HIV GENOTYPING

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DECLARATION OF INDEPENDENT WORK

I, LEZELLE BOTES, do hereby declare that this research project submitted to the Central University of Technology, Free State, for the degree DOCTOR TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY is my own independent work that has not been submitted before to any institution by me or any other person in fulfilment of the requirements for the attainment of any qualification.

SIGNATURE OF STUDENT

24/01/06

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SUMMARY

The development of viral resistance to antiretroviral drugs used for treatment of human immunodeficiency virus 1 (HIV) infection is an important cause of antiretroviral treatment (ARV) failure and limits options for alternative antiretroviral regimens. Prevention, characterisation and clinical management of such resistance are receiving increasing attention.

The primary objective of this project was to study the naturally occurring variants of HIV-1 present in a group of ARV-naïve patients. The presence of pre-existing mutations may aid clinicians in designing optimal ARV combinations for the country. The secondary objective was to choose a suitable method for doing the analysis. Published primer sequences and in-house methods for the different steps of the procedure were used, but this was changed to an established commercial system (viroseq) because of superior sensitivity and the fact that it is FDA approved. The study population consisted of 19 adult ARV-naïve AIDS patients recruited from Tsepo House, and Medi Inn, Bloemfontein.

The CD4 counts indicated that the immune systems of these patients were severely compromised, the highest count being 348 and the mean value 184 cells/mm^3 whole blood. Therefore, according to the Department of Health's criteria, all of them qualified for ARV treatment. The viral loads were high, varying from 23 000 to >750 000 RNA copies/ml plasma. This demonstrates how people differ in their response to the viral infection. All patients were in the terminal stage of disease, yet displayed up to thirty-fold differences in viral load.

After RT-PCR and sequencing, the sequences were trimmed to 99 codons of the full protease and the first 335 codons of the reverse transcriptase reading frames. These were translated to amino acids and used separately in phylogenetic analyses to study their relatedness to each other and to other isolates. A benefit of phylogenetic analysis is to indicate possible

contamination of one sample by another as this will show tight clustering of some samples. The form and branch distances of the trees found clearly eliminated this possibility. The sequences were compared to 20 other South African isolates, randomly chosen from the Los Alamos (http://www.hiv.lanl.gov/content/index) HIV-1 sequence database. Both the protease and reverse transcriptase trees revealed that the Bloemfontein sequences do not differ appreciably from those found in the rest of the country, since they tend to diffuse through the tree rather than to cluster on their own.

All the patients tested positive for subtype C, complying with the demographical data. Very few mutations were detected in the reverse transcriptase (RT) reading frame, although a mutation (K103N) which confers high level resistance against non-nucleoside RT inhibitors was found in one isolate. This may mean that a small percentage of patients may harbour a virus that is naturally resistant to these drugs. In the protease reading frame, mutations at nine amino acid positions have been designated primary or major resistance mutations. None of the primary mutations were found, although several secondary mutations (of lesser significance) contributing to reduced susceptibility (e.g., M36I and I93L) were found in 95% of our samples. This was not unexpected as these polymorphisms are extremely common in subtype C viruses. These genetic differences may be clinically relevant when considering long-term strategies for patients infected with non-B subtypes.

As the public sector ARV rollouts gather momentum, the emergence of drug-resistant isolates is sure to follow and the laboratory services must be geared to provide the backup needed by the clinicians to plan salvage therapy. This project has contributed to the baseline knowledge of the infected population enabling us to anticipate the emerging resistance mutations.

OPSOMMING

Ontwikkeling van virale weerstandigheid teen antiretrovirale middels wat gebruik word vir die behandeling van menslike immuniteitsgebrek virus 1 (MIV-1) infeksies is 'n belangrike oorsaak van mislukking van antiretrovirale behandeling en beperk die opsies vir alternatiewe behandeling. Voorkoming, karakterisering en kliniese beheer van sulke weerstandigheid geniet al hoe meer aandag.

Die primêre doelwit van hierdie projek was om die natuurlike variante van MIV-1 te bestudeer in 'n groep antiretrovirale naïewe pasiënte. Die teenwoordigheid van voorafbestaande mutasies mag geneeshere help in die daarstelling van optimale antiretrovirale kombinasies vir die land. Die sekondêre doelwit was om 'n geskikte metode vir die uitvoering van analises te kies. Gepubliseerde priemstuk nukleotiedvolgordes en metodes vir die verskillende stappe van die prosedure was gebruik, maar is later vervang met 'n gevestigde kommersiële sisteem (Viroseq) as gevolg van beter sensitiwiteit en die feit dat dit FDA goedgekeur is. Die studiepopulasie het bestaan uit 19 volwasse antiretrovirale naïewe pasiënte gewerf vanaf Tsepo Huis en Medi Inn, Bloemfontein.

Die CD4 telling toon dat die immuunsisteme van hierdie pasiënte erg gekompromitteer was met 'n hoogste telling van 348 en 'n mediaanwaarde van 184 selle/mm^3 heelbloed. Gevolglik, na aanleiding van die Departement van Gesondheid se kriteria, kwalifiseer almal vir antiretrovirale behandeling. Die virusladings was hoog en varieer van 23 000 tot >750 000 RNA kopieë/ml plasma. Dit demonstreer hoe mense verskil in hul reaksie tot virale infeksie. Alle pasiënte was in die terminale fase, maar het tog 30-voudige verskille in viruslading getoon.

Na trutranskripsie en amplifikasie is die nukleotiedvolgordes bepaal. Die volgordes is beperk tot die 99 kodons van die volle protease en die eerste 335 kodons van die trutranskriptase leesrame. Daarna is dit omgeskakel na

aminosure en afsonderlik gebruik in filogenetiese analise om die verwantskap aan mekaar en aan ander isolate te bestudeer. 'n Voordeel van filogenetiese analise is om kontaminasie van een monster deur 'n ander aan te dui aangesien dit digte groepering van sommige monsters toon. Die vorm en afstande van die bome gevind elimineer hierdie moontlikheid. Die nukleotiedvolgordes is vergelyk met 20 ander Suid-Afrikaanse isolate, lukraak gekies van die Los Alamos MIV-1 databasis. Beide die protease en die trutranskriptase bome toon dat die Bloemfonteinse nukleotiedvolgordes nie merkwaardig verskil van die in die res van die land nie, aangesien hulle deur die boom versprei eerder as om afsonderlik saam te groepeer.

Al die pasiënte het positief getoets vir subtipe C, wat ooreenstem met die demografiese data. Min mutasies is opgespoor in die trutranskriptase leesraam, alhoewel een mutasie (K103N), wat aanduidend is van 'n hoë vlak van weerstandigheid teen nie-nukleosied trutranskriptase inhibeerders in een isolaat gevind is. Dit mag beteken dat 'n klein persentasie van pasiënte 'n virus huisves wat van nature weerstandig is teen hierdie middels. In die protease leesraam word mutasies by nege aminosuur posisies aangedui as primêre of hoof weerstands-mutasies. Nie een van die primêre mutasies is gevind nie, alhoewel verskeie sekondêre mutasies (van mindere belang) wat bydra tot verminderde vatbaarheid (bv. M36I en I93L) in 95% van ons monsters gevind is. Dit was nie onverwags nie aangesien hierdie polimorfismes uiters algemeen in subtipe C virusse voorkom. Hierdie genetiese verskille mag klinies betekenisvol wees wanneer langtermyn strategieë in ag geneem word vir pasiënte wat geïnfekteer is deur nie-B subtipes.

Soos wat die publieke sektor se antiretrovirale program momentum opbou, is die verskyning van middel-weerstandige isolate onvermydelik en sal laboratoriums gerat moet wees om die nodige ondersteuning aan geneeshere te verskaf rakende terapie. Hierdie projek het bygedra tot die basislyn kennis van die geinfekteerde populasie wat ons in staat stel om verwagte weerstandbiedende mutasies te antisipeer.

ABBREVIATIONS & SYMBOLS

SYMBOLS:

ψ	psi
%	percentage
α	alpha
β	beta
≥	greater/equal to
κ	kappa
μ	mu
σ	sigma
ºC	Degrees Celsius
(-) C	negative control
(+) C	positive control
3TC	Lamivudine

A

ABC	Abacavir
AG 1549	Capravirine
AIDS	Acquired Immune Deficiency Syndrome
Ala	Alanine
ANPs	Acyclic nucleoside phosphonate analogs
APV	Amprenavir
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
AV-HRP	Avidin-Horseradish Peroxidase Conjugate
AZT	Zidovudine; 3' –azido-2'3'-dideoxythymidine

B

BIV	Bovine Immunodefieciency Virus

C

C	Cysteine
CA	Capsid protein
CCR2b	CC-chemokine receptor 2b

CCR3	CC-chemokine receptor 3
CCR5	CC-chemokine receptor 5
CD	Cluster of Differentiation
CDC	Centers for Disease Control
cDNA	complementary Deoxyribonucleic acid
CRM1	Chromosome Region Maintenance Protein 1
CTD	C-terminal domain
CXCR4	CXC-chemokine receptor 4
CypA	Cyclophilin A

D

d4T	Stavudine
ddC	Zalcitabine
ddH2O	Deionized, distilled water
ddI	Didanosine
ddNTP	2'3' dideoxyribonucleoside-5'-triphosphate
	diphenyltetrazolium bromide
DLV	Delavirdine
DNA	Deoxyribonucleic acid
dNTP's	deoxynucleoside-triphosphate
DTT	1,4-dithiothreitol

E

EFV	Efavirenz
EDTA	ethylenediaminetetraacetic acid
e.g.	example
Elisa	Enzyme-linked immunosorbent assay
env	envelope gene
ER	Endoplasmic Reticulum

F

FDA	US Food and Drug Administration

G

gag	group antigen gene
GCN4	General Control of Nitrogen gene 4
Gln	Glutamine
Glu	Glutamic Acid

Gly	Glycine
gp	glycoprotein
GTP	Guanosine triphosphate

H

h	hours
H(+)C	High positive control
HA	Hemagglutinin
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HIV-DIL	HIV Diluent
HMG	High Mobility Group
hRip/Rab	Nucleoporin-like protein
HTLV-1	Human T-cell leukaemia virus type 1
HYB	Hybridization Buffer

I

IC_{50}	50% Inhibitory concentration
IC_{90}	90% Inhibitory concentration
IDV	Indinavir
Ig	Immunoglobulin
Ile	Isoleucine
IN	Integrase

K

Kb	Kilobase
K_d	Dissociation constant
Kd/Kda	Kilodalton

L

l	litre
L(+)C	Low positive control
Lck	Lymphocyte-specific tyrosine kinase
Leu	Leucine
LiPA	Line probe assay
LPV	Lopinavir
LTR	Long Terminal Repeat

LYS	Lysis buffer
Lys	Lysine

M

MA	Matrix protein
MAP	Mitogen Activated Protein (kinase)
Mg	Magnesium
MHC	Major Histocompatibility Complex
MHR	Major Homology Region
min	minutes
MIP-1α/MIP-1β	Macrophage Inflammatory Protein 1α and 1β
MKC-442	Emivirine
ml	millilitre
Mn	Manganese
Mo-MLV	Moloney murine leukaemia virus
mRNA	mitochondrial RNA
MT	Mutant
MTT	3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyltetrazolium bromide
MuLV	Murine Leukemia Virus
MWP	Microtitration Plate

N

N	Asparagine
n	nano
NAK	Nef Associated Kinase
NC	Nucleocapsid Protein
Nef	Negative regulatory factor
NES	Nuclear Export Signal
NFV	Nelfinavir
NHP	Negative Human Plasma
NLS	Nuclear Localization Signal
NM	Nanomolar
Nm	Nanometer
NMR	Nuclear Magnetic Resonance
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NSI	Non-syncytium Inducing
NVP	Nevirapine

O

OH	Hydroxyl group
OD	Optical Density

P

PAK	P21 Kinase
PBMCs	Peripheral blood mononuclear cells
PBS	Primer Binding Site of Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFA	Foscamet
PHA	Phytohemagglutinin
PIC	Pre-integration complex
PMEA	Adefovir
PMPA	Tenofovir
pol	Polyprotein gene
PP_2A	Protein Phosphatase 2A
PR	Protease
Pro	Proline
PXXP	SH_3 domain binding sequence
Phe	Phenylalanine
PIs	Protease Inhibitors

Q

QS	Quantitation Standard

R

RANTES	Regulated an Activation, Normal T Expression and secreted
Rch1	Synonym for importin α1
RegaInst	Rega Institute
Rev	Regulator of viral expression
RNA	Ribonucleic acid
RNase	Ribonuclease
rpms	Revolutions per minute
RRE	Rev responsive element
RT	Reverse transcriptase
RTV	Ritonavir

S

SDF-1	Stomal cell-derived factor 1
sec	Seconds
SEQ	Sequence
Ser	Serine
SH3	Src-homology-3
SI	Syncytium Inducing
SIV	Simian immunodeficiency virus
SL3	Stem-loop-3 of the HIV-1 ψ-RNA
Snf5	Sucrose non-fermenting gene 5
snRNA	Small nuclear RNA
snRNPs	Small nuclear ribonuclear proteins
SQV	Saquinavir
SU	Surface Envelope Protein
Suppl	Supplement

T

TAR	Trans-activating response element
Tat	Transactivator of transcription
TBE	Tris-Borate-EDTA
TBP	TATA-box binding protein
Thr	Threonine
TM	Transmembrane
tRNA	Transfer ribonucleic acid
tRNAlys	Transfer ribonucleic acid lysine
Tyr	Tyrosine

U

U1	Unique region of the 1' LTR
U3	Unique region of the 3' LTR
U5	Unique region of the 5' LTR
UN	United Nations

V

Vif	Virion infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

W

WB	Wash Buffer
WHO	World Health Organization

X

XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Z

ZDV	Zidovudine

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CHAPTER 1

INTRODUCTION

The development of viral resistance to antiretroviral drugs used for treatment of human immunodeficiency virus (HIV) infections is an important cause of treatment failure (Saag *et al.*, 1993; Kozal *et al.*, 1994; Richman *et al.*, 1994; D'Aquila *et al.*, 1995; Schuurman *et al.*, 1995; Havlir *et al.*, 1996) and limits options for alternative antiretroviral regimens. Prevention, characterization and clinical management of such resistance are receiving increasing attention.

The virus population in a person infected with an RNA virus (e.g., HIV-1, hepatitis C virus) has been termed quasi species (Eigen, 1993), which refers to the existence of genetically distinct viral variants that evolve from the initial virus inoculum. The variants are generated because DNA proofreading mechanisms that preserve the genetic composition of organisms with double-stranded DNA genomes do not exist for RNA viruses. Thus as single-stranded RNA viruses replicate, each newly copied genome differs from the parental virus on average by a single nucleotide (Drake, 1993; Mansky & Temin, 1995). Viral polymorphisms (genetic variants with apparently equivalent fitness [replication capacity]) are commonly seen in virus populations in infected persons. Nucleotide differences may be "neutral", be deleterious, or confer replicative advantage if selective pressures such as immune responses or drug treatments change. These possibilities illustrate the survival strategy of organisms with high mutation rates that provides a large pool of genetic variants able to adapt rapidly to changing selective pressures (Coffin, 1996; Leigh Brown & Richman, 1997).

An estimated 10 billion HIV-1 virions are produced daily in established HIV infection (Perelson *et al.*, 1996). If each contains on average 1 mutation per 9200-nucleotide genome, replication-competent virus with every possible single drug-resistance mutation is likely to be generated daily. Double mutants are less likely, and the probability of 3 or more drug-resistant mutations in the same genome is very low (Perelson *et al.*, 1996). These estimates are supported by observations in infected persons. Virus or HIV-1 RNA with single drug-resistance mutations have been isolated from treatment-naïve patients or those infected before antiretroviral drug availability (Najera *et al.*, 1994; Najera *et al.*, 1995; Wong *et al.*, 1997). Mathematical modelling of the rate of resistance emergence after nevirapine treatment in previously untreated persons permits estimates of plasma prevalence of HIV-1 variants with nevirapine-resistance mutations before treatment. About 1 in 1000 copies/ml of plasma HIV-1 RNA contains the tyrosine-to-cysteine mutation at amino acid residue 181 of the reverse transcriptase conferring nevirapine resistance (Havlir *et al.*, 1996).

When antiviral drug selective pressure is applied to viral quasi species in an infected person, pre-existing minor viral species resistant to that drug rapidly become predominant and are selected as the most fit species in the presence of the drug. For some antiretroviral drugs, such as lamivudine and certain non-nucleoside reverse transcriptase inhibitors (NNRTIs), a single mutation can confer high-level resistance. When these drugs are given in combinations only partially suppressing virus replication, drug-resistant mutants predominate within weeks (Havlir *et al.*, 1996; Wei *et al.*, 1995; Van Leeuwen *et al.*, 1995). For some other drugs, such as zidovudine and certain protease inhibitors, high-level resistance requires the accumulation of 3 or more mutations in a single viral genome (Larder & Kemp, 1989; Condra *et al.*, 1995; Molla *et al.*, 1996). These highly resistant variants emerge more slowly, requiring months to predominate during less than maximum viral suppression (Condra *et al.*, 1995; Molla *et al.*, 1996; Larder *et al.*, 1989; Richmann *et al.*, 1991), supporting the prediction that

genetic variants with multiple mutations are present at much lower levels than those with single mutations in untreated patients. Development of high-level resistance to these drugs requires viral replication and selective drug pressure. Persistent viral replication permits further viral evolution leading to drug resistance by cumulative mutation acquisition.

Assuming a high degree of adherence and adequate drug absorption, the settings in which resistance testing is likely to prove helpful are (1) early after therapy initiation if only a minimal plasma HIV RNA decline occurs over the first 4 to 12 weeks, suggesting sub-optimal treatment response; (2) during early virologic breakthrough; (3) during more prolonged virologic replication in which more extensive resistance might be suspected; and (4) prior to therapy to provide a baseline for longitudinal testing, likely to become a more common component of patient monitoring. Therefore, to conclude, emerging data indicate that, despite limitations, resistance testing should be incorporated into patient management in some settings. Resistance testing is recommended to help guide the choice of new regimens after treatment failure and for guiding therapy for pregnant women. It should be considered in treatment-naïve patients with established infection, but cannot be firmly recommended in this setting. Testing should also be considered prior to initiating therapy in patients with acute HIV infection, although therapy should not be delayed pending the results.

The scale of the HIV/AIDS epidemic in South Africa needs no further explanation. The patients fall into two treatment categories, namely state and private. At the moment only a limited amount of antiretroviral treatment is being done in the state sector, where treatment is restricted to general immune system boosting and symptomatic treatment. In the private sector, however, anti-retrovirals are being prescribed on a par with the developed world. Antiretrovirals do prolong the lifespan of AIDS patients, but they do have several disadvantages. The cost of the medication is very high, and there are sometimes side-effects. The most worrying problem, however, is that of resistance to the drugs. In resistant cases,

viral loads creep up after being low for anything from one to five years, indicating impending relapse into full-blown AIDS. The resistance develops due to mutations that accumulate in the viral genes coding for the reverse transcriptase and the protease. The type of mutation can be correlated with a specific drug as each drug has a series of mutations conferring resistance to it. There are several ways of determining these mutations, but these have all been developed in Europe or North America and are specific for the virus strains occurring there. It needs to be established whether these assays will work reliably with the strains common in South Africa.

Having access to both treated and untreated patients puts us in an ideal situation to study the phenomenon of drug resistance. Therefore, the main objective of this study was to optimise and implement a method for the detection of all variants of the HIV-1 virus. In the cohort of untreated patients the naturally occurring variants of the virus, which could yield information of possible existing resistance against drugs, was established. In the process, previously-published methods for viral RNA isolation, RT-PCR and sequencing were evaluated, but the final decision was to use a commercial assay because of superior sensitivity and quality of sequence.

CHAPTER 2

LITERATURE REVIEW

2.1 HISTORICAL OVERVIEW

In 1981 the Centres for Disease Control (CDC) began to receive reports of an increasing incidence of two previously very rare conditions. One was a form of pneumonia caused by *Pneumocystis carinii* and the other a very unusual skin cancer named *Karposi's sarcoma* (Muesing *et al.*, 1985). Some patients were afflicted by both conditions. It was determined that the immune system of these patients was impaired, and in 1982 the CDC recognised a new disease called *acquired immune deficiency syndrome (AIDS)*. Unlike any other infectious disease in modern medical history, AIDS has galvanised the concern and efforts of physicians, scientists, and the lay public alike. The magnitude of the mounting AIDS problem is indeed sobering. It became clear that an infectious agent was involved and that transmission of the disease required the transfer of bodily fluids (sexual contact, exposure to infected blood or blood products, and perinatal transmission from mother to child) from an infected to an uninfected individual (Greene, 1991).

Thus while AIDS is primarily a sexually transmitted disease, some people who had received blood transfusions or blood products such as Factor VIII, used in the treatment of haemophilia, were also affected. Blood supplies are now protected, but intravenous drug users who share needles remain a major risk group (Levine, 1985). It was in 1983, only 2 years after the appearance of the previously unknown disease, that the causative agent of AIDS was isolated. It was found to be a human

retrovirus that was later named *human immunodeficiency virus (HIV-1)*. Subsequently a second virus, HIV-2, was discovered in Africa, therefore it became a catastrophic epidemic that no country, and no group of individuals, has escaped (Levy *et al.*, 1985).

Acquired immune deficiency syndrome (AIDS) ranks as one of the most important infectious diseases. Since its identification more than two decades ago, the AIDS epidemic has resulted in a total of 21.8 million deaths, including the deaths of 4.3 million children (UNAIDS/WHO, 2000). Thirty-six million people are currently living with the human immunodeficiency virus (HIV) and 5.3 million people were infected with HIV in 2000 alone. Clearly, AIDS represents an international health crisis that threatens to overwhelm even the best systems of health care delivery. The rational design and development of drug therapies to control the virus and the preparation of an effective vaccine to prevent infection represent public health goals of the highest priority. However, substantial progress in these areas will probably hinge on achieving a more complex understanding, in molecular and biochemical terms, of this pathogenic retrovirus and the nature of its cytopathic interplay with its cellular host.

2.2 IS HUMAN IMMUNODEFICIENCY VIRUS THE CAUSE OF AIDS?

Is there any evidence that can prove that AIDS is caused by the Human Immunodeficiency Virus? HIV was identified as the causative agent for AIDS in 1983 (Barré-Sinnoussi *et al.*, 1983; Gallo *et al.*, 1984). The most straightforward evidence is that HIV can be isolated from almost all patients with AIDS, and that patients with more advanced disease have higher amounts of the virus present. Also, antibodies to HIV are found in asymptomatic individuals who later go on to develop full-blown AIDS. Furthermore, recipients of blood contaminated with HIV will subsequently develop AIDS, even if they do not have any of the other risk factors

associated with AIDS. Similarly, medical and laboratory staff with needle stick injuries involving HIV-infected blood has developed AIDS. About thirty percent of HIV-infected mothers transmit the virus to their unborn children, who subsequently develop AIDS. The uninfected siblings of these children do not develop AIDS. Thus, the common link between AIDS-affected individuals in very different social groups is the presence of HIV. Although the principal modes of transmission of HIV differ in different populations, epidemiological data currently show an absolute correlation between HIV infection and the subsequent development of AIDS; AIDS does not appear in a new location without evidence of prior HIV infection. Epidemiological studies and attempts to predict the course of the epidemic are difficult because of the interval -up to 8 to 10 years - between infection with HIV and the development of AIDS. Indeed, it is not yet certain that all individuals infected with HIV will go on to develop AIDS. It is clear, however, that there is a very high mortality among individuals who develop AIDS.

2.3 HIV SUBTYPES

Like the epidemic, the viruses responsible for AIDS have proven to be more complicated and much more unpredictable than initially thought. The AIDS viruses are members of the lentivirus family of retroviruses and have been demonstrated to exhibit remarkable properties of insidious disease induction, persistence, latency, variation, recombination, and escape from immune and drug pressures.

There are two distinct types of HIV namely, HIV-1 and HIV-2. These two types are distinguished on the basis of their genome organisation and phylogenetic relationships with other primate lentiviruses. Both have been further sub-classified on the basis of phylogenetic criteria. HIV-1 consists of three distinct virus groups, which have been termed groups M (main), O

(outlier) and N (non-M, non-O). The predominant HIV-1 group M comprises 11 clades denoted subtypes (A to K). The other type of HIV is HIV-2 and comprises 6 distinct phylogenetic lineages, subtypes (A to F) (Hahn *et al.*, 2000). The elucidation of the simian origins of AIDS as well as circumstances and factors contributing to the initiation of the epidemic was achieved by the reconstruction of the phylogenetic relationships among the many strains of HIV-1 and HIV-2, as well as related viruses from African primates.

Current evidence indicates that the SIV counterparts of HIV-1 and HIV-2 were introduced into the human population at least seven times or more (Gao *et al.*, 1999; Chen *et al.*, 1997). However, the HIV-1 group M viruses, which are responsible for the great majority of all HIV infections worldwide, appear to have arisen from just one such cross-species transmission event. The human race is not the natural host of either the HIV-1 or HIV-2 types. These two types of HIV have entered the human population as a result of cross-species transmission from chimpanzees (Gao *et al.*, 1999) and sooty mangabeys (Hirsch *et al.*, 1989; Gao *et al.*, 1992), respectively.

The initial epicentres of HIV both HIV-1 and HIV-2 infection appeared to be Central Africa and West Africa, with HIV-2 primarily in West Africa. HIV-1, on the other hand, spread globally. Direct human contact with infected simian blood probably resulted in the cross-species transmission of SIV to humans, and social, economic and behavioural changes that occurred in the early- and mid-20th century provided the circumstances whereby these viruses could expand and reach epidemic proportions (Gao *et al.*, 1999; Hahn *et al.*, 2000; Kober *et al.*, 2000).

2.4 THE HIV-1 VIRION STRUCTURE

The HIV-1 virion is an icosahedral structure, (Gelderblom *et al.*, 1987) containing 72 external spikes and is about 110 nm in diameter (Figure 2.4.1). Two major viral-envelope proteins, gp120 and gp41 form the spikes. The HIV-1 lipid bilayer is studded with various host proteins, including Class I and Class II histocompatibility antigens, obtained during virion budding. Four nucleocapsid proteins, p24, p17, p9, and p7 are contained in the core of HIV-1, each of which is proteolytically cleaved from a 53-kd Gag precursor by the HIV-1 protease. The main component of the inner shelf of the nucleocapsid is formed by the phosphorylated p24 polypeptide (Figure 2.4.1), whereas the myristoylated p17 protein is associated with the inner surface of the lipid bilayer and probably stabilises the exterior and interior components of the virion. The p7 protein binds directly to the genomic RNA through a zinc-finger structural motif and, together with p9, forms the nucleoid core. Importantly, this retroviral core also contains two copies of the single-stranded HIV-1 genomic RNA that is associated with the various pre-formed viral enzymes, including the reverse transcriptase, integrase, and protease (Coffin, 1995; Parren *et al.*, 1999; Turner and Summers, 1999).

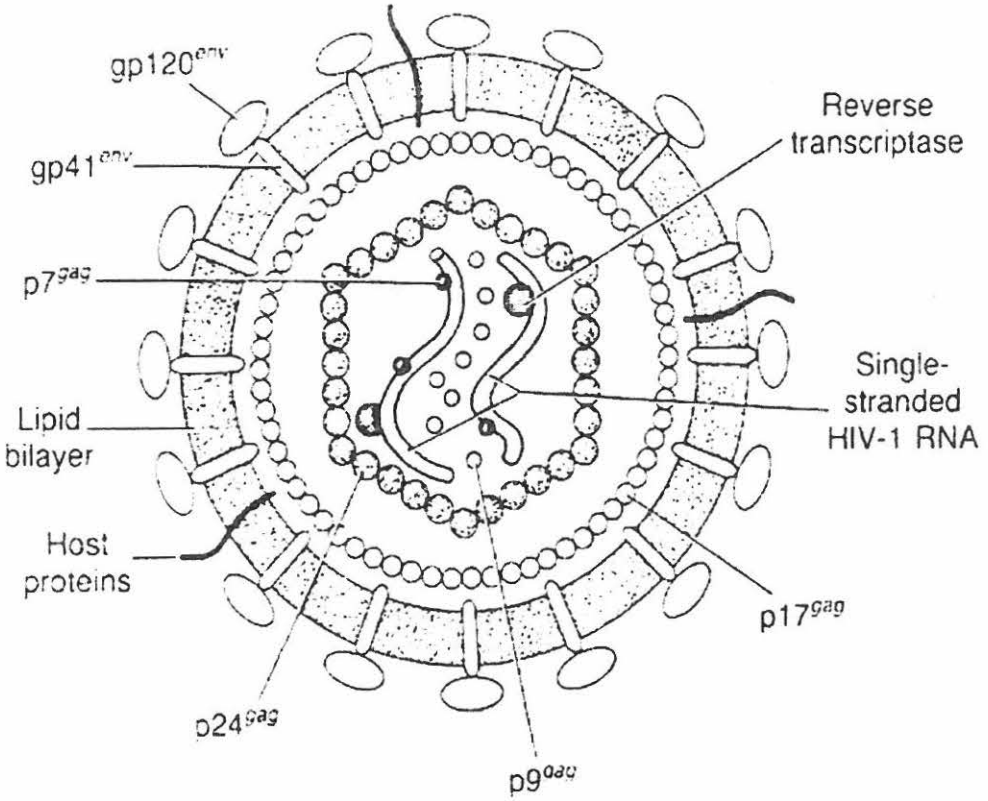

Figure 2.4.1

The HIV-1 Virion

Each of the virion proteins making up the envelope ($gp120^{env}$ and $gp41^{env}$) and nucleocapsid ($p24^{gag}$, $p17^{gag}$, $p9^{gag}$, and $p7^{gag}$) is identified. In addition, the diploid RNA genome is shown associated with reverse transcriptase.

(adapted from Greene, 1991)

2.5 THE HIV-1 GENOME STRUCTURE

Nine open reading frames are contained within the HIV-1 genome (Figure 2.5.1). Three of these reading frames encode the Gag, Pol and Env polyproteins, which are subsequently proteolysed into individual proteins, a process very common to all the retroviruses. The core of the virion and the outer membrane envelope are made up of the structural components which include the four Gag proteins MA (matrix), CA (capsid), NC

(nucleocapsid), and p6, and the two Env proteins, SU (surface or gp120) and TM (transmembrane or gp41). The three Pol proteins include the PR (protease), RT (reverse transcriptase) and IN (integrase) and are encapsulated within the particle and have essential enzymatic functions.

HIV-1 also encodes six additional proteins, often called the accessory proteins. Three of these proteins (Vif, Vpr, and Nef) are found within the viral particle. The two other accessory proteins, Tat and Rev, provide essential gene regulatory functions, and the last protein, namely Vpu, indirectly assists in the assembly of the virion. The HIV-1 genome is encoded by a ~ 9-kb RNA, and two genomic-length RNA molecules are packaged in the particle (Turner and Summers, 1999).

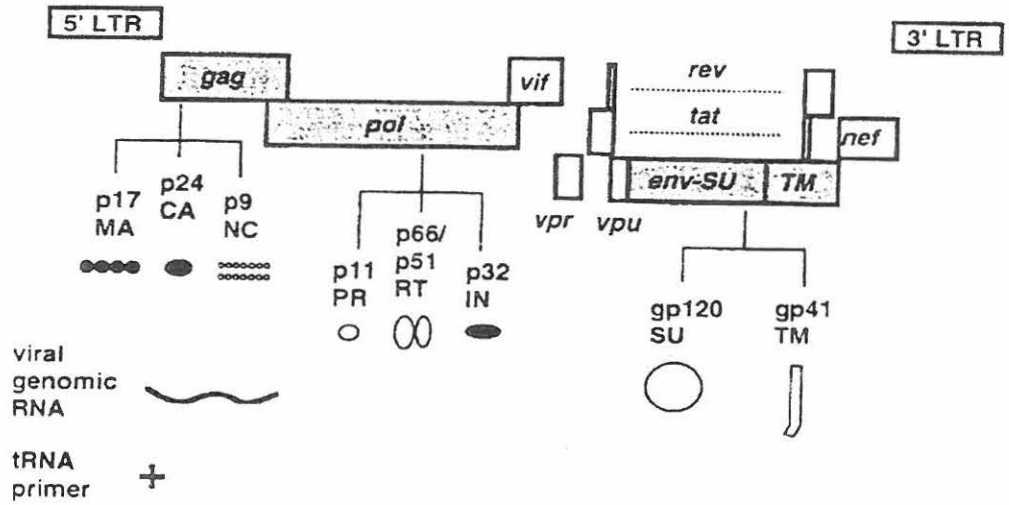

Figure 2.5.1

The HIV-1 genome and virion structure

(adapted from Luciw, 1996)

2.6 THE ENVELOPE PROTEINS

2.6.1 TM (gp41)

TM is a 345-amino acid protein located in the viral membrane (Figure 2.5.1) and its primary function is to mediate the fusion between the viral and cellular membranes following receptor binding. It has been predicted that the fusion is initiated by an N-terminal hydrophobic glycine-rich "fusion peptide" and the trans-membrane region is important for both fusion and for anchoring of Env in the viral membrane. Two crystal structures of the core region (TM_{core}) have been identified (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). In the larger of the two structures (Figure 2.6.1), residues 30-79 and 113-154 of TM were fused to a 31-residue trimeric coiled-coil from GCN4 in place of the N-terminal fusion peptide (Weissenhorn *et al.*, 1997). The TM_{core} lacks residues 80-112 of TM.

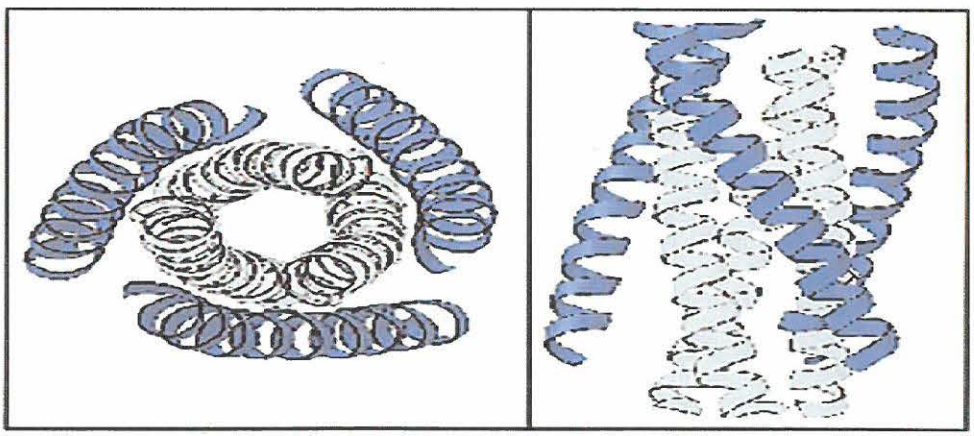

Figure 2.6.1

Helix packing of the trimeric coiled-coil structure of the HIV-1 TM ectodomain.

N- and C- terminal helices are coloured blue and purple, respectively. Viewed from top (left) and side (right).

(adapted from Chan *et al.*, 1997)

TM_{core} forms a trimer that consists of a parallel α-helical coiled-coil (residues 1-77) and an outer antiparallel α-helical layer (residues 117-154) (Weissenhorn *et al.*, 1997). The structure of the TM_{core} represents a structure formed during the fusion reaction, as suggested by the following. First, mutations at the interface between the outer and the central helical layers (including Ile62) specifically block membrane fusion. Secondly, the TM_{core} is extremely thermostable, a feature predicted for the fusion-active protein and not the native protein (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). Thirdly, the structures of the TM_{core} and a low pH fusion-active form of the influenza virus HA_2 protein are strikingly similar (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). Fourthly, the 18 C-terminal extracellular residues missing from the structure cannot span the estimated distance between the C-terminus of TM_{core} and the viral lipid. However, the distance is consistent with a conformation in which the fusion peptides and the transmembrane regions are located at the same end of the central rod structure when viral and cell membranes are brought together (Weissenhorn *et al.*, 1997). The structure helps to explain how two peptides known to inhibit fusion may act. A peptide from the C-terminus may bind to the central trimer, disrupting the structure of the N-terminal region, whereas a peptide from the N-terminus may either compete with folding of the central trimer or bind to the C-terminal region and prevent association with the central core (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). Emerging rules for the design of coiled-coils may aid in the development of new fusion inhibitors.

2.6.2 SU (gp120)

Viral entry is initiated by binding of the SU glycoprotein, located on the viral membrane surface, to specific cell surface receptors. CD4

is the major receptor for HIV-1 and is an immunoglobulin (Ig)-like protein expressed on the surface of a subset of T cells and primary macrophages. The 515-residue SU protein binds CD4 with high affinity (K_d~4 nM). Amino acids important for binding have been primarily mapped to four separate conserved regions of SU and to the C'-C'' ridge of CD4, which protrudes from the first Ig-like extracellular domain (Luciw, 1996). The structural details of the interactions are not yet known.

The SU-CD4 interaction is not sufficient for HIV-1 entry. Therefore, a group of chemokine receptors (a family of seven transmembrane G-coupled proteins) that mobilise intracellular calcium and induce leukocyte chemotaxis, serve as essential viral co-receptors (Clapham & Weiss, 1997). HIV-1 is divided into two major classes: those that are macrophage (M)-tropic and non-syncytium inducing (NSI) and those that are T-cell (T)-tropic and syncytium inducing (SI). CXCR4/Fusin was the first core-receptor identified; it permits entry of T-tropic but not M-tropic viruses. CCR5 is a major co-receptor for M-tropic but not T-tropic viruses. Other molecules, including CCR3, CCR2b, Bonzo/STRL33 and BOB/GPR15, serve as co-receptors for some HIV-1 isolates (Clapham & Weiss, 1997). The physiological ligands for CXCR4, CCR5, and CCR3 (SDF-1, RANTES/MIP-1α/MIP-1β, and eotaxin, respectively) are each able to inhibit viral entry by competing with the cognate co-receptor (Bleul *et al.*, 1996; Oberlin *et al.*, 1996; Choe *et al.*, 1996; Clapham, 1997). Some ligand derivatives have been described that block infection without activating chemokine signalling pathways and may represent a novel class of HIV-1 therapeutics (Clapham, 1997).

Binding of CD4 to SU appears to cause structural changes in Env that facilitate co-receptor binding and subsequent viral entry

(Clapham, 1997). The variable V3 loop of SU is an important determinant of viral tropism. It becomes exposed upon CD4 binding and presumably interacts with the cognate co-receptor (Figure 2.6.2) (Clapham, 1997). However, the V3 loop is probably not the sole determinant of co-receptor specificity, because HIV-1 isolates that use the same co-receptor can have highly variable V3 sequences (Choe *et al.*, 1996; Cocchi *et al.*, 1996; Oravecz *et al.*, 1996). Determinants for virus specificity are located in each of the extracellular regions of the co-receptors, and the signalling functions of these receptors are apparently not important for viral infection (Clapham, 1997).

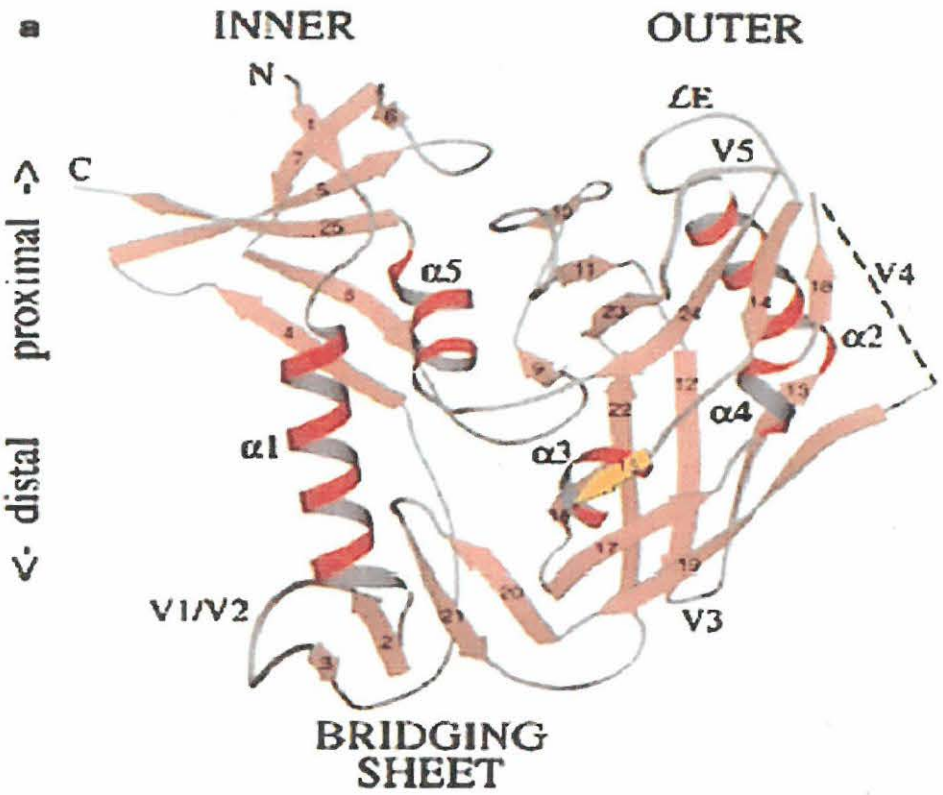

Figure 2.6.2

Structure of the HIV-1 SU core

(adapted from Kwong *et al.*, 1998)

2.7 THE STRUCTURAL PROTEINS

2.7.1 Matrix (MA)

The matrix is the terminal component of the Gag polyprotein and is important for targeting Gag and Gag-Pol precursor polyproteins to the plasma membrane prior to viral assembly. In a mature viral particle, the 132-residue MA protein lines the inner surface of the virion membrane.

There are two distinct features that play an important role in membrane targeting: an N-terminal myristoyl group and basic residues located within the first 50 amino acids (Figure 2.7.1). The crystal structure of residues 1 through 104 (Hill *et al.*, 1996) shows five α-helices capped by a three-stranded mixed β-sheet, with three monomers arranged like a triskelion. The trimeric form is of biological relevance because mutation of residues involved in trimerisation (residues 42-77) abolishes viral assembly, and because basic residues important for membrane localisation (lysines 26, 27, 30, 32) are arranged on the putative membrane – binding surface of the trimer. The MA structure suggests an obvious model for membrane binding that involves the insertion of three myristoyl groups into the lipid bilayer located directly above the trimer and interactions between basic residues of the membrane-binding surface and phospholipid head groups (Figure 2.7.1).

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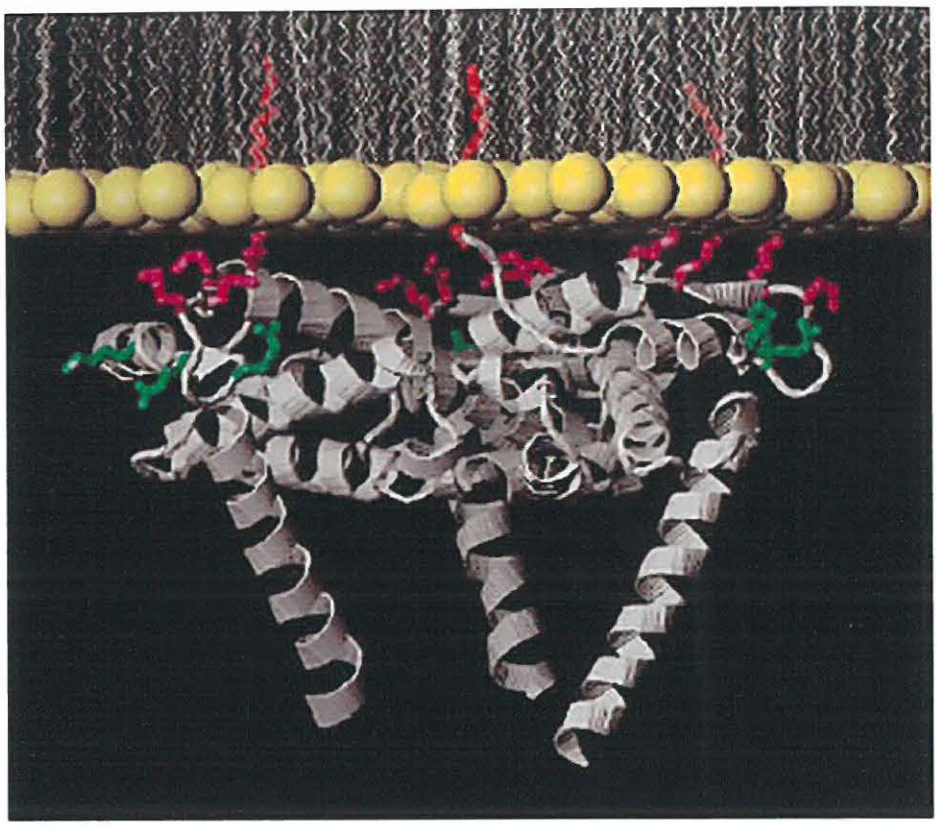

Figure 2.7.1

Model of the trimeric HIV-1 matrix protein interacting with a lipid membrane

Essential and non-essential basic residues are coloured magenta and green, respectively, and the N-terminal myristoyl groups are drawn in red (adapted from Hill *et al.*, 1996)

The N-terminal basic region is not strictly required for the formation of virus particles because non-infectious virus particles that lack MA can be produced if a myristoyl group is placed directly upstream of the capsid reading frame (Lee *et al.*, 1994). In addition to targeting Gag and Gag-Pol to the membrane, MA also appears to help incorporate Env glycoproteins with long cytoplasmic tails into viral particles (Freed *et al.*, 1995; Mammano *et al.*, 1995). The array of threefold symmetric holes located between the matrix trimers

appears to be large enough to accommodate the long cytoplasmic tails of full-length Env (Hill *et al.*, 1996; Massiah *et al.*, 1996).

In addition to the matrix function in viral assembly, MA facilitates infection of non-dividing cell types, principally macrophages. Some studies have shown that a subset of phosphorylated MA proteins are associated with viral pre-integration complexes and that MA contains a nuclear localisation signal (NLS) that interacts with Rch1, a member of the karyopherin-α family, to facilitate rapid nuclear transport (Gallay *et al.*, 1995b; Bukrinsky *et al.*, 1993; Bukrinskaya *et al.*, 1996; Gallay *et al.*, 1996). Phosphorylation of Tyr131 was shown to mediate association with IN, thereby linking MA to the pre-integration complex (Gallay *et al.*, 1995a; Trono and Gallay, 1997). Other studies, however, have found no evidence for an MA NLS and suggest that phosphorylation of Tyr131 is not important for macrophage infection (Freed *et al.*, 1995; Bukrinskaya *et al.*, 1996; Freed *et al.*, 1997). Instead, mutation of the putative MA NLS in a macrophage-tropic HIV-1 isolate decreased infectivity in both non-dividing and dividing cells and resulted in delayed proteolytic processing of the Gag polyprotein, presumably because the mutations affect association of MA with the membrane (Fouchier *et al.*, 1997). Additional studies are needed to clarify the role of MA in infection of non-dividing cells.

2.7.2 Capsid (CA)

The second component of the Gag polyprotein is the capsid (CA) which forms the core of the virus particle, with approximately 2000 molecules per virion (Figure 2.7.2). The C-terminal domain (residues 152-231) functions primarily in assembly and is important for CA dimerisation and Gag oligomerisation (Gamble *et al.*, 1997).

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Mutations in the N-terminal domain (residues 1-151) do not prevent assembly or budding. The domain is important for infectivity, apparently by participating in viral coating through its association with a putative cellular chaperone, cyclophilin A (CypA) (Figure 2.7.2).

The C-terminal domain is composed of an extended strand followed by four α-helices, with an extensive dimer interface (Gamble *et al.*, 1997). The major homology region (MHR), a 20-amino acid sequence that is one of the most highly conserved within all retroviral Gag proteins, adopts a compact fold in which the four most conserved residues (Gln155, Gly156, Glu159, and Arg167) form a stabilising hydrogen-bonding network. Additional hydrophobic residues from the MHR contribute to the hydrophobic core. The MHR is essential for particle assembly and may have a role in incorporation of Gag-Pol precursors through interactions with Gag (Srinivasakumar *et al.*, 1995), though not all mutants show this phenotype (Mammano *et al.*, 1994). Biochemical experiments have also revealed a possible role for the MHR in membrane affinity, perhaps reflecting exposure of hydrophobic residues (Ebbets-Reed *et al.*, 1996).

The structures of two dimeric forms of the N-terminal domain, one complexed to an antibody fragment and the other complexed to CypA, show the same monomeric CA structure but different subunit interfaces (Gamble *et al.*, 1996; Momany *et al.*, 1996). The CA-CA interfaces observed in the CypA complex are blocked in the antibody complex, but given that the C-terminal domain is largely responsible for dimerisation (the N-terminal domain is monomeric at mM concentrations), it still remains to be determined whether the observed N-terminal domain interfaces represent true subunit

interactions. The CA subunits are also seen to arrange in strips within a crystal, consistent with a plausible packing arrangement in the virion core (Gamble *et al.*, 1996). Residues from an extended region of CA interact with CypA, with Ala88, Gly89, and Pro90 buried in the CypA active site groove. A short spacer peptide located between CA and NC-p2-may also influence CypA incorporation into the virion (Dorfman and Göttlinger, 1996).

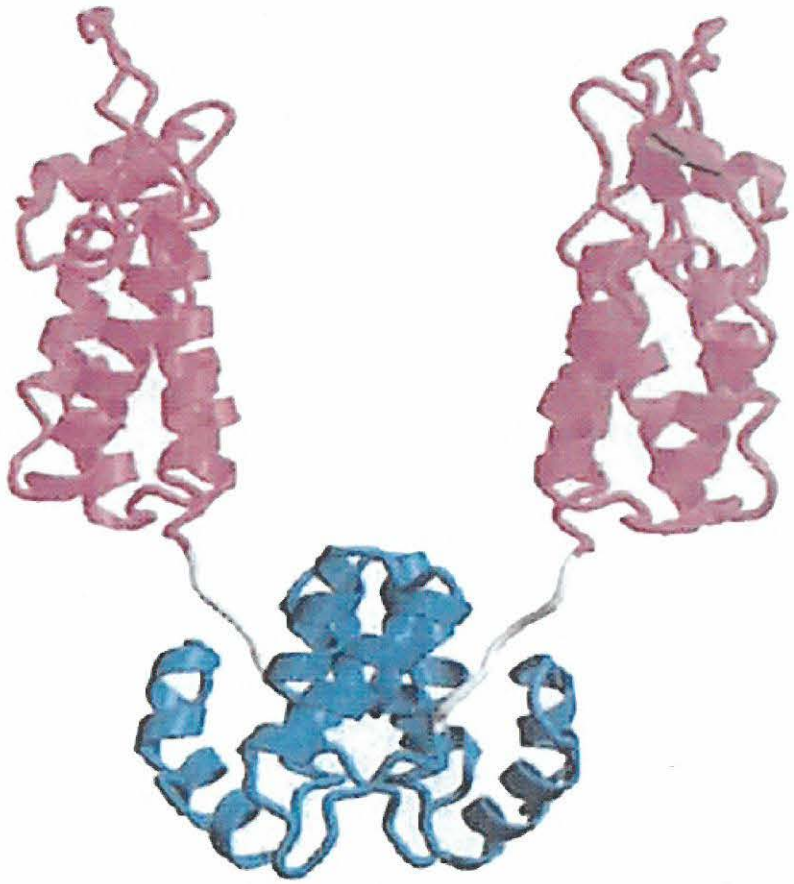

Figure 2.7.2

Model of the dimeric HIV-1 capsid protein, constructed from the independently characterised N-terminal core (purple) and C-terminal oligomerisation (cyan) domains.

The residues that link the domains are disordered in the X-ray and NMR structures of the isolated domains, and could allow ca.90° re-orientations of the N-terminal domains in the intact protein (adapted from Gamble *et al.*, 1997)

2.7.3 Nucleocapsid (NC)

The third component of the Gag polyprotein that coats the genomic RNA inside the virion core is called the nucleocapsid. The main function of the NC is to bind specifically to the packaging signal and to deliver full-length viral RNAs into the assembling virion. The packaging signal on the RNA, ψ, is not completely defined but is probably composed of three hairpins located around the major splice donor site (Clever and Parslow, 1997; Laughrea *et al.*, 1997), the first of which contains the "kissing loop" involved in RNA dimerisation (Figure 2.7.3). Studies with a chimeric Gag containing NC from HIV-1 and the remainder of Gag from Moloney murine leukemia virus (Mo-MLV), demonstrated that genomic HIV-1 RNA is preferentially packaged but that additional downstream sequences, which result in packaging of spliced RNAs, may contribute (Berkowitz *et al.*, 1995). The nucleocapsid is a basic protein that functions by means of binding single-stranded nucleic acids non-specifically, leading to coating of the genomic RNA, which presumably protects it from nucleases and compacts it within the core. The non-specific binding also results in a chaperone-like function that enhances other nucleic-acid-dependent steps in the life cycle, for example, by promoting annealing of the tRNA primer; melting of RNA secondary structures; DNA strand exchange reactions during reverse transcription (Huang *et al.*, 1997a; Guo *et al.*, 1997; Cameron *et al.*, 1997), or by stimulating integration (Carteau *et al.*, 1997).

The nucleocapsid is 55 residues long and consists of two zinc finger domains (of the CCHC type) and is flanked by basic amino acids. There are certain requirements for the NC-ψ interaction for it

requires intact fingers as well as several basic amino acids (Figure 2.7.3; Schmalzbauer *et al.*, 1996; Poon *et al.*, 1996).

The structure of the nucleocapsid has been determined by NMR (Summers *et al.*, 1992; Morellet *et al.*, 1992) and shows two well-ordered zinc domains with a relatively flexible linker in the absence of RNA. The basic residues that seem to be of particular importance for *in vitro* binding (Arg7, Arg32, and Lys33) and viral replication (Lys11 and Lys14) are indicated, though mutation of Arg32 or Lys33 seems to have little or no effect on RNA packaging *in vivo* (Poon *et al.*, 1996). Removal of zinc from the NC domains with disulphide-substituted benzamide inhibits viral replication (Rice *et al.*, 1995), providing additional evidence of the importance of these structures.

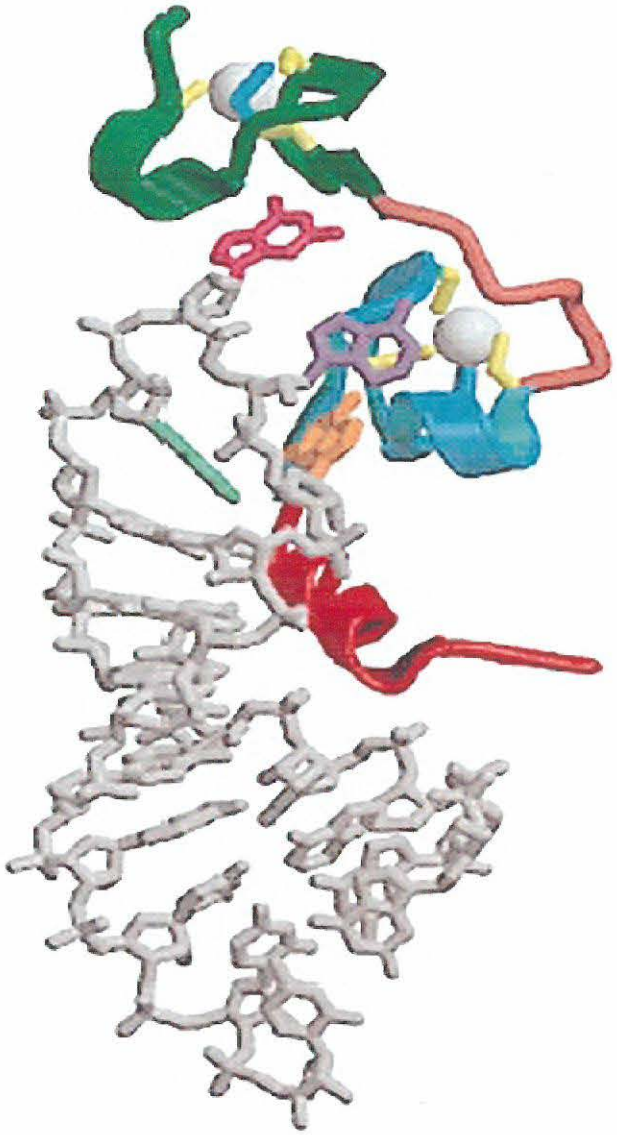

Figure 2.7.3

Structure of the complex formed between the HIV-1 nucleocapsid protein and the SL3 stem-loop recognition element of the genomic ψ-RNA packaging signal.

The colouring scheme of the tetraloop bases is as follows: G6, green; G7, pink; A8, blue; G9, orange. The colouring scheme for the Ncprotein is: N-terminal 3_{10} helix, pink; N-terminal zinc knuckle, cyan; linker, red; C-terminal zinc knuckle, green; cysteine and histidine side-chains, yellow and cyan, respectively. (adapted from Turner & Summers, 1999)

2.7.4 p6

p6 is important for the incorporation of Vpr during viral assembly and comprises the C-terminal 51 amino acids of Gag. Residues 32-39 and three hydrophobic residues within a conserved sequence motif (Leu41-X42-Ser43-Leu44-Phe45-Gly46) are important for Vpr binding (Kondo and Göttlinger, 1996; Lu *et al.*, 1995). P6 also helps to mediate efficient particle release and a region of four amino acids (Pro7-Thr8-Ala9-Pro10) has been implicated in this function (Huang *et al.*, 1995).

2.8 ACCESSORY PROTEINS

2.8.1 Nef

Nef is a 206-amino acid, N-terminally myristoylated protein that, like Vpu, reduces the level of cellular CD4. The routing of CD4 from the cell surface and golgi apparatus to the lysosomes is facilitated by Nef, resulting in receptor degradation and the prevention of inappropriate interactions with Env as well as Vpu. In the cytoplasmic tail of CD4 a dileucine-based sorting signal is located that is essential for Nef-mediated down-regulation and is presumed to interact with Nef. Nef has been proposed to serve as a direct bridge between CD4 and the cellular endocytic machinery by interacting with β-COP and adpatins, which link proteins in the golgi apparatus and the plasma membrane to clathrin-coated pits. Nef may enhance Env incorporation into virions, promote particle release, and possibly affect $CD4^+$ T-cell signalling pathways by down-regulating CD4.

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Nef can also down-regulate the expression of the MHC class I molecules, which may help in protecting infected cells from killing by cytotoxic T cells. Decreased rates of viral DNA synthesis following infection can also be attributed to a Nef mutant virus. This defect can be overcome if Nef is supplied in *trans* in virus-producing cells but not in target cells, suggesting possible roles in virus assembly, maturation or entry. These roles are consistent with the observation that ~70 Nef molecules are incorporated in each virion. These virion-associated proteins are cleaved by PR at residue 57 to generate a soluble C-terminal fragment. The mechanism of Nef incorporation has not yet been defined but is probably relatively non-specific, because Nef can also be incorporated into Mo-MLV particles (Bukovsky *et al.*, 1997). Nef contains a consensus SH3 domain binding sequence (PXXP) that mediates the binding to several Src-family proteins (e.g. Src, Lyn, Hck, Lck, Fyn), thereby regulating their tyrosine kinase activities (Figure 2.8.1) (Moarefi *et al.*, 1997). These interactions appear to be important for enhancing viral infectivity but not for down-regulating CD4. It is not yet clear which SH3-containing proteins are relevant for Nef function. The crystal structure of a Nef-SH3 complex (Figure 2.8.1) shows that the PXXP motif is in a left-handed polyproline type II helix that interacts directly with the SH3 domain (Lee *et al.*, 1996). Two residues that define the motif, Pro72 and Pro75, are important for enhancing viral replication and pack against hydrophobic residues of the SH3 domain (Lee *et al.*, 1996). The central core of Nef comprises two anti parallel α-helices packed against a layer of four anti parallel β stands (Fig 2.8.1) (Lee *et al.*, 1996). A hydrophobic crevice, which is presumably a ligand-binding site, is located between the two helices and is close to Arg 110. Arg110 has been defined as an important residue for association with NAK, a Nef-associated serine/threonine kinase

related to a p21 kinase (PAK) (Sawai *et al.*, 1997). PAKs are known to bind the p21 Rho-like GTP-binding proteins Rac-1 and CDC42hs, suggesting possible mechanisms by which Nef can interfere with both endocytosis and T-cell signalling (Cullen, 1996). However, mutations that disrupt the Nef-NAK complex do not affect Nef-mediated CD4 down-regulation (Benichou *et al.*, 1997). Nef has also been reported to bind other cellular proteins, including p53, MAP kinase, and Tease-II (Benichou *et al.*, 1997), but the significance of these interactions remains to be determined.

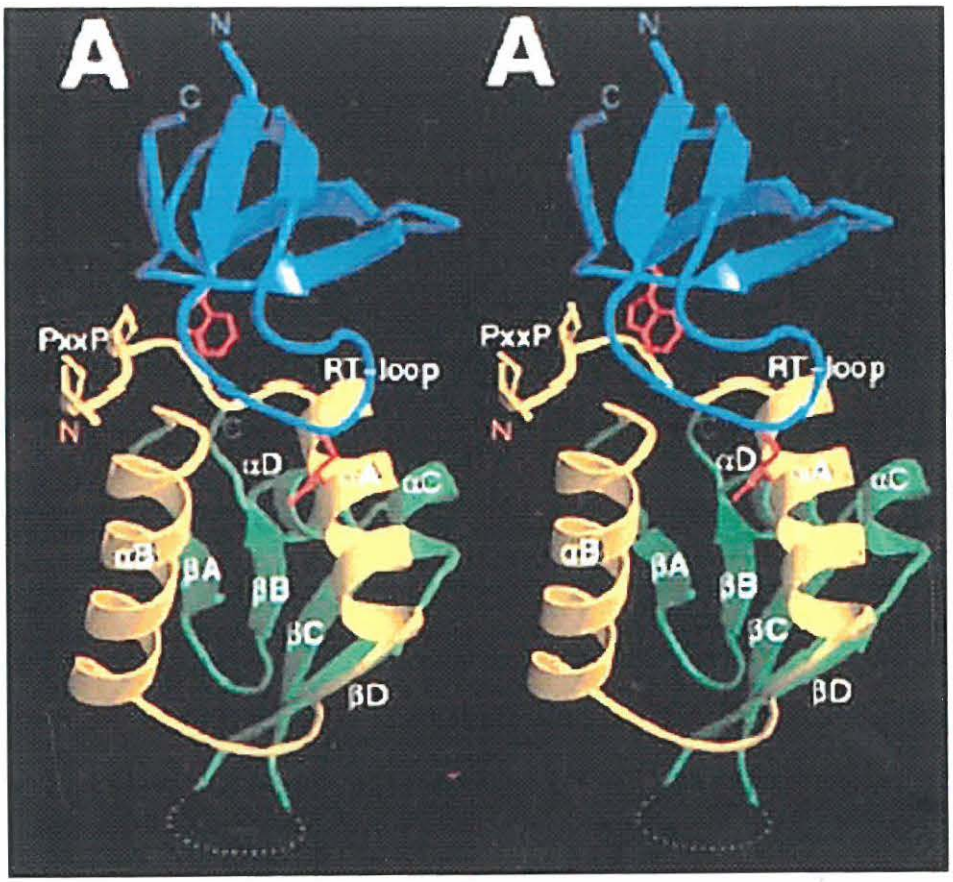

Figure 2.8.1

Stereo-view of the HIV-1 Nef core domain

Residues 71-120; N- and C-terminal residues in yellow and green respectively; the broken line represents a disordered loop bound to the Fyn(R961) SH3 domain, blue. Side-chains are drawn for the critical trytophan and isoleucine residues, red; of the SH3 domain and the polyproline helix of Nef (adapted from Turner and Summers, 1999)

2.8.2 Rev

The early stages of the HIV-1 replication cycle are marked by the exclusive expression of the fully spliced mRNA species that encode the viral regulatory proteins Tat, Rev and Nef. Later, when other viral components are needed to assemble infectious virions, singly spliced and unspliced transcripts are transported to the cytoplasm, where they are translated and where genomic RNAs are packaged.

Therefore, Rev is very important in this switch because it functions to overcome the default pathway in which mRNAs are spliced prior to nuclear export and further functions in the binding to the RRE site located in the *env* coding region (Figure 2.8.2). Whether Rev directly enhances the export of unspliced mRNAs or inhibits splicing has been unclear, but recent studies have revealed a strong tendency towards a role in export (Hope, 1997). *Xenopus oocyte* micro-injection experiments revealed that Rev is required to export unspliced RNAs that contain an RRE (Fischer *et al.*, 1995). Rev contains a leucine-rich nuclear export signal (NES) that allows it to shuttle between the nucleus and the cytoplasm (Meyer and Malim, 1994) that interacts with a nucleoporin-like protein (hRip/Rab) located at the nuclear pore (Fritz *et al.*, 1995; Bogerd *et al.*, 1995; Stutz *et al.*, 1995).

The interaction with hRip/Rab may be bridged by CRM1, a nuclear export receptor that is important for Rev export (Ullman *et al.*, 1997). Therefore, it is believed that the binding of Rev to the RRE targets the attached mRNA to the nuclear export machinery. Evidence exists that entry into the splicing pathway may also be important for Rev function due to the fact that mutating 5' splice sites on RRE-containing mRNAs eliminates Rev activity but

compensatory mutations in U1 snRNA, which bind at 5' splice sites, can restore activity (Lu *et al.*, 1990). Therefore, Rev can also directly inhibit splicing by preventing entry of additional snRNPs during the later stages of spliceosome assembly (Kjems and Sharp, 1993).

The RRE consists of several hairpins that bind several Rev monomers, nucleated by the interaction of a single monomer with a high-affinity site called the hairpin IIB (Zemmel *et al.*, 1996). The oligomeric binding function is very important for the functioning of the Rev protein, mainly because it increases the concentration of NES sites on a single mRNA. An arginine-rich domain is responsible for the mediation of binding and forms an α-helix and specifically recognises an internal loop in the IIB stem. The internal loop contains G:G and G:A base pairs that widen an otherwise narrow major groove (Figure 2.8.2). The widened groove allows amino acids on the Rev α-helix to recognise specific features of the site, primarily through hydrogen bonds between three arginines and specific bases and phosphates and between Asn40 and the G:A pair.

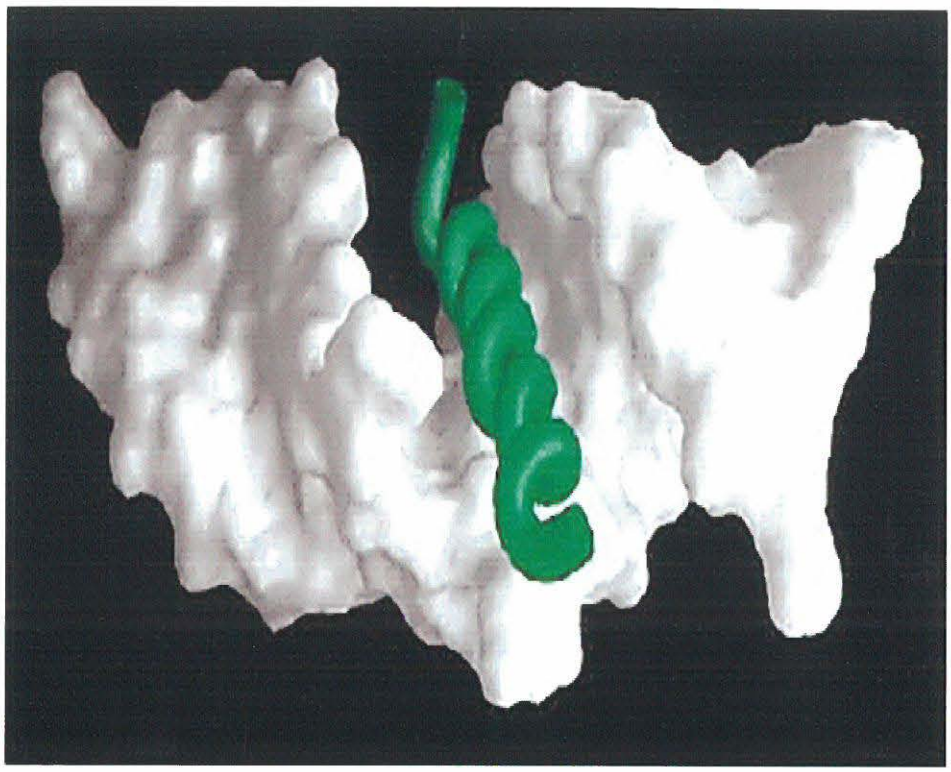

Figure 2.8.2

Space-filling representation of the Rev response element (RRE; grey) bound to the RNA-binding segment of Rev (green).

The Rev peptide forms an α-helix that binds within a widened major groove.

(adapted from Turner and Summers, 1999)

2.8.3 Tat

The HIV-1 promoter contains a number of regulatory elements important for RNA polymerase II transcription and is located in the 5'LTR. There are several sites located upstream of the start site for various cellular transcription factors, including sites for NF-κB, Sp1, and TBP (Jones and Peterlin, 1994). The function of these cellular factors is to help control the rate of transcription initiation from the integrated provirus. Their abundance in different cell types or at different times likely determines whether a provirus is quiescent or

actively replicating. Transcription complexes initiated at the HIV-1 promoter are rather inefficient at elongation and require the viral protein Tat to enhance the process of transcribing by polymerases. Under certain conditions, Tat may also enhance the rate of the initiation of transcription. Tat is essential for viral replication for it increases the production of viral mRNAs ~100-fold. It is not yet clear which features of the HIV-1 promoter cause initiating transcription complexes to be poorly possessive, but experiments in which the TATA box and downstream sequences have been interchanged with different promoters suggest an important role for these regions (Jones and Peterlin, 1994). When Tat is absent, the polymerase generally do not transcribe beyond a few hundred nucleotides, although they do not appear to terminate at specific sites. The mechanism of how Tat causes transcribing polymerases to become sufficiently possessive to completely transcribe the ~9-kb viral genome is still unknown, but experiments suggest that Tat may assemble into transcription complexes and recruit or activate factors that phosphorylate the RNA polymerase II C-terminal domain (CTD), including the general transcription factor TFIIH and other novel kinases (Zhou and Sharp, 1996; Parada and Roeder, 1996; Yang *et al.*, 1996; Garcia-Martinez *et al.*, 1997; Cujec *et al.*, 1997; Jones,1997). All these findings support a model in which Tat enhances phosphorylation of the CTD, a process known to occur as RNA polymerase II converts from an initiating to an elongation enzyme.

Tat does not bind to a DNA site but rather to an RNA hairpin known as TAR (trans-activating response element), located at the 5' end of the nascent viral transcripts. An arginine-rich domain of Tat is involved for it helps to mediate binding to a three-nucleotide bulge region of TAR, with one arginine residue being primarily

responsible for recognition. The TAR complexed to arginine (Puglisi *et al.*, 1992; Aboul-ela *et al.*, 1995) shows a base-specific contact between the arginine side chain and a guanine in the RNA major groove on NMR studies (Figure 2.8.3). The complex is stabilised by additional contacts to the phosphate backbone and by the formation of a U-A:U base triplet between a bulge nucleotide and a base pair above the bulge. NMR studies of the full-length 86-amino acid Tat protein have suggested that a hydrophobic core region of about 10 amino acids adopts a defined structure, but that the rest of the molecule, including the arginine-rich RNA-binding domain, is relatively disordered (Bayer *et al.*, 1995).

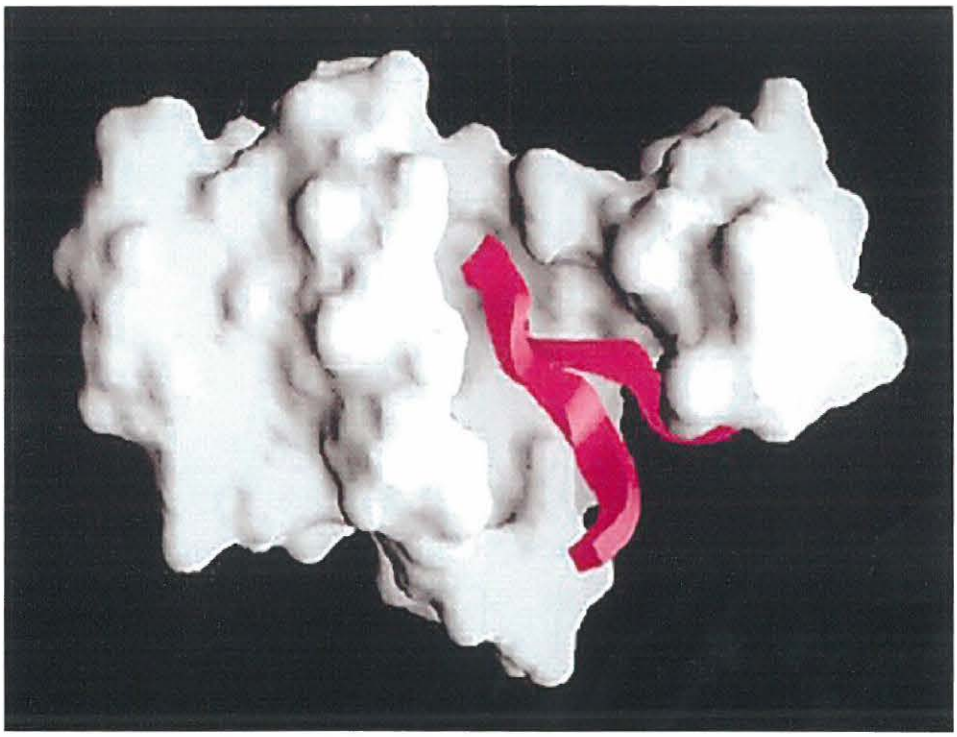

Figure 2.8.3

Space-filling representation of the bovine immunodeficiency virus (BIV) Tat peptide bound to the BIV TAR RNA.

As for the HIV-RRE, binding occurs in a widened major groove. However, the BIV Tat peptide binds in a β-hairpin conformation.

(adapted from Turner and Summers, 1999)

There is a likelihood that Tat requires interaction with cellular proteins in addition to TAR to adopt a stable structure. Another protein is needed to bind to the loop of the TAR hairpin, apparently helping to stabilise the Tat-TAR interaction (Alonso *et al.*, 1994). Functional data suggest that the loop-binding proteins are encoded by human chromosome 12. Several candidates have been identified, but none have yet been definitely shown to be essential for Tat activity.

2.8.4 Vpr

After fusion and entry, the virus is "uncoated" in the cytoplasm and the nucleoprotein complexes are rapidly transported to the host cell nucleus, mediated by the 96-amino acid Vpr protein. The components of the transported complexes are not completely defined but certainly include RT, IN, and MA (Miller *et al.*, 1997) and probably the genomic RNA and partially reverse-transcribed DNA.

The major importance of Vpr is for nuclear localisation in non-dividing cells, such as macrophages, because it contains an NLS that directs transport even in the absence of mitotic nuclear envelope breakdown (Cohen *et al.*, 1996). Vpr contains two important putative N-terminal amphipathic α-helices and not a canonical karyophilic NLS (Emerman, 1996). This unusual NLS localises Vpr to the nuclear pores rather than to the interior of the nucleus and does not use an importin-dependent pathway (Gallay *et al.*, 1996; Emerman, 1996). Vpr is incorporated into viral particles by means of interaction with p6 and may later become associated with the nucleoprotein complexes through an interaction with the C-terminal region of MA (Sato *et al.*, 1996).

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Vpr can also induce G2 cell cycle arrest prior to nuclear envelope breakdown and chromosome condensation, and sustained expression can reportedly kill T cells by apoptosis (Emerman, 1996). The Vpr will act before dephosphorylation of the $p34^{cdc2}$ cyclin-dependent kinase by CDC25, which is required to initiate mitosis (Emerman, 1996). However, the G2 arrest that takes place occurs with Vpr from proteins from different primate lentiviruses, but it is not known how the activity contributes to viral replication (Emerman, 1996). The amino acids that are important for G2 arrest are situated in the C-terminal region of the Vpr and cellular proteins have been identified that bind Vpr, including the 65-kDa regulatory subunit of protein phosphatase 2A (PP2A), a serine/threonine phosphatase that regulates the transition from G2 to mitosis (Emerman, 1996). Vpr does not only function in nuclear localisation and cell cycle arrest, but can also influence the mutation rate during viral DNA synthesis (Mansky, 1996) and has been proposed to form an ion channel (Lamb and Pinto, 1997).

2.8.5 Vpu

Newly synthesised Env glycoproteins (gp160), which are later cleaved into SU (gp120) and TM (gp41), are sometimes held in the endoplasmic reticulum through interactions with newly synthesised CD4 molecules. The function of Vpu is to promote the degradation of CD4 in these complexes, thus allowing Env transport to the cell surface for assembly into the viral particles. Vpu is an 81-residue oligomeric integral membrane protein with an N-terminal 24-residue hydrophobic membrane-spanning domain and a C-terminal cytoplasmic tail (Cohen *et al.*, 1996; Lamb and Pinto, 1997). Amino acids important for receptor binding and degradation have been

mapped to the C-terminal region of Vpu and to putative α-helices in the cytoplasmic tail of CD4 (Tiganos *et al.*, 1997). Co-immunoprecipitation experiments revealed that Vpu associates with a wild-type CD4 or with recombinant proteins containing the CD4 cytoplasmic tail, but it is not yet known if the interaction is direct or indirect (Bour *et al.*, 1995). Furthermore, these complexes are probably very relevant to CD4 degradation because there is a direct correlation between the extent of Vpu association and their relative levels of degradation (Bour *et al.*, 1995).

Post-translational modification appears to regulate the effect of Vpu on CD4 degradation. Vpu is phosphorylated on Ser52 and Ser56 by a casein kinase-2-related protein, and mutation of these positions decreases the levels of CD4 degradation (Cohen *et al.*, 1996). The mechanism by which degradation occurs is not yet clear but it seems to involve the cytoplasmic proteasome, because Vpu-mediated degradation can be blocked by proteasome inhibitors such as lactacystin (Fujita *et al.*, 1997). Vpu can also down-regulate cell surface expression of MHC class I proteins, which function in the protection of the infected cells from recognition and killing by cytotoxic T lymphocytes (Kerkau *et al.*, 1997).

In spite of its role in CD4 degradation, Vpu can also stimulate virion release, and it has also been proposed to be an ion channel (Lamb and Pinto, 1997). In Vpu mutant viruses, significantly increased numbers of particles either remain associated with the cell surface or are localised to intracellular membranes (Cohen *et al.*, 1996; Lamb and Pinto, 1997). In contrast to Vpu-mediated CD4 degradation, its effect on particle release requires the hydrophobic N-terminal domain and is not influenced by serine phosphorylation (Cohen *et al.*, 1996; Lamb and Pinto, 1997). The mechanism

appears to be relatively non-specific in that Vpu can also promote the release of heterologous retroviral particles (Cohen *et al.*, 1996; Lamb and Pinto, 1997).

2.8.6 Vif

Vif is a 192-residue protein that is important for the production of highly infectious virions. All the Vif mutant viruses show a markedly reduced level of viral DNA synthesis and produce highly unstable replication intermediates (Cohen *et al.*, 1996; Simon and Malim, 1996), suggesting that Vif functions before or during DNA synthesis. It is intriguing that Vif mutants show defects in infectivity only when produced in certain cell types, designated non-permissive or semi-permissive, but not when produced in permissive cells. There is a possibility that permissive cells produce a factor or factors that compensate(s) for a lack of Vif or that expression of Vif in permissive cells blocks an inhibitor of viral infectivity (Cohen *et al.*, 1996). Vif activity may be regulated by post-translational modification because mutation of one of three serine phosphorylation sites (Ser144) causes a defect in viral infectivity (Yang *et al.*, 1996).

Compared with mature wild-type virions, Vif mutant viruses have similar protein and RNA contents but grossly altered core structures, suggesting that Vif may play a role in viral assembly and/or maturation (Cohen *et al.*, 1996). Consistent with this role, the infectivity defect can be complemented by supplying Vif in *trans* in virus-producing cells but not in target, non-permissive cells (Cohen *et al.*, 1996), as also seen with Nef. It has been estimated that 7 to 100 molecules of Vif are packaged into the virion (Camaur and Trono, 1996; Fouchier *et al.*, 1996; Karczewski and Strebel,

1996; Liu *et al.*, 1995), suggesting that Vif may function directly within the particle. Incorporation of Vif is probably non-specific because there is no apparent requirement for any viral protein or RNA and, like Nef, Vif can be incorporated into Mo-MLV particles (Camaur and Trono, 1996).

2.9 VIRAL ENZYMES

2.9.1 Protease (PR)

The virion core is constructed to contain the Gag and Gag-Pol polyproteins, the Vif, Vpr, and Nef proteins, and the genomic RNA and, as the membrane coat containing SU and TM surrounds the particle, the virus buds from the membrane surface and is released. The immature particles that are formed are non-infectious. PR must cleave the polyproteins Gag and Gag-Pol, and conformational rearrangements must occur within the particle to produce a mature infectious virus. Although the precise timing is not clear, some of the "maturation" events may occur simultaneously with assembly and budding (Kaplan *et al.*, 1994). PR cleaves at several polyprotein sites to produce the final MA, CA, NC and p6 proteins from Gag and the PR, RT and IN proteins from Pol. The amount of Gag-Pol produced by ribosomal frame shifting and incorporation into the virion (~5-10% of Gag) will determine the final stoichiometries. Because assembly and maturation must be highly co-ordinated, factors that influence PR activity can have dramatic effects on virus production. PR, mainly functions as a dimer and is part of Pol, therefore the activity of PR is dependent on the concentration of Gag-Pol and the rate of auto-processing, which on the other hand may be influenced by adjacent p6 sequences (Zybarth and Carter, 1995). Cleavage efficiencies can vary

substantially among sites, thereby influencing the order of appearance of the different processed proteins (Dunn *et al.*, 1994). The relative cleavage rates and the infectivity may also be controlled by the p2 spacer peptide located between CA and NC (Pettit *et al.*, 1994; Kräusslich *et al.*, 1995). The processing of NC and p6 may be further influenced by RNA binding to the NC (Sheng *et al.*, 1997). Over-expression of PR can lead to aberrant rates of processing and decreased infectivity (Luukkonen *et al.*, 1995).

In drug design PR has been a prime target and the crystal structures of many PR-inhibitor complexes have been solved, including peptidomimetic and non-peptide inhibitors (Figure 2.9.1a) (Wlodawer and Erickson, 1993). The enzyme active site is located at the dimer interface, with each 99-residue monomer contributing a catalytically essential aspartic acid (Asp25). The active site resembles that of other aspartyl proteases and contains a conserved triad sequence, Asp-Thr-Gly. Within the PR dimer are flexible straps that close down on the active site upon substrate binding (Figure 2.9.1b). Surrounding the cleavage site is an amino acid side chain that binds within the hydrophobic pockets of PR, helping to explain some of the rate differences observed between different sites. Several PR inhibitors are in wide clinical use, and mutants resistant to multiple inhibitors have been observed (Condra *et al.*, 1995; Ridky and Leis, 1995). Resistant mutations are located within the inhibitor binding pockets as well as in the distant sites, and some mutants even show an increase in the catalytic activities (Schock *et al.*, 1996). An alternative approach to inhibitor design involves the use of inactive subunits that act as dominant negative inhibitors (McPhee *et al.*, 1996).

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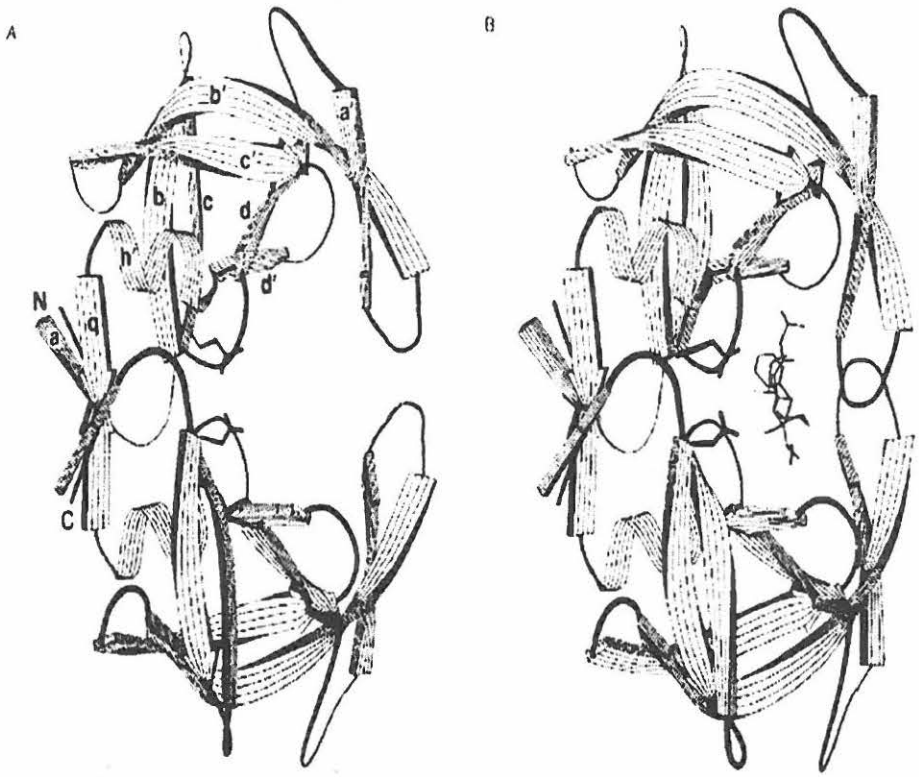

Figure 2.9.1

HIV-1 protease free (a) and complexed with an inhibitor (b). Inhibitor binding induces a significant conformational change in which the "flaps" close down over the active site

(adapted from Wlodawer and Erickson, 1993)

2.9.2 Reverse Transcriptase (RT)

Before any integration of the viral genome into the host chromosome can take place, it must first be reverse transcribed into duplex DNA. RT contains an RNase H domain that cleaves the RNA portion of RNA-DNA hybrids generated during the reaction; furthermore RT also catalyses both the RNA-dependent and DNA-dependent DNA polymerisation reactions. The reverse transcription initiates from the 3' end of a $tRNA_3^{Lys}$ primer annealed to the primer-binding site near the 5' end of the genomic RNA. RT

can make use of other tRNAs if complementary binding sites are provided, but reverse transcription is most efficient with RNA_3^{Lys} (Oude Essink *et al.*, 1996). During assembly the $tRNA_3^{Lys}$ is incorporated into virions and is often extended by several nucleotides inside the particle (Oude Essink *et al.*, 1996; Huang *et al.*, 1997b). The remainder of the reaction probably occurs after uncoating in the cytoplasm. The kinetic properties of RT during the initiation and elongation phases of the reaction are quite different, becoming highly processive during elongation, and post-transcriptional modifications of $tRNA_3^{Lys}$ enhance the formation of initiation complexes (Lanchy *et al.*, 1996). These kinetic transitions are reminiscent of those observed in transcription complexes, with $tRNA_3^{Lys}$ performing a role analogous to σ factor (Lanchy *et al.*, 1996). After tRNA-primed initiation, reverse transcription involves two DNA strand transfer reactions that are catalysed by RT and have an important function for priming the synthesis of both minus and plus strands.

RT also served as a major target for drug design and the crystal structures of the unliganded RT, an RT-DNA complex and RT-inhibitor complexes have been solved (Rodgers *et al.*, 1995; Jacobo-Molina *et al.*, 1993; Kohlstaedt *et al.*, 1992; Ren *et al.*, 1995). RT is a heterodimer and contains a 560-residue subunit (p66) (Figure 2.9.2) and a 440-residue subunit (p51) (Figure 2.9.2), and both of them are derived from the Pol polyprotein. Each subunit contains a polymerase domain composed of four sub-domains, referred to as the fingers, palm, thumb, and connection, and furthermore the p66 contains an additional RNase H domain. In spite of identical amino acid sequences, the polymerase sub-domains are arranged differently in the two subunits, with p66 forming a large active-site cleft and p51 forming an inactive closed

structure (Wang *et al.*, 1994). On the p66 polymerase active site is a catalytic triad (Asp110, Asp185, and Asp186) conserved in many polymerases, and the 3'OH group of the primer strand in an RT-DNA complex is positioned close to the active site for nucleophilic attack on the incoming nucleoside triphosphate (Jacobo-Molina *et al.*, 1993). In this complex the DNA contains both primer and template strands clamped between the palm, thumb and fingers sub-domains of p66, and is bent. Portions of the DNA near the active site adopt an A-form geometry expected of RNA-DNA hybrids or RNA duplexes bound during reverse transcription.

Two classes of RT inhibitors are clinically in use, namely nucleoside analogs such as AZT and ddI that are presumed to bind to the polymerase active site, and non-nucleoside inhibitors such as nevirapine. Several non-nucleoside inhibitor-RT complexes structurally indicate a common hydrophobic binding site near to, but distinct from, the polymerase active site that rearranges to fit the particular drug and lock RT into an inactive conformation (Kohlstaedt *et al.*, 1992; Ren *et al.*, 1995). Mutations that confer resistance to nucleoside or non-nucleoside inhibitors map to different parts of RT, including regions in and around the active site and DNA-binding cleft, suggesting that some mutations directly alter the drug-binding site while others have more indirect effects (Ren *et al.*, 1995; Tantillo *et al.*, 1994). Structures of the unliganded RT show substantial variability in the positioning of the p66 thumb sub-domain (Rodgers *et al.*, 1995), indicating that large-scale conformational rearrangements occur upon nucleic acid or drug binding. Such conformational changes may be important during reverse transcription, for example, to allow translocation of RT along the nucleic acid or to correctly position the RNase H and polymerase active sites.

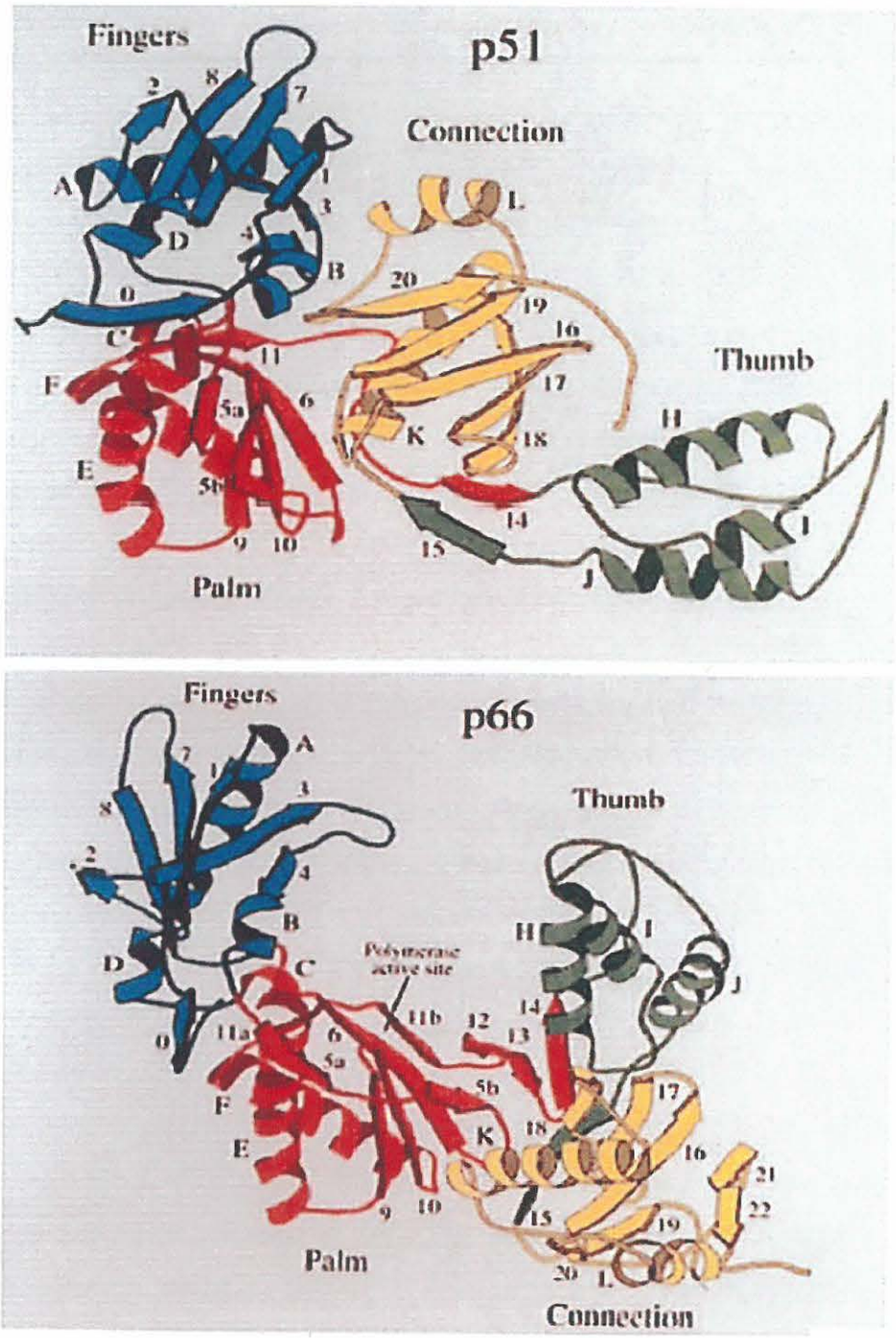

Figure 2.9.2

Ribbon drawings of the p51 and p66 proteins that form the HIV-1 RT heterodimer.

Sub-domains are coloured as follows: fingers (blue),palm (red), thumb (green), and connection (yellow).

(adapted from Jacobo-Molina *et al.*, 1993)

2.9.3 Integrase (IN)

After reverse transcription, IN catalyses a series of reactions to integrate the viral genome into a host chromosome. IN functions in the removal of two 3' nucleotides from each strand of the linear viral DNA, leaving overhanging CA-OH ends (Katz and Skalka, 1994). At the ends of many retrotransposons a CA dinucleotide is situated, and mutations of these nucleotides substantially reduces the efficiency of 3'-end processing. After this process, the processed 3' ends are covalently joined to the 5' ends of the target DNA. This step is followed by integration, which probably involves additional cellular enzymes, wherein unpaired nucleotides at the viral 5' ends are removed and the ends are joined to the target site 3'ends, generating an integrated provirus flanked by five base-pair direct repeats of the target site DNA. In integration, the viral substrate used for integration is a linear DNA molecule consisting of a complete minus strand and a discontinuous plus strand, which is presumably completed by cellular enzymes following integration (Miller *et al.*, 1995a). In vitro systems using viral pre-integration complexes, or purified IN with short oligonucleotides, have helped define important nucleotides near the viral DNA ends, which are all important features of the target DNA, and the critical amino acids of IN (Katz and Skalka, 1994). Two sequential transesterification reactions are involved in the enzymatic mechanism and require no exogenous energy source, but an appropriate metal cofactor (either Mn^{2+} or Mg^{2+}) is needed (Katz and Skalka, 1994).

Within a genome integration can take place at many target sites. *In vitro* studies revealed a preference for kinked or distorted DNA, such as found in nucleosomes, but it is not yet clear how these target sites relate to those used *in vivo* (Miller *et al.*, 1995b).

Suggestions have been made that interactions with other DNA-binding proteins might target IN to specific sites, and a yeast two-hybrid screen has identified a human Snf5-related protein with Ini1 as a possible partner (Miller and Bushman, 1995). The efficiency of integration is influenced by Ini1, but its effect on target site selection *in vivo* is unknown. HMG I (Y), another cellular factor, is associated with pre-integration complexes and plays a crucial role in integration (Farnet and Bushman, 1997).

IN, is active as an oligomer, probably a tetramer, and the 288-residue monomer can be divided into three domains whose structures have been determined (Figure 2.9.3) (Rice *et al.*, 1996). The N-terminal domain (residues 1-55) contains a zinc-binding site (coordinated by two histidines and two cysteines) and forms a dimer with a largely hydrophobic interface, as shown by NMR (Cai *et al.*, 1997). Each monomer contains a helix-turn-helix structure very similar to those found in DNA-binding proteins and exists in two closely related conformational states. Within the catalytic domain (residues 50-212) there is a D,D(35),E motif. This motif is conserved among integrases, is crucial for the processing and joining reactions, and is proposed to bind the active site metal ion (Rice *et al.*, 1996). The isolated catalytic domain cannot perform processing or joining reactions but can perform an apparent reverse reaction, termed disintegration, indicating that it contains the catalytic site for polynucleotidyl transfer. The crystal structure of the catalytic domain shows a dimeric structure, with each monomer containing a five-stranded β-sheet and six α-helices similar to other polynucleotidyl transfer enzymes (Dyda *et al.*, 1994). Asp64 and Asp116 of the D,D(35),E motif are clearly seen in the structure, but Glu152 is located on a disordered loop. The two active sites in the dimer are too far apart to permit five base

pair staggered cleavage of the target DNA, suggesting either that a very large conformational change occurs during catalysis or, more likely, that IN functions as a tetramer or other oligomeric form during some steps of the reaction (Rice *et al.*, 1996; Cai *et al.*, 1997).

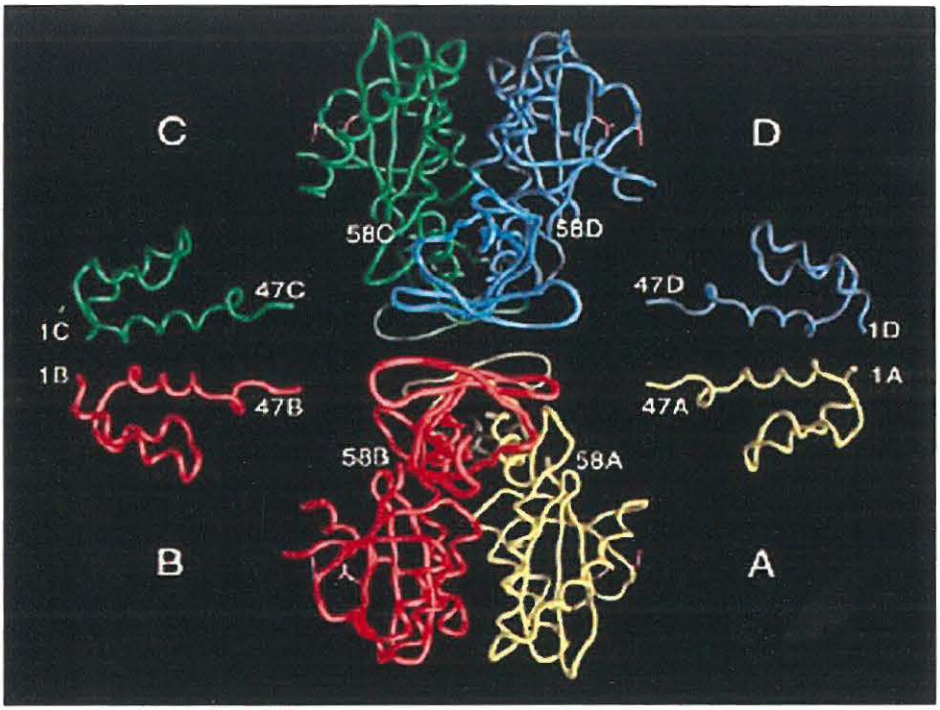

Figure 2.9.3

Model of the HIV-1 tetramer generated using independently solved structures of the N-terminal, core and C-terminal domains.

The four molecules of tetramer are displayed in different colours (adapted from Cai *et al.*, 1997)

The C-terminal domain (residues 220-270) has non-specific DNA-binding activity and forms a dimer of parallel monomers, as shown by NMR. The structure of each monomer consists of a five-stranded β barrel strikingly similar to a SH3 domain, with a saddle-shaped groove that might accommodate double-stranded DNA and

containing Lys264, an important DNA-binding residue (Eijkelenboom *et al.*, 1995; Lodi *et al.*, 1995). The relative orientations of the three IN domains remain to be established (Rice *et al.*, 1996; Cai *et al.*, 1997).

2.10 HIV-1 REPLICATION CYCLE:

Specific interactions between the viral glycoprotein gp120 and the amino-terminal immunoglobulin domain of CD4 on the cell surface initiate the viral replication cycle. T lymphocytes, monocytes and macrophages of the immune system, all of which express CD4 receptor on the cell surface, can be infected with HIV-1. However, HIV-1 requires additional cell-surface proteins to promote fusion of the viral and cellular membranes. CCR5 and CXCR4 are co-receptors for HIV-1 entry *in vivo*. Other additional chemokine receptors have been shown to serve as co-receptors for HIV-1 entry but their relevance *in vivo* remains to be confirmed (Chapham & Weiss, 1997; Doranz *et al.*, 1996; Feng *et al.*, 1996; Moore, 1997).

The membrane fusion reaction occurs between the lipid bilayer of the virus particle and the plasma membrane of the host cell and is induced by the viral glycoprotein gp41. The membrane fusion is followed by a process called uncoating, described as a series of ill-defined events. During this event, CA is lost, while at least some MA, as well as NC, IN, RT and Vpr are thought to be retained as part of a high-molecular-weight complex. Reverse transcription of the genomic viral RNA into DNA is largely completed. The accessory protein Vpr, present during the early stages of infection since it is packaged into the virion, appears to participate in the reverse transcription process by facilitating the initiation and influencing the accuracy of reverse transcription. The reverse transcription may be facilitated by Vpr by interacting with the lysine tRNA synthase, since this

reaction correlates with an inhibition of the aminoacylation of tRNAlys, the primer for HIV-1 reverse transcription (Goff 1990, Turner & Summers, 1999). Vpr is thought to modulate the mutation rate of HIV-1 by interacting with an enzyme involved in the DNA repair pathway. The process of reverse transcription is also dependent on NC. NC enhances the reverse transcription by promoting annealing of the tRNAlys primer to the PBS, melting of RNA secondary structures and DNA strand transfer through its non-specific RNA binding features.

The viral DNA is then transported to the nucleus as part of a high-molecular-weight complex, now referred to as the pre-integration complex (PIC), once it has been synthesised. Nuclear localisation of PIC is directed by Vpr, which does not contain a nuclear localisation signal (NLS) but appears to connect the PIC to the cellular nuclear import machinery (Fouchier *et al.*, 1997; Nie *et al.*, 1998). Other viral components of PIC, such as MA, IN and the central DNA flap, may facilitate nuclear localisation but their exact roles remain controversial (Miller *et al.*, 1997).

As part of the PIC, IN recognises the LTRs at the 5' and 3' ends of the viral DNA and removes the dinucleotide adjacent to a highly conserved CA dinucleotide, from the 3' strand of the U3 and U5 viral DNA LTRs. It then covalently combines the 3' ends to the 5' ends of the target cellular DNA in the nucleus (Turner & Summers, 1999). Finally, probably with the involvement of additional cellular enzymes, unpaired nucleotides at the viral 5' ends are removed and the ends are combined at the target site 3' ends, generating an integrated provirus flanked on both sides by five identical base pairs of the target cellular DNA. Once integrated, the proviral DNA remains permanently associated with the cellular DNA.

The proviral DNA serves as template for the synthesis of the unspliced and spliced mRNAs, which are transported out of the nucleus and into the

cytoplasm. Multiple-spliced mRNAs that encode Tat, Rev and Nef are synthesised and exported to the cytoplasm initially. Tat functions as an essential transcriptional activator that binds to a mRNA hairpin known as a trans-activating response element (TAR) and assembles into transcriptional complexes with cellular proteins to stimulate transcriptional elongation (Turner & Summers, 1999). Rev mediates export of the unspliced and the singly-spliced mRNAs. Rev binds as an oligomer to the rev responsive element (RRE) of nascent unspliced mRNAs and recruits the cellular nuclear shuttling protein exportin-1 (XPO) (Ohno *et al.*, 1998) and the nuclear export factor Ran guanosine triphosphatase (in its GTP-bound form). Rev, containing an NLS, directly mediates the export of these mRNAs through the CRM1 export pathway. Whether it also inhibits splicing is unclear. As Rev accumulates in the nucleus, more unspliced and singly-spliced mRNAs associated with it are shuttled out of the nucleus. In this process, Rev functions as a switch between the early synthesis of highly spliced mRNAs (encoding Tat, Rev and Nef) and the later synthesis of the singly-spliced (encoding Env, Vpu, Vif and Vpr) and unspliced (encoding the Gag and Gag-Pol proteins) mRNAs (Turner & Sumers, 1999).

The Env precursor polyprotein gp120 is synthesised in the endoplasmic reticulum (ER) and is post-translationally modified in the ER and Golgi apparatus (Turner & Summers, 1999). The polyprotein is cleaved into gp120 and gp41 by cellular endoproteases and transported to the cell membrane. Vpu binds to neosynthesised CD4 in the ER and promotes the degradation of CD4 by the ubiquitin-proteosome pathway (Crise *et al.*, 1990; Margottin *et al.*, 1998; Schubert *et al.*, 1998). In this way, the premature binding of CD4 to gp160 in the ER can be overcome and will result in the release of the gp160 for efficient processing and transport. Nef provokes the retention of neosynthesised CD4 molecules in the Golgi apparatus and the acceleration of their internalisation from the cell

surface. These CD4 molecules are then degraded in lysosomes. Similarly, Nef provokes the down-regulation of MHC-1 molecules on the surface of the infected cells by not targeting them to lysosomes but to the Golgi apparatus. While the down-regulation of CD4 may increase virion release from the cell surface and may increase the incorporation of envelope glycoproteins in virions, leading to the production of viruses with higher infectivity, the MHC-1 down-regulation protects HIV-1 infected cells from both cytotoxic T lymphocytes and natural killer cells (Mangasarian and Trono, 1997).

The Gag and Gag-Pol polyprotein precursors are synthesised on free ribosomes. The N-terminally myristoylated MA domain of the polyproteins directs binding to the cellular membrane (Bennet *et al.*, 1993; Bryant and Ratner, 1990; Chazal *et al.*, 1994; Zhou *et al.*, 1994). A basic domain near the amino terminus of MA, in conjunction with other basic residues throughout the protein, is also thought to promote membrane binding by interacting with acidic phospholipids on the inner face of the lipid bilayer. It is speculated that the MA domain of Gag interacts with the cytoplasmic tail of gp41, which should be necessary for the incorporation of the Env glycoprotein complex into the budding virion (Mammano *et al.*, 1995). During or after transport to the cellular membrane, the Gag precursor recruits two copies of unspliced viral RNA and interacts with Gag-Pol precursor. Subsequently virus budding occurs in the form of an immature particle. During or immediately after budding, the Gag and the Gag-Pol polyproteins, are cleaved by PR to produce the viral enzymes and structural proteins. The structural proteins rearrange via a process called maturation to form an infectious virion, which is now capable of initiating a new round of infection.

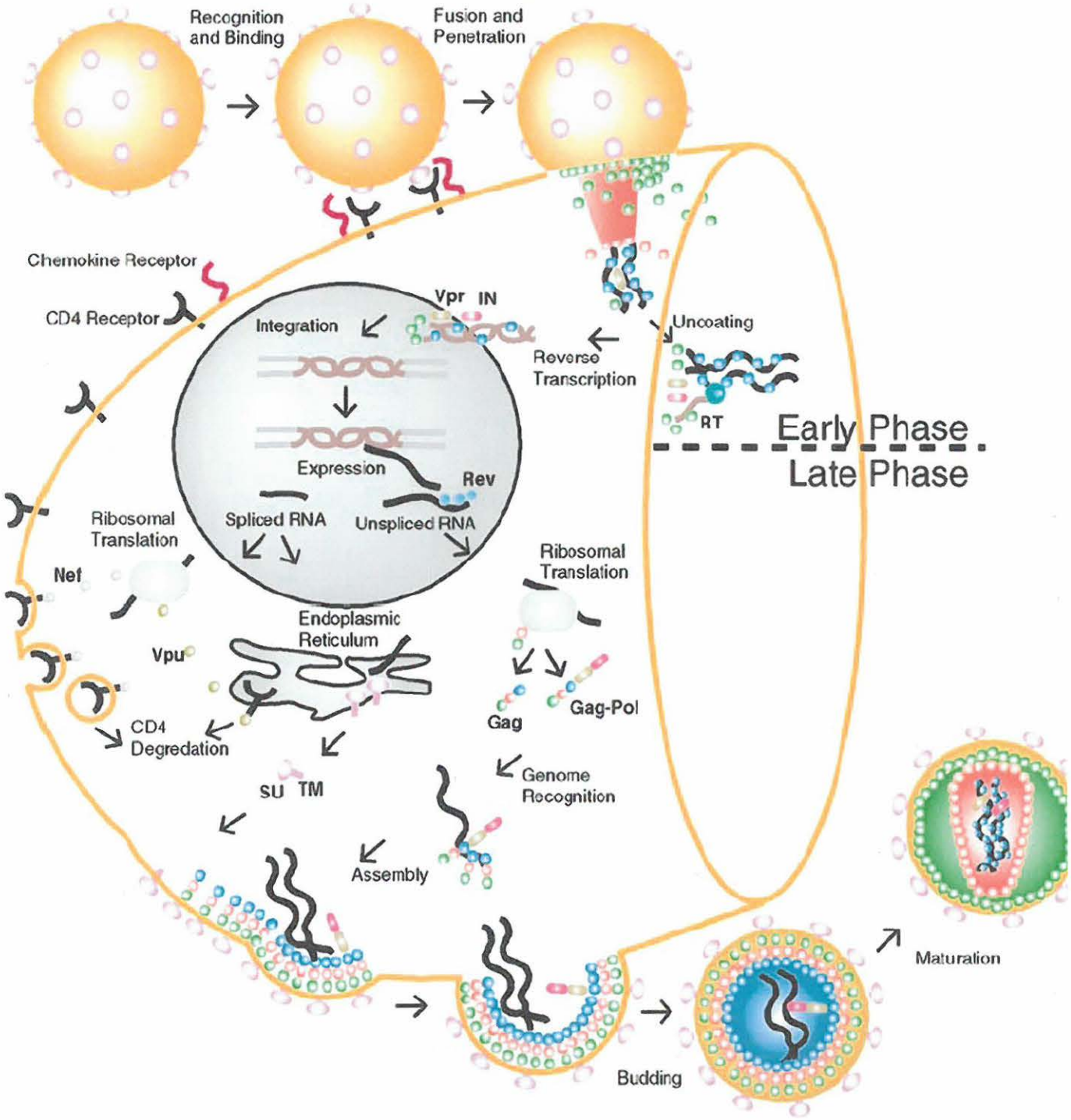

Figure 2.10

HIV-1 Replication Cycle

(adapted from Turner & Summers, 1999)

2.11 EVOLUTION AND PHYLOGENETICS

2.11.1 Phylogenetic Systematics and Classification

Charles Darwin never mentioned the word "evolution" except in his last sentence in "The Origin of Species" where he used the word "evolved". He usually referred to the phrase "descent with modification." The two central features of evolution are the basis of evolutionary classification today; groups of organisms descend from a common ancestor and, with the passage of time, acquire modifications.

Phylogenetic systematics, also known as cladistic analysis or cladistics, is the main approach for classification used in contemporary evolutionary biology. The German taxonomist Willi Hennig developed cladistics in 1950.

Phylogenetic systematics begins with the assumption that evolution is a branching process. The ancestral species split into descendant species, and these relationships are then represented much like family trees represent genealogies. The resulting trees are referred to as phylogenies. These phylogenies are then subjected to testable hypotheses, subjected to either confirmation or rejection, depending on evidence. In phylogenetic systematics, groups of organisms, referred to as taxa, are arranged into clades, which are then nested into larger clades. Each of these clades should be monophyletic, which indicates that all members share a single common ancestor, and all descendants of that ancestor are included in the clade. On the other hand, a polyphyletic group is one in which the members are derived from more than one

common ancestor. When all of the particular clade's members share a common ancestor but not all taxa share the common ancestor, it is called a paraphyletic group (http://www.learner.org/channel/courses/biology/units/compev/index.html).

2.11.2 How does Phylogenetic Systematics work?

Like most scientists, taxonomists start with the principle that the shortest, most possible, and direct path is most likely to be the correct one and this is called parsimony. One of the most commonly used methods is parsimony analysis in which the taxonomist searches for the most parsimonious tree, the one that requires the fewest number of evolutionary transitions.

The most parsimonious tree does not necessarily represent the true phylogenetic relationships. Sometimes certain types of transitions are more likely or are able to evolve more easily than others. This phenomenon is quite difficult to predict before the actual analysis is performed. Taxonomists usually resort to a fallback position, which indicates that all changes are equally likely. In molecular data in particular, a good prior knowledge of variation in the likelihoods of different changes exists. For example, certain types of mutations are more likely to occur than others. Transitions (changes from a purine –A or G- to the other purine, or a pyrimidine – C or T – to the other pyrimidine) are more likely than transversions (changes from a purine to a pyrimidine or vice versa). Statistical techniques like maximum likelihood analysis, enable the taxonomists to adjust for these situations.

Various types of trees can be used depending on the specific need. For instance, unrooted trees do not display the directionality of evolution but only patterns of relatedness. An unrooted tree can be transformed to rooted, but for any given unrooted tree, there are many possibilities of rooted trees. One approach is dependent on the identification and use of an outgroup, a taxon that is more distantly related than the taxa contained within the tree. For example, in an unrooted tree containing the great apes (humans, chimpanzees, gorillas, orangutans, and gibbons), one could use a species of monkey, such as baboons, as an outgroup. In practice the taxonomists will usually use a number of outgroups to refine the analysis (http://www.learner.org/channel/courses/biology/units/compev/index.html).

2.11.3 The Application of Phylogenetic Systematics

The methods that are used for phylogenetic systematics are similar for molecular and morphological characters but molecular data proves to be more advantageous. For instance, molecular data offers a large and essentially limitless set of characters. In theory, each nucleotide position can be considered as a character and can be assumed to be independent. The DNA of any organism has millions to billions of nucleotide positions. Furthermore, the large size of the genome makes it very unlikely that natural selection will be strongly driving changes at any particular nucleotide. Therefore, most of the nucleotide changes are "unseen" by natural selection, which is subjected only to mutation and random genetic drift. By the selection of a particular class of morphological characters, researchers may also bias the

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analysis in such a way that groups with certain characteristics cluster with others for reasons other than homology. For example, if the set of characters were weighted toward those involved in carnivory, carnivorous animals may cluster together, not because of homology but because of shared function. These problems are less likely to occur if molecular characters are used.

A second advantage of molecular data is that all known life is based on nucleic acids, thus, studies that involve any type of taxa can use DNA or RNA sequence data. Some of the genes or regions of genes evolve very quickly. These concepts are very useful in studies making use of closely related taxa. Conversely, other genes or regions of genes evolve more slowly. These concepts are more useful for studies of distantly related organisms. In extreme circumstances, some evolutionarily related genes have been found in disparate organisms such as yeast and humans. The extent of functional constraint is primarily used for the determination of the rates by which sections of DNA evolve. Genes and positions within genes that are the most useful generally evolve the slowest. The reason for this is that they are the least able to tolerate mutational change without substantially reducing the fitness of the individuals that harbour them. Many of these conserved genes play a role in development.

Phylogenetic methods can also be used to solve practical problems, like determination of the sources of infection from HIV. The rate of evolution for these retroviruses is extremely fast, owing it to their exceptionally high mutation rate. Therefore, sequences of HIV genes isolated from the same

infected individual can be as different as sequences of homologous genes in humans and birds. The rapidity of HIV's evolution is responsible for the havoc that HIV causes on the immune system. On the other hand, scientists can take advantage of that rapid evolution to study the relationships between HIV and other similar viruses.

2.11.4 Origin of HIV-1

Cross-species transmission events may have resulted in HIV infections. Five lines of evidence are used to substantiate the zoonotic origins of viruses: (a) similarities in viral genome organisation, (b) phylogenetic relatedness, (c) prevalence in the natural host, (d) geographic coincidence, and (e) plausible routes of transmission (Gao *et al.*, 1999).

The origin of HIV-1 has proven to be much more difficult to determine than that of HIV-2 (refer to 2.11.5). The criteria used were the same to establish zoonotic transmission of SIVsm to humans and have been applied to SIVcpz. (Gao *et al.*, 1999). SIVcpz and HIV-1 were initially found to be identical in genomic organisation, containing a particular gene, *vpu*, which is not present in other lentiviruses (Huet *et al.*, 1990). Therefore, the similarity in genome organisation made SIVcpz a very strong candidate for the origin of HIV-1, but some characteristics of the virus raised doubts as to its legitimacy as the immediate precursor to HIV-1. Some of the characteristics included an unexpected distant relationship between one isolate of SIVcpz (ANT) and HIV-1 (Van den Haesevelde *et al.*, 1996); seemingly low prevalence of SIVcpz infection in wild-living chimpanzees; uncertain geographic coincidence between chimpanzee habitats

and early AIDS cases (Gonder *et al.*, 1997), and questions concerning plausible routes of transmission (Figure 2.11.4.1).

According to a recent publication (Gao *et al.*, 1999) a new sequence of SIVcpz was described. According to the findings of that analysis and other data, a conclusion was drawn that the HIV-1 epidemic had arisen as a consequence of SIVcpz transmission from a particular chimpanzee subspecies, *Pan troglodytes troglodytes*, to humans. They demonstrated that HIV-1 was most closely related at a phylogenetic level to SIVcpz from *P.t. troglodytes*. Indirect evidence was presented for a higher prevalence of natural SIVcpz infection, based on the discovery of viral recombination between SIVcpz viruses of different lineages, described geographic coincidence for all groups of HIV-1 (M, N, and O) and SIVcpz from *P.t. troglodytes*, and proposed hunting and field-dressing of chimpanzees (a common practice in West Central Africa) as a plausible route of zoonotic transmission.

Another group (Corbet *et al.*, 2000) emerged with further evidence that substantially extends these conclusions. Twenty-nine captive chimpanzees from Cameroon were screened for evidence of SIVcpz infection and three animals were identified (CAM3, CAM4, CAM5) as seropositive. Two of the animals acquired their infections in the wild and the remaining one related to a cage transmission. The two animals (CAM3 and CAM5) which were naturally infected were found to be members of the *P.t. troglodytes* subspecies, and their viruses fell within the SIVcpz(*P.t.t.*) radiation (Figure 2.11.4.1 and 2.11.4.2). In the *env* region the new Cameroonian champanzee viruses were significantly more closely related to SIVcpzUS and YBF30 (the

full length representative of HIV-1 group N) than to any other virus within the HIV-1/SIVcpz*(P.t.t.)* radiation (Figure 2.11.4.2). According to this data there is a close geographical linkage of human and chimpanzee viruses and this suggested that the cross-species transmission event gave rise to HIV-1 group N occurring in Cameroon or its immediate vicinity (Corbet *et al.*, 2000; Hahn *et al.*, 2000).

Therefore it seems clear that HIV-1 arose as a consequence of SIVcpz transmission from chimpanzees to humans and that the *P.t. troglodytes* subspecies represents a natural host and reservoir for this virus (Hahn *et al.*, 2000).

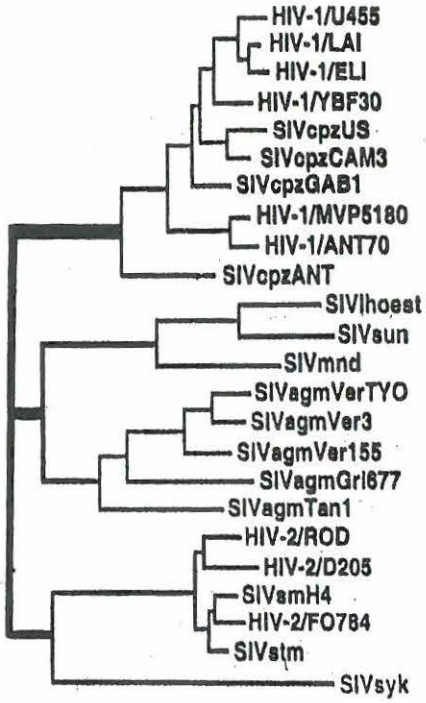

Figure 2.11.4.1

Evolutionary relationships of primate Lentiviruses based on maximum-likelihood phylogenetic analysis of full-length Pol protein sequences

The scale bar indicates 0.1 amino acid replacement per site after correction for multiple hits.

(adapted form Hahn *et al.*, 2000)

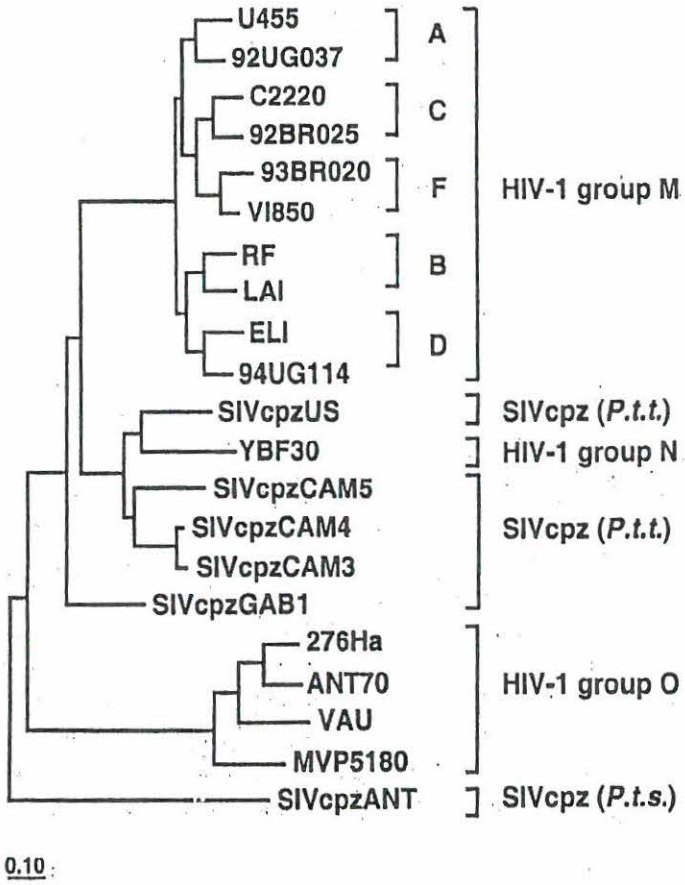

Figure 2.11.4.2

Evolutionary relationships of members of the HIV-1/SIVcpz lineage based on maximum-likelihood phylogenetic analysis of full-length Env protein sequences.

The three groups of HIV-1 (M, N, and O) are indicated in brackets on the right, as are five representative subtypes of the M group (A through F). The SIVcpz strains were isolated from either P.t. *troglodytes* (p.t.t.) or P.t. *schweinfurthii* (P.t.s.) animals. The scale bar indicates 0.1 amino acid replacement per site after correction for multiple hits.

(adapted from Hahn *et al.*, 2000)

2.11.5 Origin of HIV-2

HIV-2 was the first human lentiviral infection in which these criteria were satisfied and the simian source of the virus identified (namely sooty mangabeys). The five criteria were met

as follows: (a) HIV-2 and SIVsm share an identical genome structure, with each virus encoding an accessory protein, termed Vpx, that has not been found in any other primate lentivirus (Hirsch *et al.*, 1989). (b) SIVsm and HIV-2 strains are phylogenetically closely related and cannot be separated into distinct phylogenetic lineages according to their species of origin (Figure 2.11.4.1). In trees from partial *gag* sequences from many additional viruses, it has even been possible to find evidence of phylogenetic and geographic linkage of HIV-2 and SIVsm strains at a local level (Chen *et al.*, 1996). That is, SIVsm and HIV-2 sequences derived from animals and humans from the same immediate geographical area was found to be most related, which implicates hunting or other local activities as the route of transmission. (c) Sooty mangabeys are numerous in many West African countries and are infected with SIVsm at a substantial frequency (22% in some troops) in the wild (Chen *et al.*, 1996). (d) There is geographic coincidence between the natural habitat of the sooty mangabey and the areas where HIV-2 is endemic. The historical range of the sooty mangabey stretches from coastal West Africa south of the Casamance River in Senegal to the Sassandra River in Côte d'Ivoire. This range is in close proximity to the epicentres of the HIV-2 epidemic in Senegal, Guinea-Bissau, Guinea "Conakry" and Côte d'Ivoire, and it overlaps Sierra Leone and Liberia, where the most divergent HIV-2 strains have been identified (Chen *et al.*, 1996; Chen *et al.*, 1997). (e) Sooty mangabeys are frequently hunted for food, and orphans are kept as pets. Thus, there is the opportunity for frequent human contact with infected animals.

2.12 ANTIRETROVIRAL TREATMENT AND DRUG RESISTANCE

2.12.1 Antiretroviral Drugs in Clinical Use

The introduction of highly active antiretroviral therapy (HAART) has had a profound impact on the management of HIV infection. Suppression of HIV RNA to undetectable levels in plasma is possible for the first time. Furthermore, this implicates a decrease in morbidity and mortality through the reduction in the incidence of opportunistic infections for good survival. Currently, there are nineteen drugs approved by the FDA for the treatment of HIV, including 16 unique HIV-1 inhibitors.

The FDA divides these nineteen drugs into three classes. Despite the availability of these medications, failure to achieve complete viral suppression is common in clinical practice, occurring at a rate of 40% to 70% (Ledergerber *et al.*, 1999; Deeks *et al.*, 2000; Lucas *et al.*, 1999). The nucleoside RT inhibitors (NRTIs) inhibit HIV-1 replication in two ways. After conversion to their 5'-triphosphate form by cellular kinases, they compete with natural deoxynucleoside triphosphates for binding to RT, or for incorporation into newly synthesised viral DNA, thus resulting in DNA chain termination due to the absence of their 3'-hydroxyl group. The NRTIs currently approved for use in the clinics are zidovudine (ZDV, AZT), didanosine (ddI), zalcitabine (ddC), stuvadine (d4T), lamivudine (3TC) and abacavir (ABC) (Table 2.12.1).

Unlike the NRTIs, which function by mimicking the structure of the nucleoside bases, the non-nucleoside RT inhibitors (NNRTIs) inhibit HIV-1 RT by reversibly binding with an

allosteric site close to the active site of the enzyme. The inhibition is non-competitive with respect to template-primer and non-competitive or mixed with respect to nucleoside substrate. They are specific for the RT of HIV-1 and are not, or to a lesser extent, active against human cellular DNA polymerases or HIV-2 RT, respectively (Witvrouw *et al.*, 1999). The NNRTIs do not require intracellular modification to generate the active compound. The NNRTIs currently approved for use in clinics are nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV) (Table 2.12.1).

Protease inhibitors (PIs) inhibit the activity of PR by binding to the active site using two strategies. They either mimic the transition state during peptide cleavage, or they fit the active site as a steric complement or as a symmetric inhibitor of HIV-1 protease. The PIs presently approved are saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV) and lopinavir (LPV) in a combination with ritonavir (LPV/r) (Table 2.12.1).

Table 2.12.1

FDA-approved Antiretroviral Drugs (Youree and D'Aquila *et al.*, 2002)

Class	Generic Name (abbreviation)	Brand Name
Nucleoside Analogue Reverse Transcriptase Inhibitors (NRTI)	Zidovudine (AZT, ZDV)	Retrovir
	Didanosine (ddI)	Videx
	Zalcitabine (ddC)	Hivid
	Stavudine (d4T)	Zerit
	Lamivudine (3TC)	Epivir
	Zidovudine/Lamivudine (AZT/3TC)	Combivir
	Abacavir (ABC)	Ziagen
	Zidovudine/Lamivudine/Abacavir (AZT/3TC/ABC)	Trizivir
Nonnucleoside Reverse Transcriptase inhibitors (NNRTI)	Nevirapine (NVP)	Viramune
	Delavirdine (DLV)	Rescriptor
	Efavirenz (EFV)	Sustiva
Nucleotide Analogue Reverse Transcriptase Inhibitor	Tenofovir	Viread
Protease Inhibitors (PI)	Saquinavir (SQV, hgc)	Invirase
	Saquinavir (SQV, sgc)	Fortovase
	Ritonavir (RTV)	Norvir
	Indinavir (IDV)	Crixivan
	Nelfinavir (NFV)	Viracept
	Amprenavir (APV)	Agenerase
	Lopinavir/Ritonavir (LPV/RTV)	Kaletra

2.12.2 Antiretroviral Drugs Under Development

Various new inhibitors are under development, of which some may become available for clinical use within the next years. They can be distinguished from the currently available inhibitors by their more favourable pharmacokinetics, resistance profiles, production costs or by their new viral targets.

Promising new NRTIs are emtricitabine and DAPD. Emtricitabine is very similar to lamivudine, but it can be dosed once daily and, *in vitro*, it appears to be more potent (Molina *et al.*, 2000). DAPD is a new guanosine analog that is very well tolerated and is particularly promising because of its resistance profile (Richman *et al.*, 2000).

Acyclic nucleoside phosphonate analogs (ANPs) possess a phosphonate group attached to the acyclic nucleoside moiety. They only need two phosphorylation steps to be converted to their active metabolites. After conversion to their triphosphate form, they compete with natural deoxynucleoside triphosphates for binding to RT, or for incorporation into newly synthesised viral DNA, thus resulting in DNA chain termination (De Clercq, 1997). Adefovir (PMEA) and tenofovir (PMPA) are acyclic nucleotide analogs. Tenofovir appears promising because of its pharmacokinetic profile, activity, safety and resistance properties (Srinivas *et al.*, 1998; Miller *et al.*, 2000).

Foscarnet (PFA) is a pyrophosphate analog that inhibits viral DNA polymerases including HIV-RT. It binds to the putative pyrophosphate binding site close to the nucleotide binding site of these polymerases (Öberg, 1989). It is highly active against

HIV-1 strains with resistance to NRTIs and NNRTIs. However, its use in clinical practice is limited by a lack of oral bio-availability and by its side effects. Alkylglycerol analogs of PFA appear to have substantially greater antiviral activity than PFA and more favourable bio-availability characteristics (Hammond *et al.*, 2000).

Capravirine (AG 1549) is an important new NNRTI, primarily because of its resistance profile. According to in vitro data, more than one mutation is required for resistance to be detected and this inhibitor retains its activity against HIV-1 strains resistant to nevirapine and delavirdine (Fujiwara *et al.*, 1998). Emivirine (MKC-442) structurally resembles an NRTI but functions as an NNRTI. It is well tolerated, with a favourable adverse effect profile (Szczech *et al.*, 2000).

Tipranavir is a potent new non-peptidic PI effective against HIV-1 strains resistant against peptidometic PIs (Poppe *et al.*, 1997; Larder *et al.*, 2000; Rusconi *et al.*, 2000). BMS-232632 is a potent PI that shows a relatively unique resistance profile and may be administered once daily. It may be sensitive to HIV-1 strains resistant to nelfinavir, saquinavir and amprenavir (Colonno *et al.*, 2000). DMP-450 is a cyclic urea compound and potent inhibitor of HIV-1 PR (Sierra *et al.*, 2000). It is an especially promising compound due to its low production costs.

There has been considerable interest in the development of inhibitors targeting the fusion of HIV-1 to the host cell surface. T-20 and T-1249 are peptides that block infection by binding to a critical domain of gp41, thereby inhibiting fusion (Kilby *et al.*, 1998). Another fusion inhibitor is AMD-3100, a bicyclam that inhibits entry of HIV-1 into cells via selective blockade of the

chemokine CXCR4 co-receptor (Schols *et al.*, 1997; Donzella *et al.*, 1998).

Witvrouw *et al.* (1998) reported on one of the most active congeners of the series of QM96639 which was found to inhibit HIV type 1 replication in MT-4 cells at a concentration of 0.09 μM. This compound was toxic for the host cells only at a 1,400-fold higher concentration. The TTD derivatives proved effective against a variety of HIV-1 strains, including those that are resistant to AZT, but not against HIV-2 (ROD) or simian immunodeficiency virus. HIV-1 strains containing the L100I, K103N, V106A, E138K, Y181C, or Y188H mutations in their RT gene displayed reduced sensitivity to the compounds. Their cross-resistance patterns correlated with that of nevirapine. QM96521 enhanced the anti-HIV-1 activity of AZT and didanosine in a subsynergistic manner. HIV-1 resistant virus containing the V179D mutation in the RT was selected after approximately six passages of HIV-1 in CEM cells in the presence of different concentrations of QM96521. From structure-activity relationship analysis of a wide variety of TTD derivatives, a number of restrictions appeared as to the chemical modifications that were compatible with anti-HIV activity. Modelling studies suggest that in contrast to most other NNRTIs, but akin to nevirapine, QM96521 does not act as a hydrogen bond donor in the RT-drug complex.

2.12.3. Resistance

Development of viral resistance to antiretroviral drugs used for treatment of human immunodeficiency virus (HIV) infection is an important cause of treatment failure (Richman *et al.*,1994; Havlir *et al.*, 1996; Schuurman *et al.*, 1995; Saag *et al.*, 1993; D'Aquila *et al.*, 1995; Kozal *et al.*, 1994) and limits options for alternative antiretroviral regimens. Prevention, characterisation and clinical management of such resistance are receiving increasing attention.

The virus population in a person infected with an RNA virus (e.g. HIV-1, hepatitis C virus) has been termed a quasi species (Eigen, 1993), which refers to the existence of genetically distinct viral variants that evolve from the initial virus inoculum. The variants are generated because DNA proofreading mechanisms that preserve the genetic composition of organisms with double-stranded DNA genomes do not exist for RNA viruses. Thus, as single stranded RNA viruses replicate, each newly copied genome differs from the parental virus, on average, by a single nucleotide (Drake, 1993; Mansky and Temin, 1995).

Viral polymorphisms (genetic variants with apparently equivalent fitness ([replication capacity]), are commonly seen in virus populations in infected persons. Nucleotide differences may be "neutral" (no impact on fitness), deleterious (variants replicating less well or not at all), or confer replicative advantage if selective pressures such as immune responses or drug treatments change. These possibilities illustrate the survival strategy of organisms with high mutation rates that provide a

large pool of genetic variants able to adapt rapidly to changing selective pressures (Leigh Brown and Richman, 1997).

An estimated 10 billion (10^{10}) HIV-1 virions are produced daily in established HIV infection (Perelson *et al.*, 1996). If each contains on average 1 mutation per 9200-nucleotide genome, replication-competent virus with every possible single drug-resistance mutation is likely to be generated daily. Double mutants are less likely, and the probability of 3 or more drug-resistance mutations in the same genome is very low.

These estimates are supported by observations in infected persons. Virus or HIV-1 RNA with single drug-resistance mutations have been isolated from treatment-naïve patients or those infected before antiretroviral drug availability (Nájera *et al.*, 1995; Wong *et al.*, 1997). Mathematical modelling of the rate of resistance emergence after nevirapine treatment in previously untreated persons, permitted estimates of plasma prevalence of HIV-1 variants with nevirapine-resistance mutations before treatment. About 1 in 1000 copies/ml of plasma HIV-1 RNA contains the tyrosine-to-cysteine mutation at amino acid residue 181 (e.g. the Y181C mutation) of the reverse transcriptase conferring nevirapine resistance (Havlir *et al.*, 1996).

When antiretroviral drug selective pressure is applied to viral quasi species in an infected person, pre-existing minor viral species resistant to that drug rapidly become predominant and are selected as the fittest species in the presence of the drug. For some antiretroviral drugs, such as lamivudine and certain non-nucleoside reverse transcriptase inhibitors (NNRTIs; e.g.

nevirapine), a single mutation can confer high-level resistance. When these drugs are given in combinations only partially suppressing virus replication, drug-resistant mutants predominate within weeks (Wei *et al.*, 1995; van Leeuwen *et al.*, 1995; Havlir *et al.*, 1996). For some other drugs, such as zidovudine and certain protease inhibitors, high-level resistance requires accumulation of 3 or more resistance mutations in a single viral genome (Larder and Kemp., 1989; Condra *et al.*, 1995; Molla *et al.*, 1996). These highly resistant variants emerge more slowly, requiring months to predominate during less than maximum viral suppression (Condra *et al.*, 1995; Larder *et al.*, 1989), supporting the prediction that genetic variants with multiple mutations are present at much lower levels than those with single mutations in untreated patients. Development of high-level resistance to these drugs requires persistent viral replication and selective drug pressure. Persistent viral replication permits further viral evolution leading to high-level drug resistance by cumulative mutation acquisition.

What is known about development of resistance with potent combination therapy? Firstly, the higher the trough plasma concentrations of a protease inhibitor (e.g. ritonavir), the more slowly resistance mutations emerge (Molla *et al.*, 1996). Secondly, the lower the nadir of plasma HIV-1 RNA levels, the longer it takes for drug failure to occur (Kempf *et al.*, 1998). In patients with suppression of plasma HIV-1 RNA to below 50 copies/ml for 1 year, no resistance mutations or other evidence of virus evolution was discerned, even though HIV-1 RNA and DNA and replication-competent virus persisted (Gűnthard *et al.*, 1998; Wong *et al.*, 1997; Finzi *et al.*, 1997). Conversely,

patients with detectable HIV-1 RNA levels had ongoing virus replication and evidence of evolution.

Nucleoside analogue reverse transcriptase inhibitors (NRTIs) were the first class of antiretrovirals to be developed to provide effective antiviral therapy in the setting of HIV-1 infection (Loveday, 2001). These drugs compete with deoxynucleoside triphosphates (dNTPs) during polymerisation and act as premature chain terminators upon incorporation (Arts and Wainberg, 1996). Up to date, six NRTIs have been approved for use in the United States, e.g., zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC) and abacavir (ABC). In addition, tenofovir (TNV) is the leading drug among nucleotide reverse transcriptase inhibitors (NtRTI) and the only one of the class approved for use aged in patients. Altogether, these drugs remain the backbone of nearly all anti-HIV treatment strategies.

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are non-competitive inhibitors that bind to a hydrophobic pocket adjacent to the polymerase active site of RT (Deeks, 2001). This binding causes an allosteric change of the polymerase active site which inhibits DNA polymerisation (Esnouf *et al.*, 1995). NNRTI-associated mutations are usually in the hydrophobic pocket and a single mutation may result in high-level resistance to one or more NNRTI (Deeks, 2001). Three NNRTIs are currently approved for antiretroviral therapy in the United States: delavirdine (DLV), efavirenz (EFV) and nevirapine (NVP). The most commonly observed effect of NNRTI resistance mutations is a change in the ratio of RNase H to polymerase activities (Gerondelis *et al.*, 1999; Archer *et al.*,

2000). However, the level of drug resistance *in vitro* does not always correlate with the likelihood of a drug-resistant variant emerging *in vivo*. While NNRTIs can be potent inhibitors of HIV-1 replication, with favourable safety and pharmacokinetic parameters, rapid emergence of resistant viruses both *in vitro* (Mellors *et al.*, 1992; Richman *et al.*, 1991) and *in vivo* (Demeter *et al.*, 2000; Havlir *et al.*, 1995; Staszewski *et al.*, 1999), often as the result of single nucleotide changes, has limited the therapeutic utility of these compounds as monotherapy. However, recent clinical trials of the use of NNRTIs in combination with other antiretroviral agents have demonstrated an added benefit from inclusion of an NNRTI in such combination regimens (Carr *et al.*, 1996; Cheeseman *et al.*, 1995; Friedland *et al.*, 1999; Montaner *et al.*, 1998; Starr *et al.*, 1999). Although the viral replicative capacity of NNRTI-resistant viruses has not been extensively studied, available data suggest that NNRTI-selected single-point mutations, such as 103N or 181C plays a very important role.

High-level resistance is conferred by a variety of single-amino-acid changes within the binding pocket, including A98G, L100I, K101E, K103N, V106A, V108I, E138K, T139I, Y181C, Y188C, G190A and P236L (Buckheit *et al.*, 1997). In models of the three-dimensional structure of HIV-1 RT, these mutations cluster around the NNRTI binding site. The use of NNRTIs in patients is now dependent on defining appropriate combinations of agents which will prevent or significantly retard the selection of drug-resistant virus populations or which will result in the selection of drug-resistant virus isolates in which mutation of critical amino acid residues renders the RT less fit to support virus reproduction. These mutations do little damage to viral

fitness, creating highly resistant viruses without compromising viral replication, thus persisting during long-term virologic failure.

Several practical inferences can be derived from these principles (Table 2.12.3.1). Firstly, drugs for which only a single mutation is required for high-level resistance, e.g., lamivudine and nevirapine, should be reserved for use with other drugs in regimens designed to maximally suppress virus replication. Use in less suppressive regimens will select for high-level resistance more quickly (Richman *et al.*, 1994; Havlir *et al.*, 1996; Schuurman *et al.*, 1995). Secondly, combination regimens should be designed to confer the potency needed to maximally suppress pre-existing genetic variants and prevent replication. Regimens must establish a "genetic barrier" by suppressing minor populations with 1 or 2 mutations that could emerge with individual regimen components, permitting cumulative mutation acquisition. This requirement is more formidable in previously treated patients because prior treatment may have established a genetic archive of drug-resistant virus within peripheral blood mononuclear cells (PBMCs) and other tissue reservoirs (D'Aquila *et al.*, 1995).

Table 2.12.3.1 – Practical implications of the biology of HIV-1 drug Resistance (Richman *et al.*, 1994)

- Genetic variants of HIV with any single and probably many double mutations (although less likely) pre-exist in all patients before treatment is started. Thus, partially suppressive regimens containing lamivudine or certain NNRTIs rapidly fail because of breakthrough replication of pre-existing resistant variants.

- Genetic variants with 3 or more resistance mutations probably exist rarely, if at all, in untreated patients. Thus, potent combination regimens that require many resistance mutations for viral escape are recommended.

- Preventing cumulative acquisition of resistance mutations requires potent combination regimens that suppress virus replication to below levels of detection of the most sensitive assays available (about 50 copies/ml).

- Complex mixtures of genetic variants exist in all patients. Assays for drug resistance, both genotypic and phenotypic, may provide information only on the predominant circulating variants and may miss minor variants.

- Prior treatment may select for resistant mutants that persist in lymphoid tissues but are no longer predominant or even detectable in the absence of drug pressure. Re-treatment with the same drug may not be effective because of rapid selection of these mutants. Thus, genotypic and phenotypic assays must be interpreted in the context of drug treatment history

2.12.4 Antiretroviral Resistance Genotypes

HIV-1 resistance to antiretroviral inhibitors can be defined as any change that improves viral replication in the presence of an inhibitor. It can be determined at a genotypic level by the detection of known mutations that confer phenotypic resistance. Alternatively, it can be determined at the phenotypic level by measuring the ability of an HIV-1 isolate to grow in the presence of an inhibitor or by measuring the HIV-1 RT or PR enzyme activity in the presence of an inhibitor.

2.12.4.1 Resistance Mutations selected during Antiretroviral Treatment

Figure 2.12.4.1 lists common mutations selected by protease inhibitors (A) as well as nucleoside reverse transcriptase inhibitors (NRTIs) and NNRTIs (B), identified largely in studies of monotherapy. In general, there is good concordance between mutations seen in laboratory selection experiments and those in clinical isolates from patients with failing treatment. However, some *in vitro* mutations are not found in patients in whom that particular drug has failed, e.g., the stavudine-selected V75T (Lacey and Larder, 1994) and delavirdine-selected P236L mutation (Dueweke *et al.*, 1993). Although, they confer resistance during *in vitro* virus passage experiments, but were rarely identified in patients in whom the drugs have failed (Lin *et al.*, 1994).

Some mutations selected by antiretroviral drugs directly affect viral enzymes and cause resistance via decreased drug binding, whereas others have indirect effects (Kleim *et al.*, 1994; Chow *et al.*, 1993; Caliendo *et al.*, 1996). It is

useful to categorise resistance mutations as primary or secondary (Figure 2.12.4.1). Primary mutations are generally selected early in the process of resistance mutation accumulation, are relatively inhibitor specific, and may have a discernible effect on viral drug susceptibility. Secondary mutations accumulate in viral genomes already containing 1 or more primary mutations. Many secondary mutations alone have little or no discernible effect on resistance magnitude but may be selected for because they improve viral fitness rather than decrease drug binding to target enzymes.

The distinction between primary and secondary mutations depicted in Figure 2.12.4.1A, may help explain protease inhibitor cross-resistance. There seems to be little overlap in primary mutations selected by different protease inhibitors (e.g., saquinavir-selected L90M and G48V; nelfinavir-selected D30N; and amprenavir-selected I50V). By themselves, these primary mutations may not cause cross-resistance to other protease inhibitors. However, there is an overlapping spectrum of secondary mutations in the protease gene selected by all protease inhibitors (Figure 2.12.4.1A). Many of the secondary changes are compensatory, improving fitness of virus containing primary mutations without actually increasing inhibitor resistance (Schock *et al.*, 1996). The mutations may improve enzymatic function by altering protease catalytic activity or by affecting protease substrates (e.g., making sites in *gag* or other viral precursor polypeptides more easily cleavable).

The NRTIs can select for a single primary mutation (e.g., lamivudine), any one of a few primary mutations (e.g., didanosine and zalcitabine), or an accumulation of primary and secondary mutations (e.g., zidovudine) (Figure 2.12.4.1B). Secondary mutations that compensate for replication impairment caused by primary resistance mutations are also selected by reverse transcriptase inhibitors (Chow *et al.*, 1993; Kleim *et al.*, 1994; Caliendo *et al.*, 1996).

Cross-resistance among NRTIs can be mediated by inhibitor-specific mutations and less specific secondary mutations, especially among drugs that bind to similar or adjacent viral target residues (evident for didanosine and zalcitabine, which select for similar mutations [Figure 2.12.4.1B]). Similarly, the primary mutation commonly selected by lamivudine confers high-level phenotypic resistance to this drug as well as low-level phenotypic resistance to didanosine, zalcitabine, and abacavir in vitro. The clinical significance of cross-resistance among these drugs has not been determined.

Mutations selected by drug combinations may differ from those expected based on monotherapy experience (Richman *et al.*, 1994). A unique mutation pattern in the reverse transcriptase gene that confers broad cross-resistance to all NRTIs, includes the Q151M mutation associated with 3 or 4 additional mutations (Figure 2.12.4.1B), occasionally seen in patients with long-term exposure to NRTIs, and was first described in association with exposure to zidovudine-didanosine

combination therapy (Shafer *et al.*, 1994; Shirasaka *et al.*, 1993) or weekly alternating zidovudine-zalcitabine monotherapy supplemented briefly with didanosine (Shirasaka *et al.*, 1993).

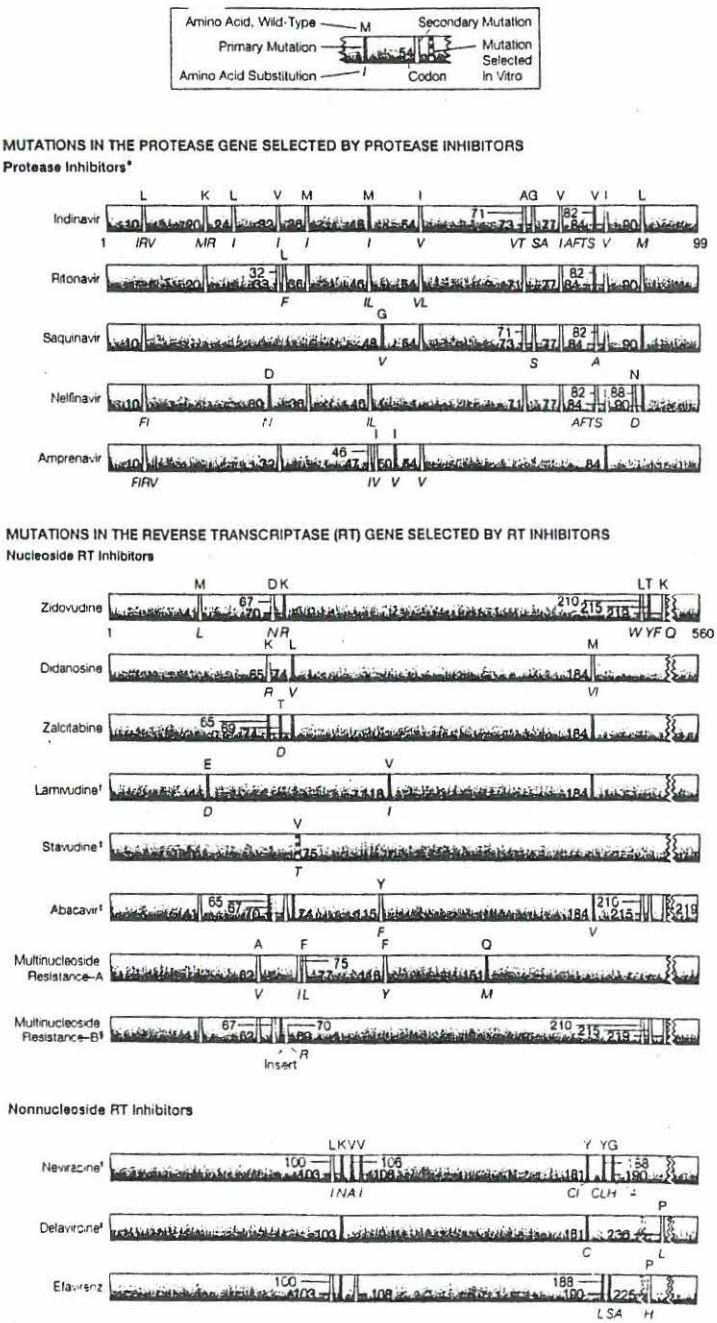

Figure 2.12.4.1 – Most Common Mutations in HIV-1 Genes Conferring Drug Resistance

For each amino acid residue, the letter above indicates the amino acid associated with wild-type virus; the italicized letters below, substitutions that confer viral resistance. Primary mutations (black bars) generally cause decreased inhibitor binding and are the first mutation selected. Secondary mutations (white bars) also contribute to drug resistance and should be considered evidence of resistance, although they may have less direct effect on inhibitor binding in vitro than primary mutations. The mutation selected in vitro (black and white bar) is rarely seen in patients having treatment failure. For indinavir, the mutations listed as primary may not be the first mutations selected, but they have selected in most patient isolates in combination with other mutations. For zalcitabine, all mutations are listed as primary because of inadequate clinical data to determine the most frequent initial mutation. Amino acid abbreviations are A, alanine; C, cysteine; D, aspartate; E, glutamine; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagines; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, Valine; W, thryptophan; Y, tyrosine. Multinucleoside resistance mutational patterns A and B each cause resistance to zidovudine, stavudine, lamivudine, didanosine, zalcitabine, and abacavir. *Mutations selcted by protease inhibitors in gag cleavage sites are not listed because their contribution to resistance is not fully defined. †A preliminary report identifies mutations E44D and V11881 as conferring moderately reduced (about 10-fold) susceptibility to lamivudine with uncertain clinical significance. This contrasts with the greater than 100-fold reduced susceptibility to lamivudine conferred by M184V or M184I, which is associated with virologic rebound (Descamps *et al.*, 2000). ‡The mutations listed for zidovudine above contribute to reduced susceptibility to abacavir in vitro and in vivo and are listed as secondary, even though they may be present before abacavir is introduced (Larder *et al.*, 1999). They have also been reported to be uncommonly selected by stavudine plus didanosine even in the absence of prior zidovudine exposure. Phenotypic resistance of these mutations to stavudine or didanosine in vitro was not identified. The clinical significance of these mutations and of V75T on in vivo response to stavudine is not known. §Several insertions of 2 amino acids have been reported following T69S (or rarely T69A), including Ser-Ser; Ser-Gly; Ser-Ala; and Thr-Ser (Larder *et al.*, 1999). ııFor nevirapine or delavirdine, each mutation can occur as an initial or subsequent mutation and affect inhibitor binding (Demeter *et al.*, 2000).
(adapted from Hirsch *et al.*, 2000)

2.12.4.2 Interactive Effects of Mutations on Drug Susceptibility

Some mutations selected by one drug suppress phenotypic effects of another mutation, e.g., suppression of zidovudine resistance by didanosine-selected L74V, NNRTI-selected Y181C, and lamivudine-selected M184V (St Clair *et al.*, 1991; Larder *et al.*, 1993; Tisdale *et al.*, 1993; Tachedjian *et al.*, 1996). Molecular mechanisms for these interactions are not well understood.

Lamivudine primarily selects for reverse transcriptase codon 184 mutations whether it is given as monotherapy or in combination. Suppression of zidovudine resistance phenotype or delay in its

emergence due to M184V is common during zidovudine-lamivudine combination therapy (Larder *et al.*, 1995). Nevertheless, additional reverse transcriptase mutations emerge with combination zidovudine-lamivudine therapy and eventually overcome the suppressive effect, resulting in high-level resistance to both drugs (Nijhuis *et al.*, 1997; Kemp *et al.*, 1998). The M184V mutation effect is thus likely to be transient and its induction less useful than maximising HIV suppression. The presence of this mutation should prompt consideration of change in therapy, unless no satisfactory therapeutic options remain.

2.12.4.3 Genotypic Resistance Assays

Assays for detecting the HIV-1 genome mutations are based upon a polymerase chain reaction (PCR) as the first methodological step (Hirsch *et al.*, 2000). The PCR amplifies an RNA fragment (after a reverse transcription step) to quantities large enough for genotyping (the second step). Most laboratories now analyse protease and reverse transcriptase gene DNA sequences but may not investigate other genome regions relevant for drug resistance (e.g., gag cleavage sites). It is still a technical challenge to amplify and genotype all regions implicated in protease and reverse transcriptase inhibitor resistance.

Similar to phenotypic assays, genotypic assays require amplification of HIV-1 sequences from plasma containing at least 500 to 1000 HIV RNA copies/ml and may detect a mutant present in plasma at a level above 10% to 50% of the total population of viruses (Schuurman *et al.*, 1999a). Resistance testing is not as likely to be useful when values are below this level. With current methods, species constituting 20% or more of amplified product can usually be detected (Hanna and D'Aquila, 2001). False positivity for mutations is possible due to carryover from

other HIV-1 samples in the laboratory or from random polymerase errors during *in vitro* nucleic acid synthesis. Also, unless molecular clones of PCR products are studied (not routinely done), it is impossible to be certain whether multiple positions in the sequence are physically linked together on the same genome; ie, it is impossible to differentiate a mixture of singly mutant genomes from a mixture having some genomes with accumulated different mutations.

2.12.4.4 Assays used for Genotypic Analysis

2.12.4.4.1 *Point Mutation Assays*

After mutations at specific codons conferring resistance have been identified, specific oligonucleotides can be synthesised to allow selective priming and DNA synthesis or to allow selective hybridisation (Kozal *et al.*, 1996).

A) *Single Nucleotide Sequencing Assay*

The PCR reaction is performed by using a biotinylated primer. The biotinylated PCR product is immobilised on a streptavidin-coated microtitre well and then denatured to a single strand. An oligonucleotide primer with its 3' end one nucleotide upstream of the point mutation under analysis, is annealed and a single labelled ddNTP is used to extend the oligonucleotide primer. If the added ddNTP is complementary to the point mutation, extension will occur. The well giving a signal indicates the complementary base in the target. In a mixed population of wild type and mutant sequences a

signal will be found in more than one well (Kaye *et al.*, 1992).

B) *Restriction Enzyme Digestion*

Vasudevachari *et al.* (1996) described a rapid screening procedure for the detection of resistance mutations based on restriction enzyme digestion. The PCR assay is performed by using two different labelled primers. Restriction digestion will only occur when no mutations are observed at the restriction site. Wild type and mutant sequences will result in different sizes of DNA fragments. After restriction digestion, the various DNA fragments are separated on a denaturing gel and the intensities of the fluorescent bands can give information on the relative proportion of wild type and mutant at the codon being analysed.

C) *Selective PCR*

Selective PCR makes use of oligonucleotide primers developed to specifically anneal at their 3' ends to either the wild type or mutant codon of interest (Larder *et al.*, 1991; Anderson *et al.*, 1994). Theoretically, during PCR, the DNA fragments are only generated when the 3' end of the oligonucleotide primers matches the target sequence. Two separate PCRs are performed, the first one to amplify the wild-type sequence and the second one to amplify the mutant sequence. After optimisation, selective PCR

can yield information on the relative proportion of wild type and mutant at the codon being analysed (Van Laethem *et al.*, 1999).

D) *RNase A Mismatch Cleavage Method*

In the RNase A mismatch cleavage method the ability of RNase A to recognise and cleave single-base mismatches in RNA-DNA hybrids is used for the detection of point mutations (López-Galindez *et al.*, 1991). An RNA fragment from a wild type sequence is synthesised and hybridised to yield a PCR product originating from a patient sample. The sequence differences between the RNA probe and the DNA target sequence generate heterohybrids, which after RNase treatment generate various RNA fragments. These RNA fragments can be separated on a denaturing gel resulting in characteristic patterns for wild type and mutant sequences respectively.

E) *Ligase Detection Reaction*

This reaction occurs by the annealing and subsequent ligation of two oligonucleotides. The probe oligonucleotide is attached to a latex bead on the 5' end, and its 3' end is complementary to either the wild type or mutant sequence. The 5' end of the detector oligonucleotide is phosphorylated and is designed to anneal to the nucleotides next to the 3' end of those bound by the probe oligonucleotide. The detector has a biotin on the 3' end. When the probe

oligonucleotide is complementary to the target sequence, the detector can be attached by ligation. Ligation of both oligonucleotides occurs only when the two bases on either side of the ligation site are complementary to the target. The probe and detector oligonucleotides are denatured from the template by heating and passing through a nylon membrane which traps the latex bead. When ligation has occurred, the biotin molecule is trapped in the membrane and can be detected with streptavidin-horseradish peroxidase, followed by colorimetric detection (Frenkel *et al.*, 1995)

F) ***Differential Hybridisation Assay***

This assay is used for the determination of proportions of wild type and mutant sequences in a sample. PCR is performed by using one biotinylated primer and subsequently dispensed in a well in which streptavidin is covalently bound. The PCR fragment is denatured and hybridised with either a wild type or mutant probe labelled with alkaline phosphatase. The detection is performed by a colorimetric or chemiluminescent method. In order to quantify the amount of PCR product bound to each well, the DNA-probe fragments are denatured again and re-hybridised with a generic probe (Eastman *et al.*, 1995 and 1998).

G) *Line Probe Assay (LiPA)*

This assay is based on reverse-hybridisation of biotinylated PCR product to the oligonucleotide probes immobilised on nitrocellulose strips. The probes are designed to contain mutations associated with resistance, or they contain wild type sequences. For every mutation of interest various probes have been immobilised on the strip to account for the variability of the background sequence in the vicinity of the codon to be analysed. Colorimetric detection of hybridisation is done by streptavidin-alkaline phosphatase (Stuyver *et al.*, 1997).

H) *GeneChip Technology*

This technology allows the probing of HIV-1 sequences using high-density oligonucleotides arrays immobilised on a solid matrix (Southern *et al.*, 1992; Günthard *et al.*, 1998a and 1998b; Anderson *et al.*, 2000). The sequence and position of each probe on the matrix is known. The fluorescent labelled PCR product anneals to a probe when it is completely complementary. Computerised detection and analysis are based upon laser fluorescence scanning of the DNA interactions on the array. The pattern of hybridisation can allow the determination of the complete target sequence. However, it can only detect variants for which probes have been immobilised on the chip and cannot detect new, previously unreported, variants. This can result in

incomplete sequences or in an over stimulation of the known variants in the total virus population.

2.12.4.4.2 *DNA Sequencing Assays*

A) *Dideoxy Chain Termination Sequencing*

Dideoxy chain termination sequencing provides information on all the nucleotides of the sequenced region. The target sequence is amplified using PCR and the sequence is determined on the incorporation of dideoxynucleotides. The various fragments differ in length and are separated by a denaturing gel. Most of the sequencing technologies are semi-automated and make use of fluorescence labelled primers or dideoxynucleotides.

Commercial versus In-House Methods

Drug resistance can either be determined phenotypically by cultivating (recombining presence of increasing drug concentrations) or genotypically by searching for mutations known to be associated with drug resistance from *in vitro* or *in vivo* data. The time-consuming phenotypic assays will remain restricted to specialised laboratories. Many assays are now performed by many laboratories with in-house techniques or are offered routine virological diagnostics. Two commercial kits for HIV genotyping exist, the HIV Genotyping System (ViroSeq HIV-1 Genotyping System), [Applied Biosystems, Foster

City, Calif.] and TruGene [Bayer Diagnostics, Fernwald, Germany]).

The ViroSeq HIV-1 Genotyping System detects mutations in the RT and protease regions of the *pol* gene and provides the physician with a report indicating genetic evidence of viral resistance. It is a complete system that provides reagents for viral RNA isolation from plasma, RT-PCR, and sequencing (Mukaide *et al.*, 2000; Cunningham *et al.*, 2001). The entire protease gene and two-thirds of the RT gene are amplified to generate a 1.8 kb amplicon. The amplicon is used as a sequencing template for seven primers that generate an approximately 1.3 kb consensus sequence. The software compares the consensus sequence with a known reference, HXB-2, to determine mutations present in the sample. Finally, the ViroSeq software uses a proprietary algorithm to analyse the mutations and generate a drug resistance report.

2.12.4.5 Phenotypic Resistance Assays

Drug-resistant virus phenotypes are detected by measuring the 50% or 90% inhibitory concentration (IC_{50} or IC_{90}) of a drug *in vitro*. In standardised drug susceptibility assays, cells are infected with a fixed amount of viral inoculum, and various drug concentrations are tested to quantitate the drug concentration required to inhibit viral replication (e.g., determine dose-response curve) compared with untreated infected control cells. The precise IC_{50} or IC_{90} values obtained depend on the assay used, cell type used, antiretroviral drug tested, input viral

inoculum, marker of viral replication selected (e.g., measurement of HIV p24 antigen or reverse transcriptase activity), and time in culture (Johnson, 1995). Therefore, IC_{50} or IC_{90} values from one type of assay should not be compared with those obtained by another method.

Drug susceptibility testing measures HIV ability to grow at different drug concentrations versus a drug-susceptible laboratory strain of virus or previous isolate from the same patient. In general, a 4-fold increase is the minimum change reliably detectable in the laboratory. Changes in IC_{50} or IC_{90} values that are clinically important regarding drug activity have not been defined. High-level HIV-1 resistance to zidovudine (e.g., isolates for which IC_{50} values are $\geq$ 1.0 μmol/L) predicted more rapid clinical progression and death in an analysis adjusting for other risk factors in patients with advanced HIV disease receiving zidovudine monotherapy (D' Aquila *et al.*, 1995; Japour *et al.*, 1993; Montaner *et al.*, 1993). The clinical relevance of IC_{50} or IC_{90} values for each multi-drug regimen component has not been defined. Also, sustained virus suppression may be seen in patients in whom drug-resistant virus has been detected (Schuurman *et al.*, 1995; Havlir *et al.*, 1995). This may result from achieving plasma drug levels *in vivo* that exceed IC_{50} or IC_{90} values for resistant virus *in vitro*.

Phenotypic assays may fail to detect evolving resistance that has not yet led to measurable increases in IC_{50} values, e.g., the K70R zidovudine resistance-conferring mutation emerges within 12 weeks in nearly half of patients receiving zidovudine monotherapy, yet its presence alone is not associated with measurable increases in zidovudine IC_{50}. Thus, detection of a mutant genotype may be expected to precede detection of an increased IC_{50} value. Moreover, a limitation of all drug susceptibility assays described to date is that only predominant circulating viral populations are sampled to yield IC_{50} or

IC_{90} values. Thus, minority drug-resistant species contributing to drug failure or transmission of resistant virus may not be detected.

2.12.4.5.1 Replication-based Assays

A) *Recombinant Virus Assay*

This assay starts with the amplification of the patient derived HIV-1 target sequence (RT, PR or PR+RT) using RT-PCR (Kellam *et al.*, 1994; Maschera *et al.*, 1995; Boucher *et al.*, 1996; Walter *et al.*, 1999). Subsequently, the PCR product is incorporated into a proviral laboratory clone by means of homologous recombination or ligation. This process generates a stock of chimeric viruses. The viruses are tested for their ability to grow in the presence of different concentrations of inhibitors. Drug resistance testing was initially performed in a HeLa plaque reduction assay. Later, it was modified by using a MT2/MT4-MTT/XTT based cythopathic protection assay (Pauwels *et al.*, 1988; Jellinger *et al.*, 1997).

B) *PBMC p24 Growth Inhibition Assay*

The assay is standardised by the AIDS Clinical Trails Group and the US Department of Defence (ACTG-DoD protocol) (Japour *et al.*, 1995). The patient's HIV-1 infected PBMCs or plasma are co-cultivated with uninfected phytohemagglutinin (PHA)-stimulated donor lymphocytes in order to grow a high-titre viral stock. After completion, the virus is tested for its

ability to grow in the presence of inhibitors by using serial dilutions of the inhibitors and measuring the production of p24 antigen by means of an ELISA test.

C) ***Hela Plaque Reduction Assay***

The assay involves the measurement of the number of plaques, reflecting virus-killed cells in a $CD4^+$ HeLa cell monolayer (Larder *et al.*, 1990). The HIV-1 isolates are co-cultivated in PBMCs and then used to infect monolayers of HeLa cells in the presence of serial dilutions of inhibitors. The viral susceptibility to inhibitors is derived from the reduction of the number of plaques in the presence of an active inhibitor.

2.12.4.5.2 Enzyme-based Assays

A) ***Amp-RT Assay***

This assay measures the HIV-1 RT activity from plasma in the presence of different RTI concentrations (Heneine *et al.*, 1995; Garcia-Lerma *et al.*, 1999; Vázquez-Rosales *et al.*, 1999). The activity of the RT in plasma is detected by measuring the ability to produce a DNA copy of a known heteropolymeric RNA template by extending a complementary DNA oligonucleotide primer. The reverse-transcribed cDNA is subsequently detected by PCR amplification. The RT susceptibility to NNRTIs and to the 5'-triphosphate form of NRTIs is

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determined based on the reduction of the PCR signal in the presence of an active inhibitor.

2.12.4.6 Genotypic versus Phenotypic Resistance Assays

Both genotypic and phenotypic assays have several advantages and disadvantages. They provide complementary information on the resistance of a HIV-1 isolate. No recommendation can be made at present in favour of or against these assays.

Genotypic resistance assays can deliver results within a few days whereas more time is required to obtain phenotypic resistance results. This turn-around time can be an important factor in some clinical situations where results are needed as soon as possible, e.g. post-exposure prophylaxis and primary HIV-1 infection.

Both resistance assays have to be performed in highly specialised laboratory facilities. This is especially true for phenotypic resistance assays as they require bio-safety level 3 facilities, whereas genotypic resistance can be performed in a dedicated molecular biology laboratory. Because both types of assays require sophisticated technology and knowledge, a strict adherence to current laboratory standards is important, and also well trained and experienced laboratory technicians are considered as an asset. A blinded genotyping proficiency panel, ENVA-2, was sent to laboratories in Europe, North America and Australia, and demonstrated that many laboratories are able to detect wild type virus but that mutant virus was often underestimated. Only half of the laboratories were able to detect 50/50 mixtures of wild type and mutant codons (Schuurman *et al.*, 1999a and 1999b).

The current resistance assays, with the exception of point mutation assays, have difficulties to detect minor variants. Sequencing and phenotypic assays may differentiate a mutant only at a level of 10% to 50% in a mixture of viruses (Günthard *et al.*, 1998a; Schuurman *et al.*, 1999a and 1999b; Van Laethem *et al.,* 1999). Point mutation assays are able to detect minor variants at a level of 1% to 5% (Stuyver *et al.*, 1997; Van Laethem *et al.*, 1999). However, point mutation assays are limited to the detection of particular key mutations and their performance can be influenced by the variability of the background sequence in the vicinity of the key mutation to be analysed. Sequencing has the advantage of detecting all known resistance-related mutations. Phenotypic assays even measure the effective sensitivity, resulting from known but also unknown resistance-related mutations.

The information provided by genotypic resistance assays may be difficult to interpret. The current knowledge on the correlation between the genotypic characteristics of a HIV-1 isolate is mainly based on *in vitro* studies or monotherapy studies. This can give only a part of the necessary information as patients are currently treated with combination therapies that change in time. This results in complex patterns of mutations. The phenotypic effects of mutations and their interactions can sometimes not be predicted. Phenotypic resistance assays give a direct measurement of susceptibility towards the inhibitors tested, which includes the effect of all mutations and their interactions. The clinical relevance of phenotypic results, however, can also be difficult to interpret because the threshold at which a specific inhibitor becomes ineffective *in vivo* has not been determined yet.

Another issue in the interpretation of genotypic results is whether the mutations that make up part of a set of multiples exist on a single clone

or are spread across multiple clones of variants. Phenotypic resistance assays resulting in low-level resistance may be caused by a small change in susceptibility of the whole virus population or a mixture of resistant and susceptible virus. The linkage of mutations could have important clinical implications, but at the moment this issue cannot routinely be elucidated with the current resistance assays.

An important limitation of currently available assays is their need of plasma samples with a HIV-1 viral load above 1000 copies/ml. This is required to achieve reliable results. Otherwise non-representative variants could be picked up due to stochastic events during the experimental procedures. However, switching therapy early after virology failure, when the HIV-1 viral load is detectable but still very low, results in a better chance of response to the salvage therapy. There are only a few studies that provide the optimal point at which therapy should be changed in terms of long-term clinical outcome, but the short-term risk of any viral replication in the presence of inhibitors is the development of resistance. HIV-1 viral load between 50 and 500 copies/ml is associated with a higher risk of resistance than levels below 50 copies/ml (Raboud *et al.*, 1998).

CHAPTER 3

METHODOLOGY

3.1 STUDY OBJECTIVE

The main objective of this study was to study the variants of HIV-1 in circulation in untreated patients in the Free State. This will establish a baseline of the variants present (including possible existing resistance against drugs) before the rollout of antiretroviral therapy in the province.

3.2 STUDY DESIGN

The study population consisted of 19 adult ARV-naïve AIDS patients recruited from Tsepo House, an AIDS hospice in Bloemfontein. Initially, samples were taken from 32 patients, but due to haemolysis (2 samples), insufficient volume of plasma (4) and non-amplification or weak amplification of the required fragment (8), most of which did not produce good quality sequences, the results of 19 patients will be presented. The duration of the study was approximately three years. Approval from the Ethics committee of the Faculty of Health Sciences, University of the Free State, was obtained (ETOVS 227/01). The necessary consent was obtained from every participant in the study (Appendix A). Every patient will also receive an information sheet to provide him/her with all the information regarding the study (Appendix A).

3.3 STUDY POPULATION

3.3.1 Number of Subjects

Nineteen HIV-positive/AIDS naïve patients were enrolled in this study. Seven of the 19 patients were male while 12 were female.

The high percentage of females represents the demographic distribution of HIV-infection prevalence in this region of South Africa and is perhaps also a reflection of the national HIV-infection prevalence.

3.3.2 Justification for Inclusion and Exclusion Criteria

The criteria were set to ensure a homogenous subject population.

3.3.2.1 Inclusion Criteria

- HIV positive/AIDS male and female patients between 18-65 years
- No previous antiretroviral treatment
- Viral Load more than 2000 copies/ml
- $CD4^+$ T-cell count of 100-350 cells/mm^3
- Only those giving informed consent will be included in the study.

3.3.2.2 Exclusion Criteria

- If consent from the patient cannot be obtained
- If the patient is not an HIV positive/AIDS sufferer
- If the viral load is less than 2000 copies/ml

- Patients previously on antiretroviral treatment
- Participation in another study with an experimental drug within eight weeks prior to the first administration of the project
- Loss of blood equal to or exceeding 500ml during the 8 weeks prior to the administration of the project
- Being on anti-retroviral therapy or any treatment for chronic diseases

3.3.3 Subject identification

- Each enrolled subject received a number (Between 01-19) and retained this number throughout the study.
- Each enrolled subject was identified by date of birth (six digits).

3.3.4 Withdrawal Criteria

Subjects had the right to withdraw from the study at any time, irrespective of the reason. The withdrawals would be handled as dropouts and would not be replaced in this study.

3.4 SAMPLING

Five ml of whole blood was collected in a sterile EDTA anticoagulant tube (Vacutainer™PPT™ Brand Tubes, Becton-Dickenson #362788 or equivalent) and the tube was immediately inverted 8-10 times to mix. The RNA was isolated from the plasma immediately after sampling and frozen at -70°C for later analysis.

The AMPLICOR HIV-1 MONITOR Test, v1.5 is for use with plasma specimens only. Another tube containing 10ml of EDTA (Lavender top, Becton Dickinson #6454) whole blood was drawn from the subjects to perform the viral load counts. The plasma was separated from the whole blood within 6 hours by centrifuging at 800-1600 xg for 20 minutes at room temperature. The viral loads were either done manually or performed by Pathcare (pathologists), Bloemfontein.

3.5 LABORATORY INVESTIGATIONS

3.5.1 Viral load counts

The AMPLICOR HIV-1 MONITOR® Test, version 1.5 (v1.5) is an *in vitro* nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma. The test is intended for use in conjunction with clinical presentation and other laboratory markers as an indicator of disease prognosis. The test can be used to assess patient prognosis by measuring the baseline HIV-1 RNA level or to monitor the effects of antiretroviral therapy by measuring changes in plasma HIV-1 RNA levels during the course of antiviral treatment. The manual Amplicor test was initially performed in the laboratories of the department of Haematology, Bloemfontein, while all samples were later re-tested by Pathcare, Bloemfontein, using the automated version of the assay. The manual method will be described.

3.5.1.1 Standard Specimen and Control Preparation

1. A 70% ethanol solution was prepared. For 12 tests, 11.0 ml 95% ethanol and 4.0 ml of deionised or distilled water were mixed.

2. One 2.0 ml screw-cap tube was labelled for each patient specimen and three additional tubes were labelled as HIV-1 Negative Control [(HIV-1 (-)C) (Tris-HCl buffer, < 0.005% Poly rA RNA (synthetic), EDTA, 0.05% Sodium azide)] ,HIV-1 MONITOR Low Positive Control [(HIV-1L(+)C) (Tris-HCl buffer, < 0.001% Non-infectious *in vitro* transcribed RNA (microbial) containing HIV-1 sequences, < 0.005% Poly rA RNA (synthetic), EDTA, 0.05% Sodium Azide)] and HIV-1 MONITOR High Positive Control [(HIV-1H(+)C) (Tris-HCl buffer, < 0.001% Non-infectious *in vitro* transcribed RNA (microbial) containing HIV-1 sequences, <0.005% Poly rA RNA (synthetic), EDTA, 0.05% Sodium azide].
3. **Preparation of Standard Working Lysis Reagent.** HIV-1 MONITOR Quantitation Standard, version 1.5 [(HIV-1 QS, v1.5), Tris-HCl buffer, < 0.001% Non-infectious *in vitro* transcribed RNA (microbial) containing HIV-1 primer binding sequences and a unique probe binding region, <0.005% Poly rA RNA (synthetic), EDTA, Amaranth Dye, 0.05% Sodium azide)] was vortexed for 5-10 seconds before use. For each batch of 12 specimens and controls, 100 µl HIV-1 QS, v1.5, was added to one bottle of HIV-1 MONITOR Lysis Reagent [(HIV-1 LYS), Tris-HCl buffer, 68% Guanidine thiocyanate, 3% Dithiothreitol, <1% Glycogen)] and mixed well.
4. 600 µl standard working lysis reagent was added to each of the labelled tubes and capped. It was necessary to check that the Working Lysis Reagent is pink to confirm that the Quantitation Standard was added to the Lysis Reagent.
5. Preparation of Standard Controls:
   - Negative Plasma (Human), [(NHP), Human plasma, non-reactive by US FDA licensed tests for antibody to HCV,

antibody to HIV-1/2, and HbsAg, 0.1% ProClin® 300)] HIV-1 (-) C, HIV-1 L(+)C and HIV-1 H(+)C were vortexed for 3-5 seconds.

- 200 µl NHP was added to each of the three control tubes. The tubes were capped and vortexed for 3-5 seconds.
- 50 µl HIV-1 (-) C was added to the tube labelled "HIV-1 (-) C" containing the Standard Working Lysis Reagent and NHP. The tube was then capped and vortexed for 3-5 seconds.
- 50 µl HIV-1 L(+)C was added to the tube labelled "HIV-1 L(+)C" containing Standard Working Lysis Reagent and NHP. Again the tube was capped and vortexed for 3-5 seconds.
- 50 µl HIV-1 H(+)C was added to the tube labelled "HIV-1 H(+)C" containing Standard Working Lysis Reagent and NHP and the tube was capped and vortexed for 3-5 seconds.

6. 200 µl of each patient's specimen was added to the appropriately labelled tubes containing standard working lysis reagent. The tubes were capped and vortexed for 3-5 seconds.
7. The specimen and control tubes were then incubated for 10 minutes at room temperature.
8. For each specimen and control tubes, the caps were removed, and 800 µl 100% isopropyl alcohol was added, after which the tubes were capped and vortexed for 3-5 seconds. **NB:** Do not have more than one tube open at a time.

9. The specimens and controls were centrifuged [(Heraeus Labofuge 400 at maximum speed (14000 xg)] for 15 minutes at room temperature making use of orientation marks.
10. By using a new, fine-tip transfer pipet for each tube, the supernatant was carefully removed and discarded from each tube, being careful not to disrupt the pellet. As much liquid as possible was removed without disturbing the pellet. The supernatant was withdrawn slowly, allowing the liquid to drain completely off the sides of the tube.
11. 1.0 ml 70% ethanol was added to each tube, which was then capped and vortexed for 3-5 seconds.
12. The tubes were then placed in a microcentrifuge with the orientation marks facing outward and centrifuged for 5 minutes at maximum speed (14000 xg) at room temperature.
13. Again a new fine-tip disposable transfer pipet was used for each tube, and the supernatant was carefully removed without disturbing the pellet. The pellet was clearly visible at this step and as much of the supernatant as possible was removed.
14. 400 µl HIV-1 MONITOR Specimen Diluent, [(HIV-DIL), Tris-HCl buffer, <0.005% Poly rA RNA (synthetic), EDTA, 0.05% Sodium azide)] was added to each tube which was then, re-capped and vigorously vortexed for 10 seconds. The reverse transcription and amplification were started within 2 hours of specimen and control preparation.
15. 50 µl of each processed specimen and control was added to the appropriately labelled reaction tubes containing Working Master Mix. The tubes were re-capped and sealed using the MicroAmp cap installing tool.
16. The positions of the controls and specimens were recorded. Reverse Transcription and Amplification was begun within

45 minutes of the time that the processed specimens and controls were added to the reaction tubes containing Working Master Mix.

17. The prepared specimens and controls were then moved in the MicroAmp Tray to the Amplification/Detection Area.

3.5.1.2 Reverse Transcription and Amplification

Performed in Post Amplification Area – Amplification/Detection

1. The tray/retainer assembly was placed in the thermocycler block.
2. The PE Applied Biosystems GeneAmp PCR System 2400 thermocycler was programmed for the AMPLICOR HIV-1 MONITOR Test, version 1.5, as follows:

HOLD Program	2 min at 50°C
HOLD Program	30 min at 60°C
CYCLE Program **(8cycles)**	10 sec at 95°C 10 sec at 52°C 10 sec at 72°C
CYCLE Program **(23 cycles)**	10 sec at 90°C 10 sec at 55°C 10 sec at 72°C
HOLD Program	15 min at 72°C

3. Specimens and controls have to be removed within 15 minutes of the start of the final HOLD program.
4. The tray from the thermocycler was removed at any time during the final HOLD program, placed in the MicroAmp base and followed immediately.
5. The caps were carefully removed from the reaction tubes to avoid creating aerosols of the amplification products. 100 µl

[1] MONITOR Denaturation Solution [([1] MONITOR DN), 1.6% Sodium hydroxides, EDTA, Amaranth dye)] was immediately added to each of the reaction tubes by using a multichannel pipettor with aerosol barrier tips and mixed by pipetting up and down 5 times. The denatured amplicon can be held at room temperature for no more than 2 hours before proceeding to Detection.

3.5.1.3 Detection

Performed in Post Amplification Area –Amplification/Detection

1. Working Wash Solution was prepared by adding 1 volume 10x Wash Concentrate [(10x WB), < 2% Phosphate buffer,< 9% Sodium chloride, EDTA, < 2% Detergent, 0.5% ProClin 300)] to 9 volumes of distilled water and mixed well.
2. The HIV-1 MONITOR Microwell Plate [(HIV-1 MONITOR MWP), MWP coated with HIV-1 specific DNA probe SK102 (Rows A to F) and QS-specific probe CP35 (Rows G and H). Twelve, 8-well strips in one re-sealable pouch with desiccant)], was allowed to warm to room temperature before removal from the foil pouch. 100 µl [2] MONITOR Hybridization Buffer [([2] MONITOR HYB), Sodium phosphate solution, < 25% Sodium thiocyanate, <0.2% Solubilizer)] was added to each well on the MWP to be tested. Rows A to F of HIV-1 MONITOR MWP are coated with the HIV-1-specific oligonucleotide probe; rows G and H are coated with the HIV-1 Quantitation Standard-specific oligonucleotide probe.

3. 25 µl of denatured amplicon was added to the HIV-1 wells in row A of the MWP and mixed up and down 10 times with a 12-channel pipettor with aerosol barrier tips. Serial 5 fold dilutions were then made in the HIV-1 wells in rows B to F by transferring 25 µl from row A to row B and repeated the mixing step. This continued to row F and row F was mixed as before, then 25 µl was removed and discarded.
4. 25 µl of denatured amplicon was then added to the Quantitation Standard wells in row G of the MWP and mixed up and down 10 times with a 12-channel pipettor with aerosol barrier tips.
5. 25 µl was transferred from row G to row H. After mixing 25 µl was removed from row H and discarded.
6. The MWP was then covered with the MWP lid and incubated for 1 hour at 37°C.
7. The MWP washer (Microtitration Plate washer, ADIL instruments F-67300 Starsbourg, France) was then used to wash the MWP with the Working Wash Solution. The MWP washer was programmed to:
   - Aspirate the contents of the wells.
   - Fill each well to the top with Working Wash Solution, soak for 30 seconds and aspirate dry.
   - Repeat the Step above an additional four times.
   - After automated washing is complete, tap the plate dry.
8. 100 µl [3] Avidin-Horseradish Peroxidase Conjugate [([3] AV-HRP), Tris-HCl buffer, <0.001% Avidin-horseradish peroxidase conjugate, Bovine gamma globulin (mammalian), Emulsit 25 (Dai-ichi Kogyo Seiyakui Co., Ltd.), 0.1% Phenol, 1% ProClin 150)] was then added to each well, the MWP covered and incubated for 15 minutes at 37°C.

9. The MWP was washed as described in Step 7.
10. The Working Wash Substrate Solution was then prepared by mixing 12 ml [4A] Substrate A [([4A] SUB A), Citrate solution, 0.01% Hydrogen peroxide, 0.1% ProClin 150)] and 3 ml [4B] Substrate B [([4B] SUB B), 0.1% 3,3',5,5'-Tetramethylbenzidine (TMB), 40% Dimethylformamide (DMF)]. The Working Substrate was prepared no more than 3 hours before use after which it was stored at room temperature and protected from exposure to direct light.
11. 100 µl of Working Substrate was added to each well.
12. The colour was allowed to develope for 10 minutes at room temperature (20-25°C) in the dark.
13. 100 µl [5] Stop Reagent [([5] STOP), 4.9% Sulfuric acid)] was then added to each well.
14. The optical density (OD) was then measured at 450 nm within 10 minutes of adding [5] STOP and the absorbance value was recorded for each well.

3.5.1.4 Result Calculation

For each specimen and control the HIV-1 RNA level was calculated as follows:

1. The appropriate HIV-1 well was chosen according to:
   - The HIV-1 wells in rows A to F represented neat 1:5, 1:25, 1:125, 1:625 and 1:3125 serial dilutions, respectively, of the HIV-1 amplicon. The absorbance values should decease with the serial dilutions, with the highest OD_{450} for each specimen and control in row A and the lowest OD_{450} in row F.

- The well with the lowest OD_{450} was chosen (≥ 0.20 and ≤ 2.0 OD units).

2. The background OD was subtracted from the selected HIV-1 OD value (background = 0.07 OD units).
3. The total HIV-1 OD was calculated by multiplying the background-corrected OD value of the selected HIV-1 well by the dilution factor associated with that well.

ROW	DILUTION FACTOR
A	1
B	5
C	25
D	125
E	625
F	3125

4. The appropriate Quantitation Standard well was chosen as follows:
   - The Quantitation Standard wells in rows G and H represented a neat and a 1:5 dilution, respectively, of the Quantitation Standard amplicon. The absorbance values would decrease with the serial dilutions, with the highest OD_{450} for each specimen or control in row G, and the lowest OD_{450} in row H.
   - The well with the lowest OD_{450} was chosen (≥ 0.20 and ≤ 2.0 OD units).
5. The background OD was subtracted from the selected Quantitation Standard OD values (Background = 0.07 OD units).
6. The total Quantitation Standard OD was calculated by multiplying the background-corrected OD value of the selected QS well by the dilution factor associated with that well.

7. For specimens and controls processed, the HIV-1 RNA copies/ml plasma were calculated as follows:

$$\frac{\text{Total HIV-1 OD}}{\text{Total QS OD}} \times \text{Input HIV-1 QS copies/PCR} \times 40 = \text{HIV-1 RNAcopies/ml}$$

Where:

Total HIV-1 OD	=	calculated Total HIV-1 OD
Total QS OD	=	calculated Quantitation Standard OD
Input HIV-1 QS Copies/PCR	=	the number of copies of Quantitation Standard in each reaction; this information is lot specific. See AMPLICOR HIV-1 MONITOR Test, version 1.5 Data Card.
40	=	Sample Volume Factor to convert copies/PCR to copies per ml of plasma.

3.5.2 HIV Genotyping

The genotyping was done by using both an in-house method and the Viroseq™ HIV-Genotyping System (supplied and supported by Abbott Diagnostics).

3.5.2.1 RNA Isolation (High Pure Viral RNA Kit, Roche) was used for both genotyping methods

1. Tubes with 0.2 ml blood were thawed on ice

2. The tubes with 40 µl poly-A were thawed on ice, and 5 ml binding buffer (1), (with 25 ml nucleic acid binding buffer consisting of 4.5 M guanidin hydrochloride, 50 mM Tris-HCl and 30% Triton X-100 (w/v), pH 6.6 (25°C) added. This solution could be used for 10 specimens and could be kept at room temperature for three months.
3. 200 µl plasma was then added to 400 µl of binding buffer (1), mixed well, and allowed to stand for 10 minutes at room temperature.
4. Every specimen was placed in a filter tube within a collection tube and numbered.
5. 600 µl was transferred to every filter tube and centrifuged for 15 seconds in an Eppendorf Minispin at 13 500 rpm.
6. The filter tube was removed and placed in a new collection tube, and the old collection tube discarded.
7. 500 µl Inhibitor Removal Buffer (3a), [(add 20 ml ethanol p.a. before first use), with 33 ml buffer consisting of 5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6 (25°C) final concentration after addition of ethanol)] was added and centrifuged for 1 minute at 13 500 rpm.
8. The filter tube was removed and placed in a new collection tube and the old collection tube discarded.
9. 450 µl wash buffer (3), [(add 40 ml ethanol p.a. to each vial before use), with 10 ml wash buffer consisting of 20 mM NaCl and 2 mM Tris-HCl, pH 7.5 (25°C) final concentration after addition of ethanol)] was added and centrifuged for 15 seconds in an Eppendorf Minispin at 13 500 rpm.
10. 450 µl wash buffer (3) was added, and centrifuged for 30 seconds in an Eppendorf Minispin at 13 500 rpm, to ensure that all the wash buffer was removed.

11. The collection tube was discarded and replaced with a 1.5 ml Eppendorf tube.
12. 50 µl Viroseq "RNA diluent"(Poly rA RNA (synthetic), Tris-HCl buffer, 0.05% Sodium Azide) was carefully placed on the filter, and centrifuged for 1 minute at 13 500 rpm in an Eppendorf Minispin.
13. The filter tube was then discarded, the Eppendorf tube closed and put at -70°C for long-term storage or kept on ice for the following step.

3.5.2.2 RT-PCR

3.5.2.2.1 Roche Titan One Tube RT-PCR System

Important: Gloves were always worn when RNA was handled to avoid any contamination with RNases.

1. The Sterile water (vial 6, H_2O redistilled PCR grade), dNTP mix (vial 2, concentration 10 mM total), DTT solution (suppl), RNase Inhibitor (vial 3, concentration 5U/µl), Control primer mix (vial 5, human β-actin upstream and downstream primer, concentration 20 µM total) downstream primer, upstream primer Control RNA (vial 4, Human control RNA (K562 total RNA with MS2 carrier RNA) concentration 2 pg/µl) and Template RNA were thawed and placed on ice.
2. All the reagents were briefly vortexed and centrifuged.
3. The Master-Mix 1 was set up in a nuclease-free microfuge tube placed on ice:

COMPONENT	VOLUME FOR CONTROL REACTION (1x)	VOLUME FOR REACTION (1x)	FINAL CONCENTRATION
Sterile Water (Vial 6)	10.5 µl	x µl up to 25 µl	
dNTP Mix (Vial 2)	4 µl	4 µl	0.2 mM
DTT Solution	2.5 µl	2.5 µl	5 mM
RNase Inhibitor (Vial 3)	1 µl	1 µl	5 U
Control Primer Mix (Vial 5	2 µl	-	0.4 µM (each)
Downstream Primer CR1	-	0.5 µl	0.4 µM
Upstream Primer AV190-1	-	0.5 µl	0.4 µM
Control RNA (Vial 4)	5 µl	-	10 pg
Template RNA	-	10µl	1 µg – 1 pg total RNA
TOTAL VOLUME	25 µl	25 µl	

(Primer detail refer to Appendix B)

4. The components for the Master Mix 2 were then set up in a separate, nuclease-free microfuge tube placed on ice:

COMPONENT	VOLUME FOR CONTROL REACTION (1X)	VOLUME FOR REACTION (1X)
Sterile Water (Vial 6)	14 µl	14 µl
5 x RT-PCR buffer (Supplemented)	10 µl	10 µl
Enzyme Mix (Vial 1, Titan [2] enzyme mix (AMV and Expand High Fidelity) in storage buffer)	1 µl	1 µl
TOTAL VOLUME	25 µl	25µl

5. 25 µl of each Master mix 1 and 2 was added to a 0.2 ml thin-walled PCR tube on ice. After it was mixed properly the tube was centrifuged briefly to collect the sample at the bottom of the tube.
6. The sample was placed in a thermocycler equilibrated at 50°C and incubated for 30 min.
7. The thermocycler was set as follows:

CYCLES	TEMPERATURE AND TIME
1 x	Denature template at 94 °C for 2 min
10 x	• Denaturation at 94 °C for 30 s • Annealing, at 55°C for 30 s • Elongation at 68°C for 2 min
25x*	• Denaturation at 94 °C for 30 s • Annealing, 55 °C for 30 s • Elongation at 68 °C for 2 min cycle elongation of 5 s for each cycle (e.g., cycle no. 11 has additional 5 s, cycle no. 12 has additional 10 s, cycle no. 13 has additional 15 s)
1x	A Prolonged elongation time of 7 min at 68 °C was used.

3.5.2.2.2 Nested PCR

8. The buffer, primers, PCR product, DNTPs and Taq were thawed on ice.
9. All the reagents were briefly vortexed and centrifuged.
10. The Master-Mix 1 was set up in a separate, nuclease-free microfuge tube placed on ice:

COMPONENTS	VOLUME FOR REACTION (X1)	FINAL CONCENTRATION
Buffer	5 µl	
Primers: • AV190-2 • CR2	 0.5 µl 0.5 µl	
PCR product	2 µl	
DNTPs	2 µl	
Taq	1 µl	
Sterile Water	40 µl	
TOTAL VOLUME	50 µl	

(For primer detail refer to Appendix B)

11. 50 µl of the Master mix was added to a 0.2 ml thin-walled PCR tube on ice. After it was mixed properly the tube was centrifuged briefly to collect the sample at the bottom of the tube.
12. The thermocycler was set as follows:

TEMPERATURE (°C)	TIME	CYCLES
95	5 min	1
95	30 sec	10
63	45 sec	
72	30 sec	
95	30 sec	30
58	45 sec	
72	30 sec	
72	10 min	1

13. The samples were then analysed on a 0.8% agarose gel.

3.5.2.3 Viroseq RT-PCR

3.5.2.3.1 Reverse Transcription

Important: This procedure was performed in pre-amplification Area 2

1. The RNA samples were thawed and vortexed for 3-5 seconds to mix.
2. One 0.2 ml MicroAmp reaction tube was labelled for each RNA sample.
3. The HIV RT Mix (Tris-KCl buffer, < 0.1% Magnesium Chloride, < 0.1% dATP, dCTP, dGTP, dTTP, < 0.1% non-infectious synthetic oligonucleotide HIV-1 primers, 0.05% Sodium Azide) and DTT (100mM, 1.4% Dithiothreitol) were thawed and vortexed for 3-5 seconds.
4. The RNAse Inhibitor (20 U/µl RNAse Inhibitor, 50% Glycerol) and Recombinant Murine Leukemia Virus
5. (MULV) Reverse Transcriptase (50 U/µl Recombinant MULV reverse transcriptase, 50% Glycerol) was removed from the kit.
6. All the reagent tubes and samples were briefly centrifuged to collect the contents at the bottom of the tubes.

3.5.2.3.2 Running the RT Reactions

1. The RT Master mix was prepared according to the following table. When finished the stock solutions were returned to –15 to -25ºC.

Reagent	Volume for 1 Reaction (µl)	Volume for 5 Reactions (µl)	Volume for 15 Reactions (µl)
HIV RT Mix	8	40	120
RNase Inhibitor	1	5	15
MuLV Reverse Transcriptase	1	5	15
DTT, 100nm	0.4	2.0	6.0
Total volume	10.4	52.0	156.0

Note: *Prepare sufficient volume for 1-2 extra reactions to compensate for pipetting loss.*

2. The RT Master mix was vortexed for 2-3 seconds to mix and centrifuged briefly to collect the contents at the bottom of the tube.

Important: Keep the RT Master mix at room temperature (15-25ºC) until you add it to the reaction tubes, but do not leave it at room temperature for more than 30 minutes.

3. 10 µl of the viral RNA was added to a 0.2 ml PCR tube. The PCR tubes were kept at room temperature and the stock viral RNA returned to –65 to –80ºC.
4. The PCR tubes were placed in a thermocycler (ABI2700) that had been set to the conditions below, and the program begun.

Temperature ºC	Time	Process
65	30 seconds	Relaxes the RNA secondary structure
42	5 minutes	Cools to the optimal enzyme activity temperature
		Press manually "pause" and perform step 6, then press "resume"
42	60 minutes	Reverse transcription
99	5 minutes	Inactivates MuLV Reverse Transcriptase
4	Hold (>10 minutes)	Holds until you are ready to proceed

Note: Set the volume on the thermocycler to 20 µL when prompted

5. After 1 minute at 42ºC "Pause" was pressed, the caps were removed one by one and 10 µl of the RT Master mix added.
6. The samples were vortexed for 3-5 seconds to mix and briefly centrifuged to collect the contents at the bottom of the tubes.
7. The samples were returned to the thermocycler (AB12700) and "Resume" pressed.

8. After the completion of the RT program, the samples in the thermocycler (AB12700) were held for at least 10 minutes at 4ºC.
9. When the program was complete the cycler was stopped and the samples stored at –15 to -25ºC until the PCR step could be performed.

3.5.2.3.3 PCR

3.5.2.3.3.1 Setting Up the PCR:

Important: This procedure was performed in pre-amplification area 2

1. The samples were thawed at room temperature (15-25°C) and briefly centrifuged to collect the contents at the bottom of the tubes.
2. The HIV PCR Mix (Tris-KCl buffer, < 0.1% Magnesium Cloride, <0.1% dATP, DCTP, dGTP, dTTP, dUTP, < 0.1% non-infectious synthetic oligonucleotide HIV-1 primers, 0.05% Sodium Azide) was thawed and vortexed for 3-5 seconds to mix.
3. The AmpliTaq Gold DNA Polymerase (5 U/µl AmpliTaq Gold, 50% Glycerol) and AmpErase UNG (1 U/µl uracyl N-glycosylase, 50% Glycerol) were removed from the kit and all the reagents briefly centrifuged to collect at the bottom of the tubes.

3.5.2.3.3.2 Performing the PCR Reaction

1. The PCR Master mix was prepared and combined in a sterile 1.5-ml microcentrifuge tube:

Reagent	Volume for 1 Reaction (µl)	Volume for 5 Reactions (µl)	Volume for 15 Reactions (µl)
HIV PCR Mix	29.5	147.5	442.5
AmpliTaq Gold polymerase	0.5	2.5	7.5
AmpErase UNG	1	5	15
Total Volume	31	155	465

2. The PCR Master mix was vortexed for 3-5 seconds to mix and briefly centrifuged to collect the contents at the bottom of the tube.
3. 30 µl of PCR Master mix was added to each RT reaction tube containing HIV-1 cDNA. The final volume was now 50 µl.
4. The thermocycler was programmed as follows:

Temperature (°C)	Time	Cycles
50	10 min	1
93	12 min	1
93	20 sec	40
64	45 sec	
66	3 min	
72	10 min	1
4	Hold	-

5. The samples were taken to the thermocycler in Area 3. Area 2 was avoided for the remainder of the day to prevent contamination with amplicon.
6. The tubes were transferred to the thermocycler, and the program started.
7. When the program was complete either the purification steps were done or the procedure was stopped and the samples stored at -15 to -25°C.

Important: Do not leave tubes on hold for more than 24 hours. The residual UNG activity may destroy the amplified DNA.

3.5.2.4 Cycle Sequencing

This procedure was performed in the post-amplification Area 3.

3.5.2.4.1 In-house Method

This was based on the ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit and the instructions in the product manual were used with a number of variations. The sequencing primers (XYZ) were added individually at a concentration of 3 pmol to 20 µl reactions to 5 µl of RT-PCR product (concentration unknown) and the following cycling program used for all reactions:

Twenty-five cycles at 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes; followed by soaking at 4 °C until ready to purify the extension products. The reactions were purified by the addition of 3 µl of 3 M sodium acetate (pH 4.6), 62.5 µl of 95% ethanol and 14.5 µl of deionised water. After mixing, the tubes were spun for 10 minutes at 14 000 rpm in a bench-top centrifuge, the supernatant was carefully aspirated and 150 µl of 70% ethanol added. After mixing, it was spun as above for 5 minutes and the supernatant aspirated. It was left open on the bench until no trace of ethanol could be seen in the tubes. The dried pellets were re-suspended in 20 µl of HiDi (ABI) formamide and loaded onto the sample tray of an ABI 310 automated sequencer. Dye set E was chosen for the 50 cm capillary and samples were run for 2.5 hours. Data was collected using Data Collection Software version 1.1 and analysed using Sequencing Analysis Software version 3.7.

3.5.2.4.2 Viroseq Method

3.5.2.4.2.1 Purifying the PCR products

1. The microconcentrator was assembled.
   a) For each sample,
      - one 1.5 ml collection tube
      - one Microcon Spin Column, was labelled
   b) Into each tube, the appropriately labelled Microcon Spin Column was inserted with the white membrane facing up.
2. 300 µl of sterile deionised water was carefully pipetted into the reservoir of each microconcentrator.

Important: Do not touch the membrane with the pipette tip.

3. The entire 50 µl PCR product of each sample was pipetted into the water-filled reservoir of the appropriately labelled microconcentrator.

Important: Do not touch the membrane with the pipette tip.

4. Each tube was firmly sealed with the attached tube cap.
5. The prepared microconcentrators were centrifuged at 450-550 xg for 15 minutes.
6. The tube caps were opened and 35 µl of sterile deionised water was carefully pipetted into the centre of each microconcentrator.

Important: Do not touch the membrane with the pipette tip.

7. The microconcentrators were inverted into newly labelled tubes.
   a. The microconcentrators were removed from the tube containing filtrate.
   b. The microconcentrator was placed upside down on top of a new, correspondingly labelled 1.5 ml tube for each sample.
   c. The old tube with the filtrate was then discarded.
8. The prepared microconcentrators were centrifuged at 450-550 xg for 5 minutes.
9. The microconcentrators were removed and discarded, retaining the samples in the tubes.
10. Each tube was firmly sealed with the attached tube cap, and either the agarose gel were run, or the procedure was stopped and the samples stored at –15 to -25°C.

3.5.2.4.2.2 Quantifying the DNA

1. A 1% agarose gel was prepared using TBE and it contained 0.5 μg/ml of ethidium bromide.
2. A 1X TBE gel buffer solution was prepared.
3. The prepared gel was placed in the gel box.
4. Enough 1X TBE gel buffer was added to cover the gel.
5. For each sample:
   - 5 μl of Agarose Gel Loading Buffer (< 0.1% Bromphenol blue, 40% Sucrose, < 3.7% EDTA, 0.5% Lauryl Sulfate Sodium) and
   - 5 μl of purified PCR product, was mixed
6. The DNA Mass Ladder solution (Tris-EDTA buffer, < 0.1% DNA mass ladder, 0.2% Lauryl Sulfate Sodium, 17% Sucrose, < 0.1% Bromphenol blue) was then loaded.

a. 6 µl in Lane 1

b. 3 µl in Lane 2

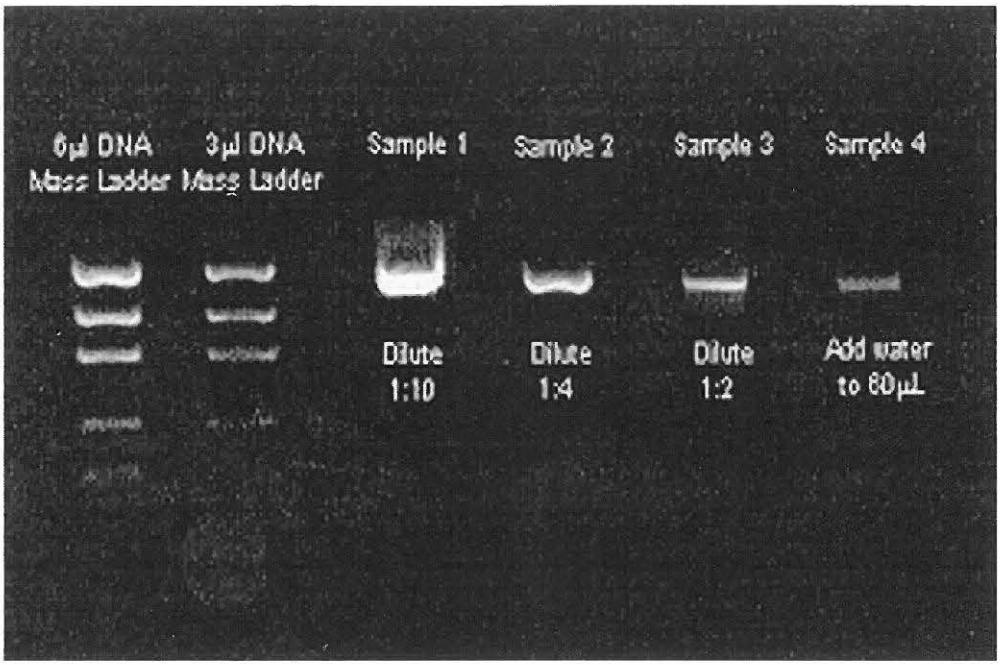
This gave the following results:

	Bands	6 µl Lane	3 µl Lane
Location	**Size (kb)**	**(ng)**	**(ng)**
Top	2.0	100	50
Second	1.2	60	30
Third	0.8	40	20

7. The remaining lanes were loaded with 10 µl of each sample.
8. Electrophoresis at 10 V/cm was performed until the bromophenol blue had migrated at least 5 cm into the gel.
9. The gel was examined with UV light.
10. The gel was photographed using an exposure time that did not saturate the film and showed the differences in intensity of the mass ladder fragments.
11. The quantities of PCR products were evaluated in each sample by comparing the intensity of each band to the intensities of the Low Mass Ladder bands (see illustration below)
12. The remaining PCR product was diluted for each sample with deionised, distilled water (ddH_2O) according to the following table.

If the band intensity is...	**Then.....**
20-40 ng	Adjust the sample volume to 60 µl.
40-60 ng	Make a 1:2 dilution with ddH_2O (1 part sample and 1 part water).
60-100 ng	Make a 1:4 dilution with ddH_2O (1 part sample and 3 parts water).
> 100 ng	Make a 1:10 dilution with ddH_2O (1 part sample and 9 parts water).

Figure 3.1 Low Mass Ladder Bands



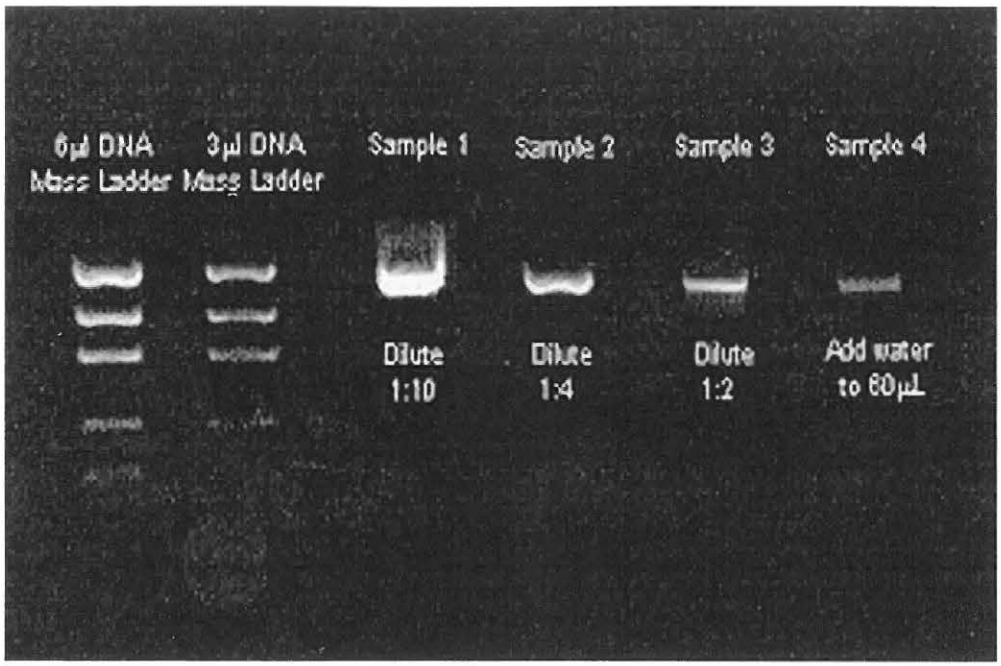
13. Each diluted sample was vortexed for 3-5 seconds to mix and briefly centrifuged to collect the contents at the bottom of the tube.
14. When the procedure was complete, we proceeded to Cycle Sequencing.

3.5.2.4.2.3 Cycle Sequencing

Important: The sequencing mixes are light sensitive. Do not expose the mixes to light for extended periods.

1. The HIV-1 sequencing mixes were thawed at room temperature.

Figure 3.1 Low Mass Ladder Bands



13. Each diluted sample was vortexed for 3-5 seconds to mix and briefly centrifuged to collect the contents at the bottom of the tube.
14. When the procedure was complete, we proceeded to Cycle Sequencing.

3.5.2.4.2.3 Cycle Sequencing

Important: The sequencing mixes are light sensitive. Do not expose the mixes to light for extended periods.

1. The HIV-1 sequencing mixes were thawed at room temperature.

2. Then the reaction format was set up. We used the following option:

- A MicroAmp optical 96-well reaction plate

A	B	C	D	E	F	G	H	I	J	K	L	M
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1-A	S2-A	S3-A	S4-A	S5-A	S6-A	S7-A	S8-A	S9-A	S10-A	S11-A	S12-A
B	S1-B	S2-B	S3-B	S4-B	S5-B	S6-B	S7-B	S8-B	S9-B	S10-B	S11-B	S12-B
C	S1-C	S2-C	S3-C	S4-C	S5-C	S6-C	S7-C	S8-C	S9-C	S10-C	S11-C	S12-C
D	S1-D	S2-D	S3-D	S4-D	S5-D	S6-D	S7-D	S8-D	S9-D	S10-D	S11-D	S12-D
E	S1-F	S2-F	S3-F	S4-F	S5-F	S6-F	S7-F	S8-F	S9-F	S10-F	S11-F	S12-F
F	S1-G	S2-G	S3-G	S4-G	S5-G	S6-G	S7-G	S8-G	S9-G	S10-G	S11-G	S12-G
G	S1-H	S2-H	S3-H	S4-H	S5-H	S6-H	S7-H	S8-H	S9-H	S10-H	S11-H	S12-H
H	S13-A	S13-B	S13-C	S13-D	S13-F	S13-G	S13-H					

A 3100 plate loaded with sample 1 (S1) beginning in column 1 of 96-well plate, S2 in column 2, and so forth. Row H is not used unless you want to run 13 samples on one plate.

3. In each well, the combinations were as follows:

Component	Volume for One Reaction (µl)
Add one of the following HIV-1 SEQ mixes to each plate well: **HIV SEQ MIX A** (BigDye™ Ready Reaction Mix, < 1% non-infectious synthetic oligonucleotide HIV-1 sequence primers) **HIV SEQ MIX B** (BigDye™ Ready Reaction Mix, < 1% non-infectious synthetic oligonucleotide HIV-1 sequence primers) **HIV SEQ MIX C** (BigDye™ Ready Reaction Mix, < 1% non-infectious synthetic oligonucleotide HIV-1 sequence primers) **HIV SEQ MIX D** (BigDye™ Ready Reaction Mix, < 1% non-infectious synthetic oligonucleotide HIV-1 sequence primers) **HIV SEQ MIX F** (BigDye™ Ready Reaction Mix, < 1% non-infectious synthetic oligonucleotide HIV-1 sequence primers) **HIV SEQ MIX G**(BigDye™ Ready Reaction Mix, < 1% non-infectious synthetic oligonucleotide HIV-1 sequence primers) **HIV SEQ MIX H** (BigDye™ Ready Reaction Mix, < 1% non-infectious synthetic oligonucleotide HIV-1 sequence primers)	12
Diluted, purified PCR product	8
Final Volume	20

4. The MicroAmp Full Plate Cover was placed over the plate and centrifuged at room temperature for 5-10 seconds.
5. The samples were then transferred to the thermocycler.
6. The thermocycler was programmed according to:

Temperature (°C)	Time	Cycles
96	10 sec	25
50	5 sec	
60	4 min	
4	Hold	-

7. The thermocycler was started.
8. When the program was completed either the purification steps were followed, or the process was stopped and the samples stored at –15 to -25°C.

3.5.2.4.2.4 Purifying the Sequences:

Note: There are three methods recommended for the purification of BigDye Terminator sequence reactions. In this case the Isopropanol precipitation method was used.

1. The MicroAmp tray was removed from the thermocycler, and the MicroAmp Full Plate Cover removed.
2. To each sample mixture was added:
   - 20 µl of deionised water
   - 60 µl of 100% isopropanol
3. The tubes were sealed with strip caps or adhesive foil tape.
4. The tray was inverted several times to mix.
5. Then the sample mixtures were incubated:
   - At room temperature
   - In the dark

- For 15 minutes

6. After incubation the tray was centrifuged at 1500-2000 x*g* for 45 minutes in a centrifuge equipped with an MTP rotor.
7. As soon as the centrifuge stopped, the tape was carefully removed from the tubes without disturbing the pellets.
8. Immediately an absorbent paper towel or Kimwipe was placed on top of the tray and the tray inverted.
9. The tray was placed in the centrifuge in the inverted position, on top of a Kimwipe or paper towel, and centrifuged at 700 x*g* for 1 minute.

Important: Perform the inverted spin immediately after centrifugation or there is a loss of signal.

10. When the centrifuge stopped, the plate was removed and either:
    - "Automated Sequence Detection" was done, or
    - the plate was sealed with aluminium tape and stored at –15 to –25°C in the dark.

3.5.2.5 Automated Sequence Detection

3.5.2.5.1 Denaturing the Samples using the ABI PRISM 3100 DNA Analyser

1. The foil from the dried sequencing reactions from procedure D4 was removed.
2. 20 µl of Hi-Di™ Formamide was added to the dried sequencing reactions and re-suspended.
3. The tray was covered with a full plate cover.
4. The samples were vortexed for 3-5 seconds to mix.

5. The tray was briefly centrifuged to collect the contents at the bottom of the tube.
6. The samples were heat denatured in a thermocycler at 95°C for 2 minutes.
7. The samples were immediately placed on ice at 2-8°C.
8. The full plate cover was removed from the tray.
9. A 3100 96-well septum was attached to the plate and the plate snapped into the 3100 analyser retainer and base.

3.5.2.5.2 The Collection Software using the ABI PRISM 3100 DNA Analyser

1. The Data Collection Software was launched.
2. From the View menu, Preferences were selected.
3. In the setting Preferences window, the Data Analysis tab was selected and the following selections made:
   - Select AutoAnalysis On
   - Clear Enable BioLIMS
   - Specify the format for the Sample File Name Prefix:
     - Sample Name
     - Well position
     - <none>
4. Click OK
5. The Plate View tab was selected, then New was clicked.
6. From the Tools menu, the Plate Editor was selected.
7. In the Plate Editor dialog box that opened:
   - Name your plate
   - Specify the application
   - Specify the plate type

Important: Use only letters and numbers or the symbols given below. Do not use any spaces.

-_(){}.+

8. OK was clicked to close the Plate Editor dialog box.
9. From the Application menu, Sequencing was selected.
10. From the Plate Type list box, a 96-well was selected.
11. Click Finish.
    The Plate Editor spreadsheet opened.

3.5.2.5.3 The Plate Editor using the ABI PRISM 3100 DNA Analyser

1. In the Sample Name column, the names of the samples were entered.

SampleName__Primer
↓
double underscore

- The sample name must be identical for all seven primer sequences of a sample.
- The sample name and primer identification must be separated by two consecutive underlines (Shift plus -).
- The primer identification contains the primer name and any other information unique to the specific sequence.
  For Example:
  Patient196__A
  Patient196__B
  Patient196__C etc.....

2. The following settings for each sample in the spreadsheet were selected:

Dye set	**E**
Mobility File	**Dt3100POP6{BD}v2.mob**
Comments	Enter your comments, if any
BioLIMS Project	**3100-Project1**
Run Module 1	**StdSeq50_POP6DefaultModule**
Analysis Mode 1	**BC-3100SR_SeqOffFtOff.saz**

3. The plate record was verified for correctness, then OK was clicked to return to the main menu.

3.5.2.5.4 Loading Samples and Starting the Run using the ABI PRISM DNA Analyser

1. The plate with the denatured sequencing reactions was placed onto the instrument.
   - Well A1 was positioned in the upper-right corner.
   - The plate position was verified and the indicator was yellow.
2. In the Pending Plate Records table, the Plate record was selected.
3. The plate position indicator for the plate intended to link to the record was selected.
   - The plate position indicator turned green.
   - The plate records moved from the Pending Plate Records table to the Linked Plate Records table.
4. The Run View tab was selected to view the run schedule.
   - Each run was selected to verify that the appropriate wells were highlighted.

Important: If the well is not highlighted, it will not be analysed.

5. The green Run Instrument button was clicked to begin the run.
6. After the run started, the fact that an electrical current was running through the system was verified by clicking the Status View tab. If there was no electrical current, there was a bubble in the polymer block and the options were:
   - Cancel the run.
   - Clear the bubble from the polymer block.
   - Restart the run.

3.5.2.5.5 The Sequence Analysis Software using the ABI Prism 3100 DNA Analyser.

1. When the run was completed, the Sequence Analysis software was launched.
2. All the files from the Run folder(s) were added to the Sample Manager.
3. The following settings for each sample were:

Basecaller	**3100SR**
Spacing	Defined by instrument
Basecaller Setting	**HIV580**
Peak 1 Location	Set by software
Start point	Set by software
Stop point	Set by software
DyeSet/Primer	**DT3100POP6{BD}v2.0**
Factura settings	-
Matrix File	-

4. The A (analyse) check box was selected for each sample.
5. The P (print) and F (Factura) columns were cleared.
6. Start was pressed.
7. After analysis, the A column was checked to see whether if a green box appeared for each sample.

Important: If a sample has a red box, the sample cannot be used in the ViroSeq software analysis. Please see the ABI Prism DNA Sequencing Analysis Software v. 3.7 User's Manual for help.

8. Proceed to "G. ViroSeq Software Analysis" followed:

3.5.2.5.6 ViroSeq Software Analysis

For detailed instructions please refer to the ViroSeq HIV-1 Genotyping System Software Version 2.5 User Guide included with the ViroSeq software. The following procedures are given:

- Creating a New Project.
- Sample Information.
- Editing Procedure
- Print Report.

3.6 PHYLOGENETIC ANALYSIS

Sequences were aligned using Bio-edit (Hall, 1999) and the same program was also used to translate the nucleic acid sequence into an amino acid sequence. The public domain phylogenetic analysis package MEGA3 (Kumar *et al.*, 2004) was used for constructing the phylogenetic trees. The reference sequence HXB2 was used as outgroup to anchor the sequences.

The UPMGA algorithm was chosen for tree construction and the final trees were the consensus of 500 bootstrap repeats.

CHAPTER 4

RESULTS and DISCUSSION

4.1 INTRODUCTION

The primary objective of this study was to study the naturally occurring variants of HIV-1 present in a group of patients from the central region of South Africa currently not on ARV treatment. The presence of pre-existing mutations may aid clinicians in designing optimal ARV combinations for a given region or country. A secondary objective was to find a suitable method for doing the analysis. To begin with, published primer sequences and in-house methods for the different steps of the procedure were used, but this was changed to an established commercial system (Viroseq by Celera Diagnostics, marketed by Abbott Diagnostics) because of superior sensitivity and the fact that it is FDA approved for diagnostic use. A major advantage of the Viroseq system is that it uses a single RT-PCR round to amplify a 1.8 kb fragment for sequencing, while the in-house method employs a nested PCR after the first RT-PCR. This can lead to preferential amplification of a subset of the viral population, creating a skewed representation of the situation in a particular patient. Even after the nested RT-PCR, bands were either faint or multiple bands were observed, which created mixed sequence when analysed (results not shown). This particular in-house system was developed in Belgium to cope with their predominantly subtype B isolates. An in-house system for local use should be adapted for subtype C sequences before it can be used here with confidence. Therefore only the sequences obtained with

the Viroseq system are presented here. The aligned raw data is shown in Appendix C.

The study population consisted of 19 adult ARV-naïve AIDS patients recruited from Tsepo House, an AIDS hospice in Bloemfontein. Initially, samples were taken from 32 patients, but due to haemolysis (2 samples), insufficient volume of plasma (4) and non-amplification or weak amplification of the required fragment (8), most of which did not produce good quality sequences, the results of 19 patients will be presented. The work reported here formed part of a study on the effect of nutritional supplements on the general health of terminal AIDS patients. The blood drawn for HIV-1/CD4 analysis was from the screening phase and so precludes any selection introduced by the treatment.

Table 4.1.1 shows a summary of the general patient information. It is clear from the CD4 counts that the immune systems of these patients were severely compromised, as the highest count was only 348 while the mean value was 184. This means that, according to the Department of Health's criteria, all of them qualify for ARV treatment. The viral loads are high, varying from 23 000 to >750 000. It demonstrates how people differ in their response to the viral infection. All patients were in the terminal stage of disease, yet displayed up to thirty-fold differences in viral load.

Table 4.1.1 Patient Information Summary

PATIENT NUMBER	SEX	BIRTH DATE	AGE	CD4	VIRAL LOAD	SUBTYPE
1	Female	1973/03/26	32	172	>750 000	C
2	Male	1968/02/23	37	284	404 400	C
3	Female	1976/11/25	29	91	>750 000	C
4	Female	1969/05/03	36	291	80 700	C
11	Male	1958/08/14	47	176	99 500	C
12	Male	1979/03/23	26	135	436 000	C
13	Male	1962/05/03	43	126	193 000	C
15	Male	1960/03/03	45	108	320 000	C
16	Male	1969/01/06	36	295	118 000	C
17	Female	1972/04/11	33	113	146 000	C
22	Female	1975/03/23	30	348	645 000	C
23	Female	1957/08/22	48	130	142 000	C
24	Female	1975/04/19	30	83	104 095	C
25	Female	1967/07/14	38	74	270 000	C
26	Male	1958/08/14	47	305	23 200	C
27	Female	1969/02/12	36	73	51 500	C
29	Female	1972/10/01	33	91	359 000	C
31	Female	1969/11/09	36	314	>750 000	C
32	Female	1973/09/24	32	232	189 000	C
Positive Control						B
Mean	**12 Female 7 Male**		**37**	**181**	**306 916**	

4.2 PHYLOGENETIC ANALYSIS

The sequences were trimmed by Viroseq to represent the 99 codons of the full protease and the first 335 codons of the reverse transcriptase reading frames. These were translated to amino acids and used separately in phylogenetic analyses to study their relatedness to each other (fig 4.1.1 & 4.1.2) and to other isolates (fig 4.1.3 & 4.1.4). As a first approach, only the group sequenced in this study were analysed. The protease phylogenetic tree shows tight clustering with sequence 4 forming a group on its own. This property is also visible in the reverse transcriptase tree. A practical benefit of phylogenetic analysis is to be an early-warning system for possible contamination of one sample by another

as this will show up as extremely tight clustering of some samples. The form and distances of the trees shown here clearly eliminate this possibility.

To see how the sequences reported in this study compare with other South African isolates, 20 ZA sequences were randomly chosen from the Los Alamos HIV-1 sequence database (http://www.hiv.lanl.gov). It is clear from both the reverse transcriptase and protease trees (Figure 4.1.3 & 4.1.4) that the Bloemfontein sequences do not differ appreciably from those found in the rest of the country, since they tend to diffuse through the tree rather than to cluster on their own. Some do form sub-clusters, but the differences are quite small. From an epidemiological perspective, it means that we largely have a single epidemic in the black community of South Africa. In white South Africans, the subtype is mostly B as in Europe and the United States (Dr P Schoeman, Pathcare, personal communication).

Although this was a small sample, the fact that no other subtypes were identified, shows that the spread of "strange" subtypes like A or D is not that prevalent in South Africa. These subtypes are more common to Central and West Africa and are sometimes difficult to analyse because of primer site mismatches. As a result of the increasing trade with and movement of people to and from the rest of the continent, spreading of these subtypes may be expected.

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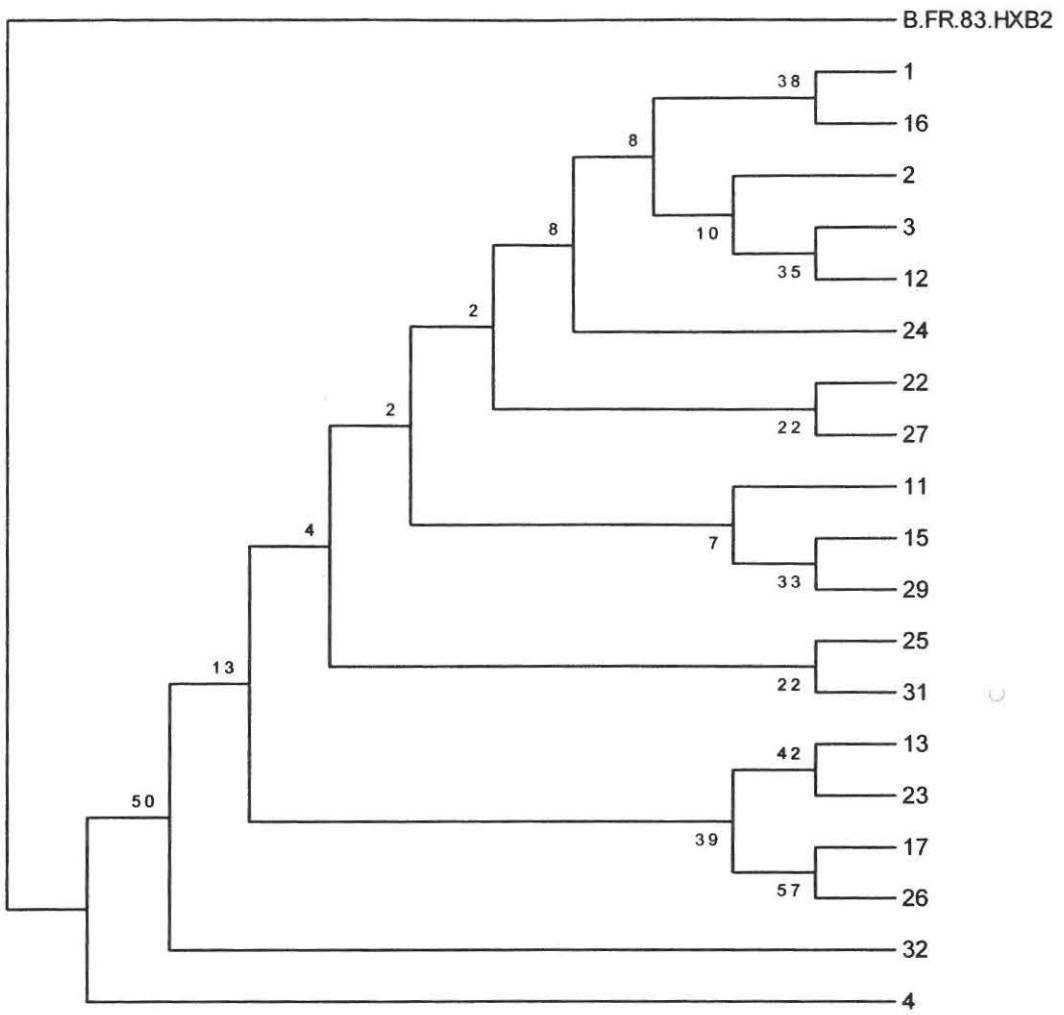

Figure 4.1.1
Phylogenetic tree of the protease amino acid sequences generated in this study.
HXB2 was used as outgroup.

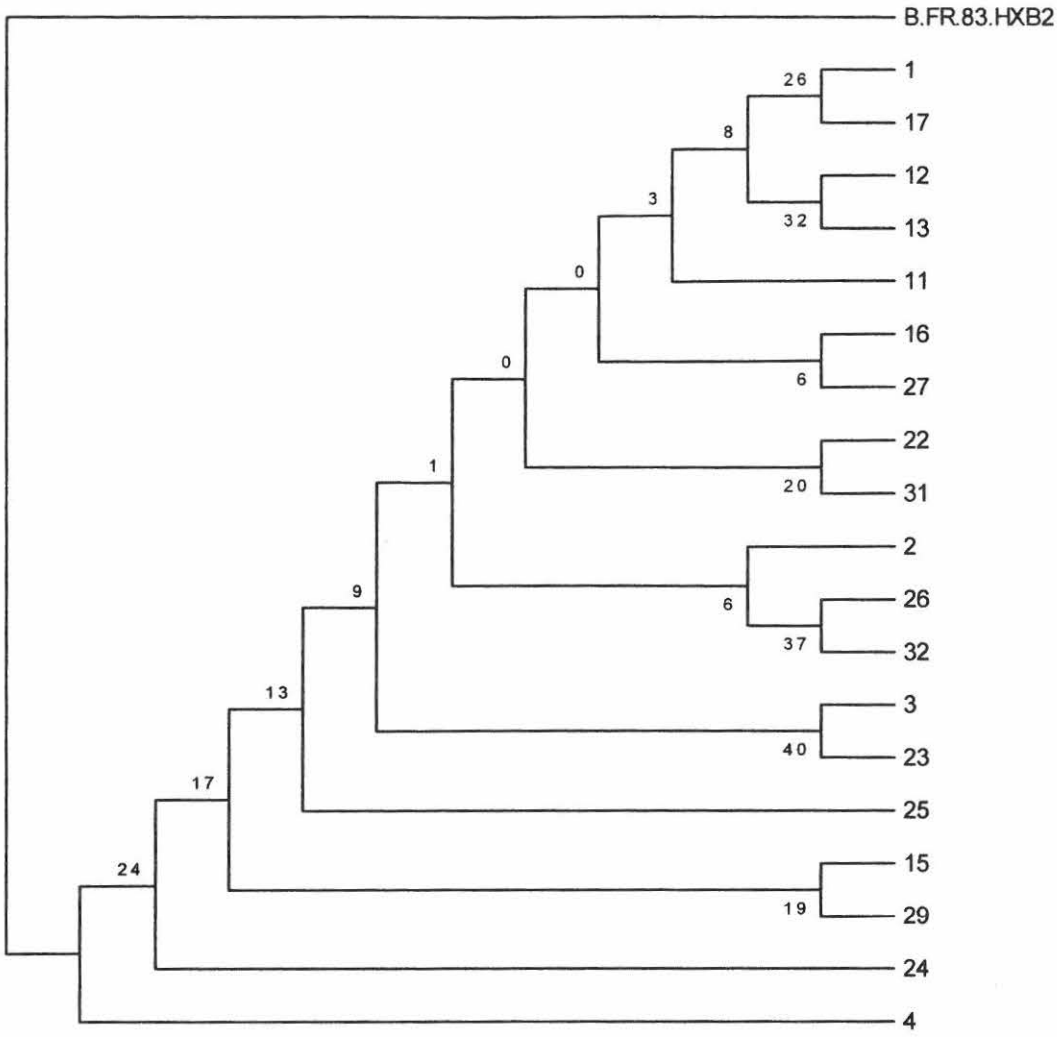

Figure 4.1.2
Phylogenetic tree of the reverse transcriptase amino acid sequences generated in this study.
HXB2 was used as outgroup.

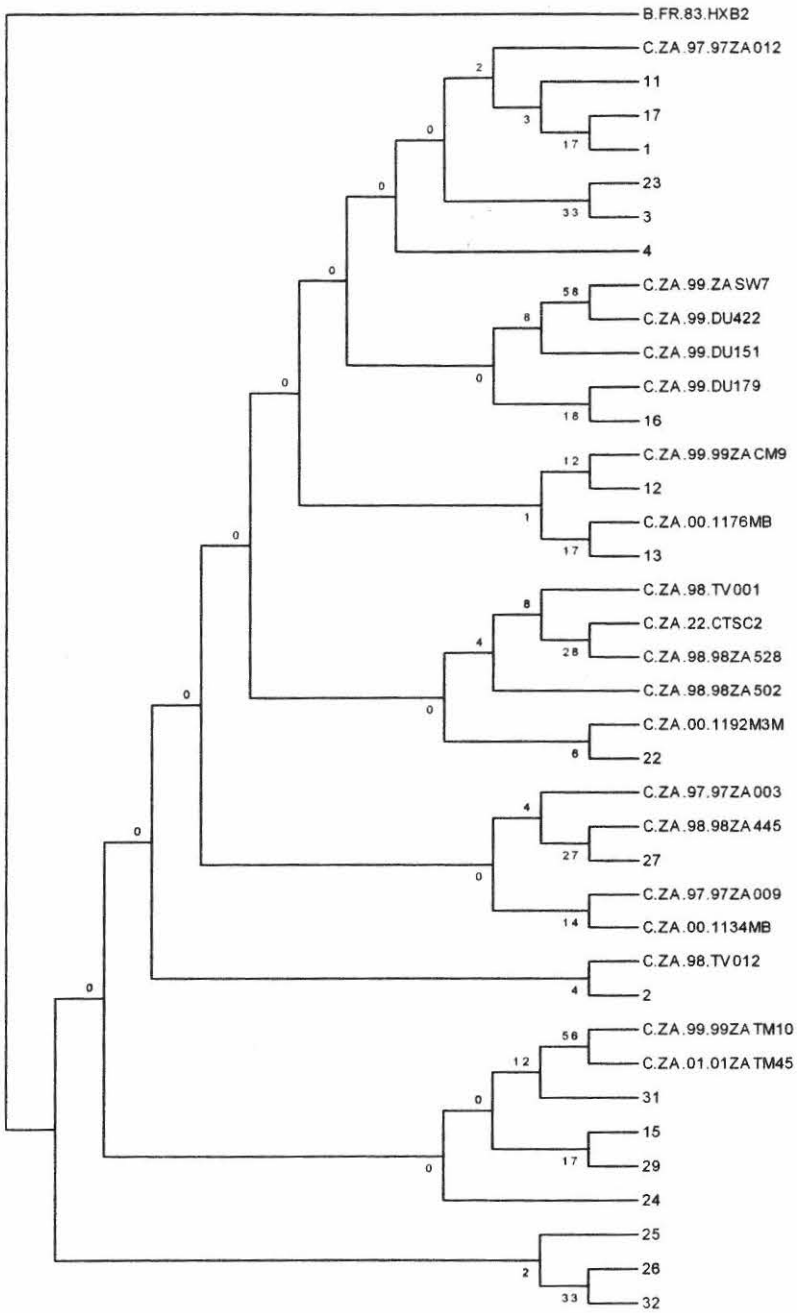

Figure 4.1.3
Phylogenetic tree of the reverse transcriptase amino acid sequences generated in this study compared to a random selection of 20 other South African isolates.
HXB2 was used as outgroup.

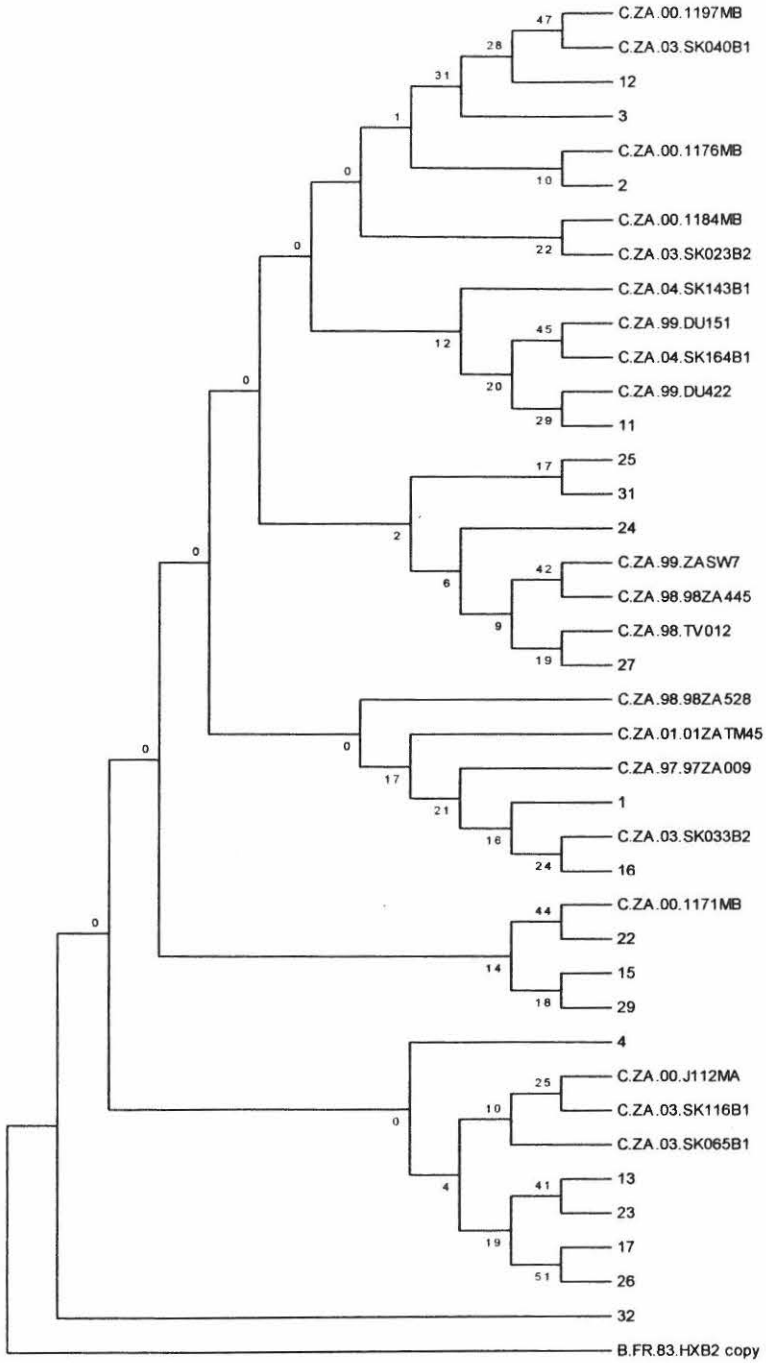

Figure 4.1.4
Phylogenetic tree of the protease amino acid sequences generated in this study compared to a random selection of 20 other South African isolates.
HXB2 was used as outgroup.

4.3 DRUG RESISTANCE MUTATIONS

The evolution of resistance against HIV-1 inhibitors within a patient depends on the generation of genetic variation and on the selection of drug-resistant variants during antiretroviral therapy. HIV-1 genetic variability is caused by the error-prone nature of HIV-1 RT and the absence of a proofreading 3' to 5' exonuclease activity (Roberts *et al.*, 1988). Substitutions are the most commonly observed genetic change, but insertions and deletions are also often observed for HIV-1. Another mechanism of HIV-1 genetic variability results from recombination during reverse transcription when viruses with different genotypes infect the same cell (Hu and Temin, 1990). The generation of genetic variation is augmented by the huge rate of HIV-1 replication *in vivo*. Studies of viral population dynamics in HIV-1 infected patients showed that during periods of clinical latency 10^8 to 10^{10} virions are produced every day (Coffin, 1995; Ho *et al.*, 1995; Wei *et al.*, 1995). Given the high rates of replication and mutation of HIV-1, and the high viral burden in HIV-1 infected patients, it was calculated that every possible single point mutation is probably generated multiple times each day (Coffin, 1995). As a result, innumerable quasispecies evolve in individuals in the months following primary infection. The majority of HIV-1 variants generated will be lethal to the virus, while many others will have little to no effect on viral function or fitness. However, some mutations result in alterations to the structure and function of important viral proteins for viral replication, such as RT and PR. Therefore, these variants often have a reduced fitness in comparison with the wild type virus and generally exist only as minor variants in the viral population of untreated patients. In the presence of antiretroviral inhibitors, minor variants with some level of resistance will gradually outcompete the wild type variants. Under the continuous selective presence of residual replication due to the insufficient potency of the

treatment, additional mutations will accumulate. These can either increase the level of resistance or they can compensate for the reduced fitness induced by the initial mutations. The number of naturally occurring variants with some level of resistance to a particular therapy decreases significantly with the number of inhibitors included in the combination, as this would require the concomitant presence of multiple mutations which are statistically very unlikely to exist in the initial population of quasispecies. Thus, resistance development will be slower when an increasing number of mutations is required for resistance to the inhibitors and when residual virus replication is lower due to more potent antiretroviral therapy (Vandamme *et al.*, 1998).

4.3.1 PROTEASE INHIBITORS AND MUTATIONS

The HIV-1 protease is the enzyme responsible for cleavage of the viral Gag and Gag-Pol polyprotein precursors during virion maturation, which yields the structural proteins and the enzymes of the viral particle (Miller, 2001; Park and Marrow, 1993). Six HIV-1 protease inhibitors have been approved to date in the United States, i.e., amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV). Many mutations associated with HIV-1 resistance to these protease inhibitors have now been identified (Miller, 2001). The protease gene has shown great plasticity, with polymorphisms detected in 49 of the 99 amino acids of the HIV-1 protease monomer and substitutions at more than 20 amino acids being associated with resistance (Miller, 2001; Figure 4.1.5A & B). Although they can be sporadically witnessed in viral quasispecies (Lech *et al.*, 1996), most of these primary mutations are rarely found in PI-naïve HIV-infected individuals, suggesting that they confer a selective

disadvantage to the virus (Kozal *et al.*, 1996). In fact, multiple mutations appear to be necessary for the development of a PI-resistant virus that is able to replicate, where most of these residues are highly conserved within the different subtypes of HIV-1 (Winslow *et al.*, 1995; Barrie *et al.*, 1996)

A

	10	20	24	30	32	33	36	46	47	48	50	54	63	71	73	77	82	84	88	90
	L	K	L	D	V	L	M	M	I	G	I	I	L	A	G	V	V	I	N	L
APV	FIRV	.	.	.	I	.	.	I	V	.	V	VM	.	.	.	.	.	V	.	M
IDV	IRV	MR	I	.	I	.	IV	IL	.	V	.	V	APQ	TV	STA	I	AFTS	V	S	M
LPV	FIRV	MR	I	.	.	.	.	I	.	.	V	.	.	TV	.	.	AFTS	V	.	M
NFV	FI	.	.	N	.	.	IV	IL	.	.	.	.	I	TV	.	I	AFTS	V	D	M
RTV	I	MR	.	.	I	FI	I	IL	.	.	.	VL	AP	TV	.	I	AFTS	V	.	M
SQV	IRV	.	.	.	I	.	.	.	.	V	.	V	.	TV	S	I	A	V	.	M

Figure 4.1.5

(A) Summary of protease mutations associated with resistance to Protease inhibitors (Krohn *et al.*, 1991).

Amino acids in red and black denote primary and secondary/compensatory mutations, respectively. Wild-type amino acids (HIV-1 subtype B) at the codons related to resistance to PI are indicated.

(Adapted from Quinones-Mateu *et al.*, 2001)

by other mutations (Martinez-Picado et al., 1999). Mutations have not only been observed within the protease but also at several protease cleavage sites (Doyon *et al.*, 1996). Growth kinetic studies have shown that these mutations improve the kinetics of the mutant enzyme and that these mutations appear to be compensatory rather than primary.

There were seven different protease mutations found in the sequences reported in this study (Table 4.1.2). The most frequently found mutations were M36I and I93L (both in 18 of the 19 sequences). The other significant mutation was L63P/V/A, which was located in 12 of the sequences analysed. K20R (8/19), D60E (3/19) and V77I (4/19) were the other mutations found. Kempf *et al.* (2001) examined the association of genotypic changes in HIV-1 protease with reduced *in vitro* susceptibility to the new protease inhibitor lopinavir (previously ABT-378). To define the genotypic correlates of reduced *in vitro* susceptibility to lopinavir, they examined the genotypes and phenotypes of 112 viral isolates from subjects experiencing virologic failure of therapy with one or more other Pis, who entered one of the lopinavir-RTV phaseI/II studies. In this analysis, mutations at 11 amino acid positions in HIV-protease (positions 54, 82, 10, 71, 46, 20, 90, 84, 24, 53, and 63, in order of greatest to least significance) were found to be statistically significantly associated with the loss of phenotypic susceptibility to lopinavir. Two statistical tests showed that specific mutations at 11 amino acid positions in protease (L10F/I/R/V, K20M/R, L24I, M46I/L, F53L, I54L/T/V, L63P, A71I/L/T/V, V82A/F/T, I84V, and L90M) were associated with reduced susceptibility. Mutations at positions 82, 54, 10, 63, 71, and 84 were most closely associated with

Table 4.1.2 Summary of the protease mutations found in the sequences reported in this study

Patient Nr	PROTEASE MUTATIONS						
	L10I,V	K20R	M36I	D60E	L63P,V,A	V77I	I93L
1			x				
2			x				x
3			x				x
4	x	x			x	x	x
11		x	x				x
12			x		x		x
13		x	x		x	x	x
15			x		x		x
16			x		x		x
17		x	x		x		x
22		x	x				x
23		x	x		x	x	x
24	x		x	x	x		x
25		x	x	x	x		x
26		x	x		x	x	x
27			x				x
29			x				x
31			x	x	x		x
32	x		x		x		x
TOTAL	3	8	18	3	12	4	18

relatively modest (4- and 10-fold) changes in phenotype, while the K20M/R and F53L mutations, in conjunction with multiple other mutations, were associated with >20- and >40-fold-reduced susceptibility, respectively. It is likely that mutations in addition to those at positions 20 and 53 will be found to contribute incrementally, in combination with multiple other mutations, to high-level *in vitro* resistance (e.g., the K20M/R and F53L mutations were only present in 8 of 16 and 5 of 16 viral isolates, respectively, in their panel with >20-fold-reduced susceptibility). As indicated in Table 4.1.2, K20R was very significant and was detected in eight of the 19 samples analysed. Each of the 16 viruses that displayed a >20-fold change in susceptibility contained mutations at residues 10, 54, 63 and 82 and/or 84, along with a median of three mutations at residues 20, 24, 46, 53, 71, and 90 (Kempf *et al.*, 2001). The number of protease

mutations from the 11 identified in these analyses (the lopinavir mutation score) may be useful for the interpretation of HIV genotypic resistance testing with respect to lopinavir-ritonavir (Kaletra) regimens, and may provide insight into the genetic barrier to resistance to lopinavir-ritonavir in both antiretroviral therapy-naïve and protease inhibitor-experienced patients.

Muzammil *et al.* (2003) characterized a mutant form of the HIV-1 protease, ANAM-11, identified in clinical isolates from HIV-1 infected patients treated with protease inhibitors. This mutant protease contains 11 mutations, 10 of which are located outside the active site (L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/L90M/I93L) and 1 within the active site (I84V). ANAM-11 lowers the binding affinity of indinavir, nelfinavir, saquinavir, and ritonavir by factors of 4000, 3300, 5800, and 80 000, respectively. Surprisingly, most of the loss in inhibitory affinity is due to the non-active site mutations, as demonstrated by additional experiments performed with a protease containing only the 10 non-active site mutations (NAM-10) and another containing only the active site mutation (A-1). Kinetic analysis with two different substrates yielded comparable catalytic efficiencies for A-1, ANAM-11, NAM-10, and the wild type protease. These studies demonstrate that non-active site mutations can be the primary source of resistance and that their role is not limited to compensate for deleterious effects of active site mutations. Analysis of the structural stability of the proteases by differential scanning calorimetry reveals that ANAM-11 and NAM-10 are structurally more stable than the wild type protease, while A-1 is less stable. Together, the binding and

structural thermodynamic results suggest that the non-active site mutants affect inhibitor binding by altering the geometry of the binding site cavity through the accumulation of mutations within the core of the protease molecule.

Rusconi *et al.* (2000) examined the anti-HIV-1 activity of a novel protease inhibitor, PHU-140690 (tipranavir), against patient-derived isolates resistant to multiple other protease inhibitors. The aim of their experiments was to investigate the genotypes and the *in vitro* phenotypes of resistance to tipranavir. Drug susceptibility tests with peripheral blood mononuclear cells and a fixed amount of infectious virus was done to determine the IC_{50} and IC_{90} levels using PCR assays for the detection of drug resistance mutations in RNA in plasma and direct sequencing of PCR products. Phenotypic resistance to PIs was invariably related to genotypic mutations, as previously pointed out by Condra *et al.* (1996). The substitutions among the amino acid residues of the protease included L10I, K20R, L24I, M36I, N37D, G48V, I54V, L63P, I64V, A71V, V77I, V82A, I84V, and L90M. Among the viral isolates described in their study, four expressed nine mutations, one expressed eight mutations, one expressed seven mutations, one expressed six mutations, and two expressed three mutations (one of the protease fragments was not amplified). Isolates from all of the patients had developed a maximal degree of resistance to indinavir, ritonavir and nelfinavir. They also compared these mutations with the amino acid changes previously described in association with *in vivo* tipranavir administration. The mutations included the following: I15V, 1 of 10 isolates; E35D, (6/10); N37D, (3/10); R41K, (2/10); D60E, (3/10) and A71T, (1/10) and did not correlate with the phenotypic susceptibility to PNU-140690. Among the patients who participated in the phase II protocol mentioned above, only

D60E, A71T, and V77G were not seen at the baseline but were seen at week 12. Tipranavir retained a sustained antiviral activity against PI-MDR clinical isolates and might be useful in combination regimens with other antiretroviral agents for patients who have already failed other PI-containing therapies.

Borman *et al.* (1996) were one of the first groups to report differences in replicative capacity in viruses resistant to the protease inhibitor ABT-77003 (which harboured the 32I mutation). Further accumulation of mutations at secondary sites (46I, 71V, and 82A) led to an improvement in fitness. A further study showed the role of the 63P/A mutation in improving the fitness of the 82A/F ritonavir resistance mutation, perhaps by compensating for the structural change around the active site of the enzyme (Eastman *et al.*, 1998).

Although numerous, the mutations found in the protease reading frame in this study are thus all secondary mutations without any evidence of causing resistance on their own, but they may be involved in increased susceptibility to developing resistance to protease inhibitors. The decision to include protease inhibitors in the treatment of South African patients should be viewed against this background and it is good to know that protease inhibitors have been reserved for second-line therapy in case of failure of the first-line regimen.

4.3.2 REVERSE TRANSCRIPTASE INHIBITORS (RTIs) AND MUTATIONS

The reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) is critical to the life cycle of HIV and is without a homologue in eukaryotic organisms. As such, it is an attractive target for selective antiviral therapy.

The number of reverse transcriptase mutations reported in this study (Table 4.1.3) is significantly less than the number of protease mutations found (Table 4.1.2). In the 19 samples analysed only 5 mutations were found. K103N and K103R were reported once each in the 19 sequences, while K101E, V179D and Y318F were found in only one of the sequences analysed. Of these mutations, K103N is the most significant. K103R does not contribute to resistance (Los Alamos website http://www.hiv.lanl.gov/content/index).

Table 4.1.3 Summary of the reverse transcriptase mutations found in the sequences reported in this study

REVERSE TRANSCRIPTASE MUTATIONS					
Patient Nr	**K101E**	**K103N**	**K103R**	**V179D**	**Y318F**
1				x	
2					
3					
4	x				
11					
12					
13					x
15					
16					
17					
22					
23					
24		x			
25					
26					
27					
29			x		
31					
32					
Total	1	1	1	1	1

Resistance to nevirapine is associated with mutations A98G, L100I, K101E/Q, K103N, V106A/I, V108I, V179D, Y181C/I, Y188C/L/H and G190S/A. Single mutations at positions L100I, K103N, V106A/I, V108I, Y181C/I, Y188C/L/H and G190S/A are sufficient to confer high level resistance towards nevirapine. The P236L mutation appears to be

relatively specific for delavirdine. However, single mutations at L100I, K103N, V106A/I, V108I, Y181C/I and Y188L also confer high-level resistance to delavirdine. A98G, K101E/Q, V179D and Y188C/H have also been observed in association with delavirdine resistance. Compared to nevirapine and delavirdine, efavirenz shows greater resilience to resistance mutations within HIV-1 RT. This is probably due to the repositioning of efavirenz in the NNRTI binding pocket of the mutant RT and to conformational rearrangements in the enzyme. High-level resistance is associated with single mutations at K103N, Y188L and G190S. Multiple mutations at A98G, L100I, K101E/Q, V106A/I, V108I, V179D, Y181C/I, Y188C/H and G190A are necessary to confer high-level resistance to efavirenz (Schinazi *et al.*, 2000).

NNRTIs inhibit HIV-1 RT by interacting with a common hydrophobic binding site in the p66 subunit. This binding site is in close proximity to but distinct from the polymerase active site of the enzyme. It has been suggested that most of the NNRTIs can adopt a conformation in which the compound assumes a "butterfly" shape, consisting of two wings. One wing is generally proximal to and the other distinct from the polymerase active site. The wings of the molecules usually contain significant π-electron systems that can interfere with amino acid side chains of the NNRTI-binding pocket. Many NNRTIs also form hydrogen bonds with the peptide main chain, "turning" the NNRTI-binding pocket. The internal surface of the NNRTI-binding pocket is mainly composed of hydrophobic amino acid residues with few hydrophilic residues in the vicinity of the inhibitor. Upon binding of NNRTIs to the hydrophobic NNRTI-binding pocket in the p66 subunit, the functional groups of the highly conserved amino acid residues Y181 and Y188 are substantially reoriented and closely mimic the conformations of the equivalent side chains observed in the inactive p51 subunit of the enzyme. Aromatic stacking interactions

between aromatic rings of the NNRTI and protein residues of the hydrophobic pocket (Y181, Y188, W229 and Y318); electrostatic forces (K101, K103 and E138); van der Waals interactions (L100, V106, V179, Y181, G190, W229, L234 and Y318) and hydrogen bonding between the NNRTI and the main chain peptide bonds (101-102 or 103-104) (Fig 4.1.6A & B), all contribute to the interaction and binding efficacies of the drugs in the NNRTI-specific pocket (Balzarini *et al.*, 1999). The K103N mutation is the most commonly observed mutation *in vivo* and gives cross-resistance to many NNRTIs.

A

	41	44	62	65	67	69	70	74	75	77	100	103	106	108	115	116	118	151	181	184	188	190	210	215	219	225	227	230	236
	M	E	A	K	D	T	K	L	V	F	L	K	V	V	Y	F	V	G	Y	M	Y	G	L	T	K	P	F	M	P
AZT	L	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	W	.	Q	.	.	.	.
ddI	.	.	.	R	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	VI	.	.	.	.	.	.	.	.	.
ddC	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
d4T	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3TC	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ABC	L	.	.	.	N	.	R	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	W	YF	Q	.	.	.	.
TNV	.	.	.	R	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DLV	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	L	.	L
EFV	.	.	.	.	.	.	.	.	.	.	I	.	A	I	.	.	.	.	Cl	.	.	.	.	.	.	H	L	.	.
NVP	.	.	.	.	.	.	.	.	.	.	I	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.
MDR-151complex	.	.	V	.	.	.	.	.	I	L	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.
MDR-69complex	L	.	V	.	N		R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	W	YF	Q	.	.	.	.

Figure 4.1.6

(A) Summary of reverse transcriptase mutations associated with resistance to RT inhibitors.

Amino acids in grey and black denote primary and secondary/compensatory mutations, respectively. Wild-type amino acids (HIV-1 subtype B) at the codons related to resistance to RTI are indicated. (Adapted from Quinones-Mateu *et al.*, 2001)

B

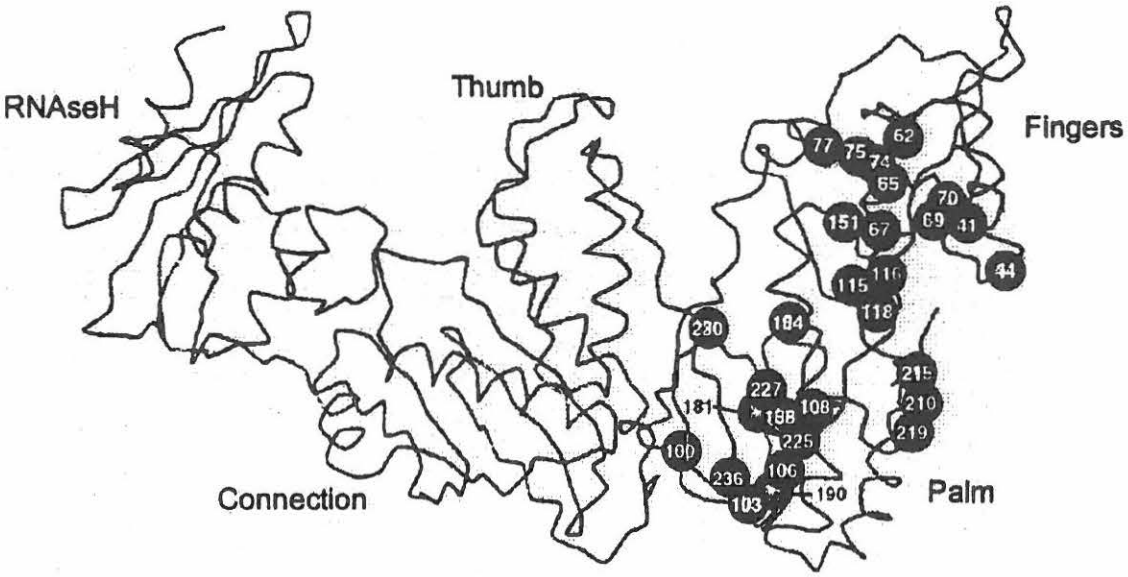

Figure 4.1.6

(B) Structure of the HIV-1 RT 166, indicating amino acids residues associated with resistance to RTI.

Each numbered circle indicates codon position and the nature of the resistance mutation (primary and secondary mutations in black and grey respectively)

(Adapted from Quinones-Mateu *et al.*, 2001)

It is suggested that the observed cross-resistance with K103N is due to the stabilizing effect of the asparagine side chain at 103 on the unliganded RT structure. This could provide resistance to a wide range of NNRTIs by giving a reduced rate of association between inhibitors and enzyme (Ren *et al.*, 2000).

Bacheler *et al.* (2000) conducted a study dealing with HIV-1 mutations selected in patients failing efavirenz combination therapy. Efavirenz is a selective and very potent nonnucleoside inhibitor of HIV-1 reverse transcriptase (RT). In cell culture, efavirenz retains significant activity against a variety of mutant strains of HIV-1 with single amino acid substitutions in the RT gene which, have been associated with resistance to other NNRTIs (Byrnes *et al.*, 1993; Young *et al.*, 1995). Cell culture selection experiments (Winslow *et al.*, 1996) demonstrated that passage of the RF strain of HIV-1 in MT-2 cells or peripheral blood mononuclear cell culture led to a selection of mutations at amino acids positions 100, 108, 179, and 181 of the HIV-1 RT gene. Nucleotide sequence analyses of the protease and RT genes (coding region for amino acids 1 to 229) of multiple cloned HIV-1 genomes, from virus found in the plasma of patients in phase II clinical studies of efavirenz combination therapy, were undertaken in order to identify the spectrum of mutations in plasma-borne HIV-1 associated with virological treatment failure. K103N was the most frequently observed NNRTI resistance mutation in samples from efavirenz-exposed patients who experienced a significant rebound in viral load, detected in more than 90% of cases of efavirenz-indinavir or efavirenz-zidovudine (ZDV)-lamivudine (3TC) treatment failure. This amino acid substitution can be achieved by a single base pair change or point mutation (AAG or AAA to AAC or AAT). V108I and P225H mutations were observed frequently, predominantly in viral genomes that also contained other nonnucleoside RT inhibitor (NNRTI) resistance mutations. L100I, K101E, K101Q, Y188H, Y188L, G190S, G190A and G190E mutations were also observed. K101E mutations were observed in 13.8% of efavirenz treatment failure patients overall, either as a single NNRTI resistance mutation (4.8% of patients) or linked to a variety of other NNRTI resistance mutations (not just K103N). V106A, Y181C, and

Y188C mutations, which have been associated with high levels of resistance to other NNRTIs, were rare in the patient samples in this study, both before and after exposure to efavirenz. The spectrum of mutations observed in cases of virological treatment failure was similar for patients initially dosed with efavirenz at 200, 400, or 600 mg once a day and for patients treated with efavirenz in combination with indanivir, stavudine, or ZDV-3TC. The proportion of patients carrying NNRTI resistance mutations, usually K103N, increased dramatically at the time of initial viral load rebound in case of treatment failure after exposure to efavirenz. Viruses with multiple, linked NNRTI mutations, especially K103N-V108I and K103N-P225H double mutants, accumulated more slowly following the emergence of K103N mutant viruses.

The spectrum of resistance mutations observed *in vivo* in patients for whom efavirenz combination therapy failed, as well as their relative frequency, was substantially different from that observed in cell culture selection experiments. The L100I- K103N double mutant selected in cell culture (Young *et al.*, 1995) was relatively infrequently observed *in vivo*, occurring in 10.6% of virological failures. In the selection experiments described by Winslow *et al.* (1996) viruses with mutations at L100, V108, V179, and Y181 were described. *In vivo*, the L100I mutation was seen only in combination with K103N, and V108I mutations were observed largely in combination with K103N. V179D and Y181C mutations were extremely rare in plasma virus from patients exposed to efavirenz. The results of characterization of the genotypic correlates of resistance to efavirenz *in vivo* emphasize that cell culture selection experiments are often not predictive of the types or frequencies of resistance mutations selected in patients receiving antiretroviral therapy.

A study conducted by Demeter *et al.* (1998) on the safety, tolerability, and the antiviral activity of atevirdine (ATV), a nonnucleoside reverse transcriptase inhibitor, took the form of a phase I/II clinical trial (ACTG 187) of patients with CD4 counts ≤ to 500/mm^3. In all, 34 HIV-1-infected patients were randomised to receive ATV for a period of twelve weeks to achieve one of three serum trough levels: 5 to 13 μM, 14 to 22 μM, or 23 to 31 μM. Most of the patients complained about a rash. There was no significant change from the baseline in HIV-1 plasma RNA mean copy number detected at week four. In addition, 2 to 4 patients with detectable baseline serum p24 antigen showed declines of >50%. HIV-1 resistance to ATV was detected in 41% of patients and it was most commonly associated with RT mutations K103N and Y181C. By contrast, the Y181C mutation was not detected in ATV-resistant isolates obtained from patients enrolled in ACTG 199, a study of ATV given in combination with zidovudine. Under the conditions of this study, ATV failed to demonstrate significant antiviral activity. However, transient *in vivo* activity might have been obscured by rapid development of resistance coupled with inadequate sampling at early time points following initiation of ATV therapy.

4.4 CONCLUSION

As mentioned earlier, although the sample size was very small, all the selected patients tested positive for subtype C, which complies with the demographical data set for subtype C. This is also indicative that the spread of "strange" subtypes like A or D is not that prevalent in South Africa. Different subtypes of HIV-1 possess distinct patterns of consensus amino acid sequences in viral proteins, including the protease, a highly polymorphic and flexible enzyme (Grossman *et al.*, 2001). Nearly 47% of

the 99 protease amino acids can vary naturally in wild type viruses (both within and between subtypes), (Kozal *et al.*, 1996). Resistance to one or more of the six presently used protease inhibitors has been associated with mutations at 45 amino acid positions (Condra *et al.*, 1995; Mammano *et al.*, 1998). Mutations at nine amino acid positions have been commonly designated primary or major resistance mutations (D30N, V32I, M46I/L, G48V, I50V, I54L/M/V, V82A/F/S/T, I84A/V, N88S, and L90M), (Wu *et al.*, 2003). Other resistance mutations considered of a lesser significance are defined as secondary or minor mutations. Although none of the primary mutations occur as polymorphysims in wild type HIV-1, several secondary mutations contributing to reduced susceptibility (e.g., M36I and I93L) are found in nearly 100% of subtype C viruses from drug-naïve patients (Cane *et al.*, 2001; Grossman *et al.*, 2001). This statement correlates very well with results achieved as 95% prevalence for both M36I and I93L was encountered. Upon antiretroviral treatment, such differences in baseline polymorphisms among subtypes may result in the evolution of drug resistance along distinct mutational pathways, or in differences in the incidence of these specific pathways (Grossman *et al.*, 2001; Brenner *et al.*, 2003). These genetic differences may be clinically relevant when considering long-term treatment strategies for patients infected with different subtypes.

However, the current ARV protocol that is employed by the State is not flawless. By looking at the First-line therapy (Schedule 1) regimens (http://www.epi.uct.ac.za/artrollout/) quite a few mutations can be associated with every drug. Unless contraindicated, all patients will commence therapy on:

(A) Stavudine (d4T) 40 mg every 12 hours (or 30 mg every 12 hours if< 60kg), plus

(B) Lamivudine (3TC) 150 mg every 12 hours, plus

(C) Efavirenz (EV) 600 mg at night (or 400 mg if < 40 kg) or Nevirapine (NVP) 200 mg daily for 2 weeks, followed by 200 mg every 12 hours. To prevent pregnancy, injectable contraception should be prescribed in addition to condoms for women of child-bearing potential who are started on efavirenz. If unable to guarantee contraception for women while on therapy, nevirapine will be substituted for efavirenz (Appendix D).

However, a great number of mutations have already been reported on the drugs mentioned above. Although the V75T mutation has been observed after *in vitro* resistance selection experiments with stavudine and it is associated with reduced susceptibility to stavudine (Lacey *et al.*, 1994), this mutation is not detected in patients failing stavudine therapy. Zidovudine resistance mutations, such as M41L, K70R and T215Y, are seen in zidovudine-naïve patients who do not have virologic suppression on stavudine-containing therapy (Coakley *et al.*, 2000; de Mendoza *et al.*, 2000). The mutation M184I/V occurs rapidly *in vitro* and *in vivo* under the selective pressure of lamivudine, and confers high-level resistance (Schinazi *et al.*, 2000). Resistance to nevirapine is associated with mutations A98G, L100I, K101E/Q, K103N, V106A/I, V108I, V179D, Y181C/I, Y188C/L/H and G190S/A. Single mutations at positions L100I, K103N, V106A/I, V108I, Y181C/I, Y188C/L/H, and G190S/A are sufficient to confer high-level resistance to nevirapine. Compared to nevirapine and delavirdine, efavirenz shows greater resilience against resistance mutations within HIV-1 RT. This is probably due to the respositioning of efavirenz in the NNRTI binding pocket of the mutant RT and to conformational rearrangements in the enzyme. High level resistance is associated with single mutations at K103N, Y188, and G190S. Multiple mutations at A98G, L100I, K101E/Q, V106A/I, V108I, V179D, Y181C/I, Y188C/H and G190A, are necessary to confer high level resistance to efavirenz (Schinazi *et al.*, 2000).

Patients may occasionally need to change a drug from the first-line regimen to one from the second-line regimen, because of a serious adverse reaction (e.g. severe rash on nevirapine, requiring a change to lopinavir/ritonavir, symptomatic peripheral neuropathy on stavudine, requiring the swap to zidovudine). However, if changing occurs the patient is very limited in his/her second-line treatment options. It is very important that the decision to change is made by a doctor with a vast amount of experience in antiretroviral treatment. If the patient continues to fail virologically despite efforts to improve adherence, his/her antiretroviral treatment may be changed to the second-line therapy (Schedule 2; http://www.epi.uct.ac.za/artrollout/). Most of the patients will commence schedule 2 as follows:

(A) Didanosine (ddI) 400 mg once a day (250 mg daily if < 60 kg), plus
(B) Zidovudine (AZT) 300 mg every 12 hours, plus
(C) Lopinavir/ritonavir (LPV/r) 400/100 mg every 12 hours (Appendix E).

There are also known mutations for second-line therapy (Schedule 2). Resistance to zidovudine is associated with six substitutions at codons M41L, D67N, K70R, L210W, T215Y/F and K219Q. These mutations appear to emerge in a characteristic sequence, with the first mutations usually appearing at codon 70. HIV-1 resistance to zidovudine results from the increased pyrophosphorolytic or nucleotide-dependent cleavage of chain-terminated viral DNA by the mutant RT combined with the increased processivity of the enzyme (Arion *et al.*, 1998; Meyer *et al.*, 1999). The most common mutation observed during didanosine monotherapy is L74V (St. Clair *et al.*, 1991). In addition to L74V, didanosine resistance occurs due to mutations at codons 65 and 184 (Winters *et al.*, 1997). The K65R has been isolated from several patients receiving long-term treatment with didanosine monotherapy. M184V also

decreases susceptibility to didanosine. However, it is only rarely observed in HIV-1 infected patients receiving didanosine.

A different protocol consisting of a first-line therapy (Schedule 1) and a second-line (Schedule 2) therapy for children < than 6 months and also > than 6 months, exists (http://www.epi.uct.ac.za/artrollout/).

The above-mentioned complications indicate that it is no easy task to treat a patient with antiretroviral drugs, bearing in mind that there are currently no further treatment options available within the public sector for patients who fail second-line therapy.

CHAPTER 5

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APPENDIX A

HIV/AIDS GENOTYPING

Date: _____

CONSENT TO PARTICIPATE IN RESEARCH

You have been asked to participate in a research study.

You have been informed about the study by .....

You may contact L Botes at 083 70 90 312 any time if you have questions about the research or if you are injured as a result of the research.

You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, UFS at telephone number (051) 4052812 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to terminate participation.

If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in this study means. I also understand that my patient information will be regarded as confidential, that my participation is voluntary and that I could withdraw at any time.

_____ _____

Signature of Participant **Date**

_____ _____

Signature of Translator **Date**

HIV/MIV GENOTIPERINGSPROJEK

Datum:_____

TOESTEMMING TOT DEELNAME AAN NAVORSING

U is versoek om aan 'n navorsingsstudie deel te neem.

U is oor die studie ingelig deur.....

U kan Me L Botes enige tyd kontak by 083 70 90 312 indien u vrae oor die navorsing het of as gevolg van die navorsing beseer is.

U kan die Sekretariaat van die Etiekkomitee van die Fakulteit Gesondheidswetenskappe, UV by telefoonnommer (051) 405 2812 kontak indien u enige vrae het oor u regte as 'n proefpersoon.

U deelname aan hierdie navorsing is vrywillig, en u sal nie gepenaliseer word of voordele verbeur as u weier om deel te neem of besluit om deelname te staak nie.

As u instem om deel te neem, sal 'n ondertekende kopie van hierdie dokument sawel as die inligtingsblad, wat 'n geskrewe opsomming van die navorsing is, aan u gegee word.

Die navorsingstudie, insluitende die bogenoemde inligting is verbaal aan my beskryf. Rk begryp wat my betrokkenheid by die studie beteken. Ek verstaan ook dat my pasiënt inligting konfidensieel hanteer sal word en dat my deelname vrywillig is en ek teen enige tyd kan onttrek.

_____ _____

Handtekening van deelnemer **Datum**

_____ _____

Hantekening van Vertaler **Datum**

MORALO WA PHEPO WA HIV/AIDS

Letsatsi: _____

TUMELLO YA HO NKA KAROLO DIPATLISISONG

O kopilwe ho nka karolo thutong ya dipatlisiso.

O ile wa tsebiswa ka thuto ena ke ..... .

O ka nna wa ikopanya le L Botes nomorong ya 083 70 90 312 nako e nngwe le e nngwe ebang o nale dipotso mabapi le dipatlisiso kapa ebang o ka tswa kotsi ka baka la dipatlisiso.

O ka nna wa ikopanya le Mongodi wa Ethics Committee ya Faculty of Health Sciences, UFS nomorong ya mohala ya (051) 4052812 ebang o nale dipotso ka ditokelo tsa hao jwaloka eo ho etswang dipatlisiso ka yena.

Ho nka karolo ha hao dipatlisisong tsena ke boithaopong ba hao, mme o keke wa fumantshwa kotlo kapa wa lahlehelwa ke menyetla ya hao ebang o ka hana kapa wa nka qeto ya ho kgaotsa ka ho nka karolo.

Ha o dumela ho nka karolo, o tla nehwa khopi e saennweng ya tokomane ena hammoho le leqhephe la ba nkang karolo e leng le ngotsweng kgutsufatso ya dipatlisiso.

Thuto ya dipatlisiso ho kenyellwa lesedi le ngotsweng ka hodimo, di ile tsa hlaloswa ho nna ka molomo.

Ke utlwisisa hore ho nka karolo ha ka thutong ena ho bolelang. Ke boetse ke utlwisisa hore tlhahiso leseding e mabapi le dintlha tse amang botho ba ka, e tla nkwa e le sephiri le hore ho nka karolo ha ka ke boithaopo le hore nka nna ka ikgula nako e nngwe le e nngwe.

_____ _____

Tshaeno ya motho ya nkang karolo **Letsatsi**

_____ _____

Tshaeno ya mofetoledi **Letsatsi**

INFORMATION DOCUMENT

STUDY TITLE:

HIV Genotyping

Dear participant, we are inviting you to be part of this clinical trail and would be very thankful if you could see your way open to be part of this study. Below you will find all the information about this study and your responsibilities as participant.

INTRODUCTION:

The development of viral resistance to antiretroviral drugs used for treatment of human immunodeficiency virus (HIV) infections is an important cause of treatment failure and limits options for alternative antiretroviral regimens. Prevention, characterization, and clinical management of such resistance are receiving increasing attention. Having access to both treated and untreated patients puts us in an ideal situation to study the phenomenon of drug resistance. Therefore, the main objective of this study was to optimize a method for the detection of all variants of the HIV-1 viruses. By investigating the untreated patients for naturally occurring variants of the virus, important information can be obtained regarding the possible existence of resistance against HIV antiretroviral drugs.

STUDY DESIGN:

One visit will be made to Tsepo House, all the information regarded the study will be explained to all the possible candidates. Consent will be obtained from the candidates to draw 5ml of blood from them to be submitted to the study. The candidates will receive an information document and must sign a form of consent.

During the initial phase of the project written approval for the protocol will be obtained from the Ethics Committee of the Faculty of Health Sciences, University of the Free State, Bloemfontein, before the first administration of study.

STUDY POPULATION:

Thirty-two volunteers living with HIV will be selected after the screening visit, who fulfilled the inclusion criteria, did not meet any of the exclusion criteria, and who gave written informed consent, will be entered into the study.

Inclusion Criteria:

- Male and female subjects from 18 to 65 years of age that are HIV/AIDS positive.
- No previous antiretroviral treatment
- A viral load of more than 2000 copies/ml
- $CD4^{+}$T-cell counts 200 – 350 cells/mm^3
- Only volunteers that give informed consent will be submitted to the study.

Withdrawal criteria:

Subjects have the right to withdraw from the study at any time, irrespective of the reason, without detriment of their medical care. These patients will be handled as drop-outs. Participation is voluntary, and refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled to.

PATIENT IDENTIFICATION:

Each enrolled subject will receive a number (01 – 32) and will retain this number throughout the study.

PARTICIPATION IS VOLUNTARY:

Participation is voluntary and refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled; the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

CONFIDENTIALITY:

Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.

SAFETY OF THE PATIENTS

The project is very safe. The patients will be well monitored during the collection of the blood specimen.

FINANCIAL IMPLICATION FOR THE PATIENTS:

There is no financial implication for the patients.

CONTACT DETAILS OF RESEARCHER(S):

Me L Botes	Cell nr	083 70 90 312 (researcher)
Dr E van den Heever-Kriek	Cell nr	082 77 05 356 (researcher)
Prof GHJ Pretorius	Cell nr	083 63 82 093 (researcher)

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INLIGTINGSDOKUMENT

TITEL VAN DIE STUDIE:

HIV Genotipering

Liewe deelnemer ons nooi u uit om deel te neem aan die kliniese navorsingstudie. Ons sal baie dankbaar wees as u u weg oopsien om deel te neem aan die studie. Die onderstaande inligting handel oor die studie en 'n uiteensetting van u verantwoordelikheid en deelname met betrekking tot die studie.

INLEIDING:

Die ontwikkeling van virale weerstandigheid teen antiretrovirale middels wat gebruik word in die behandeling van MIV infeksies is vandag 'n baie belangrike oorsaak vir die mislukking van MIV behandeling en skep beperkings rakende alternatiewe antiretrovirale behandeling. Die voorkoming, karaktirisering en kliniese bestuur van sulke weerstandigheid moet voortslepend aandag geniet. Ons toegang tot beide behandelde en onbehandelde pasiënte plaas ons in die ideale posisie om die verskynsel van middelweerstandigheid te bestudeer. Daarom, die objektief van hierdie studie was om 'n metode te optimiseer vir die opsporing van alle variante van die MIV-1 virusse. Deur te kyk na die onbehandelde pasiënte kan al die natuurlike variante van die virus opgespoor word wat waardevolle inligting kan veskaf rakende die moontlike weerstandigheid teen MIV antiretrovirale middels.

STUDIE ONTWERP:

'n Eenmalige besoek sal gebring word aan Tsepo House waar daar aan die moontlike kandidate 'n verduideling van die studie voorgelê sal word. Toestemming sal van die kandidate verkry word om 5ml bloed van hulle te trek en aan die studie te onderwerp. Die kandidate sal 'n inligtingstuk van die studie sowel as 'n toestemmings brief onderteken.

Tydens die aanvangsfase sal om skriftelike toestemming aansoek gedoen word by die Etiese Komitee van die Fakulteit Gesondheidswetenskappe, UVS, om die studie te onderneem.

STUDIE POPULASIE:

Twee en dertig vrywilligers wat MIV positief is sal gedurende die besoek geselekteer word, wat aan die insluitingskriteria voldoen en wat die skriftelike toestemmingsvorm voltooi het.

Insluitingskriteria:

- MIV positief/AIDS mans en vrouens tussen 18-65 jaar
- Geen vorige antiretrovirale behandeling
- "n Viruslading van meer as 2000 kopieë/ml
- CD_4^+ T-sel telling van 100-350 selle/mm^3
- Slegs diegene wat toestemming verleen sal in die studie ingesluit word.

Ontrekkingskriteria:

- Die pasiënt het die reg om enige tyd van die studie te onttrek, sonder dat dit tot nadeel van die pasiënt sal wees. U deelname aan hierdie navorsing is vrywillig, en u sal nie gepenaliseer word of voordele verbeur as u weier om deel te neem of besluit om deelname te staak nie. Die stakers sal hanteer word as uitvallers en nie in die studie vervang word nie.

PASIËNT IDENTIFIKASIE:

- Elke pasiënt sal 'n nommer ontvang (01-32) en sal die nommer behou deur die studie.

U DEELNAME IS VRYWILLIG:

U deelname is vrywillig, en u kan weier om deel te wees van die studie. Die pasiënt het die reg om enige tyd van die studie te onttrek, sonder dat dit tot nadeel van die pasiënt sal wees. U deelname aan hiedie navorsing is vrywillig, en u sal nie gepenaliseer word of voordele verbeur as u weier om deel te neem of besluit om deelname te staak nie.

VERTROULIKHEID:

Daar sal gepoog word om persoonlike inligting vertroulik te hou. Volkome vertroulikheid kan nie gewaarborg word nie. Persoonlike inligting kan bekend gemaak word as die wet dit vereis.

VEILIGHEID VAN PASIËNT:

Die studie is baie veilig. Die pasiënte sal deeglik gemonitor word tydens die neem van die bloedmonster.

FINANSIËLE IMPLIKASIES VIR DIE PASIËNT:

Daar is geen finansiële implikasies vir die pasiënte nie.

KONTAKBESONDERHEDE VAN DIE NAVORSERS:

Me L Botes	Cell nr	083 70 90 312 (navorser)
Dr E van den Heever-Kriek	Cell nr	082 77 05 356 (navorser)
Prof GHJ Pretorius	Cell nr	083 63 82 093 (navorser)

KONTAKBESONDERHEDE VAN NEK SEKRETARIAAT EN VOORSITTER:

Vir rappotering van klagtes/probleme

Telefoonnommer: 051 405 2812

APPENDIX B

OUTER RT-PCR PRIMERS:

- AV190-1 gctacaytagaagaaatgatgacagcat
- CR1 ttccctacaatccccaaagtcaaggagtagaattc

INNER PCR PRIMERS:

- AV190-2 tagaagaaatgatgacagcatgycagggagt
- CR2 caggaatttggnattccctacaatccccaaag

SEQUENCING PRIMERS:

- OUT5 gtacagtattagtaggacctacacctgtcaacat
- OUT3 catgagaaatcacagtaattggagagcaatg
- AV2 tgtaaactccttaggggaaccaaagcact
- AV5 aaagacagctggactgtcaat
- AV8 aataccctcccttagtgaaattatg
- AV9 cccatacaaaaggaaacat

APPENDIX C

A) ALIGNED PROTEASE SEQUENCES FROM THIS STUDY:

```
1       1  PQITLWQRPL VSIKVGGQIK EALLDTGADD TVLEEINLPG KWKPKMIGGI GGFIKVRQYD  60
2       1  .......... .......... .......... .......... ....R..... .......... 60
3       1  .......... ........V. .......... ....D..... .......... .......... 60
4       1  ..L......I ........TR .......... .....MS... R......... .......... 60
11      1  .......... .T......TR .......... ....D..... .......... .......... 60
12      1  .......... ........V. .......... .......... .......... .......... 60
13      1  .......... .T.R....TR .......... .......... .......... .......... 60
15      1  .......... .T........ .......... ....D..... .......... .......... 60
16      1  .......... .......... .......... .......... R......... .......... 60
17      1  .......... ...R.....R .......... ......A... .......... .......... 60
22      1  .......... .....E...R .......... ....D..... .......... .......... 60
23      1  .......... ...R....TR .......... .......... .......... .......... 60
24      1  .........I .......... .......... .......... .......... .........E 60
25      1  .......... ........TR .......... ....D.D... .......... .........E 60
26      1  .......... ...R.....R .......... ......S... .......... .......... 60
27      1  .......... .....E.... .......... ....D..... R......... .........E 60
29      1  .......... .T........ .......... ....D..... .......... .......... 60
31      1  .......... .T.I....V. .......... ......D... .......... .........E 60
32      1  .........V .T..I...T. .......... .......... .......... .......... 60
HXB2    1  ..V....... .T..I...L. .......... .....MS... R......... .......... 60

1       61 QILIEICGKK AIGTVLVGPT PVNIIGRNLL TQIGCTLNF 99
2       61 .......... .......... ........M. ..L...... 99
3       61 .......... .......... ........M. ..L...... 99
4       61 ..P...Y... ......I... ........M. ..L...... 99
11      61 .......... .......... ........M. ..L...... 99
12      61 ..P....... .......... ........M. ..L...... 99
13      61 ..T....... ......I... .......... ..L...... 99
15      61 ..VM...... ...S...... ........M. ..V...... 99
16      61 ..A....... .......... .......... ..L...... 99
17      61 ..T......Q .......... ........M. ..L...... 99
22      61 .......... ...S...... ........M. ..L...... 99
23      61 ..P....... ......I... ........M. ..L...... 99
24      61 ..V....... .......... ........M. ..L...... 99
25      61 ..S....... .......... ........M. ..L...... 99
26      61 ..P......Q ......I... ........M. ..L...... 99
27      61 .V........ .......... ........M. ..L...... 99
29      61 .......... ...S...... ........M. ..L...... 99
31      61 ..P....... .......... ........M. ..L...... 99
32      61 ..A....... ...A...... ........M. ..L...... 99
HXB2    61 ........H. .......... .......... ......... 99
```

B) ALIGNED REVERSE TRANSCRIPTASE SEQUENCES FROM THIS STUDY:

```
1       1   PISPIETVPV KLKPGMDGPR VKQWPLTEEK IKALTEICEE MEKEGKITKI GPENPYNTPV  60
2       1   .......... .........K .......... .....A.... .......... ..........  60
3       1   .......... .........K .......... .......... .......... .........I  60
4       1   .......... .........K .......... ....M..... .......... ..........  60
11      1   .......I.. .........K .......... .......... .......... ..........  60
12      1   .......... .......... .......... .....A.... .......... ..........  60
13      1   ...S...... .........K .......... .....A.... .......... ..........  60
15      1   .......... .........K .......... .....A.... .......... ..........  60
16      1   .......... .........K .......... .....A.... .......... .........I  60
17      1   .......... .........K .......... .......... .......... ..........  60
22      1   .......I.. .........K .......... .....A...D .......... ..........  60
23      1   .......... .........K .......... .........D. .......S.. ..........  60
24      1   .......... .........K .......... ..........D .......... ..........  60
25      1   .......... .........K .......... .....A.... .......... ..........  60
26      1   .......... .........K .......... .....I.... .......... ..........  60
27      1   .......... .........K .......... .....A.... .......... .........I  60
29      1   .......... .........K .......... .....A..K. ..E....... .........I  60
31      1   .......... .........K .......... .....A.... .......... ..........  60
32      1   .......... .........K .......... .......... .......... ..........  60
HXB2    1   .......... .........K .......... ....V...T. .......S.. ..........  60

1       61  FAIKKKDSTK WRKLVDFREL NKRTQDFWEV QLGIPHPAGL KKKKSVTVLD VGDAYFSVPL  120
2       61  .......... .......... .......... .......... .......... ..........  120
3       61  .......... .......... .......... .......... .......... ..........  120
4       61  ...R...... .......... .......... .......... E......... ..........  120
11      61  .......... .......... .......... .......... .......... ..........  120
12      61  .......... .......... .......... .......... .......... ..........  120
13      61  .......... .......... .......... .......... .......... ..........  120
15      61  .......... .......... .......... .......... .Q........ ..........  120
16      61  .......... .......... .......... .......... .......... ..........  120
17      61  .......... .......... .......... .......... .......... ..........  120
22      61  .......... .......... .......... .......... .......... ..........  120
23      61  .......... .......... .......... .......... .......... ..........  120
24      61  .......... .......... .......... .......... .QN....... ..........  120
25      61  .......... .......... .......... .......... .......... ..........  120
26      61  .......... .......... .......... .......... .......... ..........  120
27      61  .......... .......... .......... .......... .......... ..........  120
29      61  .......... .......... .......... .......... ..R....... ..........  120
31      61  .......... .......... .......... .......... .......... ..........  120
32      61  .......... .......... .......... .......... ...R...... ..........  120
HXB2    61  .......... .......... .......... .......... .......... ..........  120

1       121 DENFRKYTAF TIPSINNETP GIRYQYNVLP QGWKGSPAIF QSSMTKILEP FRAKNPEIDI  180
2       121 ..D....... .......... .......... .......... .C........ ..T.....V.  180
3       121 .......... ....R..... .......... .......... ....I..... ...Q...LV.  180
4       121 ..S....... ....R..... .......... .......... .C........ ......D.V.  180
11      121 ..G....... .......... .......... .......... .....R.... ...Q....V.  180
12      121 ..G....... .......... .......... .......... .N........ ..TQ....V.  180
13      121 ..G....... .......... .......... .......... .C........ ........V.  180
15      121 .......... ....T..G.. .......... .......... .......... ..T...D.V.  180
16      121 ..G....... ....T..... .......... .......... .......... ...Q....V.  180
17      121 ..G....... .......... .......... .......... .......... ...R....V.  180
22      121 H.S....... .......... .......... .......... .......... ........V.  180
23      121 ..D....... ....R..... .......... .......... ....I..... ...Q....V.  180
24      121 ..G....... ........S. .V........ .......... .Y........ ......D.I.  180
25      121 .KD....... .......... .......... .......... .......... ..T....VV.  180
26      121 .KD....... .......... .......... .......S.. .......... ...Q....V.  180
27      121 ..S....... .......... .......... .......... .....R.... ..KQ..N.V.  180
29      121 ..S....... ....T..... .......... .......... ....I..... ........V.  180
31      121 ..S....... .......... .......... .......... .......... ........V.  180
```

```
32     121 .KD..... ..... ..... ..... .N..... ...R....V.  180
HXB2   121 ..D..... ..... ..... ..... ..... ..KQ..D.V.  180

1      181 YQYMDDLYVG SDLEIGQHRA KIEELREHLL KWGFTTPDKK HQKEPPFLWM GYELHPDKWT  240
2      181 ..... ..... ..... ..... ..... ..... ..... .....  240
3      181 ..... ..... .V....A... ..... ..... ..... .....  240
4      181 ..... ..... ..D...A... R..... ..... ..... .....  240
11     181 ..... .....K.... ..... ..... ..... ..... .....  240
12     181 ..... ..... ......D... ..... ..... ..... .....  240
13     181 ..... ..... ..... ..... ..... ..... ..... .....  240
15     181 ..... ..... ......G... ..... ..... ..... .....  240
16     181 ..... ..... ..... ..... ..... ..... ..... .....  240
17     181 ..... ..... ..... ..... ..... ..... ..... .....  240
22     181 ..... ..... ......D... Q..... ..... ..... .....  240
23     181 ..... ..... ..... ...L.. ..... ..... ..... .....  240
24     181 ..... .....T ..... ..... ..... ..... ..... .....  240
25     181 ..... ..... ...Q..D... ..... ..... ..... .....  240
26     181 ..... ..... ......K... R..L.. ..... ..... .....  240
27     181 ..... ..... ......K... ...L.. ..... ..... .....  240
29     181 ..... ..... ......D... ...L.. ..... ..... .....  240
31     181 ..... .....M.... ..... ..... ..... ..... .....  240
32     181 ..... ..... ..... ..... ..... ..... ..... .....  240
HXB2   181 ..... .....T ......Q... R..L.. ..... ..... .....  240

1      241 VQPIQLPDKD SWTVNDIQKL VGKLNWASQI YPGIKVRQLC KLLRGTKALT DIVPLTEEAE  300
2      241 ....K..E.. N..... ..... ....Q.KH.. ..... ..I.....  300
3      241 .......E.. ..... ..... .......N.. .....A.... .....  300
4      241 .......E.. ..... ..... .A..... .....A.... .....  300
11     241 .......E.. ..... ..... ..... ..... ..... .....  300
12     241 .......E.. ..... ..... ....Q..K.. .....A.... .....  300
13     241 .......E.. ..... ..... ..... .....A.... .....  300
15     241 .......E.E A..... ..... ......KH.. .....A.... E.....  300
16     241 ....K..E.. ..... ..... ......K... ..... .....  300
17     241 .........E D..... ..... ..... .....A.... .....  300
22     241 .......E.. ..... ..... ......K... .....A.... .....  300
23     241 ....K..E.. ..... ..... ..... .....A.... .....  300
24     241 I......E.. H..... ..... .S....K... R..... .....  300
25     241 ..T.K....E ..... ..... .S..... .....A.... .....  300
26     241 .......E.. ..... ..... ....R.K... ..... .....  300
27     241 .......E.. ..... ..... ..... .....A.... ...Q.....  300
29     241 .......E.E ..... ..... ....R.K... .....A.... .V.Q.....  300
31     241 ....T..E.E ..... ..... ....R.K... .....A.... ..I...D...  300
32     241 .......E.. ..... ..... ......K... ..... .....  300
HXB2   241 ....V..E.. ..... ..... ..... ..... EVI.....  300

1      301 LELAENREIL KEPVHGVYYD PSKELIAEIQ KQGND 335
2      301 ..... ..... ...DFM.... ...H. 335
3      301 ..... ..... ...D..... ...D. 335
4      301 ..... ..... ...D....L. ...QG 335
11     301 ..... ..... ...D..... ..ECN 335
12     301 ..... ..... ...D..... ..... 335
13     301 ..... .......F.. ...D..... ..... 335
15     301 ..... ......A... ...D....L. ..... 335
16     301 ..... ..... ..... ...E. 335
17     301 ..... ..... ...D..... ...D. 335
22     301 ..... ..... ...D..... ...E. 335
23     301 ..... ..... ...D..... ...H. 335
24     301 ..... ..... ...D..... ..... 335
25     301 ..... ..... ...D..... ...QG 335
26     301 ..... ......A... ...D..... ...H. 335
```

```
27      301 .......... .......... ...D...... ...Q. 335
29      301 .......... ......A... ...D...... ..... 335
31      301 .......... .......... ...D...... ...Q. 335
32      301 .......... .......... ...D...... ...H. 335
HXB2    301 .......... .......... ...D...... ...QG 335
```

C) POSSIBLE DRUG RESISTANCE AS SCORED BY THREE DIFFERENT ALGORITHMS:

Key: S Susceptible
I Intermediate or low level resistance
R High level resistance

PATIENT NUMBER:		1
SEX:		F
AGE:		
VIRAL LOAD:		>750 000
DRUG MUTATIONS:	**PR**	M36I
	RT	V179D

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
RegaInst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		S	S
HIVDB	S	S	S
SCORED MUTATION	V179D	V179D	V179D
RegaInst	I	I	I

EI	T20
ANRS	S
HIVDB	
RegaInst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	S	S	S	S	S	S	S
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	M36I		M36I	M36I	M36I	M36I	M36I	M36I	
RegaInst	S		S	S	S	S	S	S	

PATIENT NUMBER: 2
SEX: M
AGE:
VIRAL LOAD: 404 400
DRUG MUTATIONS: PR M36I ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
RegaInst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		S	S
HIVDB	S	S	S
RegaInst	S	S	S

EI	T20
ANRS	S
HIVDB	
RegaInst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	S	S	S	S	S	S	S
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	M36I I93L		M36I I93L	M36I I93L	M36I I93L	M36I I93L	M36I I93L	M36I I93L	
RegaInst	S		S	S	S	S	S	S	

PATIENT NUMBER: 3
SEX: F
AGE:
VIRAL LOAD: >750 000
DRUG MUTATIONS: PR M36I ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
RegaInst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		S	S
HIVDB	S	S	S
RegaInst	S	S	S

EI	T20
ANRS	S
HIVDB	
RegaInst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	S	S	S	S	S	S	S
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	M36I I93L		M36I I93L	M36I I93L	M36I I93L	M36I I93L	M36I I93L	M36I I93L	
RegaInst	S		S	S	S	S	S	S	

PATIENT NUMBER: 4
SEX: F
AGE:
VIRAL LOAD: 80 700
DRUG MUTATIONS: **PR** L10I ; K20R ; L63P ; V77I ; I93L
RT K101E

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
Regalnst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		R	R
SCORED MUTATION		K101E	K101E
HIVDB	S	S	S
SCORED MUTATION	K101E	K101E	K101E
Regalnst	I	I	I
SCORED MUTATION	K101E	K101E	K101E

EI	T20
ANRS	S
HIVDB	
Regalnst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	I	S	S	S	S	S	S
SCORED MUTATIONS			L10I, K20R, L63P						
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	K20R, L10I, L63P, I93L		K20R, L10I, L63P, I93L	K20R, L10I, V77I, L63P, I93L	K20R, L10I, L63P, I93L	K20R, L10I, L63P, I93L	K20R, L10I, L63P, I93L	K20R, L10I, L63P, I93L	
Regalnst	S		I	S	S	S	I	S	
SCORED MUTATIONS			L10I, K20R, L63P, V77I, L89M				L10I, K20R, L63P		

PATIENT NUMBER: 11
SEX: M
AGE:
VIRAL LOAD: 99 500
DRUG MUTATIONS: PR K20R ; M36I ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
RegaInst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		S	S
HIVDB	S	S	S
RegaInst	S	S	S

EI	T20
ANRS	S
HIVDB	
RegaInst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	S	S	S	S	S	S	S
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	K20R, I93L, M36I		K20R, I93L, M36I	K20R, I93L, M36I,	K20R, I93L, M36I	K20R, I93L, M36I	K20R, I93L, M36I	K20R, I93L, M36I	
RegaInst	S		S	S	S	S	S	S	

PATIENT NUMBER: 12
SEX: M
AGE:
VIRAL LOAD: 436 000
DRUG MUTATIONS: PR M36I ; L63P ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS								
HIVDB	S	S	S	S		S	S	S
RegaInst								

NNRTI	DLV	EFV	NVP
ANRS			
HIVDB	S	S	S
RegaInst			

EI	T20
ANRS	
HIVDB	
RegaInst	

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS									
HIVDB	S		S	S	S	S	S	S	
Scored Mutation	L63P M36I		L63P M36I	L63P M36I	L63P M36I	L63P M36I	L63P M36I	L63P M36I	
RegaInst									

PATIENT NUMBER: 13
SEX: M
AGE:
VIRAL LOAD: 193 000
DRUG MUTATIONS: **PR** K20R ; M36I ; L63T ; V77I ; I93L
RT Y318F

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS								
HIVDB	s	s	s	s		s	S	s
RegaInst								

NNRTI	DLV	EFV	NVP
ANRS			
HIVDB	r	s	S
Scored Mutation	Y318F	Y318F	Y318F
RegaInst			

EI	T20
ANRS	
HIVDB	
RegaInst	

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS									
HIVDB	s		s	s	s	s	s	S	
Scored Mutation	K20R M36I		K20R M36I	K20R M36I	K20R M36I	K20R V77I M36I	L63P M36I	K20R M36I	
RegaInst									

PATIENT NUMBER: 15
SEX: M
AGE:
VIRAL LOAD: 320 000
DRUG MUTATIONS: **PR** M36I ; L63V ; I93V
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS								
HIVDB	S	S	S	S		S	S	S
RegaInst								

NNRTI	DLV	EFV	NVP
ANRS			
HIVDB	S	S	S
RegaInst			

EI	T20
ANRS	
HIVDB	
RegaInst	

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS									
HIVDB	S		S	S	S	S	S	S	
Scored Mutation	M36I		M36I	M36I	M36I	M36I	M36I	M36I	
RegaInst									

PATIENT NUMBER: 16
SEX: M
AGE:
VIRAL LOAD: 118 000
DRUG MUTATIONS: **PR** M36I ; L63A ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS								
HIVDB	S	S	S	S		S	S	S
Regalnst								

NNRTI	DLV	EFV	NVP
ANRS			
HIVDB	S	S	S
Regalnst			

EI	T20
ANRS	
HIVDB	
Regalnst	

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS									
HIVDB	S		S	S	S	S	S	S	
Scored Mutation	M36I		M36I	M36I	M36I	M36I	M36I	M36I	
Regalnst									

PATIENT NUMBER: 17
SEX: F
AGE:
VIRAL LOAD: 146 000
DRUG MUTATIONS: **PR** K20R ; M36I ; L63T ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS								
HIVDB	S	S	S	S		S	S	S
RegaInst								

NNRTI	DLV	EFV	NVP
ANRS			
HIVDB	S	S	S
RegaInst			

EI	T20
ANRS	
HIVDB	
RegaInst	

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS									
HIVDB	S		S	S	S	S	S	S	
Scored Mutation	K20R M36I		K20R M36I	K20R M36I	K20R M36I	K20R M36I	K20R M36I	K20R M36I	
RegaInst									

PATIENT NUMBER: 22
SEX: F
AGE:
VIRAL LOAD: 645 000
DRUG MUTATIONS: **PR** K20R ;M36I ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS								
HIVDB	s	s	s	s		s	s	s
Regalnst								

NNRTI	DLV	EFV	NVP
ANRS			
HIVDB	s	S	S
Regalnst			

EI	T20
ANRS	
HIVDB	
Regalnst	

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS									
HIVDB	s		s	s	S	s	s	s	
Scored Mutation	K20R M36I		K20R M36I	K20R M36I	K20R M36I	K20R M36I	K20R M36I	K20R M36I	
Regalnst									

PATIENT NUMBER: 23
SEX: F
AGE:
VIRAL LOAD: 142 000
DRUG MUTATIONS: **PR** K20R ; M36I ; L63P ; V77I ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS								
HIVDB	S	S	S	S		S	S	S
RegaInst								

NNRTI	DLV	EFV	NVP
ANRS			
HIVDB	S	S	S
RegaInst			

EI	T20
ANRS	
HIVDB	
RegaInst	

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS									
HIVDB	S		S	S	S	S	S	S	
Scored Mutation	L63P M36I K20R		L63P M36I K20R	L63P M36I K20R	L63P M36I K20R	L63P M36I V77I K20R	L63P M36I K20R	L63P M36I K20R	
RegaInst									

PATIENT NUMBER: 24
SEX: F
AGE:
VIRAL LOAD: 104 095
DRUG MUTATIONS: **PR** L10I ; M36I; D60E ; L63V ; I93L
RT K103N

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
Regalnst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		R	R
SCORED MUTATION		K103N	K103N
HIVDB	R	R	R
SCORED MUTATION	K103N	K103N	K103N
Regalnst	R	R	R
SCORED MUTATION	K103N	K103N	K103N

EI	T20
ANRS	S
HIVDB	
Regalnst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	S	S	S	S	S	S	S
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	L10I, L63V, I93L, M36I		L10I, L63V, I93L, M36I	L10I, L63V, I93L, M36I	L10I, L63V, I93L, M36I	L10I, L63V, I93L, M36I	L10I, L63V, I93L, M36I	L10I, L63V, I93L, M36I	
Regalnst	S		S	S	S	S	S	S	

PATIENT NUMBER: 25
SEX: F
AGE:
VIRAL LOAD: 270 000
DRUG MUTATIONS: **PR** K20R ; M36I ; D60E ; L63S ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
RegaInst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		S	S
HIVDB	S	S	S
RegaInst	S	S	S

EI	T20
ANRS	S
HIVDB	
RegaInst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	S	S	S	S	S	S	S
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	K20R, I93L, L63S, M36I		K20R, I93L, L63S, M36I	K20R, I93L, L63S, M36I	K20R, I93L, L63S, M36I	K20R, I93L, L63S, M36I	K20R, I93L, L63S, M36I	K20R, I93L, L63S, M36I	
RegaInst	S		S	S	S	S	S	S	

PATIENT NUMBER: 26
SEX: M
AGE:
VIRAL LOAD: 23 200
DRUG MUTATIONS: **PR** K20R ; M36I ; L63P ; V77I ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
RegaInst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		S	S
HIVDB	S	S	S
RegaInst	S	S	S

EI	T20
ANRS	S
HIVDB	
RegaInst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	I	S	S	S	S	S	S
SCORED MUTATION			K20R, M36I, L63P						
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	K20R, L63P, I93L, M36I		K20R, L63P, I93L, M36I	K20R, V77I, L63P, I93L, M36I	K20R, L63P, I93L, M36I	K20R, L63P, I93L, M36I	K20R, L63P, I93L, M36I	K20R, L63P, I93L, M36I	
RegaInst	S		I	S	S	I	I	S	

PATIENT NUMBER: 27
SEX: F
AGE:
VIRAL LOAD: 51 500
DRUG MUTATIONS: **PR** M36I ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
RegaInst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		S	S
HIVDB	S	S	S
RegaInst	S	S	S

EI	T20
ANRS	S
HIVDB	
RegaInst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	S	S	S	S	S	S	S
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	I93L, M36I		I93L, M36I	I93L, M36I	I93L, M36I	I93L, M36I	I93L, M36I	I93L, M36I	
RegaInst	S		S	S	S	S	S	S	

PATIENT NUMBER: 29
SEX: F
AGE:
VIRAL LOAD: 359 000
DRUG MUTATIONS: **PR** M36I ; I93L
RT K103R

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS								
HIVDB	S	S	S	S		S	S	S
Regalnst								

NNRTI	DLV	EFV	NVP
ANRS			
HIVDB	S	S	S
Scored Mutations	K103R	K103R	K103R
Regalnst			

EI	T20
ANRS	
HIVDB	
Regalnst	

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS									
HIVDB	S		S	S	S	S	S	S	
Scored Mutation	M36I		M36I	M36I	M36I	M36I	M36I	M36I	
Regalnst									

PATIENT NUMBER: 31
SEX: F
AGE:
VIRAL LOAD: >750 000
DRUG MUTATIONS: **PR** M36I ; D60E ; L63P ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS								
HIVDB	S	S	S	S		S	S	S
RegaInst								

NNRTI	DLV	EFV	NVP
ANRS			
HIVDB	S	S	S
RegaInst			

EI	T20
ANRS	
HIVDB	
RegaInst	

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS									
HIVDB	S		S	S	S	S	S	S	
Scored Mutation	L63P M36I		L63P M36I	L63P M36I	L63P M36I	L63P M36I	L63P M36I	L63P M36I	
RegaInst									

PATIENT NUMBER: 32
SEX: F
AGE:
VIRAL LOAD: 189 000
DRUG MUTATIONS: **PR** L10V ; M36I ; L63A ; I93L
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
RegaInst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		S	S
HIVDB	S	S	S
RegaInst	S	S	S

EI	T20
ANRS	S
HIVDB	
RegaInst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	S	S	S	S	S	S	S
HIVDB	S		S	S	S	S	S	S	
SCORED MUTATION	L63A, L10V, I93L, M36I		L63A, L10V, I93L, M36I	L63A, L10V, I93L, M36I	L63A, L10V, I93L, M36I	L63A, L10V, I93L, M36I	L63A, L10V, I93L, M36I	L63A, L10V, I93L, M36I	
RegaInst	S		S	S	S	S	S	S	

PATIENT NUMBER: POSITIVE CONTROL
SEX:
AGE:
VIRAL LOAD:
DRUG MUTATIONS: **PR** I93T
RT

DRUGS:

NRTI	3TC	ABC	AZT	D4T	DDC	DDI	FTC	TDF
ANRS	S	S	S	S		S		S
HIVDB	S	S	S	S	S	S	S	S
RegaInst	S	S	S	S	S	S		S

NNRTI	DLV	EFV	NVP
ANRS		S	S
HIVDB	S	S	S
RegaInst	S	S	S

EI	T20
ANRS	S
HIVDB	
RegaInst	S

PI	APV	APV_RTV	ATV	IDV	LPV	NFV	RTV	SQV	TPV_RTV
ANRS		S	S	S	S	S	S	S	S
HIVDB	S		S	S	S	S	S	S	
RegaInst	S		S	S	S	S	S	S	

APPENDIX D

NATIONAL TREATMENT GUIDELINES Version 2 APRIL 2004 (PAGES 12-13)

7. Antiretroviral regimens

7.1 Antiretroviral naïve adult patients

7.1.1 First-line therapy – Schedule 1

Unless contraindicated, all patients will commence therapy on:

1. Stavudine (d4T) 40 mg every 12 hours (or 30 mg every 12 hours if < 60 kg), plus
2. Lamivudine (3TC) 150 mg every 12 hours, plus
3. Efavirenz (EFV) 600 mg at night (or 400 mg if < 40 kg) OR
   Nevirapine (NVP) 200 mg daily for 2 weeks, followed by 200 mg every 12 hours.

- Injectable contraception should be prescribed in addition to condoms for women of child-bearing potential who are started on efavirenz. The antiretroviral clinic must check that contraceptive injections have been administered on time.
- If unable to guarantee contraception for women while on therapy, nevirapine will be substituted for efavirenz. Extra safety bloods will need to be taken as per Table 10.
- Patients may occasionally need to change a drug from the first-line regimen to one from the second-line regimen, because of a serious adverse reaction (e.g. severe rash on nevirapine, requiring a swap to lopinavir/ritonavir, severely symptomatic peripheral neuropathy on stavudine, requiring a swap to zidovudine). Swapping limits the patient's second-line treatment options. The decision to swap must be made by a doctor with antiretroviral experience.

7.1.2 Second-line therapy –Schedule 2

Patients who continue to fail virologically despite efforts to improve adherence (see section 10) may be changed to schedule 2. Before changing to schedule 2, the patient should go through the treatment readiness and education process again. Most patients will commence schedule 2 as follows:

1. Didanosine (ddI) 400 mg once a day (250 mg daily if < 60 kg), plus
2. Zidovudine (AZT) 300 mg every 12 hours, plus
3. Lopinavir/ritonavir (LPV/r) 400/100 mg every 12 hours.

- Didanosine must be taken alone (i.e. not with other medicines), on an empty stomach, at least an hour before (or 2 hours after) a meal. Tablets should be dissolved in at least 30 ml of water or clear apple juice. No other fruit juice may be used to dissolve the tablets.
- Patients should try to keep their lopinavir/ritonavir cool (< 25 degrees Celsius).

There are currently no further treatment options available within the public sector for patients who fail second-line therapy.

APPENDIX E

NATIONAL TREATMENT GUIDELINES Version 2 APRIL 2004 (PAGES 35-37)

5.1 First line therapy – Schedule 1

Unless contraindicated, all children will commence therapy on:

Children < 6 months of age:

If fridge available:

1. Stavudine (d4T), plus
2. Lamivudine (3TC), plus
3. Ritonavir

If no fridge available:

1. Zidovudine (AZT), plus
2. Lamivudine (3TC), plus
3. Ritonavir

Children > 6 months of age

If fridge available:

1. Stavudine (d4T), plus
2. Lamivudine (3TC), plus
3. Lopinavir/ritonavir

If no fridge available:

1. Zidovudine (AZT), plus
2. Lamivudine (3TC), plus
3. Lopinavir/ritonavir

- Switch to tablets or capsules from syrups or solutions as soon as possible.
- Children may occasionally need to change a drug from the first-line regimen to one from the second-line regimen, because of intolerance or a serious adverse reaction. Swapping limits the patient's second-line treatment options. The decision to swap must be made by a doctor with antiretroviral experience.
- If intolerance develops to ritonavir or lopinavir/ritonavir, switch to nelfinavir.
- Lopinavir/ritonavir needs to be kept cool (< 25 degrees Celsius).

5.2 Second-line therapy – Schedule 2

Consider a move to second-line therapy under the following conditions:

Table 13: Reasons to move to second-line antiretroviral therapy in children

Virological	Clinical	Immunological
Rebound of viral load to baseline A detectable viral load may be tolerated in children, providing that growth and elevated CD4 count are sustained	Persistent oral thrush, which is refractory to treatment New evidence of stage III disease *Note: Short intercurrent episodes of pneumonia, lower respiratory tract infection (LRTI) and gastroenteritis should not be regarded as clinical failure. Presentation with TB while on first-line therapy is NOT an indication to switch to second-line therapy* *TB can present as progression to stage III disease and must be excluded before the decision is made to switch to second-line*	A persistent decline in the CD4 percentage over 2 months in the absence of TB *Note: The CD4 percentage should NOT be measured during an intercurrent infection – but preferably a month post resolution.* *If there is a modest decline in CD4 percentage (< 5%) and if no failure to thrive, do not change medication, but monitor closely*

Procedure for introduction of second-line therapy:

- Do not rush into second-line therapy.
- First check adherence; if it is not possible to improve adherence, attempt directly observed therapy (DOT) with a health care worker or trusted family member or friend.
- Ensure second-line therapy does not include any drugs used in first-line therapy.

Most children will commence schedule 2 as follows:

Children < 3 years old or < 10 kg:

If fridge available:

1. Didanosine (ddI), plus
2. Zidovudine (AZT), plus
3. Nevirapine

If no fridge available (and child previously on zidovudine as part of first-line therapy):

1. Didanosine (ddI) tablets, plus
2. Abacavir (ABC), plus
3. Nevirapine