
**ANALYSIS OF THE EFFICACY OF SHORT HAIRPIN RNAs
TARGETED TO THE GAG OPEN READING FRAME OF HIV-1
SUBTYPE C**

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fulfillment of the requirements for the degree
of
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DECLARATION

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

Eleanor Margaret Cave

____ day of _____ 200__

**To my parents
For all their love and support**

CONFERENCE PROCEEDINGS

- Cave EM, Cilliers T, Morris L and Arbuthnot P. XV International AIDS conference, 11-16 July 2004. Targeting *gag* messenger RNA by short interfering RNAs, inhibits HIV-1 subtype C. Bangkok, Thailand.
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ABSTRACT

The human immunodeficiency virus type 1 (HIV-1) presents one of the most pressing needs for the development of effective therapies in the world to date, with the demand in developing countries being especially high. There have been many advances in the field of molecular biology, resulting in numerous, novel approaches to inhibiting HIV-1. One of the most exciting prospects is to exploit the endogenous RNA interference pathway (RNAi). RNAi is an ancient antiviral system able to induce the sequence specific degradation of viral RNA and retrotransposons. Long dsRNA are processed within the cell into short 19-21bp RNA duplexes known as short interfering RNA (siRNA) by the RNase III endonuclease dicer. These siRNAs are then incorporated into a degradative enzymatic complex known as RISC. RISC is then guided by an unwound siRNA and specifically degrades RNA with which it forms base pairs. In order to test the efficacy of the RNAi pathway in specifically inducing gag mRNA cleavage, 10 short hairpin RNA expressing plasmids, targeting the gag ORF, and expressed from an RNase III U6 promoter were created. The efficacy of the anti-gag shRNA sequences was tested against both an Enhanced Green Fluorescence Protein (EGFP) reporter plasmid system and live HIV-1 subtype C isolates. Two of the anti-gag shRNA sequences were shown to be effective in inhibiting p24 capsid production, a marker of HIV-1 replication, in both X4 and R5 isolates. The anti-gag effects were shown to occur at both the protein level (through p24 ELISA, flow cytometric/fluorescence microscope analysis of eEGFP/gag fusion peptides, and western blot analysis), and the RNA level, (through Real-time RT-PCR analysis). The most effective shRNA was shown to be processed efficiently within the cell. The efficient anti-gag shRNA mediated inhibition of capsid protein production by selected

shRNA sequences holds great promise for the development of a potential therapeutic agent to be used in conjunction with existing antiretroviral regimes.

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LIST OF ABBREVIATIONS

µg	microgram
µl	microlitre
Ag	Antigen
AIDS	Acquired immunodeficiency Syndrome
bp	Base pair
CTL	Cytotoxic T Lymphocyte
DC's	Dendritic Cells
iDC's	immature dendritic cells
DMEM	Dulbecco's modified Eagle's medium
RPMI	Roswell Park Memorial Institute medium
DNA	Deoxyribonucleic acid
dNTP's	deoxy-ribonucleoside triphosphates
ELISA	Enzyme linked immunosorbent assay
<i>Env</i>	envelope gene
Env	envelope protein
FCS	Fetal calf serum
FDA	Food and Drug Administration
<i>Gag</i>	group associated antigens gene
Gag	group associated antigens protein
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
gp	glycoprotein

Group M	Major variant
Group N	New variant
Group O	Outlier variant
EGFP	Green fluorescent protein
HAART	Highly active antiretroviral therapy
HIV-1	Human Immunodeficiency virus type 1
HRP	Horse Radish Peroxidase
IFN	Interferon
IL	Interleukin
kDa	Kilo Dalton
kB	Kilo base
LTR	Long terminal repeat
Min	minute
ml	milliliter
mM	Millimolar
nM	nanomolar
nm	nanometers
nt	nucleotides
°C	Degrees Celsius
OD	Optical Density
PBS	Phosphate buffered saline
Pg	pictogram

PIC	Pre-integration Complex
R5	CCR5 using viruses
RRE	Rev responsive element
RNA	Ribonucleic acid
RISC	RNA induced silencing complex
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SIV	Simian immunodeficiency virus
TCID ₅₀	tissue culture infectious dose
TSG-101	Tumor susceptibility gene 101
TLR	Toll like receptor
u	units
V	Volts
Vif	Viral infectivity factor
W	Watts
X4	CXCR4 using viruses

CHAPTER 1: INTRODUCTION

The Human Immunodeficiency virus type 1 (HIV-1), presents one of the greatest health challenges the world has seen in recent times. The epidemic has affected the entire world with an estimated ± 38 million people currently infected with the virus¹ (Figure 1). Developing countries however, have borne the brunt of the epidemic, particularly the countries of sub-Saharan Africa, which is home to just over 10% of the world's population, but more than 60% of people infected with HIV are living in this region¹.

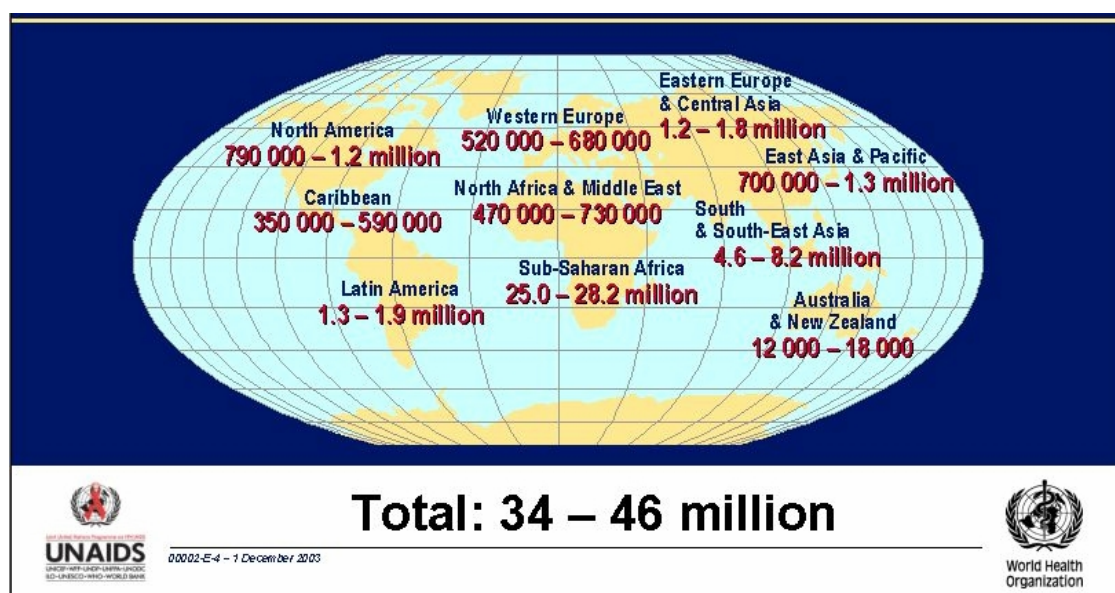


Figure 1: World HIV-1 infections statistics gathered by the UNAIDS organization for 2003 are depicted in the above map. Of the estimated 46 million people infected with HIV-1 by the end of 2003, over 28 million reside in sub-Saharan Africa¹.

HIV-1 comprises 3 distinct virus groups namely M, N and O. The predominant viral group is M, which, itself comprises of 9 clades or subtypes, with A and F further divided into A1, A2, F1 and F2². The subtypes are labeled from subtype A through to subtype K, with subtype B

being predominantly found in America and Europe and subtype C being the major subtype in sub-Saharan Africa³. Subtype C is responsible for the majority of HIV infections, making the study of subtype C essential¹.

HIV ultimately causes immune failure in patients, who subsequently succumb to opportunistic infections. This syndrome is known as acquired immunodeficiency syndrome (AIDS)⁴. Currently there are no cures or preventative vaccines to stem the flow of the virus. Therapies can prolong the onset of AIDS and the life of the patients. Multiple therapies are in existence but due to HIV's ability to mutate to evade treatment and the high cost of available therapeutics, new therapies need to be developed⁵.

Suppression of viral genes using molecular techniques, offer exciting possibilities, for the development of novel anti-HIV therapies. Gene based therapies are a promising alternative to current therapies as they will hopefully eliminate the unwanted side effects associated with current antiretroviral therapies. The discovery of siRNA, which are able to silence gene expression of genes with sequences that are complementary to that of the siRNA, has lead to the exploration of siRNAs as anti-HIV agents. The data presented to date indicate that siRNAs might hold great promise in targeting subtype C virions.

1.1 HIV Replication

The HIV epidemic was first recognized in 1980 in the United States of America, where young homosexual males presented with opportunistic infections typically associated with profound immune failure⁴. In 1983 the virus that caused the immune failure, HIV, was isolated. This

paved the way to gain important insight into the lifecycle of the virus (Figure 2), and allowed for the development of much needed therapies. HIV was shown to belong to the lentivirus genus of retroviruses, and induced a syndrome with all the properties of insidious disease including persistence, latency, variation, recombination and the ability to escape from immune and drug pressures².

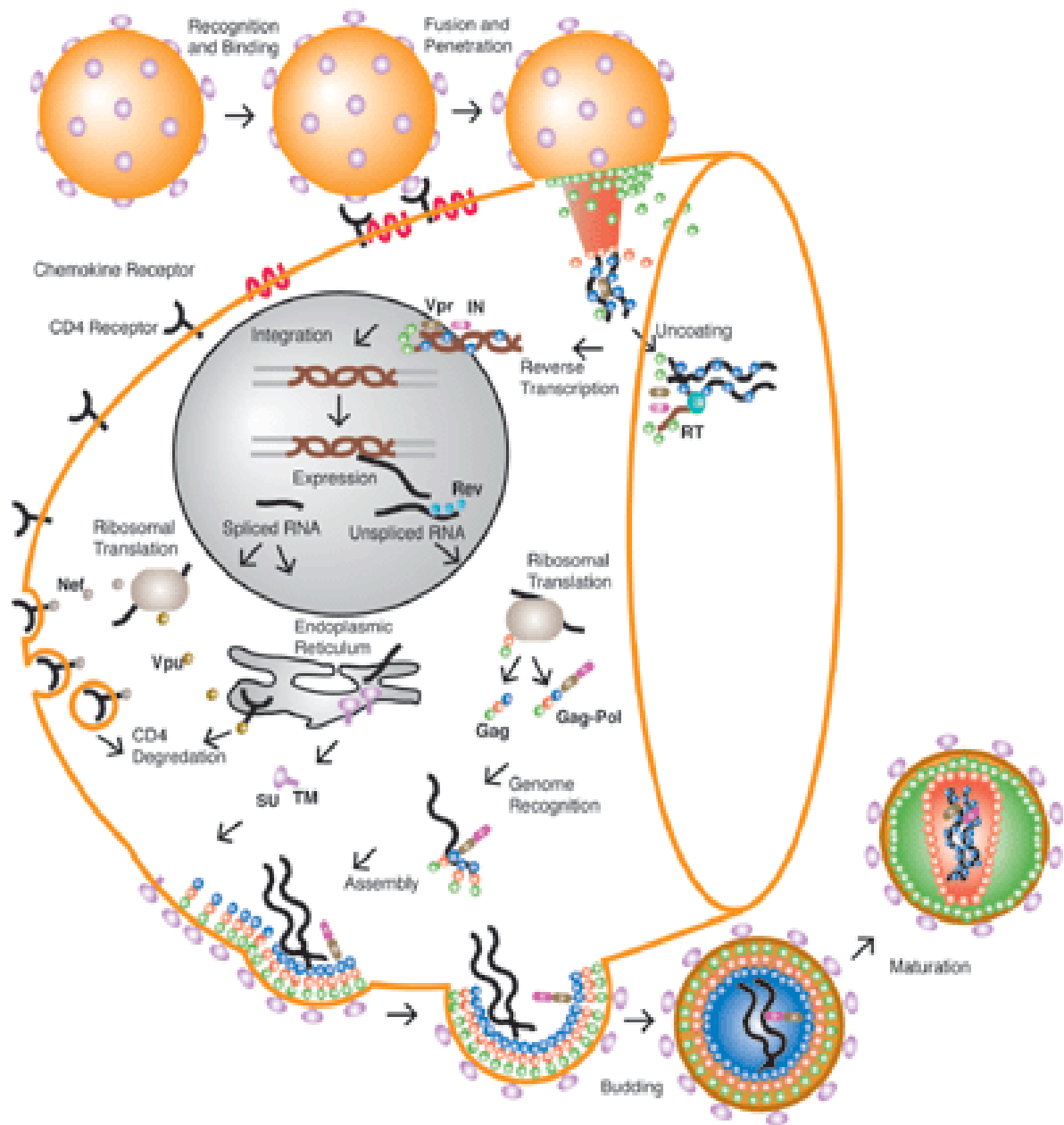


Figure 2: Diagrammatic representation of the lifecycle of HIV-1. The virus is recognized by the cell and binds to CD4 and a chemokine co-receptor. Following binding, the virus' membrane fuses with the host cell membrane. Once HIV enters the cell, it is uncoated and its RNA genome is reverse transcribed by viral error prone RT. The cDNA is then integrated into the host cell chromosome. Once integrated, viral genes are transcribed, and either included directly as viral genomic RNA or translated into viral proteins. New virions are then generated, bud from the host cell membrane and undergo maturation⁶.

The targeting and downregulation of specific HIV genes has distinct advantages, both therapeutically and in knockdown research. Interruption of the HIV lifecycle by gene knockdown could potentially be a means either to eradicate the virus, or through incomplete knockdown, the virus could, theoretically, be reduced to levels that allow the immune system to remain intact preventing the development of AIDS.

The HIV virion contains two copies of HIV-1's ssRNA genome encapsulated, together with both viral and host encoded proteins, by core proteins. Surrounding the core is an envelope with two glycoproteins visible on the outer surface, gp120 and gp41 (Figure 3). The HIV genome consists of 9 genes, falling into 2 main categories. Gag, Pol and Env are structural genes involved in virion formation and Vif, Vpu, Vpr, and Nef are accessory proteins involved in infection (Figure 4)⁷.

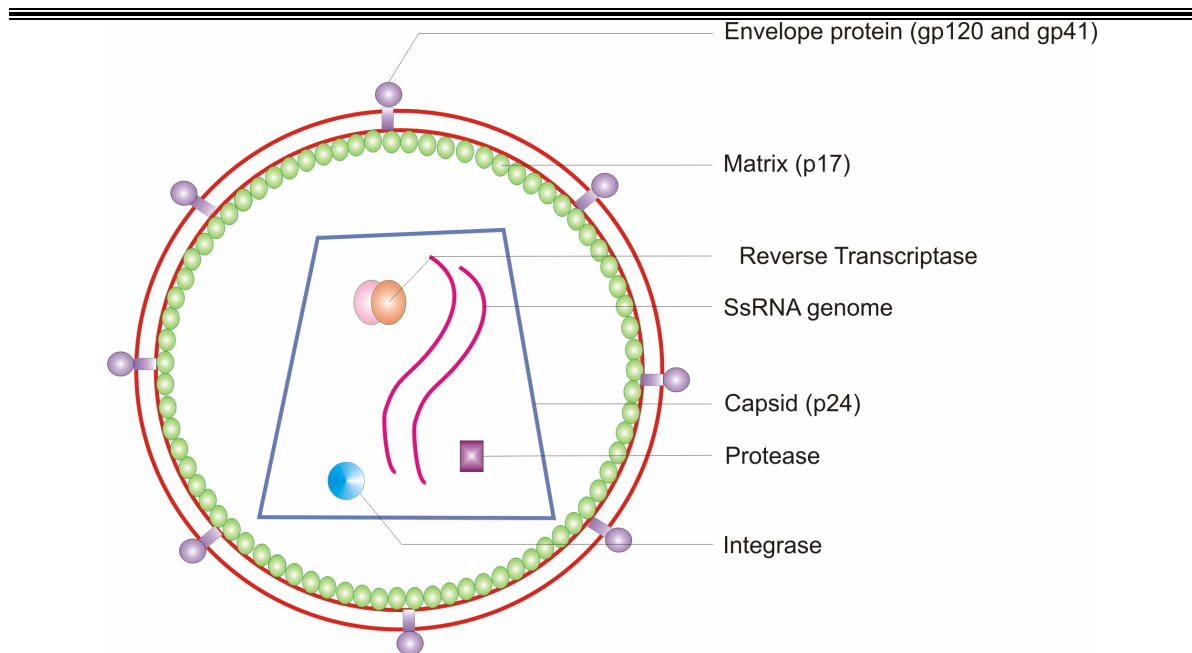


Figure 3: A diagrammatic representation of the HIV-1 virus. The virus consists of 2 ssRNA viral transcripts, RT, protease and integrase surrounded by core proteins. The viral core is encapsulated by an envelope made from the host cell membrane with viral env proteins gp120 and gp41, respectively, attached to and embedded in the membrane.

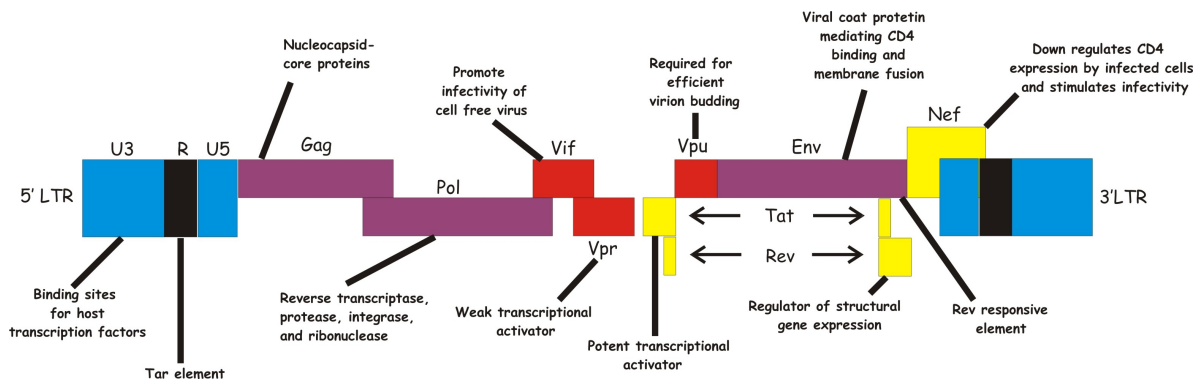


Figure 4: Schematic drawing of the 9 HIV-1 genes together with their recognized primary functions. The 5' and 3' long terminal repeats (LTR), the transactivational response element (tar) and the rev responsive element (RRE) are also depicted in the diagram⁷.

The glycoproteins mediate viral entry into the cell. HIV-1 gp120 initially binds to the CD₄ receptor, following binding gp120 undergoes conformational changes, allowing gp41 to bind, usually, to one of the two chemokine co-receptors CCR5 (viruses using this receptor are termed R5 viruses) or CXCR4 (viruses using this receptor are termed X4)^{8,9}. Other minor chemokine receptors have been shown to facilitate viral entry in the absence of CCR5 and CXCR4 (Figure 5)¹⁰. Once both CD₄ and a co-receptor are bound by the virus, fusion of the viral envelope and the cell membrane occurs and the virus is released into the cytoplasm where it is uncoated¹¹.

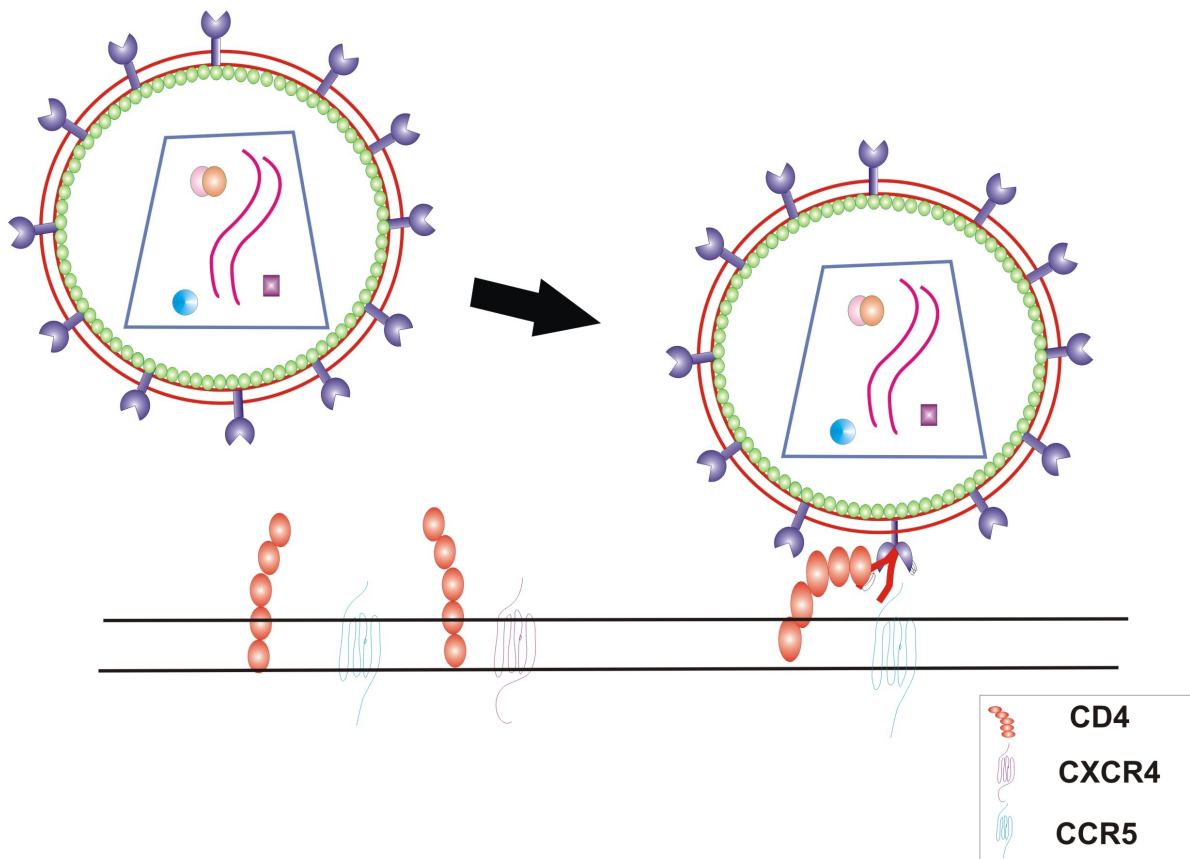


Figure 5: Viral entry into the cells. The virus is able to enter the host cell via the interaction of host proteins (CCR5 and CXCR4) and env proteins (gp120 and gp41).

Reverse transcription of the HIV genome takes place in the cytoplasm of the infected cell, and is carried out by virally encoded reverse transcriptase (RT). The RT of HIV-1 is extremely error prone in nature, inducing mutations often¹². The RT synthesizes a single strand of DNA using the ssRNA HIV-1 genome as a template. RNase H then acts to remove the RNA template, allowing the RT to synthesize the complementary strand to form a DNA duplex. Following reverse transcription a stable complex known as the pre-integration complex (PIC)

is formed. PIC consists of the viral reverse transcribed genome, together with a number of both viral and cellular derived proteins, including integrase¹³.

PIC formation results in the localization of integrase in the nucleus in an ATP dependent manner and the association of integrase with condensed chromosomes during mitosis¹³. Nup98, a human protein located in the nuclear pore complex, specifically aids the nuclear entry of PIC¹⁴.

Integrase then mediates the integration of the reverse transcribed genome into the host chromosome. Integrase accomplishes this by cleaving 2 nts from the 3' end of each end of the viral DNA duplexes, with the cleavage point on the 3' end occurring just 3' to a conserved CA dinucleotide sequence. It then cleaves the host chromosome in a similar manner, nicking the DNA twice in positions 5 bp apart. The viral DNA is inserted into the host chromosome by the addition of a covalent link at either end of the viral DNA. The cleaved 3' 2nt are transferred across to the 5' end of the LTR where integrase fills in the gaps and ligates the sequences together reconstituting the entire HIV-1 genome. Host enzymes repair the integration site. HIV-1 preferentially integrates into active genes⁴. Once HIV is integrated into the genome it becomes known as the provirus¹¹

In order for virus replication to occur, tat is required to initiate the transcription of viral genes. Tat binds to a short stem-loop structure known as the transactivational response element (TAR). TAR is located at the 5' terminus of the HIV-1 RNA. Once tat and TAR are bound, transcription of the HIV-1 genome occurs from the long terminal repeat (LTR) sequence¹⁵.

Following transcription of the genome, the resultant transcript can either be packaged directly into the new virions, or transcribed into different gene products. The genome transcript can be effectively spliced into more than 30 different mRNA species¹⁵.

There are two phases in viral gene expression, the early phase where *tat*, *rev* and *nef* are expressed and the late phase where *gag*, *pol*, *vpr*, *env*, *vif* and *vpu* are expressed. Rev action is attributed to this change in gene expression. Rev elicits this switch by binding to an RNA secondary structure known as the rev responsive element (RRE)¹⁵.

The gag ORF is translated into a 55 kDa polyprotein known as p55. During translation the N-terminus of the polyprotein is myristylated, resulting in the association of p55 with the cytoplasmic side of all cellular membranes¹⁶. Once p55 is associated with the cell membrane, it acts to recruit two copies of the viral genomic RNA, together with other proteins (both viral and cellular). These proteins trigger the budding of the newly formed virion from the infected cell¹⁵. Following budding p55 is cleaved by the viral protease into the matrix protein (p17), the capsid protein (p24), the nucleocapsid protein (p7) and p6 (Figure 6).

The matrix proteins are derived from the N-terminus of the p55 polyprotein, and mostly remain attached to the inner surface of the virion to stabilize the particle. A small group of matrix proteins becomes part of the complex that acts to escort viral cDNA to the nucleus for integration¹⁵.

p24 forms the conical core of the viral proteins, interacting with cyclophilin A and incorporating it into the virion. The nucleocapsid protein aids the inclusion of the viral genomic RNA into the virions and facilitates reverse transcription¹⁵.

The p6 protein mediates gag Vpr interactions thereby causing the incorporation of Vpr into the virion¹⁷. Along with the viral proteins, host proteins are also included in the virions. p6 is also required for the efficient release of the budding virions from the infected cells as is Tsg-101 (tumor susceptibility gene), a host protein actively recruited by the p55 polyprotein¹⁸

Nef, together with Vpu help ready the cell for the budding of new virions. They do so by down regulating the expression of the CD₄ receptor by increasing the rate of CD₄ endocytosis and subsequent lysosomal degradation. This is necessary as CD₄ inhibits env protein incorporation into the virion. Nef has a second function to down regulate the major histocompatibility complex (MHC) type 1. The decrease in MHC type 1 molecules decreases the ability of cytotoxic T-cells to 'see' and kill HIV-1 infected cells¹⁵. Vpu is also involved in the release of the virions and up regulates the rate at which newly formed virions are released from the surface of the infected cell¹⁵.

Disrupting the expression of the *gag* gene will inhibit several important steps required for virion formation. In the absence of *gag*, vital viral and cellular proteins will be excluded from the virion, as would the viral gene transcripts. The virion would be destabilized, PIC would not be able to form properly, and there would be incomplete viral budding. In addition, the targeting of the *gag* sequence within the full-length viral transcript, could theoretically destroy the primary HIV transcripts¹⁵.

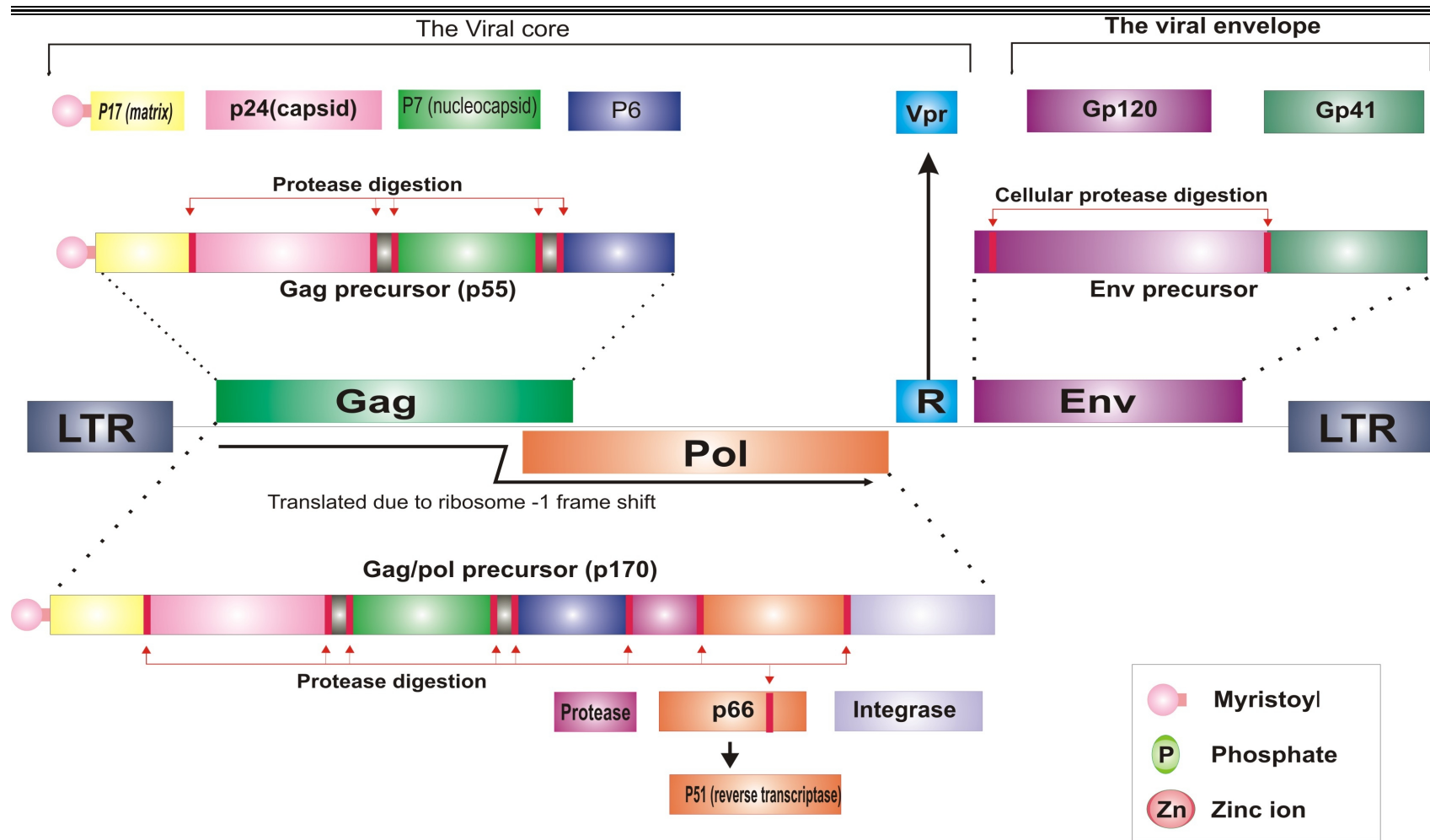


Figure 6: Schematic representation of the processing of the structural proteins required for functional virion formation. The gag and gag-pol polyproteins are processed by viral protease, whereas the env protein is processed by host proteases¹⁹

1.2 Pathogenesis

The development of novel anti-gag agents could work in conjunction with the human immune system in bringing HIV infection under control, as it is well known that the human immune system alone, is unable to control HIV-1 infection. This immune failure is presumed to be due to a lack of sufficient HIV-1 specific CD_4^+ T-cell proliferation and expansion ²⁰. HIV-1 specific CD_4^+ cells are preferentially infected by HIV-1 *in vivo* when compared to other memory CD_4^+ T-cells, this helps explain the loss of HIV-specific CD_4^+ T-cell responses and as a result the loss of immunological control of HIV replication ²¹.

Immature dendritic cells (iDC) are one of the first cells infected by HIV following mucosal exposure to the virus. Tat functions to regulate chemokine gene expression of iDCs, one of the chemokines is TRAIL (Tumour Necrosis Factor-related apoptosis inducing ligand/Apo2 ligand) which triggers apoptosis in T-cells of infected individuals (non-infected individuals are immune to the effects of TRAIL) ²².

The downregulation of HLA class 1 by Nef impairs recognition of infected cells by cytotoxic T-lymphocytes (CTL) and limits the inhibitory effects of the CTL on viral replication. CTLs that are targeted to HIV-1 are deficient in the ability to produce cytokines and perforins. $CD8^+$ cells of infected individuals are deficient in their ability to respond to Ag presentation and therefore do not proliferate in response ²⁰.

CD_4^+ T lymphocytes are an important component of the immune response. CD_4^+ T lymphocytes, function in both the cellular and humoral immune responses. As part of the cellular response, CD_4^+ T lymphocytes bind to antigens presented by macrophages and dendritic cells, then release lymphokines which attract other immune cells to the area. As part of the humoral immune response, T-helper cells bind to antigen presenting B lymphocytes, triggering clonal expansion of plasma cells²⁰. HIV-1 infection is associated with the progressive loss of CD_4^+ T cells through their destruction and subsequent decrease in cellular population, rendering the immune system deficient in its ability to control pathogenic micro-organisms, culminating in the development of AIDS²¹.

When HIV-1 infects activated CD_4^+ T-cells and macrophages, there is an abundance of cellular factors which, together with Tat, up regulate the rate of HIV-1 gene expression. The large amount of virus produced by these cells is cytopathic to the host cells, thus shortening the life span of the infected cells⁴.

HIV-1 is able to induce apoptosis in uninfected cells. It is thought that this is achieved due to the upregulation of the Fas ligand (FasL) on HIV+ cells. FasL interacts with Fas on neighboring cells, triggering apoptosis. The infected cell appears to be protected from this pro-apoptotic signal⁴.

Macrophages become infected immediately after exposure to HIV and are resistant to HIV killing. Infected macrophages continue to produce large amounts of virus, even after the virus has depleted the CD_4^+ T-cells. It is now thought that macrophages, not CD_4^+ T-cells are the most probable source of rebounding levels of HIV, even following therapy²³. Igarshi and

colleagues noted in rhesus macaques infected with a SHIV variant, that 95% of all virus producing cells in the lymph nodes and spleen were macrophages, opposed to the 1-2% of T-cells, indicating that macrophages have a more prominent role in HIV infection and pathogenesis than previously thought²³.

HIV is able to bind to dendritic cells (DC) through the receptor DC-SIGN. The newly infected DCs carry the virus to the lymph nodes. Once in the lymph nodes, HIV actively infects lymphocytes. It is through this pathway that the viral reservoir, located in the lymph nodes is formed⁴.

1.3 Prevention and treatment

Currently there is no cure for AIDS and diagnosis with HIV-1 infection usually results in death, although life expectancy is improved to approximately 10 years from infection with the administering of antiretroviral therapies (ARV). In the developing world where access to ARVs is limited, life expectancy following infection is much reduced²⁴. HIV-1 is readily able to evade the human immune response and ARVs due to an error prone RT, which rapidly introduces mutations that allows the virus to escape¹¹.

The search for a vaccine is an important one, although as yet no suitable vaccine has been developed. Research is looking into the development of two types of vaccines, one giving sterilizing immunity (a preventative vaccine), and a therapeutic vaccine, that although not able to clear the virus from the body would be able to keep the virus in check so that the HIV-1 infected individuals never develop AIDS^{25,26}.

Current antiretroviral therapies for HIV-1 effectively delay the onset of AIDS, but are not able to eliminate the virus. The problems associated with ARVs is a driving force in analyzing siRNAs and other gene-based therapies as anti-HIV agents. The first therapy against HIV-1 was approved by the FDA (Food and Drug administration) in 1987, and was called zidovudine. Several more drugs were developed to inhibit HIV-1 replication, but by 1995 it was realized that monotherapy was no longer an option due to the high rate of viral escape from the drug ²⁴.

It was following this that HAART (highly active antiretroviral therapy) was initiated. HAART consists of numerous ARVs, usually including a protease inhibitor, a nucleoside analog and a non-nucleoside reverse transcriptase inhibitor (NNRTI) ²⁷. Prolonged exposure to HAART results in an increase in naïve cells and an improvement in the functional defects in HIV-1 specific CD₄⁺ and CD₈⁺ T-cells. However, with the use of HAART, HIV-1 specific immunity is not enhanced, but instead declines ²⁰.

By 1996, the limitations of HAART were beginning to surface. Resistant HIV-1 strains were emerging, and strict dosage regimes had to be followed or the virus would rebound to high levels^{27,24}. Side effects to the treatments were also noted. These include weight redistribution (arms and legs get thinner whilst breasts, stomach and neck enlarge), lipodystrophy and metabolic complications (development of diabetes and hyperglycemia) ^{28,24}.

Administering recombinant growth hormone to patients on HAART allowed for the increase of virus specific memory CD_8^+ -T-cells, together with naïve CD_4^+/CD_8^+ cells. It also results in an increase in HIV Antigen specific CD_4^+ and CD_8^+ T-cell responses²⁹.

The use of HAART as an effective anti-HIV therapy is limited. Inevitably viruses escape from drug pressure and resistant strains rebound to high levels. This problem creates a niche for the use of molecular therapies such as siRNAs as anti-HIV agents, as they show promise as a means of inhibiting HIV without inducing the negative side effects associated with current therapies³.

1.4 Molecular Approaches to therapy

Molecular Therapies could enhance the repertoire of antiviral therapies that are currently in existence. They have the potential to be a readily producible therapy for HIV infected individuals in the developing world, where access to current antiretroviral therapies is limited. Several gene based macromolecular drugs have been investigated as promising, non-toxic alternatives to current antiretroviral therapies. These therapeutic agents included antisense oligonucleotides, ribozymes and microRNAs (miRNA).

Antisense oligonucleotides are classified as short segments of DNA (modified or not) that bind to complementary sequences of DNA or RNA with a hydrogen bond³⁰. Antisense oligonucleotides are able to cause protein reduction by two distinct mechanisms. The name of the antisense oligonucleotide is indicative of the mechanism by which they work. Cleavers are antisense oligonucleotides that bind in an antisense fashion (by Watson-Crick base pairing)

to the target RNA. This activates RNase H which cleaves the target RNA. The second group of antisense molecules is known as blockers. Blockers bind to target RNA by Watson-Crick pairing, and act as ‘roadblocks’ to ribosomes inhibiting the translation of the proteins³⁰. These antisense molecules have been shown to accumulate in the nucleus.³⁰

Antisense oligonucleotides have been shown to inhibit HIV-1 *in vitro*, and have even been shown to control chronic HIV infection³⁰. As many as ten antisense oligonucleotides against HIV have been tested in phase 1 and phase 2 clinical trials³⁰. The use of antisense technology appears to produce minimal side effects, with the exception of off target inhibition of protein translation³⁰. Although *in vitro* work has looked promising, the use of antisense oligonucleotides has had limited therapeutic success³¹.

Ribozymes are small catalytic RNA subunits which are able to cleave DNA of complementary sequence in the presence of metal ions³². They are able to act as enzymes in the complete absence of proteins.³¹ Ribozymes are able to either break or form covalent bonds with a sequence mediated specificity. Due to the sequence specificity, ribozymes can be manipulated to cleave specific target sequences as well as to cleave both *in cis* and *in trans*.³¹

Micro RNAs (miRNA) are shRNA that are transcribed by discrete loci in the genome of various organisms³³. These miRNA are able to inhibit gene expression through the RNAi pathway. The difference between miRNA and siRNA mediated gene inhibition is that where siRNA are 100% homologous to the target sequence and mediates target RNA degradation, alternatively miRNA exhibit several sequence mismatches when compared to the target and result in the inhibition of protein translation³⁴.

1.4.1.1 RNA interference

The recent discovery of the RNA interference phenomenon (RNAi), has sparked great interest in manipulating the pathway to inhibit various viral infections including HIV-1. RNAi was first described by Fire *et al*, when they noted that long double stranded RNA molecules led to sequence specific gene silencing in *Caenorhabditis elegans*³⁵. This phenomenon, has been linked to post transcriptional gene silencing (PTGS) seen in plants³⁶. It is thought that the RNAi pathway is an ancient antiviral mechanism that allows, sequence specific degradation of foreign RNA molecules such as RNA viruses and retrotransposons³⁷. Subsequently, RNAi has been found to be effective in not only plants and nematodes, but also in the fruit flies (*Drosophila*), *Trypanosoma brucei* and mammalian cells^{37,38,39}.

The finding that double stranded RNA (dsRNA) sequences of less than 22 bp can activate the RNAi pathway, whilst simultaneously evading the mammalian antiviral response, the interferon response, which once activated results in the inhibition of protein translation in the cell, has lead to the exploitation of the pathway to trigger the degradation of viral RNA transcripts⁴⁰.

siRNAs have previously been used in many different research and therapeutic settings. Many research advancements can be made using siRNA as gene knockdown tools, but perhaps the major promise of siRNAs lies in their potential therapeutic use. The sequence specific nature of siRNAs together with their ability to evade the interferon response could potentially allow viruses to be cleared from the cells without inducing cell death⁴¹.

Various viruses have been targeted by siRNAs for degradation including the polio virus ⁴², human papilloma virus ⁴³, RSV, Hepatitis B virus ⁴⁴ and Hepatitis C virus³⁸. One of the most targeted viruses is HIV-1 Subtype B, of which *gag* ^{45,46,47} *tat* ^{48,49,50} *rev*^{50,51,52,53} *vif* ⁵⁴, *env* ⁴⁷, *nef* ⁵⁴ and the *LTR*^{45,54} mRNA have been effectively degraded *in vitro* ³⁸. As yet, siRNAs have not been shown to induce HIV-1 subtype C RNA degradation

siRNA and shRNA expressed from both adenoviral and lentiviral vectors have been shown to specifically reduce the expression of stably expressed plasmids, endogenous genes and transgenes, both *in vitro* and *in vivo* ⁴⁰. Manipulation of the RNA pathway permits genes of choice to be targeted by siRNAs for degradation, and leaves the door wide open to target various HIV sequences and exploitation of the RNAi pathway will hopefully prove to be a potent antiviral tool against subtype C viruses.

1.4.1.2 siRNA and the RNA interference pathway

In the classical RNAi pathway (Figure 7), long dsRNA (typically from viruses) enter the cell and are then cleaved by an RNase III enzyme known as Dicer into short dsRNA molecules in an ATP dependent manner ⁵⁵ (short hairpin RNAs (shRNAs), can also be cleaved by dicer into short RNAs). These short RNAs, termed short interfering RNA (siRNA), are typically 19-21 base pairs (bp) in length⁵⁶. siRNAs cleaved by dicer exhibit a 2 nucleotide (nt) 3' overhang, together with a 5' phosphate and a 3' hydroxyl group, a typical RNase III cleavage pattern ³⁸,

⁵⁷.

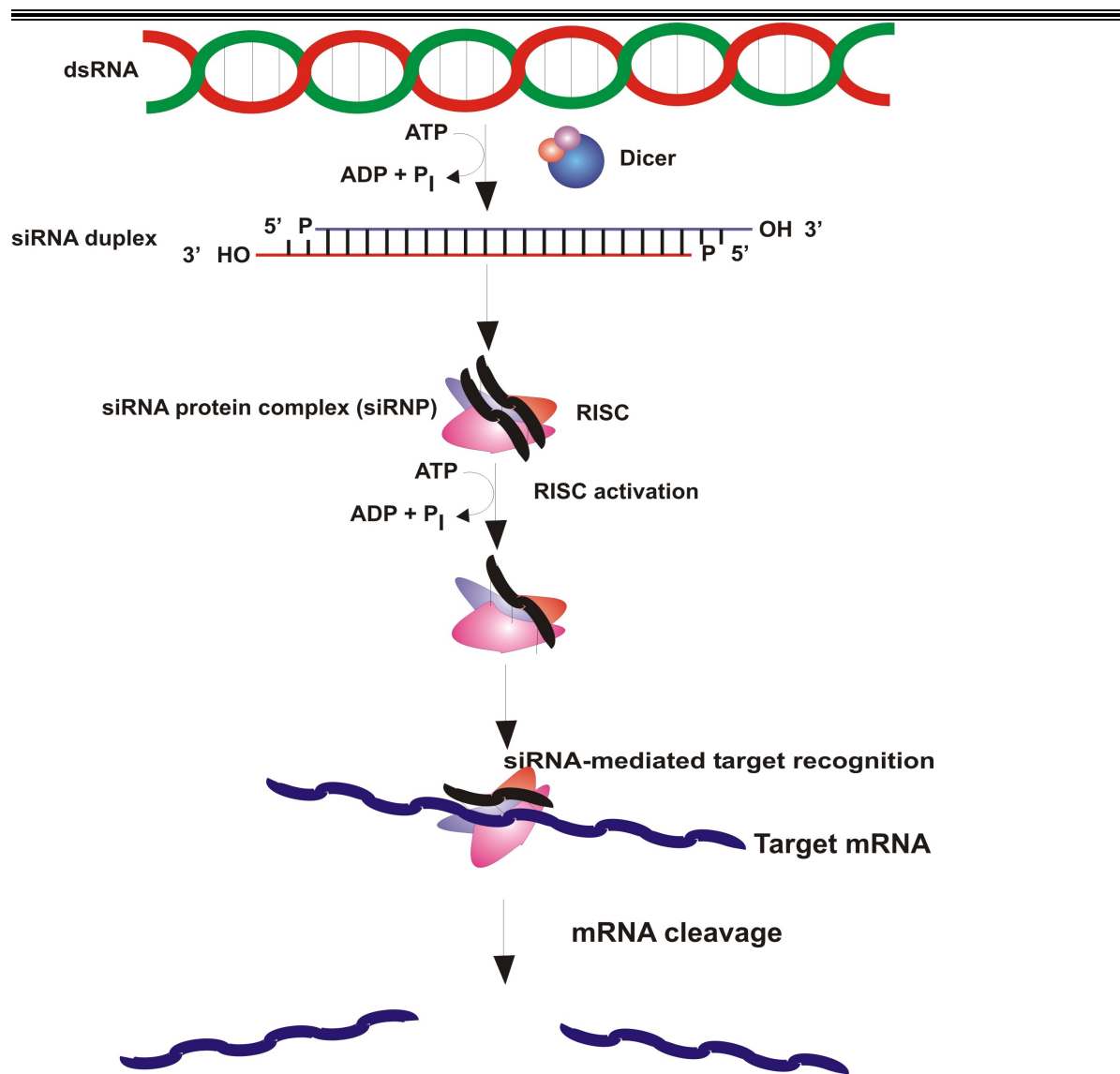


Figure 7: Schematic representation of the RNAi pathway. Long dsRNA enters the cell and is then processed by dicer into siRNA duplexes in an ATP dependent manner. The siRNA is then incorporated into RISC and the duplex is unwound (dependent on ATP). The strand that remains in RISC acts to guide RISC to target RNA of complementary sequence and mediates the cleavage of the target RNA

Following cleavage, the siRNA are incorporated into an enzymatic complex known as the RNA induced silencing complex (RISC)⁵⁸. An accessory protein known as R2D2 forms a stable complex with dicer⁵⁹. R2D2 has a role in controlling siRNA function but not siRNA

processing, and acts only to transfer siRNA molecules from dicer to RISC⁶⁰, and the R2D2 complex is then incorporated into RISC^{59, 61}. The incorporation of RNA into this complex is dependent on the siRNA being phosphorylated at its 5' end³⁸. It has been shown that exogenous synthetic siRNAs, and single stranded RNA (ssRNA), enter the pathway at this stage⁶²

RISC consists of several proteins, although not all are known. Two members of the Argonaute family of proteins eLF2C1 and eLF2C2 have been shown to be incorporated although their exact function is not yet known⁶². These proteins are not efficiently co-selected to enter RISC with biotinylated siRNAs unless there is a functional dicer/R2D2 complex present⁶⁰. The Argonaute proteins, are a vital component of the RNAi pathway, and the availability of these proteins corresponds directly to the efficiency of the RNAi response⁶².

Another component of RISC is speculated to be a kinase, which functions *in vivo* to maintain the 5' phosphate of the siRNAs. This allows them to function through multiple rounds of cleavage⁵⁵. RISC is therefore a 'multiple turnover' enzymatic complex, capable of being used numerous times. The limiting step of RISC is thought to be determined by eri-1, an enzyme related to the RNase T family of 3'-5' exonucleases. Eri-1 functions to remove the 3' overhang of the siRNAs, destabilizing the siRNA and without the 3' overhang, the siRNAs become effectively invisible to RISC.⁶³

Following incorporation into RISC, the siRNA is unwound in an ATP dependent fashion by a helicase^{55,56,62}. The helicase initiates the unwinding of the siRNA duplex at the less stable end, selecting the strand with the most 5' instability to become the strand retained in RISC^{64,65} The

antisense strand (retained in the complex), acts to 'guide' RISC to RNA with complementary sequence within the cytoplasm^{38, 65}. Once the target RNA has been located, RISC initiates endonucleolytic cleavage of the target RNA at a single site 10 nt from the 5' end of the siRNA^{37,38}. The cleavage of the target RNA molecule is not ATP dependent.⁶² The unincorporated strand of the siRNA duplex is degraded in the cell⁶⁵. siRNA inhibition of gene expression is effective, but has not been shown to completely inhibit gene expression *in vitro*⁶⁶. siRNAs that do not find a suitable target remain inert in the cell⁶⁷

The natural RNAi antiviral mechanism can be engineered to induce the knockdown of an RNA sequence of interest, such as the gag gene. The 'artificial' siRNA sequences are able to enter the RNAi pathway and result in sequence specific mRNA degradation, whilst simultaneously evading the mammalian interferon response.

Interferon (IFN) is an antiviral system found in mammalian cells, which is triggered by long dsRNA molecules (a by-product of viral replication)^{38,68,69}. The dsRNA trigger results in a non-sequence specific response involving the induction of a multi-component signaling complex that includes the enzyme PKR. PKR serves to activate a signaling cascade that up regulates the IFN genes, and acts to phosphorylate eIF2 α , a general translation factor. The phosphorylation of eIF2 α results in the arrest of protein synthesis in the cell. When the IFN system is sufficiently activated the cell undergoes apoptosis^{68,69}.

Until recently, it was thought that only dsRNA longer than 30bp have the ability to activate the interferon response³⁸. However, certain studies have indicated that dsRNA molecules of as little as 17nt can trigger the upregulation of interferon genes⁷⁰. It is now thought that the

siRNA induced interferon response results in the activation of the toll-like receptor (TLR)⁷⁰. It has been postulated that as little as 11bp, a single turn of the dsRNA helix, can activate TLR. This activation results in the production of type 1 IFN, interleukin 8 (IL-8), IFN α , NF- κ B promoter activation and the induction of sequence independent gene suppression⁷⁰.

The introduction of siRNA into cells, can be either through direct transfection of synthetic RNAs or, alternatively through the expression of siRNA from a plasmid vector. To ensure prolonged expression of anti-HIV siRNAs, plasmid based expression systems are optimal. In the study, the anti-gag siRNAs were expressed from a plasmid backbone which transcribed the sense and anti-sense sequences of the siRNA on the same transcript, joined by a miRNA loop. The transcript folds back on itself due to the complementarity between the sense and anti-sense strands of the siRNA, the miRNA loop is then cleaved away during processing of the hairpin-loop transcript into mature siRNAs.

1.5 Rationale of the investigation

There is a continuous need for the development of novel therapies targeting HIV-1, to be used alone, or in conjunction with HAART. The use of gene based therapies holds great promise in the developing world, as they are comparatively inexpensive, and easy to produce.

The RNAi pathway has been manipulated to induce the degradation of RNA of various viruses. The use of siRNAs has previously been shown to inhibit the replication of HIV-1 subtype B.

It was the aim of this study to test the inhibitory efficacy of shRNAs targeted to HIV-1 subtype C isolates. It was decided to target the gag ORF of HIV-1 subtype C as it encodes one of the most highly conserved sequences in the highly variable HIV-1 genome. Choosing a conserved region would allow numerous virus strains to be targeted by the same shRNA. The Gag proteins play an important role in the nuclear transport of the viral core, forms part of the core capsid, and binds RNA within the nucleocapsid. The removal of the Gag poly-protein would therefore result in a loss of plasma membrane targeting resulting in the loss of multimerisation and reduced binding of RNA. This would culminate in the reduction of virion particle formation and a reduced viral load, which makes it an ideal target to inhibit HIV-1 replication⁷¹.

It was decided to design plasmids capable of expressing short hairpin RNAs targeted to *gag*, apposed to synthetic RNA molecules. This was because synthetic RNA sequences are more expensive to manufacture, and it needs to be modified to become stable within the cell. A

plasmid based expression system synthesizes RNA that is naturally stable within the cell, they also allow for continued expression of the inhibiting shRNA and therefore prolonged suppression of viral genes. Prolonged expression of the therapeutic RNA could be vital for the control of chronic infections such as HIV.

1.6 Objective

The majority of studies on HIV-1 have been done on subtype B viruses, although subtype C viruses are responsible for the majority of infections world-wide. This is the first study examining the effect of the RNAi pathway on the inhibition of subtype C isolates. The specific objectives of this study were:

1. The generation of shRNA produced endogenously from cloned expression vectors targeted to the *gag* mRNA of HIV-1 subtype C isolates.
2. The construction of a reporter plasmid, including a green fluorescent protein (EGFP)/*gag* fusion transcript.
3. Testing the efficacy of the shRNA constructs against the EGFP/*gag* reporter plasmid
4. Testing the efficacy of the shRNA constructs against HIV-1 subtype C isolates.
5. Determining the ability of the shRNAs to induce an interferon response.
6. Determining if the enzyme dicer was cleaving the shRNAs correctly within the cell.

CHAPTER 2: METHODOLOGY

2.1 Construction of shRNA expressing plasmids

2.1.1 *Designing the shRNAs*

To exploit the RNAi pathway to inhibit HIV-1, sequences encoding short, hairpin RNAs were incorporated into a plasmid vector. When selecting the target gene, it was essential to opt a conserved gene, as the more conserved the area targeted the more strains of HIV could be targeted using the same shRNA, it also decreases the chance of an escape mutant developing. The gag gene was selected as it was the most highly conserved amongst the subtype C viruses.

The gag ORFs of 33 known subtype C isolates, together with the consensus sequence for subtype C viruses were obtained from the NICD (National Institute for Communicable Diseases) database. The gag sequences were then aligned and 10 of the most conserved sequences within subtype C gag mRNA were selected (Table 1). The chosen sequences were homologous to the consensus sequence, created from the NICD database for subtype C and ranged in size from 20 to 22 nt in length and spanned the entire gag mRNA (Figure 9). The shRNA were designed to meet the requirements for making a PCR product capable of expressing shRNAs, set out by Daniella Castonotto and colleagues⁷². The requirements state that the sequence targeted begin with a G (to allow the U6-promoter to function), and not end in an A (as this would create a termination signal of four adenosines due to the sequence of the miRNA that was utilized in the construct).

Table 1: The ten target sequences to which the shRNA sequences were designed to target and their position in the *gag* open reading frame of HIV-1 subtype C consensus sequence.

	HBx Accession Number	Location in the Gag consensus sequence	shRNA sequence
A	1207-1227	412-432	5' GGGCAAATGGTACACCAGGCC 3'
B	1075-1095	286-306	5' GACACCAAGGAAGCCTTAGAC 3'
C	1033-1053	244-265	5' GTAGCAACTCTCTATTGTGT 3'
D	792-182	3-24	5' GGGTGCGAGAGCGTCAATATT 3'
E	1309-1329	511-531	5' GCATTATCAGAAGGAGCCACC 3'
F	1318-1338	520-540	5' GAAGGAGCCACCCCACAAGAT 3'
G	1362-1382	567-587	5' GGGGGGAGATCAAGCAGCCAT 3'
I	1498-1518	701-721	5' GCAGGAACTACTAGTACCCTT 3'
J	1819-1839	1277-1248	5' GGCTGTTGGAAATGTGGAAAG 3'
L	2023-2024	1021-1041	5' GAAGAAATGATGACAGCATGT 3'

The constructs were designed to incorporate the U6-promoter, the sense and antisense siRNA sequences joined by a miRNA loop, a terminator sequence and a stuffer sequence containing restriction enzyme sites to allow for cloning (Figure 8).



Figure 8: Schematic Representation of the sequence encoding the shRNA together with the U6 promoter

2.1.2 Designing the PCR Primers

In order to generate the PCR product containing the U6 promoter and shRNA sequence depicted in Figure 8, a two step PCR was undertaken. The 5' primer for the initial PCR reaction was designed to flank the U6 promoter on the 5' end of the sense strand, this universal primer is complementary to the first 20 nt of the U6 promoter and has a *Xba I* and a *Xho I* site on the 5' end to allow for further cloning operations.

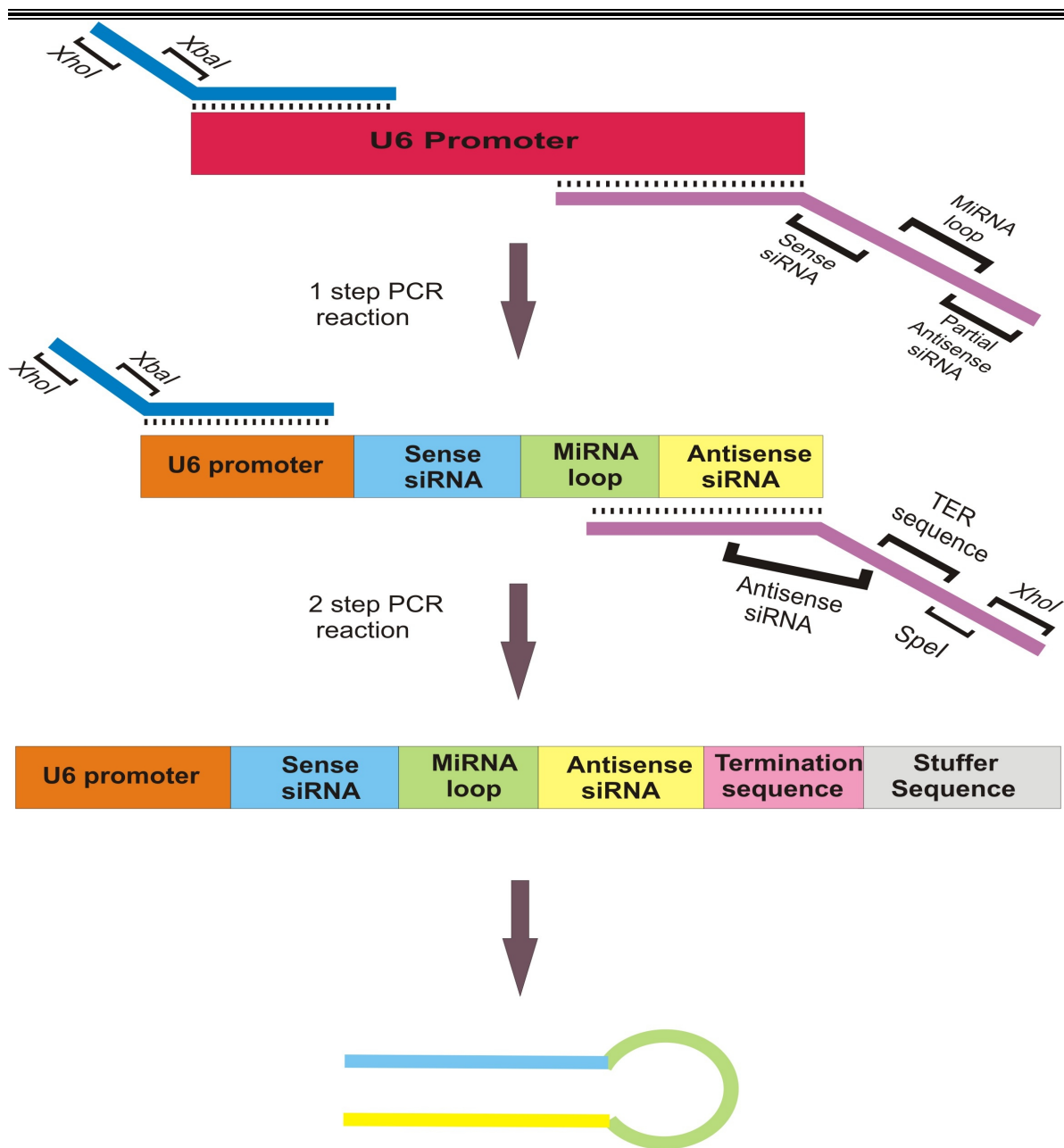


Figure 9: Schematic representation of the PCR based cloning procedure utilized to obtain a shRNA encoding sequence under the control of a U6 promoter. The resultant amplicon contains the sequence for the U6 promoter, the sense siRNA strand, the antisense siRNA strand, a miRNA loop, the signal for termination of transcription and a stuffer sequence that contains the restriction endonuclease sites *SpeI* and *XhoI* used in cloning the amplicon into a plasmid. The diagram also illustrates the final hairpin structure that will be produced from the U6 promoter.

Table 2: U6 universal primer sequence, the restriction endonuclease sequences for *XhoI* (depicted in red) and *XbaI* (depicted in blue) are indicated in the sequence

Primer Name	Primer Sequence
U6 Universal Primer	5' GCGCTCGAGTCTAGAAAGGTCGGGCAGGAAGAGGG 3'

The primer designed to flank the U6 promoter on the 3' end of the sense strand was complementary to the last 20 nt of the U6 promoters antisense sequence it also incorporated the sense sequence of the individual siRNA, the sequence for the miRNA loop and a sequence encoding a portion of the antisense strand of the siRNA (Table 3).

Table 3: Primer sequences utilized in the first step of the PCR reaction to create shRNA.

Primer Name	Primer Sequence
Gag-siRNA-2A	5' CAGGCCCTACACAAAGGCATGGTGTACCA TCTGCACGGTGTTCGTCCTTTCCACAAG 3'
Gag-siRNA-2B	5' TTAGACCTACACAAAGTCTAAGGCTTCCT TGGTGTCGGTGTTCGTCCTTTCCACAAG 3'
Gag-siRNA-2C	5' TGTGTACTACACAAATACACAATAGAGAGT TGGCTACGGTGTTCGTCCTTTCCACAAG 3'
Gag-siRNA-2D	5' ATATTACTACACACAAATAATATTGACGTT CTCCACCCGGTGTTCGTCCTTTCCACAAG 3'
Gag-siRNA-2E	5' GCCACCCTACACAAAGGTGGCTCCAACCTG

	ATAATGCGGTGTTTCGTCCTTTCCACAAG 3'
Gag-siRNA-2F	5' ACAAGACTACACAAATCTTGTGGGGTGG CTCCTTCGGTGTTTCGTCCTTTCCACAAG 3'
Gag-siRNA-2G	5' GCCATGCTACACAAACATGACTGCTTAAT CCCCCCCCGGTGTTTCGTCCTTTCCACAAG 3'
Gag-siRNA-2I	5' CCCTTCCTACACAAAGAAGGGTACTAGTA GTTCTGCGGTGTTTCGTCCTTTCCACAAG 3'
Gag-siRNA-2J	5' GGAAAGCTACACAACTTTCCACATTTCCA ACAGCCGGTGTTTCGTCCTTTCCACAAG 3'
Gag-siRNA-2L	5' GATGTCCTACACAAAGACATGCTGTCATCA TTTCTTCGGTGTTTCGTCCTTTCCACAAG 3'

Table 4: Primer sequences utilized in the second step of the PCR reaction to create shRNA. The restriction endonuclease sites *XhoI* (depicted in red) and *SpeI* (depicted in green) are indicated on the primer sequences.

Primer Name	Primer Sequence
Gag-siRNA-1A	5' GATC CTCGAG ACTAGT AAAAAAGGGCAAATGGTAC ACCAGGCCCTACACAAGGCCTG 3'
Gag-siRNA-1B	5' GATC CTCGAG ACTAGT AAAAAAGACACCAAGGAA GCCTTAGACACCTACACAAAGTCTAA 3'
Gag-siRNA-1C	5' GATC CTCGAG ACTAGT AAAAAAGTAGCAACTCTCT ATTGTGTACTACACAAATACACA 3'
Gag-siRNA-1D	5' GATC CTCGAG ACTAGT AAAAAAGGGTGCGAGAGCG TCAATATTACTACACAAATAATAT 3'

Gag-siRNA-1E	5' ATC CTCGAG ACTAGTAAAAAAGCATTATCAGAAGG TGCCACCCTACACAAAGGTGGC 3'
Gag-siRNA-1F	5'GATC CTCGAG ACTAGTAAAAAAGAAGGAGCCACCCC ACAAGACTACACAAATCTTGT 3'
Gag-siRNA-1G	5' GATC CTCGAG ACTAGTAAAAAAGGGGGGGGATTAA GCAGTCATGCTACACAAACATGGC 3'
Gag-siRNA-1I	5' GATC CTCGAG ACTAGTAAAAAAGCAGGAACTA CTAGTACCCTTCCTACACAAAGAAGGG 3'
Gag-siRNA-1J	5' GATC CTCGAG ACTAGTAAAAAAGGCAGAAGGAAA TGTGGAAAGCTACACAAACTTTCC 3'
Gag-siRNA-1L	5' GATC CTCGAG ACTAGTAAAAAAGAAGAAATGATG ACAGGATGTCCTACACAAAGACATG 3'

The U6 primer was utilized for the second PCR reaction as was a primer individualized for each shRNA sequence to be generated, consisting of a portion of sequence complementary to the miRNA loop, the antisense sequence of the individual siRNA the Termination sequence of six adenosines and finally a stuffer sequence containing a *SpeI* and a *XhoI* restriction enzyme site (Table 4).

2.1.3 PCR cloning of shRNA

Twenty-one primers were ordered (see 1.2), one being the U6 universal primer and the other 20 being individualized as described for each of the ten shRNAs (Inqaba Biotechnologies, South Africa).

The lyophilized oligonucleotides were resuspended in pure water to generate a stock at an initial concentration of 100 μ M. The stock was then diluted to form a working concentration of 15 pg/ μ l. This concentration was confirmed using A_{260} value measured on a spectrophotometer (appendix 6.1.21).

Plasmids containing the shRNA sequences in conjunction with the U6 promoter were created using a two-step PCR reaction. A plasmid vector containing the U6 promoter was used as a template and was amplified in a 30 cycle PCR reaction on the Mastercycler Gradient (Eppendorf, Germany). The PCR reaction consisted of an initial 3 minutes activation step at 94°C followed by a denaturation step at 95°C for 1 minute; an annealing step at 55°C for 1 minute and finally an extension step at 72°C for 1 minute. The PCR was carried out using 25 μ l of PCR Master Mix (Promega, WI, USA), 15 pg of the U6 universal primer, 15 pg of the reverse primer containing the sense sequence and the U6 plasmid template.

One μ l of the PCR product from the initial reaction was utilized as the template for the second PCR reaction in combination with 25 μ l PCR Master Mix (Promega, WI, USA), 15 pg of the U6 universal primer and 15 pg of the reverse primer containing the antisense and termination sequence. The reaction was carried out with the PCR program described above.

The PCR product of the second reaction was then run on a 1.5% low melting point agarose gel and the larger 362 bp fragment was eluted using the MinElute Gel Extraction Kit (Qiagen, CA, USA). The purified fragment was then ligated into the pDrive PCR cloning vector (Qiagen, CA, USA) using 4 µl of PCR fragment according to manufacturer's instructions (Qiagen, CA, USA) and left to ligate overnight at 4°C.

Five µl of the ligation mix was used to transform competent XLI-BLU *E. coli* (Stratagene, CA, USA), the transformed *E. coli* were plated out on Amp⁺ (25 µg/µl); IPTG⁺; X-gal⁺ agar plates and incubated overnight at 37°C. Ten white colonies were picked for each shRNA expressing construct and inoculated in 10 ml Amp⁺ LB medium and incubated overnight at 37°C.

The plasmids were prepared using the High Pure Plasmid Isolation Kit (Roche, Germany) (appendix 6.1.18) and digested to verify the presence of an insert with *EcoRI* (Promega, WI, USA,) and buffer H (Roche, Germany) in a 20 µl reaction volume, as *EcoRI* cuts on either side of the inserted fragment. All clones that were positive for an insert were then digested with *XbaI* (Roche, Germany) using buffer B (Roche, Germany) in a 20 µl reaction volume to determine their orientation, only clones that were in a 5' to 3' orientation were used and these gave a 260 bp fragment as opposed to a 20 bp fragment.

Three clones were sequenced by fluorescent dye terminator cycle sequencing to confirm the presence of the correct sequence (Inqaba Biotechnologies, South Africa). Each of the shRNA

expressing plasmids were prepared using the endotoxin free kit (Qiagen, CA, USA) (appendix 6.1.19) to be used in tissue culture.

2.2 Generation of a EGFP/*gag* fusion reporter plasmid

To determine the relative reduction of *gag* mRNA it was decided to institute a reporter plasmid system. To generate the reporter plasmid, the open reading frames (ORFs) of eEGFP and the *gag* ORF of the subtype C HIV-1 isolate Du422aa were combined into a plasmid capable of expressing both ORFs into a single mRNA transcript. With such a transcript, any degradation of mRNA caused by shRNA induced inhibition would result in a corresponding decrease in eEGFP expression. This system therefore allows for the direct visualization of the shRNA induced inhibition of the *gag* mRNA

2.2.1 Cloning into *pDrive PCR plasmid*

2.2.1.1 Generation of the *gag* sequence

A plasmid, *gag* 22 (Prof Lynn Morris, NICD, personal communication), containing the *gag* open reading frame of the R5 HIV-1 subtype C virus Du422a was used as the template for a PCR reaction to generate the entire *gag* ORF to insert into the reporter plasmid.

Two primers were designed to flank the ORF and were complementary to the first twenty bases on the 5' and 3' ends respectively (Table 5). The primers incorporated a *Hind III* restriction endonuclease site 5' of the ORF and an *XbaI* site 3' of the ORF. The PCR reaction was carried out using the PCR conditions described in section 1.3. The amplicon obtained

contained the entire gag ORF flanked by a 5' *HindIII* restriction endonuclease site and a 3' *XbaI* restriction endonuclease site.

Table 5: Primers utilized to generate the gag ORF

Primer	Sequence
Forward	5' GATCAAGCTTATGGGTGCGAGAGCGTCATTAT 3'
Reverse	5' GATCTCTAGATTATTGAGACAAGGGGTCGCTG 3'

2.2.1.2 Generation of the Enhanced Green Fluorescent Protein Open Reading Frame

To generate the U6 sequence, a plasmid, containing the U6 promoter sequence was used as the template for a PCR reaction. Two primers were designed to flank the EGFP ORF and were complementary to the first twenty bases on the 5' and 3' ends respectively (Table 6). The primers incorporated a *Hind III* restriction endonuclease site 3' of the ORF and a *BamHI* site 5' of the ORF. The PCR reaction was carried out using the PCR conditions described in section 2.3.1. The PCR product obtained contained the entire EGFP ORF flanked by a 5' *BamHI* restriction endonuclease site and a 3' *HindIII* restriction endonuclease site.

Table 6: Primers utilized to generate the EGFP ORF

Primer	Sequence
Forward	5' GATCGGATCCATGGTGAGCAAGGGCGAGGA 3'
Reverse	5' GATCAAGCTTCTTACTTGTACAGCTCGTCC 3'

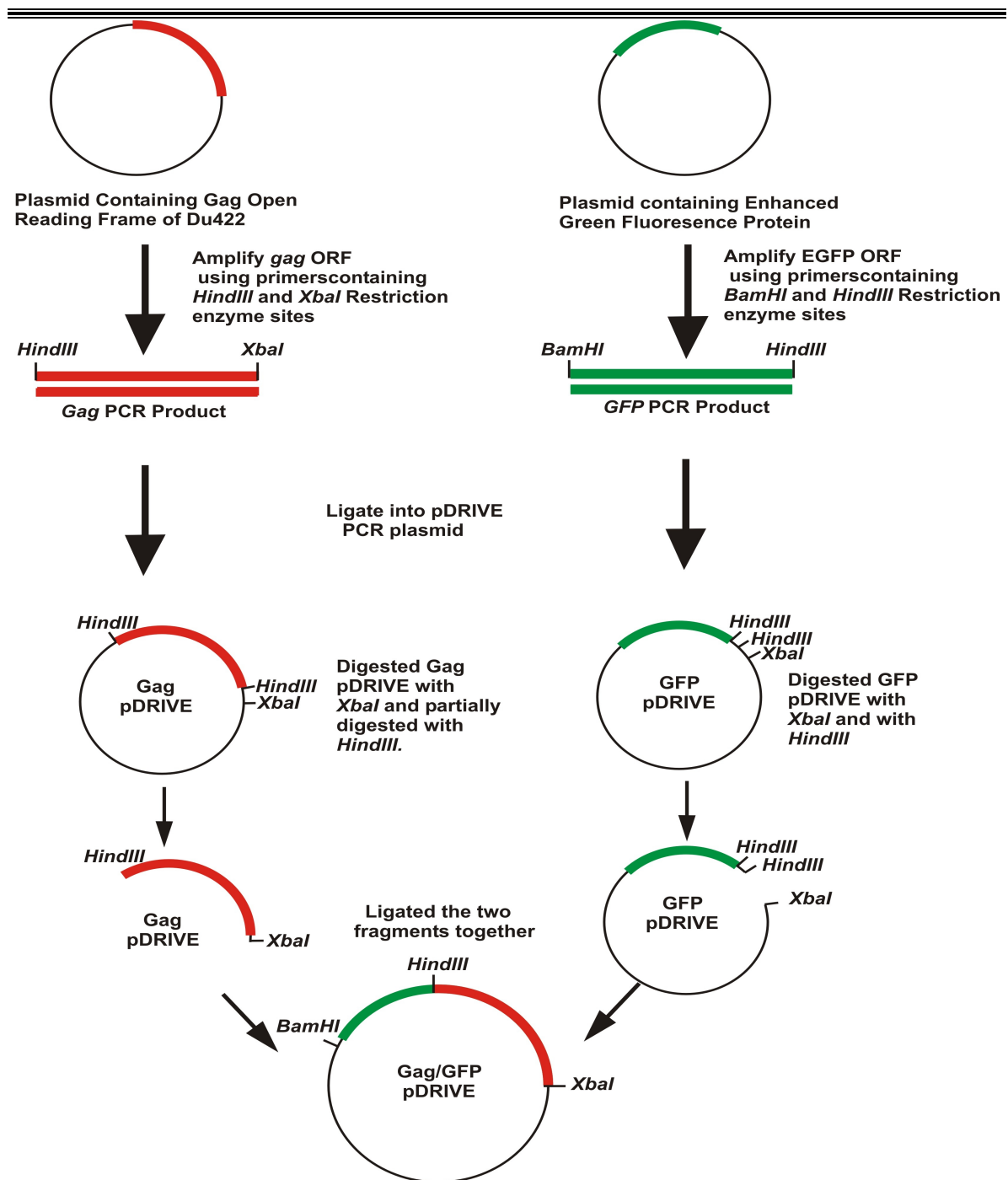


Figure 10: Schematic representation of the cloning strategy used to combine the gag and EGFP ORFs into a reporter plasmid. The ORFs of both gag and EGFP, were amplified by PCR, and were initially cloned into separate pDRIVE plasmids. The gag ORF was then eluted from gag pDRIVE and inserted into EGFP pDRIVE forming a plasmid encoding a *EGFP/gag* fusion mRNA.

2.2.1.3 Incorporating the gag and EGFP ORFs into pDrive

The eEGFP and gag ORF obtained from the PCR reaction (2:2:1.2) were run on a 1.5% low melting point agarose gel to purify the fragment. The 1200 bp gag fragment and the 700 bp eEGFP fragment were eluted separately from the gel using the MinElute Gel Extraction Kit (Qiagen, CA, USA) (appendix 6.1.17). The purified fragment was then ligated into the pDrive cloning vector using 4 µl of PCR fragment as per manufacturer's instructions (Qiagen, CA, USA) and left to ligate overnight at 4°C.

Five µl of the ligation mix was used to transform competent XLI-BLU *E. coli*, to allow for blue-white screening of colonies containing inserts, were plated out on Amp⁺; IPTG⁺; X-gal⁺ agar plates and incubated overnight at 37°C. Ten white colonies were picked for each of the presumptive eEGFP and gag clones and inoculated in 10ml Amp⁺ LB medium (appendix 6.1.1) and incubated overnight at 37°C.

The plasmids were prepared using the High Pure Plasmid Isolation Kit (Roche, Germany) (appendix 6.1.18) and digested to verify the presence of an insert with *EcoRI* (Promega, WI, USA,) and buffer H (Roche, Germany) in a 20 µl reaction volume, as *EcoRI* cuts on either side of the inserted fragment. All clones that were positive for an insert were then digested with *XbaI* (Roche, Germany) using buffer B (Roche, Germany) in a 20 µl reaction volume to determine their orientation, only clones that were in a 5' to 3' orientation were used and these gave a 260bp fragment as opposed to a 20bp fragment when run on a 1% agarose gel. The

clones lying in a 5'-3' orientation were sequenced by fluorescent dye-terminator cycle sequencing (Inqaba Biotechnology, South Africa) to ensure that the sequence was correct.

2.2.1.4 Combining the gag and EGFP plasmids

The gag and eEGFP ORFs were then combined into a single plasmid. Gag pDRIVE was digested with *XbaI* (Roche, Germany) in a 50 µl reaction volume using Buffer B (Roche, Germany). The resulting linearised product was then partially digested with *HindIII* (NEB, MA, USA) to excise the entire gag ORF. EGFP pDRIVE was digested with *XbaI* and *HindIII* in a 50 µl reaction volume using Buffer A (Roche, Germany).

The linearised EGFP pDRIVE was ligated to the gag ORF (excised from gag pDRIVE) in a 20 µl ligation reaction at 4°C overnight. Five µl of the ligation mix was then used to transform competent XLI-BLU *E. coli*, plated out on Amp⁺; IPTG⁺; X-gal⁺ agar plates and incubated overnight at 37°C. Ten white colonies were picked for each of the EGFP/gag fusion clones, inoculated in 10 ml Amp⁺ LB medium and incubated overnight at 37°C.

The plasmids were prepared using the High Pure Plasmid Isolation Kit (Roche, Germany) and digested to verify the presence of an insert with *BamHI* (Promega, WI, USA,) and *XbaI* (Roche, Germany) in buffer H (Roche, Germany) in a 20 µl reaction volume. The digestion of the plasmid, with *BamHI* and *BbaI* excises a 2100bp fragment containing both the eEGFP and gag ORF

2.3 Cloning into pTRE2 hyg

To allow the eEGFP/gag fusion mRNA to be expressed *in vitro*, the transcript needs to be under the control of a mammalian promoter system, as apposed to the bacterial promoter system found in pDRIVE. The eEGFP/gag fusion transcript was therefore cloned into the pTRE2hyg (BD Biosciences Clontech, CA, USA), which contains a mammalian promoter.

2.3.1 Preparation of pTRE2 plasmid backbone

The pTRE2hyg plasmid was digested in a 50 µl reaction volume with the restriction endonucleases *XbaI* (Roche, Germany) and *BamHI* (Roche, Germany) using buffer B (Promega, WI, USA). The digest was run on a 0.8 % agarose gel and the pTRE2 plasmid backbone eluted using the Mini-elute kit (Qiagen, CA, USA).

2.3.2 Removing the gag EGFP ORF from pDRIVE EGFP/gag

The pDRIVE EGFP/gag plasmid was digested with the restriction endonucleases *BamHI* (Roche, Germany) and *NheI*, (Promega, WI, USA) in a 50 µl reaction volume using buffer A (Roche, Germany). The digest was then run on a 0.8% agarose gel and the fragment of 2100 bp containing the EGFP/gag ORF was eluted using the Mini-elute kit (Qiagen, CA, USA)

2.3.3 Creating pTRE2Hyg EGFP/gag

The EGFP/gag 2100 bp fragment was then ligated into 1 µg of pTRE2hyg plasmid backbone in a 10 µl reaction volume together with 1 µl DNA ligase (Promega, WI, USA) and 5 µl Ligase buffer (Promega, WI, USA). The ligation was carried out at 4°C overnight.

Five µl of the ligation mix was then transformed into competent XL1-BLUE *E. coli* and plated on an AMP⁺ agar plate and incubated overnight at 37°C. Ten colonies were picked off the plate and grown up overnight at 37°C in 10ml LB medium with AMP⁺.

Plasmids were prepared from the chosen colonies using the Pure Plasmid Isolation Kit (Roche, Germany), and screened using *Bam*HI (Roche, Germany) and *Eco*RV (Promega, WI, USA) to determine the presence of a 2100 bp insert. Three of the colonies found to be positive for an insert were sequenced by fluorescent dye-terminator cycle sequencing (Inqaba Biotechnologies, South Africa). The plasmid with the correct sequence was then prepared using the endotoxin free kit (Qiagen, CA, USA) to be used in cell culture.

2.4 Transfecting Huh 7 cells with shRNAs and the reporter plasmid

2.4.1 Cell culture

Huh7 cells (donated by Dr. H. Nakabayashi) were grown to 80% confluence in 6 well (2 cm) tissue culture plates in RPMI with 2.5% fetal calf serum (FCS) with 500mg penicillin and streptomycin (PS). The cells were incubated at 37°C and 5% CO₂.

2.4.2 Transfections

Two hours prior to transfection, the Huh 7's medium was changed to RPMI without FCS and PS. The cells were then returned to the incubator (37°C and 5% CO₂).

The shRNA expressing plasmids were concentrated to 1 µg/µl. For each shRNA plasmid, 5 µl of plasmid solution was added to 250 µl of OPTIMEM medium (deficient in FCS and PS). To this solution 5 µg of the EGFP/gag reporter plasmid was added. To a second Eppendorf tube a further 250 µl of OPTIMEM was added together with 2 µl of lipofectamine 2000 (Invitrogen, CA, USA) (Table 7). Both tubes were vortexed gently and left to incubate for 5 minutes at room temperature. Once incubated the contents of the tubes were combined, vortexed gently and incubated at room temperature for 20 minutes. Following incubation, the contents of the tube were added, drop-wise to the meniscus of the RPMI medium covering the cells. The drops were added to the entire surface of the medium.

The positive and negative controls differed to the other shRNA transfections in that, in the positive control the shRNA plasmid was replaced with 5 µg of an empty pDRIVE vector. In the negative control the EGFP reporter plasmid was omitted and only the 5 µl of the pDRIVE empty plasmid were transfected into the cells. A β- galactosidase expressing plasmid was co-transfected (at 1 µg) in several wells to ensure standardized transfection efficiency occurred.

Table 7: Reaction volumes used to perform transfections with shRNA and the EGFP/Gag reporter plasmid.

Reagents	Positive Cont	Negative Control	A	B	C	D	E
ShRNA A (1 µg/µl)	-	-	5 µl	-	-	-	-
ShRNA B (1 µg/µl)	-	-	-	5 µl	-	-	-
ShRNA C (1 µg/µl)	-	-	-	-	5 µl	-	-
ShRNA D (1 µg/µl)	-	-	-	-	-	5 µl	-
ShRNA E (1 µg/µl)	-	-	-	-	-	-	5 µl
Empty pDrive Vector(1 µg/µl)	5 µl	5 µl	-	-	-	-	-
EGFP/gag Reporter plasmid (1 µg/µl)	5 µl	-	5 µl	5 µl	5 µl	5 µl	5 µl
Lipofectamine TM 2000	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
OptiMEM	500 µl	500 µl	500 µl	500 µl	500 µl	500 µl	500 µl

The cell culture plates were then left in the incubator overnight (37°C and 5% CO₂). The following morning the medium of the cells was changed to RPMI with FCS and PS.

Forty-eight hours post-transfection, the transfections were analyzed using both fluorescence microscopy and using flow cytometry. Staining for β-galactosidase was performed to ensure standard transfection efficiency occurred.

2.5 Flow cytometry

2.5.1 Preparation of cells

To prepare the cells for flow cytometry, transfections in 6-well dishes were used. Each well was incubated in 1ml of saline + EDTA (0.5M, pH8) at 37°C for 10 min on an orbital shaking incubator. One hundred µl of trypsin was then added to each well and the culture dishes were then returned to the orbital incubator for a further 3 minutes at 37°C. The trypsin was

neutralized with the addition of 900 μ l of saline + 1% FCS per well. The cells were then gently aspirated up in the 1 ml solution and transferred to a flow cytometry tube.

2.5.2 *Acquiring the data*

The flow cytometer (Beckman Coulter, CA, USA), was programmed to count cells according to size (detected measuring the forward scatter), and complexity (detected by measurement of the side scatter). The flow cytometric software was programmed to incorporate the following gates: 1) exclude all cellular debris; 2) exclude electronic noise; 3) measure fluorescence at a wavelength of 450nm; 4) measure the mean x (indicative of complexity) at approximately 70V, and 5) mean y (indicative of size) at approximately 670V. The cells collected from the EGFP transfection experiments were tested on the flow cytometer, and fluorescence was recorded for the first 10 000 cells run through the machine.

2.6 Staining for β -galactosidase

Transfected cells were fixed for 1 minute in 1 ml of fixative (appendix 6.1.13). The fixative was then removed and the cells were washed three times with 1 ml of PBS used for each wash (per well). Following washing, 3 ml of staining solution (appendix 6.1.12) was added to each well and the cells were left to incubate for 2-3 hours.

After the cells were stained, the staining solution was removed and the cells were washed with PBS. The amount of β -galactosidase expressing cells was determined by viewing the cells under a light microscope under a magnification of 40x. Cells observed to be blue under the

microscope were counted, to determine the transfection efficiency was standard throughout the individual sets of experiments.

2.7 Determining shRNA efficiency against live HIV-1 subtype C isolates

U87-CD4-CCR5 and U87-CD4-CXCR4 cells were plated out to 80% confluency in a 24 well dish and grown in DMEM with 10% FCS. The cells were transfected in the same manner as in section 2.4.2, except smaller volumes were used to accommodate the smaller well size and no reporter plasmid was added. Only 125 μ l of OPTIMEM was utilized for each tube in the transfection procedure bringing the total volume added to the cells to 250 μ l (Table 8). The β -galactosidase expressing plasmid was co-transfected at 1 μ g to ensure standard transfection efficiencies occurred throughout the experiment.

Table 8: Reaction volumes used to perform transfections with shRNA prior to live virus infection

Reagents	Positive Control	Negative Control	A	B	C	D	E
ShRNA A (1 μ g/ μ l)	-	-	3 μ l	-	-	-	-
ShRNA B (1 μ g/ μ l)	-	-	-	3 μ l	-	-	-
ShRNA C (1 μ g/ μ l)	-	-	-	-	3 μ l	-	-
ShRNA D (1 μ g/ μ l)	-	-	-	-	-	3 μ l	-
ShRNA E (1 μ g/ μ l)	-	-	-	-	-	-	3 μ l
Empty pDrive Vector (1 μ g/ μ l)	3 μ l	3 μ l	-	-	-	-	-
Lipofectamine TM 2000	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l
OptiMEM	250 μ l	250 μ l	250 μ l	250 μ l	250 μ l	250 μ l	250 μ l

The 24hrs following the transfections, live HIV-1 was added to the cells at a dose of 1000 TCID₅₀. Three different viruses were used: (1) Du151aa which uses the CCR5 co-receptor for

infection, (2) Du422a, which uses the CCR5 co-receptor for infection and (3) Sw7 which uses the CXCR4 co-receptor for infection³. Virus was added to all transfections with the exception of the negative control. Eighteen hours after infection, the excess virus was removed by ‘washing’ i.e. changing the medium of the cells.

Supernatants were analyzed on day 4, 7 and 11 following infection, by ELISA and on day 11 the cells were harvested and used for protein and RNA analysis.

2.8 p24 ELISA assay

The amount of p24 protein (expressed from the *gag* mRNA) was determined by an Enzyme linked immunosorbant assay (ELISA), which measures protein levels through the binding of the protein to antibodies (Abs), which serve to capture the proteins. Abs labeled with alkaline phosphatase bind to the primary antibody and allow for visualization of the amount of protein in a sample, by visualization of light emitted at a wavelength of 492nm. The amount of p24 produced corresponds directly to the amount of *gag* mRNA present in the cell culture.

2.8.1 Coating the ELISA plates with the anti-p24 antibody D7320

D7320 (Aalto Bio Reagents Ltd, Ireland), a sheep anti-p24 antibody was reconstituted in 2ml of pure water, a 1:200 dilution was then made, diluting the antibody in 0.1 M NaHCO₃ (Sigma-Aldrich, Germany) at a pH of 8.5. One hundred µl of the 1:200 dilution was then added to each well and incubated at room temperature overnight. The plates were then washed twice with tris buffered saline (TBS) (appendix 6.1.16). One hundred µl of

reconstituted skim milk was then added to each well; following this the plates were incubated for one hour at room temperature. The plates were stored at -20°C until used.

2.8.2 Inactivating the Virus

One hundred µl supernatant was harvested from the infected cells and added to 100 µl Empigen (Calbiochem, S.A. Scientific) on new plates and incubated at room temperature for 60 minutes to inactivate the HI virus.

2.8.3 ELISA

The coated plates were thawed and the milk discarded. The plates were then washed in 1x TBS. The inactivated sample was then added to the plate together with standard dilutions and a negative control (Empigen only). Samples were incubated on the plate for 2 hours at 37°C. Following the incubation, the plates were washed 4 times with TBS. An anti-HIV-p24 mouse monoclonal antibody, conjugated to alkaline phosphatase (Aalto Bio Reagents), with 0.05% Tween (Merck, Germany) (diluted in 100 µl TBS) and 20% sheep serum (Sigma-Aldich, Germany) was added to each well. The conjugate was then incubated for 1 hour at 37°C. the plate was then washed with a 4x Ampak buffer (Dako diagnostics, UK). Fifty µl of substrate was added and this was incubated at room temperature for 1 hour. The plate was then read at an OD of 492 nm.

2.9 Western Blot

The effect of RNAi on total proteins was determined by performing a Western blot, using HIV-1 positive serum as a primary Ab. Cells from a Du422a experiment were harvested and the cell number normalized through cell counting. The cells were then lysed with a lysis buffer (appendix 6.1.15) and equal amounts of 2x protein sample buffer (appendix 6.1.22) were added. The proteins were then denatured by boiling the sample for 5 minutes. The samples were then loaded onto a 10% SDS polyacrylamide gel and the gel was run at 40V for 2 hours.

The proteins were transferred onto a nitrocellulose membrane. Background staining was blocked by incubating the membrane overnight in 5% reconstituted skim milk powder, for 1 hour at 4°C on a shaker.

The membrane was then incubated for an hour in the primary Ab (obtained from serum from HIV positive blood) at room temperature. The membrane was washed twice in TBS and finally stained with the secondary Ab (an Anti-human goat IgG alkaline phosphatase conjugate (Sigma-Aldrich, Germany)). The Blot was developed using NBT/BCIP tablets.

2.10 Quantification of HIV-1 RNA by real-time reverse transcription PCR

RNA samples were isolated using the MagNA pure LC (Roche, Germany) from transfections using shRNA expressing plasmids and live virus. The isolation was carried out with the use of the MagNA Pure LC RNA isolation kit II (Roche, Germany), and resuspended in 50 µl.

The sample OD (appendix 7.1.20) was determined and standardized quantities of RNA were used for the real-time reverse transcription PCR (RT-PCR). The RT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen, CA, USA).

Each reaction contained 10 µl 2x QuantiTect SYBR Green RT PCR Master Mix, 10 pmol of each of the forward and reverse primers (Table 11), 0.2 µl of the QuantiTect RT mix and 1 µg of total cellular RNA in a 20 µl reaction volume. The reactions were run on a lightcycler (Roche, Germany) using the following protocol: Reverse transcription at 50°C for 20 minutes with a ramp rate of 20°C/s; an initial activation period of 15 minutes at 95°C with a ramp rate of 20°C/s; a 40-50 cycle PCR performed under the following conditions (1) denaturation for 15s at 94°C with a ramp rate of 20°C/s, (2) annealing for 20s at 50°C (ramp of 20°C/s) and finally (3) extension for 30s at 72°C (ramp of 2°C/s). Data was acquired at the end of the extension step (single acquisition). The resultant amplicon was melted by the following program: 1 minute denaturation at 95°C (ramp of 20°C/s); 30s annealing at 65°C (ramp of 20°C/s); a melt of 0s at 95°C (ramp of 0.2°C/s) with continuous data acquisition. Data was acquired at 450nm (Channel F1)

To normalize the amount of RNA assayed, an endogenous control was used. The gene selected to be amplified as the endogenous control was GAPDH as it has relatively stable expression levels. *GAPDH* RNA was quantified for all the samples in a separate capillary (using 0.5µg total RNA) using GAPDH specific primers (Table 9) and the same program as described above. The GAPDH quantities were used by the lightcycler software to generate a

ratio between the crossing points of the *gag* RNA and the *GAPDH* RNA for each sample. The ratio result for each sample could then confidently be compared with each other. The levels of total RNA were standardized, to ensure that relative increases/decreases noted are not caused by different amounts of total template RNA added.

The samples were allowed to run for between 40 and 50 cycles. A melting curve analysis was performed to ensure that there was no contamination in the reaction. The melting temperature (T_m) is defined as the temperature at which 50% of the amplicons have melted, and this value is determined by both the length of the amplicon and the nt composition. All amplicons of the same size and composition will have identical T_m , therefore any contaminant was result in the melting curve generating 2 different T_m peaks. The presence of only one peak demonstrates that there was no non-specific amplification, therefore the RNA can be accurately quantified.

Table 9: Primers used for RT-PCR reaction

Primer Name	Primer Sequence
Gag Forward (SK 145)	5' ACA TCA AGA AGC CAT GCA AAT 3'
Gag Reverse (SK 150)	5' TGT CAC TTC CCC TTG GTT CAC TC 3'
GAPDH forward	5' GAA GGT GAA GGT CGG AGT C 3'
GAPDH reverse	5' GAA GAT GGT GAT GGG ATT TC 3'

2.11 Detection of siRNA processing by dicer

To confirm that relative decreases noted in the level of *gag* mRNA and p24 protein expression were due to RNAi activity, it was determined whether the shRNA transcript was processed correctly in the cell by Dicer. To determine the processing of the shRNA, primers were designed to be complementary to the first 16nt of the shRNA transcript at both the 5' and 3' end of the transcript (Table 10). Assuming the siRNA was processed correctly, following reverse transcription, the cDNA should be approximately 21bp in length. If the strand complementary to the target sequence is retained in RISC, there should be a fragment in the reaction using the 5' primer, but the fragment should be absent from the reaction using the 3' primer.

2.11.1 Primers

Table 10: Sequences of the primers used for primer extension.

Primer Name	Primer Sequence
Probe D1	5' GGG TGC GAG AGC GTC A 3'
Probe D2	5' TAA TAT TGA CGT TTC T 3'

2.11.2 Labeling of primer

The primers were labeled using [γ - 32 P] ATP (Perkin-Elmar, MS, USA). The radiolabeled nucleotide were added to the primer in a 25 μ l reaction containing 1 μ l T4 polynucleotide kinase, 10 pmoles primer, 2.5 μ l Phosphorylation buffer, and 5 μ l [γ - 32 P]ATP. The reaction

was incubated for 30 minutes at 37°C. The kinase was then inactivated by heating the reaction at 70°C for 5 minutes.

One hundred and fifty mM of sodium acetate was added to the final concentration to help precipitate the radiolabeled primers. 87.5 µl of Cold ethanol was added to the reaction and left to incubate at -20°C for an hour. The mixture was then centrifuged at 10 000 x g, the supernatant was discarded and the pellet washed with 1ml 70% ethanol. Following the wash, the 70% ethanol was discarded and the pellet was dried under pressure using the Speedvac-SC110 (Savant, VA, USA). The pellet was then resuspended in 20 µl of water.

2.11.3 Primer extension

The radiolabeled primer was diluted 1 in 10 µl, and 1 µl of the primer dilution was utilized in the reaction. One µl of primer (1 in 10) was incubated, with 9 µl of pure water at 37°C for 20 minutes. Following the initial incubation, the primer was further incubated at room temperature for another 10 minutes. Twenty µg of total RNA was then added to the primer together with 5 µl AMV RT Buffer (Promega, WI, USA), 2.5 µl dNTPs, 1 µl AMV RT (Promega, WI, USA) and water to make a total reaction volume of 25 µl. The reaction was incubated at 42°C for 15 min. Three µl stop buffer (Epicentre Technology, CA, USA) was added to stop the reaction. The reaction was then concentrated to a 5 µl volume by ethanol precipitation.

2.11.4 Sequencing ladder

In order to determine the exact size of the siRNA generated from the hairpin structure, a sequencing reaction was performed to generate a ladder with fragments differing by only 1bp. The reaction utilized a P32 γ -labeled primer and was performed using the SequiTherm EXCEL II DNA sequencing kit (Epicentre technologies, CA, USA).

The sequencing reaction was set up as a master mix consisting of 1.5 pmol of the radio-labeled primer, 1000 fmol of dsDNA template, 7.2 μ l 3x SequiTherm EXCEL II sequencing buffer and 1 μ l SequiTherm EXCEL II DNA polymerase (5U) in a 17 μ l reaction volume. The master mix was split into 4 separate tube (4 μ l in each) containing 2 μ l of ddGTP, ddATP, ddTTP and ddCTP respectively. The Reactions were heated for 5 minutes at 95°C to denature the template, then cycled for 30 cycles of 30s at 95°C and 1 minute at 70°C. Upon the completion of the reaction, 3 μ l of stop/loading buffer was added to each reaction.

2.11.5 Running the reaction

The sequencing ladder together with the forward and reverse primer extension reaction and a non-transfected control were run on a 12% sequencing gel at 50W. After the bromophenol blue dye front had run 20 cm, the gel was fixed, dried and then exposed to Agfa CP-BU medical x-ray film for varying amounts of time. The gel was then developed to allow for analysis.

2.12 The Interferon ELISA

2.12.1 Human Interferon Beta ELISA

The human interferon Beta ELISA kit (PBL, Biomedical Laboratories, NJ, USA) was used to determine the amount of human interferon beta in the sera of cells transfected with shRNA A, B, C, D and E. A standard curve was constructed by creating serial dilutions ranging in concentration from 0 to 10 000 pg/ml of the human interferon beta solution. The dilutions were carried out using the supplied dilution buffer. One hundred µl of the samples to be tested for interferon beta were loaded, in duplicate, into the wells of the supplied microtitre plate. The microtitre plate was covered and incubated for 1 hour in a closed chamber at 24°C.

Following the initial incubation, the plate was emptied of its contents and the wells washed twice with the final wash solution (50 ml wash solution concentrate with 450 ml of distilled water). The plate was dried. One hundred µl of antibody solution (3.3 µl antibody concentrate in 1 ml of antibody diluent per strip), was added to the plate. The plate was then covered and incubated for 1 hour at 24°C.

The contents of the plate were emptied and the wells washed 3x with the final wash solution. The plate was then inverted and blotted dry. One hundred µl of horse radish peroxidase (HRP) conjugate solution (6.7 µl HRP conjugate in 1 ml of HRP conjugate diluent per strip) was added. The plate was covered and incubated for 1 hour at 24°C. The content of the plate were emptied and the wells washed 4x with the final wash solution. The plate was then dried. One hundred µl of TMB substrate solution (warmed to 24°C) was added to each well, covered and incubated for 15 minutes at 24°C in the dark.

After the incubation 100 µl of stop solution was added to each well. The solutions were mixed by swirling and tapping the plate gently. The samples were then read on a microplate reader at an absorbance of 450 nm (measurements were taken within 5 minutes of adding the stop solution).

2.12.2 Human Interferon Alpha ELISA

The human interferon Alpha ELISA kit (PBL, Biomedical Laboratories, NJ, USA) was used to determine the amount of human interferon alpha in the sera of cells transfected with shRNA A, B, C, D and E.

A standard curve was constructed by creating serial dilutions ranging in concentration from 0 to 500 pg/ml of the human interferon alpha solution. The dilutions were carried out using the supplied dilution buffer. One hundred µl of the samples to be tested for interferon alpha were loaded, in duplicate, into the wells of the supplied microtitre plate. The microtitre plate was covered and incubated for 1 hour in a closed chamber at 24°C.

Following the initial incubation, the plate was emptied of its contents and the wells washed twice with the final wash solution (50 ml wash solution concentrate with 450 ml of distilled water). The plate was then blotted dry, upside down, on lint free blotting paper. One hundred µl of antibody solution (3.3 µl antibody concentrate in 1 ml of antibody diluent per strip), was added to the plate. The plate was then covered and incubated for 1 hour at 24°C.

The contents of the plate were emptied and the wells washed 3 x with the final wash solution. The plate was then inverted and blotted dry. Twenty-five µl of HRP conjugate solution (6.7 µl HRP conjugate in 1 ml of HRP conjugate diluent per strip) was added to the plate. The plate was covered and incubated for 1 hour at 24°C. The contents of the plate were emptied and the wells washed 4x with the final wash solution. The plate was then blotted dry. One hundred µl of TMB substrate solution (warmed to 24°C) was added to each well, covered and incubated for 15 minutes at 24°C in the dark.

After the incubation 100 µl of stop solution was added to each well. The solutions were mixed by swirling and tapping the plate gently. The samples were then read on a microplate reader at an absorbance of 450 nm (measurements were taken within 5 minutes of adding the stop solution).

2.13 Statistical analysis

All statistics were calculated using the students t-test for 2 samples of unequal variance. The calculations were performed using Microsoft Excel which calculates using the following formula:

$$t = \frac{Xa - Xb}{\sqrt{\frac{\text{var } a}{na} + \frac{\text{var } b}{nb}}}$$

where X = the mean; n = the number of samples; and var = the variance of the samples

Significance was calculated at a 95% confidence interval (C.I). The samples were considered to be significantly different if the p value was less than 0.05.

CHAPTER 3: RESULTS

The use of the RNAi pathway to target the *gag* ORF is a promising alternative to current antiretroviral therapies. These results demonstrate the potential for using shRNA constructs to down regulate the expression of HIV-1 subtype C genes.

3.1.1 *shRNA expressing plasmid vectors*

HIV-1 RNA is found in the cytoplasm before reverse transcription, following provirus transcription and prior to nuclear import⁷³. This presents targets for RNAi mediated inhibition of viral gene expression. shRNAs targeting *gag* were chosen to be 100% homologous to the consensus sequence of HIV-1 subtype C viruses and spanned the entire p55 ORF (Figure 11).

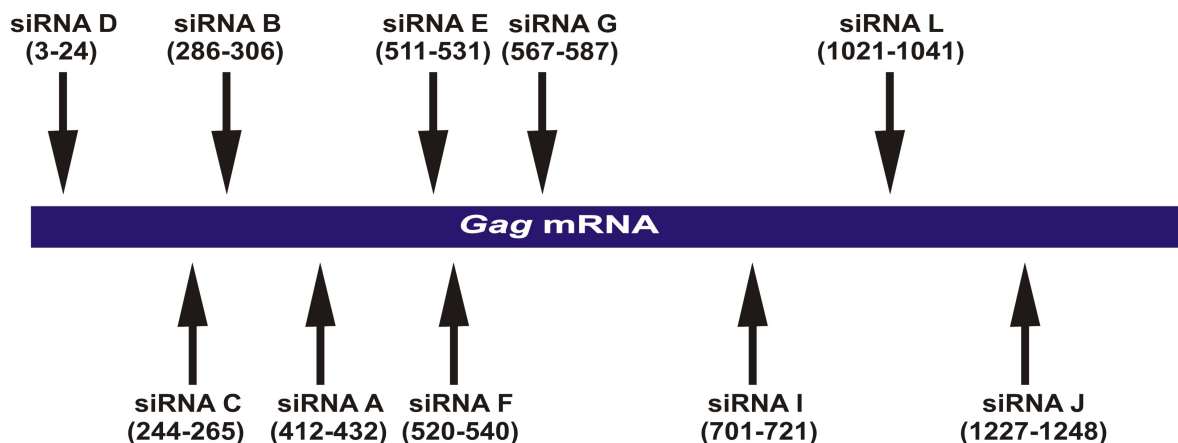


Figure 11: Schematic representation of the location, in the *gag* ORF, of the sequences targeted by the shRNAs.

The clinical use of RNAi inhibition of HIV-1 subtype C has many limitations, one being the ability of extended siRNA expression *in vitro*. To accomplish intracellular expression of siRNA, ten sequences that endogenously express shRNAs, directed against *gag*, were

successfully cloned into plasmid vectors under the control of a U6 promoter (Figure 12). An eleventh vector, devoid of gag specific shRNA sequences served as a control.

The sequences that would be targeted by the selected shRNAs were entered into BLAST (genbank) to ensure there was no homology to any human mRNA sequence to prevent off target effects. No substantial sequence homology was noted to any human genes.

The shRNA transcripts were designed to be expressed from a U6 promoter. The transcript generated from the U6 promoter contains both the sense and anti-sense sequences portions of the final siRNA. Once transcribed the transcript folds back on itself due to the homology between the sense and anti-sense sequences, forming a hairpin loop structure within the cell (Figure 12).

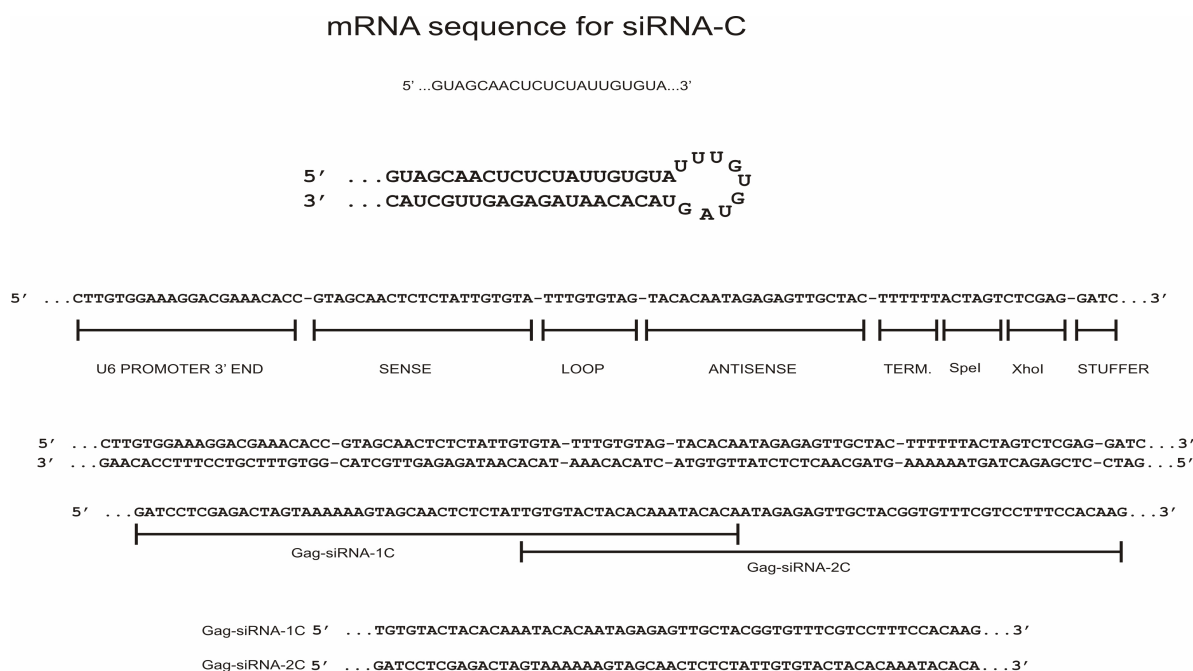


Figure 12: Schematic representation of the sequence encoding for shRNA C. The target mRNA sequences is indicated as is the sense, antisense, and miRNA sequences. Following transcription from the U6 promoter, the

resultant RNA forms a hairpin loop structure. The diagram shows the primer sequences used for cloning the shRNA into plasmid vectors

3.2 Testing the efficacy of the shRNA expressing plasmids against the EGFP/gag reporter plasmid

Co-transfection of some of the shRNA expressing vectors with the EGFP/gag reporter plasmid into the Huh 7 cell line markedly reduced the EGFP fluorescence. The efficient cellular uptake of the dsRNA is a critical step in enabling mRNA targeting. A β -galactosidase expressing plasmid was utilized to ensure standardized transfection efficiency, to allow for comparison of different transfections in an experiment as well as different experiments.

Fluorescence microscope analysis of the EGFP/gag reporter plasmid/ shRNA co-transfections indicated that shRNA A, shRNA E, and shRNA L have no visible effect on EGFP expression, when compared to the positive control, whereas shRNA C and shRNA D exhibited the most drastic decrease in the levels of EGFP expression when compared to the positive control (Figure 13). The remaining shRNA constructs appear to induce moderate levels of protein suppression.

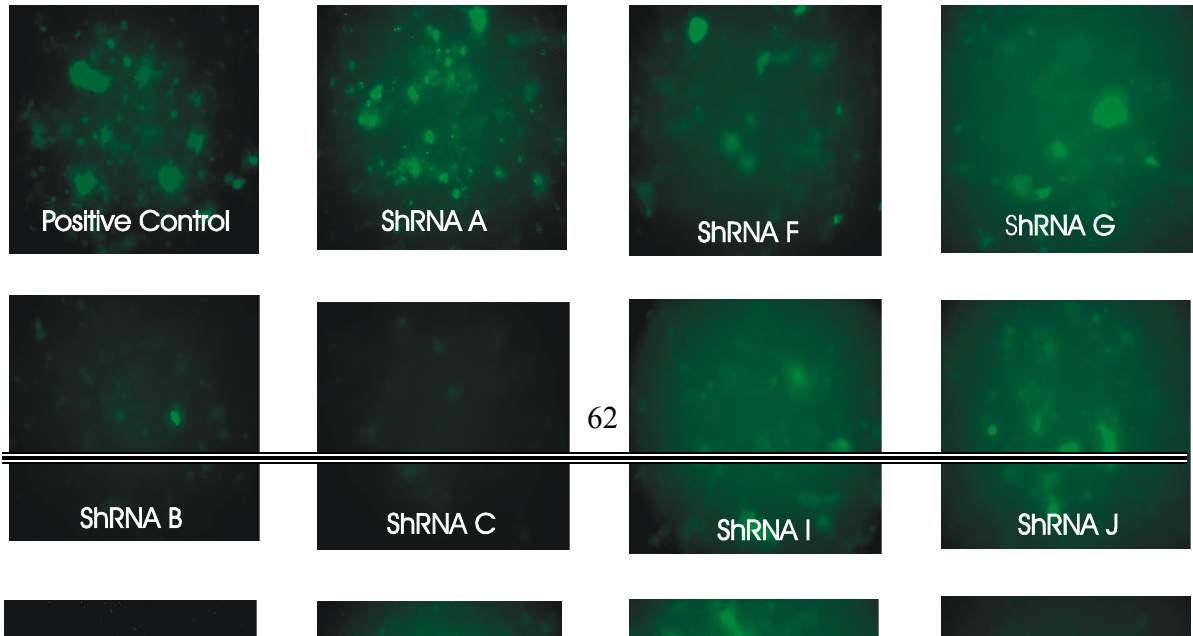


Figure 13: Fluorescence microscope images depicting the quantity of *EGFP/gag* fusion RNA knockdown induced by the various shRNAs. It is evident that shRNA C and D show the most pronounced knockdown with shRNA A, E, I and L possibly even showing an upregulation in the amount of EGFP observed when compared to the positive control.

Through flow cytometric analysis it was determined that shRNA A ($p=0.823$) and E ($p=0.167$) expression induced no significant inhibition of *gag* mRNA. shRNA C was able to reduce EGFP expression by up to 80%. shRNA D, although not as efficient as shRNA C at silencing EGFP expression, was able to induce a 68% reduction at a 95% confidence Interval (C.I), $p=0.034$. The remaining six shRNAs all inhibited EGFP expression at a statistically significant level ($p<0.05$) with relative levels of EGFP ranging from 30% and 59% (Figure 14).

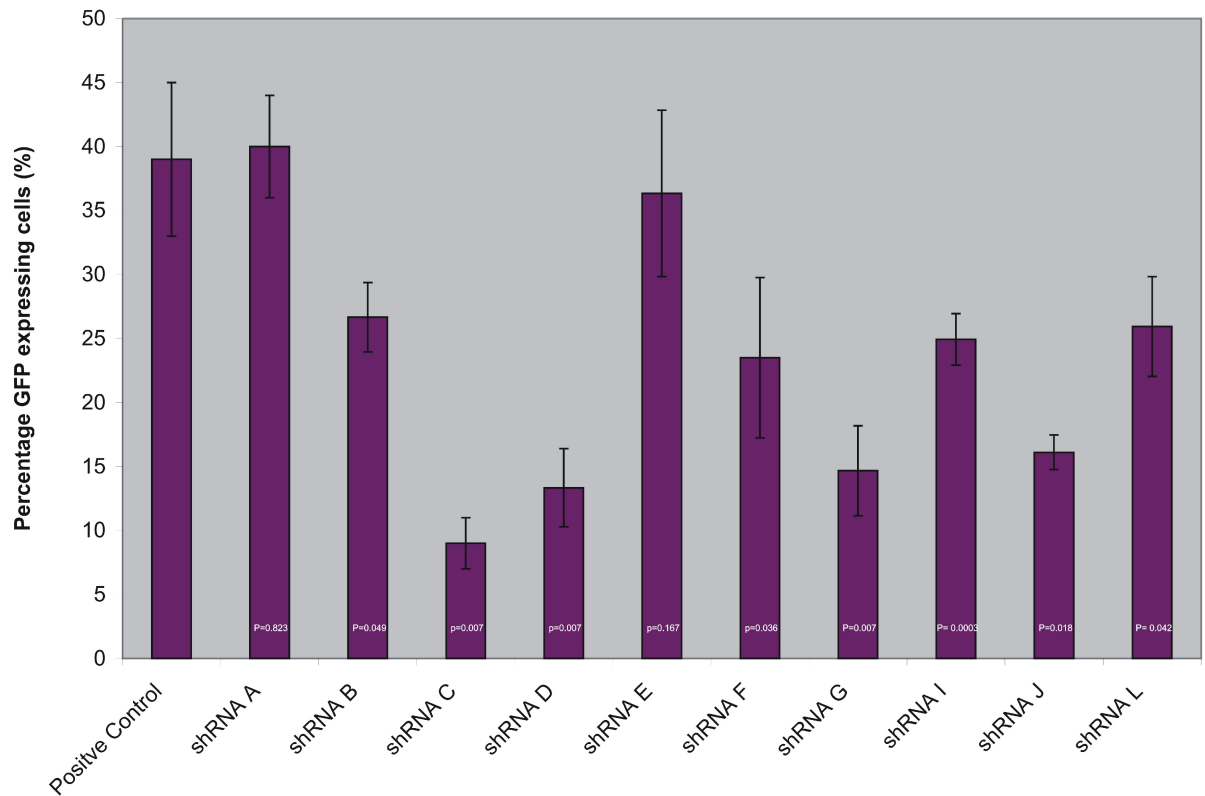


Figure 14: Flow-cytometric analysis of shRNA efficacy. The reduction of EGFP/gag mRNA derived from a reporter plasmid was quantified by measuring EGFP expression. The experiment was performed three times in triplicate. There was no noticeable reduction when comparing shRNA A and shRNA E (at a 95% CI, Students T-test) induced reduction to the positive control. shRNA C and shRNA D were the most successful in knocking down the expression of EGFP/gag fusion mRNA (all p-values are recorded on the graph). Error bars are indicated with standard error of the mean (n=3).

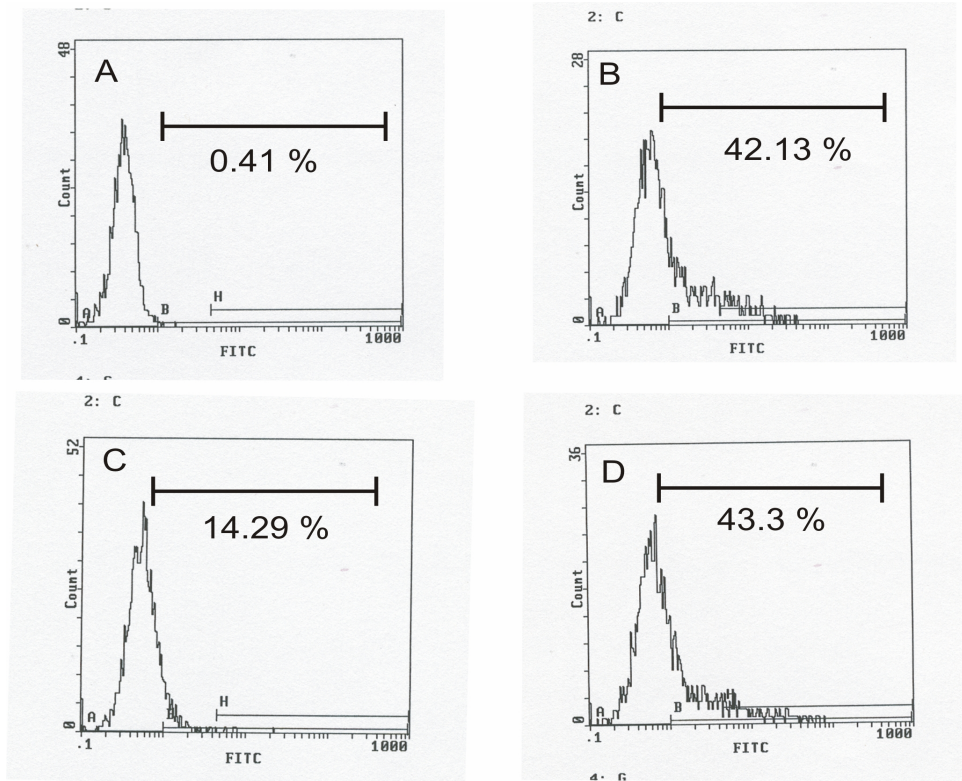


Figure 15: Flow cytometric analysis of histograms showing the effect of shRNA expressing plasmids on the levels of EGFP expressed by the reporter plasmid. A shows the negative control with only 0.41 % of the cells fluorescing at 450 nm. This fluorescence is background noise, probably due to cellular debris. B shows the positive control, C depicts shRNA D inhibition and D shows the reduction caused by shRNA E

The slightly positive results noted in the negative control of the flow cytometric analysis can be attributed to cellular debris fluorescing at 450 nm (Figure 15). The differing results obtained for shRNA L when comparing the data obtained from the flow cytometer and those noted with the fluorescence microscope, can be attributed to the flow cytometer being a sensitive instrument able to pick up weakly fluorescent cells that are undetectable under the microscope.

This study demonstrates that mRNA transcribed from a plasmid vector can be specifically targeted by shRNA mediated RNAi. The specificity of the inhibition is demonstrated by lack of β -galactosidase inhibition.

Five plasmid encoding shRNAs were chosen to be utilized in the live virus experiment based on the results from the reporter plasmid experiments according to the degree to which they inhibit EGFP expression. shRNA A and E were included as they did not appear to have any inhibitory effects of gag mRNA. shRNA C and D were incorporated due to their superior efficacy and shRNA B was included as it only exhibited modest reduction in EGFP expression.

3.3 Testing the efficacy of the shRNA expressing vectors against HIV-1 subtype C isolates

To determine whether the shRNA expressing constructs were capable of inhibiting on-going viral replication, U87-CD4⁺-CCR5⁺ and U87-CD4⁺-CXCR4⁺ cell lines, capable of supporting M and T-tropic HIV replication respectively were challenged with HIV-1 subtype C viruses 24 hours after being transiently transfected with the anti-*gag* shRNA expressing plasmids. Three viruses were selected, Du151aa and Du422a, which utilize the CCR5 co-receptor for infection and Sw7, which utilizes the CXCR4 co-receptor. Each experiment was performed in triplicate. Levels of p24 Ag were measured in the culture supernatant by an ELISA 11 days after transfection and confirmed by Western blot analysis.

When challenged by Du422a, the trends followed were almost identical to that noted when using the EGFP reporter plasmid system. This result is as expected as the reporter plasmid incorporated the *gag* ORF of the virus Du422aa. shRNA A and shRNA E were not shown to inhibit p24 protein. shRNA C, the most inhibitory sequence, reduced p24 production by 82%, with shRNA D decreasing p24 levels by 78%. shRNA B resulted in a decrease of 40 % (Figure 16).

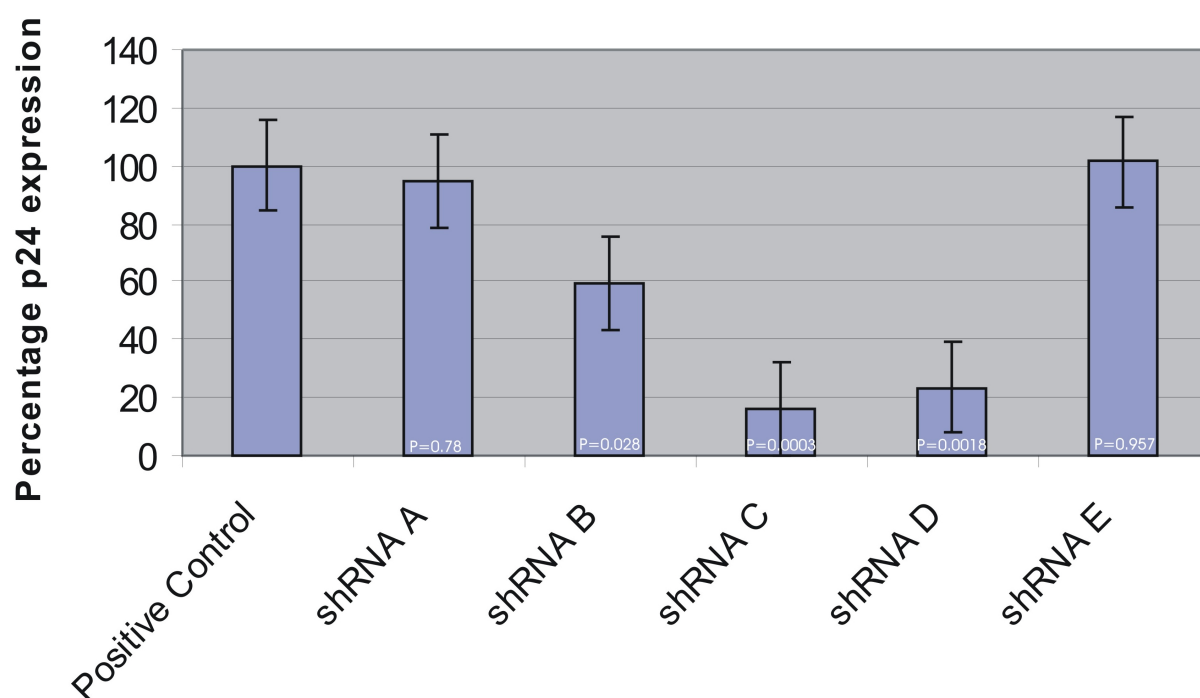


Figure 16: p24 ELISAs of supernatant samples taken 11 days post infection with Du422aa and shRNA expressing plasmid transfection. shRNA C and D significantly inhibited p24 expression , shRNA B showed modest suppression of p24 and shRNA A and shRNA E have no effect on the levels of p24 expression (all p-values are recorded on the graph). Error bars are indicated with standard error of the mean (n=3).

When the transfected cells were challenged by Du151aa (closely related to Du422aa (see appendix 6.2, Figure 33)), a slightly different trend was seen. Exposure to both shRNA A ($p=0.909$) and shRNA E ($p=0.629$) increased the amount of p24 when compared to the positive control. shRNA A generally showed a modest decrease in the amount of p24. shRNA C and shRNA D were again the most inhibitory of the interfering sequences, although when shRNA C was exposed to Du151aa, as apposed to Du422aa, the ability of the sequence to inhibit of p24 production was markedly reduced from 82% to 53% (Figure 17). shRNA D was able to induce a 81% reduction in the level of p24 (significant of a 95% C.I; $p=0.00057$)

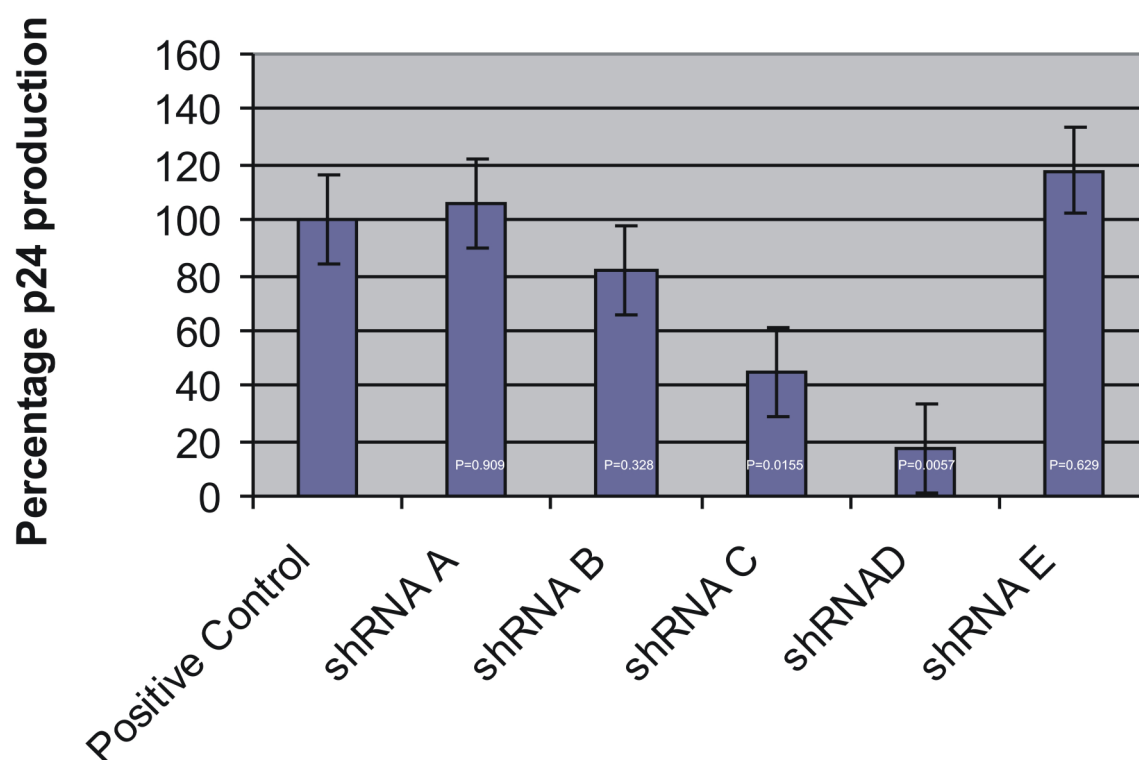


Figure 17: p24 ELISAs of supernatant samples taken 11 days post infection with Du151aa and shRNA expressing plasmid transfection. shRNA D significantly inhibited p24 expression , shRNA B and shRNA C showed modest suppression of p24 and shRNA A and shRNA E appear to promote p24 expression. Error bars are indicated with standard error of the mean ($n=3$).

Sw7, the only X4 virus tested against the shRNAs, was inhibited by only one anti-*gag* inhibitory sequence. shRNA A and shRNA E exposure, once more proved to stimulate the production of p24, as did shRNA B. shRNA C showed no significant inhibitory action ($p=0.1129$). shRNA D was the only sequence that was shown to reduce the expression of p24 when targeted to Sw7. shRNA D significantly inhibited p24 expression by 56% when compared to the positive control (95% C.I. using the students T-Test; $p=0.0048$) (Figure 18).

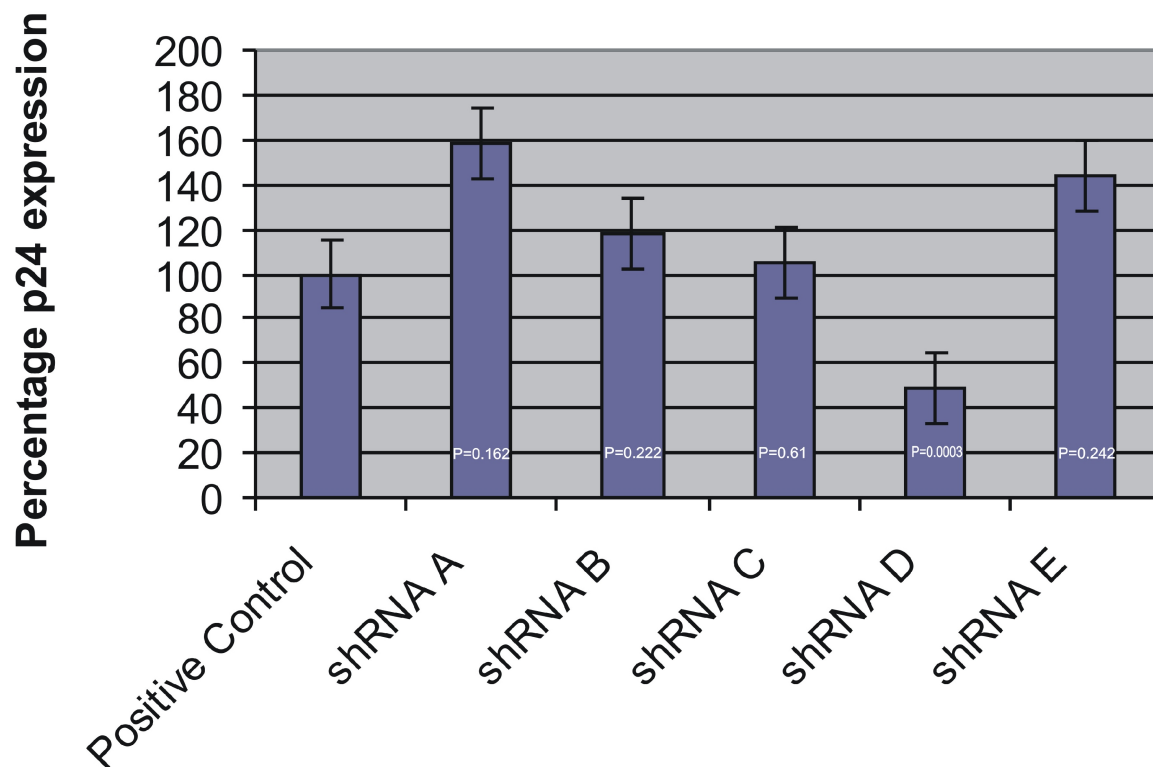


Figure 18: p24 ELISAs of supernatant samples taken 11 days post infection with Sw7 and shRNA expressing plasmid transfection. shRNA D significantly inhibited p24 expression, shRNA C showed no significant variation from the positive control and shRNA A, shRNA B and shRNA E appear to promote p24 expression. Error bars are indicated with standard error of the mean ($n=3$).

shRNA D was the only anti-*gag* inhibitory sequence tested that consistently knocked down p24 protein expression across both X4 and R5 subtype C viruses. shRNA E, contrary to shRNA D, constantly promoted the expression of p24 across all of the viruses tested. shRNA A, shRNA B, and shRNA Cs efficacy depended on the virus that was targeted.

3.4 Western Blot Analysis

Western blot analysis of a shRNA expressing plasmid transfection challenged with Du422a showed that *gag* specific shRNA C and shRNA D blocked gag protein expression, but also significantly affected the expression of all HIV-1 proteins. The blot showed the same trend as seen with the reporter plasmid and the p24 ELISAs for Du422a, confirming the previous results. The amounts of p24 of shRNA A and shRNA E were similar to the positive controls. The p24 production was modestly inhibited by shRNA B. shRNA C and shRNA D both dramatically inhibited the production of p24 protein (Figure 19).

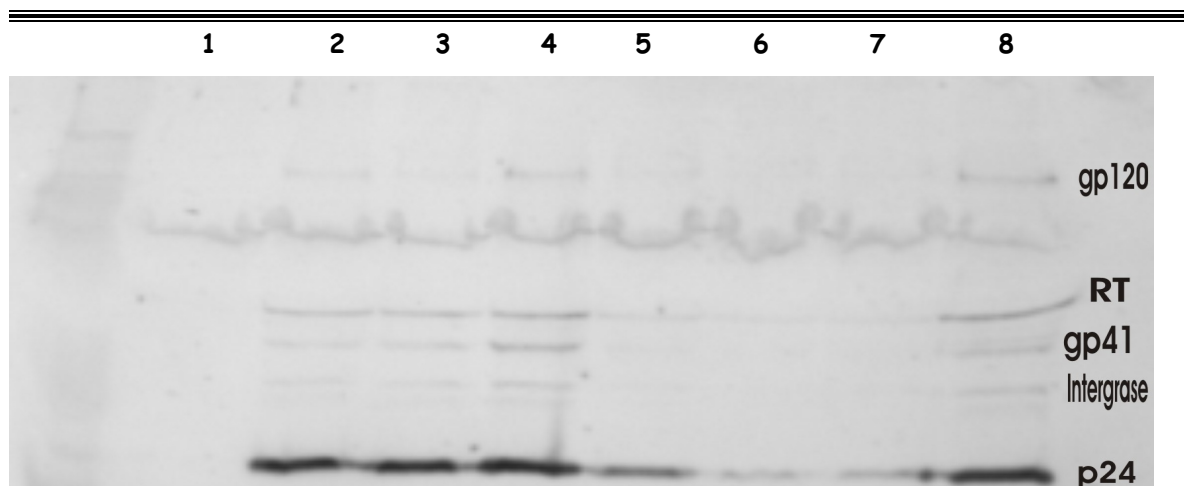


Figure 19: Western blot analysis of Du422aa challenged cells indicating the relative decrease of viral proteins induced by exposure to various inhibitory short hairpin structures. Lane 1 represents the negative control, lane 2 and 3 represent the positive controls. Lane 4 represents the relative amount of viral protein following exposure to shRNA A, which appears slightly up regulated, as does the amount of protein following exposure to shRNA E (lane 8). Exposure to shRNA B resulted in a modest decrease in HIV-1 protein expression (lane 5), whereas exposure to shRNA C (lane 6) and shRNA D (lane 7) resulted in substantial inhibition of HIV protein production.

3.5 RNA Analysis of shRNA induced *gag* inhibition

As shRNA mediated RNAi was shown to inhibit protein production from virally produced mRNA, it was then determined whether the viral infections were inhibited by mRNA degradation or protein translation suppression. U87-CD4⁺-CCR5⁺ and U87-CD4⁺-CXCR4⁺ cells were transfected with an empty, control plasmid in conjunction with HIV-1 specific shRNA expressing constructs 24 hours prior to infection of the cells with the HIV-1 subtype C isolates. Eleven days after the transfection, the cells were lysed and the effect of the shRNA sequences on the viral RNA was determined using real time SYBR green RT-PCR. The RT-PCR reactions were used to establish the amount of uncleaved HIV-1 *gag* mRNA (figure 20, 21). HIV-1 *gag* specific primers were used to amplify a 160bp fragment within the transcript.

Each reaction was standardized with the use of a calibrator (RNA that was transcribed off a Du422aa gag expressing plasmid). The cell number for each experiment condition was determined using GAPDH specific primers and the number of copies of HIV-1 subtype C *gag* mRNA was corrected for cell numbers.

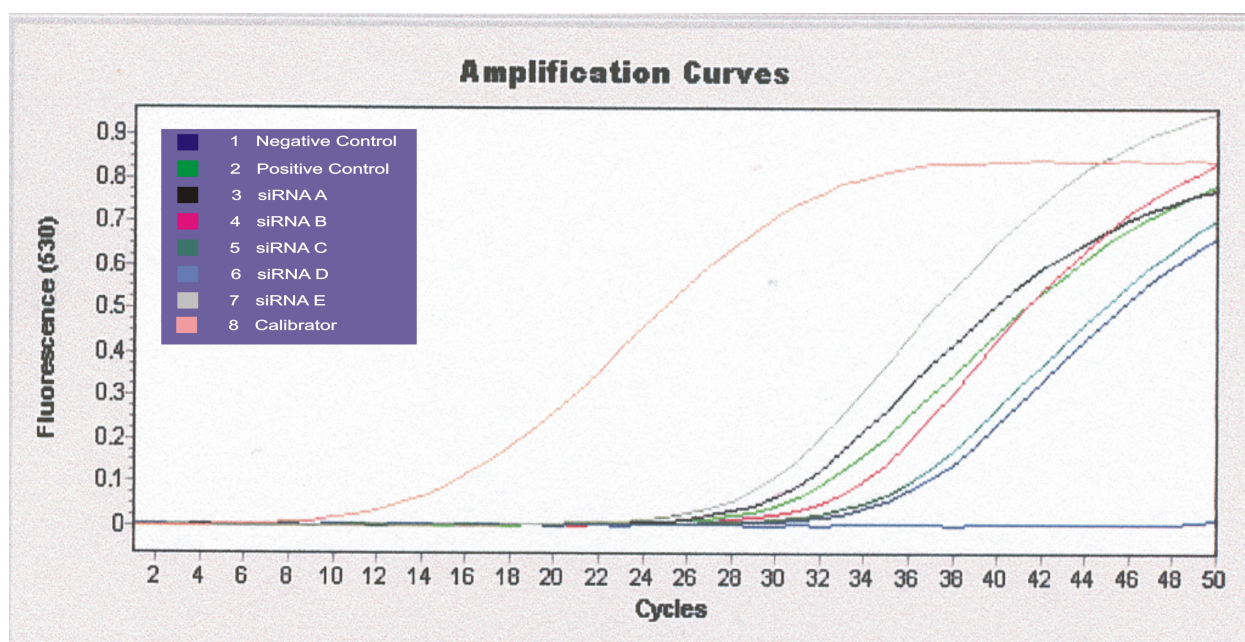


Figure 20: A Du422aa lightcycler run. The crossing point of a sample is the point where the samples fluorescence curves sharply upwards, this turning point corresponds to the first maximum of the second derivative of the curve. The amount of target RNA in the reaction is determined by the crossing point (the sooner it reaches its turning point the more RNA was in the initial sample). In the above reaction it can be seen that shRNA E had the most RNA followed by shRNA A, the positive control, shRNA B, shRNA C and finally shRNA D containing the least target RNA.

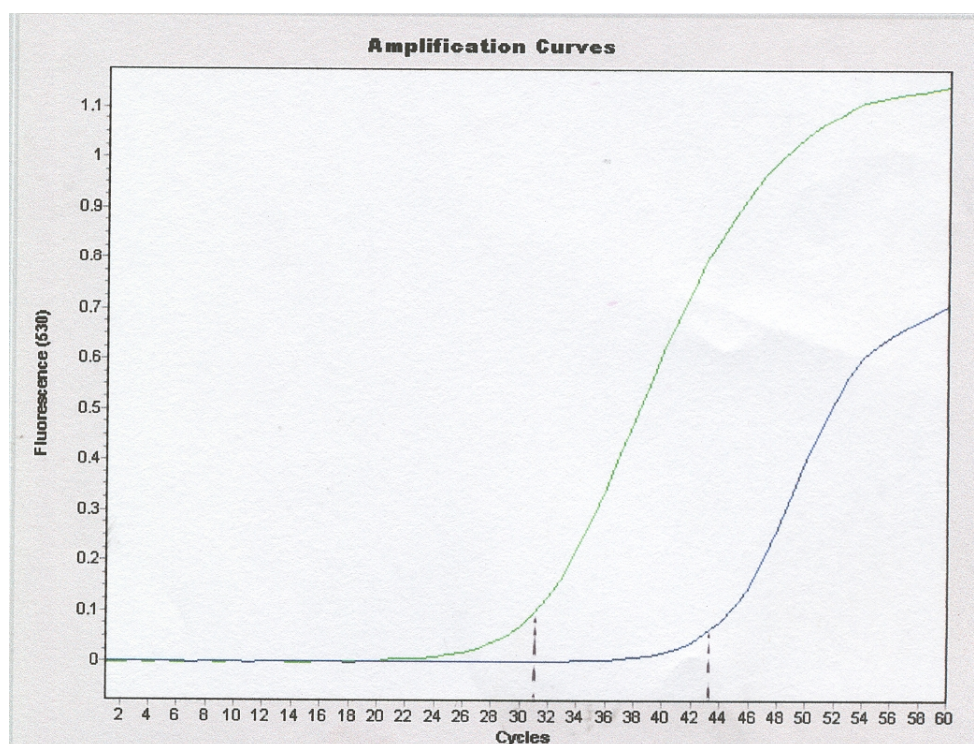


Figure 21: A depiction of the different crossing points of a positive control (green) and shRNA D (blue) on the lightcycler indicating the decrease in amount of viral *gag* RNA. The short hairpin structure elicits a 10 fold decrease in *gag* mRNA.

The relative RNA concentrations of *gag* mimicked the trends of their protein counterparts determined by p24 assay for Du422a.

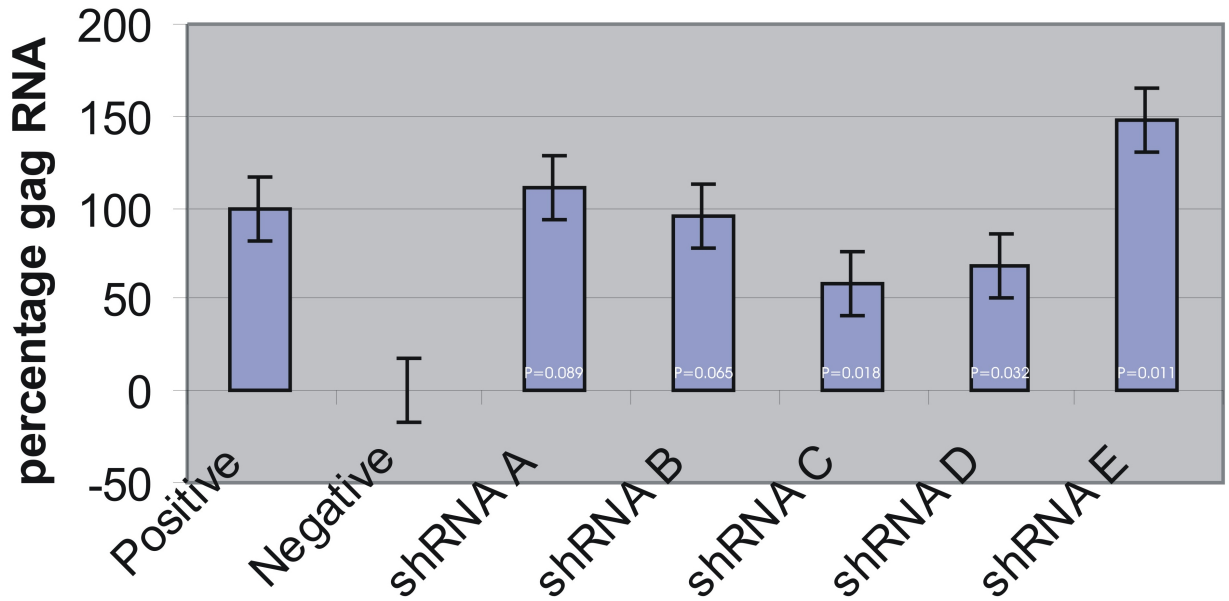


Figure 22: Relative amounts of *gag* RNA from Du422aa infected U87-CD4-CCR5 cells following shRNA challenge. shRNA A and E RNA was up regulated in comparison to the control infection. shRNA C showed the most significant decrease in viral RNA. Error bars are indicated with standard error of the mean (n=3).

Du422aa shRNA-mediated inhibition of *gag*, was capable of down regulating *gag* by a maximum of 55%. This was achieved by shRNA C. shRNA B and D were also shown to decrease *gag* RNA levels by 30% and 40% respectively. shRNA A showed no significant difference when compared to the positive control. shRNA E up regulated *gag* expression by up to 160% (Figure 22).

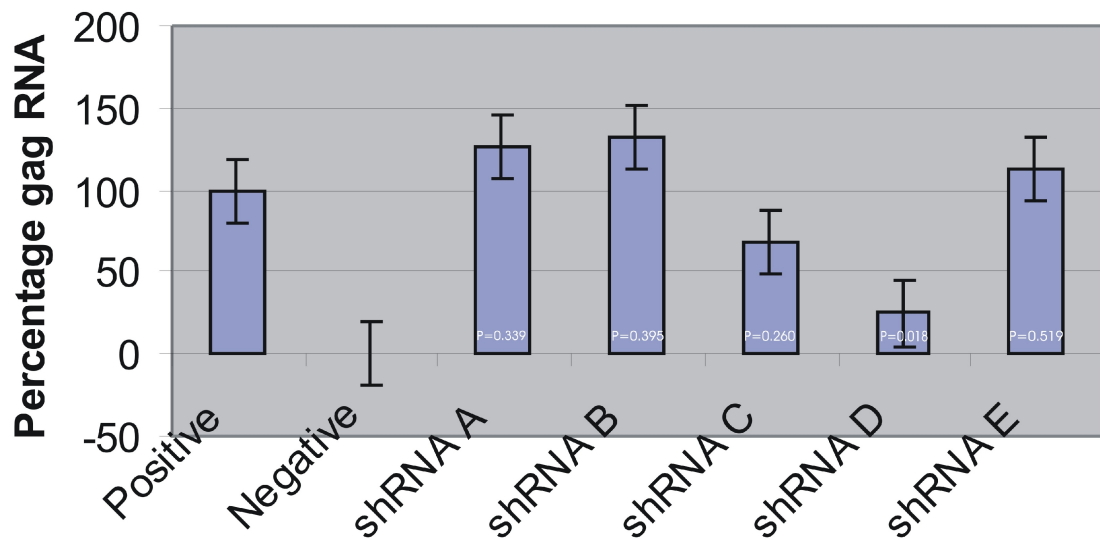


Figure 23: Relative amounts of *gag* RNA from Du151aa infected U87-CD4-CCR5 cells following shRNA challenge. shRNA A, B and E RNA was up regulated in comparison to the control infection. shRNA D showed the most significant decrease in viral RNA. Error bars are indicated with standard error of the mean (n=3).

Du151aa *Gag* was inhibited by up to 92%, determined by RT-PCR, by shRNA D. shRNA C, the only other shRNA sequence able to mediate a decrease in *gag*, reduced RNA levels by a modest 30%. shRNA A (p=0.089), and shRNA B (p=0.65) significantly up regulated the amount of RNA by significant levels (95% C.I. using a students T-test). shRNA E up regulated the amount of *gag* by approximately 15%, which is a statistically significant increase (p=0.0107) when compared to the positive control (Figure 23).

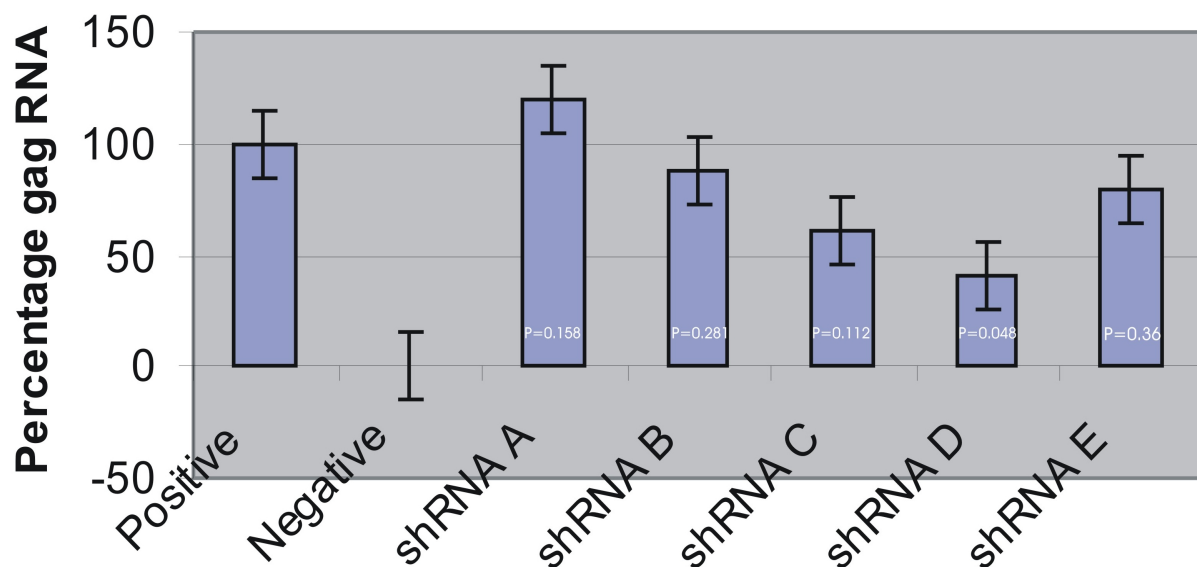


Figure 24: Relative amounts of *gag* RNA from Sw7 infected U87-CD4-CXCR4 cells following shRNA challenge. shRNA A was up regulated in comparison to the control infection. shRNA D showed the most significant decrease in viral RNA. shRNA B and E showed no significant decrease when compared to the positive control. Error bars are indicated with standard error of the mean (n=3).

Sw7 *gag* was only inhibited to a maximum level of 68% by shRNA D. shRNA C was able to induce RNA degradation of an average of only 40%. shRNA A (p=0.157), shRNA B (p=0.281) and E (0.36) showed no statistical difference when compared to the positive control. shRNA A up regulated *gag* to a maximum of 130% (Figure 24). Although shRNA appeared to induce a decrease in mRNA levels, no reduction in protein levels was noted, this could be due to an upregulation in protein expression off the existing RNA transcripts, or possibly saturation of the ribosomes, making the relative decrease in mRNA inconsequential in protein production.

All of the negative controls were negative. All statistics were worked out using the students T-test at a 95% C.I.

shRNA A Target Sequence	GGGCAAATGGTACACCAGGCC
Du422a Sequence	GGGCAAATGGTACATCAAGCC
Du151a Sequence	GGGCAAATGGTACATCAAGCC
Sw7 Sequence	GGGCAAATGGTACATCAAGCC
shRNA B Target Sequence	GACACCAAGGAAGCCTTAGA
Du422a Sequence	GACACCAAGGAAGCCTTAGT
Du151a Sequence	GACACCAAGGAAGCCTTAGT
Sw7 Sequence	GACACCAAGGAAGCCTTAGA
shRNA C Target Sequence	GTAGCAACTCTCTATTGTGTA
Du422a Sequence	GTAGCAACTCTCTATTGTGTA
Du151a Sequence	GTAGCAACTCTCTTTTGTGTA
Sw7 Sequence	GTAGCAACTCTCTATTGTGTA
shRNA D Target Sequence	GGGTGCGAGAGCGTCAATATA
Du422a Sequence	GGGTGCGAGAGCGTCAATATA
Du151a Sequence	GGGTGCGAGAGCGTCAATATA
Sw7 Sequence	GGGTGCGAGAGCGTCAATATA
shRNA E Target Sequence	GCATTATCAGAAGGAGCCACC
Du422a Sequence	GCATTATCAGAAGGAGCCACC
Du151a Sequence	GCATTATCAGAAGGAGCCACC
Sw7 Sequence	GCATTATCAGAAGGAGCCACC

Figure 25: Alignment of the sequence targeted by the respective shRNAs and various subtype C viruses

3.6 shRNA Efficacy

3.6.1 *shRNA A*

shRNA A constantly appeared to up regulate the amount of *gag* irrespective of the Viruses targeted. There was no sequence variation between the viral isolates tested (Figure 25), but there were 2 sequence mismatches at positions 15 and 18 of the target RNA. This might help explain why shRNA A was unable to inhibit *gag*. The apparent increase in the amount of *gag*

could be due to the shRNA stabilizing the RNA. shRNA A also only partially meets the requirements of a good siRNA sequence (section 4.2)

3.6.2 *shRNA B*

shRNA B's efficacy varied, depending on the strain of virus that it was inhibiting. Both the R5 viruses had a single mismatch at position 21 (Figure 25). shRNA B was able to induce a modest decrease in both Du422aa and Sw7, whereas in Du151aa it appeared to result in an upregulation of *gag*. A possible explanation for this is the folding pattern of Du151aa, made this site inaccessible to the siRNA. shRNA B only partially meets the requirement set out for an effective siRNA (section 4.2)

3.6.3 *shRNA C*

shRNA C consistently knocked down *gag* expression, for all strains tested, but to varying degrees. Du422aa and Du151aa, the R5 viruses, are phylogenetically closely related. Due to their relationship you would expect the inhibitory effects of shRNAs on these two viruses to be comparable. Unlike shRNA B, the difference in inhibition between the two R5 viruses can be explained by the fact that Du151aa, has a single mismatch at position 14 (Figure 25), which could be responsible for the partial abrogation of the effect of the shRNA. Sw7, targeted by shRNA C, also showed decreased levels of inhibition when compared to Du422aa, this could be caused by different conformations in the Sw7 RNA. shRNA C meets all the requirements of a good siRNA (section 4.2).

3.6.4 *shRNA D*

shRNA D substantially inhibited all subtype C viruses tested. shRNA D showed no sequence differences when compared to the target RNA, and meets all the requirements of a good siRNA (section 4.2).

3.6.5 *shRNA E*

shRNA E, like D is 100% homologous to the target sequences, but E has no inhibitory effects on the amount of *gag* across the viruses tested. The most likely reason for the lack of inhibition is that the secondary structure of the RNA inhibits the shRNA's access to the target site

3.7 siRNA cleavage by dicer

To determine if dicer was correctly cleaving the short hairpin into siRNA, a 15bp primer was extended along the length of the shRNA in both the forward and the reverse direction. If the shRNA was not processed correctly, the primer would extend the entire length of the hairpin generating a fragment of approximately 60 bp. Correctly processed shRNAs should yield a 21bp fragment. The presence of a fragment in the forward reaction and the absence in the reverse reaction is indicative of the antisense fragment being included in RISC.

Following primer extension, two cDNA transcripts could be recognized on the gel. The small 20 bp cDNA is most likely the correctly processed siRNA. The reason for the gel only

indicating its size as 20bp as apposed to 21bp, could be due to the positioning of the primer. It is likely that the primer binds 1 bp 5' of the start of the siRNA (Figure 26). The second band may be due to non-specific reverse transcription, or it may be a siRNA variant that was processed, by dicer, further into the miRNA loop sequence. The antisense siRNA was included in RISC.

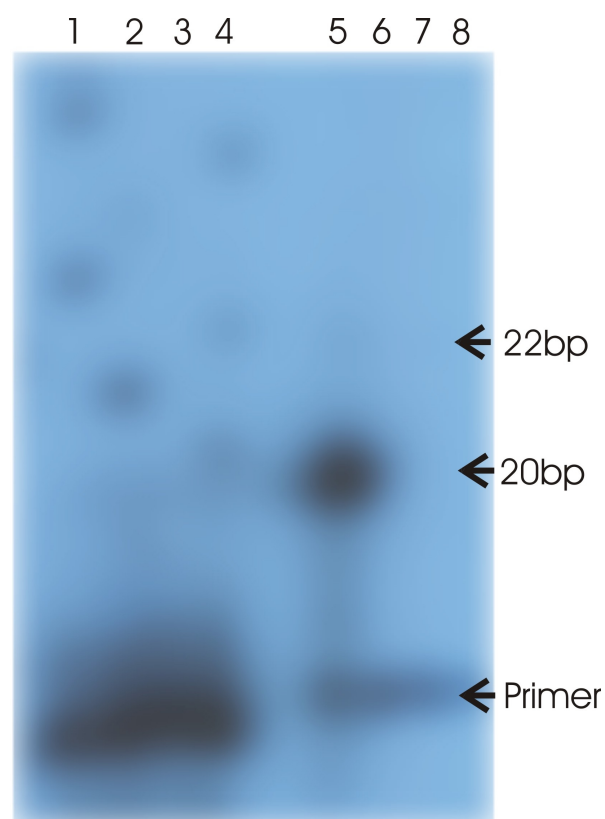


Figure 26: Determination of dicer processing of shRNA D. Dicer processes shRNA D correctly into a short RNA species of approximately 21bp. Lanes 1-4 represent the sequences ladder (GATC), lane 5 contains the forward RT reaction. The reverse reaction was run in lane 6. The forward and reverse primers were run in lanes 7 and 8 respectively. The absence of an extension in the reverse reaction, is indicative of the antisense siRNA being incorporated into RISC.

3.8 Activation of the Interferon Response

The activation of the IFN response pathway is an important consideration when designing siRNA sequences with the potential to be used in a therapeutic setting. shRNA A to E were tested using interferon ELISAs, to determine if they activate the IFN pathway.

None of the shRNAs up regulated the production of IFN alpha when compared to the blank and the negative controls included in the assay (Figure 27).

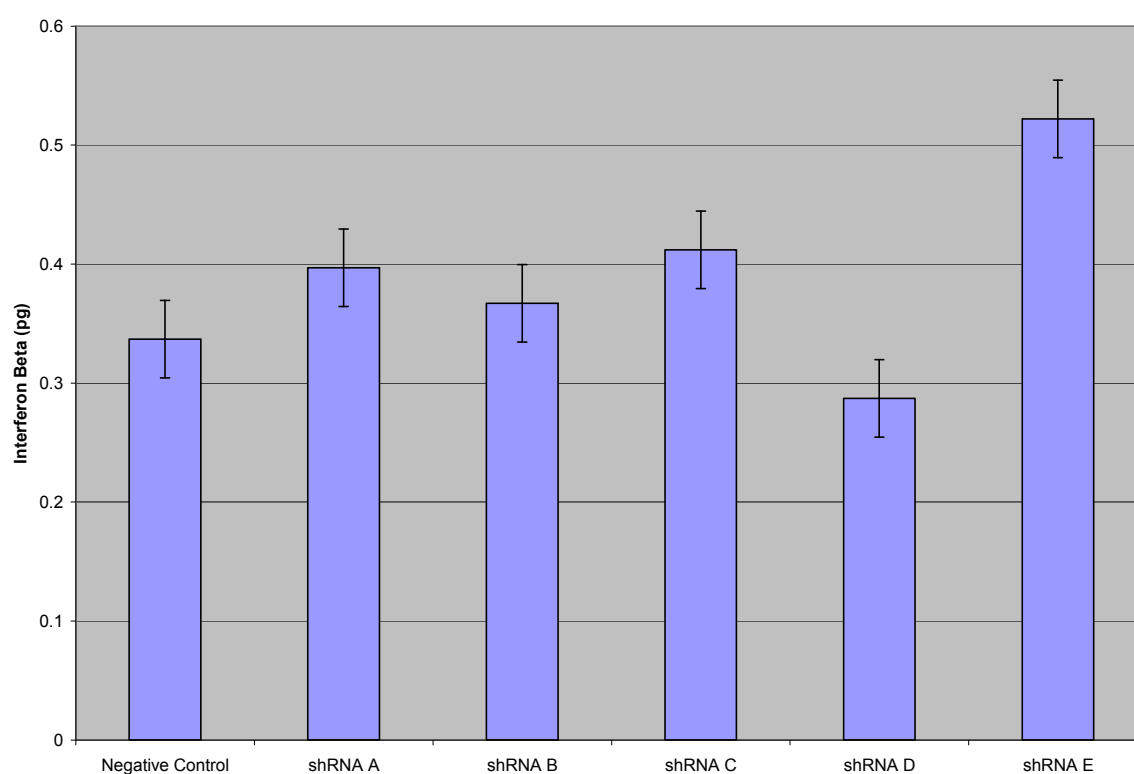


Figure 27: The expression of shRNA transcripts did not significantly up regulate the amount of interferon beta, although a slight increase in IFN B was noted when the cells were exposed to shRNA E transcripts. Error bars are indicated with standard error of the mean (n=2).

None of the shRNAs were determined to significantly induce IFN beta production. Although not statistically significant, the level of IFN beta in shRNA E transfections was found to be slightly up regulated, measuring 0.522 pg, apposed to 0.337 pg noted in the negative control.

CHAPTER 4: DISCUSSION

The involvement of the RNAi pathway in transposon silencing suggests that it is an ancient antiviral system that evolved to protect the host from invasion by genetic elements such as viruses⁷³. Several studies have indicated that the RNAi pathway can be manipulated to inhibit the replication of various viruses.

The development of drug resistant HIV-1 variants and the side effects of antiretrovirals used for the treatment of HIV-1 infection make it necessary to develop alternative therapeutic approaches for the treatment of HIV-1 infection. In this study, it was shown for the first time that shRNAs can specifically inhibit HIV-1 subtype C viral RNA. The data generated show that HIV-1 subtype C viruses' RNA and protein production can be substantially inhibited with shRNA sequences endogenously expressed from a plasmid vector, targeted to the gag ORF. This study shows that shRNA directed against a gene essential for viral replication can serve as a potential therapeutic agent for HIV-1 subtype C isolates.

There are several difficulties in using siRNA to inhibit a virus such as HIV. Firstly not all mRNA sites are equally accessible to siRNA, in fact the most conserved sequences are generally the most inaccessible⁷⁴. HIV RT exhibits rapid mutation rates allowing for escape HIV-1 viruses to be selected for⁷⁴. Optimal targets for siRNA mediated inhibition, can not only be chosen rationally but there is a certain element of trial and error involved in selecting optimal target sequences⁶⁷.

4.1 shRNA design

The U6 promoter was chosen as it is capable of producing RNA transcripts that are small, highly structured and very abundant ⁷⁵. The U6 promoter is also able to be transcribed in a variety of cell types and adds endogenous sequences which could aid in providing RNA stability ⁷⁵. It is not known yet if pol III promoters can be silenced by the cell, this might be an important consideration if long term expression of the shRNA is required ⁷⁴. The abundance of the transcripts might also lead to a disadvantage as an overabundance of shRNAs has been linked to the induction of non-specific gene silencing ⁶⁹

A U6 promoter capable of generating a hairpin structure was used as U6 governed transcripts of siRNAs individually produced remained localized in the nucleus. It has been shown that U6 transcribed sequences are efficiently transported to the cytoplasm, if they are expressed as hairpin structure containing a miRNA loop, allowing for dicer mediated cleavage to occur⁴⁸.

4.2 siRNA design Guidelines

Recently more comprehensive ‘rules’ have been proposed to aid in the development of promising therapeutic siRNAs, (1) they should have an overall GC content of between 30 and 50% to facilitate incorporation into RISC and siRNA unwinding, (2) have a low stability at the 5’ end of the antisense strand (to aid antisense incorporation into RISC), (3) form an A-form helix between the siRNA and the target RNA (to enhance RNA-RNA interactions and promote the cleavage of the target RNA), (4) lack a G at position 14 of the sense strand (aids

in unwinding) and (5) contain an A at position 3 or 19 of the sense strand (promotes antisense strand selection by RISC).⁷⁶

These guidelines were only established after the shRNAs used in this study had been designed and testing had begun. The table below indicates the features that each shRNA sequence contains (Table 11).

Table 11: Characteristics of shRNAs

	% GC Content	Has a G at position 14	Has an A at position 19	Has an A at position 3
shRNA A	62%	No	No	No
shRNA B	52%	No	No	No
shRNA C	38%	No	No	Yes
shRNA D	50%	No	Yes	No
shRNA E	52%	Yes	Yes	Yes

Using only these guidelines to predict the efficacy of the siRNAs, the best shRNAs would be shRNA C and D. shRNA A and B would be only somewhat effective and shRNA E would be extremely ineffective at inhibiting the target gene. This prediction closely matches those seen in this study and shows what promise there is in designing siRNAs according to these guidelines.

4.3 Analysis of HIV-1 Proteins

In the Western blot (Figure 19), all proteins appeared to be reduced by the introduction of effective shRNAs and not only the targeted protein. There are several possible explanations for this phenomenon. Perhaps, when the viruses first enter the cells, the initial viral transcript is targeted. Alternatively the pre-spliced mRNA in the nucleus could have been targeted (although this is unlikely as RISC is isolated from the cytoplasm) or more probably the shRNAs are inhibiting gag later on in the viral life cycle, by targeting either progeny viral genomes, or inhibiting viral capsid assembly (due to decrease in p24) ⁷⁷.

4.4 The Delivery System Problem

Similar to ribozymes and other gene therapies, many difficulties in the delivery *in vivo* of siRNA can be envisioned. A suitable delivery vehicle would reduce the susceptibility of the drugs to nucleases, have improved cellular uptake and also allow for the delivery of the drug to specific target cells ⁷⁸

Lentiviral vectors are a promising delivery system as they are able to integrate into dividing as well as non-dividing cells. These vectors are often developed from replication deficient HIV. They are highly efficient at delivering the gene of interest to the cells and are able to transduce cells rapidly, stably and uniformly ²⁸. A distinct advantage of the vectors is their ability to integrate into the genome of the host resulting in long-term expression of the shRNA. This long-term expression allows for the treatment of chronic conditions, therefore using this vector

for the delivery of shRNA targeted to HIV would be optimal. Even if the shRNAs are not able to completely eradicate HIV, they could help keep the virus in check and allow the infected individual to live a longer life. The safety issue surrounding the use of viral vector is always a high priority, but new vectors have improved safety features, such as the requirement for multiple recombination events with the transfer vectors to become replication competent, removal of all unnecessary viral genes and the removal of tat along with the inclusion of a constitutive promoter in place of the HIV sequence in the 5' LTR ^{79,78}.

4.5 Viral escape from RNAi

Due to the error prone nature of the reverse transcriptase of HIV-1, and the reliance of siRNA technology on sequence specificity, the question arises about the possibility of an “escape virus” emerging. In fact, this phenomenon has already been shown to exist, Boden *et al*, developed an shRNA targeted to the tat ORF of HIV-1 and within a month the shRNA had lost its efficacy and no longer inhibited HIV-1 replication⁸⁰. Following sequencing it was noted that a single base pair mutation at position 9 of the siRNA targeted sequence had allowed the virus the escape from the suppression caused by the shRNA ⁸⁰.

In order to overcome this hurdle, RNAi therapies could be targeted to conserved sequences in the HIV-1 genome, which, upon alteration would leave the virus replication incompetent. The use of several siRNA targeting different regions of the HIV-1 genome would also decrease the possibility of a mutant strain arising.

Although as little as a single bp change could be sufficient for the virus to escape from the RNAi pathway, this is not always the case^{81,82}, suggesting that certain sequences might be more robust to target than others. Once resistant strains do emerge it might be easier to target these strains simply by altering the therapeutic sequence. Furthermore, siRNA could be specifically targeted to known HAART escape mutants

Currently resistance to most HIV RT inhibitors, either nucleoside or non-nucleoside, has been documented. This resistance has been shown to correlate to an alteration in the nucleic acid sequence of the HIV-1 genome. In many subtype B viruses the mutations associated with the drug resistance are found in the viral populations even before the use of the drugs. Fortunately, subtype C viruses have had only limited exposure to most antiretrovirals, and the virus, as such has not developed an innate resistance to drugs⁵. This leaves open the possibility of delivering the conventional antiretrovirals together with siRNA targeted to the drug resistant strain thus preventing the existence of escape mutants and the subsequent incorporation of the escape sequence into the subtype C genome.

4.6 shRNA Efficacy

The difference between the various reduction levels noted when targeting the gag ORF might not only be due to target sequence composition, but also due to the secondary structure of the mRNA target and the binding of the RNA binding proteins³⁸. The differences can also be explained in HIV in particular, as the nucleocapsid of the incoming virus might preferentially shield certain regions of the viral RNA⁸¹

Another important restriction on the ability of the shRNAs to mediate *gag* degradation is the level of transfection efficiency. The transfection efficiency was determined to be approximately 40-50%. This would allow for the virus to replicate unchecked in the remaining untransfected cells, in spite of the siRNA inhibition. This could explain the incomplete levels of *gag* knockdown noted with even the most effective sequences.

4.7 The IFN response

If siRNAs do in fact activate the IFN response, the number of TLR on the targeted cell surface, appears to up regulate the magnitude of the IFN response ⁷⁰. This is a concern in HIV-1 treatment as cells of the immune system such as dendritic cells and macrophages have a high level of TLR on their cell surface ⁷⁰.

Even if it is true that synthetic siRNAs and plasmid based expression systems do result in the upregulation of IFN associated genes, this might not prove to be the end of siRNA based therapies. As miRNAs, also processed as dsRNA molecules, do not appear to induce the IFN response, the possibility exists that integrating siRNA producing sequences into the host cells genome, could in fact evade the IFN response ⁷⁰.

The noted IFN response that has been described in cells transfected with siRNAs might only have been induced due to the saturation of the RNAi machinery, therefore alteration of the dose of siRNA might overcome the interferon issue ⁶⁹. The IFN response described in response to siRNAs has only been found to be a modest response, and raises the question as to whether the activation of the IFN response by siRNAs would even be a problem as, apart from

the upregulation of certain IFN genes, no cytopathology has been described as a non-specific effect of siRNA treatment ⁶⁸. In this study, the interferon response was not activated to a statistically significant level in any of the shRNA transfection. Slight upregulation in interferon genes were noted, although this was not statistically significant. Either way it appears that siRNA mediated gene silencing exhibits sequence specific knockdown of target RNA without inducing apoptosis in target cells⁸³.

4.8 siRNA in therapeutic settings

An effective clinical application of siRNA technology, would be through a haemopoietic stem cells transplant, with the stem cell stably expressing siRNAs. This stem cell could then give rise to T-cells, macrophages and dendritic cells able to suppress HIV infection on replication⁷³.

The use of enzymatically derived shRNA libraries, such as EPRIL developed by Shirane *et al*, would allow for rapid screening of potentially therapeutic sequences ⁸⁴. siRNAs are relatively simple to design and synthesize, thus lowering the costs of production and development of siRNA based therapeutics.

It is hopeful that RNAi might exhibit greater promise in therapeutics than other antisense technologies as the RNAi pathway is endogenous and therefore more likely to be efficient at silencing gene expression *in vivo* ⁷⁶.

4.9 siRNA off target effects

As RNAi mediated degradation is sequence specific, it is possible that other mRNA endogenously expressed within the cell could be knocked down together with the target gene. In order to minimize off target effects, several ‘rules’ have been developed to effectively ensure safety of targeting genes with siRNAs. Preferentially the T_m difference between the desired target and possible off target genes should be maximized⁸⁵.

Specificity of siRNAs has been shown to be concentration dependent, at concentrations of approximately 100 nM non-specific gene suppression is noted, but if the concentration of siRNAs is lowered to 20 nM only specific suppression is seen⁸⁵.

The amount of siRNA delivered to the cells is crucial as not only do higher concentrations evoke non-specific gene silencing through TLR^{82,70}, but as the RNAi machinery also have innate functions and saturation of the system with therapeutic siRNAs could cause greater problems⁸². RNAi is used naturally in chromosomal architecture, any disruption of this function might result in the cells becoming defective in chromosomal function⁸².

4.10 shRNAs result in RNA degradation

As the RT-PCR results mimic the protein results, it can safely be assumed that the mechanism of inhibition of HIV-1 by the shRNA is caused by RISC-mediated RNA degradation, and not merely the suppression of protein translation. An exception to this is shRNA B in the Du151a inhibition, which appears to reduce protein synthesis, but there was no statistical difference in

the amount of RNA, implying that in this instance, protein translation, apposed to mRNA degradation was responsible for the noted decrease in p24 expression.

4.11 The Processing of shRNA D

shRNA D, the most effective shRNA sequence, was shown to be correctly processed into an approximately 21bp fragment, suitable to enter the RNAi pathway. The gel depicts a 21 bp fragment, as well as a higher fragment of 23 bp. The 21 bp fragment is the predominant siRNA resultant from Dicer mediated cleavage of the shRNA transcript. The 23 bp fragment is a variant of dicer cleavage and is unlikely to be incorporated into RISC due to its length. This also shows that the strand complementary to the target sequences was the strand retained in RISC.

CHAPTER 5: CONCLUSIONS

In summary, it was found for the first time, that shRNA transcripts, expressed from a U6 promoter system, were capable of inhibiting gene expression, in reporter plasmid, and live virus systems of HIV-1 subtype C.

shRNA D was able to specifically reduce reporter plasmid and live virus *in vitro* by RISC mediated degradation of *gag* mRNA transcripts. shRNA D was correctly cleaved by dicer *in vitro* and reduced viral p24 production by up to 85% in both X4 and R5 viruses. The ability of the remaining anti-*gag* shRNAs to inhibit HIV-1 subtype C replication was dependent on the virus strain targeted, with the exception of shRNA E which constantly up regulated the amount of p24 expressed. This could possibly due to the shRNA interacting with the target sequence and stabilizing it.

In 1988 David Baltimore proposed the concept of intracellular immunization, whereby inhibitory molecule are expressed from cells, and these molecules can protect cells from viral challenges in the future ⁶⁷. It is hopeful that the use of siRNAs can see this concept realized and revolutionize therapeutic approaches to viral diseases such as HIV-1 ⁶⁷.

CHAPTER 6: APPENDICES

6.1 Solutions

6.1.1 *Luria-Bertani medium (1 litre)*

- 10g tryptone
- 5g yeast extract
- 5g Sodium Chloride
- add water to 1 litre
- autoclave before use

6.1.2 *X-gal (10%)(for blue/white screening)*

- 100mg in 1ml dimethyl formamide

6.1.3 *12% Non-denaturing acrylamide gel (80ml)*

- 8ml TBE buffer
- 9.12g acrylamide
- 0.48g bis-acrylamide
- 40g Urea
- make up to 80ml with water
- add just before pouring :
 - 400µl APS
 - 40µl TEMED

6.1.4 *Agarose gel (1%)*

- 1ml TAE buffer
- 0.5g Agarose
- add up to 50ml with water
- heat up gel to dissolve
- add 2µl ethidium bromide

6.1.5 *TBE Buffer (10x) – 500ml*

- 54g Tris Base
- 27.5g Boric Acid
- 20ml 0.5M EDTA

6.1.6 TAE Buffer (50x)- 1 litre

- 242g Tris base
- 57.1 ml glacial acetic acid
- 100ml 0.5M EDTA

6.1.7 Ammonium Persulphate (APS)

- 0.1g Ammonium Persulphate
- 1ml water

6.1.8 0.5 M EDTA

- 186.12g/l EDTA in water
- pH to 8 with NaOH

6.1.9 Ampicillin

- 1g Ampicillin
- 5ml Ethanol
- 5ml water

6.1.10 Penicillin/streptomycin

- 0.61g penicillin (100u/ml)
- 1g streptomycin (100µg/ml)
- add 10 ml PURE water
- filter sterilize through 0.22 filter.

6.1.11 Fetal Calf Serum

- Heat inactivate at 60°C for 30 min

6.1.12 X-gal staining solution (50ml)(for cells)

- 1ml Potassium Ferricyanide (at 200mM)
- 1ml Potassium Ferrocyanide (at 200mM)
- 500µl X-Gal – DMSO
- 100µl MgCl₂·6H₂O
- make up to 50 ml with PBS

6.1.13 Fixing Solution (50ml)

- 1.35ml 37% formaldehyde
- 2ml gluteraldehyde
- 47.65ml water

6.1.14 ISE-RPMI

- 800ml PURE water
- 10.4g powdered RPMI
- 1.19g HEPES
- 1ml Na_2SeO_3 (5.19 mg/l)
- 1ml $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (27.8 mg/l)
- 1ml $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (3.71 g/ml), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.059mg/l), NH_4VO_3 (1.17mg/l) combined stock
- 1ml ethanolamine stock (1832.4mg/l)
- 2g NaHCO_3
- Adjust pH to 7.12 with HCl
- add PURE water to make up 1 litre
- add 100 μl linoleic acid (8.4mg/l stock)
- filter sterilize

6.1.15 Western Lysis Buffer

- 0.0121g Tris (10 mM Tris pH 7.5)
- 8.766g NaCl_2
- 0.744g EDTA
- 300 μl Igepal
- 0.005g Phenylmethylsulfonyl fluoride in 1 ml Ethanol
- Adjust to 100 ml with distilled water

6.1.16 Tris-buffered saline (1x)

- 6.05g Tris
- 8.76g NaCl
- 800 ml Water
- Adjust pH to 7.5 with 1 M HCl
- Adjust to 1 litre and store at 4°C

6.1.17 MiniElute Gel extraction kit protocol

- Excise DNA fragment from gel using clean sharp scalpel

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- Add 3 volumes of buffer QG to 1 gel volume (100mg gel = 100 µl buffer)
 - Incubate at 50°C for 10 min or until gel
 - Add 1 gel volume of isopropanol and mix by inverting several times
 - Place a MiniElute column in a 2 ml collection tube in a suitable rack
 - Place sample in the column and centrifuge for 1 minute
 - Discard flow through and return column to collection tube
 - Add 500 µl of buffer GQ to the spin column and centrifuge for 1 minute
 - Discard flow through and return column to collection tube
 - To wash add 750 µl of buffer PE to the spin column and centrifuge for 1 minute.
 - Discard the flow-through and centrifuge for 1 min at 13 000 rpm
 - Place the MiniElute column into a clean 1.5 ml microcentrifuge tube.
 - To elute add 10 µl buffer EB to the centre of the membrane, let the column stand for 1 minute then centrifuge for 1 minute.

6.1.18 High Pure Plasmid Isolation Kit

- Grow up plasmid containing *E. coli* in 5 ml of medium to an absorbance of A₆₀₀
- Pellet the cells by centrifugation.
- Discard the supernatant
- Resuspend the bacterial pellet in 250 µl of suspension buffer containing RNase H.
- Add 250 µl of Lysis Buffer and mix gently by inverting the tube 5 times.
- Incubate for 5 minutes at room temperature.
- Add 350 µl of chilled binding buffer, and mix by inverting the tube 5 times.
- Incubate on ice for 5 minutes
- Centrifuge for 10 minutes at 13 000 rpm.
- Insert one high pure filter tube into one collection tube and transfer the entire supernatant from the centrifugation step into the filter tube
- Centrifuge filter tube at 13 000 rpm for 60s.
- Discard flow-through.
- Add 700 µl of wash buffer II to the filter tube and centrifuge at 13 000 rpm for 60s and discard the flow-through
- Centrifuge for an additional minute at 13 000 rpm.
- Insert the filter tube into a sterile 1.5 ml microcentrifuge tube.
- Add 100 µl Elution Buffer
- Centrifuge the tube at 13 000 rpm for 1 minute to elute.

6.1.19 Qiagen Endofree Plasmid Maxi kit

- Inoculate a single colony in 5 ml of LB medium (including relevant antibiotics) for 8 hours at 37°C

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- Dilute the starter culture 1 in 500 into 25 ml selective LB medium
 - Harvest cells by centrifugation at 6 000 x g for 15 min at 4°C
 - Resuspend pellet in 4 ml buffer P1
 - Add 4 ml buffer P2 and mix by inverting 4-6 times
 - Incubate at room temperature for 5 min
 - Add 4 ml of chilled buffer P3, mix immediately by inverting the tube
 - Pour lysate into a QIAfilter cartridge and incubate for 10 min at room temperature
 - Remove the cap from the QIAfilter nozzle. Gently insert the plunger into the cartridge and filter the lysate into a convenient tube.
 - Add 1 ml of buffer ER to the filtered lysate, mix and incubate on ice for 30 minutes
 - Equilibrate a Qiagen- tip 100 by applying 10 ml buffer QBT
 - Allow the column to empty by gravity
 - Apply the ER buffered lysate to the tip and allow it to enter the resin by gravity flow
 - Wash the tip with 2x 10 ml buffer QC
 - Elute DNA with 5 ml of buffer QN
 - Precipitate the plasmids by adding 3.5 ml isopropanol. Mix and centrifuge at 15 000 g at 4°C for 30 minutes
 - Wash the pellet with 2ml endotoxin free room temperature 70% ethanol by centrifugation at 15 000 x g for 10 min
 - Air-dry pellet and resuspend in a suitable volume of endotoxin free buffer TE.

6.1.20 RNA concentration

- RNA concentration = OD260 x 40µg/µl x dilution factor

6.1.21 DNA Concentration

- DNA concentration = OD260 x 50µg/µl x dilution factor

6.1.22 2x Protein Sample Buffer

- 0.5M Tris-HCl pH 6.8
- 5% glycerol
- 2% SDS
- 100 mM DTT

6.2 Supplementary Data

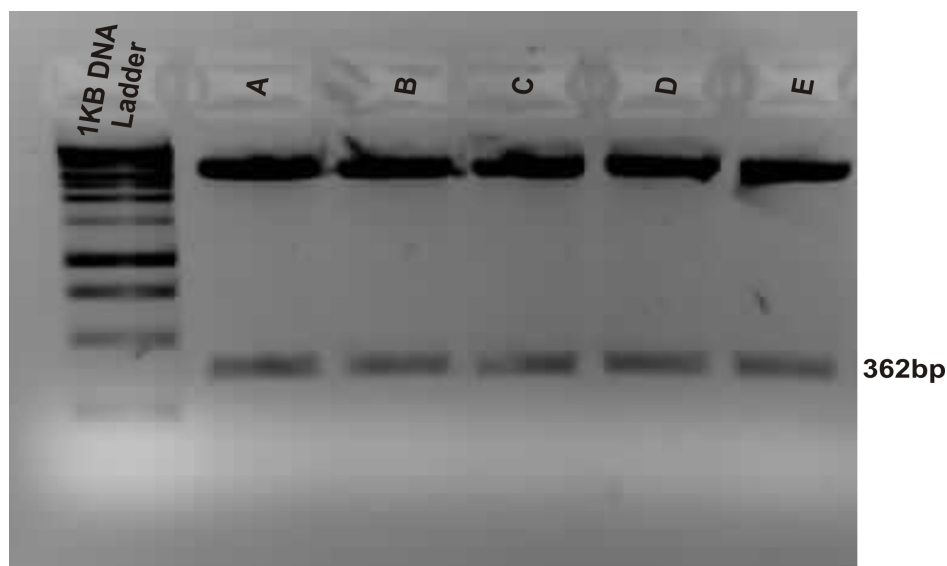


Figure 28: Restriction digest with *EcoRI* showing the anticipated 263bp fragments for shRNA A, B, C, D and E run on a 1% agarose gel together with a 1KB marker.

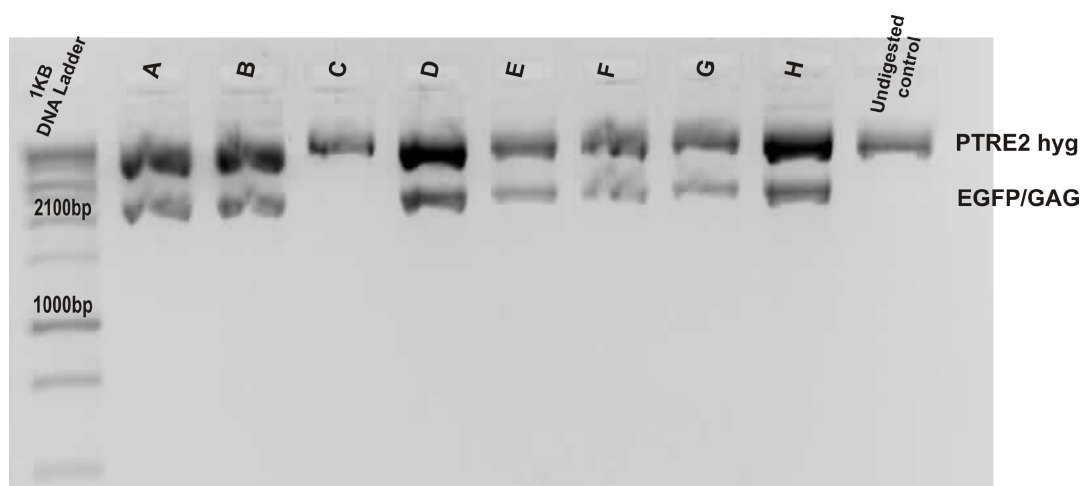


Figure 29: Restriction digest of ‘presumptive’ reporter plasmid clones, the expected fragment was approximately 2100bp. Clone C, although appearing to be a blue colony had no insert. The digest was performed with *EcoRI* and *XbaI*

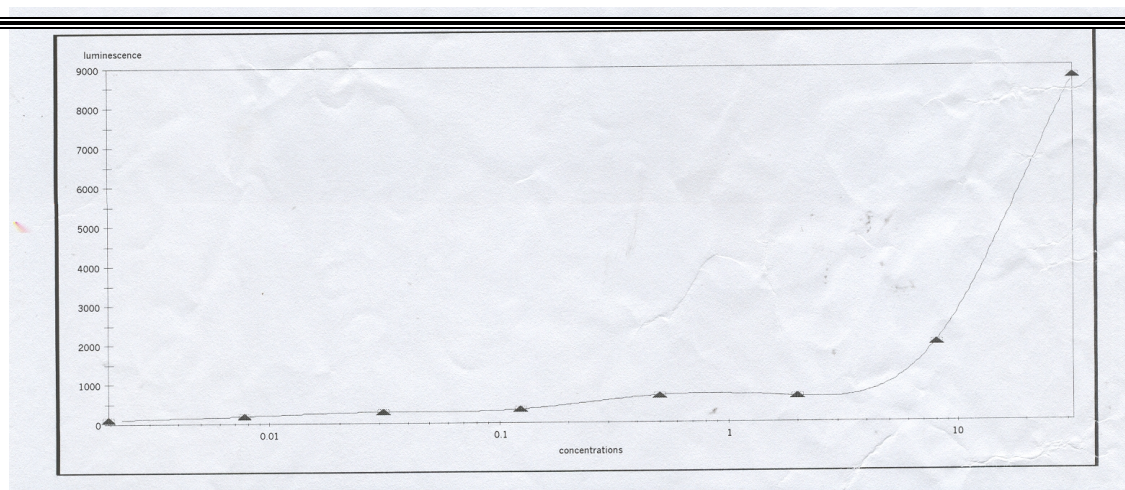


Figure 30: Standard curve used for the analysis of p24 ELISA results.

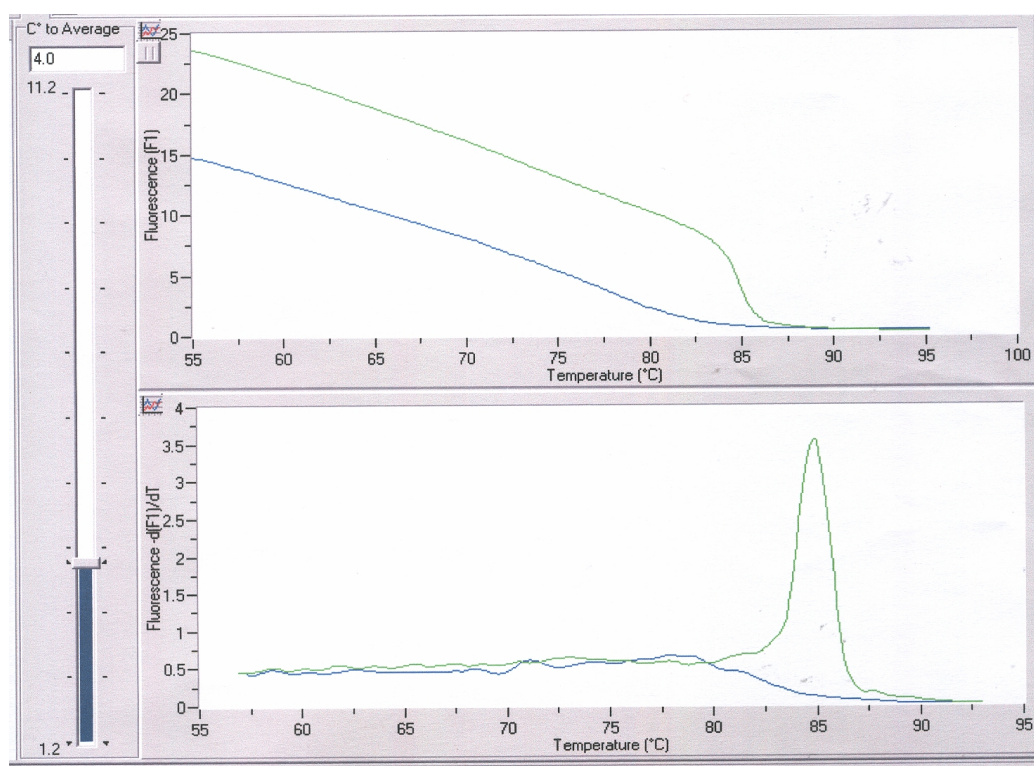


Figure 31: Melting curve analysis of Du422a amplicon and the negative control (in blue)

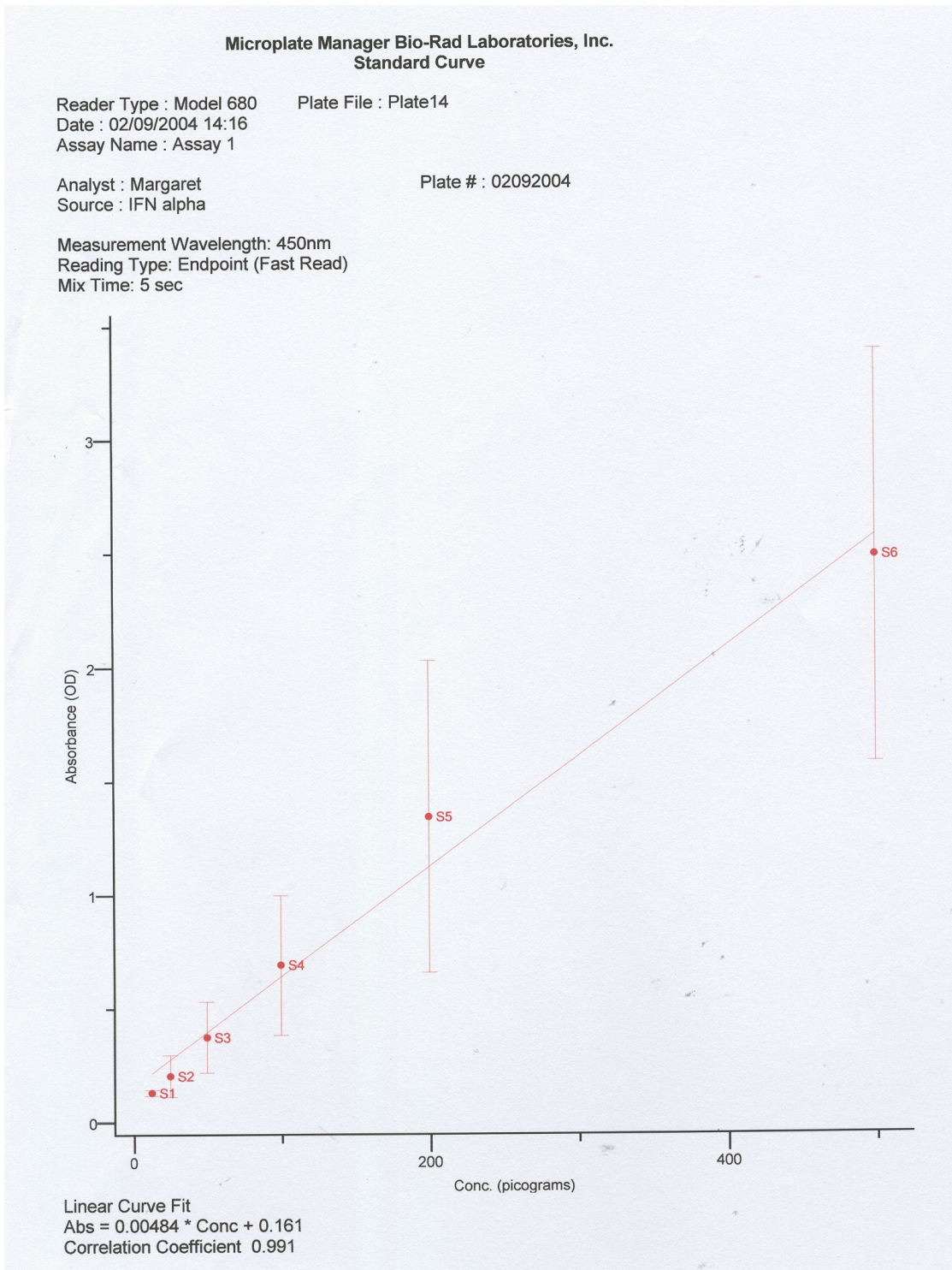


Figure 32: Standard curve utilized to analyze alpha interferon ELISA

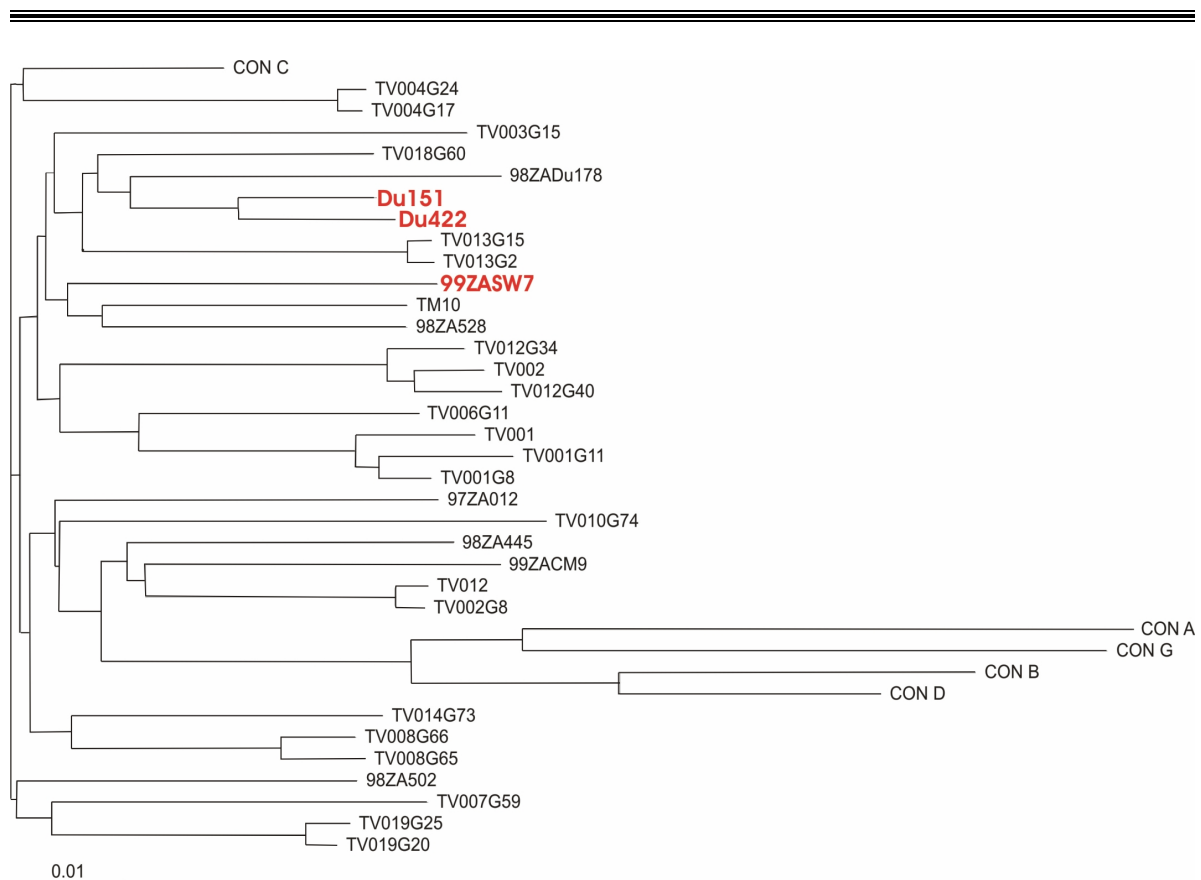


Figure 33: A phylogenetic tree indicating the relationship between the subtype C viruses used in the study. Du422a, Du151a and Sw7 have been highlighted in red.

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