Cells transfected with pCR-HBVA1 1.3x

To assess functionality of pCR-HBVA1 1.3x it was transfected into cultured mammalian cells and HBsAg secreted into culture medium measured. pCH-9/3091 was used a control positive for HBsAg secretion. The negative control included pCR-HBVA1 1x to confirm that any observed HBsAg was due to presence of BCP and pA. An EGFP expressing plasmid was utilised to standardise transfection efficiency (Figure 3.11 A). Cells transfected with pCR-HBVA1 1.3x and pCH-9/3091 strongly expressed HBsAg, whereas cells transfected with pCR-HBVA1 1x exhibited a small increase in HBsAg level (Figure 3.11E). The small increase of HBsAg may be due to a unique internal promoter that is specific for HBV surface antigen expression. No HBsAg was detected in the culture supernatant from untreated cells. The results demonstrate that pCR-HBVA1 1.3x was able to express HBsAg in cultured mammalian cells.

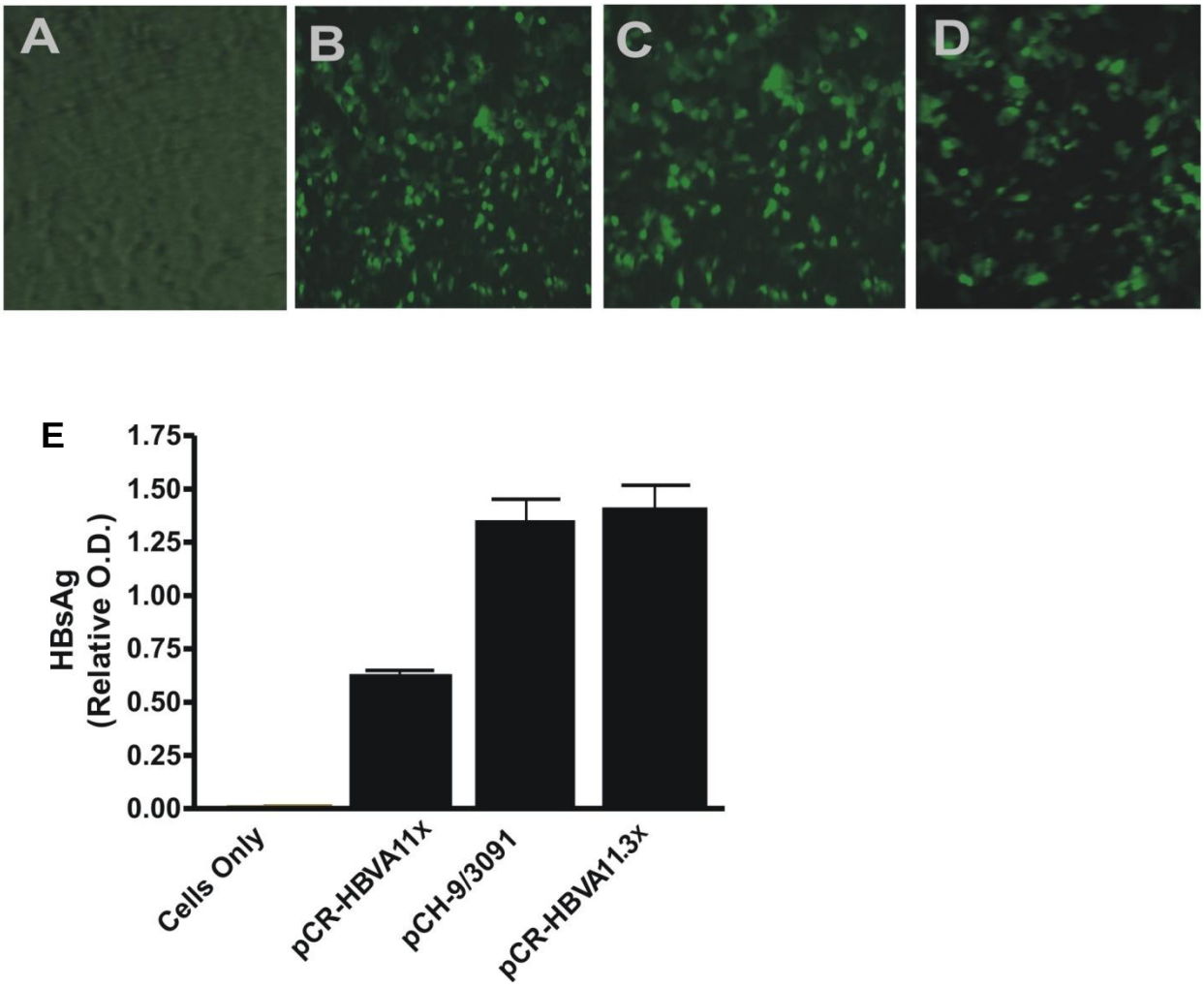


Figure 3.11: HBsAg secretion in transfected cells in culture. Fluorescence microscopy fields of (A) Huh7 cells only, and cells transfected with (B) pCR-HBVA1 1x (C), pCH-9/3091 and (D) pCR-HBVA1 1.3x. (E) HBsAg secretion from Huh7 cells transfected with HBV plasmids. HBsAg measurements by ELISA. The transfection was performed in triplicate and the bars indicate the standard error of the mean (SEM).

3.3.2 Expression of markers of HBV replication *in vivo*

3.3.2.1 HBsAg and HBeAg secretion *in vivo*

To assess HBsAg and HBeAg secretion *in vivo*, pCR-HBVA1 1.3x, pCR-HBVA1 1x, or pCH-9/3091 were delivered to the livers of mice using the hydrodynamic tail vein injection procedure. pCR-HBVA1 1.3x and pCH-9/3091 both effect secretion of HBsAg and HBeAg in the serum of mice as measured at days 3 and 5 (Figure 3.12). Mice injected with pCR-HBVA1 1x did not secrete significant amounts of HBsAg at days 3 and 5. Each experiment comprised 3 groups of animals. The data confirm that the presence of markers of HBV replication are due to the greater than genome length of pCR-HBVA1 1.3x. These results support our previous findings in transfected cells where secretion HBsAg into the culture supernatant was demonstrated. Importantly secretion of HBeAg demonstrates that pCR-HBVA1 1.3x enables viral replication and not merely gene expression to occur.

3.3.2.2 Serum viral particle equivalents *in vivo*

To assess the production of viral particle equivalents, pCR-HBVA1 1.3x, pCR-HBVA1 1x or pCH-9/3091 were delivered to the livers of mice using the hydrodynamic tail vein injection procedure. The serum viral particle equivalents (Figure 3.13) exhibited a similar trend to that seen with HBsAg and HBeAg secretion, confirming the previous results. The level of serum viral particle equivalents was increased in mice injected

with the replication competent constructs (pCR-HBVA1 1.3x) and pCH-9/3091 at days 3 and 5. However, mice injected with pCR-HBVA1 1x did not have significant detectable levels of viral particle equivalents.

3.3.2.3 HBV mRNA expression *in vivo*

The HBV genome has four primary overlapping ORFs which code for the surface, core, polymerase and HBx proteins (Figure 1.2). Transcription from the four ORFs results in formation of the pregenomic RNA and the three subgenomic mRNAs. To assess expression of these transcripts, RNA extracted from livers of mice hydrodynamically injected with pCR-HBVA1 1.3x, pCH-9/3091 and pCR-HBVA1 1x were subjected to RT-PCR. Figure 3.14 reveals that injection with pCR-HBVA1 1.3x causes expression of HBV mRNA levels equivalent to that achieved with the positive control, pCH-9/3091. Mice injected with pCR-HBVA1 1x did not exhibit significantly increased levels of *core* or s*urface* HBV mRNA at day 5.

These results demonstrate that pCR-HBVA1 1.3x was able to express HBsAg, HBeAg, viral particle equivalents, as well as *core* and *surface* HBV mRNA *in vivo.*

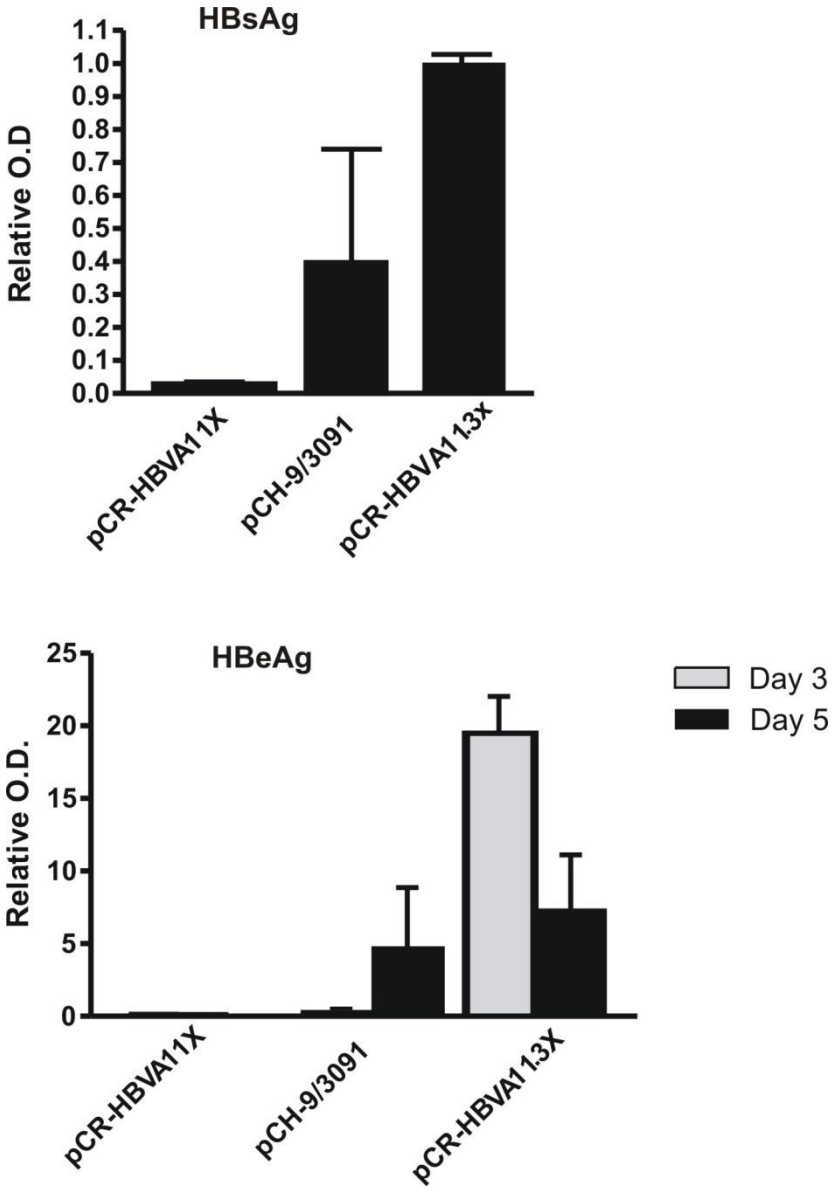


Figure 3.12: HBsAg and HBeAg measurement in the hydrodynamic injection model of HBV replication. Serum (A) HBsAg, (B) HBeAg levels were determined at day 3 and 5 after hydrodynamic injection of mice with pCR-HBVA1 1x, pCH-9/3091 and pCR-HBVA1 1.3x. Results of HBsAg (A) were normalised relative to pCR-HBVA1 1.3x. Results are expressed as the mean (SEM) from at least three mice.

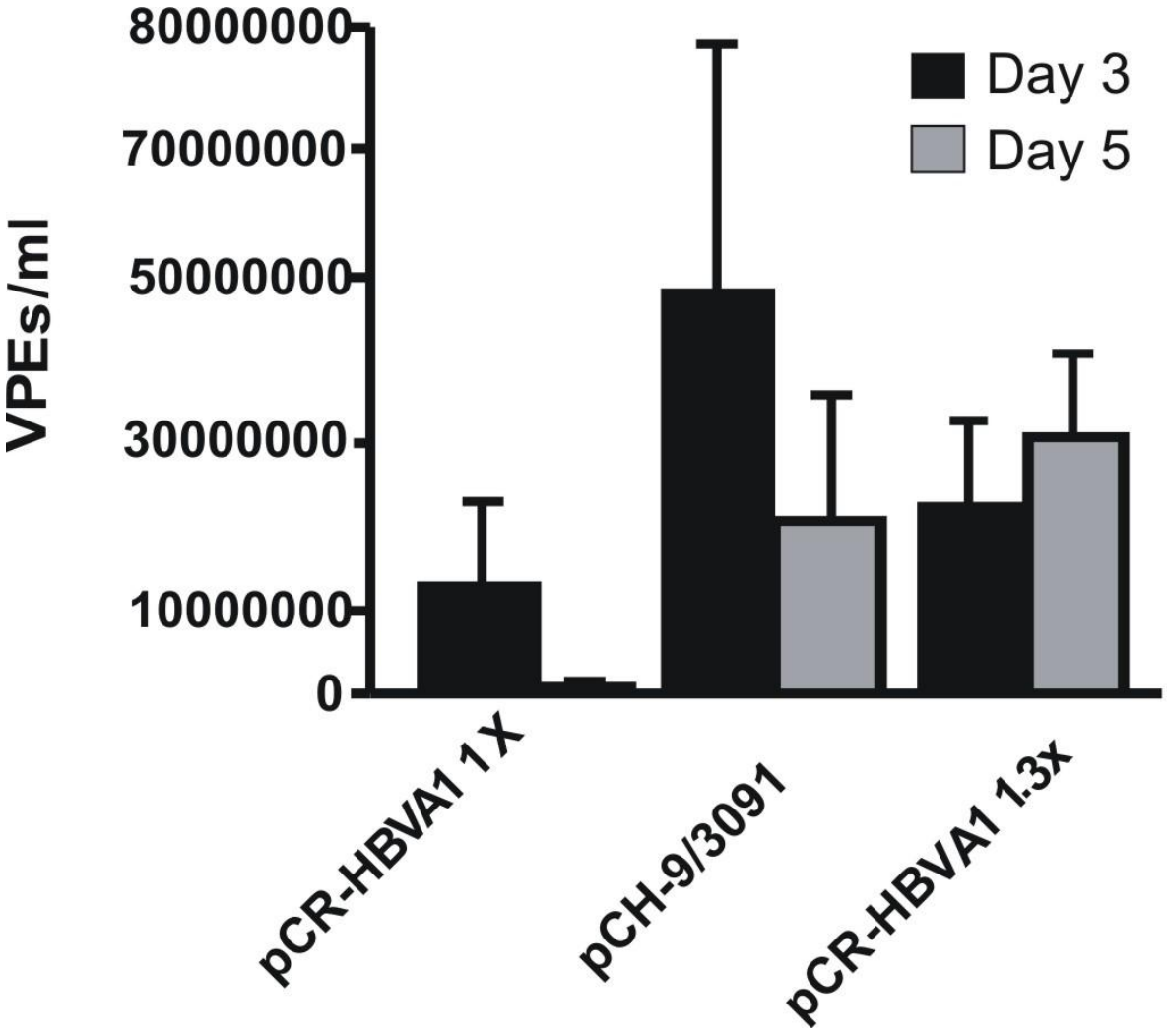


Figure 3.13: HBV serum viral particle equivalents in the hydrodynamic injection model of HBV replication. VPEs at day 3 and 5, after hydrodynamic injection with pCR-HBVA1 1x, pCR-HBVA1 1.3x and pCH-9/3091. Groups comprised 3 animals and the graphs indicate the mean and SEM for each group.

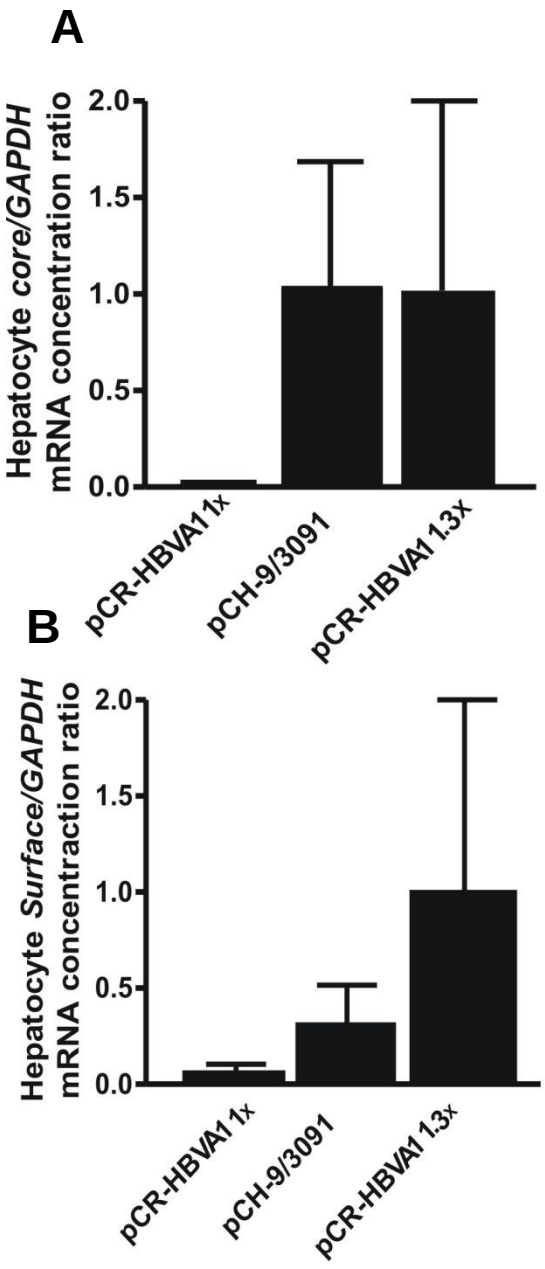


Figure 3.14: HBV mRNA measurement for *core (*A) and *surface* (B) in the hydrodynamic injection model. Hepatocyte concentrations of HBV mRNA from the *core* and *surface* regions expressed as a ratio to amount of *GAPDH* mRNA. Total RNA was isolated from liver cells at day 5 after hydrodynamic injection and subjected to quantitative real-time PCR. Groups comprised 3 animals and the graphs indicate the mean and SEM for each group.

3.4 Inhibition of HBV replication by activating RNA interference with U6 shRNA sequences

3.4.1 Inhibition of HBV replication in cultured mammalian cells

3.4.1.1 Effects of U6 shRNA sequence on luciferase activity in transfected cells

To assess the effects of U6 shRNA sequences on luciferase expression, pCH-FLuc was co-transfected with U6 shRNA 5, U6 shRNA 6 and U6 shRNA 10 at different effecter to target ratios (48). Plasmids expressing *Renilla* luciferase and EGFP were transfected to ascertain transfection efficiency. Luciferase activity decreased by approximately 50%, 70% and 80% when treated with U6 shRNA 5 and U6 shRNA 6 at the different ratios, whereas treatment with U6 shRNA 10 did not significantly alter Firefly luciferase activity (Figure 3.15). These results demonstrate a reduction in Firefly luciferase expression in cultured mammalian cells after treatment with U6 shRNA 5 and U6 shRNA 6. These results correlate with data of Carmona and colleagues (48). Firefly luciferase is not susceptible to knockdown by U6 shRNA 5 and 6. U6 shRNA 5, U6 shRNA 6 and U6 shRNA 10 all target the *HBx* ORF region. Firefly luciferase activity is diminished upon administration of expression cassettes producing these shRNAs, and indicates that reporter gene activity correlates with HBV gene expression.

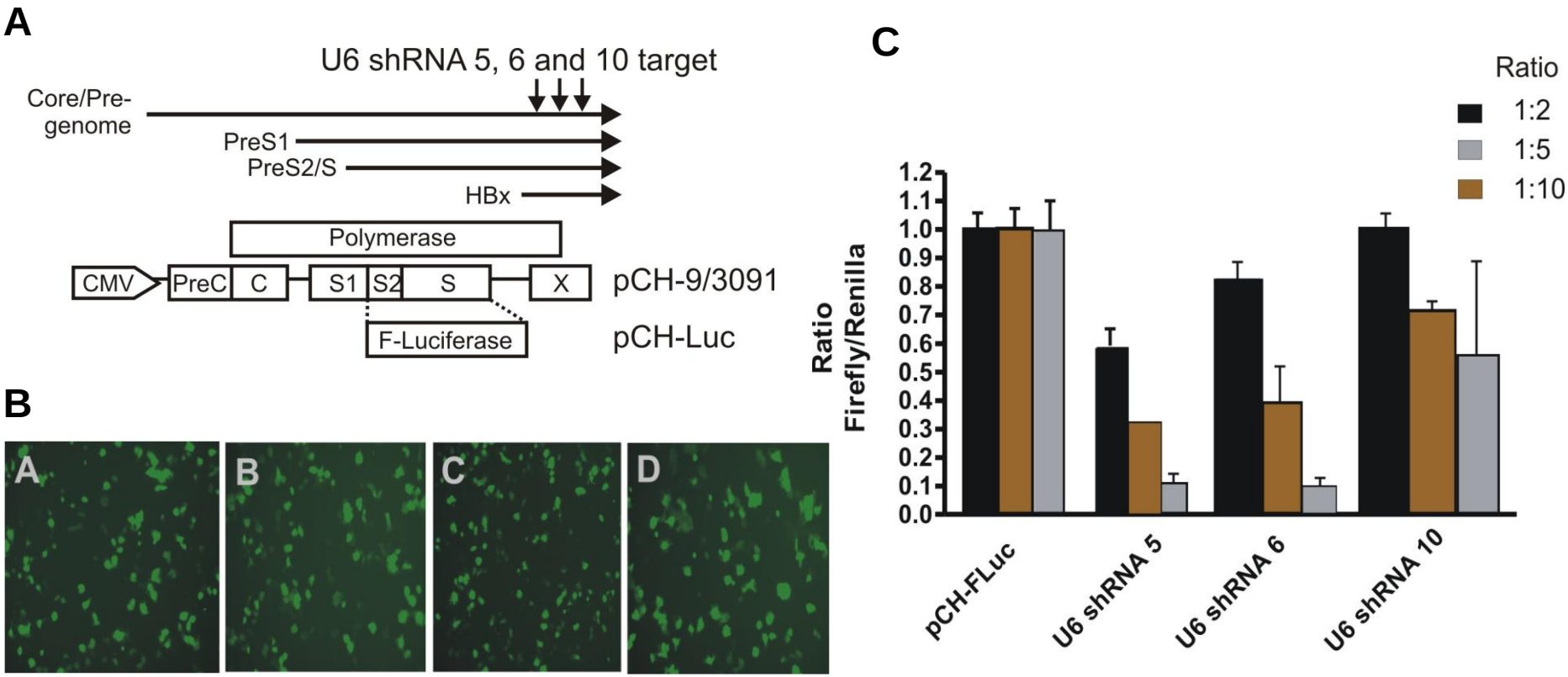


Figure 3.15: Luciferase measurements in Huh7 cells treated with shRNA sequences. Schematic illustration of plasmid construct pCH-Fluc showing ORFs, respective transcripts and sites targeted by U6 shRNA 5, U6 shRNA 6 and U6 shRNA 10 (A). Microscopy fields of Huh7 cells co-transfected with pCMV-GPF and pCH-Fluc with no U6 shRNA (BA), U6 shRNA 5 (BB), U6 shRNA 6 (BC), U6 shRNA 10 (BD). Huh7 cells were transfected with 0.4, 0.2, 0.16 µg of pCH-FLuc together with 0.9, 1.1, 1.14 µg of U6 shRNA 5 or shRNA 6 or shRNA 10. (C) Firefly luciferase activity was normalised to *Renilla* luciferase activity. The data were determined from three independent experiments and are expressed as the mean with SEM.

3.4.1.2 Effects of U6 shRNA sequence on HBsAg secretion from transfected cells

To assess the effects of shRNA sequences on HBsAg secretion in cells receiving the HBVA1 replication-competent plasmid, pCR-HBVA1 1.3x was co-transfected with U6 shRNA 5, U6 shRNA 6 or U6 shRNA 10. Cells treated with U6 shRNA 5 and U6 shRNA 6 were able to reduce HBsAg levels significantly, whereas cells treated with U6 shRNA 10 showed little or no significant knockdown of HBsAg secretion (Figure 3.16). The mock transfection, receiving no effecter, did not exhibit an effect on HBsAg concentrations, indicative of the inhibitory effects and specificity of the shRNA 5 and 6 sequences.

3.4.1.3 Effects of U6 shRNA sequence on HBV mRNA from transfected cells

To confirm that the effect of shRNA 5 and 6 on HBsAg expression was through the reduction of HBV RNA levels, we performed quantitative RT-PCR analysis with RNA extracted from Huh7 cells co-transfected with pCR-HBVA1 1.3x and U6 shRNA sequences. The observation revealed that treatment with U6 shRNA 5 and 6 resulted in a decrease of both *core* and *surface* mRNAs by 95-100%, compared with the untreated cells (Figure 3.17). Treatment with U6 shRNA 10 exhibited no significant decrease of both *core* and *surface* mRNAs. Again this observation correlates with previous work by Carmona and colleagues (48).

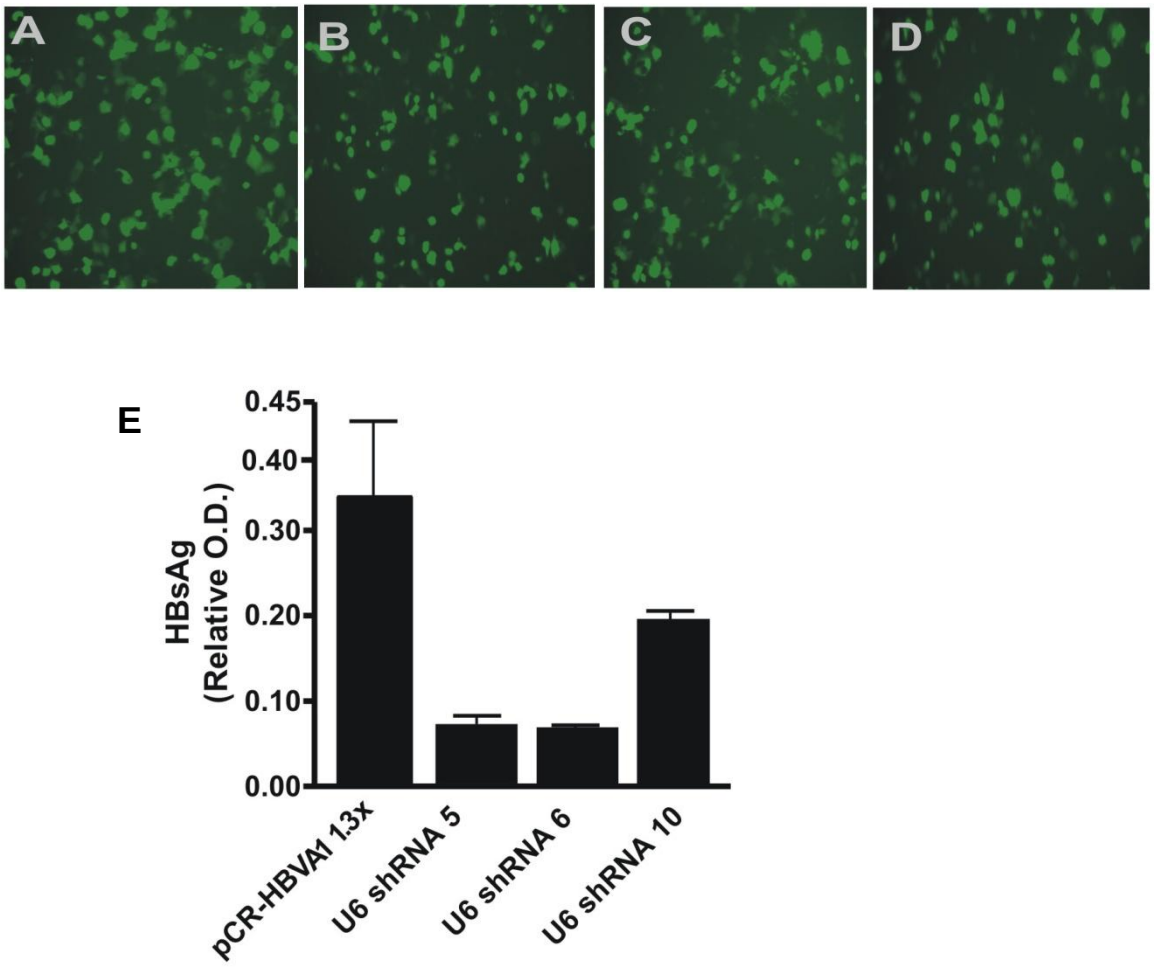


Figure 3.16: HBsAg measurement in the Huh7 cells treated with shRNA sequences. Representative fluorescence microscopy fields of Huh7 cells co-transfected with GFP expressing plasmid and pCR-HBVA1 1.3x together with U6 shRNA cassette. (A) pCR-HBVA1 1.3x, (B) U6 shRNA 5, (C) U6 shRNA 6, and (D) U6 shRNA 10. (E) HBsAg secretion as measured by ELISA. The experiments were done in triplicate and the error bars indicate the SEM.

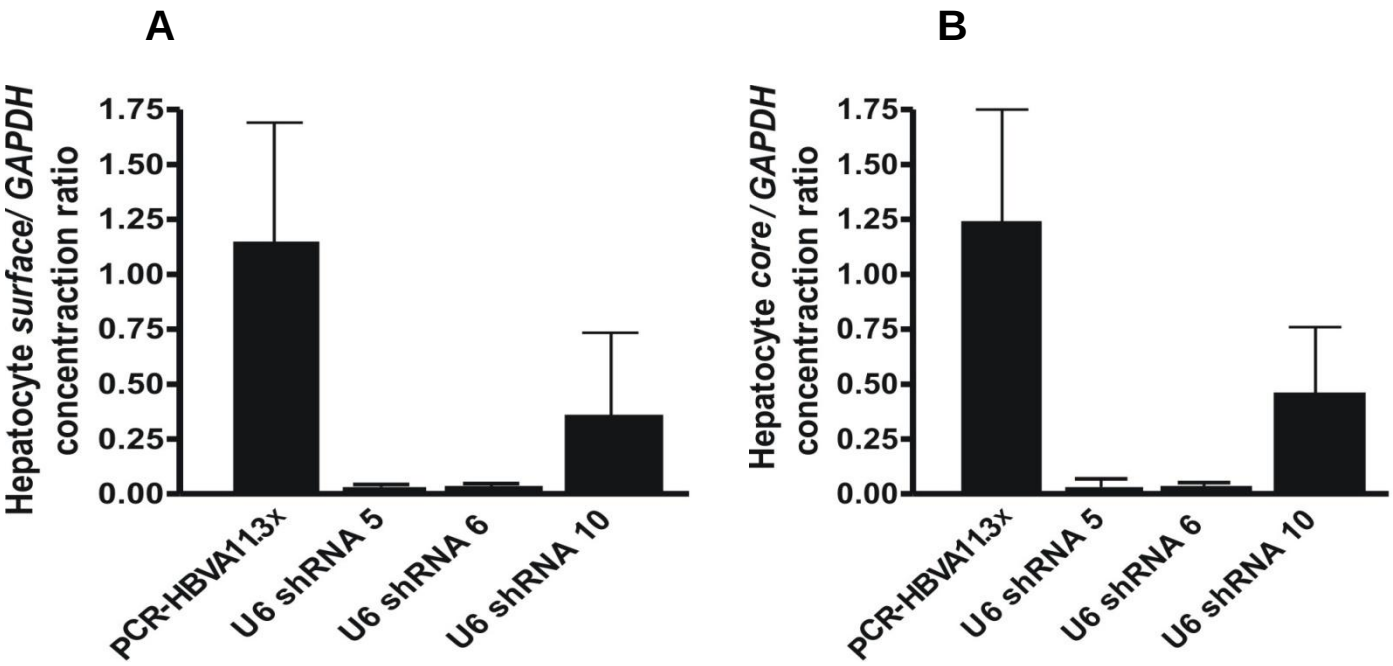


Figure 3.17: HBV mRNA measurement in the Huh7 cells treated with shRNA sequences. Hepatocyte concentrations of HBV mRNA from the (A) *surface*, and (B) *core* regions expressed as a ratio to amount of *GAPDH* mRNA. Total RNA was isolated from Huh7 cells 48 hr after transfection of pCR-HBVa1 1.3x together with shRNA sequence. The transfection was performed in triplicate and the bars indicate mean SEM.

3.4.2 Inhibition of HBV replication *in vivo*

3.4.2.1 Effects of U6 shRNA sequence on HBsAg and HBeAg secretion using the hydrodynamic mouse model of HBV replication

To determine anti HBV efficacy of U6 shRNA sequences on HBsAg and HBeAg secretion *in vivo*, pCR-HBVA1 1.3x together with U6 shRNA 5, U6 shRNA 6 or U6 shRNA 10 were delivered to the livers of mice using the hydrodynamic tail vein injection procedure. U6 shRNA 5 and 6 both show strong inhibition of HBsAg secretion at days 3, 5 and 7 (Figure 3.18). The HBeAg concentration was also significantly knocked down by U6 shRNA 5 and 6 at day 7 (approximately 90–100%) (Figure 3.19). U6 shRNA 10 exhibited no significant inhibition of HBsAg and HBeAg *in vivo*, which supports the *in vitro* results. This data correlate with the published work of Carmona and colleagues (48)

3.4.2.2 Effects of U6 shRNA sequence on HBV mRNA *in vivo*

To confirm that the effect of U6 shRNA 5 and 6 on HBsAg expression was through the reduction of HBV RNA levels *in vivo*, we performed quantitative RT-PCR analysis with RNA extracted from mouse livers, 7 days after mice were hydrodynamically injected with pCR-HBVA1 1.3x together with U6 shRNA 5, U6 shRNA 6 or U6 shRNA 10. The U6 shRNA 5 and U6 shRNA 6 vectors decreased the expression of both *core* and *surface* mRNAs by 95-100% (Figure 3.20).

3.4.2.3 Effect of U6 shRNA sequence on intrahepatic HBcAg expression

HBcAg, the nucleocapsid protein, is synthesised in infected cells and is required for HBV viral replication. To confirm that the effect of U6 shRNA 5 and 6 on HBsAg, HBeAg and mRNA expression was due to an effect of viral replication, we performed immunohistochemical staining for HBcAg in tissue sections from mice. Immunohistochemical staining for HBcAg in liver tissue from U6 shRNA 5, U6 shRNA 6 and U6 shRNA 10 infected mice allowed us to directly observe HBV clearance from hepatocytes. Fixed liver sections harvested from mice 7 days after injection were stained for HBcAg, and visualized under a light microscope. Liver sections from HBV transgenic mice were also stained. HBV transgenic mice replicate the virus at levels comparable to that in the infected livers of patients with chronic hepatitis (35). Sections from transgenic mice and mice injected with pCH-HBVA1 1.3x show numerous HBcAg positive cells, whereas mice treated with U6 shRNA 5 and 6 show no HBcAg positives cells at day 7 (Figure 3.21). Mice treated with control U6 shRNA 10 sequences did not significantly reduce the HBcAg positive cells. The results demonstrate that U6 shRNA 5 and U6 shRNA 6 were able to inhibit expression of HBsAg, HBeAg, HBcAg and mRNA of HBV *in vivo*.

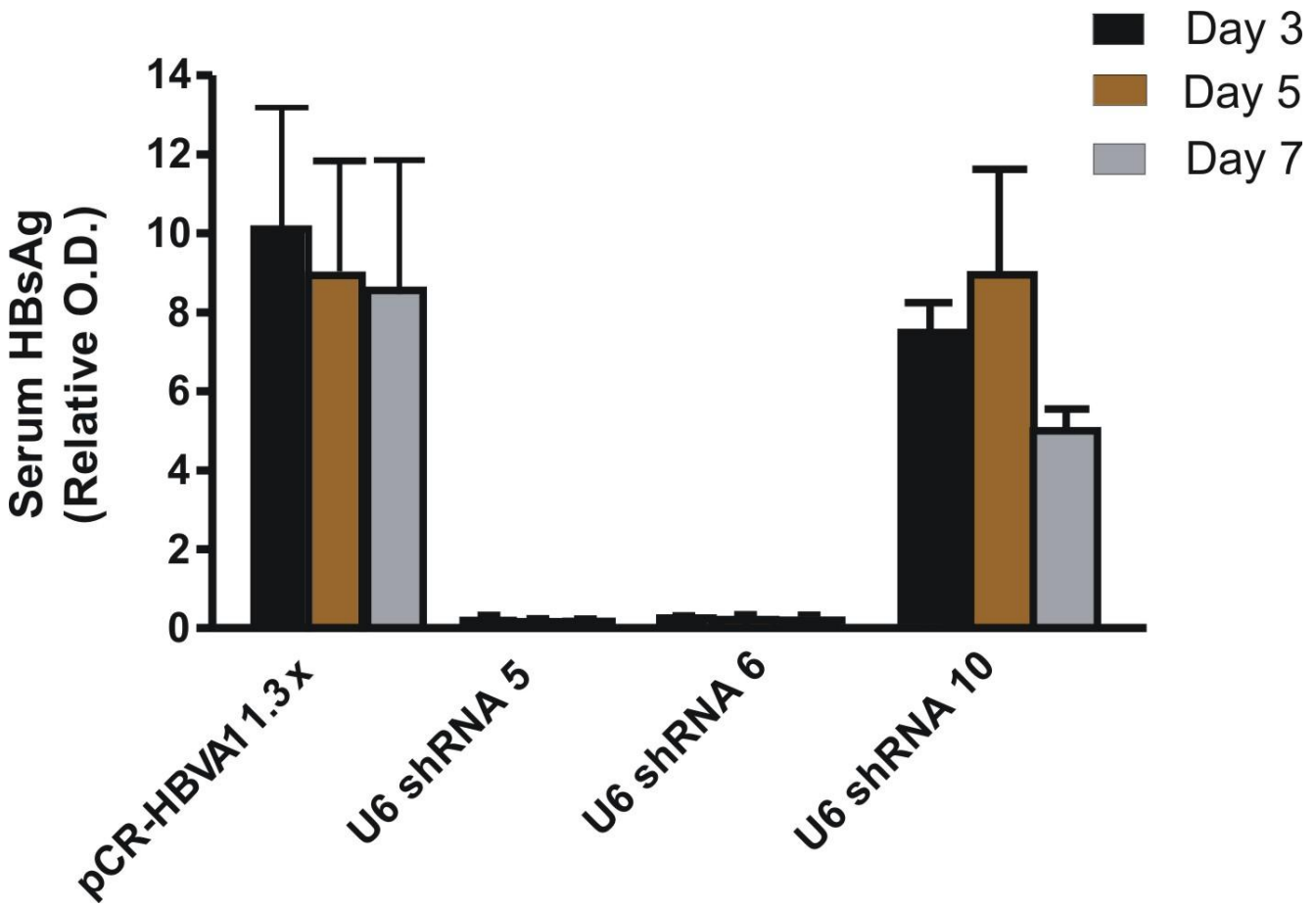


Figure 3.18: HBsAg measurement from serum of mice hydrodynamically injected with pCR-HBVA1 1.3x and shRNA sequences. Serum HBsAg concentrations were determined at days 3, 5 and 7. Groups comprised 3-5 animals and the graphs indicate the mean and SEM for each group.

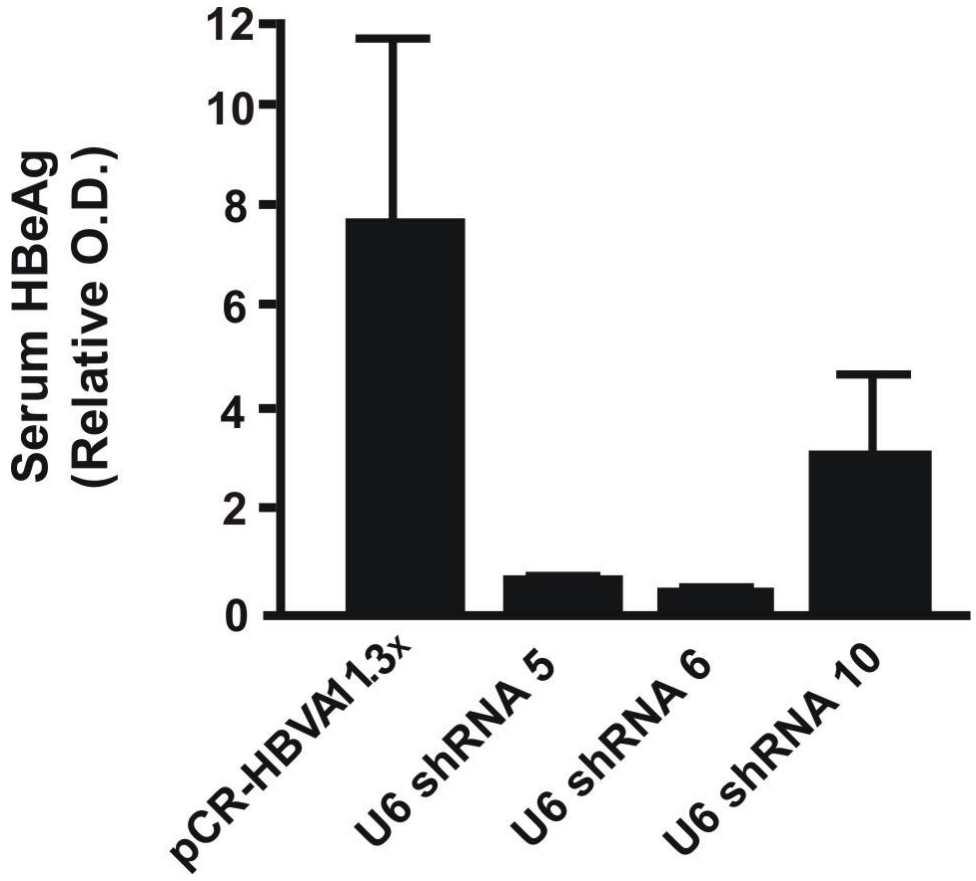


Figure 3.19: HBeAg measurement from mice hydrodynamically injected with pCR-HBVA1 1.3x and shRNA sequences. Serum HBeAg concentrations were determined at day 7 after injection. Groups comprised 3-5 animals and the graphs indicate the mean and SEM for each group.

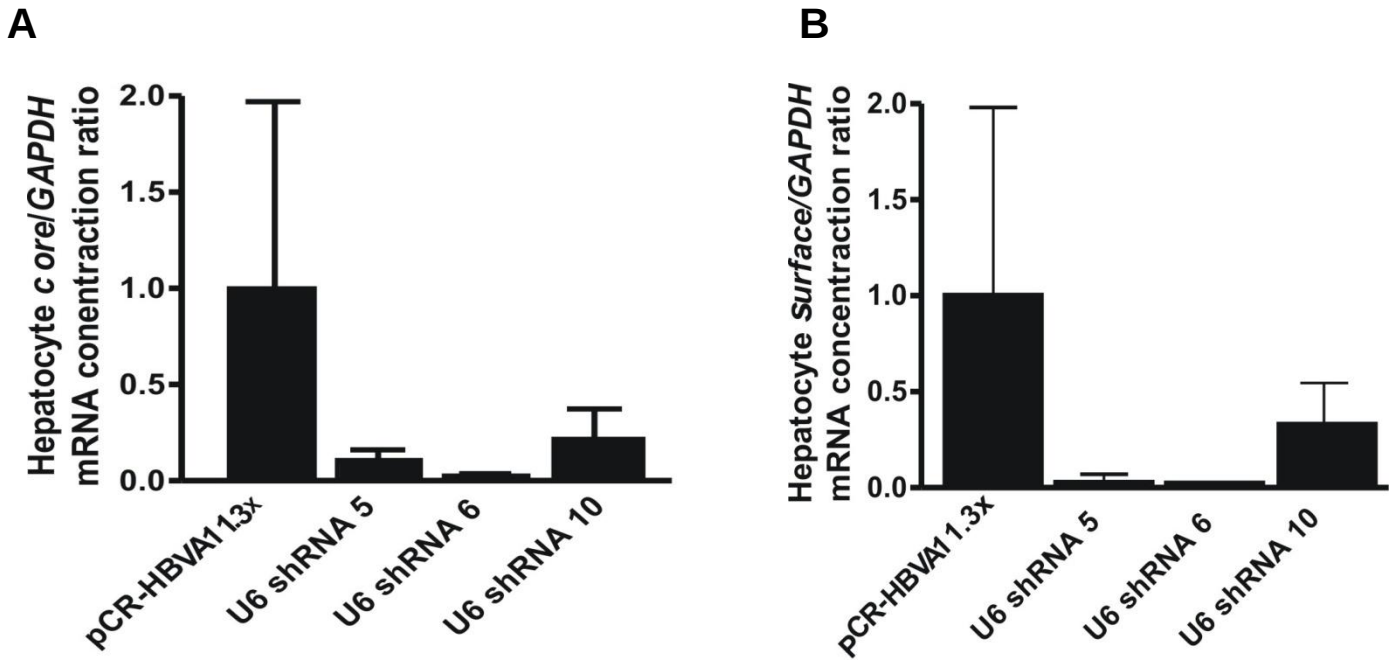


Figure 3.20: HBV mRNA measurement from mice hydrodynamic injection with pCR-HBVA1 1.3x and shRNA sequences. Hepatocyte concentrations of HBV mRNA from the (A) *core*, and (B) *surface* regions expressed as a ratio to amount of *GAPDH* mRNA. Total RNA was isolated from mouse livers at day 7 after hydrodynamic injection of pCR-HBVA1 1.3x together with shRNA sequences and subjected to RT-PCR. Groups comprised 3-5 animals and the graphs indicate the mean and SEM for each group.

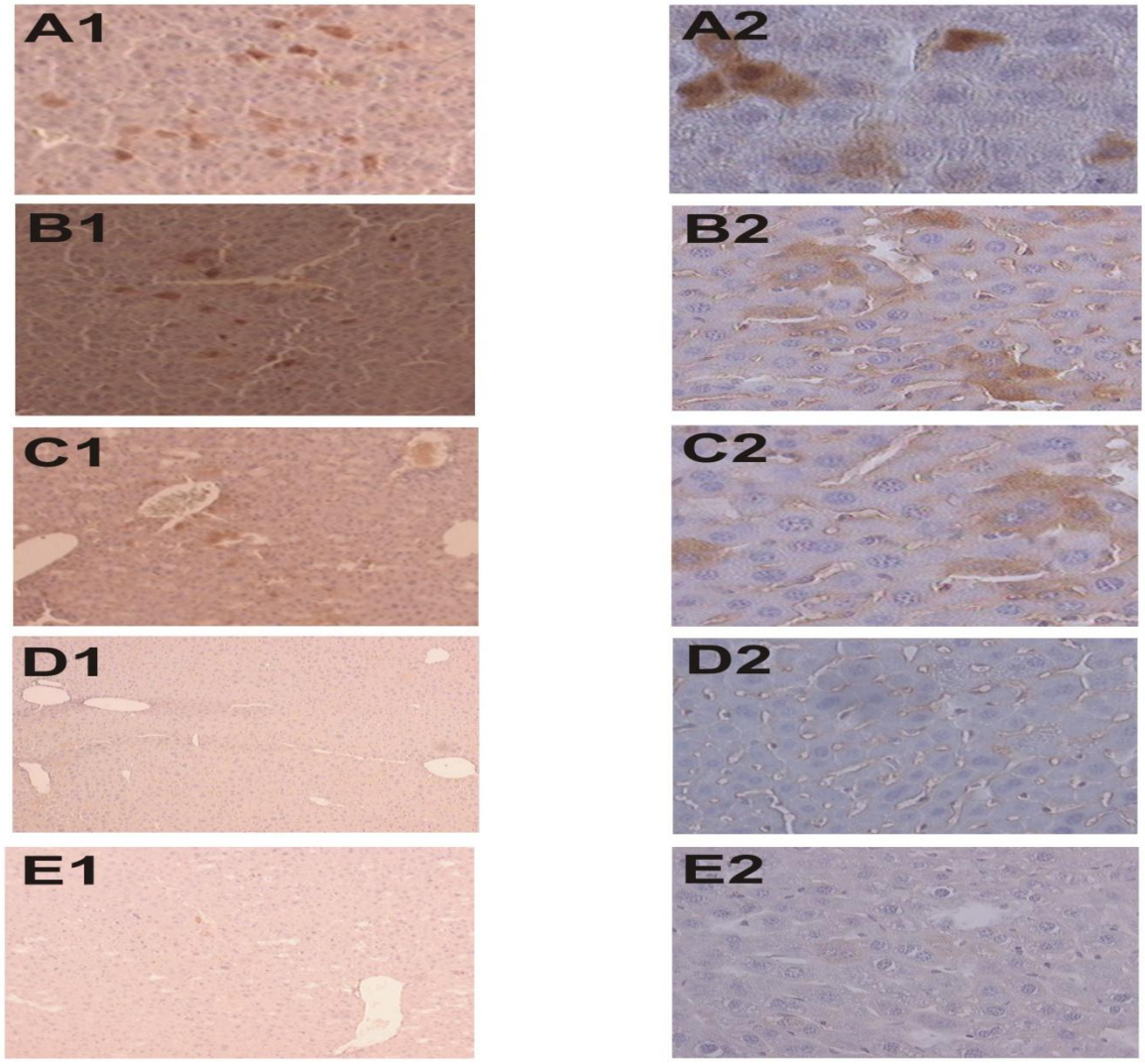


Figure 3.21: Immunohistochemical staining for HBcAg in liver sections from hydrodynamic injection model of HBV replication. (A1 and A2) HBV transgenic mouse, (B1 and B2) mouse with pCR-HBVA1 1.3x only, (C1 and C2) mouse treated with U6 shRNA 10, (D1 and D2) mouse treated with U6 shRNA 5, (E1 and E2) mouse treated with U6 shRNA 6. Mice subjected to the hydrodynamic injection procedure were sacrificed after 7 days and the livers analysed using immunohistochemistry to detect the HBcAg. Representative data, High- and low-power fields are shown for livers from pCR-HBVA1 1.3x together with shRNA sequences.

CHAPTER 4

4 DISCUSSION

The study of gene therapy against HBV infection is hampered by the lack of useful research models. This is also true for chronic HBV infection in southern Africa, in which HBV subgenotype A1 is hyperendemic and associated with high incidence of liver cancer and cirrhosis. The data presented in this study as well as other studies demonstrate the potential for the development of vectors for the study of HBV replication *in vitro* and *in vivo*. These vectors can be used to assess potential therapeutic strategies (such as RNAi against HBV) against regionally significant subgenotypes of HBV.

4.1 Replication competent vectors of HBV

The ability to generate greater than genome length constructs capable of HBV replication and gene expression at levels comparable to that in naturally infected liver has been shown to be dependent on unique cloning criteria. Guidotti and colleagues (35) identified the criteria that can increase HBV expression and replication both *in vitro* and *in vivo.* The construct was successfully generated by cloning the HBV promoter (with Enhancer I and II) immediately upstream of a complete viral genomic

sequence and the unique polyadenylation signal immediately downstream of the full genome. These criteria were also shown to be necessary for tissue specificity in that, high levels of HBV replication and gene expression were achieved in the liver tissues. Similarly, pCR-HBVA1 1.3x was generated using the same criteria to repeat the BCP sequence upstream and the polyadenylation sequence downstream of the full-length HBV sequence.

In vitro and *in vivo* analysis of pCR-HBVA1 1.3x and pCR-HBVA1 1x revealed that pCR-HBVA1 1.3x was able to increase levels of HBV expression and replication in liver whereas pCR-HBVA1 1x are defective for viral replication. In principle, HBV replication requires the BCP and pA sequences to be repeated upstream and downstream of a full length HBV sequence. pCR-HBVA1 1.3x demonstrates the potential of these vectors for the study of HBV replication and gene expression as well as the assessment of potential therapeutic agents against HBV infection.

4.2. Reporter plasmids

To facilitate studies on viral replication particularly when assessing potential therapeutic agents against HBV the generation of vectors expressing reporter proteins as markers of HBV replication can expedite analysis of therapeutic accuracy and efficiency. Numerous studies show that reporter proteins such as β-galatosidase,

EGFP and Luciferase (71) can be used to assess expression of genes of interest. The same principle can be applied to analysis of HBV gene expression.

Passman and colleagues (20) demonstrated that replacing the *surface* ORF of pCH-9/3091 with the sequence encoding enhanced Green Fluorescent Protein (EGFP) generated a vector capable of expressing EGFP as a marker of HBV replication. Similarly, in this study pCH-FLuc was generated using a technique to insert the *Firefly luciferase* gene into the position previously occupied by *surface* ORF. *In vitro* analysis of cells transfected with pCH-FLuc showed high expression of Firefly luciferase. Commercially available Dual Luciferase assays allows for the co-transfection of constitutively active *Renilla* luciferase expressing vector, which allows for normalisation of Firefly luciferase activity thereby improving the accuracy of this reporter system.

Use of this vector allows for rapid assessment of antiviral efficacy as compared to techniques such as real-time PCR. This vector by no means supplants vectors such as pCR-HBVA1 1.3x but it does allow for the rapid screening of large data sets. Employing a reporter plasmid has additional advantage in that it overcomes the problem of HBV mutants that are not detected by commercial immunoassays and allows for accurate quantitation of viral expression.

4.3 RNAi against HBV replication

That RNAi can be utilised to therapeutic effect against HBV has been well documented (9, 48, 64, 66, 67, 72). Other areas in this field still need to be explored, such as the efficacy of RNAi against regionally significant subgenotypes of HBV (e.g. A1) and the utility of using a surrogate reporter plasmid of HBV to screen efficacy of RNAi sequences.

4.3.1 shRNA against replication competent vectors of HBV

Carmona and colleagues (48) generated 10 U6 encoded short hairpin (U6 shRNAs) that target conserved sequences of the oncogenic *HBx* open reading frame of pCH-9/3091. Of these hairpins, U6 shRNA 5 and U6 shRNA 6 were selected in this study as these were shown to significantly knockdown markers of HBV replication in transfected hepatocytes and in a murine hydrodynamic injected model of HBV replication. U6 shRNA 10 was also used as it does not cause significant knockdown of markers of HBV replication. The hairpins were used to test for local SA HBV isolates (HBV subgenotype A1). Similarly, co-transfection of U6 shRNA 5 and U6 shRNA 6 with an HBV replication competent vector (pCR-HBVA1 1.3x) led to a significant reduction in viral mRNA expression and viral protein production in mammalian cells. The U6 shRNA 10 did not efficiently inhibit replication of the virus. In a murine hydrodynamic injection model of HBV replication, both U6 shRNA 5 and

U6 shRNA 6 caused significant inhibition in markers of HBV replication to near baseline levels and U6 shRNA 10 did not inhibit replication of the virus. This effect was sustained for 7 days. Immunohistochemical staining of liver sections revealed a strong association between the decline of HBV serum marker levels by U6 shRNA 5 and U6 shRNA 6. U6 shRNA 10 did not cause any significant histological changes in liver tissues from that observed in the mock treatments.

4.3.2 shRNA against reporter plasmids

The reporter plasmid (pCH-FLuc) enabled rapid assessment of antiviral efficacy *in vitro*. Co-transfection of U6 shRNA 5 and U6 shRNA 6 with the reporter plasmid led to a significant reduction of Firefly luciferase activity, whereas U6 shRNA 10 did not decrease the level of luciferase expression. *In vitro* studies transfecting pCH-FLuc with different amounts of the U6 shRNA-expressing plasmids showed significant decreases in Firefly luciferase expression by U6 shRNA 5 and U6 shRNA 6. Again U6 shRNA 10 did not decrease the level of luciferase expression at any dose. These data show that low doses of U6 shRNA 5 and U6 shRNA 6 caused significant decrease Firefly luciferase activity in mammalian cell culture. That Firefly luciferase activity is diminished upon administration of these shRNA expressing plasmids indicates that expression of these RNAi sequences correlates with HBV gene expression.

Both U6 shRNA 5 and U6 shRNA 6 were equally capable of inhibiting Firefly luciferase expression whereas U6 shRNA 10 was unable to inhibit replication of the virus. This data correlates with previous findings by Carmona and colleagues (48).

Further improvements of this model of HBV replication, such as the generation of stable cell lines and transgenic mice are necessary to assess potential therapeutic strategies against regionally significant subgenotypes of HBV. Development of delivery vehicles (viral and non viral vectors) as options for the effective deployment of siRNA or shRNA also need to be improved to increase the overall efficacy of this anti HBV treatment modality. Generation of siRNA that can induce interferon expression is also a major challenge in the field of RNAi pathways.

CHAPTER 5

5 CONCLUSION

In summary, we have demonstrated that the replication competent vectors of HBV (pCR-HBVA1 1.3x and pCH-FLuc) can recapitulate HBV replication *in vitro* and *in vivo*. Our results also show that anti HBV effecter sequences (U6 shRNA) inhibit viral gene expression, replication and reporter plasmid efficiently, both *in vitro* and *in vivo.*

The presence of the BCP and pA sequences in pCR-HBVA1 1.3x allowed this plasmid to simulate viral replication *in vitro* and *in vivo*. Mice injected with pCR-HBVA1 1.3x were able to express significant levels HBsAg, HBeAg, viral particles, as well as *surface* and *core* mRNA when compared to control vectors and indicate that the plasmid is replication competent. The ability of pCH-FLuc to express Firefly luciferase was tested in cultured mammalian cells. U6 shRNA 5 and U6 shRNA 6 (which are targeted to the *HBx* ORF) were able to specifically reduce HBV gene expression, replication and reporter gene expression both *in vitro* and *in vivo.* U6 shRNA 10 was unable to inhibit markers of HBV replication. These findings are in accordance with previous studies (48). The strategy presented in this study demonstrates the potential for the development of vectors for the study of HBV replication as well as the assessment of potential therapeutic strategies of regionally significant subgenotypes of HBV.

Future work will involve analysing intracellular viral replication intermediates, establishment of stably transfected liver cell lines producing HBV and also a transgenic mouse replicating HBV at a level similar to that found in chronic carriers of this virus.

6 APPENDICES

6.1. APPENDIX A: LABORATORY PROTOCOLS

6.1.1 Preparation of chemically competent *E.coli* and Transformation

Reagents

Luria-Bertani (LB) medium

Ten grams of Tryptone (Oxoid, UK.), 5 g Yeast Extract (Oxoid, UK) and NaCl were dissolved in 1 litre of deionised water. LB medium was sterilised by autoclaving for 20 minutes at 121°C and 1 kg/cm^2 and stored at room temperature.

Transformation buffer

Transformation buffer contained 100 mM $CaCl_2$, 10 mM PIPES-HCl and 15% Glycerol (the pH was adjusted to 7.0 with NaOH). The solution buffer was sterilised by autoclaving for 20 minutes at 121°C and 1 kg/cm^2. Transformation buffer was stored at -20°C.

Protocol

Preparing chemically competent E. coli

Luria Broth (LB, 10 ml) was inoculated with glycerol stocks of *E. coli* (50 µl). The inoculum was incubated at 37°C overnight, diluted (1:10) and grown to an

A_{600} of approximately 0.4. The cells were centrifuged (2000xg, 10 minutes) and pellets resuspended in transformation buffer (10 ml per 100 ml culture). The suspension was left on ice for 20 minutes, centrifuged (1500xg, 10 minutes) and the pellet resuspended in transformation buffer (1 ml). Aliquots (50 µl) of competent cells were stored at -70°C.

Reagents

1000^x Ampicillin or Kanamycin

One hundred milligrams of ampicillin (Roche, Germany) was dissolved in 1 ml of 50% ethanol.

Luria Bertani agar plate (Ampicillin or Kanamycin plate)

Bacterial plates were prepared containing 1% (w/v) Agar (Oxoid, UK) in LB medium. The mixture was autoclaved for 20 minutes at 121°C and 1 kg/cm^2 then cooled to 50°C. Ampicillin or kanamycin was added to a final concentration of 100 µg/ml of Luria Bertani agar solution. Luria Bertani agar plates were prepared by pouring the solution into Petri dishes and allowing agar to solidify at room temperature. Plates were stored at 4°C.

Protocol

Transforming chemically competent E. coli cells

For cell transformation, frozen stocks were thawed gently in ice and plasmid was added. The mixture was incubated on ice for 20 minutes and then heat-

shocked at 42°C for 90 seconds. The samples were transferred to ice and incubated for a further 2 minutes. Transformed bacteria were plated on agar plates containing ampicillin or kanamycin.

Reagent

***5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) stock solution*.**

Twenty milligrams of X-gal (Sigma, MO, USA) was dissolved in 1 ml dimethyl formamide. The solution is light sensitive and is therefore covered with aluminium foil and stored at -20°C.

Protocol

Preparation of X-gal positive Luria Bertani agar plates

To prepare X-gal positive Luria Bertani agar, 40 µl of X-gal and 8 µl of IPTG stock solution were added to ampicillin or kanamycin plates and spread evenly on the surface. The plates were dried in an incubator at 37°C for 30 minutes and stored at 4°C. Transformed *E.coli* cells were plated on ampicillin or kanamycin positive, X-gal, IPTG positive agar plates and incubated at 37°C overnight. IPTG induces expression of β-galactosidase, which cleaves the chromogenic substrate X-gal to yield a blue product. Successful cloning (plasmid positive for an insert) disrupts the β-galactosidase gene and therefore the protein is not functional resulting in white colonies. Unsuccessful insert of fragments leaves the β-galactosidase intact resulting in blue colonies.

6.1.2 EndoFree® plasmid maxi kit plasmid preparation (Qiagen, GmbH, Germany).

Reagents

Luria Bertani Medium *(See Appendix 6.1.1)*

1000^{x} Ampicillin or Kanamycin *(See Appendix 6.1.1)*

Protocols

Two hundred and fifty milliliters of Luria Bertani medium was inoculated with a single colony containing the plasmid of interest and incubated at 37°C with shaking (100 rpm) overnight. The cells were pelleted by centrifugation at 6000 rpm for 15 minutes at 4°C and the pellet resuspended in 10 ml Buffer P1 containing RNAse A at a concentration of 100 µg/ml. Ten millilitres of Buffer P2 (Lysis buffer) was added to the cell suspension, the solution mixed thoroughly and incubated at room temperature for no more than 5 minutes. Ten millilitres of chilled Buffer P3 (Neutralization buffer) was added to the lysate, mixed, applied to the Qiafilter cartridge and incubated at room temperature for 10 minutes. After the 10 minutes incubation, the lysate was filtered into a sterile 50 ml tube. Two and a half millilitres of endotoxin removal buffer (Buffer ER) was added to the filtrate, mixed and incubated on ice for 30 minutes. During the incubation a QIAGEN-tip 500 was equilibrated by applying the 10 ml of Buffer QBT (Equilibration buffer) to the columns and allowing the buffer to drain by gravity flow. After the incubation with endotoxin removal buffer the

solution was applied to the QIAGEN-tip and allowed to drain by gravity flow. The columns were washed twice with 30 ml of Buffer QC. The plasmids were eluted from the column with 15 ml Buffer QN (elution buffer). A 0.7x volume of isopropanol was used to precipitate the plasmids. The DNA was centrifuged at 8000 rpm for 1 hour at 4°C. The DNA pellets were washed with endotoxin-free 70% ethanol and re-centrifuged at 8000 rpm at 4°C for an additional hour. The pellets were air-dried and resuspended in an appropriate volume of endotoxin a free Tris-EDTA buffer.

6.1.3 Gel Electrophoresis

Regents

Tris Acetate EDTA Buffer (TAE)

Two hundred and forty two grams of Tris base was dissolved in 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8) .

Ethidium Bromide

One hundred milligrams of Ethidium Bromide was dissolved in 1 ml sterile water. Ethidium bromide solution was always covered with alumininium foil because it is light sensitive and stored at room temperature.

Protocol

For 0.8% agarose gel, 0.8 g of molecular grade agarose was mixed with 100 ml of 1x TAE and melted in a microwave for 2-4 minutes. The melted agarose was cooled at room temperature for 4 minutes before 5 µl of Ethidium Bromide solution was added. The mixture was then poured into a gel chamber and allowed to solidify. When it was ready to run, the gel was submerged in 1x TAE buffer in an electrophoresis chamber. The gels were run at 70 volts until the loading dye (Loading dye 6x, Fermentas, WI, USA) migrated to the edge of the gel. The DNA was visualized by placing the gel on an Ultraviolet (UV) Transilluminator.

6.1.4 Transfection

Reagents

1000x Penicillin/Streptomycin

Six hundred and ten milligrams of penicillin (100-000 U/ml, Invitrogen, Gibco-BRL, CA, USA) and 1 g of Streptomycin (100 mg/ml, Invitrogen, Gibco-BRL, CA, USA) was dissolved in 10 ml of sterile water. The solution was sterilised by filtration through a 0.22 µm filter and stored at -20°C.

Fetal calf serum (FCS)

Fetal calf serum (FCS, Highveld Biological, South Africa) was heat inactivated before use at 60°C for 30 minutes.

0.5x Trypsin

Five hundred microlitres of a 10x stock solution was made up to 50 ml and filter sterilised.

RPMI medium

RPMI medium was prepared as follows:10.4g of RPMI powder, 2g $NaHCO_3$, 1.19 g HEPES, 1 ml Na_2SeO_3 stock (5.19 mg/L), 1 ml $FeSO_4.7H_2O$ (27.8 mg/L) 1 ml $(NH_4)_6MO_7O_2$ (3.71 mg/L), $MnC_{l2}.4H_2O_2$ (0.059 mg/L), NH_4VO_3 (1.17 mg/L) and 1m ethanolamine (1832.4 mg/L) were dissolved in Sabax water (Adcock Ingram, South Africa). The solution was filter sterilized by filtration through a 0.22 µm filter and stored at 4°C.

Protocol

Human hepatoma cells, Huh7, were maintained in RPMI supplemented with 2.5% foetal calf serum, 1000x Penicillin/Streptomycin. On the day before transfection, 120 000 Huh7 cells were seeded in 1 ml per well of 4 cm^2 without antibiotics. For preparation of transfection sample, plasmid DNA was diluted in 100 µl Opti-MEM® I medium. Two microlitres of Lipofectamine™ 2000 (Invitrogen, CA, USA) was also diluted in 100 µl Opti-MEM® I medium. DNA:Opti-MEM and Lipofectamine™ 2000:Opti-MEM mixtures were incubated at room temperature for approximately 5 minutes. After the 5 minutes incubation, diluted DNA and Lipofectamine™ 2000 mixtures were combined, mixed gently, incubated for 20 minutes at room temperature. The 200 µl of

mixtures were added to each well containing Huh7 cells and growth medium. After transfection, complexes and cell medium were mixed by gentle agitation of the plate. Transfected cells were incubated for 48 hours at 37°C and 5% CO_2 prior to testing for gene expression. Medium was changed after 4-6 hours, and fresh medium (medium containing penicillin/streptomycin antibiotic) was added to transfected cells.

6.1.5 Dual-Luciferase® reporter Kit

Reagents

Preparation of Luciferase assay reagent II (LAR II)

To prepare LAR II, lypophilised luciferase assay substrate was resuspended in 10 ml of Luciferase assay buffer II, and mixed. LAR II solution (Promega, WI, USA) was always covered with aluminum foil because of its sensitivity to light and stored at -70 °C.

Preparation of 1x Passive Lysis buffer (PLB).

PLB is supplied as a 5x concentrate (Promega, WI, USA). To prepare a 1x working concentration, 1 volume of 5x PLB was mixed with 4 volumes of distilled water.

Preparation of Stop & Glo® Reagent.

Stop & Glo® Reagent is supplied at a 50x concentration (Promega, WI, USA). To prepare a working concentration, 1 volume of 50x Stop & Glo® substrate

was added to 50 volumes of Stop & Glo® Buffer in a 10 ml tube. Stop & Glo® solution was stored at -70°C.

Protocol

Briefly, growth medium was removed and cells washed with saline. Required volume of 1x PLB was added and cells were homogenized on a shaker for 15 minutes at room temperature. After the 15 minute incubation the lysates were aspirated and 20 µl of samples were transferred to a luminometer plate. To perform the Dual-Luciferase® reporter assay, the microplate was placed in the luminometer, followed by sequential auto-injection of the LAR II and Stop & Gluo® and initiate reading (Promega, WI, USA).

6.1.6 RNA Extraction

6.1.6.1 RNA extraction from cultured mammalian cells with Tri-reagent™

Regent

Tri-reagent™ (Sigma, MO, USA)

Monophase solution containing phenol and guanidine thiocyanate. This solution was stored at 4°C.

Protocol

The cells were lysed by repeated pipetting in 250 µl (per 12 well plate) of TRI reagent until a homogenous mixture was formed. The homogenate was

incubated at room temperature for 5 minutes, to promote complete dissociation of nucleoprotein complexes. Fifty microlitres of chloroform was added to the solution, vigorously mixed for 15 seconds and allowed to stand at room temperature for 2-5 minutes. After the incubation, the mixture was centrifuged for 15 minutes at 12 000xg at 4°C. Centrifugation separates the mixture into 3 phases: red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). The colorless upper aqueous phase was transferred to a new tube and the RNA was precipitated with 75 µl of isopropanol. The isopropanol mixture was stored at -20°C until ready to be used. After incubation, the mixture was centrifuged at 12 000xg for 10 minute at 4°C. The RNA pellet was re-centrifuged in 70% ethanol at 12 000xg for 10 minutes at 4°C and supernatant was discarded. The pellet was air-dried and resuspended in an appropriate volume of DEPC-treated water.

6.1.6.2 Extraction of RNA from mouse liver with guanidium thiocyanate

Reagents

Guanidium solution

Five hundred and ninety grams of guanidium thiocyanate was dissolved in 400 ml DEPC-treated water. Twenty five millilitres of 2 M Tris.HCl (pH 7.5) and 20 ml of 0.5 M Na_2EDTA (pH 8.0) were added to the solution and stirred overnight at 37^{o}C. After overnight incubation, the volume was adjusted to 950 ml with

sterile water and filtered. Fifty millilitres of 2-mercapto ethanol was added, and stored at room temperature for about three months.

2 M Sodium acetate

To prepare the sodium acetate solution, 16.42 g of sodium acetate was added to 40 ml sterile water and 35 ml of glacial acetic acid. The solution was adjusted to a pH of 4 with glacial acetic acid and the volume adjusted to 100 ml. The solution was stored at room temperature.

Water-saturated phenol

To prepare water-saturated phenol, 100 g of phenol crystals were dissolved in water at 60°C. The upper water phase was aspirated and stored at 4°C.

Diethylpyrocarbonate (DEPC) treated water

DEPC-treated water was prepared as follows: 1 ml DEPC (diethylpyrocarbonate) was added per 1000 ml of sterile water. The DEPC solution was incubated at 37°C for 16 hours and the DEPC inactivated by autoclaving for 20 minutes at 121°C and 1 kg/cm^2. DEPC treated water was stored at room temperature prior to use.

RNA Extraction protocol

The livers of the mice were harvested for total RNA preparation. One millilitre of guanidium solution (denaturation solution) was added to 100 mg of liver and tissue homogenised. The homogenised solution was transferred to a sterile 10 ml tube. One hundred microlitres of 2 M sodium acetate (pH 4) was added and

mixed thoroughly by inversion. One millilitre of water-saturated phenol was added to the solution, mixed thoroughly and 0.2 ml of 49:1 chloroform/isoamyl alcohol added. The solution was mixed and then incubated for 15 minutes at 4°C. The upper aqueous phase was removed, 1 ml of 100% isopropanol added, mixed and incubated at -20°C for about 16 hours. After incubation, the samples were centrifuged for 30 minutes at 10 000 g at 4°C. After centrifugation the supernatant was discarded. The RNA pellet was washed with 70% DEPC-ethanol, vortexed, and incubated for 15 minutes at room temperature, re-centrifuged at 10 000 g for 5 minutes at 4°C and supernatant was discarded. The pellet was air-dried and resuspended in an appropriate volume of DEPC-treated water.

6.1.7 Immunohistochemical staining

Reagent

HBcAg Antibody swell (Signet Cat)

Tris-buffered saline (TBS)

To prepare TBS buffer (1x), 6.05 g Tris and 8.7 g NaCl were dissolved in 800 ml of water. The solution was adjusted to a pH of 7.5 with 1 M HCl and the volume adjusted to 1 litre. The solution was stored at 4°C.

TBS with 0.05% Tween 20 (TTBS buffer).

Protocols

Livers were fixed in 20% paraformaldyhyde, processed, embedded in parafinwax 5 micro sections were mounted on Poly-L-Lysine coated slides. These were dewaxed, hydrated and rinsed in 0.05% Tween 20 in TBS (TTBS buffer). Using an Envision Kit (Dako Cat number K5007), sections underwent endogenous enzyme blocking for 30 minutes. The sections were rinsed again in TTBS buffer and then incubated with primary HBV core antibody, diluted 1:10 with diluting buffer for 45 minutes at room temperature. After washing with TTBS buffer the sections were labeled with secondary labeled polymer HRP and incubated for 30 minutes at room temperature. After the 30 minutes incubation, the sections were rinsed in TTBS buffer and washed in fresh solution for 5 minutes. After being washed, the sections were developed by substrate solution of Chromogen (2 drops of Diaminobenzidine in 2ml buffer) and incubated 10 minutes at room temperature. Thereafter liver sections were washed in distilled water, stained with Haematoxylin for 3 minutes. Liver sections were dehydrated, cleared and mounted. The HBcAg was observed as brown coloration under microscopy.

REFERENCES

1. Ganem D, Varmus HE. The molecular biology of the hepatitis B viruses. Annu Rev Biochem. 1987;56:651-93.
2. Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev. 2000 Mar;64(1):51-68.
3. Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. Lancet. 1981 Nov 21;2(8256):1129-33.
4. Kramvis A, Kew M, Francois G. Hepatitis B virus genotypes. Vaccine. 2005 Mar 31;23(19):2409-23.
5. Kramvis A, Weitzmann L, Owiredu WK, Kew MC. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. J Gen Virol. 2002 Apr;83(Pt 4):835-9.
6. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. CA Cancer J Clin. 1999 Jan-Feb;49(1):33-64, 1.
7. Hanazaki K. Antiviral therapy for chronic hepatitis B: a review. Curr Drug Targets Inflamm Allergy. 2004 Mar;3(1):63-70.
8. Colacino JM, Staschke KA. The identification and development of antiviral agents for the treatment of chronic hepatitis B virus infection. Prog Drug Res. 1998;50:259-322.

9. Arbuthnot P, Carmona S, Ely A. Exploiting the RNA interference pathway to counter hepatitis B virus replication. Liver Int. 2005 Feb;25(1):9-15.

10. Buendia MA. Hepatitis B viruses and cancerogenesis. Biomed Pharmacother. 1998;52(1):34-43.

11. Schaller H, Fischer M. Transcriptional control of hepadnavirus gene expression. Curr Top Microbiol Immunol. 1991;168:21-39.

12. Yuh CH, Chang YL, Ting LP. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. J Virol. 1992 Jul;66(7):4073-84.

13. Moolla N, Kew M, Arbuthnot P. Regulatory elements of hepatitis B virus transcription. J Viral Hepat. 2002 Sep;9(5):323-31.

14. Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol. 1992 May;73 (Pt 5):1201-8.

15. Kramvis A, Kew MC. Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. J Viral Hepat. 2005 Sep;12(5):456-64.

16. Kimbi GC, Kramvis A, Kew MC. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. J Gen Virol. 2004 May;85(Pt 5):1211-20.

17. Kew MC, Kramvis A, Yu MC, Arakawa K, Hodkinson J. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. J Med Virol. 2005 Apr;75(4):513-21.

18. Guha C, Mohan S, Roy-Chowdhury N, Roy-Chowdhury J. Cell culture and animal models of viral hepatitis. Part I: hepatitis B. Lab Anim (NY). 2004 Jul-Aug;33(7):37-46.

19. Lu M, Roggendorf M. Evaluation of new approaches to prophylactic and therapeutic vaccinations against hepatitis B viruses in the woodchuck model. Intervirology. 2001;44(2-3):124-31.

20. Passman M, Weinberg M, Kew M, Arbuthnot P. In situ demonstration of inhibitory effects of hammerhead ribozymes that are targeted to the hepatitis Bx sequence in cultured cells. Biochem Biophys Res Commun. 2000 Feb 24;268(3):728-33.

21. Galle PR, Hagelstein J, Kommerell B, Volkmann M, Schranz P, Zentgraf H. In vitro experimental infection of primary human hepatocytes with hepatitis B virus. Gastroenterology. 1994 Mar;106(3):664-73.

22. Sells MA, Zelent AZ, Shvartsman M, Acs G. Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. J Virol. 1988 Aug;62(8):2836-44.

23. Dandri M, Volz TK, Lutgehetmann M, Petersen J. Animal models for the study of HBV replication and its variants. J Clin Virol. 2005 Dec;34 Suppl 1:S54-62.

24. Dunsford HA, Sell S, Chisari FV. Hepatocarcinogenesis due to chronic liver cell injury in hepatitis B virus transgenic mice. Cancer Res. 1990 Jun 1;50(11):3400-7.

25. Koike K, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T, et al. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. Hepatology. 1994 Apr;19(4):810-9.

26. Barker LF, Chisari FV, McGrath PP, Dalgard DW, Kirschstein RL, Almeida JD, et al. Transmission of type B viral hepatitis to chimpanzees. J Infect Dis. 1973 Jun;127(6):648-62.

27. Bertoni R, Sette A, Sidney J, Guidotti LG, Shapiro M, Purcell R, et al. Human class I supertypes and CTL repertoires extend to chimpanzees. J Immunol. 1998 Oct 15;161(8):4447-55.

28. Shikata T, Karasawa T, Abe K. Two distinct types of hepatitis in experimental hepatitis B virus infection. Am J Pathol. 1980 May;99(2):353-67.

29. Ogata N, Cote PJ, Zanetti AR, Miller RH, Shapiro M, Gerin J, et al. Licensed recombinant hepatitis B vaccines protect chimpanzees against infection with the prototype surface gene mutant of hepatitis B virus. Hepatology. 1999 Sep;30(3):779-86.

30. Summers J, Smolec JM, Snyder R. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc Natl Acad Sci U S A. 1978 Sep;75(9):4533-7.

31. Roggendorf M, Tolle TK. The woodchuck: an animal model for hepatitis B virus infection in man. Intervirology. 1995;38(1-2):100-12.

32. Marion PL, Oshiro LS, Regnery DC, Scullard GH, Robinson WS. A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. Proc Natl Acad Sci U S A. 1980 May;77(5):2941-5.

33. Testut P, Renard CA, Terradillos O, Vitvitski-Trepo L, Tekaia F, Degott C, et al. A new hepadnavirus endemic in arctic ground squirrels in Alaska. J Virol. 1996 Jul;70(7):4210-9.

34. Mason WS, Seal G, Summers J. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J Virol. 1980 Dec;36(3):829-36.

35. Guidotti LG, Matzke B, Schaller H, Chisari FV. High-level hepatitis B virus replication in transgenic mice. J Virol. 1995 Oct;69(10):6158-69.

36. Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. Proc Natl Acad Sci U S A. 2002 Oct 15;99(21):13825-30.

37. Ueda H, Ullrich SJ, Gangemi JD, Kappel CA, Ngo L, Feitelson MA, et al. Functional inactivation but not structural mutation of p53 causes liver cancer. Nat Genet. 1995 Jan;9(1):41-7.

38. Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity. 1996 Jan;4(1):25-36.

39. Zhang G, Gao X, Song YK, Vollmer R, Stolz DB, Gasiorowski JZ, et al. Hydroporation as the mechanism of hydrodynamic delivery. Gene Ther. 2004 Apr;11(8):675-82.

40. Andrianaivo F, Lecocq M, Wattiaux-De Coninck S, Wattiaux R, Jadot M. Hydrodynamics-based transfection of the liver: entrance into hepatocytes of DNA that causes expression takes place very early after injection. J Gene Med. 2004 Aug;6(8):877-83.

41. Arbuthnot P, Kew M. Hepatitis B virus and hepatocellular carcinoma. Int J Exp Pathol. 2001 Apr;82(2):77-100.

42. Karayiannis P. Hepatitis B virus: old, new and future approaches to antiviral treatment. J Antimicrob Chemother. 2003 Apr;51(4):761-85.

43. Lin OS, Keeffe EB. Current treatment strategies for chronic hepatitis B and C. Annu Rev Med. 2001;52:29-49.

44. De Clercq E. Perspectives for the treatment of hepatitis B virus infections. Int J Antimicrob Agents. 1999 Jul;12(2):81-95.

45. Ganem D, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. N Engl J Med. 2004 Mar 11;350(11):1118-29.

46. Papatheodoridis GV, Dimou E, Papadimitropoulos V. Nucleoside analogues for chronic hepatitis B: antiviral efficacy and viral resistance. Am J Gastroenterol. 2002 Jul;97(7):1618-28.

47. Zhou DY, Cao YJ, Lin LY, Wang H, Huang JS. HBV resistant to lamivudine: experimental and clinical studies. Hepatobiliary Pancreat Dis Int. 2002 Nov;1(4):519-22.

48. Carmona S, Ely A, Crowther C, Moolla N, Salazar FH, Marion PL, et al. Effective inhibition of HBV replication in vivo by anti-HBx short hairpin RNAs. Mol Ther. 2006 Feb;13(2):411-21.

49. Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol. 2003 Jun;4(6):457-67.

50. Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK. RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev. 2003 Dec;67(4):657-85.

51. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet. 2002 Oct;3(10):737-47.

52. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998 Feb 19;391(6669):806-11.

53. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 2001 Jan 15;15(2):188-200.

54. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001 Jan 18;409(6818):363-6.

55. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. Cell. 2003 Oct 17;115(2):199-208.

56. Wu S, Kaufman RJ. A model for the double-stranded RNA (dsRNA)-dependent dimerization and activation of the dsRNA-activated protein kinase PKR. J Biol Chem. 1997 Jan 10;272(2):1291-6.

57. Lipardi C, Wei Q, Paterson BM. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell. 2001 Nov 2;107(3):297-307.

58. Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, et al. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell. 2001 Nov 16;107(4):465-76.

59. Grosshans H, Slack FJ. Micro-RNAs: small is plentiful. J Cell Biol. 2002 Jan 7;156(1):17-21.

60. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science. 2001 Oct 26;294(5543):858-62.

61. Llave C, Kasschau KD, Carrington JC. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. Proc Natl Acad Sci U S A. 2000 Nov 21;97(24):13401-6.

62. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, et al. Genes and mechanisms related to RNA interference regulate expression of the small

temporal RNAs that control C. elegans developmental timing. Cell. 2001 Jul 13;106(1):23-34.

63. Rozema DB, Ekena K, Lewis DL, Loomis AG, Wolff JA. Endosomolysis by masking of a membrane-active agent (EMMA) for cytoplasmic release of macromolecules. Bioconjug Chem. 2003 Jan-Feb;14(1):51-7.

64. Ying RS, Zhu C, Fan XG, Li N, Tian XF, Liu HB, et al. Hepatitis B virus is inhibited by RNA interference in cell culture and in mice. Antiviral Res. 2007 Jan;73(1):24-30.

65. McRobert L, McConkey GA. RNA interference (RNAi) inhibits growth of Plasmodium falciparum. Mol Biochem Parasitol. 2002 Feb;119(2):273-8.

66. McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, et al. Inhibition of hepatitis B virus in mice by RNA interference. Nat Biotechnol. 2003 Jun;21(6):639-44.

67. Giladi H, Ketzinel-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E. Small interfering RNA inhibits hepatitis B virus replication in mice. Mol Ther. 2003 Nov;8(5):769-76.

68. Nassal M, Junker-Niepmann M, Schaller H. Translational inactivation of RNA function: discrimination against a subset of genomic transcripts during HBV nucleocapsid assembly. Cell. 1990 Dec 21;63(6):1357-63.

69. Castanotto D, Li H, Rossi JJ. Functional siRNA expression from transfected PCR products. Rna. 2002 Nov;8(11):1454-60.

70. Song E, Zhu P, Lee SK, Chowdhury D, Kussman S, Dykxhoorn DM, et al. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. Nat Biotechnol. 2005 Jun;23(6):709-17.

71. Gouskos T, Wightman F, Lewin SR, Torresi J. Highly reproducible transient transfections for the study of hepatitis B virus replication based on an internal GFP reporter system. J Virol Methods. 2004 Oct;121(1):65-72.

72. Konishi M, Wu CH, Wu GY. Inhibition of HBV replication by siRNA in a stable HBV-producing cell line. Hepatology. 2003 Oct;38(4):842-50.