Mutagenesis and functional studies of the HIV-1 *vpr* gene and Vpr protein obtained from South African virus strains

By

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Declaration

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Abstract

Background: Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) is an accessory protein that interacts with a number of host cellular and other viral proteins. Vpr exerts several functions such as induction of apoptosis, induction of cell cycle G2 arrest, modulation of gene expression, and suppression of immune activation. The functionality of subtype C Vpr, especially South African strains, has not been studied. The aim of this study was to describe the diversity of South African HIV-1 subtype C *vpr* genes and to investigate selected functions of these Vpr proteins.

Methodology: The HIV-1 *vpr* region of 58 strains was amplified, sequenced, and subtyped using phylogenetic analysis. Fragments containing natural mutations were cloned in mammalian expression vectors. A consensus subtype C vpr gene was constructed and site-directed mutagenesis was used to induce mutations in postions in which no natural mutations have been described. The functionality of all constructs was compared with the wild-type subtype B Vpr, by transfecting human 293T cell line to investigate subcellular localization, induction of apoptosis and cell cycle G2 arrest. The modulation of genes expressed in the induction of apoptosis using TaqMan Low density arrays (TLDA) was also investigated.

Results: Phylogenetic analysis characterized 54 strains as HIV-1 subtype C and 4 strains as HIV-1 subtype B. The overall amino acid sequence of Vpr was conserved including motifs FPRPWL and TYGDTW, but the C-terminal was more variable. The following mutations were constructed using site-directed mutagenesis: P14I, W18C, Y47N, Q65H and Q88S. Subtype B and all natural mutants of subtype C Vpr localized to the nucleus but the W18C mutation disturbed the nuclear localization of

Vpr. The cell cycle G2 arrest activity of all the mutants, as well as consensus-C, was lower than that of subtype B Vpr. All the natural mutants of subtype C Vpr induced cell cycle G2 arrest in 54.0-66.3% of the cells, while subtype B Vpr induced cell cycle G2 arrest in 71.5% of the cells. Subtype B and the natural mutant Vpr proteins induced apoptosis in a similar manner, ranging from 95.3-98.6% of transfected cells. However, an artificially designed Vpr protein containing the consensus sequences of subtype C Vpr indicated a reduced ability to induce apoptosis. While consensus-C Vpr induced apoptosis in only 82.0% of the transfected cells, the artificial mutants of Vpr induced apoptosis in 88.4 to 96.2% of the cells. The induction of apoptosis-associated gene expression was similar for all constructs, indicated that apoptosis was efficiently induced through the intrinsic pathway by the mutants.

Conclusion: This study indicated that both HIV-1 subtype B and C Vpr display a similar ability for nuclear localization and apoptosis induction. The induction of cell cycle G2 arrest by HIV-1 subtype B Vpr may be more robust than many subtype C Vpr proteins. The natural mutations studied in the isolates did not disturb the functions of subtype C Vpr and in some cases even potentiated the protein to induce apoptosis. Naturally occurring mutations in HIV-1 Vpr cannot be regarded as defective, since enhanced functionality would be more indicative of an adaptive role. The increased potency of the mutated Vpr proteins suggests that Vpr may increase the pathogenicity of HIV-1 by adapting apoptotic enhancing mutations.

Abstrak

Agtergrond: Die virus protein R (Vpr) van Menslike Immuungebrek Virus tipe 1 (MIV-1) is ‘n bykomstige protein wat met ‘n aantal sellulêre proteine van die gasheer en ander virus proteine in wisselwerking tree. Vpr het 'n invloed op verskeie funksies onder andere die induksie van apoptose, die induksie van selsiklus G2 staking, modulering van geen uitdrukking en onderdrukking van immuun aktivering. Die funksionaliteit van subtipe C Vpr, en veral die van Suid-Afrikaanse stamme, is nie beskryf nie. Die doelwit van die studie was om die diversiteit van Suid Afrikaanse MIV-1 subtipe C *vpr* gene te beskryf en ook om selektiewe funksies van die Vpr proteine te ondersoek

Metodiek: Die MIV-1 vpr streek van 58 stamme is vermeerder, die DNA volgordes is bepaal en die stamme is gesubtipeer deur filogenetiese analise. Fragmente met natuurlike mutasies is in ekspressie vektore gekloon. ‘n Konsensus subtipe C Vpr geen is ontwerp en mutasies in posisies waar geen natuurlike mutasies beskryf is nie, is ontwerp deur mutagenese. Die funksionaliteit van die konstrukte is met die wilde tipe subtype B vergelyk deur 293T sellyn te transfekteer en te ondersoek vir subsellulêre lokalisering, induksie van apoptose, en G2 selsiklus stilstand. Die modulering van geen uitdrukking in die induksie van apoptose is deur TLDA ondersoek.

Resultate: Filogenetiese analise het 54 stamme as HIV-1 subtipe C geklassifiseer en 4 stamme as subtype B. Die Vpr aminosuur volgordes was konstant insluitend die FPRPWL en TYGDTW motiewe, maar die C-terminaal was meer variëerbaar. Deur mutagenese is die volgende mutasies ontwerp: P14I, W18C, Y47N, Q65H and Q88S. Subtipe B en al die natuurlike mutante van subtipe C het in die selkern gelokaliseer, maar die W18C mutasie het die lokalisasie versteur. Die G2 selsiklus stilstand van

alle mutante en konsensus C was laer as die van subtype B. Al die natuurlike subtipe C mutante het G2 selsiklus tot stilstand gebring in 54.0-66.3% van die selle, terwyl subtype B selsiklus tot stilstand gebring het in 71.5% van die selle. Subtipe B en die natuurlike Vpr mutante het apoptose op ‘n soortgelyke wyse geinduseer, wat wissel tussen 95.3-98.6% van getransfekteerde selle. Die protein met die kunsmatig ontwerpte konsensus C volgorde het egter ‘n verlaagde vermoë gehad om apoptose te induseer. Die konsensus subtipe C het apoptose in 82.0% van getransfekteerde selle geinduseer en die kunsmatige mutante in 88.4 – 96.2% van die selle. Die induksie van die apoptose verwante geen ekspressie deur die mutante was soortgelyk as die van konsensus C en subtipe B Vpr wat ’n aangeduiding is dat apoptose effektief veroorsaak is deur die intrinsieke roete.

Gevolgtrekking: Hierdie studie het aangetoon dat kern lokalisering en apoptose op ‘n soortgelyke wyse by beide MIV-1 subtipe B en C Vpr plaasvind. Die induksie van selsiklus G2 stilstand deur MIV-1 subtipe B Vpr is egter meer robuust as baie van die subtipe C Vpr proteïene. Natuurlike mutasies in MIV-1 Vpr kan nie as gebrekkig beskou word nie, aangesien beter funksionaliteit 'n aanduiding is vandie aanpasbare rol. Die verhoogde krag van die gemuteerde Vpr proteïen dui daarop dat Vpr die patogenisiteit van MIV-1 kan verbeter deur die aanpassing van mutasies.

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Publications:

1. Romani B, Glashoff RH, Engelbrecht S. Molecular and phylogenetic analysis of HIV type 1 *vpr* sequences of South African strains. AIDS Res Human Retroviruses. 2009: 25; 357-362.

2. Romani B, Engelbrecht S. Human immunodeficiency virus type 1 Vpr: functions and molecular interactions. J Gen Virol. 2009: 90; 1795-1805.

3. Romani B, Glashoff RH, Engelbrecht S. Functional integrity of naturally occurring mutants of HIV-1 subtype C Vpr. Virus Res, 2010: doi:10.1016/j.virusres.2010.08.020.

Presentations:

1. Romani B, Glashoff RH, Engelbrecht S. Functional integrity of naturally mutated forms of HIV-1 subtype C Vpr: comparison with subtype B and the consensus sequence of the protein. XVIII International AIDS Conference, 18-23 July 2010, Vienna, Austria.

2. Romani B, Glashoff RH, Engelbrecht S. Functional integrity of naturally mutated forms of HIV-1 subtype C Vpr. Academic Year Day, Stellenbosch University, 11 August 2010.

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Abbreviations

ºC	Degree Celsius
µg	Microgram
µl	Microlitre
7-AAD	7-amino actinomycin D
AA	Amino acid
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis-inducing factor
ANT	Adenine nucleotide translocator
AP-1	Activator protein 1
ATR	Ataxia telangiectasia-mutated and Rad3-related
bp	Base paires
CA	Capsid protein
CDK	Cyclin-dependent protein kinase
CMV	Cytomegalovirus
CTL	Cytotoxic T-lymphocyte
CUL4A	Cullin 4A
DAPI	4'-6-Diamidino-2-phenylindole
DCAF-1	DDB1- and CUL4-associated factor 1
DDB1	Damaged DNA-binding protein 1
ddNTPs	Dideoxyribonucleotide triphosphates
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates

DSBs	DNA double-strand breaks
EDTA	Ethylene diamine tetra acetic acid
env	Envelop gene
Env	Envelop protein
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
g	Gravity
gag	Group antigen gene
Gag	Group antigen protein
GFP	Green fluorescent protein
gp	Glycoprotein
GR	Glucocorticoid receptor
HAX-1	HS1-associated protein X-1
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HSPs	Heat-shock proteins
IFN	Interferon
IL	Interleukin
Impα	Importin-α
Impβ	Importin-β
IN	Integrase
Kb	Kilobases
LTR	Long terminal repeat
MA	Matrix

mg	Milligram
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
NC	Nucleocapsid protein
nef	Negative factor gene
NF-kB	Nuclear factor kB
NLS	Nuclear localization signal
NPC	Nuclear pore complex
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PI	Propidium iodide
PIC	Preintegration complex
Pol	Polymerase gene
Pol	Polymerase protein
PS	Phospholipid phosphatidylserine
PTPC	Permeability transition pore complex
RNA	Ribonucleic acid
RT	Reverse transcriptase
Taq	*Thermus aquaticus*
Th1	T-helper type 1
TNF	Tumour necrosis factor
U3	Unique 3´ region

U5	Unique 5´ region
UNG2	Uracil–DNA glycosylase 2
USA	United States of America
V	Volts
VDAC	Voltage-dependent anion channel
Vif	Virion infectivity factor
vpr	Viral protein R gene
Vpr	Viral protein R
VprBP	Vpr-binding protein
Vpu	Viral protein U
WDR	WD40-repeat
X-Gal	X-Galactosidase

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Chapter 1

1. Introduction and literature review

1.1 Introduction

Viral virulence is dependent on the interplay between virus and host. Route of virus entry, dose of the virus and the host's age, sex, immune status and species all play a role in determining virulence. The host may suppress every stage of the virus replication cycle by posing different obstacles to the progression of virus replication. A successful virus must adapt to overcome these obstacles. Viruses have evolved proteins to aid in overcoming the defensive mechanisms of the host (Tungaturthi *et al.*, 2004a). In this regard, Lentivirus genomes, such as HIV-1 genome, contain a number of accessory and regulatory genes in addition to the *gag*, *pol* and *env* genes that are commonly found in all retroviruses. Human immunodeficiency virus type 1 (HIV-1) encodes two regulatory proteins, Tat and Rev, and four accessory proteins [viral infectivity factor (Vif), viral protein R (Vpr), viral protein U (Vpu) and negative factor (Nef)]. The regulatory proteins are essential for virus replication by controlling HIV gene expression in host cells. In contrast, accessory proteins are often dispensable for virus replication *in vitro* (Anderson & Hope, 2004). All of these proteins are however implicated in viral manipulation of host defences.

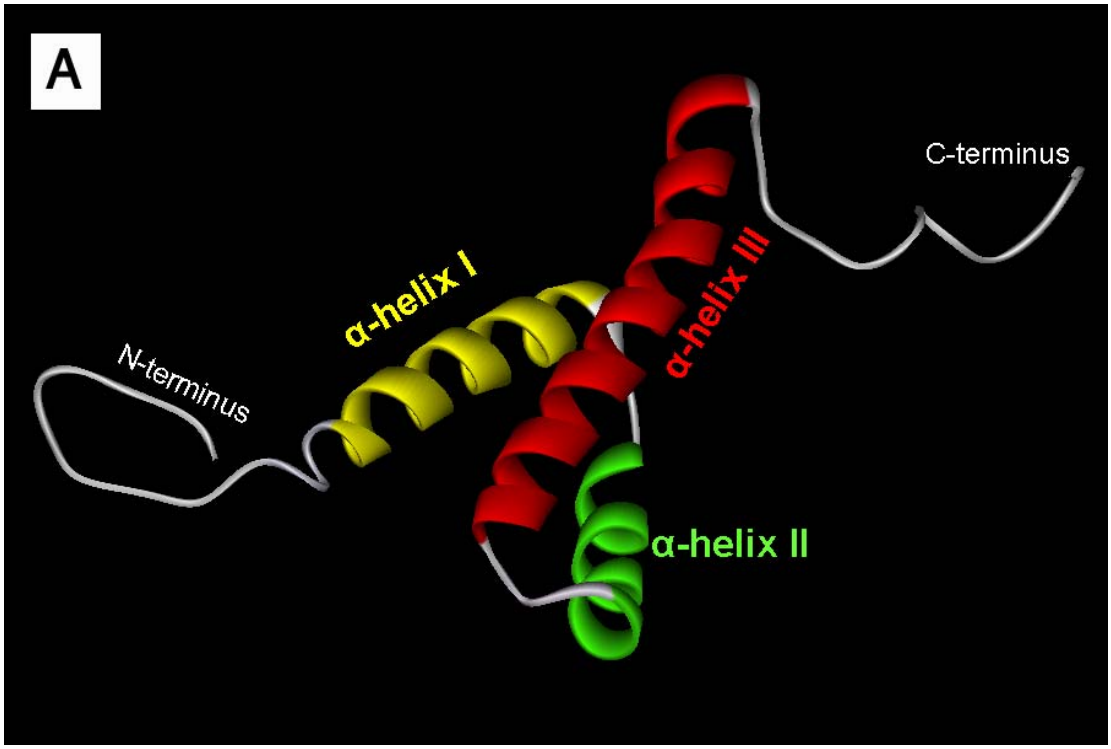
The HIV-1 genome encodes a 14 kDa accessory protein, Vpr, which is a versatile, virion-associated protein composed of 96 AA (Felzien *et al.*, 1998; Muller *et al.*, 2000; Muthumani *et al.*, 2000). Vpr is incorporated into HIV-1 virions through direct

interaction with the p6 domain of the Gag protein (Bachand *et al.*, 1999; Lavallee *et al.*, 1994). Vpr has a variety of roles in determining HIV- 1 infectivity, and the number of its newly identified roles is still increasing. Some of the functions proposed for this protein include modulation of transcription of the virus genome (Sawaya *et al.*, 2000), induction of apoptosis, disruption of cell-cycle control, induction of defects in mitosis (Chang *et al.*, 2004), nuclear transport of the HIV-1 pre-integration complex (PIC) (Vodicka *et al.*, 1998), facilitation of reverse transcription (Rogel *et al.*, 1995), suppression of immune activation (Ramanathan *et al.*, 2002) and reduction of the HIV mutation rate (Jowett *et al.*, 1999). In addition, the interactions of Vpr with a number of human proteins have been identified, but the functions of some of those interactions are not clear (Felzien *et al.*, 1998; Ramanathan *et al.*, 2002; Sawaya *et al.*, 2000). The NMR structure of Vpr indicates that Vpr contains a flexible N-terminal region, three α-helical domains with amphipathic properties and a flexible C-terminal region (Figure 1.1) (Morellet *et al.*, 2003). Each function or interaction of Vpr is attributed to one or more of its domains. Identification of the functions of Vpr domains is usually carried out by mutagenesis studies (Tungaturthi *et al.*, 2004a).

HIV-2, SIVsm and SIVmac encode two proteins that are homologous to HIV-1 Vpr, called Vpr and Vpx. HIV-2 Vpr shares the ability to induce cell cycle G2 arrest with HIV-1 Vpr, but HIV-2 Vpx has no effect on the cell cycle and, instead, is required for efficient infection of non-dividing cells such as macrophages and dendritic cells. It is believd that Vpx has originated from duplication of *vpr* within the HIV-2, SIVsm and SIVmac. The high degree of homology, and the divergent functions of Vpx with respect to HIV-1 Vpr, suggest that Vpx is a paralog of HIV-1 Vpr (Planelles & Barker, 2010).

The 3-dimentional structure of Vpr has been analyzed in the presence in pure water at low pH. The structure of Vpr is characterized by three well-defined α-helices surrounded by flexible N and C-terminal domains. The three α-helices are folded around a hydrophobic core constituted of Leu, Ile, Val and aromatic residues. This structure accounts for the interaction of Vpr with different targets (Morellet *et al.*, 2003).

The cytopathic effects induced by Vpr are mostly attributed to the N terminus, which forms ion channels in cell membranes. These effects are unrelated to the reported activities of Vpr, including virion association, G2 arrest and induction of apoptosis (Piller *et al.*, 1999; Somasundaran *et al.*, 2002). Incorporation of Vpr into HIV-1 virions is blocked by a range of mutations distributed in different regions of the protein, indicating that different domains of Vpr are involved in its incorporation into virions. Alterations in the cell cycle, including apoptosis, cell-cycle arrest and defects in mitosis, are mostly carried out by the C-terminal domain of Vpr, although alterations in the cell cycle by the other regions of Vpr have also been reported (Piller *et al.*, 1999; Roumier *et al.*, 2002; Srinivasan *et al.*, 2008).



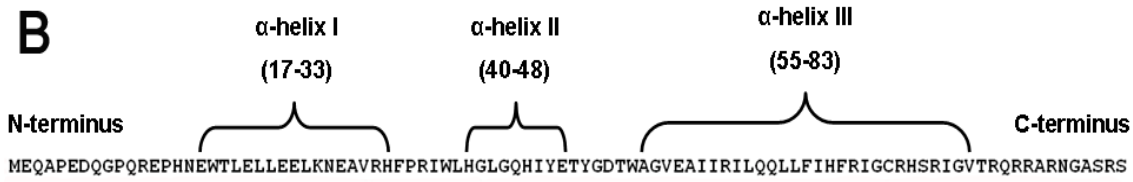


Figure 1.1: The HIV-1 Vpr protein. (A) Three-dimensional structure of HIV-1 Vpr, representing the N- and C-terminal regions in white, α-helix I in yellow, α -helix II in green and α -helix III in red. (B) Amino acid sequence of the HIV-1 Vpr according to the reference sequence HXB2 (GenBank accession no. K03455), showing the positions of the domains (Romani & Engelbrecht, 2009).

Using mutagenesis approaches, HIV-1 Vpr has been extensively studied to obtain information on the functions of different domains of this protein. Software analysis of solution structure of full length Vpr reveals that Vpr contains a flexible N-terminal region, followed by three helices connected by loops and ends with flexible C-terminus. The N-terminus of Vpr consistes of the first 16 residues Met1-Asn16 followed by the first α-helix of 17 amino acids encompassing residues Asp17 to His33. The first α-helix is connected to the second α-helix by a short inter-helical loop consisting of residues Phe34 to Leu39. The second α-helix is formed between residues His40 to Glu48, followed by a second inter-helical loop between Glu48 to Trp54

connecting to the third α-helix. The third α-helix, which is extended between Ala55 to Val83, is followed by a C-terminal domain (Thr84 to Ser96). All 3 α-helices have characteristic amphipathic properties and all are packed with hydrophobic side chains forming the core of the protein (Pandey *et al.*, 2009). The important impact of Vpr mutagenesis on functional integrity of the viral protein was the rationale for the current study. Both variability of vpr and associated mutations have not been described for the subtype C epidemic in South Africa, and this study addresses both these issues.

1.2 Literature Review

An overview of the current literature on the functions and molecular interactions of HIV-1 Vpr protein is presented. Most of the functions proposed for Vpr have been well established, however there are several functions proposed for Vpr that have not been well documented to date.

1.2.1 Nuclear import of the PIC by Vpr

DNA viruses and some RNA viruses need to access the nucleus for replication. The interior of the nucleus is isolated from the cytoplasm by a nuclear envelope that is contiguous with the endoplasmic reticulum. The nuclear envelope is composed of two lipid bilayers, the inner and outer nuclear membranes. The traffic of macromolecules such as proteins and mRNAs takes place through the nuclear pore complexes (NPCs) located in the nuclear envelope. The translocation of cargoes larger than ~40 kD usually requires signals called nuclear localization signals (NLSs). The nuclear import of basic NLS-bearing proteins is mediated by specific shuttling factors, such as

importin-α (Impα), importin-β (Impβ), small GTPase Ran/TC4, and NTF2 (Aida & Matsuda, 2009).

HIV-1 Vpr enhances the ability of HIV-1 to replicate in terminally differentiated macrophages, which is attributed to the activity of Vpr in active nuclear import of the virus PIC (Jenkins *et al.*, 1998; Le-Rouzic *et al.*, 2002; Schang, 2003; Suzuki *et al.*, 2009). The HIV-1 PIC is composed of viral proteins, such as Vpr, reverse transcriptase (RT), integrase (IN), nucleocapsid (NC) and matrix (MA), in addition to viral nucleic acids. Although the precise role(s) of some of these proteins has not been described, their importance for nuclear import of the PIC has been demonstrated. The nuclear-localization signals (NLSs) in both MA and IN have been identified, and it is believed that both utilize the classical nuclear-import pathway that includes interaction with importins. In contrast, a canonical NLS has not been identified in Vpr, but it does display karyophilic properties. It is believed that the nuclear localization of Vpr is mediated by an unknown pathway that is distinct from the classical NLS- and M9-dependent pathways. In addition, it has also been suggested that Vpr utilizes the cellular machinery that regulates nucleocytoplasmic shuttling to transfer the proviral DNA to the nucleus. For example, in the presence of importin-α, the nuclear localization of Vpr increases. It has been suggested that interaction of Vpr with importin-α facilitates its nuclear localization (Gallay *et al.*, 1997; Gallay *et al.*, 1996; Nitahara-Kasahara *et al.*, 2007).

To identify the role of Vpr in the nuclear import of PIC, green fluorescent protein (GFP) has been fused to Vpr. It has been demonstrated that the PIC is associated closely with cytoplasmic microtubules that direct it toward the nucleus, where it localizes in the perinuclear region close to centrosomes. It has also been shown that

the PIC utilizes cytoplasmic dynein to travel toward the nucleus. It has not been confirmed whether Vpr plays an active role during the transfer of the PIC along microtubules, or whether it is only associated with PIC in this step and starts its role during the next steps of the nuclear transfer of PIC (Le-Rouzic & Benichou, 2005).

Selective trafficking between the nucleus and cytoplasm is carried out through NPCs, which form aqueous channels in the nuclear envelope. NPCs are large protein complexes composed of 30 distinct nuclear-pore proteins, called nucleoporins (Nups). Some Nups that contain phenylalanine-glycine (FG) repeats are found in the filamentous structures emanating from both sides of NPCs; thereby provide docking sites for various transport factors. Importin-α is a floating receptor that binds to the NLSs of cargo proteins. FG repeats on the cytoplasmic filaments and cytoplasmic ring moiety of NPCs recognize the importin receptor. It has been shown that HIV-1 Vpr binds to the FG repeats of several Nups, including human p54 and p58. HIV-1 Vpr also binds to human CG1 (hCG1), but this interaction is not mediated by the FG repeat of this Nup. Interaction of Vpr with the N-terminal region (which does not contain FG repeats) of hCG1 is essential for docking of Vpr to the nuclear envelope (Le-Rouzic & Benichou, 2005; Vodicka *et al.*, 1998; Zeitler & Weis, 2004). It has been demonstrated that the α-helical domains of Vpr are essential for its interaction with hCG1. This interaction results in Vpr accumulation in the nuclear envelope, which is believed to be involved in active nuclear import of the PIC in nondividing cells, such as macrophages. It may also be involved in targeting the PIC to the NPC before its translocation into the nucleus. In addition to the conventional routes suggested for nuclear import of the PIC, it has also been reported that Vpr mediates transient, localized herniations in the nuclear envelope, resulting in the mixing of cytoplasmic and nuclear components. These herniations probably contribute to the

G2-arrest activity of Vpr and may also provide an unconventional route for nuclear import of the PIC. In fact, the interaction between Vpr and hCG1 could cause misassembly of the NPC, resulting in alterations of the architecture of the nuclear envelope that facilitate nuclear import of the PIC (de-Noronha *et al.*, 2001; Le-Rouzic *et al.*, 2002). Vpr-induced herniation and bursting may not be the only mechanism by which the HIV PIC enters to the nucleus because HIV vectors that lack Vpr can replicate in at least some nondividing cells (Segura-Totten & Wilson, 2001).

When the HIV-1 nucleoprotein complex reaches the nucleus, it must cross the nuclear envelope to integrate into the host genome. Theoretically, molecules of up to 9 nm in diameter can be transferred through the NPC by passive diffusion. However, the HIV-1 PIC is too large for passive transport. Although the central molecular mechanisms of PIC nuclear import are unknown, there is enough evidence to discuss several proposed theories (Figure 1. 2). The first theory proposes a role in the uncoating process. It has been shown that a small PIC (~80S) is detected in the nucleus, while a larger PIC (~100-350S) is detected in the cytoplasm. Thus, remarkable shedding of components and/or major conformational changes may occur to reduce its size, indicating that capsid (CA) dissociates from PIC. In fact, HIV-1 apparently is uncoated during infection; probably after reverse transcription is completed at the nuclear pore. Therefore, the uncoating step is essential for efficient nuclear import. The second theory proposes that although viral capsid uncoating is likely necessary for nuclear import, specific signals and import factors also likely to direct the intracellular trafficking of PIC. Importantly, MA, Vpr, and IN have also been implicated in PIC nuclear import via interaction with NLS receptors (i.e., importins or karyopherins); however, the role(s) of these interactions to HIV-1 replication in non-dividing cells is controversial. Vpr can also bind to Impα to be transported into the

nucleus by an interaction with Impα alone, but not with the Impα/Impβ heterodimer. It has been suggested that Vpr, IN and MA work either sequentially or synergistically to regulate PIC nuclear import. The third theory proposes that a new player, tRNA, which mediates translation in the cytoplasm, may be involved in PIC nuclear import. Some tRNA species lacking the 3-CCA end are essential for nuclear import. In addition, these tRNA species are packaged into budding viral particles, and tRNA has been indicated to travel in a retrograde direction from the cytoplasm to the nucleus (Aida & Matsuda, 2009).

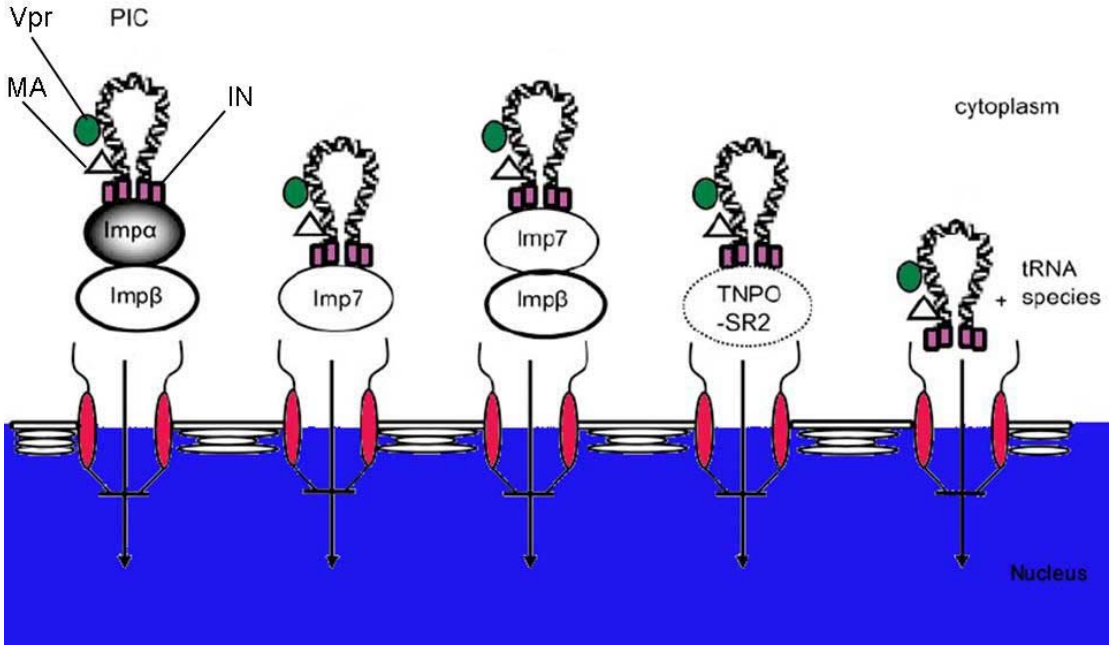


Figure 1.2: Different theories on HIV-1 PIC nuclear import. The Impα/Impβ heterodimer, Imp7, the Imp7/Impβ heterodimer, TNPO-SR2 or tRNA species contribute to the nuclear import of HIV-1 PIC (Aida & Matsuda, 2009).

1.2.2 Induction of G2 arrest by Vpr

The role of HIV-1 Vpr in inhibition of normal cell growth is well established. It is believed that the interruption of cell division by Vpr increases virus replication and induces programmed cell death. Vpr mediates cell-cycle arrest at the G2/M transition in various mammalian cells. The cell cycle G2 arrest provides a replication advantage for the virus, because the transcription level of the provirus is higher in cell cycle G2 arrested cells than in dividing cells (Belzile *et al.*, 2007; Elder *et al.*, 2001; Goh *et al.*, 1998). As the pathways regulating the cell cycle are highly conserved in eukaryotic cells (Krylov *et al.*, 2003; Warbrick & Fantes, 1988), transfection of yeast cells by vectors expressing Vpr also interrupts the cell cycle. Therefore, in studies of cell-cycle arrest induced by Vpr, the use of yeast cells is common, because they are easy to handle (Benko *et al.*, 2007; Elder *et al.*, 2001; Huard *et al.*, 2008).

The eukaryotic cell cycle is controlled by a complex network of proteins and genes. Cyclin-dependent protein kinases (CDKs) initiate the crucial events of the cell cycle by phosphorylating specific protein targets. The phosphorylation activity of CDKs is tightly dependent on binding to cyclins. Binding of cyclins to CDKs results in CDK–cyclin complexes. As there are always excess amounts of CDKs in eukaryotic cells, it is the availability of cyclins that determines the number of CDK–cyclin complexes in eukaryotic cells. CDK–cyclin complexes can be downregulated either by inhibitory phosphorylation of the CDK subunit or by binding to inhibitory molecules designated cyclin-dependent kinase inhibitors (Csikasz-Nagy *et al.*, 2006). G2 arrest is characterized by low levels of cyclin B1–p34Cdc2 activity and inhibitory phosphorylation of p34Cdc2. It has been shown that Vpr directly inhibits the *in vitro* activity of a phosphatase, Cdc25C, which normally activates cyclin B1-p34Cdc2

(Figure 1.3). Although the Vpr binding site on Cdc25C is not its catalytic site, Cdc25C is inactivated by binding to Vpr, resulting in inhibition of Cdc25C phosphatase activity. In the absence of Cdc25C phosphatase activity, cyclin B1–p34Cdc2 remains in its phosphorylated form, which is inactive (Goh *et al.*, 2004; He *et al.*, 1995). 14-3-3 proteins are also involved in this pathway. These proteins normally regulate cell-cycle progression by changing the activities of cyclins, including Cdc25C. DNA damage results in Cdc25C phosphorylation, providing the active binding site for 14-3-3. It has been shown that the C-terminal region of Vpr interacts with the C-terminal region of 14-3-3, leading to the association of 14-3-3 with Cdc25C. This complex is not able to activate cyclin B1-p34Cdc2; therefore the cell cycle is arrested (Figure 1.3) (Kino *et al.*, 2005b).

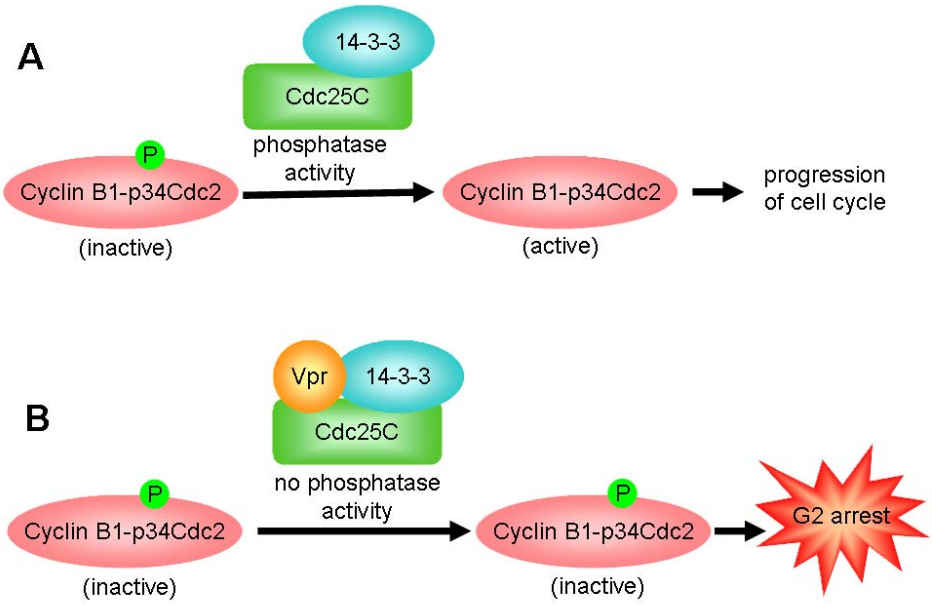


Figure 1.3: Cell-cycle arrest through inactivation of Cdc25C. (a) 14-3-3 protein binds to Cdc25C, resulting in a complex that possesses phosphatase activity. The complex removes the phosphate molecule from the inactive form of cyclin B1–p34Cdc2, altering it to the active form that drives progression of the cell cycle. (b) Vpr binds to the 14-3-3 protein and Cdc25C and inactivates this complex. In the absence of the phosphatase activity of Cdc25C, cyclin B1–p34Cdc2 remains inactive, resulting in G2 arrest (Romani & Engelbrecht, 2009).

Inactivating Cdc25C is not the only pathway utilized by Vpr to arrest the cell cycle. Several studies have documented the association of HIV-1 Vpr with a cellular protein that now is called DCAF-1 (DDB1- and CUL4-associated factor 1), also known as VprBP (Vpr-binding protein). Further research demonstrated the role of this protein in DNA replication and embryonic development. It has recently been demonstrated that Vpr exploits DCAF-1, damaged DNA-binding protein 1 (DDB1) and the cullin 4A (CUL4A) ubiquitin ligase complex to interfere with the DNA replication machinery of infected cells, resulting in cell-cycle arrest (Figure 1.4). The pathway exploited by Vpr implies that it functions as an adaptor protein in protein networks to interfere with the cell cycle and replication machinery (Giaccia & Kastan, 1998; Gieffers *et al.*, 2000; Hrecka *et al.*, 2007; Wen *et al.*, 2007). Vpr exploits DCAF-1 as an adaptor to engage DDB1 as a component of the CUL4A ubiquitin ligase complex. As this complex is involved in proteasomal degradation, it was suggested that G2 arrest is induced by Vpr through degradation of an unidentified protein that is needed for progression of cells from G2 arrest to mitosis (Wen *et al.*, 2007). In fact, the CUL4A ubiquitin ligase complex acts as a pivotal step, regulating different cellular pathways by targeting proteins for ubiquitin-dependent degradation. It has recently been demonstrated that, in this complex, DDB1 interacts with multiple WD40-repeat (WDR) proteins, which serve as the substrate-recognition subunits of the CUL4-DDB1 ubiquitin ligase. More than 150-300 WDR proteins have been identified in the human genome. Considering the variety of WDR proteins, the impact of biological processes through CUL4 ligase-mediated proteolysis can be understood. It has been reported that CDT2 interacts with the CUL4A ubiquitin ligase complex, enabling it to target CDT1 for degradation. CDT1 is a replication factor, depletion of which prevents DNA replication. Although cell-cycle arrest cannot be attributed to the

degradation of a single protein through CUL4 ligase-mediated proteolysis, which would result in G2 arrest, CDT1 can be addressed as a key protein that is ubiquitinated by the CUL4A ubiquitin ligase complex, activated by Vpr. Indeed, many other proteins may play similar roles in parallel with CDT1 (Higa & Zhang, 2007; Jin *et al.*, 2006; Rialland *et al.*, 2002).

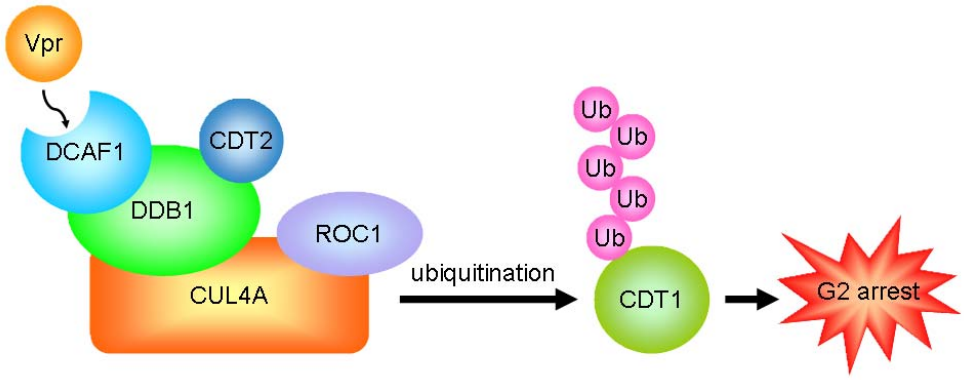


Figure 1.4: Cell-cycle arrest by triggering ubiquitination. Vpr functions as an adaptor protein to interfere with the cell cycle and replication machinery. Vpr exploits DCAF-1 as an adaptor to engage DDB1, a component of the CUL4A ubiquitin ligase complex, which is involved in proteasomal degradation. DDB1 interacts with multiple WDR proteins, including CDT2, which serve as the substrate-recognition subunits of the CUL4A ubiquitin ligase complex. CDT2 enables the complex to target CDT1 for ubiquitin-dependent degradation. CDT1 degradation results in G2 arrest (Romani & Engelbrecht, 2009).

It has been demonstrated that the expression of wild-type Vpr protein in HeLa cells induces transient herniations in the nuclear envelope. Sporadically and spectacularly, these herniations burst, releasing soluble nuclear proteins into the cytoplasm and presumably also allowing cytoplasmic proteins to enter the nucleus. The broken sections of the nuclear envelope apparently reseal within minutes. These transient ruptures of the nuclear envelope may provide an unconventional mechanism for nuclear entry that bypasses the size-restricted nuclear pore complexes. Bursting of the Vpr-induced nuclear herniations releases key cell cycle regulators, including the kinase Wee1, the phosphatase Cdc25C, and cyclin B into the cytoplasm of the host

cell. The repeated release of these proteins into the cytoplasm might explain how Vpr causes G2 arrest. Consistent with this idea, Vpr mutants that fail to cause herniations also fail to cause G2 arrest (Segura-Totten & Wilson, 2001).

1.2.3 Induction of apoptosis by Vpr

Although a variety of stimuli have been described as causes of apoptosis, apoptosis is regulated through one of two known cell death-signaling pathways: the extrinsic and intrinsic pathways. Both pathways share similar molecules (especially caspases) and features. The extrinsic pathway is initiated by external stimuli that are sensed by cell-death receptors on the cell membrane. Mitochondria play the central role in the intrinsic pathway by releasing molecules that trigger apoptosis (Budihardjo *et al.*, 1999; Green, 2000). Several studies have shown that HIV-1 Vpr induces the intrinsic pathway of apoptosis in a number of human cell lines and to promote apoptosis during HIV-1 infection. Not only does Vpr induce apoptosis in infected cells, but it has also been suggested that induction of apoptosis in uninfected bystander cells is caused by Vpr. However, neither HIV-2 nor simian immunodeficiency virus Vpr induces apoptosis in mammalian cell lines. Induction of apoptosis by several other HIV-1 proteins, including Nef, Vpu, Tat and Rev, has also been reported (Andersen *et al.*, 2008; Arokium *et al.*, 2009; Chang *et al.*, 2000; Conti *et al.*, 2000; Stewart *et al.*, 1999; Yedavalli *et al.*, 2005). While primary CD4+ T cells show high susceptibility to Vpr-inuced apoptosis, macrophages show a surprising resistance to HIV Vpr-induced apoptosis during infection and in non-virion associated forms *in vitro.* Therefor it has been suggested that Vpr may play a pivotal role in maintenance of macrophages as the reservires of HIV-1 (Thieu *et al.*, 2009).

Apoptosis studies indicate that mitochondrial intermembrane proteins, including adenine nucleotide translocator (ANT), apoptosis-inducing factor (AIF), cytochrome c, procaspases and heat-shock proteins (HSPs), are released during apoptosis and are essential for the activation of caspases and DNases. Several studies have demonstrated that HIV-1 Vpr permeabilizes the mitochondrial membrane by binding to the permeability transition pore complex (PTPC); lack of PTPC results in resistance to the apoptotic effect of Vpr. It has also been demonstrated that, in the presence of Vpr, cytochrome c is released from mitochondria, resulting in apoptosis. In fact, the mechanism responsible for mitochondrial-membrane permeabilization involves a number of proteins, including PTPC, proapoptotic members of the Bcl-2 family, ANT and the voltage dependent anion channel (VDAC) (Jacotot *et al.*, 2000).

The impact of Vpr on mitochondrial-membrane permeability is also attributed to its interaction with ANT in the inner mitochondrial membrane. It is believed that the interaction between Vpr and ANT cooperatively forms large conductance channels in the inner membrane. This suggests that the interactions of Vpr with PTPC and ANT lead to the formation of channels in the outer and inner mitochondrial membranes, respectively. These events in turn result in release of a number of molecules, such as cytochrome c, from mitochondria (Jacotot *et al.*, 2001; Yedavalli *et al.*, 2005). The released cytochrome c molecules bind to Apaf-1 (apoptotic peptidase-activating factor 1) and form apoptosome complexes that become activated by caspase 9. Activated apoptosome complexes trigger the caspase cascade and apoptosis (Figure 1.5) (Kim *et al.*, 2005; Muthumani *et al.*, 2002b; Zhu *et al.*, 2003).

It has been documented that a conserved motif in the C-terminal region of Vpr is responsible for the induction of apoptosis. Interestingly, the C-terminal peptides

containing the conserved sequence at AA 71–82 can even induce apoptosis. Extracellular addition of C-terminal peptides to human CD4+ T cells causes membrane permeabilization, DNA fragmentation and formation of apoptotic bodies, which are all signs of apoptosis. Similar effects can also be observed by adding the polypeptides to yeast, indicating that Vpr targets fundamental cellular pathways common to many eukaryotic cells (Arunagiri *et al.*, 1997).

The number of molecules that have been found to be involved in Vpr-induced apoptosis is still increasing. For example, HS1-associated protein X-1 (HAX-1) has also been demonstrated as a target for Vpr in the induction of apoptosis. HAX-1 is a proapoptotic factor that is found mainly in mitochondria. Overexpression of HAX-1 has been found to inhibit Vpr-induced apoptosis. These findings and others suggest that additional mechanisms and molecules may be utilized by Vpr for induction of apoptosis (Vafiadaki *et al.*, 2009; Vafiadaki *et al.*, 2006; Yedavalli *et al.*, 2005).

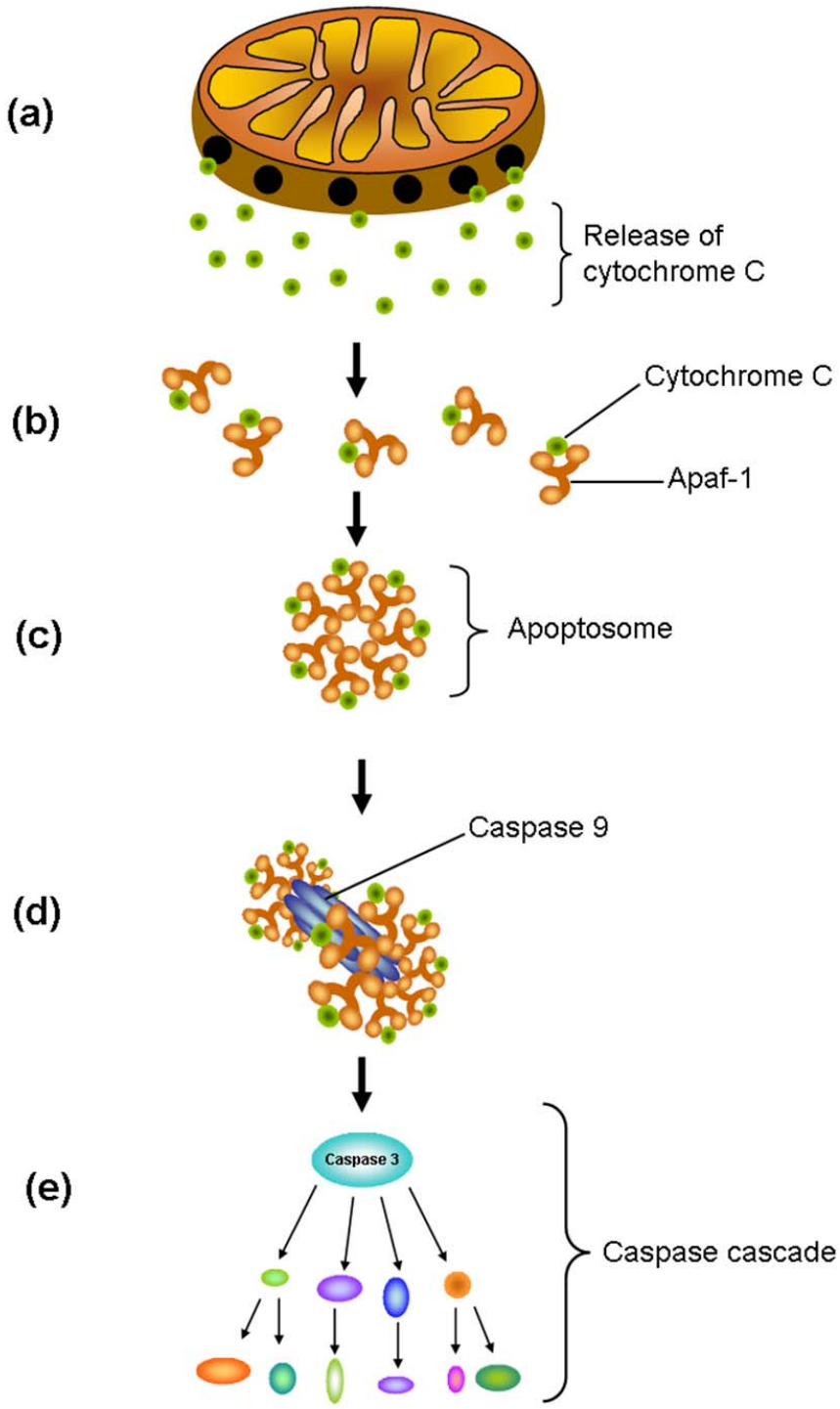


Figure 1.5: Induction of apoptosis through impact of Vpr on mitochondrial-membrane permeability and release of cytochrome *c*. (a) Vpr interaction with ANT and PTPC in mitochondrial membranes cooperatively forms large channels in the inner and outer mitochondrial membranes, respectively. Cytochrome c molecules are released from the channels. (b) The released cytochrome c molecules bind to Apaf-1 proteins and (c) apoptosome complexes are formed. (d) Caspase 9 activates apoptosomes; (e) once the apoptosome is activated; it triggers a caspase cascade, resulting in apoptosis (Romani & Engelbrecht, 2009).

The proapoptotic property of each form of Vpr (soluble, intracellular or virion-associated) has been studied. It is now clear that *de novo* expressed Vpr can induce apoptosis in various types of cells *in vitro* and *in vivo*. Recombinant Vpr has been indicated to induce apoptosis in neuronal cells, thus mimicking the potential *in vivo* effects of the extra-cellular soluble form of Vpr found in the plasma and cerebrospinal fluid of HIV infected patients. Recently, it has been shown that virion-associated Vpr is also able to trigger apoptosis. This form of Vpr induces apoptosis through caspases -3, -7 and -9 in human T cells independently of other HIV *de novo* expressed proteins. Vpr has been shown to activate caspase-8, the initiator caspase of the death receptor pathway. However, activation of caspases by virion-associated Vpr is independent of the Fas death receptor pathway. It has been suggested that virion-associated Vpr can contribute to the depletion of CD4+ lymphocytes either directly or by enhancing Fas mediated apoptosis during acute HIV-1 infection and in AIDS (Arokium *et al.*, 2009).

It is believed that Vpr-induced apoptosis contributes to the adverse effects of HIV-1 on neurons that occurs in HIV-1 infected individuals. Soluble Vpr protein is detectable in the CSF and serum of HIV-1-infected patients with neurological disorders. A number of studies have suggested that HIV-1 Vpr might be a potential toxic molecule mediating neuronal cell death during HIV-1 infection, albeit at high concentrations. *In vitro* studies of cultured rat hippocampal neurons, rat cortical and striatal neurons, or human neuronal cell lines have revealed that HIV-1 Vpr might cause neuronal apoptosis (Jones *et al.*, 2007).

1.2.4 Modulation of gene expression by Vpr

Although the first reported function for Vpr was transactivation of the virus long terminal repeat (LTR), the mechanism has not yet been elucidated completely. The ability of Vpr to induce G2 arrest has also been linked to transactivation of the LTR. Transactivation activity of the LTR during G2 is five to ten times higher than that during G1; therefore, the reproduction level of wild-type HIV-1 is five to ten times higher than that of Vpr-mutated viruses, indicating the importance of transactivation by Vpr (Zhu *et al.*, 2001). Indeed, Vpr transactivation activity is moderate as compared to HIV-1 Tat protein transactivation, which is stronger (Kino & Chrousos, 2004; Poon *et al.*, 2007). Vpr affects not only virus replication, but also cellular gene expression, proliferation and differentiation. As Vpr circulates in the blood of HIV-1-infected individuals, it may also affect the gene expression of non-infected cells (Balasubramanyam *et al.*, 2007; Xiao *et al.*, 2008).

Vpr-induced viral transcription in quiescent cells, such as macrophages, becomes more striking at low viral input states, which more closely approximates human infection. Vpr significantly increases the expression of unintegrated HIV-1 DNA that is the prevalent form of viral DNA in patients on highly active antiretroviral therapy. This can serve as latent reservoirs for HIV-1. These observations underscore the importance of Vpr in viral transcription and replication (Thieu *et al.*, 2009).

Vpr has been shown to function as a necessary factor in the primary infection of resting macrophages and to enhance viral reproduction following latency. The Vpr-induced transactivation of LTR is believed to play a pivotal role in reactivation of

latent resorvires. Since Vpr is present in the plasma of HIV-1-positive patients and it is necessary for infection of resting macrophages it is believd that Vpr could reactivate viral production from latency (Hoshino *et al.*, 2010).

The efficacy of transactivation of LTR apparently correlates with the Vpr-induced G2 arrest in host cells. It has been demonstrated that LTR activity is increased in the G2 phase of the cell cycle. Vpr mutants lacking the ability to induce G2 arrest fail to increase HIV-1 LTR expression. On the other hand, the HIV-1 and -2 strains lacking Vpr can increase LTR transcription through induction of G2 arrest via other means, thus suggesting that the ability of Vpr to prolong the G2 phase of infected cells is sufficient for LTR transactivation. It has been suggested that G2 arrest creates a cellular milieu which supports LTR activation, probably by allowing cell cycle transcription factors more time to exert their full effect on the HIV LTR (Thieu *et al.*, 2009).

Modulation of gene expression by Vpr has been shown to prevent establishment of chronic infections in T cells that could have minor effect on total virus burden. But it has been shown that Vpr-mediated transactivation of HIV-1 LTR in peripheral blood mononuclear cells results in viral gene transcription after infection. The major reservoir of infected cells *in vivo* contains latent rather than active proviruses. Therefore, the importance of Vpr in macrophages could ensure its continued selection *in vivo* (Rogel *et al.*, 1995; Vanitharani *et al.*, 2001).

As already mentioned, Vpr enhances the activity of the HIV-1 promoter located in the LTR. This region is also a target for the p53 and Sp1 transcription factors. The ability of Vpr to stimulate the HIV-1 promoter is affected negatively in the presence of p53.

Sp1 is a cellular transcription factor essential for the transcriptional activation of the LTR by Vpr (Pauls *et al.*, 2006; Sawaya *et al.*, 1998). Vpr specifically activates HIV-1 LTR-directed transcription on a minimal promoter containing a TATA box and the binding motifs for Sp1. Studies indicate that Vpr contains a leucine zipper-like domain, AA 60–81 in α-helix III, which interacts with Sp1 when it is bound to the Sp1 motifs on the HIV-1 LTR (Wang *et al.*, 1995; Wang *et al.*, 1996). It has been reported that Vpr cooperates with nuclear receptor coactivators p300/CBP and SRC-1a to activate the glucocorticoid-responsive mouse mammary tumor virus promoter (MMTV). Several studies have also reported that Vpr transactivates the HIV-1 LTR promoter by direct interaction with the p300 coactivator. It has been suggested that Vpr recruits p300/CBP to transactivate the HIV-1 LTR (Kino *et al.*, 2002).

Direct interaction between HIV-1 Vpr and the glucocorticoid receptor (GR) has also been reported to affect virus replication and gene expression. Human Vpr-interacting protein (hVIP) is also critical for the interaction of HIV-1 Vpr with GR. Glucocorticoids exert anti-inflammatory and immunosuppressive effects by interacting with their specific intracellular receptors, such as GR. Recent studies suggest that Vpr mimics some of the effects of glucocorticoids through interaction with GR (Ramanathan *et al.*, 2002). Vpr has also been suggested to bind to the LTR through interaction with GR. Vpr shares the LXXLL motif, aa 64-68, with steroid-receptor coactivators. This motif enables Vpr to bind to ligand-activated GR. These results suggest that Vpr functions as an adaptor molecule to connect the different molecules required for high promoter activity (Kino *et al.*, 2002; Shrivastav *et al.*, 2008).

HIV-1 Vpr has been demonstrated to bind specifically to the general transcription factor TFIIB. The interacting motif of TFIIB overlaps the domain of TFIIB that is involved in the intramolecular bridge between its N and C termini. Binding of Vpr may induce a conformational change in TFIIB that could possibly affect its activity (Agostini *et al.*, 1999; Agostini *et al.*, 1996).

Nuclear factor *κ*B (NF-*κ*B) is one of the main mediators of the HIV-1 LTR. The N-terminal region of NF-*κ*B mediates DNA binding, dimerization and interaction with inhibitory proteins. Activator protein 1 (AP-1) is also a transcription factor that has been suggested as a mediator for Vpr. Some studies have shown that the extracellular form of synthetic Vpr can stimulate the HIV-1 LTR via both AP-1 and NF-*κ*B activation. Extracellular Vpr is commonly found in the blood of HIV-positive individuals and may be involved in the stimulation of virus transactivation. Although no receptor has yet been identified for extracellular Vpr, studies using confocal microscopy indicate that it can enter cells easily, and it is believed that even this form of the protein enhances transcription of the promoters that interact with NF-*κ*B and AP-1 (Varin *et al.*, 2005).

Upregulation of human survivin at the transcriptional level by HIV-1 Vpr has also been reported. Human survivin is involved in inhibition of apoptosis and regulation of cell division. The survivin gene is regulated in a cell cycled-pendent manner. Vpr specifically upregulates survivin expression through a cell cycle-regulated mechanism. It is still not clear what elements on the survivin gene are used in survivin upregulation by Vpr (Zhu *et al.*, 2003).

The transactivation activity of Vpr is related to the *cis*-acting elements within the U3 region of the HIV-1 LTR, and the binding sites for several proteins, including Sp1, NF-κB, AP-1 and GRE, also lie in this region. The impact of Vpr on Nef expression from unintegrated HIV-1 has recently been demonstrated, suggesting that Vpr increases Nef expression before integration (Poon *et al.*, 2007; Varin *et al.*, 2005).

1.2.5 Role of Vpr in suppression of immune activation

Although many studies indicate that HIV-1 Env, Tat and Nef affect immune responses (Muthumani *et al.*, 2005), the immune suppression observed in HIV-1-infected patients is also partly attributed to Vpr. This protein suppresses antigen-specific CD8-mediated cytotoxic T-lymphocyte (CTL) and T-helper type 1 (Th1) immune responses. The molecular mechanisms of the suppression of CTL and antibody production by Vpr are still under debate, but it has been suggested that Vpr may prevent production of antibodies against the virus by inhibiting T-cell clonal expansion through inducing G2 arrest and suppressing T-cell proliferation. Since T cells stimulate B cells to produce antibodies, inhibition of T cell expansion may affect antibody production. Suppression of the host inflammatory responses by Vpr has also been reported. Vpr downregulates proinflammatory cytokines and chemokines, resulting in inhibition of the host inflammatory responses (Muthumani *et al.*, 2004a; Zhao *et al.*, 2005). A recent study also suggests that Vpr may alter sensitivity to insulin and thereby play a role in the development of lipodystrophy and insulin resistance (Shrivastav *et al.*, 2008).

In vitro studies have implicated the ability of Vpr to downregulate the expression of several immunologically important molecules on macrophages and dendritic cells

which are involved in stimulation of T cells, such as CD40, CD80, CD83 and CD86,. Vpr reduces the capacity of monocytes to mature to dendritic cells in tissue culture (Muthumani *et al.*, 2004b). A recent study indicates that Vpr impairs natural killer (NK) cell function *in vitro*. Production of gamma interferon is reduced in these impaired NK cells (Majumder *et al.*, 2008). It has also been shown that Vpr triggers natural killer cell-mediated lysis of infected cells through activation of the ATR-mediated DNA-damage response (Ward *et al.*, 2009).

Many of the experiments indicating that Vpr suppresses immune-cell activation and cytokine production have been performed *in vitro*. Vpr targets and suppresses NF-*κ*B activity, which is critical for T-cell activation and cytokine production. Similar results have been obtained from several *in vivo* studies (Ayyavoo *et al.*, 2002; Muthumani *et al.*, 2005). In a plasmid vaccine model using Vpr-expressing vectors, Vpr altered the induction of the CD8+ T-cell response. The mechanism of immune suppression could be complex and may be related to G2 arrest in T cells, suppression of NF-*κ*B, induction of apoptosis in T cells and the GR pathway. Even in the absence of the effect of Vpr on CD8+ T cells, the immune response is affected by targeting antigen-presenting cells and T-helper cells, indicating the multi-faceted nature of immune suppression by Vpr (Muthumani *et al.*, 2002a).

Another explanation for the possible mechanism of immune suppression by Vpr focuses on the GR pathway. As already mentioned, the direct interaction between Vpr and GR is well established. Glucocorticoids are known to control inflammation and to induce immunosuppressive signals by binding to GR (Andersen & Planelles, 2005). Interestingly, the GR–Vpr complex is also able to induce similar effects and is suppressed by GR-pathway inhibitors. Apparently, Vpr mimics the interaction of

glucocorticoids with GR to suppress immune activation (Moon & Yang, 2006; Muthumani *et al.*, 2006).

1.2.6 Induction of DNA double-strand breaks (DSBs) by Vpr

Vpr has been implicated to induce DSBs and increase gene amplification (Shimura *et al.*, 1999). Studies using fluorescence *in situ* hybridization of amplified DNA indicated that a bridge-breakage fusion cycle, a proposed mode of gene amplification triggered by DSBs, is involved in gene amplification induced by Vpr (Ishizaka *et al.*, 1995; Nakai-Murakami *et al.*, 2007). Vpr-induced DSBs have also been documented by a different approach using pulse-field gel electrophoresis (PFGE). Tachiwana *et al*. have shown that an altered migration pattern of high-molecular-weight DNA is observed in the genomic DNA prepared from HIV-1 infected cells. A similar electrophoresis pattern of DNA is also observed in cells treated with X-ray irradiation. Mutant virus lacking a functional Vpr induced slight DNA damage, suggesting that Vpr is a major viral component responsible for HIV-1-induced DSBs (Tachiwana *et al.*, 2006).

Clarifying the role DSB-sensing cellular signals in viral infection is important. It has been suggested that ataxia telangiectasia mutated (ATM), a serine/threonine-specific protein kinase, is involved in repairing the nucleotide gaps generated between host and viral DNAs. Without this repair, apoptosis would be triggered because the DNA gaps that could be recognized as DNA damage, causing viral infection to be abortive. NBS1 is a protein complex that plays essential roles in the human cellular DNA-damage response. NBS1 promotes viral transduction by associating with viral DNA and employing ATM to the integration site. In addition to ATM, ATR contributes to

the HIV-1 provirus integration during post-integration repair processes, but the recruitment of ATR proceeds independently of ATM or NBS1 (Smith *et al.*, 2008). Activation of ATM-dependent signal pathways by Vpr-induced DSBs results in the upregulation of the process which repairs the nucleotide gaps. It has been implicated that the role of ATM-dependent signaling is more prominent in resting macrophages because ATR is not expressed in resting macrophages. This confirms the proposed functions of Vpr, which is essential for HIV-1 infection of macrophages (Nakai-Murakami *et al.*, 2009).

In vitro studies provide several possible mechanisms by which Vpr may induce DSBs. Incubation of isolated nuclei with Vpr has induced DSBs. It has been shown that the highly conserved HFRIG motif present in the C-terminal region of Vpr is essential for G2 cell cycle arrest. It has also been suggested that the DNA binding activity of Vpr is linked with the induction of G2 cell cycle arrest. In contrast, it was reported that the nuclear localization of Vpr is not necessary for G2 arrest. Even Vpr mutants that localized in the cytoplasm are still able to induce G2 arrest, indicating that DNA-binding is required for the induction of DSBs but not for G2 arrest (Nakai-Murakami *et al.*, 2009). A number of studies have linked the mechanism of Vpr-induced ATR activation with impaired nucleotide excision repair. Vpr interferes with the cellular ubiquitin proteasome system by inhibiting the complex formation of factors such as DCAF1 and Cul4/DDB1 (Dehart *et al.*, 2007; Hrecka *et al.*, 2007; Schröfelbauer *et al.*, 2007).

HIV-1-infected patients suffer from a high risk for cancer development with Kaposi's sarcoma and non-Hodgkin's lymphoma being the major AIDS-related cancers (Biggar *et al.*, 1996). Vpr has been found in the serum of HIV infected patients, at a

concentration of approximately 0.7 nM (Hoshino *et al.*, 2007). Vpr has also been detected in patients who did not suffer from clinical immunodeficiency symptoms. *In vitro* experiments have shown that the physiological concentration of Vpr can induce DSBs, it seems logical to imagine that DSBs can be generated within HIV-1-positive patients. In addition, it has been observed that the frequency of aneuploidy in peripheral blood cells increased after infection with HIV-1. Recently, it has been proposed that Vpr induces DNA damage, mimicking the activity of the anticancer drug cisplatin. These observations strongly suggest that Vpr is positively involved in HIV-1-associated malignancies (Nakai-Murakami *et al.*, 2009).

1.2.7 Role of Vpr in fidelity of reverse transcription

HIV-1 RT is an error-prone enzyme that misincorporates approximately one nucleotide in every 2000–5000 polymerized nucleotides (Basavapathruni & Anderson, 2007; Li *et al.*, 1997). Reverse transcription of the HIV-1 genomic RNA is initiated in the virus core, where NC is associated with the RNA genome. The reverse-transcribed genome is directed toward the nucleus by the viral and cellular proteins forming the PIC (Gao *et al.*, 2007; Sun *et al.*, 1997). A number of studies have confirmed that Vpr colocalizes with IN and virus nucleic acids within the PIC, and remains associated with the viral DNA within 4–16 h after infection (Le-Rouzic & Benichou, 2005).

HIV-1 Vpr interacts with a cellular protein, uracil–DNA glycosylase 2 (UNG2), which is a DNA-repair enzyme involved in nucleotide-excision repair. Uracil can be introduced into DNA either by cytosine deamination or by misincorporation of dUTP (Chen *et al.*, 2004; Mansky *et al.*, 2000). If the uracil is not repaired by UNG2, after

the next round of replication, a CAT transition mutation occurs in that DNA strand and a GAA transition mutation occurs in the opposite strand of DNA. In the absence of Vpr, a fourfold increase in the rate of GAA mutations has been reported in each round of HIV-1 replication (Chen *et al.*, 2002). These GAA mutations are the main mutational force in the evolution of drug-resistant HIV-1 strains (Berkhout & de-Ronde, 2004). On the other hand, accumulation of these mutations can result in nonfunctional genomes (Keele *et al.*, 2008; Priet *et al.*, 2003). Vpr may play a role in virus evolution by balancing the level of mutations.

Several studies have established that the interaction of Vpr with UNG2 in order to incorporate it into HIV-1 virions correlates with the influence of Vpr on the HIV-1 mutation rate (Chen *et al.*, 2002). The interaction between Vpr and UNG2 has been demonstrated both *in vitro* and *ex vivo* in Vpr-expressing cells. Vpr has been found to incorporate the nuclear form of UNG2 into HIV-1 virions. HIV-1 IN may also participate in the virion incorporation of UNG2, but the role of Vpr in the virion incorporation of UNG2 seems to be more essential and is correlated with the ability of Vpr to alter the mutation rate of HIV-1 (Chen *et al.*, 2004; Mansky, 1996).

dUTPases are another class of enzymes that inhibit uracil incorporation into DNA during DNA synthesis, but utilize a mechanism distinct from that utilized by UNG2. Both UNG2 and dUTPase are encoded by some DNA viruses, such as poxviruses and herpes viruses. Some retroviruses encode only dUTPase. The genomes of non-primate lentiviruses, such as equine infectious anemia and feline immunodeficiency viruses, contain a dUTPase-encoding sequence as an integral part of the *pol* gene, lying between the RNase H and IN sequences. In contrast, the genomes of primate lentiviruses do not contain gene sequences encoding either dUTPase or UNG2. It is

believed that the interaction of HIV-1 Vpr with cellular UNG2 compensates for the lack of a viral dUTPase to correct uracil misincorporation into virus DNA (Bouhamdan *et al.*, 1996).

Vpr has been reported to interact through its N terminus with Lys–tRNA synthetase. This interaction has been observed both *in vitro* and *ex vivo*. Several cellular and viral proteins interact with Vpr and induce conformational changes that expose the N-terminal region of Vpr and thus regulate its affinity for Lys–tRNA synthetase. During the early stages of HIV replication, Vpr binds to NCp7, which may expose the N-terminal region of Vpr, resulting in a high affinity for Lys–tRNA synthetase. During the later stages of infection, the N-terminal region of Vpr interacts with the HIV-encoded p6, which may also affect Vpr interaction with the Lys–tRNA synthetase. As $tRNA_{Lys}$ acts as a primer for initiation of reverse transcription of the HIV-1 genome, it suggests that this mechanism is utilized by Vpr to potentiate HIV-1 reverse transcription (Stark & Hay, 1998).

1.2.8 Regulating RNA splicing by Vpr

Primary transcripts of eukaryotic genes usually contain exons that are interrupted by introns. Introns are removed from the pre-mRNA by splicing in the nucleus (Krämer, 1996). During splicing, recognition of splice sites is carried out through the stepwise assembly of spliceosomal complexes E, A, B, and C on the pre-mRNA (Hastings & Krainer, 2001). A complex pattern of alternative splicing generates more than 30 mRNA species from the primary mRNA transcript of the HIV-1 genome (Tang *et al.*, 1999). This sophisticated pattern of splicing is required to produce mRNAs for the viral proteins, and, undoubtedly, plays a critical role in the regulation of HIV-1 gene

expression. Viral mRNA species are classified into three groups. The 2kb-class of mRNAs, which are fully spliced and exported to the cytoplasm early in the HIV-1 replication cycle. The singly spliced 4kb-class of mRNAs, which are bicistronic, and encode Env, Vpr, Vif, and Vpu. The unspliced 9kb-class of mRNA, which encodes Gag and the Gag-Pol polyprotein, and also serves as the source of HIV-1 genomic RNA for packaging into HIV-1 virions (Zhang & Aida, 2009).

Regulating the expression of all viral genes is essential for viral replication and successful infection. Several suboptimal HIV-1 acceptor sites have been described, such as short and interrupted polypyrimidine tracts (O'Reilly *et al.*, 1995; Si *et al.*, 1997), and irregular branch point sequences (Damier *et al.*, 1997; Dyhr-Mikkelsen & Kjems, 2005). Furthermore, there is abundant evidence for the existence of several *cis*-acting elements within the HIV-1 genome such as the exonic splicing silencer (ESS), intronic splicing silencer (ISS), and exonic splicing enhancer (ESE) elements (Amendt *et al.*, 1995; Caputi *et al.*, 1999; Pongoski *et al.*, 2002; Tange *et al.*, 2001).

SAP145 is an essential component of SF3b, a key factor in spliceosome assembly. It has been shown that SAP145 is required for Vpr-induced inhibition of pre-mRNA splicing. In a recent report, Vpr was found to play a novel role as a regulator of pre-mRNA splicing both *in vivo* and *in vitro* (Hashizume *et al.*, 2007; Kuramitsu *et al.*, 2005). SAP145 binds to pre-mRNA and tethers the U2 snRNP to the branch point site, which is required for complex A assembly. Thus, in addition to the interaction between Vpr and SAP145, it was suggested that Vpr plays a role in splicing. Vpr has been shown to bind to RNA through its carboxy-terminal basic domain, which suggests the possibility of a functional association with the pre-mRNA. Vpr has been shown to inhibit the splicing of certain pre-mRNAs, such as the immunoglobulin

M pre-mRNA and the α/β-globin pre-mRNAs, and cause the accumulation of incompletely spliced forms. Because the studies showing inhibition of splicing are performed at a time in the viral life cycle when Vpr is not efficiently able to induce G2 arrest, these novel effects of Vpr must occur via a new pathway rather than arrest of the cell cycle at G2. Vpr expressed from an HIV-1 provirus is consistently sufficient to inhibit splicing of α-globin 2 pre-mRNA in infected cells (Zhang & Aida, 2009).

Vpr has also been shown to regulate Env expression by interfering with *env* pre-mRNA splicing. Vpr specifically inhibits the removal of the 3´ intron of HIV-1, but not the 5´ intron. This function of Vpr results in the accumulation of *env* mRNAs that belong to the singly spliced 4-kb class of mRNAs. Consequently, the expression of Env is elevated, which provides more Env proteins to be incorporated into the virion and maintains the high infectivity of the virus (Zhang & Aida, 2009).

It has been shown that Vpr interacts with SAP145, resulting in the inhibition of SAP145 binding to the splicing factor SAP49 which interacts specifically with both SAP145 and the pre-mRNA within the essential SF3b splicing factor complex. However, interfering with the association between Vpr, SAP145, and SAP49 may affect RNA processing, rather than leading to G2 arrest, since the R80A Vpr mutant can bind to SAP145 and inhibit pre-mRNA processing but fails to induce G2 arrest (Hashizume *et al.*, 2007). Vpr has been suggested to interfere with the assembly of the spliceosome, resulting in the inhibition of splicisome. Consistently, it has been demonstrated that Vpr binds to U1-70K, U2AF65, U2B, U1 and U2 snRNAs, as well as SAP145. These data suggest that Vpr associates with functional spliceosomes to regulate splicing (Zhang & Aida, 2009).

1.2.9 Virion incorporation of Vpr

Vpr is the major virion associated accessory protein of HIV-1. Since Vpr is not part of the p55 Gag polyprotein precursor (Pr55*gag*), its incorporation requires an anchor to associate with the assembling viral particles. Although the molecular mechanism is still unclear, the C-terminal region of the Pr55*gag* corresponding to the p6 domain appears to constitute such an anchor essential for the incorporation of Vpr into the newly assembling virions. Pr55*gag* has been implicated to physically interact with Vpr. Mutations affecting the integrity of the conserved LXSLFG motif of p6*gag* completely impair the interaction between Vpr and the Pr55*gag* and consequently prevent Vpr virion incorporation. Vpr and the Pr55*gag* demonstrate a strong interaction *in vitro* (Bachand *et al.*, 1999).

The karyophilic property of Vpr has raised the paradox why the nuclear localization of Vpr does not interfere with its virion incorporation. The answer can be found in the ability of Vpr to shuttle between the nucleus and cytoplasm. Vpr has been characterized as a nucleocytoplasmic shuttling protein which contains two novel nuclear import signals and an exportin-1-dependent nuclear export signal (NES). Mutations in this NES impair the incorporation of Vpr into newly formed virions. Vpr NES has been shown to be required for efficient HIV replication in tissue macrophages present in human spleens and tonsils. It is believed that this property of Vpr to shuttle between the nucleus and cytoplasm not only contributes to nuclear import of the HIV-1 PIC but also enables Vpr to be present in the cytoplasm for incorporation into virions, resulting in enhancement of viral spread within nondividing tissue macrophages (Sherman *et al.*, 2003b). Mutations impairing the

virion incorporation of Vpr have been reported in HIV long-term non-progressors, implying a link between efficient virion incorporation of Vpr and HIV disease progression (Caly *et al.*, 2008).

1.2.10 Additional interactions and possible functions of Vpr

HIV-1 Vpr has been observed to form ion channels in planar lipid bilayers, resulting in an inward sodium current followed by cell death in cell cultures. Mutagenesis studies have shown that the N-terminal region of Vpr is responsible for the above functions. A 40 AA peptide in the N-terminal region of Vpr is sufficient to form ion channels that are able to cause cell death. It is believed that this region is responsible for the ion-channel activity and cytotoxic effects of Vpr. These effects could be caused by the extracellular form of Vpr that is found in the serum and cerebrospinal fluid of AIDS patients (Piller *et al.*, 1999; Piller *et al.*, 1998). Extracellular Vpr can also disturb neuronal communication, resulting in neuronal dysfunction (Rom *et al.*, 2009). It also inhibits neuronal development through the induction of mitochondrial dysfunction (Kitayama *et al.*, 2008). Apparently, extracellular Vpr induces different levels of cytopathogenicity depending on the cell type (Huang *et al.*, 2000).

The interaction of HHR23A, a protein involved in nucleotide-excision repair, with HIV-1 Vpr has also been reported. It has been demonstrated that a C-terminal, 45 AA region of HHR23A binds to Vpr. Overexpression of HHR23A results in partial alleviation of the G2 arrest induced by Vpr. It has therefore been suggested that the interaction between Vpr and HHR23A could be involved in the G2 arrest induced by Vpr (Engler *et al.*, 2001; Withers-Ward *et al.*, 1997).

HIV-1 Vif expression has recently been implicated in the inhibition of Vpr-mediated G2 arrest, and deletion of Vif from the HIV-1 genome results in an increase in Vpr-induced G2 arrest. In addition, T cells infected with Vif-deleted HIV-1 express higher levels of Vpr than cells infected with the wild-type virus. It has been suggested that inhibition of Vpr by Vif is mediated by proteasomal degradation, similar to the other proteins that are directed toward proteasomal degradation by Vif (Wang *et al.*, 2008).

1.2.11 The reasons for the abundance of Vpr interactions

Interaction of a single protein with a variety of other different proteins may seem surprising. Some proteins, such as proteinases, chaperones and ubiquitin, interact with different proteins and direct them toward one pathway, e.g. proteinases only degrade proteins. The amazing property of Vpr is that this small polypeptide interacts with variety of proteins and directs them toward different pathways. Several hypotheses have been suggested to explain the capability of Vpr to exert so many effects through direct protein-protein interactions. One hypothesis suggests that Vpr possesses structural features similar to those of HSP70, a cellular chaperone, enabling Vpr to bind to many proteins with sufficient energy to cause changes in the activity of target proteins (Basanez & Zimmerberg, 2001).

In an attempt to find a common characteristic for the proteins that interact with Vpr, the WXXF motif (where X is unknown) was identified as an important criterion in some Vpr-interacting proteins. The WXXF motif of UNG2 was implicated in its interaction with Vpr. The N-terminal region of TFIIB, which binds specifically to Vpr, also contains the WXXF motif. Mutant forms of TFIIB that have a point mutation in the WXXF motif are not able to interact with Vpr. Interestingly, it was

demonstrated that, by attaching the WXXF motif to a non-human protein, chloramphenicol acetyltransferase, it was incorporated into HIV-1 virions in the presence of Vpr (Agostini *et al.*, 1999; Bouhamdan *et al.*, 1998; Yao *et al.*, 2002). Although the main functions of Vpr have been fairly well described and elucidated, there are still several interactions between Vpr and proteins of which the functions still need to be clarified.

1.2.12. Diversity of mutations in Vpr

Based on the phylogenetic analysis, HIV-1 has been classified into three groups; M, N, and O, with viral subtypes in each group (Salminen *et al.*, 1995). The genetic diversity of viral isolates has been documented throughout the genome, specifically, the *env* gene exhibits high variation. The variation observed in HIV-1 genes in the form of changes at the nucleotide level, should result in one of the possible scenarios: (1) no change in the amino acid, (2) conservative change in the amino acid, (3) nonconservative change in the amino acid, and (4) premature stop codon leading to a truncated protein (Coffin, 1995; Pang *et al.*, 1991). Therefor, it is likely that the variation may impact on the function of the protein, depending on the nature of the mutation. Several studies have provided a base for understanding the consequences of natural polymorphisms in specific regions of the Vpr molecule. However, it is also possible that other mutations (second site or compensatory mutations) may modify the effect of a specific mutation and a comprehensive analysis is needed to delineate the role of specific residues in Vpr protein. Tungaturthi *et al*. (2004a) have analyzed the sequences of HIV-1 Vpr of different subtypes available in genebanks and described the possible natural mutations that could be found in Vpr proteins. These mutations are listed in Table 1.1.

Table 1.1: Variant amino acid residues reported in HIV-1 Vpr of different subtypes (Tungaturthi *et al.*, 2004a).

Residue	Residue in NL4-3 Vpr	Variant residues reported in Vpr
1	M	no change
2	E	K
3	Q	R, L
4	A	P, S, T,V
5	P	L
6	E	A, D, G, Q
7	D	E, G, N
8	Q	A, H, R
9	G	R
10	P	S
11	Q	A, P
12	R	K
13	E	Q, V
14	P	no change
15	H	F, M, N, S, Y
16	N	A, H, I, Q, S, T
17	D	A, D, G, Q
18	W	no change
19	T	A, I, M
20	L	I, V
21	E	D, K, T
22	L	I, P, T, V
23	L	S, V
24	E	D, R
25	E	D, G, K
26	L	F, I
27	K	no change
28	N	A, E, H, I, Q, R, S, T
29	E	v
30	A	D, S
31	V	I
32	R	K
33	H	no change
34	F	Y, L
35	P	H
36	R	G, K, Q, W
37	I	D, E, G, H, L, M, P, Q, S, T, V
38	W	C, F, Y
39	L	no change
40	H	L, M, Q, Y
41	G	A, D, N, Q, S
42	L	C, I
43	G	E, R
44	Q	K, N
45	H	L, Y
46	I	V
47	Y	no change
48	E	A, D, H, N, S, T, Y
49	T	N
50	Y	H
51	G	E, K, R
52	D	no change

53	T	A, N, S
54	W	G, R
55	A	E, G, L, P, R, S, T, V
56	G	no change
57	V	A, L
58	E	G, I, K, M, Q, V
59	A	I, M, N, T, V
60	I	L, M, V
61	I	L, T, V
62	R	K
63	I	F, L, M, S, T
64	L	V
65	Q	no change
66	Q	L, R
67	L	I, M, Q
68	L	I, M, R
69	F	L
70	I	A, T, V
71	H	L, Y
72	F	Y
73	R	no change
74	I	no change
75	G	K, R
76	C	S, Y
77	R	H, L, Q
78	H	Y
79	S	N
80	R	no change
81	I	M
82	G	D, S
83	V	I
84	T	F, I, L, M, N, P, V, W
85	R	A, L, P, Q, T, V, Y
86	Q	G, R, S
87	R	G, M, S, T
88	R	E, G
89	A	E, G, I, P, R, S, T, V
90	R	G, N, S
91	N	D, K, S
92	G	A, R
93	A	F, G, M, P, S, T, V
94	S	E, F, G, N, R
95	R	A, K, P
96	S	F, P, V

Since the *vpr* gene has a conserved nature in primate lentiviruses, it has been suggested that the optimal function of Vpr is achieved by natural selection of naturally occurring Vpr mutants in the infected individuals (Macreadie *et al.*, 1995; Rouzic & Benichou, 2005; Tristem *et al.*, 1992). There are currently no data

pertaining to the functional consequences of naturally occurring mutations in Vpr, however it seems logical that naturally occurring mutations may differentially affect the functions of Vpr. It has been suggested that the mutations in Vpr represent amino acid residues with optimal functions selected *in vivo* and these mutations might be beneficial to Vpr (Tungaturthi *et al.*, 2004b). In addition to mutations, it has been shown that the subtype of Vpr also affects its functions such that the functions of Vpr are thought to be subtype-dependent. In several studies, the ability of Vpr to induce apoptosis has been compared for several subtypes and different levels of apoptosis induction have been observed (Bano *et al.*, 2009b; Jian & Zhao, 2003a). However, these studies have not sufficiently addressed different functions of subtype C Vpr. It is important to study all functions of Vpr, as well as other HIV-1 proteins, that have been established for reference sequences of Vpr as the wild-type protein. It has been shown that subtype D Vpr, for example, has no detectable apoptotic activity (Jian & Zhao, 2003a). Comparative studies of some of the HIV-1 proteins, such as Vif, Vpu, Tat, Rev, and envelope proteins, among HIV-1 subtypes have revealed slight to significant variations in a number of their functions that are subtype-dependent (Campbell *et al.*, 2007; Gupta & Benerjea, 2009; Neogi *et al.*, 2009; Sood *et al.*, 2008; Sundaravaradan *et al.*, 2007). Therefore, the established functions of Vpr cannot directly be attributed to the Vpr protein of all or other HIV-1 subtypes without experimentally confirming and measuring those functions.

1.3 Aim of this study

Most studies addressing the functionality of HIV-1 accessory proteins focus on subtype B. The functionality of subtype C Vpr, especially South African strains, has

not been studied. However, the functions shown in subtype B cannot be attributed to other subtypes without evaluating those functions independently.

The overall objective / major aim of this study was to investigate the diversity of South African HIV-1 subtype C *vpr* genes and selected functions of these Vpr proteins.

This was achieved by setting the following secondary objectives:

1. To investigate the sequence diversity of the *vpr* gene and the Vpr protein in South African HIV-1 strains by PCR amplification, sequencing and phylogenetic analysis
2. To identify the natural mutations common in these *vpr* genes and to design vectors expressing these Vpr proteins
3. To construct a consensus subtype C *vpr* gene, identify conserved amino acids in the coded protein and to induce mutations by site-directed mutagenesis
4. To transfect human cell lines with either the natural or mutated constructs and to investigate the functionality of these Vpr constructs on subcellular localization, apoptosis induction and cell cycle G2 arrest.
5. To further investigate the modulation of genes expressed in the induction of apoptosis using TaqMan Low density arrays (TLDA).

Chapter 2

2. Materials and methods

2.1 Overview of the methodology

The diversity of *vpr* gene of South African HIV-1 strains was studied using the direct sequencing of PCR-amplified products. The phylogenetic analysis was performed on the sequences. Selected sequences that contained natural mutations in different domains were cloned in eucaryotic expression vectors. Alternatively a eukaryotic expression vector was designed for the consensus sequence of subtype C Vpr and mutations were induced in different domains of Vpr. 293T cells were individually transfected with all the vectors. Subcellular localization, Induction of apoptosis, induction of cell cycle G2 arrest, and gene expression were studied for the natural and artificial mutants in the transfected cells. The diagram of the study is shown in Figure 2.1.

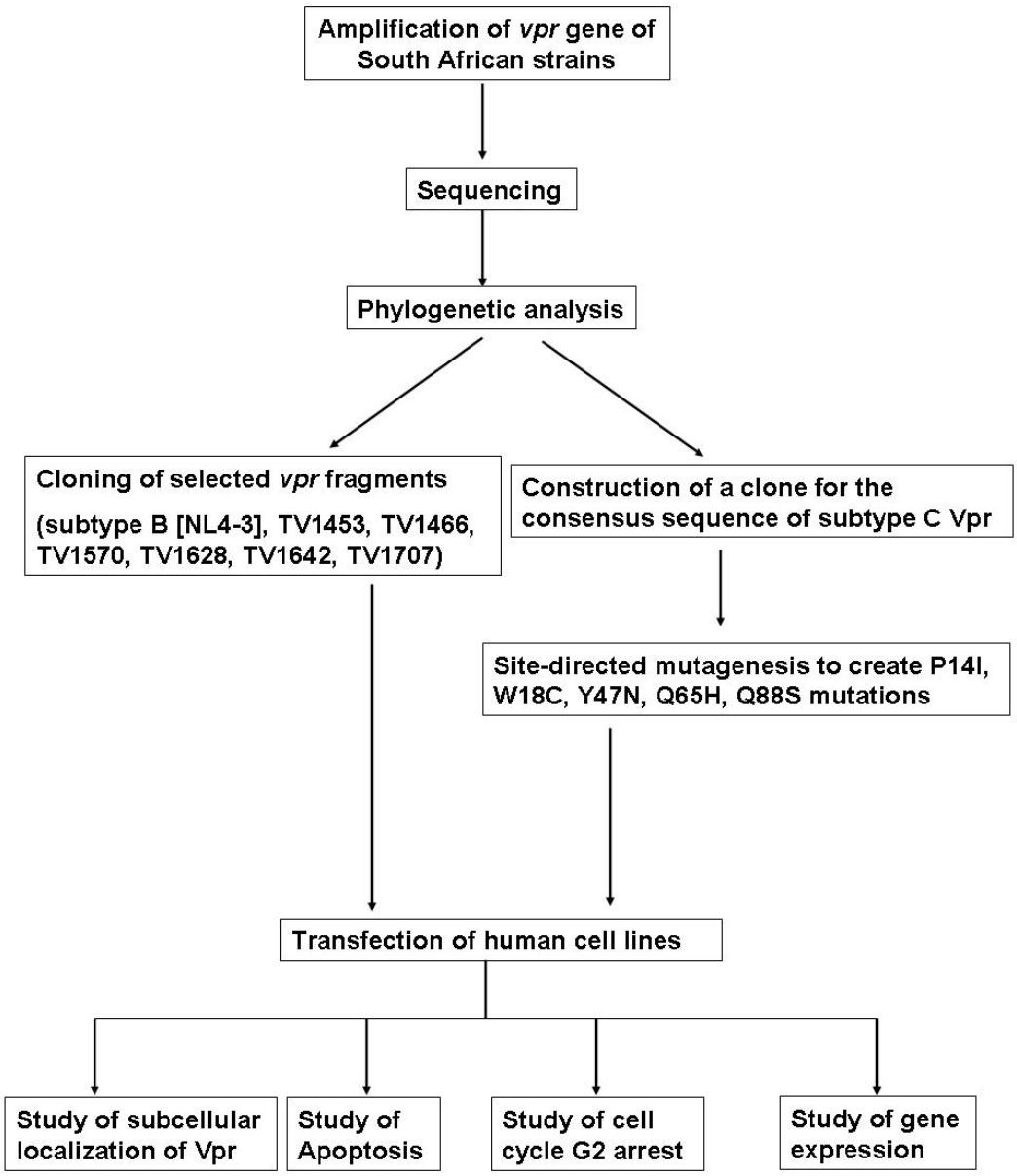


Figure 2.1: Diagram of the study design

2.2. Samples

This project was approved by the Human Research Ethics Committee (HREC) of the University of Stellenbosch on 07 February 2008. The project number is N08/01/014 (Appendix A). Plasma samples were collected from 100 HIV-1-positive patients in

the Cape Town metropole during 2002–2004. Plasma samples were stored at -80 until the viral RNA was extracted.

2.3. Molecular characterization of the *vpr* gene and Phylogenetic analysis

2.3.1. RNA extraction

Approximately 3 ml plasma was stored from 20 ml EDTA blood samples by separating it from the red blood cells, white blood cells and blood platelets. The plasma was collected after centrifugation (Beckman Coulter Allagra™ 6R, Beckman Inc., Fullerton, California, USA) at 700 × g for 10 minutes at 4ºC. The samples were stored at -80ºC for subsequent analysis. RNA was extracted from 1 ml plasma using the QIAamp® Ultrasens Virus protocol (Qiagen GmbH, Hilden, Germany). The kit protocol was followed and RNA eluted in 50 µl of low salt buffer AVE containing 0.04% sodium azide. Briefly, nucleic acids bind specifically to the QIAamp silica-gel membrane while contaminants pass through. PCR inhibitors such as divalent cations and proteins are removed in two wash steps, leaving pure nucleic acids to be eluted in either water or a buffer provided with the kit. QIAamp technology yields viral RNA and DNA from cell-free body fluids ready to use in PCR.

2.3.2. The polymerase chain reaction (PCR) for the HIV-1 *vpr* gene

Reverse transcriptase (RT) polymerase chain reaction (PCR) amplification of a 1361-bp region (HXB2 positions 4985-6346) was performed using the Access-RT PCR kit (Promega, Madison, Wiskinson, USA). The PCR mixtures were prepared in 0.2 ml

thin wall PCR® tubes (QSP, Porex BioProducts Inc., California, USA) and reactions were carried out on the GeneAMP® 9700 PCR system (Applied Biosystem, Foster City, California, USA).

For RT-PCR the primers NACC (5′-AGATAATAGTGACATAAAAGTAGTGCCAAGAAG-3′) and NACD (5′-CCATAATAGACTGTGACCCACAA-3′) were used (Zhang *et al.*, 1997). Access RT-PCR kit (Promega, Madison, Wiskinson, USA) was used with 5 µl of RNA, 200 mM of each nucleotide, 40 µmol of each primer, 1mM $MgSO_4$, 5 units each of AMV RT and Tfl DNA polymerase, and AMV/Tfl buffer in a total volume of 50 µl. After reverse transcription of 45 min at 48ºC, the reaction was held at 94ºC for 2 min, followed by 40 cycles of denaturing the DNA (94ºC; 30 s), annealing of primers (50ºC; 30 s), and extension of the annealed primers (68ºC; 2.5 min). This was followed by a final extension step of 10 min at 68ºC and the PCR product was kept at 4ºC.

The RT-PCR was followed by DNA amplification of a 714-bp region (HXB2 positions 5276–5990) using the primers HIV-vif-1F (50-GGAATTTGGGTCATGGAGTCTCCATA-30) and HIVvpr-R1 (50-GTCTCCGCTTCTTCCTGCCATAG-30), with the Promega GoTaq Flexi Kit (Promega, Madison, WI, USA). Template DNA for the nested PCR consisted of 3 µl of reaction product from the first round with 200 mM of each nucleotide, 20 µmol of each primer and 1.5mM $MgCl_2$, and 2.5 units of Taq polymerase and GoTaq buffer made up in a total volume of 50 µl. The amplification reaction was held at 94ºC for 2 min, followed by 40 cycles of denaturing the DNA (94ºC; 30 s), annealing of primers

(56ºC; 30 s), and extension of the annealed primers (68ºC; 1 min). This was followed by a final extension step of 10 min at 68ºC and the PCR product was kept at 4ºC.

The products of the nested PCR were visualized using agarose gel electrophoresis on a 0.8% agarose (Whitehead Scientific, Cape Town, South Africa) gel in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) and stained with ethidium bromide (final concentration of 0.5ug/ml), (Promega, Madison, WI, USA).

The agarose gel was run at 50 V for 50-60 minutes. The DNA bands were visualized under an ultra violet light at a wavelength of 320 nm and photographed with Syngene GeneGenius computer system (Synoptics Ltd, Cambridge, United Kingdom).

2.3.3. DNA sequencing

The PCR products were purified for sequencing. The enzymes Exonuclease 1 (*Exo*1) and Shrimp alkaline phosphatase (SAP) (Amersham Pharmacia Biotech, New Jersey, USA) were used according to the manufacturer's instructions. These enzymes are used to degrade single-strand DNA (such as excess primers) and diphosphates that might interfere with the sequencing reactions (Werle *et al.*, 1994). Ten µl of the PCR products was purified with 0.5 µl of *Exo*1 (1 unit per µl) and 0.5 µl of SAP (1 unit per µl). An incubation of 15 minutes at 37ºC followed by an enzyme inactivation step of 15 minutes at 80ºC was carried out on a Techne Gene E Thermal Cycler (Techne Ltd., Cambridge, United Kingdom) The purified aliquot was stored at -20ºC until the sequencing reactions were carried out. The DNA concentration of the PCR products was measured using the Nanodrop^{TM} ND-1000 system (Nanodrop Technologies Inc., Delaware, USA), which only requires 1-2 µl of sample input.

All purified PCR products were sequenced on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA). To perform the sequencing reactions, 4 µl of Big Dye terminator enzyme mix was added to 10 µl of purified products, approximately 50 ng DNA. Five pmol of primer, either HIV-vif-1F or HIV-vpr-R1 was added to the reaction mix. The reaction volume was brought to 20 µl by adding Nuclease-Free Water (Promega, Madison, WI, USA). The following cycle sequencing reaction was applied to the samples: denaturation at 96ºC for 10 seconds, primer annealing at 55ºC for 5 seconds, and an elongation step at 60ºC for 4 minutes. The cycles were repeated 25 times after which the samples were incubated at 4 ºC. After the sequencing reactions were accomplished, the reactions were loaded in MicroAmp® 96 well trays (Applied Biosystems, Foster City, California, USA) until they were analyzed using an ABI Prism 3130xl automated DNA sequencer (Applied Biosystems, Foster City, California, USA).

2.3.4. Sequence analysis

The ABI chromatograms of the forward and reverse primers were analyzed and overlapping fragments were assembled using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI).

2.3.5. Extracting *vpr* gene sequences from chromatograms

The positive and negative strand of DNA sequences are usually sequenced by a pair of forward and reverse primers to ensure that the string of sequences being analyzed is correct. The chromatograms of the forward and reverse primers were analyzed and

overlapping fragments were assembled using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI). Using Clustal X version 1.81 the DNA sequences were primarily aligned with HIV-1 HXB2 reference sequence (B.FR.83.HXB2) to detect the exact position of the *vpr* gene in our sequences. The *vpr* gene was manually extracted from all of the sequences.

2.3.6. Multiple alignment and phylogenetic analysis of the sequences

The *vpr* fragments of all of the DNA sequences were aligned with HIV-1 reference sequences obtained from the LANL HIV Database (http://hiv-web.lanl.gov), using Clustal X version 1.81 (Thompson *et al.*, 1997). Clustal X creates an optimal alignment and performs a global-multiple sequence alignment by the progressive method. It performs pair-wise alignment of all the sequences by dynamic programming. Multiple alignments were verified manually in BioEdit version 7.090 (Hall, 1999). The subset of the reference sequences used in the phylogenetic analysis is presented in Table 2.1.

A neighbor-joining phylogenetic tree (Saitou & Nei, 1987) was constructed with MEGA version 4 (Tamura *et al.*, 2007) using the Kimura two-parameter (Kimura, 1980). The reliability of the branching and clustering pattern was estimated from 1000 bootstrap replicates. The tree was rooted with the YBF30 reference sequence of HIV-1 group N.

To investigate the Vpr protein, the *vpr* gene sequences were translated into amino acids and the amino acid sequences were aligned with the consensus subtype C and HXB2 reference sequences using BioEdit version 7.0.9.0 (Hall, 1999). The α-helical

domains of Vpr were shaded using GeneDoc version 2.6.003 (Nicholas & Nicholas, 1997).

Table 2.1: HIV-1 subtype reference sequences used in the phylogenetic analysis

Name	Accession number	Origin of sample	Year of sampling	Subtype	Reference
Q23_17	AF004885	Kenya	1994	A1	(Neilson *et al.*, 1999)
RW008	AB253421	Rwanda	1992	A1	(Ngandu *et al.*, 2008)
CDKS10	AF286241	DRC	1997	A2	(Gao *et al.*, 2001)
CY017	AF286237	Cyprus	1994	A2	(Gao *et al.*, 2001)
HXB2	K03455	France	1983	B	(Ratner *et al.*, 1985)
BK132	AY173951	Thailand	1990	B	(Hierholzer *et al.*, 2002)
1058	AY331295	USA	1998	B	(Bernardin *et al.*, 2005)
15384	DQ853463	USA	1998	B	(Liu *et al.*, 2006)
BR025	U52953	Brazil	1992	C	(Gao *et al.*, 1996)
ETH2220	U46016	Ethiopia	1986	C	(Salminen *et al.*, 1996)
IN21068	AF067155	India	1995	C	(Lole *et al.*, 1999)
SK164B1	AY772699	Slovakia	2004	C	(Rousseau *et al.*, 2006)
01CM	AY371157	Cameron	2001	D	(Kijak *et al.*, 2004)
A280	AY253311	Tanzania	2001	D	(Arroyo *et al.*, 2004)
UG114	U88824	Uganda	1994	D	(Gao *et al.*, 1998)
VI850	AF077336	Belgium	1993	F1	(Laukkanen *et al.*, 2000)
MP411	AJ249238	France	1996	F1	(Triques *et al.*, 2000)
MP257	AJ249237	Cameron	1995	F2	(Triques *et al.*, 2000)
CM53657	AF377956	Cameron	1997	F2	(Carr *et al.*, 2001)
DRCBL	AF084936	Belgium	1996	G	(Oelrichs *et al.*, 1999)
HH8793	AF061641	Kenya	1993	G	(Salminen *et al.*, 1993)
PT2695	AY612637	Portugal	1995	G	(Ngandu *et al.*, 2008)
VI991	AF190127	Belgium	1993	H	(Janssens *et al.*, 2000)
VI997	AF190128	Belgium	1993	H	(Janssens *et al.*, 2000)
056	AF005496	Central African Republic	1990	H	(Gao *et al.*, 1998)
J_97DC	EF614151	DRC	1997	J	(Abecasis *et al.*, 2007)

SE7022	AF082395	Sweden	1994	J	(Laukkanen *et al.*, 1999)
EQTB11C	AJ249235	DRC	1997	K	(Triques *et al.*, 2000)
MP535	AJ249239	Cameron	1996	K	(Triques *et al.*, 2000)
YBF30	AJ006022	Cameron	1995	Group N	(Simon *et al.*, 1998)

2.4. Gene cloning and mutagenesis

2.4.1. Cloning of *vpr* gene – selection of samples and control

The most similar protein sequence to the consensus sequence of HIV-1 Vpr (LANL HIV Database, 2004) was identified by comparing the protein sequences translated from *vpr* gene sequences. This subtype C sequence, designated as TV1453, and 5 additional sequences (TV1628, TV1707, TV1642, TV1570, TV1466) containing naturally occurring mutations were selected for cloning experiments. The sequences were selected that contained natural mutations distributed in different domains of Vpr. Although there were other mutants that could have been chosen for the study, only a certain number of sequences had to chosen so that they could be regarded as the representative sequences of South African strains. The following sections describe the methods used to clone the *vpr* sequences and to produce the consensus sequence of Vpr.

The *vpr* gene from HIV-1 subtype B, strain pNL4-3 (NIH AIDS Research and Reagent Program, Maryland, USA), was also included in this study as a control.

2.4.2. Primer design for gene cloning

pF4A CMVd1 Flexi® Vector (Promega, Madison, Wisconsin, USA) was used as the eukaryotic expression vector. Protein-coding regions are cloned into Flexi® Vectors containing *Sgf* I and *Pme* I sites. All Flexi® Vectors carry the lethal barnase gene, which is replaced by the DNA fragment of interest that acts as a positive selection for successful ligation of the insert. Inserts can be transferred to other Flexi® Vectors following digestion with *Sgf* I and *Pme* I, which maintains insert orientation and reading frame, eliminating the need to resequence the insert after each transfer. To insert *vpr* fragments into the flexi vector, two rare-cutting restriction enzymes, *Sgf* I and *Pme* I (Promega, Madison, Wisconsin, USA), have to be appended to the protein coding regions (*vpr* fragments).

A pair of primers containing the restriction site at both sides of the *vpr* gene was designed using the online software Flexi Vector Primer Design (http://www.promega.com/techserv/tools/flexivectortool/default. aspx). The following primers were designed using the software:

Vpr-Sgf-F:

5′-GGGTGCGATCGCCATGGAACAAGCCCCAGAAGAC-3′

Vpr-Pme-R:

5′-CTTAGTTTAAACTTAGGATCTACTGGCTCCATTTCTTG-3′

The melting temperature of the primers was calculated using the online software BioMath Calculators (http://www.promega.com/biomath/).

2.4.3. Appending restriction sites to *vpr* fragments using PCR reaction

The 291 bp ORF of *vpr* gene from the samples TV1453, TV1628, TV1707, TV1642, TV1570, TV1466, and the pNL4-3 control was amplified using the primers Vpr-Sgf-F and Vpr-Pme-R and the GoTaq Flexi Kit (Promega, Madison, Wisconsin, USA). The PCR reaction consisted of 3 µl of reaction product with 200 mM of each nucleotide, 20 µmol of each primer, 1.5mM MgCl2, and 2.5 units of Taq polymerase and GoTaq buffer made up in a total volume of 50 µl. The amplification reaction was held at 94ºC for 2 min, followed by 5 cycles of denaturing the DNA (94ºC; 30 s), annealing of primers (52ºC; 30 s), and extension of the annealed primers (68ºC; 40 sec). The reaction was followed by 35 cycles of denaturing the DNA (94ºC; 30 s), annealing of primers (56ºC; 30 s), and extension of the annealed primers (68ºC; 40 sec). This was followed by a final extension step of 5 min at 68ºC and the PCR products were kept at 4ºC.

The products of the PCR were visualized using agarose gel electrophoresis on a 1% agarose as described in section 2.3.2. The Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA) was used to purify the PCR products as recommended by the manufacturer. The eluted DNA were stored at 4°C.

2.4.4. Digestion of the PCR products and the acceptor vector

pF4A CMVd1 Flexi® Vector (Promega, Madison, Wisconsin, USA) was used in this study to clone the PCR products. This vector has been designed for constitutive native (untagged) protein expression in mammalian cells using the human cytomegalovirus

(CMV) intermediate-early enhancer/promoter. The vector can be used for both stable and transient gene expression. The vector contains ampicillin resistance for selection in *E. coli*. Digestion reactions for PCR products and the acceptor Flexi® Vector can be performed concurrently. The 5X Flexi® Digest Buffer, pF4A CMVd1 Flexi® Vector and nuclease-free water were thawed and store on ice. The reaction components for digesting both the PCR products and the acceptor flexi vector with *Sgf* I and *Pme* I were separately assembled according to the protocol.

Both reactions were incubated at 37°C for 30 minutes. The reaction with the Flexi® Vector was heated at 65°C for 20 minutes to inactivate the restriction enzymes and stored on ice until the PCR product and vector were ligated. Twenty µl of Membrane Binding Solution was to the reactions with the PCR products. All reactions were stored at 4°C until they were purified as described in section 2.4.3.

2.4.5. Ligation of PCR products and acceptor vector

The ligation reactions were performed in 0.2 µl tubes. The DNA concentration of the purified PCR products was measured using Nanodrop™ ND-1000 system (Nanodrop Technologies Inc, Delaware, USA). To ligate the PCR products to pF4A CMVd1 Flexi® Vector as the acceptor vector, 5 µl of the vector (50 ng) was mixed with 100 ng DNA of the PCR products. One µl of T4 DNA Ligase (Promega, Madison, Wisconsin, USA) and 10 µl of 2X Flexi Ligase Buffer (Promega, Madison, Wisconsin, USA) were added to the reaction. The final volume of the reaction was brought to 20 µl by adding nuclease-free water (Promega, Madison, Wisconsin, USA). The reactions were incubated at room temperature for 1 hour.

2.4.6. Transformation of pF4A CMVd1 Flexi® Vectors containing the *vpr* gene

pF4A CMVd1 Flexi® Vector carries a gene for ampicillin resistance therefore selection for recombinants was performed on LB plates supplemented with 100µg/ml ampicillin. Two µl of each ligation reaction was added to a sterile 14 ml polypropylene round-bottom tube (Becton Dickinson, Franklin Lakes, USA) on ice. The tubes of frozen high-efficiency JM109 Competent Cells (Promega, Madison, Wisconsin, USA) were removed from 70°C storage and placed on ice until thawed. The cells were mixed by gently flicking the tube. Fifty µl of cells was transferred into each 14 ml polypropylene containing the ligated DNA. The tubes were placed on ice for 20 minutes. The cells were heat-shocked for 45-50 seconds in Haake L water bath with Haake D1 heating circulator (LabX, California, USA) at exactly 42°C. The tubes were immediately returned to ice for 2 minutes and 950µl of room-temperature SOC Medium (Sigma, Saint-Louis, USA) was added to each tube. The tubes were incubated for 1.5 hours at 37°C with shaking (150 rpm). After incubation, 20-50µl of each transformation was plated onto duplicate plates containing 100µg/ml ampicillin and incubated overnight at 37°C.

2.4.7. Screening the colonies for the *vpr* gene

In our study, since a sticky end restriction enzyme and a blunt-end enzyme were used, a high efficiency of the right colonies was expected. Nine colonies of each plate were chosen for screening. After overnight incubation 9 colonies were picked and cultured in 5 ml LB broth containing 100µg/ml ampicillin. The LB broth tubes were incubated for 16 hours at 37ºC and 150 rpm in shaking incubator Labcon-3081U (Labmark,

Johannesburg, South Africa). Longer incubation could damage the plasmids and shorter incubation may not yield enough plasmid DNA.

After incubation, 850 µl of the LB broth containing the bacteria was pipetted into 1.5 microcentrifuge tubes and mixed with 150 µl of glycerol and stored at -20ºC. The plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the protocol. This kit is designed for purification of up to 20 µg of high-copy plasmid DNA from 1-5 ml overnight cultures of *E. coli* in LB medium. All the protocol steps were carried out at room temperature. The plasmids were extracted from 3 ml of the bacterial cultures and stored at 4ºC until they were analyzed for the integration of the *vpr* gene. The plasmid DNA concentration was measured using Nanodrop^{TM} ND-1000 system (Nanodrop Technologies Inc., Delaware, USA).

Restriction enzyme digestion was used to identify recombinant plasmids. Briefly, a master mix was prepared by combining 0.5 µl of 10X Flexi Enzyme Blend (Promega, Madison, Wisconsin, USA), 4 µl of 5X Flexi Digest Buffer and 10.5 µl Nuclease-Free Water. Fifteen µl of master mix was added to 5µl (200-500ng) of each plasmid DNA and incubated for 2 hours at 37°C. Five µl of Blue/Orange Loading Dye (Promega, Madison, Wisconsin, USA) was mixed with the reactions and incubated at 65°C for 10 minutes. The whole reaction (20µl) was loaded onto a 0.8% agarose gel to separate fragments by electrophoresis as described in section 2.3.2. The fragments were visualized by ethidium bromide staining.

Recombinant plasmids were sequenced as described before (section 2.3.3). All the sequences were compared to the sequence of insert source and the identical sequences were chosen for the future experiments.

2.4.8. Constructing a consensus sequence of HIV-1 subtype C Vpr

The clone of TV1453, pF4A-TV1453, contained the most similar sequence to the consensus sequence of HIV-1 subtype C Vpr (LANL HIV Database, 2004) except for 1 mutation at position 45. At this position the original tyrosine in the consensus sequence of HIV-1 Vpr was mutated to histamine in TV1453 Vpr. A pair of primers was designed to mutate histamine 45 to tyrosine 45 using QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA). Site-directed mutagenesis allows site-specific mutation in virtually any double-stranded plasmid. The QuikChange® Lightning Site-Directed Mutagenesis Kit includes a derivative of *PfuUltra*® high-fidelity (HF) DNA polymerase for mutagenic primer-directed replication of both plasmid strands with the highest fidelity. The basic procedure utilizes a vector with an insert of interest and two synthetic primers, both containing the desired mutation (Figure 2.2).

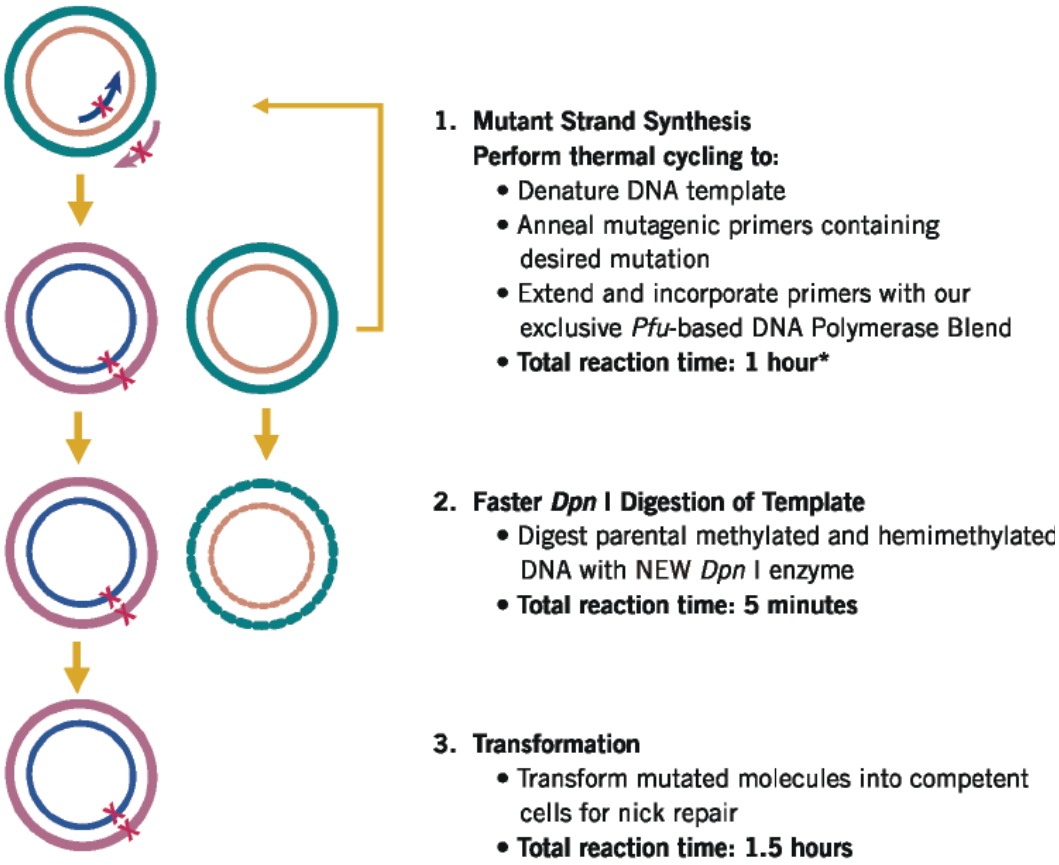


Figure 2.2: An overview of site-directed mutagenesis (http://www.promega.com)

The mutagenic primers for site-directed mutagenesis were designed individually according to the desired mutations. The mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid. The primers should be between 25 and 45 bases in length, with a melting temperature (Tm) of ≥78°C. The primer wild-mut-F: 5′-CATAGCTTAGGACAATACATCTATGAAAC-3′ and wild-mut-R: 5′-GTTTCATAGATGTATTGTCCTAAGCTATG-3′ were designed by taking all the necessary considerations into account.

The sample reaction was prepared by adding 5 µl of 10× reaction buffer to a 0.2 ml thin wall PCR® tubes (QSP, Porex BioProducts Inc., California, USA). One µl of

dNTP mix and 100 ng of the pF4A-TV1453 DNA were added to the reaction. One hundred twenty five ng of the primer wild-mut-F and wild-mut-R were added to the reaction. 1.5 µl of QuikSolution reagent was added and the volume was brought to final volume of 50 µl by adding Nuclease-Free Water (Promega, Madison, Wisconsin, USA). One µl of QuikChange® Lightning Enzyme (included in the kit) was added to the reaction. The PCR reaction was carried out on the GeneAMP® 9700 PCR system (Applied Biosystems, Foster City, California, USA) The amplification reaction was held at 95ºC for 2 min, followed by 18 cycles of denaturing the DNA (95ºC; 20 seconds), annealing of primers (55ºC; 30 seconds), and extension of the annealed primers (68ºC; 140 seconds, which corresponds to 30 seconds/kb of plasmid length). This was followed by a final extension step of 5 min at 68ºC and the PCR product was kept at 4ºC.

The PCR reaction contained the parental non-mutated plasmids and the desired mutated plasmids. To obtain the desired mutated plasmids, the parental plasmids had to be removed from the reaction. Briefly, 2 µl of the *Dpn* I restriction enzyme was directly added to the amplification reaction and then immediately incubated at 37°C for 5 minutes. The treated plasmid was transformed into XL10-Gold ultracompetent cells (Stratagene, La Jolla, USA) using a method described in section 2.4.6 except for the heat-shock (42°C for 30 seconds, instead of 45-50 seconds). The bacteria were incubated overnight at 37ºC.

2.4.9. Screening for the consensus sequence of HIV-1 Vpr

The expected colony number using QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA), depends upon the base composition and

length of the DNA template employed. Four colonies were cultured in LB broth for 16 hours at 37ºC and 150 rpm in shaking incubator Labcon-3081U (Labmark, Johannesburg, South Africa). The plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the protocol. The *vpr* fragment of the plasmids was sequenced using Vpr-Sgf-F and Vpr-Pme-R as described in section 2.3.3. All the sequences were compared to the sequence of TV1453 *vpr* to verify if the plasmids contain the desired mutation. One of the sequences containing the desired mutation that did not contain extra mutations in other positions was chosen as the consensus sequence of HIV-1 subtype C Vpr for the future experiments. This plasmid was named pF4A-Cons-C.

2.4.10. Mutating Vpr in different domains

To investigate the role of Vpr domains in different functions of the protein, pF4A-Cons-C was separately mutated in 5 highly conserved domains. In the other word, 5 different vectors were created to express 5 Vpr proteins, each one carrying 1 mutation. QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) was used to induce mutations in the expression vector (pF4A-Cons-C) for the consensus sequence of HIV-1 Vpr subtype C. The method was similar to the one described in the section 2.4.8 to induce mutations except for the primers. Five pairs of primers were manually designed to mutate the *vpr* gene of pF4A-Cons-C. The mutation positions were selected because they have never been described. As already shown in tabele 1.1, no natural mutation has been described at position 14, 18, 47, and 65. The impact of possible mutations at these positions is unknown and in this study these positions were addressed. Position 88 was also included in mutagenesis study

because it is highly conserved and the impact of mutations at this position has not been described.

The mutagenic primers were designed to mutate the original amino acids to one with different properties. For example, if the original amino acid was a polar acidic amino acid, it was mutated to a nonpolar basic amino acid. The mutagenic primers, the newly created vectors, the original mutations and the induced mutations are listed in Table 2.2. After site-directed mutagenesis, the plasmids were extracted using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) according to the protocol. The *vpr* fragment of the plasmids was sequenced according to the protocol describe in section 2.3.3.

Table 2.2: Mutagenic primers and induced mutations.

Primer (5´ to 3´)	**Original AA**	**Induced mutation**	**Vpr mutated residue**	**New vector**
P14I-F: GCAGAGGGAAATATACAATGAATGG	Proline	Isoleucine	14	pF4A-P14I
P14I-R: CCATTCATTGTATATTTCCCTCTGC				
W18C-F: CCATACAATGAATGCACACTAGAGC	Tryptophan	Cysteine	18	pF4A-W18C
W18C-R: GCTCTAGTGTGCATTCATTGTATGG				
Y47N-F: GGACAATACATCAATGAAACCTATGG	Tyrosine	Asparagine	47	pF4A-Y47N
Y47N-R: CCATAGGTTTCATTGATGTATTGTCC				
Q65H-F: GAATTCTGCACCAACTACTGTTTATTC	Glutamine	Histidine	65	pF4A-Q65H
Q65H-R: GAATAAACAGTAGTTGGTGCAGAATTC				
Q88S-F: CTTGCGACAGAGAAGTGCAAGAAATGGAG	Glutamine	Serine	88	pF4A-Q88S
Q88S-R: CTCCATTTCTTGCACTTCTCTGTCGCAAG				

2.4.11. Protein modelling

Protein modelling was used to demonstrate the position of the natural and induced mutations in the 3-dimensional structure of Vpr. The sequences of *vpr* mutants were translated into protein sequences using BioEdit version 7.090 (Hall, 1999). The proteins sequences were analyzed using the online software SWISS-MODEL workspace. The software utilizes homology modelling to build three-dimensional protein structure models using experimentally determined structures of related family members as templates. SWISS-MODEL workspace is an integrated Web-based modelling system. For a given target protein, a library of experimental protein structures is searched to identify suitable templates. On the basis of a sequence alignment between the target protein and the template structure, a three-dimensional model for the target protein is generated. Model quality assessment tools are used to estimate the reliability of the resulting models.

The 3-dimensional pictures obtained from SWISS-MODEL workspace were further analyzed using Discovery Studio Visualizer v2.0.1.7347 (Goede *et al.*, 2005). The Vpr domains were shown in different colours and the position of the mutations and the mutated amino acids were shown and annotated using the software.

2.4.12. Transfer of protein-coding regions between the Flexi® Vectors

As described, all the *vpr* fragments were cloned into the pF4A CMVd1 Flexi® Vector for expression of Vpr proteins in mammalian cells. This form of Vpr could be suitable for experiments and assays when there is no need to directly track the protein in cells. But in some of our experimentsVpr needed to be tracked in apoptotic cells or cell

cycle G2 arrested cells. A common method to track proteins in cells is tagging a protein reporter to the protein of interest. This reporter can be detected by producing fluorescent signals (e.g. green fluorescent protein) or visible light signals (e.g. luciferase). In this study, HaloTag® Technology (Promega, Madison, Wisconsin, USA) was utilized to track Vpr proteins. The HaloTag® reporter protein is an engineered, catalytically inactive derivative of a hydrolase that forms a covalent bond with HaloTag® ligands. Under physiological conditions this covalent bond forms rapidly and is highly specific and essentially irreversible, yielding a complex that is stable even under stringent conditions (Lang *et al.*, 2006; Los, 2005; Los & Wood, 2007).

To tag a reporter to the expressed Vpr protein, pFN22K CMVd1 Flexi® Vector (Promega, Madison, Wisconsin, USA) was used for cloning. The vector contains a kanamycin resistance gene that enables us to select it on a kanamycin-selective medium after transfer of protein-coding regions between Flexi® Vectors, while the donor vector (pF4A CMVd1 Flexi® Vector) cannot survive.

As described, the Vpr-coding region was cloned in pF4A CMVd1 Flexi® Vector. The purified clones including, pF4A-TV1453, pF4A-TV1628, pF4A-TV1707, pF4A-TV1642, pF4A-TV1570, pF4A-TV1466, pF4A-P14I, pF4A-W18C, pF4A-Y47N, pF4A-Q65H, pF4A-Q88S, and pF4A-Cons-C were used as donors of the Vpr-coding region to pFN22K CMVd1 Flexi® Vector. The reaction was started by digestion of the Vpr-coding regions from the donors and barnase gene from the acceptors (Figure 2.3). Briefly, 4 µl of 5X Flexi® Digest Buffer was separately mixed with 100 ng of each donor vector in 0.2 ml thin wall PCR® tubes (QSP, Porex BioProducts Inc., California, USA). One µl (100 ng DNA) of the acceptor vector and 2 µl of the Flexi®

Enzyme Blend (Promega, Madison, Wisconsin, USA) were added to the reactions. The Final volume was brought to 20 µl by adding Nuclease-Free Water (Promega, Madison, WI, USA). The reactions were incubated at 37ºC for 30 minutes and heated at 65°C for 20 minutes to inactivate the restriction enzymes. All the incubations were carried out on the GeneAMP® 9700 PCR system (Applied Biosystems, Foster City, California, USA). Ten µl of 2X Flexi Ligase Buffer was added to 10 µl of each digested DNA in 0.2 ml thin wall PCR® tubes (QSP, Porex BioProducts Inc., California, USA). One µl of T4 DNA Ligase (20 U/µl), (Promega, Madison, Wisconsin, USA) was added to the reactions and the incubated for 1 hour at room temperature. The ligation reactions were transformed into JM109 Competent Cells (Promega, Madison, Wisconsin, USA), as described in section 2.4.6. Bacteria were cultured on LB plates containing 25µg/ml kanamycin to select clones. The recombinant plasmids were extracted using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) according to the protocol. The integration of Vpr-coding regions was verified by PCR using the primer Vpr-Sgf-F and Vpr-Pme-R.

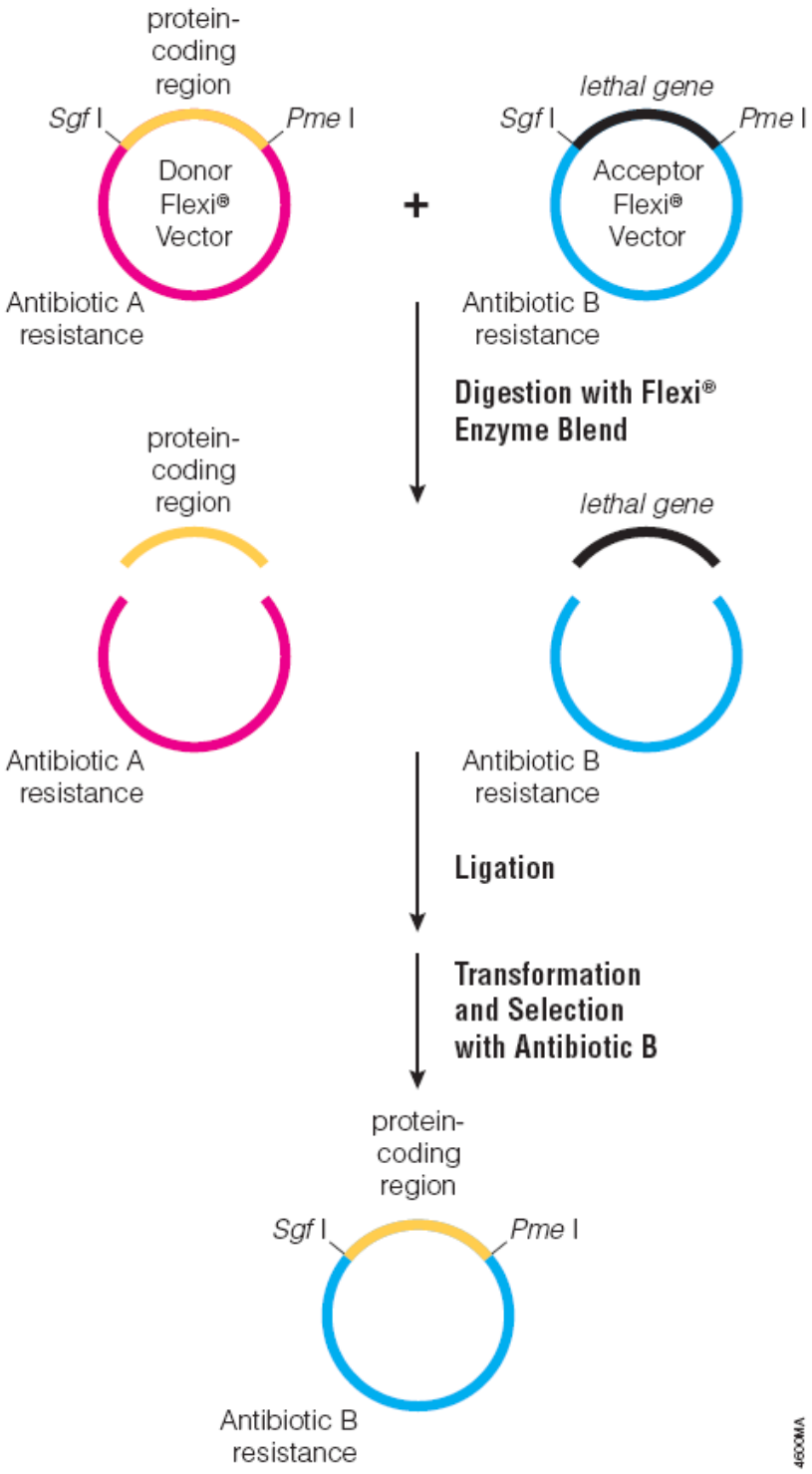


Figure 2.3: Transfer of a protein-coding region between N-terminal or native Flexi® Vectors. The donor Flexi® Vector containing the protein-coding region is mixed with an acceptor Flexi® Vector that has a different antibiotic resistance. The two plasmids are digested with *Sgf* I and *Pme* I, and the mixture is ligated and transformed into *E. coli*. The cells are plated on the appropriate selective media for the acceptor Flexi® Vector (http://www.promega.com).

2.4.13. Construction of control transfection vectors

The intact Flexi® Vectors cannot be used for transfection because of the presence of the lethal gene, barnase. To have control vectors for the experiments, barnase had to be removed from the original vectors used for cloning. The Flexi® Vectors provide the option of expressing the HaloTag® coding region either as an N-terminal or a C-terminal partner of the fusion protein. In the N-terminal vectors, as the one used in this study, the fusion protein is cloned downstream of the HaloTag® coding region, replacing the lethal gene, barnase. Hence there is no stop codon in the HaloTag® sequence. To express only the HaloTag® protein, or a vector without any coding region, it is necessary to both remove the barnase gene and introduce a stop codon for the HaloTag® coding region. This can be done by designing and annealing oligonucleotides that contain a stop codon (e.g., TAA) and replace barnase in the vector. This results in the expression of HaloTag® protein without cell death. A schematic of the HaloTag®-only control expression construct created using an oligo-based cloning strategy is illustrated in Figure 2.4. This construct has all the elements to serve as a HaloTag®-only expression control. In this example, the oligos have also been designed to recreate the SgfI and PmeI restriction sites. Thus, the construct could also be used as a cloning vector for future fusion expression projects requiring N-terminal HaloTag® fusions.

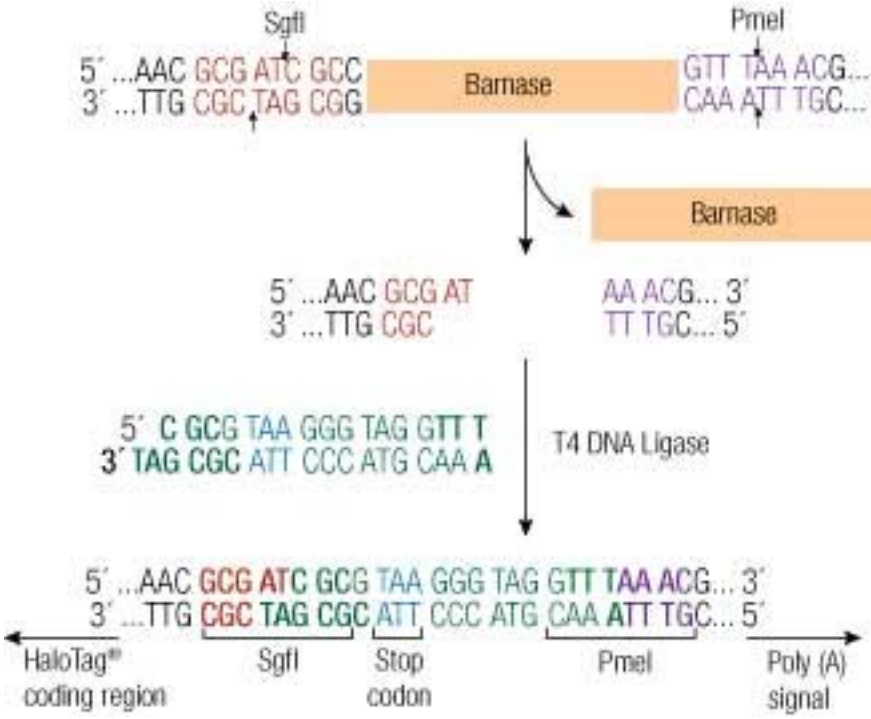


Figure 2.4: Schematic of the HaloTag® control expression construct from N-terminal Flexi® Vectors using the oligo-design strategy. N-terminal HaloTag® vectors contain the lethal barnase gene downstream of the HaloTag® coding region. The control constructs can be made by designing oligos that carry a stop codon and ends compatible for ligation. The vector is digested with *SgfI* and *PmeI* to remove the barnase gene. In a separate reaction, the oligos are annealed. The digested vector and the annealed oligos are ligated using T4 DNA Ligase (http://www.promega.com).

To construct the control vectors, 1 µl of pF4A CMVd1 Flexi® Vector and 1 µl of pFN22K CMVd1 Flexi® Vector were separately added to two 0.2 ml thin wall tubes. Four µl of Flexi® Digest Buffer and 2 µl of Flexi® Enzyme Blend (*Sgf* I and *Pme* I) were added to each tube. The final volume was brought to 20 µl by adding Nuclease-Free Water. The reactions were incubated the reaction at 37°C for 20 minutes and then heated at 65°C for 20 minutes to inactivate the restriction enzymes. The reactions were stored on ice until the ligation reaction.

In a separate reaction, 50 fmol/µl of a pair of oligo nucleotides, Flexi-strand-F: 5´-CGCGTAAGGGTAGGTTT-3´ and Flexi-strand-R: 5´-AAACCTACCCTTACGCGAT-3´ that contained the restriction site for the Flexi Enzyme Blend® were heated to 95ºC, then slowly cooled to room temperature for 15-30 minutes. The annealed oligonucleotides then had to be integrated into the control vectors to replace the barnase gene. The ligation reaction was assembled according to manufacturer's suggestions. The reaction was incubated at room temperature for 1 hour. One to two µl of the ligation reaction was transferred into high-efficiency, JM109 Competent Cells as described in section 2.4.6.

The potential control vectors were digested using Flexi Enzyme Blend® according to the manufactures protocol. The digested reactions and the intact vectors were run on 0.8% agarose gel to confirm the deletion of barnase in the control vectors.

2.5. Cell culture

2.5.1. Cell lines

Two different cell lines, 293T and HeLa cell line were used in this study. Human 293T cell line is highly tranfectable and supports the expression of high levels of viral proteins. 293T cell line has been derived from human embryonic kidney 293 cells, also often referred to as HEK 293, 293 cells, or HEK cells. The 293T cell line was

kindly provided by Professor Lynn Morris (National Institute for Communicable Diseases (NICD), Sandringham, South Africa).

The HeLa cell line [American type culture collection (ATCC) strain CCL-2] was used as an alternative cell line for transfection.

2.5.2. Cell culture maintenance

293T and HeLa cell lines were removed from the nitrogen freezer and transferred to the Labotec^{TM} Biological Safety Cabinet Class II (Labotec, Göttingen, Germany). All cell culture experiments were performed in safety cabinets to protect the cell cultures from contamination. To remove DMSO, the cells were resuspended in 10 ml of DMEM (Lonza, Basel, Switzerland) and centrifuged at 700 × g for 5 minutes. The wash was repeated and the cell pellets were resuspended in 10 ml of DMEM supplemented with 10% fetal bovine serum (FBS) (Lonza, Basel, Switzerland), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Lonza, Basel, Switzerland). The cells were seeded in 25 cm^2 Cell Culture Flasks (Corning, New York, USA) and incubated at 37ºC supplemented with 5% CO_2 in NUAIRE^{TM} US Autoflow CO_2 Water-Jacketed Incubator (Nuaire, Minnesota, USA). The medium was refreshed with complete DMEM medium after 48 hours.

The cell cultures were maintained during the study by regularly subculturing the cells in fresh medium. To obtain and maintain easily transfectable cells, regularly when the cells had 100% confluency, 5×10^5 - 1×10^6 cells were passaged to new 25 cm^2 Cell Culture Flasks (Corning, New York, USA) containing 7-10 ml of complete DMEM

medium. Briefly, all medium was removed from the flask and the cells were washed once with 10 ml PBS (Lonza, Basel, Switzerland) to remove excess medium and serum. Serum contains inhibitors of trypsin. Five ml of trypsin/versene (EDTA) solution (Lonza, Basel, Switzerland) was added to the monolayer and incubated 1 to 5 minutes at room temperature until cells detached. The cells were observed under a microscope to confirm that most of the cells have detached. Five ml of complete medium was added to the cells and the cell suspension was transferred to a sterile 15 ml High-Clarity Polypropylene Conical Tube (Becton-Dickenson, New jersey, USA). The cell suspension was centrifuged at 700 × g for 5 minutes. The supernatant was removed and the wash was repeated in 5-10 ml DMEM complete medium. The pellet was resuspended in 5ml DMEM complete medium.

To transfer 5×10^5 - 1×10^6 viable cells to a cell culture flask, the viable and total cell counts had to be determined. The cell suspension was mixed to assure an even distribution of the cells. Fifty µl of the cell suspension was mixed with the equal volume of 0.4% Trypan Blue Stain (Invitrogen, New Jersey, USA). The mix was transferred to the Neubauer Improved Bright-Line (Marienfeld Superior, Lauda-Königshofen, Germany). The viable cells (unstained cells) were counted using Eclipse E200 Microscope (Nikon, MelVille, USA). Using the following formula, the number of the viable cells per milliliter was calculated:

$$\frac{\text{Number of viable cells}}{\text{Number of squares}} \times 10^4 \times \text{dilution factor} = \text{Number of cells/ml}$$

Between 5×10^5 - 1×10^6 viable cells were transferred to a new 25 cm^2 Cell Culture Flask (Corning, New York, USA) and brought to 7-10 ml by adding fresh complete

DMEM to the flask and placed in the incubator. As already described, the cells were incubated in NUAIRE™ US Autoflow CO_2 Water-Jacketed Incubator (Nuaire, Minnesota, USA).

2.5.3. Mycoplasma screening

Mycoplasma contamination of continuous cell lines is a major problem in biological research using cultured cells. The cells were screened using RIDASCREEN® Mycoplasma IFA (R-Biopharm AG, Darmstadt, Germany) for Mycoplasma contamination. Briefly, 20,000 cells were placed in a volume of 20 to 30 µl into the well area on a coated glass microscope slide and the sample was dried at 50ºC for 45 minutes. The samples were fixed for 60 seconds by adding cold 70% ethanol. After fixation the slides were air-dried at room temperature. One drop of the Anti-Micoplasma-Conjugate was added to the fixed cells. The slides were carefully rinsed with phosphate buffered saline and washed twice for a total of 2 minutes in a bath of phosphate buffered saline. The slides were incubated at room temperature to dry. One drop of Mounting Fluid was added in the center of each well and a coverslip was placed on it. The slides were observed under fluorescent microscope.

2.6. Transfection optimization

2.6.1. Transfection control vector

Because the success of the rest of the experiments was highly dependent on transfection efficiency, it was decided to optimize the transfection method. The pSV-

β-Galactosidase Control Vector (Promega, Madison, Wisconsin, USA) was used in this study to optimize transfection.

The pSV-β-Galactosidase Control Vector vector was transformed into JM109 cells (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol and the transformed bacteria were cultured on 2 plates containing 100µg /ml penicillin. The plates were incubated at 37ºC for 24 hours and two colonies were inoculated in 2 flasks containing 100 ml of LB broth and 100µg/ml penicillin. The flasks were incubated for 16 hours at 37ºC and 150 rpm. The vector DNA was extracted using QIAgen plasmid Maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's suggestion. Eight µl of the purified vectors were run on 0.8% ethidium bromide/agarose gel to test the size and the quality of the DNA. The quantity of the vector DNA was measured using Nanodrop™ ND-1000 system (Nanodrop Technologies Inc, Delaware, USA).

2.6.2. Transfection

Transfection efficiency varies in different cell lines depending on the transfection method. To find the best combination of cell lines and transfection method 2 cell lines, including HeLa and 293T cells were used with TransFast™ Transfection Reagent (Promega, Madison, Wisconsin, USA) and FuGENE® 6 to optimize transfection.

Before transfection, 50,000 cells of each 293T and HeLa cell lines were separately seeded into 24 well cell culture plates (Corning, New York, USA). When the cells had 50-80% confluency, usually after 24 hours, they were transfected with pSV-β-

Galactosidase Control Vector. For transfection using TransFast™ Transfection Reagent, Hela and 293T cells were transfected with different DNA concentrations ranging from 250ng to 1µg according to the Promega protocol for transfection. For transfection using FuGENE® 6, Hela and 293T cells were transfected with different FuGENE® 6 Reagent:DNA ratios of 3:1, 3:2, and 6:1 (µl, for FuGENE® 6 Reagent, and µg for DNA, respectively) according to the protocol. Since it has been reported that antibiotics may have inhibitory effects on the efficiency of transfection with FuGENE® 6 (Jacobsen *et al.*, 2004), the experiments were performed in the presence or absence of antibiotics normally used in cell culture.

2.6.3. Testing transfection efficiency

Twenty four hours after transfection, the cells were fixed by replacing the medium with an equal volume of warm 4% paraformaldehhyde/0.2M sucrose/1×PBS and incubating the cells for 10 minutes at room temperature. The fixative was then replaced with an equal volume of 1×PBS/0.1% Triton X-100 and incubated for 10 minutes at room temperature. The solution was then replaced with an equal volume of 1× PBS.

The fixed cells were stained using β-Gal Staining Set (Roche, Berlin, Germany). Briefly, the PBS in each well was replaced with 0.5 ml staining solution and incubated for 1 hour at 37ºC in NUAIRE™ US Autoflow CO_2 Water-Jacketed Incubator (Nuaire, Minnesota, USA). The cells were checked for color development using the Eclipse E200 Microscope (Nikon, Melville, USA). The settings and the cell line that had the most efficient transfection were selected as our transfection setting for the future experiments.

2.7. Subcellular localization of Vpr

2.7.1. Cell labeling and Microscopic analysis

The cell line showing the most efficient transfection, 293T cells, was used to study the cellular localization of Vpr. To transfect 293T cells, 5×10^5 cells were seeded into 8-Well Lab-Tek II Chamber Slides (Fisher Scientific, Pennsylvania, USA). When the cells had 50-80% confluency, usually 24 hour after seeding, the cells were transfected with all HaloTag-Vpr expression vectors. 293T cells were individually transfected with HaloTag-Vpr expression vectors and HaloTag-only vector using FuGENE® 6 Reagent: DNA ratios of 6:1 according to the protocol. The cells in one well remained non-transfected as the negative control.

Twenty four hours after transfection, the 293T cells were labeled with HaloTag® TMR Ligand (Promega, Madison, Wisconsin, USA). Briefly, a 1:200 dilution of HaloTag® TMR Ligand was prepared in warm culture medium just prior to addition to cells. This was a 5X working stock solution. The cells were labeled by replacing one-fifth of the existing volume of medium with the 5X HaloTag® TMR ligand working solution, and mixed gently. The cells were incubated for 15 minutes at 37 °C in NUAIRE™ US Autoflow CO_2 Water-Jacketed Incubator (Nuaire, Minnesota, USA). The ligand-containing medium was replaced with an equal (or greater) volume of fresh DMEM complete medium. The wash was repeated twice, for a total of three complete rinses. The cells were incubated at 37 °C for 30 minutes to wash out unbound ligand. The medium was replaced with an equal volume of PBS.

The cells were then fixed to prevent any disturbance in Vpr localization due to the labeling treatments. Time is also another factor that can affect localization of Vpr due to the overexpression of Vpr and also apoptosis induction by Vpr if the sample preparing takes long. Cell fixation solves all of these problems and provides the free access of fluorescent dyes to cellular compartments (Kozubek *et al.*, 2000). Briefly, the 293T cells were fixed by replacing the PBS with an equal volume of warm 4%paraformaldehhyde/0.2M sucrose/1×PBS and incubating the cells for 10 minutes at room temperature. The fixative was then replaced with an equal volume of 1×PBS/0.1% Triton X-100 and incubated for 10 minutes at room temperature. The solution was then replaced with an equal volume of 1× PBS.

Since Vpr primarily trends to localize in the nucleus, the nuclei were also labelled using DAPI (4'-6-Diamidino-2-phenylindole) (Sigma, Saint-Louis, USA) to confirm the nuclear localization or cytoplasmic mislocalization of Vpr proteins. DAPI is a fluorescent dye that is excited with UV. When bound to double-stranded DNA, its absorption maximum is at 358 nm and its emission maximum is at 461 nm (Du *et al.*, 1998). To label the nuclei, a 900nM DAPI solution was prepared in 1X PBS as the working solution. The PBS in each well was replaced with 300 µl of the DAPI working solution and the cells were incubated for 3 minutes at room temperature. The cells were washed with PBS two times. The cells were observed and photographed under the Olympus IX 81 motorized inverted microscope (Olympus, Pennsylvania, USA) that was equipped with MT20 Illumination System and CellR software.

In the cases that mislocalization of Vpr was observed, to ensure the mislocalization is not due to the overexpression of the protein, the experiments was repeated as described but the transfected cells were labelled after 10, 20 and 30 hours.

2.8. Induction of apoptosis by Vpr

2.8.1. Reagents and optimization of flow cytometry

To apply a reliable method for the assay of Vpr-induced apoptosis, the flow cytometry had to be optimized for the fluorescent dyes. In this study, p-HaloTag-Cons-C was used as control to optimize the method. p-HaloTag-Cons-C is a HaloTag-Vpr expression vector that expresses the consensus sequence of HIV-1 Vpr subtype C. Using this vector, Vpr could be detected in transfected cells using the HaloTag® TMR Ligand (Promega, Madison, Wisconsin, USA) that labels HaloTag-Vpr. Using this vector we were able to assay apoptosis in all cells (transfected and non-transfected cells) and in transfected cells separately.

Camptothecin (Sigma, Saint-Louis, USA) was used to induce apoptosis for optimization of flow cytometry. Camptothecin is a strong apoptosis inducer that is widely used to induce and study apoptosis (Hsiang *et al.*, 1985; Wall *et al.*, 1966). Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, California, USA) was used in this study to assay apoptosis. Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for phospholipid phosphatidylserine (PS), and binds to cells with exposed PS. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane PS is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment.

To optimize the method and flow cytometry for apoptosis assays, 5×10^4 293T cells were seeded into 8 wells of a 24 well cell culture plate (Corning, New York, USA). After 24 hours, the 293T cells were transfected in 3 wells with p-HaloTag-Cons-C using FuGENE® 6 Reagent:DNA ratios of 6:1 according to the protocol. The 293T cells in 3 wells were treated with camptothecin (5µl final concentration) for 24 hours. Twenty four hours after transfection, the cells were harvested by washing the cells with PBS and treating with 2 mM EDTA/PBS for 1 minute. To assay apoptosis using membrane factors, such as phosphatidyl serine, adherent cells cannot be trypsinated, instead, they should be gently treated using chelating chemicals such as EDTA. The cells were labeled with HaloTag® TMR ligand (according to protocol), Annexin V FITC (according to protocol), neither or both as shown in Table 2.3. The cell suspensions were analyzed using BD FACSCalibur™ (Becton Dickinson, California, USA). The flow cytometry was set for apoptosis assay by adjusting the plots, compensation, voltage, and signal intensity.

Table 2.3: Treatments of 293T cells

Wells	Treatment	Labeling with Annexin-V FITC	Labeling with HaloTag® TMR
Well 1	Transfection with p-HaloTag-Cons-C	Yes	No
Well 2	Transfection with p-HaloTag-Cons-C	No	Yes
Well 3	Transfection with p-HaloTag-Cons-C	Yes	Yes
Well 4	5 µM Camptothecin for 24 hrs	Yes	No
Well 5	5 µM Camptothecin for 24 hrs	No	Yes
Well 6	5 µM Camptothecin for 24 hrs	Yes	Yes
Well 7	No treatment	Yes	Yes
Well 8	No treatment	No	No

2.8.2. Apoptosis assay

5×10^5 293T cells were seeded into a 24 well cell culture plate (Corning, New York, USA). When the cells had 50-80% confluency, they were transfected with all the HaloTag-Vpr expression vectors using FuGENE® 6 Transfection Reagent (Roche, Berlin, Germany) according to the protocol (the vectors were described in section 2.4.12). Twenty four hours after transfection, the cells were labeled using HaloTag® TMR ligand (Promega, Madison, Wisconsin, USA) and harvested by treating with 2mM EDTA/PBS for 1 minute. The apoptotic cells were labeled with Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, California, USA) according to protocol and analysed using BD FACSCalibur™ (Becton Dickinson, California, USA). A total of 30,000 events were collected for each sample. The main population was gated and applied to the other plots. The transfected cells (HaloTag TMR-labeled cells) were also gated and the apoptotic cells were calculated in the transfected cells. The cells transfected with HaloTag-only vector were used as control for apoptosis.

2.9. Induction of cell cycle G2 arrest by Vpr

2.9.1. Optimization of cell cycle analysis

To apply a reliable method for the assay of Vpr-induced cell cycle G2 arrest, our method had to be optimized. In this study, p-HaloTag-Cons-C (for the expression of HaloTag-Cons C Vpr) was used to optimize the method. To detect HaloTag-Vpr in the transfected cells, the HaloTag® Oregon Green® Ligand (Promega, Madison, Wisconsin, USA) was used in all the experiments. This ligand differs from HaloTag®

TMR Ligand in its emission wavelength so that it could be applied in combination with other fluorescent dyes used in cell cycle analysis.

Two fluorescent dyes were primarily used for the analysis, including propidium iodide (Sigma-Aldrich Co, St Louis, MO) and 7-amino actinomycin D (Sigma-Aldrich Co, St Louis, MO). The advantage of 7-AAD over PI is the ability to be used in conjunction with phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-monoclonal antibodies in 2-color analysis, with minimal spectral overlap between 7-AAD, PE and FITC fluorescence emissions. The 7-AAD fluorescence is detected in the far red range of the spectrum (650 nm long-pass filters).

To optimize our method, 5×10^5 293T cells were seeded into different wells of two 24 well cell culture plates. When the cells reached 50% confluency, they were transfected with p-HaloTag-Cons-C vector using our optimized transfection method. Twenty four hours after transfection, one plate was removed from the incubator for cell cycle analysis as described in the next paragraph. To find the best time for the assay, 24 hours after transfection, fresh complete DMEM medium was added to the second plate and incubated further for 24 hours (total incubation of 48 hours after transfection).

The transfected cells were labeled with 5 µM HaloTag® Oregon Green® Ligand. The cells were harvested using Trypsin-Versene Mixture (Lonza, Basel, Switzerland). The harvested cells were resuspended in 500 µl of PBS and fixed by adding 2 ml of -20ºC absolute ethanol and incubating on ice for 30 minutes. Cell fixation facilitates

labelling the DNA content by permeabilizing the cell membranes. The fixed cells were centrifuged and resuspended in PBS.

The DNA content was labelled with 1.2 µg/ml of 7-AAD or 1.2 µg/ml of PI and incubated for 10 minutes at room temperature in dark. The cell cycle was analyzed using flow cytometry within 6 hours. Briefly, multi-colour flow cytometry was performed to determine the induction of the cell cycle G2 arrest in the transfected 293T cells. Mock-transfected cells (293T cells transfected with HaloTag-only vector) were used as control. Flow cytometry analysis was performed on a BD FACSCalibour (Becton Dickinson, California, USA) equipped with a 15 mW argon-ion and 20 mW red diode lasers. A total of 50,000 events were collected for cell cycle analysis. The transfected cells were gated based on HaloTag Oregon Green. The data were analyzed using BD CellQuest Pro software version 4.0 (Becton Dickinson, California, USA).

2.9.2. Cell cycle assay

5×10^5 293T cells were seeded into 24 well cell culture plates. When the cells reached 50% confluency, they were transfected with all the HaloTag-Vpr expression vectors using FuGENE® 6 Transfection Reagent (Roche, Berlin, Germany) according to protocol. Based on our optimized method, 24 hours after transfection, the medium was replaced with fresh complete DMEM medium and 48 hours after transfection, the cells were labelled with 5µM HaloTag® Oregon Green® ligand (Promega, Madison, WI). The cells were harvested and resuspended in 500 µl of PBS and fixed by adding 2 ml of -20ºC absolute ethanol and incubating on ice for 30 minutes. Cell fixation facilitates labelling the DNA content by permeabilizing the cell membranes. The fixed cells were centrifuged and resuspended in PBS. The DNA content was labelled with

1.2 µg/ml 7-AAD and incubated for 10 minutes at room temperature in dark. The cell cycle was analyzed using flow cytometry within 6 hours as described.

2.10. Modulation of gene expression by Vpr

2.10.1. Quantitative real-time PCR

To study the modulation of gene expression by Vpr proteins, human 293T cells were plated at 400,000 cells/6 cm plate. When the cells reached 80% confluency, they were transfected using FuGENE® 6 Transfection Reagent (Roche, Indianapolis, IN) according to our optimized transfection method.

The fluorescence-based quantitative real-time PCR (qPCR) was performed using TaqMan Human Apoptosis Arrays (Applied Biosystems, Foster City, Canada). These are predesigned microfluidic cards with a 384-well format to assay the expression of 93 human genes along with 3 endogenous controls (18S, ACTB, GAPDH) for each sample, 4 samples/per card. Briefly, using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) the total RNA was extracted from the 293T cells 24 hours after transfection with Vpr-expression vectors. The total RNA was measured using Nanodrop™ ND-1000 system (Nanodrop Technologies Inc., Delaware, USA). One µg of the total RNA was reverse transcribed into cDNA using ImProm-II™ Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's suggestions. Twenty µl of each cDNA sample was added to 100 µl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Canada) and the total volume was brought to 200 µl with nuclease free water. After gentle mixing, the solution was transferred into 2 loading ports on a microfluidic card (100 µl per loading port). The microfluidic cards

were transferred to the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, Canada) and the real-time PCR conditions were set as follows: 2 min at 50°C, 10 min at 94.5°C and 30 s at 97°C, and 1 min at 59.7°C for 40 cycles. Data acquisition was done according to the manufacturer's suggestions using ABI Prism 7900HT Sequence Detection System version 2.4 (Applied Biosystems, Foster City, Canada). The housekeeping genes, 18S, ACTB and GDPH were used for validation of results. Gene expression values were then calculated based on the ΔΔCt method, with data normalized to the mock sample (293T cells transfected with barnase-removed pF4A vector). Relative quantities (RQ) were determined using the equation: $RQ=2^{-\Delta\Delta Ct}$. Only genes with reproducible amplification curves of quadruplicate determinations were analyzed and presented.

2.11. Statistical analysis

Analysis of variance (ANOVA) with Bonferroni's multiple comparison test was used to examine statistical significance in the ability of Vpr proteins in the induction of apoptosis and cell cycle G2 arrest. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad software Inc., LaJolla, Ca).

Chapter 3

3. Results

3.1. Patient samples

The patient demographics are listed in Table 3.1. The patients consist of 31 African females, 22 African males, 2 Caucasian males, as well as two males and one female of mixed race descent. Except for one patient (TV1638) who was from Kenya and was infected in Kenya, all of the other patients were South Africans infected in South Africa. The transmission pathways were, homosexual, heterosexual and unknown.

Table 3.1: Patient demographics of the PCR amplified samples.

Isolate	Date of birth	Race/Gender	Transmission
TV1325C*	1972	Mixed Race Male	Unknown
TV1345B#	1954	Caucasian Male	Homosexual
TV1421	1963	African Male	Heterosexual
TV1428B	1962	African Male	Heterosexual
TV1453	1954	African Male	Heterosexual
TV1454	1975	African Male	Heterosexual
TV1455	1979	African Female	Heterosexual
TV1459	1973	African Male	Heterosexual
TV1460	1922	African Male	Heterosexual
TV1462	1975	African Female	Heterosexual
TV1463	19-58	African Female	Heterosexual
TV1464	1977	African Female	Heterosexual
TV1466	1975	African Female	Heterosexual

TV1467	1976	African Female	Heterosexual
TV1468	1968	African Male	Heterosexual
TV1470	1964	Mixed Race Male	Heterosexual
TV1472	1978	African Female	Heterosexual
TV1526B	1967	Caucasian Male	Homosexual
TV1565	1969	African Female	Heterosexual
TV1566	1962	African Male	Heterosexual
TV1568	1968	Mixed Race Female	Heterosexual
TV1570	1957	African Male	Heterosexual
TV1571	1976	African Female	Heterosexual
TV1573	1978	African Female	Heterosexual
TV1576	1974	African Female	Heterosexual
TV1577	1970	African Female	Heterosexual
TV1578	1983	African Female	Heterosexual
TV1579	1970	African Male	Heterosexual
TV1581	1975	African Male	Heterosexual
TV1583B	1981	African Female	Heterosexual
TV1596	1955	African Male	Heterosexual
TV1597	1971	African Female	Heterosexual
TV1598	1973	African Female	Heterosexual
TV1599	1958	African Male	Heterosexual
TV1614	1963	African Male	Heterosexual
TV1616	1972	African Female	Heterosexual
TV1623	1970	African Female	Heterosexual
TV1625	1973	African Female	Heterosexual
TV1626	1952	African Female	Heterosexual
TV1627	1975	African Female	Heterosexual
TV1628	1971	African Male	Heterosexual
TV1630	1977	African Male	Heterosexual
TV1632	1971	African Female	Heterosexual
TV1633	1961	African Male	Heterosexual
TV1638	1973	African Female	Heterosexual
TV1639	1976	African Female	Heterosexual

TV1641	1981	African Female	Heterosexual
TV1642	1970	African Female	Heterosexual
TV1643	1958	African Female	Heterosexual
TV1644	1976	African Male	Heterosexual
TV1653	1984	African Female	Heterosexual
TV1654	1972	African Female	Heterosexual
TV1661	1980	African Male	Homosexual
TV1667	1963	African Female	Heterosexual
TV1681	1973	African Male	Heterosexual
TV1705	1974	African Female	Heterosexual
TV1707	1946	African Male	Heterosexual
TV1708	1975	African male	Heterosexual

* B indicates the second sample of the patient
# C indicates the third sample of the patient.

3.2. Amplification of the HIV-1 *vpr* gene

The Access RT-PCR System has been designed for the reverse transcription and PCR amplification of a specific target RNA from either total RNA or mRNA (Miller & Storts, 1995). This one-tube, two-enzyme system provides sensitive analysis of RNAs. The system uses AMV reverse transcriptase (AMV RT) from avian myeloblastosis virus for first strand DNA synthesis and the thermostable *Tfl* DNA polymerase from *Thermus flavus* for second strand cDNA synthesis and DNA amplification. The Access RT-PCR System includes an optimized single-buffer system that permits detection of RNA transcripts without a requirement for buffer additions between the reverse transcription and PCR amplification steps.

A total of 100 South African HIV-1 samples were recruited in this study but only 58 samples could be amplified. PCR amplification of a 714 bp fragment yielded sharp bands in parallel with 750bp band of the DNA ladder as observed on agarose gel (Figure 3.1).

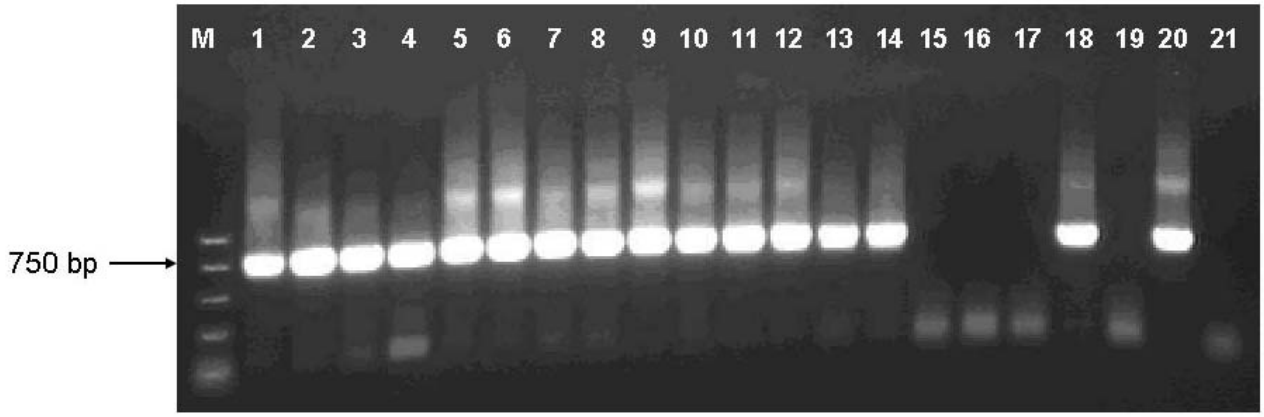


Figure 3.1: PCR amplification of South African HIV-1 isolates using primers HIV-vif-1F and HIV-vpr-R1. Fifty eight samples were amplifiable. The result of 1 run is shown in the figure. Lane M: 1 kb DNA Marker; Lane 1: TV1568, Lane 2: TV1654, Lane 3: TV1627, Lane 4: TV1639, Lane 5: TV1598, Lane 6: TV1638, Lane 7: TV1628, Lane 8: TV1641, Lane 9: TV1630, Lane 10: TV1632, Lane 11: TV1633, Lane 12: TV1428B, Lane 13: TV1566, Lane 14: TV1565, Lane 15: TV1469, Lane 16: TV1593, Lane 17: TV1624, Lane 18: TV1616, Lane 19: TV1456, Lane 20: TV1625, Lane 21-negative control.

3.3. DNA sequencing

The PCR products were purified for sequencing using enzymes Exonuclease 1 (*Exo*1) and Shrimp alkaline phosphatase (SAP). These enzymes are used to degrade single-strand DNA (such as excess primers) and diphosphates that might interfere with the sequencing reactions (Werle *et al.*, 1994).

The BigDye Terminator Cycle Sequencing Kit, which was used in this study, provides the required reagent components for the sequencing reaction in a ready reaction, pre-mixed format. The kit is based on Sanger sequencing method. This method has served as the cornerstone for genome sequencing, including eukaryotic, prokaryotic, viral sequencing, for over a decade (Goldberg *et al.*, 2006).

The sequencing of the PCR products yielded pure chromatograms of single sequences free of DNA contamination or non-specific amplification (Figure 3.2). By aligning the sequences with the reference sequences of *vpr*, the *vpr* gene was identified in the sequences. All *vpr* sequences were submitted to GenBank with accession numbers FJ039723-FJ039780.

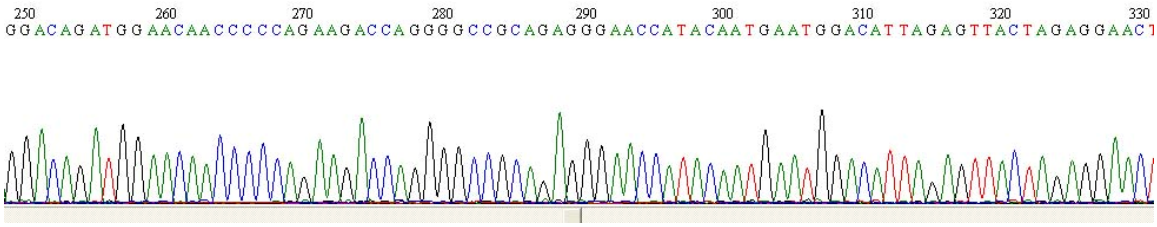


Figure 3.2: The sequencing chromatogram of a PCR product resulted in single peaks. The chromatograms do not show any traces of cross contamination of the PCR products.

3.4. Phylogenetic analysis of South African isolates

The *vpr* gene of these isolates was phylogeneticly analyzed. The phylogenetic tree constructed from the 291-bp fragment of the structural part of *vpr* gene is shown in Figure 3.3. The bootstrap values greater than 70% were included in the phylogenetic tree. Although bootstrap values greater than 95% are generally considered statistically significant, bootstrap values greater than 70% are generally accepted in the literature.

Fifty three out of 58 sequences (91%) clustered with the reference sequences of subtype C. The sequence of TV1573 was an outlier to subtype C. This sequence was analyzed using jumping profile hidden Markov model (jpHMM). The jpHMM predicts recombination breakpoints in a query sequence and assigns to each position of the sequence one of the major HIV-1 subtypes. Since incorrect subtype assignment or recombination prediction may lead to wrong conclusions in epidemiological or vaccine research, information about the reliability of the predicted parental subtypes and breakpoint positions is valuable (Schultz *et al.*, 2009). The jpHMM indicated that the *vpr* gene of TV1573 belonged to HIV-1 subtype C, however, only the complete genome sequencing could confirm the subtype of this isolate.

Four sequences (TV1526B, TV1345B, TV1626, and TV1325C) clustered with the reference sequences of subtype B from Thailand (B.TH.90.BK132), France (B.FR.83.HXB2), and the Netherlands (B.NL.00.671). Two out of four subtype B isolates had been transmitted homosexually, one through heterosexual contact, and one with an unknown route. In comparison to subtype B, only 1 (TV1661) out of 54 subtype C isolates had been transmitted homosexually and the others had a heterosexual route.

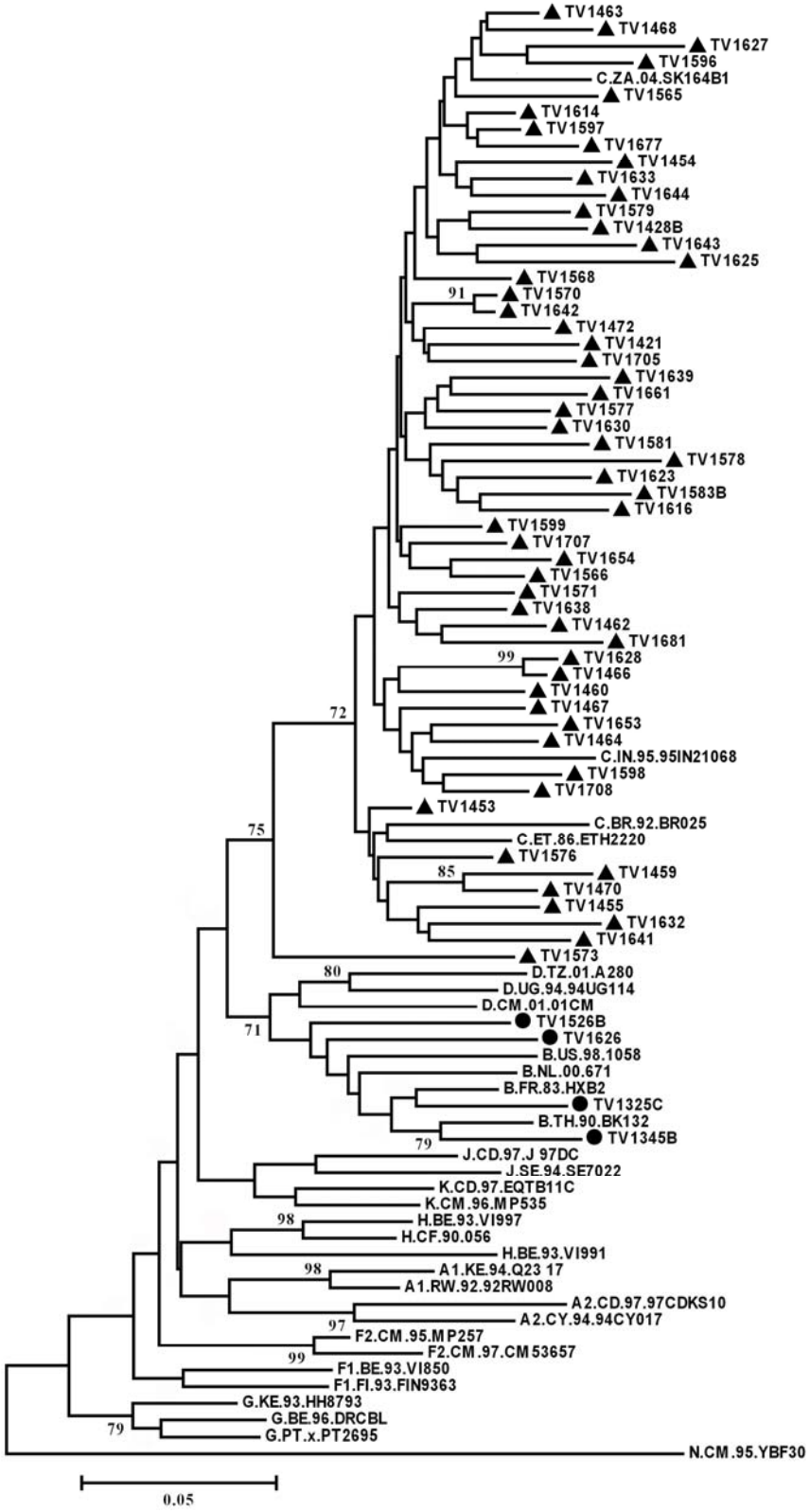


Figure 3.3: Rooted neighbor-joining tree illustrating the evolutionary relationship among vpr nucleotide sequences (291 bp) from South African HIV-1 isolates and reference sequences of HIV-1 (Los Alamos database). Fifty-eight sequences are shown with TV (Tygerberg Virology) suffixed by their respective clone numbers. Triangles indicate subtype C isolates and circles indicate subtype B isolates.

To investigate the Vpr protein, the *vpr* gene was translated and the amino acids were aligned with the consensus subtype C and HXB2 reference sequences. The α-helical domains of Vpr were shaded in gray (Figure 3.4). All of our sequences were 96 amino acid residues in length, except for TV1467. In this sequence an arginine residue had been deleted in position 90, which is located in the C-terminal domain of the protein. The patient was a 30-year-old African woman with symptoms of prurigo.

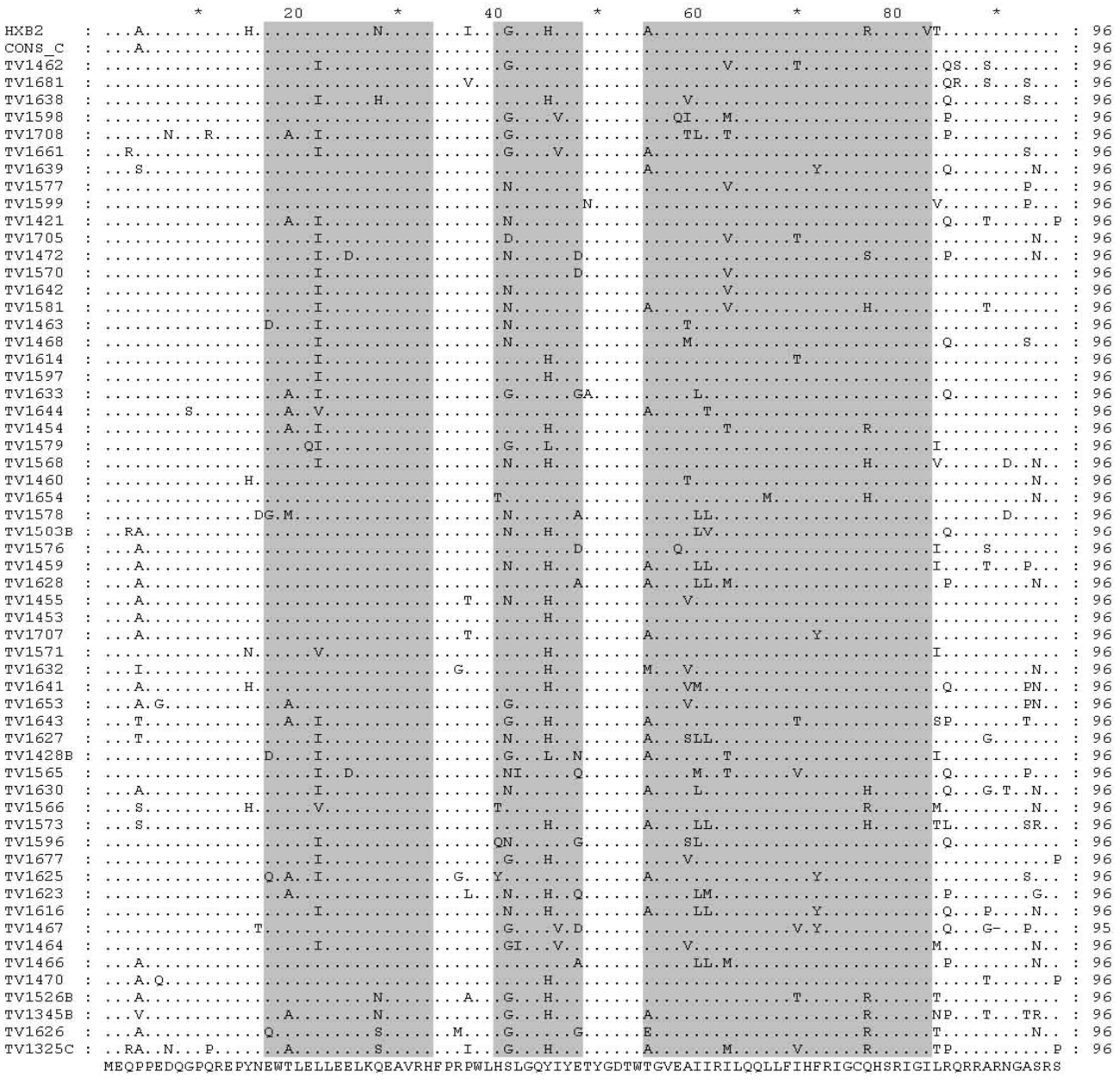


Figure 3.4: Alignment of the deduced Vpr amino acid sequences from 58 South African isolates in comparison with consensus subtype C reference and the subtype B HXB2 reference. Dots represent identity to the consensus sequence of our isolates, at the bottom, and the only dash, in TV1467, represents a gap. The α-helical domains of Vpr are shaded gray.

The consensus Vpr amino acid sequence of our strains was identical to the consensus sequence of subtype C, except for position 4. Most of the sequences had a proline residue in this position, whereas the consensus subtype C sequence had an alanine residue. No mutations were observed in positions 21 and 24 of the α-helix I for which substitutions of proline have been shown to abrogate Vpr incorporation into virus-like particles (Tungaturthi *et al.*, 2004a). A highly conserved motif in α-helix I was found in position 29-33 (EAVRH). Motif 42-44 of the α-helix II was highly conserved in our isolates of which leucine 42 and glycine are involved in Vpr incorporation (Singh *et al.*, 2001; Thotala *et al.*, 2004). Motif 55-64 in α-helix III was variable but motif 65-83 in this domain was found highly conserved. The C-terminal domain of Vpr indicated the highest variability, but even in this domain, three arginine residues in positions 87, 88, and 95 and one glycine residue in position 92 were intact in all of our isolates. The motif FPRPWL, between α-helix I and II (34-39: IH1), and the motif TYGDTW, between α-helix II and III (49-54:IH2), were highly conserved in our isolates (Tungaturthi *et al.*, 2004a).

3.5. Cloning of the *vpr* ORF

The impact of natural mutations on the established functions of Vpr and the biology of HIV-1 has not been studied. Many studies investigate that function of wild-type Vpr and the functions and significance of this protein is presented as the outcome of these studies while the natural mutants of Vpr are ignored. Since the natural Vpr mutants are frequent forms of this protein, this study addressed the functions of these proteins compared to that of the consensus sequence of Vpr that artificially was produced in this study, as well as subtype B Vpr. The sequences were selected that contained natural mutations distributed in different domains of Vpr. The impact of

these mutations on the established functions of Vpr and the biology of HIV-1 has not been studied.

The 291 bp *vpr* ORF of the South African isolates (TV1453, TV1628, TV1707, TV1642, TV1570, and TV1466) and subtype B Vpr (pNL4-3) was amplified using Vpr-Sgf-F and Vpr-Pme-R as described in chapter 2. Using these primers, the restriction sites for *Sgf*-I and *Pme*-I endonuclease were appended to the *vpr* ORFs. The PCR products were run on ethidium bromide/agarose gel as shown in Figure 3.5 and 3.6. The amplification was successful and resulted in 310 bp PCR products. After clean up of the PCR products, 60-90 ng/µl DNA was obtained from each PCR product that was used for cloning.

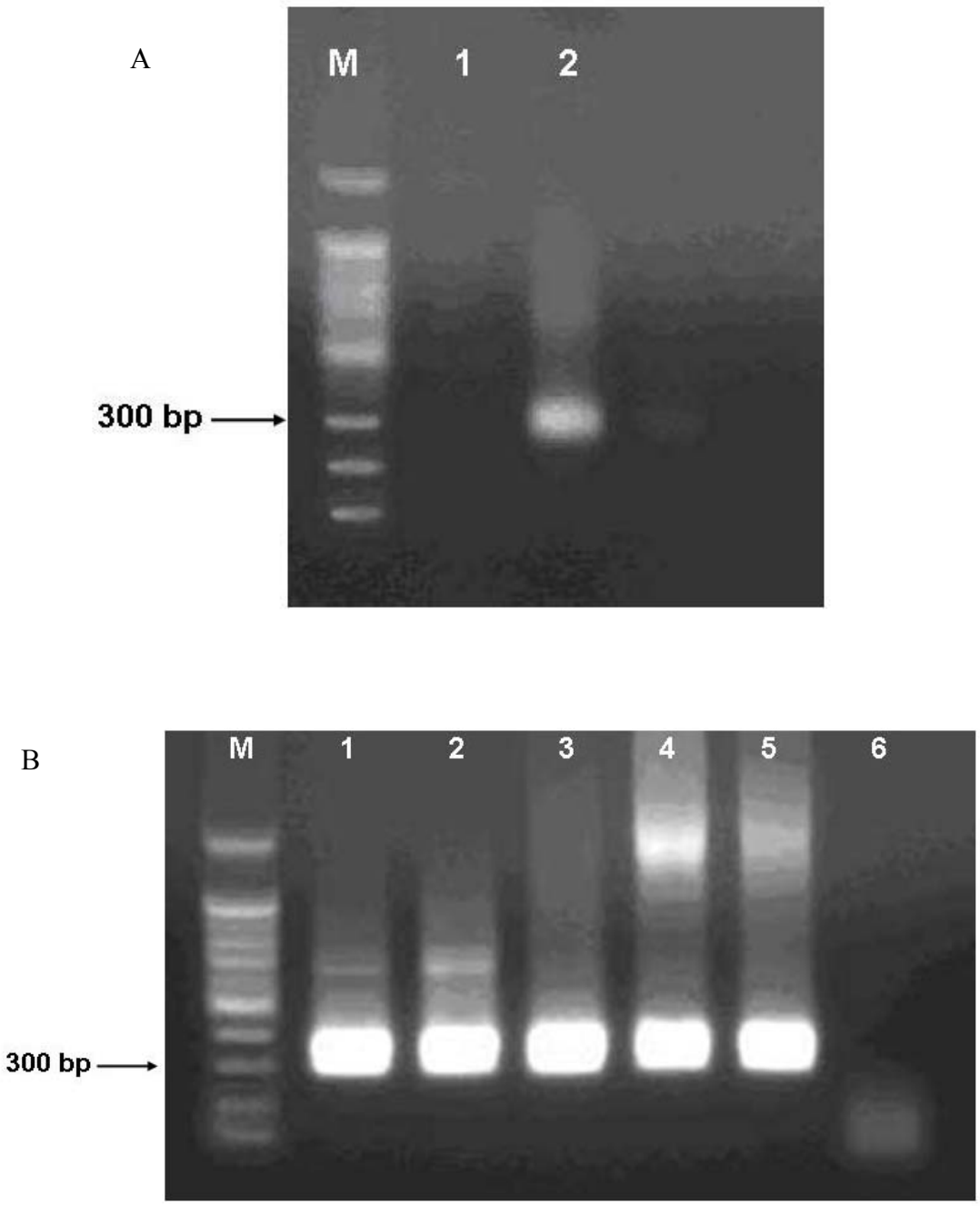


Figure 3.5: Amplification of the *vpr* ORF using the primer vpr-sgf-F and vpr-Pme-R to insert restriction sites at both sides of the *vpr* fragments. The PCR reactions resulted in 310 bp PCR products. (A) Amplification of TV1453. Lane 1-Negative control; Lane 2-TV1453. (B) Amplification of TV1628, TV1701, TV1466, TV1642, TV1570 in lane 1 to 5, respectively; Lane 6- Negative control.

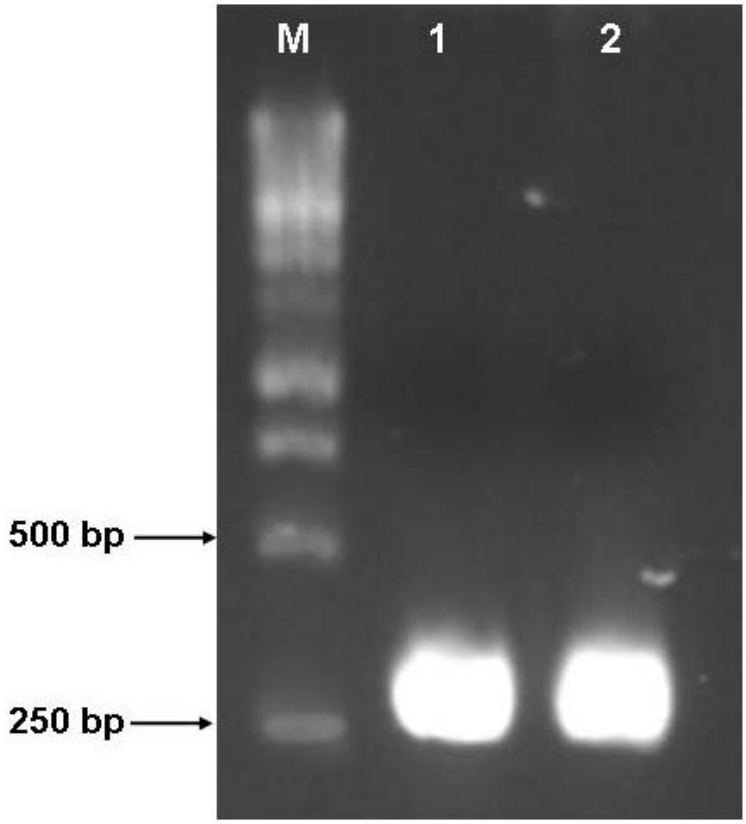


Figure 3.6: PCR products of a ~300 bp fragment containing *vpr* ORF from pNL4-3. Lanes: Lane M- 1 kb DNA Marker; Lane 1 and 2-two PCR reactions for the same sample.

The PCR products were successfully ligated to the pF4A Flexi® vector and transformed to JM109 cells. The transformants were cultured on LB medium with antibiotics and yielded 40-100 colonies for each clone. The number of the colonies grown on the plates was enough to verify the success of the cloning. For each clone 9 colonies were picked and cultured in LB broth and extracted. The plasmid purification yielded between 50-150 ng/µl DNA for each clone. The plasmids were digested using the restriction enzymes (*Sgf*-I and *Pme*-I) and run on an ethidium bromide/agarose gel. Most of the plasmids contained the fragment of interest as shown in Figure 3.7. The insertion of subtype B vpr was confirmed by PCR (Figure 3.8). Four plasmids with confirmed integration of a ~300 bp fragment, based on gel evidence, were

sequenced to verify the integration of the non-mutated *vpr* gene. The sequencing also confirmed the integration of the *vpr* gene. One vector for each plasmid expressing non-mutated fragments was stored at 4ºC for the future experiments.

At this stage we had 7 clones. The clones were named corresponding to their insert as follow:

pF4A-subtype B, pF4A-TV1453, pF4A-TV1628, pF4A-TV1707, pF4A-TV1642, pF4A-TV1570, pF4A-TV1466.

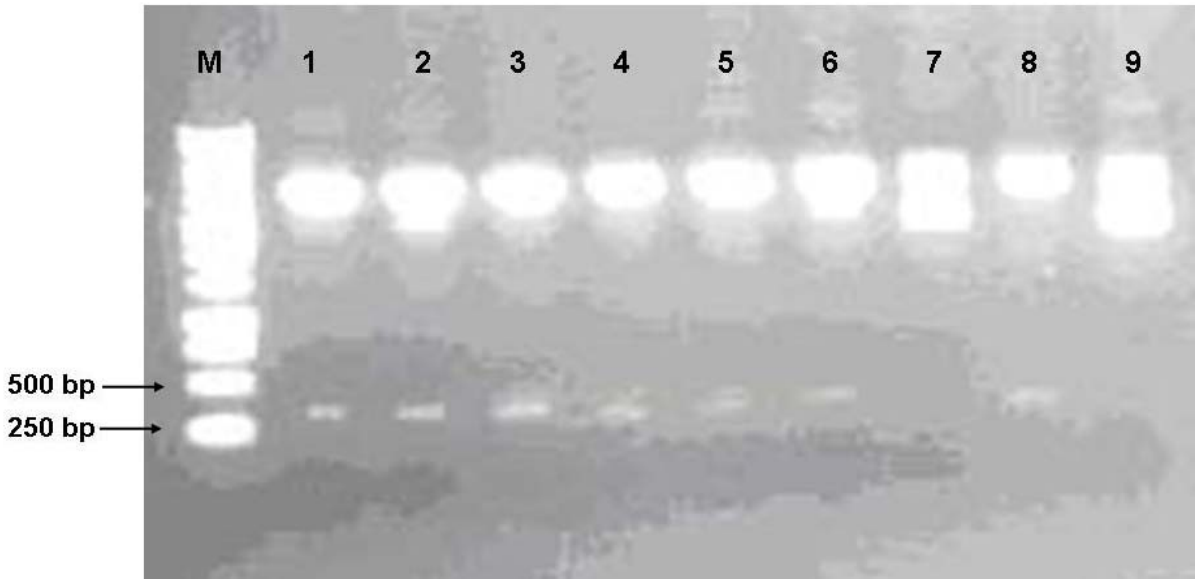


Figure 3.7: Nine plasmids were screened for each clone. The digested plasmids were run on agarose gel to screen for the integration of vpr fragments. The picture shows the clones of TV1453 in lane 1-9. Seven plasmids of TV1453 colons contained ~300 bp fragments corresponding to *vpr* gene. The exposure time was increased so that the small fragments could be observed.

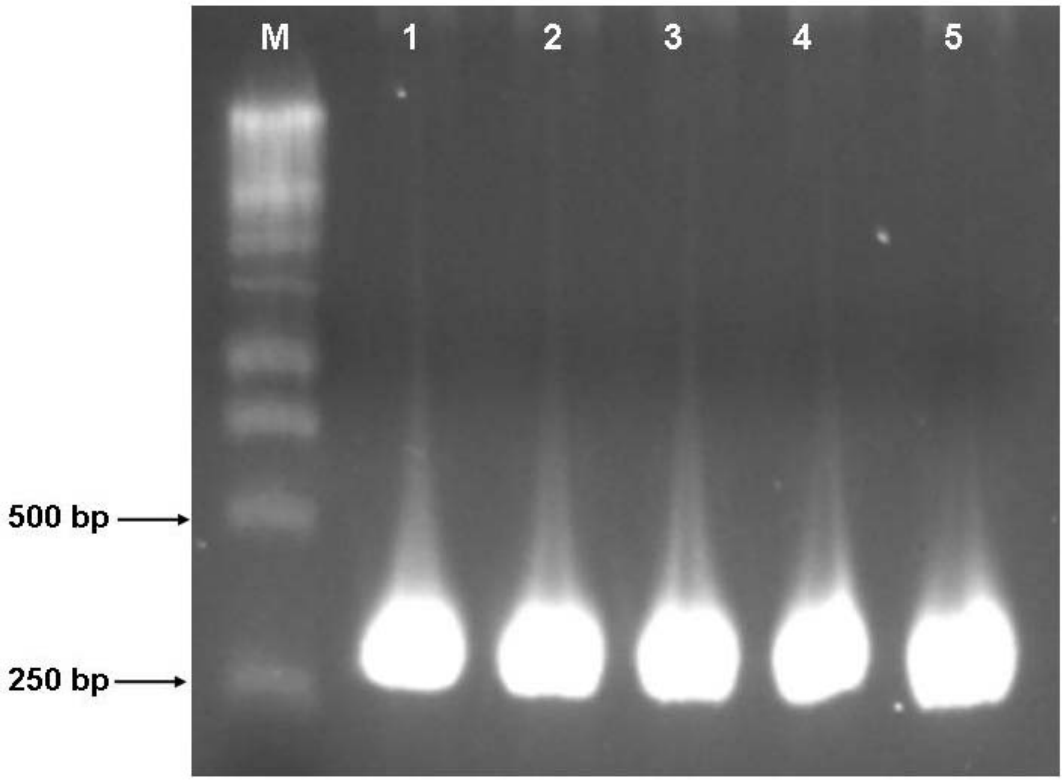


Figure 3.8: PCR of 4 clones of subtype B Vpr from pNL4-3. Lanes: Lane M-1kb DNA ladder; lane 1; positive control (pNL4-3), lane 2-5; miniprebs.

3.6. Mutagenesis experiments

Site-directed mutagenesis allows site-specific mutation in virtually any double-stranded plasmid. The QuikChange® Lightning Site-Directed Mutagenesis Kit includes a derivative of *PfuUltra*® high-fidelity (HF) DNA polymerase for mutagenic primer-directed replication of both plasmid strands with the highest fidelity. The basic procedure utilizes a vector with an insert of interest and two synthetic primers, both containing the desired mutation. The primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuUltra* HF DNA polymerase, without primer displacement. Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature

cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template.

To study the role(s) of Vpr domains in different functions of the protein, Vpr was mutated in different domains. The plasmids that were subject to site-directed mutagenesis were sequenced to confirm the mutations. According to the protocol for site-directed mutagenesis, one can expect 80% of the clones to contain the mutation of interest. The outcome of site-directed mutagenesis and sequencing of the plasmid indicated that all the sequenced plasmids contained the mutations of interest. This means the efficiency of the site-directed mutagenesis was 100% in these experiments.

The plasmids were named as follow:
pF4A-P14I, pF4A-W18C, pF4A-Y47N, pF4A-Q65H, pF4A-Q88S

3.7. Protein modelling

Homology modelling is currently the most accurate computational method to generate reliable structural models and is routinely used in many biological applications. Typically, the computational effort for a modelling project is less than 2 h. However, this does not include the time required for visualization and interpretation of the model, which may vary depending on personal experience working with protein structures (Arnold *et al.*, 2006; Kiefer *et al.*, 2009; Schwede *et al.*, 2003).

To define the position of mutations in mutated proteins, the 3-dimensional structure of proteins had to be reconstructed. These comprehensive pictures help to identify the position of the mutation(s) in the protein domains. Especially, if the study is intended for publication, these pictures help authors as a comprehensive guide to elaborate the experiments. Based on the sequencing of the plasmids, the proteins expressed by the expression plasmids were simulated using Swiss-Model and DS visualizer (Figure 3.9 & 3.10). The backbone of the proteins was shown using ribbon representation and the mutations were shown using CPK representations. The pictures clearly demonstrate the position of the mutations in the corresponding domain. Site-directed mutagenesis had created mutations in the positions that did not occur in natural mutations so that the functional study of these domains was possible. The results from protein modelling indicated that the flexible N-terminal domain was intact in all of our natural mutants. To study the role of this domain in the functions of Vpr, this domain was mutated using site-directed mutagenesis. The α-helix I was intact among the most of the natural mutants. L22I was the only mutation observed in α-helix I of two natural mutants, TV1570 and TV1642. Using site-directed mutagenesis, tryptophan was mutated to cysteine in position 18 which is situated in α-helix I. α-helix II was sufficiently covered by the natural mutations P37D, S41N, Y45H, E48A, and E48 D. α-helix III also contained natural mutations including T55A, I60L, I61L, I63M, I63V, F72Y.

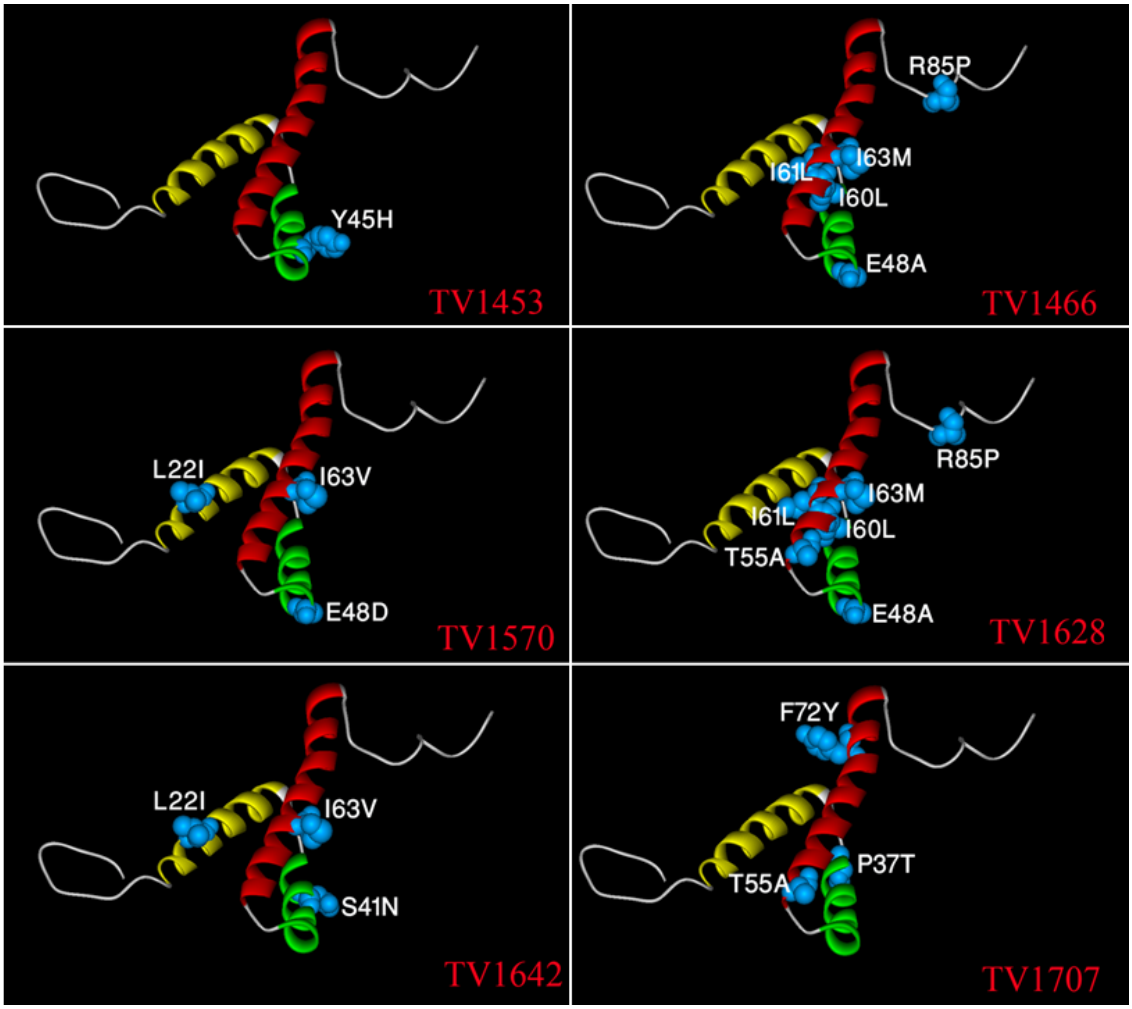


Figure 3.9: Ribbon representation of natural mutants showing the distribution of mutations in different domains of HIV-1 Vpr of our isolates. The mutations are represented in CPK (blue balls) that represent the molecular structure of the amino acids.

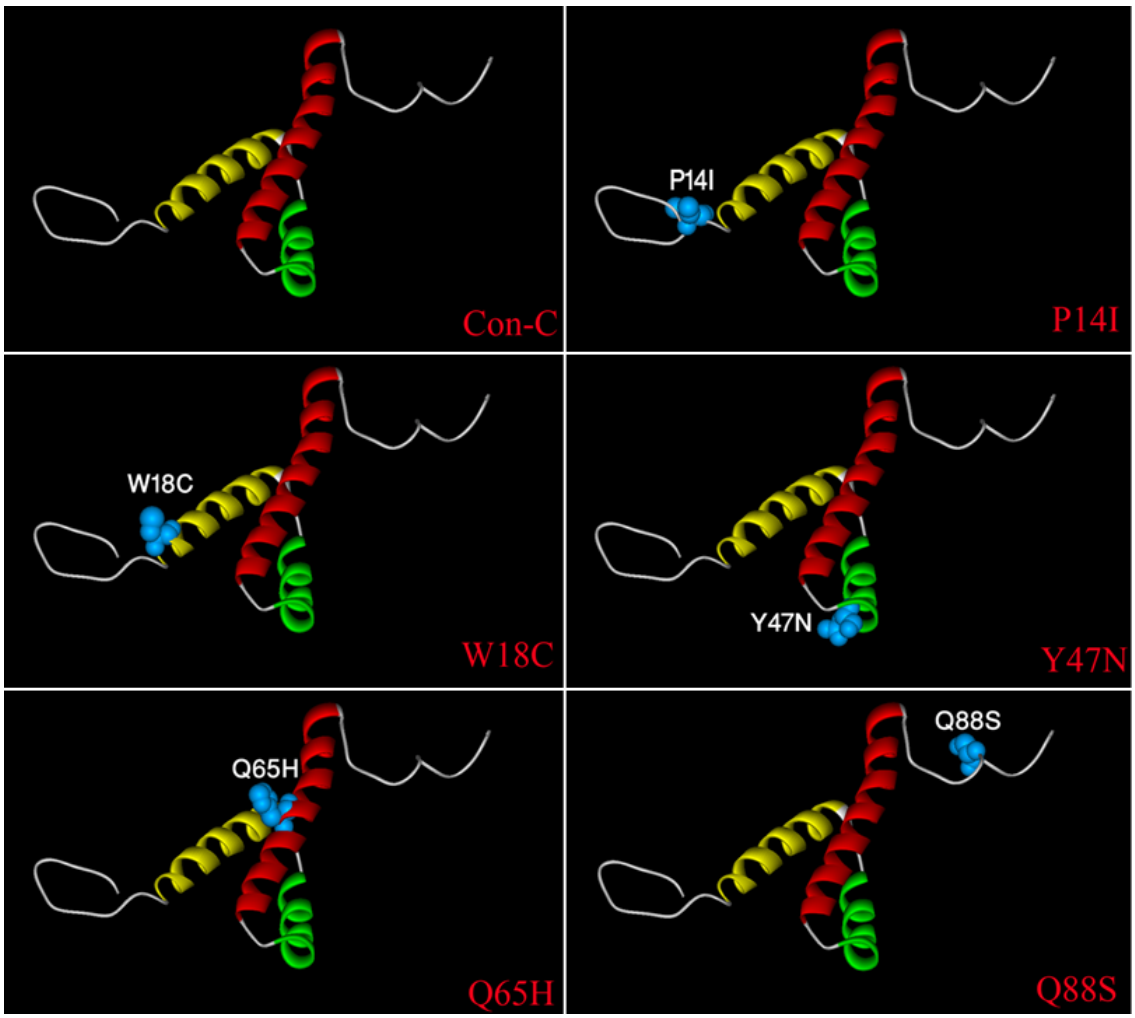


Figure 3.10: Ribbon representation of consensus sequence of HIV-1 Vpr subtype C and the mutants created by site-directed mutagenesis.

3.8. Transfer of the Vpr-coding region between the vectors

To verify the transfer of the Vpr-coding regions from the donor plasmids (clones of pF4A CMVd1 Flexi® Vector) to the acceptor plasmids (pFN22K CMVd1 Flexi® Vectors), *vpr* ORF was amplified using Vpr-Sgf-F and Vpr-Pme-R. The products of the PCR reactions were run on an agarose gel and short fragments (~300 bp) corresponding to *vpr* ORF were observed on the gel (Figure 3.11). The results indicated that *vpr* ORF had been successfully integrated in all the acceptor vectors.

The efficiency of the fragment transfer between the vectors was 100%. One vector was chosen from each clone. There was no need to confirm the vectors by sequencing at this stage because the donor vectors had not undergone PCR amplification and had been already sequenced and confirmed. The new vectors expressing the HaloTag-Vpr are listed in Table 3.2.

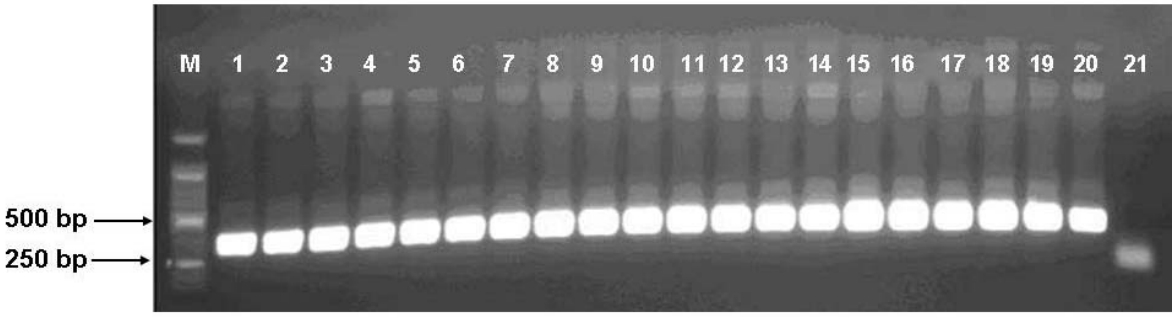


Figure 3.11: Agarose gel showing the PCR bands amplified from the acceptor vectors. The presence of this fragment in the acceptor vectors indicates the successful transfer of the Vpr-coding region between the vectors. Lane 1-4: TV1628, Lnae 5-8: TV1707, Lane 9-12: TV1642, Lane 13-16: TV1570, Lane 17-20: TV1466, Lane 21 is a negative control.

Table 3.2: The donor flexi vectors and their corresponding vectors expressing HaloTag-Vpr

The donor flexi vector	The corresponding HaloTag-Vpr expression vector
pF4A-sub-B	p-HaloTag-sub-B
pF4A-Cons-C	p-HaloTag-Cons-C
pF4A-TV1453	p-HaloTag-TV1453
pF4A-TV1628	p-HaloTag-TV1628
pF4A-TV1707	p-HaloTag-TV1707
pF4A-TV1642	p-HaloTag-TV1642
pF4A-TV1570	p-HaloTag-TV1570
pF4A-TV1466	p-HaloTag-TV1466
pF4A-P14I	p-HaloTag-P14I
pF4A-W18C	p-HaloTag-W18C
pF4A-Y47N	p-HaloTag-Y47N
pF4A-Q65H	p-HaloTag-Q65H
pF4A-Q88S	p-HaloTag-Q88S

3.9. Immunofluorescence assay for detection of Mycoplasma

The immunofluorescence assay did not show any signs of mycobacterial contamination. This means the cells were not contaminated with Mycoplasma and can be used for transfection and other experiments. It has been shown that mycoplasmas produce a variety of effects on cultured cells (e.g. changes in metabolism, immunologic or biochemical properties, growth, viability, etc.). Since mycoplasma infection in cell cultures normally is a chronic infection, which may not be obvious by visual inspection or light microscopy, periodic screening of cell cultures for mycoplasmas is important. A variety of tests to detect mycoplasmas in cell cultures

have been developed such as fluorochrome staining of DNA, monitoring toxic metabolites, culture method, etc. RIDASCREEN® Mycoplasma IFA contains a monoclonal antibody with specificity for a broad range of Mycoplasma species, including the species *Acholeplasma laidlawii*, *Mycoplasma hyorhinis*, *M. arginini*, *M. orale*, *M. fermentans* and *M. salivarium*, which account for more than 96% of cell culture infections (Blazek *et al.*, 1990; Kamla *et al.*, 1992).

3.10. Transfection optimization

Two transfection reagents, two cell lines and different transfection reagent to DNA ratios were compared with each other in this study to apply the most efficient method for transfection. TransFast™ Transfection Reagent (Promega, Madison, Wiskinson, USA) did not efficiently transfect either 293T or HeLa cells (Figure 3.12) even with different DNA concentrations of the pSV-β-Galactosidase Control Vector (Promega, Madison, Wiskinson, USA) as recommended by the manufacturer. Only a few cells appeared in blue (transfected) after staining the transfected cells with β-Gal Staining Set (Roche, Berlin, Germany). On the other hand, transfection using FuGENE® 6 Transfection Reagent (Roche, Berlin, Germany) with different concentrations of the DNA and the transfection reagent yielded generally better results. It was suspected that the presence of antibiotics in the medium may also affect the efficiency of transfection as it has been reported that in the presence of antibiotics the efficiency of transfection can be dramatically reduced (Jacobsen *et al.*, 2004). The transfection optimization experiments using FuGENE® 6 Transfection Reagent were performed in the presence or absence of penicillin/streptomycin in both 293T and HeLa cells. Antibiotics did not affect the transfection efficiency since similar results were obtained in the presence or absence of penicillin/streptomycin (Figure 3.13 and 3.14).

The highest transfection efficiency of FuGENE® 6 Transfection Reagent was obtained from the ratio of 6:1 (FuGENE® 6 Reagent:DNA ratio, respectively) in 293T cells as shown in Figure 3.13. Transfection of HeLa cells using FuGENE® 6 Transfection Reagent yielded few transfected cells (Figure 3.14). Although the transfection efficiency in HeLa cells was low, the ratio of 6:1 in HeLa cells still resulted in higher efficiency than the other ratios.

Since the highest transfection efficiency was obtained from FuGENE® 6 Transfection Reagent, it was decided to use this reagent with the optimized ratio (6:1) for the transfection of 293T cells. HeLa cells and TransFast™ Transfection Reagent were excluded from further experiments as they did not yield high transfection efficiency.

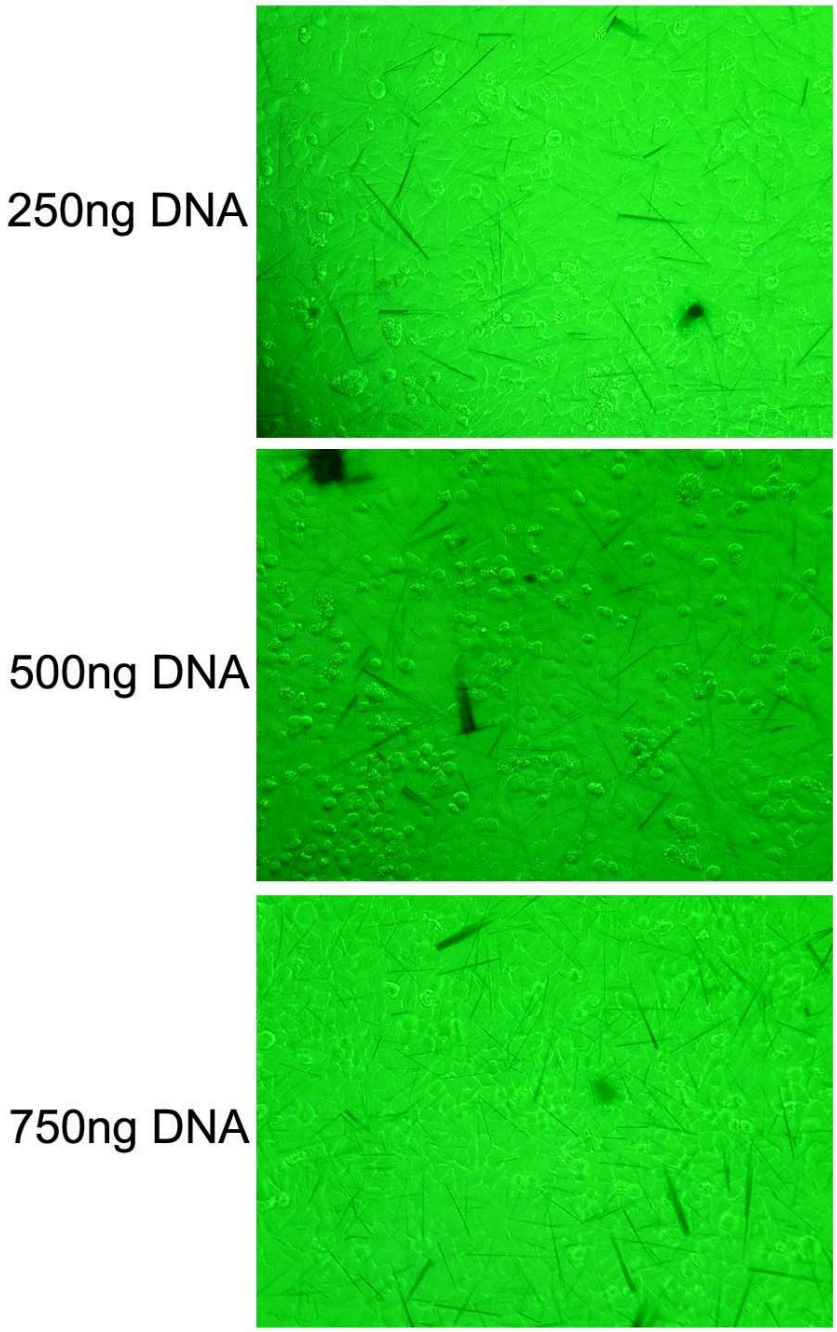


Figure 3.12: Transfection optimization of HeLa cells using TransFast™ Transfection Reagent. HeLa cells were transfected with pSV-β-Galactosidase Control Vector and after 24 hours photographed at 20X magnification using the Nikon™ Eclipse TS100 Microscope (Nicon Ltd. Surrey, UK). The dark cells indicate the transfected cells. This set up resulted in low transfection efficiency.

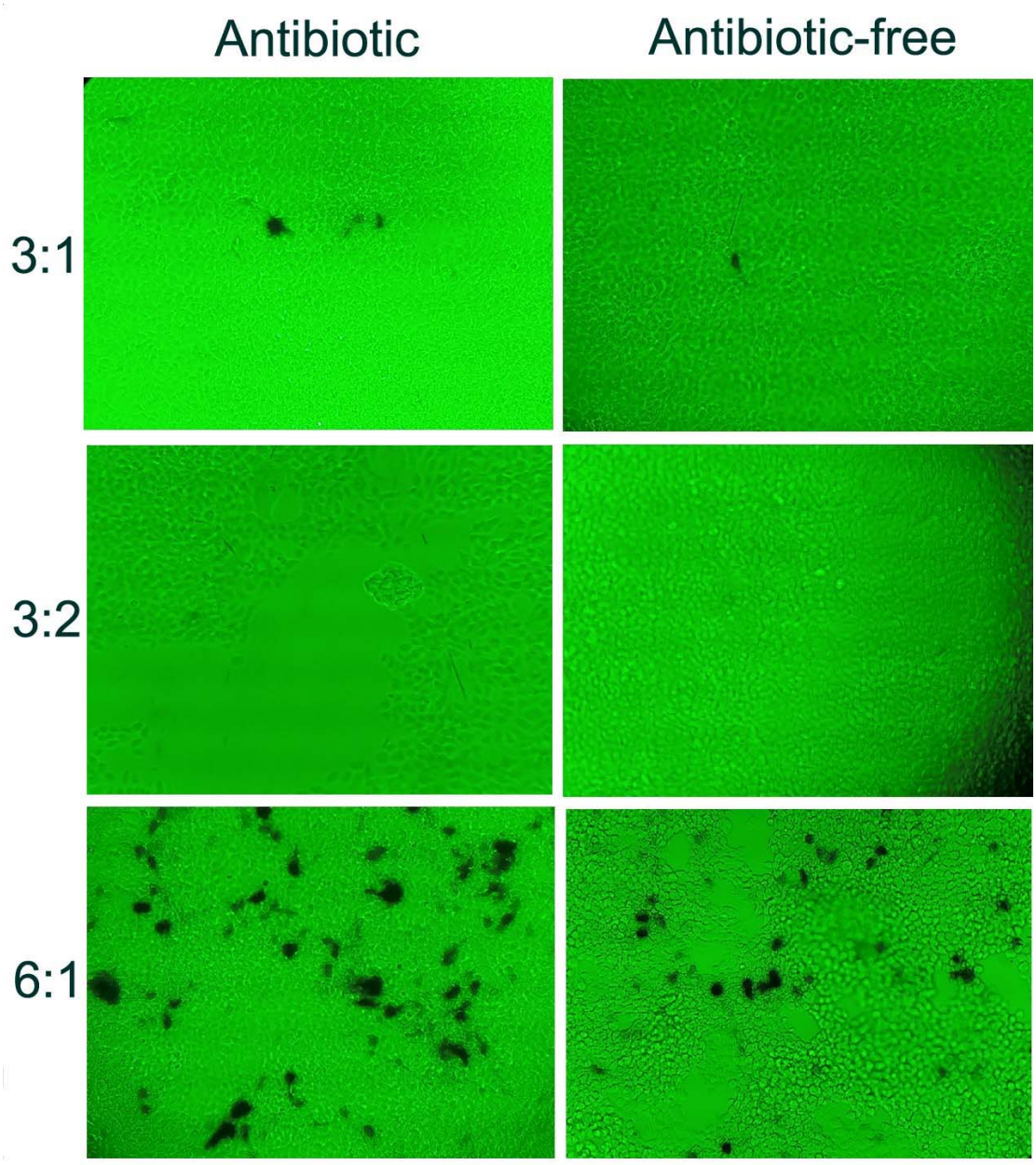


Figure 3.13: Transfection optimization of 293T cells using FuGENE® 6 Transfection Reagent. Human 293T cells were transfected with pSV-β-Galactosidase Control Vector and after 24 hours photographed at 20X magnification using the Nikon™ Eclipse TS100 Microscope (Nicon Ltd. Surrey, UK). The dark cells indicate the transfected cells. Apparently, the presence or absence of antibiotics did not significantly affect the transfection efficiency. The best result was obtained from the ratio of 6:1.

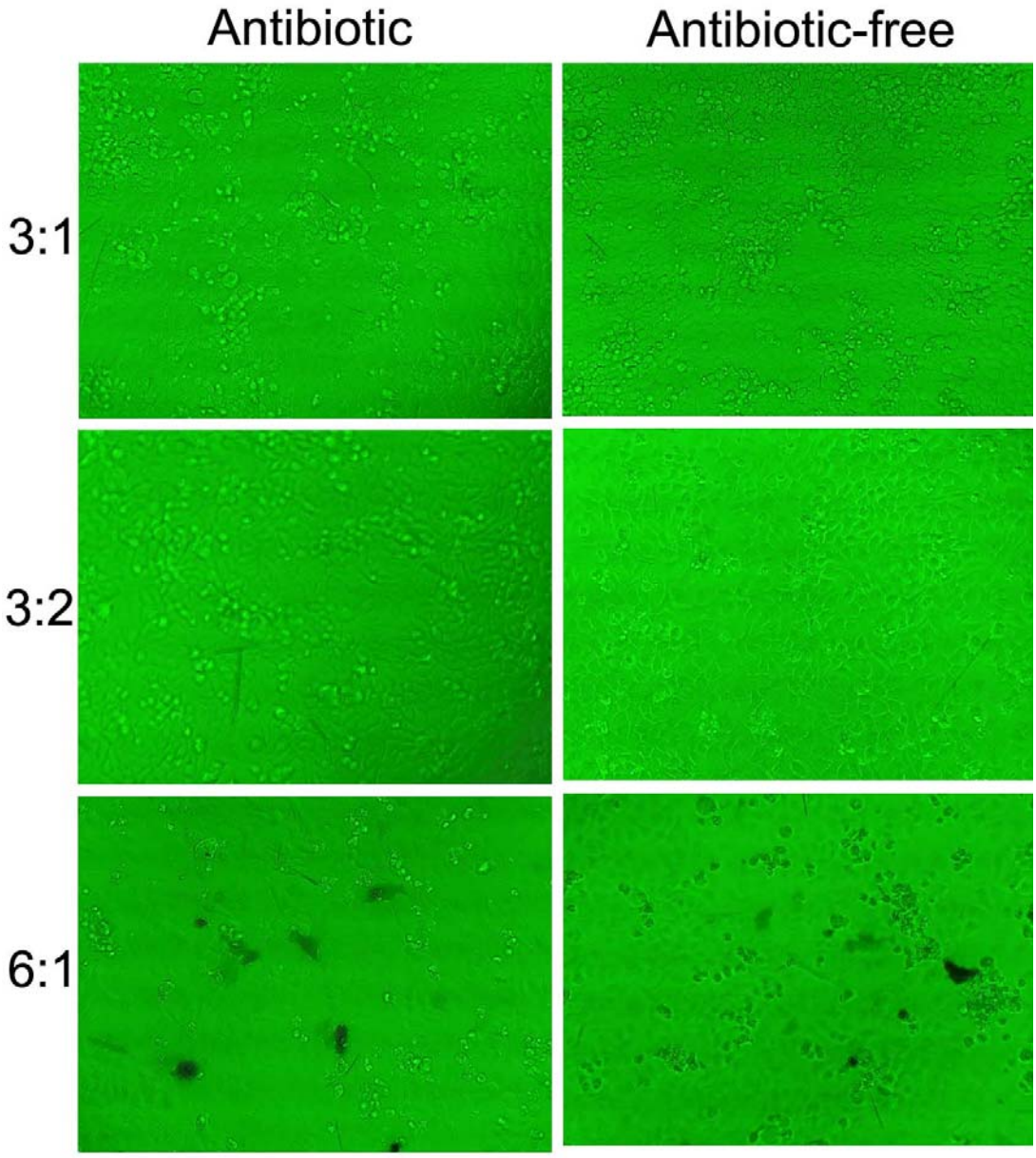


Figure 3.14: Transfection optimization of HeLa cells using FuGENE® 6 Transfection Reagent. HeLa cells were transfected with pSV-β-Galactosidase Control Vector and after 24 hours photographed at 20X magnification using the Nikon™ Eclipse TS100 Microscope (Nicon Ltd. Surrey, UK). The dark cells indicate the transfected cells. Low transfection efficiency was observed in HeLa cells using this method.

3.11. Subcellular localization of Vpr

Vpr has been shown to possess karyophilic properties and localize to the nucleus (Jacquot *et al.*, 2007). To investigate the ability of naturally mutated Vpr proteins to localize to the nucleus and the domains of Vpr involved in the nuclear localization, the subcellular localization of Vpr was studied microscopically. The nuclear localization of Vpr is closely related to its functions and the mutations affecting the subcellular localization of Vpr could thus seriously affect its functions (Nitahara-Kasahara *et al.*, 2007). For example, as Vpr has been shown to transfer HIV-1 PIC to the nucleus, disturbing the karyophilic property of Vpr could result in inhibition of PIC transfer (Jacquot *et al.*, 2007; Jenkins *et al.*, 1998; Le-Rouzic *et al.*, 2002; Suzuki *et al.*, 2009). In this study, 13 different construct expressing different forms of Vpr protein were used. Six constructs expressed naturally mutated Vpr proteins and five constructs expressed site-directed mutated Vpr proteins, one construct expressed the consensus HIV-1 Vpr subtype C, and one construct expressed the subtype B NL4-3 Vpr.

All the constructs contained a sequence to encode HaloTag protein tagged to the N-terminal region of the Vpr proteins. The HaloTag® Technology is a technology platform for understanding protein function in biochemical and cellular environments. Specific applications include imaging live or fixed cells, performing gel-based analyses including post-translational modification of labeled fusion proteins, and isolating proteins or protein complexes, all using a single genetic construct. This technology is comprised of two essential parts: the HaloTag® protein, to which a protein of interest is attached at either the N- or C-terminus, and the HaloTag® ligands, designed to covalently bind the HaloTag® protein.

The HaloTag® reporter protein is an engineered, catalytically inactive derivative of a hydrolase that forms a covalent bond with HaloTag® ligands. Under physiological conditions this covalent bond forms rapidly and is highly specific and essentially irreversible, yielding a complex that is stable even under stringent conditions (Lang *et al.*, 2006; Los, 2005; Los & Wood, 2007). The HaloTag® protein is a 33kDa monomeric protein not endogenous to mammalian, plant or *E. coli* cells, resulting in lower levels of background staining and therefore high labeling specificity (Los & Wood, 2007).

Due to the tagged reporter, tracing of the expressed proteins was possible by labelling the transfected cells with a HaloTag ligand (HaloTag TMR). 293T cells were transfected according to the method optimized for FuGENE® 6 Transfection Reagent. The subcellular localization of the Vpr proteins was studied by 2 fluorescent dyes including DAPI (blue emission spectrum) and HaloTag TMR (red emission spectrum). DAPI binds to DNA and is commonly used to label the nuclei. The method for analysis used in this study was based on the overlap of the two fluorescent dyes. If the emission spectra of the two fluorescent dyes overlap in labelled transfected cells, it means Vpr localized to the nucleus and if the red dye (HaloTag TMR) does not overlap the blue dye (DAPI), it means Vpr localized outside of the nucleus. Although it has been shown that HaloTag protein does not indicate any specific trend for subcellular localization, the non-specific localization of HaloTag protein, using HaloTag only expression, was studied to ensure the localization of Vpr is not affected by HaloTag protein.

The results indicated that all naturally mutated Vpr proteins localized to the nucleus in a similar manner to the consensus sequence of HIV-1 Vpr subtype C (Figure 3.15). This observation suggest that the mutations L22I (in α-helix I), P37T, S41N, Y45H, E48A, E48D (in α-helix II), T55A, I60L, I61L, I63M, I63V (in α-helix III), R85P, and F72Y in C-terminal region did not affect the nuclear localization of Vpr. The naturally mutated Vpr proteins were selected such that mutations in all domains were studied. However, one cannot rule out the role of other domains lodging the mutations in the nuclear localization of Vpr. There could be other amino acids in other positions of α-helix I, II and III, as well as the C-terminal region of Vpr protein that play roles in nuclear localization of Vpr.

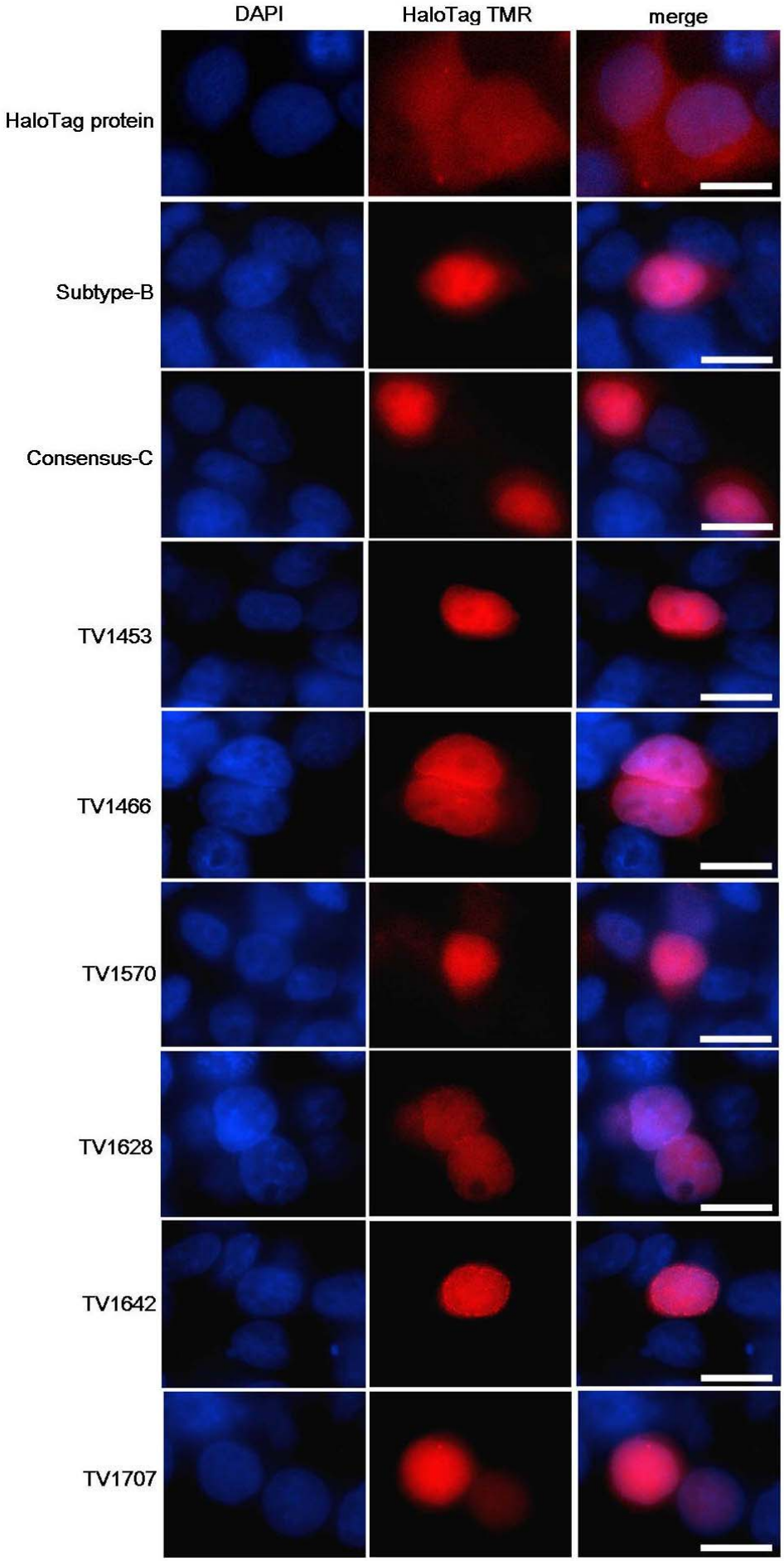

DAPI
HaloTag TMR
merge
HaloTag protein
Subtype-B
Consensus-C
TV1453
TV1466
TV1570
TV1628
TV1642
TV1707

Figure 3.15: 293T cells transfected with Vpr-expression vectors to express naturally mutated Vpr proteins. A sample was also transfected with HaloTag Expression vector as control. Twenty hours after transfection the cells werelabelled with DAPI (blue) and HaloTag TMR. The cells were photographed at 100X magnification with blue filter for DAPI and Red filter for HaloTag TMR. The merged photographs are also provided to show the overlap of the fluorescent dyes. HaloTag protein was localized non-specifically (on top of the figure). All naturally mutated Vpr proteins localized to the nucleus. The scale bars, 20 μm.

The site-directed mutated Vpr proteins containing the mutations P14I, Y47N, Q65H, and Q88S similarly localized to the nucleus (Figure 3.16) suggesting the position of the mutations does not play roles in the nuclear localization of Vpr. However, similar to the naturally mutated Vpr proteins, the role of the mutated domains in the nuclear localization of Vpr cannot be ruled out. It should be noted, however, that a Vpr protein containing the mutation W18C indicated evidence of mislocalization of Vpr to the nucleus. Time is also another factor that can affect localization of Vpr due to the overexpression of Vpr and also apoptosis induction by Vpr if the sample preparing takes long. Cell fixation solves all of these problems for us and provides the free access of fluorescent dyes to cellular compartments (Kozubek *et al.*, 2000). To ensure the mislocalization of this mutant is not due to the early expression or overexpression of the protein it was photographed at different times (Figre 3.17). In this mutated Vpr protein, tryptophan residue in position 18, which is situated at the beginning of α-helix I, had been mutated to cysteine. The mislocalization of W18C suggests that tryptophan 18 in the α-helix I of Vpr may play an essential role in the nuclear localization of Vpr and mutations in this position could affect the karyophilic property of Vpr.

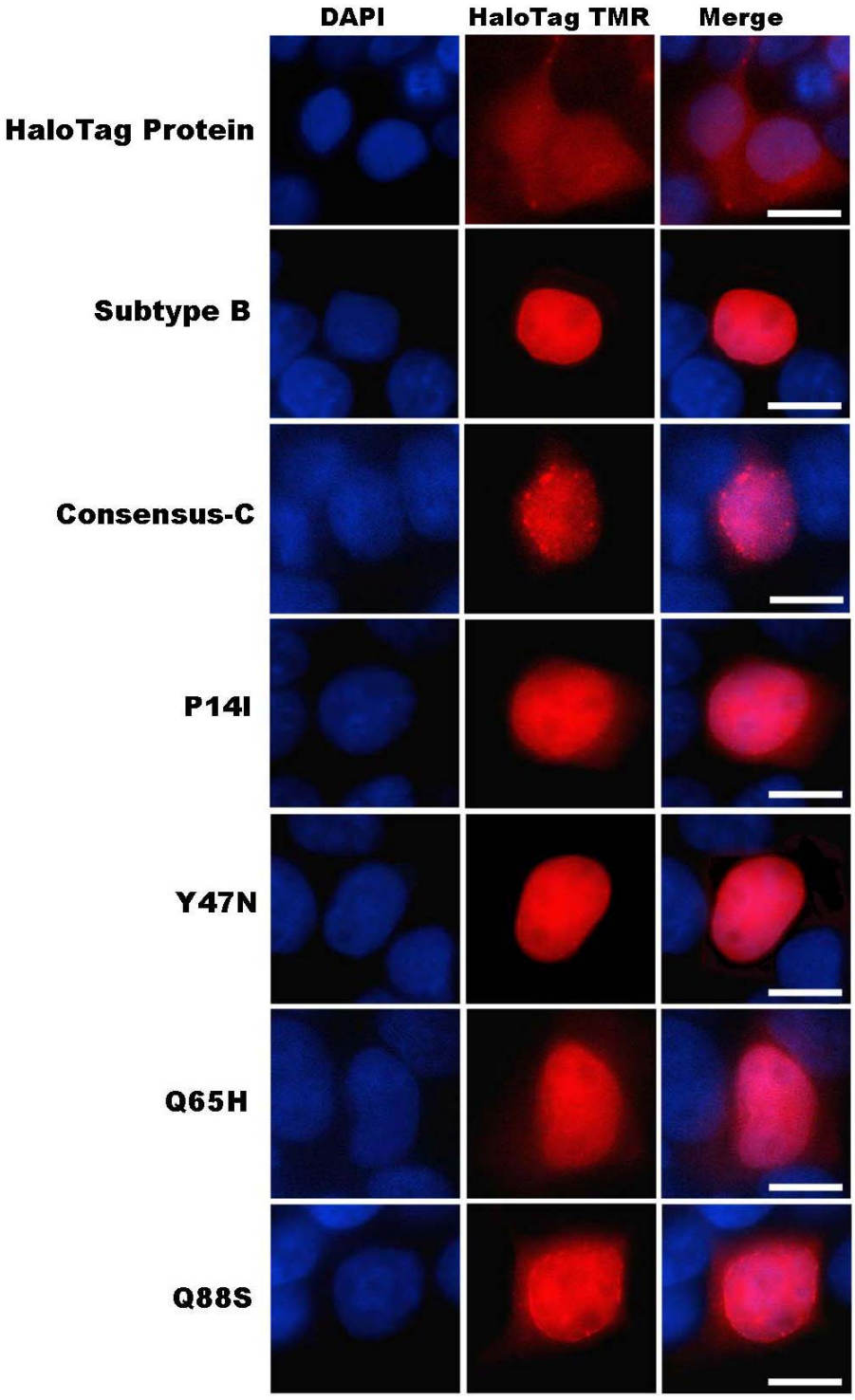


Figure 3.16: 293T cells transfected with Vpr-expression vectors to express site-directed mutated Vpr proteins. A sample was also transfected with HaloTag Expression vector as control. Twenty hours after transfection the cells were labelled with DAPI (blue) and HaloTag TMR. The cells were photographed at 100X magnification with blue filter for DAPI and Red filter for HaloTag TMR. The merged photographs are also provided to show the overlap of the fluorescent dyes. HaloTag protein was localized non-specifically (on top of the figure). The site-directed mutated Vpr proteins that localized to the nucleus are shown here. The scale bars, 20 µm.

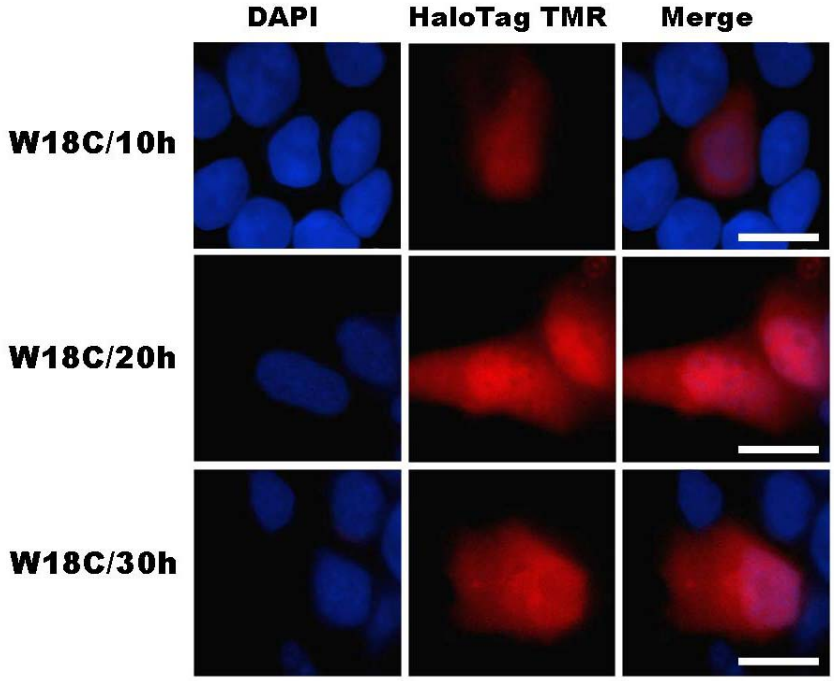


Figure 3.17: Mislocalization of W18C mutant. 293T cells were transfected with the vector to express the site-directed mutated Vpr protein W18C. To ensure the mislocalization of this mutant is not the result of early expression or overexpression of the protein, 10, 20 and 30 hours after transfection the cells were labelled with DAPI (blue) and HaloTag TMR. The cells were photographed at 100X magnification with blue filter for DAPI and Red filter for HaloTag TMR. The merged photographs are also provided to show the overlap of the fluorescent dyes. The W18C mutant localized non-specifically to all the samples. The scale bars, 20 μm.

An interesting phenomenon observed in some of the photographs is that Vpr mostly localizes to the nuclear envelope. The interaction between Vpr and nuclear pore complex (NPC) has been reported (de-Noronha *et al.*, 2001; Le-Rouzic *et al.*, 2002). The interaction of Vpr with the nuclear envelope that was observed in this study can be attributed to the interaction of Vpr with NPC. These interactions appeared as a circle or halo around the nucleus (Figure 3.16, P14I and Q88S). The nuclear localization of Vpr is a time-dependent phenomenon that can also be affected by the amount of the expressed protein. The expression level of Vpr (or any other vector expressed protein) varies in different cells within the same culture leading to slightly different localization results. The high expression levels of Vpr could result in

mislocalization of Vpr in some cells especially when this event is associated with apoptosis and/or damage to the nuclear envelope. Due to this phenomenon, the microscopic photographs of the localization of Vpr may appear slightly different. This means that even if the interaction of Vpr was clearly observed in some cells while it was not observed in others, it cannot be interpreted as mutations inhibiting the interaction of Vpr with the nuclear envelope. The results are interpretable only if Vpr does not localize to the nucleus or localizes in the both the nucleus and cytoplasm of all cells in the same culture. According to these criteria, W18C was the only mutation that disturbed the nuclear localization of Vpr. The mislocalization of the Vpr mutant shows that the mutation of tryptophan 18 in α-helix I to cysteine interferes with the nuclear localization of Vpr and indicated the importance of this residue for this function. The helical wheel of α-helix I (Figure 3.18) shows that residue 18 lies on the hydrophilic side of the α-helix I, mostly dominated by Arginine (R) and glutamic acid (E). The mutation of tryptophan to cysteine (W18C), imbalances the hydrophilic property of the chain that results in the reduction of some interactions necessary for the nuclear localization of Vpr.

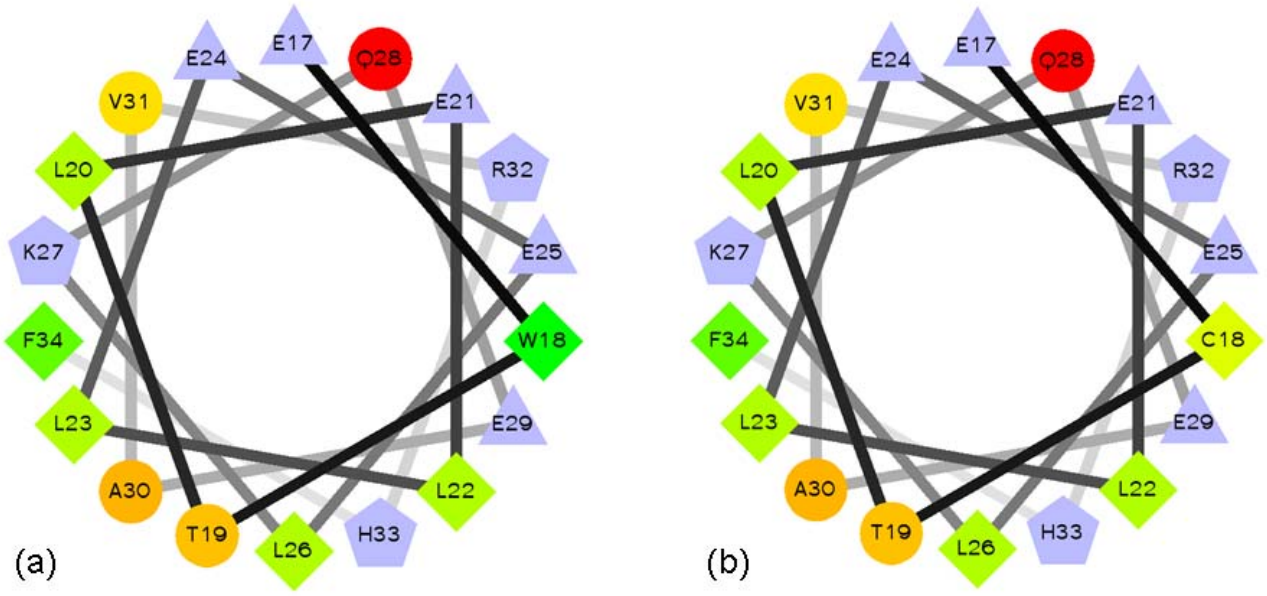


Figure 3.18: Shematic helical wheel diagram illustrating the α-helix I of (a) the consensus-C and (b) W18C. Hydrophobicity is color coded, meaning the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue. The difference between the two α-helices is residue 18 that is in the hydrophilic side of the α-helix I. Mutation of tryptophan 18 (hydrophobic) to the less hydrophobic amino acid cysteine imbalances the hydrophobicity of the peptide chain.

3.12. Assay of Vpr-induced apoptosis by flow cytometry

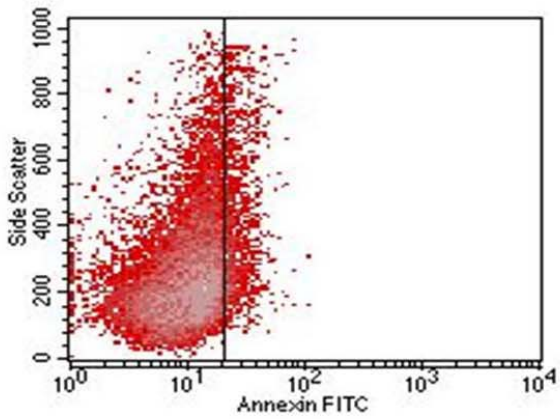
Camptothecin is a cytotoxic quinoline alkaloid which inhibits the DNA enzyme topoisomerase I (topo I). It is isolated from the bark and stem of *Camptotheca acuminata* (Camptotheca; Happy tree), a tree native in China. Camptothecin is a strong apoptosis inducer that is widely used to induce and study apoptosis (Hsiang *et al.*, 1985; Wall *et al.*, 1966). In this study camptothecin induced apoptosis in about 20% of the camptothecin treated cells 24 hours after treatment. The method for apoptosis assays was first optimized in camptothecin-induced apoptotic cells.

The apoptotic cells were also labeled with 7-AAD for late apoptotic or necrotic cells. Due to the interference of HaloTag TMR ligand with 7-AAD, this combination was not continued. Since apoptosis was induced using the Vpr-expression vectors and a very low level of apoptosis was observed in mock-transfected cells, only annexin V was used to confirm the induction of apoptosis in transfected cells.

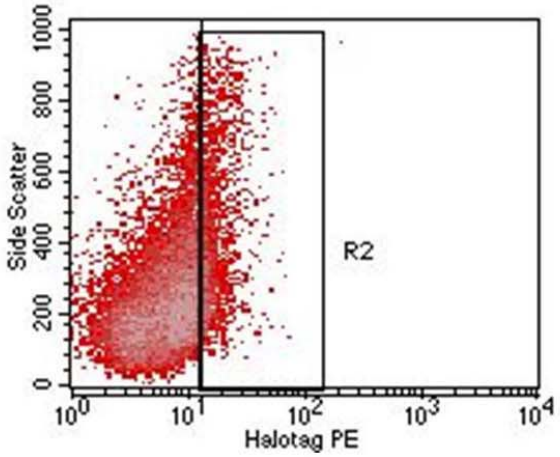
Once the method for apoptosis assay was optimized, apoptosis was assayed in all the transfected cells and compared with the mock transfected sample, 293T cells transfected with barnase-deleted pFN22K vector. The transfected 293T cells were distinguishable from untransfected cells based on the signal of HaloTag® TMR ligand. The transfected cells were gated and the percentage of apoptotic transfected cells was determined (Figure 3. 19).). In the absence of a suitable antibody for Western blot analysis, the mean fluorescence intensity of the HaloTag signal was used as a surrogate marker. The intensity of the HaloTag signal was similar in all the transfected cells (22.21±2.46), which would appear to indicate that the expression level was similar in individual transfected cells (Figure 3.20). This is important since different expression levels in individual cells could influence the level of expressed protein and thus the functional responses related to protein expression (in this case induction of apoptosis and cell cycle G2 arrest by Vpr). Based on the mean values of 3 independent experiments (Table 3.3 & 3.4), the lowest percentage of apoptotic cells was found in the mock sample with 0.08% of transfected cells being apoptotic (Figure 3.21). In all Vpr-transfected samples the percentage of apoptotic cells was close to the percentage of the transfected cells suggesting that the majority of the transfected cells are apoptotic. This was shown by gating the transfected cells and measuring of apoptosis in the gated cells. This result indicated that the EDTA treatment to harvest

the adherent cells did not result in damage to cell membranes and the consequent generation of spurious results. Interestingly, the lowest percentage of apoptotic cells among all the Vpr-transfected cells was found in the cells transfected with the consensus sequence of subtype C with 82.08% apoptotic cells ($p<0.05$). Subtype B Vpr induced apoptosis in 98.20% of the transfected cells. The percentage of apoptotic cells detected for the other Vpr variants was similar to one another and subtype B Vpr ($p>0.05$) ranging from 96.54% for TV1466 to 98.64% for TV1628. These values are highly dependent on expression level of the Vpr proteins, however in the absence of confirmatory measurement of protein expression by Western blot we have assumed similar levels of expression based on intensity of the HaloTag signal across experiments.

A



B



C

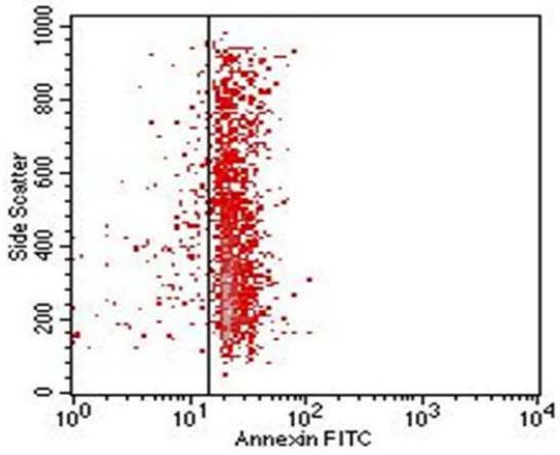


Figure 3.19: Representative dot plots showing apoptosis induction in 293T cells transfected with a Vpr-expression vector. (A) Total annexin on cells gated based on scatter (G1=R1). (B) Halotag staining of the same population, but showing the gate R2 used to define Halotag positive or transfected cells. (C) Annexin V staining of the transfected cells gated in R2.

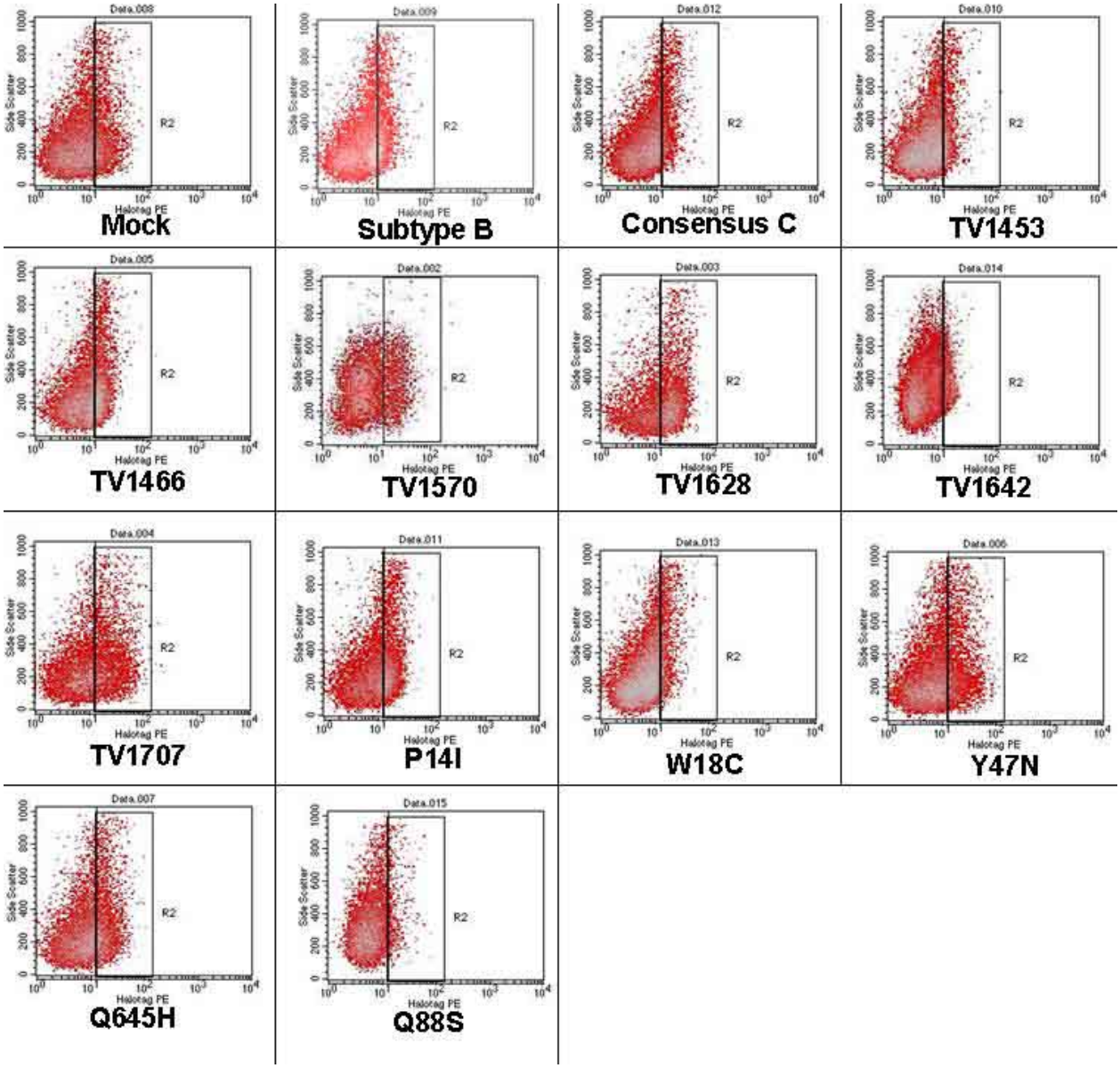


Figure 3.20: Representative dot plots showing the transfected 293T cells. 293T cells were transfected with Vpr-expression vectors. The transfected cells were labelled with HaloTag TMR ligand. Apoptotic cells were measured only in the transfected cells. The similar localization of the cells on the dot plots suggests that the intensity of HaloTag signal is similar and therefore the expression level of the vectors is comparable.

Table 3.3: Results of the flow cytometric analysis for apoptosis.

Sample	Total apoptotic cells (annexin V positive) (%)	Total non-apoptotic cells (annexin V negative) (%)	Total transfected cells (HaloTag positive) (%)	Total untransfected (HaloTag negative) (%)	Apoptotic transfected cells (%)
Mock	0.08	99.92	34.45	65.55	0.08
Subtype B	24.73	75.27	24.08	75.92	98.20
Consensus C	15.66	84.34	18.04	81.96	82.08
TV1453	16.71	83.29	16.53	83.47	95.33
TV1466	26.87	73.13	26.93	73.07	96.54
TV1570	22.78	77.22	22.28	77.72	97.56
TV1628	38.10	61.90	37.10	62.90	98.64
TV1642	20.89	79.11	19.71	80.29	91.91
TV1707	38.68	61.32	41.01	58.99	98.58
P14I	16.08	83.92	17.16	82.84	92.36
W18C	19.83	80.17	19.75	80.25	93.91
Y47N	32.29	67.71	35.67	64.33	96.25
Q65H	16.44	83.56	18.49	81.51	90.865
Q88S	15.55	84.45	16.56	83.44	88.485

Table 3.4. Results of 3 independent apoptotic assays

Sample	Average	Deviation	Assay 1	Assay 2	Assay 3
Mock sample	0.08%	0.0003	0.05%	0.07%	0.12%
Subtype B	98.20%	0.0081	98.16%	99.03%	97.40%
Consensus-C	82.08%	0.0264	80.20%	85.11%	80.94%
TV1453	95.33%	0.0074	95.69%	95.83%	94.47%
TV1466	96.54%	0.0081	97.46%	96.28%	95.89%
TV1570	97.56%	0.0020	97.52%	97.79%	97.38%
TV1628	98.64%	0.005	99.03%	98.92%	97.98%
TV1642	97.91%	0.0044	97.49%	98.38%	97.86%
TV1707	98.58%	0.0013	98.63%	98.68%	98.42%
P14I	92.36%	0.0233	90.70%	95.03%	91.35%
W18C	93.91%	0.0027	93.97%	93.61%	94.15%
Y47N	96.25%	0.0227	93.89%	98.43%	96.44%
Q65H	90.86%	0.018	89.42%	92.90%	90.25%
Q88S	88.48%	0.0317	90.55%	84.82%	90.06%

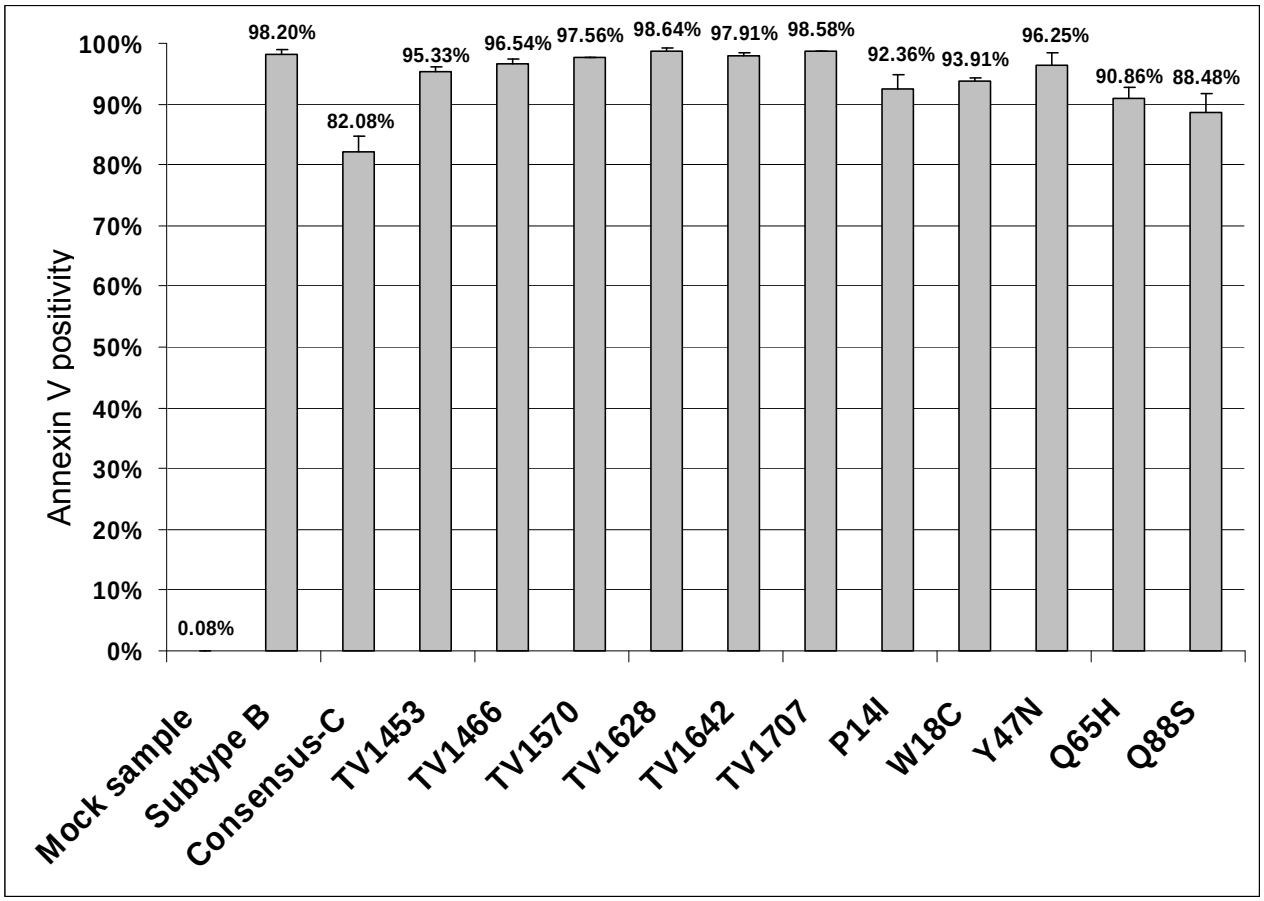


Figure 3.21: The bar graph showing apoptosis induction in 293T cells by Vpr. The first sample on the left shows the percentage of apoptotic cells in mock sample which was prepared by transfection of 293T cells with barnase-removed pFN22K vector. The error bars indicate the standard deviations in the reported measurements. Data shown represent average values of three repeats for each variant. Among the vpr mutants, the consensus sequence of subtype C induced apoptosis in 82.08% of the transfected cells which is lower than the other Vpr proteins. All the variants the induced apoptosis in a higher percentage of the transfected cells compared to the consensus-C as it was shown by ANOVA (p<0.05).

3.13. Assay of Vpr-induced cell cycle G2 arrest

Cell cycle was analyzed using 2 fluorescent dyes at different times. The optimization experiments showed that because of the overlap between PI and HaloTag® Oregon Green® Ligand that interfered with our assay, these 2 dyes could not be used in together. On the other hand our experiments using 7-AAD and HaloTag® Oregon

Green® Ligand showed no sign of spectral overlap indicating these 2 dyes could be used together in our cell cycle assays.

The assays performed 24 hours after transfection showed a low percentage of G2 arrested cells (40-50%) while 48 hours after transfection we obtained high percentage of G2 arrested cells. The optimized time for our assays was then set as 48 hours after transfection.

Different values of induction of cell cycle G2 arrest were observed in the 293T cells transfected with different Vpr-expression vectors. Observed differences could be due to different expression level in the individual cells. Therefore, in the absence of confirmatory Western blot data, these values should be cautiously interpreted. The typical results of cell cycle analysis by flow cytometry are presented in Figure 3.22 for one assay. The values of cell cycle G2 arrest are presented as bar graphs in Table 3.5 and Figure 3.23 for three independent assays. Only the transfected cells (i.e. HaloTag positive) were gated for analysis based on the signal of HaloTag® Oregon Green® ligand. The intensity of HaloTag® Oregon Green® signal was similar in the transfected cells (448.17±11.61), suggesting that the expression level in the transfected cells was similar.

The cell cycle phase was inferred based on the DNA content labeled with 7-AAD. All the results were compared with the mock transfected sample which was 293T cells transfected with barnase-removed pFN22K vector. The DNA content of the mock sample indicated that the majority of the cell population was at G1/S phase. In the mock sample only 32.45% of the cells were in the G2 phase. The highest proportion of cells in the G2 phase (71.57%) was observed for subtype B Vpr. This result shows

the majority of subtype-B Vpr transfected cells shifted to G2 phase. This result, however, was not significantly higher than that of the majority of the Vpr proteins (p>0.05). The consensus sequence of subtype C Vpr induced G2 phase in 61.65% of the transfected cells. The lowest value of G2 phase was observed for the TV1466 with 54.05% of the cells in G2 phase that was significantly lower than all naturally mutated Vpr proteins (p<0.05) except fpr TV1628. This Vpr protein had mutations in α-helix I and II and the flexible C terminus. TV1570 and TV1642 that each had one mutation in each α-helix, induced G2 phase in 66.36% and 63.84% of the transfected cells, respectively. TV1453, TV1628 and TV1707 induced G2 phase in 65.28%, 61.51% and 61.60% of the transfected cells, respectively. Except for TV1453 that had Y45H mutation in α-helix I, the two latter proteins had several mutations distributed in different domains. All variants of Vpr proteins were thus able to induce G2 arrest but at different levels. Although the induction of G2 arrest by subtype B Vpr was higher than the consensus sequence of subtype C and all the subtype C variants, however this was not statistically significant for TV1453, TV1570 and TV1642 (p>0.05). The induction of G2 arrest by consensus C was found in the same range of Vpr mutants but lower than Subtype B (p<0.05). None of the induced mutations significantly affected the G2 arrest activity of Vpr. All these values should be interpreted based on the assumption of similar expression levels of the proteins. Although some values were found significantly different, it should be noted that the uneven expression level of the proteins could increase/decrease the induction of cell cycle G2 arrest. Dissimilar expression levels of the protein could have a major impact on a functional read-out and may play a role in the less efficient induction of cell cycle G2 arrest by some of the subtype C Vpr proteins.

Except for W18C, the site-directed mutations showed similar ability to induce G2 arrest. W18C mutant induced G2 arrest in 56.65 % of the transfected cells. Although it was not found significantly lower than that of other site-directed mutations, it was significantly lower than that of subtype B Vpr.

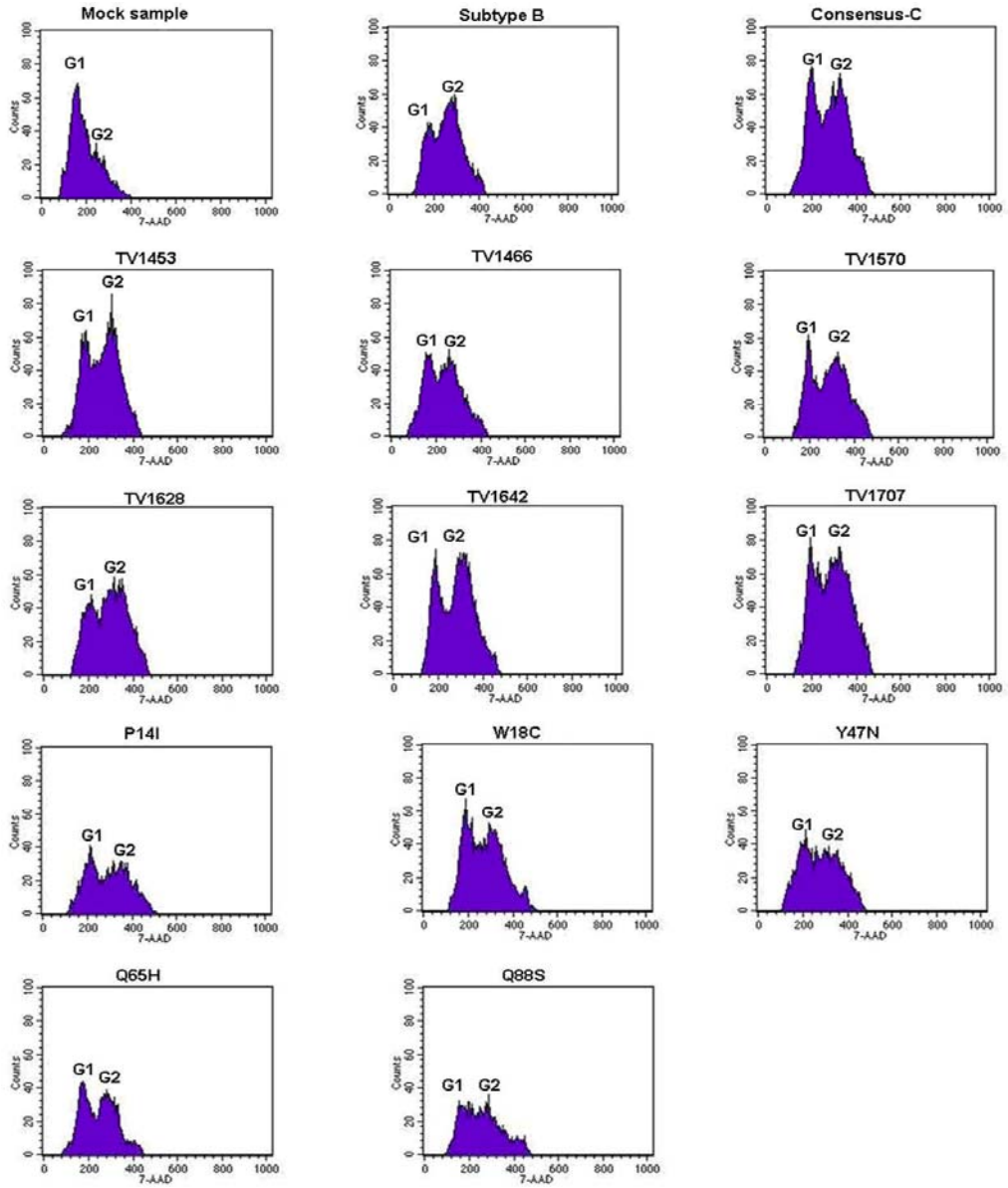


Figure 3.22: Flowcytometric analysis of cell cycle in 293T cells transfected with Vpr-expression vectors. Forty eight hours after transfection, cell cycle was analyzed in the transfected cells. The HaloTag-Vpr proteins were labaled using HaloTag® Oregon Green® Ligand and the DNA content was labelled using 7-AAD. The transfected cells were gated based on the HaloTag signal and cell cycle was analyzed only in the transfected cells. The first peak in each graph indicates the cells at G1/S phase and the second peak indicates the cells at G2 phase. Mock sample is a cell culture of 293T cells transfected with the HaloTag expression vector as control.

Table 3.5: Results of three independent assays for Vpr-induced G2 arrest

Sample	Average G2 arrested cells (%)	deviation	G2 arrest Assay 1 (%)	G2 arrest Assay 2 (%)	G2 arrest Assay 3 (%)
Mock sample	32.45	0.007	32.68	31.65	33.01
Subtype B	71.57	0.015	71.24	70.20	73.28
Consensus-C	61.65	0.011	60.46	62.68	61.81
TV1453	65.28	0.022	63.05	65.31	67.49
TV1466	54.05	0.063	52.58	48.59	60.97
TV1570	66.36	0.026	64.59	65.07	69.41
TV1628	61.51	0.034	58.09	61.40	65.03
TV1642	63.84	0.021	61.42	64.87	65.24
TV1707	61.60	0.022	60.00	60.65	64.15
P14I	65.76	0.071	60.09	63.43	73.76
W18C	56.65	0.007	56.59	55.91	57.46
Y47N	66.67	0.068	66.44	59.93	73.64
Q65H	61.26	0.022	58.84	61.62	63.33
Q88S	62.26	0.056	57.62	60.53	68.62

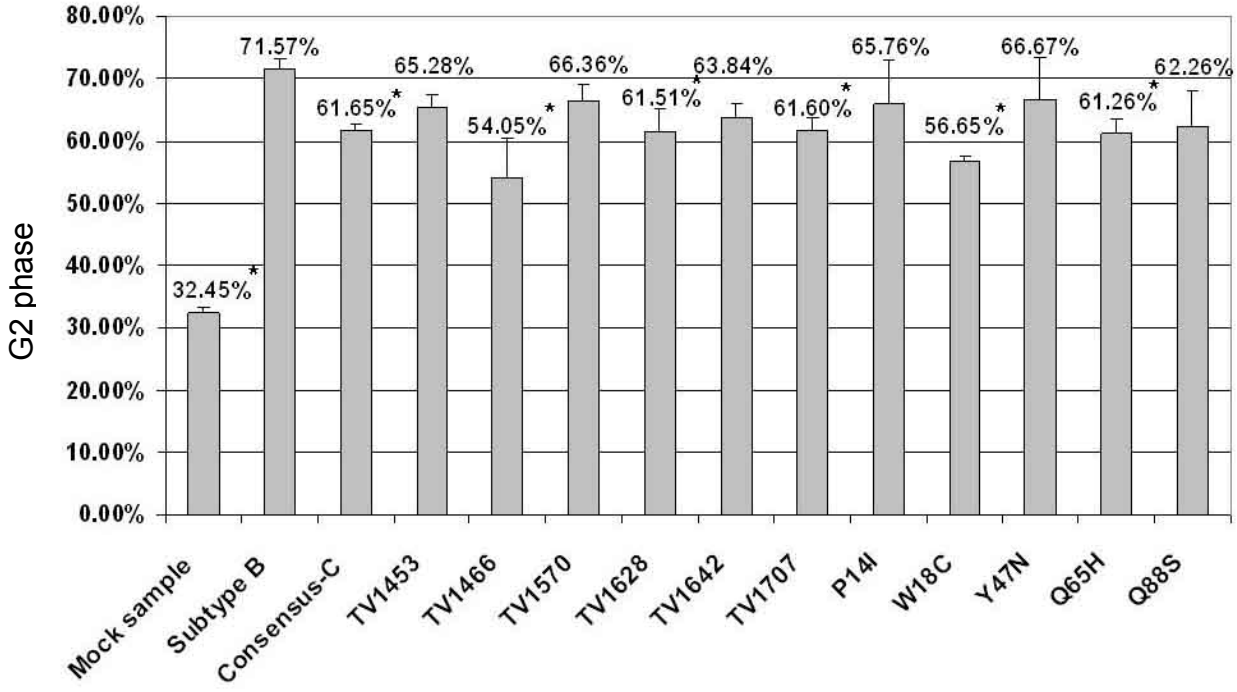


Figure 3.23: Bar graphs showing the induction of G2 arrest in 293T cells transfected with Vpr-expression vectors. The highest percentage of G2 phase was observed in the cells transfected with subtype B Vpr. The lowest percentage of G2 phase was observed in mock samples. Mock samples were cell cultures of 293T cells transfected with the HaloTag expression vector as control. The asterisks (*) indicate the variants that induced G2 arrest in a lower percentage of the transfected cells compared to subtype B Vpr as it was shown by ANOVA (p<0.05).

3.14. Modulation of gene expression by Vpr

The real-time PCR assay of several genes, with a focus on the genes involved in apoptosis, was performed for 293T cells transfected with Vpr-expression vectors. Since the values are highly dependent on the efficiency of transfection, which could vary, they cannot be directly compared. However, it is useful to indicate the overexpression of several genes triggered by Vpr proteins. The results presented in Table 3.1 show that the consensus sequence of subtype C and all the subtype C Vpr variants, similarly to subtype B, are able to modulate the examined genes.

In this study, the modulation of apoptosis-regulating genes was specifically addressed. As discussed in more detail in Chapter 1, apoptosis, in general, is triggered through one of two known cell death-signaling pathways: the extrinsic (or death receptor) pathway and the intrinsic (or mitochondrial) pathway (Fulda & Debatin, 2006; Ghobrial *et al.*, 2005). As reviewed by Romani and Engelbrecht, Vpr induces apoptosis through the intrinsic pathway (Romani & Engelbrecht, 2009). In this study, the mRNA profile of 293T cells for intrinsic and extrinsic pathway as well as anti-apoptotic genes was examined. Data is presented in Table 3.6 and Figures 3.24 and 3.25. The overexpression of caspase-3 and -6 was observed in the transfected cells. Both caspases are involved in both extrinsic and intrinsic pathways (Klaiman *et al.*, 2009; Legewie *et al.*, 2006; Zimmermann *et al.*, 2001). No significant overexpression of caspase-7, -8, and -10 by any of Vpr variants was observed. Caspase-7 has been shown in both apoptosis pathways (Bhat *et al.*, 2006; Du *et al.*, 2006) and Caspase-8 and -10 have been shown in the extrinsic pathway (Bhat *et al.*, 2006; Kischkel *et al.*, 2001). The expression level of caspase-9, which is involved in the intrinsic pathway (Du *et al.*, 2006), remained unchanged in all the transfected cells. Although the

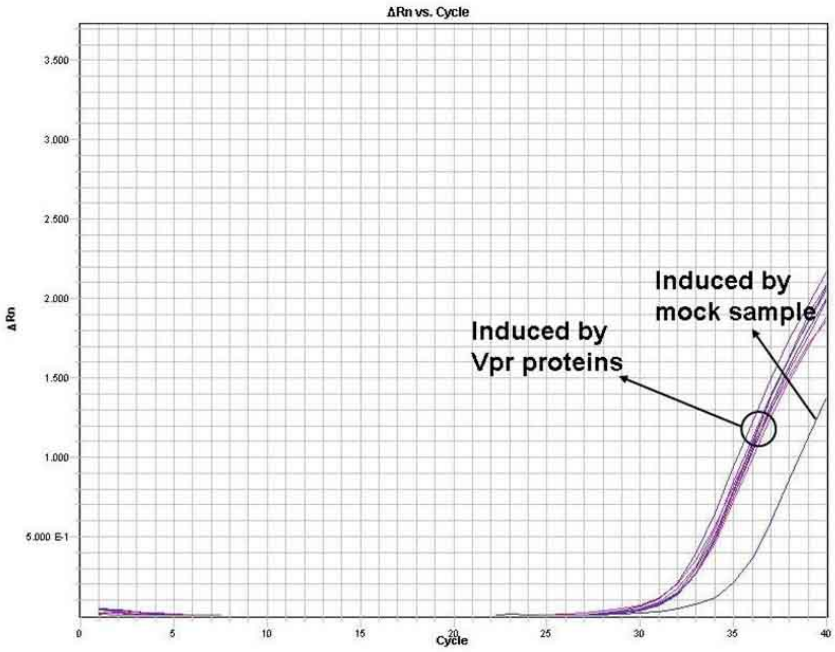
induction of apoptosis by Vpr through the intrinsic pathway was not strongly supported by the expression level of caspases, the significant overexpression of BID (Bcl-2 interacting domain) was shown in all variants. BID is a pro-apoptotic Bcl-2 family member that triggers the intrinsic pathway of apoptosis by promoting cytochrome *c* release from mitochondria (Slee *et al.*, 2000; Yi *et al.*, 2003).

Table 3.6. mRNA profile of 293T cells 24 hours after transfection with Vpr-expression vectors.

The average fold change is presented for each gene and each sample.

Accession number	Gene name	Fold change												
		Sub-B	Con-C	TV1453	TV1466	TV1570	TV1628	TV1642	TV1770	P14I	W18C	Y47	Q65H	Q88S
NM004322.3	BAD	1.60	2.54	2.02	2.91	2.38	2.21	2.84	1.60	1.25	1.56	2.50	1.98	1.78
NM001139441	BCAP31	1.95	3.52	1.40	1.07	2.09	2.1	3.64	2.0	3.20	1.23	1.56	2.54	3.75
NM207002.2	BCL2L11	1.92	2.62	1.92	3.91	1.61	2.21	2.68	2.35	2.54	2.98	3.51	1.54	1.84
NM197967.1	BID	17.22	25.12	22.14	19.69	20.36	21.56	13.83	19.82	18.56	25.63	25.60	21.42	24.78
NM001197.3	BIK	1.12	1.52	0.81	0.69	2.79	0.98	2.81	0.96	0.95	0.87	1.25	0.78	0.90
NM182962.1	BIRC3	1.52	2.32	1.14	1.52	1.61	1.49	2.52	1.26	1.27	1.84	1.56	2.71	1.58
NM004052.2	BNIP3	6.25	8.52	7.51	9.32	1.48	6.66	2.73	7.58	2.54	5.26	5.04	7.16	7.84
NM004331.2	BNIP3L	1.23	1.45	0.89	1.12	1.33	1.25	2.91	1.43	1.25	2.15	1.91	1.71	1.64
NM022162.1	CARD15	2.12	2.66	2.72	1.538	0.95	2.16	2.71	2.51	2.48	2.58	1.89	1.78	1.92
NG007497.1	CASP10	1.18	1.54	1.714	1.06	0.87	1.86	1.38	1.54	1.54	1.92	1.26	1.47	1.44
NM032991.2	CASP3	2.85	4.59	1.93	1.416	1.71	2.54	3.54	2.47	4.15	3.15	1.59	1.19	4.77
NM032992.2	CASP6	3.19	4.15	2.95	1.57	1.81	3.42	5.32	2.56	2.52	3.25	3.41	2.48	1.87
NM033338.4	CASP7	1.03	1.69	0.92	0.85	0.91	1.14	1.56	1.14	0.94	0.57	0.94	1.59	0.71
NM001228.4	CASP8	1.12	1.82	1.77	1.11	0.84	0.84	1.14	1.43	0.95	1.47	1.23	1.56	1.48
NM00113766	CASP8AP2	12.25	15.32	14.99	13.63	8.00	14.32	7.60	12.97	11.25	14.21	15.72	15.80	16.08
NM001229	CASP9	0.96	1.25	1.10	1.17	1.48	0.94	1.72	1.52	1.20	1.58	0.80	0.74	1.41
NM145074.2	HTRA2	1.96	4.53	2.00	1.69	1.33	2.01	2.73	2.13	2.46	1.42	1.05	2.08	2.44
NM014002.2	IKBKE	85.54	96.54	27.42	11.31	141.22	45.56	25.18	75.12	55.24	19.54	42.33	47.24	80.24
NM021960	MCL1	8.47	8.51	5.33	4.91	8.18	8.74	9.93	6.58	5.25	5.46	4.82	6.21	7.10
NM003998.2	NFKB1	3.71	3.65	5.75	1.56	1.75	4.25	5.74	4.29	1.23	5.23	5.21	2.35	2.02
NM00100171	NFKBIB	8.49	11.31	18.11	11.48	3.01	5.64	5.53	5.39	5.80	4.61	9.58	4.21	8.27
NM002908.2	REL	5.65	6.32	3.36	5.42	1.23	4.22	3.06	3.51	3.62	3.10	4.95	3.51	5.04
NM006509.2	RELB	14.21	17.32	22.64	14.25	6.83	11.25	14.12	8.52	10.16	15.21	9.22	11.37	9.38
NM003804.3	RIPK1	3.62	4.25	4.76	3.57	2.35	3.94	2.69	4.22	2.56	2.51	3.65	5.28	2.51

(a)



(b)

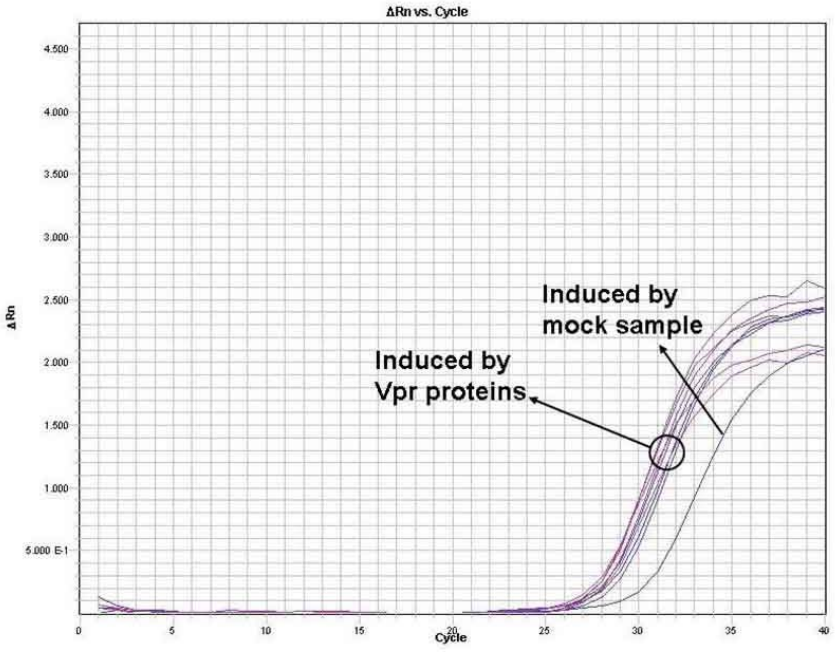


Figure 3.24: Overexpression of caspases induced by Vpr Proteins in 293T cells. (a) The expression of Vpr proteins in the transfected cells increased the expression of caspase-3. (b) The expression of Vpr proteins in the transfected cells increased the expression of caspase-6.

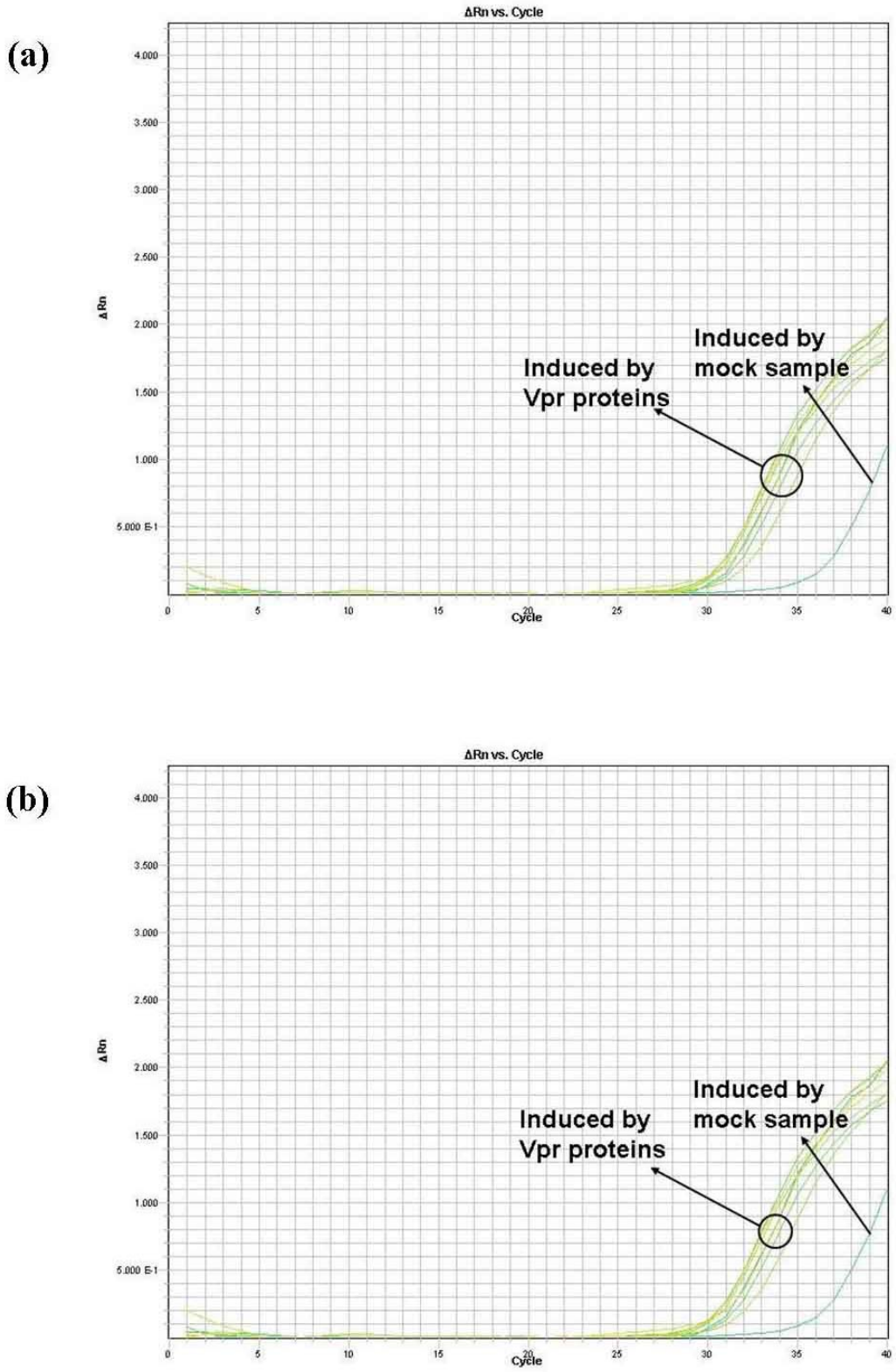


Figure 3.25: Overexpression of BID and BCL-10 induced by Vpr proteins in 293T cells. (a) Subtype B and subtype C Vpr proteins induced the overexpression of BID. (b) Subtype B and subtype C Vpr proteins induced the overexpression of BCL-10.

Chapter 4

4. Discussion and conclusion

4.1. Sequences of South African HIV-1 strains

Heterosexual contact is the major route of HIV-1 transmission in sub-Saharan Africa. In South Africa, unlike the rest of sub-Saharan Africa, HIV-1 was initially transferred by homosexual contact (Van-Harmelen *et al.*, 1997). The first two South African cases of AIDS, recorded in 1982, were male homosexuals (Ras *et al.*, 1983), and until 1987 HIV-1 was diagnosed almost exclusively in men (Van-Harmelen *et al.*, 1997). In 1988, an increasing number of HIV-infected women were reported, indicating the start of a second epidemic. Migration is one of many factors that have contributed to the AIDS epidemic in this region. Several studies have shown that people who are more mobile or who have recently changed residence tend to be at higher risk for HIV and other sexually transmitted diseases (STDs) than people in more stable living arrangements (Lurie *et al.*, 2003). In South Africa, people who had recently changed their residence were 3 times more likely to be infected with HIV than those who had not (Abdool-Karim *et al.*, 1992). It has been argued that it is not so much the movement itself but rather the "conditions and structure of the migration process" that put people at risk for HIV and other STDs (Lurie *et al.*, 2003).

Phylogenetic analyses of nucleotide sequences from the *env* gene have resulted in the classification of group M strains into at least 9 different envelope genetic subtypes, designated by letters from A to K. However, studies of the HIV-1 full-length genome sequences have demonstrated complex patterns of HIV-1 intersubtype recombination,

suggesting that the present classification requires more precision with regard to the definition of HIV-1 subtypes and intersubtype recombinants (Osmanov *et al.*, 2002). In South Africa, subtype C was the first HIV genotype that was identified in the heterosexual population and was subsequently shown to be the major virus subtype within this country (Williamson *et al.*, 1995). HIV-1 Subtype B strains, commonly found in Europe and America, are not often detected in Africa. Study of genetic heterogeneity is important in the development and application of vaccines whose efficacy could be influenced by virus variation (Mascola *et al.*, 1994).

Scriba *et al*. have studied the sequences of South African HIV-1 accessory genes, *vif*, *vpr* and *vpu*. They showed that the amino acid alignments of the Vif, Vpr, and Vpu from South African strains were well-conserved. The Vpr sequences of South African strains in this study were all shown to belong to HIV-1 subtype C (Scriba *et al.*, 2001). The finding that HIV-1 subtype C Vpr sequences were highly conserved, was confirmed in a subsequent study be Bell *et al*. (Bell *et al.*, 2007).

In the current study, phylogenetic analysis of Vpr characterized 54/58 strains (93%) as HIV-1 subtype C and 4/58 strains (7%) as HIV-1 subtype B. Except for one subtype C, Vpr was confirmed to be conserved in length in all other subtype C isolates (53 in total) and all 4 subtype B isolates. Fifty three subtype C isolates (98%) showed the heterosexual pattern which is the predominant form of subtype C transmission. Two out of 4 subtype B strains (50%) showed the homosexual pattern of transmission. This is in agreement with the homosexual pattern of transmission previously observed for subtype B in this region.

4.2 Physiological relevance of the model system used in this study

HIV-1 infects T cells and monocytes in both a latent and productive manner (Makonkawkeyoon *et al.*, 1993). Therefore *in vitro* experiments that address HIV-1 should attempt to be physiologically relevant to the *in vivo* infections. *In vitro* studies of T cell and monocyte derived cell lines may be more relevant to actual infection than 293T cells. Jurkat T cells and U937 cells, a T cell line and monocytic cell line, respectively, and also peripheral blood mononuclear cells are commonly used in *in vitro* studies of HIV-1. (Borgatti *et al.*, 2005; Ciuffi *et al.*, 2005; Furtado *et al.*, 1999; Wang *et al.*, 1999). However, the results of experiments using these lines or cells may not be representative of actual *in vivo* conditions since the complex interactions between different cells, serum proteins, cytokines, metabolites and other factors cannot be accurately reproduced.

Human 293T cell line is commonly used in functional studies of HIV-1 proteins. A major reason for this is the ability to easily transfect these cells, the same reason why they were selected for use in the current study (Gavrilescu & Van-Etten, 2007). Vpr targets fundamental cellular pathways common to many eukaryotic cells. It has been shown that Vpr could exert its functions even in yeast cells and these cells have been frequently used to study the functions of Vpr (Benko *et al.*, 2007; Elder *et al.*, 2001; Huard *et al.*, 2008). Functional studies of Vpr in 293T cells are very common (Belzile *et al.*, 2007; Felzien *et al.*, 1998; Kino *et al.*, 2005b). However, since 293T cells are not the natural hosts of HIV-1, the results of such studies should ideally be compared with results obtained using natural HIV host immune cells most accurately mimicking *in vivo* conditions. The outcomes of this study need to be reproducible and relevant to the actual infection. Although the impact of the expressed Vpr proteins on 293T cells cannot be directly linked to *in vivo* infections, the impact of mutations on *in vitro*

functionality of subtype C Vpr proteins provides an indication of cellular activity and provides a base for future experiments. Infecting T cell and monocyte derived cells with pseudotyped viruses that carry or do not carry Vpr would be one important approach for further studies.

In this study the differences observed between the Vpr proteins may not be indicative of *in vivo* infection but they do suggest that not all Vpr proteins have the same functional potential. Therefore, these results emphasize that natural Vpr mutants need to be compared *in vivo* or in natural host cells. On the other hand since in this study, Vpr proteins have been expressed using expression vectors, the results may to some extent exaggerate the impact of the virus-expressed proteins. To obtain a reliable comparison between the Vpr proteins, Vpr proteins could be compared in pseudotyped viruses that carry or do not carry the Vpr proteins.

4.3. Subtype C Vpr variants

Vpr is a dynamic mobile protein that shuttles between the nucleus and cytoplasmic compartments (Jenkins *et al.*, 2001; Sherman *et al.*, 2003a; Sherman *et al.*, 2001). The karyophilic property of subtype B Vpr and its localization to the nuclear pore complex (NPC), has been well established and it is believed to be a prerequisite for the nuclear import of PIC (Fassati *et al.*, 2003). However, the karyophilic property of Vpr needs to be addressed in other subtypes and mutants as well. Up to now, this property of Vpr has not been shown for other HIV-1 subtypes or mutants. In this study, it was shown that subtype C variants or mutants show similar affinity for the nucleus.

Induction of G2 arrest by Vpr has been documented in many studies. The detailed mechanism of this phenomenon has also been studied but remains not fully

characterized, with novel details still being revealed (Belzile *et al.*, 2007; Dehart *et al.*, 2007; Kino *et al.*, 2005a; Zhou & Ratner, 2000). However, the previous studies have investigated the cell cycle G2 arrest in subtype B Vpr and there is a lack of data regarding the induction of cell cycle G2 arrest by other HIV-1 subtypes. The ability of Vpr variants to induce G2 arrest is another question that has not been addressed in previous studies. In this study, it was shown that subtype B Vpr induces the cell cycle G2 arrest more efficiently than most subtype C Vpr variants. The consensus sequence of subtype C Vpr indicated no significant deviation from the average G2 arrest that was observed for the other subtype C variants. However, as stressed in the results section, the results of this experiment were highly dependent on the similarity of the expression level of the Vpr proteins in the individual cells. Different expression levels may affect the arresting of the cell cycle in a particular cell. Western Blot is a common method to check the expression level of proteins, but in this study the expression level of the proteins were compared based on HaloTag Oregon Green signal. Therefore the results should be evaluated within the context of similar expression levels based on the surrogate marker of expression, intensity of HaloTag signal. The intensity of the HaloTag Oregon Green signal was similar in the transfected cells, suggesting the obsereved differences were most likely not due to the different expression level. In contrast to apoptosis, mutations in Vpr did not significantly increase the ability of Vpr variants to induce G2 arrest.

Mutational analysis has shown that the C-terminal portion of Vpr, known to harbour its cell cycle-arresting activity, binds directly to 14-3-3 protein. 14-3-3 proteins regulate the G2/M transition by activating Cdc25C. The interaction of the C-terminus of Vpr promotes the association of 14-3-3 and Cdc25C, leading to the triple complex of Vpr/14-3-3/Cdc25C that is not able to propel the cell cycle (Bolton *et al.*, 2008;

Kino *et al.*, 2005b). In the current study, TV1466 and TV1628 that had R85P mutations in their C-terminus did not show lower potential to induce G2 arrest compaered to the majority of the naturall mutants of subtype C Vpr.

The C-terminal region of Vpr is probably not the only region of this protein that affects G2 arrest. Several studies have documented that the association of HIV-1 Vpr with DCAF-1 (DDB1- and CUL4-associated factor 1), also known as VprBP (Vpr-binding protein), leads to G2 arrest (Gieffers *et al.*, 2000; Hrecka *et al.*, 2007; Wen *et al.*, 2007). It has been shown that the Vpr (Q65R) mutant, which is defective in DCAF1 binding, undergoes proteasome-mediated degradation at a higher rate than wild-type Vpr. Therefore mutations outside of the C-terminal region, such as Q65R could affect the G2 arrest activity of Vpr (Le-Rouzic *et al.*, 2008). In this study, mutations outside of C-terminus region occurred in the variants but their impact could not be individually investigated since multiple mutations occurred in the Vpr proteins.

The modulation of gene expression by Vpr has been well established (Romani & Engelbrecht, 2009; Zhu *et al.*, 2003). In this study, it was shown that subtype C Vpr variants modulate the apoptotic genes in a similar manner to the subtype B Vpr. Furthermore, it was shown that both subtype B and C induce apoptosis through the intrinsic pathway. Although the overexpression of caspases specifically involved in the intrinsic pathway of apoptosis, such as caspase-9, was not detected, the induction of apoptosis might not always be dependent on overexpression of certain gene products, but available gene products. The overexpression of BID that was observed does, however, support the induction of apoptosis through the intrinsic pathway. No evidence for the induction of apoptosis through the extrinsic pathway was found for any of Vpr variants.

The pro-apoptotic activity of subtype C Vpr variants from India has been shown to be similar to that of subtype B Vpr (Bano *et al.*, 2009a). Jian and Zhao by comparing three HIV-1 subtypes demonstrated a moderate level of pro-apoptotic activity for a subtype B Vpr and an A/G subtype recombinant Vpr, whereas a subtype D Vpr had no detectable activity. It has been suggested that Vpr proteins from different HIV-1 subtypes as well as Vpr variants that emerge during HIV-1 infection may have different proapoptotic potentials and contribute to the diversity of AIDS pathogenesis (Jian & Zhao, 2003b). Bano *et al.* have also shown that several B, C and D recombinant Vpr proteins were capable of inducing apoptosis to varying extents (Bano *et al.*, 2009a).

In this current study, it was shown that South African subtype C Vpr variants were able to induce apoptosis at a similar rate to subtype B Vpr. Interestingly; it was found that the consensus sequence of subtype C Vpr induced apoptosis at a lower rate, compared to mutant Vpr proteins and subtype B Vpr. TV1453 that differs from the consensus sequence in one amino acid residue, Y45H mutation in α-helix II, indicated a higher pro-apoptotic activity ($P<0.05$). The other natural mutations at other positions also were shown in this study to potentiate the proapoptotic activity of Vpr. It has been documented that the C-terminal peptides containing the conserved sequence at amino acid 71-82 can even induce apoptosis (Arunagiri *et al.*, 1997). Many of the mutations that were shown in this study to enhance to pro-apoptotic activity of Vpr do not lie at position 71-82. Other studies have also shown that mutations outside of this motif could affect the pro-apoptotic activity of Vpr (Bolton & Lenardo, 2007; Stewart *et al.*, 1997; Tungaturthi *et al.*, 2004a). Many factors including ion channel activity of Vpr, DNA fragmentation factor activation,

interaction of Vpr with other proteins, and localization of Vpr could affect the proapoptotic activity of Vpr (Lum *et al.*, 2003; Piller *et al.*, 1999; Shostak *et al.*, 1999). Each of these functions is conducted by individual regions that could be outside of the motif 71-82.

It has been shown that substitutions of a single amino acid residue, Leu64 with Pro, Ala, or Arg, dramatically enhance the proapoptotic activity of Vpr (Jian & Zhao, 2003b). The possible consequences of particular mutations should also be examined separately for different subtypes since the same mutation has been shown to impact variably in different subtypes. The results of the current study also confirm the previous studies and suggest that the variations in subtype C Vpr cannot be regarded as defective mutations disturbing the functionality of the protein.

The mutations occurring in Vpr might have been selected under biological pressures and the evolutionary importance of these mutations cannot be ruled out. The study raises the concern as to what exactly is a wild-type HIV-1 protein? A number of studies have suggested that the consensus sequence approach could overcome the high genetic diversity of HIV-1C and facilitate an AIDS vaccine design while others have challenged this idea (Ellenberger *et al.*, 2002; Gaschen *et al.*, 2002). If the consensus sequence of subtype C Vpr does not function, at least for apoptosis induction, as effectively as some mutated Vpr proteins, could it be the case with other HIV-1 proteins as well? In addition, are the common reference sequences of HIV-1, such as the NL4-3 clone that was used in this study, the best representatives of the wild-type HIV-1? If we select the non-mutated or less mutated sequences as the wild-type sequence, are they the most functional sequences?

4.4. Artificially mutant Vpr proteins

It has been shown that substitution mutations in the N-terminus and α-helix I of Vpr disturb the nuclear localization of Vpr in human cell lines (Jacquot *et al.*, 2007; Yao *et al.*, 1995). Here we demonstrated that α-helix I contains the determinants required for the nuclear localization of Vpr. The W18C mutant that localized both to the nucleus and cytoplasm did not show a reduced activity in the induction of G2 arrest. A number of studies have found a link between the nuclear localization of Vpr and induction of G2 arrest (Jacquot *et al.*, 2007). On the other hand, it has been shown that G2 arrest activity of Vpr is induced by the residues lying at the C-terminus of Vpr but deletion of 14 amino acid from the N-terminus of Vpr reduces the extent of G2 arrest (Chen *et al.*, 1999). A possible explanation is that although C-terminal residues of Vpr are responsible for its G2 arrest activity, it is important for Vpr to localize to the nucleus to efficiently get access to the nuclear proteins regulating cell cycle. Therefore, the mutations in the N-terminus could potentially reduce G2 arrest activity without affecting the nuclear localization of Vpr. But mutations in the C-terminus and α-helix I that disturb the nuclear localization of Vpr could reduce the G2 arrest activity of Vpr. However it should be noted that the same issue as described for the expression level of natural mutants of Vpr applies to the artificial mutants. Uneven expression level could significantly affect the results therefore the values need to be cautiously compared.

The substitution mutations did not reduce the G2 arrest activity of Vpr. However, the G2 arrest activity of all the mutants, as well as consensus-C, was lower than that of subtype B Vpr. The differences in G2 arrest activity were not due to the different expression level since the expression level in individual cells (intensity of the

HaloTag Oregon Green signal) was similar in the transfected cells. In contrast to G2 arrest, the apoptotic activity of all the mutants was significantly affected by the mutations. All the substitution mutations at 5 different domains of Vpr increased the apoptotic activity of Vpr compared to the consensus-C. The results of gene expression assays show that Vpr-mutants similarly to consensus-C and subtype B Vpr induce the overexpression of certain pro-apoptotic genes. The overexpression of these genes showed that all the Vpr proteins induced apoptosis through the intrinsic pathway and the mutant proteins did not show a diminished ability to induce expression of apoptotic genes. It has been shown that the substitution of leucine residue at position 64 increases the apoptotic activity of Vpr (Jian & Zhao, 2003b) but these apoptotic-enhancing mutations have not been described further. It could be due to the use of problematic wild-type subtype B. The subtype B reference sequences already contain natural mutations that discriminate them from the consensus sequence of the corresponding subtype. These mutations could enhance the apoptotic activity of Vpr and therefore other apoptotic-enhancing mutations are not able to enhance the apoptotic activity of Vpr any further. In this study we found five apoptotic-enhancing mutations that enhance the apoptotic activity of the consensus sequence of subtype C Vpr. These mutations may not enhance the apoptotic activity of the reference sequences of wild-type subtype C Vpr or other subtypes that already contains mutations.

A conserved motif in the C-terminal region of Vpr has been implicated in its apoptotic activity. The C-terminal peptides containing the conserved sequence at amino acid 71-82 can even induce apoptosis. Extracellular addition of C-terminal peptides to human CD4+ cells causes membrane permeabilization, DNA fragmentation and formation of apoptotic bodies, which are all signs of apoptosis. Similar effects can also be observed

by adding the polypeptides to yeast, indicating that Vpr targets fundamental cellular pathways that are common among eukaryotic cells (Arunagiri *et al.*, 1997). Several studies have reported a link between Vpr-induced apoptosis and G2 arrest (Andersen *et al.*, 2008; Jacquot *et al.*, 2007) while other studies have reported that these functions are independent (Chen *et al.*, 1999; Romani & Engelbrecht, 2009). Based on the results of our study, we cannot confirm or rule out the existence of a functional link between apoptosis and G2 arrest. However, our results suggest that although apoptosis is independently induced by a short motif of Vpr, other regions of Vpr can contribute to the efficiency of apoptosis. The increase in the induction of apoptosis that was found in our mutants could be due to other factors, such as the secondary structure of the protein, domain charges and subcellular localization.

Mutations in Vpr have been shown to be subtype-dependent. It is has been hypothesized that Vpr proteins from different HIV-1 subtypes as well as Vpr variants that emerge during HIV-1 infection may have different apoptotic potentials that contribute to the diversity of AIDS pathogenesis (Jian & Zhao, 2003b). Enhancing apoptotic potential of Vpr in different subtypes and strains may be achieved through different mutations. The results of our study suggest that instead of having a highly conserved sequence that could be easily disturbed by mutations, HIV-1 Vpr has certain exchangeable residues that make substitution mutations at these residues are tolerable for the protein and could even potentially increase certain activities of the protein.

4.5. Future perspectives

Although HIV-1 subtype B has been intensively studied from different aspects, the functionality of other HIV-1 subtypes is less known. In this study several functions of

subtype C Vpr were studied and compared with subtype B Vpr. It was shown that the same functions attributed to subtype B Vpr can also be exerted by subtype C Vpr. Other proteins of HIV-1 subtype C could also be studied to identify their functionality.

The prevalence of subtype C in sub-Saharan countries may suggest the adaptation of this subtype to the genetic population of sub-Saharan countries. If there is such adaptive function or mutation in subtype C proteins, it could only be identified by functional studies of subtype C proteins of sub-Saharan strains.

Vpr interacts with many cellular proteins of which the expression level may be different in different tissues. Therefore the functions of Vpr could be studied in different cell lines. Since human 293T cells are not representative of a natural host cell of HIV-1, experiments could be designed that simulate *in vivo* infection. The use of T cell and monocyte derivatived cells could improve the physiologic relevance of the study since they are the natural hosts of HIV-1. However, any cell line is at best an approximation of a natural cell, since all cell lines are unnatural in the context of their immortality. The use of pseudotyped virus on the other hand could significantly improve the relevance of experiments as related to actual infection. Using pseudotyped viruses that contain or do not contain Vpr, it will be possible to study the role of virion-incorporated Vpr.

Determining the expression level of Vpr should be considered in future studies. Since different vectors may have different expression levels, it is important to compare the expression level of the relevant proteins as well as the size of the expressed proteins using Western-Blot. Monoclonal as well as polyclonal antibodies for detection of

South African subtype C Vpr could be produced to aid in monitoring protein expression levels in future studies..

4.6. Conclusion

In summary, the findings of this study indicate that both HIV-1 subtype B and C Vpr display a similar ability for nuclear localization and apoptosis induction. The induction of cell cycle G2 arrest by HIV-1 subtype B Vpr is, however, more robust than many subtype C Vpr proteins. The natural mutations studied in the isolates did not disturb the functions of subtype C Vpr and in some cases even potentiated the protein to induce apoptosis. This study demonstrates that naturally occurring mutations in HIV-1 Vpr cannot be regarded as defective mutations, since enhanced functionality would be more indicative of an adaptive role. The increased potency of the mutated Vpr proteins suggests that Vpr may increase the pathogenicity of HIV-1 by adapting apoptotic enhancing mutations. This is supported by the lack of pro-apoptotic activity of Vpr in non-pathogenic SIV species. The pro-apoptotic activity HIV-1 Vpr has been suggested to play a pivotal role in pathogenicity of Vpr. This study highlights the importance of studying the functionality of HIV-1 proteins from different subtypes to exclude or confirm the functional capacity of those proteins in different subtypes. The distribution of particular subtypes in some areas might be related to the adaptive mutations occurring in HIV-1 to adapt the virus to the genetic diversity of the population.

Chapter 5

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Appendix A

Letter of ethical approval

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

7 February 2008

Prof S Engelbreght
Dept of Pathology
Division of Medical Virology

Dear Prof Engelbreght

RESEARCH PROJECT: **"MUTAGENESIS ANS FUNCTIONAL STUDIES OF THE HIV-1 *VPR* GENE AND *VPR* PROTEIN OBTAINED FROM SELECTED SOUTH AFRICAN VIRUS STRAINS"**

PROJECT NUMBER : N08/01/014

At a meeting of the Committee for Human Research that was held on 6 February 2008 the above project was finally approved **for a period of one year from this date.** This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in all further correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

Patients participating in a research project in Tygerberg Hospital will not be treated free of charge as the Provincial Government of the Western Cape does not support research financially.

Due to heavy workload the nursing corps of the Tygerberg Hospital cannot offer comprehensive nursing care in research projects. It may therefore be expected of a research worker to arrange for private nursing care.

Yours faithfully

FRANKLIN WEBER
RESEARCH DEVELOPMENT AND SUPPORT (TYGERBERG)
Tel: +27 21 938 9657 / E-mail: fweb@sun.ac.za

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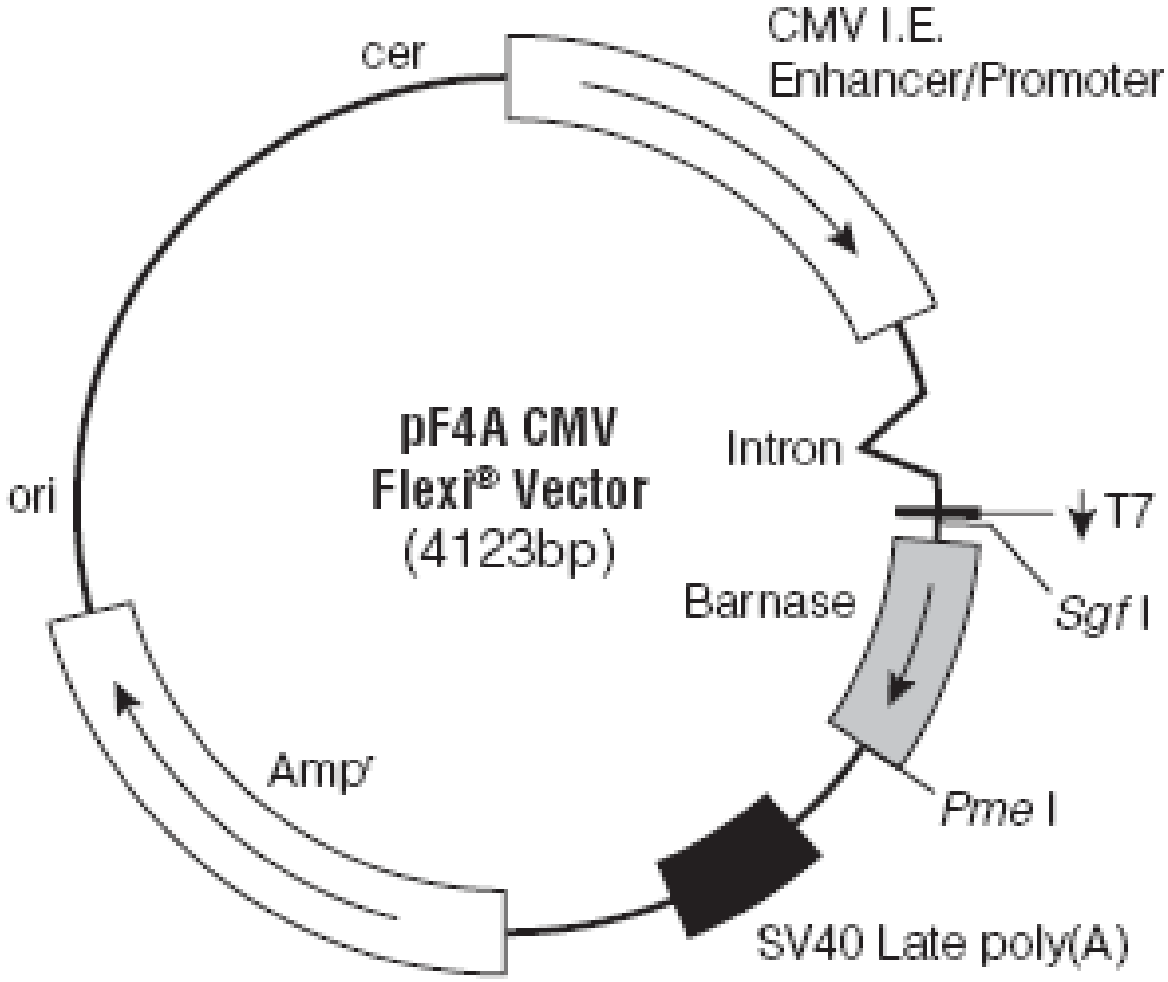
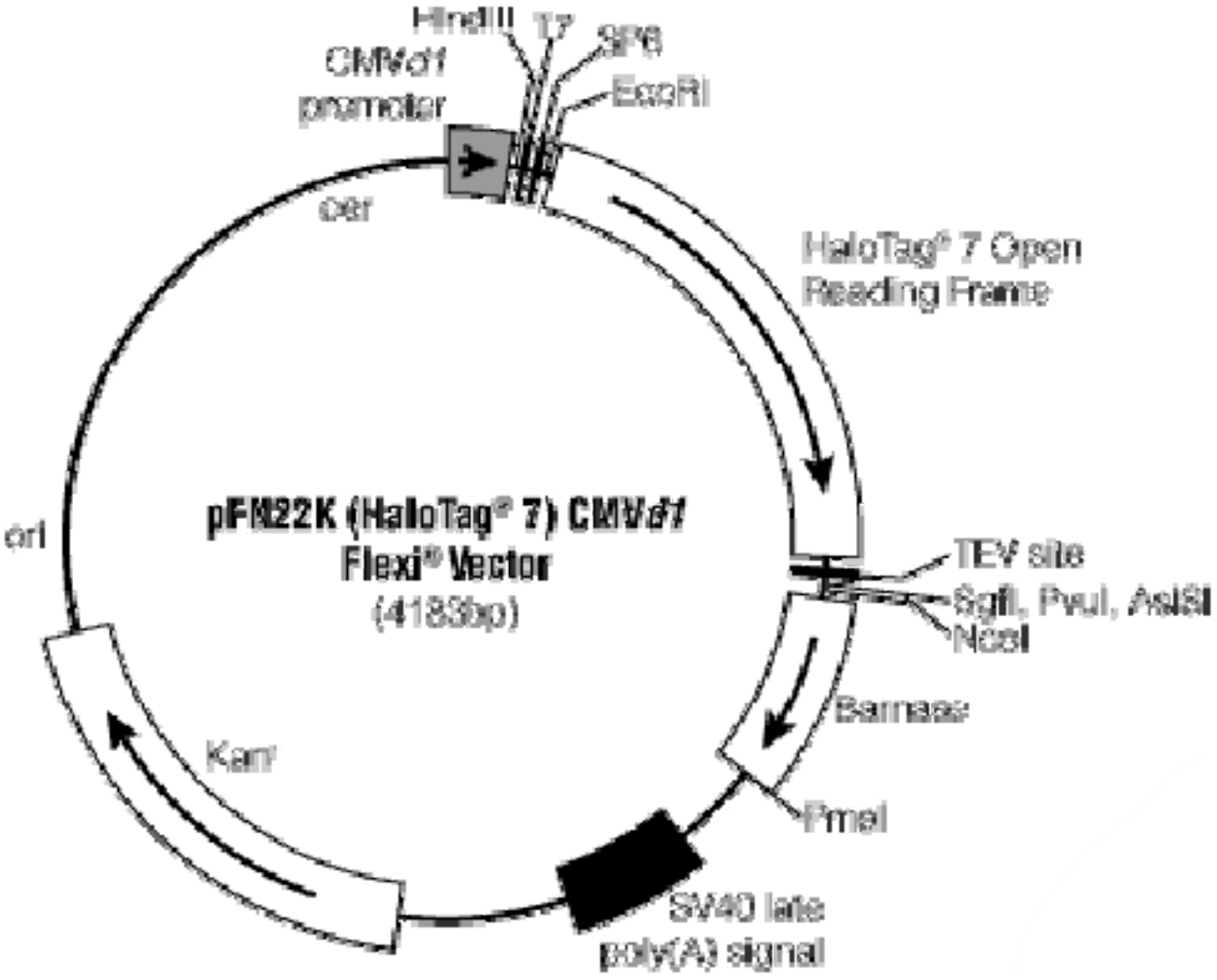
Appendix B:

pF4A CMV Flexi® Vector



Appendix C:

pFN22K (HaloTag® 7) CMV*d1* Flexi® Vector



Appendix D

Statistical analysis of the results of apoptosis assays using Bonferroni's multiple comparison test

Bonferroni's multiple comparison test	Mean diff.	t	Significant (P<0.05)
Mock sample vs Subtype B	-98.120	117.0	Yes
Mock sample vs Consensus-C	-82.000	97.77	Yes
Mock sample vs TV1453	-95.250	113.6	Yes
Mock sample vs TV1466	-96.460	115.0	Yes
Mock sample vs TV1570	-97.480	116.2	Yes
Mock sample vs TV 1628	-98.560	117.5	Yes
Mock sample vs TV1642	-97.830	116.6	Yes
Mock sample vs TV1707	-98.500	117.4	Yes
Mock sample vs P14I	-92.280	109.2	Yes
Mock sample vs W18C	-93.830	110.0	Yes
Mock sample vs Y47N	-96.170	114.8	Yes
Mock sample vs Q65H	-90.780	107.1	Yes
Mock sample vs Q88S	-88.400	103.8	Yes
Subtype B vs Consensus-C	16.120	19.21	Yes
Subtype B vs TV1453	2.870	3.41	No
Subtype B vs TV1466	1.660	1.97	No
Subtype B vs TV1570	0.6400	0.75	No
Subtype B vs TV1628	-0.4400	0.53	No
Subtype B vs TV1642	0.2900	0.34	No
Subtype B vs TV1707	-0.3800	0.45	No
Subtype B vs P14I	5.840	6.51	Yes
Subtype B vs W18C	4.290	5.10	Yes
Subtype B vs Y47N	1.950	2.05	No
Subtype B vs Q65H	7.340	8.12	Yes
Subtype B vs Q88S	9.720	10.57	Yes
Consensus-C vs TV1453	-13.250	15.79	Yes
Consensus-C vs TV1466	-14.460	17.24	Yes
Consensus-C vs TV1570	-15.480	18.46	Yes
Consensus-C vs TV1628	-16.560	19.74	Yes
Consensus-C vs TV1642	-15.830	18.87	Yes
Consensus-C vs TV1707	-16.500	19.66	Yes
Consensus-C vs P14I	-10.280	12.30	Yes
Consensus-C vs W18C	-11.830	13.78	Yes
Consensus-C vs Y47N	-14.170	17.00	Yes
Consensus-C vs Q65H	-8.780	9.42	Yes
Consensus-C vs Q88S	-6.400	7.23	Yes

TV1453 vs TV1466	-1.210	1.44	No
TV1453 vs TV1570	-2.230	2.663	No
TV1453 vs TV1628	-3.310	3.950	Yes
TV1453 vs TV1642	-2.580	3.07	No
TV1453 vs TV1707	-3.250	3.87	Yes
TV1453 vs P14I	2.970	3.59	No
TV1453 vs W18C	1.420	1.84	No
TV1453 vs Y47N	-0.920	1.17	No
TV1453 vs Q65H	4.470	5.18	Yes
TV1453 vs Q88S	6.850	7.52	Yes
TV1466 vs TV1570	-1.020	1.21	No
TV1466 vs TV1628	-2.100	2.50	No
TV1466 vs TV1642	-1.370	1.63	No
TV1466 vs TV1707	-2.040	2.42	No
TV1466 vs P14I	4.180	5.06	Yes
TV1466 vs W18C	2.630	3.31	No
TV1466 vs Y47N	0.290	0.34	No
TV1466 vs Q65H	5.680	6.33	Yes
TV1466 vs Q88S	8.060	9.20	Yes
TV1570 vs TV1628	-1.080	1.29	No
TV1570 vs TV1642	-0.350	0.41	No
TV1570 vs TV1707	-1.020	1.21	No
TV1570 vs P14I	5.200	6.13	Yes
TV1570 vs W18C	3.650	3.95	Yes
TV1570 vs Y47N	1.310	1.54	No
TV1570 vs Q65H	6.700	7.44	Yes
TV1570 vs Q88S	9.080	10.02	Yes
TV1628 vs TV1642	0.730	0.87	No
TV1628 vs TV1707	0.060	0.08	No
TV1628 vs P14I	6.280	7.14	Yes
TV1628 vs W18C	4.730	5.61	Yes
TV1628 vs Y47N	2.390	2.97	No
TV1628 vs Q65H	7.780	8.85	Yes
TV1628 vs Q88S	10.160	12.07	Yes
TV1642 vs TV1707	-0.670	0.79	No
TV1642 vs P14I	5.550	6.21	Yes
TV1642 vs W18C	4.00	4.92	Yes
TV1642 vs Y47N	1.660	1.91	No
TV1642 vs Q65H	7.050	7.96	Yes
TV1642 vs Q88S	9.430	10.33	Yes
TV1707 vs P14I	6.220	7.09	Yes
TV1707 vs W18C	4.670	5.95	Yes
TV1707 vs Y47N	2.330	3.11	No

TV1707 vs Q65H	7.720	8.72	Yes
TV1707 vs Q88S	10.100	12.26	Yes
P14I vs W18C	-1.550	1.90	No
P14I vs Y47N	-3.890	4.21	Yes
P14I vs Q65H	1.500	1.87	No
P14I vs Q88S	3.880	4.27	Yes
W18C vs Y47N	-2.340	3.29	No
W18C vs Q65H	3.050	3.64	No
W18C vs Q88S	5.430	6.44	Yes
Y47N vs Q65H	5.390	6.35	Yes
Y47N vs Q88S	7.770	8.65	Yes
Q65H vs Q88S	2.380	3.17	No

Appendix E

Statistical analysis of Cell cycle G2 arrest using Bonferroni's multiple comparison test

Bonferroni's multiple comparison test	Mean diff.	t	Significant (P<0.05)
Mock sample vs Subtype B	-38.94	16.13	Yes
Mock sample vs Consensus-C	-28.94	11.99	Yes
Mock sample vs TV1453	-32.41	13.55	Yes
Mock sample vs TV1466	-21.63	8.96	Yes
Mock sample vs TV1570	-33.94	14.06	Yes
Mock sample vs TV 1628	-28.92	11.98	Yes
Mock sample vs TV1642	-31.15	12.90	Yes
Mock sample vs TV1707	-29.05	12.03	Yes
Mock sample vs P14I	-33.310	14.48	Yes
Mock sample vs W18C	-24.200	10.14	Yes
Mock sample vs Y47N	-34.220	15.76	Yes
Mock sample vs Q65H	-28.810	11.64	Yes
Mock sample vs Q88S	-29.810	12.02	Yes
Subtype B vs Consensus-C	9.997	4.14	Yes
Subtype B vs TV1453	6.227	2.57	No
Subtype B vs TV1466	17.31	7.16	Yes
Subtype B vs TV1570	5.000	2.07	No
Subtype B vs TV1628	10.02	4.14	Yes
Subtype B vs TV1642	7.790	3.22	No
Subtype B vs TV1707	9.893	4.09	Yes
Subtype B vs P14I	5.810	2.12	No
Subtype B vs W18C	14.920	5.95	Yes
Subtype B vs Y47N	4.900	2.00	No
Subtype B vs Q65H	10.310	4.14	Yes
Subtype B vs Q88S	9.310	3.79	No
Consensus-C vs TV1453	-3.770	1.56	No
Consensus-C vs TV1466	7.310	3.02	No
Consensus-C vs TV1570	-4.997	2.07	No
Consensus-C vs TV1628	0.020	0.00	No
Consensus-C vs TV1642	-2.207	0.91	No
Consensus-C vs TV1707	-0.103	0.04	No
Consensus-C vs P14I	-4.110	1.56	No
Consensus-C vs W18C	5.000	2.08	No
Consensus-C vs Y47N	-5.020	2.08	No
Consensus-C vs Q65H	0.3900	0.10	No

Consensus-C vs Q88S	11.230	4.59	Yes
TV1453 vs TV1466	11.08	4.58	Yes
TV1453 vs TV1570	-1.227	0.50	No
TV1453 vs TV1628	3.790	1.57	No
TV1453 vs TV1642	1.563	0.64	No
TV1453 vs TV1707	3.667	1.51	No
TV1453 vs P14I	-0.4800	0.28	No
TV1453 vs W18C	8.630	3.41	No
TV1453 vs Y47N	-1.390	0.57	Yes
TV1453 vs Q65H	4.020	1.55	Yes
TV1453 vs Q88S	3.020	1.57	Yes
TV1466 vs TV1570	-12.31	5.09	Yes
TV1466 vs TV1628	-7.290	3.02	No
TV1466 vs TV1642	-9.517	3.94	Yes
TV1466 vs TV1707	-7.413	3.07	No
TV1466 vs P14I	-11.710	4.58	Yes
TV1466 vs W18C	-2.600	0.99	No
TV1466 vs Y47N	-12.620	5.12	Yes
TV1466 vs Q65H	-7.210	3.23	No
TV1466 vs Q88S	-8.210	3.44	No
TV1570 vs TV1628	5.017	2.07	No
TV1570 vs TV1642	2.790	1.15	No
TV1570 vs TV1707	4.893	2.02	No
TV1570 vs P14I	0.6000	0.45	No
TV1570 vs W18C	9.710	4.00	Yes
TV1570 vs Y47N	-0.3100	0.12	No
TV1570 vs Q65H	5.100	2.06	No
TV1570 vs Q88S	4.100	1.47	No
TV1628 vs TV1642	-2.227	0.92	No
TV1628 vs TV1707	-0.123	0.05	No
TV1628 vs P14I	-4.250	1.62	No
TV1628 vs W18C	4.860	1.87	No
TV1628 vs Y47N	-5.160	2.11	No
TV1628 vs Q65H	0.250	0.12	No
TV1628 vs Q88S	-0.750	0.41	No
TV1642 vs TV1707	2.103	0.87	No
TV1642 vs P14I	-1.920	0.79	No
TV1642 vs W18C	7.190	3.01	No
TV1642 vs Y47N	-2.830	1.30	No
TV1642 vs Q65H	2.580	1.21	No
TV1642 vs Q88S	1.580	0.62	No
TV1707 vs P14I	-4.160	1.58	No
TV1707 vs W18C	4.950	1.89	No

TV1707 vs Y47N	-5.070	2.08	No
TV1707 vs Q65H	0.340	0.13	No
TV1707 vs Q88S	-0.660	0.40	No
P14I vs W18C	9.110	3.81	No
P14I vs Y47N	-0.910	0.50	No
P14I vs Q65H	4.500	1.67	No
P14I vs Q88S	3.500	1.48	No
W18C vs Y47N	-10.020	4.13	Yes
W18C vs Q65H	-4.610	1.61	No
W18C vs Q88S	-5.610	2.08	No
Y47N vs Q65H	5.410	2.05	No
Y47N vs Q88S	4.410	1.59	No
Q65H vs Q88S	-1.000	0.49	No

Appendix F

Molecular and Phylogenetic Analysis of HIV Type 1 *vpr* Sequences of South African Strains

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Sequence Note

Molecular and Phylogenetic Analysis of HIV Type 1 *vpr* Sequences of South African Strains

Bizhan Romani,[1] Richard Glashoff,[1] and Susan Engelbrecht[1,2]

Abstract

HIV-1 subtype C is the prevalent subtype in South Africa, with non-C subtypes being sporadically detected. The accessory genes of subtype C have not been well studied in South Africa. In this study the HIV-1 *vpr* region of 58 strains was amplified, sequenced, and subtyped. Phylogenetic analysis characterized 54 strains as HIV-1 subtype C and 4 strains as HIV-1 subtype B. The amino acid sequence of the protein was also investigated. The overall amino acid sequence of Vpr was conserved as well as the motifs FPRPWL (34–39: IH1) and TYGDTW (49–54: IH2). The C-terminal was more variable. The importance of these motifs and variability needs to be addressed.

The HIV-1/AIDS pandemic is a major health problem worldwide with the majority of infected people living in sub-Saharan Africa. South Africa has the largest population of HIV-1-infected individuals in the world[1] and it is thus important to study the virus in this region. The HIV-1 genome codes for structural proteins (Gag, Pol, and Env), regulatory proteins (Tat and Rev), and accessory proteins (Vif, Vpr, Vpu, and Nef). The accessory viral protein R (Vpr) is a 96 amino acid virion-associated protein that plays an important role in virion infectivity and replication. Some of the proposed functions for Vpr include conferring nuclear transport of the preintegration complex,[2] cytopathogenicity of the virus,[3] reduction of the HIV-1 mutation rate,[4] transactivation of cellular genes, modulation of HIV-1-induced apoptosis,[5,6] cell differentiation and cycling,[7] and fidelity of reverse transcription.[8,9] Lack of a functional Vpr also decreases virion infectivity.[10] Although some studies have failed to find mutant Vpr in long-term nonprogressors (LTNPs),[11,12] it has been demonstrated in others.[13] In addition to Vpr in HIV-1, HIV-2 has a protein, Vpx, that is related to Vpr.[14,15] Other lentiviruses do not contain sequences related to *vpr* but do include small open reading frames that could perform similar functions.[16]

One of the features of HIV-1 is the extreme genetic diversity of the virus, which can be classified into three groups, M, N, and O. Group M can be subdivided into nine subtypes and at least 43 circulating recombinant forms (CRFs) (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). In South Africa, subtype C accounts for the majority of infections, but non-C subtypes are also sporadically detected.[17,18] HIV-1 accessory genes and proteins of South African subtype C viruses are an understudied area: Bell and co-workers investigated these genes in 20 primary virus isolates[19] and Scriba and co-workers in 15 primary isolates.[20] The objective of this study was the characterizing and subtyping of an additional 58 *vpr* sequences from HIV-1-infected patients in Cape Town.

Plasma samples were collected from 58 HIV-1-positive patients in the Cape Town metropole during 2002–2004. The patient demographics are listed in Table 1. The patients consist of 31 African females, 22 African males, 2 white males, as well as two males and one female of mixed race descent. Except for one patient (TV1638) who was from Kenya and was infected in Kenya, all of the other patients were South Africans infected in South Africa.

Viral RNA was extracted from the stored plasma samples using the Ultrasens Viral RNA kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol and stored at −80°C. Reverse transcriptase (RT) polymerase chain reaction (PCR) amplification of a 1361-bp region (HXB2 positions 4985–6346) was performed using the Access-RT kit (Promega, Madison, WI). This was followed by DNA amplification of a 714-bp region. For RT-PCR the primers NACC (5′-AGATAATAGTGACATAAAAGTAGTGCCAAGAAG-3′)

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Table 1. Information of Samples Used in the Study

Isolate	*Date of birth*	*Race/gender*	*Transmission*
TV1325C[a]	1972	Mixed race male	Unknown
TV1345B	1954	Caucasian male	Homosexual
TV1421	1963	African male	Heterosexual
TV1428B	1962	African male	Heterosexual
TV1453	1954	African male	Heterosexual
TV1454	1975	African male	Heterosexual
TV1455	1979	African female	Heterosexual
TV1459	1973	African male	Heterosexual
TV1460	1922	African male	Heterosexual
TV1462	1975	African female	Heterosexual
TV1463	1958	African female	Heterosexual
TV1464	1977	African female	Heterosexual
TV1466	1975	African female	Heterosexual
TV1467	1976	African female	Heterosexual
TV1468	1968	African male	Heterosexual
TV1470	1964	Mixed race male	Heterosexual
TV1472	1978	African female	Heterosexual
TV1526B	1967	Caucasian male	Homosexual
TV1565	1969	African female	Heterosexual
TV1566	1962	African male	Heterosexual
TV1568	1968	Mixed race female	Heterosexual
TV1570	1957	African male	Heterosexual
TV1571	1976	African female	Heterosexual
TV1573	1978	African female	Heterosexual
TV1576	1974	African female	Heterosexual
TV1577	1970	African female	Heterosexual
TV1578	1983	African female	Heterosexual
TV1579	1970	African male	Heterosexual
TV1581	1975	African male	Heterosexual
TV1583B	1981	African female	Heterosexual
TV1596	1955	African male	Heterosexual
TV1597	1971	African female	Heterosexual
TV1598	1973	African female	Heterosexual
TV1599	1958	African male	Heterosexual
TV1614	1963	African male	Heterosexual
TV1616	1972	African female	Heterosexual
TV1623	1970	African female	Heterosexual
TV1625	1973	African female	Heterosexual
TV1626	1952	African female	Heterosexual
TV1627	1975	African female	Heterosexual
TV1628	1971	African male	Heterosexual
TV1630	1977	African male	Heterosexual
TV1632	1971	African female	Heterosexual
TV1633	1961	African male	Heterosexual
TV1638	1973	African female	Heterosexual
TV1639	1976	African female	Heterosexual
TV1641	1981	African female	Heterosexual
TV1642	1970	African female	Heterosexual
TV1643	1958	African female	Heterosexual
TV1644	1976	African male	Heterosexual
TV1653	1984	African female	Heterosexual
TV1654	1972	African female	Heterosexual
TV1661	1980	African male	Homosexual
TV1667	1963	African female	Heterosexual
TV1681	1973	African male	Heterosexual
TV1705	1974	African female	Heterosexual
TV1707	1946	African male	Heterosexual
TV1708	1975	African male	Heterosexual

[a]B, indicates the second sample of the patient; C, indicates the third sample of the patient.

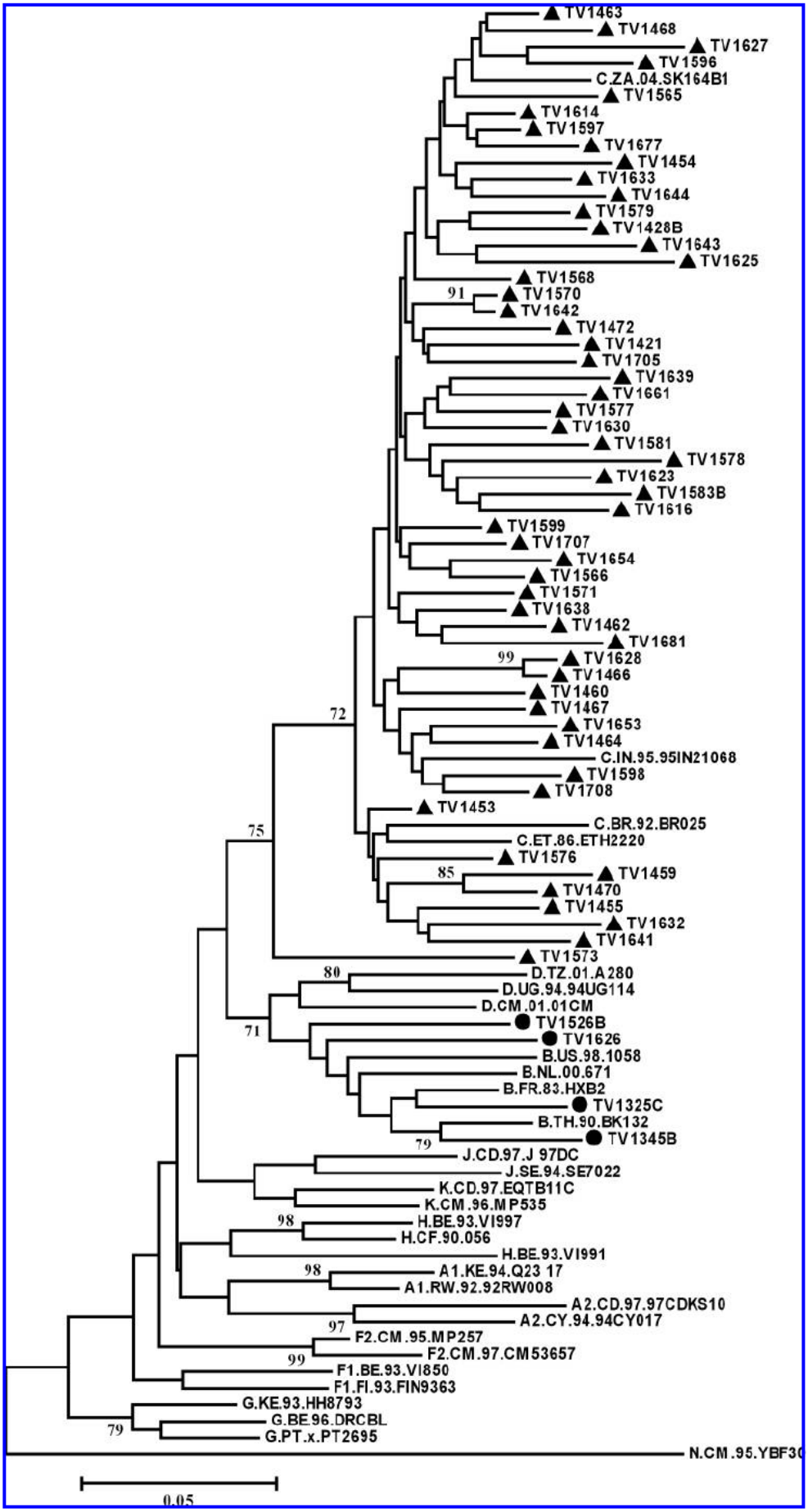
and NACD (5′-CCATAATAGACTGTGACCCACAA-3′)[21] were used. Briefly, we used the Access RT-PCR kit (Promega, Madison, WI) with 5 μl of RNA, 200 μM of each nucleotide, 39 μmol of each primer, 1 mM $MgSO_4$, 5 units each of AMV RT and Tfl DNA polymerase, and AMV/Tfl buffer in a total volume of 50 μl. After reverse transcription of 45 min at 48°C, the reaction was held at 94°C for 2 min, followed by 40 cycles of denaturing the DNA (94°C; 30 s), annealing of primers (50°C; 30 s), and extension of the annealed primers (68°C; 2.5 min). This was followed by a final extension step of 10 min at 68°C and the PCR product was kept at 4°C. This was followed by DNA amplification of the 714-bp region (HXB2 positions 5276–5990) using the primers HIV-vif-1F (5′-GGAATTTGGGTCATGGAGTCTCCATA-3′) and HIV-vpr-R1 (5′-GTCTCCGCTTCTTCCTGCCATAG-3′), with the Promega GoTaq Flexi Kit (Promega, Madison, WI). Template DNA for the nested PCR consisted of 3 μl of reaction product from the first round with 200 μM of each nucleotide, 20 μmol of each primer and 1.5 mM $MgCl_2$, and 2.5 units of *Taq* polymerase and GoTaq buffer made up in a total volume of 50 μl. The PCR cycle method used was similar to that of pre-nested PCR, except for the primer annealing step at 56°C and an extension time of 1 min. The PCR products were visualized using agarose gel electrophoresis.

To purify the PCR products, single-stranded DNA and diphosphates were degraded using the enzymes *Exonuclease* 1 (*Exo*1) and Shrimp alkaline phosphatase (SAP) (Amersham Pharmacia Biotech., NJ), respectively. All PCR products were sequenced on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed on an ABI Prism 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA).

The chromatograms were analyzed and overlapping fragments were assembled using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI). The *vpr* fragments of all of the DNA sequences were aligned with HIV-1 reference sequences obtained from the LANL HIV Database (http://hiv-web.lanl.gov), using Clustal X version 1.81.[22] Multiple alignments were verified manually in BioEdit version 7.090.[23] A neighbor-joining phylogenetic tree was constructed with MEGA version 4[24] using the Kimura two-parameter.[25] The reliability of the branching and clustering pattern was estimated from 1000 bootstrap replicates. The tree was rooted with the YBF30 reference sequence of HIV-1 group N.

The phylogenetic tree constructed from the 291-bp fragment of the structural part of *vpr* gene is shown in Fig. 1. Fifty-three out of 58 sequences clustered with the reference sequences of subtype C. The sequence of TV1573 was an outlier to subtype C. Four sequences (TV1526B, TV1345B, TV1626, and TV1325C) clustered with the reference sequences of subtype B from Thailand (B.TH.90.BK132), France (B.FR.83.HXB2), and the Netherlands (B.NL.00.671). Two out of four subtype B isolates had been transmitted homosexually, one through heterosexual contact, and one with an unknown route. In comparison to subtype B, only 1 (TV1661) out of 54 subtype C isolates had been transmitted homosexually and the others had a heterosexual route.

To investigate the Vpr protein, the *vpr* gene was translated and the amino acids, aligned with the consensus subtype C and HXB2 reference sequences using BioEdit version 7.0.9.0.[23] The α-helical domains of Vpr were shaded (Fig. 2). All of our sequences were 96 amino acid residues in length, except for


TV1463
TV1468
TV1627
TV1596
C.ZA.04.SK164B1
TV1565
TV1614
TV1597
TV1677
TV1454
TV1633
TV1644
TV1579
TV1428B
TV1643
TV1625
TV1568
91
TV1570
TV1642
TV1472
TV1421
TV1705
TV1639
TV1661
TV1577
TV1630
TV1581
TV1578
TV1623
TV1583B
TV1616
TV1599
TV1707
TV1654
TV1566
TV1571
TV1638
TV1462
TV1681
99
TV1628
TV1466
TV1460
TV1467
72
TV1653
TV1464
C.IN.95.95IN21068
TV1598
TV1708
TV1453
C.BR.92.BR025
75
C.ET.86.ETH2220
TV1576
85
TV1459
TV1470
TV1455
TV1632
TV1641
TV1573
80
D.TZ.01.A280
D.UG.94.94UG114
D.CM.01.01CM
TV1526B
71
TV1626
B.US.98.1058
B.NL.00.671
B.FR.83.HXB2
TV1325C
B.TH.90.BK132
TV1345B
79
J.CD.97.J 97DC
J.SE.94.SE7022
K.CD.97.EQTB11C
K.CM.96.MP535
98
H.BE.93.VI997
H.CF.90.056
H.BE.93.VI991
98
A1.KE.94.Q23 17
A1.RW.92.92RW008
A2.CD.97.97CDKS10
A2.CY.94.94CY017
97
F2.CM.95.MP257
F2.CM.97.CM53657
99
F1.BE.93.VI850
F1.FI.93.FIN9363
G.KE.93.HH8793
G.BE.96.DRCBL
79
G.PT.x.PT2695
N.CM.95.YBF30
0.05

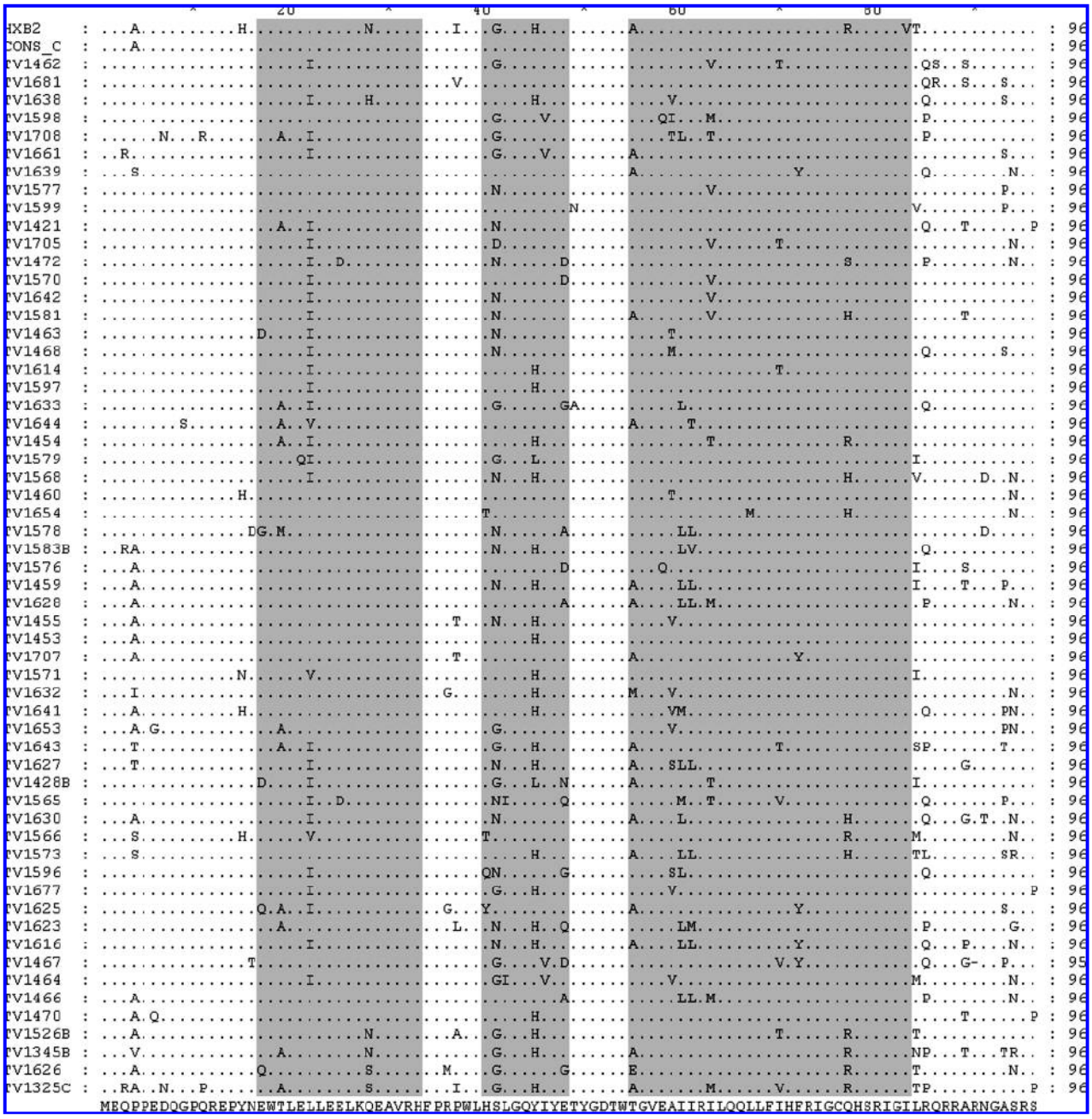


FIG. 2. Alignment of the deduced Vpr amino acid sequences from 58 South African isolates in comparison with consensus subtype C reference and the subtype B HXB2 reference. Dots represent identity to the consensus sequence of our isolates, at the bottom, and the only dash, in TV1467, represents a gap. α-Helical domains of Vpr are shaded gray.

TV1467. In this sequence an arginine residue had been deleted in position 90, which is located in the C-terminal domain of the protein. The patient was a 30-year-old African woman with symptoms of prurigo.

The consensus Vpr amino acid sequence of our strains was identical to the consensus sequence of subtype C, except for position 4. Most of our sequences had a proline residue in this position, whereas the consensus subtype C sequence had an alanine residue. No mutations were observed in positions 21 and 24 of the α-helix I for which substitutions of proline has been shown to abrogate Vpr incorporation into virus-like particles.[26] A highly conserved motif in α-helix I was found in

FIG. 1. Rooted neighbor-joining tree illustrating the evolutionary relationship among *vpr* nucleotide sequences (291 bp) from South African HIV-1 isolates and reference sequences of HIV-1 (Los Alamos database). Fifty-eight sequences are shown with TV (Tygerberg Virology) suffixed by their respective clone numbers. Triangles indicate subtype C isolates and circles indicate subtype B isolates. Branch lengths are related to the sequence dissimilarities.

position 29–33 (EAVRH). Motif 42–44 of the α-helix II was highly conserved in our isolates of which leucine 42 and glycine are involved in Vpr incorporation.[27,28] Motif 55–64 in α-helix III was variable but motif 65–83 in this domain was found highly conserved. The C-terminal domain of Vpr indicated the highest variability, but even in this domain, three arginine residues in positions 87, 88, and 95 and one glycine residue in position 92 were intact in all of our isolates. The motif FPRPWL, between α-helix I and II (34–39: IH1), and the motif TYGDTW, between α-helix II and III (49–54: IH2), were highly conserved in our isolates with the γ-turn in each.[26]

In the previous studies done in South Africa,[19,20] Vpr was found to be highly conserved in length. In our study this was confirmed in 53 subtype C isolates and 4 subtype B isolates. The functions of the α-helix and C-terminal domains of Vpr have been studied extensively, but the functions of the γ-turn regions, which in this study were found to be highly conserved, are still unknown.

The different subtypes and the variability of the *vpr* gene observed in our isolates suggest that HIV-1 infections in the Western Cape Province of South Africa did not start from a single source. It is worth studying the phylogenetic relationship of non-C subtypes in order to find the source of their introduction into the country.

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Disclosure Statement

No competing financial interests exist.

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Appendix G

Human immunodeficiency virus type 1 Vpr: functions and molecular interactions

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Review

Human immunodeficiency virus type 1 Vpr: functions and molecular interactions

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Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) is an accessory protein that interacts with a number of cellular and viral proteins. The functions of many of these interactions in the pathogenesis of HIV-1 have been identified. Deletion of the *vpr* gene reduces the virulence of HIV-1 dramatically, indicating the importance of this protein for the virus. This review describes the current findings on several established functions of HIV-1 Vpr and some possible roles proposed for this protein. Because Vpr exploits cellular proteins and pathways to influence the biology of HIV-1, understanding the functions of Vpr usually involves the study of cellular pathways. Several functions of Vpr are attributed to the virion-incorporated protein, but some of them are attributed to the expression of Vpr in HIV-1-infected cells. The structure of Vpr may be key to understanding the variety of its interactions. Due to the critical role of Vpr in HIV-1 pathogenicity, study of the interactions between Vpr and cellular proteins may help us to understand the mechanism(s) of HIV-1 pathogenicity.

Introduction

The virulence of a virus depends on the virus–host interactions, which are postulated by a variety of factors, such as route of virus entry, dose of the virus and the host's age, sex, immune status and species. The host may suppress every stage of the virus replication cycle by posing different obstacles to the progression of virus replication. A successful virus must overcome these obstacles. Viruses have evolved proteins to overcome the defensive mechanisms of the host (Tungaturthi *et al.*, 2004). Lentivirus genomes contain a number of accessory and regulatory genes in addition to the *gag*, *pol* and *env* genes that are commonly found in all retroviruses. Human immunodeficiency virus type 1 (HIV-1) encodes two regulatory proteins, Tat and Rev, and four accessory proteins [viral infectivity factor (Vif), viral protein R (Vpr), viral protein U (Vpu) and negative factor (Nef)]. The regulatory proteins are essential for virus replication by controlling HIV gene expression in host cells. In contrast, accessory proteins are often dispensable for virus replication *in vitro* (Anderson & Hope, 2004).

The HIV-1 genome encodes a 14 kDa accessory protein, Vpr, which is a versatile, virion-associated protein composed of 96 aa (Felzien *et al.*, 1998; Müller *et al.*, 2000; Muthumani *et al.*, 2000). Vpr is incorporated into HIV-1 virions through direct interaction with the p6 domain of the Gag protein (Bachand *et al.*, 1999; Lavallee *et al.*, 1994). Vpr has a variety of roles in determining HIV-1 infectivity and the number of its newly identified roles is still increasing. Some of the functions proposed for this protein include modulation of transcription of the virus genome (Sawaya *et al.*, 2000), induction of apoptosis, disruption of cell-cycle control, induction of defects in mitosis (Chang *et al.*, 2004), nuclear transport of the HIV-1 pre-integration complex (PIC) (Vodicka *et al.*, 1998), facilitation of reverse transcription (Rogel *et al.*, 1995), suppression of immune activation (Ramanathan *et al.*, 2002) and reduction of the HIV mutation rate (Jowett *et al.*, 1999). In addition, the interactions of Vpr with a number of human proteins have been identified, but the functions of some of those interactions are not clear (Felzien *et al.*, 1998; Ramanathan *et al.*, 2002; Sawaya *et al.*, 2000). Vpr contains a flexible N-terminal region, three α-helical domains with amphipathic properties and a flexible C-terminal region (Fig. 1). Each function or interaction of Vpr is attributed to one or more of its domains. Identification of the functions of Vpr domains is usually carried out by mutagenesis studies (Tungaturthi *et al.*, 2004).

The cytopathic effects induced by Vpr are mostly attributed to the N terminus, which is able to form ion channels in cell membranes. These effects are unrelated to the reported activities of Vpr, including virion association, G2 arrest, induction of apoptosis, etc. (Piller *et al.*, 1999; Somasundaran *et al.*, 2002). Incorporation of Vpr into HIV-1 virions is blocked by a range of mutations distributed in different regions of the protein, indicating that different domains of Vpr are involved in its

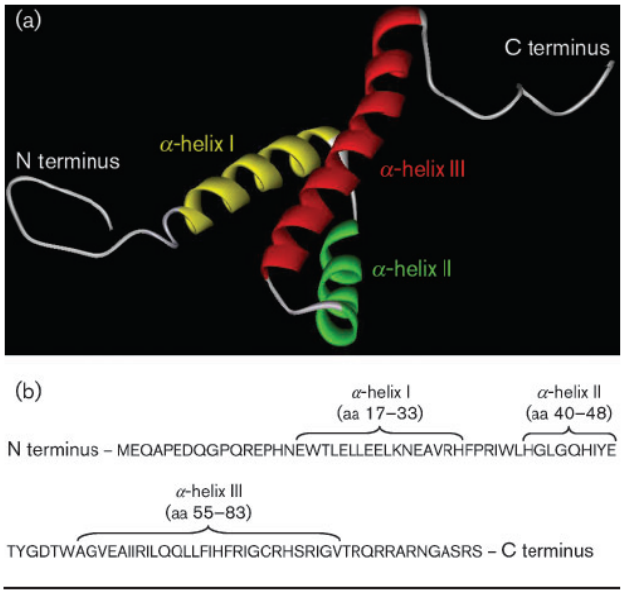

Fig. 1. The HIV-1 Vpr protein. (a) Three-dimensional structure of HIV-1 Vpr, representing the N- and C-terminal regions in white, α-helix I in yellow, α-helix II in green and α-helix III in red. (b) Amino acid sequence of the HIV-1 Vpr according to the reference sequence HXB2 (GenBank accession no. K03455), showing the positions of the domains.

incorporation into virions. Alterations in the cell cycle, including apoptosis, cell-cycle arrest and defects in mitosis, are mostly carried out by the C-terminal domain of Vpr, although alterations in the cell cycle by the other regions of Vpr have also been reported (Piller *et al.*, 1999; Roumier *et al.*, 2002; Srinivasan *et al.*, 2008).

Nuclear import of the PIC

HIV-1 Vpr enhances the ability of HIV-1 to replicate in terminally differentiated macrophages, which is attributed to the activity of Vpr in active nuclear import of the virus PIC (Jenkins *et al.*, 1998; Le Rouzic *et al.*, 2002; Schang, 2003; Suzuki *et al.*, 2009). The HIV-1 PIC is composed of viral proteins, such as Vpr, reverse transcriptase (RT), integrase (IN), nucleocapsid (NC) and matrix (MA), in addition to viral nucleic acids. Although the precise role(s) of some of these proteins has not been described, their importance for nuclear import of the PIC has been demonstrated. The nuclear-localization signals (NLSs) in both MA and IN have been identified, and it is believed that both utilize the classical nuclear-import pathway that includes interaction with importins. In contrast, a canonical NLS has not been identified in Vpr, but it displays karyophilic properties. It is believed that the nuclear localization of Vpr is mediated by an unknown pathway that is distinct from the classical NLS- and M9-dependent pathways. In addition, it has also been suggested that Vpr utilizes the cellular machinery that regulates nucleocytoplasmic shuttling to transfer the proviral DNA to the nucleus. For example, in the presence of importin-α, the nuclear localization of Vpr increases. It has been suggested that interaction of Vpr with importin-α facilitates its nuclear localization (Gallay *et al.*, 1996, 1997; Nitahara-Kasahara *et al.*, 2007).

To identify the role of Vpr in the nuclear import of PIC, green fluorescent protein (GFP) has been fused to Vpr. By using the GFP–Vpr-labelled PIC, it has been demonstrated that the PIC is associated closely with cytoplasmic microtubules that direct it toward the nucleus, where it localizes in the perinuclear region close to centrosomes. It has also been shown that the PIC utilizes cytoplasmic dynein to travel toward the nucleus. It has not still been identified whether Vpr plays an active role during the transfer of the PIC along microtubules, or whether it is only associated with PIC in this step and starts its role during the next steps (Le Rouzic & Benichou, 2005).

Selective trafficking between the nucleus and cytoplasm is carried out through nuclear-pore complexes (NPCs), which form aqueous channels in the nuclear envelope. NPCs are huge protein complexes composed of 30 distinct nuclear-pore proteins, called nucleoporins (Nups). Some Nups that contain phenylalanine–glycine (FG) repeats are found in the filamentous structures emanating from both sides of NPCs, thereby provide docking sites for various transport factors. Importin-α is a common floating receptor that binds to the NLSs of cargo proteins. FG repeats on the cytoplasmic filaments and cytoplasmic ring moiety of NPCs recognize the importin receptor. It has been shown that HIV-1 Vpr binds to the FG repeats of several Nups, including human p54 and p58. HIV-1 Vpr also binds to human CG1 (hCG1), but this interaction is not mediated by the FG repeat of this Nup. Interaction of Vpr with the N-terminal region (which does not contain FG repeats) of hCG1 is essential for docking of Vpr to the nuclear envelope (Le Rouzic & Benichou, 2005; Vodicka *et al.*, 1998; Zeitler & Weis, 2004). It has been demonstrated that the α-helical domains of Vpr are essential for its interaction with hCG1. This interaction results in Vpr accumulation in the nuclear envelope, which is believed to be involved in active nuclear import of the PIC in non-dividing cells, such as macrophages. It may also be involved in targeting the PIC to the NPC before its translocation into the nucleus. In addition to the conventional routes suggested for nuclear import of the PIC, it has also been reported that Vpr mediates transient, localized herniations in the nuclear envelope, resulting in mixing of cytoplasmic and nuclear components. These herniations probably contribute to the G2-arrest activity of Vpr and may also provide an unconventional route for nuclear import of the PIC. In fact, the interaction between Vpr and hCG1 could cause misassembly of the NPC, resulting in alterations of the architecture of the nuclear envelope that facilitate nuclear import of the PIC (de Noronha *et al.*, 2001; Le Rouzic *et al.*, 2002).

Induction of G2 arrest

The role of HIV-1 Vpr in inhibition of normal cell growth is well established. It is believed that the interruption of cell division by Vpr increases virus replication and induces programmed cell death. Vpr mediates cell-cycle arrest at the G2/M transition in various mammalian cells. The cell-cycle G2 arrest provides a replication advantage for the virus, because the transcription level of the provirus is higher during G2 (Belzile *et al.*, 2007; Elder *et al.*, 2001; Goh *et al.*, 1998). As the pathways regulating the cell cycle are highly conserved in eukaryotic cells (Krylov *et al.*, 2003; Warbrick & Fantes, 1988), transfection of yeast cells by vectors expressing Vpr also interrupts the cell cycle. Therefore, in studies of cell-cycle arrest induced by Vpr, the use of yeast cells is common, because they are easy to handle (Benko *et al.*, 2007; Elder *et al.*, 2001; Huard *et al.*, 2008).

The eukaryotic cell cycle is controlled by a complex network of proteins and genes. Cyclin-dependent protein kinases (CDKs) initiate the crucial events of the cell cycle by phosphorylating specific protein targets. The phosphorylation activity of CDKs is tightly dependent on binding to cyclins. Binding of cyclins to CDKs results in CDK–cyclin complexes. As there are always excess amounts of CDKs in eukaryotic cells, it is the availability of cyclins that determines the number of CDK–cyclin complexes in eukaryotic cells. CDK–cyclin complexes can be down-regulated either by inhibitory phosphorylation of the CDK subunit or by binding to inhibitory molecules designated cyclin-dependent kinase inhibitors (Csikasz-Nagy *et al.*, 2006). G2 arrest is characterized by low levels of cyclin B1–p34Cdc2 activity and inhibitory phosphorylation of p34Cdc2. It has been shown that Vpr directly inhibits the *in vitro* activity of a phosphatase, Cdc25C, which normally activates cyclin B1–p34Cdc2 (Fig. 2). Although the Vpr-binding site on Cdc25C is not its catalytic site, Cdc25C is inactivated by binding to Vpr, resulting in inhibition of Cdc25C phosphatase activity. In the absence of Cdc25C phosphatase activity, cyclin B1–p34Cdc2 remains in its phosphorylated form, which is inactive (Goh *et al.*, 2004; He *et al.*, 1995). 14-3-3 proteins are also involved in this pathway. These proteins normally regulate cell-cycle progression by changing the activities of cyclins, including Cdc25C. DNA damage results in Cdc25C phosphorylation, providing the active binding site for 14-3-3. It has been shown that the C-terminal region of Vpr interacts with the C-terminal region of 14-3-3, leading to the association of 14-3-3 with Cdc25C. This complex is not able to activate cyclin B1–p34Cdc2, therefore the cell cycle is arrested (Fig. 2) (Kino *et al.*, 2005).

Inactivating Cdc25C is not the only pathway utilized by Vpr to arrest the cell cycle. Several studies have documented the association of HIV-1 Vpr with a cellular protein that now is called DCAF-1 (DDB1- and CUL4-associated factor 1), also known as VprBP (Vpr-binding protein). Further research demonstrated the role of this protein in DNA replication and embryonic development. It has recently been demonstrated that Vpr exploits DCAF-1, damaged DNA-binding protein 1 (DDB1) and the cullin 4A (CUL4A) ubiquitin ligase complex to interfere with the DNA replication machinery of infected cells, resulting in cell-cycle arrest (Fig. 3). The pathway exploited by Vpr implies that it functions as an adaptor protein in protein networks to interfere with the cell cycle and replication machinery (Giaccia & Kastan, 1998; Gieffers *et al.*, 2000; Hrecka *et al.*, 2007; Wen *et al.*, 2007). Vpr exploits DCAF-1 as an adaptor to engage DDB1 as a component of the CUL4A ubiquitin ligase complex. As this complex is involved in proteasomal degradation, it was suggested that G2 arrest is induced by Vpr through degradation of an unidentified protein that is needed for progression of cells from G2 arrest to mitosis (Wen *et al.*, 2007). In fact, the CUL4A ubiquitin ligase complex acts as a pivotal step, regulating different cellular pathways by targeting proteins for ubiquitin-dependent degradation. It has recently been demonstrated that, in this complex, DDB1 interacts with

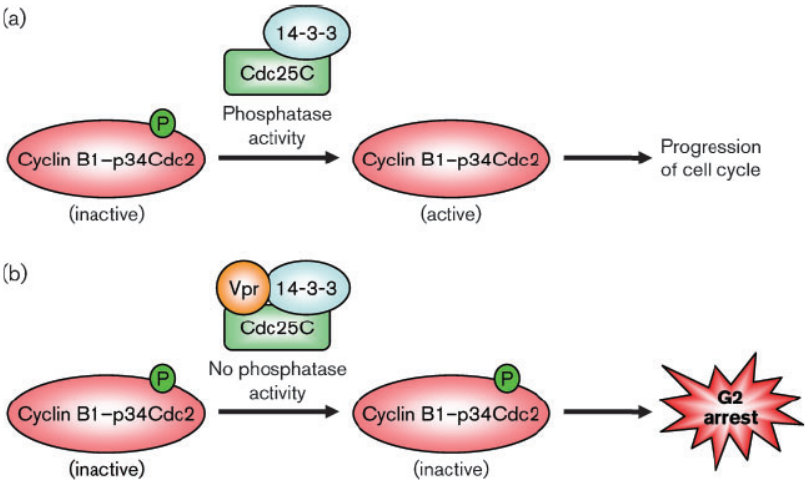

Fig. 2. Cell-cycle arrest through inactivation of Cdc25C. (a) 14-3-3 protein binds to Cdc25C, resulting in a complex that possesses phosphatase activity. The complex removes the phosphate molecule from the inactive form of cyclin B1–p34Cdc2, altering it to the active form that drives progression of the cell cycle. (b) Vpr binds to the 14-3-3 protein and Cdc25C and inactivates this complex. In the absence of the phosphatase activity of Cdc25C, cyclin B1–p34Cdc2 remains inactive, resulting in G2 arrest.

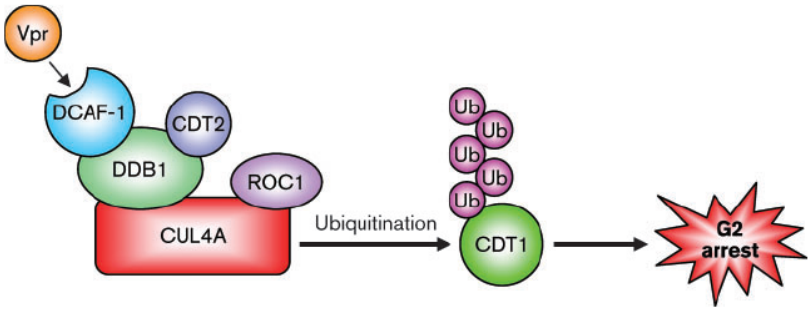

Fig. 3. Cell-cycle arrest by triggering ubiquitination. Vpr functions as an adaptor protein to interfere with the cell cycle and replication machinery. Vpr exploits DCAF-1 as an adaptor to engage DDB1, a component of the CUL4A ubiquitin ligase complex, which is involved in proteasomal degradation. DDB1 interacts with multiple WDR proteins, including CDT2, which serve as the substrate-recognition subunits of the CUL4A ubiquitin ligase complex. CDT2 enables the complex to target CDT1 for ubiquitin-dependent degradation. CDT1 degradation results in G2 arrest.

multiple WD40-repeat (WDR) proteins, which serve as the substrate-recognition subunits of the CUL4–DDB1 ubiquitin ligase. More than 150–300 WDR proteins have been identified in the human genome. Considering the variety of WDR proteins, the impact of biological processes through CUL4 ligase-mediated proteolysis can be understood. It has been reported that CDT2 interacts with the CUL4A ubiquitin ligase complex, enabling it to target CDT1 for degradation. CDT1 is a replication factor, depletion of which prevents DNA replication. Although cell-cycle arrest cannot be attributed to the degradation of a single protein through CUL4 ligase-mediated proteolysis, resulting in G2 arrest, CDT1 can be addressed as a key protein that is ubiquitinated by the CUL4A ubiquitin ligase complex, activated by Vpr. Indeed, many other proteins may play similar roles in parallel with CDT1 (Higa & Zhang, 2007; Jin *et al.*, 2006; Rialland *et al.*, 2002).

Induction of apoptosis

Although a variety of stimuli have been described as causes of apoptosis, apoptosis is regulated through one of two known cell death-signalling pathways: the extrinsic and intrinsic pathways. Both pathways share similar molecules (especially caspases) and features. The extrinsic pathway is initiated by external stimuli that are sensed by cell-death receptors on the cell membrane. Mitochondria play the central role in the intrinsic pathway by releasing molecules that trigger apoptosis (Budihardjo *et al.*, 1999; Green, 2000). Several studies have shown that HIV-1 Vpr is able to induce the intrinsic pathway of apoptosis in a number of human cell lines and to promote apoptosis during HIV-1 infection. Not only does Vpr induce apoptosis in infected cells, but it has also been suggested that induction of apoptosis in uninfected bystander cells is caused by Vpr. However, neither HIV-2 nor simian immunodeficiency virus Vpr induces apoptosis in mammalian cell lines. Induction of apoptosis by several HIV-1 proteins, including Nef, Vif, Vpr, Vpu, Tat and Rev, has also been reported (Andersen *et al.*, 2008; Chang *et al.*, 2000; Conti *et al.*, 2000; Stewart *et al.*, 1999; Yedavalli *et al.*, 2005).

Apoptosis studies indicate that mitochondrial intermembrane proteins, including adenine nucleotide translocator (ANT), apoptosis-inducing factor (AIF), cytochrome *c*, procaspases and heat-shock proteins (HSPs), are released during apoptosis and are essential for the activation of caspases and DNases. Several studies have demonstrated that HIV-1 Vpr permeabilizes the mitochondrial membrane by binding to the permeability transition pore complex (PTPC); lack of PTPC results in resistance to the apoptotic effect of Vpr. It has also been demonstrated that, in the presence of Vpr, cytochrome *c* is released from mitochondria, resulting in apoptosis. In fact, the mechanism responsible for mitochondrial-membrane permeabilization involves a number of proteins, including PTPC, proapoptotic members of the Bcl-2 family, ANT and the voltage-dependent anion channel (VDAC) (Jacotot *et al.*, 2000).

The impact of Vpr on mitochondrial-membrane permeability is also attributed to its interaction with ANT in the inner mitochondrial membrane. It is believed that the interaction between Vpr and ANT cooperatively forms large conductance channels in the inner membrane. These results suggest that the interactions of Vpr with PTPC and ANT lead to the formation of channels in the outer and inner mitochondrial membranes, respectively. These events result in release of a number of molecules, such as cytochrome *c*, from mitochondria (Jacotot *et al.*, 2001; Yedavalli *et al.*, 2005). The released cytochrome *c* molecules bind to Apaf-1 (apoptotic peptidase-activating factor 1) and form apoptosome complexes that become activated by caspase 9. Activated apoptosome complexes trigger the caspase cascade and apoptosis (Fig. 4) (Kim *et al.*, 2005; Muthumani *et al.*, 2002b; Zou *et al.*, 2003).

It has been documented that a conserved motif in the C-terminal region of Vpr is responsible for the induction of apoptosis. Interestingly, the C-terminal peptides containing the conserved sequence at aa 71–82 can even induce apoptosis. Extracellular addition of C-terminal peptides to human $CD4^+$ cells causes membrane permeabilization, DNA fragmentation and formation of apoptotic bodies, which are all signs of apoptosis. Similar effects can also be

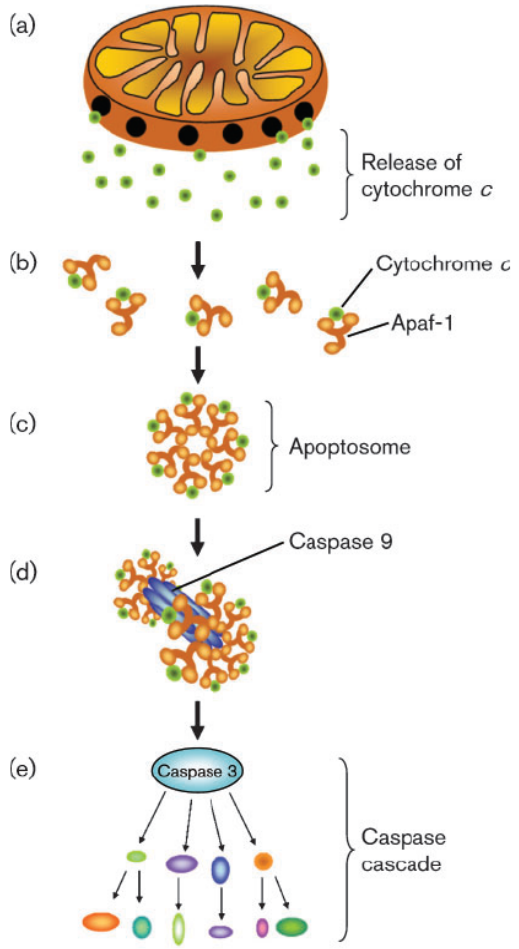

Fig. 4. Induction of apoptosis through impact of Vpr on mitochondrial-membrane permeability and release of cytochrome *c*. (a) Vpr interaction with ANT and PTPC in mitochondrial membranes cooperatively forms large channels in the inner and outer mitochondrial membranes, respectively. Cytochrome *c* molecules are released from the channels. (b) The released cytochrome *c* molecules bind to Apaf-1 proteins and (c) apoptosome complexes are formed. (d) Caspase 9 activates apoptosomes; (e) once the apoptosome is activated, it triggers a caspase cascade, resulting in apoptosis.

observed by adding the polypeptides to yeast, indicating that Vpr targets fundamental cellular pathways common to many eukaryotic cells (Arunagiri *et al.*, 1997).

HS1-associated protein X-1 (HAX-1) has also been demonstrated as a target for Vpr in the induction of apoptosis. HAX-1 is a proapoptotic factor that is found mainly in mitochondria. Current findings indicate that overexpression of HAX-1 inhibits Vpr-induced apoptosis. The number of molecules that have been found to be involved in Vpr-induced apoptosis is still increasing, suggesting that additional mechanisms and molecules may be utilized by Vpr for induction of apoptosis (Vafiadaki *et al.*, 2006, 2009; Yedavalli *et al.*, 2005).

Modulation of gene expression

Although the first reported function for Vpr was transactivation of the virus long terminal repeat (LTR), the mechanism has not yet been elucidated completely. The ability of Vpr to induce G2 arrest has also been linked to transactivation of the LTR. Transactivation activity of the LTR during G2 is five to ten times higher than that during G1; therefore, the reproduction level of wild-type HIV-1 is five to ten times higher than that of Vpr-mutated viruses, indicating the importance of transactivation by Vpr (Zhu *et al.*, 2001). Indeed, Vpr transactivation activity is moderate and that from the HIV-1 Tat protein is stronger (Kino & Chrousos, 2004; Poon *et al.*, 2007). Vpr affects not only virus replication, but also cellular gene expression, proliferation and differentiation. As Vpr circulates in the blood of HIV-1-infected individuals, it may also affect the gene expression of non-infected cells (Balasubramanyam *et al.*, 2007; Xiao *et al.*, 2008).

As already mentioned, Vpr enhances the activity of the HIV-1 promoter located in the LTR. This region is also a target for the p53 and Sp1 transcription factors. The ability of Vpr to stimulate the HIV-1 promoter is affected negatively in the presence of p53. Sp1 is a cellular transcription factor essential for the transcriptional activation of the LTR by Vpr (Pauls *et al.*, 2006; Sawaya *et al.*, 1998). Vpr specifically activates HIV-1 LTR-directed transcription on a minimal promoter containing a TATA box and the binding motifs for Sp1. Studies indicate that Vpr contains a leucine zipper-like domain, aa 60–81 in α-helix III, which interacts with Sp1 when it is bound to the Sp1 motifs on the HIV-1 LTR (Wang *et al.*, 1995, 1996). It has been reported that Vpr cooperates with nuclear-receptor coactivators p300/CBP and SRC-1a to activate the glucocorticoid-responsive mouse mammary tumor virus promoter. Several studies have also reported that Vpr transactivates the HIV-1 LTR promoter by direct interaction with the p300 coactivator. It has been suggested that Vpr recruits p300/CBP to transactivate the HIV-1 LTR (Kino *et al.*, 2002).

Direct interaction between HIV-1 Vpr and the glucocorticoid receptor (GR) has also been reported to affect virus replication and gene expression. Human Vpr-interacting protein (hVIP) is also critical for the interaction of HIV-1 Vpr with GR. Glucocorticoids exert anti-inflammatory and immunosuppressive effects by interacting with their specific intracellular receptors, such as GR. Recent studies suggest that Vpr mimics some of the effects of glucocorticoids through interaction with GR (Ramanathan *et al.*, 2002). Vpr has also been suggested to bind to the LTR through interaction with GR. Vpr shares the LXXLL motif, aa 64–68, with steroid-receptor coactivators. This motif enables Vpr to bind to ligand-activated GR. These results suggest that Vpr functions as an adaptor molecule to

connect the different molecules required for high promoter activity (Kino *et al.*, 2002; Shrivastav *et al.*, 2008).

HIV-1 Vpr has been demonstrated to bind specifically to the general transcription factor TFIIB. The interacting motif of TFIIB overlaps the domain of TFIIB that is involved in the intramolecular bridge between its N and C termini. Binding of Vpr may induce a conformational change in TFIIB that could possibly affect its activity (Agostini *et al.*, 1996, 1999).

Nuclear factor κB (NF-κB) is one of the main mediators of the HIV-1 LTR. The N-terminal region of NF-κB mediates DNA binding, dimerization and interaction with inhibitory proteins. Activator protein 1 (AP-1) is also a transcription factor that has been suggested as a mediator for Vpr. Some studies have shown that the extracellular form of synthetic Vpr can stimulate the HIV-1 LTR via both AP-1 and NF-κB activation. Extracellular Vpr is commonly found in the blood of HIV-positive individuals and may be involved in the stimulation of virus transactivation. Although no receptor has been yet identified for extracellular Vpr, studies using confocal microscopy indicate that it can enter cells easily, and it is believed that even this form of the protein enhances transcription of the promoters that interact with NF-κB and AP-1 (Varin *et al.*, 2005).

Upregulation of human survivin at the transcriptional level by HIV-1 Vpr has also been reported. Human survivin is involved in inhibition of apoptosis and regulation of cell division. The *survivin* gene is regulated in a cell cycle-dependent manner. Vpr specifically upregulates survivin expression through a cell cycle-regulated mechanism. It is still not clear what elements on the *survivin* gene are used in survivin upregulation by Vpr (Zhu *et al.*, 2003).

The transactivation activity of Vpr is related to the *cis*-acting elements within the U3 region of the HIV-1 LTR, and the binding sites for several proteins, including Sp1, NF-κB, AP-1 and GRE, also lie in this region. The impact of Vpr on Nef expression from unintegrated HIV-1 has recently been demonstrated, suggesting that Vpr increases Nef expression before integration (Poon *et al.*, 2007; Varin *et al.*, 2005).

Suppression of immune activation

This function of Vpr is related closely to the effect of Vpr on gene expression but, because suppression of immune activation could be a consequence of gene expression, it is considered an independent function. Although many studies indicate that HIV-1 Env, Tat and Nef affect immune responses (Muthumani *et al.*, 2005), the immune suppression observed in HIV-1-infected patients is also partly attributed to Vpr. This protein suppresses antigen-specific CD8-mediated cytotoxic T-lymphocyte (CTL) and T-helper type 1 (Th1) immune responses. The molecular mechanisms of the suppression of CTL and antibody production by Vpr are still under debate, but it has been suggested that Vpr may prevent production of antibodies against the virus by inhibiting T-cell clonal expansion through inducing G2 arrest and suppressing T-cell proliferation. Suppression of the host inflammatory responses by Vpr has also been reported. Vpr down-regulates proinflammatory cytokines and chemokines, resulting in inhibition of the host inflammatory responses (Muthumani *et al.*, 2004a; Zhao *et al.*, 2005). A recent study also suggests that Vpr may alter sensitivity to insulin and thereby play a role in the development of lipodystrophy and insulin resistance (Shrivastav *et al.*, 2008).

In vitro studies have implicated the ability of Vpr to downregulate the expression of several immunologically important molecules, such as CD40, CD80, CD83 and CD86, on macrophages and dendritic cells. Vpr reduces the capacity of monocytes to mature to dendritic cells in tissue culture (Muthumani *et al.*, 2004b). A recent study indicates that Vpr impairs natural killer (NK) cell function *in vitro*. Production of gamma interferon is reduced in these impaired NK cells (Majumder *et al.*, 2008).

Many of the experiments indicating that Vpr suppresses immune-cell activation and cytokine production have been performed *in vitro*. *In vitro*, Vpr targets and suppresses NF-κB activity, which is critical for T-cell activation and cytokine production. Similar results have been obtained from several *in vivo* studies (Ayyavoo *et al.*, 2002; Muthumani *et al.*, 2005). In a plasmid vaccine model using Vpr-expressing vectors, Vpr altered the induction of the $CD8^+$ T-cell response. The mechanism of immune suppression could be complex and may be related to G2 arrest in T cells, suppression of NF-κB, induction of apoptosis in T cells and the GR pathway. Even in the absence of the effect of Vpr on $CD8^+$ T cells, the immune response is affected by targeting antigen-presenting cells and T-helper cells, indicating the complexity of immune suppression by Vpr (Muthumani *et al.*, 2002a).

Another explanation for the possible mechanism of immune suppression by Vpr focuses on the GR pathway. As already mentioned, the direct interaction between Vpr and GR is well established. Glucocorticoids are known to control inflammation and to induce immunosuppressive signals by binding to GR (Andersen & Planelles, 2005). Interestingly, the GR–Vpr complex is also able to induce similar effects and is suppressed by GR-pathway inhibitors. Apparently, Vpr mimics the interaction of glucocorticoids with GR to suppress immune activation (Moon & Yang, 2006; Muthumani *et al.*, 2006).

Fidelity of reverse transcription

HIV-1 RT is an error-prone enzyme that misincorporates approximately one nucleotide in every 2000–5000 polymerized nucleotides (Basavapathruni & Anderson, 2007; Li *et al.*, 1997). Reverse transcription of the HIV-1 genomic RNA is initiated in the virus core, where NC is associated with the RNA genome. The reverse-transcribed genome is directed toward the nucleus by the viral and cellular

proteins forming the PIC (Gao *et al.*, 2007; Sun *et al.*, 1997). A number of studies have confirmed that Vpr co-localizes with IN and virus nucleic acids within the PIC, and remains associated with the viral DNA within 4–16 h after infection (Le Rouzic & Benichou, 2005).

HIV-1 Vpr interacts with a cellular protein, uracil–DNA glycosylase 2 (UNG2), which is a DNA-repair enzyme involved in nucleotide-excision repair. Uracil can be introduced into DNA either by cytosine deamination or by misincorporation of dUTP (Chen *et al.*, 2004; Mansky *et al.*, 2000). If the uracil is not repaired by UNG2, after the next round of replication, a C→T transition mutation occurs in that DNA strand and a G→A transition mutation occurs in the opposite strand of DNA. In the absence of Vpr, a fourfold increase in the rate of G→A mutations has been reported in each round of HIV-1 replication (Chen *et al.*, 2002). These G→A mutations are the main mutational force in the evolution of drug-resistant HIV-1 strains (Berkhout & de Ronde, 2004). On the other hand, accumulation of these mutations can result in non-functional genomes (Keele *et al.*, 2008; Priet *et al.*, 2003). Vpr may play a role in virus evolution by balancing the level of mutations.

Several studies have established that the interaction of Vpr with UNG2 in order to incorporate it into HIV-1 virions correlates with the influence of Vpr on the HIV-1 mutation rate (Chen *et al.*, 2002). The interaction between Vpr and UNG2 has been demonstrated both *in vitro* and *ex vivo* in Vpr-expressing cells. Vpr has been found to incorporate specifically the nuclear form of UNG2 into HIV-1 virions. HIV-1 IN may also participate in the virion incorporation of UNG2, but the role of Vpr in the virion incorporation of UNG2 seems to be more essential and is correlated with the ability of Vpr to alter the mutation rate of HIV-1 (Chen *et al.*, 2004; Mansky, 1996).

dUTPases are another class of enzymes that inhibit uracil incorporation into DNA during DNA synthesis, but utilize a mechanism distinct from that utilized by UNG2. Both UNG2 and dUTPase are encoded by some DNA viruses, such as poxviruses and herpesviruses. Some retroviruses encode only dUTPase. The genomes of non-primate lentiviruses, such as equine infectious anemia and feline immunodeficiency viruses, contain a dUTPase-encoding sequence as an integral part of the *pol* gene, lying between the RNase H and IN sequences. In contrast, the genomes of primate lentiviruses do not contain gene sequences encoding either dUTPase or UNG2. It is believed that the interaction of HIV-1 Vpr with cellular UNG2 compensates for the lack of a viral dUTPase to correct uracil misincorporation into virus DNA (Bouhamdan *et al.*, 1996).

Vpr has been reported to interact through its N terminus with Lys–tRNA synthetase. This interaction has been observed both *in vitro* and *ex vivo*. Several cellular and viral proteins interact with Vpr and induce conformational changes that expose the N-terminal region of Vpr and thus regulate its affinity for Lys–tRNA synthetase. During the early stages of HIV replication, Vpr binds to NCp7, which may expose the N-terminal region of Vpr, resulting in a high affinity for Lys–tRNA synthetase. During the later stages of infection, the N-terminal region of Vpr interacts with the HIV-encoded p6, which may also affect Vpr interaction with the Lys–tRNA synthetase. As $tRNA_{Lys}$ acts as a primer for initiation of reverse transcription of the HIV-1 genome, it suggests that this mechanism is utilized by Vpr to potentiate HIV-1 reverse transcription (Stark & Hay, 1998).

Additional interactions and possible functions of Vpr

HIV-1 Vpr has been observed to form ion channels in planar lipid bilayers, resulting in an inward sodium current followed by cell death in cell cultures. Mutagenesis studies have shown that the N-terminal region of Vpr is responsible for the above functions. A 40 aa peptide in the N-terminal region of Vpr is sufficient to form ion channels that are able to cause cell death. It is believed that this region is responsible for Vpr's ion-channel activity and cytotoxic effects. These effects could be caused by the extracellular form of Vpr that is found in the serum and cerebrospinal fluid of AIDS patients (Piller *et al.*, 1998, 1999). Extracellular Vpr can also disturb neuronal communication, resulting in neuronal dysfunction (Rom *et al.*, 2009). It also inhibits neuronal development through the induction of mitochondrial dysfunction (Kitayama *et al.*, 2008). Apparently, extracellular Vpr induces different levels of cytopathogenicity depending on the cell type (Huang *et al.*, 2000).

The interaction of HHR23A, a protein involved in nucleotide-excision repair, with HIV-1 Vpr has been reported. It has been demonstrated that a C-terminal, 45 aa region of HHR23A binds to Vpr. Overexpression of HHR23A results in partial alleviation of the G2 arrest induced by Vpr. It has been suggested that the interaction between Vpr and HHR23A could be involved in the G2 arrest induced by Vpr (Engler *et al.*, 2001; Withers-Ward *et al.*, 1997).

HIV-1 Vif expression has recently been implicated in the inhibition of Vpr-mediated G2 arrest, and deletion of Vif from the HIV-1 genome results in an increase in G2 arrest induced by Vpr. In addition, T cells infected with Vif-deleted HIV-1 express higher levels of Vpr than cells infected with the wild-type virus. It has been suggested that inhibition of Vpr by Vif is mediated by proteasomal degradation, similar to the other proteins that are directed toward proteasomal degradation by Vif (Wang *et al.*, 2008).

Concluding remarks

Interaction of a single protein with a variety of different proteins may seem surprising. Some proteins, such as

proteinases, chaperones and ubiquitin, interact with different proteins and direct them toward one pathway, e.g. proteinases only degrade proteins. The amazing property of Vpr is that this small polypeptide interacts with variety of proteins and directs them toward different pathways. Several hypotheses have been suggested to explain the capability of Vpr to exert so many effects through direct protein–protein interactions. One hypothesis suggests that Vpr possesses structural features similar to those of HSP70, a cellular chaperone, enabling Vpr to bind to many proteins with sufficient energy to cause changes in the activity of target proteins (Basanez & Zimmerberg, 2001).

In an attempt to find a common characteristic for the proteins that interact with Vpr, the WXXF motif (where X is unknown) was identified as an important criterion in some Vpr-interacting proteins. The WXXF motif of UNG2 was implicated in its interaction with Vpr. The N-terminal region of TFIIB, which binds specifically to Vpr, also contains the WXXF motif. Mutant forms of TFIIB that have a point mutation in the WXXF motif are not able to interact with Vpr. Interestingly, it was demonstrated that, by attaching the WXXF motif to a non-human protein, chloramphenicol acetyltransferase, it was incorporated into HIV-1 virions in the presence of Vpr (Agostini *et al.*, 1999; Bouhamdan *et al.*, 1998; Yao *et al.*, 2002). Although the main functions of Vpr have apparently been described, there are still several interactions between Vpr and proteins of which the functions remain to be elucidated.

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Appendix H

Functional integrity of naturally occurring mutants of HIV-1 subtype C Vpr

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ABSTRACT

HIV-1 Vpr, an accessory protein with multiple functions, is involved in the induction of apoptosis, cell cycle G2 arrest, and modulation of gene expression. Many functions of this protein have been documented for the wild-type subtype B Vpr, however the functionality of other subtypes has not sufficiently been addressed. In this study, the functionality of Subtype B Vpr, 6 subtype C mutant Vpr proteins and the consensus sequence of subtype C Vpr were compared with each other. All the subtype B and C Vpr proteins localized to the nucleus of human 293T cells. Subtype C Vpr proteins induced cell cycle G2 arrest in a lower proportion of human 293T cells compared to subtype B Vpr. Subtype B and the naturally mutant Vpr proteins induced apoptosis in a similar manner, ranging from 95.33% to 98.64%. However, an artificially designed Vpr protein containing the consensus sequences of subtype C Vpr indicated a reduced ability in induction of apoptosis. The study of mRNA profile of the transfected cells indicated that all Vpr proteins modulated the apoptotic genes triggering the intrinsic pathway of apoptosis. Our results indicate that subtype C Vpr is able to exert the same functions previously reported for subtype B Vpr. Most natural mutations in Vpr not only do not disturb the functions of the protein but also potentiate the protein for an increased functionality. The natural mutations of Vpr may thus not always be regarded as defective mutations. The study suggests the adaptive role of the natural mutations commonly found in subtype C Vpr.

1. Introduction

HIV-1 Vpr is an accessory virion-associated protein that is composed of 96 amino acids (Pandey et al., 2009). Vpr has a variety of biological functions including cell cycle G2 arrest, apoptosis induction (Jacquot et al., 2007), nuclear import of the pre-integration complex (PIC), modulation of gene expression, suppression of immune activation, ensuring fidelity of reverse transcription (Romani and Engelbrecht, 2009), and regulating RNA splicing (Zhang and Aida, 2009). Vpr contains a flexible N-terminal region, three α-helical domains and a flexible C-terminal region. Each function of Vpr is attributed to one or more of its domains. Identification of the functions of Vpr domains is usually carried out by mutagenesis studies (Romani and Engelbrecht, 2009). Mutations in the HIV-1 *vpr* gene have been shown to significantly reduce infection of terminally differentiated macrophages but have little or no effect on infection of dividing cells (Rogel and Emerman, 1995). In groups of so-called "long-term nonprogressors", mutations or truncations of Vpr have been shown to reduce or abolish the pro-apoptotic potential of Vpr. Based on these results, it has been suggested that the apoptosis induction by Vpr may contribute to the pathogenesis of AIDS (Jian and Zhao, 2003; Somasundaran et al., 2002).

The sequences of wild-type Vpr proteins reveal mutations throughout the Vpr molecule (Tungaturthi et al., 2004). Since the *vpr* gene has a conserved nature in primate lentiviruses, it has been suggested that the optimal function of Vpr is achieved by natural selection of naturally occurring Vpr mutants in the infected individuals (Macreadie et al., 1995; Rouzic and Benichou, 2005; Tristem et al., 1992). There are currently no data pertaining to the functional consequences of naturally occurring mutations in Vpr. However, it seems logical that naturally occurring mutations may differentially affect the functions of Vpr. It has been suggested that the mutations in Vpr represent amino acid residues with optimal functions selected *in vivo* and these mutations might be beneficial to Vpr (Tungaturthi et al., 2004). In addition to mutations, it has been shown that the subtype of Vpr also affects its functions such that the functions of Vpr are thought to be subtype-dependent. In several studies, the ability of Vpr to induce apoptosis has been compared for several subtypes and different levels of apoptosis induction have been observed (Bano et al., 2009; Jian and Zhao, 2003). However, these studies have not sufficiently addressed different functions of subtype C Vpr. It is important to study all functions of Vpr, as well as other HIV-1 proteins, that have been established for reference sequences of Vpr as the wild-type protein. It has been shown that subtype D Vpr, for example, has no detectable apoptotic activ-

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ity (Jian and Zhao, 2003). Bano et al. have shown that subtype B Vpr is a more powerful transactivator of LTR promoter than a subtype C Vpr from an Indian isolate (Bano et al., 2007). Comparative studies of some of the HIV-1 proteins, such as Vif, Vpu, Tat, Rev, and envelope proteins, among HIV-1 subtypes have revealed slight to significant variations in a number of their functions that are subtype-dependent (Campbell et al., 2007; Gupta and Benerjea, 2009; Neogi et al., 2009; Sood et al., 2008; Sundaravaradan et al., 2007). Therefore, the established functions of Vpr cannot directly be attributed to the Vpr protein of all or other HIV-1 subtypes without experimentally confirming and measuring those functions.

The majority of vaccine studies have been focused on subtype B HIV-1 strains predominating in developed countries of the Western world. However, by far the greatest burden of HIV-1 infection is occurring in the developing countries, where non-subtype B HIV-1 strains predominate (De-Rose et al., 2005). Although, not proven, it has been suggested that an efficient vaccine for HIV-1 should be subtype specific (Arora et al., 2001; Center et al., 2009; Chugh and Seth, 2004). Although HIV-1 subtype C is the predominant subtype in many developing countries, many aspects of this subtype have been poorly studied (Neogi et al., 2009; Romani et al., 2009). The lack of data regarding the functional impact of the naturally occurring mutations in Vpr and the shortage of data regarding HIV-1 subtype C Vpr prompted us to study the functional impact of the naturally occurring mutations in HIV-1 subtype C Vpr.

2. Materials and methods

2.1. Sequence selection and phylogenetic analysis

Viral RNA extraction, PCR and DNA sequence analysis were performed as described previously (Romani et al., 2009). The sequences were translated into protein using BioEdit version 7.090 (Hall, 1999). By comparing our amino acid sequences with one another and with the Vpr sequences obtained from LANL HIV Database (http://hiv-web.lanl.gov), 6 HIV-1 subtype C *vpr* sequences that their translated proteins contained natural mutations in different domain were selected for the study (Accession numbers: FJ039738, FJ039776, FJ039748, FJ039732, FJ039749, FJ039734). A phylogenetic analysis was carried out to indicate the phylogenetic relationship of the sequences selected in this study. Briefly, 6 selected *vpr* fragments, consensus sequence of HIV-1 subtype C (LANL HIV Database), and subtype B *vpr* (pNL4-3) were aligned with HIV-1 reference sequences obtained from the LANL HIV Database, using Clustal X version 1.81.22 (http://accelrys.com). A neighbor joining phylogenetic tree was constructed with MEGA version 4 (Tamura et al., 2007) using the Kimura two-parameter (Kimura, 1980).

2.2. Protein modeling

To indicate the mutation sites in the protein structure and to locate the mutated domains, protein structures of 6 selected sequences were primarily simulated using SWISS-MODEL protein modeling server (Arnold et al., 2006; Kiefer et al., 2009). The three dimensional structures of Vpr mutants along with their mutations were simulated as ribbon representation using Discovery Studio Visualizer version 2.0. The amino acid residues that were different from the consensus sequence of subtype C Vpr were considered mutations.

2.3. Cloning of vpr fragments

The ORF of *vpr* gene from the subtype C sequences TV1453, TV1466, TV1570, TV1628, TV1642, TV1707, and the wild-type subtype B *vpr* (pNL4-3, Accession number: AF324493) was amplified using the primers Vpr-Sgf-F (5′-GGGTGCGATCGCCATGGAACAAGCCCCAGAAGAC-3′) and Vpr-Pme-R (5′-CTTAGTTTAAACTTAGGATCTACTGGCTCCATTTCTTG-3′). The primers contain the restriction site for *SgfI* and *Pme-I*, respectively. Promega GoTaq Flexi Kit (Promega, Madison, WI) was used in the polymerase reaction. The PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The purified PCR products were individually cloned into pF4A CMVd1 Flexi® Vector (Promega, Madison, WI) following the manufacturer's protocol and transformed into JM109 Competent Cells (Promega, Madison, WI). Four individual clones for each sample were grown and plasmid DNA was extracted and automatically sequenced by ABI prism® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using Vpr-Sgf-F and Vpr-Pme-R primers. The sequencing data were analyzed using the Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI). To select the non-mutated clones, the sequences were compared with the corresponding *vpr* sequences used for cloning. One clone that contained the desired fragment was selected for each subtype C and the wild-type subtype B *vpr*.

Since the intact pF4A CMVd1 Flexi® Vector carries the lethal barnase gene, it cannot be used as a control vector for transfection. A control vector for pF4A CMVd1 Flexi® Vector was constructed by removing barnase gene according to the manufacturer's suggestion. This control vector was used for transfection in the experiments that we needed a mock-transfected sample.

2.4. Construction of the consensus sequence of HIV-1 subtype C Vpr

The clone of TV1453 of which the translated protein had the most similar sequence to the consensus sequence of subtype C Vpr (Consensus C; 2004, http://hiv-web.lanl.gov) was selected to construct the consensus sequence. The corresponding protein of this clone contained the Y45H mutation compared to the consensus sequence of subtype C Vpr. To replace tyrosine, Y, with the original amino acid, histidine or H, a pair of mutagenic primers, namely wild-mut-F (5′-CATAGCTTAGGACAATACATCTATGAAAC-3′) and wild-mut-R (5′-GTTTCATAGATGTATTGTCCTAAGCTATG-3′), was designed. The primers were complementary and contained the desired mutation in the middle. The site-directed mutagenesis was performed using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. To confirm the desired mutation has been implemented in the construct, the confirmative sequencing was carried out as described previously.

2.5. Transfer of the protein-coding regions between the constructs

The pF4A CMVd1 Flexi® Vector that was used for cloning is a vector commonly used for eukaryotic expression. In this study, Vpr had to be tracked within the transfected cells using a reporter. To tag a reporter to the expressed Vpr proteins, pFN22K CMVd1 Flexi® Vector (Promega, Madison, WI) was used for cloning. Using this vector, HaloTag protein was tagged to the N-terminus of the expressed proteins. This enabled us to track Vpr with two HaloTag® ligands (Promega, Madison, WI) depending on the emission wavelength needed in the study. The *vpr* fragments were transferred from the donor vectors, the constructs of pF4A CMVd1 Flexi® Vector, to the acceptor vectors, the pFN22K CMVd1 Flexi® Vector, following the manufacturer's protocol. The transfer of the *vpr* fragments was confirmed by PCR using Vpr-Sgf-F and Vpr-Pme-R primers.

Similarly to pF4A CMVd1 Flexi® Vector, pFN22K CMVd1 Flexi® Vector carries the lethal barnase gene that had to be removed to construct the corresponding control vector. A control vector for

pFN22K CMVd1 Flexi® Vector was constructed by removing barnase gene according to the manufacturer's suggestion.

2.6. Cell line and transfection

Human 293T cell line was kindly provided by Professor Lynn Morris (National Institute for Communicable Diseases, Sandringham, South Africa). 293T cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air in Dulbecco's modified Eagle medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland), 100 IU/ml penicillin and 100 μg/ml streptomycin. Transfection experiments were performed using FuGENE® 6 Transfection Reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. For the experiments that we needed to track the expressed protein, including subcellular localization of Vpr, cell cycle analysis and apoptosis assay, the tagged Vpr-expression vectors, the constructs of pFN22K CMVd1 Flexi® Vector, were used for transfection. For the experiments that we did not need to track Vpr, gene expression assays, the constructs of pF4A CMVd1 Flexi® Vector were used for transfection.

2.7. Subcellular localization of Vpr

To study the subcellular localization of Vpr proteins, human 293T cells were plated at 30,000 cells/chamber in 8-chamber slides. When the cells reached 80% confluency, they were individually transfected with the constructs of pFN22K CMVd1 Flexi® Vector to express Vpr protein of subtype B, consensus C, TV1453, TV1466, TV1570, TV1628, TV1642, and TV1707. To label Vpr in the transfected cells, the cells were labeled using 5 μM HaloTag® TMR ligand (Promega, Madison, WI) 20 h after transfection. The labeled cells were fixed by replacing the medium with 4% paraformaldehyde/0.2 M sucrose/phosphate buffer solution (PBS) and incubating the cells for 10 min at room temperature. The solution was replaced with PBS containing 0.1% Triton X-100 and incubated for 10 min at room temperature. The nucleus of the fixed cells was labeled using 900 nM DAPI (Sigma, St. Louis, USA) to confirm the nuclear localization or possibly cytoplasmic mislocalization of Vpr proteins. The cells were observed and photographed using Olympus IX 81 motorized inverted microscope (Olympus, PA, USA) that was equipped with MT20 Illumination System (Olympus, Pennsylvania, USA) and CellR software (Olympus, PA, USA).

2.8. Quantitative real-time PCR

To study the modulation of gene expression by Vpr proteins, human 293T cells were plated at 400,000 cells/6 cm plate. When the cells reached 80% confluency, they were individually transfected with the constructs of pF4A CMVd1 Flexi® Vector to express Vpr protein of subtype B, consensus C, TV1453, TV1466, TV1570, TV1628, TV1642, and TV1707. Using RNeasy Mini Kit (QIAGEN, Hilden, Germany) the total RNA was extracted from the 293T cells 24 h after transfection. The total RNA was measured using Nanodrop™ ND-1000 system (Nanodrop Technologies Inc., Dalaware, USA) and 1 μl of the total RNA was reverse transcribed into cDNA using ImProm-II™ Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's suggestions. The fluorescence-based quantitative real-time PCR (qPCR) was performed using TaqMan Human Apoptosis Arrays (Applied Biosystems, Foster City, Canada). These are predesigned microfluidic cards with a 384-well format to assay the expression of 93 human genes along with 3 endogenous controls (18S, ACTB, GAPDH) for each sample, 4 samples/per card. Briefly, 20 μl of each cDNA sample was added to 100 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Canada) and the total volume was brought to 200 μl with nuclease free water. After gentle mixing, the solution was transferred into 2 loading ports on a microfluidic card (100 μl per loading port). The microfluidic cards were transferred to the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, Canada) and the real-time PCR conditions were set as follows: 2 min at 50 °C, 10 min at 94.5 °C and 30 s at 97 °C, and 1 min at 59.7 °C for 40 cycles. Data acquisition was done according to the manufacturer's suggestions using ABI Prism 7900HT Sequence Detection System version 2.4 (Applied Biosystems, Foster City, Canada). The housekeeping genes, 18S, ACTB and GDPH were used for validation of results. Gene expression values were then calculated based on the $\Delta\Delta$Ct method, with data normalized to the mock sample (293T cells transfected with barnase-removed pF4A vector). Relative quantities (RQ) were determined using the equation: $RQ = 2^{-\Delta\Delta Ct}$. Only genes with reproducible amplification curves of quadruplicate determinations were analyzed and presented.

2.9. Induction of cell cycle G2 arrest by Vpr

To study the induction cell cycle G2 arrest by Vpr, human 293T cells were plated at 75,000 cells/well in 24-well tissue culture plates. When the cells reached 50% confluency, they were individually transfected with the constructs of pFN22K CMVd1 Flexi® Vector to express Vpr protein of subtype B, consensus C, TV1453, TV1466, TV1570, TV1628, TV1642, and TV1707. Twenty four hours after transfection, the medium was replaced with fresh complete DMEM medium. Forty eight hours after transfection, the cells were labeled with 5 μM HaloTag® Oregon Green® ligand (Promega, Madison, WI). The cells were harvested and resuspended in 500 μl of PBS and fixed by adding 2 ml of −20 °C absolute ethanol and incubating on ice for 30 min. Cell fixation facilitates labeling the DNA content by permeabilizing the cell membranes. The fixed cells were centrifuged and resuspended in PBS. The DNA content was labeled with 25 μg/ml of 7-amino actinomycin D (7-AAD) (Sigma–Aldrich Co., St. Louis, MO) and incubated for 10 min at room temperature in dark. The cell cycle was analyzed using flow cytometry within 6 h.

2.10. Induction of apoptosis by Vpr

To study the induction apoptosis by Vpr, human 293T cells were plated at 75,000 cells/well in 24-well tissue culture plates. When the cells reached 80% confluency, they were individually transfected with the constructs of pFN22K CMVd1 Flexi® Vector to express Vpr protein of subtype B, consensus C, TV1453, TV1466, TV1570, TV1628, TV1642, and TV1707. Twenty four hours after transfection the cells were labeled using 5 μM HaloTag® TMR ligand. Since harvesting the cells using trypsinase could damage the cellular membrane and lead to false results for apoptosis assay, the cells were gently harvested by treating with 2 mM EDTA/PBS for 1 min. The membrane phospholipid phosphatidyl serine (PS) is a component of inner membrane that is translocated from inner to outer leaflet in apoptotic cells (Iguchi et al., 2004; Mirnikjoo et al., 2009). Phosphatidyl serine was labeled in the apoptotic cells using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, CA, USA) according to the manufacturer's protocol. The cells were analyzed using flow cytometry within 1 h.

2.11. Flow cytometric analysis

Multi-colour flow cytometry was performed to determine the induction of the cell cycle G2 arrest and apoptosis in the transfected 293T cells. Mock-transfected cells were used as control. Flow cytometry analysis was performed on a BD FACSCalibour (Becton Dickinson, CA, USA) equipped with a 15 mW argon-ion and 20 mW red diode lasers. A total of 50,000 and 30,000 events were collected

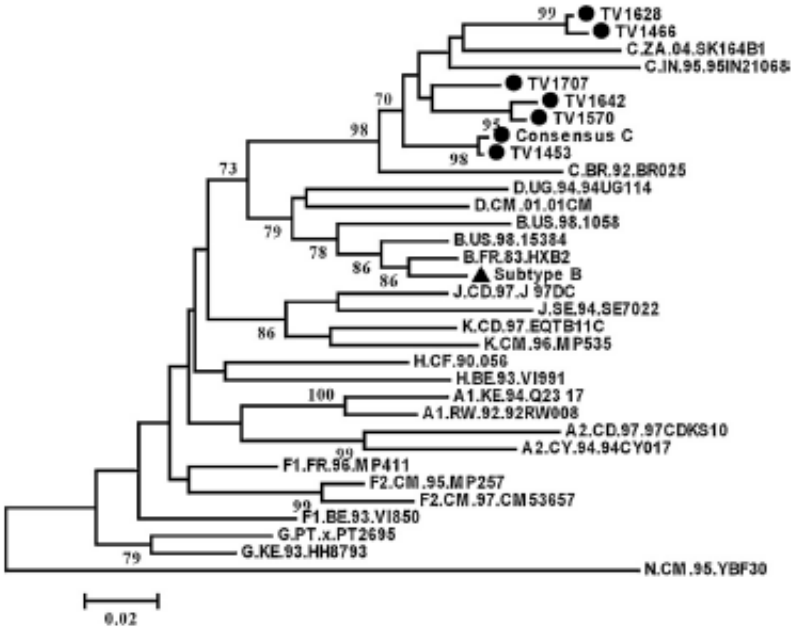

Fig. 1. The neighbor joining phylogenetic tree of the DNA sequences recruited in this study. Circles indicate subtype C isolates that were used in this study and the triangle indicates the subtype B sequence used in this study. The scale bar represents 0.02 substitutions per base position. Bootstrap values above 70% from 1000 replicates are shown for each node.

for cell cycle and apoptosis assay, respectively. The transfected cells were gated based on HaloTag Oregon Green and HaloTag TMR ligand for cell cycle analysis and apoptosis assay, respectively. The data were analyzed using BD CellQuest Pro software version 4.0 (Becton Dickinson, CA, USA).

2.12. Statistical analysis

Analysis of variance (ANOVA) with Bonferroni's multiple comparison test was used to examine statistical significance in the ability of Vpr proteins in the induction of apoptosis and cell cycle G2 arrest. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad software Inc., LaJolla, CA).

3. Results

3.1. Phylogenetic analysis and the distribution of the mutations

The DNA sequences of the *vpr* gene of 6 HIV-1 strains were analyzed and the phylogenetic tree of the isolates along with subtype B *vpr* was constructed. As shown in Fig. 1, all the isolates cluster with subtype C reference sequences showing the similarity of these sequences with subtype C *vpr*. The consensus sequence of subtype C that was constructed in this study has also been included in the phylogenetic analysis. It should be noted that only the translated protein of this construct resembles the consensus sequence of HIV-1 subtype C Vpr protein and the DNA sequence, as indicated in the phylogenetic tree, resembles TV1453 as it was derived from this isolate.

The sequence alignment of the proteins is shown in Fig. 2. Interestingly, some of the mutations of subtype C variants are also found in subtype B Vpr. For example, S41N occurs in both TV1642 and subtype B Vpr, Y45H mutation occurs in TV143 and subtype B Vpr, T55A mutation occurs in TV1628, TV1707 and subtype B Vpr.

The protein simulation indicated that the mutations are distributed in different domains of the Vpr proteins (Fig. 3). L22I mutation is located in α-helix I of TV1570 and TV1642. α-Helix II in all the subtype C mutants contains one mutation including P37T, S41N, Y45H, E48A, and E48D. Except for TV1453, all the mutants contained 1–4 mutations in α-helix III showing the diversity of mutations in this domain. TV1466 and TV1628 contained R85P mutation in the flexible C-terminus.

3.2. Karyophilic property of Vpr proteins

The α-helical domains of Vpr are essential for its interaction with the nuclear pore complex. This interaction results in Vpr accumulation in the nuclear envelope, which is believed to be involved in active nuclear import of the PIC in nondividing cells, such as macrophages (Depienne et al., 2000; Romani and Engelbrecht,

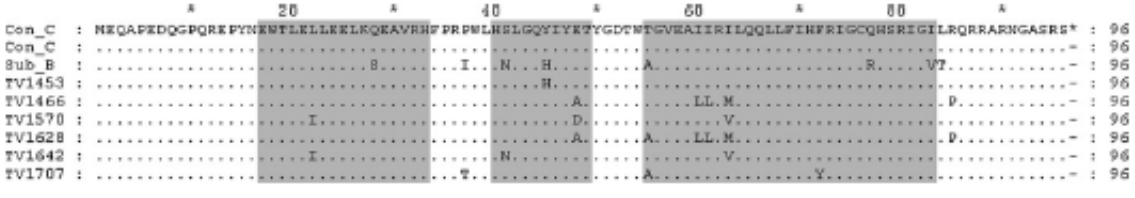

Fig. 2. Sequence alignment of the Vpr proteins. Dots indicate amino acids similar to the consensus sequence of subtype C Vpr, which is shown on top of the picture. The position of three α-helical domains is shaded in gray.

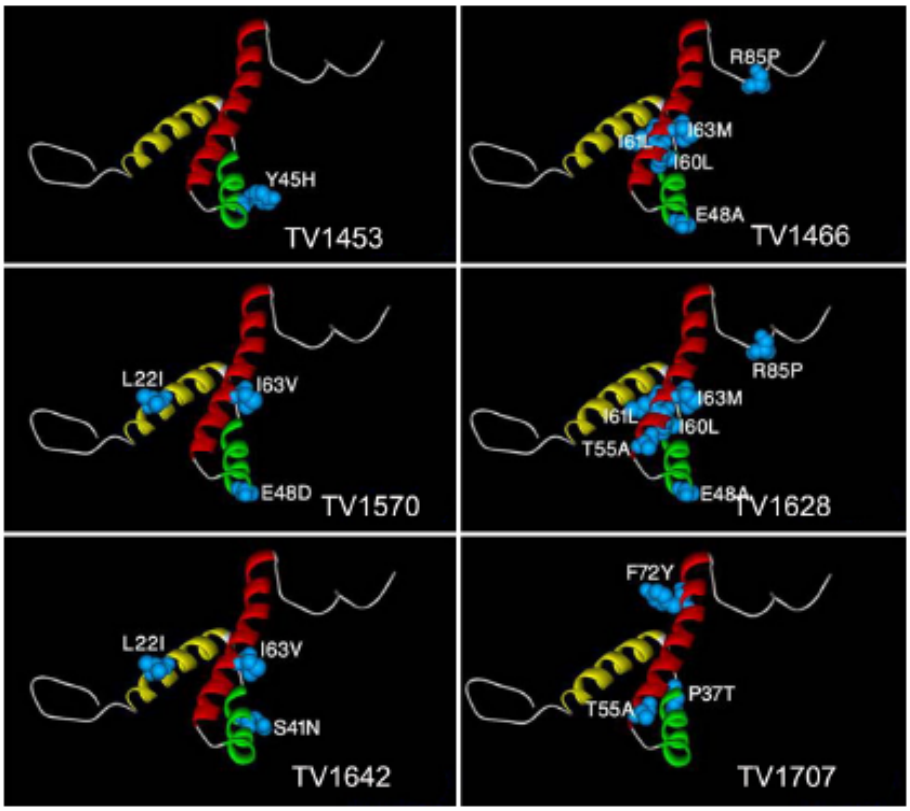

Fig. 3. Ribbon representation of the subtype C isolates containing natural mutations. Mutations are indicated in blue. α-helix I is shown in yellow, α-helix II is shown in green and helix III is shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

2009). To investigate the nuclear localization of the Vpr variants, Vpr proteins were tagged at their N-terminus to a reporter protein called HaloTag protein. This is a hydrolase that forms a covalent bond with HaloTag® ligands (So et al., 2008; Svendsen et al., 2008; Zhang et al., 2006). The ligand used for this part of the study was HaloTag TMR ligand® that has emission wavelength of 585 nm (red) in the fluorescent light. The study was designed to follow the red emission wavelength of the ligand that binds to HaloTag-Vpr. The nuclei were labeled with DAPI that binds to DNA and emits a blue light. The overlap of blue and red was interpreted as the nuclear localization of Vpr variants. The results showed that all the Vpr variants similarly localize to the nucleus (Fig. 4). No mislocalization of Vpr variants was observed when the transfected cells were examined using a fluorescent microscope. Although this cannot be interpreted as ability of Vpr variants to transfer PIC to the nucleus, the karyophilic property of Vpr is necessary for the nuclear import of PIC. These results show that the natural mutations frequently found in Vpr do not disturb the karyophilic property of Vpr.

3.3. Modulation of gene expression by Vpr

The real-time PCR assay of several genes, with a focus on the genes involved in apoptosis, was performed for 293T cells transfected with Vpr-expression vectors (Table 1). Since the values are highly dependent on the efficiency of transfection, they cannot be compared. However, it is useful to indicate the overexpression of several genes triggered by Vpr proteins. The results presented in Table 1 show that the consensus sequence of subtype C and all the subtype C Vpr variants, similarly to subtype B, are able to modulate the examined genes.

In this study, the modulation of apoptosis-regulating genes was investigated. Apoptosis, in general, is triggered through one of two known cell death-signaling pathways: the extrinsic (or death receptor) pathway and the intrinsic (or mitochondrial) pathway (Fulda and Debatin, 2006; Ghobrial et al., 2005). Vpr has been shown to induce apoptosis through the intrinsic pathway (Romani and Engelbrecht, 2009). In this study, the mRNA profile of 293T cells for intrinsic and extrinsic pathway as well as anti-apoptotic genes was examined. The overexpression of caspase-3 and -6 was observed in the transfected cells. Both caspases are involved in both extrinsic and intrinsic pathways (Klaiman et al., 2009; Legewie et al., 2006; Zimmermann et al., 2001). No significant overexpression of caspase-7, -8, and -10 by any of Vpr variants was observed. However, the significant overexpression of caspase 8 associated protein 2 (CASP8AP2), a positive regulator of apoptosis (Flotho et al., 2007), was observed in all the transfected cells. Caspase-7 has been shown in both apoptosis pathways (Bhat et al., 2006; Du et al., 2006) and Caspase-8 and -10 have been shown in the extrinsic pathway (Bhat et al., 2006; Kischkel et al., 2001). The expression level of caspase-9, which is involved in the intrinsic pathway (Du et al., 2006), remained unchanged in all the transfected cells. In summary, the expression pattern of caspases in all the transfected cells was similar, indicating that all the Vpr proteins similarly impacted the expression of caspase genes.

The overexpression of NFKBIB, REL and RIPK1 was observed in the transfected cells. Previous studies have shown the overexpression of NFKBIB, REL in apoptotic cells (Abbadie et al., 1993; Zhuang

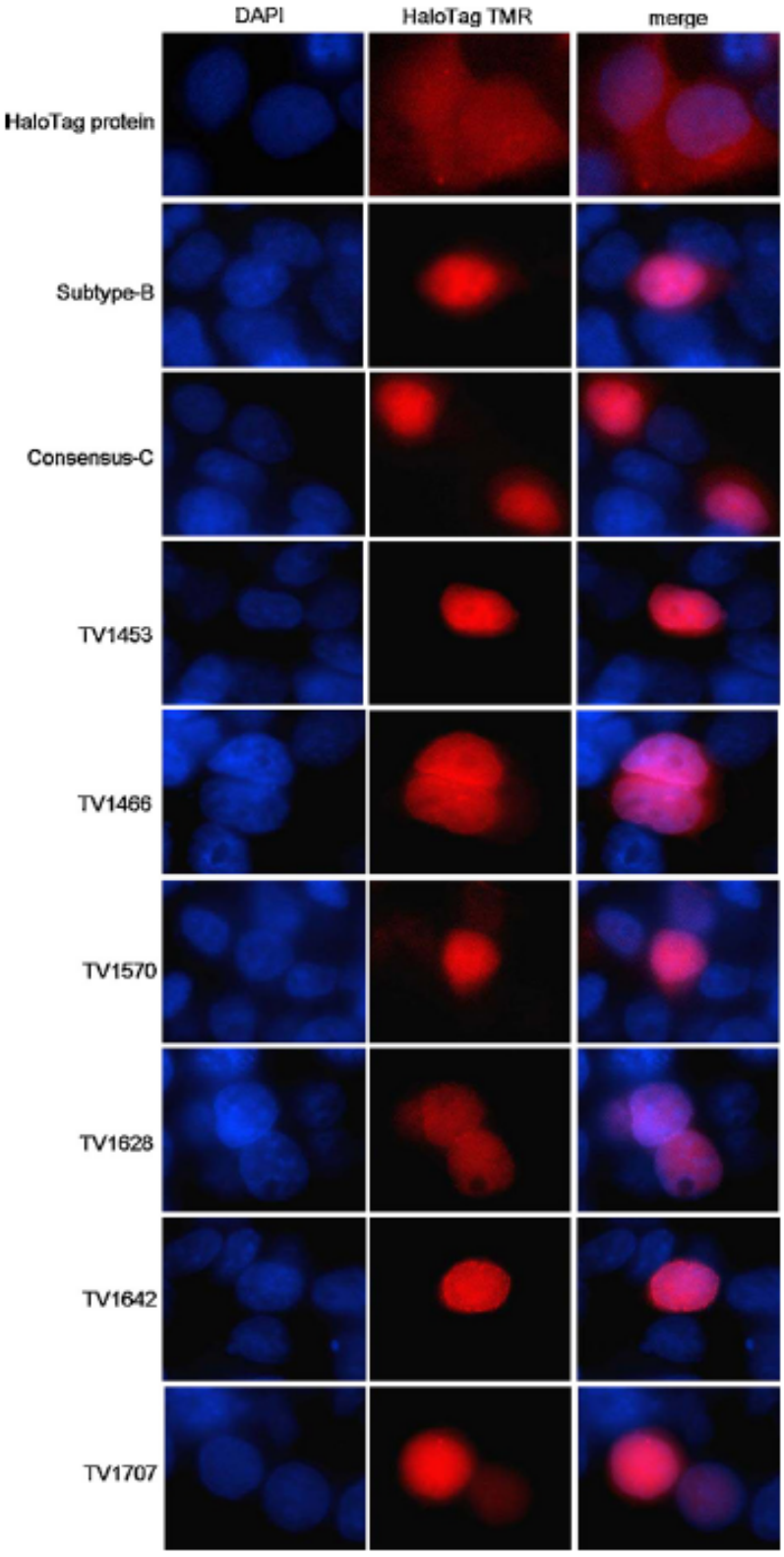

Fig. 4. Nuclear localization of Vpr proteins. The DNA of each sample is labeled with DAPI (blue) and the HaloTag-Vpr proteins are labeled with HaloTag TMR ligand® (red). The merged images of DAPI and HaloTag TMR ligand® are shown to indicate the overlap of two fluorescent dyes. Where the blue and Red dyes overlap, it means Vpr protein is localized to the nucleus which is the case with all the Vpr variants. The nonspecific localization of HaloTag protein is shown on top of the picture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 1
mRNA profile of 293T cells 24 h after transfection with Vpr-expression vectors. The average fold change is presented for each gene and each sample.

Accession numbers	Gene name	Fold change							
		Sub-B[a]	Con-C[b]	TV1453	TV1466	TV1570	TV1628	TV1642	TV1707
NM004322.3	BAD	1.60	2.54	2.02	2.91	2.38	2.21	2.84	1.60
NM001139441	BCAP31	1.95	3.52	1.40	1.07	2.09	2.1	3.64	2.0
NM207002.2	BCL2L11	1.92	2.62	1.92	3.91	1.61	2.21	2.68	2.35
NM197967.1	BID	17.22	25.12	22.14	19.69	12.36	21.56	13.83	19.82
NM001197.3	BIK	1.12	1.52	0.81	0.69	2.79	0.98	2.81	0.96
NM182962.1	BIRC3	1.52	2.32	1.14	1.52	1.61	1.49	2.52	1.26
NM004052.2	BNIP3	6.25	8.52	7.51	9.32	1.48	6.66	2.73	7.58
NM004331.2	BNIP3L	1.23	1.45	0.89	1.12	1.33	1.25	2.91	1.43
NM022162.1	CARD15	2.12	2.66	2.72	1.538	0.95	2.16	2.71	2.51
NG007497.1	CASP10	1.18	1.54	1.714	1.06	0.87	1.86	1.38	1.54
NM032991.2	CASP3	2.85	4.59	1.93	1.41	1.71	2.54	3.54	2.47
NM032992.2	CASP6	3.19	4.15	2.95	1.57	1.81	3.42	5.32	2.56
NM033338.4	CASP7	1.03	1.69	0.92	0.85	0.91	1.14	1.56	1.14
NM001228.4	CASP8	1.12	1.82	1.77	1.11	0.84	0.84	1.14	1.43
NM001137668.1	CASP8AP2	12.25	15.32	14.99	13.63	8.00	14.32	7.60	12.97
NM001229	CASP9	0.96	1.25	1.10	1.17	1.48	0.94	1.72	1.52
NM004938.2	DAPK1	1.65	2.13	1.85	1.84	1.79	1.97	2.24	2.03
NM145074.2	HTRA2	1.96	4.53	2.00	1.69	1.33	2.01	2.73	2.13
NM001145255.1	IKBKG	1.42	2.35	2.86	2.82	2.89	3.94	1.53	2.04
NM003998.2	NFKB1	3.71	3.65	5.75	1.56	1.75	4.25	5.74	4.29
NM002529.2	NFKBIA	1.52	2.12	0.97	1.71	1.34	0.85	2.74	1.41
NM001001716.1	NFKBIB	8.49	11.31	18.11	11.48	3.01	5.64	5.53	5.39
NM002908.2	REL	5.65	6.32	3.36	5.42	2.23	4.22	3.06	3.51
NM003804.3	RIPK1	3.62	4.25	4.76	3.57	2.35	3.94	2.69	4.22

[a] Subtype B.
[b] Consensus C

et al., 2004). Downregulation of RIPK1 has been shown to rescue cells from apoptosis (Petersen et al., 2007).

Although the induction of apoptosis by Vpr through the intrinsic pathway was not strongly supported by the expression level of caspases, the significant overexpression of BID (Bcl-2 interacting domain) was shown in all variants. BID is a pro-apoptotic Bcl-2 family member that triggers the intrinsic pathway of apoptosis by promoting cytochrome *c* release from mitochondria (Slee et al., 2000; Yi et al., 2003).

3.4. Cell cycle G2 arrest

Different values of induction of cell cycle G2 arrest were observed in the 293T cells transfected with different Vpr-expression vectors. The results of cell cycle analysis by flow cytometry and the corresponding bar graph representation are presented in Fig. 5. Only transfected cells were gated for analysis based on the signal of HaloTag® Oregon Green® ligand. The cell cycle phase was inferred based on the DNA content labeled with 7-AAD. All the results were compared with the mock-transfected sample which was 293T cells transfected with barnase-removed pFN22K vector. The DNA content of the mock sample indicated that the majority of the cell population was at G1/S phase. The highest ratio of G2/M:G1 (2.52) was observed in the cells transfected with subtype B Vpr. This result shows the majority of subtype B Vpr-transfected cells shifted to G2 phase. The G2/M:G1 ratio for the consensus sequence of subtype C Vpr was 1.61. The lowest ratio, 1.21, was observed in the cells transfected with TV1466. This Vpr protein had mutations in α-helix I and II and the flexible C-terminus. TV1570 and TV1642 that each had one mutation in each α-helix, induced G2 phase in the same range as the consensus C Vpr ($p > 0.05$). The G2/M:G1 ratio for TV1453, TV1628 and TV1707 was 1.89, 1.61 and 1.61, respectively. Except for TV1453 that had Y45H mutation in α-helix I, the two latter proteins had several mutations distributed in different domains. All variants of Vpr proteins were thus able to induce G2 arrest but at different values. The induction of G2 arrest by subtype B Vpr was higher than that of the consensus sequence of subtype C and all the subtype C variants ($p < 0.05$). The induction of G2 arrest by consensus C was found in the same range of Vpr mutants ($p > 0.05$).

3.5. Apoptosis assay

Apoptosis was assayed in all the transfected cells and compared with the mock-transfected sample, 293T cells transfected with barnase-removed pFN22K vector. The transfected 293T cells were distinguishable from untransfected cells based on the signal of HaloTag TMR ligand®. The transfected cells were gated and the results of apoptosis assay are presented as bar graph in Fig. 6. Based on the mean values of 3 independent experiments, the lowest percentage of apoptotic cells was found in the mock sample with 0.08% of cells being apoptotic. This result indicated that the EDTA treatment to harvest the adherent cells did not result in damage to cell membranes and the consequent generation of spurious results. Interestingly, the lowest percentage of apoptotic cells among all the Vpr-transfected cells was found in the cells transfected with the consensus sequence of subtype C with 82.08% apoptotic cells ($p < 0.05$). Subtype B Vpr-induced apoptosis in 98.20% of the transfected cells. TV1453 that had only one mutation, Y45H, induced the second lowest level of apoptosis with 95.33% apoptotic cells. The percentage of apoptotic cells detected for the other Vpr variants was similar to one another and subtype B Vpr ($p > 0.05$) ranging from 96.54% for TV1466 to 98.64% for TV1628.

4. Discussion

Vpr is a dynamic mobile protein that shuttles between the nucleus and cytoplasmic compartments (Jenkins et al., 2001; Sherman et al., 2003, 2001). The karyophilic property of Vpr and its high affinity for the nuclear pore complex (NPC), has been well established and it is believed to be a prerequisite for the nuclear import of PIC (Fassati et al., 2003). However, the karyophilic property of Vpr needs to be addressed in other subtypes and mutants as well. Up to now, this property of Vpr has not been shown for other HIV-1 subtypes or mutants. In this study, it was shown that subtype C variants or mutants show similar affinity for the nucleus,

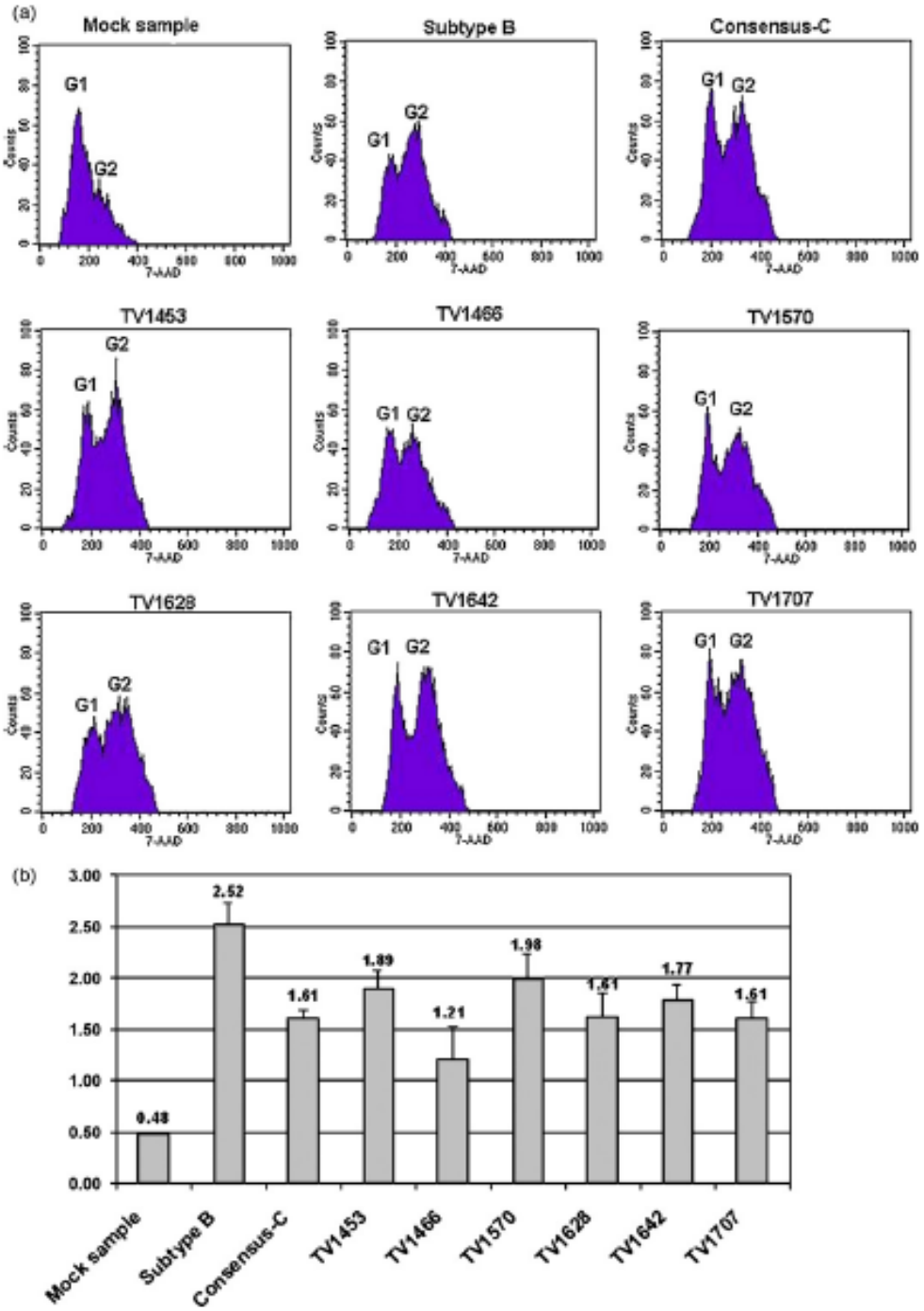

Fig. 5. Induction of cell cycle G2 arrest by Vpr. (A) Flow cytometric plots show the DNA content of the 293T cells, 48 h after transfection. The first plot on the top left shows the cell cycle in mock sample which was prepared by transfection of 293T cells with barnase-removed pF4A vector. The first peak in each plot shows the cells occurring at G1/S phase. The second peak shows the cells occurring at G2/M phase. (B) The bar graph showing the G2/M: G1 ratio for 293T cells transfected with the control or Vpr-expression vectors. Data shown represent average ratios of three repeats for each variant. The error bars indicate the standard deviations in the reported measurements.

Induction of G2 arrest by Vpr has been documented in many studies. The detailed mechanism of this phenomenon has also been studied and although not fully characterized, novel details are still being revealed (Belzile et al., 2007; DeHart et al., 2007; Kino et al., 2005a; Zhou and Ratner, 2000). However, the previous studies have investigated the cell cycle G2 arrest in subtype B Vpr and there is a lack of data regarding the induction of cell cycle G2 arrest by other HIV-1 subtypes. The ability of Vpr variants to induce G2 arrest is another question that has not been addressed in previous studies. In this study, it was shown that subtype B Vpr induces the cell cycle G2 arrest more efficiently than subtype C Vpr variants. The consensus sequence of subtype C Vpr indicated no significant deviation from the average G2 arrest that was observed for the other subtype C variants. In contrast to apoptosis, mutations in Vpr did not significantly increase the ability of Vpr variants to induce G2 arrest and even TV1466 indicated a reduced ability to induce the G2 arrest.

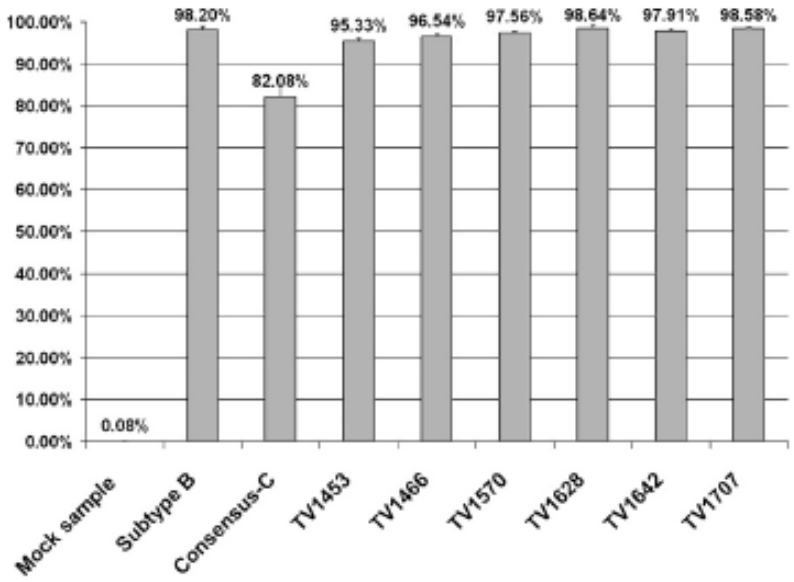

Fig. 6. The bar graph showing apoptosis induction in 293T cells by Vpr. The first sample on the left shows the percentage of apoptotic cells in mock sample which was prepared by transfection of 293T cells with barnase-removed pFN22K vector. The error bars indicate the standard deviations in the reported measurements. Data shown represent average values of three repeats for each variant. Among the vpr variants, the consensus sequence of subtype C induced apoptosis in 82.08% of the transfected cells which is lower than the other variants.

Mutational analysis has shown that the C-terminal portion of Vpr, known to harbor its cell cycle-arresting activity, binds directly to 14-3-3 protein. 14-3-3 proteins regulate the G2/M transition by activating Cdc25C. The interaction of the C-terminus of Vpr promotes the association of 14-3-3 and Cdc25C, leading to the triple complex of Vpr/14-3-3/Cdc25C that is not able to propel cell cycle (Bolton et al., 2008; Kino et al., 2005b). In our study, TV1466 and TV1628 had R85P mutation in their C-terminus. Both proteins possessed lower potential to induce G2 arrest. Our results also confirm the importance of C-terminal portion of Vpr in the induction of G2 arrest. However, the C-terminal region of Vpr is probably not the only region of this protein that affects G2 arrest. Several studies have documented that the association of HIV-1 Vpr with DCAF-1 (DDB1- and CUL4-associated factor 1), also known as VprBP (Vpr-binding protein), leads to G2 arrest (Gieffers et al., 2000; Hrecka et al., 2007; Wen et al., 2007). It has been shown that Vpr(Q65R) mutant, which is defective in DCAF1 binding, undergoes proteasome-mediated degradation at a higher rate than wild-type Vpr. Therefore mutations outside of the C-terminal region, such as Q65R could affect the G2 arrest activity of Vpr (Le-Rouzic et al., 2008). In this study, mutations outside of C-terminus region occurred in the variants but their impact cannot be individually investigated since multiple mutations occurred in our Vpr proteins.

The modulation of gene expression by Vpr has been well established (Romani and Engelbrecht, 2009; Zhu et al., 2003). In this study it was shown that subtype C Vpr variants modulate the apoptotic genes in a similar manner to the subtype B Vpr. Although the overexpression of caspases specifically involved in the intrinsic pathway of apoptosis, such as caspase-9, was not detected, the induction of apoptosis might not be always dependent on overexpression of certain gene products, but available gene products. This study indicates that certain apoptotic genes are similarly upregulated or remain unchanged by the expression of subtype B and subtype C Vpr variants. Although our results indicate that Vpr modulates the apoptotic genes triggering the intrinsic pathway of apoptosis, these results do not provide sufficient evidence for the pathway of Vpr-induced apoptosis. Apoptosis pathways are usually studied at biochemical level.

The pro-apoptotic activity of subtype C Vpr variants from India has been shown to be similar to that of subtype B Vpr (Bano et al., 2009). Jian and Zhao by comparing three HIV-1 subtypes demonstrated a moderate level of pro-apoptotic activity for a subtype B Vpr and an A/G subtype recombinant Vpr, whereas a subtype D Vpr had no detectable activity. It has been suggested that Vpr proteins from different HIV-1 subtypes as well as Vpr variants that emerge during HIV-1 infection may have different pro-apoptotic potentials and contribute to the diversity of AIDS pathogenesis (Jian and Zhao, 2003). Bano et al. have shown that a Vpr protein derived from an Indian C isolate exhibited a higher level of apoptosis than subtype B Vpr (Bano et al., 2007). They also showed that several B, C and D recombinant Vpr proteins were capable of inducing apoptosis to varying extents (Bano et al., 2009).

In this study, it was shown that South African subtype C Vpr variants are able to induce apoptosis at a similar rate to subtype B Vpr. Interestingly; it was found that the consensus sequence of subtype C Vpr induces apoptosis at a lower rate. TV1453 that differs from the consensus sequence in one amino acid residue, Y45H mutation in α-helix II, indicated a higher pro-apoptotic activity. The other natural mutations at other positions also were shown in this study to potentiate the pro-apoptotic activity of Vpr. It has been documented that the C-terminal peptides containing the conserved sequence at amino acid 71–82 can even induce apoptosis (Arunagiri et al., 1997). Many of the mutations that were shown in this study to enhance to pro-apoptotic activity of Vpr are not located at position 71–82. Other studies have also shown that mutations outside of this motif could affect apoptosis and some studies have even linked the pro-apoptotic activity of Vpr with its ability to induce the cell cycle arrest (Bolton and Lenardo, 2007; Jacquot et al., 2007; Stewart et al., 1997; Tungaturthi et al., 2004). It could be due to the different factors contributing to the cytopathicity of Vpr. Many factors including ion channel activity of Vpr, DNA fragmentation factor activation, interaction of Vpr with other proteins, and localization of Vpr could affect the pro-apoptotic activity of Vpr (Lum et al., 2003; Piller et al., 1999; Shostak et al., 1999). Each of these functions is conducted by individual regions that could be outside of the motif 71–82.

It has been shown that substitutions of a single amino acid residue, Leu64 with Pro, Ala, or Arg, dramatically enhance the pro-apoptotic activity of Vpr. The possible consequences of mutations should also be examined separately for different subtypes since the same mutation has been shown to impact variably in different subtypes (Jian and Zhao, 2003). The results of our study also confirm the previous studies and suggest that the variations in subtype C Vpr cannot be regarded as defective mutations disturbing the functionality of the protein.

The mutations occurring in Vpr might have been selected under biological pressures and the evolutionary importance of these mutations cannot be ruled out. The study raises the concern as to what exactly is a wild-type HIV-1 protein? If the consensus sequence of subtype C Vpr does not function, at least for apoptosis induction, as effectively as some mutated Vpr proteins, could it be the case with other HIV-1 proteins as well? In addition, are the common reference sequences of HIV-1, such as the NL4-3 clone that was used in this study, the best representatives of the wild-type HIV-1? If we select the non-mutated or less mutated sequences as the wild-type sequence, are they the most functional sequences? How many mutations, what mutations, and in what conditions potentiate the protein?

5. Conclusion

In summary, our results indicate similar ability of HIV-1 subtype B and C Vpr for nuclear localization and apoptosis induction. But the induction of Cell cycle G2 arrest by HIV-1 subtype B Vpr is more robust than many subtype C Vpr proteins. The natural mutations studied in our isolates did not disturb the functions of subtype C Vpr and in some cases even potentiate the protein to induce apoptosis. This study demonstrates the functionality and the adaptive role of naturally occurring mutations in HIV-1 Vpr that cannot be regarded as defective mutations. In addition, the functionality of HIV-1 proteins from different subtypes should be thoroughly studied to exclude or confirm the functionality of those proteins in different subtypes. The distribution of particular subtypes in some areas might be related to the adaptive mutations occurring in HIV-1 to adapt the virus to the genetic diversity of the population.

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