POLYCISTRONIC PRI-miRNA ANALOGUES TRANSCRIBED FROM RNA POLYMERASE II PROMOTERS THAT SILENCE EXPRESSION OF HBV.

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Master of Molecular Medicine and Haematology

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Be who you are and say what you feel because those who mind don't matter and those who matter don't mind

Declaration

DECLARATION

I, Tanusha Naidoo declare that this dissertation is my own work. Where assistance has been provided, this is acknowledged. The dissertation is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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Publications and Presentations

Publications

1. Ely, A., Naidoo, T., Arbuthnot, P. (2009) Efficient silencing of gene expression with modular trimeric Pol III expression cassettes comprising microRNA shuttles. *Nucleic Acids Res*, 37, e91.
2. Ely A, Naidoo T, Mufamadi S, Crowther C, Arbuthnot P. Expressed anti-HBV primary microRNA shuttles inhibit viral replication efficiently in vitro and in vivo. Mol Ther. 2008 Jun;16(6):1105-12
3. Arbuthnot PB, Longshaw VM, Naidoo T and Weinberg MS. Opportunities for treatment of chronic hepatitis B and C virus infection using RNA interference. Journal of Viral Hepatitis volume 14 Issue 7 Page 447-459, July 2007

Conference Proceedings

1. Ely A, Naidoo T, Mufamadi S, Crowther C and Arbuthnot P. Trends in Virology, Christophe Merieux Conference, Annecy, France, 24-26 June 2007.
2. Ely A, Naidoo T, Mufamadi S, Crowther C and Arbuthnot P. MicroRNA-like hairpin sequences capable of inhibiting HBV replication efficiently *in vitro* and *in vivo*. ASGT 10th Annual Meeting, 2007 May 30 – June 3, Washington, USA
3. Naidoo T, Longshaw VM and Arbuthnot PB. microRNA Analogues that target silencing of expression from the HCV NS5B sequence. 2006 SASBMB, Pietermaritzburg, South Africa.

4. Naidoo T, Longshaw VM and Arbuthnot PB. microRNA Analogues that target silencing of expression from the HCV NS5B sequence. 2006 Wits Faculty of Health Science Research Day, University of the Witwatersrand, South Africa.

Patents

1. Ely A, Naidoo T, Arbuthnot P; inventors. Anti HBV micro RNA shuttle expression cassettes inhibit viral replication efficiently in vitro and in vivo. RSA Provisional Patent Application No. 2007/04435

ABSTRACT

Globally, the hepatitis B virus (HBV) is the primary cause of acute and chronic infections of the liver. Despite an effective vaccine, HBV related liver cancer results in 600 000 deaths per year. This study focused on the development of novel RNA interference (RNAi) therapeutic modalities. Cassettes transcribing primary microRNA (pri-miRNA) shuttles that target HBV when expressed from Pol II or Pol III promoters were generated. The pri-miRNA constructs resulted in at least 88% knockdown efficacy *in vitro* and *in vivo*. The approach was further developed by generating polycistronic pri-miRNA constructs, containing three pri-miRNA in series under the control of Pol II CMV promoters. These constructs displayed specific, efficient knockdown efficacy without inducing an interferon response. This multi-targetting approach can potentially be used to target any virus and holds the hope of preventing viral escape, a great problem when using RNAi therapeutics against viruses.

DEDICATION

To my husband,
Arthur Kirsten and my son,
Connor Sen Kirsten.

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LIST OF ABBREVIATIONS

1. A	Adenine
2. AAV	Adeno-associated virus
3. Ago2	Argonaute2
4. ALT	alanine transferase
5. bp	base pair
6. cccDNA	covalently closed circular DNA
7. CMV	cytomegalovirus
8. C	Cytosine
9. DGCR8	DiGeorge syndrome critical region gene 8
10. dsRNA	double-stranded RNA
11. dsRBP	double-stranded RNA binding protein
12. DEPC	diethyl pyrocarbonate
13. DNA	deoxyribonucleic acid
14. dsRNA	double-stranded RNA
15. EDTA	ethylene diamine tetra-acetic acid
16. EGFP	enhanced green fluorescent protein
17. ELISA	enzyme linked immunosorbent assay
18. Exp 5	exportin 5
19. FCS	foetal calf serum

20. G	Guanine
21. HBV	hepatitis B virus
22. HBcAg	hepatitis B virus core antigen
23. HBeAg	hepatitis B virus e antigen
24. HBsAg	hepatitis B virus surface antigen
25. HBx	hepatitis B virus X protein
26. HCC	hepatocellular carcinoma
27. HCV	hepatitis C virus
28. HEK293	human embryonic kidney cell line
29. HIV	human immunodeficiency virus
30. Huh7	human hepatoma cell line 7
31. IFN	interferon
32. IPTG	isopropyl-β -D-thiogalactopyranosid
33. IRF	interferon regulatory factor
34. kb	kilobase
35. MHI	murine hydrodynamic injection
36. miRNA	microRNA
37. mRNA	messenger RNA
38. NFκB	Nuclear factor-kB
39. nm	nanometer
40. nt	nucleotide
41. OAS	oligoadenylate synthetases

42. OligodT	oligodeoxythymidylic acid
43. ORF	open reading frame
44. pdsRNA	partially double stranded RNA
45. pgRNA	pregenomic RNA
46. PKR	protein kinase R
47. Pol II	polymerase II
48. Pol III	polymerase III
49. pre-miRNA	precursor miRNA
50. pri-miRNA	primary precursor miRNA
51. Ran GTP	RAs-related Nuclear GTP binding protein
52. RIG 1	retinoid-inducible gene 1
53. RISC	RNA induced silencing complex
54. RNA	ribonucleic acid
55. RNAi	RNA interference
56. RNase	ribonuclease
57. rpm	revolutions per minute
58. RPMI medium	roswell Park Memorial Institute medium
59. shRNA	short hairpin RNA
60. siRNA	short interfering RNA
61. SNALP	stable nucleic acid-lipid particle
62. ssRNA	single stranded RNA
63. TLR	toll-like receptor

64. U	uracil
65. X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
66. YMDD	tyrosine – methionine – aspartate - aspartate

CHAPTER ONE – INTRODUCTION

1.1 Hepatitis B Virus

1.1.1 Hepatitis B virus biology

According to the most recent World Health Organisation (WHO) estimate, 2 billion people have been infected with the hepatitis B virus (HBV) of which 360 million account for chronic infections (Shepard *et al*, 2006). Despite an effective vaccine being available, the virus is estimated to be responsible for 600 000 deaths per year (WHO, 2000).

HBV is a partially double-stranded DNA (pdsDNA) virus that is classified under the virus family of hepadnaviridae (Seeger, Mason, 2000). The compact 3.2 kb genome encodes four open reading frames (ORFs): the Pre-S/S, precore/core, X and polymerase ORFs. The pre-S/S ORF encodes the three envelope glycoproteins that are known as the large (L), middle (M) and small (S) Hepatitis B surface Antigen (HBsAg). The precore/core ORF generates the precore polypeptide, which is the precursor of the soluble hepatitis B e antigen (HBeAg), and the nucleocapsid or core protein. The remaining two ORFs encode the X and polymerase proteins (Seeger, Mason, 2000).

Humans are the only known host of the virus. Hepatitis can manifest as an asymptomatic, acute or chronic infection. There appears to be an inverse relationship between age and risk of developing a chronic infection (Shepard *et al*, 2006).

Hepatitis related symptoms for new, acute infections are directly related to age (McMahon *et al*, 1985). The trend shows that most perinatal infections are asymptomatic whereas older children and adults more readily display symptoms

(McMahon *et al*, 1985). Typical symptoms for acute infections include: nausea, abdominal pain, vomiting, fever, jaundice, dark urine, changes in stool colour, hepatomegaly and splenomegaly (Shepard *et al*, 2006). Newly acquired acute infections are typically characterised by detectable amounts of hepatitis B surface antigen (HBsAg) and antibodies to hepatitis B core antigen (HBcAg). Upon recovery, HBsAg seroclearance is achieved and antibodies to surface antigen develop (Shepard *et al*, 2006).

Chronic HBV sufferers are defined by the presence of HBsAg and hepatitis B e antigen (HBeAg), which is a marker of viral replication. Chronic carriers can be asymptomatic, but it should be noted that a greater portion of the virus burden is attributed to chronic infection. Infections that occur early on in life (0-5 years) lead to chronicity which is a major risk factor for the development of hapatocellular carcinoma (HCC) or cirrhosis. Mortality as a result of these complications are estimated to be 15-25% of total carriers (Shepard *et al*, 2006).

HBV is stable for at least 7 days and is highly transmissible through percutaneous or mucosal exposure to body fluids. Vertical transmission from mother to child also accounts for a large number of infections. Five to twenty percent of vertical infections occur from HBsAg positive mothers and 70-90 % infection occurs from HBeAg positive mothers (Beasley *et al*, 1977).

1.1.2 Global Epidemic

The global HBV epidemic is categorised into regions of high, intermediate and low HBsAg prevalence. Regions with an HBsAg seroprevalence greater than or equal to 8% are regarded as highly endemic. Seroprevalence of HBsAg antigenaemia of 2-7%

and less than 2% define regions of intermediate and low endemicity, respectively (Shepard *et al*, 2006).

A correlation between the level of endemicity and the transmission route has been observed. The modes of transmission in highly endemic regions are of the horizontal and perinatal nature. Areas of intermediate endemicity are characterised by horizontal and vertical mode of transmission as well as health-care related and sexual forms of transmission. New infections in low endemic regions are mostly due to intravenous drug use and sexual transmission (Shepard *et al*, 2006; Custer *et al*, 2004).

Eight genotypes of HBV have been characterised (genotypes A–H). Okamoto *et al* (1988) identified genotypes A-D. Genotypes E, F (Norder *et al*, 1992), G (Stuyver *et al, 2000)* and H (Arauz-Ruiz *et al*, 2002) were described later. Subgenotypes have been found within genotypes A (Bowyer *et al*, 1997), B (Sugauchi *et al*, 2002), C (Sugauchi *et al*, 2001), D (Norder *et al*, 2004) and F (Arauz-Ruiz *et al*, 1997). Of these genotypes, A, D, and G commonly occur throughout the world. Genotypes B and C have a more specific distribution and are found predominantly in east and southeast Asia. Genotype E is found in west Africa, and genotypes F and H are found among various population groups, including indigenous peoples in Central and South America.

In addition to the characterised genotypes, cases of recombinant genotypes have been reported. Recombination between the eight genotypes results in novel variants, making HBV genetically diverse. These recombination events have been attributed to modes of transmission, change in frequencies of persistence and HBeAg expression (Simmonds, Midgley, 2005).

Recombinant variants have important implications for management of HBV infection. Current treatment will definitely be affected as resistant strains come to the fore. The

need for alternative and combination therapies is thus essential for effective management and treatment of HBV.

1.1.3 African Epidemic

Sixty five million of the world's 360 million chronic HBV carriers reside in Africa. In terms of mortality, approximately 250 000 out of 1.3 million global HBV related deaths, occur in Africa (Kramvis, Kew, 2007a).

The exposure rate of HBV is defined as the prevalence of anti-HBcAg antibodies in a population. The African population displays an exposure rate of 1.8-98%, with blood donors and children at the lower range and patients with chronic liver disease and HCC at the higher end of the range (Kramvis, Kew, 2007a). Geographically this rate differs within the African continent (Amazigo, Chime, 1990; Chiaramonte *et al*, 1991; Pellizzer *et al*, 1994).

Chronic HBV infection is characterised by the presence of HBsAg six months after exposure. The prevalence of HBsAg within Africa follows the same trend as the prevalence of HBcAg. In Africa, three levels of chronic infection exist (with respect to HBsAg). At greater than eight percent infection, sub-Saharan Africa, on average, has the highest level of chronic HBV carriers. Areas showing an exception to this trend include Kenya, Zambia, Cote d`Ivoire, Liberia, Sierra Leone and Senegal. At two to eight percent HBsAg prevalence, these areas represent an intermediate level of chronic infection. Northern African countries, at less than two percent display low endemicity (Kramvis, Kew, 2007a).

HBeAg is commonly associated with active viral replication, except in some cases of precore mutations. Compared to global adult HBsAg carriers Africa has a low rate of HBeAg positivity (Kramvis, Kew, 2007a).

The prevalence of HBV markers within the African population imparts a better understanding to the transmission pattern within the region. Most studies show that South Africa for instance displays a horizontal route of transmission in children ages one to five years old (Vardas *et al*, 1999). Vertical transmission has been noted to make up a very low percentage of infection (Prozesky *et al*, 1983). Studies regarding the transmission rates in South African children aided the decision to include HBV in the immunisation of South African infants (Prozesky *et al*, 1983; Di Bisceglie *et al,* 1986; Schoub *et al*, 1991; Schoub *et al*, 1993) . However ongoing studies are required as the current immunisation programme is based on the notion that early transmission occurs at a low rate. Vardas *et al* (1999) have showed that the rate of early transmission is increasing which may mean that the vaccine schedule needs to be re-evaluated.

Africa, like the rest of the world, displays a distinct geographic distribution of the eight HBV genotypes. Genotypes A, D and E are found in Africa. Genotype A is predominant in southern, eastern and central Africa. The dominant genotypes in northern and western Africa are D and E, respectively. Sequence analysis of genotypes A, D and E shows genotype A was the initial genotype endemic to Africa, followed by genotype D and genotype E was the most recently introduced (Kramvis, Kew, 2007a).

Of particular interest in South Africa is subgenotype A1 which is also found within the African countries of Malawi, Uganda, Tanzania, Somalia, Yemen, as well as the Indian subcontinent and the Philippines (Kramvis, Kew, 2007b). The sequence characteristics of subgenotype A1 gives rise to characteristic clinical outcomes.

Lower HBV DNA levels is one such characteristic. Mutations within regulatory elements of subgenotype A1 that affect viral replication are thought to ultimately result in lower HBV DNA levels (Kramvis, Kew, 2007b). Another noted

characteristic is that black South African sufferers have a higher rate of serconversion from an HBeAg positive to an HBeAg negative state (compared to other hyperendemic areas in the world). HBeAg negativity is a result of mutations and substitutions occurring within subgenotype A1 (Kramvis, Kew, 2007b). The higher rate of HCC and earlier onset of cancer that is seen in genotype A as compared to non-A genotypes have been attributed to these characteristics of subgenotype A1. In the scenario of reduced serum HBeAg levels cellular and humoral immune responses are no longer diverted from the liver. Immune targeting of hepatic HBcAg can lead to necrosis of hepatocytes and liver damage which is a significant factor in the development of HBV-related HCC (Kramvis, Kew, 2007b).

Further studies are required to better understand and elucidate the details of the sequence characteristics of the African HBV genotypes and the clinical manifestations of these characteristics. This will pave the way for designer therapeutics and go some way into better managing the African HBV burden.

1.1.4 Treatment

A recombinant and a plasma-derived HBV vaccine are currently available. Both vaccines are usually administered in a three dose series. But the dosing can be altered into two or four dose series. The measure of protection offered by the vaccine is the level of Anti-HBs. The level declines in the years post vaccination (more than 10 years) and a booster is sometimes needed to increase the Anti-HBs back to a protective level. Vaccine failure has been seen in patients with small surface protein mutations. There has been success in countries that have integrated the hepatitis B vaccine into their national immunisation programmes. Such countries include the USA, Taiwan, Gambia, South Africa and Malaysia (Shepard *et al*, 2006).

Immunisation is only effective in the uninfected population though. For the 360 million chronic carriers, treatment is the only hope of suppressing viral replication and remission of liver disease. Licensed treatments for HBV infection which include interferon alpha (IFN-a), nucleoside (lamivudine) and nucleotide (adefovir) analogues, are partially effective (Karayiannis, 2003). Treatment response in HBeAg negative patients, which is typical of sub-Saharan African carriers, is usually not durable.

First line treatment of chronic HBV entails the use of lamivudine. Long-term lamivudine therapy can suppress HBV replication, however, prolonged monotherapy leads to the emergence of lamivudine resistant HBV mutants (Lau *et al*, 2000). An example of this is the tyrosine-methionine-aspartate-aspartate (YMDD) mutation of the viral polymerase which is associated with high baseline levels of HBV DNA and elevated Alanine aminotransferase (ALT) (Lau *et al*, 2000).

Adefovir dipivoxil can be used as a therapy for lamivudine-resistant HBV patients (Schiff *et al*, 2003). Though less common adefovir resistance often coincides with existing lamivudine resistance (Lee *et al*, 2006). Sequential monotherapy should thus be avoided due to the induced occurrence of viral mutations. Combination therapy could reduce the rate of viral resistance but it will by no means eradicate the number of global chronic infections (Kwon *et al,* 2008). Current therapies must be taken consistently as withdrawal of therapy results in recovery of viral infection.

It is for this reason that there is ongoing research into alternative therapies against chronic HBV. Recent discovery of the naturally occurring RNA interference (RNAi) pathway has paved the way for many studies involving the development of novel effective nucleic acid-based HBV therapies. RNAi is viewed as a potential therapeutic option due to its specificity and can be used to impair viral replication. Viral eradication is achieved without activating non-specific cellular responses (Elbashir *et al*, 2001) and with minimal side effects. A number of studies have displayed the utility of RNAi as a potential therapeutic against HBV (Giladi *et al*,

2003; Moore *et al*, 2005; Uprichard *et al*, 2005; Carmona *et al*, 2006; Grimm *et al*, 2006; Zhao *et al*, 2006).

1.2 RNAi

RNAi is best described as a process by which double-stranded RNA (dsRNA) directs the sequence–specific silencing of gene expression. The evolutionary conserved mechanism is present in eukaryotic organisms. The mechanism by which RNAi effects silencing of genes was elucidated in nematode worms (Fire *et al*, 1998) and the pathway has quickly become the tool of choice for functional genomics. RNAi potentially holds the key to specific and targeted treatment of a variety of diseases.

1.2.1 Mechanism

Short dsRNAs trigger a cascade of biochemical events which results in the degradation of a target mRNA. Naturally RNAi involves processing of endogenously produced double-stranded RNA precursors to effecter sequences (microRNAs or miRNAs) that mediate gene silencing. miRNAs are transcribed by RNA polymerase II (Pol II) as part of a long primary precursor miRNA (pri-miRNA) (Cullen, 2005; Lee *et al*, 2003). These sequences are expressed as hairpin-like precursors which may exist as mono- or polycistronic structures on the Pol II transcript (Cullen, 2004). miRNAs regulate expression of genes that are involved in important cellular processes, such as proliferation, differentiation and apoptosis. Typically miRNAs bind to imperfectly matched or non-homologous targets and exert silencing through suppression of translation without cleavage of target mRNA (Bartel, 2004).

Mature miRNAs are the products of two sequential cleavage reactions (Cullen, 2005; Zeng, Yi, Cullen, 2005; Zhang *et al*, 2004; Zeng, Cullen, 2005). The first cleavage is

catalysed by Drosha (an RNAse III enzyme) together with DGCR8, which is its double stranded RNA binding (dsRBD) partner. The molecular cropping of the pri-miRNA stem results in the generation of pre-miRNAs (~65 nt long) (Zeng, Yi, Cullen, 2005 ; Zeng, Cullen, 2005). This cleavage reaction occurs two helical turns (~22 nt) away from the terminal loop of the pri-miRNA (Zeng, Yi, Cullen, 2005 ; Zeng, Cullen, 2005, Lee *et al*, 2004; Zeng, Cullen, 2003). Pri-miRNA cleavage by Drosha is a critical event in miRNA biogenesis. Drosha defines one end of the mature miRNA and leaves characteristic 2 nt 3' overhangs which are required for further processing (Lee *et al*, 2003). Pre-miRNAs are then exported to the cytoplasm by the nuclear export factor Exportin-5 (Exp5) and the Ran-GTP cofactor (Lund *et al*, 2004). The pre-miRNA interacts with a second RNAse III enzyme, Dicer, which performs the second cleavage reaction. Dicer binds to the 2 nt 3' overhang created by Drosha, and cleaves ~22 nt away from this base (Lee *et al,* 2004). This catalytic event effectively removes the terminal loop and leaves another 2 nt 3' overhang (Lee *et al,* 2004). The resultant duplex interacts with the RNA-induced silencing complex (RISC), of which Argonaute-2 (Ago2), an RNA endonuclease, is a component (Figure 1.1). Ago2 is responsible for the cleavage action of RISC (Liu *et al*, 2004). The RNA strand having a less stable 5' end, is selected by RISC and guides the complex to perfectly homologous mRNAs for degradation or translational suppression if there is incomplete complementarity to the target mRNA (Zeng, Yi, Cullen, 2003; Doench, Sharp, 2004).

In the miRNA biogenesis pathway, discussed above, the three distinct RNA intermediates produced during miRNA biogenesis (i.e. the initial pri-miRNA transcript, the pre-miRNA hairpin, and the miRNA duplex) can be used as entry points to allow programming of RISC with exogenous sequences (Cullen, 2005; Zeng, Yi, Cullen, 2005; Han *et al*, 2004). Typically the most commonly used exogenous RNAi inducing sequences have been synthetic small interefering RNA (siRNA) and expressed short hairpin RNA (shRNA). siRNAs are identical in structure to Dicer cleavage products and cause gene silencing by direct incorporation into and activation

of RISC. The main disadvantages associated with this type of RNAi effecter are the high costs involved and transient silencing. Expressed shRNAs enter into the miRNA biogenesis pathway earlier than siRNAs as they are similar to pre-miRNA hairpins. Pol III promoters are commonly used to transcribe shRNAs as a result of their ability to generate short, defined transcripts. Expressed shRNAs offer the advantage of prolonged silencing. Unfortunately expressed RNAi effecters, under the transcriptional control of Pol III promoters, do not lend themselves to regulation. More recently Pol III expressed shRNA effecters have displayed toxicity *in vivo* due to saturation of the endogenous miRNA pathway (Grimm *et al*, 2006). The entry point of pri-miRNA's into the miRNA biogenesis pathway has not been largely exploited but pri-miRNAs mimics under the transcriptional control of Pol II or Pol III promoters have been successfully used (Silva *et al*, 2005; Dickins *et al*, 2005). This type of effecter has the potential to be the ideal approach to activate the RNAi pathway as it has been shown to be more potent than other effecters (Silva *et al*, 2005) and can be regulated when under the control of Pol II promoters (Dickins *et al*, 2005).

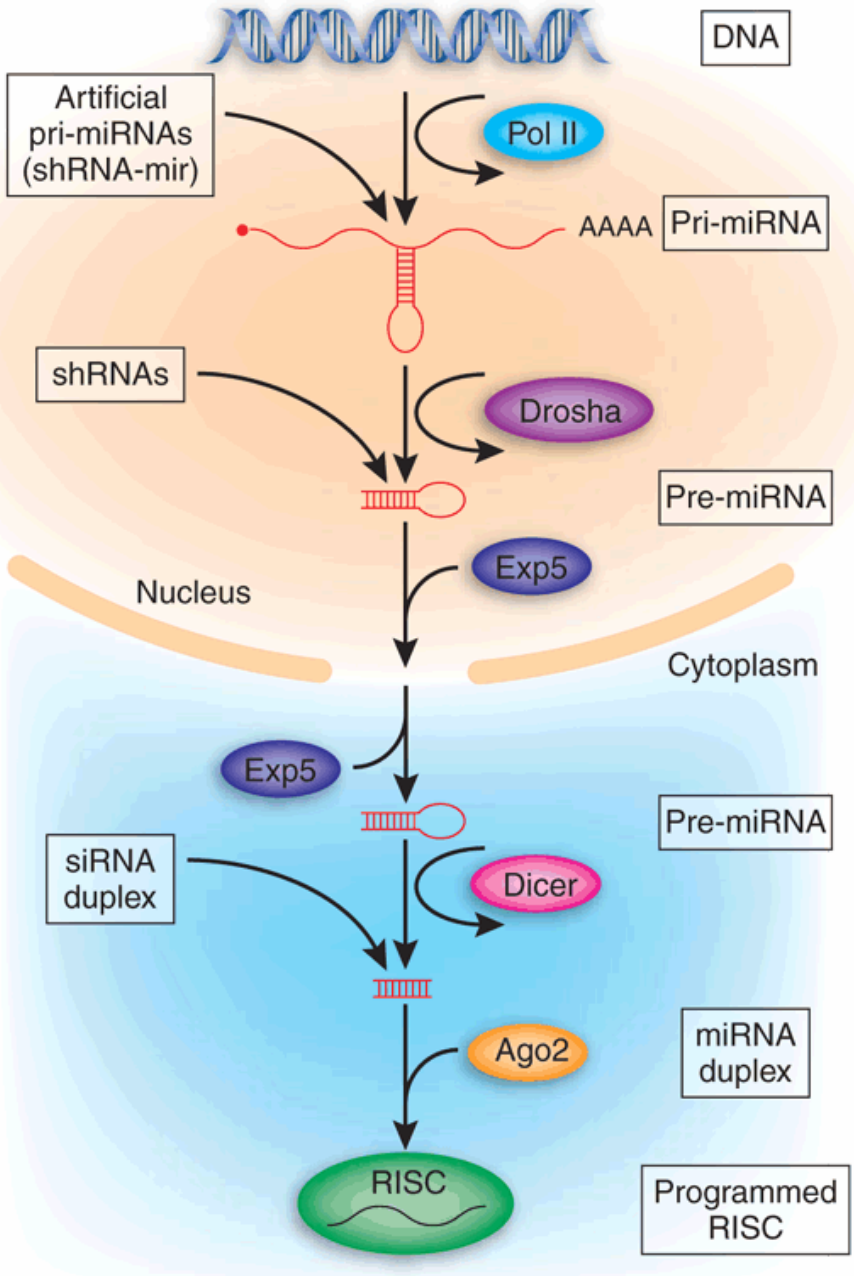

Figure 1.1 The miRNA biogenesis pathway in vertebrate cells.
This pathway may be exploited by inserting synthetic siRNA duplexes, shRNAs transcribed by Pol III or artificial pri-miRNAs. Ago 2 represents Argonaute-2 and Exp5 represents Exportin 5.
Figure reproduced from Cullen, 2005.

1.2.2 Exploiting the natural RNAi pathway

The first demonstration of RNAi was in the nematode worm by Fire and colleagues who showed that introduction of long dsRNA caused silencing of genes homologous to the introduced RNA (Fire *et al*, 1998). Both Fire (Timmons, Fire, 1998) and Mello (Tabara, Grishok, Mello, 1998) have also shown that *Caenorhabditis elegans* has the ability to respond in a gene specific manner to dsRNA encountered in its immediate environment. They elucidated that the RNAi response could be induced through injecting worms with dsRNA (Fire *et al*, 1998), soaking worms in a solution of dsRNA (Tabara, Grishok, Mello, 1998) or feeding worms bacteria that expressed the dsRNA (Timmons, Fire, 1998).

Attempts at inducing RNAi in mammalian cells were hampered by the fact that long dsRNA has the property of activating a sequence non-specific interferon response (Bridge *et al*, 2003; Sioud, 2006). The discovery that the long dsRNA activators of RNAi are processed into 21-23 nt siRNA (Zamore *et al*, 2000) led Elbashir *et al* (2001) to assess the ability of siRNA to induce RNAi in mammalian cells. They demonstrated that 21 nt siRNA containing 2 nt 3' overhangs, were small enough to be able to evade the non-specific interferon response while simultaneously inducing sequence-specific gene silencing (Zamore *et al*, 2000; Elbashir *et al,* 2001).

siRNA effecters as used by Tuschl and colleagues (Elbashir *et al,*2001) are commercially available, and although these effecters make delivery and dosage simple, the problems associated with these chemically synthesised effecters are two-fold. The costs to generate these molecules are high and once transfected into a cell, the concentration of the effecter becomes diluted as a result of successive cell divisions and degradation. Both problems may be avoided by using DNA cassettes that express silencing effecters (shRNA, pre-miRNA or pri-miRNA). Intracellular

generation of interfering RNA molecules is usually achieved by inserting an RNAi effecter DNA sequence downstream of a Pol III promoter. shRNA sequences enter the RNAi pathway as Dicer substrates, and mimic the processing of endogenous pre-miRNAs. The small nuclear RNA U6 (Beck, Nassal, 1995; Brummelkamp, Bernards, Agami, 2002) or human H1 promoters (Hannon, 2002) are the most commonly used Pol III promoters. Unlike Pol II promoters, these transcription regulators have the advantage of containing all of their *cis* sequence elements, with the exception of the first transcribed nucleotide, upstream of the transcription initiation site. Pol III termination is typically defined by a poly dT sequence (usually dTdTdTdT) in the sense sequence of the transcription template.

Several algorithms have been described to assist with optimising RNAi effecters, and general properties of effective siRNA/shRNA sequences have been described, which help with design.

The main design parameters to consider when designing expressed RNAi effecter sequences are derived from the design parameters of siRNA and analysis of miRNA structure. These parameters are noted below :

1. The effecter should have a G/C content of 30-52 %.
2. To make one of the strands more favourable for selection, it should be designed so that it is less tightly bound to its complementary strand at its 5' end. Thus incorporating at least three A/U bp in positions 15-19 of the sense strand increased functionality of the effecter.
3. Absence of internal repeats.
4. Terminal loop derived from a naturally occurring miRNA (Zeng, Cullen, 2003; Zeng, Yi, Cullen, 2005).

In addition to the above-mentioned criteria, Reynolds *et al* (2004), further defined base-specific criteria for the sense strand that confer functionality to the effecter sequences:

5. There is a base preference for an A at positions 3 and 19 (sense strand).
6. The presence of U at position 10.
7. Absence of a G/C at positions 13 and 19

Using these criteria, Reynolds *et al* (2004) assigned scores to their panels of RNAi sequences. Thus an effecter received one point for conforming to criteria one to five, extra points were awarded for every A/U between positions fifteen to nineteen (point two) and one point was deducted if a G/C was present at position thirteen or nineteen (point six). When the sequences were sorted by score, it was shown that sequences receiving six or more points were functional. These rational design criteria were then used to design siRNA against six different genes. Of the thirty rationally designed siRNA, twenty nine were shown to be functional. This increased the probability of selecting a functional RNAi effecter from 46.5% (random selection) to 96.6%.

Although these design criteria may confer increased functionality, effecter sequences will have to be assessed for other unwanted affects. Unintended immunostimulation, cross-reaction with cellular RNA and disruption of the endogenous miRNA pathway may complicate development of RNAi-based therapy.

The above mentioned criteria have been successfully employed to generate functional and specific anti-HBV shRNA vectors that are effective *in vivo* (Uprichard *et al*, 2005; Carmona *et al*, 2006). The guide sequences from three of the anti-HBV vectors designed by Carmona *et al* (U6shRNA 5, U6shRNA 8 and U6shRNA 9) were utilized in this study to generate pri-miRNA.

1.3 HBV Genome and Replication

Classified in the hepadnaviridae family, HBV is a small, enveloped virus containing a 3.2 kilobase (kb) pdsDNA genome with a virally encoded polymerase (Figure 1.2).

HBV replication can be divided into three distinct phases. The first phase is marked by the presence of infectious virions containing the pdsDNA viral genome. Phase two occurs during infection and is characterised by the conversion of the pdsDNA to covalently closed circular DNA (cccDNA). In the final phase the cccDNA is used as a template for viral RNA transcription. The transcripts are packaged and form new infectious virions (Beck, Nassal, 2007). The virus has a compact organisation with four overlapping open reading frames (ORFs) that encode for the viral polymerase, core and precore proteins, the three envelope or surface proteins, and the X protein (HBx) (Seeger, Mason, 2000). The core and the polymerase proteins are essential for viral DNA replication (Seeger, Mason, 2000). Pre-core protein is processed and secreted via the Golgi pathway into the serum as HBeAg, which is a marker of viral replication. The surface proteins are essential for the envelopment of nucleocapsids (Seeger, Mason, 2000). The function of HBx has not been fully elucidated but it is thought to have transcriptional transactivating properties. Both the pre-core and X proteins are classified as accessory proteins.

Following viral entry into the hepatocyte, the viral genome is released and is converted to cccDNA (Tuttleman, Pourcel, Summers, 1986). The cccDNA serves as a template for transcription of the viral RNA. Transcription is unidirectional and results in the generation of four viral transcripts (Seeger, Mason, 2000). The largest being the 3.5 kb pregenomic RNA (pgRNA), which serves as the template for reverse transcription as well as three subgenomic mRNAs which encode the HBV envelope proteins (preS1, preS2 and S) and the X protein (Robinson, 1994). On completion of transcription, the pgRNA moves to the cytosol where the nucleocapsid is formed via encapsidation of the pgRNA and attached polymerase with core protein (Summers, Mason, 1982). While maturation of the core particle occurs, the pgRNA is reverse transcribed into a relaxed partially double-stranded DNA. Viral assembly ensues, following which virions are released to continue the cycle of hepatocyte invasion.

The compact nature of the HBV genome with overlapping reading frames, viral replication through RNA-directed (pgRNA) DNA synthesis and regions of high sequence conservation make the virus vulnerable to RNAi modalities.

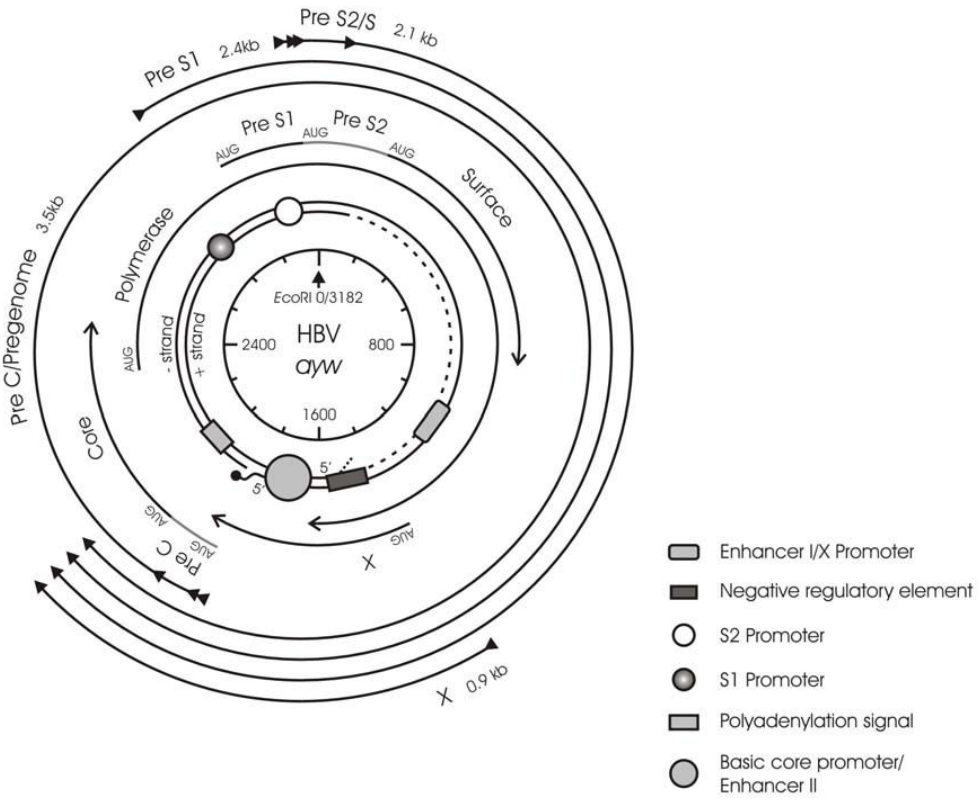

Figure 1.2 Organisation of the HBV genome.
Co-ordinates are relative to the single *Eco*RI restriction site. The partially double-stranded HBV DNA (pdsDNA) comprises of + and – strand that have cohesive complementary 5' ends. *Cis*-elements that regulate HBV transcription are denoted by circular and rectangular symbols. Arrows following the pdsDNA represent the viral ORFs with initiation codons. The outer arrows on the periphery show the HBV transcripts which have common 3' ends that all include the *HBx* ORF. Adapted from Arbuthnot *et al*, 2007).

1.4 Use of RNAi against hepatitis B virus Infection

The lack of a model system for HBV infection hampers the progress of RNAi based HBV therapeutic. Despite this obstacle RNAi-mediated sequence-specific knockdown of HBV has been successfully demonstrated in a number of studies (Giladi *et al*, 2003; Moore *et al*, 2005; Uprichard *et al*, 2005; Carmona *et al*, 2006; Grimm *et al*, 2006; Zhao *et al*, 2006). These studies have shown different sites within the virus to be feasible targets for effective silencing.

In the absence of a model of HBV infection the above mentioned studies used replication competent plasmids that encode greater than genome length sequences and/or transgenic mice to simulate HBV replication. Replication competent plasmids can be used *in vitro* via transfection into cell culture or *in vivo* via murine hydrodynamic tail vein injection (MHI), to evaluate the efficacy of RNAi effecters. Transgenic mice contain greater than genome length HBV sequence stably integrated into their genomes.

McCaffrey and colleagues assessed the anti-HBV efficacy of a panel of seven U6 promoter cassettes that encoded shRNAs (McCaffrey *et al*, 2003). Different regions within each of the four viral transcripts were targeted with the shRNAs. Six of the seven shRNAs displayed an antiviral effect. The most potent shRNA effecter being targeted to the surface and overlapping polymerase ORFs. The findings from the study showed a reduction in HBsAg in cell culture medium and mouse serum, reduced viral RNA in mouse liver, undetectable levels of HBV DNA in mouse liver and a reduction in HBcAg as seen by immunohistochemical staining of mouse liver (McCaffrey *et al*, 2003).

Another study by Giladi and colleagues, also displayed the *in vitro* and *in vivo* efficacy of RNAi effecters against HBV (Giladi *et al*, 2003). The siRNAs targeted the small surface antigen and overlapping polymerase ORFs within the virus. The study

showed sustained, dose-dependant inhibition of HBV. siRNA-1 displayed the highest level of HBV inhibition and reduced HBV DNA to undetectable levels. The siRNA used in this study, unlike nucleoside analogues, did not require active viral replication. This was proven through the use of replication deficient HBV plasmids (Giladi *et al*, 2003).

In one study, hepatotropic recombinant adenovirus vectors expressing shRNAs from Pol III promoters effected successful inhibition of viral replication in HBV transgenic mice (Uprichard *et al*, 2005; Carmona *et al*, 2006). To use an RNAi effecter in a therapeutic context it is important to regulate the dosage. A recent finding by Grimm *et al* has shown that high amounts of an RNAi effecter in an Adeno-associated virus (AAV) vector caused fatality in HBV transgenic mice (Grimm *et al*, 2006). The use of lower amounts of the RNAi effecters resulted in the inhibition of viral replication without any toxicity. In addition to dosage, the effects of virus mutations should also be considered when generating RNAi effecters. Silencing is attenuated by mismatches within the target region of the RNAi effecter. The risk of viral escape mutants can be significantly reduced by targeting multiple regions.

The above mentioned findings thus highlight ways of preventing some of the issues associated with RNAi, demonstrates its utility and serves as proof of concept for the use of RNAi effecters as a potential anti HBV therapy.

1.5 Challenges for developing RNAi-based HBV therapy

The broad applicability, specificity and low toxicity associated with RNAi have encouraged research into developing the technology into a potential therapeutic against HBV. However the limitations of RNAi remain a barrier that needs to be overcome before clinical application of RNAi antivirals.

1.5.1 Immunostimulatory effects of siRNAs and expressed hairpin sequences

For safe clinical use, the type of RNAi effecter used should not activate the host cell's innate immune response. Activation occurs when pathogens or nucleic acids are detected as foreign by the host cell. Figure 1.3, depicts the ways in which an RNAi effecter can stimulate the interferon response. Activators of the interferon response include: dsRNAs longer than 30 bp, single-stranded RNAs (ssRNAs) and blunt ended siRNAs (Kim, Rossi, 2007; Marques *et al*, 2006). dsRNAs longer than 30 bp stimulate the interferon response through the activation of protein kinase receptor (PKR). Alvarez, Ridenour, Sabatini (2006) have shown that shRNA are capable of stimulating an interferon response through the activation of PKR. Specific immunostimulatory sequence motifs contained within ssRNAs, siRNAs or dsRNAs may also stimulate the interferon response when recognised by endosomal toll-like receptors (TLRs). Blunt ended siRNAs activate the interferon response through the activation of retinoic acid gene 1 encoded RIG 1 helicase (Marques *et al*, 2006). PKR, RIG 1 and TLR's trigger a signaling cascade resulting in the translocation of nuclear factor Kb (NFKB), Interferon regulatory factor (IRF) and activating transcription factor 2 into the nucleus (Seth, Sun, Chen, 2006). This signaling cascade results in the induction of a Type I interferon response which causes non-specific shutdown of protein synthesis and global RNA degradation (Manche *et al*, 1992).

Functional studies on RNAi have led to a better understanding of the features and characteristics that make some RNAi effecters immunostimulatory. To evade the host response the following design considerations should be adhered to:

1. Restrict the length of the dsRNA to less than 30 base pairs (bp),
2. Ensure the inclusion of 2 nt 3' overhangs, and
3. Avoidance of 5′-GUCCUUCAA-3′ and 5′-UGUGU-3′ sequence motifs.
4. Model shRNA constructs on enogenously expressed miRNA.

In addition to the above mentioned design parameters it has been shown that chemical modifications can be added to RNAi effecters to abrogate immunostimulatory effects. Judge *et al*, 2006 showed that the addition of 2'O-methyl (2'OMe) uridine or guanosine into one strand of an siRNA duplex reduced immunostimulation.

A recent study by Bauer *et al*, 2009 showed that the design of the shRNA passenger stand is a vital feature in preventing interferon related responses. The study demonstrated the manner wherein shRNA constructs trigger the interferon response. The shRNA constructs were then modified to mimic natural miRNA resulting in circumventing the interferon response.

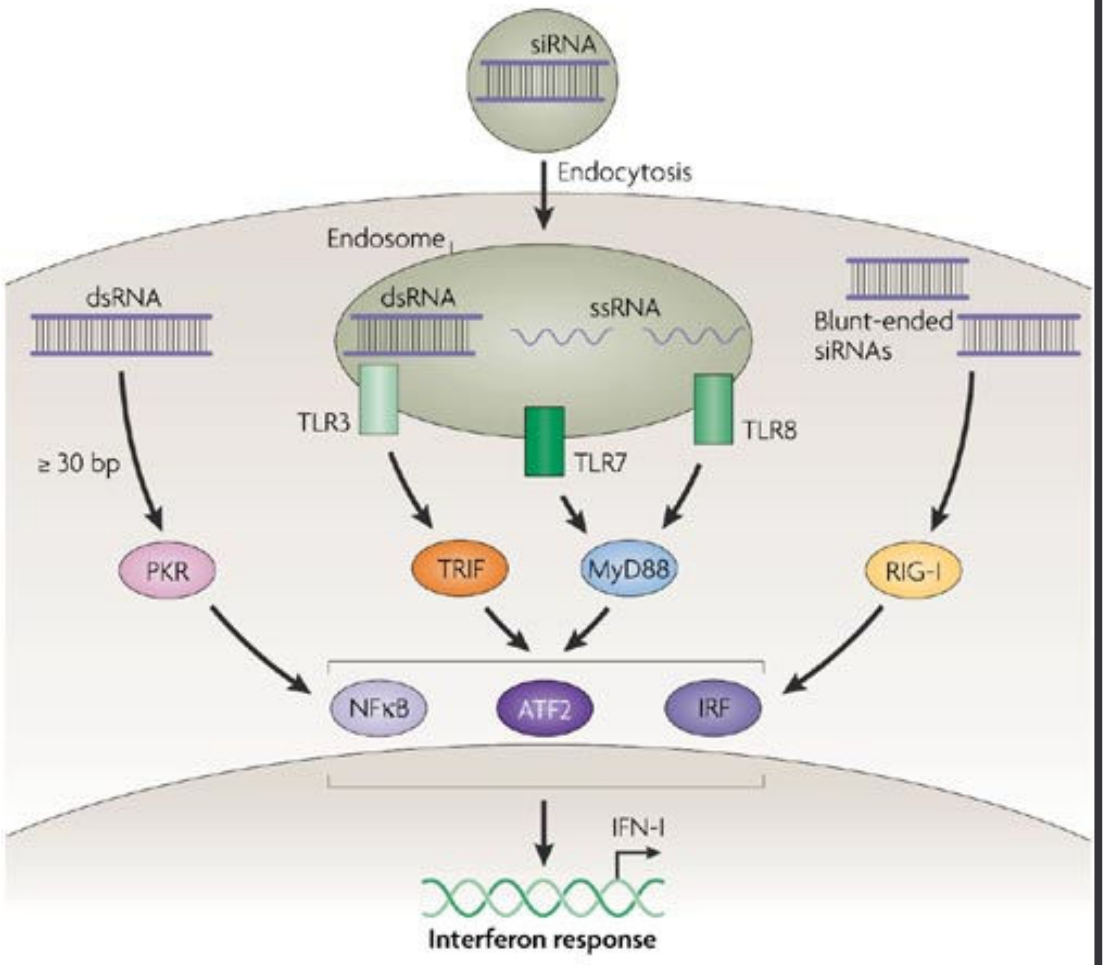

Figure 1.3 RNAi mediated activation of innate cellular immune response.
dsRNA greater than 30 bp can activate PKR and stimulate the interferon response. Blunt-ended siRNAs activate cell immunity by stimulating RIG-I. Alternatively ssRNA's and siRNA's can be incorporated within endosomes. Activation of TLR's ensue if there are immunostimulatory sequence motifs present. This results in a signaling cascade culminating in the stimulation of transcription factors NFkB, ATF2 and IRF (Kim , Rossi, 2007).

1.5.2 Nonspecific interaction of silencing molecules with cellular sequences

Processed RNAi effecters are approximately 21-23 nt in length. Since complete complementarity is not required for interference to occur it is possible for part of the 21-23 nt processed RNAi effecter to interact, non-specifically, with an unintended target. The resulting off-target effect will diminish the specificity of the RNAi effecter molecule and have great implications for the development of RNAi-based therapy. Such silencing of non-targeted sequences has been reported by Jackson *et al* (2003). Careful analysis of unintended silencing of cellular transcripts will need to be undertaken as a prerequisite when using RNAi-based therapy against HBV and other infections.

Characterisation of off-target effects can be analysed by microarray expression profiling and will become commonplace in development of therapeutic RNAi effecters.

1.5.3 Optimising delivery vectors

One of the most important challenges for development of RNAi-based HBV therapy is optimisation of delivery methods. Current delivery strategies are divided into non-viral and viral methods. Non-viral delivery methods can be cell specific or non-selective.

One non-selective systemic approach entails intravenous injection of RNAi effecter sequences attached to a lipid carrier. A lipid carrier is needed to aid in the movement of negatively-charged effecter sequence through the cellular membrane (Kim, Rossi, 2007).

Stable nucleic acid-lipid particles (SNALPs), comprising lipid bilayers with an outer layer of polyethylene glycol have been used to encapsulate siRNAs (Morrissey *et al*, 2005). Highly effective, non-toxic therapeutics responses have been achieved with the use of these particles (Zimmermann *et al*, 2006). The off-target effects associated with SNALP delivery vehicles are yet to be determined. It should be noted that non selective systemic approaches utilise higher doses of RNAi effecters compared to cell specific approaches. As mentioned previously, dosage of RNAi effecters must be administered for safe, effective therapeutic use. For this reason selective targeting approaches are more attractive as the lower dosage requirements could potentially reduce the number of off-target effects (Kim, Rossi, 2007). Examples of selective targeting include the coupling of RNAi effecters to antibody fragments and aptamers or nanoparticles coated with receptor-targeting ligands (Kim, Rossi, 2007). Antibody fragments have been used in HIV studies to facilitate the specific delivery of siRNAs to HIV infected cells (Song *et al*, 2005). Aptamers, specific for prostate-specific membrane antigens, have been conjugated to siRNAs (McNamara *et al*, 2006; Chu *et al*, 2006) and resulted in the specific delivery of the effecter sequence to prostate cancer cells. Nanoparticles coated with transferring ligands have been successfully utilised to target Ewing sarcoma tumours (Hu-Lieskovan *et al,* 2005). These specific non-viral strategies function by allowing the incorporation of effecters into specific cells through endocytosis.

The specific and non-specific non viral vectors are commonly used in acute virus infections. Viral delivery vectors are preferred when working with chronic diseases that require RNAi effecters present for extended periods of time (Kim, Rossi, 2007). The prolonged response is achieved by viral delivery of RNAi expression cassettes to cells for genomic integration or episomal expression. Potential toxic effects are the main concern associated with viral delivery vectors. Despite initial reservations both lentiviral and AAV vectors have been used successfully (Grimm, Kay, 2006). Since lentiviral vectors integrate into the genome they may be used to stably express RNAi effecters. AAV vectors are non-integrating and are strong inducers of innate and

adaptive immune responses. Though episomal expression of transgenes delivered by AAV vectors may be maintained for prolonged periods of time (in non dividing cells), the effect will eventually be lost (in dividing cells) as the episomal DNA is not replicated with the host cell DNA. Repeated administration may therefore be necessary but is limited as a result of the adaptive immunity triggered by these vectors (Alba, Bosch, Chillon, 2005).

1.5.4 Alternative targets

Typically anti HBV RNAi effecters have been designed to specifically target regions within the viral genome and are aimed at reducing viral replication. These strategies have been successful but issues such as viral escape mutants and recombination of viral genomes may impact on the efficacy of these strategies.

Non-viral targets may prove to be more essential in the future. Such strategies are already in use. Endogenous miR-122, which has recently been shown to be required for HCV replication, is a cellular target that may be silenced to counter viral infection (Jopling *et al*, 2005). The Fas cell death receptor, known to mediate T-cell hepatocyte toxicity, caused by viral infection, has also efficiently silenced using RNAi. Zender and colleagues demonstrated that inhibition of caspase-8 prevented acute liver failure in mice (Zender *et al*, 2003). Although promising, alternative targeting will require in depth studies into the effects of inhibiting essential cellular components. Especially in the case of chronic viral hepatitis which requires prolonged therapy.

1.5.5 Regulating dose

A recent study has demonstrated that high amounts of shRNA driven by a U6 promoter in an AAV vector, caused saturation of Exp5 (Grimm *et al*, 2006). Saturation of a key component of the RNAi machinery led to the inhibition of nuclear

export of endogenous pre-miRNA and resulted in the death of 23 out of 49 mice (Grimm *et al*, 2006). The findings from the study were that 25 mer shRNA at high doses caused toxicity and ultimately death. Smaller sized shRNA (19 mer) at lower doses resulted in efficient and sustained knockdown. This study highlighted the importance of dose dependant studies when testing the efficacy of RNAi effecters.

In another study An *et al* showed that the use of a weaker H1 promoter instead of U6 promoter eliminated cytotoxicity in lymphocytes (An *et al*, 2006). These findings suggest that certain components within a cell's RNAi machinery are in limited supply. Thus the testing of RNAi effecter molecules should include dose assays in which the least amount of effecter that generates the greatest knockdown is determined.

The type of promoter used has enormous bearing on the dose of RNAi effecter generated. Constitutively active Pol III promoters, such as U6 and H1, make dose regulation very difficult. For better regulation of expression, Pol II promoters can be utilised. Pol II promoters lend themselves to tissue specificity and regulation and are thus a better option when designing a potential RNAi therapeutic sequence.

1.5.6 Targeting multiple viral sequences with RNAi

A concern with the use of sequence-specific technologies such as RNAi is that many viruses are able to mutate and would thus evade silencing. In addition to viral mutation, recombinant genotypes are becoming more common.

Thus vectors that deliver several siRNAs or expressed hairpin cassettes that target multiple viral sequences is potentially useful to overcome viral escape. Long hairpin RNA are an example of multiple targeting effecters that have been shown to be effective against HCV (Akashi *et al*, 2005; Watanabe *et al*, 2006).

In addition to prevention of viral escape mutants, a multi–targeting approach may result in greater inhibitory effects due to cumulative inhibition. The areas of concern for this type of targeting approach are the same as that for monomeric RNAi effecters. In fact the concerns with a multi-targeting approach will be magnified due to the increase in RNAi effecter sequences. Unwanted effects such as the silencing of non-targeted genes and disruption of the endogenous miRNA pathway may result. An alternative to using multiple RNAi effecters for targeting different sites within a viral genome to limit escape, would be the combination of a single RNAi effecter with established licensed drugs that have a different mechanism of action.

1.6 AIMS

Exploiting RNAi using exogenous sequences to achieve specific gene silencing is an attractive HBV therapeutic option as viral transcripts and hence proteins can be specifically targeted, thus impairing viral replication and promoting viral eradication. Viral eradication is achieved without activating non-specific cellular responses and with minimal side effects.

The *HBx* ORF has been chosen as a target within the HBV genome as it allows for simultaneous targeting of all viral transcripts. shRNA expression cassettes have already been successfully used to knockdown HBV. The main objective of the project was to design, generate and evaluate the efficacy of polycistronic pri-miRNA expression cassettes against HBV.

Pol III (U6) and Pol II (CMV) pri-miRNA constructs were generated to allow for a direct comparison of promoter activity. The generated polycistronic pri-miRNA were expressed from a Pol II promoter (the natural promoters of miRNA), as opposed to a Pol III promoter that has traditionally been used to express shRNA. Pol II promoters

lend themselves to tissue specificity and regulation, which has been a problem for Pol III driven shRNA that are expressed constitutively and ubiquitously.

The monomeric pri-miRNA units within the polycistronic structures were based on shRNA that have been shown to knockdown markers of HBV replication effectively. The generated Pol III and Pol II transcribed pri-miRNA-31 constructs were compared to U6 shRNAs generated by Carmona *et al* (2006). The comparative evaluation included luciferase knockdown assays to determine the potency of the pri miRNA constructs, HBsAg assays to confirm the knockdown results achieved in the luciferase assays, assessment of the interferon response by q-PCR to determine whether any non-specific cellular responses were activated and northern blots to show the formation of mature miRNA. To validate the *in vitro* results of the pri-miRNA constructs, it was necessary to test the RNAi effecters in an *in vivo* system such as the mouse model.

The generated polycistronic pri-miRNA contain three pri-miRNA in series that target different regions within the *HBx* ORF. This multi-targeting approach could potentially result in increased knockdown of target, multiple target knockdown and decrease the risk of viral escape mutants.

CHAPTER TWO – MATERIALS AND METHODS

2.1 RNAi effecter constructs

2.1.1 Construction of U6 pri-miRNA and CMV pri-miRNA cassettes

Anti-HBV pre-miRNA DNA shuttles were generated by annealing partly complementary pre-miR-31/5, pre-miR-31/8 and pre-miR-31/9 forward (F) and reverse (R) oligonucleotides. This first round of PCR was followed by a second round of PCR to generate completely double-stranded DNA (Figure 2.1). Oligodeoxynucleotides used to generate the anti-HBV pre-miRNA shuttles (Table 2.1) were synthesised using phosphoramadite chemistry (Inqaba Biotech, South Africa). Primer extension PCR's were performed using Promega' s PCR Master Mix (Promega, WI, USA) as per manufacturers instructions. The thermal cycling conditions were as follows: Initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 10 seconds, annealing at 50°C for 10 seconds and extension at 72°C for 10 seconds and a final extension step at 72°C for 10 minutes. The primer extended products were subjected to agarose gel electrophoresis (1% gel). The desired fragments for pre-miR/5, pre-miR/8 and pre-miR/9 (78 bp) were excised and extracted from the gel slices using Qiagen's MinElute™ Gel Extraction Kit (Qiagen, Germany) (Appendix 6.1.5). Approximately 100ng of purified extended pre-miRNA DNA was used as template to generate the pri-miRNA shuttle sequences in a second round of PCR. The oligonucleotides in the second round of PCR are shown in Table 2.2. The pri-miRNA-31 sequences were then inserted into the PCR cloning vector, pTZ57R/T (InsTAcloneTM PCR cloning Kit, Fermentas, MD, USA) to generate pTZ pri-miRNA-31/5, pTZ pri-miRNA-31/8 and pTZ pri miRNA-31/9. The ligation reaction containing pri-miRNA [2 µl]; pTZ vector [1 µl]; 10 x Ligation Buffer [3 µl]; PEG [3 µl]; PCR grade water [20 µl] and T4 DNA ligase [1 µl] into a final volume of 30µl. Five microlitres of the ligation mix was used to transform 100 µl of chemically competent *E. coli* (DH5α, Invitrogen, CA, USA) (Appendix 6.1.1).

The transformed *E. coli* were plated on Luria Bertani ampicillin positive, X-gal, IPTG positive agar plates (Appendix 6.1.2) and incubated at 37°C overnight. Colonies containing an insert (white colonies) were selected and screened by M13 Forward and Reverse primers (IDT, IA, USA) to determine insert orientation. The plasmid DNA from the correct clones was purified according to the methods described in Appendix 6.1.3 and the sequence of the inserts confirmed (Inqaba Biotechnology, Sunnyside, South Africa).

To produce U6-driven pri-miRNA expression plasmids (pTZ U6-pri-miRNA-31/5, pTZ U6-pri miRNA-31/8 and pTZ U6-pri-miRNA- 31/9), pTZ pri-miRNA-31/5, pTZ pri-miRNA-31/8, pTZ pri miRNA-31/9 and pTZ U6 were digested with NheI [2 µl] and ScaI [2 µl] in buffer 2 (50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT, pH 7.9, and 0.1mg/ml BSA) at 25ºC for 1 hour. The digested fragments were subjected to agarose gel electrophoresis and the desired fragments (2295 bp for pTZ-U6 and 1067 bp for pTZ-pri-miRNA) were excised and extracted using Qiagen's MinElute™ Gel Extraction Kit (Qiagen, Germany) (Appendix 6.1.5). Purified products were ligated in reactions containing NheI / ScaI digested pri-miRNA [2 µl]; NheI / ScaI digested U6 [1 µl]; 10 x Ligation Buffer [3 µl]; PEG [3 µl]; PCR grade water [20 µl] and T4 DNA ligase [1 µl] into a final volume of 30µl. Separate ligation reactions were set up for pri-miRNA-31/5, pri-miRNA-31/8 and pri-miRNA-31/9. The ligation mix was incubated at 22 º C for one hour and then transformed into competent DH5α and plated onto Luria Bertani ampicillin positive, X-gal, IPTG positive agar plates as previously mentioned (Appendix 6.1.1 and 6.1.2). Colonies containing an insert (white colonies) were selected and screened by M13 Forward and Reverse primers (IDT, IA, USA) to determine PCR insert orientation. The plasmid DNA of the correct clones was purified as per Appendix 6.1.3. The clones with the correct inserts were verified by sequencing at Inqaba Biotechnology, South Africa.

Pol II-driven pri-miRNA expression plasmids (pCI-CMV-pri-miRNA- 31/5, pCI-CMV-pri-miRNA- 31/8 and pCI-CMV-pri-miRNA- 31/9) were generated by

digesting pTZ pri-miRNA-31/5, pTZ pri-miRNA-31/8 and pTZ pri miRNA-31/9 with XbaI [2 µl] and SalI [2 µl] in buffer Tango (33mM Tris-acetate, pH 7.9, 10mM Mg-acetate, 66mM K-acetate and 0.1mg/ml BSA) at 37℃ for 1 hour. pCI-neo HBx was digested with XhoI [2 µl] and XbaI [2 µl] in buffer Tango (33 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate and 0.1 mg/ml BSA) at 37℃ for 1 hour. The digested fragments were subjected to agarose gel electrophoresis and the desired fragments (5472 bp for pCI-neo and ~200 bp for pTZ-pri-miRNA) were excised and extracted using Qiagen's MinElute™ Gel Extraction Kit (Qiagen, Germany) (Appendix 6.1.5). Purified products were ligated in reactions containing XbaI / SalI digested pri-miRNA [4 µl]; XhoI / XbaI digested pCI-neo HBx [1 µl]; 10 x Ligation Buffer [3 µl]; PEG [3 µl]; PCR grade water [18 µl] and T4 DNA ligase [1 µl] into a final volume of 30µl. The ligation mix was incubated at 22 º C for one hour and then used to transform competent DH5α and plated onto Luria Bertani ampicillin positive, X-gal, IPTG positive agar plates as previously described (Appendix 6.1.1 and 6.1.2). Colonies containing an insert were selected and screened by M13 Forward and Reverse primers (IDT, IA, USA) to determine PCR insert orientation. The plasmid DNA of the correct clones was purified as per Appendix 6.1.3. The clones with the correct inserts were verified by sequencing at Inqaba Biotechnology, South Africa.

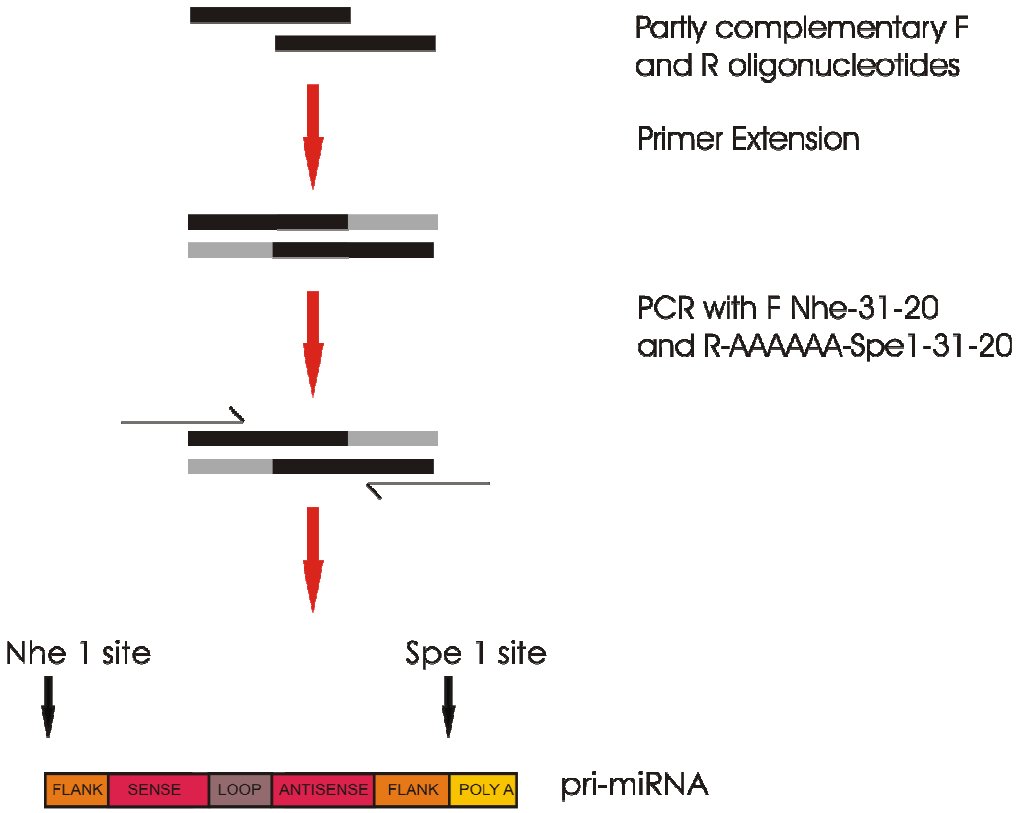

Figure 2.1 Generation of pri-miRNA constructs
pri-miRNA 31-5, pri-miRNA 31-8 and pri-miRNA 31-9 were all constructed using a two-step PCR. All the constructs contained an miR 31 loop, mismatches within the guide, loops within the stem and 20 nucleotide single stranded flanking regions.

Table: 2.1 Oligonucleotide sequences used in the generation of anti –HBV pre-miRNA DNA shuttles

pre-miR-31/5 F **pre-miR-31/5 R**	5’-GTA ACT CGG AAC TGG AGA GGG GTG AAG CGA AGT GCA CAC GGG TTG AAC TGG GAA CGA CG-3’ 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 **pre-miR-31/8 R**	5’-GTA ACT CGG AAC TGG AGA GGC AAG GTC GGT CGT TGA CAT TGG TTG AAC TGG GAA CGA AA-3’ 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 **pre-miR-31/9 R**	5’ GTA ACT CGG AAC TGG AGA GGA TTT ATG CCT ACA GCC TCC TAG TTG AAC TGG GAA CGA AG-3’ 5’-CTG CTG TCA GAC AGG AAA GCC TTT ATT CCT TCA GCC TCC TTC GTT CCC AGT TCA ACT AGG-3’

Table 2.2 Oligonucleotide sequences used in the generation of anti –HBV pri-miRNA DNA shuttles

pri-miR-31 F	5’-GCT AGC CAT AAC AAC GAA GAG GGA TGG TAT TGC TCC TGT AAC TCG GAA CTG GAG
pri-miR-31 R	AGG-3’
	5’-AAA AAA ACT AGT AAG ACA AGG AGG AAC AGG ACG GAG GTA GCC AAG CTG CTG
	TCA GAC AGG AAG C-3’

2.1.2 Construction of polycistronic CMVpri-miRNA cassettes

Trimeric shuttle cassettes, containing pri-miRNA-31 were formed by inserting combinations of pri-miRNA-31/5, pri-miRNA-31/8 and pri-miRNA-31/9 sequences downstream of the U6 promoter and the CMV immediate early promoter enhancer. A total of six trimeric cassettes was generated (pri-miRNA-31/5-8-9, pri-miRNA-31/5-9-8, pri-miRNA-31/8-5-9, pri-miRNA-31/8-9-5, pri-miRNA-31/9-5-8 and pri-miRNA-31/9-8-5).

Similar cloning strategies were used to propagate each of the trimers. As an example (Figure 2.2), to generate the pri-miRNA-31/5-8-9 cassette, pri-miRNA-31/8 was excised from pTZ pri-miRNA-31/8 with NheI and EcoRI and ligated to pTZ pri-miRNA-31/5 that had been digested with SpeI and EcoRI to create pTZ pri-miRNA-31/5-8. Similarly, the sequence encoding pri-miRNA-31/9 was then excised from pTZ pri-miRNA-31/9 with NheI and EcoRI and ligated to SpeI and EcoRI sites of pTZ pri-miRNA-31/ 5-8. Successful ligation resulted in formation of pTZ primiRNA-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 (Inqaba Biotechnology, South Africa).

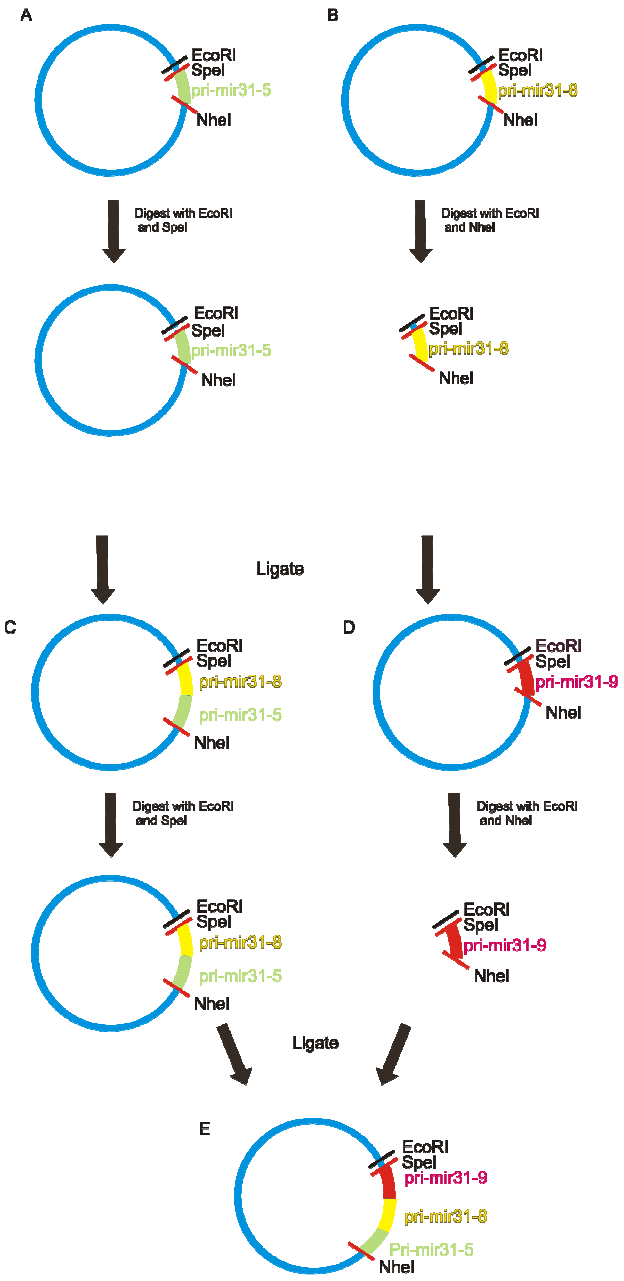

Figure 2.2 Cloning Strategy to generate polycistronic pri-miRNA.

In A, pri-miR31/5 is digested with EcoRI and SpeI, whereas in B, pri-miRNA 31/8 is digested with EcoRI and NheI. These are ligated together to generate C (primiR31/58). C is then digested with EcoRI and SpeI and D is digested with EcoRI and NheI. The desired fragments are ligated together to form the polycistronic pri-miR31/589.

2.2 Generation of HBV target plasmids.

2.2.1 Construction of luciferase target plasmids : psiCHECK-5T, psiCHECK-8T and psiCHECK-9T

To produce dual luciferase reporter plasmids containing sites individually targeted by pri miRNA-31/5, pri-miRNA-31/8 and pri-miRNA-31/9, primers were designed to amplify specific HBV coordinates as seen in Table 2.3 (Genbank accession J02203). The oligonucleotide sequences, which also introduced a SpeI site at the 3' end of the amplicons are shown in Table 2.3. The targets for the desired regions (Table 2.3) were amplified with target specific forward and reverse primers using Promega's PCR Master Mix (Promega, WI, USA). The thermal cycling conditions were as follows: Initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 10 seconds, annealing at 50°C for 10 seconds and extension at 72°C for 10 seconds and a final extension step at 72°C for 10 minutes. The amplicons were subjected to agarose gel electrophoresis (1% gel), excised and extracted from the gel slice using Qiagen's MinElute™ Gel Extraction Kit (Qiagen, Germany) (Appendix 6.1.5). Purified fragments were incorporated into the pTZ57R/T PCR cloning vector (InsTAcloneTM PCR cloning Kit, Fermentas, MD, USA) using the standard ligation reaction followed by transformation of competent DH5α cells as previously described. Positive clones were selected and screened by M13 Forward and Reverse primers (IDT, IA, USA) to determine PCR insert orientation. Inserts from the positive clones were removed by restriction endonuclease digestion with SalI [1 µl] and SpeI [1 µl] in buffer R (10mM Tris-HCl, pH 8.5, 10mM magnesium acetate, 66mM K-acetate and 0.1mg/ml BSA) at 37°C for 1 hour. These inserts were ligated into the XhoI and SpeI sites of psiCHECK2.2 that had been previously modified from psiCHECK-2 (Promega, WI, USA). Transformation of competent DH5α cells with ligation mixes and screening for the desired clones generated

psiCHECK-5T, psiCHECK-8T and psiCHECK-9T with the target sites downstream of the Renilla luciferase reporter open reading frame (ORF).

2.2.2 Construction of luciferase target plasmid psiCHECKHBx

The psiCheck-HBx target plasmid was generated by restriction endonuclease digestion of pCI-neo HBx (Weinberg *et al*, 2000) with XhoI [1 µl] and NotI [1 µl] in buffer R (10mM Tris-HCl, pH 8.5, 10mM magnesium acetate, 66mM K-acetate and 0.1mg/ml BSA) at 37℃ for 1 hour. psiCheck2.2 was simultaneously restriction endonuclease digested with XhoI [1 µl] and NotI [1 µl] in buffer R (10mM Tris-HCl, pH 8.5, 10mM magnesium acetate, 66mM K-acetate and 0.1mg/ml BSA) at 37℃ for 1 hour. The XhoI/NotI restricted pCI-neo HBx and the XhoI/NotI restricted psiCheck2.2 were subjected to agarose gel electrophoresis (1% gel). The desired fragments were excised and extracted from the gel slice using Qiagen's MinElute™ Gel Extraction Kit (Qiagen, Germany) (Appendix 6.1.5). The purified fragments were ligated and transformed as previously mentioned to generate psiCheck-HBx containing the HBx ORF within the 3' untranslated region of the Renilla Luciferase cassette.

2.2.3 Construction of target plasmid pCH-9/3091

The construction of the target plasmid pCH-9/3091 has been previously described by Nassal (1992). The pCH-9/3091 vector contains the wild-type HBV genome with terminal repeats under the control of the CMV immediate early promoter-enhancer. During transcription, pCH-9/3091 generates a greater than genome length pregenomic RNA. pdsDNA may then be reverse transcribed from the pregenomic RNA template. Transcription from pCH-9/3091 therefore simulates HBV replication.

2.2.4 Construction of luciferase target plasmid pCH Firefly Luc

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, in a standard reaction, from pGL4 (Promega, Madison, WI) using PCR. In the PCR reaction 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 are given in Table 2.3. The PCR amplified product was cloned, using the previously mentioned methods of ligation and transformation, into pTZ57R/T to generate pTZ Firefly Luc. The *Firefly luciferase* sequence was then excised from pTZ Firefly Luc using restriction endonuclease digestion 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.

Table2.3 Oligonucleotide sequences used in the generation of dual luciferase reporter plasmids

HBV-1575–1599	
5T F	5’-CCG TGT GCA CTT CGC TTC AC-3’
5T R	5’-ACT AGT CAG AGG TGA AGC GA-3’
HBV-1678–1702	
8T F	5’-CAA TGT CAA CGA CCG ACC TT-3’
8T R	5’-ACT AGT GCC TCA AGG TCG GT-3’
HBV-1774–1798	
9T F	5’-TAG GAG GCT GTA GGC ATA AA-3’
9T R	5’-ACT AGT ACC AAT TTA TGC CT-3’
pCH Firefly Luc	
F	5′-ACT GCT CGA GGA TTG GGG ACC CTG CGC TGA ACA TGG AAG ACG CCA AAA AC-3
R	5′ ACT GAC TAG TTT ACA CGG CGA TCT TTC C-3′

2.3 pri-miRNA mediated inhibition of HBV gene expression in cell culture models of HBV replication.

2.3.1. Plasmid Preparation

U6 pri-miRNA, CMV pri-miRNA and CMV polycistronic miRNA as well as all the target and reporter plasmids were prepared using the EndoFree® Plasmid Maxi Kit (Qiagen, CA, USA) (refer to Appendix 6.1.5).

2.3.2 Transfection of cultured cells with plasmids encoding moncistronic and polycistronic miRNA.

The Human hepatoma cell line, Huh7 were maintained in RPMI medium supplemented with 2.5% fetal calf serum (FCS), penicillin (50 IU/ml) and streptomycin (50 g/ml) (Gibco BRL, UK) (Appendix 6.1.6). The Human embryonic kidney cells, HEK293 were propagated in DMEM supplemented with 10% FCS, penicillin (50 IU/ml) and streptomycin (50 g/ml) (Gibco BRL, UK) (Appendix 6.1.6). On the day prior to transfection, 1 500 000 HEK293 cells or 1 000 000 Huh7 cells were seeded in dishes of 10 cm diameter.

2.3.3 Evaluation of HBV knockdown by RNAi effecter sequences.

2.3.3.1 Luciferase Assays

To assess HBV knockdown efficacy of the Pol III and Pol II pri-miRNA shuttles, Lipofectamine 2000TM (Invitrogen, CA, USA) was used to co-transfect 80 ng pCH-FLuc, 800 ng of the relevant pri-miRNA shuttle plasmid, together with effecter plasmid or vector control plasmid according to previously described methods (Seeger, Mason, 2000) (Appendix 6.1.6). 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 (Appendix 6.1.7).

To determine the efficacy of individual pri-miRNA 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) (Appendix 6.1.6). Luciferase activity was assayed using the Dual- Luciferase Reporter Assay System (Promega, WI, USA) and Renilla luciferase to Firefly luciferase activity was determined (Appendix 6.1.7). Silencing of mutant HBx sequences was assayed similarly by using psiCHECK-HBx and psiCHECK-mHBx dual luciferase reporter vectors.

2.3.3.2 HBsAg Secretion

Knockdown of HBV replication was assessed in cells co-transfected with pCH-9-3091 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) (Appendix 6.1.8).

2.3.4 Assessment of specificity of RNAi effecter sequences

The cell line utilized for assessment of specificity of the RNAi effecters was Human Embryonic Kidney cells, HEK293. The HEK293 cell are more fragile that the Huh 7 cell line. Saline washes were not performed on HEK293 cells. This amendment to routine cell maintenance minimized the loss of cells. Cells were maintained in DMEM supplemented with 10% FCS, penicillin (50 IU/ml) and streptomycin (50 g/ml) (Gibco BRL, UK). On the day prior to transfection, 250 000 HEK293 cells were seeded in dishes of 2 cm diameter. Transfection was carried out with 80 ng of

pCH-FLuc target plasmid, 40 ng of pCI-neo eGFP (expresses enhanced green fluorescent protein) (Passman *et al*, 2000) and 800 ng of shRNA- or miRNA-encoding plasmid using Lipofectamine (Invitrogen, CA, USA) according to the manufacturer's instructions. As a positive control for the induction of the interferon (IFN) response, cells were also transfected with 800 ng poly (I:C) (Sigma, MI, USA). Two days after transfection, cells were checked for the expression of eGFP using a fluorescence microscope. RNA was then extracted with Tri Reagent (Sigma, MI, USA) according to the manufacturer's instructions. The RNA extracts were DNase treated (Fermentas) according to manufacturers instructions. cDNA's were sysnthesized by incubating 10 x RT buffer (Qiagen) [1 µl], 5mM dNTP mix [1 µl], 10 µM oligo d(T) [1 µl], sensiscript RT (Qiagen) [0.5µl], RNase-free water [2.5 µl] and template RNA [4 µl] at 37°C for 1 hour.

To amplify interferon (IFN) response genes *IFNβ* and *GAPDH* cDNA, the procedures described by Song *et al* (2005) were used. All quantitative PCRs (q-PCRs) were carried out using the Roche Lightcycler V.2. Controls included water blanks and RNA extracts that were not subjected to reverse transcription. LightCycler Faststart DNA master Sybr Green (Roche, Germany) was used as per manufacturer's instructions to amplify and detect DNA during the reaction. Thermal cycling parameters consisted of a hotstart for 30 sec at 95°C followed by 50 cycles of 58°C for 10 sec, 72°C for 7 sec and then 95°C for 5 sec. Specificity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. The primer combinations used to amplify IFN response-related mRNA of human HEK293 cells are shown in Table 2.4.

Table 2.4 Oligonucleotide sequences used in the interferon response q-PCR

IFN-ß	
***IFN-ß* F**	5’ – TCCAAATTGCTCTCCTGTTGTGCT 3’
***IFN-ß* R**	5 – CCACAGGAGCTTCTGACACTGAAAA 3’
GAPDH	
***GAPDH* F**	5’ – AGGGGTCATTGATGGCAACAATATCCA 3’
***GAPDH* R**	5’ – TTTACCAGAGTTAAAAGCAGCCCTGGTG 3’

2.3.5 Evaluation of processed products of expressed RNAi effecter sequences

HEK293 cells were maintained in DMEM supplemented with 10% FCS, penicillin (50 IU/ml) and streptomycin (50 µg/ml) as described in Appendix 6.1.6. One day prior to transfection 3000 cells were seeded into 10 cm^2 Costar® tissue culture plates (Corning Inc, NY, USA). The transfection was carried out using 16 µg of each RNAi effecter plasmid, 3 µg of pCH-9/3091 and 1 µg of pCI-neo eGFP, mixed into a DNA cocktail. Five hundred microlitres Opti-MEM I was added to the DNA cocktails. The DNA was transfected into the cells with Lipofectamine™ 2000 (Invitrogen, CA, USA) according to manufacturer's instructions. Two days after transfection total RNA was extracted using Tri-Reagent (Sigma, MO, USA) according to manufacturer's instruction. The extracted RNA was quantified spectrophotometrically (using a Nanodrop) and approximately 25 µg was separated on a 15% denaturing polyacrylamide gel. Radioactively labelled DNA oligonucleotides (18 and 30 bases) were run alongside the cellular RNA and used as size indicators. 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 by exposure to 200 000 $\mu J/cm^2$ 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, 8 and 9 guide sequences and the U6 snRNA sequence were prepared by labelling oligonucleotides (Table 2.5) according to standard procedures using polynucleotide kinase and γ-32P ATP. After the prehydrisation step membranes were hybridised overnight at 42°C with the relevant probe at a final concentration of 10

ng/ml. Following overnight hybridization 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. To verify equal loading of the lanes with cellular RNA, the blots were stripped and reprobed with an oligonucleotide complementary to endogenous U6 snRNA.

Table 2.5 Oligonucleotide probes used to detect processed RNAi effecters

5 guide	5’ – CCGTGTGCACTTCGCTTC 3’
8 guide	5’ – CAATGTCAACGACCGACC 3’
9 guide	5’ – TAGGAGGCTGTAGGCATA 3’
U6snRNA guide	5’ – TAGTATATGTGCTGCCGAAGCGAGCA 3’

2.4 Testing of anti HBV efficacy of miR sequences in vivo using the hydrodynamic injection model of HBV replication.

2.4.1 Hydrodynamic injection of mice.

The murine hydrodynamic tail vein injection (HDI) method (Yang *et al*, 2002) was employed to determine the effects of pri-miRNA and polycistronic pri-miRNA 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. The MHI method was performed by Prof Arbuthnot and Abdullah Ely. A saline solution comprising 10% of the mouse's body mass was injected via the tail vein over 5-10 seconds. This saline solution included a combination of three plasmid vectors: 5 μg target DNA (pCH-9/3091) or 5 μg pCI-neo plasmid DNA (Promega WI, USA) that lacks HBV sequences; 5 μg anti HBV sequence or mock (pTZ backbone); and 5 μg pCI neo EGFP (a control for hepatic DNA delivery, which constitutively expresses the EGFP marker gene). After discarding data from injections that were suboptimal each experimental group comprised 5-8 mice. Blood was collected under anaesthesia by retroorbital puncture on days 3 and 5 after HDI. Serum HBsAg concentration was measured using the Monolisa (ELISA) immunoassay kit (BioRad, CA, USA) according to the manufacturer's instructions.

CHAPTER THREE - RESULTS

3.1 Design of pri-miRNA sequences targeted against the HBx ORF

The pri-miRNA expression cassettes were designed by replacing the guide sequences of pri-miRNA-31 with the guide sequence of U6 shRNA 5, U6 shRNA 8 and U6 shRNA 9 (Carmona *et al*, 2006). The wild-type sequences of the pri-miRNA were maintained as far as possible except for the guide and antiguide sequences such that the predicted secondary structure of the pri-miRNA cassettes did not differ significantly from that of its respective wild-type pri-miRNA. Figure 3.1 shows the predicted structures of the wildtype pri-miRNA-31 sequences together with their anti HBV derivatives (pri-miRNA-31/5, pri-miRNA-31/8 and pri-miRNA-31/9).

The final cassettes contained 51 nucleotides of wild-type sequences flanking either end the pre-miRNA (Zeng, Yi, Cullen, 2005). Cloning of the pri-miRNA into expression cassettes was facilitated by the inclusion of *Nhe* I and *Spe* I restriction sites at their 5’ and 3’ ends, respectively. A schematic illustration of the targeted HBV regions is indicated in Figure 3.2.

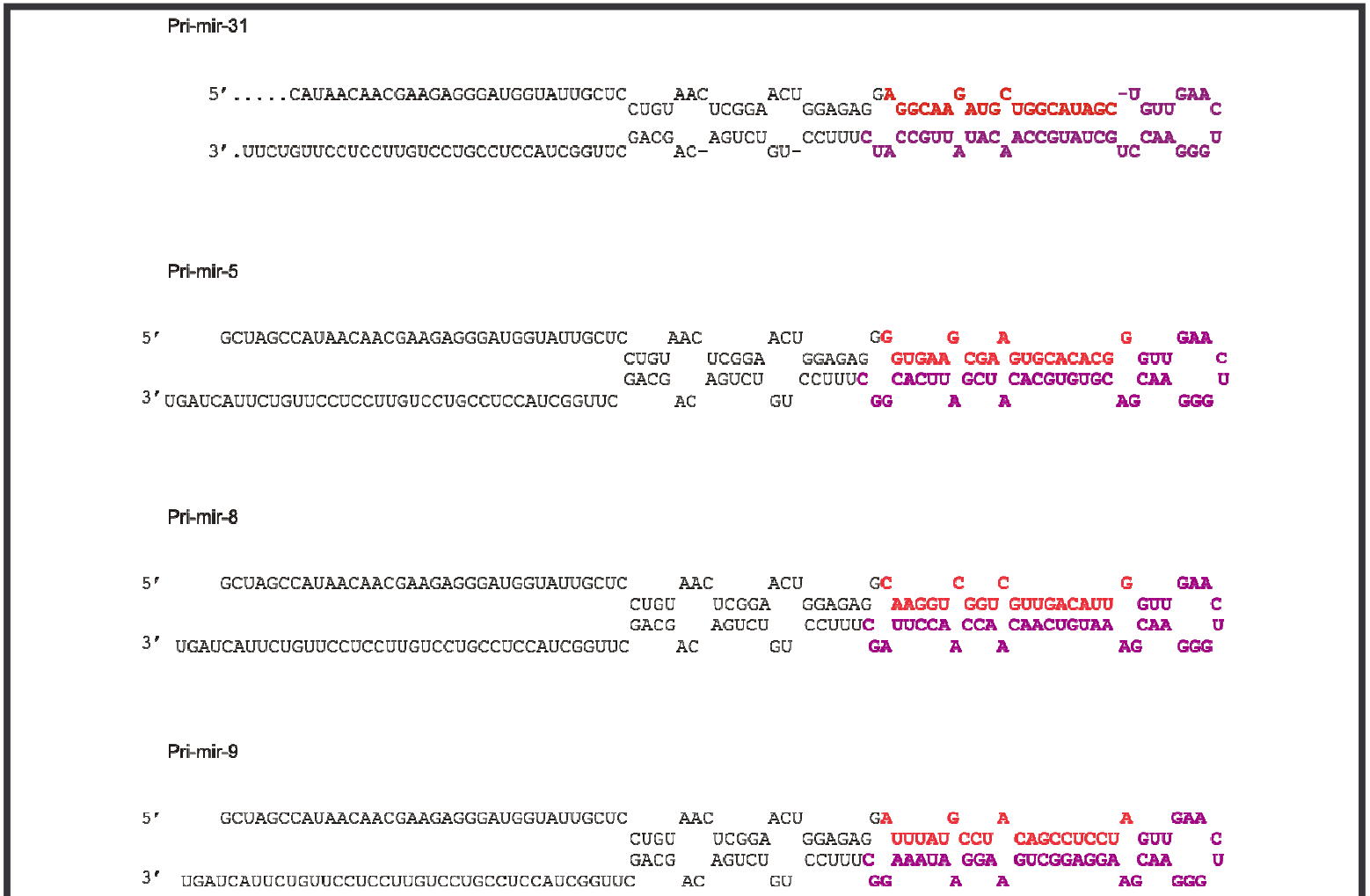

Figure 3.1 Predicted structure and sequences of pri-miR-31 with its anti HBV derivatives pri-miR-31/5, pri-miR-31/8 and pri-miR-31/9. Following Drosha/DGCR8 processing, the sequences indicated in black are cleaved generating pre-miRNA (red and purple sequences). The mature processed guide sequences that are selected after Dicer processing and strand selection by RISC are indicated in red.

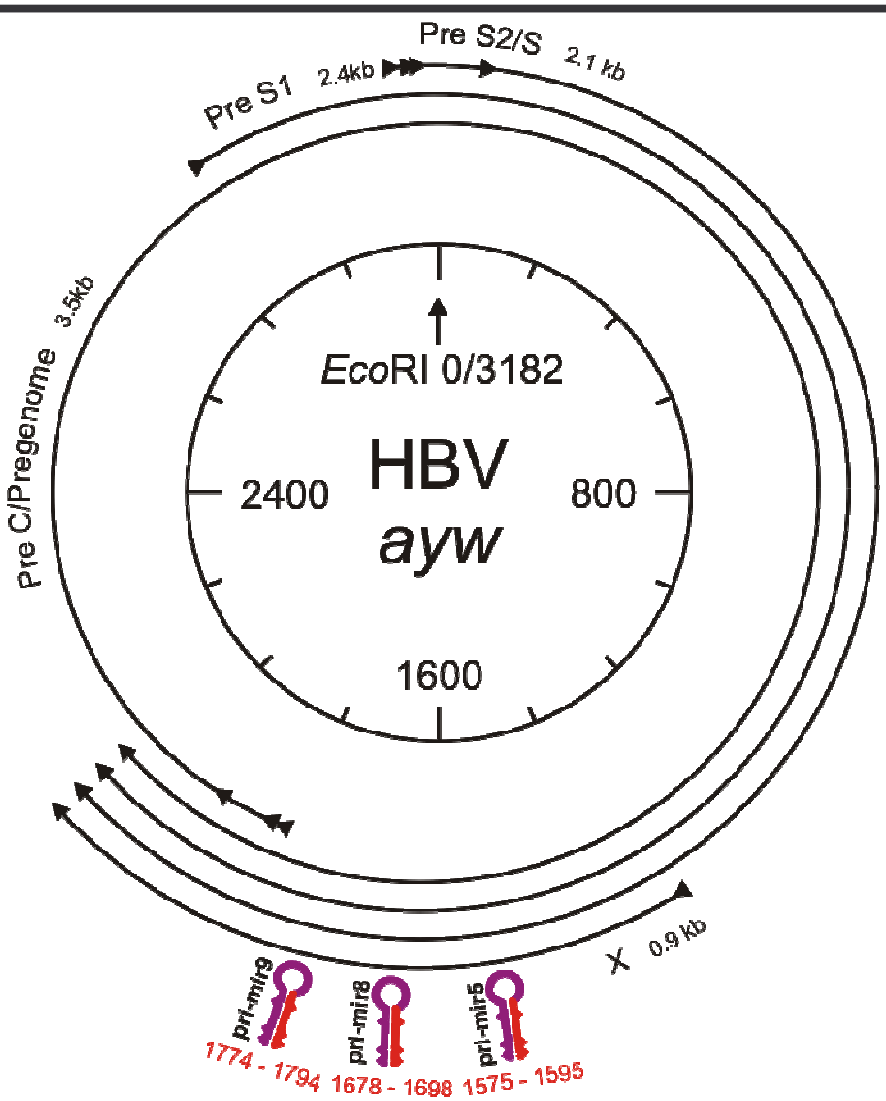

Figure 3.2 Schematic Representation of the regions targeted within the HBV genome.

pri-miRNA 5 targets nucleotides 1575 – 1598, pri-miRNA 8 targets nucleotides 1678 – 1698 and pri-miRNA 9 targets nucleotides 1774 – 1794, relative to the single EcoRI restriction site. All the generated pri-miRNA target the X ORF and subsequently also target the Pre C, Pre S1 and Pre S2, ORF's as they overlap with the X ORF.

3.2 pri-miRNA mediated inhibition of HBV gene expression in cell culture models of HBV replication.

3.2.1 Evaluation of HBV knockdown by pri-miRNAi effecter sequences

3.2.1.1 Luciferase assays

Target sites of the pri-miRNA cassettes are located within the HBV X (HBx) ORF (Figure 3.2). This sequence is conserved, common to all HBV viral transcripts and has been shown to be a good target for RNAi-based HBV silencing (Carmona *et al*, 2006).

Co-transfection of pCH-FLuc (surface ORF of pCH-9/3091 was substituted with a Firefly luciferase ORF) and pri-miRNA-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 pri-miRNA-31/5, U6 pri-miRNA-31/8, U6 pri-miRNA-31/9, CMV pri-miRNA-31/5, CMV pri-miRNA-31/8 and CMV pri-miRNA-31/9 containing vectors.

Compared to the positive control the RNAi effecters resulted in the following knockdown of pCH-FLuc: 84% by U6 shRNA 5, 80% by U6 pri-miRNA-31/5, 95% by U6 pri-miRNA-31/8, 88% by U6 pri-miRNA-31/9, 92% by CMV pri-miRNA-31/5, 88% by CMV pri-miRNA-31/8 and 90 % by CMV pri-miRNA-31/9 (Figure 3.4).

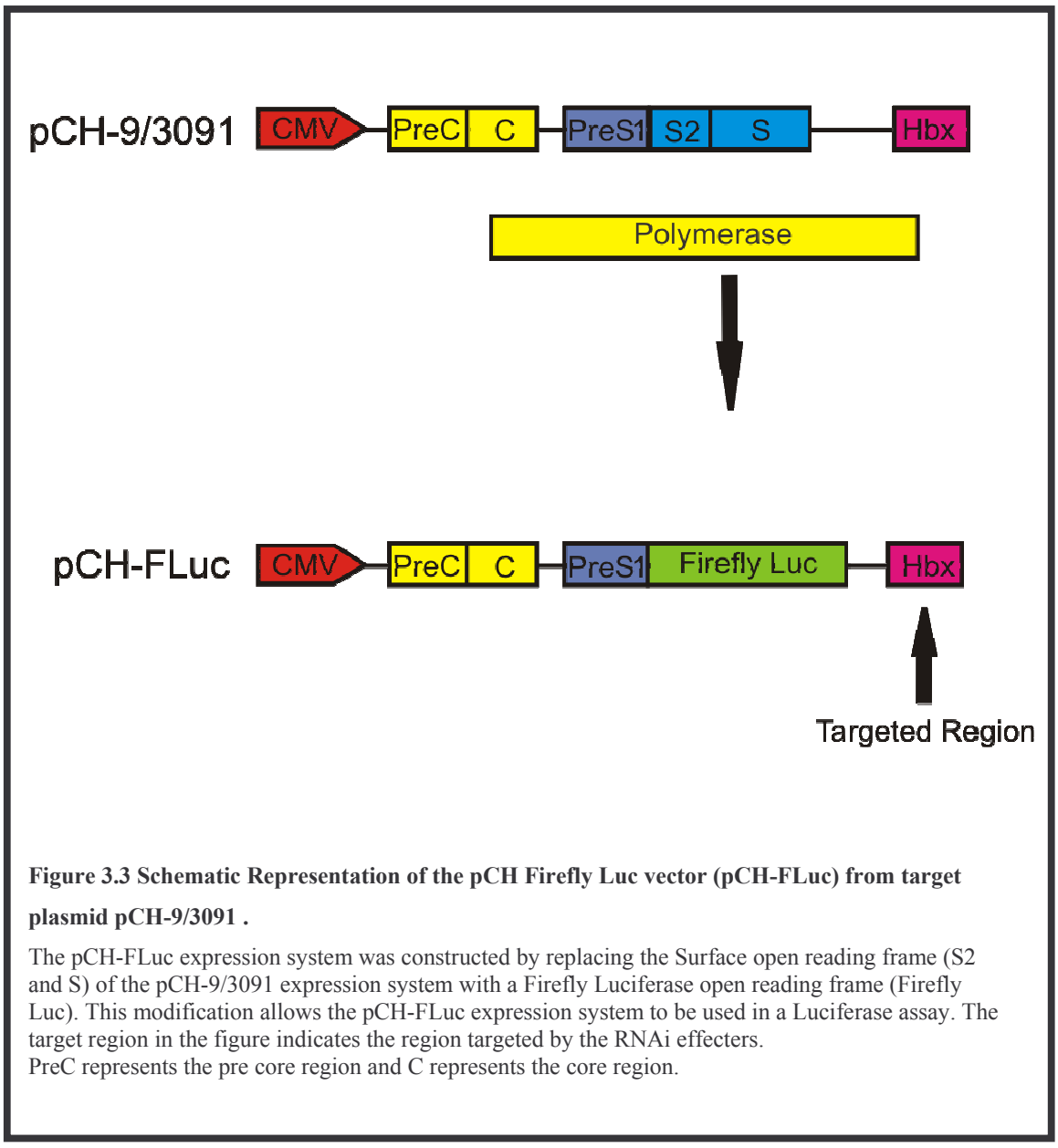

Figure 3.3 Schematic Representation of the pCH Firefly Luc vector (pCH-FLuc) from target plasmid pCH-9/3091 .

The pCH-FLuc expression system was constructed by replacing the Surface open reading frame (S2 and S) of the pCH-9/3091 expression system with a Firefly Luciferase open reading frame (Firefly Luc). This modification allows the pCH-FLuc expression system to be used in a Luciferase assay. The target region in the figure indicates the region targeted by the RNAi effecters.
PreC represents the pre core region and C represents the core region.

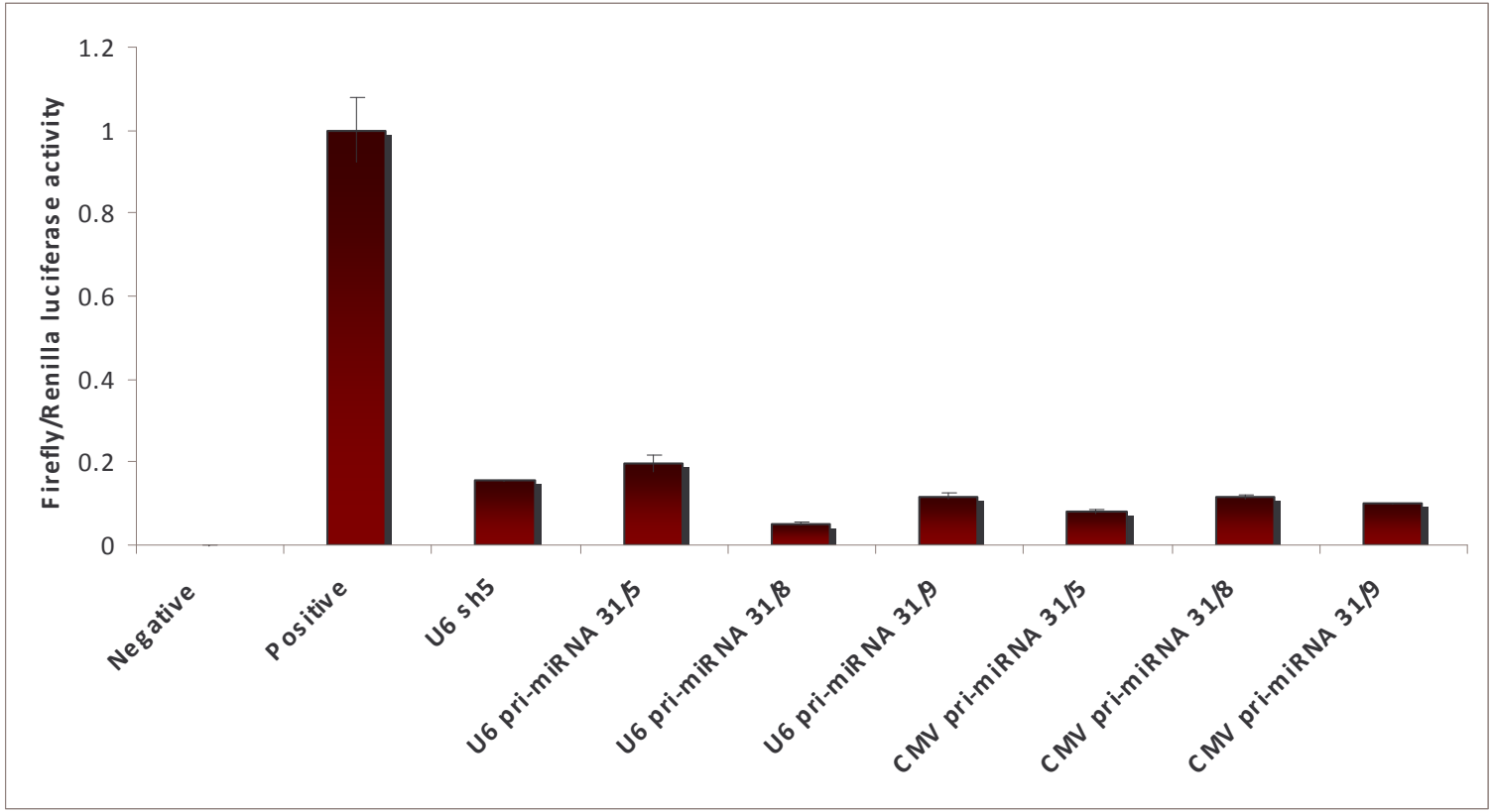

Figure 3.4 Dual Luciferase Assay to evaluate knockdown efficacy of pri-miRNA constructs.
The positive control contained the target plasmid, pCH-FLuc. The negative control did not contain target plasmid or pri-miRNA effecter constructs. The U6 sh5 construct was used as a control to compare the knockdown efficacy of the U6 pri-miRNA and the CMV pri-miRNA constructs.
Huh 7 cells were cotransfected with plasmids containing the indicated RNAi expression cassettes, pCH-FLuc and a plasmid constitutively expressing Renilla luciferase. Results are given as ratios of Firefly to Renilla luciferase activity. The plotted data is given as a normalized mean relative to the corresponding measurements from mock-treated cells (positive control). The results are from three independent transfections, and the bars indicate the normalized SEM.

3.2.1.2 HBsAg

HBsAg is a marker of HBV and an ELISA for HBsAg serves as a suitable method to support the data obtained from the Luciferase knockdown assays. Huh7 cells were co-transfected with pri-miRNA-encoding vectors together with the pCH-9/3091 HBV replication competent target plasmid. Controls included a positive which only contained pCH-9/3091 target, a negative which contained neither pCH-9/3091 target nor pri-miRNA-encoding vectors and U6 shRNA 5, which we have previously shown to be effective against HBV (Carmona *et al*, 2006).

Analysis showed that viral antigen secretion was diminished significantly by U6 shRNA 5, U6 pri-miRNA-31/5, U6 pri-miRNA-31/8, U6 pri-miRNA-31/9, CMV pri-miRNA-31/5, CMV pri-miRNA-31/8 and CMV pri-miRNA-31/9 containing vectors. Compared to the positive control the RNAi effecters resulted in the following reduction of HBsAg: 93% by U6 shRNA 5, 92% by U6 pri-miRNA-31/5, 91% by U6 pri-miRNA-31/8, 90% by U6 pri-miRNA-31/9, 91% by CMV pri-miRNA-31/5, 87% by CMV pri-miRNA-31/8 and 82 % by CMV pri-miRNA-31/9 (Figure 3.5). The data obtained further validated the Luciferase knockdown results.

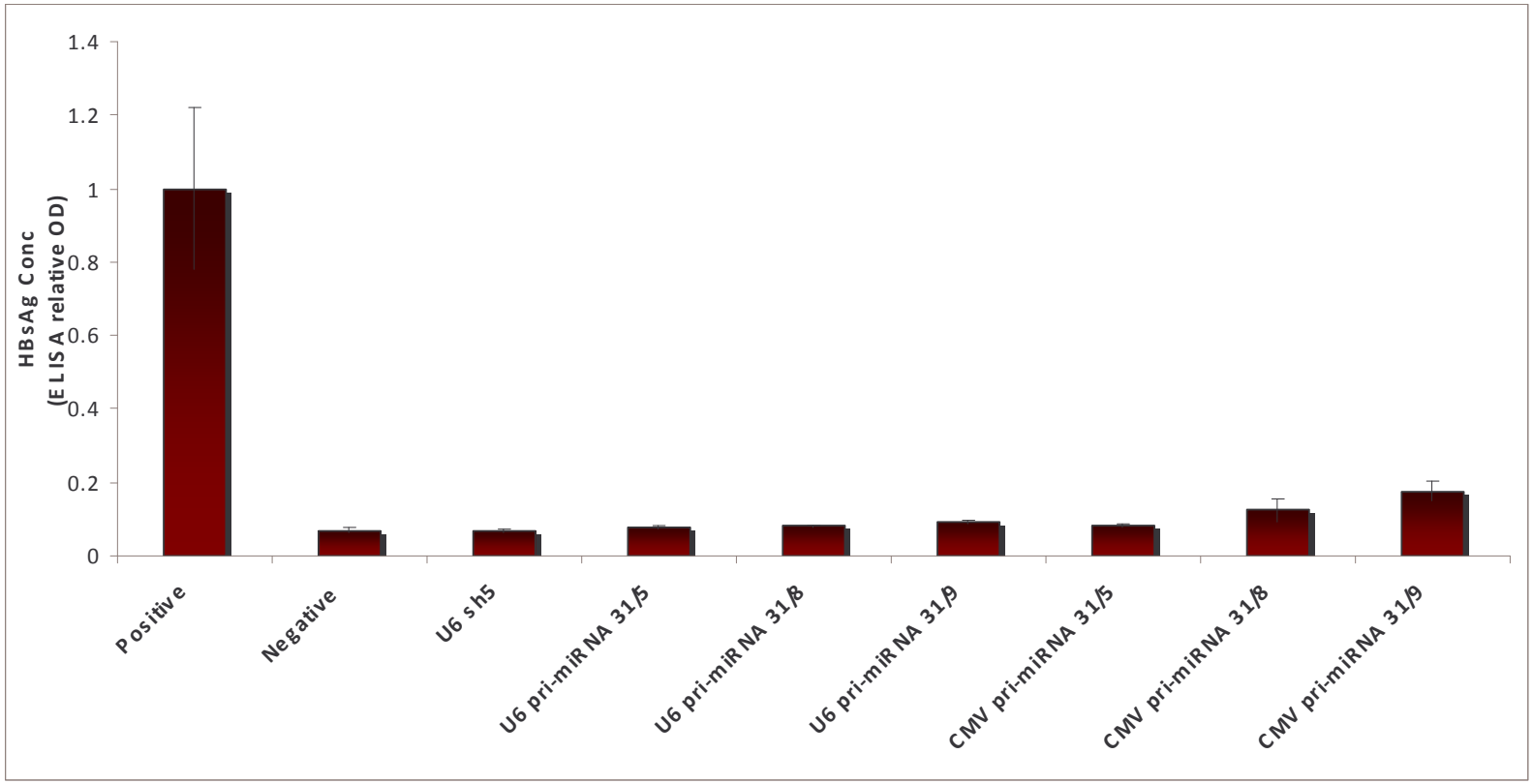

Figure 3.5 HBsAg serum secretion to evaluate knockdown efficacy of pri-miRNA constructs.
Huh7 cells were co-transfected with plasmids encoding the miR- or shRNA-encoding cassettes, together with the HBV target plasmid, pCH-9/3091. HBsAg measurements from quantitative enzyme-linked immunosorbent assay are given as a normalized mean relative to the corresponding measurements from mock-treated cells (positive control). The results are from three independent transfections, and the bars indicate the normalized SEM.

3.2.2 Assessment of activation of the interferon response by of pri-miRNA-expressing constructs

The presence of duplex RNA within cells may lead to activation of a type 1 interferon (IFN) response which results in programmed cell death. Despite causing gene silencing, this effect is not desirable as a result of the non specific nature of the process. 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 3.6). *IFN- β* messenger RNA was not significantly induced in any of the cell groups that had been transfected with pri-miRNA-encoding 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 as it has been observed that the IFN response is attenuated in this liver-derived line (Li *et al*, 2005). A plasmid expressing eGFP was included in the transfections to control for transfection efficiency. Two days post transfection, cells were visually assessed for the expression of eGFP using a fluorescence microscope. The absence of eGFP indicated a suboptimal transfection whereas the presence of eGFP indicated a successful transfection. All suboptimal transfections were repeated as it was assumed that an absence of eGFP indicated an absence of the other transfected components (pCH-FLuc, poly I:C, shRNA and pri-miRNA-encoding vectors).

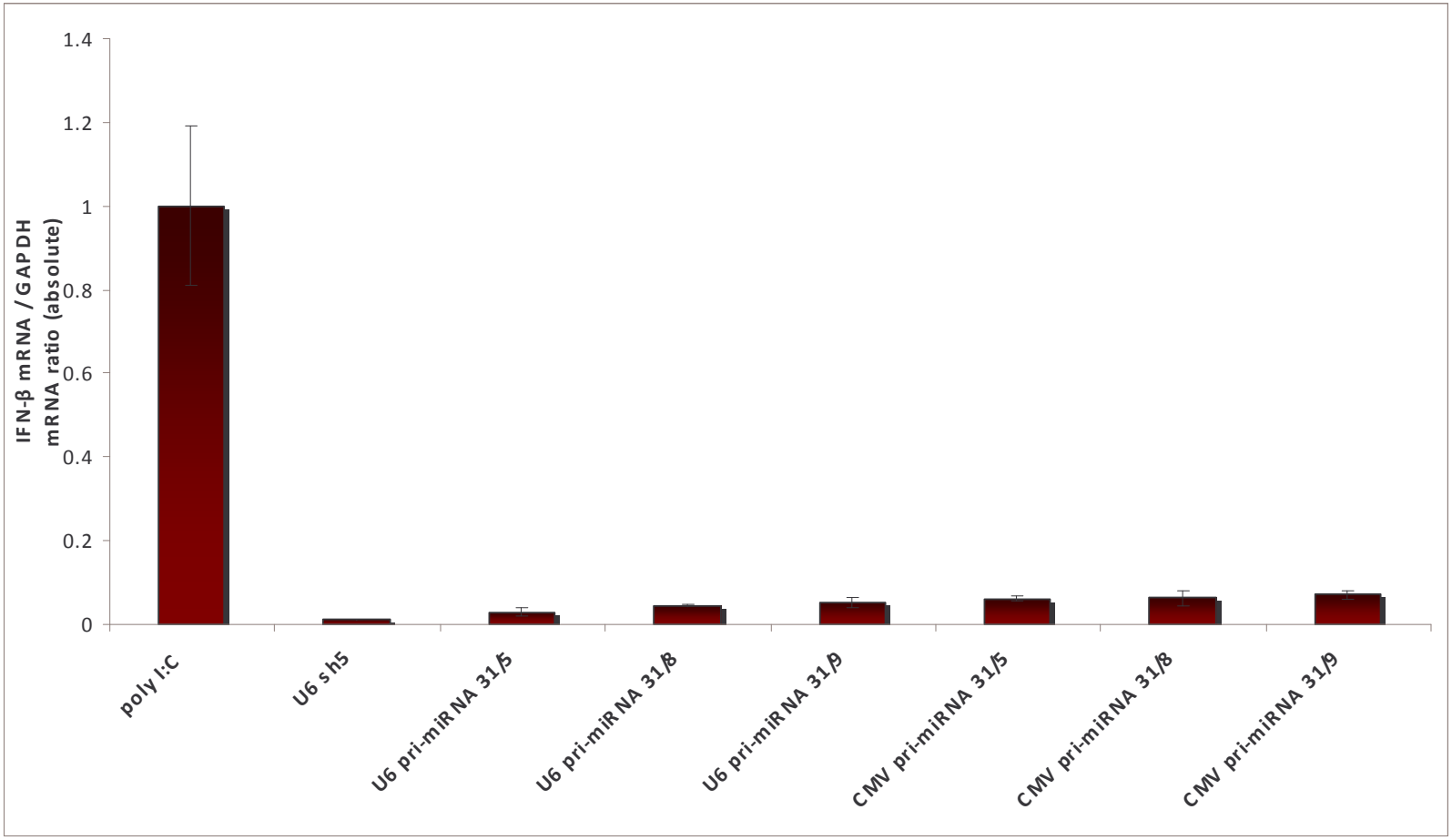

Figure 3.6 Assessment of off-target effects induced by pri-miRNA constructs.
HEK293 cells were transfected with plasmids encoding the indicated miR- or shRNA-encoding cassettes or with poly (I:C). RNA was extracted from the cells 48 hours later and subjected to quantitative real-time PCR to determine concentrations of *IFN-β* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) messenger RNA (mRNA). The ratio of IFN-β mRNA / GAPDH mRNA is plotted as a normalized mean relative to the corresponding measurements of poly (I:C). The results are from three independent transfections, and the bars indicate the normalized SEM.

3.2.3. Evaluation of processed products of pri-miRNA constructs

Northern blot hybridization analysis was carried out to detect processed products of the anti-HBV pri-miRNA expression cassettes. RNA was extracted from transfected cells and Figure 3.7 shows the signals obtained after hybridization to a probe that was complementary to the putative mature processed CMV pri-miRNA-31/5, CMV pri-miRNA-31/8, CMV pri-miRNA-31/9 or U6 shRNA 5 guides. No signal was detected in any of the mock controls which contained only pCH-9/3091. 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 pri-miRNA-31 and pri-miRNA-30 (shRNA 5 contains a miRNA 30 terminal loop) (Zeng, Cullen, 2005). Larger-molecular-weight processed intermediates were detected in RNA extracted from cells transfected with U6 promoter–containing vectors but not from cells expressing the CMV pri-miRNA-31/5, CMV pri-miRNA-31/8 or CMV pri-miRNA-31/9 cassettes. Figure 3.7 shows that U6 pri-miRNA-31/5, U6 pri-miRNA-31/8, U6 pri-miRNA-31/9, CMV pri-miRNA-31/5, CMV pri-miRNA-31/8 and CMV pri-miRNA-31/9 is processed into mature miRNA. This processing is similar albeit on a lower scale when compared to the processing of the corresponding U6 HBV shRNA derived guides (5, 8 or 9). The U6 HBV shRNAs were included as a positive control of known high level expression. It should be noted that the mature pri-miRNA-31 sequences were detected at up to 85-fold lower concentration than the processed U6 HBV shRNAs. The specificity of the guide strands were assessed by lack of signal detected in the mock controls.

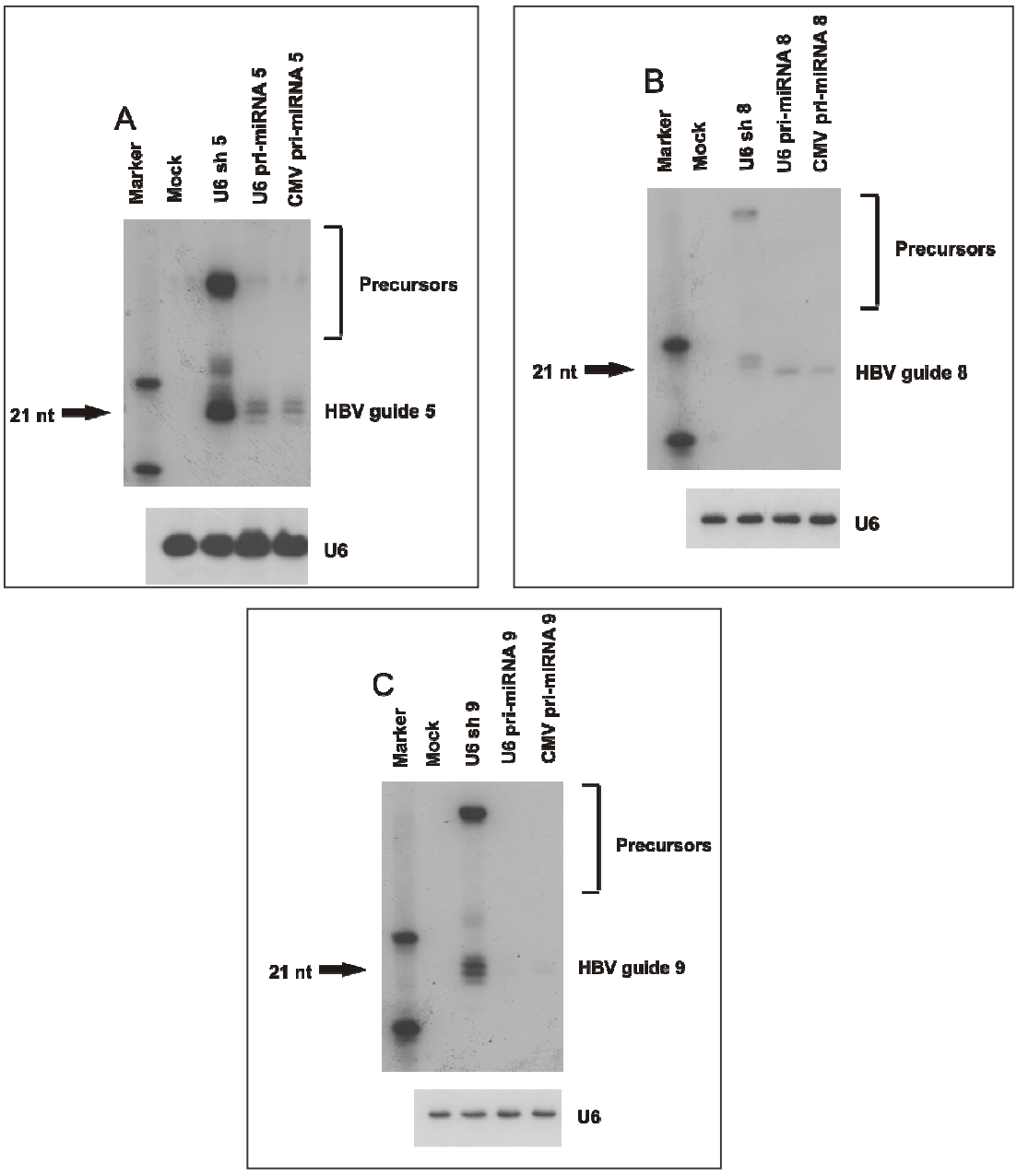

Figure 3.7 Evaluation of cleavage products of pri-miRNA constructs.
HEK293 cells were cotransfected with pCH-9/3091 and plasmids encoding the indicated RNAi effecter sequences. The mock control contained only pCH-9/3091 target plasmid. Northern Blot analysis ensuedthen carried out. Hybridization was to a radiolabeled probe complementary to the putative mature anti-HBV guide 5 (A), guide 8 (B) or guide 9 (C) strands. In A, B and C, 18 and 30 nt markers were utilized to assess the size of the processed RNAi effecters. Fragments greater than the expected 21 nt are precursor cleavage products. Blots were stripped and rehybridized to a probe complementary to endogenous U6 shRNA to confirm equal loading of cellular RNA.

3.3 Testing the anti HBV efficacy of pri-miRNA sequences in vivo using the hydrodynamic injection model of HBV replication.

The concentrations of HBsAg were measured in the sera of mice that had been subjected to hydrodynamic tail-vein injection (HDI) (Yang *et al*, 2002). Mice were co-injected with pCH-9/3091 replication competent plasmid together with a selection of vectors encoding CMV pri-miRNA-31 shuttles. Five groups of mice were utilized and each group comprised 5 mice. The results (Figure 3.8) show the means and are normalized with respect to the mock control (containing pCH-9/3091 and CMV containing vector). Each of the U6 shRNA 5, CMV pri-miRNA-31/5, CMV pri-miRNA-31/8 and CMV pri-miRNA-31/9 containing plasmids demonstrated a reduction in the serum viral antigen concentration (Figure 3.8). This was observed when measurements were taken at day 3 post HDI. Compared to the mock control, the RNAi effecters resulted in the following reduction of HBsAg: 97% by U6 shRNA 5, 94% by CMV pri-miRNA-31/5, 96% by CMV pri-miRNA-31/8 and 74 % by CMV pri-miRNA-31/9 (Figure 3.8). With the exception of CMV pri-miRNA-31/9, both CMV pri-miRNA-31/5 and CMV pri-miRNA-31/8 demonstrated efficacy which is comparable with those of the U6 shRNA 5 sequences.

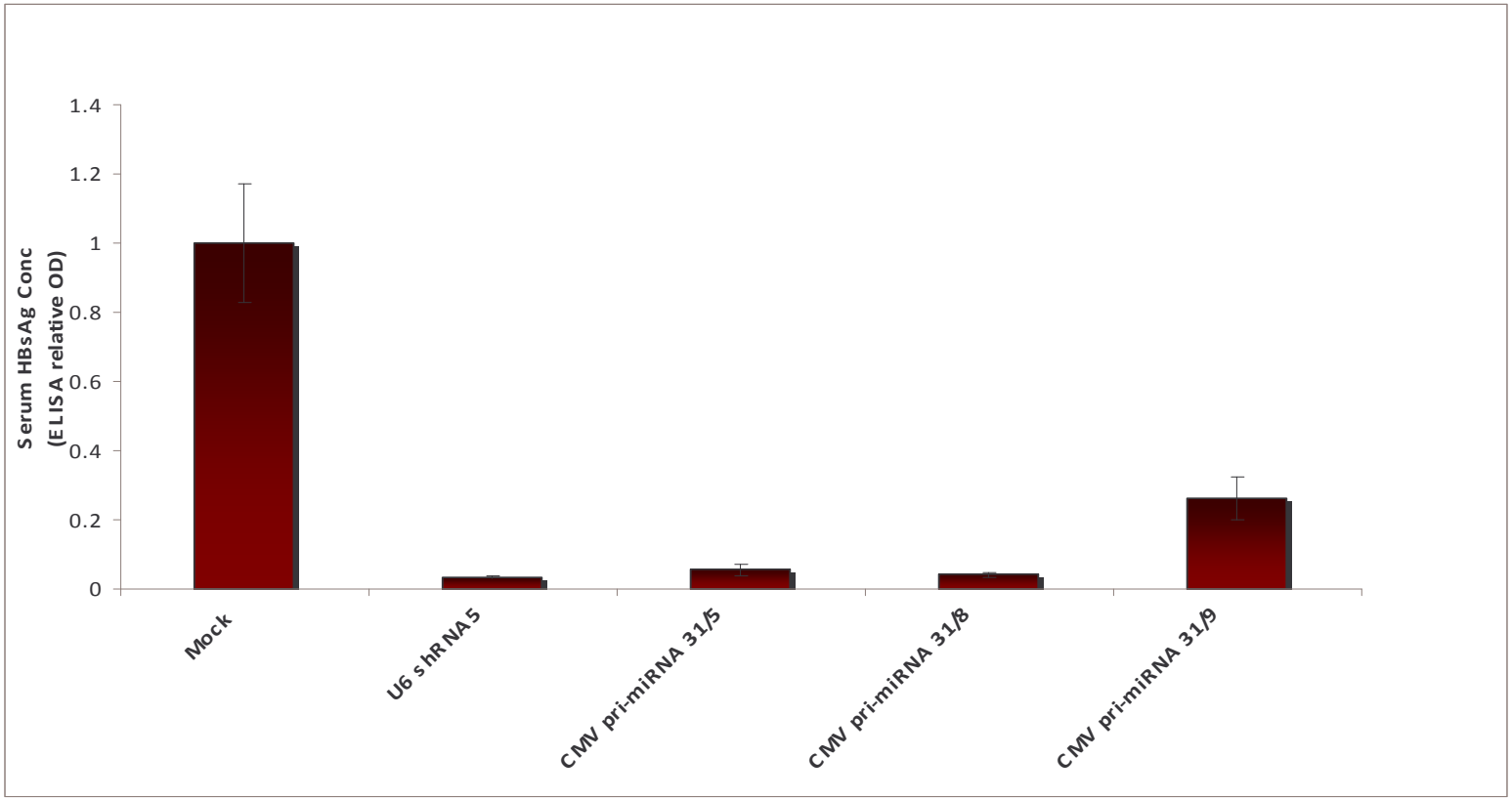

Figure 3.8 Measurement of HBsAg secretion to evaluate knockdown efficacy of pri-miRNA constructs *in vivo*.
Serum concentration of HBsAg was measured at Days 3 after hydrodynamic injection of mice with pCH-9/3091 and plasmids expressing the indicated miR- or shRNA-encoding sequences. Mock injections included control backbone plasmids containing CMV promoters that did not express anti-HBV RNAi effecters. HBsAg measurements from quantitative ELISA are given as a normalized mean relative to the corresponding measurements from mock-treated cells (positive control). The results are from five independent injections, and the bars indicate the normalized SEM.

3.4 Polycistronic pri-miRNAi effecter constructs

3.4.1 Design of Polycistronic pri-miRNA expression cassettes.

Polycistronic cassettes were designed such that pre-miRNAs comprised 59 nt and were flanked by 51 nt of natural pri-miR-31-derived sequences. According to this scheme, the mature anti-HBV pri-miRNAs were predicted, using the MFold algorithm (Zuker, 2003), to have a similar structure to that of naturally occurring pri-miR-31. To assess the modular nature of the cassettes, six different polycistronic expression cassettes were generated using all possible ordering combinations of the three pri-miRNA-31 shuttles. Computer-based predictions indicated that the intended miR-31-like structures of the polycistronic cassettes were energetically most favourable and similar for each of the six different combinations. These predicted structures resembled natural pri-miRNA and augur well for favourable functioning of the designed pri-miRNA. The calculated ΔG value of each combination was approximately -195kcal/mol (Figures 3.9 – 3.14).

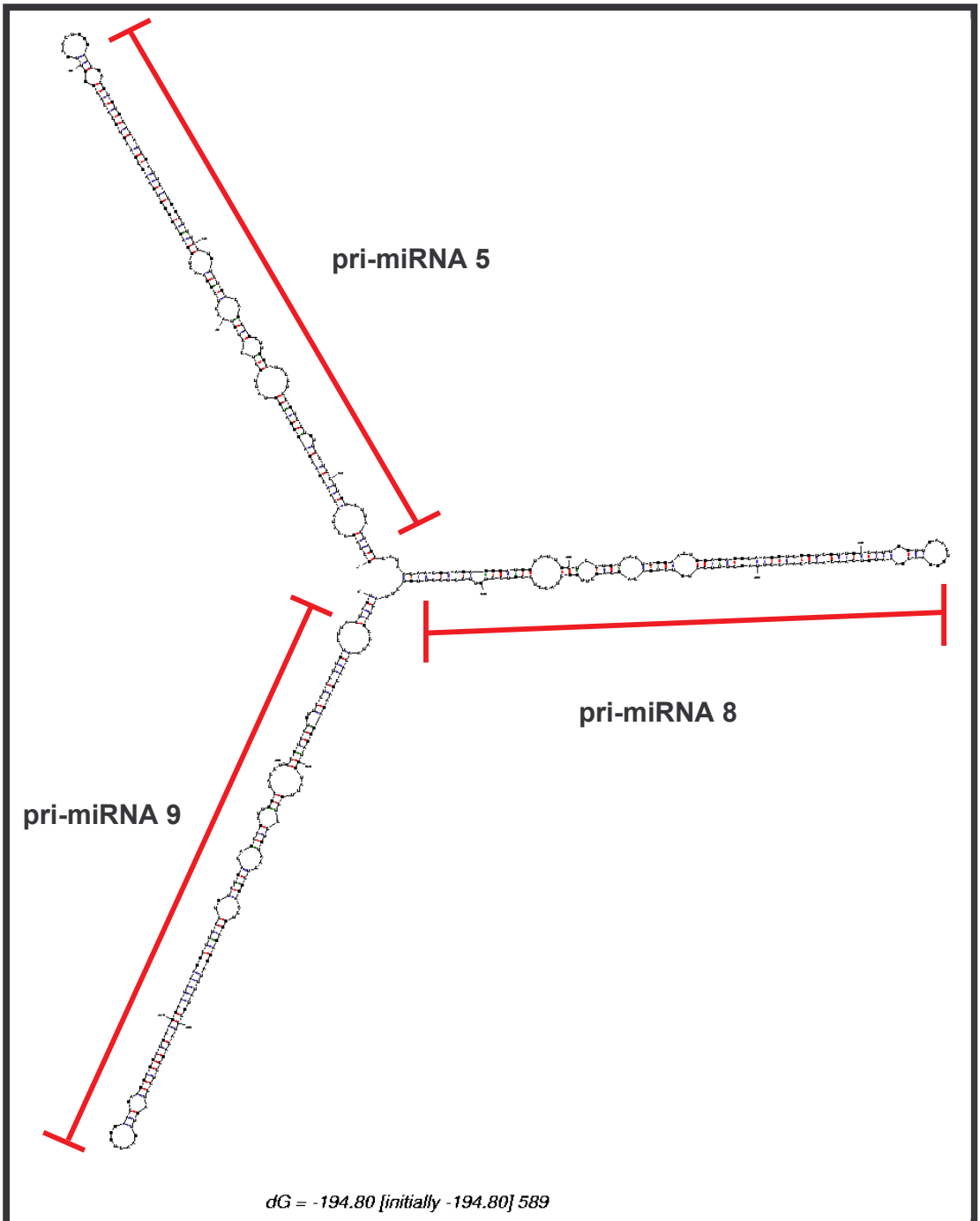

Figure 3.9 MFOLD prediction of polycistronic pri-miRNA 5-8-9.

Polycistronic pri-miRNA 5-8-9 contains pri-miRNA 5, pri-miRNA 8 and pri-miRNA 9 in series. The MFold algorithm predicts that the structures of the individual pri-miRNA are maintained when the polycistronic pri-miRNA 5-8-9 structure is generated. The above structure is the most energetically favourable prediction with a ΔG value of -194.80 kcal/mol

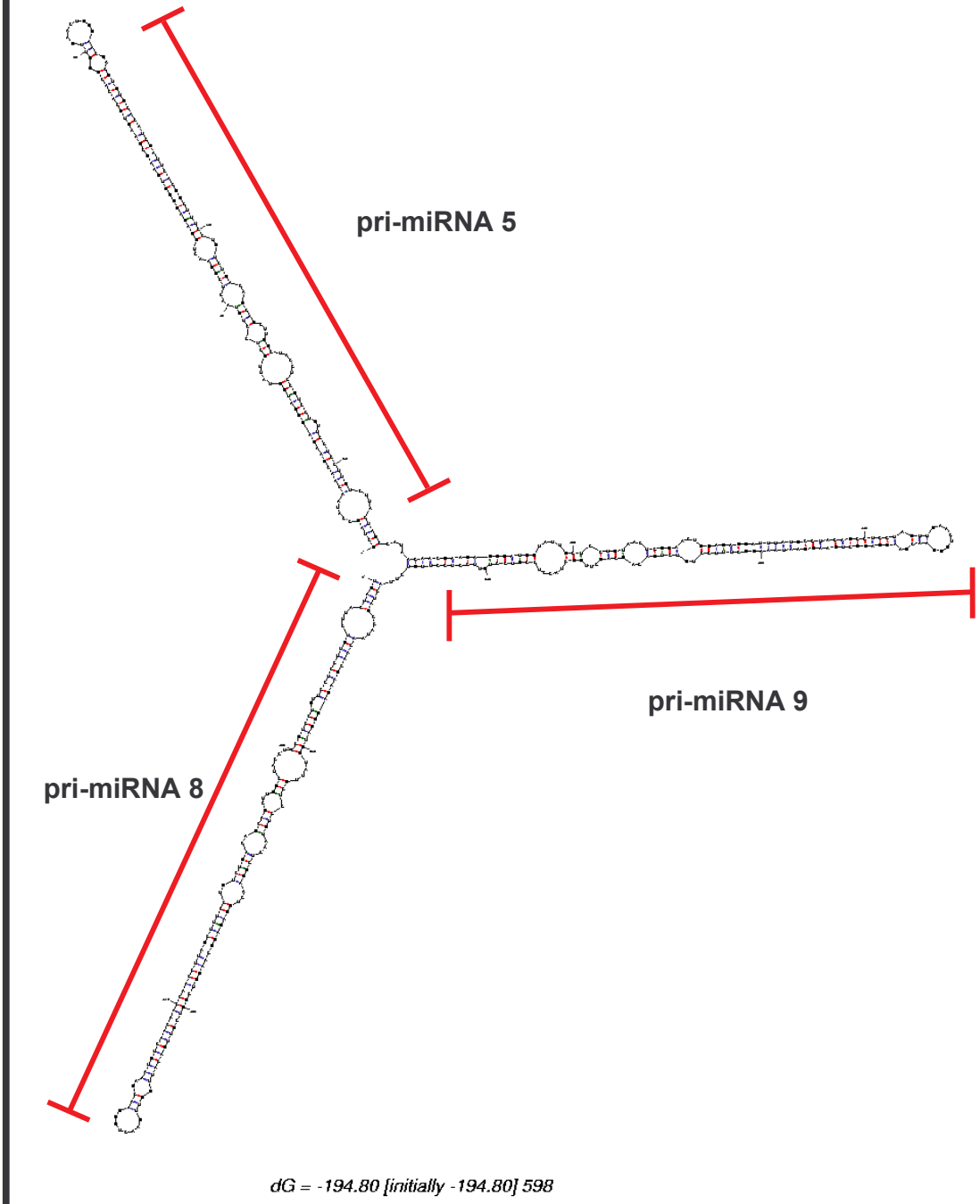

Figure 3.10 MFOLD prediction of polycistronic pri-miRNA 5-9-8.

Polycistronic pri-miRNA 5-9-8 contains pri-miRNA 5, pri-miRNA 9 and pri-miRNA 8 in series. The MFold algorithm predicts that the structures of the individual pri-miRNA are maintained when the polycistronic pri-miRNA 5-9-8 structure is generated. The above structure is the most energetically favourable prediction with a ΔG value of -194.80 kcal/mol

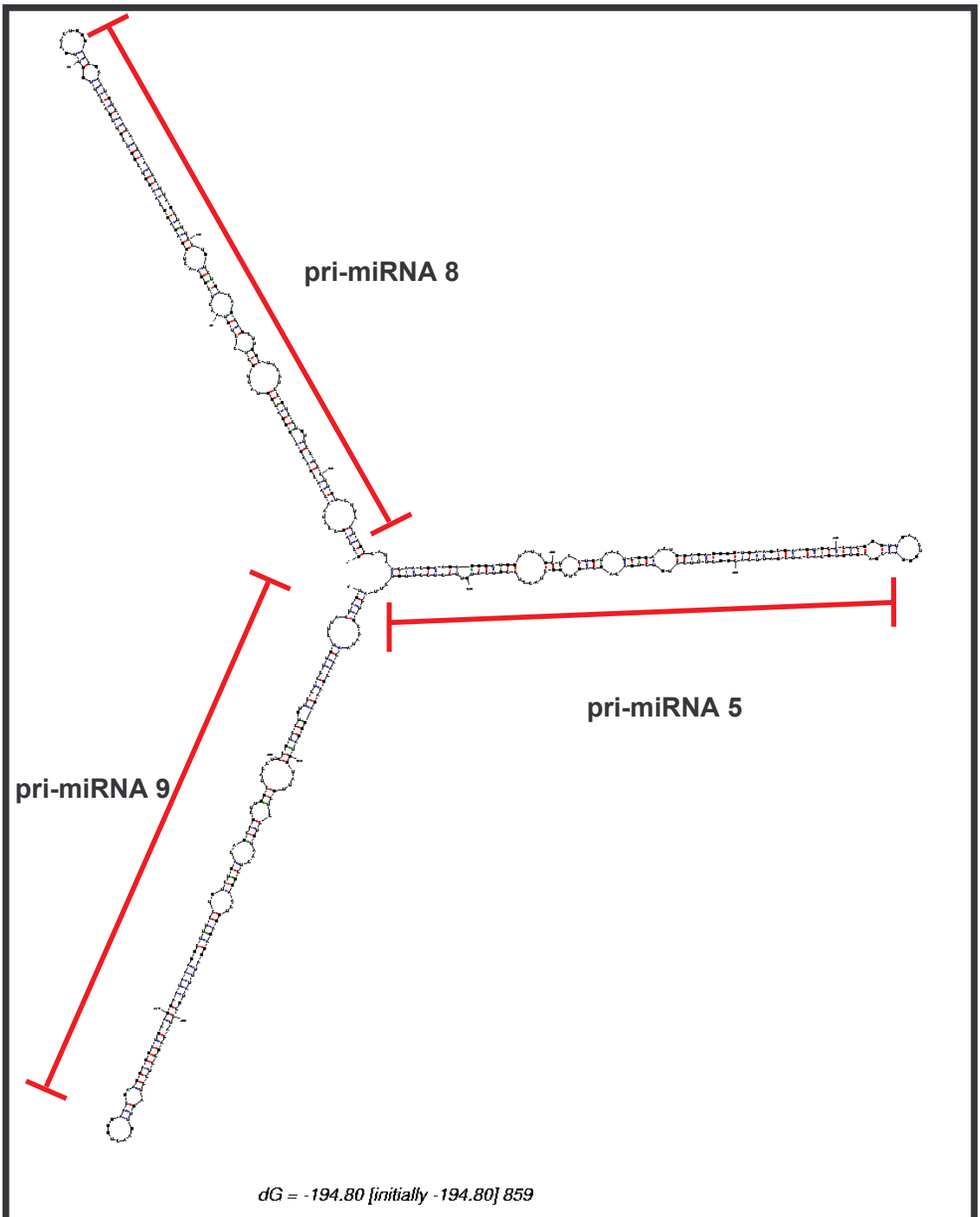

Figure 3.11 MFOLD prediction of polycistronic pri-miRNA 8-5-9.

Polycistronic pri-miRNA 8-5-9 contains pri-miRNA 8, pri-miRNA 5 and pri-miRNA 9 in series. The MFold algorithm predicts that the structures of the individual pri-miRNA are maintained when the polycistronic pri-miRNA 8-5-9 structure is generated. The above structure is the most energetically favourable prediction with a ΔG value of -194.80 kcal/mol

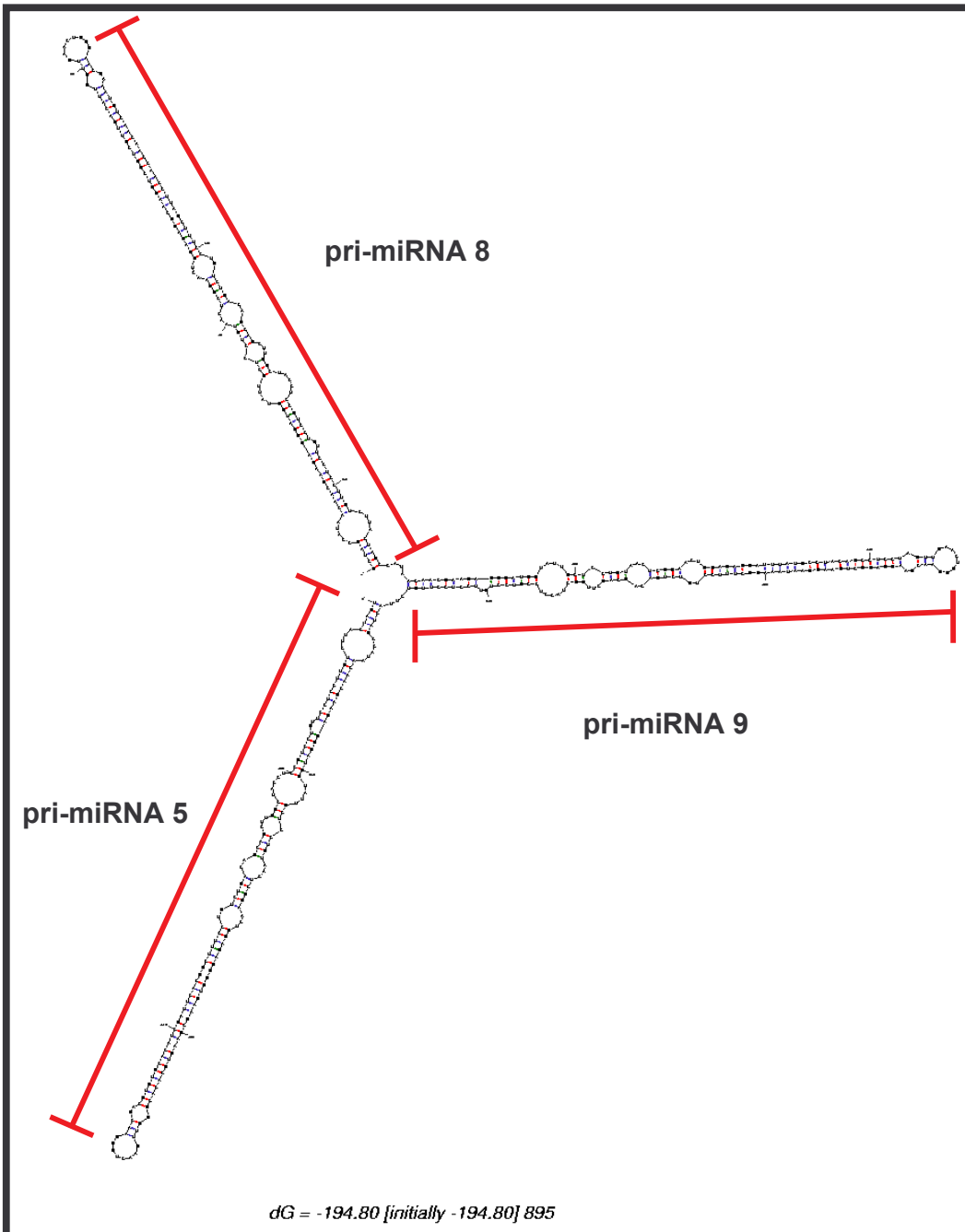

Figure 3.12 MFOLD prediction of polycistronic pri-miRNA 8-9-5.

Polycistronic pri-miRNA 8-9-5 contains pri-miRNA 8, pri-miRNA 9 and pri-miRNA 5 in series. The MFold algorithm predicts that the structures of the individual pri-miRNA are maintained when the polycistronic pri-miRNA 8-9-5 structure is generated. The above structure is the most energetically favourable prediction with a ΔG value of -194.80 kcal/mol

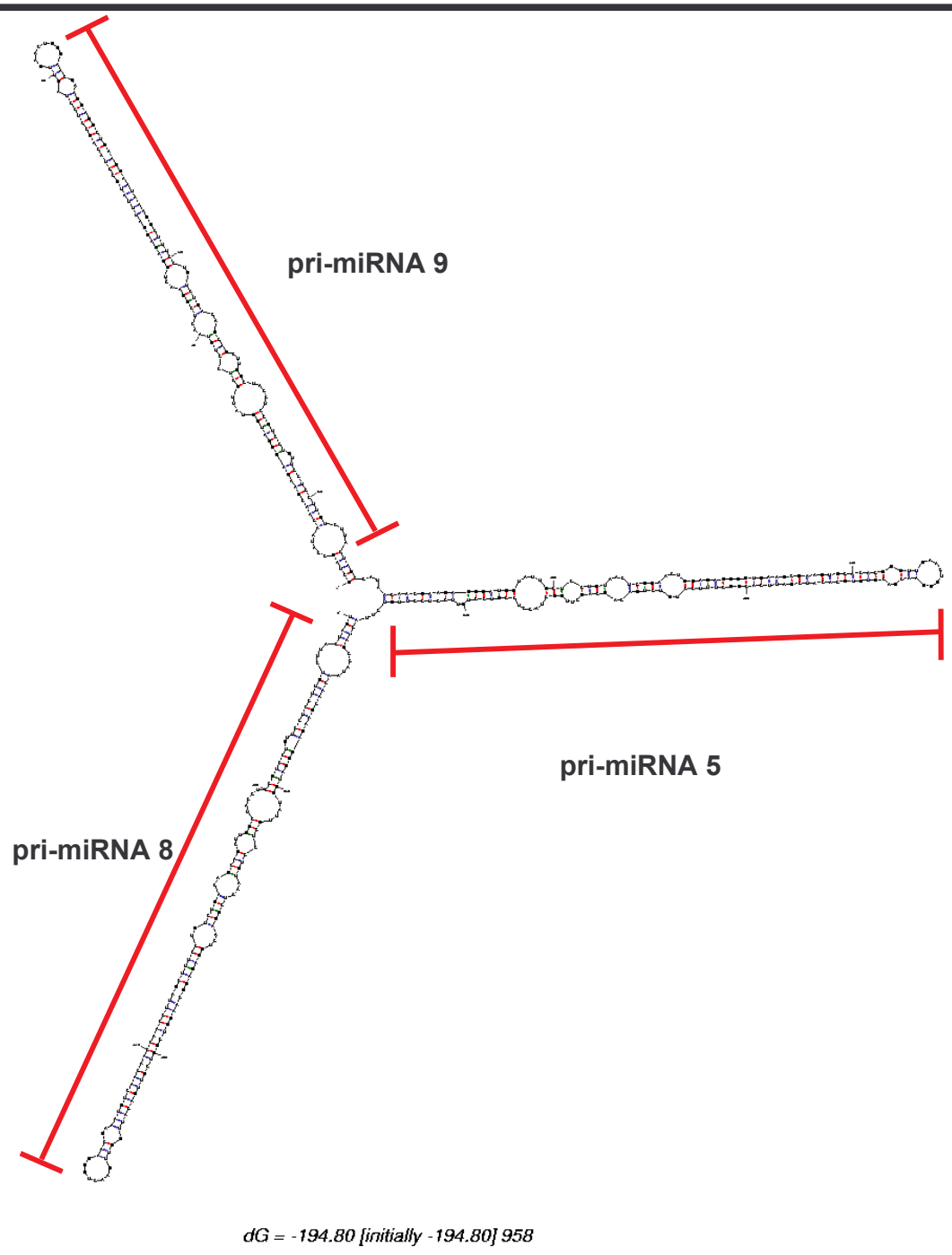

Figure 3.13 MFOLD prediction of polycistronic pri-miRNA 9-5-8.

Polycistronic pri-miRNA 9-5-8 contains pri-miRNA 9, pri-miRNA 5 and pri-miRNA 8 in series. The MFold algorithm predicts that the structures of the individual pri-miRNA are maintained when the polycistronic pri-miRNA 9-5-8 structure is generated. The above structure is the most energetically favourable prediction with a ΔG value of -194.80 kcal/mol

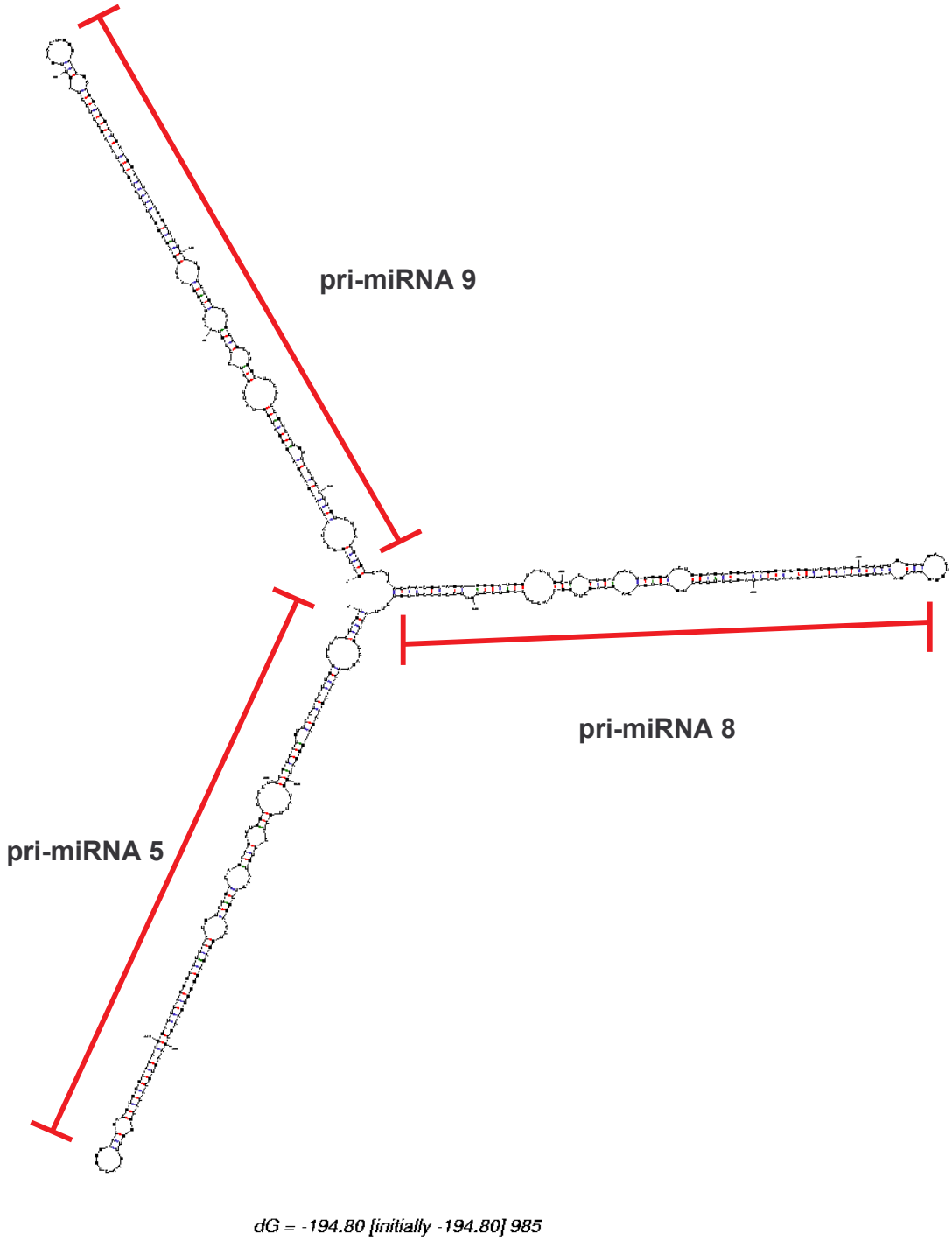

Figure 3.14 MFOLD prediction of polycistronic pri-miRNA 9-8-5.

Polycistronic pri-miRNA 9-8-5 contains pri-miRNA 9, pri-miRNA 8 and pri-miRNA 5 in series. The MFold algorithm predicts that the structures of the individual pri-miRNA are maintained when the polycistronic pri-miRNA 9-8-5 structure is generated. The above structure is the most energetically favourable prediction with a ΔG value of -194.80 kcal/mol.

3.5 Polycistronic CMV pri-miRNA mediated inhibition of HBV gene expression in cell culture models of HBV replication.

3.5.1 Evaluation of HBV knockdown by polycistronic CMV pri-miRNA effecter sequences

3.5.1.1 Luciferase assays

A potential advantage of employing polycistronic cassettes to inhibit viral replication is that viral escape resulting from emergence of evading mutations is limited. As with the individual pri-miRNA constructs, dual luciferase assays were performed to assess the knockdown efficacy of the polycistronic cassettes.

Co-transfection of pCH-FLuc and polycistronic CMV pri-miRNA-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, CMV pri-miRNA 5-8-9, CMV pri-miRNA 5-9-8, CMV pri-miRNA 8-5-9, CMV pri-miRNA 8-9-5, CMV pri-miRNA 9-5-8 and CMV pri-miRNA 9-8-5 containing vectors. Compared to the positive control the RNAi effecters resulted in the following knockdown of pCH-FLuc: 84% by U6 shRNA 5, 94% by CMV pri-miRNA 5-8-9, 96% by CMV pri-miRNA 5-9-8, 95% by CMV pri-miRNA 8-5-9, 79% by CMV pri-miRNA 8-9-5, 95% by CMV pri-miRNA 9-5-8 and 94% by CMV pri-miRNA 9-8-5 (Figure 3.15).

In addition to a luciferase assay using the pCH-FLuc target plasmid, luciferase assays using individual targets (psiCHECK-5T, psiCHECK-8T and psiCHECK-9T) was also performed. The individual target plasmids contain a target sequence complementary to guide 5, 8 or 9, inserted downstream of the Renilla luciferase reporter ORF of psiCHECK2.2. Co-transfection of psiCHECK-5T, psiCHECK-8T or psi-CHECK-9T with polycistronic CMV pri-miRNA-encoding vectors allows for the determination of the specificity of each pri-miRNA monomer within the polycistronic construct to

knockdown its complementary target. The percentage knockdown of individual targets by the polycistronic pri-miRNA constructs can be seen in Table 3.1 and Figure 3.16 depicts the reduction of Renilla luciferase relative to firefly luciferase.

Table 3.1 Percentage knockdown of individual targets by polycistronic CMV pri-miRNA

CMV pri-miRNA	psiCHECK-5T	psiCHECK-8T	psiCHECK-9T
CMV pri-miRNA 5-8-9 Normalize SEM	88% 0.005	93% 0.001	52% 0.01
CMV pri-miRNA 5-9-8 Normalize SEM	90% 0.005	0% 0.004	80% 0.005
CMV pri-miRNA 8-5-9 Normalize SEM	86% 0.001	93% 0.001	46% 0.01
CMV pri-miRNA 8-9-5 Normalize SEM	85% 0.002	93% 0.002	51% 0.01
CMV pri-miRNA 9-5-8 Normalize SEM	88% 0.001	93% 0.001	54% 0.02
CMV pri-miRNA 9-8-5 Normalize SEM	91% 0.001	0% 0.01	80% 0.01

The percentage knockdown is calculated as a normalized mean relative to a positive control.

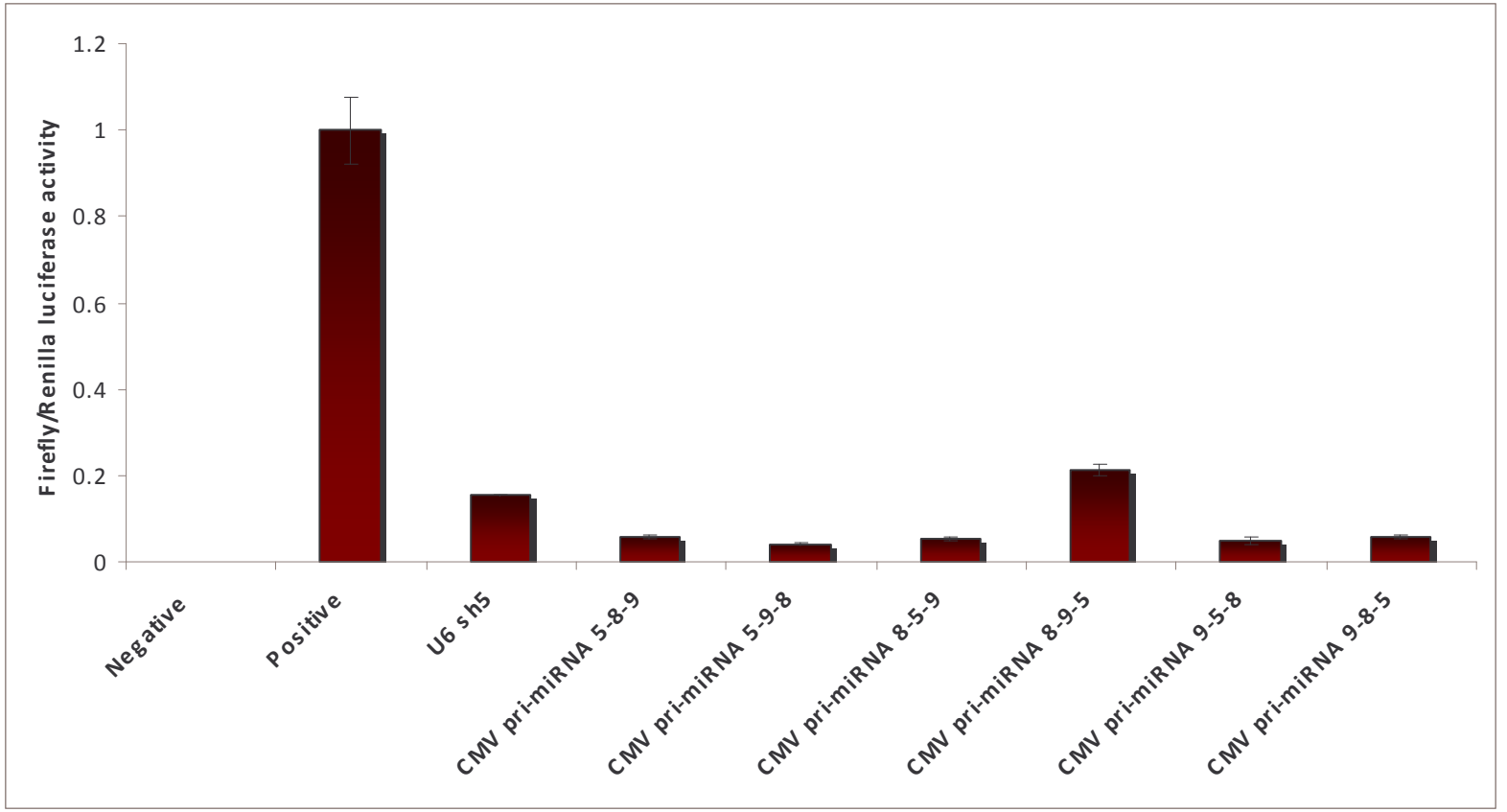

Figure 3.15 Dual Luciferase Assay to evaluate knockdown efficacy of polycistronic pri-miRNA constructs.
The positive control contained the target plasmid, pCH-FLuc. The negative control did not contain target plasmid or polycistronic pri-miRNA effecter constructs. The U6 sh5 construct was used as a standard to compare the knockdown efficacy of the CMV polycistronic pri-miRNA constructs.
Huh 7 cells were cotransfected with plasmids containing the miR- or shRNA-encoding cassettes, pCH-FLuc and a plasmid constitutively expressing Renilla luciferase. Results are given as ratios of Firefly to Renilla luciferase activity. The plotted data is given as a normalized mean relative to the corresponding measurements from mock-treated cells (positive control). The results are from three independent transfections, and the bars indicate the normalized SEM.

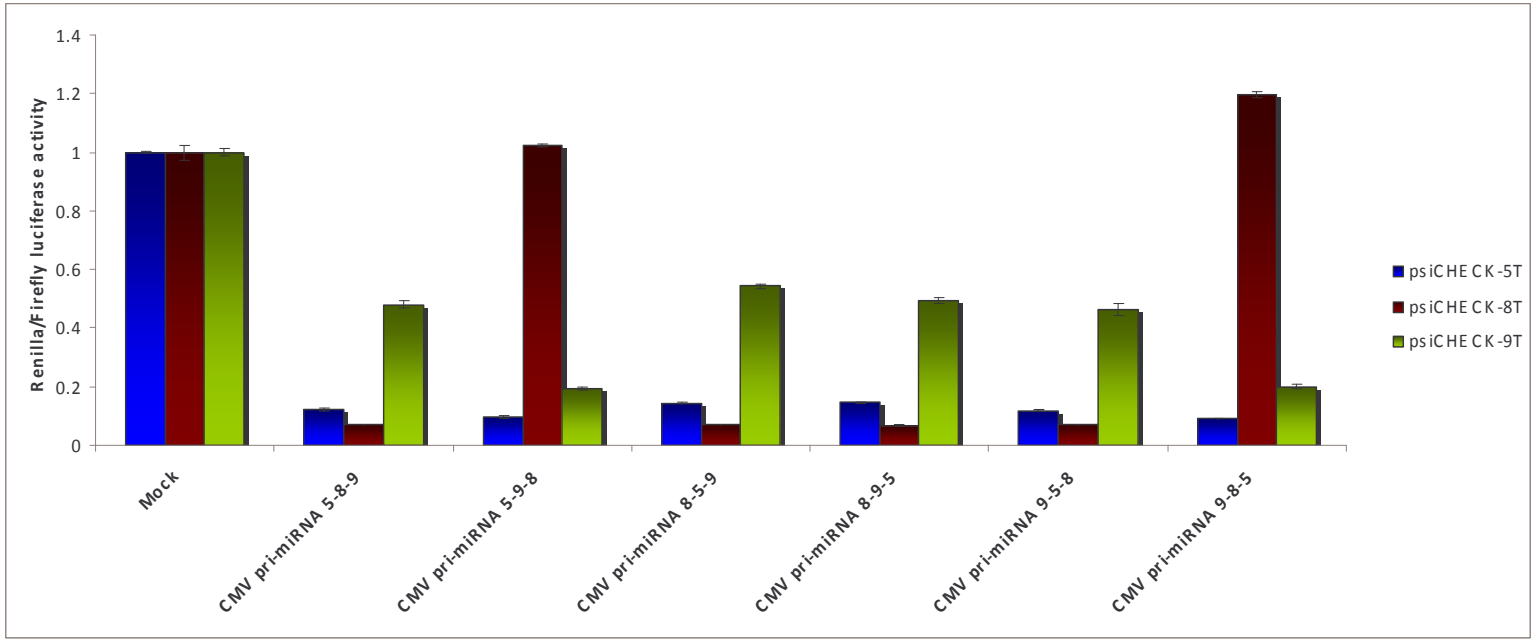

Figure 3.16 Dual Luciferase Assay to evaluate knockdown efficacy of individual targets by polycistronic pri-miRNA constructs.
The mock control contained the target plasmid, psi-CHECK2.2. Huh 7 cells were cotransfected with plasmids containing the indicated polycistronic pri-miRNA expression cassettes and either psiCHECK-5T, psiCHECK-8T or psiCHECK-9T. Results are represented as ratios of Renilla to Firefly luciferase activity. The plotted data is given as a normalized mean relative to the corresponding measurements from mock-treated cells (positive control). The results are from three independent transfections, and the bars indicate the normalized SEM.

3.5.1.2 HBsAg

The data obtained from the Luciferase knockdown assays was corroborated with the ELISA for HBsAg. Huh7 cells were co-transfected with polycistronic CMV pri-miRNA-encoding vectors together with the pCH-9/3091 HBV replication competent target plasmid. Controls included a positive which only contained pCH-9/3091 target, a negative which did not contain pCH-9/3091 target or polycistronic CMV pri-miRNA-encoding vectors and U6 shRNA 5.

Analysis showed that viral antigen secretion was diminished significantly by U6 shRNA 5, CMV pri-miRNA 5-8-9, CMV pri-miRNA 5-9-8, CMV pri-miRNA 8-5-9, CMV pri-miRNA 8-9-5, CMV pri-miRNA 9-5-8 and CMV pri-miRNA 9-8-5 containing vectors. Compared to the positive control the RNAi effecters resulted in the following reduction of HBsAg: 90% by U6 shRNA 5, 91% by CMV pri-miRNA 5-8-9, 96% by CMV pri-miRNA 5-9-8, 91% by CMV pri-miRNA 8-5-9, 91% by CMV pri-miRNA 8-9-5, 90% by CMV pri-miRNA 9-5-8 and 88% by CMV pri-miRNA 9-8-5 (Figure 3.17). The data obtained further validated the Luciferase knockdown results.

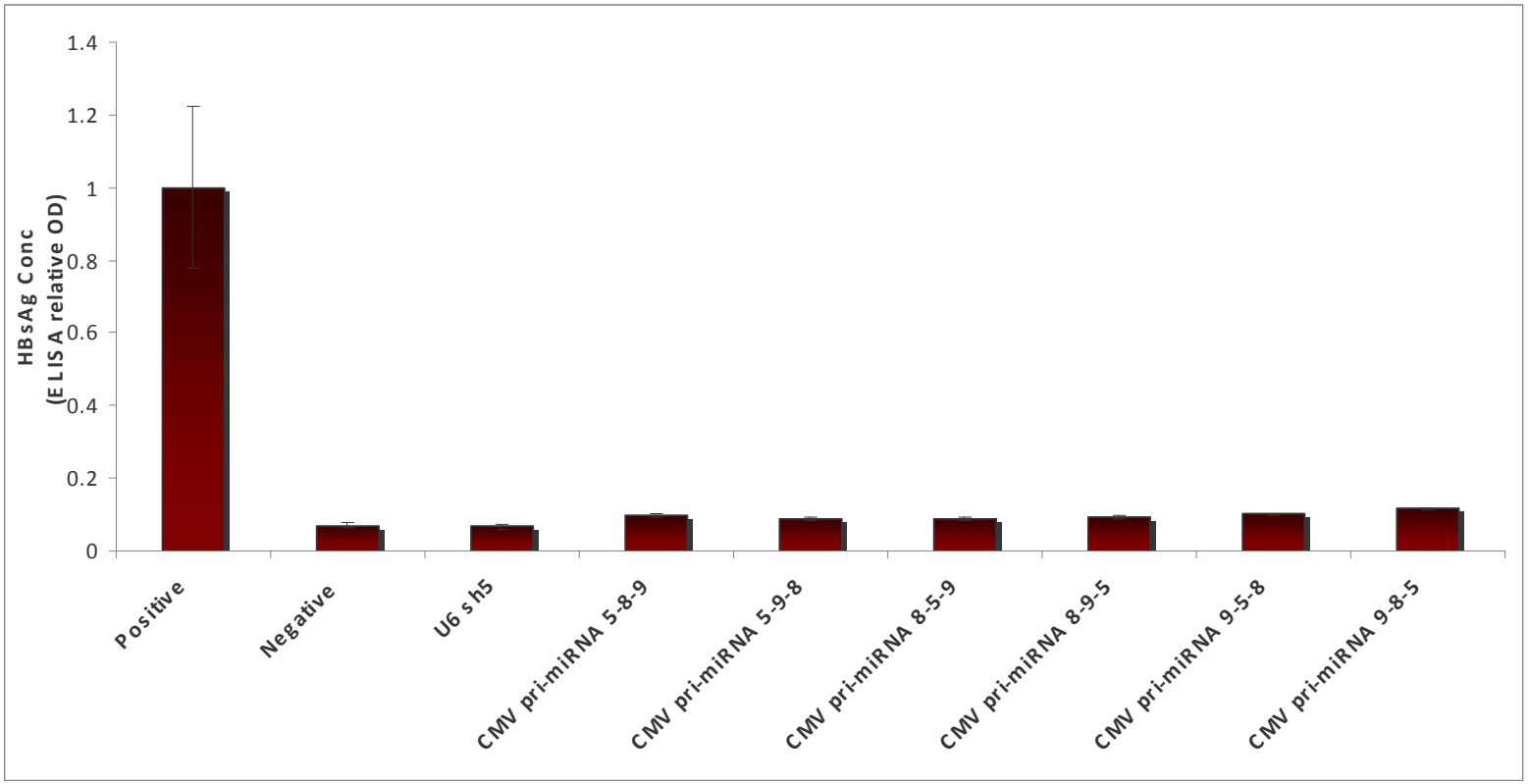

Figure 3.17 HBsAg serum secretion to evaluate knockdown efficacy of polycistronic CMV pri-miRNA constructs.
Huh7 cells were co-transfected with plasmids encoding the indicated miR- or shRNA-encoding cassettes, together with the HBV target plasmid, pCH-9/3091. HBsAg measurements from quantitative enzyme-linked immunosorbent assay are given as a normalized mean relative to the corresponding measurements from mock-treated cells (positive control). The results are from three independent transfections, and the bars indicate the normalized SEM.

3.5.2 Assessment of specificity of polycistronic CMV pri-miRNA effecter sequences

Section 3.2.2 showed that the generated pri-miRNA effecter monomers are specific and do not induce an interferon related response. The arrangement of these pri-miRNA effecter monomers in series results in a larger construct and may activate the interferon response. The disruption of the endogenous miRNA pathway and stimulation of the innate IFN response by polycistronic CMV pri-miRNA cassettes were assessed. 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 3.18). *IFN- β* messenger RNA was not significantly induced in any of the cell groups that had been transfected with polycistronic CMV pri-miRNA cassettes, while treatment with poly (I: C) (positive control) resulted in activation of *IFN- β* expression. A plasmid expressing eGFP was included in the transfections to control for transfection efficiency. Two days post transfection, cells were visually assessed for the expression of eGFP using a fluorescence microscope. The absence of eGFP indicated a suboptimal transfection whereas the presence of eGFP indicated a successful transfection. All suboptimal transfections were repeated as it was assumed that an absence of eGFP indicated an absence of the other transfected components (pCH-FLuc, poly I:C, shRNA and polycistronic CMV pri-miRNA cassettes).

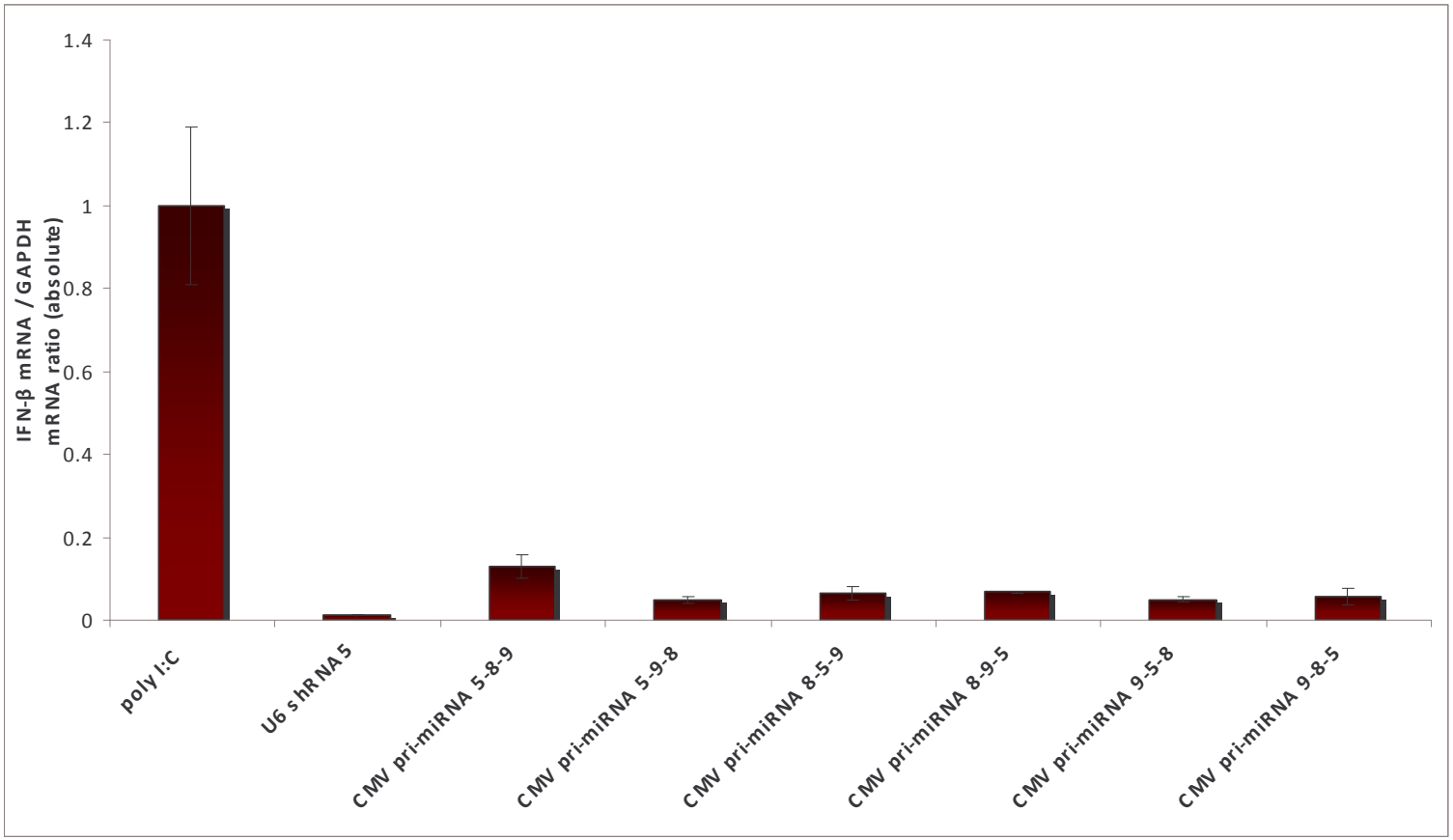

Figure 3.18 Assessment of off-target effects induced by polycistronic CMV pri-miRNA constructs.
HEK293 cells were transfected with plasmids encoding the miR- or shRNA-encoding cassettes or with poly (I:C). RNA was extracted from the cells 48 hours later and subjected to quantitative real-time PCR to determine concentrations of *IFN-β* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) messenger RNA (mRNA). The ratio of IFN-β mRNA / GAPDH mRNA is plotted as a normalized mean relative to the corresponding measurements of poly (I:C). The results are from three independent transfections, and the bars indicate the normalized SEM.

3.5.3 Evaluation of processed products of polycistronic CMV pri-miRNAi effecter sequences

The generated polycistronic CMV pri-miRNA constructs contain three pri-miRNA monomers in series. The design of these constructs is such that Drosha cleavage will separate each of the pri-miRNA and result in its entrance into the RNAi pathway. Northern blot hybridization analysis was carried out to detect processed products of the anti-HBV polycistronic CMV pri-miRNA expression cassettes. RNA was extracted from HEK293 cells transfected with DNA-expressing polycistronic CMV pri-miRNA constructs (Figure 3.19). All six polycistronic CMV pri-miRNA constructs were hybridized with probes complementary to guide strand 5, 8 and 9. Hybridization to a probe complementary to guide strand 5 showed heterogenous processing to form guide sequences of 20–22 nt in length (Figure 3.19, panel A). No signal was detected in any of the mock controls which contained only pCH-9/3091. The mature miRNA produced from the individual pri-miRNA 5 (Figure 3.7, panel A) and polycistronic CMV pri-miRNA cassettes (Figure 3.19, panel A) was of a similar size and was not affected by the position of the pri-miRNA 5 monomer within the anti-HBV polycistron. Precursor processed product are evident in the U6 shRNA 5-expressing plasmid and the CMV pri-miRNA constructs when hybridized to a probe complementary to guide 5. It should be noted that guide band intensities were considerably higher in cells transfected with U6 shRNA 5-expressing plasmid when compared to cells transfected with the polycistronic CMV pri-miRNA constructs. Northern blot analysis to detect guide 8 revealed a single band of 21 nt (Figure 3.19, panel B), which was distinct in appearance when compared to the mature miRNA generated in Figure 3.19, panel A. No mature pri-miRNA 8 was detectable in RNA extracted from cells transfected with CMV pri-miRNA 9-8-5 and CMV pri-miRNA 5-9-8. Precursor processed product are not present in any of the CMV pri-miRNA constructs when hybridized to a probe complementary to guide 8. The guide produced from the U6 shRNA 8 cassette was slightly larger than that of the polycistronic CMV pri-miRNA constructs. Northern Blot analysis to detect guide 9 revealed very faint

bands of ~ 21 nt (Figure 3.19, panel C). Precursor processed product are not present in any of the CMV pri-miRNA constructs when hybridized to a probe complementary to guide 9.

The U6 HBV shRNAs were included as a positive control of known high level expression. The specificity of the guide strands were assessed by lack of signal detected in the mock controls. The presence of precursor processed product suggests inefficient processing whereas the absence of precursor processed product is indicative of efficient processing and suggests that smaller doses are required to obtain the desired effect.

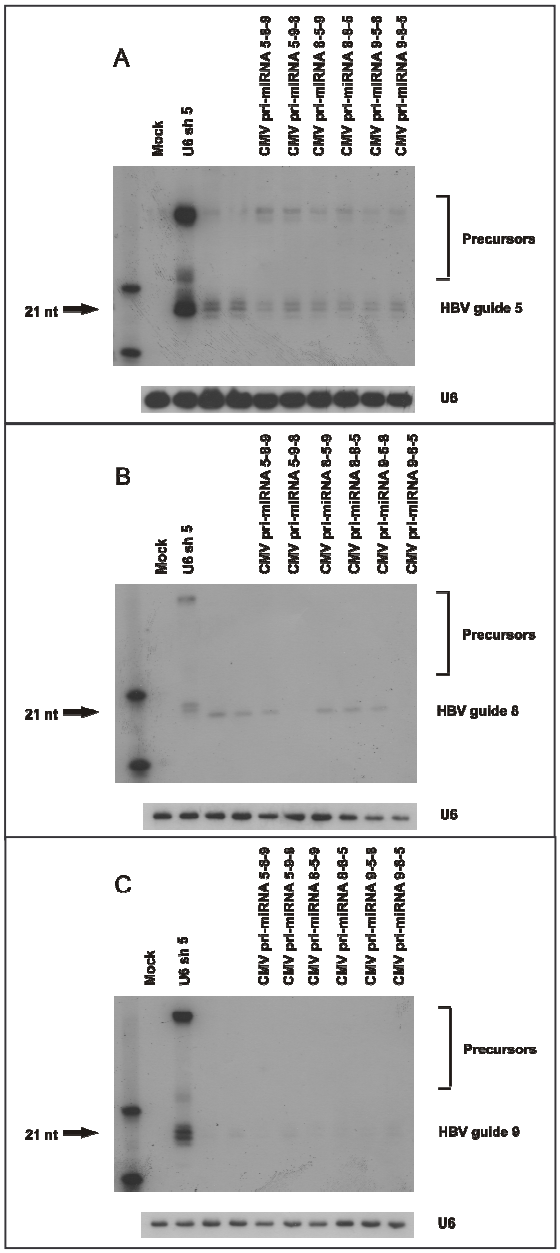

Figure 3.19 Evaluation of cleavage products of polycistronic CMV pri-miRNA constructs.
HEK293 cells were cotransfected with pCH-9/3091 and plasmids encoding the miR- or shRNA-encoding sequences. The mock control contained only pCH-9/3091 target plasmid. Northern Blot analysis was performed. Hybridization was to a radiolabeled probe complementary to the putative mature anti-HBV guide 5 (A), guide 8 (B) or guide 9 (C) strands. In A, B and C, 18 and 30 nt markers were utilized to assess the size of the processed RNAi effecters. Fragments greater than the expected 21 nt are precursor cleavage products. Blots were stripped and rehybridized to a probe complementary to endogenous U6 shRNA in order to confirm equal loading of cellular RNA.

3.6 Testing of anti HBV efficacy of polycistronic CMV pri-miRNA constructs in vivo using the hydrodynamic injection model of HBV replication.

To determine silencing of target genes *in vivo* in a model that simulates HBV replication, mice were co-injected with a pCH-9/3091 together with a selection of vectors encoding polycistronic CMV pri-miRNA constructs using the hydrodynamic procedure. Five groups of mice were utilized and each group comprised of 5 mice. The results (Figure 3.20) are thus averaged and normalized with respect to the mock control (containing pCH-9/3091 and CMV containing vector). 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 3.20). On Day 3 post HDI, compared to the mock control, the RNAi effecters resulted in the following reduction of HBsAg: 97% by U6 shRNA 5, 94% by CMV pri-miRNA-31/5, 92% by CMV pri-miRNA 5-8-9 and 95 % by CMV pri-miRNA 8-5-9 (Figure 3.20). On Day 5 post HDI, compared to the mock control, the RNAi effecters resulted in the following reduction of HBsAg: 86% by U6 shRNA 5, 94% by CMV pri-miRNA-31/5, 95% by CMV pri-miRNA 5-8-9 and 92 % by CMV pri-miRNA 8-5-9 (Figure 3.20).

These findings confirm that the polycistronic CMV pri-miRNA constructs are capable of silencing HBV replication and verify that they are active against transcripts that are produced during viral replication *in vivo*.

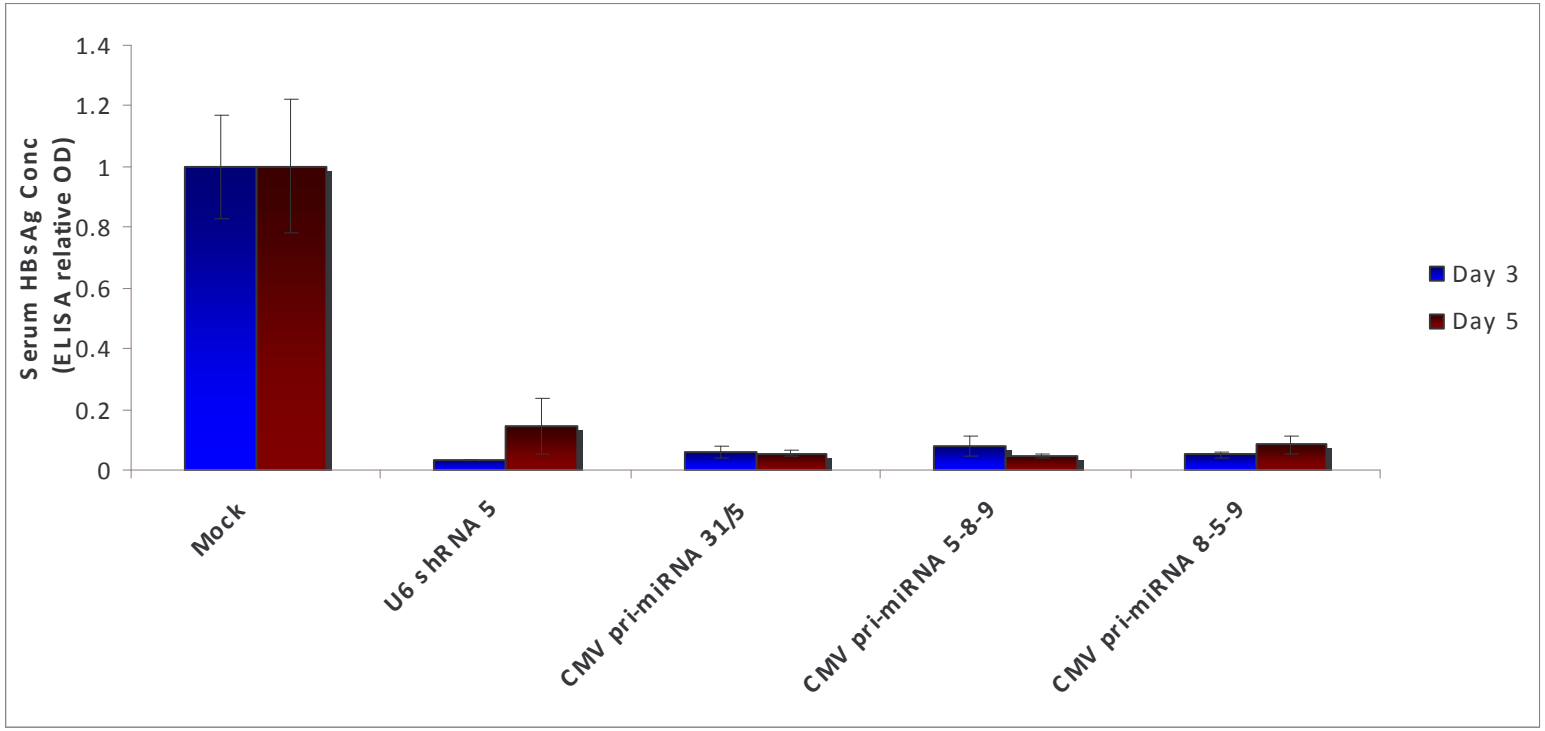

Figure 3.20 HBsAg secretion to evaluate knockdown efficacy of polycistronic CMV pri-miRNA constructs *in vivo*.
Serum concentration of HBsAg was measured at Days 3 and 5 after hydrodynamic injection of mice with pCH-9/3091 and plasmids expressing the indicated miR- or shRNA-encoding sequences. Mock injections included control backbone plasmids containing CMV promoters that did not express anti-HBV RNAi effecters. HBsAg measurements from quantitative enzyme-linked immunosorbent assay are given as a normalized mean relative to the corresponding measurements from mock-treated mice (positive control). The results are from five independent injections, and the bars indicate the normalized SEM.

CHAPTER FOUR - DISCUSSION

The powerful gene silencing that can be achieved by harnessing RNAi has facilitated the development of new approaches to inhibition of pathology-causing genes and the study of gene function. 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 (Scherer, Rossi, 2003).

The main aim of the study presented here was to design, generate and test the efficacy of Pol II expressed polycistronic anti-HBV cassettes. This aim was achieved and all combinations of the Pol II expressed polycistronic cassettes demonstrate potent silencing of HBV targets.

4.1 Design of pri-miRNA sequences targeted against the HBx ORF

The pri-miRNA's generated in this study utilize the guide sequence of U6 shRNA 5, U6 shRNA 8 and U6 shRNA 9 designed by Mr Abdullah Ely (Carmona *et al*, 2006). These U6 shRNA displayed significant knockdown of HBV (*in vitro* and *in vivo*) and it was concluded that the target regions of these shRNA are very sensitive to RNAi (Carmona *et al*, 2006). For this reason the guide sequences of the mentioned shRNA were incorporated in the design of the pri-miRNA for this study. With significant progress being made in understanding the mechanisms of pri-miRNA processing, the design parameters outlined by Zeng, Cullen, (2005) formed the basis for the design of anti-HBV pri-miRNA expression cassettes.

The generated pri-miRNAs were based on the sequence of natural miRNA-31. Zeng, Cullen (2005) have shown that this terminal loop as well as miRNA-30 and miRNA-223 terminal loop serve as good Drosha substrates. Long single-stranded sequences of 51 nucleotides flanked the 5'and 3' termini of the generated structures. It is important to note that the single-stranded flanking sequences contain minimal secondary structure. Using the mentioned parameters, three pri-miRNA contructs were generated: pri-miRNA-31/5, pri-miRNA-31/8 and pri-miRNA-31/9. The designed pri-miRNAs maintain the structure of wild-type pri-miRNA-31. Each generated pri-miRNA was cloned downstream of a Pol III promoter (U6 promoter) and downstream of a Pol II promoter (CMV promoter).

Although other studies have reported silencing by pre-miRNA or shRNAs expressed from Pol II promoter elements, (McCaffrey *et al*, 2003; Giladi *et al*, 2003; Zhao *et al*, 2006) these cassettes are ofhave variable and sometimes poor silencing efficiency. The reasons for this could be that these RNAi effecter sequences are recognized as synthetic and not belonging to the natural RNAi pathway. Or perhaps flanking single-stranded RNA sequences are of importance in the nuclear processing of pri-miRNA, (Zeng, Cullen,2005) in which case it is likely that pre-miRNA and shRNA expression from Pol II promoters will not be as efficient as pri-miRNA mimics. The pri-miRNAs designed in this project are predicted to be recognized as natural and as a consequence of become incorporated into the endogenous RNAi pathway.

4.2 pri-miRNA mediated inhibition of HBV gene expression in cell culture models of HBV replication.

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 (Grimm *et al*, 2006). The Pol III and Pol II transcribed pri-miRNA-31 constructs designed in this project were compared to U6 shRNAs generated by Carmona *et al* (2006), that were shown to be effective. The comparative evaluation included luciferase knockdown assays, HBsAg assays, assessment of the interferon response by q-PCR and northern blots to show formation of the mature miRNA.

The Luciferase knockdown assay (Figure 3.4) showed that U6 shRNA 5, Pol III (U6) and Pol II (CMV) pri-miRNA-31s effectively knockdown markers of HBV replication. This assay did not conclusively show an improvement in knockdown when using Pol II promoters as CMV pri-miR-31/5 achieved higher knockdown than U6 pri-miR-31/5 but knockdown with the U6 and CMV variants of pri-miR-31/8 and pri-miR-31/9 were not significant enough.

The assay was able to show however that the incorporation of an anti-HBV sequence into miRNA-like structures of miR-31 enables expression of the silencing sequence from a Pol II or Pol III promoter without compromising silencing efficacy. When compared to the 84% knockdown achieved by U6 shRNA 5 the averaged 89% knockdown achieved by the pri-miRNA constructs (U6 and CMV) is considered to be good. The inhibitory effect of the pri-miRNA constructs was verified with a HBsAg ELISA assay.

Secreted HBsAg, which is a reliable indicator of HBV replication was measured and the results are shown in Figure 3.5. An average knockdown of 89% was achieved by the pri-miRNA constructs (U6 and CMV). The result was comparable to the 93% knockdown achieved by U6 shRNA 5. With the knockdown efficacy of the pri-miRNA constructs being proven the next point of interest was to verify the specificity of the generated constructs.

An advantage of using RNAi based technologies is the specificity of interaction between the guide strand of the RNAi effecter and it cognate target. The lack of specificity of a designed RNAi effecter could have disasterous implications and is one of the obstacles that needs to be overcome before this technology can be used therapeutically. The generated pri-miRNA constructs were evaluated in a q-PCR for the induction of an interferon-related response. A positive control, Poly I:C, was included in the assay as it induces an interferon-related response. Relative amounts of *IFN-* β to GAPDH (housekeeping gene) were quantified (Figure 3.6). Compared to the Poly I:C positive control, none of the generated pri-miRNA (U6 and CMV) induced an interferon-related response. This result indicates that the generated constructs are both effective and specific. Specificity however is not the only feature that requires investigation. An effective pri-miRNA should be specific and functional.

To determine whether the generated pri-miRNA are functional and yet are cleaved to form 21-22 nt mature miRNA northern blot analysis of RNA extracted from cells transfected with the constructs and the HBV target was executed (Figure 3.7). Figure 3.7 shows the hybridization of a radiolabeled probe complementary to the putative mature anti-HBV guide 5 (A), guide 8 (B) or guide 9 (C) strands. All the pri-miRNA produce mature miRNA of 21 – 22 nt in size. The amounts of the processed products produced by the pri-miRNA are less than that produced by the cognate U6 shRNA. Despite the decreased amount of mature miRNA the pri-miRNA constructs are able to knockdown markers of HBV replication with an efficiency comparable to that of U6 shRNA 5. This suggests that complete processing of the pri-miRNA transcripts occurs more efficiently than that of the shRNA transcripts. The demonstration that the anti-HBV miRNAs generate an intended guide strand of ~21 nucleotides confirms that these sequences function as pri-miRNA mimics and are processed in a manner similar to those of natural pri-miRNAs.

4.3 Testing the anti HBV efficacy of pri-miRNA sequences in vivo using the hydrodynamic injection model of HBV replication.

To back up or validate the *in vitro* results of the pri-miRNA constructs, it was necessary to test the RNAi effecters in an *in vivo* system such as the mouse model. As a result of the regulatory problems associated with Pol III promoters it was decided to only test the Pol II (CMV) pri-miRNA constructs in mice. Compared to the mock control the CMV pri-miR-31 shuttles on average resulted in 88% reduction in the HBsAg levels. This result is considered to be good especially when compared to the 97% reduction caused by the U6 shRNA 5 construct.

This study thus 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-miRNA mimics that are transcribed from a Pol II promoter. These results together with literature on the advantages of the use of Pol II promoters compared to Pol III promoters formed the basis of the decision to continue the work using only Pol II promoters.

4.4 Design of polycistronic CMV pri-miRNA sequences targeted against the HBx ORF

A problem associated with most RNAi based technologies against viruses is that the sequence inflexibility of the RNAi molecules renders them ineffective when escape mutants come to the fore. To evade the problem of escape mutants the expression of silencing sequences that efficiently target multiple sites would be a particularly useful attribute to enhance knockdown. Ideally this should be achieved using a single expression cassette to control for equal dosage of RNAi effecter. With this in mind the polycistronic pri-miRNA cassettes described here were designed to provide a suitable method to attain these objectives. The cassettes were generated using pri-miRNA- 31-derived constructs that were proven to be effective *in vitro* and *in vivo*

(Figures 3.4 – 3.8). Each pri-miRNA-31 monomer within the poycistronic cassette follows the design parameters of Zeng, Cullen, (2005). One design parameter (Zeng, Cullen, 2005), that supported the idea of an expressed polycistronic pri-miRNA cassette stated that free pri-miRNA ends are not required for Drosha processing. Thus each pri-miRNA that was joined together to form the polycistronic should still undergo Drosha processing.

The pri-miRNAs were combined in different orders, as trimers and expressed from a Pol II (CMV) promoter as most endogenous pri-miRNA's are expressed from Pol II promoters. The areas targeted by the pri-miRNAs do not overlap, the importance of this is evident when assessing whether the knockdown effect is due to multiple copies of the same pri-miRNA effecter or the cumulative effect of different pri-miRNAs. The idea of combining the individual pri-miRNAs to form polycistronic structures (clusters) stemmed from the fact that many miRNA occur naturally in clusters (Lagos-Quintana, 2001).

Although computer-predicted structures of the shuttles were similar (Figures 3.9 – 3.14), empirical characterization of the processing of expressed RNAi effecters remains critically important. The HBV genome comprises overlapping ORFs with embedded viral cis elements (Seeger, Mason, 2000). 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. 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.

In addition to potentially inhibiting escape mutants, the versatility of a multi-targetting RNAi system allows for numerous other applications. In a scenario of an existing mutated virus it would be possible to target multiple strains of the virus

simultaneously. This would allow for better complementarity between RNAi guide and viral target and achieve optimal target knockdown. Moreover, a multi-targetting system as mentioned in this study can potentially target different viruses simultaneously. In countries such as Africa this would be desirable as virus co-infection is rife.

4.5 Polycistronic CMV pri-miRNA mediated inhibition of HBV gene expression in cell culture models of HBV replication.

The Pol II transcribed polycistronic pri-miRNA-31 constructs designed in this project were compared to U6 shRNAs generated by Carmona *et al* (2006) that were shown to be effective. As the individual pri-miRNA-31 constructs were established to have an efficacy comparable to that of U6 shRNA it would also be important to evaluate the efficacy of the polycistronic cassettes compared to U6 shRNA. The comparative evaluation included luciferase knockdown assays, HBsAg assays, assessment of the interferon response by q-PCR and northern blots to show formation of the mature miRNA.

A Luciferase knockdown assay (Figure 3.15) showed that U6 shRNA 5 and Pol II (CMV) polycistronic pri-miRNA-31s effectively knockdown markers of HBV replication. With the exception of CMV pri-miRNA 8-9-5, this assay showed an improvement in knockdown when using the Pol II polycistronic pri-miRNA cassettes compared to U6 shRNA 5 cassette. The knockdown achieved by these cassettes (CMV pri-miRNA 5-8-9/5-9-8/8-5-9/9-5-8/9-8-5) was on average 11% more effective that U6 shRNA 5. The knockdown achieved by CMV pri-miRNA 8-9-5 was reduced at 79%. This reduction in efficacy may be for a number of reasons that will be covered. The assay was able to show that the CMV polycistronic cassettes were capable of knockdown and in most instances resulted in greater knockdown than U6 shRNA 5. The achieved knockdown data is in line with published data for multi-targetting miRNA-like vectors expressed from a Pol II promoter (Chung *et al*, 2006;

Snyder *et al*, 2008). The study by Chung et al (2006) demonstrated the simultaneous knockdown of two targets using a single Pol II expressed miRNA-like system. Snyder *et al* (2008) confirmed the knockdown of two targets simultaneously and demonstrated a knockdown of four targets using a single Pol II multi-targetting pri-miRNA system.

Following the knockdown of HBx (Figure 3.15), the knockdown efficacy of the individual pri-miRNA within the polycistronic cassette was assessed. This was done by performing a luciferase assay with the polycistronic constructs and the individual targets (psi-CHECK 5T, psi-CHECK 8T or psi-CHECK 9T). The result (Figure 3.16) showed that all the polycistronic cassettes except CMV pri-miRNA 5-9-8 and CMV pri-miRNA 9-8-5 result in efficient knockdown of psi-CHECK 5T, psi-CHECK 8T and psi-CHECK 9T. As seen in Table 3.1 with the exception of the above mentioned polycistronic cassettes that did not knock down psi-CHECK 8T, on average the knockdown of psi-CHECK 5T was 88% whilst knockdown of psi-CHECK 8T was 93% and psi-CHECK 9T displayed 61 % knockdown. An important finding from this experiment was that in polycistronic constructs with pri-miRNA 9 followed by pri-miRNA 8 it was noted that psi-CHECK 8T was not knocked down. Additionally these constructs also resulted in improved knockdown of psi-CHECK 9T as the average knockdown efficiency improved from 51% to 80% (Table 3.1). Also of importance was the finding that although CMV pri-miRNA 5-9-8 and CMV pri-miRNA 9-8-5 do not knockdown psi-CHECK 8T, they have displayed very good knockdown of markers of HBV replication (96% and 94% respectively as seen in Figure 3.15). It has been shown (Snyder et al, 2009) that the position of miRNA-like hairpins within a polycistronic transcript can affect the silencing potency of each individual miRNA-like hairpin. In this study it was noted that miRNA sometimes performed better in different positions within the polycistronic construct.

The ability of the CMV polycistronic pri-miRNA in this study to knock down a mutated target was not evaluated as part of this study but was investigated by Ely,

Naidoo, Arbuthnot (2009). In the study a luciferase assay was performed with the polycistronic cassettes and either a wild type HBx target or a mutated HBx target. The results showed that CMV pri-miRNAs 5-8-9/8-5-9/8-9-5/9-5-8 resulted in comparative similar knockdown of the wildtype and mutated HBx targets. In CMV pri-miRNAs 5-9-8/9-8-5 the knockdown of wild type and mutated HBx targets was not comparative. Again it is shown that knockdown efficacy is reduced when pri-mRNA 9 is follwed by pri-miRNA 8 in a polycistronic cassette. Ely, Naidoo, Arbuthnot (2009) also demonstrated an improvement to CMV pri-miRNA 5-9-8 by changing the scaffold of one of the pri-miRNA monomers to that of 30a or 122. However it should be noted that the knockdown efficacy of the other individual targets may be compromised by changing the scaffold. The work described here showed that when psi-CHECK 8T was not knocked down, the knockdown efficacy of psi-CHECK 9T was greatly increased. Thus it may be possible that the improvement of CMV pri-miRNAs 5-9-8/9-8-5 by changing the scaffold could result in impaired knockdown of psi-CHECK 9T. This is an aspect that will be investigated in the future.

The inhibitory effect of the polycistronic constructs was verified with a HBsAg ELISA assay that validated the results achieved from the luciferase assays (Figure 3.15 and 3.16).

It should be noted that CMV pri-miRNA 8-9-5 that displayed knockdown efficiency of 79% (Figure 3.15) reduced HBsAg by 91% when compared to the positive control. This may suggest that more testing should be done on CMV pri-miRNA 8-9-5. With the knockdown efficacy of the pri-miRNA constructs being proven the next point of interest was verifying the specificity of the generated constructs.

Section 4.2 shows that none of the individual pri-miRNA constructs developed in this project result in a non-specific response. It is thus important to assess the specificity of the polycistronic cassettes as it may be more likely to induce an immune response due to the larger size of the constructs. The generated polycistronic constructs were

evaluated in a q-PCR for the induction of an interferon-related response. The result (Figure 3.18) showed the knockdown of the generated constructs to be not only effective but also specific. With the specificity of the constructs being proven the next parameter to assess was the processing of the polycistronic cassettes.

Based on the design parameters used in this project (Zeng, Cullen, 2005), the polycistronic cassettes containing three pri-miRNA monomers in series should not affect the processing of the monomers into mature miRNA. The design should allow for Drosha cleavage which would result in separation of the polycistronic into monomeric subunits (precursor miRNAs). These precursor miRNAs would then be cleaved by dicer to form mature miRNA and proceed to RISC to induce silencing of its cognate target. To determine whether the generated polycistronic constructs get cleaved according to the above mentioned process northern blot analysis of RNA extracted from cells tranfected with the constructs and the HBV target was executed (Figure 3.19). From Figure 3.19, panel A shows that mature miRNA 5 was detected in all the polycistronic cassettes regardless of the position of pri-miRNA 5 within the cassette. Panel B shows that pri-miRNA 8 mature product was detected in all the polycistronic cassettes except CMV pri-miRNA 5-9-8 and CMV pri-miRNA 9-8-5. This result corroborates the result from the Luciferase assay in which these same polycistronics were not able to knowdown psi-CHECK 8T (Table 3.1). Thus the 0% knockdown result was due to no mature pri-miRNA 8 being produced by CMV pri-miRNA 5-9-8 and CMV pri-miRNA 9-8-5. Panel C shows that pri-miRNA 9 mature miRNA is detected in all the polycisronics although at small amounts. Despite the decreased amount of mature miRNA the polycistronic pri-miRNA constructs are able to knockdown markers of HBV replication with an efficiency comparable to that of U6 shRNA 5. This suggests that the polycistronic cassettes (excluding the above mentioned instances) are processed into mature miRNA as per the design parameters utilized. This resultsresult shows the applicability of these constructs in RNAi, the benefits of this multi-targetting system compared to previously used systems and its potential use against other viruses.

The result also show that although the polycistronic is delivering more miRNA (three different pri-miRNA), the amounts of each pri-miRNA is reduced (Figure 3.19) without compromising knockdown efficacy (Figure 3.15).

4.6 Testing the anti HBV efficacy of polycistronic CMV pri-miRNA sequences in vivo using the hydrodynamic injection model of HBV replication.

To back up or validate the *in vitro* results of the polycistronic constructs it was necessary to test the RNAi effecters in an *in* vivo system such as the mouse model. It was decided to only test two polycistronic pri-miRNA as a result of the controls needed and the number of mice per sample group. Polycistronic pri-miRNAs 5-8-9 and 8-5-9 were selected for the *in vivo* experiments. It was decided to include controls would give a complete data set on the functioning of RNAi effecters that contain the guide for 5. In addition to this the two selected polycistronic pri-miRNA cassettes include 5 in different positions within the construct thus allowing us to assess the *in vivo* effect of positioning of a pri-miRNA within the polycistronic construct. Compared to the mock control the polycistronic cassettes achieved an average of 94% reduction in HBsAg on days 3 and 5 post infection. This result is considered to be good especially when compared to the 92% average reduction caused by the U6 shRNA 5 construct. The reduction of HBsAg achieved by the polycistronic cassettes was comparable to the reduction achieved by the pri-miRNA-31/5 construct. Thus showing that the efficacy can be maintained when using the polycistronic constructs and it allows for the added benefit of targeting multiple regions. Figure 3.20 also shows that the reduction in HBsAg levels is maintained when using the polycistronic cassettes as the meaurements from days 3 and 5 are comparable. This result is especially important when comparing it to reduction of HBsAg achieved by U6 shRNA 5 which achieved 97% reduction on day 3 and 86% reduction on day 5 post infection. This suggests that the pri-miRNA and polycistronic miRNA constructs

produce a more sustained knockdown result. On comparing this result to similar published work it should be noted that the multi-targetting miRNA-like studies by Chung *et al*, 2006; Snyder *et al*, 2008; Hu *et al*, 2009 and Snyder *et al*, 2009 did not include any in vivo experiments.

This study thus shows that potent knockdown of markers of HBV replication is attained *in vitro* and *in vivo* when three pri-miRNA-31 constructs are arranged in series to form a polycistronic cassette that is transcribed from a Pol II promoter.

Work that can be conducted to further this study includes, using different promoters to assess any improvement in knockdown efficacy. It has been shown (Giering *et al*, 2008) that a liver-specific promoter induces targeted silencing in hepatoma cells and in mice livers. Such an effect is desirable as it would minimize off target effects. Work regarding polycistronic cassettes with different scaffolds has already been completed (Ely, Naidoo, Arbuthnot, 2009). The work described here did not go into delivery vehicles but different delivery systems could be tested. And lastly it would be useful to show that a polycistronic system as described here, can be modified by changing the guide sequences and used against a different virus. That would demonstrate the flexibility and utility of the muti-targetting system.

CHAPTER FIVE - CONCLUSION

The work described here involves the design, development and testing of polycistronic anti-HBV pri-miRNA effecters. The polycistronic pri-miRNA effecters comprised of pri-miRNA monomer constructs that were based on the U6 shRNA that were shown to be effective by Carmona *et al* (2006).

The results from the work described here show that the incorporation of miR 31 features to generate a pri-miRNA mimic results in effective knockdown of markers of HBV replication when compared to anti-HBV U6 shRNA. Potent and specific silencing of markers of HBV replication was observed, with no evidence of toxicity. Also, data presented here shows that anti-HBV pri-miRNA sequences that target different sites within the virus are capable of efficient silencing. These results were further developed by incorporating the effective pri-miRNA into a polycistronic cassette. Each polycistronic cassette contained three pri-miRNA in tandem, in different combinations and was expressed from a Pol II promoter. The six different polycistronic cassettes were tested *in vitro* and *in vivo*. The results showed that the polycistronic cassettes achieved effective knockdown, *in vitro* and *in vivo*, comparable to that of U6 shRNA and CMV pri-miRNA-31.

Multi-targetting appraches as described here have been effectively used in a number of studies. Initially multi-targetting shRNA vectors were developed. Zhu *et al* (2007) generated multi targetting shRNA vectors targetting isoforms of cAMP-dependant protein kinase. This study was one of the first to show effective knockdown using a multi-targetting system.

Another effective multi-targetting approach utilising shRNA, (Song *et al*, 2007) developed multi-targetting shRNA cassettes against key HIV viral genes. This work

demonstrated the benefit of having a vector targetting two sites simultaneously verses a vector targetting a single site.

A study by Liu *et al* (2008) showed that four siRNA in series (single vector) was able to prevent escape mutants within HIV. Each siRNA had different promoters that lacked sequence conservation (important to prevent recombination and thus deletion of some shRNA). The above mentioned work all illustrate the potential and the capabilities of multi-targetting systems however these studies did not assess the efficacy of the constructs *in vivo* and did not evaluate whether an interferon response was mounted. These are critical factors that validate the effectiveness of any RNAi effecter constructs and are obstacles in the path of realising the utility of these constructs into a therapeutic setting.

More recently, work utilizing microRNA-like multi-targetting systems have been favoured over multi-targetting shRNA vectors as the incorporation of microRNA features reduces toxicity of the constructs (Cullen, 2006).

Snyder *et al* (2008) were one of the first groups illustrating the use of microRNA-like multi-targetting systems. The work demonstrated the efficacy of a anti-HBV microRNA-like system capable of targetting four HBV targets simultaneously. The work has yet to be tested in an *in vivo* system but did show that the vectors avoided stimulating the interferon response.

Aagaard *et al* (2008) demonstrated the utility of an altered miRNA cluster (miR-106b) to generate a multiplexed anti-HIV-1 RNAi effecter.

The above mentioned work all verify the utility of muti-targetting systems and more recent work has demonstrated an improvement in these vectors by incorporating microRNA features.

The findings from the presented study are in line with observations from the latest literature. A specific, non toxic, multi-targetting RNAi modality is greatly anticipated in the field of RNAi. The implications of such a modality is multi faceted as it can be used to prevent escape mutants, allow for direct targetting of multiple virus strains or can be used to target different viruses simultaneously. The latter would be of significant use in regions like Africa where viral co-infection is a common occurance.

As a proof of concept the polycistronic miRNA constructs in this study were designed and tested against HBV however this model can be designed to target other viruses. Further research will be conducted to test the utility of this model against other viruses.

Although in its infancy, RNAi therapeutics has quickly become a multi million dollar industry. In addition to the current RNAi therapeutic masrket there are numerous potential RNAi therapies in various phases of clinical trials. The unprecedented move of this technology from research laboratory to clinical setting augurs well for the future of this technology and its recognition as an accepted therapy.

Analysis of the results from the work described here provides evidence of the potential utility of the novel system as a multi-targetting RNAi approach.

These findings suggest that the design of polycistronic Pol II pri-miRNA shuttles described here is potentially valuable for treating HBV infection and may be useful as a generic application in RNAi-based therapy.

CHAPTER SIX - APPENDIX

6.1 Laboratory Techniques

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

Reagents

Luria Bertani medium

The final volumes were made up with deionised sterilized water. LB was then autoclaved for 20 minutes at 121℃.

	5L	1L	500ml	100ml
Tryptone	50g	10g	5g	1g
Yeast Extract	25g	5g	2.5g	0.5g
NaCl	50g	10g	5g	1g

Transformation buffer

The Buffer was prepared with 100 mM $CaCl_2$, 10 mM PIPES-HCl and 15% Glycerol. The Buffer was titrated to pH 7.0 using NaOH. Thereafter the Transformation Buffer was autoclaved for 20 minutes at 121℃.

Protocol

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

Luria Bertani media (10ml) was inoculated with 100µl untransformed bacterial stock (DH5α). The inoculated LB was then incubated overnight at 37℃. The overnight culture was used to inoculate 100ml Luria Bertani media. A 90 minute incubation of the newly inoculated LB was set up. The time period is necessary to get the cells in the logarithmic growth phase (A600 = 0.6-1). After the stipulated time the culture was centrifuged at 2000RPM for 15 minutes at 4℃.The resulting pellet was resuspended in Transformation Buffer and incubated on ice for 20 minutes. After the time period the suspension was centrifuged for 10 minutes at 4℃. Thereafter the bacteria were resuspended in 2ml Transformation Buffer. The cell suspension was then stored as 100µl aliquots at -70℃.

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

Ligation products [10µl] were added to competent DH5α cells [100µl] and incubated on ice for 30 minutes. After the incubation the cells were heat shocked at 42℃ for 90s. The heat shock was followed with an additional incubation in ice for 5 minutes. The transformed cells were then plated onto Amp positive Luria Bertani agar plates (containing 40µl X-gal).

6.1.2 α-Complementation

Reagents

1000× Ampicillin

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

Ampicillin positive Luria Bertani agar plates

Twelve grams of Bacteriological agar (Oxoid, England) was added per 100ml Luria Bertani media (as previously described). The solution was autoclaved for 20 minutes at 121℃. Ampicillin (1000x) was added to a final concentration of 100µg/ml. It is important to note that the Ampicillin was added once the Luria Bertani-Agar had cooled sufficiently. The LB agar was then poured into Petri dishes and allowed to set.

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

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

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

Protocol

Preparing IPTG, X-gal positive Luria Bertani agar plates

At least thirty minutes prior to use, 40 µl of X-gal stock solution and 8 µl of IPTG stock solution were added to an ampicillin positive Luria Bertani agar

plate and spread evenly on the surface. The plates were dried in an incubator at 37°C.

Transformed *E. coli* are plated on ampicillin positive, X-gal, IPTG positive agar plates and incubated at 37°C overnight for α-complementation. IPTG induces expression of β-galactosidase, which cleaves the chromogenic substrate X-gal yielding a blue product. Unsuccessful insertion of fragments leaves the β-galactosidase intact resulting in blue colonies. Successful cloning (plasmids positive for an insert) disrupts the β-galactosidase gene and therefore the protein is not functional resulting in white colonies. These successful clones still need to be screened for the correct orientation and sequence fidelity.

6.1.3 High Pure Plasmid Isolation Kit (Roche)

Reagents

LB (10ml per miniprep)
High Pure Plasmid Isolation Kit

Protocol

Overnight cultures (10ml) were grown with shaking in grenier tubes at 37℃. Cultures were harvested in a standard benchtop centrifuge at 4000RPM for 30 minutes. The supernatants were discarded and the bacterial pellets were resuspended in 250µl Suspension Buffer [50mM Tris-HCl, 2.5mg RNase A, 10mM EDTA, pH 8.0 (25˚C)]. The suspensions were transferred into 1.5ml microfuge tubes and were centrifuged at 9000rpm for 30s. After the spin, 250µl Lysis Buffer [0.2 M NaOH and 1% SDS] was added to the samples. The samples were mixed by gentle inversion and incubated at room temperature for 5 minutes. Following the incubation, 350µl of chilled Binding

Buffer [4M guanidine hydrochloride and 0.5 M potassium acetate, pH 4.2] was added. Samples were mixed by gentle inversion and incubated on ice for 5 minutes. The addition of the Binding Buffer results in the samples forming a cloudy precipitate. The samples were centrifuged at 14000rpm for 10 minutes. After the spin the lysate was transferred to the upper reservoir of the High Pure filter tube. Samples were then centrifuged at 14000rpm for 1 minute. During the spin the DNA in the lysate binds to the High Pure filter tube. All other undesirable material moves straight through the filter and collects in the tube beneath the filter.

After the first spin the flow through in the collection tube is discarded. The DNA (bound to the filter) is then washed with 700µl Wash Buffer II [20mM NaCl, 2 mM Tris-HCl, 40 ml absolute ethanol, pH 7.5 (25˚C)] . The samples were centrifuged at 14000rpm for 1 minute. The flow through was once again discarded. In order to remove any residual Wash Buffer, the samples were centrifuged again (same speed). After this spin the collection tubes were discarded and the High Pure filter was inserted into a 1.5ml eppendorf. Elution Buffer (100µl) [10 mM Tris-HCl, pH 8.5 (25˚C)] was added to the centre of the filter. Samples were then centrifuged at 14000rpm for 1 minute. The eluted DNA was stored at -20˚C.

6.1.4 EndoFree® Plasmid Maxi Kit plasmid preparation

Reagents

Luria Bertani medium (250ml per Maxi Prep)

See Appendix 6.1.1

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

Protocol

Luria Bertani media (250ml) was inoculated with a colony containing a plasmid of interest. Overnight cultures were grown with shaking in Ehrlenmeyer flasks at 37℃. Cells were then harvested in large buckets at 4000RPM for 30 minutes. The resulting pellets were resuspended in 10ml Buffer P1 [50 mM Tris-Cl, pH8.0; 10 mM EDTA; 100µg/ml RNase A]. Thereafter, 10ml Buffer P2 [200 mM NaOH, 1% SDS] was added to lyse the cells. The solution was mixed by gentle inversion and was incubated at room temperature for 5 minutes. After the incubation 10ml chilled Buffer P3 [3.0 M potassium actetate, pH 5.5] was added to the solution. Buffer P3 is a neutralizing solution and causes the solution to form snowflakes. The solution with added Buffer P3 (lysate) was mixed by gentle inversion. The lysate was then poured through the prepared Qiafilter Cartridge. The lysate was incubated for 10 minutes in the cartridge. After the stipulated incubation a plunger was inserted into the cartridge and the lysate was filtered into a 50ml Falcon tube. ER buffer (2.5ml) was added to the filtered lysate. The ER Buffer assists in precipitating the DNA. The filtered lysate with the added ER Buffer was incubated on ice for 30 minutes. Following the incubation period, the lysate was applied to an equilibrated Qiagen Tip 500 Column. The lysate was allowed to pass through the column by gravity flow. After filtration of the lysate, the column was washed twice with 30ml Buffer QBT Buffer [750 mM

NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100]. After the second wash the DNA was eluted into a new tube. Buffer QN (15ml) [1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol] was used to elute the DNA. Isopropanol (10.5ml) was added to the precipitated DNA. This solution was mixed and centrifuged at 4000RPM for 1hour. After the spin the supernatant was carefully decanted. The resulting pellet was washed with 5ml Endofree 70% ethanol (from kit). This solution was centrifuged at 4000RPM for 30 minutes. The supernatant was removed as before. The pellet was air-dried and thereafter resuspended in a suitable amount of Endotoxin Free sterile deionised water. An aliquot of the DNA was quantified and the remainder was stored at -20℃.

6.1.5 MinElute Gel Purification Kit (Qiagen)

Reagents

Plasmid DNA

Agarose Gel

The sample DNA [20µl] was added to agarose gel loading buffer [5µl] (Fermentas) and resolved on an agarose gel [50 ml] (1% w/v) gel containing ethidium bromide (0.5 µg/ml), in 1x Tris Acetate EDTA buffer (Tris-HCl, 0.25 mol/L EDTA, Glacial Acetic Acid, pH8.0) at 100 V for 2 hours.

Protocol

The desired fragment is excised from a standard or low-melting agarose gel. The weight of the excised fragment must be measured. Three volumes of Buffer QG are then added to 1 volume of gel. This mixture is incubated at 50˚C for 10 minutes or until the agarose gel fragment has melted. Once the solution is homogenous, 1 volume of isopropanol is added. The sample is mixed by gentle inversion and is added to a prepared MinElute column. Addition of the sample to the column causes the DNA to bind to the column. The column is centrifuged at max (14000rpm) for 1 minute. The flow through that is collected is discarded and 500µl of Buffer QG is added to the upper chamber of the column. Another spin (max speed for 1 minute) is done. The flow through is discarded and 750µl of Buffer PE is added to the column. Buffer PE is needed to wash the bound DNA. The column with added Buffer PE is centrifuged at max speed for 1 minute. After the spin, the flow through is discarded. An additional spin is done to remove any residual Buffer PE. Buffer EB (10µl) is used to elute the DNA. It is important to add Buffer EB to the centre of the membrane in the MinElute column. After standing for ~1 minute, the column is centrifuged at max speed for 1 minute. The eluted DNA is stored at -20˚C.

6.1.6 Tissue culture

Reagents

RPMI medium (RPMI)
(Invitrogen, CA, USA)

DMEM medium (DMEM)
(Invitrogen, CA, USA)

Foetal Calf Serum (FCS)

FCS (delta bioproducts, South Africa) was used to supplement the RPMI. A 2.5% FCS concentration was used for maintaining cell cultures and 10% was used before transfections.

1000×Pen/Strep

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

Saline + 0.01% EDTA

0.5× Trypsin

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

Protocol

Cell maintenance

Huh7 and HEK293 are the main cell lines that were used in experimental procedures. Huh7 cells require RPMI (supplemented with FCS) whereas HEK293 was maintained in DMEM (supplemented in FCS).

Cell lines were maintained in a humidified incubator at 37℃ and 5% CO_2. When cell reached ~80% confluency the cells were passaged. Passaging the cells entailed removing the growth media and washing the cells in saline. After removing the saline another wash with saline was performed. The cells were incubated for 5 minutes at 37℃ in the second saline wash. After the incubation the saline was removed. Cells were dislodged by the addition of trypsin (500µl). The cells were incubated for 1-3 minutes at 37℃ in trypsin. Inactivation of the trypsin was achieved by the addition of 2 ml of medium (RPMI or DMEM). Media must be aspirated around the culture plate in order to dislodge all the cells. Once all the cells are dislodged the media in the culture plate is divided into 3 equal volumes. Each volume is inserted into a new culture plate. Ten milliliters of media (RPMI or DMEM) is then added to the new plates. The culture plates are incubated at 37℃ and 5% CO2 in a humidified incubator. Cell media was changed depending on the growth rate of the cells.

Protocol

Transfection in Huh 7

Huh7 cells were maintained in a humidified incubator at 37°C and 5% CO_2 in RPMI growth medium supplemented with 2.5% FCS and antibiotics. Huh 7

cells were seeded once a confluency of 70-80% had been reached. Seventy thousand cells were seeded per well in 12-well plates. The first step in the procedure was the removal of the growth media. Cells were washed twice with saline. The second wash was incubated for 5 minutes at 37℃ in saline. After removing the last saline wash, 500 µl of 0.5× trypsin was added to the cells. The trypsinized cells were incubated for 5 minutes at 37℃. The trypsin was inactivated by the addition of 2 ml media. The media was aspirated around the plate to ensure the complete displacement of the cells off the plate. Cells were counted on a haemocytometer. Once the cell number was determined the desired amount of cells could be added to growth media. The growth media with cells were aspirated and 1 ml aliquots were added per well. Plates were incubated for 24 hours before transfecting the cells.

On the day of the transfection the DNA to be transfected was diluted into the required amounts needed. The required amount of Lipofectamine 2000 (Invitrogen, CA, USA), as per manufacturers' instructions, was diluted in 100 µl of Opti-MEM I and incubated at room temperature for 5 minutes. After the 5 minute incubation the diluted DNA was mixed with the diluted Lipofectamine 2000 and the mixture incubated for 20 minutes at room temperature to allow the DNA-Lipofectamine 2000 lipid complexes to form. Thereafter, 200 µl of transfection sample was added per well to the 12 well plate seeded with Huh7 cells. The cells were incubated for 24-48 hours at 37°C in a humidified incubator with 5% CO2.

Protocol

Transfection in HEK293

HEK293 cells were maintained in DMEM supplemented with 10% FCS, penicillin (50 IU/ml) and streptomycin (50 µg/ml). These cells are more

fragile that the Huh 7 cell line. Cells were handled very carefully as they came off very easily. HEK293 cells were seeded once a confluency of 70-80% had been reached. The first step in the procedure was the removal of the growth media. The cells were dislodged using saline wih EDTA. The saline containing EDTA was aspirated around the plate to ensure the complete displacement of the cells off the plate. Cells were counted on a haemocytometer. Once the cell number was determined the desired amount of cells could be added to growth media. The growth media with cells were aspirated and 1 ml aliquots were added per well. Plates were incubated for 24 hours before transfecting the cells. Seventy thousand cells were seeded per well in 12-well plates. One day prior to transfection cells were seeded in the desired culture wells or plates.

For interferon response transfections, cells were seeded into 24well plates. The transfections were carried out using 800 ng of each RNAi effecter plasmid, 610 ng of pCH-9/3091 and 370 ng of pCI-neo eGFP, mixed into a DNA cocktail. One hundred and fifty microlitres Opti-MEM I was added to the DNA cocktails. The DNA was transfected into the cells with Lipofectamine™ 2000 (Invitrogen, CA, USA) according to manufacturer's instructions.

For the Northern Blot transfections, cells were seeded into 10cm plates. The transfections were carried out using 16 µg of each RNAi effecter plasmid, 3 µg of pCH-9/3091 and 1 µg of pCI-neo eGFP, mixed into a DNA cocktail. Five hundred microlitres Opti-MEM I was added to the DNA cocktails. The DNA was transfected into the cells with Lipofectamine™ 2000 (Invitrogen, CA, USA) according to manufacturer's instructions.

6.1.7 Dual-Luciferase Reporter Assay System (Promega)

Reagents

Transfected cells

Passive Lysis Buffer 1x (PLB)

The PLB solution is provided in the kit as a 5x concentrate. PLB is used as a 1x concentration. This is prepared by adding 1 volume of 5x PLB to 4 volumes of sterile deionised water and mixing well. In a 6-well plate 500µl 1x PLB is used per well. This amount per well is halved when using 12-well plates.

PLB is used to promote rapid lysis of cultured mammalian cells. It is especially used for passive cell lysis applications but it can also be used for active cell lysis. Unlike other lytic reagents, PLB is formulated to minimize background autoluminescence.

Luciferase Assay Reagent II (LAR II)

LAR II is prepared by resuspending the provided Luciferase Assay Substrate in 10ml of the supplied Luciferase Assay Buffer II. A volume of 100µl LAR II per sample is used.

Stop and Glo Reagent

The Stop and Glo substrate is supplied at a 50x concentration. One volume of Stop and Glo substrate is added to 50 volumes of Stop and Glo Buffer. In the assay 100µl of Stop and Glo Reagent is used per sample.

Protocol

The growth medium is removed from the cultured cells. Wash the cells in phosphate buffered saline (PBS). Remove the PBS and add the required volume of 1x PLB to each well. The culture plates are then placed on a rocking platform or orbital shaker with gentle rocking/shaking to ensure that all the cells are covered in 1x PLB. Rock the culture plates for 15 minutes at room temperature. After the stipulated time, the lysates are transferred into eppendorf tubes. An aliquot of 20µl per sample lysate is added to the Luminometer plate. Once all the samples are loaded, the plate is inserted into the Luminometer. The Promega protocol for two injectors is selected. LAR II and the Stop and Glo reagents are inserted into the allocated holders in the Luminometer. The injectors are primed with the reagents. After the injectors have been correctly primed the protocol can be started. Data appears in the form of an excel spreadsheet. This data is plotted on the appropriate graph.

6.1.8 Monolisa Ag HBs PLUS (Biorad)

Reagents

Serum, plasma or supernatant from transfected cells

Microplate

Comprises of strips of wells that are coated with a monoclonal anti-HBs antibody (mouse, IgG 2b).

Concentrated washing solution (10x)

The washing solution is diluted 1:10 in distilled water. Each well requires 0.370ml of ready to use washing solution per wash. A minimum of 5 washes is performed per well.

Negative Control

The negative control should give an optical density reading of lower than or equal to 0.080 units. Sample optical densities that are equal to or lower than that of the negative control are reported as negative for HBsAg.

Positive Control

The positive control should give an optical density reading of more than or equal to 1. Sample optical densities more than or equal to that of the positive control are reported as positive for HBsAg.

Conjugate working solution

The conjugate working solution is prepared by adding the conjugate diluent to the lyophilized conjugate. The working solution is left to stand for 10 minutes with gentle shaking and inversion from time to time.

Enzymatic development solution

This solution is prepared by diluting the chromogen reagent 1:11 with the peroxidase substrate buffer. 100µl of enzymatic development solution is required per well.

Stop solution

This solution is ready to use and is required to stop the enzymatic development reaction. 100µl of stop solution is required per well.

Protocol

All the reagents were left to stabilize at room temperature for 30 minutes. 100µl of negative control was aliquoted into well A1. Unknown samples were aliquoted (100µl per well) into the subsequent wells (A2, A3, A4, etc). 100µl

of positive control was aliquoted into the last well. 50µl of working conjugate solution was added to each well. The plate was then covered with an adhesive film and incubated at 37ºC for 2 hours. Following the incubation time, the adhesive film was removed and the contents of the wells were aspirated out. The wells were washed with washing solution (0.370ml per well per single wash). Each well was washed a minimum of five times. 100µl of enzymatic development solution was added to each well. The plate was incubated in a dark room for 30 minutes. The development reaction was stopped by the addition of 100µl stopping solution into each well. The optical densities of the wells were measured at 450/620-700nm in a plate reader.

6.2 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

Works 2000/Iain0015/AESCCert.wps

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