CHARACTERISATION OF NEF FROM HIV-1 SUBTYPE C-INFECTED INDIVIDUALS

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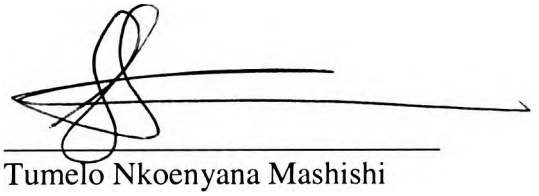
ABSTRACT

This dissertation examines HIV-1 subtype C nucleotide and amino acid Nef sequences from South African recently-infected (n=12) and chronically-infected individuals (n=9) and from AIDS patients (n=11). The overall aim is to determine which regions of subtype C Nef are variable and which are conserved and to associate these regions with targeting by the immune system. Phylogenetic analysis and sequence alignments showed that there was no association of Nef variations with stage of disease. Nef amino acid alignments of 32 sequences showed a high degree of conservation in areas of functional and structural importance. The mean intrasubtype variation from these isolates was 15.8%, with sequences from the AIDS cohort being significantly ($p<0.05$) more variable than those from recent or chronic infection. Moreover, the mid-portion of subtype C Nef was shown to be significantly ($p<0.001$) more conserved (with intrasubtype percentage amino acid variation of 8.2%) than either the amino (percentage variation=17.1%) or carboxy termini (20.0%). Previous studies of the Nef molecule have shown the mid- and COOH- regions to be rich in CTL epitopes. Using the IFNγ ELISPOT, seven out of nine HIV-1 subtype C infected individuals showed a response to subtype B-based peptides. The majority of these (86%) responses were in the highly conserved middle regions of Nef, confirming this region to be rich in CTL epitopes. This project is the first to report on a combined sequence and immunological analysis of subtype C Nef from HIV-1 infected South Africans.

DECLARATION

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



Tumelo Nkoenyana Mashishi

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LIST OF ABBREVIATIONS USED IN THE TEXT

Ab	antibody
Ag	antigen
AIDS	acquired immunodeficiency syndrome
AMV	avian myeloblastosis virus
bp	base pair
°C	degrees celcius
CDC	Centers for Disease Contol
cDNA	complementary deoxyribose nucleic acid
CTL	cytotoxic T lymphocyte
CTR	control
dATP	deoxy-adenosine triphosphate
dCTP	deoxy-cytosine triphosphate
ddATP	dideoxy-adenosine triphosphate
ddCTP	dideoxy-cytosine triphosphate
ddGTP	dideoxy-guanosine triphosphate
ddNTPs	dideoxy-nucleotide triphosphate
DDT	dithiothreitol
ddTTP	dideoxy-thymidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxy-guanidine triphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
dNTPs	deoxynucleotide triphosphates

dTTP	deoxy-thymidine triphosphate
Du	Durban
EDTA	Ethylenedinitrilotetraacetic acid disodium salt
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
env	envelope gene
Env	envelope protein
FCS	foetal calf serum
HAART	highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HMA	heteroduplex mobility assay
H_2O	water
hr	hour
HRP	horseradish peroxidase
IFN	interferon
IL	interleukin
kDa	kiloDalton
LTNP	long term non-progressor
M	Molar
mAb	monoclonal antibody
mg	milligram
min	minute
ml	millilitre
mM	millimolar

mRNA	messenger RNA
$MgCl_2$	magnesium chloride
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
pmol	picomole
RNA	ribose nucleic acid
RNAsein	RNAse inhibitor
rpm	revolutions per minute
rt	room temperature
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
sec	seconds
SIV	simian immunodeficiency virus
Sw	Sizwe Hospital for Tropical diseases
TB	tuberculosis
TBE	tris boric EDTA
TEMED	N,N,N',N',-Tetramethylethylenediamine
Th	T-helper
TNF	tumour necrosis factor
Tris	Tris-(hydroxymethyl)-aminoethane
Triton X-100	polyethyleneglycol (9-10)-p-t-octylphenol
µg	microgram

µl	microlitre
µM	micromolar
V	volts
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

1.1 Human Immunodeficiency Virus Epidemic and Infection

1.1.1 The Global Epidemic

The HIV/AIDS epidemic first emerged about 20 years ago. It was first seen in young homosexual men who were treated for Kaposi's sarcoma (CDC, 1981a) and *Pneumocytis caninii* pneumonia (CDC, 1981b). It was observed that these individuals had a dramatic decline in CD4+ cells. The pathogenic agent responsible for this syndrome was later isolated and shown to grow to high titres in CD4+ cells and, identified as a retrovirus (Montagnier *et al.*, 1984) (Gallo *et al.*, 1984). In 1986, the International Committee on Taxonomy of Viruses termed the virus, human immunodeficiency virus (HIV)(Coffin, 1990). This virus belongs to the family of Retroviridae, subfamily lentivirinae, which are slow acting, cytopathic viruses associated with long incubation periods and infection of the central nervous system (CNS)(Hehlmann, 1991). Two types of HIV have been characterised, termed HIV-1 and HIV-2. Phylogenetic analysis of HIV envelope sequences resulted in classification of two groups within HIV-1, the major group (M), which is further divided into at least 10 genetically distinct subtypes (A-J) and comprises the majority of HIV-1 isolates, and the outlier group (O), which represents a number of highly divergent strains. There have been five genetically distinct subtypes of HIV-2, designated HIV-2 A to E (O'Shaughnessy, 1995).

By the end of 1999, it was estimated that a total of 34.3 million people were infected with HIV globally. Over two thirds of these cases (24.5 million) are thought to be residing in sub-Saharan Africa (UNAIDS/WHO, 2000), see Figure 1.1. It is also estimated that since the beginning of the epidemic, there have been about 18.8 million

AIDS deaths globally, with the majority of these, about 15 million, from sub-Saharan Africa. The WHO estimates that by the end of year 2000, about 40 million people were infected with HIV globally, with more than 90% of these from developing countries in sub-Saharan Africa, Asia and Latin America. In developed countries such as Europe and North America, the use of effective, but expensive, antiretroviral therapies, has significantly increased the life expectancy of HIV-infected individuals (Palella *et al.*, 1998). In sub-Saharan Africa, however, the majority of infected individuals cannot afford antiretroviral therapy, contributing to the high AIDS deaths seen in this region. Subsequently, the epidemic is spreading rapidly in sub-Saharan Africa, where there were about 11 000 new infections per day and over 6 000 AIDS deaths per day in 1999 (UNAIDS/WHO, 2000).

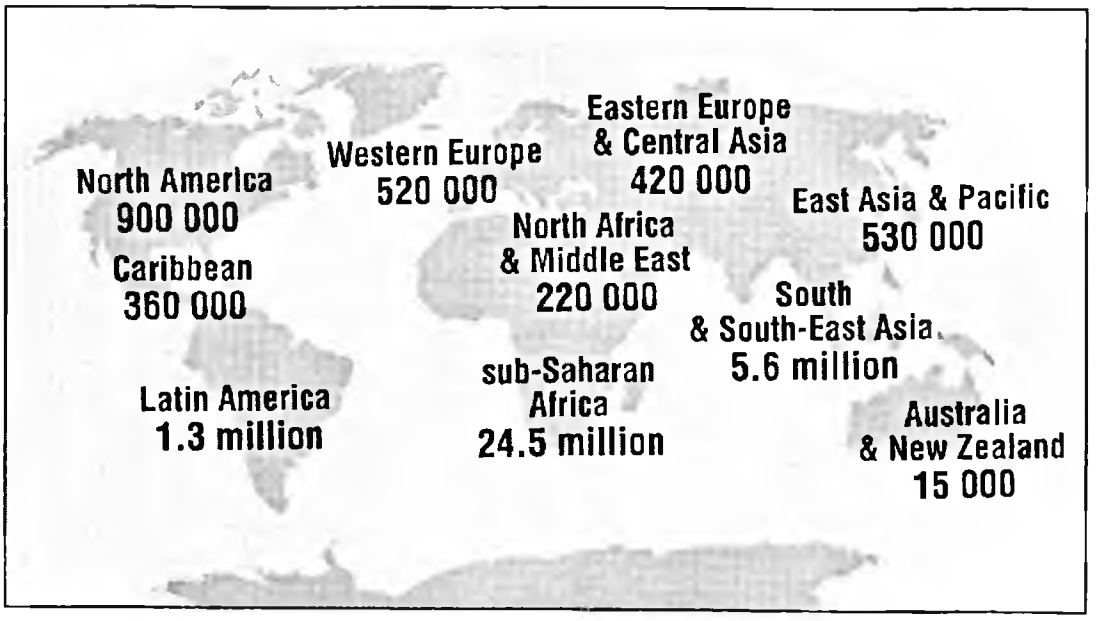

Figure 1.1: Global distribution of the HIV-1 epidemic(*Reprinted from UNAIDS, 2000*)

1.1.2 The South African Epidemic

The HIV epidemic has rapidly increased in the 1990s in South Africa. The country currently has the fastest rate of new infections (UNAIDS/WHO, 2000), where the

virus is mainly transmitted heterosexually (Schoub, 1990). By the end of 1999, it was estimated that HIV prevalence in South Africa was 10% of the whole population (UNAIDS/WHO, 2000). The number of deaths attributed to HIV in 1999 was 250 000 (UNAIDS/WHO, 2000). The majority of infected individuals are in the economically productive age group (15-49), which has major social and economic implications. The most prevalent clade in South Africa is subtype C (Williamson *et al.*, 1995); (Van Harmelen *et al.*, 1999), which is also the fastest spreading clade globally. Figure 1.2 shows HIV-1 prevalence of women attending antenatal clinics in South Africa from 1990 to 1999 (Department of Health Report, 2000). From this figure, there is a marked increase within this period in the percentage of HIV-1 prevalence. Between 1990 and 1998, there was an exponential increase in the number of HIV-1 infected women attending antenatal clinics, which appears to be doubling every one to two years, but seems to levelling off from 1999. From the antenatal surveillance, it was also observed that the KwaZulu-Natal province in South Africa has the highest HIV-1 prevalence rate (32.5% of women attending antenatal clinics), with Western Cape having the lowest (7.1%).

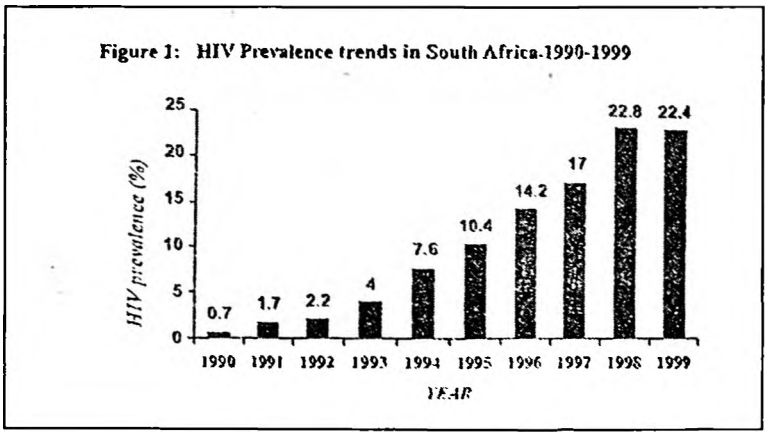

Fig 1.2: HIV-1 prevalence trends in South Africa between 1990 to 1999. This data was obtained from pregnant women attending antenatal clinics across the country.

It is now thought that subtype C is now the most prevalent subtype globally, accounting for 54% of all infections (UNAIDS/WHO, 2000). Subtype C viruses are associated with new, rapidly growing epidemics in southern Africa, India and China, and have also been reported to occur in other parts of the world, including eastern Europe, Russia and Brazil (Leitner, 1996).

1.2 Strategies for Medical interventions

1.2.1 Microbicides

Over 95% of HIV-1 transmissions in South Africa are thought to be through heterosexual intercourse (Schoub, 1990). Therefore, development of effective chemical and physical barriers against HIV infection can help prevent acquisition and transmission of HIV. Studies have been undertaken to determine the feasibility of using topical microbicides in containing the spread of HIV (Howes and Bass, 1999); (Kawamura *et al.*, 2000). These include agents as surfactants (such as detergents) and natural agents that kill HIV *in vitro* by disrupting the virus membrane, as well as inhibitors of viral entry that can either specifically or non-specifically block HIV attachment and fusion to a host cell (Mori *et al.*, 1997). The use of microbicides in humans has been hindered by lack of true associations between *in vitro* findings, animal models and their efficacy in humans. Negative attributes of microbicides include potential negative side effects in both the user and the sexual partner and the reluctance of the general public to use them (Junker *et al.*, 1998); (Xu *et al.*, 2000).

1.2.2 Anti-retroviral Therapy

The use of highly active antiretroviral therapy (HAART) has significantly reduced the mortality and morbidity in HIV-1-infected individuals in developed countries (Gallant, 2000). The life expectancy of HIV-1-infected individuals is dramatically increased in infected individuals on a HAART regimen (Palella *et al.*, 1998). Antiretroviral drugs are very expensive and are therefore not widely used in developing countries, where the epidemic is growing very rapidly. It has also been shown that the administration of zidovudine and nevirapine for a short course before delivery, significantly reduces the risk of mother-to-child HIV transmissions (Lallemant *et al.*, 2000); (Mirochnick *et al.*, 2000).

In infected individuals on HAART, viral load can decline to levels undetectable using ultrasensitive assays (< 50 RNA copies per ml) that measure viral RNA copies. Upon cessation of treatment, however, there was a rebound in viraemia suggesting that virus was not cleared by the treatment (reviewed by Ho and Zhang, 2000). The rebound was later attributed to latent reservoirs comprising infectious provirus integrated quiescently within resting memory CD4+ T cells (Chun *et al.*, 1997); (Finzi *et al.*, 1997); (Cohen and Fauci, 1998). Anti-retroviral drugs were not effective in these reservoirs. It is now clear that complete eradication of the virus cannot be achieved using the current regimen of HAART (Ho and Zhang, 2000).

1.2.3 Vaccines

Vaccines have in the past been very successful in combating several lethal diseases such as small-pox (Darekar, 1966). Vaccines are generally inexpensive and can be administered widely. The use of such vaccines has had a major impact in reducing

both morbidity and mortality for a variety of bacterial and viral diseases (Baxby, 1991); (Fennerty, 1996); (Niemialtowski *et al.*, 1996). The main reason for the success of preventative vaccines in many bacterial and viral infections, is that primary protection has been shown to be mediated by long-lived humoral immune responses through the production of neutralising antibodies. However, for infections such as HIV-1, there is currently no effective vaccine. Furthermore, although humoral immunity could be important in preventing infection by HIV-1, it is the cellular immune response that is thought to be important in mediating protection. The aim of developing an effective vaccine should therefore include a means to elicit a good CTL response. Vaccines are thought to be the most feasible means of controlling the spread of HIV-1 and lowering the rate of transmission.

1.2.4 Challenges to vaccine development

The major challenge for HIV vaccine design is the capacity of HIV-1 to evade the immune system. Viral sequence variation in different proteins is the most studied mechanism of viral escape from CTL- and antibody-mediated immune pressure. The combination of high viral turnover and the error-prone reverse transcriptase generates a high level of genetic variation in the HIV-1 genome (Mansky and Temin, 1995). These variations could result in mutations within CTL (Phillips *et al.*, 1991) and T helper epitopes (Hadida *et al.*, 1998), which can lead to evasion of the host immune system. Such sequence variations can abrogate 1) binding of the epitope to the presenting HLA molecule and/or 2) recognition of the epitope by the T cell receptor on the CTL (Borrow *et al.*, 1997); (Klenerman *et al.*, 1994).

Viral evasion has also been demonstrated using monkey models of HIV/AIDS (SIV) (Walker and Goulder, 2000); (Allen *et al.*, 2000). During the early phase of infection, many of the CTL generated locate and kill infected cells by recognising MHC class I-restricted epitopes from Tat (Allen *et al.*, 2000). These Tat specific CTL are very efficient at controlling the strain of virus that first infects the host. However, mutations within the Tat epitopes develop during the initial period of immune reactivity, leading to establishment of a long lasting infection (Allen *et al.*, 2000). During disease progression, there is selection for viral mutants which cannot be recognised by CTL, resulting in unchallenged viral replication, increase in viraemia and finally disease progression. It is thought that rapid progression to AIDS can be associated with increased mutation arising in HIV epitopes while in slow progression, there is control of virus and the likelihood of mutations arising during acute infection is low (Walker and Goulder, 2000). Therefore, the ability of HIV to rapidly mutate, and subsequently lead to evasion of a cellular immune response is the one biggest challenge in developing an effective preventative vaccine if there is a breakthrough infection. The challenging virus might rapidly mutate and not be recognised by vaccine-specific CTL.

1.3 The Human Immunodeficiency Virus

1.3.1 Structure of the virion

HIV is an icosahedral virus that is 100 to 150 nm in diameter and containing 72 external spikes (Gelderblom *et al.*, 1987); (Greene, 1991); (Connor *et al.*, 1997) (Figure 1.3). The external spikes are formed by the two major envelope proteins, an external glycosylated protein gp120 and a transmembrane protein gp41, that are embedded in the lipid membrane (Montagnier and Clavel, 1994). Mature particles

have a cone-shaped capsid, which contains the major capsid protein, p24, nucleocapsid proteins, p7 and p9, a diploid single-stranded RNA genome and three viral enzymes, protease, reverse transcriptase and integrase. The viral capsid is surrounded by a matrix protein p17, which is located underneath the lipid bilayer envelope membrane. The viral genomic RNA encodes for a series of structural (*env* and *gag*), enzymatic (*pol*) and regulatory (*rev*, *tat*, *nef*, *vpu* and *vif*) proteins that are required to complete the viral life cycle efficiently (Barre-Sinoussi, 1996).

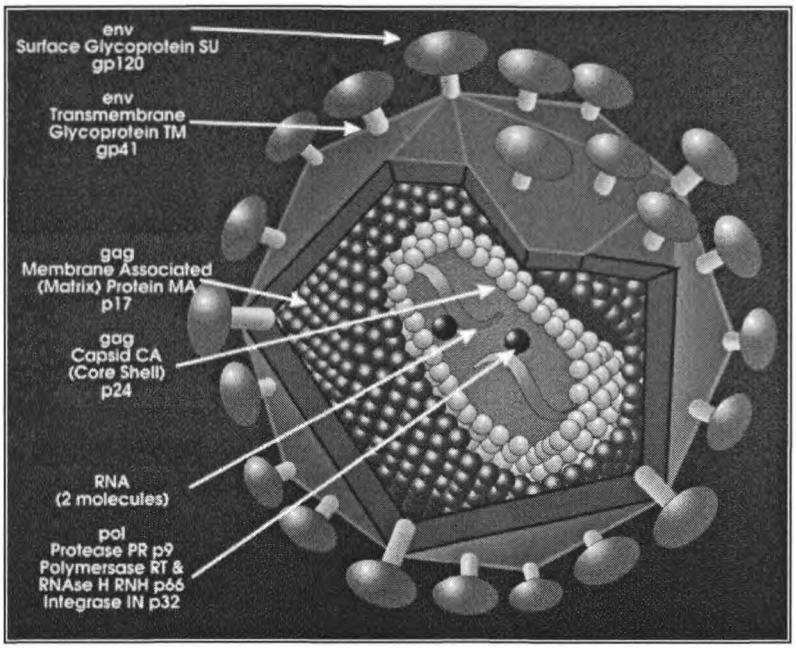

Fig 1.3: Structure of mature HIV-1 virion (*Reprinted from the ARC AIDS library website*)

1.3.2 Genomic structure of HIV

The HIV-1 genome is approximately 10 kb in length and has open reading frames coding for several viral proteins (see Figure 1.4). The genome consists of three structural genes: *gag*, *pol* and *env* as well as six regulatory genes: *tat*, *rev*, *nef*, *vpr*, *vpu* (*vpx* in HIV-2) and *vif*. The proteins encoded by these accessory genes regulate

transcription and translation of viral proteins, and enhance assembly and release of particles from infected cells (Connor *et al.*, 1997).

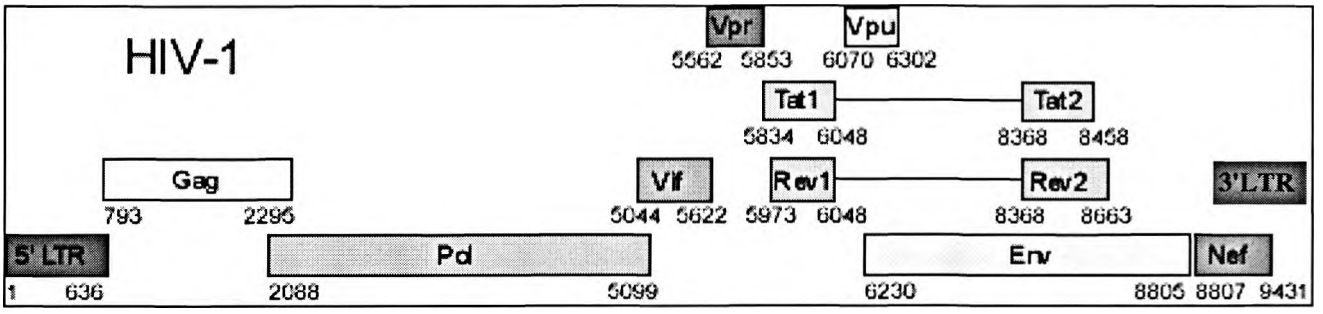

Fig 1.4 Genomic organisation of HIV-1 *(From Los Alamos Database)*

1.3.3 HIV-1 Life Cycle

Human CD4+ T lymphocytes, macrophages, dendritic cells and monocytes are the major cellular targets for HIV-1 *in vivo* (Fauci, 1988). The HIV-1 gp 120 envelope protein binds to CD4 with high affinity when HIV-1 infects T-lymphocytes as well as macrophages. For viral fusion and entry into host cell, HIV-1 also requires a co-receptor on the cell surface (Vodicka *et al.*, 1997). HIV-1 strains that infect macrophages (M-tropic) utilise CCR5 as a co-receptor and are designated R5 viruses, and those that infect T lymphocytes (T-tropic) utilise CXCR4 and are termed X4 viruses (Berger, 1998a); (Vodicka *et al.*, 1997); (Scarlatti *et al.*, 1997). Viruses that are able to utilise both co-receptors are termed R5/X4 variants (Berger, 1998b). CCR5 and CXCR4 are chemokine receptors expressed on the surface of human cells. CCR5 is expressed on lymphocytes, macrophages and brain cells, while CXCR4 is expressed on monocytes and T lymphocytes (Signoret *et al.*, 1997).

Receptor bound HIV-1 virions are internalised inside the cell by either classic receptor-mediated endocytosis (Maddon *et al.*, 1986) or virus-mediated membrane fusion (Stein *et al.*, 1987). Recent studies have shown that membrane fusion is the dominant mechanism for virion entry (Bedinger *et al.*, 1988). The membrane fusion involves the gp 41envelope protein (Veronese *et al.*, 1985), which is noncovalently associated with gp 120. gp 41 mediates fusion of the viral and host cell membranes leading to internalisation. After entry into the cell, the virion is uncoated and generation of a first-strand DNA copy of the viral RNA is mediated by reverse transcriptase (enzymatic product of *pol* gene). When complete, the reverse transcription results in a double-stranded DNA replica of the viral RNA genome. After reverse transcription, the viral double-stranded DNA is translocated to the nucleus where it is inserted into the host genome by the viral integrase (another product of *pol* gene). The integrated viral DNA is termed proviral DNA. After integration into the host genome, HIV-1 establishes a latent or persistent form of infection.

1.3.3.1 Early expression of HIV-1 regulatory genes

Activation of the proviral DNA is induced by binding of host transcriptional factors to sites in the HIV-1 long terminal repeat (LTR). This binding of host transcriptional factors stimulates a low but important level of expression of HIV-1 regulatory genes. Regulatory genes expressed early in the course of infection include *tat*, *nef*, and *vpr* (Kim *et al.*, 1996). Tat protein activates the transcription of all other HIV-1 genes (Laspia, Rice, and Mathews, 1989). Another regulatory gene product, Nef, is expressed in high proportions and constitutes about 75% of all viral mRNA during early infection (Schwartz *et al.*, 1996). The effects of this early expression of Nef are

discussed in section 1.5 of this chapter. The precise functions of the other regulatory gene product Vpr, are not clearly understood, but recent studies have suggested that Vpr functions as a weak transcriptional activator (Cohen *et al.*, 1990), capable of a moderate stimulation of HIV-1 LTR.

1.3.3.2 Late expression of structural genes

As described above, the early phase of expression of HIV-1 genes is characterised by expression of regulatory proteins. For generation and assembly of infectious HIV-1 virions, however, the viral structural and enzymatic proteins must be produced. The transition from expression of early regulatory genes to late structural and enzymatic genes appears to be regulated by the Rev protein. The Rev protein exerts this regulatory activity at the post-transcriptional level (Rimsky *et al.*, 1988). It activates the cytoplasmic expression of unspliced and singly spliced forms of HIV-1 RNA that encode the products of the *gag*, *pol*, and *env* genes (Malim *et al.*, 1988).

The products of the HIV-1 *gag* and *pol* genes form the core of the mature HIV-1 virion, whereas products of the *env* gene are the principal exterior coat proteins. These coat proteins are synthesised as a gp160 precursor that is subsequently cleaved by a cellular protease to yield gp 120 and gp 41. Similarly, the HIV-1 Gag proteins are derived from a 53-kDa protein that is specifically cleaved by the HIV-1-derived protease yielding the p24, p17, p9 and p7 Gag proteins. The *pol* gene product is responsible for production of several important viral enzymes, including reverse transcriptase, integrase, ribonuclease, and an aspartyl protease (Jacks *et al.*, 1988). Other HIV-1 genes expressed late in infection include *vif* and *vpu*. The functions of these two gene products is not clearly understood, but it is thought that Vpu protein

promotes efficient release of the budding virions from the surface of the cell (Klimkait *et al.*, 1990). Vpu protein is also thought to be necessary for full infectivity of the released HIV-1 virions (Sodroski *et al.*, 1986); (Strebel *et al.*, 1987).

1.4 Host Factors in HIV Infection

1.4.2 MHC Class I Complex

Major histocompatability complex (MHC) or human leukocyte antigen (HLA in humans) regulate the immune response to foreign antigens. Antigen presenting cells (APC), such as monocytes, macrophages, B cells and dendritic cells, carry high concentrations of highly polymorphic MHC class II genes encoding gene products that present antigenic peptides to the T cell receptor on CD4+ cells, which in turn augment production of specific antibodies and CTL. Additionally, all nucleated somatic cells, express MHC class I genes that encode molecules that present endogenously processed host- or virally- derived antigenic peptides to cytotoxic CD8+ cells, which can then attack and kill the infected cells.

In MHC class I presentation, translated viral proteins are processed in the cytosol by proteosomes into short peptides. These short peptides are then transported across the endoplasmic reticulum by products of transporter for antigen presentation (TAP) genes (which are also part of the MHC), and are assembled with class I heavy and light chains for presentation on the cell surface (Townsend *et al.*, 1989); (Williams and Cloyd, 1991). These presented epitopes can be recognised by CD8+ CTL resulting in killing of the infected cell (discussed in section 1.4.3). The presentation of epitopes by MHC class I is considered to be important in determining the outcome of HIV-1 infection (Lederman, 1995); (Roos *et al.*, 1992); (Zhu *et al.*, 1993).

Many studies have showed that there is a direct influence of MHC class I on HIV-1 disease progression. It has been shown that haemophilic siblings, sharing one or both MHC class I haplotypes, showed a concordant risk to AIDS (Goulder *et al.*, 1997); (Mackewicz and Levy, 1992), and there has subsequently been attempts to find specific alleles that are associated with relatively favourable or unfavourable disease outcomes (Whetsell *et al.*, 1992); (Harrowe and Cheng-Mayer, 1995). Several MHC class I alleles have been associated with rapid progression to AIDS. Kaslow *et al.* (1996) have shown that MHC class I alleles such as B27 and B57 are associated with slow progression to disease, while others like B37, B35 and B49 are associated with faster progression. It was also shown that the rate of disease progression is to a great degree determined by combinations of different MHC class I haplotypes (Kaslow *et al.*, 1996), reviewed by Hill and Littman (1996). It has been demonstrated that some HLA supertypes, such as A2/6802, are associated with protection against HIV-1 infection in sex-workers (MacDonald *et al.*, 2001). Knowing which supertypes are associated with resistance or slow progression may be important for vaccine design as more than one allele would present the same epitope.

1.4.3 T Helper cells

T helper (CD4+) responses are important for viral control during the chronic phase of HIV-1 infection. CD4+ T cell depletion is the most obvious marker of HIV-1-induced immunosuppression (Lane and Fauci, 1985); (Clerici *et al.*, 1989). In the past, studies of HIV-infected humans have indicated that T helper cells are critical in maintenance of effective CTL control (Zajac *et al.*, 1998). CD4+ lymphocytes "help" CTL function by recognising viral peptides on antigen presenting cells (APC). The

peptides recognised by CD4+ T cells are processed through the exogenous pathway by APC (such as dendritic cells, macrophages and B cells) expressing MHC class II molecules. After recognition of antigen restricted by MHC class II molecules, CD4+ T cells become activated and differentiate into functional subsets called T helper 1 (T_H1) and T helper 2 (T_H2) cells. These two subsets are differentiated by the types of cytokines they produce (Seder and Hill, 2000). The signatory cytokine produced by T_H1 is IFNγ while that of T_H2 is IL-4. T_H1 cells, through IFNγ production, mediate killing of organisms responsible for a variety of intracellular infections (such as HIV-1). The induction of T_H1 is also dependant on another cytokine, IL-12, which is produced by APC after exposure to HIV-1. Therefore, in response to HIV-1 (and other intracellular infections), IL-12 is the inducer cytokine of T_H1 cells, and IFNγ is the effector cytokine.

1.4.4 Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes specific against HIV-1 are considered to play a crucial role in the immune response against HIV- 1 infection (Borrow *et al.*, 1994); (Brander and Walker, 1999); (Kalams *et al.*, 1999). During untreated acute HIV-1 infection, the increase in virus-specific CTL activity is associated with the initial decrease in viraemia (Borrow *et al.*, 1997); (Koup *et al.*, 1994). The high levels of HIV-specific CTL are also detectable during the long asymptomatic phase of infection (Walker *et al.*, 1987); (Carmichael *et al.*, 1993). It is thought that during this stage, there is an equilibrium between replicating virus and responses mounted by CTL (Klein *et al.*, 1995). With progression to disease, there is a rapid increase in viraemia, associated with a decrease in CD4+ T cells as well as in CD8+ T cells (Fauci, 1996). Figure 1.5 shows the disease course of AIDS with relation to viraemia and CD4+ T cell count.

The progression to disease is characterised by a marked increase in viraemia and a significant decrease in CD4+ T cells. With decreased CD4+ cells, there is not sufficient help for the immune system to control the virus, allowing for opportunistic infections to occur.

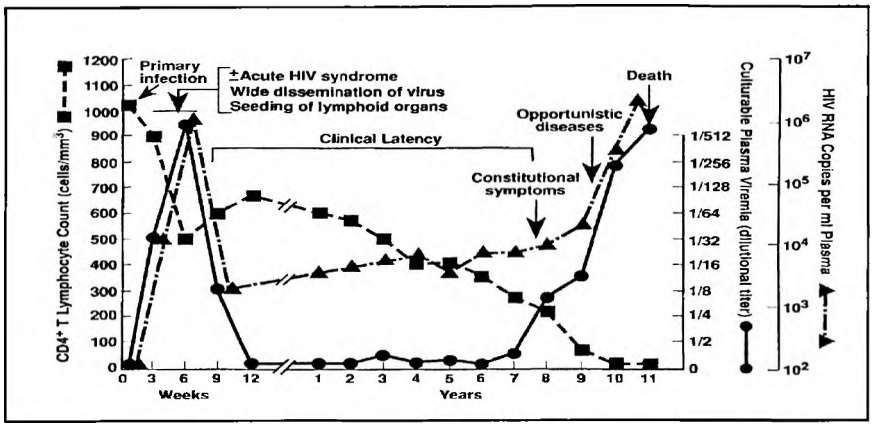

Fig 1.5: Disease course of HIV-1 infection. The figure illustrates the CD4+ cell count and viral load in three distinct phases of disease progression; acute phase, asymptomatic phase (clinical latency) and AIDS which is associated with marked increase in viral load, severe depletion of CD4+ cells and onset of opportunistic infections.

CTL detect infected cells through their T cell receptors and CD8+ molecules which recognise foreign molecules in the antigen binding grooves of MHC class I proteins (Elliott *et al.*, 1994). CTL are able to lyse cells expressing foreign antigens by secretion of granzymes and perforin. In addition to the traditional cytolytic response, activated anti-HIV CTL secrete soluble substances such as the antiviral cytokines IFNγ (Wong *et al.*, 1991) and TNFα (Mestan *et al.*, 1986) which can effectively inhibit infection by HIV (Mestan *et al.*, 1986); (Yang *et al.*, 1996); (Wagner *et al.*, 1998).

1.5 Viral Factors In HIV Infection: Nef

1.5.1 Role of Nef in HIV infection

Three HIV-1 genes *tat*, *rev* and *nef*, are transcribed very early in viral infection as multiply spliced mRNAs, but the Nef protein is expressed at a much higher level than both Tat and Rev (discussed in section 1.3.3.1). The *nef* gene of HIV-1 encodes a protein of approximately 27 kDa, which is covalently modified with the amino terminal myristic acid from multiply spliced mRNA transcripts (Allan *et al.*, 1985); (Guy *et al.*, 1987).

Nef has been shown to be an important determinant of viral pathogenecity in humans and monkeys, and is responsible for high titre viral replication *in vivo* (Kestler *et al.*, 1991). This was shown by observations that Nef-deleted variant of SIV lead to low viral loads and AIDS does not subsequently develop in these monkeys. Moreover, certain long-term survivors of HIV infection showed a lack of disease progression, which has been associated with deletions within the *nef* gene (Deacon *et al.*, 1995); (Kestler and Jeang, 1995) or a defective *nef* (Mariani *et al.*, 1996). The Nef protein is highly conserved in primate lentiviruses and forms homomeric oligomers and intramolecular disulphide bonds (Guy *et al.*, 1987). It has been documented that in primary lymphocytes and macrophages, Nef promotes HIV replication (Miller *et al.*, 1994) and stimulates HIV proviral synthesis (Aiken and Trono, 1995).

1.5.2 Structural Features of Nef

Nef is a phosphorylated and myristolated protein of about 206 amino acids. It associates with cellular membranes via the myristic acid by attaching to the CD4 cytoplasmic tail (Niederman *et al.*, 1993a). The soluble structure of Nef has been

determined by nuclear magnetic resonance (NMR) (Grzesiek *et al.*, 1996), and was shown to consist of a type II polyproline helix (amino acids 70-77) which represents the main binding site for the *src* family kinases. This domain is followed by two alpha helices, a four-stranded anti-parallel beta-sheet (amino acids 121-186), and two additional alpha helices (amino acids 187-203). Nef also possesses a proline-rich (PxxP) region that is able to associate *in vitro* with tyrosine kinases Hck and Lyn, presumably as an SH3-binding site (Saksela, Cheng, and Baltimore, 1995).

1.5.3 Functions of Nef in HIV Virulence

1.5.3.1 CD4 downregulation

Nef-induced CD4 downregulation is the most well understood function of Nef in HIV-1 pathogenesis. CD4 is a 55 kDa cell surface glycoprotein and a component of the T cell receptor on MHC class II-restricted T cell (helper T lymphocytes) (Parnes, 1989). It also serves as the cellular receptor for HIV and SIV (Klatzmann *et al.*, 1984); (Littman, 1996). The CD4 molecule is physically associated with the lymphocyte-specific protein tyrosine kinase $p56^{lck}$ (Lck), an essential participant for antigen-specific signal transduction and thymic maturation of T cells. Several studies, using PMA-induced internalisation of CD4 (Hurley and Sefton, 1989), demonstrated that dissociation of the CD4 molecule from Lck occurred prior to rapid internalisation of CD4. In resting lymphocytes, endocytosis of surface CD4 is very slow, whereas in nonlymphoid cells, which do not express Lck, it occurs much faster at rates of 2-3% of the cell surface pool per minute and is quickly recycled back to the cell surface (Marsh, Armes, and Pelchen-Matthews, 1990); (Pelchen-Matthews *et al.*, 1991). These findings suggest that the association of Lck to CD4 molecules on the cell surface prevents them from being internalised.

CD4 contains four hydrophobic amino acids and a dileucine motif in the membrane proximal region of its cytoplasmic tail that are necessary for its endocytosis (Aiken *et al.*, 1994); (Salghetti, Mariani, and Skowronski, 1995). Nef itself contains a dileucine motif necessary for binding to the adapter complexes. Nef was shown to displace Lck from CD4 and thus induce internalisation of CD4 by directly binding to Lck (Saksela, Cheng, and Baltimore, 1995); (Collette *et al.*, 1996). Recently, it has been suggested that Nef does not only induce internalisation of CD4, but also directs the internalised CD4 molecules to lysosomes for degradation, as undegraded CD4 molecules will be recycled back to the cell membrane. It was later demonstrated that Nef recruits AP-2 via the AP-μ chain (Greenberg *et al.*, 1997); (Piguet *et al.*, 1998). The AP-2 adapter complex is responsible for recruiting membrane proteins to the coated pits of the endoplasmic reticulum for their endocytosis.

The significance of Nef-induced CD4 downregulation is thought to be important for the virus in three ways. Firstly, by removing CD4 on the cell surface, Nef prevents superinfection of an infected cell, which could kill the cell before production of free virions. This would significantly reduce the spread of the infection. Secondly, Nef mediated downmodulation of CD4 alters T cell activation. This is because increased levels of free Lck (displaced from CD4 by Nef) could promote T cell activation, thereby creating a milieu favourable for viral gene expression, and thus virion production. This will aid in spreading the virus. Thirdly, it has been shown that high levels of surface CD4 molecules could block the release of newly formed virions from the infected cell (Ross, Oran, and Cullen, 1999), again, hindering the spread of the virus.

1.5.3.2 MHC class I downregulation

Nef-induced downregulation of MHC class I is less well understood than the downmodulation of CD4. Le Gall and colleagues (1998) demonstrated that a tyrosine found in the cytoplasmic tails of HLA-A and HLA-B but not HLA-C alleles, is critical for response to Nef downregulation. In the presence of Nef, MHC class I molecules were shown to be internalised more rapidly and transported to the trans Golgi apparatus (Greenberg *et al.*, 1997); (Le Gall *et al.*, 1998). Internalised MHC class I complexes were shown to accumulate in endosomal vesicle, where they are degraded (Schwartz *et al.*, 1996). Although Nef mediated downregulation of MHC class I is not well understood, it is thought to be dependant on an intact SH3 binding motif and a cluster of acidic amino acids within the Nef protein. Mutations in these SH3 and acidic motifs in Nef abolish rapid internalisation of MHC class I molecules (Greenberg *et al.*, 1997). Direct interaction between Nef and the MHC class I complex has not been shown and it is now speculated that Nef might result in alterations in signal transduction pathways, particularly via the *src* kinase family members, resulting in phosphorylation of a critical tyrosine in MHC class I molecules. This in turn could lead to an enhanced endocytosis of the MHC complex (Greenberg *et al.*, 1997).

The biological significance of Nef-mediated MHC downregulation is that it allows HIV-infected cells to evade attack by CTL (Collins *et al.*, 1998). An important feature of Nef mediated MHC class I downregulation, is that Nef does not induce HLA-C downregulation. The significance of this is that it would allow infected cells to escape natural killer (NK) cells which would attack a cell that is completely devoid of surface MHC class I molecules (Cohen, 1999). Evasion of the immune system

through this manner is crucial in the course to HIV disease (reviewed by McMichael, 1998).

1.5.3.3 Nef induces alterations in common signal transduction pathways

Nef has some amino acid similarity to the family of GTP-binding proteins. Since GTPases are involved in intracellular signal transduction processes, it has been speculated that Nef might be able to interfere with these pathways (Guy *et al.*, 1987) and thereby alter cell function. It has been demonstrated that Nef inhibits induction of the platelet-derived growth factor (PDGF) and bombesin receptor-mediated proliferation pathways (De and Marsh, 1994). These two receptors activate phospholipase Cγ1 and Cβ1, respectively, through separate pathways (reviewed by Berridge (1993). Both phospholipases then hydrolyse phophatidylinositol to yield diacyglycerol (DAG) and inositol triphosphate (IP_3). IP_3 binds to a specific receptor on the endoplasmic reticulum and induces the release of stored calcium ions from vesicle pools via Ca^{2+} ATPase, thereby stimulating cell growth and proliferation. In NIH3T3 cells transfected with Nef, the growth factor-stimulated calcium release is blocked (Graziani *et al.*, 1996). In contrast, it was shown that Nef can enhance calcium release (Skowronski, Parks, and Mariani, 1993) and IL-2 synthesis in murine T cells (Rhee and Marsh, 1994), which would stimulate cell growth and proliferation. These conflicting findings suggest that Nef can alter biochemical pathways both negatively and positively. The general consensus is that Nef activity in *in vivo* viral infection does not function as a suppressor, as originally thought, but has cellular activating properties. Induction of cell growth and proliferation is beneficial for the virus as it encourages virion production.

1.6 Combined Effects of Nef and Host Factors

Most of the characterisation of Nef has been performed on subtype B isolates. Not much is known about the role of Nef in subtype C HIV-1 infection nor about how subtype C HIV-1-infected individuals immunologically target Nef. In this project, Nef has been characterised from subtype C HIV-1 infected individuals was characterised to determine sequence conservancy and variation and to identify regions that are targeted by cellular immunity. The rationale for analysing Nef is that since it is expressed early in large quantities, T cell epitopes within Nef would be targeted early before new virions are produced. This has important implications in development of an effective HIV-1 vaccine. It is hypothesised that Nef contains several highly conserved domains which would contain CTL epitopes and thereby demonstrate cross-clade reactivity.

1.7 Specific Objectives of This Project

This project deals with virological and immunological characterisation of HIV-1 subtype C Nef. Most studies relating to Nef have been performed on subtype B. This work is the first study to analyse subtype C Nef from South Africa. The specific objectives of this study are:

1. To develop and optimise PCR conditions for detection of subtype C *nef*.
2. To sequence *nef* genes from different subtype C HIV-1 infected cohorts to identify regions within subtype C Nef that are well conserved.
3. To align predicted protein regions from subtype C Nef and identify areas that are conserved and variable.

4. To identify regions within subtype C Nef that are targeted by subtype C HIV-1 infected individuals using the ELISPOT assay.

CHAPTER 2: PATIENT COHORTS AND SUBTYPING OF HIV-1

2.1 INTRODUCTION

This project used established cohorts of HIV-1 infected individuals. Three cohorts representing different stages of disease progression were analysed. The first cohort consisted of recently-infected individuals who were recruited in a microbicidal trial (col 1492). The second cohort consisted of chronically-infected individuals before recruitment into drug trials at the Perinatal HIV Research Unit, Chris Hani-Baragwanath hospital. The third cohort consisted of AIDS patients who were recruited from Sizwe Tropical Diseases Hospital.

This chapter describes 1) the cohorts analysed in this project, 2) analysis of HIV-1 from a multiply-exposed cohort to subtype virus and measure the incidence of recombination between *gag* and *env* genes.

It has been well documented that subtype C is the predominant circulating HIV-1 strain in South Africa (Williamson *et al*, 1995), (Van Harmelen, 1999). However, because of the presence of other subtypes besides C, albeit in low frequencies (Bredell *et al.*, 2000), it is possible that recombination can occur between viruses belonging to different subtypes (for example C/B recombinants). Inter-subtype recombination can result when the host cell is infected with two different viruses (from different subtypes), either sequentially (super-infection) or simultaneously (co-infection). Subtyping was performed on viral isolates derived from commercial sex-workers, most of whom were recently-infected with HIV-1. Samples were subtyped using the heteroduplex mobility assay (HMA) which has been shown to be a quick and effective method of subtyping HIV-1 (Delwart *et al.*, 1993). In an HMA reaction, there is

amplification of various known subtypes by PCR (reference strains) as well as unknown samples. Reference strains and sample DNA are denatured by heating and reannealled by cooling to allow for formation of heteroduplexes and homoduplexes. Heteroduplexes occur when a strand of the reference anneals to a strand of the sample. If there is a high degree of variation between the strand and the sample (when each belongs to a different subtype) there will be mismatches which will retard the migration of the heteroduplex in a polyacrylamide gel. If the two strains are more related (belonging to the same subtype), there will be less mismatches and the heteroduplex will migrate further down the gel. HIV-1 strains can be subtyped in this manner

As individuals in this cohort were all sex-workers and multiply exposed to HIV-1 (see section 2.2.1), the incidence of recombinant viruses would likely reflect the maximum prevalence of recombination events between *env* and *gag* found in circulating subtype C viruses in the heart of the South African epidemic. If recombination is not found within in this cohort, it is unlikely that it will be found in other cohorts where subtype C is predominantly transmitted.

2.2 Cohorts

2.2.1 Recently-Infected Individuals (Du)

This cohort consisted of individuals with recent infection of HIV-1 (spanning 1.6-12 months after seroconversion). The cohort consisted of women sex workers who work along the truck stops between Johannesburg and Durban. The majority of these patients were asymptomatic with varying viral loads, ranging from 161 to >500 000 copies/ml, with a median of 15 556 copies/ml (Table 2.1). This cohort was designated

as Du and was chosen for subtyping by HMA in *env* and *gag* to assess for the prevalence of recombination.

Table 2.1 Viral loads, CD4 counts and clinical staging of recently HIV-1 infected individuals (Du cohort)

Patient ID	Time from Sero-conversion (months)	Viral Load (copies/ml)	CD4 Count (cells/μl)	CDC staging
Du 151	2.1	>500 000	217	A2
Du 156	0.8	22 122	372	A2
Du 281	4.7	24 689	346	A2
Du 467	3.2	6 658	750	A1
Du 115	3.9	7 597	782	A1
Du 123	3.4	19 331	432	A2
Du 179	16.1	1 359	368	A2
Du 258	15.8	9 114	508	A1
Du 285	2.1	161	559	A1
Du 368	8.3	13 993	628	A1
Du 422	2.0	17 118	397	A2
Du 457	3.5	19 268	524	A1
Median	3.5	15 556	470	
25% IQR	2.1	7 128	351	
75%	5.6	20 727	611	

2.2.2 Chronically-infected HIV Patients (B)

The second cohort consisted of patients from Chris Hani-Baragwanath hospital, Johannesburg, and consisted of patients chronically infected with HIV-1. Viral loads ranged from 579 to > 500 000 copies/ml with a median viral load of 99341 copies/ml (see Table 2.2). Due to low cell material available from this cohort, CD4 counts were not determined (see Table 2.2).

Table 2.2 Viral loads and clinical staging of chronically infected patients (B)

Patient ID	Viral Load (copies/ml)	CD4 Count (cells/μl)	CDC staging
B296	1 090	ND	–
B304	17 717	ND	–
B383	17 936	ND	–
B384	37 607	ND	–
B299		ND	–
B399	56 670	ND	–
B400	16 452	ND	–
B401	116 852	ND	–
B402	37 410	ND	–
Median	27 673		
25% IQR 75%	17 084 47 138		

ND – Not determined
As CD4 counts were not determined in these individuals, CDC staging could not be achieved.

2.2.3 AIDS patients (Sw)

The third cohort consisted of individuals with late stage infection. These individuals had progressed to AIDS and had an active episode of pulmonary *Mycobacterium* tuberculosis. The patients were recruited from Sizwe Hospital for Tropical Diseases, Johannesburg and had a median viral load of 327719 copies/ml.

Table 2.3 Viral loads, CD4 counts and clinical staging of AIDS patients (Sw)

Patient ID	Viral Load (copies/ml)	CD4 Count (cells/ml)	CDC staging
Sw 2	339 478	84	C3
Sw 5	274 847	40	C3
Sw 7	290 440	10	C3
Sw 8	>500 000	67	C3
Sw 9	301 605	65	C3
Sw 10	353 833	89	C3
Sw 15	>500 000	5	C3
Sw 20	43 595	2	C3
Sw 23	>500 000	10	C3
Cot 2	ND	ND	_
Cot 6	ND	ND	_
Median	327 719	25	
25%	286 542	9	
IQR			
75%	500 000	71	

Figure 2.1 shows a summary of the median differences of $\log_{10}$ viral loads between the three cohorts studied in this project. It can be seen that the median viral load of the AIDS (Sw) cohort was much higher than that of the recently-infected cohort and the chronically-infected cohort.

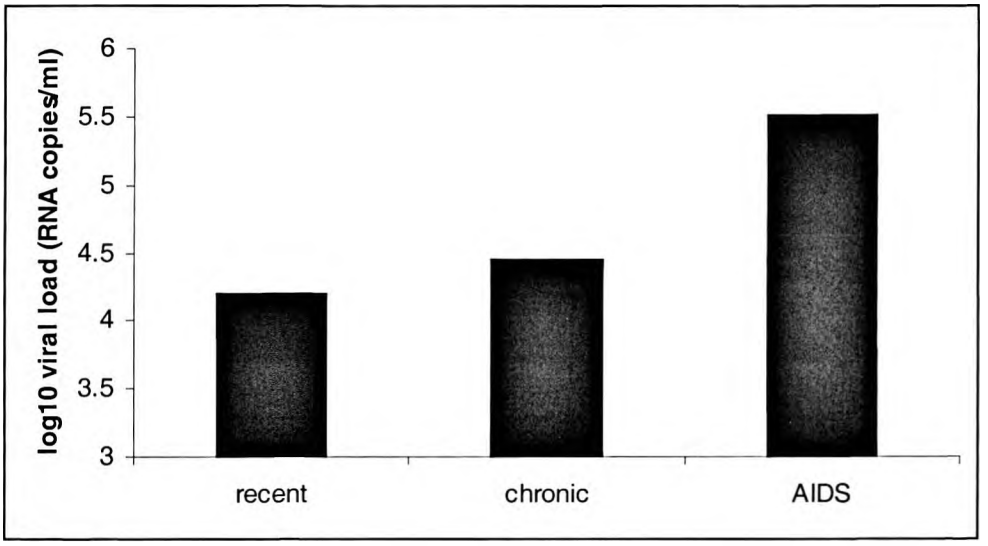

Fig 2.1: Summary of $\log_{10}$ viral loads between cohorts

The overall aim of this chapter is to describe from where patient material was obtained and how viral isolates from a selected cohort were characterised by HMA.

2.3 MATERIALS AND METHODS

2.3.1 Isolation of Plasma and PBMC from whole blood

Plasma was isolated from whole blood (anticoaggulated in Acid Citrate Dextrose) by centrifugation at 1200 rpm for 10 min. Plasma was aspirated from the top layer and immediately stored at -70°C. The remaining blood was diluted with 1%FCS-RPMI to 50ml. The solution was divided into two and layered onto 15ml Ficoll-Hypaque density gradient. The tubes were centrifuged at 2000 rpm for 30 min without applying the centrifuge brake. The lymphocyte layer was aspirated from the interface between Ficoll and diluted plasma and washed twice with 1%FCS-RPMI. Cells were counted on a haemocytometer using Trypan blue and cryopreserved in liquid nitrogen.

2.3.2 Isolation of HIV-1 RNA and DNA

2.3.2.1 Isolation of viral RNA from plasma and co-culture supernates

Fresh or frozen samples were used to isolate viral RNA. The QIAamp viral RNA Mini Spin (QIAGEN) was used to isolate viral RNA from plasma and viral co-culture. Firstly, 560 µl of prepared Buffer AVL containing Carrier RNA was pipetted into a 1.5 ml microcentrifuge tube. Plasma or culture supernate (140 µl) was added to the Buffer AVL and mixed by pulse vortexing for 15 seconds. The mixture was then incubated at room temperature for 10 min. After incubation, 560 µl of ethanol (96-100%) was added to the sample. A portion of this preparation (630 µl) was centrifuged at 6000 g for 1 min in a spin column in a 2 ml collection tube. As the maximum volume of the spin column was 650 µl, this was repeated with the remaining solution. Buffer AW1 (540 µl) was added to the spin column, in a clean collection tube, and centrifuged for 1 min at 6000 g. Buffer AW2 (500 µl) was then

added to the spin column and centrifuged at 20 000 g for 3 min. The spin column was placed in a sterile 1.5 ml microcentrifuge tube and 60 µl of elution Buffer AVE was added and incubated at room temperature for 1 min, followed by centrifuging at 6000 g for 1 min. The isolated viral RNA was either stored at -70°C or used immediately as template for RT-PCR

2.3.2.2 Isolation of proviral and total DNA from PBMC

Fresh or frozen samples were used to isolate proviral DNA from PBMC. Cryopreserved PBMC were thawed and 9 ml of 10% FCS-RPMI (final volume of 10 ml) added. The cell suspensions were centrifuged at 1000 rpm for 10 min and supernatant discarded. Pellets were washed twice by resuspending in 1 ml PBS and centrifuged at 1000 rpm for 10 min. The pellets were resuspended in 50 µl of lysing buffer (10mM Tris-HCl pH 8.3, 1mM EDTA, 0.5% Triton X-100, 0.001% SDS) and 3.75 µl Proteinase K (Roche) was added and the mixture incubated overnight at 56°C. After the overnight incubation, Proteinase K was inactivated by incubating the tubes at 95°C for 15 min. The isolated DNA was either stored at 4°C or used immediately as template for PCR.

2.3.3 PCR and RT-PCR

2.3.3.1 Env region PCR and RT-PCR

Nested PCR reactions were used to generate sufficient quantities of *env* DNA for heteroduplex mobility assay (HMA) subtyping. For analysis of HIV-1 *env* gene, the following primers were used: first round reactions, primer ED5 (5'-ATG GGA TCA AAG CCT AAA GCC ATG TG; positions 6556-6591) and primer ED12 (5'-GTG CTT CCT GCT GCT CCC AAG AAC CCA AG; positions 7822-7792) were used.

The second round primers were ES7 (5'-CTG TTA AAT GGC AGT CTA GC; positions 7001-7020) and ES8 (5'-CAC TTC TCC AAT TGT CCC TCA; positions 7359-7380). The PCR reaction mixtures contained 10 pmol primers, 0.625 U of Supertherm Taq polymerase with appropriate 10 X buffer, 0.08 mM dNTPs, 1.5 mM $MgCl_2$ and 5 µl input of DNA/RNA template (depending on whether the source of the template was from PBMC or plasma/culture supernates) to make a final volume of 50 µl. For RT-PCR, the reaction contained 5 U of AMV-RT and 10 U RNAsin with appropriate 10 X buffer. The thermal cycling conditions used were an initial 42°C for 60 min for the reverse transcriptase stage, followed by 3 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min followed by 30 cycles of 94°C for 90 sec, 55°C for 45 sec, 72°C for 90 sec, with a final extension step at 72°C for 5 min. The second round reaction (100 µl) was initiated with 5 µl of the first round reaction as the template. The thermal cycling conditions for the second round were similar to those of the first round, except that the 42°C RT stage was omitted and the primer concentrations were increased to 20 pmol. Seven reference (kindly provided by Helba Bredell, National Institute for Virology) isolates were used in the *env* HMA. These included a subtype A reference isolate, two subtype B (b_1 and b_2), two subtype C (c_1 and c_2), a D and an E isolate. The env PCR products would result in a 647bp fragment. Reference products were amplified as described.

2.3.3.2 Gag region PCR and RT-PCR

Nested PCR reactions were performed to generate sufficient *gag* DNA for HMA subtyping. The first round primers used were MSF12 (5'-AAA TCT CTA GCA GTG GCG CCC GAA CAG) and SK431 (5'-CCT GCT ATG TCA CTT CCC CTT GGT TCT C). The second round primers were LTR236 (5'-CGC AGG ACT CGG CTT

GC) and Gag778 (5'-CCC ATG CGT TCG TTC TAG GTG). The PCR reaction mixtures for the first round contained 10 pmol primers, 0.08 mM dNTPs, 2.8 mM $MgCl_2$ and 0.625 U of Supertherm Taq polymerase with the appropriate 10X buffer. The RT-PCR reactions contained 5 U of RNAse and 10 U of RNAsin. The thermal cycling conditions for the first round were 42°C for 60 min, 3 cycles of 94°C for 2 min, 45°C for 2 min, 72°C for 4 min, followed by 30 cycles of 94°C for 45 seconds, 45°C for 1 min, 72°C for 90 seconds, with a final extension step of 72°C for 7 min. The second round thermal cycling conditions were similar to those of the first round except omitting the initial RT stage of 42°C for 60 min and the primer concentrations were increased to 20 pmol. Five reference isolates (Helba Bredell, NIV) were used in the gag HMA, including subtype A, B, two C (c_1 and c_2) and D. Gag products were amplified in the manner described. The gag PCR product would result in a 550bp fragment.

2.3.3.3 Product electrophoresis

Amplified products were analysed on a 1% agarose (D-1 LE Agarose) gel containing 0.5mg/ml ethidium bromide. Ten microlitres of amplified PCR product were mixed with 2 µl of Bromophenol blue and Xylenexynol loading dye and electrophoresed at 100 V for 45 min in TBE buffer. DNA was detected by ultraviolet illumination and pictorial records were taken using the GelDoc system.

2.3.4 Heteroduplex Mobility Assay

Seven and five PCR products of known subtypes were prepared as references for the *env* and *gag* genes respectively. Products for each *env* and *gag* genes were generated as described in sections 2.3.3.1 and 2.3.3.2 respectively. For each HMA reaction, 7.5

µl of the unknown isolate (PCR product) was added to 2.5 µl each known reference as well as 1.1 µl of 10X HMA annealing buffer. The tubes were then heated at 94°C for 2 min and immediately put on ice for 10 min. Samples were mixed with Bromophenol blue/xylenexynol loading dye and electrophoresed on a 5% polyacrylamide gel at 200 V for 6 hrs. The gels were stained with ethidium bromide and heteroduplexes visualised under ultraviolet illumination.

2.4 RESULTS

2.4.1 *env* and *gag* PCR and RT-PCR

As previously described, only samples from the recently-infected cohort were amplified in the *env* and *gag* regions for HMA. Both the *env* and *gag* PCR/RT-PCR resulted in products of good yield and high purity that was required for HMA. Figure 2.2a and 2.2b below show representative electophoretograms of *env* and *gag* PCR.

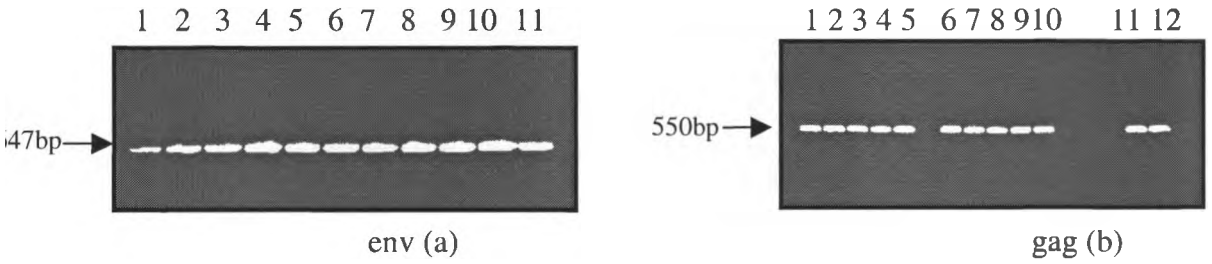

Fig 2.2 Electrophoretograms depicting *env* (b) products (lanes 1-11) and *gag* (b) products (lanes 1-12)

2.4.2 Heteroduplex mobility assay

HMA was performed using products shown in the above electrophoretograms. Figure 2.3a and 2.3b show representative *gag* and *env* HMA electrophoretograms respectively.

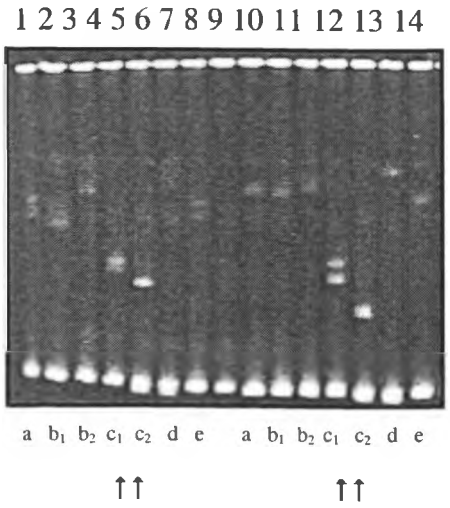

Fig 2.3a Electrophoretogram depicting env HMA results of two Du samples. The letters below show reference subtypes. Arrows show heteroduplexes that migrated the furthest and therefore were more homologous to reference sequences in those lanes.

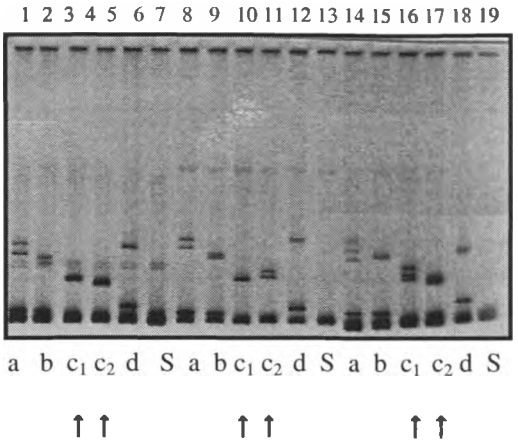

Fig 2.3b Electrophoretogram depicting gag HMA results of three Du isolates. The letters below show reference subtype. S denotes the isolate hybridised against itself.

Figure 2.3a shows that the heteroduplexes that migrated the furthest (shown by arrows) were in lanes representing references c_1 and c_2, showing that the envelope region of these two isolates was grouping with clade C reference isolates. Figure 2.3b shows the same scenario for the *gag* HMA, showing that the isolates were belonged to clade C *gag* gene. All samples from recent infection were subtyped in this manner.

All eleven Du isolates were found to be of clade C both in the *env* and *gag* regions (Table 2.4.) This data is in agreement with previously published data showing that subtype C is the predominant HIV-1 subtype in South Africa and recombination of different subtypes is very rare (Bredell *et al.*, 2000).

Table 2.4: HMA subtyping in gag and env regions from PCR products derived from the Du cohort

P.I.D	**Subtype in *env* region**	**Subtype in *gag* region**
Du 115	C	C
Du 123	C	C
Du 151	C	C
Du 156	C	C
Du 172	C	C
Du 179	C	C
Du 258	C	C
Du 281	C	C
Du 368	C	C
Du 422	C	C
Du 457	C	C

2.6 DISCUSSION

In this chapter three cohorts have been described as well as the genetic diversity of viral isolates from one cohort comprising of individuals with recent HIV-1 infection. Viral isolates from recently-infected sex-workers were chosen to examine the subtype of HIV-1 and the occurrence of gene recombination between *env* and *gag* genes in South Africa. Occurrence of recombinant events in the viral populations derived from multiply exposed individuals provides a maximum likelihood of detecting the prevalence of recombination. The data show that all viral isolates were subtype C and that recombination is a rare event. These data do not preclude the possibility that superinfection with more than one subtype C virus has occurred. In fact, there is evidence from Du 151 that there was a dual infection with a second subtype C virus about one year after seroconversion (Grobler, personal communication, University of Cape Town). Data from this chapter confirms previously published data showing that subtype C is the predominant subtype in South Africa (Williamson *et al.*, 1995); (Van Harmelen *et al.*, 1997), and although other subtypes (A, B and D) have been identified, inter-subtype recombination occurs rarely (Bredell *et al.*, 2000).

The remaining chapters of this thesis extend the genetic analysis to characterising subtype C *nef* gene and which regions of the Nef protein are targeted by the immune system. Chapter 3 provides details of the optimised conditions for detecting subtype C *nef* and for sequencing the full-length *nef* gene.

CHAPTER 3: DEVELOPMENT AND OPTIMIZATION OF NEF AMPLIFICATION FROM SUBTYPE C HIV-1

3.1 INTRODUCTION

The *nef* gene lies towards the 3' end of the HIV-1 genome, overlapping a portion of the 3' long terminal repeat (LTR) and the C-terminus of the *env* gene (Ratner *et al.*, 1985). Nef has been credited with several important functions, which are crucial for the pathogenesis of HIV-1 (described in detail in chapter 1, section 1.5). Many structural and functional motifs, as well as CTL epitopes have been identified within the Nef protein. These regions are also important for viral pathogenesis and it is likely that they are highly conserved. To genetically characterise subtype C *nef* in detail, optimal conditions have to be established that will enable successful PCR and subsequent sequencing.

Most of the research and understanding of the role of *nef* in HIV-1 pathogenesis has been performed on subtype B sequences isolated from North America and Europe. Although subtype C Nef sequences have been obtained from Ethiopia, Botswana and Brazil, there has been no analysis of subtype C *nef* derived from South African HIV-1 infected individuals. The previous chapter confirmed that selected viral isolates were subtype C and this chapter will extend the analysis to successful amplification of the *nef* gene.

The approach was to perform a nested PCR using three sets of primers resulting in two short fragments (540 bp and 441 bp), overlapping by 96 bases, and combined would cover the full *nef* gene. Figure 3.1 is a schematic diagram of the nested *nef* PCR that was designed for this project. The three sets of primers are shown as small

boxes, with arrows showing direction of amplification of each primer. Because newly designed primers were used, it was unlikely that the established *nef* PCR protocols (Ahmad *et al*, 1998) would effectively amplify products from subtype C HIV-1-infected South Africans. This chapter provides a detailed account of the procedure used to optimise PCR conditions for effective amplification of subtype C *nef*. The aim was to generate high yields of PCR product from plasma containing low viral RNA copy numbers.

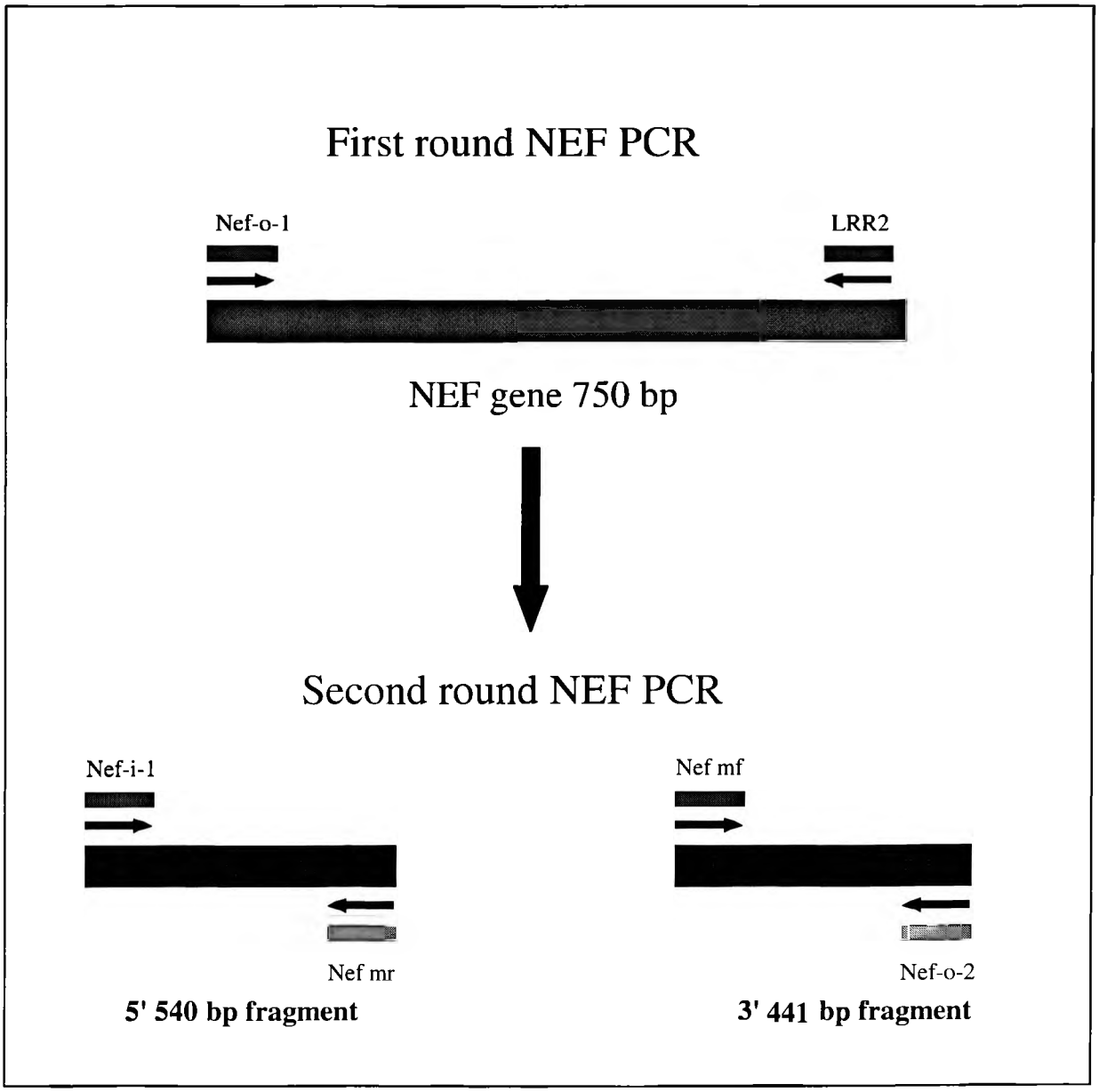

Fig 3.1: Schematic diagram showing the design of *nef* amplification. The names of primers used are shown next to the boxes representing PCR products.

3.2 MATERIALS AND METHOD

3.2.1 STANDARD NEF PCR

First round PCR reactions were performed using plasma, PBMC co-culture supernates or baseline PBMC. The first round reaction contained 10 pmol each of the primers Nef-O-1 (5' CGC TTG AGA GAC TTA CTC TTG ACT 3', position 7908-7931) and LRR2 (5' CCC TCA GAT GCT GCA TAT AAG C 3', 8897-8918). The reaction also contained 10mM dNTPs, 1.5 mM $MgCl_2$, as well as 0.625 U of Supertherm Taq polymerase, with the appropriate 10X buffer. Total DNA (5 µl) isolated from PBMC (section 2.3.2.1, chapter 2), was used to initiate the reaction. The thermal cycling conditions for the first round reaction were 94°C for 2 min, 50°C for 2 min and 72°C for 4 min, followed by 35 cycles of 94°C for 45 sec, 50°C for 1 min, 72°C for 1:30 min, with a final extension step at 72°C for 7 min. The second round PCR reaction (100 µl), was initiated with 5 µl of the first round reaction as the template using the following pairs of primers, nef-i-1 (5' AGT CC{AC} AGC {AG}GA AAG TC 3', 8094-8114), for the 5' fragment and nef-mr (5' CCA GTT GAC CCA AGT GAA GTG G 3', 8634-8656) as well as nef-i-2 (5' CAC T{GT}G GGC TTC CAG G 3', 8881-8902) and nef-mf (5' TAA GGC AGC ATT CGA TCT CAG C 3', 8438-8460) for the 3' fragment, curly brackets represent degenerate bases. Degenerate bases are for variable sequences, therefore, either one of the nucleotides can bind to the template. The thermal cycling conditions used in the second round PCR were similar to those used for the first round, except the primer concentrations were increased to 20 pmol.

3.2.2 NEF REVERSE TRANSCRIPTASE PCR

3.2.2.1 nef RT-PCR using AMV-RT

Reverse transcriptase PCR was performed on specimens with highest viral loads (Du 151 and Du 281, > 500 000 and 24 689 RNA copies/ml respectively). For the first round PCR, a 50µl reaction was set up containing 10pmol of each primer (Nef-0-1 and LRR2), 4mM dNTPs, 0.7 mM $MgCl_2$, 3.125 U AMV-RT (ROCHE), 8 U of RNAsin (ROCHE), 0.625 U Taq polymerase (with appropriate 10X buffer), and the reaction initiated with 5µl of isolated viral RNA (chapter 2 section 2.3.2.1). The reaction was incubated at 42°C for 60 min for reverse transcription, followed by thermal cycling conditions similar to those described in section 3.2.1. The PCR products were electrophoresed and bands visualised as described in chapter 2 section 2.3.3.3.

3.2.2.2 nef RT-PCR using Thermoscript RT

Thermoscript RT™ (GIBCO) was also used in *nef* RT-PCR. This is a heat-stable reverse transcriptase and would thus be more suitable for the high annealing temperatures in *nef* PCR. cDNA was generated as described in the manufacturer's protocol (see Appendix A). The kit allows for using gene-specific primer, random hexamers or oligodTs for cDNA synthesis. Briefly, 6µl isolated viral RNA was denatured at 65°C for 5 min in the presence of 20pmol outer reverse primer (LRR2) and 2 µl of DEPC-treated water, with a final volume of 10µl. After the denaturation step, tubes were placed on ice. In a separate microcentrifuge, the reverse transcriptase reaction (10µl) was prepared (see Appendix A). This reaction contained 15 U of Thermoscript RT, 40 U of RNAse inhibitor, 20mM dNTPs, 0.01mM DDT as well as 4µl of appropriate 5X cDNA buffer (provided with the RT) and 1µl of DEPC-treated

water. The reaction was mixed with the denatured viral RNA/primer mixture and incubated at 58°C for 50 min and stopped by heating the tube at 85°C for 5 min. One microlitre of RNAse H was added to each tube to degrade any secondary RNA molecules that might be inhibitory to the PCR reactions. The generated cDNA (2µl) was used as template for *nef* nested PCR, using the thermal cycling conditions described in section 3.2.1.

3.2.2.3 Modification of viral RNA isolation from plasma and co-culture supernates

The above RT-PCR reaction resulted in sufficient product only on specimens with extremely high viral loads (above 20 000 copies/ml), and not those with moderate to low viral loads (between 500 and 20 000 copies/ml). To increase the amount of the viral RNA isolated, a modification of the Viral RNA Isolation Kit (QIAgen) protocol (described in section 2.3.2.1) was made. The initial plasma/supernate volume for isolation was increased along with the corresponding buffers. Briefly, 2240 µl of prepared Buffer AVL containing Carrier RNA was pipetted into a sterile 1.5 ml microcentrifuge tube. Instead of 140 µl of plasma/supernate, 560 µl of plasma/supernate was added to the Buffer AVL and the solution mixed by pulse vortexing for 15 sec. The tube was incubated at room temperature (15-25°C) for 10 min. After incubation, 2240 µl of ethanol (96-100%) was added to the sample and again the solution mixed by pulse-vortexing for 15 sec. The rest of the methods followed were similar to those described in section 2.3.2.1 of chapter 2, resulting in viral RNA suspended in 60 µl of elution buffer. This resulted in a higher concentration of isolated viral RNA, which was used in Nef RT-PCR described in section 3.2.2.2.

3.2.3 Optimization Reactions

3.2.3.1 Annealing Temperature

The annealing temperature for the sets of primers used for *nef* sequencing was mathematically calculated to be 50°C (see Appendix A). This is not accurate and only gives an approximation based on the G-C ratio of each primer sequence. Experimental conditions were established to determine the correct annealing temperature for the primers. A temperature gradient from 42°C to 56°C, with 2°C changes, was set up on the RoboCycler (Stratagene) PCR machine. The other reagents ($MgCl_2$ and template concentration) and thermal cycling conditions were kept constant (similar to those described in section 3.2.1). The temperature that resulted in the highest yield of product would be optimal for the primers.

3.2.3.2 Magnesium chloride concentration

The magnesium chloride ($MgCl_2$) concentration used for PCR reactions described in section 3.2.1 was 1.5mM. This did not result in a high yield of Nef product. A $MgCl_2$ concentration titration was set up to determine the optimum concentration for Nef primers. A series of eight PCR reactions were set up with $MgCl_2$ concentrations from 1.5-5mM $MgCl_2$ (using 0.5mM concentration gradient). The thermal cycling conditions and other components of the reaction were kept constant and an optimum annealing temperature identified above (section 3.2.2.1) was used.

3.2.3.3 Nef PCR using two different PCR machines

To determine whether different sized tubes resulted in differences in heat distribution, and thus differences in amplification, Nef nested PCR was set up using two thermocyclers. The GeneAmp PCR system 2400 (Perkin-Elmer) and the RoboCycler

(Stratagene) PCR machines were used for comparisons in amount of PCR product formed. The GeneAmp PCR system uses 200µl microcentrifuge tubes while the RoboCycler uses 500µl microcentrifuge tubes. For both reactions, the same thermal cycling conditions were used. The optimised annealing temperature of 48°C (identified in the annealing temperature titration - section 3.2.3.1) and $MgCl_2$ concentration of 3mM (identified in section 3.2.3.2) were used. Amplification was performed as described in section 3.2.1 in the two PCR machines. This would give an indication as to whether Nef PCR was machine-dependent and if the reaction was optimal for a specific machine.

3.2.4 Sequencing

3.2.4.1 Automated Sequencing

The method used for sequencing was the dideoxyterminator (Sanger) sequencing from PCR products. Gel electrophoresis was used to separate labelled oligonucleotides that differ in length by only one nucleotide. The different sized oligonucleotides result from interruption of DNA synthesis caused by the presence of 2',3'-dideoxyribonucleoside triphosphates (ddNTPs). The ddNTPs (corresponding to each of the four dNTPs - dATP, dTTP, dCTP, dGTP) can be incorporated into a growing (extending) DNA chain, but lack the 3'-hydroxyl group necessary to form a bond to another nucleoside. Therefore, when a ddNTP is incorporated into a growing DNA chain, there was termination of growth at that point.

3.2.4.2 PCR product purification

Before the PCR products could be sequenced, they had to be purified from the reaction components (primers, dNTPs etc.), as these would inhibit the sequencing reaction. This was achieved using the High Pure PCR product Purification Kit (Roche) according to the manufacturer's instructions (see appendix A). A 100µl of PCR product was purified and the pure PCR product was eluted with 100µl of SABAX water (pH 8.0).

3.2.4.3 Sequencing reaction

Sequencing was performed using the ABI PRISM 377 sequencer. The sequencing protocol performed was that of the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) according to the manufacturer's instructions. Briefly, 6 µl of the purified PCR product was used and the sequencing reaction mixtures were placed in 100µl PCR tubes. The sequencing reaction cycles consisted of 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min and rapid cooling to 4°C and then held at this temperature for further use.

3.2.4.4 Sequencing reaction purification

The sequencing reaction was purified using the Centri-Sep Spin-Column (Perkin-Elmer) purification kit. The procedure was performed according to the manufacturer's protocol to result in a 20µl volume. The solution was then dried by speed-vacuuming and the pellet resuspended in 6µl of loading dye before loading on a gel.

3.3 RESULTS

3.3.1 Optimisation of Nef PCR and RT-PCR

3.3.1.1 Annealing temperature titration

The electrophoretogram shown in Figure 3.2 depicts Nef product derived from different annealing temperatures. Lanes 1 and 2 (representing 42°C and 44°C, respectively) show that there was some Nef product generated from these annealing temperatures, although these were relatively low. The darkest band of *nef* product was seen in lanes 3 and 4 (representing annealing temperatures 46°C and 48°C, respectively). Lane 5 shows that from 50°C upward, the amount of product decreases. From this data, the optimal annealing temperature for *nef* primers was deduced to be 48°C (lane 4).

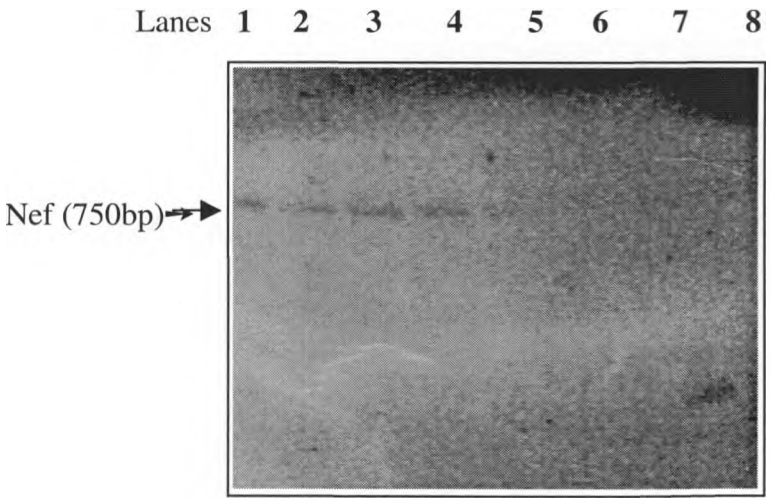

Fig 3.2: Electrophoretgram depicting eight *nef* RT-PCR reactions using viral RNA isolated from Du 151 plasma at different annealing temperatures (from 42°C to 56°C, lanes 1 to 8, correspondingly). The temperature gradient was 2°C.

3.3.1.2 Magnesium chloride concentration titration

Figure 3.3 shows a series of eight PCR reactions set up using a range of $MgCl_2$ concentrations, from 1.5-5mM $MgCl_2$ (0.5mM concentration gradient). The other components of the reaction were kept constant and an optimum annealing temperature (48°C) identified above (section 3.2.3.1) was used. The electrophoretogram (fig 3.3) showed that lanes 5 and 6 (corresponding to 3.5 and 4.0 mM $MgCl_2$, respectively) showed the brightest band of *nef* product, but non-specific amplification was also observed. This meant that the two $MgCl_2$ concentrations were too high. Lane 4 (3mM $MgCl_2$) had the highest amount of clean Nef product and was subsequently taken as the optimum $MgCl_2$ concentration for Nef PCR.

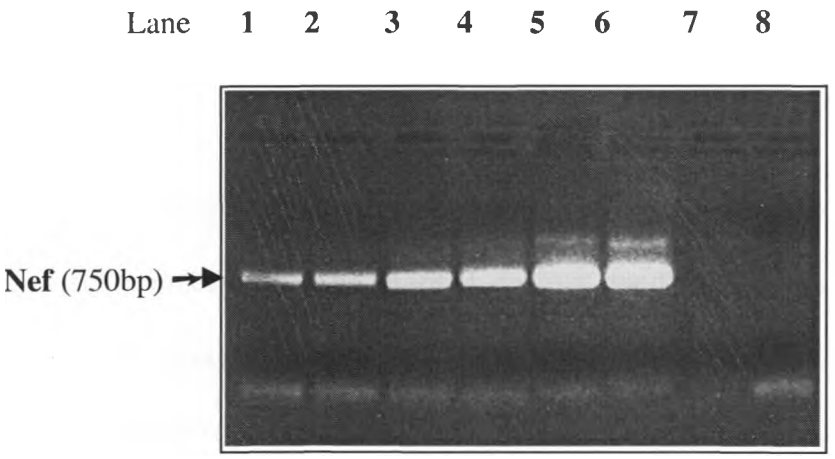

Fig 3.3: Viral RNA isolated from plasma of Du 151 were reverse transcribed and amplified in the full length Nef region with different amounts of $MgCl_2$ added (from 1.5 mM to 5 mM, from lane 1 to 8, with 0.5 mM gradient).

3.3.1.3 nef PCR using two different PCR machines

Figure 3.4 shows *nef* PCR using two different machines. Lane 1 represents Nef PCR using the Perkin Elmer PCR Machine and lane 2 represent Nef PCR using the

Stratagene Robocycler machine. From the electrophoretogram, it can be seen that both PCR machines were able to amplify *nef* to the same degree. Although lane 1 seems to have less product than lane 2, this was not as a result of differences in amplification, but due to a damaged well on the gel.

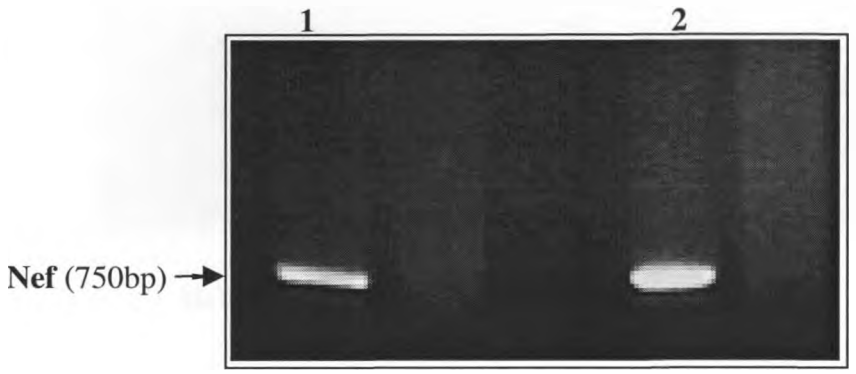

Fig 3.4: *nef* was amplified from Du 151 with the newly optimised conditions. Lane 1 shows Nef product obtained using the Perkin-Elmer machine and lane 2 represents Nef amplification using the Robocycler.

3.3.1.4 Optimised nef PCR

The new thermal cycling conditions identified from the above optimisation reactions are provided in appendix A. The thermal cycling conditions for *nef* PCR (after reverse transcription using Thermoscript RT) were 92°C for 2 min, 48°C (identified in the annealing temperature titration) for 2 min, 72°C for 4 min, followed by 30 cycles of 94°C for 45 sec, 55°C for 30 sec, 72°C for 1 min, with a final extension step at 72°C for 5 min. The reactions contained 10 pmol of each primer, 10mM dNTPs, 3 mM $MgCl_2$ (identified in the $MgCl_2$ optimization reaction), 0.625 U Taq polymerase, with the appropriate 10X buffer. These were used to generate 5' and 3' Nef fragments ready to be sequenced. Figure 3.5 depicts *nef* RT-PCR reactions from six Du samples, using the above PCR conditions. Lane 1 represents the molecular weight markers. For each specimen, there was a 5' product (540 bp) and a 3' fragment (441

bp). An example is shown for Du 281 in lanes 10 and 11, which represent the 5' product and 3', respectively.

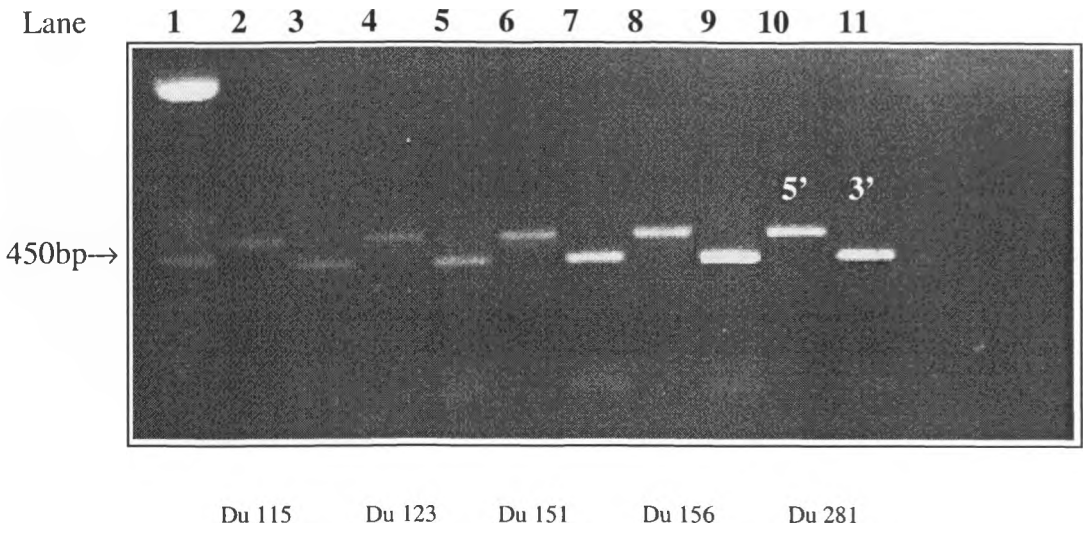

Fig 3.5 Electrophoretogram showing the results of *nef* RT-PCR for six Du samples. Lane 1 is the molecular weight marker, lanes 2 and 3 depicts 5' and 3' *nef* products for Du 115.

3.3.2 Sequencing

The 5' and 3' *nef* products of each isolate shown in Figure 3.5 were sequenced in both directions using primers depicted in Figure 3.1. Figure 3.6 shows a representative example of a sequencing electrophoretogram. The ddNTPs were distinguished by different fluorescent labels, where ddATP was green, ddTTP was pink, ddGTP was black and ddCTP was blue (fig 3.6). In the case where the computer could not distinguish between different colours, an ambiguous nucleotide would result, represented by a red N. This could be a result of low signals due to mutations from Taq polymerase or low amounts of PCR product. After electrophoresis, the dideoxynucleoside of each oligonucleoside was detected by a computer by identifying its characteristic spectrum.

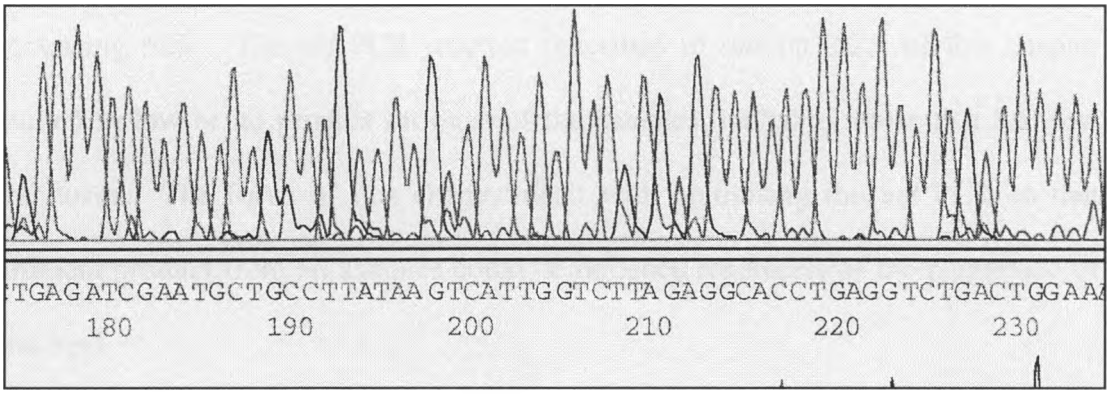

Fig 3.6 A representative graph showing the colour spectrum of each of the four nucleotides from the ABI 377 sequencer.

Each colour peak in Fig 3.6 represents the specific nucleotide that was recognised. Below the graphs is the nucleotide sequence obtained from the peaks. The letters show the exact sequence interpreted by the computer.

3.4 DISCUSSION

As sequencing was performed directly from PCR products, rather than using cloned material, the products had to be of high purity and quantity to achieve successful sequencing runs. The *nef* PCR reaction described in section 3.2.1 of this chapter resulted in low or no product on most of the samples, including those that had low viral loads. The focus of this chapter dealt with optimising the *nef* PCR so that sufficient product from all samples could be obtained regardless of the magnitude of viral load.

Subtype C *nef* has previously been sequenced from infected individuals outside South Africa (Novitsky *et al.*, 1999). So far, there have been no *nef* sequences obtained from isolates derived from HIV-1 infected South Africans, except from full-length HIV-1 sequences (Rodenburg *et al.*, 2001). Intrasubtype and intersubtype *nef* variations have been observed between isolates from different countries. Therefore, it was unlikely that primers used to sequence *nef* isolates from other geographical areas would be able to amplify subtype C isolates from South Africa. Also, reaction conditions used to amplify *nef* from India (Ahmad *et al.*, 1998), were not successful in amplifying isolates from South Africa, therefore new sets of primers were designed and reaction conditions optimised for South African subtype C isolates. Subtype C primers were designed based on full length HIV-1 isolate from Botswana (96BW0504) (Novitsky *et al.*, 1999). The mean amino nucleotide percentage difference between the isolate from Botswana and 5 subtype B isolates was 18.9%, and between seven Indian subtype C isolates was 11.7%. Consequently, it was considered that primers should amplify nef based on an isolate geographically close to South Africa. Two sets of primers were designed to incorporate the whole *nef*

genome (approximately 640 bp). Sequencing was be achieved directly from PCR products derived from plasma, and proviral DNA. Sequencing from plasma measures the recent viral progeny that was replication competent and from proviral DNA measures integration competent virus and gives an indication into the historical archives of infecting virus. For the purpose of this study, sequencing was not performed on cloned material, which for quasispecies analysis and suitable for identifying selection of viral strains.

The optimised *nef* PCR reaction described in this chapter can be used to successfully sequence *nef* from viral RNA (plasma) or pro-viral DNA (PBMC) and is suitable for use when RNA copies are <20000 copies/ml. This is important since this procedure can be used to sequence isolates from any stage of disease, including the chronic phase where the viral load has reached a low set-point. It can also be used to follow *nef* sequences when viral replication is suppressed by antiretroviral therapies.

CHAPTER 4: CHARACTERISATION OF SUBTYPE C NEF SEQUENCES FROM SOUTH AFRICA

4.1 INTRODUCTION

Many studies have been performed to gain further understanding of the functional properties of Nef in viral replication and pathogenesis (Niederman *et al.*, 1993a), (Niederman, Hastings, and Ratner, 1993b); (Lee *et al.*, 1996); (Reiss *et al.*, 1989). Earlier studies described Nef as a negative regulatory factor (hence the name; **N**egative **F**actor), suppressing both viral replication and transcriptional activity of the LTR and helping to maintain viral latency (Ratner *et al.*, 1987); (Le Gall *et al.*, 1998). However, recent studies have demonstrated that Nef has a positive effect on HIV-1 replication in PBMC (Miller *et al.*, 1994); (Spina *et al.*, 1994). The importance of Nef in viral pathogenesis was further shown in rhesus monkeys infected with SIV containing a mutated Nef sequences (Kestler *et al.*, 1991). The *nef* mutants containing a premature stop codon reverted to a full open reading frame *in vivo* and caused disease, whereas *nef*-deleted virus replicated only to low levels and did not produce clinical disease. Many regions within *nef* have subsequently been identified as being responsible for several pathogenic functions of *nef*, including the PKC site, polypurine tract and proline motif. Because of the importance of these regions, it is likely that such regions will be well conserved within subtype C isolates and between clades and that aberrations at these sites could possibly alter the pathogenic nature of HIV-1.

This chapter will show data from the analysis of *nef* sequences using the optimised conditions outlined in chapter 3. The aim of this chapter is: 1) to determine how South African *nef* sequences cluster with reference *nef* sequences from other clades.

2) To determine the phylogenetic relationship between South African *nef* sequences from isolates obtained during different stages of disease and 3) to determine the degree of Nef amino acid conservation and variability in subtype C HIV-1. The hypothesis being explored is that *nef* is highly conserved within subtype C and between subtypes in regions that span the important functional domains of the molecule.

4.2 MATERIALS AND METHOD

4.2.1 Generation of Nef nucleotide sequences and translation to amino acid sequences

Sequences of individual isolates (from the PCR designed in chapter 3) were joined in the overlapping region and the flanking regions cut using DNASIS (Hitachi). This resulted in full-length nef sequences for each isolate. Nucleotide sequences were translated to amino acid sequences using DNASIS software.

4.2.2 Construction of Phylogenetic trees

The nucleotide sequences of all isolates were aligned using DNAMAN software (Lynnon BioSoft). Dashes were incorporated into the sequences to obtain optimum alignments. Genetic distances between the sequences were calculated using the DNADIST program in the PHYLIP package (version 3.5) (Felsenstein, 1990) employing the Kimura two-parameter method (Kimura, 1980). Phylogenetic trees were constructed using a 100 bootstrap replicates of neighbour-joining algorithm (PHYLIP programs SEQBOOT and CONSENSE), and bootstrap values of greater than 75% were taken as significant grouping. Visualisation of the trees was achieved using TREEVIEW Win32 software.

4.2.3 Generation of Subtype C Nef consensus sequences

Nef amino acid amino acid sequences were aligned using DNAMAN and the alignments saved in the PHYLIP format. Consensus sequences were generated from all the Nef sequences using the SeqPublish program from the Los Alamos HIV Sequences database (Myers *et al.*, 1994).

4.2.4 Calculation of amino acid variations

Percentage amino acid variations were calculated using PROTDIST program in PHYLIP.

4.2.5 Statistical analysis

Significant differences were measured using one-way analysis of variance between groups and the Student's t-test. A combination of Microsoft Excel and Sigmastat were used to analyse the samples.

4.3 RESULTS

4.3.1 Phylogenetic analysis of nef sequences from three different cohorts

To show how South African subtype C *nef* (bold font) sequences related to other published sequences, a nucleotide neighbour-joining tree (Figure 4.1) was constructed using reference sequences representing subtype A, B, D, C (from India, Brazil, Botswana and Ethiopia), F, G, J and J. The tree was rooted to an SIV (CPZgab) *nef* sequence. All subtype C *nef* sequences clustered significantly together with a bootstrap value of 100% (Figure 4.1). There was no significant clustering of nef sequences from viruses isolated from persons at different stages of disease. For example, sequences from Du467 and Du457 (recently-infected) clustered with B402 and B401 (chronically-infected) and Sw10, Sw5, and Sw15 (AIDS) clustered with B304, B383, B296 and B384. In this cross-sectional analysis, there was no distinct difference in *nef* sequences obtained from different stages of disease progression. The Indian subtype C nef sequences were highly clustered (bootstrap values of 99%) and separate from other subtype C sequences.

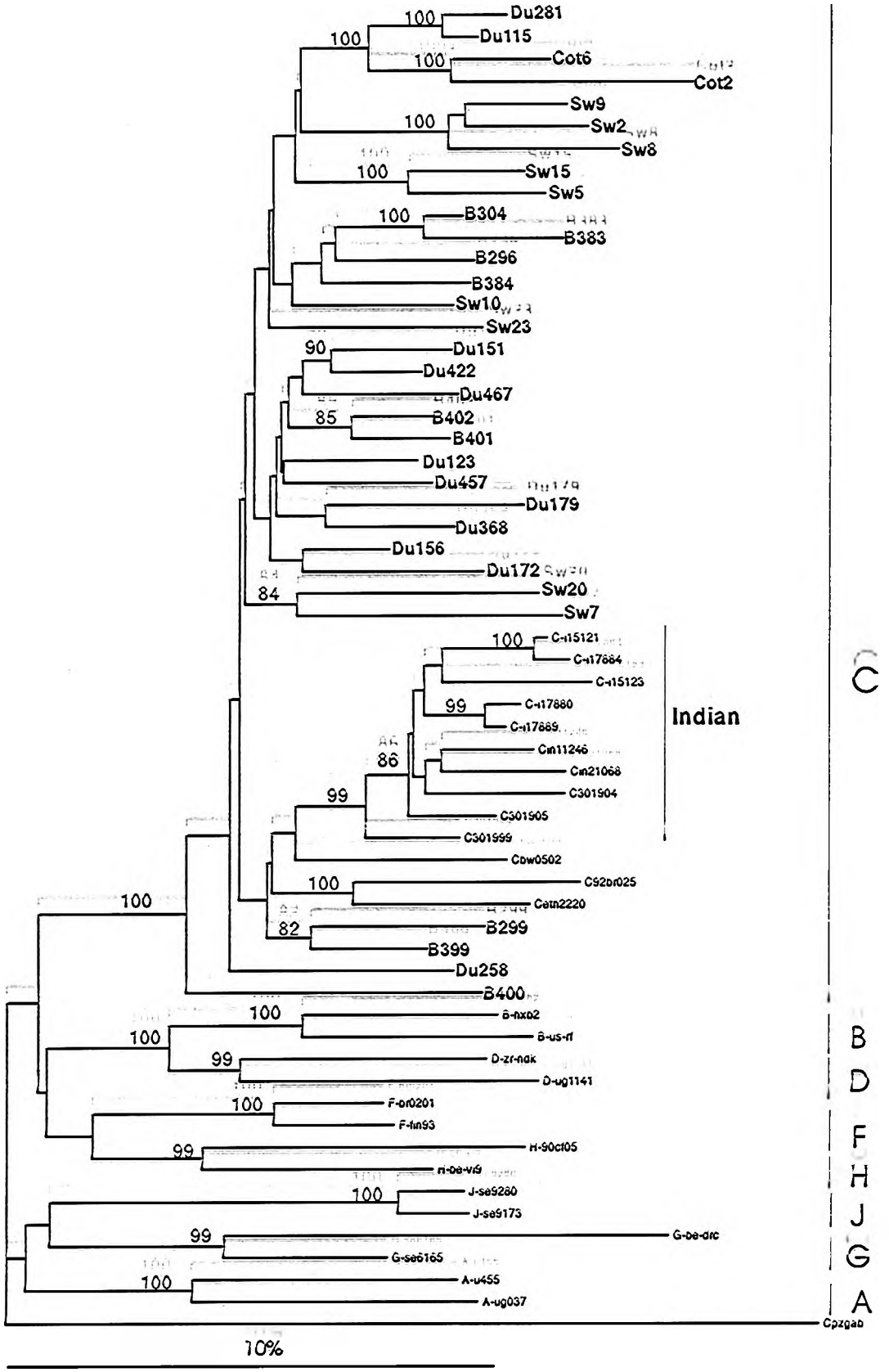

Fig 4.1: Neighbour-joining tree of *nef* nucleotide sequences from acute seroconverter (Du), asymptomatic (B) and AIDS patient (Sw/Cot) cohorts. The tree is rooted with *nef* sequence of SIVcpz

4.3.2 Comparisons *nef* and V3 loop nucleotide sequences from the same isolate

To gauge the degree of sequence variation between gene regions within each viral isolate, comparisons were made between the V3 loop *nef* nucleotide sequences. The rationale for this analysis was to determine whether the observed conserved nature of *nef* in the Du isolates was a reflection of relatedness in the transmitted viruses within this cohort. Neighbour-joining phylogenetic trees were constructed from *nef* and V3 loop nucleotide sequences and shown in Figure 4.2. The scales of the two trees are shown and both represent 10% variation. Collectively, the mean branch lengths (representing variation) for the Du *nef* sequences was 7.9 +/- 2.1% while that of the autologous V3 loop sequences was 11.5 +/- 3.8%, being significantly different ($p<0.01$).

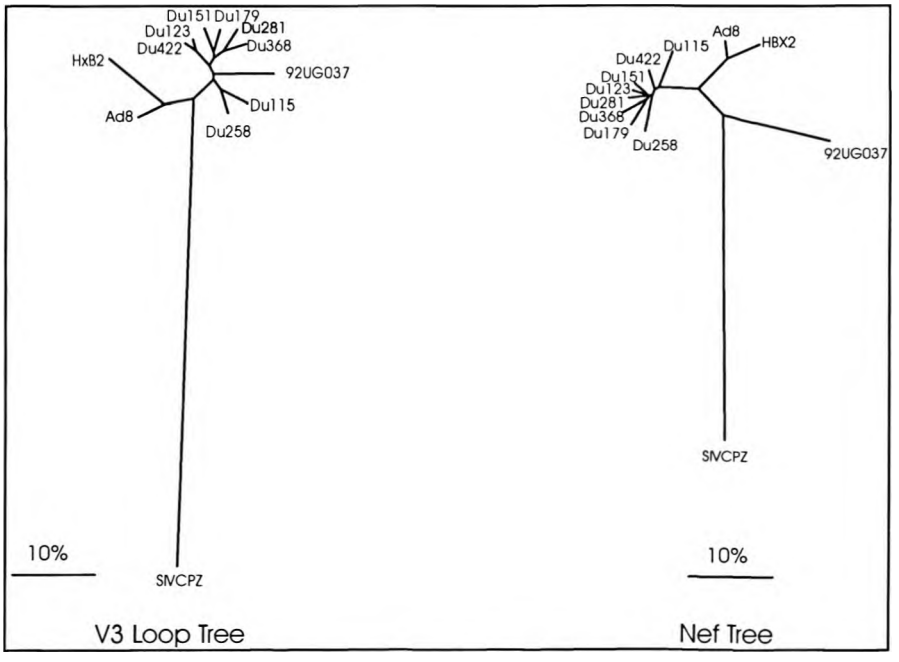

Fig 4.2 Neighbour-joining trees of autologous amino acid sequences for the V3 loop region of env (tree on the left) and Nef (tree on the right). The scales for both trees are the same in length. The trees were rooted with SIVcpz sequences and three reference sequences were included, two subtype B (HxB2 and Ad8) and one subtype A (92UG037).

These data show that the V3 loop sequences are more variable that the autologous nef sequences.

4.3.3 Comparison of sequences from plasma, proviral DNA derived from fresh and co-cultured PBMC

The variability of *nef* between replication competent virus (plasma), integration competent virus (pro-viral DNA), and freely replicating virus (in co-cultured PBMC) was measured from 9 isolates in parallel.

Figure 4.3 shows an alignment of representative amino acid sequences derived from plasma, PBMC and co-culture. The figure depicts that Nef amino acid sequences do not vary greatly when sequenced from products isolated from the three sources. The few changes observed were in the carboxy end of the polypeptide, no changes were observed in the middle and amino terminal regions. The same pattern was observed for Du samples and summarised in table 4.1.

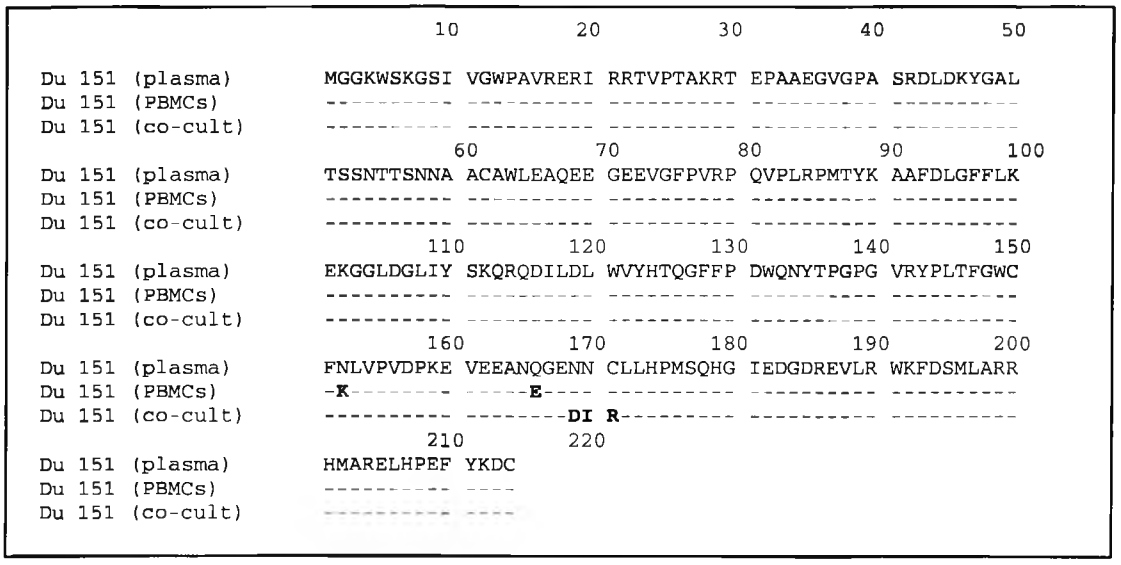

Fig 4.3 An alignment of amino acid sequences of Du 151 sequenced from different sources; plasma, baseline PBMCs and co-cultured PBMCs. The dashes represent regions of homology to the reference sequence (plasma). Bold font represents amino acid substitutions.

These data show that Nef does not vary greatly between replication competent virus (from plasma) and integration competent virus (from PBMC) and remains stable when allowed to replicate freely during co-culture.

Table 4.1: Percentage amino acid variation of sequences obtained from plasma (n=12), PBMC (n=12), and co-cultured PBMC (n=12)

		Plasma	PBMC
Plasma	Mean SD		
PBMC	Mean SD	0.9 0.6	
Co-cult	Mean SD	1.8 0.4	2.3 0.7

4.3.4 Amino acid alignment of subtype C Nef sequences from the three cohorts

Nef amino acid sequences were translated from the 32 nucleotide sequences and aligned (Figure 4.4). All sequences were aligned against a generated subtype C consensus generated from the sequences described and used to examine more closely intra-cohort variation of Nef, especially within functional and structural motifs.

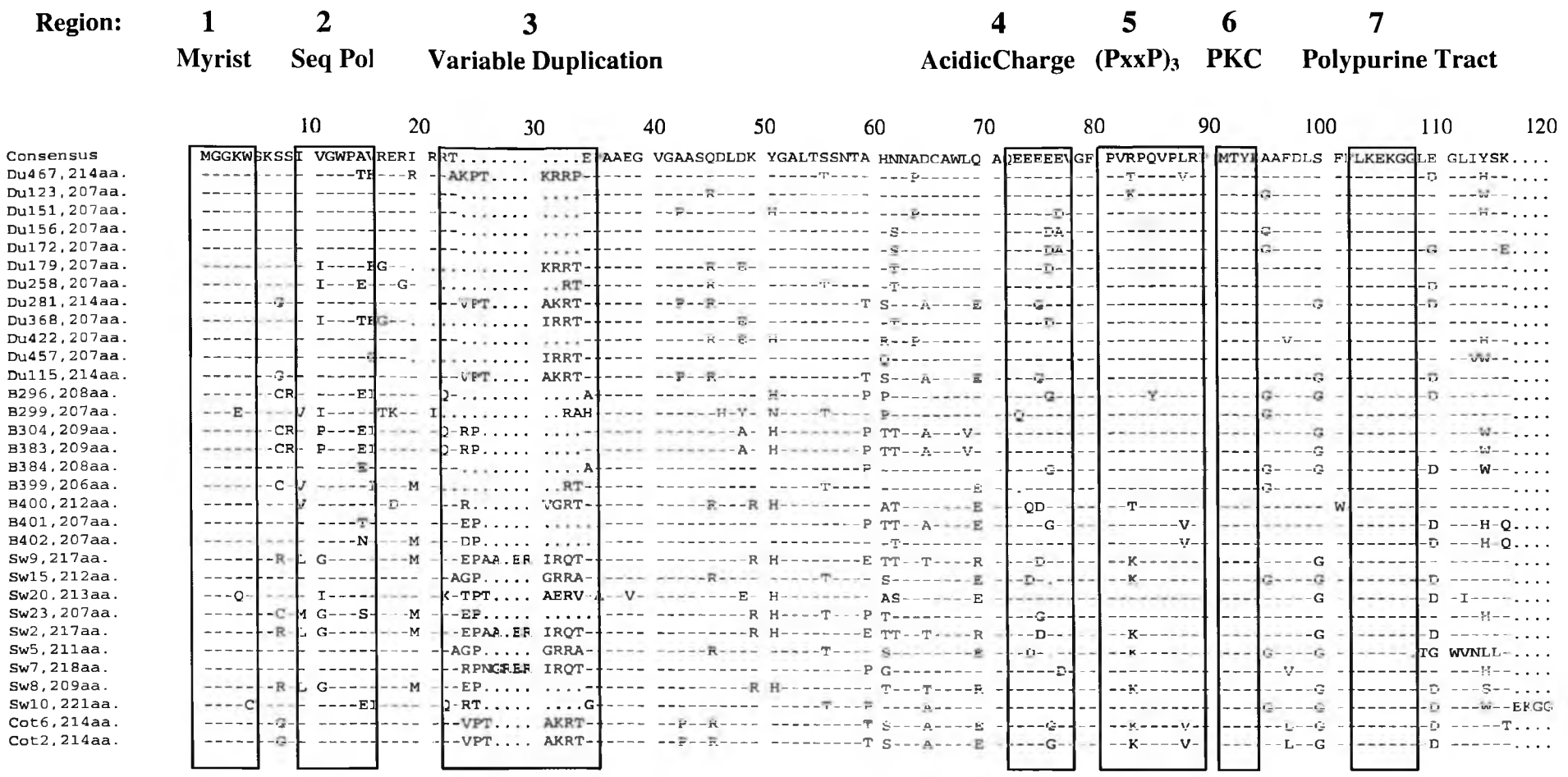

Fig 4.4 Amino acid alignment of Nef sequences from Du, B and Sw patients. The sequences were aligned to a consensus sequence from all thirty sequences. The shaded boxes represent the Nef characteristic motifs; myristolation signal, sequence polymorphism, protein kinase C site as well as polypurine tract.

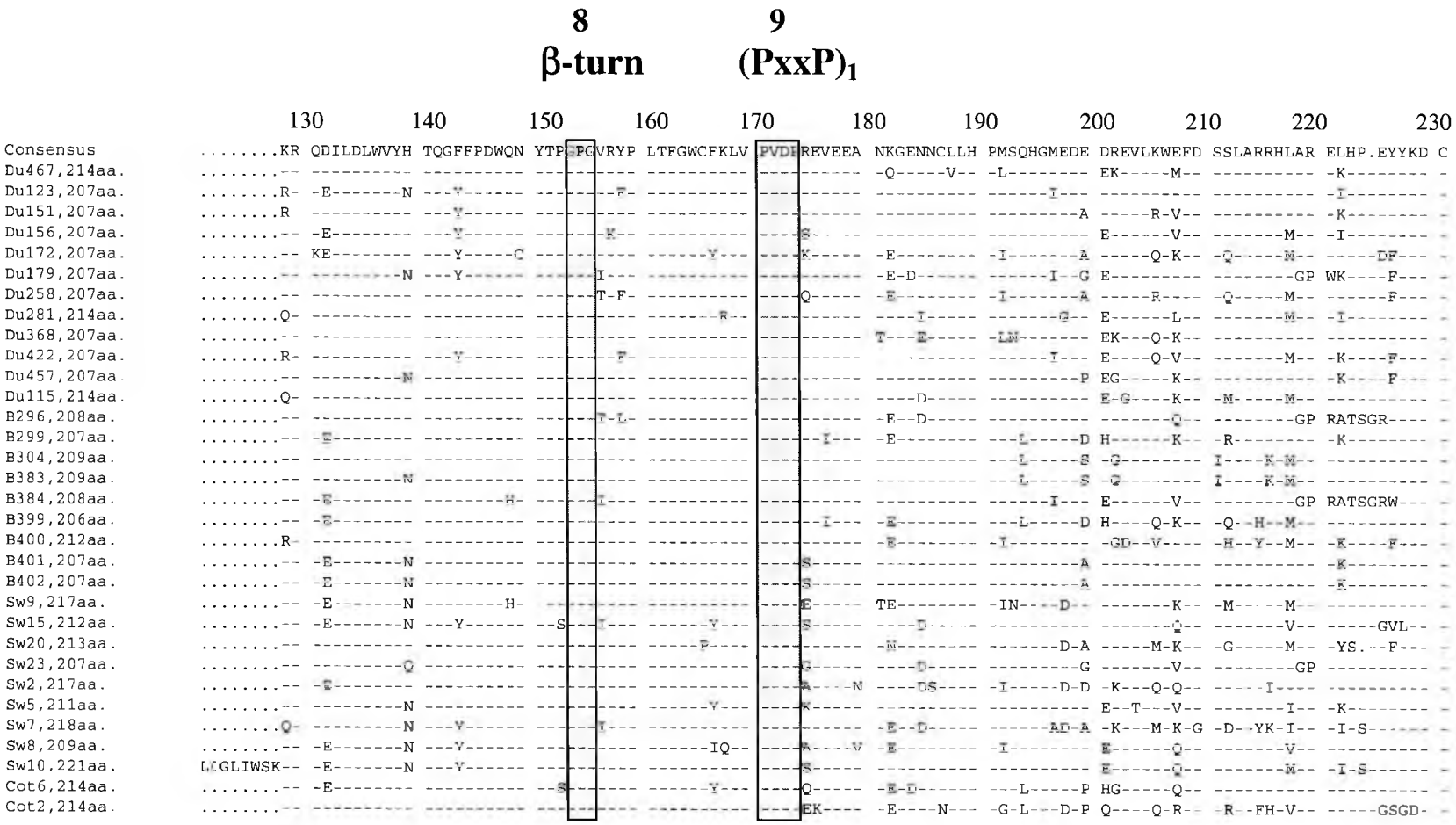

Fig 4.4: Continued

Previous studies have identified highly variable regions within the N- terminus of Nef, including the sequence polymorphism (region 2) and the variable duplication (region 3), which were also present in subtype C sequences from South Africa (Figure 4.4). Most of the functional domains, shown by the highlighted boxes, were well conserved across subtype C sequences (Figure 4.4). The myristolation signal (region 1), proline motif (PxxP) (regions 5 and 9), protein kinase C (PKC) site (region 6), polypurine tract (region 7) and β-turn (region 8) were highly conserved between isolates. There were several single and double amino acid substitutions in the acidic region (^{73}EEEEE^{77}) (region 4), replacing Glutamic acid (E) with either Aspartic acid (D), Glutamine (Q), Glycine (G) or Alanine (A). These substitutions probably do not alter the acidic nature of this region because G, Q and G are neutral amino acids, while A is acidic.

There was no significant sequence variations associated with disease, although in one AIDS patient (SW-10) there was a duplication inserted at positions: ^{116}EKGGLDGLIWSK^{128}, being a duplication of ^{105}EKGGLEGLIYSK^{114} which partly spans the polypurine tract. This duplication was only observed in one individual and it was therefore not a feature of isolates from the AIDS patients studied in this thesis.

4.3.5 Percentage Nef amino variations between SA sequences

Percentage Nef amino acid variations were calculated to quantify the degree of variability within subtype C isolates and to ascertain if stage of disease was associated with changes in Nef. Table 4.2 shows the percentage amino acid variation subtype C isolates from South Africa compared with variations to Nef sequences from Indian subtype C and other subtypes.

Table 4.2: Intra- and inter- cohort mean percentage variations compared with Indian subtype C sequences and subtype A, B and D reference sequences

		Recent (n=12)	Chronic (n=9)	AIDS (n=11)	Indian C (n=10)	Subtype A (n=10)	Subtype B (n=10)	Subtype D (n=10)
Recent								
	Mean	13.3			18.3	26.3	32.1	32.7
	SD	4.2			3.8	4.9	5.2	5.4
Chronic								
	Mean	16.8	14.2		19.1	27.1	24.7	27.1
	SD	4.4	3.8		4.4	2.8	5.3	4.1
AIDS								
	Mean	18.3	19.9	19.9*	19.7	27.9	26.2	28.3
	SD	4.0	4.5	4.3	3.9	3.1	4.7	4.2

* - ($p < 0.01$)

The mean percentage amino acid variation for all subtype C isolates was 17.1% for the three cohorts. Nef variations from recently-infected individuals were least variable, with a percentage variation of 13.3 ±4.2%, followed by chronically-infected, 14.2 ±3.8% and then AIDS patients: 19.9 ±4.3% being significantly ($p<0.01$) more variable (Table 4.2). This finding suggests that Nef becomes more variable as the stage of disease progresses. Subtypes A, B, D and Indian subtype C Nef sequences were included as references and show that there was a higher degree of amino acid variation between different subtypes. The variation between South African subtype C sequences and Indian sequences (at any stage of disease) was not significantly different, with the average percentage variation between these regions being 19.0%.

4.3.6 Percentage amino acid variations between different regions

The alignment in Figure 4.4 showed qualitatively that the middle region of South African Nef had the highest degree of conservation compared to the N- and C-termini. To quantitatively measure the degree of conservation between the three regions, Nef sequences were arbitrarily divided into three regions. The N-terminus was from amino acid 1 to 80, middle region from position 81 to 160 and the C-terminus was from position 161 to the end of the molecule. Percentage variations were then calculated for each region to measure if the middle region was significantly more conserved that either the N- or C-termini.

Table 4.3: Nef amino acid percentage variations in three different regions of the Nef molecule between HIV-1 infected individuals at different stages of disease

		N terminus	Middle region	C terminus
Du (recent infection)	Mean	13.0	7.8	17.1
	SD	4.5	2.6	4.7
B (chronic)	Mean	18.0	7.4	20.8
	SD	6.3	2.9	6.0
Sw (AIDS)	Mean	20.1	9.5	22.0
	SD	6.5	3.4	5.3
Total		17.1	8.2*	20

* - ($p<0.001$)

Table 4.3 shows the mean +/- SD amino acid percentage variations between the N-terminus, the middle region, and the C-terminus of Nef for each cohort. It is clear that the middle region of Nef has significantly lower amino acid variation and supports the data in Figure 4.5 showing that the middle region of Nef has the least variation. The difference in percentage variations between the N- and the C-termini was not significant, but that of the middle region was significantly ($p<0.001$) lower than both termini.

4.3.7 Nef amino acid alignment between subtypes

Alignment of Nef amino acid sequences between different subtypes is shown in Figure 4.5.

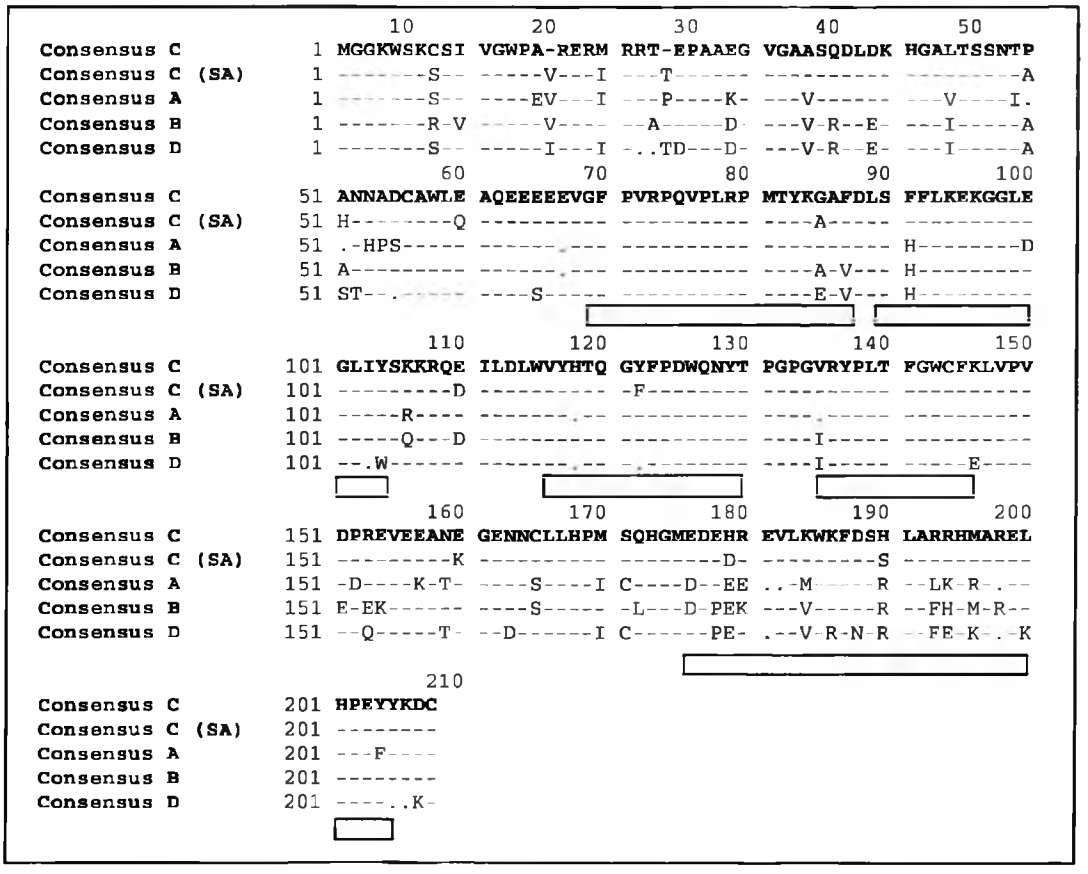

Fig 4.5: Alignment depicting an alignment of consensus sequences from clade C, B, D and A. consensus C (SA) represent consensus sequence generated from South African isolates sequenced in this project. The other sequences were obtained from the Los Alamos database. All the sequences were aligned to the published consensus C.

The subtype C Nef consensus sequence generated from the infected cohorts in this project, was aligned with a published consensus C Nef from the Los Alamos database (Myers *et al.*, 1994) and consensus sequences for subtypes B, A and D (Figure 4.5). These alignments confirm that Nef is least variable in the middle region between clades and that conserved functional domains are present across different clades. The brown shaded boxes below the sequences depict areas known to be rich in epitopes recognised by cytotoxic T lymphocytes (CTL) across a spectrum of HLA alleles. This information has been derived exclusively from analysing responses to subtype B Nef and is comprehensively reported in the Los Alamos database. Figure 4.5 shows that most of CTL-rich regions in Nef are in the conserved middle region. However, CTL epitopes have also been found in the highly variable carboxy terminus. It is therefore likely that CTL epitopes presented by subtype C-infected individuals from the C-terminus will be different from those presented by subtype B-infected.

4.3.8 CTL epitopes and Nef sequence variation

Known Nef CTL epitopes (Los Alamos database) restricted by a range of HLA class I molecules are shown in Table 4.4. As previously mentioned, these epitopes have been defined from studies using subtype B-infected individuals. The epitope sequences were aligned with the corresponding subtype C consensus sequence generated from 32 sequences reported in this project. It can be seen from Table 4.4 that HLA-A2 or –B8 restricted epitopes, such as QVPLRPMTYK, PLTFGWCFKL or FLKEKGGL are conserved when aligned with subtype C consensus sequences. It is therefore likely that cross-clade CTL epitopes would exist between subtype C and subtype B-infected individuals. In some epitopes, however, there were several amino acid substitutions observed between the defined epitope and consensus C sequence.

Table 4.4: defined HLA-restricted CTL epitopes identified from subtype B infected individuals aligned with the corresponding subtype C consensus sequence from South African isolates.

	HLA-A1	HLA-A2	HLA-A3	HLA-A11	HLA-B8	HLA-B7	HLA-B57	HLA-B18	HLA-B49
defined	VGFPVTPQVPLRM	QVPLRPMTY	QVPLRPMTY	QVPLRPMTY	WPTVRER	HSQRRQDILDLWI	HTQGYFPDW	GVRYPLTFGWCYKL	YPLTFGWC
sub C	T----R------	K---------	K---------	K---------	M-A----	Y-KK--------	Q---F----	VP----------F--	Y-------
defined	EKGGLEGLIHSQR	DLSHFLKEKGGLEG	VPLRPMT	VPLRPMT	VGFPVTPQVPLRM	NYTPGPGVRYPL	GPGVRYPLTFGWC	RYPLTFGWC	F
sub C	R---------Y-	L--F----------	Y-------	Y-------	T-----R------	T----------	Y----------	F---------	
defined	WIYHTQGYFPDWQN	PLTFGWCFK	AFHHVARE	AAVDLSHFLKE	EKGGLEGLIHSQR	TPGPGVRYP	VRYPLTFGWCYKLV	YPLTFGWC	
sub C	V-----F------	L---------	K-RR-L----	K-F---F-----	R---------Y-	L---------	PV---------F---	Y-------	
defined	GVRYPLTFGWCYKL	AFHHVARE	L	AVDLSHFL	GVRYPLTFGWCYKL	FPVTPQVPL	YFPDWQNY	F	
sub C	VP----------F--	L-RR-L----		K-F---F--	VP----------F--	R---R------	T--------		
defined	HHVARELHPEYFKN				FLKEKGG	RPMTYKAA			
sub C	R-L---------Y-				L-------	L--------			
	D-					F			

4.4 DISCUSSION

Phylogenetic analysis was used to determine the variability of subtype C nef from isolates representing different stages of disease progression. South African *nef* sequences were shown to cluster with subtype C isolates from other geographical areas which is consistent with previous findings that South Africa has a subtype C epidemic (Van Harmelen *et al.*, 1999); (Williamson *et al.*, 1995) and that recombination with other subtypes is very rare (Bredell *et al.*, 2000). From the phylogenetic analysis, there was no distinct grouping of sequences according to disease status. It is important to note that this analysis was not from a longitudinal study but cross-sectional. These findings should be extended by looking at the stability of Nef within an individual as disease progresses. Also, analysis of subtype C infected long-term nonprogressors would give a better indication into the role of Nef in subtype C infection. Previous studies of other clades have shown significant Nef deletions in long-term nonprogressors when compared to those with rapid progression (Kirchhoff *et al.*, 1995); (Huang, Zhang, and Ho, 1995); (Salvi *et al.*, 1998).

To discount the possibility that the conserved and stable nature of *nef* was not due to related viruses being transmitted within the Du cohort, comparisons were made between *nef* and V3 loop sequences from the same isolates. The findings showed that autologous V3 loop variation, which is known to be a highly variable region of the *env* gene (Korber *et al.*, 1994); (Albert *et al.*, 1992); (Engelstad, 1996) was greater ($p<0.001$) than Nef.

This chapter describes the first characterisation of Nef subtype C sequences from South African HIV-1 infected individuals. Virological aspects and implications of Nef sequence variability were analysed. The third part of this chapter looked at the

sequence variations between proviral DNA and plasma. The data in this chapter suggest that Nef does not vary between free virions (plasma) and the integrated genome (proviral DNA). The analysis was taken further to show that Nef is stable when virus was allowed to replicate freely in culture. The stability of Nef that was observed could be a reflection of the importance of Nef in the virulence and function of the virus.

The variability of Nef at the amino acid level was also analysed to identify conserved and variable regions within Nef. Previous studies on subtype B (Shugars *et al.*, 1993); (Saksena *et al.*, 1997); (Dwyer *et al.*, 1997) and subtype C (from Indian isolates) Nef amino acid sequences (Ahmad *et al.*, 1998)) have shown that Nef has highly conserved regions as well as variable domains. The functional domains of Nef were found to be highly conserved (Jubeir-Maurin *et al.*, 1999). The myristolation signal was found to be highly conserved in subtype C sequences. The conservation of the myristolation signal in subtype C sequences was hypothesised since subcellular targeting of Nef proteins to the cytoplasmic membranes depends on the presence of an intact myristolation signal (You and Felted, 1992). Also, the conservation within *nef*-defining regions was anticipated as a result of the functional importance of these regions for viral pathogenesis. Amino acid alignments of subtype C Nef also showed highly variable domains in the amino terminal of Nef which include the sequence polymorphism and variable duplication. These features are common amongst Nef sequences across clades (Ratner *et al.*, 1996); (Huang, Zhang, and Ho, 1995); (Blumberg *et al.*, 1992).

Amino acid alignments were also used to determine which regions within subtype C Nef were variable. As alluded to before, the amino terminus of subtype C Nef was highly variable, mainly due to the presence of the sequence polymorphism and variable duplication. The carboxy terminus of subtype C Nef also showed a high degree of variability. This variability was not only present in subtype C sequences, but was also seen in intersubtype alignments. This region has been shown to be rich in CTL epitopes from subtype B analysis (Haas *et al.*, 1996); (Hadida *et al.*, 1995); (Cheynier *et al.*, 1992). These variations could be translated to mean that there are possibly novel subtype C Nef CTL epitopes in this region. The middle region of subtype C was significantly ($p<0.001$) more conserved than the other regions, even when aligned with consensus sequences representing other clades. Several Nef CTL epitopes have been identified in this region from subtype B analysis (Shiga *et al.*, 1996); (Robertson *et al.*, 1993); (Rowland-Jones *et al.*, 1995); (Couillin *et al.*, 1995) and it is hypothesised that cross-clade recognition from subtype C-infected individuals would occur in the middle region of Nef.

CHAPTER 5: USING THE ELISPOT ASSAY TO MEASURE IMMUNE RECOGNITION OF NEF FROM SUBTYPE C-INFECTED INDIVIDUALS

5.1 INTRODUCTION

As discussed in the previous chapter, several Nef epitopes have been reported from analysing subtype B infected individuals. It was shown that the middle region and C-terminus of Nef is rich in CTL epitopes and it is hypothesised in this chapter that subtype C infected individuals will recognise conserved regions of subtype B-based peptides Nef from the middle region of the molecule. To explore this hypothesis, the IFNγ ELISPOT assay was employed to measure which Nef peptide regions were recognised by recently infected individuals from the Du cohort.

The enzyme-linked immunospot (ELISPOT) assay is based on the same principle as the enzyme-linked immunosorbent assay (ELISA). It has successfully been used to measure the frequency of antigen-specific CD8+ T cells (Miyahira *et al.*, 1995). The assay can be used to measure cells that release any specific molecule after antigen stimulation, such as TNFα (Mestan *et al.*, 1986) or granzyme-B (Rininsland *et al.*, 2000). CD8+ T cells also release IFNγ upon recognition of antigen and the assay has been adopted to measure the frequency of IFNγ-releasing cells as a result of antigen (peptide) stimulation (Czerkinsky *et al.*, 1988); (Quiding *et al.*, 1991). For this project, the assay was employed to identify regions within Nef that contain possible CTL epitopes.

In the IFNγ ELISPOT assay, nitrocellulose membrane plates are coated with the primary anti-IFNγ mAb. PBMC are then incubated in the presence of a specific stimulus, such as viral peptides or recombinant vaccinia virus expressing an HIV-1

gene (Larsson *et al.*, 1999) or a laboratory strain of HIV (Zhang *et al.*, 1996). After an overnight incubation, cells are discarded and the plate incubated with a secondary biotinylated anti-IFNγ mAb. An appropriate detector system, such as horseradish peroxidase (HRP) or alkaline phosphatase (ALP) allows for detection each IFN-g-producing cell as a separate spot. These spots represent the location of the cell that secreted the cytokine and can be regarded as spot-forming cells. The spots can be counted by eye under a dissecting microscope, or by an automated computer system, and expressed as spot-forming units (sfu) per million.

The aim of this chapter is: 1) to determine if subtype C-infected individuals would have an immune response to subtype B-based Nef peptides, and 2) To determine the degree of conservation between subtype B peptides evoking the response and autologous subtype C sequences.

5.2 MATERIALS AND METHOD

Figure 5.1 shows a schematic diagram of the ELISPOT assay procedure. The procedure is described in more detail in sections 5.2.2, 5.2.3 and 5.2.4 of this chapter

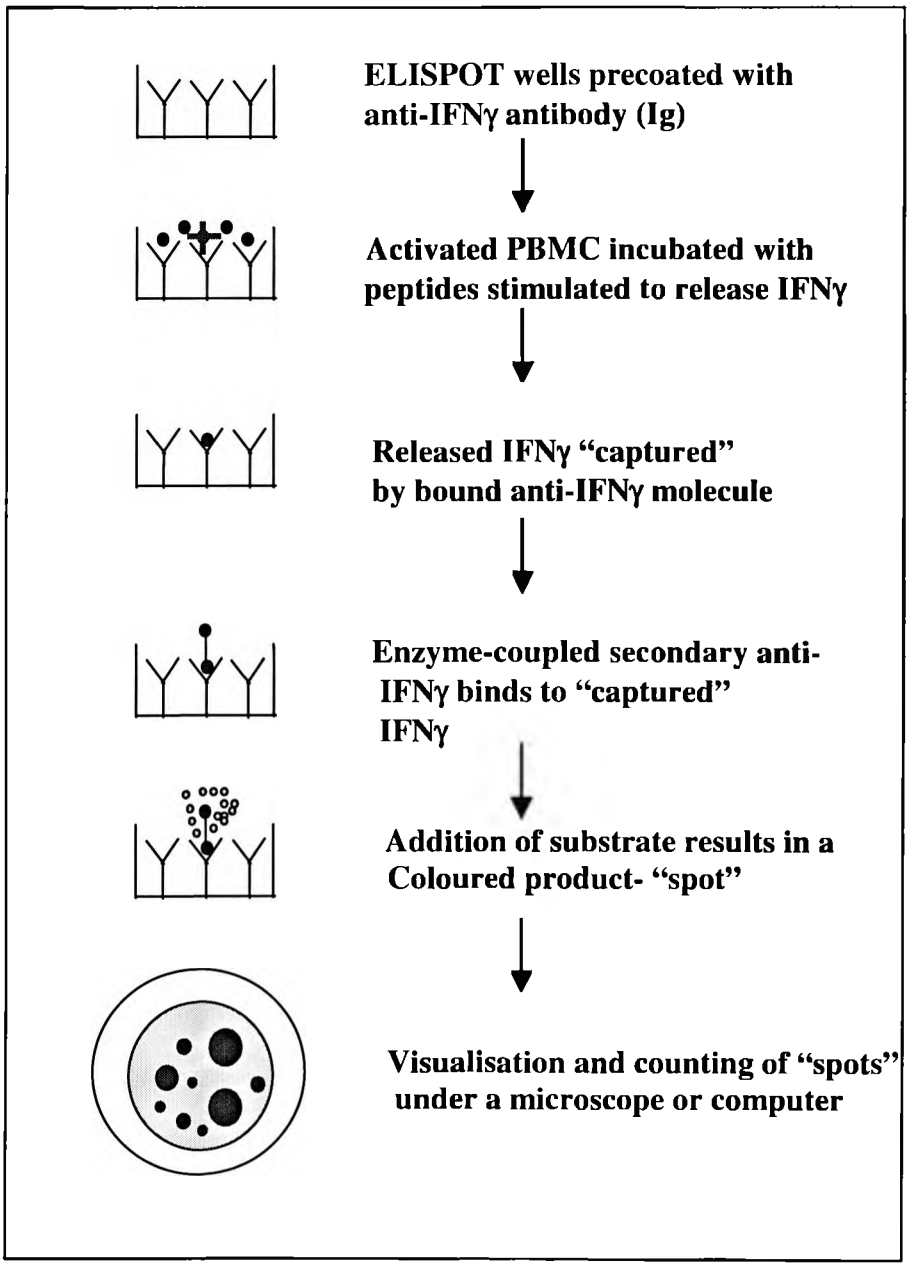

Fig 5.1 : Schematic diagram representing IFNγ ELISPOT assay

5.2.1 Preparation of Nef peptides pools

Twenty subtype B-based peptides covering the whole Nef open reading frame (Nef peptides 1 to 20) were used in this project. These consisted of 20-mers overlapping by 10 amino acids and were based on the BRU subtype B strain of HIV-1. Peptide pools and matrices were established so that each peptide appears twice in a different mixture of peptides, ie. each peptide is found in one pool (consisting of 5 peptides) and one matrix (consisting of 4 peptides, only one of each is common to a pool). Table 5.1 shows the compositions of the peptide pools.

Table 5.1: Peptide composition of each pool and matrix

	Matrix 1	**Matrix 2**	**Matrix 3**	**Matrix 4**	**Matrix 5**
Pool 1	1	2	3	4	5
Pool 2	6	7	8	9	10
Pool 3	11	12	13	14	15
Pool 4	16	17	18	19	20

For example: pool 1 contained peptides 1, 2, 3, 4, 5 and matrix 1 contained peptides 1, 6, 11 and 16. The common peptide being peptide 1. The advantage of using peptide pools in this manner is to screen for specific peptide responses without assaying each single peptide, which saves reagents, time and the number of cells used in the assay. Each lyophilised peptide was reconstituted to 1mg/ml using DMSO. For setting up peptide pools, 25 µl of each peptide (peptides 1 to 5 for pool 1) was placed in one vial, to result in 125 µl (for pools) and 100 µl each peptide at concentrations of 1mg/ml. In order to have a working peptide concentration of 40 µg/ml, a 1:25 dilution was made using PBS and appropriate volume of DMSO to make 10% final

DMSO concentration. The pools were aliquoted at 50 µl and stored at –70°C until further use.

5.2.2 Preparation of cells and coating of plates

Frozen cells were thawed and resuspended in 25 ml of warm (37°C) 10% FCS-RPMI and centrifuged at 1200 rpm for 10 min. After the initial spin, the cells were again resuspended in 25 ml 10% FCS-RPMI and 500 µl of 0.002% DNAse (Sigma,) was added to the suspension. The cells were centrifuged (1200 rpm) again, resuspended and counted using Trypan blue on a haemocytometer. After counting, cells were resuspended to a concentration of $2x10^6$/ml in 10% FCS-RPMI and incubated at 37°C, 5% CO_2 overnight. During the incubation, 96 well plates (Millipore MAIPS4510, Microsep) were coated with 50 µl anti-human IFNγ capture antibody (mAb 1-D1K, Mabtech) diluted to 5µg/ml with PBS. The plates were incubated overnight at 4°C.

5.2.3 Stimulation of cells

After the overnight incubation, coated plates were washed three times with 200 µl/well of sterile PBS. The plates were blocked with 100 µl/well of 10% FCS-RPMI at room temperature for at least 30 min. During the blocking, cells were counted and resuspended at either $4x10^6$/ml $2x10^6$/ml or $1x10^6$/ml to plate 200 000, 100 000 or 50 000 cells/well, respectively. The amount of cells used depended on the initial number of cells stored, although it was aimed at using 100 000 and 200 000 cells/well as the preferred cell number. Peptide pools/matrices or individual peptides (40 µg/ml) were thawed diluted to 4 µg/ml using 10% FCS-RPMI. In each well, either 200 000, 100

000 or 50 000 cells/well (50 µl) were plated with 50 µl of the peptide pools and incubated for 16-18hr.

5.2.4 Development of the plate

After 16-18hr incubation of cells with peptides, the wells were washed 3 times with 200 µl PBS followed by 3 times with 200 µl PBS-Tween (0.0005% Tween-20, 1% FCS in RPMI). To each well, 50 µl of the biotinylated anti-IFNγ detection mAb (Mabtech, mAb7-B6-1), diluted to 2 µg/ml in 10% FCS-PBS and incubated at room temperature for 3 hrs. The plates were then washed 6 times with PBS-Tween. Following washes, 50 µl/well of Streptavidin-HRP, diluted to 2 µg/ml in 10% FCS-Tween was added to each well and incubated at room temperature for 1 hr. The plates were again washed 6 times with 200 µl/well PBS-Tween. Novared (Vector) developing substrate was then prepared according to the manufacturer's instructions and added to the wells (100 µl/well). The developing reaction was stopped after 40 min by rinsing the plates in cold water. Spots were counted using the CTL Immunospot or Zeiss ELISPOT automated plate readers.

5.3 RESULTS

A representative example of an ELIPSOT plate depicting results of an assay is shown in Figure 5.2. The figure depicts responses to Nef peptides using cells isolated from 4 recently infected (Du cohort) individuals as well as a subtype B-infected individual, which was used as a positive control. Column 1 shows the negative control (CTR), where 10% FCS-RPMI was added in place of peptide. Column 2 is the positive control stimulus, PHA, which is a non-specific mitogen that will stimulate cells to release IFNγ. Columns 3 to 6 represent Nef pools 1 to 4 and columns 7 to 11 represent Nef matrices 1 to 5. Row 1 to 3 represents assays using cells from Du 179, Du 281 and Du 457, respectively. Rows 4 and 5 represent the subtype B-infected individual (positive control) plated out at 100 000 cells/well. Row 6 was Du 467 and the final two rows represent the positive control at 50 000 cells/well.

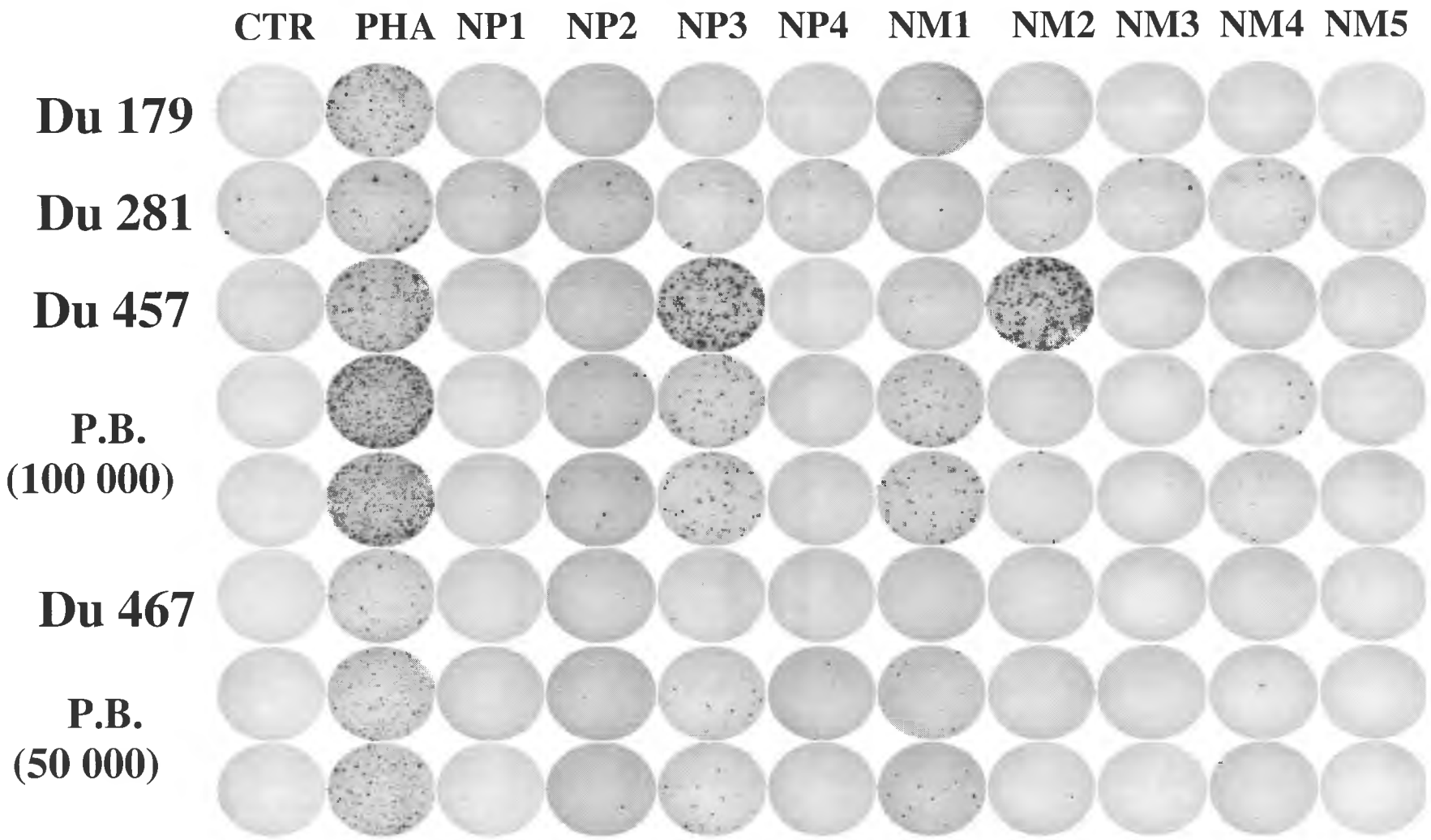

Fig 5.2 A representative example showing results of Nef ELISPOT plate

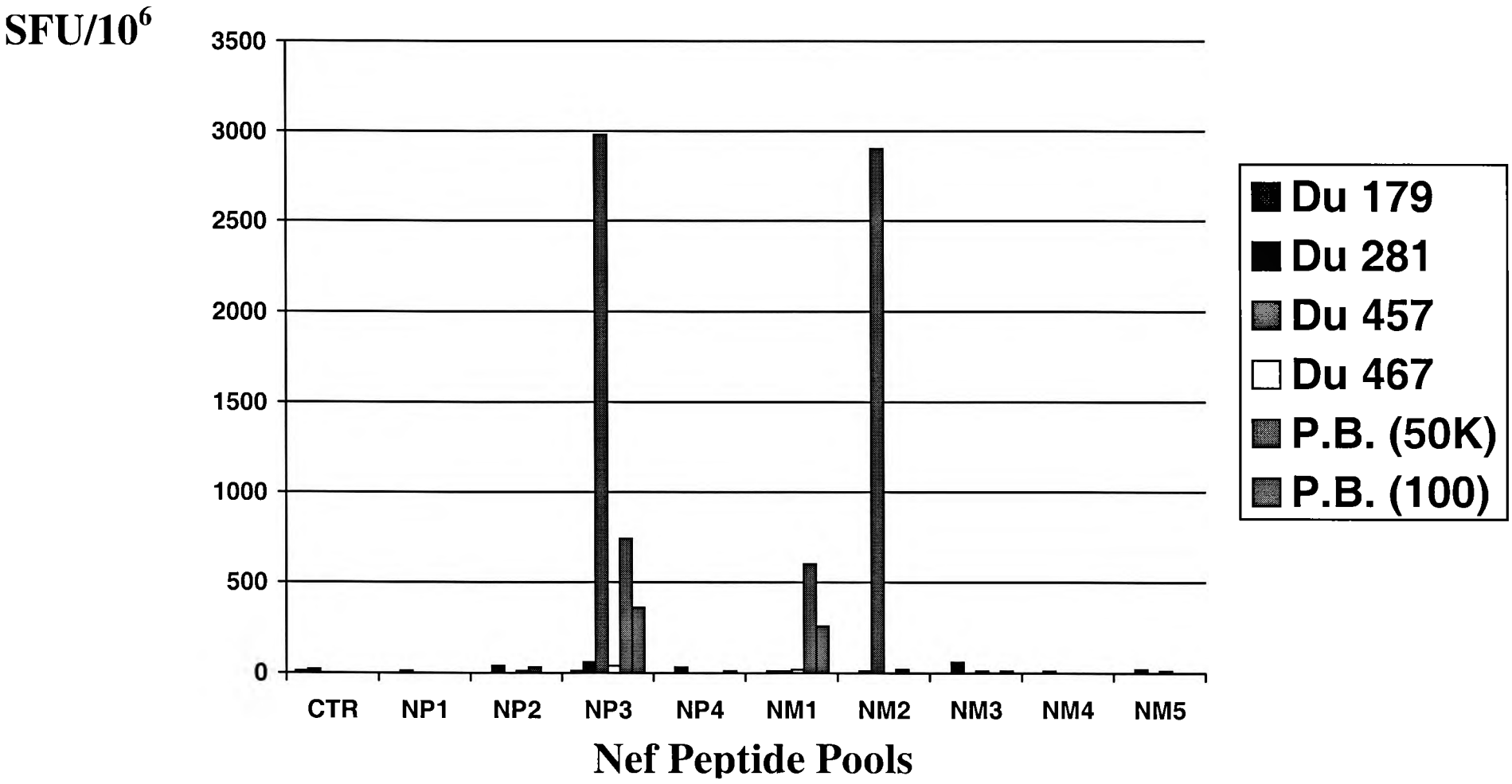

Fig 5.3: Graphical quantification of the ELISPOT results for the assay in figure 5.2. The graph shows the response of each pool and matrix and spots are expressed as SFU/10^6

For the assay shown in Figure 5.2, only Du 457 showed a response to the Nef peptides, whereas Du 179 and Du 281 showed no response. Du 467 in this assayed was later repeated as the assay failed because the PHA response was very low. There were no spots seen in the control well for Du 457, and the low background was seen in all the wells. It is clear from the figure that Du 457 cells responded well to subtype B peptides in pool 3 and matrix 2. The common peptide within the pools was peptide 12, see table 5.1. Figure 5.3 shows quantitatively the results of the assay in Figure 5.2. The frequency of CTL specific for a particular peptide is expressed as spot-forming units per million input cells (SPU/10^6 cells). These were calculated by multiplying the initial cell input by a factor to result in a million cells; therefore for 200 000, 100 000, 50 000 input cells, the factors were 5, 10 and 20 respectively. From the graph, it can be seen that Du 457 had a positive (5 times the background number spots) in pool 3 and matrix 2. The magnitude of the responses in pool 3 and matrix 2 were 2980 and 2880 SPU/10^6 cells respectively. The closeness of these values again show that the response was elicited from the same peptide (peptide 12), which was common in the two wells. The graph also shows that none of the other Du samples in this assay had a positive response to subtype B peptides.

Responses from six other Du samples were successfully identified in the same manner. The magnitude of responses for each of the seven samples is depicted in Figure 5.4. Du 115 had the lowest response (fig 5.4a) while the highest response was from Du 457 (fig 5.4g). All the graphs in this figure have the same scale to give an indication in the variation of the magnitude of responses observed.

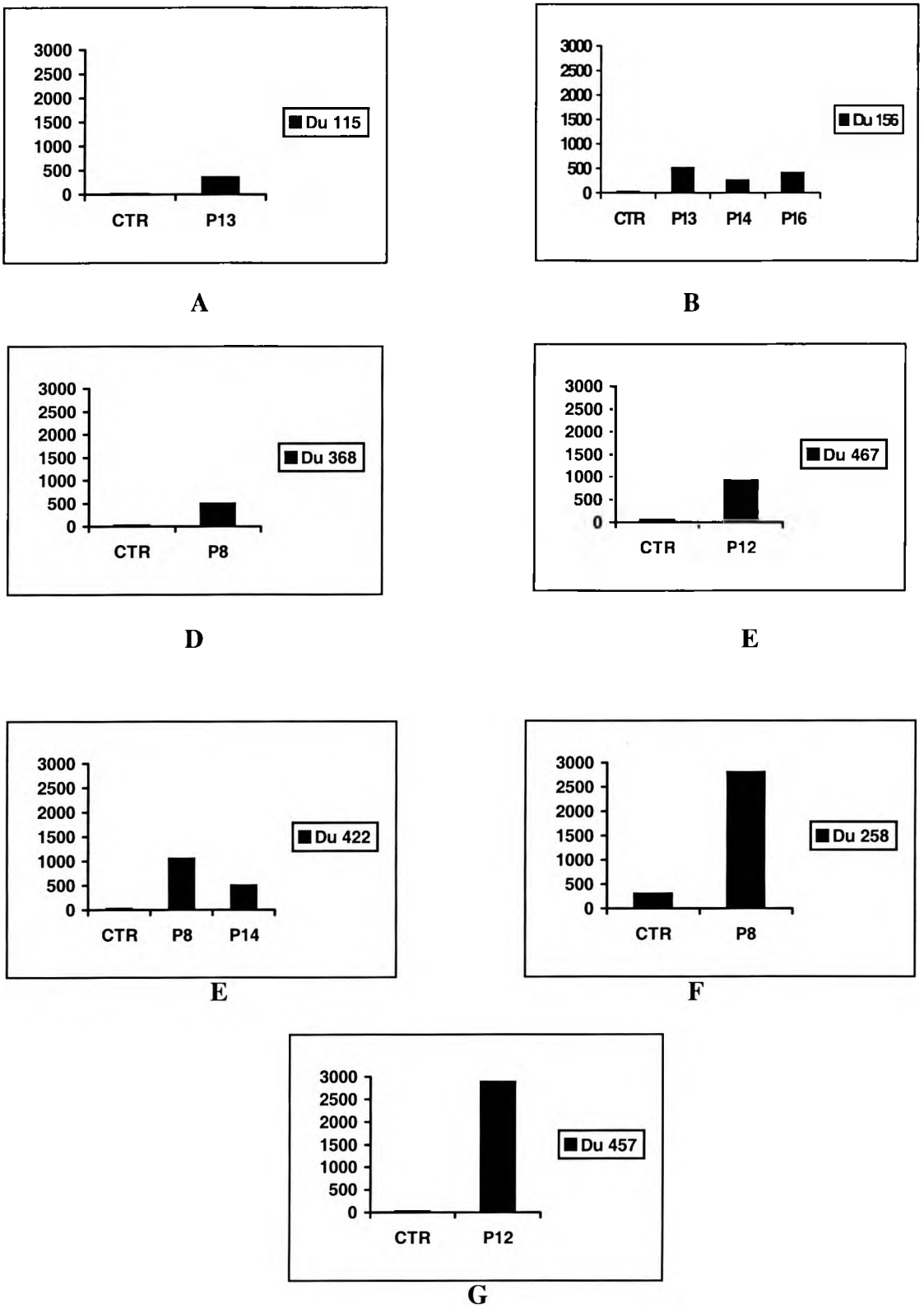

Fig 5.4: The graphs of each of the seven Du samples that gave a response to Nef. The graphs also show the magnitude of each of the response

Table 5.2 shows the overall ELISPOT results for each of the seven subtype C-infected individuals. For each specimen, the peptide giving a response was identified using the pools and matrices. Only Du 156 and Du 422 had a response to more than one peptide, responding to three and two peptides respectively. The number of spot-forming units per million cells for each specimen are shown along with spu/10^6 PBMC. The sequences for the subtype B peptides have been aligned to the autologous viral sequence identified in each Du individual. The subtype B peptides being recognised were aligned to the autologous viral sequence for the individual.

Table 5.2:Recognition of subtype B Nef peptides using PBMC from 7 subtype CHIV1infectedIndividuals using the IFNg ELISPOT assay.

	Cd4 Count	sfc/10^b PBMC	Viral load	Peptide #		amino-acid sequence	HLA haplotype
Du115	782	360	7597	13	subtype B peptide	^{121}PDWQNYTPGPGVRYPLTFGW^{140}	ND
					autologous HIV-1	----------	
Du156	372	510	22122	13	subtype B peptide	^{121}PDWQNYTPGPGVRYPLTFGW^{140}	A30, A43; B8, B58
					autologous HIV-1	----------	
		262		14	subtype B peptide	^{131}GVRYPLTFGWCYKLVPVEPD^{150}	
					autologous HIV-1	----------D----	
		410		16	subtype B peptide	^{151}KVEEANKGENTSLLHPVSLH^{170}	
					autologous HIV-1	E---------NC----M-Q-	
Du258	508	2800	9114	8	subtype B peptide	^{71}PQVPLRPMTYKAAVDLSHFL^{90}	
					autologous HIV-1	----------F--	
Du368	628	520	13993	8	subtype B peptide	^{71}PQVPLRPMTYKAAVDLSHFL^{90}	A2, A34; B13, B81
					autologous HIV-1	----------F--	
Du422	397	500	17118	8	subtype B peptide	^{71}PQVPLRPMTYKAAVDLSHFL^{90}	A24, A66; B7, B58
					autologous HIV-1	----------F--	
		1056		14	subtype B peptide	^{131}GVRYPLTFGWCYKLVPVEPD^{150}	
					autologous HIV-1	---F----------	
Du457	524	2880	19268	12	subtype B peptide	^{111}LWIYHTQGYFPDWQNYTPGP^{130}	A30, -; B18, B39
					autologous HIV-1	----------	
Du467	750	920	6658	12	subtype B peptide	^{111}LWIYHTQGYFPDWQNYTPGP^{130}	ND
					autologous HIV-1	----------	

ND – not determined

Figure 5.5 shows the specific regions that were targeted by subtype C-infected individuals. The figure shows that most of the responses were found in the middle region of Nef, where it was shown in chapter 4 that this region was the most conserved. It is likely that responses observed in this region are from known subtype B epitopes, as the degree of conservation between the subtype B peptides and subtype C sequences was very high. Only Du 156 responded to a peptide found in the more variable region of the C-terminal. The peptide sequence conferring the response was more variable when aligned with the same sequence from Du 156, with five amino acid substitutions observed. From this analysis it cannot be ascertained whether the response was from a known epitope or from a novel subtype C epitope.

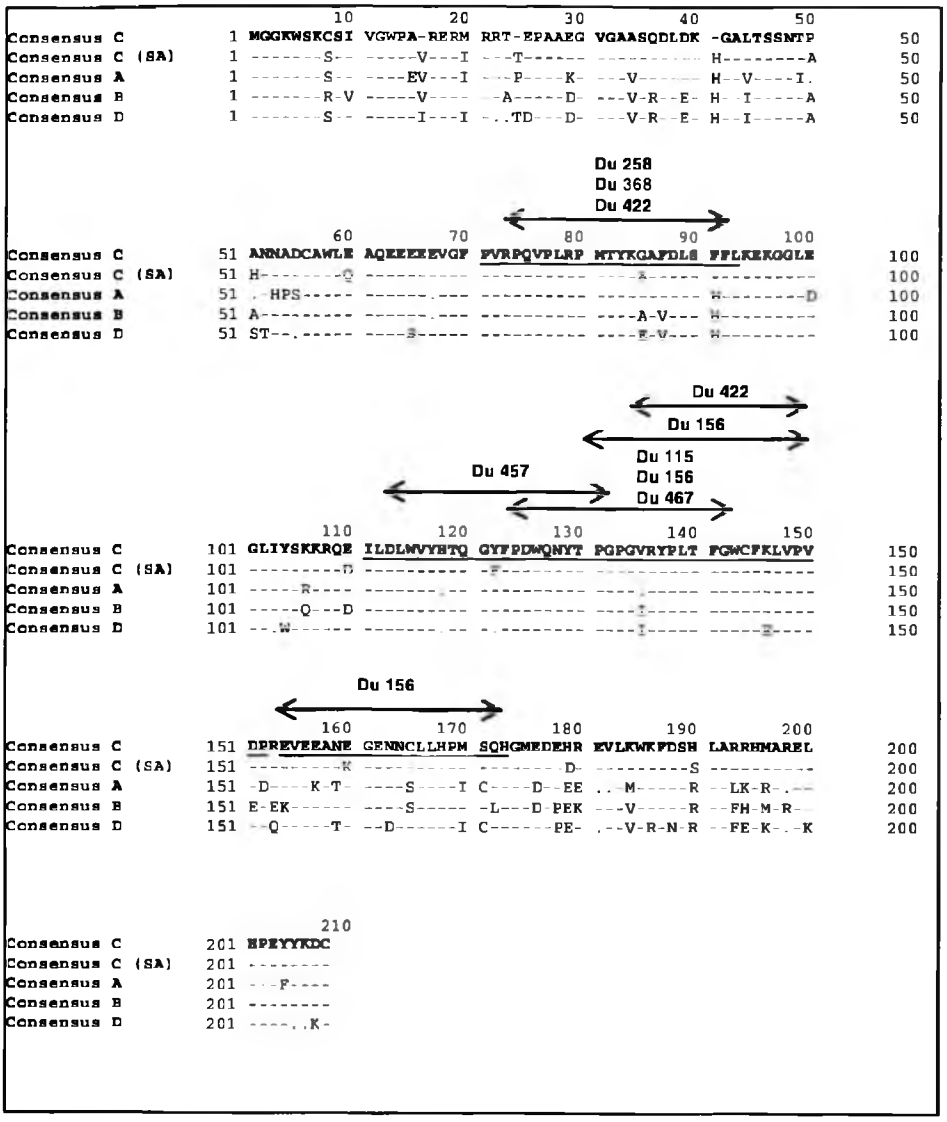

Fig 5.5: Nef regions targeted by seven recently infected individuals. The figure shows an alignment of consensus sequences (A, B, C, D and consensus C of South African sequences.

5.4 DISCUSSION

The ELISPOT assay serves as a rapid screening method for identifying immunogenic regions within viral proteins. It is a quick assay that has successfully been used in this assay to screen for immunogenic region in subtype C Nef protein. There are several advantages of using the ELISPOT assay to measure antigen-specific CTL frequency as opposed to other described assays. The assay is relatively rapid to perform, taking about 2 days rather than 12-14 days by limiting dilution (LDA) or Bulk CTL assays (Tan *et al.*, 1999). The IFNγ ELISPOT frequencies are also around 2-5 fold higher than those measured using LDA. The ELISPOT assay has been shown to be able to detect frequencies of antigen-specific responses to a frequency of 1/50000 input PBMC (Altman *et al.*, 1996), which makes it a highly sensitive assay. The major disadvantage of the assay is that it does not discriminate between the phenotype of the T lymphocyte, as CD4+ T cells also release IFNγ as a result of antigen-stimulation (Moss *et al.*, 2000). Likewise, the IFNγ ELISPOT assay does not necessarily give a measurement of killing by CD8+ T cells as other cells might be stimulated to release IFNγ upon antigen stimulation and cytokine production may not equate with killing potential.

The results in this chapter show that 7 out of 9 individuals infected with subtype C HIV-1 are able to respond to subtype B-based peptides. This is likely due to the high degree of conservation seen between subtype B and subtype C Nef sequences, particularly in the middle region which is known to be rich in Nef CTL epitopes

It was shown in chapter 4 that there was a high degree of amino acid conservation within the middle region and it is likely that these infected individuals are recognising

known epitopes. As the HLA background of these individuals is not known, it is not clear whether these peptide responses do contain known epitopes. Several epitopes identified in subtype B analysis were present in some of the peptide sequences giving responses. For example, the sequence QVPLRPMTY (restricted by HLA class I –A2, A3, A11 and B 35) is a well-known subtype B Nef epitope (Koenig *et al.*, 1990); (Culmann *et al.*, 1991); (DiBrino *et al.*, 1993). It is possible that responses to the peptide (peptide 8 – P**QVPLRPMTY**KAAVDLSHFL) containing this epitope were responding to this particular epitope as it is restricted by a variety of HLA class I molecules. Only one subtype C infected individual (Du 156) showed a response in the highly variable C-terminus. The response was from peptide 16 (KVEEANKGENTSLLHPVSLH) which had 5 amino acid substitutions to the autologous viral sequence. For this response, it is possible that there was a novel subtype C epitope presented by this individual. Or that the substitutions were not found in the anchor residues necessary for HLA restriction

From this immunological analysis, it was observed 2 out of 9 subtype C infected individuals gave no responses and those that gave responses were varied in magnitude and frequency. The range of responses could be due differences in the viral loads and CD4 counts. However, from table 5.2, however, there was no association between viral load and magnitude of responses. For example, Du 156 had a high viral load of 22122 RNA copies/ml, but had a weak and broad response ranging from 262 to 510 spu/10^6 cells. In contrast, Du 258 had a relatively lower viral load of 9114 copies/ml, but a high response of 2800 spu/10^6 cells.

In this chapter, the ELISPOT assay was successfully used to screen for sequences that contain Nef CTL epitopes in subtype C-infected individuals. It was shown that because of the high degree of Nef conservation in some regions, it was possible to use subtype B-based peptides for this purpose. The use of subtype C-based peptides, however, could lead to more responses, particularly in the C-terminus, where there is high variation. Using matched sequences would lower the possibility of selecting responses from conserved regions such as the middle part of Nef.

CHAPTER 6: SUMMARY AND CONCLUDING REMARKS

In this study established cohorts were used to characterise HIV-1 subtype C *nef*. A cohort consisting of individuals multiply-exposed to HIV-1 was subtyped by HMA to measure the prevalence of intersubtype recombination. All subtyped viruses from the Du cohort were subtype C in both the *gag* and *env* genes and served as the basis for extending the analysis to subtype C *nef*.

A *nef* PCR reaction was optimised using subtype C primers designed using a *nef* sequence from a Botswana isolate. The redesign of primers was necessary to achieve optimal amplification and accurate sequencing of subtype C infected individuals. PCR conditions were re-calibrated based on these primer designs and it was possible to amplify clean product of *nef* from isolates with viral loads as low as 161 copies/ml. Nef was subsequently amplified from 32 HIV-1 infected South Africans and sequenced.

Phylogenetic analysis of the 32 sequences obtained in this study showed that all sequences from South Africa clustered significantly (75%) together along with other subtype C reference sequences from India, Brazil, Ethiopia and Botswana. No association between Nef variability to disease stage was observed in HIV-1 subtype C infected South African individuals studied in this project.

Amino acid alignments and measure of variability showed that the middle region of Nef was significantly more conserved than the N- or the C-terminus of the polypeptide. All the functional structural motifs within subtype C Nef were highly conserved with the C-terminus of Nef being highly variable. Variability in this region

could be lack of any structural or functional motifs within this region. Amino acid alignments of consensus subtype C sequence with subtypes A, B and D consensus sequences highlighted homologous regions which are also known from reported from subtype B analysis. It was therefore hypothesised in this project that cross-clade recognition from subtype C-infected individuals would occur in the middle region of Nef. This hypothesis was explored using overlapping peptides based on a subtype B isolate. These peptides were able to evoke peptide responses in 7 out of 9 subtype C-infected individuals which were targeting the middle region of Nef.

Regions that were targeted spanned many well-known epitope regions and with further knowledge of the HLA background, epitopes can be determined. However, for the purpose of this project, HLA-independent peptide responses were identified. The highly conserved nature of Nef in the middle region along with detected responses in this region, suggest that Nef could be included into a vaccine construct. Such vaccine responses would elicit cross-clade anti-Nef responses which would be appropriate for use globally.

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APPENDIX A: Composition of buffers, gels and PCR reactions

1 Reagents for agarose gel electrophoresis

1% agarose gel

1g D-1 LE agarose (molecular grade) in 100 ml TBE buffer. The solution was heated until agarose dissolved. The solution was allowed to cool to room temperature and 5 µl 10 ml/ml ethidium bromide added.

10X TBE stock buffer:

Tris HCl	108 g
Boric Acid	55 g
0.5M EDTA	40 ml

Make up to 1 l with distilled water. Dilute 1:10 for 1X TBE and 1:20 for 0.5X TBE buffers

2. Reagents for HMA

10X Heteroduplex annealing buffer:

1M NaCl	5.84 g
1 M Tris base (pH 7,8)	10 ml
0.5M EDTA	4 ml

Make up to 100 ml with sterile, deionized, distilled water

5%PAGE gel:

30% acrylamide:bis acrylamide (30:0.8)	8.3 ml
10X TBE	5 ml
Distilled water	36.7 ml
10% ammonium persulphate	500 µl
TEMED	33 µl

3. PCR reactions

Env Nested PCR

Reagent	volume (µl)
DNA template	5
ED 5 (5 pmol)	2
ED 12 (5 pmol)	2
H2O	29.075
10x Buffer	5
dNTPs (1mM)	4
MgCl2 (25mM)	2.8
Taq polymerase	0.125
Total	50

Env RT-PCR

Reagent	volume (µl)
DNA template	5
ED 5 (5 pmol)	2
ED 12 (5 pmol)	2
H2O	29.07
10x Buffer	5
dNTPs (1mM)	4
MgCl2 (25mM)	2.8
AMV-RT	0.125
RNAsin	0.2
Taq polymerase	0.125
Total	50

For RT-PCR, the thermal cycling conditions were an initial 42°C for 60 minutes for the reverse transcriptase stage.

Gag Nested PCR

Reagent	volume (µl)
DNA template	5
MSF12 (5 pmol)	2
SK431 (5 pmol)	2
H2O	29.075
10x Buffer	5
dNTPs (1mM)	4
MgCl2 (25mM)	2.8
Taq polymerase	0.125
Total	50

Gag RT-PCR

Reagent	volume (µl)
DNA template	5
LTR 236	2
GAG 778	2
H2O	29.075
10x Buffer	5
dNTPs (1mM)	4
MgCl2 (25mM)	2.8
AMV-RT	0.125
RNAsin	0.2
Taq polymerase	0.125
Total	50

Nef PCR reactions after optimization reactions

Reagent	**Volume (µl)**	
	First round	**Second round**
10x Reaction Buffer	5	10
MgCl2	6	12
dNTP (1mM)	10	20
Primers (5 pmol)	2	4
Taq Polymerase	0.125	0.25
H20	19.875	48.75
DNA template	5	5
Total	50	100

Nef Reverse transcriptase PCR using AMV-RT (ROCHE)

Reagent	Volume (µl)
RNA template	5
Nef-0-1 (5 pmol)	2
LRR2 (5 pmol)	2
H2O	29.075
10X Buffer	5
dNTPs (1mM)	4
MgCl2 (25 mM)	2.8
AMV-RT	0.125
RNAsin	0.2
Taq polymerase	0.125
Total	50

Nef RT-PCR using Thermoscript RT

Reverse transcription using Thermoscript RT requires generation of cDNA first before amplification.

RNA degradation

Reagent	Vol(µl)
Outer nef reverse (LRR2)	1
DEPC-treated H_2O	4
Viral RNA	5
Total	10

Master Mix (RT reaction)

Reagent	vol (µl)
5x cDNA buffer	4
0.1 mM DDT	1
RNAsin	1
DEPC H_20	1
dNTPs(10 mM)	2
Thermoscript RT	1
Total	10

Ten microlitres of the master mix was then added to the RNA mix and mixed gently. The tube was then incubated at 58oC for 50 min to generate cDNA and the reaction stopped by incubating at 85oC for 5 min.

Sequencing Reaction

The sequencing reaction was prepared on ice in the absence of fluorescent light.

Reagent	**Volume (µl)**
Terminator Ready Reaction Mix	8
PCR product	6
Primer (3.2 pmol)	1
dH20	5
Final volume	20

APPENDIX B: Reagents and Suppliers

Acrylamide	Roche Diagnostics, USA
ABI PRISM 377 DNA Sequencer	Perkin Elmer Applied Biosystems, USA
Ammonium persulphate	Pharmacia Biotech, Sweden
AMV-RT	Roche Diagnostics, USA
Baxter water	Adock Ingram Limited, South Africa
Bis-Acrylamide	Roche Diagnostics, USA
Boric acid	Merck, Germany
Bromophenol blue	Merck, Germany
CentriSep Spin Columns	Applied Biosystems, Perkin Elmer, Great Britain
D-1 LE Agarose	Whitehead Scientific, South Africa
DMSO	Sigma-aldrich GmbH, Germany
DNASIS Sequencing Software	Hitachi Software Engineering, USA
dRhodamine Terminator Cycle Sequencing kit	Applied Biosystems, Perkin Elmer, Great Britain
dNTP	Roche Diagnostics, USA
ddNTP	Roche Diagnostics, USA
EDTA	Roche Diagnostics, USA
Ethidium Bromide	Promega, USA
Ethanol	Merck, Germany
Ficoll Hypaque	Amersham Pharmacia Biotech AS, Sweden
Foetal Calf Serum	Sterilab, UK

GeneAmp PCR System 2400 thermocycler	Applied Biosystems, Perkin Elmer, Great Britain
PBS	Merck, Germany
PHA	Roche Diagnostics, USA
Primers	Department of Biochemistry, University of Cape Town, South Africa
Proteinase K	Roche diagnostics, USA
QIAamp Viral RNA Isolation kit	QIAGEN GmbH, Germany
RNAse Inhibitor	Roche Diagnostics, USA
SDS	Merck Germany
Sequenase PCR product Sequencing kit	Amersham Life Science Inc., USA
Streptomycin	Sigma-Aldrich GmbH, Germany
SuperTherm Taq Polymerase (including 10X buffer and $MgCl_2$)	Advanced Biotechnologies Limited, UK
TEMED	Stratagene cloning system, USA
Thermoscript RT	GibcoBRL, Life Technologies, Scotland
Tris-HCl	Roche diagnostics
Triton X-100	Roche diagnostics, USA
Tween 20	Merck GmbH, Germany
Treeview Analysis Program	Roderick Page, Scotland
Xylenexyanol	Fluka AG, Switzerland

APPENDIX C

PRESENTATIONS AND MANUSCRIPTS ARISING FROM THIS PROJECT

1999

Presented a Poster the University of the Witwatersrand Faculty of Health research day

Title "Prediction and Characterisation of HLA Class I-restricted Cytotoxic T Lymphocyte Epitopes from Regions Within *Nef* in Hiv-1 Subtype C-Infected Individuals".

2000

Gave an oral presentation at the XIII International AIDS Conference in Durban

Title "Conservation of South African HIV-1 Subtype C Nef sequences at different stages of disease progression: implications for vaccine strategies".

Abstract Number: WeOrA598

2001

Gave an oral presentation at the Scientific discoveries from the Du cohort meeting in Durban

Title "Molecular and immunological characterisation of HIV-1 subtype C Nef"

2001

Mashishi TN, van Harmelen J, Puren AJ, Morris L, Gray G, Ballard R, Ramjee G, Abdool Karim S, Williamson C and Gray CM. Immune recognition of conserved regions of South African subtype C HIV-1 Nef: Implications for Vaccine design. (In Preparation)

APPDENDIX D: Ethical Clearance