HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 DISTRIBUTION IN SOUTH AFRICA AND THE RELEVANCE OF GENETIC DIVERSITY ON VACCINE DESIGN

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CONTENTS

ABSTRACT

The overall aim of this project was to investigate HIV-1 genetic diversity in South Africa and to characterise the immune response in mice to a South African subtype C gp120.

To investigate the relationship between subtype and mode of transmission, samples were collected from individuals infected by heterosexual and male homosexual transmission from patients attending local HIV/AIDS clinics in Cape Town (n=49) and Bloemfontein (n=4). Isolates were subtyped using heteroduplex mobility assay (HMA) based on the V3-V5 region of the *env* geneusing reference plasmids (2 B, 2 C and 1 D) representative of local subtypes. HMA identified four *env* subtypes: A, B, C and D. Subtype B viruses were found in 92.9% (26/28) of the male homosexual/bisexual group and subtype C viruses in 77.2% (17/22) of the heterosexual group. Subtype B viruses were also identified in two heterosexual patients, one patient infected by blood transfusion and in two patients with unknown mode of transmission. Subtype D viruses were found in one male homosexual patient and one heterosexual patient and a husband and wife couple were infected with subtype A viruses. A significant association between subtype and mode of transmission (p=<0.0001) was identified, confirming two independent epidemics.

To determine the subtype distribution of HIV within urban heterosexual populations throughout South Africa, samples were collected from women attending antenatal clinics in Johannesburg (n=34), Pretoria (n=5) and Durban (n=20). Samples from Bloemfontein (n=24) were taken from individuals attending an HIV/AIDS clinic. All eighty-three samples were subtyped by HMA in the *env* region as before. The predominant subtype circulating within the urban heterosexual population throughout South Africa was identified as subtype C (92.8%) although subtype B was also detected (7.2%). It may thus be beneficial if a HIV vaccine for South Africa is based on a subtype C model.

In addition, a rapid method for identification of HIV-1 *gag* subtypes was developed based on restriction fragment length polymorphism (RFLP) analysis of 400bp (p17) or 650bp (p17 and 5' p24) long PCR fragments. This strategy was applied to eighty-six samples (Cape Town n=47, Johannesburg n=20, Bloemfontein n=17 and Durban n=2) previously subtyped by either sequence analysis of the *gag* p17 region (n=31), heteroduplex mobility assay (HMA) based on the *env* gene (n=76), or both (n=21). RFLP analysis identified two subtype A, twenty-five subtype B, fifty-eight subtype C and one subtype D isolates. There were no discrepancies between RFLP and sequence *gag* subtypes, demonstrating the reliability of this method and no discordance between *gag* RFLP subtypes and *env* HMA subtypes, indicating no recombinant viruses in the genomic regions analysed.

The first phylogenetic analysis of a gp120 from a macrophage tropic, non-syncitium inducing (NSI) subtype C isolate from South Africa (97ZA347TS) was performed in this study and the isolate was found to cluster within the subtype C lineage with a bootstrap value of 100%. The gp120 protein of the 97ZA347TS contained twenty-two *N*-linked glycosylation sites, including an additional site within the CD4 binding site, characteristic of subtype C isolates. The phylogenetic relationship between 117 published subtype C V3 loop sequences, including three isolates sequenced in this study, was also investigated. No sub-clustering was identified within South African isolates, or with any country of origin. The isolates were found to be 14% divergent from each other. The majority of isolates retained the characteristic GPGQ tetrapeptide crown and the loss of a *N*-linked glycosylation site 5' to the V3 loop at position 265-267. Argenine substitutions indicative of a syncitia inducing phenotype, were present in only two of the southern African subtype C isolates, NOF and 89ZA067, at position, 276.

The gp120 from 97ZA347TS was cloned into the pcDNA3.1/Zeo DNA to determine the immunogenicity of the gp120 in comparison with the gp120 from a NSI subtype B isolate (SF162). The DNA vaccines were immunised intramuscularly into female BALB/c mice and boosted at weeks 2, 4 and 6. The antibody response to both DNA vaccines peaked at five to seven-fold higher levels than the negative control mice, immunised with the DNA vaccine vector alone.

To determine the potential cross-reactive response between subtype B and C isolates, DNA vaccines expressing 97ZA347TS and SF162 were used to immunise female BALB /c mice once intramuscularly, followed by an intraperitoneal challenge with recombinant vaccinia virus (VV) expressing the gp120 from subtype C, 97ZA347TS, after nine days. The VV titres in the mouse ovaries is an indirect measurement of the CD8+ CTL (cytotoxic T lymphocyte) response in the mouse. The CD8+ CTL response was determined, by clearance of the recombinant VV from the mouse ovaries in comparison to unimmunised mice. The subtype C immunised mice had a recombinant VV ovarian titre over 500-fold lower than the negative control mice and the subtype B immunised mice had an ovarian titre of recombinant VV 100 fold lower than the negative control mice. A greater control of replication by homologous subtype C recombinant VV was obtained and a cross-reactive CTL response was demonstrated. Comparison of the amino acids in the two isolates indicated 24% difference, which would have an effect on the CTL epitopes in common between the isolates. A region with less variability than the gp120 would possibly be better in eliciting a cross-reactive CTL response. It is thus possible that with an optimised South African macrophage tropic subtype C vaccine, CTL cross-reactivity between subtypes may be achieved. This was the first study utilising a murine *in vivo* challenge method of determining the cross-reactive CTL response between different HIV-1 subtypes.

This study has resulted in the following publications:

i) **J. van Harmelen**, E. van der Ryst, R. Wood S.F. Lyons and C. Williamson. Restriction fragment length polymorphism analysis for rapid *gag* subtype determination of Human Immunodeficiency Virus Type 1 (HIV-1) in South Africa. *J Virol Methods* 1999, 78:1-2, 51-59.

ii) **J.H. van Harmelen**, E. van der Ryst, A.S. Loubser, D. York, S. Madurai, S. Lyons, R. Wood and C. Williamson. A predominately subtype C restricted epidemic in South African urban populations. *AIDS Res Hum Retroviruses* 1999, 15:4, 395-398.

iii) R. Cheingsong-Popov, C. Williamson, S. Lister, L. Morris, **J. van Harmelen**, H. Bredell, R. Wood, P. Sonnenberg, E. Van der Ryst, D. Martin and J. Weber. Usefulness of HIV-1 V3 serotyping in studying the HIV-1 epidimic in South Africa. *AIDS* 1998, 12:8, 949-966.

iv)**J. van Harmelen**, R. Wood, M. Lambrick, E. Rybicki, A-L. Williamson, C. Williamson. An Association Between HIV-1 Subtype and Mode of Transmission in Cape Town, South Africa. AIDS 1997, 11:1, 81-87.

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ABBREVIATIONS

A	adenine
aa	amino acid(s)
Abs_{260}	absorbance reading at wavelength 260nm
AIDS	acquired immune deficiency syndrome
AP	alkaline phosphatase
APS	ammonium persulphate
ATP	adenosine triphosphate
b	bases
BCG	*M. bovis* bacille Calmette-Guerin
β-gal	beta-D-galactosidase
bp	base pair(s)
BUdR	5-bromodeoxyuridine
°C	degrees celsius
C	cytosine
$CaCl_2$	calcium chloride
cpe	cytopathic effect
CSP	circumsporozoite protein
CTL	cytotoxic T lymphocytes
Da	Daltons
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanidine triphosphate
dTTP	deoxythymidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytosine triphosphate
ddGTP	dideoxyguanisine triphosphate
ddTTP	dideoxythymidine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
(d)dNTP	(di)deoxynucleoside triphosphate
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl ammoniummethylsulphate
ds	double stranded
dUTP	deoxyuracil triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein isothyocyanate
G	grams
g	gravitational acceleration
G	guanine
h	hours
HBS	Hepes-buffered saline
HCl	Hydrochloric acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
H_3PO_4	hydrogen pyrophosphate
HRP	horseradish peroxidase
HSV	herpes simplex virus
IFN	interferon
IL	interleukin
IPTG	isopropyl-β-D-thio galactosidase
kB	kilobases
KCl	pottasium chloride
kDa	kiloDaltons
KOAc	pottasium acetate
LA	Luria-Bertani agar
LB	Luria-Bertani broth
LTR	long terminal repeat
M	Molar
µg	micrograms
µl	microlitres
Mab	monoclonal antibody
MCS	multiple cloning site
mg	milligrams

$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
min	minutes
ml	millilitres
mM	millimolar
mm	millimetres
(m)RNA	messenger ribonucleic acid
N	normal
NA	not applicable
NaOAc	sodium acetate
NaOH	sodium hydroxide
NaCl	sodium chloride
ng	nanograms
NIH	National Institutes of Health
nm	nanometres
nt	nucleotide
OD	optical density
OPD	1,2 phenylenediamine dihydrochloride
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pH	hydrogen potential
rBCG	recombinant BCG
RNase	ribonuclease
rpm	revolutions per minute
RSA	Republic of South Africa
^{35}S-dATP	deoxyadenosine triphosphate labelled with radioactive ^{35}S isotope
SDS	sodium dodecyl sulfate
(s)IgA,G,M	(secretory) immunoglobulin A, G, M
ss	single stranded
STD	sexually-transmitted disease
T	thymidine
TAE	Tris-acetate
TBE	Tris-borate
TBS	tris-buffered saline
TE	Tris-EDTA
TEMED	N,N,N^{I},N^{I} tetramethyl-ethylenediamine
Tm	melting temperature
TNF	tumor necrosis factor
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
U	units
UCT	University of Cape Town
UK	United Kingdom
UNAIDS	Joint United Nations Programme on HIV/AIDS
USA	United States of America
UV	ultraviolet
V	volts
v/v	volume per volume
VLP	virus-like particles
WHO	World Health Organisation
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
YT	yeast-tryptone

CHAPTER 1 : INTRODUCTION

1.1) Historical perspective

Acquired immunodeficiency syndrome (AIDS), as a clinical entity, was first recognised in 1981. In 1983, the causative agent of AIDS was identified by a number of scientific groups: lymphadenopathy-associated virus (LAV) was first isolated in 1983 by Montagnier and Barré-Sinoussi (Barré-Sinoussi *et al.*, 1983) and a human T-cell lymphotropic retrovirus (HTLV-III) was isolated by Gallo *et al* (Popovic *et al.*, 1984) in 1984. In addition, an AIDS-associated retrovirus (ARV) was isolated in the same year by Levy from an AIDS patient (Levy *et al.*, 1984). LAV and HTLV-III were subsequently sequenced and found to be only 1-2% divergent (Wong-Staal *et al.*, 1985) leading to the renaming of the virus as human immunodeficiency virus (HIV) in 1986 (Coffin *et al.*, 1986).

1.2) Origin of HIV

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are retroviruses belonging to the lentivirus family. There are five recognised or known groups of primate lentiviruses (Barré-Sinoussi, 1996):

I) HIV-1 and simian immunodeficiency virus, or SIVcpz, from a wild-caught chimpanzee

II) HIV-2 (from humans), SIVsm and SIVmac (from captive macaques and feral sooty mangabeys)

III) SIVagm (from african green monkeys)

IV) SIVmnd (from wild-caught mandrills)

V) SIVsyk (from sykes monkeys)

HIV-1 and HIV-2 are presumed to have entered the human population by cross-species (zoonotic) infections (Gao *et al.*, 1999). The sooty mangabey (*Cercocebus atys*) has been identified as the primate reservoir of HIV-2 (Hirsch *et al.*, 1989; Gao *et al.*, 1992 and Chen *et al.*, 1996). Until recently, the origin of HIV-1 was not known. However, by sequence analysis of the mitochondrial DNA of all chimpanzees known to be infected with SIVcpz, two chimpanzee subspecies were identified in Africa and the SIVcpz virus infecting them was phylogenetically characterised (Gao *et al.*, 1999). *Pan troglodytes* troglodytes (central Africa) and *P. t.* schweinfurthii (eastern Africa) were found to harbour SIVcpz viruses which form two highly divergent and subspecies-specific phylogenetic lineages.

The HIV-1 strains that infect man (including Groups M; subtypes A to J, and O) are closely related to the SIVcpz lineage found in *P. t.* troglodytes (see figure 1.1). In addition, a new lentivirus

isolate was recently identified from a Cameroonian woman and subsequently found in a further two individuals. The highly divergent isolates (as divergent from HIV-1 Group M as SIVcpz-gab) have been subsequently been named Group N (Simon *et al.*, 1998). HIV-1 Group N was found to be a mosaic of SIVcpzUS (a new SIVcpz identified) and HIV-1-related sequences, which indicates an ancestral recombination event in the chimpanzee host (Gao *et al.*, 1999). The lentivirus, SIVcpz is thought to have been introduced into the human population 40 to 60 years ago (Zhu *et al.*, 1998).

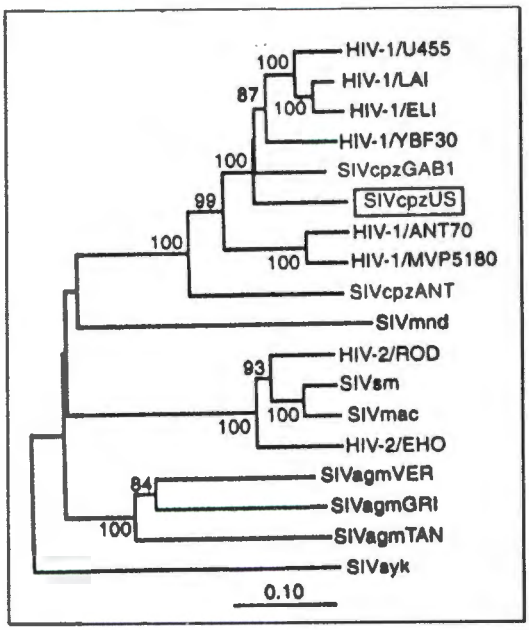


Figure 1.1: A phylogenetic tree taken from Gao *et al.* (1999) showing the relationship between the HIV-1 group M (U455, LAI, ELI), group O (ANT70, MVP5180) and group N (YBF30) isolates in comparison to HIV-2 and SIV lentiviruses, including a recently identified SIV, SIVcpzUS. Branch lengths are indicated by the bar (0.1 amino acid replacements per site).

The origin of HIV-1 subtypes has been analysed by sequencing strains of HIV-1 from the start of the epidemic. Phylogenetic analysis from an isolate obtained from Gabon in 1959 showed that the origin of the isolate was near the ancestral node of subtypes B, D and F. These subtypes are thus thought to have diverged from a common ancestor shortly before 1959 (Leitner *et al.*, 1997 and Zhu *et al.*, 1998).

1.3) Virus structure and organisation

Lentiviruses are enveloped viruses, 80-100nm in diameter with conical cores and Mg^{2+}-dependent reverse transcriptase enzymes. All *Retroviridae* have two copies of single-stranded (ss) RNA, approximately 9.2Kb in length, encoding the *gag*, *pol* and *env* genes, which are converted into double-stranded (ds) DNA early in infection by the reverse transcriptase enzyme. In addition, the complex lentivirus genome incorporates accessory and regulatory genes (see figure 1.2) (Fields, 1996). The DNA provirus is integrated into the host chromosome and is then transcribed into new viral RNA genomes and shorter sub-genomic RNAs by the cellular DNA-dependent RNA

polymerases. Viral structural proteins are translated from unspliced or single spliced viral RNAs, whereas regulatory and accessory proteins are translated from multiply spliced viral RNAs (Barré-Sinoussi, 1996).

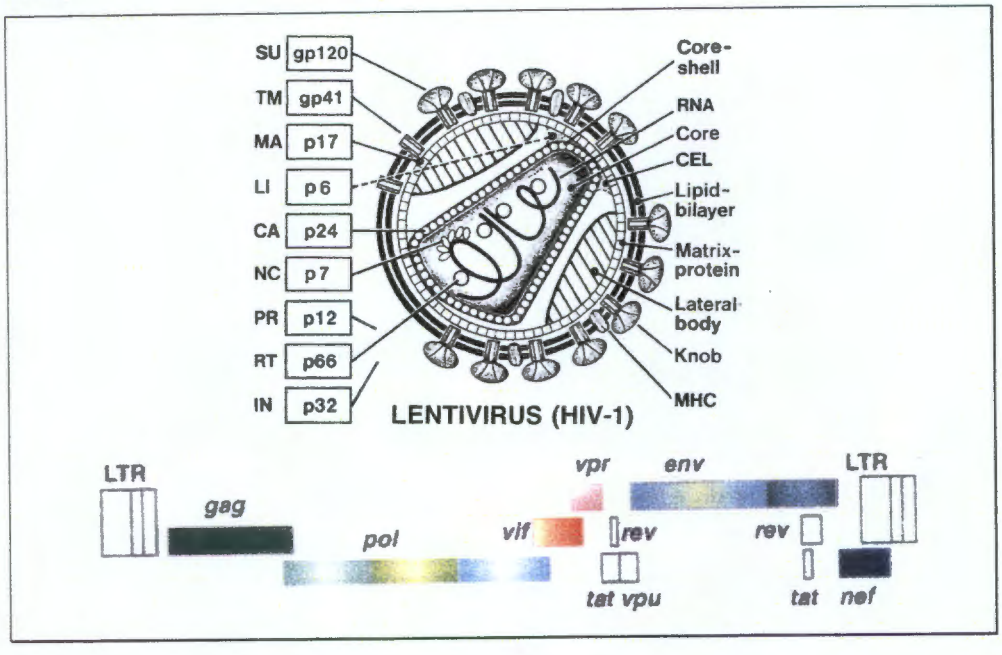


Figure 1.2: Figure illustrating the structure of the HIV virion, including the structural proteins making up the outer coat (gp120 and gp41) and inner capsid (p17 and p24) as well as the two ssRNA's and the reverse transcriptase enzymes. The genome of the virus is also shown, with the different open reading frames of structural, regulatory and accessory genes indicated in different colours (Gelderblom, 1997 and UNAIDS, 1998).

1.3.1) Gag proteins

The Gag proteins form the major structural proteins of the virus. They are initially produced as $Pr55^{gag}$ which is targeted to the cell membrane for budding, where a viral protease cleaves it into smaller Gag proteins. The matrix protein (MA), p17 is situated at the N-terminal of $Pr55^{gag}$ and undergoes co-translational myristoylation, allowing membrane targeting of the $Pr55^{gag}$ protein (Mervis *et al.*, 1988 and Fields, 1996).

The MA protein is a trimer, with each monomer consisting of five α-helices and a β-sheet (Cannon *et al.*, 1997). Lysine residues along the hydrophobic face of the protein stabilise the MA trimer during viral assembly at the plasma membrane (Spearman *et al.*, 1997). The MA protein is involved in the early replication cycle and, independently to Vpr, facilitates transport of the pre-integration complex (PIC) into the host cell nucleus. In this way, HIV, and other lentiviruses, are able to infect both dividing and non-dividing cells (Cannon *et al.*, 1997). The MA protein surrounds the viral capsid, CA (p24), and is internal to the envelope. It is also involved in binding to gp41, incorporating the envelope into the virus (Zhou and Resh, 1996). In addition, it was recently suggested that the MA protein binds to human cellular factors found on CD4+ and CD8+ cells, resulting in increased proliferation of peripheral blood mononuclear cells (PBMC's), thereby enhancing HIV replication (De Francesco *et al.*, 1998).

The other $Pr55^{gag}$ cleavage products, p7/p9 (nucleocapsid proteins, NC) and the proline-rich p6 are all localised in the CA protein core of the virus. CA surrounds the viral genome and enzymes, NC binds to the RNA genome by two zinc fingers (Gelderblom, 1997) and p6 is needed for complete ribonucleoprotein formation and virus budding (Mervis *et al.*, 1988 and Fields, 1996).

1.3.2) The pol gene

The *pol* gene encodes the protease, reverse transcriptase, integrase and RNAse H enzyme precursor. The reverse transcriptase consists of p51/p66 subunits and incorporates RNAse H which is an RNA specific ribonuclease for RNA/DNA hybrids. The protease is released from the Gag-Pol polyprotein by autocatalytic cleavage, after which it targets other sites in the polyprotein. Integrase, which forms part of the pre-integration complex (PIC), mediates the covalent linkage of viral dsDNA into the host genome (Fields, 1996).

1.3.3) Regulatory and accessory genes

Primate lentiviruses also have regulatory and accessory proteins (see figure 1.2), encoded by *tat*, *rev*, *vif*, and *nef* genes. HIV-1 and SIVcpz have *vpu*, whereas HIV-2, SIVsm, SIVmac and SIVagm contain *vpx* and all primate lentiviruses but SIVagm contain *vpr*. *Vpr*, *vpx* and *vif* gene products are packaged into the virions, but *tat*, *rev*, *vpu* and *nef* are not (Fields, 1996).

The Tat protein regulates viral gene expression by binding to the *trans*-activating response (TAR) sequence in nascent RNA (Fields, 1996 and Majello *et al.*, 1998). Tat is released by infected cells in large amounts and high levels of Tat have been detected in HIV infected patients. This Tat protein is taken up by both infected and non-infected cells and is translocated to the cell nucleus where transcription of virus in infected cells is enhanced. In both infected and non-infected cells factors such as TNF (tumour necrosis factor), IL6 (interleukin 6) and IL10 (interleukin 10) are activated. Prevention of Tat uptake by antibodies to Tat, leads to an attenuation of HIV infection *in vitro* (Goldstein, 1996).

The Rev protein facilitates the export of unspliced or single spliced viral RNA from the nucleus to the cytoplasm by binding to a *rev* responsive element (RRE) in viral transcripts. In HIV-1, the RRE is a RNA sequence in the *env* gene 3' to sequences encoding the gp120/gp41 junction. These RNA's are then translated into the Gag-Pol and Env proteins. Without Rev, no structural proteins are made (Fields, 1996).

Production of the Vif protein is dependent on Rev function and although the role of the Vif (viral infectivity) protein is not known for certain, two functions have been proposed. During early infection, it may transport virions to the nucleus by interaction with the intermediate filaments in the

cell cytoskeleton. It may also stabilise the newly synthesised DNA intermediates and function in provirus formation (Miller and Sarver, 1997).

Vpr (viral protein R) is translated late in infection from a single-spliced RNA and is dependent on Rev. It is associated with the nucleocapsid in mature virions, mediated by NC p7 and p6 in a protein-protein interaction, or with the nucleus or cytoplasmic membranes in infected cells (de Rocquigny *et al.*, 1997). The 96 amino acid protein regulates cellular processors in the HIV life cycle by interruption of the host cell division in the G2 phase. It is also involved in the targeting of the PIC to the nucleus of non-dividing cells (the first cells to be infected) (de Rocquigny *et al.*, 1997).

Vpu (viral protein U) is a single-spliced Rev dependent viral mRNA, which is translated into an amphipathic, 9.2 kDa membrane protein associated with the endoplasmic reticulum or Golgi system. It enhances virus release by formation of ion-conductive channels in its oligomerised form, degrades the CD4 receptor and down-regulates MHC class I molecules on the infected cell surface (de Rocquigny *et al.*, 1997 and Kerkau *et al.*, 1997).

Nef (negative factor) is translated from two multiply-spliced mRNA's, independant of Rev and is a 27kDa protein, produced in high amounts during early replication. It is important for replication and pathogenicity of the virus and may enhance viral infectivity, activate T-cells (Miller and Sarver, 1997), down-regulate CD4 and MHC class I molecules on the cell surface (Xu *et al.*, 1997) and be associated with cellular serine kinase (Fields, 1996). The functions of Nef are discussed in more detail in section 1.7.2.6.

1.3.4) The HIV env glycoprotein

The *env* gene encodes the gp160 precursor protein that is processed by cellular proteases into the gp41 and gp120 glycoproteins. All synthesis and processing of the gp160 occurs in the secretory pathway at the endoplasmic reticulum (ER). Glycosylation influences both immunological and functional properties of the highly variable gp160 and occurs in the Golgi body of the cell (Fields, 1996). The viral spikes, or knobs occur in low numbers on the surface of mature virions (Layne *et al.*, 1992) and are made up of three heterodimers of the gp120 (surface subunit) and gp41 (transmembrane subunit). The gp120 and gp41 are non-covalently linked, and the gp120 is weakly anchored to the surface of the virus or to the plasma membrane of infected cells by the gp41. The spikes are arranged in a T=7 levo-rotational symmetry and are shed spontaneously (half-life of about 30 hours) from the virus. Macrophages infected with HIV have been found to produce virions that are depleted of spikes, whereas T-cells make abundant amounts of gp120 (Gelderblom *et al.*, 1987; Meltzer *et al.*, 1990 and Layne *et al.*, 1992). The gp120 contains the CD4 receptor binding domain as well as the co-receptor binding domains (Fields, 1996) (see section 1.4.2 for a more detailed description).

The gp41 is less glycosylated and more conserved than the gp120 and is glycine-rich and hydrophobic at the N-terminal (20 amino acids) which is the region fusing the virion membrane to the plasma membrane during virus entry. A second hydrophobic region spans the virion and cell membranes, anchoring the glycoprotein heterodimer. It is postulated that CD4 binding of the gp120 leads to a conformational change in the gp41, making it more accessible for fusion (Tan *et al.*, 1997). A triple coil-coil α-helix structure in the gp41 N-terminal ectodomain (external domain) is the fusion active region that penetrates the target cell membrane (Chan *et al.*, 1997; Tan *et al.*, 1997 and Weissenhorn *et al.*, 1997).

1.3.4.1) Gp120 V3 domain

The gp120 contains conserved (C1-C5) and variable regions (V1-V5) interspersed. Much attention has been focused on the third variable domain (V3 domain), a loop of 35 amino acids (aa). It governs cell tropism, cytopathicity and co-receptor usage of the virus. B and T-cell responses are aimed at the V3 loop and it is known as the principle neutralising determinant. The tetrapeptide crown of the loop is fairly conserved amongst subtypes, as are the cysteine residues at the base of the loop that form a disulphide bond. Sequence changes within the loop are conservative, substituting with amino acids that have similar chemical properties (Fields, 1996). Amino acid changes in the V3 loop from acid to basic, influence viral tropism from macrophage and primary T-cell tropic towards the ability to replicate in continuous T-cell lines. Specifically, the region between the V3 and C2 is important for syncitia formation, tropism and infectivity, C4 is part of the CD4 binding site and interacts with the V3 loop, affecting the gp120 conformation (Fields, 1996).

1.4) HIV infection and disease progression

1.4.1) Routes of transmission

HIV is transmitted by various routes, including sexual, parenteral (including blood transfusion and intravenous drug use), perinatal transmission and via the breastmilk (WHO Report, 1998). HIV is transmitted cell-associated in the semen and in vaginal secretions. Perinatal transmission can occur at a number of different stages during pregnancy, delivery and during breast-feeding (Dunn *et al.*, 1992; Newell *et al.*, 1996; Newell *et al.*, 1997 and Newell *et al.*, 1998). The highest risk of transmission is during delivery (Newell *et al.*, 1996). Approximately 25% of babies born to HIV positive mothers in the developing world are infected with a further 14% of infections occurring during breast-feeding (Gray *et al.*, 1996 and Leroy *et al.*, 1998).

1.4.2) Co-receptor Usage

Although the main receptor for HIV is CD4, it has long been known that HIV requires more than CD4 for entry into the host cells. However, only in 1996 were other co-receptors identified (see figure 1.3).

Fusin, or LESTR, now known as CXCR4 was first identified as being needed for fusion of the virus to the host cell (Feng *et al.*, 1996). The receptor is found in many human cells and is a member of the 7-transmembrane G-protein coupled receptor family which activate calcium ion fluxes or cyclic AMP in response to certain extracellular ligands (Alkhatib *et al.*, 1996 and Paxton *et al.*, 1996). CXCR4 is an α-chemokine receptor and is specific, binding only one ligand (Premack and Schall, 1996 and de Roda Husman and Schuitemaker, 1998). CXCR4 is the receptor used by T-cell adapted syncitia inducing (SI) virus variants which form syncitia when grown in cell culture.

CCR5 was identified later and is also a member of the G-protein coupled receptor family (see figure 1.3). CCR5 is a β-chemokine receptor and belongs to the C-C group that binds more than one ligand (Premack and Schall, 1996 and de Roda Husman and Schuitemaker, 1998). It was determined that the CCR5 receptor was the binding site for primary, non-syncitium inducing (NSI) HIV isolates that are macrophage or monocyte tropic (Alkhatib *et al.*, 1996). These primary isolates and their associated co-receptor CCR5 have been associated with sexually transmitted HIV variants (Zaitseva *et al.*, 1997; Paxton *et al.*, 1998a and Paxton *et al.*, 1998b). HIV binds to CD4 first, after which there is a conformational change in the gp120 protein allowing co-receptor binding and then fusion of the viral envelope and the host cell membrane. The CCR5-binding region is conserved in a number of primate lentiviruses, including HIV-1 subtype A, B, C and E, as well as SIV (Nyambi *et al.*, 1998). There are also dual tropic, NSI/SI variants that can utilise both the CCR5 and CXCR4 receptors.

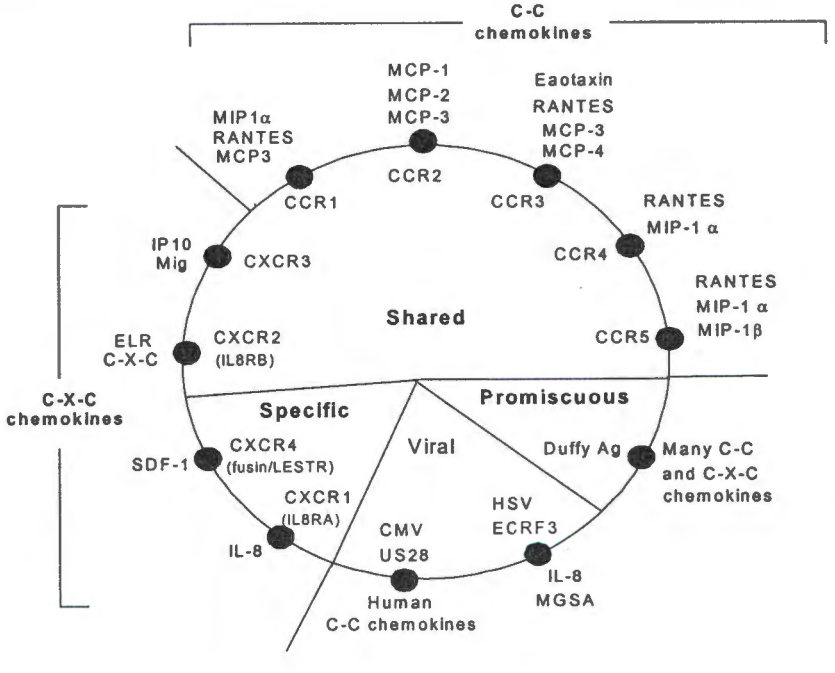


Figure 1.3: The chemokine receptors and ligands to which they bind are illustrated as well as the four major chemokine classes. HIV can utilise CCR5, CCR3, CCR2 and CXCR4 co-receptors, amongst others (Premack and Schall, 1996).

A new classification system for SIV/HIV strains based on co-receptor usage has been proposed (Berger *et al.*, 1998). Viruses using the CCR5 co-receptor are designated R5, viruses using the CXCR4 co-receptor are designated X4 and dual tropic viruses using both receptors are classified X4R5. In addition, other co-receptors which may be utilised can also be incorporated into the classification system, such as CCR3, which would be designated R3. This classification system is more flexible and accurate than the current SI (rapid/high) and NSI (slow/low) classification system, especially as more co-receptors are identified (Doms and Moore, 1996 and Fenyö *et al.*, 1996).

Natural ligands to these co-receptors inhibit HIV replication by blocking virus attachment to the host cells. Macrophage inflammatory protein's (MIP)1α and MIP1β, and regulated-on-activation, normal T cell-expressed and secreted (RANTES) are ligands produced by CD8+ T-cells which block the attachment of HIV isolates utilising the CCR5 co-receptor (Trkola *et al.*, 1996 and Wu *et al.*, 1996). It has been found that people who have increased levels of MIP1α, MIP1β, and RANTES are more resistant to HIV infection by primary, macrophage-tropic isolates (Paxton *et al.*, 1996). Stromal cell-derived factor 1 (SDF-1), on the other hand, is the only ligand identified which can inhibit the replication of CXCR4-binding isolates.

Other co-receptors, or orphan receptors such as CCR2b, CCR3, CCR8, V28, STRL33 (Bonzo), GPR1, GPR15 (BOB), Chem23 (which is expressed by dendritic cells) and virally encoded chemokine receptor analogue, US28, have also been identified (Choe *et al.*, 1996; Doranz *et al.*, 1996; Deng *et al.*, 1997; Farzan *et al.*, 1997; Liao *et al.*, 1997; Pleskoff *et al.*, 1997; Reeves *et al.*, 1997; Rucker *et al.*, 1997; Pleskoff *et al.*, 1998 and Samson *et al.*, 1998). The significance of the alternative co-receptors *in vivo* has not yet been determined, although they may be involved in viral pathogenesis. CCR3, for example is expressed in microglia along with CCR5 and usage of this receptor may result in viral neurotropism (He *et al.*, 1997).

It has recently been shown that in certain cases there is a switch from the exclusive use of CCR5 during early infection, to the use of a broader range of co-receptors such as CCR1, CCR2b, CCR4 and BOB in rapid and late progressors. This increase in co-receptor utilisation by the virus usually coincides with a decrease in CD4 levels in the host and subsequent progression to AIDS. Long term non-progressors (LTNP's), on the other hand, tend to maintain CCR5 as the major co-receptor and produce higher levels of β-chemokines than rapid or late progressors (Xiao *et al.*, 1998).

People who remain HIV-negative, despite repeated exposure to the virus have been identified. Sequence analysis of the CCR5 gene has shown that there is a 32 base pair deletion (Δ32) leading to a truncated protein in certain individuals. If the individual is homozygous for the 32 base pair deletion, it leads to a non-functional CCR5 receptor and in most cases, partial resistance to macrophage tropic primary isolates (Liu *et al.*, 1996 and Samson *et al.*, 1996). Individuals

heterozygous for the Δ32 deletion are not protected from infection, but have slower disease progression (Dean *et al.*, 1996 and Huang *et al.*, 1996). It has been established that the frequency of the homozygous Δ32 deletion is higher in populations of European descent and the further North, the higher the frequency. For example, in Finland 2.6% out of 194 HIV-negative people are homozygous for the Δ32 mutation (Pastinen *et al.*, 1998), whereas in Cypress only 0.1% of 1002 HIV-negative people are homozygous for the Δ32 deletion (Christodoulou *et al.*, 1997). No homozygous Δ32 deletions have been identified in non-Caucasians in Venezuela (Dean *et al.*, 1996), Japan, or Central Africa (Samson *et al.*, 1996).

A second mutation has been detected in the CCR5 gene that results in a non-functional protein. It is a T → A point mutation, causing an early stop codon, designated m303. The m303 mutation, together with a Δ32 deletion on the other allele may render PBMC's resistant to primary isolates. In a study of 209 blood donors in France, under 1% had the mutation, making it 10 to 20 times less frequent than the Δ32 deletion (Quillent *et al.*, 1998). In addition, an A/G polymorphism in the CCR5 promoter has been identified which may affect the rate of disease progression after infection. The polymorphism, termed 59029-G (glycine instead of alanine at base pair 59029), when homozygous, appears to lead to decreased CCR5 mRNA production, thereby facilitating a decrease in the amount of CCR5 receptor which is expressed. Individuals homozygous for 59029-G/G progress to AIDS 3.8 years slower on average than those homozygous for 59029-A/A (McDermott *et al.*, 1998).

In 1997 the CCR2b-64I mutation (caused by valine to isoleucine substitution) in the transmembrane domain of CCR2b was identified. The CCR2b-64I mutation was associated with a delay in disease progression by two to four years in the first study performed (Smith *et al.*, 1997). It was subsequently shown that delay in disease progression associated with the CCR2b-64I mutation may be linked to CCR5-restricted NSI HIV-1 viruses, as the delay was less in individuals homozygous for the mutation who were infected with SI variants. In addition, no delay in disease progression was observed in individuals heterozygous for the CCR2b-64I mutation, who were infected with SI HIV-1 variants (van Jij *et al.*, 1998). However, in a later study, no delay in disease progression in intravenous drug users (IVDUs) heterozygous for the CCR2b-64I mutation infected with CCR5-restricted NSI viruses was shown either. It was postulated that immunologic differences may exist between the IVDUs from the later study and the homosexual men in the prior study, where a delay in disease progression was shown. There may also be differences in the HIV variants that infect IVDUs as compared with homosexual men, or possibly the different routes of transmission account for the lack of a delay in disease progression (Schinkel *et al.*, 1999). Clearly more work needs to be done in order to investigate the effect of the CCR2b-64I mutation.

Mutations associated with a change in disease progression may also be in the natural ligand that binds the receptor. Stromal cell-derived factor 1 (SDF-1), the major ligand for CXCR4 may have a polymorphism in the 3' untranslated region of the gene. Individuals homozygous for the SDF-1-3'A

mutation were found to have delayed disease progression, possibly by up-regulation of the production of the protein. The protective effect is more pronounced in late stage AIDS, possibly due to most of the viruses being SI/X4 viruses (Balter *et al.*, 1998 and Winkler *et al.*, 1998). High frequency of the 3'A mutation of the SDF-1 gene was detected in Cambodia (Rousset *et al.*, 1999).

1.4.3) Mechanisms of infection and spread of the virus

It is thought that the first cells to be infected are cells of the dendritic macrophage lineage, such as Langerhans' cells and lymphocytes. Immature dendritic cells (DCs) have been shown to allow only R5 HIV to replicate, although mature DCs can support both M or T tropic HIV replication when co-cultured with CD4+ T cells (Zaitseva *et al.*, 1997 and Granelli-Piperno *et al.*, 1998). It has been determined by the use of CCR5Δ32 engineered DCs that although no viral replication occurs in these DCs, they are able to pass the virus on to CCR5 wild-type CD4+ T-cells. The resistance of people with homozygous CCR5Δ32 mutations is thus not DC-mediated. HIV in infected DCs can survive without proliferating for the time it takes for the DCs to migrate from the mucosa to the draining lymph nodes, allowing infection of CD4+ T-cells in the lymph node (Dybul, *et al.*, 1998).

Latently infected folicular dendritic cells in the germinal centres of the lymph organs (Panteleo and Fauci, 1996) are long term reservoirs for the virus, allowing viral replication when the cell is activated by infection. These latent reservoirs have been shown to be small, 10^4 to 10^6 infected cells per host although there is a low turnover of 60 to 6000 cells per day, allowing low levels of HIV replication in the reservoirs (Ho, 1998).

1.4.4) HIV-1 disease progression

From the lymphoid cells the virus disseminates in the blood, or lymph as free virus, or in cell-associated form (Miller *et al.*, 1989; Spira *et al.*, 1996; Dittmar *et al.*, 1997 and Pope *et al.*, 1997). During primary infection, there is a high level of viraemia (±10^7 copies of RNA/ml) and the cell-associated virus allows spread of the infection and the initiation of the immune response (Panteleo and Fauci, 1996). Both bone derived, and lymphoid dendritic cells can carry virus, thereby infecting the CD4 lymphocytes.

HIV initiates a humoral and cellular immune response to the virus, which leads to the initial decrease in the levels of viral load. The clinical course of infection can be divided into three phases; primary infection, clinical latency and AIDS (see figure 1.4). The majority of HIV-infected people are typical progressors, that is, they progress to AIDS within eight to ten years without therapy. Three other types of progression have also been identified:

i)rapid progressors, who progress to AIDS within two to three years

ii)long term nonprogressors (LTNPs), who may not reach progression to AIDS even after ten years

iii)long term survivors, who progress to disease in a similar time frame to typical progressors, but who remain clinically stable for a long time (Panteleo and Fauci, 1996).

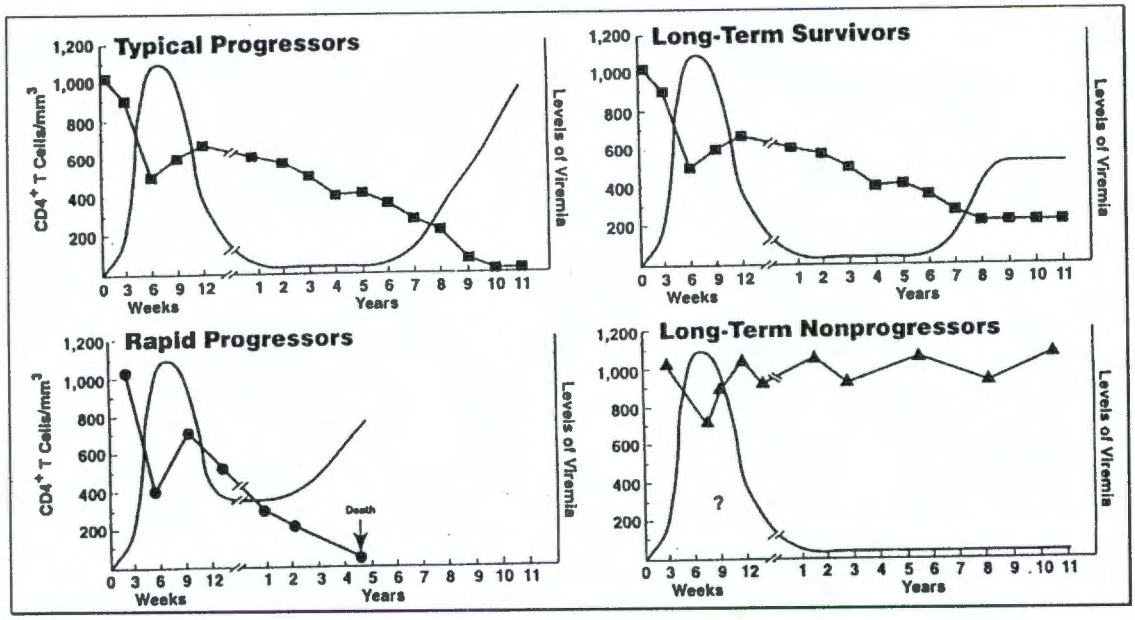


Figure 1.4: An illustration of the four types of disease progression in individuals infected with HIV, illustrated by the levels of CD4+ T-cells (lines with symbols) and viraemia (straight lines). In all cases there is a rise in viraemia at six to nine weeks, with a concomitant drop CD4+ T-cells. The CD4+ T-cell count then rises and the level of HIV viraemia decreases up until approximately twelve weeks for all groups, except the LTNPs who maintain CD4+ levels at approximately 900-1000 cells/mm^3 and low viraemia. The level of viraemia in rapid progressors steadily increases and CD4+ T-cells decrease until death after approximately five years, whereas in typical progressors the level of viraemia only increases after seven to eight years. Long term survivors have a similar increase in viraemia after seven to eight years, but their CD4+ T-cell count does not decease. Although the level of viraemia increases after seven to eight years in long term survivors, their CD4+ T-cell count does not drop below approximately 300 cells/mm^3 for an extended period of time (Pantaleo and Fauci, 1996).

1.4.5) The replication cycle, virion assembly and budding

The gp120 envelope protein of HIV initially binds to the CD4+ receptor on the host cell, which induces a conformational change in the gp120 protein, allowing it to bind the relevant co-receptor molecule. There is a further conformational change in the gp120, exposing the three coiled coil N-terminal fusion peptides (ectodomain) of the gp41 transmembrane protein (see section 1.3.4). The actual mechanism whereby the gp41 fusion peptides insert themselves into the host cell membrane, initiating fusion is not known. After the HIV virion and host cell membranes fuse, however, the viral core enters the host cell (see figure 1.5) (Chan *et al.*, 1997; Tan *et al.*, 1997; Weissenhorn *et al.*, 1997 and Kwong *et al.*, 1998).

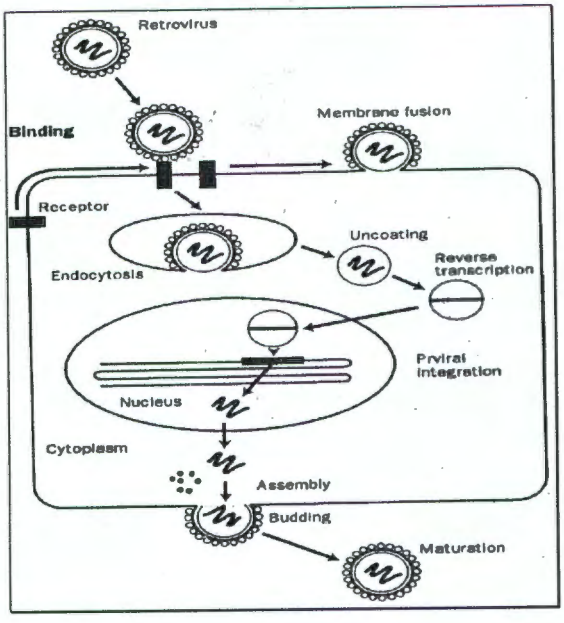


Figure 1.5: The replication cycle, assembly and budding of the HIV virion is illustrated (Barré-Sinoussi, 1996). After entry viral particles uncoat, releasing the nucleoprotein complex. Reverse transcriptase initiates DNA synthesis from the $tRNA^{lys}$ primer, bound to the viral RNA. Proviral DNA is integrated into the host genome near the LI and Alu retroposon elements. Viral RNA is synthesised in the nucleus with viral *cis*-acting elements, transactivators and several host cellular proteins. Full length HIV transcripts produce progeny viral RNA, mRNA for Gag and Gag-Pol polyproteins in the cytoplasm and precursors for more than 30 spliced mRNA's producing the Env proteins and accessory proteins. Multiply spliced mRNA's are produced early, encoding Tat, Rev and Nef (non-viral proteins) and later the unspliced and single spliced mRNA's encoding virion structural proteins are produced (Fields, 1996).

The Gag polyprotein ($Pr55^{gag}$) and Gag-Pol polyproteins ($Pr160^{gag\text{-}pol}$) interact with the cell plasma membrane via the C-terminal of the MA protein where self-assembly of the spherical ribonucleoprotein (RNP) core occurs (Yu *et al.*, 1992 and Dorfman *et al.*, 1994). Once the RNP is completely sealed, incorporating the diploid RNA genome bound to the NC protein, the immature virion buds out of the cell, tightly surrounded by a lipid bilayer. The Vpu and p6 (C-terminal region of the $Pr55^{gag}$) facilitate budding of the immature virion (Göttlinger *et al.*, 1989) which is densely studded with gp120 oligomers that are spontaneously shed as the virion matures (Gelderblom, 1997). Host cell proteins are also present in the virion; β-microglobulin, HLA-α, HLA-β (which exceeds the numbers of gp120 and gp41, thereby masking the virus from the immune system), CD55 (decay-accelerating factor) and cyclophilin-A (which may play a role in virion uncoating). The cyclophilin A has been found to be necessary for in vitro growth of the Group M HIV-1 and SIVcpz, but not Group O HIV-1 viruses (Braaten *et al.*, 1996).

1.5) Epidemiology

1.5.1) The Global HIV Epidemic

The HIV epidemic is persistently spreading, with 33.4 million people estimated to be living with HIV/AIDS as of the end of 1998 (see figure 1.6) and more than 16 000 new infections per day. In 1997 alone, 5.8 million new infections occurred, of which 4 million were in sub-Saharan Africa. More than 95% of infections occur in developing countries and sub-Saharan Africa has the highest prevalence of infected people (7.4%) with 22.5 million individuals infected, of which approximately 50% are women. This reflects the main mode of transmission, which is by heterosexual contact, and in turn leads to the staggering numbers of new infections by vertical transmission. In 1997, out of 590 000 new infections in children under the age of fifteen, 530 000 occurred in sub-Saharan Africa (UNAIDS and WHO, 1998).

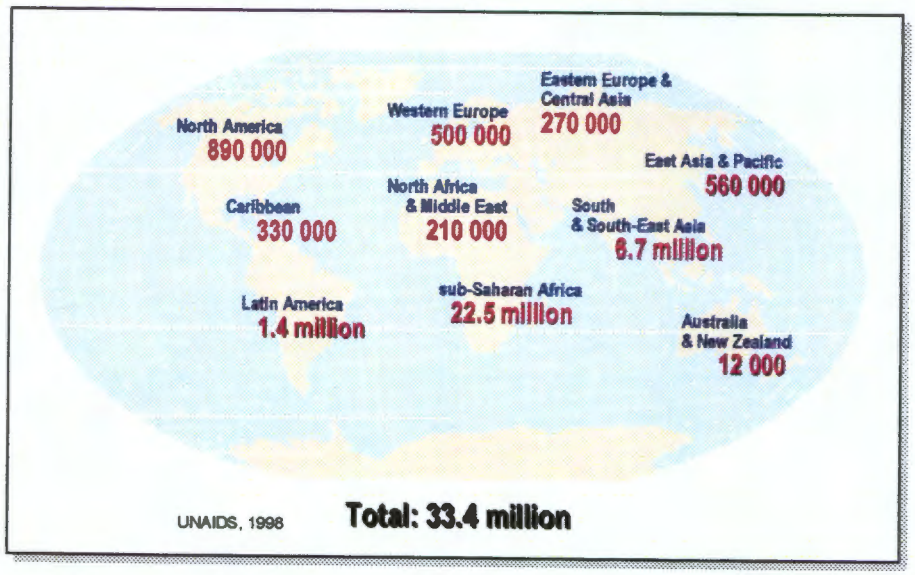


Figure 1.6: The total number of adults and children living with HIV as of December 1998. Sub-Saharan Africa has the greatest number of infections with 22.5 million people living with HIV (UNAIDS, 1998).

1.5.2) Sub-saharan Africa

The African HIV epidemic started in the early 1980's as a broad belt stretching across the equatorial regions of the continent. By 1987, however, the epidemic in Africa had spread southwards. At present, the highest HIV prevalence on the continent occurs in southern Africa. Rates of HIV infection in West African countries have plateaued at levels lower than in East and southern Africa, except in certain countries, such as Côte d'Ivoire and Nigeria. Unfortunately, these countries have some of the highest populations in Africa, and even with only 4.1% prevalence in Nigeria, it amounts to 2.2 million people infected (MAP, 1997 and UNAIDS and WHO, 1998).

The most devastating epidemics to date occur in southern Africa, however. In South Africa, out of a total of 3.6 million infected people, 900 000 were infected in 1998 alone. Indeed, over 50% of the new infections occurring in southern Africa are in South Africa (UNAIDS, 1998). The epidemic in South Africa has increased logarithmically from a prevalence of just under 1% of women attending antenatal clinics infected in 1990, to 22.8% at the end of 1998, a 33.8% national increase since 1997 (Department of Health, 1999). The South African epidemic will be discussed in more detail in Chapter 2.1 and Chapter 3.1. There are other countries in southern Africa which have even higher seroprevalence in women attending antenatal clinics. In Francistown, in Botswana, 45% of women were HIV-positive at the end of 1997 and in Beit Bridge, a large agricultural centre in Zimbabwe, HIV prevalence rose in pregnant women from 32% in 1995 to 59% in 1996 (MAP, 1997).

Urban areas tend to have higher HIV infection rates than rural areas, although this is not true in all cases. For example, in Uganda, one of the first countries to be affected by the epidemic, HIV seroprevalence has decreased in both rural and urban areas due to an intensive prevention and education programme. It is important that the education and prevention programmes continue once the levels of infection have declined, however. In neighbouring Tanzania, which also had declining HIV infection rates in 1996, there is now an explosive epidemic (Renjifo *et al*,. 1998). Most east African countries are not showing decreases in rates of infection. This is, at least in part, due to the trans-African highway that runs up the east coast of the continent which fuels the epidemic. In countries such as Kenya, seroprevalence in pregnant women is up to 25% in urban regions such as the capital, Nairobi (MAP, 1997 and UNAIDS and WHO, 1998).

1.5.2.1) Determinants of the spread of HIV in Africa

There are a number of possible explanations why sub-Saharan Africa has over 60% of the worlds HIV/AIDS cases and why certain regions in Africa have more explosive epidemics than other regions. In Africa, over 95% of HIV infections are due to heterosexual or vertical transmission. Blood transfusion, however, is also a concern. It was determined that in sub-Saharan Africa in 1995 nearly a quarter of the 2.5 million blood transfusions had not been screened for HIV antibodies (MAP, 1997). Cultural, behavioural, biological, socio-economical and political factors also play a role in the spread of HIV. In addition, the stigma of being infected with HIV and the resulting shame and denial of the infected person has also contributed to the continued spread of the disease (UNAIDS, 1998).

Cultural aspects include religion, the use of contraception and condoms, male circumcision as well as certain sexual practices. Sexual behaviour plays a major role in the spread of HIV and the use of condoms or a decrease in the number of sexual partners in certain countries, such as Senegal, has lead to a lower rate of infection. Certain vaginal products or the use of drying agents as well as anal intercourse or intercourse during menses may lead to a higher risk of infection from an infected partner. In addition, biological factors including the disease stage of the partner, which impacts on the viral load, the type of HIV (HIV-1 or HIV-2) and the presence of other sexually

transmitted diseases (STDs) all influence the chance of HIV infection during intercourse (MAP, 1998). Genital ulceration has been shown to lead to an increased risk of transmission and therefore treatment of STDs could to some extent prevent the transmission of HIV by the sexual route (Grosskurth *et al.*, 1995; Laga *et al.*, 1995 and Wawer *et al.*, 1998).

Socio-economic factors also contribute to the spread of HIV and in West Africa, for example in the Great Lakes region and especially southern Africa where there are mass population movements, mostely due to migrant male workers. These men move away from their homes in search of work and may spend weeks or even months living and working away from home. Many of these dislocated men turn to commercial sex workers (CSWs) while away from home and upon their return spread HIV to their wives and partners (Quinn, 1996). In Abidjan, the capital of Côte d' Ivoire, about 80% of the CSWs were HIV-positive in 1995 (Decosas, 1995 and Quinn, 1996). It has been shown that populations, where sex with CSWs is common, have rapid spread of HIV. In addition, it has been found that many concurrent partnerships leads to the more efficient spread of HIV than serial monogamy (MAP, 1997). Movement of people by urbanisation is also increasing significantly and by the year 2000 it is expected that as much as a third of the population may be residing in urban centres for many African countries (Quinn, 1996). Migration to and from South Africa will be discussed in more detail in Chapter 3.1.

Political upheaval and war are other reasons for mass movement of people allowing the spread of HIV (Quinn, 1996 and UNAIDS and WHO, 1998). In Rwanda, for example, the HIV prevalence in people who had lived in refugee camps during the conflict was 8.5%, in comparison to 1.3% in the same population when they were living in their rural settlements (UNAIDS, 1998).

1.5.2.2) Prevention of infection in Africa

Education programs have played a role in the decline of HIV in certain countries in Africa such as Uganda and Senegal. These include: the encouragement of condom use and a decrease the number of sexual partners, as well as education about risky sexual behaviour and education of pregnant women about the risks of breast-feeding (Robinson *et al.*, 1995; Ngugi *et al.*, 1996 and Jackson *et al.*, 1997). It has been determined that the treatment of sexually transmitted diseases (for example in Mwanza, Tanzania) also has a positive impact limiting the spread of HIV (Laga *et al.*, 1995 and Wawer *et al.*, 1998). In addition, a short-term zidovudine, reverse transcriptase inhibitor, (ZDV) regimen was shown to decrease perinatal transmission by 51% in a Thailand study (Marseille *et al.*, 1998). This is more applicable for developing countries than the expensive long term regimen (Sperling *et al.*, 1996) which decreases transmission by 66% ($50 versus $800 for the long term regimen).

1.5.3) Asia

The Asian epidemic only started in the early 1990s in Thailand's intravenous drug using (IVDU) and CSW populations, but by 1997 had expanded throughout south-east Asia. South-east Asia and India, which has its own predominantly heterosexual epidemic, are the worst affected regions in Asia. China, which is at the start of its epidemic, has over 400 000 HIV/AIDS cases and two separate epidemics; one infecting IVDUs in the south-west China and the other heterosexuals along the eastern coast-line, where prostitution is common. India, with four million infected people, has the highest number of HIV infections in any country in the world. Infections are mostly spread through heterosexual transmission and IVDU. In 1996, in IVDUs in Manupur, HIV rates reached 73% in some drug clinics (UNAIDS and WHO, 1998).

Thailand has embarked on a campaign of awareness in IVDUs and heterosexual populations to decrease the number of HIV infections, although levels are increasing in other countries. Cambodia, despite an increase in condom use, Viet Nam and Myanmar all show rapid increases in infection rate, especially in CSW and IVDU's. Just over one-fifth of the worlds population infected with HIV live in Asia and the pacific and this is expected to reach one quarter by the year 2000 (UNAIDS and WHO, 1998).

1.5.4) South America and the Caribbean

Men who have sex with men (MSM) and IVDUs are the major populations infected with HIV in South America, but increasing rates of transmission in women show an increase in the number of heterosexual infections. In Brazil one quarter of AIDS cases are female. Heterosexual transmission, reflected by the prevalence rates in pregnant women in Haiti and Dominican republic, are much higher, at 8%. Surveillance is limited and data fragmented, but HIV prevalence in all countries in South America and the Caribbean is increasing (UNAIDS and WHO, 1998).

1.5.5) Eastern Europe

Although the epidemic in eastern Europe was one of the latest epidemics, levels of HIV prevalence are rising rapidly. Extremely low infection levels (30 000 infections) were detected in mass blood screening of approximately 450 million people up until the start of 1995, however newly diagnosed infections increased from 196 in 1995 to 4 399 in at the end of 1997. The Soviet Union countries of Belarus, Moldova, the Russian Federation and Ukraine have all shown explosive epidemics due to IVDUs. The epidemic moves into the general population after heterosexual contact with IVDUs and a strong overlap has been demonstrated between infected IVDUs and infected CSWs. Other sexually transmitted diseases (STDs) are increasing dramatically as well and indicate the ease with which an HIV epidemic could spread through eastern Europe (UNAIDS and WHO, 1998).

1.5.6) The developed world

Due to the HIV epidemic being measured as AIDS cases in the industrialised world, rather than HIV infections, with the advent of effective treatment of HIV, the numbers of AIDS cases has declined. There are still infections occurring, however, especially in disadvantaged populations, such as African Americans and in Hispanics as well as in IVDUs (UNAIDS and WHO, 1998).

1.6) Genetic diversity and geographical distribution of HIV-1 subtypes

Initially, HIV was classified into only two groups, "US" and "African". Currently, however, with the abundance of sequencing data and the ongoing global effort to characterise HIV-1, the virus has been reclassified into a number of groups and subtypes. HIV-1 has been divided into three groups; Group M (major group), Group O (outlier group) (Carr *et al.*, 1998) and the newly identified Group N (Simon *et al.*, 1998). Group M is responsible for the majority of infections to date and has been classified into 10 *env* subtypes (A-J), with subtypes A to E being the most common (UNAIDS and WHO, 1998). The percentage DNA difference in the M group of this highly mutable pathogen ranges from approximately 15 to 30% between subtypes in the *env* region (Bobkov *et al.*, 1994b), growing by 1% per year (UNAIDS, 1998) and between 7 and 14% in the *gag* region (Louwagie *et al.*, 1993).

Group O, or subtype O consists of highly divergent HIV-1 viruses that are up to 50% divergent from both the Group M viruses as well as from each other, hence Group O, or outlier group. These viruses are too divergent to be divided into discrete subtypes as yet. (Korber *et al.*, 1996). In addition, isolate YBF-30 was recently identified, which led to the classification of the new HIV-1 virus group, Group N (Simon *et al.*, 1998) (see section 1.2).

1.6.1) The variability of HIV-1

The great genetic variability of HIV-1 is as a result of two major factors, the error-prone mechanism of the reverse transcriptase enzyme and recombination between viruses co-infecting a host cell. The reverse transcriptase enzyme introduces, on average, one error per genome per replication cycle, which results in approximately 1% of genetic drift each year (Balfe *et al.*, 1990). Recombination, on the other hand, which occurs at a rate of about 2% per kilobase per replication cycle has been frequently identified amongst the M group of HIV-1 and selects for the most beneficial combinations of genes (Cornelissen *et al.*, 1996 and Subbarao *et al.*, 1998). Recombination occurs in host cells super-infected with more than one HIV virion, during replication of the viral genome. There are random cross-over events which occur between homologous regions of the two viral genomes, resulting in mosaic progeny HIV virions, with regions of the genome incorporated from each of the viruses infecting the host cell. Another factor resulting in the fast

evolution of HIV is the rapid production of virions in the host (10^9 per day) and the large number of replication cycles, (320 per year) (Ho, 1996).

1.6.2) Recombinant viruses and the HIV epidemic

Full-length genome sequences have revealed that members of certain subtypes are exclusively recombinant viruses. For example, subtype E is a subtype A in the *gag/pol* and part of gp41 and subtype E in the rest of the genome. The parental subtype E virus has not been found. Subtype IbNG is a *gag* subtype A and subtype G in portions of the *pol* and in the LTR (Carr *et al.*, 1998). It was suggested that since recombinant viruses were becoming more common, the criteria used for classification of these recombinant subtypes be changed. Recombinant viruses such as E and IbNG are now classified as circulating recombinant forms (CRF) AE and AG IbNG, respectively, (Carr *et al.*, 1998). It is now important to look at different regions of the genome or preferably to sequence the entire genome in order to designate a subtype. CRF AE and AG IbNG are established subtypes with widespread prevalence. CRF AE is predominant in central Africa and Asia, whereas CRF AG IbNG, first described in Nigeria, is prevalent throughout West and West Central Africa (Mastro *et al.*, 1996; Yu *et al.*, 1997; Carr *et al.*, 1998; Kusagawa *et al.*, 1998; Loue *et al.*, 1998; Loussert-Ajaka *et al.*, 1998; Paladin *et al.*, 1998; Subbarao *et al.*, 1998; Carr *et al.*, 1999 and McCutchan *et al.*, 1999). The subtype A in the *gag* region from these two viruses is distinct from each other and from the non-recombinant subtype A virus, which occurs predominantly in East Africa.

There are a number of new epidemic outbreaks that have occurred globally due to recombinant viruses, for example in China, Russia and Africa, that will be discussed later (Liitsola *et al.*, 1998; Peeters *et al.*, 1998; Renjifo *et al.*, 1998; Shao *et al.*, 1998 and Yu *et al.*, 1998). Triple recombinants have also been identified, for example subtype I, which is an A/G/I recombinant (Gao *et al.*, 1998). Although co-infections with Group M and Group O viruses have been reported (Zekeng *et al.*, 1998), no recombinant viruses have yet been identified, possibly due to the great diversity between these subtypes.

1.6.3) HIV subtypes and population migration

Although most HIV-1 strains fall into a discrete phylogenetic lineage, increasing divergence is becoming evident within these subtypes (Kuiken *et al.*, 1993; Myers *et al.*, 1995 and Rencher *et al.*, 1997). In addition, recombinant viruses are being detected more frequently (Liitsola *et al.*, 1998; Peeters *et al.*, 1998; Renjifo *et al.*, 1998 and Shao *et al.*, 1998).

Subtype surveillance can be used to track the HIV-1 epidemic and together with a knowledge of political, economical, social and cultural dynamics of spread, may allow transmission

patterns to be understood, future epidemic trends to be predicted and may provide information on the history of the HIV epidemic. This data is important for effective development of vaccines. It is possible that there may be a relationship between disease progression and subtype and this may also be investigated. In addition, the evolution of highly divergent strains must be monitored in order to facilitate their detection, as illustrated by the inability of many of the commercial kits for HIV to detect subtype O viruses when they were first identified (Schable *et al.*, 1994).

In many cases it is the movement of people that promotes the spread of HIV. For example in China, where the initial subtype C infections were shown to be due to needle sharing with IVDUs from India (Luo *et al.*, 1995). It has been further determined that there are two subtypes associated with IVDU in China. In a recent study on IVDUs in Guangxi Province which borders Yunnan in the West and Vietnam in the South, subtype C was associated with IVDU in Baise city, in western China, whereas subtype AE has been associated with IVDU in Pingxiang city in southern China (Yu *et al.*, 1998). In addition, the recent recombinant subtype B/C epidemic in China follows the drug trafficking route from south to west (Shao, *et al.*, 1998). In Africa one of the major factors influencing the spread of the HIV epidemic is migration for socio-economic reasons (see section 1.5.2.1). Also, the deployment of army personnel to various countries has also resulted in the spread of subtypes to different countries, for example in the United States, subtypes A, D, and AE were detected in five US service men who were infected whilst in Uganda, Kenya or Thailand respectively (Brodine *et al.*, 1995). Non-B subtypes have also been spread to French military personnel (Lasky *et al.*, 1997). In addition, subtype AE was detected in five out of six Uruguayan soldiers infected whilst in Cambodia (Artenstein *et al.*, 1995).

One of the largest population migrations at any one time involved the emigration of approximately 17 000 Jewish Ethiopians to Israel in 1991, due to political unrest. Whereas initially the predominant subtype circulating in Israel and Palestine was subtype B, associated with homosexual transmission, a later study indicated that subtype C had entered the country and was associated with heterosexual transmission in Ethiopian refugees (Gehring *et al.*, 1997). The same subtypes have been associated with independent introduction of HIV-1 into the homosexual (subtype B) and heterosexual populations (subtype C) in South Africa (Williamson *et al.*, 1995; van Harmelen *et al.*, 1997; van Harmelen *et al.*, 1999a) (see Chapter 2).

Subtype AE is primarily responsible for the burgeoning heterosexual epidemic in Thailand. Full-length subtype AE sequences from Thailand and the Central African Republic showed similar mosaic patterns, indicating that the recombination event occurred prior to its introduction into Thailand (Gao *et al.*, 1996b). Subtype AE is now spreading across South East Asia and has been detected in Myanmar, Vietnam, Japan and Malaysia (Menu *et al.*, 1996 and Kondo *et al.*, 1998). It has also been detected in certain groups in France, Sweden, England, Russia, and USA (Arnold *et al*, 1995; Brodine *et al.*, 1995; Lukashov *et al.*, 1996; Simon *et al*, 1996; Bobkov *et al.*, 1997; Brown *et al.*, 1997 and Sönnerburg *et al.*, 1997).

1.6.4) Shifts in subtype distribution

1.6.4.1) South and south-east Asia

The importance of continual monitoring of genetic diversity is highlighted by the shift in HIV-1 subtype distribution that occurred in Thailand. Subtype AE accounts for 95% of infections in the heterosexual population, followed by Thailand B' and then subtype B (Subbarao *et al.*, 1998 and Mastro *et al.*, 1996). A significant shift in subtype distribution in intravenous drug users (IVDU) has been shown. From 1992 to 1995, the proportion of subtype AE in IVDUs increased from 25% to 68% in the central region, and from 16% to 52% in the south (Mastro *et al.*, 1996). A shift in subtype distribution has also been observed in IVDU in South West China (Shao *et al.*, 1996): from 1990 to 1993, the proportion of prototype subtype B decreased from 90% to 40% and Thailand B' subtype increased from 10% to 59%. In 1995, 8 out 26 IVDU users were infected with subtype C, probably as a result of drug trafficking from India, where this subtype is predominant (Luo *et al.*, 1995). Subtypes A, D and AE have also been identified, with subtype AE spread mostly by IVDU and heterosexual transmission. Subtypes A, B and AE have also been detected in India (Weniger *et al*, 1994 and Tripathy *et al.*, 1996).

A new epidemic which has started in China, following the drug trafficking route from the South, through central China to the far western region is caused by a recombinant subtype B/C virus. Two recombinant subtypes have been identified with a breakpoint in the *tat* gene. The subtype B/C recombinant has been shown to increase in prevalence; with subtype C occurring in the South, to rare subtype B/C recombinants identified in central China, to common subtype B/C recombinants identified in West China and finally to the subtype B/C recombinant being exclusively found in the far West region (Shao *et al.*, 1998).

1.6.4.2) Europe and America

The presence of non-B subtypes in previously subtype B-restricted areas of Europe and North America indicate that eventually multiple subtypes will occur in most countries see figure 1.7. Recent studies in Sweden, have recorded the presence of A, B, C, D, G, H, J, and an A/D recombinant (Letiner *et al.*, 1995; Alaeus *et al.*, 1996 and Sönnerberg *et al.*, 1997). Although most of these infections were associated with African immigrants to Sweden, there is evidence of limited transmission of non-B subtypes within Sweden (Alaeus *et al.*, 1997). In Norway, subtype C was identified in the heterosexual population in 1996 (Engelstad *et al.*, 1996) and two epidemiologically-linked cases of subtype O infection were also identified, dating back as early as 1979 (Jonassen *et al.*, 1997). In non-Dutch populations living in the Netherlands, subtypes A, B, C, D, AE, F and G have been identified (Lukashov *et al.*, 1996) and subtypes A, B, C, D and AE were found in England (Arnold *et al.*, 1995 and Brown *et al.*, 1997). In a German study, 15% of new infections (three AEs and one C) were non-B subtypes (Dietriech *et al.*, 1997). In addition, in France different HIV-1 subtypes are emerging. Subtypes A to H were identified in French blood donors and in patients

attending a hospital in Northern Paris, including patients originating from the Central African Republic (CAR), Caribbean, West Africa and South East Asia (Simon *et al.*, 1996 and Barin *et al.*, 1997).

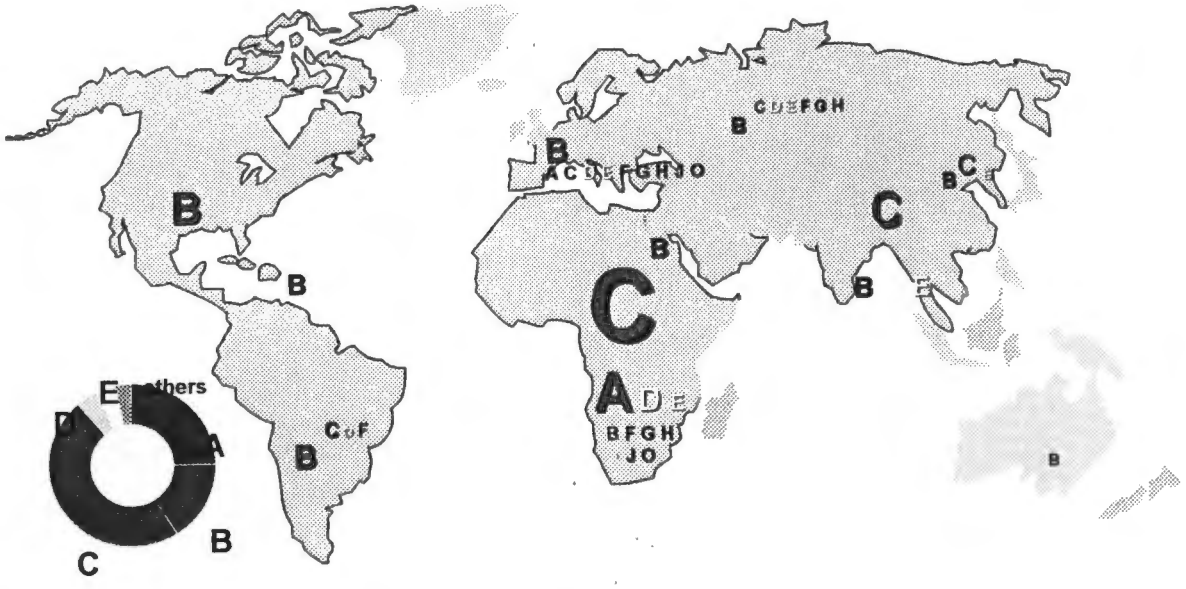


Figure 1.7: The global distribution of HIV-1 subtypes, indicating the prevalent subtypes occurring in the western hemisphere in comparison to Africa and Asia. Subtype B is predominant in Europe and the United States, whereas this subtype is fairly rare in Africa. Africa has the greatest diversity of HIV subtypes, reflecting the age of the epidemic on this continent. In addition, the global prevalence of subtypes is indicated by the circle in the left bottom corner, with subtype C the most prevalent subtype in the world (UNAIDS, 1998).

In Athens, Greece, a study of eighty patients from different risk groups identified 89% were subtype B isolates, although five subtype A, two subtype C and four subtype D viruses were also detected (Nasioulas *et al.*, 1998). In addition, a subtype I virus was identified which is the first subtype I to be found outside of Cyprus where the first two subtype I viruses were identified (Kostrikis *et al.*, 1995). It has recently been discovered from the full-length sequence of provirus from a Cyprus subtype I, that subtype I is a recombinant subtype A/G/I virus. The triple recombinant has more than eleven cross-over points. Segments of the subtype I clone grouped closely with 2321B (A/G/?) and MAL (A/D/?), indicating a possible African origin for subtype I (Gao *et al.*, 1998).

A high degree of genetic diversity has also been observed in the former Soviet Union where subtypes A, B, C, D, AE, F, G and H have been identified (Bobkov *et al.*, 1994a; Bobkov *et al.*, 1994b; Lukashov *et al.*, 1995; Bobkov *et al.*, 1996a; Bobkov *et al.*, 1996b; Bobkov *et al.*, 1997; Bobkov *et al.*, 1998; Liitsola *et al.*, 1998 and Novitsky *et al.*, 1998). In Russia, all of the above subtypes have been identified in heterosexuals, while male homosexuals were predominantly infected with subtype B (Bobkov *et al.*, 1996b). In Russia, Belarus and the Ukraine an explosive epidemic in the IVDU population has occurred since the early 1990s. Phylogenetic analysis showed that a closely related subtype A epidemic had been introduced into the IVDU population (Bobkov *et*

al., 1998 and Novitsky *et al.*, 1998). A new epidemic is taking hold in the IVDU's in Kalingrad in the Russian Federation. Out of 33 isolates from the Ukraine, Lithuania and Kalingrad 82% (all from Kalingrad) were subtype A/B recombinant viruses, with the *gag* A region derived from a subtype A prevalent in IVDUs in the Ukraine. Three subtype A and three subtype B viruses were also identified (Liitsola *et al.*, 1998). In addition, a closely related nosocomial epidemic occurred in Russia in the early 1990's by subtype G (Bobkov *et al.*, 1994a). The subtype F viruses in Romania were shown to be closely related, probably as a result of a founder effect, followed by spread amongst hospitalised children in this region. The Romanian subtype F viruses are not thought to be linked to the subtype F isolates from Brazil (Bandea *et al.*, 1995).

Non-B subtypes have been recorded in America in recent study using V3 serotyping. Out of a total of 40 samples from individuals attending a hospital in the Bronx, New York 15 % were from non-B serotypes, including 3 serotype A's 1 serotype B and 2 serotype C's (Irwin *et al.*, 1997). In Brazil, subtype B is the predominant subtype associated with different modes of transmission (Sabino *et al.*, 1996; Morgado *et al.*, 1998 and Tanuri *et al.*, 1999), although subtypes F, and to a lesser extent, C have also been identified (Brigido *et al.*, 1998). In Rio de Janeiro dual infections (6.5%) and recombinants (4.7%) make up an integral part of the HIV-1 epidemic. Dual infections of subtypes B and F, F and D, B and D and B and C were present, but only subtype B/F recombinants were identified (Pieniazek *et al.*, 1998). Subtype B is also predominant subtype in the Honduras (Lara *et al.*, 1997;) Argentina (Campondonico *et al.*, 1998), Chile (Desgranges *et al.*, 1998) and Venezuela (Quinones-Mateu *et al.*, 1995), but subtype F, C and A have also been detected in Argentina (Campodonico, *et al* , 1998) and subtype A in Santiago, Chile (Desgranges *et al.*, 1998).

1.6.5) Geographical distribution of subtypes in Africa

Monitoring the shift and drift of HIV within Africa is more complicated than in regions with limited genetic diversity, since HIV has been circulating within Africa the longest of all the continents. All subtypes have been detected, or connected, to Africa with the greatest genetic diversity occurring in central Africa (see figure 1.7). A study in west and central Africa showed that up to seven subtypes are present in some countries, although subtypes AE, F, G and H were only identified in central Africa (Peeters *et al.*, 1998). Unfortunately, the lack of representative sampling from most regions in Africa makes it difficult to determine the exact subtype diversity in many countries. In addition, seroconversion dates are often not known, thus subtyping data may not reflect the current subtypes circulating and the place of sampling often may not reflect the origin of the infection.

Although subtype A is still the predominant subtype in west Africa and may be increasing in countries such as Kenya (Poss *et al.*, 1997), its prevalence is decreasing in central Africa. Subtypes A and D are common in east Africa, and screening of a large number of samples in Uganda by oligonucleotide probing demonstrated a ratio of approximately 2:1 of subtypes A:D, respectively (Luo *et al.*, 1998 and Rayfield *et al.*, 1998).

Recombination is now playing a major role in many infections in Africa. A study by Peeters *et al* (1998) in central and west Africa identified 20% of the isolates were *env/gag* recombinants. In addition, dual infections in Cameroon have been identified in a number of samples, including subtype AE and subtype O dual infections. Triple infections of subtype A, subtype D and subtype O were also identified (Peeters *et al*, 1998 and Zekeng *et al.*, 1998), although recombinant subtype O/Group M viruses have not been identified to date, possibly due to the genetic diversity between the two groups. In Tanzania, where subtypes A and D were prevalent from the late 1980's to early 1990's, subtype C is now present in 32% of samples analysed. In addition, 11% of the isolates were *gag/env* recombinant viruses; two C/A, seven C/D and two A/D recombinants (Renjifo, *et al.*, 1998).

Subtype C is the predominant virus in Africa, occurring in more than 50% of samples (UNAIDS 1998) in southern Africa including Malawi, Zimbabwe, Mozambique and South Africa (Engelbrecht *et al.*, 1995; Williamson *et al.*, 1995; van Harmelen *et al.*, 1997; Bredell *et al.*, 1998; van Harmelen *et al.*, 1999a and van Harmelen *et al.*, 1999b) (see Chapters 2 and 3). This subtype is spreading rapidly, however and is present in 99% of 94 isolates sequenced from Ethiopia (Abebe *et al.*, 1997). Subtype C was also found in more than 90% of samples from infected Ethiopian immigrants living in Israel (Gehring *et al.*, 1997). In addition, it has also spread globally and is the most common HIV-1 subtype, making up 48% of the HIV infections world wide (UNAIDS, 1998).

1.6.6) Subtype O distribution

The highly divergent subtype O viruses appear to have originated in central Africa and studies have shown seroprevalences of 10.2% in Cameroon, 6% in Niger , 4.6% in Gabon and 0.5% in Nigeria (Peeters *et al.*, 1994). The seroprevalence is given as a percentage of the total HIV infections in these countries. Subtype O has also been detected in Equatorial Guinea (Hunt *et al.*, 1997), in eight Cameroonians living in France and one French national (Charneau *et al*, 1994 and Loussert-Ajaka *et al.*, 1994), as well as in Spain (Mas *et al.*, 1996), USA (Rayfield *et al.*, 1996), Germany (Dietrich *et al.*, 1997), Norway (Jonassen *et al.*, 1997) and Belgium (de Leys *et al.*, 1990).

1.6.7) Relationship between age of the epidemic and genetic diversity of HIV-1 subtypes

In Africa, the countries bordering the equatorial belt were the first affected by the HIV-1 epidemic. The greatest genetic diversity occurs in the central regions of Africa where almost all subtypes were detected (Janssens *et al.*, 1997). Presumably, this diversity is due to the limited spread of the virus in isolated areas or restricted sexual networks, as well as the age of the epidemic (Janssens *et al.*, 1997). In contrast to this, countries with rapidly emerging, or "explosive," epidemics such as South Africa, Botswana, Malawi and Zimbabwe have low subtype diversity. Over 90% of heterosexually infected individuals in these countries are infected with subtype C viruses (Orloff *et al.*, 1993; Becker *et al.*, 1995; Williamson *et al.*, 1995; Gao *et al.*, 1996; Janssens *et al.*, 1997; Shafer *et al.*, 1997; van Harmelen *et al.*, 1997; Engelbrecht *et al.*, 1998; Novitski *et al.*, 1999; Tien *et al.*,

1999; van Harmelen *et al.*, 1999a and van Harmelen *et al.*, 1999b) (see Chapters 2 and 3).

A similar effect has been seen in Europe and America as well, where subtype B accounts for the majority of HIV-1 infections in the homosexual population, although non-subtype B viruses have been identified in IVDU and heterosexual populations (see section 1.6.4.2). In addition, in south-east Asia, subtypes C and AE account for the majority of infections in the heterosexual populations (Ou *et al.*, 1993; Grez *et al.*, 1994; Yu, *et al.*, 1995; Mastro *et al.*, 1996; Tripathy *et al.*, 1996 and Subbarao *et al.*, 1998). Finally, in eastern Europe subtype A accounts for the majority of IVDU infections (Novitsky *et al.*, 1998), apart from Kalingrad, where the predominant subtype is an A/B recombinant virus (Liitsola *et al.*, 1998).

1.6.8) Monitoring the HIV epidemic

The global pattern of HIV genetic diversity is dynamic, as people move within and between countries, although subtypes A to AE remain predominant world-wide. Subtype C makes up 48% of global infections, subtype A is second, at 25%, followed by subtype B, 16% and subtypes D and AE at 4% each. The other subtypes make up the remaining 3% (UNAIDS 1998). It is not yet known if the proliferation of certain subtypes, such as A in west Africa, AE in Thailand and C in India and southern Africa, is due to selective advantages with respect to viral transmissibility or due to chance introduction and expansion in permissive social networks.

The multiple introduction of non-B subtypes into B restricted areas has been shown to occur and although there is not significant spread of these non-B subtypes within these countries yet, it is important to monitor their progress (see section 1.6.4.2). Regions such as south and south-east Asia as well as eastern Europe, have illustrated the possible extent of spread after introduction of single subtypes into populations with high risk behaviour, such as CSWs and IVDUs (1.6.4.1).

The biological and immunological characteristics of all subtypes must be studied because ultimately, vaccines will be needed that are effective again all HIV subtypes.

1.7) HIV vaccine development

1.7.1) Introduction

HIV vaccine development is beset with problems. HIV exhibits a high mutation rate, leading to a large degree of genetic variation. Serum antibodies from participants in the initial vaccine trials failed to neutralise "field" isolates, partly due to phenotypic differences between laboratory adapted, T-cell (SI, CXCR4) and primary (NSI, CCR5) isolates (Burton *et al.*, 1997). It is possible that initial vaccines may not induce sterilising immunity. Vaccines that decrease the viral load and thus reduce

transmission may be acceptable, especially in developing countries that have the highest rates of transmission (Haynes, 1996).

1.7.1.1) Correlates of immunity

Both a neutralising antibody response and a cytotoxic T lymphocyte response are known to be important components of the immune response to HIV. The neutralising antibody response binds free virus and prevents initial infection (Emini *et al.*, 1990) and the CTL response prevents the spread of infection by eliminating cell-associated virus (Borrow *et al.*, 1997 and Goulder *et al.*, 1997). However, despite infected individuals mounting both a humoral and cellular response to HIV, the virus persists.

Humoral response

There are a number of mechanisms enabling HIV to foil the efforts of the antibody response to eliminate the virus before it is internalised within the cells of the immune system. Although maintained at high levels during infection, anti-Env antibodies are often only weakly neutralising against primary isolates (Letvin, 1998). The exposed gp120 trimer on the mature virion is only weakly immunogenic, due to the release of gp120 monomers and unprocessed gp160 during infection. Antibodies are preferentially directed against CD4, V3, V4, C1 or C5 regions on this "viral debris", but are unable to bind to these regions on the mature oligomer, as they are poorly accessible. The B-cells then inhibit the production of antibodies to the mature oligomers in a process which has been named the "original antigenic sin" (Burton *et al.*, 1997 and Parren, 1997).

The crystal structure of the gp120 "core" from the laboratory strain HxBc2 bound to CD4 and 17b monoclonal antibody has recently been elucidated (Kwong *et al.*, 1998 and Wyatt *et al.*, 1998). This structure mimics the HIV-CCR5/CXCR4 co-receptor binding. This new evidence from the crystal structure has provided valuable insight as to how the virus evades the immune response.

The variable loops on the surface of the gp120, as well as heavy glycosylation (40-50%), bury and protect the conserved functional domains such as CD4 (shielded by the V1 and V2 loops) and the chemokine receptor binding site (shielded by the V2 and V3 loops) to which neutralising antibodies may be produced. The extensive glycosylation allows the regions covered to be seen as 'self' by the immune system and thus the face of the outer domain has also been termed the "silent face" of gp120 (Kwong *et al.*, 1998 and Wyatt *et al.*, 1998).

The conserved stem of the V1/V2 stem-loop and the fourth conserved region make up what are known as the CD4i (inducible) epitopes which are only exposed after CD4 binding. The gp120 is divided into two domains (inner and outer) and the inducible epitopes are part of the bridging sheet connecting the two domains of gp120. CD4 binding induces changes within the bridging sheet and between the two gp120 domains, allowing the bridging sheet to be exposed and revealing the co-receptor binding site, thereby facilitating co-receptor binding (see figure 1.8). It is thought that the

charge or conformation of the V3 loop may be the factor influencing whether CCR5 or CXCR4 co-receptors are bound (see section 1.4.2) (Moore and Binley, 1998).

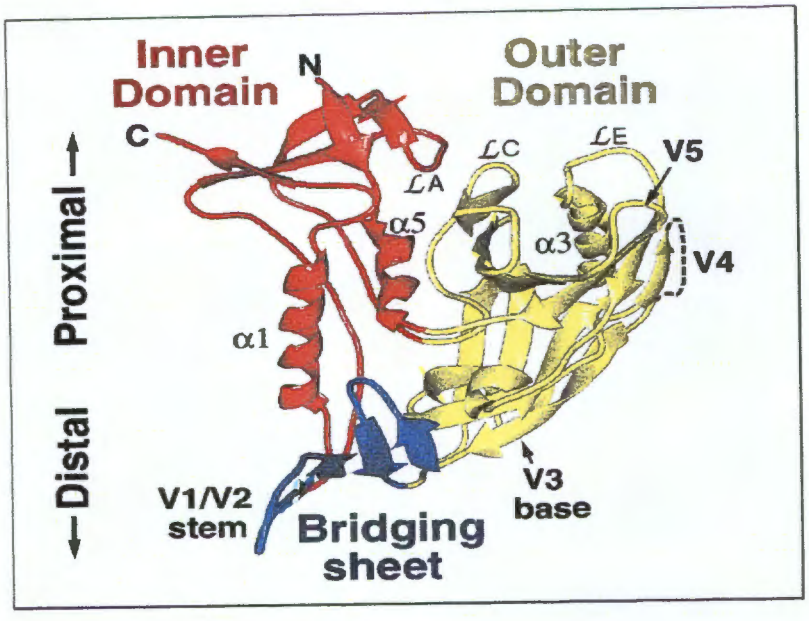


Figure 1.8: The X-ray crystal structure of the gp120 core which was derived from the HXBc2 strain of HIV-1, subtype B, in a ternary complex with two-domain CD4 and a neutralising antibody, shown from the perspective of the CD4 molecule (Wyatt *et al.*, 1998 and Kwong *et al.*, 1998).

Later in infection, more broadly reactive neutralising antibodies are produced, possibly due to the change in conformation of the T-cell adapted viruses, which have a more "open" conformation than the primary isolates, thus exposing neutralisation epitopes (Burton, 1997). Some of these antibodies, such as IgG1b12, are able to access the CD4 binding site, preventing gp120 and CD4 binding (Moore and Trkola, 1997) whilst other antibodies, which are more rarely found, can bind to the CD4i epitopes, preventing binding to the co-receptor. A monoclonal antibody which is able to bind to the outer domain, or "silent face" of the gp120, preventing gp120-CCR5 binding, is 2G12 (Muster *et al.*, 1993 and Moore and Trkola, 1997). This is unusual, since the antibody binds to the more conserved carbohydrate moieties on the silent face which are usually be seen as "self" (Wyatt and Sodroski, 1998).

Higher neutralising antibody titres (arising after one to four years) have been found in long term non-progressors (LTNP's) than in rapid progressors (Carotenuto *et al.*, 1998), although this is not true in all cases (Harrer *et al.*, 1996b). LTNP's with high CD4 levels and efficient T-cell function are preferentially able to produce neutralising antibodies against heterologous isolates. The ability to elicit neutralising antibodies may thus be related to the integrity of the immune system (Carotenuto *et al.*, 1998). The importance of neutralising antibodies has been illustrated by the fact that they have been found to protect against infection in babies born to HIV-infected mothers (Hengel *et al.*, 1998). Chimpanzees have been protected from HIV infection by monoclonal antibodies to the V3 loop, or

gp41; SCID mice were protected from HIV-1 infection by monoclonal antibodies to the CD4-binding domain and V3 loop and macaques infected with SIV had decreased morbidity and mortality when given immunoglobulin (Zolla-Parzner, 1997). It was recently demonstrated by Frankel *et al* (1998) that monoclonal antibodies 2G12, IgGb12 and 2F5 are able to prevent infection of mature DC/T-cell cultures after mucosal infection. CCR5, CXCR4 and dual tropic viruses were all blocked from infecting.

Poignard *et al* (1998), on the other hand, showed that hu-PBL-SCID mice infected with primary isolates of HIV-1 and then treated with IgG1b12 at concentrations higher than 90% *in vitro* neutralisation titres were not protected. Approximately 70% of the viruses rescued were escape mutants, thus antibodies to HIV-1 may play a limited role in the control of HIV-1 infection *in vivo*. A study where chimpanzees were protected against SHIV (a SIV/HIV chimera, replacing the SIV Env protein with HIV Env) infection by neutralising serum administered before challenge, but not after, indicates that neutralising antibodies may have a prophylactic role, but not a therapeutic one. This is due to the inability of neutralising antibodies to prevent cell-to-cell spread of the virus (Foresman *et al.*, 1998).

Cytotoxic T lymphocyte (CTL) response

After primary infection, the suppression of HIV replication is associated with a strong CD8+ CTL response. A broad CTL response against multiple epitopes leads to stable, low viral load and a delay in disease progression (Borrow *et al.*, 1997). The CTL response may be associated with protection from infection in exposed, uninfected individuals as well as in seronegative infants of seropositive mothers (Rowland-Jones *et al.*, 1993; Rowland-Jones *et al.*,1995; Cao *et al.*, 1997 and Letvin, 1998).

HLA (human leukocyte antigen) haplotype also plays a role in the CTL response to HIV. Since HIV is processed in the cytoplasm, there is a vigorous HLA-restricted MHC class-I CTL response to the virus. Since the HLA allele frequency varies between different populations (Hammond, 1997 and Sette and Sidney 1998) not all HIV peptides will elicit a strong CTL response in all populations. It is thus important to determine HLA allele frequencies for different populations, in order to determine which HIV epitopes in a vaccine will give the most effective CTL response for that population (Brander and Walker, 1995).

Certain regions of the HIV-1 genome have been identified as containing more broadly recognised CTL epitopes. For example, gp41 (position 589-598) contains epitopes presented by three different HLA-class I molecules, A24, B8 and B14 (Gnann *et al.*, 1987 and Hammond *et al.*, 1991); reverse transcriptase (RT) (position 476-485) contains epitopes restricted by HLA-A2 and -Bw62 (Johnson *et al.*, 1994) and the Nef protein contains two regions, the C-terminal end (position 183-196) which contains HLA-A2, A52 and B35 (Hadida *et al.*, 1995) as well as position 73-82 which contains epitopes restricted by HLA-A3, A11 and B35 (Johnson *et al.*, 1994; Brander and Walker,

1995). In addition, p24 contains multiple cross-reacting epitopes between different subtypes and different HLA haplotypes (Korber *et al.*, 1997).

In addition, a number of HLA-class I molecules which share similar peptide binding motifs have been identified and have been termed HLA-class I supertypes or superfamilies. There are four superfamilies which have been identified thus far; the A2-like family (del Guercio *et al.*, 1995), B7-like family (Sidney *et al.*, 1995), the B44-like family and the A3-like superfamily (Sidney *et al.*, 1996a and Sidney *et al.*, 1996b). These superfamilies may make the construction of vaccines which have broad CTL activity easier, since vaccines could contain a limited number of epitopes and due to the degenerate epitope-binding of these HLA families, a large portion of the population would still elicit CTL responses to the vaccine (Threlkeld *et al.*, 1997). Incorporating A2, A3 and B7 superfamily epitopes into a vaccine may allow a coverage of 83-89% of the population, regardless of ethnicity (Sette and Sidney, 1998).

1.7.1.2) Cross reaction between subtypes

HIV-1 is highly variable and the significance of genetic diversity on vaccine efficacy is not yet known.

Humoral response

Antibody cross-neutralisation between HIV subtypes does occur, but varies between isolates, and genotype does not correspond to neutralisation serotype (Moore and Trkola, 1997 and Nkengasong *et al.*, 1998). Certain isolates are more easily neutralised, whereas others are difficult to neutralise. In addition, certain human sera may also have broader neutralising capacity than others (Zolla-Parzner, 1997).

There are certain epitopes on the V3 loop of HIV-1 that share the same antigenic features between subtypes A, B and C. Five monoclonal antibodies (mAbs) to HIV-1 were selected using the V3 peptide of HIV_{SF} and mapped to three epitopes in the hypervariable, N-terminus side of the V3 loop. These mAbs had broad cross-reactivity, binding to four subtype A, nine subtype B and two subtype C laboratory adapted viruses, as well as four subtype B primary isolates. The antigenic relatedness was mapped to overlapping epitopes in the KSITKGP peptide region (Gorny *et al.*, 1997). A similar study showed that an epitope on the β region of the V3 loop was shared between subtype A, B and AE (Moore *et al.*, 1995) and mAb, 19b, can bind to a discontinuous epitope in V3, cross-reacting to subtypes A, B, C, AE and F. It is thus possible that vaccines will be able to elicit cross-subtype humoral immunity if the correct epitopes are presented (Gorny *et al.*, 1997).

There are also, however, broadly resistant viruses. Four subtype B viruses (three of which were isolated from paediatric patients) were recently identified which showed a high level of resistance to neutralisation by neutralising monoclonal antibodies, 2G12, 2F5 or IgG1b12. The same

isolates were also resistant to neutralisation by two sera found to be broadly neutralising against a diverse panel of primary isolates belonging to different subtypes. The resistance may be due to escape from the maternal antibodies by the infecting viruses in the infants. The development of vaccines to elicit neutralising antibodies may thus not be effective, due to the possible evolution of such broadly resistant viruses (Parren *et al.*, 1998).

A novel approach to the induction of neutralising antibodies was recently reported, however. In this study, "fusion-competent" (FC) HIV immunogens were generated by capturing the envelope-CD4-co-receptor structures which arise during HIV binding and fusion to the host cell. The FC immunogens were produced by the addition of 0.2% formaldehyde after five hours to a co-culture of COS-7 cells expressing the envelope of 168P (SI, CCR5 and CXCR4 primary subtype B virus) and human U87 glioma cells (expressing CXCR4 and CCR5). The formaldehyde cross-linked the envelope-CD4-co-receptor structures that are produced before syncytium formation between the cells could occur, producing an inactivated whole cell, FC vaccine. The FC vaccine appears to target the exposed, highly conserved regions of the HIV envelope during the binding process and prevent binding of the virus to the host cell. The neutralising antibodies were able to prevent infection by twenty-three out of twenty-four primary virus isolates from subtypes A to AE, as well as the homologous 168P subtype B virus, indicating that FC immunogens may be developed in order to achieve a broadly neutralising vaccine to the majority of subtypes (LaCasse *et al.*, 1999).

Cytotoxic T lymphocyte response

Cross-reactive cytotoxic T lymphocyte (CTL) responses have been demonstrated in a number of studies in both naturally infected individuals and in vaccinees.

Gag, Env and reverse transcriptase (RT) specific CTLs were studied from individuals infected with subtype B in the United States and from Senegalese patients, infected with subtypes A (n=10), G (n=3) or C (n=1). Eight of the nine subtype B CTL clones (recognising two p17, one p24, two RT and four Env epitopes) which were tested against all subtypes (A to O) were found to be cross-reactive at 100μg/ml peptide concentration. It was also determined that up to three nucleotide variations in the epitope can be tolerated. Fourteen Senegalese patients were found to have CTLs to at least one subtype B vaccinia virus construct expressing Gag, Env, RT, or Nef. The MHC class I restricted allele, A3 allowed broad cross-reactivity between subtypes in the p17 and Nef regions from the Senegalese patients (Cao, *et al.*, 1997). A second study was performed by Durali *et al* (1997), in which patients from France (subtype B) and Central African Republic (CAR) (subtype A and one subtype A/G recombinant) were studied for CTL cross-reactivity. It was found that there was frequent CTL cross reactivity with conserved epitopes between subtype A and B especially in the Gag p24, p18, the central region of Nef and in the integrase gene. CTL cross-reactivity was also demonstrated between subtype A, G and B when the A/G recombinant was studied (Durali *et al.*, 1998). A study examining the cross-subtype CTL recognition of subtype C infected Zambian patients

to autologous target cells infected with vaccinia virus (VV)-subtype B (IIIB) Gag/Env/Pol or Nef showed that out of eight patients, six elicited a CTL response to Gag/Env/Pol, but only three to Nef. Target cells infected with VV subtype B Gag, Pol or Env showed that one individual had CTL recognising only Gag and one recognising only Pol (Betts *et al.*, 1997).

The HLA background of the individuals to be vaccinated has also been studied. Out of 19 recently subtype B infected patients studied, CTL reactivity to subtype B $gp120_{LAI}$ and subtype C $gp160_{92BR025}$ was detected in 13. Nine patients also showed CTL cross-reactivity to subtype A $gp160_{90UG037}$. Two HLA class 1 B7 donors were able to elicit a CTL response to $gp160_{LAI}$, subtype C and A, but not to $gp120_{MN}$. In addition, five patients PBMCs could elicit cross-reactive CTL responses to subtype A Gag^{p55}, Pol and Nef (Wilson *et al.*, 1998). A recent study of the CTL responses to the Pol-RT, Env, Gag and Nef HIV-1 proteins from twelve Thai and nine North American HLA-characterised patients infected with subtype AE or B respectively, was also performed (Lynch *et al.*, 1998). The Thai and North American patients had different HLA profiles and although cross reactive CTL responses were fairly common, they were not uniform across the two groups. The most frequent CTL responses from both groups of patients were to the Gag protein. It is thus important to determine HLA alleles in different populations in order to prepare for global vaccine trials.

The first cross-reactive CTL responses elicited in vaccinated humans was after immunisation with canarypox-HIV vectors. Using ALVAC-$gp160_{MN}$ and $rgp120_{SF2}$ prime-boost protocol, or ALVAC vCP205 (containing the *env*, *gag* and *pol* from MN and LAI) alone, two different patterns of CTL reactivity were elicited dependant on the HLA haplotype of the volunteer. One volunteer could elicit broadly cross-reacting CTL response to primary isolates from subtypes A to F, but another, although eliciting a CTL response to the autologous T cell-adapted HIV_{LAI}, could not elicit CTLs to any primary isolates. Vaccination with vCP205 increased the breadth of the CTL response and greater number of primary isolates were able to be recognised by CTLs from the volunteers receiving vCP205, regardless of their HLA haplotype (Ferrari *et al.*, 1997).

The type of CTL response obtained after immunisation with a monovalent vaccine of $p55^{gag}$ was studied in HIV-infected patients from the United Kingdom (UK) and Uganda. Target cells were infected with recombinant VV expressing the $p55^{gag}$ protein from subtypes A, B, C and D. Five out of six Ugandan patients infected with diverse HIV-1 subtypes (which were not determined) had inducible CTL responses to subtypes A to D. Five out of seven UK patients were infected with subtype B and had CTL which recognised two or more of the four HIV subtypes (McAdam, *et al.*, 1998).

The results from these studies of naturally infected and vaccinated individuals indicate that genetic subtype may not play a role in the CTL response to either a vaccine, or to the virus. It would

appear that by selecting a vaccine strain with broadly recognised CTL epitopes, a number of populations with different HLA haplotypes could be protected against a number of HIV subtypes.

1.7.1.3) No absolute animal model

No ideal animal model has yet been identified. High levels of neutralising antibody have protected chimpanzees from challenge, although macaques with similar levels were infected. CTL responses have lead to protection in certain animal models but not in others (Johnston, 1997). In macaques SIV is used instead of HIV as the challenge virus. In an attempt to make SIV more similar to HIV, SIV/HIV chimeric viruses (SHIVs) have been developed. These chimera's have replaced the *env*, *rev* and *tat* genes of SIV with the corresponding genes from HIV-1. Challenge of macaques with SHIVs leads to persistent infection and development of disease (Bogers *et al.*, 1995; Cranage *et al.*, 1997; and Shibata *et al.*, 1997).

An additional problem is the variability in the results obtained from animal trials due to differences in the animal models and challenge systems used, as well as the type of adjuvant or antigen. The inoculation regime and timing and the dosage of the challenge has also varied, making interpretation of results difficult. These factors must all be standardised (Kuiken and Pillai, 1997).

1.7.1.4) Non-vaccine prevention of infection

In addition to vaccines, other factors contribute towards keeping the epidemic in check. The use of condoms for protection against STDs and HIV as well as the treatment of STDs will reduce the transmission of HIV (Laga, 1995; Figueroa, *et al.*, 1998 and Wawer *et al.*, 1998). In addition, improvement of the economic conditions and hygiene may provide for better nutrition and general health, thereby decreasing the spread of the epidemic. In developed countries, the treatment of HIV positive people with drugs to decrease viral load and thus the risk of transmission may also check the spread of HIV. In developing countries, the short-term treatment of pregnant women with anti-retroviral drugs may decrease the numbers of HIV-infected babies born (Marseille *et al.*, 1998).

Behavioural modification must also take place, such as changes in sexual behaviour (Robinson *et al.*, 1995; Ngugi *et al.*, 1996 and Jackson *et al.*, 1997) and the empowerment of women. Education of mothers to prevent transmission of HIV to their babies by breast feeding and general education of people to inform them about prevention of transmission are also important (Montagnier, 1996).

1.7.2) Vaccine candidates

The major types of vaccines thus far developed include: subunit vaccines, live vectors, whole virus (inactivated or attenuated), DNA vaccines or mixtures of the above. These vaccines have all been tested in non-human primates and many have also reached clinical trials in humans. Most give

CD4 T-cell proliferation and a CD8+ CTL response and many elicit neutralising antibodies to T-cell adapted strains, but it is rare to elicit neutralising antibodies to primary isolates.

1.7.2.1) Recombinant subunit vaccines

Initial HIV vaccines were envelope-based, as most neutralising epitopes are found in this protein (Letvin, 1998) (see Tables 1.1-1.4). Baculovirus-derived recombinant gp160 was used in early trials, since it is a relatively cheap way to manufacture large quantities of protein. After Phase I testing, it was found to elicit only weakly neutralising antibodies, in part due to an altered glycosylation of the baculovirus-derived gp160 that did not match the natural virus (Keefer *et al.*, 1994). Even mammalian-derived gp120 or gp160 from T-cell adapted viruses were of low immunogenicity and inefficient at eliciting cross-neutralisation even between virus strains of the same subtype (see section 1.7.1.1).

Although rare, rgp120 or rgp160 can elicit cross-neutralising antibodies and T-cell proliferative responses, however. Chuenchitra *et al* (1998) presented results from a Phase I trial in Bangkok, Thailand, after immunisation with a Chiron $rgp120_{SF2}$ (subtype B) vaccination. After one month the $gp120_{SF2}$ (subtype B) immunisation elicited a 90% T-cell proliferation with the homologous protein, whereas the heterologous $gp160_{Chiang\text{-}Mai}$ (subtype E) protein produced 53% proliferation. After 4 months the T-cell proliferative response had dropped to 85% for $gp120_{SF2}$ and 30% for $gp160_{Chiang\text{-}Mai}$. T-cell proliferation is measured as the percentage increase in the number of T-cells after peptide stimulation in comparison with a mitogen stimulant which primes all T-cells, such as phytohaemoglutinin (PHA).

A comparison of HIV_{IIIB} accidentally infected laboratory workers with $rgp120_{IIIB}$ or $rgp160_{IIIB}$ vaccinated individuals showed that the overall antibody response, especially neutralising response, was higher in the naturally infected people. Three differences were found between laboratory infected individuals and vaccinees: antibodies were to different V3 peptide conformations, there was a lack of an antibody response to the V1 peptide in vaccinees and an increase in antibodies to gp120, blocking CD4-gp120 binding in infected workers (Pincus *et al.*, 1997).

A number of vaccinees participating in phase I/II, $rgp120_{MN}$ subunit trials have become infected with breakthrough viruses. In a study by Berman *et al* (1997), seven out of a total of 499 volunteers participating in phase I/II trials of MN-rgp120 (recombinant gp120 subunit vaccine) became infected with breakthrough viruses. Analysis of the infecting viruses showed that there were differences in neutralising epitopes in the V2 and C4 (CD4-blocking domain) domains. Some infecting viruses had a greater number of glycosylation sites than the vaccinating MN strain, and were NSI phenotype, unlike the MN vaccine strain which is an SI virus (Berman *et al.* 1997). In a separate trial, eighteen individuals participating in phase I/II clinical trials for recombinant gp120 subunit vaccine constructed from SF-2 and MN became infected with HIV-1 during the trials. No neutralising antibody or CTL responses were elicited. Viral burden was comparable to unvaccinated

of viral replication by prior vaccination with the subunit. The majority of infecting viruses were found to be NSI, CCR5-utilising subtype B, although two isolates were SI, one of which utilised the CCR5, CCR2b, CCR3 and CXCR4 co-receptors. No selective pressure for a more pathogenic virus by immunisation with the subunit vaccine was thus found (Connor *et al.*, 1998). Similar results were shown in a study by Graham *et al* (1998) where twenty-three volunteers who became infected were selected from a total of 2099 volunteers involved in a number of phase I/II vaccine trials. Four of the twenty-three patients had received placebo's, and a further six of the volunteers had not completed their vaccination course before they became infected. The vaccinations included: recombinant proteins ($gp120_{MN}$ given with alum adjuvant, or with QS21 and alum, $gp120_{SF-2}$ in MF59 or MPL, $gp160_{MN}$ given with alum and deoxycholate) and live recombinant vaccinia virus vector expressing $gp160_{LAI}$, followed by a $rgp160_{LAI}$ boost. There was no difference in viral load or CD4 count between any of the infected vaccinees and non-vaccinated controls and no major differences between the V3 loop regions of the viruses infecting vaccinated and non-vaccinated patients. It was also established that although some of the isolate $HIV\text{-}1_{MN}$ vaccinated volunteers had elicited high neutralising antibodies *in vitro* at the time of infection, there were no differences in the V3 loop regions of their infecting viruses that would have indicated immune escape from the vaccine elicited antibodies. None of the vaccine formulations gave significant CTL responses, even the live VV recombinant vaccine (Graham *et al.*, 1998).

Oligomeric Env is more similar to the native Env protein than monomeric Env, as the oligomeric form expresses functional epitopes that can induce protection. Thus, current work now focuses on the use of oligomeric gp120 from both primary and T-cell adapted viruses, in an attempt to elicit stronger neutralising antibodies (Petry *et al.*, 1998) (see Table 1.1).

Apart from the conformation of the immunogen; the route of administration, number of immunisations and the adjuvant used all have an effect on the potency of the neutralising antibody response (VanCott *et al.*, 1997). In rare cases, even a CTL response may be induced using a subunit vaccine with appropriate epitopes in conjunction with adjuvant formulations favouring a Th1-type response (see Table 1.1).

1.7.2.2) Peptide vaccines

It is possible that peptide or epitope vaccines may be more successful than proteins, especially if delivered in efficient carrier molecules, or with immune-enhancing adjuvants. These peptides may represent specific epitopes, targeted to be cross-neutralising, or to elicit a CTL response.

More research must be undertaken to identify efficient epitopes that may be included in these peptide vaccines. There are currently a number of Phase 1 trials in progress for peptide vaccines in different formulations (Jordan report, 1998) (see Table 1.1). Recombinant protein

vaccines are likely to be most efficient when used in a "prime-boost" approach in combination with a vaccine eliciting a strong CTL response, such as live recombinant vectors or a DNA vaccine. This type of vaccination schedule involves an initial immunisation designated the prime, eliciting a weak, transient, or incomplete immune response and the subsequent vaccinations are termed boosts, increasing the duration of protection, the breadth of the immune response (humoral and cellular) as well as the strength of the immune response.

Table 1.1: Table of subunit and peptide HIV-1 vaccine candidates in development and clinical trial to date (modified from the Jordan Report, 1998).

Vaccine candidate	Expression system/ Production method	Adjuvant or delivery system	Stage of development/Results	Ref
Subunits				
rgp160 LAI	Baculovirus/insect cell	alum	PhI (P), PhI, II(T)	1, 2
rgp160 IIIB or MN	Vaccinia/monkey kidney cell	Alum, DOC	PhI (P), PhI, II(T)	3, 4
rgp160 MN/LAI	Vaccinia/mammalian cell	Alum or IFA	PhI (P), PhI (T)	5, 6, 7
rgp160 LAI	Vaccinia/mammalian cell	Oil, water, 3-deacyl monophosperyl Lipid A	Preclinical, chimpanzees	8
Oligomeric rgp160 LAI	Baculovirus/insect cell	Alum, MPL,IFA	Preclinical, small animals, monkeys	9, 10
Oligomeric rgp160 IIIB, 451	Mammalian cell	-	Preclinical, elicited neutralising antibodies to a number of primary isolates in small animals	11
rgp120 (Env 2-3) SF2	Yeast	MF59±MTP-PE	PhI (P), PhI (T)	12
rgp120 SF2	CHO cells	MF59±MTP-PE	PhI,II (P), PhI (T)	13
rgp120 IIIB	CHO cells	alum	PhI (P), PhI (T)	14, 15
rgp120 MN	CHO cells	Alum or QS21 or both	PhI, II (P), PhI, II (T)	14, 16
rgp120 W61D (primary, dual tropic)	-	Alum, SBAS-1 (QS21 and MPL-A) or SBAS-2 (QS21, MPL-A and an emulsion)	Preclinical, elicited CTL, T-cell proliferative and antibody responses in macaques, mice and chimpanzees	17
rgp120 W61D (primary, dual tropic)	-	SBAS-2	PhI (P), elicited antibody and cross-neutralising responses, not CD8 CTL	18
rgp120 coupled to *N*-linked carbohydrate	Vaccinia	*N*-linked carbohydrate to reduce the net positive charge	Preclinical, broad neutralisation response to usually non-neutralising V1 domain epitope, antibody elicited to C1 and C5 regions in guinea pigs.	19
rp24 LAI	Baculovirus/insect cell	alum	PhI (P), PhI, II (T)	20
RT-VCG LAI	*E. coli* / *Vibrio cholerae*	*V. cholerae* ghosts	Preclinical, small animals	21

Table 1.1: continued...

Vaccine candidate	Expression system/ Production method	Adjuvant or delivery system	Stage of development/Results	Ref
Peptides				
V3 peptide MN	Synthetic peptide	Alum or IFA	PhI (P)	22
V3-MAPS MN	Synthetic peptide	Alum, microparticulate	PhI (P)	23, 24
V3-PPD MN	V3 peptide coupled to PPD		PhI (P)	25
V3 MN - Toxin A	V3 peptide coupled to Pseudomonas aeruginosa toxin A	None	PhI (P)	26
V3 crown and 2F5 epitope coupled to MHC class II specific antibody	Synthetic chimeric peptide	Coupled to FR3-H region of the Tib92 antibody - presented on APC's	Preclinical, high titre anti-HIV gp160 antibodies elicited in guinea pigs	27
V3 (T helper epitope (PCLUS 3-18MN, PCLUS 6-18MN)	Synthetic chimeric peptides	IFA	PhI (T)	28, 29
C4-V3 peptides MN, RF, CANO, EV91	Synthetic chimeric peptides	IFA	PhI (P), PhI (T)	30
V3 MN -HA	Recombinant baculovirus	Influenza virus haemagglutinin	Preclinical, elicited neutralising antibodies against homologous HIV strain in small animals	31
HBVsAg-gp41 LAI Katinger epitope	Recombinant *Pichia pastoris*	HBVsAg	Preclinical, small animals	32
V3-CD4, gag peptide (VC1) IIIB, Thai A, Thai B, LAI	Synthetic chimeric	CFA	Preclinical, small animals	33
HGP-30 SF2	Synthetic p17 peptide	alum	Preclinical, elicited cross-reacting serum in mice to subtype B, C, A and E. Elicited similar response in humans, Ph1(P).	34
lipopeptide tail to C-terminal V3 peptide	Synthetic V3 peptide	hexadecanoic acid tail	Preclinical, elicited CTL response in small animals	35
MPL tail to V3 peptide, ENV302-336	Synthetic V3 peptide	Montanide ISA51, Nε-palmitoyllysine (MPL) tail	Preclinical, elicited a CTL response maintained up to eight months in mice	36
gag-lipopeptide LAI	Synthetic peptide	lipopeptide	PhI (P)	37
p24-hsp70 fusion LAI	Recombinant protein	*M. tuberculosis* heat shock protein (hsp) 70	Preclinical, small animals	38
p24 23mer peptide Conserved across clades A-G	Synthetic peptide	CFA	Preclinical, small animals	39

1, Keefer *et al.*, 1994; 2, Valentine *et al.*, 1996; 3, Belshe *et al.*, 1993; 4, Gorse *et al.*, 1994; 5, Pialoux *et al.*, 1995; 6, Fleury *et al.*, 1996; 7, Achour *et al.*, 1996; 8, Bruck et al., 1994; 9, Broder *et al.*, 1994; 10, Richardson *et al.*, 1996; 11, VanCott *et al.*, 1997; 12, Keefer *et al.*, 1996; 13, Kahn *et al.*, 1994; 14, Berman *et al.*, 1994; 15, Lasky *et al.*, 1986; 16, Belshe *et al.*, 1994; 17, Mooij *et al.*, 1998; 18, Weber Cent Gardes pp287-292, 1997; 19, Garrity *et al.*, 1997; 20, Zunich *et al.*, 1992; 21, Eko *et al.*, 1994; 22, Salmon-Ceron *et al.*, 1995; 23, Gorse *et al.*, 1996; 24, Kelleher *et al.*, 1997; 25, Rubinstein *et al.*, 1995; 26, Cryz *et al.*, 1995; 27, Cook and Barber, 1997; 28, Ahlers *et al.*, 1996; 29, Ahlers *et al.*, 1997; 30, Staats *et al.*, 1996; 31, Kalyan *et al.*, 1994; 32, Eckhart *et al.*, 1996; 33, Bukawa *et al.*, 1995; 34, Zimmerman *et al.*,1998; 35, Deprez *et al.*, 1996; 36, Sauzet *et al.*, 1998; 37, Nixon *et al.*, 1996; 38, Suzue *et al.*, 1996; 39, Nakamura *et al.*, 1997.

1.7.2.3) Live recombinant vectors

Genes from HIV can be engineered into the genome of viruses or bacteria, in order for the proteins to be expressed. The live vectors will allow processing of the antigens and presentation via the MHC class 1 pathway thereby eliciting a CTL response. There are limitations to the technique, in that the recombinant organism must be non-pathogenic, stabley express the foreign genes and should be able to replicate at least a limited number of times in order to stimulate an adequate immune response. Unfortunately, the greater the capacity to replicate, the more pathogenic the vector tends to be (Letvin, 1998).

Pox virus vectors

One of the most common viral vectors used as delivery vehicles for HIV proteins are pox viruses. The initial vectors were constructed from vaccinia virus (VV), the live, attenuated smallpox vaccine, expressing either the gp120 or gp160 (Katz and Moss, 1997 and Letvin, 1998). Although VV recombinants have elicited efficient CTL responses (Tartaglia, 1997), there are safety concerns for the use of VV in potentially immunocompromised individuals, thus attenuated strains such as NYVAC and ALVAC have been designed for vaccine delivery. NYVAC virus strain, derived from the Copenhagen VV had the open reading frames implicated in virulence removed, attenuating the virus; whereas ALVAC is derived from Kanapox, a canarypox virus and can only replicate in avian tissues. In addition, an attenuated vaccinia strain, Ankara MVA (modified Ankara virus) was derived from the serial passage of Ankara VV through chick fibroblasts 500 times, thereby changing the host range of the virus to avian origin only (Tartaglia, 1997). Of all the poxvirus vectors, the canarypox recombinant has progressed the furthest in clinical trials thus far (Jordan Report, 1998).

Alternative live recombinant vectors for HIV vaccination

A number of viral and bacterial vectors have also been researched as possible HIV vaccines (see Table 1.2). These vectors are often studied in an attempt to find a vaccine capable of eliciting a mucosal response, for example, BCG and Adenovirus (see section 1.7.2.7).

Table 1.2: Vaccine candidates and the immune response elicited in preclinical and phase 1 and 2 trials for recombinant poxvirus vaccines (modified from the Jordan Report, 1998).

Vaccine candidate	Expression system/ Production method	Stage of development	Results	Ref
VV gp120 IIIB	gp120 / gpB5R cytoplasmic transmembrane VV chimera	Preclinical in mice and rabbits	Immunogenisity improved by greater expression of gp120 on the VV surface Increased anti-HIV antibody titre	1
ALVAC gp160 SF2	Fowlpox and canarypox - HIV	Preclinical in small animals	Gp160 correctly expressed on the virus surface, T-cell proliferation and antibody response, No cross-neutralisation of heterologous strains	2,3
vCP125 gp160	ALVAC-HIV	Ph 1(P)	Generates an efficient CTL response and cross neutralising response in a 'prime-boost' protocol	4
vCP205 gp120TM+ gag/pro	ALVAC-HIV	Ph 1,2(P)	Elicits a broader CTL response with additional Gag and protease proteins	4

Table 1.2: continued...

Vaccine candidate	Expression system/ Production method	Stage of development	Results	Ref
vCP300	vCP250 + separate Pol-Nef CTL epitopes	Ph 1(P)	Elicits a broader CTL response but decreased replication efficiency in chick embryo fibroblasts	4
vCP1433	vCP300 Pol and nef engineered into a single cassette resulting in chimeric (string-of-beads) Pol-Nef protein	Ph 1(P)	Improved replicative capasity to vCP300	4
vCP1452	E3L + VVK3L leads to enhanced VV gene expression	Ph 1(P)	Enhanced expression in human cell lines	4
VV gp160 LAI	VV-HIV	Ph1(P)	CTL response and neutralising antibodies	5, 6
VV gp160 LAI	VV-HIV	Ph1(P), Ph1(T)	CTL response and neutralising antibodies	7
VV *env, gag, pol* LAI (TBC-3B)	VV-HIV	Ph1(P)	CTL response and neutralising antibodies	8
VV *env, gag, pol* NYVAC LAI, MN	VV-HIV	Preclinical, small animals	CTL response and neutralising antibodies	9, 10
Modified VV Ankara (MVA) SIV *env, gag, pol*	Attenuated VV-SIV	Preclinical, monkeys	CTL response and neutralising antibodies	11
VV gp140, 30 isolates	VV-HIV	Preclinical, small animals, Ph1(P)	CTL response and neutralising antibodies	12

1, Katz and Moss, 1997; 2, Radaelli *et al.*, 1994a; 3, Radaelli *et al.*, 1994b; 4, Tartaglia, 1997; 5, Graham *et al.*, 1992; 6, Graham *et al.*, 1993; 7, Achour *et al.*, 1996; 8 Daniel *et al.*, 1994; 9, Abimiku *et al.*, 1995; 10, Paoletti, 1996; 11, Moss *et al.*, 1995; 12, Rencher *et al.*, 1997.

Table 1.3: Vaccine candidates and the immune response elicited in preclinical trials for live recombinant viral vaccines (Modified from the Jordan Report, 1998).

Vaccine candidate	Expression system/ Production method	Stage of development	Results	Ref
Semliki Forest Virus (SFV) HIV IIIB gp160	Recombinant SFV	Preclinical, monkeys	Strong cellular and humoral immune response, protects against homologous SIV challenge	1
Venezuelan equine encephalitis (VEE)-HIV MA/CA,	Recombinant VEE	Preclinical, small animals	Anti-Gag IgG elicited and IgA antibodies in vaginal washes. Strong CD8+ CTL in mice	2
Vesicular Stomatitis Virus (VSV) HIV CD4 receptor and CXCR4 co-receptor	VSV glycoprotein is replaced with HIV receptor genes in order to target and kill HIV-infected cells	BR&D (T)	HIV titres in an HIV-infected T-cell line was decreased by 10^4	3, 4
Rabies Virus HIV CD4 and CXCR4 receptors	Rabies G protein is replaced with HIV receptor genes to target and kill HIV-infected cells	BR&D (T)	-	5
Poliovirus-HIV *env* LAI	Recombinant Poliovirus	BR&D	Neutralising antibodies	6

1, Berglund *et al.*, 1997; 2, Caley, 1997; 3, Schnell *et al.*, 1997; 4, Johnson *et al.*, 1997; 5, Mebatsion *et al.*, 1997; 6, Evans *et al.*, 1989.

1.7.2.4) Whole, inactivated viruses

Whole, inactivated vaccines have been successfully used as poliomyelitis and influenza vaccines and have elicited long-lasting protection, thus were initially attractive as an HIV vaccine despite possible safety issues (Letvin, 1998). A number of HIV or SIV variants have been inactivated to form different vaccine formulations for different subtypes. SIV, inactivated by either formalin or β-propiolactone, has been shown to protect against both intrarectal and intravenous challenge with

either homologous or heterologous virus (Stott *et al.*, 1990; Cranage *et al.*, 1992). In addtion, digitonin was used as an adjuvant for the β-propiolactone, binary ethylenamine and formaldehyde inactivated-HIV immunisation. Immunisation of rats, guinea pigs and rabbits elicited neutralising antibodies to both homologous and heterologous virus, as well as T-cell proliferation and a CTL response (Race *et al.*, 1995).

An inactivated vaccine which has been shown enhance the immune response in HIV-infected patients is REMUNE™. REMUNE™ is constructed from an early HIV-1 isolate, *gag*G/*env*A from Zaire (HZ321), with the gp120 removed. The vaccine is completely inactivated by β-propiolactone and γ-radiation, and the protein conformation is retained. The vaccine has been administered in Incomplete Freunds adjuvant (IFA) and in phase II trials has been shown to elicit a broad lymphoproliferative response to different HIV subtypes. Subtypes B, E, Gag G peptides have been recognised after immunisation, due to the conserved immunodominant *gag* gene of REMUNE™.

It has also been suggested that REMUNE™ may be used to restimulate the immune response in HIV-infected patients who have undergone highly active antiretroviral therapy (HAART) which lowers the viral load and may preserve the immune functions of the patient. In addition, REMUNE™ stimulated the production of β-chemokines, RANTES, MIP-1α and MIP-1β that are indicative of a Th1 response. However, levels of proinflammatory cytokines such as TNFα, which at high levels lead to a decrease in CD4+ cells, were found to decrease (Moss *et al.*, 1998).

The novel fusion-competent whole cell vaccine strategy (LaCasse *et al.*, 1999) has been discussed in section 1.7.1.2.

1.7.2.5) Virus-like particles as vaccines

Virus-like particles (VLP's) as vaccines were considered as an alternative, in an attempt to elicit a broader immune response than with subunit vaccines, but without the safety issues of whole-killed vaccines. Most of the HIV VLP's utilise core proteins, such as p24 or the $Pr55^{Gag}$ precursor. The p24 C-terminal contains a CTL epitope which had the highest CTL precursor frequency in an asymptomatic patient studied and it is the most conserved region amongst all retroviruses (Harrer *et al.*, 1996a). Also, escape variants to this epitope have not been found.

Table 1.4: Vaccine candidates and the immune response elicited in preclinical and phase 1 trials for virus like particles (VLP's) as vaccine vectors (modified from the Jordan Report, 1998).

Vaccine candidate	Expression system/ Production method	Adjuvant or delivery system	Stage of development	Results	Ref
P17/p24 LAI Ty-VLP	P17/p24 and yeast transposon product	Alum/none	Ph1(P), Ph1,2(T)	*Elicited anti-p17 or p24 T-cell proliferation above baseline, 2/8 patients had enhanced CTL reponses against Gag.	1, 2, 3
HIV-1 LAI, RF, others *env, gag, pro* pseudovirions	MoMLV/mammalian cells	CFA, IFA	Preclinical, small animals, monkeys	Antibody, CTL response	4
HIV-1 LAI *env, gag, pol* pseudovirions	Vaccinia/mammalian cells	Alum	Preclinical, small animals, monkeys	Antibody, CTL response	5
Gag-V3 LAI VLP	Baculovirus/insect cells		Preclinical, small animals	Antibody, CTL response	6, 7
Pr55-^{Gag} gp160 or oligomeric gp120	Baculovirus/insect cells	none	Preclinical, small animals	Neutralising antibody, CTL response	8, 9
HIV LAI VLP without RNA, containing MN V3 loop in C2 region	Chimeric HIV LAI/MN in mammalian cells	none	Preclinical, small animals	Neutralising antibodies recognised both MN and LAI V3 loop epitopes	10
HbcAg-V3 particles LAI	E. coli	-	Preclinical, small animals	Antibody, CTL response	11
Tobacco mosaic virus (TMV)/alfalfa mosaic virus (AlMV) chimera, V3 loop MN	Transgenic plants, V3 loop expressed in AlMV coat protein	IFA, CFA	Preclinical, small animals	Neutralising antibody response in mice. Possible mucosal immune response if edible vaccine is used	12

*Two of the eight patients had increased viral RNA (at greater levels than the unvaccinated control patients) similar to the activation phenomenon, indicating that vaccination with the VLP may be hazardous.
1, Weber *et al.*, 1995; 2, Veenstra *et al.*, 1996; 3, Klein *et al.*, 1997; 4, Rovinski *et al.*, 1995; 5, Daniel *et al.*, 1994; 6, Truong *et al.*, 1996; 7, Brand *et al.*, 1995; 8, Deml *et al.*, 1997; 9, Tobin *et al.*, 1997; 10, Rovinski *et al.*, 1992; 11, Grene *et al.*, 1997; 12, Yusibov *et al.*, 1997.

1.7.2.6) Live attenuated vaccines

The *nef*-attenuated vaccines were initiated after the discovery of a cohort of people infected by blood transfusion in Sydney, Australia who were infected with a relatively non-pathogenic Δ*nef* HIV-1 virus which rendered the patients long term non-progressors (LTNPs) (Deacon *et al.*, 1995). Follow-up on the cohort has shown increasing *nef* deletions in the evolving progeny viruses which leads to decreasing replication efficiency in comparison to wild-type viruses (Rhodes *et al.*, 1998). *Nef* is necessary for HIV to replicate efficiently and, along with *vpu*, has been linked to pathogencity of the virus (see section 1.2). Both proteins down-regulate the surface expression of MHC class I molecules, thereby reducing the CD8+ mediated CTL response and in addition, Nef upregulates the expression of the Fas-ligand (FasL) thereby increasing the binding to Fas on activated CTL's and initiating apoptosis. *Nef* deletions therefore abrogate these effects and Δ*nef* isolates are less pathogenic (Xu *et al.*, 1997).

Vaccines from live virus which has been genetically altered in order to attenuate the pathogenicity have successfully been used against polio, chicken pox and measles. The immune

response obtained is similar and as potent as the immune response to the wild type virus (Letvin, 1998).

Initial studies in macaques with a nef-deleted SIV_{mac239} were encouraging. Attenuated $SIV_{mac239\Delta nef}$ was not pathogenic after immunisation and protected macaques from subsequent intravenous challenge with pathogenic SIV_{mac239} (Daniel *et al.*, 1992). Also, immunisation of macaques with Δnef SIV_{macC8} or non-pathogenic SHIV89.6 (a SIV chimera containing the HIV-1 89.6 *env* gene) provided protection against rectal (Cranage *et al.*, 1997) and vaginal (Miller *et al.*, 1997) challenge with pathogenic SHIV/HXB2 or SIV_{mac239} respectively. Protection was associated with a CTL response and cervicovaginal or rectal secretions, although not neutralising antibodies. Other studies have also shown a lack of protection by neutralising antibodies, including Shibata *et al* (1997) where no neutralising antibodies were elicited by immunisation or after challenge. Almond *et al* (1997) showed that transfer of immune serum from protected macaques did not confer protection to pathogenic SIV_{macJ5} in naïve macaques (Almond *et al.*, 1997). In addition, Δ*nef* SIV vaccines in macaques with wild-type SIV or SHIV intravenous, cell-free, or cell-associated challenge provided protection, or decreased viral infectivity substantially in a mechanism which was CD8+ CTL mediated (Johnson *et al.*, 1997 and Gaudin *et al.*, 1998).

Live attenuated HIV vaccines pose major safety concerns and as a result no live attenuated vaccines have reached clinical trials in humans (Letvin, 1998). HIV mutates rapidly and it is possible that the attenuated virus may regain its pathogenic potential, such as SIV_{macC8}, (Whatmore *et al.*, 1995). In addition, the possible long-term pathogenic effects make it difficult to perform safety trials. Δnef strains with further deletions, such as *vpr*, *vpx* and portions of the LTR (Desrosiers *et al.*, 1998) have been developed in an attempt to prevent the reversion of the live attenuated vaccine to a pathogenic strain. If too many genes are removed, however, the vaccine virus will lose its ability to replicate and at the same time its immunogenic potential. No protection from intravenous challenge by pathogenic strains have been elicited to these highly attenuated strains. In addition, a recent study by Baba *et al.* (1999) and earlier work by Baba *et al.* (1995) and Ruprecht *et al.* (1996) showed that although adult monkeys are protected from pathogenic challenge after nef-deletant immunisation, long term infection may eventually lead to disease. Also, neonatal monkeys immunised with nef-deletant strains were infected and became ill which lead to death in a number of cases.

1.7.2.7) Mucosal immunity

Since over 80% of HIV infections occur via heterosexual transmission, a vaccine to elicit mucosal immunity is essential (UNAIDS and WHO, 1998) (see Table 1.5).

Table 1.5: Vaccine candidates tested in preclinical trials using small animals, monkeys or chimpanzees for the activation of a mucosal immune response (modified from the Jordan Report, 1998).

Vaccine candidate	Expression system/ Production method	Adjuvant or delivery system	Results	Ref
Subunit Vaccines				
SIV_{mac251} rgp120, rp27, Pr55 and SIV_{mac239} gp130	Immunogen injection into the posterior wall of the pelvis , drained by the iliac lymph nodes (TILN).	Alum	Protection elicited after rectal but not vaginal challenge with SIV_{mac239} in macaques, although high IgG and lower IgA levels were produced	1
Peptide Vaccines				
gp120 C4/V3 regions MN	Synthetic peptide containing immunogenic epitopes for antibody, T-cell proliferation and CTL responses	I.n., CT, boosted twice via rectum, gastric intubation, or vagina	Elicited anti-HIV specific vaginal IgA and IgG response, serum IgG, neutralising antibodies to homologous strain and T-cell proliferative responses in mice	2
Synthetic multideterminant peptide from IIIB	Synthetic peptide	i.r., i.n., i.g. or i.d. with or without CT adjuvant	Th1 type, IL12 and IFN-γ needed for CTL response. Mice challenged with VV-$gp160_{IIIB}$ recombinant intrarectally cleared the virus from their ovaries. Faecal IgG and IgA and serum IgG detected	3
macromolecular peptide V3	Synthetic peptide containing 4 V3 peptides from different subtypes, 1 CD4 binding domain and 1 *gag* region	CT, oral route	Elicited high titre gut-associated sIgA able to neutralise three heterologous HIV-1 strains (IIIB, SF-2 and MN) in mice. High serum IgG antibodies and faecal sIgA detected.	4
VLP Vaccine				
$Pr55^{Gag}$ SIV	Baculovirus/insect cells	CT, oral route	Elicited systemic and mucosal IgG and IgA in rectal washes and saliva, but IgG only in vaginal wash*. T-cell proliferative Th1 response also obtained	5
Live Recombinant Vaccines				
Adenovirus *env/gag*- IIIB	Recombinant adenovirus	Intranasal	Nasal, vaginal, salivary and rectal IgG detected with low IgA levels. Short-lived neutralising antibodies stimulated by baculovirus rgp160 boost in chimps	6
Adenovirus gp160 MN rgp120 SF2	Recombinant adenovirus, CHO cells	Intranasal, recombinant protein boost	Chimps protected from low dose HIV_{SF-2} challenge and 1 year later from high dose challenge. Neutralising antibody response elicited against primary and T-cell isolates after low dose challenge	7
VV-gp160, then rgp160 IIIB subunit protein.	VV/monkey kidney cells	CT, intragastric or intranasal with subunit boost	Elicited IgA, long lasting systemic CTL response and weak T-cell proliferation, improved by boost in mice	8
Salmonella-HIV V3 LAI	Recombinant attenuated *Salmonella typhimurium aroA* strain	oral	CTL response and mucosal IgA	9
Salmonella-SIV p27	*Salmonella typhimurium aroA* mutant	oral	CTL response and mucosal IgA	10
M. bovis bacillus Calmette-Guérin (BCG) Gp110 SIVmac251	Recombinant BCG	oral	Strong CTL response, IgG neutralised homologous SIV *in vitro*, high levels of IgA to gp110 detected in faeces. IgA levels higher after oral than subcutaneous immunisation	11
BCG-HIV *env* LAI	Recombinant BCG	oral	CTL response, mucosal IgA	12
BCG-SIV *nef*	Recombinant BCG	oral	Elicited anti-Nef T cell proliferative response and a strong systemic and mucosal CTL response in mice	13
BCG-SIV Nef, Env and Gag	Recombinant BCG	different mucosal routes	Elicited antibody and mucosal CTL response in mice	14
BCG-HIV V3 Japanese consensus	Recombinant BCG	oral	CTL response, mucosal IgA	15

*Lack of IgA in the vaginal washes may have been due to the hormonal cycle of the female macaques, which can effect secretory IgA production in the genital tract. The low IgA production in the macaques mirrors the secretory antibody response in HIV-infected women.

1, Lü *et al.*, 1998; 2, Staats *et al.*, 1997; 3, Belyakov *et al.*, 1998; 4, Bukawa *et al.*, 1995; 5, Kubota *et al.*, 1997; 6, Lubeck *et al.*, 1994; 7, Lubeck *et al.*, 1997; 8, Brühl *et al.*, 1998; 9, Charbit *et al.*, 1993; 10, Valentine *et al.*, 1996; 11, Lim *et al.*, 1997; 12, Yasutomi *et al.*, 1993; 13, Lagranderie *et al.*, 1997; 14, Lagranderie *et al.*, 1998; 15, Honda *et al.*, 1995.

1.8) DNA vaccination

1.8.1). Introduction

Naked DNA vaccines are constructed by cloning immunogenic DNA sequences into eukaryotic expression vectors, which are amplified in bacteria and purified (Whalen, 1997). DNA vaccines can be used against a wide variety of bacterial, viral and parasitic pathogens and give long lasting, broadly reactive responses. Human immunodeficiency virus (HIV), Hepatitus B virus (HBV) and malaria DNA vaccines are in phase I trials (DNA vaccine web, 1998; MacGregor *et al.*, 1998; Ugen *et al.*, 1998; Wang *et al.*, 1998 and Boyer *et al.*, 1999).

Usually DNA is introduced into the skin, muscle or intravenously and introduction can be by injection in saline, or with various adjuvants, or by gene gun bombardment with the DNA attached to gold particles. Expression of the DNA is under the control of an eukaryotic promotor, allowing expression in the hosts' transfected cells (see Chapter 4.2.4). Both a CTL response and protective immunity is elicited by the small amounts (nanograms) of proteins which are expressed. A long lasting antibody response can also be stimulated (Robinson, 1997a).

1.8.2) Type of immune response to DNA vaccines

DNA injected intramuscularly is expressed in skeletal muscle and when introduced into the skin, is expressed by keratinocytes. These cells may then present the antigen to the immune system. The intramuscular route elicits more of a Th1 type response, whereas the gene gun inoculation elicits a Th2 response. The antigen presenting cells (APCs) at the two sites are significantly different, but were both found to be derived from bone marrow. These bone marrow-derived cells either obtain the antigen presented by the muscle cells and keratinocytes, or they are transfected directly by DNA at the inoculation site or as the DNA moves through the blood or lymph (Robinson, 1997a and Iwasaki *et al.*, 1997).

Naïve antigen-specific T and B lymphocytes are converted to memory and effector cells in the lymphoid tissue and germinal centers develop where immature B-cells mature to high affinity B-cells and antigen-antibody complexes are deposited on follicular dendritic cells. Effector B lymphocytes then localise in the bone marrow and produce long term antibody, whilst the memory B and T lymphocytes circulate, maintaining the immune response. Lymphokines such as gamma-interferon (IFN-γ) and interleukins (ILs) are also stimulated by T lymphocytes and these support the immune response. Intramuscular injection of DNA elicits a Th1 response with the production of IFN-γ, IL2 and IL12. The Th1 response is complement-dependant and phagocytic, recruiting and activating macrophages and monocytes. Bombardment of the DNA vaccine attached to gold particles uses much less DNA and tends to elicit a Th2 response, where granulocyte-monocyte

colony stimulating factor (GM-CSF) and IL 4, 5 and 10 are produced. The response is complement-independent and recruits eosinophils and mast cells which secrete toxins to counter pathogens (see figure 1.9) (Robinson, 1997a).

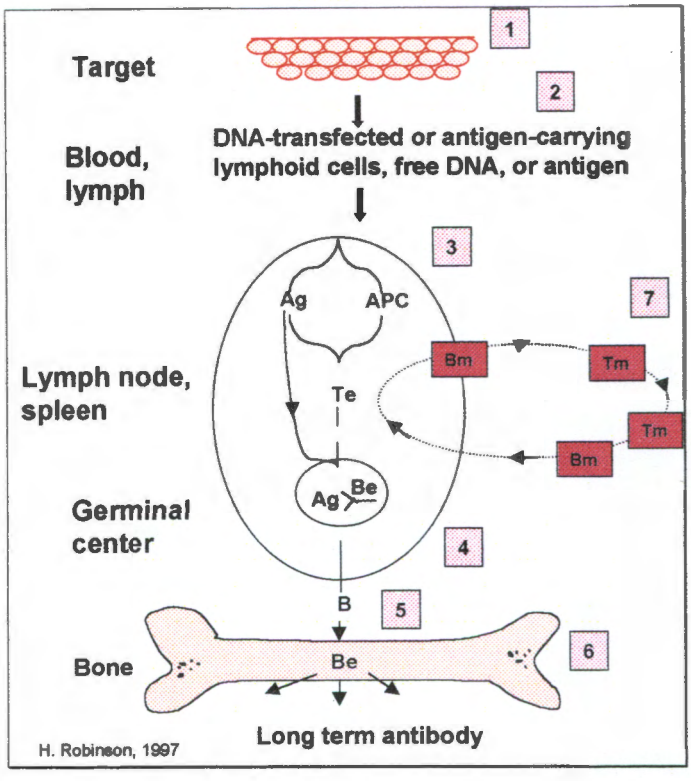


Figure 1.9: An illustration of the mechanism whereby DNA immunisation elicits an immune response. The DNA is initially taken up by the epithelial or muscle cells, in which the immunogen is expressed. The expressed antigen then travels through the blood or lymph to the lymph node or spleen where T- and B-cells are activated (Robinson, 1997a).

1.8.3) Factors influencing the the immune response

A number of factors influence the effectiveness and the type of the immune response to DNA vaccines:

- the inoculation method and the DNA concentration,
- co-factors and adjuvants used,
- the type of host animal (genotype and age) and
- the type of antigen (toxicity, expression level and intracellular biology).

The gene gun gives a better Th2 response with more DNA expressed. It has been found that decreasing the amount of DNA administered elicits a better CTL response, whereas increasing the amount elicits a better antibody response. Intramuscular and intradermal immunisation uses

about 100-fold more DNA than the gene gun, but not as much reaches the cells due to extra-cellular nuclease digestion, thus less DNA is expressed and a better CTL response is obtained (Robinson, 1997a). It is also possible that the large amount of bacterial vector DNA may act as an adjuvant, leading to a Th1 response (Whalen, 1996).

Inserting an intron 5' to the gene or addition of GM-CSF increases the expression of the gene, thus increasing the antibody response. This is not so for toxic proteins, however, such as rabies, which kills the cells if expression is too high (Barry and Johnson, 1997).

DNA vaccination with a plasmid encoding antigen-ligand fusion proteins; L-selectin or cytotoxic T-lymphocyte antigen 4 (CTLA4) which allows targeting to the lympoid organs and antigen presenting cells (APCs) respectively allowed an enhancment of both the humoral and cellular immune response in mice. CTLA4 is a more potent enhancer, with eight-fold higher T-cell proliferation and 10 000-fold higher antibody-binding response. The ligand acts in a similar but more direct way to GM-CSF, which enhances both cellular and humoral responses by recruiting APCs to the site of DNA vaccine expression. The CTLA4 method targets these APCs directly, increasing the potency of the immune response (Boyle *et al.*, 1998).

DNA can either be injected in saline, or complexed with various compounds. Lipids, cationic liposomes and microparticles have all been used for injection of DNA vaccines (Ishii *et al.*, 1997; Jones *et al.*, 1997 and Norman *et al.*, 1997). Cationic liposomes in a number of delivery systems (intramuscular, intravenous, intraperitoneal and intranasal) have elicited good antibody and CTL responses (Ishii *et al.*, 1997 and Norman *et al.*, 1997) and have no side effects (Norman *et al.*, 1997). Microparticles make use of poly (DL-lactide-co-glycolide) DNA complexes to elicit an antibody response (Jones *et al.*, 1997).

Vaccination schedules and the addition of co-stimulatory cytokines can be manipulated to induce better antibody or CTL responses. Increasing time between immunisations and fewer immunisations as well as a protein boost increases the antibody response (Fuller *et al.*, 1997). Cytokines like GM-CSF and IL4 can increase the antibody response whereas IL2, or IL12, or IFN-γ can increase the CTL response (Kim *et al.*, 1997).

The haplotype of the animal and the MHC epitopes recognized also effect the immune response. Four week mice have been found to elicit a better immune response than six to eight week mice when inoculated with DNA vaccines.

1.8.4) Advantages and limitations of DNA vaccination

The advantages of DNA vaccines are numerous:

- the plasmid encoding the immunogenic sequence allows the body to elicit an immune response close to that of a natural infection without the infectious pathogens,
- both a humoral and cellular immune response are obtained,
- the vaccines work efficiently in infant immune systems without interference from maternal antibodies,
- a number of pathogenic antigens can be combined in a cocktail and
- the vaccines are easy to produce, cheap and stable (Robinson, 1997b and Special conference issue, 1997).

There are safety issues, however, although as yet, they have not been proven. Tolerance induction, an antibody response against the tissues expressing the protein or the DNA itself and the possibility of integration into the chromosome are concerns. Integration does not appear to occur, however (Special conference issue, 1997) and up to 175µg of DNA did not elicit anti-DNA antibodies (Liu *et al.*, 1997).

Injecting a mouse with DNA vaccine plasmid and co-stimulatory cytokines such as GM-CSF or IL12 does not convert non-haemopoitic cells in the muscle to APCs. This is encouraging for the use of DNA vaccines in humans, since APCs cannot be created from non-APCs by DNA vaccination, thereby possibly inducing auto-immune disease. Instead, the bone marrow-derived APCs are either directly transfected, or pick up the antigen from other cells which are transfected with the DNA vaccine (Iwasaki *et al.*, 1997).

1.8.5) DNA vaccine vectors

The most common promoter in use for DNA vaccine vectors is the human cytomegalovirus (HCMV) immediate/early promoter that induces high expression in many cell types although Rous Sarcoma Virus (RSV) has also been used. It has been reported that the CMV promoter works better than the RSV promoter in the DNA vaccine vector for inoculation of mice with the influenza NP (Raz *et al.*, 1994). Terminator sequences, bovine growth hormone poly-A (BGHpA) or simian virus 40 poly-A (SV40pA) can be included in the vector 3' to the foreign gene, as can an intron 5' to the gene, increasing transcription efficiency. BGHpA is more efficient than SV40pA (Norman, 1997). Immunostimulatory DNA sequences; CpG palendromic dinucleotides, also increase the effectiveness of transcription and give rise to a better CTL response (Sato *et al.*, 1996).

1.8.6) HIV-1 DNA vaccines

Numerous HIV-1 genes have been utilised in DNA vaccines including structural, accessory and regulatory genes (Kim *et al.*, 1997; Hinkula *et al.*, 1997 and Boyer *et al.*, 1999). Using more

genes gives a broader immune response. Both *nef* and *gag* give CTL and antibody responses (Hinkula *et al.*, 1997) with a DNA vaccine. *Tat*, *nef* and *rev* proteins give a better antibody response than DNA, whereas DNA gives a better CTL response than protein (Hinkula *et al.*, 1997). HIV DNA vaccines will be discussed further in section 4.1.

1.8.7). Infant immunisation

Infant immunisation is limited by factors such as: neonatal immunodeficiency, immature neonatal immune system and the presence of maternal antibodies. As a result, there is defective B-cell activation by bacterial polysacchrides, slow B-cell activation by protein antigens and reduced T-cell helper activities. Neonates are thus easily infected by encapsulated bacteria and need multiple vaccines for their early years (Siegrist, 1997).

Nucleic acid vaccines can overcome these limitations by helping in the identification of antigens and can induce a longer-lived antibody response, thus decreasing the number of vaccinations necessary. The vaccination process can be simplified by utilising combination DNA vaccines and missing cytokines can be replaced by immunising with genes expressing cytokines. Th1 responses can be triggered to assist in intracellular pathogen destruction and APC's can be transfected directly, thereby circumventing maternal antibody interference (Siegrist, 1997).

Animal trials in infant immunisation have started. New-born mice vaccinated with murine leukaemia virus (MuLV) *env* gene were protected from challenge and had a strong CTL response. Immunisation with the whole genome gave a long-lasting CTL response and CD8+ cell transferral confers protection. The whole genome immunisation gave a higher immune response than that to the *env* gene alone (Sarzotti *et al.*, 1997). New-born chimps were immunised on their day of birth against Hepatitus B surface antigen (HbsAg), boosted at six and 24 weeks, were protected against challenge with HBV, even though a transient antibody response to the challenge virus was found (Prince *et al.*, 1997).

1.8.8). Mucosal immunisation with DNA vaccines

A good system for delivery of vaccines to mucosal surfaces is necessary. Mucosal adjuvants to enhance the immune response are also needed. It was found that intramuscular injection of the H1 protein of influenza virus with two boosts, protected mice against challenge and elicited an IgG and CTL response. Intranasal inoculation with cholera toxin adjuvant gave slight protection and elicited low IgA levels in the lungs (Ban *et al.*, 1997).

Mice have also been immunised intranasally with plasmid encoding the luciferase gene. A high serum IgG and low titre vaginal IgA response was elicited by immunisation with cationic

liposomes. The spleen and iliac lymph nodes showed a potent CTL response (Klavinskis *et al.*, 1997). Microparticles of poly(DL-lactide-co-glycolide) were administered orally and intraperitoneally with the luciferase plasmid to elicit mucosal antibodies. Good IgG, IgA and IgM responses were obtained with as little as 1µg of DNA although the intraperitoneal route gave better IgG and IgM responses than oral. Good stool IgA levels were measured after oral immunisation, as opposed to intraperitoneal immunisation (Jones *et al.*, 1997). Mucosal immunity is important for sexually transmitted disease prevention and intravaginal inoculation of gp160 induced IgG, secretory IgA and neutralising antibodies in vaginal secretions of mice (Wang *et al.*, 1997).

By oral immunisation immunogens are taken up by "M" cells in the intestine and transported to the Peyer's patch where T and B-cell responses are elicited. Activated cells travel through the lymph system and elicit systemic and mucosal immunity. Cochelates are phopholipid-calcium precipitates that form scroll-like structures, which are multi-layered and exclude water, protecting the centre from harsh environmental conditions such as pH or lipid digesting enzymes. These cochelates are able to trap DNA plasmids within the lipid bilayers and peptides can be covalently cross-linked to the phospholipid layers, thereby producing a novel vaccine delivery system that can protect immunogens from the harsh environment of the gastrointestinal tract.

Immunisation via a number of different mucosal and routes and intramuscular immunisation allowed the induction of a humoral and cell mediated immune response to HIV-1_{IIIB} *env*, *tat* and *rev* expressing plasmid. The DNA is able to enter the cell by fusion of the cochelate with the cell membrane, increasing the efficiency of DNA uptake (Gould-Fogerite, 1997).

1.8.9). Non-HIV DNA vaccines

A number of DNA vaccines are being tested in preclinical trials using different delivery protocols (see Table 1.6). The most promising vaccination schedule for HIV thus far appears to be the use of a prime-boost approach, however, using a DNA vaccine prime, followed by a recombinant fowlpox virus boost (Kent *et al.*, 1998). HIV DNA vaccines will be discussed in section 4.1.1.

Table 1.6: DNA vaccine candidates and the immune responses elicited in preclinical trials for a number of non-HIV DNA vaccines.

Vaccine candidate	Expression system/ Production method	Adjuvant or delivery system	Results	Ref
Hepatitus B surface antigen (HBsAg)	Plasmid	Intramuscular, cardiotoxin, bupivacaine	Elicited long-lasting humoral response and T-cell response in mice	1
Hepatitus B/ Hepatitus C (HBV/HCV) fusion plasmids	Fusion plasmid	Intramuscularly	Elicited humoral and CTL response to HBV and HCV in mice	2
HSV Glycoproteins B and D	Plasmid	Intramuscularly	Elicited humoral response and neutralising antibodies in guinea-pigs	3

Table 1.6: continued...

Vaccine candidate	Expression system/ Production method	Adjuvant or delivery system	Results	Ref
Influenza NP, H1 or H7	Plasmid	Intramuscular, intranasal and intravenous of stabley transfected myoblasts transplanted	MHC class 1, CTL response with high antibody titres and protected the mice from cross strain challenge	4
HA, NP and M1 influenza variant cocktail	Plasmid	Intramuscular	Elicited protection against genetic drift in ferrets as effective as the licensed vaccine homologous for the challenge strain	5
Rabies virus glycoprotein G (gpG)	Plasmid	i.m., i.d., MPL (monophosphoryl lipid A[9])	Elicited strong antibody response with protective neutralising antibodies against a global spectrum of rabies variants in mice	6
Rotavirus virus protein (VP) 4, VP6, VP7 genes	Plasmid	Gene gun	Elicited IgA and neutralising antibodies to the outer capsid proteins (VP4,VP7) and mice were protected against homologous challenge.	7
Ebola nucleoprotein (NP) and secreted (sGP) or transmembrane (GP) glycoprotein	Plasmid	Intramuscular	Elicited a T-cell and antibody response for sGP and GP which protected guinea pigs against challenge	8
P. yoelii genes homologous to *P. falciparum*	Multi-gene plasmid	intramuscular	Antibody and CTL response elicited in mice	9
[1]PbTRAP and [2]PbCS DNA vaccines followed by modified virus Ankara (MVA)-PbTRAP or MVA PbCS boost	Plasmid with recombinant MVA-malaria boost, not vice versa, however.	Intramuscular DNA vaccine, followed by intravenous MVA boost 18 days later	Elicited a potent CD8+ CTL response and protected BALB/c and C57BL/6 mice from homologous *P. berghei* sporozoite challenge	10

[1]DNA vaccine expressing the *Plasmodia berghei* thrombospondin-related adhesive protein
[2] DNA vaccine expressing the *Plasmodia berghei* circumsporozoite protein
1, Davis *et al.*, 1997; 2, Inchauspé *et al.*, 1997; 3, McClements *et al.*, 1997; 4, Fynan *et al.*, 1993; 5, Donnelly *et al.*, 1997; 6, Ray *et al.*, 1997; 7, Chen *et al.*, 1997; 8, Xu *et al.*, 1998; 9, Hoffman *et al.*, 1997; 10, Schneider *et al.*, 1998.

1.8.10). Clinical trials

A prophylactic vaccine for hepatitus B virus (HBV) is currently in Phase I clinical trials being conducted by PowderJect and Glaxo Wellcome. The PowderJect immunisation is by gene gun that bombards gold particles carrying the DNA into the epidemis using a jet of helium. The DNA enters the epidermal cells and detaches from the gold particles, after which it is expressed. Thus far, eleven out of twelve patients immunised using the PowderJect system have elicited anti-hepatitus B surface antigen (HBsAg) antibodies at 10 milli-International Units per millilitre, which is considered protective (DNA vaccine web, 1998).

In addition, Vical's DNA vaccine with Allovectin-7, which uses a lipid-DNA complex to immunise against cancer cells, is in phase II/III testing in patients with metastatic melanoma and phase II in patients with head and neck cancer. Leuvectin, also a lipid-DNA complex which stimulates an immune response to cancer cells, is in phase II testing in patients with kidney cancer (DNA vaccine web, 1998). HIV clinical trials are discussed in section 4.1.1.

Phase I trials for a malaria DNA vaccine have started. A DNA vaccine encoding the *P falciparum* circumsporozoite protein (*Pf*CSP) was used to immunise 20 healthy, malaria naïve volunteers. Of the 20 people, 11 were able to elicit antigen-specific, CD8+ CTL responses to the

DNA vaccine. The CTL responses were also determined to be HLA class-I-restricted by CTL assays with target cells which had matched or mis-matched HLA epitopes. Mis-matched targets were not recognised (Wang *et al.*, 1998).

1.9) Motivation for the project

The initial objective of this study was to define the genetic diversity of the South African HIV-1 epidemic. The only South African HIV-1 isolate which had been identified at this time was Nof, belonging to subtype C, which was found to be closely related to the Indian subtype C isolates (Dietrich *et al.*, 1993). Knowledge of the genetic diversity of the HIV epidemic in a country can shed light on the origin of the subtypes circulating and may also have implications on vaccine efficacy.

In the early 1980s, the majority of HIV infections in South Africa occurred in the male homosexual or bisexual populations, with very limited infections detected in the heterosexual population. However, by the late 1980s, there were increasing numbers of HIV infections appearing in the female population and by 1994, at the initiation of this project, there were more female infections than male infections, heralding the start of the heterosexual epidemic (see Chapter 2). Since the HIV epidemic in South Africa appeared to be separated according to time course and mode of transmission, we wanted to define the HIV-1 subtypes in individuals infected by different modes of transmission. An investigation whether there was any relationship between mode of transmission and HIV-1 subtype was important, as the two populations may have needed to be targeted with different vaccine subtypes. Finally, the HIV epidemics in the different populations could be tracked and possible shifts in the epidemics, such as occurred in Thailand and China (see section 1.6.4.1) could be identified.

The initial study was performed on patient samples with known mode of transmission from Cape Town, a port city in the south-west province of South Africa and Bloemfontein, a central province in the country (see chapter 2). To identify the subtype of a large number of samples, a rapid subtyping technique, heteroduplex mobility assay (HMA) (Delwart *et al.*, 1993), was utilised. The second aim of the project was to determine whether similar subtypes were circulating in the urban heterosexual populations in the rest of South Africa (see chapter 3). To do this, HMA was adapted to include South African reference plasmids. Patients were selected from three major urban centres in geographically distinct regions in South Africa and subtypes identified were compared with subtypes circulating in the heterosexual population in Cape Town.

As increasing numbers of recombinant viruses were being identified (Korber *et al.*, 1997), the third aim of the study was to subtype HIV-1 in more than one region of the genome in order to detect recombinant viruses (Chapter 3). To do this, a second rapid screening technique was successfully developed for subtype analysis of the *gag* region (see chapter 3). Restriction fragment length polymorphism (RFLP) analysis in the *gag* region was used in conjunction with HMA in the *env* region, in order to detect any viruses recombinant in the two regions. RFLP was chosen as it is a simple technique to perform, allowing the identification of large numbers of isolates.

After establishing that subtype C was overwhelmingly responsible for the majority of HIV-1 infections in South Africa, the second phase of the project aimed to analyse the gp120 from a representative South African isolate (see chapter 4). No macrophage tropic subtype C isolates from South Africa had been characterised at this time. The gp120 from a local macrophage tropic, primary subtype C isolate was thus chosen for characterisation in order to compare the envelope region of this isolate with other HIV isolates. In addition, the majority of vaccines at that time had been based on the use of gp120 from T-cell tropic subtype B viruses, whereas the predominant virus infecting the heterosexual population in South Africa was subtype C. Also, it had only recently been discovered that primary isolates were not neutralised by the initial vaccines based on T-cell adapted viruses due to phenotypic differences between them and the recombinant proteins utilised had failed to elicit effective CTL responses (Berman *et al.*, 1997 and Connor *et al.*, 1998).

The final aim of this study was thus to determine the immunogenicity of the gp120 from a predominant South African HIV-1 subtype and to determine the potential CTL cross-reactivity between this isolate and a macrophage tropic subtype B isolate utilising an *in vivo* recombinant Vaccinia Virus (VV) challenge system in mice (Chapter 4).

In order to elicit a CTL response, a DNA vaccine vector was chosen. DNA vaccines are easy to construct and can be injected intramuscularly eliciting an effective immune response (Iwasaki *et al.*, 1997 and Robinson, 1997a) (see section 1.8.9). The DNA vaccine was then used to immunise BALB/c mice in order to determine the possibility of a cross-reactive CTL response between subtypes B and C (Chapter 4).

A murine *in vivo* model, involving challenge with a recombinant VV expressing the relevant immunogen (subtype C gp120), was utilised for the detection of a cross-reactive CTL response in the DNA vaccine immunised BALB/c mice (Chapter 4). It has been established that a CTL response is more important than a humoral response for prevention of HIV replication (see section 1.7.1.2). A cross-reactive CTL response would prove to be valuable, since a single HIV vaccine may be developed for use in all infected populations in South Africa, regardless of the infecting HIV-1 subtype. In addition, the DNA vaccine system may be utilised for the assessment of other potential HIV vaccine proteins in conjunction with the VV challenge system. The VV challenge may also be used for determination of the optimal vaccine vector and immunisation strategy.

CHAPTER 2: AN INVESTIGATION OF THE ORIGIN OF HIV-1 IN SOUTH AFRICA AND THE RELATIONSHIP BETWEEN SUBTYPE AND MODE OF TRANSMISSION

2.1) Introduction

It has been estimated that there are currently approximately 3.6 million HIV-positive persons in South Africa (Department of Health, 1999). The HIV-1 epidemic in South Africa has spiralled in recent years and fully half of the new southern African (including Angola, Zambia, Malawi, Mozambique, Zimbabwe, Botswana, Namibia, Swaziland, Lesotho and South Africa) infections occur here. It is estimated that there are up to 50 000 new infections occurring every month, with the black community in South Africa the worst affected (Department of Health, 1999).

Since 1990, the South African Department of Health has performed an annual survey of women attending antenatal clinics of the public health services, whereby routine antenatal blood samples are collected consecutively during October and November from over 12 000 patients. At the end of 1998, it was found that 22.8% of the women attending public antenatal clinics were infected with HIV, which is a national increase in prevalence of almost 34% since 1997 (see figure 2.1) (Swanevelder, 1995 and Department of Health, 1999).

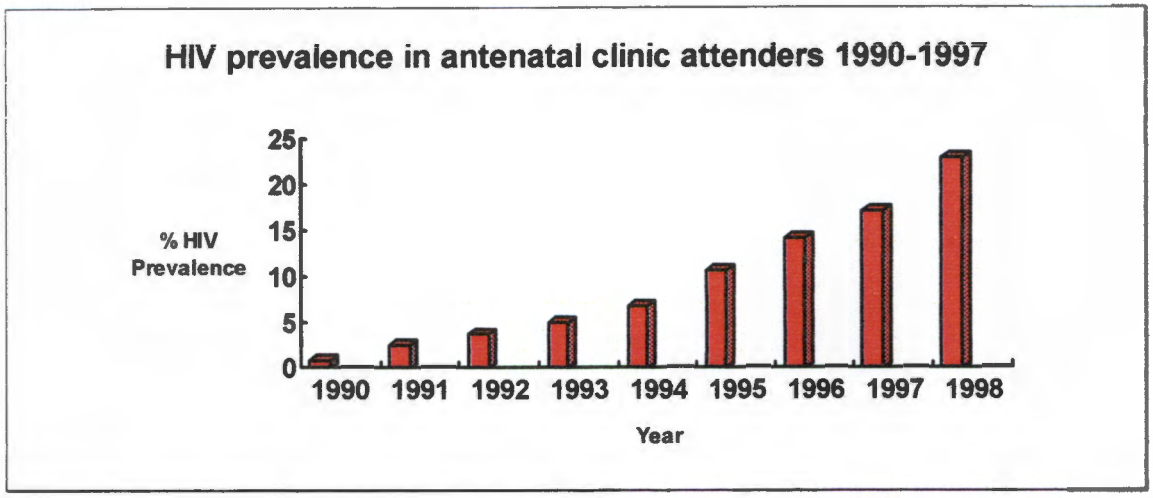


Figure 2.1: Graph indicating the increase in HIV prevalence in women attending antenatal clinics in South Africa from 1990 to 1998 (Swanevelder, 1995 and Department of Health, 1999).

The antenatal clinic survey is limited to women of child-bearing age, from 15 to 49 years, with the highest rates of infection occurring in the most sexually active age groups, from 20-24 (26.1%) and 25-29 (26.9%) years. Women who are older have lower rates of infection, at 19.1% for 30-34 age group, 13.4% for the 35-39 age group, 10.5% in the 40-44 age group and 10.2% in women aged 45-49 years (see figure 2.2). It must be noted that since fertility decreases after 40 years, the sample sizes in age groups over 40 are small and the percentage prevalence may not be accurate. In the past year, the prevalence in the youngest age group from 15-19 years has increased substantially from 12.7% in 1997 to 21.0% in 1998; a rate of increase of 65.4% (Department of Health, 1999). Clearly, awareness campaigns and prevention education need to target this age group.

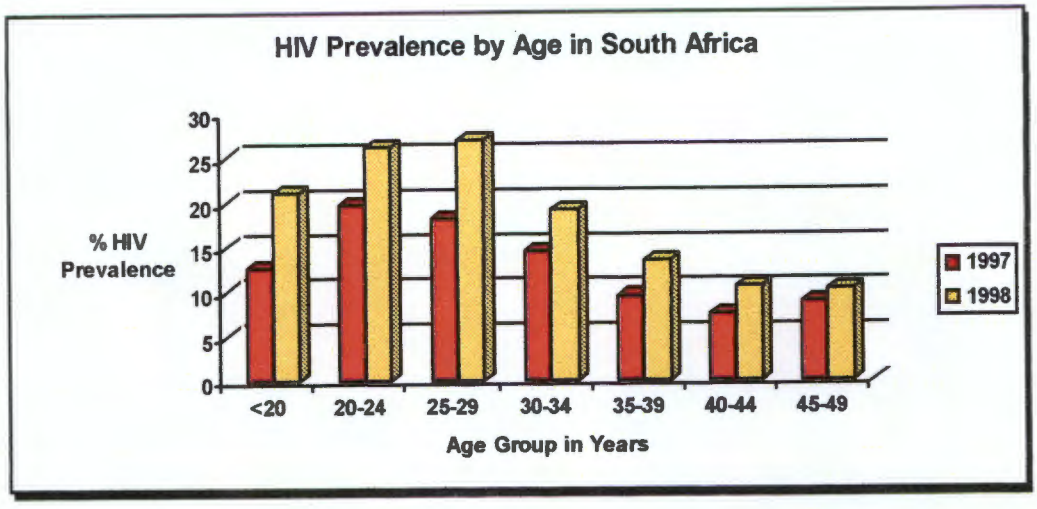


Figure 2.2: HIV prevalence by age group in women attending antenatal clinics in South Africa from 1997 to 1998 (Department of Health, 1999).

The 1997 antenatal clinic survey gave an indication of the seroprevalence in women attending antenatal clinics from different population groups (Department of Health, 1998). The highest seroprevalence was in the black population with 65% of the HIV positive women being black, followed by the mixed race population with 9.2% HIV positive, whites at 0.3% HIV positive and Asians at 0.1% HIV positive. The remaining 25.4% of HIV positive women were of unknown race. The white and Asian population statistics are under-represented, however, since the antenatal clinic survey is from antenatal clinics of the public health services and the white and asian population groups tend to use private facilities (Department of Health, 1998). Reasons for the high HIV prevalence in the black population in South Africa have been discussed in detail in section 1.6.2 and include social, behavioural, political and economic factors.

The HIV epidemic in South Africa started in the early 1980s in the male homosexual population (Sher, 1989). This was unlike the HIV epidemic occurring in the rest of sub-Saharan Africa at this time, where the epidemic was predominantly spread by heterosexual transmission (Sher, 1989; McCutchan *et al.*, 1992 and Louwagie *et al.*, 1995). In order to determine whether HIV infection had been occurring in South Africa prior to the early 1980s, 2574 samples which had been collected during a pneumococcal vaccine trial in black South African mine workers from 1970-1974, were screened in 1986 for HIV. Participants in the trial came from Mozambique (n=1191), Malawi (n=1080), South Africa (n=171), Lesotho (n=55), Botswana (n=32), Angola (n=29) and Swaziland (n=16). No conclusive evidence was found that there were any HIV infections in the samples obtained during this time period (Sher *et al.*, 1987). This was in keeping with results obtained from studies by Levy *et al* (1986) and Lyons *et al* (1985).

The first two reported cases of AIDS in South Africa, recorded in 1982, were male air

stewards infected by homosexual transmission (Ras *et al.*, 1983). Until 1988, the predominant number of AIDS cases were found to be in men infected by homosexual or bisexual mode of transmission (Sher, 1989). A retrospective study in Cape Town on all adult HIV-positive patients presenting at Somerset Hospital and Groote Schuur Hospital was performed on samples taken over an eleven year period (1984-1995). Somerset Hospital is the major HIV referral clinic in Cape Town, with a second clinic at Groote Schuur Hospital. Two patterns of infection were identified (see figure 2.3). It was determined that from 1984-1989, the HIV-positive outpatient population at these two hospitals consisted mainly of white males, infected by homosexual mode of transmission. This is in contrast to 1990-1995, where there was a significant increase in the outpatients infected by heterosexual transmission, especially in the numbers of females and members of the black population (Wood *et al.*, 1996). The second, larger epidemic, occurring in the heterosexual population from the late 1980's has now spread significantly and is responsible for the majority of HIV infections to date (WHO, 1998).

We were interested in characterising the HIV subtype diversity within two population groups (homosexual/bisexual versus heterosexual) in South Africa, in order to track the epidemic. Determination of genetic diversity may be important for the development of vaccines since we do not, as yet, know which epitopes are needed for protective immunity and whether subtype-specific proteins will have to be used (see section 1.7.1.2). In addition, if genotype is important for vaccine design, it is necessary to constantly monitor circulating subtypes of HIV-1 in order to detect possible shifts in genetic diversity such as have been observed in other parts of the world, for example, Thailand and China (see section 1.6.4).

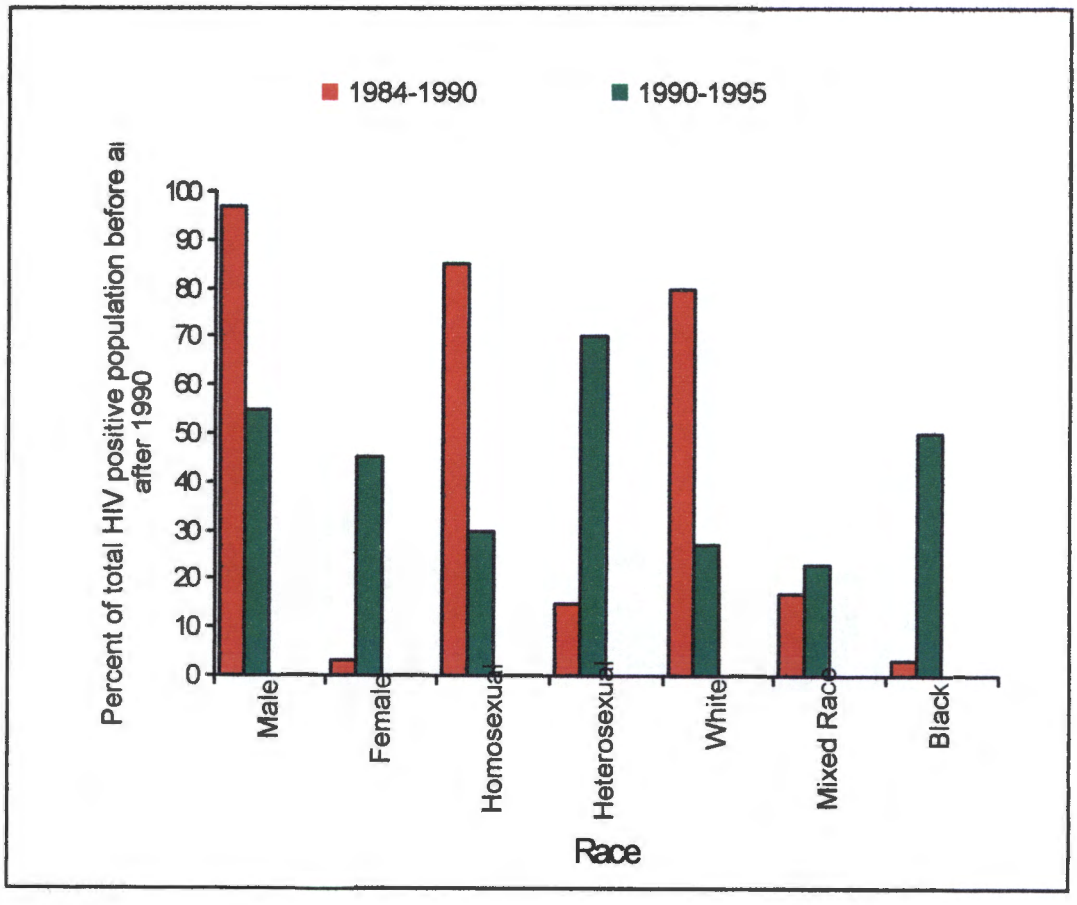


Figure 2.3: Graph showing the demographics of HIV positive patients attending the HIV/AIDS clinics in Cape Town from 1984-1995 (Wood *et al.*, 1996).

In addition, the movement of HIV subtypes globally must be monitored. HIV-1 subtypes occurring in Africa are now detected in other parts of the world where they have become predominant. For example, subtype C has spread to India where it is now the prevalent virus and is responsible for the majority of heterosexual infections (Dietrich *et al.*, 1993 and Grez *et al.*, 1994). In addition, subtype C is the predominant virus in China associated with heterosexual mode of transmission as well as IVDU (Shao *et al.*, 1996; Shao *et al.*, 1998 and Yu *et al.*, 1998). It was speculated that the virus entered China via IVDUs from India, since the average DNA distance between the Indian subtype C sequences and the Chinese subtype C sequences is only 4.5% (Luo *et al.*, 1995). Unlike the burgeoning epidemics in the Far East, such as in Thailand and China, in South Africa infection due to IVDU is rare (Sher *et al.*, 1996 and WHO, 1998).

In order to monitor circulating HIV subtypes within a country, a number of different techniques have been used. Sequencing is the most informative technique, however it is expensive and time consuming, thus rapid screening techniques for HIV subtype elucidation have been developed. For example, heteroduplex mobility assay (HMA) (Delwart *et al.*, 1993) and V3 serotyping (Cheingsong-Popov *et al.*, 1993 and Cheingsong-Popov *et al.*, 1998) have been used to analyse the *env* region of HIV-1.

HMA was developed by Delwart *et al* (1993) as a rapid subtyping technique in order to identify HIV-1 *env* subtypes. Unknown samples of DNA are amplified by polymerase chain reaction (PCR), as are reference plasmids for each subtype. The size of the fragment may vary, although for the HIV *env* genome, the 700bp V3 to V5 region is most commonly used (Delwart *et al.*, 1993; Bobkov *et al.*, 1996b; van Harmelen *et al*, 1997; Bredell *et al.*, 1998; van Harmelen *et al.*, 1999a and van Harmelen *et al.*, 1999b). The technique involves the formation of heteroduplex DNA fragments by denaturation and annealing of an equal amount of unknown amplified DNA with a reference subtype DNA. The denaturation separates the unknown and reference sample dsDNA to single strands and when cooled on ice, heteroduplexes of mixed reference and unknown sample dsDNA will form along with homoduplexes of the parent strands that have re-annealed (Appendix A9). Due to sequence mismatches between the unknown DNA and reference plasmid DNA, bulges occur in the heteroduplexes. The heteroduplex fragments are electrophoresed through a 5% non-denaturing polyacrylamide gel. Samples that are highly divergent have more bulges and thus move slower through the gel than samples with high similarity. By creating heteroduplexes with a number of different subtype references, the unknown isolate can be identified as the isolate that moves the fastest after heteroduplex formation.

The method also provides a means of determining quasi-species diversity within a sample by melting and re-annealing the unknown sample alone. Any intrasample diversity will cause the formation of heteroduplexes that are resolved in the polyacrylamide gel. This intrasample diversity is then compared with the heteroduplex patterns obtained with reference subtypes so that the quasi-species bands do not interfere with the identification of samples using the reference plasmids.

No published data was available on the relationship of subtype to mode of transmission at the time of initiation of this study. The only South African subtype which had been published was isolate Nof subtype C (Dietrich *et al.*, 1993) and sequence comparison of Nof by Dietrich *et al* (1993) showed that the sequence was genetically related to the subtype C sequences in India.

Thus, in this study, 49 samples obtained from patients infected by different modes of transmission from Cape Town in the Western Cape Province and four samples from Bloemfontein in the Free State Province, were analysed in the V3-V5 region of the *env* gene by HMA in order to determine infecting HIV-1 subtypes. It was determined that distinct HIV-1 subtypes were associated with either heterosexual or male homosexual modes of transmission in the samples analysed.

2.2) Materials and methods

2.2.1) Patient data

EDTA-blood was obtained from patients attending the HIV/AIDS clinic at Somerset Hospital (n=49) (obtained from Robin Wood, University of Cape Town, Department of Medicine). In addition, extracted DNA was obtained for four samples from the Bloemfontein HIV/AIDS clinic (obtained from Elna van der Ryst, Department of Virology, University of the Free State). Ethical approval was obtained by Dr Carolyn Williamson (University of Cape Town) and blood was taken with informed consent. The patients were anonymous and samples were coded, with only the treating health care workers knowing the patient identification. Patients were not obliged to participate in the study, or to fill in the questionnaire. Participation in the study did not affect treatment of the patient.

The following patient demographic data was requested: age, sex, race, place of residence, speculated geographical origin of infection, travel history within the previous five years and route of infection. In addition, CD4 count of the patient was obtained. When indicated as possible source of infection, the travel history of sexual partner was also recorded. Samples were named by year of isolation, country of origin and patient identification number.

Patient clinical and demographic data from the Cape Town samples analysed (n=49) is shown in Table 2.1. Year of diagnosis was between 1984 and 1994. The average age of the Somerset Hospital patients (n=46) was 35.3 with a range of 20 to 56 years and three patients with unknown ages. The male to female ratio was 37:12. There were 17 white patients of which 14 were infected by homosexual mode of transmission, two by heterosexual mode of transmission and one by blood transfusion. Of the 19 mixed race patients, ten were infected by homosexual transmission, seven by heterosexual mode of transmission and two by unknown mode of transmission. All 13 of the black patients were infected by heterosexual transmission. Out of the total 49 patients studied, 24 had CD4 counts under 200 ($X10^6$/l), with five patients having unknown CD4 counts. Five of the Cape Town patients infected by homosexual transmission and four infected by heterosexual transmission reported their speculated geographical origin of infection, due to travel within five years prior to the study, having been in outside of South Africa (see Table 2.6). Four Bloemfontein patient samples were taken in 1994. Two of the patients were black males infected by bisexual transmission and two of the patients were white males infected by either homosexual or bisexual transmission. Only one of the patients had a CD4 count above 200 ($X10^6$/l). See Table 2.3 for the Bloemfontein patients' demographic data.

2.2.2) DNA extraction.

DNA was extracted from 5 to 10ml of EDTA-blood by Maureen Lambrick, (Clinical Virology, UCT) as described (Kawasaki, 1990) (Appendix A1). Briefly, 100µl of whole blood was

mixed with 0.5ml of 1XTE buffer (10mM Tris-HCl, 1mM EDTA , pH8.0) in a microfuge tube and centrifuged at 13 000g for 10 seconds. The pellet was resuspended in 0.5ml of 1XTE buffer by vortexing and again pelleted. This was repeated twice until a clean nuclei pellet was obtained. The pellet was resuspended in 100µl of lysis buffer K (1XPCR buffer (Promega, Madison, WI, USA), 0.5% Tween 20, 100µg/ml fresh proteinase K) by vortexing and then spun down before being incubated at 56°C for 45 minutes. The protease was inactivated at 95°C for 10 minutes and the cell/nuclei lysate spun down briefly. Ten microlitres of the lysate was used for a 50µl PCR master mix volume.

2.2.3) Polymerase chain reaction (PCR)

A 700bp and 1.2Kb DNA fragment were both amplified by nested polymerase chain reaction (PCR). The 700bp DNA fragment encompassing the V3-V5 region of the *env* gene was amplified by nested PCR from patient samples using outer primers ED5/12 and inner primers ES7/8 (NIH AIDS Research and Reference Reagent Program, USA) (see Table 2.1) as described (Delwart *et al.*, 1993) (Appendix A2).

The 50µl master mixes contained:

- 5µl of PCR buffer (500mM KCl, 100mM Tris-HCl (pH9.0), 1% Triton X-100) (Promega, Madison, WI, USA),
- 1% DMSO, 1% glycerol,
- 200µM each of dNTPs,
- 1.5mM $MgCl_2$ (outer reaction) and 1.8mM $MgCl_2$ (inner reaction),
- 400nM each primer; ED5 and ED12 (outer reaction) or ES7 and ES8 (inner reaction),
- 2.5U *Taq* DNA Polymerase (outer reaction) and 2U *Taq* DNA Polymerase (Promega, Madison, WI, USA) (outer reaction) and
- 10µl patient DNA (outer reaction) or 3µl of the outer reaction PCR product (inner reaction).
- If reference plasmids (NIH AIDS Reference and Reagent Program, USA) were amplified, 2µl of plasmid DNA (20ng) was added to the inner reaction mix.

The PCR reaction was carried out using the Techne PHC-2 PCR machine with the following cycling conditions:

Outer reaction:

- initial denaturation at 94°C for 2 minutes,
- 94°C for 1 minute, 45°C for 1 minute and 72°C for 2 minutes (5 cycles)
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (35 cycles)
- final extension at 72°C for 6 minutes.

Inner reaction:

- initial denaturation at 94°C for 2 minutes,
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (35 cycles)

- final extension at 72°C for 6 minutes.

A water control and HIV-negative patient DNA was also included in order to determine primer specificity and to monitor for contamination. All PCR master mix reactions were made up in a laminar flow hood. DNA was added in a separate cubical with no DNA products allowed to enter the master mix flow hood. DNA was transferred to nested reactions in a third cubical in order to prevent contamination. Amplified product was stored at -20°C. The 700bp fragment was used for subtyping of patient samples by HMA.

The 1.2Kb fragment of the *env* gene from seven samples (94ZA060, 94ZA061, 93ZA004, 93ZA009, 93ZA040, 94ZA067, 93ZA034) already subtyped by HMA, was amplified using primers ED3/14 for the outer reaction, and ED5/12 (see Table 2.1) for the inner reaction (NIH AIDS Research and Reference Reagent Program, USA) as descibed (Delwart *et al.*, 1993) (see Appendix A2). Briefly, the PCR reactions for the inner and outer amplifications were were as above for ED5/12. Ten microlitres of patient DNA was added to the outer reaction master mix, and 3µl of the outer reaction amplified product transferred to the inner reaction for amplification of the final product. Water and HIV-negative patient controls were included as before. The product was stored at -20°C.

PCR amplified fragments were separated by electrophoresis in a 1% agarose gel in 1XTAE and visualised with ethidium bromide (10ng/ml) at 256nm with UV transillumination (Appendix A3).

Table 2.1: Primers (Delwart *et al.*, 1993) constructed for PCR amplification of the *env* region for HMA and sequence analysis.

Name	#Position	Sequence
ED3	5956bp-5985bp	5'TTAGGCATCTCCTATGGCAGGAAGAAGCGG3'
ED14	7960bp-7931bp	5'TCTTGCCTGGAGCTGTTTGATGCCCCAGAC3'
ED5	6556bp-6581bp	5'ATGGGATCAAAGCCTAAAGCCATGTG3'
ED12	7822bp-7792bp	5'AGTGCTTCCTGCTGCTCCCAAGAACCCAAG3'
ES7*	7001bp-7020bp	5'CCCGGCTGTTRAATGGYAGYCTAGC3'
ES8*	7667bp-6747bp	5'GCCGCCATAATTCATTTCYCCAATTG3'
ED33*	7359bp-7380bp	5'TTACAGTAGAAAAATTCCCCTC3'

*Sequencing primers for the V3-V5 fragment.
Position was taken from the HIV-1 HXB2 genome (Genebank acession number K03455)
Degenerate bases are shown using the IUPAC degenerate codes described by the DNAMAN Version 4, computer package (Lynnon BioSoft, 1994-1997); R = A or G and Y = C or T.

2.2.4) Cloning and sequencing

The 1.2Kb (ED5/ED12) fragment of the *env* gene amplified from seven samples (94ZA060, 94ZA061, 93ZA004, 93ZA009, 93ZA040, 94ZA067, 93ZA034) was cloned by TA cloning into pMos-blue T-vector polycloning site (Amersham, Buckinghamshire, UK), as recommended by the manufacturers (Appendix A5). Ligated DNA was transformed into competent *Escherischia coli* strain DH5α (Appendix A6) and white colonies containing putative clones screened for inserts by mini-preparation of DNA and *Pst*I/*Eco*RI double digestion (Appendix A4 and A7). Digested fragments were visualised after electrophoresis in a 1% agarose gel with ethidium bromide (10ng/ml) staining under 256nm UV light (Appendix A3).

DNA from positive clones was purified using a commercial silica-based anion-exchange column DNA purification kit (Nucleobond® AX100, Machery-Nagel, Germany) according to manufacturers instructions (Appendix A7). The V3-V5 region (±700bp) of the 1.2Kb *env* fragments (n=7) were sequenced in both directions with forward and reverse primers, ES7 and ED33 (Delwart *et al.*, 1993) (see Table 2.2) until unambiguous, by standard dideoxy chain termination method (Sanger *et al.*, 1977) using ^{35}S-dATP label and the Sequenase® II kit (United States Biochemical Corporation, Amersham®, USA) (Appendix A8.1).

2.2.5) Sequence analysis

Sequences were initially analysed by performing a BLAST (Basic Logical Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST) search (Altschul *et al.*, 1990) to confirm the HIV identity of the sequence. Sequences were aligned with reference sequences from subtypes A to H (see Table 2.3) using Clustal X (Thompson, 1997), with a final manual adjustment. Subtype J reference sequences were SE91733 and SE92809 from recent immigrants from the former Zaire (Democratic Republic of the Congo) residing in Sweden. Phylogenetic analysis generated trees based on the distance matrix neighbour-joining method in TREECONW 1.0 (van der Peer and de Wachter, 1994). Tree topology was inferred using the Kimura two-parameter correction for multiple substitutions, ignoring insertions and deletions (Kimura, 1980). Reliability of the tree topology was assessed using 1000 bootstrap replicates with the SIVcpz-gab sequence as outgroup. No interpatient sequences were found to be identical and there was no tight clustering of patient sequences with reference plasmid sequences. DNA sequences were translated to amino acid sequences using Genepro (Version 6.10, Riverside Scientific Enterprises WA, USA). The seven *env* sequences were named by year of isolation, country of origin and patient identification number. The sequences have been deposited in the Genbank database (accession numbers AF095825 - AF095831).

2.2.6) Heteroduplex mobility assay (HMA)

Heteroduplex mobility assay (HMA) was performed on the 700bp *env* gene V3-V5 region of a total of 53 samples (Appendix A9) as described (Delwart *et al.*, 1993). For subtype designation, initial unknown samples were compared with reference plasmids representative of subtypes A to D, using any one subtype A, two subtype B, two subtype C and one subtype D reference plasmids for each isolate. The reference plasmids were supplied by the HMA kit and are shown in Table 2.2) (NIH AIDS Research and Reference Reagent Program, USA). Any isolates which were ambiguous, or identified as a subtype A or subtype D were compared with all HMA reference plasmids, from subtype A to G. Prior to the use of the HMA kit, all reference plasmids were subjected to HMA analysis against each other and compared to a reference HMA gel supplied in the kit. This procedure was necessary in order to maintain that there was no cross-contamination between reference plasmids for each subtype.

Table 2.2: Reference subtypes provided in the NIH AIDS Research Reference and Reagent Program (USA) HMA kit.

Subtype	Sequence Name	Country of Origin	Fragment Cloned
A1	RW20	Rwanda	gp160
A2	IC144	Ivory Coast	ED5-ED12
A3	SF170	Rwanda	gp160
B1	BR20	Brazil	gp160
B2	TH14	Thailand	gp160
B3	SF162	USA	6.6kb 3'end
C1	MA959	Malawi	gp160
C2	ZM18	Zambia	ED5-ED12
C3	IN868	India	ED5-ED12
D1	UG21	Uganda	gp120
D3	UG46	Uganda	gp120
E1	TH22	Thailand	gp160
E2	TH06	Thailand	gp160
E3	CAR7	Central African Republic	ED5-ED12
F1	BZ162	Brazil	gp120
F2	BZ163	Brazil	gp120
G1	RU131	Russia	ED5-ED12
G3	VI525	Gabon	gp160
H1	CA13	Cameroon	ED5-ED12
H2	VI557	Zaire	ED5-ED12

Five sequenced isolates were used as the South African reference plasmids instead of

the subtype reference plasmids from the HMA kit (NIH AIDS Research and Reference Reagent Program, USA) (see section 2.2.4). These were; subtype B, 93ZA004 and 93ZA009; subtype C, 93ZA040 and 94ZA067 and subtype D, 93ZA034. In addition, one of the three subtype A isolates from the HMA kit (NIH AIDS Research and Reference Reagent Program, USA) was used.

Briefly, heteroduplexes of reference strain and unknown PCR amplified DNA were formed in a 500µl eppendorf PCR tube by mixing 4.5µl of PCR product (approximately 100-250ng of DNA), 4.5µl reference PCR plasmid (approximately 100-250ng of DNA) and 1µl heteroduplex annealing buffer (100mM NaCl; 10mM Tris, pH7.8; 2mM EDTA). In order to determine the genetic diversity of each PCR amplified unknown sample, 4.5µl of sterile distilled water was added instead of reference plasmid DNA. The tube was heated to 94°C for 2 minutes in the PHC-2 PCR Techne thermocycler in order to denature the DNA strands and the tubes were then rapidly cooled by placing on wet ice. The heteroduplex reaction mixture was then mixed with 2µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in deionised water) and loaded into the wells of a 5% non-denaturing vertical polyacrylamide gel.

The heteroduplexes were separated by electrophoresis on a Hoefer SE600, 19cm/16cm, 1.5mm thick (Hoefer Scientific Instruments, San Francisco, USA) 5% non-denaturing polyacrylamide gel in 1XTBE at 200V for five hours (see Appendix A9). The DNA fragments were visualised by ethidium bromide (10ng/ml) staining in 1XTBE for 30 minutes and UV transillumination at 256nm. Although it may be necessary to dilute the patient sample prior to amplification if there is high quasi-species diversity, this was not necessary for any of the samples analysed in this study. By diluting the sample DNA to be amplified, fewer quasi-species are amplified which may interfere with the analysis of the heteroduplexes formed with the reference plasmid DNA. Samples ambiguous with the full range of HMA kit reference plasmids (NIH AIDS Research and Reference Reagent Program, USA) were analysed by sequence analysis of the V3 loop region.

2.3) Results

2.3.1 Subtype designation

HMA of the V3-V5 region of the *env* gene was used to subtype a total of 49 samples from Cape Town and four samples from Bloemfontein. Four different subtypes were identified; subtypes A, B, C and D (see table 2.3). In addition, five of the Cape Town isolates; 93ZA004, 93ZA009 (subtype B), 93ZA040, 94ZA067 (subtype C) and 93ZA034 (subtype D) were sequenced in the V3-V5 region of the *env* gene for subtype confirmation and utilised as South African reference plasmids for HMA. The V3-V5 region of the *env* gene from an additional two samples (94ZA060 and 94ZA061) was sequenced in order to confirm subtype.

An initial 26 of the 53 isolates were analysed by HMA using the HMA kit reference plasmids (NIH AIDS Research Reference and Reagent Program, USA) as described in the methods, section 2.2.3. However, South African subtype reference isolates were postulated to be more similar to the unknown South African samples being studied than the reference isolates from the HMA kit (NIH AIDS Research Reference and Reagent Program, USA). Five South African reference plasmids were thus constructed, in order to increase the specificity of the HMA technique. The five isolates were selected as South African reference plasmids after identification by HMA using the HMA kit reference plasmids, from subtype A to G (NIH AIDS Research Reference and Reagent Program, USA): subtype B (93ZA004, 93ZA009), C (93ZA040, 94ZA067) and D (93ZA034). Subtype identity was subsequently confirmed by sequence analysis of the V3-V5 region of the *env* gene (see section 2.3.2). The remaining 27 samples were thus identified using these South African reference plasmids. Although Delwart *et al* (1993) reported that it was possible to calculate DNA distances based on the mobility of unknown samples, we found that the technique was unreliable. This was partly due to samples not migrating according to their DNA distances, but also because we did not have sufficient numbers of reference plasmids to accurately determine the DNA distance standard curve.

When used for HMA analysis, it was found that although the South African reference plasmids did not increase the mobility shift between subtypes for South African unknown samples in comparison to the reference plasmids, all but two of the samples studied were still able to be successfully identified. Isolates not subtyped as B or C were rare, (n=4) and were further analysed using the NIH AIDS Research Reference and Reagent Program (USA) HMA kit reference plasmids.

Patient 94ZA050 had a speculated origin of infection in Thailand. After unsuccessful HMA identification using the South African reference plasmids, the isolate was analysed using subtype A (RW20), subtype B (BR20 and TH14), subtype C (MA959), subtype D (UG21) and subtype E (TH06) from the HMA kit (NIH AIDS Research Reference and Reagent Program, USA). The heteroduplex formed with the Thailand subtype B reference plasmid, TH14, migrated the fastest (see figure 2.4), identifying the isolate as a subtype B.

The isolate from patient 94ZA061 gave anomalous results, using the South African reference plasmids, thus a more detailed analysis was performed on the isolate, using the reference plasmids from subtype A to F in the HMA kit (see Table 2.3). The heteroduplexes formed between isolate 94ZA061 and subtype E reference plasmids (TH22 and TH06) migrated similar distances as heteroduplexes formed with subtype A reference plasmids (RW20 and SF170) (see figure 2.5), although the fastest migration occurred with subtype E plasmid TH22 (Thailand). In order to clarify the HMA results, a second sample was obtained for both patient 94ZA061 and his wife, 94ZA060. Both samples were analysed by HMA using the reference plasmids from the HMA kit, including all of the subtype A (RW20, SF170 and IC144) and all of

the subtype E plasmids (TH06, TH22 and CAR7). Isolate 94ZA060 was identified as a subtype A by HMA, migrating fastest with the subtype A reference plasmids. No heteroduplex shifts were observed with any of the subtype E reference plasmids. Isolate 94ZA061, however, again shifted both with the subtype E reference plasmids as well as the subtype A reference plasmids. The fastest migration was observed with TH22, the subtype E Thailand isolate, as before. To confirm the sequence identity of both isolates, they were sequenced in the V3 to V5 region of the *env* gene and it was determined that both patients were infected with HIV-1 subtype A (see figure 2.6).

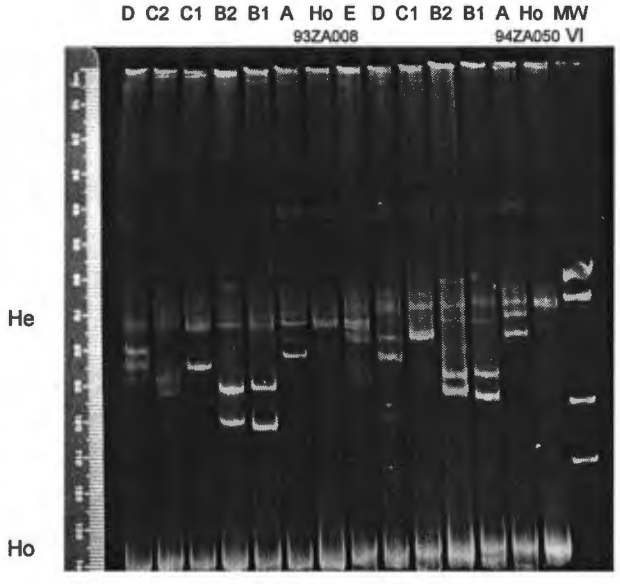


Figure 2.4: HMA gel of two subtype B isolates, 93ZA008 and 94ZA050. The faster mobility of the heteroduplexes when annealed to a subtype B reference isolate is clearly shown. He (heteroduplexes), Ho (homoduplexes), A (RW20), B1(BR20), B2 (TH14), C1 (MA959), C2 (ZAM18), D (UG21) and E (TH06). MW VI (Boehringer Mannheim, Hamburg, Germany).

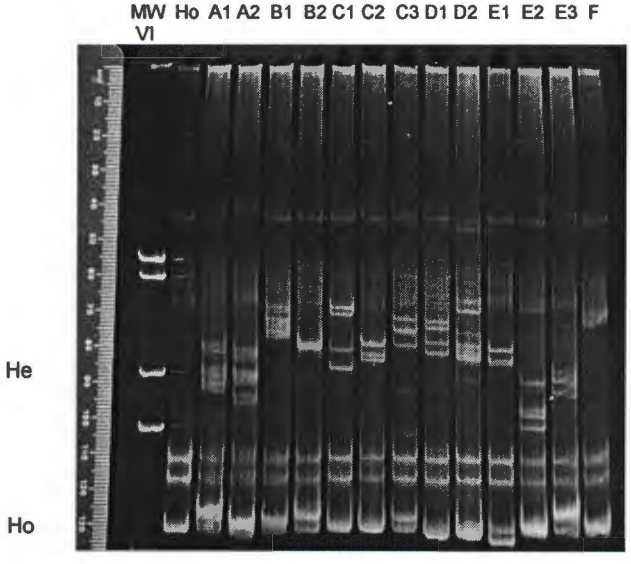


Figure 2.5: HMA gel of 94ZA061, showing the dual shift with subtype A and subtype E reference plasmids. Subsequent sequence analysis confirmed that the isolate was a subtype A. He (heteroduplexes), Ho (homoduplexes), A1 (RW20), A2 (SF170), B1 (BR20), B2 (TH14), C1 (MA959), C2 (ZAM18), C3 (I868), D1 (UG21), D2 (UG46), E1 (TH06), E2 (TH22), E3 (CAR7), F (BZ162), MW VI (Boehringer Mannheim, Hamberg, Germany).

Table 2.3: Demographic and patient clinical data according to mode of transmission for the patient samples studied from Somerset Hospital in Cape Town in the Western Cape Province and from the HIV/AIDS clinic in Bloemfontein in the Free State Province.

Sample name	Age (years)	Sex	Race	CD4 count ($x10^6$/l)	Origin of infection	Origin of infection of partner	Subtype
Cape Town							
Heterosexual Transmission							
93ZA006	30	male	white	342	RSA	-	C
93ZA010	41	female	black	530	RSA	-	C
93ZA020	33	female	mixed race	5	Zambia	Zambia	C
93ZA023	48	male	black	169	RSA	-	C
93ZA024	41	male	black	200	RSA	Namibia	C
93ZA029	29	female	black	172	RSA	-	C
93ZA031	31	male	black	154	Zimbabwe	-	C
93ZA032	34	female	black	72	RSA	-	C
93ZA035	52	male	mixed race	140	RSA	-	C
93ZA037	32	male	mixed race	418	RSA	-	C
93ZA040	34	female	black	269	RSA	-	C
93ZA042	27	male	mixed race	54	RSA	-	C
93ZA043	32	female	mixed race	443	RSA	-	C
93ZA046	21	female	black	404	RSA	-	C
93ZA047	24	male	black	373	RSA	-	C
93ZA048	40	male	black	111	RSA	-	C
93ZA049	20	female	black	517	RSA	-	C
94ZA058	37	female	white	357	RSA	-	D
#94ZA060	42	female	mixed race	421	RSA	-	A
94ZA061	33	male	black	138	RSA	-	A
94ZA063	24	male	mixed race	ND	RSA	-	C
94ZA067	30	female	black	ND	RSA	-	C

Table 2.3 continued...

Sample name	Age (years)	Sex	Race	CD4 count ($x10^6$/l)	Origin of infection	Origin of infection of partner	Subtype
Homosexual Transmission							
93ZA001	37	male	white	504	RSA	-	B
93ZA002	35	male	white	52	RSA	-	B
93ZA003	32	male	white	84	Unknown	-	B
93ZA004	30	male	white	564	USA/Europe	-	B
93ZA005	42	male	mixed race	231	RSA	USA/Europe	B
93ZA007	41	male	white	0	RSA	Europe	B
93ZA009	31	male	mixed race	499	RSA	-	B
93ZA011	25	male	mixed race	481	RSA	-	B
93ZA017	25	male	white	8	RSA	-	B
93ZA021	44	male	mixed race	245	RSA	-	B
93ZA022	49	male	white	69	RSA/Aus	-	B
93ZA033	36	male	white	374	Aus/USA	-	B
93ZA034	42	male	white	21	RSA	-	D
93ZA039	37	male	mixed race	746	RSA	-	B
93ZA041	30	male	mixed race	54	RSA	-	B
93ZA044	48	male	white	208	RSA	-	B
93ZA045	26	male	mixed race	397	RSA	-	B
94ZA050	39	male	mixed race	32	RSA	Thailand	B
94ZA059	55	male	mixed race	77	RSA	-	B
94ZA073	47	male	white	23	RSA	-	B
94ZA076	ND	male	white	ND	RSA	-	B
94ZA077	ND	male	mixed race	ND	Thailand	-	B
Bisexual Transmission							
93ZA038	38	male	white	0	RSA	-	B
94ZA072	56	male	white	45	RSA	-	B
Other Transmission							
*93ZA008	28	female	white	6			B
[&]94ZA074	42	male	mixed race	11			B
[&]94ZA078	ND	male	mixed race	ND			B
Bloemfontein							
Homosexual transmission							
95ZA25FS	38	Male	White	10	RSA	-	B
Bisexual transmission							
95ZA06FS	22	Male	Black	130	RSA	-	C
95ZA14FS	22	Male	Black	240	RSA	-	B
95ZA17FS	30	Male	White	120	RSA	-	B

ND, not determined
*Patient infected by blood transfusion
Isolate sequenced in the *env* region
[&] Patients infected whilst incarcerated in Pollsmoor prison
RSA, Republic of South Africa; USA, United States of America; UK, United Kingdom; Aus, Australia.

2.3.2) Sequence analysis of the env V3-V5 region

The V3-V5 (700bp) region from isolates 94ZA060, 94ZA061, 93ZA004, 93ZA009, 93ZA040, 94ZA067 and 93ZA034 were sequenced using the Sanger dideoxy termination method (Sanger *et al.*, 1977) and subjected to phylogenetic analysis with reference sequences from subtypes A to J with SIVcpz-gab as the root (see figure 2.6). All of the subtypes designated by HMA were found to cluster with their expected reference subtype sequences, with both intrasubtype and intersubtype DNA distances shown in Table 2.4.

Table 2.4: The intrasubtype and intersubtype DNA distance percentages for South African subtypes A to D with two reference sequences for each subtype.

		Subtype A		**Subtype B**		**Subtype C**		**Subtype D**
Isolates		94ZA060	94ZA061	93ZA004	93ZA009	93ZA040	94ZA067	93ZA034
Subtype A	94ZA060	0.0	9.1	25.8	24.7	23.4	24.6	26.7
	94ZA061	9.1	0.0	27.2	27.4	25.0	26.2	27.3
	RW20	15.4	16.5	28.3	25.1	21.1	21.7	27.3
	SF170	11.9	11.4	25.7	24.1	21.7	22.2	24.7
Subtype B	93ZA004	25.8	27.2	0.0	13.2	28.8	28.5	22.1
	93ZA009	24.7	24.7	13.2	0.0	25.5	26.7	22.1
	TH14	26.2	27.2	15.1	10.6	24.5	26.7	20.3
	SF162	24.7	26.8	13.2	8.9	21.6	22.8	20.6
Subtype C	93ZA040	23.4	25.0	28.8	25.5	0.0	4.5	22.1
	94ZA067	24.6	26.2	28.5	26.7	4.5	0.0	23.9
	ZM18	23.8	26.0	25.2	22.5	8.1	7.9	21.8
	MA959	26.1	27.0	28.2	26.7	8.1	9.1	23.3
Subtype D	93ZA034	26.7	27.3	22.1	22.1	22.1	23.9	0.0
	UG21	29.3	28.6	25.6	24.2	21.3	23.7	15.9
	UG38	32.3	33.3	28.7	27.1	26.0	27.6	17.4

In our comparison of the V3 loop region from subtypes A to AE (figure 2.7), including our reference plasmid sequences (HIV sequence database, 1998), the V3 loop of all South African isolates, except 93ZA034 (subtype D) was 35 amino acids in length. Isolate 93ZA034 had a V3 loop of 36 amino acids.

Both of the subtype B viruses had the characteristic GPGR tetrapeptide crown. The two subtype A, two subtype C viruses and one subtype D virus had the GPGQ tetrapeptide crown motif. Both of the subtype C isolates sequenced in this study, as well as all of the reference subtype C isolates were missing the *N*-linked glycosylation site at position 265-267, proximal to the start cycteine of the V3 loop, that is usually absent in subtype C isolates (Korber *et al.*, 1997 and Abebe *et al.*, 1997). The argenine substitutions at positions 276, 290 and 297 that result in an increased positive charge and are indicative of a syncitium-inducing phenotype was only present in sample 94ZA067 (Fouchier *et al.*, 1992 and De Wolf *et al.*, 1994). Unfortunately, the CD4 count for this patient was not available, thus the disease stage could not be determined. The second V3 loop glycosylation site at position 271-273 was present in all subtype C isolates.

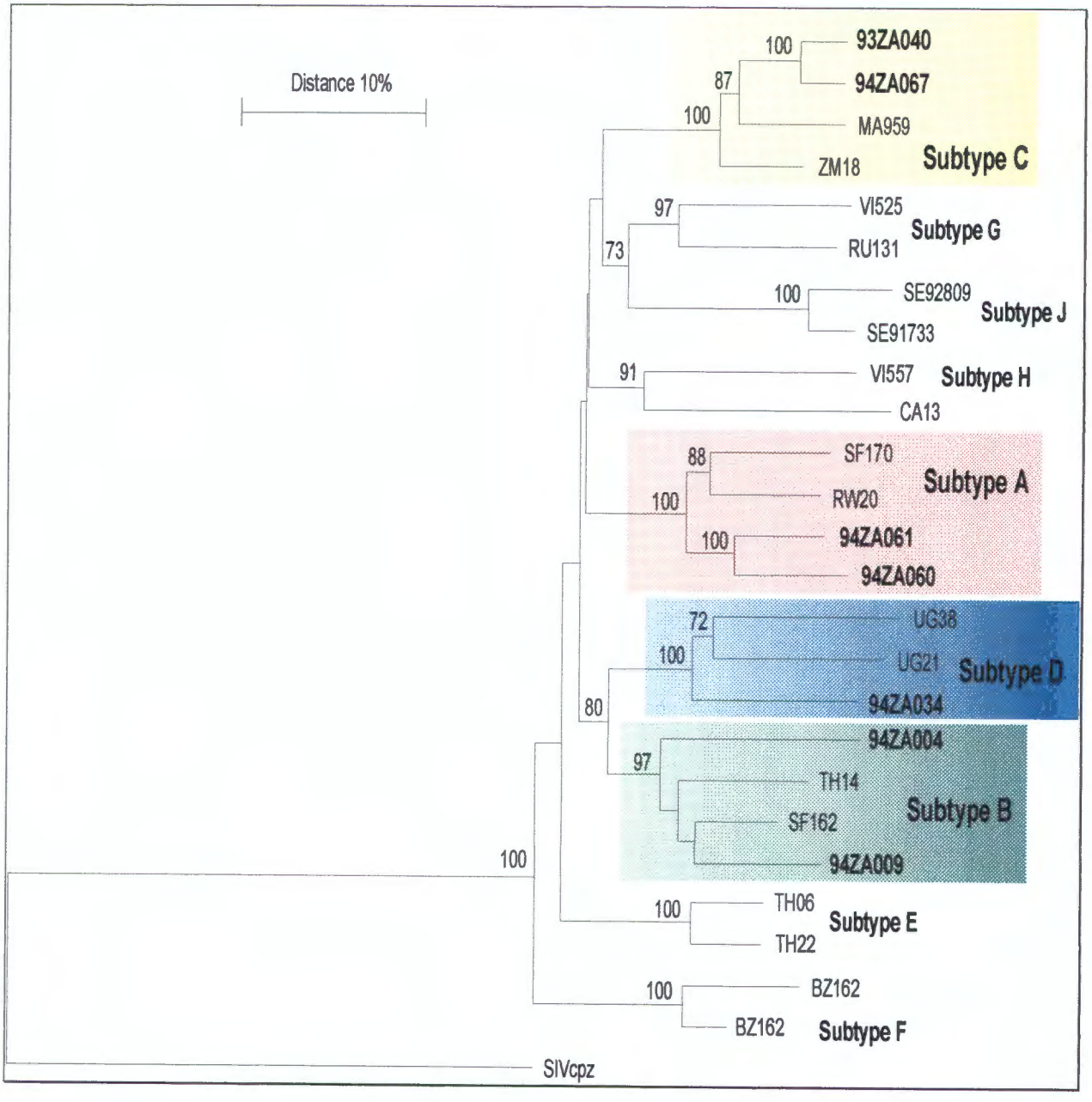


Figure 2.6: Phylogenetic tree of the V3 to V5 region of the *env* gene for isolates 94ZA060, 94ZA061, 93ZA004, 93ZA009, 93ZA040, 94ZA067 and 93ZA034 in comparison to at least two reference sequences from subtypes A to J. Isolates 94ZA060 and 94ZA061 cluster with the subtype A sequences with a bootstrap value of 100%; isolates 93ZA004 and 93ZA009 group with the subtype B sequences with a bootstrap value of 97%; isolates 93ZA040 and 94ZA067 belong to subtype C, clustering with the subtype C isolates with a bootsrap value of 100%; and isolate 93ZA034 clusters with the subtype D reference sequences with a bootstrap value of 100%.

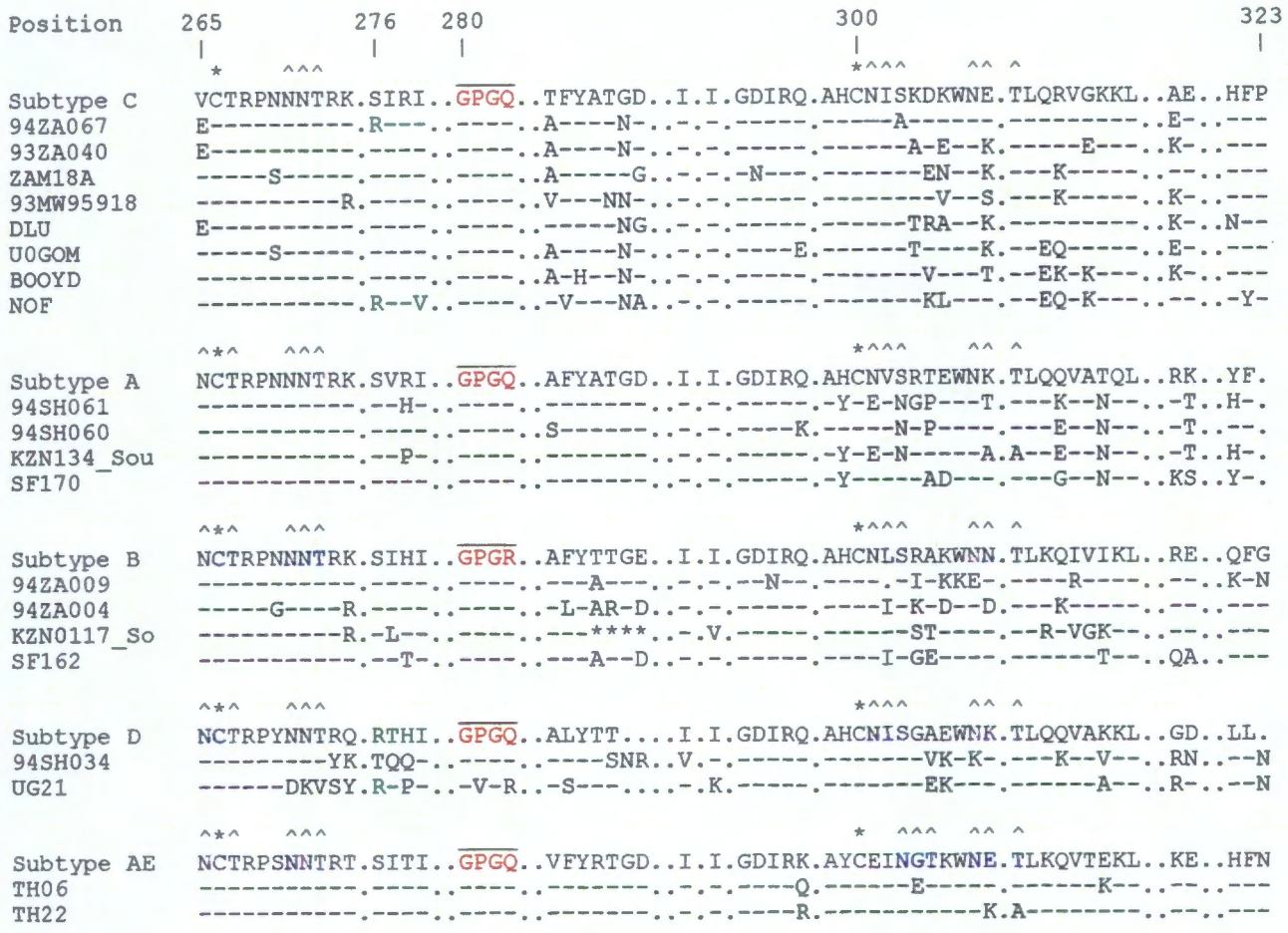


Figure 2.7: An alignment of the V3 loop for subtypes A to AE. The top sequence is a consensus sequence for the subtype taken from Myers *et al* (1996). Potential *N*-linked glycosylation sites are shown in blue with carets (^) above, the conserved cysteine residues are indicated with asterisks (*) and the V3 crown tetrapeptide is shown in red. The arginine residue at position 276, indicative a SI phenotype is indicated in green (Fouchier *et al*., 1992 and De Wolf *et al*., 1994).

2.3.3) HIV-1 subtype in South Africa according to mode of transmission

The HIV-1 subtypes in South Africa were found to segregate according to mode of transmission, with subtype C the prevalent virus in the heterosexual population and subtype B responsible for the majority of homosexual and bisexual infections.

2.3.3.1) Heterosexual Transmission

Of the 22 patients infected by heterosexual transmission, 17 were infected with subtype C, two with subtype A, two with subtype B and one with a subtype D virus (see figure 2.8). The majority of the patients infected with subtype C in this study were black (n=11), although four patients were of mixed race and two patients were white. The single subtype D-infected patient was a woman, residing in South Africa, with no record of travel history to any other country within the last five years before sampling.

2.3.3.2) Homosexual transmission

Of the 24 male patients analysed from Cape Town, infected by homosexual or bisexual transmission, 23 were infected with subtype B and one with a subtype D virus (see figure 2.4). The majority of men infected by homosexual or bisexual transmission (n=24) were white males (n=14), although 10 males of mixed race were also infected. In the Cape Town study, no men infected by homosexual or bisexual transmission were infected with a subtype C virus, the subtype predominant in the heterosexual population. The single subtype D virus was detected in a white male residing in South Africa who had no travel history to a country where subtype D was prevalent, within the last five years prior to the study.

In Bloemfontein two white males infected by homosexual and bisexual mode of transmission and one black male infected by bisexual mode of transmission were infected with subtype B. The remaining sample, taken from a black male infected by bisexual transmission was found to be infected with the first subtype C virus identified in the bisexual/homosexual population.

2.3.3.3) Other modes of transmission

Three additional subtype B viruses were identified; two transmitted by unknown route in men currently incarcerated at Pollsmoor prison and one transmitted to a woman by blood transfusion (see figure 2.8).

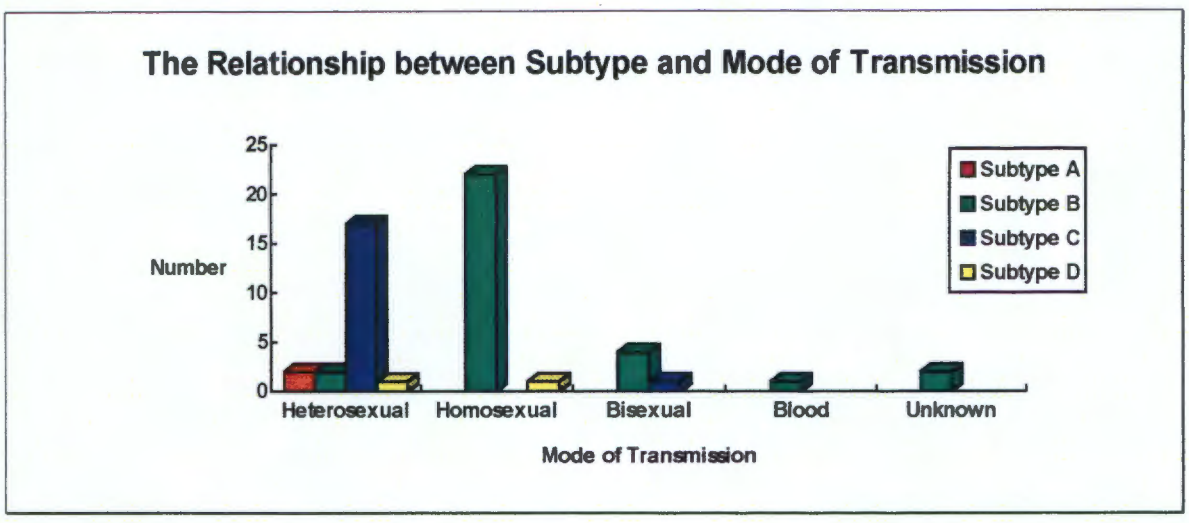


Figure 2.8: A graph indicating the relationship between subtype and mode of transmission. The predominant subtype infecting the heterosexual population is subtype C and the predominant subtype infecting the homosexual population is subtype B.

2.3.3.4) The relationship of HIV-1 subtype to speculated origin of infection

Although the patients involved in the study all resided in South Africa, a number of patients from the Cape Town study reported having had sexual contact outside of the country, or their partners had had sexual contact abroad within five years prior to sampling (see section 2.2.1). All of these individuals infected by homosexual transmission were infected with subtype B viruses, including 93ZA004, 93ZA005 and 93ZA033 (presumed origin of infection, USA); 93ZA004 and 93ZA007 (Europe), 93ZA022, 93ZA033 (Australia) and 94ZA050 and 94ZA077 (Thailand). In addition, a number of Cape Town patients infected by heterosexual contact speculated that their origin of infection was in a nearby Southern African country, or the patients had previously resided in these countries. These include 93ZA020 (presumed origin of infection Zambia), 93ZA024 (Namibia) and 93ZA031 (Zimbabwe). All of these individuals were infected heterosexually with subtype C viruses (see Table 2.5).

Table 2.5: The HIV subtype identified in each patient according to race group and speculated origin of infection of the patients or their sexual partner within five years of sampling.

Race	*[a] HMA subtype designation according to presumed geographical of origin of infection				
#Homosexual/bisexual transmission					
	South Africa	United States	Europe	Australia	Thailand
White	10B, 1D	2B	1B	2B	-
Black	1B, 1C	-	-	-	-
Mixed race	7B	1B	1B	-	2B
Heterosexual transmission					
	South Africa	Zimbabwe	Zambia	Namibia	
White	1C, 1D	-	-	-	
Black	10C, 1A	1C	-	1C	
Mixed race	3C, 2B, 1A	-	1C	-	

One white male patient had an unknown origin of infection.
* Predicted geographical origin of infection for either the patient or their partner. In addition, two mixed race, male patients were infected whilst in Pollsmoor prison and one white female was infected by blood transfusion, all with subtype B viruses.
[a] Note: patients may have a speculated origin of infection in more than one country.

2.4) Discussion

A comparison of the number of AIDS cases occurring in men and women from the early 1980's until the present indicates that there were two separate patterns of HIV infection in South Africa. In the early 1980s, HIV was predominantly detected in white or mixed race homosexual men, whereas HIV infection in women was rare (Sher, 1989 and Wood *et al.*, 1996). A 1983 study performed in Johannesburg showed that the majority of homosexual infections were as a result of sexual contact with men abroad in Europe or the USA (Sher *et al.*, 1985). However, many (23 out of 35) of patients infected homosexually in our study, in the mid- to late 1980s, reported that they had not had sexual contact whilst abroad, or with any partners who had been abroad five years prior to sampling for this study.

The numbers of HIV infected women started rising by 1988 and in 1992, the number of infected females was approximately equivalent to the number of infected males (Sher *et al.*, 1989; Swanevelder, 1995 and Wood *et al.*, 1996). By 1994, the number of female AIDS cases in South Africa was higher than AIDS cases in men with large numbers of infections in the sexually active female population (see figure 2.2) (Swanevelder, 1995).

In order to understand the epidemic occurring in South Africa, the global HIV epidemic must be taken into consideration. HIV-1 subtype B was first identified in men infected by homosexual transmission in the United States and countries in Europe and is now the

predominant virus in these countries. Subtype B is, however, rarely detected in Africans residing on the African continent. Although all of the subtypes are present in Africa, subtypes A, C and D are the prevalent viruses. Subtype A is common in west Africa (Poss *et al.*, 1997), subtypes A and D in central and east Africa (Luo *et al.*, 1998; Rayfield *et al.*, 1998) and subtype C in southern and north Africa (Abebe *et al.*, 1997; Björndal *et al.*, 1999; Novitski *et al.*, 1999 and Tien *et al.*, 1999). The predominant mode of transmission is heterosexual (MAP, 1997 and WHO, 1998).

In this study, four subtypes were identified in the 53 samples analysed in the *env* region of the HIV-1 genome, including two subtype A, 31 subtype B, 18 subtype C and two subtype D viruses. The subtypes segregated according to mode of transmission, with 92.9% (26 out of 28) of the male homosexual/bisexual group infected with subtype B viruses and 77.2% (17 out of 22) of the heterosexual group infected with subtype C viruses. Three patients were infected by unknown node of transmission. There is a significant association between subtype and mode of transmission ($p=<0.0001$), which suggests that the origin of the second, heterosexual epidemic is mostly independent to the first wave of infections.

The genetic diversity of two South African subtype B reference isolates, 93ZA004 and 93ZA009 were investigated by sequence analysis of an approximately 700bp region of the *env* gene, incorporating the V3 loop. It was determined that the intrasubtype DNA distance between the reference sequence from the United States (SF162) was equal to (93ZA004, 13.2%) or less than (93ZA009, 8.9%) the DNA distance between the two South African subtypes (13.2%)(see Table 2.4). This is in comparison to the DNA distance (9.6%) between the reference sequence from the United States (SF162) and from Thailand (TH14). The South African subtype B isolates were thus as divergent from each other as from at least one of the HMA kit (NIH AIDS Research Reference and Reagent Program, USA) reference subtype B isolates. This high genetic diversity may be an indication as to why the South African reference plasmids were not more efficient in the identification of South African subtype B samples than the kit reference subtypes.

Although the predominant subtype infecting the homosexual population in our study was subtype B, subtype D was also identified. An earlier study on fourteen samples from Tygerberg Hospital, obtained between 1984 and 1989 by Engelbrecht *et al* (1995) reported on the identification of subtype D in five men infected by homosexual or bisexual mode of transmission. The subtype D isolates may have epidemiological linkage, with an intrasubtype DNA distance of only 4.6% to 6.5% in the V3 loop of the *env* gene. This is in comparison to a maximum intrasubtype DNA distance of 13.9% when the Zairean reference sequence, ELI, was included. However, in our study, out of 28 male homosexual patients diagnosed in the late-1980's to early-1990's (up to seven years after the Tygerberg patients) only one was infected with a subtype D virus. It would thus appear that the subtype D epidemic has failed to proliferate in the homosexual population.

Subtype C was identified as the predominant virus infecting the heterosexual population in Cape Town by HMA. Results from a concurrent study on the p17 region of the *gag* gene from Cape Town patient isolates gave an indication of the DNA distances and genetic diversity between Cape Town subtype C isolates (Williamson *et al.*, 1995 and van Harmelen *et al.*, 1997). The high intrasubtype DNA distance in the *gag* region (6.8%), together with the recent introduction of subtype C into the country indicated the multiple entry of this subtype at different times (Williamson *et al.*, 1995 and van Harmelen *et al.*, 1997). These results were confirmed by a more recent study, performed by Bredell *et al* (1998) on 43 mine workers working in the Gauteng Province gold mines. Mine workers included migrant workers from Lesotho, Botswana, Mozambique and Swaziland, as well as South African men. The average intrasubtype DNA distance between subtype C isolates sequenced in the V3 to V5 region was over ten percent (Bredell *et al.*, 1998).

Epidemiological data supports the hypothesis that subtype C was introduced into the heterosexual population in South Africa on a number of different occasions from neighbouring countries. Some of the patients in this study reported having had sexual contact in Zambia, or Zimbabwe, where subtype C is commonly found (McCutchan *et al.*, 1992 and Tien *et al.*, 1999). It would thus appear that the later epidemic was as a result of the regional spread of the HIV-1 from neighbouring countries into South Africa. Due to the close socio-economic ties between South Africa and its neighbouring countries as well as the extensive migrant labour, there is a constant movement of people, facilitating the spread of HIV-1 to South Africa.

The two South African subtype C reference isolates sequenced in the V3-V5 region were only 4.5% divergent, in comparison to the Zambian and Malawian subtype C sequences to which they were at least 8.1% divergent (93ZA040 and ZM18 or MA959) and at most 9.1% divergent (94ZA067 and MA959). The low DNA difference between the two subtype C isolates is not representative of the subtype C epidemic in South Africa, however, as illustrated in the *gag* and *env* regions (see Chapter 3) (Williamson *et al.*, 1995; van Harmelen *et al.*, 1997 and Bredell *et al.*, 1998). The low genetic diversity of the South African reference plasmids, in comparison to the majority of highly divergent subtype C isolates may explain why the reference plasmids did not yield greater mobility shifts by HMA with unknown South African samples than the kit subtype C reference plasmids from Zambia and Malawi. The genetic diversity of subtype C in South Africa and factors influencing the spread of the heterosexual epidemic will be discussed in more detail in Chapter 3.

The heterosexual population in Cape Town had the highest diversity of HIV subtypes, with subtypes A, B and D also identified. Although subtype B was the subtype associated with the initial homosexual epidemic, it failed to spread significantly within the heterosexual population in Cape Town, where only two patients were found to be infected with subtype B.

The two subtype A viruses, 94ZA060 and 94ZA061, were sequenced in the V3-V5 region of the *env* gene. Although they had a fairly high DNA distance (9.1%), the two sequences grouped together in the phylogenetic tree, with a bootstrap value of 100%. The average DNA distance of 94ZA060 and 94ZA061 was 12.5% in comparison to the two reference subtype A samples, RW20 and SF170. The migration of isolate 94ZA061 with TH22, the Thailand reference plasmid, by HMA could not be explained by the DNA distance (21.1%) between the sequences. The *gag* region of these isolates was also sequenced and will be discussed in Chapter 3.

In addition, subtype D was identified in a white female infected by heterosexual transmission. There is no indication as to where the patient may have been infected, since the patient resided in South Africa, with no reported travel, or contact with a sexual partner who had travelled to central or east Africa, where subtype D is common.

There is an association between subtype and mode of transmission in a number of other countries; including China, India, Thailand and Russia (Dietrich *et al.*, 1993; Ou *et al.*, 1993; Luo *et al.*, 1995; Wasi *et al.*, 1995; Lukashov *et al.*, 1997 and Liitsola *et al.*, 1998; Shao *et al.*, 1998) (see detailed discussion in section 1.7.4). It is necessary to maintain surveillance over the prevalent subtypes, since genetic shifts between subtypes have been identified (see section 1.6.4). In South Africa, the first subtype C virus infecting the bisexual population was identified in the Bloemfontein study. This could be an indication of the beginning of a shift in the subtypes infecting the homosexual/bisexual population, with subtype C now entering this population. The limited sample numbers make an accurate analysis impossible, and a larger study of men infected by homosexual/bisexual transmission should be performed in order to monitor the possible spread of subtype C within this population.

Although it is possible that certain subtypes may have a selective advantage in transmission, in South Africa, however, it is more probable that social and cultural reasons influence the segregation of subtype with mode of transmission. Besides mode of transmission, there may be other segregating factors between the two epidemics. The early homosexual epidemic started during the years when apartheid was still the political policy in South Africa and it is possible that the forced racial segregation played a role in the segregation between the two epidemics. However, our study was not designed to investigate this.

In conclusion, the initial homosexual epidemic in South Africa was at least in part, a result of the introduction of HIV-1 into South Africa from other continents, such as North America and Europe. Molecular evidence confirms this, whereby the predominant subtype infecting the homosexual population is subtype B, which is common in Europe and the United States, but rare in Africa. The heterosexual epidemic, on the other hand, is predominantly caused by subtype C

infections. This devastating epidemic appears to have been introduced into South Africa on a number of occasions and at different times, and probably originated from neighbouring countries where subtype C is known to be prevalent (Bredell *et al.*, 1998; Engelbrecht *et al.*, 1998; Novitski *et al.*, 1999 and Tien *et al.*, 1999). The genetic diversity of the heterosexual epidemic in South Africa will be discussed in detail in the following chapter.

CHAPTER 3: THE GEOGRAPHICAL DISTRIBUTION OF HIV-1 SUBTYPES IN SOUTH AFRICA

3.1) Introduction

Until December 1988 only 166 AIDS cases were reported in South Africa. The majority of these cases were in the homosexual/bisexual population (75.3%), although there were a small number of cases in the heterosexual population (14.5%). In addition, 8.4% of the cases were by infection with HIV-contaminated blood product and 1.8% of cases were in babies infected by vertical transmission (Sher, 1989). 1988 was a pivotal year, however, and in an analysis of over 700 000 blood donor samples collected up until October, 244 tested positive for HIV antibodies, of which 101 were women and 143 were men (Sher, 1989). This increase in the numbers of female HIV infections was indicative of the heterosexual epidemic to come, which was subsequently documented by the annual antenatal clinic surveys, initiated in 1990 (Swanevelder, 1995). There was a steady rise in the numbers of AIDS cases in women, starting in 1987 and increasing until 1994 where more females than males were diagnosed (see figure 3.1) (Swanevelder, 1995).

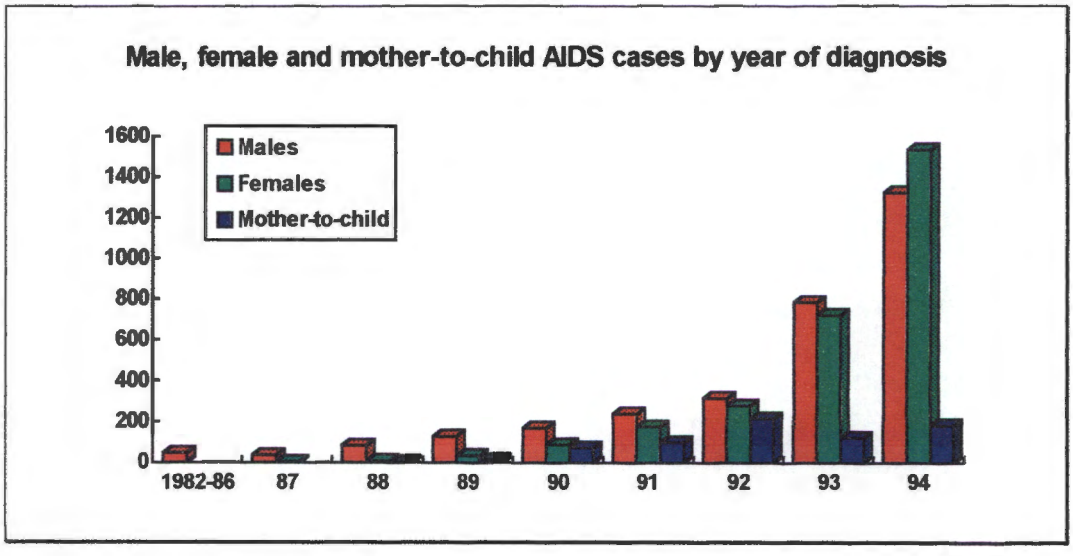


Figure 3.1: The number of AIDS cases reported by year of diagnosis in South Africa from 1982-1994 illustrating the increase in AIDS cases in the female population from 1987 until 1994 (Swanevelder, 1995).

The first epidemic occurred in the homosexual population, as described by Sher *et al.* in the early Johannesburg study in 1985, as well as in the Cape Town study by Wood *et al.* in 1996. These provinces, Gauteng and Western Cape respectively, had the highest numbers of AIDS cases in 1988. As AIDS is not a notifiable disease, the number of AIDS cases are not a good representation of HIV prevalence, however the numbers do give an indication of the epidemic in different regions. As the second, heterosexual epidemic started, seroprevalence in provinces such as KwaZulu/Natal, Mpumalanga, Free State and Gauteng increased (Dusheiko *et al.*, 1989).

There is geographical variation in the timing and severity of the heterosexual epidemic in the country. The annual antenatal clinic survey shows that by 1991 KwaZulu/Natal Province had the highest HIV prevalence at 2.87%, whereas Transvaal and Cape Provinces had 1.11% and 0.37%, respectively. It is not possible to directly compare provincial figures from this time to current figures, due to changes in provincial boundaries in 1994. The most recent antenatal clinic survey (performed in 1998), illustrated in Table 3.1, indicates that the Western Cape Province, the only province with a decrease in HIV prevalence, still has the lowest HIV prevalence at 5.2%, although this may be an under-representation. KwaZulu/Natal Province still has the highest prevalence at 32.5% (see figure 3.2) (Department of Health, 1999).

Table 3.1 : The annual antenatal clinic survey of HIV-positive women attending public health services antenatal clinics from 1997-1998 (Department of Health, 1999).

Province	HIV+ (95%CI) 1997	HIV+ (95%CI) 1998
Western Cape	6.3 (5.2-7.5)	5.2 (3.2-7.2)
Northern Cape	8.6 (6.4-11.3)	9.9 (6.4-13.4)
Northern Province	8.2 (6.9-9.7)	11.5 (9.2-13.7)
Eastern Cape	12.6 (11.0-14.4)	15.9 (11.8-20.0)
North West	18.1 (16.2-20.1)	21.3 (19.1-23.4)
Gauteng	17.1 (15.1-19.2)	22.5 (19.2-25.7)
Free State	20.0 (17.1-22.2)	22.8 (20.2-25.3)
Mpumalanga	22.6 (20.5-24.8)	30.0 (24.3-35.8)
KwaZulu/Natal	26.9 (24.9-29.0)	32.5 (29.3-35.7)
National	**17.04**	**22.8**

The HIV-1 epidemic in sub-Saharan Africa is influenced by a number of factors; including economic, social, cultural and political factors as was discussed in detail in section 1.6.2. The same factors play a role in the spread of the heterosexual epidemic in South Africa. Migration between countries on the African continent is frequent and has been occurring for many generations. Migration plays a major role in the spread of HIV by the movement of people throughout the African continent. Since many countries in Africa are poverty stricken, or plagued by continual civil wars, migrant workers and refugees are constantly on the move in search of work or asylum (Quinn, 1996).

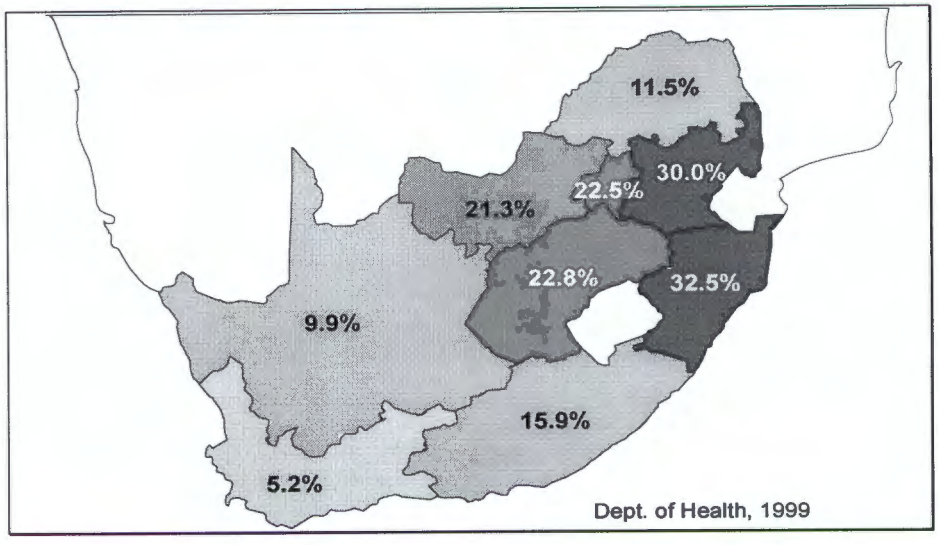


Figure 3.2: The percentage prevalence of HIV positive women attending antenatal clinics at the end of 1998 for each of the nine Provinces in South Africa (Department of Health, 1999).

In South Africa, the mining industry has played a major role in the movement of people from rural to urban areas. Whilst working on the mines, the majority of migrants reside away from their families, in single sex hostels which are often serviced by commercial sex workers (CSWs). In Carltonville, Gauteng Province, an area where 88 000 mine workers are resident, serviced by 400-500 CSWs, there is an HIV prevalence of 22% (UNAIDS, 1998). The large numbers of concurrent sexual partners allows a rapid spread of the HIV epidemic in the migrant mine worker population which is then spread to the rural areas when the men return home. The migrant workers on the mines are mostly men from neighbouring countries such as Lesotho, Botswana, Mozambique and up until the late 1990's, Malawi, as well as South African men (Williams and Campbell, 1996). The spread of HIV into South Africa, may thus have been facilitated by migrants from these neighbouring countries. Evidence of this was illustrated in 1986 by Sher where the HIV prevalence in migrant mine workers was 3.71% in Malawians and 0.07% in Mozambicans, a time when the majority of South African infections were occurring in the homosexual population (Sher, 1989).

In addition to migrant labourers who enter the country for work, there are also large numbers of illegal immigrants who enter the country from Lesotho, Mozambique, Swaziland and Zimbabwe, as well as further afield such as Zaireans, Ugandans and Zambians (van Haldenwang, 1996). Although the figures are rough estimates, it was suggested that in 1994 from two to eight million illegal immigrants entered the country, with Gauteng and KwaZulu/Natal Provinces having the highest numbers of illegal immigrants (van Haldenwang, 1996).

In the previous chapter we established that the HIV epidemic is segregated according to mode of transmission, with subtype B primarily associated with homosexual or bisexual mode of transmission and subtype C with heterosexual transmission. A concurrent study performed in Cape Town, based on *gag* p17 gene sequences (Williamson *et al.*, 1995), indicated that according to the age of the HIV epidemic (approximately four to six years at the time of sampling) and the DNA distance between subtype C *gag* sequences (6.8%), there appear to have been multiple introductions of subtype C into the country.

Rapid screening techniques for subtype elucidation often utilise the *env* region for subtype analysis. These techniques include heteroduplex mobility assay (HMA) and V3 loop serotyping. The *env* region represents less than 10% of genome however, and it is important to define subtypes using more than one region in order to detect recombinant viruses.

Restriction fragment length polymorphism (RFLP) analysis involves the identification of unique cutting restriction endonucleases that are able to distinguish between subtypes or strains. The unknown sample DNA is amplified by polymerase chain reaction after which the DNA is digested using the selected restriction endonucleases. The fragments produced are separated by agarose or polyacrylamide gel electrophoresis in order to visualise the patterns specific for each subtype or strain. A number of different virus strains have successfully been differentiated using this technique, including; dengue virus (Vorndam *et al.*, 1994), infectious bursal disease virus (Liu *et al.*, 1994) as well as HIV-1 in the protease gene (Pieniazek *et al.*, 1995, Heyndrickx *et al.*, 1996 and Janini *et al.*, 1996) and p24 region (Janini *et al.*, 1996). No radioactive labelling is required as for ^{35}S sequencing and the technique is faster and easier to perform than HMA.

In this study, in order to assess the geographical distribution of HIV-1 subtypes within South Africa, 83 samples from Johannesburg and Pretoria (in the Gauteng Province), Bloemfontein (in the Free State Province) and Durban (in the KwaZulu/Natal Province) were analysed by heteroduplex mobility assay (HMA) in the *env* region. Thirty-nine of these samples were also analysed in the *gag* region by restriction fragment length polymorphism (RFLP) analysis. In addition, 47 samples from Cape Town and four samples from Bloemfontein that had been analysed in the V3-V5 region of the *env* gene (see Chapter 2), sequenced in the p17 region of the *gag* gene (Williamson *et al.*, 1995 and van Harmelen *et al.*, 1997), or subtyped in both regions, were analysed by RFLP analysis of the *gag* gene. Samples subtyped in the *env* and *gag* regions were then compared in order to determine the presence of recombinant viruses in the regions studied.

A phylogenetic study taking into account all published Southern African subtype C sequences would give an indication of the genetic diversity within the South African heterosexual epidemic, as well as whether there is any subclustering with a country of origin. In this work, we thus phylogenetically compared all published South African subtype C V3 loop sequences with sequences from other southern African countries as well as at least two reference sequences from each subtype from A to H and J.

3.2) Materials and methods

3.2.1) Patient data

Convenience samples were collected between 1994 and 1996. Extracted proviral DNA was obtained from 83 samples collected in three geographically distinct urban centres in South Africa: Johannesburg (n=34) (obtained from Sue Lyons, National Institute of Virology, Johannesburg) and Pretoria (n=5) (obtained from Elna van der Ryst, University of the Free State) in Gauteng Province; Bloemfontein (n=24) (obtained from Elna van der Ryst, University of the Free State) in Free State Province; and Durban (n=20) (obtained from Dennis York, University of Natal) in KwaZulu/Natal Province. Samples from Johannesburg and Durban were obtained from women attending antenatal clinics, whereas the Bloemfontein samples were taken from individuals attending an HIV/AIDS clinic. Ethical approval was obtained by each institute from which the samples were donated.

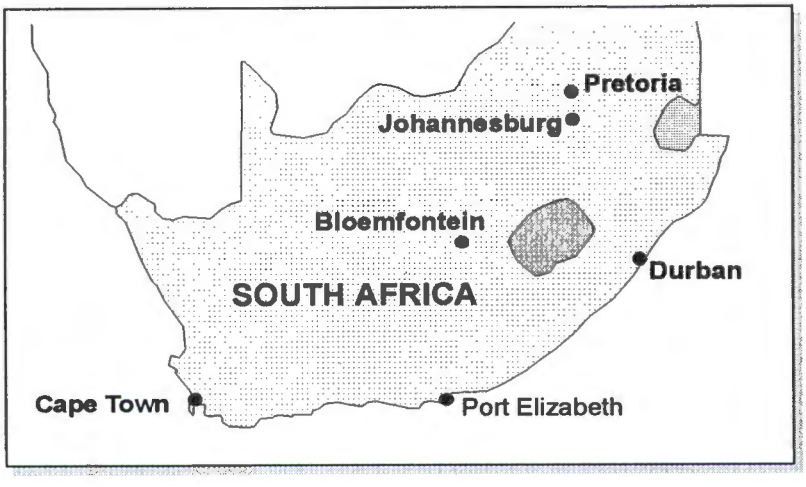


Figure 3.3: Map of South Africa, showing the five cities in distinct geographical locales sampled for subtyping.

We obtained demographic data for the analysed samples including: age, sex, race, date

of first serodiagnosis, CD4 count and sexual orientation (see Table 3.7). Demographic data from the Cape Town samples were reported previously in Chapter 2. The ages of the Johannesburg and Pretoria women were not recorded. Out of the 34 women from Johannesburg, 30 women were black and four were of Asian descent and all five of the Pretoria women were black. The Durban women were between the ages of 20 and 35 years, except for one baby that was born to an HIV positive mother who had attended the antenatal clinic. Of the 20 patients, 18 were black, one was of mixed race and one was Asian. The Bloemfontein samples were from both men and women at a ratio of 5:19 men to women, all infected by heterosexual transmission. All of the patients were black, except one woman who was of mixed race. The average age was 24 years with a range of 15-47 years. Eighteen out of the 24 patients had a CD4 count above 200 ($X10^6/l$). In addition, four Bloemfontein samples were infected by homosexual of bisexual mode of transmission (94ZA06FS, 94ZA14FS, 92ZA17FS and 91ZA25FS; analysed by HMA in the *env* region in Chapter 2). Samples were numbered by date of diagnosis, country of residence and sample number.

In addition, 5-10ml of EDTA-blood was collected from 47 patients attending the HIV/AIDS clinic at Somerset Hospital in Cape Town (n=42) and five patients sampled in the routine diagnostic laboratory at Groote Schuur hospital in Cape Town. We had previously subtyped twenty-one of the samples by both HMA in the V3-V5 region of the *env* gene (see Chapter 2) and by sequence analysis in the p17 region of the *gag* gene (Williamson *et al.*, 1995 and van Harmelen *et al.*, 1997) (see Chapter 2). In addition, we had analysed 16 samples by HMA alone in the *env* region and 10 samples by sequence analysis alone in the p17 region (van Harmelen *et al.*, 1997) (see Chapter 2).

Out of ten Cape Town patients previously subtyped by sequence analysis (van Harmelen *et al.*, 1997) and analysed by RFLP in the *gag* region in this study, eight reported their presumed origin of transmission due to travel or residence within five years prior to sampling, to be in South Africa. However, patient 93ZA019, a white male, infected by homosexual transmission reported having a possible origin of infection in Europe. Patient 93ZA030, a black male infected by heterosexual transmission, reported a presumed origin of infection in Zambia.

3.2.2) DNA extraction

DNA was extracted from peripheral blood mononucleocytes for the Johannesburg and Pretoria samples (n=39) or directly from blood in the Cape Town (n=47), Bloemfontein (n=28) and Durban (n=20) samples. DNA was isolated from Cape Town (Maureen Lambrick, Department of Clinical Virology, UCT Medical School) and Bloemfontein (Elna van der Ryst,

Department of Virology, University of the Free State) samples as described (Kawasaki, 1990 and Appendix A1). Johannesburg samples were extracted using Ficoll (Sigma, St Louis MO, USA) gradient separation and Proteinase K digestion in lysis buffer (TE lysis buffer, 10mM Tris-HCl pH7.5, 1mM EDTA, 0.5% Triton X-100, 1% Tween 20) (Sue Lyons, National Institute of Virology, Johannesburg and Appendix A1). Durban sample proviral DNA was extracted from 300µl of whole blood containing EDTA, using the Genomix extraction kit (Talent, SRL, Italy) (Savathree Madurai and Dennis York, Department of Virology, University of Natal and Appendix A1).

3.2.3) Polymerase chain reaction (PCR) amplification of patient samples for HMA analysis

For HMA analysis in this study, a 700bp V3 to V5 fragment of the *env* gene was amplified by nested polymerase chain reaction (PCR) using primers ED5/12 for the outer reaction and ES7/8 (NIH AIDS Research and Reference Reagent Program, Rockville MD, USA) for the inner reaction as described in Chapter 2 (section 2.2.3 and Appendix A2). Ten microlitres of patient DNA was added to the outer reaction, except for the Durban samples, which were more concentrated, where 5µl was added. For the inner reaction of the nested PCR, 3µl of the outer PCR was tranferred to the inner master mix (Delwart *et al.*, 1993). All PCRs were performed with the appropriate controls for contamination (see section 2.2.3); a water control as well as a sample from a HIV-negative patient. PCR amplified samples were electrophoresed on a 1% agarose gel in order to detect positive samples. The DNA was stained with ethidium bromide (10ng/ml) and visualised under a 256nm UV transilluminator (Appendix A3).

3.2.4) Heteroduplex mobility assay (HMA) of env sequences

Heteroduplex mobility assay (HMA) was performed on amplified DNA from all 83 samples (Delwart *et al.*, 1993, see Appendix A9) in this study. Unknown isolates were compared with South African reference plasmids developed (two subtype B, two subtype C and one subtype D, see chapter 2) as well as one subtype A reference plasmid from the NIH AIDS Research Reference and Reagent Program (USA) (see section 2.2.6). Any ambiguous isolates would have been compared with the full range of HMA reference plasmids, from subtype A to G (NIH AIDS Research and Reference Reagent Program, USA). The plasmids are shown in Table 2.5 (see Chapter 2). Samples still unidentified would have been subjected to sequence analysis. Heteroduplexes were subjected to non-denaturing polyacrylamide gel electrophoresis at 200V for five hours through the 5%, 19cm/16cm, 1.5mm thick (Hoefer SE600, Hoefer Scientific Instruments, San Francisco, USA) gel (see section 2.2.6). Gels were stained for 30 minutes with 10ng/ml ethidium bromide and visualised with 256nm UV transillumination (Appendix A9).

3.2.5) Polymerase chain reaction (PCR) amplification of patient samples for RFLP analysis

In this study, two fragments from the *gag* region of HIV-1 (400bp and 650bp respectively) were amplified for RFLP analysis. The 400bp fragment contained the complete p17 gene coding for the matrix protein of HIV-1. The 650bp fragment contained the p17 gene as well as part of the p24 gene coding for the viral capsid protein. A total of 86 patient samples were amplified for RFLP analysis (Cape Town n=47, Johannesburg n=20, Bloemfontein n=17 and Durban n=2). PCR conditions are recorded in detail in Appendix A2.

Proviral DNA was amplified using nested PCR primers; LTRu5 and Pol2 (van Harmelen *et al.*, 1999) as outer primers to amplify a 1.5Kb fragment and LTR310j (van Harmelen *et al.*, 1999) and SK431 (Lynch *et al.*, 1992) (650bp fragment) or LTR310j and *gag*778 (van Harmelen *et al.*, 1999) (400bp fragment) (see Table 3.2). In addition, a 400bp fragment from cloned, previously sequenced, p17 gag sequences (Williamson *et al.*, 1995) was amplified using the inner primers only (LTR310j/*gag*778) in order to determine the reliability of the RFLP technique.

Table 3.2: Primers for PCR amplification of the *gag* region for RFLP analysis.

Name	#Position	*Sequence
LTRu5	608bp-630bp	5'ATCTCTAGCAGTGGCGCCCGAAC3'
Pol2	2265bp-2279bp	5'**CGGAATT**CAGGGTCGTTGCCAAAG'3
LTR310j	753bp-773bp	5'**TAGTCGAC**GACTAGCGGAGGCTAGAAG3'
SK431	1472bp-1499bp	5'TGCTATGTCAGTTCCCCTTGGTTCTCT3'
*gag*778	1232bp-1255bp	5'CACCTAGAACTTT[A/G]AA[T/C]GCATGGG3'

#Sequence positions were taken from HXB2 (Genebank acession number K03455)
*Bold letters represent restriction enzyme sites, *EcoR*1 and *Sal* I that could be used for cloning purposes.

The PCR master mixes (50µl) contained:

- 5µl 10X PCR (500mM KCl, 100mM Tris-HCl (pH9.0), 1% Triton X-100) (Promega, Madison, WI, USA),
- 1% DMSO, 1% glycerol,
- 200µM each of dNTPs,
- 1.5mM $MgCl_2$, (outer reaction) and 1.8mM $MgCl_2$, (inner reaction),
- 400nM each primer; LTRu5 and Pol2 (outer reaction) and LTR310j and SK431 or *gag*778 (inner reaction),
- 2.5U *Taq* DNA Polymerase (outer reaction) and 2U *Taq* DNA Polymerase (Promega, Madison, WI, USA) (inner reaction) and

- 10µl of patient DNA (outer reaction) or 3µl of outer PCR reaction (inner reaction).

If cloned and already sequenced reference *gag* plasmid DNA was used, 20ng of DNA was added.

The PCR amplification was performed using the Techne PHC-2 thermocycler under the following conditions:

Outer reaction:

- initial denaturation at 94°C for 2 minutes,
- 94°C for 1 minute, 45°C for 1 minute and 72°C for 2 minutes (5 cycles)
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (30 cycles)
- final extension at 72°C for 6 minutes.

Inner reaction:

- initial denaturation at 94°C for 2 minutes,
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (30 cycles)
- final extension at 72°C for 6 minutes.

A water control, as well as an HIV-negative patient DNA control, was included for all PCR reactions in order to monitor for possible contamination and all necessary precautions were taken in order to avoid contamination (see section 2.2.3). PCR amplified DNA was electrophoresed in a 1% agarose gel in 1XTAE and visualised with ethidium bromide (10ng/ml) at 256nm with UV transillumination (Appendix A3).

3.2.6) Restriction endonuclease site analysis (RESA) of HIV-1 gag sequences

In order to determine unique cutting restriction endonucleases for RFLP analysis of HIV-1 subtypes, we obtained *gag* HIV-1 consensus sequences for subtypes A, B, C and D from the HIV sequence database (http://hiv-web.lanl.gov/). The unique restriction sites and predicted product length for each subtype was determined using a computer program (Genepro Version 6.1, Riverside Scientific Enterprises, WA, USA). I have named the process of determining unique cutting restriction endonuclease sites using Genepro restriction endonuclease site analysis (RESA).

In addition, the following published full length *gag* sequences (n=25) from the HIV sequence database were analysed with Genepro to determine RESA patterns for this study: subtype A, VI32 (Gabon), 92UG037.1, U455 (Uganda) and K89 (Kenya); subtype B, JRFL, HXB2, RF (USA) and OYI (Gabon); subtype C, Nof, Kon (South Africa), DJ259 (Djibouti),

ETH2220 (Ethiopia), LBV10-5, VI1313 (Gabon), SM145 (Somalia), UG268 (Uganda), ZAM18, ZAM19, ZAM20, ZAM716-17 (Zambia) and 92BR025 (Brazil), and subtype D, 94UG114.1 (Uganda), ELI, Z2Z6 and NDK (Democratic Republic of Congo, formerly Zaire).

South African sequences of the p17 *gag* region (Williamson *et al.*, 1995) (n=31) and the sequences from isolates 94ZA060 and 94ZA061 were also analysed using Genepro to determine RESA patterns for our study: subtype A, 94ZA060, 94ZA061; subtype B, 88ZA001, 93ZA008, 91ZA011, 90ZA012, 89ZA017, 87ZA019, 91ZA021, 93ZA022, 92ZA025, 93ZA037, 92ZA045, 93ZA051, subtype C, 93ZA006, 93ZA010, 92ZA023, 93ZA029, 91ZA030, 93ZA032, 93ZA035, 92ZA036, 90ZA040, 93ZA046, 93ZA048, 94ZA063, 93ZA200, 93ZA201, 92ZA202, 93ZA204, 92ZA205, 93ZA207 and subtype D, 91ZA034.

3.2.7) Restriction fragment length polymorphism (RFLP) analysis of gag sequences

In this study, the amplified *gag* fragment from a total of 86 samples was analysed by RFLP analysis. Eight microlitres of amplified PCR product were digested with with 10U of restriction endonuclease (Boehringer Mannheim, Hamburg, Germany) for two hours at 37°C (Appendix A4). All subtype C isolates were confirmed by *Acc*I digestion, since *Acc*I is able to digest subtype B, but not subtype C. Tracking dye (2μl, 0.25% bromophenol blue, 0.25% xylene cynol FF and 30% glycerol in water) was then added and the sample was electrophoresed in a 4% agarose (MetaPhore, USA) horizontal gel at 5 Volts/cm using TAE buffer (40mM Tris-acetate, 10mM EDTA, pH8.0) (Appendix A3). Molecular weight markers VI and VIII (Boehringer Mannheim, Hamburg, Germany) were used as DNA fragment size markers. The gels were stained with 10ng/ml of ethidium bromide and the DNA fragments visualised at 256nm with UV transillumination.

3.2.8) Cloning and sequencing of the gag sequences

The 650bp *gag* fragment from two isolates, 94ZA060 and 94ZA061, a husband and wife pair were amplified by PCR (see section 3.2.5) and cloned the *gag* fragments into the pMos-blue T-vector polycloning site (Amersham, Buckinghamshire, UK) by TA cloning, as recommended by the manufacturers (Appendix A5). Ligated DNA was transformed into competent *Escherischia coli* strain DH5α (Appendix A6) and white colonies containing putative clones screened for inserts by minipreparation of DNA and *Pst*I/*Eco*RI double digestion (Appendix A4 and A7). DNA fragment electrophoresis was performed in a 3% agarose gel containing ethidium bromide (10ng/ml) and visualised by UV transillumination at 256nm (Appendix A3).

The two 650bp *gag* fragments (94ZA060 and 94ZA061) were sequenced in both directions until unambiguous using forward and reverse primers T7 (5'AATACGACTCACTATAGGG3') and U19 (5'ACGTCGTGACTGGGAAAACC3') (pMOS-blue-T vector, Amersham, Buckinghamshire, UK) respectively, by the Pharmacia™ ALFexpress® automated sequencer using fluorescent Cy5-labelled primers (Appendix A8.2). DNA sequences were translated to amino acid sequences for V3 loop analysis using Genepro (Version 6.10, Riverside Scientific Enterprises WA, USA).

3.2.9) Sequence analysis of the env V3 region

Thirty-nine subtype C reference isolate V3 sequences from African countries, India and Brazil were obtained from the HIV sequence database (http://hiv-web.lanl.gov/) for comparison with the South African sequences (see Table 3.3). Seventy-eight South African V3 sequences included those taken from studies in Gauteng Province (Bredell *et al.*, 1998) (n=43), KwaZulu/Natal Province (Moodley *et al.*, 1998) (n=22) and Western Cape Province (n=7) (Becker *et al.*, 1995 and Engelbrecht *et al.*, 1995).

Table 3.3: Subtype C reference sequences obtained from the HIV sequence database, 1998 for phylogenetic comparison with all published South African subtype C sequences, according to subtype and country of origin.

	Country												
	ZW	ZM	MW	MZ	BW	ZR	DJ	UG	SO	SN	IN	BR	ZA
No.	4	4	9	8	1	1	2	1	1	1	6	1	78

Zimbabwe (ZW), Zambia (ZM), Malawi (MW), Mozambique (MZ), Botswana (BW), Zaire (ZR), Djibouti (DJ), Uganda (UG), Somalia (SO), Senegal (SN), India (IN), Brazil (BR) and South Africa (ZA). Number of sequences, No.

The seven V3 loop sequences from subtypes A to D from our study in Chapter 2 were included for comparison, as were five subtype C mother-baby sequences from Mr Shayne Loubser (Department of Medical Microbiology, UCT) (Genbank numbers, AF095834 - AF095838) and sequence 97ZA347TS (Genbank number, AF095831), which will be discussed in more detail in Chapter 4. Two reference V3 sequences for each subtype from A, B, D to H and J were taken from the NIH AIDS Reference and Reagent Program kit (USA) (subtypes A, B and D to H) and the HIV sequence database (1998) (subtype J). The names of the reference sequences from subtypes A, B and D to H are illustrated in Table 2.5. Subtype J is represented by sequences SE91733 and SE92809 from former Zairean individuals residing in Sweden. In addition, a single subtype A and subtype B sequence from South Africa was included in the phylogenetic analysis (Moodley *et al.*, 1998) (see figure 3.7).

All 140 V3 sequences were aligned using Clustal X (Thompson, 1997) and final adjustments were made manually. The distance matrix neighbour joining method was used to generate the phylogenetic tree with insertions and deletions ignored. The tree topology was inferred using the Kimura two-parameter algorithm (Kimura, 1980) and the reliability of the tree topology was assessed by doing 100 bootstrap replicates in TREECONW (van der Peer and de Wachter, 1994). The phylogenetic tree was drawn using the TreeView program (Page, 1996) with the SIVcpz-gab sequence as the outgroup. All 117 subtype C V3 loop DNA sequences were translated into amino acid sequences using Genepro (Version 6.10, Riverside Scientific Enterprises, WA, USA).

3.2.10) Sequence analysis of the gag 650bp fragments from 94ZA060 and 94ZA061

The 650bp sequences obtained from the husband and wife samples, 94ZA060 and 94ZA061 in this study were aligned with at least two reference sequences from subtypes A to H and J, using Clustal X (Thompson, 1997) and final adjustments were made manually. Reference sequences are shown in table 3.4. The phylogenetic tree was constructed using the distance matrix neighbour joining method and insertions and deletions were ignored. The topology of the phylogenetic tree was inferred using the Kimura two-parameter algorithm (Kimura, 1990) and 1000 bootstrap replicates were performed to assess the reliability of the tree topology using the TREECONW program (van der Peer and de Wachter, 1994). The phylogenetic tree was drawn using TREECONW (van der Peer and de Wachter, 1994) with the SIVcpz-gab sequence as the outgroup.

3.3) Results

In this study, a total of 83 samples taken from heterosexually infected individuals from Johannesburg, Bloemfontein and Durban were subtyped using heteroduplex mobility assay (HMA) in the *env* V3-V5 region, identifying 77 subtype C and six subtype B viruses. RFLP analysis was developed as a tool for the rapid identification of *gag* subtypes. Thirty-nine of the above eighty-three samples were analysed in the p17 region of the *gag* gene. A further 47 samples from patients infected by heterosexual or homosexual transmission in Cape Town were also analysed by RFLP in the p17 region of the *gag* gene. These samples had either been previously subtyped in the *env* region by HMA (see Chapter 2), sequenced in the *gag* p17 region (Williamson *et al.*, 1995 and van Harmelen *et al.*, 1997), or both. Out of the total 86 isolates subtyped by RFLP in the *gag* region, two belonged subtype A, 25 to subtype B, 58 to subtype C and one to subtype D. Finally, the phylogenetic relationship between 117 subtype C sequences

was also investigated.

Table 3.4: Reference sequences obtained from the HIV sequence database, 1998 for phylogenetic comparison with sequences from isolates 94ZA060 and 94ZA061 from Cape Town.

	Name of sequence	Country of origin
subtype A	U455	Uganda
	92UG037	Uganda
	K89	Kenya
	VI32	Ivory Coast
subtype B	RF	Haiti
	HXB2	United States
	JRFL	United States
	OYI	Gabon
subtype C	92BR025	Brazil
	ETH2220	Ethiopia
	ZAM18	Zambia
	UG268	Uganda
	DJ259	Djibouti
	SM145	Somalia
	VI1313	Gabon
	Nof	South Africa
	Kon	South Africa
subtype D	NDK	DRC, formerly Zaire
	Z2Z6	DRC, formerly Zaire
	ELI	DRC, formerly Zaire
subtype AE	CM240	Thailand
	93TH253	Thailand
	90CF402	Central African Republic
subtype F	93BZ162	Brazil
	93BR020	Brazil
	VI69	Belgium
subtype G	SE6165	Sweden
	HH8793-11	Finland
	DRCBL	Belgium
subtype H	VI997	Belgium
	VI991	Belgium
	90CF056	Central African Republic
	VI557	DRC, formerly Zaire
Subtype J	SE92809	Sweden
	SE91733	Sweden

DRC, Democratic Republic of the Congo

3.3.1) Computer aided restriction endonuclease site analysis (RESA) of HIV-1 gag sequences

Four restriction endonucleases that would generate unique fragment patterns for each subtype were identified by RESA of the *gag* gene consensus sequences for subtypes A to D (HIV sequence database, 1998). Restriction endonucleases; *Swa*I, *Acc*I, *Alu*I and *Xmn*I were predicted to distinguish subtypes A, B, C and D respectively (see figure 3.4).

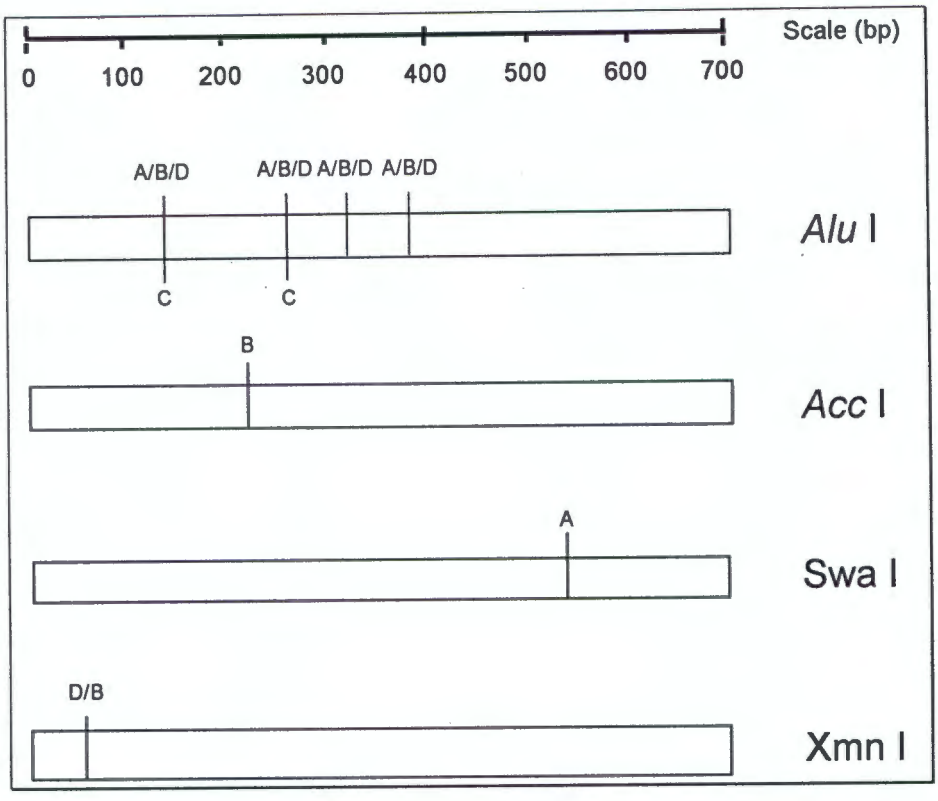


Figure 3.4 : The restriction endonuclease site analysis (RESA) of the partial *gag* consensus sequences for subtypes A, B, C and D in this study, showing where the identifying restriction endonucleases digested and the fragment sizes in base pairs which are produced.

3.3.3) Determination of endonuclease sites in published gag sequences

In order to determine the reproducibility of the technique, 33 South African p17 *gag* sequences (Williamson *et al.*, 1995 and unpublished data) were analysed using RESA to determine the fragment sizes which would be produced after digestion with *Alu*I, *Acc*I, *Swa*I and *Xmn*I respectively. Samples included 18 subtype C isolates, 12 subtype B isolates, two subtype A isolates and one subtype D isolate. All of the sequences were correctly subtyped using the four restriction endonucleases.

In addition, to determine whether the RFLP strategy would identify isolates from outside of South Africa, twenty-five published international sequences (HIV sequence database, 1998) were analysed by RESA (see section 3.2.6). Samples included thirteen subtype C full-length *gag* sequences and RESA analysis determined that the 400bp fragment was more reliable than the 650bp fragment for subtype C detection. Twelve out of the thirteen subtype C sequences would have been correctly identified by *Alu*I digestion in the 400bp p17 fragment. The remaining subtype C isolate (LBV10-5 originating from Libreville) contained an additional *Alu*I site,

producing a restriction fragment pattern similar to subtypes A, B and D, but did not have the specific restriction sites for the identification of the other subtypes and was classified as non-typable.

Subtype B isolates (n=4) would be detected using either the 400bp or 650bp fragments after *Acc*I digestion. The four subtype A sequences, however, would be detected by *Swa*I digestion in the 650bp fragment only.

Finally, only three of the four subtype D isolates would have been correctly identified using *Xmn*I restriction endonuclease in either the 400bp or 650bp fragments, since one of the subtype D isolates (Z2Z6) contained an *Acc*I site incorrectly identifying the sample as a subtype B. Such a sample would be identified as a B/D isolate. The remaining three isolates were correctly identified as subtype D since *Xmn*I alone and not *Acc*I would be able to digest the isolates. The single subtype D isolate sequenced from South Africa was correctly identified as a subtype D by RESA using either the 400bp or 650bp fragment since no *Acc*I site was present.

3.3.4) Restriction fragment length polymorphism (RFLP) strategy for subtype identification

Since the majority of isolates from South Africa identified at the time of the study were subtype C (Williamson *et al.*, 1995; van Harmelen *et al.*, 1997 and Bredell *et al.*, 1998), a strategy for identification of local isolates was developed based on the predicted restriction endonuclease sites by RESA (see figure 3.5).

A total of 86 isolates from Johannesburg (n=20), Bloemfontein (n=17), Durban (n=2) and Cape Town (n=47) were analysed by RFLP in the p17 (400bp) or p17, partial p24 region (650bp) of the *gag* gene in this study. PCR amplified 400bp fragments would be initially digested with *Alu* I in order to detect subtype C. Samples identified as subtype C would then be re-amplified and digested with *Acc* I that is able to digest subtype B, but not subtype C, as confirmation of subtype. A 400bp or 650bp fragment would then be amplified from any unidentified samples, and digested with *Acc* I in order to detect subtype B. Remaining unidentified isolates would be reamplified and the 650bp fragment subjected to *Swa* I amplification in order to detect subtype A. Finally, unidentified samples would be PCR amplified and either the 400bp or 650bp fragment digested with *Xmn* I for the identification of subtype (see figure 3.5).

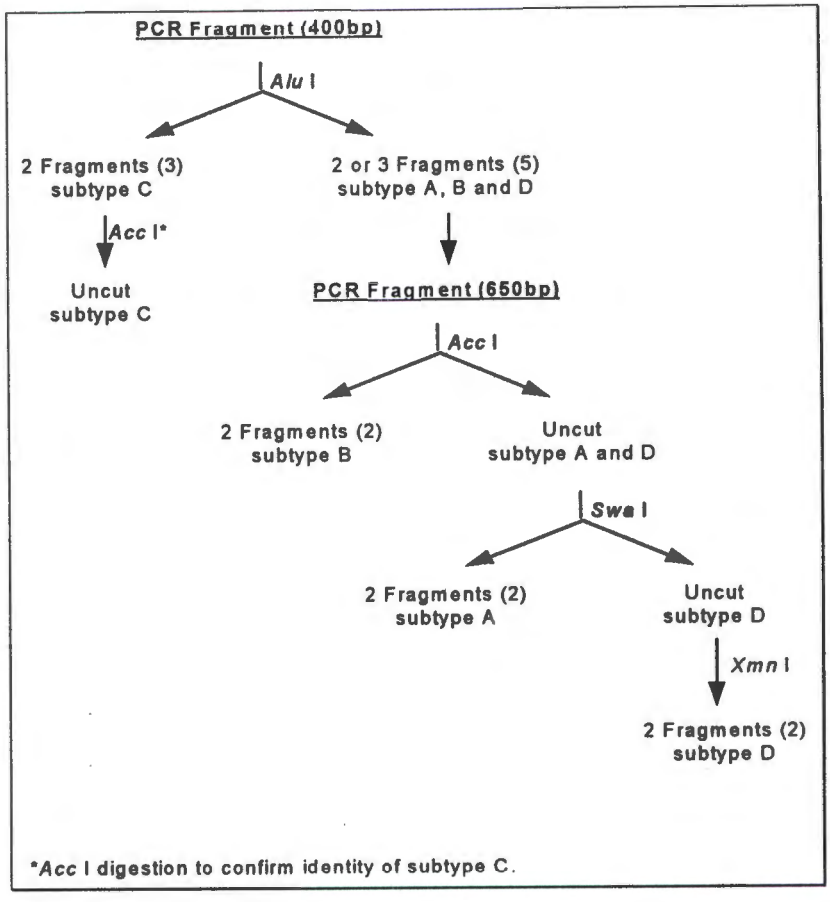


Figure 3.5: The RFLP strategy developed in this study, starting with digestion of the 400bp *gag* PCR product with *Alu* I, to detect subtype C's, followed by amplification of a 650bp fragment and digestion with *Acc*I, to detect subtype B's, *Swa* I to detect subtype A's and finally *Xmn*I to detect subtype D's, which are digested with *Xmn*I, but not *Acc*I. The predicted fragments from the consensus sequence analysis are shown in brackets.

Initially, 31 of the 33 previously sequenced HIV-1 p17 isolates analysed by RESA (Williamson *et al.*, 1995 and unpublished data) were subtyped by RFLP in a blinded fashion in this study, in order to determine the reliability of the technique (see Table 3.5). Two were subtype A, 11 were subtype B, 17 were subtype C and one was a subtype D. No subtype C isolates were digested with *Acc*I, confirming their identity. Figure 3.6 shows the RFLP patterns obtained using *Acc*I to detect subtype B (see figure 3.6a) and *Alu*I to detect subtype C (see figure 3.6b) in unknown samples. Figure 3.6 shows the RFLP patterns obtained using *Acc*I to detect subtype B (see figure 3.6a) and *Alu*I to detect subtype C (see figure 3.6b) in unknown samples.

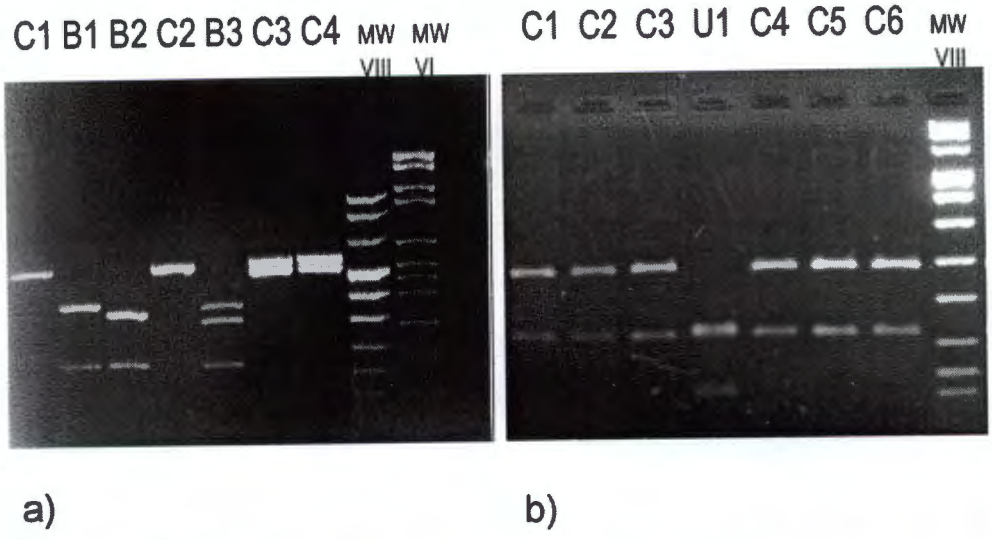


Figure 3.6: RFLP gels showing the unique restriction fragment patterns produced by digestion of the gag p17 fragment with *Acc*I (a) and *Alu*I (b). a) Three subtype B isolates identified by *Acc*I which only digests subtype B. Four subtype C isolates identified by *Alu*I digestion are confirmed as subtype C by *Acc*I digestion, which does not digest subtype C. The variations in size and fragment number are caused by insertions and deletions in the *gag* gene, but do not hinder subtype designation. b) Six subtype C isolates identified by *Alu*I digestion which results in only two visible fragments for subtype C, but multiple smaller fragments for subtypes A to D (see figure 3.4). Molecular weight markers are MW VI and MW VIII (Boehringer Mannheim, Hamburg, Germany).

Table 3.5: *Gag* subtype designation by RFLP analysis and RESA compared to the genetic subtype identified by *gag* sequencing.

	South Africa (n=33)		**International (n=25)**
	RFLP	RESA	RESA
Subtype A	2A	2A	4A
Subtype B	11B, 1ND	12B	4B
Subtype C	17C, 1ND	18C	12C, 1NT
Subtype D	1D	1D	3D, 1MT

ND, Not determined
NT, Not typable
MT, Mis-typed as a subtype B

3.3.5) Subtype designation by restriction fragment length polymorphism (RFLP) of the gag gene

Once the reliability of the RFLP technique had been determined, 16 Cape Town samples that had been subtyped by HMA in the *env* region in the previous chapter were analysed by RFLP. Analysis of the 400bp *gag* fragment identified four subtype C isolates. In addition, 12 subtype B isolates were identified using the 650bp fragment. No subtype C samples were digested by *Acc*I, confirming their subtype. Although all subtypes could be successfully identified using the four restriction endonucleases, minor variations in the

fragment sizes between samples of the same subtype were observed due to insertions and deletions.

In addition, 39 patient samples from Johannesburg, Bloemfontein and Durban, identified by HMA in the *env* region (see section 3.3.6) were subtyped by RFLP of the partial *gag* gene fragment. The majority of these samples belonged to subtype C (37 out of 39), including one male Bloemfontein patient infected by bisexual transmission, although two male Bloemfontein patients infected by homosexual/bisexual transmission (92ZA17FS and 91ZA25FS) were identified as being infected with a subtype B virus. The homosexual/bisexual Bloemfontein patients subtyped by HMA were analysed in Chapter 2 and the results used for comparison with RFLP subtyping in this chapter.

3.3.6) Subtype identification by heteroduplex mobility assay (HMA) of the env gene

It was established in this study that subtype C predominated in the three major urban centres in South Africa where an average of 92.8% of the 83 isolates (n=77) analysed by HMA were classified as subtype C (see figure 3.7). The patients were specifically selected as having been infected by heterosexual transmission in order to determine the genetic diversity of HIV in the urban heterosexual populations in South Africa. A total of 7.2% of the samples tested: two of the 34 samples from Johannesburg (94ZA267J and 94ZA263J), one of the 24 samples from Bloemfontein (94ZA20FS) and three of the 20 samples from Durban (94ZA025DBN, 94ZA065DBN, 94ZA115DBN) were infected with subtype B viruses (see figure 3.7).

All of the samples could be subtyped using the South African reference plasmids, 93ZA004, 93ZA009 (subtype B), 93ZA040, 94ZA067 (subtype C) and 93ZA034 (subtype D), as well as one of the three subtype A reference plasmids (RW20, IC144, or SF170) from the HMA kit (NIH AIDS Research Reference and Reagent Program, USA). None of the amplified samples needed to be analysed against additional plasmids, in order to successfully identify them.

There were no discrepancies in subtype designation between the *env* and *gag* regions using HMA or RFLP in these isolates, indicating no recombinant viruses in the regions studied (see Table 3.6).

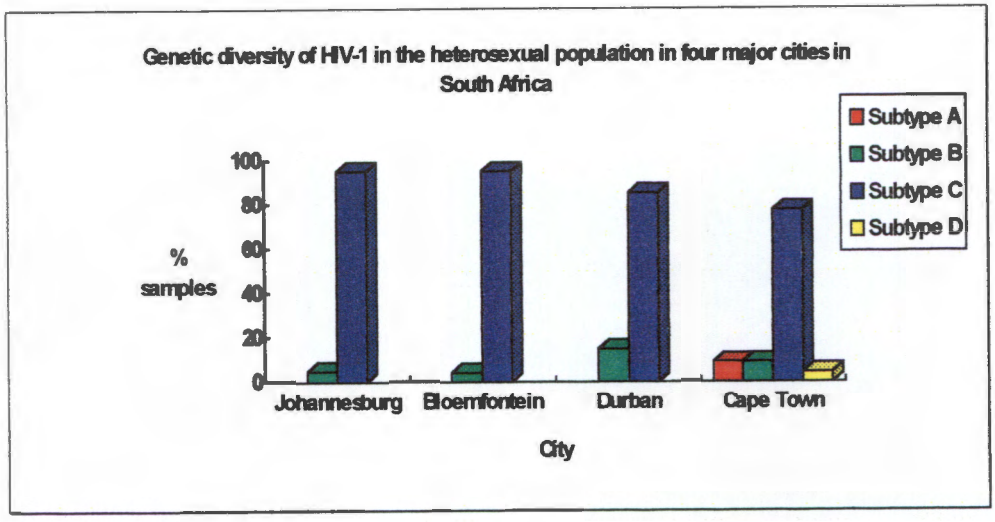


Figure 3.7: Graph illustrating the genetic diversity within the heterosexual urban populations in four major cities in South Africa, as determined in this study. Cape Town data from the previous chapter has been included for comparitive purposes. Similar subtype distribution between subtypes B and C occurs in all four cities in the heterosexual population, with subtype C making up 77% to 96% of the samples (Cape Town to Bloemfontein) and subtype B only 4% to 15% of the samples (Bloemfontein to Durban). Greater subtype diversity occurs in Cape Town with subtypes A, B, C and D present in the heterosexual population.

Table 3.6: A comparison of the subtype obtained by RFLP in the *gag* region and HMA in the *env* region for samples from Johannesburg (n=20), Durban (n=2), Bloemfontein (n=17) and Cape Town (n=38), indicating no discrepancies were found between subtype designation in the *env* and *gag* regions studied.

		***env* HMA**							
		Johannesburg	Durban	#Bloemfontein		#Cape Town			
	Subtype	C	C	B	C	A	B	C	D
	A	-	-	-	-	2	-	-	-
gag	B	-	-	2	-	-	21	-	-
RFLP	C	20	2	-	15	-	-	14	-
	D	-	-	-	-	-	-	-	1

* An additional ten samples were analysed by RFLP in the *gag* region from Cape Town samples which had been previously sequenced in the p17 region.
# A number of samples from Bloemfontein and Cape Town were analysed in Chapter 2.

3.3.7) Genotypic analysis of the V3 loop region of the env gene

In order to determine the genetic diversity within South African subtype C sequences and to determine whether there was any clustering of South African sequences with a southern African country of origin, all published Southern African subtype C sequences were

phylogenetically compared. Phylogenetic analysis of the 105 subtype C sequences (see figure 3.8) from southern African countries gave no indication of clustering with a country of origin, however. Only a small number of subtype C samples grouped together with bootstrap values of over 70%. The average intrasubtype DNA difference for the South African subtype C's was 14.06%, which was similar to the average intrasubtype DNA distance of 13.97% for all 117 of the subtype C sequences analysed. The South African sequences are thus as divergent from each other as they are from the remaining subtype C isolates from southern Africa and the rest of the world.

The V3 loop amino acid sequence that was generated for all 117 of the subtype C sequences analysed in this study was found to be 35 amino acids in length for 111/117 of the isolates (see figure 3.9). In 16 samples a single amino acid deletion had occurred at different positions in the V3 loop, resulting in a V3 loop of 34 amino acids. In three samples; 92BR025 (Brazil), 93MW960 and 93MW956 (Malawi) there was an insertion of isoleucine at position 280 and an amino acid deletion at position 291, maintaining the V3 loop 35 amino acid length. Out of the 117 isolates, 114 maintained the characteristic GPGQ tetrapeptide crown. One South African isolate (95ZA961ZA) had a GPGR tetrapeptide crown and one South African isolate (95ZA742ZA) and one Zimbabwean isolate (SE9337) had a RPGQ crown motif. The octameric tip was predominantly RIGPGQTF, followed by RIGPGQAF, although a small number of other variations were identified. Positively charged argenines in positions 276, 290 and 297 are associated with a syncitia-forming phenotype (Fouchier *et al.*, 1992 and De Wolf *et al.*, 1994). Position 276 had an argenine substitution present in only two of the southern African subtype C isolates, NOF and 89ZA067. No data was available on the stage of disease for either of these isolates.

Subtype C viruses characteristically lack the first glycosylation site proximal to the start cysteine of the V3 loop at position 265-267 and only 17 of the 117 isolates retained this glycosylation site (Abebe *et al.*, 1997 and Korber *et al.*, 1997). In the remaining isolates, valine was the most common substitution. Eleven of these isolates were from the KwaZulu/Natal Province of South Africa. The first glycosylation site within the V3 loop, at positions 271-273 was absent in five isolates, all originating from South Africa. No additional glycosylation sites were identified within the V3 loop.

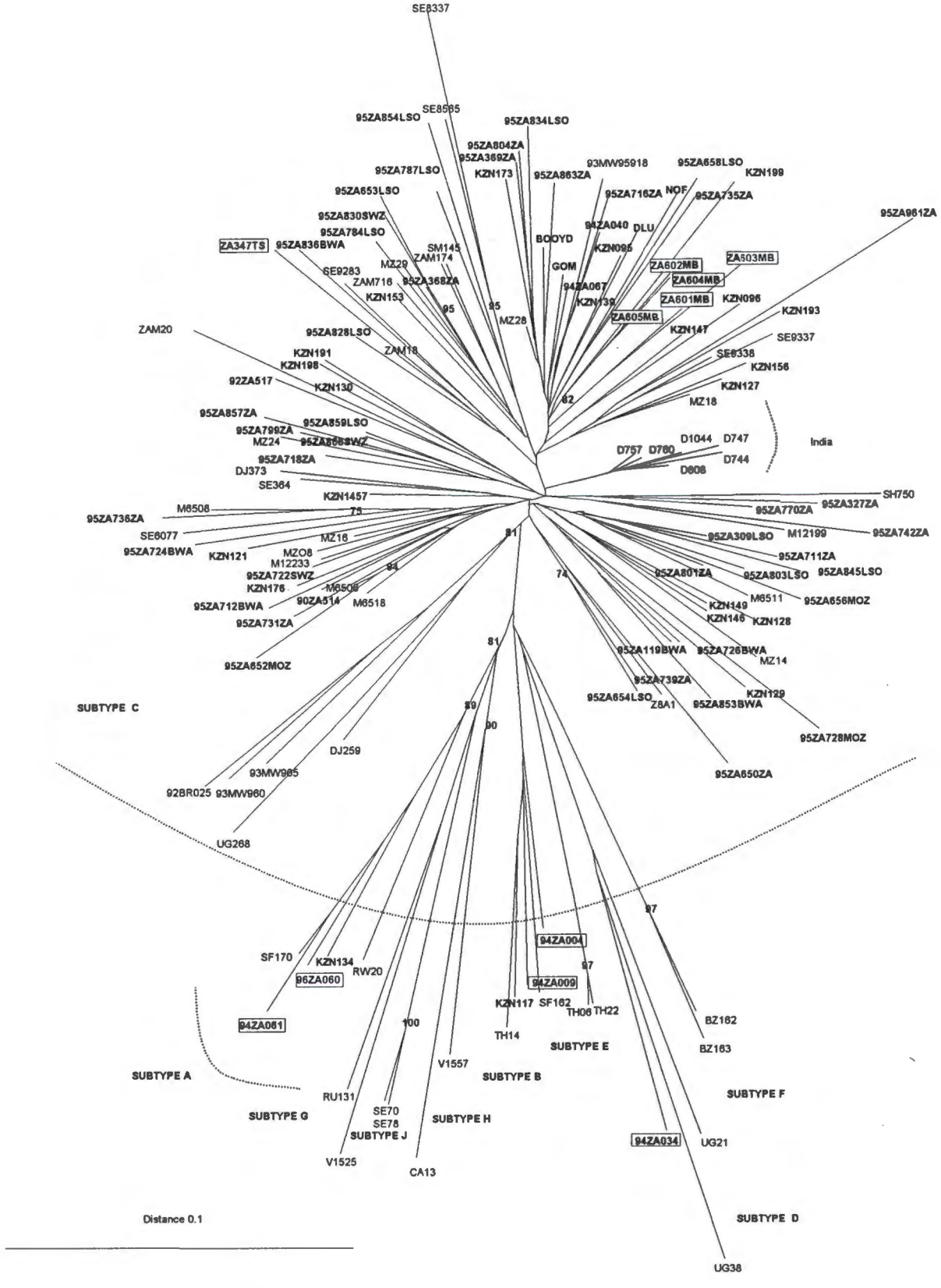


Figure 3.8: Phylogenetic tree of all published southern African V3 loop sequences showing no South African subclusters. The South African sequences are in bold and isolates sequenced in our laboratory are indicated in blocks. The DNA distance is indicated by the bar as 0.1 amino acid replacements per site.

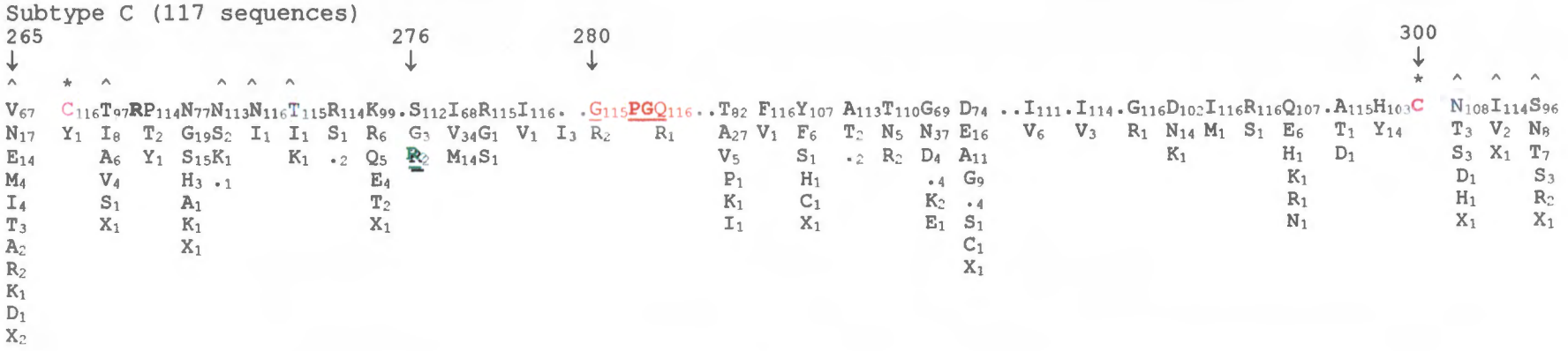


Figure 3.9: An alignment of the amino acids comprising the V3 loop for 117 subtype C sequences, including the two South African sequences analysed in the previous chapter (93ZA040 and 94ZA067) and isolate 97ZA347TS which will be discussed in more detail in Chapter 4. The sequence shown at the top of the alignment is the consensus subtype C sequence, taken from Korber *et al* (1997). A period (.) indicates a deletion and bold letters in the consensus sequence show conservation in all 117 sequences. An "X" denotes ambiguous or no sequence with no amino acid assigned. *N*-linked glycosylation sites are indicated with carets and the start and end cysteine residues are shown with asterisks. The V3 crown is shown underlined. An argenine substitution at positions 276, 290, or 297 that increases the positive charge of the V3 loop and may indicate the ability to form syncitia in two isolates is shown in bold and underlined. The other arginine substitutions indicative of an SI phenotype, at positions 290 and 294 were not present in any of the isolates (Fouchier *et al*., 1992 and De Wolf *et al*., 1994).

3.3.8) Phylogenetic analysis of the 650bp gag region of 94ZA060 and 94ZA061

Isolates 94ZA060 and 94ZA061 that were analysed in the *gag* region were found to group together with a bootstrap value of 96%. In addition, the two sequences group with the subtype A reference sequences, U455, 92UG037, K89 and VI32, as well as the subtype AE reference sequences, CM240, 93TH253 and 90CF402 with a bootstrap value of 100%. The subtype AE sequences form their own sub-cluster with a bootstrap value of 100% (see figure 3.10). The average intrasubtype DNA distance of the South African subtype A sequences with the reference subtype A sequences determined in this study, is 9.57%, with a range of 6.4% (94ZA061 and 92UG037) to 11.0% (94ZA060 and U455).

3.4) Discussion

The HIV epidemic in South Africa was shown to segregate according to mode of transmission in the previous study (see Chapter 2). The heterosexual epidemic started later than the homosexual epidemic and is now responsible for the majority of infections occurring monthly in South Africa (Swanevelder, 1995; WHO, 1998 and Department of Health, 1999). The current study involved the sampling of females attending antenatal clinics in Johannesburg, Pretoria and Durban, in an attempt to select samples specifically from heterosexually infected patients. In addition, male and female patients infected heterosexually were sampled from a Bloemfontein HIV/AIDS clinic and heterosexually infected patients from the Cape Town study (see Chapter 2) were all analysed in order to identify the predominant subtype(s) causing the overwhelming heterosexual epidemic in South African urban centres.

Out of a total of 109 isolates taken from heterosexually infected patients from four major urban centres in South Africa; Johannesburg and Pretoria (Gauteng Province), Bloemfontein (Free State Province), Durban (KwaZulu/Natal Province) and Cape Town (Western Cape Province) and analysed in the *env* region, *gag* region, or both, the majority of individuals (92%) were found to be infected with subtype C. The results from this study are similar to results from our Cape Town study reported in the previous chapter, where subtype C was responsible for the majority (77.2%) of the heterosexual infections and subtype B for only 9%. A greater subtype diversity was detected in Cape Town, with two subtype A and one subtype D viruses detected in the heterosexual population (see figure 3.7 and Table 3.6). However, a greater number of samples were analysed, thus it was more likely that low prevalence subtypes would be detected. Subtypes A and D, the most common subtypes in Central and East Africa, were not detected outside of Cape Town, although subtype A has subsequently been reported in Durban (Moodley *et al.*, 1998 and van Harmelen *et al.*, 1999) (see Chapter 2). Although subtype B

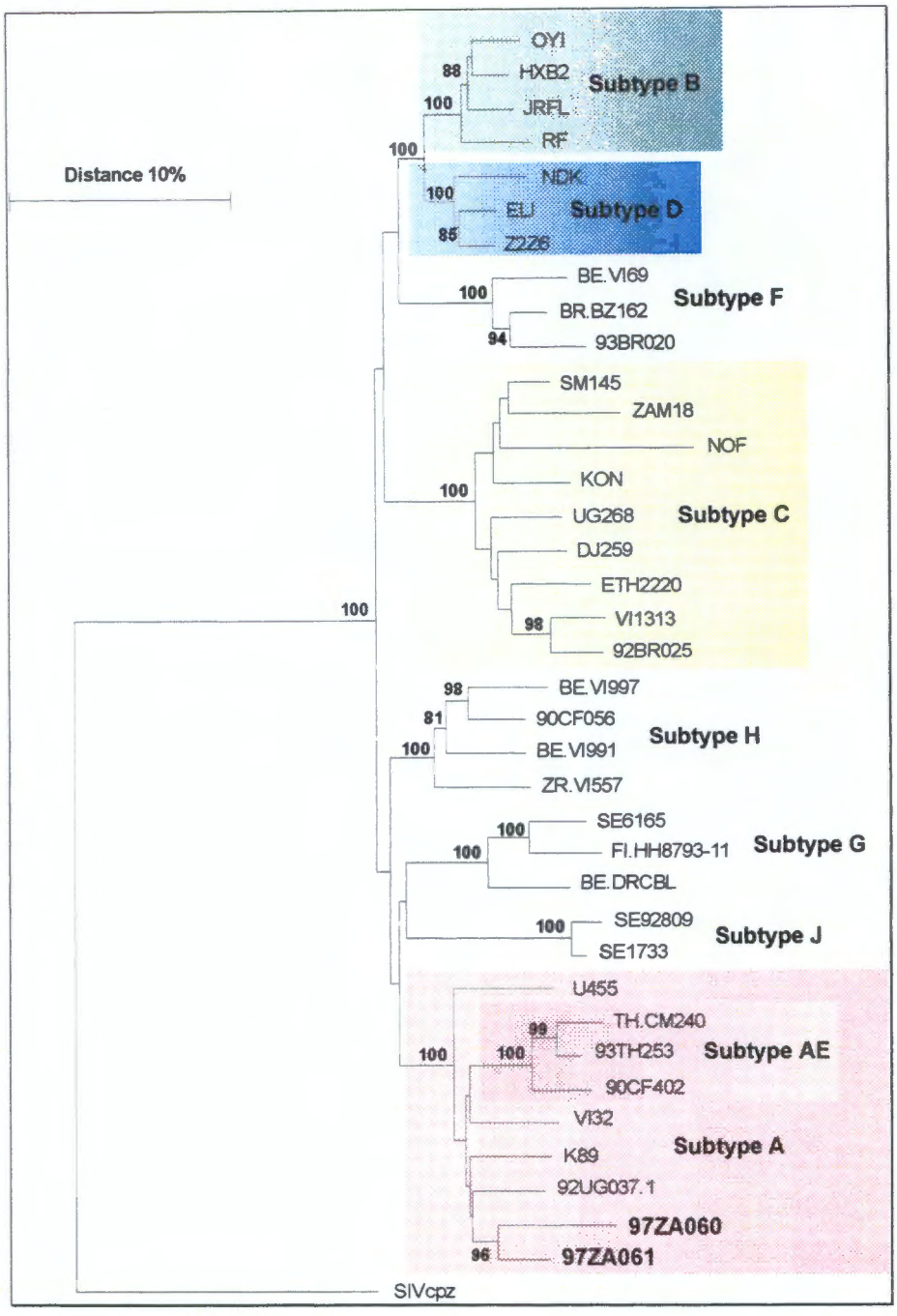


Figure 3.10 : Phylogenetic analysis of 94ZA060 and 94ZA061, indicating their subtype and their relationship to at least two reference sequences from each subtype from A to H and J as determined by this study. The two sequences, from a husband and wife pair group together on the phylogenetic tree with a bootstrap value of 96%, as well as with the subtype A lineage, clustering with a bootstrap value of 100% with the subtype A sequences from Uganda, Kenya and the Ivory Coast, as well as three subtype AE sequences from Thailand and the Central African Republic.

was associated with the initial homosexual epidemic in South Africa (Engelbrecht *et al.*, 1995; Williamson *et al.*, 1995 and van Harmelen *et al.*, 1997) it has failed to proliferate significantly in the subsequent heterosexual epidemic. This is illustrated by the low percentage of subtype B

infections (8%), a total of nine subtype B viruses; two from Johannesburg, one from Bloemfontein, three from Durban and three from Cape Town patients.

Table 3.7: Collective demographic and patient clinical data for this study, as well as subtype designation for the Johannesburg, Bloemfontein, Durban and Cape Town patients.

Sample name	Age (years)	Sex	Race	CD4 count ($x10^6$/l)	Subtype
Johannesburg					
Heterosexual Transmission					
95ZA256GP	ND	Female	Black	ND	C
95ZA259GP	ND	Female	Black	ND	C
95ZA261GP	ND	Female	Black	ND	C
95ZA263GP	ND	Female	Black	ND	C
95ZA265GP	ND	Female	Black	ND	C
95ZA267GP	ND	Female	Black	ND	B
95ZA269GP	ND	Female	Black	ND	C
95ZA271GP	ND	Female	Black	ND	C
95ZA274GP	ND	Female	Black	ND	C
95ZA279GP	ND	Female	Black	ND	C
95ZA281GP	ND	Female	Black	ND	C
95ZA283GP	ND	Female	Black	ND	B
95ZA286GP	ND	Female	Black	ND	C
95ZA287GP	ND	Female	Black	ND	C
95ZA292GP	ND	Female	Black	ND	C
95ZA294GP	ND	Female	Black	ND	C
95ZA298GP	ND	Female	Black	ND	C
95ZA300GP	ND	Female	Black	ND	C
95ZA302GP	ND	Female	Black	ND	C
95ZA305GP	ND	Female	Black	ND	C
95ZA001GP	ND	Female	Black	ND	C
95ZA002GP	ND	Female	Asian	ND	C
95ZA003GP	ND	Female	Asian	ND	C
95ZA004GP	ND	Female	Asian	ND	C
95ZA005GP	ND	Female	Asian	ND	C
95ZA0065GP	ND	Female	Black	ND	C
95ZA007GP	ND	Female	Black	ND	C
95ZA008GP	ND	Female	Black	ND	C
95ZA009GP	ND	Female	Black	ND	C
95ZA010GP	ND	Female	Black	ND	C
97ZA347TS	ND	Male	Black	<200	C

Table 3.7: Continued...

Sample name	Age (years)	Sex	Race	CD4 count ($x10^6$/l)	Subtype
98ZAUN01GP	20-40	ND	Black	<200	C
98ZAUN02GP	20-40	ND	Black	<200	C
98ZAUN03GP	20-40	ND	Black	<200	C
Pretoria					
Heterosexual Transmission					
98ZAUN04GP	20-40	ND	Black	<200	C
98ZAUN05GP	20-40	ND	Black	<200	C
98ZAUN06GP	20-40	ND	Black	<200	C
98ZAUN07GP	20-40	ND	Black	<200	C
98ZAUN08GP	20-40	ND	Black	<200	C
Bloemfontein					
Heterosexual Transmission					
90ZA01FS	37	Female	Black	150	C
91ZA02FS	31	Female	Black	260	C
94ZA03FS	24	Male	Black	600	C
94ZA04FS	27	Male	Black	370	C
93ZA05FS	47	Male	Black	110	C
94ZA07FS	23	Female	Black	600	C
94ZA08FS	17	Female	Black	430	C
93Z09FSA	25	Female	Black	260	C
90ZA10FS	23	Female	Black	110	C
93ZA11FS	20	Female	Black	210	C
92ZA12FS	35	Female	Black	40	C
94ZA13FS	26	Female	Mixed race	280	C
91ZA15 FS	38	Female	Black	100	C
94ZA16FS	39	Male	Black	180	C
94ZA18FS	25	Female	Black	660	C
94ZA19FS	28	Female	Black	200	C
94ZA20FS	15	Female	Black	600	B
94ZA21FS	31	Male	Black	460	C
94ZA22FS	25	Female	Black	340	C
Homosexual transmission					
95ZA25FS	38	Male	White	10	B
Bisexual transmission					
95ZA06FS	22	Male	Black	130	C
95ZA14FS	22	Male	Black	240	B
95ZA17FS	30	Male	White	120	B
Durban					
Heterosexual Transmission					
95ZA008DBN	20-35	Female	Black	ND	C
95ZA009DBN	20-35	Female	Black	ND	C
95ZA014DBN	20-35	Female	Black	ND	C
95ZA015DBN	20-35	Female	Asian	ND	C
95ZA022DBN	20-35	Female	Black	ND	C
95ZA023DBN	20-35	Female	Black	ND	C
95ZA025DBN	20-35	Female	Black	ND	B
95ZA031DBN	20-35	Female	Black	ND	C
95ZA033DBN	20-35	Female	Black	ND	C
95ZA048DBN	<0.1	ND	Black	ND	C
95ZA049DBN	20-35	Female	Black	ND	C
95ZA052DBN	20-35	Female	Black	ND	C
95ZA056DBN	20-35	Female	Black	ND	C
95ZA058DBN	20-35	Female	Mixed race	ND	C
95ZA059DBN	20-35	Female	Black	ND	C
95ZA065DBN	20-35	Female	Black	ND	B
94ZA115DBN	20-35	Female	Black	ND	B
94ZA116DBN	20-35	Female	Black	ND	C
94ZA117DBN	20-35	Female	Black	ND	C
94ZA118DBN	20-35	Female	Black	ND	C

Table 3.7: Continued...

Sample name	Age (years)	Sex	Race	CD4 count ($x10^6$/l)	Subtype
Cape Town					
Heterosexual Transmission					
93ZA006	30	Male	White	342	C
93ZA010	41	Female	Black	530	C
93ZA020	33	Female	Mixed race	5	C
93ZA023	48	Male	Black	169	C
93ZA024	41	Male	Black	200	C
93ZA025	24	Female	Mixed race	375	B
93ZA029	29	Female	Black	172	C
93ZA030	58	male	Black	154	C
93ZA031	31	Male	Black	154	C
93ZA032	34	Female	Black	72	C
93ZA035	52	Male	Mixed race	140	C
93ZA036	42	male	Black	625	C
93ZA037	32	Male	Mixed race	418	C
93ZA040	34	Female	Black	269	C
93ZA042	27	Male	Mixed race	54	C
93ZA043	32	Female	Mixed race	443	C
93ZA046	21	Female	Black	404	C
93ZA047	24	Male	Black	373	C
93ZA048	40	Male	Black	111	C
93ZA049	20	Female	Black	517	C
94ZA058	37	Female	White	357	D
94ZA060	42	Female	Mixed race	421	A
94ZA061	33	Male	Black	138	A
94ZA063	24	Male	Mixed race	ND	C
94ZA067	30	Female	Black	ND	C
94ZA201	38	Female	Mixed race	ND	C
94ZA202	44	Female	Black	ND	C
94ZA204	0.1	Male	Black	ND	C
94ZA205	21	Female	Black	ND	C
94ZA207	1	Unknown	Black	ND	C
Homosexual Transmission					
93ZA001	37	Male	White	504	B
93ZA002	35	Male	White	52	B
93ZA003	32	Male	White	84	B
93ZA004	30	Male	White	564	B
93ZA005	42	Male	Mixed race	231	B
93ZA007	41	Male	White	0	B
93ZA009	31	Male	Mixed race	499	B
93ZA011	25	Male	Mixed race	481	B
93ZA017	25	Male	White	8	B
93ZA021	44	Male	Mixed race	245	B
93ZA022	49	Male	White	69	B
93ZA033	36	Male	White	374	B
93ZA034	42	Male	White	21	D
93ZA039	37	Male	Mixed race	746	B
93ZA041	30	Male	Mixed race	54	B
93ZA044	48	Male	White	208	B
93ZA045	26	Male	Mixed race	397	B
94ZA050	39	Male	Mixed race	32	B
94ZA059	55	Male	Mixed race	77	B
94ZA073	47	Male	White	23	B
94ZA076	ND	Male	White	ND	B
94ZA077	ND	Male	Mixed race	ND	B
93ZA012	26	Male	Mixed race	550	B
93ZA019	40	Male	White	534	B

Table 3.7: Continued...

Sample name	Age (years)	Sex	Race	CD4 count ($x10^6$/l)	Subtype
Bisexual Transmission					
93ZA038	38	Male	White	0	B
94ZA072	56	Male	White	45	B
Other Transmission					
*93ZA008	28	Female	White	6	B
&94ZA074	42	Male	Mixed race	11	B
&94ZA078	ND	Male	Mixed race	ND	B

ND, not determined
*Patient infected by blood transfusion
& Patients infected whilst incarcerated in Pollsmoor prison

The epidemiological data suggests that migrant labour and illegal immigrants play a role in the introduction of HIV into the country (Sher, 1989; Williams and Campbell, 1996 and van Haldenwang, 1996). Neighbouring southern African countries have some of the most explosive HIV epidemics within sub-Saharan Africa, with HIV prevalence ranging from 25% to over 40% in women attending some antenatal clinics in 1998 (MAP, 1997). Within South Africa, the spread of the virus is rapid due to both economic and behavioural factors (see section 1.6.4).

Our molecular data supports the epidemiological inference that the heterosexual epidemic in South Africa was as a result of regional spread of HIV from neighbouring countries. Subtyping studies in Malawi (Orloff *et al.*, 1993 and Gao *et al.*, 1996a), Zimbabwe (Shafer *et al.*, 1997 and Tien *et al.*, 1999), Botswana (Novitski *et al.*, 1999) and Mozambique (Engelbrecht *et al.*, 1998) have shown that subtype C is predominant in these countries. In addition, a study performed on 43 mine workers in Gauteng Province of South Africa identified only subtype C in migrant labourers from Botswana and Lesotho, although it is not certain that these men were infected in their country of origin, or within South Africa (Bredell *et al.*, 1998). Isolates from these countries were included in the 117 subtype C sequences phylogenetically compared in the V3 loop in this study. No subclusters with a country of origin were identified, in this study, however. This is in contrast to 93 sequences analysed in the V3 region of the *env* gene from Addis Ababa in Ethiopia, where a subcluster of 32 isolates were identified with an average 7.4% DNA distance, in comparison to the remaining 61 isolates which were on average 11.5% divergent (Abebe *et al.*, 1997).

In addition, the high DNA distance demonstrated in this study between the southern African V3 loop sequences is an indication that the HIV subtype C epidemic in South Africa is not a clonal epidemic and it is probable that HIV entered the country on a number of occasions from different countries of origin. As was indicated in the previous chapter (see section 2.3.3.4), a number of the patients infected with subtype C reported having a possible origin of infection in a

neighbouring country where subtype C is known to occur. Multiple subtype C introductions into South Africa have also been indicated by sequencing results of isolates taken from mine workers in the Gauteng province, mentioned above (Bredell *et al.*, 1998). In these isolates the *env* V3-V5 region had an intrasubtype genetic diversity of 11%. In addition, high intrasubtype DNA distances for subtype C were also determined in a study of the p17 region of the *gag* gene from Cape Town isolates sampled during the start of the epidemic (Williamson *et al.*, 1995 and van Harmelen *et al.*, 1997), providing evidence of multiple subtype C introductions into the country. Here, intrasubtype DNA distances from the subtype C isolates analysed in the *gag* gene were 6.8% divergent, with a range of 1 to 11.8%.

High genetic diversity within subtype C isolates has also recently been reported by Novitsky *et al.* (1999). In a phylogenetic analysis of eight full length subtype C isolates from Botswana, an interpatient DNA distance of 9.1% was determined. The DNA distance in the envelope gene was higher, at 12.4%. This group give a number of reasons as to why there is such high genetic diversity within the subtype C lineage, including that the viral load in patients infected with subtype C may be higher and that there may be faster viral transcription and replication within subtype C viruses (Neilson *et al.*, 1998 and Novitski *et al.*, 1999). They also do not discount the possibility that multiple introductions of subtype C into Botswana may account for the great genetic diversity within the subtype.

The South African epidemic (and Botswana epidemic) can be contrasted to subtype C epidemics in India and China that have much less genetic diversity. It has been suggested that the Indian epidemic was started by a single ancestor virus that then spread throughout India. The greatest DNA distance recorded in the Indian epidemic is 6.8% in the *env* region in Indian patients in Bombay (Grez *et al.*, 1994). The Chinese subtype C epidemic was traced back to Chinese IVDUs sharing needles with Indian IVDU's (Shao *et al.*, 1996) and an analysis of the sequence diversity of the Chinese subtype C isolates and Indian subtype C isolates indicated only 4.5% distance between the viruses in the *env* region (Luo *et al.*, 1995). Intrasubtype sequence diversity of subtype C isolates from China show an average DNA distance of only 2.25% (Shao *et al.*, 1996) to 2.6% (Luo *et al.*, 1995).

It has recently been shown by a number of studies that subtype C viruses rarely evolve to utilise the CXCR4 co-receptor (Doms and Moore, 1997; Tscherning *et al.*, 1998 and Björndal *et al.*, 1999). An indication of the phenotype of the isolate can be obtained from the V3 loop where more basic (positively charged) arginine residues at positions 276, 290 and 297 (see figure 3.8) are indicative of a fast/high, SI virus. A recent study of nine Ethiopian AIDS patients showed that all isolates were CCR5-restricted (Björndal *et al.*, 1999). The results from our study

of 117 southern African subtype C isolates also only shows two isolates to have one of the arginine substitutions indicative of an SI phenotype, NOF and 94ZA067. Unfortunately no information on the disease stage of the patient was available for the isolates.

Recently, the number of reports of recombinant viruses has been increasing, especially in central Africa where there many different subtypes circulating (Liitsola *et al.*, 1998; Peeters *et al.*, 1998; and Renjifo *et al.*, 1998) (see section 1.6.2) and it no longer sufficient to subtype only one region of the viral genome. The only way to identify complete recombinant viruses is by sequence analysis of the entire genome. However, sequencing is both time consuming and costly, thus rapid methods are needed for subtyping different regions of the genome to allow a general screening of large numbers of samples, in order to identify potential recombinant viruses, which can then be characterised.

Restriction fragment length polymorphism (RFLP) analysis is a rapid method of subtyping that has been used in the protease and p24 regions of HIV genome. A useful strategy to ensure the reliability of the technique, and simultaneously screen for recombinants, would be to analyse different regions of the genome by RFLP, including p17, p24 and protease encoding regions and to combine this with V3 serotyping, or HMA of the *env* gene. RFLP analysis of the highly variable HIV *env* region was not possible, since analysis of the 700bp V3-V5 *env* region gave discrepant results in comparison to *env* HMA subtypes. V3 serotyping has also not proved as reliable as HMA in the identification of subtype B isolates in the South African epidemic, however. A 1998 study reported that 15 out of 119 samples, including 47 of the Cape Town isolates identified by HMA in the envelope region in this study, were not able to be typed using the V3 serotyping technique. In addition, three out of 26 subtype B samples were incorrectly serotyped as A/C (n=1), or C (n=2) and two subtype C samples were incorrectly serotyped as A, or A/C. It is possible, however, that with the use of alternative peptides the specificity of the technique may be improved (Cheingsong-Popov *et al.*, 1998).

In this study RFLP analysis was used in the HIV-1 *gag* p17 region in order to identify the *gag* subtype of South African isolates. In combination with HMA in the *env* region, a rapid screening of large numbers of samples would allow the detection of recombinant viruses in the *gag* and *env* regions and strengthen our knowledge of virus diversity. Although RFLP analysis is cheaper and easier to perform than sequence analysis on large numbers of samples, the technique does have some limitations, since HIV is a rapidly evolving virus and identification is based on the analysis of a limited number of informative sites. Even so, all 33 of the Cape Town samples of known sequence analysed by restriction endonuclease site analysis (RESA) in this study were correctly identified and 16 Cape Town isolates subtyped by RFLP analysis in a

blinded fashion were all correctly identified. In addition, when 25 sequences from other regions of Africa and the world were analysed by RESA, only two samples were problematic: a subtype C from Gabon was, which was untypable and a subtype D from the Democratic Republic of Congo which was inaccurately typed as a subtype B. It has been shown in a number of studies (Engelbrecht *et al.*, 1995; Williamson *et al.*, 1995; van Harmelen *et al.*, 1997; Bredell *et al.*, 1998; Moodley *et al.*, 1998; van Harmelen *et al.*, 1999a and van Harmelen *et al.*, 1999b) that at present there is limited subtype diversity in South Africa, although there is high variability within subtype C and RFLP analysis has proven to be reliable in this setting.

No discordance in subtype designation was identified in 77 samples from Johannesburg (n=20), Bloemfontein (n=17), Durban (n=2) and Cape Town (n=38) subtyped in both the *gag* gene (by RFLP) and *env* gene (by HMA) (see Table 3.6), indicating that no recombinants were identified in the regions of the genome analysed. In South Africa the greatest numbers of HIV-1 infections are in the heterosexual population, with over 90% of the viruses to date being subtype C, although subtypes A, B and D have also been rarely identified (van Harmelen *et al.*, 1997 and Bredell *et al.*, 1998). The homosexual epidemic is almost exclusively subtype B (Williamson *et al.*, 1995 and van Harmelen *et al.*, 1997). Recombinant viruses are thus not common, as opposed to Uganda, for example, where subtypes A and D co-circulate, leading to frequent co-infection and recombinant formation (European Commission and UNAIDS Workshop Report, 1997).

It has not been determined why there is a predominance of subtype C in South Africa, India and China; whether it is by selective advantage of the virus due to phenotypic differences, allowing enhanced transmission, or due to increased transmissibility of the virus, or whether it is merely chance. Thus far, the only report of selection for growth of subtype C (and AE) was in Langerhans' cells (Soto-Ramirez *et al.*, 1996 and Essex *et al.*, 1997), but has since been refuted (Dittmar *et al.*, 1997 and Pope *et al.*, 1997). In addition, disease progression and survival rate was found to be similar between African and non-African HIV-positive individuals tested at an HIV/AIDS clinic in London (del Amo, 1996). However, a recent study of CSWs in Senegal reported that there may be faster disease progression in women infected with non-A subtypes of HIV-1 than subtype A infected women. The study was small, but suggests that further work may be warranted in order to investigate the possible relationship between subtype and pathogenicity (Kanki *et al.*, 1999).

Subtype C has spread throughout Africa to as far north as Ethiopia, Senegal and Djibouti (Louwagie *et al.*, 1995; Abebe *et al.*, 1997 and Björndal *et al.*, 1999). In addition, subtype C has spread globally, reaching epidemic proportions in India (Dietrich *et al.*, 1993 and Grez *et al.*,

1994) and China (Luo *et al.*, 1995; Shao *et al.*, 1998 and Yu *et al.*, 1998) and can now be found in every continent (European Commission (EC) and the Joint United Nations Programme on HIV/AIDS (UNAIDS), 1998).

In this study it has thus been shown that subtype C is the predominant virus infecting the heterosexual population in South Africa and although subtypes A, B and D have been identified, the distribution of these subtypes is limited. Since South Africa is a potential site for vaccine trials (International AIDS Vaccine Initiative (IAVI Report, 1998), however, it is important to continually monitor genetic diversity within the country.

CHAPTER 4: THE GENOTYPIC CHARACTERISATION OF A MACROPHAGE TROPIC SOUTH AFRICAN SUBTYPE C GP120 AND AN EVALUATION OF THE IMMUNOGENICITY OF THE GP120 USING A DNA VACCINE SYSTEM

4.1) Introduction

As was established in Chapters 2 and 3, the predominant HIV-1 subtype infecting the heterosexual population in South Africa is subtype C, and subtype B is prevalent in the homosexual population (Engelbrecht *et al.*, 1995; Williamson *et al.*, 1995; van Harmelen *et al.*, 1997; Bredell *et al.*, 1998; Moodly *et al.*, 1998; van Harmelen *et al.*, 1999a and van Harmelen *et al.*, 1999b). Since South Africa is actively involved in HIV vaccine trials (IAVI Report, 1998), it would be beneficial if studies pertaining to the effect of virus diversity on vaccine design could be done on South African isolates. It has been shown by a number of studies that a cellular immune response is more important than a humoral response in limiting the spread of HIV in the body (Pantaleo *et al.*, 1995; Rinaldo *et al.*, 1995; Rowland-Jones *et al.*, 1995 and Harrer *et al.*, 1996). It is possible that for the most effective cytotoxic T lymphocyte (CTL) response, the vaccine should be constructed from virus subtypes endemic to the region. This is because the most conservation between CTL epitopes is within a subtype, although some CTL cross-reaction between subtypes has been shown (see sections 1.7.1.1 and 1.7.1.2).

4.1.1) DNA vaccines

A vaccine simple to construct and efficient at eliciting a CTL response would be ideal for the determination of immunogenicity of potential HIV vaccine proteins. DNA vaccines are known to elicit a potent CTL response to a number of foreign genes incorporated into DNA vaccine vectors (see Table 4.1). HIV-1 structural, accessory and regulatory genes have all been included in DNA vaccines (Kim *et al.*, 1997, Boyer *et al.*, 1997 and Hinkula *et al.*, 1997). See section 1.8 for a detailed description of DNA vaccines.

Table 4.1: A survey of the published results of DNA vaccines containing HIV antigens in order to elicit an immune response in non-human primates and small animals. Advantages and disadvantages are appraised.

Genes/ Proteins	Modifications	Results	References
$gp120_{HXB2}$ $gp140_{HXB2}$ $gp160_{HXB2}$	• V1/V2, V3 regions removed, or not. • removal of the variable regions exposes the CD4-binding domain	• removal of the variable regions increased the immunogenicity of gp140 and gp160 in New Zealand White rabbits • antibody responses were obtained to regions of the Env not usually expressed • neutralising antibody response did not improve	Lü *et al.*, 1998
$gp120_{HIV-1}$ $gp140_{HIV-1}$	• boost with either HIV*env* DNA vaccine or rgp160	• neutralising antibody and CTL responses obtained in chimpanzees • chimpanzees protected against SHIV-HXBc2 challenge	Letvin *et al.*, 1997
gp120		• produces a MHC class-II response more closely mimicking natural infection than a recombinant protein would in macaques • PBLs of the macaques recognised gp120 and secreted IFN-γ and TNF-α	Lekutis *et al.*, 1997
HIV *env* subtype D		• subtype D vaccine elicited cross-neutralising antibodies and cross-reactive CTLs against subtype B detected *in vitro* in cynomologous macaques	Wang *et al.*, 1995
HIV *env* and *gag-pol*	• rFPV-HIV *gag-pol* and *env* boost used in mice and macaques	• T-cell proliferation and CTL responses were substantially enhanced in comparison to unimmunised macaques. • intravenous challenge by a non-pathogenic $SHIV_{LAI}$ was withstood in macaques • mice completely protected from intracerebral challenge with vaccinia virus (VV)-*gag-pol* indicating CD8+ CTL response	Kent *et al.*, 1998
gp120 or gp160	• intramuscular DNA vaccine immunisation	• Th1 response with IFN-γ and IL2 production in mice and rhesus macaques	Shiver *et al.*, 1997
gp120	• gene gun immunisation • gene gun immunisation of primary and T-cell isolates	• Th2 response in rabbits which was blocked with IL2, IL7 or IL12 co-delivery • IFN-γ levels increased and IL4 and IgG1 response suppressed by IL2, IL7 or IL12 • primary and T-cell adapted isolates gave rise to neutralising antibodies in the rabbits, although neutralising antibodies from primary isolates are more broadly reactive	Richmond *et al.*, 1997
$HIV\text{-}1_{IIIB}$ *env*	• mannan-coated liposomes of a DC-Chol (3β[*N(N',N'*-dimethylaminoethane)carbamoyl]cholesterol), DOPE (dioleoylphasphtidylethanolamine) and mannan mixture added	• increased the delayed-type hypersensitivity and CTL; inhibited by anti-IFN-γ Ab indicating a Th1 response in BALB/c mice • intranasal immunisation increased IgG, mucosal IgA and faecal IgA • mannan coated liposomes are taken up by macrophages with mannan receptors for carbohydrates in bacteria, yeast and protozoa to activate them, hence the Th1 response	Toda *et al.*, 1997

Table 4.1 continued..

Genes/ Proteins	Modifications	Results	References
HIV-1_{IIIB} *env*	• mannan-coated diC14-amidine (N-t-butyl-N'-tetradecyl-3-tetradecylamino-propion-amidine), a diphasic cationic lipid used	• enhanced the Th1 response and elicited vaginal IgA in BALB/c mice • in humans anti-mannan Ab may be a problem, thus mannopentose was used instead • the Th1 response was as good and no immunogenicity or toxicity was found	Sasaki *et al.*, 1997
HIV-2_{ROD} *env* and *rev*	• rgp140 boost • co-administration of IL12 expressing plasmid	• enhanced cellular response and cross-reactive T-cell proliferative response in mice • cross-reactive antibodies elicited to SIV_{1A11} and SIV_{239}	Agadjanyan *et al.*, 1997

A number of animal trials have been performed demonstrating the efficiency of DNA vaccines in eliciting both a humoral and cellular immune response (see Table 4.1). In addition, HIV DNA vaccines have entered clinical trials. In a phase I study of nine HIV-infected, but symptom-free patients, DNA vaccines containing the *nef, rev* or *tat* regulatory genes of HIV were injected intramuscularly into the patients' deltoid muscle. Although the patients had low or no CTL or antibody reaction to these HIV regulatory proteins before DNA immunisation, eight patients elicited MHC class-I restricted CD8+ CTLs to the protein expressed by the DNA vaccine. CTLs to regulatory proteins, which are produced early in the replication cycle of HIV, may eliminate infected cells before progeny virus is released (Calarota *et al.*, 1998).

Phase I clinical trials are ongoing with a therapeutic *env/rev* DNA vaccine (APL 400-003) to determine the safety and effectiveness of this vaccine. No anti-DNA antibodies, muscle enzyme elevations, or local or systemic reactions to the vaccine have been recorded, indicating the safety of the vaccine. Fifteen asymptomatic HIV infected patients were divided into three groups, immunised with three doses at ten week periods: 30μg, 100μg, or 300μg of DNA vaccine and all five of the patients in the 100μg and 300μg groups elicited anti-gp120 antibodies greater than double their baseline antibody level after 21 weeks. None of the patients in the 30μg group were able to elicit an antibody response greater than their baseline level to gp120, however. CTLs were obtained in 2/3 patients analysed from the 100μg group, although no CTL response was detected in any of the other patients (n=7) tested from the other groups. All patients tested for T-cell proliferation showed an increase in stimulation index (SI), however, which was not dose-related. The vaccine thus appears to be safe and potentially immunogenic, encouraging further studies (MacGregor *et al.*, 1998).

DNA vaccines would thus provide a useful model for determination of the immunogenicity of potential HIV vaccine proteins. The effective CTL response elicited may be used in studies to determine CTL cross-reactivity between HIV subtypes.

4.1.2) The in vivo Vaccinia Virus challenge system: a critical review

In order to evaluate the possibility of a cross-reactive CTL response to predominant South African HIV-1 subtypes, an *in vivo* recombinant vaccinia virus (VV) murine challenge system was utilised. A number of papers have reported on the use of this murine challenge system in order to determine the *in vivo* CTL response to different vaccine formulations (Doherty *et al.*, 1989; Binder and Kündig, 1991; Bachmann *et al.*, 1994, Belyakov *et al.*, 1998; and Kent *et al.*, 1998). Here, the pertinent literature on this challenge model is critically appraised.

In mice, the primary infection by VV is controlled by the CD8+ CTL response, whereas antibodies play a role in assisting the CTL response in the complete clearance of the virus as well as preventing the spread of the virus in a secondary infection (Hirsch *et al.*, 1968). The recombinant VV progeny is produced within the cytoplasm of the infected cell and the endogenously expressed proteins can thus enter the MHC class 1 pathway and be expressed on the surface of the cells, eliciting the CD8+ CTL response (Binns and Smith, 1992). It has been shown that after vaccination of mice with a specific antigen, followed by challenge with a similar antigen expressed by the VV recombinant, if the mice are depleted of CD8+ T cells, the virus is able to initiate an infection equivalent to that in unimmunised mice. Depleting the challenged mice of CD4+ T cells, however, has no effect and the immunised mice are able to clear the recombinant VV infection (Binder and Kündig, 1991, Bachmann *et al.*, 1994; Belaykov *et al.*, 1998 and Kent *et al.*, 1998).

Since the CD8+ CTL response to the recombinant VV allows the mouse to eliminate the recombinant VV (Hirsch *et al.*, 1968; Binder and Kündig, 1991; Bachmann *et al.*, 1994; Belyakov *et al.*, 1998 and Kent *et al.*, 1998), the challenge system gives an *in vivo* indication of the CD8+ CTL response in the mouse to the immunogenic protein.

Vaccinated mice have been challenged with recombinant vaccinia virus (VV) expressing foreign genes from the vaccine strain via a number of different inoculation routes including; intraperitoneal (Bachmann *et al.*, 1994), intrarectal (Belyakov *et al.*, 1998), intracranial (Doherty *et al.*, 1989) or intravenous (Binder and Kündig, 1991 and Kent *et al.*, 1998). The earliest paper studied the susceptibility (as the percentage mortality) of BALB/c, CBA/H and C57B1 mice to recombinant VV expressing the influenza virus nucleoprotein and haemagglutanin proteins after intracranial challenge. It was established that male and older female mice were more resistant to the recombinant VV, as were C57B1 mice (Doherty *et al.*, 1989).

A second study investigated the recombinant VV titres in C57BL/6 mouse organs

(spleen, liver, lung, thymus, ovaries and brains) after intravenous challenge with a VV expressing the nucleoprotein of vesicular stomatitis virus Indiana, Mudd-Summer isolate (VV-IND-NP), showing the highest titres to be in the mouse ovaries after three to seven days. Mice challenged with recombinant VV-IND-NP after priming with VSV were shown to have over 5 $\log_{10}$ lower titres of VV-IND-NP in their ovaries in comparison to mice immunised with unrelated influenza virus PR8 (Binder and Kündig, 1991). Recombinant VV clearance and the level of the CTL response can thus be determined by calculating the VV titres in the mouse ovaries and comparing the titres to those in unimmunised, control mice.

A further three studies have used the murine VV challenge model to detect CTLs. The first group (Bachmann *et al.*, 1994) used the technique in order to illustrate that a CTL response was elicited to their VSV N (nucleoprotein) vaccine. The group were able to show that immunisation of mice with recombinant viral proteins from VSV Indiana produced in a baculovirus system elicited a CD8+ CTL response after intraperitoneal challenge with recombinant VV-VSV-N. The CTL response was measured as a 5.6 $\log_{10}$ decrease in VV-VSV-N titres twenty-five days after immunisation in the mouse ovaries in comparison to the control, unimmunised mice. A second study used the *in vivo* challenge model in order to compare the effectiveness of different inoculation routes, followed by recombinant VV challenge. Similar levels of protection to Bachmann's group were obtained after intrarectal challenge with recombinant VV expressing HIV-1_{IIIB} gp160 in BALB/c mice immunised intrarectally with a HIV-1 peptide vaccine, PCLUS3-18IIIB (Belyakov *et al.*, 1998). The recombinant challenge technique has also been used to determine the most effective vaccination strategy, using a prime-boost protocol. Intramuscular immunisation with a DNA vaccine expressing the *gag* and *env* proteins of HIV-1_{SF2}, followed by a fowlpox-HIV-1_{SF2}-*gag/pol* boost, completely protected CBA/H mice from challenge with a VV expressing the Gag and Pol proteins of HIV-1_{SF2}. The protection was measured as a decrease in recombinant VV titre from 6.8 $\log_{10}$ to undetectable levels (<2 $\log_{10}$) (Kent *et al.*, 1998).

The murine recombinant VV challenge technique thus gives an indirect measurement of the *in vivo* CD8+ CTL response to the immunogenic proteins in the vaccinated mice.

4.1.3) Objectives of this study

At the time of initiation of this study, the gp120 of HIV was widely used in vaccine design (see section 1.7). Limited studies had been performed on the identification of CTL epitopes in HIV proteins or on CTL cross-protection between subtypes (see section 1.7). The gp120 from two HIV-1 isolates belonging to subtypes B and C were thus chosen for genotypic

characterisation and immunogenicity studies, since the two subtypes predominate in the homosexual male and heterosexual populations in South Africa respectively (see Chapters 2 and 3). Subtype C was also chosen, as at the time of initiation of the study, vaccine development had mostly been based on T-cell tropic, subtype B isolates (IAVI, 1998). The gp120 from a NSI, CCR5-restricted, primary subtype B reference isolate, SF162 (USA) and subtype C from a macrophage tropic, primary virus isolate, 97ZA347TS, from South Africa were selected. To determine the immunogenicity of the gp120 from the two subtypes, the gp120 protein from each was cloned into pcDNA3.1/Zeo DNA vaccine vectors (Invitrogen, USA). In addition, the CTL cross-reactivity between the subtype B and subtype C gp120s was determined using the *in vivo* murine VV challenge system.

4.2) Materials and Methods

4.2.1) Source of the gp120

The gp120 from a NSI reference plasmid, SF162, from the United States of America (USA) (obtained from NIH AIDS Research Reference and Reagent Program, USA) was used for the subtype B DNA vaccine. In addition, the gp120 from a local subtype C isolate was selected for the subtype C DNA vaccine. The subtype C isolate, 97ZA347TS, was cultured from an individual who had been working on a mine in the Gauteng Province (obtained from Sue Lyons, National Institute of Virology, Johannesburg). The isolate was shown to be non-syncitium inducing and macrophage tropic, unable to grow in U-937 or MT-2 cell lines (Sue Lyons, personal communication).

4.2.2) DNA extraction of 97ZA347TS and PCR amplification of 97ZA347TS, SF170 and SF162

Proviral DNA was extracted from the cultured 97ZA347TS isolate as in described (Kawasaki, 1990 and Appendix A1). The gp120 from 97ZA347TS was amplified by nested PCR using primers ED3/14, (Delwart *et al.*, 1993), in the outer reaction and *envJ*/*env*B (Gao *et al.*, 1994) as the inner primers as described (Appendix A2). The gp120 from the reference plasmid, SF162, was amplified by the inner primers only. Briefly, the 2.5Kb outer reaction and 1.6Kb inner reaction fragments were amplified by nested PCR amplification with 50µl master mixes containing:

- 5µl 10X PCR buffer (Promega, Madison, WI, USA), (500mM KCl, 100mM Tris-HCl (pH9.0), 1% Triton X-100),
- 1.5mM $MgCl_2$,

- 1% DMSO, 1% glycerol,
- 200μM each of dNTP's,
- 400nM each of ED3/ED14 (outer reaction) and *env*B/*env*J (inner reaction) and
- 2.5U *Taq* DNA Polymerase (outer reaction) and 2U *Taq* DNA Polymerase (inner reaction)

The outer reaction mixes were made up to 40μl and the inner reactions to 47μl with sterile, deionised water and were overlayed with 30μl of mineral oil. Ten microlitres of DNA isolated from viral culture was added to the outer reaction and 3μl of the outer reaction product was transferred to the inner reaction mix. For amplification of the plasmid DNA, 2μl of plasmid DNA was added to the inner reaction mix. Negative controls included HIV negative patient DNA and sterile distilled water in order to monitor contamination. The amplification conditions in the Techne PHC-2 PCR thermocycler for the outer reactions were:

- Initial denaturation at 94°C for 2 minutes,
- 94°C for 1 minute, 45°C for 1 minute and 72°C for 2 minutes (5 cycles),
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (35 cycles) and
- Final extension at 72°C for 6 minutes.

Amplification conditions for the inner reactions were:

- Initial denaturation at 94°C for 2 minutes,
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (35 cycles) and
- Final extension at 72°C for 6 minutes.

The amplified product was stored at -20°C before cloning into the pMOS-blue vector (Amersham, Buckinghamshire, UK).

4.2.3) Cloning and sequencing of the gp120 from subtypes B and C

The amplified gp120 was cloned into the pMOS-blue (Amersham, Buckinghamshire, UK) vector by TA cloning according to manufacturers' instructions (Appendix A5). The ligated plasmids were transformed into competent *E. coli*, strain DHα (Appendix A6) and DNA from putative clones extracted by mini-preparation (Appendix A7). The clones were then screened by restriction endonuclease digestion with *Hinc*II (subtype B) and *Hind*III (Boehringer Mannheim, Hamburg, Germany) (subtype C) in order to determine the correct orientation for sub-cloning into pcDNA3.1/Zeo (Invitrogen, USA) (Appendix A4).

The ATG start codon of the gp120 from the isolates had to be adjacent to the *Eco*RI site of pMOS-blue (Amersham, Buckinghamshire, UK) for directional cloning into the

pcDNA3.1/Zeo+ vector (Invitrogen, USA) (see figure 4.1). However, for cloning of the subtype C isolate into the pSC65 VV vector (obtained from Bernard Moss, NIH, USA), the ATG start codon had to be in the opposite orientation. Two pMOS-blue plasmid vectors for 97ZA347TS were thus constructed, one in each orientation (see figure 4.1). The digested fragments were electrophoresed through a 1% TAE agarose gel and stained with ethidium bromide (10ng/ml) (Appendix A3) in order to visualise the fragments using the UVP transilluminator at 256nm.

The pMOS-97ZA347TS plasmid DNA was extracted by maxi-preparation using the Nucleobond® AX100 anion-exchange kit (Machery-Nagel, Germany) according to manufacturers instructions (Appendix A7) and sequenced using forward and reverse primers, T7 (5'AATACGACTCACTATAGGG3') and U-19 (5'ACGTCGTGACTGGGAAAACC3') respectively (pMOS-blue-T vector, Amersham, Buckinghamshire, UK), as well as internal primers, E135 and E155 (Myers *et al.*, 1995), shown in Table 4.2. Sequencing was performed using the Pharmacia™ ALFexpress® automated sequencer using fluorescent Cy5-labelled primers (Appendix A8.1) with both strands sequenced until unambiguous.

Table 4.2: Sequencing primers for the HIV-1 *env* gene used for internal sequencing of the 97ZA347TS isolate gp120.

Primer	*Location	Sequence (5'-3')
E135	<<836	AGCTGTACTATTATGGTTTTAGCATTGT
E136	<<281	CTGTTCTACCATGTTATTTTTCCACATGT

Nucleotide position is indicated on the 5' end of the primer and is taken from the reference isolate HXB2 (Genbank accession number, K03455). Reverse primers are indicated by "<<".

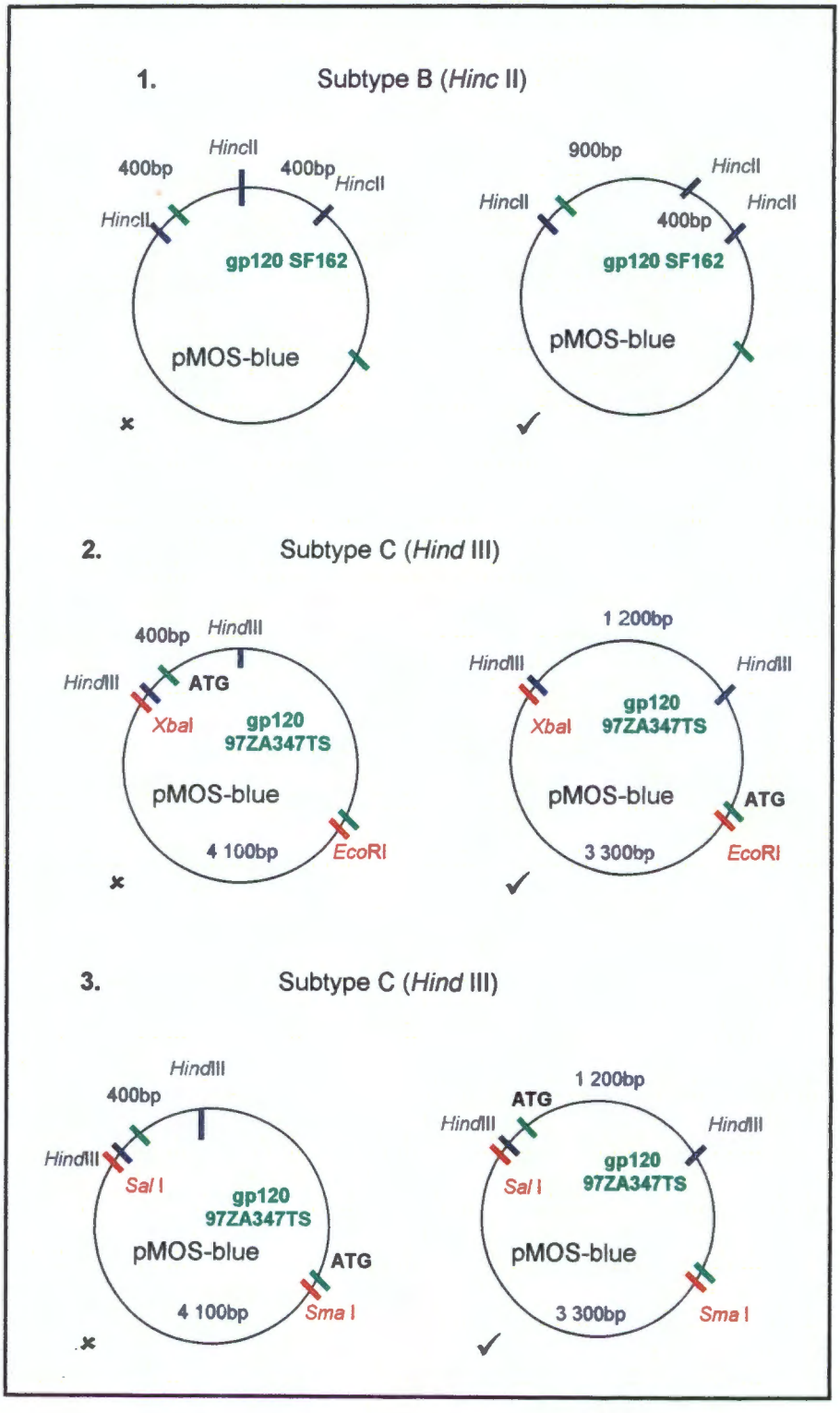


Figure 4.1: An illustration of the restriction endonucleases used and the expected fragment sizes for identification of pMOS-blue clones containing the inserted gp120 from subtype B (SF162) and C (97ZA347TS). Clones in the correct orientation, indicated by a tick, were used for subcloning into pcDNA 3.1/Zeo and pSC65.

Phylogenetic analysis of the gp120 from 97ZA347TS was performed with at least two reference sequences each, from subtypes A to J, using Clustal X (Thompson, 1997) to align sequences, with additional manual alignment. The tree was drawn using TREECONW (van der Peer and de Wachter, 1994) using the distance matrix neighbour joining method, ignoring insertions and deletions. The Kimura two-parameter algorithm (Kimura, 1990) was used to infer tree topology and the reliability of the tree topology was assessed by doing 1000 bootstrap replicates with the SIVcpz-gab sequence as the outgroup in TREECONW (van der Peer and de Wachter, 1994) (see section .2.9). The entire gp120 sequence was translated using the computer programme, Genepro (Version 6.10 Riverside Scientific Enterprises WA, USA). Variable loop regions, conserved cysteines and *N*-linked glycosylation sites were determined and compared with reference sequences belonging to subtype A, B, C, D and AE (HIV sequence database, 1998) (see section 4.3.1).

4.2.4) Directional cloning of the gp120 into pcDNA3.1/Zeo

The DNA vaccine vector, pcDNA3.1/Zeo+ (Invitrogen, USA) is a 5.0Kb plasmid with a polycloning site containing restriction endonuclease sites for 16 enzymes (See Appendix C.1 for the pcDNA3.1/Zeo+ map). The foreign gene is inserted with the start ATG codon downstream of a human cytomegalovirus (HCMV) immediate/early promoter. Downstream of the foreign gene is the bovine growth hormone polyA terminator sequence (BGHpA), which has been shown to increase transcription efficiency (Norman, 1997). The vector contains a zeocin resistance gene for selection of stable transfectants, upstream of a simian virus 40 (SV40) origin of replication, and downstream of a SV40pA sequence. The plasmid also contains a ColE1 origin of replication for growth in *E coli*, as well as an ampicillin resistance gene for selection of recombinant bacteria.

The subtype B and C gp120 fragments and digested pcDNA3.1/Zeo (Invitrogen, USA) vectors were ligated overnight at 16°C using T4 DNA ligase (Boehringer Mannheim, Hamburg, Germany) (Appendix A5) and the ligated product was used to transform competent *E. coli* strain DH(cells (Appendix A6) with the appropriate controls. Putative clones were screened by miniprep DNA purification (Appendix A7) and restriction endonuclease analysis with *Eco*RI and *Xba*I (Appendix A4). Incorporation of gp120 was also detected by PCR analysis with *env* V3-V5 primers, ES7and ES8, as performed in section 2.2 (Appendix A2). See figure 4.2 for a graphic representation of the cloning protocol.

4.2.5) Transfection of HeLa cells with pcDNA- SF162 and –97ZA437TS

In order to determine whether the gp120 proteins from the two DNA vaccines were expressed, immunofluorescent detection of the gp120 was performed after transfection of the DNA vaccines into HeLa cells.

The pcDNA3.1-HIV subtype B and C vectors were amplified by growth in *E. coli* DHα cells and the DNA purified by maxi-preparation using the Nucleobond® AX100 anion-exchange kit (Machery-Nagel Germany) according to manufacturers' instructions (Appendix A7). DNA was further purified by ethanol precipitation (Appendix A4) and purity and concentration measured by spectrophotometric analysis using the Beckman DU-40 spectrophotometer scanning from 220nm to 310nm wavelengths. Purified DNA at a 260/280nm ratio of between 1.7 and 2 was stored in aliquots at –20°C (Appendix A7).

HeLa cell stocks (obtained from the American Type Culture Collection, Rockville, MA, USA) frozen in liquid nitrogen were thawed and centrifuged at 1 000 rpm for five minutes to remove DMSO (Appendix B1). The cell pellet was re-suspended in 5ml of Dulbecco's Modified Eagle's Medium (DMEM) medium containing 10% fetal calf serum (FCS), penicillin, streptomycin (PS) and Fungizone (F) (GibcoBRL, Paisley, UK) (Appendix B1). The HeLa cell culture was then incubated at 37°C with 5-10% CO_2, and the cell culture was split when the cells had reached 100% confluency (Appendix B1).

Ten micrograms each of pcDNA3.1$_{SF162}$ (subtype B) and pcDNA3.1$_{97ZA346TS}$ (subtype C) was transfected separately into 60-70% confluent HeLa cells in a six-well plate containing a glass microscope cover-slip in each well, using 30µl of the DOTAP (Boehringer Mannheim, Hamburg, Germany) transfection reagent according to manufacturers instructions (Appendix B2). The cells were able to grow on the cover-slip, which was then removed for immunofluorescence detection. Two microlitres of positive control eukaryotic expression plasmid containing the β-D

galactosidase gene, pSVNeo-βgal (Stratagene, La Jolla, CA, USA), was also added, since the pcDNA3.1/Zeo vector does not have the β-D galactosidase marker gene, allowing detection of transfected cells by 5-bromo-4-chloro-indolyl β-D-galactoside (X-gal) staining. After a six hour incubation at 37°C with 5-10% CO_2, the 2ml of medium containing the DOTAP and DNA mixture was removed and 2ml of fresh DMEM (10% FCS, PS and F) media added.

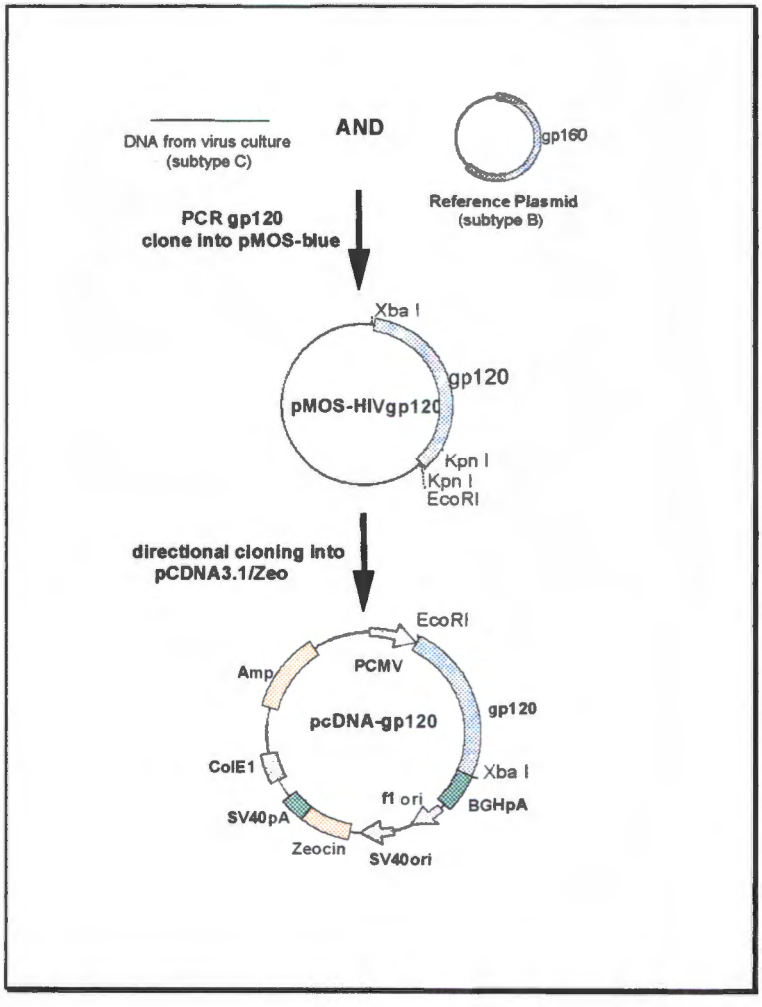


Figure 4.2: Flow diagram illustrating the method used for construction of the DNA vaccines. The gp120 (shown in blue) was cloned into pMOS-blue (Amersham, Buckinghamshire, UK) in the correct orientation (see figure 4.1). The gp120 was then excised and directionally cloned into pcDNA3.1/Zeo (Invitrogen, USA) using *Eco*RI and *Xba*I restriction endonucleases. The technique was repeated for subtypes B and C.

4.2.6) Immunofluorescent detection of the gp120 protein

The transfected cells were incubated for a further 24-48 hours after which the medium and cover-slips were removed and the remaining cells in the six well plate were fixed with 4% para-formaldehyde for five minutes at room temperature. At the same time the cover-slips were submerged in acetone at 4°C for ten minutes for fixation of the cells to the glass, after which they were allowed to dry at room temperature. The cells remaining in the six well plate were stained with X-gal stain (Appendix B5) and the number of blue cells counted to give an indication of the effectiveness of the transfection protocol. The cover-slips were subjected to immunofluorescent detection (Appendix B6) using a goat anti-HIV gp120 (Biogenesis, Poole, England), followed by a rabbit anti-sheep FITC conjugate (DAKO, Glostrup, Denmark). Immunofluorecent cells expressing the gp120 were visualised using the SM-LUX fluorescent microscope (Leitz-Wetzlar, GMBL, Germany) with a tungsten halogen lamp (100W, 12V) at 450-490nm wavelength and photographed.

4.2.7) Immunisation of BALB/c mice with DNA vaccine and antibody detection

Six to eight week male BALB/c mice ($H\text{-}2^b$) were obtained from the UCT animal unit after approval of the study by the UCT ethics committee (ethical approval number 97/026). Mice were divided into three groups, with five mice in each group (see Table 4.3).

Table 4.3: The DNA vaccine and gp120 subtype which was inoculated into the five mice in each group.

Mouse Group	DNA vaccine	Subtype
Group 1 (5 mice)	pcDNA3.1$_{SF162}$	Subtype B gp120
Group 2 (5 mice)	pcDNA3.1$_{97ZA346TS}$	Subtype C gp120
Group 3 (5 mice)	pcDNA3.1/Zeo alone	Negative control

For immunisation, the mice were immunised with 100µl of 0.25% bupivacaine-HCl (Adcock Ingram Pharmaceuticals) into each *tibialis anterior* muscle, 48 hours prior to the DNA vaccine immunisation (Kim *et al.*, 1997). The bupivacaine-HCl is a local anaesthetic that causes the localised muscle tissue to necrotise. After approximately 48 hours, the muscle fibres begin to regenerate and allow a more efficient uptake of the naked DNA. Fifty micrograms of the relevant DNA vaccine diluted to a concentration of 1µg/µl in sterile 1XPBS for immunisation, was thus injected intramuscularly into the regenerating muscle at the same position (100µg per mouse) (Whalen, 1997) (Appendix B7). Mice were immunised on weeks 0, 2, 4 and 8 and 200µl of blood was taken prior to the first immunisation, prior to each bupivacaine-HCl injection and a

final 200µl was obtained nine days after the final DNA immunisation. Blood was allowed to clot at 4°C overnight and then microfuged at 8 000rpm for one minute to remove red blood cells. The serum was transferred to a fresh eppendorf tube and stored at -20°C until antibody detection.

Antibodies to the expressed gp120 were detected using an enzyme-linked immunosorbant assay (ELISA) with a rabbit anti-mouse HRP (horseradish peroxidase) conjugate (Dako, Glostrup, Denmark) (Appendix B8). Briefly, ninety-six well microtitre plates (Nunc-Immuno(, Delta, Denmark) were coated overnight at 4°C with 2µg of HIV_{IIIB} gp120 protein (obtained from the National Institute for Biological Standards and Control (NIBSC), Hertfordshire, UK). After blocking with ELITE® milk powder, the mouse serum diluted 1/50 in 1XPBS containing 0.05% Tween-20™ was added. The plate was incubated at 37(C for an hour, followed by incubation with the rabbit anti-mouse HRP conjugate diluted 1/600 in blocking solution (1XPBS, 0.05% Tween-20™, 1% ELITE® (milk powder) for an hour. The plate was washed with 1X PBS containing 0.05% Tween-20™ between each incubation and before the addition of the substrate. After the addition of freshly made up OPD substrate (DAKO, Glostrup, Denmark), the ELISA plate was read on the Anthos ELISA plate reader (Labtech Instruments) at 492nm OD with a filter of 620nm.

4.2.8) 97ZA347TS gp120 subcloning into pSC65

The pSC65 shuttle vector (obtained from Bernard Moss, NIH, USA) is a vector for the construction of recombinant VVs containing foreign genes. The shuttle vector contains a polycloning site consisting of seven restriction endonuclease sites, which is downstream of the synthetic early/late poxvirus promoter (See Appendix C.2 for the pSC65 map). The vector also contains a (β-D galactosidase (LacZ) gene, which is under the control of the poxvirus early/late P7.5 promoter. Poxvirus promoters must be used in poxvirus vectors, as cellular promoters will not be recognised by poxvirus transcriptional enzymes (Binns and Smith, 1992). The foreign gene and LacZ gene are flanked by regions of the poxvirus thymidine kinase (TK) gene in order to allow homologous recombination with the wild type VV. The TK gene interruption by these inserts also provides a selection mechanism for the recombinant poxviruses. This is due to the TK gene being involved in the recycling of nucleotides needed for the replication of the dsDNA poxvirus genome. *In vitro*, TK competent viruses allow the phosphorylation of BUdR (5-bromodeoxyuridine), which is lethally incorporated into the virus genome. After interruption of the TK gene in recombinant VVs, addition of BudR has no effect and only the recombinant TK^- viruses will survive (Chakrabarti *et al.*, 1985 and Binns and Smith, 1992). The pSC65 vector also contains a ColE1 origin of replication for growth in *E. coli*, as well as an ampicillin resistance

gene for selection of *E. coli* clones.

In order to construct the $pSC65_{97ZA347TS}$ recombinant vector containing the gp120 from isolate 97ZA347TS, the gp120 with the ATG start codon adjacent to the *Sal* I site (see figure 4.1), was excised from pMOS-blue (Amersham, Buckinghamshire, UK) using the restriction endonuclease, *Sma I* (Boehringer Mannheim, Hamburg, Germany) (Appendix A4). The DNA was purified by ethanol precipitation (Appendix A4). The pMOS-blue-gp120 vector was then digested with *Sal* I in a different buffer (Boehringer Mannheim, Hamburg, Germany) in order to release the gp120 fragment with one sticky and one blunt end (Appendix A4). The pSC65 vector was digested in the same manner in order to release the stuffer fragment with complementary sticky/blunt ends. The vector and gp120 fragments were gel purified by excision from 1% TAE gel after electrophoresis as in section 4.3.2.1 (Appendix A3) and extracted by phenol squeeze (Appendix A5). The vector and gp120 fragment was ligated overnight at 16°C using Boehringer Mannheim (Hamburg, Germany) T4 DNA ligase (Appendix A5) and the ligation mix was used to transform competent *E. coli* strain DH(cells (Appendix A6) with the appropriate controls. Putative clones were screened by miniprep DNA purification (Appendix A7) and restriction endonuclease analysis with *Eco*RI and *Xba*I to release the gp120 fragment (Appendix A4). The gp120 was also detected by PCR analysis with *env* V3-V5 primers, ES7and ES8, as performed in section 2.2.3 (Appendix A2). See figure 4.3 for a flow diagram of the cloning protocol utilised in this study.

4.2.9) Recombinant $VV_{97ZA347TS}$ selection

Two cell types were used in the formation of recombinant $VV_{97ZA347TS}$: CV1 and 143B (TK^-). Cell stocks (American Type Culture Collection, Rockville, MA, USA, Appendix B1) stored in liquid nitrogen, were thawed and washed as before (see section 4.2.5). Cells were incubated at 37°C with 5-10% CO_2 until 100% confluent after which they were split and fresh medium added every two days.

VV-infected CV1 cells were the initial cells used for transfection of the $pSC65_{97ZA347TS}$ plasmid (Appendix B3). Briefly, the cells were grown to 60-80% confluence in a six well plate and then infected with 10^5 plaque forming units (pfu) of wild-type VVwr, (VV strain western reserve) (obtained from John Williamson, St Mary's Hospital, London) in 500µl of virus diluent (DMEM, 1% 10mM HEPES) for one hour, with shaking every 30 minutes. The virus was removed and 2ml of DMEM (10%FCS, PS and F) containing 3µg of *Sca* I linearised $pSC65_{97ZA347TS}$ DNA and 4µl of FuGene 6 (Boehringer Mannheim, Hamburg, Germany) were

added according to manufacturers' instructions (Appendix B2). The infected CV1 cells were incubated for two to three days, until cytopathic effect (cpe) was detectable. The CV1 cells were then scraped from the six well plate using a rubber policeman, transferred to Nunc cryotubes (Delta, Denmark) and freeze/thawed three times. The cells were frozen by plunging them into an ethanol, dry ice bath and thawed at 37°C. The infected cells were then diluted 1/2 in virus diluent for infection of 70-80% confluent 143B (TK⁻) cells for one hour, shaking every 30 minutes.

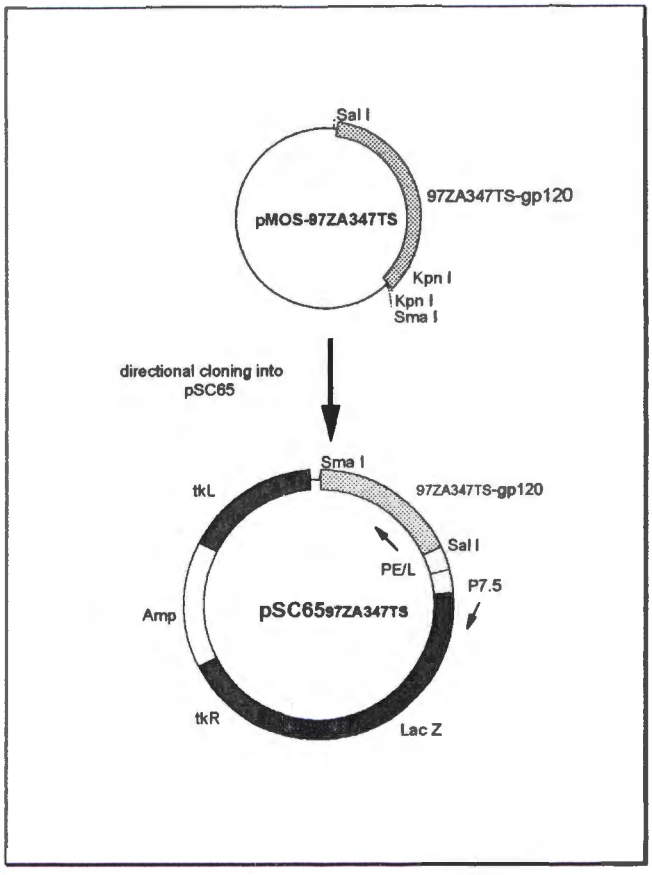


Figure 4.3: Flow diagram illustrating the method used for construction of the recombinant VV vector, pSC65 containing the 97ZA347TS isolate gp120 ($pSC65_{97ZA347TS}$). The gp120 gene was excised from pMOS-blue (Amersham, Buckinghamshire, UK) using *SmaI* and *SalI* restriction endonucleases (see figure 4.1) and directionally cloned into pSC65 (obtained from Bernard Moss, NIH, USA).

TK⁻ cells, lacking the thymidine kinase gene are used in the selection of recombinant VVs. Selection medium consisting of BUdR (5-bromodeoxyuridine) (5mg/ml) incorporated into 2ml of DMEM medium at 1/200 (25μg/ml) concentration was added to the TK⁻ cells after removal of the virus. After two to three days, the medium was removed and stored at -70°C, and a 2% agarose overlay, containing 25(g/ml BUdR and 250(g/ml X-gal in 2XDMEM at 42°C, was added. Blue plaques indicative of recombinant $VV_{97ZA347TS}$ were picked, resuspended in 200μl of virus diluent and freeze/thawed as before. A 1/5 dilution of virus in diluent was used to infect TK⁻ cells

and fresh selection medium containing 25μg/ml BUdR was added. The infected TK^- cells were incubated for two days until cpe had formed and then overlayed with agarose as before (Appendix B3). The recombinant viruses were passaged a total of 13 times before pure recombinant $VV_{97ZA347TS}$ was obtained.

A pure $VV_{97ZA347TS}$ stock was grown on the amniotic membrane of (five to ten day) fertilised eggs at 37°C (Appendix B4). Three days after infection of the membranes, the $VV_{97ZA347TS}$ was harvested (Appendix B4). The virus titre was determined by serial dilution of virus from 10^{-3} to 10^{-9} in virus diluent and infection of 70% confluent CV1 cells (Appendix B4). The virus was grown for three days at 37°C until plaques had formed and then pfus were counted after Ziehl Neelson Carbol Fuchsin staining. The $VV_{97ZA347TS}$ stock was stored in aliquots at -70°C in McIlvaine's buffer. Purified $VV_{97ZA347TS}$ was confirmed by immunofluorescence of infected cells using goat anti-HIV gp120 (Biogenesis, Poole, England) and a rabbit anti-sheep FITC conjugate (DAKO Glostrup, Denmark) as in section 4.2.3.3.

4.2.10) Challenge of DNA vaccine-immunised female BALB/c mice with recombinant $VV_{97ZA347TS}$

A preliminary study to confirm that the recombinant $VV_{97ZA347TS}$ would replicate in the mouse ovaries was performed. Eight female BALB/c mice (six to eight weeks) were injected intraperitoneally with 100μl of: PBS; $5X10^6$ pfu; $5X10^7$ pfu; and $1X10^8$ pfu (two mice per group) of virus. After five days, the mice were sacrificed and their ovaries removed. The ovaries were ground up in McIlvaine's buffer (10(l/mg of mouse ovary) using ten-broek grinders (Appendix B9). The virus titre was measured by serial dilution from 10^{-3} to 10^{-9} in virus diluent, infection of 70% confluent CV1 cells and detection of virus plaques after three days as above (Appendix B4).

Once it was determined that the recombinant $VV_{97ZA347TS}$ was able to grow in BALB/c mouse ovaries, two experiments were performed. In both, female BALB/c mice (six to eight weeks) were divided into three groups with two mice in each group. Group 1 contained two mice immunised with $pcDNA3.1_{SF162}$, group 2 contained two mice immunised with $pcDNA3.1_{97ZA346TS}$ and the final group contained two mice that were immunised with the pcDNA3.1 (Invitrogen, USA) vector alone. Mice were only given one DNA vaccine immunisation as described in section 4.2.4. (Appendix B7) and nine days later challenged intraperitoneally (Appendix B7). For the first experiment, 100μl of $1X10^7$ pfu of $VV_{97ZA347TS}$ was delivered as the challenge dose and for the second experiment $5X10^7$ pfu of $VV_{97ZA347TS}$ was delivered. Mice receiving $1X10^7$ pfu of virus (low challenge dose) were sacrificed eight days later and their ovaries removed. Mice receiving $5X10^7$ pfu of $VV_{97ZA347TS}$ (high challenge dose) were sacrificed after five days and their ovaries removed. Ovaries from both mice in each group were pooled and ground up in

McIlvaine's solution as above (Appendix B9). Virus titres were determined as above (Appendix B4) and the level of virus in each group was compared to the negative control, pcDNA3.1 immunised mice.

4.3) Results

As subtype C was demonstrated to be the predominant subtype in South Africa in the previous chapter, we aimed to characterise the gp120 in more detail. An isolate that was macrophage tropic and NSI was selected (97ZA346TS) as it was considered to be more closely related to the sexually transmitted phenotype. Co-receptor usage was not available at this time and is therefore unknown. For comparative purposes, a parallel study utilising the gp120 from a NSI, CCR5-restricted subtype B isolate (SF162) was undertaken. In order to determine the immunogenicity of the gp120, the genes were cloned and expressed by the pcDNA3.1/Zeo DNA vaccine (pcDNA3.1$_{SF162}$ and pcDNA3.1$_{97ZA346TS}$) and used to immunise BALB/c mice. The *in vivo* CTL cross-reactivity obtained in BALB/c mice between subtypes B and C was also investigated, using the murine *in vivo* challenge system (Binder and Kündig, 1991, Bachmann *et al.*, 1994; Belyakov *et al.*, 1998 and Kent *et al.*, 1998), utilising the VV-HIV recombinant, VV$_{97ZA347TS}$, as the challenge virus.

4.3.1) Characterisation of the gp120 from 97ZA347TS

The DNA sequence encoding the complete gp120 (and 5' end of the gp41) of 97ZA347TS is shown in figure 4.4. Phylogenetic analysis of 97ZA347TS in figure 4.5 shows that the sequence clusters within the subtype C lineage with a bootstrap value of 100%. The average intrasubtype DNA distance between 97ZA347TS and the nine reference subtype C sequences is 10.1% with a range of 8.5% to 10.8% (see Table 4.4). No South African sequences of the complete gp120 were available for comparison and the South African sequence, 97ZA347TS, was found to be most related to ZAM18, from Zambia with a DNA difference of 8.5%.

The gp120 amino acid divergence between the two isolates being immunogenically compared, 97ZA347TS and SF162, was also determined. In addition, the gp120 amino acid sequence of ZAM18 was included for comparison. The amino acid divergence between SF162 and 97ZA347TS is 24% and 25% between SF162 and ZAM18, whereas ZAM18 is 15% divergent from 97ZA347TS.

97ZA347TS DNA sequence

ATGAGAGTGAGGGGGATACGGAAGAATTGTCAACAATGGTGGATATGGGGCATCTTAGGC

signal peptide\ /gp120

←vpU

TTTTGGATGCTAATGATTTGTAATGGGGAGGGCTTGTGGGTCACAGTCTATTATGGGGTA
CCTGTGTGGAAAGAAGCAAAAACTACTCTATTTTGTGCATCAGATGCTAAAGCATATGAG
AGAGAAGTGCATAATGTTTGGGCTACACATGCCTGTGTACCCACAGACCCCGATCCACAA
GAAATAGTTTTGGAAAATGTAACAGAAAATTTTAACATGTGGAAAAATGATATGGTGGAT
CAGATGCATGAGGATATAATCAGTTTATGGGATCAAAGTCTAAAGCCATGTGTAAAGTTG
ACCCCACTCTGTGTCACTTTAAACTGTACTAATGTTAACAGAACTGTTGATACTGTTGAC
AATGATACCATGAGTGGAGGAATGAAAAATTGCTCTTTCAATATAACCACAGATCTAAGA
GATAAGAGAAAGAAGGAATATGCACTTTTTTATGGACTTGATATAGTACCACTTAATGAG
GAGAAGAGTGAGTATAGATTAATAAGTTGTAATACCTCAACCGTAACACAAGCTTGTCCA
AAGGTCTCTTTTGACCCAATTCCTATACATTATTGTGCTCCAGCTGGTTATGCGATTCTA
AAGTGTAATAATAAGAAATTCAATGGGACAGGACCACGCAATAATGTCAGCACAGTCCAA
TGTACACATGGAATTAAGCCAGTGGTATCAACTCAACTACTGTTAAATGGTAGTCTAGCA
GAAGAAGAGATAATAATTAGCTCTGAAAATCTGACAGACAATGCTAAGCCAATATTAGTA

↓ V3 loop start

CAACTTAATGGGTCTGTAGAAATTGTGTGTACAAGACCCAGCAATAATACAAGAAAAAGT
ATAAGGATAGGACCAGGACAAGCATTCTATGCAACAGGTGACATAATAGGTAACATAAGG

↓ V3 loop end

CAAGCATATTGTAACATTAGTGAAGAGAAGTGGAATGAAACTTTACAAGAGGTAGTAAAA
AAATTAAAAGAATACTTCCAACAAAAAACAATAATATTGAACCATCACTCAGGAGGGGAC
CTAGAAATTCCAACCCATAGCTTTAATCGCAGAGGGGAATTTTTCTATTGCAATACATCA
AAACTGTTTAAGGGTACATATGATACAAATGGTACAGAAAGTAATTCAAGCTCAACCATC
ATACTCCCATGCAGAATAAACCAAATTATAAACATGTGGCAGGGGGTAGGACGAGCAATG
TATGCCCCTCCCATTGCAGGAAACATAACATGTAAATCAAATATCACAGGCCTACTATTG
ACACGTGATGGAGGTGGATATGGAAATAACACAACCACAACAGAGATATTCAGACCTGGA
GGAGGAGATATGAGGGACAATTGGAGAAGTGAATTATATAAATATAAAGTGGTAGAAATT

env gp120/ *env* gp41

AAGCCATTAGGAGTAGTACCCACTAAGGCAAAAAGGAGAGTGGTGGAGAGAGAGAAAAGA
GCAGTGGGAATAGGAGCTGTGTTCCTTGGGTTCTTGGGAGCAGCCGGAAGCACTATGGGC
GCAGCATCAACAACGCTGACGGTACAGGCCAGACAATTGTTGTCTGGTATAGTGCAACAG
CAAAGCAATTTGCTGAGGGCTATAGAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGG
GGCATCAAACAGCTCCAGGCAAGA

Figure 4.4: *The complete* gp120 DNA sequence and 5' end of the gp41 for the macrophage tropic, primary subtype C isolate, 97ZA347TS indicating the start and end of the gp120 as well as the V3 loop sequence.

Table 4.4: The percentage DNA distance between subtype C, 97ZA347TS and reference sequences from subtypes A to AE, including nine subtype C reference sequences from southern Africa (n=4) and globally (n=5). No full length gp120 subtype C sequences from South Africa have been published and could thus not be included.

	A	B	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	D	AE
C1	16.5	16.1	0	10.5	10.6	10.4	8.5	10.8	10.4	10.0	10.0	10.0	17.1	17.7
C2	17.3	17.6	10.5	0	10.7	10.5	10.0	11.3	11.3	10.2	9.9	9.9	18.0	18.3
C3	17.1	16.8	10.6	10.7	0	10.4	9.8	12.3	11.0	9.0	8.3	9.4	18.1	18.3
C4	16.8	16.5	10.4	10.5	10.4	0	9.3	11.0	10.0	9.4	8.3	9.6	17.3	17.7
C5	16.3	17.0	8.5	10.0	9.8	9.3	0	19.7	9.4	7.7	7.4	9.1	17.6	18.2
C6	16.5	16.6	10.8	11.3	12.3	11.0	10.7	0	9.9	11.2	10.7	8.5	17.1	18.4
C7	16.5	16.5	10.4	11.3	11.0	10.0	9.4	9.9	0	10.3	9.6	9.1	17.5	17.5
C8	16.8	16.7	10.0	10.2	9.0	9.4	7.7	11.2	10.3	0	5.8	9.5	17.9	17.7
C9	17.0	16.5	10.0	9.9	8.3	8.3	7.4	10.7	9.6	5.8	0	8.6	17.6	17.9
C10	15.3	15.8	10.0	9.9	9.4	9.6	9.1	8.5	9.1	9.5	8.6	0	17.1	16.9

A - K89, SF170; B – OYI, LAI, SF162; C1 – 97ZA347TS; C2 – 96BW0408; C3 – 96BW0502; C4 – 96BW01B22 C5– ZAM18; C6 – ETH2220; C7 – 92BR025; C8 – IN.11246; C9 – IN.D1024; C10 – UG268A2; D – NDK, Z2Z6; and AE – CR402, KH03.

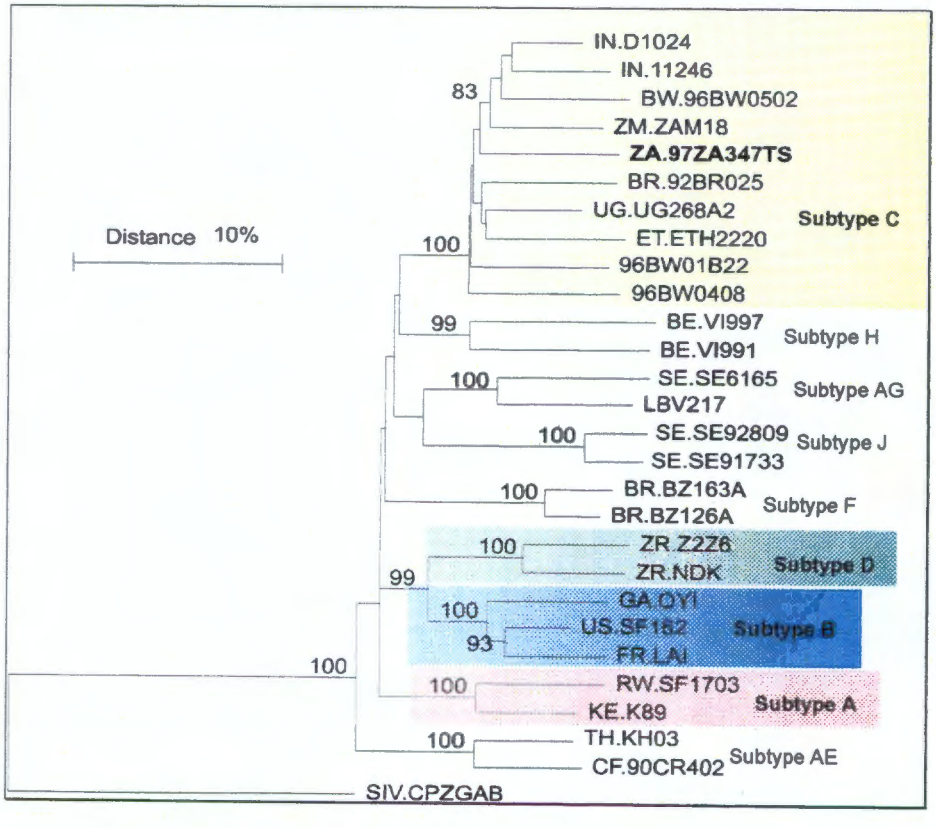
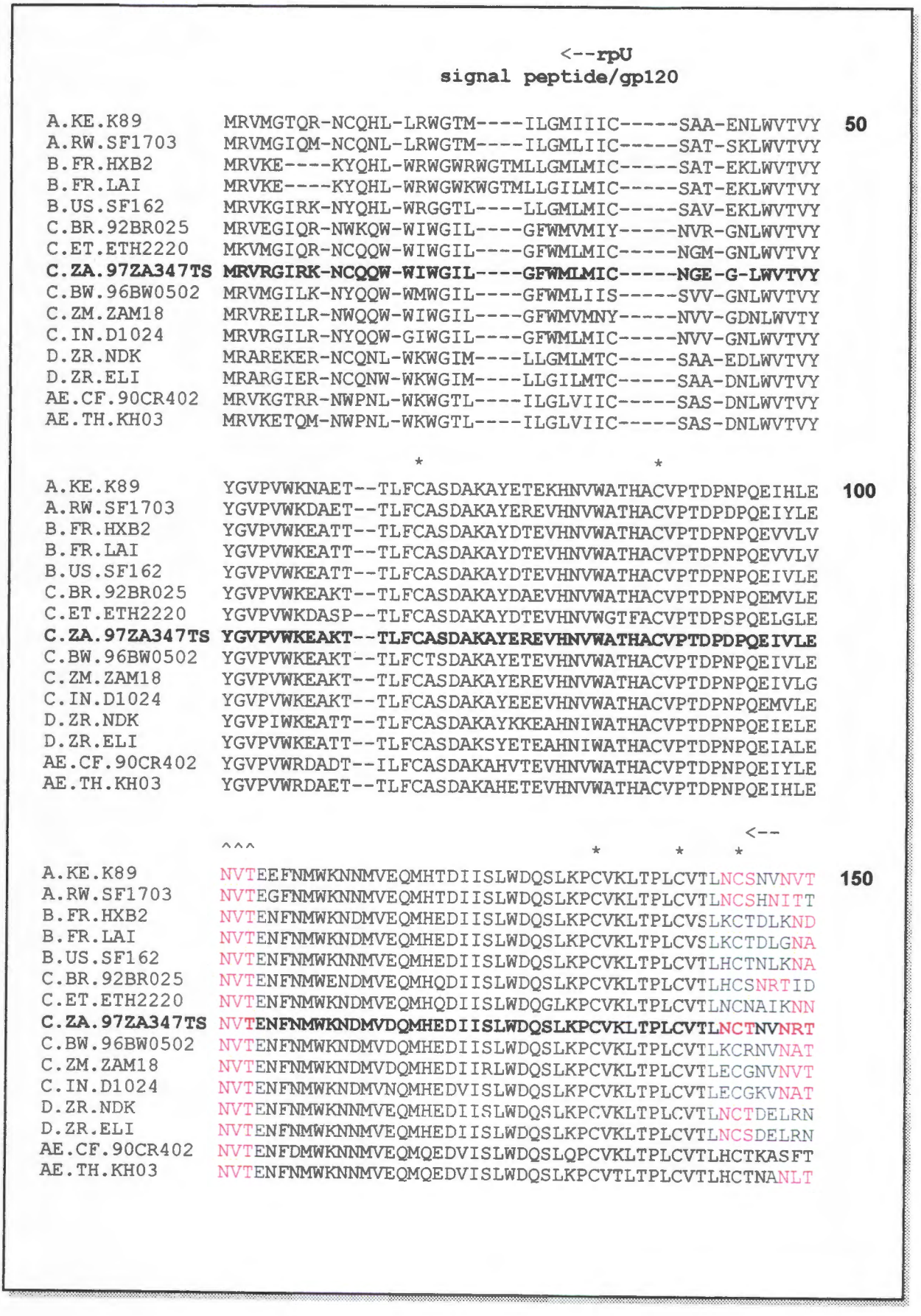


Figure 4.5: Phylogenetic tree of the gp120 and 5' portion of the gp41 from isolate 97ZA347TS in comparison with reference sequences from subtypes A to J. The sequence from macrophage tropic isolate SF162 was also included for comparison. Isolate 97ZA347 clusters within the subtype C lineage with a bootstrap value of 100%.


<--rpU
signal peptide/gp120
A.KE.K89 MRVMGTQR-NCQHL-LRWGTM----ILGMIIIC-----SAA-ENLWVTVY 50
A.RW.SF1703 MRVMGIQM-NCQNL-LRWGTM----ILGMLIIC-----SAT-SKLWVTVY
B.FR.HXB2 MRVKE----KYQHL-WRWGWRWGTMLLGMLMIC-----SAT-EKLWVTVY
B.FR.LAI MRVKE----KYQHL-WRWGWKWGTMLLGILMIC-----SAT-EKLWVTVY
B.US.SF162 MRVKGIRK-NYQHL-WRGGTL----LLGMLMIC-----SAV-EKLWVTVY
C.BR.92BR025 MRVEGIQR-NWKQW-WIWGIL----GFWMVMIY-----NVR-GNLWVTVY
C.ET.ETH2220 MKVMGIQR-NCQQW-WIWGIL----GFWMLMIC-----NGM-GNLWVTVY
C.ZA.97ZA347TS MRVRGIRK-NCQQW-WIWGIL----GFWMLMIC-----NGE-G-LWVTVY
C.BW.96BW0502 MRVMGILK-NYQQW-WMWGIL----GFWMLIIS-----SVV-GNLWVTVY
C.ZM.ZAM18 MRVREILR-NWQQW-WIWGIL----GFWMVMNY-----NVV-GDNLWVTY
C.IN.D1024 MRVRGILR-NYQQW-GIWGIL----GFWMLMIC-----NVV-GNLWVTVY
D.ZR.NDK MRAREKER-NCQNL-WKWGIM----LLGMLMTC-----SAA-EDLWVTVY
D.ZR.ELI MRARGIER-NCQNW-WKWGIM----LLGILMTC-----SAA-DNLWVTVY
AE.CF.90CR402 MRVKGTRR-NWPNL-WKWGTL----ILGLVIIC-----SAS-DNLWVTVY
AE.TH.KH03 MRVKETQM-NWPNL-WKWGTL----ILGLVIIC-----SAS-DNLWVTVY
A.KE.K89 YGVPVWKNAET--TLFCASDAKAYETEKHNVWATHACVPTDPNPQEIHLE 100
A.RW.SF1703 YGVPVWKDAET--TLFCASDAKAYEREVHNVWATHACVPTDPDPQEIYLE
B.FR.HXB2 YGVPVWKEATT--TLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLV
B.FR.LAI YGVPVWKEATT--TLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLV
B.US.SF162 YGVPVWKEATT--TLFCASDAKAYDTEVHNVWATHACVPTDPNPQEIVLE
C.BR.92BR025 YGVPVWKEAKT--TLFCASDAKAYDAEVHNVWATHACVPTDPNPQEMVLE
C.ET.ETH2220 YGVPVWKDASP--TLFCASDAKAYDTEVHNVWGTFACVPTDPSPQELGLE
C.ZA.97ZA347TS YGVPVWKEAKT--TLFCASDAKAYEREVHNVWATHACVPTDPDPQEIVLE
C.BW.96BW0502 YGVPVWKEAKT--TLFCTSDAKAYETEVHNVWATHACVPTDPNPQEIVLE
C.ZM.ZAM18 YGVPVWKEAKT--TLFCASDAKAYEREVHNVWATHACVPTDPNPQEIVLG
C.IN.D1024 YGVPVWKEAKT--TLFCASDAKAYEEEVHNVWATHACVPTDPNPQEMVLE
D.ZR.NDK YGVPIWKEATT--TLFCASDAKAYKKEAHNIWATHACVPTDPNPQEIELE
D.ZR.ELI YGVPVWKEATT--TLFCASDAKSYETEAHNIWATHACVPTDPNPQEIALE
AE.CF.90CR402 YGVPVWRDADT--ILFCASDAKAHVTEVHNVWATHACVPTDPNPQEIYLE
AE.TH.KH03 YGVPVWRDAET--TLFCASDAKAHETEVHNVWATHACVPTDPNPQEIHLE
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C.BW.96BW0502 NVTENFNMWKNDMVDQMHEDIISLWDQSLKPCVKLTPLCVTLKCRNVNAT
C.ZM.ZAM18 NVTENFNMWKNDMVDQMHEDIIRLWDQSLKPCVKLTPLCVTLECGNVNVT
C.IN.D1024 NVTENFNMWKNDMVNQMHEDVISLWDQSLKPCVKLTPLCVTLECGKVNAT
D.ZR.NDK NVTENFNMWKNNMVEQMHEDIISLWDQSLKPCVKLTPLCVTLNCTDELRN
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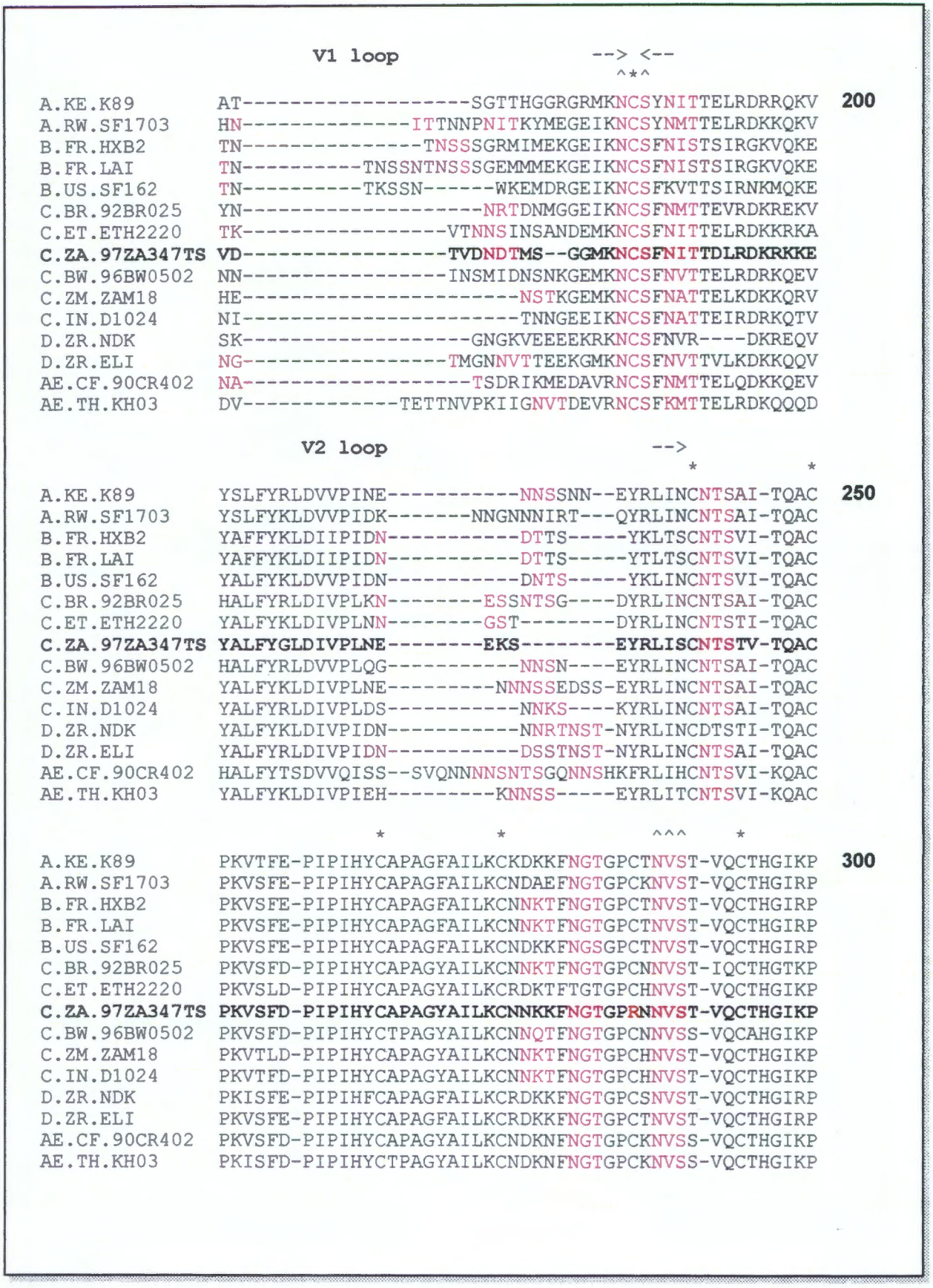
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B.FR.LAI TN----------TNSSNTNSSSGEMMMEKGEIKNCSFNISTSIRGKVQKE
B.US.SF162 TN----------TKSSN------WKEMDRGEIKNCSFKVTTSIRNKMQKE
C.BR.92BR025 YN----------NRTDNMGGEIKNCSFNMTTEVRDKREKV
C.ET.ETH2220 TK----------VTNNSINSANDEMKNCSFNITTELRDKKRKA
C.ZA.97ZA347TS VD----------TVDNDTMS--GGMKNCSFNITTDLRDKRKKE
C.BW.96BW0502 NN----------INSMIDNSNKGEMKNCSFNVTTELRDRKQEV
C.ZM.ZAM18 HE----------NSTKGEMKNCSFNATTELKDKKQRV
C.IN.D1024 NI----------TNNGEEIKNCSFNATTEIRDRKQTV
D.ZR.NDK SK----------GNGKVEEEEKRKNCSFNVR----DKREQV
D.ZR.ELI NG----------TMGNNVTTEEKGMKNCSFNVTTVLKDKKQQV
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B.FR.LAI YAFFYKLDIIPIDN----------DTTS-----YTLTSCNTSVI-TQAC
B.US.SF162 YALFYKLDVVPIDN----------DNTS-----YKLINCNTSVI-TQAC
C.BR.92BR025 HALFYRLDIVPLKN--------ESSNTSG----DYRLINCNTSAI-TQAC
C.ET.ETH2220 YALFYKLDIVPLNN--------GST--------DYRLINCNTSTI-TQAC
C.ZA.97ZA347TS YALFYGLDIVPLNE--------EKS--------EYRLISCNTSTV-TQAC
C.BW.96BW0502 HALFYRLDVVPLQG----------NNSN----EYRLINCNTSAI-TQAC
C.ZM.ZAM18 YALFYKLDIVPLNE---------NNNSSEDSS-EYRLINCNTSAI-TQAC
C.IN.D1024 YALFYRLDIVPLDS----------NNKS----KYRLINCNTSAI-TQAC
D.ZR.NDK YALFYKLDIVPIDN----------NNRTNST-NYRLINCDTSTI-TQAC
D.ZR.ELI YALFYRLDIVPIDN----------DSSTNST-NYRLINCNTSAI-TQAC
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AE.TH.KH03 YALFYKLDIVPIEH---------KNNSS-----EYRLITCNTSVI-KQAC
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C.ET.ETH2220 PKVSLD-PIPIHYCAPAGYAILKCRDKTFTGTGPCHNVST-VQCTHGIKP
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C.BW.96BW0502 PKVSFD-PIPIHYCTPAGYAILKCNNQTFNGTGPCNNVSS-VQCAHGIKP
C.ZM.ZAM18 PKVTLD-PIPIHYCAPAGYAILKCNNKTFNGTGPCHNVST-VQCTHGIKP
C.IN.D1024 PKVTFD-PIPIHYCAPAGYAILKCNNKTFNGTGPCHNVST-VQCTHGIKP
D.ZR.NDK PKISFE-PIPIHFCAPAGFAILKCRDKKFNGTGPCSNVST-VQCTHGIRP
D.ZR.ELI PKVSFE-PIPIHYCAPAGFAILKCRDKKFNGTGPCTNVST-VQCTHGIRP
AE.CF.90CR402 PKVSFD-PIPIHYCAPAGYAILKCNDKNFNGTGPCKNVSS-VQCTHGIKP
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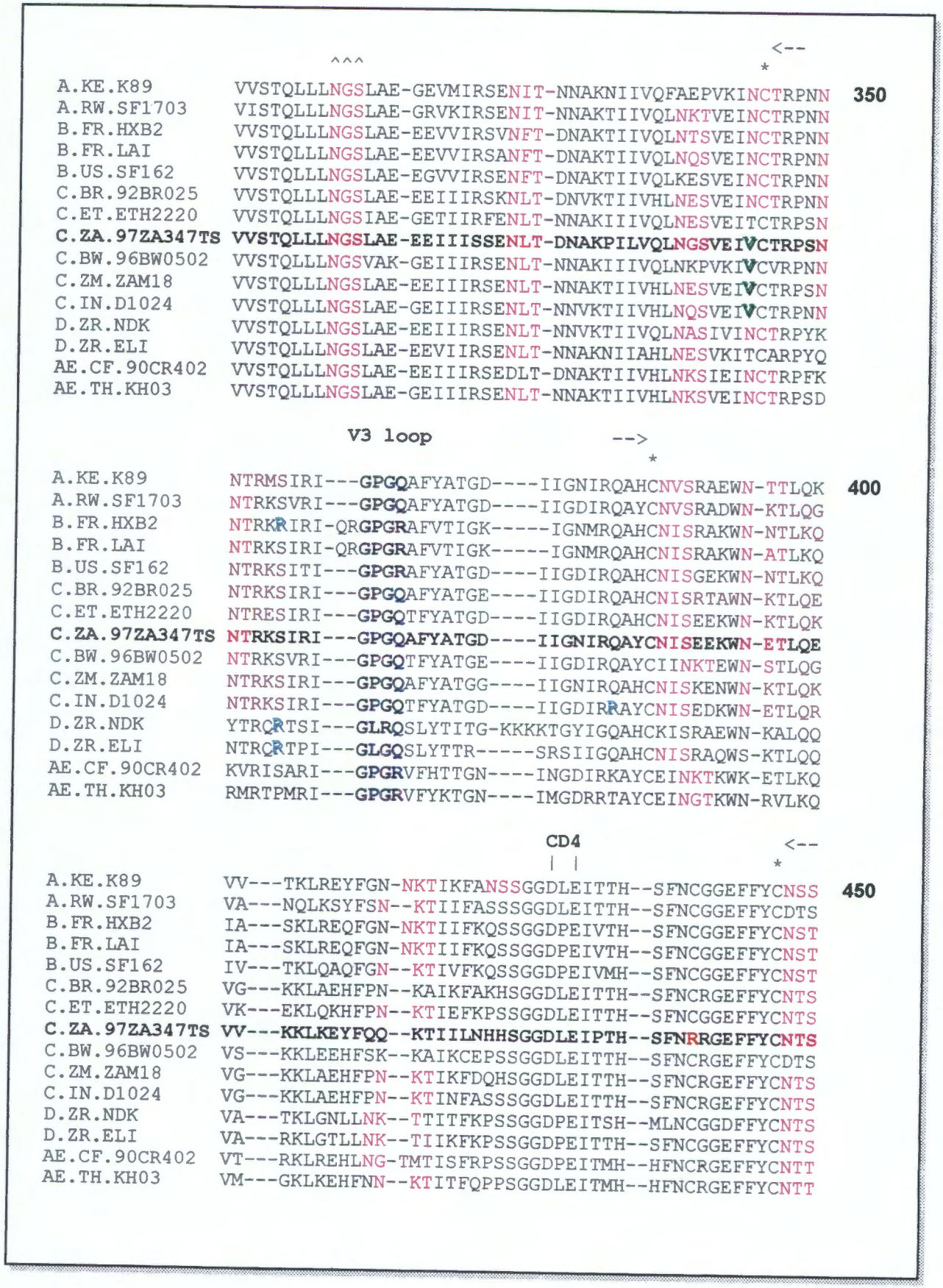
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B.FR.HXB2 VVSTQLLLNGSLAE-EEVVIRSVNFT-DNAKTIIVQLNTSVEINCTRPNN
B.FR.LAI VVSTQLLLNGSLAE-EEVVIRSANFT-DNAKTIIVQLNQSVEINCTRPNN
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C.ET.ETH2220 VVSTQLLLNGSIAE-GETIIRFENLT-NNAKIIIVQLNESVEITCTRPSN
C.ZA.97ZA347TS VVSTQLLLNGSLAE-EEIIISSENLT-DNAKPILVQLNGSVEIVCTRPSN
C.BW.96BW0502 VVSTQLLLNGSVAK-GEIIIRSENLT-NNAKIIIVQLNKPVKIVCVRPNN
C.ZM.ZAM18 VVSTQLLLNGSLAE-EEIIIRSENLT-NNAKTIIVHLNESVEIVCTRPSN
C.IN.D1024 VVSTQLLLNGSLAE-GEIIIRSENLT-NNVKTIIVHLNQSVEIVCTRPNN
D.ZR.NDK VVSTQLLLNGSLAE-EEIIIRSENLT-NNVKTIIVQLNASIVINCTRPYK
D.ZR.ELI VVSTQLLLNGSLAE-EEVIIRSENLT-NNAKNIIAHLNESVKITCARPYQ
AE.CF.90CR402 VVSTQLLLNGSLAE-EEIIIRSEDLT-DNAKTIIVHLNKSIEINCTRPFK
AE.TH.KH03 VVSTQLLLNGSLAE-GEIIIRSENLT-NNAKTIIVHLNKSVEINCTRPSD
V3 loop
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*
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B.FR.LAI NTRKSIRI-QRGPGRAFVTIGK-----IGNMRQAHCNISRAKWN-ATLKQ
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C.BR.92BR025 NTRKSIRI---GPGQAFYATGE----IIGDIRQAHCNISRTAWN-KTLQE
C.ET.ETH2220 NTRESIRI---GPGQTFYATGD----IIGDIRQAHCNISEEKWN-KTLQK
C.ZA.97ZA347TS NTRKSIRI---GPGQAFYATGD----IIGNIRQAYCNISEEKWN-ETLQE
C.BW.96BW0502 NTRKSVRI---GPGQTFYATGE----IIGDIRQAYCIINKTEWN-STLQG
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C.IN.D1024 NTRKSIRI---GPGQTFYATGD----IIGDIRRAYCNISEDKWN-ETLQR
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D.ZR.ELI NTRQRTPI---GLGQSLYTTR-----SRSIIGQAHCNISRAQWS-KTLQQ
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AE.TH.KH03 RMRTPMRI---GPGRVFYKTGN----IMGDRRTAYCEINGTKWN-RVLKQ
CD4
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| |
*
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B.FR.HXB2 IA---SKLREQFGN-NKTIIFKQSSGGDPEIVTH--SFNCGGEFFYCNST
B.FR.LAI IA---SKLREQFGN-NKTIIFKQSSGGDPEIVTH--SFNCGGEFFYCNST
B.US.SF162 IV---TKLQAQFGN--KTIVFKQSSGGDPEIVMH--SFNCGGEFFYCNST
C.BR.92BR025 VG---KKLAEHFPN--KAIKFAKHSGGDLEITTH--SFNCRGEFFYCNTS
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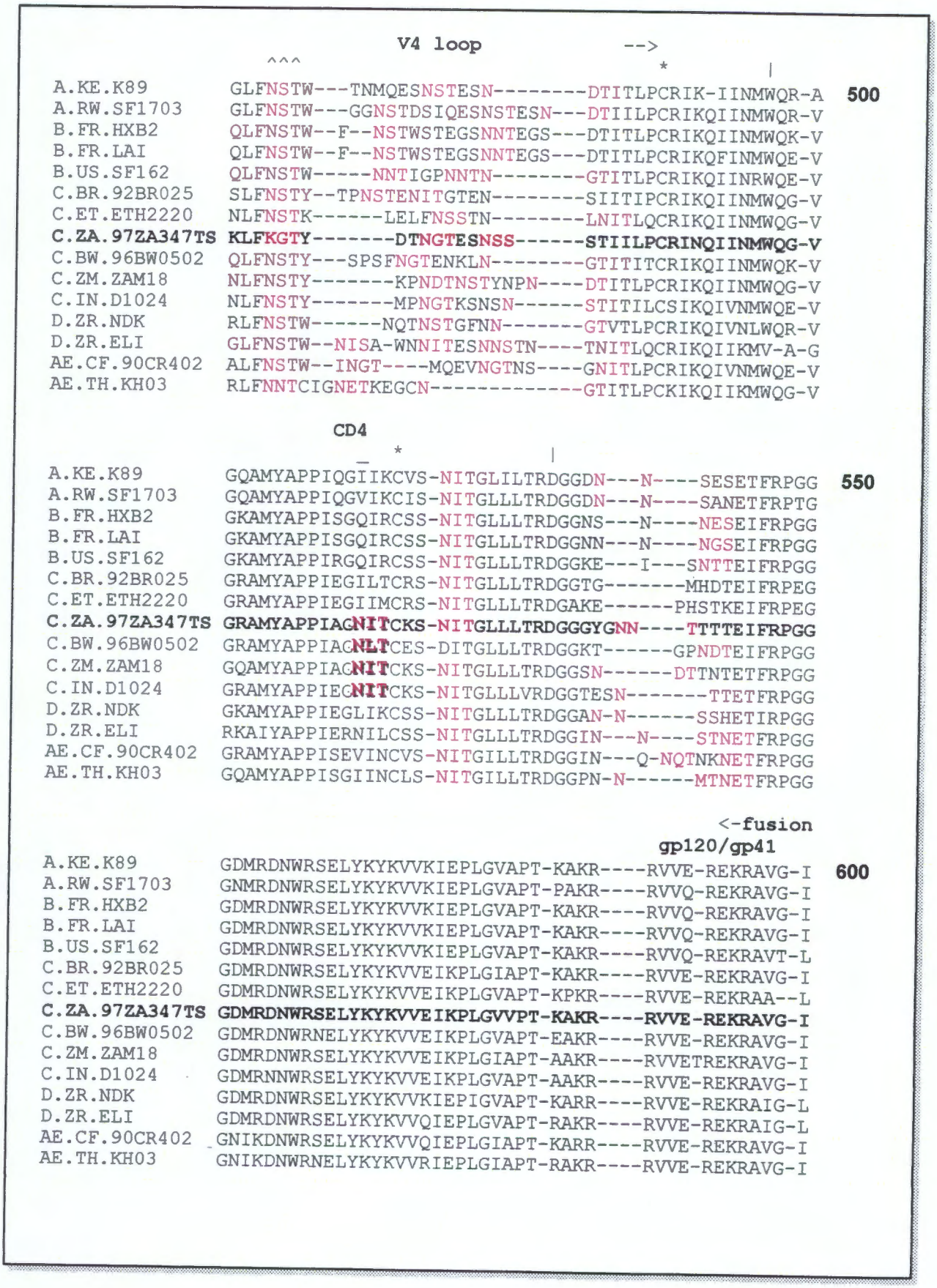
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B.FR.LAI QLFNSTW--F--NSTWSTEGSNNTEGS---DTITLPCRIKQFINMWQE-V
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C.ET.ETH2220 NLFNSTK------LELFNSSTN--------LNITLQCRIKQIINMWQG-V
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C.BW.96BW0502 QLFNSTY---SPSFNGTENKLN--------GTITITCRIKQIINMWQK-V
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C.IN.D1024 NLFNSTY-------MPNGTKSNSN------STITILCSIKQIVNMWQE-V
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AE.TH.KH03 RLFNNTCIGNETKEGCN----------GTITLPCKIKQIIKMWQG-V
CD4
*
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AE.TH.KH03 GQAMYAPPISGIINCLS-NITGILLTRDGGPN-N------MTNETFRPGG
<-fusion
gp120/gp41
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B.FR.HXB2 GDMRDNWRSELYKYKVVKIEPLGVAPT-KAKR----RVVQ-REKRAVG-I
B.FR.LAI GDMRDNWRSELYKYKVVKIEPLGVAPT-KAKR----RVVQ-REKRAVG-I
B.US.SF162 GDMRDNWRSELYKYKVVKIEPLGVAPT-KAKR----RVVQ-REKRAVT-L
C.BR.92BR025 GDMRDNWRSELYKYKVVEIKPLGIAPT-KAKR----RVVE-REKRAVG-I
C.ET.ETH2220 GDMRDNWRSELYKYKVVEIKPLGVAPT-KPKR----RVVE-REKRAA--L
C.ZA.97ZA347TS GDMRDNWRSELYKYKVVEIKPLGVVPT-KAKR----RVVE-REKRAVG-I
C.BW.96BW0502 GDMRDNWRNELYKYKVVEIKPLGVAPT-EAKR----RVVE-REKRAVG-I
C.ZM.ZAM18 GDMRDNWRSELYKYKVVEIKPLGIAPT-AAKR----RVVETREKRAVG-I
C.IN.D1024 GDMRNNWRSELYKYKVVEIKPLGVAPT-AAKR----RVVE-REKRAVG-I
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AE.CF.90CR402 GNIKDNWRSELYKYKVVQIEPLGIAPT-KARR----RVVE-REKRAVG-I
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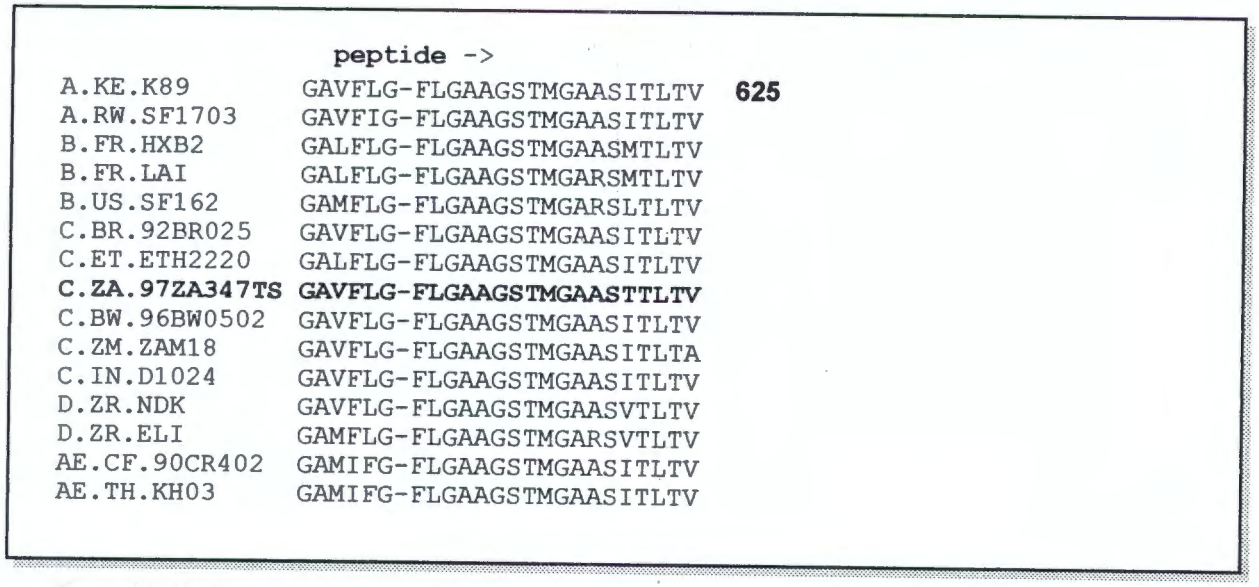


Figure 4.6: The alignment of amino acids making up the gp120 and part of gp41 from the South African, macrophage tropic, primary isolate, 97ZA347TS (in bold type) sequenced in this study compared with two reference subtypes from A to AE (HIV sequence database, 1998). Also, the amino acid sequence from SF162, the NSI reference subtype B isolate included in the DNA vaccine study has been included for comparative purposes. Conserved cysteine residues are indicated with an asterisk (*), whilst potential *N*-linked glycosylation sites are shown in pink (conserved potential *N*-linked glycosylation sites indicated with carets [^]). The tetrapeptide crown is shown bold and in blue. The amino acid positions are also shown (starting from zero at the start of the gp120), as are the variable loop positions and CD4 binding domains.

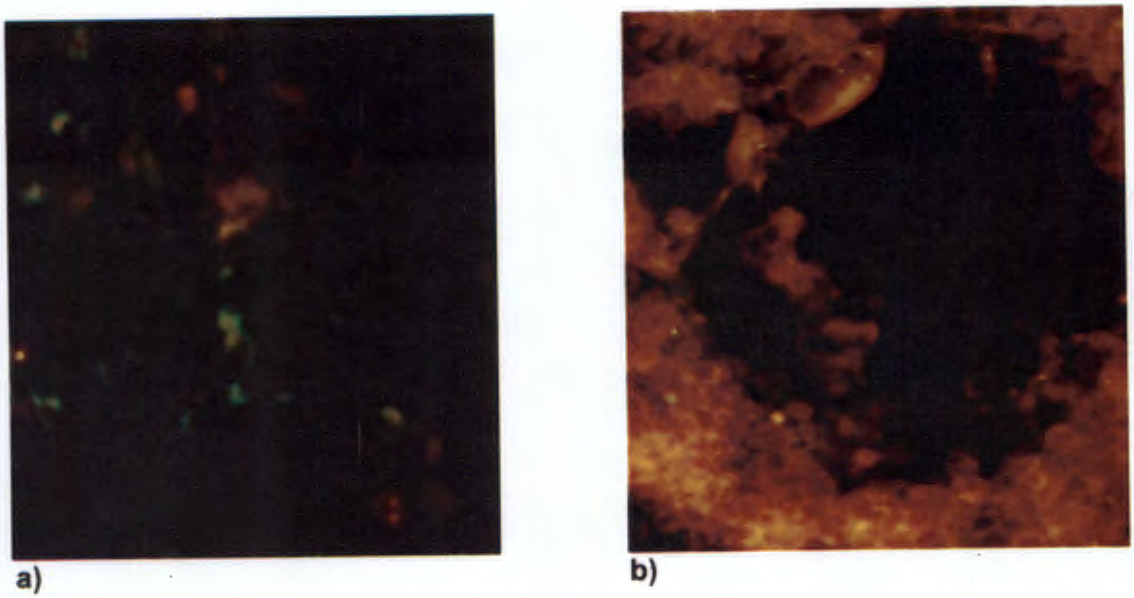
Figure 4.6 shows an alignment of the amino acids making up the gp120 and partial gp41 from 97ZA347TS as well as reference subtypes from subtypes A to AE (HIV sequence database, 1998) indicating conserved cysteine residues, *N*-linked glycosylation sites, variable loop regions and CD4 binding regions. The South African isolate 97ZA347TS gp120 contains 22 potential *N*-linked glycosylation sites, in comparison to an average of 24 in five macrophage tropic subtype C isolates analysed, 23 in macrophage tropic subtype B isolates (n=5), 28 in T-cell tropic subtype C isolates (n=2) and 26 in T-cell tropic subtype B isolates (n=6) (HIV sequence database, 1998 and Doms and Moore, 1997). In two positions, at 285 and 440, the usually conserved cysteine residue is replaced with an arginine residue (shown in red). The characteristic loss of a glycosylation site at position 344-346 in subtype C viruses was present in 97ZA347TS, with a valine replacing the asparagine residue at position 344 (shown in green). In addition, an extra *N*-linked glycosylation site, shown in bold maroon, common in subtype C isolates, was present at position 512 in 97ZA347TS. The molecular weight of the gp120 from 97ZA347TS is 56 303 (499 amino acids) and has a net charge of +6 with 51 acidic and 57 basic residues. No arginine residues, indicative of a syncitium inducing phenotype (Fouchier *et al.*, 1992 and De Wolf *et al.*, 1994), were present at positions 355, 369 or 376 (shown in turquoise) in isolate 97ZA347TS, although the arginine substitution was present in position 355 in isolates HXB2, NDK and ELI and position 376 in D1024.

D1024.

4.3.2) Immunofluorescent detection of the expressed gp120 protein

In order to determine the expression of gp120 from both pcDNA3.1$_{SF162}$ and pcDNA3.1$_{97ZA347TS}$, immunofluorescent staining of the gp120 was performed. Immunofluorescent detection showed gp120 expressed by the DNA vaccines in the cell cytoplasm in HeLa cells after 24 hours, but not after 48 hours. The same pattern of fluorescence was detected for the gp120 proteins of both subtypes. No fluorescence was observed in cells that were transfected with pcDNA3.1/Zeo alone.

The gp120 from 97ZA347TS cloned into pSC65 was transfected into VV-infected CV1 cells and the recombinant VV expressing gp120$_{97ZA347TS}$ selected by serial passage in TK$^-$ cells. After thirteen passages in TK$^-$ cells, the gp120, stabley expressed by recombinant VV$_{97ZA347TS}$, was detected by immunofluorescence, showing the fluorescent labelled gp120 both in the cell cytoplasm and on the cell membranes of TK$^-$ cells (see figure 4.7a). No fluorescence was observed in the TK$^-$ cells infected with wild-type VV (see figure 4.7b).



a) b)

Figure 4.7: Immunofluorescent staining of TK$^-$ cells infected with VV$_{97ZA347TS}$ (a) or VVwr (b) at 40X magnification. a) TK$^-$ cells infected with VV$_{97ZA347TS}$ are stained fluorescent yellow-green by the anti-HIV gp120 FITC conjugate which binds to the gp120 expressed in the cell cytoplasm by the recombinant VV$_{97ZA347TS}$ as well as on the cell membranes. b) Negative control of TK$^-$ cells infected with VVwr. The virus plaque is visible, and no fluorescent staining can be observed.

4.3.3) DNA immunisation of BALB/c mice

DNA vaccines expressing the gp120 from SF162 and 97ZA347TS were inoculated into BALB /c mice in order to determine the immune response. The BALB/c mice were immunised intramuscularly into the TA muscle with the DNA vaccine (100μg per mouse), followed by three intramuscular boosts, one every two weeks. The antibody response to gp120 in mice immunised with pcDNA3.1$_{SF162}$ (subtype B) and pcDNA3.1$_{97ZA346TS}$ (subtype C), as well as pcDNA3.1/Zeo alone, was determined by indirect ELISA with gp120 from laboratory isolate, HIV$_{IIIB}$, as the capture antigen. Peak antibody levels were reached 2 weeks after the first immunisation in mice immunised with pcDNA3.1$_{97ZA346TS}$, an increase of seven-fold above the levels detected in mice immunised with pcDNA3.1/Zeo plasmid alone (negative control mice) (see figure 4.8). The antibody levels then dropped to three-fold above negative control mice, remaining stable throughout the two subsequent boost immunisations. The mice immunised with pcDNA3.1$_{SF162}$ had slowly increasing antibody levels from three-fold to almost five-fold above the negative control mice, from the start of the immunisation regimen until the final immunisation. The pre-bleed serum was non-reactive, with an OD_{492} of 0.001 and mice immunised with the pcDNA3.1/Zeo plasmid alone had antibody levels of 0.028 OD_{492}. Mice with antibody levels greater than two-fold that of the pcDNA3.1/Zeo immunised mice were considered to have a positive antibody response.

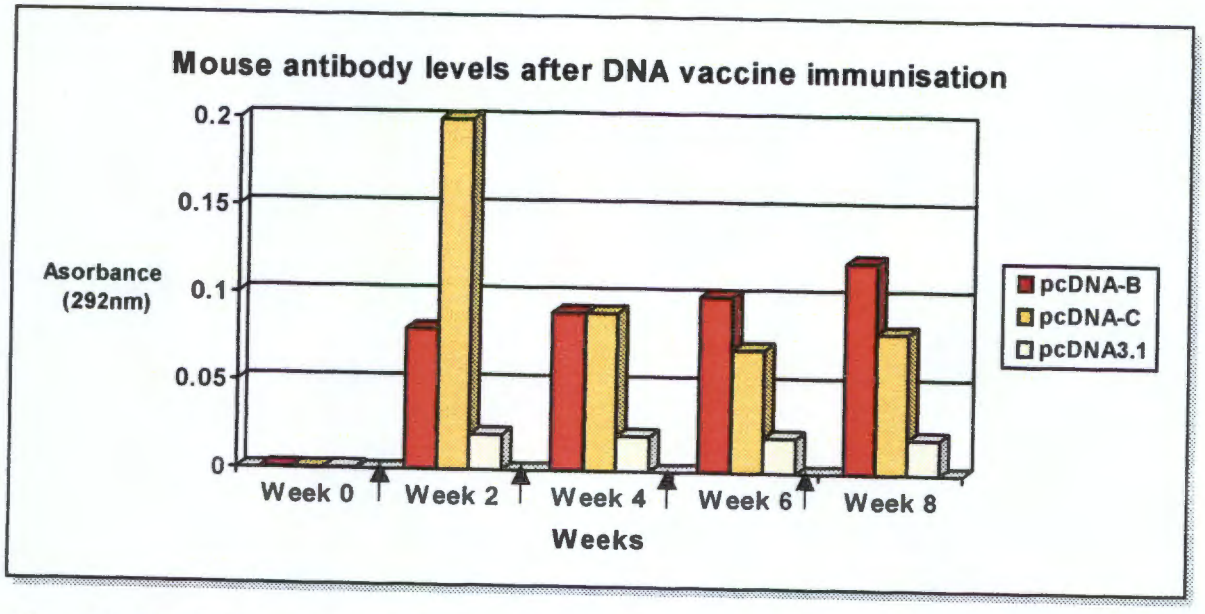


Figure 4.8: Antibody levels obtained in BALB /c mice in our study after immunisation with DNA vaccine: pcDNA-B; pcDNA-C; or pcDNA3.1/Zeo. The immunisation times are indicated with arrows (weeks 0, 2, 4 and 6) and blood was taken prior to each immunisation as well as two weeks after the final immunisation.

4.3.3) Determination of a cross-reactive in vivo CTL response to subtype B and C DNA vaccines

It was previously established that unimmunised BALB /c mice were not protected from VVwr and the virus was able to replicate to a titre of 9 to 10 $\log_{10}$ pfu per mouse (see section 4.2.10). The unimmunised BALB /c mice challenged with $VV_{97ZA347TS}$ did not have as high viral ovarian titres, since the recombinant VV is attenuated, with the gp120 interrupting the TK^- gene. In order to determine the possible CTL cross-reaction obtained between subtype B and C gp120, BALB /c mice inoculated once with a DNA vaccine expressing either subtype B, or subtype C gp120, were challenged nine days later with recombinant VV expressing the gp120 from the homologous subtype C isolate.

Table 4.5 shows the $VV_{97ZA347TS}$ titres in the ovaries of two female BALB /c mice immunised once with either pcDNA3.1_{SF162} (subtype B), pcDNA3.$1_{97ZA346TS}$ (subtype C) or pcDNA3.1/Zeo (negative control). The CTL response was measured as the decrease in $VV_{97ZA347TS}$ titre ($\log_{10}$ of the pfu) in the subtype B and C immunised mouse ovaries, in comparison to the titres obtained in the negative control mice (see figure 4.9). As the negative control mice had not been immunised, there was limited protection against the $VV_{97ZA347TS}$ challenge and these mice had higher ovarian titres of VV.

Two experiments were performed. In the first experiment, mice immunised with pcDNA3.1_{SF162} and $pcDNA_{97ZA347TS}$ were given an intraperitoneal challenge of $1X10^7$ pfu in 100(l of sterile 1XPBS and sacrificed after eight days. The negative control mice had a titre of 6.58 $\log_{10}$ per mouse, in comparison to the mice immunised with the homologous subtype C gp120, pcDNA3.$1_{97ZA346TS}$, which had a 1.9 $\log_{10}$ decrease in viral titre per mouse. The subtype B DNA vaccine (pcDNA3.1_{SF162}), on the other hand, elicited a 1.4 $\log_{10}$ pfu decrease. This was approximately 0.5 $\log_{10}$ higher titre than in mice vaccinated with the homologous subtype C DNA vaccine (see Table 4.5 and figure 4.9).

In the second experiment, a higher challenge titre ($5X10^7$ pfu) was used and the mice were sacrificed after five days. The ovaries from the negative control mice in this experiment had a higher titre of recombinant $VV_{97ZA347TS}$ (8.70 $\log_{10}$), than in the first experiment, due to the higher initial titre infecting the mice. The levels of protection, on the other hand, were also greater than in the first experiment, possibly because the timing of the second experiment lead to a more efficient CTL response being detected than in the first experiment. Mice immunised with the homologous subtype C DNA vaccine (pcDNA3.$1_{97ZA346TS}$) had a viral titre of 6.10 $\log_{10}$ pfu per mouse, which was 2.6 $\log_{10}$ pfu lower than for the negative control mice. The subtype B (pcDNA3.1_{SF162}) immunised mice had a virus titre 2.0 $\log_{10}$ lower than the negative control mice

(see figure 4.9).

Table 4.5: The cross-reactive *in vivo* CTL response as measured by $VV_{97ZA347TS}$ titres in mouse ovaries. BALB /c mice were immunised with DNA vaccines expressing the gp120 from subtypes B, C, or the DNA vaccine alone (negative control), followed by challenge with the recombinant $VV_{97ZA347TS}$. The CTL response was measured as a decrease in $VV_{97ZA347TS}$ titres in the mouse ovaries* between immunised and negative control mice.

DNA vaccine	Ovary weight	[1]Titre/ml	Titre/mouse
Experiment 1			
[2]$pcDNA3.1_{SF162}$	50mg/0.5ml	$1.8X10^5$ pfu	$1.5X10^5$ pfu
[3]$pcDNA3.1_{97ZA346TS}$	50mg/0.5ml	$2X10^5$ pfu	$4.5X10^4$ pfu
pcDNA3.1/Zeo	50mg/0.5ml	$1.5X10^7$ pfu	$3.8X10^6$ pfu
Experiment 2			
[2]$pcDNA3.1_{SF162}$	90mg/0.9ml	$1X10^7$ pfu	$5X10^6$ pfu
[3]$pcDNA3.1_{97ZA346TS}$	70mg/0.7ml	$5X10^6$ pfu	$1.25X10^6$ pfu
pcDNA3.1/Zeo	90mg/0.9ml	$2X10^9$ pfu	$5X10^8$ pfu

[1] The minimal level of detection was 100pfu.
[2-3] DNA vaccines encoding the gp120 from subtypes B and C respectively. There were two mice in each group.
*The virus titres were determined by pooling the mouse ovaries for both mice in each group and calculated as titre per mouse in pfu.

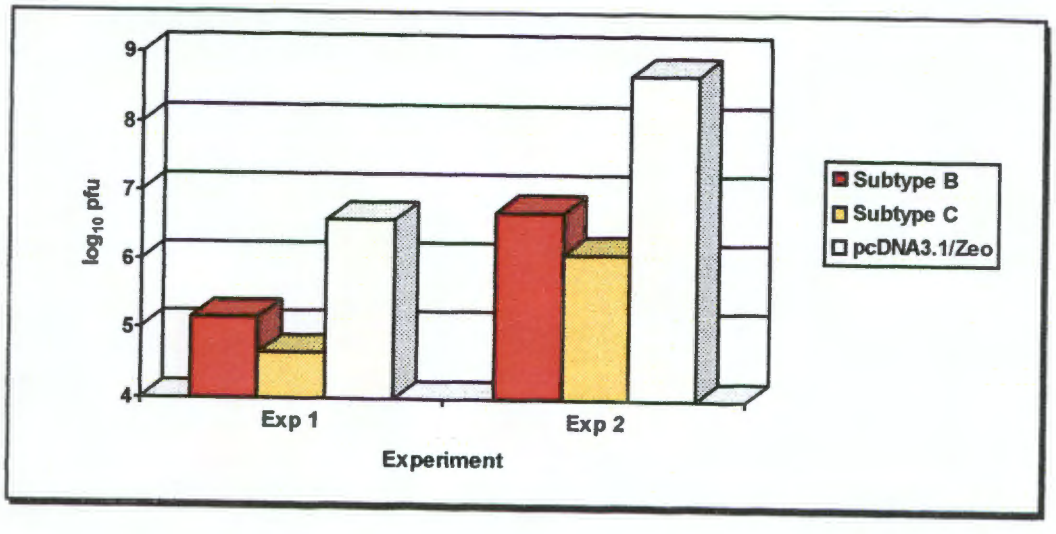


Figure 4.9: The cross-reactive CTL response between subtypes B and C measured as the decrease in VV97ZA347TS in the mouse ovaries between subtype B and C immunised mice and negative control mice.

The human and murine gp120 epitopes for consensus subtype B and C isolates were

compared (Korber *et al.*, 1997). In addition, the gp120 epitopes from the macrophage tropic South African isolate 97ZA347TS, and the NSI subtype B reference isolate, SF162 were deduced and compared with the gp120 epitopes in the subtype B and C consensus sequences (see figure 4.10). Unfortunately, only a limited number of murine CTL epitopes were available for the HIV gp120 protein. The only murine BALB /c *H-2*^{d} epitope recorded from gp120 was epitope 40, KQFINMWQEVGKAMYA, which had two amino acid mismatches in the sequence from isolate SF162 and four in the sequence from isolate 97ZA347TS. CTL epitopes with greater than two mismatches are unlikely to be functional (Cao *et al.*, 1997). It is thus unlikely that 97ZA347TS would have elicited a CTL response to epitope 40 in the BALB /c mice, however other CTL epitopes which have not yet been identified may have played a role in the murine CTL response.

A better indication of the possibility of cross-reactive CTL epitopes is obtained by a determination of the amino acid distance between isolates 97ZA347TS and SF162 (see section 4.3.1). Isolate SF162 is 24% divergent from 97ZA347TS. This divergence is reflected in the gp120 CTL epitopes for the two isolates, where twenty-two of the identified forty-two gp120 epitopes (Korber *et al.*, 1997) are conserved in isolate SF162, whereas only five of the gp120 epitopes are conserved in isolate 97ZA347TS.

Gp120 Epitope 1 (2-10)

```
Epitope/HLA   :RVKEKYQHL /B8
Subtype B     :rvkgirkny
SF162         :---------
Subtype C     :--r--qr-c
97ZA347TS     :--R-----C
```

Gp120 Epitope 2 (29-39)

```
Epitope/HLA   :AANELWVTVYY /B44
Subtype B     :a?ae?klwvtvyy
SF162          CS-VE--------
Subtype C     :v-mg.n-------
97ZA347TS     :NG-G.--------
```

Gp120 Epitope 3 (32-41)

```
Epitope/HLA   :KLWVTVYYGV /A2
Subtype B     :klwvtvyyGV
A3
SF162         :----------
Subtype C     :n---------
97ZA347TS     :G---------
```

Gp120 Epitope 4-6 (35-45)

```
Epitope/HLA   :VTVYYGVPVWK /A11
              : TVYYGVPVWK /A3.1,

Subtype B     :vtvyyGVPVWk
SF162         :----------
Subtype C     :----------
97ZA347TS     :----------
```

Gp120 Epitope 7-8 (41-51)

```
Epitope/HLA   :VPVWKEATTT  /B55
               VPVWKEATTTL /B35
Subtype B     :VPVWkeatTTL
SF162         :----------
Subtype C     :-------k---

97ZA347TS     :-------K---
```

Gp120 Epitope 9 (51-60)

```
Epitope/HLA   :LFCASDAKAY /B38
Subtype B     :LFcASdakay
SF162         :----------
Subtype C     :----------
97ZA347TS     :---------E
```

Gp120 Epitope 10-12 (77-85) B51,B35,

```
Epitope/HLA   :DPNPQEVVL /B*3501
Subtype B     :dPnPqEvvl
SF162         :------I--
Subtype C     :------m--
97ZA347TS     :--D---I--
```

Gp120 Epitope 13 (104-116)

```
Epitope/HLA   :HEDIISLWDQSLK /A2
Subtype B     :hEdiisLWdqslK
SF162         :-------------
Subtype C     :-------------
97ZA347TS     :-------------
```

Gp120 Epitope 14 (120-128)

```
Epitope/HLA   :KTLPLCVTL /A2
Subtype B     :ktlPlcVtL
SF162         :---------
Subtype C     :---------
97ZA347TS     :---------
```

Gp120 Epitope 15 (160-169)

```
Epitope/HLA   :NCSFNISTSI /Cw8
Subtype B     :ncsfnittsi
SF162         :----KV----
Subtype C     :-----a--el
97ZA347TS     :------T-DL
```

Gp120 Epitope 16-17 (196-204)

```
Epitope/HLA   :KLTSCNTSV /A2, A2.1
Subtype B     :rliscntsv
SF162         :K--N-----
Subtype C     :--in----a
97ZA347TS     :R-I------
```

Gp120 Epitope 18 (243-251)

```
Epitope/HLA   :CTNVSTVQC /Cw8
Subtype B     :ctnvStvqc
SF162         :---------
Subtype C     :-h-------
97ZA347TS     :RN-------
```

Gp120 Epitope 19-21 (256-264)

```
Epitope:  RPVVSTQLLLNGSLAEEEVV/B7
Epitope/HLA:SVEINCTRPNNNTRKSI/A2
/HLA      RPVVSTQLL /B35, B*3501
RPNNNTRKSI/B7,?
Subtype B:rpvvstqlllNGSlaeeevv

SF162     :-----------------G--
Subtype C:k-----------------ii

97ZA347TS:K-----------------DI
```

Gp120 Epitope 22-24 (295-311)

```
Subtype B   :sveinCtRpnnntrksi

SF162       :-----------------
Subtype C   :----v------------

97ZA347TS   :----V----S-------
```

Gp120 Epitope 25-30 (310-324)

```
Epitope/HLA  :   RGPGRAFVTI    /A2.1,A2
              RIQRGPGRAFVTIGK /A2,A3,A11
                   GRAFVTIGK /B27
Subtype B    :sihigpgrafyttge
SF162        :--T--------A--D
Subtype C    :s-ri---Qt-yat-d
97ZA347TS    :S-RI---Q--YAT-D
Murine       :RIQRGPGRAFVTIGK/H-2d,p,u,q,H-2Dd,Dd
              RIHIGPGRAFYTTKN/H-2Dd,Dd
              SITKGPGRVIYATGQ/Dd
                 IGPGRAFYTT  /H-2Dd ,Dd
                 RGPGRAFVTI  /Dd,Ld,D,H-2d,p,u
                 IGPGRAFHT   /H-2Dd
```

Gp120 Epitope 31

```
Epitope/HLA   :NNTLKQIDSKLREQFG/CD4+
Subtype B     :nntLkqivkklreqfg
SF162         :-----------QA---
Subtype C     :-k--qrVgk--a-h-p
97ZA347TS     :-E--QEVVK--K-Y-Q
```

Gp120 Epitope 32 (373-379)

```
Epitope/HLA   :PEIVTHS /A2
Subtype B     :peivmhs
SF162         :-------
Subtype C     :l--tt--
97ZA347TS     :L--P---
```

Gp120 Epitope 33-34 (379-387)

```
Epitope/HLA   :SFNCGGEFF /Cw4
/A29
Subtype B     :sfnCggeff
SF162         :---------
Subtype C     :----r----
97ZA347TS     :---RR----
```

Gp120 Epitope 35-37 (380-391)

```
Epitope/HLA   :FNCGGEFFY

                FNCGGEFFYCNS /A2
                 NCGGEFFYCNS /A2
Subtype B     :fnCggeffyCnt
SF162         :-----------S
Subtype C     :---r-------t
97ZA347TS     :--RR-------T
```

Gp120 Epitope 38-40 (417-436)

```
Epitope/HLA   :LPCRIKQFINMWQEVGKAMY /A2
               LPCRIKQFINMWQE       /DR4
                    KQFINMWQEVGKAMY /A2
Subtype B     :lpCrikqiinmwQevgkamy
SF162         :----------R---------
Subtype C     :----------------r---
97ZA347TS     :-----N-------G--R---
Murine        :     KQIINMWQEVGKAMYA/H-2a,b,f
```

Gp120 Epitope 41 (422-437)

```
Epitope/HLA   :KQIINMWQEVGKAMYA /A2
Subtype B     :kqiinmwQevgkamya
SF162         :----------------
Subtype C     :----------r----
97ZA347TS     :N-------G--R----
Murine        :            MYAPPIGGQI/H-2Kd
```

Gp120 Epitope 42 (491-510)

```
Epitope/HLA   :VKIEPLGVAPTKAKRRVVQR /A2
Subtype B     :vkieplgvAptk.akrRvvqr
SF162         :-----------.--------
Subtype C     :-e-k--------.------e-
97ZA347TS     :-E-K----V---.------E-
```

Key

RED: Conserved
BLUE: 1 mismatch
GREEN: 2 Mismatches
Black: >2 Mismatches

Figure 4.10: An alignment of the gp120 CTL epitopes, taken from the HIV Molecular Immunology Database (Korber *et al.*, 1997) is illustrated. The epitope number and amino acid site in the gp120 protein is shown above each alignment, followed by the epitope amino acid sequence and the human HLA allele for that epitope. The subtype B and C consensus amino acid sequences for each epitope are also shown. In addition, the gp120 epitopes for the two DNA vaccine isolates in this study (97ZA347TS and SF162) are included for comparative purposes. Dashes indicate regions of conservation with the consensus subtype B epitope, dots indicate amino acid deletions and question marks indicate stop codons in the consensus sequence. The level of conservation with the human epitope is indicated by the colour of the amino acid sequences. Red indicates conservation with the epitope, blue indicates a single amino acid mismatch to the epitope and green indicates two amino acid mismatches in comparison with the epitope. The equivalent murine gp120 epitopes are also included for comparison.

4.4) Discussion

Isolate 97ZA347TS is the first South African, known macrophage tropic isolate to be genotypically characterised in the complete gp120. The only published South African subtype C gp120 was NOF (Dietrich *et al.*, 1993), but this sequence lacks the first approximately 500bp of the gp120. Although there is an arginine substitution at position 276 in the V3 loop that has been demonstrated to be indicative of a syncitium inducing phenotype (Fouchier *et al.*, 1992 and De Wolf *et al.*, 1994), the disease stage of the infected patient and phenotype of isolate NOF was not published. At the time of initiation of this study, little work had been performed to identify cross-reactive CTL epitopes in HIV and the majority of vaccines were based on the envelope region of HIV-1 (see section 1.7.2). The gp120 protein from isolate 97ZA347TS was thus selected as the protein to be analysed for vaccine design.

It is possible that the most effective CTL response will be elicited by a vaccine representative of the circulating subtypes in the area, although cross-reaction between different subtypes has been demonstrated (see section 1.7.1.2). The complete gp120 from 97ZA347TS was thus phylogenetically compared with other subtype C isolates, as well as a number of reference sequences, from subtypes A to J and SIVcpz-gab. The average DNA distance between isolate 97ZA347TS and the other subtype C isolates was 10.2%, however the subtype C sequences clustered together in the phylogenetic tree with a bootstrap value of 100%. The high genetic diversity of subtype C within South African isolates has been discussed in Chapter 3.

The number of potential *N*-linked glycosylation sites in the macrophage tropic isolate, 97ZA347TS was also determined, since the great degree of glycosylation in the envelope region makes the HIV gp120 less immunogenic (Kwong, 1998 and Wyatt, 1998). This is due to the glycosylated areas being seen as "self" by the immune system. The macrophage tropic subtype C isolate from South Africa, 97ZA347TS, contains 22 potential *N*-linked glycosylation sites in its gp120, which is below the average number of *N*-linked glycosylation sites (24 potential sites) in other macrophage tropic subtype C isolates studied from the HIV sequence database (1999). It has been postulated (Graziosi *et al.*, 1998) that a lack of glycosylation in certain regions of the gp120, such as the C2 and V2, as well as a decrease in the total number of potential *N*-linked glycosylation sites may correlate with the ability of the patient to neutralise the infecting virus. Out of three patients analysed, two infected with viruses having fewer glycosylation sites (23 and 24) were able to develop a neutralising response to autologous virus as well as T-cell adapted strains *in vitro*. On the other hand, the patient infected with a virus containing 27 *N*-linked glycosylation sites in the gp120 did not elicit a neutralising response. It may thus be important that the isolate chosen for vaccine development have a low degree of *N*-linked glycosylation,

especially in the V2 and C2 regions.

Isolate 9ZA347TS also had the GPGQ V3 loop crown sequence characteristic of subtype C viruses and the asparagine replacement at position 344 with valine, common in subtype C isolates. This results in the loss of the potential *N*-linked glycosylation site just downstream of the V3 loop. The characteristic addition of an *N*-linked glycosylation site at position 512 in subtype C isolates was also present in 97ZA347TS. It is possible that this additional glycosylation site, which is within the CD4 binding site (Myers *et al.*, 1996), may have an effect on binding affinity or antigenicity. The argenine substitutions at positions 355, 369 or 376 (see figure 4.6) resulting in an increased positive charge and indicative of a syncitium-inducing phenotype were not present in isolate 97ZA347TS (Fouchier *et al.*, 1992; De Wolf *et al.*, 1994). This is to be expected, since the isolate is a primary, macrophage tropic isolate. As was discussed in chapter 3, this substitution was only detected in 2 out of 117 southern African subtype C isolates analysed, NOF and 94ZA067. It has been documented that subtype C isolates rarely evolve to CXCR4, syncitium inducing isolates (Doms and Moore, 1997; Korber *et al.*, 1997; Tscherning *et al.*, 1998 and Björndal *et al.*, 1999)

The pcDNA3.1$_{SF162}$ and pcDNA3.1$_{97ZA347TS}$ immunised BALB /c mice elicited an anti-gp120 antibody response. Peak antibody levels of between five- (pcDNA3.1$_{SF162}$) and seven-fold (pcDNA3.1$_{97ZA347TS}$) greater than the negative control mice were detected. Similar antibody levels were documented by Wang *et al* (1993), where BALB /c mice were immunised with gp160 from a T-cell isolate, HXB2. A strong humoral response was not expected from the mice immunised intramuscularly with the DNA vaccine. It is known that intramuscular immunisation with DNA yields a predominantly Th1 type immune response with subsequent inhibition of the Th2 response and lower antibody levels (Robinson, 1997a). Although not the aim of this study, it is possible to enhance the antibody response in the mice by using alternative methods of inoculation, for example with a gene gun (Lu *et al.*, 1995 and Lu *et al.*, 1998), or with the addition of co-stimulatory cytokines such as GM-CSF, that will induce a stronger humoral immune response. However, a drawback of these strategies is that the cellular response will be down-regulated.

The DNA vaccine system with intramuscular immunisation was chosen in order to induce the Th1 response in mice which would allow CTL cross-reactivity between subtypes B and C to be elucidated. In this study, the cross-reactive CTL response elicited to the two subtypes was determined in two experiments, with recombinant VV$_{97ZA346TS}$ (subtype C) as the challenge virus. Our study is the first utilising the *in vivo* challenge technique in order to determine the CTL cross-reactivity between subtypes.

Mice inoculated with the pcDNA3.1/Zeo plasmid alone were used as the control mice since they were not protected from the $VV_{97ZA346TS}$ challenge. These mice were found to have increased replication of the challenge virus in their ovaries, with viral titres of 6.58 and 8.70 log_{10} pfus per mouse in the two experiments, respectively. The subtype B and C immunised mice, however, were able to clear the $VV_{97ZA346TS}$ infection by at least 50-fold and at most over 500-fold in comparison to the negative control mice (see figure 4.9), indicating a cross-reactive CTL response (Binder and Kündig, 1991; Bachmann *et al.*, 1994; Belyakov *et al.*, 1998 and Kent *et al.*, 1998). This cross-reactive CTL response was obtained with only a single DNA vaccine immunisation.

The initial experiment was performed with a lower intraperitoneal $VV_{97ZA346TS}$ challenge dose of $1X10^7$ pfu, which resulted in lower viral titres in the mouse ovaries. The second experiment used $5X10^7$ pfu of $VV_{97ZA346TS}$ as the intraperitoneal challenge dose. In the first experiment, the difference in $VV_{97ZA346TS}$ viral titre between the control, pcDNA3.1/Zeo-immunised mice and the homologous pcDNA3.$1_{97ZA346TS}$-immunised mice was only 1.9 log_{10}, whereas in the second experiment with the higher challenge titre, the difference was 2.6 log_{10}. Similar results were seen in mice immunised with the heterologous subtype B DNA vaccine, where in the first challenge experiment, there was a 1.4 log_{10} decrease in viral titre compared to the negative control mice and in the second experiment, a 2 log_{10} decrease in titre. Although the heterologous subtype B DNA vaccine was not able to elicit as effective a CTL response as the homologous subtype C DNA vaccine, possibly due to differences in the CTL epitopes between the two viruses (see figure 4.10), we were still able to demonstrate CTL cross-reaction using the *in vivo* challenge system (see figure 4.9).

The levels of protection elicited by the single DNA vaccine immunisation in our study were lower than those reported in other studies where a decrease in recombinant VV titre of between 5 and 7 log_{10} per mouse were reported. It must be noted that in the other studies, the challenge technique was used after an optimal vaccination regimen in order to determine the effectiveness of the vaccination strategy or different routes of inoculation. In our study, however, the technique was used in order to determine the extent of CTL cross-reactivity between subtypes B and C, which was achieved.

In hindsight, the gp120 may not be the optimal region for eliciting a broadly cross-reactive CTL response, possibly due to the high variability in the region (HIV sequence database, 1998). It was determined in a study by Lole *et al.* (1999), that CTL epitope conservation in subtype C viruses is less in the *env* region than other regions, such as the Gag, Pol and Nef, and

these more conserved regions are likely to elicit strong, broadly cross-reactive CTL responses. Future studies should involve the construction of DNA vaccines expressing these alternative proteins as well as recombinant VVs expressing the corresponding proteins in order to characterise the *in vivo* CTL cross-reactivity.

The high genetic variability within South African subtype C isolates was discussed in the previous chapter and was confirmed in studies by Williamson *et al* (1995) and Bredell *et al* (1998). The CTL epitopes, within the gp120, for the subtype C isolate used in our study were thus identified for all human leucocyte antigen (HLA) haplotypes. The gp120 epitopes were compared for isolate 97ZA347TS, SF162 and the consensus sequence epitopes for subtypes B and C. The results confirm that the envelope region is not the optimal region for the induction of a CTL response (see figure 4.10).

Out of the fourty-two human CTL epitopes identified for the gp120, isolate SF162 has twenty-two conserved epitopes and thirty-three with up to a two amino acid mismatch. In addition, only five of the epitopes are conserved in isolate 97ZA347TS and thirteen have up to a two amino acid mismatch. It is important to note that the gp120 epitopes that have been identified were based on a subtype B isolate and as the CTL epitopes for subtype C gp120 have not been defined, it may be more pertinent to look at the amino acid difference between isolates SF162 and 97ZA347TS as an indication of potential CTL epitope conservation. It was determined that the two isolates are 24% divergent, in comparison with only 15% divergence between isolate 97ZA347TS and the closest related sequence available, ZAM18, from Zambia. This high divergence between isolates SF162 and 97ZA347TS is a possible indication why the CTL response elicited in the BALB/c mice was not strongly cross-reactive between the two subtypes.

The mice used in our study were inbred BALB/c mice ($H\text{-}2^b$) defined murine MHC (murine histocompatability complex) class I molecules and it is questionable how relevant the *in vivo* CTL detection technique is to the CTL response elicited in outbred humans. Unfortunately, only limited data is available on murine CTL epitopes for the gp120 of HIV (see figure 4.10) (Korber *et al.*, 1997). Preliminary studies have started to identify common HLA haplotypes in indigenous South African populations, in order to determine the best CTL epitopes for eliciting a broad and effective CTL response. Studies based on the Xhosa population in South Africa (Brain and Hammond, 1972 and Omar *et al.*, 1984) have been performed. The distribution of HLA showed that the most common HLA-class I, HLA-A alleles are A30, A28, A2 and A23 respectively. HLA-B alleles screened showed that the most common alleles are Bw58, Bw70, Bw42 and B7, with B27 being extremely rare. The most frequently detected Cw alleles were

Cw7, Cw6, Cw2 and Cw4 (du Toit *et al.*, 1988 and Hammond *et al.*, 1997). If the gp120 epitopes recognised by these alleles are determined from figure 4.10, it can be seen that the allele which has the broadest recognition gp120 epitopes for both SF162 and 97ZA347TS is A2. It must be noted, however, that the A2 haplotype has been the most characterised. Only six of the gp120 CTL epitopes in 97ZA347TS would potentially be recognised in indigenous populations in South Africa based on the gp120 epitopes identified and the alleles identified thus far. Thirteen of the gp120 CTL epitopes from isolate SF162 would be recognised. More human HLA genotypes need to be analysed in order to broaden our knowledge of what will be needed for an efficient, broadly cross-reactive CTL response.

For future studies, DNA vaccines should be constructed containing other HIV proteins such as Gag, RT, and Nef in order to determine which will elicit a strong and broadly cross-reactive CTL response. The *in vivo* murine CTL model used in this study can be used to determine the immunogenicity of these potential vaccine proteins as well as to assess alternative vaccine types and regimens. In this way, the best proteins to elicit a strong, broadly cross-reactive CTL response can be identified and utilised in an HIV vaccine.

CHAPTER 5: CONCLUSIONS AND FUTURE STUDIES

This thesis presents a study of HIV-1 genetic diversity according to different modes of transmission and geographical distribution of HIV-1 subtypes in South Africa. The complete gp120 from a macrophage tropic, NSI isolate, representative of the predominant subtype infecting the heterosexual population in South Africa was genotypically characterised. In addition, the immunogenicity of the gp120 was determined by DNA vaccine immunisation and CTL cross-reactivity investigated using a recombinant vaccinia virus challenge system in BALB/c mice.

5.1) The association of HIV-1 subtype with mode of transmission in South Africa

Although the HIV epidemic in Africa is the oldest, with all of the HIV-1 subtypes occurring on this continent, the majority of infections are caused by viruses belonging to subtypes A, C and D (Orloff *et al.*, 1993; Williamson *et al.*, 1995; Abebe *et al.*, 1997; Poss *et al.*, 1997; Shafer *et al.*, 1997; van Harmelen *et al.*, 1997; Bredell *et al.*, 1998; Engelbrecht *et al.*, 1998; Luo *et al.*, 1998; Rayfield *et al.*, 1998; Novitski *et al.*, 1999; Tien *et al.*, 1999; van Harmelen *et al.*, 1999). Subtype B, although the predominant subtype detected in Europe and the United States, is rarely identified on the African continent. At the time of initiation of this study, in 1994, only one isolate from South Africa had been genetically characterised.

Although based on a limited number of samples, epidemiological data showed two modes of transmission in the South African HIV-1 epidemic, male homosexual and heterosexual. It was also reported that the majority of men infected by homosexual or bisexual transmission during the early stages of the epidemic (early 1980s) had had sexual contact with men whilst abroad in the United States or Europe (Sher *et al.*, 1985). It was thus not surprising that in our initial study of patients infected by homosexual or bisexual mode of transmission from Cape Town and Bloemfontein, 92.9% (26 out of 28) of the isolates identified by HMA in the *env* region belonged to subtype B (see chapter 2). The subtype B epidemic was not limited to men who reported having had homosexual contact abroad, however, as 22 of the 28 patients in the study reported no sexual contact abroad, or with a sexual partner who had been abroad, up to five years prior to the study. The homosexual, subtype B epidemic had thus continued to spread within the homosexual population in South Africa.

In addition to subtype B, a single *env* subtype D virus was identified in the homosexual population from the Cape Town/Bloemfontein study. Subtype D was also identified in a separate study by Engelbrecht *et al*, (1995) in five homosexually or bisexually infected men attending Tygerberg hospital, who were infected between 1984 and 1989. The subtype D epidemic does not appear to have spread, however, since out of 28 male

homosexual patients in our study (with patients diagnosed up to seven years after the Tygerberg patients), only one, 93ZA034, was infected with a subtype D virus.

By 1994, at the start of this study, there were more AIDS cases reported in women than in men (Swanevelder, 1995), which was indicative of the increase in numbers of heterosexual infections. Although subtype B was identified as the predominant virus infecting the homosexual and bisexual populations, subtype C was identified by HMA in 77.2% (17 out of 22) of the heterosexual infections in Cape Town and Bloemfontein patients sampled. The origin of the subtype C epidemic was probably regional, supported by patient epidemiological data, whereby a number of patients infected by heterosexual transmission reported having sexual contact whilst in neighbouring countries such as Zambia, or Zimbabwe, where subtype C is common (McCutchan *et al.*, 1992; Korber *et al.*, 1997; Shafer *et al.*, 1997 and Tien *et al.*, 1999). Subtypes A and D were rarely detected in South Africa.

The heterosexual population in Cape Town had the greatest HIV-1 subtype diversity, with subtypes A, B and D identified, as well as the major subtype, C. Subtype B, associated with the initial homosexual epidemic, failed to spread significantly within the heterosexual population in Cape Town, where only two patients were found to be infected with subtype B. It must be noted, however, that one of the patients from the Bloemfontein cohort, a bisexual male, was infected with a subtype C isolate. This is the first subtype C isolate reported to be transmitted via homosexual or bisexual contact.

A significant association between subtype and mode of transmission ($p=<0.0001$), was identified, suggesting that the two epidemics in South Africa are indeed independent of each other and related to mode of transmission. The distribution of subtypes in all populations must continue to be monitored, however, as genetic shifts in subtype may occur.

5.2) The genetic diversity and geographical distribution of HIV-1 in the heterosexual population of South Africa

The second part of this study was to identify the predominant subtype responsible for the heterosexual epidemic in the rest of the country and to determine the genetic diversity of HIV-1 in South Africa.

As in the initial Cape Town/Bloemfontein study, the majority (92%) of isolates analysed by HMA (out of a total of 109) from four major urban centres in South Africa belonged to subtype C (see Chaper 3). Only 8% of the heterosexual patients sampled from these centres were infected by subtype B. Subtype A and subtype D, detected in the heterosexual population in Cape Town were not identified in any of the four cities sampled in this study, even though a greater number of samples were analysed, making it more likely

that low prevalence subtypes would be detected. Subtype A has subsequently been reported in Durban, however (Moodley *et al.*, 1998).

To determine the genetic diversity within subtype C, 117 published subtype C V3 loop sequences (up until 1998), including 78 from South Africa were obtained from HIV sequence database (1998) and these sequences were phylogenetically compared.

The South African subtype C sequences were as divergent to each other as they were to subtype C isolates from countries further North in Africa, and subtype C from other continents, with an average DNA distance of 14.06% and no subclusters within South Africa, or with a country of origin were detected. Similarly high DNA distances have been reported in a Gauteng Province study by Bredell *et al* (1998), where HIV isolates from 43 mine workers were found to be 11% divergent in the V3-V5 region of the *env* gene. A recent study by Novitski *et al* (1999) in Botswana compared the DNA distance in the complete genome between subtype C isolates in Botswana with subtype B isolates. The study indicates that there is a greater degree of divergence within the subtype C lineage (12.4% in the envelope region) in Botswana than between isolates belonging to subtype B.

The high DNA distance demonstrated in the V3 loop sequences in our study is an indication that the HIV subtype C epidemic in South Africa is not a clonal epidemic and it is probable that HIV entered the country on multiple occasions. As was determined in the initial Cape Town study, where patients infected with subtype C reported sexual contact in neighbouring countries, Bredell *et al* (1998) also reported that a number of the mine workers infected with subtype C in their study were residents of neighbouring countries where this subtype has been identified.

It has been shown by a number of studies that subtype C viruses are usually restricted to the use of the CCR5 co-receptor and remain NSI, despite being able to cause the patient to progress to AIDS (Doms and Moore, 1997; Tscherning *et al.*, 1998 and Björndal *et al.*, 1999). An indication of the phenotype of the isolate can be obtained from the V3 loop where more basic (positively charged) arginine residues at positions 276, 290 and 297 indicate a SI phenotype. The 117 subtype C isolates analysed in our study also shows that the arginine substitutions indicative of an SI phenotype are rare, only being identified in two isolates, NOF and 94ZA067.

Increasing numbers of recombinant viruses are being reported, especially in central Africa where there many different subtypes circulating (Peeters *et al.*, 1998 and Renjifo *et al.*, 1998) (see section 1.6.2) and it is now necessary to sequence more than just one region of the viral genome. Although the entire genome of the virus should be sequenced, this technique is expensive and time consuming, thus methods are needed for rapid screening of

a number of different regions of the genome, in order to identify recombinant viruses, which can then be characterised by sequence analysis.

Seventy-seven samples from Johannesburg, Bloemfontein, Durban and Cape Town which had been subtyped in the *env* gene by HMA, were thus subtyped in the *gag* gene by RFLP. No discordance was identified between subtype designation in the *gag* region in comparison to the *env* region, indicating that none of the viruses were recombinants in the regions analysed. It will be necessary to continue to screen for recombinant viruses, even through there is a segregation of subtype with mode of transmission. In China, for example, it has recently been reported that there is a new epidemic in the IVDU population caused by a subtype B/C recombinant (Shao *et al.*, 1998) and in Russia, another IVDU epidemic has been caused by a subtype A/B recombinant (Liitsola *et al.*, 1998).

Although it has not been established why subtype C is the predominant HIV-1 subtype world wide, with 48% of infection caused by this subtype, some suggestions have been put forward. A number of studies have shown that there may be a relationship between subtype and viral pathogenicity (Kanki *et al.*, 1999 and Neilson *et al.*, 1999), with certain subtypes associated with faster disease progression than others, although this has been disputed by others (Aleus *et al.*, 1999 and Hu *et al.*, 1999). Thus far, the only report of phenotypic selection for subtype C (and AE) was in Langerhans' cells (Soto-Ramirez *et al.*, 1996 and Essex *et al.*, 1997), but was subsequently refuted by a number of studies (Dittmar *et al.*, 1997 and Pope *et al.*, 1997). Although there have been multiple introductions of HIV into the country, the majority of the viruses belong to subtype C. Whether this subtype has a selective advantage allowing easier transmission than other subtypes, or whether it is merely the predominance of this virus in southern Africa which has led to its almost exclusive entrance into South Africa, is not certain.

It was thus shown in this part of our study that subtype C is the predominant virus infecting the heterosexual population throughout South Africa and although subtypes A, B and D have been identified, the distribution and numbers of these subtypes are limited. South Africa is actively involved in vaccine trials (International AIDS Vaccine Initiative (IAVI) Report, 1998) and it is essential to constantly monitor the genetic diversity of HIV-1 subtypes circulating within the country.

5.3) Characterisation of the macrophage tropic South African subtype C gp120 and a determination of the immunogenicity of the gp120 protein

The majority of vaccines being developed at the time of initiation of this study were based on the envelope region of T-cell tropic subtype B isolates and little work had been performed to characterise cross-reactive CTL epitopes. In addition, the only South African

gp120 sequence that had been published was isolate NOF and this sequence did not include the complete gp120. We thus set out to characterise the complete gp120 of a macrophage tropic subtype C isolate from South Africa in order to determine the immunogenicity of this isolate in comparison with a macrophage tropic subtype B isolate (see Chapter 4).

Isolate 97ZA347TS is the first to be genotypically characterised from a known macrophage tropic South African isolate and clustered within the subtype C lineage, with an average DNA distance of 10.2%. The amino acid sequence for isolate 97ZA347TS was generated and the macrophage tropic isolate was found to lack any of the arginine substitutions in the V3 loop increasing the positive charge and indicative of a SI phenotype, suggesting that the isolate is NSI (Fouchier *et al.*, 1992; De Wolf *et al.*, 1994), which was expected. Isolate 97ZA347TS contains 22 *N*-linked glycosylation sites, including an additional site in the CD4 binding region at position 512, common in subtype C isolates, which may influence CD4 binding affinity or antigenicity (Myers *et al.*, 1996).

The immunogenicity of the gp120 from isolate 97ZA347TS (subtype C) and the reference isolate, SF162 (subtype B) was determined in BALB/c mice. As the vaccination was intramuscular by DNA vaccine, a predominantly Th1 type response was expected (Robinson, 1997a) and antibody levels peaked at between five- (pcDNA3.1$_{SF162}$) and seven times (pcDNA3.1$_{97ZA347TS}$) greater than the negative control mice.

BALB/c mice immunised once with the DNA vaccine and challenged with VV$_{97ZA346TS}$ showed a decrease in ovarian titre of 100 to over 500 fold in mice immunised with the homologous subtype C DNA vaccine, whereas the mice immunised with the heterologous subtype B DNA vaccine had a 50 to 100 fold decrease in ovarian VV$_{97ZA346TS}$ titre. This is the first study utilising an *in vivo* technique in order to determine the cross-reactive CTL response between different subtypes, although other studies have shown cross-reactive CTL responses by conventional *in vitro* methods.

The difference in efficiencies between the two DNA vaccines to elicit an *in vivo* CTL response to VV$_{97ZA347TS}$ in the BALB/c mice is probably due to the differences in their CTL epitopes. It is assumed that greater than two amino acid mismatches in the CTL epitope of the virus will not be recognised by the host (Cao *et al.*, 1997), although it also depends where the amino acid mismatch occurs in the epitope. In this study, a comparison of the identified fourty-two human CTL epitopes in the gp120 with the gp120 from isolate 97ZA347TS showed that only 11.9% of the gp120 epitopes were conserved, indicating that the gp120 may not be the optimal region for the induction of a CTL response.

As the CTL epitopes for subtype C have not been determined, a better indication of the possibility of CTL cross-reactivity between isolates 97ZA347TS and SF162 may be the

amino acid divergence between the two isolate gp120s. The amino acid similarity between the two isolates should be a reflection of the similarity between potential CTL epitopes as well. The two isolates are highly divergent, with almost a quarter of the amino acids making up the gp120 protein differing between them. Even ZAM18 is 15% divergent from 97ZA347TS and it is possible that a broader CTL response will be obtained using a more conserved region for an HIV vaccine.

5.4) Future investigations

Although subtype C is currently the predominant HIV-1 subtype circulating within the heterosexual population of South Africa, with subtypes A, B and D rarely detected or with limited distribution (subtypes A and D), it is essential to continue to monitor the South African epidemic for emerging subtypes. Seroconvertors from high risk groups may be good sentinel populations to monitor. Since the African continent has all of the HIV-1 subtypes present, as well as HIV-2, and with a constant traffic of people from one country to another for reasons such as work, famine or war (see section 1.5.2.1) it is highly likely that additional subtypes may be introduced into the country. In addition, recombinant viruses are becoming more prevalent and are responsible for some of the burgeoning epidemics in China, Russia and Tanzania (Shao *et al.*, 1998; Liitsola *et al.*, 1998 and Renjifo *et al.*, 1998). Although at present there is limited subtype diversity in South Africa, it is possible that recombinant viruses may enter the country, or may form between the subtype B and C viruses circulating in regions or populations where both subtypes have been identified.

The first man infected by bisexual transmission with a subtype C virus was identified in this study The bisexual population should be monitored, as it is possible that greater numbers of subtype C infections may enter the bisexual and homosexual populations. Shifts in subtype distribution according to mode of transmission have been reported in Thailand (Mastro *et al.*, 1996) and China (Shao *et al.*, 1996) and it is important to continue monitoring possible "transition populations" in order to detect similar shifts in South Africa.

The genetic diversity of the prevalent subtypes circulating within South Africa may have an effect on vaccine design for the country. As South Africa is involved in developing vaccine trial sites (IAVI Report, 1998) it is important to keep abreast with the HIV subtypes circulating within the country and the genetic diversity within these subtypes as they may influence vaccine efficacy. This study has laid down a basis for the investigation of subtype C isolates from different centres in South Africa. Regions other than the Env and Gag must be analysed, in order to determine the diversity between South African subtype C isolates in the whole HIV genome. For future studies, alternative regions with more conserved CTL epitopes such as Gag, Pol or Nef, may be used in a DNA vaccine system in order to determine which will elicit a strong and broad CTL response. The *in vivo* murine CTL challenge model can

then be used to determine the immunogenicity of these potential vaccine proteins. This model may also be used to compare vaccines and immunisation schedules, in order to determine their efficacy in eliciting a CTL response, as has been utilised in a number of other studies (Binder and Kündig, 1991; Bachmann *et al.*, 1994; Belyakov *et al.*, 1998 and Kent *et al.*, 1998). In this way, the best vaccine schedule to elicit a strong, broadly cross-reactive CTL response will be identified prior to testing in more expensive primate models.

APPENDICES

APPENDIX A: DNA TECHNIQUES

A1 DNA extraction

Cape Town and Bloemfontein samples:

DNA was extracted from 5-10ml of blood as described (Kawasaki, 1990) by Maureen Lambrick, Clinical Virology, UCT and Elna van der Ryst, Department of Virology, University of the Free State). 100µl of whole blood was mixed with 0.5ml of 1XTE buffer (10mM Tris-HCl, 1mM EDTA , pH8.0) in a microfuge eppendorf tube and centrifuged at 13 000g for 10 seconds. The pellet was resuspended in 0.5ml of 1XTE buffer by vortexing and again pelleted by microfugation. The process was repeated twice until a clean nuclei pellet was obtained. The pellet was resuspended in 100µl of lysis buffer K [1XPCR buffer (Promega, Madison, WI, USA), 0.5% Tween-20, 100µg/ml fresh proteinase K] by vortexing and then spun down by microfugation before being incubated at 56°C for 45 minutes. The protease was inactivated at 95°C for 10 minutes and the cell/nuclei lysate spun down briefly. Ten microlitres of the lysate was used for a 50µl PCR master mix volume.

Johannesburg samples :

Samples were collected and DNA extracted by Sue Lyons (National Institute of Virology, Johannesburg). Peripheral blood mononucleocytes were purified on a Ficoll gradient (Sigma, St Louis, USA) after which cells were pelleted by centrifugation and washed 2-4 times with 1X phosphate buffered saline (PBS). PBS (pH7.2) is made by adding 17.2g of KH_2PO4 (solution A) and 104.4g of $Na_2HPO_4{\cdot}12H_2O$ (solution B) to 1000ml of distilled water. The cells were then lysed using ammonium chloride to remove the red blood cells if necessary and re-suspended in 100 µl lysis buffer (TE lysis buffer; 10mM Tris HCl pH 7.5, 1mM EDTA, 0.5% Triton X-100, 1% Tween 20™) and 6µl Proteinase K (stock 20mg/ml). The tube was then incubated at 56°C for three hours (or overnight), followed by 95°C for 15 min, then stored until use at -70°C.

Durban samples:

HIV positive samples were collected and proviral DNA was extracted directly from 300µl of whole EDTA-blood using the Genomix extraction kit (Talent, SRL, Italy) by Savathree Madurai and Dennis York (Department of Virology, University of Natal). The protocol was as described by the manufacturer but with the following addition: the final pelleted DNA (100µl) was re-precipitated with 255µl absolute ethanol and 10µl 3M sodium acetate (pH5.2) and washed with 70% ethanol. The pellet was re-suspended in 50µl of distilled water and stored at -70°C until use.

A2 Polymerase chain reactions (PCR)

V3-V5 region:

The V3 loop was amplified by nested PCR of 1.2Kb and 700bp fragments respectively using primers ED5/12 for the outer reaction and ES7/8 for the inner reaction (Delwart *et al.*, 1993).

The outer reaction master mixes (50µl) contained:

- 5µl 10X PCR buffer (Promega, Madison, WI, USA), (500mM KCl, 100mM Tris-HCl (pH9.0), 1% Triton

X-100), 1.5mM $MgCl_2$,

- 1% DMSO, 1% glycerol,
- 200µM each of dNTP's,
- 400nM each ED5 and ED12 and
- 2.5U *Taq* DNA Polymerase (Promega, Madison, WI, USA)

The reaction mixes were made up to 40µl with sterile, distilled water and overlayed with 30µl of mineral oil and 10µl of patient DNA (or 5µl of patient DNA for Durban samples, topped up with 5µl of sterile, deionised water) was added. Samples were amplified using the following amplification conditions in the Techne PHC-2 PCR thermocycler:

- Initial denaturation at 94°C for 2 minutes,
- 94°C for 1 minute, 45°C for 1 minute and 72°C for 2 minutes (five cycles),
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (35 cycles) and
- Final extension at 72°C for 6 minutes.

The inner reaction master mixes (50µl) contained:

- 5µl 10X PCR buffer (Promega, Madison, WI, USA), (500mM KCl, 100mM Tris-HCl (pH9.0), 1% Triton X-100), 1.8mM $MgCl_2$,
- 1% DMSO, 1% glycerol,
- 200µM each of dNTP's,
- 400nM each ES7 and ES8 and
- 2U *Taq* DNA Polymerase (Promega, Madison, WI, USA)

The PCR reaction mixes were made up to 47µl with sterile, distilled water and overlayed with 30µl of mineral oil. For the inner reaction, 3µl of outer reaction DNA was transferred, or 2µl of plasmid DNA was added. Amplification conditions using the Techne PHC-2 PCR thermocycler were:

- Initial denaturation at 94°C for 2 minutes,
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (35 cycles) and
- Final extension at 72°C for 6 minutes.

Gag p17 and partial p24 region

The *gag* p17 region and partial p24 and p17 region were amplified by nested PCR for RFLP analysis. Primers used are shown in Table 3.2. The nested PCR amplified 1.5Kb and 400bp or 650bp fragments respectively, using primers LTRu5/Pol2 (van Harmelen *et al.*, 1999a) for the outer reaction and LTR310j (van Harmelen *et al.*, 1999a)/SK431 (Lynch *et al.*, 1992) or *gag*778 (van Harmelen *et al.*, 1999a) for the inner reactions. PCR master mixes (50µl) contained:

- 5µl 10X PCR (500mM KCl, 100mM Tris-HCl (pH9.0), 1% Triton X-100) (Promega, Madison, WI, USA),
- 1% DMSO, 1% glycerol,
- 200µM each of dNTP's,
- 1.5mM $MgCl_2$, (outer reaction) and 1.8mM $MgCl_2$, (inner reaction),
- 400nM each primer; LTRu5 and Pol2 (outer reaction) and LTR310j and SK431 or *gag*778 (inner

reaction) and

- 2.5U *Taq* DNA Polymerase (outer reaction) and 2U *Taq* DNA Polymerase (Promega, Madison, WI, USA) (inner reaction).

The reaction mixes were made up to 40µl (outer reaction), or 47µl (inner reaction) with sterile deionised water and overlayed with 30µl of mineral oil. 10µl of patient DNA was added to the outer reaction. For the inner reaction, 3µl of outer PCR product was transferred to the outer reaction mix. If cloned and already sequenced reference *gag* plasmid DNA was used, 20ng of DNA was added. Amplification conditions for the outer reaction using the Techne PHC-2 PCR thermocycler were:

- Initial denaturation at 94°C for 2 minutes,
- 94°C for 1 minute, 45°C for 1 minute and 72°C for 2 minutes (5 cycles),
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (30 cycles) and
- Final extension at 72°C for 6 minutes.

For the inner reaction, PCR conditions were:

- Initial denaturation at 94°C for 2 minutes,
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (30 cycles) and
- Final extension at 72°C for 6 minutes.

gp120 fragment:

The gp120 was amplified from a primary, macrophage tropic isolate belonging to subtype C (97ZA347TS) in order to characterise the gp120. Primers ED3/ED14 were taken from Delwart *et al* (1993) and envB/env J from Gao *et al* (1994) and used to amplify a 2.5Kb outer reaction and 1.6Kb inner reaction by nested PCR amplification. The PCR master mixes (50µl) contained the following:

- 5µl 10X PCR buffer (Promega, Madison, WI, USA), (500mM KCl, 100mM Tris-HCl (pH9.0), 1% Triton X-100),
- 1.5mM $MgCl_2$,
- 1% DMSO, 1% glycerol,
- 200µM each of dNTP's,
- 400nM each of ED3/ED14 (outer reaction) and *env*B/*env*J (inner reaction) and
- 2.5U *Taq* DNA Polymerase (outer reaction) and 2U *Taq* DNA Polymerase (Promega, Madison, WI, USA)

The reaction mixes were made up to 40µl (outer reaction) or 47µl (inner reaction) with sterile, deionised water and overlayed with 30µl of mineral oil. 10µl of cultured isolate proviral DNA was added to the outer reaction and 3µl of the outer reaction product was transferred to the inner reaction mix. Negative controls included HIV negative patient DNA and sterile distilled water in order to monitor contamination. The amplification conditions in the Techne PHC-2 PCR thermocycler for the outer reactions were:

- Initial denaturation at 94°C for 2 minutes,
- 94°C for 1 minute, 45°C for 1 minute and 72°C for 2 minutes (5 cycles),
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (35 cycles) and

- Final extension at 72°C for 6 minutes.

Amplification conditions for the inner reactions were:

- Initial denaturation at 94°C for 2 minutes,
- 94°C for 20 seconds, 55°C for 45 seconds and 72°C for 1 minute (35 cycles) and
- Final extension at 72°C for 6 minutes.

All samples were stored at -20°C after amplification and the appropriate controls (HIV negative patient DNA and sterile distilled water) were included in order to detect possible contamination.

Samples were stored at -20°C after amplification.

A3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using horizontal gel apparatus (Hoefer Scientific Instruments, San Francisco, USA) as described in Sambrook *et al* (Sambrook, 1989). The agarose powder was melted with frequent swirling to prevent clumping in 1XTAE (50XTAE/liter; 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA, pH 8.0) at concentrations of 1 to 4%, depending on the size of the DNA fragments to be separated. Once melted, ethidium bromide (10mg/ml) stain was added to a final concentration of 1µg/100ml of agarose. The agarose gel slabs were allowed to set at room temperature for 30 minutes after which the well combs were removed. Samples were mixed with 1/5 their volume of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in deionised water) and added to the 1XTAE submerged wells. The agarose gel was electrophoresed at 5V/cm until the relevant fragments were separated. The DNA fragments were then visualised using the UVP transilluminator at 256nm wavelength and photographed either with a M645 1000S polaroid camera or with the UVP computerised gel imager. Molecular weight markers were used to determine DNA fragment sizes; including Marker VI and VIII (Boehringer Mannheim, Hamburg, Germany) and the 1Kb ladder (Promega, Madison, WI, USA).

A4 Restriction endonuclease digestion

Restriction digests were carried out using the 10X buffer supplied by the manufacturer (Boehringer Mannheim, Hamburg, Germany). Two restriction enzymes were combined in a single digest if their buffers were compatible. If not, the digestion was done sequentially and the DNA was purified to remove the first buffer using ethanol precipitation, before addition of the second enzyme and buffer. The restriction enzyme digests were performed as described in Sambrook *et al* (1989). Most of the restriction endonucleases digested at 37°C, except *Sma* I that had an optimal temperature of 25°C. The restriction endonucleases were all supplied at 10U/µl (Boehringer Mannheim, Hamburg, Germany). Fragments were electrophoresed in a 1% agarose gel for detection and/or ligation purification. For a single digestion reaction, the following reaction components were included in a 10µl reaction mix:

1µl 10X restriction endonuclease buffer

8µl of PCR product or 1µg/8µl of plasmid DNA

1µl 10U/µl restriction endonuclease

Ethanol precipitation

The reaction volume was made up to 90µl with TE pH 8.0 (10mM Tris·Cl pH 8.0; 1mM EDTA, pH 8.0) in an eppendorf tube. One volume of phenol was added and the solution emulsified by shaking, followed by centrifugation in a benchtop microfuge at 14 000rpm for 2 minutes. The upper phase was transferred to a fresh eppendorf tube. This process was repeated and then one volume of chloroform/isoamyl alcohol (24:1) was added and the solution emulsified by shaking. The upper aqueous phase was recovered by centrifugation as before. 1/10 the volume of 3M sodium acetate pH 4.8 and 2.5 volumes of absolute alcohol was added, the solution mixed and incubated on dry ice for 10 minutes. The DNA pellet was collected by centrifugation at 14 000rpm for 20 minutes, washed with 70% ethanol and allowed to dry at room temperature for 5 minutes before re-suspending in the appropriate amount of water or TE buffer.

A5 Ligation

TA ligation

TA ligation was performed as per manufacturers' instructions using the pMOS-blue TA cloning kit (Amersham, Buckinghamshire, UK). The ligation mix contained:

1µl 10X DNA ligase buffer

0.5µl 100mM DTT

0.5µl 10mM ATP

1µl 50ng/µl pMOS vector

2-3µl PCR product

0.5µl T4 DNA ligase

Made up to 10µl with nuclease-free water.

The ligation mix was incubated at 16°C for 2 hours, 1µl of ligation mix was transformed into 100µl of competent DHα cells and 350µl was plated onto 2XYT agar (Appendix A6).

Subcloning

The restriction endonuclease digested vectors were electrophoresed in 1% low melting point agarose to separate out the gp120 and stuffer fragments as described by Sambrook *et al* (1989). The relevant fragments were excised from the gel visualised under low intensity UV light. The agarose was removed using the phenol freeze method (modified from Sambrook *et al.*, 1989). The vector and inserts were mixed together at 3:1 ratios and the following was added to a total of 60µl:

6µl 10X DNA ligase buffer (Boehringer Mannheim, Hamburg, Germany)

2. 5µl 100mM DTT (Amersham, Buckinghamshire, UK)

2.5µl 10mM ATP (Amersham, Buckinghamshire, UK)

2µl (1U) DNA ligase (Boehringer Mannheim, Hamburg, Germany)

Made up to 60µl with nuclease free water (Amersham, Buckinghamshire, UK)

The ligation mix was incubated at 16°C overnight and 5 volumes of ice cold 0.1M $CaCl_2$ added. Twenty microlitres were used to transform DHα cells and 350µl was plated onto 2XYT agar (Appendix A6).

Phenol freeze

The relevant DNA fragments were excised from the low melting point agarose gel, transferred to an eppendorf and finely chopped up with a scalpel. One volume of phenol was added, the solution mixed and frozen at -70°C for 10 minutes, followed by centrifugation in a microfuge for 10 minutes at 14 000rpm. The upper aqueous phase was transferred to a fresh eppendorf tube and 1 volume of phenol and 1 volume of chloroform:isoamyl alcohol (24:1) added. The solution was emulsified by mixing and microfuged as before. The upper aqueous phase was again transferred to a new eppendorf and 1.5 volumes of absolute ethanol and 1/10 the volume of sodium acetate (3M, pH4.8) added to precipitate the DNA. The tube was placed on dry ice for 10 minutes followed by centrifugation for 20 minutes at 14 000rpm to pellet the DNA. The DNA was then washed with 70% ethanol and re-suspended in an appropriate volume of distilled, deionised water.

A6 Transformation of DNA into competent *E. coli* cells

$CaCl_2$ shock procedure (Dagert and Ehrlich, 1979)

E coli strain DHα was used for manufacture of competent cells. Five milililtres of DHα bacteria were grown overnight at 37°C in 2xYT broth, pH7.0 (Per liter, 900ml deionised water, 16g bacto-tryptone 10g bacto-yeast extract, 5g NaCl) in a McCartney bottle placed flat on the shaker for good aeration of the culture. The bacterial culture was then diluted 1/100 to 1/200 in 50 or 100ml 2xYT broth in a flask five to ten times the culture volume. The bacterial culture were grown to early log phase (OD_{600} 0.2-0.4) and the cells were harvested by centrifugation at 4 000-5 000rpm for 5 minutes at 4°C in the Beckman J2-21 centrifuge. The cell pellet was re-suspended in 1/2 the culture volume of 0.1M ice cold $CaCl_2$ and then placed on ice for 1 to 2 hours. The cells were collected by centrifugation as before and then re-suspended in 1/10 the original culture volume of 0.1M ice cold $CaCl_2$. The competent cells were then aliquoted in 100µl aliquots into ice cold eppendorf tubes and 10% (v/v) ice-cold sterile glycerol was added. The cells were mixed and left on ice for 30 minutes and then stored at -70°C.

Transformation of $CaCl_2$ competent cells

The relevant plasmid DNA (1-10ng) containing ampicillin resistance was mixed with 100µl of competent DHα cells and the mixture swirled to mix and left on ice for 30 minutes. The bacteria were then heat-shocked at 42°C for 2 minutes. The cells were removed and 900µl of 37°C 2xYT broth was added after which the cells were incubated at 30 to 60 minutes to allow plasmid ampicillin resistance gene, β-lactamase expression before plating. The transformed bacteria were plated on 2xYT agarose (2xYT broth with 15g bacto-agar added before autoclaving) plates containing 0.5ml of X-gal (5ml DMSO, 0.4g X-gal, mixed and 5ml of distilled water added), 50µl of IPTG (10ml distilled water, 0.24g IPTG) and ampicilin (100µg/ml) per 100ml of agar. Colonies were grown overnight at 37°C and transformed bacteria identified (Appendix A2, A4, A7).

If the marker gene, *lacZ* was present on the plasmid transformed into the bacteria (pMOS-blue and pSC65) the transformed bacteria appeared blue. The defective *lacZ* gene in the plasmid and the defective *LacZ* gene in the bacteria complement each other, allowing the expression of β-galactosidase, induced by

the IPTG in the medium. The β-galactosidase is able to metabolise X-gal, a chromogenic substrate, thus producing blue colonies. Selection of recombinant clones can occur if the multiple cloning site is within the *LacZ* gene of the plasmid, disrupting it. The recombinant bacteria are therefore not able to express β-galactosidase and the colonies are white. The recombinant pMOS-blue vectors were identified in this way.

Controls were also run for all ligation and transformation experiments. Negative control, non-transformed bacteria were unable to grow, since they did not contain the plasmid-carried ampicillin resistance gene. Positive control, non-recombinant pMOS-blue was plated in order to determine the transformation efficacy. In addition, digested, non-ligated vector (pMOS-blue, pcDNA, or pSC65) was transformed and plated to ensure that the vector had not re-circularised; re-ligated vector (pMOS-blue) was added in order to determine that the T4 ligase (Boehringer Mannheim, Hamburg, Germany) was efficient; and digested, DNA fragments were ligated and transformed to ensure that no plasmid DNA was present. Putative clones were identified by restriction endonuclease digestion (Appendix A4) of miniprepped clones, followed by agarose gel electrophoresis, as well as V3-V5 PCR using primers ES7 and ES8 (Appendix A2).

A7 Plasmid DNA extraction

Plasmid DNA minipreperation

The protocol was based on Sambrook *et al.* (1989). The selected recombinant bacteria were inoculated into 3-5ml of 2xYT broth containing 100μg/ml ampicillin and grown overnight at 37°C with shaking. Two mililitres of culture was transferred to a 2ml eppendorf tube and centrifuged at 14 000rpm for 2 minutes in a microfuge. The supernatant was removed and the pellet re-suspended in 100μl of Buffer 1 (50mM glucose, 10mM EDTA, 25mM Tris-Cl, pH8.0). After re-suspension, 400μl of Buffer 2 (8.8ml distilled water, 0.2ml 10M NaOH, 1ml 10% SDS) was added and the tube placed on ice for 5 minutes after gentle mixing. The protein and genomic DNA was then precipitated by the addition 300μl of Buffer 3 (3M KAc and 1.3M formic acid) and placed on ice for 30 minutes. The flocculant was removed by centrifugation at 14 000 rpm for 15 minutes in a micro-centrifuge and the clear supernatant transferred to a fresh eppendorf tube. The DNA was further purified by the addition of 200μl of phenol and vortexed to emulsify, followed by the addition of 400μl of chloroform:isoamyl alcohol (24:1). The solution was vortexed and then centrifuged for 2 minutes. The upper aqueous phase was transferred to a new eppendorf and 500μl of absolute ethanol was added. The solution was mixed and placed on dry ice for 10 minutes. Precipitated plasmid DNA was pelleted by centrifugation in a microfuge at 14 000rpm for 20 minutes and washed with 70% ethanol. The DNA pellet was re-suspended in an appropriate amount of TE (pH 7.6) or sterile, distilled water.

Plasmid DNA maxipreperation

For amplification of up to 100μg of plasmid, the Nucleobond® AX PC-Kit 100 (Machery-Nagel, Germany) was used according to manufacturers' instructions. The AX100 column is a silica-based, anion-exchange column for purification of plasmid DNA from 10 to 100ml of broth. The desired transformed bacterial cells were grown in 50ml of 2xYT broth containing 100μg/ml of ampicillin overnight at 37°C with shaking. The bacterial cells were then pelleted at 5 000rpm for 5 minutes at 4°C using the Beckmann J2-21 centrifuge. The supernatant was removed and the bacterial pellet carefully re-suspended in 4ml of 4°C

buffer S1 (50mM Tris/HCl, 10mM EDTA, 100µg Rnase A/ml, pH8.0). The re-suspended bacteria were then gently mixed with 4ml of room temperature buffer S2 (200mM NaOH, 1%SDS) and incubated at room temperature for exactly 5 minutes. Buffer 3 (2.8M KAc, pH5.2) (4ml) at 4°C was then added and the suspension mixed gently by inverting the tube 6 to 8 times, until a homogeneous suspension was formed. The suspension was incubated for 5 to 10 minutes on ice and then centrifuged in a Beckmann J2-21 centrifuge at 11 000rpm for 25 minutes at 4°C. The supernatant was carefully removed from the white precipitate and loaded onto the AX 100 column, equilibrated with 2ml of buffer N2 (100mM Tris/H_3PO_4, 15% ethanol, 900mM KCl, pH6.3). Once the supernatant had run through, the column was washed with 2x 4ml of buffer N3 (100mM Tris/H_3PO_4, 15% ethanol, 1150mM KCl, pH6.3). The purified plasmid DNA was then eluted from the column with 2ml of buffer N5 (100mM Tris/H_3PO_4, 15% ethanol, 1000mM KCl, pH8.5), although one bed volume (0.35ml) was discarded at the beginning to reduce the volume containing the plasmid. The eluted plasmid DNA was then precipitated with 0.8 volumes of room temperature isopropanol and immediately centrifuged in a micro-centrifuge at 14 000rpm in a microfuge for 10 minutes. The pellet was washed with 70% ethanol and re-suspended in an appropriate volume of TE (pH7.8). The DNA was then further purified by ethanol precipitation (Appendix A4), or for pcDNA vaccine purification, by addition of 2.5 volumes of absolute ethanol and 1/10 volume of 3M sodium acetate. The tube was then incubated at -20°C overnight, or on dry ice for 5 minutes to precipitate the DNA and centrifuged at 14 000rpm in a microfuge for 20 minutes. The DNA pellet was washed and re-suspended in sterile 1X PBS (pH 7.2) and stored at -20°C.

Determination of DNA purity and concentration

The re-suspended DNA was diluted one in one hundred in distilled water and scanned using the Beckman DU-40 spectrophotometer from 310nm to 220nm wavelengths. The spectrophotometer was blanked against distilled water after which the samples were read. To determine the purity of the DNA, the 260nm peak (DNA) was divided by the 280nm (protein) value and if the ratio was between 1.7 and 2.0 the DNA was deemed pure. The DNA concentration was determined by multiplication of the A_{260} by 50 (the factor for double stranded DNA) multiplied by one hundred, the dilution factor.

A8 Sequence analysis

A8.1 Radioactive DNA sequencing

The five reference plasmids and two subtype A isolates from Cape Town were sequenced in the V3 to V5 region of the *env* gene using the Sanger dideoxy termination method (Sanger *et al.*, 1977) with primers ES7, ES8 and ED33 shown in Table 2.2. The Sequenase® Version 2.0 DNA Sequencing Kit was used as per manufacturers' instructions (United States Biochemical Corporation (USB™) Amersham, Illinois, USA). The kit was stored at -20°C.

Alkaline denaturation of the dsDNA template

Nucleobond columns were used to purify the DNA for sequencing (Appendix A7), after which the pellet was further purified by ethanol precipitation (Appendix A4). Approximately 10-15µg of purified DNA was added to 18µl of deionised MilliQ® water in a 0.5ml eppendorf tube. Two microlitres of 2M NaOH was

added and the eppendorf incubated at 37°C for 30 minutes. The denatured DNA in the eppendorf was then placed on ice and 3µl of 3M sodium acetate and 100µl of absolute ethanol was added to precipitate the DNA. The tube was placed at -70°C for 5 minutes after which the DNA was pelleted in a microfuge at 14 000rpm for 20 minutes. The supernatant was removed and the DNA pellet washed with 70% ethanol, after which it was air-dried.

Annealing reaction

The denatured DNA pellet was resuspended in 7µl of deionised MilliQ® water and the following added:

2µl 5X Reaction Buffer (supplied by kit)

2µl primer (20ng/µl or 2 pmol)

The DNA was then annealed by heating at 65°C for 2 minutes, then cooled slowly to <35°C over 15 to 30 minutes. The DNA was pelleted by microfugation and placed on ice.

Note: 5X Reaction Buffer (1.0ml): 200mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 250mM NaCl

While cooling, 1.0ml eppendorf tubes were labelled and filled with 2.5µl of each Termination mixture, ddGTP, ddATP, ddTTP and ddCTP, respectively. The tubes were then pre-warmed to 37°C.

Termination mixes contain 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 50mM NaCl and:

ddG termination mix (250µl): 8µM ddGTP

ddA termination mix (250µl): 8µM ddATP

ddT termination mix (250µl): 8µM ddTTP

ddC termination mix (250µl): 8µM ddCTP

The Labelling mix (7.5µM dGTP, dCTP and dTTP) was diluted 5-fold to the working concentration, typically:

2µl dGTP

8µl deionised MilliQ® water

Labelling reaction

To the ice-cold annealed DNA mixture (10µl) was added:

1µl DTT (0.1M)

2µl Diluted Labelling mix

0.5µl [35S] dATP

2µl diluted T7 DNA polymerase (diluted freshly 1:8 in ice cold Enzyme Dilution Buffer)

The total 15.5µl was incubated at room temperature for 2-5 minutes after thorough mixing, avoiding bubbles.

Note: Enzyme Dilution Buffer (1.0ml): 10mM Tris-HCl, pH7.5, 5mM DTT, 0.5mg/ml BSA

Sequenase Version 2.0 T7 DNA Polymerase (25µl; 13 units/µl) in: 20mM KPO_4, pH7.4, 1mM DTT, 0.1mM EDTA and 50% glycerol.

Termination reactions

3.5µl of labelling reaction was added to each termination tube (G, A, T and C) and mixed after which the eppendorf tubes were incubated at 37°C for a further 5 minutes. The reactions were then stopped by adding 4µl of Stop Solution. The samples were heated to 75°C for two minutes immediately before loading onto the sequencing gel. 2-3µl was loaded onto each well.

Note: Stop Solution (2X 1.25ml): 95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cynol FF.

Preparation of the 6% non-denaturing polyacrylamide gel

Two glass plates, one notched, were washed thoroughly in warm water using a soft wash cloth and non-scratch detergent. The surface of one glass plate was made hydrophobic using silane in order to facilitate separation of the gel. The insides of the plates were wiped with ethanol in order to remove dust particles and the plates were placed on top of each other with 0.5mm spacers along both sides and the bottom of the plates. The plates were clamped together using "bulldog paper clamps" making sure that there were no gaps between the spacers.

40% acrylamide stock solution was made by dissolving 38g of acrylamide and 2g N',N'-methylene bis-acrylamide in 100ml of distilled deionised water. The 6% acrylamide was made by mixing:

- 15ml 40% acrylamide
- 50ml 10XTBE (88mM Tris-borate, 89mM Boric acid, 2mM EDTA)
- 46g molecular grade urea
- made up to 100ml with distilled deionised water

The mix was heated to 37°C in order to allow the gel to set more rapidly and just prior to pouring the gel the following was added:

- 100µl aliquot of ammonium persulphate
- 50µl N,N,N',N'-tetramethyl-1,2-diaminoethane (TEMED)

The clamped glass plates were held slightly upright and the acrylamide gel was poured between the glass plates, making sure that no bubbles were trapped between the plates, maintaining a steady flow. Once the gel casting apparatus was filled, it was rested with the open end slightly raised to prevent leakage and the "sharks-tooth" comb inserted backwards approximately 5mm into the gel in order to seal the open end. The gel was then clamped over the comb to prevent leakage and the gel left for 30 minutes one hour to set. If left overnight, Gladwrap® was used to seal the gel to prevent evaporation and drying.

Polyacrylamide gel electrophoresis of the sequencing reactions

The Gladwrap®, "Bulldog clamps" and the spacer at the bottom of the gel were removed. The polymerised gel was then transferred to the electrophoresis tank with the notched plate at the back, facing the upper buffer chamber. The electrophoresis tanks were filled with 0.5XTBE in the upper tank and 1XTBE in the lower tank. The "shark's tooth" comb was then removed carefully and inserted evenly with the teeth 0.5-1mm in the gel surface. Before loading the sequencing reactions, the DNA was denatured at 95°C for 5

minutes and then placed on ice. The wells were washed using buffer from the upper electrophoresis tank and a 20ml syringe with a needle in order to remove any diffuse urea that would lead to uneven band formation. A P20 PIPETMAN Gilson® micropipette (Gilson Medical Electronics, France) was used to layer 3µl of denatured sequencing reaction onto each well. Each template is run in four wells, ending in A, C, G and T). The DNA was then electrophoresed at 30V/cm. The power supply was set at 2000-2300V, 30-35mA and 80-85W for 1.5-2 hours for 100-200bp to be read and 3.5-4 hours for 150-400bp to be read.

Gel drying and autoradiography

After electrophorsis, the gel apparatus was dismantled and the plates pried apart using a thin wedge. The gel remained on the un-notched plate, due to the silane coating of the notched plate. The electrophoresis buffer containing radioactive waste was disposed of as recommended by the laboratory radioactive safety manual. Whatman 3MM chromatography paper was cut to the size of the gel and was lowered onto the polyacrylamide gel, pressing carefully to ensure that the entire gel was in contact with the absorbent paper. The gel was lifted from the glass plate by removal of the paper and covered in Gladwrap®. The gel was dried on a 70°C gel drier (make) attached to a vacuum pump for 1-2 hours after which the Gladwrap® was removed and the gel placed on top of Agfa Curix P1 X-ray film (Agfa-Gevaert™), in a dark room. The X-ray film was exposed for 1-4 days, depending on the strength of the ^{35}S used. The X-ray film was then removed and developed by submerging for 3 minutes in developer (1 part Novolith™Liquid A to 3 parts water mixed with an equal volume of 1 part Novolith™ Liquid B to 3 parts water) (May and Baker, Essex, UK), followed by 1 minute in stop solution (2% acetic acid) and then 3 minutes in fixative (Agfa G334C (Bayer, RSA) 1 part solution A to four parts water, mixed with 1 part solution B to 20 parts water). After a water rinse the developed X-ray was air-dried and the A, C, T and G lanes for each template were read manually and recorded for analysis.

A8.2 Automated sequencing

Automated sequencing was performed on the 650bp *gag* p17, partial p24 regions of 94ZA060 and 94ZA061 as well as on the entire gp120 from the primary, macrophage tropic isolate, 97ZA347TS. The Pharmacia™ ALFexpress® sequencer was used to detect fluorescently labelled dideoxy chain terminated fragments after electrophoresis. Sequencing was performed by Di James (Department of Microbiology, UCT). The sequencing reaction is based on the detection of non-radioactive Cy5-labelled primers in Thermo Sequenase® cycle sequencing reactions (see Pharmacia ALF Express manual and the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit). The primers used were U19 (5'ACGTCGTGACTGGGAAAACC3')and T7 (5'AATACGACTCACTATAGGG3') (pMOS-blue-T vector, Amersham, Buckinghamshire, UK) in the outer *gag* and gp120 sequencing reactions and internal primers, E135 and E155 (Myers *et al.*, 1995), shown in Table 4.2. The primers were labelled with Cy5 amidite using an oligonucleotide synthesiser. Thermo Sequenase is an exonuclease-free thermostable DNA polymerase that was engineered for cycle sequencing.

Thermo Sequenase cycle sequencing using the fluorescent dye-labelled primer

5µg of template DNA was added to 20µl of sterile MilliQ water in an eppendorf tube. The reaction

mix contained:

- 1µl Methyl violet-labelled primer (1-2pmol/µl)
- 5µl Template DNA
- 2µl A, C, G or T reagent (Tris-HCl (pH9.5, MgCl2, Tween™20, Nonidet™ P-40, 2-mercaptoethanol, dATP, dCTP, dTTP, dGTP and ddATP, thermostable pyrophosphatase and Thermo Sequenase DNA polymerase in addition to the respective ddATP, ddCTP, ddGTP or ddTTP) supplied by kit.

The reaction was mixed by pipetting up and down twice after which the following thermocycler conditions were used:

- Initial denaturation at 98°C for 5 minutes,
- 98°C for 30 seconds, 60°C for 30 seconds and 72°C for 40 seconds (25-30 cycles) and
- Final extension at 72°C for 40 seconds.

The DNA was then ethanol precipitated by addition of 1/10 volume of 3M sodium acetate, pH5.2 and 2.5 volumes of absolute ethanol and mixed. The DNA was pelleted at 12 000rpm for 10 minutes at room temperature and the pellet washed with 70% ethanol. After air-drying, the pellets were re-suspended in formamide loading dye (formamide, EDTA and methyl violet) supplied by the kit and DNA denatured at 90°C for 2 minutes before gel electrophoresis.

Electrophoresis and detection

A vertical gel cassette was used for electrophoresis. The temperature was controlled by water circulation through the ALFexpress automated sequencer thermoplate. Samples were loaded onto wells at the top of the gel. Detection is by a fixed laser beam that passes through the glass spacer located between the glass and thermoplate of the gel cassette. The beam passes through the gel perpendicular to the direction of band migration and exits the fluorescently-labelled DNA bands. The light emitted is detected by photodetectors which are behind the gel. Signals are collected, digitised and stored on a computer for processing. The raw data was displayed as a chromogram on a computer monitor during electrophoresis. The ALF Express software, AM V.0 stores the data during electrophoresis and performs a post-electrophoresis evaluation of the sequencing data.

A9 Heteroduplex Mobility Assay (HMA)

Heteroduplex formation (Delwart et al., 1993)

In order to form heteroduplexes of reference strain and unknown PCR amplified DNA the following was mixed in a 500µl eppendorf PCR tube:

4.5µl PCR product (approximately 100-250ng of DNA),
4.5µl reference PCR plasmid (approximately 100-250ng of DNA) and
1µl heteroduplex annealing buffer (100mM NaCl; 10mM Tris, pH7.8; 2mM EDTA).

In order to determine the genetic diversity of each PCR amplified unknown sample, 4.5µl of sterile distilled water was added instead of reference plasmid DNA. The tube was heated to 94°C for 2 minutes in the Techne PHC-2 PCR thermocycler in order to denature the DNA strands and the tubes were then rapidly cooled by placing on wet ice. The rapid cooling is important for facilitating stable heteroduplex formation

between highly divergent sequences, although not as important for more closely related sequences. The heteroduplex reaction mixture was then mixed with 2µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in deionised water) and loaded onto the wells of a 5% non-denaturing vertical polyacrylamide gel.

Polyacrylamide gel electrophoresis

Polyacrylamide gel (5%)was mixed (per 50ml):

8.3ml	30% acrylamide stock (1% bis-acrylamide)
5ml	10X TBE (88mM Tris-borate, 89mM Boric acid, 2mM EDTA)
36.7ml	distilled water
50mg	ammonium persulphate
33µl	TEMED

The 5% polyacrylamide gel was poured into the 190mm/160mm, 1.5mm thick Hoefer SE600 vertical gel casting tray (Hoefer Scientific Instruments, San Francisco, USA) to set at 37°C for 30 minutes. The set polyacrylamide gel was then transferred to the electrophoresis apparatus containing 1X TBE (10X, 108g Tris-HCl, 55g boric acid, 20ml of 0.5M EDTA made up to a litre) for electrophoresis of the samples. The heteroduplexes were electrophoresed at 200V for 5 hours at room temperature. DNA was visualised by ethidium bromide (1ng/ml) staining and visualisation on a 254nm wavelength UVP transilluminator.

APPENDIX B: TISSUE CULTURE TECHNIQUES

B1 Mammalian cell propagation

Three different mammalian cell lines were obtained from American Type Tissue Culture Collection (Rockville, MA, USA); HeLa (a human cervical epitheloid carcinoma cell line), CV1 (African Green monkey [*Cercopithecus aethiops*] kidney cell line) and 143B cells (human osteosarcoma cells, resistant to 5-bromo-2'-deoxyuridine, due to defective a thymidine kinase gene). Frozen stocks were thawed from liquid nitrogen storage by melting the cells in a 37°C water bath. The cells were pelleted by centrifugation at 1 000rpm for 5 minutes and the storage medium containing 10% DMSO was removed. The cells were washed twice with 1XDMEM (Dulbecco's Modified Eagle's Medium) (Dulbecco *et al.*, 1959) (Highveld Biological, South Africa) containing 10% fetal calf serum (FCS), penicillin (P), streptomycin (S) and Fungizone (F) (GibcoBRL, Paisely, UK) and then re-suspended in a 25ml flask (Nunclon, Nunc, Delta, Denmark) in 5ml of DMEM with 10% FCS and PSF. The cells were then incubated at 37°C with 5-10% CO_2 atmosphere for two to three days, until the cells formed a confluent layer. Cells were split by adding trypsin to lift them for 5 minutes at 37°C and then diluting them 1/5 in fresh DMEM with 10% FCS and PSF. The trypsin working solution was made by mixing 1ml of 5% trypsin with 10ml of 10X Trypsin base, in 89ml of sterile distilled water.

The 10 trypsin base was made by adding 30g NaCl, 2g KCl, 1.2g Na_2HPO_4 and 5g glucose to 700ml of sterile distilled water. Two grams of EDTA was added, followed by 25ml of 0.4% phenol red and the solution stirred. The trypsin base was adjusted to a pH of 7.8 with 1N NaOH and the solution made up to 1l. The trypsin base was then filtered through a 0.2 micron Cameo 25AS pore filter (Westboro Medical, USA) and stored at –20°C.

The 5% trypsin solution was made by adding 10ml of trypsin base to 90ml of sterile distilled water and 0.5ml of PSF. 1N HCl was added drop-wise until the solution turned bright yellow and the solution was then heated to 37°C for 2 minutes. The trypsin powder (5g) was then sprinkled slowly on top of the solution and swirled gently to dissolve it, without stirring. The solution was left at room temperature for 1-4 hours to dissolve the trypsin completely and then filtered through a 0.2 micron pore filter. The 5% trypsin solution was stored at –20°C.

Cell stocks were prepared by trypsinising a 10ml culture of confluent cells and centrifuging at 1 000rpm for 5 minutes to remove additional trypsin. The cell pellet was re-suspended in 800µl of FCS with 10µl of DMEM. The Nunc cryotubes (Nunc, Delta, Denmark) of cells were placed on ice and 10µl of DMSO was immediately added. The cryotubes were then thickly wrapped in paper towel and placed at -70°C for 2 to 3 days, after which they were transferred to liquid nitrogen for storage.

B2 Transfection using DOTAP and Fugene6

HeLa cells were grown in a Nunc six well plate (Delta, Denmark) until they were 60-80% confluent. The cells were transfected with 30µl of DOTAP (Boehringer Mannheim, Hamburg Germany), 2µl of pSVNeo-βgal (Stratagene La Jolla, CA, USA) and 10µl of either pcDNA3.1$_{SF162}$ or pcDNA3.1$_{97ZA346TS}$, according to manufacturers' instructions. The pSVNeo-βgal plasmid expresses β-galactosidase. The DOTAP was

incubated for 5 minutes at room temperature in HEPES buffer making up a total of 100µl. The pSVNeo-βgal and DNA vaccines were also added to HEPES buffer, made up to 50µl and the DNA and DOTAP mixed by gently tapping the tube and incubated at RT for 10-15 minutes. The pSVNeo-βgal and DNA vaccine, DOTAP mixture was added to the HeLa cells growing in 2ml of fresh DMEM with 10% FCS and PSF and the transfected cells were incubated at 37°C with 5-10% CO_2 overnight. The DOTAP, DNA mixture was then removed and 2ml of DMEM with 10% FCS and PSF added. The cells were incubated at 37°C with 5-10% CO_2 for two days and X-gal stained (Appendix B5).

B3 Recombinant formation

CV1 cells were grown to 60-80% confluence in a six well plate. The vaccinia virus (VV) western reserve (wr) stock (obtained from John Williamson, St Mary's Hospital, London) was diluted to $3X10^5$ viruses/ml in virus diluent (DMEM and 1% 1M HEPES) and 0.5ml added to each well. The infected cells were incubated at 37°C with 5-10% CO_2 for 1 hour with occasional shaking. Instead of DOTAP, FuGene6 was used as the transfection reagent. Four microlitres of Fugene6 was added to 97µl of serum-free medium and incubated at room temperature for 5 minutes. Three microlitres of *Sca* I linearised $pSC65_{97ZA347TS}$ plasmid, expressing the HIV gp120 gene, was added to the FuGene6 transfection reagent and incubated at room temperature for 15 minutes. The VVwr in diluent was removed from the wells and the transfection mix was added in 2ml fresh DMEM with 10% FCS and PSF. The transfected, infected cells were incubated at 37°C with 5-10% CO_2 for two to three days until cytopathic effect (cpe) were detected. After cpe were detected, the cells were scraped off the plate with a rubber policeman and re-suspended in 200µl of virus diluent in cryotubes (Nunc, Delta, Denmark).

The infected cells were frozen in dry ice and ethanol and thawed at 37°C three times to lyse the cells and release the virus. The virus was then diluted 1/2 in 500µl of virus diluent and added to 80% confluent TK^- cells. The cells were incubated at 37°C with CO_2 for 1 hour with occasional shaking as before and the virus removed. 25µg/ml BUdR selection medium (Sigma, St Louis, USA) in 2ml DMEM with 10% FCS and PSF was added and the cells incubated for 2 to 3 days until cpe were evident. The medium was removed and the infected cells overlayed with 2% low melting point agarose at 42°C containing 25µg/ml BudR and 250µg/ml X-gal to detect recombinant plaques. The agarose mixture was set at RT and the cells incubated overnight at 37°C with 5-10% CO_2.

Blue recombinant plaques were picked using a Pasteur pipette with a bulb and added to 200µl of virus diluent. The infected cells were freeze/thawed as before, diluted 50µl into 500µl virus diluent and used to infect a second plate of 80% confluent TK^- cells (Passage 2). The recombinant virus, $VV_{97ZA347TS}$ was passaged until purely blue recombinant plaques were detected (11 passages) and then used to infect eggs for the production of large quantities of virus (see Appendix B4). After virus from eggs was extracted, wild type VVwr revertants were detected, thus the virus was passaged a further two times and the virus re-grown in eggs. Pure $VV_{97ZA347TS}$ was obtained and confirmed by immunofluorescence (see Appendix B6).

B4 Vaccinia virus propagation in eggs

Infection of eggs with VV

A 25ml flask of TK$^-$ cells in 5ml of DMEM with 10% FCS and PSF was grown until the cells were 80% confluent. The cells were infected with 5X10^{5}pfu/ml of $VV_{97ZA347TS}$ recombinant and incubated for 3 days, or till cpe were evident. The infected cells were scraped off the bottom of the flask with a rubber policeman and saved in the supernatant. The infected cells in supernatant were freeze/thawed three times, as before, to release the virus and stored at –70°C.

In order to infect the eggs, the virus was diluted ½, 1/5 and 1/10 in 1X PBS and 5 day to 10 day old eggs used for infection. The eggs were placed on a rack and marked, using 6 eggs per dilution factor. The virus is inoculated onto the amniotic membrane of the egg and to determine where the inoculation site was, the eggs were held to a light bulb in the dark. The air sac on round side was identified and marked with a pencil. It is best to inoculate near a vein, thus a vein on the top of the egg was also marked with a cross. The eggs were returned to the rack and a small hole made in the cross on top of the egg, being careful not to break the membrane. A hole was also made in the air sac. The air sac hole was opened with a sterile, sharp fountain pen. A drop of sterile 1XPBS containing PS was placed onto the top hole using a Pasteur pipette and a drop of melted wax was placed above the hole. The top membrane was loosened with a curved, sterile fountain pen to let the PBS run in. The eggs were again looked at over a light and a rubber bulb used to suck at the air sac hole making the top membrane drop. The PBS should run onto the top amniotic membrane inoculation site. The eggs are incubated for 1.5 hours in order for the chick to settle. 600µl of recombinant $VV_{97ZA347TS}$ of each dilution factor was sucked into a MODRI 1ml syringe with a BIOCOR 25 gauge needle (Disposmedicor, South Africa) and 100µl per egg injected into top hole just below surface without puncturing the membrane. The wax was scraped over the hole to seal the egg, the eggs were swirled slowly to disperse the virus and then replaced in the 37°C incubator for 3 days.

Extraction of virus

The desk was lined with a paper towel and sterile forceps and scissors, a petri dish and two bottles (100ml each) of physiological saline placed on top. McIlvaine's buffer and Arklone® (AECI, South Africa) were placed on ice. McIlvaine's buffer was made by adding 1.83ml of solution A (0.1M citric acid) and 18.17ml of solution B (0.2M $Na_2HPO_4 \cdot 12H_2O$) to 800ml of sterile distilled water. The pH was adjusted to 7.4 with the two solutions and made up to 1l with distilled water. The solution was then autoclaved and stored at 4°C. In addition, 4 McCartney bottles were filled with glass beads up to 2cm from the bottom of the bottle and the bottles placed on ice. A metal bucket was lined with a bio-waste bag and placed next to the paper towel.

The pair of scissors was used to cut around sides of the egg from air sac hole, working over the bucket. The shell was flapped up and the chick and bottom half of the egg cut away. The dropped membrane should be visible with the poxes seen as white dots. The egg shell around the dropped membrane was cut away and discarded. The infected membrane was removed with sterile forceps and dropped into the first bottle of saline to rinse, followed by a second rinse in the second bottle of saline. The

infected membrane was placed in the McCartney bottle with glass beads, using one bottle for 4 membranes. One millilitre of McIlvaine's and 250µl of Arklone® per membrane was added. The McCartney bottle lids were tightened and the bottles wrapped in paper towel in case of leakage. The bottles were shaken vigorously for two minutes to disrupt the infected membrane.

The disrupted membranes were centrifuged at 1 000rpm for 10mins at 4°C and the supernatant transferred to 2 fresh McCartney bottles on ice. Four millilitres of McIlvaine's buffer was added to the glass beads and debris and the bottles shaken again for 2 minutes. The membranes were re-centrifuged as before. The supernatant was again transferred to the McCartney bottles on ice and the supernatant incubated on ice for 1.5 hours. The two McCartney bottles were then centrifuged at 2 000rpm for 15 minutes to remove debris and the supernatant transferred to Beckman® centrifuge bottles. Slowly, 500µl of 36% sucrose was allowed to run down the side of the tube to form a layer at the bottom and the virus was then centrifuged at 11 000rpm for 1 hour in the Beckman J2-21 rotor. The supernatant was removed and the pellet re-suspended gently overnight in 1ml McIlvaine's buffer at 4°C. The re-suspended virus was diluted 10^{-3} to 10^{-9} in virus diluent for virus titration in triplicate.

Virus titration

The re-suspended virus diluted 10^{-3} to 10^{-9} was used to infect a 24 well plate (Nunc, Delta, Denmark) of 80% confluent CV1 cells. 200µl of diluted virus was added to each well, using three wells per dilution factor. The cells were incubated for 1 hour with occasional shaking at 37°C after which the virus was removed and 1ml of fresh DMEM with 10% FCS and PSF was added. The infected cells were incubated at 37°C with 5-10% CO_2 for two to three days until cpe was visible after which they were stained with Ziehl Neelson Carbol Fuchsin stain for one minute, and the plaques counted. The number of plaques was multiplied by 5 to obtain a value per 1ml.

B5 X-gal stain

Transfected cells were incubated for 2 days at 37°C with 5-10% CO_2 after which the medium was removed and the cells washed in 1X PBS. 1ml of 4% para-formaldehyde was added to each well for 10 minutes at room temperature to fix the cells. The para-formaldehyde was removed and 1ml of the X-gal stain (working solution in 10mls; 9.2ml 1X PBS, 0.2ml of 50mg/ml X-gal, 0.2ml of 200mM ferricyanide, 0.2ml of 200mM ferrocyanide and 0.2ml of 200mM $MgCl_2$) was added. The cells were incubated at 37°C overnight and the number of blue cells counted.

B6 Immunofluorescence

Transfected or infected cells were grown on a glass cover-slip in a 6 well plate for 2 to 3 days. The cells were fixed to the cover-slip with acetone at 4°C for 10 minutes and the cover-slip air dried and washed with 1X PBS. 2% ovalbumin was added as blocking reagent for 20 minutes at RT with shaking and then the cover-slips were washed twice with 1X PBS for 10 minutes each. A 1/300 dilution of the anti-HIV gp120 goat polyclonal antibody (Biogenetics, Poole, England) containing 1.5% BSA in 1X PBS was added and the cells incubated for 1 hour at 37°C. The cover-slip was washed twice with 1X PBS for 10min each and 1/40

dilution of rabbit anti-sheep FITC conjugate (DAKO, Glostrup, Denmark) and 200µl of Evan's blue counter stain in 1X PBS was added and the cover-slip incubated for 1 hour at 37°C. The cells were washed twice with 1X PBS for 10 minutes each, rinsed in distilled water and allowed to air dry. The cover-slips were then mounted cells down on a slide for fluorescent microscopy using the SM-LUX (Leitz-Wetzlar, Germany) fluorescent microscope at 450-490nm wavelength and 40X magnification. Photographs were taken using Fugi® Sensia II IL400 film with a 2-3 minute exposure.

B7 Immunisation and challenge of mice

Direct gene transfer to the tibialis anterior muscle in mice

Six to eight week BALB/c males were used for antibody detection, whilst 6-8 week female BALB/c mice were used for the *in vivo* CTL experiment. Forty-eight hours prior to the DNA vaccine immunisation the mice were injected with 100µl of 0.25% bupivacaine-HCl (Adcock Ingram Pharmaceuticals) using a MODRI 1ml syringe with a BIOCOR 25 gauge needle (Disposmedicor, South Africa) with a collar to limit penetration to 2mm into the *tibialis anterior* (TA) muscle, causing localised necrotisation of the muscle.

After 48 hours, the muscle was regenerating which facilitates uptake of the DNA injected into the same site. Mice were anaesthetised using an ether nose-cone, since awake mice contract their muscles and squeeze the DNA solution out. Once the mice were asleep, the DNA prepared in endotoxin-free injectable 1X PBS at 1mg/ml was injected. Fifty microlitres was injected into each TA muscle using the MODRI 1ml syringe with BIOCOR 25 gauge needle (Disposmedicor, South Africa). The injection was 3mm lateral to the anterior tibial tuberosity (half way between the knee and the ankle), keeping the needle almost perpendicular to the tibia. Once the needle was in place, the DNA was injected slowly, over approximately 10 seconds, the needle held in place for another 5-10 seconds and then removed slowly.

Recombinant $VV_{97ZA347TS}$ challenge of the mice

Mice immunised once with DNA vaccine were challenged after nine days with the recombinant $VV_{97ZA347TS}$. The mice were injected with 100µl of either $1X10^7$ or $5X10^7$ pfu/ml of virus intraperitoneally using the MODRI 1ml syringe with a BIOCOR 25 gauge needle (Disposmedicor, South Africa). After 5 to 8 days the mice were sacrificed by lethal inhalation of ether and their necks were broken. Their ovaries were removed and extracted (see Appendix B9).

B8 Detection of mouse antibodies

ELISA plate (Nunc-Immuno™, Delta, Denmark) wells were coated with 50µl of 2µg/ml of HIV_{IIIB} gp120 protein (obtained from the National Institute for Biological Standards and Control (NIBSC) AIDS Reagent Project, Hertfordshire, UK) in 0.1 M, pH 7.6 carbonate buffer (sodium carbonate, 1.36 g; sodium bicarbonate, 7.35 g and distilled water, 950 ml) overnight at 4°C. The wells were washed with 1X PBS containing 0.05% Tween-20® and blocked for 1 hour at 37°C in blocking solution (1% Elite® milk powder, 0.05% Tween-20® in 1X PBS made up fresh weekly), filling the wells to the top. The plate was then washed 3 times using LP55 Sanofi plate washer (Pasteur Diagnostics, Paris, France) with 10 second waits in

washing buffer (1XPBS with 0.05% Tween-20®). Fifty microlitres of mouse serum diluted to 1/50 in 1X PBS was added to duplicate wells and the plate was incubated covered at 37°C in a water bath for 1 hour. Controls consisted of coated wells with no serum, and non-coated wells with serum. The plate was washed as before using the plate washer. Fifty microlitres of rabbit anti-mouse-HRP conjugate (DAKO, Glostrup, Denmark) diluted to 1/600 in blocking solution was added to the wells, including the control wells, in order to detect background or contamination. The plate was incubated for 1 hour at 37°C in a water bath and then washed as before in the plate washer. Fifty microlitres of freshly made up substrate (DAKO, Glostrup, Denmark) (12ml distilled water, 4 OPD tablets and 5µl H_2O_2) was added to each sample well, and to two non-coated, empty wells and the plate was incubated at room temperature for 25 to 30 minutes in the dark. Fifty microlitres of 1N H_2SO_4 was added to stop the reaction and the plate was read using the Anthos ELISA plate reader (Labtech Instruments) at 492nm with a 620nm filter.

B9 $VV_{97ZA347TS}$ extraction from mouse ovaries

The two ovaries from each mouse were pooled and weighed once all fat had been removed. The ovaries were then placed in a petri dish and finely chopped using a sterile scalpel. The chopped ovaries were transferred to a ten broek grinder in McIlvaine's buffer, using 10µl of buffer per 1mg of mouse ovary tissue and subjected to 30 strokes with the grinder. The ground ovary solution was transferred to a 2ml eppendorf tube and the ovary extract freeze/thawed three times to lyse any cells which may have remained whole. The ovary cell extract was then centrifuged on a benchtop microfuge for 1 minute to remove debris and the supernatant transferred to a fresh eppendorf. The $VV_{97ZA347TS}$ recombinant was titrated as before (see Appendix B4) and the stock virus stored in aliquotes at –70°C in McIlvaine's buffer.

APPENDIX C: VECTOR MAPS

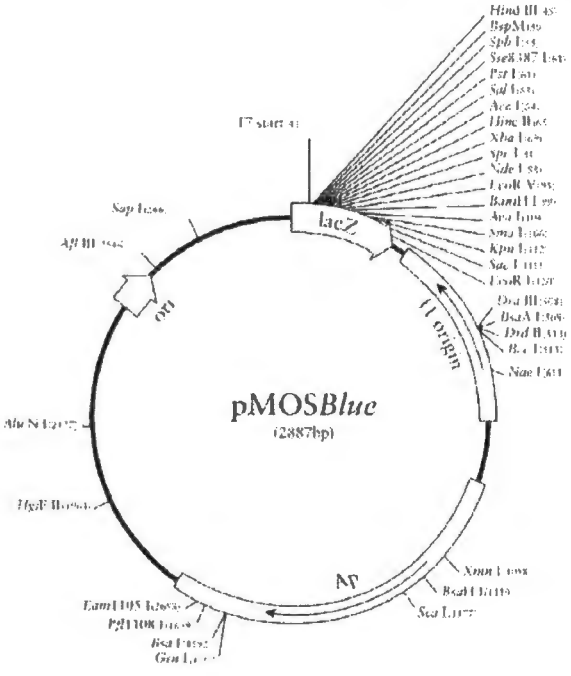
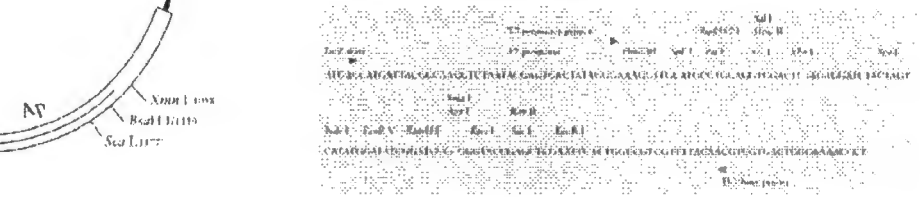
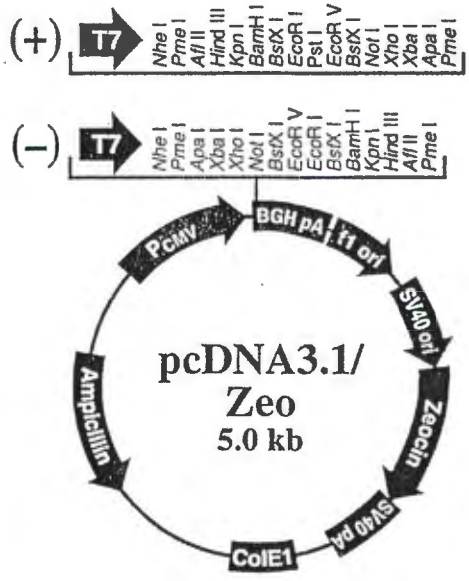
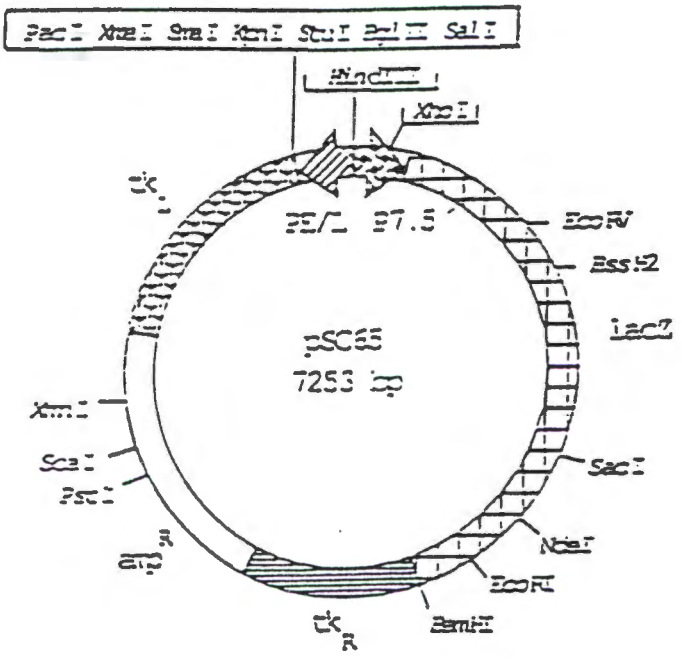


FIGURE 10
pMOSBlue multiple cloning region






pSC65
7253 bp
EcoRV
Sac I
Nhe I
EcoR I
BamH I
Pst I
Sca I
Xma I
Sal I

APPENDIX D1: AMINO ACID CODE

Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

APPENDIX D2: CODONS

Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid
AAA	K	CAA	Q	GAA	E	TAA	*
AAC	N	CAC	H	GAC	D	TAC	Y
AAG	K	CAG	Q	GAG	E	TAG	*
AAT	N	CAT	H	GAT	D	TAT	Y
ACA	T	CCA	P	GCA	A	TCA	S
ACC	T	CCC	P	GCC	A	TCC	S
ACG	T	CCG	P	GCG	A	TCG	S
ACT	T	CCT	P	GCT	A	TCT	S
AGA	R	CGA	R	GGA	G	TGA	*
AGC	S	CGC	R	GGC	G	TGC	C
AGG	R	CGG	R	GGG	G	TGG	W
AGT	S	CGT	R	GGT	G	TGT	C
ATA	I	CTA	L	GTA	V	TTA	L
ATC	I	CTC	L	GTC	V	TTC	F
ATG	M	CTG	L	GTG	V	TTG	L
ATT	I	CTT	L	GTT	V	TTT	F

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