USING SODIUM BISULPHITE TREATMENT AND PCR TO CONSTRUCT MAMMALIAN ANTI-HIV-1 LONG HAIRPIN RNA EXPRESSION CASSETTES.

MASIXOLE YVONNE LUGONGOLO

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DECLARATION

I, Masixole Yvonne Lugongolo declare that this dissertation is my own work. It is being submitted for the degree of MTech in the University of Johannesburg, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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ABSTRACT

RNA interference (RNAi) is a gene silencing mechanism that uses short RNA duplexes to block gene expression. This mechanism has been widely explored to determine functions of genes. Furthermore, this phenomenon has been used to silence unwanted genes such as viral genes. RNAi has been successfully employed in non-mammalian organisms such as plants, where long dsRNAs (more than 30 bp) have been used without inducing non-specific effects. However, in mammalian cells, cytoplasmic dsRNAs of more than 30 bp trigger non-specific induction of many genes, which may result from the activation of dsRNA-dependent protein kinase (PKR) and 2',5'-oligoadenylate synthetase (2',5'-OAS), via the interferon response pathway. In this study, we describe a novel and simple strategy to overcome nonspecific effects induced by longer RNA duplexes. This strategy uses sodium bisulphite which is a mutagen that deaminates cytosine residue to uracil residues in order to introduce mutations in the sense strand of the duplex. Introduction of these mutations results in the formation of G:U pairings between the sense and antisense strands of the long hairpin RNA. RNA duplexes with mismatches have been shown to be able to prevent interferon induction in mammalian cells. According to the obtained results, long hairpins RNA with and without mismatches were unable to inhibit the expression of the target region, which was the U5 region of the HIV-1 subtype C LTR. The U5 region of the LTR is actively involved in the reverse transcription of HIV-1. Therefore silencing of this region would have led to the inhibition or reverse transcription blockage. Furthermore, data showed that the interferon response was induced when using these long hairpin RNA duplexes. Due to the sensitivity of mammalian cells, the action of sodium bisulphite could have stimulated certain genes of the interferon pathway. Even though hairpins constructed in this study were unable to prevent the induction of the interferon response pathway and also could not silence the target, this strategy of using sodium bisulphite has a great potential as shown by its ability to induce changes in cytosine residues and leaving other nucleotides unchanged.

ACKNOWLEDGEMENTS

DEDICATION

To my late grandmother Kholiswa Lugongolo, my mother Nomatolo Lugongolo, my late uncle, Fezekile Mlaza, my aunt Nombhedesho Mlaza and my late sister Zonke Maciki.

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allow for the hairpin structure to occur naturally *in vivo.*

desired orientations, as indicated by the direction of the arrow. G. shows pTZDuLTRc-S/AS containing long hairpin sequence, which is 260 bp after head to cloning, and the M13 primers binding sites used for the screening of positive clones. H. Shows the M13 PCR product which is a 367 bp fragment. I. Shows a 260 bp fragment, which is the product of a restriction digest with *Xho*I and *Not*I to be introduced into a eukaryotic vector, pCiNeo, for hairpin synthesis. J, K and L show the possible orientations (other than the head to head orientation in G) in which the sense and antisense sequences would have joined.

(http://www.ncbi.nlm.nih.gov/nuccore/119662088). U5 working sequence is the target region used in designing the hairpins, pTZ-LTRc-T1 and pTZ-LTRc-T4 are the treated sequences at 10 and 150 minutes, and pTZ-LTRc-U is the untreated PCR product. The bases with a white background are the newly converted T's and the arrows show columns where conversion of bases occurred.

LIST OF ABBREVIATIONS

A	Adenine
Ago2	Argonaute 2
AIDS	Acquired Immune Deficiency Syndrome
As	Antisense
bp	Base pair
C	Cytosine
DMEM	Dulbeco's modified eagles medium
dsRNA	Double stranded RNA
Exp 5	Exportin-5
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEK 293	Human embryonic kidney 293
HIV-1	Human Immunodeficiency Virus Type 1
IFN I	Interferon Type I
LB	Luria Bertani
lhRNA	Long hairpin RNA
LTR	Long terminal repeat
MCS	Multiple cloning site
miRNA	Micro RNA
nt	Nucleotide

OAS1	2', 5'-oligoadenylate synthase 1
Pbs	Primer binding site
PCR	Polymerase Chain Reaction
PKR	dsRNA-dependent RNA protein kinase
Pol II	RNA Polymerase II
Pol III	RNA Polymerase III
Poly I:C	Polyinosinic:polycytidylic acid
PTGS	Post-transcriptional gene silencing
Pri-miRNA	Primary RNA
qRT-PCR	Quantitative Reverse Transcriptase-Polymerase Chain Reaction
R	Repeat
RE	Restriction Enzyme
RISC	RNA induced silencing complex
RNAi	RNA interference
RRE	Rev-response element
RT	Reverse transcriptase
S	Sense
siRNA	Small Interfering RNA
SS	Single stranded RNA
T	Thymine
TLR 3	Toll-like receptor

TRBP	TAR RNA binding protein
U3	Unique 3'
U5	Unique 5

CHAPTER 1

1. INTRODUCTION

1.1 RNA interference

RNA interference (RNAi) is a conserved biological response present in a range of organisms, whereby double-stranded RNAs (dsRNAs) direct sequence specific degradation of mRNA (Sharp, 2001 and Hutvanger, 2005). This mechanism was first observed in unrelated plant studies (Napoli *et al*., 1990 and van der Krol *et al*., 1990) and the groundbreaking discovery was made in *Caenorhabditis elegans*, when a dsRNA homologue to the endogenous gene was injected into the nematode leading to loss of gene activity (Fire *et al*., 1998). It is a natural process in which small RNA molecules play important roles in embryonic development, cell proliferation, apoptosis and cell differentiation in living organisms (Ambros, 2004 and Bartel, 2004). One of the main functions of RNAi is to defend the host against molecular parasites such as viruses, retrotransposons, and transgenes in a wide range of organisms (Ketting and Plasterk, 2000 and Aravin *et al*., 2001).

1.1 1 Endogenous RNAi pathway (microRNA biogenesis)

The endogenous process of RNAi is mediated by microRNAs (miRNAs), which are single-stranded RNA (ssRNA) molecules that are 21-25 nucleotides (nt) in length that regulate gene expression in plants and animals (Bartel, 2004). Animal miRNAs are transcribed by RNA polymerase II (pol II) as long primary transcripts (pri-miRNAs) (Fig. 1.1A) (Lee *et al*., 2004). These pri-miRNAs have a 5'-methyl guanosine cap (5'cap) and a 3'-poly Adenosine tail (poly A tail) (Fig. 1.1A). They are enzymatically processed into shorter, ~70-nt stem loop forms called pre-miRNAs. This cleavage occurs in the nucleus by an RNAse III enzyme Drosha, and a cofactor DGCR8 in humans, as shown in figure 1.1A (Lee *et al*., 2003). The action of Drosha on dsRNA results in the generation of a pre-miRNA with a 5'-phosphate group and a 2-nt 3'overhang (Lee *et al*., 2003). Pre-miRNAs are then transported to the cytoplasm by the action of Exportin-5 (Exp 5) and the GTP-bound form of its cofactor Ran. Together they recognize and bind the signal motif, 2-nt 3' overhang on the pre-miRNAs, to transport it to the cytoplasm (Fig. 1.1A) (Lund *et al*., 2004).

In the cytoplasm (Fig. 1.1B) pre-miRNAs are processed into ~22-nt miRNA duplexes by another RNase III enzyme, called Dicer (Hutvagner *et al*., 2001, Ketting *et al*., 2001 and Bernstein *et al*., 2001), which also has a preference for the terminus of dsRNA with 2-nt 3'overhangs (Stewart *et al*., 2003). Dicer interacts with the dsRNA binding protein, TAR RNA binding protein (TRBP) in mammals (Chendrimada *et al*., 2005). The miRNA duplex starts unwinding at the duplex end with lowest thermodynamic stability and the miRNA strand that has its 5' terminus at this end is the guide strand (Hutvagner, 2005). Dicer facilitates the incorporation of the miRNA strand of the duplex into an RNA induced silencing complex (RISC) (Hammond *et al*., 2000), which contains a protein called Argonaute 2 (Ago 2) (Chendrimana *et al*., 2005). Argonaute proteins are the catalytic components of RISC that bind to these small RNA fragments and have endonuclease activity directed against mRNA strands that are complementary to their bound siRNA fragments. It is these Argonaute proteins that are partly responsible for the selection of the guide strand and destruction of the target strand (Rand *et al*., 2005). In RISC, miRNAs mediate gene silencing by two modes: either translational inhibition or target mRNA cleavage. The choice on which mode to use is based on the degree of complementarity between the miRNA and target gene in combination with an Ago protein. Translational inhibition is due to partial complementarity between the target and the miRNA, whereas near-perfect complementarity results in cleavage leading to the degradation of the target RNA (Hutvanger and Zamore, 2002b and Zeng *et al*., 2002).

These silencing events by miRNAs take place in large cytoplasmic aggregates that serve as sites of mRNA degradation, which are known as P-bodies. They contain translationally masked mRNA, and a general block of translation elongation in cells leads to disassembly of P-bodies (Teixeira *et al*., 2005), whereas a block in translation initiation results in increased P-body formation (Teixeira *et al*., 2005).
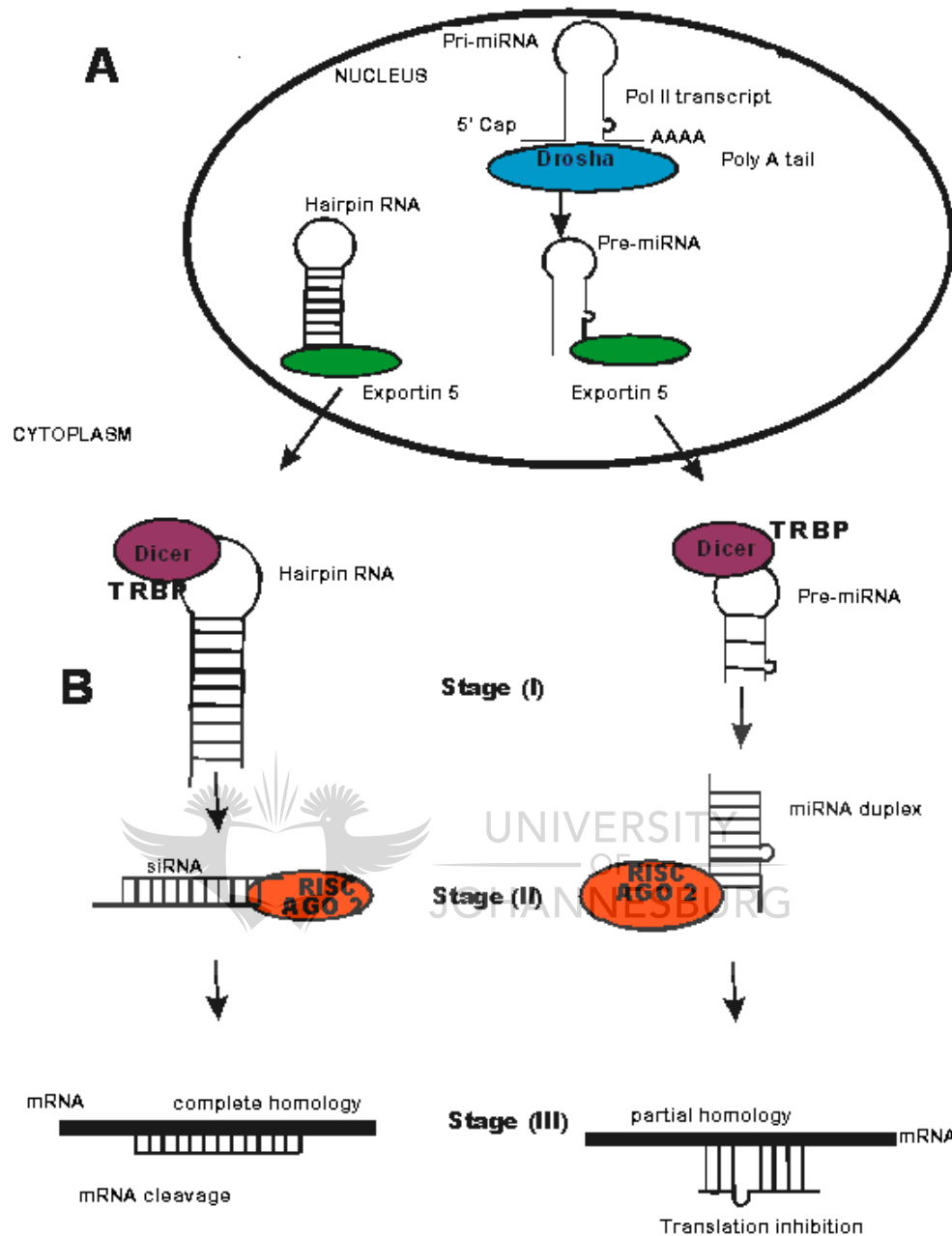
Figure 1.1 RNA interference pathway in mammals: A: Primary-miRNAs transcribed by pol II are processed to pre-miRNA (which are ~70 nt stem loop forms) by Drosha in the nucleus. The pre-miRNA and hairpin RNA (exogenous dsRNA) are transported to the cytoplasm by Exportin 5 (green ellipses). **B**. In the cytoplasm, RNA duplexes unwind and DICER (purple ellipses) incorporates the antisense strands of the duplexes into RISC/AGO 2 complex (orange ellipse). The endonuclease activity of Ago 2 proteins is responsible for the destruction of the target strand. Pre-miRNA (RIGHT) leads to gene silencing via translational inhibition. The exogenous dsRNA (LEFT) leads to gene silencing via mRNA degradation.

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1.1.2 RNAi induction

Unlike miRNAs, small interfering RNAs (siRNAs) are generated from exogenous dsRNA molecules (Zamore *et al*., 2000 and Elbashir *et al*., 2001b). siRNAs are short RNA duplexes that are 21 to 23 nt in length. They enter the RNAi pathway after the Dicer processing step in the cytoplasm. A hairpin RNA is an RNA molecule with two strands that are complementary to each other and these two strands are joined by a loop of four to ten nucleotides. Unlike the chemically synthesized siRNAs, hairpin RNAs are generated from expression cassettes under the influence of an RNA polymerase promoter. Hairpins are introduced into the nucleus and transported to the cytoplasm by Exp 5 (Fig. 1.1A-LEFT). siRNA duplexes are then incorporated into RISC where they unwind (Nykanen *et al*., 2001). An ATP independent unwinding of siRNA duplex is necessary for RISC activation (Duxbury *et al*., 2005). The antisense strand, which is a strand that is complementary to the target region, locates mRNA targets by Watson-Crick base pairing. The degradation of mRNA is due to the catalytic activity of Ago 2 (Rand *et al*., 2005). Nucleolytic degradation of the target mRNA by the enzyme RNase H results in gene silencing (Hammond, 2005).

1.1.3 Exploiting the RNAi pathway

Since its discovery, RNAi has been globally explored for therapeutic applications. Initially, the induction of RNAi in mammalian cells was achieved by the use chemically synthesized siRNAs. The chemical synthesis of siRNAs was too costly and took a long time to prepare, which led to the development of other methods of RNAi induction. The alternatives are enzymatically generated siRNAs and the use of expression systems.

Generation of siRNAs by *in vitro* transcription (enzymatic generation of siRNAs) is a cost effective method that takes short periods to prepare, it can be performed in any laboratory and it mimics the endogenous process. *In vitro* transcription is mediated by T7 phage RNA polymerase from short double stranded oligo cassettes containing the promoter sequence upstream of the siRNA template sequence to be transcribed (Yu *et al*. 2002, Sohail *et al*. 2003 and Donze and Picard, 2002). However, down-regulation of gene expression using this method is transient, lasting not more than 5 days in cell culture

(Holen *et al*., 2002). Also, T7 phage polymerases add phosphate groups to 5' end of RNA and this causes immuno-stimulation and is toxic (Kim, 2004).

The transient expression of single siRNAs is not sufficient for therapeutic purposes and the problem can be solved by stable expression of RNAi effector molecules using expression cassettes, delivered from plasmid and viral vectors. Several groups have used strong promoters such as that of RNA polymerase II (pol II) e.g. cytomegalovirus (CMV) and pol III promoters e.g. U6 and H1, for the transcription of hairpin RNAs (Brummelkamp *et al*., 2002, Miyagishi and Taira, 2002, Paddison and Hannon, 2002a and Sui *et al*., 2002). However, the most commonly used approach is the use of pol III promoters for the transcription of a short hairpin RNA structure, which is a short RNA duplex of 19-29 bp with a loop of 4-10 nt between the two strands (Figure 1.2A) (Brummelkamp *et al*., 2002 and Paddison *et al*., 2002b). In some cases the two strands are transcribed from separate cassettes or two different plasmids (Figure 1.2B) (Yu *et al*., 2002) and the two strands hybridize forming siRNA duplexes that become incorporated into RISC and silence homologous mRNA. Pol III expression cassettes produce short RNAs with precise 5' and 3' termini and this quality makes it possible to use DNA templates to synthesize *in vivo* small RNAs with structural features close to what has been found to be required for active siRNAs synthesized *in vitro* (Elbashir *et al*., 2001a). Expression of RNA duplexes using pol II promoters has a major drawback as extra sequences (such as 5' methyl guanosine cap and poly Adenosine tail) are added to the transcript resulting in a transcript that is difficult to be recognized by the cellular RNAi machinery, thereby interfering with siRNA production (Paddison *et al*., 2002c). Despite the difficulties for these transcripts to be recognized by the RNAi machinery, some pol II transcripts may be processed as endogenous pri-mRNAs (Zeng *et al*., 2002). This gives pol II transcript an advantage as they are treated as endogenous pri-miRNA.
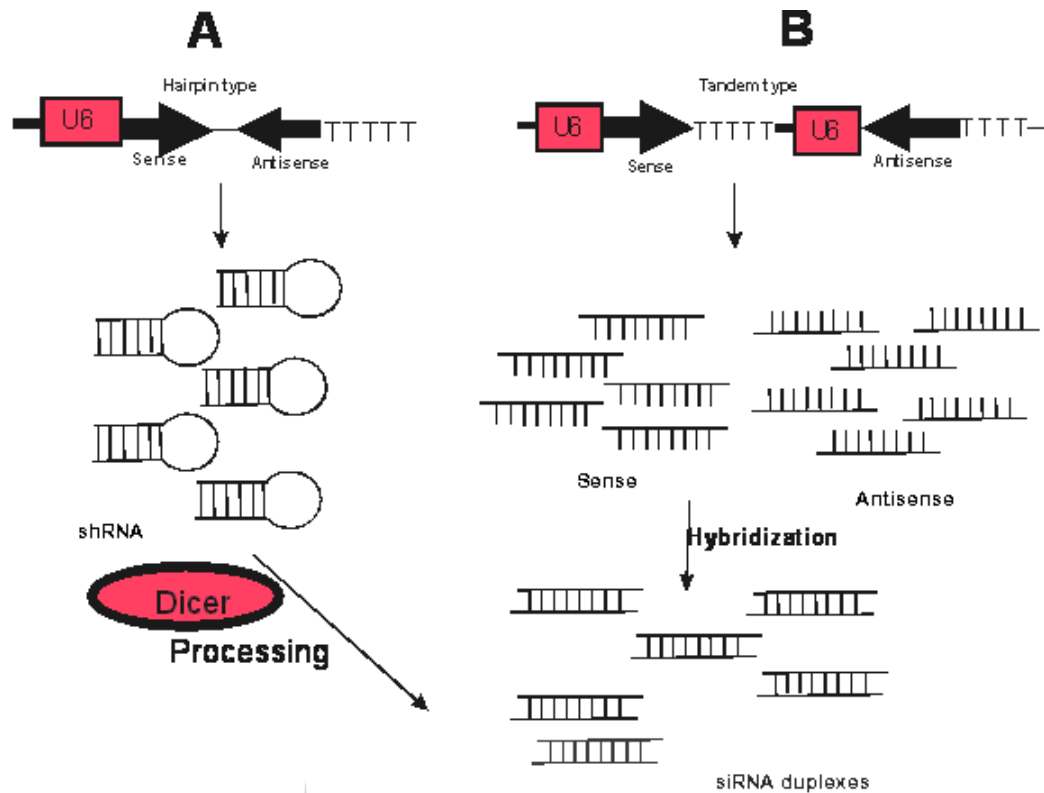
Figure 1.2 Stable expression of effector molecules using expression cassettes. **A**. Pol III (U6) promoter used for the transcription of a short hairpin RNA (shRNA). The hairpin is then processed by Dicer to form siRNA duplexes. **B**. Sense and antisense strands are transcribed from two different cassettes. The two complementary strands are then hybridized to form siRNA duplexes.

In addition to the use of these expression systems, RNAi molecules can also be delivered by viral vectors. The advantage of using viral vectors in delivering transgenes is their specificity to cell types as they only deliver to the cells that they normally infect. When using viral vectors, the expression of RNAi effector molecules is expressed for longer periods (Nishitsuji *et al*., 2004).

1.1.4 Long hairpin RNAs in mammalian systems

Short dsRNAs (siRNAs or shRNAs) have been shown to effectively silence various Human Immunodeficiency Virus type 1 (HIV-1) genes in culture experiments (Qin *et al*., 2003, Jacque *et al*., 2002, Capodici *et al*., 2002, and Nishitsuji *et al*., 2004). However, the use of these small RNA duplexes has a major limitation which is the rapid emergence of escape mutants when used in silencing viral genes (Das *et al*., 2004). This challenge could be solved by using RNA duplexes that are more than 30 bp in length. These RNA duplexes can potentially generate multiple siRNAs, thereby targeting longer and multiple sequences, resulting in the delay of the emergence of viral escape mutants. However, the silencing of unwanted genes by long RNA duplexes may be hindered by the induction of non-specific type I interferon (IFN) response (Stark *et al*., 1998). The induction of the interferon response causes the inhibition of translation, thereby silencing non-specific genes. The IFN response is caused by the interaction of dsRNAs with either of the two cellular proteins involved in the IFN pathway: dsRNA-dependent RNA protein kinase (PKR) and 2', 5'-oligoadenylate synthase 1 (OAS1) (Stark et al., 1998). This non-specific silencing by interferons is unwanted in therapeutics as it hinders the silencing ability of the therapeutic substance such as siRNAs and long hairpin RNAs (lhRNA). In several invertebrate species like *C. elegans* and *Drosophila*, efficient specific silencing of gene expression has been obtained by the use of long exogenous dsRNAs (Fire *et al*., 1998, Tuschl *et al*., 1999). Unlike mammalian cells, these organisms lack the protein based adaptive immunity that is found in higher vertebrates (Tijsterman *et al*., 2002), therefore the use of long dsRNAs induce RNAi without triggering an interferon response is effective in these organisms.

According to the observations in previous studies, the induction of interferons by dsRNAs is cell line dependent (Harada *et al*., 1990, Billy *et al*., 2001 and Elbashir *et al*., 2001a). These studies showed that RNAi can be induced by long dsRNAs in undifferentiated embryonic cells without any non-specific effects (Billy et al., 2001), whereas non-specific silencing due to interferon induction occurred when using long dsRNAs in differentiated cells (Elbashir *et al*., 2001a). The use of undifferentiated cell lines only when using long RNA duplexes is not the solution as viruses infect

differentiated cell types. Therefore, a strategy to prevent interferon induction in a wide range of cell lines should be the focus.

Endogenously expressed RNA duplexes have been shown to silence unwanted genes without any adverse effects in cells (Robbins *et al*., 2006). This suggests that the cells respond differently to endogenously expressed dsRNAs as compared to the exogenous dsRNA. Exogenous RNAs induce IFN response whereas the endogenously expressed do not (Robbins *et al*., 2006). Therefore, interferon induction may be prevented by endogenous expression of RNA duplexes used in gene silencing studies (Bhargava *et al*., 2004, and Robbins *et al*., 2006). Also, multiple mutations within the sense strand of the dsRNA region can be included to generate mismatches between the two strands of lhRNAs to inhibit interferon response (Akashi *et al*., 2005). Mutations within a hairpin have additional advantages such as stability in mammalian genomes, easy sequencing (Akashi *et al*., 2005) and stability of plasmids in *E. coli* (Miyagishi and Taira, 2003). In studies where lhRNAs were used in viral gene silencing, lhRNAs were effectively designed to include G:U mismatches (Akashi *et al*., 2005, Konstantinova *et al*., 2006, Weinberg *et al*., 2007, Barichievy *et al*., 2007 and Sano *et al*., 2007).

1.1.5 Construction of mismatched long hairpin RNAs

As mentioned previously, the presence of mismatches within an lhRNA may prevent the induction of interferon response in mammalian cells. Therefore, when generating lhRNAs with mismatches synthetic oligonucleotides have been efficiently used for hairpins that are not more than 65 bp in length. However, this approach seems to be difficult and expensive for hairpin RNAs longer than 65 bp. An alternative method was devised in order to overcome the problems associated with the use of synthetic oligonucleotides. This new approach involves treating the DNA encoding the hairpin RNA with a mutagenic chemical, sodium bisulphite which deaminates cytosine residues to uracil residue.

The main objective of the present study was to test the efficiency of sodium bisulphite for the generation of mismatched duplexes of dsRNA. Sodium bisulphite is a mutagen which causes deamination of cytosine residues in DNA through the formation of a 5, 6-dihydrocytosine-6-sulphonate intermediate to uracil (Shapiro *et al.*, 1973). The

introduction of mismatches using this mutagen would be simpler and cost effective. Sodium bisulphite is widely used in molecular biology for the analysis of cytosine methylation status in DNA using a method known as the bisulphite genomic sequencing (Frommer *et al*., 1992), as it was observed that a portion of the pyrimidine base cytosine is methylated at position 5 in animal DNA. The methylated cytosine residue is referred to as 5-methylcytosine, and it is not altered by sodium bisulphite treatment (Frommer *et al*., 1992). When the bisulphite-modified DNA is subjected to PCR, the uracil residues will become thymine residues, while 5-methycytosine is not changed by bisulphite treatment. The amplification will produce nucleotides in which cytosine residues represent the 5-methylcytosine residues of the original (Shiraishi *et al*., 2002 and Frommer, *et al*., 1992).

To design and construct a lhRNA, the target gene needs to be selected. The sequence of the target region is amplified using PCR to produce large quantities of DNA (Fig. 1.3.A). A fraction of the PCR product gets treated with sodium bisulphite to obtain a treated PCR product with cytosines converted to thymines so that G: U pairing occurs in the hairpin duplex (Fig. 1.3B). The mRNA gene to be used as a target for lhRNA binding is known, by convention, as the sense strand. Hence, the DNA strand with the similar sequence obtained by PCR amplification will be known as the sense or plus strand. The complementary sequence is therefore termed the antisense or minus strand. Since it is the antisense strand of the lhRNA molecule that is directed against the sense mRNA for catalytic degradation by RISC; the sense and antisense terminology will be used to differentiate the complimentary sequences of the stem structure of the lhRNA which are inverted repeats. Since each of these strands will be synthesized and cloned separately, this terminology will be used to prevent confusion.

To generate sense sequence with mutations, treated DNA is subjected to PCR using primers which are specific for the treated sequence. Two different sets of primers are used to amplify the untreated DNA in order to produce sense and antisense sequences. One primer set has linkers that are the same as those used in mutated sense sequence and this is used to generate a sense sequence without mismatches. Also, a different set of primers with different linkers is used for the generation of the antisense sequence (Fig. 1.3 C).

Section D of Fig. 1.3 shows the amplicons and the location of restriction enzyme sequences before head to head cloning. The two PCR products are arranged in a head to head orientation, with RE1 being the sequence that forms the loop, which is a restriction enzyme sequence that is common in both sense and antisense sequence as represented by section E of Fig. 1.3. The loop encoding sequence is formed by a sequence of 6-10bp. The head to head construct may be placed within an expression cassette such that the resulting transcript generates a hairpin sequence with stem components that are encoded by the untreated and the treated PCR products. The long dsRNA results in G: U pairing occurring in the stem, between the sense and the antisense fragments of the duplex, as they are inverted repeats, and they will form the stem loop structure as shown in Fig. 1.3F.
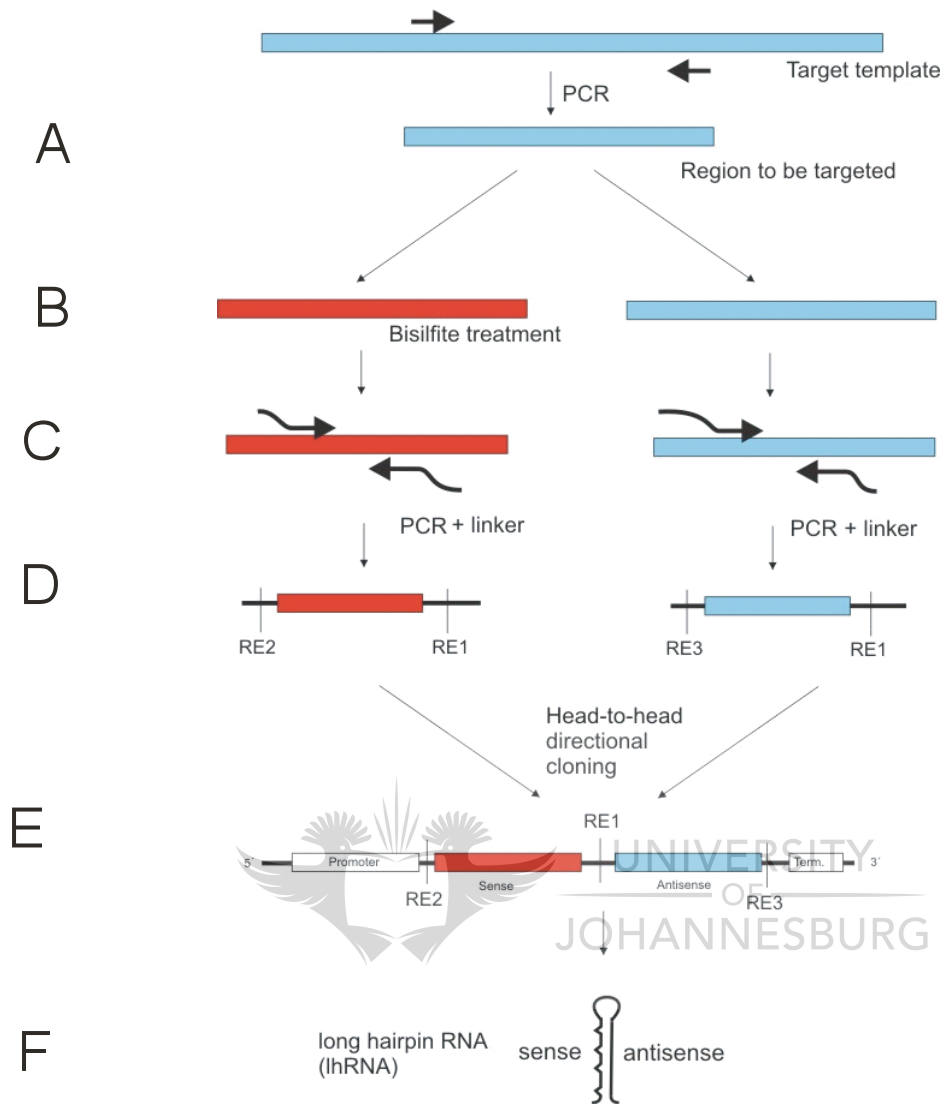
Figure 1.3 Construction of long hairpins with mismatches induced by sodium bisulphite. **A**. The target sequence was amplified using PCR. **B**. A fraction of the PCR product is treated with sodium bisulphite. **C**. Both treated and untreated PCR products were used as templates for another PCR. PCR was performed using primers with different linkers for the generation of sense and antisense sequences. **D**. The black short lines indicate the location of different restriction enzyme sequences for head to head cloning. **E**. Long hairpin sequence with RE1 representing restriction enzyme sequence that forms the hairpin loop. **F**. Long hairpin RNA with mismatches as the sense strand has been treated. The bump-like features on the sense strand are the positions with G: U base pairing. Complementarity of the sense and antisense strands will subsequently allow for the hairpin structure to occur naturally *in vivo*.

The G: U base pairing is due to the conversion of cytosine to uracil and instead of guanidine pairing with cytosine it pairs with uracil and these mismatches result in an imperfect hairpin as illustrated in figure 1.4B. The transcripts are under the control of RNA pol II with poly A termination signal.
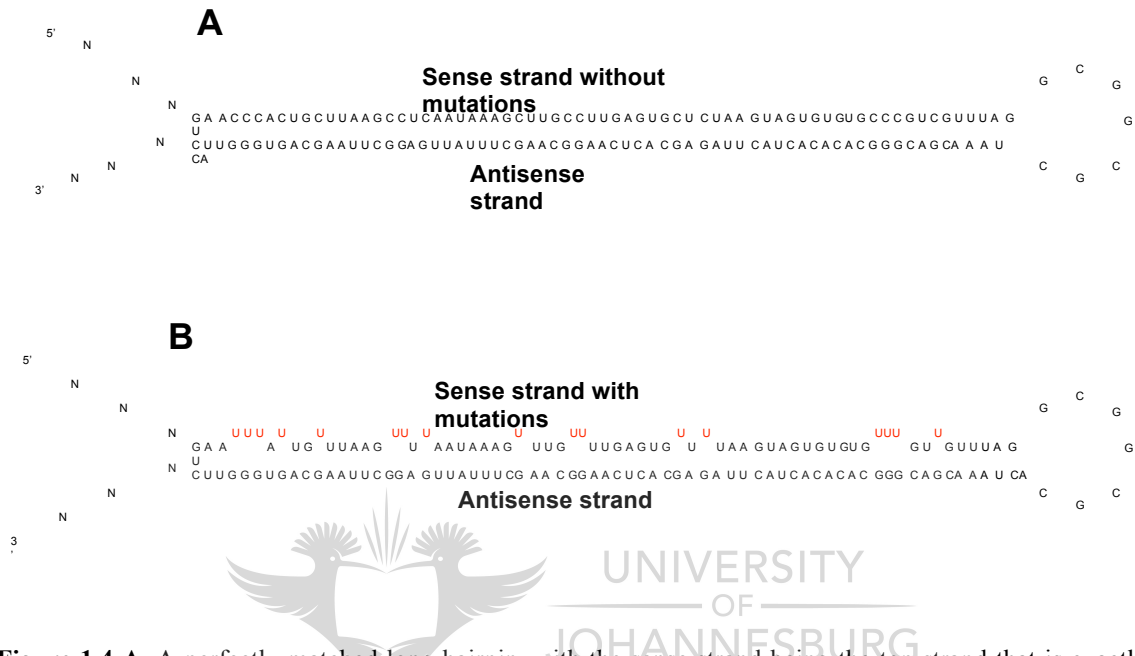
Figure 1.4 A. A perfectly matched long hairpin, with the sense strand being the top strand that is exactly the same as the target sequence and it gets degraded during the cleavage of the target. **B**. an imperfectly matched hairpin, with changes on the sense strand, where C has been converted to U resulting in a dsRNA molecule with G: U wobble mismatches. Red U's on B are the converted C's.

1.2 Therapeutic applications of RNAi against HIV-1

The potential of RNAi as a means of therapy has been explored in a number of diseases ranging from genetic disorders to viral infections like Hepatitis C Virus, Hepatitis B Virus and HIV-1. HIV-1 is the AIDS causative agent that has claimed millions of people's lives worldwide. Its genome contains nine genes (*gag, pol, env, tat, rev, vpu, vif, vpr,* and *nef*) that are flanked by long terminal repeats (LTRs) on both ends of the genome. These LTRs are subdivided in the U3 (unique 3'), R (repeat) and U5 (unique 5') domains. Within these LTRs there are promoter sequences and the transcript polyadenylation signal (Buchschacher, 1993). In this study, the ability of expressed

lhRNAs (longer than 100 bp) to silence unwanted genes was tested on the U5 region of the 5' LTR of HIV-1 subtype C, Du151 strain.

1.2.1 HIV life cycle

HIV-1 infects human cells that express CD4 receptors on their surfaces. These cells include T-helper lymphocytes, macrophages, dendritic cells and brain microglial cells (Bour *et al*., 1995). The reduction in the number of CD4 molecules in the body leads to the weakening of the immune system, therefore the body becomes unable to fight infections (Douek *et al*., 2003). HIV-1 enters the cell by the interaction of the viral surface protein, gp120 with the CD4 receptor (Wu *et al*., 1996). The viral trans-membrane protein, gp41 fuses with the cell membrane by utilizing a co-receptors either CCR5 and CXCR4 (Alkhatib *et al*., 1996 and Doranz *et al*., 1996). The viral core enters into the cytoplasm and forms a pre-integration complex with various host factors (Anderson and Hope, 2005). The viral core contains viral enzymes and the viral genome, mRNA which gets converted to DNA by the process of reverse transcription mediated by the viral reverse transcriptase (RT) enzyme (Telesnitsky and Goff, 1997). Reverse transcription requires the formation of a complex between RT, a cellular $tRNA_3^{Lys}$; which serves as a primer and the viral RNA genome. The primer initiates negative strand DNA synthesis by binding to the primer binding site (PBS) region, located 3' to the U5 region (Joshi and Lamothe, 2000). The newly transcribed cDNA is translocated to the nucleus where the viral DNA is integrated into the host genome by the action of one of the viral enzyme, integrase (IN) (Asante-Appiah and Skalka, 1997). The integrated viral DNA is used as a template for the production of viral mRNAs. Cellular transcription machinery is responsible for the low level of HIV-1 transcription. In addition to the cellular machinery, the viral protein Tat is required for high level HIV-1 transcription and activates HIV-1 transcription by greater than 100 fold (Cullen, 1986 and Feng and Holland, 1988). In the absence of Tat, most transcripts produced from the HIV-1 promoter are short due to the early transcription termination, whereas in the presence of Tat the number of transcripts that are full length increases (Keen *et al*., 1996). Tat induces elongation of HIV-1 transcription by recruiting transcriptional co-activators that include Positive Transcription Elongation Factor b (P-TEFb), an RNA polymerase II C-terminal domain kinase

(Herrmann and Rice, 1995, Yang *et al*., 1997 and Zhu *et al*., 1997), and histone acetyl transferases (Kierman *et al*., 1999 and Ott *et al*., 1999). While P-TEFb induces HIV-1 transcription from non-integrated HIV-1 template (Herrmann and Rice, 1995, Yang *et al*., 1997 and Zhu *et al*., 1997), histone acetyl transferases transactivate integrated HIV-1 provirus (Kierman *et al*., 1999 and Ott *et al*., 1999). Tat may also increase initiation of HIV-1 transcription by enhancing phosphorylation of SP1, a transcription factor involved in basal HIV-1 transcription (Chun *et al*., 1998). Activation of HIV-1 transcription by Tat depends on a trans-activation response (TAR) RNA element. TAR is a 59 nucleotide RNA stem loop structure that forms the 5' end of all HIV-1 transcripts and is found in the LTR. TAR has two stems, a four nucleotide bulge, and a six nucleotide loop (Colvin and Garcia-Blanco, 1992 and Harper and Logsdon, 1991).

The newly transcribed mRNA is spliced multiple times by the cellular splicing machinery. It is from these multiply spliced transcripts that the Tat, Rev and Nef proteins are produced. As Tat activates transcription, Rev regulates transport of HIV-1 transcripts from the nucleus to the cytoplasm. Rev binds to a major groove in the Rev-response element (RRE), which is a 210 nucleotide RNA sequence found in HIV-1 transcripts (Daly *et al*., 1989 and Battiste *et al*., 1996). As Rev levels reach a critical level of expression, a shift in viral mRNA production from multiply spliced (encoding Tat, Rev and Nef) to singly spliced and unspliced transcripts occurs (Cullen, 1992). Nef is important for maintenance of high viral loads and for the development of AIDS (Kestler *et al*., 1991). Once in the cytoplasm the unspliced viral mRNA is translated into a Gag-Pol polyprotein by a process that involves ribosomal frame shifting. The Gag-Pol polyprotein is cleaved by the viral protease into the mature virion structural proteins (matrix, capsid, and nucleocapsid) as well as the virion enzymes (protease, reverse transcriptase and integrase). The viral envelope proteins are synthesized as the gp160 precursor polyprotein which is then cleaved by cellular proteases into gp120 and gp41. Viral assembly seems to be regulated at least in part by the accessory proteins Vpu and Vif (Chen *et al*., 1996 and Simon *et al*., 1997). Efficient packaging of genomic RNA of retroviruses into viral particles requires a specific nucleotide sequence, termed the packaging sequence (psi) (Mann and Baltimore, 1985). Packaging of HIV-1 RNA into virus particles then takes place, a process which involves an interaction between psi and

the zinc binding domains of the nucleocapsid protein (Rice *et al*., 1995) and new virions are released from the surface of the host cell by means of budding and a new cycle can start.

According to the HIV-1 life cycle, infection can either be blocked or inhibited depending on the target gene. HIV infection can be blocked by targeting cellular proteins that play significant roles in viral entry into the cell like CD4 receptor, a primary receptor that HIV uses to enter into its target cells (Novina *et al*., 2002), CCR5 (Qin *et al*., 2003) a major co-receptor that is used by this virus to get into target cells, CXCR4 (Anderson *et al*., 2003) and DC-SIGN (Madhavan *et al*., 2005), which is a CD4 independent attachment receptor that is involved at the initial stages of HIV-1 infection (Geijtenbeek *et al*., 2000). Besides targeting cellular proteins, HIV-1 infection can be inhibited by targeting different points in the life cycle, such as the reverse transcriptase, integrase and protease processes.

1.2.2 RNAi in HIV-1 therapy

The use of siRNAs in controlling HIV-1 infection is effective and makes RNAi a promising technology to be used in HIV-1 gene therapy (Das *et al*., 2004 and Westerhout *et al*., 2005). However, this technology faces a major limitation, namely the rapid emergence of viral escape mutants. Only short regions of the virus can be targeted by siRNAs or shRNAs thereby providing the virus an opportunity to mutate and escape, causing the silencing effect of siRNAs to lose its efficacy rapidly (Das *et al*., 2004 and Westerhout *et al*., 2006). RNAi is highly specific as one nucleotide mismatch would abolish the whole silencing process (Das *et al*., 2004 and Westerhout *et al*., 2006). However, siRNAs have been successfully used to inhibit HIV-1 replication *in vivo* by targeting different viral genes within the HIV-1 genome with the intention of inhibiting viral replication (Capodici *et al*., 2002, Novina *et al*., 2002, Boden *et al*., 2004, Lee *et al*., 2005). In addition to the use of siRNAs and hairpin RNAs, natural occurring small RNA molecules, miRNAs that are natural components responsible for RNAi have been shown to be more efficient in silencing target genes when compared to siRNAs (Boden *et al*., 2003). The high efficacy of miRNAs could be attributed to the fact miRNAs are natural triggers of RNAi.

AIMS

The main objective of this project was to develop a method of introducing mismatches within long hairpin RNA constructs in order to down-regulate HIV-1 subtype C LTR and subsequently prevent the induction of interferon response.

Specific aims:

(i) Designing a long hairpin sequence targeting the HIV-1 subtype C LTR.

(ii) Introducing mismatches by sodium bisulphite treatment.

(iii) Determination of the mutation efficiency of sodium bisulphite.

(iv) Assembling long hairpin constructs containing mutated sense and a wild type antisense arm against the U5 region of HIV-1 subtype C, Du151, by head to head cloning.

(v) Cloning of the mismatched long hairpin construct into the pCi-Neo expression vector.

(vi) Testing the effect of hairpin constructs against the target region.

(vii) Testing the effect of the constructs in mammalian cells by doing interferon studies.

CHAPTER 2

2. MATERIALS AND METHODS

2.1. Generation of long hairpin RNAs

2.1.1. Construction of the target plasmid

The plasmid containing the target sequence, pTZDuLTR-Luci was constructed by Samantha Barichievy of the Antiviral Gene Therapy Research Unit, University of the Witwatersrand (Barichievy *et al*., 2007). The construct contains the LTR sequence from a proviral sample of the HIV-1 isolate Du151 (GenBank: AY043173), a subtype C HIV-1 strain from Durban, South Africa. The LTR sequence is upstream from a Firefly luciferase gene.

2.1.2. Amplification of long hairpin sequences

To generate long hairpin RNA sequences, pTZDuLTR-Luci was used as a template for the amplification of the target sequence in order to produce large amounts of DNA for the construction of both sense and antisense components of the stem structure of the long hairpin RNAs. The target sequence (118 bp) which is the U5 LTR (GAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTCTAAGTAGTG TGTGCCCGTCTGTTGTATGACTCTGGTAACTAGAGATCCCTCAGACCCTTTGT GTTAGTGTGGAAA) was amplified with unmodified LTRc forward (5' GGGAGCTCTCTGGCTATCTAG 3') and reverse primers (5' GGGCGCCACTGCTAGAGA 3') synthesized by Inqaba Biotechnology (South Africa). These primers were designed flanking the118 bp target region and generated 168 bp product. The amplification reaction contained 1 ng of the template (pTZDuLTR-Luci), each primer had a final concentration of 0.375 μM, dNTPs mixture to a final concentration of 0.25 mM, *Taq* buffer to a final concentration of 1X and 2 U *Taq* polymerase (Promega, USA Water was added to a final volume of 40 μl. Reactions were carried out in a thermal cycler at 95°C for 5 minutes to activate *Taq polymerase*, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and primer extension at 72°C for 30 seconds and a final extension step of 72°C for 10 minutes. Amplification products were analyzed by gel electrophoresis on a 2% agarose gel (Appendix 7.4) (Biorad, USA) containing 1 μg/ml ethidium bromide.

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Electrophoresis was carried out in a 1X boric acid buffer (Appendix 7.4) at 100 V. Amplification products were purified using the Qiagen MinElute™ Gel Extraction kit (Qiagen, CA, USA) and eluted in a final volume of 50 µl of water (see Appendix 7.5).

2.1.3. *Introduction of mutations on the sense sequence of a long hairpin RNA by sodium bisulphite treatment.*

Bisulphite treatment of the amplicon was carried out using the EZ DNA methylation Gold Kit (Zymo Research, CA, USA) to catalyze the deamination of cytosine residues. The purified amplicons were subjected to sodium bisulphite treatment by adding 130 µl of freshly prepared CT conversion reagent (containing sodium bisulphite) to PCR tubes containing 20 µl of the PCR product. CT conversion reagent was prepared by adding 900 µl of water, 50 µl M-dissolving buffer, and 300 µl M-dilution buffer to the CT conversion reagent tube according to the manufacturer's instructions. This mixture was allowed to dissolve at room temperature for 10 minutes with brief vortexing. DNA and sodium bisulphite mixtures were placed in a thermal cycler which was set to denature DNA at 98°C for 10 minutes then deamination at 64°C for 150 minutes. To determine whether time would have an effect on the C to U transversion, samples were incubated at 64°C for 10 and 150 minutes. Immediately after removing each tube out of the thermal cycler, DNA was purified by loading the treated samples into Zymo-Spin columns with 600 µl M-Binding buffer. The solutions mixed by inverting for 5 minutes. These columns contain silica, which is positively charged and allowing negatively charged DNA molecules to bind to the column. Furthermore, the addition of binding buffer to the column creates a high salt condition, which allows the DNA to bind tightly. The columns were centrifuged at 10,000 xg for 30 seconds in a bench-top centrifuge and the flow-through was discarded. To the columns, 200 µl of M-desulphonation buffer was added and incubated at room temperature for 20 minutes followed by centrifugation at 13,000 rpm for 30 seconds in a bench-top centrifuge. Following centrifugation, 200 µl of wash buffer containing ethanol was added to the column and centrifuged for an additional 30 seconds at 10,000 xg. Due to the strength of the bond between silica and DNA, DNA is not eluted during the washing, but all the contaminants are removed. The wash step was performed twice and the DNA was eluted in 10 µl of M-elution buffer by centrifuging at

10,000 xg for 30 seconds. The elution buffer has low ionic strength and this allows for easy elution of the purified DNA that had been bound to the silica.

2.1.4. Amplification of treated DNA by PCR and cloning

Following the sodium bisulphite treatment, treated DNA was amplified using the unmodified primers that were used in the first PCR and the amplification conditions as described previously. *Taq* polymerase has non-specific terminal deoxynucleotidyl transferase activity that introduces nucleotides, specifically ddA at the 3' terminus of a PCR product. The amplified fragments hybridize to the 5' ddT overhangs of T/A cloning vectors for efficient ligation. Amplicons were purified using the Qiagen MinElute^TM Gel Extraction Kit (Qiagen, CA, USA) (Appendix 7.5). Purified amplicons were ligated into a PCR T/A cloning vector, pTZ-57R/T (Fermentas, WI, USA) (Fig. 2.1) at a molar ratio of 1:4 (vector: insert).
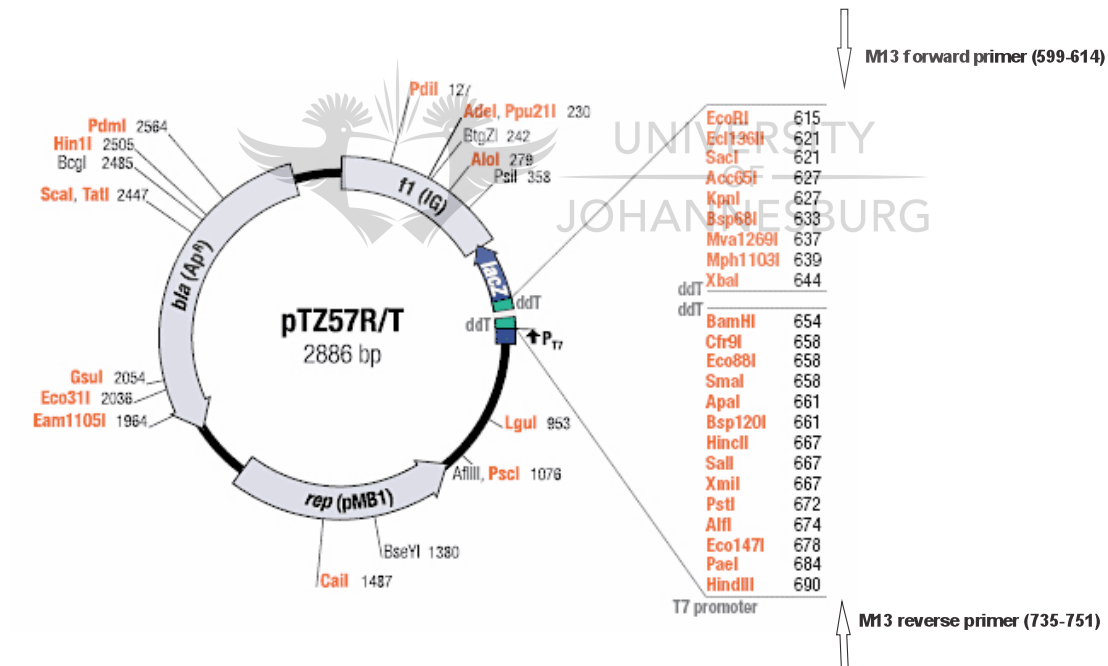
Figure 2.1 T/A cloning vector, pTZ57R/T (Fermentas, WI, USA) with a T/A cloning site within the *lacZ* gene and various restriction enzyme sites are used to facilitate the cloning process. Sites where M13 primers bind, flanking the MCS are indicated
http://www.fermentas.com/pdf/flyers/fl_transforminsta2006.pdf.

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The pTZ-57R/T vector is a commonly used T/A vector that has ampicillin resistance, the *β*-galactosidase gene for blue/white colony screening and a multiple cloning site (MCS). The ligation mixture contained 10% v/v PEG4000, 1X ligation buffer, 4 U T4 ligase (Fermentas, WI, USA), and nuclease free water to a final volume of 30 μl. In addition, a ligation reaction, without an insert, was used as a negative control. The ligation mixes were incubated at 16°C overnight, after which 10 μl of each ligation mix was used to transform 100 μl of chemically competent DH5*α*, *E. coli* cells, (see Appendix 7.1). The transformation mix was plated on Luria Bertani (LB) agar plates with 100 μg/ml ampicillin (Roche, Germany) (Appendix 7.2.1 and 7.2.4), 20 μg/ml X-gal (Sigma, MO, USA) (Appendix 7.2.2 and 7.2.5), 200 μg/ml IPTG (Roche, Germany) (Appendix 6.2.3 and 6.5.5). Agar plates were incubated at 37°C overnight and blue/white screening was used to select for clones with inserts (white colonies), as described in Appendix 6.2. Several white colonies were randomly selected and grown overnight at 37°C at 200 rpm in 4 ml of LB broth with ampicillin (see Appendix 7.2.1).

2.1.5. Screening for positive clones

Four milliliter overnight cultures containing plasmids with untreated and sodium bisulphite treated amplicons isolated, using a high pure plasmid isolation kit (Roche Applied Science, Germany) as described in Appendix 7.3. Bacterial cells were lysed under alkaline conditions established by the sodium hydroxide present in the lysis buffer, while all the bacterial RNA is degraded by the RNase treatment. In the presence of high salt (binding buffer), DNA binding conditions are established, and all the contaminants (genomic DNA, and cellular debris) were removed by centrifugation. The supernatant, which contain the plasmid DNA was transferred to the columns (with glass fiber fleece). Nucleic acids (RNA and DNA) bind to the surface of the glass fiber fleece and get immobilized due to the high ionic strength. Contaminants are then removed during the wash steps, using wash buffers that contains ethanol to ensure the removal of salts. The purified plasmid was eluted using the Elution buffer with low ionic strength, enabling the plasmid to be unbound from the column. The isolated plasmids were screened for the presence of correct sized inserts (243 bp) by double digests with *Eco*RI and *Hin*dIII (New England BioLabs, UK), at 37°C for 1 hour. The reaction contained 1.5 μg DNA, 1X

concentration of *Eco*RI buffer, 2 U *Eco*RI and 2 U *Hin*dIII to a final volume of 20 μl. The enzymes have unique sequences that are found on the MCS of pTZ57R/T, and they are not present in the insert. Restriction fragments were examined by electrophoresis on a 1 % agarose under the same conditions as previously described. Based on restriction fragment size, clones with correct size inserts (243 bp) were selected and sequenced (Inqaba Biotech, South Africa) using the universal M13/pUC reverse primer (5' CAGGAAACAGCTATGAC 3') flanking the MCS of the pTZ57R/T corresponding to the N-terminus of the *β*-galactosidase gene. Raw sequencing data was edited using Finch TV 14.0 (Geospiza, WA). The sequences were subsequently aligned using the Vector NTI Suite 9, Align X (Invitrogen, WA) to determine the conversion of cytosines in the treated DNA. Clones containing 10 minutes treated inserts were named pTZ-LTRc-T1, those with 150 minutes treated inserts were termed pTZ-LTRc-T4, and the ones with the untreated insert were referred to as pTZ-LTRc-U.

2.1.6. Generation of sense and antisense strands by PCR.

The constructed lhRNAs would consist of a stem region containing an untreated antisense strand and a bisulphite treated sense strand. This would allow for the antisense strand to hybridize completely with the LTR target sequence of HIV-1, thereby allowing target mRNA cleavage by RISC. The treated sense strand would serve to generate a mismatched lhRNA *in vivo* that would preferably not induce interferon response in mammalian cells. In the construction of the lhRNAs with mismatches, it was also necessary to have a control hairpin (hairpin that would have an untreated sense strand). This would be important in determining the effects of the treated hairpins on the target, in comparison to the untreated hairpins, as well as determining whether these hairpin constructs would have the ability to induce interferon response in the cells. Furthermore, generation of the specific strands that would form a hairpin was facilitated by designing primers with linkers, like *Spe*I sequence (Fig. 2.2C), which was the sequence responsible for loop formation between the sense and the antisense strands of the lhRNA. In addition, *Xho*I sequence on the sense strand (Fig. 2.2 A and B) and *Not*I sequence on the antisense strand (Fig. 2.2 C) were used in cloning the hairpin sequence into a mammalian expression vector, pCiNeo (Fig. 2.5). This sequence would be cut from the T/A vector

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(pTZ57R/T) using these two restriction enzymes and be ligated into the eukaryotic vector pCiNeo, that had been digested by these restriction enzymes, as they are present on the vector.
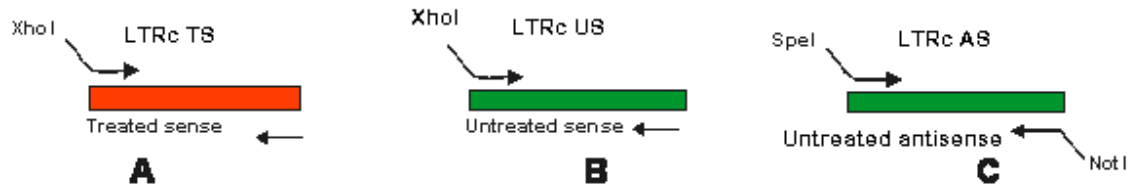
Figure 2.2 Generation of specific long hairpin RNA sequences by PCR. (Red indicates sodium bisulphite treated sequence and green indicates untreated sequence) A. Treated sequence for the generation of mutated sense strand, was amplified with LTRc TS F & R primers with linkers (*Xho*I on the forward primer) **B.** Untreated sequence for the generation of untreated sense strand was amplified with LTRc US F & R primers with linkers (*Xho*I on the forward primer) and **C**. Untreated sequence for the generation of antisense strand was amplified with LTRc AS F & R with linkers (*Spe*I on the forward primer and *Not*I on the reverse primer).

The generation of specific of hairpin strands required three sets of primers, each consisting of a forward (F) and reverse (R) (Fig.2.2). LTRc AS F and R (primers that were used in the generation of the untreated antisense (AS) strand, Fig. 2.2C) that would generate 133 bp PCR product consisting of 118 bp target sequence and 15 bp from the linkers. LTRc US F and R (primers that were used for the generation of the untreated sense (US) strand, Fig. 2.2 B) that would generate 125 bp PCR product consisting of 118 bp target region and LTRc TS F and R (primers that were used in the generation of treated sense (TS) strand, (Fig. 2.2A), which would also generate a 125 bp PCR products. To generate sense sequences with mutations, treated DNA was amplified with LTRc TS primers with a linker (the linker sequence is the section of the primer highlighted in bold) containing the *Xho*I restriction enzyme recognition site on the LTRc TS F (5'***GCTCGAG***GAA<u>T</u>CCA<u>T</u>TG<u>T</u>TTAAGC<u>T</u>TCA 3') and LTRc TS R (5'TTTC<u>T</u>ACA<u>T</u>TAACA<u>T</u>AAAGGGTCTG 3') (Fig. 2.2A). The sense sequence without mutations was generated by amplifying the original PCR product with LTRc US primers containing *Xho*I restriction sites on the LTRc US F (5' ***CTCGAG***GAACCCACTGCTTAAGCCTCA 3') and LTRc US R

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(5' TTTCCACACTAACACAAAGGGTCTG 3') (Fig. 2.2B). The primers used in the generation of treated and untreated sequences differed partially. On the LTRc TS primers, the T residues highlighted by being underlined, are C residues on the LTRc US. C residues were replaced by T residues so that the primers could bind on the treated DNA, which had converted C residues.

To generate an antisense sequence, the original amplicon product that was not treated with sodium bisulphite was amplified with LTRc AS primers (primers that are specific for the untreated DNA) with linkers, *Spe*I restriction site on LTRc AS F (5' ***GACTAGT***GAACCCACTGCTTAAGCCTCA 3') and *Not*I restriction site on LTRc AS R (5'***GGCGGCCGC***TTTCCACACTAACACAAAGGGTCTG3') (Fig. 2.2C). All three PCR reactions contained 1 ng of the original PCR product, each primer to a final concentration of 0.375 μM, dNTPs mixture to a final concentration of 0.25 mM; *Taq* buffer to a final concentration of 1X and 2 U *Taq* polymerase (Promega, USA) and the amplification process was carried out under the same conditions as in section 2.1.2

Amplification products were purified using the Qiagen MinElute^{TM} Gel Extraction kit (Qiagen, CA, USA), as described in Appendix 7.5. Amplicons were used as inserts in a T/A cloning process using pTZ-57R/T vector, as previously described. The products of this cloning process were pTZ-LTRc-US (plasmid with untreated sense sequence), pTZ-LTRc-TS1 (plasmid with sense sequence treated for 10 minutes), pTZ-LTRc-TS4 (plasmid with sense sequence treated for 150 minutes) and pTZ-LTRc-AS (plasmid with antisense sequence).

2.1.7 Generation of a complete long hairpin RNA sequences by head to head cloning

When performing head to head cloning, the fragments to be joined must be of a specific orientation so as to produce the desired long hairpin sequence. When cloning into a T/A vector, the PCR products with ddA overhangs are introduced in either a forward or reverse orientation as directionality cannot be established in T/A cloning. Here, the antisense sequence had to be in the reverse orientation and the sense in the forward orientation, as shown by inserts direction in Fig. 2.3 C and D, while A and B show inserts in the incorrect orientation. Orientation of the insert is very important as head to head cloning would generate a hairpin with complementary stem sequences to trigger the

RNAi pathway. In this study, the plasmid with the antisense sequence pTZ-LTRc-AS was double digested with *Eco*RI (NEB, UK) found on pTZ57R/T MCS, and *Spe*I (Fermentas, WI, USA) which was incorporated into the antisense strand through a linker (Fig. 2.2 C), in a 50 µl reaction volume with 2 µg DNA, 1X *Eco*RI buffer, 1X BSA, 2 U *Spe*I and 2 U *Eco*RI. This digestion had two possible results in different orientations as shown in Fig. 2.3 A and C. The desired fragment size was 163 bp, which contained 133 bp of the antisense sequence and 30 bp of the MCS (between *Eco*RI and *Not*I in Fig. 2.3 C). Another possible result would be the one shown in Fig. 2.3 A, which would generate a 133 bp fragment. Both results contain the fragment of interest but in different orientation. The fragment obtained in Fig.2.3 C would be the one in the correct orientation for creating the head to head construct (Fig. 2.3 G). The digest reaction was then examined on 2% agarose gel.

Plasmid pTZ-LTRc-S in Fig. 2.3 B and D is a representative of all the plasmids containing the sense sequence at different treatment time points, namely: untreated (pTZ-LTRc-US); treated for 10 minutes (pTZ-LTRc-TS-1); and treated for 150 minutes (pTZ-LTRc-TS-4). pTZ-LTRc-S plasmids were double digested with *Xba*I (Fermentas, WI, USA) and *Eco*RI (New England BioLabs, UK), in a 50 µl reaction volume with 0.5-1 µg DNA, 1X buffer 2, 1X BSA, 2 U *Eco*RI and 2 U *Xba*I. Both restriction enzyme sites are found on the MCS of the vector. Fig. 2.3 B and D show two possible products when digesting with these two restriction enzymes, and D is the correct one, as it would have the fragment of interest in the correct orientation for head to head cloning. The desired fragment size would be 2981 bp, which contained the 125 bp sense sequence fragment in addition to the vector sequence. Fig. 2.3 B would also have the insert but it would be in an incorrect orientation. This fragment was examined on 0.8% agarose gel. The antisense (163 bp) and sense (2981 bp) fragments (Fig. 2.3 E and F) were excised from the gels and DNA was extracted using the MinElute Gel Extraction kit as described in Appendix 7.5. The two fragments were ligated together on 1:1 molar ratio to produce a plasmid pTZ-LTRc-S/As (Fig. 2.3 G) encoding for the hairpin sequence. The loop of the hairpin was formed by the ligation of the sense and antisense fragments through *Spe*I and *Xba*I sequences, which produce complementary 5' overhangs. Ligation reactions were used to transform *E. coli* cells, DH5α.
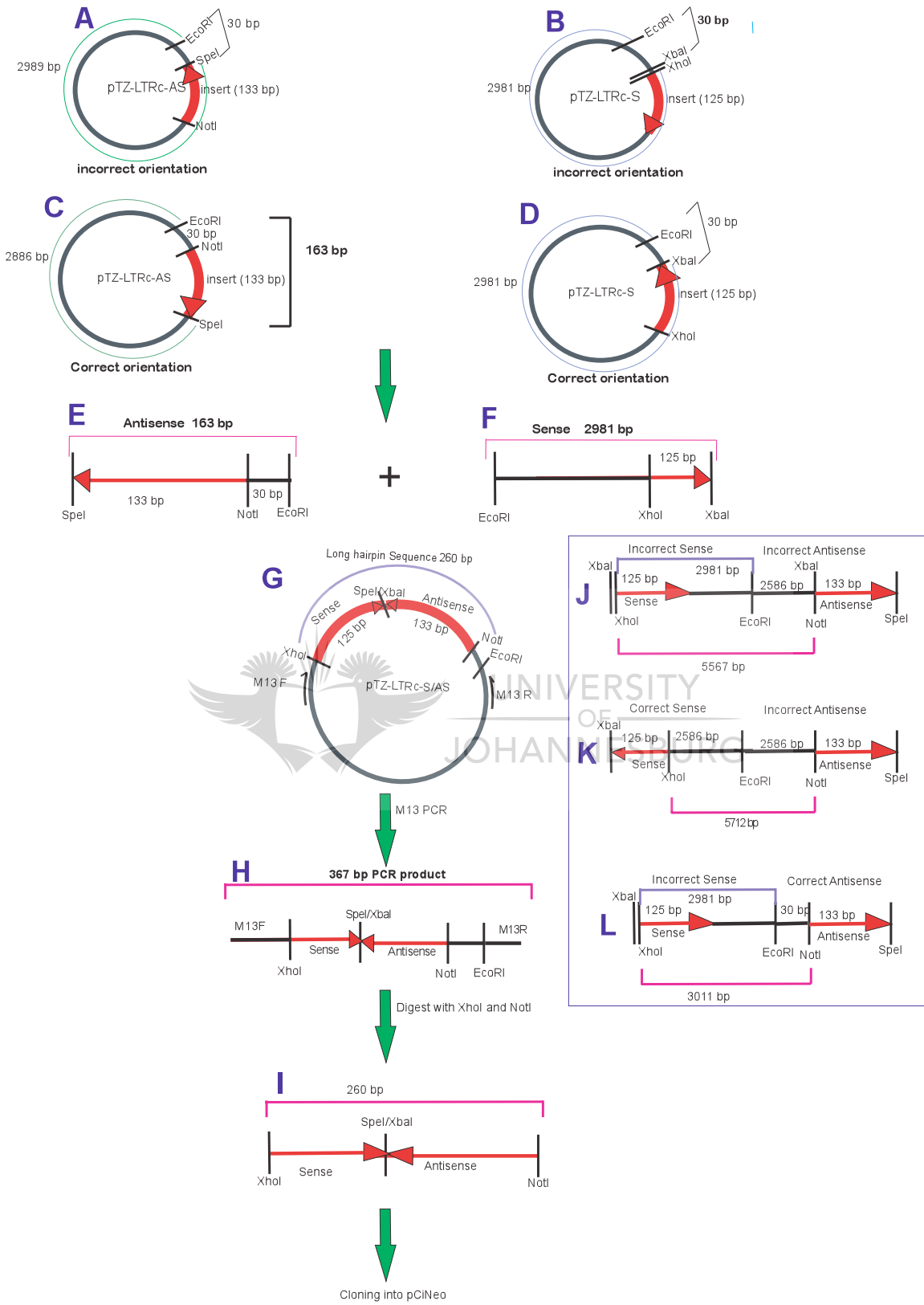
Figure 2.3 **Head to head cloning of hairpin sequences.** A and B pTZ-LTRc-AS and pTZ-LTRc-S showing orientations that would not allow for the two sequence to join head to head. The sense and antisense inserts are indicated in red and the direction of the arrows indicate the head of the product. C and D pTZ-LTRc-AS and pTZ-LTRc-S showing the orientation of the sense and antisense sequences in correct orientations that would allow them to join head to head. The green and the purple outer semi-circle indicate the region that forms the 2886 bp fragment for the antisense and 2981 bp for the sense sequence. E. and F antisense and sense fragments containing the inserts in desired orientations, as indicated by the direction of the arrow. G. shows pTZDuLTRc-S/AS containing long hairpin sequence, which is 260 bp after head to cloning, and the M13 primers binding sites used for the screening of positive clones. H. Shows the M13 PCR product which is a 367 bp fragment. I. Shows a 260 bp fragment, which is the product of a restriction digest with *Xho*I and *Not*I to be introduced into a eukaryotic vector, pCiNeo, for hairpin synthesis. J, K and L show the possible orientations (other than the head to head orientation in G) in which the sense and antisense sequences would have joined.

Transformed cells were plated on LB agar with ampicillin and incubated overnight at 37°C. To select for positive clones, colonies were screened by PCR using M13 forward primer (5' GTTTTCCCAGTCACGAC 3') and reverse primer (5' CAGGAAACAGCTATGAC 3') (Fig. 2.3 H). These primers were chosen as their sites flank the T/A cloning site of pTZ57R/T (Fig. 2.1) which contained the long hairpin cassette. The expected PCR product was 367 bp, which contained the sense and the antisense sequences with *Spe*I/*Xba*I loop, sequences of the linkers (*Xho*I and *Not*I), MCS region and M13 primer sequences and additional sequences between MCS and M13 primer sites. A number of clones were randomly selected by touching a colony with a pipette tip and diluting the contents of the tip in 10 μl of ultrapure water (SABAX, South Africa). Five microlitres of the diluted colony was used as a PCR template and another 5 μl was used to inoculate 4 ml LB broth. Reaction mixtures contained 0.2 μM of each primer, 0.1 mM dNTPs, 1X *Taq* buffer and 2 U *Taq* polymerase (Promega, USA). Reactions were cycled in thermal cycler at 95°C for 2 minutes to activate *Taq* polymerase, followed by 20 cycles of denaturation at 95 °C for 15 seconds, annealing at 55°C for 15 seconds, primer extension at 72°C for 15 seconds and a final extension of 72°C for 10 minutes. Amplicons were examined on 1% agarose gel. Using the remaining 5 μl of the diluted colony, the PCR positive clones were used for plasmid isolation. To select for plasmids containing both sense and antisense sequences, isolated

plasmids were double digested with *Xho*I and *Not*I (Fig. 2.3 I) in a 20 µl reaction volume with 1-1.5 µg DNA, 1X NEB buffer 3 (New England BioLabs, UK) and 2 U of *Not*I (New England BioLabs, UK) and 2 U *Xho*I. These restriction enzymes were used as their sequences are found on the hairpin sequence; they were introduced by linkers that were present on the primers used in the generation of specific strand sequences. They are also found on the pCiNeo and they would be used in cloning the hairpin sequence into this vector. The expected fragment size of 260 bp was examined by gel electrophoresis on 1% gel with 1 µg/ml ethidium bromide. The 260 bp fragment with sense and antisense sequences was excised from the gel and purified using Qiagen gel extraction kit (Qiagen, CA, USA) see Appendix 7.5**.** Constructs with incorrect orientation of the inserts would have yielded fragments much bigger than the expected 260 bp. Fig. 2.3 J indicates a potential head to tail orientation (both sequences in incorrect orientation as per Fig.2.3 A and B), where restriction digest with *Xho*I and *Not*I would have generated a 5567 bp fragment, which would contain 2981 bp fragment highlighted in blue in Fig. 2.3 B and J; and 2586 bp vector sequence without the 133 bp antisense fragment. Fig. 2.3 K indicates a potential tail to tail orientation (sense in the correct orientation (Fig.2.3 D) and antisense in the incorrect orientation (Fig. 2.3 A)), which would have generated 5712 bp fragment without sense and antisense sequences but with vector sequences from both A and D. Fig. 2.3 L indicates another potential head to tail orientation, which would have generated 3011 bp fragment with only the sense sequence without the 133 bp antisense sequence.

2.1.8 Cloning of long hairpins into a mammalian expression vector, pCINeo and screening of hairpin cassette clones

pCI neo (Promega, WI, USA) (Fig. 2.5), a eukaryotic expression vector, was used for the transcription of the long hairpin sequences under the control of the CMV promoter and an SV40 poly-adenylation signal. This vector also contains an SV40 enhancer/promoter, which drives neomycin resistance. Two micrograms of the vector was linearized by digesting with *Xho*I and *Not*I in a 20 µl reaction volume with 1X NEB buffer 3 (New England BioLabs, UK) and 2 U of *Not*I (New England BioLabs, UK) and 2 U *Xho*I, and dephosphorylated with calf intestinal phosphatase (CIP) (Boehringer Mannheim) to

prevent self ligation of the vector. The dephosphorylation reaction contained 3 U of the CIP and 1 X CIP buffer (1 mM ZnCl, 1 mM MgCl, 10 mM Tris, pH 8.3). The reaction was incubated at 37°C for 1 hour and examined on a 0.8% agarose gel. Single bands of approximately 5.4 kb (vector size) were excised from the gel and purified as previously described. The purified vector was ligated with purified long hairpin sequences, as both the vector and the hairpin sequence were digested with *Xho*I and *Not*I, which had compatible cohesive ends.
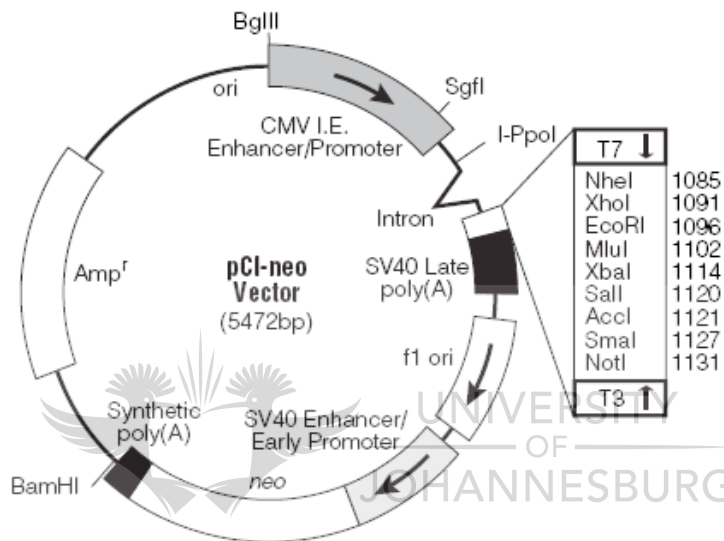
Figure 2.4 pCiNeo vector map showing the long hairpin sequence that was cloned in this mammalian expression vector-between T7 and T3 regions. Also this map shows the CMV region, SV40 promoter region and different restriction enzyme sites that are used in different cloning experiments.(Promega, WI, USA) http://www.promega.com/tbs/tb215/tb215.

The ligation reaction was at a 1:5 molar ratio (vector: insert) in the presence of T4 ligase and incubated at 16°C for 4 hours and this was used to transform *E. coli*, SURE cells, (see appendix 7.1).

To screen for positive clones, colonies were randomly selected and used to inoculate 4 ml of LB broth with ampicillin and incubated overnight. Plasmids were isolated using the Endotoxin Free maxi-prep kit (Qiagen, USA) as described in Appendix 7.3. The isolated plasmids were double digested with the same enzymes used in the cloning process,

namely *Xho*I and *Not*I under the same conditions as described previously. The digested plasmids were examined for the correct fragment (260 bp) by agarose gel electrophoresis. The long hairpin RNAs were named as follows: pCi-LHRNA-1 (hairpin with no mismatches), pCi-LHRNA-2 (hairpin with 10 minutes treated sense) and pCi-LHRNA-3 (hairpin with the 150 minutes treated sense).

2.2 Testing the efficiency of hairpins and checking for interferon induction

2.2.1 Cell culture

The human embryonic kidney 293 (HEK293) cell line was maintained in Dulbeco's modified eagles medium (DMEM, BioWhittaker, Walkersville, MD) supplemented with 10 % heat inactivated faetal calf serum (FCS, Delta Bioproducts, Johannesburg, SA) at 37°C, 5% CO_2 and 1% humidity.

2.2.2 Transfection

Transfection is a technique in which exogenous DNA is introduced into eukaryotic cells. This process is performed by introducing DNA to a cationic lipid as a non-invasive means to deliver DNA into the cell. In this study, long hairpins were introduced into HEK293 cells using Lipofectamine2000 (Invitrogen, USA).

To assess the effect of long hairpin RNAs against the target (pTZ-DuLTR-Luci) *in vitro*, HEK 293 cells were co-transfected with long hairpin constructs, namely pCi-lhRNA-1 (hairpin with an untreated sense sequence), or pCi-lhRNA-2 (hairpin with a sense sequence that was treated for 10 minutes) or pCi-lhRNA-3 (hairpin with a sense sequence that was treated for 150 minutes) in addition to the target plasmid (pTZ-DuLTR-Luci) shown in Fig. 2.5. The expression of Firefly luciferase to be controlled is in the target plasmid, as the LTR promoter sequence (target region) is located upstream from the Firefly luciferase gene. Transcription initiated by the LTR will generate a luciferase mRNA with the TAR secondary structure at the 5' terminus. Transfection of *in vitro* transcribed LTR RNA into Dicer producing cells such as HEK293 should allow processing of the TAR stem loop by cellular Dicer, thereby downregulating Firefly luciferase expression.
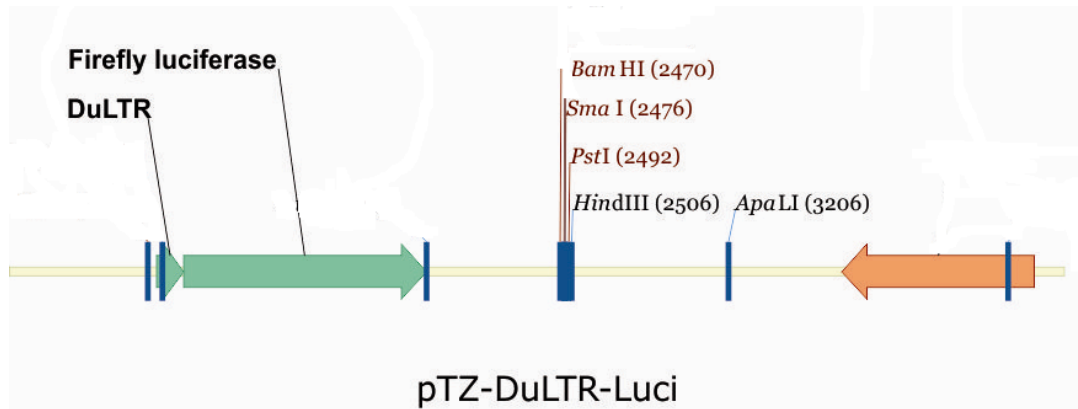
Figure 2.5. pTZ-DuLTR-Luci showing the target region indicated as DuLTR and found upstream of Firefly luciferase gene

Empty vectors, pTZ-U6+1 and pCiNeo served as negative controls. Negative controls were used as references, i.e. any value that is the same or above that of the control would mean that there was no target knockdown, and values below that of the control would indicate that hairpins were able to silence the target sequence. Negative controls were not expected to silence gene expression as they could not express products that would downregulate expression of Firefly luciferase from the co-transfected target plasmid, hence maximum luminescence was expectd. The pTZ-lhRNA-TAR-2 and pTZ-lhRNA-TAR-3 plasmids were used as positive controls because they had been previously shown to be effective against TAR (Barichievy *et al*., 2007). As TAR is situated in the LTR, these hairpins were expected to silence the target by cleaving on the TAR region of the LTR target sequence.

HEK293 cells were seeded 24 hours prior to transfection at a density of 1.2×10^5 cells per well in 24-well culture dishes. To determine the effects of lhRNAs encoding plasmid on a target, HEK293 cells were transfected with 90 ng of target plasmid (pTZDuLTR-Luci), 5 ng pCMV-Renilla (donated by Samantha Barichievy) and 900 ng of long hairpin RNA encoding plasmid vector. To compare the effect of Tat on the target, 90 ng of pCMV-Tat (donated by Samantha Barichievy) was also added in some wells. *Renilla* (pCMV-Renilla) was included so that the Firefly luciferase activity could be normalized to *Renilla* luciferase activity to control for differences in transfection efficiency. *Renilla* luciferase is under the control of the CMV promoter and should therefore not be affected by the long hairpin constructs. Transfections were carried out

using 1 µl Lipofectamine 2000 (Invitrogen, USA) to 1 µg DNA per well with OptiMem (Gibco, BRL). All experiments were performed in triplicate.

2.2.3 Dual luciferase assay

The dual luciferase assay is a sensitive method used to express and measure two luciferase reporter enzymes, Firefly luciferase and *Renilla* luciferase within a single system. The Firefly luciferase reporter is measured first by means of Luciferase assay reagent (LAR II) to generate luminescent signal. Once the luminescent signal is quantified with a luminometer, the signal is quenched by the addition of Stop and Glo reagent. This reagent, however, simultaneously activates the *Renilla* luciferase reaction and the signal is quantified by a luminometer to normalize the Firefly luciferase activity. Renilla luciferase encoded by the control plasmid is used to normalize the Firefly luciferase enzyme activity. This is achieved by dividing Firefly luciferase activity with *Renilla* luciferase activity. Luciferase assays were performed using the Dual Luciferase Reporter Assay kit (Promega, WI, USA). Briefly, 48 hours post transfection media was aspirated off the cells to be assayed, 100 µl of 1X passive lysis buffer (Appendix 7.7) was added per well of a 24-well dish and incubated for 30 minutes at 37°C and 5% CO_2. Following incubation, 10 µl per sample was pipetted into a plate reader tray and assayed in a Veritas Luminometer (Turner Biosystem, CA, USA) using 50 µl each of Firefly reagent (LARII) (Appendix 7.7) and *Renilla* reagent (Stop and Glo) (Appendix 7.7) per sample. Target-specific Firefly luciferase expression was normalized to background *Renilla* luciferase expression. Average expression ratios for pCiNeo (empty vector) were set to 100% and relative expression levels for other samples were set accordingly. Two independent experiments in triplicate were performed and the data were expressed as the mean ± standard error of the mean. Using Student T-test, statistical difference was considered significant for $P < 0.05$.

2.2.4 Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) is generally used to detect and quantitate mRNA sequences within a cell sample. qRT-PCR was used to determine the expression of

IFN-β genes that are involved in the interferon pathway, which may have been induced by the lhRNAs used in this study. Primers specific for IFN-β and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (a housekeeping gene) genes were used in a PCR reaction.

Twenty four hours prior to transfection, HEK293 cells were seeded in 24 well plates at a density of 1.5×10^5 cells per well. Cells were transfected with either 1 μg or 2 μg of Poly I:C (positive RNA control), 1 μg of pCI neo (empty vector, negative control) or 1 μg of each lhRNA (pCi-LHRNA-1, pCi-LHRNA-2, and pCi-LHRNA-3). Poly I: C is a synthetic analogue of dsRNA that has been demonstrated to have immunostimulatory effects resembling that of viral dsRNA. It triggers the immune response by interacting with Toll like Receptor 3 (TLR3), which is a dsRNA ligand that is highly expressed in immature dendritic cells (Alexopoulou *et al.*, 2001). In this study, it was used as a positive control as it is known to stimulate the immune response, so as to compare it's level of IFN β expression with that of the hairpins' and determine whether the long hairpin have the ability to trigger IFN response. To measure concentrations of IFN response related genes (IFN β), total RNA from HEK 293 cells transfected with positive control (Poly I: C) or lhRNAs or an empty vector acting as a negative control (pCINeo) were extracted using TriReagnet™ (Sigma, MO, USA) (Appendix 7.6). Resuspended RNA pellets were DNAse treated for 60 minutes at 37°C and reverse transcribed using Sensicript (Qiagen, GmbH, Germany) and oligo-dT primer to a final concentration of 1μM. The cDNA was then used as a template for PCR with the following primer sets (IDT, USA) to amplify IFN-β and GAPDH mRNA: IFN-β Forward: 5' TCC AAA TTG CTC TCC TGT TGT GCT 3', IFN-β Reverse: 5' CCA CAG GAG CTT CTG ACA CTG AAA A 3', GAPDH Forward: 5' AGG GGT CAT TGA TGG CAA CAA TAT CCA 3' and GAPDH Reverse: 5' TTT ACC AGA GTT AAA AGC AGC CCT GGT G 3'. All real time PCRs were carried out using the Roche Lightcycler V.2 (Germany). Controls included water blanks and RNA extracts that were not subjected to reverse transcription. *Taq* Readymix with SYBR green (Sigma, MO, USA) was used to amplify and detect DNA during the reaction. Thermal cycling parameters consisted of a hot start for 30 seconds at 95°C followed by 50 cycles of denaturation at 95°C for 10 seconds, annealing

at 58°C for 10 seconds and extension at 72°C for 10 seconds. Quantification of the PCR product was verified by SYBR green fluorescence intensity.

CHAPTER 3

3. RESULTS

3.1. Construction of long hairpin RNAs

The construction of lhRNAs used in this study was a multi-step process that involved target sequence selection, PCR, sodium bisulphite treatment, addition of specific linkers to sequences by PCR, cloning using pTZ57R/T T/A vector, sequencing, head to head cloning, and cloning into a mammalian expression vector pCi Neo.

3.1.1. Amplification of long hairpin sequences

The amplified pTZ-DuLTR-Luci plasmid was subjected to agarose gel electrophoresis to screen whether the region of interest was amplified; to test whether the primers were specific to their binding sequence (U5 region of the LTR) and that they annealed efficiently at 60°C. The fragment shown in Fig. 3.1, lane 2 shows the PCR product, which is 168 bp in length, indicated as ~170 bp on the figure. This fragment size resulted from amplifying 118 bp the target sequence with the unmodified LTRc that flank the target.
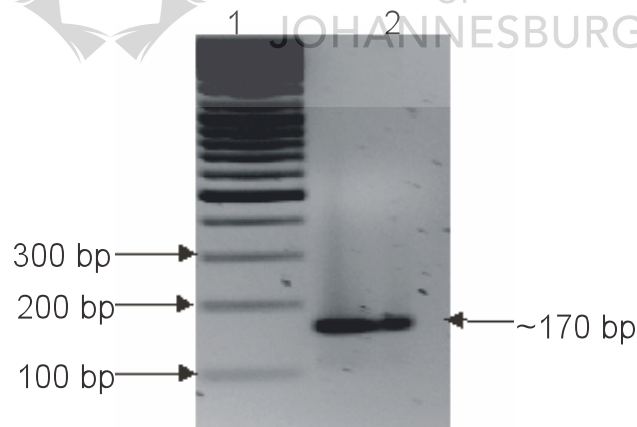
Figure 3.1 2% agarose gel with ethidium bromide showing amplified pTZ-DuLTR-Luci: Lane 1 is the molecular weight marker; lane 2 is the PCR product, which is ~170 bp in length.

3.1.2. Screening for positive clones containing sodium bisulphite treated DNA and sequencing

After treating the PCR product with sodium bisulphite and inserting the treated and untreated amplicons into pTZ57R/T, the success of the cloning procedure was established by performing a double digest with *EcoR*I and *Hin*dIII that cleave within the MCS of the plasmid, to screen for positive clones.
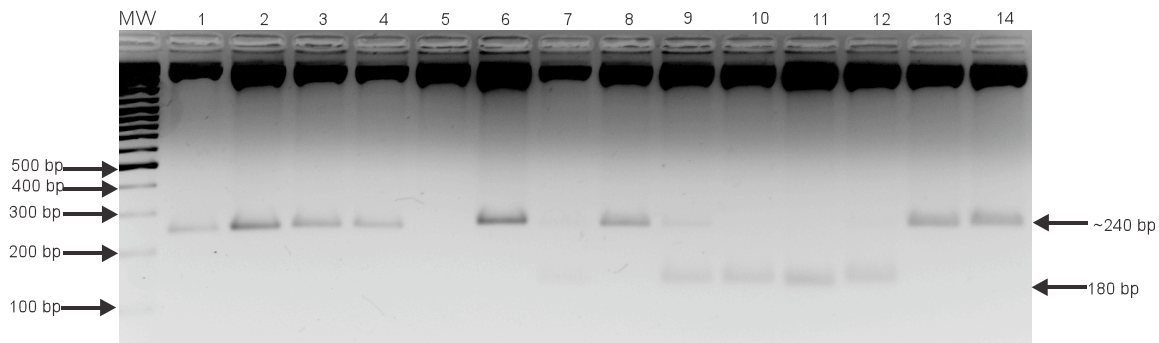
Figure 3.2 1% agarose gel showing the screening of positive clones by digesting with *EcoR*I and *Hind*III. Lane MW is a molecular weight marker, lanes 2, 3, 4, 6, 8, 13 and 14 have positive clones with an insert of 243 bp, indicated as ~240 bp on the figure, lanes 5 and 7 indicate clones that had no inserts, while lanes 7, 9, 10, 11 and 12 have clones with 180 bp fragment, which is an artifact, as the fragment without an insert could have been ~70 bp.

According to the agarose gel screening (Fig. 3.2), only 7 out of the 14 screened clones had the expected insert size of 243 bp. This fragment contains the 168 bp fragment shown in Fig. 3.1 and the MCS sequence, which is 75 bp long, which is restricted by *EcoR*I and *Hind*III. However, 5 clones showed negative results (absence of the 243 bp fragment), but 180 bp fragments. The 180 bp fragment is unknown and could be an artifact, as the size of the fragment without an insert is 75 bp, which is the MCS of the pTZ57R/T vector. Four of the 7 clones were selected and confirmed by sequencing.
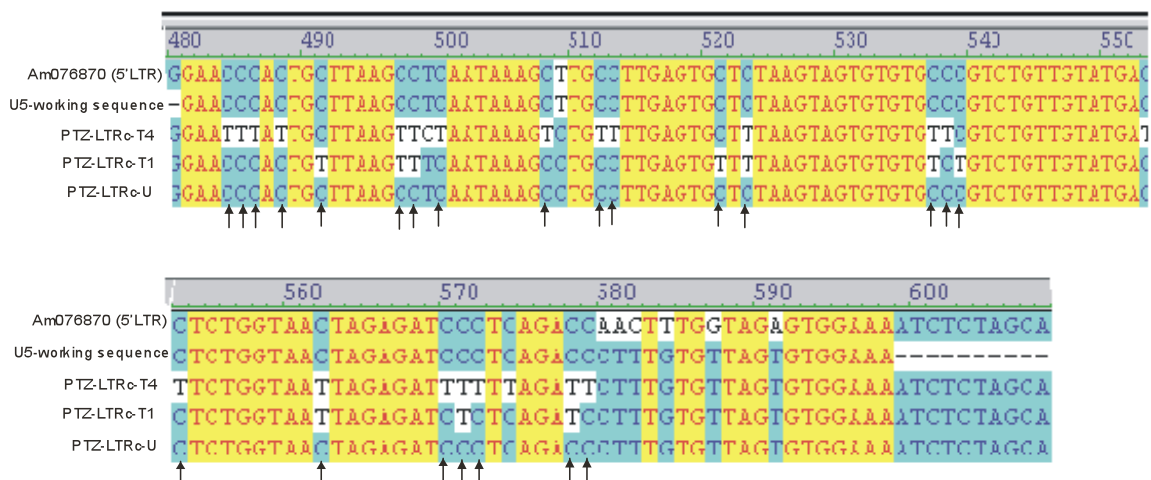
Figure 3.3 Sequencing results showing the conversion of cytosine residues to thymine residues on the sense sequence of the bisulphite treated amplicons. AM076870 is the 5' LTR region of Du151 from the GenBank (http://www.ncbi.nlm.nih.gov/nuccore/119662088). U5 working sequence is the target region used in designing the hairpins, pTZ-LTRc-T1 and pTZ-LTRc-T4 are the treated sequences at 10 and 150 minutes, and pTZ-LTRc-U is the untreated PCR product. The bases with a white background are the newly converted T's and the arrows show columns where conversion of bases occurred.

The sequencing results in Fig. 3.3 show that the pTZ-LTRc-T1, plasmid containing the insert treated for 10 minutes, had 10 cytosine residues (C's) converted to thymine (T's) residues, whereas the one that was treated for 150 minutes, pTZ-LTRc-T4 had 22 C's converted to T's out of a total of 27 cytosines. The sequencing results show 118 bp sequence, which is the actual length of the target sequence (lacking extraneous primer sequences) used in designing the hairpin that were constructed in this study.

3.1.3. Generation of sense and antisense strands by PCR

Clones produced by amplifying treated and untreated DNA with LTRc US, LTRc TS and LTRc AS primers for the generation of sense and antisense sequences resulted in the production of pTZ-LTRc-US (plasmid with untreated sense), pTZ-LTRc-TS1 (plasmid with sense strand treated for 10 minutes), pTZ-LTRc-TS4 (plasmid with sense strand treated for 150 minutes), and pTZ-LTRc-AS (plasmid containing untreated antisense strand) these results are not shown.

3.1.4. Generation of a complete long hairpin RNA sequence by head to head cloning

Having generated the sense and the antisense sequences, the products needed to be cloned together to generate a hairpin after transcription. This was achieved by digesting the antisense sequence with *Eco*RI and *Spe*I (*Eco*RI site is found on the MCS of the vector and *Spe*I was incorporated on the antisense sequence through a linker, whereas the sense sequence was digested with *Eco*RI and *Xba*I (which are both found on the MCS of the vector). The digested fragments were 163 bp (indicated as ~170 bp on the gel) from the antisense sequence and 2981 bp fragment from the sense sequence (this fragment contained the pTZ-57R/T vector sequence).
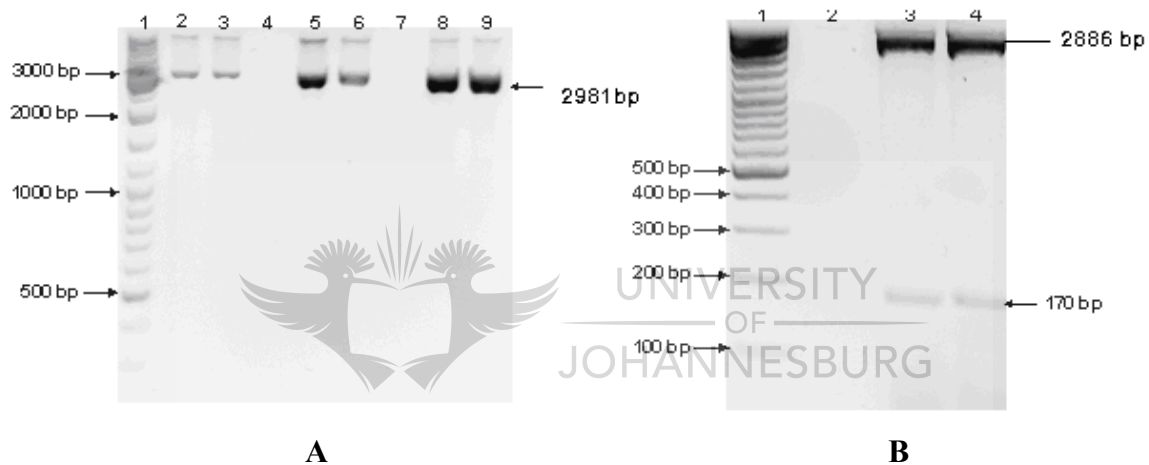
Figure 3.4 Agarose gels showing sense and antisense cassettes: **A.** Sense sequence digested with *Eco*RI and *Xba*I showing a 2981 bp fragment on a 0.8% agarose gel. Lane 1 represents the molecular weight marker, lanes 2 and 3 untreated sense sequences, Lanes 5, 6, 8 and 9 are the treated sense sequences at 10 minutes and 2.5 hours, respectively. **B.** Antisense sequence digested with *Eco*RI and *Spe*I showing a 170 bp fragment and 2886 bp on 2% agarose gel. Lane 1 represents the molecular weight marker, whereas lanes 3 and 4 represent fragments containing antisense sequence.

The two fragments were ligated to form one fragment with both sense and antisense sequences in head to head orientation. To screen for clones with both sequences, PCR using M13 universal primers was performed and positive clones had fragments of 367 bp, indicated as 400 bp in Figure 3.5 (left).
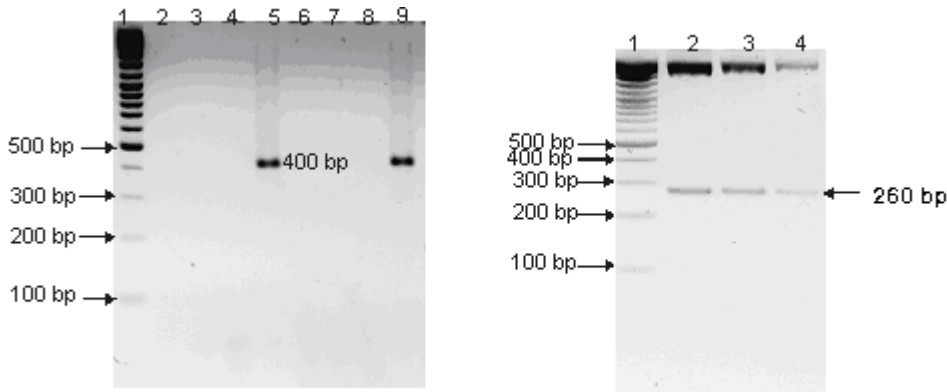
Figure 3.5 PCR screening of clones using M13 generic primer 1% agarose: (LEFT GEL): Lane 1 is a molecular weight marker, lanes 2, 3 and 4 are the negative clones, lane 5 is a positive clone with an expected fragment size of 367 bp containing 10 minutes treated sense and antisense sequences, lane 6 to lane 8 are the negative clones and lane 9 is a positive clone with an expected fragment size of 400 bp containing 150 minutes treated sense and antisense sequences. **(RIGHT GEL):** Long hairpin cassettes digested with *Xho*I and *Not*I, with lane 2 containing the long hairpin sequences (260 bp) with an untreated sense and lanes 3 and 4 with treated sense at 10 and 150 minutes time intervals, respectively.

The 367bp (indicated as 400 bp on the gel) fragment contained the sense (125 bp) and antisense (133 bp) sequences, MCS sequence (75 bp), and M13 primer sequences (34 bp). Subsequently, positive clones were digested with *Xho*I and *Not*I (linkers on the sense and antisense sequences) to cleave off the exact long hairpin sequences as shown in (Fig. 3.5, right), which was a 260 bp fragment on lanes 2, 3 and 4.

3.1.5 Cloning of long hairpins into a mammalian expression vector, pCiNeo and screening of hairpin cassette clones

To express the long hairpin sequences, *Xho*I and *Not*I fragments were purified from a gel and cloned into a mammalian expression vector, pCiNeo under the control of CMV, polymerase II promoter. CMV enhancer/promoter is a powerful promoter as it allows for strong, constitutive expression in a wide range of cell types. Clones were digested with *Xho*I and *Not*I, to screen for positive clones (Fig. 3.6).
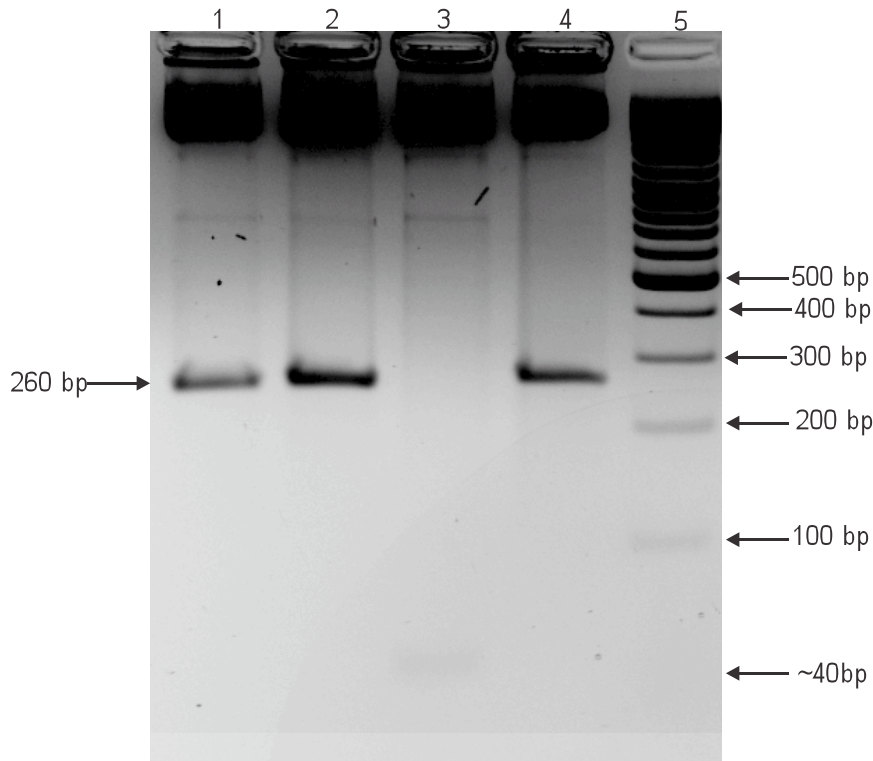
Figure 3.6. 1% agarose showing long hairpin sequences digested with *Xho*I and *Not*I (260 bp). Lane 1: pCi-LHRNA-3, lane 2: pCi-LHRNA-2, lane 3: pCiNeo (expression vector), lane 4 pCi-LHRNA-1 lane 5 is the molecular weight marker.). In lane 1 there is pCi-LHRNA-3, lane 2 has pCi-LHRNA-2, lane 3 has the pCiNeo that had been digested with *Xho*I and *Not*I; dephosphorylated using CIP; and lane 4 has pCi-LHRNA-1.

According to figure 3.6, the hairpin sequence was ~260 bp, which includes the sense and the antisense sequences, *Spe*I recognition site (loop sequence), and the primer sequences with linkers, *Xho*I and *Not*I sequences. These results were comparable to those obtained when the hairpin sequence was excised from the T/A cloning vector using *Xho*I and *Not*I (Fig. 3.5). The 3 clones that were constructed were pCi-LHRNA-1 (hairpin with untreated sense sequence), pCi-LHRNA-2 (hairpin with 10 minutes treated sense sequence) and pCi-LHRNA-3 (hairpin with the 150 minutes treated sense sequence).

3.2. Testing the efficiency of long hairpin RNAs against a target sequence.

3.2.1. Testing the effect of the long hairpin RNAs on the target and dual luciferase assay

The effect of long hairpin RNAs against the target was assessed by co-transfecting long hairpin constructs, which were either pCi-lhRNA-1 or pCi-lhRNA-2 or pCi-lhRNA-3 and the target plasmid (pTZ-DuLTR-Luci). The expression of Firefly luciferase to be controlled is in the target plasmid, as the LTR promoter sequence which is the target for the lhRNAs, is located upstream from the Firefly luciferase gene. Therefore, if the target sequence is knocked down, there would be no Firefly luciferase expression. The dual luciferase assay uses both Firefly luciferase and *Renilla* luciferase, and their ratios were normalized to determine the target knockdown. Empty vectors, pTZ-U6+1 and pCiNeo served as negative controls, whereas pTZ-lhRNA-TAR-2 and pTZ-lhRNA-TAR-3 were used as positive controls, which had been shown to be effective against TAR (Barichievy *et al.*, 2007). The pTZ-lhRNA-TAR-2 and pTZ-lhRNA-TAR-3 hairpin constructs target different regions within the TAR sequence of HIV-1. They were generated by amplifying DNA encoding these regions and ligated into a PCR cloning vector, pTZ57R/T.

The experiments were performed both in the presence and in the absence of Tat to determine the influence of Tat. Tat plays an important role in the HIV-1 transcription process as described in chapter 1 and is expected to upregulate Firefly luciferase expression. Two days post transfection, Firefly and *Renilla* luciferase expression was measured and the ratio was used to determine relative luciferase activity. Luciferase expression in the presence of pCiNeo was set at 100% as this was the vector in which the hairpins were expressed. Averages and standard deviations represent three independent transfections.

Fig. 3.7 shows that pTZ-U6+1 and pCiNeo, did not knockdown gene expression as expected, as they did not have the hairpin sequence that would bind to the target, thereby blocking expression of the LTR sequence. This was observed by ~190% Firefly luciferase expression in the presense of pTZ-U6+1 as shown in Fig. 3.7 A, which was the post-transcriptional gene silencing (PTGS) experiment performed in the absence of Tat. Therefore, 100-190% is the range of Firefly luciferase expression that lies between the

negative controls used. This range is therefore indicative of a lack of gene silencing. Also, Fig. 3.7 B, which was the PTGS experiment in the presence of Tat, showed that Firefly luciferase expression in the presence of pTZ-U6+1 was upregulated to ~290 %, therefore 100-290% would be the range of Firefly luciferase expression that lies between the negative controls used under the influence of Tat. The high expression of Firefly luciferase in Fig. 3.7 B in the use of pTZ-U6+1, pTZ-lhRNA-TAR-2 or pTZ-lhRNA-TAR-3 could be attributed to Tat's ability to increase transcription when compared to it's absence (Fig. 3.7A) as it was reported that the presence of Tat activates HIV-1 transcription by greater than 100 fold (Cullen, 1986 and Feng and Holland, 1988). Activation of HIV-1 transcription by Tat depends on TAR, as it Tat stimulates polymerase elongation by recognizing TAR (Jones and Peterlin, 1994; Jones, 1997).

One of the positive control hairpins, pTZ-lhRHA-TAR-2, knocked down the expression of the target in the absence of Tat as shown in figure 3.7 A. However, the same hairpin could not downregulate target gene expression in the presence of Tat (Fig. 3.7B). The difference in the role of pTZ-lhRNA-TAR-2 in the absence and presence of Tat may be explained by variability of transcription rates of the lhRNA compared to the target transcript expression. In the absence of Tat, basal levels expression of both lhRNA and LTR-luciferase transcript occur which would influence RNAi to target and downregulate luciferase expression. However, in the presence of Tat, the levels of the target gene are upregulated whereas the pTZ-lhRNA-TAR-2 transcript remains unchanged (relative to Tat level), which may consequently allow gene silencing by RNAi to occur at levels that may not significantly affect the increase LTR transcripts. The second positive control hairpin, pTZ-lhRHA-TAR-3 did not silence the target in both experiments. This could be due to sequence variations between the target and the hairpin as RNAi is sequence sensitive and one base mismatch between the target and the hairpin could abolish the whole gene silencing process (Das et al., 2002 and Westerhout et al., 2006). According to Fig. 3.7 A, which was the PTGS in the absence of Tat, pCi-LHRNA-2 showed the highest Firefly luciferase expression at ~180%, when compared to the other two hairpins (pCi-LHRNA-1 and pCi-LHRNA-3), which had ~130% and 150% luminescence ($p < 0.05$).
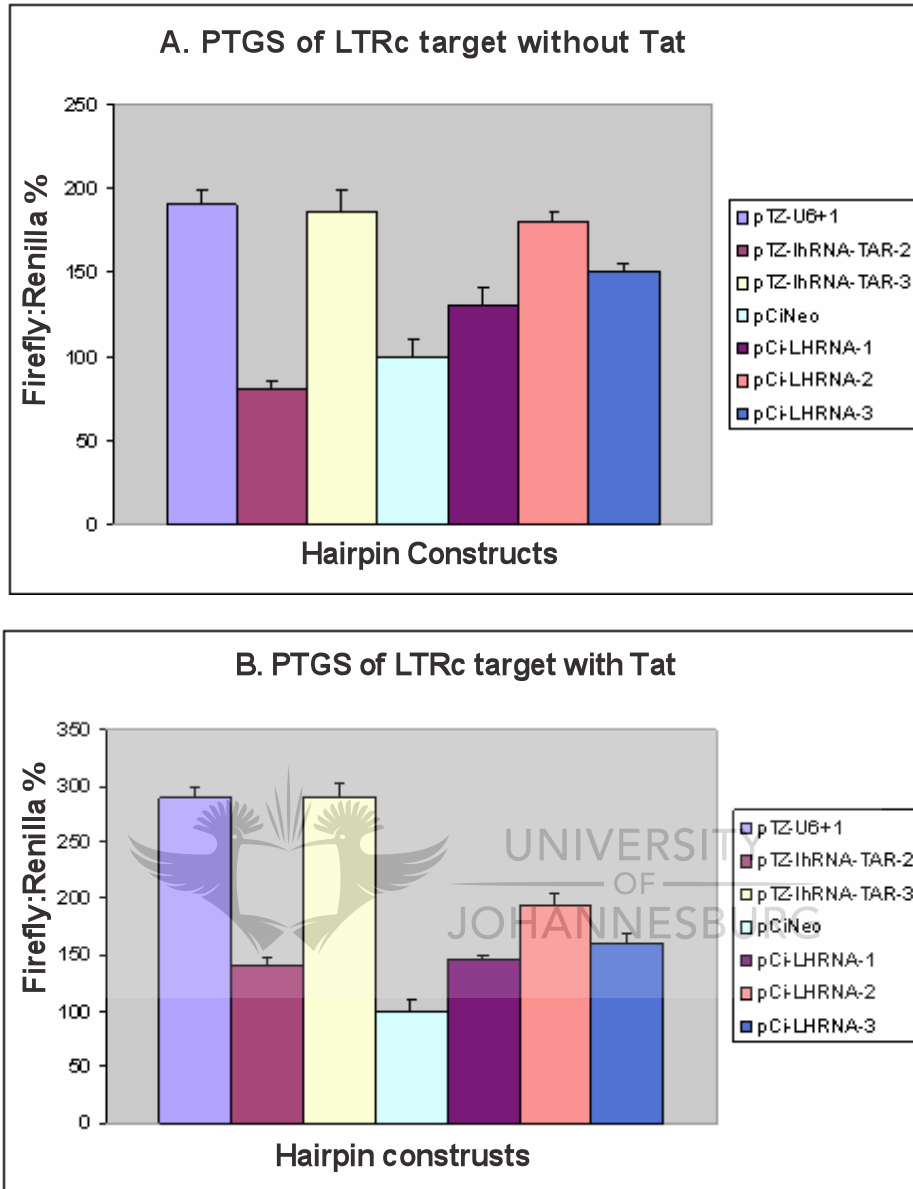
Figure 3.7. **Testing of the hairpins against a target**: **A.** Long hairpin RNAs targeted against the LTR of subtype C in the absence of Tat. pTZ-U6+1 is an empty vector and was used as a negative control, pTZ-lhRNA-TAR-2 and pTZ-lhRNA-TAR-3 were used as positive controls. The y-axis represents Firefly luciferase activity relative to background *Renilla* luciferase activity normalized to empty pCiNeo. pCiNeo is an expression vector used for the expression of long hairpin, it was used for normalizing Firefly and *Renilla* ratios. pCi-LHRNA 1 is the hairpin with an untreated sense sequence, whereas pCi-LHRNA 2 and pCi-LHRNA 3 are the hairpins with treated sense sequences for 10 minutes and 2.5 hours, respectively. The y-axis represents the average of 3 ratios (FF:*Renilla*) normalized to the empty pCi-Neo. **B.** LhRNAs targeted against the LTR of the subtype C in the presence of Tat. The error bars represent the standard error of the mean (SEM).

A similar pattern was observed in the presence of Tat, as pCi-LHRNA-2 showed ~195% Firefly luciferase expression, and pCi-LHRNA-1 and pCi-LHRNA-3 showed ~150% and ~160% of Firefly luciferase expression. Therefore, these results show that that both in the presence and in the absence of Tat, all the three long hairpins constructed in this study could not inhibit the expression of the target region in HEK 293 cells.

3.2.2. Assessing the effect of long hairpin RNAs in mammalian cells (IFN response) by qRT-PCR

Long RNA duplexes in mammalian cells activate the IFN response, which results in non-specific gene silencing. To assess the activation of IFN response in cells that were transfected with lhRNAs, relative cellular levels of IFN-β mRNA were measured by quantitative RT-PCR.
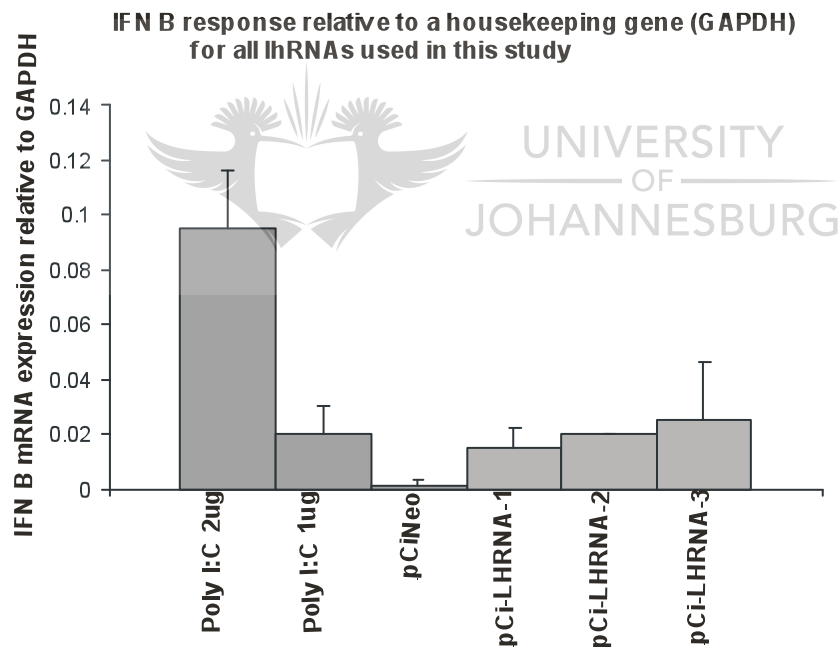
Figure 3.8. Long hairpin RNAs induce interferon response: Poly I:C is a double stranded RNA that is known to induce interferon response in mammalian cell; it was used in this study as a positive control. pCiNeo is an empty vector it was used as a negative control and it is a vector that was used for the expression of long hairpin sequences. pCi-LHRNA-1 is the hairpin with untreated sense sequence; pCi-LHRNA-2 and pCi-LHRNA-3 are the hairpins with treated sense sequences for 10 minutes and 150 minutes, respectively. The y-axis represents IFN-β mRNA expression relative to background GAPDH mRNA expression.

According to Fig. 3.8, the results of the poly I: C, which is a positive control that triggers immune response were as expected. A 1 µg dose of Poly I: C transfected into HEK293 cells resulted in four fold decrease of INF-β mRNA production relative to a 2 µg dose of Poly I:C. GAPDH, the housekeeping gene, aids in normalizing the expression of IFN-β. The importance of the normalization process was significant, as the levels of GAPDH in cells should be similar in all transfections. Hence, any variations of IFN response should be reflected in housekeeping gene expression. pCiNeo, the empty vector, was used as a negative control, as no transcripts that could potentially influence interferon production were expressed from it. Relative to the negative control, all 3 synthesized hairpins induced the IFN response. The hairpin containing no mismatches between sense and antisense strands (pCi-LHRNA-1) was expected to induce an immunostimulatory response as it had been previously reported that dsRNA longer than 30 bp induce IFN response in mammalian cells (Akashi et al., 2005). Approximately 0.018 units of IFN-β were expressed in cells transfected with this hairpin. Surprisingly, mismatched hairpins, pCi-LHRNA-2 and pCi-LHRNA-3 induced IFN-β mRNA production of 0.019 and 0.022 respectively. This is contrary to what was previously reported that the introduction of mismatches on the sense strand of the lhRNA prevents IFN response (Akashi *et al.*, 2005). Since pCi-LHRNA-3, which was treated for 150 minutes with sodium bisulphite had more mismatches (22 cytosine residues converted to thymine residues) than pCi-LHRNA-2, which was treated with sodium bisulphite for 10 minutes and had 10 cytosine residues converted to thymine), it was expected to induce less IFN response than the pCi-LHRNA-2.

CHAPTER 4

4. DISCUSSION

Gene silencing in mammalian systems has been achieved by using small RNA duplexes (Elbashir *et al*., 2001a and Brummelpkamp *et al*., 2002). However, their use has a major limitation, which is the rapid emergence of viral escape mutants. This challenge has been solved by using lhRNAs that target longer and multiple sequences, thereby delaying the emergence of escape mutants. Although the use of RNA duplexes longer than 30 bp solves the challenge of using short RNA duplexes, their use brought another challenge, the induction of interferon responses (Stark, 1998). However, it has been documented (Akashi *et al*., 2005) that the introduction of mismatches on the sense strand of the lhRNAs can prevent the interferon induction. Since RNAi is a sequence specific process in which a single point mutation mismatch between the antisense sequence and the target could abolish the silencing process; mutations need to be introduced on the sense strand. Mutations on the sense strand result in lhRNAs that have G:U mismatches, instead of G:C, allowing the cytosine residues to be replaced by a uracil residues. Mutations in lhRNAs have been successfully introduced using a conventional method involving the use of synthetic oligonucleotides for duplexes that are 65 bp or less (Barichievy *et al*., 2007 and Weinberg *et al*., 2007). Although this method works well for 65 bp constructs and less, its use is expensive and difficult for duplexes longer than 65 bp.

This study was therefore aimed at solving the problem associated with the use of synthetic oligonucleotides in the introduction of mismatches in long RNA duplexes by using sodium bisulphite. Due to its specific mutagenic effect, it was chosen in the present study for the introduction of G:U mismatches in lhRNAs, as it deaminates cytosine residues leaving guanosine and adenosine residues unaltered (Shapiro and Klein, 1966). Only uracil and cytosine residues have ring systems that make them susceptible to nucleophilic attack at the 5, 6 double bond (Shapiro *et al.,* 1970).

According to the results obtained in this study (Fig. 3.3), sodium bisulphite was able to induce mutation by converting cytosine residues to thymine residues, thereby introducing mismatches in long hairpin constructs. The ability of sodium bisulphite to introduce mutations resulting in lhRNAs with mismatches makes it a cheaper and an easy way of introducing mismatches in hairpin RNAs. Furthermore, results showed (Fig. 3.3,

pTZ-LTRc-T4, pTZ-LTRc-T1, and pTZ-LTRc-U) that sodium bisulphite deamination process is time dependent, as the longer the treatment period, the higher the number of converted cytosine residues. This property of sodium bisulphite makes it an even more attractive tool, as a desired number of converted cytosines can be achieved by treating DNA for shorter periods instead of having all cytosines converted while one wanted to convert only a few. This also depends on the number of cytosines a hairpin encoding DNA has.

According to Fig. 3.9, the constructed lhRNAs induced interferon response, which means that these constructs did not prevent the induction of interferon response as intended. It could be that mismatches introduced by sodium bisulphite have different properties from those introduced by conventional method, and therefore they are still recognized as dsRNA and they trigger IFN response. This could be prevented by endogenous expression of lhRNAs, instead of using transfection procedure to introduce these lhRNAs to the target region (Akashi et al., 2005).

The method used in the production of hairpin RNA molecules has a significant influence in cells, as expressed hairpin RNA have different immunostimulatory properties compared to the synthetic RNA or exogenously introduced RNAs (Robbins *et al*., 2006). Synthetic or *in vitro* synthesized dsRNAs might be recognized directly by a receptor for exogenous RNAs, such as a Toll-like receptor 3 or Toll-like receptor 7 to activate downstream genes (Alexopoulou *et al*., 2001). In addition to the method of RNA production, the type of promoter used in endogenous expression also has a significant influence in the transcription process. In this study, the pol II promoter, CMV, was used. This promoter is used in the expression of long transcripts, and due to its ability to express RNA in a wide range of cell lines, makes it the best promoter choice when expressing lhRNAs. Compared to pol III promoters, pol II promoters are cell/tissue specific (Lagos-Quintana *et al*., 2003). However, pol II promoters add extra sequences to their transcripts (Paddison *et al*., 2002a) which have a great influence in the processing of the hairpin, thereby leading to poor efficiency of produced siRNAs. The processing of pol II transcripts in the nucleus by Drosha which leaves a product with 2 nt 3' overhang, allows Dicer to be able to recognize pol II transcripts as substrates that they can process. However, it has been shown that not all siRNAs generated from lhRNAs silence their

targets efficiently. This unequal silencing has been shown in a study where siRNAs originating from the duplex at the loop terminus side of the hairpin have poor efficacy, whereas those siRNAs derived from the stem base have been shown to be the most effective (Weinberg *et al.*, 2007). The unequal silencing of siRNAs derived from lhRNAs is likely due to the Dicer cleavage that is initiated at the stem base of the lhRNAs where there is a 2nt 3' overhang that Dicer recognizes (Provost *et al.* 2002 and Zhang *et al.*, 2002).

Long hairpin RNA constructs used in gene silencing experiments may cause cell death and are often deleted or rearranged rapidly in *E. coli*. Such constructs recovered from *E. coli* have partial or complete deletion of either the sense or antisense arm (Brunier *et al.*, 1988). In this study, we successfully assembled these constructs using a simple T/A cloning vector-based strategy. These constructs were replicated and maintained in standard *E. coli,* DH5α. In addition, sequencing was done using standard primers and conditions. Subsequently, the constructs were sub-cloned into an expression vector and they were unable to replicate in DH5α and JM101 cells. SURE cells were then used to solve the problem experienced with DH5α and JM101 cells, as they are known to enhance the stability of unstable plasmids, like pCiNeo.

Long hairpin RNAs have the potential of delaying the rapid emergence of viral escape mutants when used in antiviral studies as they target longer and multiple sequences, simultaneously. In the present study, data revealed that the three lhRNAs were unable to silence the target region both in the absence and presence of Tat (Fig. 3.8). Although lhRNA constructs induced IFN response, there are other factors that can cause the inability of lhRNAs to silence their target genes, such as the inability of Dicer to process constructs. However, this was not checked as northern blot experiments were not done. Performing northern blots would have shown whether the constructs were expressed and then processed to siRNAs or not, as northern blots would show different RNA species (sizes) present in cells. Also, the inability of Dicer to process constructs can be due to a non-functioning Dicer. It could be checked by using two sets of cells, the first set can be transfected with a plasmid encoding Dicer the other without Dicer, including hairpin constructs and the target. If the constructs are not processed in the presence of

Dicer encoding plasmid it would mean that the problem was with constructs but not Dicer.

Interferons function as antiviral cytokines in mammalian systems as the first line of defense against viral infection. This is usually mediated by dsRNA as most viruses produce dsRNA at some point in their replicative life cycle. As already described in the introduction, the binding of these dsRNAs to PKR activates and induce its autophosphorylation and further activates eIF-2α which leads to the global shutdown of protein synthesis (Williams, 1999). Also, 2'-5'OAS gets activated upon binding to dsRNA and catalyze the polymerizayion of ATP into 2'-5' linked oligoadenylates (Hartmann *et al*., 2003). The oligoadenylates in turn bind and activate RNaseL, which leads to the non-specific degradation of mRNA.

Initial RNAi experiments in mammalian cells were hampered by the induction of the interferon response as long dsRNA molecules were being used. Since dsRNA greater than 30 bp in size induce the interferon response, only the use of siRNAs was allowed for use of RNAi in mammalian cells (Elbashir *et al*., 2001a). In recent years a solution to overcome this limitation of only using siRNAs instead of longer dsRNAs has been found. This is achieved by introducing mismatches on the sense strand of the long dsRNA as previously reported that mismatches lead to the avoidance of interferon response (Akashi *et al*., 2005).

CHAPTER 5

5. CONCLUSIONS AND FUTURE STUDIES

In the current study, mismatched hairpin constructs were successfully created, showing that sodium bisulphite has a great potential in solving the problems encountered when using synthetic oligonucleotides. However, the use of sodium bisulphite did not prevent the induction of interferon response. This would therefore require further studies to be conducted in order to determine how the deamination property of sodium bisulphite can be used to solve the problem of interferon induction in the use of lhRNAs in mammalian gene silencing, and that such lhRNAs can be endogenously expressed instead of using transfection experiments.

In conclusion, lhRNA constructs used in the current study were unable to silence the target and failed to prevent interferon induction. As mentioned in the discussion, the inability of the constructs to knock down the target could not only be attributed to the induction of interferon response, as there is a possibility that Dicer could not process the constructs efficiently due to the poor alignment between the two complementary arms. In future studies, a method used by plant scientists to use 1 kb introns can also be employed for mammalian studies, as the splicing process by splicosomes aligns complementary arms, resulting to the production of active siRNAs.

CHAPTER 6

6. REFERENCES

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7. APPENDIX

7.1. Preparation of chemically competent bacterial cells (*E. coli*) and transformation.

1. Luria Bertani medium (broth)

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

2. Transformation buffer

Transformation buffer was prepared as follows: 100 mM Calcium chloride (CaCl$_2$) (Merck), 10 mM PIPES-HCl (Sigma, USA) and 15% (v/v) Glycerol (Merck). The pH was adjusted to 7.0 with Sodium hydroxide (NaOH). The solution was autoclaved for 30 minutes at 121°C and 1 kg/cm^2. Transformation buffer was stored at -20°C.

3. Preparation of chemically competent bacterial cells

Eight milliliters of ml Luria Bertani medium was inoculated with bacterial cells. The broth culture was incubated overnight in a shaker at 37° C. In the morning, the 8 ml culture was used to inoculate 200 ml of LB broth which was incubated for two hours at 37° C in a shaker. The cells were centrifuged at 3500 rpm for 10 minutes and the pellet was resuspended in 20 ml transformation buffer and incubated on ice for 20 minutes. After 20 minutes of incubation cells were centrifuged at 1500 rpm for 15 minutes and the pellet was resuspended in 1ml transformation buffer. Aliqouts of 100 µl were transferred to sterile microcentrifuge tubes and stored at -70°C.

4. Transformation of chemically competent bacterial cells

Ten microlitres of ligation mix was mixed with 100 µl of chemically competent cells and incubated on ice for 30 minutes. After the 30 minutes of incubation the cells were heat shocked at 42° C for 90 seconds and placed on ice for an additional 2-5 minutes. Transformed bacteria were then plated on Luria Bertani agar plates containing selective antibiotics.

7.2. Alpha-Complementation

1. 1000x Ampicillin

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

2. 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) stock solution

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

3. Isopropyl-beta-D-thiogalactopyranoside (IPTG) stock solution

Two hundred milligrams of IPTG (Roche, Germany) was dissolved in 1 ml sterile water. The solution was stored at -20° C.

4. Ampicillin positive Luria Bertani (LB) agar plates

Ten grams of Bacteriological agar (Oxoid), 10 g Bacto-tryptone (Oxoid), 5 g Yeast extract (Oxoid) and 5 g Sodium chloride (NaCl) were dissolved per litre of distilled water. The medium was autoclaved for 30 minutes at 121°C and 1 kg/cm^2. Follwing autoclaving, the agar was allowed to cool down to approximately 65°C. To the cooled agar, 1 ml of 1000x ampicillin was added to a final concentration of 100 µg/ml. Luria Bertani agar was poured into petri dishes and allowed to solidify at room temperature.

5. Preparing IPTG, X-gal positive LB agar plates

Forty microlitres of X-gal stock solution and 8 µl of IPTG stock solution were added to an ampicillin positive LB agar plate and spread evenly on the surface. The plates were dried by placing them in an incubator at 37° C for 10 minutes before use. Transformed bacterial cells are plated on ampicillin, X-gal and IPTG positive plates and incubated at 37° C overnight for α-complementation. IPTG induces expression of β-galactosidase, which cleaves the chromogenic substrate X-gal yielding a blue product. Successful cloning (plasmids positive for an insert) disrupts the β-galactosidase gene and therefore

the protein is not functional which results in white colonies. Unsuccessful insertion of fragments leaves the β-galactosidase intact resulting in blue colonies.

6.3. Plasmid Isolation

Luria Bertani see Appendix 7.1.

1. High Pure Plasmid Isolation Kit-Plasmid Preparation (Roche, Germany)

Four milliliters of Luria Bertani was inoculated with a single colony containing the plasmid of interest and incubated at 37°C at 200 rpm for 16 hours. At the end of the incubation period the broth culture was centrifuged 10,000xg at 4°C in a bench-top centrifuge for 15 minutes. The pellet was resuspended in 250 μl of Suspension buffer/RNase. To the cell suspension 250 μl of Lysis buffer was added and mixed gently. This mixture was incubated at room temperature for 5 minutes. Following the incubation period 350 μl of the Binding buffer was added and mixed. The mixture was incubated for 5 minutes on ice and centrifuged for 13,000 rpm in a bench-top centrifuge for 10 minutes. The supernatant was transferred into columns with collection with collection tubes and centrifuge for 1 minute at 13,000 rpm for 1 minute. After discarding the flow-through 750 μl of wash buffer II was added to the column and centrifuged at 10,000xg. Centrifugation step was repeated to completely remove wash buffer. The columns were then placed into clean microtubes and the plasmid was eluted by adding 100 μl of the Elution buffer (10 mM Tris-HCl, pH 8.5) and centrifuged for 1 minute at 10,000xg.

2. EndoFree Plasmid Maxi Kit plasmid preparation (Qiagen, CA, USA)

Too hundred millilitres of LB broth was inoculated with a single colony containing the plasmid of interest and incubated at 37°C in a shaker for 16 hours. The culture was centrifuged at 4000 xg for 30 minutes. The pellet was resuspended in 10 ml of Resuspension buffer (Buffer P1; 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing RNAse A at a concentration of 100 μg/ml. To the cell suspension 10 ml of Lysis buffer (Buffer P2; 200 mM NaOH; 1% SDS) was added and thoroughly mixed and incubated at room temperature for 5 minutes. While incubating, QIAfilter cartridges were set up by screw capping the outlet nozzle and placing them in 50 ml plastic tubes. Following the 5 minute of incubation period, 10 ml of chilled Neutralization buffer (Buffer P3, 3 M KAc,

pH 5.5) was added and mixed by inverting the flask. Contents of the flask were transferred to the Qiafilter Cartridge and incubated at room temperature until the lysate has cleared with the solid white part on at the top of the cartridge. After incubation the lysate was filtered into a sterile 50 ml tube and 2.5 ml of Endotoxin removal buffer (Buffer ER) was added to the filtrate, mixed and incubated on ice for 30 minutes. During incubation Qiagen-tip 500 was equilibrated by applying 10 ml of the Equilibration buffer (Buffer QBT; 750 mM NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol; 0.15% Triton X-100) to the column and allowing the buffer to drain by gravity flow. At the end of 30 minutes the lysate is transferred into the equilibrated column and allowed to filter. The column was washed twice with Wash buffer (Buffer QC; 1 M NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol). After the second wash, DNA was eluted from the column into a sterile tube with 15 ml of Elution buffer (Buffer QN; 1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol). The eluted DNA was precipitated with 10.5 ml of isopropanol and left at -70°C for 1 hour. After 1 hour it was mixed and centrifuged at 4000XG for 1 hour at 4°C. The pellet was washed with 5ml of endotoxin free 70% ethanol from the kit and centrifuged at 4000XG for 30 minutes. The supernatant was carefully removed and the pellet was air-dried and re-suspended in 200 µl endotoxin free sterile water. Stored at -20°C.

7.4. Gel electrophoresis

1. Preparation of 10x Boric acid buffer (Tris-Borate-EDTA)

One hundred and eight grams of Tris base, 55 g of Boric acid and 9.3 g EDTA was dissolved in 1 litre of distilled water.

2. Preparation of ethidium bromide

To prepare ethidium bromide solution, 10 mg of ethidium bromide powder (Roche) was dissolved in 1 ml of distilled water. The tube with ethidium bromide in it was wrapped with foil as ethidium bromide is light sensitive.

3. Agarose gel

To prepare 2% agarose gel, 1 g of agarose powder (Biorad, USA) was dissolved in 50 ml of TBE buffer by heating in a microwave until the gel was completely dissolved. After dissolving the agarose, the solution is allowed to cool down to ~ 60°C by gently swirling and ethidium bromide was added to a final concentration of 1 μg/ml. The solution was poured into a gel tray with combs to form wells.

7.5 DNA purification-Qiagen MinElute PCR Purification and Gel Extraction Kit (Qiagen, CA,USA)

1. Gel extraction

The DNA fragment was excised from the gel and 900 μl Buffer QG were added to a 300 mg gel mass. This was dissolved by incubating at 50°C for 10 minutes with vortexing every 3 minutes during incubation. After the gel had dissolved completely, 300 μl of isopropanol was added to the sample and mixed by inverting the tube. MinElute columns were placed in a 2 ml collection tube and the dissolved DNA sample was poured into the column and centrifuged for 1 minute at 13,400 rpm in a conventional bench-top micro centrifuge. After spinning and discarding the flow-through 500 μl of QG was added and centrifuged for 1 minute. The contents of the column were washed by adding 750 μl of Buffer PE and centrifuged for 1 minute. After discarding the flow-through the column was centrifuged to completely remove Buffer PE. The column was then placed in a

micro-centrifuge tube and DNA was eluted by adding 10 μl of Buffer EB to the centre of the column membrane and incubated for 1 minute then centrifuge for 1 minute at 13,400 rpm in a bench-top centrifuge.

2. PCR product purification

Four hundred and fifty micro-litres of QG buffer were mixed with 100 μl of the PCR product. Isopropanol was also added and mixed. The mixture was then transferred to the column and centrifuged at 13,400 rpm for 1 minute. The supernatant was discarded and 750 μl of the PE buffer was added to the column and centrifuged at 13,400 rpm for one minute. Supernatant was discarded and the column was further centrifuged for another minute to remove excess liquid from the column, which was also discarded. The column was then transferred onto an empty tube and the DNA was eluted by adding 50 μl of SABAX best quality water and collecting the DNA by spinning the tube with the column.

7.6. RNA extraction using TRI Reagent (sigma,)

Twenty four hours post-transfection of cells with long hairpin RNAs, RNA was extracted from the cells: One hundred and twenty five micro-litres of the TRI-Reagent was added per well and incubated at room temperature for 5 minutes. Following the incubation period cells were mixed by pipetting up and down. The cell mixture was transferred to a clean 1.5ml tube. To the tube with cell mixture, 25 μl of chloroform was added and vortexed. The mixture was incubated at room temperature for 5 minutes and centrifuged at 13,000 rpm in a refrigerated bench-top centrifuge for 30 minutes. Following the centrifugation process the contents of the tubes had three layers:

- Upper clear aqueous layer with RNA,
- Interphase (thin layer) with DNA and
- Lower pink organic layer with protein.

The upper layer was transferred to another tube and 80 μl of isopropanol was added to the tube contents and mixed by inverting the tube. The mixture was incubated at -70°C overnight. Following overnight incubation, the tubes were left at room temperature for 15 minutes and centrifuged at 13,000rpm for 1 hour. The supernatant is removed and the pellet is air-dried by allowing the tube to stand

upside down. To the dried pellet 10 µl of SABAX best quality water was added and newly extracted RNA can be stored at -70°C until needed.

7.7. Luciferase Assay

**Reagents**

1X Passive Lysis Buffer:

For 96 samples 9, 6 ml of 1X passive lysis buffer was used, and 10 ml was prepared by diluting 2 ml of 5 % lysis buffer in 8 ml of distilled water and vortexed.

1X Stop and Glo Reagent:

For 96 samples, 4, 8 ml was needed and 5ml was prepared by diluting 0.1 ml of 50X Stop & Glo Substrate in 4, 9 ml of Stop & Glo buffer.

Luciferase Assay Reagent II (LARII)

It was prepared by resuspending the lyopholised Luciferase Assay Substrate in Luciferase Assay Buffer II and 1ml aliquots were made and stored at -70°C. For the assay 4.8 ml of LARII was used.
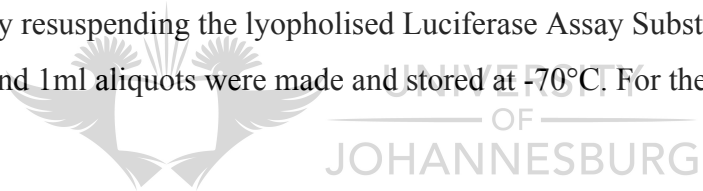
7.8 Dephosphorylation of pCI Neo with calf intestinal phosphatase

Four microlitres of 0.5 µg/µl pCI Neo was digested with each of *Not*I and *Xho*I (NEB, UK) to a final volume reaction of 30 µl. The thirty micro-litre digest reaction was dephosphorylated with CIP in the presence of phosphatase. The reaction was incubated at 37°C for 1 hour.