INHIBITING HEPATITIS B VIRUS REPLICATION WITH SHORT HAIRPIN RNA SEQUENCES THAT TARGET THE VIRAL X OPEN READING FRAME

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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

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DECLARATION

I, Abdullah Ely declare that this dissertation is my own work. It is being submitted for the degree of Master of Science 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 ……, 2005

To my parents,

Gava and Yagya Ely

PUBLICATIONS AND PRESENTATIONS

Publications

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

Conference Proceedings

1. Ely A, Weinberg MS, Moolla N, Carmona S, Arbuthnot PB. Inhibiting HBV Gene Expression in Cultured Cells using Micro-RNA. International Meeting of the Molecular Biology of the Hepatitis B Viruses; 2003 Sep 7-10; Bergamo, Italy.

2. Ely A. Pol II and Pol III promoter-derived short hairpin RNA mediate silencing of the hepatitis B virus. The Faculty of Health Sciences Research Day; 2004 Aug 4th; Parktown, South Africa.

3. Ely A, Carmona S, Moolla N, Crowther C, Passman M, Weinberg MS, Arbuthnot PB. Using Short Hairpin RNAs that Target the Hepatitis B Virus X Open Reading Frame to Inhibit Viral Replication. RNAi Europe; 2004 Oct 18-19; London, England.

4. Carmona S, Moolla N, Ely A, Crowther C, Passman M, Weinberg MS, Arbuthnot PB. Effective Inhibition of HBV Replication with Short Hairpin RNAs that Target the Viral X Open Reading Frame. Keystone Symposia; 2005 Jan 8-14; Colorado, USA.

5. Carmona S., Ely A., Moolla N., Crowther C., Weinberg M. and Arbuthnot P. Inhibiting HBV replication with short hairpin RNA and siRNAs that target the viral X open reading frame. The 5th anniversary International Symposium for Gene Design and Delivery, 2005 May 20-21, Tokyo, Japan.

Patents

1. Arbuthnot PB, Weinberg MS, inventors. Interference and inhibition of viral gene expression using expression cassettes that comprise a combination of ribozymes with either siRNA or miRNA. Patent Number PCT/IB2004/002816. PCT application filed 1 September 2004.

ABSTRACT

Chronic infection with the hepatitis B virus (HBV) is endemic to sub-Saharan Africa and south-east Asia where it is a major risk factor for the development of cirrhosis and hepatocellular carcinoma (HCC). Currently available therapy is only effective in a small subset of chronic carriers. The development of novel treatment modalities for the management of HBV therefore remains an important global medical objective. Sequence plasticity of the HBV genome is limited by its small size and the overlapping nature of its open reading frames (ORFs). These features make HBV an ideal target for therapy based on nucleic acid hybridization. The use of ribozymes (RNA enzymes) and antisense molecules to inhibit gene expression is well documented. The recent discovery of RNA interference (RNAi) has added to the arsenal of therapy based on nucleic acid hybridization. RNAi is the process whereby short RNA duplexes (called short interfering RNA or siRNA) mediate the sequence-specific post-transcriptional silencing of genes homologous in sequence to the siRNA. siRNA function by guiding a protein complex (RNA Induced Silencing Complex or RISC) to target mRNA for degradation or translational repression. The protein X ORF (*HBx* ORF) is a conserved region of the HBV genome and is common to all viral transcripts. HBx is required for infection by the virus and plays an important role in the establishment of chronic infections *in vivo* as well as in the development of HCC. RNAi targeted against the *HBx* ORF may therefore prove useful as treatment of chronic HBV infection.

Plasmid based expression cassettes capable of endogenously generating short hairpin RNA (shRNA) targeted to the *HBx* ORF were constructed. The shRNA function as substrates for the RNAi machinery and are processed into siRNA. The ability of the expression cassettes to knockdown markers of HBV gene expression was tested in a human hepatoma cell line. A panel of 10 U6 promoter-driven shRNA expression vectors was generated. The U6 promoter (an RNA polymerase III promoter) is normally involved in the transcription of small nuclear RNA and as such is ideal for the generation of shRNA of precisely defined length. Three cytomegalovirus (CMV) promoter-driven shRNA expression cassettes incorporating ribozymes that produce defined hairpin sequences were also generated. The CMV promoter (an RNA polymerase II) promoter is involved in the transcription of large messenger RNA. Two hammerhead ribozymes lying 5' and 3' of the shRNA encoding sequence were incorporated into the cassette. *Cis*-cleavage by the ribozymes releases a shRNA of defined length thereby overcoming the limitations imposed by extraneous sequences from CMV promoter-driven transcription. U6 promoter-driven shRNA expression vectors efficiently knocked down markers of HBV replication in liver cells. The CMV promoter-driven expression vectors were incapable of inhibiting HBV gene expression; however shRNA generated *in vitro* from these vectors mediated efficient knockdown of HBV replication. shRNA-mediated inhibition of gene expression therefore holds promise as a novel treatment strategy for the management of HBV and other mobile genetic elements.

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1. ATP	-	adenosine triphosphate
2. bp	-	base pair
3. cccDNA	-	covalently closed circular DNA
4. CMV	-	cytomegalovirus
5. Da	-	Dalton
6. ddATP	-	dideoxyadenosine triphosphate
7. ddCTP	-	dideoxycytosine triphosphate
8. ddGTP	-	dideoxyguanosine triphosphate
9. ddNTP	-	dideoxynucleoside triphosphate
10. ddTTP	-	dideoxythymidine triphosphate
11. DEPC	-	diethyl pyrocarbonate
12. DNA	-	deoxyribonucleic acid
13. dsRNA	-	double-stranded RNA
14. EDTA	-	ethylene diamine tetra-acetic acid
15. EGFP	-	enhanced green fluorescent protein
16. ELISA	-	enzyme linked immunosorbent assay
17. FCS	-	foetal calf serum
18. FITC	-	fluorescein isothiocyanate
19. HBV	-	hepatitis B virus
20. HBcAg	-	hepatitis B virus core antigen

21. HBeAg	-	hepatitis B virus e antigen
22. HBsAg	-	hepatitis B virus surface antigen
23. HBx	-	hepatitis B virus X protein
24. HCC	-	hepatocellular carcinoma
25. Huh7	-	human hepatoma cell line 7
26. IPTG	-	isopropyl-β -D-thiogalactopyranosid
27. kb	-	kilobase
28. miRNA	-	microRNA
29. miRNP	-	miRNA ribonucleoprotein
30. mRNA	-	messenger RNA
31. nm	-	nanometer
32. nt	-	nucleotide
33. OAS	-	oligoadenylate synthetases
34. ORF	-	open reading frame
35. PKR	-	protein kinase R
36. pre-miRNA	-	precursor miRNA
37. pri-miRNA	-	primary precursor miRNA
38. pmol	-	picomole
39. rATP	-	riboadenosine triphosphate
40. rCTP	-	ribocytosine triphosphate
41. RdRP	-	RNA-dependant RNA polymerase
42. RISC	-	RNA induced silencing complex

43. rGTP	-	riboguanosine triphosphate
44. RNA	-	ribonucleic acid
45. RNase	-	ribonuclease
46. rpm	-	revolutions per minute
47. RPMI medium	-	Roswell Park Memorial Institute medium
48. rUTP	-	ribouridine triphosphate
49. Rz	-	ribozyme
50. shRNA	-	short hairpin RNA
51. siRNA	-	short interfering RNA
52. stRNA	-	small temporal RNA
53. X-gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LIST OF SYMBOLS

1. α - alpha
2. β - beta
3. μ - mu (μl - microlitre)

CHAPTER 1

1 INTRODUCTION

1.1 The Hepatitis B Virus

1.1.1 Hepatitis B virus biology

The hepatitis B virus (HBV) causes both chronic and acute infections of the liver. Chronic infection with HBV is a global medical problem as it is estimated that 350 million people worldwide are chronically infected with HBV (1). Chronic infection with HBV is a major risk factor for the development of cirrhosis and hepatocellular carcinoma (HCC). HCC is the fifth most important cancer worldwide in terms of sheer numbers (5.4% or 437,000 of new cancer cases) and fourth in mortality rates (8.2% or 427, 000 of total cancer-related deaths) (2). HBV and hepatitis C virus, are estimated to be the cause of 75% of HCC cases in developed countries and 85% of HCC cases in developing countries (2). An effective vaccine has been available for 20 years and in areas where HBV immunization has been introduced the incidence of chronic HBV infections and HCC has decreased (2). Vaccination however does not treat established infections. Currently, interferon-α (an immunomodulator) and the two nucleoside analogues, lamivudine and adefovir dipivoxil, are the only antiviral strategies licensed for the treatment of chronic HBV infection (3). The use of interferon-α and nucleoside analogues is only effective in a subset of chronic HBV carriers (i.e. HBV e antigen (HBeAg)-positive carriers). In addition, the use of nucleoside analogues leads to the

development of resistant strains of HBV (4). This highlights the need for the development of novel treatment strategies for the management of chronic HBV infection. The use of RNA interference (RNAi) to knockdown gene expression holds promise as a novel anti-HBV treatment strategy. The current investigation is aimed at developing treatment strategies capable of exploiting the RNAi pathway to inhibit HBV gene expression.

1.1.2 HBV life cycle

HBV belongs to the *Hepadnaviridae* family of viruses (1), and as the name suggests these viruses have DNA genomes and exhibit hepatotropism. HBV is an enveloped virus that has its partially double-stranded DNA genome encapsidated in a nucleocapsid together with the viral polymerase. Early events in the life cycle of HBV, including entry, uncoating and translocation of the viral genome to the nucleus (Figure 1.1) are poorly understood due to the lack of cell lines susceptible to HBV infection. HBV is thought to interact with an as yet unidentified receptor and enter its host cell by receptor-mediated endocytosis. Once the virus has entered the cell it is uncoated and translocated to the nucleus, where the partially double-stranded DNA genome undergoes DNA repair to form covalently closed circular DNA (cccDNA) (5). The cccDNA then serves as a template for the transcription of viral RNA by host RNA polymerase II. Transcription under the control of four internal promoters and the two viral enhancer elements, En1 and En2, produces pregenomic RNA as well as three classes of subgenomic mRNA from the cccDNA template (1). The subgenomic mRNA codes for the

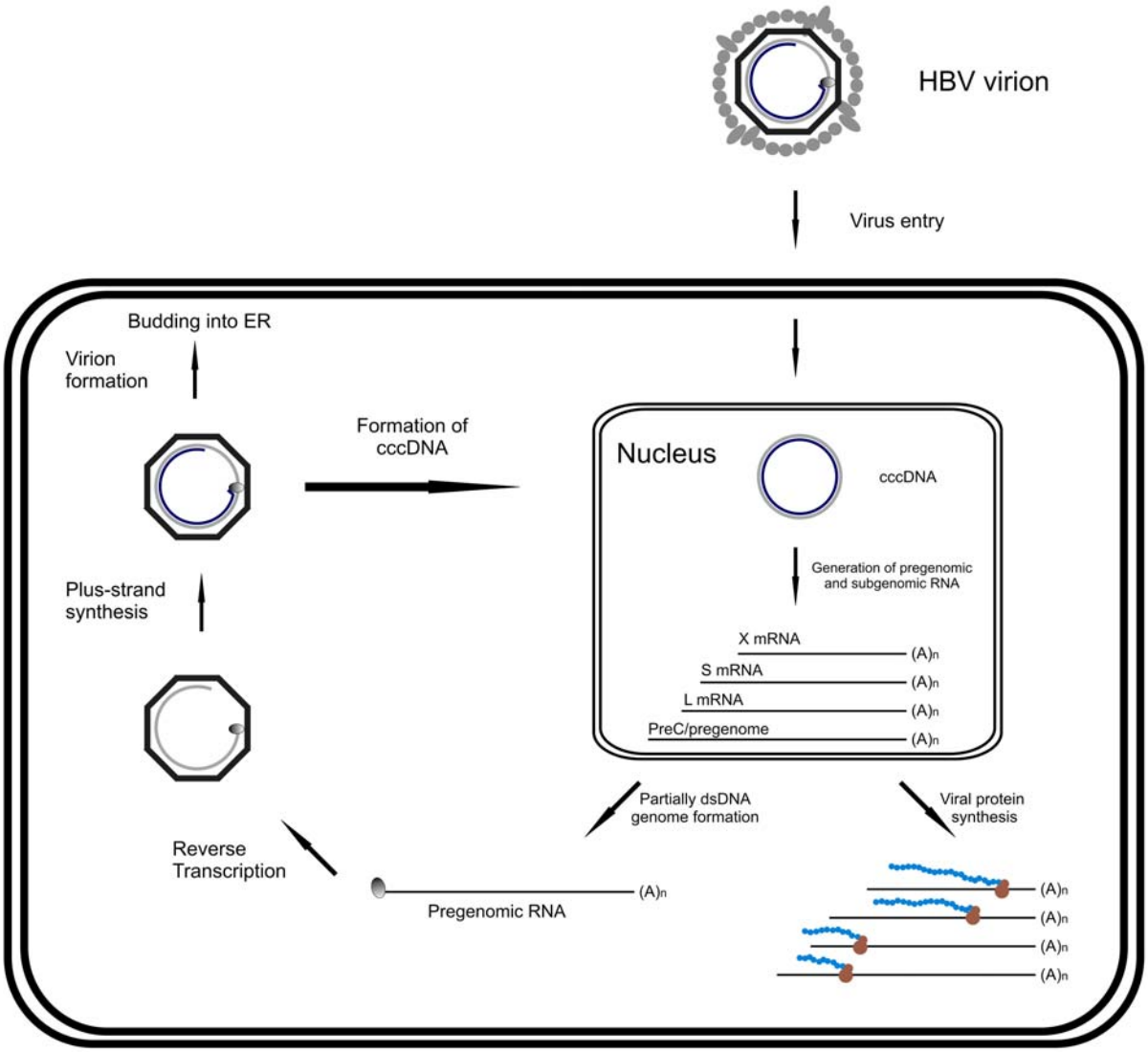


Figure 1.1: **Hepatitis B Virus life cycle.** Upon entry into the host cell the partially double-stranded DNA genome is converted to cccDNA. cccDNA functions as template for the transcription of viral RNA. Pregenomic RNA is translated into polymerase, which first reverse transcribes the pregenome to generate the minus strand of the genome followed by plus strand synthesis. The DNA genome can re-enter the nucleus to form more cccDNA or be enveloped for secretion by Golgi apparatus. (Adapted from Seeger and Mason (1)).

three surface antigens and the X protein (HBx). Pregenomic RNA is a greater than genome length RNA with terminal sequence duplication, and codes for the viral polymerase and precore proteins (6). The pregenomic RNA also functions as a replication intermediate for the reverse transcription of the HBV genome. Pregenomic RNA is encapsidated into immature nucleocapsids together with the viral polymerase. Within these nucleocapsids the pregenome is reverse transcribed to generate the partially double-stranded DNA genome. Once reverse transcription is complete the nucleocapsids undergo maturation when an outer envelope is acquired and the mature virion is released from the cell. Alternatively, the nucleocapsids may be redirected to the nucleus for the formation of cccDNA.

1.1.3 HBV genome structure and viral proteins

HBV contains an approximately 3.2 kilobase (kb) partially double-stranded DNA genome (Figure 1.2). Viral particles mainly contain a complete minus strand with the viral polymerase covalently attached to its 5' end. The circular structure of the HBV genome is maintained by the incomplete plus strand, which sits astride the 5' and 3' ends of the minus strand. The genome of HBV is organized into four open reading frames (ORFs) encoding the precore/core proteins, viral polymerase, HBx and the three HBV surface antigens (HBsAg), PreS1, PreS2 and S. Transcription from the four ORFs give rise to the pregenomic RNA and the three subgenomic mRNAs. A single polyadenylation signal results in all viral transcripts having identical 3' ends. The 3.5 kb pregenomic RNA contains the ORFs for the translation of viral polymerase and the precore/core proteins. Translation of the precore/core ORF is

initiated at two different initiation sites. Precore protein is processed via the Golgi apparatus and secreted as HBeAg. Translation from the second initiation codon of the precore/core

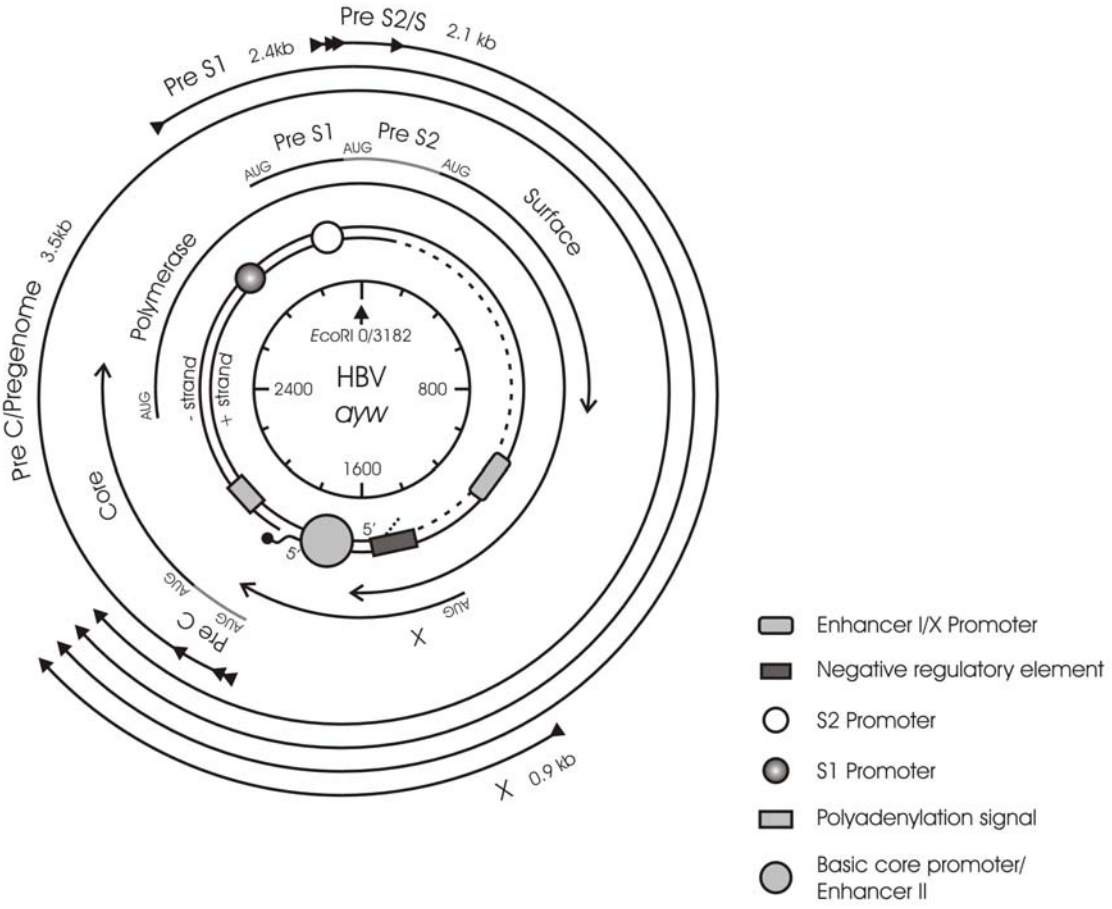


Figure 1.2: <u>Hepatitis B Virus genome organization</u>. Co-ordinates are shown relative to the single *Eco*RI restriction site. The partially double-stranded DNA genome is shown with the viral polymerase covalently attached. Different transcription initiation sites are represented on the HBV genome by circular and rectangular objects. Arrows immediately surrounding the HBV genome indicate the four open reading frames. The four outer arrows represent viral transcripts. Adapted from Passman, Weinberg, Kew and Arbuthnot (7).

ORF generates core proteins (HBcAg), which forms the nucleocapsid. The 2.4 kb RNA transcript encodes the PreS1 antigen whereas the 2.1 kb encodes the PreS2 and S antigens. The 0.9 kb RNA transcript codes for HBx.

1.2 HBV Management

An effective vaccine is at present available (1), vaccination however does not treat established infections. The immunomodulator, interferon-α, and the nucleoside analogues lamivudine and adefovir are currently the only licensed treatment strategies for the management of chronic HBV infection (4). Patients chronically infected with HBV have attenuated T cell responses, whereas the rapid clearance of HBV during an acute infection is attributed to a robust T cell response (3). Therefore, the immunomodulator, interferon-α is used to boost the T cell response against HBV and in so doing aid in clearance of the virus (3). Interferon-α, however is only effective in chronic carriers with a high replicative state (i.e. in HBeAg positive carriers). The use of interferon-α is also associated with a number of adverse side effects, of which leucopoenia, fatigue and depression are the most common. Nucleoside analogues are chemically synthesized molecules that are structurally similar to naturally occurring nucleosides. The structural similarity of nucleoside analogues to nucleosides allow these molecules to be incorporated into HBV DNA during DNA polymerization (3). Incorporation of nucleoside analogues into the nascent DNA chain terminates polymerization, thereby inhibiting viral replication. Lamivudine has been shown to be equally effective in

HBeAg-negative carriers as in HBeAg-positive carriers. The emergence of drug-resistant strains of HBV following treatment with lamivudine represents a major drawback to the use of nucleoside analogues. HBV polymerase like other RNA-dependant DNA polymerases contains the YMDD (tyrosine-methionine-aspartate-aspartate) motif in its catalytic site. Mutations within this motif (methionine to lysine (YVDD) and methionine to isoleucine (YIDD)) are known to confer resistance to lamivudine (3).

The development of novel treatment strategies for the management of chronic HBV therefore remains an important medical objective. One such approach is the use of ribozymes or RNA enzymes. Ribozymes are naturally occurring RNA molecules capable of sequence-specific cleavage and splicing (8). Target site recognition is dictated by the ribozyme sequence, which is designed to be complementary to that of the target RNA. The anti-HBV activity of ribozymes have been demonstrated (7, 9). In recent years the use of RNA interference (RNAi) as a novel antiviral treatment modality (10-15) has become quite popular. RNAi is based on nucleic acid hybridization, where a 'guide' RNA binds to a complementary region in the 'target' RNA thereby mediating inhibition of the target RNA. HBV has a very compact genome consisting of overlapping ORFs with many of the viral transcripts coding for more than one protein. These characteristics severely limit the sequence plasticity of the HBV genome making it an ideal target for therapy based on nucleic acid hybridization. RNAi therefore represents a potentially effective treatment strategy for the management chronic HBV infections.

1.3 RNA Interference

The term RNA interference or RNAi was first coined in 1998 when Andrew Fire and colleagues demonstrated that injection of double-stranded RNA (dsRNA) into the nematode worm *Caenorhabditis elegans* leads to the potent and specific silencing of genes homologous in sequence to the injected dsRNA (16). Not only was gene silencing effected throughout the body of the worm but RNAi was also observed in the first generation offspring. The effects of injecting dsRNA into the worms were reversible and therefore did not reflect a stable genetic change. Later the same year it was demonstrated that the dsRNA effectors of RNAi could also be delivered by feeding nematode worms with bacteria expressing dsRNA from recombinant plasmids (17). With the discovery that dsRNA was the effector molecule of RNAi two previously unexplained gene silencing phenomenon termed co-suppression in plants and quelling in fungi were linked to RNAi (18, 19).

1.3.1 The mechanism of RNAi

The development of a cell-free system capable of recapitulating RNAi *in vitro* provided a biochemical framework for experimental investigation of the mechanism of RNAi (20). RNAi is initiated by the introduction of dsRNA into cells (Figure 1.3). The dsRNA is processed into short RNA fragments (called short interfering RNA or siRNA) that are 21 to 22 nucleotides (nt) in length with 2 nt 3' overhangs (21). siRNA are processed from larger dsRNA by the ribonuclease (RNase) III, Dicer (22) and incorporated into a protein complex

called the **R**NA **I**nduced **S**ilencing **C**omplex (RISC). The sense strand of the siRNA is removed in an ATP-dependent manner and the antisense strand (i.e. the strand complementary to the target mRNA) is retained (23, 24). The antisense strand of the siRNA functions by guiding RISC to mRNA with complementary sequence, where RISC mediates endonucleolytic cleavage and subsequent degradation of the target RNA. That siRNA mediate RNAi proved to be significant in the context of mammalian RNAi as dsRNA greater than 30 base pairs (bp) in size causes sequence non-specific knockdown of gene expression through the induction of the interferon response (25, 26). The discovery that 21 nt synthetically produced siRNA, which are small enough to avoid the interferon response, are able to induce RNAi in mammalian cells (27, 28) paved the way for mammalian RNAi studies.

1.3.1.1 The random degradative PCR pathway

In the nematode worm, *C. elegans*, induction of RNAi leads to the potent silencing of the targeted gene throughout the entire body of the worm. The RNAi effect may also be transferred to the first generation offspring. An additional step during which amplification of siRNA occurs has been proposed to explain the spreading effect seen in *C. elegans* (29, 30). The antisense strand of a siRNA is proposed to act as a primer for an RNA-directed RNA polymerase (RdRP). Hybridization of the antisense strand of a siRNA to the target mRNA initiates RNA polymerization to generate a dsRNA, which is processed by Dicer to form secondary siRNA. The secondary siRNA may in be incorporated into RISC to function as guides or re-enter the random degradative PCR pathway. If random degradative PCR does

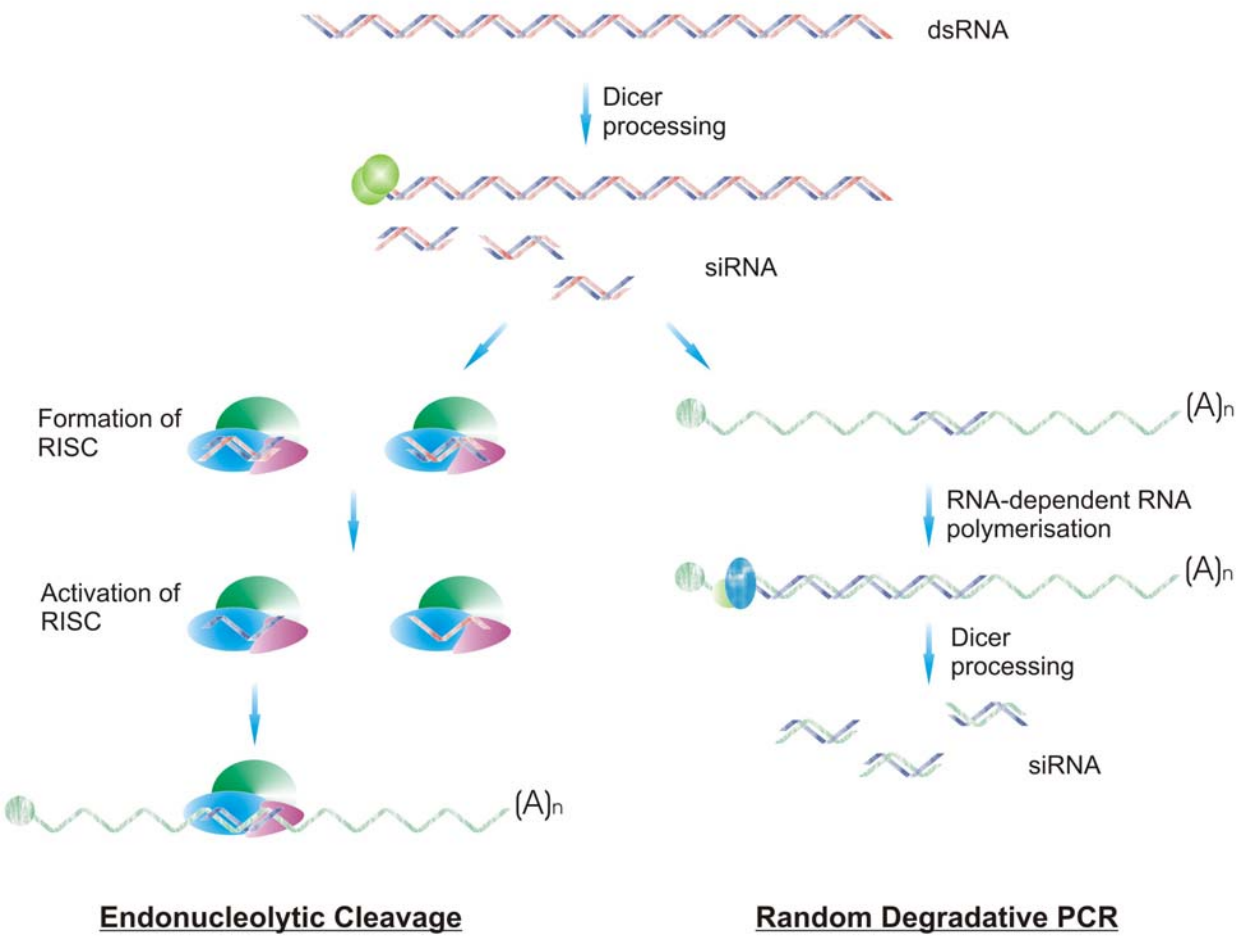


Figure 1.3: **The RNA interference pathway.** Long dsRNA is cleaved by Dicer into siRNA. The siRNA are incorporated into RISC and guide the protein complex to target mRNA for endonucleolytic cleavage. In plants and nematodes the siRNA may also function as primers for the RNA-dependant RNA polymerization of target mRNA to generate dsRNA, which in turn is processed by Dicer to produce secondary siRNA.

hold true it may be predicted that the secondary siRNA would target a region of the mRNA that lies upstream of the primary siRNA target site (i.e. by transitive RNAi). Indeed, this proves to be the case (29, 30). When the gene for green fluorescent protein (*gfp*) is cloned 5' of the gene for β-galactosidase (*lacZ*), siRNA targeted against *lacZ* leads to *gfp* silencing. On the contrary, when *gfp* is cloned 3' of the *lacZ* gene, siRNA targeted against *lacZ* leads to the silencing of *lacZ* only. These results indicated that transitive RNA does occur, at least in the nematode worm, and leads to an amplification of the siRNA. The same cannot be said of RNAi in the fruitfly, *Drosophila melanogaster* or in mammals where siRNA have been shown to function as guides only and not as primers (31).

1.3.1.2 MicroRNA

MicroRNA (miRNA) are a family of small, non-coding RNA encoded within the genome that regulate sequence-specific gene expression at the post-transcriptional level. The first microRNAs to be identified, *lin-4* and *let-7* were initially classified as small temporal RNA (stRNA) (32). *lin-4* and *let*-7 function in embryogenesis by repressing expression of their target genes. Repression is achieved through inhibition of translation (33). Certain characteristics were shared between stRNA and siRNA; both siRNA and stRNA are 21 nt in length and are processed from larger dsRNA precursors. The similarities between these two species of non-coding RNA prompted the investigation into the link between RNAi and stRNA expression (34-36). It was discovered that genes involved in RNAi regulate the

expression of stRNA. These observations were followed by the identification of a whole host of microRNA (37-39).

With the identification of the miRNA sequences an elucidation of their biogenesis soon followed (Figure 1.4). The primary precursors of miRNA (pri-miRNA) are transcribed in the nucleus (40) and are processed into precursor miRNA (pre-miRNA) by the RNase III, Drosha (41, 42). Following processing by Drosha, the pre-miRNA is exported to the cytoplasm by the Ran-GTP dependent cargo transporter Exportin-5 (43). In the cytoplasm the pre-miRNA is processed into the mature miRNA, which is incorporated into a miRNA nucleoprotein (miRNP) (44). In much the same way that siRNA function the mature miRNA guides the miRNP to target mRNA for translational repression (40). The miRNP was later shown to be the same protein complex involved in RNAi, i.e. RISC (45). This begs the question as to why do siRNA function by degrading mRNA when miRNA repress translation. It was later demonstrated that the different mechanism of action can be attributed to the degree of complementarity between the siRNA or miRNA and the target mRNA (46-48). If the siRNA or miRNA is completely complementary to the target mRNA inhibition will be by mRNA degradation. On the other hand if incomplete complementarity exists between the siRNA or miRNA and the target RNA silencing will occur by translational repression.

Although most miRNA do not have an ascribed function they appear to play important roles in developmental regulation (49). The importance of miRNA for correct development has also been highlighted recently by the discovery of human cancers that can be attributed to

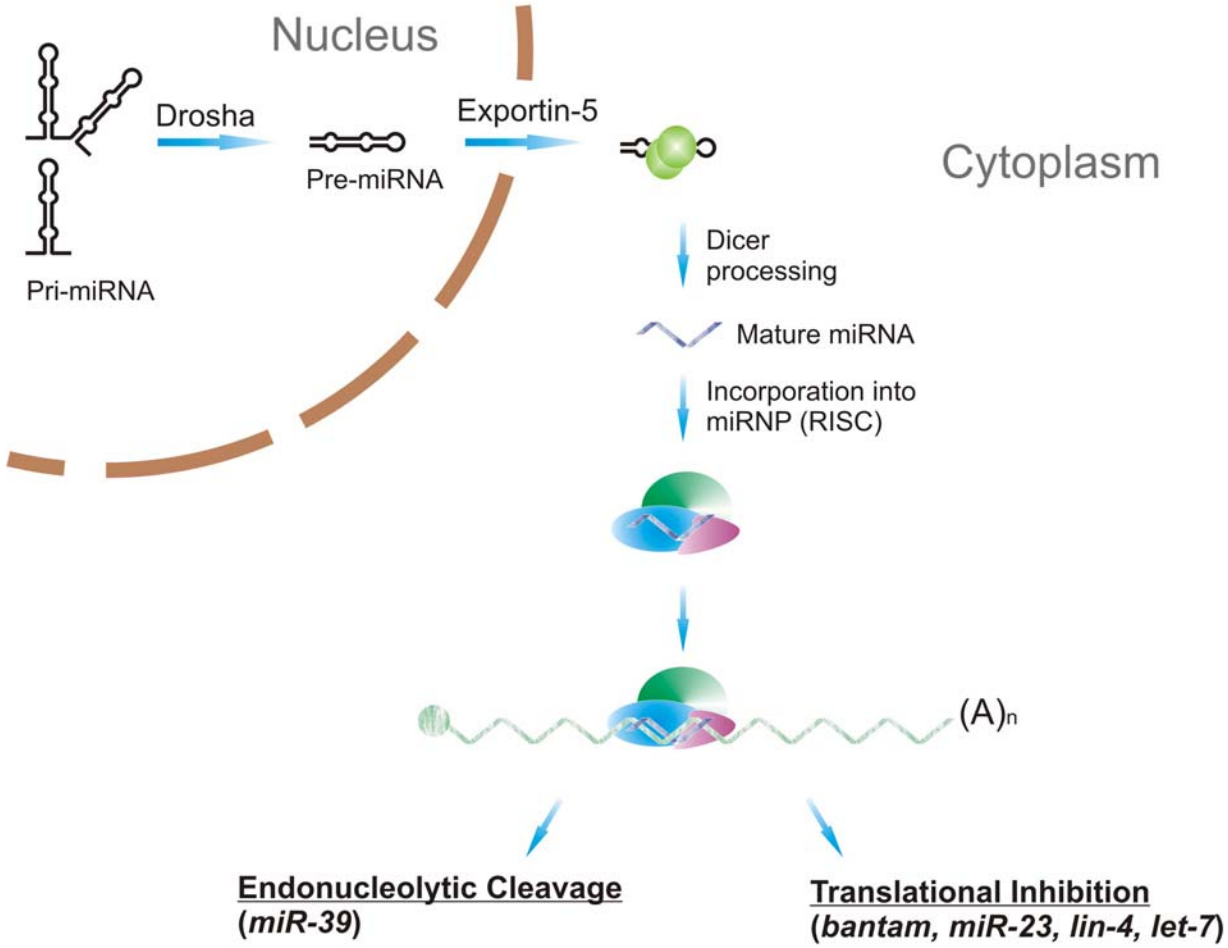


Figure 1.4: **MicroRNA biogenesis.** Polycistronic and monocistronic pri-miRNA are transcribed in the nucleus and processed in ~70 nt pre-miRNA. The pre-miRNA is exported to the cytoplasm by an exportin-5 mediated pathway, where it is processed by Dicer into mature miRNA. The mature miRNA associates with RISC and guides the complex to target mRNA for silencing by endonucleolytic cleavage or translational repression.

loss of miRNA function (50, 51) (for review see (52)). In addition to this, miRNA encoded by the Epstein-Barr virus have recently been identified, which are thought to function as viral regulators of host and/or viral gene expression (53).

1.3.2 Inducing RNAi

In order to induce RNAi in mammalian cells the use of chemically synthesized siRNA is generally employed. Chemically synthesizing siRNA however is not always feasible due to the costs incurred. Therefore numerous methods of inducing RNAi have been developed. These can be broadly divided into *in vitro* generated siRNA and systems capable of expressing siRNA in mammalian cells.

In vitro *generation of siRNA*. The first method employs run-off transcription of a short sequence of DNA encoding separate strands of the siRNA (54). Double-stranded DNA sequences 38-39 nt in length are synthesized such that the first 18 nt encode the T7 RNA polymerase promoter followed by the sequence encoding either strand of the siRNA. Run-off transcription with the T7 RNA polymerase generates 20-21 nt fragments, which are annealed to form the siRNA. *In vitro* transcription from phage polymerases generates transcripts with a 5' nucleotide triphosphate. This characteristic is thought to be recognized by mammalian cells as foreign and has been demonstrated to lead to the induction of the interferon response (55). The second method uses the products of hydrolysis of recombinant Dicer or *Escherichia coli* RNase III to induce RNAi (56, 57). Large dsRNA homologous to the target gene is transcribed

in vitro and treated with recombinant human Dicer or *E. coli* RNase III to produce a pool of siRNA capable of mediating efficient RNAi. Using a pool of siRNA is not ideal as it increases the probability of one of the siRNA exhibiting off-target effects. The use of deoxyribozymes (catalytic DNA molecules) to digest *in vitro* transcribed RNA to produce siRNA has also been described (58). Since siRNA-mediated post-transcriptional gene silencing takes place in the cytoplasm the use of RNA has the advantage that only the plasma membrane needs to be crossed during delivery for the siRNA to be functional. The disadvantage in using chemically synthesized or *in vitro* transcribed siRNA lies in the instability of RNA and is therefore prone to degradation. The high turnover rate of RNA also means that the knockdown achieved will only be maintained for a limited time period. If RNAi is to be used for the management of chronic infections knockdown should ideally be maintained for longer periods of time than can be achieved when using siRNA. The use of siRNA expression systems would allow for knockdown to be maintained for longer periods of time since the siRNA could be expressed continuously.

siRNA expression systems. Expression systems under the control of RNA polymerase II (59) and RNA polymerase III (60-65) promoters have been developed. These expression systems are capable of endogenously transcribing either individual strands of a siRNA (60) or short hairpin RNA (shRNA) precursors, which function as substrates for Dicer (59, 61-65). RNA polymerase III promoters (pol III promoters) are generally favoured as these are normally involved in the transcription of small RNA species and are therefore ideal for the production of siRNA or shRNA. Expression from pol III promoters however is ubiquitous (i.e.

expressed in all cell types) and therefore expression cannot be controlled in a tissue specific manner. Transcription from pol III promoters is also constitutive and as such dosage cannot be controlled. RNA polymerase II promoters (pol II promoters) on the other hand exhibit tissue-specific expression and may be employed to express shRNA in specific tissues. Pol II promoters are normally involved in the transcription of large messenger RNA. Generation of shRNA under the transcriptional control of a pol II promoter therefore yields RNA transcripts containing unwanted sequences, which may interfere with the processing of the shRNA. Expression systems generating shRNA that mimic the structure miRNA precursors have also been described (66, 67). These systems also employ the pol II promoter and require processing by Drosha to release the shRNA from the larger RNA transcript. An additional disadvantage in using DNA expression systems to effect knockdown of a gene of interest is that the DNA needs to be delivered to the nucleus. The DNA encoding the siRNA or shRNA expression systems therefore has to cross the plasma membrane as well as the nuclear membrane before transcription can occur.

1.4 RNAi-mediated inhibition of HBV

Several sites of the HBV genome have been targeted in studies to assess efficiency of using RNAi against HBV (10, 12, 14). To date, siRNA sequences have been evaluated *in vitro* and *in vivo* in a murine model of HBV replication. McCaffrey and colleagues (14) assessed the anti-HBV efficacy of a panel of six U6 promoter cassettes that encoded hairpin RNA sequences. The data demonstrated inhibition of markers of HBV gene expression with some

variation in the efficiency of the hairpin-encoding cassettes. Giladi and colleagues (10) assessed the efficacy of five synthetic siRNA duplexes that targeted different sites within the surface ORF of HBV. The siRNA sequences diminished HBsAg production from a polymerase deficient HBV plasmid. This is an important property that is distinct from anti-HBV nucleoside analogues, which have inhibitory effects on viral DNA synthesis and require viral replication to be effective. In another study (12), inhibition of HBsAg and HBeAg secretion was observed in mice treated with synthetic RNA duplexes, which targeted the core and surface genes of HBV. HBV antigen secretion and also the production of HBV RNA (as measured by RT-PCR) were diminished by both siRNA duplexes, and the siRNA that targeted the surface region was a more effective inhibitor of antigen secretion. Comparison of anti-HBV activity of synthetic and pol III-derived small hairpin RNA shows that, although both types of sequence effectively inhibit HBV gene expression, synthetic siRNA duplexes may have a more rapid effect (10). However, small hairpin constructs generated endogenously from pol III promoters may have more sustained effects. Complete characterization of the differences between these two classes of interfering RNA is yet incomplete.

This project entailed the construction and evaluation of two shRNA expression cassettes. The first expression cassette employs the U6 promoter to drive expression of shRNA with a precisely defined size. The shRNA function as substrates for processing by Dicer into siRNA, which are targeted against the *HBx* ORF. A PCR-based technique for the rapid generation of U6 promoter driven shRNA expression cassettes (65), was initially employed to identify sequences capable of effectively inhibit markers of HBV replication. The second cassette

employs an RNA polymerase II promoter (the cytomegalovirus (CMV) promoter) to drive the production of RNA transcripts containing ribozymes lying on either side of a shRNA sequence. *Cis*-cleavage by the ribozymes releases the shRNA, which in turn enters the RNAi pathway to mediate knockdown of HBV gene expression.

The ribozyme–shRNA (Rz-shRNA) expression cassette incorporates the most favourable features of the use of RNA or the use of DNA into its design. The fact that it is a plasmid-based expression cassette allows for the continuous expression of shRNA thereby prolonging knockdown of the targeted gene. Use of a pol II promoter allows for tissue-specific expression of shRNA. The problem of unwanted sequences generated under pol II promoter-driven transcription is overcome by the incorporation of ribozymes in the design of the expression cassette to allow for the production of shRNA of defined length. Alternatively the expression cassette could be used for the exogenous production of shRNA. The shRNA generated from *cis*-cleavage by the ribozymes do not contain 5' triphosphates and would therefore not induce the interferon response.

The expression cassettes were designed to endogenously (and exogenously in the case of the Rz-shRNA cassette) generate shRNA targeted against the *HBx* ORF. Transcription from the HBV genome is under the control of four internal promoters but ends with a single termination signal. All viral transcripts therefore share a common 3' end. Since the *HBx* ORF generates the smallest transcript it represents the sequence common to all viral transcripts. RNAi mediated against the *HBx* ORF would therefore target all viral transcripts. HBx plays a

vital role in the establishment of a successful viral infection *in vivo*. HBx has also been implicated in the development of HCC. These factors make HBx an ideal target for the development of antiviral therapy.

A total of ten sites within the *HBx* ORF were chosen as target sites for RNAi-mediated inhibition of HBV. These sites represent the most conserved regions of the *HBx* ORF as determined by alignment of South African isolates of HBV. To establish whether the shRNA targeted to these sites might inadvertently target unrelated genes within the human genome a BLAST search was performed. *HBx* coordinates 1168-1192 received 98 hits. The single hit showing significant homology (91%) with a human cDNA had 2 nt mismatches occurring towards the center of the sequence and is therefore unlikely to be silenced. Similar search results were obtained for most of the target sites. The exception ($HBx_{1678\text{-}1702}$) received 98 hits with one showing a 95% sequence homology and a single nucleotide mismatch occurring towards the 3' end of the sequence. The shRNA targeted against $HBx_{1678\text{-}1702}$ may therefore cross react with a human cDNA clone.

1.5 Aims

The aim of this project was the construction and evaluation of two expression cassettes capable of generating shRNA sequences targeted to the *HBx* ORF. The first expression cassette employs the U6 promoter to drive expression of shRNA of defined sequence and size.

shRNA are processed by Dicer into siRNA, which enter the RNAi pathway to mediate knockdown of HBV gene expression. The second expression cassette is a self-cleaving construct under the control of the cytomegalovirus (CMV) promoter, an RNA polymerase III promoter. Transcription from the CMV promoter produces a transcript containing self-cleaving ribozymes lying on either side of a shRNA sequence. *Cis*-cleavage by the ribozymes releases the shRNA sequence, which in turn enters the RNAi pathway. The ability of these expression cassettes to mediate inhibition of HBV replication was assessed in the human hepatoma cell line, Huh7.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Target and Reporter Plasmids

2.1.1 The target plasmid

The construction of the target plasmid pCH-9/3091 has been previously described by Nassal and colleagues (68). Briefly, pCH-9/3091 contains wild-type HBV genome with terminal repeats under the control of the CMV immediate early promoter-enhancer. The terminal repeats functionally mimic the circular nature of the HBV genome. As would occur normally during HBV replication, transcription from pCH-9/3091 generates a greater than genome length pregenomic RNA. cccDNA may then be reverse transcribed from the pregenomic RNA template. Transcription from pCH-9/3091 therefore simulates HBV replication.

2.1.2 The reporter plasmid

Construction of the reporter plasmid expressing enhanced green fluorescent protein (EGFP) as a marker of HBV replication has been previously described by Passman and colleagues (7). The reporter plasmid, pCH-EGFP, was generated by replacing the preS2/S

ORF of pCH-9/3091 with a sequence encoding EGFP. To generate pCH-EGFP, the DNA sequence coding for EGFP was amplified by PCR from the plasmid vector pBI-EGFP (Clontech, Palo Alto, USA). The amplicon was digested with *Xho*I and *Xba*I and cloned into the equivalent sites of the mammalian expression vector, pCI-neo (Promega, WI, USA) to generate pCI-EGFP. The DNA sequence encoding EGFP was then digested out of pCI-EGFP with *Xho*I and *Xba*I and cloned into the *Xho*I and *Spe*I sites of pCH-9/3091 to generate pCH-EGFP.

2.2 U6 shRNA Expression Vectors

2.2.1 Design of shRNA sequences targeted against the *HBx* ORF

Full genome sequences of South African HBV isolates were aligned with the Bioinformatics programme GeneDoc (69) and the most conserved regions of the *HBx* ORF were chosen as target sites. A panel of 10 shRNA targeted against the most conserved regions of the *HBx* ORF were designed. The shRNA were designed with 25 nt stems and the loop sequence of miR-23. Hairpins with stems greater than 21 nt exhibit improved processing by Dicer and miRNA loop sequences improve translocation of the hairpin to the cytoplasm (64). To aid in cloning, GU or CA mismatches were incorporated into the sequence of the stem structures. The mismatches were introduced into the sequence encoding the sense strand in order to maintain complete complementarity between the antisense (guide) strand and its

target site within the *HBx* ORF.

2.2.2 Construction of U6 shRNA expression vectors targeted against the *HBx* ORF

Expression cassettes capable of endogenously generating shRNA targeted against the *HBx* ORF were constructed in accordance with the PCR-based technique developed by Castanotto, Li and Rossi (65) with some modifications. The expression cassettes were incorporated into the pGEM®-T Easy PCR Cloning vector (Promega, WI, USA). Oligonucleotides encoding shRNA sequences were synthesized by phosphoramidite chemistry (Inqaba Biotechnology, South Africa). Each shRNA was encoded for by two staggered oligonucleotides (refer to Table 2.1). A universal primer complementary to the 5' end of the U6 promoter was also synthesized (5'-CTA ACT AGT GGC GCG CCA AGG TCG GGC AGG AAG AGG G-3').

The human U6 promoter was amplified by PCR (PCR Master Mix; Promega, WI, USA) using the universal U6 primer and the primer encoding the first half of the shRNA (U6 $shRNA_{x.1}$) in the first step of the two-step PCR (Figure 2.1). PCR products from the first step were then amplified by PCR using the universal U6 primer and the primer encoding the second half of the shRNA (U6 $shRNA_{x.2}$) during the second step of the two-step PCR to

Table 2.1: **Oligonucleotide sequences used in the generation of shRNA expression constructs.**

HBx$_{1168\text{-}1192}$	
U6 shRNA$_{2.1}$	5′-**TGACGTGACAGGAAGCGTTA**GCAGACACTTGGCATAGGCC*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{2.2}$	5′-CCCAGATCTACGCGTAAAAAAGGTCTGTGCCAAGTGTTTGC**TGACGTGACAGGAAGCGTTA**-3′
HBx$_{1432\text{-}1456}$	
U6 shRNA$_{6.1}$	5′-**GGACGTGACAGGAAGCGTTC**GTGGGATTCAGCGTCGATGG*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{6.2}$	5′-CCCAGATCTACGCGTAAAAAACCGTCGGCGCTGAATCCCGC**GGACGTGACAGGAAGCGTTC**-3′
HBx$_{1514\text{-}1538}$	
U6 shRNA$_{7.1}$	5′-**CTTTATGACAGGAAGCAAAG**AGAGATGCGCCCCATGGCCG*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{7.2}$	5′-CCCAGATCTACGCGTAAAAAACGACCACGGGGCGCACCTCT**CTTTATGACAGGAAGTAAAG**-3′
HBx$_{1518\text{-}1542}$	
U6 shRNA$_{8.1}$	5′-**ACGCGTGACAGGAAGCGTGT**GAAGAGAGGTGTGCCCTGTG*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{8.2}$	5′-CCCAGATCTACGCGTAAAAAACACGGGGCGCACCTCTCTTT**ACGCGTGACAGGAAGCGTGT**-3′
HBx$_{1575\text{-}1599}$	
U6 shRNA$_{10.1}$	5′-**CTCTGTGACAGGAAGCAGAG**GCGAAGCAAAGCGCACACGA*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{10.2}$	5′-CCCAGATCTACGCGTAAAAAACCGTGTGCACTTCGCTTCAC**CTCTGTGACAGGAAGCAGAG**-3′

HBx$_{1580\text{-}1604}$	
U6 shRNA$_{11.1}$	5′-**CACGTTGACAGGAAGATGTG**TAGAGGTGAAGCGAGGTGTA*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{11.2}$	5′-CCCAGATCTACGCGTAAAAAATGCACTTCGCTTCACCTCTG**CACGTTGACAGGAAGATGTG**-3′
HBx$_{1640\text{-}1664}$	
U6 shRNA$_{12.1}$	5′-**GGACTTGACAGGAAGAGTTC**TTTTATGTAGGACTTTGGGC*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{12.2}$	5′-CCCAGATCTACGCGTAAAAAAGCCCAAGGTCTTACATAAGA**GGACTTGACAGGAAGAGTTC**-3′
HBx$_{1678\text{-}1702}$	
U6 shRNA$_{14.1}$	5′-**GAGGCTGACAGGAAGGCTTC**AAGGTTGGTTGTTGACGTTG*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{14.2}$	5′-CCCAGATCTACGCGTAAAAAACAATGTCAACGACCGACCTT**GAGGCTGACAGGAAGGCTTC**-3′
HBx$_{1774\text{-}1798}$	
U6 shRNA$_{17.1}$	5′-**TTGGTTGACAGGAAGACTAA**TTTGTGCCTACAGCTTCTTA*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{17.2}$	5′-CCCAGATCTACGCGTAAAAAATAGGAGGCTGTAGGCATAAA**TTGGTTGACAGGAAGACTAA**-3′
HBx$_{1863\text{-}1887}$	
U6 shRNA$_{20.1}$	5′-**CTTGGTGACAGGAAGCCAAA**GCACAACTCGGAGGCTCGAA*CGGTGTTTCGTCCTTTCCACA*-3′
U6 shRNA$_{20.2}$	5′-CCCAGATCTACGCGTAAAAAATTCAAGCCTCCAAGCTGTGC**CTTGGTGACAGGAAGCCAAG**-3′

Complementary sequences are indicated in bold.
The region complementary to the U6 promoter is italicized.
HBx target sites are underlined.

generate a complete U6 shRNA expression cassette. The completed cassette consists of a U6 promoter followed by the sequence encoding the shRNA and the terminator sequence (TTTTTT). For each PCR the reaction conditions were as follows: Initial denaturation at 95°C for 2 minutes; followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute; a final extension step was carried out at 72°C for 7 minutes.

The amplicons were run on 0.8% agarose gels, excised and purified using the MinElute™ Gel Extraction Kit (Qiagen, CA, USA). The purified fragments were ligated into the pGEM®-T Easy Cloning vector according to the manufacturer's instructions (Promega, WI, USA) (Figure 2.2). Purified U6 shRNA expression cassettes at a 20× molar excess to pGEM®-T Easy vector were incubated at 15°C overnight in a 10 µl reaction volume containing T4 DNA Ligase (Promega, WI, USA). Five microlitres of the ligation mix was used to transform 100 µl of chemically competent *E. coli* (DH5α, Invitrogen, CA, USA) (for preparing and transforming chemically competent *E. coli* see Appendix 6.1.1). The transformed *E. coli* were plated on Luria Bertani ampicillin positive, X-gal, IPTG positive agar plates (see Appendix 6.1.2) and incubated at 37°C overnight. Colonies containing an insert (white colonies) were selected and screened by restriction enzyme digestion with *Bgl*II and *Spe*I. Plasmids positive for the correct sized insert (pG-U6 shRNA) were sequenced (Inqaba Biotechnology, South Africa) to ensure that correct sequence fidelity was maintained.

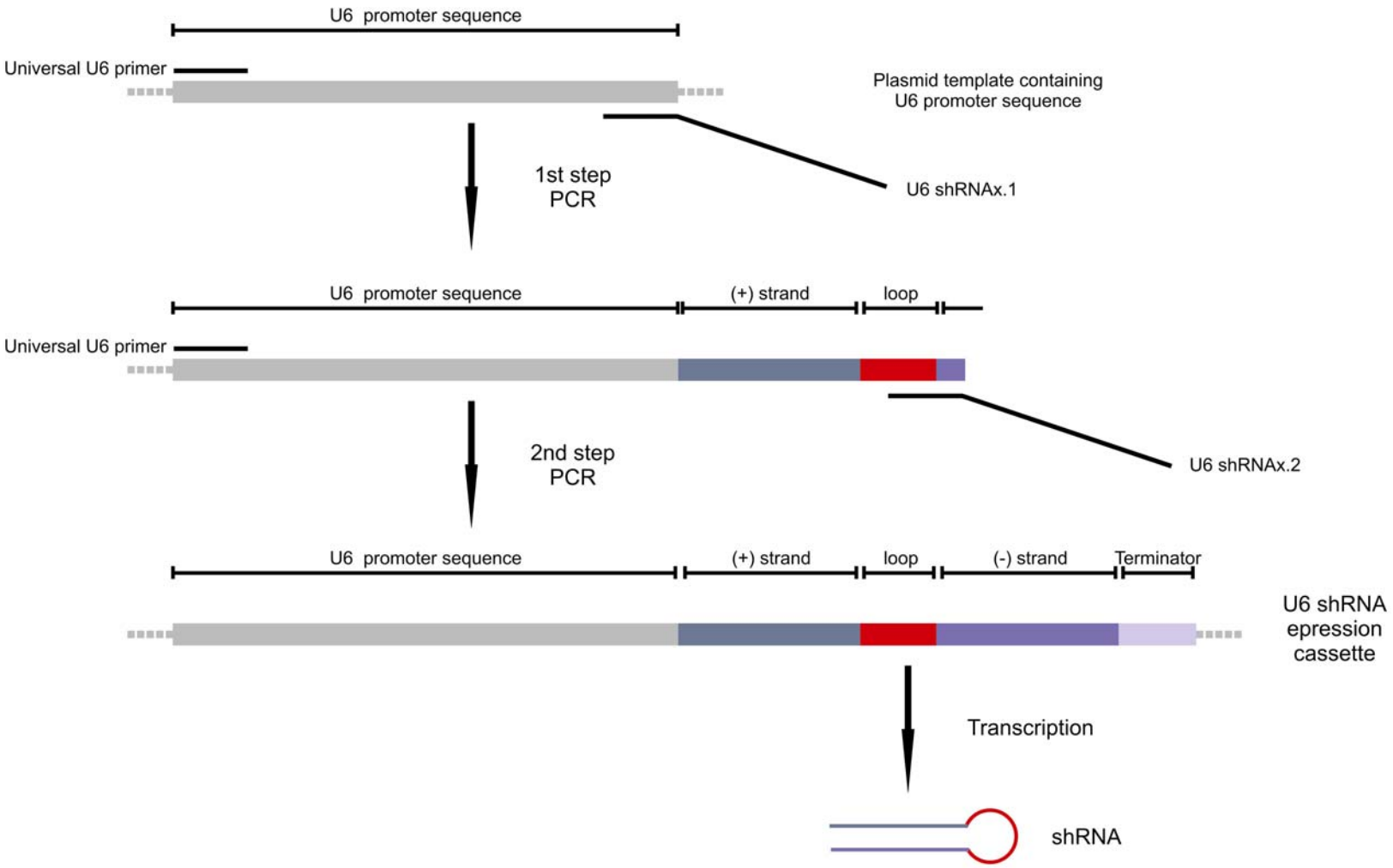


Figure 2.1: **Schematic representation of PCR-based technique for the generation of U6 shRNA expression cassette.** Transcription from the expression cassette generates a shRNA sequence.

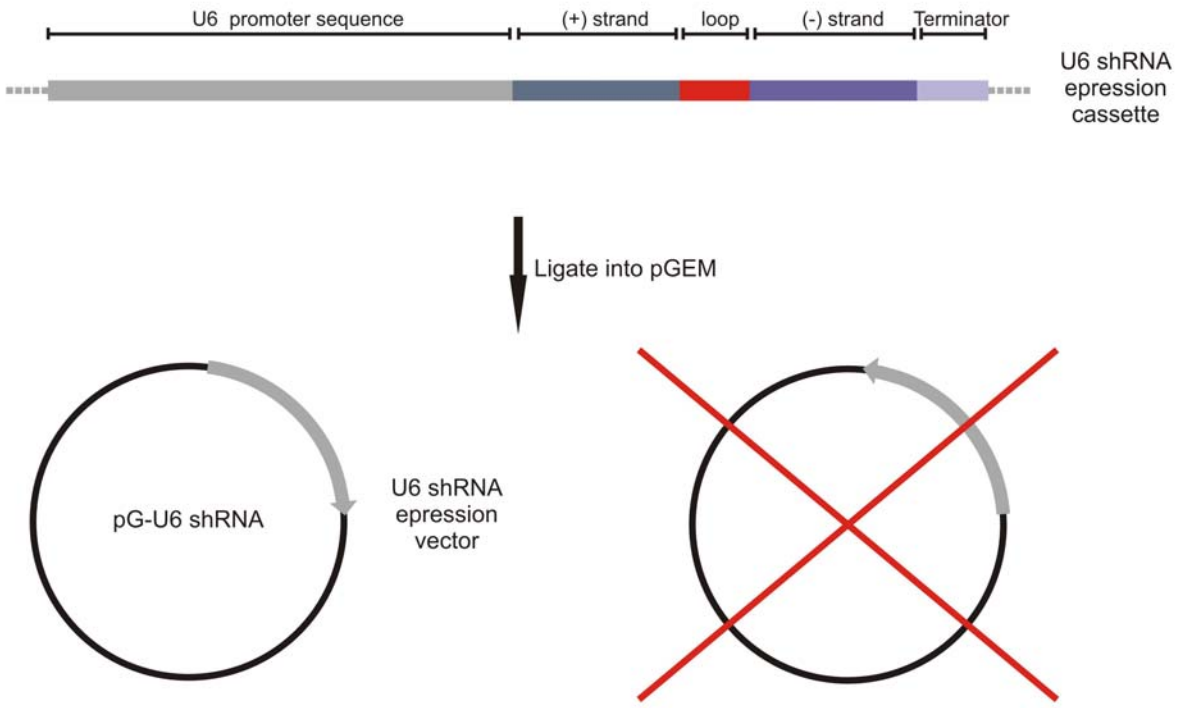


Figure 2.2: Schematic representation of cloning procedure for the generation of U6 shRNA expression plasmids.

2.3 U6 shRNA-mediated Inhibition of HBV Gene Expression

2.3.1 Plasmid preparation

U6 shRNA as well as target and reporter plasmids were prepared with the EndoFree® Plasmid Maxi Kit (Qiagen, CA, USA) (refer to Appendix 6.1.3).

2.3.2 Transfection of cultured human hepatocytes with U6 shRNA expression cassettes

The human hepatoma cell line, Huh7 was maintained in RPMI growth medium (Appendix 6.1.4) supplemented with 2.5% foetal calf serum (FCS), penicillin (100 000 U/ml) and streptomycin (100 µg/ml) (Gibco BRL, UK). One day before transfection, 2.0-2.5 × 10^5 cells were seeded in 2 cm diameter dishes (Costar®, Corning Inc, NY, USA) in 2 ml RPMI growth medium. Five hours prior to transfection the RPMI growth medium was replaced with 2 ml RPMI supplemented with 10% FCS but without any antibiotics.

Each transfection sample was prepared as follows: 3 µg of pG-U6 shRNA and 6 µg pCH-9/3091 or pCH-EGFP were thoroughly mixed in 250 µl Opti-MEM I (Invitrogen, CA, USA). Three microlitres of Lipofectamine 2000 (Invitrogen, CA, USA) was diluted in 250 µl of Opti-MEM I and incubated at room temperature for 5 minutes. After the 5 minute incubation the diluted DNA was mixed with the diluted Lipofectamine 2000 and the mixture incubated for 20 minutes at room temperature to allow the DNA-Lipofectamine 2000 lipid complexes to form. Thereafter, 500 µl of transfection sample was added per well to the 6-well plate seeded with Huh7 cells. The cells were incubated for 24-48 hours at 37°C in a humidified incubator with 5% CO_2.

2.3.3 Assay for markers of HBV expression

2.3.3.1 EGFP expression

Cells co-transfected with pCH-EGFP were replenished with RPMI growth medium 24-hours post-transfection and incubated for an additional 24 hours at 37°C in a humidified incubator with 5% CO_2. Forty eight hours post-transfection cells were assayed for EGFP expression by Fluorescence Microscopy and Flow Cytometry.

The cells were analysed by confocal microscopy (Axiovert 100M, Zeiss, Germany) under a FITC filter to identify green fluorescent cells.

Growth medium was removed and cells washed with shaking in saline containing 0.01% EDTA at 37°C for 10 minutes. The saline was removed, 100 µl of 0.5× trypsin added and the cells incubated for an additional 5 minutes at 37°C. After the 5 minute incubation, 900 µl of saline containing 1% FCS was added and the cells aspirated to ensure complete dissociation from culture plate. Cells were transferred to flow cytometry tubes and green fluorescent cells analysed with a Beckman-Coulter EPICS XL Flow cytometer (Beckman, CA, USA). Cells were identified by forward scatter and side scatter with the mean channel number within the 60-80 range. EGFP fluorescence was detected at 450 nm.

2.3.3.2 HBsAg secretion

Twenty-four hours post-transfection the medium was collected from cells co-transfected with pCH-9/3091 and HBsAg secretion measured (National Health Laboratory Services, South Africa). HBsAg secretion was measured by the **e**lectro**c**hemi**l**uminescence

immuno**a**ssay or ECLIA (Roche, Germany). Briefly, 50 µl of the growth medium is incubated with a biotinylated monoclonal HBsAg-specific antibody and a monoclonal HBsAg-specific monoclonal antibody labelled with ruthenium to form a sandwich complex with HBsAg. Streptavidin-coated microparticles are added to this mixture to bind the complex to the microparticles by the biotin-streptavidin interactions. The microparticles are magnetically captured on the electrode of the measuring cell. Chemiluminescence is induced by applying voltage to the electrode, the emission is measured and HBsAg quantified.

2.4 CMV Ribozyme-shRNA Expression Vectors

2.4.1 Designing Ribozyme-shRNA expression cassettes targeted against the *HBx* ORF

A panel of three Ribozyme-shRNA (Rz-shRNA) expression cassettes targeting the same regions targeted by pG-U6 $shRNA_7$, pG-U6 $shRNA_{10}$ and pG-U6 $shRNA_{20}$ were designed. The Rz-shRNA expression cassette consisted of a sequence of DNA encoding a shRNA flanked on its 5' and 3' ends by two hammerhead ribozymes (Figure 2.3). The hairpins were designed to mimic the structure of miR-30 (66, 67). The sequence of the basal stem and the loop of miR-30 were retained but the sequence of the internal stem was altered to target regions within the *HBx* ORF.

2.4.2 Construction of Rz-shRNA expression vectors targeted against the *HBx* ORF

2.4.2.1 Generation of Ribozyme component of Rz-shRNA expression cassette

A generic cloning cassette containing the 3' and 5' ribozymes separated by a spacer of 9 nt was generated in the mammalian expression vector pCI-neo (Figure 2.4). This was used for the subsequent insertion of shRNA-encoding sequences. Oligonucleotides coding for the 5' and 3' ribozymes of the Rz-shRNA cassette were synthesized by standard phosphoramidite chemistry (Inqaba Biotechnology, South Africa) (Table 2.2). Double-stranded DNA sequences encoding the 5' ribozyme fragment (5'Rz*) was produced by amplifying the 5'Rz* forward and reverse primers by PCR (PCR Master Mix, Promega, WI, USA). Similarly, the 3' ribozyme fragment (3'Rz*) was amplified as a double-stranded sequence by subjecting 3'Rz* forward and reverse primers to PCR. The PCR conditions were as follows: Initial denaturation at 95°C for 2 minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 48°C for 30 seconds and extension at 72°C for 30 minutes; and a final extension step at 72°C for 2 minutes.

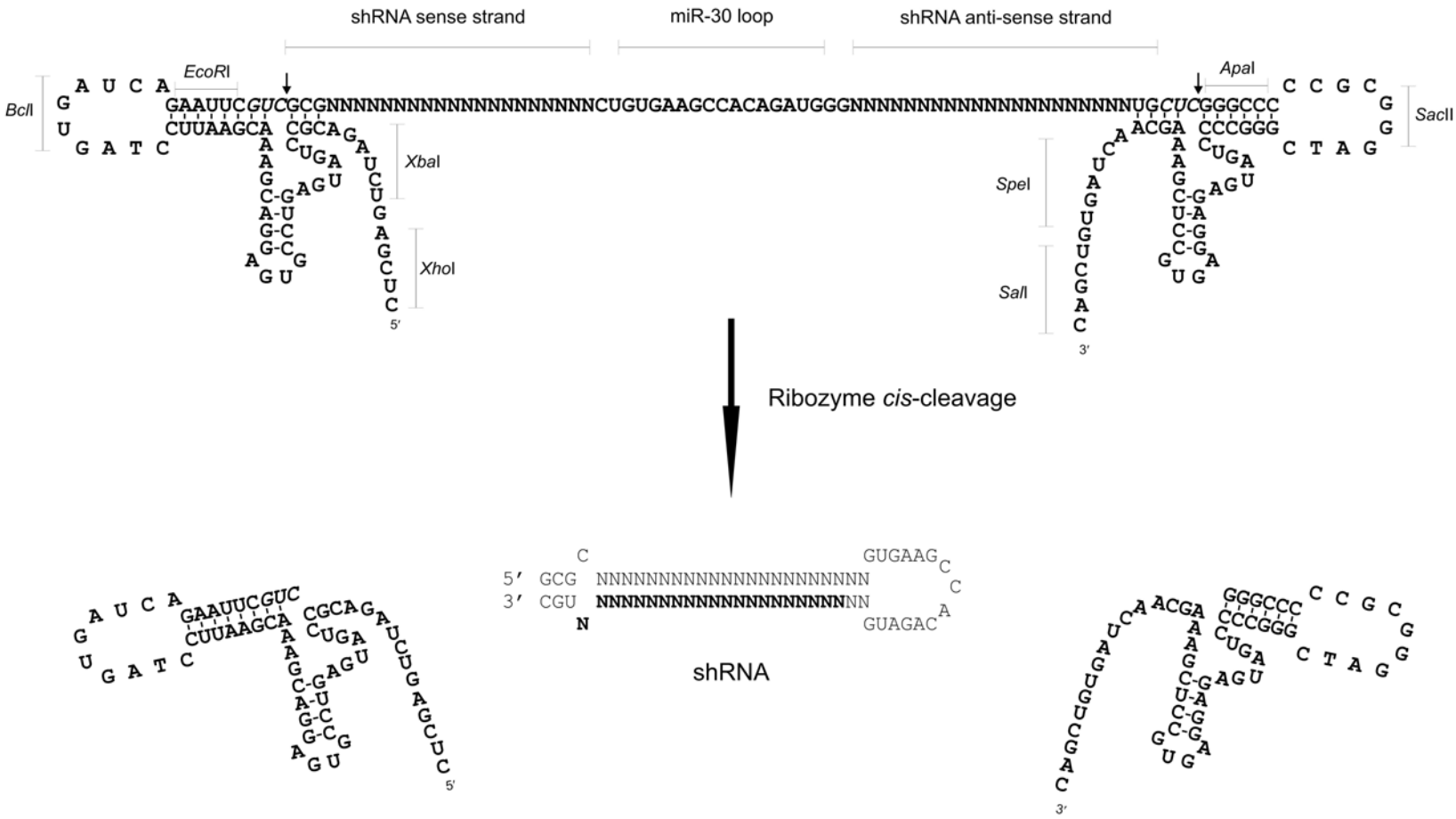


Figure 2.3: **Ribozyme shRNA expression cassette.** Transcription generates a full length transcript which folds into its active conformation. *Cis*-cleavage by the ribozymes releases the shRNA sequence.

Table 2.2: Oligonucleotide sequences for the generation of double-stranded 5' and 3' ribozyme fragments.

5'Rz* F	5′-GATCCTCGAGTCTAGACGCCTGATGAGT**CCGTGAGGACGAAACGAAT**-3′
5'Rz* R	5′-GATCTTGGATCCTTGAATTCTGATCAGA**ATTCGTTTCGTCCTCACGG**-3′
3'Rz* F	5′-GATCAAGGATCCAAGGGCCCCCGCGGGG**GCCCCTGATGAGAGGAGT**-3′
3'Rz* R	5′-GATCGTCGACACTAGTTGCTTTCGAGGC**ACTCCTCTCATCAGGGGC**-3′

Complementary regions are indicated in bold.
Ribozyme encoding sequences are underlined.

The 5' and 3' ribozyme fragments were subjected to agarose gel electrophoresis and eluted with Qiagen's MinElute™ Gel Extraction Kit. The purified fragments were ligated into the pGEM®-T Easy PCR cloning vector at 20× molar excess overnight at 15°C in a 10 µl reaction volume. Chemically competent *E. coli* (DH5α) were transformed with 5 µl of the ligation mix and plated on Luria Bertani ampicillin positive, X-gal, IPTG positive agar plates. The plates were incubated overnight at 37°C. Clones positive for the 5'Rz* insert (pG-5'Rz*) were screened for correct orientation by restriction with *Bam*HI and *Spe*I. Clones positive for the 3'Rz* insert (pG-3'Rz*) were screened for correct orientation by restriction with *Bam*HI and *Xba*I. Clones in the correct orientation were sequenced (Inqaba Biotechnology, South Africa).

To combine the 5' and 3' ribozyme sequences into a single vector, pG-5'Rz* and pG-3'Rz* were each digested with both *Sca*I and *Bam*HI. The vector fragments containing the

ribozyme components were ligated together in a 20 µl reaction volume containing T4 DNA Ligase (New England Biolabs, MA, USA). The ligation reaction was incubated at 15°C overnight. Chemically competent DH5α *E. coli* were transformed with 10 µl of the ligation mix and plated on Luria Bertani ampicillin positive, X-gal, IPTG positive agar plates and incubated at 37°C overnight. Clones positive for both inserts (pG-Rz) were screened with *Eco*RI and sequenced to confirm that sequence fidelity was maintained.

To incorporate the DNA sequence encoding the 5' and 3' ribozymes into a mammalian expression vector, pCI-neo was digested with *Xho*I and *Sal*I. After a 2 hour incubation period, 10 U of calf intestinal phosphatase (Roche, Germany) was added to the digestion reaction and incubated at 37°C for 30 minutes. The pCI-neo backbone was resolved on a 0.8% agarose gel, excised and purified from the gel slice with Qiagen's MinElute™ Gel Extraction Kit. The DNA sequence encoding the 5' and 3' ribozymes was digested out of pG-Rz with the restriction enzymes *Xho*I and *Sal*I, subjected to agarose gel electrophoresis, excised and purified from the gel. A ligation reaction was set up with the 5'/3' ribozyme fragment at a 50× molar excess to pCI-neo vector backbone in a 20 µl reaction volume containing T4 DNA Ligase (New England Biolabs, MA, USA). The ligation mixture was incubated at 15°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°C overnight. Clones positive for single unit inserts (pCI-Rz) were screened with *Hind*III. Single unit clones were screened for correct orientation with *Xho*I and *Sal*I.

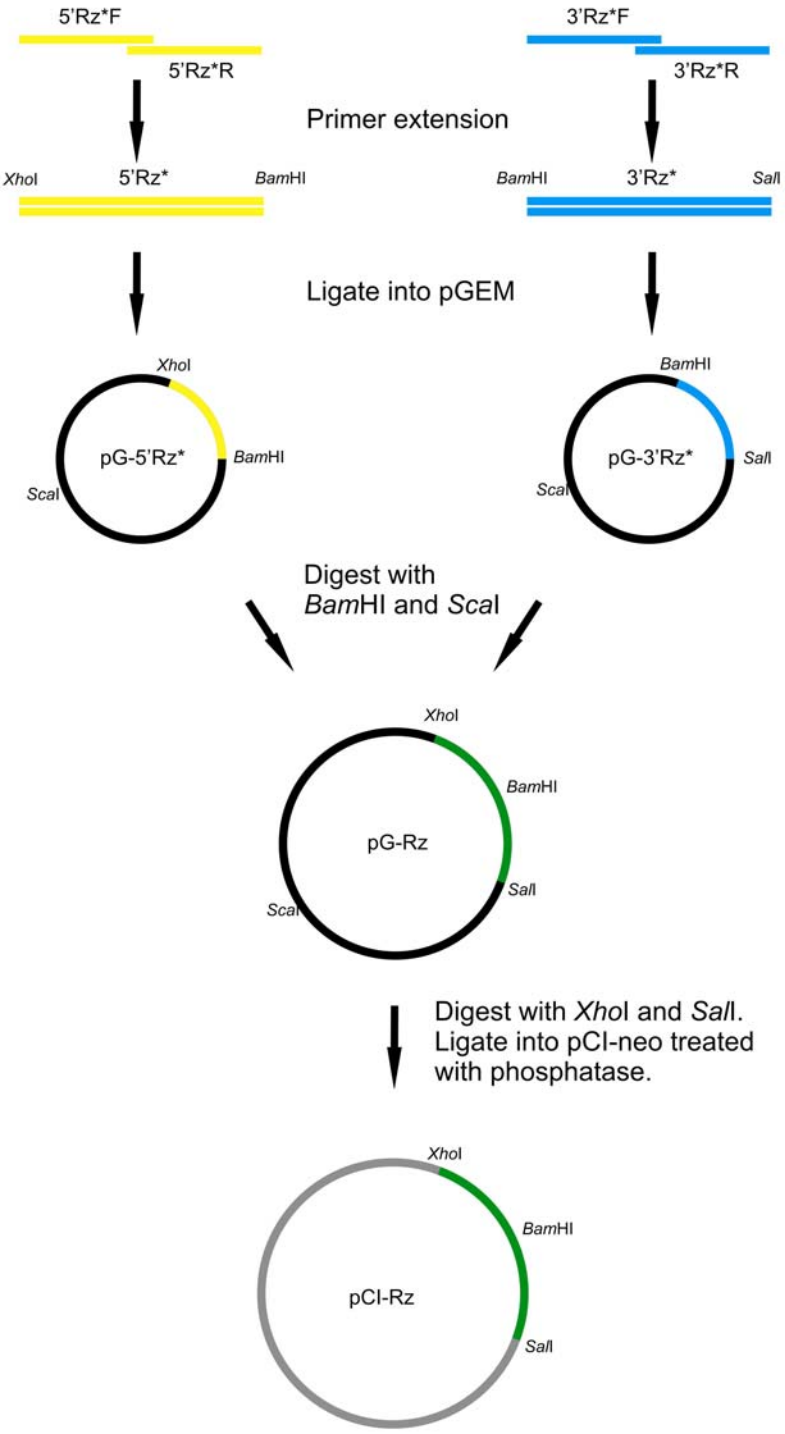


Figure 2.4: Diagrammatic illustration of cloning strategy for the generation of the ribozyme component of the Rz-shRNA expression cassette.

2.4.2.2 Generation of shRNA component of Rz-shRNA expression cassette

DNA sequences encoding the three shRNA were initially cloned into the pGEM®-T Easy PCR cloning vector (Figure 2.5A). Oligonucleotides encoding the shRNA were synthesized by standard phosphoramidite chemistry (Inqaba Biotechnology, South Africa). The oligonucleotides encoding $shRNA_1$, $shRNA_2$ and $shRNA_3$ (Table 2.3) were amplified by PCR (PCR Master Mix, Promega, WI, USA) to generate double-stranded sequences. The shRNA fragments (shRNA*) were run on a 0.8% agarose gel, excised and purified. A ligation of purified shRNA* at a 20× molar excess to pGEM®-T Easy cloning vector was set up in a 10 µl reaction volume and incubated overnight at 15°C. Five microlitres of the ligation mix was used to transform chemically competent *E. coli* (DH5α). Transformed *E. coli* were plated on Luria Bertani agar plates positive for ampicillin, X-gal and IPTG. Agar plates were incubated overnight at 37°C. Plasmids produced from individual bacterial colonies (clones) were screened with *Eco*RI to identify plasmids positive for hairpin encoding sequences. Positive clones were sequenced (Inqaba Biotechnology, South Africa).

To clone the shRNA sequences into pCI-Rz the restriction endonuclease sites *Bcl*I and *Sac*II were incorporated into the hairpin sequence by amplifying pG-$shRNA_1$*, pG-$shRNA_2$* and pG-$shRNA_3$* with primers containing the sequence for these sites (BCL-Fwd 5'- GAT CTG ATC AGA TCG AAT TCG TC -3' and SAC-Rev 5'- GAT CCC GCG GGA TCG GGC CCG AG -3'). The shRNA amplicons (shRNA**) were cloned into the pGEM®-T Easy cloning vector. Clones positive for an insert were sequenced (Inqaba Biotechnology, South

Africa).

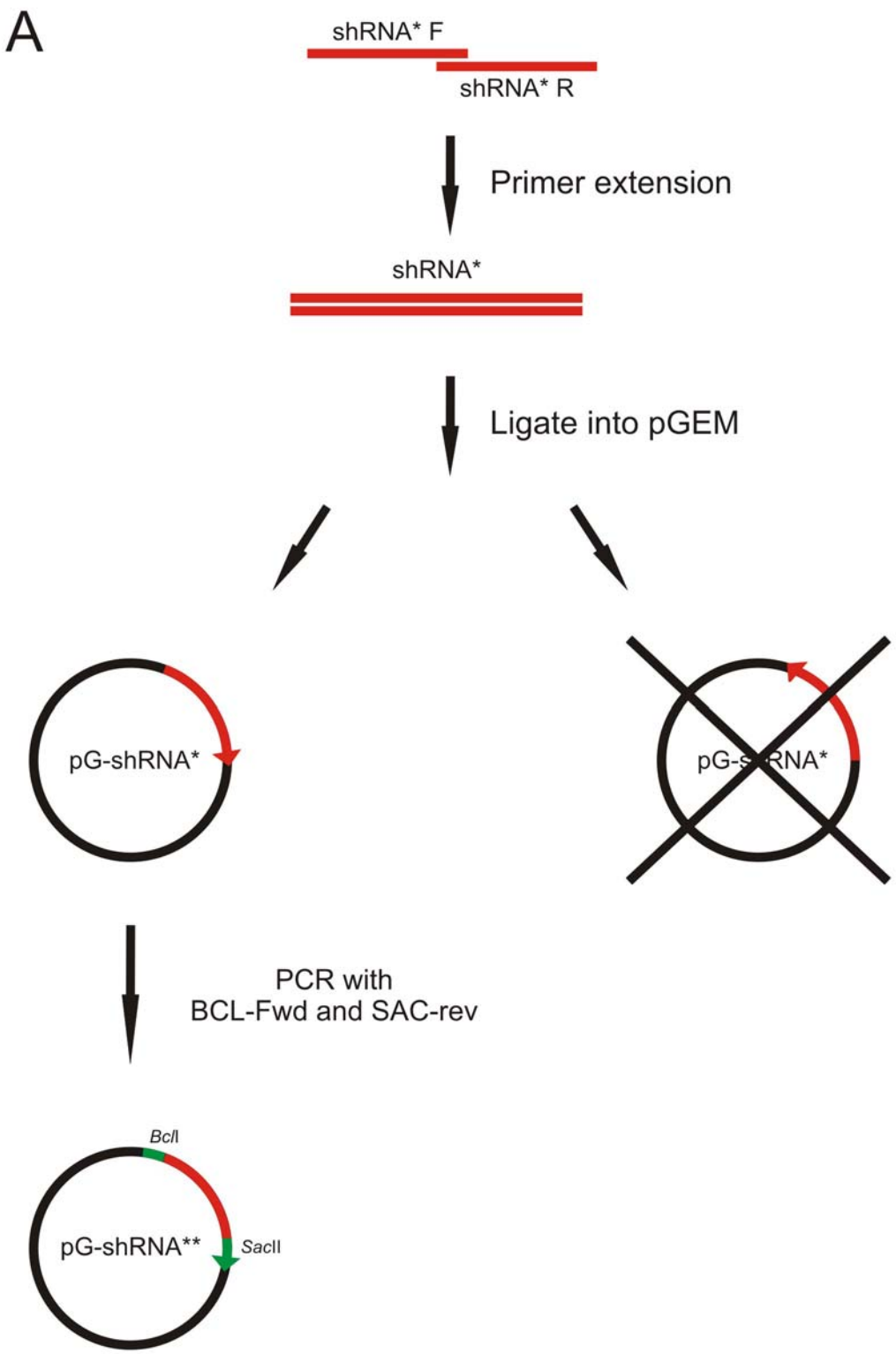
Table 2.3: Oligonucleotides encoding shRNA sequences.

HBx$_{1514\text{-}1538}$ shRNA$_1$* F	5′-GATCGAATTCGTCGCGCCCGCGGGGCGCACTTCTCTT**CTGTGAAGCCACAGATGG**-3′
shRNA$_1$* R	5′-GATCGGGCCCGAGCAACCACGGGGCGCACCTCTCTTC**CCATCTGTGGCTTCACAG**-3′
HBx$_{1575\text{-}1599}$ shRNA$_2$* F	5′-GATCGAATTCGTCGCGACGTGTGTACTTCGCTTCGCC**CTGTGAAGCCACAGATGG**-3′
shRNA$_2$* R	5′-GATCGGGCCCGAGCACCGTGTGCACTTCGCTTCACCC**CCATCTGTGGCTTCACAG**-3′
HBx$_{1863\text{-}1887}$ shRNA$_3$* F	5′-GATCGAATTCGTCGCGCTCAGGCCTCCAAGTTGTGCC**CTGTGAAGCCACAGATGG**-3′
shRNA$_3$* R	5′-GATCGGGCCCGAGCATTCAAGCCTCCAAGCTGTGCCC**CCATCTGTGGCTTCACAG**-3′

Complementary regions are indicated in bold.
HBx target sites are underlined.

2.4.2.3 Generation of Rz-shRNA combination expression vector

The complete Rz-shRNA combination cassette was generated in the mammalian expression vector pCI-neo (Figure 2.5B). pCI-Rz, pG-shRNA$_1$**, pG-shRNA$_2$** and pG-shRNA$_3$** were passaged through the *dam* negative, *dcm* negative strain of *E. coli*, GM161 (for preparation and transformation of chemically competent *E. coli* refer to Appendix 6.1.1) . The shRNA sequences were digested out of pG-shRNA$_1$**, pG-shRNA$_2$** and pG-shRNA$_3$** with the restriction enzymes *Bcl*I and *Sac*II. Following digestion with *Bcl*I and *Sac*II the shRNA sequences were cloned into the equivalent sites of pCI-Rz to generate pCI-Rz-shRNA$_1$, pCI-Rz-shRNA$_2$ and pCI-Rz-shRNA$_3$. Sequence fidelity was checked by manual


A
shRNA* F
shRNA* R
Primer extension
shRNA*
Ligate into pGEM
pG-shRNA*
PCR with
BCL-Fwd and SAC-rev
BclI
pG-shRNA**
SacII

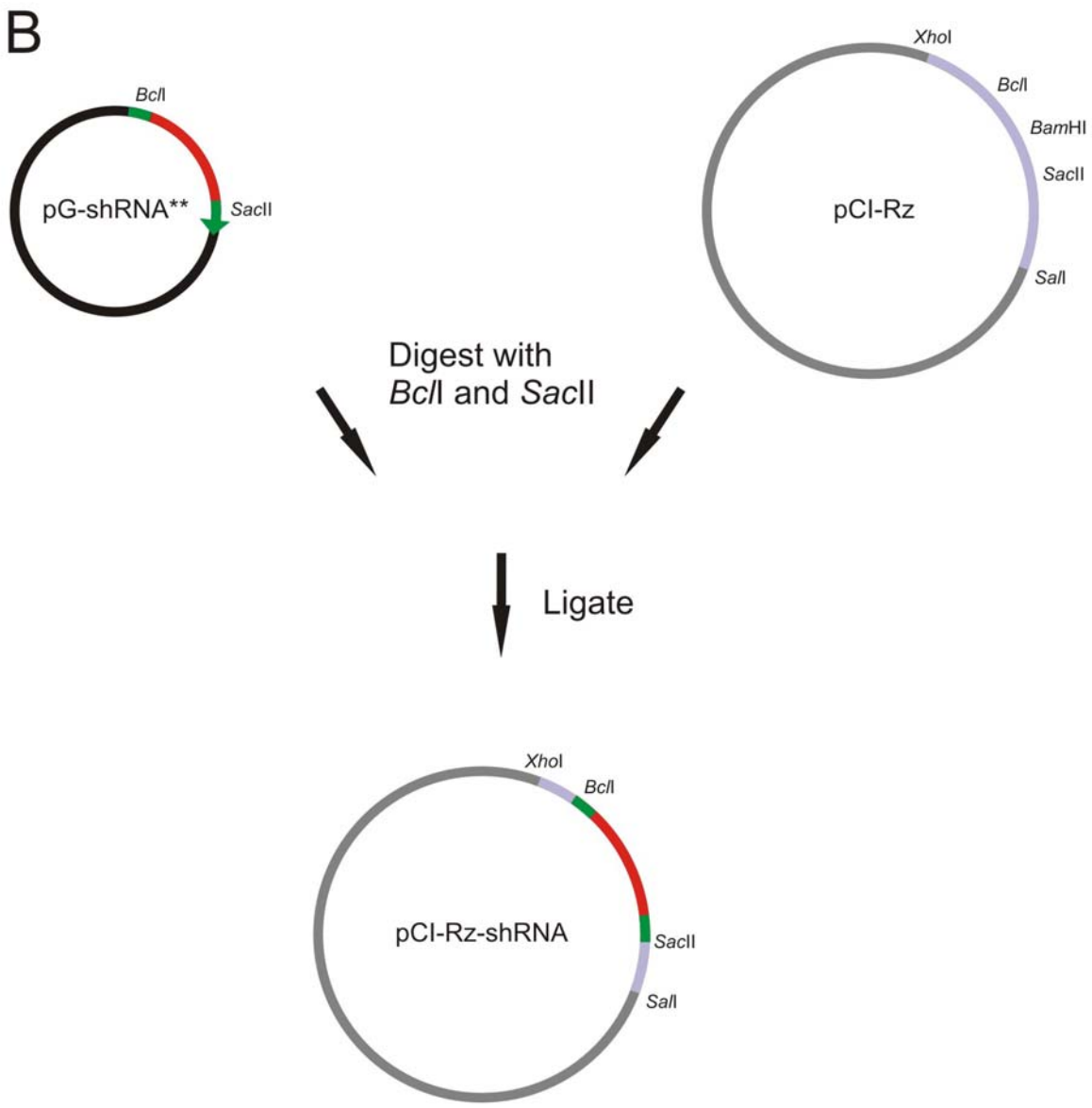


Figure 2.5: Schematic representation of cloning strategy for the generation shRNA component of the Rz-shRNA expression cassette (A) and subsequent insertion of shRNA sequences into pCI-Rz to generate the complete Rz-shRNA expression cassette (B).

sequencing (Appendix 6.1.5).

2.4.3 Testing Rz-shRNA Combination Cassette

2.4.3.1 *In vitro* transcription assay and RNA purification

The Rz-shRNA expression vectors were linearized with *Sal*I to generate template for transcription from the T7 promoter and *Xho*I to generate template for transcription from the T3 promoter. Radiolabelled RNA was transcribed from linearized template DNA using the Riboprobe® Combination System - T3/T7 RNA Polymerase (Promega, WI, USA) according to manufacturer's instructions. Briefly, 2 μg of template DNA, 10× Transcription buffer (Promega, WI, USA), 20 U RNasin, 2.5 mM rATP, 2.5 mM rGTP, 2.5 mM rUTP, 100 μM rCTP and 20 μCi of α-^{32}P rCTP (3000 Ci/mmol; Perkin Elmer, MA, USA) was incubated at 37°C for 30 minutes with 20 U of T7 (*Sal*I template) or T3 (*Xho*I template) RNA Polymerase in a 20 μl reaction volume. The reaction was stopped by addition of 10 μl of RNA loading buffer (Promega, WI, USA). Five microlitres of the transcription reaction was run on a 41 cm 10% denaturing polyacrylamide gel until the xylene cyanol dye front had migrated 30 cm down the gel. Gels were subjected to autoradiography for 30 minutes at -70°C.

The remaining T7 transcription reaction was run on a 20 cm 10% polyacrylamide gel until the xylene cyanol dye front had run approximately 15 cm. The gel was subjected to

autoradiography for 5-10 minutes and the autoradiograph used to locate full- and intermediate-length transcripts. The RNA fragments were excised and recovered from the gel slice using the crush and soak method (see Appendix 6.1.6).

2.4.3.2 Ribozyme *cis*-cleavage assays

The purified RNA was resuspended in 62.5 mM Tris-HCl (pH 8.0). Twenty microlitres of resuspended RNA was heated at 95°C for 5 minutes and allowed to cool to room temperature. *Cis*-cleavage was initiated by addition of 5 µl of 250 mM $MgCl_2$ and incubation at 37°C. Aliquots of 5 µl were removed at time points 0 and 20 minutes and *cis*-cleavage stopped by addition of 2 µl of RNA loading dye. The *cis*-cleavage reactions were run on a 41 cm 10% denaturing polyacrylamide gel until the xylene cyanol dye front had run approximately 30 cm down the gel. The gel was subjected to autoradiography for 24 hours at -70°C.

2.4.3.3 Testing efficacy of Rz-shRNA expression cassette in cultured cells

Transfections of Huh7 cells were carried out as described in Section 2.3.2, 6 µg of target/reporter plasmid (pCH-9/3091 for HBsAg quantitation and pCH-EGFP for green fluorescence detection) was co-transfected with 3 µg of pCI-Rz-shRNA vectors or empty pCI-neo vector as a control. Quantification of markers of HBV replication was carried out as described in Section 2.3.3.

2.4.3.4 *In vitro* transcription of shRNA for transfection

Transcription template was prepared by digesting pCI-Rz-shRNA vectors with *Bgl*II and *Sal*I. Transcription reactions were carried out with the MEGAshortscript™ Transcription Kit (Ambion, TX, USA) according to the manufacturer's instructions. Two to four micrograms of template was incubated with 10× Transcription buffer (Ambion, TX, USA), 150 mM rGTP, 150 mM rATP, 150 mM rUTP, 150 mM rCTP and 2 µl Enzyme mix at 37°C for 24 hours.

The transcription reaction was terminated by addition of 15 µl ammonium acetate, precipitated with ethanol and the RNA pellet resuspended in 50 µl of 100 mM Tris-HCl (pH 8.0). Ten microlitre aliquots were heated to 95°C and allowed to cool to room temperature. The RNA sample was subjected to ribozyme *cis*-cleavage by addition of 10 µl of 100 mM $MgCl_2$ and incubation at 37°C for 1 hour. Ribozyme *cis*-cleavage was terminated by addition of 5 µl RNA loading buffer. *Cis*-cleaved RNA was resolved on a 10% denaturing polyacrylamide gel until the xylene cyanol dye front was within 1.5-2.0 cm of the end of the gel. The gel was stained with SYBR gold (Invitrogen, CA, USA) and viewed on a UV transilluminator. Bands corresponding to the shRNA sequences were recovered from the gel by the crush and soak method (Appendix 6.1.6).

2.4.3.5 Transfection of cells with *in vitro* transcribed shRNA

To test the efficacy of *in vitro* transcribed shRNA, Huh7 cells were co-transfected with 4 µg pCH-9/3091 and 20 pmol of purified shRNA (70). Five hours before transfection Huh7 growth medium was replaced with RPMI containing 10% FCS only. Four micrograms of the target vector and 20 pmol of the shRNA were diluted into 250 µl of Opti-MEM I. Three microlitres of Lipofectamine 2000 was diluted into 250 µl of Opti-MEM I and incubated at room temperature for 5 minutes. The diluted Lipofectamine was mixed with the diluted nucleic acid for 20 minutes at room temperature to allow nucleic acid-lipid complexes to form. Five hundred microlitres of the nucleic acid-lipid complex mixture was added per well to a 6-well plate and mixed. The cells were incubated overnight at 37°C in a humidified incubator with 5% CO_2. Fifteen hours post-transfection the medium was harvested and replaced with RPMI supplemented with 2.5% FCS and antibiotics. Medium was harvested again at 24 hours post-transfection. The medium was used to measure HBsAg secretion with the HBV Monolisa HBsAg ELISA (Bio-Rad, CA, USA), HBeAg secretion (Axsym immunoassay kit, Abbot Laboratories, IL, USA) and human interferon alpha and beta (Promega, WI, USA).

CHAPTER 3

3 RESULTS

3.1 U6 shRNA Expression Vectors Inhibit Markers of HBV Gene Expression

3.1.1 U6 shRNA expression vectors inhibit EGFP expression

Transient transfection of cultured cells with pCH-EGFP allowed for the use of fluorescence microscopy and flow cytometry in the detection of green fluorescent protein as a marker of HBV gene expression. The positive control included pCH-EGFP co-transfected with empty pGEM®-T Easy vector to confirm that any observed inhibition cannot be attributed to vector backbone. The cells shown in Figure 3.1 indicate representative fluorescence microscope fields of transfected cells. Cells transfected with pG-U6 $shRNA_2$, pG-U6 $shRNA_8$, pG-U6 $shRNA_{10}$, pG-U6 $shRNA_{11}$, pG-U6 $shRNA_{12}$, pG-U6 $shRNA_{14}$ and pG-U6 $shRNA_{17}$ were able to significantly reduce EGFP expression. Transfection with pG-U6 $shRNA_6$ and pG-U6 $shRNA_7$ exhibited a moderate reduction in EGFP expression and transfection with pG-U6 $shRNA_{20}$ did not significantly alter EGFP expression.

Flow cytometry allows for more accurate quantification of green fluorescent cells, therefore the transfected cells were analysed by flow cytometry. Transfections performed in

triplicate were averaged and the averages normalized to 1, where the number of green cells in the positive control equals 1 (Figure 3.2). The total numbers of green fluorescent cells indicate a statistically significant reduction at a confidence interval of 95% however the degree of reduction in EGFP was only moderate (30-50%).

siRNA function by mediating the degradation of target mRNA. EGFP has a very long half-life; therefore any reduction in EGFP mRNA levels would not immediately be translated into a reduction in the protein levels. Using EGFP as a marker of HBV gene expression therefore underestimates the inhibitory effect of the U6 shRNA expression vectors. The cotransfection efficiency may also contribute to the moderate reduction in inhibition. The U6 shRNA vectors may not have transfected with the same efficiency as the reporter plasmid. Some cells therefore may only contain the reporter plasmids and as such would be positive for EGFP. The degree of inhibition observed is therefore a function of cotransfection efficiency.

3.1.2 U6 shRNA expression vectors reduce HBsAg secretion

To assess the effects of U6 shRNA on HBsAg secretion growth medium was harvested from cells transiently transfected with the target plasmid, pCH-9/3091, and U6 shRNA expression vectors. Transfections were performed in triplicate, averaged and the values normalized to 1 (where 1 equals the total amount of secreted HBsAg from the positive control). HBsAg secretion reflect EGFP results; pG-U6 $shRNA_2$, pG-U6 $shRNA_8$, pG-U6

shRNA$_{10,}$ pG-U6 shRNA$_{11}$, pG-U6 shRNA$_{12}$, pG-U6 shRNA$_{14}$ and pG-U6 shRNA$_{17}$ significantly inhibited HBV gene expression, pG-U6 shRNA$_6$ and pG-U6 shRNA$_7$ have moderate anti-HBV activity, whereas pG-U6 shRNA$_{20}$ does not inhibit HBV gene expression (Figure 3.3). The high turnover of HBsAg gives a more accurate indication of the inhibitory effects of the U6 shRNA expression vectors. At a 95% confidence interval HBsAg was reduced by 80-90%.

Results obtained from EGFP expression and HBsAg secretion were corroborated by Northern blotting. pG-U6 shRNA$_{10}$ significantly knocked down levels of HBV RNA whereas pG-U6 shRNA$_7$ only moderately knocked down HBV RNA and pG-U6 shRNA$_{20}$ did not significantly reduce levels of HBV RNA (Carmona S, personal communication).

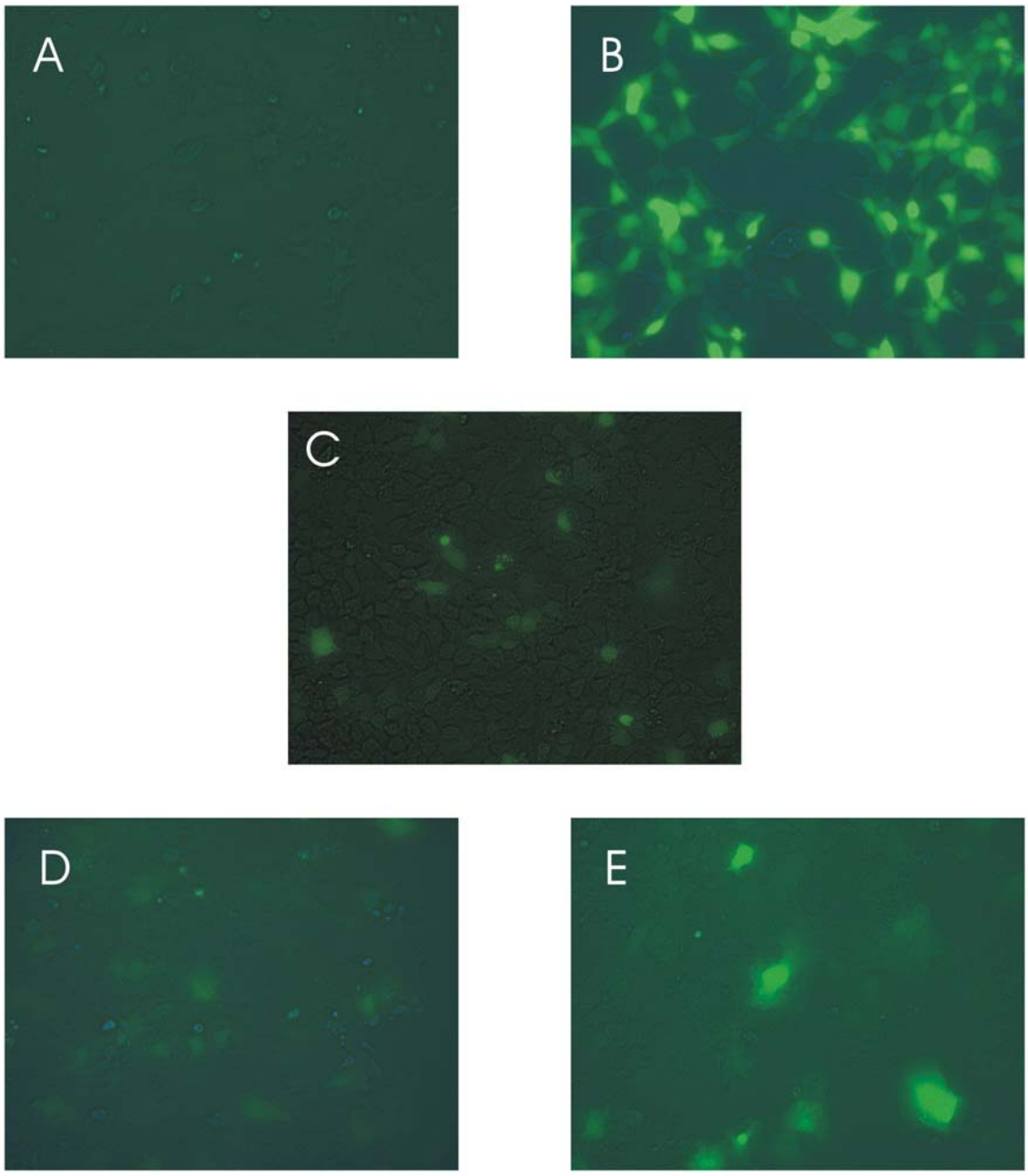


Figure 3.1: Representative fluorescence microscopy fields of Huh7 cells transfected with pCI-neo (A) pCH-EGFP only (B), and pCH-EGFP co-transfected with pG-U6shRNA$_7$ (C), pG-U6shRNA$_{10}$ (D) and pG-U6shRNA$_{20}$ (E).

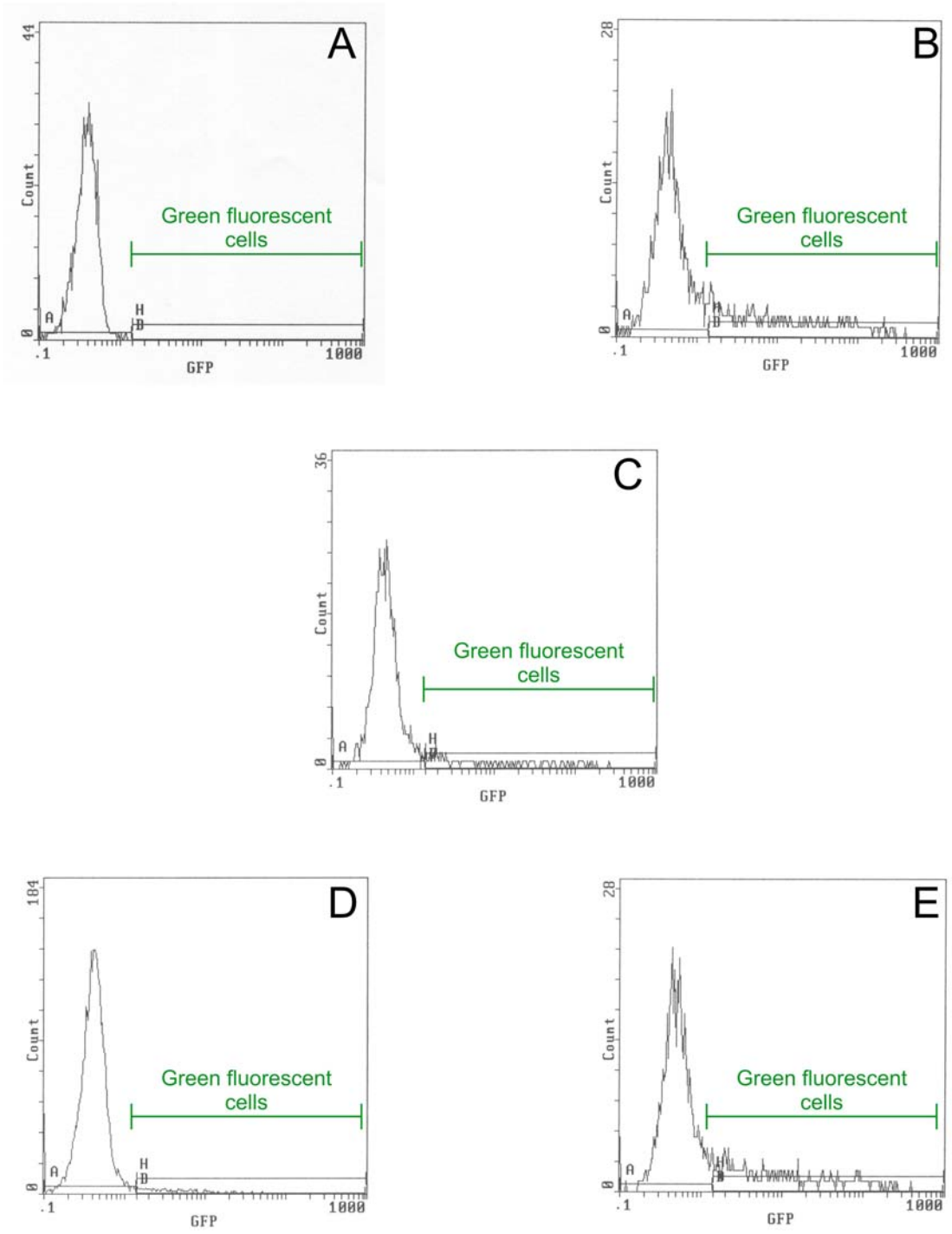


Figure 3.2: Representative flow cytometry histograms of Huh7 cells transfected with pCI-neo (A) pCH-EGFP only (B), and pCH-EGFP co-transfected with pG-U6shRNA$_7$ (C), pG-U6shRNA$_{10}$ (D) and pG-U6shRNA$_{20}$ (E).

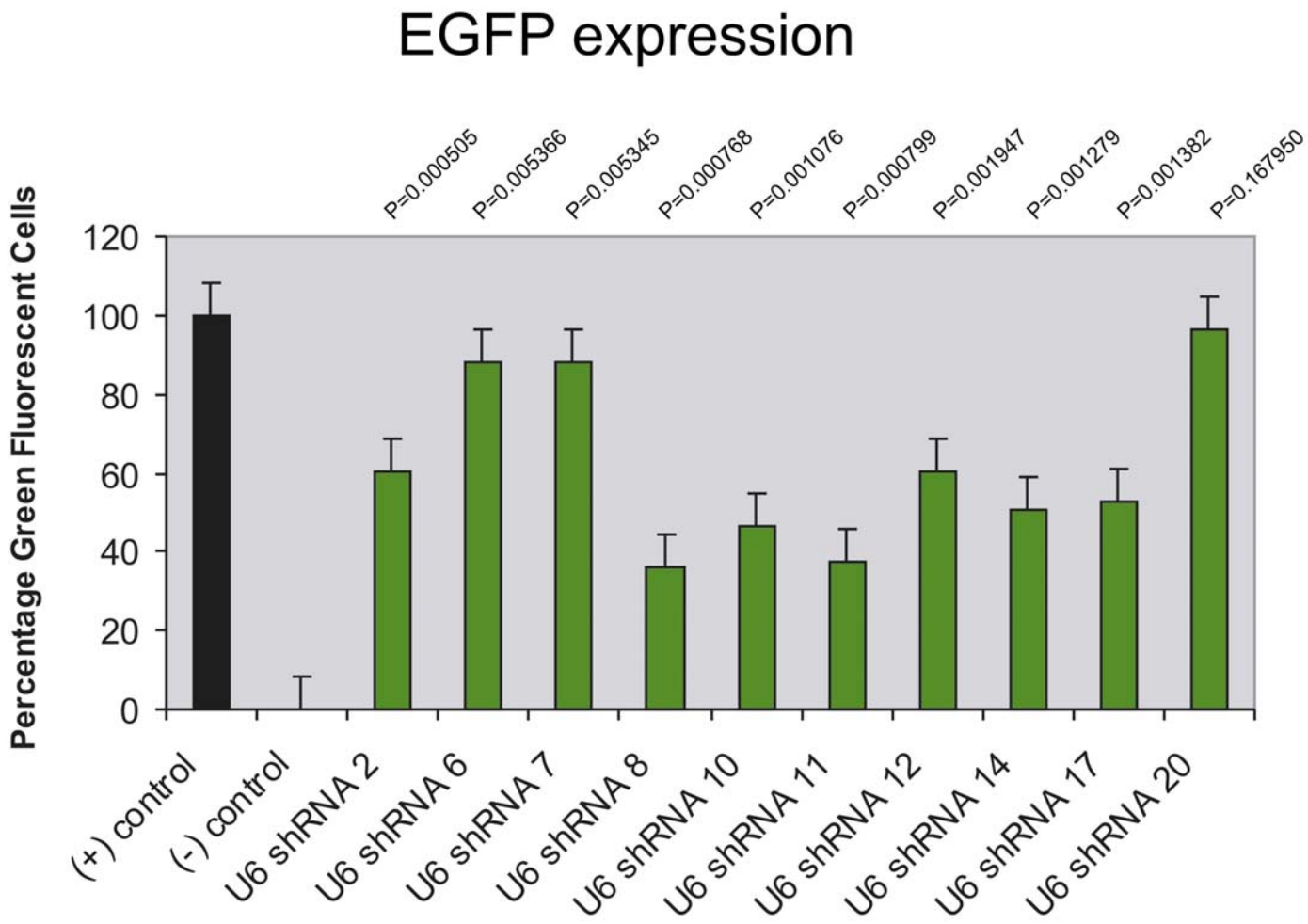


Figure 3.3: U6 promoter-derived shRNA mediated inhibition of EGFP expression as analysed by Flow Cytometry. Mean percentage of green fluorescent cells are indicated with the standard error of the mean.

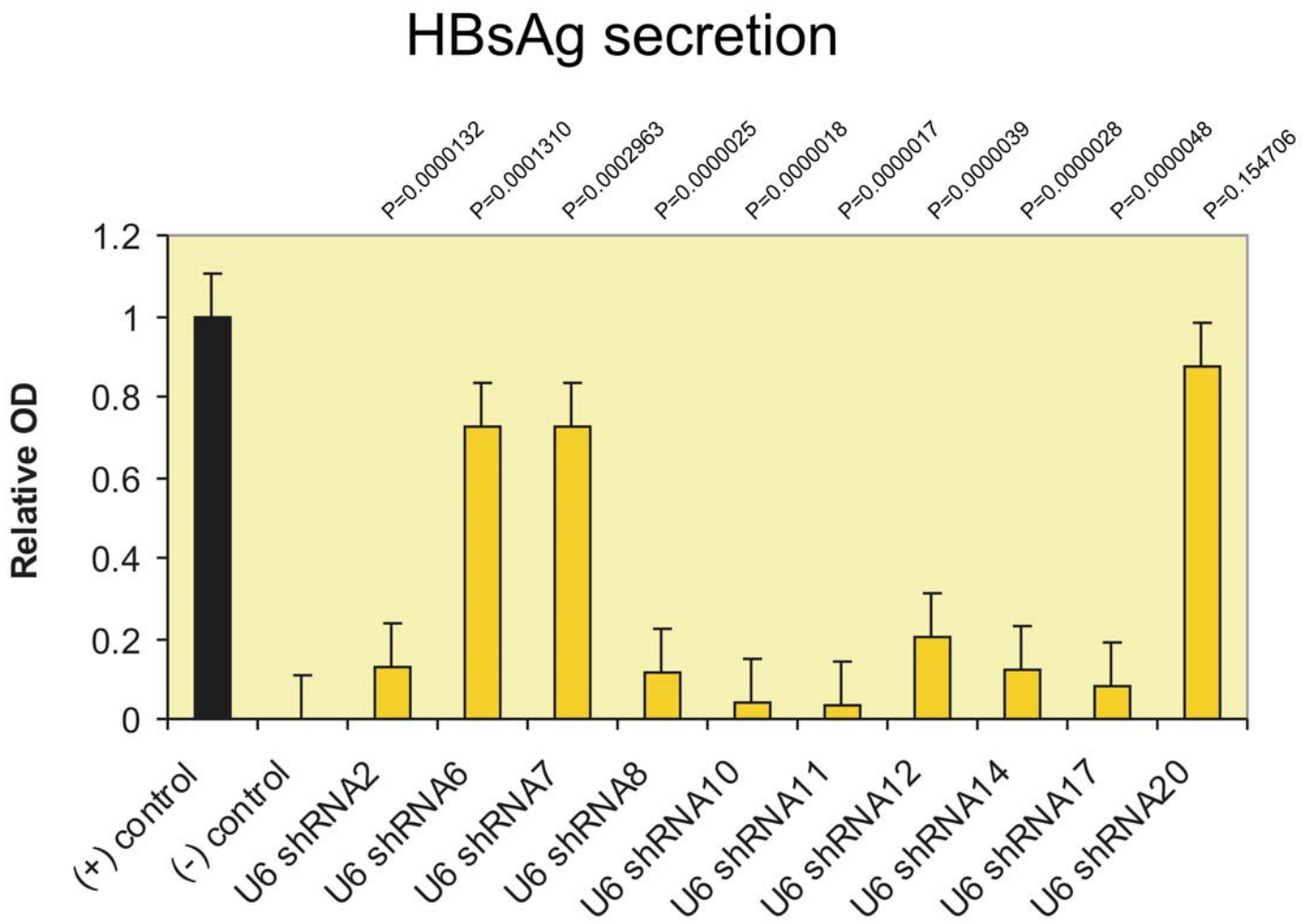


Figure 3.4: U6 promoter-derived shRNA mediated inhibition of HBsAg. Means of HBsAg secretion are indicated with the standard error of the means.

3.2 Selection of Rz-shRNA Sequences

Approximately two in ten randomly chosen siRNA are effective at knocking down gene expression by 95% (71). Construction and validation of the Rz-shRNA expression cassettes is time consuming and labour intensive. Therefore a rapid technique for the validation of target sites is required. Algorithms are available for the rational design of effective synthetic siRNA sequences (71, 72); however these do not necessarily apply to shRNA sequences. The technique developed by Castanotto et al. (65) allows for the rapid generation and validation of shRNA expression cassettes. To test the Rz-shRNA expression cassette three U6 shRNA sequences were chosen; a highly effective shRNA (U6 $shRNA_{10}$), a moderately effective shRNA (U6 $shRNA_{7}$) and an ineffective shRNA (U6 $shRNA_{20}$). U6 $shRNA_{7}$, U6 $shRNA_{10}$ and U6 $shRNA_{20}$ are equivalent to Rz $shRNA_{1}$, Rz $shRNA_{2}$ and Rz $shRNA_{3}$, respectively.

3.3 *In vitro* Transcription and Ribozyme *Cis*-cleavage Assays

3.3.1 *In vitro* transcribed RNA undergo *cis*-cleavage

To determine if the hammerhead ribozymes undergo *cis*-cleavage, RNA was transcribed from the three pCI-Rz shRNA expression vectors linearized with *Sal*I and *Xho*I to produce the sense and antisense transcript, respectively (Figure 3.5). Hammerhead ribozymes require magnesium ions to undergo *cis*-cleavage. Ribozyme *cis*-cleavage is effected when the oxygen

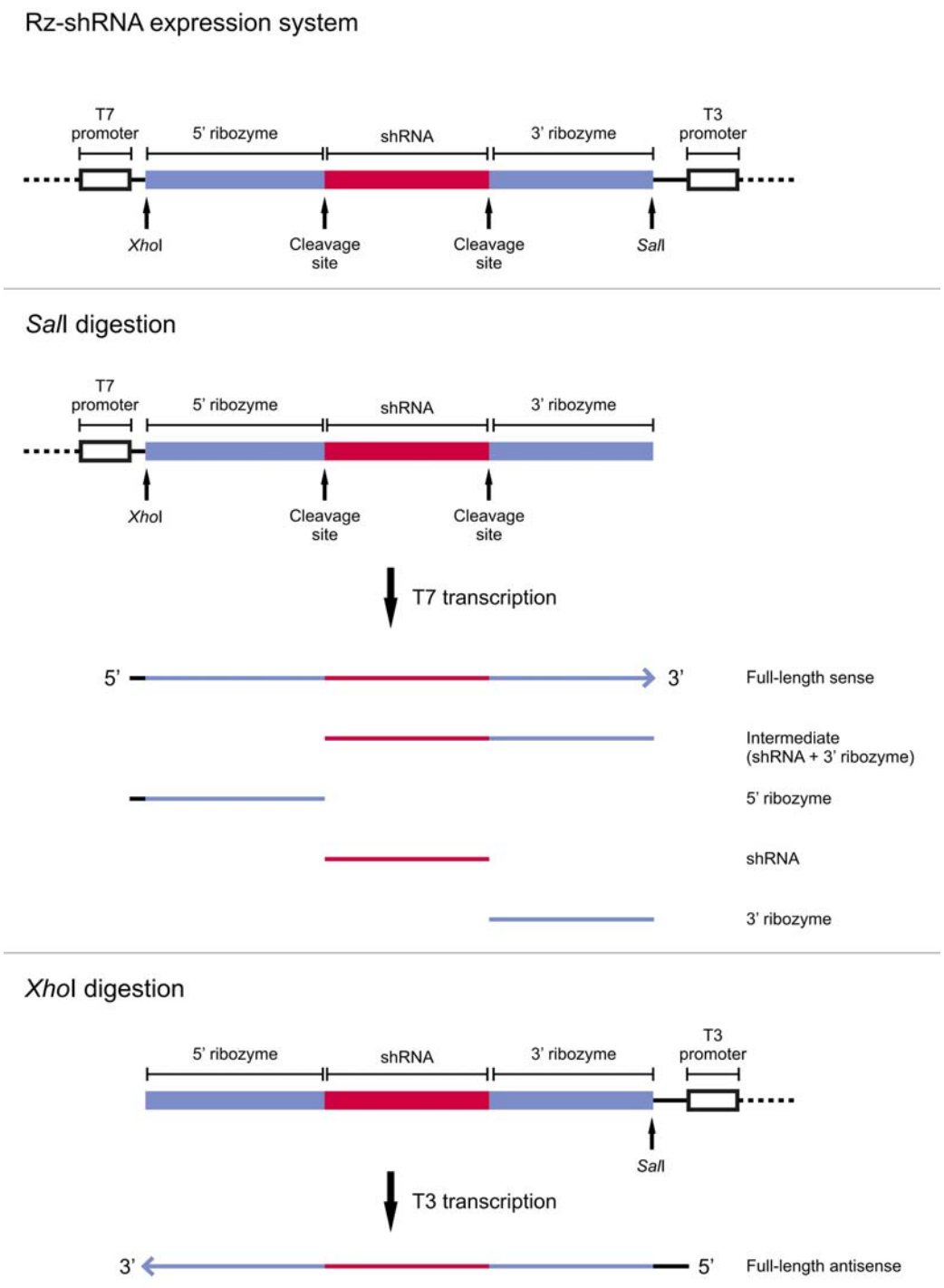


Figure 3.5: Schematic representation of products generated from *in vitro* transcription using T7 and T3 RNA polymerases.

of the 2'-OH group acts as a nucleophile to form a bond with the phosphorus atom thereby breaking the bond between the phosphorus atom and the 5'-OH (73). Magnesium ions facilitate ribozyme *cis*-cleavage by stabilizing the intermediates between cleaved and uncleaved states. Since the transcription reaction is supplemented with $MgCl_2$ ribozyme *cis*-cleavage occurs as the RNA is transcribed. After an hour of *in vitro* transcription, the cleavage products were visible (i.e. 5' ribozyme, shRNA and 3' ribozyme) (Figure 3.6). Since the antisense transcripts have the complementary sequences for the hammerhead ribozymes no cleavage products were observed. The shRNA generated from *cis*-cleavage of Rz-$shRNA_2$ is larger than those generated from *cis*-cleavage of Rz-$shRNA_1$ and Rz-$shRNA_3$. The resolution of the 10% polyacrylamide gel was high enough to resolve slight differences in the molecular weights of the three shRNA ($shRNA_1$ = 21 844 Da, $shRNA_2$ = 21 889 Da and $shRNA_3$ = 21 849 Da)

3.3.2 *Cis*-cleavage of full-length transcripts yields cleavage products

To confirm that the lower RNA bands were as a result of ribozyme cleavage, full-length and intermediate cleavage products were excised from the gel and subjected to *cis*-cleavage assays. The *cis*-cleavage assay reveals the full-length and intermediate RNA fragments decrease over time with a reciprocal increase in the cleavage products (Figure 3.7). Although the 5' and 3' hammerhead ribozyme undergo *cis*-cleavage the reaction is very inefficient since the full-length transcript was still present after a 20 minute incubation period

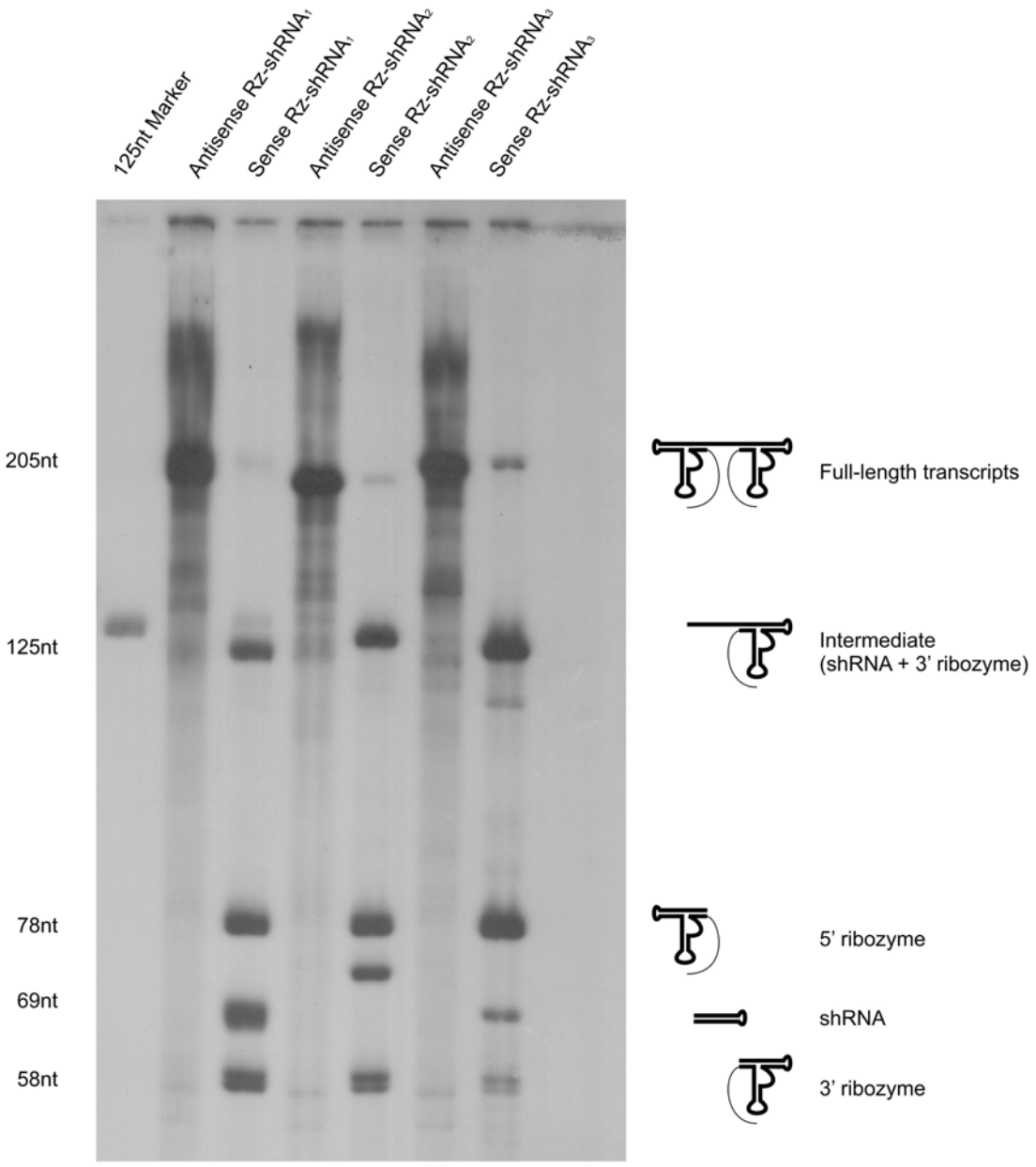


Figure 3.6: Autoradiograph of *in vitro* transcription. Transcription from the T7 promoter generates sense strand RNA with catalytically active ribozymes whereas transcription with the T3 RNA polymerase generates catalytically inactive ribozyme sequences. Active ribozymes undergo *cis*-cleavage to yield RNA fragments corresponding to the 5' ribozyme, the shRNA and the 3' ribozyme. Inactive ribozyme do not undergo *cis*-cleavage and remain as full-length transcripts.

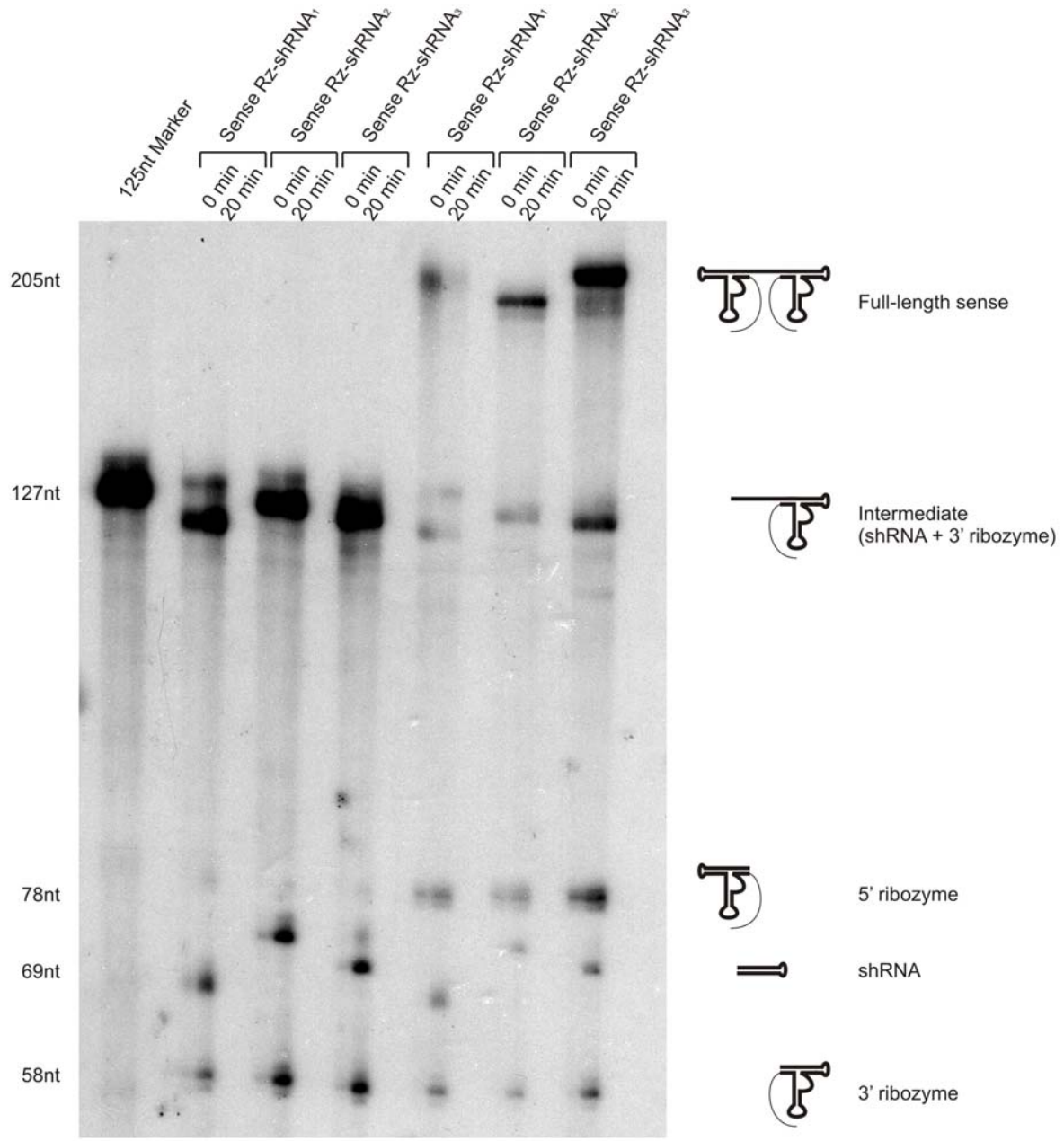


Figure 3.7: Ribozyme *cis*-cleavage assay. Full-length and intermediate sense transcripts were purified and subjected to a ribozyme c*is*-cleavage reaction. Over time the intensity of cleavage products (i.e. 5' ribozyme, shRNA and 3' ribozyme) increase with a concomitant decrease in the intensity of the full-length and intermediate transcripts.

in 50 mM $MgCl_2$. In order to achieve efficient *cis*-cleavage at least five Watson-Crick base pairing is required in helix I. The inefficiency of ribozyme *cis*-cleavage may be attributed to the instability of helix I of the ribozyme, which only has three Watson-Crick base pairs (see Figure 2.3). This may prevent the ribozymes, especially the 3' ribozyme from assuming the desired conformation thereby preventing *cis*-cleavage from occurring.

3.3.3 Rz-shRNA expression vectors do not inhibit markers of HBV gene expression

To test the ability of the Rz-shRNA expression to inhibit HBV gene expression pCI-Rz $shRNA_1$, pCI-Rz $shRNA_2$ and pCI-Rz $shRNA_3$ were co-transfected with pCH-9/3091 and HBsAg secretion measured. Transfections done in triplicate were averaged and the averages normalized to 1, where 1 equals total HBsAg secreted by the positive control. None of the Rz-shRNA expression vectors were capable of inhibiting HBV gene expression (Figure 3.8). This may be due to the fact that the vectors would endogenously produce a >1 kb fragment. Since the Rz-shRNA sequences are only approximately 200 nt in length the endogenously produced transcript also contains extraneous sequences. The extraneous sequences may not be conducive to proper folding of the ribozymes. Physiological conditions may also not favour ribozyme activity. Hammerhead ribozymes require divalent metal ions such as Mg^{2+} for efficient cleavage to occur. Intracellularly the free Mg^{2+} concentration is estimated to range between 0.1 and 0.5 mM (74). Ribozyme *cis*-cleavage assays were carried out at a Mg^{2+} concentration of 50 mM. After a 20 minute incubation full-length and intermediate transcripts

were still present demonstrating the inefficiency of the ribozymes especially the 3' ribozyme. The inefficiency of the ribozymes coupled with the limited availability of Mg^{2+} in the physiological context contributed to the inability of the Rz-shRNA expression to suppress markers HBV replication.

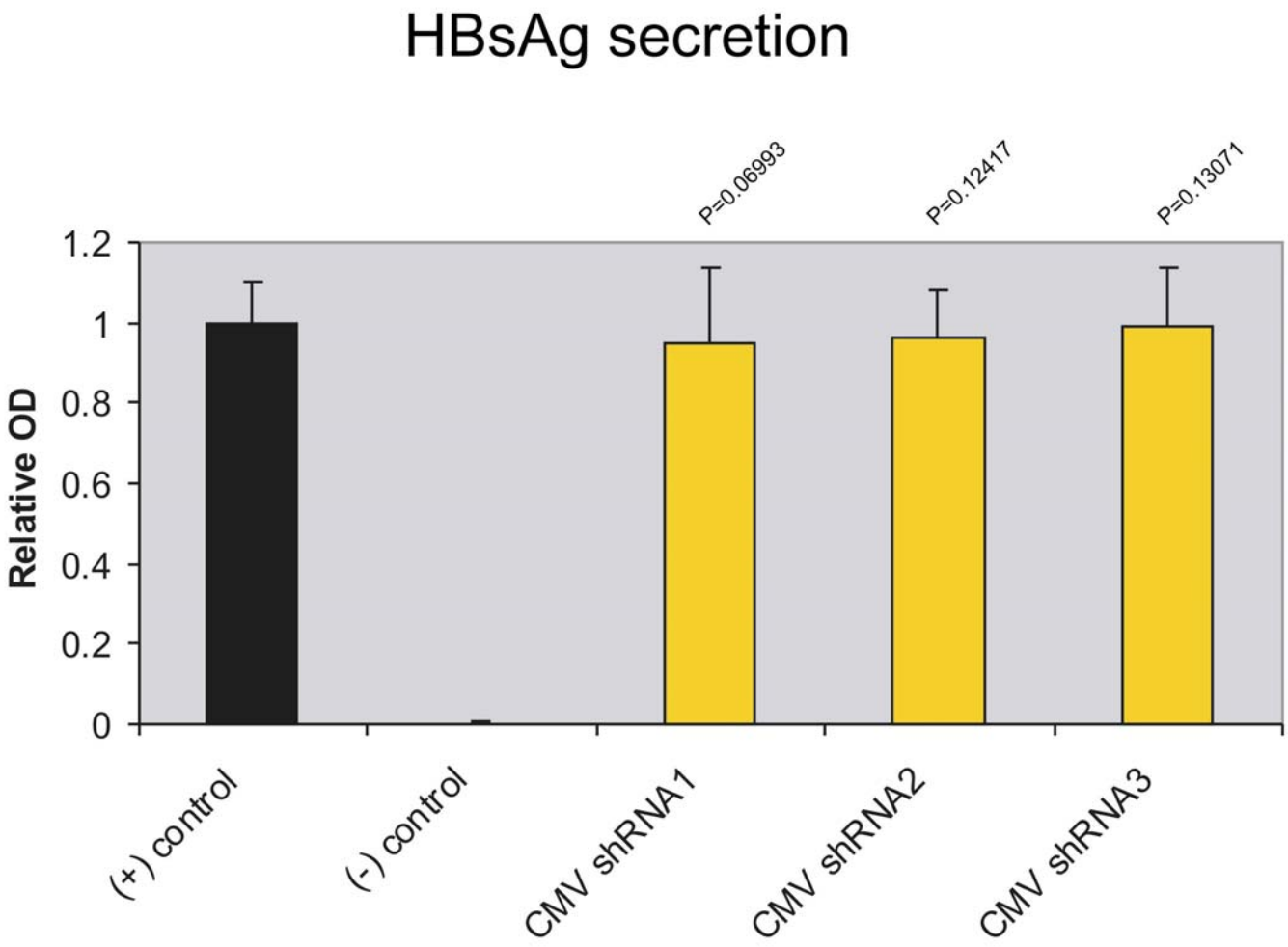


Figure 3.8: CMV Rz-shRNA mediated inhibition of HBsAg secretion. Cells were co-transfected with pCI-Rz shRNA and pCH-3091. HBsAg secretion was normalized to 1, where 1 equals the total HBsAg secreted by the positive control. Means are indicated with SEM.

3.3.4 *In vitro* transcribed Rz-shRNA inhibits HBV gene expression

To confirm that the inability of the Rz-shRNA expression cassettes to inhibit HBV gene expression was as a result of properties of the expression vector and not the shRNA sequence, Rz-shRNA$_1$, Rz-shRNA$_2$ and Rz-shRNA$_3$ were prepared by *in vitro* transcription of the expression vectors (Figure 3.9). Huh7 cells were co-transfected with combinations of pCH-9/3091, Rz-shRNA$_1$, Rz-shRNA$_2$ and Rz-shRNA$_3$. As expected Rz-shRNA$_1$, which is equivalent to U6 shRNA$_7$ only modestly knocked down HBsAg expression. Rz-shRNA$_2$, which is equivalent to U6 shRNA$_{10}$, was able to reduce HBsAg secretion to near baseline levels whereas Rz-shRNA$_3$ (equivalent to U6 shRNA$_{20}$) was unable to reduce HBsAg secretion significantly (Figure 3.10).

HBsAg can be expressed directly from pCH-9/3091 and therefore although HBsAg secretion from cells transfected with pCH-9/3091 correlates well with HBV replication strictly speaking it is not a direct marker of HBV replication. pCH-9/3091 is designed such that HBeAg is not expressed directly from the plasmid. Cells transfected with pCH-9/3091 only express HBeAg if cccDNA is formed (i.e. if replication occurs) (68). HBeAg secretion is therefore a direct marker of HBV replication. The effect of *in vitro* transcribed Rz-shRNA on HBeAg secretion was therefore also measured (Figure 3.11). HBeAg secretion did not correlate well with HBsAg secretion. The differences in suppression may be an indication that the 3.5 kb message encoding HBeAg has a different susceptibility profile to

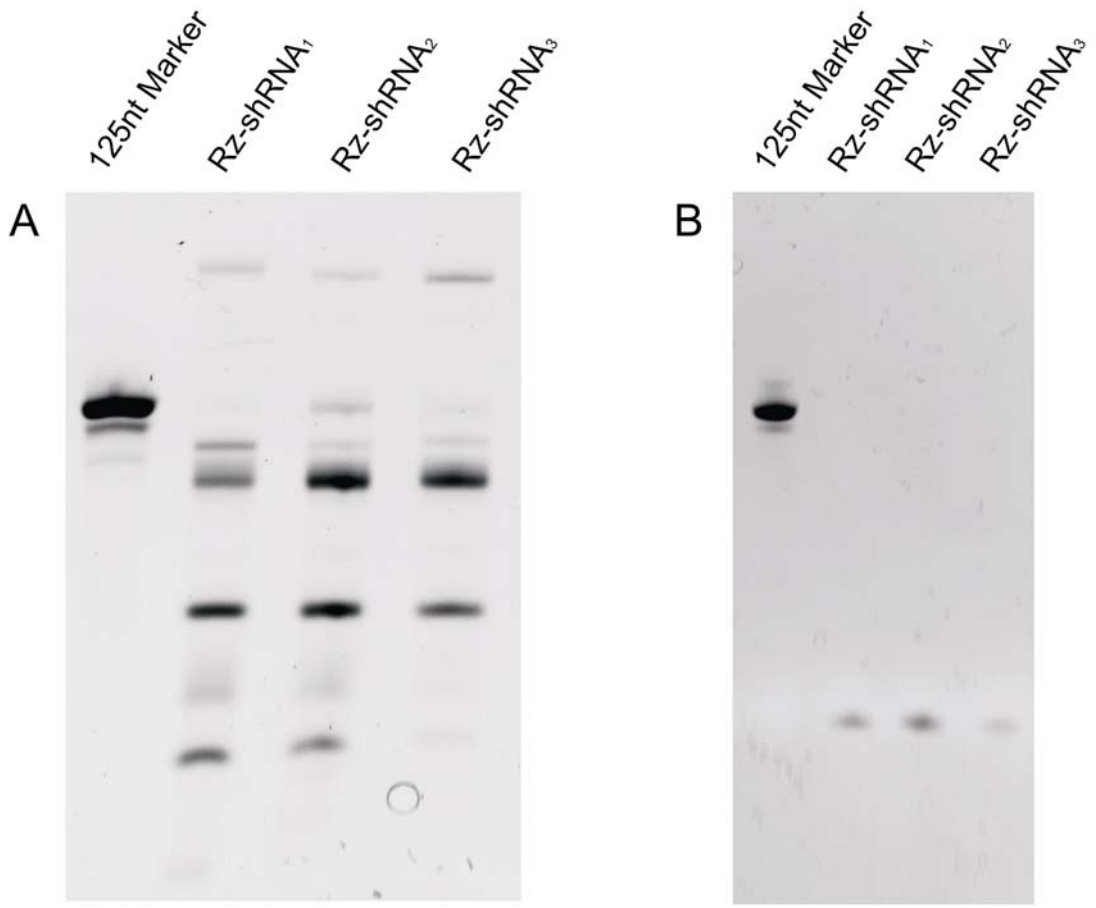


Figure 3.9: Rz-shRNA purification. RNA produced from run-off transcription of linearized pCI-Rz shRNA were subjected to polyacrylamide gel electrophoresis (A), the bands corresponding to the Rz-shRNA identified and purified by the crush and soak method. The purified Rz-shRNA were subjected to polyacrylamide gel electrophoresis to check RNA quality (B).

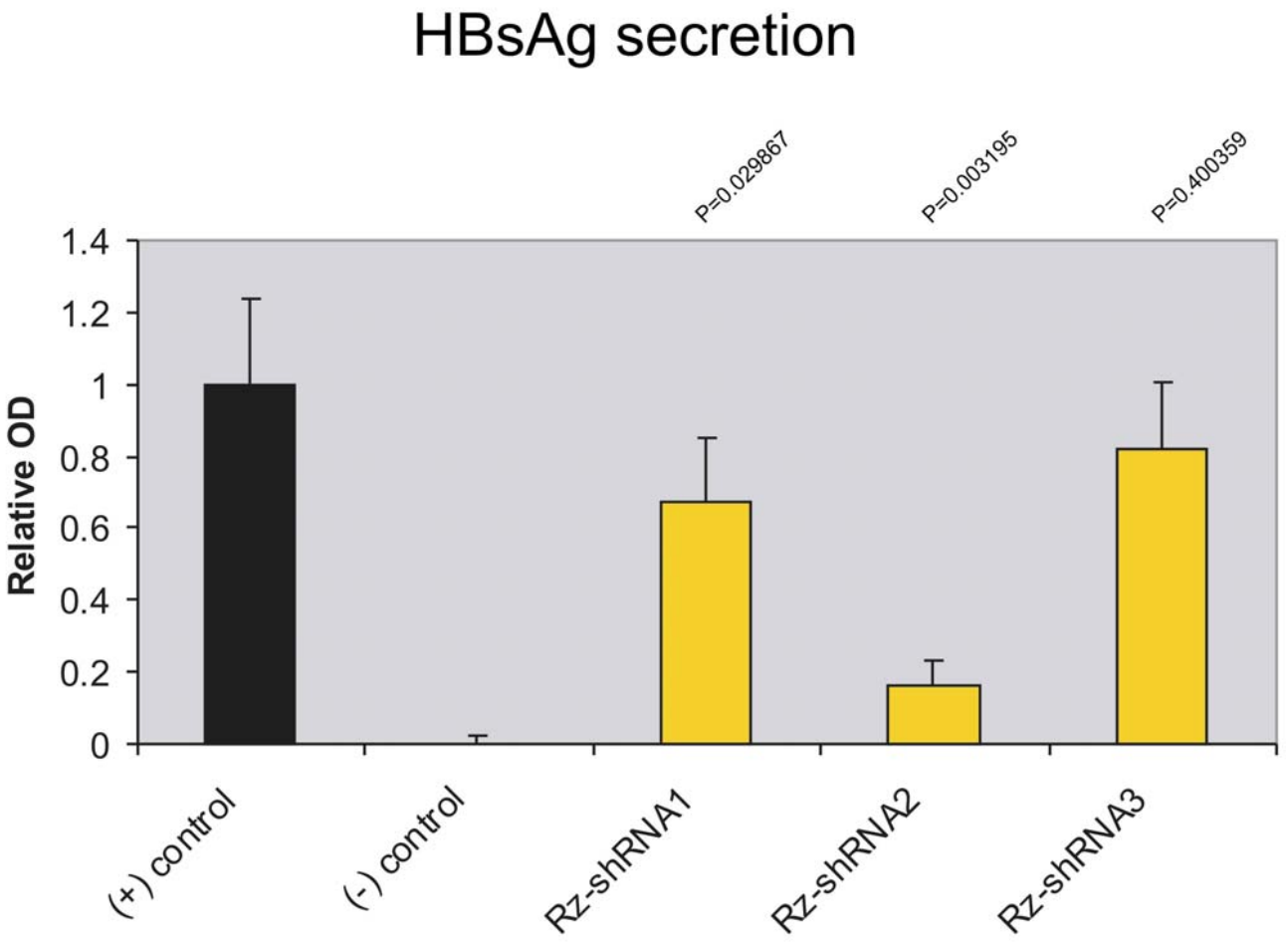


Figure 3.10: Rz-shRNA mediated inhibition of HBsAg secretion. Cells were co-transfected with *in vitro* transcribed Rz-shRNA and pCH-3091. HBsAg secretion was normalized to 1, where 1 equals the total HBsAg secreted by the positive control. The means are indicated with the SEM.

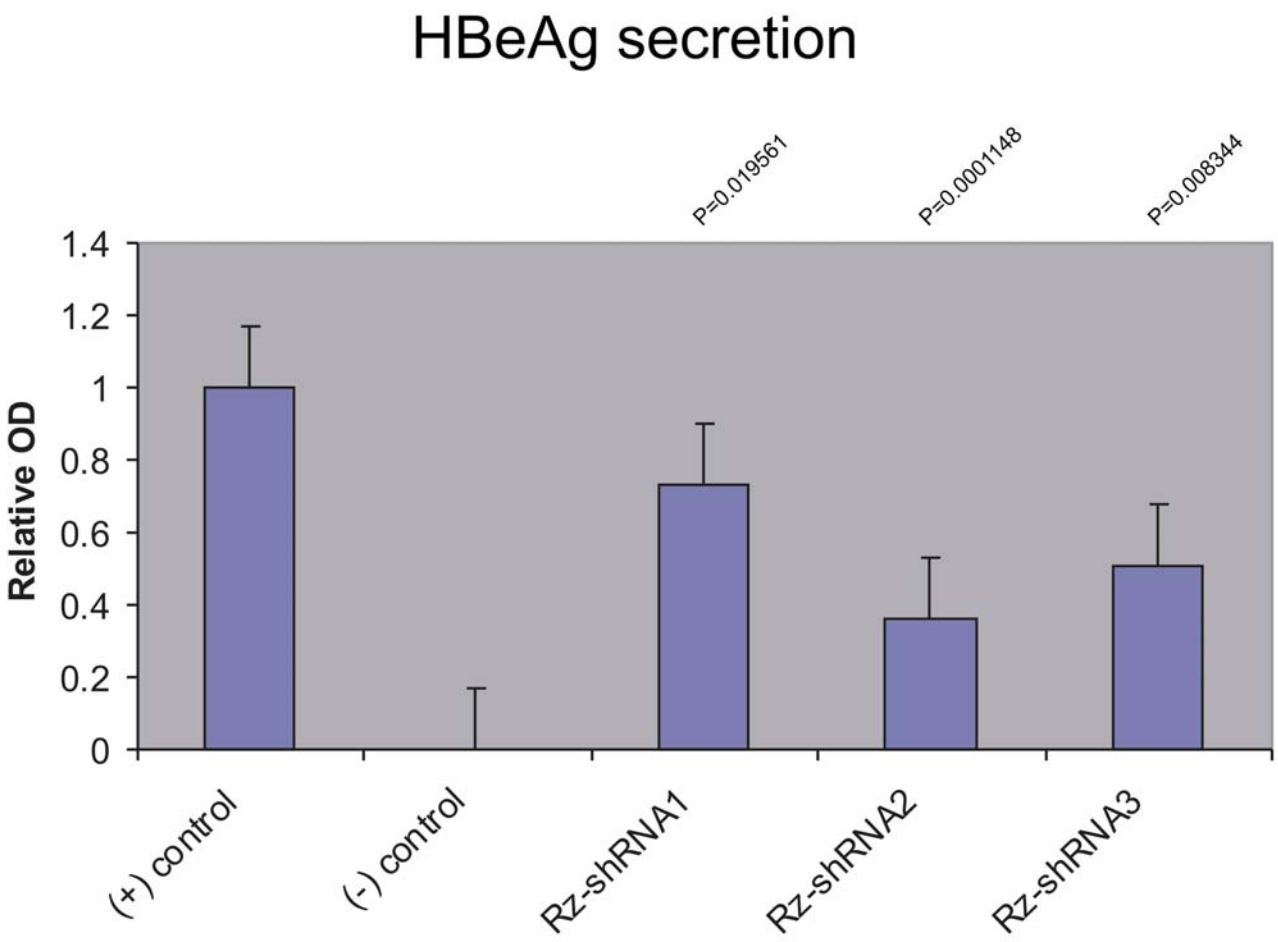


Figure 3.11: Rz-shRNA mediated inhibition of HBeAg secretion. *In vitro* transcribed shRNA and pCH-9/3091 were used to co-transfect Huh7 cells, the medium harvested at 24 hours and HBeAg secretion measured. HBeAg secretion was normalized to 1, where 1 equals the total HBeAg secreted by the positive control. The means are indicated with the SEM.

shRNA mediated inhibition than the 2.1 kb message encoding HBsAg (75).

3.4 Interferon-alpha and interferon-beta secretion

The use of RNAi to knockdown gene expression in mammalian cells can only be achieved through the use of siRNA as dsRNA greater than 30 bp induces the interferon response. Chemically synthesized siRNA, shRNA expression vectors as well as *in vitro* transcribed siRNA have been demonstrated to induce the interferon response (55, 76, 77). To determine whether the observed effects (i.e. knockdown of HBV gene expression) could be attributed to a specific RNAi-mediated pathway or to a non-specific interferon response interferon-α and interferon-β secretion from cells co-transfected with pCH-9/3091 and *in vitro* transcribed shRNA measured (Figures 3.11 and 3.12). Interferon secretion was either not significantly different from baseline (interferon-α) or significantly lower (interferon-β) than baseline. The significant decrease in interferon-β cannot be explained however, since interferon secretion (α and β) was not raised it may be concluded that the observed knockdown of HBV gene expression is not due to a non-specific interferon response.

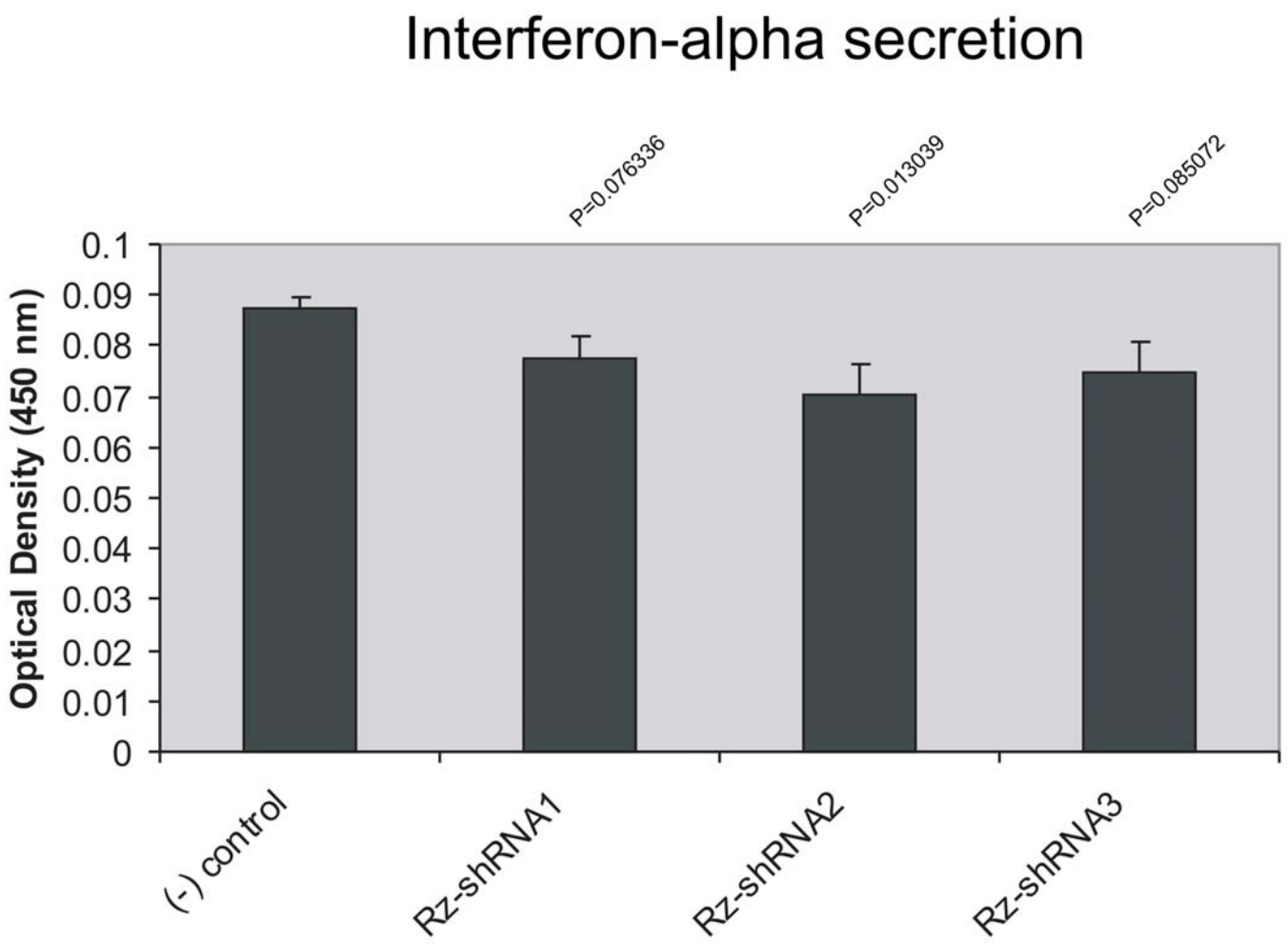


Figure 3.12: Interferon-alpha secretion. Interferon-α secretion from Huh7 cells co-transfected with pCH-9/3091 and *in vitro* transcribed shRNA was measured 24 hours post-transfection.

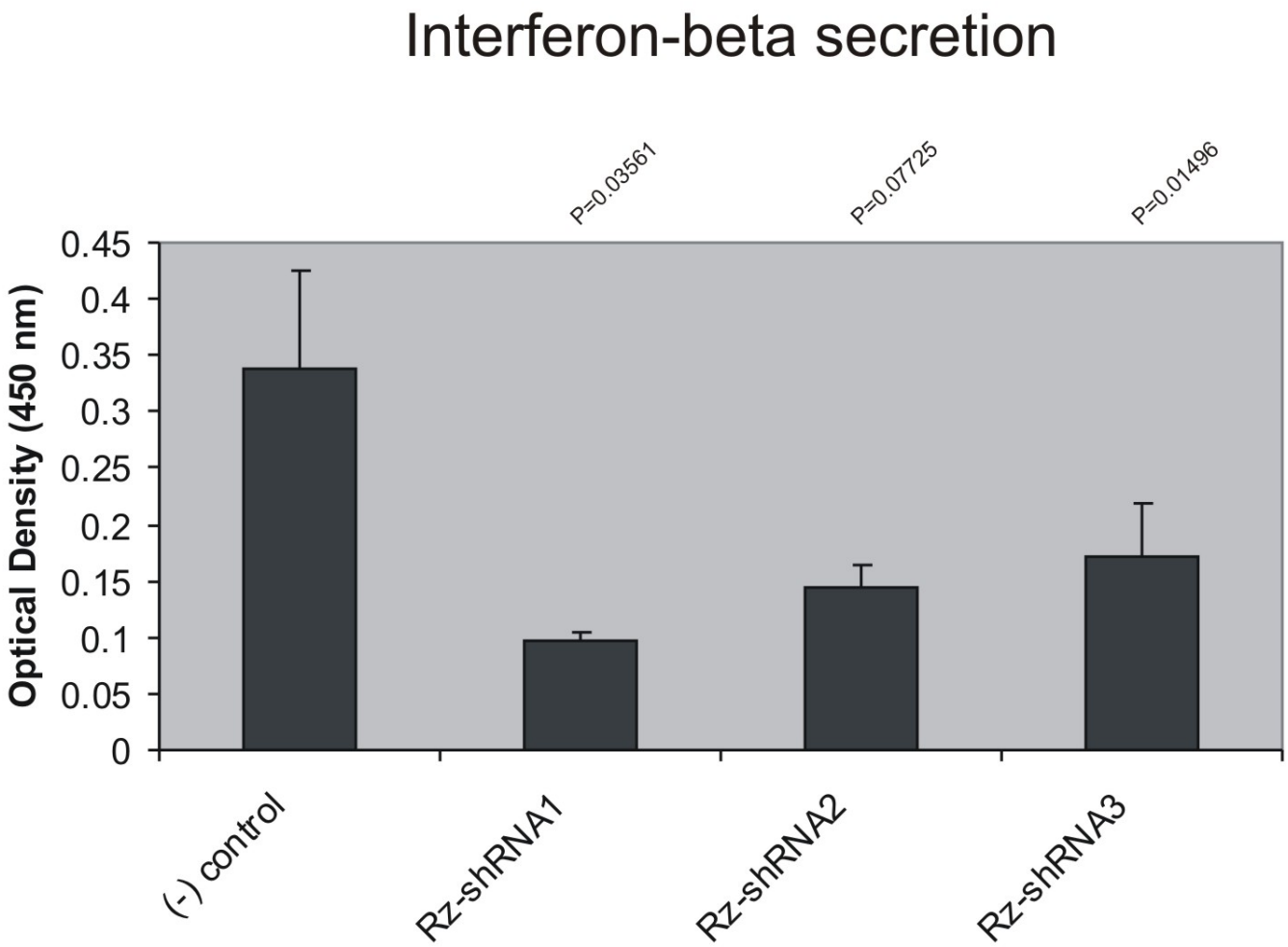


Figure 3.13: Interferon-beta secretion. Growth medium from Huh7 cells co-transfected with *in vitro* transcribed shRNA and pCH-9/3091 were analysed for interferon-β secretion at 24 hours post-transfection.

CHAPTER 4

4 DISCUSSION

Results presented in this study as well as other studies demonstrate that siRNA (10-13) and vector-expressed shRNA (14, 15) are capable of efficiently mediating RNAi against HBV. The use of RNAi as a treatment modality for the management of chronic HBV infection therefore holds much promise.

4.1 Efficacy of shRNA

The ability of any given siRNA to mediate efficient gene knockdown has been shown to be dependent on several sequence-specific criteria (71). Reynolds and colleagues identified eight criteria that confer functionality to a siRNA. These include a GC content of 30%-52%, 3 or more A/U pairs at positions 15-19, lack of internal repeats with $T_m < 20^{\circ}C$, an A base at positions 3 and 19, a U base at position 10, an A or U base at position 19 and an A, U or C at position 13 (with the positions of the bases given with reference to the sense strand). The first four criteria are important during the initial steps of RNAi (i.e. siRNA uptake by RISC, strand selection and duplex unwinding). The remaining criteria identify base preferences, which may be important in the final stages of RNAi (i.e. target recognition and mRNA cleavage). The sequences of the U6 shRNA and Rz-shRNA were analysed and scored based on the eight criteria (see Table 4.1 and Figure 4.1).

Table 4.1: Scoring table for predicting shRNA efficacy

	30-50% GC Content	3 AU bases at positions 15-19	Absence of Internal Repeats	A at position 19	A at position 3	U at position 10	A or U at position 19	A, U or C at position 13	Total Score
U6 shRNA$_2$	**1** (52%)	**3**	**1**	**1**	**1**	**0**	**1**	**1**	**9**
U6 shRNA$_6$	**0** (76%)	**0**	**1**	**1**	**0**	**0**	**1**	**1**	**4**
U6 shRNA$_7$ Rz-shRNA$_1$	**0** (72%)	**1**	**0**	**0**	**1**	**0**	**1**	**1**	**4**
U6 shRNA$_8$	**0** (62%)	**3**	**1**	**0**	**0**	**1**	**0**	**1**	**6**
U6 shRNA$_{10}$ Rz-shRNA$_2$	**0** (57%)	**3**	**1**	**0**	**0**	**0**	**1**	**1**	**6**
U6 shRNA$_{11}$	**0** (57%)	**2**	**1**	**1**	**0**	**0**	**1**	**1**	**6**
U6 shRNA$_{12}$	**1** (43%)	**5**	**0**	**1**	**1**	**0**	**1**	**1**	**10**
U6 shRNA$_{14}$	**1** (48%)	**3**	**0**	**1**	**0**	**0**	**1**	**1**	**7**
U6 shRNA$_{17}$	**1** (43%)	**4**	**0**	**0**	**0**	**0**	**1**	**1**	**7**
U6 shRNA$_{20}$ Rz-shRNA$_3$	**0** (57%)	**2**	**1**	**0**	**0**	**0**	**1**	**1**	**5**

T_m of internal loops determined with Gene Runner (78)

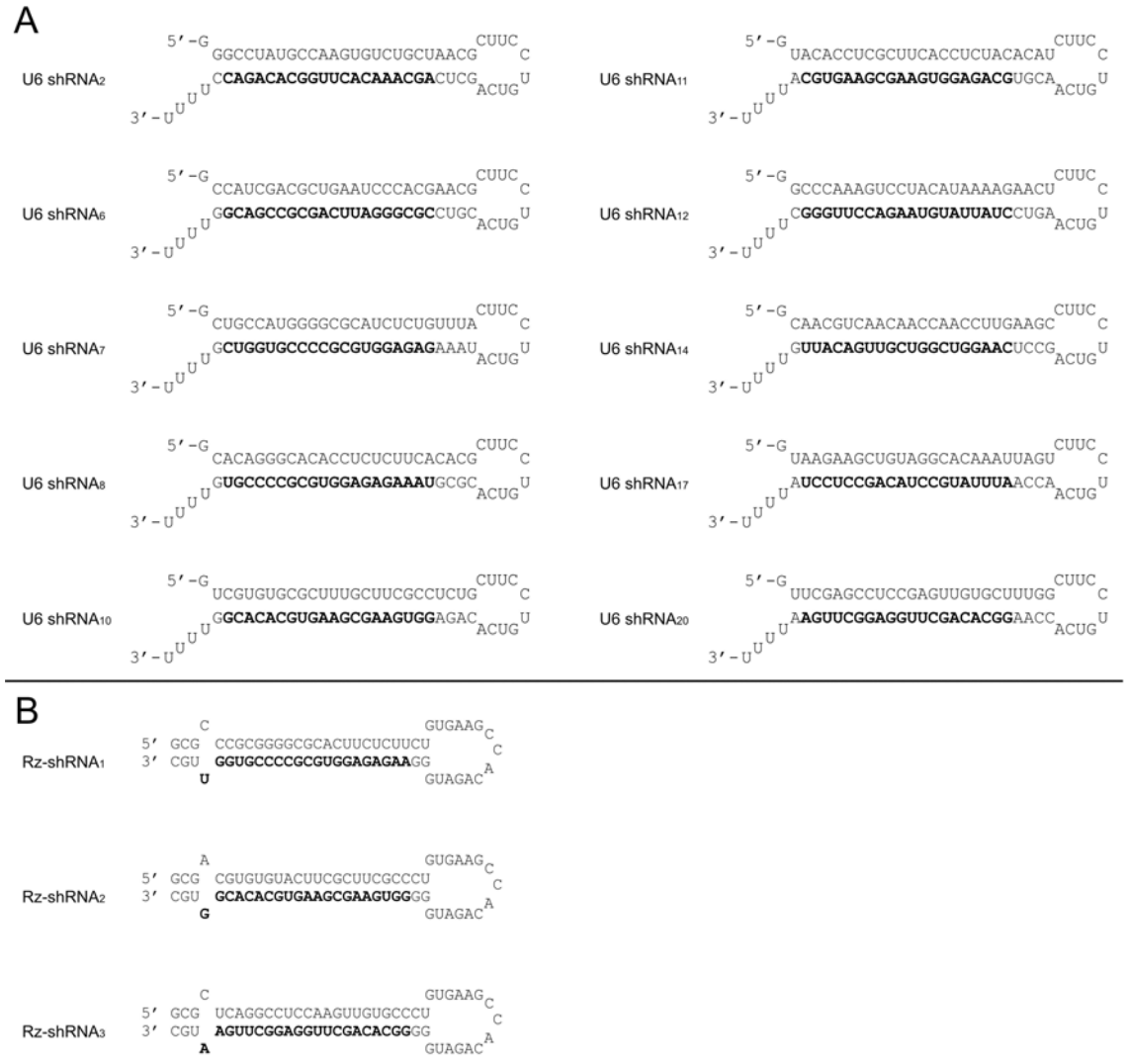


Figure 4.1: Predicted secondary structures of (A) U6 shRNA and (B) Rz-shRNA. Scores were generated against the sequences indicated in bold. Secondary structures predicted from design.

The eight criteria identified by Reynolds and colleagues were determined through the analysis of synthetically produced siRNA 19 bp in length. These criteria may therefore not be accurate at predicting effective shRNA sequences. This point is illustrated by the sequences of U6 $shRNA_{10}$ and its equivalent Rz-$shRNA_2$, which were effective at inhibiting HBV replication but scored low (Table 4.1). Similarly, U6 $shRNA_8$, U6 $shRNA_{11}$, U6 $shRNA_{14}$ and U6 $shRNA_{17}$ although effective at knocking down HBV replication received low scores. Other criteria, not necessarily excluding the eight listed, may be more important in determining efficacy of shRNA.

Primer extension analysis of cells transfected with pG-U6 $shRNA_{10}$ and pG-U6 $shRNA_{20}$ revealed that U6 $shRNA_{10}$ was processed efficiently into a siRNA whereas U6 $shRNA_{20}$ remained unprocessed (Carmona S, personal communication). Processing of shRNA by Dicer is therefore an important factor in determining the efficacy of a shRNA. The sequence requirements facilitating processing by Dicer are as yet unknown.

4.2 Endogenous versus Exogenous shRNA production

This project adopted two approaches for the generation of shRNA targeted to the *HBx* ORF of HBV. The first expression cassette involved the endogenous production of shRNA driven by the U6 promoter whereas the second expression cassette allowed for the *in vitro* production of shRNA from a phage promoter (i.e. the T7 promoter). Each expression cassette

has its own advantages as well as shortcomings. Neither expression cassette influenced the anti-HBV modulatory power of the expressed shRNA sequence. A shRNA sequence expressed endogenously from the U6 promoter was equally capable of inhibiting HBV replication as the same shRNA sequence produced from *in vitro* transcription. Both U6 $shRNA_7$ and Rz-$shRNA_1$ exhibited moderate anti-HBV activity, both U6 $shRNA_{10}$ and Rz-$shRNA_2$ were capable of inhibiting HBV replication to near baseline levels and U6 $shRNA_{20}$ and Rz-$shRNA_3$ were unable to inhibit replication of the virus. The observed degree of HBV gene knockdown was therefore determined wholly by the shRNA sequence without any contribution from the cassette it was expressed from.

The merit of using either approach is therefore defined by the innate advantages as well as the disadvantages of endogenous or exogenous shRNA production. RNAi-mediated gene silencing can only be maintained as long the DNA or RNA inducing RNAi remains stable. DNA has a longer half-life than RNA and as such the use of expression cassettes to endogenously generate shRNA maintain gene knockdown for longer periods than shRNA produced exogenously. DNA expression cassettes also allow for versatility of delivery in that both viral and non-viral vectors may be used to deliver the expression cassette whereas only non-viral vectors may be used for the delivery of exogenously produced shRNA. siRNA-mediated post-transcriptional gene silencing occurs in the cytoplasm therefore the shRNA, whether produced endogenously or exogenously, needs to reach the cytoplasm. The use of exogenously produced shRNA therefore only requires that the plasma membrane be crossed whereas expression cassettes need to cross the plasma membrane as well as the nuclear

membrane to exert an effect. The amount of shRNA generated from the endogenous transcription of the expression cassettes cannot be controlled therefore the dose cannot be controlled. The use of exogenously produced shRNA however does allow for the dosage to be controlled. Although the Rz-shRNA expression cassette could only knockdown HBV replication through the exogenous production of shRNA it has the potential to be used for both the endogenous and exogenous production of shRNA.

4.3 Off-target Effects

To identify sites within the human genome, which may be targeted by the panel of shRNA, the *HBx* target sites were subjected to a BLAST search. None of the *HBx* target sites were completely homologous to regions within the human genome. The search results returned 98 hits for $HBx_{1168\text{-}1192}$ (U6 $shRNA_2$ target site) of which 5 were significant. The first hit shows a 22/24 nt match, with the two mismatches lying close to the centre of the sequence, and is therefore unlikely to be silenced as mismatches close to the centre of the siRNA/mRNA duplex abolish silencing (79). The remaining 4 hits showed 18/19 nt matches. $HBx_{1640\text{-}1664}$ (U6 $shRNA_{12}$ target site) and $HBx_{1575\text{-}1599}$ (U6 $shRNA_{20}$ target site) were not significantly homologous to any region within the human genome. $HBx_{1618\text{-}1542}$ (U6 $shRNA_8$ target site) is 95% homologous to a region of a human cDNA clone (19/20 nt match) however the mismatch lies close to the centre of the target site U6 $shRNA_8$ is therefore unlikely to silence this mRNA. $HBx_{1678\text{-}1702}$ (U6 $shRNA_{14}$ target site) is significantly homologous to a human

expressed sequence tag (23/24 nt match) and may exhibit off-target effects. The remaining shRNA from the panel exhibit 18/19 nt matches with human cDNA clones.

Post-transcriptional gene silencing of a target mRNA by siRNA and miRNA is achieved in one of two ways, mRNA degradation or translational repression. The degree of complementarity between the siRNA or miRNA and the target mRNA determines whether silencing is through mRNA degradation or translational repression (45-48). If the siRNA or miRNA is completely complementary to the target silencing will occur through mRNA degradation. If incomplete complementarity exists between the target and the siRNA or miRNA silencing will be mediated through translational repression. A siRNA designed to be completely complementary to its target could theoretically exhibit incomplete complementarity to an undesired target. siRNA could therefore be predicted to mediate off-target gene silencing through translational repression.

Off-target gene regulation has been reported (80), however the results are questionable. Expression profiles demonstrated silencing of genes sharing as little as 8 complementary nucleotides with the siRNA. Silencing was demonstrated to occur by rapid kinetics indicating degradation events and not, as would be predicted, translational repression. Similar gene expression profiling done by two separate groups contradicted the first study (81, 82). A recent study argues against off-target effects (83). mRNA contain numerous sites targeted by miRNA. Only when single copies of different miRNA species or multiple copies of a single miRNA function synergistically is translational repression achieved. Therefore a siRNA

incompletely complementary to a single site on an mRNA sequence is unlikely to repress its translation.

4.4 The Interferon Response

Interferons function as antiviral cytokines in mammalian and avian systems as the first line of defence against viral infection. This is usually mediated by dsRNA as most viruses produce dsRNA at some point in their replicative life cycle. dsRNA binds and activates enzymes, which induce the interferon response. Binding of dsRNA to the dsRNA-activated Protein Kinase, PKR, activates the kinase by inducing autophosphorylation of this dimeric protein (25). Activated PKR phosphorylates and activates the α subunit of the eukaryotic translation initiation factor 2 (eIF-2α). Activated eIF-2α rapidly leads to the global shutdown of protein synthesis. 2'-5' Oligoadenylate synthetases (2'-5' OASs) are also activated upon binding to dsRNA and catalyze the polymerization of ATP into 2'-5' linked oligoadenylates (26). The oligoadenylates in turn bind and activate RNase L, which leads to the non-specific degradation of mRNA.

Initial RNAi experiments in mammalian cells were hampered by the induction of the interferon response as large dsRNA molecules were being used. The discovery that Dicer processing of the large trigger dsRNA could be bypassed (21), demonstrated that RNAi could be induced without inducing the interferon response. Since only dsRNA greater than 30 bp in

size induce the interferon response the use of siRNA, which are 21-22 nt in length, allowed for the use of RNAi in mammalian cells (27, 28).

Recently it was demonstrated that siRNA produced from *in vitro* transcription using phage polymerases (T7, T3 and SP6 polymerases) induce the interferon response (55). *In vitro* transcription from phage polymerases generates RNA with 5' triphosphates. RNA species containing 5' triphosphates are recognized by mammalian cells as viral in nature and therefore induce an interferon response. The Rz-shRNA cassettes described here allows for the *in vitro* production of shRNA from the T7 phage polymerase. However unlike previously described methods for the *in vitro* production of siRNA this method requires a ribozyme *cis*-cleavage step. As a result the 5' phosphate (which occurs on the 5' ribozyme) does not occur on the shRNA. The Rz-shRNA cassette therefore improves on the design of *in vitro* transcribed siRNA or shRNA as it bypasses induction of the interferon response through removal of the 5' triphosphate.

4.5 Delivery

In order to mediate inhibition of HBV the siRNA, shRNA or systems expressing the siRNA/shRNA sequences need to be effectively delivered to the liver. Delivery is the major hurdle facing any form of gene therapy.

Recombinant viral vectors have been generated carrying shRNA expression vectors (84, 85) and allow for the endogenous of shRNA capable of mediating RNAi. The use of recombinant viral vectors cannot be used in conjunction with exogenously produced siRNA or shRNA. The risks associated with recombinant viruses severely limit their application in gene therapy. Retroviruses, for example, are able to integrate into the genome of the host cell. Should the integration event disrupt normal gene expression, especially of genes involved in cellular proliferation, tumourogenesis could occur. As a fail-safe mechanism recombinant viruses are usually devoid in a gene or genes essential for viral replication making the recombinant virus replication-defective. Packaging cell lines complement replication-defective viruses since these cells express the gene or genes that the virus is deficient in. The possibility therefore still exists that a recombination event could occur within the packaging cell line, which would generate a replication-competent virus.

A safer option is the use of cationic lipids to deliver nucleic acids to cells. This method allows for the use of exogenously produced siRNA or shRNA as well as expression systems capable of endogenously generating siRNA or shRNA sequences. The development of lipid-based delivery systems exhibiting cell tropism remains an important objective. A novel approach to the development of cell tropic lipids was demonstrated recently. Numerous enveloped viruses exhibit cell tropism, due to specific interactions between viral receptors embedded within the viral envelope and host cell receptors. Yamada and colleagues generated nanoparticles (called L particles) by transfecting yeast cells with plasmids expressing the large surface antigen of HBV (86). The L particles were virion free. These particles were shown to

exhibit hepatotropism and were capable of efficiently delivering nucleic acids in addition to other molecules to hepatocytes. A major drawback to this technology was that the L particles were unable to transduce human HCC cells (PLC/PRF/5 cells). HBsAg particles containing L protein released by the cell line out competing L particles for host cell receptor sites is thought to be the reason for the inability of these nanoparticles to efficiently deliver to HCC cells.

The use of steroid and lipid conjugates linked to the 3' ends of synthetic siRNA have been demonstrated to enhance uptake of the siRNA by liver cells (87). Chemically modified siRNA molecules with conjugated cholesterol moieties have also been used as delivery systems *in vivo* after systemic administration (88). Since the liver is an important site of cholesterol biogenesis it has numerous receptors dedicated to the uptake of cholesterols. The receptors are presumably responsible for the increased uptake of cholesterol bound nucleic acids (89).

CHAPTER 5

5 CONCLUSION

In this study the construction and evaluation of expression cassettes capable of the endogenous and exogenous generation of shRNA is described. This study is the first to describe a self-cleaving cassette capable of endogenously and exogenously generating shRNA of precisely defined length.

The work presented here details a novel method for the *in vitro* production of shRNA by phage polymerases. The use of phage polymerases for the *in vitro* generation of siRNA or shRNA is complicated by the incorporation of 5' triphosphates in the RNA transcript. The presence of 5' triphosphates on RNA transcripts has been shown to induce the sequence non-specific interferon response. *In vitro* production of shRNA from the Rz-shRNA expression cassette requires ribozyme *cis*-cleavage events, which generates a shRNA sequence devoid of any 5' triphosphates. Although the Rz-shRNA expression cassettes were unable to mediate efficient knockdown of HBV replication, shRNA produced from *in vitro* transcription of the Rz-shRNA expression cassette were equally capable of inhibiting HBV replication as shRNA generated from conventional U6 promoter-driven expression.

siRNA expression systems favour the use of RNA polymerase III promoter as these are normally involved in the transcription of short RNA species and as such are ideal for the

generation of siRNA. The Rz-shRNA expression cassette employs ribozyme *cis*-cleavage to generate shRNA of exact size and is therefore not limited to the use of RNA polymerase III promoters. Since RNA polymerase II promoters exhibit tissue-specificity the Rz-shRNA expression cassette lends itself to tissue-specific expression of shRNA.

Knockdown of gene expression could not be achieved through the use the Rz-shRNA expression cassette. This may be due to the inability of the hammerhead ribozymes to undergo *cis*-cleavage in a physiological context. Free Mg^{2+} concentration within cells is well below the level required for hammerhead ribozyme to undergo catalysis. The Rz-shRNA expression cassette can be improved upon by the incorporation of ribozyme sequences capable of efficient *cis*-cleavage at physiological Mg^{2+} concentrations.

The Rz-shRNA expression cassette expands and improves on the repertoire of siRNA expression systems. The results presented in this study demonstrate the potential effectiveness in using the Rz-shRNA expression cassette for the knockdown of gene expression.

6 APPENDIX

6.1 Laboratory Techniques

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

Reagents

Luria Bertani medium

Ten grams of Bacto-tryptone (Oxoid, England), 5 g Yeast extract (Oxoid, England) and 5 g NaCl were dissolved per litre of deionized water. The medium was autoclaved for 30 minutes at 121°C and 1 kg/cm^2.

Transformation buffer

Transformation buffer was prepared as follows: 100 mM $CaCl_2$, 10 mM PIPES-HCl and 15% Glycerol. The pH was adjusted to 7.0 with NaOH and the solution autoclaved for 30 minutes at 121°C and 1 kg/cm^2. Transformation buffer is stored at -20°C.

Protocol

***Preparing chemically competent* E. coli**

Fifty millilitres of Luria Bertani medium was inoculated with a single colony of the desired strain of *E. coli* (DH5α or GM161). The culture broth was incubated at 37°C with shaking until the Absorbance read at 600 nm was 0.4. The cells were centrifuged at 2500 rpm for 5 minutes and the pellet resuspended in 10 ml Transformation buffer followed by a 20 minute incubation on ice. After the 20 minute incubation period the

cells were centrifuged at 2500 rpm for 5 minutes and the pellet resuspended in 1 ml of Transformation buffer. Aliquots of 100 µl were transferred to sterile microcentrifuge tubes and stored at -70°C.

***Transforming chemically competent* E. coli**

One hundred microlitres of competent *E. coli* was added to 5-10 µl of ligation mix and incubated on ice for 30 minutes. After the 30 minute incubation period the cells were heat shocked at 42°C for 90 seconds and placed on ice for an additional 2-5 minutes. Transformed bacteria were then plated on Luria Bertani agar plates containing selective antibiotics.

6.1.2 α-Complementation

Reagents

1000× Ampicillin

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

Ampicillin positive Luria Bertani agar plates

Ten grams Bacteriological agar (Oxoid, England), 10 g Bacto-tryptone (Oxoid, England), 5 g Yeast extract (Oxoid, England) and 5 g NaCl were dissolved per litre of deionized water**.** The solution was autoclaved for 30 minutes at 121°C and 1 kg/cm^2. 1000× ampicillin was added to a final concentration 100 µg/ml. Luria Bertani agar was poured into Petri dishes and allowed to solidify at room temperature.

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.

Isopropyl-β -D-thiogalactopyranosid (IPTG) stock solution

Two hundred milligram of IPTG (Roche, Germany) was dissolved in 1 ml sterile water.

Protocol

Preparing IPTG, X-gal positive Luria Bertani agar plates

Forty microlitres of X-gal stock solution and 8 µl of IPTG stock solution were added to an ampicillin positive Luria Bertani agar plate 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* are plated on ampicillin positive, X-gal, IPTG positive agar plates and incubated at 37°C overnight for α-complementation. IPTG induces expression of β-galactosidase, which cleaves the chromogenic substrate X-gal yielding a blue product. Successful cloning (plasmids positive for an insert) disrupts the β-galactosidase gene and therefore the protein is not functional resulting in white colonies. Unsuccessful insertion of fragments leaves the β-galactosidase intact resulting in blue colonies.

6.1.3 EndoFree® Plasmid Maxi Kit plasmid preparation

Reagents

Luria Bertani medium

See Appendix 6.1.1

EndoFree® Plasmid Maxi Kit (Qiagen, CA, USA)

Protocol

Three hundred millilitres of Luria Bertani medium was inoculated with a single colony containing the plasmid of interest and incubated at 37°C with shaking (100 rpm) for 18 hours. The cells were pelleted by centrifugation at 4000 rpm for 20 minutes at 4°C and the pellet resuspended in 10 ml Resuspension buffer (Buffer P1; 50 mM Tris-HCl, pH 8.0; 10 mM EDTA) containing RNAse A at a concentration of 100 µg/ml. Ten millilitres of Lysis buffer (Buffer P2; 200 mM NaOH; 1% SDS) was added to the cell suspension, the solution mixed thoroughly and incubated at room temperature for no more than 5 minutes. Ten millilitres of the Neutralization buffer (Buffer P3; 3 M KAc, pH 5.5) was added to the lysate, mixed, applied to the Qiafilter Cartridge and incubated at room temperature for 10 minutes. After the 10 minute 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 on ice a QIAGEN-tip 500 was equilibrated by applying the Equilibration buffer (Buffer QBT; 750 mM NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol; 0.15% Triton® X-100) to the column and allowing the buffer to

drain by gravity flow. After the incubation in Endotoxin removal buffer the solution was applied to the QIAGEN-tip and allowed to drain by gravity flow. The column was washed with 30 ml Wash buffer (Buffer QC; 1 M NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol), twice. The plasmid was eluted from the column with 15 ml Elution buffer (Buffer QN; 1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol). A 0.7× volume of isopropanol was used to precipitate the plasmid. The DNA was centrifuged at 8000 rpm for 1 hour at 4°C. The DNA pellet was washed with endotoxin-free 70% ethanol and re-centrifuged at 8000 rpm at 4°C for an hour. The pellet was air-dried and resuspended in an appropriate volume of endotoxin-free TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

6.1.4 Tissue culture

Reagents

RPMI medium (RPMI)

One litre of RPMI was made as follows: 10.4 g of RPMI-1640 (Gibco BRL, United Kingdom), 5.0×10^{-3}M HEPES, 3.0×10^{-8}M Na_2SeO_3, 3.0×10^{-9} $(NH_4)_6Mo_7O_2 \cdot 4H_2O$, 1.0×10^{-7} M $FeSO_4 \cdot 7H_2O$, 3.0×10^{-10}M $MnCl_2 \cdot 4H_2O$, 1.0×10^{-8} M NH_4VO_3, 3.0×10^{-9} M linoleic acid, 3.0×10^{-9} M oleic acid, 3.0×10^{-5} M ethanolamine and 2.4×10^{-2} M $NaHCO_3$. The medium was sterilized by filtration.

FCS (delta bioproducts, South Africa)

1000× Pen/Strep

0.61 g of Penicillin and 1 g of Streptomycin was dissolved in 10 ml of deionized water and the solution filter sterilized.

Saline + 0.01% EDTA

0.5× Trypsin

Five hundred microlitres of a 10× stock solution was made up to 10 ml and filter sterilized.

Protocol

Huh7 cells were maintained in a humidified incubator at 37°C and 5% CO_2 in RPMI growth medium supplemented with 2.5% FCS and antibiotics. When cells reached a density covering 90% of the culture dish surface the cells were passaged. The cells were washed once with saline followed by a 5 minute incubation at 37°C in saline containing 0.01% EDTA. Following the 5 minute incubation the saline-EDTA solution was removed, 500 µl of 0.5× trypsin added and the cells incubated for an additional 5 minutes. The cells were then dislodged from the culture dish by gentle aspiration. An equal volume of conditioned medium was added to inactivate the trypsin. Cells were added to a sterile 10 cm^2 tissue culture dish at the desired density (30-40%). Ten millilitres of RPMI was added and the cells incubated at 37°C and 5% CO_2 in a humidified incubator. Growth medium was replenished at 48 hour intervals until cells needed to be passaged.

For transfections, Huh7 cells were grown to a density of 90-100%. The growth medium was removed and the cells washed with saline. Following the wash the cells were incubated in saline containing 0.01% EDTA for 5 minutes. The saline-EDTA was

removed and the cells trypsinized at 37°C for 5 minutes. Two hundred- to two hundred and fifty-thousand cells were seeded per well in a 6-well plate and the cells grown overnight at 37°C and 5% CO_2 in RPMI supplemented with 2.5% FCS and antibiotics. Five hours before transfection the growth medium was removed and replaced with RPMI supplemented with 10% FCS and no antibiotics.

6.1.5 Manual sequencing

Reagents

SequiTherm EXCEL™II DNA Sequencing Kit (Epicentre®, WI, USA).

T7/T3 unlabeled primer

T7 primer sequence: 5'- TTA ATA CGA CTC ACT ATA -3'

T3 primer sequence: 5'- CAT TAA CCC TCA CTA AAG -3'

α-^{32}P dATP (3000 Ci/mmol; Amersham, United Kingdom)

10× Tris-Borate EDTA (TBE) buffer

Fifty four grams of Tris, 27.5 g of Boric acid and 20 ml of 0.5M EDTA (pH 8.0) were dissolved in 500 ml of deionized water and sterilized by autoclaving at 121°C and 1 kg/cm^2.

8% denaturing polyacrylamide gel

Eight grams of Acrylamide and bis-acrylamide (at a ratio of 19:1) per 100 ml

7 M Urea

Ten millilitres of 10× TBE

Protocol

In a 17 µl reaction volume, a master mix containing 1.5 pmol of unlabeled primer (T7 or T3), 0.5-1.0 µg of plasmid DNA (pCI-Rz-shRNA), 7.2 ml 3.5× SequiTherm EXCEL™ II Sequencing Buffer, 10 µCi α-^{32}P dATP, and 5 U of SequiTherm EXCEL™ II DNA Polymerase. Two microlitres of ddGTP, ddATP, ddTTP and ddCTP were added separately to four sterile 0.5 ml microcentrifuge tubes. Four microlitres of the master mix was added to each of the tubes containing ddNTPs. The mixture was denatured at 95°C for 5 minutes. The cycling parameters were as follows: 30 cycles of 30 seconds at 95°C and 1 minute at 70°C. Upon completion of the cycling, 3 µl of Stop/Loading Dye was added to the sequencing reactions. Before loading the sequencing reactions the samples were heated at 95°C for 5 minutes. Three microlitres of the sequencing reactions were loaded per well in the order G-A-T-C and resolved on an 8% denaturing polyacrylamide gel at 60 W. The samples were run until the bromophenol blue dye front reached the end of the gel. The gel was removed from the plates and subjected to autoradiography at -70°C for 30 minutes to an hour.

6.1.6 RNA recovery from polyacrylamide gel

Reagents

Diethyl pyrocarbonate (DEPC)-treated water

One millilitre of DEPC was added to a litre of deionized water. DEPC treatment was allowed to proceed overnight. DEPC was inactivated by autoclaving at 121°C and 1 kg/cm^2 for 30 minutes.

Elution buffer

The Elution buffer was prepared as follows: 20 mM Tris (pH 7.5), 0.5 M Sodium acetate (pH 5.2), 10 mM EDTA (pH 8.0) and 1% SDS. All solutions were prepared with DEPC-treated water.

Protocol

A sharp, clean blade was used to excise the gel slice containing RNA of interest. The gel slice was transferred to a sterile microcentrifuge tube and crushed with a filtered pipette tip. Two hundred microlitres of the Elution buffer was added per gel slice and incubated at 37°C with agitation overnight. After the overnight incubation the solution was centrifuged at 10 000 rpm for 10 minutes. The supernatant was carefully removed to a clean microcentrifuge tube and the RNA ethanol precipitated. A 0.1× volume of 3M Sodium acetate and an equal volume of 100% ethanol was added to the supernatant and incubated at -20°C for an hour. After the hour incubation the RNA was precipitated at 13 000 rpm for 30 minutes at 4°C. The RNA pellet was washed in 70% ethanol and re-centrifuged at 13 000 rpm for 10 minutes at 4°C. RNA to be used for ribozyme *cis*-cleavage assays was resuspended in 62.4 mM Tris-HCl. The RNA to be used in transfections was resuspended in Endotoxin free TE buffer (Qiagen, CA, USA).

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