Functional Analyses of HIV-1 Glycoproteins: Fusogenic Potential in Relation to Viral Pathogenesis

A Thesis submitted to the University of London for the Degree of Doctor of Philosophy

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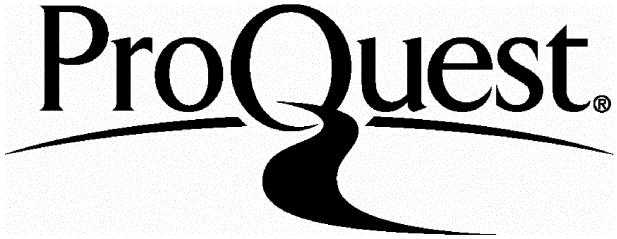

Abstract

HIV-1 entry into host cells is mediated by the viral surface glycoprotein (gp160 or Env) that is processed into gp120 and gp41, carrying receptor-binding and membrane-fusion activities respectively. Interplay between these two activities determines the overall fusogenic potential of processed Env. Comparison of full-length *env*-genes recovered directly from a patient cohort has revealed amino acid differences across Env between long-term non-progressor and rapid progressor patient groups that could affect the efficiency of membrane-fusion. To assess this the aim of my project was to develop a cell-cell fusion assay for HIV-1 Env.

The ability of the assay described in this thesis to distinguish non-functional and functional Envs was determined with HIV-1_{NL43} Env constructs either mutated in the gp160 KAKRRVVQREKR processing site, or progressively truncated from the C-terminus. A novel attenuating single residue amino acid substitution in the KAKRR motif has been identified and the results have been corroborated by virus infectivity assays following insertion of the *env*-genes into C2, an *env*-gene cosseting infectious molecular clone. Other mutations in the VVQ motif highlight qualitative differences in the function of Env when expressed either on a cell surface or on a virus surface, suggesting additional roles of the KAKRR motif in Env function. The study of Env truncation mutants, in the context of recent revised models of the gp41 cytoplasmic domain, provided functional evidence for regions adjacent to and involving the gp41 Kennedy domain playing a role in membrane fusion.

The application of the cell-cell fusion assay to longitudinal samples of *env*-genes derived from four patients who are progressing to AIDS after 12 or more years of asymptomatic HIV-1-infection, as determined by declining $CD4^+$ cell numbers and increasing viral load, indicated a lack of Envs with high fusogenic capacities. The C2 system indicated that many of these Envs were unable to support chimeric virus infection of PBMCs. The assay developed could thus provide a rapid screen for functional Envs to study in the C2 infectious molecular clone. The developed cell-cell fusion assay is complementary to existing methods of analyses in our laboratory, and could be adapted in the future for assessing other aspects related to clinical treatment of patients, such as surveillance for the emergence of Envs that are resistant to entry inhibitors.

Declaration

I, Carlum Shiu, declare that the work described in this thesis was, except where otherwise indicated in the text, entirely my own.

I have not submitted any portion of the work referred to in this thesis in support of any other qualification at this or any other institute of higher learning.

Carlum Shiu, April 2004

To my parents

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Finally, I would like to thank my parents, my brother Carlo and especially Eriko, for their love and support throughout this trying period.

Glossary Of Terms

°C	degrees Celsius
µg	microgram(s), 10^{-6}grams
µl	microlitre(s), 10^{-6} litre
AIDS	Acquired Immune Deficiency Syndrome
CCR5	CC chemokine receptor 5
CD4	Receptor for IL-16
CPE	cytopathic effects
CXCR4	CXC chemokine receptor 4
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Env	Viral surface glycoprotein
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
GFP	green fluorescent protein
g	grams
gp120	receptor binding subunit of Env (SU)
gp41	membrane anchor and fusion peptide subunit of Env (TM)
h	hour(s)
HA	haemagglutinin
HIV	Human Immunodeficiency Virus
HRP	horseradish peroxidase
Ig	immunoglobulin
kb	kilobase(s)
kDa	kilodaltons
l	litre
LTNP	long-term non-progressor
LTR	long terminal repeat promoter
M	molar
MAb	monoclonal antibody
min	minute(s)
ml	millilitre, 10^{-3} litre
mM	millimolar
m.o.i.	multiplicity of infection.

Glossary of Terms (*Continued*)

ng	nanogram(s), 10^{-9}grams
NP	nucleoprotein
nt	nucleotides
PCR	polymerase chain reaction
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pfu	plaque forming unit
Pfu polymerase	*Pyrococcus furiosus* polymerase
pg	picogram(s), 10^{-12}grams
RNA	ribonucleic acid
RP	rapid progressor
sec	second(s)
SDS	sodium dodecyl sulphate
SOE	splice-overlap extension
SP	standard progressor
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
$TCID_{50}$	tissue culture infectious dose
vRNA	viral ribonucleic acid
$vSIMB_{E/L}$	recombinant vaccinia virus encoding SP6 RNA polymerase
vTF7-3	recombinant vaccinia virus encoding T7 RNA polymerase

Amino Acid Nomenclature

Standard one and three letter amino acid codes are used throughout the text. The table below describes the three and single letter amino acid designations.

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

Chapter 1

Introduction

1.1 Introduction

1.1.1 A Brief History of Acquired Immune Deficiency Syndrome

Acquired Immunodeficiency Syndrome (AIDS) was first identified as a syndrome in a number of young homosexual males manifesting *Pneumocystis* pneumonia in 1981 (Pneumocystis pneumonia--Los Angeles, 1981), and the causative agent, human immunodeficiency virus type-1 (HIV-1) was finally isolated by various groups shortly after (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Levy and Shimabukuro, 1985). The result of HIV infection is the depletion of $CD4^+$ T lymphocytes. By leading to the destruction and/or functional impairment of modulatory cells of the immune system, HIV progressively destroys the body's ability to fight infections and certain cancers (Yeargin *et al.*, 2003). HIV-1 can be traced to simian immunodeficiency virus of chimpanzees (SIV_{cpz}), indicating its origin as a zoonotic infection of humans (Bailes *et al.*, 2003). Since the discovery of HIV-1, another type, HIV-2 has been described that appears more closely related to a SIV strain in sooty mangabeys (SIV_{sm}) and provides stronger evidence for a separate jump from non-human primates to humans. HIV-1 can be categorised into phylogenetically distinct major (M), outlier (O) and non-M/non-O (N) groups (Gurtler *et al.*, 1994; Simon *et al.*, 1998; reviewed in Robertson *et al.*, 1999). While all three groups are present on the African continent, group M has managed to spread across and establish itself on the other continents, (Figure 1.1) (Louwagie *et al.*, 1995; reviewed in Perrin *et al.*, 2003). Within group M itself, the virus can be subdivided into distinct subtypes A-D, F-H, J and K and circulating recombinant forms (CRFs) based upon analyses of full-length genomes (Robertson *et al.*, 1999). The continual evolution of subtypes through an error-prone viral reverse transcriptase is driven by recombination, immune responses and anti-viral drug selection. Within an infected individual, the genetic drift may be less than 3%, enabling epidemiological and evolutionary studies through the application of molecular methods to viral genes such as *gag* and *env* (Douglas *et al.*, 1996; Essajee *et al.*, 2000; Louwagie *et al.*, 1995; Nelson *et al.*, 1997; Penny *et al.*, 1996).

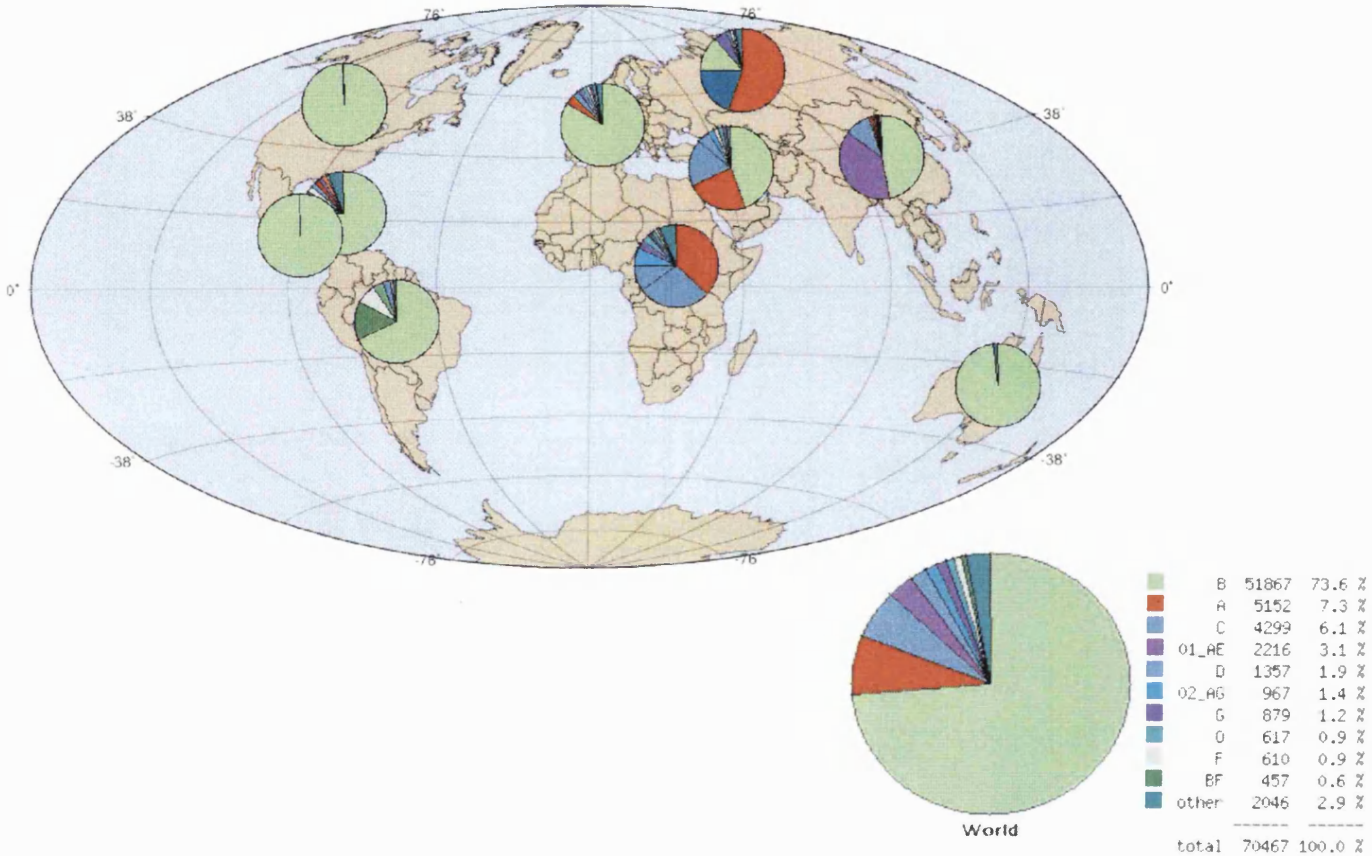


Figure 1.1. Global distribution of HIV-1 Subtypes.

World map representing subtype frequencies of sequence depositions in the LADB 2004, not populations. Adapted from the Los Alamos National Laboratory HIV Database:http://www.hiv.lanl.gov/content/index

HIV-1 is transmitted by homosexual and heterosexual intercourse, perinatally and postnatally (breast feeding), contaminated blood products and by the sharing of needles by intravenous drug users. Despite global efforts to prevent the spread of the virus, it is estimated that over 40 million individuals worldwide are infected with HIV, with 5 million new infections and 3 million deaths having occurred in 2003 alone. Of the estimated 14000 new infections that occur daily, more than 95% of these are in developing countries, with those in sub-Saharan Africa bearing much of the burden (UNAIDS, http://www.unaids.org/en/resources/epidemiology/epidemicupdateslides.asp (UNAIDS, 2004)).

1.1.2 Clinical Course of Disease

HIV-1 infection is characterised by the selective depletion of $CD4^+$ T lymphocytes leading to symptomatic AIDS. Following infection with HIV-1, there is an initial peak in viremia associated with a reduction in $CD4^+$ T lymphocytes and the onset of clinical symptoms. The usual course of disease in an infected individual in the absence of anti-retroviral therapy is illustrated in figure 1.2. Although $CD4^+$ T lymphocyte numbers may recover to near normal levels after acute infection, the numbers will continue to decline during the asymptomatic period (Lum *et al.*, 2003; Yeargin *et al.*, 2003). It is estimated that the daily turnover of CD4 cells is of the order of 10^9 (Wei *et al.*, 1995). The selective depletion of $CD4^+$ T cells is a direct result of HIV-1 replication, as the introduction of HAART results in recovery of the $CD4^+$ lymphocyte numbers to some degree (Ho *et al.*, 1995; Wei *et al.*, 1995). Direct cell killing can occur through syncytium formation, single-cell lysis or by antibody dependent cellular cytotoxicity (ADCC) directed at the surface expressed viral glycoprotein, Env. Although syncytium formation has never been observed directly *in vivo*, it can result in apoptosis *in vitro*. It is suggested that apoptosis may be delayed by HIV-1 Vpr (Medema and Medema, 2002). Whilst HIV-1 replication is optimal in activated antigen-specific $CD4^+$ T lymphocytes, resting bystander cells can be productively infected by HIV-1 at a lesser extent (Scales *et al.*, 2001).

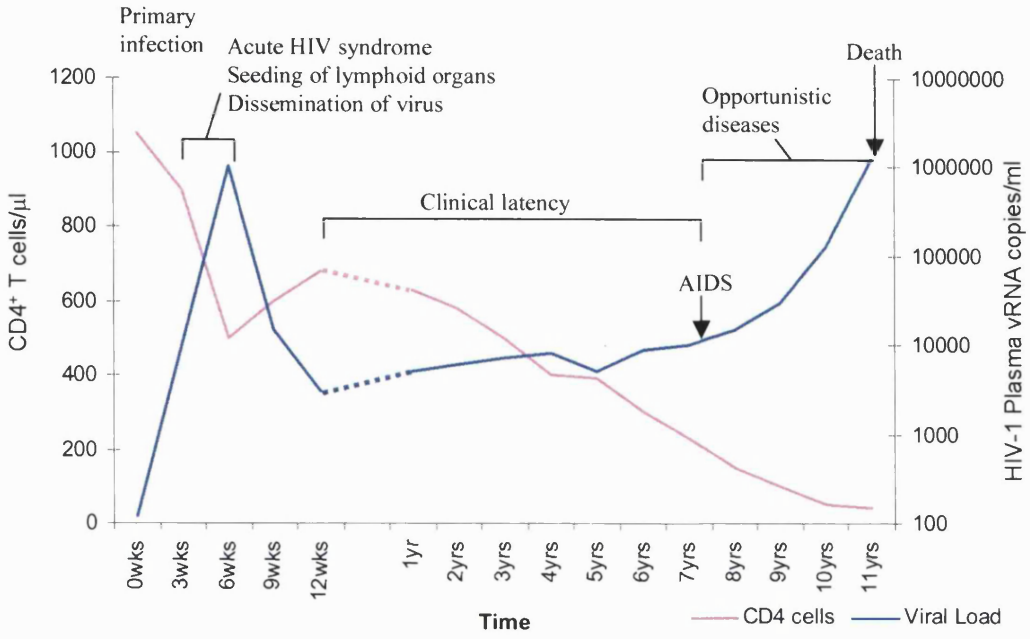


Figure 1.2. Model of the expected course of disease progression following initial infection with HIV-1.

The median time from seroconversion to AIDS-related death is approximately 10 years, assuming the absence of anti-retroviral therapy. The clinical latency period persists until $CD4^+$ T lymphocyte numbers fall below 500 cells/µl and viral load in the blood increases. AIDS manifests when cell numbers fall below 200 cells/µl, rendering the patient susceptible to opportunistic pathogens.

The patient is considered symptomatic for AIDS when $CD4^+$ T lymphocytes are depleted to less than 200 cells/μl; this eventually leads to AIDS-related death as the destruction of the immune system portends the onset of fatal infections caused by opportunistic pathogens.

1.2 HIV-1 Morphology

1.2.1 Taxonomic Classification

HIV belongs to the *Lentivirus* genus within the *Retroviridae* family, according to the International Committee on Taxonomy of Viruses (ICTV, http://www.mcb.uct.ac.za/ictv/ICTV.html). Members of this family have the defining trait of reverse transcribing their genomic RNA to double-stranded proviral DNA that integrates into the host cell genome. This allows the viruses to establish a persistent infection despite a host immune response.

1.2.2 HIV-1 Genome

HIV-1 virions encapsulate two copies of positive-sense single-stranded RNA approximately 9.8kb in length with 5'methyl cap structures and 3'polyadenylated tails. Comprehensive reviews of the HIV-1 genome and lifecycle are available (Fields *et al.*, 2001; Levy, 1998; Zack *et al.*, 1990). Each genomic RNA copy is individually encapsidated by the viral p7 nucleoprotein (NC) to form ribonucleoprotein complexes (RNP). After reverse transcription, identical long terminal repeats (LTR) flank either end of the proviral genome (Figure 1.3).

All retroviral genomes possess *gag*, *pol* and *env* genes. The *gag* and pol genes encode large precursor proteins ($Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ respectively) that are proteolytically cleaved into mature products. Viral structural proteins matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), p6 and the spacer proteins p1 and p2 are derived from $Pr55^{Gag}$ precursor. The $Pr160^{Gag\text{-}Pol}$ precursor is also cleaved into the viral enzymes protease (PR, p11), integrase (IN, p31) and reverse transcriptase (RT, p51 and p66). Complex retroviruses like HIV and SIV possess additional proteins encoded by the *vif*, *vpr* (restricted to HIV-1 and SIV_{cpz}), *vpu*, *tat*, *rev* and *nef*-genes, whilst HIV-2 and SIV has *vpx* and *vpu*.

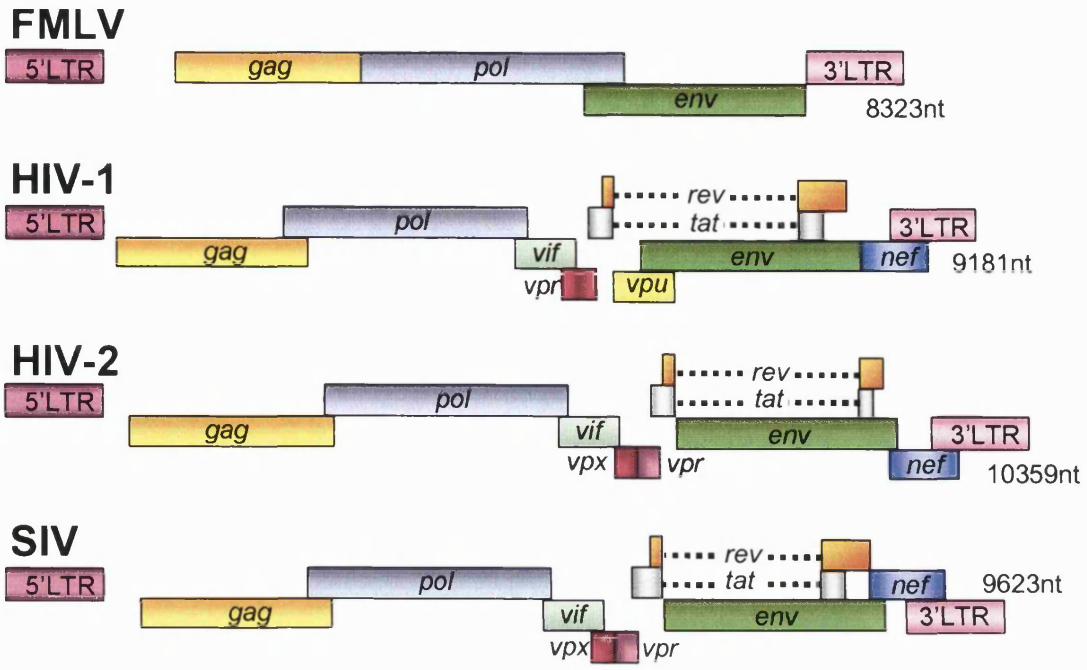


Figure 1.3. Schematic representation of proviral genomes of simple and complex retroviruses.

Friend murine leukaemia virus (FMLV, accession number Z11128), typical of a simple retrovirus, possesses only *gag*, *pol* and *env* genes. Complex retroviruses, such as the lentiviruses HIV-1 (Accession number AF033819), HIV-2 (Accession number M30502) and SIV (Accession number M58410), possess *tat* and *rev* regulatory genes as well as other auxiliary genes. The *vpu* gene is only present in HIV-1, whilst both HIV-2 and SIV possess *vpx*. Diagram is not to scale, however, the lengths of the viral genomes are indicated in nucleotides (nt) according to their reference genomes (Accession numbers as indicated) available from the National Centre for Biotechnology Information (NCBI) genome database (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Genome). Adapted from (Fields *et al.*, 2001).

These additional proteins may be part of the virion (Vif, Vpr/Vpx, Nef), regulate viral gene expression (Tat and Rev) or interfere with host cellular processes to enhance virus propagation (Vif, Vpu and Nef).

1.2.3 HIV-1 Morphology

The morphology of HIV-1 is schematically presented in figure 1.4. The RNPs are protected within a cone-shaped capsid shell made up from many units of the capsid protein. Outside the capsid is an outer protein shell, composed of the myristoylated matrix protein, which lies beneath the lipid envelope derived from the host cell. On the virion surface is the viral glycoprotein, Env, which has been processed into 2 non-covalently linked subunits (gp120/SU and gp41/TM). The fusogenic gp41 subunit anchors the receptor binding gp120 subunit to the surface, and the Env exists in an oligomeric state as trimers, amongst host-derived membrane proteins. Structural analyses are available for Env, MA, CA, RT, IN, PR (Reviewed in Turner and Summers, 1999).

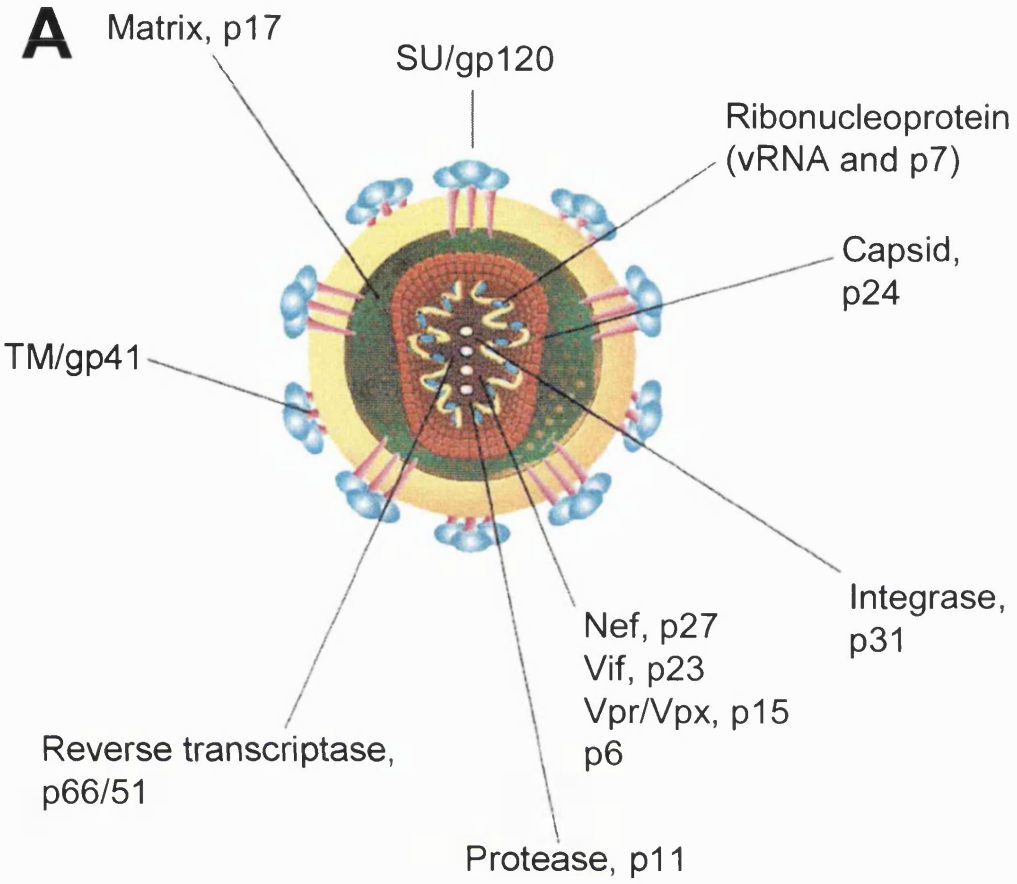
1.2.4 Viral Enzymes

A mature virion will carry three viral enzymes within the capsid: a magnesium-dependent RT with RNaseH activity (p66/51); IN and PR.

1.2.5 Auxiliary Proteins

HIV-1 virions also package a number of auxiliary proteins (Reviewed in Janvier *et al.*, 2000).

Vif is a highly conserved protein amongst all lentiviruses except Equine Infectious Anaemia Virus (EIAV). HIV-1 virions deficient in *vif* are unable to replicate in primary peripheral blood mononuclear cells (PBMCs) and some non-permissive T cell lines, but are replication competent in other permissive T cell lines. PBMCs and non-permissive cell lines express apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like-3G (APOBEC3G), which functions to deaminate deoxycytosine to deoxyuridine, affecting the reverse transcription process when incorporated into virions. Vif protects the viral genome from APOBEC3G by excluding it from the virion and mediating its ubiquitination for degradation in infected cells (Sheehy *et al.*, 2002; Sheehy *et al.*, 2003).



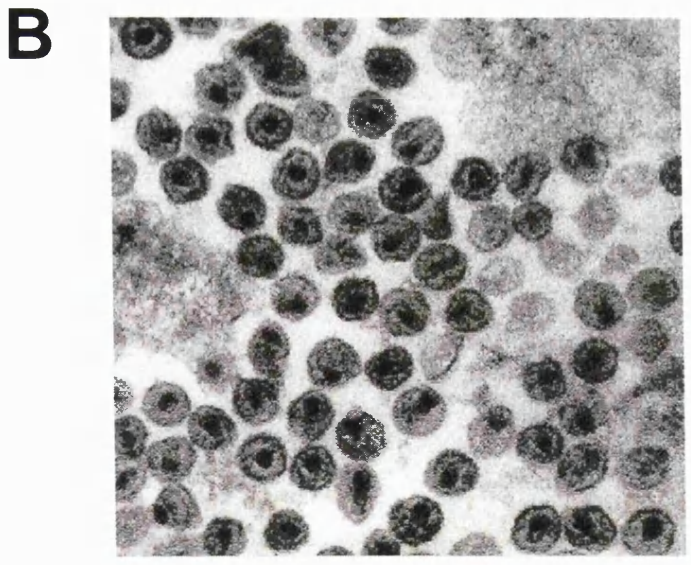


Figure 1.4. Schematic representation of HIV structure.

The structural and non-structural proteins in the HIV virion are indicated in (A) (Adapted from Turner and Summers, 1999). Transmission electron micrograph (50, 000x magnification) of mature HIV-1 particles, showing the dense capsid core (B). HIV virions are approximately 100nm in diameter. Adapted from Phillips, 1995.

The *nef* gene encodes a 27 kDa myristoylated phosphoprotein associated with membranes. The functions of Nef in HIV and SIV include the down-regulation of surface CD4 and MHC-1, leading to the enhancement of virus infectivity, modulation of serine/threonine and tyrosine kinases to perturb cellular activation pathways, and some mutations are associated with different stages of disease (Aiken and Trono, 1995; Kirchhoff *et al.*, 1999; Luo and Peterlin, 1997; Mangasarian *et al.*, 1999).

Vpr is involved in the transport of the pre-integration complex (PIC) into the nucleus (particularly non-dividing cells such as macrophages) and the arrest of infected cells in the G2 phase, providing an environment conducive to the stimulation of gene expression from the HIV-LTR (Mahalingam *et al.*, 1997; Popov *et al.*, 1998). In the case of HIV-2 and SIV, the nuclear translocation function is encoded by the additional protein Vpx (Fletcher *et al.*, 1996). Vpr can induce caspase-mediated apoptosis through disruption of mitochondrial membrane permeability (Lum *et al.*, 2003). In addition, the fidelity of the reverse transcription process is improved by Vpr recruitment of uracil DNA glycosylase into the virion (Mansky, 1996).

The HIV-1 p6 is a proline-rich 6 kDa protein derived from the C-terminal of the Gag precursor, it is essential for virion release from the infected cell and the incorporation of Vpr into virions (Jenkins *et al.*, 2001; Lu *et al.*, 1995).

1.3 HIV-1 Lifecycle

1.3.1 Host Cell Receptors

1.3.1.1 CD4 Receptor

HIV-1 infection of target cells is mediated by the gp120 subunit interacting with the primary CD4 receptor and chemokine co-receptors (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1986; Pauza and Price, 1988; reviewed in Clapham and McKnight, 2001; Doms, 2000; Goldsmith and Doms, 2002; Popik and Pitha, 2000). CD4 is a member of the immunoglobulin (Ig) superfamily, with four extracellular immunoglobulin (Ig)-like domains (D1-4), a transmembrane domain and a cytoplasmic tail that can associate with kinase $p56^{lck}$, which can regulate endocytosis and signalling (Pelchen-Matthews *et al.*, 1995). Although CD4 is a receptor for the chemoattractant interleukin (IL)-16 (Cruikshank *et al.*, 1998), it is primarily involved in T-helper (T_H) lymphocyte recognition of foreign antigens through interaction with the major

histocompatibility complex (MHC)-II β2 subunit on antigen presenting cells (APCs). CD4 is also expressed on cell types that do not express the T cell receptor (TCR), such as monocytes and dendritic cells (DCs). Differences in replication kinetics and selection of viral variants indicate differing levels of CD4 expression between individuals (Bannert *et al.*, 2000; Spira and Ho, 1995), and the redox state of the disulphide bond of the D2 domain has been shown to be a determinant in blocking HIV-1 infection of T cell lines (Matthias *et al.*, 2002).

1.3.1.2 Chemokine Receptors

Prior to the discovery of chemokine receptors (CKRs) as co-receptors for primate immunodeficiency viruses, phenotypes of HIV and SIV isolates could be described as syncytium inducing (SI) or non-syncytium inducing (NSI). The CKRs employed by HIV and SIV for entry enabled isolates to be characterised with regard to specific co-receptor usage *in vitro*, as reviewed in (Dragic, 2001; Pelchen-Matthews *et al.*, 1999; Zaitseva *et al.*, 2003). The vast majority of NSI/M-tropic HIV isolates preferentially use CCR5, and are designated as R5-tropic (Choe *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996), whereas SI/T-tropic isolates exhibit a preference for CXCR4, and are thus X4-tropic (Feng *et al.*, 1996).

The chemokine receptors are members of the family of seven-transmembrane domain G-protein coupled receptors (GPCRs), expressed on a variety of cells such as leukocytes, neurones and endothelial cells. The chemokine receptors exhibit 4 surface exposed domains, the N-terminus and 3 external loops (EC-I-III), likewise there are three intracellular loops and a short C-terminal cytoplasmic tail. The N-terminal head associates with EC-III, whereas EC-I associates with EC-II via disulphide bonds. Different CKRs are likely to adopt different conformations depending on the cell type, effecting dimerisation, associations with heterologous chemokine receptors or with other molecules such as CD4 and the ability to support HIV infection (McKnight *et al.*, 1997).

1.3.1.3 CKR Surface expression

CCR5 is expressed on immature DCs, monocytes, macrophages, brain microglia cells and T lymphocytes, and functions in chemotaxis during inflammatory responses. CCR5 responds to the chemoattractant ligands: regulated-upon-activation, normal T cell expressed and secreted (RANTES); macrophage inflammatory protein (MIP)-1α and MIP-1β (Cocchi *et al.*, 1995; Deng *et al.*, 1996). Although CCR5 is capable of

supporting infection at trace concentrations, it has been suggested that membrane fusion by R5 Envs involved the cooperation of multiple molecules of CCR5 (Kuhmann *et al.*, 2000), hence increased surface expression of CCR5 renders cells more susceptible to R5-tropic variants (Deng *et al.*, 1996). Surface expression of CCR5 at sufficient concentration can permit HIV-2 and SIV infection in the absence of CD4 (Bannert *et al.*, 2000; Reeves *et al.*, 1999). CXCR4 is expressed on neutrophils, monocytes, B cells, $CD4^+$ and $CD8^+$ T lymphocytes and across a wide range of tissues (Bleul *et al.*, 1996; Forster *et al.*, 1998). CXCR4 and its ligand, stromal cell-derived factor-1 (SDF-1) (Bleul *et al.*, 1996), are essential for maturation and development of T lymphocytes. CXCR4 can be detected in intracellular compartments in some cells, and upregulated upon stimulation of cells with IL-4 or in the absence of SDF-1 (Jourdan *et al.*, 1998; Zaitseva *et al.*, 1997). In Th-1 differentiated $CD4^+$ T lymphocytes, exposure to IL-4 can induce susceptibility to HIV-1 infection; therefore the wrong cytokine environment in a HIV-infected subject may subvert efficient control of HIV (Jourdan *et al.*, 1998).

CCR5 and CXCR4 may be co-expressed on blood-derived DCs, macrophages and $CD4^+$ T lymphocytes (van Rij *et al.*, 2000; Zaitseva *et al.*, 1997). The predominance of R5/M-tropic strains isolated from macrophages is surprising as these cells also express CXCR4. However, CD4 has a higher affinity for association with CCR5, therefore low levels of surface CD4 preferentially form functional complexes with CCR5 to support infection with R5-tropic strains (Lee *et al.*, 2000). This is clearly observed in the THP-1 monocytic cell line, whereby undifferentiated monocytes are susceptible to X4-tropic viruses, but after stimulation the differentiated cells down-modulate CD4 and cells become restricted to R5 viruses (Konopka and Duzgunes, 2002). Blocking of CCR5 on the surface of monocyte-derived macrophages (MDM) through its cognate chemokine ligands or specific MAbs enabled infection of MDM with X4-tropic variants (Lee *et al.*, 2000). Endogenous association of CD4 with CXCR4 may be observed at low levels, but is markedly increased by the presence of gp120 (Forster *et al.*, 1998; Mbemba *et al.*, 2002).

On $CD4^+$ T cells CCR5 surface expression is mainly restricted to a small $CD45RO^+$ memory subset population of $CD4^+$ T lymphocytes, whilst CXCR4 is widely expressed on both the $CD45RO^+$ memory and $CD45RA^+$ naïve subsets of $CD4^+$ T lymphocytes (van Rij *et al.*, 2000). Although R5-tropic viruses predominantly infect the memory subset, their replication is significantly restricted compared to X4-viruses, however, this

low level of replication of R5-viruses may contribute to the persistence of R5-tropic variants throughout the entire course of disease (van Rij *et al.*, 2000).

Surface expression of chemokine receptors is modulated predominantly by clathrin-dependent endocytosis upon ligand binding (Signoret et al., 1997; Signoret et al., 1998). Endocytosis of the chemokine receptors rather than competitive blocking by their cognate ligands provides a greater protective mechanism against HIV infection; resistance to infection with R5-tropic HIV-1 strains has been associated with individuals homozygous for a short 32 bp deletion in CCR5 (CCR5Δ32)(Dean *et al.*, 1996). Endocytosed CXCR4 may either be degraded or recycled back to the surface (Forster et al., 1998; Signoret et al., 1997; Signoret et al., 1998). The alternative trafficking of CXCR4 to the degradation pathway may be mediated by ubiquitination (Marchese and Benovic, 2001).

1.3.2 Infection

The HIV-1 lifecycle is typical for retroviruses, as depicted in figure 1.5 and described in the literature (Fields *et al.*, 2001; Turner and Summers, 1999). HIV-1 productively infects macrophage and T-helper cell subsets of the host immune system, using the surface expressed primary receptor CD4 and a CKR co-receptor such as CXCR4 or CCR5. Following membrane fusion at the cell surface, the virion core is delivered into the host cell cytoplasm. Internalisation of HIV has been shown to occur by either endocytosis or phagocytosis in particular cell types, but the latter may not represent an infectious pathway (Pauza and Price, 1988).

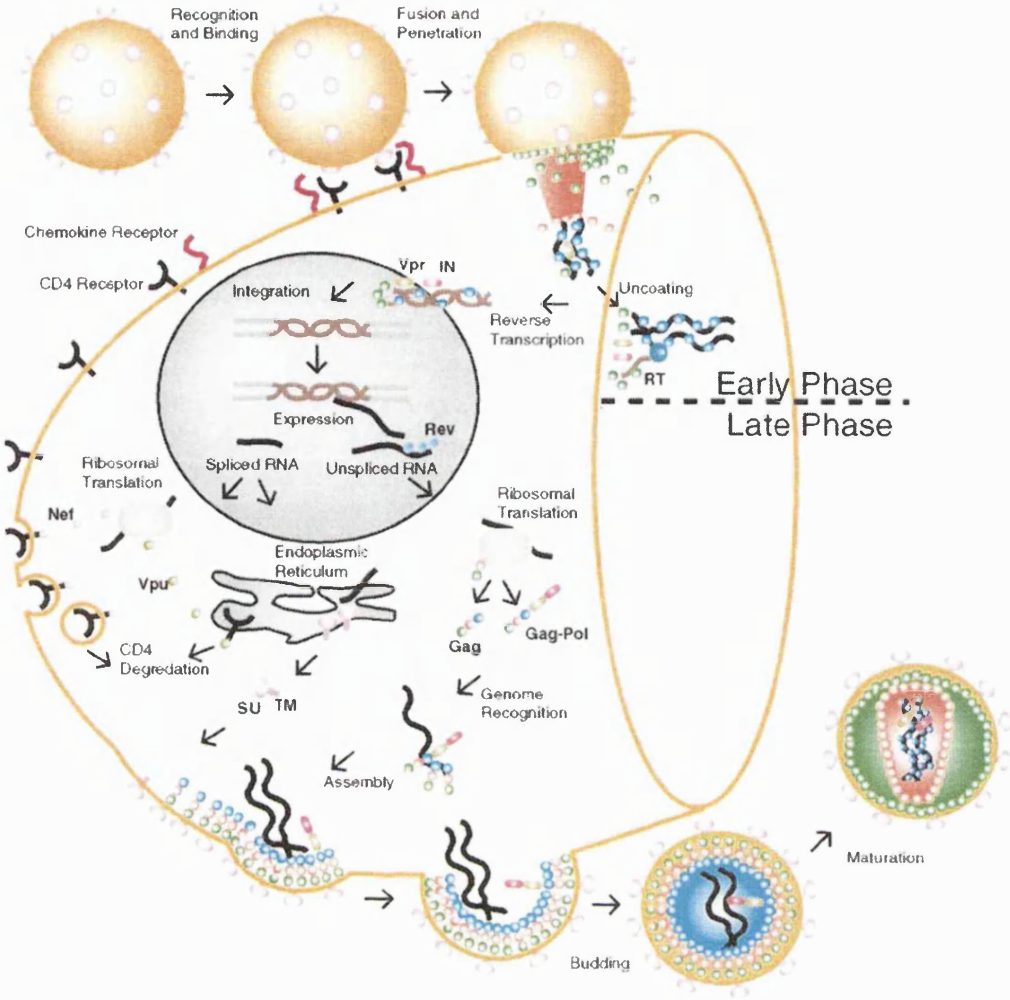


Figure 1.5. Schematic representation of HIV-1 lifecycle.

The early phase of the lifecycle is characterised by the penetration of the target cell using CD4 and a chemokine co-receptor at the plasma membrane. Reverse transcription occurs as the preintegration complex is released into the cytoplasm and traffics to the nucleus. The proviral DNA is integrated into the host genome where it can remain latent. Upon activation of the infected cell, the late phase of the lifecycle proceeds as transcription and translation of viral gene products. CD4 is downregulated from the host cell and assembly of the virus occurs at a membrane site, in this case, the plasma membrane. Maturation occurs after budding of the virion. Adapted from (Turner and Summers, 1999).

The viral ribonucleoprotein, in association with the viral proteins MA, Vpr, RT and IN (Bukrinsky *et al.*, 1993; Depienne *et al.*, 2000; Popov *et al.*, 1998), constitute the PIC required to initiate reverse transcription of the vRNA to linear double-stranded DNA. The signal(s) to activate the viral RT is not known, although exposure of cell-free virus to deoxyribonucleotides (dNTPs) is sufficient according to some studies (McDonald *et al.*, 2002; Zhang *et al.*, 1996). A study of the intracellular trafficking of HIV-1 following penetration of the cell membrane suggests the initial use of actin to access the microtubule network (MTN), and then the use of dynein and the MTN to deliver the PIC to the nucleus (McDonald *et al.*, 2002). Nuclear localisation signals on integrase and Vpr may aid targeting of the PIC to the nucleus (Depienne *et al.*, 2000; Pluymers *et al.*, 1999; Sherman *et al.*, 2001). Integration of the viral DNA creates a consistent proviral structure, consisting of viral sequences flanked by complete LTRs (Figure 1.3).

1.3.3 Transcription

Although low levels of replication may take place following integration, the provirus will essentially remain silent (latent) until activation of the host cell transcription apparatus. Studies suggest the maintenance of latency may involve cis- or trans-acting factors depending on the cell type (Chen *et al.*, 1994). The HIV-1 LTR is a powerful promoter, however its activation is dependent on host cell general transcription factors (GTFs) binding core promoter sequences and enhancers such as Sp1 binding sites, a Pol II TATA box, and recognition sites for the NF-κB/Rel family of transcription factors (Berkhout and Jeang, 1992; Perkins *et al.*, 1993) (Figure 1.6). $CD4^+$ cells may be activated by the specific binding of gp120 to induce the translocation of NF-κB into the nucleus via $p56^{lck}$ (Briant *et al.*, 1998).

The HIV-1 Tat product is a powerful transactivator of gene expression essential for productive virus infection; in the absence of Tat, transcription complexes terminate prematurely (Kessler and Mathews, 1992; Rice and Mathews, 1988). Tat is secreted by HIV-1 infected cells and can be taken up by neighbouring cells, thus extracellular Tat represents a mechanism to stimulate latent genomes and contribute to the dissemination of HIV (Ensoli *et al.*, 1993; Huigen *et al.*, 2004).

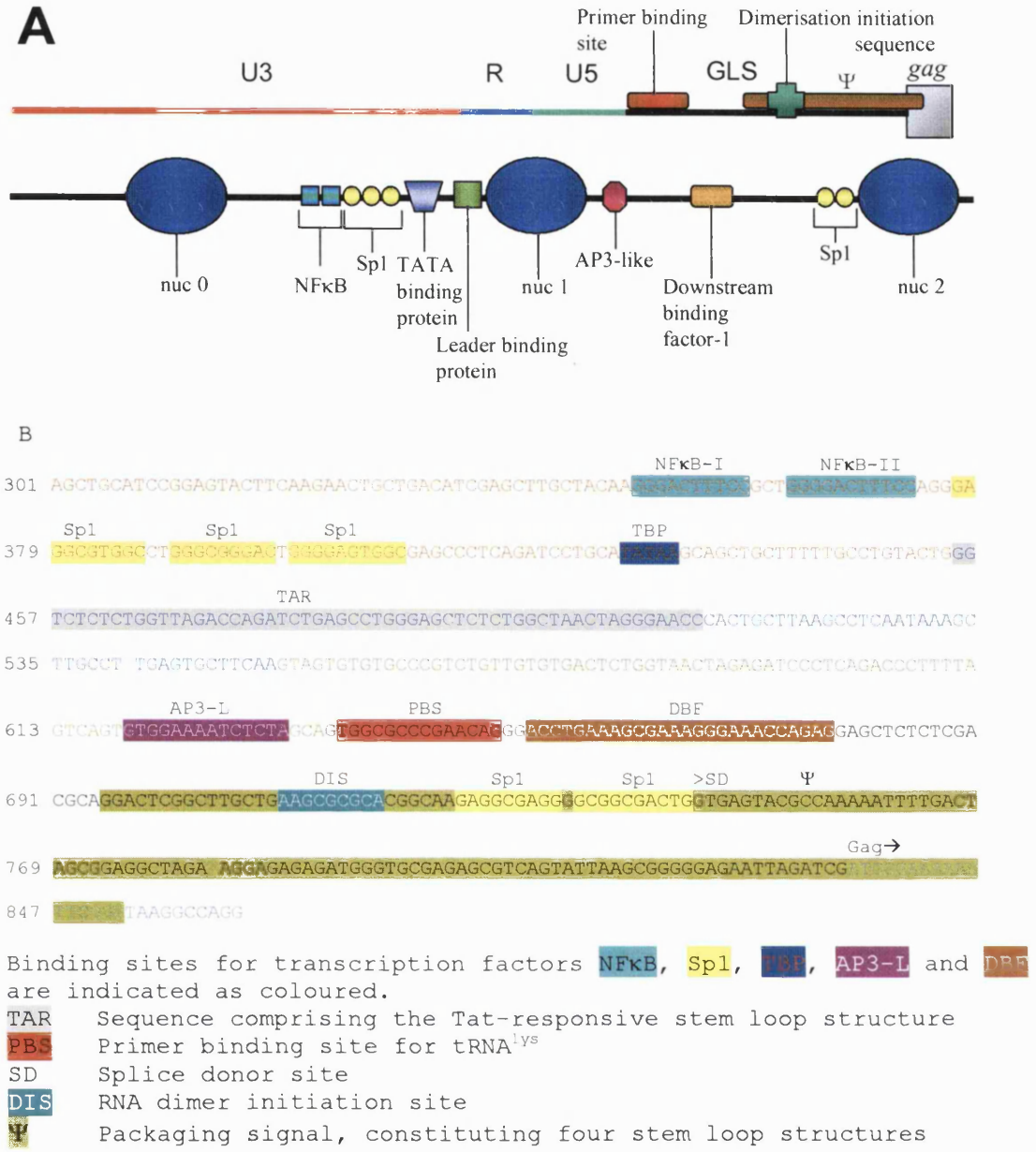


Figure 1.6. Organisation of the HIV-1 long-terminal repeat (LTR) promoter.

The HIV-1 LTR is schematically represented in (A), and the representative HIV-1_{HXB2} nucleotide sequence of the U3, R, U5, GLS and *gag*-gene regions are indicated as coloured in (B). Binding sites for transcription factors and nucleosomes (nuc), and sequences important for the formation of secondary structures across the LTR and the Gag leader sequence (GLS) upstream of the *gag*-gene are indicated. Diagram is not to scale, but features correspond to LTR and GLS domains as indicated. Adapted from (Fields *et al.*, 2001; Van Lint *et al.*, 1997) and the Los Alamos HIV sequence database (Accession number AF033819 from www.hiv.lanl.org).

Tat binding to a hairpin loop transactivator region (TAR) at the 5' of all viral transcripts enables recruitment of a cyclin T/CDK9 heterodimer to drive translation (Wei *et al.*, 1998). The ensuing full-length transcript is multiply spliced into three classes of HIV mRNAs: (a) unspliced genomic RNA encoding the Gag and Gag-Pol precursors; (b) partially spliced mRNAs translated into Vif, Vpr, Vpu and Env products; and (c) multiply/completely spliced mRNAs encoding Tat, Rev and Nef (Schwartz *et al.*, 1990).

1.3.4 Translation

The HIV Rev protein regulates the nuclear export of unspliced HIV-1 mRNAs (Malim *et al.*, 1989). Rev interacts with the Rev response element (RRE) in all unspliced and partially spliced messages located at the gp120-gp41 junction. Initially multiply spliced viral mRNAs are exported from the nucleus by the host export mechanisms. The proteins Tat, Rev and Nef are all synthesised in the cytoplasm from these mRNAs (Purcell and Martin, 1993). Rev binds importin β in the cytoplasm and this heterodimer is translocated across the nuclear pore and into the nucleus where binding of RanGTP to importin β induces dissociation of Rev. The exposure of the nuclear export signal (NES) on Rev multimers bound to unspliced mRNA enables the specific binding of the heterodimeric complex of chromosome region maintenance 1 (CRM1)-RanGTP to the Rev/RRE complex. The entire complex is then exported to the cytoplasm through the nuclear pore, whereupon the export complex is disassembled. The unspliced viral mRNA can then be translated or transported to the site of virion assembly, and Rev and importin β can cycle through the export process again (Henderson and Percipalle, 1997; Izaurralde *et al.*, 1999; Meyer and Malim, 1994).

The *gag* gene product is translated from a full-length mRNA transcript in the cytoplasm to produce the myristoylated 55 kDa precursor $Pr55^{Gag}$, which is localised to the host cell membrane. $Pr55^{Gag}$ is processed by the viral protease during virion maturation to produce the mature proteins MA, CA, NC, p6 and the spacer proteins p1 and p2. The location of the p6 protein in the virion is undefined. The *gag* and *pol* ORFs of HIV-1 are in different reading frames, so the *pol* gene is expressed by a frameshift to produce the $Pr160^{Gag\text{-}Pol}$ precursor (Vickers and Ecker, 1992). This strategy of expression ensures that proteins are made in appropriate ratios and enables the simultaneous targeting of many proteins to site of virion assembly (Karacostas *et al.*, 1993). The viral proteins Vif, Vpr, Vpu and Nef are primary products of spliced transcripts.

1.3.5 Env Synthesis

1.3.5.1 Translation

Type-I viral fusion proteins are usually synthesised as inactive precursors, requiring a cleavage event to generate the active subunits, SU, which carries receptor-binding determinants, and TM, which mediates membrane fusion. The subunits may be associated by means of disulphide bridges, as in the case of Influenza HA_1-HA_2, and the F1-F2 fusion proteins of human respiratory syncytial virus (HRSV) and Newcastle Disease Virus (NDV), or by non-covalent means as in the case of HIV-1 gp120/41 (Moulard and Decroly, 2000).

The *env* gene is expressed from a distinct singly spliced Vpu/Env bicistronic mRNA (Schwartz *et al.*, 1990).For many viral glycoproteins, translation of the signal peptide on membrane bound polysomes directs the transcript to the endoplasmic reticulum (ER) for the synthesis of the inactive precursor glycoprotein. The signal peptide features are conserved amongst HIV/SIV Env, although HIV-1 signal sequences are longer, more positively charged and have greater side chain volume than HIV-2/SIV. The HIV-1 endogenous signal peptide effects a low rate of synthesis and secretion, the N-terminus shares the *vpu* coding region (absent in HIV-2/SIV). Early folding of gp160 occurs within 30mins of synthesis in the ER, during this time disulphide-bond formation, oligomerisation and addition of carbohydrate side chains to Asn of glycosylation sequons (N-X-S/T, where X is any amino acid) residues occur. In all cases, maturation occurs in the Golgi with regard to proteolytic processing, trimming of N-linked oligosaccharides, terminal glycosylation and acylation prior to trafficking to the cell surface (Anderson *et al.*, 1992; Kuroda *et al.*, 1991; Webster and Rott, 1987). Failure to oligomerize can prevent or retard exiting from ER compartment. The mis-folded precursor may be directed to the lysosomes or proteasome for degradation by calcium-dependent proteases, or even degraded within the ER (Courageot *et al.*, 1999).

1.3.5.2 Env Trafficking

In the case of HIV, the gp160 precursor is glycosylated by enzymes in the rough endoplasmic reticulum (RER) at up to 32 potential N-linked sequons in the gp120 subunit and at 4 sites in the gp41 subunit. The disturbance of initial N-linked glycosylation can also prevent trafficking of the precursor out of the ER, thereby preventing Env processing and function (Fenouillet *et al.*, 1993; Fenouillet and Jones, 1995; Fenouillet *et al.*, 1997). The cluster of glycans in the ectodomain of gp41 has

been shown to regulate trafficking, processing and the fusogenic potential of gp160 in the context of a molecular clone derived *env*-gene (Dash *et al.*, 1994; Fenouillet *et al.*, 1993; Fenouillet and Jones, 1995; Perrin *et al.*, 1998). The gp160 precursor takes nearly 2h to exit the ER as it traffics along the constitutive secretory pathway towards the *cis*-Golgi (Moulard and Decroly, 2000; Willey *et al.*, 1991). After maturation, the glycoprotein is exported to the cell surface of infected $CD4^+$ T lymphocytes, however the Tyr-based YXXϕ (where X represents any amino acid and ϕ is an amino acid with a large side chain) endocytosis motif proximal to the gp41 membrane spanning domain rapidly induces the endocytosis of Env (Rowell, Ruff et al., 1995; Rowell, Stanhope et al., 1995).

1.3.5.3 Env Processing

In all avirulent avian and mammalian strains of Influenza A, and in Influenza B, activation of the glycoprotein requires cleavage of the precursor. Infectious virus is produced when the HA0 precursor is processed by a trypsin-like enzyme at an exposed loop in the cells of the respiratory tract or the enteric tract, resulting in a local infection (Kido et al., 1999; Kido et al., 1996) (Figure 1.7). In humans, coincident bacterial infections of the respiratory tract induce the local production of inflammatory factors such as plasminogen, urokinase and thrombin that are also capable of activating HA on the surface of viruses (Webster and Rott, 1987). Highly pathogenic natural isolates of avian Influenza, typified by H5 and H7 haemagglutinins possess either insertions of basic residues at the cleavage site, or are deficient for an N-linked oligosaccharide near the cleavage site. It has been demonstrated that insertion of basic residues into HA0s derived from avirulent strains bestow intracellular processing properties (Ohuchi *et al.*, 1991; Walker and Kawaoka, 1993; Webster and Rott, 1987).

Studies of paramyxoviruses, lentiviruses, herpesviruses and filoviruses reveal the presence of a basic motif at proteolytic processing sites. In the case of Newcastle Disease Virus (NDV), pathogenicity is correlated in a similar fashion to that of Influenza HA (Gorman *et al.*, 1988; Morrison, 2003), thus it is expected that other type I viral glycoproteins require proteolytic processing for activation of the membrane fusion function. An exception to this appears to be the Ebola virus GP_0 precursor, where studies have demonstrated that mutation of the basic cleavage motif does not abrogate glycoprotein function (Neumann *et al.*, 2002; Wool-Lewis and Bates, 1999).

All HIV and SIV Envs have a processing site and the effects of processing are clearly demonstrable for HIV, uncleaved forms of gp160 are able to mediate aggregation of $CD4^+$ cells, but syncytium formation is not observed (Moulard and Decroly, 2000). Processing of gp160 to the oligomeric gp120/41 occurs within the Golgi, concomitant with maturation of the glycoprotein with respect to oligosaccharide trimming and extension by α-glucosidases. Sensitivity of gp160 and gp120 to the glycosidase EndoH indicates that the processing event occurs in the *cis-medial* Golgi where sialylation occurs. The acidity of the processing environment is also a contentious issue, although it is known that the *trans*-Golgi network (TGN), secretion vesicles and the lysosomes are acidic compartments, whereas the optimal pH for some proteases are in the neutral range (Stein and Engleman, 1990). The use of different Env expression methods could well have clouded any certainty as to the compartment in which processing occurs, and the absence of CD4 and the MHC-like human leukocyte antigens (HLA) host proteins and other viral proteins such as Rev, Nef and Vpu may not accurately reflect the exact events of gp160 maturation in a virally infected cell.

The presence of a cleavage motif does not guarantee processing. For natural isolates, the variation in the use of target cells and ability to mediate syncytia formation *in vitro* is attributed to the considerable variation in the Env sequence. Adams *et al.* studied the natural variation around the cleavage site of Envs derived from primary HIV-1 isolates with varying cytopathogenicity (Adams *et al.*, 2000). The majority of variations were observed in the N-terminal fusion peptide (FP) of gp41, flanked by the REKR and dual FLG motifs; certain mutations in the FP blocked gp160 processing and reduced the association of the subunits.

1.3.6 Downregulation of CD4

For successful HIV release from infected $CD4^+$ cells, downregulation of CD4 is necessary to prevent interactions with nascent Env within the cell and at sites of virion assembly. Nef mediates the downregulation of surface CD4 via endocytosis from the cell surface using the clathrin machinery and sorting into the degradative pathways (Piguet *et al.*, 1999; reviewed in Janvier *et al.*, 2000). In the ER, phosphorylated HIV-1 Vpu interacts with Env-CD4 complexes to direct the degradation of CD4 in the proteasome (Fujita *et al.*, 1997; Schubert and Strebel, 1994).

1.3.7 Structural Protein Assembly

The Gag and Gag-Pol precursor proteins are responsible for establishing the structural framework of the virion (Reviewed in Turner and Summers, 1999), encapsidation of the genome, acquisition of the viral envelope and incorporation of viral proteins as the virion assembles at the plasma membrane (Ono *et al.*, 2000), or the late endosomes of primary macrophages (Pelchen-Matthews *et al.*, 2003). Although conclusive evidence remains lacking, many studies suggest Env-MA interactions in assembly (Cosson, 1996; Hammonds *et al.*, 2003; Hourioux *et al.*, 2000). In support of this are observations that Influenza HA and HIV-1 Env exhibit a preference for accumulation at lipid raft domains where virion budding occurs (Rousso *et al.*, 2000; Takeda *et al.*, 2003), and glycoprotein expression in polarised cells dictates basolateral budding in the systems of Marburg virus, HIV-1, HIV-2 and SIV (Ball *et al.*, 1997; Lodge *et al.*, 1997; Sanger *et al.*, 2001). Oligomerisation of the Vpu N-terminal transmembrane domain regulates virion release that may involve the formation of an ion channel (Schubert *et al.*, 1996; Schubert and Strebel, 1994). The PTAP motif located within the HIV-1 p6 domain, and also in Ebola VP40, is involved in virus-cell separation by recruitment of the tumour susceptibility gene 101 (Tsg101) to the site of virion assembly (Demirov *et al.*, 2002; Luban, 2001; Martin-Serrano *et al.*, 2001). Tsg101 is a part of the endosomal protein sorting complex (ESCRT-1) that traffics ubiquitinated proteins between the *trans* Golgi, plasma membrane and endosome, thus, p6 is associated with ubiquitin. The p6 product also specifically incorporates Vpr into virions (Lu *et al.*, 1995).

1.3.8 RNA packaging

The Ψ packaging sequence near the 5' end of the vRNA (Figure 1.6) and other sequences in adjacent regions modulate interactions with the NC domain of Gag to package the vRNA into the virion (Lawrence *et al.*, 2003; reviewed in Turner and Summers, 1999). Packaging of the vRNA is coincident with the dimerisation of vRNA via the dimerisation initiation sequence (DIS) found on the first stem loop of the Ψ site (Figure 1.6). Host $tRNA^{lys}$ is also incorporated into virions to serve as a primer for reverse transcription.

1.3.9 Maturation

The $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ precursors must be proteolytically processed by the active protease, PR during or after virion release from the host cell. Processing of the $Pr160^{Gag\text{-}Pol}$ precursor is necessary to produce PR, the heterodimeric RT, and IN. The RT undergoes additional cleavage to produce the p66 and p51 subunits (Reviewed in Turner

and Summers, 1999). The $Pr55^{Gag}$ precursor is cleaved into its constituent products MA, CA, NC and p6. The protease-induced maturation process allows the condensation of CA to form a cylindrical or conical core in lentiviruses (von Schwedler *et al.*, 1998).

1.4 Immunological Response to HIV-1 Infection

1.4.1 Role of Antigen Presenting cells

For HIV-1 to disseminate in the host tissues following sexual transmission, it must be able to traffic from the mucosal surfaces into the target cell-rich lymphoid compartment. Macrophages and immature DCs associated with mucosal surfaces are presumed to be the first cells to encounter sexually transmitted HIV-1 (Zaitseva *et al.*, 1997). DCs are professional APCs, following antigen uptake, DCs migrate to the lymph nodes where they interact with $CD4^+$ and $CD8^+$ T lymphocytes. Studies have identified a role for the surface expressed lectin, the DC-specific intercellular adhesion molecule (ICAM) grabbing non-integrin (DC-SIGN), in the transmission of HIV-1 from immature DCs and certain monocyte subsets to T cell populations (Kwon *et al.*, 2002; Tsunetsugu-Yokota *et al.*, 1997; Vanham *et al.*, 2000). DC-SIGN interacts with ICAM-2 and ICAM-3 expressed on T cells and endothelial cells respectively, and is able to specifically bind HIV-1 gp120 with higher affinity via the carbohydrate recognition domain (Cambi *et al.*, 2004; Su *et al.*, 2004).

On immature DCs, DC-SIGN is located in discrete microdomains associated with lipid rafts on the cell surface (Cambi *et al.*, 2004). The clustering of DC-SIGN, and possibly its organisation into higher ordered oligomers may enhance binding of virions (Su *et al.*, 2004). Following DC-SIGN binding and internalisation, virions accumulate in early endosomes (Kwon *et al.*, 2002). DC-SIGN binding does not induce conformational change in the viral glycoprotein, and so they can remain in the DC until transmission to T lymphocytes (Kwon *et al.*, 2002; Nobile *et al.*, 2003). Although DCs express low levels of CD4, CCR5 and CXCR4 and they are capable of binding and internalising both R5 and X4 viruses, only R5 viruses are able to replicate to a low degree in DCs. Infection of DCs is not necessary for transmission to T lymphocytes, but it is associated with Nef-mediated upregulated surface expression of DC-SIGN (Sol-Foulon *et al.*, 2002), enhancing contact with ICAM-2 expressed on the surface of $CD4^+$ T cells. This can result in the formation of an immunological synapse, and exocytosis of internalised virions in a regulated manner at these junctures would release a concentrated amount of virions onto susceptible cells (McDonald *et al.*, 2003; Sol-Foulon *et al.*, 2002). Thus the

antigen presenting ability of the DCs can lead to the establishment of HIV-1 infection of $CD4^+$ T lymphocytes.

A similar mechanism of HIV transfer to $CD4^+$ T lymphocytes is proposed to occur with macrophages that are susceptible to HIV-1 infection. It has been demonstrated in MDM that assembly of HIV occurs in late endosomes and multi-vesicular bodies as opposed to the plasma membrane (Pelchen-Matthews *et al.*, 2003). The accumulation of virions into MHC-II compartments may allow regulated secretion of a large number of particles upon macrophage interactions with $CD4^+$ T lymphocytes. Thus, it appears that HIV-1 has taken advantage of the antigen presenting machinery of APCs for translocation into the lymphoid compartments and subsequent transmission to susceptible activated target cells.

1.4.2 Non-Specific Control of HIV-1 Infection

The initial viremia is controlled by the host immune system and the viral load is reduced to a low level for an indeterminate period of time, as the infected individual enters the asymptomatic phase (Reviewed in Hoffman *et al.*, 2003; Levy, 2001). The innate immune response may contribute to the control of HIV-1 following initial infection. The production of soluble antiviral factors such as interferon and tumour necrosis factor α (TNF-α) may directly modulate HIV-1 replication, chemokines can attract natural killer cells, macrophages and T lymphocytes to the site of infection, and cytokine profiles can directly affect the development of subsequent cell-mediated (T helper 1 -Th1) or humoral (Th2) adaptive immune responses.

1.4.3 HIV-Specific Cellular Immune Response

Cell mediated immunity is dependent on $CD4^+$ T_H cells and is sensitive to a dominant IL-2 cytokine profile. $CD8^+$ T lymphocytes play an important role in controlling HIV-1 infected cells through cytolytic responses (CTL) and a non-cytotoxic antiviral response (NCAR). While HIV-specific $CD8^+$ T lymphocytes are present in all HIV-1 infected patients to varying degrees, studies suggest they are not significantly different between long-term non-progressor (LTNP) and slow progressor patients (Migueles *et al.*, 2002). In LTNP patients, $CD8^+$ T lymphocytes exhibit a greater proliferative response, coupled with perforin expression, to autologous infected $CD4^+$ T lymphocytes. This specific response is independent of plasma viral load, suggesting that a diminished proliferative ability in patients progressing to disease may not be recovered through highly active

anti-retroviral therapy (HAART) to reduce viral load (Migueles *et al.*, 2002). Interestingly, productive infection of $CD8^+$ T lymphocytes has been noted from some virus isolates in a co-receptor independent manner (Saha *et al.*, 2001), the ability of Vpr to induce cell-cycle arrest may be a contributing factor to the reduced proliferative ability of infected $CD8^+$ T lymphocytes in progressors.

In NCAR, HIV replication is blocked even at low levels of $CD8^+$ T lymphocytes. HIV suppressive factors produced by stimulated $CD8^+$ T lymphocytes from HIV-1 infected individuals include the chemokines RANTES, MIP-1α and MIP-1β as well as another soluble cellular antiviral factor (CAF). In some uninfected/non-progressing individuals, the relative resistance of $CD4^+CCR5^+$ T lymphocytes to infection is due in part to competitive inhibition by endogenous β-chemokines (Vyakarnam *et al.*, 2001). Stromal cell-derived factor (SDF)-1α production along mucosal membranes can be extensive, coincident with CXCR4 down-regulation. Although this is supportive of the important role of $CD8^+$ T lymphocytes in controlling HIV replication *in vivo*, production of these chemoattractants are likely to provide new targets of stimulated $CD4^+$ cells for HIV infection (Cocchi *et al.*, 1995). Efficient suppression of HIV-1 replication by direct $CD8^+$ T cell contact and by CAF has been documented (Chang *et al.*, 2003; Chun *et al.*, 2001; reviewed in Vella and Daniels, 2003), however, the continued presence of HIV in the plasma shows that cell-mediated immunity is insufficient for absolute control.

1.4.4 HIV-Specific Humoral Response

The surface expression of Env makes it a target for the host Th2-type humoral response, mediated by B cells. The dominant immunogenic sites of gp120 are the principal neutralising domain on the V3 loop and the cluster I, principle immunogenic domain (PID) near the cysteine residues on the gp41 ectodomain. Epitopes from these domains for a variety of HIV-1 subtypes are recognised by antisera from HIV-1 infected patients, suggesting common structural organisation (Nyambi *et al.*, 2000). Effective antibodies can be categorised as neutralising, cytotoxic, or enhancing (Subbramanian *et al.*, 2002). Neutralising antibodies directly inactivate the virus in the presence or absence of complement (C-NA and NA respectively) by direct binding of a specific epitope, however, very few neutralising antibodies exhibit cross-subtype activity. The broad reactivity of the 2G12 neutralising antibody is attributable to its unique dimeric structure. This allows recognition of N-linked glycans in close proximity on the gp120 silent face (Calarese *et al.*, 2003). Another neutralising antibody 2F5 is able to recognise

a conformation dependent epitope on gp41 that is lost upon CD4 and CKR binding (Barbato *et al.*, 2003).

In the face of neutralising antibodies, the dissemination of HIV-1 can occur by other avenues. Cell-cell transmission is the major route of dissemination of HTLV-1, as infected cells produce very few virions. Upon contact with a susceptible cell, the surface expressed Env is able to signal polarisation of HTLV-1 vRNA, Gag and Env to the cell-cell junction via microtubules and transfer the genome into the susceptible cell (Igakura *et al.*, 2003). In the case of HIV-1, *in vitro* cell-cell transmission is also more successful than cell-free virus (Dimitrov *et al.*, 1993).

Cytotoxic antibodies eliminate virus and infected cells through the process of antibody dependent cellular cytotoxicity (ADCC), mediated by natural killer (NK) cells, monocytes and PBMCs, or complement dependent cytolysis (CDC) following complement activation (Hezareh *et al.*, 2001). Soluble Env can induce cytolysis of uninfected cells by binding to surface CD4; this may represent a mechanism for $CD4^+$ cell depletion during the asymptomatic phase of HIV-1 infection. ADCC clearance of infected cells has also been correlated to the viral protein Nef (Yamada *et al.*, 2004).

Complement dependent- and independent-infection enhancing antibodies (C-IEA and IEA respectively) facilitate binding of opsonised viral particles to Fc receptor bearing cells, thereby allowing infection if appropriate receptors are expressed on the cell surface. A longitudinal study of autologous Abs following acute HIV-1 infection found no correlation between the development of NAs and resolution of viremia, although anti-Env antibodies able to recognise autologous and heterologous antigens were detected very early on following acute infection (Aasa-Chapman *et al.*, 2004). Other studies found the early appearance of C-IEA showed strong positive correlation with plasma viral load (Szabo *et al.*, 1999), and total antibody titres were lowest in patients with less than 200 $CD4^+$ cells/μl (Subbramanian *et al.*, 2002), suggesting an important role of antibodies in the control of HIV.

Efficient control of HIV-1 following infection thus requires all arms of the immune system acting in concert to control infection mediated by viral particles and by cell-cell transmission.

1.5 Viral Glycoproteins

1.5.1 Role in Virus Lifecycle

The major role of viral glycoproteins is to target the virion to an appropriate cell that supports replication and further dissemination of the virus. As such, the glycoproteins on the surfaces of enveloped viruses are an essential component, as without penetration of the host cell, replication cannot take place (Peisajovich and Shai, 2003). The extensive structural studies of the Influenza A virus HA glycoprotein make it a model structure for the study of other type-I viral fusion glycoproteins (Figure 1.7). The metastable pre-fusion conformation of HA is stabilised by the major α-helices of HA2 via the formation of a triple-stranded coiled coil, mediated by van der Waals contacts between non-polar residues at the N-terminus of the helices and electrostatic interactions between the polar and charged residues at the C-terminus. Recent studies of Dengue virus and Semliki Forest virus glycoproteins are beginning to shed light on the mechanisms of action of the type-II viral fusion proteins (Gibbons *et al.*, 2004; Modis *et al.*, 2004).

1.5.2 SU/gp120 Structure

The SU/gp120 subunit can be delineated into conserved and variable domains (Figure 1.8) (Reviewed in (Clapham and McKnight, 2002; Douglas *et al.*, 1997). The conserved domains play an important role in determining the conformation of the gp120 core structure, as shown by the co-crystallisation of extensively deglycosylated monomeric HIV-1_{HXBc2} and HIV-1_{YU2} (Subtype B, X4- and R5-tropic respectively) gp120 core structures with CD4 domains D1D2 and the Fab fragment of the neutralising MAb 17b (Figure 1.9) (Kwong *et al.*, 2000; Kwong *et al.*, 1998). The major architecture of gp120 consists of an inner and an outer domain connected by a bridging sheet (Figure 1.10) (Kwong *et al.*, 2000; Wyatt *et al.*, 1998), the resultant structure is representative of a non-native, CD4-induced (CD4i) structure (Kwong *et al.*, 1998). Omitted from the core structures were terminal residues from the C1 and C5 domains, and much of the V1/V2 and V3 loops. Residues in C1 and C5 are important for association with the gp41 subunit, and indeed both domains appear to extend away from the core towards the viral membrane (Kwong *et al.*, 2000; Kwong *et al.*, 1998; Yang *et al.*, 2003).

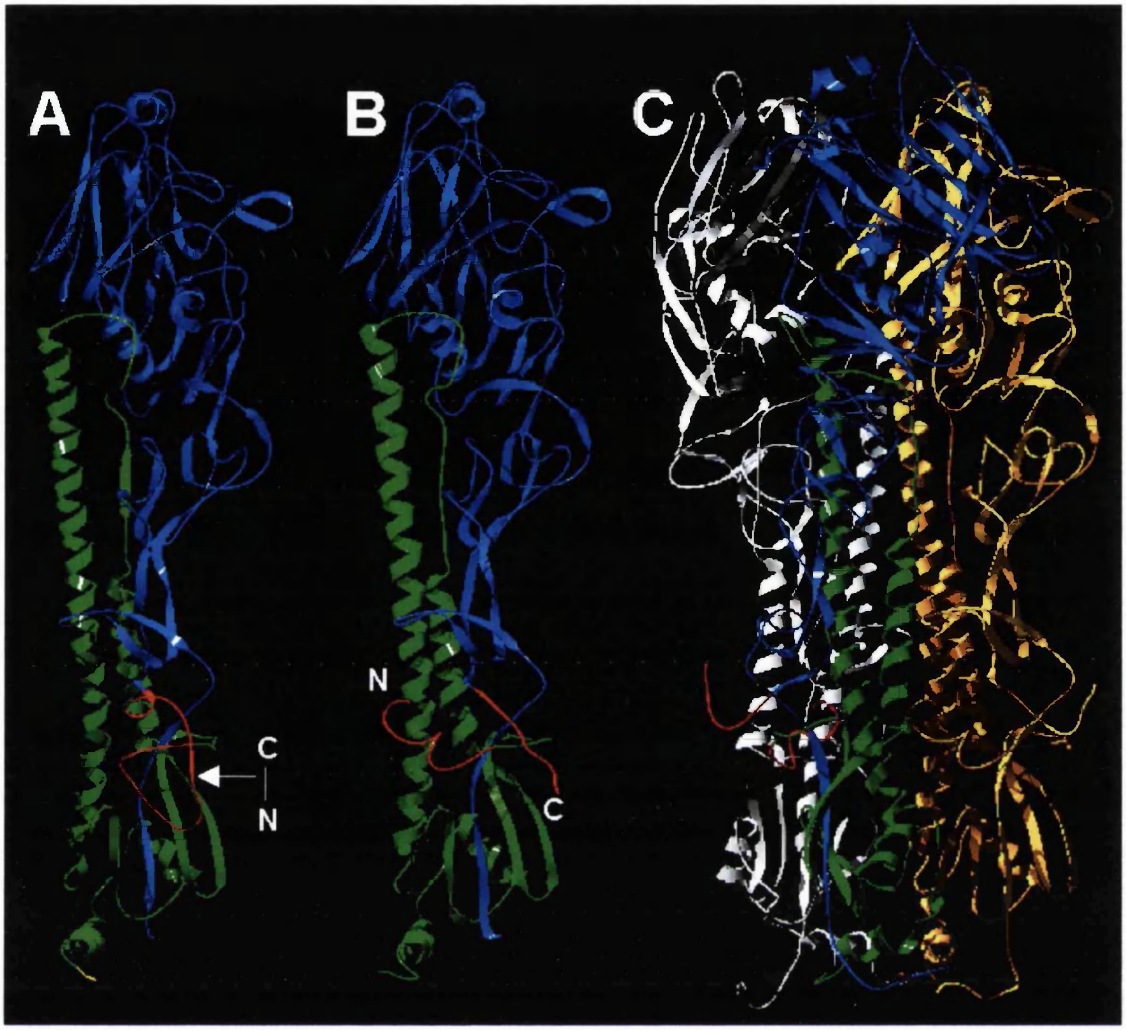


Figure 1.7. Exposure of the Influenza A X31 HA0 cleavage loop.

Monomeric HA0 with the mutated cleavage loop (R→Q substitution) constituting residues 323-329 of HA1, and 1-12 of HA2, the HA1 and HA2 subunits are coloured accordingly (A). The C-terminus of HA1 and N-terminus of HA2 are indicated. Following processing, rearrangement of the C-terminal residues of HA1 and the N-terminal residues of HA2 occurs (B). Trimeric projection of cleaved HA (C). Glycans were omitted from these structures. For further details see (Chen *et al.*, 1998; Wilson *et al.*, 1981).

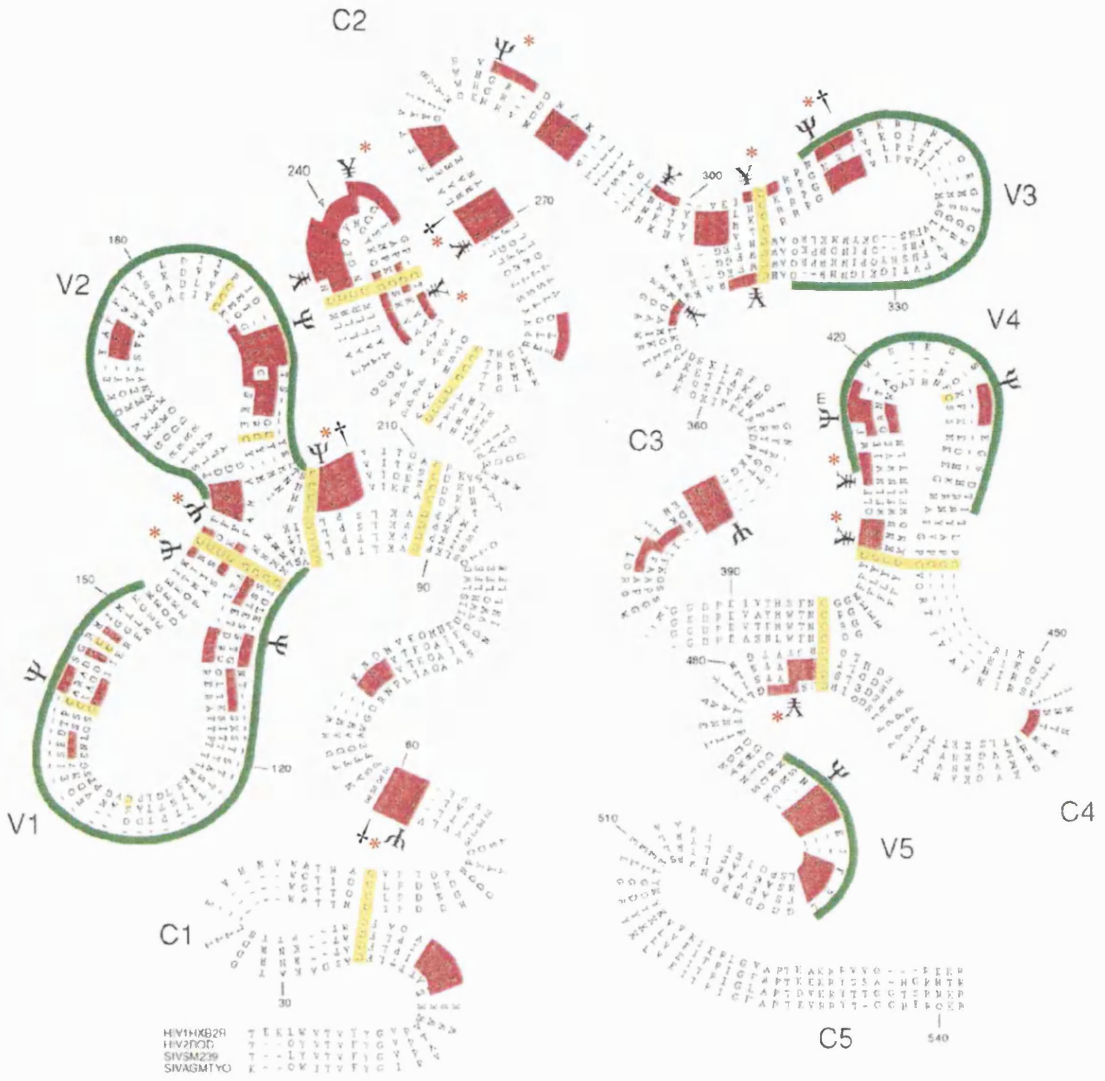


Figure 1.8. Schematic representation of gp120 constant and variable domains.

The sequences of HIV-1_{HXB2R}, HIV-2_{ROD} and SIV_{SM239} and SIV_{agmTYO} isolates are aligned. Variable domains are indicated by green N-linked glycosylation sequons are highlighted in red (psi), and conserved Cys residues are highlighted in yellow. Complex type (ψ) and high mannose and/or hybrid types of oligosaccharides are indicated (¥). Highly conserved sequons across HIV-1 isolates (*) and between HIV and SIV (†) are indicated accordingly. Adapted from (Douglas *et al.*, 1997).

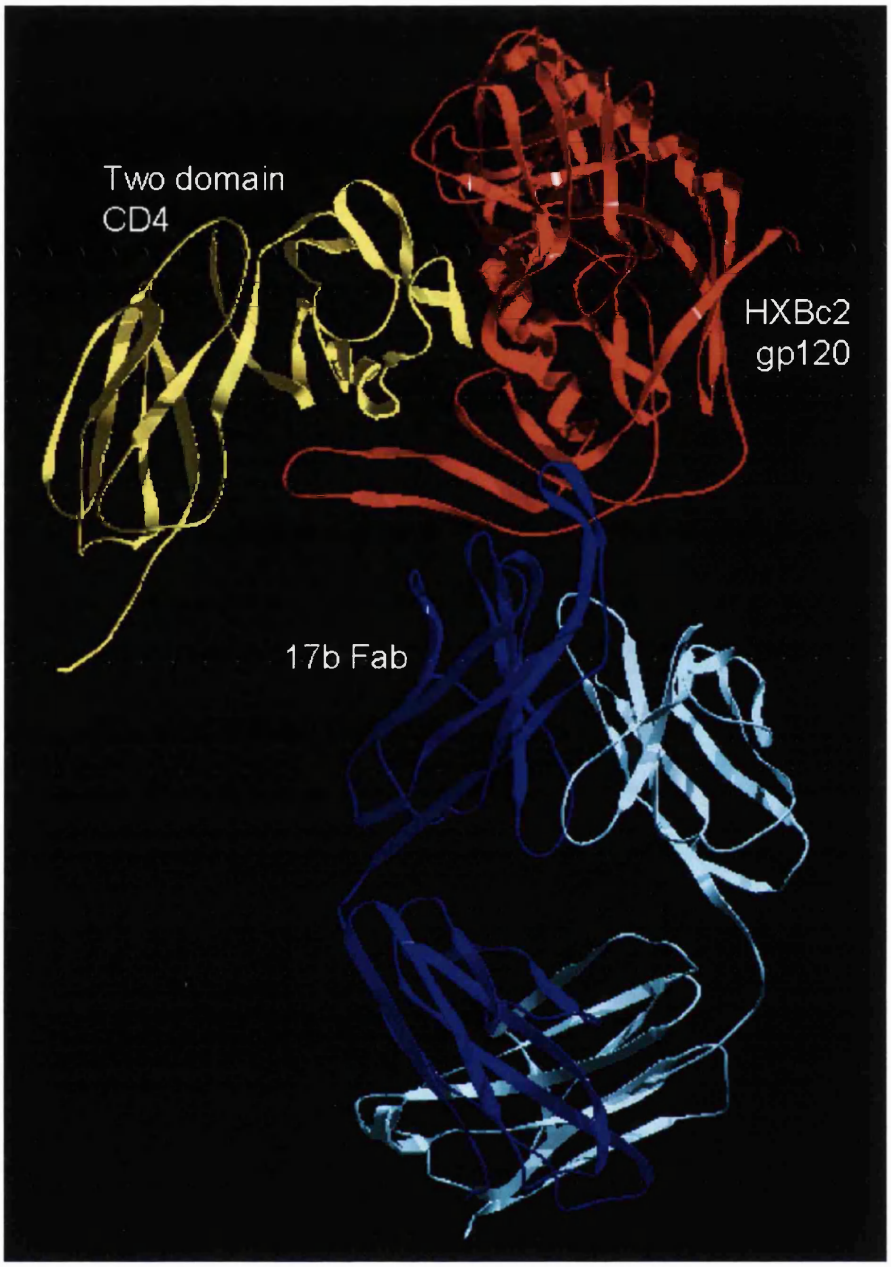


Figure 1.9. X-ray crystal structure of HIV-1_{HXBc2} gp120 core.

The gp120 core is complexed with a two domain soluble CD4 and the Fab fragment of the MAb 17b as determined by Kwong (Kwong *et al.*, 1998). Much of the V1/V2 and V3 loop was deleted from the gp120 construct, and the V4 domain lacked sufficient stability for its structure to be determined. In this representation, the gp120 core is closest to the viral membrane and the 17b Fab fragment is closest to the host cell membrane. For additional details see (Wyatt *et al.*, 1998).

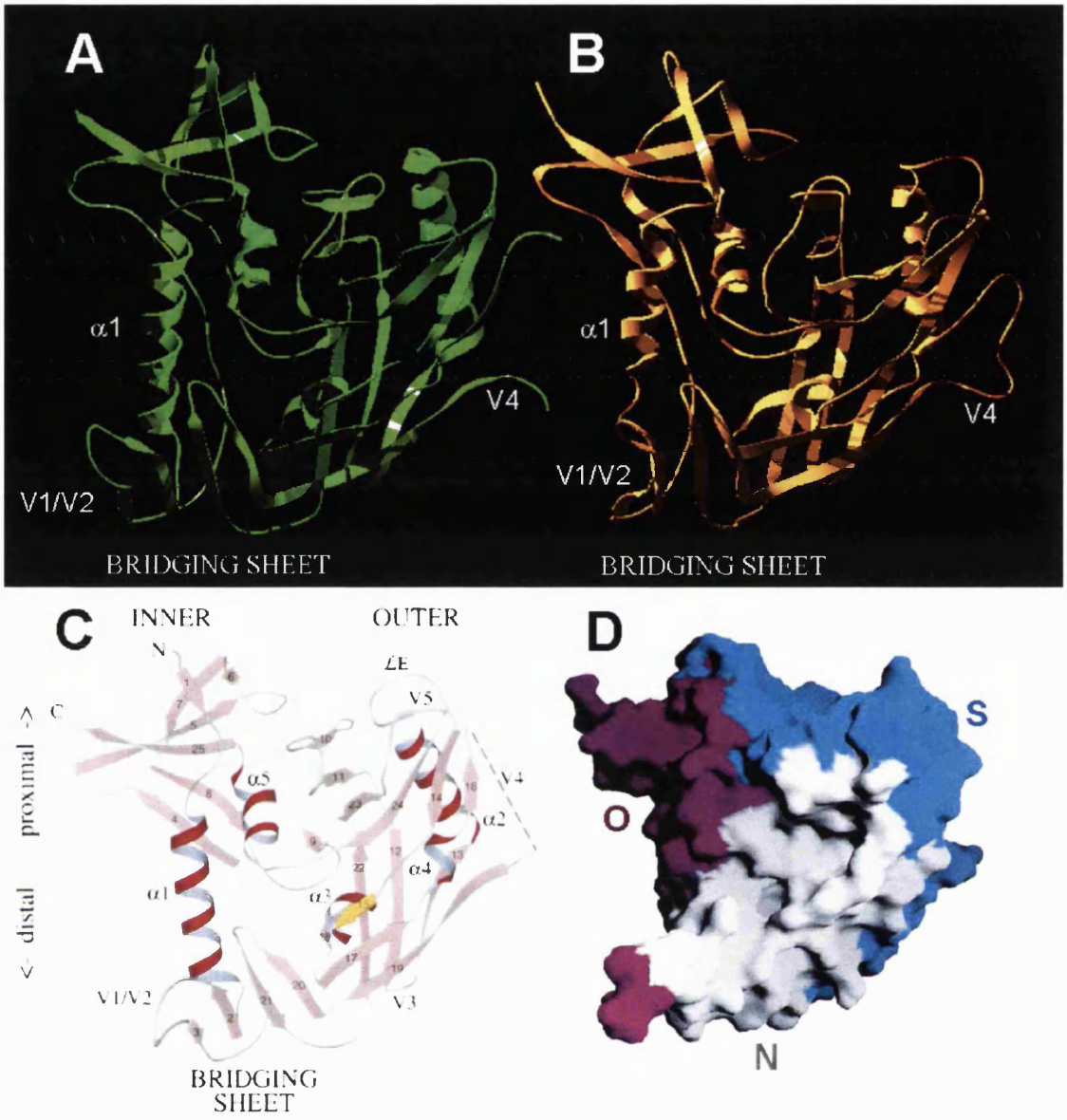


Figure 1.10. Comparison of gp120 core structure.

The gp120 cores from the X4-tropic HXBc2 (A) and the R5-tropic YU2 (B) strains are similar despite different tropisms, as determined by Kwong (Kwong *et al.*, 2000). Differences in the α1 helix length, the bridging sheet orientation and the resolution of the V4 loop are apparent in the YU2 structure. The inner and outer domains and the bridging sheet of HXBc2 gp120 are indicated in (C), as are the positions of the variable loops. The immunologically silent (S), neutralising (N) and non-neutralising (O) faces are indicated in (D) as coloured. Figures C and D were adapted from (Kwong *et al.*, 1998). See references for further details.

In the native gp160, the C5 region terminates in the charged processing site. It is conceivable that this conformation may expose the processing site structure on the oligomeric surface in a similar manner to that of Influenza HA0 (Figure 1.7) (Chen *et al.*, 1998), and indeed, antisera from HIV-1 exposed patients are able to recognise peptides corresponding to this domain (Brown *et al.*, 1999; Chang *et al.*, 2000). The highly conserved gp120 core structures observed between HXBc2 and YU2 (Figure 1.10), despite different tropisms and sensitivity to neutralisation, indicate that the variable loops play a greater role in determination of phenotype and resistance to neutralisation (Kwong *et al.*, 2000). The V1/V2 domain is a target for neutralising Abs; local glycosylation can affect efficient recognition of epitopes, whereas insertions/deletions and point mutations can affect CD4 binding, highlighting the importance of this domain in gp120 function (Fox et al., 1997; Kolchinsky, Kiprilov, Bartley et al., 2001; Sullivan et al., 1993; Wu et al., 1995). SI isolates have greater positive charge than NSI in this region, and can also dictate tropism and cytopathicity independently of the V3 domain (Palmer *et al.*, 1996). Due to the conformational changes induced by CD4/CKR binding, changes in the V1/V2 can affect membrane fusion.

HIV-1 V3 alterations can result in changes to tropism, infectivity and growth kinetics. SI variants are associated with higher net positive charge in V3 in certain subtypes (De Jong *et al.*, 1992). The V4 and V5 domains are also surface exposed and glycosylated, however, few neutralising antibodies target these HIV-1 regions suggesting little function in these domains associated with receptor binding or membrane fusion (Simmonds *et al.*, 1990). In SIV studies, neutralising epitopes involving the V3 and V4 are conformation-dependent (Cole *et al.*, 2001; Glamann *et al.*, 1998).

1.5.3 Oligomerisation

The accepted organisation of functional HIV-1 Env is a homotrimer. The overall trimeric structure of Env projects the neutralising face containing the CKR binding site at the tip of the trimer towards the target cell membrane (Figure 1.9) (Wyatt *et al.*, 1998).

The immunologically 'silent' face approximates to the outer domain, the highly glycosylated and persistent variation in sequences constituting this exposed surface enable evasion of neutralising antibodies and occlusion of critical domains (Figure 1.10). The non-neutralising face is poorly accessible on trimeric forms and elicits non-neutralising antibodies. This region equates to the inner domain and is buried in the trimeric state (Schulke *et al.*, 2002; Wyatt *et al.*, 1998). Studies of HIV-1 and Rous Sarcoma Virus suggest conformational changes could be directly transmitted to other subunits of the oligomer in a cooperative manner (Damico *et al.*, 1998; Salzwedel and Berger, 2000). Oligomerisation is mediated by the gp41 subunit, however, there are conflicting studies whether the leucine zipper like domain (LZL) is involved (Chen, 1994; Chen et al., 1993; Wild, Dubay et al., 1994).

1.5.4 TM/gp41 Ectodomain

The HIV-1 gp41 is non-covalently associated with the gp120 subunit after proteolytic processing during maturation. The gp41 ectodomain contains the fusion peptide, the leucine zipper-like domain (LZL) and an assembly domain (Figure 1.11A). The fusion peptide of HIV-1 carries a double FLG motif, whereas only one motif is apparent in HIV-2 and SIV_{sm} isolates (Douglas *et al.*, 1997). Changes that affect the hydrophobicity of this motif are not well tolerated, and substitutions that increase hydrophobicity can increase syncytium formation (Douglas *et al.*, 1997). The highly conserved nature of the LZL domain indicates its important role in membrane fusion (Bernstein et al., 1995; Cao et al., 1993; Chen, 1994; Chen et al., 1993; Dubay, Roberts, Brody et al., 1992; Wild, Dubay et al., 1994). The principle immunogenic domain (PID), adjacent to HR1, contains a disulphide-bonded loop featuring a CX_2K/RX_2C motif (Earl *et al.*, 1997; Merat *et al.*, 1999). Intersubtype variation is observed across the HR1 and the PID sequences, however, this region affects oligomerisation and association with the gp120 subunit; thus only conservative variation in and around the PID is tolerated (Douglas *et al.*, 1997; Earl *et al.*, 1997; Maerz *et al.*, 2001; Merat *et al.*, 1999). Downstream of the PID motif is the assembly domain. Well-conserved N-glycosylation sequons are present near the PID and in the assembly domain, and O-linked glycosylation is likely to occur, these glycans affect the function and immunogenicity of the glycoprotein (Dash *et al.*, 1994; Fenouillet *et al.*, 1993; Fenouillet and Jones, 1995; Perrin *et al.*, 1998). The ELDKWAS neutralising epitope for MAb 2F5 is also localised to the ectodomain region proximal to the membrane spanning domain (MSD) (Figure 1.11A) (Barbato *et al.*, 2003). The TM subunit is anchored to the virus/cell membrane via the MSD. The

glycoproteins of complex lentiviruses possess an exceptionally large cytoplasmic tail of around 150 residues compared to the short tails of simple retroviruses and other enveloped viruses. The native structure of metastable gp41 is as yet unknown, although antigenic mapping of oligomeric Env indicate the principle exposed epitopes of gp41 correspond to the PID, residues constituting the assembly domain C-helix, and the ELDKWA epitope recognised by the neutralising antibody 2F5, while the fusion peptide is likely to be occluded by gp120 associations (Earl *et al.*, 1997).

1.5.5 TM/gp41 Structural Studies

Sequences in the LZL and assembly domain exhibit a propensity to form amphipathic helices (N- and C-helices or heptad repeats, HR, respectively) like Influenza HA2. Helical wheel projections predict interactions between residues in successive layers on the N-helices at a and d positions on the threefold axis of symmetry (Lu *et al.*, 2001; Wang *et al.*, 2002), the C-helices pack in an anti-parallel manner into the hydrophobic grooves formed between the N-helices, interacting via a and d residues with residues at the e and g positions of the N-helix (Figure 1.11B). The resultant six-helix bundle structure is confirmed by X-ray crystallographic and NMR studies on SIV (Caffrey *et al.*, 1998; Malashkevich *et al.*, 1998; Yang *et al.*, 1999) and HIV-1 (Chan *et al.*, 1997; Tan *et al.*, 1997; Weissenhorn *et al.*, 1997) peptides derived from these domains (Figure 1.12). Non-conservative substitution of residues at a and d positions affect glycoprotein function through destabilisation of the trimer, whilst mutations at the e and g positions affect packing of the C-helices into the hydrophobic grooves (Caffrey *et al.*, 1998; Chan *et al.*, 1997; Malashkevich *et al.*, 1998; Tan *et al.*, 1997; Weissenhorn *et al.*, 1997; Yang *et al.*, 1999). As such, without knowledge of the native structure, the models do not invoke the spring-loaded mechanism of fusion peptide insertion as determined for Influenza HA2.

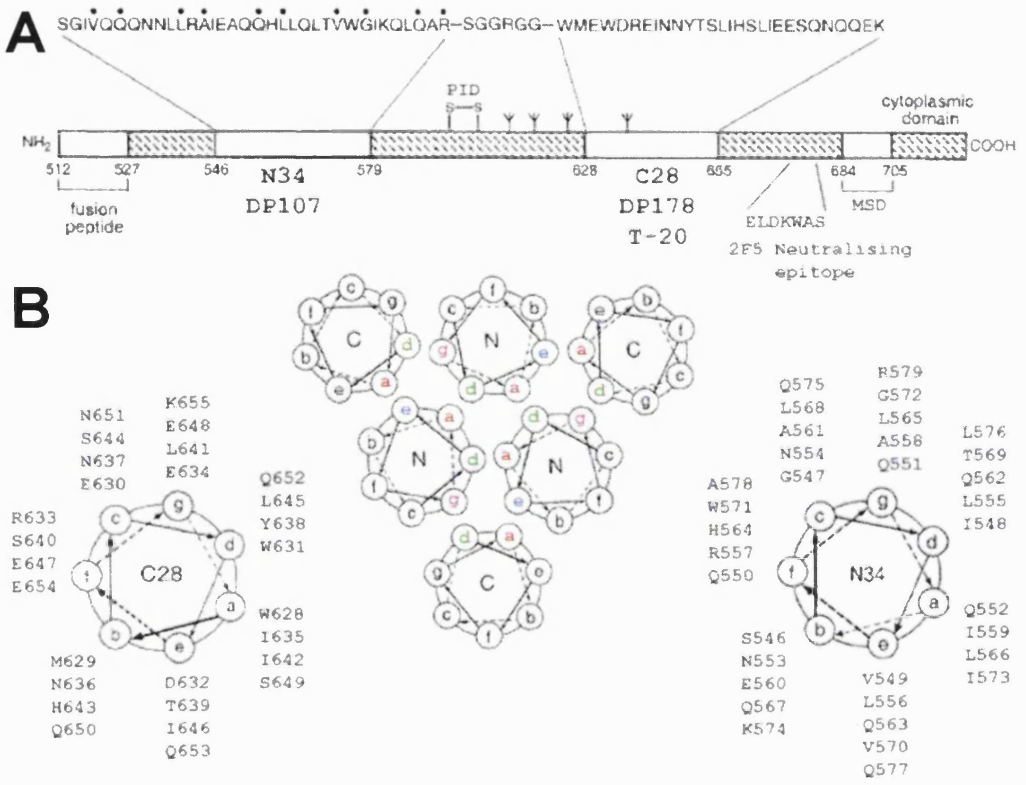


Figure 1.11. Alignment of the HIV-1_{HXBc2} gp41 N- and C-helices according to helical wheel projections.

The sequences comprising discrete domains of the gp41 ectodomain, such as the fusion peptide, N34 and C28 helices (DP107 and DP178/T-20 respectively), the principal immunogenic domain (PID), membrane spanning anchor (MSD), 2F5 neutralising epitope and glycosylation sites (ψ) are indicated (A). The alignment of residues comprising the N- and C-helices according to a helical wheel projection indicates that hydrophobic interactions occur at buried positions a and d between the N-helices, whereas residues at e and g positions interact with residues a and d of the outer helices (B). Adapted from (Lu *et al.*, 2001; Wang *et al.*, 2002)

Figure 1.12. Comparison of the structures adopted by HIV and SIV peptides derived from TM ectodomains.

Peptides corresponding to the N- and C-helices of gp41 associated with a short linker (A) or with the GCN4 (B) are homologous to corresponding peptides from SIV, which show a propensity to associate as six-helix bundles as determined by NMR (C) or X-ray crystallography (D), consistent with helical wheel predictions. Further details are available from references (Malashkevich *et al.*, 1998; Tan *et al.*, 1997; Weissenhorn *et al.*, 1997; Yang *et al.*, 1999).

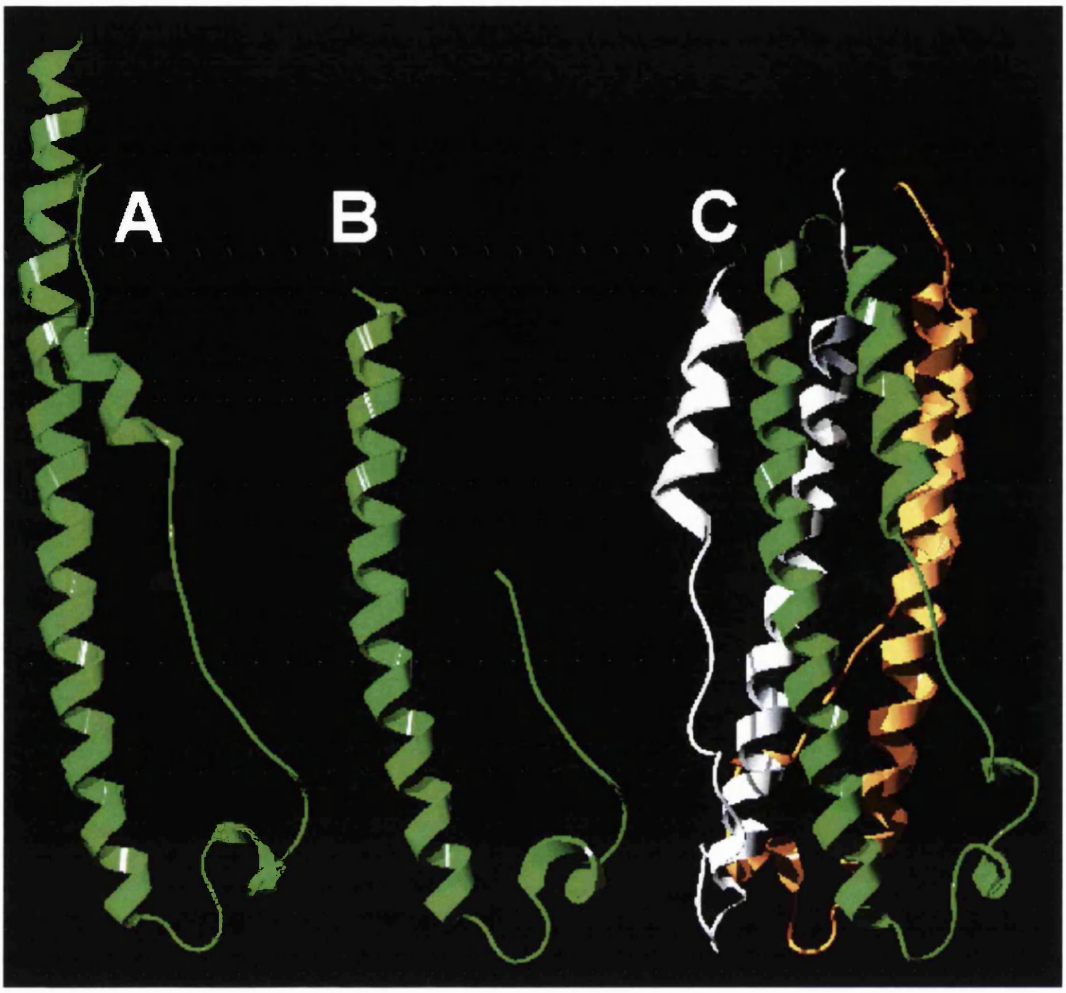


Figure 1.13. TM structures of other viruses.

Illustration of structures adopted by HTLV-1 (A), MoMLV (B) and Ebola (C) peptides derived from TM ectodomains. Ebola TM peptides show a propensity to associate as a six-helix bundle similar to SIV. The similarity of monomeric HTLV-1 (A) and MoMLV TM ectodomain peptides suggest homologous structures in these systems are highly likely. Further details can be obtained from these references (Fass *et al.*, 1996; Kobe *et al.*, 1999; Malashkevich *et al.*, 1999).

Homologous structures are observed using synthetic peptides corresponding to the TM subunits of Moloney murine leukaemia virus (Mo-MuLV) (Fass *et al.*, 1996), Ebola (Malashkevich *et al.*, 1999) and HTLV-1 (Kobe *et al.*, 1999), in the case of HA2, Mo-MLV, Ebola and HTLV-1, the C-terminal residues form a short six-helix bundle with the N-helices at the base of the structure, followed by an extended and disordered chain (Figures 1.13-14). Overall the structures suggest evolution of a common mechanism for membrane fusion in type-1 viral fusion proteins. The six-helix bundle of the TM represents a stable post-fusion structure, in all cases, anti-parallel packing of the C-helices against the N-helices results in the transmembrane anchor and fusion peptide being positioned at the same ends of the molecule.

1.6 Interactions Leading to Membrane Fusion

Upon infection, Influenza virus is endocytosed following binding of sialic acid on the cell surface. The ensuing reduction in pH in the endosome induces a conformational change in the HA molecule from the native state to a 'fusion active' state that is responsible for fusing the viral and endosome membranes. Structure of a trypsin/thermolysin-treated soluble fragment of HA (Bullough *et al.*, 1994) consisting of HA1 (residues 1-27) and HA2 (residues 38-175) at pH5.0 indicated a major conformational change in the HA2 subunit (Figure 1.14). Residues at the N-terminus of HA2, usually a loop structure, rearrange into α-helix at low pH to extend the existing α-helix, projecting the fusion peptide towards the endosome membrane. Meanwhile, a conformational change in the HA2 C-terminus induces movement of these α-helices away from the viral membrane and towards the endosome membrane, the resultant change in length of HA2 is from 75Å to 110Å. For pH-independent viruses, such as HIV and SIV, interaction of Env with receptors is the trigger for conformational change to induce membrane fusion (Damico *et al.*, 1998; McClure *et al.*, 1988).

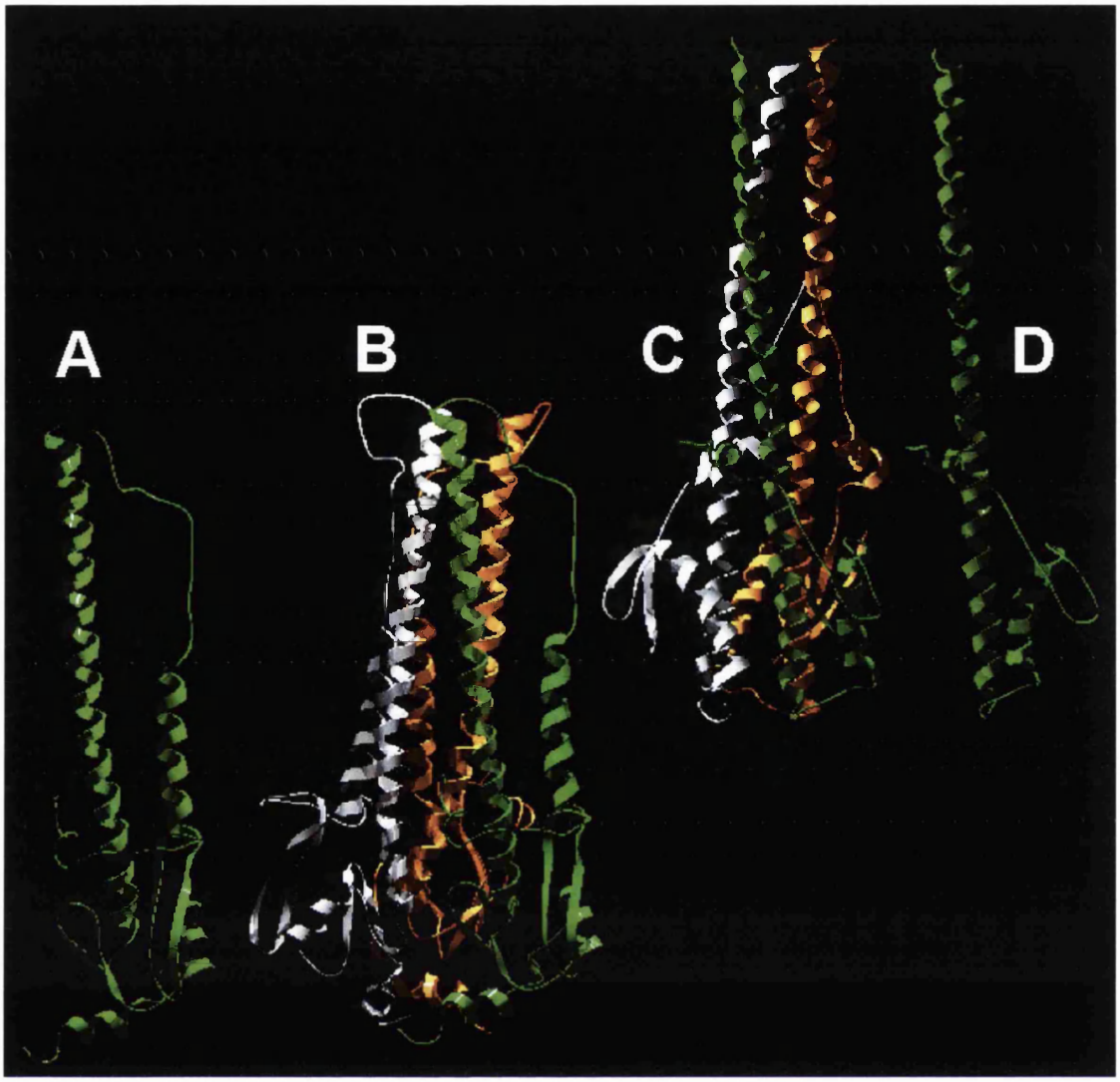


Figure 1.14. Comparison of Influenza virus HA2 native and low-pH structures.
The native monomeric (A) and trimeric (B) HA2 structures are associated by means of an internal triple stranded coiled-coil. The fusion active low-pH conformation is shown as a trimer (C) or monomer (D), the internal triple coiled-coil is extended towards the target membrane (top). Structures are reviewed in detail in (Bullough *et al.*, 1994).

1.6.1 Initial Adsorption of HIV to Cells

Many *in vitro* studies suggest that the initial adsorption of HIV-1 to cells involves Env interaction with surface proteoglycans such as heparan sulphate (HS) or chondroitin sulphate (CS), as removal of these proteoglycans can inihibit HIV-1 infection (Argyris et al., 2003; Bobardt et al., 2004; Herold et al., 1997; Patel et al., 1993). This adsorption is likely to be mediated by ionic interactions between sulphated proteoglycans and charged Env domains such as the V3 loop. Adsorbed particles would be more likely to encounter CD4 and CKRs on the target cell surface. However, some studies with CHO cell lines deficient in surface proteoglycans suggest some primary isolates do not require this initial adsorption to facilitate infection (Zhang et al., 2002). The importance of proteoglycans in the infection process of HIV-1 and other sexually transmitted pathogens has been targeted in developing therapeutic compounds to block transmission, whereby HS analogues were able to block infection (Herold et al., 1997). Primary cultures of human genital epithelial cells have been shown to sequester HIV-1 particles using HS, and successfully transmit virus to permissive cells in subsequent co-culture; this may represent a pathway to capture sexually transmitted HIV-1 and to infect underlying dendritic cells or infiltrating cells via cell-cell contact (Dezzutti et al., 2001; Wu et al., 2003). Studies also indicate that transcytosis of HIV-1 across the blood brain barrier is dependent on proteoglycans as opposed to CD4 or CKRs, as following transmigration virions remain infectious (Argyris et al., 2003; Bobardt et al., 2004).

1.6.2 gp120 Interactions with CD4

The gp120 subunit first binds to domain 1 of CD4, the most distal from the target cell membrane (Esser *et al.*, 2000). Different Envs are likely to possess different binding affinities for CD4 due to variation in the amino acid sequences surrounding the binding site causing local structural alterations (Bannert *et al.*, 2000). The CD4 binding site on gp120 is a recessed pocket between the inner and outer domains, and the bridging sheet.

Although the recessed nature of the CD4 binding pocket will provide a protective mechanism against neutralisation, the V1/V2 loop may occlude both CD4 and CKR binding sites in the native state (Fox *et al.*, 1997; Kwong *et al.*, 1998; Wyatt *et al.*, 1998). Twenty-two CD4 residues (mainly spanning residues 25-64) and twenty-six gp120 residues are involved in direct interactions, crucial interactions are mediated between CD4 residues Phe43 and Arg59, and gp120 residues Asp368, Glu370 and Trp427. The conservation of hydrophobic residues surrounding the binding pocket

highlights the functional nature of this region (Kwong *et al.*, 1998). The conformational change following CD4 binding stabilises gp120, particularly with regard to the bridging sheet and other CD4i epitopes. Contact between CD4 and the V2 stem is consistent with the notion of CD4-induced repositioning of the V1/V2 loop and exposure of the CKR binding site (Jones *et al.*, 1998). The flexibility of the four-domain CD4 is required to bring gp120 closer to the cell surface to interact with a specific CKR (Kwong *et al.*, 1998). During exposure of the CKR binding site, the neutralising face will also be exposed but it may be protected from antibody binding by steric hindrance due to the close proximity to the target cell membrane (Kwong *et al.*, 1998; Wyatt *et al.*, 1998).

CD4 can undertake conformational changes in the flexible region between D2 and D3 and between D4 and the transmembrane anchor. For HIV-1 infection it is necessary to orientate the gp120 CKR binding domain towards the cell surface where co-receptors lie. MAbs to the D2-3 hinge can inhibit HIV infection (Healey *et al.*, 1990), and perturbations in the D4 membrane flexible region can affect gp120 V3 loop exposure and subsequent infection (Moir *et al.*, 1996).

The integrity of the cellular microfilament network must be maintained for membrane fusion, suggesting mobility of the membrane domain or of the Env-CD4 complexes is required (Frey *et al.*, 1995), the latter is likely to enable movement of the complexes to interact with co-receptors. Studies suggest multiple CCR5 molecules act in a cooperative manner to facilitate virus infection (Kuhmann *et al.*, 2000), as trace levels of CCR5 on the cell surface is sufficient to permit virus infection.

1.6.3 gp120 Interactions with CKRs

Following CD4-binding, a co-receptor binding site is exposed on gp120 through conformational changes in the orientation of the V1/V2, V3 loops and the bridging sheet (Abrahamyan *et al.*, 2003; Cormier *et al.*, 2001). CKR binding involves gp120 residues Lys121, Arg419, Lys421 and Gln422 that also constitute the CD4i 17b epitope on the bridging sheet, thus the 17b Fab fragment reflects CKR binding to some degree (Kwong *et al.*, 1998). Other residues in the gp120 C1, C2, C3 and C4 domains have been identified as participants in binding CCR5 regions distinct from the N-terminus; this may stabilise the gp120-CD4-CCR5 complex prior to triggering conformational changes required for membrane fusion (Cormier *et al.*, 2001).

Interaction of gp120 with CKRs involves a two-step interaction with the CKR N-terminal domain and ECL-I and ECL-II domains (Cormier *et al.*, 2001; Doranz *et al.*, 1997; Kuhmann *et al.*, 2000). The gp120 binding site of the CCR5 N-ter domain has been delineated to residues 10-18 (Cormier *et al.*, 2001). Charged residues on CCR5 such as Asp11, Glu18, Lys197 and Asp276 are likely to interact with the gp120 V3 loop (Doranz *et al.*, 1997). Two N-linked glycosylation sites are observed in CXCR4, and one in CCR5 (Chabot *et al.*, 2000). N-linked glycosylation of the CCR5 N-ter domain adversely affects infection by R5 variants (Kuhmann *et al.*, 2000); instead, sulfation of tyrosines and O-linked glycosylation of the N-ter domain is required for usage by R5-tropic strains (Chabot *et al.*, 2000; Cormier *et al.*, 2001). Structural modelling of CCR5 (model based upon bovine rhodopsin) to fit the HXBc2 (X4-tropic) gp120 core supports an initial interaction between the bridging sheet and the N-terminus of CCR5, then conformational change in CCR5 may permit a subsequent interaction of ECL-II with the base region of the gp120 V3 loop, suggesting a two-step interaction between gp120 and CCR5 involving electrostatic interactions (Liu *et al.*, 2003). The sulfation of CXCR4 and the glycosylation at Asn11 is important for specific gp120 binding of X4-tropic strains, removal of this glycan enabled usage by R5 primary isolates of clades A, B and B/F (Chabot *et al.*, 2000). It is possible that variations in expression of CXCR4 glycoforms may drive the switch to CXCR4 usage, moreover monomeric and dimeric forms of CXCR4 were observed. Disruption of the disulphide bond between CXCR4 N-ter and ECL-III also enabled usage by R5-tropic variants. These observations suggest that CCR5 and CXCR4 share structural homology, but have different post-translational modifications (Chabot *et al.*, 2000).

1.6.4 gp120 Interaction with Protein Disulphide Isomerase

Following engagement of CD4, the conformational change in gp120 results from disulphide bond exchange catalyzed by the enzyme protein disulphide isomerase (PDI). PDI normally traffics in the ER and possesses chaperone functions to assist protein folding and disulphide bond formation, however, the activity of PDI in HIV-1 infection is specifically associated with the target cell surface membrane. Inhibition of the thiol/disulphide exchange activity of PDI on the target cell surface, using specific inhibitors or MAbs, can prevent HIV-1 Env-mediated membrane fusion and virus infection (Fenouillet et al., 2001; Gallina et al., 2002; Ryser et al., 1994). Studies suggest two disulphide bonds in gp120 are cleaved post-CD4 and CXCR4 binding (Barbouche et al., 2003). Whilst co-immunoprecipitation experiments suggest an association between CD4 and PDI molecules in lymphocytes, only marginal co-

localisation between these molecules are observed by immunohistochemistry studies in the absence of gp120 and CKRs (Fenouillet et al., 2001; Markovic et al., 2004). Thus, the role of PDI in HIV-1 Env mediated fusion is likely to destabilise gp120 through disulphide bond exchange, transducing a signal to the gp41 subunit to undergo conformational change to adopt the six-helix bundle conformation (Markovic et al., 2004).

1.6.5 Membrane Fusion

Following CD4 and CKR binding, gp120 transmits conformation changes to the gp41 subunit, resulting in the insertion of the fusion peptide into the host cell membrane (Finnegan *et al.*, 2002; reviewed in Gallo *et al.*, 2003). The gp120 subunit dissociates to enable the transition of gp41 to the stable six-helix bundle, bringing the transmembrane anchor and fusion peptide to the same end of the molecule to induce fusion pore formation (Figure 1.15) (de Rosny *et al.*, 2001). Stabilisation and enlargement of the pore then allows delivery of the viral capsid into the cell. The conformational change in HIV-1 gp41 is dependent on the presence of glycosphingolipids (GSLs) in the target membrane. GSL-rich domains on the membrane surface may organise the accumulation of CD4, CXCR4 and CCR5 molecules, while secondary interactions between the polar head-groups of GSL molecules may play a role in the dissociation of gp120 and exposure of hydrophobic domains to induce membrane fusion (Hug *et al.*, 2000).

Stabilisation of the chain reversal in forming the six-helix bundle is likely to be a key feature of the transition from the pre-fusion to post-fusion conformation (Figure 1.15C-F). The chain reversal motif of HTLV-1 is CX_4EXCCF (Single amino acid code, where X_n denotes any amino acids) and disulphide-bonding between the first and second Cys residues forms the most stable structures (Kobe *et al.*, 1999). The Glu residue interacts with an upstream basic residue in the N-helix. For HIV and SIV, the basic residue in their CX_2K/RX_2C motif within the PID may interact with the E residue in a corresponding upstream position. In the full-length gp41 it is expected that the C-terminal residues between the C-helix and the MSD would be flexible (Figures 1.11-12). This is supported by the loss of the highly conserved epitope recognised by the cross-reactive neutralising MAb 2F5 in the post-fusion conformation (Barbato *et al.*, 2003; Gorny and Zolla-Pazner, 2000). The extent of membrane fusion is also influenced by the degree of glycosylation of the gp41 ectodomain at a cluster of residues near the PID, the presence of these glycans may aid the transition to a favourable conformation of the gp41 to induce membrane fusion (Perrin *et al.*, 1998).

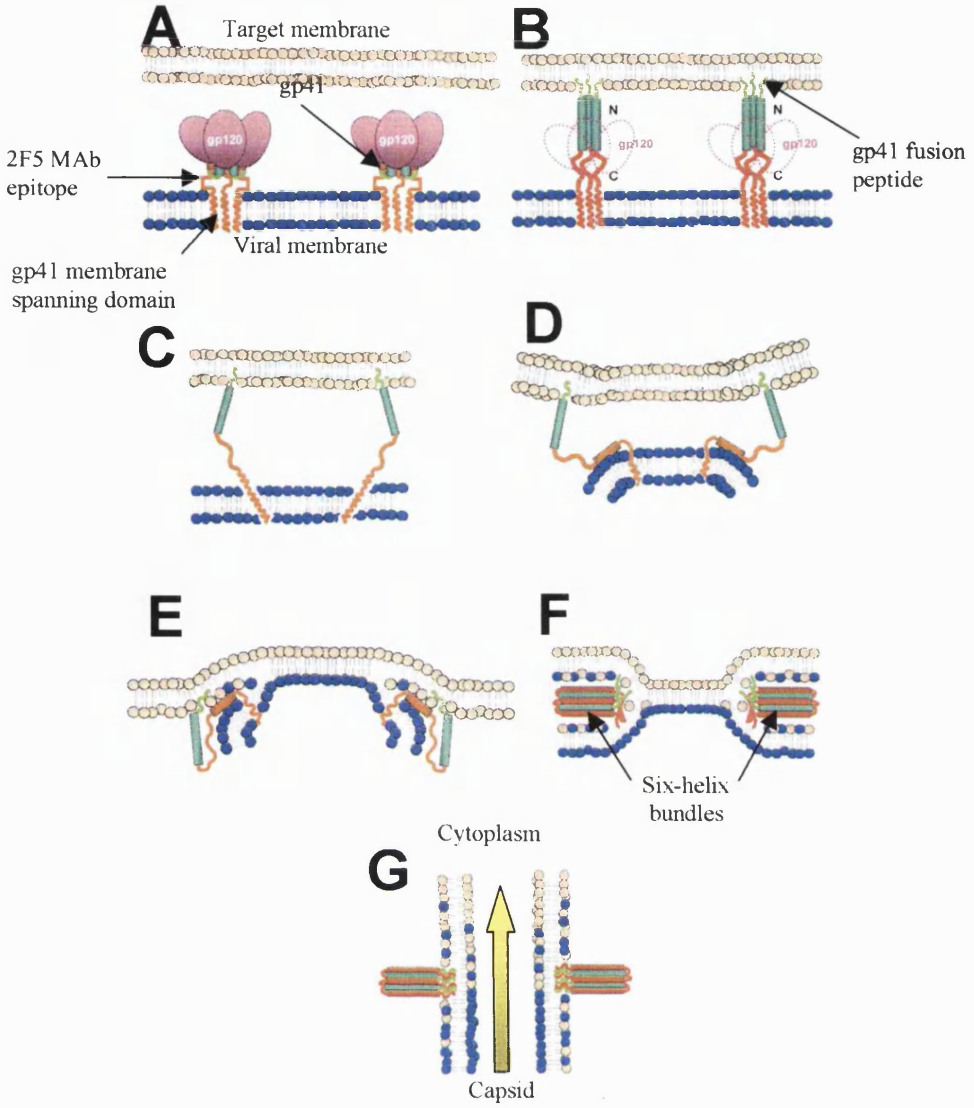


Figure 1.15. Proposed mechanism of gp41-induced membrane fusion.

Native Env in oligomeric form on the viral surface membrane (A) interacts with CD4 and CKR to induce a conformational change in gp120. This is transmitted to gp41, leading to insertion of the fusion peptide into the target cell membrane (B). Following the dissociation of gp120, conformational change in each of the gp41 subunits of the oligomer brings the membrane spanning domain into close proximity with the fusion peptide (C-E, illustrated as a monomer for clarity), assisted by the flexible 2F5 MAb conformational epitope near the membrane spanning domain. Hemifusion and pore formation occurs with the formation of the six-helix bundle (F), enlargement of the fusion pore allows delivery of the viral capsid into the cell (G). Adapted from Barbato (Barbato *et al.*, 2003).

1.6.6 Inhibition of Membrane fusion

The formation of the six-helix bundle is essential for gp41-induced membrane fusion. N- and C-peptides (DP107 and DP178 respectively) derived from the respective gp41 HR regions exhibit potent anti-viral activity (Chan et al., 1998; Jiang et al., 1993; Wild et al., 1993; Wild, Shugars et al., 1994; reviewed in Chen *et al.*, 2002; Wild *et al.*, 1992). Biological and structural studies confirm the inhibitory action of these peptides via competitive binding in the pre-six-helix bundle conformation, preventing the formation of the stable structure (Chan *et al.*, 1997; Malashkevich *et al.*, 1998). The potency of the C-peptides are related to the ability of hydrophobic residues such as Trp628, Trp631 and Ile635 to insert into the large hydrophobic cavity formed in the coiled coil (Chan *et al.*, 1998). The activity of the peptides is dependent on the α-helical structure, as scrambled peptides have little effect (Jin *et al.*, 2000; Wild *et al.*, 1993). The highly conserved hydrophobic residues required for the formation of the six-helix bundle structure explains the broad range of activity against homologous and heterologous HIV-1 isolates, whilst much higher concentrations were required to block HIV-2 membrane fusion (Jiang et al., 1993; Wild, Shugars et al., 1994). It was also observed that a higher concentration of peptide was required to block virus infection than cell-cell fusion (Wild, Shugars et al., 1994). Similar peptides derived from the HR sequences of human parainfluenza virus F protein have similar activity, again highlighting a common mechanism of membrane fusion among unrelated viruses (Yao and Compans, 1996).

The effectiveness of the fusion inhibitors is inversely correlated to the affinity of primary isolate Envs for co-receptors, as the gp41 transitional conformation may only exist for a short time in Envs with high affinities for co-receptors, although studies suggest there is a 15min window for blocking following receptor interactions (Gallo *et al.*, 2001). Small molecule inhibitors have been demonstrated to directly block CXCR4 and CCR5 interaction with HIV-1 Envs (Dragic et al., 2000; Schols, Este et al., 1997; Schols, Struyf et al., 1997), thereby preventing the CKR-induced conformational change preceding membrane fusion. Increased affinity for CKRs (CCR5) can reduce sensitivity of primary isolates to these antagonists (Gorry *et al.*, 2002). Primary resistance to DP178, associated with G→S and V→M mutations in the highly conserved GIV motif of HR1 (1.11A), is yet to be detected in long-term infected or HAART-treated patients (Zollner *et al.*, 2001).

1.7 Role of Envelopes in Pathogenesis

1.7.1 Structural-Function Relationship

The viral glycoprotein is thus essential for the viral lifecycle; however, the surface exposure of the glycoprotein also renders it a target for the host immune response. HIV and Influenza continually change the nature of exposed epitopes. The extensive database of immunological epitopes at the Los Alamos National Laboratory (www.hiv.lanl.gov) is testament to the ability of HIV-1 to evade immunological control through substitutions, insertion/deletions and alteration in glycosylation of the surface glycoprotein. However, determinants of pathogenesis are closely correlated to structural features of viral glycoproteins. For Influenza the susceptibility of HA0 precursor to proteolytic processing at the HA1-HA2 juncture is directly correlated to the pathogenicity of the strain, although other gene products do have the potential to modulate pathogenicity (Ohuchi *et al.*, 1991; Walker and Kawaoka, 1993; Webster and Rott, 1987; reviewed in Steinhauer, 1999). With pH-independent virus like HIV and SIV, binding of the cellular receptor(s) is the primary trigger for membrane fusion at the cell surface. It is thus expected that variations in Env domains affecting structure and functionality will be limited *in vivo* in order to preserve replication competence.

HIV-1 *env*-genes derived from other subtypes indicate variation from prototypic subtype B strains with regard to cysteine residues, N-linked glycans, length polymorphisms in the hypervariable regions and premature truncations in gp41 (Gao *et al.*, 1996; Penny *et al.*, 1996). Notably, insertions and deletions in the HIV-1 gp120 V2 domain affect the accessibility of the CD4 binding site and neutralisation sensitivity (Fox *et al.*, 1997). An example of this in the MLV model is the common G100R substitution in its SU that results in enhanced receptor binding, leading to increased internalisation and viral titre (Lu *et al.*, 2003). Substitution of conserved residues involved in maintaining the gp120-gp41 association on the viral surface, resulting in increased gp120 shedding can affect membrane fusion and viral entry (Yang *et al.*, 2003). Additionally, substitutions of gp120 residues involved in transmitting signals of conformation change to the gp41 subunit, such as I225 and T244 (HIV-1_{HXBc2} numbering) can also affect membrane fusion (Yang *et al.*, 2003). Increasing the α-helical nature of the fusion peptide of Simian Virus 5 (Paramyxoviridae) can increase the rate of membrane fusion (Bagai and Lamb, 1997). Neutralisation escape mutants exhibiting changes in HIV-1 gp41 LZL domain and regions affecting gp120-gp41

association can result in increased efficiency of fusion, leading to enhanced infectivity (Park *et al.*, 2000).

1.7.2 Phenotypic Switch

HIV-1 produced in an infected individual has an estimated half-life of less than 3 days. An estimated 10^9 viral particles may be produced and eliminated daily from a HIV-1 infected individual, the majority of virions being produced from recently infected cells as opposed to long-lived or latently infected cells (Ho *et al.*, 1995; Perelson *et al.*, 1996; Wei *et al.*, 1995). The error-prone reverse transcription mechanism results in the production of a mixed population of related but distinct viral quasispecies. While a population equilibrium may be established within the spectrum of quasispecies, continual immune clearance results in selection for competitively dominant variants at any given time, this is most apparent in the plasma compartment (Wei *et al.*, 1995). The arisal of beneficial mutations or introduction of antiretroviral therapy can allow a variant constituting a relatively minor population to suddenly become the dominant strain (Ho *et al.*, 1995; Wei *et al.*, 1995). During the course of infection a heterogeneous population is observed and can lead to the establishment of dominant SI strains in 50% infected individuals. (Connor *et al.*, 1993; Connor *et al.*, 1997; Koot *et al.*, 1992; van Rij *et al.*, 2000; van 't Wout *et al.*, 1998). The presence of 2 distinct gp120 binding sites on CCR5 suggests a mechanism for the co-receptor switch as mutations accumulate in Env during immune selection. A single additional N-glycosylation identified in the V1/V2 loop in a primary isolate from a LTNP has been associated with acquisition of a dual-tropic phenotype (Jekle *et al.*, 2002). Dual-tropic Envs are sensitive to the post-translational modifications of the CCR5 N-terminus (See section 1.6.2), whilst modifications of ECL-I and ECL-II of CXCR4 affect usage by X4 viruses. The gp120 residues that restrict interactions to specific chemokine receptors are principally located on the V3 loop, as direct substitution of the V3 loop is sufficient to alter co-receptor tropism (Bagnarelli *et al.*, 2003; Choe *et al.*, 1996; Connor *et al.*, 1997; Polzer *et al.*, 2002), however, other studies suggest efficient interactions are likely to involve all variable domains (Cho *et al.*, 1998). The SI phenotype is associated with positively charged residues in the V3 region, which may aid interaction with the negatively charged CXCR4 receptor (Kuiken *et al.*, 1992; van Rij *et al.*, 2000; Wang *et al.*, 2000). Some V3 sequences are unable to facilitate infection when inserted into a constant gp120 backbone, suggesting additional residues in other domains of gp120 are required for co-receptor interaction (Bagnarelli *et al.*, 2003). The presence of a complex glycan on the V3 loop is an important determinant for CCR5 usage. The loss of this glycan

enhances infection by dual-tropic and X4-tropic variants, and is associated with increased susceptibility for neutralisation, however, there may be a lack of neutralising antibodies during the late stages of disease (Cormier *et al.*, 2001; Li *et al.*, 2001; Polzer *et al.*, 2002).

SI/X4-tropic variants emerging from $CXCR4^+$ $CCR5^+$ $CD45RO^+$ $CD4^+$ and $CXCR4^+$ $CD45RO^+$ $CD4^+$ T lymphocytes are able to adapt to a new reservoir of naïve $CD45RA^+$ $CXCR4^+$ $CD4^+$ T lymphocytes, thereby avoiding competition with the large plasma population of NSI/R5-tropic variants for target cells (van Rij *et al.*, 2000). This adaptation enables NSI and SI variants to occupy distinct niches in $CD4^+$ T lymphocyte populations and provides an explanation for the persistence of R5 variants throughout the course of disease, although HIV-1 populations in non-lymphoid tissue are predominantly associated with cells of the monocyte/macrophage lineage (Wang *et al.*, 2001). The adaptation to X4-tropism may occur at the expense of viral fitness, hence dual-tropic variants exhibit lower virulence, and may explain why the switch is not observed in all patients progressing to AIDS (de Roda Husman *et al.*, 1999; Koot *et al.*, 1996; van Rij *et al.*, 2000; van 't Wout *et al.*, 1998). SI variants exhibit faster replication kinetics and increased cell killing; these features appear prior to or concurrent with CD4 cell depletion and correlate with disease progression (Connor *et al.*, 1993; Koot *et al.*, 1996; van 't Wout *et al.*, 1998).

1.7.3 CD4-independent Infection

As AIDS manifests, the loss of immunologic control allows further dissemination of HIV-1, HIV-2 and SIV in host tissues through the arisal of variants capable of using other CKRs in *in vitro* studies (Reviewed in Clapham and McKnight, 2002), although in general, alternative CKR usage is in addition to CCR5 or CXCR4. The dissemination of HIV-1 into other tissues may induce further pathologies, for example, infection of brain derived cells by brain-cell tropic $HIV\text{-}1_{GUN\text{-}1}$ using CCR8 may result in the development of AIDS dementia complex (Jinno *et al.*, 1998). In addition, it has been demonstrated that HIV-1 infection of $CD4^+/CXCR4^-$ cell lines could be facilitated by the transfer of CXCR4 to these cells by platelet- and megakaryocyte-derived microparticles (Rozmyslowicz *et al.*, 2003). The loss of N-linked glycosylation sites in the V1/V2 stem has been associated with CD4-independent infection through exposure of the CCR5 binding site, but this is likely to be strain dependent (Gorry et al., 2002; Kolchinsky, Kiprilov, Bartley et al., 2001; Kolchinsky, Kiprilov and Sodroski, 2001; Liu et al.,

2000). The glycolipid galactosyl ceramide mediates CD4-independent entry by certain HIV-1 isolates (Harouse *et al.*, 1991).

In cell-cell fusion assays using HIV-2_{ROD} Env, other chemokine receptors may be used in a CD4-dependent manner, whereas for virus infection, CXCR4 was primarily used, and CCR3 to a lesser extent (Bron *et al.*, 1997). This suggests that: (a) the Env-receptor interactions are different between virus-cell and cell-cell membrane fusion; (b) structural homology exists between these chemokine receptors to support the diverse tropism observed; (c) cell-cell fusion may represent an alternative and more efficient mechanism of virus dissemination within the infected host.

Some HIV-2 and SIV isolates are able to infect cells using CKRs, such as CCR1, CCR2, CCR3, CCR4, CCR5, CXCR2 or CXCR4, independently of CD4 (Endres *et al.*, 1996; Liu *et al.*, 2000). Some mutations conferring CD4-independence upon HIV-2 Env are located in the V3 and C4 domains in SU, and upstream of the N-helix in the TM. These TM substitutions increase the sensitivity of Env to sCD4, suggesting reduced threshold energy is required to induce conformational change (Reeves and Schulz, 1997). Greater exposure of the CKR binding site also increases the neutralisation susceptibility of isolates (Gorry et al., 2002; Kolchinsky, Kiprilov and Sodroski, 2001; Reeves and Schulz, 1997). The absence of neutralising antibodies in late stages of infection or immunologically distinct compartments may encourage the emergence of CD4-independent isolates; this is particularly obvious in SIV models of infection (Ryzhova *et al.*, 2002).

1.8 Objectives

To date our laboratory has reported sequence analyses of HIV-1 *env*-genes from infected patients that provides epidemiological information with regard to quasispecies in infected individuals, transmission of variants between individuals and populations, and the evolution of variants within populations (Balfe et al., 1998; Breuer et al., 1995; Cavaco-Silva et al., 1997; Cavaco-Silva et al., 1998; Daniels et al., 2003; Daniels et al., 1991; Novelli et al., 2000; Novelli et al., 2002; Penny et al., 1996; Rice et al., 1999; Vella, Gregory et al., 1999; Vella, King et al., 1999; Vella et al., 2002; Wike et al., 1992; Zheng and Daniels, 2001). Whilst the Env DNA and amino acid sequences provide putative information with regard to tropism and neutralisation (Douglas *et al.*, 1997), investigations of the biological properties of the *env*-gene products in the context of virus replication and infectivity requires workers to clone *env*-genes into an

infectious cassette under containment 3 conditions (Zheng and Daniels, 2001). This process is time consuming and the number of samples that can be processed is limited by equipment and the expense of quantitative reagents. The aim of this study was to develop a rapid, inexpensive, and practical biological assay, complementary to existing studies, and capable of screening multiple samples using existing laboratory equipment. This thesis describes the development and validation of a cell-cell fusion assay capable of dissecting structural determinants of HIV-1 Env-mediated membrane fusion, and its complementation of existing methods for the study of the biological properties of primary isolate *env*-gene products derived from LTNP patients.

Chapter 2

Methodology

2.1 Materials and Reagents

2.1.1 Suppliers of Reagents

Details of the common laboratory equipment, reagents, consumables, plasmids, bacterial cells, mammalian cells and viruses used in this thesis can be found in the Appendix section A1.

Except where specified, all commercially available reagents used are analytical grade and obtained from the Sigma-Aldrich Company Ltd. (Poole, UK), or BDH Laboratory Supplies (Poole, UK).

The NIMR Media Services department produced in-house supplied reagents, and the composition of each reagent is indicated in Appendix A1. Where reagents were self-prepared, protocols were followed as detailed in (Sambrook *et al.*, 1989).

The manipulations of all microorganisms described in this study were conducted in appropriate containment conditions according to the codes of practice outlined by the NIMR Safety Committee.

2.2 Methodology

2.2.1 Construction of Mutant HIV-1 Envs

2.2.1.1 Processing Site Mutants

Processing site mutants were generated from a HIV-1_{NL43} *env* template in a pQ7 vector supplied by N. Zheng. The basic cleavage motifs in this Env are characterised by the amino acid sequence KAKRRVVQREKR, containing the site 1 and site 2 motifs as indicated by colour. The cleavage site mutations were generated by mutant oligonucleotide-primed polymerase chain reaction as outlined in figure 2.1 (Primer sequences detailed in Appendix A1.9.1). Products were amplified in a PTC-100 thermal cycler (MJ Research Inc.) under the following PCR conditions: 1x native *Pfu* polymerase buffer (Stratagene); 250µM each dNTP (1.0mM final, Pharmacia); 0.5µM each primer (Oswell); 500ng template DNA; 2.5U native *Pfu* polymerase (Stratagene) and made up to 50µl with sterile water. Template DNA was denatured at 95°C/90s followed by 20 cycles of: denaturation 95°C/90s; annealing 40°C/90s; extension 72°C/5min followed by a final extension step consisting of denaturation 95°C/90s; annealing 40°C/90s and extension at 72°C/10min and a 4°C hold. Products were electrophoresed on a 1% low melting point (LMP) agarose (SeaPlaque) Tris-acetate EDTA (TAE) gel at 90V/2h at 4°C. Products were visualised under a low power UV transilluminator, fragments of appropriate length were excised and purified using a gel purification kit (QIAGEN). Products were eluted in 30µl sterile water.

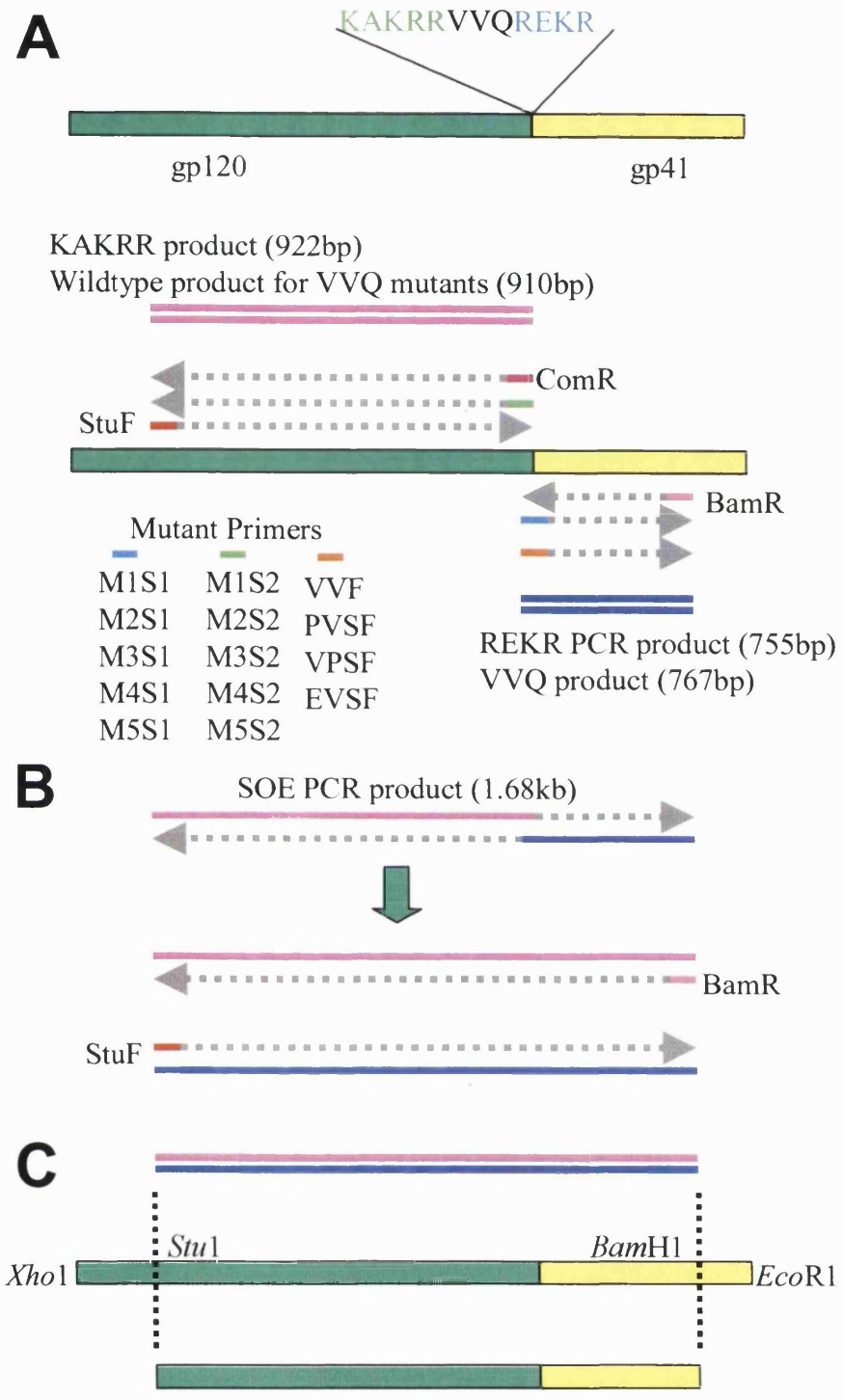


Figure 2.1. Construction of processing site mutants.

Mutant oligonucleotide-primed PCR generated 5' and 3' fragments using an $NL43_{WT}$ *env*-gene template (A). 5' and 3' PCR fragments were joined together by SOE PCR (B). Primers StuF and BamR were added to amplify the fragments. $NL43_{WT}$ sequences were replaced with the mutant subgenomic fragments using *Stu*1 and *Bam*H1 restriction sites (C). Full-length HIV-1 *env*-genes can be cloned into other vectors using *Xho*1 and *Eco*R1 restriction sites. Diagram not to scale.

Purified products were used to generate subgenomic 1.677kb mutant *env*s by splice - overlap extension (SOE) PCR in a PTC-100 thermal cycler under the following conditions: 1x native *Pfu* reaction buffer; 250µM each dNTP (1.0mM total); 5U native *Pfu* polymerase; 0.5µM each primer, 500ng template DNA and made up to 100µl volume with sterile water. Template DNA was denatured at 95°C/90s followed by 20 cycles of: denaturation 95°C/90s; annealing 39°C/90s; extension 72°C/10min. There was a final extension step of 72°C/10min and then products were held at 4°C. Full-length products of 1662bp were purified using phenol/ chloroform working solution and chloroform/isoamyl alcohol mix (ratio 24:1 v/v) according to standard methodology (Sambrook and Russell, 2001). PCR products were dissolved in 30µl sterile water and then digested with 25U *Bam*H1 and 25U *Stu*1 restriction enzymes (Roche) in 1x SuRE/Cut buffer B in 50µl volume at 37°C/1h. PCR products were gel purified as before. 5µg of pSP-*luc*$^+$.NL43 vector was digested with *Bam*H1 and *Stu*1, and dephosphorylated with 1U calf intestinal phosphatase (CIP, Roche) in 1x CIP buffer at 37°C/30min and then gel purified. PCR products were ligated into this digested pSP-*luc*$^+$NL43$_{WT}$ vector using a rapid ligation kit (Roche) according to the manufacturer's instructions in a 4:1 insert:vector ratio (as judged by product intensity on agarose gels) to generate the 5 mutants in each basic motif and 4 mutants in the VVQ motif of the processing site. Plasmids amplified by transformation into competent bacterial cells.

2.2.1.2 Cloning HIV-1 *env*-genes

Escherichia coli DH5α competent cells were transformed with plasmids containing HIV-1 *env*-genes by the heat-shock method (Sambrook *et al.*, 1989). Transformed cells were plated onto L-agar plates containing 100µg/ml each of Ampicillin and Nafcillin (Amp/Naf, Sigma-Aldrich) and incubated at 30°C/48h. 2ml L-broth (100µg/ml each of Amp/Naf) miniprep cultures were grown at 30°C/overnight in a G24 Environmental Incubator Shaker and processed using a Rapid Pure Miniprep kit (Qbiogene). Plasmids were eluted in 50µl sterile water and digested using 5U *Eco*R1 and 5U *Xho*1 enzymes (Roche) in 1x SuRE/Cut buffer H in 10µl volume at 37°C/1h. Products were electrophoresed on a 0.8% agarose tris-borate-EDTA (TBE) gel and analysed using an IS550 Kodak imager and 1D software on a PC. Frozen stocks (held at –80°C) were made of complete clones with 20% (v/v) glycerol. Midiprep cultures in 50ml volumes of L-broth (100µg/ml each of Amp/Naf) were grown at 30°C overnight and processed with a Midiprep kit (QIAGEN) according to manufacturer's instructions. Plasmids were reconstituted in 80-100µl sterile water and 0.5µl of each midiprep was digested as

before with 5U *EcoR*1 and 5U *Xho*1 in 10µl volume reactions to excise the 2.56kb *env*-fragment from the vector. The concentrations of complete plasmids were quantified by measuring absorbance at 260nm/280nm wavelengths using an Eppendorf Biophotometer, plasmids stocks were stored at –20°C.

2.2.1.3 Insertion into pQ7 Vector

5µg of each pSP-*luc*$^+$*env* construct, and also 5µg of pQ7.NL43$_{WT}$ was digested with 25U each of *Xho*1 and *Eco*R1 restriction enzymes in 1x SuRE/Cut buffer H in 50µl sterile water at 37°C/2h. The *env*-gene fragments from the pSP-*luc*$^+$*env* constructs were gel purified as before. The digested pQ7.NL43$_{WT}$ vector was treated with 1U CIP in 1x CIP buffer at 37°C/30min prior to gel purification. The purified *env*-gene fragments were inserted into pQ7 using a rapid ligation kit as before. DH5α *E. coli* competent cells were transformed with the pQ7.*env* plasmids by the heat-shock method, subsequent miniprep and midiprep procedures were conducted as described to obtain viable pQ7.NL43 processing site mutant clones.

2.2.1.4 Env Truncation Mutants

Using the HIV-1$_{NL43}$ *env*-gene in pQ7 vector as the template, each truncation mutant was generated using a common forward primer, FENV, and a distinct reverse primer TXRem where X (1-10) denotes the mutant primer (Appendix A.1.9.2). The reverse primers contain an *Eco*R1 restriction site to enable cloning back into pQ7 vector. Products were produced in a PTC-100 thermal cycler using the following PCR conditions: 1x native *Pfu* polymerase buffer; 250µM each dNTP (1.0mM final); 0.1µM each primer; 220ng template DNA; 5U native *Pfu* polymerase and made up to 100µl with sterile water. Template DNA was denatured at 95°C/90s followed by 20 cycles of: denaturation 95°C/90s; annealing 40°C/90s; extension 72°C/5min followed by a final extension step consisting of denaturation 95°C/90s; annealing 40°C/90s and extension at 72°C/10min and a 4°C hold. Products were electrophoresed on 0.5% LMP/TAE gels at 90V/2h at 4°C. Appropriate fragments were gel purified as before. and ligated into the digested pQ7 vector as before using a rapid ligation kit. DH5α E. coli competent cells were transformed with the pQ7.*env* plasmids by the heat-shock method as before, and subsequent miniprep and midiprep procedures were conducted as described to obtain viable pQ7.NL43 truncated *env*-gene clones.

2.2.1.5 DNA sequencing

HIV-1 *env*-genes in plasmids were sequenced using primers listed (Appendix A1.9.3). Sequencing products were produced using ABI PRISM® Big Dye Terminator Cycle Sequencing Ready Reaction Kits (ABI Prism®). Reactions consisted of: 200ng plasmid DNA; 2pmole primer; 1x Half BD buffer (Genpak) and MilliQ water in a volume of 10µl. Thermocycling was carried out in a Hybaid Omn-E thermocycler using 20 cycles of: denaturation 95°C/30s; annealing 50°C/15s; extension 60°C/4min and a final hold at 4°C. Sequencing products were purified by sodium acetate/ethanol precipitation according to the protocol described in the Big Dye terminator kit. Final precipitated pellets were air-dried and reconstituted in 4µl loading buffer (Formamide:Dextran Blue 4:1 ratio) and denatured at 95°C in a hotblock immediately prior to electrophoresis on a 5% polyacrylamide gel [1x TBE, 5% acrylamide solution, 36% w/v urea (Sigma-Aldrich), 0.005% ammonium persulphate (Sigma-Aldrich), 0.0007% TEMED (Sigma-Aldrich)] in an ABI 377 DNA Sequencer. Sequences were assembled on a SUN Systems platform using the Staden package programmes Gap4 and Genetic Data Environment (GDE) (Smith *et al.*, 1994).

2.2.2 HIV-1 Env Expression

2.2.2.1 Vaccinia Virus Stock Production

Recombinant vaccinia virus stocks of vTF7-3 and $vSIMB_{E/L}$ were produced using CV-1 cells. Cells were infected using a multiplicity of infection (m.o.i) of 5 plaque-forming units (pfu) per cell at 37°C/24h. Cells were subjected to a freeze-thaw cycle to detach monolayers and then disrupted in a sonicating waterbath in 2x 1min bursts, with 1min incubation on ice in between. The supernatant was clarified by centrifugation at 1500rpm/5min in a Beckman GPR centrifuge. The concentration of virus was determined by plaque assay and aliquots of the viruses were stored at –70°C.

2.2.2.2 Plaque Assay

Recombinant vaccinia virus constructs were serially diluted 10-fold to 10^{-10} using serum-free DMEM. 6 well plates were seeded with 3.3 x 10^5 CV-1 cells per well and incubated overnight at 37°C. Monolayers were washed twice with PBS and cells were infected with 1ml diluted vaccinia virus at 37°C/1h. Monolayers were washed twice with PBS and then cells were fed with 2ml DMEM/10% FCS and incubated at 37°C/48h. Medium was aspirated from the monolayers and cells were fixed with 1ml

PBS/0.25% glutaraldehyde (BDH) at room temperature/30min. The fixative was removed and then cells were stained using 70% ethanol/2% (w/v) crystal violet (Sigma-Aldrich) at room temperature/30min. Monolayers were carefully washed with water and dried before plaques were counted. The plaque forming unit (pfu) titre of the stock was determined by the mean number of plaques, between duplicate wells, in the range of 50-200 at a given dilution. The stock pfu was determined by the equation:

$$d(y \times 10^{x})$$

where:

d = initial dilution (e.g. 200µl virus stock in 1ml medium = 5)

y = mean number of plaques

x = dilution factor to yield a mean number of plaques in the range of 50-200

2.2.2.3 Transfection of 293T cells

HIV-1 *env* genes cloned into pQ7 were assayed for expression competence in 293T cells seeded in 60mm dishes at a density of 2×10^{6} cells per dish. Cells were transfected with appropriate pQ7.*env* plasmids by the calcium phosphate method using a Profection transfection kit (Promega) according to manufacturer's instructions. Cells were incubated at 37°C/4h then infected with the recombinant vaccinia vTF7-3 at an m.o.i. of 5pfu/cell at 37°C/1h. Cells were washed with fresh medium then fed with 2ml fresh medium and incubated at 37°C/16h. The cells were washed once with PBS and lysed in 100µl lysis buffer (150mM sodium chloride, 50mM Tris-HCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and sterile water) for10min at room temperature and stored at –70°C.

2.2.2.4 Polyacrylamide Gel Electrophoresis (PAGE)

Lysates were clarified by centrifugation at 13000rpm/1min in a microfuge prior to protein quantification. Protein content of lysates was quantified using Bradford Reagent (Bio-Rad) according to the manufacturer's instructions and read at a wavelength of 595nm on an Eppendorf Biophotometer. Standardised amounts of protein in 1x sample loading buffer (Invitrogen)/10% (v/v) β-mercaptoethanol (Sigma-Aldrich) were denatured at 70°C/10min in a hotblock and then electrophoresed on pre-cast NuPAGE®

Novex 4-12% Bis-Tris polyacrylamide gels (Invitrogen) at 200V/60min in 1x NuPAGE® MOPS SDS buffer (Invitrogen).

2.2.2.5 Western Blotting

Protein was transferred to Hybond C nitrocellulose membrane (Amersham) using a semi-dry transfer cell at 350mA/90min. Membranes were blocked using 10% (w/v) milk powder (Marvel) in PBS/0.1% Tween20 for at least 1h at room temperature or 4°C overnight. Membranes were washed twice with PBS/0.1% Tween20 after blocking, and probed with primary antibodies ARP301, ARP401, DP7324 or T-30 (at relevant dilutions in PBS/0.1% Tween20) by incubating on an orbital mixer at room temperature/1h. Membranes were washed 4 times with PBS/0.1% Tween20 and then probed with an appropriate IgG-peroxidase conjugate (Sigma-Aldrich), diluted 1:5000 in PBS/0.1% Tween20 (see Appendix A1.10), by incubation at room temperature/40min. Membranes were washed 4 times with PBS/0.1% Tween20 and then treated with 2ml ECL Western blotting detection reagents (Amersham). Membranes were then exposed to Kodak MXB film and processed using an X-ray film processor.

2.2.6 Cell Surface Immunofluorescence

2.2.6.1 Coating Coverslips

Sterile 13mm round coverslips were transferred aseptically into 24 well plates and treated with 2% 3-aminopropyltriethoxysilane (APTS, Sigma-Aldrich) diluted with industrial methylated spirits (IMS, BDH). Coverslips were incubated at room temperature/20min. The 2% 3-APTS/IMS was removed and coverslips were washed with 96% ethanol, twice with sterile deionised water, and then finally with sterile PBS. The PBS was removed and plates were stored at 4°C until used.

2.2.6.2 HIV-1 Env expression

293T cells were used to seed 24 well plates containing 3-APTS-treated coverslips at a concentration of 3x 10^5 cells/well. Cells were transfected with 6μg of each pQ7.*env*, pSP-*luc*$^+$ and phRL-TK(Int$^-$) plasmids by the calcium phosphate transfection method using a Profection Transfection kit. Transfected cells were incubated at 37°C/4h and then infected with vTF7-3 at 5pfu/cell in 300μl volume of medium at 37°C/1h. The inoculum was removed and cells were fed 1ml fresh medium and incubated at 32°C/overnight.

2.2.6.3 Antibody Staining of Cells

293T cells were washed 3x with PBS and then fixed with 500µl 4% paraformaldehyde (BDH)/PBS at room temperature/20min. Cells were permeabilised with 300µl 0.2% Triton X-100/PBS at room temperature/20min and then washed 3x with PBS. Cells were incubated in 300µl 3% BSA (w/v)/PBS at room temperature/30min and then washed once with PBS. Cells were probed with EVA 3012 in 1% BSA (w/v)/PBS at room temperature/30min, washed 3x with PBS and then incubated with 300µl of FITC-conjugate diluted in 1% BSA (w/v)/PBS/DAPI (1µg/ml) at room temperature/30min in the dark. Cells were washed 3x with PBS, then coverslips were mounted onto glass slides with 5µl Citifluor and analysed using a Nikon Labophoto 2 microscope and IPLab software (Scanalytics Inc.) on a G4 Macintosh computer.

2.2.3 Cell-Cell Fusion Assay

2.2.3.1 Effector And Target Cells

Cell-cell fusion assay was set up based on a protocol described by Isaacs *et al.* (Isaacs *et al.*, 1999). In our modified assay, 60mm dishes were seeded with 293T cells at a density of 2 x 10^6 cells/dish and incubated at 37°C overnight. 6µg each of the plasmids pSP-*luc*$^+$, phRL-TK (Int$^-$) and pQ7.*env* (where *env* denotes an HIV-1 *env*-gene) were transfected into 293T cells using a Profection kit and incubated at 37°C/4h. Cells were infected with vTF7-3 at an m.o.i. of 5pfu/cell in 1.5ml medium and incubated at 37°C/1h. The virus inoculum was removed, and cells were fed 2ml of medium supplemented with rifampicin (100µg/ml, Sigma-Aldrich) and incubated at 32°C overnight.

For the NP2 target cell lines, separate T175cm^2 flasks were seeded with NP2/CD4, NP2/CD4/CXCR4 and NP2/CD4/CCR5 cell lines at a density of up to 14.4 x 10^6 cells/flask and incubated at 37°C overnight. Cells were infected with vSIMB$_{E/L}$ at an m.o.i. of 5pfu/cell at 37°C/1h in medium supplemented with cytosine arabinoside (AraC, 40µg/ml, Sigma-Aldrich). The virus inoculum was removed and cells were fed with 50ml medium supplemented with AraC (40µg/ml) and rifampicin (100µg/ml) and incubated at 32°C overnight.

Luciferase background expression controls were set up for each HIV-1 *env*-gene in the cell-cell fusion assays whereby a population of NP2/CD4 target cells was not infected with $vSIMB_{E/L}$. A negative control was included in each assay by omission of pQ7.*env* plasmid from the effector cells.

2.2.3.2 Cell-cell Fusion

Each dish of effector cells was washed once with PBS and then detached from the dishes with 0.5mM EDTA/PBS. Cells were washed twice with PBS and resuspended in 1ml DMEM/10% FCS supplemented with AraC (40µg/ml) and rifampicin (100µg/ml). Each effector cell suspension was used to seed 20 wells of a 96 well flat-bottomed plate at a density of $1x10^5$ cells in a volume of 50µl.

Target cell lines were washed twice with PBS and then detached with 6ml 0.5mM EDTA/PBS. Cells were washed again with PBS and resuspended with DMEM/10% FCS supplemented with AraC (40µg/ml) and rifampicin (100µg/ml) to a concentration of $13.33x\ 10^6$ cells/ml. $2x\ 10^5$ cells (150µl) of each target cell line were added to 4 separate wells of each HIV-1 Env-specific effector cell population, thus allowing quadruplicate tests. Plates were incubated at 37°C/7h. The supernatant was removed from each well and cells were lysed in 25µl 1x passive lysis reagent (Promega) at room temperature/10min. Lysates were stored at –70°C.

2.2.3.3 Luciferase assay

Lysates were processed using a Victor Wallac Luminometer, Luciferase Assay System and Renilla Assay System kits (Promega). 20µl of lysate was added to 100µl of *Renilla* or *Photinus* luciferase substrate (Promega) at room temperature. Luciferase activity was determined by measuring the counts per second (CPS) with a 2s delay followed by a 10s read. The net *Photinus* luciferase expression was calculated by subtraction of the mean background activity from all the samples for each HIV-1 Env-specific fusion.

The relative ratio of expression of *Photinus* luciferase activity in relation to $NL43_{WT}$ or $JRFL_{WT}$ Envs was calculated as detailed by Promega using the equation:

$$\frac{E_P / E_R}{C_P / C_R}$$

Where:

E = Sample Env

C = Control Env ($NL43_{WT}$ or $JRFL_{WT}$)

P = Net *Photinus* luciferase activity

R = *Renilla* luciferase activity

Expression of relative fusogenic potential allows inter-assay comparison of results for each Env.

2.2.4 Chimeric Virus Studies

2.2.4.1 Transfection of pC2.*env*

The required *env*-genes were excised from pQ7.*env* clones by *Xho*1/*Eco*R1 restriction enzymes and ligated into a similarly digested and CIP-treated pC2 cassette to yield pC2.*env* clones. 60mm dishes were seeded with 293T cells at a density of 2 x 10^6 cells/dish and incubated overnight. Cells were transfected with 10µg of pC2.*env* and incubated at 37°C/16h. The supernatant was removed and cells were fed 3ml fresh medium and incubated at 37°C/72h. Supernatants were filtered through a sterile 0.45µm syringe filter and 0.5ml aliquots were frozen at –70°C. Cells were washed once with PBS and then lysed in 0.5ml lysis reagent (as for PAGE) at room temperature/10min. Lysates were stored at –70°C until analysed by western blotting. Filtered supernatant was serially diluted 10-fold to 10^{-6} and the 3 highest dilutions of each virus preparation were assayed for p24 content with a Murex HIV Antigen MAb kit according to manufacturer's instructions. Results were analysed on a Multiskan Ascent plate reader using Ascent Software version 2.6 on a Dell Intel Pentium 4 PC.

2.2.4.2 Chimeric Virus $TCID_{50}$ Determination using PBLs

PBMCs were resuscitated from LN_2 stocks and stimulated with 5µg/ml PHA-P at 37°C/72h. Cells were washed once with PBS and incubated in medium with 20U/ml IL-2 at 37°C/24h. Chimeric virus stocks were serially diluted 10-fold to 10^{-5} dilution. 1 x

10^6 PBLs were infected with each dilution of virus at 37°C/3h, a negative control consisting of medium was included. PBLs were washed twice with PBS and resuspended in 1ml medium. 200µl of infected PBLs were added to each of 4 wells of a 96 well plate. PBS was added to external wells to maintain the incubation temperature of edge wells. Cells were incubated at 37°C and were fed 150µl fresh medium after four days. On day seven, the supernatants of wells were tested for HIV-1 p24 using a Murex kit according to the manufacturer's instructions. The $TCID_{50}$/ml of the chimeric virus stock was calculated using the following equation:

$$-\log TCID_{50} = [-I-d(S-0.5)]$$

$$TCID_{50}/ml = f(10^{-\log TCID50})$$

where:

I = negative log of first virus dilution

d = log of each dilution step

S = sum of the proportion of positive wells

f = initial dilution factor

2.2.4.3. Chimeric Virus $TCID_{50}$ Determination using NP2 Cell Lines

NP2/CD4/CCR5 and NP2/CD4/CXCR4 cell lines were used to seed 24 well plates at 1 x 10^5 cells/well and incubated at 37°C/overnight. Chimeric virus stocks were serially diluted 10-fold to 10^{-5} dilution in 1ml NP2 cell medium and 200µl of each virus dilution was used to infect 4 replicate wells of appropriate NP2 cells at 37°C/3h, a negative control consisting of medium was included. Cells were washed 3 times with PBS and fed 1ml fresh medium. Cells were incubated at 37°C and were fed 150µl fresh medium after four days. On day seven, the supernatants of wells were tested for HIV-1 p24. $TCID_{50}$/ml was determined as described earlier.

2.2.4.4. Chimeric Virus Infection of Ghost Cell Lines

24 well plates were seeded with separate Ghost cell lines expressing CD4, CD4/CCR5 or CD4/CXCR4 surface receptors at a density of 6 x 10^4 cells/well. Cells were infected with 100ng p24 of chimeric virus stocks in 100µl volume of serum-free DMEM at 37°C/16h. Cells were washed once with PBS, fed fresh medium and incubated at

37°C/48h. Cells were washed once with PBS and incubated with 0.5ml trypsin/EDTA at 37°C/5min. Detached cells were transferred to FACS tubes containing 3ml PBS/4% formaldehyde and centrifuged at 1500rpm/5min at 4°C in a Beckman GPR benchtop centrifuge. Cells were resuspended in 50µl PBS/4% formaldehyde (BDH) and incubated on ice/4h or overnight.

2.2.4.5. Flow Cytometry

Infected Ghost cells were analysed by fluorescence activated cell scanning (FACS) using a Calibur FACScan flow cytometer and CellQuest software on a Macintosh G3 computer. Using uninfected Ghost cells, live and dead cells were sorted according to side scatter and forward scatter and a live gate (R1) encompassed live cells. Live cells were gated for GFP detection on the FL-1 channel and 10000 events were accumulated.

Chapter 3

Development Of Cell-Cell Fusion Assay: Analyses of Processing Defective HIV-1 Glycoprotein

3.1 Introduction

3.1.1 Cell-Cell Fusion Assays

Infection of a cell by HIV-1 involves the interaction of gp120 on the virus surface with CD4 and β-chemokine co-receptors on the target cell surface, followed by pH-independent fusion of the virus and target cell membranes. In the natural lifecycle of HIV-1, unspliced *env*-gene transcripts require the Rev protein for nuclear export, and in the absence of Rev, transcripts remain in the nucleus (Fischer *et al.*, 1994; Henderson and Percipalle, 1997; Malim *et al.*, 1989). Many groups have used recombinant vaccinia virus to drive expression of HIV-1 gp160 *in vitro* because of the lower containment category for manipulation, high level of uniform expression, broad host cell tropism, and the cytoplasmic localization of proteins necessary for polyadenylation and capping of transcripts (Fields *et al.*, 2001; Nussbaum *et al.*, 1994). However, the cloning of glycoprotein genes into recombinant vaccinia and purification of the clones is a cumbersome process if one needs to analyse a large number of primary isolates. The construction of recombinant vaccinia viruses encoding bacteriophage T7 or SP6 RNA polymerases enabled the expression of other proteins to be specifically driven through their promoters (Ashorn *et al.*, 1993; Fuerst and Moss, 1989; Studier and Moffatt, 1986; Usdin *et al.*, 1993). In the case of HIV-1 gp160, the level of expression can be greater than from recombinant vaccinia (Fuerst *et al.*, 1987). It can be argued that the artificially high level of glycoprotein expression in these systems does not reflect that observed in a natural HIV-1 infection, however, studies have proved the glycoprotein is correctly processed and functional (Ashorn *et al.*, 1993; Fuerst *et al.*, 1987). Adaptation of these methods has led to the evolution of cell-cell fusion assays whereby vaccinia expressed RNA polymerases drive expression of genes carried on separate plasmids. HIV-1 infected cells *in vitro* are able to form syncytia with target cells expressing the appropriate receptors, thus cell-cell fusion assays are regarded as model systems for dissecting the membrane fusion requirements of HIV-1 Envs and the kinetics of entry (Etemad-Moghadam *et al.*, 2001; Gallo *et al.*, 2001; Lineberger *et al.*, 2002; Nussbaum *et al.*, 1994; Reeves *et al.*, 2002; Singh *et al.*, 2001; Yi *et al.*, 1999; Yi *et al.*, 2001), thus demonstrating that value of these biological studies in complementing and extending DNA sequence and phylogenetic analyses.

3.1.2 HIV-1 Env Processing

For HIV-1 Env to be functional, there is a requirement for protease activation of the precursor. Processing of Influenza HA0 involves the minimal basic motif R-X-K/R-R and is influenced by the presence of a nearby oligosaccharide at Asp22, thus it appears that efficient processing by ubiquitous proteases requires exposure of the cleavage loop (Walker and Kawaoka, 1993). Influenza B is closely related to Influenza A, however, insertion of multiple basic residues into the cleavage site of the HA did not result in increased proteolytic processing, suggesting a different local structure at the site of cleavage, indeed, studies have suggested the presence of two N-linked oligosaccharides near the cleavage site (Brassard and Lamb, 1997), explaining why Influenza B causes milder disease. Processing of HIV-1 gp160 is also dependent on the presence of a highly conserved motif characterised by the sequence KAKRRVVQREKR. The sequence encompasses two basic K/R-X-K/R-R motifs that are recognised by the subtilisin-like proteases, however, cleavage after the terminal arginine of the REKR motif generates the hydrophobic N-terminus of gp41, thus REKR is designated as site 1, the primary processing site, and KAKRR is designated as site 2. Mutational studies dissecting the minimum requirements for site 1 (Table 3.1) indicate the terminal basic residue is essential while the penultimate residue can tolerate conservative changes (Bosch and Pawlita, 1990; Bosch and Pfeiffer, 1992; Dubay *et al.*, 1995; Freed *et al.*, 1989; Guo *et al.*, 1990; McCune *et al.*, 1988; Rovinski *et al.*, 1995; Willey *et al.*, 1991). Mutation of site 1 to a multi-basic cluster similar to that in pathogenic Influenza HAs can increase the proportion of processed gp160 (Binley *et al.*, 2002). The remarkable conservation of the second basic motif in all HIV-1 isolates raises questions as to its necessity for Env function. Mutational analyses of site 2 (Table 3.1) indicate its importance in the primary processing event (Bosch and Pawlita, 1990; Dubay *et al.*, 1995; Kieny *et al.*, 1988). The selective processing of gp160 at the REKR motif underline potentials role of the site 2 motif in recruiting processing enzymes to the cleavage loop or adopting a structural configuration that exposes the cleavage loop (Moulard *et al.*, 1998; Moulard and Decroly, 2000; Oliva *et al.*, 2002), similar to diphtheria toxin where cleavage by furin occurs at an exposed loop flanked by α-helices (Oliva *et al.*, 2002). The degree of exposure of the fusion peptide remains to be confirmed, as the crystallographic data for gp120 is based upon a non-native monomeric structure lacking many variable loop domains and existing data regarding the gp41 structure reflects a post-fusion state (as indeed the gp120 may be), and both structures lack the gp41 fusion peptide (Kwong *et al.*, 1998), however, studies with antisera from

HIV-1 exposed patients suggest exposure of the gp120 C-terminal domain (Brown *et al.*, 1999; Chang *et al.*, 2000).

3.1.3 Processing Enzyme

The exact enzyme responsible for gp160 processing is not fully established. Of the total gp160 that is produced in a HIV-1 infected cell, only a small proportion is processed (Willey *et al.*, 1991), suggesting a low incidence of proper folding, or that there are low saturable levels of processing enzyme(s) in the secretory pathway. The ability of the yeast protease kexin, to specifically process gp160 implicates a processing role for calcium-dependent kexin-like proteases in mammalian cells (Moulard, Achstetter, Ikehara *et al.*, 1994; Moulard, Achstetter, Kieny *et al.*, 1994). These proprotein convertases (PCs) involved in prohormone maturation process precursors at characteristic K/R-X-K/R-R motifs. PCs include furin, PACE4, PC1/3, PC2, PC4, PC5/6 and PC7/LPC (Hallenberger *et al.*, 1992). The most likely enzymes involved are furin and PC7, as mRNA transcripts were found to be upregulated in $CD4^+$ T lymphocytes and $CD4^+$ cell lines (Decroly *et al.*, 1997). Moreover, PC2 and PC1/3 traffic in the regulated secretory pathway, while Env transits in the constitutive secretory pathway along with furin, PACE4 and PC7. The use of decanoyl-peptides to inhibit gp160 processing indicated their interactions occur with furin and PC7 enzymes (Bahbouhi *et al.*, 2002). Expression of gp160 in furin-deficient cell lines does not prevent proteolytic processing (Gu *et al.*, 1995; Ohnishi *et al.*, 1994); supporting the notion that gp160 employs redundant enzymes for processing. The requirement for calcium in the processing event may be more relevant for the trafficking of gp160 to the processing compartment, as opposed to being a enzyme co-factor (Moulard, Montagnier *et al.*, 1994). Two specific calcium-independent endoproteases have been isolated from a $CD4^+$ T cell line and PBMCs; viral envelope glycoprotein maturase (VEM) (Kido *et al.*, 1993) and VLP (VEM-like protease) respectively (Bendjennat *et al.*, 2001). In this study I have developed a cell-cell fusion assay based upon the protocol described by Issacs *et al.* (1999). To determine the specificity of a cell-cell fusion assay, it is necessary to distinguish between functional and non- functional HIV-1 glycoproteins. For HIV-1 Env, non-functional glycoproteins were created by mutation of the processing site. These mutants would prevent or limit proteolytic processing of the gp160 precursor, thereby preventing or limiting generation of the hydrophobic fusion peptide at the N-terminus of gp41 that inserts into the host cell membrane. Mutations were designed in both of the basic motifs and in the short intervening sequence (VVQ),

based upon published literature (Table 3.1), ranging from single amino acid changes to multiple changes (Table 3.2).

Cleavage Site Sequence[a]																Cleavage[b]	Fusion[b]	Infection[b]	Reference
K	A	K	R	R	V	V	Q	R	E	K	R	A	V	G	I				
-	-	-	-	-	-	-	-	G	-	E	F	-	-	-	-	-	-	(McCune *et al.*, 1988)
-	-	-	-	-	-	-	-	-	R	R	K	K	R	-	-	+++	ND	ND	(Binley *et al.*, 2002)
-	-	-	-	-	-	-	-	-	R	R	-	K	R	-	-	+++	ND	ND	
-	-	-	-	-	-	-	-	-	R	R	-	R	R	-	-	+++	++	+	
-	-	-	-	-	-	-	-	K	K	R	K	K	R	-	-	+++	ND	ND	
-	-	-	-	-	-	-	-	-	-	R	-	R	K	K	R	+++	ND	ND	
-	-	L	-	L	R	L	R	L	R	L	-	L	R	-	-	-	ND	ND	(Willey *et al.*, 1991)
-	-	N	I	-	-	-	-	-	-	P	-	G	P	-		-	ND	ND	
-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	++	++	ND	(Freed *et al.*, 1989)
-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	++	++	ND	(Guo *et al.*, 1990)
-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	ND	(Rovinski *et al.*, 1995)
-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-	++	++	ND	
-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	++	++	++	(Dubay *et al.*, 1995)
-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	++	
-	-	E	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	
-	-	E	-	S	-	-	-	-	-	-	-	-	-	-	-	++	++	++	
-	-	E	-	S	-	-	-	-	-	E	-	-	-	-	-	-	-	-	
-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	++	++	++	
-	-	-	S	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	
-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	
-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	ND	ND	(Bosch and Pawlita, 1990)
-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	+	ND	ND	(Bosch and Pfeiffer, 1992)
-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	+	ND	ND	
-	-	-	-	-	-	-	-	-	S	-	N	-	-	-	-	-	ND	ND	
-	-	-	-	-	-	-	-	-	S	-	-	S	-	-	-	-	ND	ND	
-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	++	ND	ND	
-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	++	ND	ND	
N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	ND	ND	
N	-	N	S	S	-	-	-	-	-	-	-	-	-	-	-	-/+	ND	ND	
N	-	I	S	S	-	-	-	-	-	-	-	-	-	-	-	-/+	ND	ND	
-	-	-	-	-	-	-	-	-	N	-	H	Q	-	-	-	-/+	-	ND	(Kieny *et al.*, 1988)
-	-	Q	N	H	-	-	-	N	-	H	Q	-	-	-	-	-	-	ND	

Table 3.1. Summary of studies on the HIV-1 processing site motifs.

[a] Identity with the $NL43_{WT}$ processing site is indicated by (-) and engineered substitutions are indicated by the single letter amino acid code.

[b] The cleavage, fusion or infection of Env mutants were: (+++) better than wildtype; (++) equivalent to wildtype; (+) less than wildtype; (-/+) ambiguous; (-) not observed; or ND - Not done.

3.2 Methodology

3.2.1 Construction of Processing-Defective Envs

The mutations in the cleavage sites of a HIV-1_{NL43} backbone (Table 3.2) were generated by mutant oligonucleotide-primed PCR as outlined in figure 3.1 and as described in Chapter 2. Briefly, to generate the mutations in either basic cleavage motif, two subgenomic *env* fragments were generated with an overlap of common 9 nucleotides constituting the intervening VVQ sequence. The 922bp 5' subgenomic fragment contained the KAKRR motif, and the 755bp 3' subgenomic fragment contained the REKR motif. For the mutants at the VVQ motif, only one 910bp 5' fragment and 4 mutant 767bp 3' fragments were required. Splice-overlap extension (SOE) PCR using a mutant fragment and a wildtype fragment generated a large 1.67kb subgenomic fragment (Figure 3.1).

Mutant fragments replaced the wildtype sequences in a pSP-*luc*$^+$.$NL43_{WT}$ vector using *Stu*1 and *Bam*H1 restriction sites and the presence of mutations were verified by DNA sequencing. Full-length mutant *env*-genes could then be excised and cloned into pQ7 and pC2 using *Xho*1 and *Eco*R1 restriction sites.

3.2.2 Expression of Processing-Defective Envs

293T cells were transfected with pQ7.$NL43_{WT}$ or mutant plasmids as detailed in Chapter 2. Expression of *env*-genes was driven by the recombinant vaccinia construct vTF7-3. Cell surface expression was detected using EVA3012 MAb and anti-mouse FITC-conjugated antibody, cytoplasmic expression was evaluated by western blotting using ARP401 and D7324 antisera, detected with secondary donkey anti-sheep IgG-peroxidase conjugate. Both procedures are detailed in Chapter 2.

HIV-1 Env	Processing Site[a]
$NL43_{WT}$	KAKRRVVQREKR
$JRFL_{WT}$	KAKRRVVQREKR
NL43.M1S1	KAKRRVVQREER
NL43.M2S1	KAKRRVVQRNER
NL43.M3S1	KAKRRVVQREKT
NL43.M4S1	KAKRRVVQSHEQ
NL43.M5S1	KAKRRVVQSEHN
NL43.M1S2	KAKERVVQREKR
NL43.M2S2	KAKEGVVQREKR
NL43.M3S2	KAKSRVVQREKR
NL43.M4S2	NAISSVVQREKR
NL43.M5S2	KAQNGVVQREKR
NL43.VVF	KAKRR--QREKR
NL43.PVSF	KAKRRPVQREKR
NL43.VPSF	KAKRRVPQREKR
NL43.EVSF	KAKRREVQREKR

Table 3.2. The gp160 processing site of $NL43_{WT}$ and mutant Envs.

Mutated residues are indicated in red. Mutant VVF had both valine residues deleted. Primer sequences are detailed in Appendix A1.9.1-2.

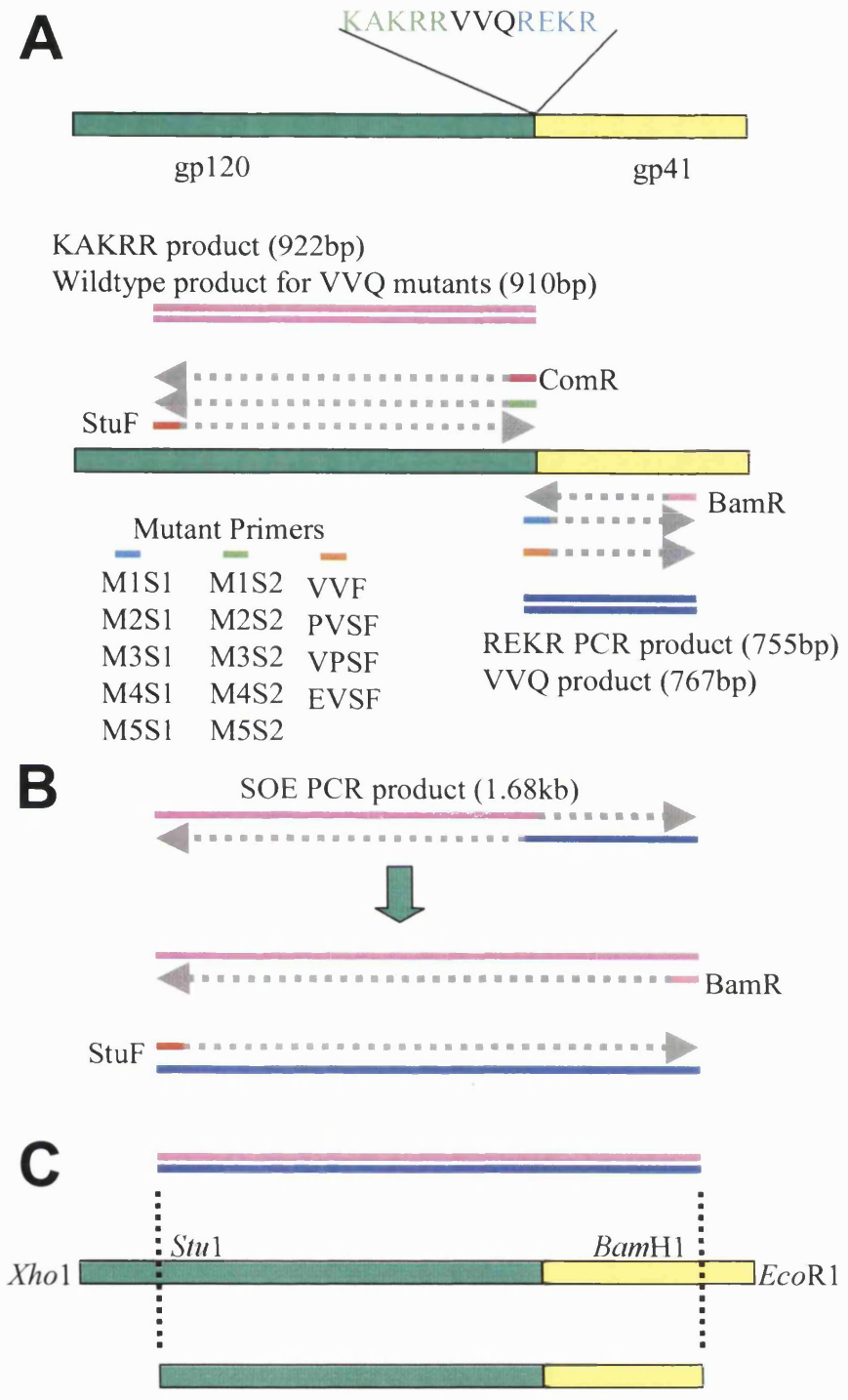


Figure 3.1. Construction of processing site mutants.

Mutant oligonucleotide-primed PCR generated 5' and 3' fragments using the $NL43_{WT}$ *env*-gene as the template (A). 5' and 3' PCR fragments were joined together by SOE PCR (B). Primers StuF and BamR were added to amplify the fragments. $NL43_{WT}$ sequences were replaced with the mutant subgenomic fragments using *Stu*1 and *Bam*H1 restriction sites (C). Full-length HIV-1 *env*-genes can be cloned into other vectors using *Xho*1 and *Eco*R1 restriction sites. Diagram not to scale.

3.2.3 Development of the Cell-Cell Fusion Assay

The protocol described in Chapter 2 represents the final working protocol; many alterations from the published protocol (Isaacs *et al.*, 1999), were necessary. 293T cells readily detached from 24 well plates during washing procedures, so transfections were carried out in 60mm dishes. The macrophage target cells were substituted with cell lines expressing CD4 only or in conjunction with CCR5 or CXCR4 (R5 and X4 respectively) chemokine receptors. Ghost cell lines were used initially (Vodros *et al.*, 2001) but were substituted for NP2/CD4 cell lines (Shimizu *et al.*, 1999; Soda *et al.*, 1999) due to the endogenous expression of CXCR4 on all Ghost cell lines, thereby producing false positive signals with X4 expression in supposedly CD4/CCR5 expressing cells. Assays were conducted in a 96 well plate format to allow each Env-mediated fusion assay to be performed in quadruplicate.

Initially, HIV-1 *env* genes were cloned into pSP-*luc*$^+$ plasmid downstream of the T7 RNA polymerase promoter reading in the opposite direction of the *Photinus pyralis* luciferase reporter enzyme so that there was an equimolar gene dosage of HIV-1 *env* and *luc*. Unfortunately, this elicited high backgrounds of luciferase expression in the absence of SP6 RNA polymerase, indicating read-through due to the T7 RNA polymerase (Studier and Moffatt, 1986) and the presence of cryptic promoter sequences upstream of the luciferase gene (personal communication with Promega technical support staff, UK). Thus, HIV-1 *env*-genes were kept in the pQ7 plasmid separate from the luciferase reporter gene on pSP-*luc*$^+$, resulting in a dramatic decrease in the luciferase background in the absence of SP6 RNA polymerase. Another plasmid, phRL-TK(Int$^-$), was included to address the issue of transfection efficiency.

The methodology published by Isaacs *et al.* (1999) suggested infection prior to transfection. However, greater cytopathic effects were observed in effector cells post-transfection by this method. To address if the efficiency of vTF7-3 infection is a major variable, a 8 x 10^6 293T cells in a T175cm^2 flask were infected with recombinant vaccinia vTF7-3 at an m.o.i. of 5pfu/cell at 37°C/1h. Cells were then washed twice with PBS and then detached with 0.5mM EDTA/PBS. 60mm dishes were seeded with 2 x 10^6 vTF7-3-infected 293T cells in 4ml medium and incubated at 37°C/3h prior to transfection with 6μg each of plasmids pQ7.NL43, pSP-*luc*$^+$ and phRL-TK(Int$^-$) by the calcium phosphate method using a Profection Kit (Promega) according to manufacturer's instructions. In parallel, 60mm dishes containing 2 x 10^6 293T cells

were transfected with the same set of plasmids accordingly, and then after 4h were infected with vTF7-3 at an m.o.i. of 5pfu/cell using similar conditions. NP2 target cells were prepared as described in chapter 2. All cells were all incubated at 32°C/overnight, then processed for cell-cell fusion as described in chapter 2.

Quantification of *Photinus* luciferase activity in lysates was measured in counts per second (CPS). The *Renilla* luciferase activity was used as a measure of transfection efficiency and used to adjust the *Photinus* luciferase expression for that sample. The relative ratio of fusion of each cleavage site mutant *env*-gene in relation to $NL43_{WT}$ and $JRFL_{WT}$ Envs was calculated as described in Chapter 2. The significance of luciferase activities in relation to background levels (background CPS vs sample CPS) and to $NL43_{WT}$ Env (Sample net CPS vs $NL43_{WT}$ net CPS) was determined by the Student's T-test using Microsoft® Excel.

3.2.4 Chimeric Virus Production

$NL43_{WT}$ and mutant *env* genes were cloned into pC2 vector using *Xho*1 and *Eco*R1 restriction sites. Plasmids were transfected into 293T cells and the supernatants were harvested 48h later. Samples were assayed for HIV-1 p24 by ELISA and for gp160/gp120 expression with ARP401 or D7324 polyclonal antibodies by western blotting.

3.2.5 Ghost Cell Line Infection Assay

Ghost CD4, CD4/CCR5 and CD4/CXCR4 cell lines were infected with equivalent amounts (300ng p24) of chimeric viruses in duplicate assays. Cells were analysed by flow cytometry after 48h, a minimum of 10,000 live cells were gated for expression of the green fluorescent protein (GFP) reporter gene product as described in Chapter 2.

3.2.6 $TCID_{50}$ Determination

Chimeric viruses were serially diluted 10-fold and used to infect monolayers of NP2/CD4/CCR5 and NP2/CD4/CXCR4 cells in 24 well plates as described in Chapter 2. The plates were scored for cytopathic effects on a daily basis and supernatants were assayed for HIV-1 p24 by ELISA after 7 days.

3.3 Results

3.3.1 Construction of Processing Defective HIV-1 *env*-Genes

The required 5' and 3' fragments were successfully joined together by SOE-PCR to generate the 1.67kb fragments containing the site 1, site2 and VVQ mutations (Figure 3.2A). Downstream cloning of the full-length 2.65kb *env*-gene into pQ7 and pC2 vectors was verified by restriction enzyme analysis (Figure 3.2B). DNA sequencing confirmed that mutant fragments replaced the corresponding $NL43_{WT}$ *env*-gene sequence (Appendix A2.1).

3.3.2 Cell Surface Immunofluorescence

Expression of HIV-1 Env on the surface of effector cells is essential to mediate membrane fusion with a target cell expressing the appropriate receptors. Cell-surface immunofluorescence was used to assess the cell-surface expression of the potentially cleavage defective-Envs. Surface staining was compared with intracellular staining and results are shown for a selection of mutants (Figures 3.3 and 3.4). Similar results were obtained for $NL43_{WT}$ and all mutants, with only a small proportion of cells exhibiting Env expression on the surface of transfected/vTF7-3 infected 293T cells. The extent of expression varied with regard to the mutant Env, but this may reflect differences in efficiencies of transfection rather than a genuine feature of particular Envs. The inefficient transfection may be attributable to the fixation of 293T cells onto coverslips, as after seeding the cells are unable to divide. Additionally, non-uniform infection of the 293T cell monolayer by vTF7-3 could result in higher expression in cells infected with more viruses and is something beyond control. In all cases greater immunofluorescence was observed when transfected/vTF7-3 infected cells were permeabilised suggesting constitutive production of Env in all cases.

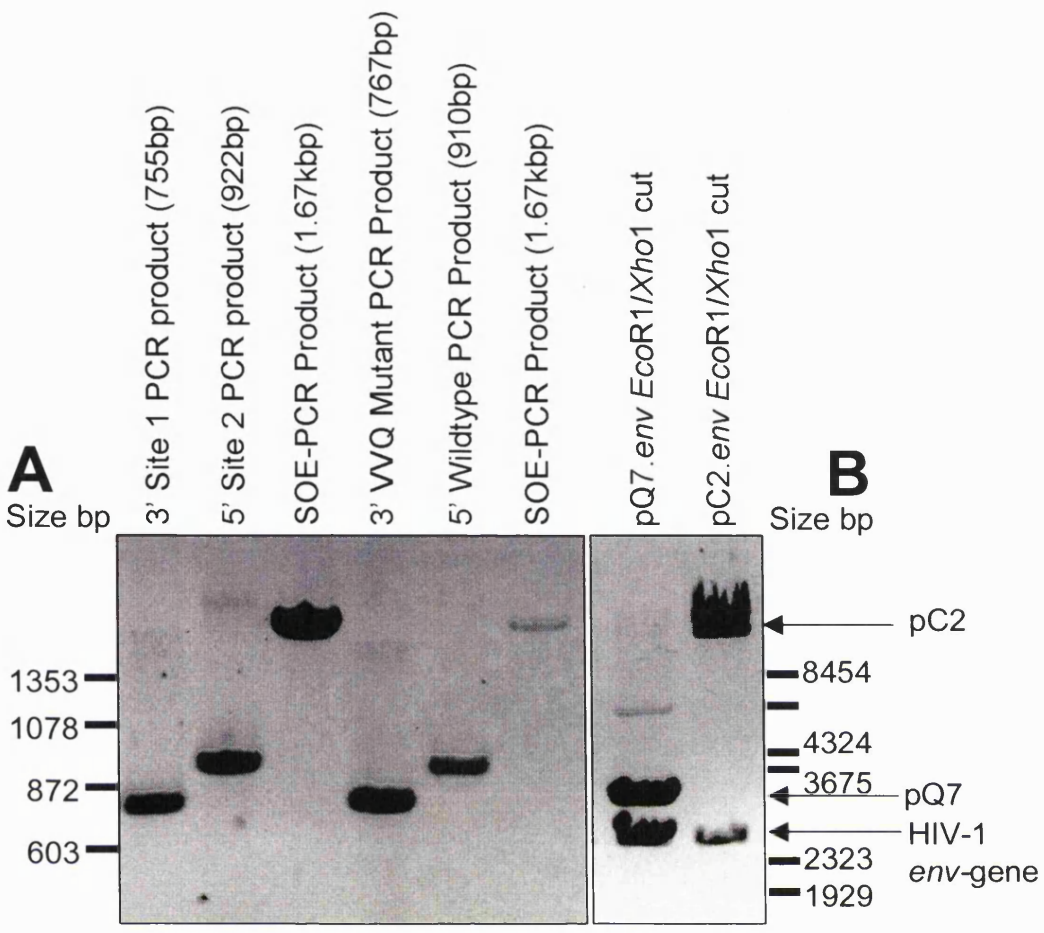


Figure 3.2. Mutant PCR products and SOE PCR products.

The 3' and 5' fragments were joined together by SOE PCR to generate a 1.67kbp subgenomic fragment (A). Restriction digestion of pQ7.*env* and pC2.*env* with *Xho*1 and *Eco*R1 enzymes (B). The full-length HIV-1 *env*-gene is 2.65kbp. Linearised pC2, pQ7 and HIV-1 *env*-gene fragments are by arrows.

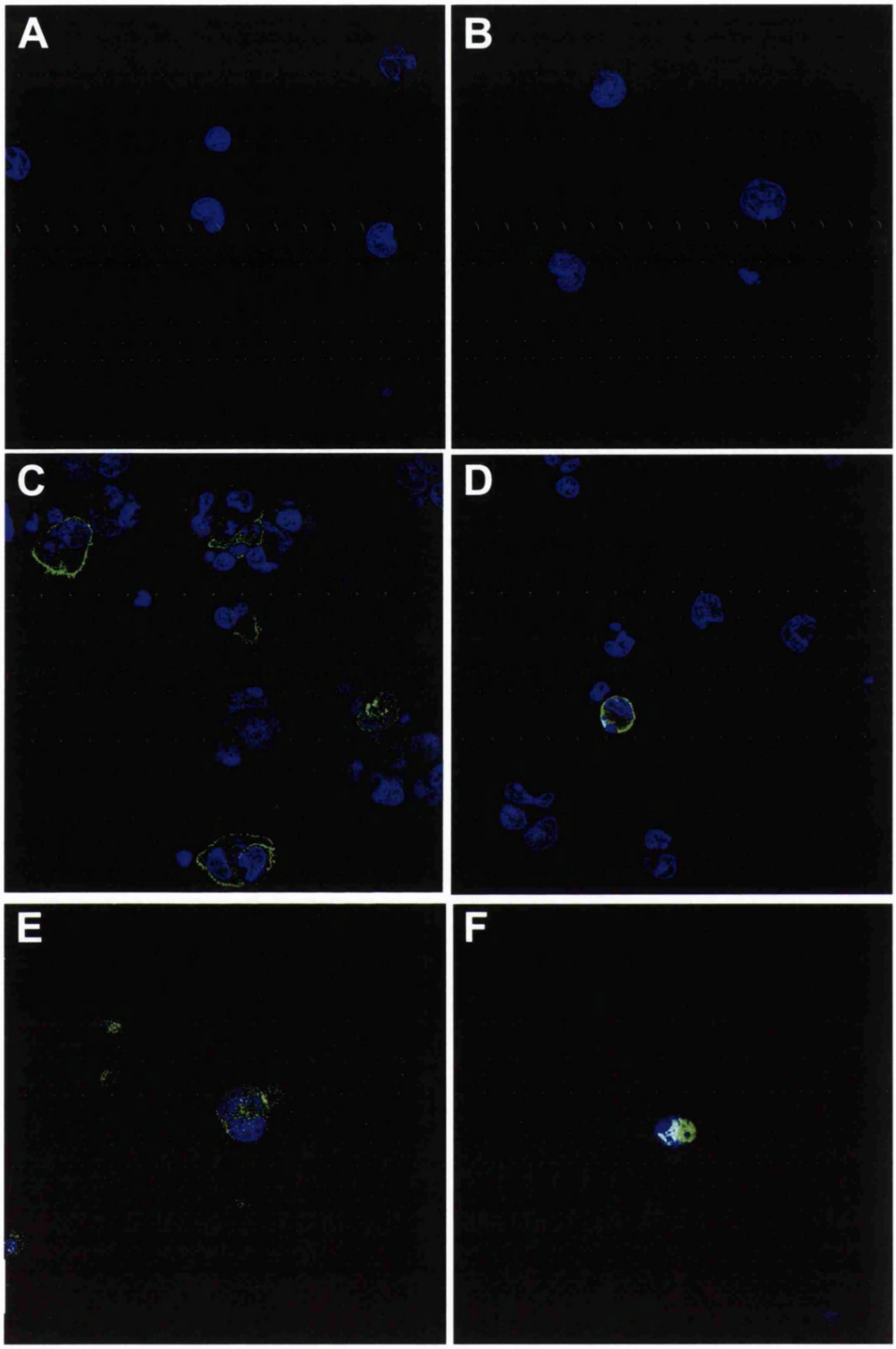


Figure 3.3. Cell surface immunofluorescence.

The results show mock-transfected cells (A,B) and cells expressing $NL43_{WT}$ (C,D) and NL43.VVF Envs (E,F). Panels A, C and E show surface expression, and panels B, D and F show permeabilised cells. Each panel represents an area of $1243\mu m^2$ viewed at 200x magnification

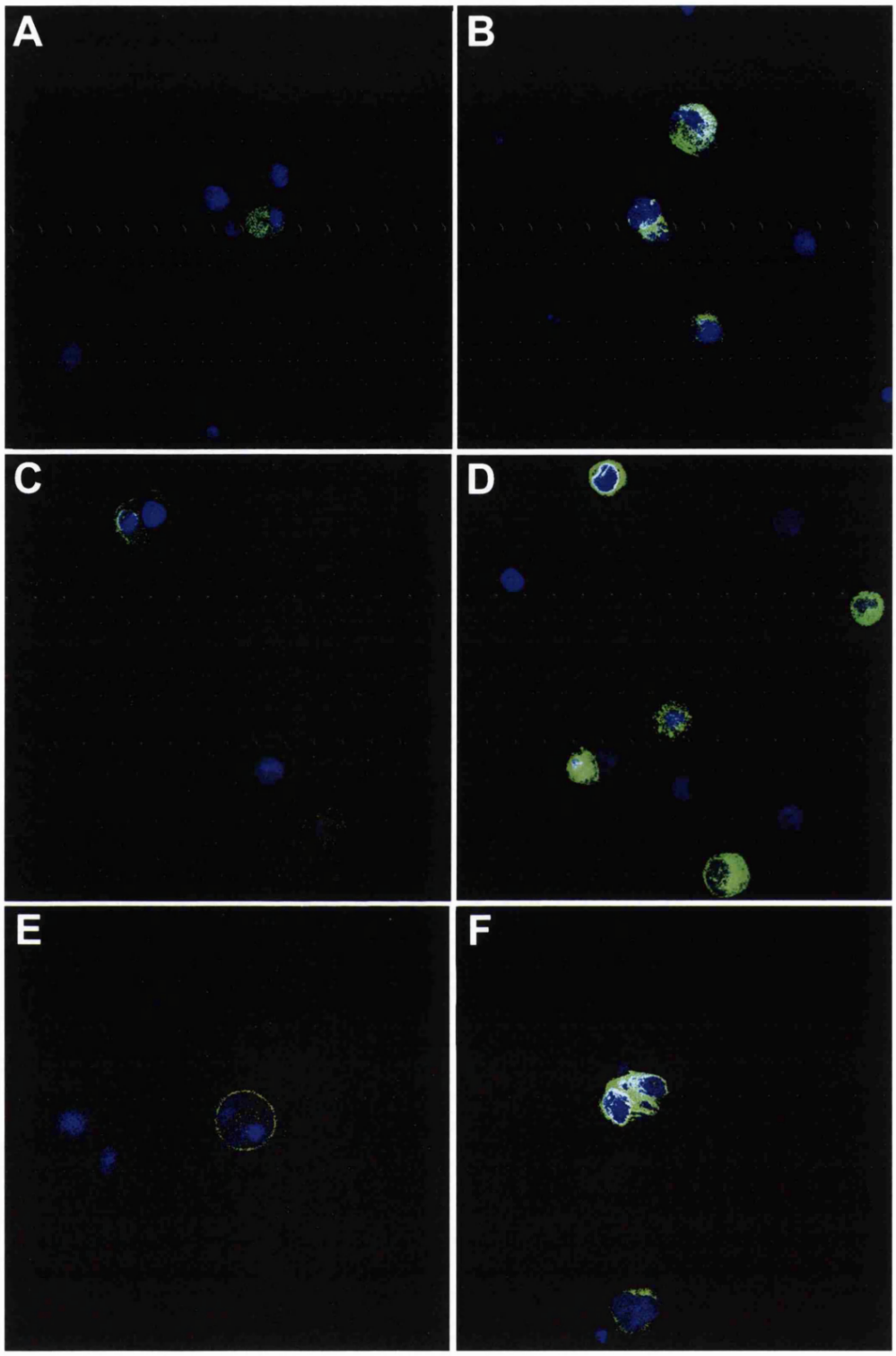


Figure 3.4. Cell surface immunofluorescence.

The results show cells expressing NL43.M1S1 (A,B) NL43.M2S1 (C,D) and NL43.M1S2 Envs (E,F). Nuclei are stained with DAPI (Blue) and Env is indicated by FITC (Green). Panels A, C and E show surface expression, and panels B, D and F show permeabilised cells. Each panel represents an area of 1243μm^2 viewed at 200x magnification.

3.3.3 Cell-Cell Fusion Assay

The major variables with the methodology of the cell-cell fusion assay are the transfection efficiency and the infection by vTF7-3. To address the transfection/infection strategies with regard to efficiency of Env expression on effector cell populations, effector cells were either infected before transfection according to Isaacs *et al.* (1999), or transfected before infection. The transfection efficiency, as determined by *Renilla* luciferase expression, was greater in samples transfected prior to infection (Figure 3.5); moreover, greater cytopathic effects (CPE) were evident in samples infected prior to transfection. The results also show variation between independent transfection of identical sets of plasmids into similar effector cell populations, suggesting non-uniform uptake of phRL-TK(Int$^-$) or variations in the intracellular environment conducive to constitutive *Renilla* luciferase expression across the effector population.

Comparison of *Photinus* luciferase expression show that in cells infected before transfection, reporter enzyme activity was higher, suggesting a greater extent or more rapid fusion (Figure 3.6A). Considering infected/transfected effector cells exhibited greater CPE, it is possible that the vTF7-3 and transfection affected the condition of those cells, rendering them more prone to membrane destabilisation to facilitate fusion. Greater variation in *Photinus* luciferase expression was observed compared to transfected/infected effector cells, indicating standardisation of the proportion of infected cells does not lead to consistency in Env-mediated fusion.

Adjustment of the raw *Photinus* luciferase expression data with *Renilla* luciferase activity indicated that transfected/infected effector cells produced more consistent fusion than infected/transfected cells (Figure 3.6B). The strategy of transfection prior to infection was thus adopted for subsequent assays.

Effector 293T cells expressing wildtype and mutant NL43 Envs were co-cultured with NP2 target cells expressing CD4 alone or with CCR5 or CXCR4 chemokine receptors.

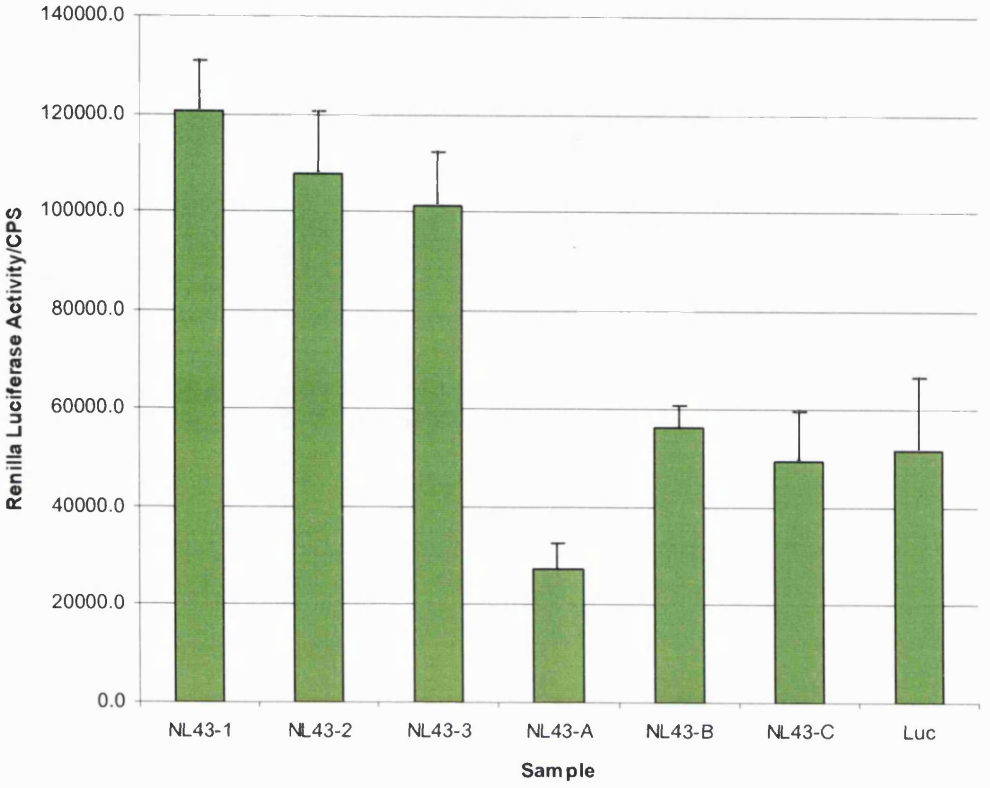


Figure 3.5. The effect of vTF7-3 infection upon transfection efficiency.

The efficiency was measured by *Renilla* luciferase expression. Samples NL43-1, 2 and 3 were transfected prior to infection with vTF7-3, whereas samples NL43-A, B and C were treated conversely. The Luc sample indicates a pQ7.NL43 negative control, but containing both reporter plasmids.

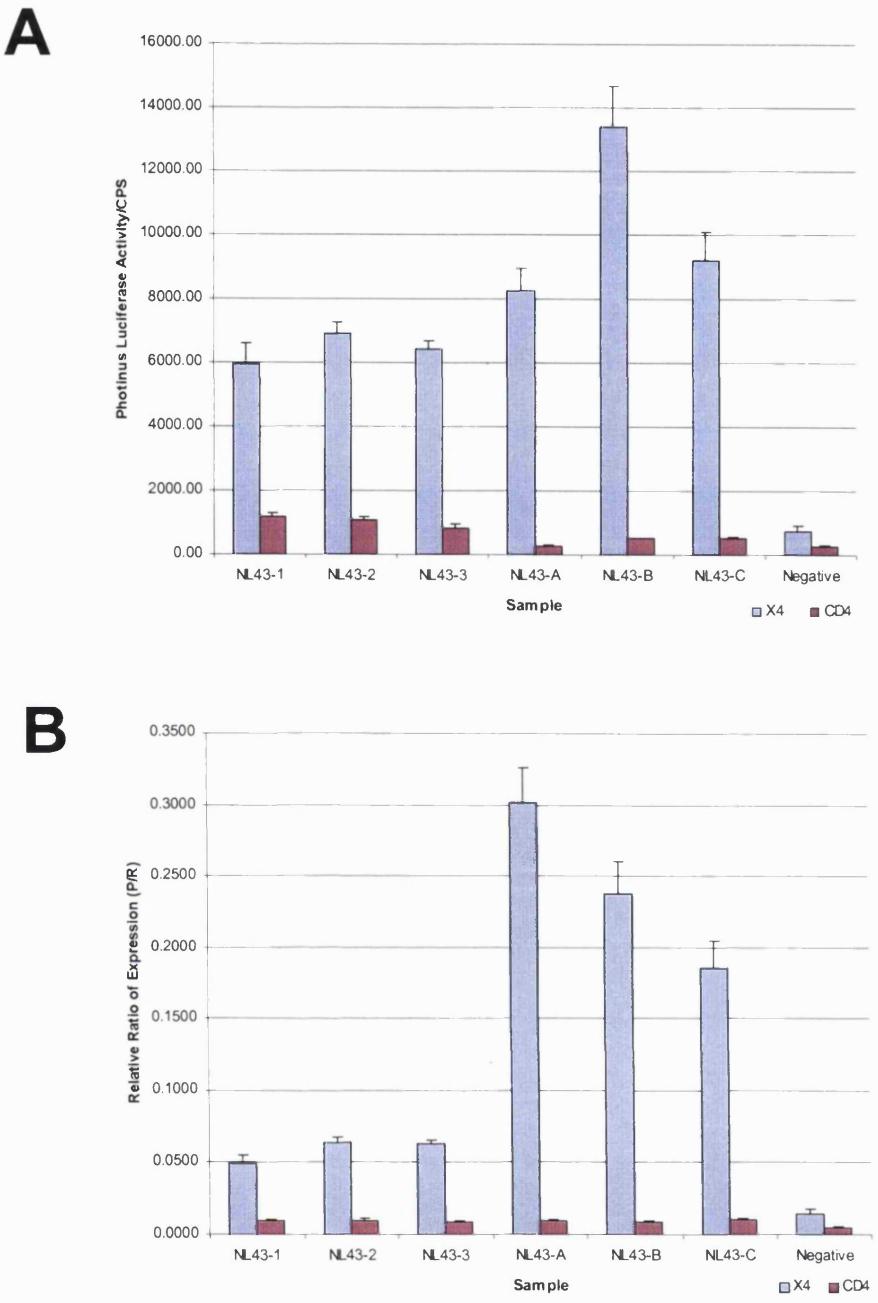


Figure 3.6. Relative expression of luciferase by NL43 Env.

NL43-1 to NL-3 represent independent transfections and infections, while NL43-A to NL43-C represent independent transfection of pooled infected cells to assess standardisation of vTF7-3 infection. The results show that net *Photinus* luciferase production is specific to $NL43^{+}$ effector cells co-cultured with NP2/CD4 cells expressing CXCR4, and all results were significantly greater than the background ($P<0.05$) (A). The effects of adjusting *Photinus* expression with *Renilla* expression are shown in (B). Results indicate independent transfection and infection does not affect expression levels as much as transfecting a previously infected population of effector cells.

Following 7h incubation, the wells were scrutinised for cell fusion events using light microscopy. Syncytia were not observed in the absence of Env expression (figures 3.7-9). The syncytia forming ability of the $NL43_{WT}$ (Figure 3.8) and $JRFL_{WT}$ (3.9) control Envs were specific for the correct co-receptor expressing NP2 target cells, and considerably more apparent in $JRFL_{WT}$-expressing effector cells co-cultured with R5 target cells than cells expressing $NL43_{WT}$ Env co-cultured with X4 target cells.

The determination of luciferase activity in the cell-cell fusion assay shows that in relation to the $NL43_{WT}$ and $JRFL_{WT}$ Envs as the wildtype controls, the activity of cleavage defective NL43 mutants exhibited lower activity (Figure 3.10 and Table 3.3). The cell-cell fusion assay supported microscopic observations that fusogenic capacity of $JRFL_{WT}$ Env was greater than $NL43_{WT}$ Env (Figure 3.8-9). The observed specificity of fusion exhibited by the control Envs is representative of multiple experiments, demonstrating retention of their co-receptor specificity and reproducibility across independent experiments.

It is therefore expected that the $NL43_{WT}$-derived mutant Envs would exhibit activity less than or equal to $NL43_{WT}$ depending on the effects of the mutations in limiting gp160 processing. Mutants involving the central residues of the REKR (Site 1) motif exhibited reduced fusion (M1S1, M2S1), consistent with literature, while the other 3 exhibited negligible activity (Figure 3.11). None of the mutants in the KAKRR (Site 2) exhibited any obvious fusion activity. In the case of the Envs mutated in the conserved VVQ residues between the basic sites, all 4 mutants were able to mediate membrane fusion. The PVSF and EVSF mutants were able to elicit levels of luciferase comparable to $NL43_{WT}$, whereas the VPSF and VVF mutants had significantly reduced fusogenic capacity.

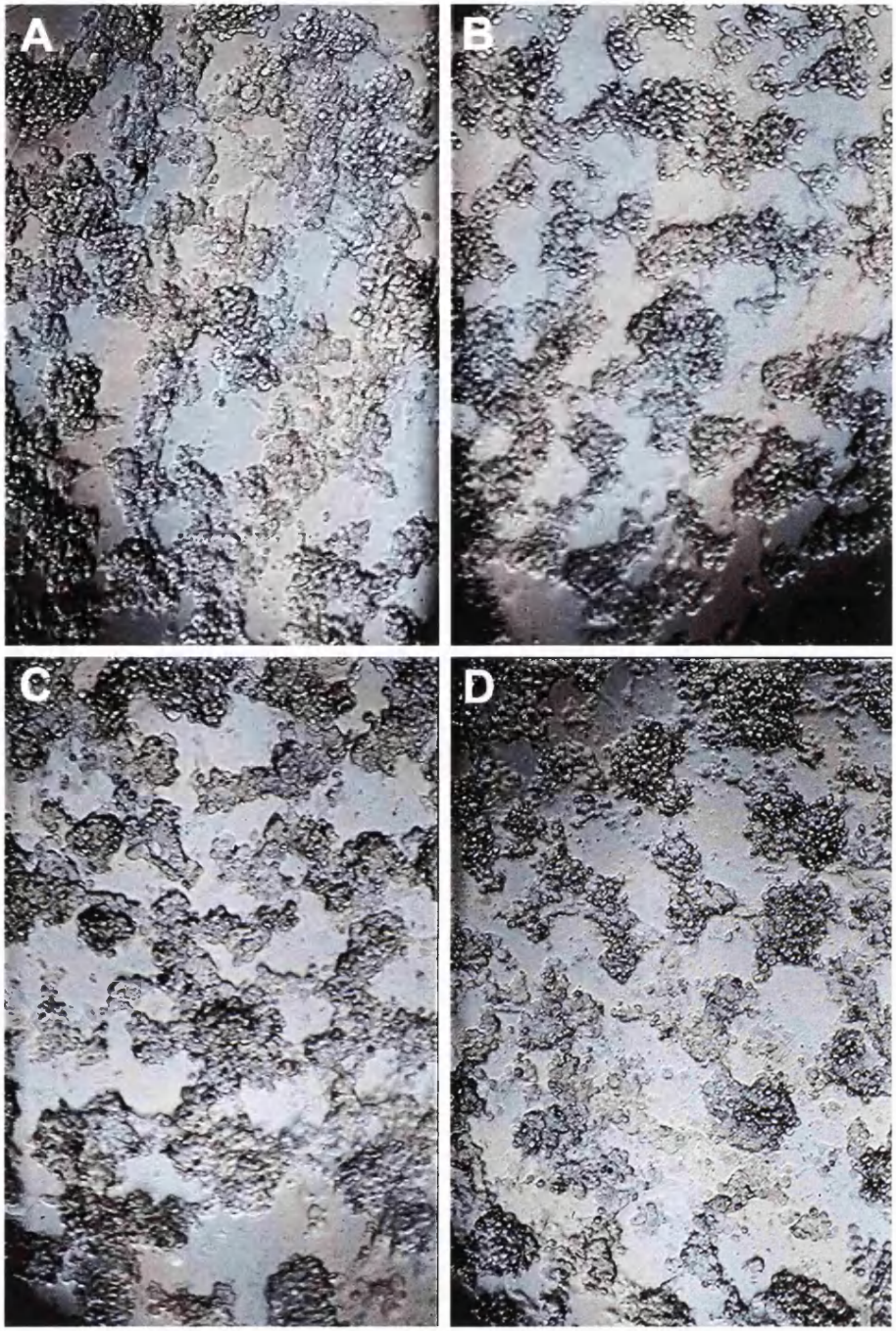


Figure 3.7. Co-culture of NP2 target cells with effector cells not expressing Env.
The targets cells in the different panels are: NP2/CD4 (A); NP2/CD4 (B); NP2/CD4/CCR5 (C) and NP2/CD4/CXCR4 (D). Cells were not infected with $vSIMB_{E/L}$ in panel A. Each panel represents an area of 12631μm^2 viewed at 40x magnification.

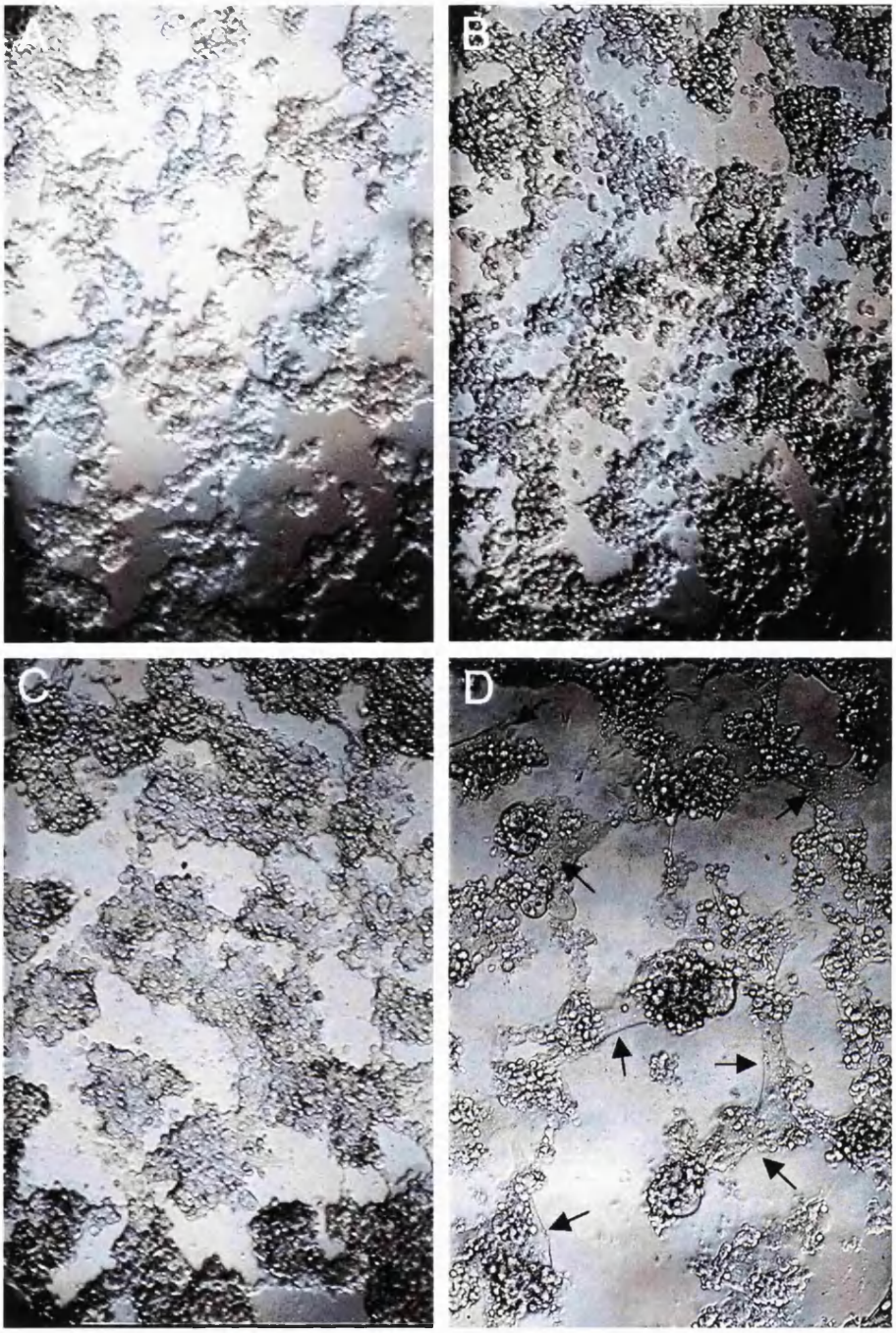


Figure 3.8. Co-culture of NP2 target cells with effector cells expressing $NL43_{WT}$ Env. The targets cells in the different panels are: NP2/CD4 (A); NP2/CD4 (B); NP2/CD4/CCR5 (C) and NP2/CD4/CXCR4 (D). Cells were not infected with $vSIMB_{E/L}$ in panel A. Syncytia are indicated by arrows. Each panel represents an area of 12631μm^2 viewed at 40x magnification.

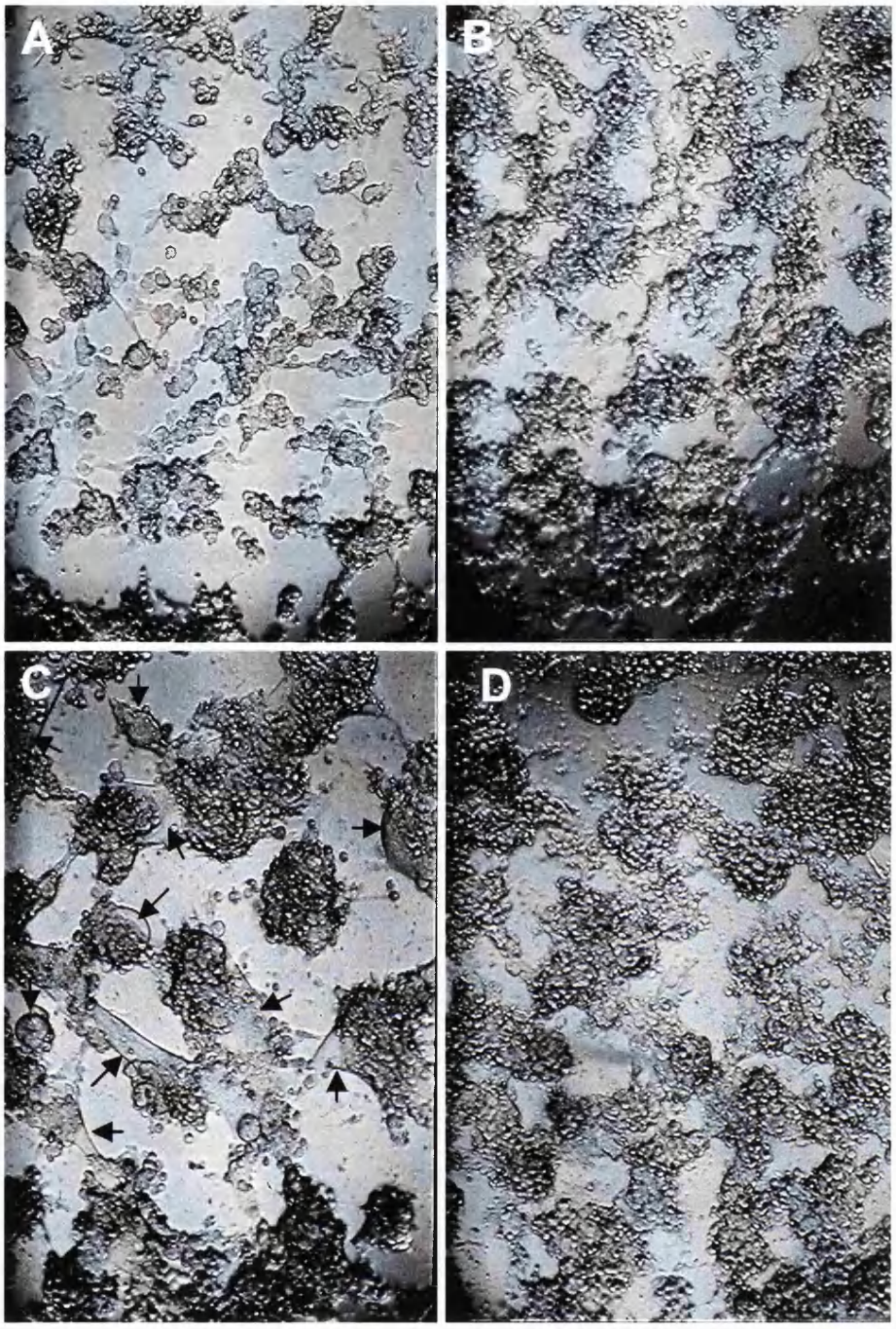


Figure 3.9. Co-culture of NP2 target cells with effector cells expressing $JRFL_{WT}$ Env. The targets cells in the different panels are: NP2/CD4 (A); NP2/CD4 (B); NP2/CD4/CCR5 (C) and NP2/CD4/CXCR4 (D). Cells were not infected with $vSIMB_{E/L}$ in panel A. Syncytia are clearly observed in panel C (Arrows). Each panel represents an area of 1263 1μm^2 viewed at 40x magnification.

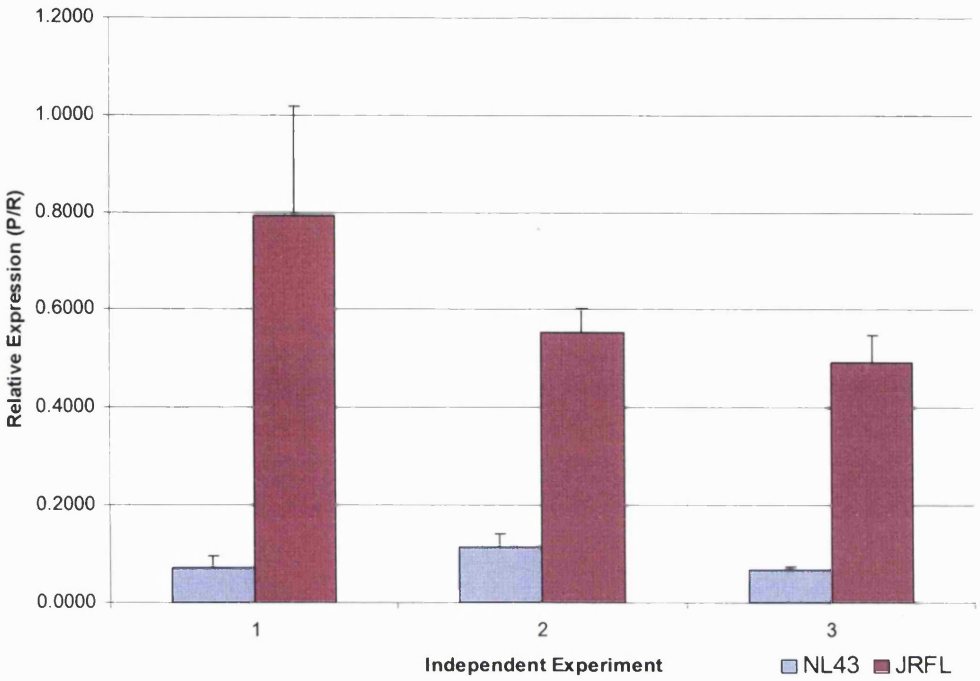


Figure 3.10. Fusogenic capacity of $JRFL_{WT}$ and $NL43_{WT}$ Envs.

The relative expression of mean *Photinus* luciferase/mean *Renilla* luciferase elicited by NL43 and JRFL Envs across independent experiments is shown. Results represent quadruplicate samples and bars indicate standard deviations. All results are significantly greater than background expression levels as determined by the Student's T-test ($P<0.05$).

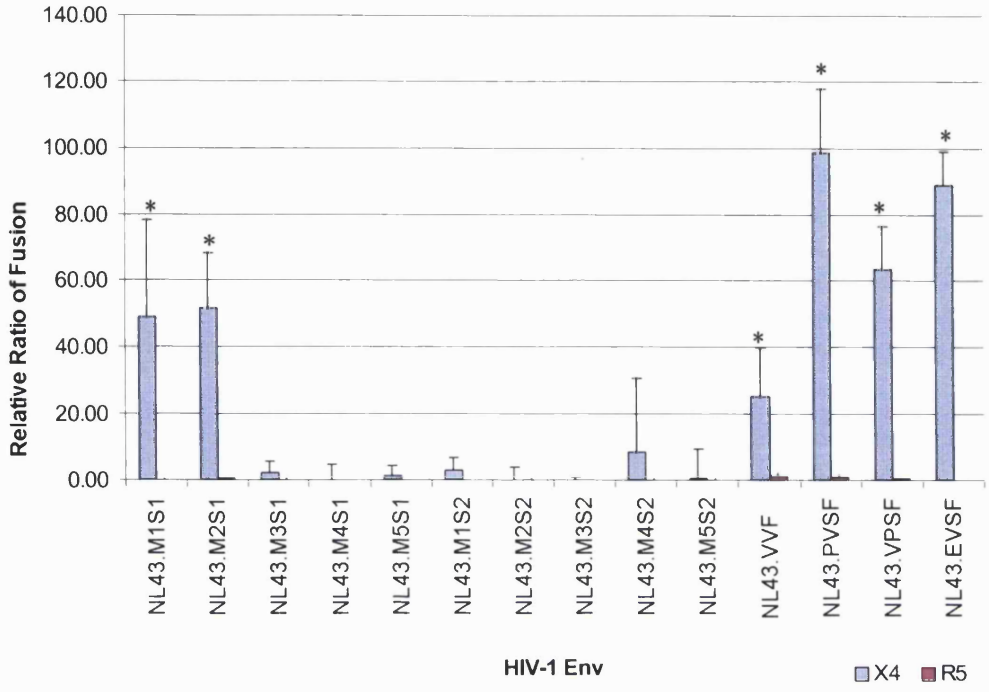


Figure 3.11. Fusogenic capacities of NL43 mutant Envs.

The relative ratio of fusion in relation to the activities of $NL43_{WT}$ and $JRFL_{WT}$ wildtype Envs. Results represent the average of quadruplicate assays and standard deviations.

* indicates expression significantly greater than background.

HIV-1 Env	P(≠BG)	P(≠NL43$_{WT}$)
NL43.M1S1	0.0209	0.0313
NL43.M2S1	0.0034	0.0281
NL43.M3S1	0.1645	0.0048
NL43.M4S1	0.2954	0.0037
NL43.M5S1	0.2420	0.0048
NL43.M1S2	0.1474	0.0049
NL43.M2S2	0.3132	0.0041
NL43.M3S2	0.2012	0.0038
NL43.M4S2	0.2445	0.0028
NL43.M5S2	0.4709	0.0037
NL43.VVF	0.0191	0.0071
NL43.PVSF	0.0008	0.4723
NL43.VPSF	0.0003	0.0573
NL43.EVSF	0.0000	0.2780

Table 3.3. Student's T-test determining the significance of fusion elicited by Env mutants relative to background (BG) or NL43$_{WT}$ Env.
Significant fusogenic activity above background is indicated in red, while fusogenic potential similar to NL43$_{WT}$ is indicated in blue.

3.3.4 Ghost cell Infection Assay

Chimeric viruses expressing $NL43_{WT}$ and $JRFL_{WT}$ Envs were able to infect R5 or X4 Ghost cell lines to elicit GFP production. The extent of cytopathic effects (CPE) was considerably greater in Ghost cell cultures infected with $C2.JRFL_{WT}$ than $C2.NL43_{WT}$ chimeras after 48h. While $C2.JRFL_{WT}$ exhibited specific tropism for the R5 Ghost cells, it was observed that the $C2.NL43_{WT}$ chimeric virus was able to infect all three Ghost cell lines; this is due to the previously mentioned endogenous expression of CXCR4. FACS analysis of the Ghost cell infection assay after 48h of culture showed $C2.NL43_{WT}$ and $C2.JRFL_{WT}$ cultures to contain low percentages of GFP positive cells (Table 3.4), however, only live cells were monitored and they represented a minor population of the total cell number. Of the 10 C2.NL43 constructs with mutations in site 1 or site 2, only C2.NL43.M1S1 and C2.NL43.M2S1 were able to infect X4 Ghost cells and at a reduced level compared to $C2.NL43_{WT}$ (Table 3.4). Very little CPE was observed in cultures infected with the other mutant chimeric viruses.

Lysates of chimeric viruses were probed with ARP401 antisera to assess gp160 processing and incorporation into virions (Figures 3.12). Whilst gp160 was detectable in all viruses, gp120 was observed only in the viruses that are capable of infecting Ghost X4 cells. Hence mutations in the gp160 processing sites do not appear to prevent incorporation of the unprocessed precursor into virions. Although cell lysis may release gp160 into the supernatant, our virion preparations are unlikely to be contaminated with non-virion associated gp160 as pellets were washed during preparation. Moreover, the preparation of virus lysates in the presence of PEG-8000 indicated additional proteins of similar or lower mass to gp160 are precipitated (Figure 3.12), indicating exogenous gp160 is unlikely to be recovered.

The VVQ mutant Envs show discordant results between cell-cell fusion and chimeric virus infection (Figure 3.11 and Table 3.4).

Chimeric Virus	Processing Site[a]	Ghost Cell Lines/ % GFP Positive[b]		
		CD4	CD4/CXCR4	CD4/CCR5
C2.NL43	KAKRRVVQREKR	0.27	3.01	0.09
C2.JRFL	KAKRRVVQREKR	0.05	0.00	3.06
C2.NL43.M1S1	KAKRRVVQREER	0.10	1.61	0.03
C2.NL43.M2S1	KAKRRVVQRNER	0.12	0.88	0.06
C2.NL43.M3S1	KAKRRVVQREKT	0.02	0.00	0.00
C2.NL43.M4S1	KAKRRVVQSHEQ	0.01	0.02	0.00
C2.NL43.M5S1	KAKRRVVQSEHN	0.03	0.03	0.00
C2.NL43.M1S2	KAKERVVQREKR	0.03	0.02	0.00
C2.NL43.M2S2	KAKEGVVQREKR	0.02	0.01	0.00
C2.NL43.M3S2	KAKSRVVQREKR	0.02	0.00	0.01
C2.NL43.M4S2	NAISSVVQREKR	0.00	0.01	0.00
C2.NL43.M5S2	KAQNGVVQREKR	0.00	0.00	0.00
C2.NL43.VVF	KAKRR--QREKR	0.01	0.02	0.00
C2.NL43.PVSF	KAKRRPVQREKR	0.01	0.00	0.00
C2.NL43.VPSF	KAKRRVPQREKR	0.01	0.01	0.00
C2.NL43.EVSF	KAKRREVQREKR	0.01	0.01	0.00
Negative	-	0.01	0.01	0.00

Table 3.4. Determination of chimeric virus infection of Ghost cell lines by FACS analysis.

The processing site of the mutant Env expressed by the chimeric virus is indicated. Ghost cell lines were infected with 300ng p24 of each chimeric virus and harvested 48h post-infection. 10,000 live cells were analysed. Results are representative of duplicate experiments.

[a] Mutated residues are indicated in red.

[b] Ghost cell lines expressing CD4, or CD4 with CXCR4 or CCR5.

In the case of the chimeric viruses expressing VVQ mutant Envs, the levels of incorporation appear to be less than for C2.NL43$_{WT}$ (Figure 3.13). Analysis of the lysates of 293T cells producing chimeric viruses with ARP401 polyclonal antisera indicate adequate gp160 was produced intracellularly (Figure 3.14A), whereas the D7324 antisera were unable to detect gp160 suggesting that the VVQ motif is an important determinant of epitopes recognised by the D7324 antisera. A small amount of gp120 in mutants NL43.VVF, NL43.PVSF and NL43.VPSF is observed (Figure 3.14B), however, the amount of processed gp160 is far less than that observed in chimeric viruses expressing NL43$_{WT}$ and JRFL$_{WT}$ Envs, and explains why these mutant viruses are not able to infect Ghost cells. Considering 293T cells were used as effector cells in the fusion assay and as producer cells for chimeric viruses, it is expected that the Envs would be processed in a similar manner and hence, function similarly. The results suggest a difference in gp160 processing in these two systems.

3.3.5 Processing of VVQ Mutant Env in Cell-Cell Fusion Assays

The expression of VVQ mutant Envs in 293T cells exhibited a high level of gp160 when driven by vTF7-3, similar to that observed with NL43$_{WT}$ (Figure 3.15A). Whilst it is not easy to discern gp120 from gp160 in the cells expressing NL43$_{WT}$, gp120 usually runs with the 97kDa marker, and in the lysates of cells expressing VVQ mutant Envs, there does appear to be less product there than in the NL43$_{WT}$ Env expressing cells. The reduced processing in this system is concordant with that observed in the lysates of chimeric viruses expressing these Envs (Figure 3.13B).

Post-cell-cell fusion lysates were probed with ARP401 antibodies to determine if Env processing had occurred during the fusion assay. Fusions assays involving JRFL$_{WT}$ Env show nearly equal amounts of gp160 and gp120 in the lysates, while cell fusions with NL43$_{WT}$ Env show more gp160 precursor than processed gp120 (Figure 3.15B). This may be a contributing factor as to why the fusogenic capacity of JRFL Env is greater than NL43. In the case of the VVQ mutants, greater gp160 expression is apparent. A product corresponding to gp120 is observed at high levels in the VVF and PVSF mutants, indicating processing had occurred (Figure 3.15B).

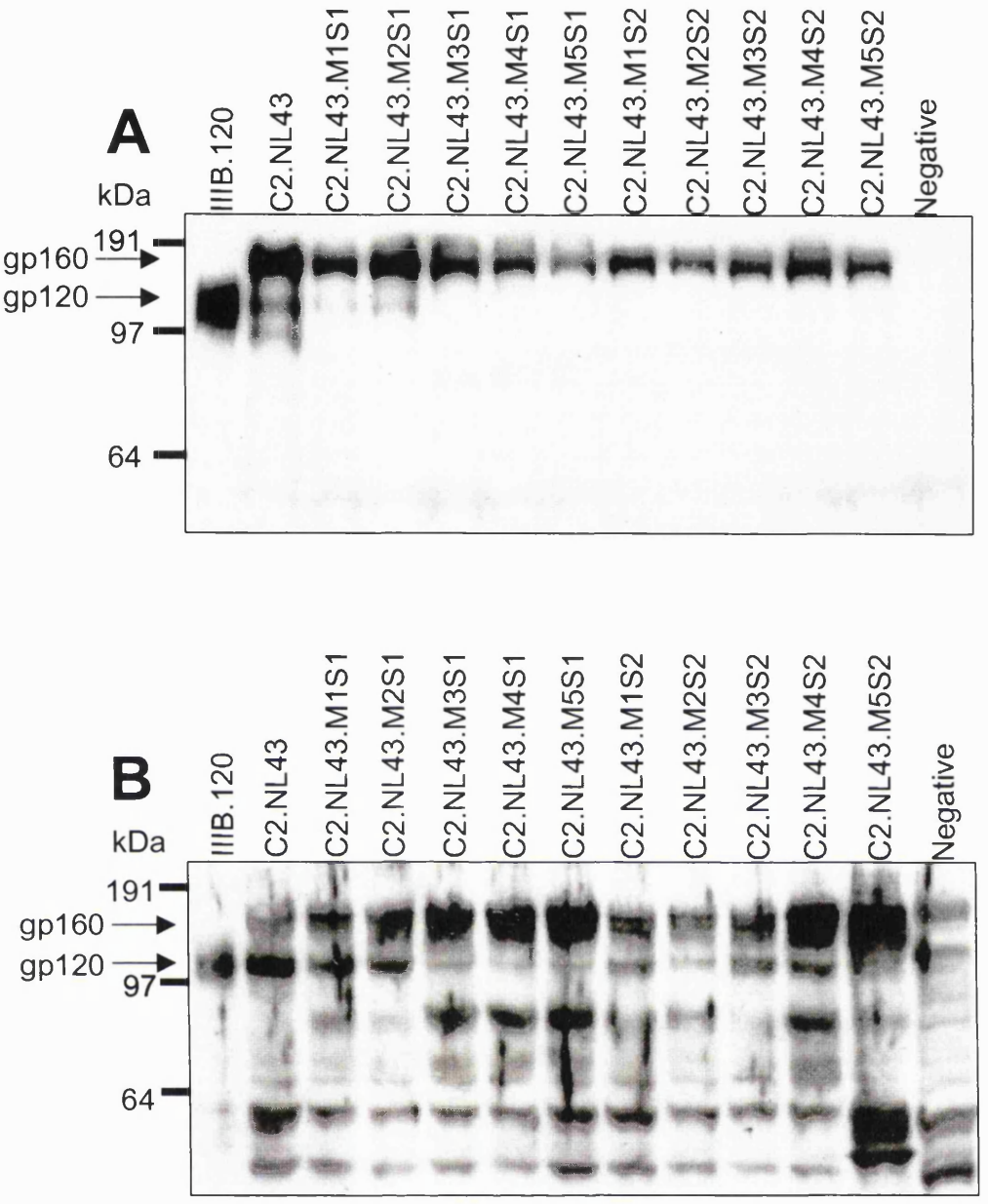


Figure 3.12. Detection of gp160 and gp120 in chimeric virus lysates.

Virus particles were harvested by centrifugation of 0.45mm filtered supernatants from 293T cells transfected with pC2.*env* plasmids in the absence (A) or presence of PEG 8000 (B). Chimeric virus lysates were probed with ARP401. Infection was observed with $C2.NL43_{WT}$ and mutants C2.NL43.M1S1 and C2.NL43.M2S1, coincident with detection of gp120 in these viruses in PEG 8000-free preparations. The sizes of gp160 and gp120 are indicated.

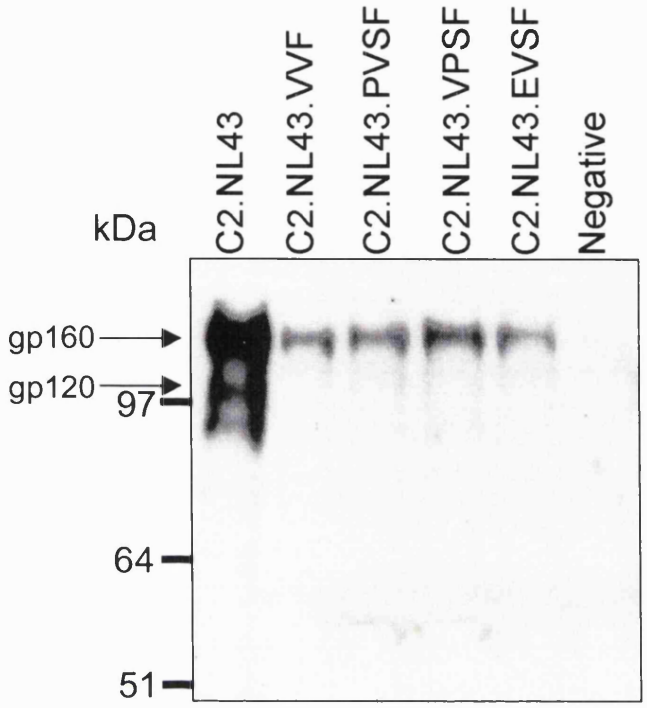


Figure 3.13. Detection of gp160 in lysates of chimeric viruses expressing NL43.VVQ mutant Envs.

Lysates were probed using ARP401 polyclonal antisera. Lysates were prepared in the absence of PEG 8000. Only NL43WT exhibited gp160 processing, moreover, there appears to be less Env incorporated into the virions as equivalent levels of p24 were prepared. The sizes of gp160 and gp120 are indicated.

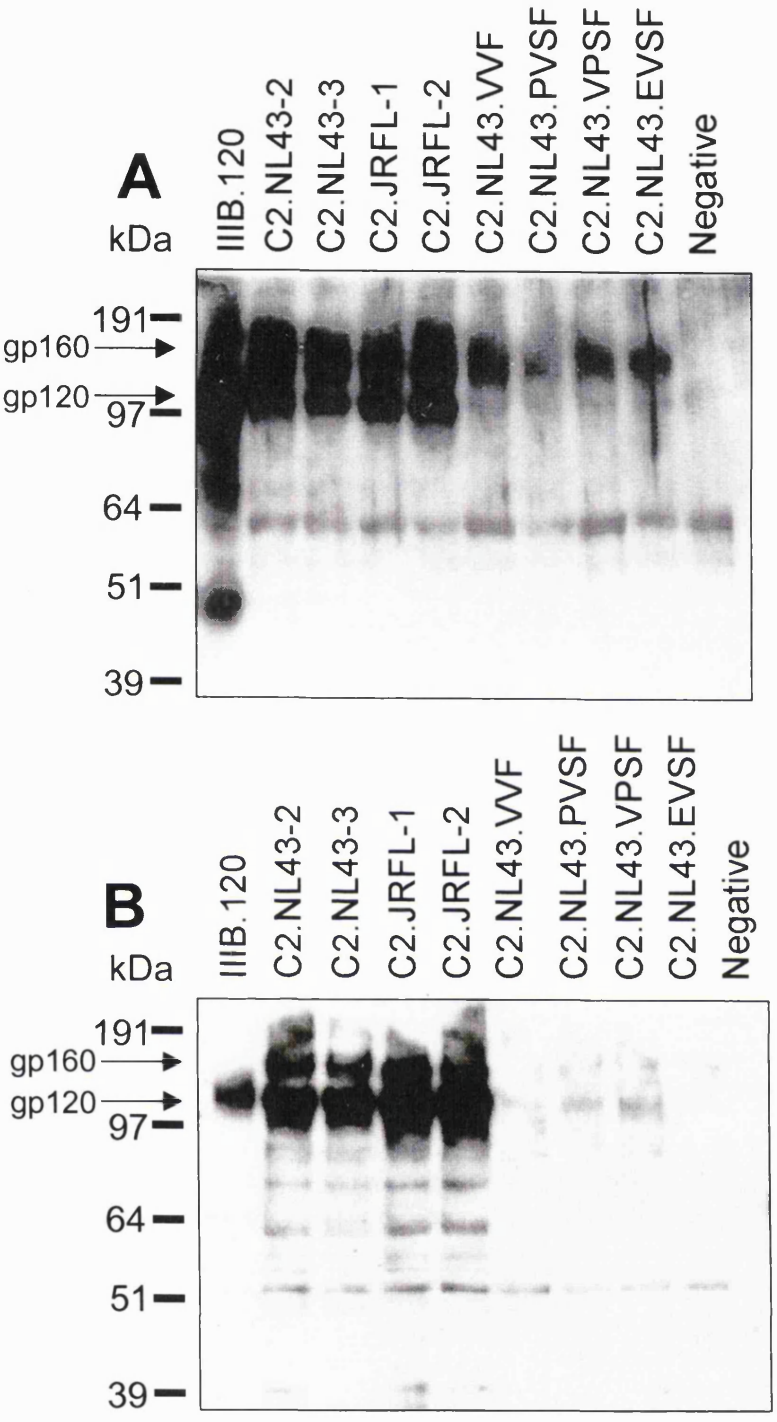


Figure 3.14. Detection of gp160 and gp120 in lysates of 293T cells producing chimeric viruses expressing NL43.VVQ mutant Envs.

Probing with ARP401 antibodies (A), probing with D7324 antibodies raised against a peptide containing the KAKRRVVQREKR sequence (B).

The greater extent of gp160 processing of the PVSF construct correlates with its higher fusogenic capacity amongst these mutants, although the results suggest processing is not specific to the receptor. These results suggest processing of the VVQ mutant Envs occurs at some point during co-culture with NP2 target cells during the cell-cell fusion assay, as there appears to be minimal Env processing in the 293T cells in the absence of NP2 target cells (Figure 3.15A).

3.3.6 $TCID_{50}$ Determination

It is possible that there may be some factor unique to NP2 cell lines but not Ghost cell lines that allows the VVQ mutants to mediate membrane fusion, thus C2 chimeras were used to infect NP2 cell lines. $TCID_{50}$ assays were conducted to determine the infectious titres of the chimeric viruses expressing $NL43_{WT}$ and mutant Envs in the NP2/CD4/X4 cell line (NP2/CD4/CCR5 for $JRFL_{WT}$).

As determined by HIV-1 p24 ELISA, $TCID_{50}$ titrations were only obtainable for those chimeric viruses expressing the wildtype Envs and mutants NL43.M1S1 and NL43.M2S1 (Table 3.5) and coincident with cytopathic effects in the cultures. A small number of syncytia were observed in the cultures of the VVQ mutants, but cytopathic effects were absent, and p24 was minimal. Thus, it appears that the chimeric virus stock did contain a few infectious particles, but these viruses were not able to mediate a productive infection.

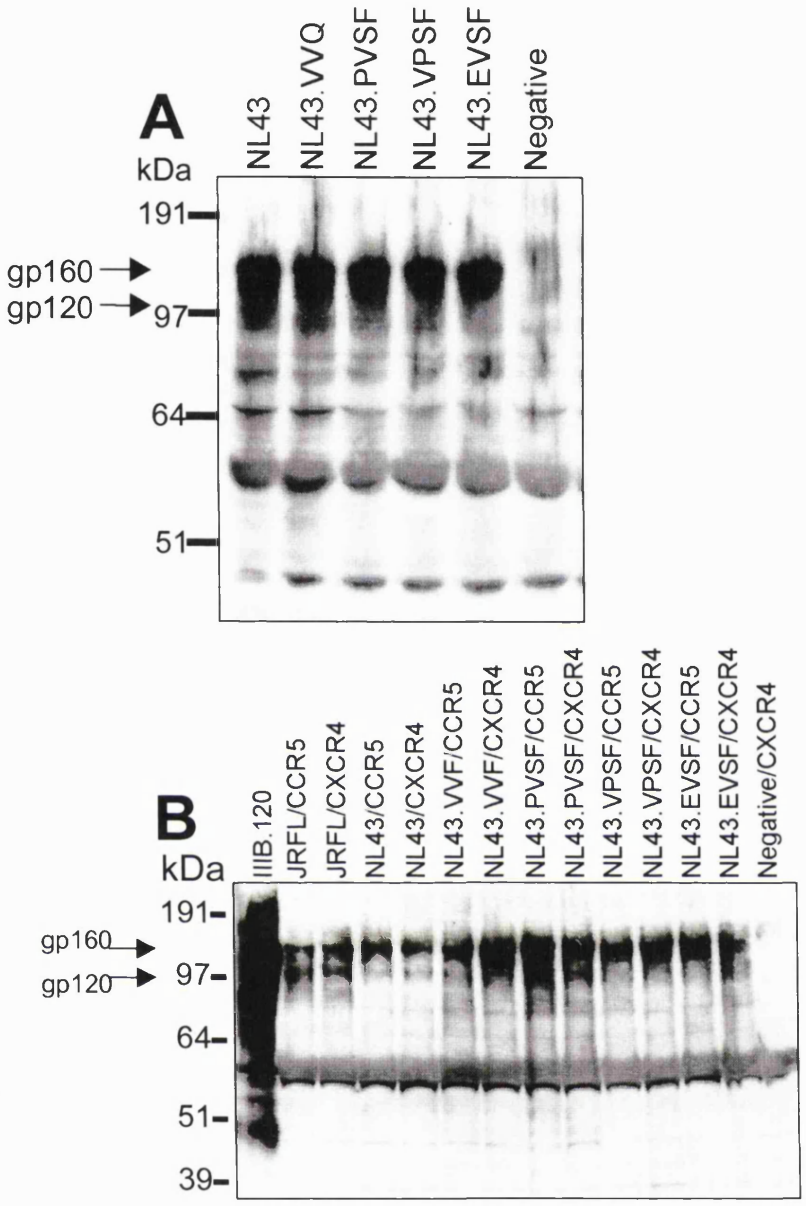


Figure 3.15. Analysis of vTF7-3 driven Env expression in lysates of 293T cells effector cells.

In prefusion effector cell lysates (A), gp160 is predominantly observed in the NL43.VVQ mutants. Postfusion effector and target cell lysates (B), the co-receptor expression of the target cell is indicated.

Chimeric Virus	$TCID_{50}$/ml	Syncytia[b]
C2.JRFL[a]	100000	Abundant
C2.NL43.	1000000	Abundant
C2.NL43.M1S1	56234	Abundant
C2.NL43.M2S1	56234	Abundant
C2.NL43.M3S1	0	None
C2.NL43.M4S1	0	None
C2.NL43.M5S1	0	None
C2.NL43.M1S2	0	None
C2.NL43.M2S2	0	None
C2.NL43.M3S2	0	None
C2.NL43.M4S2	0	None
C2.NL43.M5S2	0	None
C2.NL43.VVF	0	Few
C2.NL43.PVSF	0	Few
C2.NL43.VPSF	0	Few
C2.NL43.EVSF	0	Few

Table 3.5. $TCID_{50}$ of chimeric virus stocks titrated in NP2/CD4/CKR cell lines.

[a] Infection of NP2/CD4 cell line expressing CCR5 co-receptor.

[b] Observation of syncytia at 10^{-1}-10^{-2} dilutions of virus.

3.4 Discussion

The cell-cell fusion assay for assaying HIV-1 *env*-genes has been verified using processing-defective *env*-gene products. The laboratory adapted HIV-1_{NL43} and HIV-1_{JRFL} *env*-genes served as positive controls for specific CXCR4 and CCR5 usage respectively. The limiting factors of the assay would be the infection of cells with an appropriate m.o.i. of recombinant vaccinia to produce sufficient RNA polymerases and the transcription rate of the reporter enzyme following membrane fusion. The amount of recombinant vaccinia virus required for gp160 expression and luciferase expression is determined by titration between batches, and experiments indicate that the transfection/infection strategy elicits reproducible levels of normalised reporter enzyme activity across independent assays. Given that SP6 RNA polymerase is active in the cytoplasm, thereby negating the need for nuclear translocation of the reporter plasmid so the latter concern should not be a problem.

The use of HIV-1 Envs mutated in the basic motifs of the KAKRRVVQREKR processing site demonstrated the assay could discern between functional and non-functional Envs, *Photinus* luciferase was only expressed in the event of cytoplasmic mixing following Env-mediated cell-cell fusion. In support of many studies, mutations in either of the basic motifs were able to abrogate processing, and therefore membrane fusion (Binley *et al.*, 2002; Bosch and Pawlita, 1990; Bosch and Pfeiffer, 1992; Dubay *et al.*, 1995; Freed *et al.*, 1989; Guo *et al.*, 1990; Kieny *et al.*, 1988; McCune *et al.*, 1988; Rovinski *et al.*, 1995; Willey *et al.*, 1991). Alteration of the terminal arginine residue of site 1 prevented gp160 processing, indicating the importance of this residue in interacting with the protease active site. Mutation of the penultimate lysine residue to glutamic acid reduced the efficiency of processing and fusogenic activity, and this was reflected in the reduced infectivity of the chimeric virus. The other residues in site 1 are also involved in interactions with the protease active site, as shown when multiple changes were introduced.

In support of other studies, multiple changes in site 2 abrogated the processing of gp160 (Bosch and Pawlita, 1990; Bosch and Pfeiffer, 1992; Kieny *et al.*, 1988). Our studies consistently failed to detect any processing at site 2 despite using the same D7324 antisera as Fenouillet and Gluckman (Fenouillet and Gluckman, 1992). It is possible that aberrant folding and maturation of gp160 may occur in different cell lines and using

different expression systems, thereby rendering the second site more accessible than the primary cleavage site in those gp160 species. This point may be exemplified by our observations that a single amino acid substitution in the site 2 motif of M1S2 and M3S2 (KAKRR to KAKER and KAKSR respectively) was able to abrogate processing and function in our system, yet contrasted those results published by Dubay *et al.* (1995) using a similar mutant. However, our use of chimeric viruses to assess infectivity and therefore fusion reflected observations of the cell-cell fusion assays indicating the manner of expression did not affect the fusogenic capacity of these particular Env mutants in our system.

In the case of the VVQ mutants, reduction in the fusogenic capacity observed is not unexpected, as the non-conservative change of Gln to an acidic Asp residue in the EVSF mutant may exert charge-related interference with a processing enzyme, similar to the M1S2 mutant. The VVF, PVSF and VPSF mutants most likely perturb the helical nature of the local region, thus disrupting the exposure of site 1. However, despite the observed fusogenic capacities of these Envs, they were not able to mediate infection by chimeric viruses. Lineberger *et al.* (2002) have shown that the density of gp120/41 at the surface determines the extent of cell-cell fusion, and it is likely to be an important parameter in determining the ability of a virion to infect a cell. 293T cells transfected with pC2 encoding these chimeric Envs indicate a lack of Env processing, yet neither was processing to any obvious extent observed in vTF7-3-driven Env expression off the pQ7 plasmid. Whilst it cannot be excluded that vTF7-3-driven transcription in the cytoplasm of 293T cells may misdirect a proportion of Env transcripts through a translation and maturation pathway different than normal, enabling the precursor to encounter other proteolytic enzymes, processing must still occur at the primary site to generate functional glycoprotein. The fact that the other eight unprocessed mutants exhibited less extreme structural perturbation at the cleavage site suggests that this is not the explanation. Protease(s) encoded by the recombinant vaccinia vTF-7 are unlikely to be involved in processing, as other members of our laboratory group routinely use vTF7-3 to express Env in processing-deficient CV-1 cells and processing is never observed.

Overexpression of the mutant Envs may saturate cellular chaperones and other proteins (such as enzymes involved in glycosylation) involved in protein folding and maturation, resulting in a proportion of mis-folded Envs that may adopt a conformation exposing

the processing loop to processing enzymes. The HIV-1 LTR-driven expression of Env from pC2 is reduced compared to vTF7-3-driven pQ7 expression. By this hypothesis, reduced Env production by pC2 would not saturate processes involved in its maturation, thus less mis-folded Env is produced, and subsequently non-infectious chimeric viruses are produced.

Qualitative differences in the Env species between cell-cell fusion assays and virus infection assays must relate to some factor that is permissive for Env function in the former. The presence of gp120 in the lysates of the effector cells expressing PVSF and VVF mutant Envs following cell-cell fusion indicates processing of gp160 occurs at some point during co-culture with NP2 target cells. It is thus possible that uncleaved gp160 is exported to the cell surface, where interaction with CD4 induces a conformational change in gp160 to expose the primary cleavage site to a cell surface protease. Studies have demonstrated that plasmin is able to process gp160 correctly into gp120/41 (Okumura *et al.*, 1999), thrombin can cleave within the V3 loop and it is possible for furin to be exported to the cell surface (Moulard and Decroly, 2000; Staropoli *et al.*, 2000). It has been documented similarly for Influenza A virus that exogenous factors, such as plasmin, are capable of activating glycoproteins (Steinhauer, 1999). Notably, the neuraminidase of the WSN isolate of Influenza has been shown to bind plasmin to effect processing of the HA0 precursor on the viral surface (Goto and Kawaoka, 1998).

Further support for extracellular processing of Env comes from groups who have demonstrated that synthetic multibranched peptides and linear peptides derived from the processing region are able to inhibit a range of laboratory-adapted Envs in mediating syncytium formation and HIV-1 infection (Bahbouhi *et al.*, 2002; Barbouche *et al.*, 2002; Sabatier *et al.*, 1996). The inhibitory activity of these peptides is conditional on the presence of both basic motifs, and in particular the integrity of the site 2 KAKRR motif (Bahbouhi *et al.*, 2002; Barbouche *et al.*, 2002; Sabatier *et al.*, 1996). The post-CD4/CXCR4 binding block against processed gp120/41 observed by Barbouche *et al.* (2002) suggests an interference with the membrane fusion event. These inhibitory peptides are able to bind to a molecule on the lymphocyte surface, and the low inhibitory concentrations required to inhibit syncytium formation in vaccinia-driven Env expression systems suggests that the peptides block a surface molecule, not CD26,

thrombin or plasmin, that is present in low concentrations. In relation to this, the size of a virus particle limits the amount of Env oligomers present on the surface, and heterogeneity in functionality of oligomers contributes to a reduced likelihood of interaction with host factors present at low concentrations on the cell surface. Additionally, the observation that synthetic peptides corresponding to the C helix of gp41 are able to inhibit cell-cell fusion 15 min after co-culture of cells leaves a time frame for an additional event after CD4/co-receptor binding to trigger conformational change in the gp41 ectodomain into the six-helix bundle, such as further processing of gp120 at the cell surface, since acid activated glycoproteins are able to trigger fusion rapidly (Gallo *et al.*, 2001).

Thus, evidence is accumulating to suggest the site 2 KAKRR motif has a dual-role in the virus lifecycle; in the intracellular processing of site 1, and its processing to trigger release of the fusion peptide. Cleavage at this site in gp120/41 may induce release of the gp120 subunit and allow gp41 to undergo the final conformation changes to insert the fusion peptide into the target cell membrane. Studies have described processing occurring at a low frequency at the N-terminus of site 2 (Fenouillet and Gluckman, 1992; Morikawa *et al.*, 1993), yielding monomeric gp41 possessing polar amino acids at the head of the fusion peptide. The F_0 glycoprotein precursor of human Respiratory Syncytial Virus (HRSV) is involved in membrane fusion possesses two basic cleavage motifs separated by 22 amino acids (Begona Ruiz-Arguello *et al.*, 2002; Gonzalez-Reyes *et al.*, 2001). The F_0 precursor requires processing at both sites to activate the fusion function, although cleavage at the downstream site only is necessary to liberate the fusion peptide. The double cleavage leads to an observable conformational change that may be representative of a post-activation form. Questions arise as to the activation trigger, as intracellular processing at both sites would lead to the surface expression of 'triggered' F protein. Hypothetical solutions are that F protein oligomers are in different states of processing, or the G protein oligomers are involved in modulating the activation. While the expression of F protein alone is sufficient for mediating cell-cell fusion, HRSV has a slow rate of cell infection (Begona Ruiz-Arguello *et al.*, 2002; Gonzalez-Reyes *et al.*, 2001). Perhaps in the case of HRSV, processing occurs at one site intracellularly, and then interaction of the G protein with the target cell receptor elicits an interaction between a host surface protease with the other cleavage site on the F protein to trigger activation of the fusion peptide.

This study is the first to describe a single residue substitution in the site 2 motif, KAKRR, capable of abrogating Env processing and function when studied independently of other viral proteins or in the context of an infectious molecular clone. This provides further support for roles of this motif in addition to presentation of the site 1 REKR motif. Hitherto undescribed non-conservative substitutions in the highly conserved VVQ motif also raise questions regarding possible processing of gp160 in the external milieu.

Chapter 4

Analyses of the Effects of Step-Wise Deletions in the HIV-1_{NL43} gp41 Cytoplasmic Tail upon the Fusogenic Capacity of Env

4.1. Introduction

4.1.1. HIV-1 gp41 Cytoplasmic Tail

The primary functions of the TM subunit of lentiviral glycoproteins are to anchor the glycoprotein to the virion membrane and to mediate membrane fusion. To fulfil these requirements, there is a hydrophobic membrane spanning domain (MSD) and a hydrophobic fusion peptide at the N-terminus of the ectodomain (Figure 4.1). Lentiviruses are distinguished from simpler retroviruses by virtue of the length of their cytoplasmic tail (CT), around 150-200 amino acids for the former compared to around 30-50 residues for the latter. Compared to the ectodomain and membrane anchor the CT displays more variation between HIV-1 subtypes, despite the constraints imposed by the overlapping reading frames of *tat* and *rev* (Douglas *et al.*, 1997). The functional importance of the length of the CT is indicated by the rare isolation of HIV-1 isolates with gp41 truncations. The culture of certain SIV strains in human cell lines results in the acquisition of a stop codon to truncate the CT to enable increased growth kinetics (Johnston *et al.*, 1993). The subsequent inoculation of these mutants into either macaques or their cultured T cells results in the reversion to full-length CTs, further indicating the necessity of a full-length TM subunit for the pathogenesis of SIV-AIDS (Luciw et al., 1998; Shacklett et al., 1998; Shacklett et al., 2000).

4.1.2. Features of the C-terminal Tail

The HIV-1 gp41 CT has a predominantly hydrophobic character and possesses 3 conserved domains: a hydrophilic 'Kennedy' domain (Kennedy *et al.*, 1986) and two amphipathic helices (AH1 and AH2) (Figure 4.2). Additional properties such as Env internalisation, induction of pore formation, calmodulin interaction, accumulation on basolateral membranes in polarised cells and incorporation into virions are modulated by features of the CT (Douglas *et al.*, 1997).

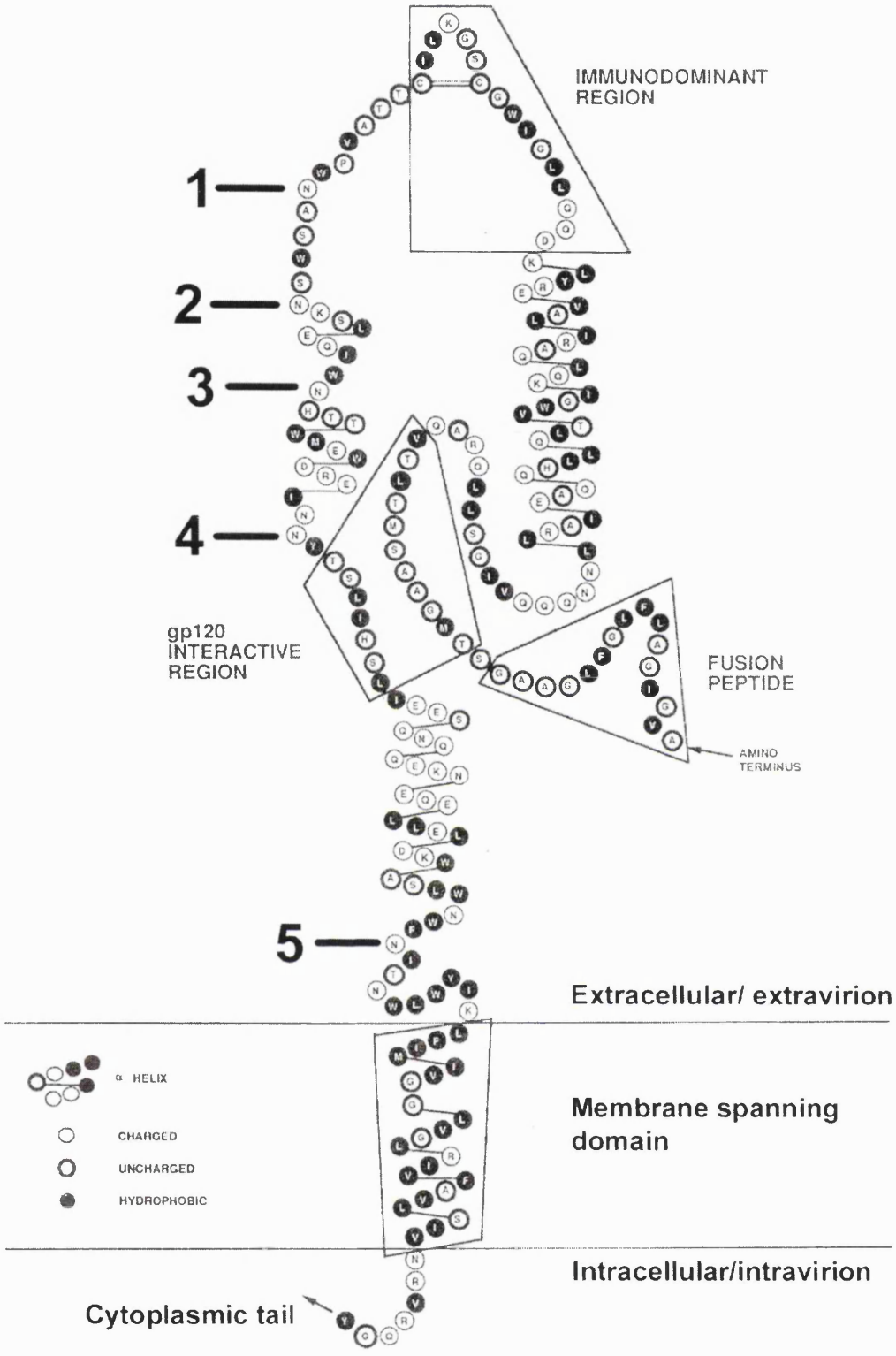


Figure 4.1. Schematic representation of the HIV-1 gp41 ectodomain and transmembrane anchor.

Numbers represent putative N-linked glycosylation sites. The diagram is truncated in the cytoplasmic tail, in HIV-1_{NL43} it is 151 residues. Adapted from Levy, 1998.

4.1.2.1. Membrane Anchor

The hydrophobic MSD is essential for anchorage of the TM subunit to the viral membrane (Haffar *et al.*, 1988). There is a conserved Arg residue in the middle of the MSD that is critical for surface expression and syncytium formation (Owens *et al.*, 1994), as are charged residues flanking the MSD (a Lys residue at the N-terminus and two Arg residues at the C-terminus; Figures 4.1 and 4.2) for the latter. In SIV, progressive truncation of the MSD modulated the efficiency of membrane fusion without affecting surface anchorage, thus indicating a role of the MSD in membrane destabilisation during the fusion event (Lin *et al.*, 2003).

4.1.2.2. Endocytosis

There are two conserved endocytosis motifs YXXϕ (where X indicates any residue and ϕ indicates an amino acid residue with a large side group), one is immediately proximal to the MSD and the other is proximal to the AH2 domain (Figure 4.2). It has been shown that the MSD proximal motif plays a greater role in the endocytosis of Env from the infected cell surface (Egan et al., 1996; Ohno et al., 1997; Rowell, Stanhope et al., 1995). The endocytosis of Env is mediated through interactions with the adaptor chain family of host factors involved in intracellular sorting events. It is possible these may be involved in the targeting of Env species to the basolateral membrane, or mis-folded species to the lysosome and MHC-II compartments (Ohno *et al.*, 1997). Two motifs, a hydrophobic *is*1 and a di-Leu rich *is*2, located downstream of the Kennedy domain and overlapping the AH2 domain (Figure 4.2), have been identified as capable of retaining Env in the Golgi (Bultmann *et al.*, 2001). Thus, mechanisms exist to ensure incorporation of Env into virions and the downregulation of surface expression to avoid detection by the host immune system. Curiously, whilst substitution of the Tyr residue in the membrane proximal YXXϕ motif with Cys or Phe residues increases Env surface expression and confers faster fusion kinetics upon the virus, the virus itself is unable to sustain a productive infection *in vitro*, suggesting an additional role for the conserved YXXϕ motif in the virus lifecycles (West *et al.*, 2002). Basolateral budding in the polarised MDCK cell line is also dictated by the integrity of this particular motif (Lodge *et al.*, 1994).

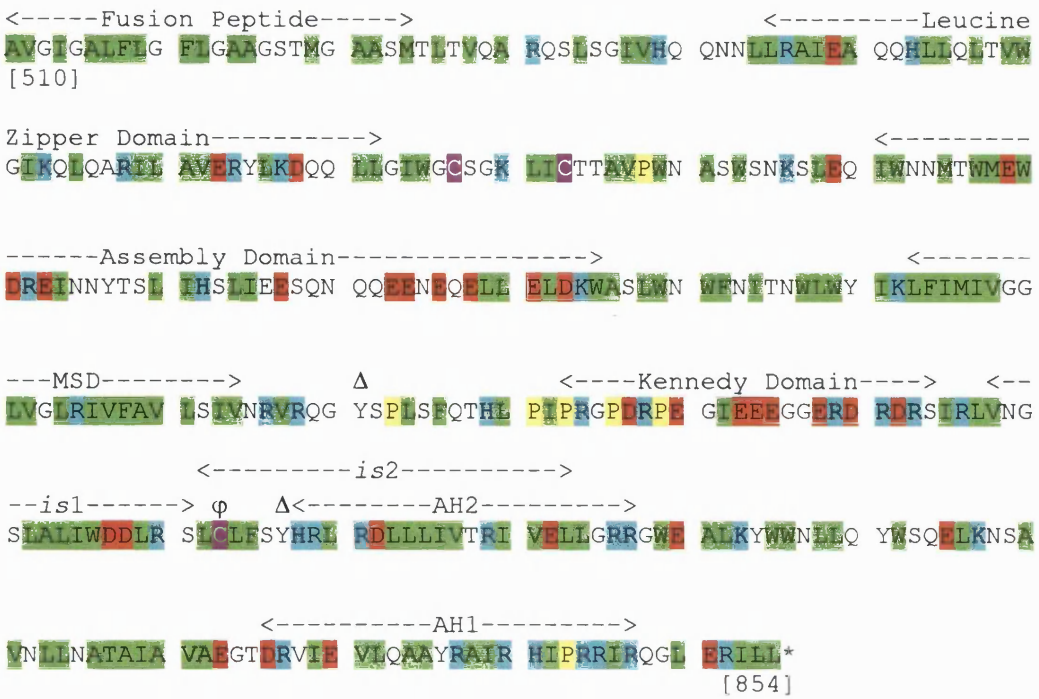


Figure 4.2. Features of HIV-1_{NL43} gp41 subunit.

The membrane spanning domain (MSD), Kennedy domain, inhibitory sequences (*is*) and amphipathic helices (AH) are indicated. YXXϕ endocytosis motifs are marked Δ, and the putative palmitoylation site is marked φ. Hydrophobic, acidic and basic residues are coloured accordingly, as are proline and cysteine residues.

4.1.2.3. Kennedy domain

The conserved Kennedy domain represents a hydrophilic sequence with a predominant negatively charged character (Kennedy *et al.*, 1986). This domain is characterised by the neutralising epitope ERDRD, an immunodominant but non-neutralising epitope IEEE, and a neutralisation escape epitope PDRPEG (Figure 4.2) (Abacioglu *et al.*, 1994; Cleveland *et al.*, 2000; Cleveland *et al.*, 2003; McLain *et al.*, 1995; Vella *et al.*, 1993). The conservation of this domain is also attributed to the presence of the splice acceptor site for the second exon of *tat* and *rev* transcripts (Douglas *et al.*, 1997; Fields *et al.*, 2001).

4.1.2.4. Amphipathic Helices

Sequences downstream of the Kennedy domain are proposed to form two conserved amphipathic helices (AH1 and AH2) according to structural predictions (Kalia *et al.*, 2003; Venable *et al.*, 1989). When expressed independently of the rest of gp41 these peptides were able to form membrane pores in bacterial and mammalian cells (Koenig *et al.*, 1999), hence their alternative nomenclature of lentiviral lytic peptides (LLPs). Another domain downstream of AH2 is believed to form a leucine zipper capable of disrupting phospholipid membranes (Arroyo *et al.*, 1995; Chen *et al.*, 2001; Chernomordik *et al.*, 1994; Douglas *et al.*, 1997).

AH1 has been the subject of intensive study with regard to its role in the virus lifecycle; the motif is required for permeabilising virions to exogenous dNTPs, permitting natural endogenous reverse transcription (NERT) that may augment infection of quiescent cells such as macrophages (Zhang *et al.*, 1996). The AH1 domain also possesses calmodulin binding properties (Tencza *et al.*, 1997; Tencza *et al.*, 1995). Calmodulin binding to Env would impact upon cellular enzymes dependent on calcium-bound calmodulin for activation, probably contributing to the pathogenesis observed. While single mutations of basic or hydrophobic residues in the AH1 domain of molecular clones are capable of attenuating calmodulin binding and cell lysis, natural isolates exhibit conservative variation in this domain, suggesting maintenance of the overall charge and structure is a requisite for function (Tencza *et al.*, 1997). Truncations and mutations in the AH1 domain can result in degradation of the glycoprotein, resulting in reduced trafficking to the cell surface, reduced incorporation of the mutant Envs into virions and slower replication kinetics (Kalia *et al.*, 2003; Lee *et al.*, 2002; Piller *et al.*, 2000). It is likely that disruption of the native AH1 conformation destabilises Env, possibly in the context

of oligomerisation, leading to degradation. The block during infection appears to occur during virus entry, indicating a role of this region in membrane destabilisation for efficient fusion (Gabuzda *et al.*, 1992), (Yu *et al.*, 1993). Unusually, antisera from HIV-1 infected patients are able to recognise corresponding AH1 peptides derived from a subtype E Env (Chang *et al.*, 2000). This suggests possible exposure of this domain in another subtype, perhaps reflective of a different structural organisation.

Viruses expressing Envs with mutations of Arg residues in the AH2 motif are able to replicate in culture, however there is a marked reduction in syncytia. This suggests the cell-cell fusion mechanism is distinct from virus-cell fusion, and ascribes a function for the AH2 motif in virus propagation (Kalia *et al.*, 2003). The work of Murakami and Freed (2000) implicates an interaction between the AH2 and the matrix protein (MA), as a compensatory change in MA overcame the suppression of Env incorporation into virions caused by deletion of some residues in AH2.

4.1.2.5. Palmitoylation

In AH1 and upstream of AH2 are two cysteine residues that are palmitoylated in some HIV-1 isolates. It has been shown that palmitoylation of at least one of the cysteines is required for targeting the glycoprotein to lipid raft domains in some cell types, and the mutation of both sites can reduce the infectivity of the virus (Rousso *et al.*, 2000). Virus assembly has been shown to occur preferentially at lipid rafts enriched for palmitoylated or GPI-linked glycoproteins, however, truncated Env products can be incorporated into virions. Thus it is likely that these palmitoylation signals are required, in the case of full-length cytoplasmic tails, to prevent exclusion of Env from these membrane domains. The observed substitution of the AH1 Cys for Ser in a natural isolate also resulted in an increased affinity for calmodulin (Tencza *et al.*, 1997).

4.1.3. Env Incorporation into Virus Particles

In HIV-1, the myristylation of $Pr55^{Gag}$ targets the precursor to a cell membrane. Maturation of virus particles results in a MA shell underlying the membrane with Env homotrimers projecting from the surface of the virus (Figure 1.1) (Fields *et al.*, 2001). The incorporation of Env into virions occurs either via a specific interaction with the MA portion of the $Pr55^{Gag}$ precursor, or via a passive means of incorporation. In support of MA-Env interactions it has been shown that the expression of $Pr55^{Gag}$ prevents the endocytosis of Env from the cell surface, although this may be attributed to Gag interactions with the host proteins involved in clathrin-mediated endocytosis. MA

mutants that prevent Env incorporation do not prevent Env endocytosis (Egan *et al.*, 1996; Freed and Martin, 1995), and also changes in the AH2 domain can overcome this suppression. Moreover, the distribution of Env in polarised cells dictates basolateral assembly and budding of virions (Lodge *et al.*, 1994).

Pseudotyping HIV-1 particles with Gibbon ape Leukaemia Virus (GaLV) required the substitution of the GaLV CT with that of murine leukaemia virus (MLV) (Stitz *et al.*, 2000), suggesting that some elements of the GaLV CT are incompatible with HIV-1 MA. In contrast, truncation of the HIV-1 and HIV-2 TM intracytoplasmic domains to 7 amino acids is necessary to facilitate pseudotyping of MLV particles (Hohne *et al.*, 1999; Lee *et al.*, 2002; Mammano *et al.*, 1997; Nack and Schnierle, 2003; Schnierle *et al.*, 1997; Thaler and Schnierle, 2001). The inability of simple retroviruses to be pseudotyped with full-length lentiviral glycoproteins suggests steric parameters as a barrier for non-specific-glycoprotein incorporation into certain virus particles. Moreover, it has been documented that a variety of host cell surface proteins, with no common cytoplasmic sequences, can be incorporated into virions (Rousso *et al.*, 2000).

How much of the cytoplasmic domain is required for virus infection remains contentious. Step-wise C-terminal truncations of gp41 resulted in impaired expression, syncytium formation and incorporation into viruses (Dubay, Roberts, Hahn et al., 1992; Owens et al., 1994; Yu et al., 1993). Conversely, other studies showed that certain truncated mutants exhibit increased surface Env expression, efficiency of incorporation into virions and syncytium formation (Earl et al., 1991; Murakami and Freed, 2000). Similarly in SIV, certain Env truncations increase virion expressed Env, resulting in faster replication kinetics in human cell lines (Johnston *et al.*, 1993; Manrique *et al.*, 2001).

4.1.4. Conventional Structure

The conventional view of the gp41 C-terminal domain is that it is an entirely cytoplasmic feature (Figure 4.3). Downstream of the conventional MSD is the hydrophilic Kennedy domain, and both amphipathic helices associate with the cytoplasmic side of the plasma membrane. Helical wheel predications also suggest the formation of distinct hydrophilic and hydrophobic faces on AH1 and AH2 in support of the conventional model (Kalia *et al.*, 2003), allowing the simultaneous alignment of the helices along the plane of the membrane and the interaction with host factors.

MAbs directed against the Kennedy domain are capable of reacting with and neutralising HIV and non-HIV virions containing this sequence (Kennedy *et al.*, 1986; Vella *et al.*, 1993); moreover, antisera derived from HIV-1 infected patients are also capable of recognising peptides derived from a subtype E Env overlapping this region (Chang *et al.*, 2000). In the absence of lytic conditions, it would be unusual to observe antibody recognition of epitopes if they are not surface exposed. However, other studies were unable to detect either IEEE or ERDRD motifs (Buratti *et al.*, 1997; Haffar *et al.*, 1988; Sattentau *et al.*, 1995), this has led to the suggestion that antibodies are directed instead against the corresponding IEEE epitope on MA (Buratti *et al.*, 1997; Papsidero *et al.*, 1989; Shang *et al.*, 1991), suggesting exposure of the MA at some point in the virus lifecycle.

Cleveland *et al.*(2003) have recently provided evidence of the exposure of the Kennedy domain in mature virions without prior interaction with the primary CD4 receptor and have proposed alternative conformations of the gp41 C-terminal tail (Figure 4.4). In this revised model, the Kennedy domain is exposed and the AH2 and AH1 helices are inserted into or associated with the virion/cell membrane in support of their ability to initiate NERT in virions. Despite extensive study, definitive conclusions regarding the conformation of the HIV-1 gp41 cytoplasmic tail remain lacking. The purpose of this investigation was to determine if Env expression and cell-cell fusion studies with Env constructs truncated at specific gp41 domains could provide functional data that would support one of the revised conformations (Cleveland *et al.*, 2003).

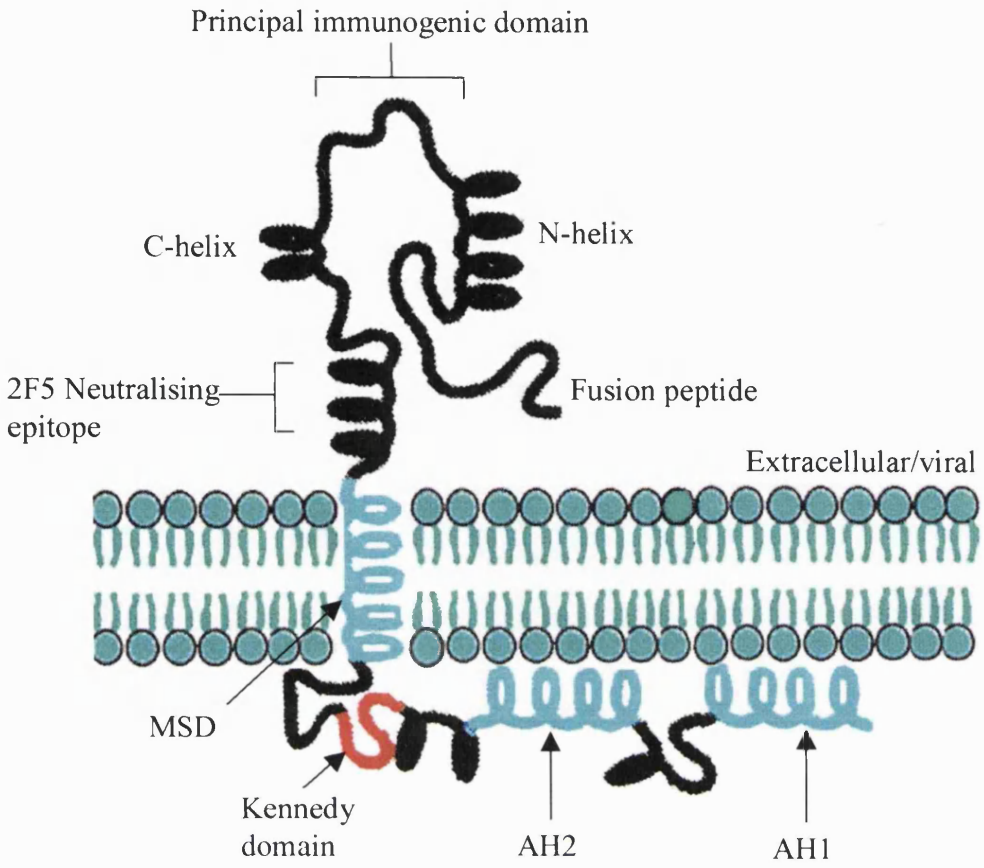


Figure 4.3. Schematic representation of the organisation of the monomeric HIV-1 gp41 subunit.

Features of the gp41 ectodomain are indicated, the structure depicted is arbitrary. The Kennedy domain is coloured in red. Both AH2 and AH1 amphipathic helices in the cytoplasmic tail are represented as membrane-associated helices. Diagram not to scale. Adapted from (Kalia *et al.*, 2003).

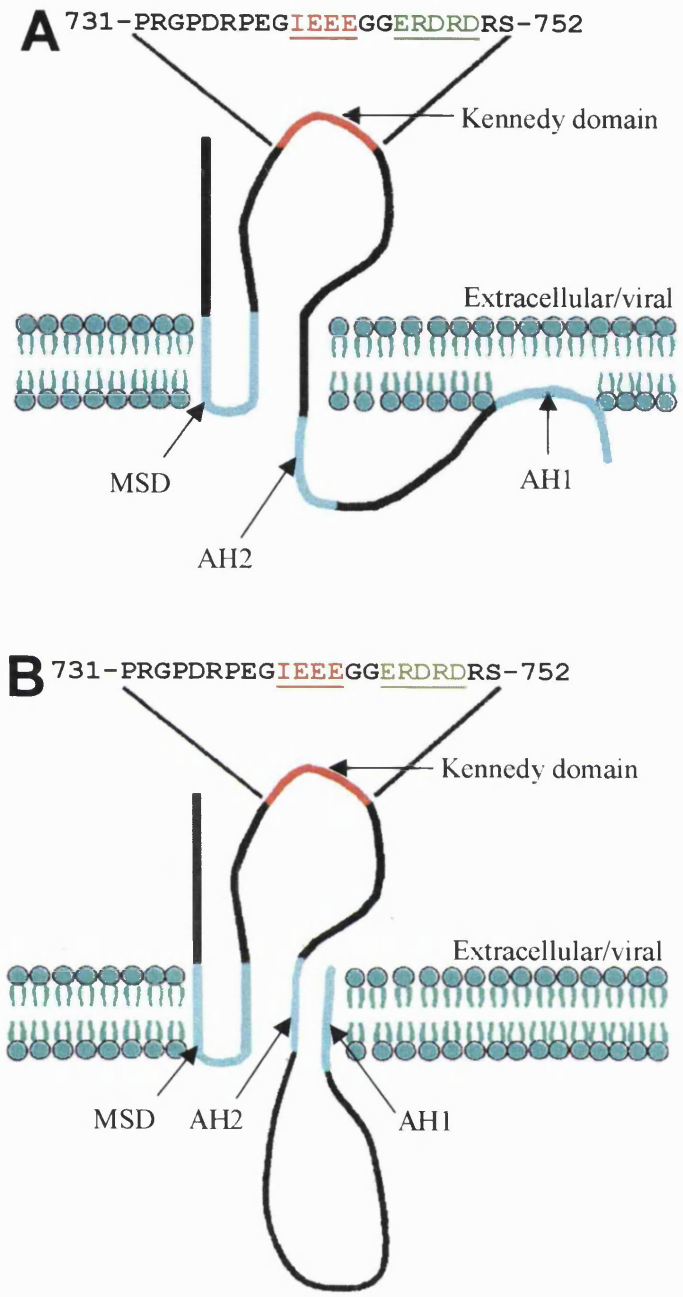


Figure 4.4. Revised organisation of the HIV-1 gp41 cytoplasmic domain.

In the models proposed by Cleveland *et al.*, 2003, the Kennedy domain is projected into the extracellular/extravirion milieu, the immunodominant and neutralising epitopes recognised by antibodies are indicated by colour and underlining. In (A) the AH1 domain is associated with the cytoplasmic plane of the plasma membrane. Hydrophobic residues other than those in AH2 are implicated in traversing the plasma membrane in this depiction. In (B) both AH2 and AH1 domains traverse the plasma membrane, possibly forming a pore. Adapted from (Cleveland *et al.*, 2003).

4.2. Methodology

4.2.1. Construction of gp41 C-terminal Truncation Mutants

Dr Rod Daniels designed and constructed ten 3' deletion mutants of the HIV-1_{NL43} *env*-gene. Mutant oligonucleotides were used to direct the synthesis of stepwise deletions of the *env*-gene by PCR as detailed in Chapter 2 (The C-terminal truncations are indicated in Figures 4.5 and 4.6) and products were ligated into pQ7 vector using *Xho*1 and *Eco*R1 restriction sites. DH5α competent cells were transformed by the heat-shock method, and viable clones were determined after analyses of plasmid miniprep extracts as described in Chapter 2. DNA sequencing was conducted by Z. Xiang to confirm the integrity of the engineered truncations.

4.2.2. Western blotting

Cell lysates of 293T cells expressing truncated Envs were reduced and denatured for western blot analyses to assess the production of truncated Env species within transfected cells. Mouse ascitic fluid from which ARP301 polyclonal antibodies were derived, and T30 MAb were used with a goat anti-mouse IgG-peroxidase conjugate as described in Chapter 2 to detect intracellular Env expression.

4.2.3. Cell Surface Immunofluorescence Assay

Extracellular and intracellular expression of the truncated Env constructs in 293T cells was assessed as described in chapter 2. Intracellular and surface expression was assessed using EVA3013 MAb. Surface bound antibodies were detected using a sheep-anti-mouse FITC-conjugate. Permeabilising cells with 0.2% Triton X-100/PBS post-fixing enabled the detection of truncated *env*-gene products inside 293T cells.

4.2.4. Cell-Cell Fusion Assay

Cell-cell fusion assays with truncated Envs were conducted as described in chapter 2.

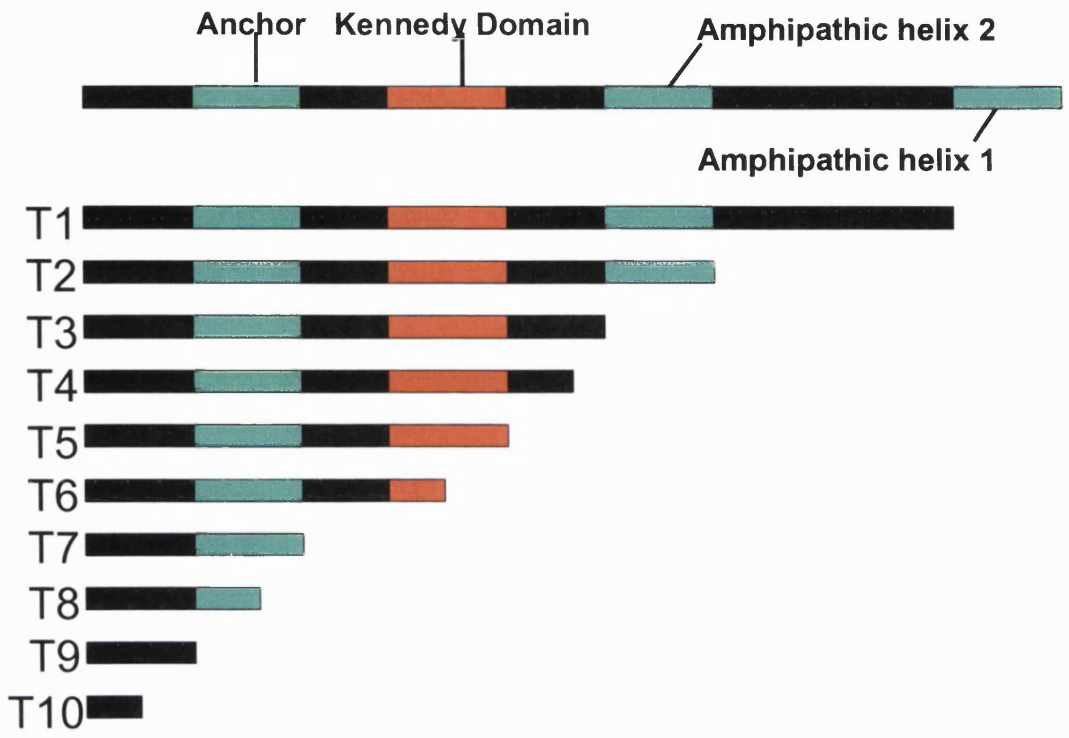


Figure 4.5. Schematic representation of HIV-1_{NL43} Env truncation mutants.
Step-wise truncation mutants of $NL43_{WT}$ *env*-gene (T1-10) were generated by PCR. Colouring complements figures 4.2 and 4.3. Diagram not to scale.

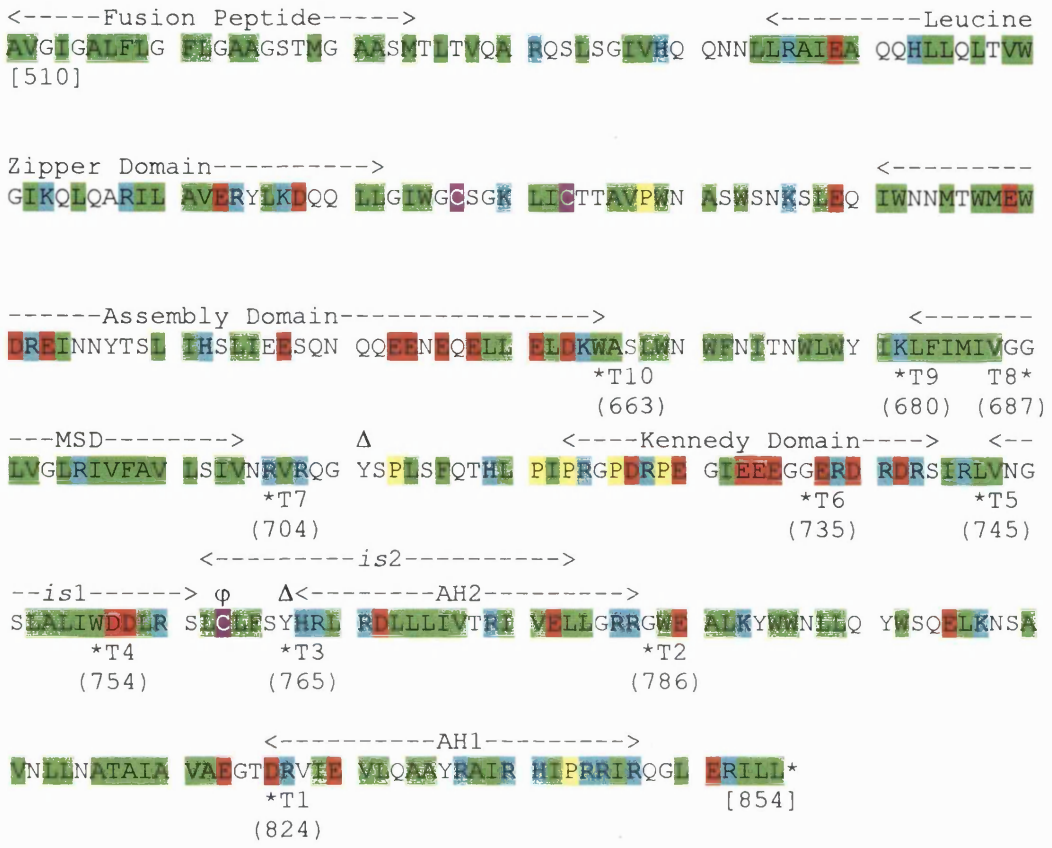


Figure 4.6. Truncation sites of HIV-1_{NL43} gp41 mutant constructs.

The MSD, Kennedy domain, inhibitory sequences (*is*) and amphipathic helices (AH) are indicated. YXXϕ endocytosis motifs are marked Δ, and the putative palmitoylation site is marked φ. Hydrophobic, acidic and basic residues are as coloured. Proline and cysteine residues are indicated. The terminal residues of the truncation mutants are indicated by *TX and the overall length of the truncated Env is indicated in parentheses.

4.3. Results

4.3.1. Construction of Mutants

To determine if Env function is related to the structure proposed by Cleveland *et al.*, 2003, a series of constructs were made with progressive deletions of the $NL43_{WT}$ *env*-gene from the 3' end. The truncations were designed to delete in a stepwise manner from the C-terminus, the amphipathic helices, the inhibitory sequences, the Kennedy domain and the MSD (T1-9) (Figures 4.4 and 4.5). The T10 truncation is at a position analogous to a gp140 construct yielding soluble protein for ongoing protein purification and structural studies in our group. Generation of truncated PCR products were confirmed by agarose gel electrophoresis to detect shifts in DNA fragment sizes. Purified PCR products were cloned into pQ7 vector and verified by restriction enzyme digestion to assess insert sizes. The results of enzyme digestion illustrate a stepwise increase in *env*-gene mobility correlating with stepwise truncations engineered in the 3' end of the $NL43_{WT}$ *env*-gene (Figure 4.7). DNA sequencing confirmed there is no frameshift permitting any extension of the cytoplasmic tail (Appendix A2.2). The pQ7.*env* clones were used for expression studies and cell-cell fusion assays; however, it was considered non-viable to clone these genes into pC2 as the second exons of *tat* and *rev* were affected in mutants T6-T10.

4.3.2 Expression of Truncated Envs

Western blotting of 293T cell lysates using T-30 MAb confirmed stepwise truncations in gp41 (Figure 4.8), consistent with the plasmid analyses and DNA sequencing. Studies with ascitic fluid-derived ARP301 MAb indicated the truncations were not sufficient to generate mobility shifts in the gp160 species, and also highlighted a deficiency in gp160 processing of Env mutants T8-T10.

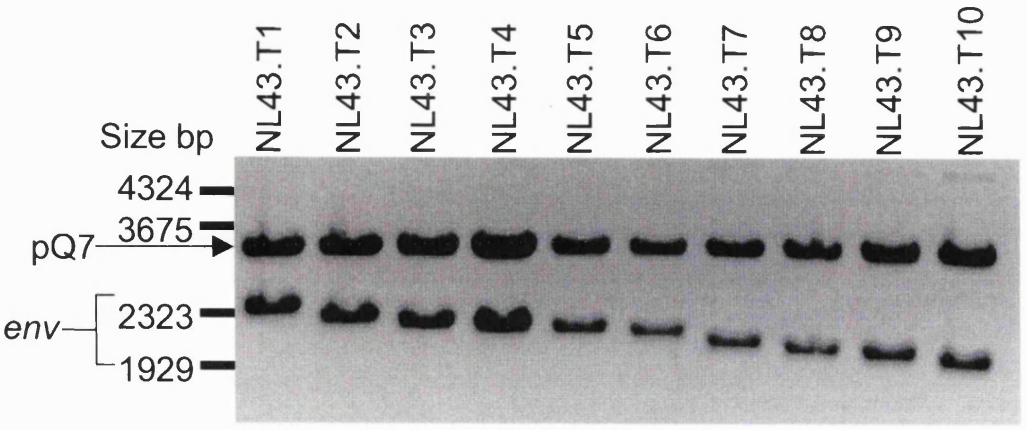


Figure 4.7. Restriction digests confirming truncation of $NL43_{WT}$ *env*-gene. *Xho*1/*Eco*R1 digestion of pQ7.*env* plasmids exhibit shifts in electrophoretic mobility of the *env*-gene, indicating successful truncation.

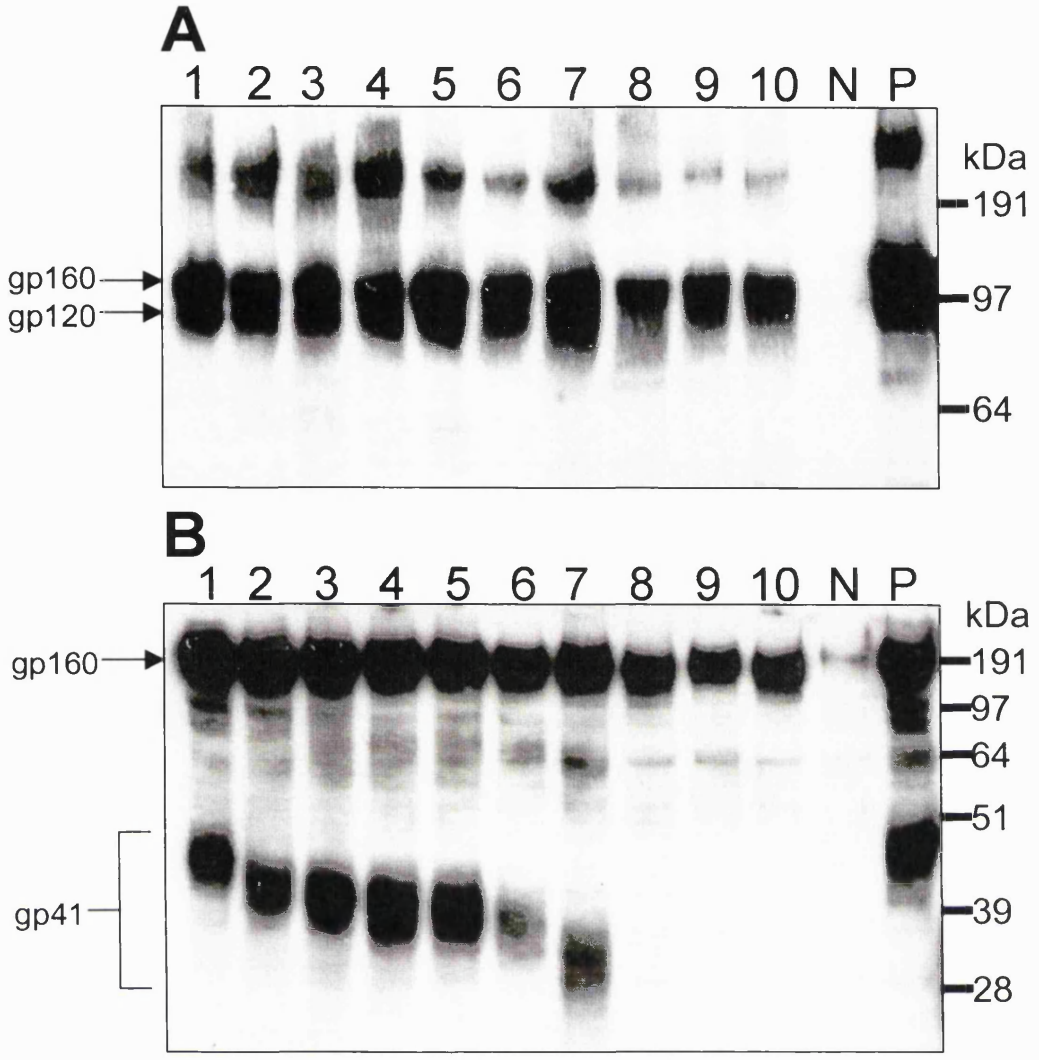


Figure 4.8. Western blotting of 293T cell lysates.

Non-reduced 293T cell lysates were probed with ascitic fluid-derived ARP301 MAb (A). Reduced 293T cell lysates were probed with anti-gp41 T30 monoclonal antibody (B). Lanes: 1-10 corresponds to truncation mutants T1-T10; N, negative control; P, $NL43_{WT}$ control. The sizes of gp160, gp120 and gp41 are indicated. An increase in the electrophoretic mobility of gp41 species is observed, consistent with step-wise truncations from the C-terminus.

4.3.3. Cell Surface Expression of Truncated Envs

The use of EVA3013 MAb reveals high levels of intracellular and cell surface Env expression for the $NL43_{WT}$ Env, and no staining for untransfected cells (Figure 4.9). The scarcity of cells expressing Env indicates low transfection efficiency. Intracellular expression for all mutants was detected (Figures 4.10-11). Mutants T2 and T4 exhibited less intracellular Env than the other mutants (Figure 4.10). It is not certain if this is physiologically relevant, if so it could indicate that these truncations have an effect on the rate of Env degradation. Surface expression was only deficient in mutants T8 and T9; truncation of mutant T8 is close to the N-terminus of the MSD, rendering the hydrophobic MSD too short to traverse the membrane, whilst mutant T9 is truncated N-terminal to the MSD. Thus, it is to be expected that these two truncations would affect Env surface expression due to the loss of the MSD. In contrast, the truncation of mutant T10 was engineered at a position upstream of the conventional transmembrane anchor past the Trp-rich domain, yet this mutant Env remains detectable on the cell surface at considerable intensity. Analysis of upstream sequences suggest that a stretch of hydrophobic residues in the gp120 C1 domain, characterised by the sequence LWVTVYYGVPVW, could serve as a membrane anchor (Douglas et al., 1997).

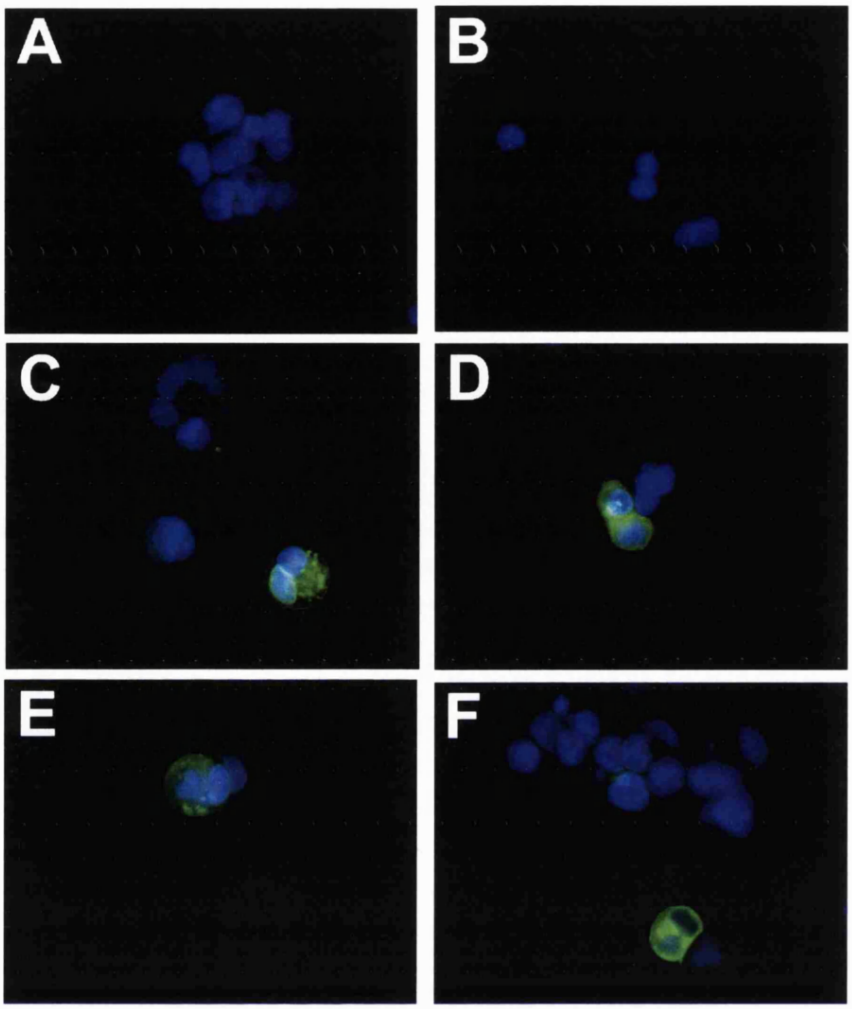


Figure 4.9. Expression of HIV-1_{NL43} Env in 293T cells driven by vTF7-3.

The cell surface (Panels A, C and E) and intracellular (Panels B, D and F) distribution of Env was assessed using EVA3013 MAb. Panels: AB, negative controls; C-F, $NL43_{WT}$. Each panel represents an area of 263.5μm^2 viewed at 400x magnification.

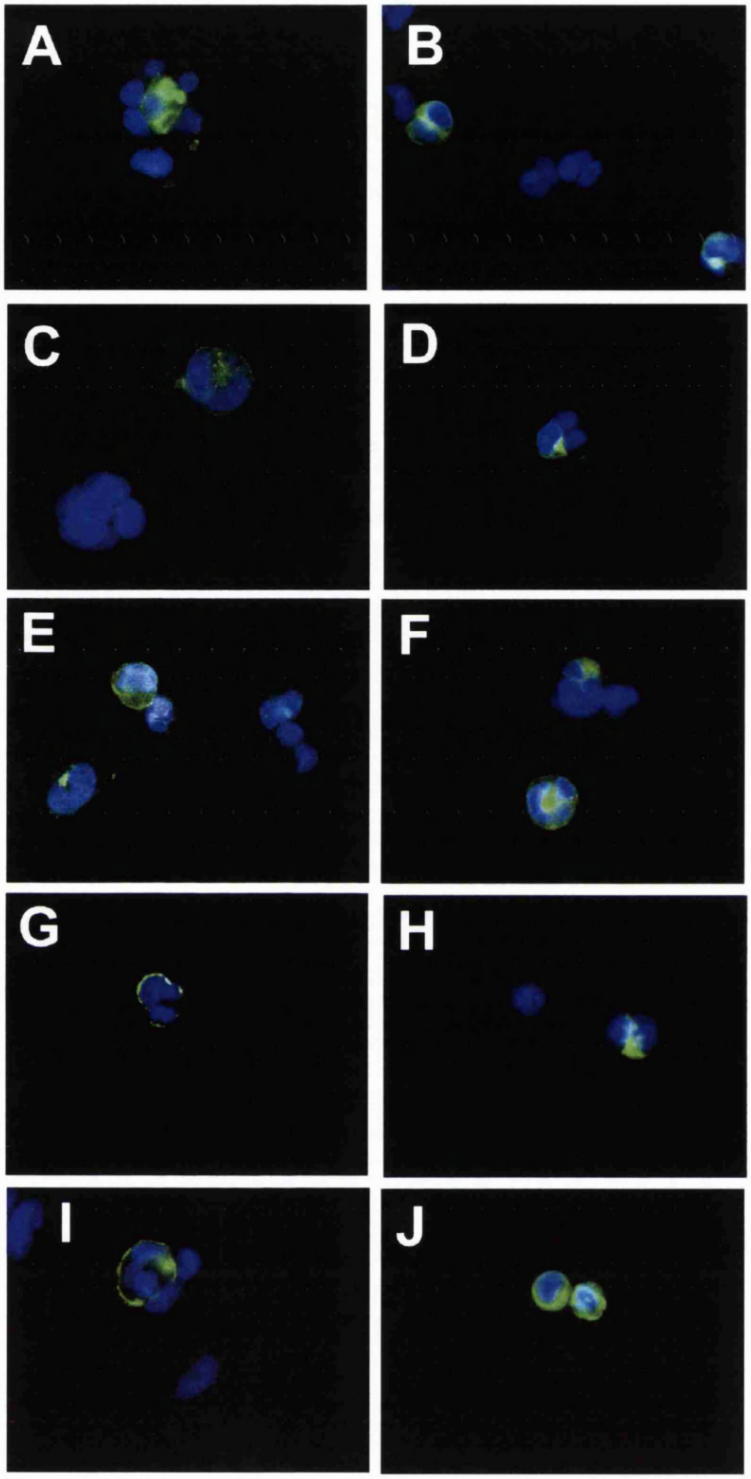


Figure 4.10. Expression of HIV-1_{NL43} Env truncation mutants T1-T5 in 293T cells driven by vTF7-3.

The cell surface (Panels A, C, E, G and I) and intracellular (Panels B, D, F, H and J) distribution of Env was assessed using EVA3013 MAb. Panels: AB, T1; CD, T2; EF, T3; GH, T4 and IJ, T5. Each panel represents an area of 263.5μm^2 viewed at 400x magnification.

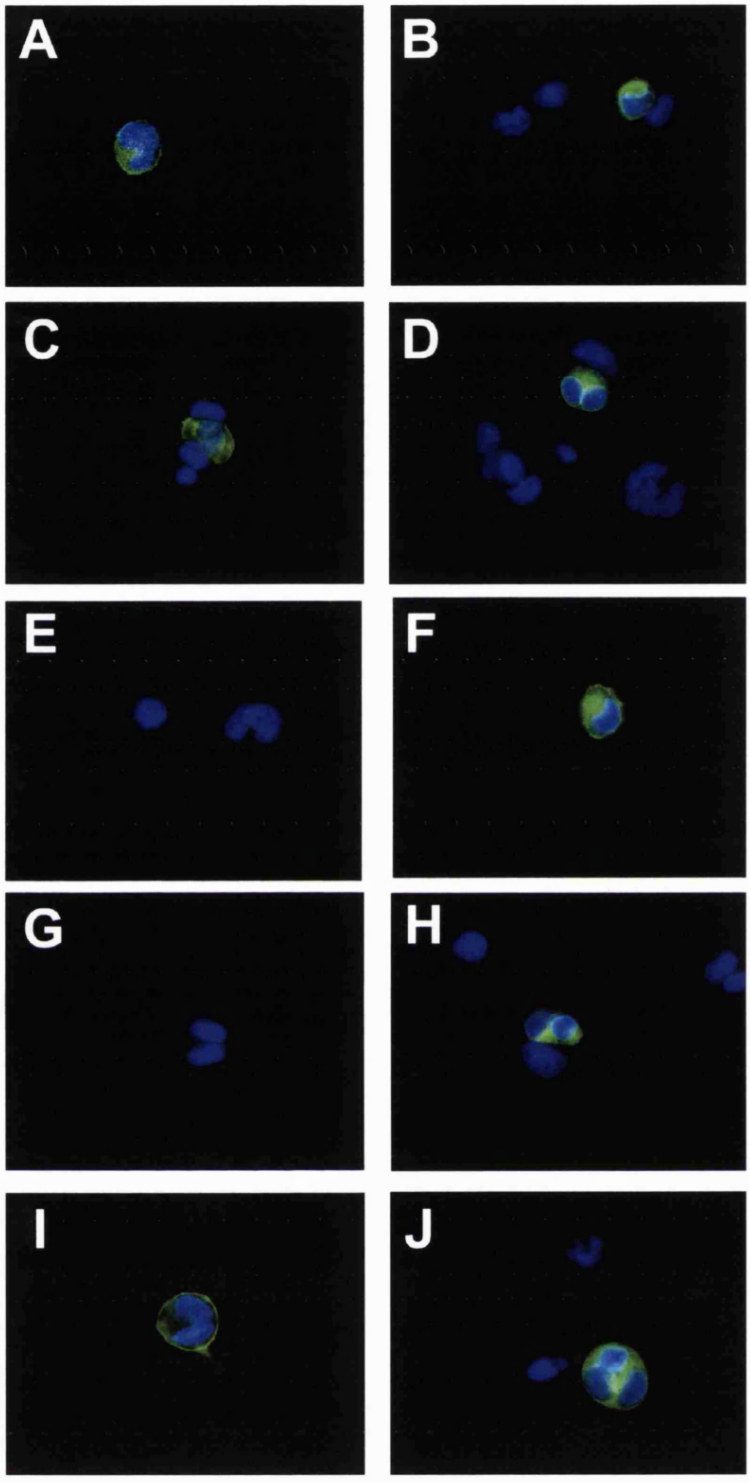


Figure 4.11. Expression of HIV-1_{NL43} Env truncation mutants T6-T10 in 293T cells driven by vTF7-3.

The cell surface (Panels A, C, E, G and I) and intracellular (Panels B, D, F, H and J) distribution of Env was assessed using EVA3013 MAb. Panels: AB, T6; CD, T7; EF, T8; GH, T9 and IJ, T10. Each panel represents an area of 263.5μm^2 viewed at 400x magnification.

4.3.4. Cell-Cell Fusion Assay

The effect of the truncations on the fusogenic capacity of the glycoprotein was assessed in cell-cell fusion assays with NP2/CD4 cells expressing CXCR4 or CCR5 (Figure 4.12 and Table 4.1). Luciferase expression results show that only truncation mutant T6 significantly enhanced the fusogenic capacity of $NL43_{WT}$, whilst the enhancement observed with mutants T4 and T5 approached significance. Other constructs (T1-3 and T7) exhibited fusogenic capacities similar to $NL43_{WT}$.

Microscopic analyses of syncytia formation indicated the greatest extent was observed with cells expressing mutant Envs T5 and T6, resulting in blebs of membrane (Figure 4.13). Results for mutants T1, T2, T3, T4 and T7 indicate that truncations at these regions of gp41 do not significantly perturb the fusogenic capacity of Env.

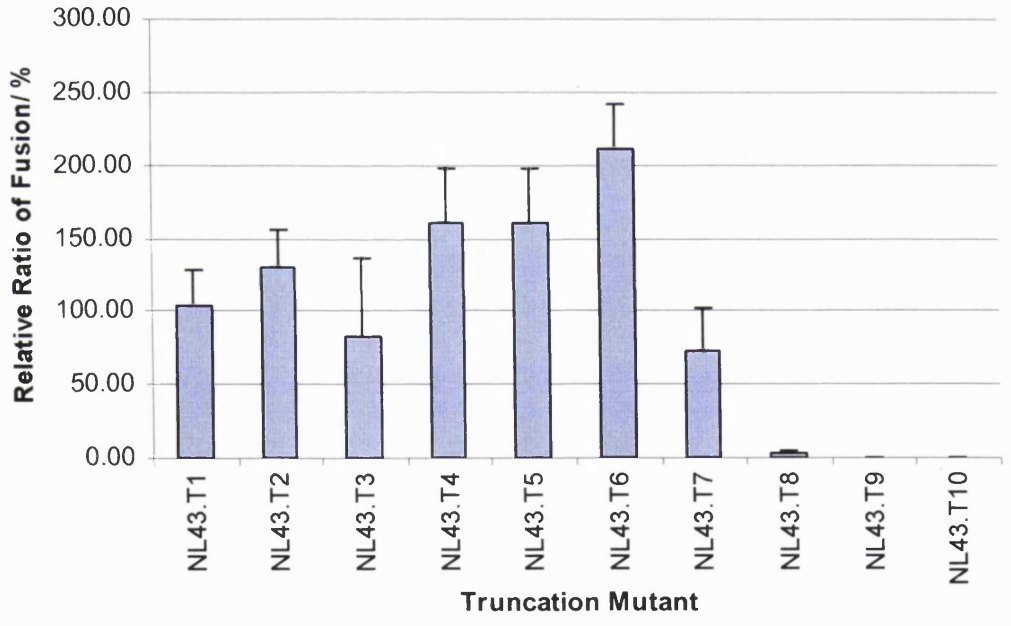


Figure 4.12. Fusogenic capacity of truncated Envs.
The relative ratio of expression of the Env mutants in relation to $NL43_{WT}$ is illustrated. No fusion was observed in the absence of CXCR4 expression on NP2 target cell lines. The average of three assays is shown and bars represent the standard deviations.

HIV-1 Env	% NL43 Expression	*P*(>NL43)
NL43.T1	104.51	0.38817
NL43.T2	129.01	0.09695
NL43.T3	83.10	0.31979
NL43.T4	160.85	0.05185
NL43.T5	160.43	0.05301
NL43.T6	210.38	0.01303
NL43.T7	73.76	0.12418
NL43.T8	3.08	0.00005
NL43.T9	0.49	0.00000
NL43.T10	0.00	0.00001

Table 4.1. Significance of fusogenic potential in relation to $NL43_{WT}$.

The fusogenic capacity of the T6 truncation mutant was significantly greater than the wildtype as determined by the Student's T-test, whilst mutants T4 and T5 approached significance ($P<0.05$). The fusogenic capacities of the truncation mutants T8-T10 were significantly less than $NL43_{WT}$.

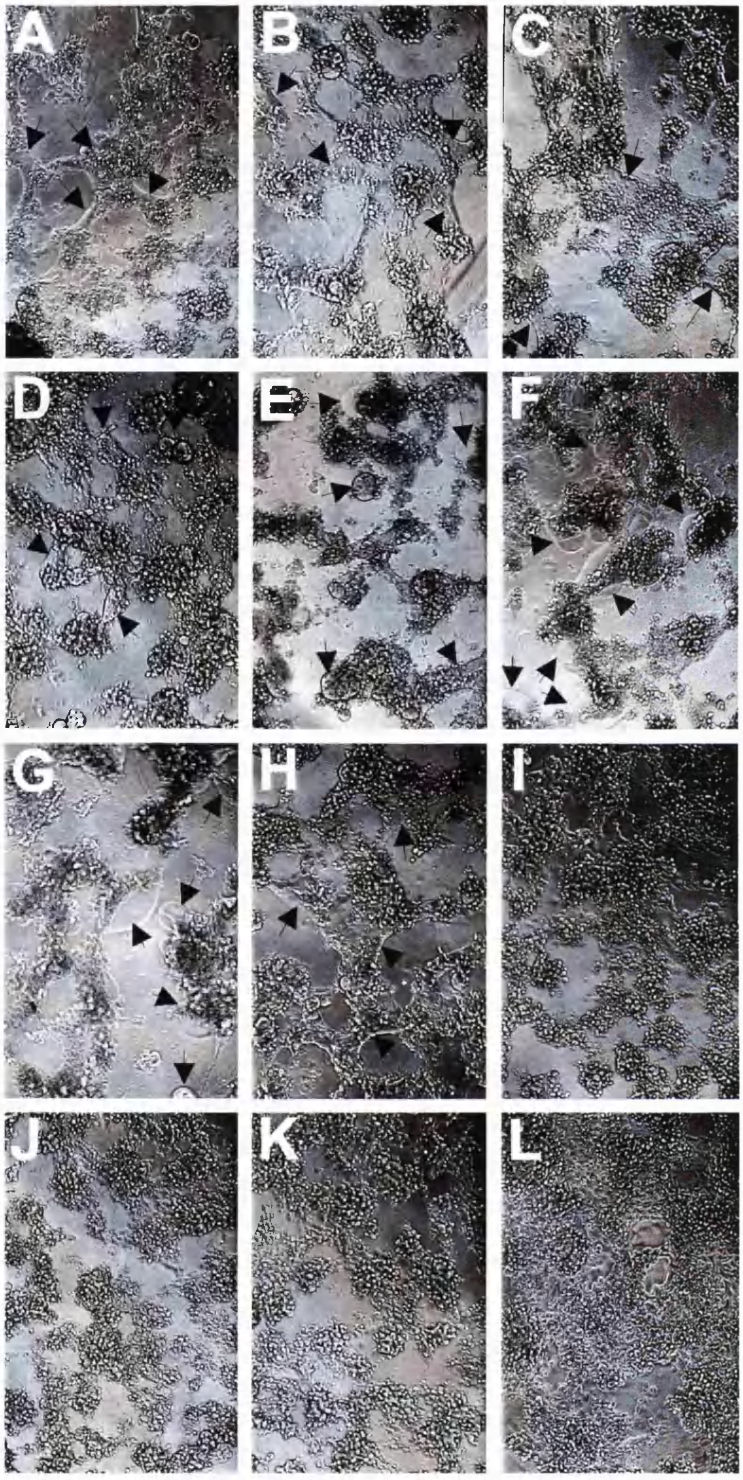


Figure 4.13. Syncytia formation by NL43 truncation mutants.

In alphabetical order, the panels depict effector cells expressing: (A) $NL43_{WT;}$ (B-K) mutants T1-T10; (L) Env negative control. In all cases the target cells are NP2/CD4/CXCR4 cells. Arrows indicate membrane fusion. Each panel represents an area of 12631μm^2 viewed at 40x magnification.

4.4. Discussion

The commonly held view of the topology of HIV-1 gp41 is that all residues downstream of the MSD (151 in the case of $NL43_{WT}$, Figure 4.2) are located entirely on the cytoplasmic side of the membrane (Figure 4.14). The two amphipathic helices in the long CT are predicted to associate with the plasma membrane, and the calmodulin binding properties of AH1(Tencza *et al.*, 1997), the MSD proximal YXXϕ motif modulating endocytosis (Rowell, Stanhope et al., 1995), and palmitoylated Cys residues involved in targeting the Env to lipid raft domains (Rousso *et al.*, 2000) are all indicative of the cytoplasmic location of the relevant structures in the CT.

However, the conventional model addresses neither the neutralisation sensitivity of the Kennedy domain (Cleveland *et al.*, 2003; Vella *et al.*, 1993), nor the membrane permeabilising properties of the amphipathic helices (Arroyo *et al.*, 1995; Koenig *et al.*, 1999; Zhang *et al.*, 1996). The neutralisation studies and subsequent models of the gp41 CT proposed by Cleveland *et al.*, 2003 place the Kennedy domain on the surface of the virion without pre-exposure to CD4 (Figure 4.15). Moreover, they have shown that protease treatment of virions destroys the neutralising ERDRD epitope, arguing that the conformational change in Env following CD4 and co-receptor binding is not a pre-requisite for exposure of this domain (Cleveland *et al.*, 2003). The lack of reactivity of the MA with MAbs in their studies further argues that the immunodominant IEEE epitope is not that of MA. These revised models are compatible with the observation that peptides derived from AH1 and AH2 can multimerise and permeabilise virions through the formation transmembrane pores (Chen et al., 2001; Tencza et al., 1995; Zhang et al., 1996).

In this investigation we sought to test Cleveland's model through studying the function of Env mutants truncated at relevant domains as depicted (Figure 4.5). Our results found that the fusogenic capacity of NL43 Env was unaffected by truncations at those positions in mutants T1-3 and T7, enhanced in mutants T4-6, and abrogated in mutants T8-10. Mutant T4 Env was considerably less represented on the surface than the other mutants, despite the truncation removing most of the *is* motifs, the second YXXϕ motif and di-Leu motifs that signal endocytosis or Golgi retention.

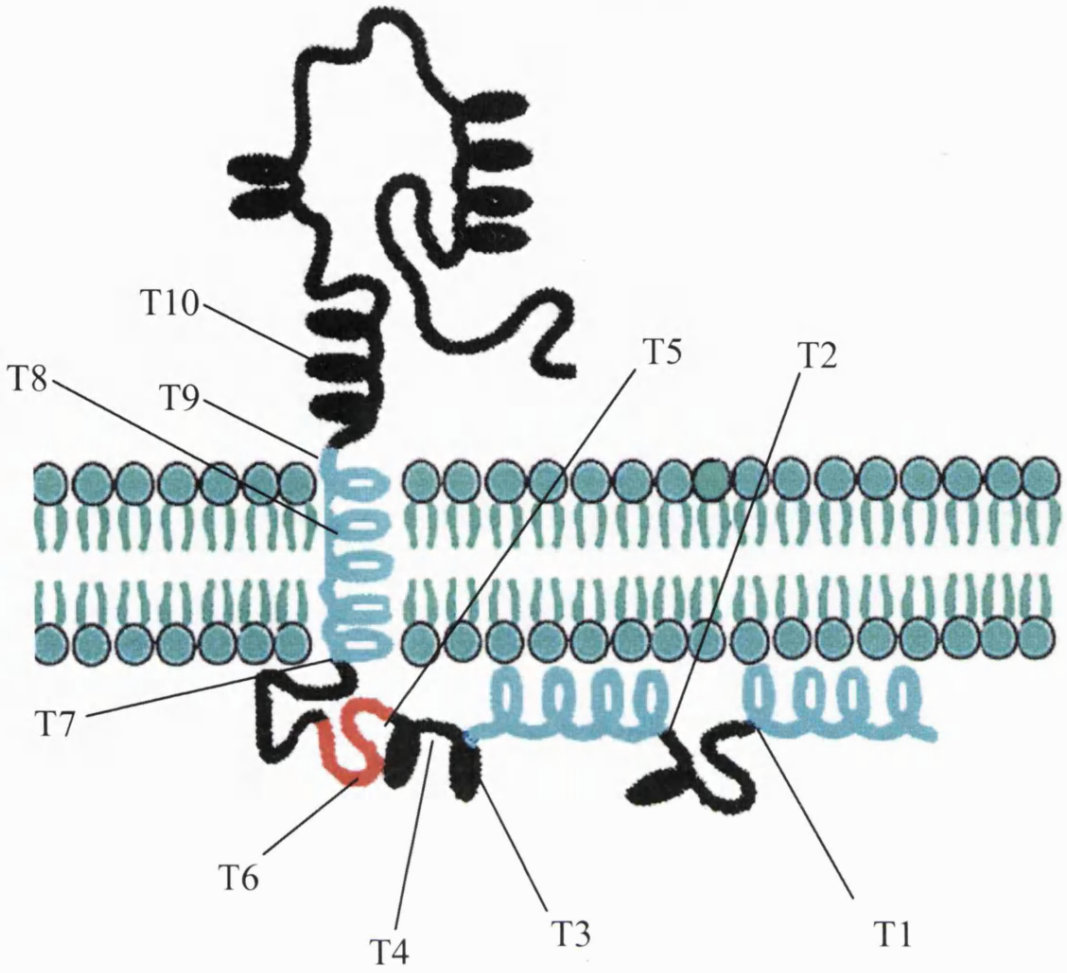


Figure 4.14. Conventional view of gp41 structure.

The large cytoplasmic tail is maintained entirely beneath the basal surface of the lipid membrane. The truncations generated in this study are indicated on this model. The colouring is consistent with figure 4.5. Figure adapted from (Kalia *et al.*, 2003).

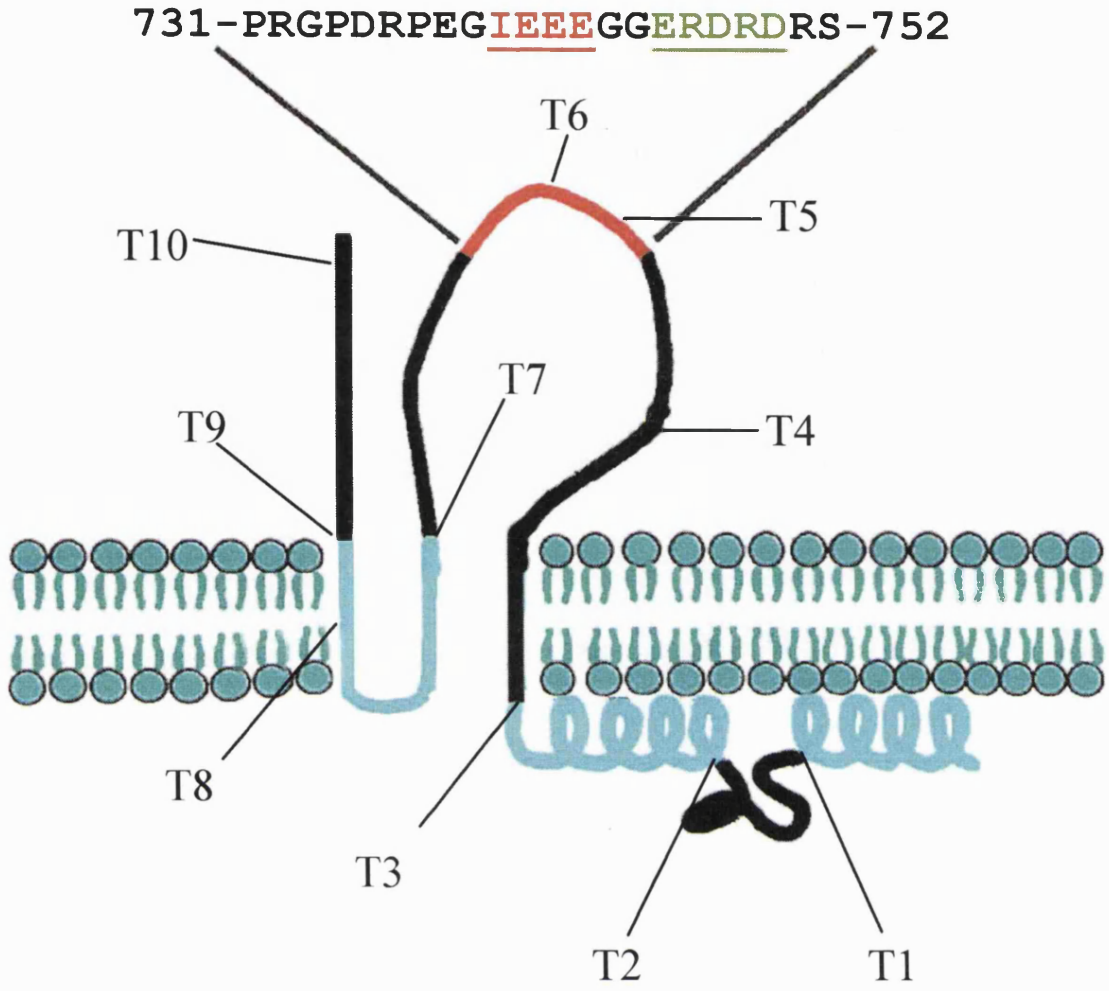


Figure 4.15. Schematic representation of a revised gp41 CT structure.
In this representation, the Kennedy domain is extracellular/viral, the immunodominant and neutralising epitopes are indicated as coloured. Whilst the AH2 and AH1 helices are depicted as associating with the membrane, it is possible that AH1 may associate to form a transmembrane channel with other subunits in the functional homotrimer, consistent with the literature (see text for details). The truncation sites of each mutant are indicated, and the colouring is consistent with figure 4.5. Figure adapted from (Cleveland *et al.*, 2003; Kalia *et al.*, 2003).

In contrast, mutant T2 possesses all these motifs and expresses a greater amount of Env on the surface. Many studies indicate that perturbations of the AH1 domain, whether through truncation or residue substitution, can drastically affect Env incorporation into virions with the ensuing decrease in Env density on the virion surface resulting in reduced infectivity (Kalia et al., 2003; Manrique et al., 2001; Murakami and Freed, 2000). Mutant T1 exhibited discordant results with other studies that indicate the importance of this domain in membrane fusion and in syncytium formation (Dubay, Roberts, Hahn et al., 1992; Lee et al., 2002; Piller et al., 2000; Zhang et al., 1996), however, differences in cell lines used and the mode of Env expression may be factors in this discrepancy. It has been demonstrated that the loss of either of two palmitoylated Cys residues in the CT results in a reduction of Env in lipid rafts where HIV-1 assembles (Rousso *et al.*, 2000). However, our original $NL43_{WT}$ Env lacks the Cys residue within the AH1 domain whilst maintaining the upstream Cys proximal to the AH2 domain, and we have demonstrated that the wild type Env is capable of being incorporated into virions (Chapter 3).

Mutants T10 and T9 directly eliminate the MSD from the Env, while T8 truncates the MSD, leaving 7 anchoring residues. The absence of fusogenic activity in mutants T8 and T9 correlated with the absence of cell surface expression. The conserved Trp-rich ectodomain sequence proximal to the MSD is predicted to form an amphipathic helix (Barbato *et al.*, 2003), suggesting a possibility that this domain may associate with membranes, but our surface labelling suggests otherwise in the absence of Gag and in a non-CD4i state. These results are thus consistent with literature reporting that the MSD is essential for destabilisation of membranes during fusion (Dubay, Roberts, Hahn et al., 1992; Lin et al., 2003; Owens et al., 1994). The deletion of the MSD in mutant T10 makes its detection on the surface surprising. The major hydrophobic domains on full-length Env identified by Haffar were the signal peptide, the fusion peptide and the MSD (Haffar *et al.*, 1988), however, only the MSD possessed a stop-transfer signal. Our results suggest there are sufficient hydrophobic residues upstream of the MSD, most likely a stretch of residues in the gp120 C1 domain, to facilitate anchorage of Env to the cell membrane. In all three mutants, Env processing is abrogated, consistent with observations that membrane anchorage of Env via the MSD is a requisite for efficient processing by relevant proteases during maturation (Decroly *et al.*, 1997; Morikawa *et*

al., 1993). The lack of processing further correlates with the absence of fusogenic activity observed with these constructs.

The T6 truncation showed significantly greater fusogenic capacity than $NL43_{WT}$, whilst the enhancement observed with mutants T4 and T5 approached significance (Figure 4.12 and table 4.1). Mutants T5 and T6 exhibited consistently larger syncytia than $NL43_{WT}$ and all other mutants (Figure 4.13). In the context of the conventional model (Figure 4.14), all truncation mutants, bar T7-10, possess a cytoplasmic tail. It is therefore uncertain how in this model the T4-T6 mutants enhance fusogenic activity compared to other mutants. In the context of the revised model of the gp41 CT (Figure 4.15) the extracellular/viral Kennedy domain would be anchored to the membrane by hydrophobic sequences that correspond to terminal regions of Env mutants T2/T3 and T7. Consistent with this model are the observations that of all the truncated Envs that are anchored to the surface membrane via the MSD (T1-7), mutant T7 is the only one lacking an extracellular component containing a proportion of the Kennedy domain and it exhibits slightly reduced fusogenic capacity compared to $NL43_{WT}$. Mutants T1-3, where the extracellular/viral location of the Kennedy domain is maintained, do not show significantly altered the fusogenic capacity. In the case of mutants T4-6, the charged residues of the Kennedy domain (EEEGGERDRDRSIR) on a extracellular/viral loop are no longer tethered to the virion/cell membrane by C-terminal hydrophobic sequences. This highly charged domain may be able to interact with the gp120 subunit or some other part of the gp41 ectodomain, destabilising the native/CD4i state of Env or facilitating the rapid formation of the six-helix bundle to induce rapid fusion. The significantly enhanced fusogenic capacity associated with mutant T6 compared to T5 suggests either the IEEEG motif or the loss of the ERDRD motif is involved in the increased fusogenic capacity. The IEEE motif is an immunodominant but non-neutralising motif, whereas the ERDRD motif is a neutralising epitope (Cleveland *et al.*, 2000; Cleveland *et al.*, 2003; Vella *et al.*, 1993), which adds support for it's involvement in Env-mediated fusion. Conversely, Edwards *et al.*, 2002, demonstrated that truncations in gp41 CT, terminating at a position corresponding to the Gly residue in the DRPEGIEEE sequence of the Kennedy domain are capable of transmitting conformational changes to gp120, resulting in exposure of CD4i epitopes. Such conformational changes are more likely if the resultant Kennedy domain is extracellular, one could then envisage the charged Kennedy domain interacting with either some of

the many N-linked oligosaccharides on gp120, and/or one of the variable loops to mediate exposure of the CKR binding sites.

Cleveland *et al.*, 2003, concede that it is possible that the Env CT may exist in the conventional and revised conformations, or that particular species of Env may adopt different CT structures as dictated by their sequences. Some features of the CT remain incompatible with their revised models, such as the membrane proximal YXXϕ motif; extracellular positioning of this motif would not allow it to interact with host proteins involved in endocytosis (Ohno *et al.*, 1997). It is possible that endocytosis at a local region of the membrane surface requires less than 100% of Env oligomers presenting endocytosis signals on the cytoplasmic side of the membrane. In this scenario, Envs that do not present YXXϕ motifs on the cytoplasmic side of the membrane could be endocytosed, therefore only a proportion of Envs need to adopt a conventional structure. The distinct efficiencies of virus-cell and cell-cell membrane fusion, and neutralisation of virions, may be attributable to heterogeneity in Env populations in addition to surface density (Poignard *et al.*, 2003). Depending on the Env and the cell type, a variable proportion of gp160 is processed in the cell and expressed on the surface or incorporated into virions. Our own observations that virions can package processed and unprocessed Envs (Chapter 3) indicate that at least these two different species of Envs may be presented on the cell surface. Studies have shown that the expression of $Pr55^{Gag}$ prevents the endocytosis of Env from the cell surface (Egan *et al.*, 1996). While $Pr55^{Gag}$ interactions with the host cell proteins involved in clathrin-mediated endocytosis are likely to play a role in modulating endocytosis, it is possible that during virion assembly a Gag-Env interaction may preferentially occur with particular species of Env lacking the YXXϕ motif on the cytoplasmic side of the membrane, such as those adopting either of the conformations proposed by Cleveland *et al.*, 2003. In support of this, studies show that in the case of MA mutants suppressing Env incorporation into virions, substitution of the Tyr residue in the YXXϕ motif for Phe or Cys residues overcomes this block to allow packaging of Env at greater densities and even increase the infectivity of virions, indicating that the motif is not necessary for incorporation (West *et al.*, 2002).

Thus, a system may exist whereby the CT of a majority of Envs is folded according to the conventional model and the cytoplasmic YXXϕ motif would be involved in downregulation of surface Env expression to guard against immune surveillance;

another proportion of processed Env is folded according to a revised model, retained on the surface at the site of $Pr55^{Gag}$ assembly and preferentially, but not exclusively, packaged into virions. Our results have identified gp41 CT truncations that enhance the fusogenic capacity of Env, supportive of a revised model of the gp41 CT, in which the extracellular/viral exposure of the Kennedy domain is involved in the process of membrane fusion. With regard to the recent revised model of the gp41 CT, further work is necessary to address the role of the Kennedy domain in cell-cell fusion and the effects of the truncations on virus assembly and infectivity.

Chapter 5

Analyses of HIV-1 Envs Derived from Long-Term Non-Progressor Patients Who Have "Switched" to Progressor Status

5.1 Introduction

5.1.1 Disease Progression

Clinical progression can be predicted by determination of plasma viral load and $CD4^+$ T cell numbers; rapidly increasing viral load concurrent with decreasing $CD4^+$ T cell counts are prognostic for disease progression. The interval from seroconversion to development of AIDS and death can be extremely variable. In developed countries where clinical support is readily available, the median interval is approximately 10 years for a HIV-1 infected individual classified as a standard progressor (SP). In less developed countries where the population is burdened by chronic parasitic infections and poor medical infrastructures, this interval can be considerably reduced. In extreme cases it has been observed that AIDS can develop in a matter of months, while in others AIDS does not develop even after 10 years of infection (Demarest *et al.*, 2001; Easterbrook, 1999). The duration of infection coupled with determinations of $CD4^+$ cell counts enables classification of patients according to the rate of disease progression.

5.1.2 Categorisation of Infected Patients

5.1.2.1 Rapid Progressors

Patients who exhibit a rapid decline in $CD4^+$ T lymphocytes to less than 500 cells/μl within 2-5 years of initial infection, characterised by an accelerated course of disease progression leading to death, are classified as rapid progressors (RP) (Liu *et al.*, 2002). In these individuals, the viral load following the acute phase of HIV infection is not resolved by the immune response, allowing the persistent proliferation of a homogeneous virus population. In SIV models, a high viral load set point, coupled with a lack of SIV-specific humoral response is also prognostic for rapid disease progression (Dehghani *et al.*, 2003; Marthas *et al.*, 1995; Ryzhova *et al.*, 2002).

5.1.2.2 Long-Term Non-Progressors

Asymptomatic HIV-1 infected adults who maintain normal $CD4^+$ T cell counts above 500 cells/μl and low viral load in the absence of antiretroviral therapy for over 10 years are classified as long-term non-progressors (LTNP) (Easterbrook, 1999; Levy, 1998). The low viral loads after seroconversion are suggestive of efficient immune control of HIV. It is not known how long the asymptomatic period could be, as HIV/AIDS has been a significant problem in humans for two decades only, but LTNP patients are certain to eventually progress to AIDS as defined by a decline in $CD4^+$ cells and increasing viral load.

In the case of paediatric HIV sufferers and SIV neonatal-macaque models, RP and LTNP patient types can be similarly distinguished (Marthas *et al.*, 1995). The median time for survival is about 8 years, infected children that remain asymptomatic after 8 years are considered non-progressors (Easterbrook, 1999), while RP patients may succumb to AIDS by their second year of life (Essajee *et al.*, 2000). The clinical course in the HIV-1^+ mother may determine the rate of progression through the transmission of virulent strains or a higher inoculum (Essajee *et al.*, 2000).

5.1.2.3 Other Patient Groups

A distinct group of patients are highly exposed but persistently seronegative individuals (HEPS) observed in homosexual and heterosexual communities such as commercial sex workers (CSW). These individuals remain uninfected (as determined by HIV-1 specific PCR and serology) despite repeated exposure to HIV (Bird *et al.*, 2002; Fowke *et al.*, 1998; Kaul *et al.*, 2000; Kaul *et al.*, 1999; Rowland-Jones *et al.*, 1995). These individuals have been the subjects of intense study; the disparity in disease progression has been attributed to the efficiency of the immune response (Kaul *et al.*, 2000; Kaul *et al.*, 1999; Rowland-Jones *et al.*, 1995) rather than any genetic traits such as β-chemokine receptor polymorphisms (See below) (Fowke *et al.*, 1998).

An additional patient group exhibiting slow disease progression are asymptomatic individuals with $CD4^+$ T cell counts less than 200 cells/μl. These patients usually exhibit a slower rate of decline in $CD4^+$ cells than SPs despite the lack of treatment; it is likely that other mechanisms control HIV replication in these individuals (Easterbrook, 1999).

5.1.3 Genetic Determinants of HIV-1 Resistance

5.1.3.1 Chemokine Receptors

As discussed in the introduction, the susceptibility of a cell to HIV-1 is dependent on the expression of appropriate receptors in sufficient quantity on the target cell surface. The majority of primary HIV-1 isolates obtained during the early and asymptomatic stages of infection preferentially use CCR5 as a co-receptor. It has been demonstrated that the density of CCR5 on the cell surface can be a major determinant for disease progression (Reynes *et al.*, 2001), and polymorphisms in chemokine receptors can affect their surface expression. Of the many polymorphic CCR5 alleles, the CCR5Δ32 mutant allele is the most effective in restricting the entry of HIV-1 into $CD4^+$ cells of

homozygous individuals (Cohen *et al.*, 1998), and heterozygotes are associated with a delayed onset of $CD4^+$ cell decline (Balfe *et al.*, 1998). CCR5 is physiologically dispensable, as individuals homozygous for CCR5Δ32 suffer little ill health as a direct result. However, this mutation is not necessarily observed in all individuals exhibiting resistance to HIV-1 infection (Greenough *et al.*, 1999; Magierowska *et al.*, 1999), and as this mutation is predominantly observed in the Caucasian population, LTNP patients of other ethnicities are likely to retard disease progression by other mechanisms.

Of the other CKRs employed by HIV-1, the CCR2b-64I mutant allele has been associated with slower disease progression, although this has less of an effect than CCR5Δ32 (Easterbrook *et al.*, 1999; Greenough *et al.*, 1999; Magierowska *et al.*, 1999). Polymorphisms are rarely described for the CXCR4 α-chemokine co-receptor used by the SI HIV-1 strains isolated during the later stages of AIDS (Cohen *et al.*, 1998). This may be due to CXCR4 being required for a manner of processes such as cellular maturation, trafficking and co-stimulation of T lymphocytes (Soriano *et al.*, 2002). CXCR4 surface expression is downregulated by its constitutively expressed ligand, stromal cell-derived factor 1 (SDF-1) (Soriano *et al.*, 2002). A polymorphic allele, SDF1-3'A, is affected by the stability of the mRNA transcripts, reducing the amount of translated SDF-1, thus homozygous individuals for this mutant allele are likely to have less plasma SDF-1 (Soriano *et al.*, 2002). It has been suggested that a high level of plasma SDF-1 in combination with a low level of cell surface CXCR4 could offer a protective effect against X4-tropic strains (Soriano *et al.*, 2002), although other studies did not find an association between this mutant allele and the rate of disease progression (Easterbrook *et al.*, 1999). CXCR4 has been noted to be at a higher level in some HEPS individuals, so the lack of infection observed in these individuals presumes that the primary HIV strains encountered by these individuals are not X4-tropic (Fowke *et al.*, 1998).

5.1.3.2 Human Leukocyte Antigen Types

The human leukocyte antigen (HLA) gene products, the equivalent of the murine major histocompatibility complex (MHC), are involved in the presentation of foreign antigenic peptides to cells of the immune system. The recognition of these peptides can be restricted to subsets of immune cells depending on the HLA-type they are presented on. Studies of LTNP patients from various cohorts and also chimpanzees have identified the presence or combinations of certain HLA genes associated with disease progression

(Table 5.1) (Balla-Jhagjhoorsingh *et al.*, 1999; Carrington *et al.*, 1999; Kaslow *et al.*, 1996; Magierowska *et al.*, 1999). Chimpanzees, as do some LTNP patients, recognise identical Gag epitopes despite differences in the HLA binding pockets, suggesting a correlation between the type of epitope recognised and disease progression (Balla-Jhagjhoorsingh *et al.*, 1999). Heterozygosity of the HLA type-1 loci has been implicated as an important determinant of non-progression in some LTNP cohort studies but not others (Carrington *et al.*, 1999; Greenough *et al.*, 1999). In terms of an individual, all their type-1 HLA molecules are constitutively expressed on all cell surfaces. Interestingly, the presence of HLA-B*35 or HLA-Cw*04 has been associated with disease progression. It is thought that these particular alleles may be involved in modulating natural killer (NK)cell function; and it has been suggested that a loss of NK cell function is associated with rapid disease progression (Carrington *et al.*, 1999). Transporters associated with antigen processing (TAP) act with type-1 HLA for efficient presentation of antigenic peptides to cytotoxic T lymphocytes (CTL), and are therefore involved in disease progression in conjunction with certain HLA combinations (Table 5.1) (Demarest *et al.*, 2001; Kaslow *et al.*, 1996)

Patient Category	Associated HLA Haplotypes
Rapid progressors	A1, A11, A23+TAP2.3, A28+TAP2.3, A29+TAP2.1, B8+DR3, B35+Cw4, DR2
Long-term non-progressors	A25+TAP2.3, A26, A3, A32, B5, B14, B17, B18, B27, B51, B57, BW4, DR6, DR7, BRB1*0702+DQA1*0201, DR13
Highly exposed, persistently seronegative individuals	A2, A28, DR13

Table 5.1. HLA haplotypes associated with differential rates of disease progression. Compiled from (Balla-Jhagjhoorsingh *et al.*, 1999; Carrington *et al.*, 1999; Demarest *et al.*, 2001; Greenough *et al.*, 1999; Levy, 1998).

5.1.4 Immunologic Control of HIV-1

Longitudinal studies have identified an inverse relationship between HIV-specific CTL and viral load, indicating that the ability to mount an effective CTL response is correlated with disease suppression (Alexander *et al.*, 2000; Demarest *et al.*, 2001; Greenough *et al.*, 1999; Liu *et al.*, 1997; Ogg *et al.*, 1999; Rosenberg *et al.*, 1997). The Th-1 response (T_H cell mediated CTL response, and IL-2 production) is able to control HIV viral load better than the Th-2 humoral response (IL-10 production and B cells) (Rosenberg *et al.*, 1997), although the role of neutralising antibodies in retarding disease progression is significant in SIV models (Dehghani *et al.*, 2003; Marthas *et al.*, 1995; Ryzhova *et al.*, 2002). In the case of HEPS CSW, the cervical mucosa is the most likely site of initial interaction between HIV and the host immune system. Indeed active CTL and HIV-specific IgA are correlates of protection in these individuals (Kaul *et al.*, 2000; Kaul *et al.*, 1999; Rowland-Jones *et al.*, 1995). RP patients commonly exhibit a near complete absence of HIV-specific CTL, despite the presence of the competent naïve subset of $CD8^+$ lymphocytes. In SIV- and HIV-infection, the lack of neutralising antibodies against endogenous variants has strong correlations with rapid disease progression (Demarest *et al.*, 2001; Liu *et al.*, 2002; Marthas *et al.*, 1995).

5.1.5 Role of HIV-1 Variants in Disease Progression

The nature of the virus is demonstrably important in disease progression, infection of neonatal macaques with different strains of SIV induces different rates of disease progression from LTNP-like to RP-like (Marthas *et al.*, 1995), while other SIV studies indicate that isolates from later stages of disease have increased virulence (Dehghani *et al.*, 2003). In the case of HIV-1 infected LTNP patients, virus isolates exhibit reduced growth kinetics (Connor *et al.*, 1996; Greenough *et al.*, 1999). Many studies correlate slow progression with the development of a broad genetic diversity in HIV strains highly diverged from the initial parental strains (Essajee *et al.*, 2000; Liu *et al.*, 1997; Ross and Rodrigo, 2002). Neutralising antibodies and an active cell-mediated response are commonly observed in LTNP patients, these factors most likely drive the selection of escape variants over a long period of time, hence the higher frequency of non-synonymous changes observed in isolates from these patients (Liu *et al.*, 1997). In contrast, progressive disease is correlated with a lack of HIV diversification and divergence. The accumulation of synonymous changes observed in isolates obtained from RP patients suggests purifying selection for increasingly replication competent strains in the absence of specific immune responses (Demarest *et al.*, 2001; Essajee *et*

al., 2000; Ross and Rodrigo, 2002). In a RP case study of an individual infected with two distinct variants, recombination was detected between the two isolates leading to the establishment of dominant recombinant variants; however, the accumulation of non-synonymous variation was extremely low consistent with the lack of selection for immune escape mutants (Liu *et al.*, 2002; Liu *et al.*, 1997).

Many studies have documented mutations in the virus genome that attenuate the virus to varying degrees. Studies of SIV lacking the entire, or part of, the *nef* gene in rhesus macaques suggest the possibility of generating live-attenuated HIV vaccine strains. Such clones exhibit attenuated replication kinetics *in vitro* and in adult monkeys. Vaccinees exhibit normal CD4 cell counts and varying degrees of specific humoral and cellular responses to various SIV proteins (Almond et al., 1995; Baba et al., 1995; Cranage et al., 1997; Daniel et al., 1992; Gauduin et al., 1999; Gundlach et al., 1998; Igarashi et al., 1997; Norley et al., 1996; Tenner-Racz et al., 2004; Titti et al., 1997). Challenges with pathogenic SIV strains applied via systemic or mucosal routes have demonstrated successful long-term protection against development of SIV-AIDS in the vaccinees compared to unvaccinated controls in all studies. The mode of protection elicited by SIV vaccine strains lacking part of or the entire *nef* gene appears to be efficient control of replication of the challenge strain, as secondary immune responses specific to the challenge strain are rarely detected, although reactivation of the vaccine strain may be observed. Protection is unlikely to be dependent upon neutralizing antibodies directed at the viral Env, as vaccinees are protected against challenge with SIV strains, SHIV and SIV-MuLV hybrid constructs bearing unrelated Envs (Cranage et al., 1997; Gundlach et al., 1998; Igarashi et al., 1997; Norley et al., 1996).

However, despite their attenuation, vaccine strains can persist in the host for a long duration (Khatissian et al., 2001), and it has been shown that protection against homologous and heterologous challenge strains is not absolute (Khatissian et al., 2001; Nilsson et al., 1998). Further, although studies suggest that breakthrough viruses exhibit somewhat limited diversity in vaccinees (Sodora et al., 1999), adult macaques, vaccinated with attenuated SIV constructs, do manifest clinical symptoms of disease progression and AIDS during long-term follow-up studies (Baba et al., 1999; Hofmann-Lehmann et al., 2003). In other studies, spontaneous repair of *nef* defects were observed, resulting in persistent viremia in vaccinated animals and increased virulence (Carl et al., 1999; Cranage et al., 1997; Norley et al., 1996; Whatmore et al., 1995). Converse to the

results in adult macaques, administration of attenuated SIV to neonates induced fatal SIV-AIDS (Baba et al., 1995; Baba et al., 1999). Hence there are considerable hurdles to be overcome before a live-attenuated HIV vaccine becomes a viable option for human treatment.

In a longitudinal study of a LTNP, premature stop codons in the viral p17, p24 and RT proteins were implicated in arresting viral evolution (Wang *et al.*, 2003), whereas in a study of haemophiliacs, unusual polymorphisms were detected in p17, p2, p6, gp41, Vpu and Nef proteins and the 3'LTR, yet practically all viral isolates were replication competent (Alexander *et al.*, 2000). Perturbations of Nef or the *nef*/LTR juncture observed in LTNP-derived viral isolates can impair infection of macrophages and viral expansion, although these mutations are not absolutely essential for slower disease progression (Alexander *et al.*, 2000; Carl *et al.*, 2000; Geffin *et al.*, 2000; Mourich *et al.*, 1999; Rhodes *et al.*, 2000). Particular Nef motifs may be associated with modulation of $CD4^+$ cell numbers, in one LTNP case study, dual-tropic variants with *nef* perturbations emerged in later stages of disease, capable of inducing apoptosis to a greater extent than earlier isolates (Jekle *et al.*, 2002; Kirchhoff *et al.*, 1999). Primary isolates exhibiting deficiencies in promoter sequences in the LTR have been demonstrated to have reduced expression of viral genomes (Fang *et al.*, 2001). The identification of truncated Vpr in chronically infected T cell lines compared to latently infected T cell lines, suggested a possible role for this HIV protein in modulating HIV pathogenesis (Masciotra *et al.*, 2002). Analyses of the Los Alamos database and a LTNP cohort revealed further that an R77Q substitution in the C-terminal functional domain of Vpr is over-represented in LTNP sequences. The inoculation of this mutant form of Vpr into mice did not, compared to that of the wildtype Vpr, result in a $CD4^+$ cell loss implicating Vpr in influencing disease progression (Lum *et al.*, 2003).

The Env surface glycoprotein of HIV mediates entry into the host cell, so it is a highly exposed protein on the viral surface and is a major target for neutralising antibodies. Extensive glycosylation and variation via amino acid residue substitution, insertion and/or deletion is employed by HIV to mask neutralising epitopes, making the Env the most variable protein of HIV. Some studies suggest that selection in *env* occurs at T-helper lymphocyte epitopes as well as CTL and antibody (Ross and Rodrigo, 2002). Moreover, other studies suggest CTL escape variants are more likely to emerge as the virus adapts to a novel host population with restricted HLA haplotypes (Daniels *et al.*,

2003). E*nv*-gene sequence and glycoprotein analyses of HIV isolates indicate there may be defects in protein synthesis or processing of Env, suggesting impairment of the infection process is a contributory factor to low viral load and slower disease progression (Adams *et al.*, 2000; Connor *et al.*, 1996). In a SIV study, only a minority of Envs derived from RP macaques are processed, but these are fusion competent (Dehghani *et al.*, 2003). The integrity of the processing sites are usually maintained in many of the defective *env*-gene products, suggesting that other structural determinants are involved in Env processing (Adams *et al.*, 2000; Connor *et al.*, 1996). The transmission kinetics of cell-free viruses are also less efficient than co-culture of HIV-infected PBMCs with uninfected PBMCs. It is possible that processing may occur at extremely low frequency in infected cells, allowing dissemination of virus by cell-cell spread rather than virus-cell spread, as in the case of HTLV, and as discussed in chapter 3 (Connor *et al.*, 1996).

The majority of isolates exhibit the greatest diversity in the gp120 variable loops, affecting their affinities for host cell receptors to varying degrees (Bagnarelli *et al.*, 2003; Fang *et al.*, 2001; Masciotra *et al.*, 2002). The co-receptor switch, from CCR5 to CXCR4 usage, observed in some AIDS patients is commonly related to substitutions in the V3 loop for basic residues (Milich *et al.*, 1997; Xiao *et al.*, 1998), with a greater diversity observed in X4-tropic strains than R5-tropic strains (Masciotra *et al.*, 2002; Nelson *et al.*, 2000; Shankarappa *et al.*, 1999). Although SI strains are associated with disease progression, the switch from NSI strains is observed in just over 50% of symptomatic AIDS patients (Liu *et al.*, 1997). SIV isolates from infected macaques similarly exhibit considerable evolution in the Env variable domains with the exception of the V3 loop equivalent, which is well conserved (Dehghani *et al.*, 2003; Rudensey *et al.*, 1998), the variation is likely to be driven by immune selection for escape variants (Rudensey *et al.*, 1998). Although a switch in co-receptor usage is not commonly observed in SIV AIDS, rapid disease progression in macaque models of SIV infection correlates with the emergence of CD4-independent macrophage-tropic variants, associated with mutations in gp120 of a conserved CD4-binding GGDPE motif in the C4 domain and the loss of an N-linked glycosylation site in the V1V2 loop (Dehghani *et al.*, 2003; Ryzhova *et al.*, 2002). Although the appearance of X4-tropic HIV-1 strains portends disease progression in many cases, R5-tropic variants are still maintained in the host (Nelson *et al.*, 2000). The lack of neutralising antibodies in RP patients is reflected in the lack of diversity observed in the *env*-genes of their HIV-1 isolates

(Lukashov *et al.*, 1995). In SIV RP models, the absence of neutralising antibodies permits the increased exposure of co-receptor binding epitopes as the fitness of the virus evolves to increase its affinity for the CKRs (Dehghani *et al.*, 2003; Ryzhova *et al.*, 2002).

5.1.6 Study Objectives

Although disease progression is a multi-factorial process, it is considered that the replicative capacity of the virus plays an important role. From previous cohort studies within our laboratory, analyses of consensus *env*-gene sequences derived from proviruses in RP and LTNP patients has revealed physico-chemical differences associated with particular amino acid substitutions in HIV-1 glycoproteins derived from these groups (Rod Daniels, personal communication). It is plausible that variation at specific sites can impair the fusogenic capacity of Env with regard to infection of $CD4^+$ cells. This could translate to a reduced rate of spread during the asymptomatic period, and hence a delay in the manifestation of AIDS associated with a LTNP phenotype. Over time, the emergence of aggressive variants prior to or at the point of accelerated $CD4^+$ cell depletion (and notably as immune control is lost), and their expansion throughout the symptomatic AIDS phase could be prognostic for disease progression. Thus, we propose that the apparent evolution of HIV-1 *env*-genes in LTNP patients may lead to variants better able to infect $CD4^+$ cells, resulting in their depletion and thus disease progression. To study the biological properties of our cohort-derived *env* genes, we employed cell-cell fusion assays and virus infection assays to determine if there is any association between the fusogenic capacities of the viral Envs derived from LTNP patients and disease progression.

5.2 Methodology

5.2.1 Patient Cohort

A cohort of patients was established at the Chelsea and Westminster Hospital in 1995 and managed from King's College Hospital (Easterbrook, 1998). The demographics of the cohort, consisting of RP, SP and LTNP patients are detailed in table 5.2. Patient selections for each progression group were intended to provide matched demographic data with regard to age, race, sexual orientation and median time of HIV-1 infection. Other research groups, involved in the study of the mechanisms underlying slow disease progression, use samples provided from this cohort (Figure 5.2) (Carl *et al.*, 2000; Kirchhoff *et al.*, 1999). Our group at NIMR is primarily interested in the *env*- and *gag*-genes with regard to epidemiology and biological function (Zheng *et al.*, 2002).

Clinical samples were provided consisting of Ficoll-Hypaque separated buffy coats at specific time points following a patient's enrolment in the cohort. Samples were not accepted for study following the onset of anti-retroviral therapy to prevent drug-induced bias of the *env*-gene data being collected.

	LTNP	SP	RP
Number of patients (n)	51	82	33
Median age at infection (Years)	28.9	31.7	29.3
Median duration of infection (Years)	10.2	10.1	9.8
% Homosexual	84	91	97
% Caucasian	92	96	94
% AIDS[a]	0	11	100
Mean $CD4^+$ T cell count (x $10^6/\mu l$)	646	286	144
Mean Viral RNA copies (x 10^3/ml)	14	29	72

Table 5.2. Demographic data of patients enrolled in the Chelsea and Westminster Cohort. Data represents 1995 sampling.

[a] The percentage of patients classified as manifesting symptomatic AIDS at enrolment.

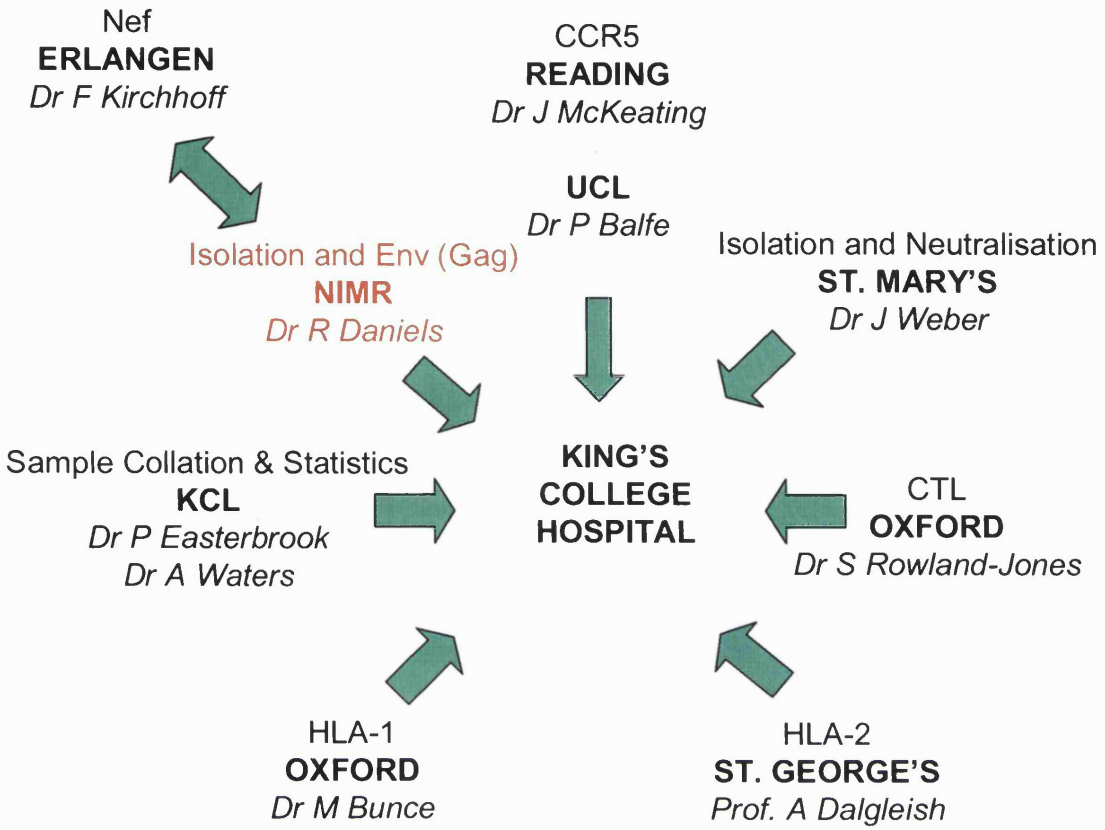


Figure 5.1. Research groups involved in the study of the Chelsea and Westminster Cohort of HIV-1 infected patients in 1995.

While the majority of LTNP patients in the cohort have remained asymptomatic for over 20 years since initial exposure, 4 patients have been identified as progressing to symptomatic AIDS within the last few years as determined by their rates of $CD4^+$ cell decline (Figures 5.2 and 5.3). The aim of this study was to determine if HIV-1 *env*-gene evolution could be involved in the switch to AIDS progression in these LTNP patients. Sampling of patient blood had been conducted at irregular intervals on the dates indicated on the graphs, and termination of sampling is attributed to the onset of anti-retroviral therapy. The HLA-haplotype of each patient is detailed in table 5.3. LTNP patients 012 and 028 are heterozygous for the CCR5Δ32 mutant allele, and LTNP patient 048 is heterozygous for the SDF1-3'A allele. The $CD4^+$ and $CD8^+$ T lymphocyte numbers and plasma viral RNA copies indicated in figures 5.3 and 5.4 were determined in hospital laboratories. Unfortunately, incomplete information was available to include in this study, notably for viral load, which is limited to the symptomatic phase in all patients.

Patient	HLA Typing:	CCR5Δ32	SDF1-3'A	CCR2b 64I
001	A*01, A*03, B*1402, B*57, Bw*4, Bw*6 DRb1*0701, DRb1*0701, DQa1*0201 DQa1*0201, DQb1*0201, DQb1*03032	wt/wt	wt/wt	wt/wt
012	A*02, A*3201-B*07, B*0801-, Bw*6, Bw* DRb1*0103, DRb1*03011, DQa1*0102, DQa1*0501, DQb1*0501, DQb1*0201	wt/mut	wt/wt	wt/wt
028	A*02, A*, B*27, Bw*4, B*73, Bw*6, Cw*01, Cw*1505 DRb1*0101, DRb1*0405, DQa1*0101, DQa1*0301, DQb1*0501, DQb1*0302	wt/mut	wt/wt	wt/wt
048	A*02, A*68, B*07, B*62, Bw*, Bw*6, Cw*0304, Cw*1203 DRb1*03011, DRb1*15021, DQa1*0501, DQa1*0102, DQb1*0201, DQb1*0602	wt/wt	wt/mut	wt/wt

Table 5.3. Genetic information of LTNP-switcher patients.

The patient HLA haplotypes, wildtype (wt) and mutant (mut) alleles of SDF-1 and chemokine receptors CCR5 and CCR2b are indicated. Unidentified HLA alleles are highlighted.

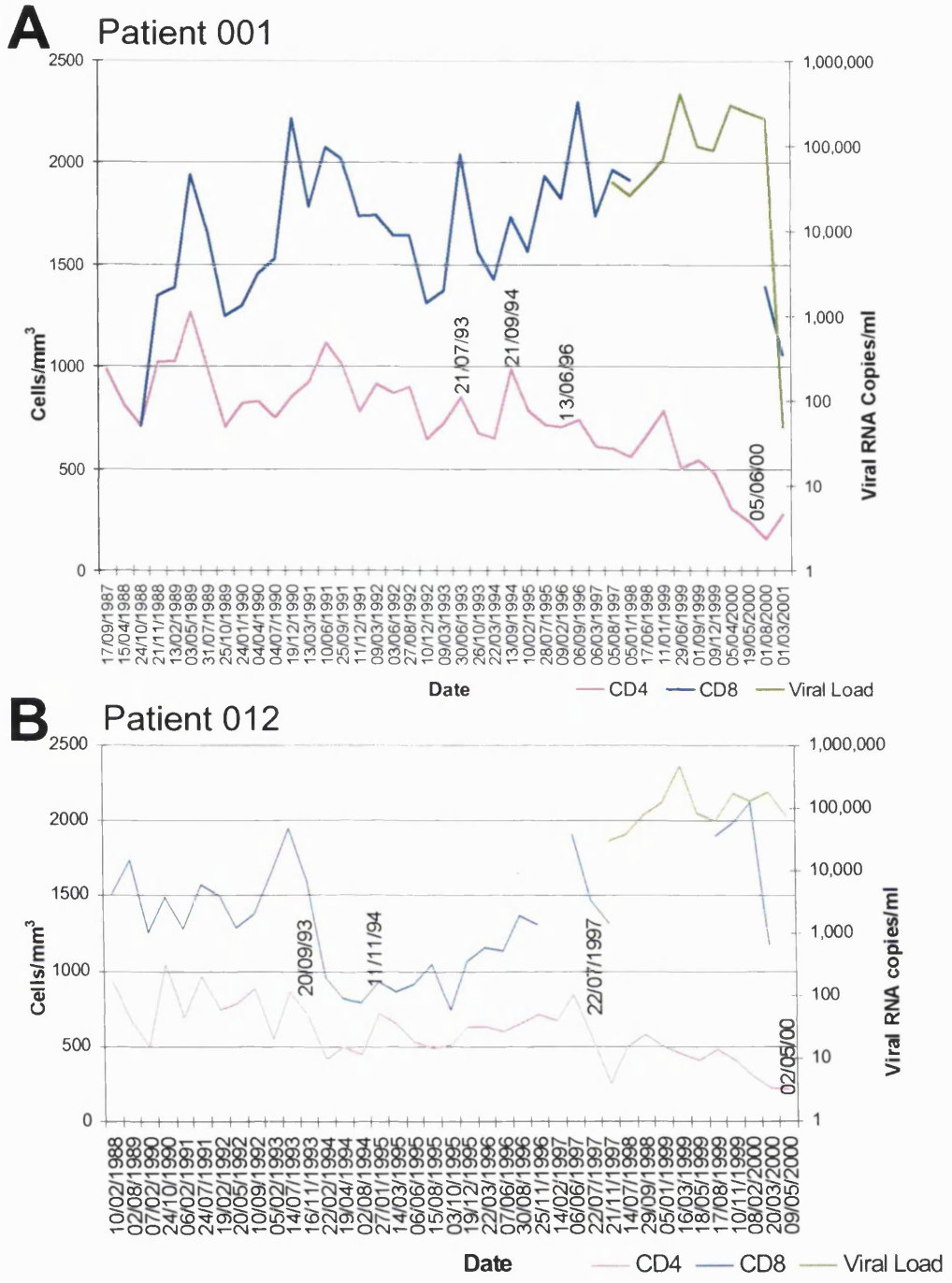


Figure 5.2. Longitudinal measurement of mean $CD4^+$, $CD8^+$ T lymphocytes numbers and mean viral load in LTNP-switcher patients.

Patient 001 (A) and Patient 012 (B). Time points where HIV-1 *env*-genes were isolated are indicated on the graphs. The data for $CD8^+$ T lymphocyte numbers and viral load are incomplete. The x-axis is a non-linear scale and dates represent hospital visits by the patient. The *env*-gene clones derived for each sampling period are assigned the sequential nomenclature indicated later in the chapter (e.g. 001:1B, 001:2B, 012:12B, etc.).

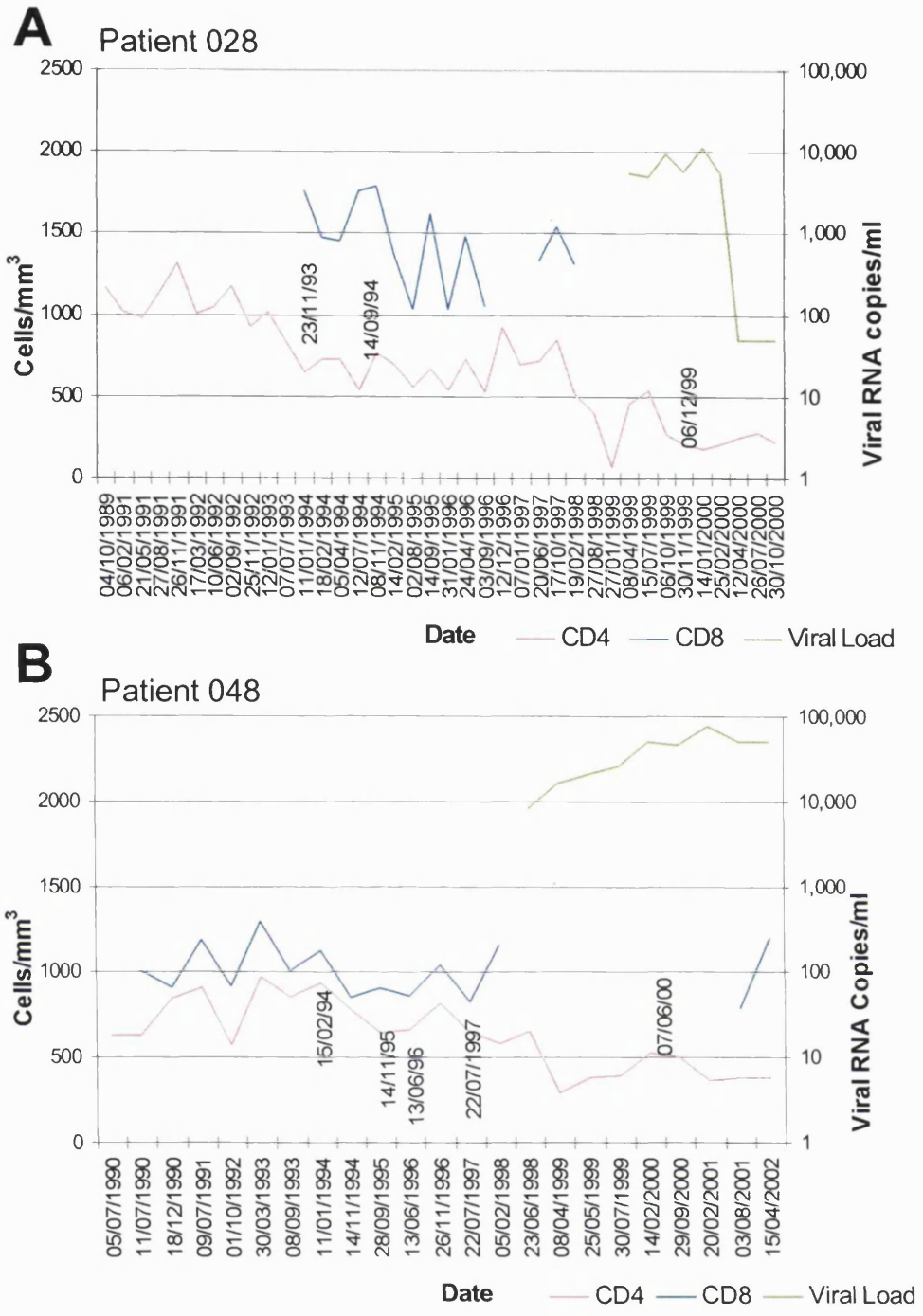


Figure 5.3. Longitudinal measurement of mean $CD4^+$, $CD8^+$ T lymphocyte numbers and mean viral load in LTNP-switcher patients.

Patient 028 (A) and Patient 048 (B). Time points where HIV-1 *env*-genes were isolated are indicated on the graphs. The data for $CD8^+$ T lymphocyte numbers and viral load are incomplete. The x-axis is a non-linear scale and dates represent hospital visits by the patient. The *env*-gene clones derived for each sampling period are assigned the sequential nomenclature indicated later in the chapter (e.g. 028:16B, 028:17B, 048:21B etc.).

5.2.2 Rescue of HIV-1 *env*-genes

Previous workers in the group conducted the rescue of proviral *env*-genes. Approximately 2x 10^6 PBMCs isolated from buffy coats were provided for each time point. Total cellular DNA was extracted from these samples and used as templates to rescue proviral HIV-1 *env*-genes as described (Douglas *et al.*, 1996; Penny *et al.*, 1996; Zheng *et al.*, 2002). Briefly, nested PCR using TATF, NEFR, FENV and RENV primer sets (Appendix A1.9.3) amplified full-length *env*-genes that were inserted into pQ7 vector downstream of a T7 RNA polymerase promoter by virtue of the *Xho*1 and *EcoR*1 restriction sites encoded by the 5' and 3' primers respectively. Resultant clones were screened for expression competence in the CV-1 cell line using T7 RNA polymerase expressed by the recombinant vaccinia construct vTF7-3 and ARP401 polyclonal antisera in subsequent western blots. Expression competent clones for each time point were sequenced by Z. Xiang using a panel of HIV-1 *env*-gene specific primers (Appendix A1.9.3). The effectiveness of this procedure has been demonstrated across a range of HIV-1 subtypes (Douglas et al., 1996). Phylogenetic analyses of the aligned *env*-genes were conducted by the Neighbour-Joining method (Pearson *et al.*, 1999) and the output visualised using TreeTool. All programs were contained in the Genetic Data Environment package (Smith *et al.*, 1994) and executed on a UNIX (SUN Systems) platform by R. Daniels.

5.2.3 Cell-Cell Fusion Assay

The ability of the patient-derived *env*-gene glycoprotein products to mediate cell-cell fusion was studied using the method described in Chapter 2. Results of quadruplicate assays are shown.

5.2.4 Chimeric Virus Production

Selected LTNP switcher-derived *env*-genes (based upon their activity in the fusion assay) were inserted into the pC2 infectious cassette using *Xho*1 and *Eco*R1 restriction sites as described in Chapter 2. Plasmids were transfected into 293T cells to produce clonal chimeric viruses.

5.2.5 Analyses of Env Processing

Chimeric viruses were harvested from the culture medium of transfected 293T cell by centrifugation and lysed. Lysates were analysed by myself and R. Chung using western blotting techniques, probing with ARP401 and ARP301 antisera and appropriate IgG-peroxidase conjugates, to detect the presence of gp160/gp120 species as described in Chapter 2.

5.2.6 Determination of $TCID_{50}$

The ability of chimeric viruses to infect PBMCs was determined by measurement of the $TCID_{50}$/ml of harvested virus in quadruplicate cultures of 5x 10^4 ($CD8^+$ T lymphocyte depleted) activated PBMCs, previously prepared by L. Whittaker and C. Vella. Measurement of HIV-1 p24 was performed as described in Chapter 2.

5.3 Results

5.3.1 Amino Acid Sequence Analysis

Previous Phylogenetic analyses of *env*-genes derived from the cohort have shown all patients to be infected with HIV-1 subtype B strains (R. Daniels personal communication). Such analyses for the four patients under study here, revealed discrete clustering of the *env*-genes within patients (Figure 5.4), indicating the variants are unique to each patient, and that no cross-contamination had occurred during isolation and cloning procedures. Divergence of variants into early and late lineages is observed in patients 001 and 048.

Comparison of the amino acid sequences of all full-length Env clones indicated extensive variation in the amino acid sequences of the gp120 subunit in all isolates (Appendix A3.1-4). Changes were characterised by substitutions, insertions and deletions across the V1/V2, C3, V4, C4 and V5 regions. A highly conserved residue Asp207 (HIV-1_{HXB2} numbering) was substituted for Asn in clone 028:17Ba. Analysis of the V3 loop exhibited sequences characteristic of the NSI phenotype except for isolates 028:22Rb and 028:22Rc which exhibit SI-like sequences. Considerable variation in the number and positions of putative N-linked glycosylation sites is observed between isolates from each patient (Table 5.4). The loss of a complex glycan on the V3 loop, implicated in defining phenotype, is observed in clones 001:18Rc, 048:22Ba and 048:23Ba.

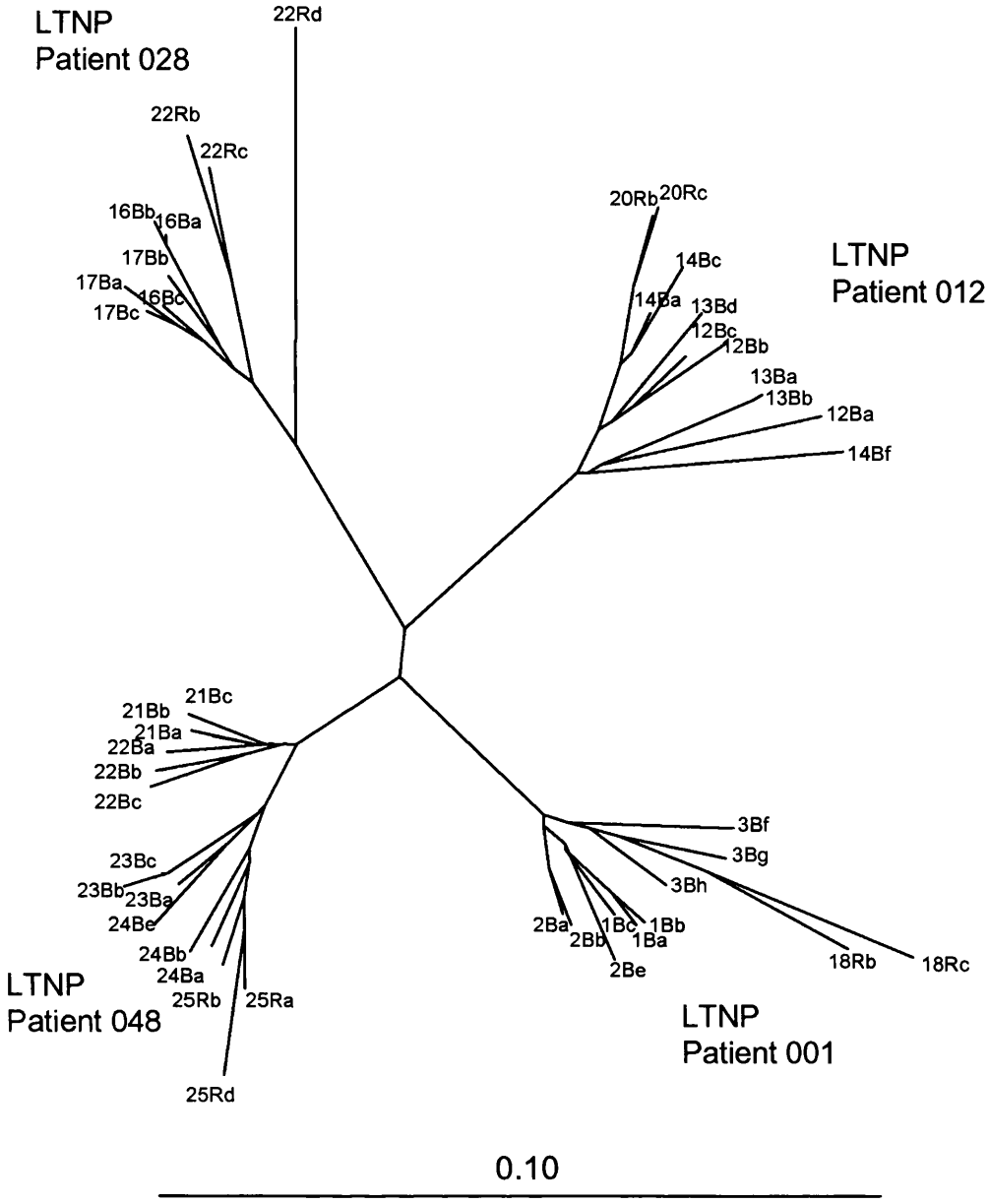


Figure 5.4. Neighbour-Joining phylogenetic tree.

This figure illustrates the relationships between full-length HIV-1 *env*-genes derived from the LTNP-switcher patients, and indicates no cross-contamination during isolation and cloning of the *env*-genes.

Patient	HIV-1 Env Clone	gp120 Domains										Net Change[a]	gp41 sites[b]
		C1	V1	V2	C2	V3	C3	V4	C4	V5	C5		
001	1Bc												4
	1Ba			-1		+1		-1				-1	4
	1Bb					+1		-1					4
	2Ba			-1					-1			-2	4
	2Bb			-1					-1			-2	4
	2Be			-1			-1	+1				-1	3
	3Bf					+1				+1		+2	4
	3Bg		-1					+1					4
	3Bh		-1	-1		-1	-1					-4	4
	18Rb	+1				+1				+1		+3	3
	18Rc	+1						-1		+1		+1	3
012	12Bb												5
	12Ba	+1		-1			-1					-1	4
	12Bc	+1					-1	-1				-1	5
	13Ba		+1	+1								+2	4
	13Bb		+1	+1								+2	4
	13Bd		+1					-1					5
	14Ba				+1			-1					4
	14Bc				+1			-1					4
	14Bf		+2		+1	+1	-1					+3	4
	20Rb				+1			-1					4
	20Rc				+1			-1					4
028	16Ba												4
	16Bb						-1					-1	4
	16Bc							-1				-1	4
	17Ba						-1	-1				-2	4
	17Bb				-1							-1	4
	17Bc						-1	-1				-2	4
	22Rb		-1		-2							-3	4
	22Rc		-1		-1							-2	4
	22Rd		+1	-1			-2	-2				-4	4
048	21Ba												4
	21Bb		-1					+1					4
	21Bc							+1				+1	4
	22Ba					-1		+1					4
	22Bb		-2					+1				-1	4
	22Bc							+1				+1	3
	23Ba		-1			-1	-1	+1	+1			-1	3
	23Bb							+1	+1	+1		+3	2
	23Bc							+1	+1	+1		+3	3
	24Ba		-1			-1	-1	+1	+1			-1	4
	24Bb		-1									-1	3
	24Be		-1						+1	-1		-1	2
	25Ra		-1					+1	+1	-1			4
	25Rb		-1					+1	+1	-1			4
	25Rd							+1	+1	-1		+1	4

Table 5.4. Variation in the number of putative N-linked glycosylation sequons across Env clones derived from LTNP-switcher patients.

[a] Indicates relative change in the number of gp120 N-linked glycosylation sequons with respect to the first clone indicated for each patient.

[b] Indicates the total number of putative N-linked glycosylation sequons in gp41.

Analysis of the KAKRRVVQREKR processing site revealed non-conservative substitutions in isolates from patients 001, 028, and 048 (Appendix A3.1-4). Variation in gp41 was predominantly observed in the sequences surrounding the assembly domain and in the number of N-linked glycosylation sequons near the principal immunodominant domain of the chain-reversal loop, moreover, a putative N-linked glycosylation sequon proximal to the gp41 MSD was only detected in clones 012:12Ba, 012:12Bc and 012:13Bd (Table 5.4).

Substitutions that may impact the surface expression of Env are observed in a number of clones: 001:2Bb exhibited a non-conservative R→G substitution within the MSD, and clone 012:13Bd exhibited a single residue change in the MSD-proximal YXXϕ endocytosis motif. The putative palmitoylated Cys residue proximal to the AH2 domain is detected in all isolates bar clones 012:20Rb and 012:20Rc, whereas the Cys residue in the AH1 domain is only detected in 001:1Ba and all the 001:2B clones. An additional Cys residue is observed between the AH2 and AH1 domains in clones 001:2Bb and 001:2Be, and adjacent to the AH2 Cys in 048:22Bb.

Other non-synonymous changes that could affect Env function are observed; such as an R→G substitution immediately downstream of the MSD in clone 028:17Bb. Non-synonymous changes in the immunogenic epitopes of the Kennedy domain are detectable in clones from all patients, and more variation occurs across the AH1 domain than the AH2 domain.

5.3.2 Cell-cell Fusion Assays

Env-genes were isolated from patient 001 at three time points in the asymptomatic phase ($CD4^+$ T cells >500 cells/μl) and one in the symptomatic stage ($CD4^+$ T cells <500 cells/μl; Figure 5.3A). Of the 11 Envs encoded by these genes, 9 possessed significant fusogenic activity ($P<0.05$, Table 5.5 and Figure 5.5). Only one of the clones, 18Rc, was isolated during the symptomatic stage when viral load was high. R5-tropic Envs with high fusogenic capacity were isolated in the second (2Ba) and third sampling periods (3Bf and 3Bg), coincident with a decline in $CD4^+$ cells (Figure 5.3A). The dual-tropic or X4-tropic Envs isolated during the asymptomatic phase did not exhibit high fusogenic capacity, whereas 18Rc isolated after the $CD4^+$ T cell count had decreased from approximately 800 cells/μl to less than 240 cells/μml (Figure 5.3A), exhibited the highest fusogenic capacity of all X4-tropic Envs (Figure 5.6).

For patient 012, *env*-genes were isolated from three sampling points during the asymptomatic period, and one in the symptomatic period (Figure 5.3B). Six of eleven Envs had significant fusogenic activity ($P<0.05$): 12Ba; 12Bb; 13Bb; 14Ba; 20Rb and 20Rc (Table 5.5 and Figure 5.6). Env 12Bb had the highest fusogenic capacity of all R5-tropic Envs, while 12Ba had the highest fusogenic capacity of X4 Envs isolated from this patient, and this sampling point is marked by a rapid reduction in $CD4^+$ cells from more than 800 cells/μl to approximately 400 cells/μl (Figure 5.3B). A precipitous decrease in $CD4^+$ cells to 252 cells/μl follows the third sampling period when 14Ba was isolated. Both the X4-tropic Envs 20Rb and 20Rc isolated in the final sampling period exhibited significant activity coincident with the low $CD4^+$ cell count and high viral load in the patient, which at this point exceeded 100,000 RNA copies/ml (Figure 5.3B).

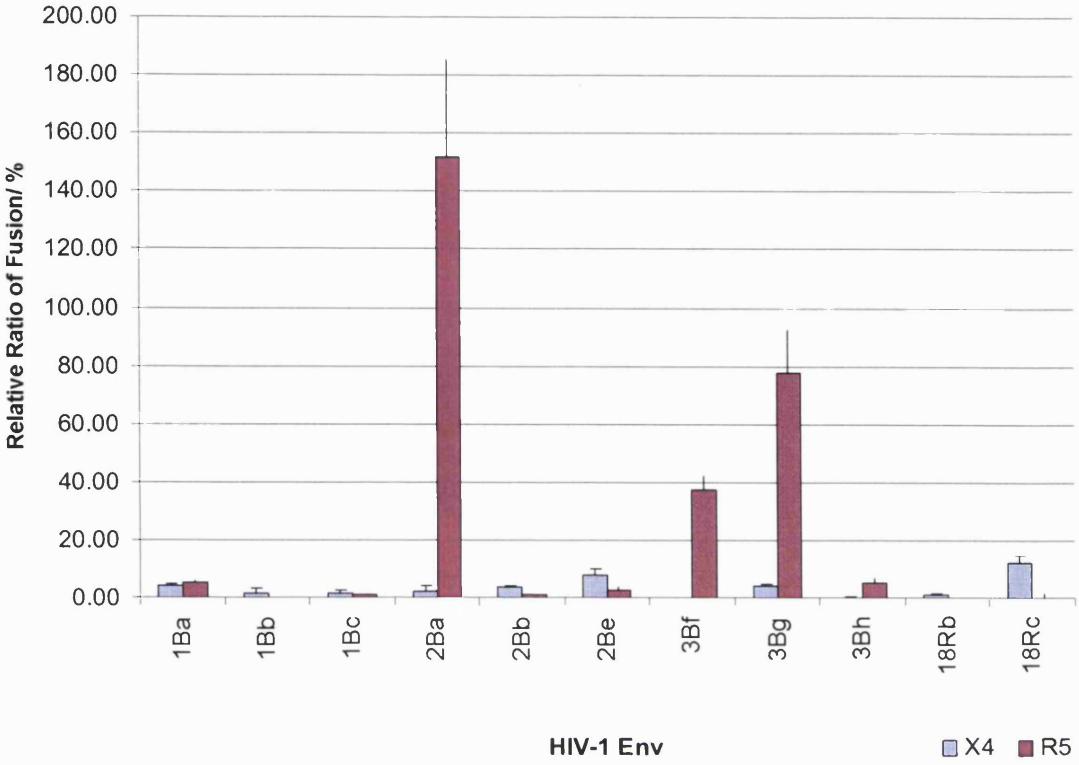


Figure 5.5. Fusogenic capacity of HIV-1 *env*-genes derived from patient 001.
The relative ratio of fusion compared to the $NL43_{WT}$ and $JRFL_{WT}$ Envs was plotted. Bars represent standard deviations.

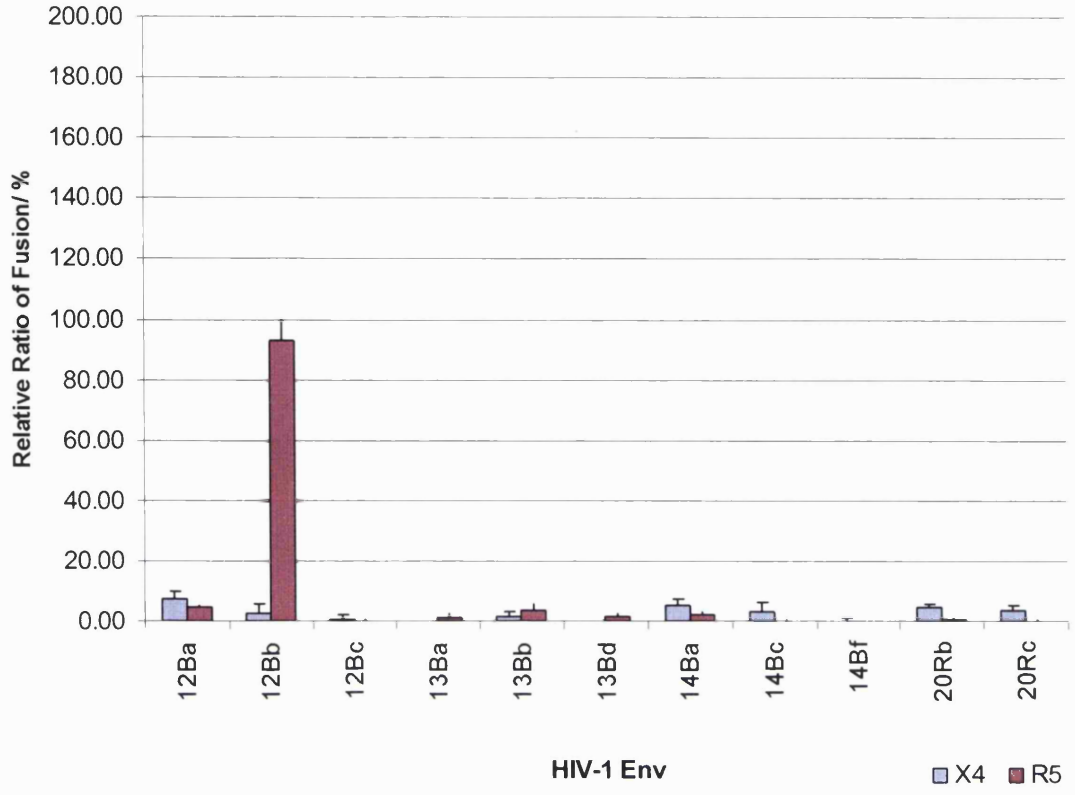


Figure 5.6. Fusogenic capacity of HIV-1 *env*-genes derived from patient 012.
The relative ratio of fusion compared to the $NL43_{WT}$ and $JRFL_{WT}$ Envs was plotted. Bars represent standard deviations.

In the case of patient 028, *env*-genes were derived from two sampling points in the asymptomatic period and one in the symptomatic period (Figure 5.4A). The relative ratio of fusogenic activity of recovered Envs were the lowest for the four patients studied and only Envs 17Ba, 17Bb and 22Rb show significant activity (Table 5.5 and Figure 5.7). The R5-tropic Envs 17Ba and 17Bb were isolated during the asymptomatic phase. Notably at the latter sampling point, the viral load was approximately a log lower than that seen in the other patients. The $CD4^+$ cell counts following the first and second sampling periods rise and fall frequently, until 10/1997 when there is a drop from 849 cells/μl to 65 cells/μl within 17 months (Figure 5.4A). Unfortunately, the irregular sampling meant that *env*-genes could not be recovered during this period.

All the samples obtained from patient 048 were during the asymptomatic phase, although the last sampling period was during a period of $CD4^+$ cell recovery prior to a lapse into the symptomatic stage (Figure 5.4B). Fifteen *env*-genes were isolated and of these, 8 had significant fusogenic activity with R5 cells and only 2 had exclusive significant fusogenic activity with X4 cells (Table 5.5 and Figure 5.8). The X4-tropic Envs (24Be and 25Rd) were isolated during the fourth and final sampling periods. The final sampling from patient 048 was during a period of $CD4^+$ cell recovery (Approximately 400 cells/μl) in contrast to the sampling periods in other patients where low levels of $CD4^+$ cells were observed, although high viral load was observed in this case (Figure 5.4B).

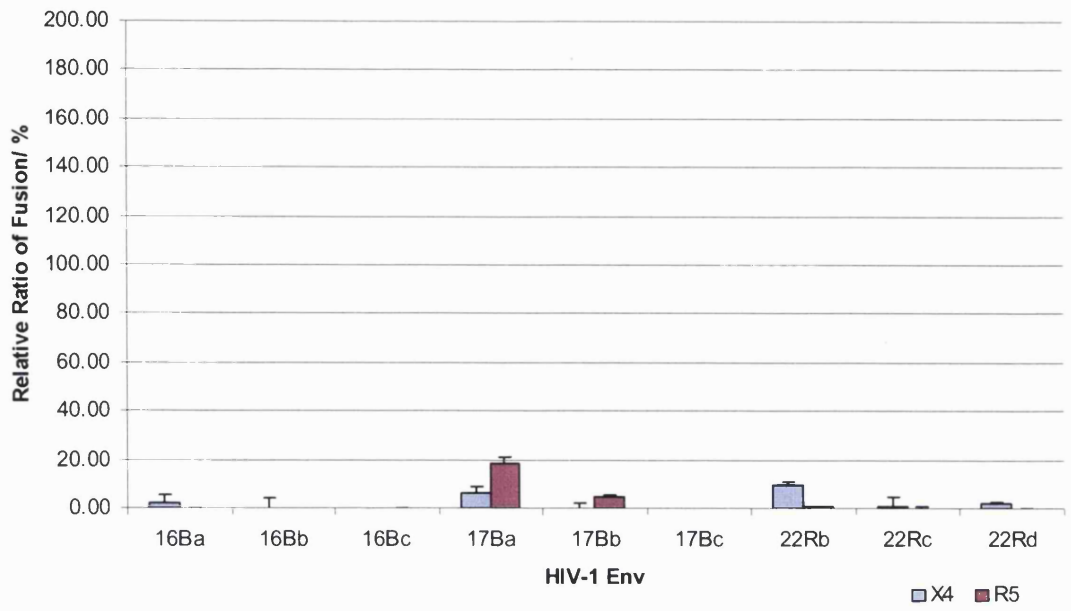


Figure 5.7. Fusogenic capacity of HIV-1 *env*-genes derived from patient 028.
The relative ratio of fusion compared to the $NL43_{WT}$ and $JRFL_{WT}$ Envs was plotted. Bars represent standard deviations.

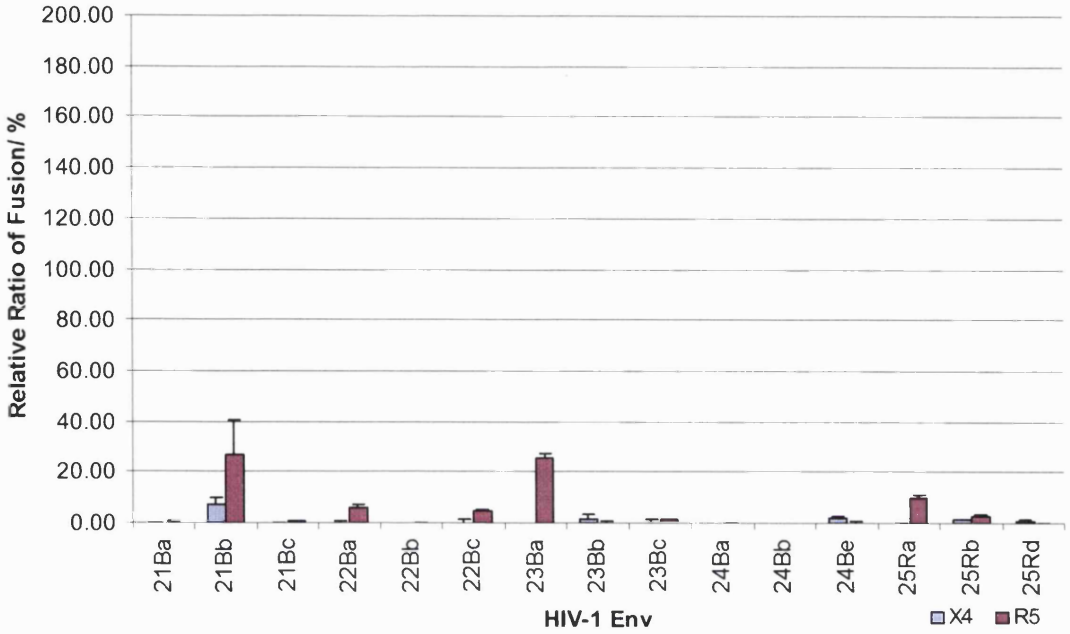


Figure 5.8. Fusogenic capacity of HIV-1 *env*-genes derived from patient 048.

The relative ratio of fusion compared to the $NL43_{WT}$ and $JRFL_{WT}$ Envs was plotted. Bars represent standard deviations.

HIV-1 Env	%NL43	P(>BG)[a]	%JRFL	P(>BG)[a]	P(X4≠R5)[b]	Tropism[c]
1Ba	**3.95**	0.0009	**5.21**	0.0000	0.0000	D/R5
1Bb	**1.51**	0.2644	**0.00**		0.0514	X4
1Bc	**1.82**	0.0015	**1.01**	0.0003	0.1722	D
2Ba	**2.31**	0.0721	**151.60**	0.0014		R5
2Bb	**3.52**	0.0008	**0.99**	0.0050	0.0265	D/X4
2Be	**7.84**	0.0029	**2.84**	0.0017	0.1336	D
3Bf	**0.00**		**37.20**	0.0002		R5
3Bg	**4.03**	0.0004	**77.70**	0.0009	0.0009	D/R5
3Bh	**0.00**		**5.09**	0.0049		R5
18Rb	**5.21**	0.0396	**4.06**	0.0028	0.0770	D
18Rc	**12.01**	0.0004	**0.09**	0.4594		X4
12Ba	**7.47**	0.0036	**4.73**	0.0002	0.0002	D/R5
12Bb	**2.37**	0.1313	**92.87**	0.0001		R5
12Bc	**0.78**	0.2209	**0.18**	0.2148	0.4750	D
13Ba	**0.00**		**0.88**	0.1786		R5
13Bb	**1.56**	0.3033	**3.89**	0.0095		R5
13Bd	**0.00**		**1.41**	0.0551		R5
14Ba	**5.40**	0.0031	**1.89**	0.0233	0.1477	D
14Bc	**3.39**	0.0705	**0.04**	0.4622	0.0639	D
14Bf	**0.00**		**0.00**			
20Rb	**4.73**	0.0091	**0.72**	0.0244	0.0905	D
20Rc	**3.84**	0.0196	**0.23**	0.2275		X4
16Ba	**2.06**	0.1926	**0.12**	0.2696	0.2838	D
16Bb	**0.00**		**0.00**			
16Bc	**0.00**		**0.00**			
17Ba	**6.29**	0.0372	**18.08**	0.0001	0.0005	D/R5
17Bb	**0.00**		**4.65**	0.0003		R5
17Bc	**0.00**		**0.00**			
22Rb	**9.79**	0.0001	**0.61**	0.0052	0.0011	D/X4
22Rc	**0.99**	0.4758				X4
22Rd	**2.07**	0.1145	**0.05**	0.4019	0.0503	D
21Ba	**0.00**		**0.03**	0.4371		R5
21Bb	**6.86**	0.0127	**26.50**	0.0069	0.0001	D/R5
21Bc	**0.00**		**0.63**	0.0085		R5
22Ba	**0.00**		**5.79**	0.0014		R5
22Bb	**0.00**		**0.00**			
22Bc	**0.00**		**4.33**	0.0017		R5
23Ba	**0.00**		**25.26**	0.0001		R5
23Bb	**1.49**	0.1289	**0.17**	0.2899	0.2152	D
23Bc	**0.05**	0.4669	**1.22**	0.0001		R5
24Ba	**0.00**		**0.00**			
24Bb	**0.00**		**0.00**			
24Be	**2.20**	0.0246	**0.30**	0.1237		X4
25Ra	**0.00**		**9.71**	0.0003		R5
25Rb	**1.17**	0.0001	**2.71**	0.0022	0.0033	D/R5
25Rd	**0.87**	0.0087	**0.07**	0.1882		X4

Table 5.5. Fusogenic capacities of Envs derived from LTNP-switcher patients expressed as the percentage activity of the control Envs $NL43_{WT}$ and $JRFL_{WT}$.

[a] Student's T-test *P* value determining is significantly greater than the background. Samples with no values exhibit no activity above background levels. Significant values are indicated in red.

[b] Student's T-test determining if the relative ratio of expression significantly differs between X4 and R5 target cells if both values for background expression have equivalent significance. Significant values ($P<0.05$) are indicated in red.

[c] X4, CXCR4-tropic; R5, CCR5-tropic; D, Dual-tropic. Dual-tropism is conferred in the event of matched significance for X4 or R5 target cells. Preference is indicated if *P* (X≠R) value is significantly different, according to the relative ratio of expression.

Tropism[a]	Number of Envs	Number showing Significant Activity[b]
R5	15	12
X4	6	4
Dual-tropic	18	13
No activity	7	0
Total	46	29

Table 5.6. Summary of the fusogenic capacities of HIV-1 Envs derived from LTNP-switcher patients.

[a] Dual-tropic Envs includes those Envs with significant preference for either X4 or R5 target cells as determined in Table 5.5.
[b] The number of each Env phenotype expressing significant fusogenic activity in cell-cell fusion assays.

Overall 39/46 Envs demonstrated fusogenic activity, and of them 29 were significant (Table 5.6). R5- and Dual-tropic Envs were predominantly isolated from LTNP-switcher patients, whereas X4-tropic Envs were isolated at a low frequency. However, it was observed that X4-tropic Envs were isolated from the later sampling periods (5/6). A greater proportion of Envs lacking significant activity were isolated from patients 012 and 028. Dual-tropic Envs exhibited a preference for R5-target cells (6/18) compared to X4-target cells (2/18). Although dual-tropic and X4-tropic Envs were predominantly isolated during the final sampling period in all patients, the number of isolates studied is too small for definite conclusions regarding a co-receptor switch to be drawn.

5.3.3 Chimeric Virus Studies

5.3.3.1 Chimeric Virus Production

A selection of Envs exhibiting fusogenic activity and closely related Envs (as determined by amino acid sequence) were studied in the pC2 infectious cassette system. To generate virus stocks, 293T cells were transfected with the pC2-constructs. Quantification of the HIV-1 p24 content of chimeric virus stocks indicated successful production of chimeric viruses in all cases bar those clones expressing 012:12Bb and 012:12Bc Envs (Figure 5.9).

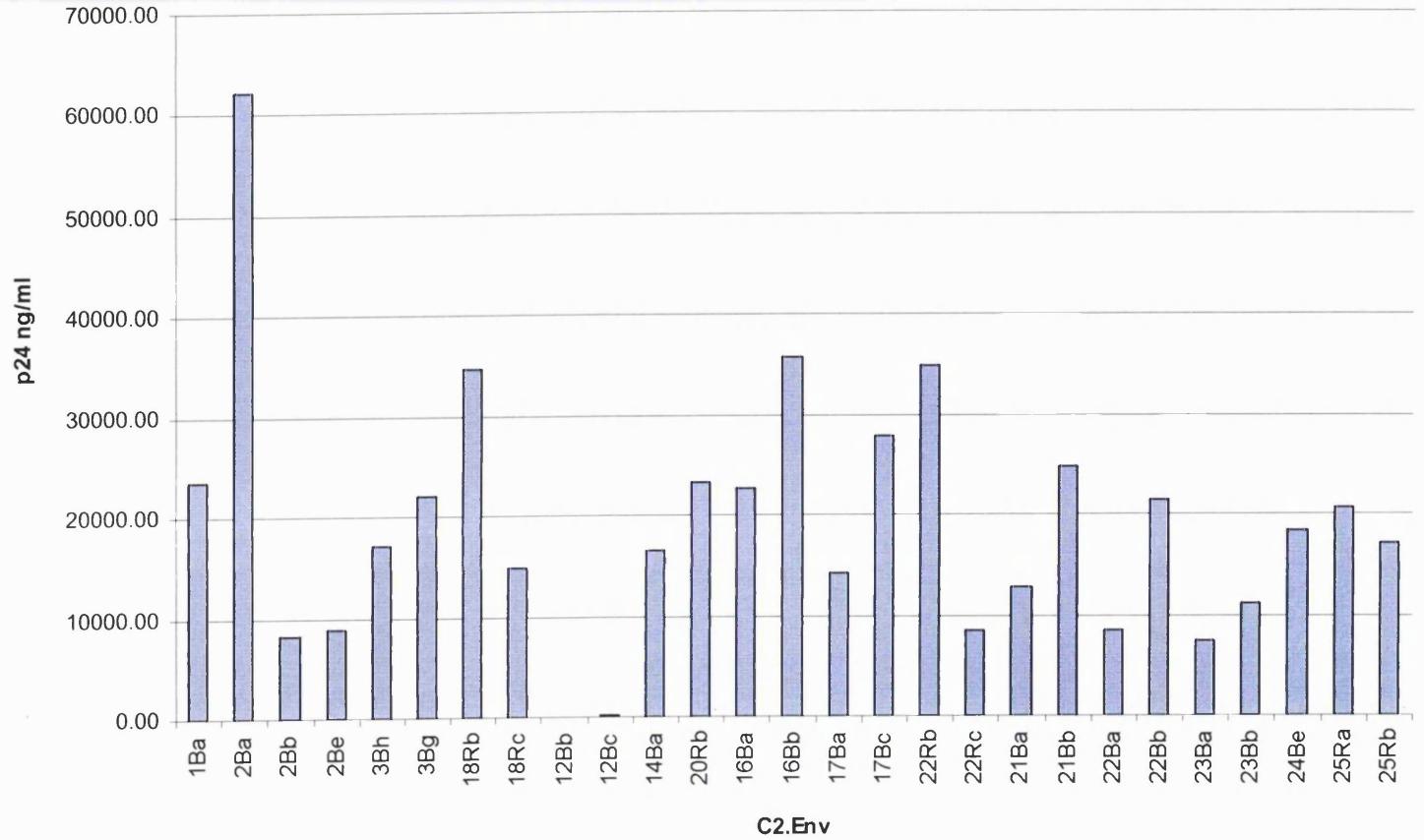


Figure 5.9. HIV-1 p24 content of chimeric virus stocks.

Analyses of cell lysates using ARP301 and ARP401 antisera indicated the presence of gp160 in all 293T cell lysates bar those cells transfected with pC2.12Bb and pC2.12Bc clones (Figures 5.10-12).

5.3.3.2 Env Processing

Analyses of the processing sites of these glycoproteins reveal minor variations around the dibasic processing site (Appendix A3.1-4 and summarised in Table 5.7). Compared to the KAKRRVVQREKR consensus motif, 17/19 Envs from 3 patients (012, 028 and 048) showed substitutions. 16 of the substitutions occurred at the first Lys residue of site 2 (KAKRR), the most common being a conservative K-R substitution. Although this is not expected to alter the structure or charge of the processing site, processing was only observed in clones 048:21Bb and 048:23Ba possessing this substitution. A non-synonymous K-E substitution at the same position was only observed in Envs isolated from patient 028. Clone 028:22Rb was the only clone from patient 028 that possessed an additional non-conservative E-G substitution within site 1 (REKR). None of the Envs from patient 028 that were studied in the pC2 cassette exhibited gp160 processing or high fusogenic capacities. Other non-conservative substitutions observed at this site 2 position were K-Q and K-M, in clones 048:23Bb and 048:24Be respectively; whilst 048:23Bb did not exhibit gp160 processing, 048:24Be did.

A non-conservative R-G substitution was observed in the penultimate residue of site 2 in clone 048:22Bb, and no processing was detected, consistent with our observation that processing is abrogated by non-conservative substitutions at this position (Chapter 3). However, as with other studies, the conservation of the processing motifs is not the only requisite for successful processing (Adams *et al.*, 2000; Connor *et al.*, 1996). The increased electrophoretic mobility of 001:2Bb indicates either premature truncation or an internal cleavage, such as the the presence of an R-X-K-K/R motif that is observed in the V3 loop of certain strains (Morikawa *et al.*, 1993). However, sequence analysis indicates no such motif in the V3 domain of 001:2Bb.

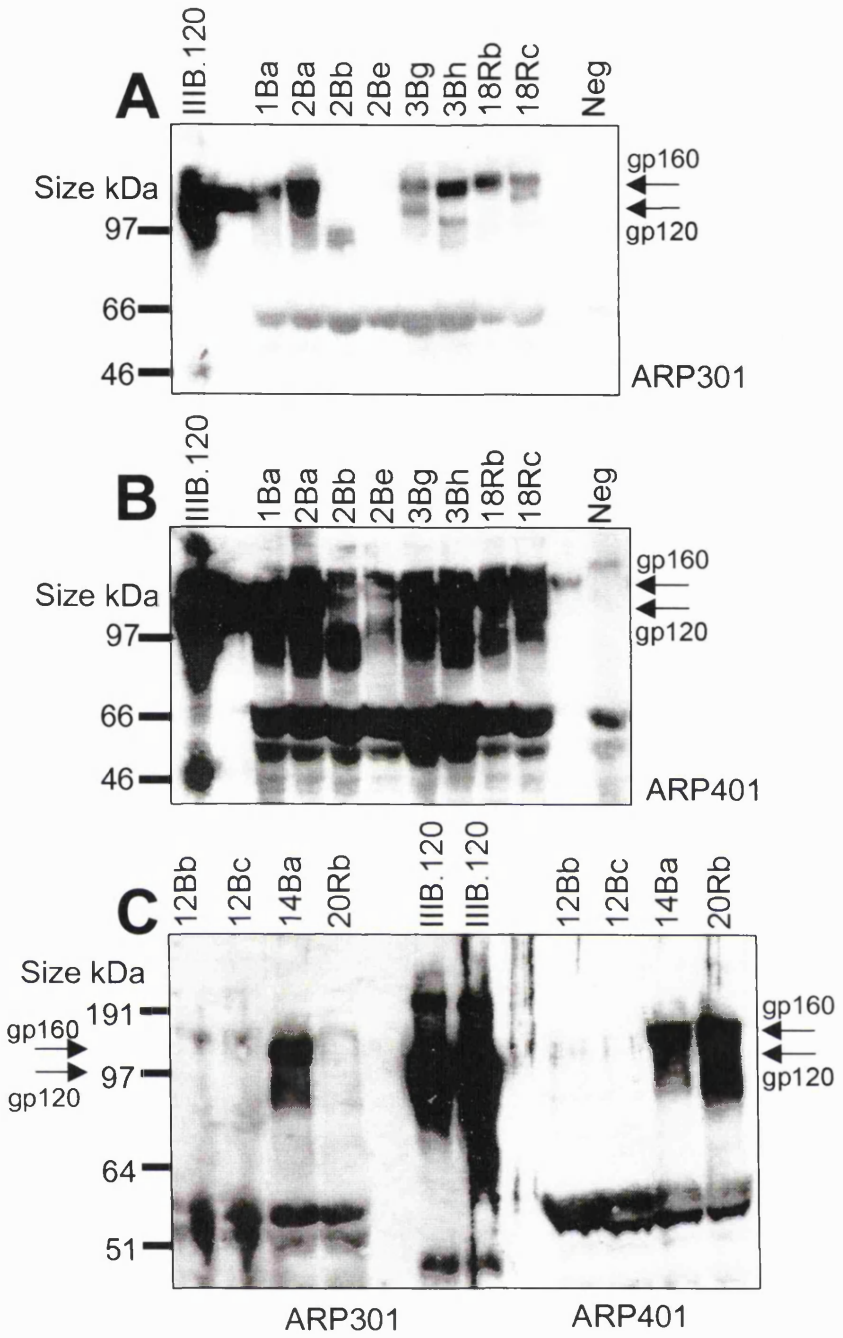


Figure 5.10. Detection of gp160/gp120 in the lysates of 293T cells following transfection with pC2.*env* constructs.

The *env*-genes were derived from patients 001 (A and B) and 012 (C). Western blotting was conducted using ARP301 or ARP401 anti-gp120 antisera as indicated. The expected sizes of gp160 and gp120 are indicated. 50µg of reduced cellular protein, as determined by the Bradford reagent assay, were electrophoresed across all samples.

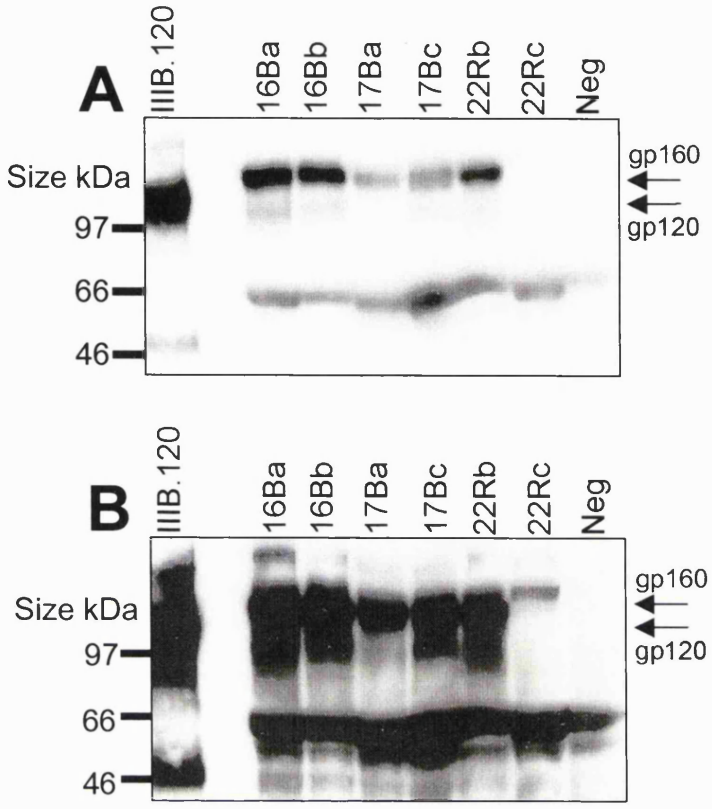


Figure 5.11. Detection of gp160/gp120 in the lysates of 293T cells following transfection with pC2.*env* constructs.

The *env*-genes were derived from patients 028. Western blotting was conducted using ARP301 (A) or ARP401 (B) anti-gp120 antisera. The expected sizes of gp160 and gp120 are indicated. 50μg of reduced cellular protein, as determined by the Bradford reagent assay, were electrophoresed across all samples.

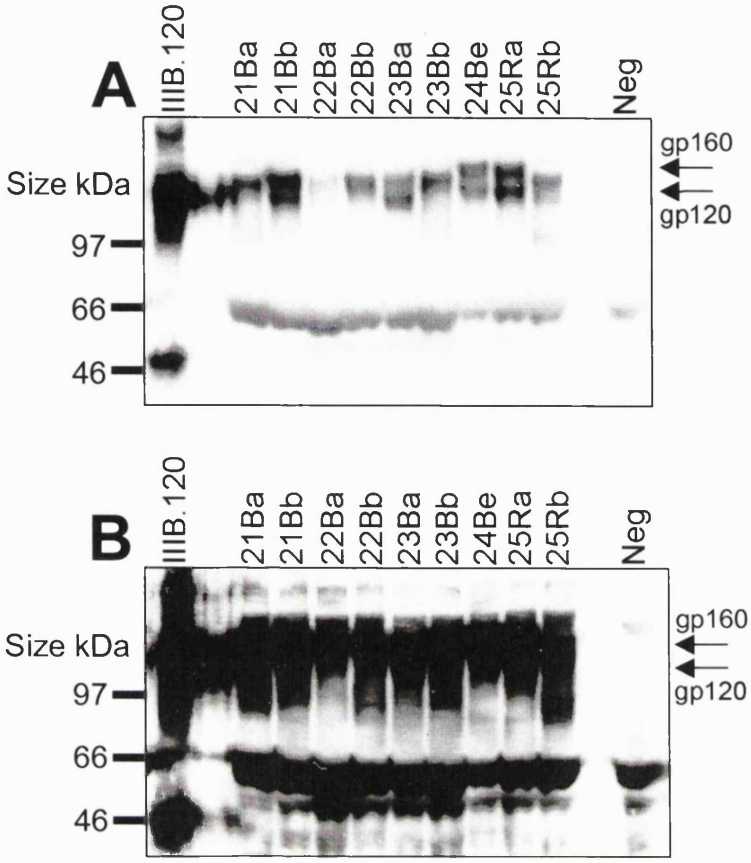


Figure 5.12. Detection of gp160/gp120 in the lysates of 293T cells following transfection with pC2.*env* constructs.

The *env*-genes were derived from patients 048. Western blotting was conducted using ARP301 (A) or ARP401 (B) anti-gp120 antisera. The expected sizes of gp160 and gp120 are indicated. 50µg of reduced cellular protein, as determined by the Bradford reagent assay, were electrophoresed across all samples.

Analyses of the lysates of chimeric viruses (Figures 5.13-14) revealed the presence of gp160 in all clones except those of pC2.2Bb and pC2.2Be. Viruses expressing Envs 012:12Bb, 012:12Bc and 012:14Ba and 028:22Rc exhibited low amounts of gp160 that can be attributed to poor incorporation of Env during virion assembly as equivalent amounts of p24 across all samples were used for western blotting. Low levels of products corresponding to gp120 could be detected in viruses expressing Envs 028:16Ba, 028:17Bc, 048:21Bb and 048:22Ba.

5.3.3.3 Infection of PBMCs with Chimeric Viruses

Of the 27 chimeric virus clones tested, only 9 were capable of productive infection of PBMCs as determined by $TCID_{50}$/ml titrations (Table 5.7). PBMCs were inoculated with 10^{-1} to 10^{-6} dilutions of each harvested chimeric virus and seven days later, tissue culture supernatant was diluted from 1/20 to 1/100 and assayed for HIV-1 p24. Discordant results between cell-cell fusion assays and $TCID_{50}$/ml determinations were observed with chimeric virus expressing Env 028:16Bb, where high $TCID_{50}$/ml titres were obtained despite the absence of significant fusogenic capacity, and in the case of 012:12Bb, which exhibited high fusogenic capacity but was unable to productively infect PBMCs (compare tables 5.5 and 5.7).

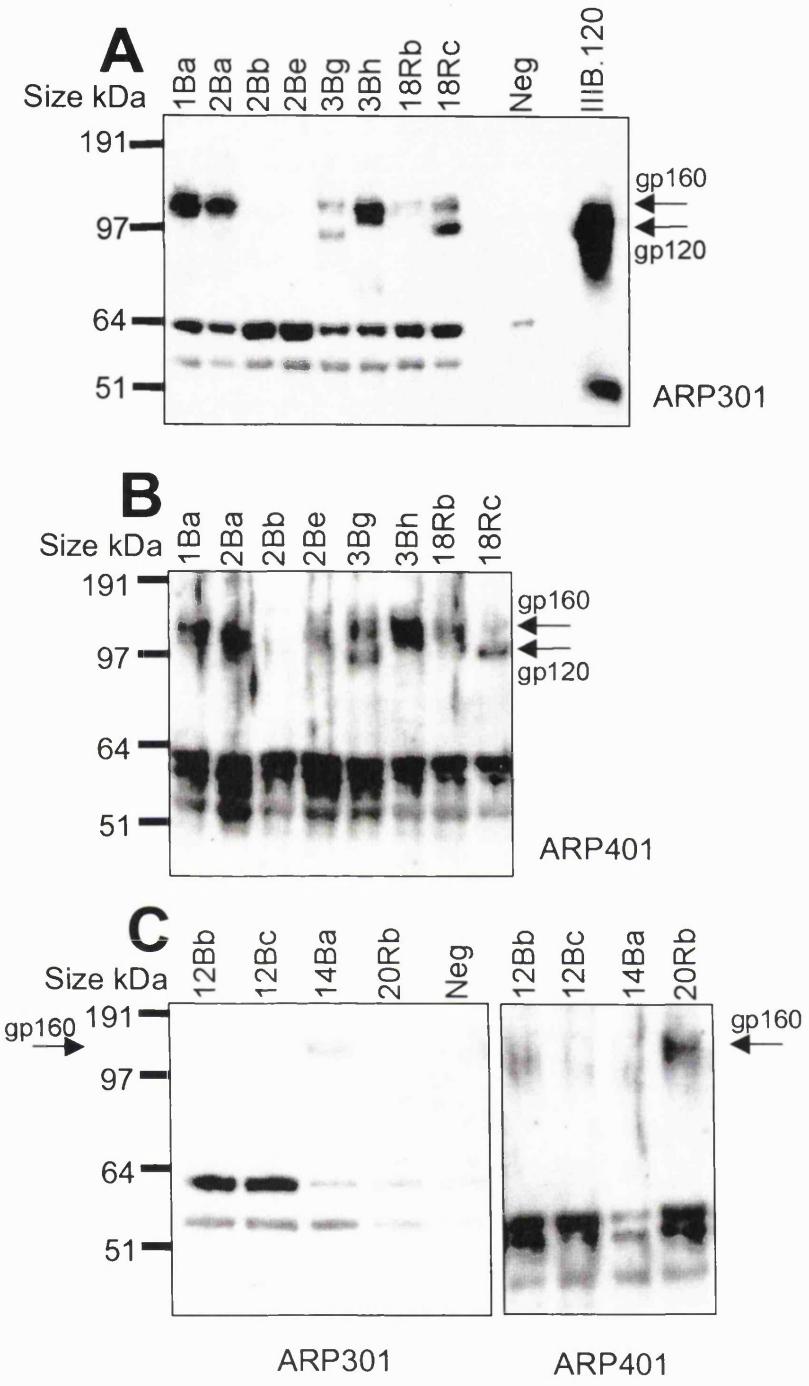


Figure 5.13. Detection of gp160/gp120 in chimeric virus lysates.

Lysates of chimeric viruses containing *env*-genes from patients 001 (A and B) or 012 (C) were reduced and probed with ARP301 or ARP401 antisera (as indicated) to assess Env incorporation and processing. The expected sizes of gp160 and gp120 are indicated. Equivalent amounts of virus, as assessed by 4μg of p24, were analysed across all samples as determined by HIV-1 specific ELISA.

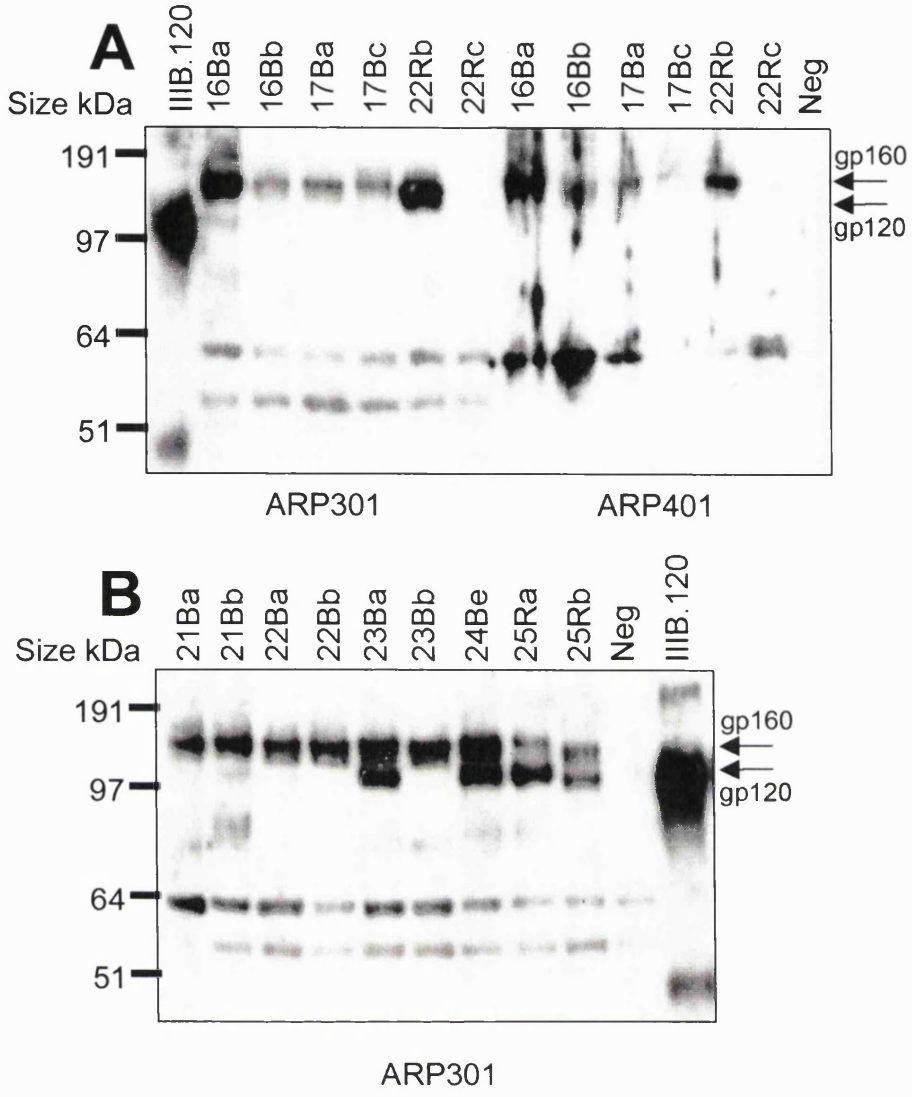


Figure 5.14. Detection of gp160/gp120 in chimeric virus lysates.

Lysates of chimeric viruses containing *env*-genes from patients 028 (A) or 048 (B) were reduced and probed with ARP301 or ARP401 antisera (as indicated) to assess Env incorporation and processing. The expected sizes of gp160 and gp120 are indicated. Equivalent amounts of virus, as assessed by 4μg of p24, were analysed across all samples as determined by HIV-1 specific ELISA.

Patient	HIV-1 Env	Cleavage Site Sequence[a]	Env Cells[b]	Env virus[c]	p24[d]	$TCID_{50}$/ml
	NL43	KAKRRVVQREKR	+++	+++	+	31622
	JRFL	----------	++++	+++	+	31622
001	1Ba	----------	++	+++	+	
	2Ba	----------	+++	+++	+	316
	2Bb	----------	++		+	
	2Be	----------	+		+	
	3Bg	----------	++	++	+	316
	3Bh	----------	+++	+++	+	
	18Rb	----------	+++	+	+	
	18Rc	----------	++	++	+	1778
0012	12Bb	R---------	-	-	-	
	12Bc	R---------	-	-	-	
	14Ba	R---------	++	+	+	
	20Rb	R---------	+++	++	+	
028	16Ba	R---------	+++	+++	+	
	16Bb	R---------	+++	++	+	562
	17Ba	E---------	++	++	+	5623
	17Bc	E---------	++	++	+	
	22Rb	E-------G--	++	+++	+	
	22Rc	E---------	+		+	
048	21Ba	R---------	++	++	+	
	21Bb	R---------	+++	++	+	
	22Ba	R---------	+	++	+	
	22Bb	---G------	++	++	+	
	23Ba	R---------	++	+++	+	31622
	23Bb	Q---------	++	++	+	
	24Be	M---------	++	+++	+	1000
	25Ra	----------	+++	++	+	17782
	25Rb	----------	++	+	+	17782

Table 5.7. Summary of Env processing and function in chimeric virus studies.

The amino acid sequence of each Env processing site is shown with indications of processing and incorporation into chimeric viruses. The ability of chimeric viruses to infect PBMCs are indicated by the $TCID_{50}$/ml determinations of the virus stocks.

[a] Identity is indicated by a dash (-), conserved substitutions are indicated in black and non-conservative substitutions are indicated in red.

[b] The presence and relative quantity of gp160 in cell lysates is indicated by (+). Red (+) indicates processing.

[c] The presence and relative quantity of gp160 in chimeric virus lysates is indicated by (+). Red (+) indicates processing.

[d] Refer to Figure 5.10 for p24 data.

5.3.3.4 Regulatory Proteins

The absence of p24 and gp160 from pC2.12Bb and pC2.12Bc clones suggest either failure in the transfection, or inhibition of chimeric virus production as a direct result of the *env*-gene sequences in these clones. Alternative causes of dysfunction in pC2.*env* clones may exist because the *env*-gene overlaps the second exons of both Tat and Rev regulatory proteins, the reading frames of which may have been altered. The Tat second exon product may be involved in the binding of host-derived transcription factors necessary for the activation of the viral genome. The amino acid sequences of the Tat second exon encoded by the pQ7.*env* clones derived from the patients under study are shown in Appendix A3.5.1. Products isolated from patients 028 and 048 exhibited the greatest variation from the amino acid sequence of the second exon of clone 001:1Ba. Non-conservative amino acid substitutions at the C-terminus (VD→DA) are present in all patient 028 clones. Other non-conservative substitutions at the N-terminus do not suggest a role for the Tat second exon in the ability of clone 028:16Bb to mediate a productive infection compared to other chimeric viruses expressing *env*-genes derived from this patient.

In the case of the Rev second exon product (Appendix A3.5.2), the pQ7.*env* clones derived from all LTNP-switcher patients exhibited few non-conservative changes of charged residues. Extensions of three amino acids are observed in 012:14Ba, 012:20Rb and 048:24Be, the significances of which are not certain. Clone 001:2Be exhibits a non-conservative substitution in the RNA binding/nuclear localisation domain. Clones 048:22Ba and 048:25Ra exhibit substitutions in the activation/nuclear export domain that may affect Rev function. Premature stop-codons are observed in clones 012:12Bb and 028:16Bb that are likely to affect viral mRNA export from the nucleus, but not in 012:12Bc.

The Tat and Rev second exons of the pC2 clones were sequenced to determine if any alterations in the *env*-gene sequences had occurred during sub-cloning that could affect Env expression. Sequencing analyses showed no differences in the truncated 012:12Bb Tat or Rev products (Appendix A3.5.3-6). Thus, the reason for the lack of chimeric virus production for pC2.12Bb clones is attributable to the truncated Rev product. Two transversions, a C→G and an A→T were observed in the sequences of clone 028:16Bb (Appendix A3.5.3). These result in a Pro>Ala substitution proximal to the gp160 cleavage site, and a non-conservative Glu→Val substitution in the gp41 AH2 domain

(Appendix A3.5.4). The former change is likely to alter the local structure of the cleavage loop, perhaps increasing accessibility to processing enzymes. The latter substitution to a hydrophobic residue is observed in the HIV-1_{NL43} gp41 AH2 domain, and in the context of the Rev second exon, the transversion results in a change from a termination codon into a Cys codon (Appendix A3.5.5). Thus, the Rev product for 028:16Bb is full-length, and coupled with substitutions in the Env, is likely to enable production of infectious chimeric viruses. No changes in the Tat or Rev sequences were observed between the pQ7.12Bc and pC2.12Bc clones; therefore the lack of viral p24 expression is attributable to other features of the *env*-gene.

5.4 Discussion

The slow disease progression exhibited by HIV-1 infected LTNP patients is a phenomenon associated with host genetic factors, effective immune control and viral factors. The efficient control of HIV-1 replication has been attributed to certain combinations of HLA-haplotypes (Balla-Jhagjhoorsingh *et al.*, 1999; Carrington *et al.*, 1999; Demarest *et al.*, 2001; Greenough *et al.*, 1999; Levy, 1998), efficient $CD4^+$ and $CD8^+$ lymphocyte recognition of HIV-1 antigens (Alexander *et al.*, 2000; Demarest *et al.*, 2001; Greenough *et al.*, 1999; Liu *et al.*, 1997; Ogg *et al.*, 1999; Rosenberg *et al.*, 1997), and the presence of neutralising antibodies (Dehghani *et al.*, 2003; Kaul *et al.*, 2000; Kaul *et al.*, 1999; Marthas *et al.*, 1995; Rowland-Jones *et al.*, 1995; Ryzhova *et al.*, 2002). Moreover, attenuated viral variants, caused by defects in viral genes and the LTR promoters, are implicated in slower disease progression (Alexander *et al.*, 2000; Fang *et al.*, 2001; Lum *et al.*, 2003; Masciotra *et al.*, 2002; Wang *et al.*, 2003). HIV-1 Env is likely to play a direct role in disease progression as it is responsible for binding host cell receptors and mediating membrane fusion, thereby initiating infection and allowing virus dissemination in the host. The localisation of Env on the viral surface means it is a major target for the host immune system, so it must avoid neutralisation through mutating or masking critical epitopes. This study sought to determine the functional role of HIV-1 *env*-genes in disease progression in LTNP patients.

Four LTNP patients were studied whom had undergone switching from asymptomatic disease to symptomatic disease as determined by $CD4^+$ T lymphocyte counts of less than 500 cells/μl and high plasma viral load (Figures 5.2 and 5.3). Determination of the HLA-haplotypes of each subject revealed the presence of alleles associated with HEPS patients and slow progression (Table 5.3). Some determinations were absent, it would

be of interest to acquire the complete set of HLA-genotypes as studies have suggested maximum heterozygosity at the HLA-type 1 loci, as observed so far in all patients studied here, is associated with slower disease progression (Carrington *et al.*, 1999). It was noted that LTNP patients 012 and 028 are heterozygous for the mutant CCR5Δ32 allele, and LTNP 048 is heterozygous for the SDF1-3'A mutant allele. Thus, the LTNP-switcher patients possess HLA or the β-chemokine receptor alleles associated with slower disease progression (Balfe *et al.*, 1998; Cohen *et al.*, 1998; Greenough *et al.*, 1999; Magierowska *et al.*, 1999; Soriano *et al.*, 2002).

The study patients provided PBMCs at irregular intervals during their asymptomatic and symptomatic periods, and sampling had been terminated upon the commencement of anti-retroviral therapy. HIV-1 *env*-genes were amplified from proviral DNA in PBMCs as opposed to plasma vRNA. It has been suggested that the plasma vRNA is representative of dominant circulating strains at that point, and PBMCs proviral DNA represents older sequences (Fang *et al.*, 2001; Liu *et al.*, 1997). However, isolation of *env*-genes from plasma vRNA does not indicate if the particular variant is neutralised or defective for entry, whereas proviral DNA in PBMCs is indicative of the variant having had an *env*-gene encoding a fusogenic Env product, though the provirus will be one reverse transcription step removed from the original variant that infected the cell.

Significant biological activity in cell-cell fusion assays was detected in 29/46 (63%) of the clones (Tables 5.5-6), but only 6/29 (21%) of these Envs possessed high fusogenic activity (fusogenic activity >20% of $NL43_{WT}$ or $JRFL_{WT}$), and these were all R5-tropic. Of the 17 Envs without significant activity, seven exhibited no activity (012:14Bf, 028:16Bb, 028:16Bc, 028:17Bc, 048:22Bb, 048:24Ba and 048:24Bb), five were assigned dual-tropic status (012:12Bc, 012:14Bc, 028:16Ba, 028:22Rd and 048:23Bb), and five exhibited R5- or X4-tropism (001:1Bb, 012:13Ba, 012:13Bd, 028:22Rc and 048:21Ba). Of the 6 Envs that were exclusively X4-tropic, 4/6 exhibited significant fusogenic potential, and these were all isolated in the later sampling periods.

Human and macaque studies, of HIV-1 and SIV infections respectively, have documented viral divergence and diversity influencing the rate of disease progression (Dehghani *et al.*, 2003; Demarest *et al.*, 2001; Essajee *et al.*, 2000; Fowke *et al.*, 1998; Lukashov *et al.*, 1995; Rudensey *et al.*, 1998). Our longitudinal analyses of a limited set of 46 HIV-1 *env*-genes derived from four LTNP-switcher patients during the

asymptomatic stage and near the symptomatic stage of disease is concordant with these studies. The majority of sequence variation is observed in the gp120 subunit, particularly in the variable loops as observed in other studies (Dehghani *et al.*, 2003; Fowke *et al.*, 1998; Masciotra *et al.*, 2002; Rudensey *et al.*, 1998). An Asp→Asn substitution in clone 028:17Ba at the highly conserved position 207 in the V2 domain could affect infectivity of the chimeric virus (Wang *et al.*, 1995). Rudensey *et al.* (1998) documented increases in putative glycosylation sites in the V4 domain during later stages of disease and this is apparent in isolates from patient 048. Extensive variation in the gp120 subunit is likely to modulate the efficiency of CD4 and co-receptor binding steps, hence this would be reflected in varying fusogenic capacities. However, other features of Env could modulate its fusogenic capacity. Many of the studies referred to above had been conducted in the context of *env*-genes of laboratory-adapted strains of HIV-1, hence they may not be entirely applicable to Envs derived from primary isolates.

When a selection of 27 *env*-genes (19/27 exhibiting significant fusogenic capacity) was analysed in the context of chimeric viruses, only nine clones were able to mediate productive infection of PBMCs (Table 5.7). Of these nine clones, five Envs exhibited significant fusogenic capacity greater than 10% of $NL43_{WT}$ or $JRFL_{WT}$ Envs (Table 5.5). Thus, significant fusogenic capacity of the Env in cell-cell fusion assays is not an accurate indication of viral fitness. Although it is likely that co-receptor densities on NP2 target cell surfaces differ from that of PBMCs, the analyses of Envs in virus lysates are suggestive of defects in incorporation and processing (Figures 5.13-14). Env targeting to the lipid raft domain is correlated with the presence of at least one palmitoylated Cys residue in the AH1 or AH2 domains (Rousso *et al.*, 2000), which is observed in all isolates except Envs 012:20Rb and 012:20Rc. However, the requirement of cysteine palmitoylation for Env incorporation is questioned by the detection of Env 012:20Rb in virus lysates. Thus, it is likely that the Env backbone plays a role in determining the requirement for palmitoylation during Env trafficking in the virus lifecycle. An additional Cys residue was observed in clones 001:2Bb and 001:2Be between the AH2 and AH1 domains, Env was under-represented in these chimeric viruses, whereas clone 048:22Bb possessed an extra Cys residue two residues upstream of the conserved Cys near the AH2 site and this particular Env was able to be efficiently incorporated into virions. The presence of an additional Cys residue between AH2 and AH1 has been detected in the *env*-genes of other subtype B primary isolates (Daniels *et*

al., 2003), the biological effects of Cys residues at these positions on Env trafficking and virus assembly has not been described, but could be investigated in the context of laboratory adapted Envs such as $NL43_{WT}$ or $JRFL_{WT}$.

The second exons of *tat* and *rev* are encoded by the *env* sequences, thus their products could be affected by *env* sequence variation. Tat is required for upregulating HIV-1 mRNA synthesis, and Rev is essential for export of RRE-containing mRNA from the nucleus. While the second exon of *tat* does not encode functional domains, the *rev* second exon has nuclear localisation and export signals. Our analyses show the *tat* sequences are conserved in practically all clones, although a previous study identified truncations in other clones isolated from 012 (Zheng *et al.*, 2002). Env 012:12Bc is the only clone exhibiting a Pro-Gln substitution in the second-exon of *tat*, which may affect Tat structure (Appendix A3.5.1). The non-conservative D→N substitution in the second-exon of *rev* encoded by clone 048:22Ba does not appear to adversely affect the synthesis of Env (Figures 5.12 and 5.14). An additional triplet of residues extending the Rev product was observed in clones 012:14Ba, 028:20Rb, in agreement with Zheng *et al.* (Zheng *et al.*, 2002), and also 048:24Be, this is unlikely to be functionally relevant as pC2 clones containing these *env*-genes were able to produce chimeric virus with gp160. The lack of chimeric virus synthesis with pC2 expressing 012:12Bb Env is attributable to the truncated Rev product preventing viral mRNA export from the nucleus. The process of cloning 028:16Bb *env* into pC2 caused a T→A transversion that resulted in the rescue of the truncated Rev product. This transversion also resulted in a D→V substitution in the Env Kennedy domain, and another upstream transversion resulted in a P→A substitution immediately proximal to the gp120/41 processing motif. These changes may have local effects on Env structure that may enhance the fusogenic capacity in the chimeric virus compared to the relatively non-functional pQ7.16Bb clone. It would be necessary to assess the ability of the original pQ7.16Bb *env*-gene in the pC2 cassette to determine if the truncated Rev product could mediate viral mRNA export, also it would be interesting to study the pC2.16Bb *env*-gene in the cell-cell fusion assay to ascertain if the changes in Env result in increased fusogenic capacity.

Of the nine chimeric virus clones able to infect PBMCs, only 6 exhibited processed gp120 in the virion (Figures 5.13-14 and Table 5.7). Chimeric viruses expressing 001:2Ba, 028:16Bb and 028:17Ba Envs were able to infect PBMCs without exhibiting gp160 processing. We have previously discussed extracellular/viral proteolytic

processing of gp160 as a prerequisite for membrane fusion in Chapter 3. Analysis of the processing site across all mutants revealed conservation of the site except in isolates derived from patients 028 and 048 (Table 5.7), indicating that other regions of gp120 are involved in determination of processing. Of the Envs isolated from patient 048 that exhibit non-conservative substitution in the KAKRR motif (Table 5.7), only clone 048:24Be, exhibiting a structurally similar (with regard to side chain length) Met substitution for Lys, was able to mediate chimeric virus infection.

In agreement with other studies of *env*-genes derived from LTNP patients (Adams *et al.*, 2000; Connor *et al.*, 1996), our results show that 293T cells producing chimeric virus are unable to efficiently process many Envs despite conservation of processing sites 1 and 2 (Figures 5.10-14). It is likely that for primary isolate Envs, extensive variation in amino acid sequence and glycosylation sites of gp120 during immune evasion may structurally hinder enzyme processing during intracellular maturation. In the case of Newcastle Disease Virus F_0 glycoprotein, mutations in other regions of the molecule can prevent processing (Morrison, 2003) and in the Influenza A HA0, oligosaccharides near the cleavage loop sterically hinder enzyme access (Ohuchi *et al.*, 1991; Steinhauer, 1999). Thus, significant activity of unprocessed Envs may be associated with extracellular cleavage, and further reflects qualitative differences between virus infection and cell fusion studies.

Other studies have highlighted the importance of N-linked glycans in the gp41 ectodomain in Env processing and routage in the cell during maturation (Dash *et al.*, 1994; Fenouillet *et al.*, 1993; Fenouillet and Jones, 1995; Perrin *et al.*, 1998). Perturbations in the cluster of three putative N-linked glycosylation sites near the immunodominant region of gp41 are observed in the unprocessed Envs derived from the first 2 sampling stages of patient 048 (Appendix A3.4), these Envs have lesser fusogenic capacities than Env 048:21Bb, which is processed to some degree (Figures 5.12 and 5.14 and Tables 5.5 and 5.7). It is uncertain if the lack of gp160 processing is attributable to the loss of an N-linked glycosylation site in this region. However, only those Env clones derived from patient 048 that exhibit the cluster of 3 N-linked glycans in gp41 are able to infect PBMCs. Analyses of the gp41 sequences reveal the presence of an additional N-linked glycosylation sequon immediately upstream of the MSD in Envs 012:12Ba, 012:12Bc, 012:13Bd, 048:21Ba, 048:21Bb, 048:21Bc, 048:22Ba and 048:22Bb (Appendix A3.2 and A3.4), although it is unknown whether or not this site is

glycosylated due to it's close proximity to the MSD. This additional sequon has also been observed in subtype B isolates from Korean patients (Daniels *et al.*, 2003).

Of interest is that patients 012 and 028, heterozygous for the CCR5Δ32 allele, possess a smaller proportion of Envs with significant fusogenic activity with $CCR5^+$ target cells (5/11 and 3/9 respectively) compared to patients 001 and 048 who are homozygous for the CCR5 wildtype allele (9/11 and 8/15 respectively). None of the Envs from patients 012 and 028 selected for study in the C2 infectious cassette exhibited processing. Reduced CCR5 surface expression has been correlated with protection from R5-tropic strains (Reynes *et al.*, 2001). Our results suggest that R5-tropic variants are less established in patients heterozygous for the CCR5Δ32 mutation, and viral variants within these patients have probably evolved to use co-receptors other than R5, hence the higher frequency of dual-tropic and non-functional Envs. The latter were assigned by deficiencies of processing in 293T cells, of incorporation into virions, and of cell-cell fusion determined with target cells expressing either CXCR4 or CCR5 co-receptors only. In support of this is the observation that some NSI isolates with rapid growth kinetics were able to be recovered from with PBMCs of patient 012 in other studies (Zheng *et al.*, 2002).

During disease progression, a switch in the usage of co-receptors may be observed, coincident with a reduction in the numbers of $CD4^+$ cells. It is possible that the dual-tropic Envs represent intermediate variants adapting to X4-usage during the NSI-SI switch, although the small sample sizes most likely under-represent the viral phenotypes that arise throughout the course of asymptomatic disease. All patients yielded X4-tropic Envs from the final sampling period. Variation in the V3 loop is primarily associated with co-receptor switching (Dehghani *et al.*, 2003; Liu *et al.*, 2002; Milich *et al.*, 1997; Nelson *et al.*, 2000; Polzer *et al.*, 2002; Xiao *et al.*, 1998), and an example of differential glycosylation in the V1/V2 domain associated with the acquisition of dual-tropism has been described in late isolates from a LTNP case study (Jekle *et al.*, 2002). However, correlation between V3 sequences associated with an SI-phenotype and significant X4-tropism in cell-cell fusion assays was only observed in Envs 028:22Rb and 028:22Rc, and the loss of the complex glycan in the V3 loop and association with CXCR4 usage was only observed with clone 001:18Rc. In the case of patient 048, the $CD4^+$ cell numbers at the final sampling point was marginally less than 400 cells/μl, whilst for the other patients it was less than 250 $CD4^+$ cells/μl (approximately <243

cells/μl, 218 cells/μl and 212 cells/μl in patients 001, 012 and 028 respectively, see figures 5.2-3). The co-receptor switch is not necessarily observed in all patients progressing to disease, nor does it prevent the persistence of R5-tropic variants during late stages of disease (Nelson *et al.*, 2000; van Rij *et al.*, 2000). It remains possible that the appearance of X4-tropic variants was still suppressed in patient 048 until the $CD4^+$ cell numbers declined further.

In this study, we have sought to determine if the fusogenic capacity of HIV-1 *env*-gene products influences disease progression in LTNP patients. Our results are concordant with other studies describing dysfunctional Envs isolated from LTNP patients (Connor *et al.*, 1996). Although the number of *env*-genes studied was too small to obtain definitive data, there is a trend for dysfunctional or R5-tropic variants to be isolated from the asymptomatic stages of all LTNP patients. The predominance of dual-tropic and X4-tropic variants during the final sampling stages in all patients, coincident with the decline of $CD4^+$ cells and increasing viral load, is suggestive of a co-receptor switch being a feature of disease progression. The effects of other factors cannot be discounted in the slow disease progression exhibited by these patients. Data pertaining to the immune specificities observed in these patients is not available to us at this stage, but would be useful to determine the level of immune control of circulating viral variants. It would also be necessary to screen isolates for mutations in other viral regions implicated in attenuating HIV-1. One study, based on samples from early asymptomatic stages, suggests that there are no significant perturbations in the *nef* alleles derived from the four patients under study here (Kirchhoff *et al.*, 1999). Changes in the *nef* sequences were observed in other asymptomatic patients progressing to disease in other cohorts, thus it would be interesting to screen our patients with regard to this aspect. Moreover, it would be of interest to assess the frequency of the R77Q mutation in Vpr among isolates from these patients to determine if progression is associated with reversion to the wildtype residue. The study of LTNP-switcher patient derived HIV-1 *env*-genes in the context of infectious chimeric viruses using PBMCs confirmed the predominance of dysfunctional Envs in these patients. This indicates that defective Envs are likely to be a factor in slow disease progression. A mode of cell-cell dissemination as opposed to viral infection may be favoured in these patients, as many of the *env*-genes recovered exhibited fusogenic activity in cell-cell fusion studies.

Chapter 6

General discussion

6.1 General Discussion

As discussed in the introduction, the HIV-1 Env plays a critical role in the infectious lifecycle; to deliver the viral genome and replicative machinery into the target cell, membrane fusion must occur. The fusogenic capacity, or ability to mediate membrane fusion, is dictated by the Env's affinity for receptors and it's ability to undergo rapid conformational change. These processes are intimately associated with the Env structure as determined by its amino acid sequence. Mutations were engineered across the KAKRRVVQREKR processing site of HIV-1_{NL43} Env to abrogate cleavage of the gp160 precursor into functional gp120/gp41 subunits, thereby preventing insertion of the hydrophobic fusion peptide into the target cell membrane. Discordant results between cell-cell fusion and chimeric virus studies with mutations in the VVQ motif suggest extraneous events occurring at the cell surface. One proposal is that gp160 processing is also possible at the cell surface, in support of other studies (Barbouche et al., 2002). The ability to disrupt processing with a single non-conservative residue substitution in the site 2 motif (KAKER or KAKSR) further supports the notion that site 2 plays a more interactive role with the processing enzyme(s) during the cleavage event and may even be a requisite for triggering membrane fusion as discussed (Chapter 3.4). The observation that reduced processing in two mutants (M1S1 and M2S1) results in reduced infectivity of chimeric viruses lend support to studies opining heterogeneity of Env species incorporated into virions (Poignard *et al.*, 2003). Future work will be directed at investigations into the role of cell surface processing through the use of protease inhibitors in the biological assays. Other members of our group involved in HIV glycoprotein crystallisation studies have incorporated the KAKSR and REKR mutations into gp140 constructs that may hopefully shed light on the nature of the defect imposed.

Studies of the biological properties of truncated Env were prompted by the work of others that suggests a revision of the conventional cytoplasmic localisation of the gp41 150-residue tail (Cleveland et al., 2003). Extensive study of the gp41 cytoplasmic domain has delineated it into discrete continuous regions exhibiting a range of hydrophobic and hydrophilic characteristics (Douglas *et al.*, 1997). The location of the hydrophilic Kennedy domain remains contentious; many studies suggest an intracellular location, while others suggests an extracellular location (Cleveland *et al.*, 2003; Kennedy *et al.*, 1986; Vella *et al.*, 1993). Other studies even suggest the immunodominant IEEE epitope is cross-reactive with a similar epitope on the matrix

protein (Buratti et al., 1997). The conflicting data may be indicative of a dual conformation of Env, depending on functionality or co-expression with other viral proteins such as Gag. Our functional studies are supportive of a revised model of gp41 structure as discussed in Chapter 4, with indications of a significant association between the Kennedy domain and the fusogenic capacity of the truncated Env mutants. Investigations are currently underway to investigate the effects of Env processing and Gag expression upon the exposure of the Kennedy domain at the cell and virion surface through immunofluorescence and EM studies.

Immune selection at exposed residues, or changes in glycosylation pattern affecting exposure/restricting adoption of appropriate conformation may thus impact upon the fusogenic capacity of Env. As the rate of virus infection of susceptible target cells, rather than viral load, is a stronger measure of virus spread within a host, it is thus expected that mutations affecting the fusogenic capacity of Env is a major determinant in the progression of disease as discussed in Chapter 5. While initial screening of *env*-genes isolated from LTNP-switcher patients identified full-length and expression competent clones, the cell-cell fusion assay was able to identify Envs with significant fusogenic capacity with regard to CXCR4 and CCR5 chemokine receptors in the presence of CD4. A number of Envs did not exhibit significant activity with regard to these two CKRs. This may relate to *in vitro* studies that have demonstrated the use of other CKRs, although such usage was additional to CXCR4 or CCR5 (Michael *et al.*, 1998; reviewed Clapham and McKnight, 2001). Thus, use of other NP2 target cells expressing alternate CKRs (Soda *et al.*, 1999) would be useful investigative tools to determine the existence of other viral phenotypes that arise in LTNP patients during the asymptomatic stages and progressive stages of HIV-1 infection.

Further studies of the *env*-gene products in the context of chimeric viruses indicated that productive infection of PBMCs could be achieved when viruses expressed Envs that had significant fusogenic activities in the cell-cell fusion assay. However, one exception was identified where the original *env*-gene clone was truncated in the second exon of Rev. Acquisition of full-length Rev and two further non-conservative substitutions were observed during the process of cloning into the pC2 infectious cassette that are likely to have contributed to the gain of function. Discrepancies classified as significant fusogenic activity in the cell-cell fusion assay but lack of infectivity of C2-chimeric virus particles are attributable to variations in the ability of viruses to incorporate

processed Env species, and probably reflect the complexities of interactions of the surface of cells with virions. It was observed that gp160 was detectable in the lysates of cells transfected with all infectious cassette constructs that were studied bar two clones, one possessing a truncated Rev. Processing of Env was not observed in all infectious clones, which would suggest an extracellular cleavage event as discussed in chapter 3. The observations reaffirm the importance of overall Env structure in dictating maturation and function rather than discrete domains.

In SP patients, a phenotypic switch in viral variants may be observed in 50% of patients at the time of or prior to disease progression, marked by the decline of $CD4^+$ cells. In our longitudinal study, the majority of Envs isolated from LTNP-switcher patients exhibited significant fusogenic activity with CCR5, whilst later isolates in all patients exhibited dual-tropism or X4-tropism, however, the numbers of Envs studied were too small for conclusions regarding a phenotypic switch to be associated with disease progression in the patients studied. However, viral variants carrying defective genes are likely to have low infectivity for $CD4^+$ cells such that they would not contribute significantly to the proviral load. Detailed interpretation of our results would require collation of data from other groups investigating other aspects of this particular cohort with regard to other viral genes and patient immunology coincident with our samples. The presence of a potent immune response is highly likely to effectively control such variants. Moreover, our initial results suggest patients heterozygous for the CCR5Δ32 mutation exhibited isolates possessing *env*-genes with less biological function.

In developed countries, treatment of the majority of LTNP patients is delayed until markers for disease progression are detected. Patients treated with HAART routinely undergo surveillance for drug-resistant strains of viruses, enabling modifications of therapy to combat these strains. The introduction of the expensive drug class of entry inhibitors means it is more prudent than ever to guard against the selection of resistant variants and their transmission to the general population. Ongoing epidemiological surveillance of *env*-genes is one such sentinel system to detect such variants in the general populace; however, simple analyses of the DNA sequence would not be sufficient to identify such variants. Routine screening of individual patients for drug-resistant mutations could thus be complemented by cell-cell fusion assays to provide a rapid screen to enable monitoring for emerging Env variants resistant to the new class of entry inhibitors. This would prevent patients being treated unnecessarily with such

drugs, thereby reducing the cost of therapy. The association of less fusogenic *env*-gene products with slow disease progression would provide another marker to determine the onset of therapy. Further studies are warranted to compare LTNP and RP patient groups. Analyses of consensus sequences between these two groups using the Variate program (Douglas *et al.*, 1997) revealed physico-chemical differences across the whole of Env that may have implications for the role of Env fusogenic capacity in rapid disease progression (R. Daniels personal communication).

The primary objective of this study was to develop a rapid cell-cell fusion assay for characterising the biological properties of HIV-1 *env*-gene products isolated from patient samples. As shown in this study, the combination of cell-cell fusion assays and virus-infection assays are complementary to our existing surveillance methods. Further optimisation of the assay is necessary. Although results suggest independent transfection and infection of effector cells does not greatly impact the fusion events with regard to the positive controls, Env expression is still dependent upon two variables. Moreover, the recombinant vaccinia vTF7-3 driven expression system results in over-expressed Env that may be a contributing factor in the qualitative difference observed between cell-cell fusion assays and chimeric virus infectivity. Co-transfecting additional plasmids autonomously expressing T7 RNA polymerase and HIV-1 Rev with pQ7.*env* clones may be able remove vTF7-3 from the system and result in a more physiologically relevant level of Env expression in the cell. Despite the discordant results observed with certain mutants between the cell-cell fusion assay and virus infection assays, the cell-cell fusion assay developed retains uses in studying the biological function of *env*-gene products in our laboratory group. The assay obviates unnecessary downstream cloning of biologically defective *env*-genes into the pC2 infectious cassette, saving time and valuable resources. The absence of a complete crystal structure of the native Env, either as a monomer or homotrimer means the effects of structural changes, as a direct result of immune selection or purifying selection in a HIV-1 infected patient, can only be inferred from existing data, the majority of which has been generated using laboratory adapted strains. Our observations that primary isolate Envs can exhibit biological function in contradiction to many mutational studies carried out with reference HIV Envs illustrate that effects of mutations depends on the backbone into which they are introduced, and highlights the necessity to back-up inferences from sequence analyses with studies of biological function.

Appendix

A1 Equipment and Materials

A1.1 Major Equipment

- ABI Prism 377 Sequencer (Applied Biosystems, UK).
- Balance BD202 (Mettler Toledo).
- Benchtop spinmixer (Gallenkamp).
- BD FACSCalibur™ System (BD Biosciences, USA).
- Centrifuges: IEC Centra-4R (Damon, UK); Eppendorf Centrifuge 5415R (Eppendorf GMBH, Germany); Beckman GPR Centrifuge (Beckman); Hermle Z400K (Hermle).
- DNA electrophoresis apparatus: made in-house.
- Eppendorf Biophotometer (Eppendorf GMBH, Germany).
- FPM 2100 X-ray Film Processor (Fuji, Japan).
- Freezers: K40-8 Kelvinator –20°C freezer; Ultima II Revco –70°C; Lab Impex Research –70°C.
- Incubators: MCO-17A1 CO_2 Incubator (Sanyo); CO_2 Incubator (Leec); Cabinet Incubator (Gallenkamp); Economy Incubator Size 1 (Gallenkamp); Luckham R300 Incubator Shaker (Luckham); G24 Environmental Incubator Shaker (New Brunswick Scientific).
- IS550 Imager (Kodak)
- Magnetic stirrer/hotplate MR3001 (Heidolph).
- MegaBACE Capillary Sequencer (Amersham Biosciences, UK).
- Microscopes: Nikon Eclipse TS100 (Nikon, Japan); Nikon Labophot 2 with HB10101AF mercury lamp (Nikon, Japan); Nikon Diaphot microscope with mounted Nikon F301 Camera (Nikon, Japan).
- Multiskan Ascent Plate Reader (Thermo Labsystems, UK).
- Pipetteboy acu (Integra Biosciences, UK).
- Pipettes: Pipetteman (P2, P10, P20, P200 and P1000)(Gilson); Multipette® Plus (Eppendorf GMBH, Germany); Multichannel pipette (Labsystems).
- Powerpac 200 power pack (BioRad).
- Thermal Cyclers: PTC-100 (MJ Research Inc.); Hybaid Omn-E Thermocycler (Hybaid); Robocycler® Gradient 96 (Stratagene).
- Trans-Blot SD semidry transfer apparatus (BioRad).
- UV transilluminator (BDH).

- Victor Wallac Multilabel Counter.
- Water baths (Grant Instruments Ltd, UK).
- Xcell SureLock™ protein electrophoresis apparatus (Novex).

A1.2 Laboratory Consumables

- 0.2ml Thin walled PCR tubes (The Perkin Elmer Corporation, USA).
- 0.5ml flip-top tubes (Treff Lab, Switzerland).
- 1.0mm thick SuperFrost glass slides (BDH).
- 1.5ml flip-top tubes (Treff Lab, Switzerland).
- Filtered pipette tips 10µl, 20µl, 200µl and 1000µl. (Rainin, UK)
- Pipette tips 10µl, 20µl, 250µl and 1000µl (Rainin, UK)
- Disposable Pipettes (1ml, 5ml, 10ml and 25ml. Costar, UK)
- 13mm round coverslips (BDH)
- 14ml Falcon tubes (Becton Dickinson).
- 15ml Centrifuge tubes (Corning).
- 2.5ml Screw top tubes (Sarstedt).
- 20ml Universal tubes (Sterilin).
- 2ml Cryotubes (Nunc).
- 50ml Centrifuge tubes, skirted (Corning).
- 60mm Tissue culture dishes (Orange)
- 96 well black flat-bottomed plates (Costar)
- 96 well flat-bottomed plates (Costar)
- Bijoux (Sterilin).
- Combitips Plus 2.5ml and 5.0ml (Eppendorf GMBH, Germany)
- Cuvettes (Merck)
- Disposable sterile scalpels no.11 (Swann-Morton, UK)
- FACS tubes (Falcon)
- Hybond™ C Extra nitrocellulose membrane (Amersham Biosciences, UK)
- Kodak MXB film (Kodak)
- Kova Glasstic Slides (Bio-Stat Diagnostics)
- Minisart filters 0.45µm and 0.20µm (Sartorius)
- Pre-cast 15 well 4-12% Bis-Tris polyacrylamide gels (Novex)
- Syringes 5, 10, 20 and 50ml (Becton Dickinson Labware, UK)

- Tissue culture flasks T25cm^2, T80cm^2 , T175cm^2 and triple layered T175cm^2 (Nunc)
- Uvettes (Eppendorf GMBH, Germany)

A1.3 Complete Kits

- ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits (Cat# 4304150, Applied Biosystems, UK)
- Half BD Buffer (Cat# K1104, GenPak)
- Luciferase Assay System (Cat# E1501, Promega)
- Murex HIV Antigen Mab kit (Cat# VK86, Abbott)
- Profection Transfection Kit (Cat# E1200, Promega)
- QIAGEN Gel Extraction Kit (Cat# 28706, QIAGEN, UK)
- QIAGEN Midiprep kit (Cat# 12143, QIAGEN, UK)
- Rapid DNA Ligation kit (Cat# 1 635 379, Roche)
- Rapid Pure Miniprep Kit (Cat# 2070-400, Qbiogene, USA)
- *Renilla* Luciferase Assay System (Cat# E2820, Promega)

A1.4 DNA plasmid vectors

- pC2 infectious molecular cassetting vector was held in-house. Details of pC2 have been published (Zheng and Daniels, 2001).
- phRL-TK (Int$^-$) codon optimised plasmid carrying *Renilla reniformis* luciferase reporter gene downstream of a human herpes virus thymidine kinase promoter (Promega).
- pQ7 cloning vector for the insertion and expression of PCR-rescued HIV-1 *env*-genes was held in-house. Details have been published (Douglas *et al.*, 1996).
- pSP-*luc*$^+$ carrying *Photinus pyralis* luciferase reporter gene downstream of a SP6 RNA polymerase promoter (Promega).

A1.5 Enzymes

- Calf Intestinal Alkaline Phosphatase (CIAP) (Invitrogen Life Technologies, UK).
- *pfu* polymerase (Stratagene).
- Restriction enzymes and buffers (Roche, UK).
- RNAse A (QIAGEN, UK).

A1.6 Standards and Controls

- 1kb λ DNA BstE II digested ladder, 50μg/ml (New England Biolabs).

- ΦX174 DNA HaeIII digested ladder, 50μg/ml (New England Biolabs).
- HIV-1_{IIIB} Lectin purified gp120 supplied by R. Chung (NIMR, UK).
- SeeBlue® Plus 2 protein marker (Invitrogen Life Technologies, UK).

A1.7 Commercial Reagents

All general chemicals were analytical or ultrapure grade, supplied by Sigma-Aldrich, UK or BDH Ltd., UK unless otherwise stated.

- 96% Ethanol (BDH).
- β-Mercaptoethanol (Sigma-Aldrich, UK).
- Bovine serum albumin (BSA) (Sigma-Aldrich, UK).
- Bradford Reagent (BioRad).
- Citifluor (UKC Chem. Lab.).
- Chloroform (Rectapur).
- Crystal Violet (Sigma-Aldrich, UK).
- Electro-chemiluminescence (ECL) development reagent (Amersham Pharmacia Biotech).
- Ethidium bromide tablets (Amresco, Ohio, USA), used at 1μg/ml working concentration.
- Formaldehyde 36% (PROLABO).
- Glutaraldehyde (BDH).
- Glycerol (BDH).
- Hydrochloric Acid (11.6N) (BDH).
- Isoamyl alcohol (BDH).
- Milk powder (Marvel).
- NuPAGE® MOPS SDS buffer (Invitrogen).
- NuPage™ LDS Sample buffer 4x (Invitrogen).
- Paraformaldehyde (BDH).
- Phenol/ chloroform (Amresco, Ohio, USA).
- Polyoxyethelenesorbitan monolaurate (Tween20) (Sigma-Aldrich, UK).
- Romil SpS™ water (Romil Ltd., UK) autoclaved for reconstitution or elution of DNA.
- Sodium dodecyl sulphate (SDS) (BioRad).

A1.8 In-House Supplied Reagents

Reagent	Composition	Amount/litre
L Broth/Agar	Bacto-tryptone	10.000g
	Yeast Extract	5.000g
	NaCl	10.000g
	Distilled water	1.000l
Phosphate Buffered Saline	NaCl	10.000g
	KCl	0.250g
	Na_2HPO_4	1.437g
	KH_2PO_4	0.250g
	Distilled water	1.000l
SOC medium	Deionised water	950.000ml
	Bacto-tryptone	20.000g
	Bacto yeast	5.000g
	NaCl	0.500g
	250mM KCl	10.000ml
	2M $MgCl_2$ solution	5.000ml
	1M sterile filtered glucose solution	20.000ml
Tris-acetate-EDTA (50x)	Tris Base	242.000g
	Glacial acetic acid	57.100ml
	EDTA	18.612g
	Distilled water	1.000l
Trypsin in Versene	NaCl	8.000g
	KCl	0.200g
	Na_2HPO_4	1.150g
	KH_2PO_4	0.200g
	EDTA	0.200g
	Trypsin	10.000g
	Phenol Red	0.015g
	Distilled water	1.000l
Tris-borate EDTA (10x)	Trizma base	121.100g
	Boric acid	61.830g
	EDTA	18.600g
	Distilled water	1.000l

Detailed preparation protocols can be found in (Sambrook *et al.*, 1989).

A1.9 Oligonucleotides

- All oligonucleotides were synthesised and supplied lyophilised by Oswel. Oligonucleotides were reconstituted using Romil SpS™ water.

A1.9.1 Oligonucleotide Primer Sequences for the Generation of HIV-1_{NL43} Env Processing Site Mutants

Reaction Set	Mutant	Cleavage Motif	Primer	Sequence	Length/nt	Tm(°C)
Set 1			StuF	TTACAC*AGGCCT*GTCCAAAGG	21	64
KAKRR	WT	KAKRRVVQREKR	NormR	CTCTGCACCACTCTTCTCTTTGC	23	70
Site 2	M1S2	KAKERVVQREKR	M1S2R	CTCTGCACCACTCTTTCCTTTGCCTTCCTGGGTG	34	54
	M2S2	KAKEGVVQREKR	M2S2R	CTCTGCACCACTCCTTCCTTTGCCTTCCTGGGTG	34	54
	M3S2	KAKSRVVQREKR	M3S2R	CTCTGCACCACTCTTGACTTTGCCTTCCTGGGTG	34	54
	M4S2	NAISSVVQREKR	M4S2R	CTCTGCACCACGCTGCTTATTGCATTCCTGGGTGCTAC	38	44
	M5S2	KAQNGVVQREKR	M5S2R	CTCTGCACCACTCCATTCTGTGCCTTCCTGGGTGC	35	50
			BamR	AAGTGCTAA*GGATCC*GTTCACTAA	24	68
Set 2			StuF	TTACAC*AGGCCT*GTCCAAAGG	21	64
REKR	WT	KAKRRVVQREKR	NormF	GAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAATAGG	22	62
Site 1	M1S1	KAKRRVVQREER	M1S1F	GAGTGGTGCAGAGAGAAGAAAGAGCAGTGGGAATAGG	37	56
	M2S1	KAKRRVVQRNER	M2S1F	GAGTGGTGCAGAGAAAAGAAAGAGCAGTGGGAATAGG	37	56
	M3S1	KAKRRVVQREKT	M3S1F	GAGTGGTGCAGAGAGAAAAAACTGCAGTGGGAATAGGAG	39	50
	M4S1	KAKRRVVQSHEQ	M4S1F	GAGTGGTGCAGAGCCATGAACAAGCAGTGGGAATAGGAG	39	52
	M5S1	KAKRRVVQSEHN	M5S1F	GAGTGGTGCAGAGCGAACATAATGCAGTGGGAATAGGAG	39	50
			BamR	AAGTGCTAA*GGATCC*GTTCACTAA	24	68
Set 3			StuF	TTACAC*AGGCCT*GTCCAAAGG	21	64
VVQ motif	WT	KAKRRVVQREKR	ComR	TCTTCTCTTTGCCTTGGTG	19	49
	VVF	KAKRR--QREKR	VVF	CACCAAGGCAAAGAGAAGAC------AGAGAGAAAAAAGAGCAGT	39	66
	PVSF	KAKRRPVQREKR	PVSF	CACCAAGGCAAAGAGAAGACCGGTGCAGAGAGAAAAAAGAG	41	68
	VPSF	KAKRRVPQREKR	VPSF	CACCAAGGCAAAGAGAAGAGTGCCGCAGAGAGAAAAAAGAGCAG	44	70
	EVSF	KAKRREVQREKR	EVSF	CACCAAGGCAAAGAGAAGAGAGGTGCAGAGAGAAAAAAGAG	41	67
			BamR	AAGTGCTAA*GGATCC*GTTCACTAA	24	68

Mutant residues are indicated in red. *Stu*I and *Bam*HI restriction sites indicated.

A1.9.2 Oligonucleotide Primer Sequences for the Generation of HIV-1_{NL43} Env Truncation Mutants

Truncation Mutant	Primer	Sequence	Length/nt	Tm(°C)	Product Size/bp	Product Size/ amino acids
	FENV	TAA*GAGCTC*GAGCAGAAGATAGTGGCAATGATAGTGA	37	>100		
T1	T1Rem	CTTCTAT*GAATTC*TTATGTCCCCTCAGCTACTGC	34	58	2466	821
T2	T2Rem	TGAGGGC*GAATTC*TTACCTGCGTCCCAGAAGTTC	34	58	2352	783
T3	T3Rem	CTCTCAA*GAATTC*TTAGCTGAAGAGGCACAGGCT	34	62	2289	762
T4	T4Rem	TCCGCAG*GAATTC*TTAGATAAGTGCTAAGGATCCGT	36	60	2256	751
T5	T5Rem	AGGATCC*GAATTC*TTATCGAATGGATCTGTCTCTGT	36	60	2229	742
T6	T6Rem	CTCTGTC*GAATTC*TTAACCTTCTTCTTCTATTCCTTC	37	58	2199	732
T7	T7Rem	ATCCCTG*GAATTC*TTAATTCACTATAGAAAGTACAGC	37	56	2106	701
T8	T8Rem	TTAAACC*GAATTC*TTATCCTACTATCATTATGAATAATT	39	56	2058	685
T9	T9Rem	TCATTAT*GAATTC*TTATATATACCACAGCCAATTTGT	37	56	2037	678
T10	T10Rem	TCCACAA*GAATTC*TTATTTATCTAATTCCAATAATTCTT	39	56	1983	660

The *Xho*1 and *Eco*R1 restriction sites are indicated as coloured.

A1.9.3 Oligonucleotide Primers Used for the Rescue of HIV-1 Proviral *env*-genes and Subsequent DNA Sequencing

Reaction[a]	Sequence[b]	HXB2c region[c]
RT-PCR, PCR		
TATF - 1st round	GAGCCCTGGAASCAYCCRGGRAGTCAGCC	5854-5882
NEFR - 1st round	GTCATWGGYCTYARAGGTACCTGDGG	9009-9035
FENV - 2nd round	TAAGAGCTCGAGCAGAAGAYAGTGGCAATGARAGYGA	6199-6235
RENV - 2nd round	ACCACAGAATTCTTTGACCAYTTGCCACCCATBTTA	8792-8817
Sequencing, forward primers		
Q7F	TCGAGCCCGCGGTGATCATCCGGATATCG	
M2A	TGGGCCACACATGCCTGTGTACC	6428-6450
M5	GTCAGCACAGTACAATGTACA	6947-6967
M7R	TGTRGAGGRGAATTTTTCTAYTG	7355-7377
M11R	GGGATKTGGGGYTGCTCTGG	8003-8022
M22R	GTTAGGCAGGGATAYTCACC	8345-8364
Sequencing, reverse primers		
SEQ1	GGGGTTAATTTTACACATGG	6575-6594
V3-1R	TTCTGCTAGRCTRCCATT	7007-7024
V3-3R	TAGAAAAATTCYCCTCYACARTTAA	7350-7375
R20R	TTGAGGRYTTCCCACCCC	8587-8604
R22N	GTTTCCAGAGCAACCCA	8009-8027
Q7R	AATTCGATATCCGGATGATCACCGCGGC	

[a] The RT-PCR and nested PCR primers serve to rescue HIV-1 *env*-genes from proviral/viral genomes, the forward and reverse primers were used for DNA sequencing full-length *env*-genes. Q7F and Q7R are located on the pQ7 vector.
[b] Nucleotide code is according to the internationally accepted nomenclature.
[c] Locations of the primers in the HIV-1_{HXB2c} genome are indicated.

A1.10 Antibodies

- ARP401 Sheep-derived polyclonal antiserum, raised against HIV-1_{IIIB} gp120 was obtained from the National Institute for Biological Standards and Controls, Centralised Facility for AIDS Reagents (NIBSC CFAR), UK. The antiserum was used at a 1:1000 dilution in western blotting.
- ARP301 (221) anti-gp120 mouse derived IgG1 MAb recognising a HIV-1_{IIIB} peptide (ARP740.45, NIBSC CFAR, UK), was generated in-house (Bristow *et al.*, 1994). The ascites fluid was used at a 1:2500 dilution in western blotting.
- D7324 (Cat# D7324, Aalto Bio Reagents Ltd, Dublin, Ireland) affinity purified sheep polyclonal antibodies, recognising the HIV-1_{IIIB} gp120 carboxy-terminal amino acid sequence APTKAKRRVVQREKR, were reconstituted from a lyophilised form to 1mg/ml with Romil water and used at a dilution of 1:500 in western blotting.
- EVA3012 (NIBSC CFAR, UK) mouse derived IgM-isotype monoclonal antibody recognising HIV-1_{LAI} gp120 V3 loop, was supplied as cell culture supernatant and used at a 1:20 dilution in cell surface immunofluorescence assays (IFA).
- EVA3013 (NIBSC CFAR, UK) mouse derived IgM-isotype monoclonal antibody recognising HIV-1_{LAI} gp120 V3 loop, was supplied as cell culture supernatant and used at a 1:20 dilution in cell surface immunofluorescence assays (IFA).
- T-30 mouse derived MAb, raised against HIV-1_{BH8} oligomeric Env and recognising a gp41 epitope (Earl *et al.*, 1994), was held in-house and used at a 1:1000 dilution in western blotting.
- Donkey anti-sheep IgG-peroxidase conjugate (Cat# A3415, Sigma-Aldrich, UK) was diluted 1:5000 to detect ARP401 and D7324 in western blotting experiments.
- Anti-mouse IgG-FITC conjugate (Cat# N1031, Amersham Life Science) was used at a 1:200 dilution in 1%(w/v) BSA/PBS to detect EVA3012 and EVA3013 in cell surface immuno-fluorescent assays.
- Goat anti-mouse IgG-peroxidase conjugate (Cat# W4021, Promega, UK) was used at a 1:5000 dilution to detect ARP301 in western blotting experiments.

A1.11 Bacteria

A1.11.1 Bacterial Strains

- DH5α *Escherichia coli* library efficiency competent cells (Invitrogen Life Sciences, UK).

A1.11.2 Bacterial media supplements

- Ampicillin 100mg/ml stock (Sigma-Aldrich, UK).
- Nafcillin 100mg/ml stock (Sigma-Aldrich, UK).

A1.12 Mammalian Cells

A1.12.1 Media and supplements

- (3-Aminopropyl)triethoxysilane (3-APTS) (Sigma-Aldrich, UK).
- Cytosine 1-β-D-arabinofuranoside (AraC) (Sigma-Aldrich, UK).
- Rifampicin (Sigma-Aldrich, UK) .
- Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies, UK).
- Foetal Calf Serum (FCS) (PAA Laboratories), screened by in-house large-scale laboratory department for mycoplasma. Subsequently heat-inactivated at 56°C/30min and filtered through 0.20μm syringe filters prior to use.
- G418 Sulphate (Invitrogen Life Sciences, UK).
- Hygromycin B (Sigma-Aldrich, UK).
- Interleukin-2 (IL-2) (Roche) 10000U/ml.
- L-glutamine 200mM stock (Invitrogen Life Technologies, UK).
- Panserin 401 medium (PAN Biotech GmbH).
- Penicillin/Streptomycin (Pen/Strep) supplied as a 100x stock solution of 10000U/ml Penicillin and 10g/ml Streptomycin (Sigma-Aldrich, UK).
- *Phaseolus vulgaris* phytohaemagglutinin (PHA-P) (Sigma-Aldrich, UK).
- Puromycin (Sigma-Aldrich, UK).
- RPMI 1640 L-glutamine free (Invitrogen Life Technologies, UK).

A1.12.2 Peripheral Blood Mononuclear Cells

Frozen peripheral blood mononuclear cells (PBMCs) stocks were prepared from blood packs obtained from the National Blood Service (Colindale, London) by C. Vella and L. Whittaker. Ficoll-Hypaque was used to isolate buffy coats and $CD8^+$ cells were depleted using Dynal beads (Dynal). Cells were maintained using 1:1 Panserin 401/RPMI 1640 media supplemented with 10% FCS, 100U/ml penicillin, 100μg/ml Streptomycin and 2mM L-glutamine.

A1.12.3 Cell Lines

All cell lines were incubated at 37°C in a 5% CO_2 atmosphere incubator unless otherwise stated.

- 293T cells are human embryo kidney cells transformed with SV40 large T antigen. Supplied by E. Yamada of the Edward Jenner Institute for Vaccine Research, Compton, UK. Cells were maintained in DMEM supplemented with 10% heat inactivated foetal calf serum.
- CV-1 cells are African Green monkey kidney cells. CV-1 cells were already held in-house. Cells were maintained in DMEM supplemented with 10% heat inactivated FCS.
- Ghost reporter cell lines expressing CD4 and CD4 with CCR5 or CXCR4, were obtained from NIBSC CFAR, UK. Ghost cell lines are human osteosarcoma (HOS) cell lines carrying a green fluorescent protein (GFP) gene controlled by an HIV-2 long terminal repeat promoter. Cell lines were maintained in DMEM supplemented with 10% FCS, G418 sulphate (500μg/ml), hygromycin B (100μg/ml), and for those cell lines expressing β-chemokine receptors, puromycin (1μg/ml).
- Dr Aine Mcknight of the Wohl Virology Centre, UCL, UK, kindly supplied NP2 glioma cell lines with permission from Dr Hiroo Hoshino. Cells were maintained in DMEM supplemented with 5% FCS, G418 sulphate (500μg/ml), and puromycin (1μg/ml) in the case of cell lines expressing chemokine receptors.

A1.13 Viruses

A1.13.1 Recombinant Vaccinia Viruses

- vTF7-3, expressing T7 RNA polymerase, was held in-house. The recombinant vaccinia construct vTF7-3 is derived from the Western Reserve vaccine strain (Fuerst *et al.*, 1987).
- $vSIMB_{E/L}$, encoding SP6 RNA polymerase under the control of a synthetic hybrid early/late vaccinia promoter (Usdin *et al.*, 1993), was kindly donated by Dr B. Moss at the National Institute for Allergy and Infectious Diseases, Bethesda, Maryland, USA.

A1.13.2 HIV-1 *env*-genes

- HIV-1_{NL43} *env*-gene was excised from an in-house pQ7 plasmid vector using *Eco*R1 and *Xho*1 restriction sites.
- HIV-1_{JRFL} *env*-gene was excised from an in-house pQ7 plasmid vector using *Eco*R1 and *Xho*1 restriction sites.
- Primary isolate HIV-1 *env*-genes derived from proviral DNA were supplied by Dr R. Daniels and Dr Z. Xiang

A2 Amino Acid Sequences of HIV-1 env*-genes*

A2.1 HIV-1_{NL43} Env Processing Site Mutant Clones

```
  NL43_1     1  MRVKEKYQHL WRWGWKWGTM LLGILMICSA TEKLWVTVYY GVPVWKEATT TLFCASDAKA YDTEVHNVWA THACVPTDPN PQEVVLVNVT ENFNMWKNDM VEQMHEDIIS LWDQSLKPCV KLTPLCVSLK CTDLKNDTNT NSSSGRMIM  149
NL43_M1S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_M2S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_M3S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_M4S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_M5S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_M1S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_M2S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_M3S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_M4S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_M5S     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_VVF     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_PVS     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_VPS     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
NL43_EVS     1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149

  NL43_1   150  EKGEIKNCSF NISTSIRDKV QKEYAFFYKL DIVPIDNTSY RLISCNTSVI TQACPKVSFE PIPIHYCAPA GFAILKCNNK TFNGTGPCTN VSTVQCTHGI RPVVSTQLLL NGSLAEEDVV IRSANFTDNA KTIIVQLNTS VEINCTRPN  298
NL43_M1S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_M2S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_M3S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_M4S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_M5S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_M1S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_M2S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_M3S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_M4S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_M5S   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_VVF   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_PVS   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_VPS   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
NL43_EVS   150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298

  NL43_1   299  NNTRKSIRIQ RGPGRAFVTI GKIGNMRQAH CNISRAKWNA TLKQIASKLR EQFGNNKTII FKQSSGGDPE IVTHSFNCGG EFFYCNSTQL FNSTWFNSTW STEGSNNTEG SDTITLPCRI KQFINMWQEV GKAMYAPPIS GQIRCSSNI  447
NL43_M1S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_M2S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_M3S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_M4S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_M5S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_M1S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_M2S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_M3S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_M4S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_M5S   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_VVF   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_PVS   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_VPS   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
NL43_EVS   299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
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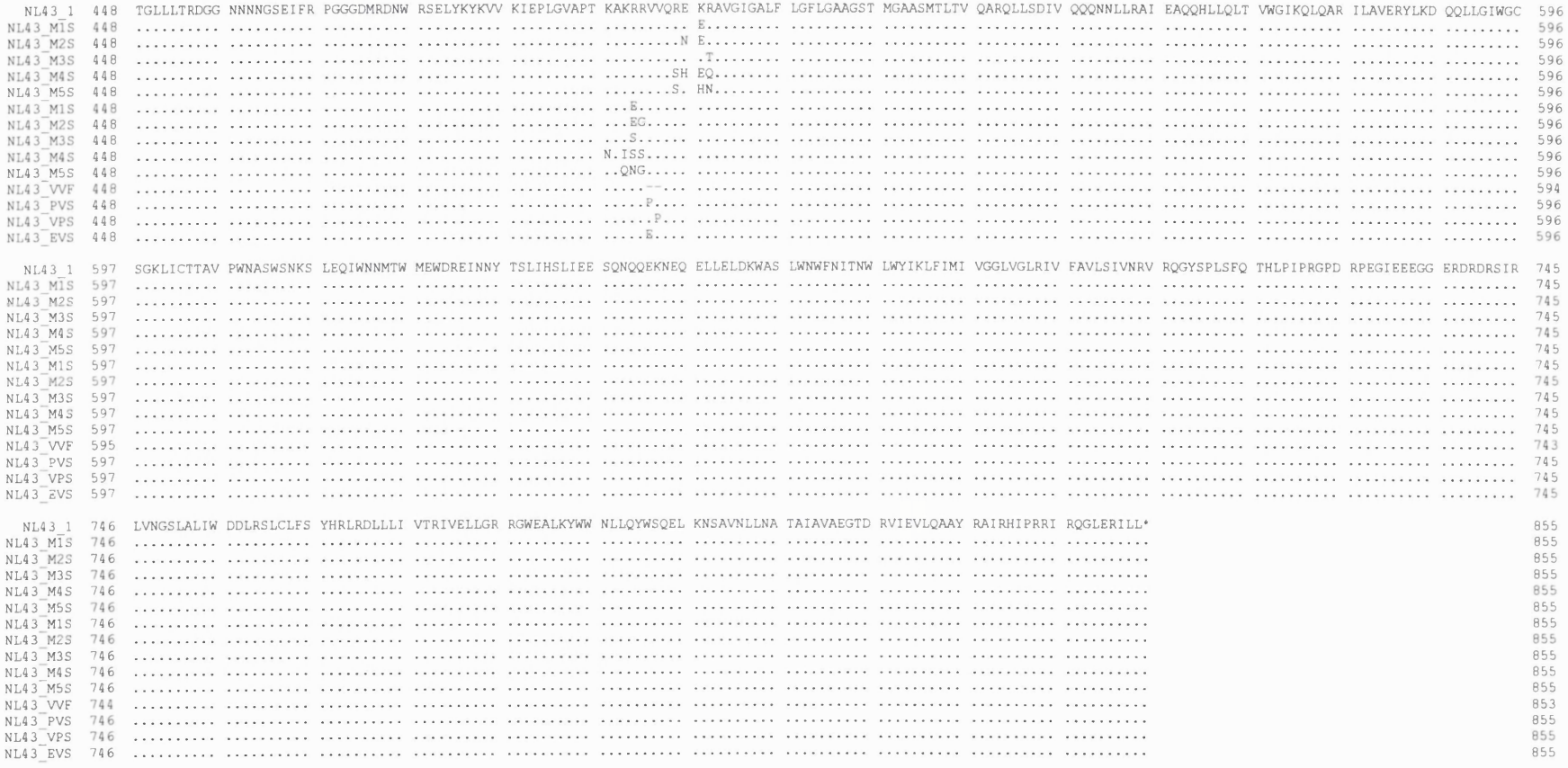
Identity to the $NL43_{WT}$ Env sequence is indicated by (.).



A2.1. Continued. Identity to the NL43$_{WT}$ Env sequence is indicated by (.),deletions in the VVQ motif by (-), and termination codons by (*).

A2.2 HIV-1_{NL43} Env Truncation Mutant Clones

```
     NL43    1  MRVKEKYQHL WRWGWKWGTM LLGILMICSA TEKLWVTVYY GVPVWKEATT TLFCASDAKA YDTEVHNVWA THACVPTDPN PQEVVLVNVT ENFNMWKNDM VEQMHEDIIS LWDQSLKPCV KLTPLCVSLK CTDLKNDTNT NSSSGRMIM  149
   NL43_T1    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
   NL43_T2    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
   NL43_T3    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
   NL43_T4    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
   NL43_T5    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
   NL43_T6    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
   NL43_T7    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
   NL43_T8    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
   NL43_T9    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149
  NL43_T10    1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  149

     NL43  150  EKGEIKNCSF NISTSIRDKV QKEYAFFYKL DIVPIDNTSY RLISCNTSVI TQACPKVSFE PIPIHYCAPA GFAILKCNNK TFNGTGPCTN VSTVQCTHGI RPVVSTQLLL NGSLAEEDVV IRSANFTDNA KTIIVQLNTS VEINCTRPN  298
   NL43_T1  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
   NL43_T2  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
   NL43_T3  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
   NL43_T4  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
   NL43_T5  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
   NL43_T6  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
   NL43_T7  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
   NL43_T8  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
   NL43_T9  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298
  NL43_T10  150  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  298

     NL43  299  NNTRKSIRIQ RGPGRAFVTI GKIGNMRQAH CNISRAKWNA TLKQIASKLR EQFGNNKTII FKQSSGGDPE IVTHSFNCGG EFFYCNSTQL FNSTWFNSTW STEGSNNTEG SDTITLPCRI KQFINMWQEV GKAMYAPPIS GQIRCSSNI  447
   NL43_T1  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
   NL43_T2  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
   NL43_T3  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
   NL43_T4  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
   NL43_T5  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
   NL43_T6  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
   NL43_T7  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
   NL43_T8  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
   NL43_T9  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447
  NL43_T10  299  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  447

     NL43  448  TGLLLTRDGG NNNNGSEIFR PGGGDMRDNW RSELYKYKVV KIEPLGVAPT KAKRRVVQRE KRAVGIGALF LGFLGAAGST MGAASMTLTV QARQLLSDIV QQQNNLLRAI EAQQHLLQLT VWGIKQLQAR ILAVERYLKD QQLLGIWGC  596
   NL43_T1  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
   NL43_T2  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
   NL43_T3  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
   NL43_T4  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
   NL43_T5  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
   NL43_T6  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
   NL43_T7  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
   NL43_T8  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
   NL43_T9  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
  NL43_T10  448  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  596
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Identity to the $NL43_{WT}$ Env sequence is indicated by (.).

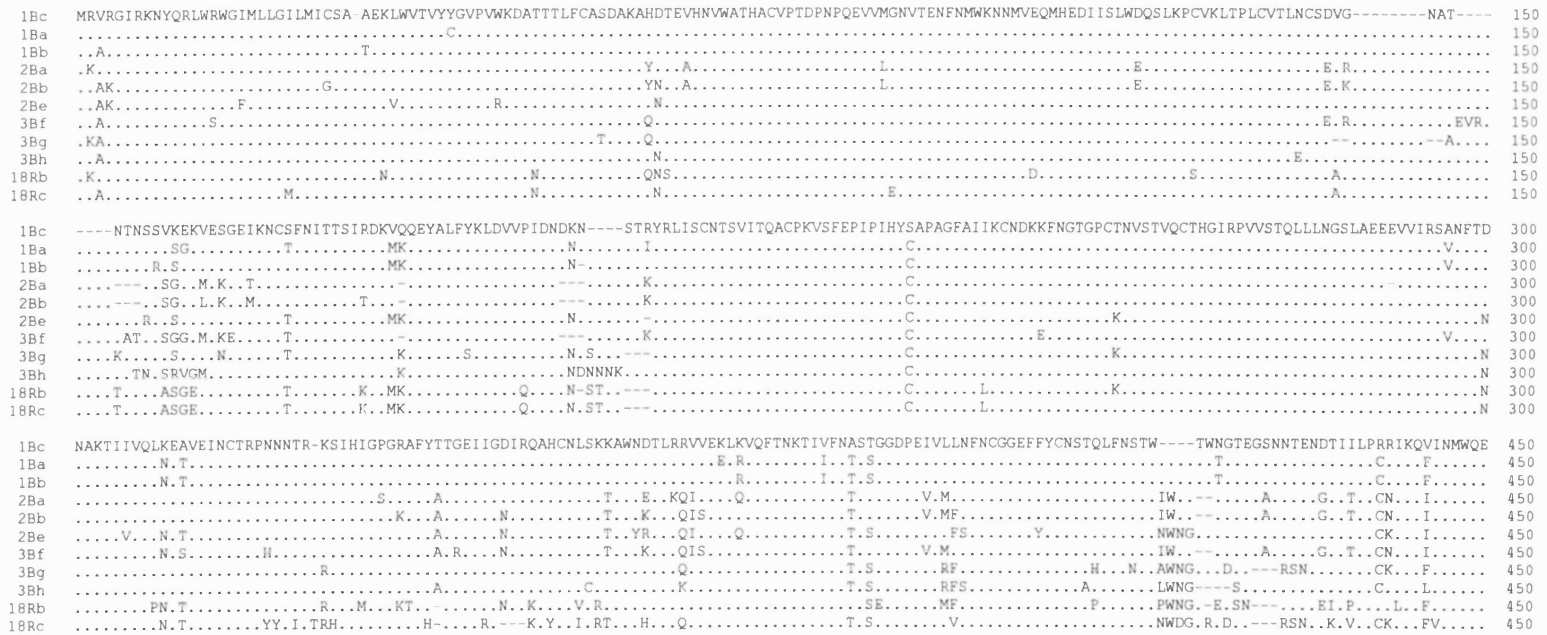
```
    NL43  597  SGKLICTTAV PWNASWSNKS LEQIWNNMTW MEWDREINNY TSLIHSLIEE SQNQQEKNEQ ELLELDKWAS LWNWFNITNW LWYIKLFIMI VGGLVGLRIV FAVLSIVNRV RQGYSPLSFQ THLPIPRGPD RPEGIEEEGG ERDRDRSIR  745
NL43_T1  597  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  745
NL43_T2  597  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  745
NL43_T3  597  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  745
NL43_T4  597  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  745
NL43_T5  597  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........  745
NL43_T6  597  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........*          736
NL43_T7  597  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........*                                                          705
NL43_T8  597  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..*                                                                                          689
NL43_T9  597  .......... .......... .......... .......... .......... .......... .......... .......... .......... ....*                                                                                                681
NL43_T10 597  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......*                                                                                                664

    NL43  746  LVNGSLALIW DDLRSLCLFS YHRLRDLLLI VTRIVELLGR RGWEALKYWW NLLQYWSQEL KNSAVNLLNA TAIAVAEGTD RVIEVLQAAY RAIRHIPRRI RQGLERILL*                                                                                                  855
NL43_T1  746  .......... .......... .......... .......... .......... .......... .......... .......... .......... .........*                                                                                                  825
NL43_T2  746  .......... .......... .......... .......... .*                                                                                                                                                                  787
NL43_T3  746  .......... .......... *                                                                                                                                                                                          766
NL43_T4  746  .........*                                                                                                                                                                                                       755
NL43_T5  746  *                                                                                                                                                                                                               746
```

A2.2. Continued. Identity to the $NL43_{WT}$ Env sequence is indicated by (.) and termination codons by (*).

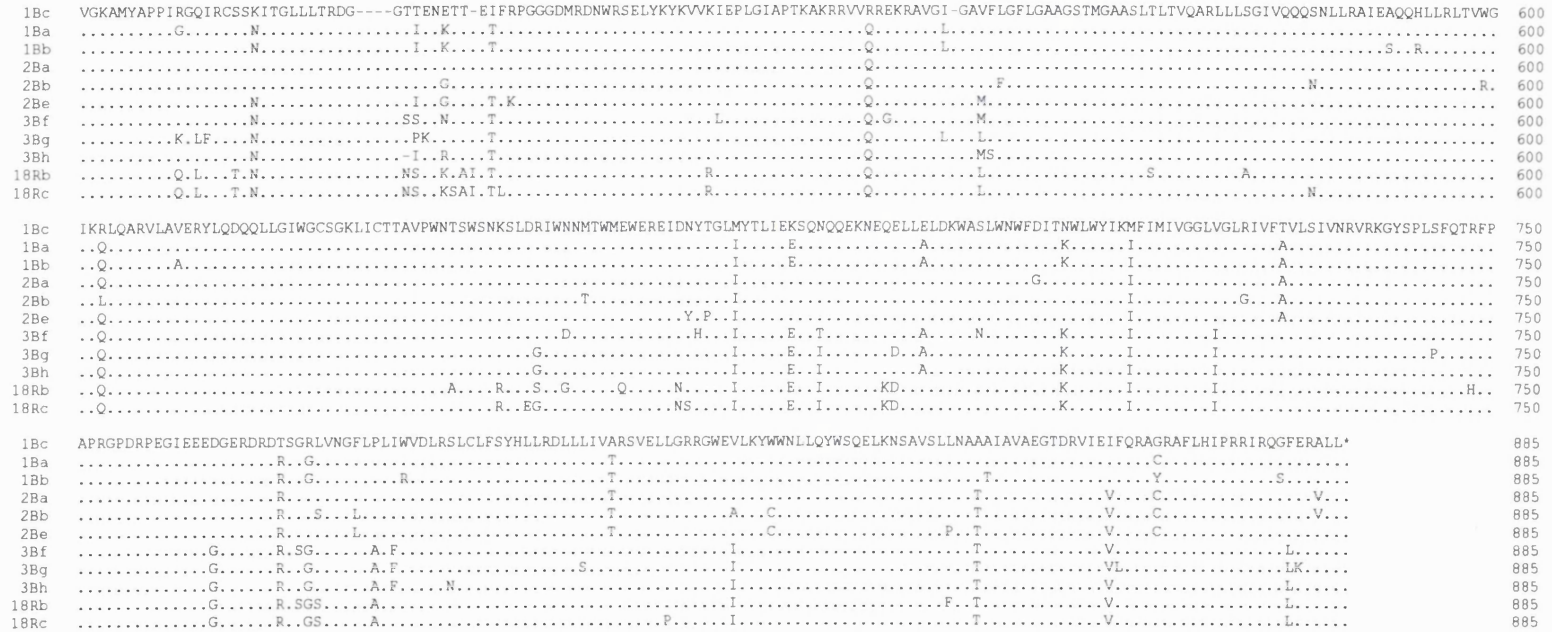
A3 LTNP-Switcher Patients HIV-1 env*-gene Clones*

The alignments shown for each patient are derived from a master alignment for all patient samples, generated by Clustal W within the GDE suite of programmes (Smith *et al.*, 1994).

A3.1 Amino Acid Sequences of HIV-1 *env*-gene Clones Derived From Patient 001

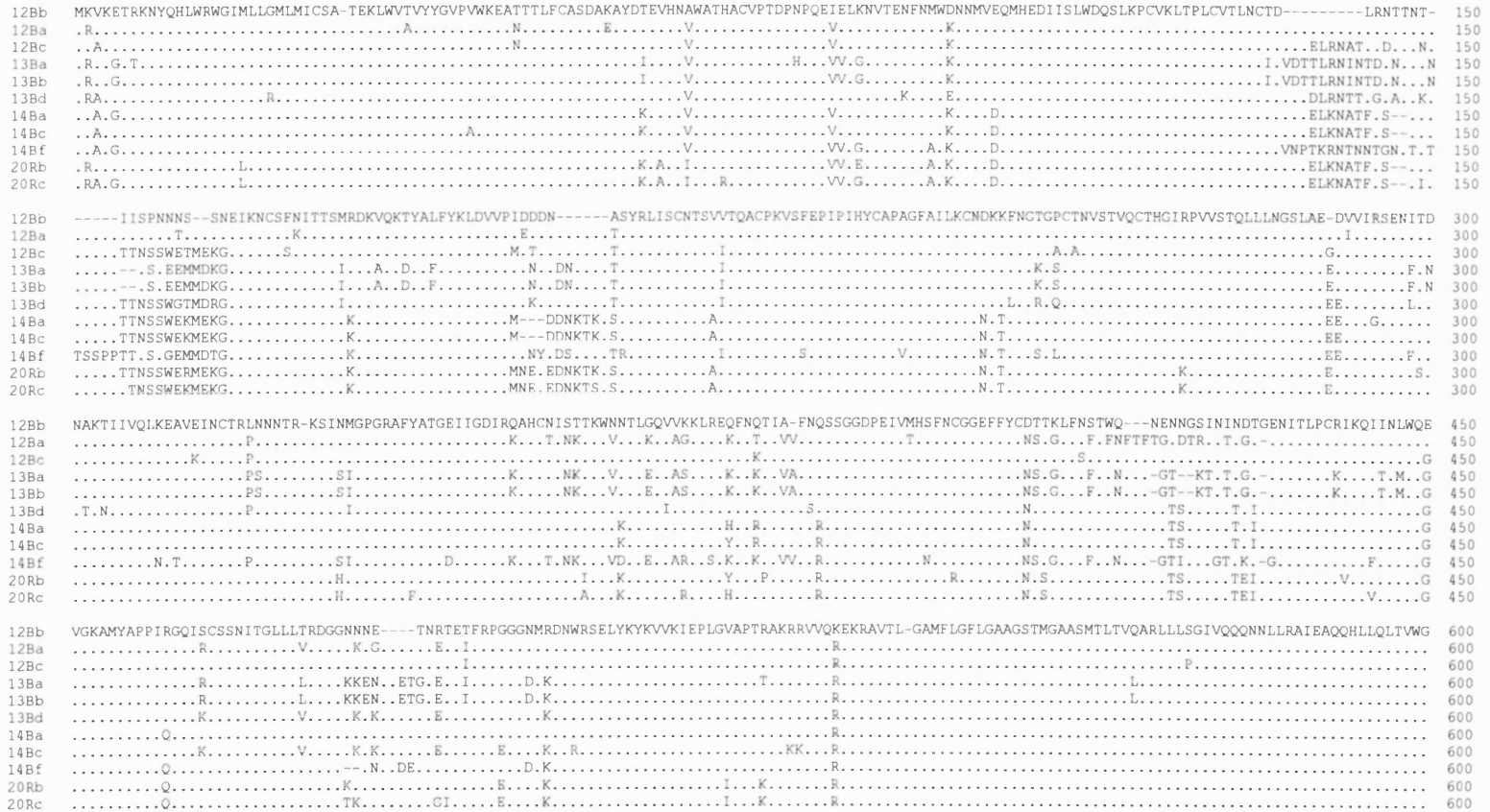


Identity with the 1Bc sequence is indicated by (.) and gaps introduced to improve the alignment are indicated by (-).

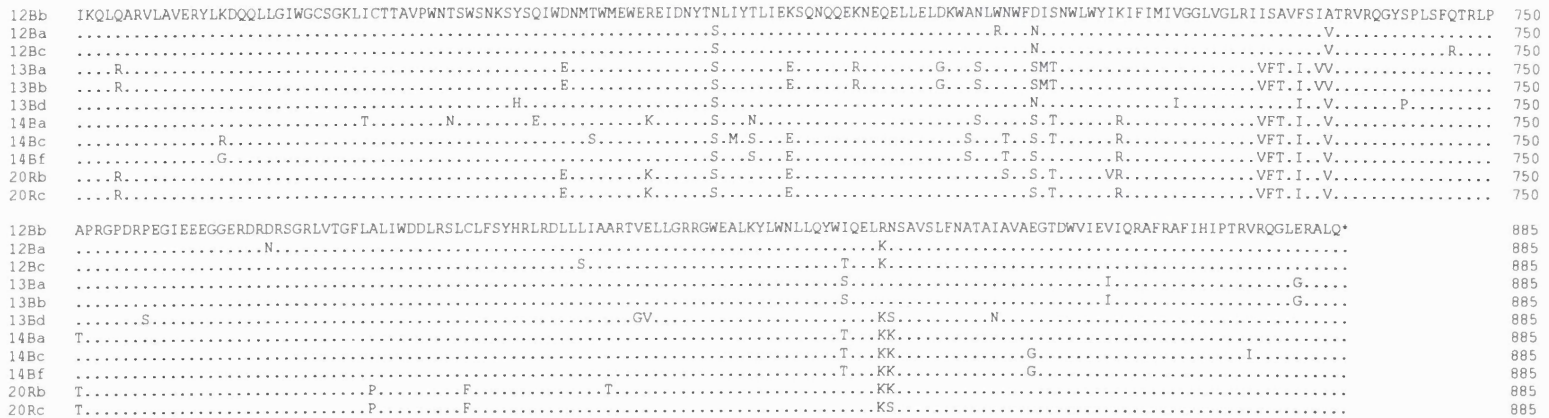


A3.1. Continued. Identity with the 1Bc sequence is indicated by (.) and gaps introduced to improve the alignment are indicated by (-).

A3.2 Amino Acid Sequences of HIV-1 *env*-gene Clones Derived From Patient 012

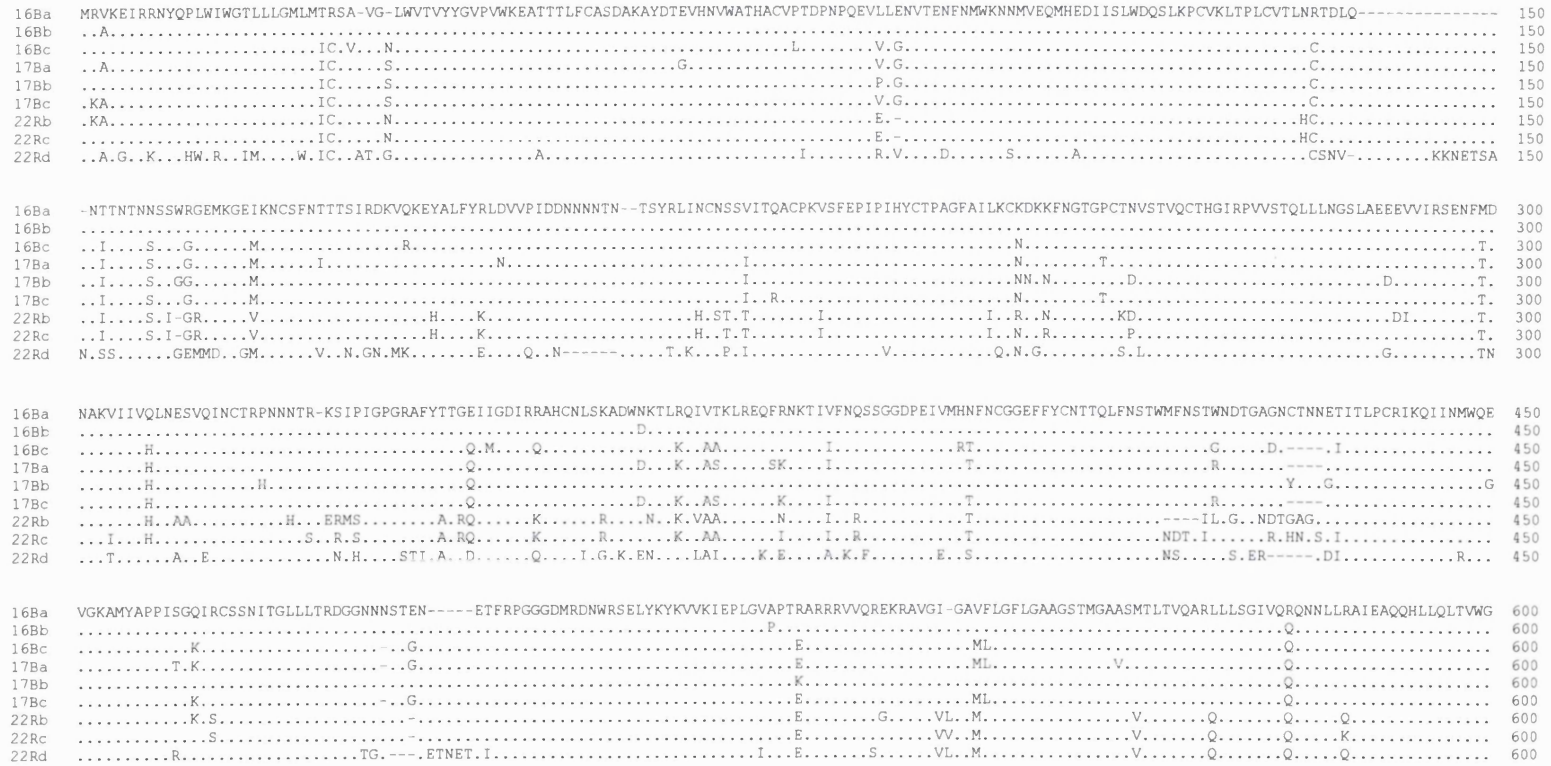


Identity with the 12Bb sequence is indicated by (.) and gaps introduced to improve the alignment are indicated by (-).



A3.2. Continued. Identity with the 12Bb sequence is indicated by (.) and gaps introduced to improve the alignment are indicated by (-).

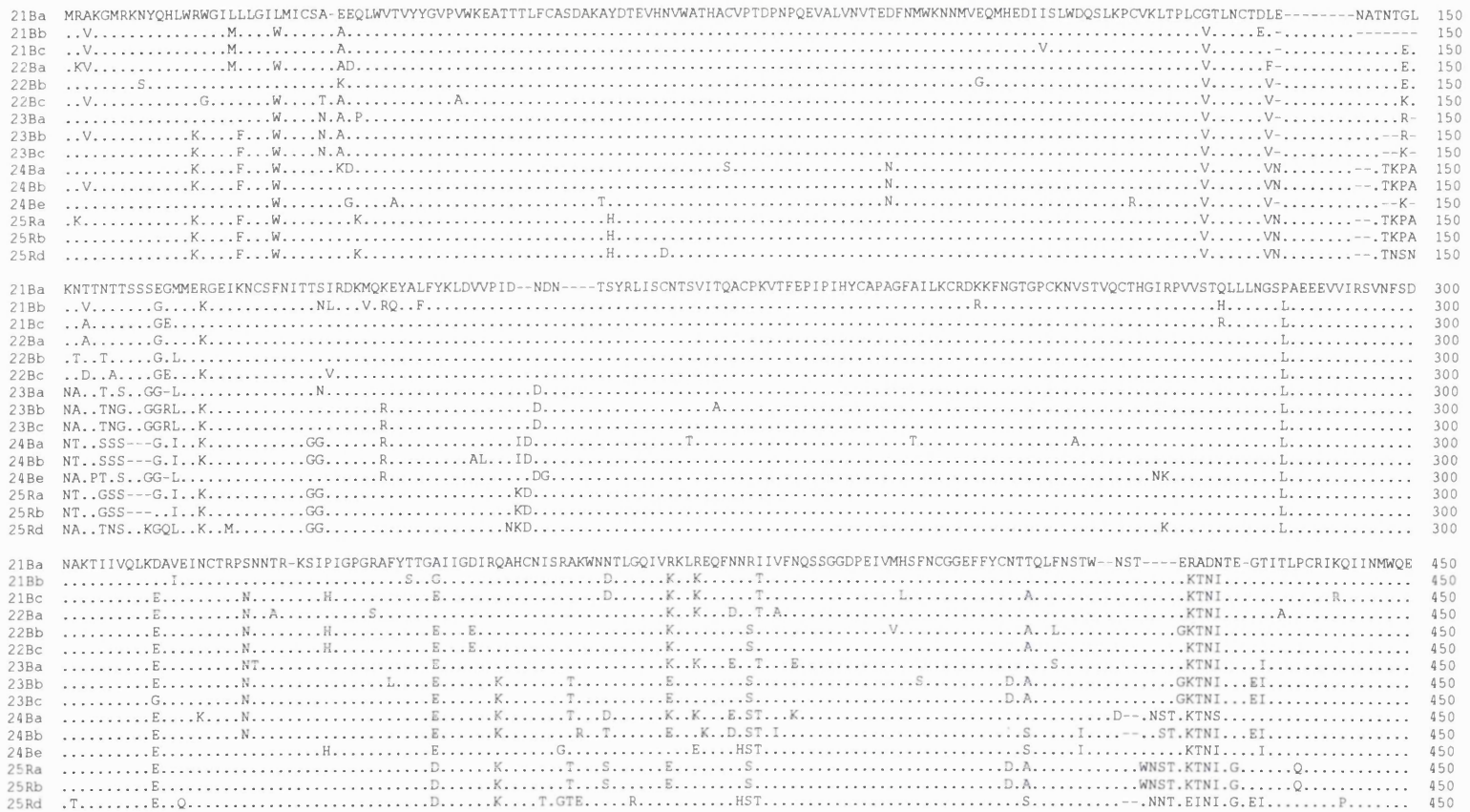
A3.3 Amino Acid Sequences of HIV-1 *env*-gene Clones Derived From Patient 028



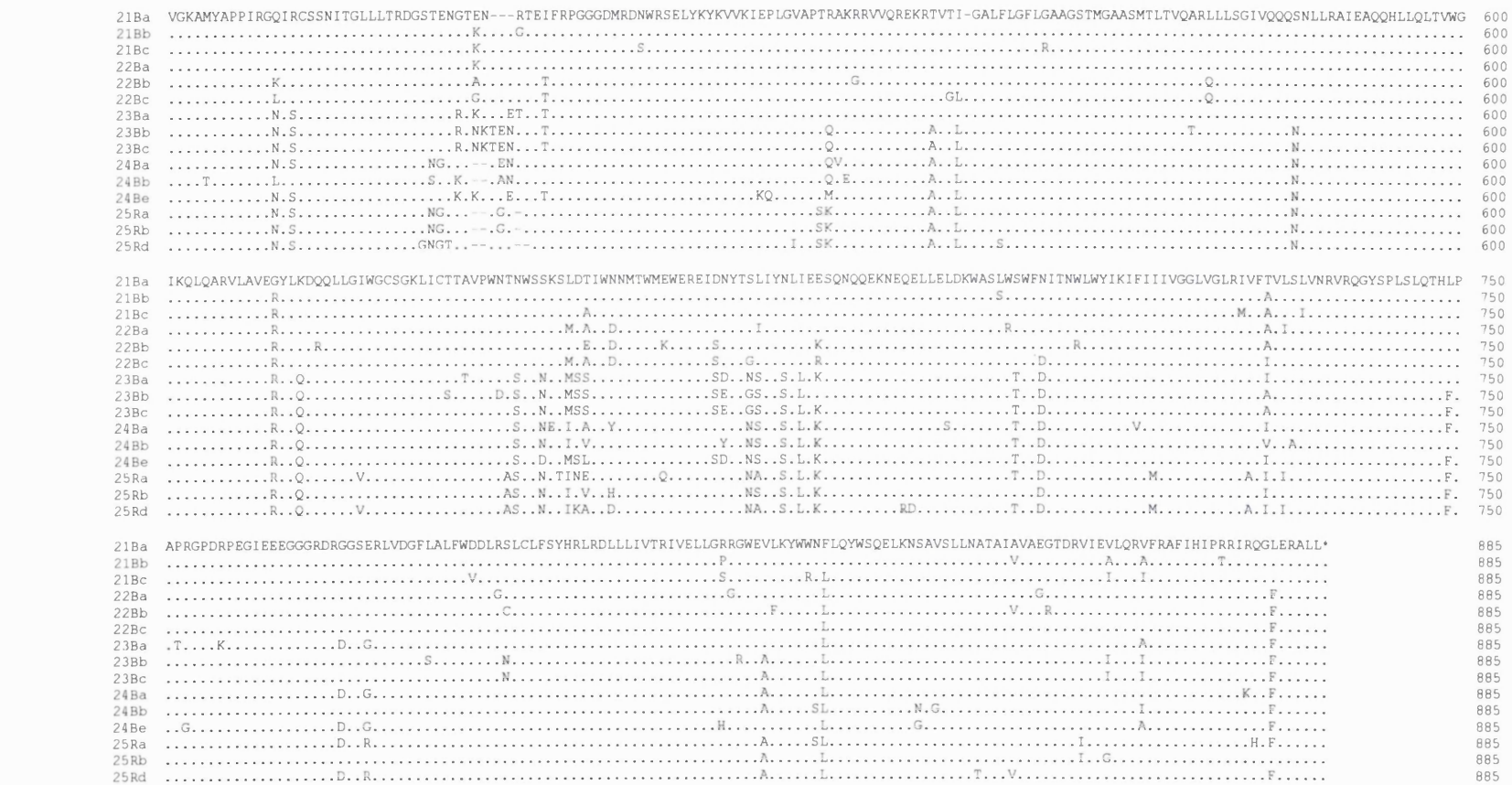
Identity with the 16Ba sequence is indicated by (.) and gaps introduced to improve the alignment are indicated by (-).

A3.3. Continued. Identity with the 16Ba sequence is indicated by (.) and gaps introduced to improve the alignment are indicated by (-).

A3.4 Amino Acid Sequences of HIV-1 *env*-gene Clones Derived From Patient 048



Identity with the 21Ba sequence is indicated by (.) and gaps introduced to improve the alignment are indicated by (-).



A3.4. Continued. Identity with the 21Ba sequence is indicated by (.) and gaps introduced to improve the alignment are indicated by (-).

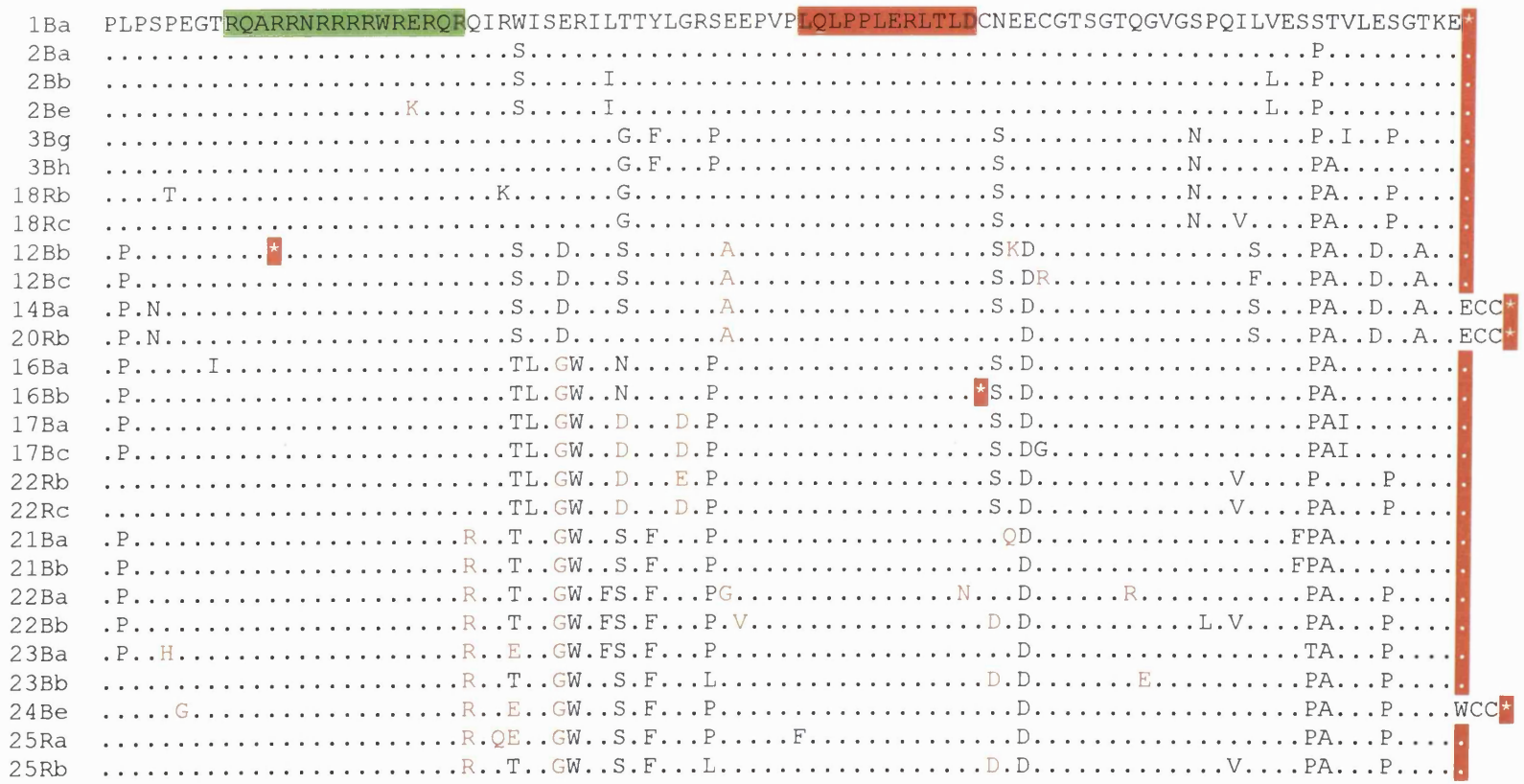
A3.5 Regulatory Protein Amino Acid Sequences

A3.5.1 Tat Second Exon Sequences of Clones Derived From LTNP-Switcher Patients.

```
 1Ba_ PASQPRGDPTGPKESKKKMERETETDPVD*-   30
 2Ba_ ............................      31
 2Bb_ ............................Q*    31
 2Be_ ....................K.........    31
 3Bg_ ..................V...........    31
 3Bh_ ..................V...........    31
18Rb_ .T..H.............V.......Q..Q*   31
18Rc_ ..................V..........Q*   31
12Bb_ ...........L......V...........    31
12Bc_ ...........Q......V...........    31
14Ba_ ..................V...........    31
20Rb_ ..................V...........    31
16Ba_ .P....R...........V........DA..   31
16Bb_ .P....R...........V........DA..   31
17Ba_ .P..S.............V........DA..   31
17Bc_ .S..S..N..........V........DA..   31
22Rb_ .P..S..N..........V........DA..   31
22Rc_ .P..S..N..........V........DA..   31
21Ba_ .T................V.G...A..N...   31
21Bb_ .T................V.G...A..N...   31
22Ba_ .T................V.G...A..N...   31
22Bb_ .T................V.G...A..N...   31
23Ba_ .T........S.......V.G......G...   31
23Bb_ .T................V.G...A..N...   31
24Be_ .T................V.G......G...   31
25Ra_ .T................V.G......G...   31
25Rb_ .T................V.G...A..N...   31
```

Non-conservative charged amino acid substitutions are indicated in red. Identity with the 1Ba_ sequence is indicated (.), as are the positions of the termination codons (*).

A3.5.2 Rev Second Exon Sequences of Clones Derived From LTNP-Switcher Patients.



The RNA binding/nuclear localisation domain and the activation/nuclear export domains are indicated. Non-conservative charged amino acid substitutions are shown in red. Identity with the 1Ba_ sequence is indicated (.), as are the positions of the termination codons (*).

A3.5.3 Comparison of 012:12Bb and 028:16Bb *env*-Gene Sequences Between pQ7.*env* and pC2.*env* Clones

The fidelity of all *env*-gene sequences were assessed following transfer from pQ7 to pC2. Only 16Bb exhibited alterations during the cloning process. Sequences shown here are for those two clones exhibiting premature truncations in the *rev* second exon. Identity with the pQ7.12Bb DNA sequence is indicated by (.) and gaps introduced to improve the alignment are indicated by (-). Transversions are highlighted.

A3.5.4 Comparison of 012:12Bb and 028:16Bb Env Sequences Between pQ7.*env* and pC2.*env* Clones

```
12Bb_pQ7   394  GSININDTG- --ENITLPCR IKQIINLWQE VGKAMYAPPI RGQISCSSNI TGLLLTRDGG NNNETNRTET FRPGGGNMRD  470
12Bb_pC2   394  .......... .......... .......... .......... .......... .......... .......... ..........  470
16Bb_pQ7   400  WNDTGAGNCT NN.T...... ......M... .......... S...R..... .......... ...S.-EN.. ......D...  478
16Bb_pC2   400  WNDTGAGNCT NN.T...... ......M... .......... S...R..... .......... ...S.-EN.. ......D...  478

12Bb_pQ7   471  NWRSELYKYK VVKIEPLGVA PTRAKRRVVQ KEKRAVTLGA MFLGFLGAAG STMGAASMTL TVQARLLLSG IVQQQNNLLR  550
12Bb_pC2   471  .......... .......... .......... .......... .......... .......... .......... ..........  550
16Bb_pQ7   479  .......... .........P ....R..... R.....GI.. V......... .......... .......... ..........  558
16Bb_pC2   479  .......... .......... ....R..... R.....GI.. V......... .......... .......... ..........  558

12Bb_pQ7   551  AIEAQQHLLQ LTVWGIKQLQ ARVLAVERYL KDQQLLGIWG CSGKLICTTA VPWNTSWSNK SYSQIWDNMT WMEWEREIDN  630
12Bb_pC2   551  .......... .......... .......... .......... .......... .......... .......... ..........  630
16Bb_pQ7   559  .......... .......... .....L.... .......... .........N ....A..... .QD...G... .........N.  638
16Bb_pC2   559  .......... .......... .....L.... .......... .........N ....A..... .QD...G... .........N.  638

12Bb_pQ7   631  YTNLIYTLIE KSQNQQEKNE QELLELDKWA NLWNWFDISN WLWYIKIFIM IVGGLVGLRI ISAVFSIATR VRQGYSPLSF  710
12Bb_pC2   631  .......... .......... .......... .......... .......... .......... .......... ..........  710
16Bb_pQ7   639  ..D...N... E......R.. L......... S.......TK .......... .......... VFT.L..VNK ..........  718
16Bb_pC2   639  ..D...N... E......R.. L......... S.......TK .......... .......... VFT.L..VNK ..........  718

12Bb_pQ7   711  QTRLPAPRGP DRPEGIEEEG GERDRDRSGR LVTGFLALIW DDLRSLCLFS YHRLRDLLLI AARTVELLGR RGWEALKYLW  790
12Bb_pC2   711  .......... .......... .......... .......... .......... .......... .......... ..........  790
16Bb_pQ7   719  ..P....K.. .......... .......... ..D...T... V......... .......... E......... ........W.  798
16Bb_pC2   719  ..P....K.. .......... .......... ..D...T... V......... .......... V......... ........W.  798

12Bb_pQ7   791  NLLQYWIQEL RNSAVSLFNA TAIAVAEGTD WVIEVIQRAF RAFIHIPTRV RQGLERALQ*                          850
12Bb_pC2   791  .......... .......... .......... .......... .......... ..........                          850
16Bb_pQ7   799  S.....S... K......I.T .......... R....VL.VG ..IL.....I ........L.                          858
16Bb_pC2   799  S.....S... K......I.T .......... R....VL.VG ..IL.....I ........L.                          858
```

Identity with the pQ7.12Bb sequence is indicated by (.), gaps introduced to improve the alignment are indicated by (-) as is the termination position (*). Changes between the pQ7.*env* and pC2.*env* clones are highlighted.

A3.5.5 Comparison of 012:12Bb and 028:16Bb Rev Second Exon Sequences Between pQ7.*env* and pC2.*env* Clones

```
12Bb_pQ7   PPPSPEGT RQA*RNRRRR WRERQRQIRS ISDRILSTYL GRSAEPVPLQ LPPLERLTLD CSKDCGTSGT QGVGSPQISV ESPAVLDSGA KE*
12Bb_pC2   ........ ...*...... .......... .......... .......... .......... .......... .......... .......... ..*
16Bb_pQ7   ........ ...R...... .........T L.GW..N... ..PE...... .......... *.E....... ........L. ......E..T ..*
16Bb_pC2   ........ ...R...... .........T L.GW..N... ..PE...... .......... ..E....... ........L. ......E..T ..*
```

Identity with the pQ7.12Bb sequence is indicated by (.), and termination positions are indicated by (*).

A3.5.6 Comparison of 012:12Bb and 028:16Bb Tat Second Exon Sequences Between pQ7.*env* and pC2.*env* Clones

```
12Bb_pQ7   PASQPRGDPT GLKESKKKVE RETETDPVDQ*
12Bb_pC2   .......... .......... .........*
16Bb_pQ7   .P....R... .........V. .......DA*
16Bb_pC2   .P....R... .........V. .......DA*
```

Identity with the pQ7.12Bb sequence is indicated by (.), and termination positions by (*)

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