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OPTIMISATION OF EXPRESSED RNA INTERFERENCE EFFECTERS FOR THE INHIBITION OF HEPATITIS B VIRUS REPLICATION

Abdullah Ely

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

of

Doctor of Philosophy

Johannesburg, 2009

DECLARATION

I, Abdullah Ely declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy 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 ……., 2009

DEDICATION

طلب العلم فريضة علي كل مسلم (سُنن ابن ماجه)

Taulabul ilmi faridatun ala kulli Muslimeen (Sunan ibn Majah)

Seeking knowledge is an obligation on every Muslim (Collections of ibn Majah)

To my family

My parents, Gava and Yagya.

My brother, Moegammad and sisters, Madenia, Rhodah and Godeejah.

My nieces, Mary-oleen, Ameerah and Zainab and nephew, Mu'ain.

And to future additions.

PUBLICATIONS AND PRESENTATIONS RELATED TO THESIS

Journal Publications

1. Ely, A., Naidoo, T., and Arbuthnot, P. (2009). Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles. Nucleic Acids Res. 2009 Jul; 37(13):e91.
2. Arbuthnot, P., Ely, A and Weinberg M. (2009) Hepatic delivery of RNA interference activators for therapeutic application. Curr Gene Ther, Apr; 9(2):91-103.
3. Crowther, C., Ely, A., Hornby, J., Mufamadi, S., Salazar, F., Marion, P., and Arbuthnot, P. (2008). Efficient inhibition of hepatitis B virus replication in vivo, using polyethylene glycol-modified adenovirus vectors. Human Gene Therapy, Nov; 19:1325-1332.
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1. Ely, A and Arbuthnot, P (2009) Advances in the use of RNA interference to treat chronic hepatitis B virus infection, In: RNAi and Viruses, Current Innovations and Future Trends, Ed, Martinez MA, Horizon Scientific Press, UK, In Press.

Conference Proceedings

1. Abdullah Ely, Tanusha Naidoo and Patrick Arbuthnot, Efficient silencing of hepatitis B virus gene expression with modular trimeric Pol II expression cassettes comprising microRNA-31 shuttles, 12th Annual Meeting of the American Society of Gene Therapy, May 27-30, 2009, San Diego, California, USA.
2. Justin Hean, Rafique Ul Islam, Carol Crowther, Abdullah Ely, Willem van Otterlo, Charles de Koning, Piet Herdewijn and Patrick Arbuthnot, Silencing hepatitis B virus replication in cell culture and *in vivo* using altritol-modified siRNAs, 12th Annual Meeting of the American Society of Gene Therapy, May 27-30, 2009, San Diego, California, USA.
3. Abdullah Ely, Carol Crowther, Tanusha Naidoo, Justin Hean, Marc Weinberg, Nicolas Ferry and Patrick Arbuthnot, Harnessing RNAi to counter hepatitis B virus replication *in vivo*, 3rd European Scientific Foundation Functional Genomics & Disease Conference, Innsbruck, Austria, 1-4 Oct 2008.

4. Abdullah Ely, Carol Crowther, Tanusha Naidoo, Steven Mufamadi and Patrick Arbuthnot, optimising RNAi-mediated silencing of hepatitis B virus replication, RIGHT Symposium - RNAi for therapy, 9 November 2008, Lisbon, Portugal.
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18. Abdullah Ely. Pol II and Pol III promoter-derived short hairpin RNA mediate silencing of the hepatitis B virus. The Faculty of Health Sciences Research Day, Parktown, South Africa, 4 August 2004.
19. Abdullah Ely, Marc Weinberg, Naazneen Moolla, Sergio Carmona, Patrick Arbuthnot. Inhibiting HBV Gene Expression in Cultured Cells using Micro-RNA. International Meeting of the Molecular Biology of the Hepatitis B Viruses, Bergamo, Italy, 7-10 September 2003.

Patents

Case 1.	A self-cleaving RNA expression cassette.
Patent Number	PCT/IB2004/002816, RSA No 2006/01368, USA Patent Application 10/568,933
Inventors	Patrick Arbuthnot, Marc Weinberg, Abdullah Ely and Sergio Carmona.
Brief Description	The invention relates to interference and inhibition of viral gene expression using expression cassettes that comprise a combination of ribozymes with either siRNA or miRNA.
Current Status:	PCT application filed before 1 September 2004. South African and South African national filings carried out 1 March 2006. RSA patent

	awarded and US patent pending.
Case 2.	A primary microRNA expression cassette
Patent Number	RSA Provisional Patent Application No. 2007/04435, PCT/IB2008/052103
Inventors	Abdullah Ely, Tanusha Naidoo and Patrick Arbuthnot
Brief Description	Expression constructs containing sequences derived from endogenous micro RNAs (miRs) are used in the method to generate silencing sequences that are specific to HBV sequences.
Current Status:	PCT review

ABSTRACT

Chronic infection with the hepatitis B virus (HBV) is a major risk factor for cirrhosis and hepatocellular carcinoma, which is the sixth most common cancer worldwide. Available treatment for chronic HBV infection has limited efficacy in preventing associated complications. The compact and multifunctional nature of the viral genome limits its mutability making HBV an ideal candidate for therapy based on nucleic acid hybridisation. The potent and specific gene silencing that can be achieved with RNA interference (RNAi) has fueled interest in exploiting this pathway as a therapeutic modality. Synthetic and expressed RNA sequences have been used to activate RNAi. These engineered sequences mimic natural substrates of the RNAi pathway, which allows them to enter and reprogramme the pathway to effect silencing of intended targets. Tradionally expressed RNAi activators have been transcribed as short hairpin RNA (shRNA) sequences from RNA polymerase III (Pol III) promoters. These shRNA mimic precursor microRNA (pre-miRNA) and consequently enter the RNAi pathway at a relatively late stage. Overexpression of shRNA sequences from Pol III promoters, specifically the U6 promoter, has been associated with toxic side effects and has raised concerns about the use of expressed RNAi activators. Another concern of developing therapeutic RNAi expression cassettes is the emergence of HBV mutants that are resistant to silencing by a single expressed RNAi effecter. These points have highlighted the need for the development expressed RNAi activators that are effective at low concentrations and capable of combinatorial silencing. To address these issues the aim of this study was to assess the feasibility of anti HBV effecter sequences that mimic an early substrate (viz. primary miRNA or pri-miRNA) of the RNAi pathway. Pri-miRNA expression is typically under the transcriptional control of Pol II promoters. Consequently RNAi activators that

mimic pri-miRNA, so-called pri-miR shuttles, may be expressed from Pol II promoters. Initially a panel of shRNA expression cassettes driven by a Pol III promoter was constructed and silencing of HBV replication assessed. Pri-miR shuttles were then designed by incorporating guide sequences of the most effective anti HBV U6 shRNA into naturally occurring pri-miR-122 and pri-miR-31. Potent inhibition of viral replication was observed with both Pol III and Pol II-driven pri-miR shuttle expression cassettes *in vitro* and *in vivo*. Subsequently liver-specific pri-miR-122 and multimeric pri-miR-31 shuttle expression cassettes were created. Pri-miR-122 shuttle sequences expressed from the alpha-1 antitrypsin promoter and HBV basic core promoter exhibited the best liver-specific silencing. Polycistronic pri-miR-31 shuttle sequences were shown to produce multiple RNAi activators capable of silencing multiple target sequences. Silencing by the pri-miR shuttle sequences was independent of toxic effects that arise from induction of the interferon response or saturation of the endogenous miRNA pathway. Pri-miR shuttles clearly represent an improved option for the use of expressed shRNA and brings therapeutic RNAi technology a step closer to clinical application.

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LIST OF ABBREVIATIONS

A1AT	-	alpha 1 antitrypsin
AAV	-	adeno-associated virus
ATP	-	adenosine triphosphate
BCP	-	basic core promoter
cccDNA	-	covalently closed circular DNA
CMV	-	cytomegalovirus
DGCR8	-	DiGeorge syndrome chromosomal region 8
DMEM	-	Dulbecco's Modified Eagle's Medium
dsDNA	-	double-stranded DNA
dsRNA	-	double-stranded RNA
ECLIA	-	electrochemiluminescence immunoassay
eGFP	-	enhanced green fluorescent protein
FCS	-	foetal calf serum
FVIII	-	factor VIII
GAPDH	-	glyceraldehyde-3-phosphate dehydrogenase
HBsAg	-	hepatitis B virus surface antigen
HBV	-	hepatitis B virus
HBx	-	hepatitis B virus X protein
HCC	-	hepatocellular carcinoma
HCV	-	hepatitis C virus
HIV	-	human immunodeficiency virus
HNF	-	hepatocyte nuclear factor
IFN	-	interferon

kb	-	kilobase
lhRNA	-	long hairpin RNA
miRNA	-	microRNA
MxA	-	Myxovirus A
nt	-	nucleotide
OAS1	-	oligoadenylate synthetase 1
ORF	-	open reading frame
PEG	-	polyethylene glycol
pgRNA	-	pregenomic RNA
PKR	-	protein kinase R
Pol	-	RNA polymerase
pre-miRNA	-	precursor miRNA
pri-miRNA	-	primary miRNA
qRT-PCR	-	quantitative reverse transcriptase PCR
rcDNA	-	relaxed circular DNA
RIG-1	-	retinoic-acid-inducible protein 1
RISC	-	RNA induced silencing complex
RNAi	-	RNA interference
RPMI	-	Roswell Park Memorial Institute medium
SEM	-	standard error of the mean
shRNA	-	short hairpin RNA
siRNA	-	small interfering RNA
SNALPs	-	stable nucleic-acid-lipid particles
snRNA	-	small nuclear RNA
stRNA	-	small temporal RNA

TLR	-	toll-like receptor
TRBP	-	HIV transactivating response RNA-binding protein
ZFP	-	zinc finger protein

LIST OF SYMBOLS

α	-	alpha
β	-	beta
μ	-	micro

1 INTRODUCTION

1.1 HEPATITIS B VIRUS EPIDEMIOLOGY

The hepatitis B virus (HBV) is a human pathogen that infects the liver and causes both acute and chronic hepatitis. An estimated 2 billion people worldwide have been exposed to this pathogen and 350 million individuals are chronic carriers of the virus (1). Virus spread is parenteral which may occur by perinatal transmission from an infected mother to the new-born, horizontally between children or through sexual contact between adults. Chronic HBV infection is endemic to sub-Saharan Africa, east and south east Asia and the western Pacific islands. Individuals chronically infected with HBV are at an increased risk of developing the severe complications of cirrhosis and hepatocellular carcinoma (HCC). Worldwide HCC is ranked as the sixth most common cancer, accounting for 5.7% of all new cancer cases (2). HCC prognosis is very poor and annual incidence is practically equal to annual mortality (3). In 2002, 95% of the total number of new HCC cases ended in mortality making it the third most common cause of cancer-related death (2). Hepatocarcinogenesis is multifactorial and may be influenced by viral genotype and age and gender of chronic carriers. Sub-genotype A1 for example, which is hyperendemic to South Africa, is associated with an increased risk for the development of HCC (4). An effective anti HBV vaccine is available and universal vaccination of infants was first implemented in Taiwan in 1984 (5). Since the introduction of universal vaccination there has been a progressive decrease in HBV seroprevalence (5). Furthermore a steady decrease in the incidence of HCC in Taiwan since 1984 has also been reported and is attributed to the efficacy of the vaccination programme (6). South Africa introduced anti HBV vaccination into its Expanded Programme on Immunisation in 1995. A study to assess the impact of immunisation on HBV-associated nephropathy, which

equates to HBV incidence, demonstrated a reduction in disease burden in the post-immunisation period (7). Progress in realising universal HBV vaccination however has been hampered and new cases of infection are still reported (8). Furthermore vaccination is prophylactic and offers little therapeutic benefit to existing chronic carriers. Immunomodulators and nucleotide and nucleoside analogues remain the only licensed therapies for management of chronic infections. Current treatment regimens though are plagued by expense, side effects, development of resistance and limited efficacy. The advancement of novel treatment strategies to limit severe sequelae of chronic HBV infection therefore remains an important medical goal.

1.1.1 HBV biology

HBV is a prototypical member of the *Hepadnaviridae* family of viruses (9). As the name suggests these viruses have DNA genomes and infect hepatocytes. The viral genome has a circular, partially double-stranded DNA (relaxed circular DNA or rcDNA) arrangement (10) (Figure 1.1). The long minus strand DNA is approximately 3.2 kilobases (kb) in length and covers the entire viral genome. The circular structure of the viral genome is maintained by the plus strand DNA which is variable in length and spans the 5' and 3' ends of the minus strand. Upon infection rcDNA is translocated to the nucleus where it is converted to covalently closed circular DNA (cccDNA) (11).

Transcription from the cccDNA template is under the control of four viral promoters and two enhancer sequences (reviewed in (12)). Four major viral transcripts are produced during infection, three subgenomic mRNA (2.4, 2.1 and 0.9 kb species) and the greater than genome length pregenomic RNA (pgRNA). The large surface protein is translated from the 2.4 kb viral RNA and the middle and major surface proteins are

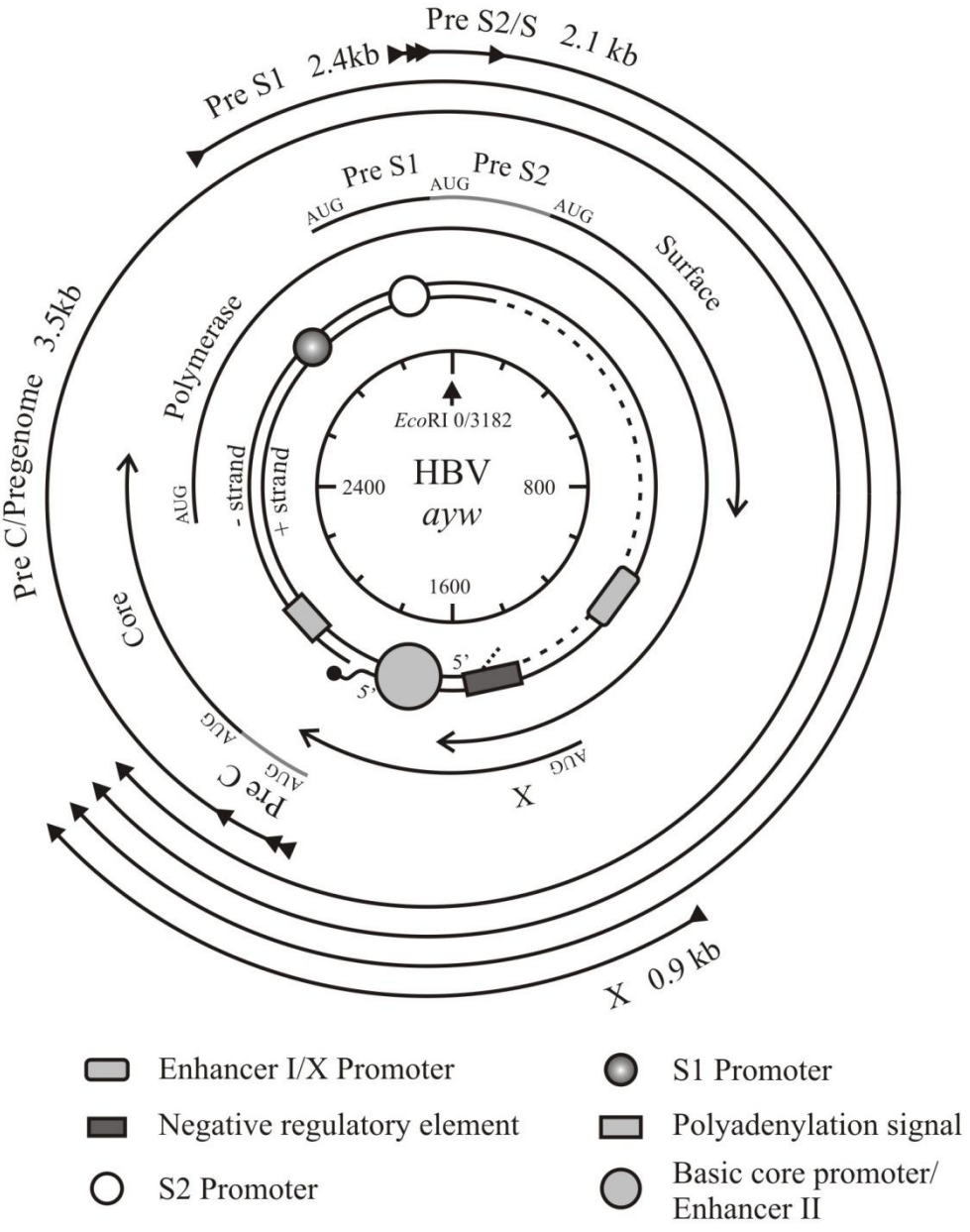

Figure 1.1: <u>HBV genome organisation.</u>

The partially double stranded genome is indicated with transcription regulatory elements and nucleotide co-ordinates. Viral open reading frames are shown as arrows immediately surrounding the genome. The outermost arrows represent the four major viral transcripts that terminate at a single polyadenylation site.

translated from the 2.1 kb mRNA. The HBV X protein (HBx) is translated from the 0.9 kb viral transcript. Replication of viral genomic DNA proceeds from reverse transcription of the 3.5 kb pgRNA. Encapsidation of polymerase bound pgRNA initiates reverse transcription to generate the minus strand. Additionally the 3.5 kb transcript may exist with a 5' extension. These transcripts serve as template for the translation of core and precore proteins (13). Terminal repeat sequences at the 5' and 3' end of the pgRNA facilitate circularisation of the nascent reverse transcribed minus strand DNA permitting synthesis of the plus strand to proceed (14). Methods that interfere with the stability of pgRNA therefore have the potential to limit viral replication by disruption of viral genome formation.

1.1.2 Current HBV treatment

The chronic nature of HBV infection requires effective antiviral therapy to ideally exhibit a sustained effect. Currently immunomodulators (interferon-α (IFN-α) and polyethylene glycol (PEG)-modified IFN-α (PEG-IFN-α)), nucleoside (lamivudine, telbivudine and entecavir) and nucleotide (adefovir and tenofovir) analogues are the only licensed therapies for managing chronic HBV infection (15, 16).

Once inside cells nucleoside and nucleotide analogues are phosphorylated and exert their antiviral effects by inhibiting viral DNA synthesis. As a result of structural similarities shared with naturally occurring nucleotides these analogues are incorporated into nascent viral DNA strands and cause termination of chain elongation. Advantages of the use of nucleoside and nucleotide analogues include tolerance and ease of administration (oral route), however long-term monotherapy is associated with the emergence of viral escape mutants (17, 18). Resistance to nucleoside and nucleotide

analogues commonly entails mutations within the viral polymerase sequence. Mutation of the YMDD (tyrosine-methionine-aspartate-aspartate) motif of HBV polymerase is one example often reported to confer resistance to lamivudine (19, 20). The YMDD motif represents the active site of the viral polymerase and mutations to this motif may confer resistance if binding of nucleoside or nucleotide analogues is impaired.

A weak immune response to HBV is implicated in the development of chronicity (21). IFN-α has been used widely to augment anti HBV immune responses (22, 23) and was the first therapy to be licensed for the management of chronic viral infection (16). The therapeutic effect of IFN-α is thought to be two-fold. It exerts its antiviral effect by inducing antiviral genes and stimulating the immune cells to eradicate viral infection (24). Induction of the Protein Kinase R (PKR), RNase L and orthomyxovirus systems are some of the well known pathways of the innate immune system induced by IFN-α. Immunomodulatory effects of IFN-α include stimulation of B cells to secrete protective antibodies and cytotoxic T cells for the elimination of infected cells. Improvement in efficacy and long-term maintenance of a therapeutic effect has resulted in unmodified IFN-α being supplanted with PEG-IFN-α. Side effects, which range from mild flu-like symptoms to bone marrow suppression, thyroid dysfunction and depression, associated with the use IFN-α and PEG- IFN-α have limited the use of these therapeutics (15). The development of innovative approaches to manage chronic HBV infection therefore remains an important medical objective. Exploiting the RNA interference (RNAi) pathway to achieve potent and specific gene silencing offers a potentially useful therapeutic modality for the management of chronic HBV infection.

1.2 RNA INTERFERENCE

The RNAi regulatory pathway was first described in 1998 by Fire et al. (25). Injection of long double-stranded RNA (dsRNA) into the nematode worm, *Caenorhabditis elegans* effected potent and specific suppression of homologous genes. Remarkably the suppressive effect was observed throughout the body of injected animals and was also passed on to the first generation offspring. The discovery that dsRNA initiated genetic interference in *C. elegans* linked two previously described sequence-dependent gene-silencing mechanisms (co-suppression in plants (26) and quelling in fungi (27)) with RNAi. Subsequent reports demonstrated that RNAi also exists in other metazoan organisms (28-30). Though evidence of the pathway existed in early mouse embryonic cells (31, 32) the applicability of RNAi in somatic mammalian cells remained in doubt. This is as a consequence of stimulation of the sequence non-specific IFN response in somatic mammalian cells by long dsRNA. Elucidation of the mechanism of RNAi in *Drosophila* embryo lysates identified the production of small RNA duplexes from introduced long dsRNA during RNAi (33-35). These RNA duplexes, termed small interfering RNA (siRNA), which were shown to be central to activating RNAi were 21-23 nucleotides (nt) in length and characterised by 5'-terminal phosphates and 2 nt 3' overhangs. These findings led to the landmark study that demonstrated induction of RNAi with chemically synthesised siRNA in somatic mammalian cells without stimulation of the innate immune system was possible (36). The small size of the chemically synthesised siRNA allowed these molecules to evade recognition by the IFN response. As a result of this study numerous investigators have exploited the use of synthetic siRNA or siRNA precursor expression systems to silence the expression of genes in mammalian systems.

Similarities shared between siRNA and the previously described small temporal RNA (stRNA), *lin-4* and *let-7*, led researchers to investigate a possible connection between these two classes of small RNA (37-39). *Lin-4* and *let*-7 function in embryogenesis by targeting homologous mRNA for translational inhibition (40, 41). Findings indicated that stRNA and siRNA share the processing machinery of the RNAi pathway. These studies were soon followed by reports identifying a large number of endogenously encoded small RNA species called microRNA (miRNA) (42-44). It has since become evident that endogenous miRNA are the natural activators of the RNAi pathway.

1.2.1 MicroRNA biogenesis

Diverse biological functions ranging from developmental timing (40, 41) to haematopoiesis and differentiation (45, 46) to tumour suppression (47) are regulated by miRNA (reviewed in (48)). Biogenesis of these small RNA species (Figure 1.2) is effected in a two-step process which entails the sequential processing of an RNA polymerase (Pol) II-derived transcript to generate the final mature effecter sequences. miRNA are transcribed as class II gene products that contain single or multiple hairpin-motifs (the primary miRNA or pri-miRNA transcript) (49). Nuclear processing of pri-miRNA by the microprocessor complex yields an approximately 70 nt hairpin sequence called precursor miRNA (pre-miRNA) (50). The microprocessor, which comprises the nuclear RNase III Drosha and the dsRNA-binding protein DGCR8 (DiGeorge syndrome chromosomal region 8) (50, 51), binds to the stem-loop structure of pri-miRNA and cleaves approximately two helical turns from the loop (~22 nt) to release the pre-miRNA (48). Export of the pre-miRNA to the cytoplasm is mediated by the Ran-GTP dependent nuclear export factor, Exportin-5 (52, 53). In the cytoplasm pre-miRNA undergoes the second step of processing by the cytoplasmic RNase III Dicer and its dsRNA-binding partner TRBP (the HIV

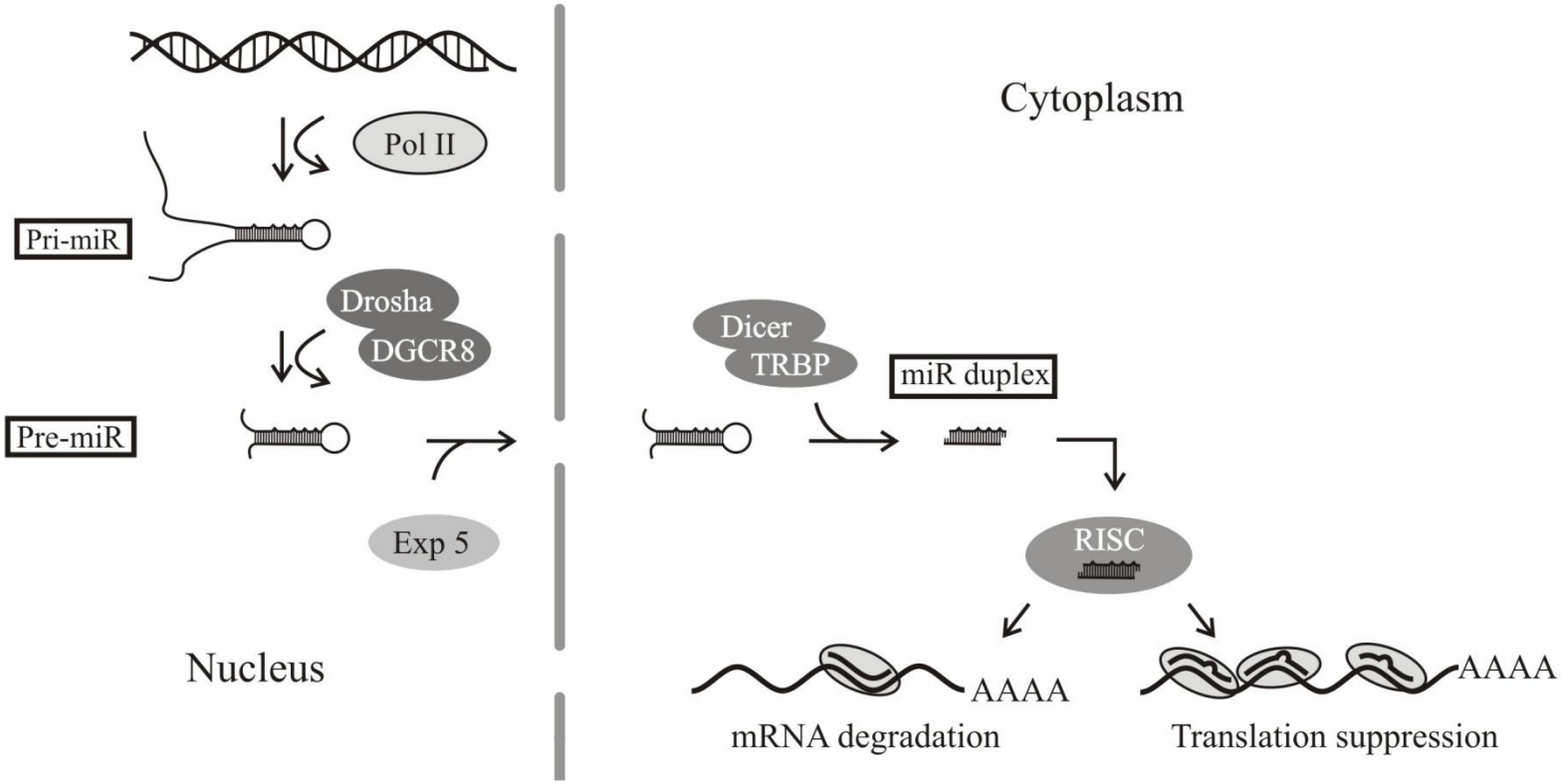

Figure 1.2: <u>MicroRNA biogenesis pathway.</u>

In the first of two steps ~70 nt pre-miRNA stem-loop sequences are derived from processing of the pri-miRNA transcripts. Exportin-5 mediates export of the pre-miRNA to the cytoplasm where the second processing step produces the mature miRNA duplex. Incorporation of the duplex into RISC and activation of the complex follows. The strand that is retained guides RISC to target mRNA to effect silencing.

transactivating response RNA-binding protein) (54). Dicer cleaves the pre-miRNA two helical turns (~22 nt) from the 3' end of the stem-loop to remove the loop and generate the mature miRNA duplex with 5'-terminal phosphates and 3' overhangs of 2 nt (48). Following incorporation of the miRNA duplex into the RNA induced silencing complex (RISC) one strand (guide) is retained while the other strand (antiguide or passenger) is removed (55-57). Removal of the passenger strand activates RISC, which is directed to target mRNA that is complementary to the guide strand. Activated RISC effects silencing through degradation or suppressing translation of the target mRNA. Perfect complementarity between the guide strand and the target site is required to achieve transcript degradation (58, 59). The requirement for translational suppression is less rigorous. Hybridisation of nucleotides 2-8 of the guide sequence (the so-called seed region) to mRNA is sufficient for silencing through translational repression (60). Recent reports have provided insight into the mechanism of translational repression (61, 62). Localisation of miRNA-containing RISC together with target mRNA within cytoplasmic processing bodies (or P-bodies) has been shown to be important for translational repression. Furthermore translational repression was shown to be dependent on binding of Argonaute components of RISC to GW182, a subunit of P-bodies (62). Sequestration of target mRNA within P-bodies by the RNAi machinery is thought to lead to repression as the translational machinery cannot access P-bodies.

1.2.2 RNAi as an antiviral strategy

Synthetic or expressed RNA sequences that mimic intermediates produced during miRNA biogenesis are typically utilised to reprogramme the RNAi pathway to achieve suppression of genes of interest (63). Numerous reports have demonstrated the utility of exploiting the RNAi pathway for potent and specific inhibition of various genes.

Investigation of the feasibility of RNAi as a therapeutic strategy has received considerable attention as it provides a tool against diseases that are not amenable to conventional therapy and could potentially improve on available treatments. Application of RNAi to limit pathology-causing viral elements is supported by evidence from plants where RNA silencing functions in antiviral defence (64). In theory all viruses can be targeted by RNAi-based therapeutics (65). Viruses with RNA genomes or that employ RNA intermediates for replication may be targeted directly with RNAi. Alternatively, viruses may be targeted indirectly by silencing viral genes or host factors required for replication. Silencing DNA viruses with RNAi of necessity entails adopting such an indirect approach. As a consequence RNAi may effectively knock down replication of DNA viruses but have a limited effect on persistence of the viral genome. A number of early studies reporting on the potent antiviral effects of RNAi (66-70) have spurred research efforts in the therapeutic RNA field.

1.3 RNAI AGAINST HBV

The HBV genome is extremely compact in nature with each of its four ORFs partially overlapping at least one other ORF (Figure 1.1 (above)). Together the four ORFs cover the entire viral genome and all viral regulatory elements are contained within protein coding regions (12). These properties severely limit the mutability of the virus making it a good target for therapy based on nucleic acid hybridisation. HBV is therefore an ideal candidate for the development of RNAi-based treatment strategies. Ultimately the success of such development efforts will depend heavily on the availability of model systems that simulate chronic HBV infection. The paucity of cell lines and convenient animal models infectable by HBV has necessitated the development of surrogate models of viral

replication. This is in the form of replication-competent plasmid vectors that recapitulate viral replication in cultured mammalian cells. To address the lack of *in vivo* models the murine hydrodynamic injection model of HBV replication and transgenic HBV mice have been developed. Hydrodynamic tail vein injection is an efficient way of delivering nucleic acids to mouse hepatocytes and entails rapid injection of a large volume (10% body weight) of nucleic acid-containing saline (71). This procedure has been adapted to deliver replication-competent HBV vectors to the livers of mice recreating viral replication *in vivo* (72). Since an episomal plasmid is used to drive viral gene expression the model by nature is transient. Additionally the procedure itself elicits an immune response complicating differentiation of the cause of inhibitory effects. Nevertheless the hydrodynamic model of HBV replication is convenient for short-term efficacy studies. In contrast, HBV transgenic mice have a copy of a replication-competent viral sequence integrated within their genomes. Consequently viral replication continues for the life-time of the mice which more closely resembles the chronic carrier state in human patients. As such the transgenic HBV mouse model is a more rigorous model for assessing potentially beneficial synthetic or expressed RNAi sequences (73, 74).

1.3.1 Synthetic RNAi activators

Both synthetic (75-79) and expressed (80-87) activators of the RNAi pathway have been used to silence HBV replication *in vitro* and *in vivo*. Advantages of the use of chemically synthesised siRNA include ease of cellular delivery and regulation of dose. Initial studies utilising chemically synthesised siRNA targeted to various regions of HBV efficiently limited viral replication demonstrating the utility of RNAi-based therapeutics (75-79). Importantly anti HBV activity of synthetic siRNA was observed *in vivo* using the murine hydrodynamic injection model (75, 77). Suppression was at the protein, DNA and

RNA levels indicating disruption not only of gene expression but also of viral replication. In contrast to the inhibitory effects of nucleotide and nucleoside analogues, RNAi-mediated silencing did not require viral replication (75). This is not unexpected as nucleotide and nucleoside analogues need active replication to be incorporated into nascent viral DNA and exert their effect whereas the RNAi sequences exert their effects by targeting viral RNA. An additional advantage to targeting HBV with RNAi as opposed to nucleotide and nucleoside analogues is that a single RNAi effecter sequence can target multiple viral RNAs. This is as a consequence of the overlapping nature of the viral ORFs and the single polyadenylation signal which creates significant sequence commonality between the four major viral transcripts. Multiple viral transcripts that share the region targeted by a single siRNA were therefore silenced. However knockdown achieved with synthetic siRNA is transient, which is likely to be as a result of susceptibility to degradation by RNases. Subsequent research therefore explored chemical modification to improve stability and efficacy of synthetic siRNA.

Typically chemically synthesised siRNA are duplexed RNA molecules with a 19-21 nt base sequence and 2 nt 3' overhangs. These structural features are important for recognition by RISC and consequently play a significant role in the function of siRNA. Limited chemical modification of siRNA is tolerated as demonstrated by a loss of silencing upon complete substitution of ribonucleotides of either strand with 2'-deoxy or 2'-*O*-methyl residues (88). Typically changes to the 2' hydroxyl of the ribose moiety of ribonucleotides are used to improve stability, specificity and efficacy of siRNA (88-90). Interestingly various modifications to anti HBV siRNAs were shown to improve *in vivo* silencing efficacy in the murine injection model (89). Modifications included inverted abasic residues at the 5' and 3' ends of siRNA, phosphorothioate linkages, and substitution

of 2’ hydroxyl groups with flouro, methyl or H groups. Notably the combination of modifications that resulted in improved efficacy differed with siRNA sequence. Intravenous administration under normal pressure of these extensively modified naked siRNA caused significant suppression of viral replication, however the dose required to achieve silencing makes this approach impractical. To improve *in vivo* delivery of the modified siRNA, lipid encapsulation was used to form stable nucleic-acid-lipid particles (SNALPs) (91). Anti HBV SNALP formulations were administered to immune compromised mice that had received a replication-competent plasmid by the hydrodynamic injection procedure. The use of immune compromised mice allows HBV replication to proceed for an extended period of time. Suppression of viral replication was sustained for a period of 6 weeks. This represents a significant step towards the development of potent and long-lasting synthetic anti HBV siRNA sequences. Importantly the technology required for efficient hepatic delivery of synthetic RNAi effecter sequences have also developed rapidly. In addition to enhancing stability and efficacy of siRNA, chemical modifications have also proved to be important to limit off-target effects.

Off-target effects may result from stimulation of the innate immune response or unintentional silencing of non-targeted genes. Immunostimulation by siRNA in a sequence-dependent and -independent manner has been described. Sequence-dependent stimulation generally occurs through interaction of siRNA with endosomal toll-like receptors (TLRs), specifically TLR7, TLR8 and TLR9 (92, 93). So-called danger motifs have been identified (5’ GUCCUUCAA 3’ (92) and 5’ UGUGU 3’ (93)) that confer potent immunostimulatory properties to siRNA. Immune stimulation however was abrogated by modifying siRNA with locked nucleic acids (92). A recent study demonstrated stimulation of the IFN response by naked siRNA in a sequence-independent manner through activation

of cell-surface TLR3 (94). Conventional 2'-*O*-methyl modification of siRNA did not alleviate immunostimulatory effects however cholesterol conjugation was shown to avoid TLR3 recognition by aiding cellular entry of siRNA and incorporation into RISC. Immune stimulation by synthetic siRNA needs to be taken into consideration if these sequences are to be used for therapeutic purposes. The safety profile of synthetic siRNA can further be improved with chemical modifications to limit unintentional silencing of non-target genes as a result of seed region complementarity and selection of passenger strand sequences. Incorporation of the passenger strand of an siRNA into RISC could potentially lead to off-target silencing. The phosphorylation status of siRNA is an important factor for strand selection by RISC (95). Preventing phosphorylation of the terminal ribonucleotide through the introduction of a 5'-O-methyl group impaired incorporation of the modified strand into RISC. Asymmetric modification of an siRNA duplex therefore facilitates selection of the unmethylated guide strand. Chemical modification has also been shown to limit off-target silencing that results from seed region complementarity (96). Modification of the second ribonucleotide from the 5' end of the guide sequence with a 2'-*O*-methyl group alleviates off-target silencing. Initially chemical modification of synthetic siRNA was explored as a means of improving stability and consequently efficacy of effecter sequences. While chemical modification of synthetic siRNA has achieved this purpose it has also proven to be beneficial towards improving the safety of potential therapeutic sequences. The convenience of using synthetic siRNA to achieve silencing of gene expression has certainly focused research efforts on this class of RNAi activator. Expressed effecter sequences belong to the other equally important class of RNAi activators and offer a number of advantages over the use of synthetic siRNA.

1.3.2 Expressed RNAi activators

DNA exhibits increased stability compared to RNA and continuous effecter production occurs from DNA cassettes. Consequently RNAi expression systems are capable of long-term silencing. Additionally expression cassettes are compatible with recombinant viral vectors. Utilisation of RNAi expression systems therefore offer sustained silencing and effective means of delivery. Pol III promoters such as the U6 small nuclear RNA (snRNA) and H1 RNA promoters are commonly used to generate expressed activators of the RNAi pathway (97, 98). Typically Pol III expression cassettes are designed to transcribe short hairpin RNA (shRNA) sequences which mimic pre-miRNA and enter the RNAi pathway as Dicer substrates. With the exception of the first transcribed nucleotide, the U6 and H1 promoters contain all *cis* regulatory elements upstream of the transcription start site (97-100). Furthermore termination of transcription can be easily achieved by inserting a poly dT sequence within the expression cassette. These properties are convenient for the transcription of precisely defined RNA sequences and have resulted in the preference for RNAi expression cassettes driven by Pol III promoters. An additional advantage of using DNA cassettes is that expression of RNAi sequences does not exhibit the same immunostimulatory effects seen with synthetic siRNA. Expressed RNAi activators do not enter the cell through endocytosis and are therefore unlikely to come in contact with endosomal TLRs and cause stimulation of the IFN response. Also, expressed sequences undergo processing by Dicer to yield products with characteristics of self RNA which are inherently non-immunostimulatory (101). The 2 nt 3’ overhangs produced by Dicer processing for example interfere with RNA helicase RIG-1 (retinoic-acid-inducible protein I) recognition and subsequent IFN activation. Though expressed RNAi effecter sequences may avoid the innate immune system plasmid-based expression vectors themselves may have immunostimulatory potential. Unmethylated CpG dinucleotide

motifs are common to bacterially derived DNA and activate the immune system by stimulating TLR9 (102). Complete elimination of CpG motifs from plasmid-based expression cassettes is necessary to avoid interferon stimulation and sustain transgene expression (103). DNA expression cassettes embedded within recombinant viral vectors naturally would not be subject to similar limitations.

Viral Vectors. The liver is a very good target for the delivery of therapeutic sequences and progress in this field of study has advanced at a rapid pace. Both viral and non-viral vectors have been utilised to deliver RNAi effecter sequences to the liver. Non-viral vectors are typically used to deliver synthetic siRNA sequences as these vectors tend to be better at delivering siRNA than plasmid DNA. Adenoviruses and certain strains of adeno-associated viruses (AAVs) exhibit natural liver tropism and as a consequence have been extensively used as vehicles for the delivery of anti HBV expression cassettes. Adenoviruses are capable of efficiently infecting the liver but do not integrate into the host genome. These features make recombinant adenoviruses appealing for efficient delivery of therapeutic sequences to the liver without causing disruption of host gene expression upon integration. Typically as a precautionary measure recombinant viral vectors are designed to be replication defective. Removal of the E1 sequence generates recombinant adenoviral vectors that are capable of efficiently infecting target cells but are sterile (104). Propagation of these first generation recombinant adenoviruses is possible in HEK293 cells that provide the E1 sequence *in trans*. Efficacy of expressed anti HBV sequences has been extended to an *in vivo* setting with the administration of recombinant adenoviruses containing U6-driven shRNA cassettes to transgenic HBV mice (105, 106)[1]. Potent and sustained suppression of viral DNA, RNA and antigen levels was achieved in mice that

[1] See Appendix A2 for reference 105 (Carmona et al.)

received anti HBV recombinant adenoviral vectors. These studies were significant in that effective silencing *in vivo* in a model that simulates persistent HBV infection was demonstrated. PEGylation of recombinant adenovirus has also been shown to reduce immune stimulation and thereby allow repeat administration (107). Exploring second and third generation adenoviruses for the delivery of therapeutic RNAi effecter sequences in terms of safety may offer a more preferable option.

Further improvements to the first generation adenoviral vectors have been made by deletion of the E2b and E3 sequences (108). These second generation vectors provide an improved safety profile as recombination events that could reconstitute a replication competent virus are less likely to occur. Third generation or helper-dependent adenoviral vectors have most viral sequences deleted and are thought to exhibit fewer cytotoxic effects than first or second generation viruses. A single study thus far has employed a helper-dependent adenovirus to deliver an anti HBV shRNA sequence (109). A limitation of the study was that the anti HBV sequence used was not effective and little could be concluded about the feasibility of these vectors. As described earlier HBV therapy will most likely need to be maintained over a prolonged period of time. Since recombinant adenoviral vectors exhibit transient transgene expression repeated administration will in all probability be necessary. Adenoviruses however are potent stimulators of the immune system making repeated administration of recombinant adenoviral vectors problematic. This issue may be addressed by polyethylene glycol modification of recombinant adenoviruses to reduce the immunogenicity of these vectors and allow for repeated administration (107).

The low immunogenicity and improved safety of AAVs has garnered interest in these vectors for the delivery of therapeutic anti HBV sequences (110, 111). The commonly used AAV-2 genome has been pseudotyped with the capsid of the liver-specific AAV-8 to create hepatotropic recombinant AAV vectors. Pseudotyped AAV-2/8 vectors have been shown to efficiently deliver Pol III-driven expression cassettes to the livers of transgenic HBV mice and effect potent and persistent viral suppression (110). Eventual loss of suppressive activity was observed and attributed to clearance of recombinant virus from livers of mice by the immune response. Sequential administration of pseudotyped AAV-2/7 or AAV-2/9 to mice that were pre-immunised with AAV-2/8 was shown to overcome the adaptive immune response and further extend suppressive activity (111). Improving the safety profile of recombinant viral vectors is extremely important for the development of expressed therapeutic sequences.

The preceding studies demonstrate potent and specific inhibition of viral replication when targeting single sites within the HBV genome. A single-targeted approach may create selective pressure that leads to the emergence of escape mutants and indeed several reports describe HIV-1, poliovirus and HCV mutants that evade RNAi-mediated silencing (112-114). Like retroviruses HBV replicates its genome by reverse transcription. Reverse transcriptase does not have proofreading activity resulting in a high error rate during replication (115, 116). However due to the overlapping nature of the viral ORFs not all mutants produced are viable and as such HBV is not as mutable as HIV, HCV or the poliovirus. To assess site-specific RNAi silencing Wu et al. identified and characterised a viable HBV mutant isolated from a chronically infected individual (117). A silent mutation within the *polymerase* and *surface* ORFs was shown to confer resistance to an H1 promoter derived shRNA targeted to the region spanning the point mutation. The authors

demonstrated that the mutant isolate was selected over time from a heterogenous HBV population challenged with the H1 shRNA. Notably, mutating the H1 shRNA sequence to match the mutant target site perfectly restored silencing activity. This study underscores the importance of developing a multi-targeted RNAi-based therapeutic approach as a means to prevent the emergence of viral escape mutants. Improving efficacy of RNAi activators is an important objective however limiting toxic effects is equally important.

Safety concerns of the use of expressed RNA sequences to activate the RNAi pathway were raised by a recent study (118). Severe toxicity and eventually death were observed in HBV transgenic mice that received recombinant adeno-associated virus containing U6-driven anti HBV shRNA. Lethality was attributed to saturation of the RNAi pathway as a result of overexpression of the shRNA. Specifically saturation of Exportin-5 was shown to cause dysregulation of miRNA biogenesis by disrupting export of pre-miRNA to the cytoplasm. The loss of liver miRNA function resulted in liver failure and ultimately in the death of mice. Synthetic siRNA enter the RNAi pathway downstream of miRNA biogenesis and would therefore not be expected to cause any interference. Indeed synthetic siRNA delivered to the livers of mice does not disrupt endogenous miRNA levels (119). As such, avoiding saturation of the miRNA biogenesis pathway is mostly only of concern for the development of RNAi expression cassettes. Since saturation is a result of overexpression of shRNA sequences, ideally RNAi expression cassettes should be designed to produce effecter sequences at the lowest amount necessary to achieve knockdown. Consequently expressed RNAi activators have to be very potent to achieve desired silencing at relatively low effecter sequence levels. Additional safety concerns that need to be considered for the development of both synthetic and expressed RNAi activators include limiting unwanted off-target effects. Regulating levels and tissue

expression of RNAi activators could be of potential benefit to restricting off-target effects. The development of DNA expression cassettes under tight transcriptional control offers a means of achieving the goals of limiting off-target effects and circumventing saturation effects. Finally an important element of RNAi expression cassettes, particularly antiviral expression cassettes, is the ability to express multiple effecter sequences. In the context of antiviral therapy preventing the emergence of viral escape mutants is crucial for the ultimate success of the therapeutic sequences.

1.4 AIMS

The objective of the current set of research endeavours has been the development of potent therapeutic sequences that exploit the RNAi pathway to silence HBV replication. This entailed the initial identification of effective RNAi activators that are targeted to HBV (or target sites within HBV that are especially susceptible to RNAi-mediated silencing). The generation of U6 shRNA cassettes offers reliable means to create a large panel of RNAi expression cassettes that can be used for the rapid identification of functional effecter sequences. U6 shRNA expression cassettes however have a number of limitations, which include constitutive and ubiquitous expression. Nevertheless the use of U6 shRNA expression cassettes is a necessary step to the identification of effective anti HBV RNAi effecter sequences. Once identified these sequences were subjected to further development to create expression systems that can be regulated temporally as well as tissue-specifically. Furthermore development of expression cassettes that are capable of producing multiple RNAi effecter sequences was also explored. Generation of these expression cassettes entailed exploiting characteristics of naturally occurring miRNA sequences to aid in Pol II-driven effecter expression. Achieving effective RNAi activator expression from a Pol II

promoter is the first step in achieving regulatable and multi-effecter expression for RNAi-based therapy of HBV infection to be realised.

2 Pol-III shRNA AND lhRNA EXPRESSION VECTORS

2.1 INTRODUCTION

As with synthetic siRNA, Pol III-driven shRNA expression represents a very rapid and reliable method of identifying effective expressed RNAi effecter sequences. An effective synthetic siRNA sequence may not necessarily be effective as an expressed RNAi effecter. Development of expressed RNAi effecter sequences that are regulatable and multi-targeted is therefore dependent on the identification of effective expressed RNAi sequences. To this end a panel of 10 Pol III (U6)-driven anti HBV shRNA expression cassettes targeted to various regions within the *HBx* ORF was assessed for efficacy in silencing viral replication. As a consequence of overlapping viral sequences a number of regions of the HBV genome have multiple functions. The *HBx* sequence encodes the HBx protein, which has been associated with the development of hepatocellular carcinoma (120) and plays an important role in the establishment of viral infection *in vivo* (121). Additionally the *HBx* sequence overlaps the 3' end of the viral polymerase ORF, sequences necessary for viral replication (direct repeats) and the basic core promoter. *HBx* is therefore a multifunctional sequence. Finally the *HBx* ORF is common to all viral transcripts as the single polyadenylation signal terminates transcription after the *HBx* sequence. All viral transcripts therefore may be silenced simultaneously by therapy targeted to *HBx*. These properties make the *HBx* sequence an ideal target for U6 shRNA-mediated gene silencing. These first generation U6 shRNA sequences may then be developed to further improve efficacy and safety of RNAi-based therapeutics. Furthermore two U6-driven anti HBV long hairpin RNA (lhRNA) cassettes were assessed as a multi-targeted approach for the

inhibition of HBV gene expression.

2.2 MATERIALS AND METHODS

2.2.1 Target and Reporter plasmids

2.2.1.1 pCH-9/3091

Construction of the target plasmid pCH-9/3091 has been described previously (122). pCH-9/3091 contains a wild-type HBV genome sequence with terminal repeats under control of the cytomegalovirus (CMV) immediate early promoter-enhancer. The terminal repeats functionally mimic the circular nature of the HBV genome such that transcription from the CMV promoter generates a greater that genome length pgRNA as would normally be transcribed from viral cccDNA. The pgRNA may then be reverse transcribed to propagate the virus in transfected cells. Transcription from pCH-9/3091 therefore may be used to simulate HBV replication in transfected cells in culture or *in vivo*.

2.2.1.2 pCH-eGFP

The reporter plasmid, pCH-eGFP, was generated by replacing the *preS2/S* ORF of pCH-9/3091 with a sequence encoding enhanced green fluorescent protein (eGFP) (123).

2.2.2 Design of U6 shRNA expression vectors

Complete genome sequences of South African HBV isolates were aligned against the full HBV genome sequence contained in pCH-9/3091 with the bioinformatics software GeneDoc (124). The most conserved sequences within the *HBx* ORF of HBV genotype A variants were chosen as potential target sites for RNAi effecter sequences. A panel of 10

shRNA was designed to target these conserved regions of the *HBx* ORF. The shRNA sequences were designed to be transcribed from a Pol III promoter (the U6 promoter) and encode a 25 bp stem and the loop sequence derived from miR-23. In addition expression cassettes were designed to produce shRNA with stem structures containing G:U or C:A mismatches. Introducing mismatches proved to be necessary to prevent primer sequences encoding the passenger strand from binding to the guide strand encoding sequences during PCR. To maintain complete complementarity between the intended guide strand of the shRNA and its target site within the *HBx* ORF, the sequence encoding the passenger strand was mutated to generate a mismatched stem.

2.2.3 Construction of U6 shRNA expression vectors

The PCR-based method used for the generation of the U6 shRNA cassettes was first described by Castanotto et al. (125). Each complete individual anti HBV shRNA sequence was encoded by two overlapping reverse primers (Table 2.1). The primers sequentially add the sequence encoding the shRNA and a Pol III terminator to the 3' end of the U6 promoter in a two-step PCR protocol (Figure 2.1). A universal primer (5'- CTA ACT AGT GGC GCG CCA AGG TCG GGC AGG AAG AGG G -3') binding to the 5' end of the human U6 promoter was used as the forward primer in PCR for the generation of all anti HBV U6 shRNA cassettes. Oligonucleotides were synthesised by standard phosphoramidite chemistry (Inqaba Biotech, South Africa).

pU6 (125), which contains the human U6 promoter sequence, was used as template in the first round of PCR amplification. The first portion of the U6 shRNA expression cassettes were amplified using the universal U6 primer with the first of the shRNA reverse primers (U6 shRNA n.1). Purified fragments were used in a second round of PCR to

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

Vector	Primers	Sequences
pU6 shRNA 1	*HBx*$_{1168\text{-}1192}$[1]	
	U6 shRNA 1.1	5′-TGACGTGACAGGAAGCGTTAGCAGACACTTGGCATAGGCC*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 1.2	5′-CCCAGATCTACGCGTAAAAAAGGTCTGTGCCAAGTGTTTGCTGACGTGACAGGAAGCGTTA-3′
pU6 shRNA 2	*HBx*$_{1432\text{-}1456}$	
	U6 shRNA 2.1	5′-**GGACGTGACAGGAAGCGTTC**GTGGGATTCAGCGTCGATGG*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 2.2	5′-CCCAGATCTACGCGTAAAAAACCGTCGGCGCTGAATCCCGC**GGACGTGACAGGAAGCGTTC**-3′
pU6 shRNA 3	*HBx*$_{1514\text{-}1538}$	
	U6 shRNA 3.1	5′-**CTTTATGACAGGAAGCAAAG**AGAGATGCGCCCCATGGCCG*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 3.2	5′-CCCAGATCTACGCGTAAAAAACGACCACGGGGCGCACCTCT**CTTTATGACAGGAAGTAAAG**-3′
pU6 shRNA 4	*HBx*$_{1518\text{-}1542}$	
	U6 shRNA 4.1	5′-**ACGCGTGACAGGAAGCGTGT**GAAGAGAGGTGTGCCCTGTG*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 4.2	5′-CCCAGATCTACGCGTAAAAAACACGGGGCGCACCTCTCTTT**ACGCGTGACAGGAAGCGTGT**-3′
pU6 shRNA 5	*HBx*$_{1575\text{-}1599}$	
	U6 shRNA 5.1	5′-**CTCTGTGACAGGAAGCAGAG**GCGAAGCAAAGCGCACACGA*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 5.2	5′-CCCAGATCTACGCGTAAAAAACCGTGTGCACTTCGCTTCAC**CTCTGTGACAGGAAGCAGAG**-3′
pU6 shRNA 6	*HBx*$_{1580\text{-}1604}$	
	U6 shRNA 6.1	5′-**CACGTTGACAGGAAGATGTG**TAGAGGTGAAGCGAGGTGTA*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 6.2	5′-CCCAGATCTACGCGTAAAAAATGCACTTCGCTTCACCTCTG**CACGTTGACAGGAAGATGTG**-3′

pU6 shRNA 7	*HBx*$_{1640\text{-}1664}$	
	U6 shRNA 7.1	5′-**GGACTTGACAGGAAGAGTTC**TTTTATGTAGGACTTTGGGC*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 7.2	5′-CCCAGATCTACGCGTAAAAAAGCCCAAGGTCTTACATAAGA**GGACTTGACAGGAAGAGTTC**-3′
pU6 shRNA 8	*HBx*$_{1678\text{-}1702}$	
	U6 shRNA 8.1	5′-**GAGGCTGACAGGAAGGCTTC**AAGGTTGGTTGTTGACGTTG*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 8.2	5′-CCCAGATCTACGCGTAAAAAACAATGTCAACGACCGACCTT**GAGGCTGACAGGAAGGCTTC**-3′
pU6 shRNA 9	*HBx*$_{1774\text{-}1798}$	
	U6 shRNA 9.1	5′-**TTGGTTGACAGGAAGACTAA**TTTGTGCCTACAGCTTCTTA*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 9.2	5′-CCCAGATCTACGCGTAAAAAATAGGAGGCTGTAGGCATAAA**TTGGTTGACAGGAAGACTAA**-3′
pU6 shRNA 10	*HBx*$_{1863\text{-}1887}$	
	U6 shRNA 10.1	5′-**CTTGGTGACAGGAAGCCAAA**GCACAACTCGGAGGCTCGAA*CGGTGTTTCGTCCTTTCCACA*-3′
	U6 shRNA 10.2	5′-CCCAGATCTACGCGTAAAAAATTCAAGCCTCCAAGCTGTGC**CTTGGTGACAGGAAGCCAAG**-3′

[1] Numbers indicate co-ordinates on the HBV genome (accession number J02203.1).

Overlapping sequences are indicated in bold.

The region complementary to the U6 promoter is italicised.

HBx target sites are underlined.

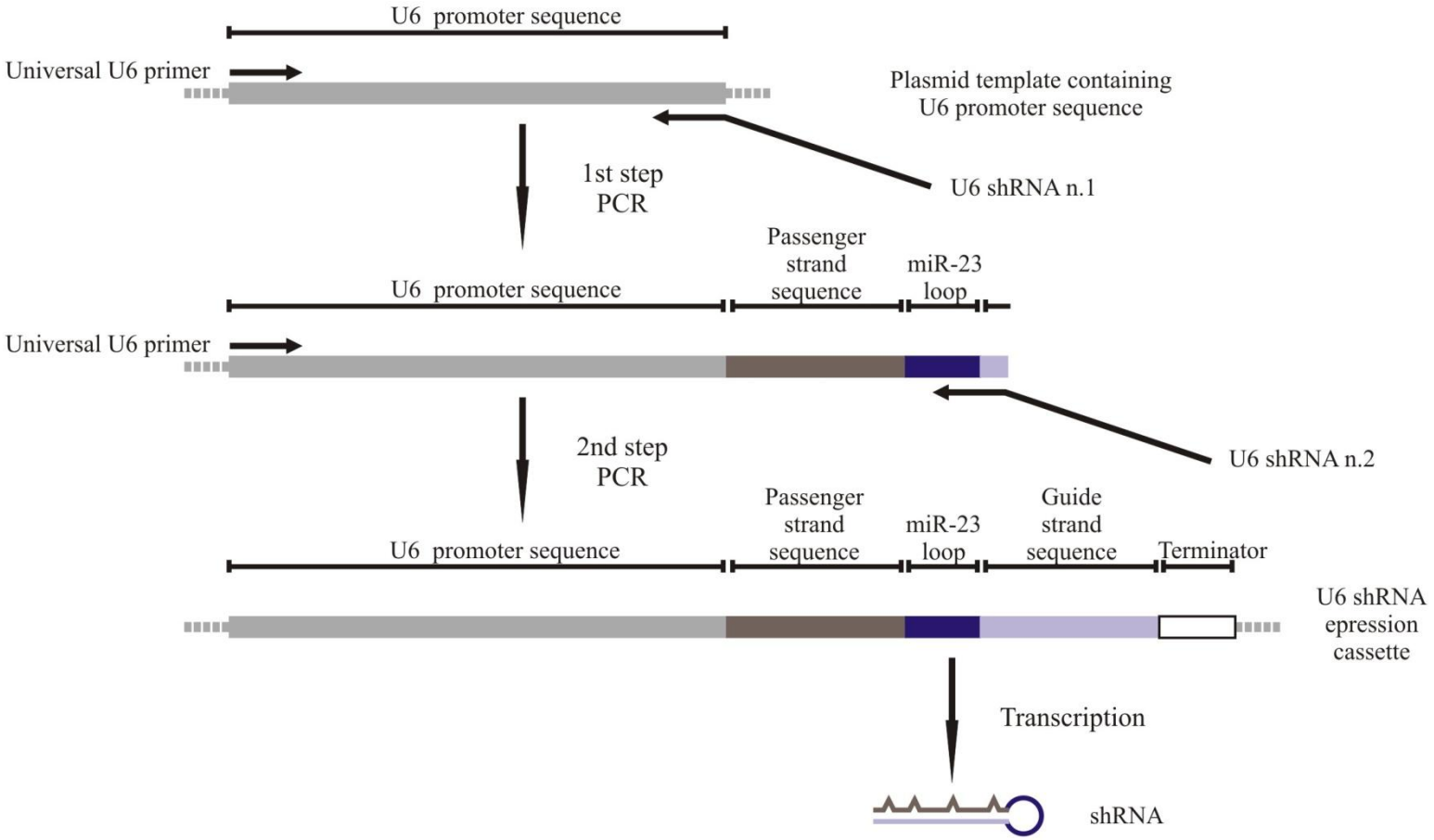

Figure 2.1: <u>Diagrammatic representation of PCR-based method for the generation of U6 shRNA expression cassettes.</u>

The sequences encoding the shRNA were introduced immediately downstream of the U6 promoter by a two-step PCR amplification procedure. Transcription from the U6 expression cassette produces a transcript which folds on itself to form a shRNA with a 25 bp stem and miR-23 loop.

generate the complete U6 shRNA expression cassettes with the universal U6 forward primer and the second of the shRNA reverse primers (U6 shRNA n.2). The amplicons were ligated into the PCR cloning vector pGEM®-T Easy (pGEM®-T and pGEM®-T Easy Vector Systems, Promega, WI, USA) to generate the pG-U6 shRNA vectors (pU6 shRNA 1-10). The prepared plasmids were subjected to restriction enzyme digestion to screen for correct sized inserts (*Spe*I and *Bgl*II) and orientation (*Spe*I). Plasmids containing inserts of the expected size were sequenced by automated cycle sequencing (Inqaba Biotech, South Africa) to confirm sequence fidelity.

2.2.4 Assessing efficacy of U6 shRNA expression vectors

2.2.4.1 Transfection of cultured mammalian cells

The human hepatoma cell line, Huh7 (126) was maintained in RPMI growth medium and cells seeded in 6-well tissue culture plates (Appendix A1-1). Three micrograms of the pU6 shRNA vectors was co-transfected with 6 µg pCH-9/3091 or pCH-eGFP with Lipofectamine™ 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions (Appendix A1-1).

2.2.4.2 HBsAg concentration

Forty eight hours post-transfection the culture medium was collected from cells transfected with pCH-9/3091 and HBsAg concentration measured (National Health Laboratory Services, South Africa). HBsAg secretion was measured by the electrochemiluminescence immunoassay or ECLIA (Roche Diagnostics GmbH, Germany). Briefly, 50 µl of the growth medium was incubated with two HBsAg-specific monoclonal antibodies (the first antibody is biotinylated and the second monoclonal antibody is labelled with ruthenium) forming antibody-antigen complexes with HBsAg sandwiched

between the two antibodies. Streptavidin-coated microparticles were added to this mixture to bind the complexes to the microparticles via the biotin-streptavidin interactions. The microparticles are magnetically captured on the electrode of a measuring cell and chemiluminescence induced by applying an electrical current. The chemiluminescent emission was measured and HBsAg quantified.

2.2.4.3 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. Forty eight hours post-transfection 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% foetal calf serum (FCS) was added and the cells aspirated repeatedly to ensure complete dissociation from culture plate. Cells were transferred to flow cytometry tubes and green fluorescent cells counted 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.

2.2.5 Design of lhRNA expression vectors

Two U6-driven lhRNA expression cassettes that are targeted to conserved regions within the *HBx* ORF were generated (127)[2]. Additionally a lhRNA expression cassette targeted to a sequence unrelated to HBV was constructed. The U6 lhRNA cassettes were designed according to methodologies similar to those described for the U6 shRNA

[2] Weinberg et al. (Appendix A2)

cassettes. The lhRNA sequences comprised a 62 bp stem with 12 G:U wobble base pairs interspersed within the stem. In addition to facilitating PCR-based construction of hairpin-encoding DNA cassettes, incorporation of G:U wobble base pairing has been reported to allow lhRNA sequence to avoid inducing the IFN response (128).

2.2.6 Generation of lhRNA expression vectors

The PCR-based method described in Section 2.2.3 for the generation of the U6 shRNA expression cassettes was also used to construct the U6 lhRNA expression cassettes. pU6 was used as template in the initial round of PCR with the U6 universal primer together with the first lhRNA reverse primers (U6 lhRNA n.1) (Table 2.2). The amplicons were purified and used as template in the second round of PCR amplification with the U6 universal primer and the second lhRNA reverse primers (U6 lhRNA n.2). PCR products from the second round of PCR were purified and ligated into the PCR cloning vector pTZ57R/T (InsTAclone™ PCR Cloning Kit, Fermentas, MD, USA) to create the lhRNA vectors (pU6 lhRNA 1-3).

2.2.7 Assessing *in vivo* efficacy of lhRNA expression vectors

The murine hydrodynamic injection procedure was used to assess *in vivo* efficacy of anti HBV lhRNA constructs. Animal experimentation was conducted in accordance with procedures approved by the University of the Witwatersrand Animal Ethics Screening Committee (Appendix A3). The procedure was carried out by injecting mice with a saline solution containing 10 µg pCH-9/3091, 10 µg anti HBV or control expression cassette and 10 µg of pCI-neo eGFP. All plasmids were prepared using the EndoFree® Plasmid Maxi Kit (Qiagen, GmbH, Germany) (Appendix A1-2). As a positive control for the induction of

Table 2.2: Oligonucleotide sequences used in the generation of lhRNA expression constructs.

Vectors	Primers	Sequences
pU6 lhRNA 1	*HBx*$_{1581–1640}$[1]	
	U6 lhRNA 1.1	5′- **ACTCTCTTGAAGCGCAAAG**GCGAAGCAAAGTACACACGATCCGACAGACGAGAAGACACAAA CAGGAAGT*CGGTGTTTCGTCCTTTCCACAA* -3′
	U6 lhRNA 1.2	5′- GATCTCTAGAAAAAAGACTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGC TTCACCTCTGC**ACTCTCTTGAAGCGCAAAG** -3′
pU6 lhRNA 2	*HBx*$_{1372-1431}$	
	U6 lhRNA 2.1	5′- **CATCTCTTGAATGCCGGTA**CGCAAACAACTTACGCCCACAACCTCCCAGCACAAAGACCCTCA ACCCAAT*CGGTGTTTCGTCCTTTCCACAA* -3′
	U6 lhRNA 2.2	5′- GATCTCTAGAAAAAAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCATAAATTGT CTGCGCACCAG**CATCTCTTGAATGCCGGTA** -3′
pU6 lhRNA 3	Control	
	U6 lhRNA 3.1	5′- **CCTCTCTTGAAGAGTCCCC**TAAATAACCAGAGAACCCCCGGACTCAGATCCGGTCCACCCAGA AAGAAC*CGGTGTTTCGTCCTTTCCACAA* -3′
	U6 lhRNA 3.2	5′- GATCTCTAGAAAAAAGGGTCTCTCTAGGTAGACCAGATCTGAGCCCGGGAGCTCTCTGGCTAT CTAGGGAAC**CCTCTCTTGAAGAGTCCCC**--3′

[1] Numbers indicate HBV genome co-ordinates (accession number J02203.1).

Overlapping regions are indicated in bold.

The region complementary to the U6 promoter is italicised.

IFN response-related genes mice were injected with 100 µg of poly I:C using the hydrodynamic procedure. The saline solution comprised 10% of the mouse's body weight and was injected via the tail-vein over a period of 5-10 seconds. Blood was collected from mice 2 and 5 days post-injection.

2.2.7.1 Serum HBsAg concentration

Mouse serum was diluted 1:4 with saline and HBsAg levels quantified using the MONOLISA® HBs Ag Assay kit (Bio-Rad, CA, USA). Briefly, 100 µl of diluted mouse serum was added per well to an ELISA plate coated with mouse monoclonal anti-HBs antibodies. To each well 50 µl of Conjugate Solution was added containing monoclonal and polyclonal anti-HBs antibodies bound to peroxidase. The samples were incubated at 37°C for 90 minutes to allow sandwich complexes to form. After the incubation period unbound antibodies were removed by washing the well with Washing Solution (Tris NaCl buffer, pH 7.4; 0.04% ProClin™ 300) at least 5 times. One hundred microlitres of Development Solution (Citric acid and Sodium acetate solution, pH 4.0; 0.015% H_2O_2; 4% DMSO and tetramethyl benzidine) was dispensed into each well and the plate incubated at room temperature in the dark for 30 minutes. The enzymatic reaction was stopped with the addition 100 µl of Stopping Solution (1 N H_2SO_4) and the optical density of the samples measured at 420/690 nm on a Bio-Rad Microplate Reader, Model 680 (Bio-Rad, CA, USA).

2.2.7.2 Serum HBV DNA quantitation

Total DNA was extracted from 50 µl of mouse serum using the Total Nucleic Acid Isolation Kit and MagNA Pure LC system (Roche Diagnostics, GmbH, Germany). Serum viral DNA levels were determined by real-time PCR analysis using SYBR® Green *Taq* ReadyMix™ (Sigma, MO, USA). The primer set, HBV surface forward (5'- TGC ACC

TGT ATT CCC ATC -3') and HBV surface reverse (5'- CTG AAA GCC AAA CAG TGG -3') was used to amplify HBV DNA. PCR was carried out on the Roche Lightcycler v.2 (Roche Diagnostics, GmbH, Germany) with the following thermocycling parameters: a 30 second hotstart at 95°C and 50 cycles of annealing at 57°C for 10 seconds, extension at 72° for 7 seconds and denaturation at 95°C for 5 seconds. Melting curve analysis was performed on the PCR products to confirm specificity of amplification. Viral particle equivalents were calculated using a standard curve generated with the Eurohep HBV DNA standard (129).

2.2.7.3 Quantitative reverse transcriptase PCR (qRT-PCR) of IFN response genes

Total RNA was extracted from mouse liver and reverse transcribed with the Sensiscript® Reverse Transcription Kit (Qiagen GmbH, Germany) and an oligo-dT primer. Primer sets described by Song et al. (130) were used for the amplification of murine *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *2', 5'-oligoadenylate synthetase 1* (*OAS1*) and *IFN-β* mRNA. SYBR® Green *Taq* ReadyMix™ was used for the PCRs and run on the Lightcycler v.2 using with the following cycling parameters: a hotstart at 95°C for 30 seconds and 50 cycles of annealing at 58°C for 10 seconds, extension at 72° for 7 seconds and denaturation at 95°C for 5 seconds. Specificity of the amplicons was confirmed by melting curve analysis. IFN response-related gene mRNA levels were normalised to *GAPDH* mRNA.

2.3 RESULTS

2.3.1 U6 shRNA expression vectors inhibit markers of HBV replication

To assess the efficacy of the U6 shRNA expression cassettes targeted to the *HBx* ORF, Huh7 cells were co-transfected with the hairpin-producing vectors and pCH-9/3091. The panel of anti *HBx* shRNA exhibited variable knockdown efficiency of HBsAg secretion (Figure 2.2). Of the panel, U6 shRNA 4, U6 shRNA 5, U6 shRNA 6, U6 shRNA 8 and U6 shRNA 9 were most efficient reducing HBsAg concentrations. U6 shRNA 10 did not significantly alter HBsAg secretion as compared to mock treated cells. A similar trend was observed when U6 hairpin vectors were co-transfected with the reporter plasmid, pCH-eGFP (Figure 2.3). The knockdown achieved against pCH-eGFP was not as marked as against pCH-9/3091 and reflects the stability of eGFP. Though the mRNA is efficiently targeted for degradation the eGFP protein is not turned over rapidly and the degree of knockdown underestimated.

Northern blot analysis on RNA extracted from cells transiently transfected with shRNA expression vectors and HBV target plasmid (pCH-9/3091) confirmed reduction in HBV transcript levels (105)[3]. Furthermore the knockdown achieved was independent of immune stimulation as measured by induction of the IFN response genes *IFN-β* and *OAS1*. To assess processing of the shRNA sequences, radiolabelled oligonucleotides complementary to the putative guide and passenger strand sequences of shRNA 5 or shRNA 10 were hybridised to total RNA extracted from cells transfected with the relevant U6 expression vector and extended by reverse transcription. An extended product was generated from the primer complementary to the guide strand of shRNA 5 but not from the

[3] The data presented here was carried out by Dr S. Carmona (Carmona et al. Appendix A2)

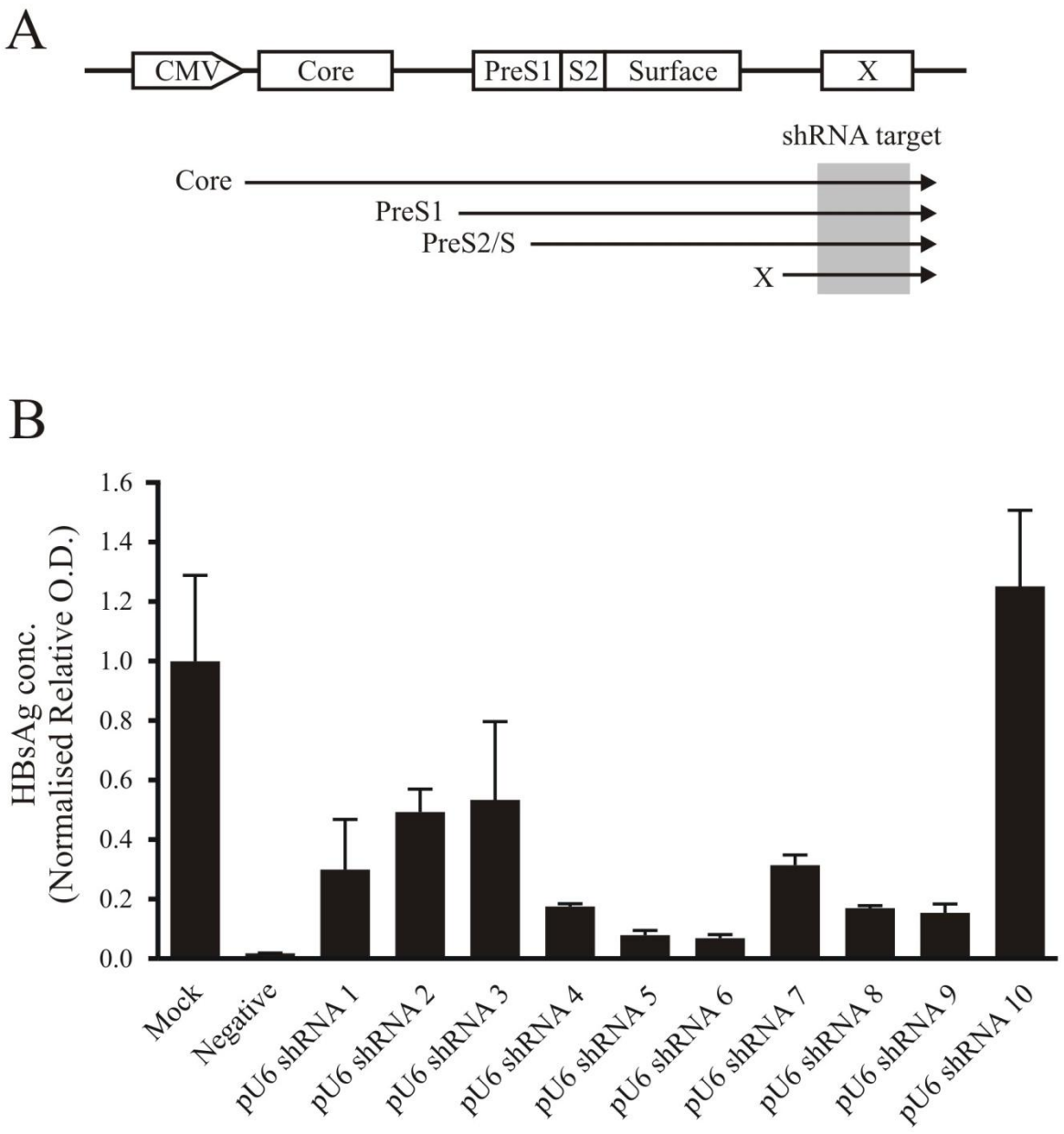

Figure 2.2: <u>Inhibition of HBsAg secretion from cultured cells.</u>

(A) Schematic representation of replication-competent plasmid, pCH-9/3091 showing ORFs. (B) HBsAg secretion was measured in the culture supernatant of cells co-transfected with the indicated U6 shRNA constructs and the HBV replication-competent plasmid, pCH-9/3091. Measurements are indicated as averaged values from at least three independent transfections normalised to mock treated cells. Error bars indicate standard error of the mean (SEM).

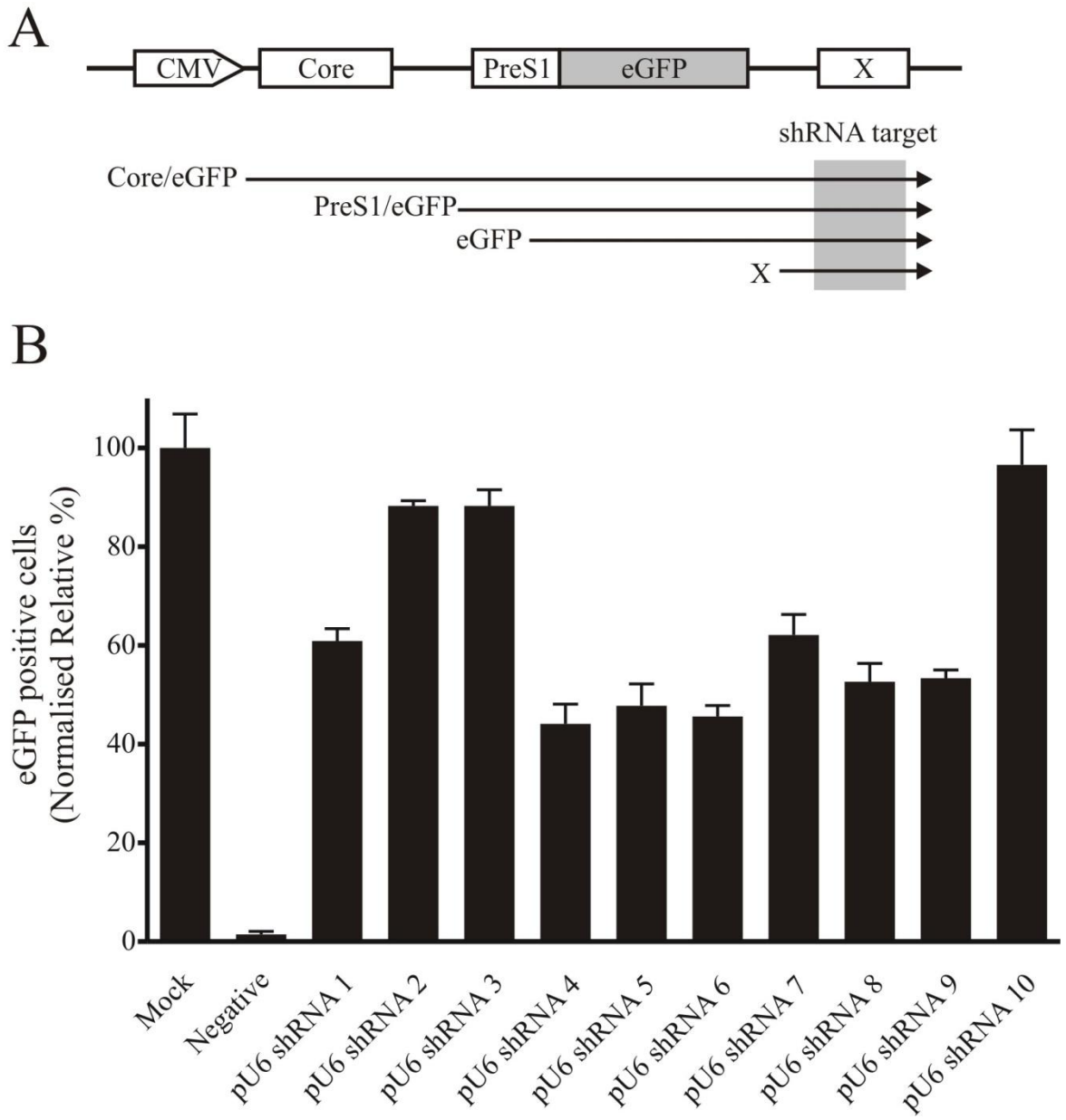

Figure 2.3: <u>Inhibition of eGFP reporter gene expression.</u>

(A) Diagrammatic representation of pCH-eGFP reporter plasmid, showing ORFs. (B) The number of eGFP positive cells as quantified by flow cytometry are shown after co-transfection of the indicated expression cassettes with the HBV reporter plasmid, pCH-eGFP. Triplicate experiments are shown normalised to the mock treated cells and with SEM indicated by error bars.

primer complementary to the passenger strand. In contrast, extended products were generated from primers complementary to both the guide and passenger strands of shRNA 10. The primer extension analysis data demonstrates that processing of a shRNA sequence impacts on its ability to effect knockdown (105)[4]. *In vivo* analysis of efficacy using the hydrodynamic injection model and HBV transgenic mice corroborate knockdown data from cultured cells. Together the data presented here demonstrate that U6-driven shRNA expression cassettes are capable of efficient suppression of viral replication *in vitro* and *in vivo*.

2.3.2 U6 lhRNA cassettes knock down HBV replication without inducing IFN response genes *in vitro* and *in vivo*

Co-transfection of Huh7 cells with anti HBV lhRNA expression vectors and pCH-9/3091 or luciferase target vector caused significant knockdown of markers of viral replication (127)[5]. Knockdown achieved, as measured by HBsAg secretion and luciferase expression, with the anti HBV lhRNA cassettes was equivalent to that of the previously described U6 shRNA 5. Viral replication was not significantly different between mock-treated cells and cells treated with the control lhRNA cassette targeted against a sequence unrelated to HBV. The observed knockdown was also demonstrated to be independent of stimulation of the IFN response genes *OAS1*, *IFN-β* or *Myxovirus A* (*MxA*).

Efficacy of the lhRNA cassettes were assessed *in vivo* using the hydrodynamic tail vein procedure to co-deliver HBV DNA and RNAi vectors to the livers of mice. HBsAg levels in the serum of mice that received U6 shRNA 5, U6 lhRNA 1 or U6 lhRNA 2 were

[4] Carmona et al. (Appendix A2)
[5] Weinberg et al. (Appendix A2)

decreased 90% as compared to mock injected animals (Figure 2.4B). Mice that received the control lhRNA cassette (U6 lhRNA 3) did not differ significantly from mock treated mice with respect to HBsAg secretion. Similarly, serum viral particle equivalents, as quantified by real-time PCR, were also reduced in mice that received U6 shRNA 5 and U6 lhRNA 1 (Figure 2.5B). Inhibitory effects on serum DNA concentrations by U6 lhRNA 2 however were less robust. These observations were corroborated by qRT-PCR analysis of intrahepatic viral mRNA levels (127)[6]. HBV *core* and *surface* mRNA concentrations relative to *GAPDH* mRNA were reduced 60-70% in mice receiving anti HBV shRNA or lhRNA expression cassettes. Efficient knockdown using lhRNA expression cassettes was therefore demonstrated at protein, DNA and RNA levels.

To determine if lhRNA cassettes stimulate the IFN response *in vivo*, *OAS1* and *IFN-β* levels were measured by qRT-PCR on mouse liver samples 5 days after hydrodynamic injection. The positive control for induction of IFN response-related genes included hepatic RNA extracted from mouse livers 6 hours after hydrodynamic injection of poly (I:C). Quantitation at 6 hours post-injection proved necessary as IFN stimulation was not observed at 5 days after injection, which is likely to be a result of the transient effects of poly (I:C). An increase in *OAS1* levels was observed in the mock injected group 6 hours after injection which may reflect the immunostimulatory effects of the injection procedure itself. Nevertheless, no significant induction of *OAS1* or *IFN-β* was detected in any of the mice receiving shRNA or lhRNA cassettes (Figure 2.6). This indicates that the *in vivo* anti HBV effects of the lhRNA cassettes were not as a result of non-specific immune response.

[6] Weinberg et al. (Appendix A2)

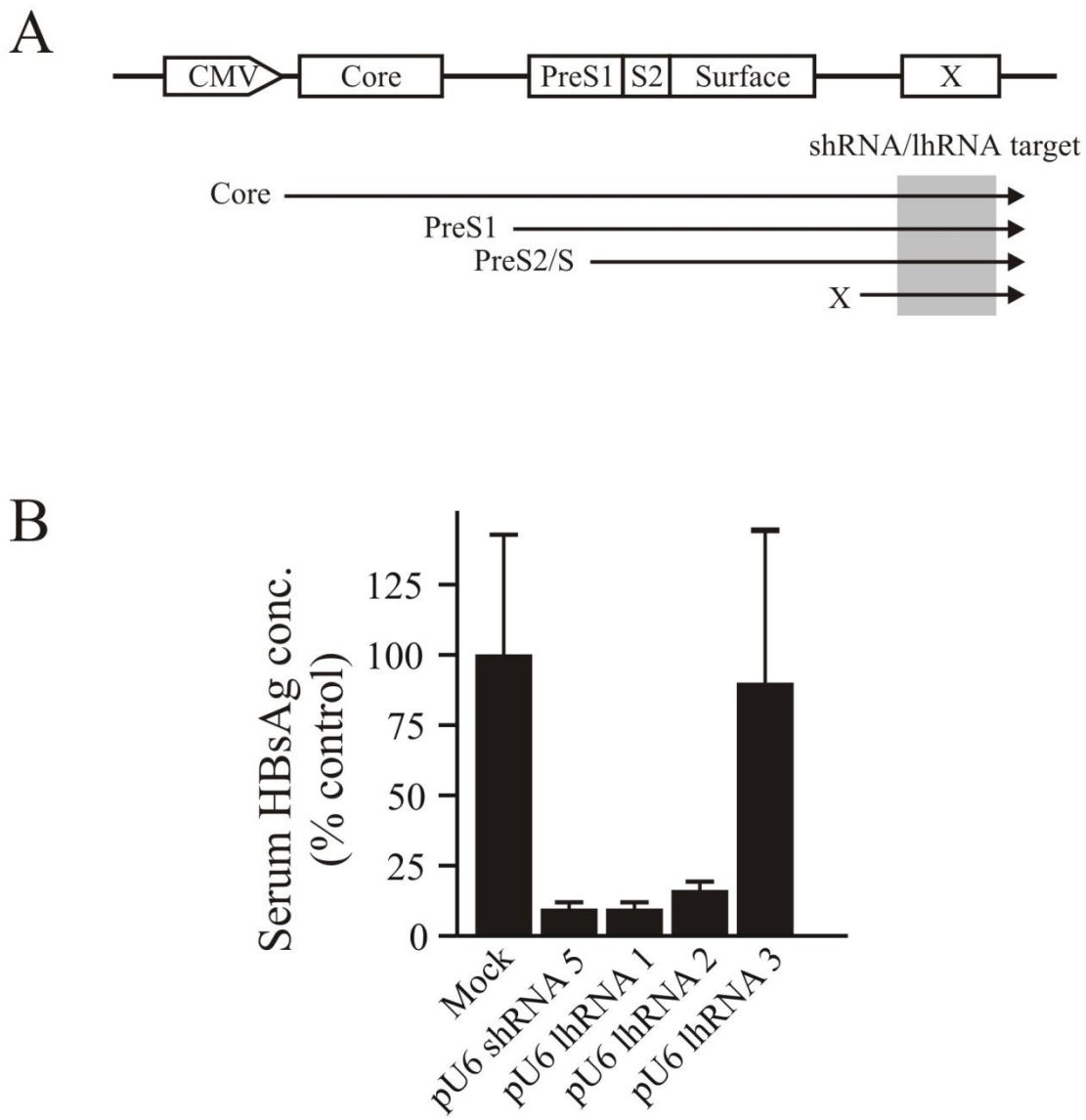

Figure 2.4: *In vivo* inhibition of HBsAg serum concentrations by U6-driven lhRNA.

(A) Schematic representation of pCH-9/3091. (B) Serum HBsAg concentrations were determined from mice 5 days after co-injection with HBV DNA (pCH-9/3091) and shRNA or lhRNA expression cassettes. Means from 5 independent experiments are shown with SEM.

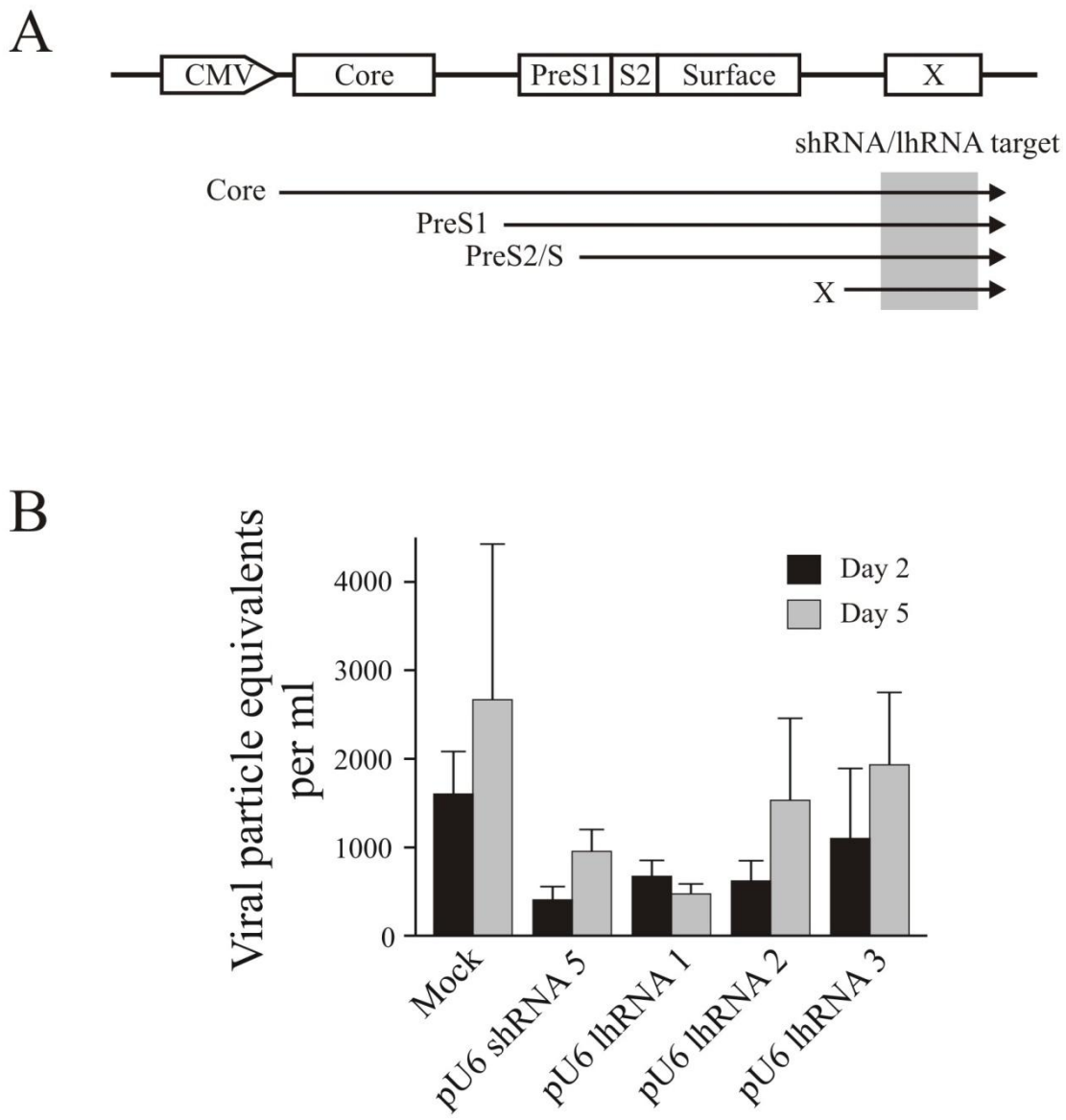

Figure 2.5: <u>Suppression of circulating HBV particle equivalents</u>

(A) Schematic representation of pCH-9/3091. (B) Viral particle equivalents were determined using real-time quantitative PCR from mouse serum samples collected 2 and 5 days after hydrodynamic injection. Averages were normalised to the mock and are indicated with SEM.

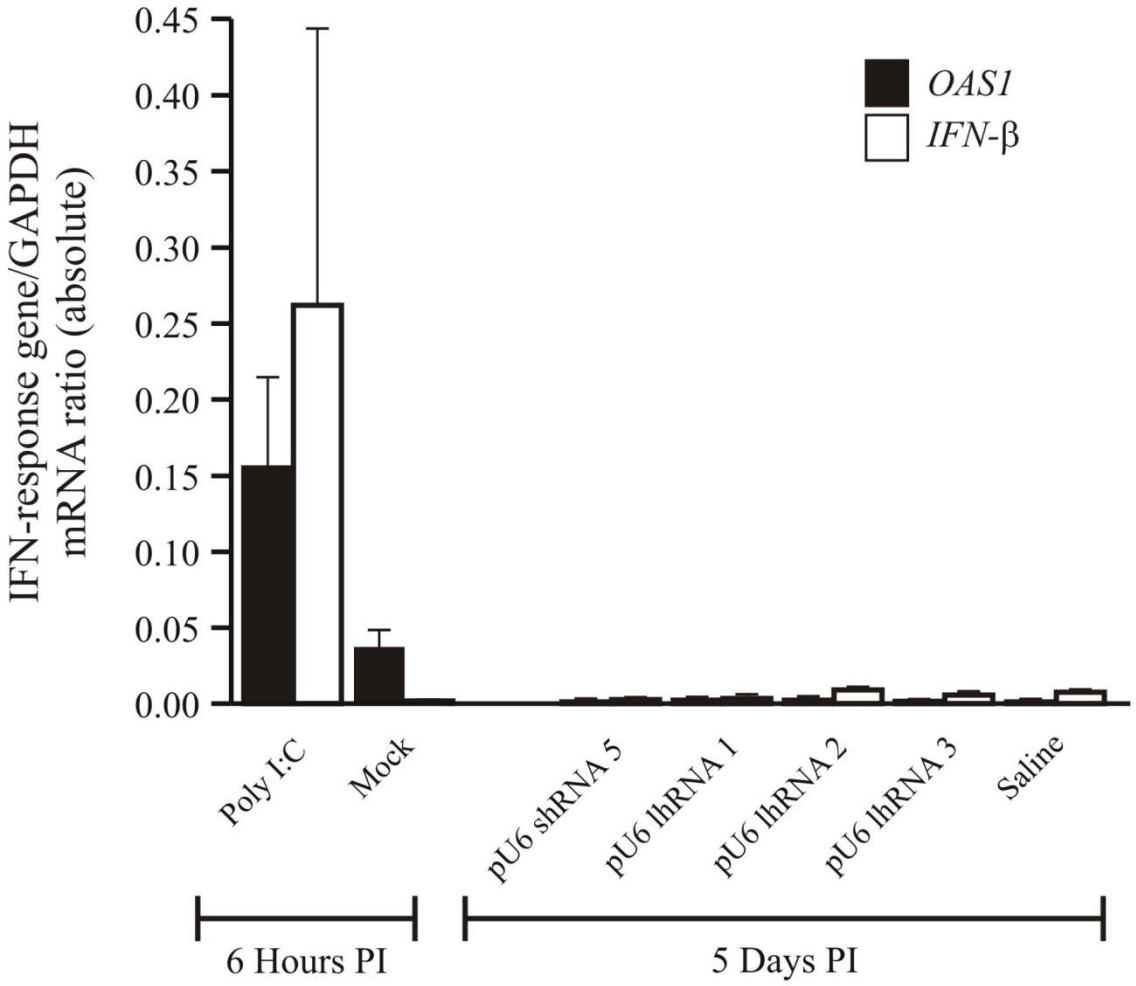

Figure 2.6: <u>Intrahepatic stimulation of IFN response-related genes.</u>

RNA extracted (6 hours or 5 days post-injection) from livers of mice injected with the indicated plasmids and controls was subjected quantitative RT-PCR to determine murine *OAS1* and *IFN-β* mRNA levels and normalised to *GAPDH* mRNA. Average ratio of IFN response gene to housekeeping gene is shown with SEM.

2.3.3 U6 lhRNA produce multiple siRNA sequences

Long hairpins have the potential advantage to be processed into multiple siRNA sequences, which could theoretically silence multiple targets. To assess this, wild-type and mutant luciferase target vectors were generated (127)[7]. U6 shRNA 5 could only silence the wild-type reporter whereas U6 lhRNA 1 was able to silence expression from the wild-type and mutant vectors. These data indicated that multiple siRNA sequences were produced from a single lhRNA sequence. To confirm this observation Northern blot hybridisation was carried out to detect the three putative guide sequences that could be produced from lhRNA 1. Three oligonucleotide probes were designed to span the full-length sequence of the lhRNA. Northern blot analysis revealed that only two of the three potential siRNA guide sequences were produced (127). This and data presented elsewhere (131) suggested that siRNA closer to the loop end of the lhRNA sequences were inefficiently processed by Dicer. Indeed functional analysis of U6 lhRNA 1 against luciferase vectors containing sites along the entire extent of lhRNA sequence showed that silencing efficacy diminished from the base to the loop of the hairpin (127). Together the Northern blot and functional analysis data indicate that loss of processing along the stem base of the lhRNA correlates with loss of activity.

2.4 DISCUSSION

A panel of 10 anti HBV U6 shRNA was designed with stem regions of 25 bp and the loop sequence of human miR-23. shRNA with larger stem regions (up to 29 bp in length) have been demonstrated to exhibit improved processing by Dicer while

[7] Weinberg et al. (Appendix A2)

incorporation of a miRNA loop sequence in the design of a shRNA has been shown to improve cytoplasmic localisation of the effecter sequence (132). Effective inhibition of markers of HBV replication was achieved with a number of these U6 shRNA expression cassettes. These data demonstrate the reliability of the Pol III shRNA expression cassette approach for the generation of effective RNAi-mediated silencing sequences. The fact that these sequences exhibit potent antiviral activity in the transgenic mouse model of HBV replication (105)[8] is particularly noteworthy. The data are very impressive and highlight the important feature of RNAi expression cassettes being compatible with recombinant viral vectors. The lack of *in vitro* and *in vivo* models of HBV infection however places limitations of studies of efficacy. Although assessing RNAi therapeutics in transgenic HBV mice yields data of substantial importance, emergence of viral escape mutants to administered therapeutics cannot be evaluated in this or any other model of HBV replication. Nevertheless developing expressed RNAi therapeutics that are capable of simultaneously targeting multiple sites within HBV is an important objective.

Effective RNAi activator sequences identified from the panel of U6 shRNA constructs were further developed to create multi-targeting lhRNA cassettes. lhRNA 1 was designed to target the same region targeted by the U6 shRNA 5 and U6 shRNA 6 sequences. As predicted this cassette caused silencing of viral expression equally efficiently when compared to its shRNA counterparts and suppression of markers of viral replication was observed *in vitro* and *in vivo*. Multiple siRNA could potentially be produced from a single lhRNA which can simultaneously target multiple sites within a virus. The need for a multi-targeted approach to silence HBV was highlighted by Wu et al. (117) who demonstrated emergence of a mutant virus resistant to a single RNAi effecter

[8] Carmona et al. (Appendix A2)

sequence. Though the anti HBV lhRNA 1 described here was able to inhibit wild-type and mutated viral sequences, loss of silencing activity was observed against targets complementary to the loop end of the lhRNA. The observed loss of functionality was as a result of inefficient processing of the entire lhRNA sequence. Improvements in the anti HBV effecter sequences identified from the panel of U6 shRNA is therefore still possible.

The data presented here contributes significantly to the growing body of evidence that exploiting the RNAi pathway is a feasible approach for the development of novel anti HBV therapeutics. The study by Carmona et al. (105)[9] was one of the first to demonstrate anti HBV efficacy in a clinically relevant model by recombinant adenoviral delivery of the shRNA expression cassettes described here. Also, although the lhRNA expression cassettes need further development, the data clearly indicate the potential for RNAi-based therapeutics to limit viral resistance. A shortcoming of U6-driven RNAi expression cassettes was demonstrated recently by fatality in mice as a consequence of saturation of the endogenous miRNA pathway (118). Consequently this has raised concerns about the safety of expression-based RNAi therapeutics, specifically of U6 shRNA expression cassettes. Endogenous miRNA are normally expressed from Pol II promoters and often as polycistronic transcripts. Exploiting characteristics of naturally occurring miRNA therefore offer new avenues for improving efficacy (through polycistronic expression) and importantly safety (by means of regulatable expression) of RNAi expression cassettes. The following Chapter will explore the adaptation of features of naturally occurring miRNA to the development of anti HBV expression cassettes as a means of improving expressed RNAi activators.

[9] Appendix A2

3 PRI-MIR SHUTTLE EXPRESSION VECTORS

3.1 INTRODUCTION

U6 promoter driven transcription yields RNA sequences of precisely defined size and sequence and for this reason has been the preferred method for the expression of shRNA. The preceding Chapter and reports by others (80-87) have demonstrated the utility of exploiting anti HBV shRNA expressed from Pol III promoters and more specifically the U6 promoter. Strong, constitutive shRNA expression from the U6 promoter may however not be desirable for therapeutic purposes. Pol II regulatory elements offer greater transcriptional control than Pol III promoters. Preliminary efforts to express an effective shRNA from a Pol II regulatory element however have met with limited success. Expressing the shRNA 5 sequence, which has been shown to be effective when expressed from the U6 promoter, from a CMV promoter results in poor anti HBV activity. This is likely as a result of sequences in the Pol II transcript that occur upstream and downstream of the shRNA which may impair processing. Naturally occurring miRNA are normally expressed from Pol II promoters (49) and therefore contain the necessary features for recognition and processing by the RNAi machinery.

Exploiting sequence and structural elements of miRNA may offer the means to design effective Pol II-driven RNAi expression cassettes. To this end RNAi effecter sequences that mimic naturally occurring pri-miRNA (or pri-miR[10] shuttles) were designed by embedding anti HBV guide sequences within pri-miR-31 (42, 133-135) and pri-miR-122 (133, 136-138) sequences. *In vitro* characterisation of pri-miR-31 indicated that this

[10] Pri-miRNA, pre-miRNA and miRNA refer to naturally occurring sequences, whereas pri-miR and pre-miR refer to designed shuttle sequences.

sequence is efficiently processed by Drosha (134) and miR-122 is a well-established liver-specific miRNA (133, 136-138). Efficacy of the pri-miR shuttles was initially assessed by expressing these sequences from the U6 promoter. Expression from a promoter that is known to drive transcription of effective RNAi effecters validated the design of the pri-miR shuttle sequences. U6 shuttle cassettes efficiently silenced HBV expression which indicated that the design of the pri-miR were effective. Once validated the next goal was to create pri-miR shuttle sequences driven from a Pol II promoter. CMV promoter-driven pri-miR shuttle expression cassettes were equally capable of suppressing viral gene expression as their U6 promoter counterparts. Both Pol III- and Pol II-driven pri-miR shuttle sequences were processed according to intended design. Processing indicated that concentrations of guide sequences derived from pri-miR shuttles was much lower than guide sequences derived from U6 shRNA. Moreover silencing was achieved without induction of the innate immune response or disruption of the miRNA biogenesis pathway.

3.2 MATERIALS AND METHODS

3.2.1 Design of Pol-III and Pol-II pri-miR shuttle cassettes

To assess the functionality of anti HBV pri-miR shuttles, the major guide sequences of naturally occurring human pri-miR-31 and human pri-miR-122 were substituted with the guide sequences of anti *HBx* U6 shRNA (Figures 3.1 and 3.2). Pri-miR-31/5, pri-miR-31/8 and pri-miR-31/9 shuttle sequences were designed by replacing the guide sequence of pri-miR-31 with guide sequences of U6 shRNA 5, U6 shRNA 8 and U6 shRNA 9, respectively. Similarly, the putative guide sequences of U6 shRNA 5, U6 shRNA 6 and U6 shRNA 10 were used to the replace the guide sequence of pri-miR-122 to generate pri-miR-122/5, pri-miR-122/6 and pri-miR-122/10, respectively. The mature

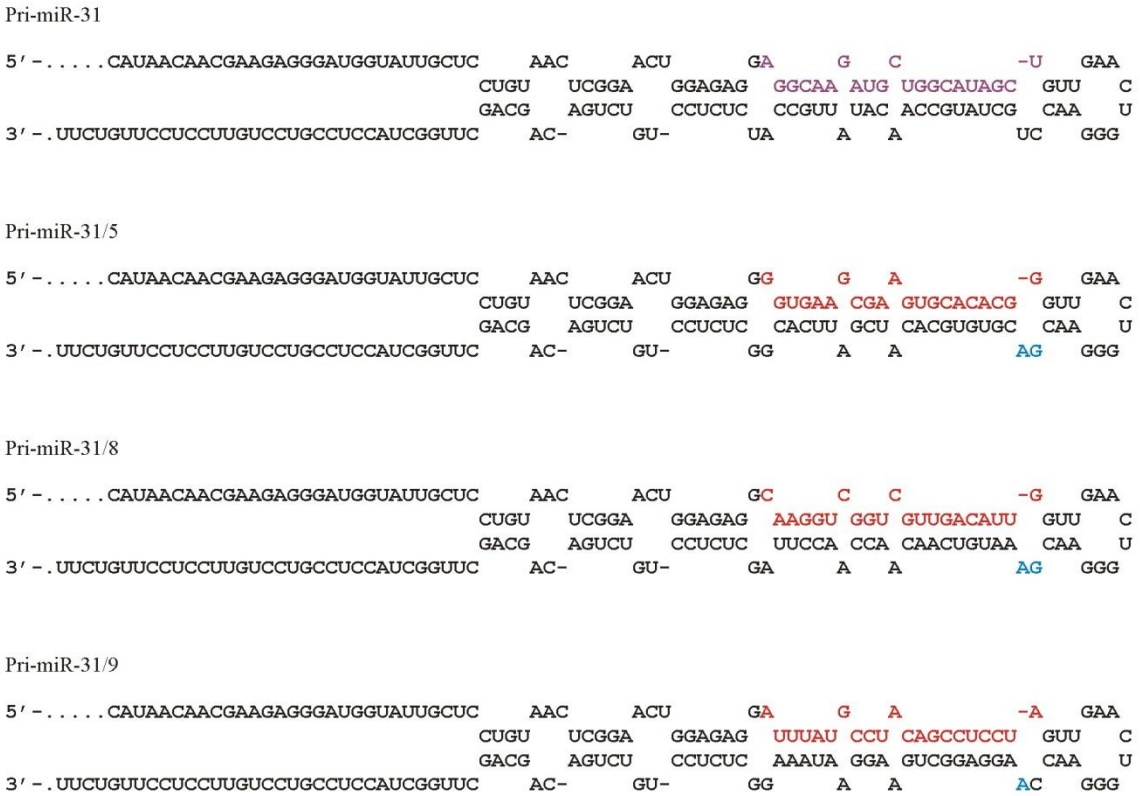

Figure 3.1: <u>Design of pri-miR-31 shuttle sequences.</u>

The putative guide sequence derived from the 5' arm of hsa-miR-31 (indicated in purple) was replaced with anti HBV guide sequences (indicated in red). Wild-type pri-miR sequences were maintained so long as secondary structure of the pri-miR shuttle sequences were not compromised (nucleotides indicated in blue). Complete pri-miR shuttles contained 51 nt of wild-type pri-miRNA sequences flanking the pre-miR shuttle sequences.

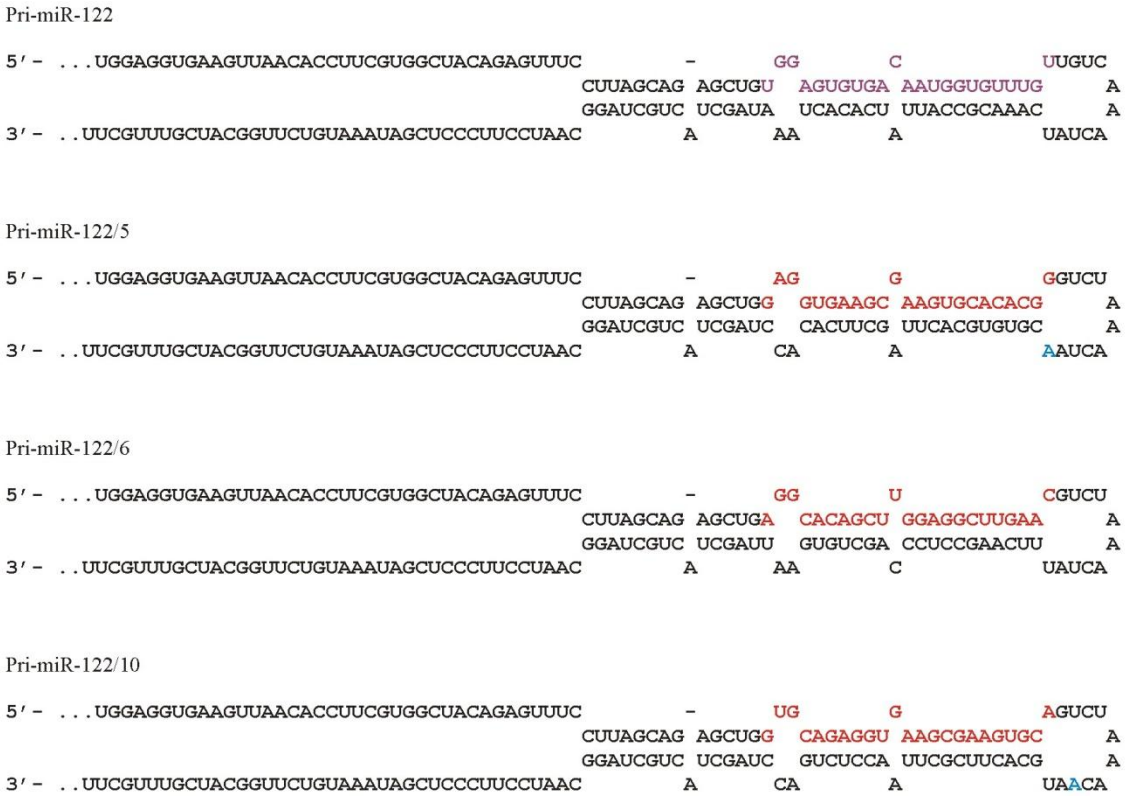

Figure 3.2: <u>Design of pri-miR-122 shuttles</u>

The putative guide sequence derived from the 5' arm of hsa-miR-122 (indicated in purple) was replaced with anti HBV guide sequences (indicated in red). Wild-type pri-miR sequences were maintained so long as secondary structure of the pri-miR shuttle sequences were not compromised (nucleotides indicated in blue). Complete pri-miR shuttles contained 51 nt of wild-type pri-miRNA sequences flanking the pre-miR shuttle sequences.

human miR-31 (hsa-miR-31) is a 21 nt RNA sequence therefore only 21 nt of the U6 shRNA guide were incorporated into the pri-miR-31 shuttles. Thus, pri-miR-31/5 is targeted to HBV co-ordinates 1575-1595, pri-miR-31/8 targets co-ordinates 1678-1698 and pri-miR-31/9 targets co-ordinates 1774-1794. In contrast, mature hsa-miR-122 is a 23 nt RNA sequence and therefore the pri-miR-122 shuttles contained 23 nt of the U6 shRNA guide. Accordingly, the HBV genomic co-ordinates 1575-1597 were targeted by pri-miR-122/5, co-ordinates 1580-1602 were targeted by pri-miR-122/6 and co-ordinates 1863-1885 were targeted by pri-miR-122/10. The pri-miR shuttles were designed to have incompletely complementary stem sequences such that the shuttles retained the secondary structure of the wild-type pri-miRNA as determined by *in silico* prediction (139, 140) (see Appendix A4-1).

The presence of non-structured sequences flanking pre-miRNA hairpins has been demonstrated to be essential for efficient processing by Drosha (134). In cultured cells the most efficient processing was achieved when at least 51 nt of natural RNA sequences flanked each arm of a pre-miRNA hairpin (134). The complete pri-miR-31 and pri-miR-122 shuttles therefore consisted of pre-miR-31 or pre-miR-122 hairpin shuttles flanked 5' and 3' by 51 nt of natural hsa-miR-31 or hsa-miR-122 sequence, respectively. A *Nhe*I restriction site was introduced 5' and a *Spe*I site introduced 3' of the pri-miR shuttle sequence to facilitate cloning. The Pol III pri-miR expression cassettes contained the shuttle sequences downstream of the U6 promoter whereas the Pol II pri-miR cassettes consisted of the shuttle sequences located within an exonic sequence of a CMV expression system.

3.2.2 Generation of Pol III and Pol-II pri-miR shuttle cassettes

3.2.2.1 Generation of pri-miR sequences

Oligonucleotides encoding pre-miR-31 or pre-miR-122 shuttle sequences were synthesised by standard phosphoramidite chemistry (Table 3.1). The pre-miR-31/n F and R and pre-miR-122/n F and R were subjected to primer extension to generate the pre-miR-31/n and pre-miR-122/n shuttle sequences as double-stranded (dsDNA) (Figure 3.3). The primer extension reaction was performed as a PCR and contained 200 pmol of pre-miR forward and reverse primers. Purified pre-miR-31 and pre-miR-122 fragments were subsequently used for the PCR amplification of the pri-miR-31 and pri-miR-122 shuttle sequences using the relevant primer set (Table 3.1). The pri-miR-31 and pri-miR-122 shuttle fragments were ligated to the PCR cloning vector pTZ57R/T according to the manufacturer's instructions to generate pTZ-pri-miR-31 or pTZ-pri-miR-122 vectors. Plasmids containing inserts of the expected size and orientation (reverse with reference to the *lacZ* gene) were sequenced.

3.2.2.2 Generation of U6 pri-miR expression vectors

To generate Pol III driven pri-miR expression vectors the pri-miR-31 and pri-miR-122 shuttle sequences were cloned downstream of the human U6 promoter. First the U6 promoter sequence was cloned into the pTZ57R/T PCR cloning vector to create pTZ-U6. To this end oligonucleotides were designed to amplify the U6 promoter sequence and introduce a *Bgl*II restriction site and a *Nhe*I restriction site on its 5' and 3' ends, respectively. The U6 (*Bgl*II) F (5'- GAT CAG ATC TAA GGT CGG GCA GGA AGA GGG -3') and U6 (*Nhe*I) R (5'- GAT CGC TAG CGG TGT TTC GTC CTT TCC ACA AG -3') primers were synthesised by standard phosphoramidite chemistry (Inqaba Biotech, South Africa). The U6 promoter sequence from pU6 was amplified with the

Table 3.1: Oligonucleotide sequences for the generation of pri-miR-31 and pri-miR-122 shuttle cassettes

Primer	Oligonucleotide sequences	Manufacturer
HBx$_{1575\text{-}1595}$ [1]		Integrated DNA Technologies, CA, USA.
Pre-miR-31/5 F	5′- GTAACTCGGAACTGGAGAGGGGTGAAGCGAAGTGCACACGGGTTGAACTGGGAACGACG -3′	
Pre-miR-31/5 R	5′- CTGCTGTCAGACAGGAAAGCCGTGAATCGATGTGCACACGTCGTTCCCAGTTCAACCCTG -3′	
HBx$_{1678\text{-}1698}$		
Pre-miR-31/8 F	5′- GTAACTCGGAACTGGAGAGGCAAGGTCGGTCGTTGACATTGGTTGAACTGGGAACGAAA -3′	
Pre-miR-31/8 R	5′- CTGCTGTCAGACAGGAAAGCTAAGGTTGGTTGTTGACATTTCGTTCCCAGTTCAACCAAT -3′	
HBx$_{1774\text{-}1794}$		
Pre-miR-31/9 F	5′- GTAACTCGGAACTGGAGAGGATTTATGCCTACAGCCTCCTAGTTGAACTGGGAACGAAG -3′	
Pre-miR-31/9 R	5′- CTGCTGTCAGACAGGAAAGCCTTTATTCCTTCAGCCTCCTTCGTTCCCAGTTCAACTAGG -3′	
Pri-miR-31 F	5′- GCTAGCCATAACAACGAAGAGGGATGGTATTGCTCCTGTAACTCGGAACTGGAGAGG -3′	
Pri-miR-31 R	5′- AAAAAAACTAGTAAGACAAGGAGGAACAGGACGGAGGTAGCCAAGCTGCTGTCAGACAGGAAGC -3′	

HBx$_{1575\text{-}1597}$		
Pre-miR-122/5 F	5′- GAGTTTCCTTAGCAGAGCTGGAGGTGAAGCGAAGTGCACACGGGTCTAAACTAACGTGTGCA -3′	
Pre-miR-122/5 R	5′- GGATTGCCTAGCAGTAGCTAGGTGTGAAGCTAAGTGCACACGTTAGTTTAGACCCGTGTGCA -3′	
HBx$_{1580\text{-}1602}$		
Pre-miR-122/6 F	5′- GAGTTTCCTTAGCAGAGCTGGTGCAGAGGTGAAGCGAAGTGCAGTCTAAACAATGCACTTCG –3′	
Pre-miR-122/6 R	5′- GGATTGCCTAGCAGTAGCTAGGTCAGAGGTTAAGCGAAGTGCATTGTTTAGACTGCACTTCG -3′	
HBx$_{1863\text{-}1885}$		
Pre-miR-122/10 F	5′- GAGTTTCCTTAGCAGAGCTGAGGCACAGCTTGGAGGCTTGAACGTCTAAACTATTTCAAGCC -3′	Inqaba Biotech, South Africa
Pre-miR-122/10 R	5′- GGATTGCCTAGCAGTAGCTAATTCACAGCTGGGAGGCTTGAAATAGTTTAGACGTTCAAGCC -3′	
Pri-miR-122 F	5′- GACTGCTAGCTGGAGGTGAAGTTAACACCTTCGTGGCTACAGAGTTTCCTTAGCAGAGCTG -3′	
Pri-miR-122 R	5′- GATCACTAGTAAAAAAGCAAACGATGCCAAGACATTTATCGAGGGAAGGATTGCCTAGCAGTAGCTA -3′	

[1] Numbers indicate co-ordinates on the HBV genome (accession number J02203.1)

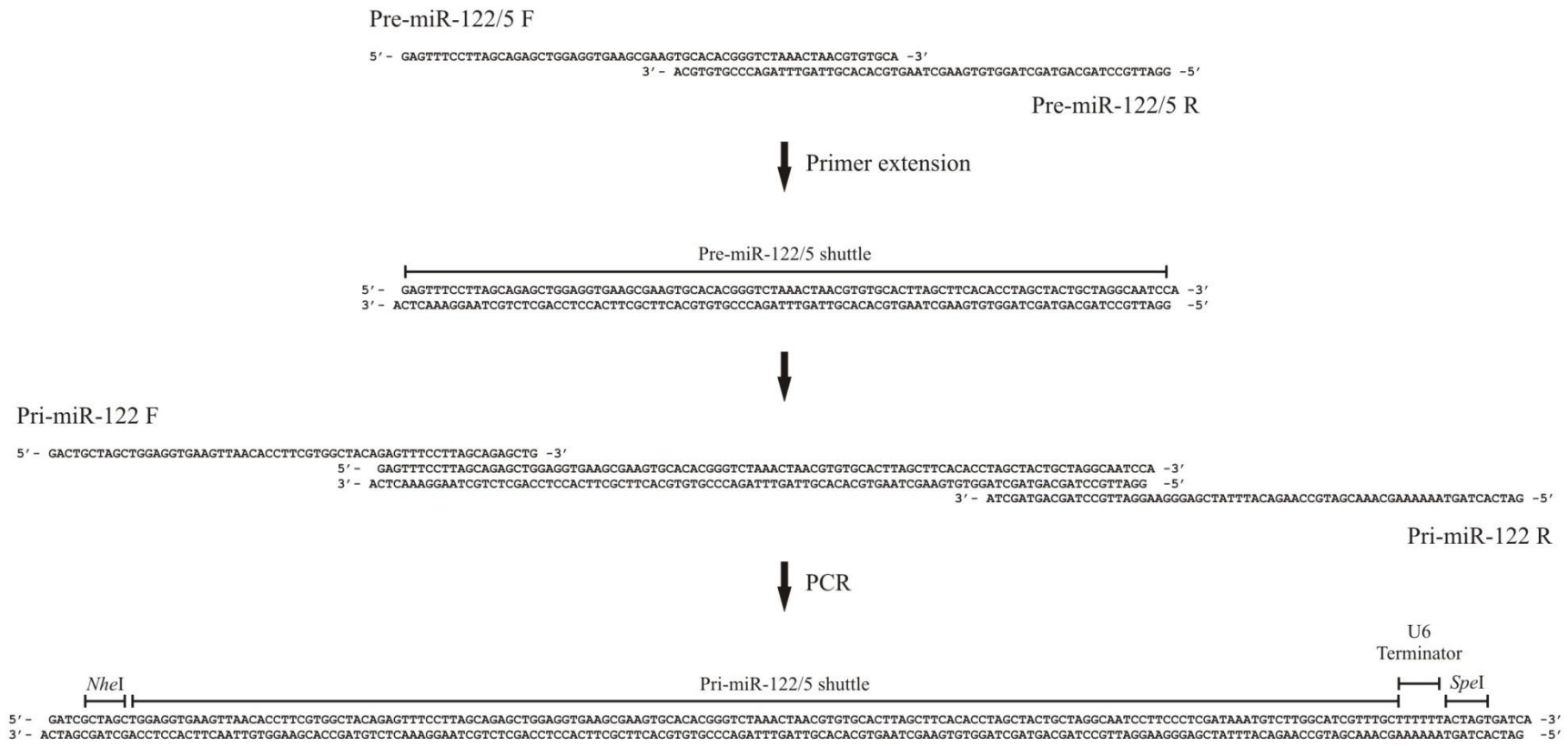

Figure 3.3: <u>Generation of pri-miR shuttle sequences.</u>

Pre-miR shuttle sequences were generated as dsDNA by primer extension of partially complementary oligonucleotides. Generic pri-miR-31 or pri-miR-122 primer sets were used in the amplification of relevant pre-miR shuttle sequences to generate the complete pri-miR shuttle cassettes.

aforementioned primers and ligated to the pTZ57R/T PCR cloning vector. Clones containing inserts of the correct size and desired orientation (reverse with reference to the *lacZ* gene) were sequenced.

To clone the pri-miR-31 shuttle sequences downstream of the U6 promoter the pTZ-pri-miR-31 and pTZ-U6 vectors were digested with *Sca*I and *Nhe*I. The 1262 bp pri-miR-31 fragments were ligated with the 2080 bp pTZ-U6 fragment to generate the pU6 pri-miR-31 expression vectors. The pri-miR-122 shuttle sequences were inserted downstream of the U6 promoter of pTZ-U6 by first digesting the pTZ-pri-miR-122 vectors and pTZ-U6 with *Nhe*I and *Eco*RI. Finally, the pU6 pri-miR-122 expression vectors were generated by ligating the 211 bp pri-miR-122 fragments to the 3132 bp pTZ-U6 fragment. All plasmids were sequenced.

3.2.2.3 Generation of CMV pri-miR expression vectors

The Pol II pri-miR expression cassettes were generated by inserting the pri-miR-31 and pri-miR-122 shuttle sequences into the exonic sequence of the CMV expression cassette contained in the mammalian expression vector, pCI-neo. To insert the pri-miR-31 shuttle sequences into pCI-neo, the pTZ-pri-miR-31 vectors were digested with *Sal*I and *Xba*I. The resultant pri-miR-31 fragments were ligated to compatible *Xho*I and *Xba*I sites of the pCI-neo backbone. The pri-miR-122 shuttle sequences were ligated to the *Nhe*I and *Xba*I sites of pCI-neo after restriction with *Nhe*I and *Bcu*I (an isoschizomer of *Spe*I). Clones positive for correct insertion were sequenced.

3.2.3 Luciferase Reporter plasmid (pCH-FLuc)

The pCH-FLuc reporter plasmid was generated by replacing the *preS2/S* ORF of pCH-9/3091 with a sequence encoding Firefly luciferase, which was similar to the procedure employed to propagate pCH-eGFP. Using PCR, a Firefly luciferase encoding sequence was amplified from the plasmid pGL4 (Promega, WI, USA) with primer sequences, FLuc F: 5'- ACT GCT CGA GGA TTG GGG ACC CTG CGC TGA ACA TGG AAG ACG CCA AAA AC -3' and FLuc R: 5′- ACT GAC TAG TTT ACA CGG CGA TCT TTC C -3′. FLuc F spanned 31 nt of the HBV genome from the *Xho*I restriction site at position 129 to the initiation codon of the middle HBs protein at position 159 and finally ending with sequence complementary to the 5' end of *Firefly luciferase*. The reverse primer was complementary to the 3' end of the *Firefly luciferase* ORF and included the sequence for a *Spe*I restriction site. PCR-based amplification of pGL4 (Promega, WI, USA) yielded a *Firefly luciferase* sequence with its initiation codon equivalent to that of the middle HBs protein. The PCR product was cloned into pTZ57R/T to generate pTZ-FLuc. The *Firefly luciferase* sequence was restricted from pTZ-FLuc with *Xho*I and *Spe*I and inserted into the *Xho*I and *Spe*I sites of pCH-9/3091 to generate pCH-FLuc.

3.2.4 Assessing efficacy of pri-miR shuttles *in vitro*

3.2.4.1 Transfection of cultured mammalian cells

Inhibition of markers of HBV replication

Huh7 cells were maintained as described (Appendix A1-1) and seeded in 24-well tissue culture plates (Nunclon™ Δ Surface, Nunc, Denmark) on the day prior to

transfection. To assess effect on HBsAg secretion 80 ng of pCH-9/3091 was co-transfected with 800 ng of effecter plasmid and 20 ng of pCI-neo eGFP. *In situ* knockdown of luciferase expression was evaluated by co-transfecting Huh7 cells with 80 ng of pCH-FLuc, 800 ng of RNAi effecter plasmid, 80 ng of the phRL-CMV plasmid (Promega, WI, USA) which expresses *Renilla* luciferase from a CMV promoter and 40 ng of pCI-neo eGFP. Lipofectamine™ 2000 was used for transfection of DNA according to manufacturer's instructions (Appendix A1-1). Approximately 48 hours after transfection supernatant from cells transfected with pCH-9/3091 was assayed for HBsAg secretion and lysates from cells transfected with pCH-FLuc assayed for luciferase activity.

3.2.4.2 Quantification of markers of viral replication

Quantitation of HBsAg secretion

HBsAg secreted into the growth medium of cells transfected with pCH-9/3091 was quantified with the MONOLISA® HBs Ag ULTRA Assay kit following the previously described procedure (Section 2.2.7.1). One hundred microlitres of undiluted culture supernatant of each sample was used for the ELISA.

Quantification of *in situ* target knockdown

Cells transfected with pCH-FLuc were assayed for *in situ* luciferase activity using the Dual-Luciferase® Assay System (Promega, WI, USA). Growth medium was removed and cells lysed with agitation for 15 minutes at room temperature in Passive Lysis Buffer. Ten microlitres of the lysates were dispensed per well into a Costar® 96-well assay plate (Corning Inc, NY, USA) and Firefly luciferase and *Renilla* luciferase activities measured with the Veritas Dual Injection Luminometer (Turner BioSystems, CA, USA). Firefly luciferase activity was measured immediately after 50 µl of Luciferase Assay Reagent II

was added to each sample. Addition of 50 µl of Stop & Glo® inhibits Firefly luciferase activity and allows the reaction catalysed by *Renilla* luciferase to be measured independently. Firefly luciferase activity was normalised to *Renilla* luciferase activity.

3.2.5 Evaluation of pri-miR shuttle processing

3.2.5.1 Transfections

HEK293 cells were maintained in DMEM supplemented with 10% FCS, penicillin (100 000 U/ml) and streptomycin (100 µg/ml) as described in Appendix A1-1. Cells were seeded at 60% confluency in 10 cm^2 Costar® tissue culture plates (Corning Inc, NY, USA) 24 hours before transfection in antibiotic-free medium. Sixteen micrograms of each RNAi effecter plasmid was mixed with 3 µg of pCH-9/3091, 1 µg of pCI-neo eGFP and diluted in 500 µl of Opti-MEM I. The DNA was transfected into the cells with Lipofectamine™ 2000 according to manufacturer's instructions. The transfection mixes were left on the cells for 48 hours at 37°C and 5% CO_2 in a humidified incubator.

3.2.5.2 Polyacrylamide gel electrophoresis and Northern blot analysis

Total RNA was extracted from transfected cells using Tri-Reagent (Sigma, MO, USA) and approximately 25 µg separated on a 15% denaturing polyacrylamide gel. As a molecular weight marker 10 pmol each of 18 and 30 base deoxyoligonucleotides labelled with 20 µCi of [γ-^{32}P]-ATP with T4 polynucleotide kinase (Fermentas, MD, USA). The polyacrylamide gel was stained in 0.5× Tris-Borate EDTA (TBE) containing ethidium bromide at a final concentration of 4 µg/ml for 5 minutes with shaking. RNA was visualised on a UV transilluminator to confirm equal loading and RNA quality. The RNA was transferred to a positively charged nylon membrane (Hybond-N+, Amersham, NJ, USA) by semi-dry blotting using the Semi-Dry Electroblotting Unit Z34,050-2 (Sigma-

Aldrich, MO, USA). Electroblotting proceeded at 3.3 mA/cm^2 for an hour at 4°C in 0.5× TBE. After RNA transfer the nylon membrane was crosslinked with 200 000 µJ/cm^2 of energy using a UV crosslinker (UVP, Inc., CA, USA) and baked at 80°C for an hour.

The membranes were prehybridised in 10 ml/100 cm^2 of Rapid-hyb (Amersham, NJ, USA) at 42°C for at least 15 minutes. Probes against putative 5, 6, 8, 9 and 10 guide sequences and the U6 snRNA sequence were prepared by labelling oligonucleotides (Table 3.2) with 20 µCi of [γ-^{32}P]-ATP using T4 polynucleotide kinase (Fermentas, MD, USA). After the prehybridisation step membranes were hybridised overnight at 42°C with the relevant probe at a final concentration of 10 ng/ml. Following overnight hybridisation membranes were subjected to a low stringency wash with a 5× SSC (20× SSC (3M NaCl, 0.3M sodium citrate, pH 7.0)), 0.1% SDS solution at room temperature and 2 high stringency washes with a 1× SSC, 0.1% SDS solution at 42°C. The probed membranes were subjected to autoradiography for at least 7 days. Membranes were stripped and reprobed with a ^{32}P labelled oligonucleotide to detect U6 snRNA.

3.2.6 Assessing off-target effects of pri-miR shuttles

3.2.6.1 IFN response assay

Transfection of IFN responsive cell line

HEK293 cells (human embryonic kidney cell line) were maintained as described (Appendix A1-1) and seeded into 24-well culture plates 24 hours before transfection. Cells were transfected with 800 ng of pCH-9/3091, 800 ng of RNAi effecter plasmid, 80 ng of pTZ57R and 40 ng of pCI-neo eGFP. As a positive control for induction of IFN genes 800 ng of poly (I:C) (Sigma, MO, USA) was co-transfected with 80 ng of pCH-9/3091, 80 ng

Table 3.2: Oligonucleotides used as probes against putative shRNA and miR guide sequences and U6 snRNA

Probe	Sequence	Manufacturer
5 guide	5′- CCGTGTGCACTTCGCTTC -3′	Inqaba Biotech, South Africa.
6 guide	5′- TGCACTTCGCTTCACCTC -3′	
8 guide	5′- CAATGTCAACGACCGACC -3′	
9 guide	5′- TAGGAGGCTGTAGGCATA -3′	
10 guide	5′- GTTCAAGCCTCCAAGCTG -3′	
U6 snRNA guide	5′- TAGTATATGTGCTGCCGAAGCGAGCA -3′	

of pTZ57R and 40 ng of pCI-neo eGFP. Transfections were carried out with Lipofectamine™ 2000 (Appendix A1-1).

IFN-β qRT-PCR

Total RNA was extracted from transfected cells with the aid of Tri-Reagent (Sigma, MO, USA) and treated with RQ1 RNase-free DNase (Promega, WI, USA). Approximately 30 ng of RNA was reverse transcribed with the Sensiscript® Reverse Transcription Kit and an oligo-dT primer. *IFN-β* and *GAPDH* mRNA were quantified by real-time PCR of cDNA samples in the Roche Lightcylcer v.2 using the SYBR® Green Jumpstart Taq Ready Mix (Sigma. MO, USA). The primer sequences were *IFN-β* F: 5'-TCC AAA TTG CTC TCC TGT TGT GCT -3', *IFN-β* R: 5'- CCA CAG GAG CTT CTG ACA CTG AAA A -3', *GAPDH* F: AGG GGT CAT TGA TGG CAA CAA TAT CCA -3' and *GAPDH* R: 5'- TTT ACC AGA GTT AAA AGC AGC CCT GGT G -3'. qRT-PCR proceeded as described (Section 2.2.7.3).

3.2.6.2 Saturation assay

Assays were designed to assess saturation of the miRNA biogenesis pathway to determine potential toxic effects of the pri-miR shuttle sequences. Interference with the ability of an exogenous pri-miR shuttle (CMV pri-miR-31/8) or an endogenous miRNA (miR-16) to silence their respective targets was used as a measure of saturation.

Disruption of independent target silencing

Pri-miR-31/8 target plasmid (psiCHECK-8T)

A pri-miR-31/8 luciferase target vector was generated by inserting the sequence corresponding to nucleotides 1678-1702 (i.e. target of guide 8) of the HBV genome (accession number J02203.1) downstream of the Renilla *luciferase* ORF of psiCHECK2.2.

PCR of pCH-9/3091 with 8T F (5'- CAA TGT CAA CGA CCG ACC TT -3') and 8T R (5'- ACT AGT GCC TCA AGG TCG GT -3') yielded the 8 target sequence with a *Spe*I site at its 3' end. The amplicon was purified and ligated to the pTZ57R/T PCR cloning vector. Thereafter the target sequence was removed with *Sal*I and *Spe*I and ligated to the *Xho*I and *Spe*I sites of psiCHECK2.2 to generate psiCHECK-8T.

Assessing saturation of an exogenous pri-miR in cultured cells

To assess potential disruption of pri-miR-31/8 target silencing by the pri-miR-31/5 and pri-miR-122/5 shuttles Huh7 cells were co-transfected with 80 ng of psiCHECK-8T, 40 ng of pCMV pri-miR-31/8 and 780 ng of pU6 shRNA 5, pU6 pri-miR-122/5, pCMV pri-miR-122/5, pU6 pri-miR-31/5 or pCMV pri-miR-31/5. Forty eight hours after transfection *Renilla* and Firefly luciferase activity was measured.

Endogenous hsa-miR-16 saturation

miR-16 sponge/target sequence

A sponge vector capable of expressing an RNA sequence that hybridises to and interferes with miR-16 function was generated to assess saturation of endogenous miRNA. Sponge and target sequences of hsa-miR-16 were designed as described by Ebert et al. (141). A U6 promoter-driven miR-16 sponge (pU6-miR-16S×7) was generated by cloning 7 copies of an imperfectly complementary target of miR-16 within the U6+27 sequence (98, 142, 143). Inserting the same sequence into psiCHECK™-2 (Promega, WI, USA) created a miR-16 luciferase target vector (psi-miR-16T×7). Oligonucleotides encoding a single copy of the miR-16 target site (miR-16S) which anneal as dsDNA with 3' adenine overhangs were synthesised by standard phosphoramidite chemistry (Inqaba Biotech, South Africa). The sequences of the oligonucleotides were miR-16S F: 5'- CTC GAG

CGC CAA TAT TAT GTG CTG CTA GTC GAC GCG GCC GCA -3' and miR-16S R: 5'- GCG GCC GCG TCG ACT AGC AGC ACA TAA TAT TGG CGC TCG AGA -3'. An *Xho*I restriction site occurs immediately upstream of the hsa-miR-16 target sequence while *Sal*I and *Not*I restriction sites were introduced immediately downstream of the target site.

The miR-16S F and miR-16S R oligonucleotides were annealed and ligated into pTZ57R/T to generate pTZ-miR-16S×1. Sequencing revealed a single clone with the insert in the reverse orientation with respect to the *lacZ* gene. To facilitate formation of tandem repeats of the miR-16S×1 sequence it was initially inserted into pGEM®-T Easy to generate pG-miR-16S×1 (Figure 3.4). pTZ-miR-16S×1 was digested with *Apa*I and *Pvu*II to yield restriction fragments 2513 bp, 235 bp and 181 bp in size while digestion of pU6 shRNA 5 with *Apa*I and *Hin*cII yielded 2936 and 443 bp fragments. The 181 bp miR-16S×1 fragment was ligated into the 2936 bp pGEM®-T Easy fragment to produce pG-miR-16S×1.

To create a vector with tandem copies of the miR-16S sequence, pG-miR-16S×1 was digested with *Xho*I and *Sca*I and separately with *Sal*I and *Sca*I (Figue 3.5). *Xho*I-*Sca*I restriction gives rise to a 1962 bp fragment and a 1151 bp fragment whereas *Sal*I-*Sca*I digestion yields fragments 1935 bp and 1178 bp in length. The 1962 bp and 1178 bp fragments were purified and ligated together to generate pG-miR-16S×2. By similar procedures pG-miR-16S×3 and pG-miR-16S×4 were generated. Finally the vectors containing 3 and 4 tandem copies of the miR-16S sequence were used to create pG-miR-16S×7.

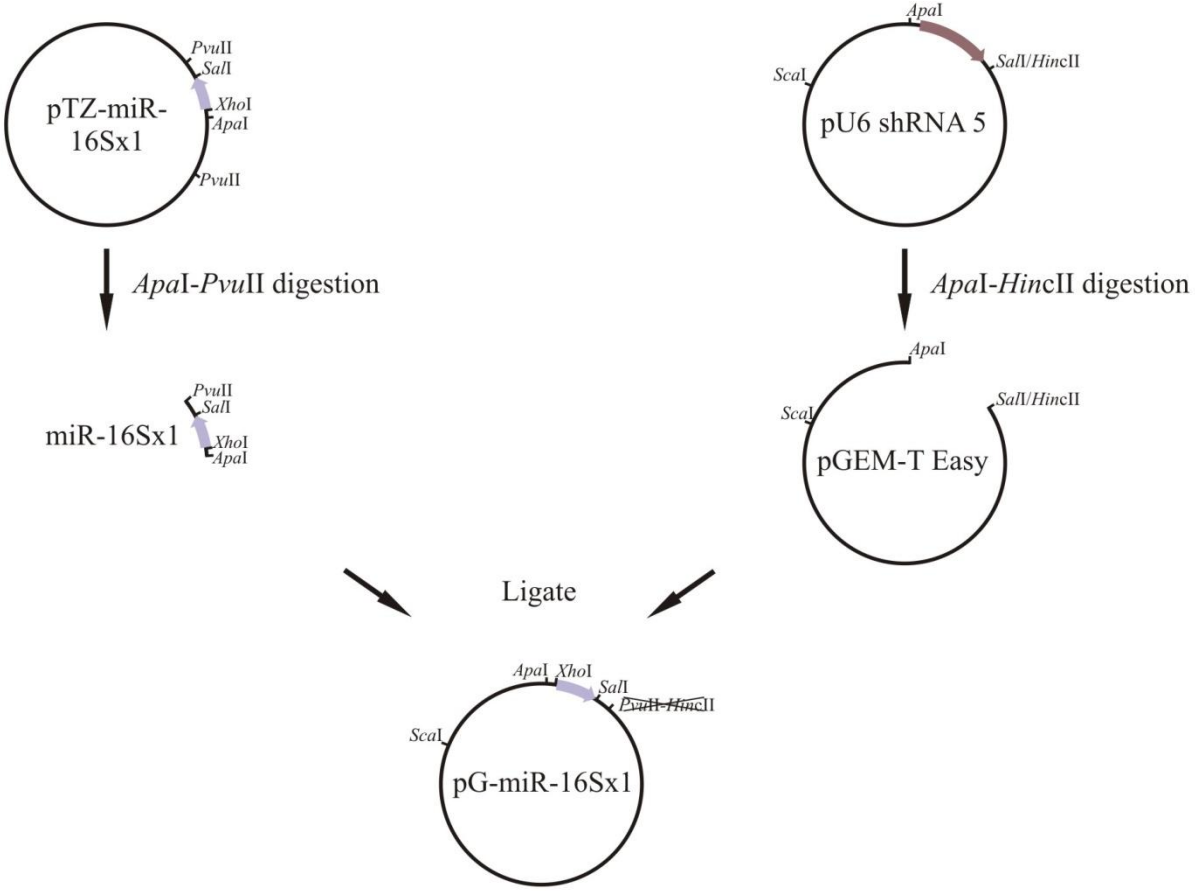

Figure 3.4: <u>Strategy for generation of the miR-16 sponge.</u>

The miR-16S sequence was initially inserted into the pGEM-T Easy vector to produce pG-miR-16S×1. The miR-16-1 sequence was excised from pTZ-miR-16S×1 by *Apa*I and *Pvu*II digestion and inserted into pG-U6 shRNA5 that had been digested with *Apa*I and *Hinc*II to create pG-miR-16S×1.

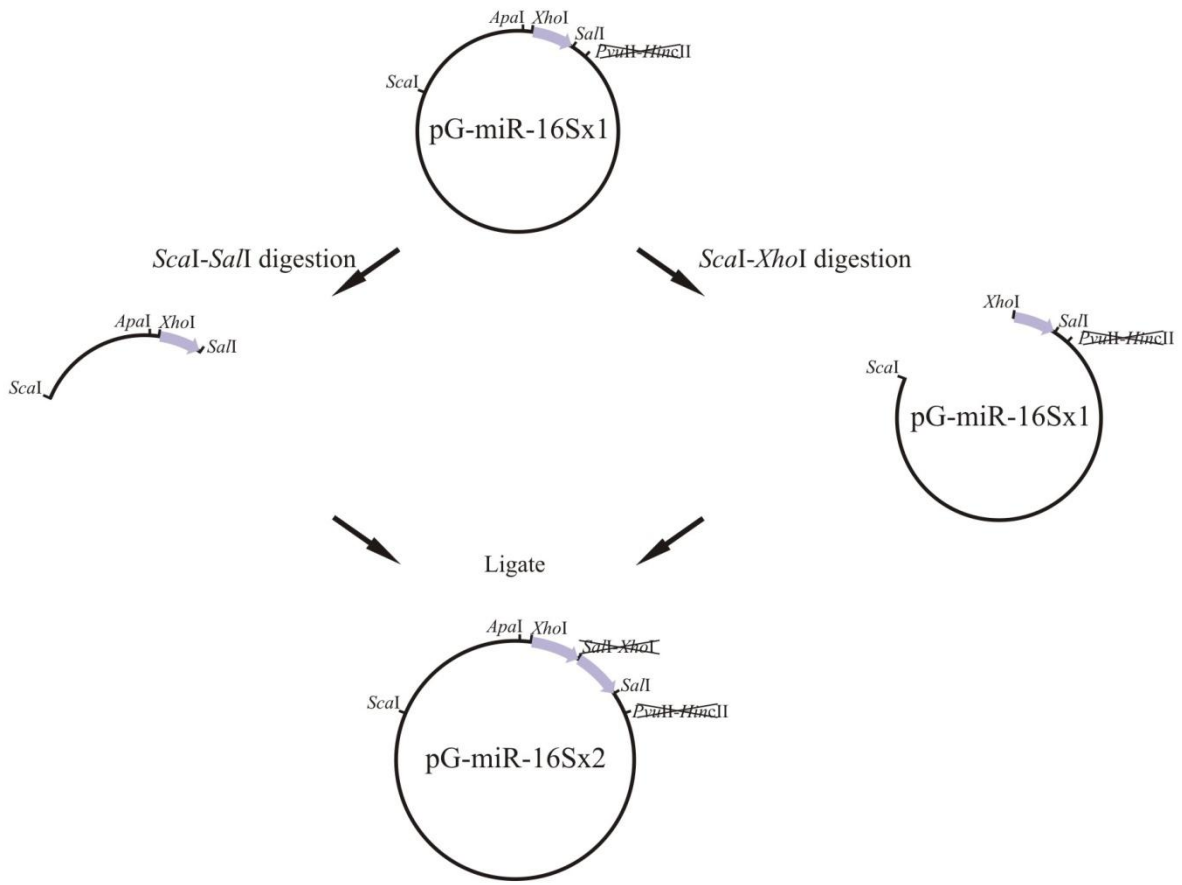

Figure 3. 5: <u>Strategy for concatemerisation of the miR-16 sponge sequences.</u>

To create a plasmid with two copies of the sponge sequence, pG-miR-16S×1 was separately digested with *Sca*I and *Sal*I or *Sca*I and *Xho*I. The two fragments from each restriction containing the sponge sequence were ligated together to form pG-miR-16S×2. Using similar procedures pG-miR-16S×3, pG-miR-16S×4 and ultimately pG-miR-16S×7 were constructed.

Generation of miR-16 sponge vector

To create the U6 promoter-driven miR-16 sponge vector the U6+27 sequence was firstly cloned into pTZ57R/T. The U6+27 cassette (Figure 3.6) contains the entire U6 promoter sequence including the first 27 nucleotides of the U6 snRNA, followed by two restriction sites (*Xho*I and *Xba*I), a strong stem sequence and a termination signal (98, 142, 143). The U6+27 sequence was generated in a two-step PCR of the human U6 promoter. The primer sequences U6 (*Xba*I) F (5'- GAT CTC TAG AAA GGT CGG GCA GGA AGA GGG -3'), U6+27 R1 (5'- CTC GAG TAG TAT ATG TGC TGC CGA AGC GAG CAC GGT GTT TCG TCC TTT CCA C -3') and U6+27 R2 (5'- GAT CAA AAA AGC GGA CCG AAG TCC GCT CTA GAC TCG AGT AGT ATA TGT GCT -3') were synthesised by standard phosphoramidite chemistry. The U6+27 sequence was cloned into pTZ57R/T to generate pTZ-U6+27. The miR-16S×7 sequence was restricted from pG-miR-16×7 with *Xho*I and *Sal*I and cloned into pTZ-U6+27 that had been linearised with *Xho*I to produce pU6-miR-16S×7.

miR-16 dual luciferase target vector

The miR-16 target vector (psi-miR-16T×7) was generated by cloning the miR-16S×7 sequence into the 3' UTR of the Renilla *luciferase* ORF of psiCHECK™-2. pG-miR-16S×7 was digested with *Xho*I and *Not*I and the miR-16S×7 fragment ligated into a psiCHECK™-2 fragment that had been linearised with *Xho*I and *Not*I.

In vitro *saturation assay*

To assess whether any of the pri-miR shuttle sequences cause saturation of the endogenous miRNA biogenesis pathway, Huh7 cells were co-transfected with RNAi effecter expression cassettes and the miR-16 target vector, psi-miR-16T×7. Huh7 cells were seeded at a density of 50% in 24-well tissue culture plates (Appendix A1-1). Cells

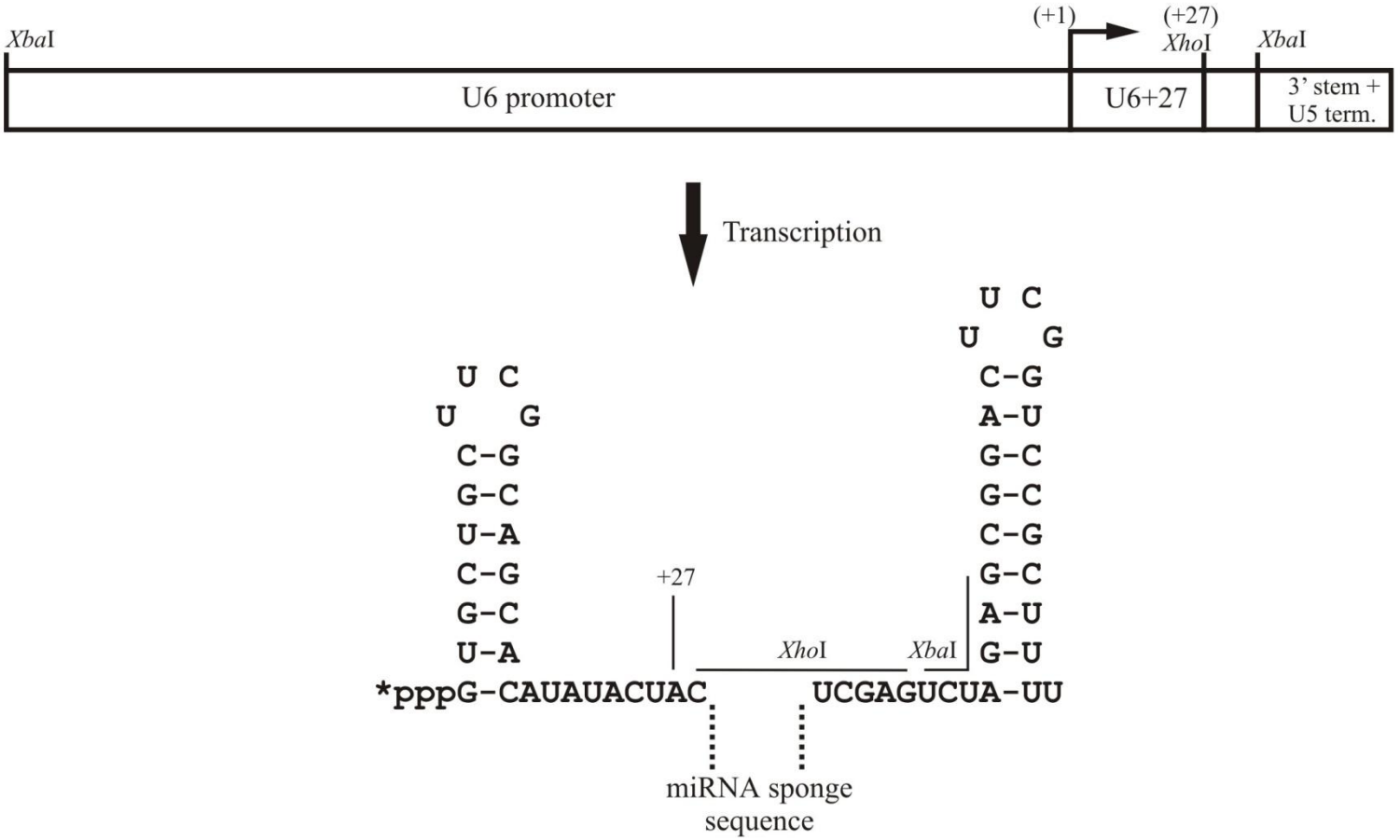

Figure 3.6: <u>Diagrammatic representation of the U6+27 expression cassette and transcript.</u>

The U6+27 expression cassette was designed to express 27 nt of U6 snRNA followed by a sequence encoding a strong 3' stem. *Xho*I and *Xba*I restriction sites were included between the two stem regions to facilitate insertion of miRNA sponge sequences.

were co-transfected with 80 ng of psi-miR-16T×7, 800 ng of RNAi effecter or sponge (pU6-miR-16S×7) vectors and 120 ng of pCI-neo eGFP using Lipofectamine™ 2000 (Appendix A1-1).

Luciferase Assay

Forty eight hours after transfection Firefly luciferase and *Renilla* luciferase activities in lysates of transfected cells were measured as described in Section 3.4.2.4. However, with these assays *Renilla* luciferase activity was normalised to Firefly luciferase activity.

3.2.7 Assessment of *in vivo* efficacy of pri-miR shuttle sequences

3.2.7.1 Delivery of HBV DNA and miR shuttle cassettes to mouse hepatocytes

The *in vivo* efficacy of the pri-miR shuttle sequences was assessed using the murine hydrodynamic model of HBV replication. Procedures for conducting animal experimentation were approved by the University of the Witwatersrand Animal Ethics Screening Committee (Appendix A3). Mice were co-injected with 5 µg of pCH-9/3091, 5 µg of control (pTZ57R) or anti HBV expression vector (pU6 shRNA 5, pCMV pri-miR-31/5 and pCMV pri-miR-122/5) and 5 µg of pCI-neo eGFP. Blood was collected from mice at 3 and 5 days post-injection. Mice were sacrificed on the fifth day after injection and livers harvested.

3.2.7.2 Quantitation of markers of HBV replication

Serum HBsAg levels and viral particle equivalents were determined as described in Section 2.2.7.1 and 2.2.7.2, respectively.

Southern blot analysis

Ten micrograms of total DNA was isolated from livers of mice and subjected to agarose gel electrophoresis. The DNA was transferred to a positively charged nylon membrane (Hybond-N+) by capillary transfer according to standard procedures (144). Following transfer the nylon membrane was crosslinked with 200 000 μJ/cm^2 of energy and baked at 80°C for an hour. To prepare probe the *HBx* sequence was initially amplified from pCH-9/3091 with the HBx forward (5'- GAT CAA GCT TTC GCC AAC TTA CAA GGC CTT T -3′) and HBx reverse (5′-GAT CTC TAG AAC AGT AGC TCC AAA TTC TTT A-3′) primer set. The PCR product was purified and used as template for random-primed labelling using the HexaLabel™ DNA Labeling Kit (Fermentas, MD, USA). The membrane with immobilised DNA was prehybridised at 65°C for at least 15 minutes followed by hybridisation with random-primed probes overnight. Post-hybridisation, the membrane was washed in a 5× SSC, 0.1% SDS solution at room temperature followed by 2 washes in a 1× SSC, 0.1% SDS solution at 65°C. X-ray film was exposed to the DNA blot for 24 hours at -70°C and developed.

3.3 RESULTS

3.3.1 Pri-miR shuttle sequences effectively reduce markers of viral replication *in vitro*

To assess ability of the shuttle sequences to silence viral replication *in vitro*, Huh7 cells were co-transfected with pri-miR-31 and pri-miR-122 expression vectors pCH-9/3091 or pCH-FLuc. pU6 shRNA 5 was included as a positive control for knockdown. With the exception of the pri-miR-122/10 shuttle sequences significant inhibition of HBsAg secretion was observed with all Pol III- and Pol II-driven pri-miR shuttles (Figure 3.7). Poor efficacy of the pri-miR-122/10 shuttles was not unexpected as its shRNA counterpart

(U6 shRNA 10) has been shown to be ineffective (Chapter 2). These data were corroborated using the reporter vector pCH-FLuc (Figure 3.8), which expresses Firefly luciferase as a measure of viral replication *in situ*. Firefly luciferase activity was significantly reduced in Huh7 cells co-transfected with the pri-miR shuttle expression vectors and pCH-FLuc. In accordance with assessment of HBsAg knockdown, the pri-miR-122/10 shuttle sequences silenced reporter gene expression poorly. These data therefore demonstrate that incorporating effective guide sequences within naturally occurring pri-miR-31 and pri-miR-122 structures allows for the generation of RNAi effecter sequences capable of potent gene silencing. However, embedding guide sequences within a miRNA context does not necessarily lead to an improvement in efficacy as demonstrated by the pri-miR-122/10 shuttle sequences. Importantly pri-miR shuttle sequences allow expression of these sequences from Pol II promoters.

3.3.2 Intended guide strands are processed from pri-miR shuttle sequences

Northern blot analysis was carried out on total RNA extracted from cells transfected with the pri-miR-31/5 and pri-miR-122/5 shuttle expression cassettes (Figure 3.9). Analysis revealed that the predicted 21-23 nt guide sequences that correspond to the U6 shRNA 5 guide were produced from the pri-miR shuttle sequences. Interestingly, the concentrations of the putative guides processed from the pri-miR shuttle sequences were at least 85-fold lower than that produced from their U6 shRNA counterparts. This was true for shuttle sequences expressed from both the U6 and CMV promoter. Though the concentrations of pri-miR shuttle derived guides were significantly lower than that of the U6 shRNA 5 guide strand, equivalent knockdown was achieved with the pri-miR shuttle

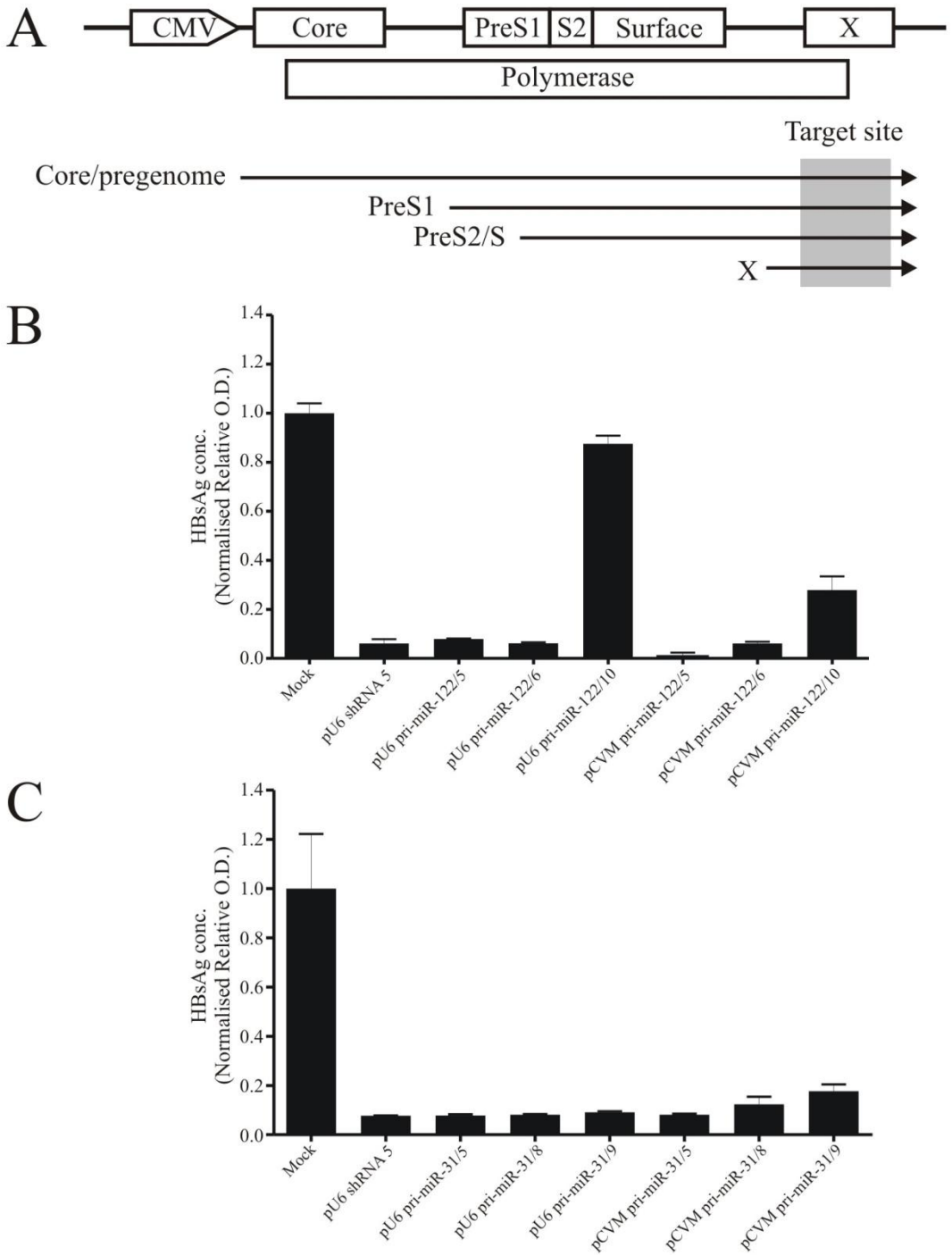

Figure 3.7: <u>Pri-miR shuttle vector mediated inhibition of HBsAg concentration.</u>

(A) Schematic representation of replication-competent plasmid, pCH-9/3091. HBsAg concentration was measured in culture supernatants of cells transfected with pCH-9/3091 and pri-miR-122 shuttle vectors (B) or pri-miR-31 shuttle vectors (C). Average HBsAg levels from three independent experiments are shown with SEM.

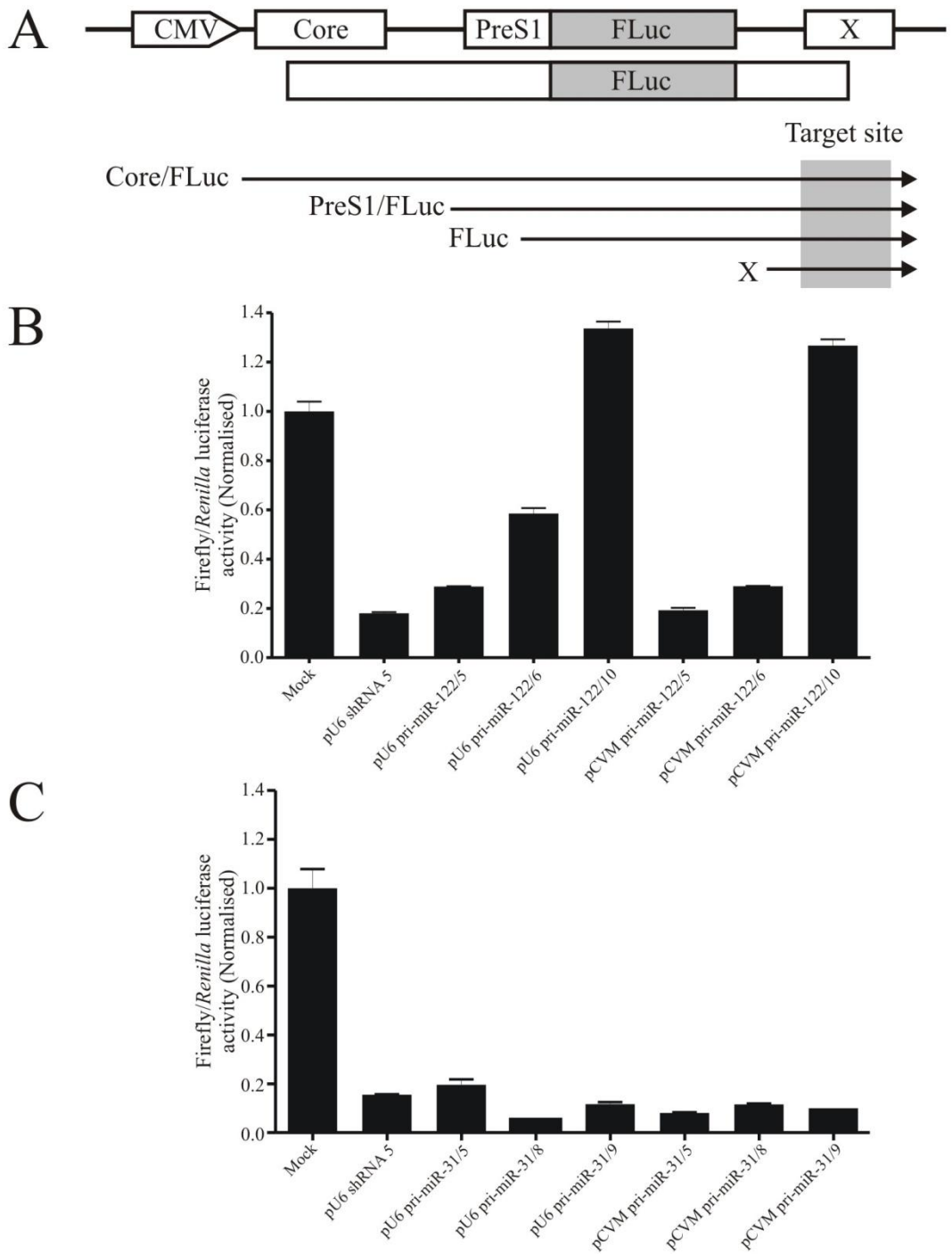

Figure 3.8: <u>Inhibition of viral replication *in situ*</u>.

(A) Diagrammatic representation of the Firefly luciferase reporter plasmid, pCH-FLuc. Firefly and *Renilla* luciferase activity was measured in the lysates of cells co-transfected pCH-FLuc, phRL-CMV and the pri-miR-122 shuttle vectors (B) or pri-miR-31 shuttle vectors (C). Ratio of Firefly to *Renilla* luciferase activity is shown with SEM.

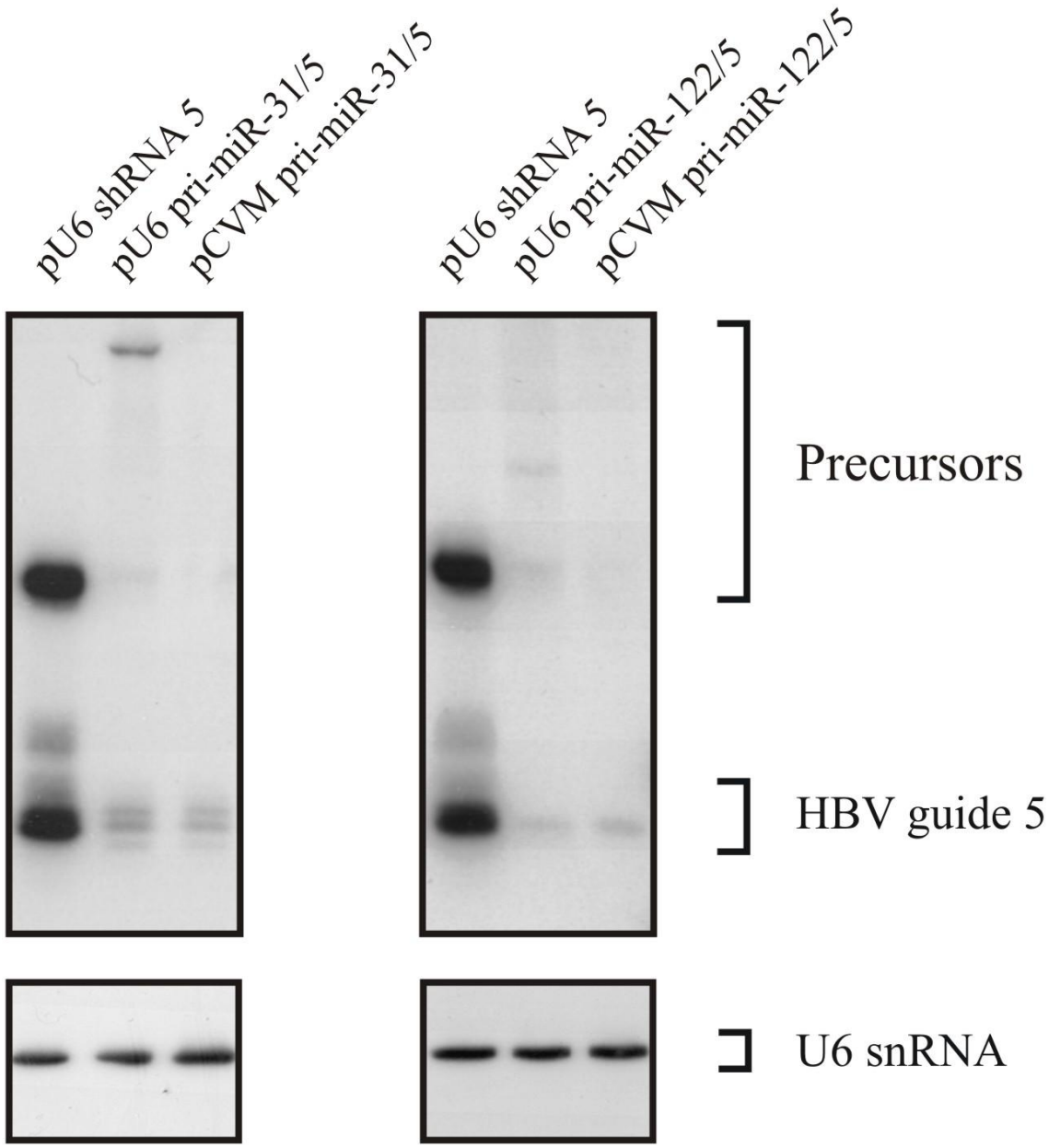

Figure 3.9: <u>Northern blot analysis of pri-miR shuttle sequences.</u>

Total RNA extracted from HEK293 cells transfected with the indicated plasmids was subjected to polyacrylamide electrophoresis, transferred to a positively charged membrane and immobilised. Membranes were probed for 5 guide sequence, stripped and reprobed for U6 snRNA to confirm equal loading and transfer of RNA.

sequence. This suggests that shRNA expression from the U6 promoter is in excess of the amount required to achieve effective silencing. Alternatively guide sequences derived from the pri-miR shuttles may enter the RNAi pathway more efficiently. Low guide sequence concentrations from the U6 pri-miR shuttle sequence also indicates that a mechanism other than promoter expression is responsible for the difference seen between the shuttle sequences and the shRNA sequences. One possibility is that Drosha processing of pri-miR shuttle transcripts limits the amount of substrate available for further processing thereby limiting amount of guide sequence produced.

3.3.3 Pri-miR shuttles do not stimulate IFN response or cause saturation of the RNAi pathway

3.3.3.1 Silencing is independent of immune stimulation

To rule out non-specific antiviral effects as a result of stimulation of the IFN response, qRT-PCR was used to determine *IFN-β* mRNA concentrations in cells transfected with the pri-miR shuttle cassettes. HEK293 cells transfected with the various anti HBV expression cassettes were not significantly elevated in *IFN-β* mRNA relative to *GAPDH* levels (Figure 3.10). In contrast, transfection of poly (I:C) resulted in strong induction of *IFN-β* mRNA levels. The pri-miR shuttle sequences therefore do not exhibit immunostimulatory behaviour.

3.3.3.2 Pri-miR shuttle sequences do not saturate the RNAi pathway

Lethality in mice as a result of overexpression of shRNA sequences from the U6 promoter has highlighted potential fatal consequences of saturating the endogenous

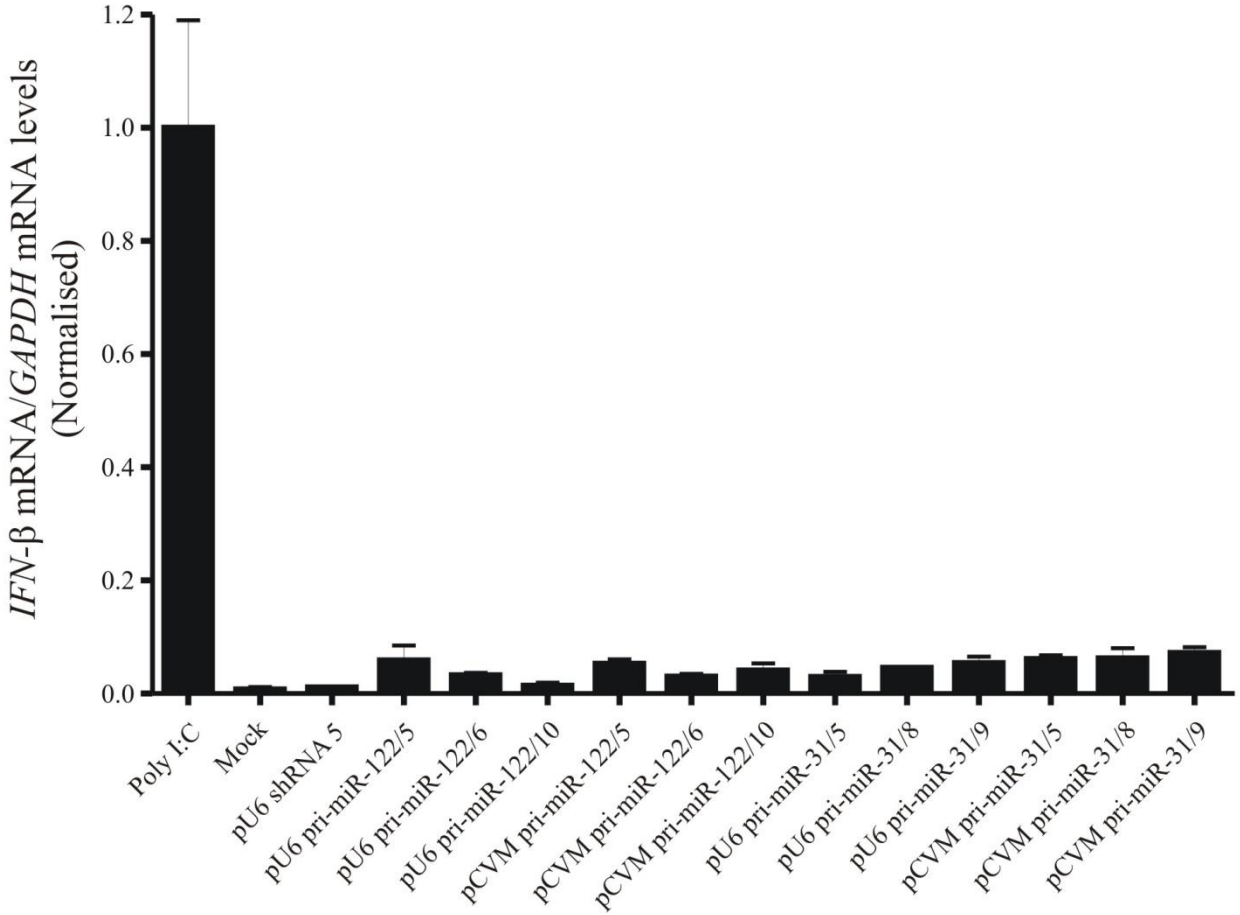

Figure 3.10: *IFN-β* stimulation by pri-miR shuttle sequences.

qRT-PCR analysis of *IFN-β* stimulation in HEK293 cells 48 hours after transfection with the indicated plasmids. Poly (I:C) was included as a positive control for stimulation of IFN-response genes. Average *IFN-β* mRNA levels from transfections performed in triplicate are shown normalised to *GAPDH* mRNA and with SEM.

miRNA pathway (118). Two assays were used to determine whether pri-miR shuttle sequences disrupt miRNA function. The first assay involved co-transfection of pCMV pri-miR-31/8 and its dual luciferase target vector (psiCHECK-8T) with the shRNA 5, pri-miR-31/5 or pri-miR-122/5 expression cassettes. Co-transfection of the pri-miR-31/5 or pri-miR-122/5 shuttle sequences did not interfere with pri-miR-31/8 silencing (Figure 3.11) indicating that these sequences do not interfere with independent pri-miR-mediated silencing. U6 shRNA 5 expression however significantly derepressed pri-miR-31/8 silencing, which is in accordance with the findings of Grimm et al. (118).

The second assay was designed to assess saturation of the endogenous miRNA pathway by detecting perturbations in miR-16 function (i.e. the ability of endogenous miR-16 to knock down an exogenous target sequence). A *Renilla* luciferase reporter containing 7 copies of an imperfect miR-16 target (psi-miR-16T×7) was constructed to measure miR-16 function. As a positive control for loss of miRNA function a miR-16 sponge (141), a stable RNA sequence containing 7 imperfect copies of the miR-16 target sequence, was also created. Co-transfection of the pri-miR shuttle expression cassettes with psi-miR-16T×7 revealed no significant disruption of miR-16 function (Figure 3.12). In contrast the sponge vector caused a significant loss of miR-16 silencing activity. Though the U6 promoter driven shRNA 5 sequence derepressed silencing of an exogenous miRNA (pri-miR-31/8) it did not affect the activity of an endogenous miRNA (miR-16). Together the data from the two saturation assays indicate that the pri-miR shuttle design represents an improvement in terms of safety over the traditional U6 shRNA expression cassettes.

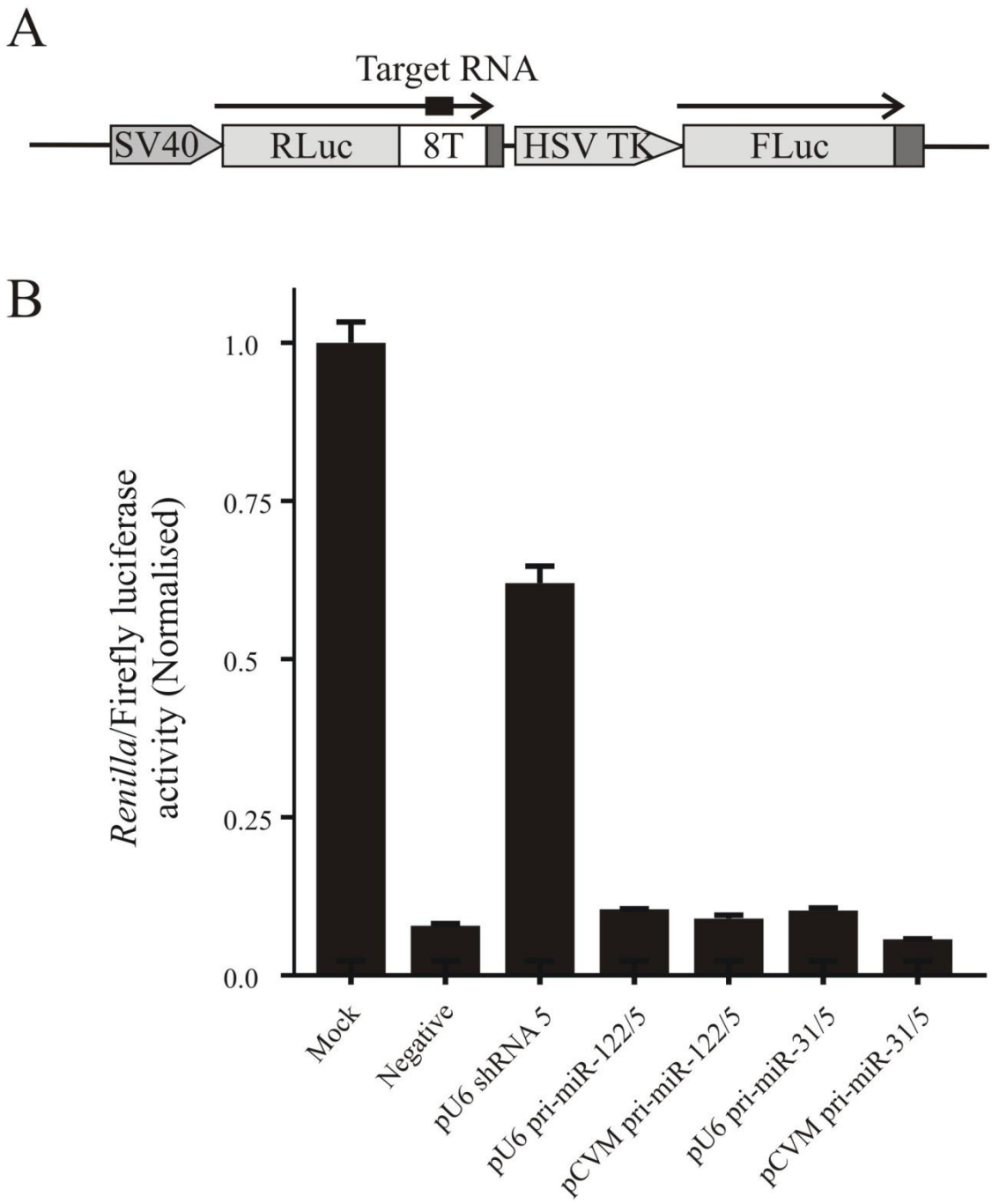

Figure 3.11: <u>Saturation of an exogenous pri-miR effecter sequence.</u>

(A) Schematic representation of pri-miR-31/8 target plasmid, psiCHECK-8T. (B) Huh7 cells were co-transfected with psiCHECK-8T, pCMV pri-miR-31/8 and the indicated expression vectors. *Renilla* to Firefly ratio was used to measure derepression of pri-miR-31/8 silencing.

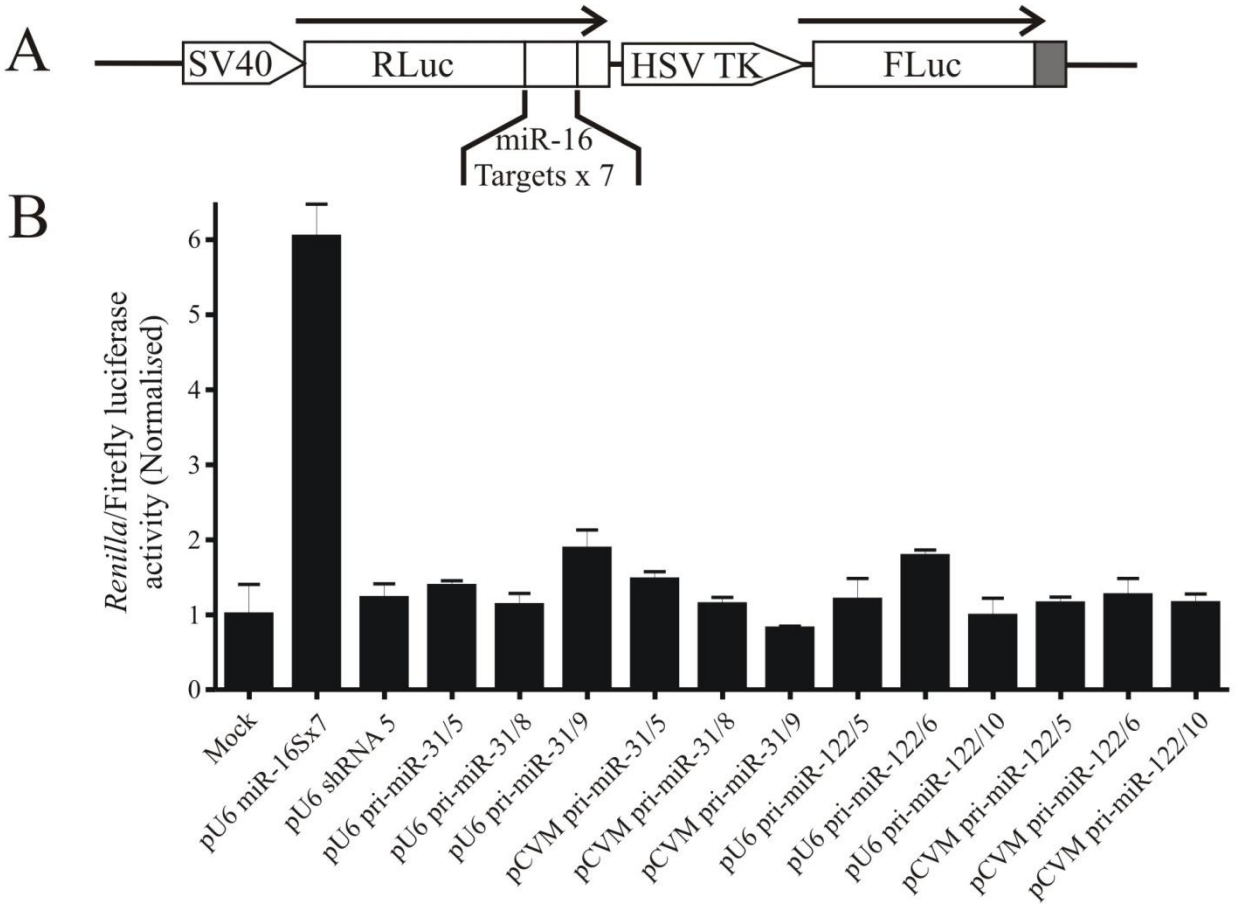

Figure 3.12: <u>Saturation of the endogenous miRNA biogenesis pathway.</u>

(A) Schematic representation of miR-16 target plasmid, psi- miR-16T×7. (B) Huh7 cells were co-transfected with the indicated plasmids and psi-miR-16T×7. The ratio of *Renilla* to Firefly luciferase activity is indicative of effect of the various constructs on miR-16 silencing.

3.3.4 Pri-miR shuttle sequences silence HBV *in vivo*

In vivo efficacy of the pri-miR shuttle sequences was assessed in the hydrodynamic injection model of HBV replication. Serum HBsAg concentrations, measured at day 3 and 5 post-injection, were significantly reduced in mice that received pCMV pri-miR-31/5 or pCMV pri-miR-122/5 as compared to mock-treated animals (Figure 3.13B). Though U6 shRNA 5 exhibited the greatest degree of knockdown, the pri-miR shuttle sequences nevertheless achieved impressive silencing. Quantitative real-time PCR analysis of circulating viral particle equivalents supported HBsAg data (Figure 3.13C) and indicated significantly reduced serum HBV DNA levels 3 and 5 days post-injection. Southern blot analysis of intrahepatic HBV DNA levels also revealed significant suppression (Figure 3.14). HBV DNA intermediates were only detectable in livers of mock-treated animals 5 days post-injection. Although RNAi-mediated silencing only occurs at the viral mRNA level, suppression of HBV replication was observed at multiple levels (i.e. viral protein synthesis, viral particle formation and viral DNA synthesis).

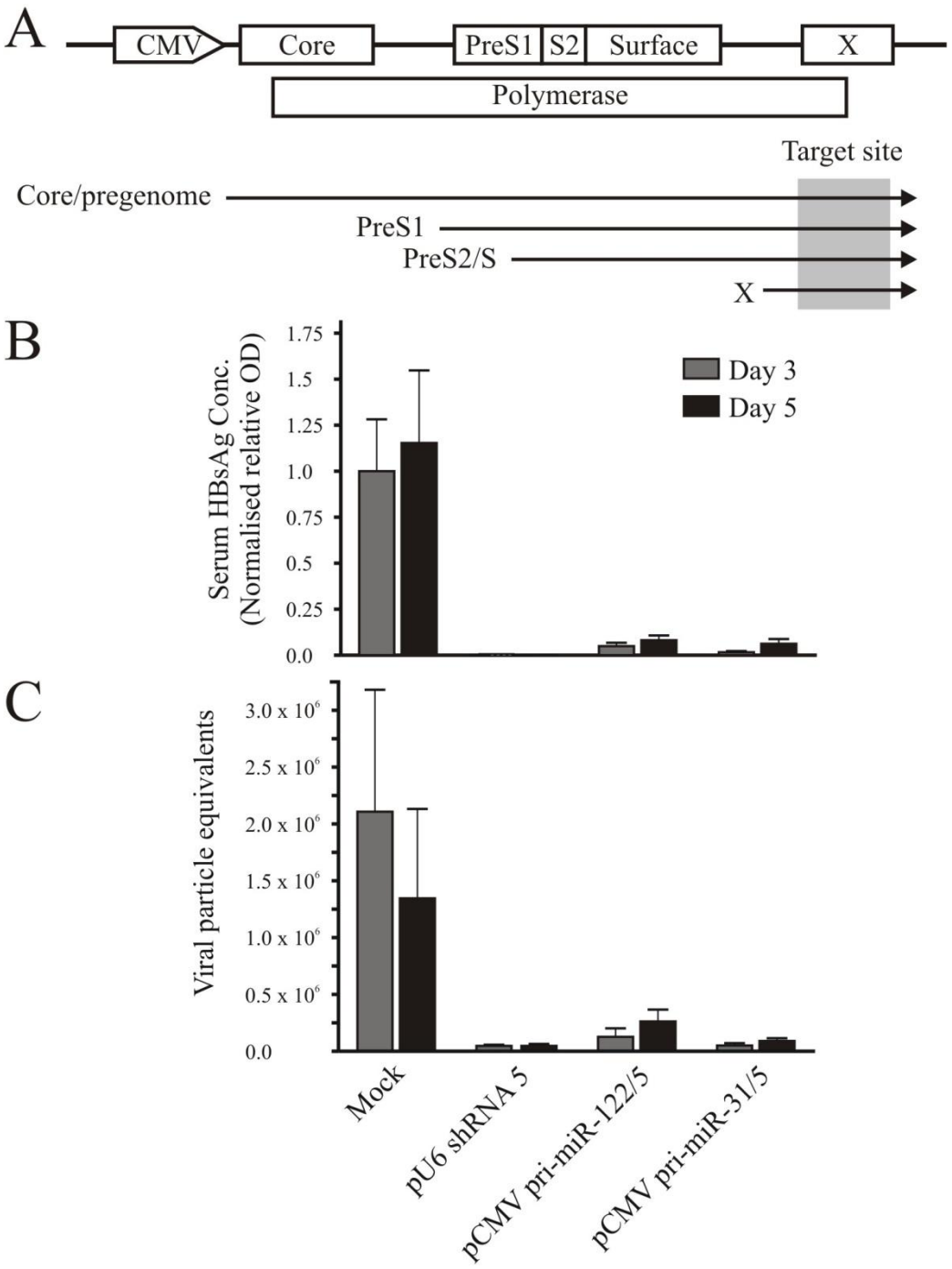

Figure 3.13: <u>Suppression of HBV replication *in vivo*.</u>

(A) Schematic representation of pCH-9/3091. Serum viral antigen (A) and DNA (B) determinations from mice 3 and 5 days after co-injection with pCH-9/3091 and the indicated plasmids. The columns indicate average readings taken from at least four mice and error bars show SEM.

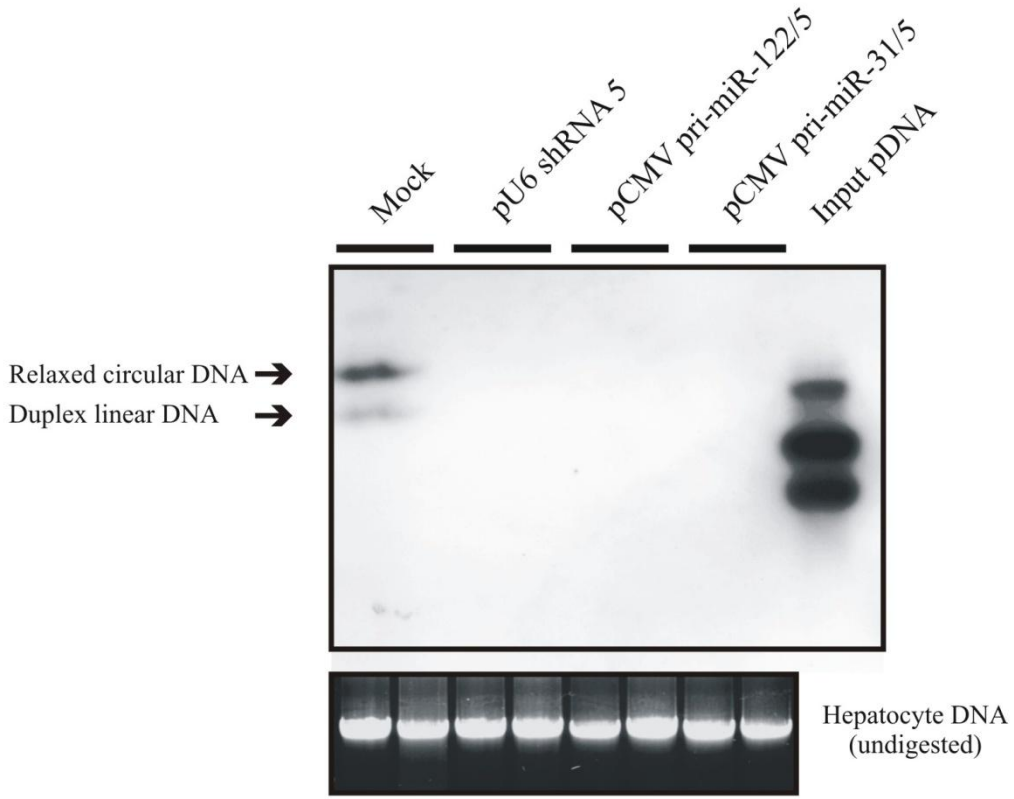

Figure 3.14: <u>Intrahepatic suppression of viral replicative intermediates.</u>

Total DNA extracted from livers of mice that were co-injected with pCH-9/3091 and the indicated vectors was subjected to Southern blot analysis to detect HBV DNA replicative intermediates.

3.4 DISCUSSION

Embedding anti HBV guide sequences within the structure of naturally occurring pri-miRNA allowed for the creation of pri-miR shuttle sequences that target HBV. The guide sequences of pri-miR-31 and pri-miR-122 were replaced with the guide sequence of previously described U6 shRNA (Chapter 2). The antiguide sequences of the pri-miR shuttle were redesigned to maintain wild-type secondary structure. To determine whether a functional RNAi effecter sequence was created by replacing the guide of an endogenous pri-miRNA with an anti HBV guide, transcription of these pri-miR shuttles was initially driven from the U6 promoter. The data demonstrated that Pol III-driven anti HBV pri-miR shuttle sequences are capable of efficiently knocking down viral replication. Similar results have been reported with U6-driven pre-miR-30 shuttle sequences (145, 146). Since the pri-miR shuttle sequences mimic naturally occurring pri-miRNA, which are normally expressed as RNA Pol II transcripts, the efficacy of Pol II promoter-driven anti HBV pri-miR shuttles were assessed. An RNAi expression cassette that enables expression from a Pol II promoter improves on current systems by allowing tissue-specific, inducible and multimeric effecter expression. Both pri-miR-31 and pri-miR-122 shuttle sequences incorporated within the exonic sequence of a CMV-derived transcript were capable of effective knockdown of markers of HBV replication. Furthermore the degree of knockdown achieved by the Pol III and Pol II pri-miR shuttle sequences was equivalent to that of the highly effective U6 shRNA 5 sequence. Northern blot analysis revealed that the pri-miR shuttles were processed to form 21-23 nt guide sequences. This clearly indicates that processing of the pri-miR shuttle sequences by the miRNA biogenesis pathway proceeded as intended and therefore that incorporation of an anti HBV guide within a miRNA backbone creates an effective RNAi activator. Interestingly, concentrations of guide sequences derived from U6 pri-miR shuttle cassettes were significantly lower than

their U6 shRNA derived counterparts. This correlates well with data from other groups employing pre-miR-30 shuttle expression cassettes (145, 146). Despite lower concentrations of guide sequence derived from the pri-miR shuttles silencing, efficacy was equivalent to the U6 shRNA, indicating that the guide sequences are especially potent. This has proved to be of particular importance as low levels of RNAi effecter sequences are crucial to avoid toxic side-effects that arise from saturation of the endogenous miRNA pathway. This represents a significant step in improving the safety of expressed RNAi effecters for eventual clinical application.

Since the discovery of RNAi, significant progress has been made in the development of synthetic and expressed effecters as potential therapeutic sequences. Numerous studies have exploited and subsequently demonstrated efficacy of Pol III-driven shRNA expression cassettes to limit HBV gene expression *in vitro* and *in vivo*. However, to advance expressed RNAi activators as an antiviral therapeutic option, further improvements in safety (through dose regulation) and efficacy (limiting viral escape) of current systems is necessary. Demonstration that pri-miR shuttle sequences expressed from Pol II are capable of effecting potent gene silencing lays the foundation for further development of these RNAi effecters. Pri-miR shuttle sequences permit the creation of regulatable RNAi expression cassettes as Pol II promoters are amenable to tissue-specific and inducible expression. Expression cassettes such as these may potentially limit off-target effects from constitutive expression but more importantly also avoid saturation of the RNAi pathway. Transcripts derived from Pol II transcription are typically large RNA molecules which makes it possible to incorporate multiple RNAi effecters within a single expression cassette. Indeed numerous endogenous miRNA are polycistronic and are expressed as a cluster on a single Pol II transcript. Employing a multi-targeted approach,

such as polycistronic pri-miR shuttles, to silence viral replication may prevent the emergence of viral escape mutants. In the following Chapter the feasibility of pri-miR-122 shuttles expressed from liver-specific promoters and multimeric pri-miR-31 shuttles is explored.

4 LIVER-SPECIFIC AND MULTI-TARGETING PRI-MIR SHUTTLE EXPRESSION VECTORS

4.1 INTRODUCTION

The preceding Chapter demonstrated that embedding anti HBV guides into the backbone of hsa-pri-miR-31 or hsa-pri-miR-122 yielded RNAi effecters capable of efficient knockdown of markers of HBV replication *in vitro* and *in vivo*. Furthermore silencing efficacy was not compromised when the pri-miR shuttle sequences were transcribed from a Pol II promoter (specifically the CMV promoter). Demonstration that silencing efficacy is not compromised when a pri-miR shuttle sequence is expressed from a CMV promoter indicates that effective pri-miR shuttle sequences may be expressed from other Pol II promoters. From the data presented in Chapter 2 it can be inferred that pri-miR shuttle sequences are effectively processed within the context of a Pol II transcript. Sequences occurring upstream or downstream of the pri-miR shuttle sequence therefore do not interfere with processing of the shuttle sequences. It may be possible to include multiple shuttle sequences within a single pri-miR shuttle cassette, which will not interfere with processing of each individual effecter sequence. The aims of the next set of exercises were to generate pri-miR-122 shuttles under the transcriptional control of liver-specific promoters and multimeric pri-miR-31 shuttle expression cassettes.

The liver-specific and liver-abundant expression of miR-122 is well characterised (136) and expressing anti HBV pri-miR-122 shuttle sequences could potentially benefit from tissue-specific properties of natural pri-miR-122 processing. The human Factor VIII (FVIII) and alpha-1-antitrypsin (A1AT) promoters and HBV preS2 and core promoters

were chosen as potential candidates to drive liver-specific expression of the pri-miR-122 shuttle sequences. Typically liver-specific expression is conferred on promoters by sequence elements within promoters and enhancers that are recognised by transcription factors enriched in the liver. The promoter of FVIII for example contains sequence elements necessary for recognition by the liver-enriched transcription factor hepatocyte nuclear factor 1 (HNF-1) (147), which confers liver-specificity to the promoter. Similarly liver-specific expression of the A1AT promoter is regulated by the liver transcription factors HNF-1α and HNF-4 (148). FVIII is a glycoprotein cofactor with an important role in the coagulation process and mutation or deficiencies in FVIII leads to haemophilia A (reviewed in (149)). Evidence that the liver is a major site of FVIII production came in the form of clinical data which demonstrated that liver transplantation reverses heamohpilia (147, 150). A1AT belongs to a family of serine proteinase inhibitors (serpins) that are involved in diverse biological roles. The liver is the major site for the synthesis of this serpin and expression of A1AT is driven from a liver-specific promoter (reviewed in (148)). Although extra-hepatic expression of A1AT does take place, transcription in other tissues occurs from alternate promoters. The liver-specificity of the FVIII and A1AT promoters is well-characterised making these ideal candidates for assessing the tissue-specific expression of pri-miR shuttle sequences. Similarly employing HBV regulatory elements for the liver-specific expression of pri-miR shuttle sequences is an appealing alternative as the liver tropism exhibited by HBV is conferred in part by its transcription regulatory elements (reviewed in (12)).

The pri-miR-31 shuttle sequences were further developed as a multi-targeted approach to silence HBV replication. The initial description of miRNA indicated that approximately 50% of these sequences occur in close proximity to each other and raised

the possibility that these are transcribed as polycistronic units (48). Elucidation of the miRNA biogenesis pathway soon revealed that miRNA clusters are indeed expressed from a single transcriptional unit. The fact that miRNA are naturally expressed as Pol II transcripts (49) allows for polycistronic units which of necessity need to be large transcriptional products. The monocistronic miR-31 was chosen as the basis for creating a multimeric pri-miR shuttle expression cassette. This facilitates a more convenient modular approach to creating multimeric expression cassettes than to embed guide sequences within an existing miRNA cluster.

In this Chapter cassettes capable of the tissue-specific expression of a transgene were initially created as a precursor to the development of liver-specific pri-miR-122 shuttle expression cassettes. Additionally the development of a modular, multimeric pri-miR shuttle expression cassette is described. The efficacy and safety of these cassettes was initially assessed *in vitro* followed by demonstration of silencing efficacy *in vivo*.

4.2 MATERIALS AND METHODS

4.2.1 Liver-specific promoter driven pri-miR-122 shuttle cassettes

4.2.1.1 Generation of liver-specific expression vectors

The human A1AT (Genbank accession number D38257.1) and FVIII (Genbank accession number NT167198.1) promoter sequences were amplified from total human genomic DNA extracted from Huh7 cells using the primer sets in Table 4.1. The viral Basic Core Promoter (BCP) and PreS2 promoter sequences (Genbank accession number J02203.1) were amplified from the plasmid pCH-9/3091 using the primer sets in Table 4.1.

All oligonucleotides were synthesised by standard phosphoramidite chemistry (Inqaba Biotech, South Africa). Primers were designed such that amplification introduced a *Bgl*II (*Bcl*I in the case of A1AT) site at the 5' end and a *Hind*III site at the 3' end of the amplicons. Promoter sequences were amplified using the Expand High Fidelity PCR^{PLUS} System (Roche Diagnostics GmbH, Germany) according to manufacturer's instructions. Purified amplicons were ligated to the linearised PCR cloning vector pTZ57R/T. Plasmids were subjected to restriction enzyme digestion and clones yielding desired results were sequenced (Inqaba Biotech, South Africa).

Next, the CMV immediate early enhancer promoter sequence within pCI-neo was substituted with the sequences of the liver-specific promoters (Figure 4.1). The new expression vectors created would therefore initiate transcription from the liver-specific promoters instead of the ubiquitous CMV promoter. The sequences encoding the FVIII, BCP and PreS2 promoters were removed from their respective plasmids (pTZ-FVIII, pTZ-BCP and pTZ-PreS2) with *Bgl*II and *Hind*III restriction. *Bcl*I is sensitive to methylation, therefore pTZ-A1AT was propagated through the *dcm*- and *dam*-methylase deficient strain of *E.coli*, GM2929. The sequence encoding the A1AT promoter was then restricted from pTZ-A1AT with *Bcl*I and *Hind*III. pCI-neo was digested with *Hind*III and *Eco*RI to yield 3815 bp, 1317 bp and 340 bp fragments. Secondly, pCI-neo was digested with *Eco*RI and *Bgl*II to yield 4371 and 1101 bp fragments. The liver-specific promoter sequences were ligated with the 340 bp *Hind*III-*Eco*RI and the 4371 bp *Eco*RI-*Bgl*II fragments to generate the new liver-specific expression vectors (pCI-A1AT, pCI-FVIII, pCI-BCP and pCI-PreS2). *Bcl*I and *Bgl*II generated complementary overhangs thus allowing the A1AT promoter sequence to be ligated to the pCI-neo backbone.

Table 4. 1: Oligonucleotide sequences for amplification of liver-specific promoters

Primer	Oligonucleotide sequence
A1AT F	5′- GATC**TGATCA**TTCCCTGGTCTGAATGTGTG -3′
A1AT R	5′- GATC**AAGCTT**ACTGTCCCAGGTCAGTGGTG -3′
FVIII F	5′- GATC**AGATCT**GAGCTCACCATGGCTACATT -3′
FVIII R	5′- GATC**AAGCTT**GACTTATTGCTACAAATGTTCAAC -3′
BCP F	5′- GATC**AGATCT**GCATGGAGACCACCGTGAAC -3′
BCP R	5′- GATC**AAGCTT**CACCCAAGGCACAGCTTGGA -3′
PreS2 F	5′- GATC**AGATCT**GCCTTCAGAGCAAACACCGC -3′
PreS2 R	5′- GATC**AAGCTT**ACAGGCCTCTCACTCTGGGA -3′

Restriction sites are indicated in bold (*Bgl*II or *Bcl*I in the forward primer and *Hind*III in the reverse primers).

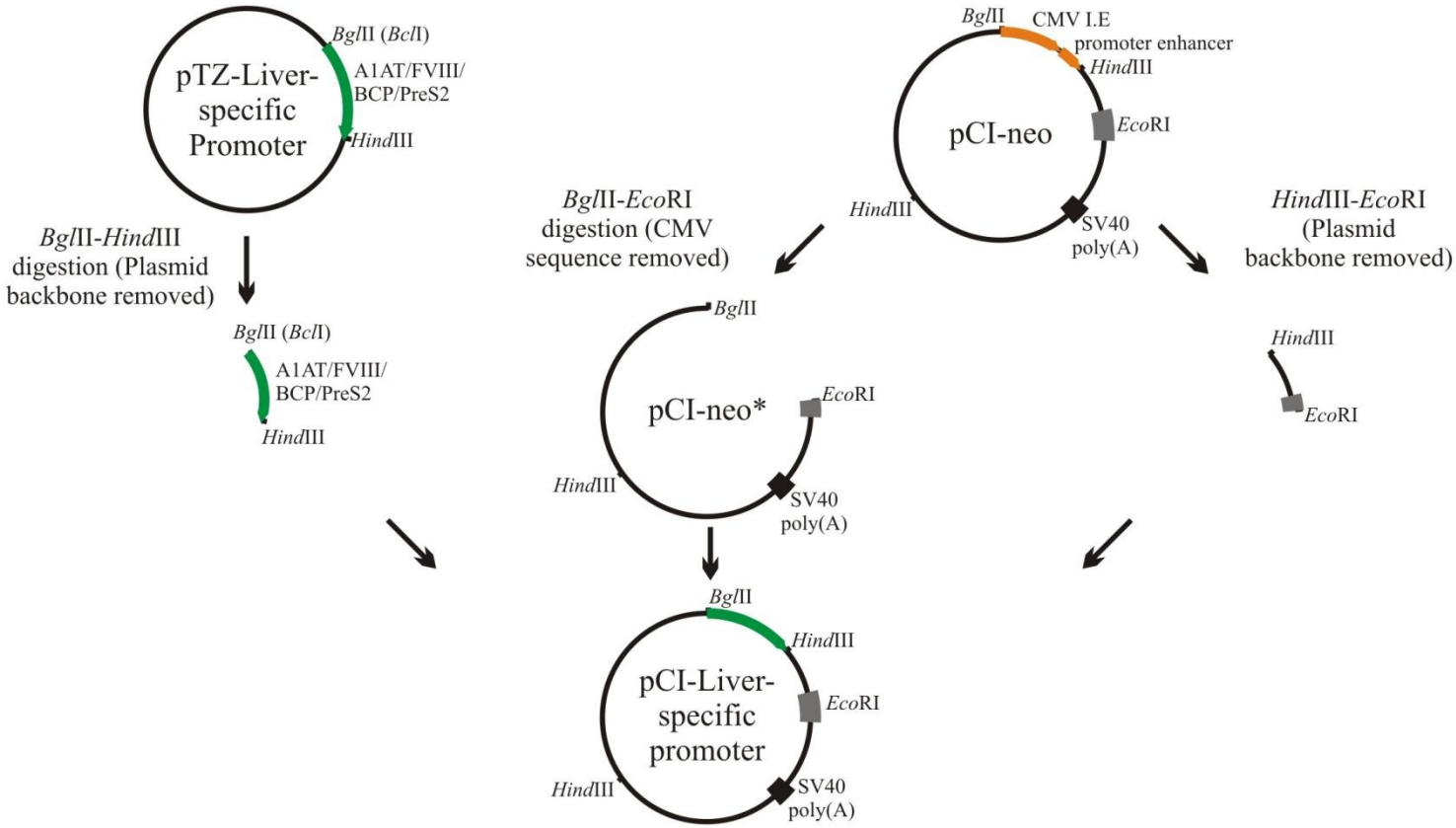

Figure 4.1: <u>Schematic representation of cloning strategy for the generation of liver-specific expression vectors.</u>

The liver-specific promoter (A1AT, FVIII, BCP and PreS2) sequences that had been inserted into the pTZ57R/T vector and indicated as green arrows were excised with *Hind*III and *Bgl*II (or *Bcl*I in the case of the A1AT promoter). pCI-neo was digested with *Hind*III and *Eco*RI or *Bgl*II and *Eco*RI. The relevant fragments were combined in a 3-way ligation to generate the liver-specific expression vectors containing the A1AT, FVIII, BCP or PreS2 promoter sequences.

4.2.1.2 Assessing functionality and tissue-specificity of the chosen promoter sequences

To assess the functionality and tissue-specificity of the designed expression vectors the sequence encoding Firefly luciferase was cloned downstream of the promoter sequences. First pCI-neo FLuc was generated by *Xho*I and *Spe*I restriction digestion of pTZ-FLuc and cloning the resultant DNA fragment into pCI-neo that had been digested with *Xho*I and *Xba*I. The Firefly luciferase sequence was then removed from pCI-neo FLuc with *Nhe*I and *Sma*I and ligated into the equivalent sites of the liver-specific expression vectors to generate pCI-A1AT FLuc, pCI-FVIII FLuc, pCI-BCP FLuc and pCI-PreS2 FLuc.

Transfections

To assess functionality of liver-specific expression cassettes Huh7 cells and the HEK293-derived 116 cells were transfected with 100 ng of the different Firefly luciferase expression vectors, 100 ng of phRL-CMV and 100 ng of pCI-neo eGFP using Lipofectamine™ 2000 (Invitrogen, CA, USA) according to manufacturer's instructions (Appendix A1-1). Firefly luciferase and *Renilla* luciferase activities were measured as described in Section 3.4.2.4. Firefly luciferase activity was normalised to *Renilla* luciferase activity. Detection of Firefly luciferase activity exclusive to the liver-derived cell line was taken as liver-specific expression.

4.2.1.3 Generation of liver-specific pri-miR shuttle vectors

To generate liver-specific shuttle cassettes the pri-miR-122 sequences were excised from pCMV pri-miR-122/5, pCMV pri-miR-122/6 and pCMV pri-miR-122/10 with *Nhe*I and *Sma*I and ligated to the equivalent sites of pCI-A1AT and pCI-BCP.

4.2.1.4 Assessing silencing of markers of HBV replication by liver-specific pri-miR shuttles in vitro

To assess the tissue-specificity of the liver-specific pri-miR shuttles Huh7 and 116 cells were transfected with 80 ng of target plasmid (pCH-FLuc), 800 ng of the A1AT- and BCP-driven pri-miR shuttle vectors, 50 ng of phRL-CMV and 50 ng of pCI-neo eGFP. Plasmid DNA was made up to a total of 1 µg with pCI-neo and transfected with Lipofectamine™ 2000 (Appendix A1-1). Forty eight hours post-transfection cells were lysed and measurement of Firefly and *Renilla* luciferase activities was carried out as described in Section 3.4.2.4.

4.2.1.5 Assessing saturation of the endogenous miRNA pathway

To assess disruption of the miRNA biogenesis pathway by the A1AT and BCP pri-miR-122 shuttle expression cassettes the miR-16 saturation assay was carried out as described in Section 3.2.6.2.

4.2.1.6 In vivo *efficacy of liver-specific pri-miR shuttle sequences*

In vivo efficacy of the A1AT- and BCP-driven pri-miR-122 shuttle expression cassettes was further assessed in the hydrodynamic injection model of HBV replication. Five micrograms of anti HBV expression cassettes were co-injected with 5 µg each of pCH-9/3091 and pCI-neo eGFP. Blood samples were collected from mice 3 and 5 days post-injection and HBsAg concentrations determined by ELISA (Section 2.2.7.1).

4.2.2 Polycistronic pri-miR shuttles

4.2.2.1 Generation of polycistronic pri-miR-31 expression cassettes

Polycistronic pri-miR-31 shuttle cassettes were generated by inserting combinations of pri-miR-31/5, pri-miR-31/8 and pri-miR-31/9 sequences downstream of the CMV immediate early promoter enhancer. A total of 6 trimeric cassettes were generated (pri-miR-31/5/8/9, pri-miR-5/9/8, pri-miR-8/5/9, pri-miR-8/9/5, pri-miR-9/5/8 and pri-miR-9/8/5). To generate the pri-miR-31/5/8/9 cassette, the pri-miR-31/8 sequence was excised from pTZ pri-miR-31/8 with *Nhe*I and *Eco*RI and ligated into pTZ pri-miR-31/5 that had been digested with *Spe*I and *Eco*RI to create pTZ pri-miR-31/5/8. Subsequently, the sequence encoding pri-miR-31/9 was excised from pTZ pri-miR-31/9 with *Nhe*I and *Eco*RI and ligated into pTZ pri-miR-31/5/8 that had been digested with *Spe*I and *Eco*RI. Successful ligation generated pTZ pri-miR-31/5/8/9. The remaining 5 trimeric cassettes were constructed using a similar cloning strategy. The trimeric pri-miR-31 cassettes were excised with *Nhe*I and *Xba*I and cloned into equivalent sites of pCI-neo to produce the CMV pri-miR-31 expression vectors. All plasmids were sequenced by automated sequencing (Inqaba Biotech, South Africa).

4.2.2.2 Individual pri-miR target vectors

To assess the ability of the polycistronic pri-miR shuttles to create multiple effecter sequences, luciferase reporter vectors with individual target sites downstream of the Renilla *luciferase* ORF were made. Dual luciferase reporter plasmids containing a site targeted by pri-miR-31/5 or pri-miR-31/9 were generated as described in Section 3.2.5.2. Primers were designed to amplify HBV co-ordinates 1575-1599 (5T) (5T F 5’- CCG TGT GCA CTT CGC TTC AC -3’ and 5T R 5’- ACT AGT CAG AGG TGA AGC GA -3’) and co-ordinates 1774-1798 (9T) (9T F 5’- TAG GAG GCT GTA GGC ATA AA -3’ and 9T R

5'- TAG GAG GCT GTA GGC ATA AA -3') and introduced a *Spe*I site at the 3' end of the target sequences. The target sequences were amplified by PCR, purified then ligated to the pTZ57R/T PCR cloning vector. Subsequently, the target sequences were excised with *Sal*I and *Spe*I and ligated to the *Xho*I and *Spe*I sites of psiCHECK2.2 to create psiCHECK-5T and psiCHECK-9T.

4.2.2.3 Knockdown of individual luciferase target sequences

Huh7 cells were co-transfected with 80 ng of each individual target vector (psiCHECK-5T, psiCHECK-8T and psiCHECK-9T), 800 ng each polycistronic pri-miR-31 shuttle vector and 100 ng of pCI-neo eGFP. Plasmid DNA was made up to a total of 1 µg with pCI-neo and transfected using Lipofectamine™ 2000 (Appendix A1-1). Firefly and *Renilla* luciferase activities were determined as described in Section 3.4.2.4.

4.2.2.4 Processing of polycistronic pri-miR shuttles

HEK293 cells seeded in 10 cm^2 culture dishes were transfected with 16 µg of U6 shRNA or pri-miR-31 shuttle vectors, 3 µg of pCH-9/3091 and 1 µg of pCI-neo. Transfections proceeded as described (Appendix A1-1) using Lipofectamine™ 2000. Total RNA was isolated from cells 48 hours post-transfection using Tri-Reagent (Sigma, MO, USA). Northern blot analysis of extracted RNA was carried out as described in Section 3.2.5.2.

4.2.2.5 Assessing efficacy of polycistronic pri-miR shuttles in cultured cells

Transfections

Huh7 cells were maintained in DMEM containing 10% FCS and antibiotics as described in Appendix A1-1. To assess knockdown of HBsAg secretion 80 ng of pCH-9/3091 was diluted with 800 ng of RNAi effecter plasmid and 120 ng of pCI-neo eGFP and

transfected using Lipofectamine™ 2000 (Appendix A1-1). The effect of polycistronic pri-miR shuttles on *in situ* viral marker expression was determined by co-transfection of 80 ng of pCH-FLuc with 800 ng of RNAi effecter plasmid, 80 ng of phRL-CMV and 40 ng of pCI-neo eGFP. HBsAg secretion into culture medium from cells transfected with pCH-9/3091 was assessed 48 hours post-transfection as described in Section 2.2.7.1 using the MONOLISA® HBs Ag ULTRA Assay kit. Firefly and *Renilla* luciferase activity in lysates prepared from cells transfected with pCH-FLuc were determined as described previously (Section 3.4.2.4). Firefly luciferase activity was normalised to *Renilla* luciferase activity.

4.2.2.6 Assessing efficacy of polycistronic shuttles against a mutant HBx

Target vectors

Generation of the luciferase reporter plasmid psiCHECK-*HBx*, which has the complete *HBx* sequence inserted downstream of the Renilla *luciferase* ORF of psiCHECK™-2, has been described before (127) [11]. A mutant luciferase reporter (psiCHECK-m*HBx*) was derived from the parental psiCHECK-*HBx* vector by PCR. Nucleotides 1882 to 2214 of psiCHECK-*HBx* were amplified with the m*HBx* F (5'- GAT CCG GTC CGT CTG CAG TTC GGT TGT CCT CTG CAC GTT GCA TGG AG -3') and R (5'- GAT CGC GGC CGC CCG GGT CGA CTC -3') primer set. The forward and reverse primers were designed to include the *Rsr*II and *Not*I restriction sites of psiCHECK-*HBx*, respectively. Amplification introduced five point mutations (underlined in m*HBx* F above) within the region targeted by anti HBV guide 5. The m*HBx* amplicon was inserted into the PCR cloning vector pTZ57R/T to produce pTZ-m*HBx*. After sequence verification, the m*HBx* sequence was excised from pTZ-m*HBx* with *Rsr*II and *Not*I and inserted into equivalent sites of psiCHECK-*HBx* to generate psiCHECK-m*HBx*.

[11] Weinberg at al. (Appendix A2)

Substitution of nucleotides 1890 and 1895 converted the *Apa*LI restriction site in the wild-type sequence to a *Pst*I restriction site in the mutant sequence. Restriction of potential psiCHECK-m*HBx* clones with *Pst*I was used to verify insertion of the mutant sequence.

Transfection of cultured mammalian cells

Huh7 cells were maintained in DMEM containing 10% FCS and antibiotics (Appendix A1-1). Knockdown of wild-type and mutant *HBx* sequences was assessed by co-transfecting 800 ng of polycistronic pri-miR-31 plasmids and 40 ng of pCI-neo eGFP with 80 ng of psiCHECK-*HBx* or psiCHECK-m*HBx*. Transfections were carried out with Lipofectamine™ 2000 (Appendix A1-1). Forty eight hours after transfection Firefly and *Renilla* luciferase activity in lysates of transfected cells was determined as described in Section 3.4.2.4. *Renilla* luciferase activity was normalised to Firefly luciferase activity.

4.2.2.7 Assessing off-target effects of polycistronic pri-miR shuttle sequences

IFN response assay

To assess possible induction of interferon response genes qRT-PCR analysis was performed on total RNA extracted from HEK293 cells transfected with the polycistronic pri-miR shuttle expression cassettes. As a positive control for interferon induction cells were also transfected with poly I:C. Transfection and analysis was carried out as described in Section 3.2.5.1.

Disruption of endogenous miR-16 function

To assess potential disruption of the miRNA biogenesis pathway by the multimeric pri-miR shuttle expression vectors the miR-16 saturation assay was carried out as described in Section 3.2.6.2.

4.2.2.8 In vivo *efficacy of polycistronic pri-miR-31 shuttle sequences*

Efficacy of polycistronic pri-miR-31 shuttles was assessed in the hydrodynamic model of HBV replication. Ten micrograms of pCH-9/3091 was co-injected with 10 µg RNAi effecter plasmid and 10 µg of psiCHECK2.2. Blood was collected from mice 3 and 5 days post-injection and HBsAg concentrations determined as described previously (Section 2.2.7.1).

4.2.2.9 Generation of polycistronic pri-miR cassettes containing pri-miR-30a/8

Design of pri-miR-30a/8 shuttle sequence

The pri-miR-30a/8 shuttle was designed along similar lines to the pri-miR-31/8 sequence. The major guide sequence of human pri-miR-30a (42, 133) was substituted with the guide sequence of U6 shRNA 8 (Figure 4.2). Since the mature miR-30a guide sequence is 22 nt in length the same number nucleotides from the U6 shRNA 8 guide sequence was incorporated into the pri-miR-30a/8 shuttle. This shuttle is therefore targeted to HBV co-ordinates 1575-1596. As described previously computer aided prediction ((139, 140) Appendix A4-1) was employed to design the stem sequence of the pri-miR-30a/8 shuttle sequence such that secondary structure of wild-type pri-miR-30a was retained.

Construction of pri-miR-30a/8 shuttle sequence

Oligonucleotides encoding the pri-miR-30a/8 shuttles were synthesised using standard phosphoramidite chemistry (Inqaba Biotech, South Africa). The pre-miR-30a/8 sequence was generated as dsDNA by primer extension of pre-miR-30a/8 F (5’- TGC TGT TGA CAG TGA GCG ACT CAA GGT CGG TCG TTG ACA TTG CTG TGA AGC CAC AGA TGG GC -3’) and pre-miR-30a/8 R (5’- GAA GTC CGA GGC AGT AGG CAG CTC AAG GTC GGT TTG ACA TTG CCC ATC TGT GGC TTC ACA G -3’) primer set (Figure 3.3 (above)). The primer extended product was used as template for the

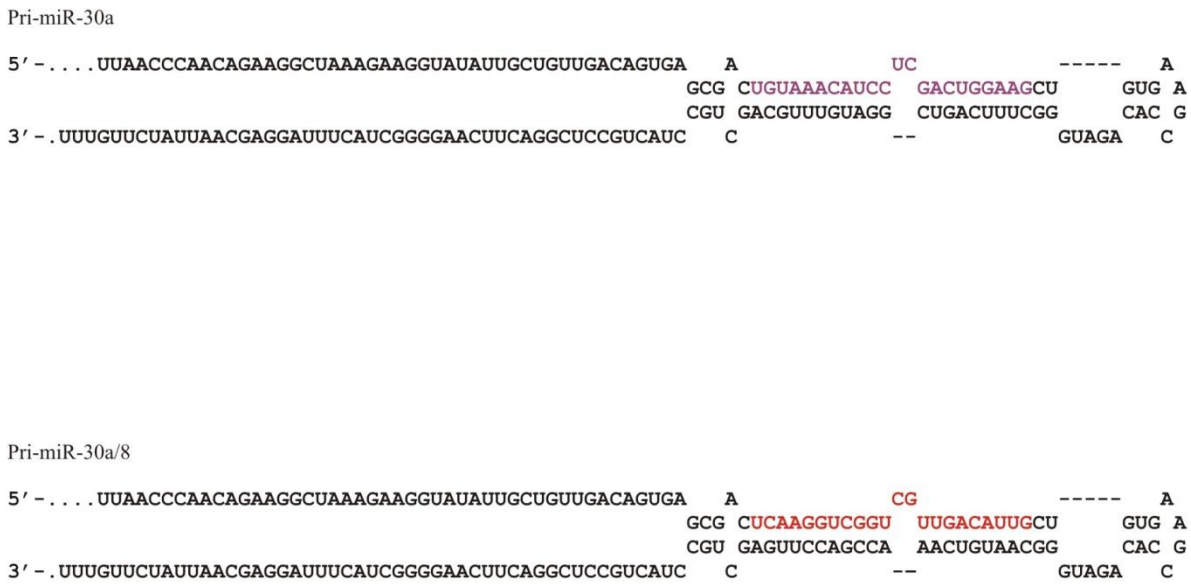

Figure 4.2: <u>Design of pri-miR-30a/8 shuttle sequence.</u>

The major guide sequence derived from the 5' arm of hsa-miR-30a (indicated in purple) was replaced with anti HBV guide sequence 8 (indicated in red) and complementary sequences altered to retain the secondary structure of the wild-type pri-miRNA stem region. Complete pri-miR shuttles contained 51 nt of wild-type pri-miRNA sequences flanking the pre-miR shuttle sequences.

amplification of the pri-miR-30a/8 shuttle sequence with pri-miR-30a F (5'- GAT CGC TAG CTT AAC CCA ACA GAA GGC TAA AGA AGG TAT ATT GCT GTT GAC AGT GAG CGA C -3') and pri-miR-30a R (5'- GAT CAC TAG TAA AAA ACA AGA TAA TTG CTC CTA AAG TAG CCC CTT GAA GTC CGA GGC AGT AGG CA -3'). The amplified pri-miR-30a shuttle sequence was inserted into the PCR cloning vector pTZ57R/T to produce pTZ-pri-miR-30a/8. Sequence of plasmid clones yielding correct restriction maps and that contained inserts in the desired orientation, reverse with respect to that of the *lacZ* gene, was verified by automated sequencing.

Generation of CMV polycistronic pri-miR shuttle sequences containing pri-miR-30a/8

To assess whether substituting the pri-miR-31/8 sequence of pri-miR-31/5/9/8 with pri-miR-30a/8 restores silencing to the 8 guide sequence, pri-miR-31/5/9-30a/8 and pri-miR-122/5-31/9-30a/8 trimers were constructed. First the pri-miR-31/5/9 and pri-miR-122/5-31/9 shuttle sequences were generated by restricting pTZ-pri-miR-31/5 and pTZ-pri-miR-122/5 with *Sca*I and *Spe*I and pTZ-pri-miR-31/9 with *Sca*I and *Nhe*I. Ligation of the fragments containing pri-miR-31/5 or pri-miR-122/5 with the fragments containing pri-miR-31/9 yielded pTZ-pri-miR-31/5/9 or pTZ-pri-miR-122/5-31/9, respectively. Similarly fragments produced from restriction of pTZ-pri-miR-31/5/9 and pTZ-pri-miR-122/5-31/9 with *Sca*I and *Spe*I were ligated with fragments from *Sca*I and *Nhe*I digestion of pTZ-pri-miR-30a/8 to produce pTZ- pri-miR-31/5/9-30a/8 and pTZ-pri-miR-122/5-31/9-30a/8. Finally, the trimeric pri-miR shuttle sequences were excised with *Nhe*I and *Xba*I and inserted into equivalent sites of pCI-neo to produce pCMV pri-miR-31/5/9-30a/8 and pCMV pri-miR-122/5-31/9-30a/8.

Assessing silencing by the pri-miR-30a/8 shuttle sequence

Individual target 8 and mutant* HBx *target silencing

Knockdown of the individual luciferase target vector psiCHECK-8T and the wild-type and mutant *HBx* sequences was used to determine whether the pri-miR-30a/8 shuttle sequence was effective within the context of a polycistron. Huh7 cells were maintained in DMEM supplemented with FCS and antibiotics (Appendix A1-1). Knockdown of the individual target was assessed by co-transfecting 800 ng of the effecter plasmids with 80 ng of psiCHECK-8T and 40 ng of pCI-neo eGFP. Knockdown of wild-type and mutant *HBx* target sequences by the modified polycistronic shuttle sequences was determined by transfecting 800 ng of shuttle expression plasmids, 80 ng of psiCHECK-*HBx* or psiCHECK-m*HBx* and 40 ng of pCI-neo eGFP. Transfections were carried out with Lipofectamine™ 2000 (Appendix A1-1). Firefly and *Renilla* luciferase activity in lysates of transfected cells were determined 48 hours post-transfection (Section 3.4.2.4). Firefly luciferase activity was used as a normalisation control for *Renilla* luciferase activity.

4.3 RESULTS

4.3.1 Liver-specific pri-miR-122 shuttle expression cassettes

4.3.1.1 Identified promoter sequences are functional and capable of tissue-specific expression of a Firefly luciferase transgene

To establish whether the promoter sequences selected for eventual use in the liver-specific pri-miR shuttle expression vectors are functional and exhibit tissue-specificity, the ability of these sequences to express a transgene that is easily quantifiable was first assessed. Quantification of Firefly luciferase may be achieved with relative ease and was

used as a surrogate to identify functional promoter sequences that may be used for the liver-specific expression of the pri-miR-122 shuttle sequences. The CMV immediate early promoter enhancer element of pCI-neo was replaced with the sequences encoding the liver-specific promoters of human A1AT and FVIII and the HBV PreS2 and BCP/enhancer II sequences. Subsequently, the *Firefly* luciferase sequqnce was inserted downstream of the various Pol II promoters of pCI-neo, pCI-A1AT, pCI-FVIII, pCI-BCP and pCI-PreS2. Firefly luciferase activity was assessed in human liver-derived (Huh7) and human kidney-derived (116) cells, which were transfected with the CMV-driven expression plasmid (pCI-neo FLuc) and the liver-specific promoter-driven expression plasmids (pCI-A1AT FLuc, pCI-FVIII FLuc, pCI-BCP FLuc and pCI-PreS2 FLuc). Firefly luciferase was strongly expressed in both Huh7 and 116 cells transfected with pCI-neo FLuc (Figure 4.3). This is not unexpected as the ubiquitously active and powerful nature of the CMV immediate early promoter enhancer is well-characterised (151). The detection of Firefly luciferase in Huh7 cells transfected with the liver-specific expression plasmids indicated that these promoter sequences were functional. In contrast to the expression from the CMV immediate early promoter enhancer, which strongly expressed Firefly luciferase in Huh7 and 116 cells, transgene activity was not detected in kidney-derived cells transfected when expressed from any of the liver-specific promoters indicating that these cassettes exhibited tissue-specific expression. The CMV promoter sequence however was significantly stronger than that of the liver-specific promoters. Indeed Firefly luciferase expression from the FVIII and PreS2 promoters was barely detectable. Consequently these promoters were excluded from further investigation as it is unlikely that sufficient levels of pri-miR shuttle sequence would be expressed from these promoters.

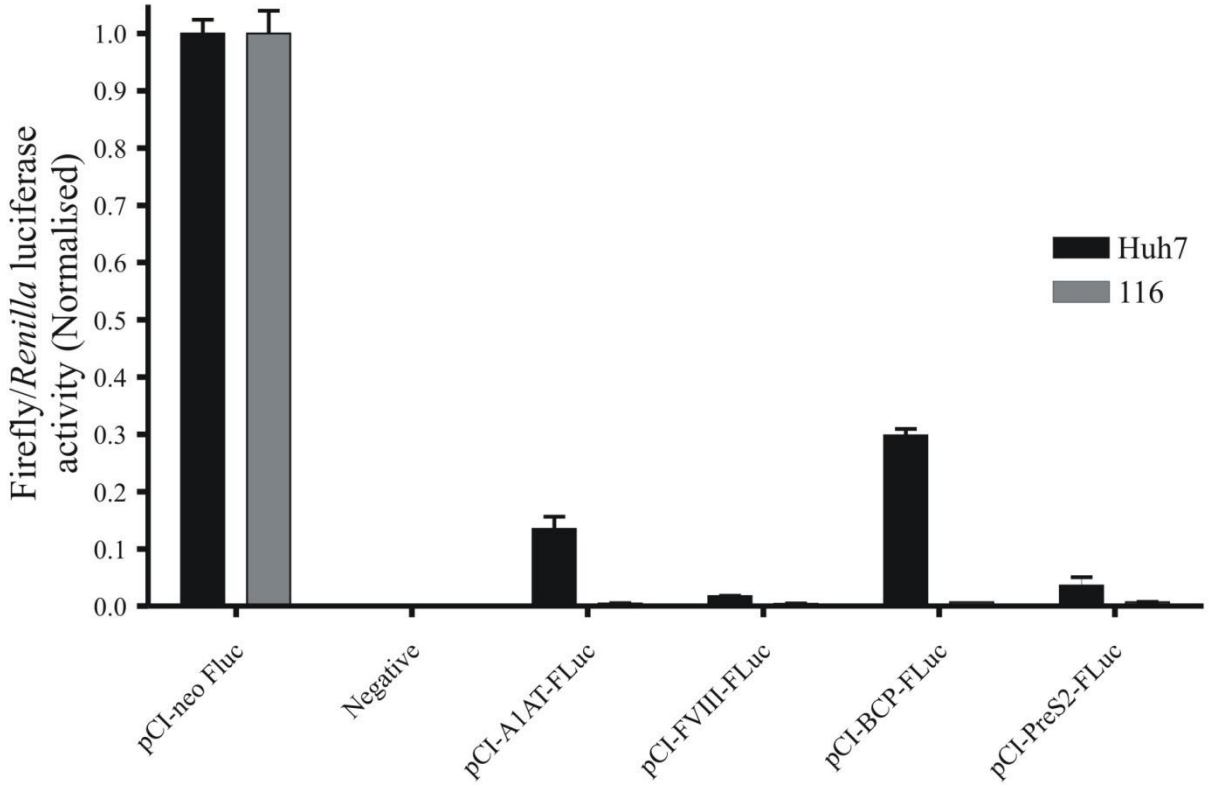

Figure 4.3: <u>Expression of the *Firefly luciferase* transgene from the various promoter elements.</u>

The functionality and tissue-specificity of the putative liver-specific promoters was assessed by measuring expression of the *Firefly luciferase* transgene. Firefly luciferase activity was measured in liver-derived (Huh7) and kidney-derived (116) cells. Bars indicate means of relative Firefly to *Renilla* luciferase activity in Huh7 (black bars) and 116 (gray bars) cells. Error bars indicate SEM.

4.3.1.2 Pri-miR shuttle sequences driven from the A1AT and HBV core promoters are capable of tissue-specific inhibition of HBV gene expression

Demonstration that the A1AT and HBV core promoter sequences are able to express a transgene tissue-specifically indicated that these sequences could potentially be employed to achieve liver-specific expression of the pri-miR-122 shuttle sequence. A1AT- and BCP-driven shuttle cassettes were therefore generated to determine whether these cassettes could tissue-specifically express the pri-miR-122 sequences. Silencing of a marker of HBV gene expression (relative Firefly luciferase activity) was used as a measure of pri-miR-122 shuttle sequence expression (Figure 4.4). Whereas pri-miR-122/5 shuttle sequences expressed from the CMV promoter caused knockdown of HBV gene expression in liver- and kidney-derived cell lines, equivalent knockdown by the A1AT and BCP shuttle vector was only achieved in the liver-derived cell line. Knockdown in the kidney-derived cell line by the tissue-specific pri-miR shuttle vectors was marginal but not comparable to knockdown by the CMV pri-miR shuttles. Interestingly CMV pri-miR-122/6 did not cause significant silencing in the kidney-derived cell line and is likely as a result of the lower efficacy of the pri-miR-122/6 sequence. The pri-miR-122/10 shuttle sequence, which is known to be ineffective at achieving knockdown of HBV, was incapable of silencing irrespective of the promoter from which it was expressed. CMV pri-miR-122/10 seemed to induce HBV expression and is in accordance with observations with U6 shRNA 10 (data not shown). Taken together these data indicate that expressing pri-miR shuttle sequences from liver-specific promoters limits silencing activity to liver-derived cell lines. These constructs may be developed further to ultimately limit expression of therapeutic sequences to the liver.

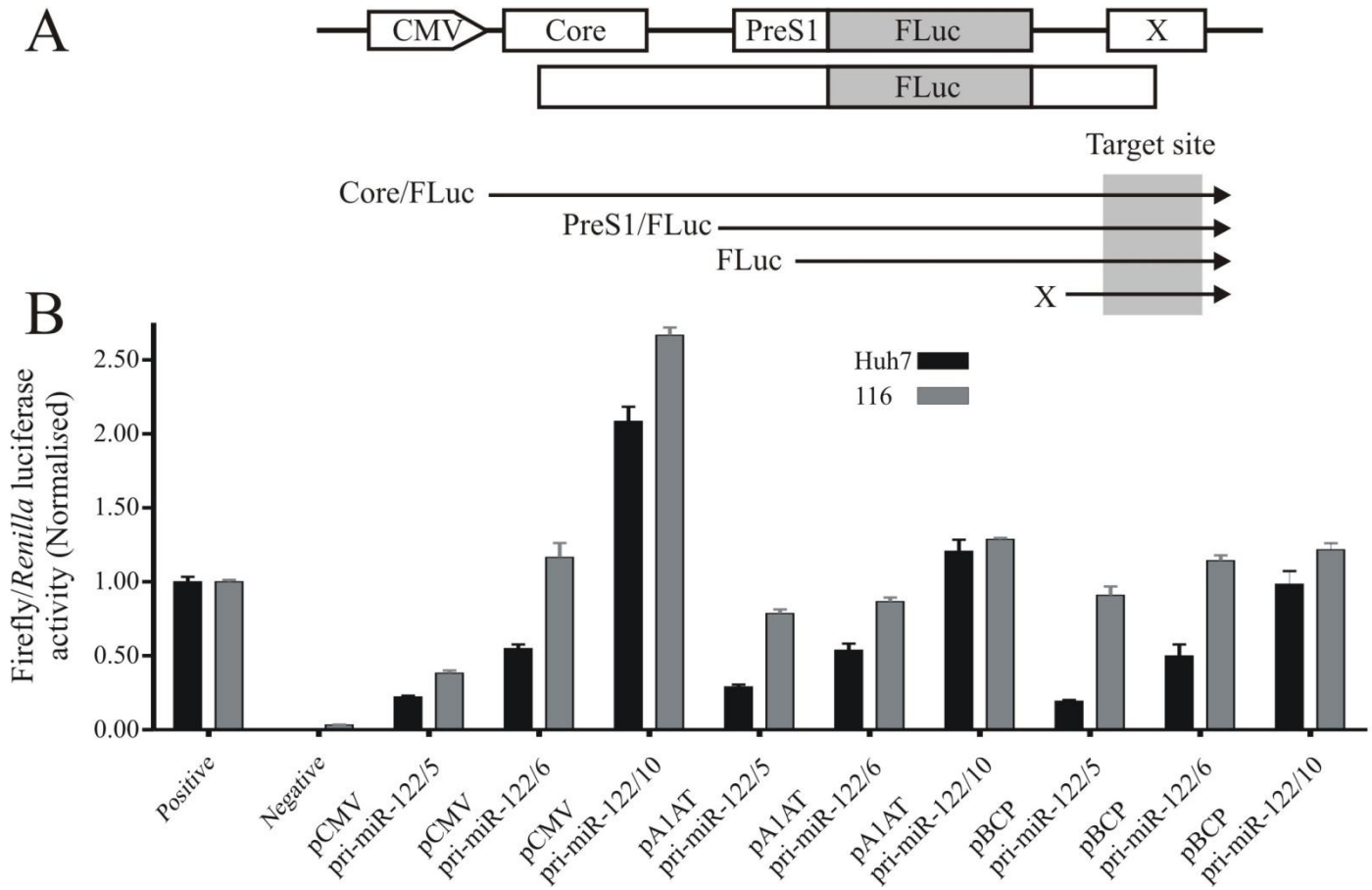

Figure 4.4: <u>Inhibitory activity of pri-miR-122 shuttle sequences expressed from various promoter sequences.</u>

(A) Schematic representation of pCH-FLuc. (B) Efficacy of liver-specific pri-miR shuttles was assessed by co-transfection of Huh7 and 116 cells with pCH-FLuc and the various Pol II expression cassettes. Bars indicate means of relative Firefly to *Renilla* luciferase activities from Huh7 (black bars) and 116 (grey bars) cells. Error bars indicate SEM.

4.3.1.3 Liver-specific pri-miR-122 shuttle expression cassettes do not disrupt the endogenous miRNA pathway

To assess whether the liver-specific pri-miR-122 shuttle expression cassettes cause disruption of the miRNA biogenesis pathway the previously described miR-16 saturation assay was carried out. Co-transfection of the miR-16 sponge plasmid, pU6 miR-16S×7, with the psi-miR-16T×7 target plasmid (Figure 4.5A) was included as a positive control for disruption of miR-16 function. pU6 miR-16S×7 co-transfected with psi-miR-16T×7 caused a significant increase in *Renilla* luciferase activity as compared to mock-transfected cells (Figure 4.5B) indicating that the sponge plasmid interfered with endogenous miR-16 silencing of the *Renilla* luciferase target. In contrast, none of the liver-specific pri-miR shuttle sequence expression cassettes derepressed *Renilla* luciferase activity. The pri-miR shuttle sequences therefore do not interfere with endogenous miR-16 function. This result is not unexpected as the liver-specific promoters are not as powerful as the CMV promoter (Figure 4.3 (above)), which has previously been shown to not cause disruption of the miRNA biogenesis pathway. The relatively low levels of pri-miR shuttle sequences produced from the liver-specific promoters are therefore unlikely to outcompete naturally occurring miRNA for processing by the RNAi processing machinery.

4.3.4 A1AT-driven pri-miR shuttle sequences knock down HBV replication in vivo

In vivo efficacy of the A1AT- and BCP-driven pri-miR shuttle expression cassettes was assessed in the hydrodynamic injection model of HBV replication. Serum HBsAg concentration was measured at day 3 and 5 post-injection to determine silencing of HBV replication (Figure 4.6). Silencing achieved with pA1AT pri-miR-122/5 was equivalent to

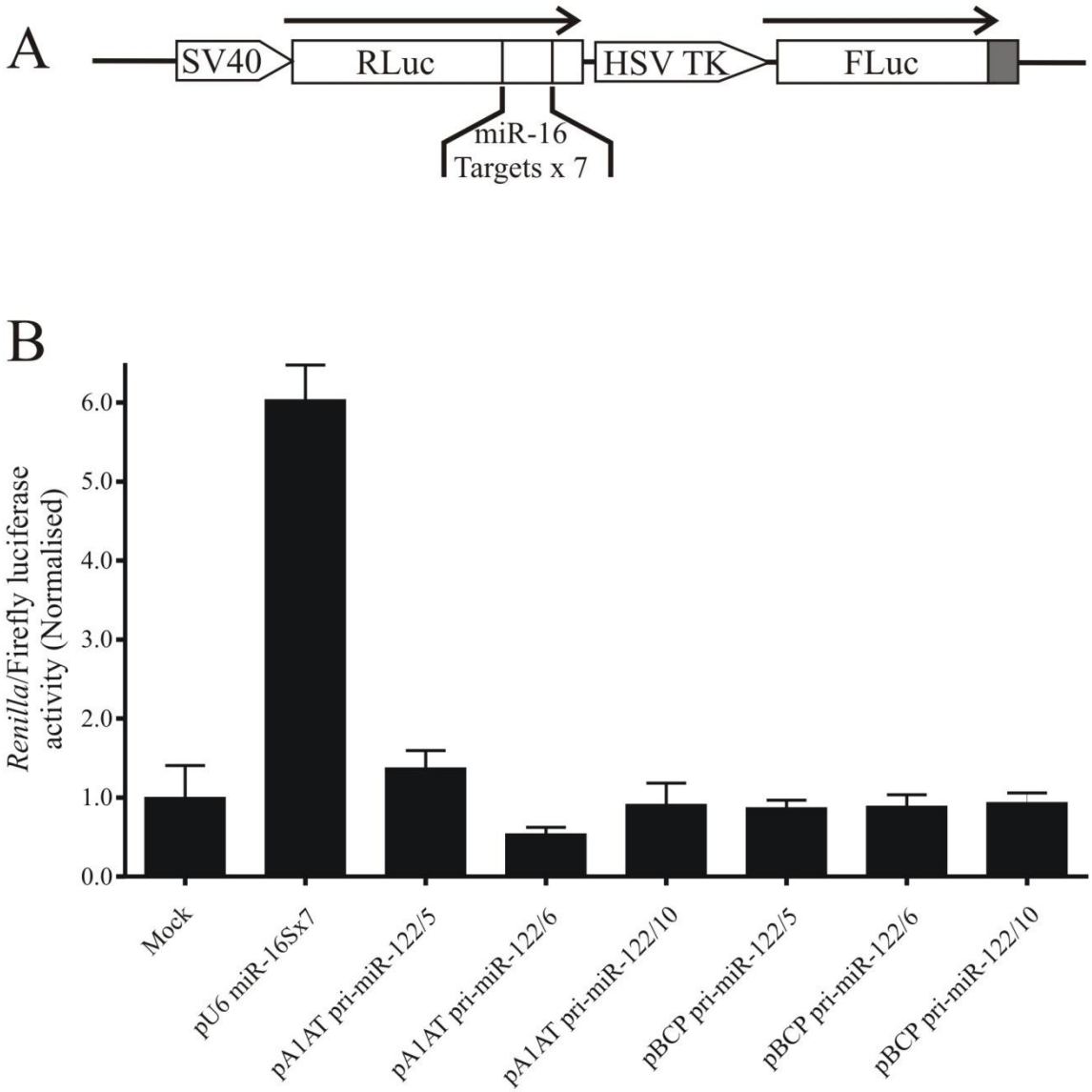

Figure 4.5: <u>Assessing saturation of the miRNA biogenesis pathway.</u>

(A) Schematic representation of the psi-miR-16T×7 target plasmid, which expresses the *Renilla* luciferase transcript with seven imperfect miR-16 target sites within its 3' UTR. (B) Huh7 cells were co-transfected with the indicated plasmids and psi-miR-16T×7 in triplicate and the cells analysed for luciferase activity 48 hours later. Means of the relative *Renilla* to Firefly luciferase activity are indicated with SEM.

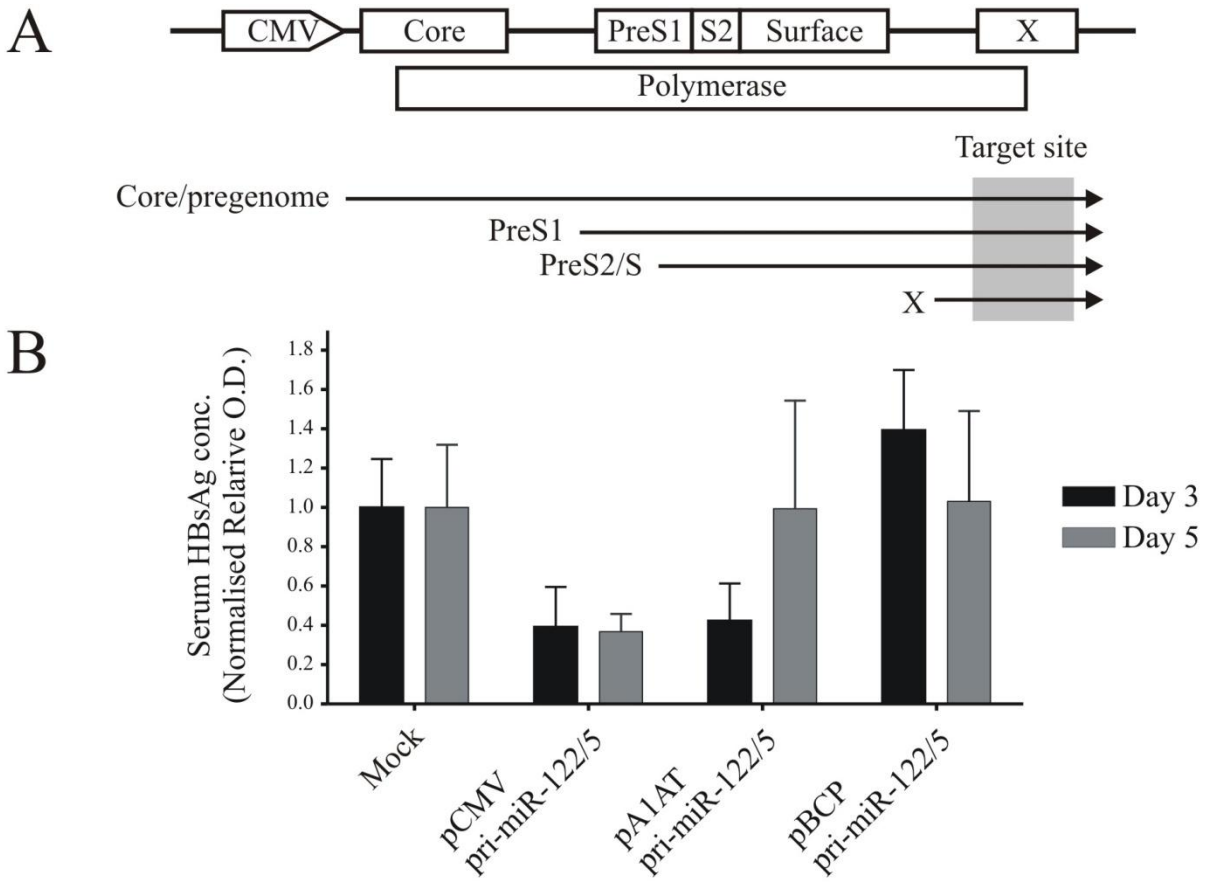

Figure 4.6: *In vivo* inhibitory effects of liver-specific promoter pri-miR-122 expression cassettes.

(A) Schematic representation of pCH-9/3091. (B) Mice were injected using the hydrodynamic tail vein injection procedure with the indicated plasmids and pCH-9/3091. Each group comprised at least 4 mice. Serum was collected 3 and 5 days post-injection and HBsAg concentrations determined by ELISA. Mean HBsAg concentrations are indicated with SEM.

that of its CMV promoter-driven counterpart, however silencing was only maintained until day 5 in mice that received pA1AT pri-miR-122/5. This may be as a result of the relative strengths of the A1AT and CMV promoters. No silencing of HBV replication was observed in mice that received BCP-driven pri-miR-122 shuttle expression cassettes.

4.3.2 Polycistronic CMV pri-miR-31 expression cassettes

4.3.2.1 Polycistronic pri-miR-31 expression cassettes produce multiple effecter sequences

A functional assay was initially used to determine whether the multimeric pri-miR-31 expression cassettes produce all the expected guide sequences. Dual luciferase reporter plasmids were generated that are targeted by each individual effecter sequences (i.e. pri-miR-31/5, pri-miR-31/8 or pri-miR-31/9) (Figure 4.7A). The ability of an expression cassette to silence *Renilla* luciferase expression from individual reporter plasmids is therefore indicative of the production of a functional effecter sequence. Each of the six multimeric pri-miR-31 shuttle expression cassettes was co-transfected with the three different individual psiCHECK target plasmids. *Renilla* luciferase expression from psiCHECK-5T was significantly inhibited by all of the different polycistronic pri-miR-31 shuttle expression cassettes (Figure 4.7B). All the polycistronic pri-miR-31 shuttle expression cassettes therefore produced functional pri-miR-31/5 shuttle sequence. Of the six multimeric cassettes only pCMV pri-miR-31/5/9/8 and pCMV pri-miR-31/9/8/5 did not inhibit *Renilla* luciferase expression from psiCHECK-8T which indicates that these cassettes lack the ability to produce a pri-miR-31/8 shuttle sequence. Though all the polycistronic cassettes significantly silenced *Renilla* luciferase expression from psiCHECK-9T, the inhibitory effects were not as marked as that achieved against the 5 or

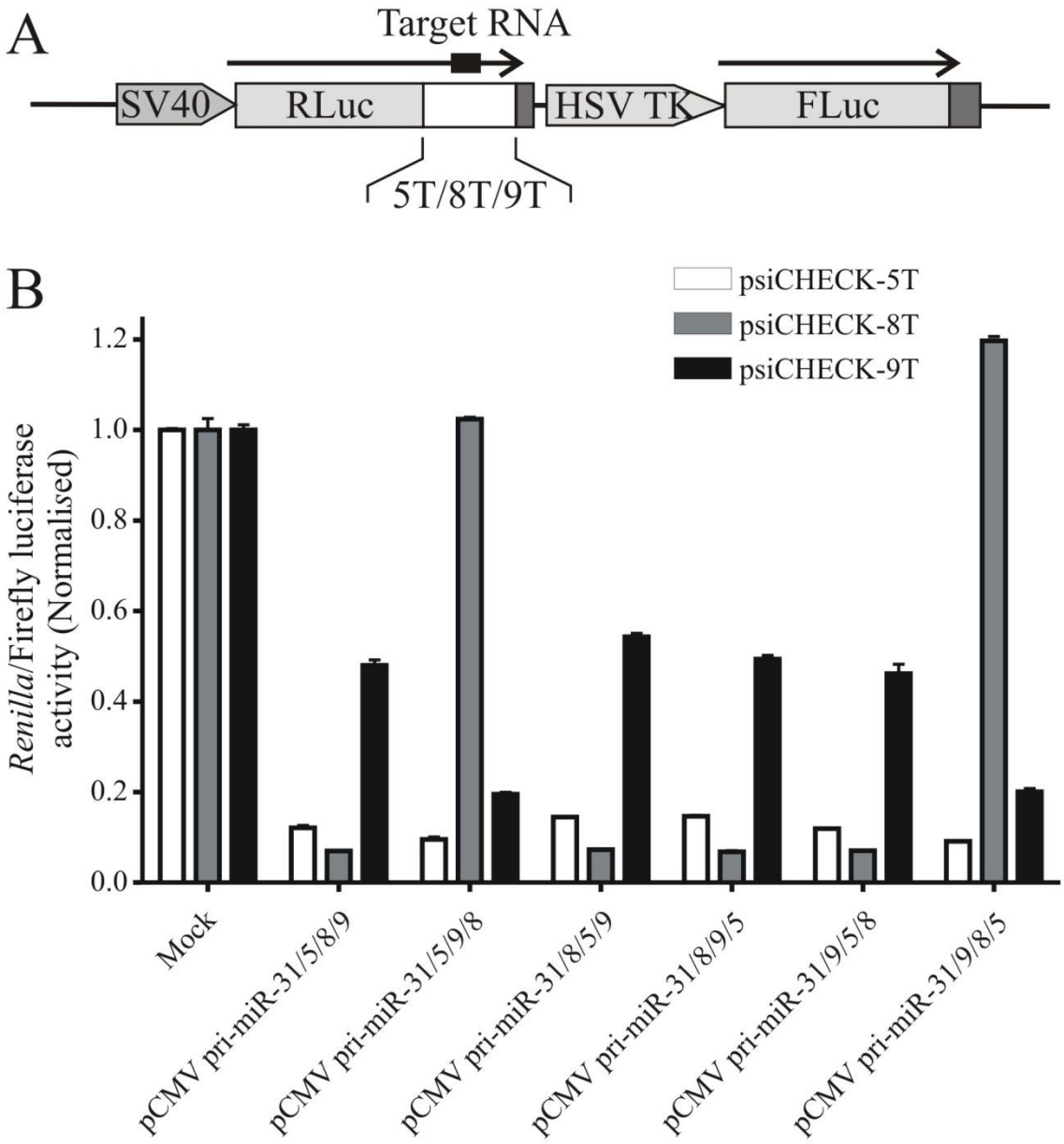

Figure 4.7: <u>Determination of effecter sequence production by a functional assay.</u>

(A) Schematic representation of the individual luciferase target vectors. (B) Huh7 cells were co-transfected with psiCHECK-5T, psiCHECK-8T or psiCHECK-9T and the indicated trimeric pri-miR shuttles plasmids in triplicate. Forty eight hours post-transfection *Renilla* and Firefly luciferase activity was measured from the lysates of transfected cells. Indicated are the relative averages of *Renilla* to Firefly luciferase activity with SEM.

8 target plasmids and may indicate that the pri-miR-31/9 shuttle sequences produced from these polycistronic cassettes are not highly effective.

The data from the functional analysis were corroborated by Northern blot hybridisation to detect processed guide sequences (Figure 4.8). In accordance with the functional analysis using psiCHECK-5T, guide sequences corresponding to miR-31/5 were produced from all the multimeric pri-miR-31 expression cassettes (Figure 4.8A). Guide sequences corresponding to miR-31/8 were not detected in cells transfected with pCMV pri-miR-5/9/8 or pCMV pri-miR-9/8/5 (Figure 4.8B) which clarifies the results observed with the functional assay. The miR-31/9 guide sequence was produced from all the different pri-miR-31 shuttle expression cassettes (Figure 4.8C), however guide concentration was lower than that observed for miR-31/5 and miR-31/8 and may explain the lower inhibitory effects observed against the individual luciferase target.

4.3.2.2 Multimeric pri-miR-31 shuttles effectively knock down markers of HBV replication in vitro

Next, silencing efficacy of polycistronic pri-miR shuttle expression cassettes against more relevant viral targets was assessed. Huh7 cells were co-transfected with the various multimeric pri-miR-31 shuttle expression cassettes and the target vectors, pCH-9/3091 and pCH-FLuc. HBsAg concentrations in supernatants of cells transfected with the different polycistronic cassettes were significantly reduced as compared to mock-transfected cells (Figure 4.9). Furthermore the degree of silencing was comparable to that achieved with the highly effective U6 shRNA 5 sequence. Similar results were observed with the polycistronic pri-miR-31 shuttles targeted against the Firefly luciferase reporter pCH-FLuc (Figure 4.10). These results together with the previous data (Section 4.3.2.1) indicate that at least one of the effecter sequences that are produced from the multimeric

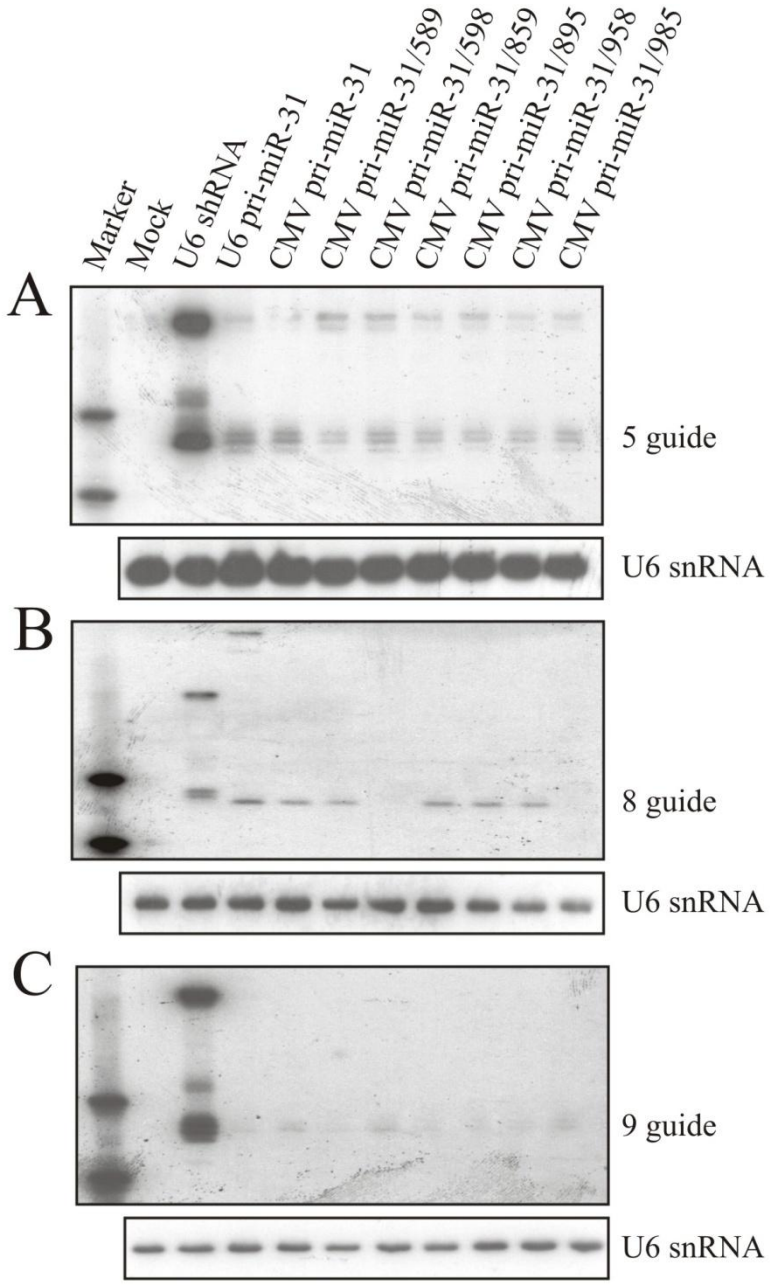

Figure 4.8: <u>Northern blot analysis for the detection of guide sequences from multimeric cassettes.</u>

Total RNA extracted from cells transfected with the different shRNA or pri-miR-31 expression cassettes were hybridised to nylon membranes and relevant guide sequences detected with radioactively labelled probe. Membranes were stripped and reprobed for U6 snRNA to confirm equal loading.

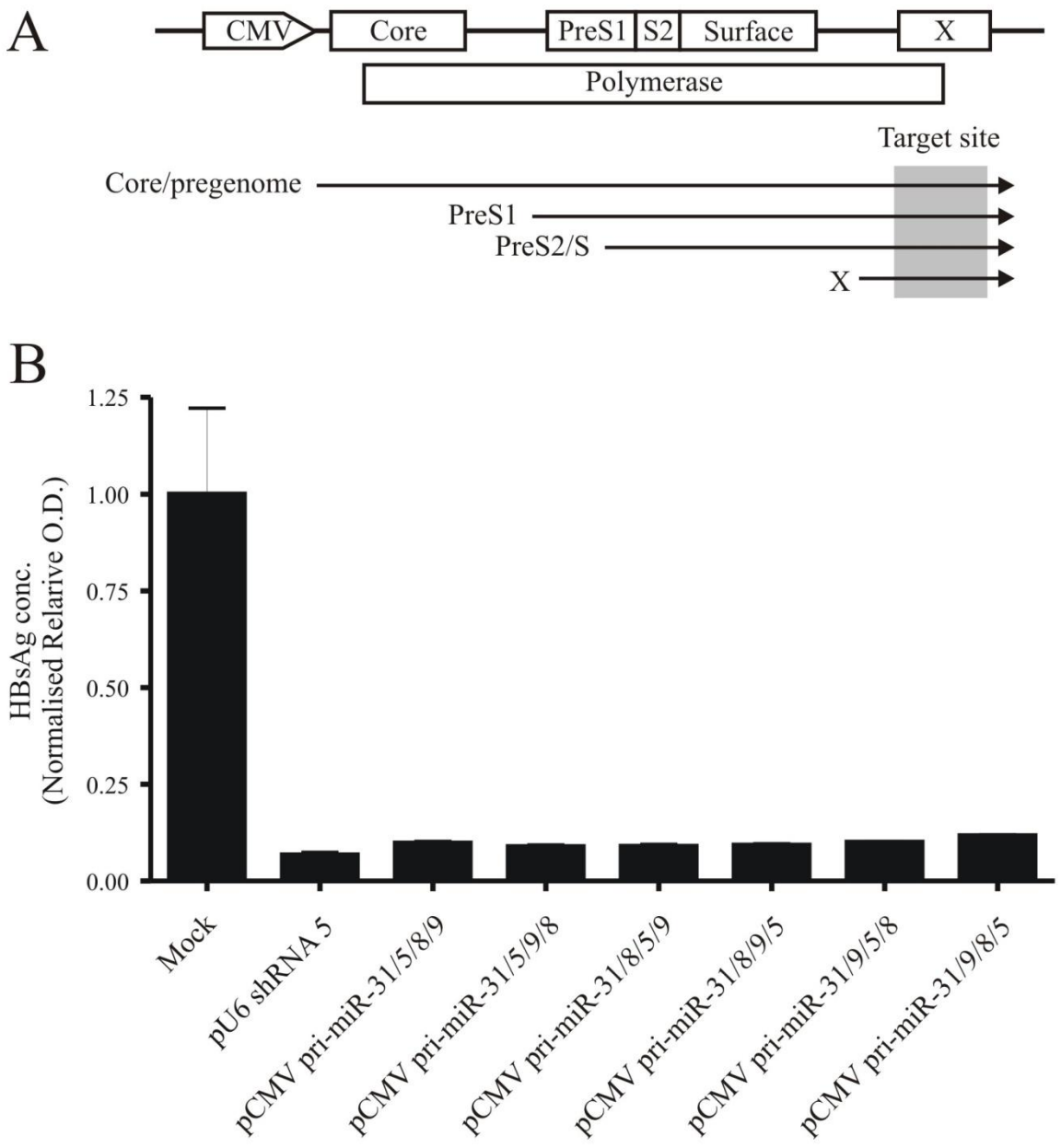

Figure 4.9: <u>Inhibition of HBV replication in cultured mammalian cells.</u>

(A) Schematic representation of pCH-9/3091. (B) Huh7 cells were co-transfected with pCH-9/3091 and the indicated plasmids in triplicate. HBsAg concentration in culture supernatant was determined by ELISA. Bars indicate normalised averages of HBsAg levels with SEM.

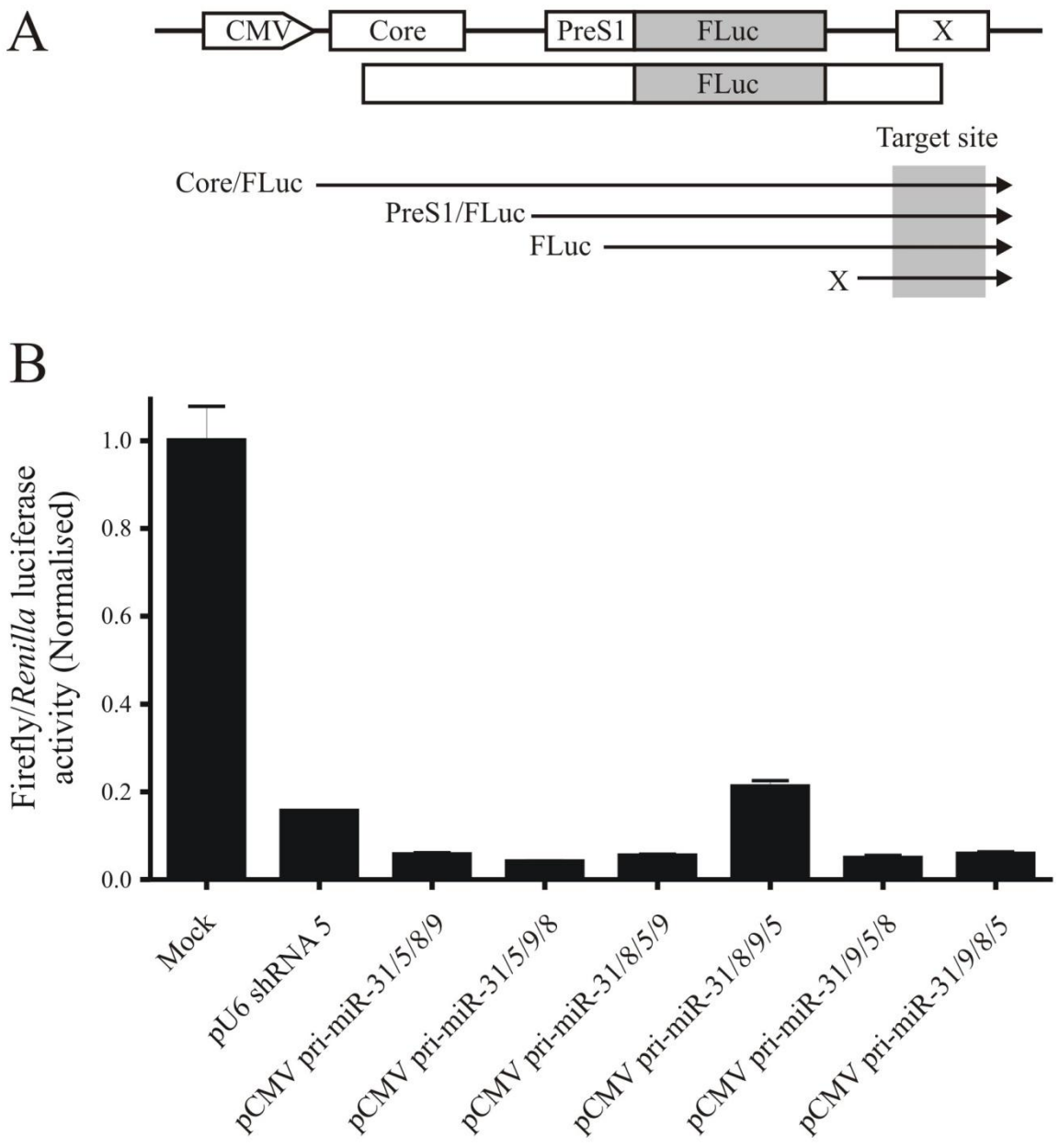

Figure 4.10: *In situ* inhibition of a marker of HBV replication.

(A) Schematic representation of pCH-FLuc. (B) To assess silencing activity of the multimeric pri-miR-31 expression cassettes within cells the Firefly luciferase reporter plasmid, pCH-FLuc was co-transfected with the indicated effecter plasmids. Firefly and *Renilla* luciferase activities were assessed in the lysates of transfected cells and are indicated graphically with SEM.

shuttle expression cassettes is causing effective knockdown of viral gene expression.

4.3.2.3 Polycistronic pri-miR-31 shuttes are capable of silencing mutant **HBx** *target sequences*

To assess whether the polycistronic pri-miR-31 shuttle expression cassettes could potentially prevent the emergence of a viral escape mutant, a dual luciferase reporter vector was constructed that has a mutated pri-miR-31/5 target. The psiCHECK-*HBx* vector, which contains a copy of the complete *HBx* ORF downstream of the Renilla *luciferase* sequence, was used to derive the mutant target vector psiCHECK-m*HBx* (Figure 4.11A). Using a PCR-based approach five point mutations were introduced into the pri-miR-31/5 target site. Huh7 cells were transfected with the polycistronic pri-miR-31 expression cassettes and the wild-type or mutant *HBx* target vectors The pri-miR-31/5/8/9, pri-miR-31/8/5/9, pri-miR-31/8/9/5 and pri-miR-31/9/5/8 shuttle cassettes were capable of efficiently reducing expression of wild-type and mutant *HBx* targets (Figure 4.11B). Unsurprisingly the pri-miR-31/5/9/8 and pri-miR-31/9/8/5 expression cassettes, though capable of efficiently silencing the wild-type *HBx* target, only moderately knocked down the mutant *HBx* target. Northern hybridisation analysis (Figure 4.8 (above)) revealed that the 8 guide sequence was not produced from the pri-miR-31/5/9/8 or pri-miR-31/9/8/5 shuttle sequences and as a consequence was not functional against an 8 target (Figure 4.7 (above)). Additionally the 9 guide sequence produced from the polycistronic cassettes was shown to be only moderately effective (Figure 4.7 (above)). Consequently only the 5 guide produced from the pri-miR-31/5/9/8 or pri-miR-31/9/8/5 shuttle sequences is potentially effective and since its cognate is mutated, these cassettes cause poor silencing of the *mHBx* target. Nevertheless, the modular nature of the polycistronic pri-miR-31 shuttle sequences allowed rearraging the order of each individual shuttle to produce four multimeric cassettes that are effective and potentially capable of limiting viral escape mutants.

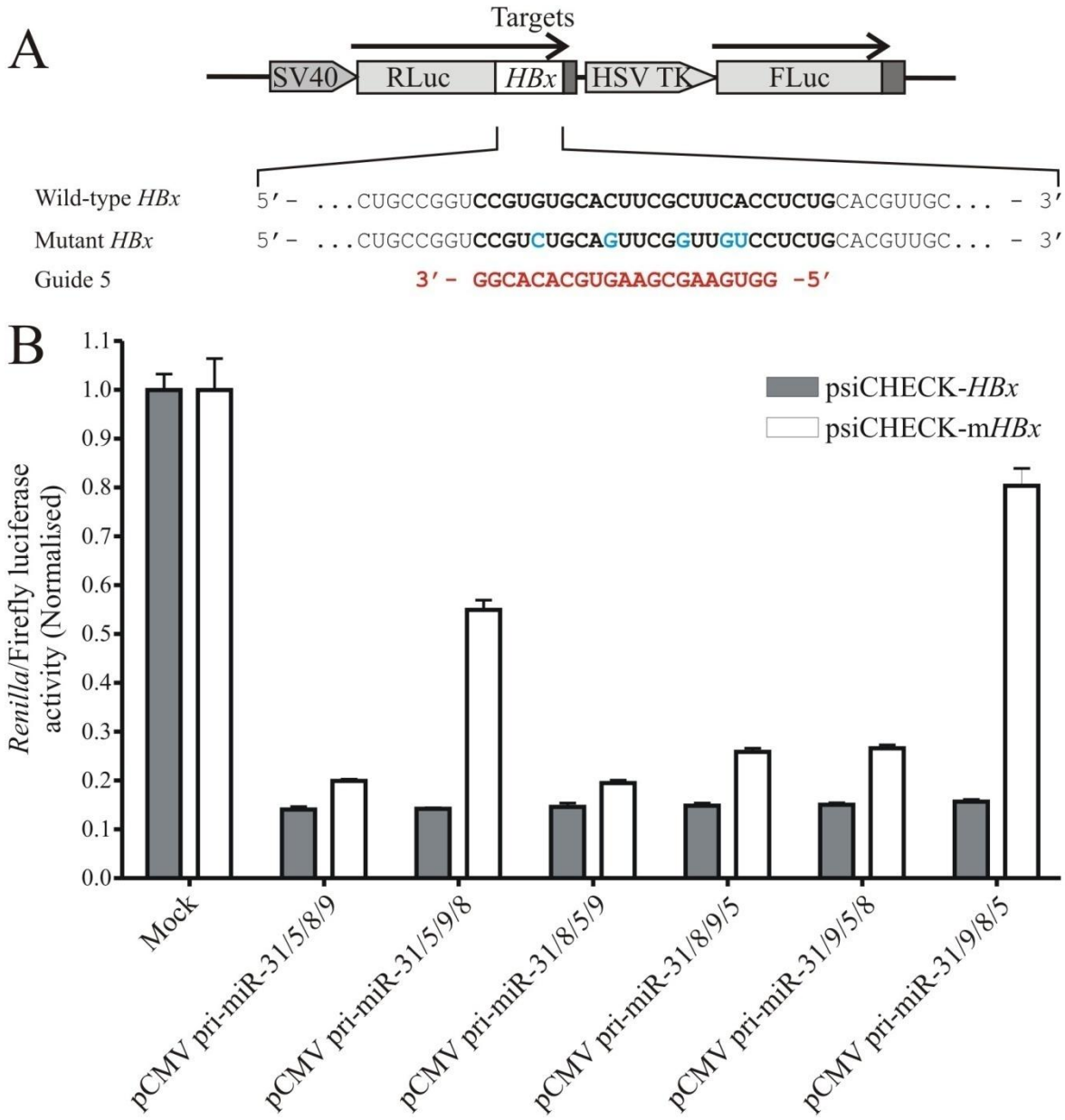

Figure 4.11: <u>Silencing of mutant HBx sequence by polycistronic pri-miR-31 shuttles.</u>

(A) Schematic representation of psiCHECK-*HBx* and psiCHECK-m*HBx* target vectors. Wild-type and mutant *HBx* sequences occur downstream of the Renilla *luciferase* ORF with the pri-miR-31/5 guide sequence that target these sites indicated in red. (B) psiCHECK-*HBx* (grey bars) or psiCHECK-m*HBx* (white bars) were co-transfected with the indicated polycistronic pri-miR-31 shuttle plasmids. Mean ratios of *Renilla* to Firefly luciferase activity from transfected cells are shown with SEM indicated by error bars.

4.3.2.4 Polycistronic pri-miR-31 shuttle sequences do not exhibit toxic effects

To exclude toxic effects as factors contributing to the observed viral inhibition, induction of the interferon response was measured. *IFN-β* was determined in HEK293 cells transfected with the panel of multimeric pri-miR-31 shuttles. Compared to the control positive for induction of the interferon response (poly (I:C) control) none of the polycistronic pri-miR-31 shuttle sequences caused a significant increase in *IFN-β* mRNA levels (Figure 4.12). The silencing effects observed are therefore independent of immune stimulation.

Again to assess possible disruption of the endogenous miRNA biogenesis pathway, the miR-16 saturation assay was carried out with the pri-miR-31 expression cassettes. Whereas the miR-16 sponge plasmid co-transfected with the dual luciferase target plasmid clearly disrupted endogenous miR-16 function as can be seen by derepression of *Renilla* luciferase activity, none of the multimeric cassettes had a negative effect on miR-16 function (Figure 4.13). Therefore the polycistronic pri-miR-31 expression cassettes neither cause induction of the IFN response nor do these cassettes saturate the endogenous miRNA pathway.

4.3.2.5 Polycistronic pri-miR-31 shuttles efficiently knock down HBV replication **in vivo**

To assess efficacy of the polycistronic pri-miR-31 expression cassettes for silencing HBV replication *in vivo*, the murine hydrodynamic injection procedure was used to co-deliver effecter plasmid with the HBV replication-competent plasmid, pCH-9/3091. In comparison to mock-injected mice, serum HBsAg concentrations were significantly reduced in mice that received the multimeric pCMV pri-miR-31/5/8/9 or pCMV pri-miR-

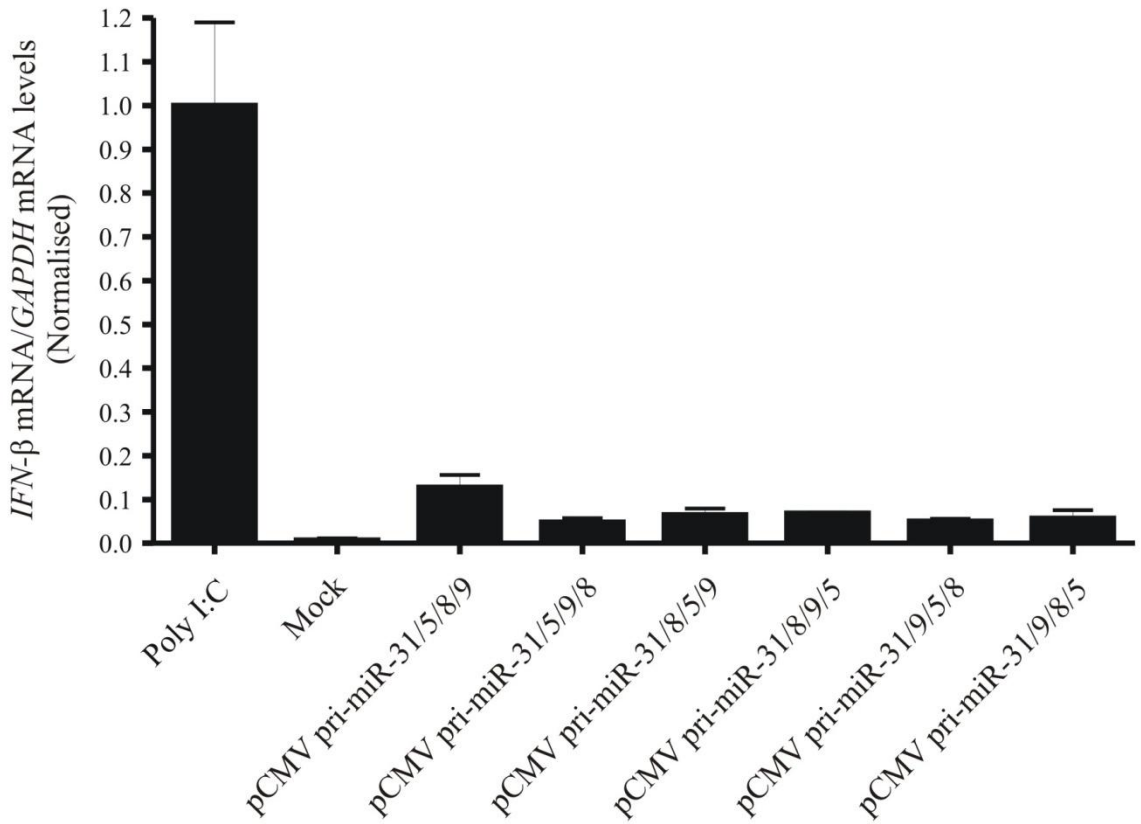

Figure 4.12: <u>Stimulation of IFN response genes by polycistronic pri-miR-31 shuttle sequences.</u>

Real-time qRT-PCR analysis was carried out on total RNA extracted from HEK293 cells transfected with the indicated plasmids. *IFN-β* mRNA levels were normalised and averages from triplicate experiments are indicated with SEM.

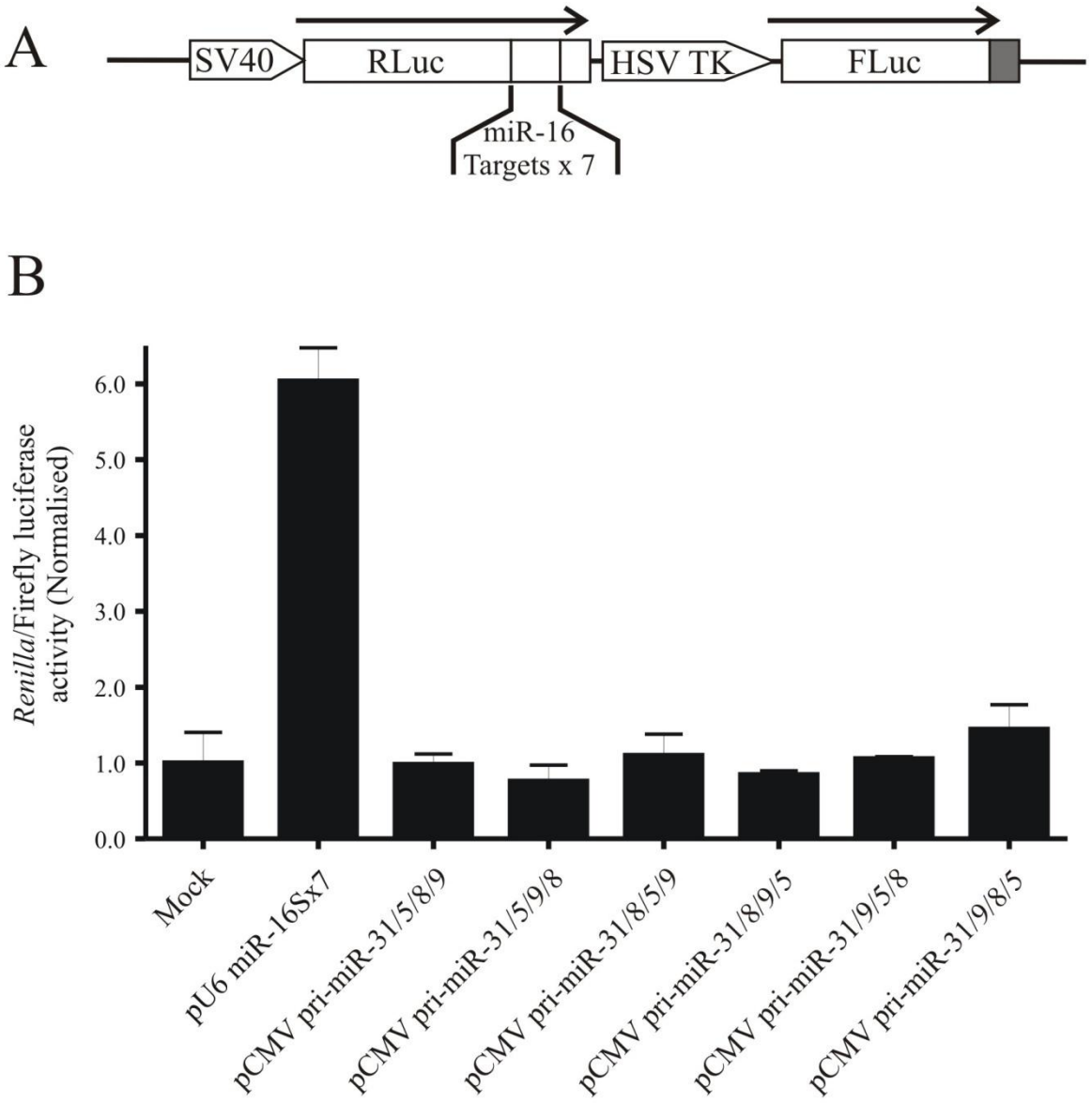

Figure 4.13: <u>Saturation assay to assess disruption of endogenous miR-16 function.</u>

(A) Schematic representation of the dual luciferase target plasmid, psi-miR-16T×7, which contains seven imperfect miR-16 target sites within the 3' UTR of the *Renilla* luciferase transcript. (B) Huh7 cells were co-transfected with the indicated plasmid and *Renilla* and Firefly luciferase activities determined 48 hours post-transfection. Relative *Renilla*/Firefly activities are indicated as averages from triplicate experiments with error bars representing SEM.

31/8/5/9 (Figure 4.14). Knockdown achieved with the two multimeric expression cassettes was equivalent to the degree of inhibition achieved with the CMV-derived monomeric shuttle sequence, pri-miR-31/5 and the U6 shRNA 5 effecter sequence. In addition knockdown was sustained for up to five days post-injection. Polycistronic pri-miR shuttle sequences are therefore capable of efficiently silencing HBV replication in an *in vivo* model that simulates viral replication.

4.3.2.6 Replacing pri-miR-31/8 shuttle with a pri-miR-30a/8 shuttle sequence restores silencing to the 5/9/8 trimer

To assess whether replacing the pri-miR-31/8 shuttle sequence of CMV pri-miR-31/5/9/8 with a different pri-miR shuttle backbone would restore repressive activity to the trimeric cassette, pCMV pri-miR-31/5/9-30a/8 and pCMV pri-miR-122/5-31/9-30a/8 were created. Initially silencing by the pri-miR-30a/8 shuttle sequence within the context of a polycistronic sequence was assessed by co-transfecting the pCMV pri-miR-31/5/9-30a/8 and pCMV pri-miR-122/5-31/9-30a/8 cassettes with the individual 8 target vector. As shown previously (Figure 4.7 (above)) the pCMV pri-miR-31/5/9/8 and pCMV pri-miR-31/9/8/5 shuttle expression cassettes do not knock down *Renilla* luciferase expression from the psiCHECK-8T target vector (Figure 4.15). In contrast both the pCMV pri-miR-31/5/9-30a/8 and pCMV pri-miR-122/5-31/9-30a/8 cassettes effectively knock down *Renilla* luciferase activity. Similarly the pCMV pri-miR-31/5/9-30a/8 and pCMV pri-miR-122/5-31/9-30a/8 cassettes were effective against a mutated *HBx* target whereas the pri-miR-31/5/9/8 and pri-miR-31/9/8/5 shuttles exhibited limited silencing efficacy (Figure 4.16). These results clearly indicate that substituting the pri-miR-31/8 sequence for the pri-miR-30a/8 shuttle sequence restores repressive activity to the 5/9/8 trimer.

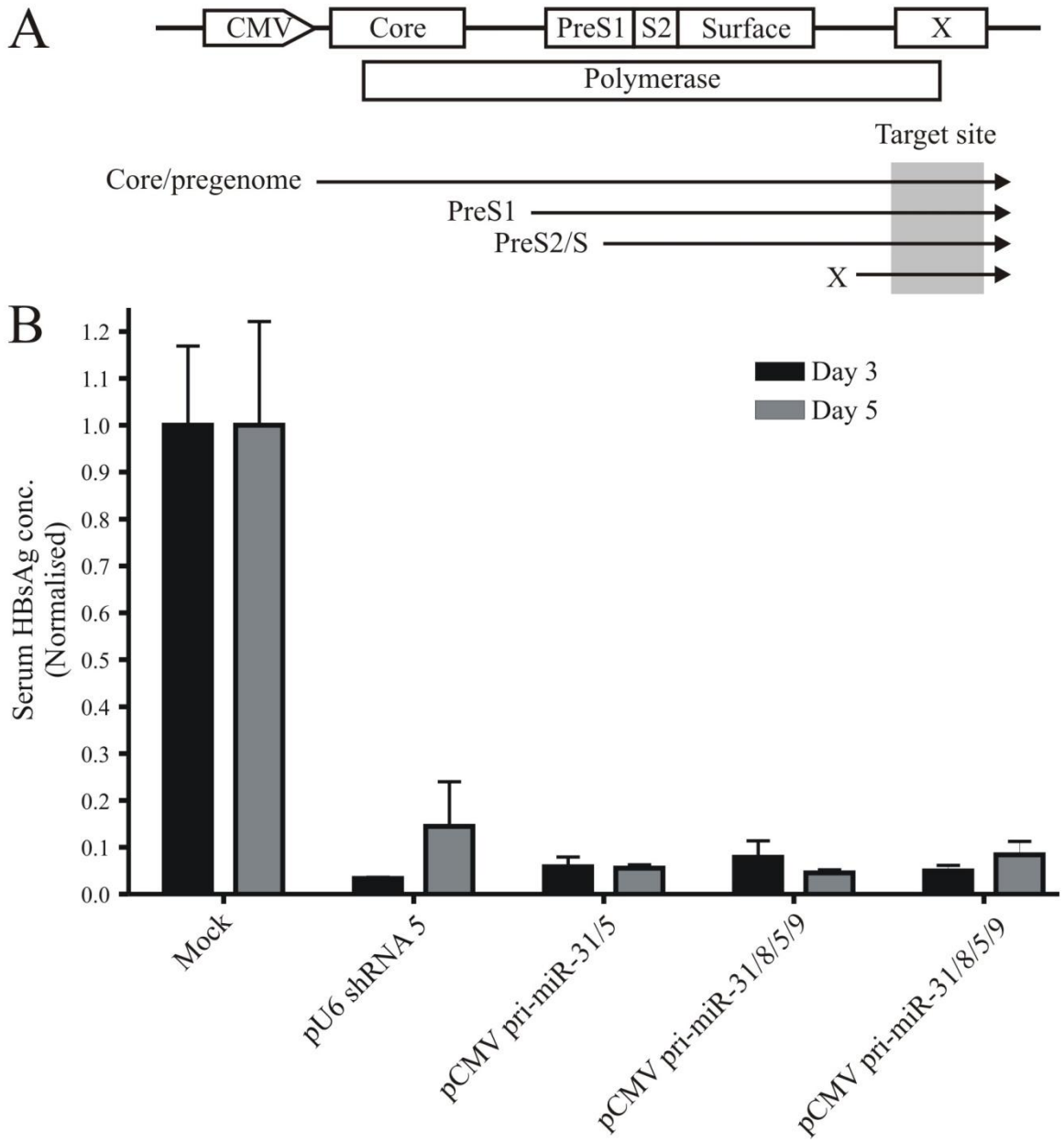

Figure 4.14: <u>Inhibitory potential of polycistronic pri-miR-31 shuttle expression cassettes *in vivo*.</u>

(A) Schematic representation of pCH-9/3091. (B) Mice were injected hydrodynamically with the indicated plasmids and blood collected at days 3 and 5 post-injection. Serum HBsAg concentrations were determined by ELISA and are indicated as the average from at least four experiments. Error bars represent SEM.

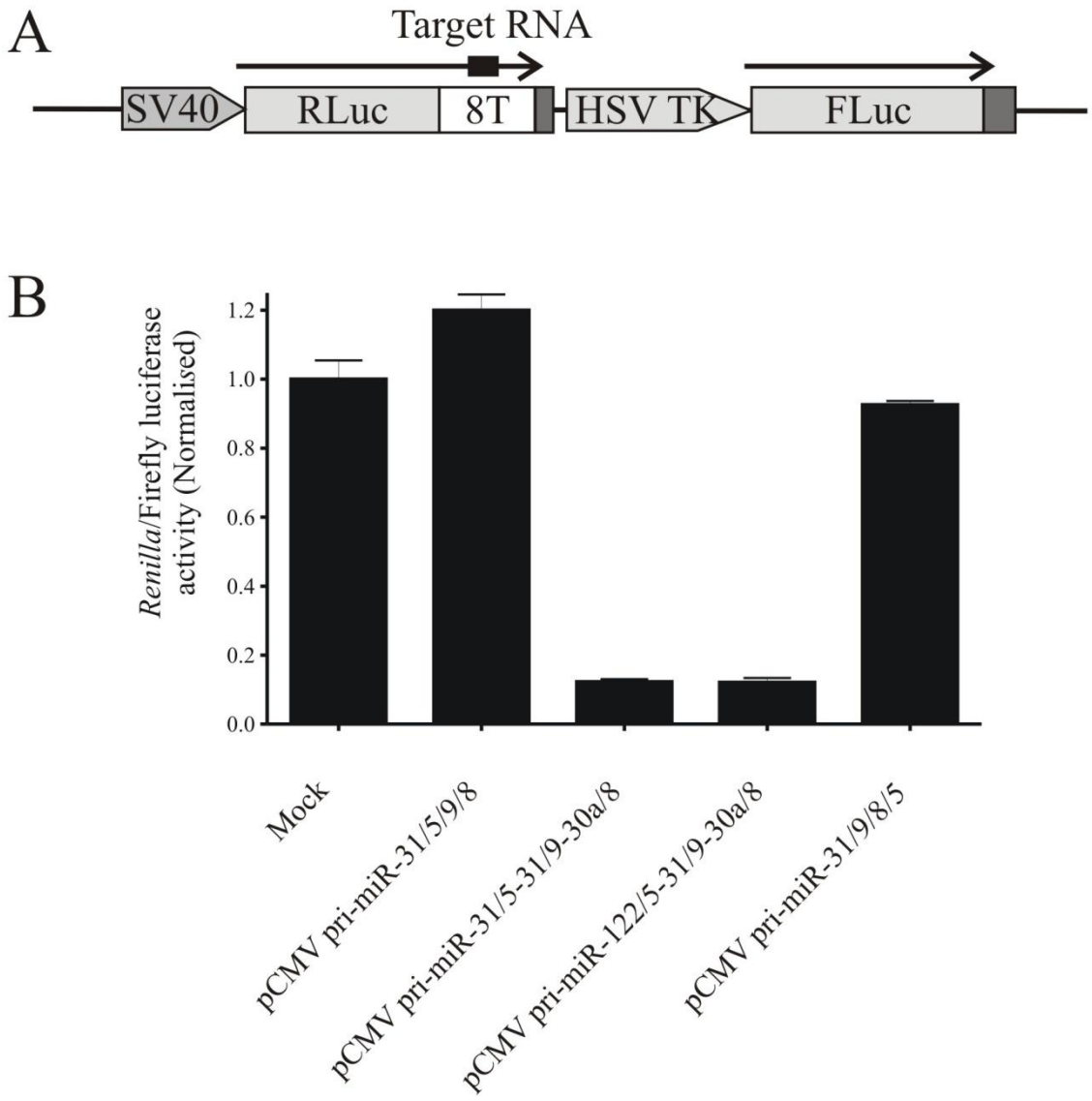

Figure 4.15: <u>Indvidual target silencing by modified pri-miR shuttle sequences.</u>

(A) Schematic representation of individual 8 target vector. (B) psiCHECK-8T was co-transfected with the indicated polycistronic pri-miR shuttle cassettes. Ratios of *Renilla* to Firefly luciferase activity is shown with SEM.

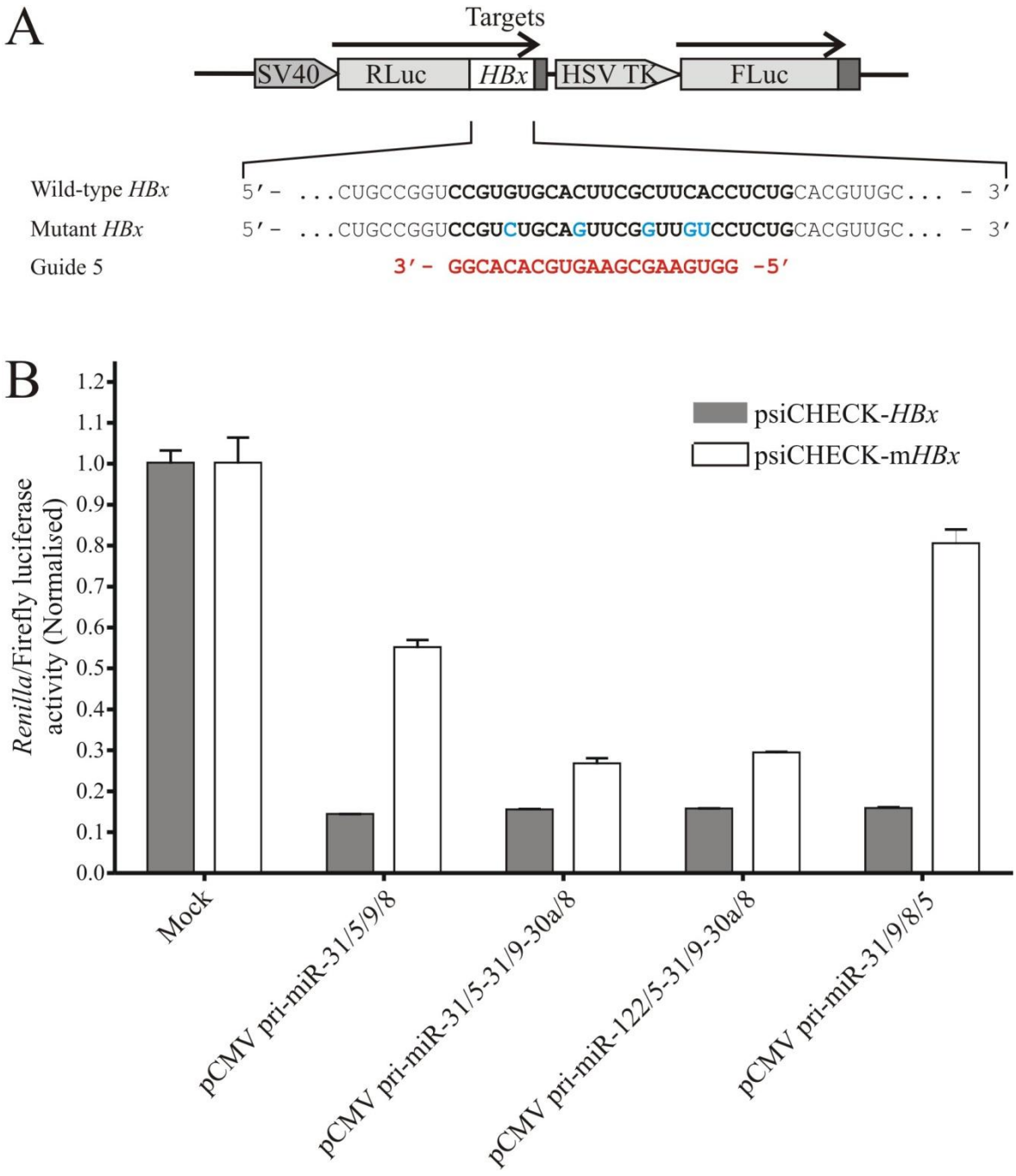

Figure 4.16: Mutant target silencing by modified pri-miR shuttle sequences.

(A) Diagrammatic representation of the wild-type and mutant *HBx* target vectors. (B) psiCHECK-*HBx* (grey bars) or psiCHECK-mHBx (white bars) was co-transfected with the indicated polycistronic expression plasmids. Average *Renilla* to Firefly luciferase activity is shown with SEM indicated by the error bars.

4.4 DISCUSSION

Until recently attempts to create RNAi expression cassettes that express effecter sequences from Pol II promoters have yielded constructs that exhibit variable efficacy and applicability (152-154). This is likely as a result of attempting to express traditional shRNA sequences from Pol II promoters. The development of pre- and pri-miR shuttle sequences as RNAi effecters has allowed other important characteristics of natural miRNA biogenesis to be exploited. First pri-miRNA are typically transcribed from Pol II regulatory elements and consequently pri-miR shuttle sequences have the potential for Pol II transcription. In the previous Chapter embedding anti HBV guides within RNAi effecters that mimic naturally occurring pri-miRNA was shown to enable these sequences to be transcribed from Pol II promoters without compromising silencing activity. Subsequently liver-specific human and viral (HBV) Pol II regulatory elements were assessed for their ability to express the pri-miR-122 shuttle sequences in a tissue–specific manner. Though expression of the *Firefly luciferase* transgene was greatest from the constitutively active CMV immediate early promoter enhancer, expression of the transgene from the liver-specific promoters was limited to the liver-derived cell line Huh7. The A1AT and HBV core promoter, which exhibited the strongest transgene expression of the liver-specific promoters, were further analysed for pri-miR-122 shuttle sequence expression. Silencing of HBV gene expression was only observed in a liver-derived cell line when HBV DNA had been co-transfected with the liver-specific pri-miR-122 expression cassettes. This strongly implies that in cultured mammalian cells liver-specific expression of the pri-miR-122 shuttle sequences was achieved. *In vivo*, only the A1AT expression cassette was able to knock down HBV replication, however silencing was not maintained for longer than 5 days. Further characterisation of candidate liver-specific Pol II regulatory elements is

therefore necessary to improve tissue-specific expression of these pri-miR shuttle sequences (e.g. by improving promoter strength). One option that has been explored was to combine a murine alpha-fetoprotein enhancer with a minimal albumin promoter sequence (155). This chimaeric promoter was shown to exhibit improved strength and liver-specificity and was demonstrated to effectively express anti HBV pre-miR-30 shuttle sequences. Numerous naturally occurring miRNA such as the liver-abundant miR-122 exhibit tissue-specificity and the promoter elements of these miRNA may offer an alternative for the design of tissue-specific pri-miR shuttle sequences. Furthermore Pol II cassettes can be designed to only express the pri-miR shuttle sequence upon HBV infection (through the transactivation potential of HBx) to limit effecter expression to actively infected cells. Preliminary data to assess transactivation by HBx of liver-specific promoters (A1AT, BCP and murine transthyretin (mTTR) promoter) driving Firefly luciferase expression indicate that further development of promoter sequences is necessary (P. Arbuthnot, personal communication).

The second feature conferred to pri-miR shuttle sequences is the potential for the generation of polycistronic expression cassettes. Multiple miRNA are often processed from a number of pre-miRNA structures that are clustered on a single pri-miRNA transcript (156). This characteristic therefore enables multiple shuttle sequences to be clustered together as a single polycistronic pri-miR shuttle sequence. To assess the feasibility of such an approach, trimeric Pol II-driven pri-miR-31 shuttle expression cassettes were generated from the individual pri-miR-31/5, pri-miR-31/8 and pri-miR-31/9 shuttle sequences. As predicted, multiple effecter sequences were produced from each of the single multimeric expression cassettes. Furthermore functional analysis revealed that each of the polycistronic pri-miR-31 shuttle expression cassettes was able to effectively silence

multiple targets. Interestingly some degree of interference was observed within pri-miR-31 clusters of a particular order. Whenever the pri-miR-31/8 shuttle sequence occurred downstream of the pri-miR-31/9 sequence the miR-31/8 guide was not produced. Absence of pri-miR-31/8 processing within these clusters corroborated functional analysis. Computer-aided prediction of secondary structure of the pri-miR-31/5/9/8 and pri-miR-31/9/8/5 clusters did not reveal any difference as compared to the other polycistronic shuttle sequences. One possibility is that preferential processing of the pri-miR-31/9 shuttle sequence causes subsequent RNA folding which interferes with further pri-miR-31/8 processing. Nevertheless the modular nature of the polycistronic pri-miR-31 shuttle design allowed for the creation of a number of constructs that produce multiple RNAi effecter sequences. Furthermore replacing the pri-miR-31/8 shuttle sequence with that of a pri-miR-30a shuttle restored functionality to the 5/9/8 trimer.

The present study describes the vast potential presented by the generation of pri-miR shuttle expression cassettes capable of producing effective silencing effecter sequences from RNA Pol II transcription. Achieving tissue-specific expression of RNAi effecter sequences for example mitigates the need for strategies that are designed to specifically deliver expression cassettes to target tissues. At the least tissue-specific expression cassettes would complement such strategies. In addition to tissue-specific expression of RNAi effecters, Pol II regulatory elements also allow inducible regulation of expression and may prove to be important to limit toxic effects that arise from the expression of foreign sequences. A multi-targeted approach to silence viral genes has become especially important since the description of the emergence RNAi escape mutants (112-114, 117). An RNAi approach that prevents emergence of resistant viral mutations would be an invaluable therapeutic tool. The polycistronic pri-miR-31 shuttle expression

cassettes described here were designed to specifically target HBV. The application of this polycistronic approach to other highly mutable viruses like HIV, HCV and poliovirus is just as feasible. The value of combining a polycistronic pri-miR shuttle with a tissue-specific promoter remains to be assessed.

5 CONCLUSION

In recent years considerable resources have been invested in the development of RNAi-based therapeutics to treat genetic and infectious diseases. Initial studies established the feasibility of exploiting the RNAi pathway to silence pathology-causing genes and further development of expressed anti HBV RNAi effecters is reported here. Subsequently these sequences were further developed to improve RNAi as an anti HBV therapeutic strategy. Specifically guide sequences of naturally occurring pri-miRNA were replaced with anti HBV guide sequences of U6 shRNA described here. Pol III- and Pol II-driven pri-miR shuttle sequences were shown to effectively silence viral replication *in vitro* and *in vivo*. Concentrations of anti HBV guide sequences produced from the pri-miR shuttles were significantly lower than guide strands produced from U6 shRNA cassettes. Viral knockdown however was comparable to that achieved by the shRNA sequences indicating the potency of the pri-miR shuttles. The Pol II-driven pri-miR shuttle expression cassettes were adapted as liver-specific expression cassettes as well as multimeric pri-miR shuttle expression cassettes. Liver-specific effecter expression was achieved by placing the pri-miR-122 shuttles under the transcriptional control of the human A1AT promoter as well as the HBV BCP. Polycistronic pri-miR shuttles were created from individual pri-miR-31, pri-miR-30a/8 and pri-miR-122/5 sequences and shown to be capable of silencing multiple target sequences. Significant progress has been made in the development of RNAi-based therapeutics for the management of chronic HBV infection. However, for the promise of this therapeutic modality to be fully realised in clinical application, a number of hurdles need to be overcome. Persistence of cccDNA, efficient delivery of therapeutic sequences, limiting off-target effects and refining dose of effecter sequences are key issues that need to be addressed.

The stability of cccDNA has significant implications for the development of anti HBV RNAi-based therapeutics. Using a surrogate model of HBV infection Starkey et al. demonstrated that a U6 shRNA administered post-infection resulted in suppression of viral replication, however established cccDNA levels remained unaffected (157). This study has important implications for HBV therapeutic interventions that target viral replication. One implication is that silencing may need to be maintained for prolonged periods of time as viral replication would be reinitiated from the pool of stable cccDNA when the therapeutic is removed. The pri-miR shuttles described in this study are compatible with the use of recombinant helper-dependent adenoviral vectors, lentiviral vectors and AAVs. Long-term silencing that can be achieved with the use of these vectors may, given enough time, diminish cccDNA levels. RNAi-mediated transcriptional gene silencing (158, 159), though debatable in mammalian systems, may offer a means of inactivating episomal HBV cccDNA. Transcriptional inactivation of cccDNA would allow viral replication to be permanently silenced. Zinc finger proteins (ZFPs) represent an alternative gene therapy strategy that has the potential silence cccDNA transcription (160). Typically ZFPs are designed to bind a specific DNA sequence and regulate the gene of interest by interfering with its transcription (160). Artificial ZFPs are designed with up to six zinc finger domains each composed of two β sheets and an α helix (161). Each zinc finger domain recognises a DNA sequence of 3 bp and as such ZFPs can be designed to specifically recognise an 18 bp DNA sequence. Zinc finger nucleases, artificial ZFPs that are capable of recognising and cleaving a specific DNA sequence, also holds potential as an anti HBV therapeutic. ZFPs and RNAi-mediated transcriptional gene silencing however, are still in their infancy and further development is required before application of these gene silencing mechanisms can be realised.

Immune stimulation, saturation of the miRNA biogenesis pathway and off-target effects are some of the safety issues surrounding the use of RNAi activators for therapy. Immune stimulation, most notably by synthetic siRNA, is well characterised and means of avoiding activation of the innate immune response by chemical modification of siRNAs has been thoroughly researched (89, 90, 92, 95, 96). In contrast saturation of the miRNA pathway is a potential complication of expressed RNAi effecters, specifically effecter sequences produced in excessive amounts. The present undertaking and work by others that employ pre-miR shuttle expression cassettes (146, 155, 162, 163) represent significant advances in limiting toxic effects of expressed RNAi effecters. Non-specific silencing of gene expression as a result of off-target effects by RNAi effecter sequences remains a concern. Off-target effects may occur through unintended passenger strand selection. Naturally occurring miRNA exhibit strand selection bias, which may improve guide selection of shuttle sequences. Additionally off-target effects may occur as a result of complementarity of cellular genes to the seed region of effecter sequences (60, 164). The pri-miR shuttle cassettes described here are capable of potent silencing. These cassettes may therefore reduce the risk of off-target effects as the concentration at which the shuttle sequences are effective is very low. Avoiding disruption of the endogenous miRNA pathway, negligible immunostimulation and potential reduced off-target effects through dose regulation are therefore some of the benefits of expressed pri-miR shuttle sequences.

Expressed miR shuttle sequences offer a number of advantages over the traditional Pol III-driven shRNA effecter sequences. Importantly miR shuttles can be expressed from Pol II regulatory elements. In this study pri-miR shuttle expression cassettes were shown to relieve saturation effects observed with U6 promoter-driven shRNA sequences. Additionally pri-miR shuttle sequences allowed for the generation of multimeric effecter

expression cassettes. The multi-targeted approach afforded by polycistronic pri-miR shuttle cassettes is of potential benefit to limit emergence of viral escape mutants. The identification of an HBV mutant strain that is resistant to single RNAi effecter sequences has already been described (117). Though the pri-miR shuttle sequences in this study have been generated to target HBV the process described here is applicable to other viral targets. The polycistronic pri-miR shuttle system may be of benefit to limit emergence of HCV, HIV and poliovirus resistant mutants. Computational analysis has revealed that four effective RNAi effecter sequences acting simultaneously are necessary to prevent emergence of HIV-1 escape mutants (165). HBV is not as mutable as HIV and as a consequence fewer than four effecter sequences are likely necessary to limit emergence of resistant mutants. In addition to the multimeric system, tissue-specific and inducible expression of RNAi effecters that can be achieved with pri-miR shuttles may also be applied to silence pathology causing genes other than those of HBV.

Mature miRNA are invariably produced from at least one pri-miRNA sequence. miR-16 for example is processed from two different precursors, the pri-miR-16-1 and pri-miR-16-2 stem loop sequences (133). Both pri-miR-16-1 and pri-miR-16-2 produce miR-16 as the major product and miR-16-1 and miR-16-2 as minor products, respectively. Since tertiary structure, and therefore sequence, plays an important role in miRNA biogenesis pre- and pri-miRNA sequence likely depends on the sequence of the miRNA duplex. The method adopted in this study was to replace the major guide sequence of naturally occurring miRNA with anti HBV guide sequences. Pre- and pri-miR shuttles therefore rely on existing miRNA scaffolds/backbones to ferry guide sequences for RNAi activation. The next stage in the evolution of expressed RNAi activators will be the creation of completely artificial pri-miR shuttles with the scaffold tailor-made for the guide sequence it needs to

ferry. Furthermore a growing body of evidence suggests that tissue-specific expression of miRNA may not rely exclusively on promoter activity (166, 167). Sequence motifs within certain miRNA precursors have been identified that are necessary for binding of a co-factor, KSRP (K homology domain-type splicing regulatory protein), which in turn facilitates Drosha binding and processing (166). The presence or absence of KSRP determines whether the mature miRNA accumulate or not. Exploiting these features of endogenous miRNA may therefore be used to further improve regulation of pri-miR shuttles. Achieving these goals will require comprehensive understanding of the structural and sequence requirements of miRNA and their precursors and interaction of these sequences with the RNAi machinery. The benefits of expressed RNAi activators will only be realised with the implementation of concerted efforts to develop effective delivery vehicles. Discoveries in RNAi are constantly being made and continue to drive research efforts to overcome these hurdles.

6 APPENDIX A

A1 STANDARD LABORATORY TECHNIQUES

A1-1 Tissue culture

Reagents

RPMI medium (RPMI)

One litre of RPMI was made as follows: 10.4 g of RPMI-1640 (Gibco BRL, United Kingdom), HEPES (5.0×10^{-3}M), Na_2SeO_3 (3.0×10^{-8}M), 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 $NaHCO_3$ were dissolved in water. The medium was sterilised by filtration.

1000× Pen/Strep

One gram of Streptomycin and 0.61 g of Penicillin were dissolved in 10 ml of deionised water and the solution filter sterilised.

DMEM supplied in liquid form by Sigma-Aldrich, MI, USA.

FCS (Gibco BRL, United Kingdom)

Saline + 0.01% EDTA

0.5× Trypsin

Five hundred microlitres of a 10× Trypsin (Gibco BRL, United Kingdom) stock solution was made up to 10 ml and filter sterilised.

Opti-MEM (Gibco BRL, United Kingdom)

Protocol for propagation of cells

Huh7, HEK293 and 116 cells were maintained in a humidified incubator at 37°C and 5% CO_2 in RPMI or DMEM growth medium supplemented with 2.5-10% FCS and antibiotics. The cells were subcultured upon reaching a density of 90-100% by washing once with saline and subsequently incubating for 5 minutes 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. Trypsinisation was omitted for HEK293 and 116 cells. The cells were then dislodged from the culture dish by gentle aspiration. An equal volume of growth medium was added to inactivate the trypsin. Cells were added to a sterile 56 cm^2 tissue culture dishes or 60 cm^2 tissue culture flasks at the desired density (30-40%). Ten millilitres of RPMI or 15 ml of DMEM were 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.

Transfection of cells

For transfections cells were seeded at a density of 40-50% per well (see Table A1 below for cell numbers) and allowed to grow overnight at 37°C and 5% CO_2 in growth medium supplemented with 2.5-10% FCS and antibiotics. Prior to transfection the growth medium was replaced with fresh RPMI or DMEM supplemented with 2.5-10% FCS but lacking antibiotics.

On the day of transfection DNA:Opti-MEM and Lipofectamine:Opti-MEM mixes were prepared as indicated in Table A1. The mixes were incubated at room temperature for 5 minutes added together and incubated for an additional 20 minutes. The DNA:Lipofectamine mixes were added to cells and the transfections were allowed to proceed at 37°C in a humidified incubator for a minimum of 5 hours.

Table A1: Seeding of cells and amounts of DNA and reagents used for transfections.

Cell Type	6-well plate (per well)	12-well plate (per well)	24-well plate (per well)
Huh7	300 000 cells	150 000 cells	75 000 cells seeded
HEK293/116	500 000 cells seeded	250 000 cells seeded	120 000 cells seeded
Transfection Mix			
DNA **Opti-MEM**	4-9 µg 200 µl	2 µg 100 µl	1 µg 50 µl
Lipofectamine™ 2000 **Opti-MEM**	4-9 µl 200 µl	2 µl 100 µl	1 µl 50 µl

A1-2 EndoFree® Plasmid Maxi Kit plasmid preparation

Reagents

Luria Bertani medium

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

1000× Ampicillin

One hundred milligrams of ampicillin (Roche Diagnostics GmbH, Germany) was dissolved in 1 ml of 50% ethanol. Stored at -20°C.

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

Protocol

Three hundred millilitres of Luria Bertani medium containing 100 µg/ml ampicillin 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 collected by centrifugation at 4000 rpm for 20 minutes at 4°C and the pellet resuspended in 10 ml 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 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 Buffer P3 (3 M Potassium acetate, 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 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 30 ml of 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 500 and allowed to drain by gravity flow. The column was washed with 30 ml Buffer QC (1 M NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol), twice. The plasmid was eluted from the column with 15 ml 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 and the solution centrifuged at 8000 rpm for 1 hour at 4°C. The DNA pellet was washed with endotoxin-free 70% ethanol and centrifuged for an additional hour at 8000 rpm and 4°C then air-dried and resuspended in an appropriate volume of endotoxin-free TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

A2 PUBLICATIONS

A2-1 Selected Research Publications derived from work presented in this Thesis

doi:10.1016/j.ymthe.2005.10.013

ARTICLE

Effective Inhibition of HBV Replication *in Vivo* by Anti-*HBx* Short Hairpin RNAs

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Exploiting the RNA interference pathway has shown promise for developing novel and effective treatment of hepatitis B virus (HBV) infection. To advance this approach, we analyzed the antiviral efficacy of a panel of 10 Pol III U6 promoter-encoded short hairpin RNAs (shRNAs) that target conserved sequences of the oncogenic *HBx* open reading frame. To facilitate intracellular processing, the shRNAs included mismatches in the 25-bp stem region and a terminal loop of miRNA-23. Two shRNAs (shRNA 5 and shRNA 6) showed knockdown of HBV markers by 80–100% in transfected hepatocytes and also in a murine hydrodynamic injection model of HBV replication. Intracellular processing of hairpin RNA with the intended strand bias correlated with antiviral efficacy. Moreover, markers of HBV replication were inhibited without inducing genes associated with the nonspecific interferon response. To assess the antiviral efficacy of the shRNAs in a context that is similar to natural HBV infection, shRNA-encoding cassettes were tested against the virus in a HBV transgenic murine model. When delivered using recombinant adenovirus vectors, U6 shRNA 5 and U6 shRNA 6 mediated significant HBV knockdown. Collectively, these observations indicate that U6 shRNA 5 and U6 shRNA 6 are promising candidates for therapy of chronic HBV infection.

Key Words: RNAi, short hairpin RNA, HBV, hydrodynamic injection, HBV transgenic mice, recombinant adenovirus, interferon response

INTRODUCTION

Persistent hepatitis B virus (HBV) infection remains an important global public health problem with an estimated 6% of the world's population chronically infected with the virus [1–3]. The clinical course of HBV infection is not constant and may be influenced by properties of individual viral variants [4,5]. For example, chronic infection with HBV subgenotype A1, which is hyperendemic to South Africa, is associated with a particularly high risk of hepatocellular carcinoma [6]. Licensed treatments for HBV infection, which include interferon-α and nucleoside (lamivudine) and nucleotide (adefovir) analogues, produce a long-term response in only a minority of chronic carriers [7,8]. The continued search for an effective therapy to prevent life-threatening complications thus remains a priority. HBV has a compact genome (Fig. 1) that makes it well suited to developing antiviral therapies that are based on nucleic acid hybridization. Overlapping open reading frames (ORFs) cover the entire viral genome [9] and conserved regions may encode more than one protein as well as HBV *cis* elements required for viral replication. *HBx* is an example of a multifunctional HBV sequence. It encodes the HBx protein, which has been implicated in HBV-mediated hepatocarcinogenesis (reviewed in [1]) and is required for HBV replication [10,11]. *HBx* also overlaps with the 3′ end of the polymerase ORF, direct repeats that are required during virus reverse transcription, and regions important for transcription control (basic core promoter and negative regulatory elements). HBV transcripts are usually unspliced and have a common 3′ end that includes *HBx* (Fig. 1). The key role of HBx in viral replication and hepatocarcinogenesis, together with the presence of the conserved *HBx* sequence in all of the HBV transcripts, makes it a good target to develop nucleic acid hybridization-based therapy.

Exploiting the RNA interference (RNAi) pathway has shown exciting promise for the development of novel

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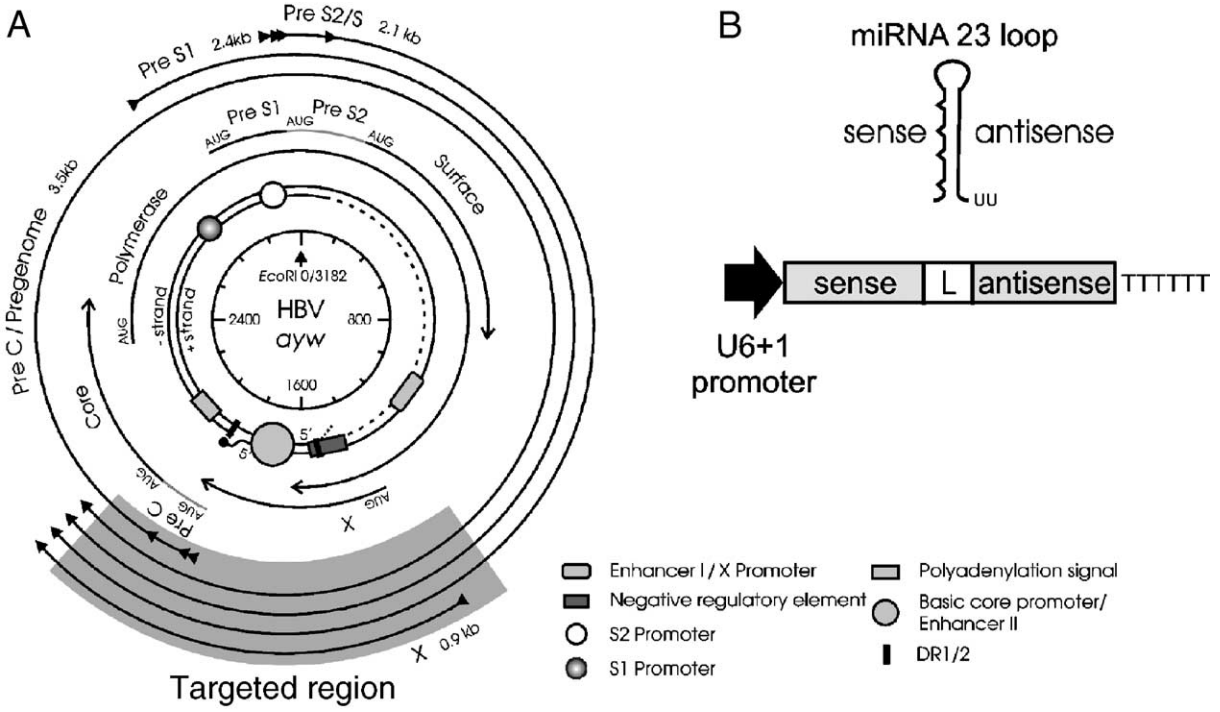

FIG. 1. HBV target sites and shRNA-encoding vectors. (A) Organization of the hepatitis B virus genome showing sites targeted by shRNA sequences. Coordinates of the genome are given relative to the single *Eco*RI restriction site. Partially double-stranded HBV DNA comprises + and − strands with cohesive complementary 5′ ends. The *cis* elements that regulate HBV transcription are represented by the circular and rectangular symbols. Immediately surrounding arrows indicate the viral open reading frames (with initiation codons) that encompass the entire genome. Four outer arrows indicate the HBV transcripts, which have common 3′ ends that include *HBx*. (B) (Top) Schematic illustration of anti-HBV shRNA indicating mismatches in the sense strand, miRNA 23 loop, and sequence of two U residues that are derived from the transcription termination signal. The DNA cassette with U6 promoter, sense, miRNA 23 loop (L), and antisense-encoding sequences is indicated below.

antiviral therapy. Naturally, the mechanism involves processing of larger dsRNA by Dicer to form short interfering RNA (siRNA) duplexes [12–14]. One of the strands of the siRNA is incorporated into the RNA-induced silencing complex (RISC) and acts as a guide to target degradation of complementary cytoplasmic RNA. Exogenous silencing is typically induced by synthetic RNA or transcripts produced from Pol III cassettes [12,15–17]. A number of studies have demonstrated that activation of RNAi has potential for the development of novel treatments of HBV infection [18–27]. As the pathway of RNAi becomes better understood, more rational approaches to the design and characterization of potentially therapeutic RNAi inducers are being sought. Avoidance of the interferon response, efficacy at a low concentration of RNAi-inducing sequences, specificity for the viral cognate, and processing of the duplexes with appropriate strand bias are important considerations. In this study, we report that anti-subgenotype A1 short hairpins RNAs (shRNAs) targeted to *HBx*, which have features of endogenous micro RNA (miRNA) that allow for influencing strand bias, are powerful and specific inhibitors of HBV replication. Inhibition of HBV replication was observed *in vitro* in transfected cells, *in vivo* after gene transfer using hydrodynamic DNA injection, and in HBV transgenic models. Sequences found to be most effective also have homology to other HBV genotypes and potentially have broad therapeutic applications.

RESULTS AND DISCUSSION

HBV Targets and Design of DNA Encoding Antiviral shRNA Sequences

We generated a panel of 10 U6 shRNA cassettes, which target the most conserved sites of the *HBx* sequence from South African genotype A1 isolates of the virus [28]. We designed hairpins to comprise a 25-bp stem with four GU or CA mismatches in the shRNA strand of HBV sense polarity, and the *HBx* antisense sequence was perfectly complementary to the viral target (Fig. 1 and Table 1). The 10-nt loop sequence was common to all of the hairpins and was derived from miRNA-23 [29]. These features were incorporated to facilitate intracellular processing and may also diminish non-specific effects of activating the interferon response [30,31].

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TABLE 1: Nucleotide sequences of PCR reverse primers used to generate HBV U6 shRNA cassettes

HBV target[a]	Name	Primer sequence
1168–1192	U6 shRNA 1.1	5′-**TGACG**TGACAGGAAGCGTTAGCAGACACTTGGCATAGGCCCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 1.2	5′-CCCAGATCTACGCGTAAAAAAGGTCTGTGCCAAGTGTTTGCTGACGTGACAGGAAGCGTTA-3′
1432–1456	U6 shRNA 2.1	5′-**GGACG**TGACAGGAAGCGTTCGTGGGATTCAGCGTCGATGGCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 2.2	5′-CCCAGATCTACGCGTAAAAAACCGTCGGCGCTGAATCCCGCGGACGTGACAGGAAGCGTTC-3′
1514–1538	U6 shRNA 3.1	5′-**CTTTA**TGACAGGAAGCAAAGAGAGATGCGCCCCATGGCCGCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 3.2	5′-CCCAGATCTACGCGTAAAAAACGACCACGGGGCGCACCTCTCTTTATGACAGGAAGTAAAG-3′
1518–1542	U6 shRNA 4.1	5′-**ACGCG**TGACAGGAAGCGTGTGAAGAGAGGTGTGCCCTGTGCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 4.2	5′-CCCAGATCTACGCGTAAAAAACACGGGGCGCACCTCTCTTTACGCGTGACAGGAAGCGTGT-3′
1575–1599	U6 shRNA 5.1	5′-**CTCTG**TGACAGGAAGCAGAGGCGAAGCAAAGCGCACACGACGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 5.2	5′-CCCAGATCTACGCGTAAAAAACCGTGTGCACTTCGCTTCACCTCTGTGACAGGAAGCAGAG-3′
1580–1604	U6 shRNA 6.1	5′-**CACGT**TGACAGGAAGATGTGTAGAGGTGAAGCGAGGTGTACGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 6.2	5′-CCCAGATCTACGCGTAAAAAATGCACTTCGCTTCACCTCTGCACGTTGACAGGAAGATGTG-3′
1640–1664	U6 shRNA 7.1	5′-**GGACT**TGACAGGAAGAGTTCTTTTATGTAGGACTTTGGGCCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 7.2	5′-CCCAGATCTACGCGTAAAAAAGCCCAAGGTCTTACATAAGAGGACTTGACAGGAAGAGTTC-3′
1678–1702	U6 shRNA 8.1	5′-**GAGGC**TGACAGGAAGGCTTCAAGGTTGGTTGTTGACGTTGCGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 8.2	5′-CCCAGATCTACGCGTAAAAAACAATGTCAACGACCGACCTTGAGGCTGACAGGAAGGCTTC-3′
1774–1798	U6 shRNA 9.1	5′-**TTGGT**TGACAGGAAGACTAATTTGTGCCTACAGCTTCTTACGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 9.2	5′-CCCAGATCTACGCGTAAAAAATAGGAGGCTGTAGGCATAAATTGGTTGACAGGAAGACTAA-3′
1863–1887	U6 shRNA 10.1	5′-**CTTGG**TGACAGGAAGCCAAAGCACAACTCGGAGGCTCGAACGGTGTTTCGTCCTTTCCACA-3′
	U6 shRNA 10.2	5′-CCCAGATCTACGCGTAAAAAATTCAAGCCTCCAAGCTGTGCCTTGGTGACAGGAAGCCAAG-3′

Overlapping sequences are underlined. Mismatches incorporated into the sense strand of the hairpin stems are indicated with a double underline. The region complementary to the U6 promoter is italicized. Sequences encoding *HBx* antisense RNA are bold.

[a] HBV coordinates are relative to the single *Eco*RI site as indicated in Fig. 1.

Effects of shRNA on HBsAg Secretion from Transfected Cells and Assessment of Efficacy *in Situ*

Initially, to assess efficacy against HBV, we cotransfected Huh7 cells with hairpin-encoding sequences and the pCH-9/3091 HBV target plasmid [32]. Variable efficacy of knockdown of viral antigen secretion was achieved by each of the sequences (Fig. 2A). U6 shRNA 5 and U6 shRNA 6 were most effective and plasmids encoding these hairpins decreased HBsAg concentration in the culture supernatant to less than 5% of the mock-treated cells' level. Transfection of the vector encoding U6 shRNA 10 had little if any effect on HBsAg secretion. We corroborated these data using a reporter gene plasmid (pCH-eGFP) to measure knockdown *in situ* (Fig. 2B) [33]. In pCH-eGFP, the preS2/S sequence of pCH-9/3091 was replaced with the enhanced green fluorescent protein (eGFP) ORF, with the targeted *HBx* ORF remaining intact. Cotransfection of pCH-eGFP with shRNA-encoding vectors allows for the convenient measurement of anti-HBV shRNA efficacies using flow cytometry and fluorescence microscopy. Analysis showed that the number of cells expressing eGFP was diminished significantly by U6 shRNA 5 and U6 shRNA 6, while U6 shRNA 10 was least effective (Fig. 2C). The sites targeted by these shRNAs 5 and 6 overlap each other, which suggests that this region of *HBx* is particularly susceptible to RNAi-mediated knockdown. Activators of the RNAi pathway can potentially stimulate nonspecific inflammatory effects by activating the interferon response, which in turn is capable of inducing cell death by apoptosis [34,35]. To exclude this effect, we measured mRNA from three genes that are key components of the interferon response: IFN-β, OAS1, and MxA (Supplemental Fig. 1). Expression of interferon response genes was not increased in Huh7 and HEK293 cells that were transfected with shRNA-expressing plasmids. These observations indicate that the knockdown of HBsAg secretion and eGFP marker expression was specific to activation of RNAi and not a result of causing interferon response-mediated programmed cell death.

Effects of shRNAs on HBV RNA Concentrations

We extracted total cellular RNA from Huh7 cells that had been transiently transfected with shRNA-encoding plasmids together with HBV target DNA. Analysis using Northern blot hybridization confirmed that shRNA 5 efficiently reduced the concentration of HBV RNA (Fig. 3A). When normalized for loading differences using the GAPDH mRNA band intensity, U6 shRNA 5 diminished HBV transcript concentration to approximately 35% of the control value. Concentrations of HBV transcripts in cells cotransfected with the U6 shRNA 10 vector were 90–100% of the controls. Cotransfection of Huh7 cells with U6 shRNA 9 had an intermediate knockdown effect on HBV RNA and diminished concentrations by approximately 40%. These data correlate with the observations on the shRNA effects on HBsAg secretion and *in situ* marker gene expression.

shRNA Processing

To assess processing of expressed shRNA sequences, we carried out primer extension analysis on total RNA that was extracted from the transfected Huh7 liver cell line.

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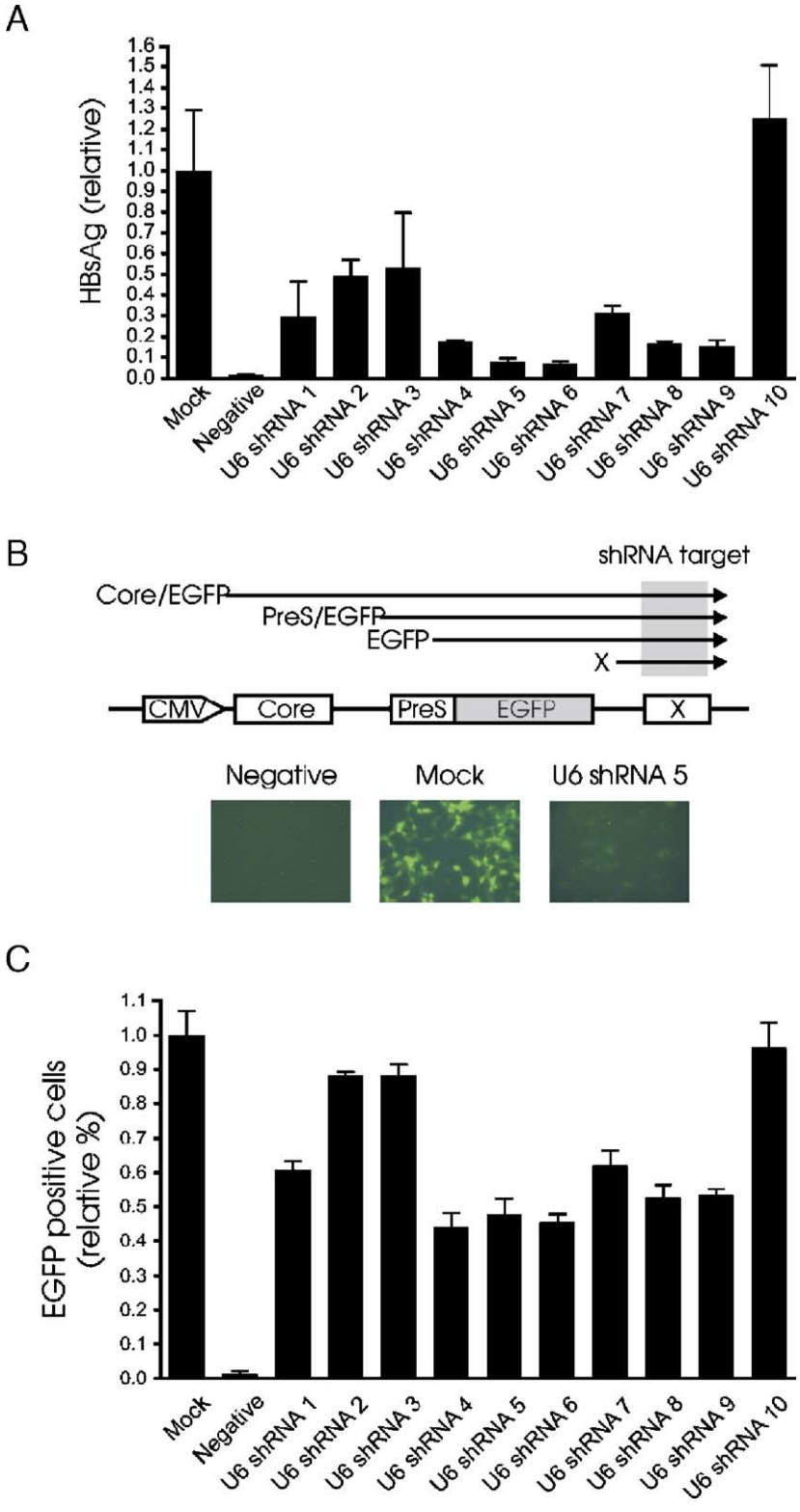

FIG. 2. shRNA-mediated inhibition of HBsAg secretion and HBV–eGFP fusion marker protein expression in transfected cells. (A) Measurement of HBsAg secretion from Huh7 cells cotransfected with indicated shRNA-encoding plasmids together with HBV target plasmid. HBsAg measurements from quantitative ELISA are given as a normalized mean relative to the mock-treated cells. Results are from four independent transfections and the bars indicate the standard error of the mean (SEM). (B) Schematic illustration of plasmid construct pCH-eGFP showing open reading frames, respective transcripts, and sites targeted by shRNAs. The disrupted polymerase ORF is not indicated. Representative fluorescence microscopy fields of Huh7 cells transfected with pCH-eGFP and indicated shRNA-expressing construct are also shown. (C) Quantitative comparison of the percentage of eGFP-positive Huh7 cells detected using flow cytometry after transfection with indicated shRNA-encoding expression vectors. Number of eGFP-positive cells is given as a normalized mean relative to the mock-treated cells, which represents approximately 45% eGFP-positive cells in the total population. Results are depicted as means from four independent transfections (each counting 100,000 events) with the SEM indicated.

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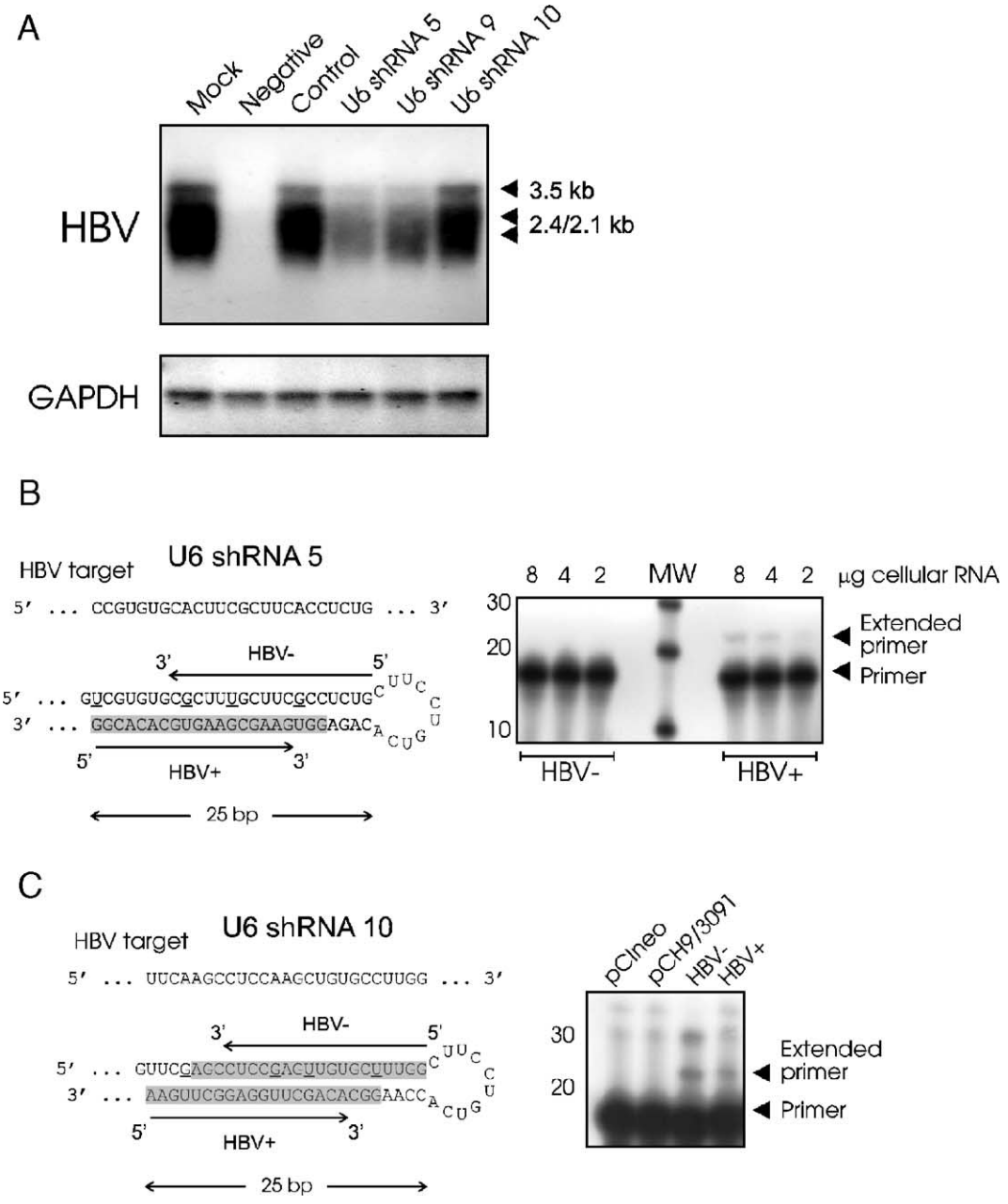

FIG. 3. RNA analysis. (A) Huh7 hepatocytes were transfected with the indicated shRNA-encoding plasmids. Two days after transfection, total RNA was extracted from the cells and analyzed by Northern blot hybridization. The control lane represents analysis of RNA extracted from cells that had been transfected with a plasmid encoding shRNA 5 sequences under transcriptional regulation of the Pol II CMV immediate early promoter/enhancer. Blots were probed for HBV RNA and also GAPDH as a loading control. (B and C) Either radiolabeled HBV^- or radiolabeled HBV^+ oligonucleotides targeting the relevant sequences of shRNA 5 or shRNA 10 were hybridized to total RNA extracted from transfected Huh7 cells and then subjected to primer extension analysis using reverse transcriptase. Sizes of extended fragments were then detected using autoradiography after resolution with denaturing PAGE. The amount of template cellular RNA subjected to reverse transcription is indicated above the lanes and RNA molecular weight marker sizes (nt) are indicated on the left of the autoradiographs.

We radiolabeled oligonucleotide primers that were complementary to the HBV sense or antisense sequence of the hairpin RNA encoded by U6 shRNA 5 or U6 shRNA 10 at their 5′ end, hybridized them to complementary cellular RNA from transfected cells, and then extended them using reverse transcriptase. Extension of end-labeled primers complementary to the U6 shRNA 5 antisense strand generated a 21-nt product that corresponds to the

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putative guide sequence that effects HBV gene silencing (Fig. 3B). A similar primer extension to detect a shRNA-derived *HBx* sense sequence did not generate a signal, and this was confirmed in an overexposed autoradiograph from the same gel. Conversely, when we subjected RNA extracted from U6 shRNA 10-transfected cells to primer extension analysis, both sense and antisense labeled oligonucleotides generated extended products 21 nt in length (Fig. 3C). Moreover, the U6 shRNA 10-encoded sense strand sequence is present at a slightly higher concentration compared to the intended antisense guide. Thus, intracellular processing of the hairpin encoded by U6 shRNA 10 has a slight bias for the nonhybridizing sequence with the same polarity as its viral target. Conversely, the only single-stranded RNA sequence that is detectable from U6 shRNA 5-transfected cells is that which corresponds to the intended antisense guide RNA. Several factors that affect processing and functionality of RNAi effectors have been identified. Included among these is a GC content of 30–52% that is thought to be optimal for efficient unwinding of the duplex while at the same time retaining sufficient stability of interaction of the siRNA guide strand with its target [36–38]. Based on primer extension analysis of shRNA 5, the siRNA derived from this hairpin has 47% GC pairs (9 of 19 pairs in the duplex region). However, the proportion of GC pairs between the shRNA 5-derived guide sequence and the HBV target is higher (62%), and this is a result of the GU mismatch pairs of the shRNA stem that are not present in the hybrid with the HBV target. Thus, it is likely that the relatively lower GC content of the Dicer-processed product facilitates unwinding of the duplex, while the higher GC content of the guide and target pair stabilizes this interaction. Another important factor that determines the processing of RNA duplex is the stability at the 5′ end of the intended guide sequence within the double-stranded RNA [36–38]. With shRNA 5, there is a 5′ GU mismatched base pair at the 5′ end of the antisense strand, and this is likely to facilitate the intended strand bias. Together, these factors are likely to improve processing of shRNA 5 and advance our understanding of the properties of effective anti-HBV RNA sequences.

Efficacy of shRNA-Encoding Plasmids *in Vivo* Using the Murine Hydrodynamic Injection Procedure

To determine anti-HBV efficacy of shRNA *in vivo*, we initially used the hydrodynamic injection procedure to deliver target and shRNA-encoding DNA simultaneously. Using this approach, shRNA 5-encoding plasmid DNA continuously knocked down HBsAg in the serum of mice to a background level over a period of 4 days (Fig. 4A). We also observed an inhibitory effect when we measured HBV core antigen (HBcAg) using immunohistochemical staining in liver sections from mice that had been subjected to hydrodynamic injections (Fig. 4B). The control for DNA delivery efficiency showed that uptake and expression of the *lacZ* marker gene were similar in treated and untreated mice. Comparing efficacies of U6 shRNAs 5, 6, and 10 on day 4 after injection, the production of HBsAg was suppressed completely in mice treated with shRNA 5- or shRNA 6-encoding plasmids (Fig. 4C). However, as was observed in transfected cells, HBsAg concentrations in the serum of mice that were injected with shRNA 10-encoding DNA were not significantly diminished. Similarly, on day 4 HBV viral loads were knocked down to background levels in the mice treated with DNA encoding shRNA 5 or shRNA 6 (Fig. 4D). Again U6 shRNA 10 was less effective, indicating that the knockdown is a sequence-specific phenomenon and is unrelated to generic features of the anti-*HBx* hairpin cassettes. Efficient inhibition of viral replication markers was confirmed when we administered shRNA 5-encoding DNA at a fifth of the molar concentration of HBV target DNA (not shown). Simultaneous injection of 25 μg of synthetic siRNA 5, which has a sequence equivalent to that encoded by U6 shRNA 5, inhibited secretion of HBsAg and viral particle concentration in the serum significantly ($P < 0.05$) (Figs. 4C and 4D). However, the synthetic RNA was also significantly less effective against markers of viral replication than U6 shRNA 5 and U6 shRNA 6 ($P < 0.05$). Sustained production of shRNA from a constitutively active DNA cassette as well as the shorter half-life of RNA compared to plasmid DNA is likely to account for this. Moreover, improved silencing by coupled Dicer processing prior to loading of guide sequences onto the RISC has been reported [39] and may contribute to the enhanced silencing effected by DNA-encoded shRNA compared to synthetic siRNA.

Efficacy of shRNA-Encoding Recombinant Adenovirus Vectors *in Vivo* in HBV Transgenic Mice

We assessed the efficacy of shRNAs against HBV in the context of established constitutive replication of the virus in HBV transgenic mice [40], which mimic natural infection more closely than does the hydrodynamic murine injection model. We incorporated U6 shRNA 5, 6, and 10 expression cassettes into recombinant adenovirus vectors to produce ADV shRNA 5, ADV shRNA 6, and ADV shRNA 10, respectively. Control adenoviruses included a U6 shRNA cassette targeted to the β-galactosidase marker (ADV shRNA LacZ) or no U6 shRNA sequence (ADV eGFP). The recombinant vectors coexpressing eGFP and fluorescence microscopy revealed that approximately 60–80% of hepatocytes expressed adenovirus DNA after tail vein injection with 5×10^9 infectious particles (not shown). Higher doses of adenovirus vectors caused significant hepatic toxicity. Compared to controls and ADV shRNA 10, a single dose of ADV shRNA 5 and ADV shRNA 6 significantly diminished the concentration of HBsAg concentration in the serum over a period of 12

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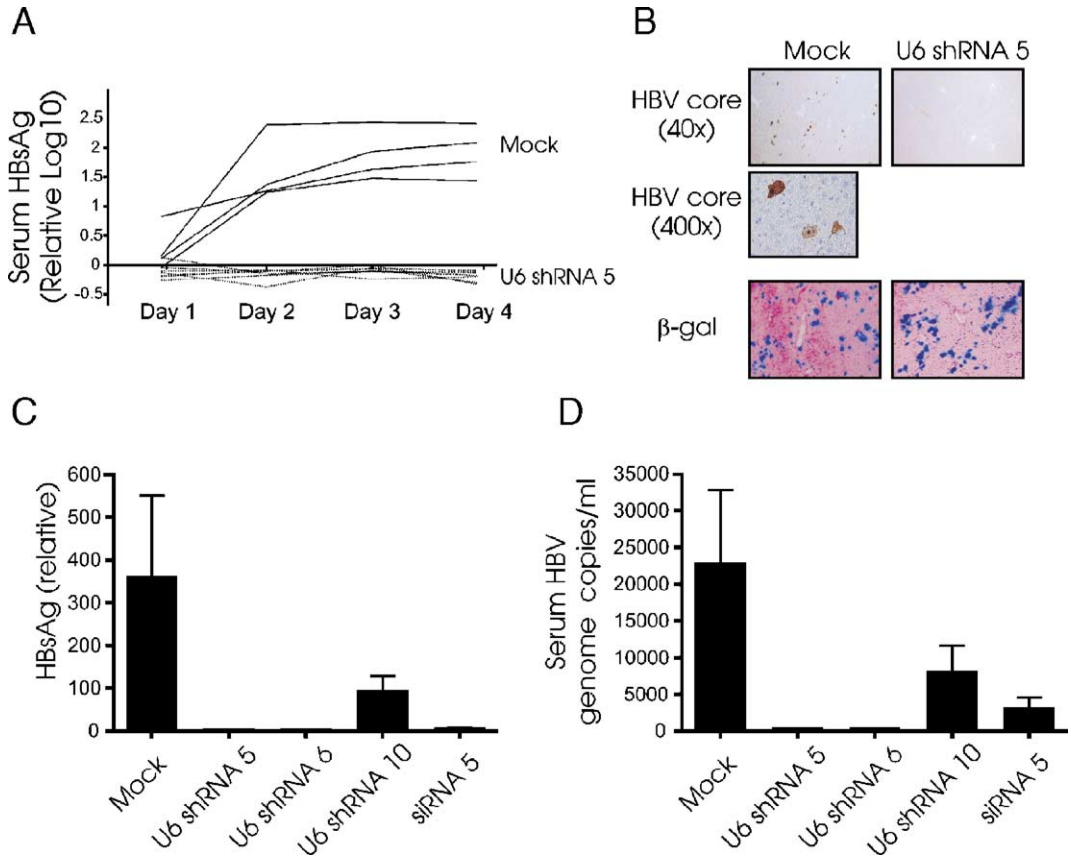

FIG. 4. Effects of shRNA sequences on HBV antigen production in the hydrodynamic injection model of HBV replication. (A) Time course of relative serum HBsAg concentrations, measured using quantitative ELISA, in mice treated with hydrodynamic injection. Graphical depictions of individual measurements of the viral antigen are given on a log scale for four mock- and four U6 shRNA 5-treated animals. (B) Mice subjected to the hydrodynamic injection procedure were sacrificed after 4 days and the livers analyzed using immunohistochemistry to detect the HBcAg. Representative low- and high-power fields are shown for livers from mock- and U6 shRNA 5-treated animals. Frozen sections from the same animals were stained for LacZ activity to confirm similar delivery of DNA to hepatocytes. (C) Serum HBsAg concentrations and (D) viral loads on day 4 after injection of mice using the hydrodynamic injection procedure. Mice were injected with the indicated shRNA-encoding plasmids or synthetic RNA duplex equivalent to shRNA 5 (siRNA 5) together with pCH/9-3091 HBV target DNA. Mock-treated animals received backbone plasmid (pGEM-T Easy) without shRNA-encoding sequences. Viral loads were determined using real-time quantitative PCR and included EuroHep standards to calibrate the virion copy numbers. Groups comprised six to eight animals and the graphs indicate the mean and SEM for each group.

days ($P < 0.05$) (Fig. 5A). Preliminary data also indicate that the inhibition of HBsAg secretion persists for at least 28 days (not shown). This is comparable to a recently reported observation that adenovirus vectors expressing shRNA sequences effected a sustained inhibition of markers of HBV replication [25]. Similarly, compared to control adenovirus vectors, serum HBeAg concentrations were significantly diminished by both ADV shRNA 5 and ADV shRNA 6 at day 12 after administration of the viral vectors ($P < 0.05$) (Fig. 5B). Interestingly, the effects of ADV shRNA 5 and ADV shRNA 6 on HBeAg secretion were less marked than those on HBsAg (approx 2-fold compared to 10-fold inhibition, compare Figs. 5A and 5B). This correlates with the previously reported observation that suggests the 3.5-kb HBV transcript encoding HBeAg is relatively more resistant to RNAi-mediated knockdown [25]. Circulating virion counts were diminished in animals treated with ADV shRNA 5 and ADV shRNA 6, although the effect was less significant than on serum HBeAg and HBsAg concentrations (Fig. 5C). Although target sites of shRNA 5 and shRNA 6 are conserved in the transgenic mice, silencing is less marked than that observed in the hydrodynamic model. There are two possible explanations for this observation. First, levels of HBV in the transgenic mice greatly exceed those of the hydrodynamic injection model (compare viral loads of Figs. 4D and 5C) and complete silencing may be more difficult to achieve when HBV replication is high. Second, under experimental conditions described here, the adenovirus vectors infected 60–80% of hepatocytes. Incomplete delivery of the shRNA-encoding cassettes to all of the HBV-producing cells is likely to account for

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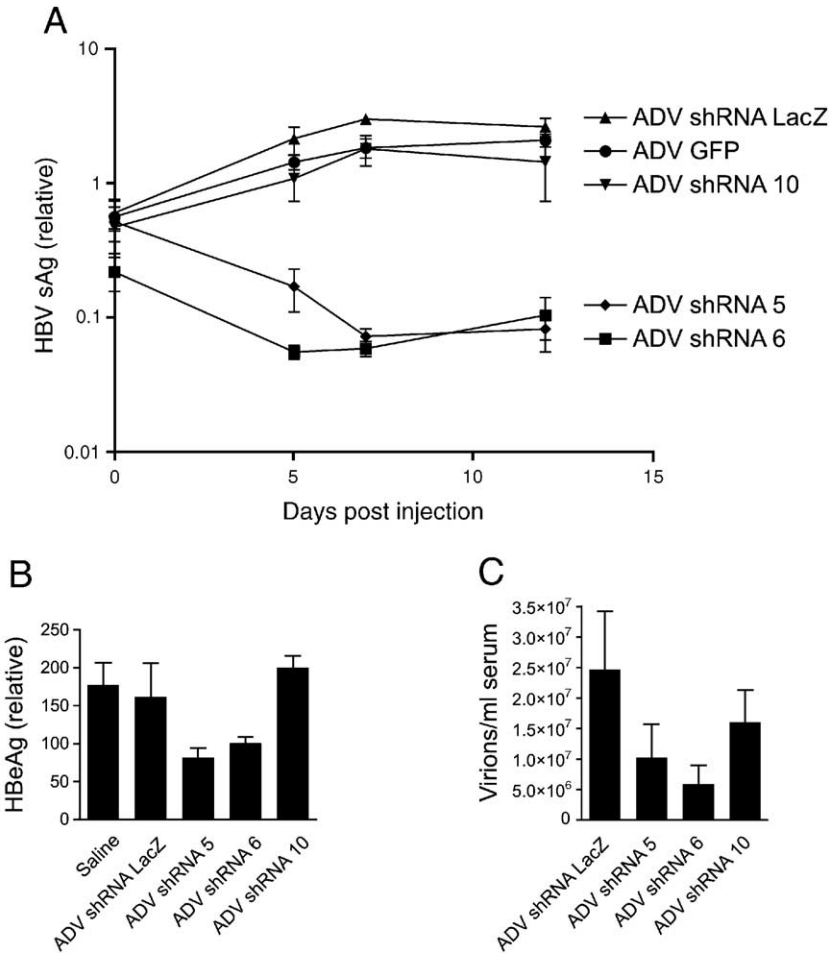

FIG. 5. Effects of recombinant adenovirus-mediated shRNA delivery on viral replication markers in the HBV transgenic mouse model. (A) Time course of relative mouse serum HBsAg concentrations, measured using quantitative ELISA, after tail vein injection of indicated recombinant adenovirus vectors. Relative serum HBsAg concentrations over a period of 12 days are shown. Comparison of (B) serum HBeAg and (C) viral loads at day 12 after injection of animals with saline or indicated recombinant adenovirus.

lower silencing efficiency. Taken together, these data indicate that recombinant adenoviruses incorporating U6 shRNA 5 and U6 shRNA 6 are capable of sustained inhibition of gene expression *in vivo* during constitutive replication of HBV. Moreover, HBV RNA is susceptible to RNAi-mediated silencing and is not protected from silencing by viral proteins.

The demonstration that the anti-*HBx* shRNAs used in this study can successfully inhibit HBV replication indicates that this target sequence is potentially useful for therapeutic application. Effective inhibition of HBV antigen production by U6 shRNA 5 and U6 shRNA 6 is an important advantage over available inhibitors of HBV reverse transcription, which do not reduce viral antigen secretion directly. HBV antigenemia may attenuate the host immune response and compromise eradication of the virus by these drugs [41]. RNAi-based inhibition of viral antigenemia may thus induce a more vigorous anti-HBV immune response to eliminate HBV during chronic infection. Unlike many other viruses, plasticity of the HBV genome is restricted because of its compact arrangement. Target sites of shRNA 5 and shRNA 6 are conserved in most genotypes, suggesting that these RNAi effectors may have broad applicability against different HBV genotypes. Although the results reported here and elsewhere augur well, there are concerns that need to be addressed before effective RNAi-based therapy of HBV infection will be realized. In particular, off-target effects of anti-HBV RNA sequences, which include activation of the interferon response and nonspecific interactions between guide sequences and alternative cellular targets, remain a concern. These effects require further characterization, and optimal therapeutic concentrations need to be defined. An important technical hurdle related to developing RNAi-based antiviral therapy is the efficient delivery of nucleic acid sequences to infected cells in the liver. Although DNA expression cassettes have been shown to be effective in the context of recombinant adenoviruses tested in this study and by others [25], these vectors are unlikely to be suitable for clinical application. Potential toxicity and the limited number of administrations caused by adenovirus immunity are of concern.

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Incorporation of U6 shRNA 5 and U6 shRNA 6 into hepatotropic nonviral vectors with limited immunogenicity is an objective of current work.

MATERIALS AND METHODS

shRNA expression cassettes and synthetic siRNA. Conserved target sequences within the *HBx* open reading frame of the HBV A1 subgenotype were identified by aligning sequences of 27 viral isolates of South African origin. To generate the panel of 10 shRNA expression constructs, oligonucleotides were designed to produce Pol III U6 shRNA cassettes from a U6 DNA template [42] in a two-step amplification reaction. The reverse oligonucleotide sequences used during PCR are indicated in Table 1. U6 shRNA X.1 primers were complementary to part of the U6 promoter and included the mismatched *HBx* sense sequences of the short hairpin, together with the hairpin loop. U6 shRNA X.2 primers included the overlapping loop, *HBx* antisense sequence, and transcription termination sequence. Each PCR step was performed with a U6 universal forward primer (5′-CTAACTAGTGGCGCGCCAAGGTCGGGCAGGAAGAGGG-3′). The PCR products from the final amplification step were purified and ligated to a PCR cloning vector (pGEM-T Easy, Promega, WI, USA) to generate pG-U6shRNA plasmids (e.g., pG-U6shRNA 5 and pG-U6shRNA 6). The sequences were confirmed by standard manual or automated sequencing procedures involving dideoxy chain termination. siRNA 5, which has a sequence equivalent to that of the duplex stem of shRNA 5, was synthesized using 2′-O-ACE-RNA phosphoramidites (Dharmacon, CO, USA). The sequences of the oligoribonucleotides were 5′-UCGUGUGCGCUUUGCUUCGCCUCUG-3′ (sense) and 5′-CAGAGGUGAAGCGAAGUGCACACGG-3′ (antisense).

Target vectors. pCH-9/3091 has been described previously [32]. It contains a greater than genome length HBV sequence, which is similar to the HBV A1 subgenotype consensus. pCH-eGFP is derived from pCH-9/3091 and has the eGFP sequence substituting for the preS2/S ORF [33].

Cell culture. Huh7 cells were maintained in RPMI medium supplemented with 2.5% fetal calf serum (FCS), penicillin (50 IU/ml), and streptomycin (50 μg/ml) (Gibco BRL, UK). HEK293 cells were propagated in DMEM supplemented with 10% FCS, penicillin (50 IU/ml), and streptomycin (50 μg/ml) (Gibco BRL). On the day prior to transfection, 250,000 HEK293 cells or 150,000 Huh7 cells were seeded in wells 2 cm in diameter. Transfection was carried out using Lipofectamine (Invitrogen, CA, USA) according to the manufacturer's instructions. To determine the effects of shRNA-encoding plasmids, Huh7 cells were transfected with a combination of 6 μg of pCH-9/3091 and 2 μg of hairpin-encoding pGEM-derived plasmid or control plasmid lacking the shRNA cassette. HBsAg secretion into the culture supernatants was measured daily using the Monolisa (ELISA) immunoassay kit (Bio-Rad, CA, USA). To determine *in situ* effects of the pG-U6 shRNA series of plasmids, Huh7 cells were cotransfected with 6 μg pCH-eGFP instead of pCH-9/3091. Cells labeled with eGFP were detected using flow cytometry and confirmatory fluorescence microscopy 48 h after transfection. The mean number of fluorescent cells as well as the standard error of the mean was calculated from four independent experiments. A plasmid vector that constitutively produces β-galactosidase [43] was included in each cotransfection and equivalent transfection efficiencies were verified by staining for activity of this marker gene [44].

Northern blot hybridization. Huh7 cells were harvested 4 days after transfection and total RNA was extracted using Tri Reagent (Sigma, MI, USA) according to the manufacturer's instructions. The RNA was resolved using formaldehyde agarose gel electrophoresis and blotted onto nylon membranes. An HBV sequence from the surface region was radiolabeled with [α-^{32}P]dCTP using the multiprime technique (Megaprime kit; Amersham, UK) and then hybridized to blotted RNA and detected using autoradiography. As a control for equal loading, the same blot was stripped and rehybridized to a radiolabeled GAPDH-specific probe.

Expression and processing of shRNA. DNA oligonucleotides, which were complementary to 18 nucleotides of each of the strands of the U6-encoded hairpins, were labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase. After purification using standard procedures, labeled DNA oligonucleotides were hybridized to 2 to 8 μg of total Huh7 cellular RNA and then extended for 20 min at 42°C with AMV reverse transcriptase (Promega) according to the conditions recommended by the supplier. Products were analyzed by autoradiography after resolution using 8 M urea denaturing 15% polyacrylamide gel electrophoresis. The oligonucleotides used in the primer extension assays were as follows: shRNA 5 HBV+, 5′-CCGTGTGCACTTCGCTTC-3′; shRNA 5 HBV−, 5′-CAGAGGCGAAGCAAAGCG-3′; shRNA 6 HBV+, 5′-TGCACTTCGCTTCACCTC-3′; shRNA 6 HBV−, 5′-ATGTGTAGAGGTGAAGCG-3′; shRNA 10 HBV+, 5′-TTCAAGCCTCCAAGCTGT-3′; shRNA 10 HBV−, 5′-CCAAAGCACAACTCGGAG-3′.

Quantitative PCR. To measure the effects of shRNA sequences on circulating virion DNA (see below), total DNA was isolated from 50 μl of mouse serum using the Total Nucleic Acid Isolation Kit and MagNApure instrument from Roche Diagnostics. Controls included water blanks and HBV-negative serum. DNA extracted from the equivalent of 8 μl of mouse serum was amplified using SYBR Green *Taq* Readymix (Sigma, MO, USA). Crossing-point analysis was used to measure virion DNA concentrations and standard curves were generated using EuroHep calibrators [45]. The HBV surface primer set was HBV surface forward, 5′-TGCACCTGTATTCCATC-3′, and HBV surface reverse, 5′-CTGAAAGCCAAACAGTGG-3′. PCR was carried out using the Roche Lightcycler v.2. Capillary reaction volume was 20 μl and thermal cycling parameters consisted of a hot start for 30 s at 95°C followed by 50 cycles of 57°C for 10 s, 72°C for 7 s, and then 95°C for 5 s. Specificity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis.

Assessment of in vivo efficacy of anti-HBV shRNA constructs. The murine hydrodynamic tail vein injection method was initially employed to determine the effects of shRNA plasmid vectors on the expression of HBV genes *in vivo*. Experiments on animals were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. A saline solution comprising 10% of the mouse's body mass was injected via the tail vein over 5–10 s. The saline solution included a combination of three plasmid vectors: 10 μg target DNA (pCH-9/3091) or 10 μg pCI-neo plasmid DNA (Promega), which lacks HBV sequences; 10 μg anti HBV sequence (shRNA-encoding plasmid) or mock (pGEM backbone); and 10 μg pLTR β-gal [43] (a control for hepatic DNA delivery, which encodes the β-galactosidase marker gene under control of an LTR promoter). In some investigations, the amount of shRNA-encoding plasmid was reduced to 5 and 1 μg. To test the efficacy of synthetic siRNA 5 against HBV, 25 μg of this duplex was coinjected via the tail vein. Blood was collected daily from the tail vein over a period of 5 days and HBsAg was measured using the electrochemiluminescence assay (ECLIA) from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. Animals were sacrificed after 4 days. Fixed and unfixed frozen liver sections were processed respectively for immunohistochemical HBcAg detection or for β-galactosidase staining [44]. A rabbit polyclonal antibody against HBcAg (Signet Laboratories, Inc., MA, USA) and horseradish peroxidase-conjugated secondary antibody (Dako, Denmark) were used to detect the viral antigen in paraffin-embedded sections according to standard procedures.

Adenovirus vectors. The procedure described by He *et al*. [46] was followed for the preparation of the ADV shRNA 5, ADV shRNA 6, ADV shRNA 10, ADV eGFP, and ADV shRNA LacZ adenovirus vectors. ADV shRNA LacZ is a control adenovirus that includes a U6 shRNA cassette with hairpin that targets the β-galactosidase reporter gene. ADV eGFP is another control that includes the backbone sequence without U6 hairpin cassette. The pG shLacZ vector was propagated using the two-step PCR procedure described above. The U6 universal forward primer, with 5′-CTGTTTGACAGGAAGAACAAGTATCCGCTAGTCACTTCGACGGTGTTTCGTCCTTTCCACA-3′ and 5′-CCCAGATCTACGCGTAAAAAATCGAAGTGACCAGCGAATACCTGTTTGACAGGAAGAACAA-3′ as reverse primers, was used in sequential amplifications. To generate adenovirus shuttle vectors containing the shRNA cassettes, pG-U6shRNA vectors were

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digested with *Not*I and *Hin*dIII. The U6 shRNA-containing fragment was purified and ligated to equivalent sites of pAdTrack to generate pAd-Track U6 shRNA. After verifying the sequence of the inserts, pAdTrack U6 shRNAs were digested with *Pme*I and homologous recombination with the pAdEasy-1 adenoviral backbone plasmid was carried out in *Escherichia coli* BJ55183 cells. Propagation of the adenovirus in HEK293 cells was then done according to the described procedures [46]. Absence of E1A sequences from each adenovirus was confirmed using PCR. Failure to observe a cytopathic effect in A549 cells, which support adenovirus replication, but which do not provide E1 sequences *in trans*, verified that wild-type revertants were not present in any of the adenovirus preparations.

HBV transgenic mice. HBV transgenic mice with greater than genome length HBV sequence stably integrated into their genomes, which constitutively generates HBV particles [40], were used to assess the antiviral efficacy of shRNA-encoding adenoviral vectors. All procedures were approved by the Animal Care Committee at Stanford University. A dose of 5×10^9 adenovirus infectious particles was injected via the tail vein. Serum HBsAg was measured using a quantitative sandwich ELISA from Abbott Laboratories, and HBeAg was determined using the ECLIA from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. Viral loads were determined using real-time PCR according to procedures described above. Adenovirus gene transduction, assessed by detection of eGFP, was determined using fluorescence microscopy of liver sections.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical difference was considered significant when $P < 0.05$ and was determined according to the Dunnett multiple comparison test and calculated with the GraphPad Prism software package (GraphPad Software, Inc., CA, USA).

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymthe.2005.10.013.

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Specific Inhibition of HBV Replication *In Vitro* and *In Vivo* With Expressed Long Hairpin RNA

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Activating RNA interference to achieve specific gene silencing has shown promise for the development of RNA-based treatment of chronic hepatitis B virus (HBV) infection. To further this approach, we assessed the efficacy of expressed long hairpin RNAs (lhRNAs) that target the conserved *HBx* open reading frame of HBV. As substrates for Dicer, lhRNAs have the potential to generate multiple short interfering RNAs (siRNAs) to enable simultaneous targeting of different sites. Two U6 Pol III vectors were constructed that encode anti-HBV lhRNAs with a 62 base pair stem sequence containing multiple G:U pairings. Assessment in transfected cultured cells and also *in vivo* using the murine hydrodynamic injection model showed that one of the lhRNA vectors (lhRNA 1) diminished markers of virus replication by 70–90% without evidence of interferon response induction. Greatest silencing efficacy was observed for targets that are complementary to sequences located at the base of the hairpin stem and this correlated with a higher concentration of siRNAs derived from this region of the lhRNA. Although lhRNA 1 has the advantage of targeting a greater viral sequence, incomplete cellular processing may result in unequal silencing across the span of the viral target RNA.

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INTRODUCTION

Infection with hepatitis B virus (HBV) is an important global medical problem. Available therapy is only partially effective against the virus[1,2] and development of improved treatment modalities remains an important medical priority. Recent demonstrations that activation of the RNA interference (RNAi) pathway can achieve specific silencing of HBV genes[3–8] have prompted the development of novel RNA-based antiviral agents. RNAi usually involves processing of precursor double-stranded RNA (dsRNA) by Dicer to form short interfering RNA (siRNA) duplexes of 21–23 base pairs (bp).[9,10] The siRNA is incorporated into the RNA-induced silencing complex, where one of the strands is selected and acts as a guide to target degradation of complementary cytoplasmic RNA.[11] Silencing by exogenous potentially therapeutic sequences is typically induced by short synthetic duplex RNA or expressed Pol III-driven short hairpin RNA (shRNA).[12–15] Although several viruses have been shown to be susceptible to knockdown by RNAi effector sequences,[16,17] an important concern is escape from silencing effects by viruses that are prone to a high mutation rate. Selection of RNAi escape mutants has been reported *in vitro* for poliovirus,[18] human immunodeficiency virus 1 (HIV-1),[19,20] and hepatitis C virus (HCV).[21] However, with HCV, resistant viral sequences were susceptible to silencing by siRNAs that targeted alternative sites.[21] Using a vector that produces a Dicer substrate that generates multiple siRNAs would have the advantage of limiting escape and targeting a range of sequences found in different viral genotypes or quasispecies.

Use of dsRNA Dicer substrates with a duplex region greater than 30 bp may be complicated by the induction of the nonspecific type 1 interferon (IFN) response in mammalian cells.[22] This may ultimately cause translation suppression and mRNA degradation, which would be undesirable in a therapeutic context. The IFN response is elicited by interaction of dsRNA with cellular proteins, such as dsRNA-dependent RNA protein kinase (PKR) and triggers activation of interferon-β (*IFN-β*), *Myxovirus A* (*MxA*), and *2′,5′-oligoadenylate synthase 1* (*OAS1*) genes among others. Recent studies showed that expressed hairpins evade activation of the IFN response more efficiently than exogenous synthetic RNA.[23] Also, modified long hairpin RNAs (lhRNAs) comprising 50–100 bp with multiple G:U pairings in the stem sequence have been reported to silence HCV targets in cell culture without an immunostimulatory effect.[24,25] Although promising for therapeutic application, it has not been established whether expressed lhRNA vectors are capable of efficient silencing across the extent of the hairpin duplex and whether specific viral gene silencing can be achieved *in vivo*. To address this, we examined the silencing of HBV replication by expressed lhRNAs targeting *HBx*. This multifunctional sequence encodes the HBx protein, which is required for viral replication and has been implicated in HBV-related hepatocarcinogenesis. We show that an anti-lhRNA cassette is capable of specific inhibition of markers of viral replication.

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RESULTS

lhRNA-encoding cassettes

Two U6 Pol III lhRNA cassettes were generated, which target conserved *HBx* sites of HBV genotype A1 isolates[26] (**Figure 1**). Hairpins were designed to comprise a 62 bp stem with 12 G:U wobble pairs in the stem sequence. The lhRNA antisense sequence was perfectly complementary to the viral target, whereas the sense sequence included mismatches. It has been suggested that inclusion of G:U wobble pairings evades induction of the IFN response by a mechanism that involves structural simulation of endogenous microRNA.[24,25] Although the lhRNAs described here included G:U pairings, they were incorporated primarily as a technical aid to improve the efficiency of the two-step polymerase chain reaction (PCR) method for generating expression cassettes.

lhRNA-mediated target silencing in transfected cells without evidence for induction of an IFN response

Initially, to assess efficacy against HBV *in vitro*, Huh7 cells were co-transfected with lhRNA 1 and lhRNA 2 vectors together with the pCH-9/3091 HBV target plasmid.[27] The *HBx* sequence is common to all HBV transcripts (**Figure 1b**) and inhibition of HBV surface antigen (HBsAg) secretion correlates with RNAi-mediated silencing of HBV replication.[3,28,29] Controls included an shRNA-encoding plasmid (shRNA 5), which we have previously shown to be effective against HBV,[3] and also a long hairpin directed against an unrelated HIV-1 trans-acting response element (TAR) sequence. Compared to mock-treated cells, a knockdown of approximately 90% of viral antigen secretion was achieved by lhRNA 1 and shRNA 5 (**Figure 2a**).

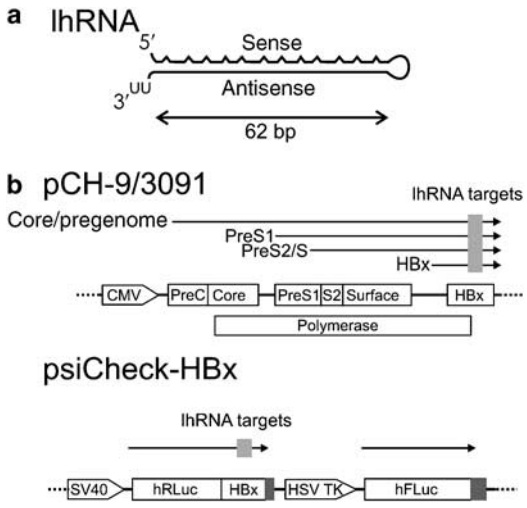

Figure 1 lhRNA sequences and HBV target sites. (**a**) Schematic illustration of lhRNA comprising 62 bp in the stem. G:U pairings are shown as well as a sequence of 2 U residues that are derived from the transcription termination signal. The antisense strand is perfectly complementary to its HBV target. (**b**) Organization of the HBV genome showing ORFs and sites within the pCH-9/3091 target vector that are complementary to antisense components of lhRNA. Four arrows indicate the HBV transcripts, which have common 3′ ends, and include the lhRNA targets. The psiCheck-*HBx* vector includes the entire *HBx* target sequence downstream of the *Renilla* luciferase reporter ORF and firefly luciferase is constitutively produced from a separate expression cassette.

The vector encoding lhRNA 2 caused less impressive inhibition of HBsAg secretion (approximately 50%) and control HIV lhRNA TAR had no significant effect on HBsAg secretion. A series of experiments was also carried out in which psiCheck-*HBx*, a dual luciferase reporter vector (**Figure 1b**), was co-transfected with hairpin plasmids. Firefly luciferase is expressed constitutively in psiCheck-*HBx*, whereas *Renilla* luciferase is susceptible to silencing by hairpin-expressing sequences. A ratio of *Renilla* to firefly luciferase activity was thus used as an indicator of target knockdown. A very similar inhibition of the ratio of *Renilla* to firefly luciferase acivity, and HBsAg secretion was demonstrated (**Figure 2b**). These observations are in accordance with previously reported data, which showed that the viral sequence targeted by lhRNA 1 is particularly susceptible to RNAi-mediated silencing, whereas the downstream lhRNA 2 site is less so.[3]

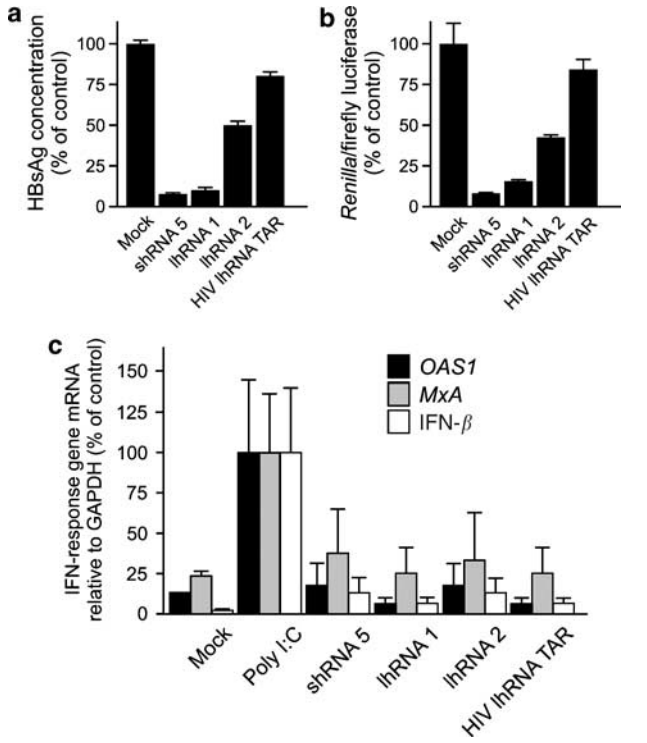

Figure 2 HBsAg secretion and IFN response-related gene expression in transfected cells in culture. (**a**) HBsAg secretion from Huh7 cells co-transfected with indicated hairpin RNA-encoding plasmids together with HBV target plasmid. HBsAg measurements from quantitative ELISA (enzyme-linked immunosorbent assay) are presented as a normalized mean relative to the mock-treated cells. Results are from seven independent transfections and the bars indicate the SEM. (**b**) *Renilla* luciferase reporter gene activity in Huh7 cells co-transfected with indicated hairpin RNA-encoding plasmids together with psiCheck-*HBx* reporter plasmid. Measurements are given as a normalized ratio (±SEM) of *Renilla* to firefly luciferase activity and were determined from three independent experiments. (**c**) Assessing activation of the IFN response in transfected cells. Quantitative real-time PCR was used to measure *IFN-β*, *MxA*, or *OAS1* mRNA. HEK293 cells were transfected with the indicated hairpin RNA-encoding plasmids and the positive control group was transfected with poly (I:C). Means (±SEM) of *IFN-β*, *MxA*, or *OAS1* to *GAPDH* mRNA were calculated from the positive control and were determined from four independent experiments.

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Activation of the IFN response, which can induce unintended programmed cell death and nonspecific gene silencing, may be effected by duplex RNA in mammalian cells (reviewed in ref. 22). To assess activation of the IFN response in cells that were transfected with lhRNAs, relative levels of cellular *2′,5′-OAS1*, *MxA*, and *IFN-β* mRNA were measured using a sensitive quantitative PCR assay. Data revealed that IFN response genes were not significantly induced in HEK293 cells that had been transfected with lhRNA-encoding sequences (**Figure 2c**), whereas cells treated with poly (I:C) (positive control) mounted an expected IFN response. Activation of *2′,5′-OAS1*, *MxA*, and *IFN-β* was not observed in Huh7 cells after lhRNA vector transfection or poly (I:C) treatment (data not shown), supporting previous findings that the IFN response is attenuated in this liver-derived line.[30] Also, transfection efficiency and cell viability were unaffected by transfection with lhRNA-encoding plasmids (data not shown). Together these data indicate that silencing of reporter gene expression and HBsAg secretion from cells that were co-transfected with lhRNA-encoding cassettes was specific and not a result of induction of the IFN response.

Effects of lhRNA on viral replication *in vivo*

To determine anti-HBV efficacy of shRNA *in vivo*, HBV replication competent plasmid together with lhRNA-encoding vectors were delivered to the liver using the hydrodynamic tail vein injection method. lhRNA 1- and shRNA 5-encoding plasmids knocked down HBsAg in the serum of mice by approximately 90% when measured at day 5 after administering this DNA ($P<0.05$) (**Figure 3a**). lhRNA 2 effected a less significant decrease in HBsAg secretion ($P<0.1$) and HIV lhRNA TAR did not diminish serum HBsAg concentration. These data were supported by measuring the number of viral particle equivalents in the serum at 2 and 5 days after coinjecting HBV target and hairpin-encoding DNA (**Figure 3b**). lhRNA 1 and shRNA 5 inhibited production of viral particle equivalents *in vivo* by 60–70%. Furthermore, measurement of intrahepatic knockdown of HBV gene expression corroborated these findings. Concentrations of HBV mRNA containing core and surface sequences were diminished by lhRNA 1 and shRNA 5 by approximately 60–70% (**Figure 3c**). Three separate lines of evidence, HBsAg concentration determination, viral loads, and intracellular HBV mRNA determination, establish that lhRNA 1 substantially decreases markers of HBV replication *in vivo*.

IFN response *in vivo*

To determine whether lhRNA-induced activation of the IFN response pathway occurs *in vivo*, induction of *OAS1* and *IFN-β* genes in hepatocytes was determined after murine hydrodynamic injection. Expression of both genes was increased at 6 h after injection with poly (I:C), which confirms activation of the IFN response in the positive control (**Figure 4**). This effect was not observed after 5 days and is likely to be a result of the transient nature of the effect of poly (I:C) (data not shown). There was a small increase in *OAS1* mRNA concentration in mock-treated animals at 6 h, which may be due to effects of the hydrodynamic injection procedure itself. Five days after administration of

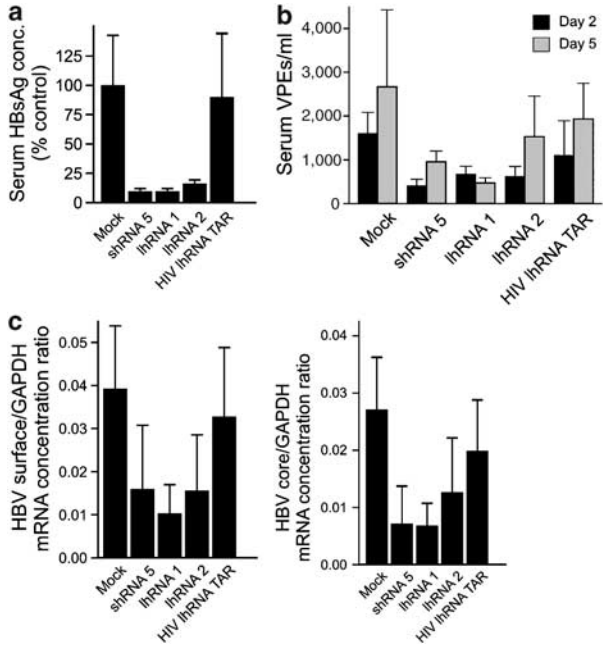

Figure 3 Effects of hairpin sequences on markers of HBV replication in the hydrodynamic injection model of HBV replication. (**a**) Serum HBsAg concentrations were determined at day 5 after hydrodynamic injection of mice with pCH-9/3091 HBV target and indicated hairpin-encoding sequences. Results were normalized relative to the mock-treated mice and are expressed as the mean (±SEM) from at least five mice. (**b**) Serum circulating viral particle equivalents (VPEs) at days 2 and 5 after hydrodynamic injection with the target vector together with hairpin-encoding sequences. (**c**) Hepatocyte concentrations of HBV mRNA from the core and surface regions expressed as a ratio to amount of *GAPDH* mRNA. Total RNA was isolated from liver cells at day 5 after hydrodynamic injection and subjected to quantitative real-time PCR. Groups comprised 5–8 animals and the graphs indicate the mean and SEM for each group.

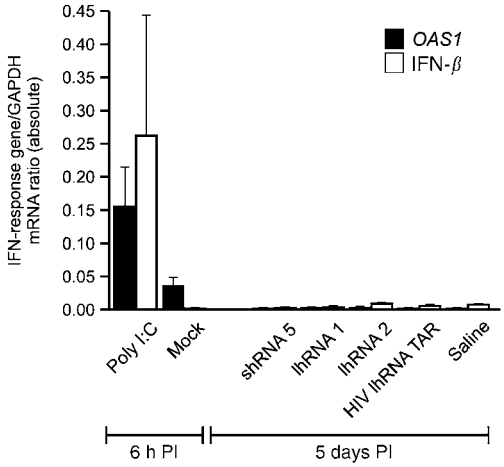

Figure 4 Assessment of *in vivo* IFN response. Mice were mock treated, injected with indicated hairpin-encoding cassettes, or with poly (I:C) using the hydrodynamic procedure. RNA was extracted from the livers and then subjected to quantitative real-time PCR to determine concentrations of *OAS1*, *IFN-β*, and *GAPDH* mRNA. Analysis was carried out after 6 h to verify that an IFN response was induced using the poly (I:C) positive control. In the remaining animals, which had been treated with anti-HBV hairpins, markers of induction of the IFN response were determined after 5 days.

lhRNA-encoding vectors, activation of *OAS1* and *IFN-β* was not observed in any of the groups of mice.

Silencing of mutant targets by lhRNA 1

An advantage of using long hairpin sequences to effect silencing is their potential to serve as substrates for the production of several siRNAs, which can target different viral sequences. This is of importance for inhibition of expression of viral mutants that evade the silencing mechanism. To assess this, we inserted wild-type and mutant targets downstream of the sequence encoding the *Renilla* luciferase gene within the psiCheck2 dual reporter vector. The mutations were located within the target of shRNA 5 (**Figure 5a**). Transfection of cultured cells demonstrated that both lhRNA 1 and shRNA 5 were capable of silencing the normal target (**Figure 5b**). However, the lhRNA sequence silenced mutant targets more effectively than shRNA 5. Interestingly, the shRNA vector remained capable of significant mutant sequence knockdown when compared to the control transfected cells. This may have resulted from translational suppression that did not require perfect complementarity with the HBV cognate. Although lhRNA 1-mediated knockdown of the mutant targets was better than that of shRNA 5, this observation does not establish that there is equally efficient silencing of the target across the extent of the lhRNA 1 duplex.

siRNA guide sequences derived from lhRNA 1

Northern blot hybridization was carried out to determine whether the siRNA guide sequences from lhRNA 1 were produced in equal amounts. RNA extracted from cells transfected with lhRNA 1- and shRNA 5-encoding plasmids was hybridized to each of three labelled oligonucleotide probes (**Figure 6a** and **b**). The sequences of the probes were complementary to, and together spanned the extent of the lhRNA HBV antisense region. Precursor unprocessed shRNA 5 sequences were detectable as a band of approximately 60 nucleotides (nt). Probe A, which is located at the 3′ end of the lhRNA, detected a putative guide sequence of approximately 23 nt in the cells that had been transfected with lhRNA 1, whereas no hybridizing RNA was detectable in cells that had been transfected with shRNA 5. RNA sequences of approximately 23 nt, when detected by probe B, were found in both lhRNA 1- and shRNA 5-transfected cells. As expected, probe C detected a prominent siRNA sequence in shRNA 5-transfected cells. Interestingly, a 23 nt band was not detectable in RNA from lhRNA 1-transfected cells when hybridized to probe C. This was confirmed in a duplicate blot and when hybridized blots were overexposed (data not shown). Thus, siRNAs generated from the loop side of the lhRNA 1 sequences are present in lower concentration than those from the stem base. These observations may reflect initial efficient Dicer cleavage at the stem base of lhRNA 1 and less efficient processing toward the loop side of the hairpin.

Greater silencing efficacy of siRNAs derived from the lhRNA 1 stem base sequence

To determine whether silencing by lhRNA 1 along the extent of the viral target sequence correlated with siRNA concentrations, a tiling assay was performed in which the entire lhRNA 1 target and segments of this sequence spanning from 5′ to 3′

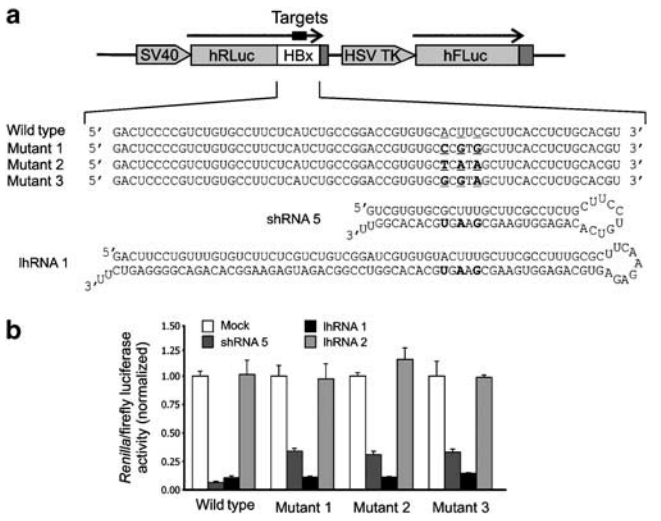

Figure 5 Assessment of silencing efficacy of shRNA 5, shRNA 6, and lhRNA 1 against mutant and wild-type HBV targets. (**a**) HBV sequences were inserted downstream of the *Renilla* luciferase ORF within the psiCheck2 plasmid vector. Nucleotides that are mutated within the targets are indicated in bold as are the affected complementary bases within the putative shRNA 5 and lhRNA 1 structures. (**b**) Knockdown efficiency of shRNA 5 and lhRNA 1 vectors against wild-type and mutant targets. Values are normalized to the ratio of *Renilla* to firefly luciferase activity in the mock transfected cells. Columns indicate the average values from four separate experiments and error bars show the SEM.

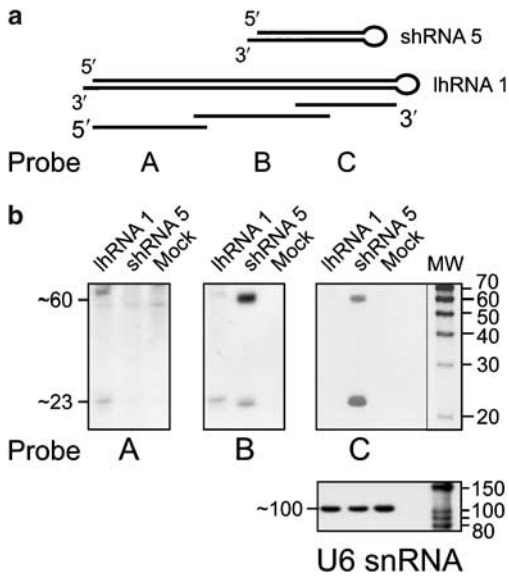

Figure 6 Detection of siRNA sequences produced from expressed lhRNA 1 and shRNA 5. (**a**) Schematic illustration of shRNA 5 and lhRNA 1 with relative positions of probes A, B, and C. These oligonucleotides comprise single-stranded DNA sequences complementary to the HBV antisense strand of the hairpins. (**b**) Hybridization of radiolabelled probes A, B, C, and U6 snRNA (small nuclear RNA) oligonucleotides to RNA from transfected HEK293 cells using Northern blot analysis. Approximate sizes (nt) of hybridizing bands and molecular weight markers are indicated. Blots were sequentially hybridized, stripped, and rehybridized. Repeated hybridization analysis showed data to be reproducible.

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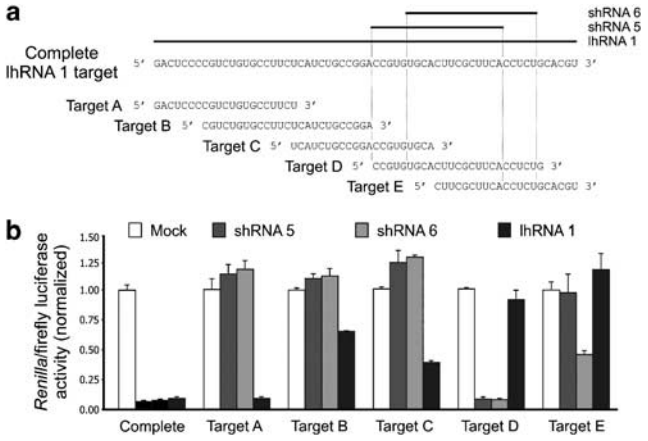

Figure 7 Silencing efficiency across the span of the lhRNA1 target sequence. (**a**) The complete lhRNA1 as well as individual short target sequences (targets A–E) were inserted downstream of the *Renilla* luciferase ORF within the psiCheck2 vector. Areas that are complementary to the sequences located within shRNA 5, shRNA 6, and lhRNA 1 are indicated above. (**b**) Knockdown by shRNA 5, shRNA 6, and lhRNA 1 of individual target sequences. Values are normalized to the ratio of *Renilla* to firefly luciferase activity in the mock-transfected cells. Columns indicate the average values from four separate experiments and error bars show the SEM.

(progressively targets A–E) were inserted downstream of the *Renilla* luciferase open reading frame (ORF) of psiCheck2 (**Figure 7a**). Knockdown of each of the targets was determined according to the ratios of *Renilla* and firefly luciferase activity (**Figure 7b**). Predictably, each of the hairpin-encoding sequences caused knockdown of the complete target. Only lhRNA 1 inhibited *Renilla* luciferase expression when target A was placed downstream of the reporter. Neither shRNA 5 nor shRNA 6 had any effect on *Renilla* luciferase expression when the reporter vector contained targets A, B, or C, whereas lhRNA 1 induced silencing at targets B and C, but with lower efficacy than against target A. Target B straddles the putative initial Dicer cleavage site of lhRNA 1. This means that siRNAs from the long hairpin are likely to have only partial sequence complementarity to target B and poor silencing efficacy against this sequence. More effective lhRNA 1 silencing of target C than target B may be a result of improved hybridization of a putative second Dicer-generated siRNA from the lhRNA 1 sequence. Target D, which encompasses shRNA 5 and shRNA 6 cognates, was a poor substrate for silencing by lhRNA 1, but efficient knockdown was achieved by the shRNA vectors. Target E was also poorly silenced by lhRNA 1 and partially knocked down by shRNA 6. Taken together with the analysis of concentrations of RNA fragments from lhRNA 1 (**Figure 6**), these data suggest that siRNAs from the loop side of the long hairpin are formed less efficiently and are less effective silencers of target RNA than those from the stem base. Similar investigation of knockdown of HIV-1 targets by lhRNAs (data not shown) corroborates this.

DISCUSSION

Several studies have demonstrated that synthetic siRNA and expressed shRNA can be used to silence HBV gene expression.[3–8] A range of different sites within the viral genome has been targeted, and variable efficiency of knockdown has been observed. Although algorithms have been devised that distinguish effective RNAi inducers, predictions that identify optimal targets are often difficult to make.[31] Typically, screening of several candidate silencing sequences and empirical validation is required to aid in identifying the optimal RNAi effector. Use of a combination of siRNAs should thus facilitate the process of achieving successful silencing. This was confirmed in a study which showed that transfecting cells with a panel of siRNAs produced *ex vivo* using recombinant Dicer is a useful means of attaining reliable silencing.[32,33] Generating several siRNAs from one expressed lhRNAs sequence is analogous and should improve the probability of assembling effective silencing complexes.[34] An important concern of applying RNAi to the treatment of virus infections, particularly viruses such as HBV which replicate using error-prone reverse transcriptase, is the evasion of silencing effects as a result of mutation. Selection of poliovirus,[18] HIV-1,[19,20] and HCV[21] RNAi escape mutants has been reported and, in the case of HCV, resistant sequences were susceptible to siRNAs that targeted alternative sites.[21] Using lhRNA to target several sites simultaneously should overcome viral escape and also improve the probability of successful silencing. Although these are important theoretical advantages, our data show that silencing is not equally efficient across the extent of the lhRNA duplex. siRNAs originating from the duplex at the loop terminus side of the hairpin have poor silencing efficacy. Knockdown was most effective by siRNAs derived from the stem base and siRNAs from this part of the lhRNA were also present in higher concentration. Importantly, this bias in knockdown efficiency has implications for design and use of lhRNAs to counter viral escape and optimize target sequence silencing. A likely reason for unequal silencing by lhRNA-derived siRNAs is that Dicer cleavage is initiated at the stem base end of the lhRNA where 2 nt 3′ overhangs are recognized by the enzyme.[9,10] Processing capacity of intracellular Dicer may be incomplete, resulting in a relatively higher concentration of siRNAs arising from the stem base of the lhRNA. The rate of lhRNA transcription and differences in the stability of nascent and partially diced hairpin RNA are also likely to contribute to our findings.

Several properties of RNAi effectors have been described that may cause unwanted activation of an innate immune response. These include duplex sequences that are longer than 30 bp,[35] presence of 5′ triphosphates,[36] "danger" sequence motifs,[37] and lack of 2 nt 3′ overhangs.[38] Induction of immunostimulatory effects, particularly the IFN response, has thus been a concern of using lhRNA sequences to effect gene silencing. Although long dsRNA induces the IFN response through activation of PKR and interaction with endosomal Toll-like receptors, this effect may be of greater significance when using perfectly matched exogenous synthetic RNA to cause gene silencing.[24,25,39] Expressed hairpin RNA does not have the same immunostimulatory properties as synthetic dsRNA.[23] Importantly, RNA that is transcribed within cells does not typically traverse the endosomal compartment and interact with Toll-like receptor 3, Toll-like receptor 7, and Toll-like receptor 8 to stimulate downstream activators of the innate immune response. Moreover, lhRNA products of Dicer

processing have characteristic 2 nt 3′ overhangs, which are a structural feature that suppresses immunostimulation by RIG-I (retinoic-acid-inducible protein I).[38] Collectively, these factors, and possibly the presence of G:U wobbles in the lhRNA duplex, are likely to limit induction of the IFN response in cultured cells or *in vivo*.

Specificity of RNAi effectors for their cognate targets is also critical for development of therapeutic RNA. Minimal or no unintended crossreaction of silencing sequences with cellular RNA will be an important requirement for therapeutic application of lhRNAs. A concern of using lhRNAs is that Dicer may generate multiple overlapping siRNAs with unpredictable guide strand bias, resulting in an increased probability of off-target effects. The length and specific sequences of the duplex are likely to be the major contributors to these nonspecific side effects. Although Dicer predominantly generates duplex siRNAs containing 21 nt RNA strands, the size ranges from approximately 21 to 23 nt.[9,10] Thus, with longer dsRNA substrates, formation of siRNAs is likely to proceed with decreasing precision, which may contribute to off-target effects. In this study, we designed an lhRNA expression cassette that would potentially target three adjacent HBV siRNA targets. This is considerably shorter than the duplex region of other lhRNAs that have been reported to cause specific silencing of target genes.[34] Nevertheless, as with eventual therapeutic application of any RNAi effectors, it is likely that detailed analysis, including use of microarrays, will be required to verify specificity of lhRNA1.

The recent demonstration that saturation of the natural microRNA pathway by exogenous expressed RNA causes toxicity in hepatocytes is a caveat for development of RNAi-based HBV therapy.[40] This is of concern for application of constitutively expressed lhRNAs, and refined transcription control to regulate intracellular concentration of RNAi effectors will be required. Investigations are currently in progress in our laboratory, which are aimed at assessing efficacy of hairpin sequences expressed from liver-specific Pol II promoters.

Results from our study have shown that expressed lhRNA is capable of inhibiting production of markers of viral replication in cell culture and *in vivo* models of HBV. This effect was observed without induction of the IFN response. Although unequal silencing across the extent of the lhRNA duplex was observed, the approach offers promise for the use of expressed sequences containing long duplexes that generate multiple siRNAs to silence HBV gene expression.

MATERIALS AND METHODS

lhRNA expression plasmids. Generation of the Pol III U6 shRNA 5 cassette has been described.[41] A similar two-step PCR approach was used to produce the lhRNA vectors complementary to HBV coordinates 1581–1640 (lhRNA 1) and 1372–1431 (lhRNA 2) (accession J02203). The first amplification was carried out with a universal U6 forward primer and first lhRNA reverse primer with U6 promoter plasmid DNA as template. The amplified product was used as template for a PCR step with a second lhRNA reverse primer and again the universal U6 forward primer. The sequence of the U6 universal forward (F) primer was 5′-CTAACTAGTGGCGCGCCAAGGTCGGGCAGGAAGAGGG-3′. Sequences of the reverse (R) primers for the amplifications were as follows: lhRNA HBV1-R1 5′-ACTCTCTTGAAGCGCAAAGGCGAAGCAAAGTACACAC GATCCGACAGACGAGAAGACACAAACAGGAAGTCGGTGTTTCGTC CTTTCCACAA-3′ (92 nt), lhRNA HBV1-R2 5′-GATCTCTAGAAAAAA GACTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTC GCTTCACCTCTGCACTCTCTTGAAGCGCAAAG-3′ (92 nt), lhRNA HBV2-R1 5′-CATCTCTTGAATGCCGGTACGCAAACAACTTACGCC CACAACCTCCCAGCACAAAGACCCTCAACCCAATCGGTGTTTCGT CCTTTCCACAA-3′ (92 nt), lhRNA HBV2-R2 5′-GATCTCTAGAAA AAAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCATAAA TTGTCTGCGCACCAGCATCTCTTGAATGCCGGTA-3′ (92 nt), lhRNA HIV TAR-R1 5′-CCTCTCTTGAAGAGTCCCCTAAATAACCAGAGAAC CCCCGGACTCAGATCCGGTCCACCCAGAAAGAACCGGTGTTTCGT CCTTTCCACAA-3′ (91 nt), and lhRNA HIV TAR-R2 5′-GATCTCTA GAAAAAAGGGTCTCTCTAGGTAGACCAGATCTGAGCCCGGGAGCT CTCTGGCTATCTAGGGAACCCTCTCTTGAAGAGTCCCC-3′ (91 nt). Each pair of primers had an overlapping sequence of 19 bases that enabled extension of the PCR product to generate a U6 promoter lhRNA cassette with transcription termination signal.[41] Amplified DNA was ligated to a PCR cloning vector (pTZ57R/T, Fermentas, Madison, WI) to generate pTZ-U6 lhRNA plasmids (pTZ-U6 lhRNA HBV 1, pTZ-U6 lhRNA HBV 2, and pTZ-U6 lhRNA TAR). The sequences were confirmed by standard procedures.

Target plasmids. pCH-9/3091 has been described previously.[27] It contains a greater than genome length HBV sequence, which is similar to the HBV A1 subgenotype consensus. The psiCheck-*HBx* target plasmid was prepared by directed insertion of the *Xho*I-*Not*I digested *HBx* fragment from pCI-neo *HBx*[29] into the plasmid psiCheck2 (Promega, Madison, WI) such that the *HBx* ORF is within the 3′ untranslated region of the *Renilla* Luciferase cassette. The lhRNA HBV 1 target sequence was amplified by PCR amplification using HBx lhRNA-1 F (5′-GATCTCGAGGACTCCCCGTCTGTGCCTTCT-3′) and HBx lhRNA-1 R (5′-GATCGCGGCCGCACGTGCAGAGGTGAAGCGAAGT GCACACGG-3′) primer combinations. The mutagenic reverse primer HBx lhRNA-1m R (5′-GATCGCGGCCGCACGTGCAGAGGTGAAGC NANGNGCACACGG-3′), which contains three random nucleotides, was used to generate mutated targets. PCR products were cloned into the PCR cloning vector as described above and, for the mutated targets, a mixed plasmid population was used to extract target fragments for insertion into corresponding *Xho*I-*Not*I sites within the *Renilla* 3′ untranslated region of psiCheck2. To generate multiple short targets that "tile" the entire HBx lhRNA 1 target, complementary oligonucleotides were treated with polynucleotide kinase (Promega, WI), annealed, and cloned directly into the *Xho*I-*Not*I sites of psiCheck2. To facilitate screening, an *Eco*RV site was inserted within each annealed dsDNA insert. The complementary oligonucleotide sequences used were lhRNA 1a F 5′-TCGAGATATCGACTCCCCGTCTGTGCCTTCTGC-3′ and lhRNA 1a R 5′-GGCCGCAGAAGGCACAGACGGGGAGTCGATA TC-3′; lhRNA 1b F 5′-TCGAGATATCCGTCTGTGCCTTCTCATCTG CCGGAGC-3′ and lhRNA 1b R 5′-GGCCGCTCCGGCAGATGAGA AGGCACAGACGGATATC-3′; lhRNA 1c F 5′-TCGAGATATCTCATCTG CCGGACCGTGTGCAGC-3′ and lhRNA 1c R 5′-GGCCGCTGCACACG GTCCGGCAGATGAGATATC-3′; lhRNA 1d F 5′-TCGAGATATCCCGTG TGCACTTCGCTTCACCTCTGGC-3′ and lhRNA 1d R 5′-GGCCGCCA GAGGTGAAGCGAAGTGCACACGGGATATC-3′; and lhRNA 1e F 5′-TCGAGATATCCTTCGCTTCACCTCTGCACGTGC-3′ and lhRNA 1e R 5′-GGCCGCACGTGCAGAGGTGAAGCGAAGGATATC-3′.

Cell culture. Huh7 cells were maintained in Roswell's Park Memorial Institute medium supplemented with 2.5% fetal calf serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml) (Gibco BRL, Paisley, UK). HEK293 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml) (Gibco BRL, UK). On the day before transfection, 250,000 HEK293 cells or 150,000 Huh7 cells were seeded

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in wells of 2 cm diameter. Transfection was carried out using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To determine the effects of lhRNA-encoding plasmids, Huh7 cells were transfected with a combination of 6 μg of pCH-9/3091[27] or psiCheck-*HBx* target vector and 2 μg of long hairpin-encoding pTZ-derived plasmid or plasmid lacking the lhRNA/shRNA cassettes. HBsAg secretion into the culture supernatants was measured using the Monolisa (enzyme-linked immunosorbent assay) immunoassay kit (BioRad, Hercules, CA). A plasmid vector that constitutively produces enhanced green fluorescent protein[28] was included in each co-transfection and equivalent transfection efficiencies were verified by fluorescence microscopy. The activities of *Renilla* and firefly luciferase were measured with the dual luciferase assay kit (Promega, WI) and using the Veritas dual injection luminometer (Turner BioSystems, Sunnyvale, CA).

Assessment of* in vivo *efficacy of lhRNA constructs. The murine hydrodynamic tail vein injection method was employed according to previously described methods to determine the effects of hairpin-encoding plasmid vectors on the expression of HBV genes *in vivo*.[3] Experiments on animals were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. As a positive control for the activation of the IFN response *in vivo*, mice were injected with 100 μg poly (I:C) (Sigma, St Louis, MO) and killed 6 h thereafter for gene expression analysis. After discarding data from injections that were suboptimal each experimental group comprised 5–8 mice.

Quantitative PCR. To measure the effects of lhRNA sequences on circulating viral particle equivalents, total DNA was isolated from 50 μl of the serum of mice on days 3 and 5 after hydrodynamic injection and viral DNA was determined using quantitative PCR according to previously described methods[3] with EuroHep calibrating standards.[42] To measure concentrations of mRNA encoding HBV and IFN response-related genes, total RNA from HEK293 cells or mouse liver was reverse transcribed using Sensiscript (Qiagen, GmbH, Germany) and oligo d-T primer according to the manufacturer's instructions. HBV mRNA containing core and surface sequences was amplified using the following primer combinations: core F 5′-ACCACCAAATGCCCCTAT-3′, core R 5′-TTCTGCGACGCGGCGA-3′, surface F 5′-GCCAAAATTCGCAGT CC-3′, and surface R 5′-ACGGGCAACATACCTT-3′. The primer combinations listed below were used to amplify IFN response-related mRNA of human (Huh7 and HEK293 cells) origin: *IFN-β* F (h): 5′-TCCAAATTGCTCTCCTGTTGTGCT-3′; *IFN-β* R (h): 5′-CCACAGGA GCTTCTGACACTGAAAA-3′; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) F (h): 5′-AGGGGTCATTgATGGCAACAATATCCA-3′; *GAPDH* R (h): 5′-TTTACCAGAGTTAAAAGCAGCCCTGGTG-3′; *OAS1* F (h): 5′-CGAGGGAGCATGAAAACACATTT-3′; *OAS1* R (h): 5′-GCAG AGTTGCTGGTAGTTTATGAC-3′; *MxA* F (h): 5′-CTGGTGCTGAAAC TGAAGAAAC-3′; and *MxA* R (h): 5′-ATCTCAATCTCGTAGTCCTGG TA-3′. To amplify murine *OAS1*, *IFN-β*, and *GAPDH* complementary DNA, the procedures and primer combinations described by Song *et al.*[43] were used. All quantitative PCRs were carried out using the Roche Lightcycler V.2. Controls included water blanks and RNA extracts that were not subjected to reverse transcription. Taq readymix with SYBR green (Sigma, St Louis, MO) was used to amplify and detect DNA during the reaction. Thermal cycling parameters consisted of a hotstart for 30 s at 95°C followed by 50 cycles of 58°C for 10 s, 72°C for 7 s, and then 95°C for 5 s. Specificity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis.

Northern blot analysis. HEK293 cells were harvested 4 days after transfection and total RNA was extracted using Tri Reagent (Sigma, MI) according to the manufacturer's instructions. Twenty-five micrograms of RNA was resolved on urea denaturing 15% polyacrylamide gels and blotted onto nylon membranes. Decade RNA molecular weight markers (Ambion, Austin, TX), which were labelled radioactively as described below, were run alongside the cellular RNA. Blots were hybridized to three DNA oligonucleotides (probes A, B, and C) to detect products of hairpin processing. These were complementary to regions spanning the HBV antisense sequence of the long hairpin. Probes were labelled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase. After purification using standard procedures, they were hybridized to immobilized RNA, exposed to X-ray film and then stripped and reprobed. An oligonucleotide sequence complementary to U6 small nuclear RNA was used as a control to verify equal loading of the cellular RNA. Probe oligonucleotide sequences were as follows: probe A: 5′-GACTCCCC GTCTGTGCCTTCTCA-3′; probe B: 5′-TCATCTGCCGGACCGTGTG CACT-3′; probe C: 5′-TGCACTTCGCTTCACCTCTGCACTC-3′; and U6 small nuclear RNA probe: 5′-TAGTATATGTGCTGCCGAAGCGAG CA-3′.

Statistical analysis. Data are expressed as the mean ± SE of the mean. Statistical difference was considered significant when $P < 0.05$ and was determined according to Dunnett's multiple comparison test and calculated with the GraphPad Prism software package (GraphPad Software, San Diego, CA).

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Expressed Anti-HBV Primary MicroRNA Shuttles Inhibit Viral Replication Efficiently *In Vitro* and *In Vivo*

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The use of RNA interference (RNAi) to inhibit gene expression is potentially applicable in the treatment of viral infections such as hepatitis B virus (HBV) persistence. Although efficient HBV gene silencing by short hairpin RNA (shRNA) expressed from RNA polymerase (Pol) III promoters has been reported, constitutive high-level transcription may cause harmful side effects. Here, we report an approach that allows the use of a Pol II promoter to improve transcription regulation of expressed RNAi effecters. Pol II [cytomegalovirus (CMV)] or Pol III (U6) promoter cassettes that transcribe anti-HBV primary microRNA (pri-miR)-122 and pri-miR-31 shuttles were generated. In cultured cells both types of pri-miR-like sequences effected knockdown of markers of viral replication (>80%) and were processed to form intended 21-nucleotide guides. The concentration of CMV-expressed miRs was ~85-fold lower than the U6 shRNA-derived guide RNA. When cells were co-transfected with pri-miR expression cassettes, attenuation of independent RNAi–mediated gene silencing was not observed, which is in contrast to the action of U6 shRNA expression cassettes. The efficacy of the anti-HBV pri-miR shuttles *in vivo* was verified using the murine hydrodynamic injection model. Employing Pol II–expressed pri-miR mimics may be useful in the treatment of HBV infection, and potentially also for generic application in RNAi-based therapy.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection, which continues to be endemic to sub-Saharan Africa and parts of Asia, is often complicated by cirrhosis and hepatocellular carcinoma (reviewed in ref. 1). Licensed therapies are only partially effective, and developing improved treatment strategies to prevent the life-threatening sequelae of virus persistence remain an important medical priority.[2] Activation of the RNA interference (RNAi) pathway to effect specific HBV gene silencing[3–8] has prompted enthusiasm for the potential of nucleic acid–based HBV treatment. RNAi involves specific and powerful gene silencing through predictable complementary interaction between RNAi effecters and their targets.[9] Naturally, RNAi plays an important role in regulating gene expression through the processing of endogenous miRs (reviewed in ref. 10), which control several cellular processes including organogenesis, apoptosis, cell proliferation, and tumorigenesis. miRs are transcribed by Pol II[11] as pri-miR hairpin-like structures, which are then processed to form precursor miRs (pre-miRs) within the nucleus. This step is catalyzed by Drosha (an RNAse III enzyme) together with DGCR8, which is its double-stranded RNA–binding domain partner. After export from the nucleus, pre-miRs are processed by Dicer with associated double-stranded RNA–binding domain TAR RNA–binding protein. The resulting 19–24-base pair duplex is handed on to the RNA-induced silencing complex before selection of one strand as the mature miR guide. miRs are usually not entirely complementary to their targets, and bind to the 3′ untranslated regions of cognate messenger RNA to induce translational suppression. When base pairing between guide and target is perfectly matched, the Ago2 component of RNA-induced silencing complex exerts silencing through site-specific cleavage of the guide complement.[12,13]

The specific and powerful gene silencing that may be induced by RNAi has prompted investigation of RNAi-based therapeutic modalities to inhibit expression of pathology-causing genes, which include those of viruses such as HBV. Typically, exogenous RNAi-inducing sequences have been either synthetic short interfering RNA (siRNA) duplexes or expressed short hairpin RNA (shRNA) sequences.[14] Synthetic siRNAs are similar to Dicer cleavage products and cause gene silencing by direct activation of RNA-induced silencing complex. shRNAs enter the RNAi pathway at an earlier stage and act as pre-miR mimics. Constitutively active Pol III promoters have been favored for transcribing shRNAs because of their ability to generate short, defined transcripts with a minimal requirement for regulatory elements within the transcript-encoding sequences. Several sites of the HBV genome have been targeted with synthetic and expressed RNA sequences, and impressive knockdown of markers of viral replication has been demonstrated.[3–8] However, a recent finding that U6 Pol III–expressed anti-HBV shRNAs cause serious toxicity *in vivo* by saturating the endogenous miR pathway, is an important concern

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while considering the therapeutic application of expressed RNAi sequences.[15] Tissue-specific and inducible Pol II promoters may therefore be preferable to Pol III regulatory elements, because they provide a better means of transcription control and dose regulation of expressed RNAi effecters. Some reports have demonstrated efficient silencing by Pol II RNAi expression cassettes, but this approach has been hampered by unpredictable and variable silencing efficacy of conventional hairpin sequences.[16] Sequences upstream and downstream of the hairpins, which are incorporated into Pol II–derived transcripts, may interfere with processing of the silencers. In order to improve transcription control of potentially therapeutic sequences, we have taken advantage of the natural Pol II–mediated transcriptional control of cellular miRs. Anti-HBV sequences were incorporated into expression cassettes that encode mimics of pri-miR-31 (refs. 17,18) or pri-miR-122.[19] Potent silencing of markers of viral replication was achieved *in vitro* and *in vivo* when anti-HBV pri-miR shuttle expression cassettes were placed under the control of Pol II or Pol III promoters.

RESULTS

Design of anti-HBV pri-miR–expressing plasmids

Pri-miR expression cassettes were designed by replacing guide and complementary sequences of natural miR-31 (refs. 17,18) and miR-122 (ref. 19) with those of an effective anti-HBV shRNA (U6 shRNA 5) described earlier.[3] The wild-type sequences of the miRs were maintained as far as possible, and computer-aided prediction[20] of the secondary structure of the transcripts did not differ significantly from that of their respective wild-type miRs (**Figure 1a**). The final cassettes contained 51 nucleotides of wild-type sequence flanking either end of the pre-miR[17,19] that was located downstream of a U6 promoter or within an exonic sequence of a cytomegalovirus (CMV) immediate early promoter enhancer expression cassette (**Figure 1b**). Unlike typical cellular miRs, the intended guide sequence was perfectly complementary to its HBV target, and was therefore expected to effect cleavage of all HBV transcripts.

miR-mediated inhibition of HBV surface antigen secretion from transfected cells

Initially, in order to assess efficacy against HBV *in vitro*, Huh7 cells were co-transfected with miR-31/5- and miR-122/5-expressing vectors together with the pCH-9/3091 HBV replication competent target plasmid[21] (**Figure 2a**). Controls included a U6 shRNA-encoding plasmid (U6 shRNA 5), which we have previously shown to be effective against HBV,[3] and also a vector in which the CMV promoter transcribed the shRNA 5 sequence. Compared with mock-treated cells, knockdown of 95–98% of viral antigen secretion was achieved by U6 shRNA 5-, U6 miR-31/5-, U6 miR-122/5-, CMV miR-31/5-, and CMV miR-122/5-expressing vectors (**Figure 2b**). CMV miR-122/5 was slightly less effective than the other miR vectors (85–90% knockdown). The vector encoding shRNA 5 derived from the CMV promoter caused the least efficient silencing (~60%).

miR-mediated inhibition of Firefly luciferase activity in transfected cells

The HBsAg secretion data were corroborated using a reporter gene plasmid (pCH Firefly Luc) to measure knockdown efficiency *in situ* (**Figure 2c**). In pCH Firefly Luc, the *preS2/S* sequence of pCH-9/3091 (ref. 21) was replaced with the *Firefly luciferase* open reading frame (ORF) with the targeted *HBx* ORF remaining intact (**Figure 2a**). Co-transfection of pCH Firefly Luc with miR-encoding vectors allows for the convenient quantitative measurement of anti-HBV efficacy *in situ* by determining luciferase reporter gene activity. Analysis showed that the Firefly luciferase activity was diminished significantly by U6 shRNA 5-, U6 miR-31/5-, U6 miR-122/5-, CMV miR-31/5-, and CMV miR-122/5–containing vectors. Each of these plasmids caused knockdown of ~75% as compared with controls (**Figure 2c**). Again, the CMV shRNA 5 vector did not inhibit Firefly luciferase activity significantly. Taken together, these data indicate that the incorporation of an anti-HBV sequence into miR-like structures of miR-31 or miR-122 enables expression of the silencing sequence from a Pol II or Pol III promoter without compromising silencing efficacy.

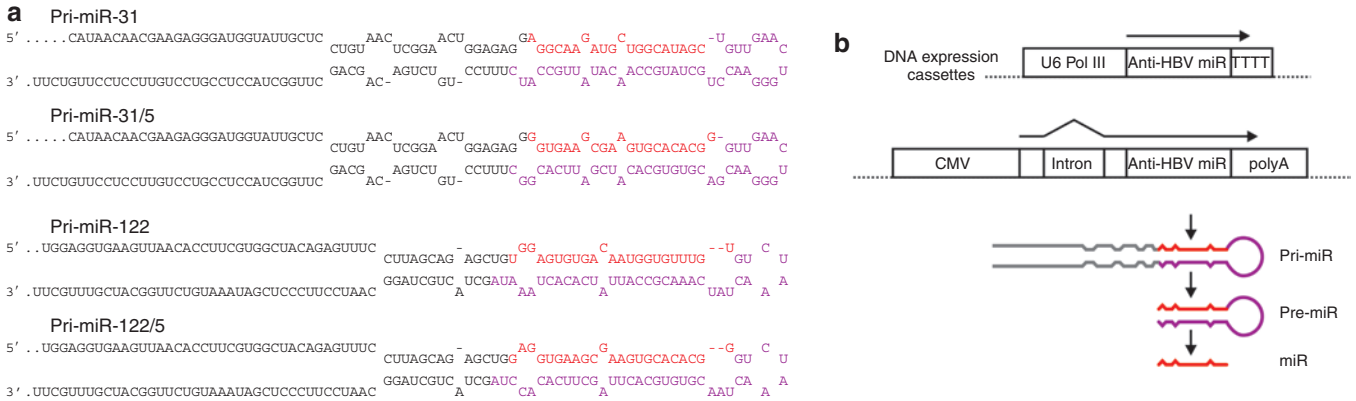

Figure 1 Pri-miR-31/5 and pri-miR-122/5 anti-HBV sequences. (**a**) Predicted structure and sequences of pri-miR-31 and pri-miR-122 (available at http://microrna.sanger.ac.uk/) with their anti-HBV derivatives, pri-miR-31/5 and pri-miR-122/5. The sequence of the putative pre-miRs generated after Drosha/DGCR8 processing is indicated in color (purple and red) and mature processed guide sequences are indicated in red only. (**b**) Schematic illustration (not to scale) of anti-HBV miR DNA expression cassettes showing putative processing of pri-miR and pre-miR to generate mature miR. The arrangement of the Pol II [cytomegalovirus (CMV)] or Pol III (U6) promoter, pri-miR-31/5- or pri-miR-122/5–encoding sequences, together with the transcription termination sequences are indicated. HBV, hepatitis B virus.

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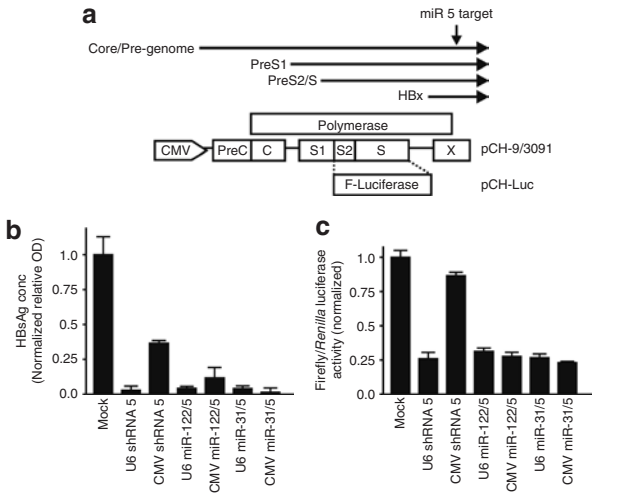

Figure 2 HBsAg secretion and reporter gene expression in Huh7 cells co-transfected with miR-encoding plasmids. (**a**) Organization of the hepatitis B virus (HBV) genome with open reading frames (ORFs) and sites within the pCH-9/3091 target vector. Four parallel arrows indicate the HBV transcripts which have common 3′-ends and include the miR-31/5, miR-122/5, and U6 short hairpin RNA 5 (shRNA 5) targets. The structure of the pCH-9/3091–derived pCH Firefly Luc target vector containing the *Firefly luciferase* ORF (F-luciferase) is indicated below. (**b**) HBsAg secretion from Huh7 cells co-transfected with plasmids encoding the indicated miR or shRNA cassettes, together with the HBV target plasmid. HBsAg measurements from quantitative enzyme-linked immunosorbent assay are given as a normalized mean relative to the corresponding measurements from mock-treated cells. The results are from three independent transfections, and the bars indicate the SEM. (**c**) Firefly luciferase reporter gene activity in transfected Huh7 cells. The measurements are given as a normalized ratio (±SEM) of Firefly activity to constitutively expressed *Renilla* luciferase activity, and were determined from three independent experiments. CMV, cytomegalovirus; OD, optical density.

Detection of processed miR-31/5 and miR-122/5 sequences

Northern blot hybridization analysis was carried out to detect processed products of the anti-HBV miR expression cassettes. RNA was extracted from transfected cells and **Figure 3** shows the signals obtained after hybridization to a probe that was complementary to the putative mature processed miR-31/5, miR-122/5, or shRNA 5 guides. The dominant processed product was detectable as a band of ~21 nucleotides in size, which is a length similar to that of naturally occurring mature miR-31 and miR-122 products.[17,19] Interestingly, bands corresponding to RNA of 20 and 22 nucleotides in length were also detected in cells transfected with CMV miR-31/5 and U6 miR-31/5 (**Figure 3a**), which implies that processing of anti-HBV guide strands in the context of the miR-31 shuttle may be heterogenous. Larger-molecular-weight miR/shRNA intermediates were detected in RNA extracted from cells transfected with U6 promoter–containing vectors but not from cells expressing the CMV miR-31/5 or CMV miR-122/5 cassettes. This suggests that complete processing of the CMV Pol II transcripts occurs more efficiently than that of the Pol III–expressed RNA. When compared with the U6 HBV shRNA 5–derived guide, which was included as a positive control of known high level expression, the mature miR-31 and miR-122 sequences were detected at up to 85-fold lower concentration. Interestingly, intracellular concentrations of miR-derived guides from U6 cassettes

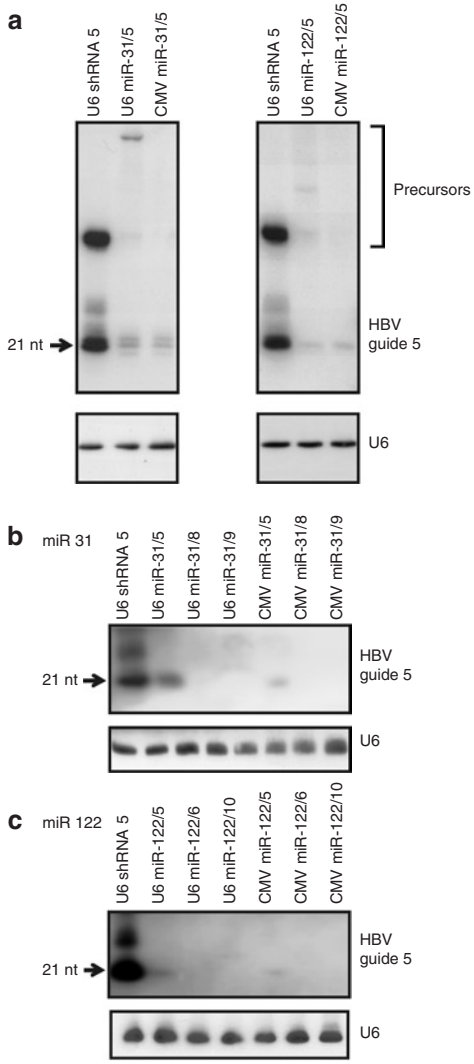

Figure 3 Northern blot hybridization analysis of expressed miR shuttle sequences that were extracted from HEK293 cells after transfection with plasmids encoding the indicated miR or short hairpin RNA (shRNA) cassettes. Hybridization was to a radiolabeled probe complementary to the putative mature anti-HBV guide 5 strand. Representative hybridization signals to detect (**a**) precursors of mature miRs and comparative concentrations of (**b**) mature miR-31/5 or (**c**) miR-122/5 sequences are shown. Blots were stripped and rehybridized to a probe complementary to endogenous U6 shRNA in order to confirm equal loading of cellular RNA (lower panels of **a**, **b**, and **c**). CMV, cytomegalovirus; HBV, hepatitis B virus.

were lower than for U6 shRNA 5. This could be the result of lower Pol III transcription efficiency of the longer miR-122/5 and miR-31/5 sequences. The detected guide strand signal was specific, as no bands were detectable when the probe was hybridized to RNA that had been extracted from cells transfected with similar pri-miR expression vectors that target different HBV sites (**Figure 3b** and **c**).

Assessment of off-target effects of miR-expression cassettes

The presence of duplex RNA within cells may lead to activation of a type 1 interferon (IFN) response, resultant programmed cell

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death, and nonspecific gene silencing (reviewed in ref. 22). In order to assess activation of the IFN response, the ratios of cellular *IFN-β* to *GAPDH* messenger RNA concentrations were measured in transfected cells using a sensitive quantitative real-time PCR assay (**Figure 4a**). *IFN-β* messenger RNA was not significantly induced in any of the cell groups that had been transfected with miR-expressing vectors, while treatment with poly (I:C) (positive control) resulted in activation of *IFN-β* expression. *IFN-β* activation was not tested in Huh7 cells, because we (data not shown) and others[23] have observed that the IFN response is attenuated in this liver-derived line. In order to control for transfection efficiency, a plasmid expressing enhanced green fluorescent protein (eGFP) was also included in the assessment of IFN induction. No decrease in eGFP expression was observed in the presence of the miR-expressing vectors (data not shown), which further supports the idea that these shuttles are not toxic to cells.

The effect of miR-expressing vectors on independent RNAi–mediated gene silencing was also assessed. To determine this, a dual luciferase reporter plasmid (psiCHECK-8T) containing an independent HBV miR-31/8 target sequence downstream of the Renilla *luciferase* ORF was transfected together with pCMV miR-31/8 and each of the shRNA 5-, miR-31/5-, or miR-122/5-expressing vectors (**Figure 4b**). In accordance with previous observations that overexpression of shRNA from U6 Pol III promoter causes disruption of the endogenous miR pathway,[15] the silencing of psiCHECK-8T target by pCMV miR-31/8 was diminished in the presence of pU6 HBV shRNA 5. This effect was, however, not observed when miR-122/5- or miR-31/5–expressing plasmids were co-transfected. These consequences are likely to be dependent on RNAi effecter concentration, and this is in keeping with our finding that the intracellular pri-miR-derived guide sequences are present at lower concentrations than U6 shRNA 5 guides (**Figure 3a**). In order to corroborate this, knockdown was measured using decreasing concentrations of co-transfected pU6 HBV shRNA 5 with constant amounts of CMV miR-31/8 and psiCHECK-8T target (**Figure 4c**). Efficient miR-31/8-mediated knockdown was achieved at low concentrations of pU6 HBV shRNA 5. However, when the amount of pU6 HBV shRNA 5 was increased, the efficacy against HBV target 8 was diminished. Co-transfecting a similar range of pU6 HBV shRNA 5 concentrations with pCH-9/3091 HBV replication competent plasmid confirmed that potent silencing of HBsAg secretion is achieved by the RNAi effecter (**Figure 4d**). These data further support the notion that disruption by pU6 HBV shRNA 5 of independent pCMV miR-31/8 silencing is influenced by the concentration of expressed shRNA 5.

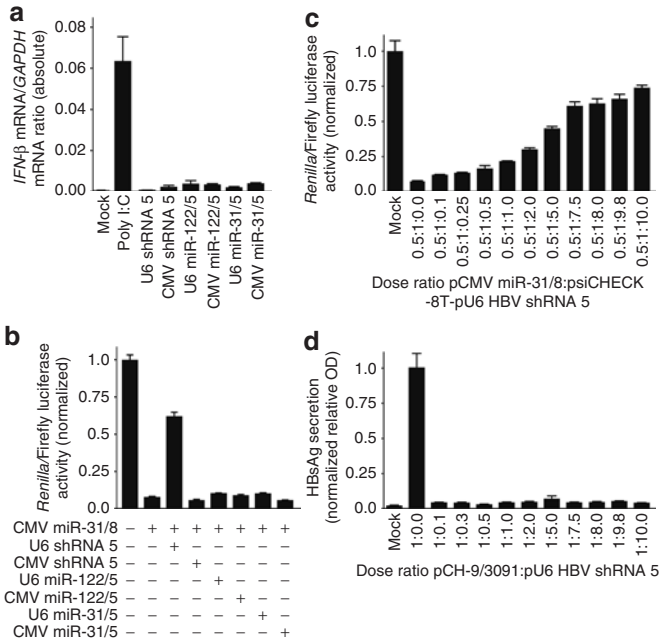

Figure 4 Assessment of off-target effects of miR-expression cassettes. (**a**) Interferon (IFN) response was assessed in HEK293 cells that were transfected with plasmids encoding the indicated miR-encoding cassettes or with poly (I:C). RNA was extracted from the cells 24 hours later and subjected to quantitative real-time PCR to determine concentrations of *IFN-β* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) messenger RNA (mRNA). The mean values (±SEM) of the normalized ratios of *IFN-β* to *GAPDH* mRNA concentrations are indicated from three independent experiments. (**b**) Attenuation of independent RNA interference–mediated silencing was assessed by co-transfection of Huh7 cells with plasmids expressing the indicated short hairpin RNA (shRNA) or miR cassettes, together with pCMV miR-31/8 and a psiCHECK-8T dual luciferase vector. The reporter plasmid contained the independent hepatitis B virus (HBV) miR-31/8 cognate sequence downstream of the Renilla *luciferase* open reading frame. Measurement of *Renilla*:Firefly luciferase activity was used for assessing the effects of shRNA 5-, miR-31/5-, or miR-122/5–expressing plasmids on miR-31/8 silencing of its target. (**c**) Effect of the amounts of pU6 HBV shRNA 5 used for transfection on attenuation of cytomegalovirus (CMV) miR-31/8 silencing. The indicated mass ratios of pCMV miR-31/8 to psiCHECK-8T to pU6 HBV shRNA 5 vectors were co-transfected. Again, measurements of *Renilla*:Firefly luciferase activities (±SEM) were used for assessing the effects of decreasing amounts of pU6 HBV shRNA 5 on miR-31/8 silencing of its target. (**d**) Inhibition of HBsAg secretion from Huh7 cells that were transfected with decreasing amounts of pU6 HBV shRNA 5. The indicated mass ratios of pU6 HBV shRNA 5 to HBV replication competent pCH-9/3091 vectors were used for transfection and the HBsAg concentrations in the culture supernatants were determined 48 hours thereafter. Normalized mean relative optical density (OD) readings (±SEM) from enzyme-linked immunosorbent assays are represented.

Inhibition of markers of HBV replication *in vivo*

Concentrations of HBsAg were measured in the sera of mice that had been subjected to hydrodynamic tail-vein injection (HDI).[24] Each of the shRNA 5-, miR-31/5-, and miR-122/5-containing plasmids knocked down the serum viral antigen concentration by at least 95% (**Figure 5a**). This was observed when measurements were taken at both 3 and 5 days after HDI. Of the three plasmid vectors, pU6 shRNA 5 was the most efficient, and HBsAg was not detectable in the sera of mice injected with this plasmid. The number of circulating viral particle equivalents in the same mice was also measured using quantitative real-time PCR at days 3 and 5 (**Figure 5b**). The results corroborate the HBsAg determinations, in that pU6 shRNA 5, pCMV miR-31/5, and pCMV miR-122/5, each decreased the number of circulating viral particle equivalents by at least 95%. HBV DNA replication intermediates were also measured in the liver tissues of representative animals that had been subjected to HDI experimentation (**Figure 5c**). HBV duplex linear and relaxed circular DNA were detectable only in the mock-treated animals, but not in any of the mice that had been co-injected with CMV miR-31/5, CMV miR-122/5, or U6 shRNA 5 plasmids. The size of the HBV DNA bands from mock-treated mice did not correspond to any of the

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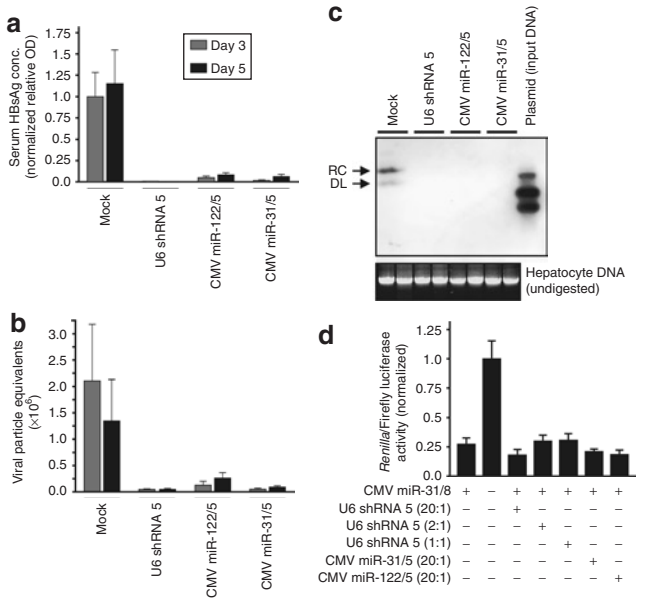

Figure 5 Effects of miR sequences on markers of hepatitis B virus (HBV) replication *in vivo* using the hydrodynamic tail-vein injection (HDI) model of HBV replication. (**a**) Serum HBsAg concentrations and (**b**) circulating viral particle equivalents were determined at days 3 and 5 after HDI of mice with pCH-9/3091 HBV target and plasmids encoding cytomegalovirus (CMV) miR-31/5, CMV miR-122/5, or U6 short hairpin RNA 5 (shRNA 5) sequences. The results are expressed as mean values (±SEM) from at least four mice. (**c**) Southern blot analysis of HBV DNA replication intermediates extracted from two representative animals from each of the groups of mice that had been subjected to the HDI procedure (upper panel). HBV duplex linear (DL) and relaxed circular (RC) replication intermediates were detectable only in the mock-treated animals. pCH-9/3091 was loaded as a control to verify that HBV replication intermediates did not correspond to input DNA. The lower panel is a representation of the separated extracted DNA after ethidium bromide staining and before Southern transfer and hybridization. (**d**) In order to assess the effects of miR shuttles on independent silencing *in vivo*, mice were subjected to HDI with the psiCHECK-8T vector, together with the indicated RNA interference expression cassettes. Where relevant, the ratios of the CMV miR-31/5, CMV miR-122/5, and U6 shRNA 5 to CMV miR-31/8 vectors are indicated in parentheses. The normalized mean values of *Renilla*:Firefly luciferase activities (±SEM) were determined in liver homogenates 3 days after plasmid injection. mRNA, messenger RNA; OD, optical density.

pCH-9/3091 bands, thereby signifying that the detected double-stranded and relaxed circular HBV DNA were not the same as input plasmid DNA.

For the purpose of assessing possible disruption of independent RNAi-mediated silencing and toxicity *in vivo* caused to hepatocytes by pri-miR shuttles, mice were also injected with the psiCHECK-8T dual luciferase reporter plasmid and various anti-HBV expression cassettes (**Figure 5d**). Firefly and *Renilla* luciferase activities in liver homogenates were measured 3 days after HDI. Selective and efficient silencing of *Renilla* luciferase activity was achieved with pCMV miR-31/8. This knockdown was not attenuated by co-injection of 20-fold excess amount of U6 shRNA 5, CMV miR-122/5, or CMV miR-31/5. This indicates that, under the experimental conditions described here, independent silencing was unaffected by U6 shRNA or miR shuttle expression. When using the HDI model, direct assessment of hepatotoxicity caused by miR mimics is complicated by the damage to liver cells caused by the release of enzyme markers that is inherent to the injection procedure itself. As a surrogate indicator to assess damage to liver cells caused by pri-miR shuttles, untargeted and constitutively active psiCHECK-8T-derived Firefly luciferase activity was independently evaluated in the groups of mice. When compared with the animals receiving no RNAi effecter, the mice that had received the miR shuttles showed undiminished Firefly luciferase activity in liver homogenates (data not shown). Collectively, these data show that miR mimics generated from CMV miR-31/5 and CMV miR-122/5 are specific silencers of HBV replication *in vivo* with negligible effects on independent RNAi-mediated silencing. Moreover, the efficacy of the miR-expression cassettes is comparable with those of the U6 shRNA 5 sequences.

DISCUSSION

Although recent studies have demonstrated efficient silencing of HBV replication by activating the RNAi pathway,[3–8] some hurdles remain before the goal of therapeutic application of this approach is realized. Particularly important is limiting of unintended effects, which include off-target silencing of cellular RNA, disruption of the endogenous miR pathway and immunostimulation. Both synthetic and expressed sequences are being widely used to achieve RNAi-mediated HBV gene knockdown.[14] Unlike with DNA expression cassettes, dose regulation and delivery is easier to achieve with synthetic siRNAs. Nonetheless, expressed RNAi sequences have significant advantages, which include better stability of DNA templates, ability to achieve sustained silencing by continuously transcribed RNAi effecters, as well as compatibility of expression cassettes with incorporation into both recombinant viral and nonviral vectors. These properties render expression cassettes well suited for the treatment of chronic diseases such as persistent HBV infection. Constitutively active Pol III promoters have traditionally been used for expressing RNAi effecters. However, these regulatory elements have been reported to have serious toxic effects *in vivo*, which result from shRNA overexpression and saturation of the endogenous miR pathway.[15] Against this background, the use of Pol II promoters to express RNAi effecters offers the advantage of being able to refine transcription control and thereby limit unwanted effects. This study shows that potent knockdown of markers of HBV replication is attained *in vitro* and *in vivo* when an antiviral guide is incorporated into exonic pri-miR mimics that are transcribed from a Pol II promoter. miR-122 and miR-31 backbones were selected because these sequences were predicted to favor intrahepatic processing of adapted anti-HBV shuttles. miR-122 is liver-specific[19] and pri-miR-31 is efficiently processed by Drosha.[17] The demonstration that the anti-HBV miRs do generate an intended guide strand of ~21 nucleotides confirms that these sequences function as pri-miR mimics and are processed in a manner similar to those of natural pri-miRs. Potent silencing of markers of HBV replication was observed, with no evidence of toxicity or disruption of independent miR-mediated silencing. Also, data from our investigations have shown that anti-HBV pri-miR sequences that target different sites within the virus are capable of efficient silencing (**Figures 4b** and **5d**). These findings suggest that the design of Pol II pri-miR shuttles described here is potentially valuable for treating HBV infection and may be useful for generic application in RNAi-based therapy.

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Significant progress has been made in understanding the mechanisms of pri-miR processing and we aimed to utilize these recent insights to ensure optimal design of anti-HBV RNAi expression cassettes. Zeng *et al.*[17,25] have shown that Drosha substrate preference is for RNA hairpins that bear long terminal loops, and that pre-miRs are generated by cleavage that occurs two helical turns from the junction of the pri-miR loop and stem sequences. By contrast, Han *et al.*[26] reported that the terminal loop sequence is not essential. According to this alternative mechanism, the initial binding of DGCR8 to pri-miRs requires single-stranded hairpin-flanking regions. Thereafter, Drosha is recruited to cleave the pri-miR ~11 nucleotides from the stem–single-stranded RNA junction. The reason for the apparent differences between these proposed pri-miR processing mechanisms is unclear, but it is possible that both operate naturally. The anti-HBV pri-miR shuttles that we used here accommodate Drosha/DGCR8 processing by either of the mechanisms. Although other studies have reported silencing by pre-miR or shRNAs expressed from Pol II promoter elements,[27–36] these cassettes are of variable and sometimes poor silencing efficiency, and therefore this approach has not gained widespread acceptance. Importantly, if flanking single-stranded RNA sequences are of importance in the nuclear processing of pri-miR,[26] then it is likely that pre-miR and shRNA expressions from Pol II promoters will not be as efficient as those from the pri-miR mimics described here.

Recently, in an attempt to improve efficacy and overcome problems of viral escape mutation, long hairpin RNA–expressing sequences have been used for silencing HBV.[37] These cassettes were intended to be capable of generating different siRNAs to target independent HBV sites simultaneously. However, the use of long hairpin RNA expression cassettes seems to be limited by intracellular Dicer processivity and difficulties associated with controlling guide strand bias of multiple siRNAs. In addition to their production from Pol II promoters, another interesting property of miRs is that they are often naturally derived from polycistronic precursor sequences.[10] This is a characteristic that could be adapted for the formation of multimeric therapeutic silencing sequences,[36] which would be useful for overcoming viral escape. Current investigations in our laboratory are aimed at assessing the use of liver-specific Pol II promoters and production of multimeric pri-miR shuttles that target different HBV sequences.

MATERIALS AND METHODS

Anti-HBV miR sequences. Initially, DNA encoding pre-miR-31 and pre-miR-122 sequences with a guide targeting HBV were generated by PCR-based primer extension of partially complementary pre-miR-31/5 and pre-miR-122/5 forward and reverse primers. The anti-HBV guide of miR-31/5 targets HBV coordinates 1,575–1,595, and miR-122/5 targets HBV coordinates 1,575–1,597.

The oligonucleotide sequences were pre-miR-31/5 forward: 5′-GTAA CTCGGAACTGGAGAGGGGTGAAGCGAAGTGCACAC GGGTTGAACTGGGAACGACG-3′, pre-miR-31/5 reverse: 5′-CTGCT GTCAGACAGGAAAGCCGTGAATCGATGTGCACACGTC GTTCCCAGTTCAACCCTG-3′, pre-miR-122/5 forward: 5′-GAGTTT CCTTAGCAGAGCTGGAGGTGAAGCGAAGTGCACAC GGGTCTAAACTAACGTGTGCA-3′ and pre-miR-122/5 reverse: 5′-GG ATTGCCTAGCAGTAGCTAGGTGTGAAGCTAAGTGCAC ACGTTAGTTTAGACCCGTGTGCA-3′. The pre-miR-31/5 and pre-miR-122/5 products were purified and used as the template for a second round of PCR amplification with forward and reverse pri-miR primers, respectively. The primer sequences were pri-miR-31 forward: 5′-GCTAGCCA TAACAACGAAGAGGGATGGTATTGCTCCTGTAACTCGGAACTG GAGAGG-3′, pri-miR-31 reverse: 5′-AAAAAAACTAGTAAGACAAG GAGGAACAGGACGGAGGTAGCCAAGCTGCTGTCAGACAG GAAGC-3′; pri-miR-122 forward: 5′-GACTGCTAGCTGGAGGTGAAG TTAACACCTTCGTGGCTACAGAGTTTCCTTAGCAGAGCTG-3′ and pri-miR-122 reverse: 5′-GATCACTAGTAAAAAAGCAAACGATGCCA AGACATTTATCGAGGGAAGGATTGCCTAGCAGTAGCTA-3′.The U6 promoter was also amplified using standard PCR conditions from a U6 promoter–containing plasmid.[38] The primer sequences were U6 forward: 5′-GATCAGATCTGGTCGGGCAGGAAGAGGGCC-3′ and U6 reverse: 5′-GCTAGCGGTGTTTCGTCCTTTCCACA-3′. DNA fragments encoding pri-miR-31/5, pri-miR-122/5, or the U6 promoter were each ligated into pTZ57R/T (InsT/Aclone PCR Cloning Kit; Fermentas, Hanover, MD) to generate pTZ pri-miR-31/5, pTZ pri-miR-122/5, and pTZ-U6 respectively. For generating Pol III–driven pri-miR vectors, pri-miR-31/5 and pri-miR-122/5 fragments were cloned downstream of the U6 promoter in pTZ-U6. Pri-miR-31/5 was excised from pTZ pri-miR-31/5 by digesting with NheI and ScaI, and pri-miR-122/5 was removed from pTZ pri-miR-122/5 after NheI and EcoRI restriction. These fragments were inserted into SpeI and ScaI, or SpeI and EcoRI sites of pTZ-U6 to produce pU6 pri-miR-31/5 and pU6 pri-miR-122/5, respectively. The method of generation of the pU6 shRNA 5 vector has been described earlier.[3] Pol II–driven pri-miR vectors were generated by inserting pri-miR-31/5, pri-miR-122/5, and shRNA 5 sequences downstream of the CMV of pCI-neo (Promega, Madison, WI). pri-miR-31/5 was excised from pTZ pri-miR-31/5 with SalI and NheI and ligated to complementary overhangs of pCI-neo (which had been digested with XhoI and XbaI) to form pCMV pri-miR-31/5. pCMV pri-miR-122/5 was generated by inserting a NheI and XbaI restriction fragment from pTZ pri-miR-122/5 into a pCI-neo backbone that had been digested with the same restriction enzymes. pTZ pri-miR-31/8 (HBV target 1,678–1,700) and pCMV pri-miR-31/8 (HBV target 1,678–1,700) expression cassettes targeting different sites within the HBx ORF were generated using similar procedures.

Target plasmids. pCH-9/3091 has been described earlier.[21] The pCH Firefly Luc vector was prepared by replacing the *preS2/S* ORF of pCH-9/3091 with Firefly luciferase–encoding DNA. A *Firefly luciferase* sequence was amplified from pGL4 (Promega, Madison, WI) using PCR. The forward primer comprised sequences complementary to HBV sequences from coordinates 129–159 (including a naturally occurring *Xho*I restriction site) and 5′ *Firefly luciferase* sequences. In this primer, the position of the *Firefly luciferase* initiation codon is equivalent to that of the translation initiation codon of the middle HBs protein. The reverse primer included sequences complementary to the 3′-end of the *Firefly luciferase* ORF, as well as a *Spe*I restriction site. The PCR primer sequences were Firefly Luc forward: 5′-ACTGCTCGAGG ATTGGGGACCCTGCGCTGAACATGGAAGACGCCAAAAAC-3′ and Firefly Luc reverse: 5′-ACTGACTAGTTTACACGGCGATCTTTCC-3′. The PCR product was cloned into pTZ57R/T to generate pTZ Firefly Luc. The *Firefly luciferase* sequence was then excised from pTZ Firefly Luc with *Xho*I and *Spe*I and inserted into the *Xho*I and *Spe*I sites of pCH-9/3091 to generate pCH Firefly Luc. For generating psiCHECK-8T, containing the miR 8 target, primer 8T forward 5′-CAATGTCAACGACCGACCT-3′ and primer 8T reverse 5′-ACTAGTGCCTCAAGGTCGGT-3′ were used for amplifying nucleotides 1,678–1,702 of the HBV genome and for introducing a *Spe*I site at the 3′-end of the amplicon. The purified fragment was ligated into the pTZ57R/T PCR cloning vector, and the insert was removed with *Sal*I and *Spe*I and then ligated into the *Xho*I and *Spe*I sites of the psiCHECK 2.2 derivative of psiCHECK 2 (Promega, WI). The resulting plasmid, psiCHECK-8T, contained the HBV target site downstream of the Renilla *luciferase* ORF. The psiCHECK 2.2 plasmid, a gift from Dr. Marc Weinberg, contained additional *Sac*I, *Eco*RI, *Sma*I, *Spe*I, and *Sal*I sites between the *Not*I and *Xho*I sites of psiCHECK 2. All the plasmid sequences

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were verified in accordance with standard dideoxy chain termination protocols (Inqaba Biotechnology, Pretoria, South Africa).

Cell culture. Culture and transfection of Huh7 and HEK293 lines were carried out as described earlier.[3] For determining the effects of miR-31/5- and miR-122/5–encoding plasmids, cells were transfected with a combination of 80 ng of pCH-9/3091 (ref. 21) or pCH Firefly Luc target vector and 800 ng of shRNA 5, miR-31/5- and miR-122/5–derived plasmids, or vector lacking the miR cassettes. For assessing the effects of miR-31/5- and miR-122/5–expressing plasmids on independent RNAi-mediated silencing, cells were seeded into 24-well dishes at a density of 35–40% and transfected with 80 ng of psiCHECK-8T, 40 ng of pCMV miR-31/8, and 780 ng of shRNA 5 or miR 5 expression plasmids. In the case of transfections with the pCH Firefly Luc target vector, a plasmid that constitutively produces *Renilla* luciferase under control of the CMV promoter (phRL-CMV; Promega, Madison, WI) was included in order to control for transfection efficiency. The effects of the plasmid dose of pU6 shRNA 5 on independent silencing by pCMV miR-31/8 were determined by transfecting with pU6 shRNA 5 in amounts ranging from 0 to 800 ng. Similarly, for determining the silencing potency of pU6 shRNA 5 against pCH-9/3091, pU6 shRNA 5 was used for transfection in amounts ranging from 0 to 800 ng. A constant amount of 80 ng of psiCHECK-8T and pCH-9/3091 were used for transfection in each well. Backbone plasmid was included in each case so as to ensure that equal amounts of total plasmid DNA were used for transfection. The measurement of IFN response, HBsAg, and the activities of *Renilla* and Firefly luciferase were determined as described earlier.[37] A plasmid vector that constitutively produces eGFP[39] was also included in each co-transfection so as to verify equivalent transfection efficiencies using fluorescence microscopy. Northern blot analysis of total RNA extracted 3 days after transfection was carried out as described earlier.[37] The miR-31/5, miR-122/5, and shRNA 5 guide probe oligonucleotide was 5′-GACTCCCCGTCTGTGCCTTCTCA-3′.

Testing of anti-HBV efficacy of miR sequences* in vivo *using the HDI model of HBV replication. The murine HDI method[24,37] was employed for determining the *in vivo* effects of miR-expressing vectors on the markers of HBV replication and reporter gene expression. All experiments on animals were carried out in accordance with protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. For the purpose of assessing the effects on viral replication, the injected solutions included a combination of three plasmid vectors: 5 μg target DNA (pCH-9/3091); 5 μg anti-HBV sequence (pU6 shRNA 5, pCMV miR-31/5, or pCMV miR-122/5 plasmid) or mock (pTZ backbone); and 5 μg pCI-neo eGFP (a control for hepatic DNA delivery, constitutively expressing the eGFP marker gene[39]). Serum HBsAg concentration and circulating viral particle equivalents were measured as described.[3] The mice were killed at day 5 after HDI, and the livers were removed. Total DNA was extracted[40] and subjected to agarose gel electrophoresis without restriction digestion before being processed for Southern blot analysis using Rapid-hyb solution (Amersham, Piscataway, NJ). pCH-9/3091 (ref. 21) was run alongside as a control for input DNA. For generating a probe, HBx DNA was amplified with HBx forward (5′-GATCAAGCTTTCGCCAACTTACAAGGCCTTT-3′) and HBx reverse (5′-GATCTCTAGAACAGTAGCTCCAAATTCTTTA-3′) primers. The PCR products were purified and used as the template for random-primed labeling with the HexaLabel DNA Labeling kit (Fermentas, WI), in accordance with the manufacturer's instructions. In order to determine the effects of miR on reporter gene activity *in vivo*, mice were administered 0.5 μg reporter target DNA (psiCHECK-8T), 5 μg pCH-9/3091, combinations of anti-HBV plasmids (pCMV miR-31/8, pU6 shRNA 5, pCMV miR-31/5, or pCMV miR-122/5), or mock (pCI-neo backbone). The mice were killed 3 days after HDI, and their livers were removed and homogenized in phosphate-buffered saline; thereafter, the activities of *Renilla* and Firefly luciferase were determined as described earlier.[37]

Statistical analysis. Analysis of statistically significant differences was carried out using the Student's paired two-tailed *t*-test.

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Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles

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ABSTRACT

Expressed polycistronic microRNA (miR) cassettes have useful properties that can be utilized for RNA interference (RNAi)-based gene silencing. To advance their application we generated modular trimeric anti-hepatitis B virus (HBV) Pol II cassettes encoding primary (pri)-miR-31-derived shuttles that target three different viral genome sites. A panel of six expression cassettes, comprising each of the possible ordering combinations of the pri-miR-31 shuttles, was initially tested. Effective silencing of individual target sequences was achieved in transfected cells and transcribed pri-miR trimers generated intended guide strands. There was, however, variation in processing and silencing by each of the shuttles. In some cases the monomers' position within the trimers influenced processing and this correlated with target silencing. Compromised efficacy could be compensated by substituting the pri-miR-31 backbone with a pri-miR-30a scaffold. Inhibition of HBV replication was achieved *in vivo*, and in cell culture without disruption of endogenous miR function or induction of the interferon response. A mutant HBV target sequence, with changes in one of the guide cognates, was also silenced by the trimeric cassettes. The modular nature of the cassettes together with compatibility with expression from Pol II promoters should be advantageous for gene silencing applications requiring simultaneous targeting of different sites.

INTRODUCTION

The powerful and specific gene silencing that may be achieved by harnessing the RNA interference (RNAi) pathway is potentially useful for developing new therapies required to treat a variety of diseases. In addition, application of RNAi has utility for the study of gene function. Both synthetic and expressed sequences are being developed to activate RNAi (1). Exogenous expression cassettes achieve this by transcribing mimics of intermediates of the microRNA (miR) processing pathway (2). Short hairpin RNAs (shRNAs), which are typically expressed from Pol III promoters, simulate precursor miR (pre-miR) products of Drosha/DGCR8 processing. Primary miR (pri-miR) shuttles are analogues of nascent miR transcripts and their processing is compatible with expression from Pol II transcription regulatory elements (3–7). This important property provides the means of improving control of production of RNAi activators and thereby limiting unwanted off target effects caused by saturating the endogenous miR pathway (8). Pri-miR-like shuttles are also thought to effect superior silencing by simulating natural miR processing more closely. Processing of pri-miR shuttles by Drosha/DGCR8, which is bypassed by shRNAs, may improve entry into the RNAi pathway (4). pri-miR-30 was initially the most widely utilized (9–11) backbone, but other pri-miR shuttles such as miR-155 (6) miR-31 and miR-122 (3) have since been used successfully to generate exogenous RNAi effecters. The polycistronic arrangement of some naturally occurring miR clusters is an additional property that may be exploited to generate combinatorial multitargeting RNAi expression cassettes (4,6,7). This is particularly useful to improve knockdown efficacy and overcome attenuation of silencing caused by target site mutation such as often occurs during chronic viral infection. Recently, the miR-106 (7) and miR-17-92 (4) polycistronic clusters have been used successfully to generate multiplexed anti-HIV-1 RNAi activators. To improve use of expressed multimeric RNAi effecters, a system that allows convenient assembly, modification to improve silencing efficacy and which causes knockdown without disrupting the endogenous miR pathways would be valuable. We demonstrate these attributes in a panel of anti-hepatitis B virus (HBV) Pol II trimeric pri-miR cassettes, which are

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capable of inhibiting viral replication in transfected cells and *in vivo*.

MATERIALS AND METHODS

miR expression plasmids

Anti-HBV pre-miR DNA shuttles were generated by annealing partly complementary pre-miR-31/5, -31/8, 31/9 and pre-miR30a/8 forward (F) and reverse (R) oligonucleotides, which was followed by primer extension to generate completely double-stranded DNA. The oligonucleotide sequences were pre-miR-31/5 F: 5′-GTA ACT CGG AAC TGG AGA GGG GTG AAG CGA AGT GCA CAC GGG TTG AAC TGG GAA CGA CG-3′, pre-miR-31/5 R: 5′-CTG CTG TCA GAC AGG AAA GCC GTG AAT CGA TGT GCA CAC GTC GTT CCC AGT TCA ACC CGT-3′, pre-miR-31/8 F: 5′-GTA ACT CGG AAC TGG AGA GGC AAG GTC GGT CGT TGA CAT TGG TTG AAC TGG GAA CGA AA-3′, pre-miR-31/8 R: 5′-CTG CTG TCA GAC AGG AAA GCT AAG GTT GGT TGT TGA CAT TTC GTT CCC AGT TCA ACC AAT-3′, pre-miR-31/9 F: 5′-GTA ACT CGG AAC TGG AGA GGA TTT ATG CCT ACA GCC TCC TAG TTG AAC TGG GAA CGA AG-3′, pre-miR-31/9 R: 5′-CTG CTG TCA GAC AGG AAA GCC TTT ATT CCT TCA GCC TCC TTC GTT CCC AGT TCA ACT AGG-3′, pre-miR-30a/8 F: 5′-TGC TGT TGA CAG TGA GCG ACT CAA GGT CGG TCG TTG ACA TTG CTG TGA AGC CAC AGA TGG GC-3′ and pre-miR-30a/8 R: 5′-GAA GTC CGA GGC AGT AGG CAG CTC AAG GTC GGT TTG ACA TTG CCC ATC TGT GGC TTC ACA G-3′. Extended pre-miR DNA was used as template to generate the pri-miR shuttle sequences using PCR with pri-miR-31 F: 5′-GCT AGC CAT AAC AAC GAA GAG GGA TGG TAT TGC TCC TGT AAC TCG GAA CTG GAG AGG-3′, pri-miR-31 R: 5′-AAA AAA ACT AGT AAG ACA AGG AGG AAC AGG ACG GAG GTA GCC AAG CTG CTG TCA GAC AGG AAG C-3′, pri-miR-30a F: 5′-GAT CGC TAG CTT AAC CCA ACA GAA GGC TAA AGA AGG TAT ATT GCT GTT GAC AGT GAG CGA C-3′ and pri-miR-30a R: 5′-GAT CAC TAG TAA AAA ACA AGA TAA TTG CTC CTA AAG TAG CCC CTT GAA GTC CGA GGC AGT AGG CA-3′ primers. The pri-miR-31 and pri-miR-30a sequences were then inserted into the PCR cloning vector, pTZ57R/T (InsTAclone™ PCR cloning Kit, Fermentas, MD, USA) to generate pTZ pri-miR-31/5, pTZ pri-miR-31/8, pTZ pri-miR-30a/8 and pTZ pri-miR-31/9. Propagation of pTZ pri-miR-122/5 has been described previously (3). To produce U6-driven pri-miR expression plasmids (pTZ U6-pri-miR-31/5, pTZ U6-pri-miR-31/8 and pTZ U6-pri-miR-31/9), the pri-miR-31 shuttle sequences were excised with NheI and ScaI then inserted into equivalent sites downstream of the U6 promoter in the pTZ-U6 vector (12). Pol II-driven miR expression plasmids (pCI-pri-miR-31/5, 31/8 and 31/9) were constructed by excising the pri-miR-31 shuttle sequences with SalI and XbaI and ligating these fragments to XhoI and XbaI sites of pCI-neo (Promega, WI, USA).

Trimeric shuttle cassettes, containing pri-miR-31, pri-miR-30a and pri-miR-122 sequences, were formed by inserting combinations of pri-miR-31/5, pri-miR-31/8, pri-miR-30a/8, pri-miR-31/9 and pri-miR-122/5 sequences downstream of the CMV immediate early promoter enhancer. A total of eight trimeric cassettes was generated (pri-miR-31/5-8-9, pri-miR-31/5-9-8, pri-miR-31/8-5-9, pri-miR-31/8-9-5, pri-miR-31/9-5-8, pri-miR-31/9-8-5, pri-miR-31/5-31/9-30a/8 and pri-miR-122/5-31/9-30a/8). Six of the trimeric cassettes comprised pri-miR-31-derived sequences exclusively, while pri-miR-31/5-31/9-30a/8, and pri-miR-122/5-31/9-30a/8 also included pri-miR-30a and pri-miR-122 scaffolds. Similar cloning strategies were used to propagate each of the trimers. As an example, to generate the pri-miR-31/5-8-9 cassette, pri-miR-31/8 was excised from pTZ pri-miR-31/8 with NheI and EcoRI and ligated to pTZ pri-miR-31/5 that had been digested with SpeI and EcoRI to create pTZ pri-miR-31/5-8. Similarly, the sequence encoding pri-miR-31/9 was then excised from pTZ pri-miR-31/9 with NheI and EcoRI and ligated to SpeI and EcoRI sites of pTZ pri-miR-31/5-8. Successful ligation resulted in formation of pTZ pri-miR-31/5-8-9. The trimer cassettes were excised with NheI and XbaI and inserted at equivalent sites of pCI-neo to generate the CMV panel of multimeric cassettes. Sequences were verified using standard automated dideoxy chain termination reactions.

miR target plasmids

To produce dual luciferase reporter plasmids containing sites individually targeted by pri-miR-31/5, pri-miR-31/8, pri-miR-31/9, pri-miR-30a/8 and pri-miR-122/5 primers were designed to amplify HBV coordinates 1575–1599 (5T), 1678–1702 (8T) and 1774–1798 (9T) (Genbank accession J02203). Oligonucleotide sequences, which also introduced a SpeI site at the 3′ end of the amplicons, were 5T F: 5′-CCG TGT GCA CTT CGC TTC AC-3′, 5T R: 5′-ACT AGT CAG AGG TGA AGC GA-3′, 8T F: 5′-CAA TGT CAA CGA CCG ACC TT-3′, 8T: R 5′-ACT AGT GCC TCA AGG TCG GT-3′, 9T F: 5′-TAG GAG GCT GTA GGC ATA AA-3′ and 9T R: 5′-ACT AGT ACC AAT TTA TGC CT-3′. Purified fragments were incorporated into the pTZ57R/T PCR cloning vector (InsTAclone™ PCR cloning Kit, Fermentas, MD, USA) and the insert was removed with SalI and SpeI then ligated to the XhoI and SpeI sites of psiCHECK2.2 (3) that had been previously modified from psiCHECK™-2 (Promega, WI, USA), to generate psiCHECK-5T, psiCHECK-8T and psiCHECK-9T with the target sites downstream of the Renilla *luciferase* reporter open reading frame (ORF). The reporter target vector psiCHECK-HBx, which contains an intact *HBx* target sequence downstream of the Renilla ORF within psiCHECK™-2 (Promega, WI, USA), has been described previously (13). The derivative with mutant *HBx* target, psiCHECK-mHBx, was propagated using PCR. Briefly *mHBx* was amplified from psiCHECK-HBx using mHBx F (5′ GAT CCG GTC CGT **CTG CAG** TTC GGT TGT CCT CTG CAC GTT GCA TGG AG 3′) and mHBx R (5′ GAT CGC GGC CGC CCG GGT

CGA CTC 3′) primers. Mutant bases of the target 5 sequence (underlined) were incorporated within the forward PCR primer. The resultant amplicon included RsrII and NotI sites at the 5′ and 3′ ends, respectively. After insertion into the pTZ57R/T PCR cloning vector (InsTAclone™ PCR cloning Kit, Fermentas, MD, USA) and sequence verification, *mHBx* was excised with RsrII and NotI then used to replace *HBx* in psiCHECK-HBx and generate psiCHECK-mHBx. Presence of a PstI restriction digestion site (bold font in mHBx F primer), which is absent from wild-type *HBx*, was used to verify insertion of the intended mutant sequences. The HBV target plasmids, pCH-9/3091 (14) and pCH-FLuc (3), have been described previously.

miR-16 sponge and dual luciferase target

The U6-driven miR-16 sponge (15) was generated by cloning seven copies of an imperfectly complementary target of miR-16 into the U6 + 27 sequence (16–18). A single copy of duplex DNA, comprising annealed oligonucleotides encoding a single copy of the miR-16 target site (miR-16S) with single nucleotide 3′ A overhangs, was initially ligated to pTZ57R/T to create pTZ-miR-16S×1. The resulting target sequence included an XhoI site that was 5′ of the target and SalI and NotI sites 3′ of this sequence. Oligonucleotide sequences used to generate the inserts were miR-16S F: 5′-CTC GAG CGC CAA TAT TAT GTG CTG CTA GTC GAC GCG GCC GCA-3′ and miR-16S R: 5′-GCG GCC GCG TCG ACT AGC AGC ACA TAA TAT TGG CGC TCG AGA-3′. The miR-16S×1 sequence was restricted from pTZ-miR-16S×1R (insert in reverse orientation with respect to the *β-galactosidase* gene) with ApaI and PvuII and cloned into the ApaI and HincII sites of pGEM®-T Easy (Promega, WI, USA) to create pG-miR-16S×1. To generate vectors with tandem copies of the miR-16S sequence, pG-miR-16S×1 was digested with XhoI and ScaI and separately with SalI and ScaI. The fragments containing the miR-16S sequence from each digestion were ligated to create pG-miR-16S×2. pG-miR-16S×3 and pG-miR-16S×4 were generated using similar procedures. Finally, the vectors containing three and four tandem copies of the miR-16S sequence were used to create pG-miR-16S×7.

The U6 + 27 sequence (18) was produced using a two-step PCR of the human U6 promoter. U6 forward (U6 F, 5′-GAT CTC TAG AAA GGT CGG GCA GGA AGA GGG-3′) and U6 + 27 reverse 1 (U6 + 27 R1, 5′-CTC GAG TAG TAT ATG TGC TGC CGA AGC GAG CAC GGT GTT TCG TCC TTT CCA C-3′) primers used in the first round of amplification. Amplicons from this reaction were used as template for the second round of PCR using the U6 + 27 R2 primer (5′-GAT CAA AAA AGC GGA CCG AAG TCC GCT CTA GAC TCG AGT AGT ATA TGT GCT G-3′) and U6 F primer. The complete U6 + 27 sequence was inserted into the PCR cloning vector pTZ57R/T to generate pTZ-U6 + 27. The miR-16S×7 sequence was removed from pG-miR-16S×7 with XhoI and SalI and ligated to the XhoI site of pTZ-U6 + 27 to produce the pTZ-U6-miR-16S×7 sponge plasmid. To generate the psiCHECK-miR-16T×7 target vector containing 7 miR-16 sites downstream of the Renilla *luciferase* ORF, the miR-16S×7 sequence was restricted from pG-miR-16S×7 with XhoI and NotI and inserted into equivalent sites of psiCHECK™-2 (Promega, WI, USA).

Cell culture, transfection, northern blot analysis and dual luciferase assay

Huh7 cells were cultured in DMEM (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (Gibco BRL, UK). To determine the efficacy of individual pri-miR monomers in the context of multimeric cassettes, each trimeric plasmid (800 ng) was co-transfected with psiCHECK-5T, psiCHECK-8T or psiCHECK-9T (80 ng). Luciferase activity was assayed using the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) and *Renilla* luciferase to Firefly luciferase activity was determined. Silencing of mutant HBx sequences was assayed similarly by using psiCHECK-HBx and psiCHECK-mHBx dual luciferase reporter vectors. To assess HBV knockdown efficacy of the Pol III and Pol II pri-miR shuttles, Lipofectamine 2000™ (Invitrogen, CA, USA) was used to co-transfect 80 ng pCH-FLuc, 800 ng of the relevant pri-miR shuttle plasmid, together with effecter plasmid or vector control plasmid according to previously described methods (3). phRL-CMV (Promega, WI, USA), a plasmid constitutively expressing *Renilla* luciferase, was included in all transfections. Forty-eight hours after transfection cells were assayed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) and the ratio of Firefly luciferase to *Renilla* luciferase activity was calculated.

Northern blot analysis was performed on RNA extracted from cells transfected with the various miR-31 shuttle constructs according to previously described methods (3). The probes for the 5, 8 and 9 guide sequences were 5′-CCG TGT GCA CTT CGC TTC-3′, 5′-CAA TGT CAA CGA CCG ACC-3′ and 5′-TAG GAG GCT GTA GGC ATA-3′, respectively. Scanned autoradiographs were used to quantitate guide bands using KODAK MI Software.

Knockdown of HBV replication was assessed in cells co-transfected with pCH-9-3091(14) and relevant RNAi effecter plasmid. Forty-eight hours after transfection growth medium was harvested and HBsAg secretion measured by ELISA using the MONOLISA® HBs Ag ULTRA kit (Bio-Rad, CA, USA).

Activation of the interferon (IFN) response was assessed using previously described methods (19). Assays to assess saturation of the endogenous miR pathway were performed in Huh7 cells cotransfected with 80 ng psiCHECK-miR-16T×7 and 800 ng RNAi effecter plasmids or miR-16 sponge plasmid. Luciferase assays were performed as described above.

Assessment of efficacy of pri-miR-31 shuttles *in vivo*

Mice were injected using the hydrodynamic injection procedure with a combination of 5 μg pCH-9-3091 (14), 5 μg of RNAi expression vector, 5 μg of control U6 (pTZ-U6 vector (12)) or CMV (pCI-neo, Promega, WI, USA)

promoter-containing backbone plasmid and 5 μg of psiCHECK2.2. Blood was collected 3 and 5 days post-injection. Experiments were carried out according to protocols approved by the University of the Witwatersrand Animal Ethics Screening Committee. ELISA for HBsAg levels was performed on serum samples using the MONOLISA® HBs Ag ULTRA kit from Bio-Rad.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical difference was considered significant when $P < 0.05$ and was determined according to the Student's paired two-tailed *t*-test. Calculations were done with the GraphPad Prism software package (GraphPad Software Inc., CA, USA).

RESULTS

Design of trimeric pri-miR expression cassettes

Structure of the expression cassettes producing trimeric anti-HBV pri-miR-31 mimics is depicted schematically in Figure 1A. The pri-miR-31 backbone was initially selected as we have previously shown that single unit shuttles with this scaffold can be used to generate efficient Pol II anti-HBV expression cassettes (3). Sequences encoding the pri-miR-31 trimers were located within an exon and downstream of a CMV Pol II transcription controlling element and intron sequence. Trimeric cassettes were designed such that pre-miRs comprised 59 nt and were flanked by 51 nt of natural pri-miR-31/derived sequences (Figure 1B). According to this scheme, the mature anti-HBV miRs were predicted, using the MFold algorithm (20), to have a similar structure to that of naturally occurring pri-miR-31. To assess the modular nature of the cassettes, six different trimeric expression cassettes were generated using all possible ordering combinations of the three pri-miR-31 shuttles. Computer-based predictions indicated that the intended miR-31-like structures of the trimeric cassettes were energetically most favourable and similar for each of the six ordering combinations. The calculated ΔG values of each was approximately –195 kcal/mol.

Detection of processed pri-miR sequences and silencing of individual targets

To verify formation of individual guide sequences, northern blot analysis was carried out on RNA extracted from Huh7 liver-derived cells transfected with DNA-expressing pri-miR-31 shuttles (Figure 2A–C). Hybridization to a probe complementary to the intended miR-31/5 HBV guide showed heterogenous processing to form guide sequences of 20–22 nt in length. Guide strand 5 production was similar when generated from monomeric and trimeric cassettes and was not affected by shuttle position within the anti-HBV polycistron. As expected, measurement of relative guide band intensities showed that the 20–22 nt HBV anti-sense sequence was present in considerably higher amounts in cells transfected with U6 shRNA 5-expressing plasmid when compared to cells transfected

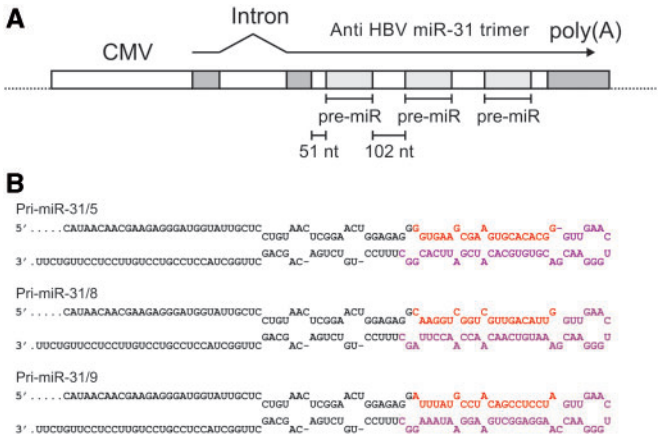

Figure 1. Trimeric pri-miR-31 expression cassettes. (**A**) Schematic illustration of pri-miR-31 shuttle expression cassettes showing upstream CMV promoter, intron and three miR mimic sequences with downstream transcription termination signal [poly(A)]. Pre-miR shuttles were inserted in an exon and were flanked by 51 nt of pri-miR-31-derived sequences. (**B**) Pri-miR-31/5, pri-miR-31/8 and pri-miR-31/9 anti-HBV sequences. Predicted structures and sequences of anti-HBV pri-miR-31 derivatives. The sequences of the putative pre-miRs generated after Drosha/DGCR8 processing are indicated in colour (purple and red) and the mature processed guide sequences that are selected after Dicer processing and strand selection by RISC are indicated in red only.

with the pri-miR trimer shuttles. Specific knockdown of target 5 sequence, assessed using a dual luciferase reporter system, was similar and highly effective (~90%) for the U6 shRNA 5 and each of pri-miR-31 shuttle expression cassettes (Figure 2D). Northern blot analysis to detect guide 8 revealed a single band of 21 nt (Figure 2B), which was distinct from the heterogenous mature pri-miR-31/5 sequences. Interestingly, no mature pri-miR-31/8 was detectable in RNA extracted from cells transfected with CMV pri-miR-31/9-8-5 and CMV pri-miR-31/5-9-8. This suggests that pri-miR-31/8 shuttle position within the trimer affects processing, and presence of pri-miR-31/9 immediately upstream of pri-miR-31/8 may be responsible for compromised guide 8 production. Assay of knockdown using a dual luciferase assay with miR-31/8 target alone, confirmed that knockdown of reporter expression correlated with detection of mature miR sequences (Figure 2E). Interestingly, guide produced from the U6 shRNA 8 cassette was slightly larger than that of the CMV miR-31-derived sequences. It is likely that the differences in secondary structure of the shRNA 8 and pri-miR-31/8 RNA, as well as the involvement of Drosha in miR shuttle processing, are responsible for generation of guide strands of different molecular weight. Analysis of silencing and processing of pri-miR-31/9 sequences showed less efficient knockdown (45–80%) of ORF containing pri-miR-31/9 target (Figure 2F), which correlated with lower efficiency of pri-miR-31/9 guide production (Figure 2C). Although there is variation in the efficiency of individual guide strand production and knockdown, these data indicate that the pri-miR-31 scaffold is useful for production of Pol II trimeric cassettes.

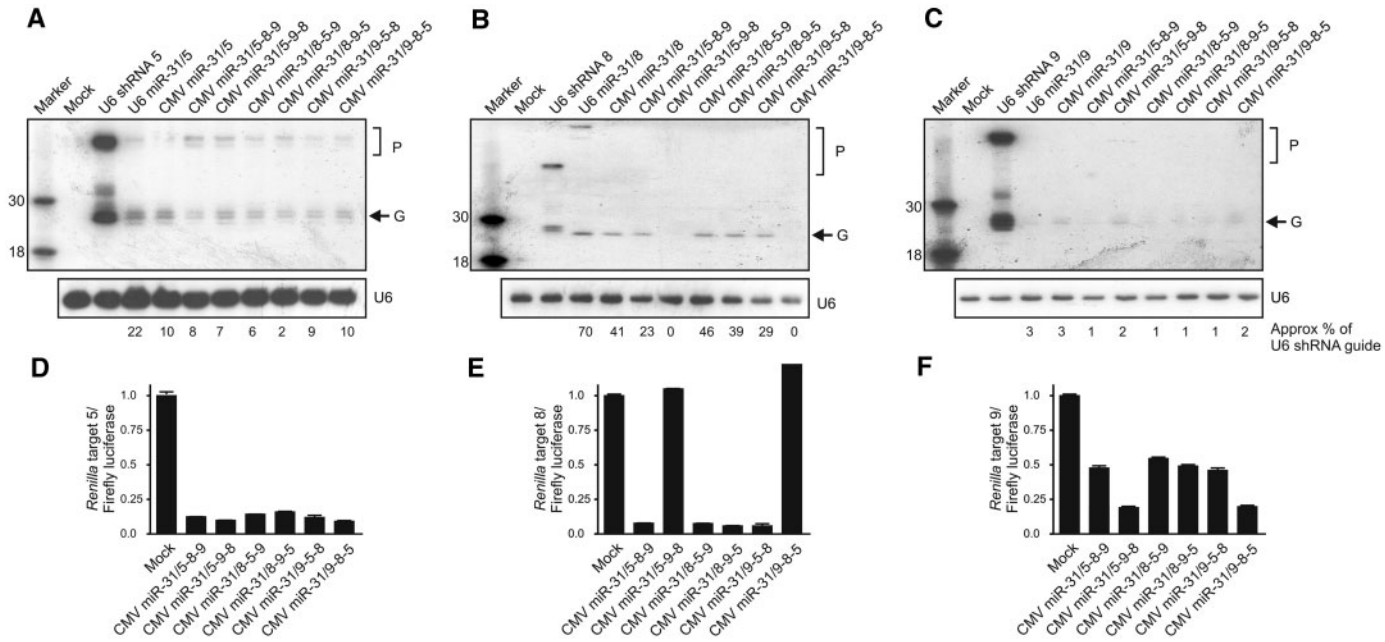

Figure 2. Processing and knockdown efficacy of trimer shuttles. Northern blot analysis of RNA extracted from cells that had been transfected with indicated expression cassettes. Hybridization was carried out with probes complementary to putative guide 5 (**A**), guide 8 (**B**) or guide 9 (**C**). Bands corresponding to guide sequence (G) and precursors (P) are indicated by the arrow and square bracket respectively. Re-hybridization of blots to U6 snRNA was carried out to confirm equal RNA loading in each of the lanes (lower panels). Oligodoexynucleotide length (nt) of labelled single-stranded DNA markers is indicated on the left of each panel. Approximate band intensities of miR-derived guides relative to the U6 shRNA guides (%) are indicated below. Assessment of knockdown efficacy of trimer shuttles using a dual luciferase reporter gene assay in which a target sequence complementary to guide 5 (**D**), 8 (**E**) or 9 (**F**) only was inserted downstream of the Renilla *luciferase* reporter ORF of psiCHECK 2.2. Data are represented as mean ratios of *Renilla* to Firefly luciferase activity (±SEM) and are normalized relative to the cells treated with backbone plasmid lacking RNAi effecter sequences (mock).

Incorporating a pri-miR-30a monomer scaffold improves silencing of HBV target 8

Sequence-specific properties of the individual anti-HBV pri-miR-31 shuttles as well as position of monomer shuttles within the trimers are likely to influence their processing and silencing efficiency. To assess the effect of pri-miR backbone scaffold sequences within the expression cassettes, silencing of target 8 sequences by an expanded panel of trimeric expression cassettes that included pri-miR-122/5 and pri-miR-30a/8 shuttles was measured (Figure 3). Each of the six pri-miR-31 trimers together with pCMV pri-miR-31/5-31/9-30a/8 and pCMV pri-miR-122/5-31/9-30a/8 were co-transfected with psiCHECK 8T. As described before (Figure 2E), knockdown of *Renilla* luciferase activity was poor with cassettes containing pri-miR-31/5-9-8 and pri-miR-31/9-8-5. However, efficient inhibition of reporter gene activity was achieved with pCMV pri-miR-31/5-31/9-30a/8 and pCMV pri-miR-122/5-31/9-30a/8 (Figure 3B). This indicates that substitution of the pri-miR-31 scaffold with a pri-miR-30a backbone restores target 8 silencing. In addition, inclusion of the pri-miR-122/5 monomer, which we have previously shown to act efficiently against HBV (3), does not compromise silencing by the pri-miR-30a/8 monomer. Thus, in addition to allowing improved silencing efficiency by changing monomer positions within the trimers, the cassettes described here have the added advantage of permitting the changing of pri-miR shuttle backbones to compensate for any compromised silencing efficacy of pri-miR-31 scaffolds.

miR-mediated inhibition of markers of HBV replication in transfected cells and *in vivo*

Target sites of the individual miR cassettes are located within the HBV *X* (*HBx*) ORF (Figure 4A). This sequence is conserved, common to all viral transcripts and has been shown to be a good target for RNAi-based HBV silencing (19). A dual luciferase assay, in which the surface ORF of pCH-9/3091 was substituted with a *Firefly luciferase* ORF, demonstrated that each of the trimeric cassettes achieved good knockdown when all three cognates of the intended miR-31/5, miR-31/8 and miR-31/9 guides were present (Figure 4B). As an initial assessment of pri-miR-31 trimer-mediated inhibition of HBV replication, Huh7 liver-derived cells were transfected with the pCH-9-3091 HBV replication competent plasmid (14), together with the panel of RNAi expression plasmids. Secreted HBV surface antigen (HBsAg), which is a reliable indicator of HBV replication in our hands (3,19,21), was determined thereafter in the culture medium (Figure 4C). Knockdown of ~90% was achieved. This correlated with the inhibitory effect that was observed when using the dual luciferase reporter system to measure silencing of individual targets (Figure 2D–F) and also inhibition of a *Firefly luciferase-HBx* reporter gene construct (Figure 4B). To determine silencing of target genes *in vivo* in a model that simulates

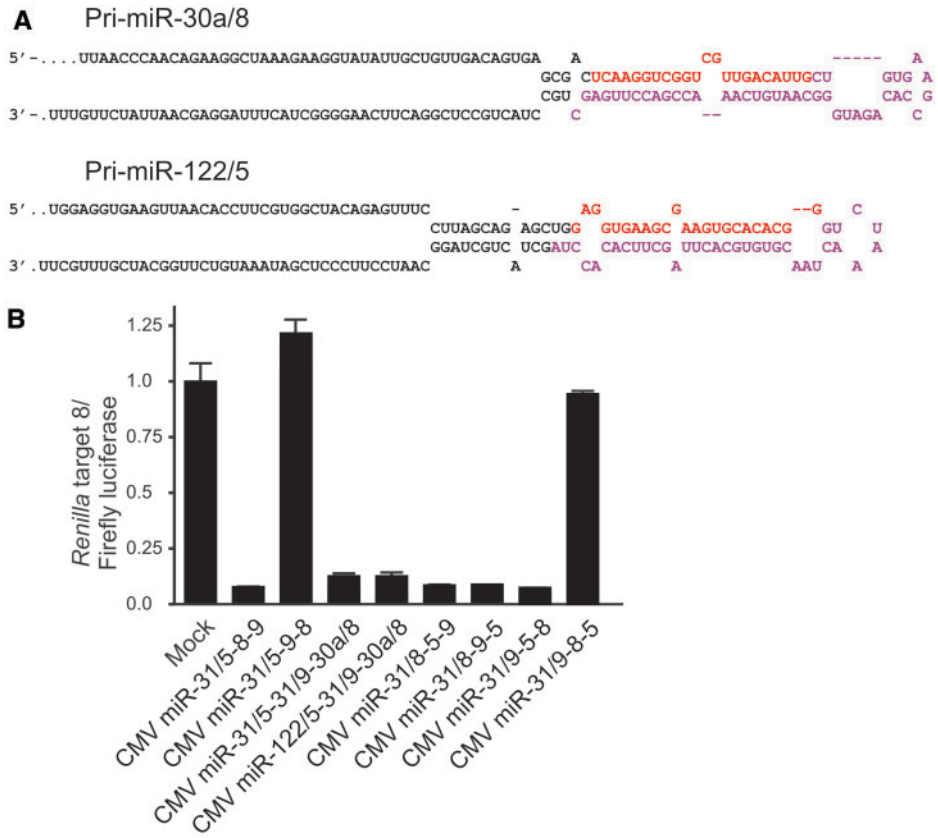

Figure 3. Use of shuttles containing pri-miR-31, pri-miR-30a and pri-miR-122 to improve silencing of HBV target 8 sequence. (**A**) Predicted structures and sequences of pri-miR-30a/8 and pri-miR-122/5 anti-HBV sequences. Colour coding of the sequences representing putative pre-miRs and mature guides are as indicated in Figure 1B. (**B**) Assessment of knockdown efficacy of trimer shuttles using a dual luciferase reporter gene assay in which a target sequence complementary to guide 8 was inserted downstream of the *Renilla* luciferase reporter ORF of psiCHECK2.2. Data are represented as mean ratios of *Renilla* to Firefly luciferase activity (±SEM) and are normalized relative to the mock-treated cells.

HBV replication, mice were co-injected with an HBV replication competent plasmid together with a selection of vectors encoding pri-miR-31 shuttles using the hydrodynamic procedure (22). Significant knockdown of HBsAg was observed at Days 3 and 5 after the injection, and the effects appeared to be independent of promoter interference (Figure 4D). These findings confirm that trimeric pri-miR-31 shuttles are capable of silencing HBV replication and verify that they are active against transcripts that are produced during viral replication *in vivo*.

A potential advantage of employing multimeric cassettes to inhibit viral replication is that viral escape resulting from emergence of evading mutations is limited. A dual luciferase assay was undertaken to assess whether HBV target silencing occurred when mutations were introduced into the target 5 site of HBV (Figure 5A). Co-transfection of cells was carried out with each plasmid encoding the panel of eight trimeric expression cassettes together with wild-type or mutant *HBx* target. Silencing of reporter gene expression was achieved with all trimeric expression cassettes with the exception of mutant target silencing by CMV pri-miR-31/5-9-8 and CMV pri-miR-31/9-8-5. This was expected as these two expression cassettes are known to generate antiHBV guide 9 in low amounts and be defective with respect to guide 8 production (Figure 2). Importantly, mutant target silencing was restored in these cassettes by changing the pri-miR-31/8 monomer for a pri-miR-30a/8 unit, and confirms earlier observations (Figure 3) that target 8 silencing may be improved by substituting the pri-miR scaffold of its guide. Thus trimeric pri-miR cassettes are capable of efficiently silencing targets containing one mutant guide cognate and defective silencing may be overcome by changing the order of the miR units or scaffold sequence within the monomers.

Exclusion of non-specific effects induced by pri-miR-31 trimer cassettes

Verification that the pri-miR-31 trimer cassettes are indeed non-toxic and induce gene silencing by an RNAi-mediated mechanism is important to establish. To address this, disruption of the endogenous miR pathway and stimulation of the innate IFN response by pri-miR-31 trimer cassettes were assessed. Measurement of *IFN-β* mRNA concentration in cells transfected with trimer expression cassettes showed no elevation of this transcript, indicating that little or no immunostimulation is caused by IFN pathway induction (Figure 6). To assess disruption of the endogenous miR pathway, we adapted the recently described method that utilizes miR sponges as a control to verify derepressive effects of endogenous miR

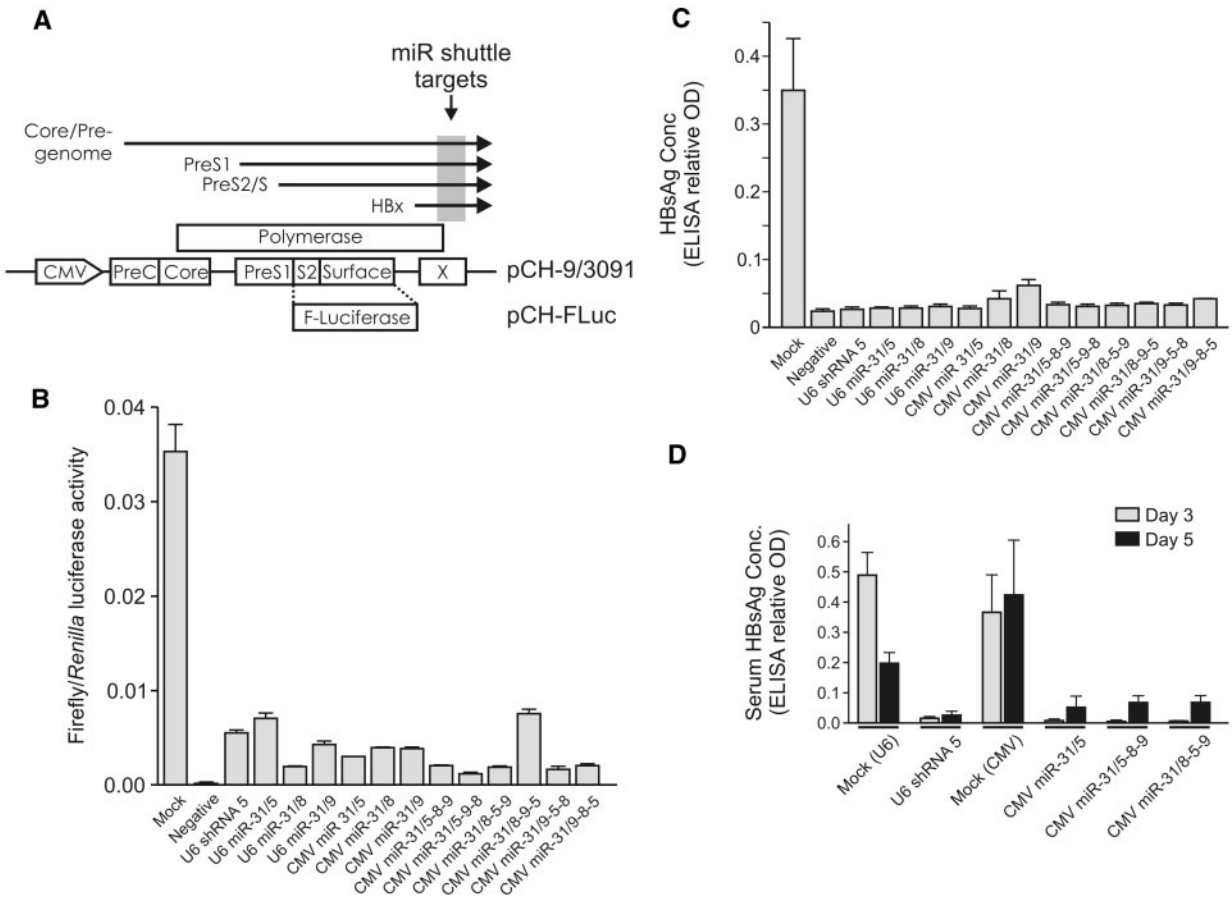

Figure 4. Trimer expression cassette-mediated knockdown of HBV replication in cultured cells and *in vivo*. (**A**) Organization of the HBV genome with ORFs and sites within the pCH-9/3091 vector that are targets complementary to processed products of pri-miR-31/5, pri-miR-31/8 and pri-miR-31/9 expressing vectors. Four parallel arrows indicate the HBV transcripts, which have common 3′ ends, and include the pri-miR-31/5, pri-miR-31/8 and pri-miR-31/9 targets. The pCH-9/3091-derived pCH-FLuc target vector has the *Firefly luciferase* ORF substituted for the preS2/S HBV sequence. (**B**) Luciferase reporter gene-based assay of knockdown efficacy *in situ*. pCH-FLuc was cotransfected with plasmids containing indicated RNAi expression cassettes in addition to a plasmid constitutively expressing *Renilla* luciferase. Results are given as ratios of Firefly to *Renilla* luciferase activity. Column-labelled negative represents data from transfections that excluded the pCH-FLuc plasmid. (**C**) Concentration of HBsAg in culture supernatants of Huh7 cells 48 h after transfection with pCH-9/3091 HBV replication-competent plasmid together with indicated anti-HBV expression cassettes. Column-labelled negative represents data from transfections that excluded the pCH-9/3091 HBV plasmid. (**D**) Silencing of HBV replication *in vivo*. Serum concentration of HBsAg was measured at Days 3 and 5 after hydrodynamic injection of mice with replication-competent vector and plasmids expressing anti-HBV RNAi sequences. Mock injections included control backbone plasmids containing U6 or CMV promoters that did not express anti-HBV RNAi effecters.

function (15). A dual luciferase reporter vector was generated in which seven copies of an imperfectly matched endogenous miR-16 target were inserted downstream of the Renilla *luciferase* ORF (Figure 7A). Perturbations in miR-16 translational suppression could be detected sensitively by measuring *Renilla*/Firefly luciferase reporter gene activity. miR-16 was selected for this assay as it is expressed in a variety of tissues (23) and can be conveniently used to determine disruption of natural miR function. A miR sponge expression cassette that encodes seven tandemly repeated miR-16 target sites was used to control for endogenous miR derepression (Figure 7B). Analysis revealed that co-expression of each of the trimeric constructs within transfected cells did not cause derepression of miR-16 inhibition of its cognate in the reporter fusion sequence (Figure 7C). The Pol II promoter-controlled expression of trimeric anti-HBV miR-31 shuttles, therefore, cause no detectable toxicity that results from IFN response induction or disruption of the endogenous miR pathway.

DISCUSSION

The powerful gene silencing that can be achieved by harnessing RNAi has facilitated development of new approaches to inhibition of pathology-causing genes and the study of gene function (1). Although synthetic siRNAs have been favoured as RNAi activators for many such applications, use of expressed silencing sequences has several advantages. These include achievement of sustained knockdown, compatibility with recombinant viral vectors and evasion of some of the immunostimulatory properties of exogenous synthetic sequences (24). Convenient expression of silencing sequences that efficiently target multiple

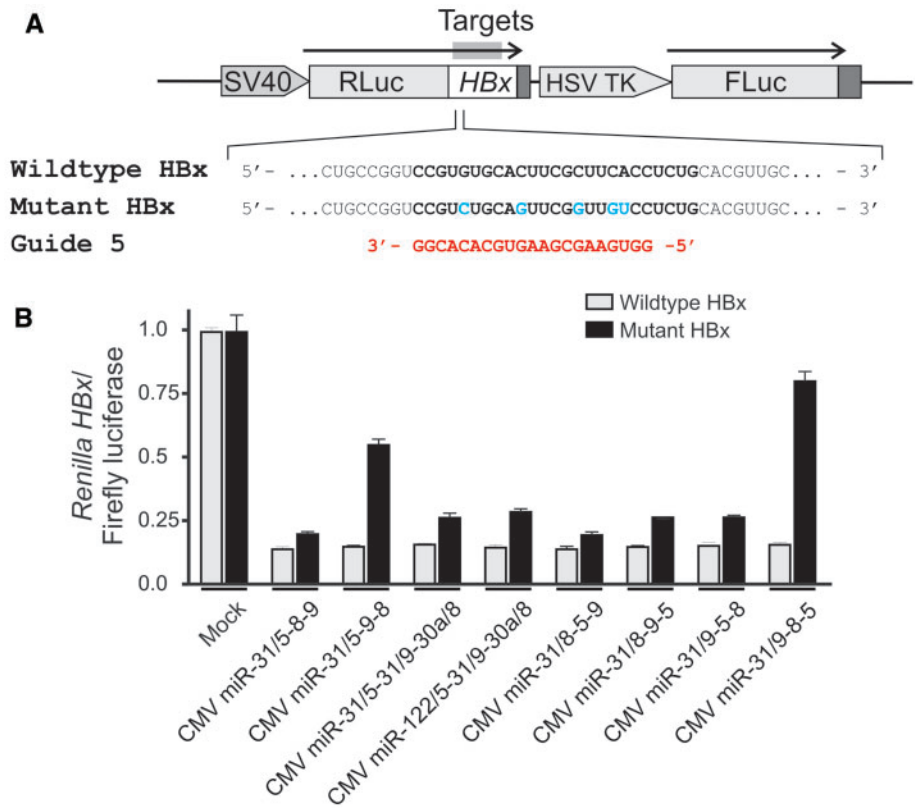

Figure 5. Dual luciferase reporter assay to detect silencing of wild-type *HBx* and mutant *HBx* target sequences using pri-miR expression cassettes. (**A**) The dual luciferase reporter vectors include the entire wild-type or mutant HBx target sequence downstream of the Renilla *luciferase* ORF. Guide 5 sequence is indicated in red, and mutations within its mutant *HBx* cognate are indicated in blue. (**B**) Assessment of knockdown efficacy of trimer shuttles using the psiCHECK-based dual luciferase reporter gene assay in which wild-type *HBx* or mutant *HBx* had been inserted downstream of the *Renilla* ORF. Data are represented as mean ratios of *Renilla* to Firefly luciferase activity (±SEM) and are normalized relative to the mock-treated cells.

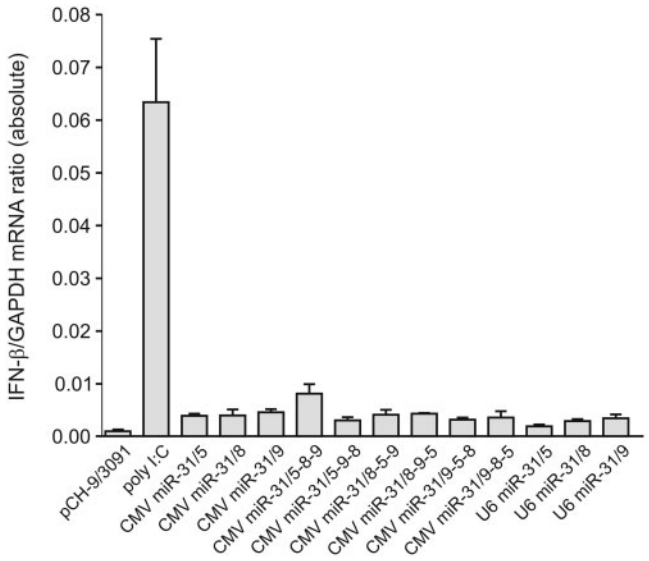

Figure 6. Assessment of IFN response induction by miR-expression cassettes. *IFN-β* mRNA concentrations were determined in HEK293 cells, which were transfected with the indicated miR-encoding cassettes, or with poly(I–C) as positive control. RNA was extracted from the cells 24 h later and then subjected to quantitative RT-PCR to determine concentrations of *IFN-β* and *GAPDH* mRNA. Means (±SEM) of the normalized ratios of *IFN-β* to *GAPDH* mRNA concentrations are indicated from three independent experiments.

sites would be a particularly useful attribute to enhance knockdown and counter evading target mutations. Achieving this without causing unintended off target effects and needing to utilize complex systems that require multiple expression cassettes is desirable. The engineered polycistrons described here provide a suitable method to attain these objectives. Cassettes were generated using pri-miR-31-, pri-miR-30a- and pri-miR-122-derived modules, which were combined as trimers and expressed from a Pol II promoter. Efficient processing of the shuttles and silencing of HBV cognates was observed without evidence for disruption of the endogenous miR pathway.

Detailed analysis revealed variation in efficacy of individual units that was dependent on specific sequences of the monomers as well as their position within the engineered polycistrons. Despite simultaneous production of three RNAi effecters from a single transcript, the mature guide sequences were not formed in equimolar amounts. Although knockdown of single targets may be compromised as a result of poor processing of individual guides, the modular nature of the cassettes facilitates improvement of defective silencing. Rearranging the order of the pri-miR units, which is not easily achieved with polycistronic miR cluster mimics, may restore function of individual miR shuttles. In addition, the cassettes described here allow improvement of efficacy to be achieved by

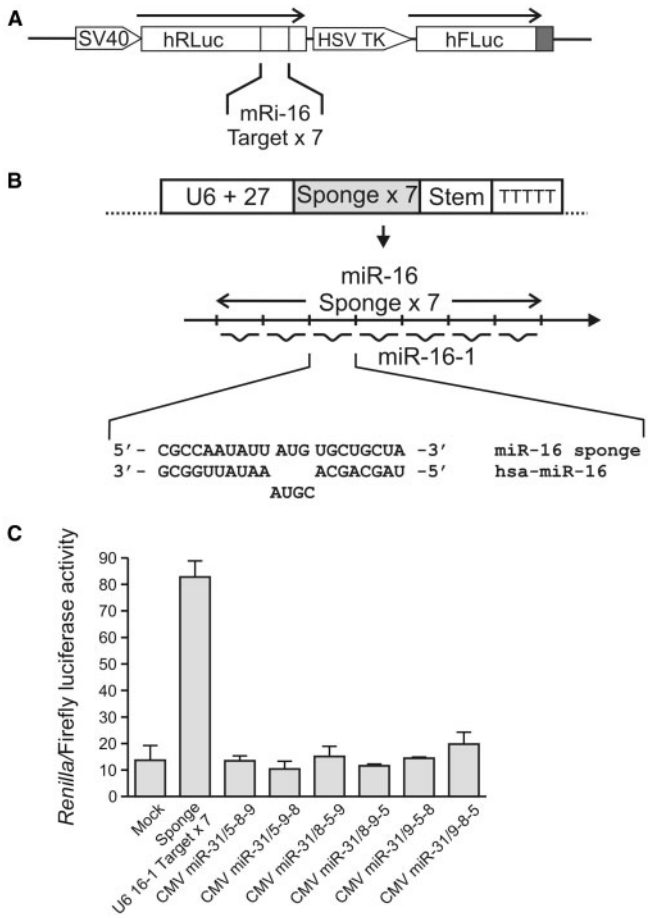

Figure 7. Assessment of effects of pri-miR-31 shuttles on endogenous miR-16 function using dual luciferase reporter and sponge vectors. (**A**) Schematic illustration to show dual luciferase psiCHECK-derived vector with seven copies of miR-16 target inserted downstream of the Renilla *luciferase* ORF. Firefly luciferase constitutively expressed from the same plasmid was used to normalize data. (**B**) Schematic illustration of expression cassette that generates a transcript containing seven copies of an imperfectly matched miR-16 target. The transcript contains 5′ U6+27 and 3′ stem sequences, which are thought to improve stability of U6 Pol III transcripts. (**C**) Analysis of effects of pri-miR-31 expression cassettes on endogenous miR-16 repression of target reporter sequence using a dual luciferase assay. Co-transfection of reporter plasmid, containing seven copies of miR-16 target inserted downstream of the *Renilla* luciferase ORF, was carried out together with RNAi expression cassettes, empty backbone plasmid (mock) or miR-16 sponge plasmid. Ratio of *Renilla* to Firefly luciferase activity was measured to assess derepression of endogenous miR-16 by coexpressed miR shuttles.

substituting poorly acting pri-miR-31 scaffolds with other backbone shuttles, such as those derived from pri-miR-30a and pri-miR-122. The sequence-specific differences in individual guide processing and target knockdown that we observed are not surprising but currently difficult to explain. Although computer-predicted structures of the shuttles were similar, empirical characterization of the processing of expressed RNAi effecters remains critically important.

Previous investigations have demonstrated that a combinatorial approach to knockdown of HIV-1 replication augments silencing and prevents the emergence of viral escape mutants (25). It has been calculated that four optimally acting individual antiviral guide sequences are required to prevent HIV-1 escape from RNAi (26), and anti-HIV-1 RNAi activators have been designed accordingly (4,7). Unlike with HIV-1, the HBV genome comprises overlapping ORFs with embedded viral *cis* elements (27). This highly compact arrangement of the genome restricts ability of HBV to mutate without compromising its replication fitness. The number of RNAi effecters within a combinatorial cassette that is required to prevent emergence of HBV escape mutants is not established, but it is likely to be fewer than the four that are required for HIV-1. Nevertheless, although only three pri-miR-31 shuttles were tested in the polycistronic cassettes described here, it is likely that a larger number of monomeric modules can be accommodated.

An important concern for the development of RNAi-based therapy is avoidance of off target effects. Unintended consequences may result from disruption of endogenous miR functions and silencing of normal cellular genes that have partial sequence complementarity to exogenous RNAi activators. We have shown that endogenous miR-16-mediated repression of a target reporter fusion is unaffected by expression of the Pol II pri-miR-31 trimer cassettes. Generating multiple silencing sequences from an engineered polycistronic cassette potentially increases the likelihood of causing unintended off target effects. Interaction of a guide seed region, comprising nucleotides 2–8 from the 5′ end, is potentially sufficient to effect translational suppression of a cellular target. This weak sequence restraint emphasizes the importance of utilizing potent expressed silencing sequences that are effective at low concentration, and also restricting the expression of RNAi effecters to target tissues. Compatibility of polycistronic pri-miR shuttles with expression from Pol II promoters, and efficacy that is equivalent to that of shRNA transcribed from a U6 promoter, are useful features that may be harnessed to diminish unintended effects. Compared to Pol III promoters, Pol II transcription regulatory elements have greater versatility that facilitates production of mature RNAi effecters without perturbing endogenous miR function. In the case of developing RNAi-based HBV therapy, expression cassettes containing liver-specific promoters that are induced by target-encoded transcription activators, e.g. the HBV X protein (28,29), should facilitate transcription regulation with consequent attenuation of off target effects.

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Conflict of interest statement. None declared.

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A3 ANIMAL ETHICS CLEARANCE CERTIFICATE

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2007/47/3

APPLICANT: Dr P Arbuthnot

SCHOOL: Molecular medicine and Haematology
DEPARTMENT:
LOCATION: Medical School

PROJECT TITLE: **Assessment of efficacy of anti hepatitis B virus RNA interference expression cassettes using the murine hydrodynamic injection model**

Number and Species

384 male and female mice

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20071127. This approval remains valid until 20091127

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

Report % of animal deaths under this protocol, volume injected must be in proportion to body size with 3ml maximum, retro-orbital bleeds twice only, liaison with CAS vet for injections and retro orbital bleeds, all animals that die other than euthanasia for experimental procedure must be made available to the CAS in line with normal procedure.

Signed: _____ Date: 30/11/2007
(Chairperson, AESC)

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

Signed: _____ Date: 30/11/2007
(Registered Veterinarian)

cc: Supervisor:
Director: CAS

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A4 SUPPLEMENTARY FIGURES

A4-1 Predicted secondary structures of pri-miR and pri-miR shuttle sequences

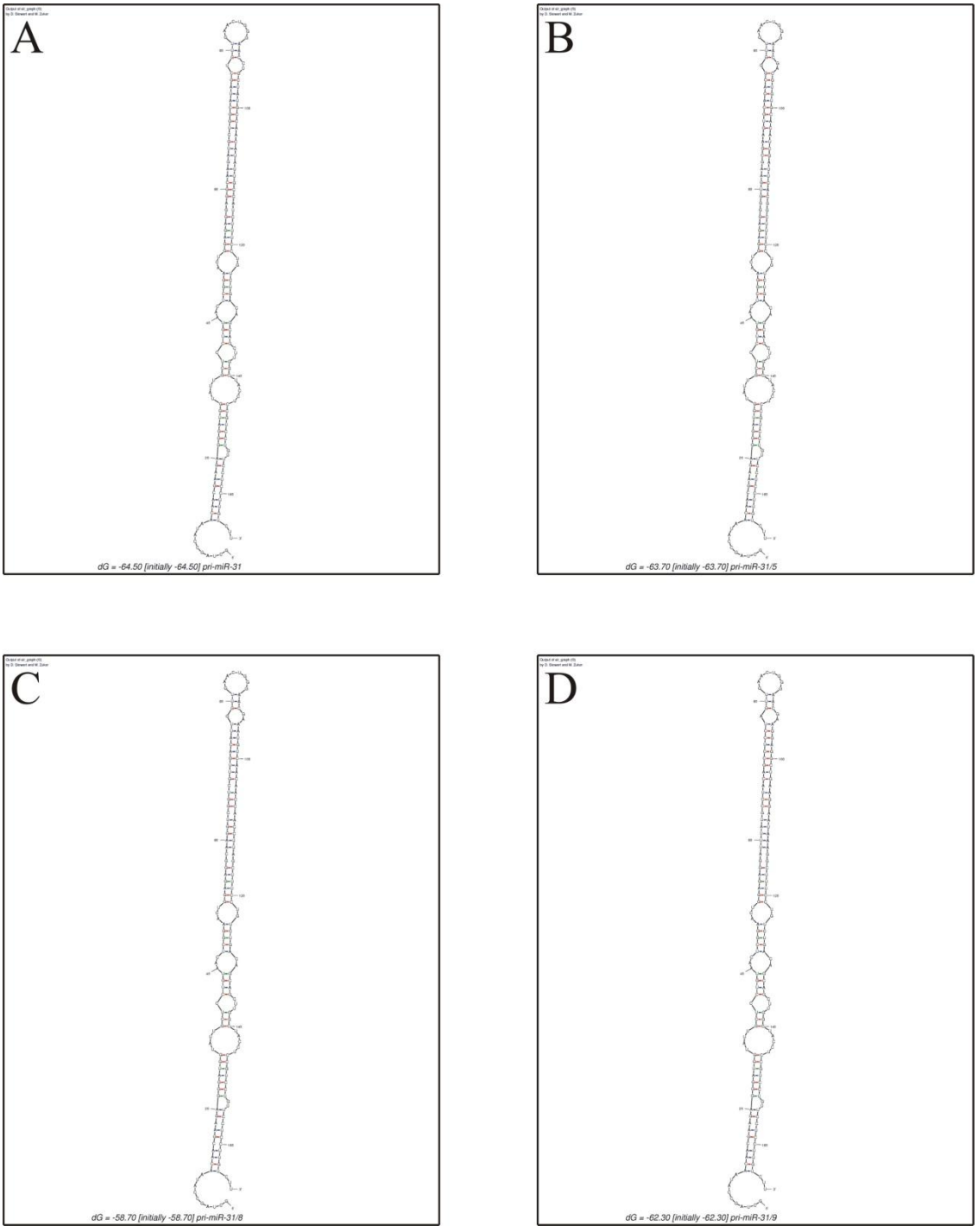

Figure A1: <u>Predicted secondary structures of pri-miR-31 wild-type (A) and shuttle (B-D) sequences</u>.

Computer aided prediction was carried out on RNA sequence encoding wild-type pre-miR-31 and 51 nt flanking sequences.

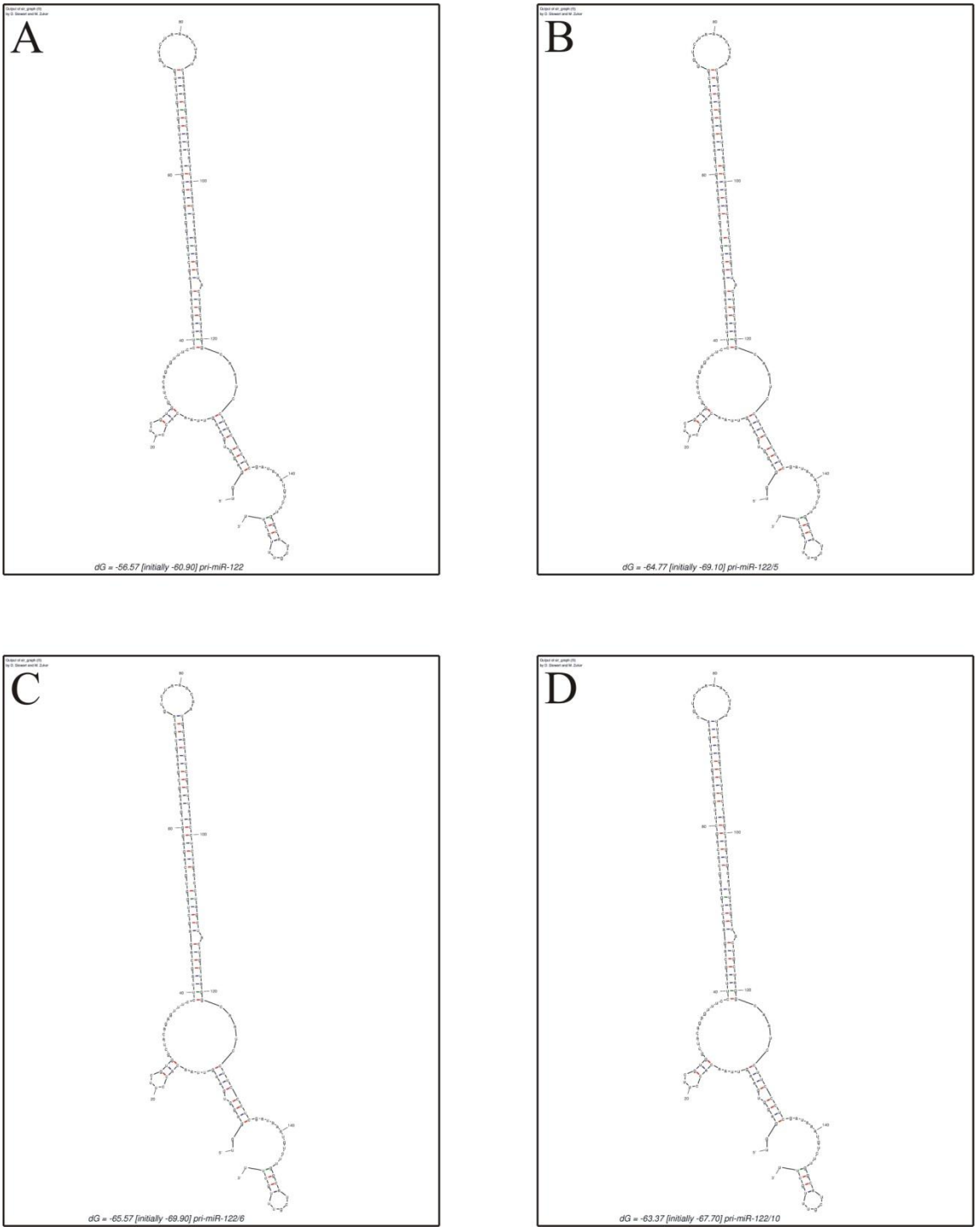

Figure A2: <u>Predicted secondary structures of pri-miR-122 wild-type (A) and shuttle (B-D) sequences.</u>

Computer aided prediction was carried out on RNA sequence encoding wild-type pre-miR-122 and 51 nt flanking sequences.

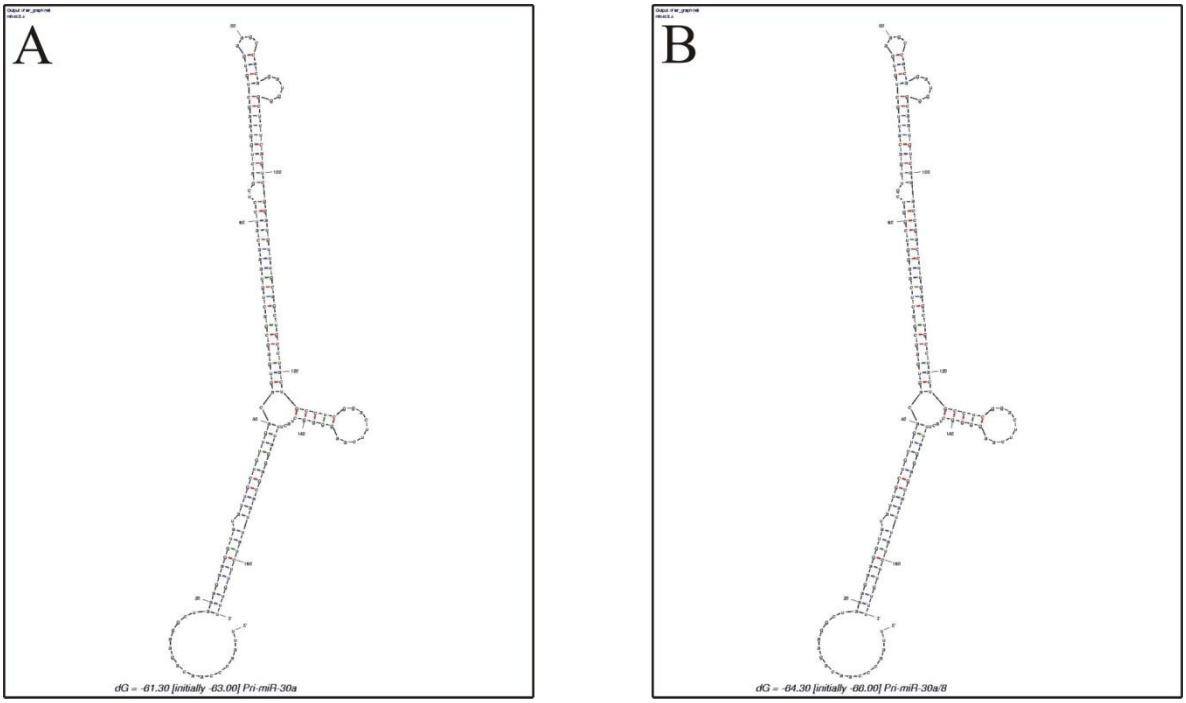

Figure A3: <u>Predicted secondary structure of pri-miR-30a wild-type (A) and shuttle (B) sequences.</u>

Computer aided prediction was carried out on RNA sequence encoding wild-type pre-miR-30a and 51 nt flanking sequences.

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