In Vitro Studies of SIV *nef* Function

Thesis

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Shamim Akhtar Karim

In Vitro Studies of SIV *nef* Function

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Abbreviations

A_{600}	Absorbance at 600 nm
AD	activation domain
AIDS	acquired immundeficiency syndrome
Anti-HRP	anti-horse radish peroxidase
bp	base pair(s)
β-gal	β-galactosidase
BD	binding domain
BLV	bovine leukaemia virus
C8	SIVmacC8 virus
CCPs	clathrin coated pits
DNA	deoxyribonucleic acid
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immunosorbant assay
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
Facscan	fluorescence activated cell scanner
HIV	human immunodeficiency virus
HTLV	human T cell lymphotrophic virus
IPTG	isopropyl β-D-thiogalactoside
J5	SIVmacJ5 virus
Kb	kilo base(s)
KDa	kilo Daltons

LTR	long terminal repeat
LB	Luria-Bertram broth
Lbamp	Luria-Bertram broth containing 0.1 mg/ml ampicillin
Mbq	mega becqueral
mRNA	messenger RNA
mA	milli amps
MAb	monoclonal antibody
MW	molecular weight
ONPG	O-Nitrophenyl β-D-galactopyranoside
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
p/i	post infection
RMM	relative molecular mass
RNA	ribonucleic acid
SIV	simian immunodeficiency virus
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf9 cells	Spodoptera frugiperda (fall armyworm) cells
TMB	3, 3', 5, 5' tetramethylbenzidine
Triton X-100	t-octylphenoxypolyethoxyethanol
Tween-20	polyoxyethylene-sorbitan monolaurate
TGN	trans golgi network
TCA	Trichloroacetic acid
TAE	Tris-acetic acid-EDTA buffer

TBE	Tris- boric acid-EDTA buffer
TE	Tris-EDTA buffer
UV	ultra violet
V	volts
-WHAUL	-tryptophan, -histidine, -adenine, -uracil, -leucine media or agar
x-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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'It is desire and belief, not just ability, that determines success'

Abstract

There is strong evidence that a functional Nef protein of human and simian immunodeficiency viruses is a critical factor in contributing to viral pathogenesis. A variety of properties have been ascribed to Nef that may contribute towards these effects. However, the exact mechanisms by which Nef functions remain unclear.

A number of clones of SIV *nef* have been described which were generated after spontaneous evolution both *in vivo* and *in vitro* based upon changes relative to the wild type SIVmacJ5 virus (Rud *et al.*, 1994, Arnold *et al.*, 1999, N. Almond, personal communication). Furthermore, additional clones exist which were based upon the repair mutants reported by Whatmore *et al.* (1995) after infection of macaques with the attenuated SIVmacC8 virus (Rud *et al.*, 1994).

The aims of this study were two-fold. First, to utilise a panel of SIV Nef specific monoclonal antibodies (Arnold *et al.*, 1999) to elucidate structural differences between wild type and attenuated SIV Nef clones and further information on the epitope recognised by the conformational dependant monoclonal antibody, KK70 (Arnold *et al.*, 1999). Second, by utilising yeast-2-hybrid technology to investigate interactions of the SIV Nef clones with selected cellular components and relate these findings to the manner in which the virus behaves *in vivo*.

The results indicate that the alpha helical region around which the attenuation in SIVmacC8 lies is a key component of the epitope recognised by KK70 as well as being a key region for interaction of cellular components with SIV Nef. The repair mutants reported by Whatmore *et al.* (1995) did not regain the epitope recognised by KK70 as the amino acids evolved to a near wild type sequence. This indicates that it may be an overall structural effect that these mutations are having which is affecting

antigenicity. Interestingly, one of the repair mutants (J5(EIYL)) did regain the ability to interact with the cellular components. This was lost when this repair was presented in conjunction with the R191E mutation (J5(EIYL+R>E)). Interestingly, the R191E mutation alone (J5(R>E)) caused non-specific trans-activation of the GAL4-AD. Furthermore, GX2, which harbours a 66 nucleotide deletion at the 5' end of Nef (disrupting the binding of KK75, a monoclonal antibody that recognises a linear epitope) also caused non-specific trans-activation of the GAL4-AD. These results suggest that the mutations in GX2 and J5(R>E) may have a significant effect on the overall structure of SIV Nef.

This study describes the use of a novel technique for the fine mapping of novel antigenic epitopes using monoclonal antibodies. The structural and yeast-2-hybrid data together highlight important observations of SIV Nef which can be related to structure and function *in vivo*, as well as being used as a basis for future *in vivo* studies.

1. Introduction

1. Introduction

1.1. History of Retroviruses

'It was a radical proposal that ran counter to the generally accepted central dogma of molecular biology; that there is a unidirectional flow of genetic information from DNA to RNA to protein. In this setting, Temins' hypothesis that RSV replicates by transfer of information from RNA to DNA not only failed to win acceptance from the scientific community but was met with general derision'.

Geoffrey M. Cooper
The DNA Provirus: Howard Temins' Scientific legacy

Retroviruses were discovered in 1908, when two scientists Vilhelm Ellermann and Oluf Bang demonstrated that chicken leukosis, a form of leukemia and of lymphoma, was caused by a virus (Ellermann & Bang, 1908). Shortly afterwards, in 1911, cell free transmission of a sarcoma to chickens was reported by Peyton Rous (1911). The viruses causing these chicken leukoses have been classified as avian leukosis viruses (ALV), whilst the virus observed by Peyton Rous is now known as Rous sarcoma virus (RSV). These viruses make up the avian sarcoma/leukosis virus genus (ASLV) within the retrovirus family.

The discovery of the mode of replication characteristic of retroviruses dramatically altered the general concept of the widely accepted 'Central Dogma'; that is, the unidirectional flow of genetic information from DNA to RNA to protein, as described in the passage above. The retrovirus life cycle involves the copying of RNA into DNA and is mediated by a virus encoded polymerase enzyme. This process, which

results in a reversal of the normal flow of genetic information, is termed reverse transcription, hence the term 'retrovirus'.

Retroviruses consist of a large and highly diverse family of enveloped RNA viruses. Retroviral virions are 80-100 nm in diameter, with an outer lipid envelope incorporating the viral glycoprotein. The viral genome consists of 7-12 kb, linear, single stranded, nonsegmented RNA, of a positive polarity and diploid in nature. Retroviruses are divided into two categories, simple and complex, which are distinguished by the organisation of their genomes. Simple retroviral genomes consist of three or four major coding domains, *gag*, *pol*, *env* and *pro*, which code only for the viral structural proteins (Figure 1.1). Complex retroviral genomes code for a variety of accessory (non-structural proteins) in addition to the structural viral proteins. The details of the genes encoding for non-structural proteins will be discussed later.

1.2. Genomic Organisation & Virion Structure

1.2.1. *gag*

The *gag* gene was named because it was recognised that the proteins it encodes acted as group specific *a*ntigens (the Gag proteins). Proteolytic cleavage of the Gag polyprotein produces the matrix (MA), capsid (CA) and nucleocapsid (NC) viral proteins. The minimum Gag protein unit reads MA-CA-NC (Figure 1.2), although additional segments can exist between these proteins which can be cleaved into smaller proteins or peptides, the precise functions of which remain unknown (for review see Wills & Craven, 1991).

The amino terminal of Gag consists of the *m*embrane-*a*ssociated, or *ma*trix protein (MA). The presence of a 14-carbon fatty acid myristoyl group at the amino terminus of Gag suggests that Gag may localise to the plasma membrane. Mutation studies

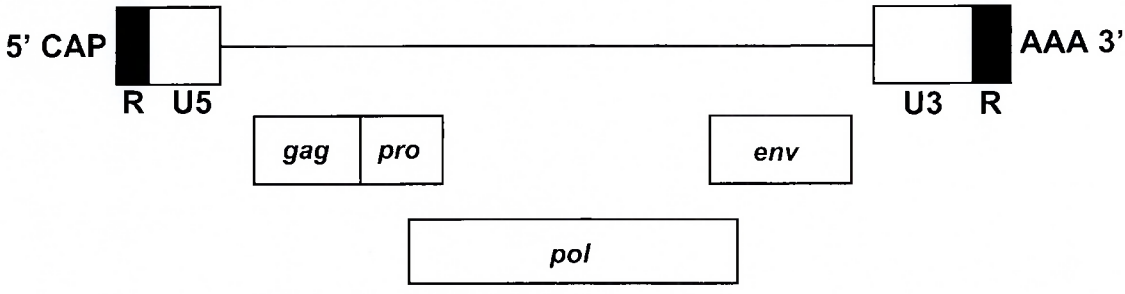


Figure 1.1. A simple retroviral genome. The genetic map of ALV contains four major coding regions *gag, pro, pol* and *env*. Different reading frames are indicated by vertical displacement of the coding region. The *pro* gene is encoded in the *gag* reading frame. The terminal noncoding sequences include two direct repeats (R), a U5 (5' unique) and a U3 (3' unique) sequence.
Adapted from: Retroviruses, Cold Spring Harbor Laboratory Press, 1997.

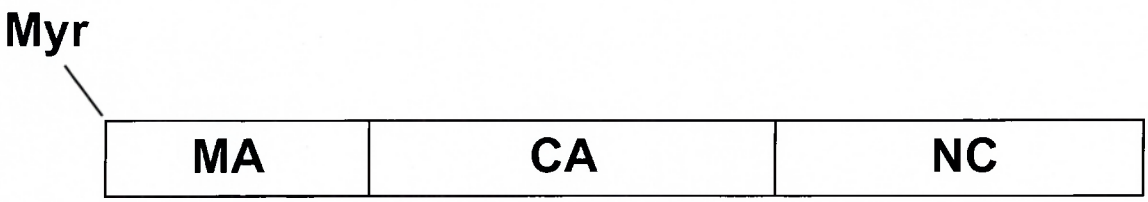


Figure 1.2. The organisation of a 'minimum' Gag protein unit. The vertical solid lines and the cleavage sites for viral protease. The sequences representing the mature proteins MA, CA and NC are shown. A myritylation motif (Myr) at the amino terminus is shown.
Adapted from: Retroviruses, Cold Spring Harbor Laboratory Press, 1997

have indicated that myristoylation is essential for retroviral assembly and may allow proteins to associate with plasma membrane. (Rein *et al.,* 1986). However, the Gag proteins of some retroviruses, including ASLV, are not myristoylated. Therefore the presence of a myristoyl group may not be the only mechanism for membrane association. ASLV Gag is acetylated at the amino terminus, and although the exact consequence of this modification is unknown it may contribute to membrane association (Palmiter *et al.,* 1978).

The *ca*psid protein (CA) forms a protective shell (capsid) surrounding the ribonucleoprotein-genomic RNA containing complex. It is possible that other Gag proteins (MA and NC) may also be contained in the capsid. The capsid, together with the components it encloses, is known as the 'core'.

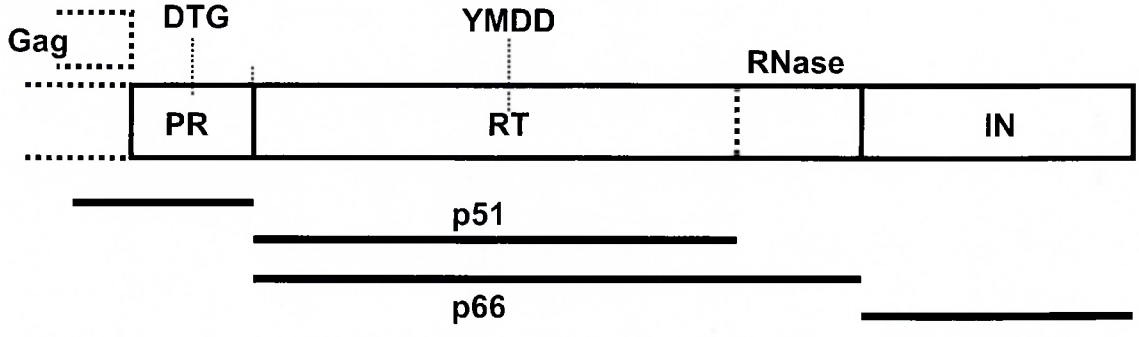
The *n*ucleo*c*apsid protein (NC) is a small basic protein which contains sequences required for assembly or budding of the virion. It is associated with the genomic RNA (Davis & Rueckert 1972, Fleissner & Tress 1973).

1.2.2. *pro & pol*

Infectious retroviruses carry three enzymes; reverse transcriptase (RT), integrase (IN) and protease (PR). The *pro* (*pro*tease) gene encodes PR, which is responsible for the cleavage of the *gag* and *pol* polyproteins and sometimes part of *env* as well. The *pol* (*pol*ymerase) gene encodes IN and RT proteins, which are essential for the integration of the virus genome into the host cell DNA early during infection and for the process of reverse transcription, which will be discussed later (section 1.3) (Figure 1.3).

Figure 1.3. The organisation of the Pro and Pol proteins of HIV-1. Shown are the mature Pro and Pol proteins and their precursors, Sequences for the mature proteins PR, RT and IN are shown. The solid vertical lines show major cleavage sites and the thick horizontal bars show mature proteins. YMDD and DTG indicate conserved active site residues in PR and RT. The location of the RNase H domain of RT is indicated.

Adapted from: Retroviruses, Cold Spring Harbor Laboratory Press, 1997.


Gag
DTG
YMDD
RNase
PR
RT
IN
p51
p66

1.2.3. *env*

The viral membrane of retroviral virions is studded with glycoproteins, encoded by the *env* gene, (*env*elope or Env proteins). These are involved are involved in attachment and subsequent entry of virus into host cells. All enveloped viruses have glycoproteins protruding from the viral membrane which bind to specific receptors. Retroviruses express two envelope glycoproteins, derived from a common precursor polypeptide. These are the *su*rface (SU) protein, responsible for recognition of specific cell-surface receptors and the *trans*membrane (TM) protein which anchors the SU-TM complex to the virion envelope and contains domains responsible for fusion of viral and cellular membranes.

Env polyproteins are synthesised from a spliced version of the genomic RNA from which *gag*-, *pro*- and *pol*-coding sequences have been removed. The Env polypeptide becomes associated with the endoplasmic reticulum (ER) by binding to a signal recognition particle via its amino terminal leader segment (Figure 1.4). Further translation results in the polypeptide extruding into the lumen of the ER, with the carboxy-terminal tail of Env remaining in the cytoplasmic compartment. The leader sequence is cleaved off and Env is transported through the Golgi apparatus to the plasma membrane by vesicular traffic. In the Golgi, Env is cleaved further to yield the mature TM and SU proteins that are present in virions. Cleavage activates the fusion potential of the SU-TM complex, required for viral entry into the host cell. The SU-TM proteins remain attached to each other by noncovalent interactions or in some cases by disulphide bonds. Both SU and TM proteins are incorporated into the budding viral particle. The Env protein determines the type of cell a retrovirus can infect as it is responsible for recognition of specific cell surface proteins (the viral receptors) (Coffin *et al.*, 1997).

Figure 1.4. The organisation of the Env protein of HIV-1. Shown are the sequences representing the mature proteins SU and TM. Vertical lines, separating the leader peptide (L) and SU and separating SU and TM, mark cleavage sites for cellular proteases. The hydrophobic fusion domain of TM is highlighted in yellow and the membrane anchor is coloured orange. The triangles indicate sites of predicted N-linked glycosylation addition.
Adapted from: Retroviruses, Cold Spring Harbor Laboratory Press, 1997.

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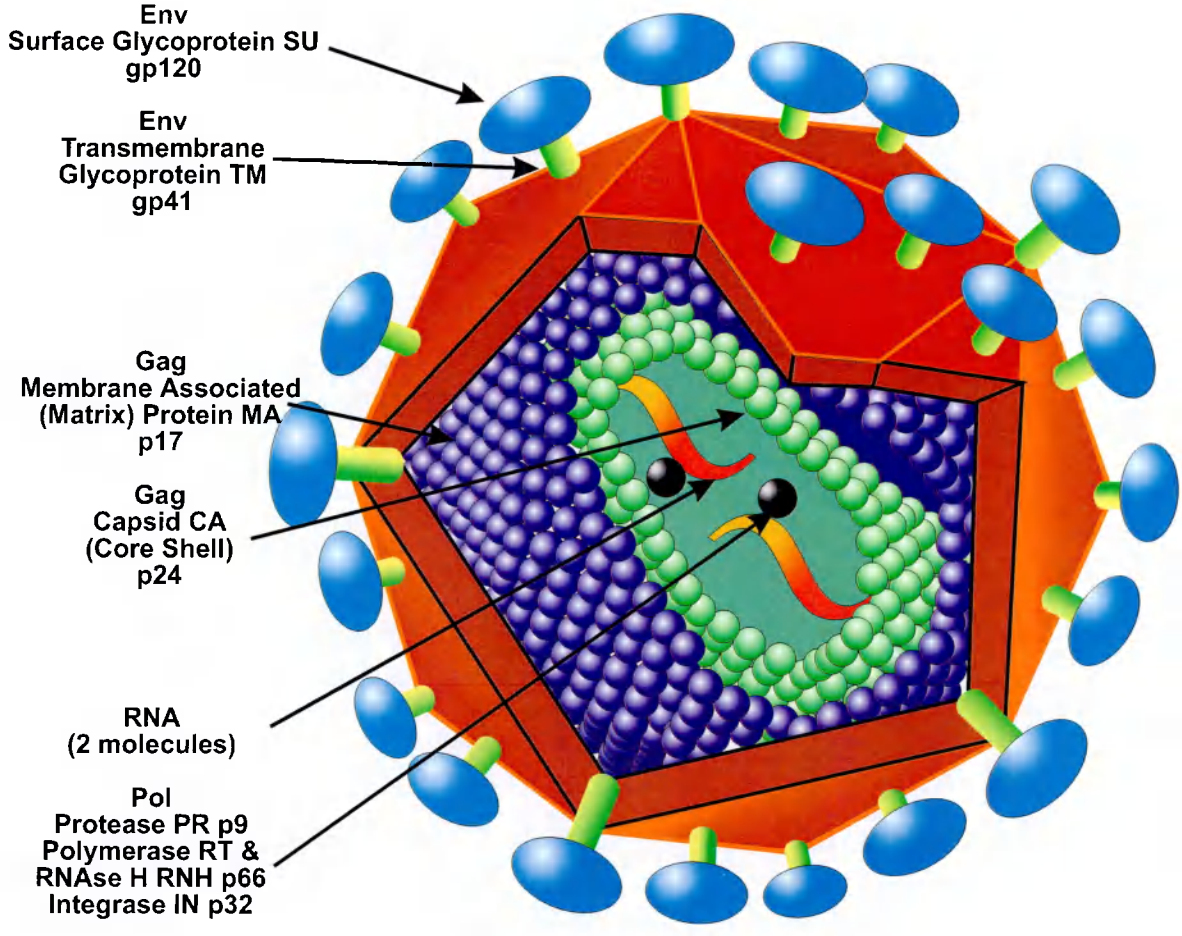
The basic retrovirus virion structure and location of the proteins is shown in Figure 1.5. Complex retroviruses encode additional regulatory nonvirion proteins derived from multiply spliced messenger RNA, which will be described later with reference to Lentiviruses.

1.3. Viral Replication Cycle

The retroviral life cycle is unique and distinguishes this family of viruses from others. The unique stages are the transcription of the virion RNA into linear double-stranded DNA and the subsequent integration of this DNA into the genome of the cell. Retroviral DNA synthesis is dependent upon the two distinct enzymatic activities of the reverse transcriptase (RT) enzyme (a DNA polymerase that can use RNA or DNA as a template) and of ribonuclease H (RNase H), a nuclease that is specific for the RNA strand of RNA: DNA duplexes.

Initial attachment of the virus occurs to the target cell through the interaction of the SU protein of Env with a specific cell-surface receptor protein (Figure 1.6a).
Upon binding of the SU protein to its receptor, the virus envelope and host cell membrane fuse together allowing the virion core to be internalised into the cytoplasm of the cell. Reverse transcription of the RNA genome into double stranded DNA occurs in the cytoplasm (Figure 1.6b). Sequences essential for the reverse transcription process are present at the ends of the retroviral genomic RNA. These include direct repeats (R) which lie at the 5' and 3' ends of the RNA, and the U5 and U3 sequences (which lie at the 5' and 3' ends respectively) and which are internal to the R sequences (Figure 1.7). The process of reverse transcription involves duplication of the U5 and U3 region, therefore generating a DNA product that is

Figure 1.5. A schematic cross section through a retroviral particle. The viral envelope is formed by a cell derived lipid bilayer into which proteins encoded by the *env* region of the viral genome are inserted. These consist of the transmembrane (TM) and the surface (SU) components linked together by disulphide bonds. Internal nonglycosylated structural proteins are encoded by the *gag* region of the viral genome. They are, matrix protein (MA), capsid protein (CA) and nucleocapsid protein (NC). The major products of the *pol*-coding region are reverse transcriptase (RT) and integrase (IN). The protease (PR) is derived from the *pro* gene between *gag* and *pol*. Graphics: Andrew Davies, NIBSC


Env
Surface Glycoprotein SU
gp120
Env
Transmembrane
Glycoprotein TM
gp41
Gag
Membrane Associated
(Matrix) Protein MA
p17
Gag
Capsid CA
(Core Shell)
p24
RNA
(2 molecules)
Pol
Protease PR p9
Polymerase RT &
RNAse H RNH p66
Integrase IN p32

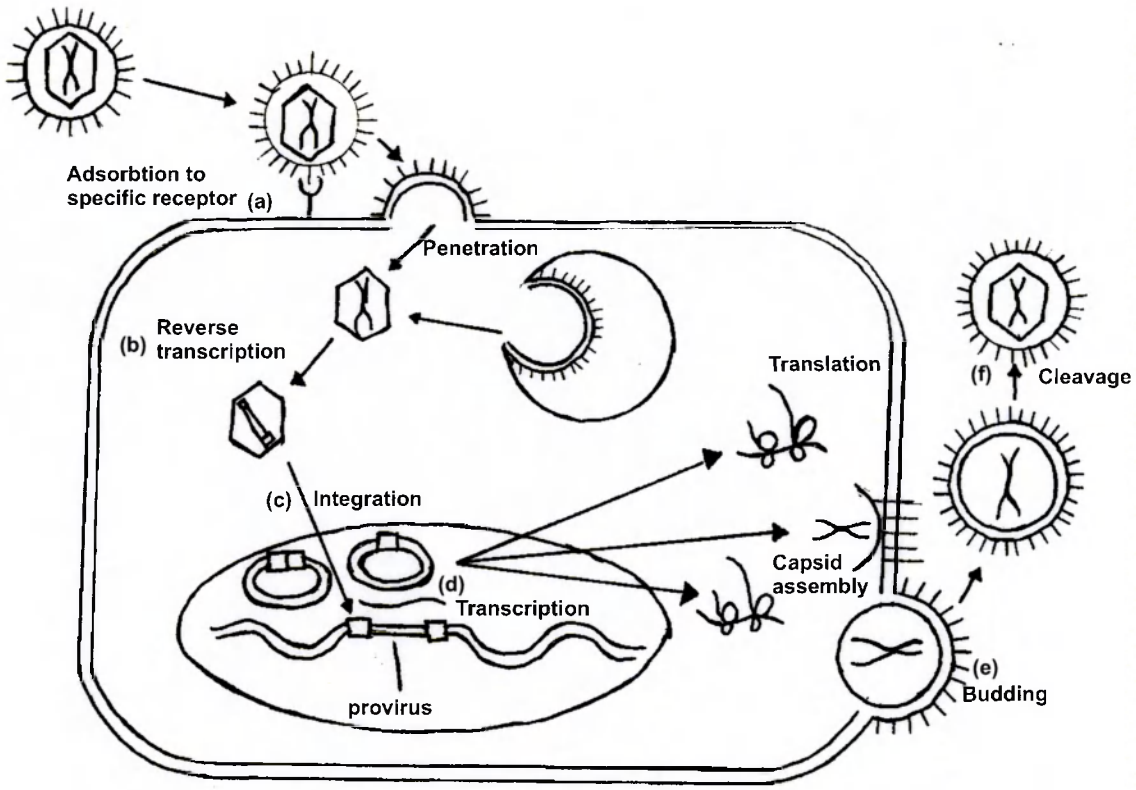


Figure 1.6. A schematic representation of the retroviral life cycle. In brief, the virion attaches to a specific cell surface receptor (a) and the virion core penetrates into the cell after fusion of the viral and cell plasma membranes. Reverse transcription within the core structure occurs within the cytoplasm, copying the genome RNA into DNA (b). The viral DNA locates to the nucleus, where it integrates into the cellular DNA to form the provirus (c). The viral RNA is synthesised by cellular RNA polymerase II using the integrated provirus as a template (d). The transcripts are processed into genomic and mRNAs and virion proteins are synthesised from the mRNAs in he cytoplasm. Virions are assembled and bud (e) at the plasma membrane and the capsid proteins are cleaved (f).
Adapted from: Virology, Volume 2, 3rd edition. Fields, Knipe & Howley, 1996.

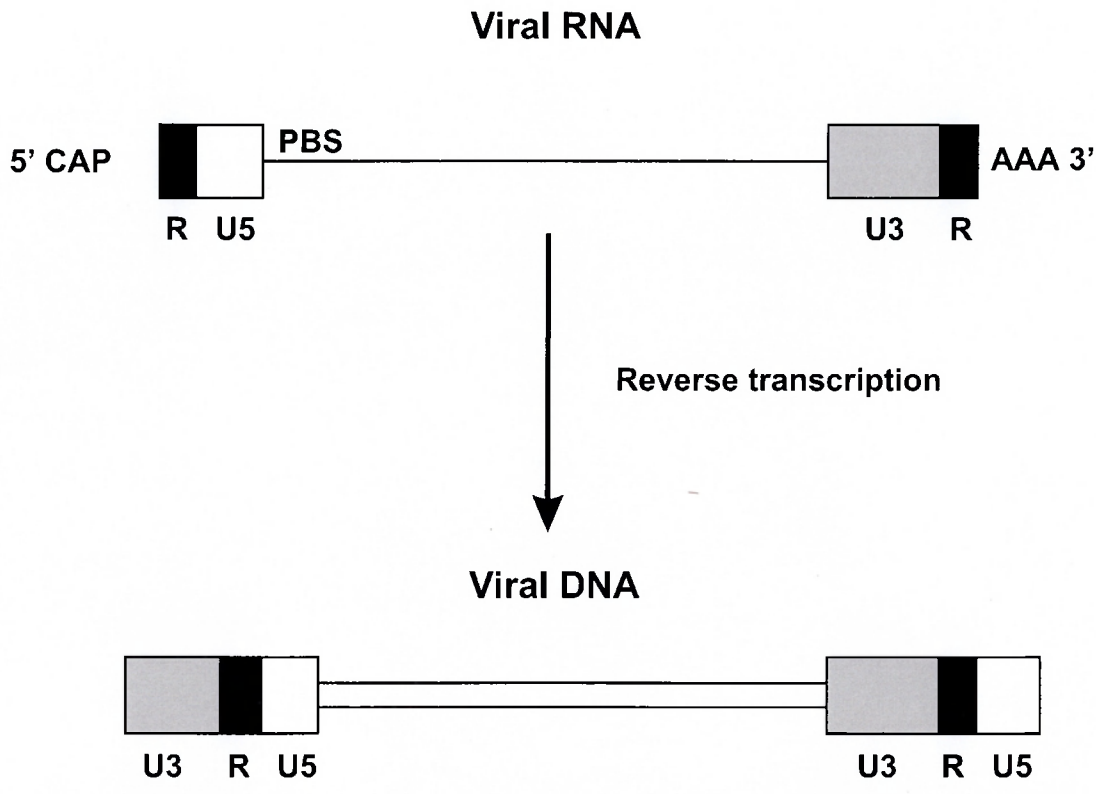
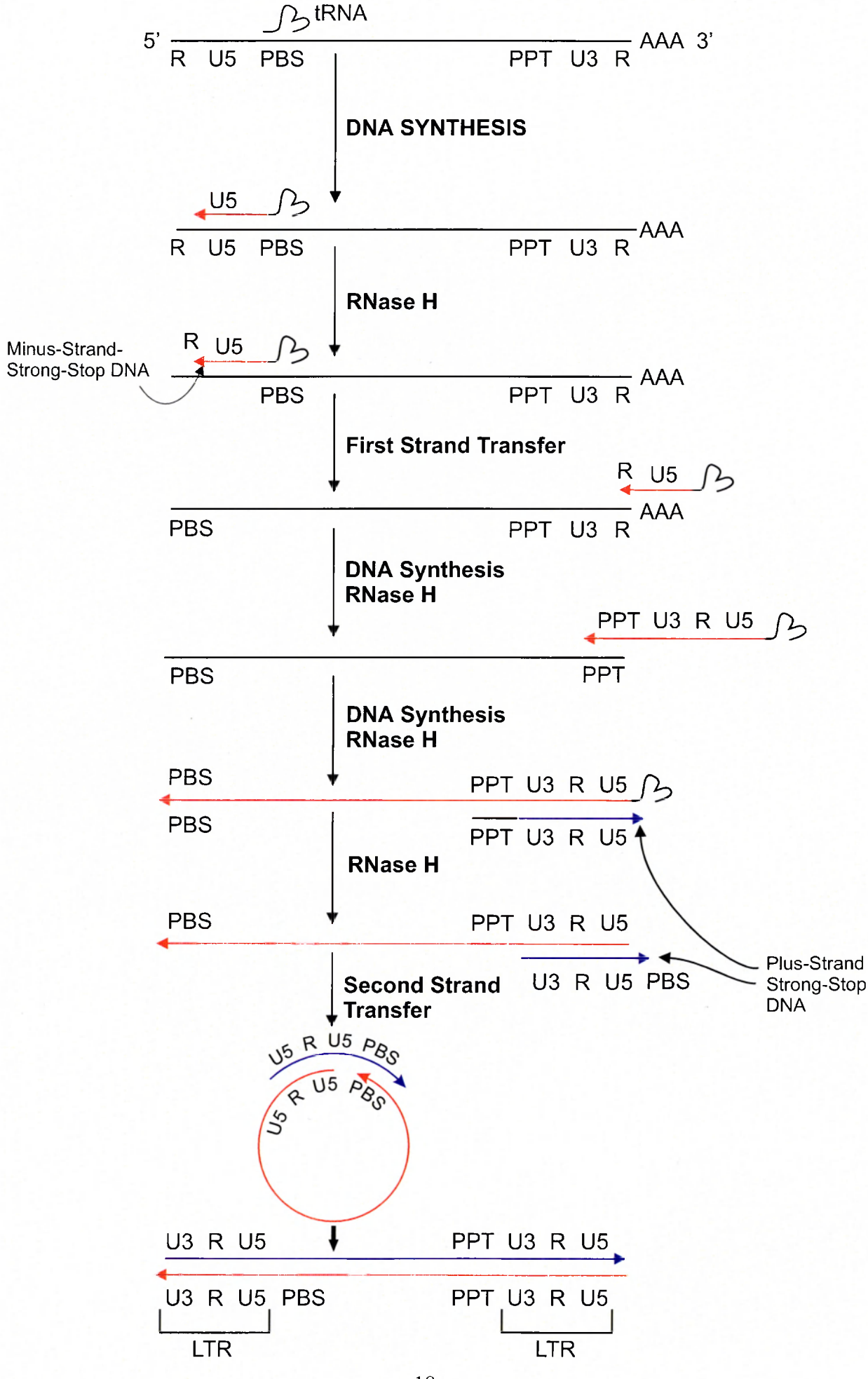


Figure 1.7. Reverse transcription of the viral RNA genome, generating a linear DNA duplex. Reverse transcription generates duplications of the U5 and U3 regions, resulting in a DNA product that is longer than the RNA at both ends, which is the origin of the two long terminal repeats (LTRs), each consisting of the U3/R/U5 regions.
Adapted from: Retroviruses, Cold Spring Harbor Laboratory Press, 1997.

longer than the RNA at both ends. This is the origin of the two long terminal repeats (LTRs) which are characteristic of the DNA form of the viral genome. The stages of reverse transcription can be summarised as follows (Figure 1.8).

1. Minus strand synthesis is initiated using the 3' end of a specific host derived transfer RNA which anneals to the primer binding site (PBS) in genomic RNA, as a primer. Synthesis of the minus strand DNA continues until the 5' end of the genomic RNA is reached, generating a DNA intermediate known as minus-strand strong-stop DNA (-sssDNA). This –sssDNA is between 100-150 bp long.
2. RNase-H then degrades the RNA strand of the RNA:-sssDNA duplex.
3. The first strand transfer then occurs, resulting in the –sssDNA annealing to the 3' end of a viral genomic RNA. This process is mediated by the R sequences present at the 5' and 3' ends of the RNA genome. The 3' end of the –sssDNA was copied from the R sequences at the 5' end of the viral genome and hence contains sequences complementary to R. After removal of the RNA template the –sssDNA can anneal to the R sequences at the 3' end of the RNA genome.
4. Minus strand DNA synthesis resumes, accompanied by Rnase H digestion of the template strand.
5. The RNA genome contains a short polypurine tract (PPT) which is relatively resistant to RNase H degradation.
6. An RNA segment derived from the PPT primes plus-strand DNA synthesis. Plus strand synthesis stops after a portion of the primer tRNA is reverse transcribed, yielding a DNA known as plus-strand strong-stop DNA (+sssDNA).
7. RNase H removes the primer tRNA, exposing sequences in +sssDNA that are complementary to sequences at or near the 3' end of plus strand DNA.

Figure 1.8. The process of reverse transcription. For details, see text.
Adapted from: Retroviruses, Cold Spring Harbor Laboratory Press, 1997.


tRNA
5'
AAA 3'
R U5 PBS
PPT U3 R
DNA SYNTHESIS
U5
AAA
RNase H
Minus-Strand-
Strong-Stop DNA
First Strand Transfer
DNA Synthesis
RNase H
PPT U3 R U5
DNA Synthesis
RNase H
RNase H
Plus-Strand
Strong-Stop
DNA
Second Strand
Transfer
U5 R U5 PBS
LTR
LTR

8. The complementary PBS segments in the +sssDNA and minus-strand DNA anneal and result in the second strand transfer.
9. Plus- and minus-strand synthesis are completed, with plus and minus strands of DNA serving as a template for the other strand (Figure 1.8).

The double stranded DNA is transported into the nucleus and integrates into chromosomal DNA (Figure 1.6c). The integrated virus is now referred to as a provirus. The provirus is stable and all further replication occurs via the transcription of the provirus into RNA by cellular RNA Polymerase II (Figure 1.6d). This RNA is the source of both new genomes and the mRNA species. In all retroviruses, full length transcripts are packaged as new viral genomes or used as mRNA-coding for the *gag*, *pro* and *pol* genes. Upon transport to the cytoplasm, the spliced RNAs serve as mRNA for *env* and for regulatory genes. The translation products are assembled at the cell periphery into viral particles that are released from the cell by budding of the plasma membrane (Figure 1.6e). Following budding, the virion polyproteins are subjected to proteolytic cleavage by the viral protease and cellular proteases (Figure 1.6f).

1.4. Nature and Classification of Retroviruses

The retroviral genomes may be exogenous or endogenous and many retroviruses are involved in cancer induction. These features and taxonomic classification are described below.

Exogenous & Endogenous retroviruses

Exogenous retroviruses integrate into the genome of a host cell, forming a provirus, resulting in a permanent association between the cell and retrovirus. These are transmitted through infection.

The integration of a retrovirus into the chromosome of a germ cell allows the retrovirus to exist as a stable proviruses for multiple generations. Viral genomes that are transmitted by proviral DNA in eggs and sperm are known as endogenous retroviruses. Once a germline infection is established, it can found a multicopy family of proviruses by further gene duplication within the germline compartment. Endogenous retroviruses have been found in all vertebrates.

Oncogenic retroviruses

Cancer inducing retroviruses (oncogenic retroviruses) are prevalent in nature. They can be categorised into non-acute transforming retroviruses, acute transforming retroviruses and trans-acting transforming viruses.

The non-acute retroviruses are abundant in nature and have been isolated from many species, including humans. They cause a variety of cancers, of which leukaemias and lymphomas are the most common, and contain a full set of viral replicative and structural genes. Cancer induction occurs by insertional mutagenesis, whereby the viral DNA integrates at or near to host proto-oncogenes or close to genes involved in growth control. This results in the host proto-oncogenes becoming transcriptionally activated or deregulated. The structure of the proto-oncogene product could also be altered as a result of viral DNA insertion. Alternatively, a tumour suppressor gene can be deactivated. All of these features contribute to genetic alterations that can affect growth control and result in oncogenesis.

Acute retroviruses cause polyclonal tumour growth within days. They transform normal cells *in vitro* and induce rapidly growing tumours *in vivo*, a property which is associated with the viral oncogenes present in their genomes. Such viral oncogenes are derived from host proto-oncogenes through recombination. During the recombination process many of the viral oncogenes acquire mutations and deletions that enhance the oncogenicity of the proto-oncogene.

Trans-acting retroviruses, such as HTLV and BLV, induce cancers by a different mechanism to that of the acute and nonacute retroviruses. HTLV encodes additional genes to those in the genome of acute and non-acute retroviruses. In addition to *gag*, *pol* and *env*, it has a unique pX region, which encodes for 6 polypeptides, of which Tax and Rex are the most understood. It is the Tax protein that is involved in the immortalisation or transformation of T cells. This protein is a transactivator that enhances the transcription of the viral LTR and other cellular genes. Tax augments the expression of a variety of genes, including protooncogenes c-*jun*, c-*fos*, c-*myc*, c-*sis* and c-*rel*. Thus HTLV can produce a viral product that can activate proto-oncogenesis in *trans*.

Taxonomic Classification

Retroviruses are divided into seven genera defined by nucleotide sequence relationship and genome structure. These are the avian leukosis-sarcoma virus genus, the mammalian C-type virus genus, the B-type virus genus, the D-type virus genus, the HTLV-BLV genus, spumaviruses and lentiviruses. These are described in more detail below.

The Avian Leukosis-Sarcoma Virus (ALSV) Genus

The avian leukosis-sarcoma virus group consists of exogenous and closely related endogenous viruses of birds (Payne 1992). This group has C-type virions and simple genomes that encode only *gag*, *pol*, *env* and *pro* genes. Many isolates of exogenous viruses have acquired oncogenes, such as *src* in Rous Sarcoma Virus. They are divided into ten subgroups according to their receptor usage, denoted A through to J. Subgroups A-D are characteristic of exogenous viruses from chickens, subgroup E belongs to endogenous viruses of chickens and subgroups F and G are from endogenous viruses of pheasants. The virus from Hungarian partridge belongs to subgroup H (Hanafusa *et al.*, 1976), whilst subgroup I consists of Gambel's quail (Troesch & Vogt 1985). Subgroup J is the most recently discovered and has been isolated from chickens (Payne & Howes 1991a, Payne *et al.*, 1991b).

The Mammalian C-Type Virus Genus

This genus incorporates a large number of endogenous and exogenous viruses from a diverse group of mammals including rodents (Kozak & Ruscetti 1992), carnivores (Hardy 1993), primates, reptiles and some exogenous viruses of birds. No replicating human viruses of this group have been isolated.

The B-Type Virus Genus

The only infectious agent within this group is the mouse mammary tumour virus (MMTV) which has been isolated as both endogenous and exogenous viruses. These viruses contain *gag*, *pol* and *env* genes, as well as an additional coding region, *sag*, which encodes a superantigen activity. No oncogenic variants within this group have been described (Coffin *et al.*, 1997).

The D-Type Virus Genus

The D-type viruses group comprises both endogenous and exogenous isolates from primates, and includes the Mason-Pfizer virus. Recent isolates include a virus associated with an immunodeficiency syndrome in some captive monkey colonies (sAIDS) (Fine & Schochetman 1978, review). There is no evidence that this group can exhibit any oncogenic capacity.

The HTLV-BLV Genus

The human T-cell lymphotropic virus-bovine leukemia virus group (HTLV-BLV) includes exogenous viruses associated with B-cell lymphoma in cattle and T-cell lymphoma as well as some neurological diseases in humans (Clapham *et al.*, 1983, Ghysdael *et al.*, 1985). Viruses from this group have a complex genome structure and contain at least two additional genes, *tax* and *rex*, which encode non-structural proteins important for gene expression.

Spumavirus Genus

Spumaviruses were discovered contaminating cells cultured from healthy monkeys in 1954 (Enders & Peebles 1954, Rustigan *et al.*, 1955). They induce a characteristic foamy appearance (*spuma*, meaning foam in Latin) in the cytoplasm of cells cultured from a number of mammalian species, including monkeys, cattle, cats and humans (Flugel 1991, Loh 1993).

Lentivirus Genus

The discovery of lentiviruses can be dated back to 1904 (Valle & Carre 1904), with the description of equine infectious anaemia, although the actual classification of this

virus as a retrovirus within the lentivirus family occurred much later. In 1957, Visna, a neurological disease in sheep caused by a virus was described. This gave rise to the concept of slow viral infections: hence the current terminology *lenti*virus, which was derived from *lentus,* the latin for slow. Lentiviruses have subsequently been isolated from sheep, goats, horses, cattle, cats, monkeys and humans. The nonprimate lentiviral group consists of the original slow virus, visna/maedi virus (VMV), caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV), feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV). The Lentiviruses morphology is characterised by a cone-shaped or cylindrical nucleoid in the mature virion.

1.5. Human Immunodeficiency Retroviruses, HIV-1 and HIV-2

Human immunodeficiency virus-1 (HIV-1) and Human immunodeficiency virus-2 (HIV-2) are members of the Lentivirus genus. They constitute important human pathogens that cause immunodeficiency in their hosts. HIV-1 and HIV-2 cause a lifelong infection, exhibiting complex interactions within the host and inducing a slowly progressing and chronically degenerative disease. Infection with HIV can remain asymptomatic for years or can be characterised by an initial dramatic weight loss and 'flu-like symptoms or persistent diarrhoea. Progression of the viral infection (over years or decades) is characterised by an increase in susceptibility to opportunistic infections, a condition known as acquired immunodeficiency syndrome (AIDS). Globally it is estimated that approximately 34.3 million adults and children are infected with HIV (UNAIDS, 2000). The primary receptor for these viruses is the CD4 receptor, an integral component of the T cell receptor (TCR) complex on MHC-

class II restricted T-cells, and one of the characteristics of AIDS is a decrease in the number of T cells carrying this receptor.

HIV-1 has spread throughout the world, whilst HIV-2 is concentrated in West African countries, such as Guinea Bissau, Ivory Coast and Senegal, with cases identified in America and Europe. Isolates of each type exhibit a high level of variance in their *env* gene, in specific hypervariable sites. Based on *env* sequences HIV-1 can be classified into two main groups, group M ('main') which represents the majority of infections worldwide and group O ('outgroup') which is rare and has only been isolated in Cameroon, Gabon and France. Group M can be further divided into nine subtypes or clades, denoted A-I. HIV-2 can be divided into five subtypes (A to E).

The first case of human AIDS was recognised in 1981. Evidence from epidemiological studies implicated an infectious agent. In 1983, Barre-Sinoussi and colleagues (Barre-Sinousi *et al.,* 1983) described the isolation of a previously unknown human retrovirus, described as lymphadenopathy-associated virus (LAV), from an AIDS patient. This was supported by subsequent reports by Gallo (*et al.*, 1984) and Levy (*et al.,* 1984), of a virus isolated from AIDS patients, called human T-cell lymphotropic virus type III (HTLV-III) and AIDS-associated virus (ARV) respectively. These viruses were subsequently classified as members of the Lentivirus genus, further defined as the causative agents of AIDS and renamed human immunodeficiency virus (HIV). The transmission of HIV by direct sexual contact (homosexual or heterosexual), by blood or blood products and from an infected mother to infant, either intrapartum, perinatally, or through breast milk (Fauci & Lane 1991) has now been demonstrated by extensive epidemiological studies.

The first case of a simian immunodeficiency virus (SIV, initially described as simian T-cell lymphotropic virus type III (STLV-III)), (Daniel *et al.,* 1985, Letvin *et al.,*

1985) was observed in 1962. An outbreak of lymphomas, now associated with simian AIDS (sAIDS) was observed in captive rhesus macaques (*Macaca mulatta*) at the California Regional Primate Research Centre (CRPRC) in Davis, California (Mansfield *et al.*, 1995). SIV was subsequently observed to induce symptoms in all other Asian macaque species (cynomolgus and nemestrina macaques) similar to those of AIDS in humans. Within their natural hosts however, the various isolates of SIV (SIVagm (african green monkey), SIVsm (sooty mangabey), SIVmnd (mandrill), SIVsyk (sykes) and SIVcpz (chimpanzees)) do not cause disease. The viruses isolated from some of these captive animals (SIVmac (macaque), SIVcyn (cynomolgus) and SIVmne (nemestrina)) appear to be closely related to SIVsm, implicating a common origin for all (Mansfield *et al.*, 1995). The likelihood of cross transmission from the naturally infected sooty mangabeys to macaques is therefore high.

1.6. Receptor usage

Receptor usage varies amongst enveloped viruses. The HIV Env protein recognises and binds to CD4 present on the surface of macrophages and helper T lymphocytes (Dalgleish *et al.*, 1984). More recent studies have indicated that an additional coreceptor is required to mediate efficient entry into cells (Berger 1997). These 'fusion cofactors' are present on CD4+ target cells and determine the specificity of Env-mediated fusion. HIV exhibits a high mutation rate resulting in a high degree of variability in the *env* gene. One of the consequences of such mutations is that individual HIV-1 isolates, tested *in vitro*, have distinct cytotropisms for infection of different CD4+ human cell types. Some isolates replicate in continuous T cell lines, but only poorly in macrophages. Others infect macrophages more efficiently than T cell lines. This difference in tropism has led to isolates being designated T-cell tropic

(TCL-tropic) or macrophage tropic (M-tropic). Further analysis investigated the determinants responsible for cytotropism and it was concluded that the *env* gene (specifically a region of gp120 including the V3 loop) determined whether a virus would exhibit a TCL- or M-tropic phenotype. To explain why Env mediates fusion with only a limited number of CD4+ target cells, it was suggested that the target cell contained an essential cofactor required for Env-dependant fusion. A cofactor which fused to Env from the IIIB isolate was identified as a seven-transmembrane segment G-protein coupled receptor and named 'fusin', later renamed CXCR4 (Feng *et al.*, 1996). This was established to be an essential cofactor for TCL-tropic HIV-1 isolates. The identification of fusin lead to a focus on the G-protein coupled receptor superfamily in the search for a cofactor for the M-tropic isolates. Finally, CCR5 was identified as a fusion cofactor for M-tropic HIV-1 strains (Berger *et al.*, 1997.)

1.7. Genomic Organisation of Immunodeficiency Viruses

The Lentivirus genus has a complex genomic structure. The genomes of HIV and SIV consist of three major open reading frames, *gag*, *pol* and *env*, which encode the core proteins (p24, p17, p9 and p7), the viral replication enzymes (reverse transcriptase, RNase H, protease and integrase) and the envelope glycoproteins (Env) respectively (see section 1.2). HIV and SIV also have two regulatory genes, *tat* and *rev*. HIV and SIV differ in their additional accessory genes. HIV-1 encodes *vif*, *vpr*, *vpu* and *nef*, whilst HIV-2 and SIV encode *vif*, *vpx*, *vpr* and *nef* (Figure 1.9). These genes are sometimes referred to as non-essential, as they are not required for viral replication in tissue culture. Proteins produced from the *vif*, *vpr* and *nef* genes are assembled into virions.

Figure 1.9. The genomes of HIV-1, HIV-2 and SIV. The start of the gene is indicated by the small number from the upper left hand corner of each rectangle and is the position of the a in the atg start codon for that gene. The number in the lower right is the last position of the stop codon.

Shaded boxes denote *tat* and *rev* spliced exons.

Red boxes denote 5'LTR and 3'LTR.

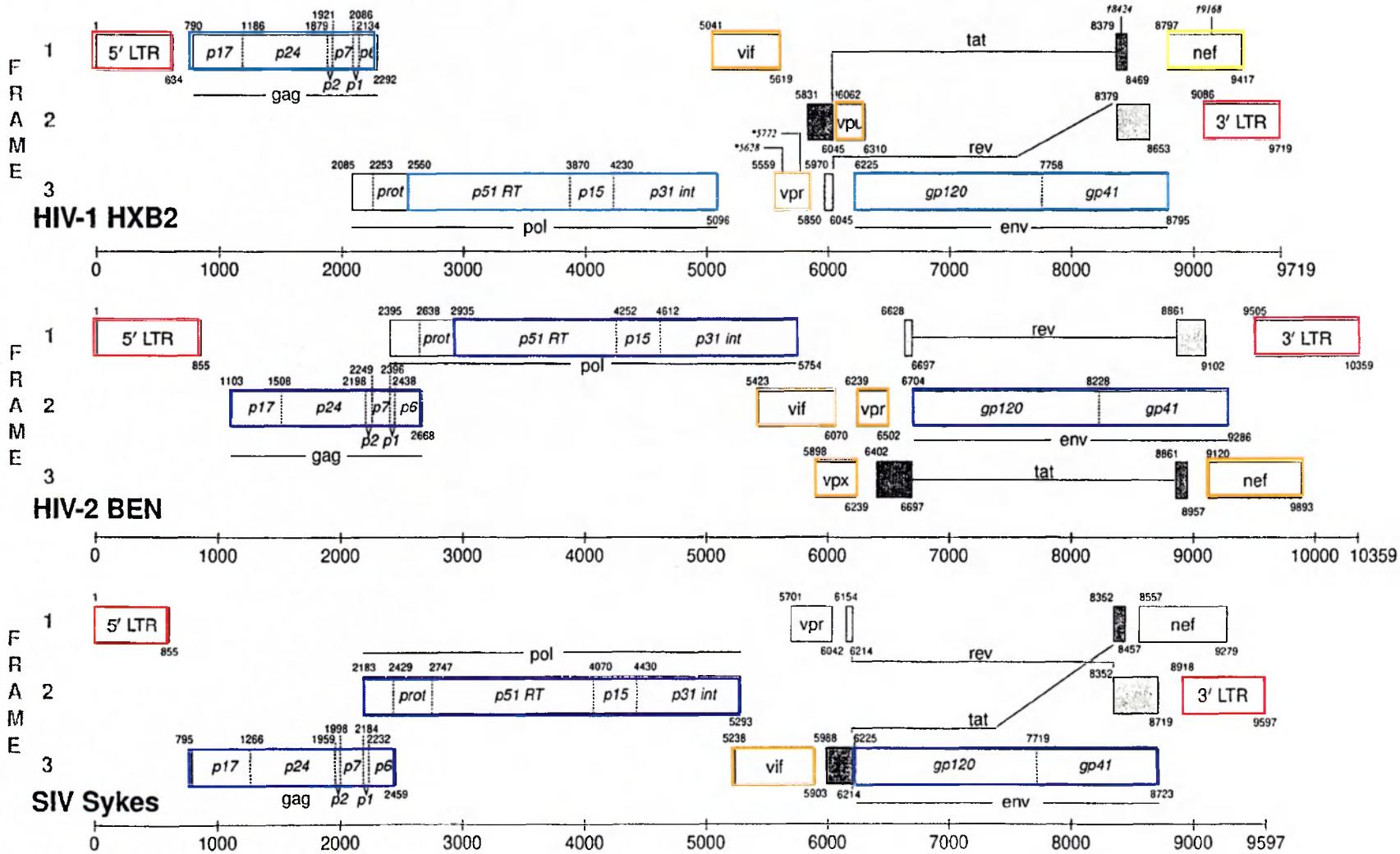
Blue boxes denote standard retroviral genes, *gag*, *pol* and *env*.

Orange boxes denote accessory genes, *vif*, *vpx*, *vpr/vpu*.

Yellow box denotes the *nef* gene.

In the HIV-1 HXB2 genome, the **5628* and **5772* mark the positions of the frameshifts in the *vpr* gene; !6062 indicates a defective acg start codon in *vpu*; *f8424* and *f9168* mark premature stop codons in *tat* and *nef*.

Adapted from: Human Retroviruses and AIDS (1999). Ed; Kuiken C, Foley B, Hahn B, Marx P, McCutchan F, Mellors J, Mullins J, Wolinsky S, Korber B.


FRAME
1
2
3
HIV-1 HXB2
5′ LTR
p17
p24
p7
p6
p2
p1
gag
prot
p51 RT
p15
p31 int
pol
vif
vpr
vpu
tat
rev
env
gp120
gp41
nef
3′ LTR
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
9719
HIV-2 BEN
vpx
10000
10359
SIV Sykes
9597

1.7.1. Regulatory Genes

a. *tat*

The *tat* gene (*trans*activator of *t*ranscription) is located between the *vpr* and *env* genes. Tat is expressed early in the viral life cycle and is essential for viral gene expression, replication and pathogenesis (Dayton *et al.*, 1986, Fisher *et al.,* 1986). Tat is translated from multiply spliced viral transcripts that do not require Rev for export from the nucleus to the cytoplasm (Jones & Peterlin, 1994).

An arginine rich motif (ARM) within Tat binds to the 5' bulge region of the trans-activation response element (TAR), which is located within the LTR and is transcribed within the 5' end of all viral transcripts. Tat regulates the process of elongation (Jones & Peterlin 1994, Kao *et al.*, 1987), therefore, in its absence, transcription complexes assemble on the viral promoter (LTR), but RNA polymerase II synthesises only short transcripts (Kao *et al.*, 1987). When Tat binds to TAR the RNA polymerase II is then competent for elongation (Kao *et al.*, 1987, Laspia *et al.*, 1989, Feinberg *et al.*, 1991). The highly specific interaction between Tat and TAR is mediated by the cyclin subunit of the positive elongation factor b (P-TEFb). By interacting with the human cyclin T1 (hT1), Tat recruits the cyclin dependant kinase 9 (CDK9), which phosphorylates the C-terminal domain (CTD) of RNA polymerase II. The conversion of RNA polymerase II from unphosphorylated to phosphorylated form is the point of transition from initiation to elongation (Jones, 1997).

b. *rev*

In order to encode nine different genes in an approximately 9 kb genome, HIV and SIV utilise alternative reading frames and complex patterns of RNA splicing (Gallo *et al.*, 1988, Schwartz *et al.* 1990). The Rev protein (*r*egulator of *e*xpression of *v*irion

proteins), expressed early in the viral replication cycle, is involved in the regulation of viral gene expression during the replication cycle (Kim *et al.*, 1989, Pomerantz *et al.*, 1990). Rev is responsible for the efficient export of unspliced and singly spliced viral mRNAs (Feinberg *et al.* 1986). In an infected cell, Rev binds to the *R*ev *R*esponsive *E*lement (RRE) in viral transcripts and shifts the balance from multiply spliced transcripts (encoding *tat*, *rev* and *nef* early in the viral replication cycle) to both singly spliced and unspliced transcripts (encoding viral structural proteins Gag, Pol, Env and the accessory proteins Vif, Vpu, Vpr and Vpx expressed in the late stages of replication). Hence, the genes expressed by HIV and SIV can be divided into two groups. The Rev independent genes are expressed from RNAs that are completely spliced and are exported to the cytoplasm by the normal mRNA export pathway. The Rev dependent genes are the intron containing messages that are retained in the nucleus and are expressed only when the Rev protein is present. The Rev protein itself is expressed from a completely spliced message, therefore expression of Rev dependent messages is delayed until a threshold level of Rev is attained (Felber *et al.*, 1990). Hence, the intron containing messages are known as the late genes, whilst the Rev independent messages are the early genes.

Rev interacts with a specific loop in the RRE via an arginine rich RNA binding motif (ARM) which also serves as the Rev nuclear localisation signal (NLS) (Zapp & Green, 1989, Malim *et al.*, 1990). A leucine rich domain between amino acids 75 and 84 in Rev acts as a nuclear export signal (NES) which can induce the efficient nuclear export of linked substrate proteins (Fischer *et al.*, 1995, Wen *et al.*, 1995).

Overall, Rev is responsible for mediating temporal regulation of viral gene expression and influencing transport of mRNA from the site of synthesis in the nucleus to the cytoplasm.

1.7.2. Viral Accessory Genes

Although the term 'accessory' implies that these genes are dispensable for viral pathogenesis, it is in fact the opposite which is true. It has been shown, on many occasions, that deletion or attenuation of any of these genes has a dramatic effect on the viruses' ability to replicate *in vivo*, revealing an altogether more critical requirement for these genes *in vivo*.

a. *vif*

The *vif* gene (*v*iral *i*nfectivity *f*actor) is located downstream of the *pol* gene. Early studies on the requirement of the HIV-1 and HIV-2 *vif* gene for viral replication revealed that the infectivity of *vif* deleted virus is reduced up to 1,000 fold relative to wild type HIV in certain CD4+ T-cell lines (H9) (Fisher *et al.*, 1987, Strebel *et al.*, 1987). More recently, the HIV-1 *vif* gene has been demonstrated to be required for replication in T-cell lines such as CEM, H9 and peripheral blood lymphocytes, but not in others, such as SupT1, C8166 and Jurkat (Fan & Peden 1992, Gabuzda *et al.*, 1992). Vif has been implicated in playing a role in virion maturation, as well as in uncoating and/or internalisation of virus in restrictive cells, thereby enabling the reverse transcription process to produce linear viral DNA molecules (Coffin *et al.*, 1997). Furthermore, Desrosiers *et al.* (1998) revealed that attempts to recover virus from PBMCs of rhesus macaques infected with SIVmac239Δ*vif* (SIVmac239 virus with a deleted *vif* gene) proved unsuccessful, as were attempts to detect SIV DNA by PCR from these animals. The relative virulence of the virus, due to the deletion in *vif*, was reduced to 10^{-5} in comparison with wild type SIVmac239 virus. More recently, the cellular protein CEM15 was identified as the critical homologue for Vif (Sheehy *et al.*, 2002).

Small amounts of Vif have been demonstrated in virus particles. Initially, Vif packaging appeared to be non-specific, however, it has now been shown that Vif is specifically packaged into virions as a component of the viral nucleoprotein complex, a process which is dependent upon the packaging of viral genomic RNA in permissive and restrictive HIV-1 target cells. Mutations in the zinc finger domains which abrogated packaging of viral genomic RNA also abolished packaging of Vif (Khan *et al.*, 2001).

b. *vpr* & *vpx*

*V*iral *p*rotein *r* (*vpr*) is present in HIV-1, SIV_{CPZ}, SIV_{SYK}, SIV_{AGM} and SIV_{MND}. By contrast, HIV-2 and several other strains of SIV, such as SIV_{MAC} and SIV_{SMM}, contain both *vpr* and *vpx* (Myers *et al.,* 1994). These genes are homologous, suggesting that *vpx* may be a duplication of *vpr*. The presence of *vpr* and *vpx* in the viral particle suggests that they may function during the early stages of viral replication.

Mutagenic studies of HIV-1, HIV-2 and SIVmac have revealed that a virus containing mutations within *vpr* can still replicate to wild type levels in CD4+ T-cell lines and cultured primary lymphocytes (Dedera *et al.,* 1989). However, at low multiplicities of infection HIV-1 *vpr* mutants replicate poorly in T-cell lines in comparison to wild-type viruses.

Studies have implicated a role for *vpr* in nuclear localisation of the pre-integration complex (Heinzinger *et al.*, 1994). This gene has also been implicated in the regulation of viral and cellular gene expression (Levy *et al.*, 1993). Using *in vitro* transient expression assays, a plasmid expressing the HIV-1 *vpr* gene was shown to activate its homologous LTR, as well as promoters of several heterologous viruses. Other studies based on analysis of stable transfectants of a human muscle tumour cell

line harbouring the HIV-1 *vpr* gene, have implicated a nuclear role for *vpr*, as *vpr* was observed to block cell proliferation and induce expression of muscle cell markers. This suggests that *vpr* could influence viral gene expression by altering host cell regulatory mechanisms which subsequently enhance viral replication.

The levels of Vpr incorporated into virions, by comparison to Vif and Nef are significantly high. For the packaging of Vpr, a leucine rich element in the p6 region of p55 Gag polyprotein and a region of Vpr that overlaps a predicted α-helical domain (amino acids 16-33) have been described to be critical (Di Marzio *et al.*, 1995, Kondo *et al.*, 1996, Lu *et al.*, 1995, Mahalingham *et al.*, 1997). It has been described that HIV-1 Vpr is primarily cytoplasmic, which could facilitate the packaging of Vpr into virions (Jenkins *et al.*, 2001).

It has been demonstrated that the carboxyl terminus of Gag is required for the incorporation of Vpx into virions (Wu *et al.*, 1994). An interaction of Vpx with p27 CA in the virion has been described (Horton *et al.*, 1994). Furthermore, for the uptake of SIVmac Vpx and SIVmac Vpr into virus like particles a dileucine containing motif in the N-terminal half of $p6^{gag}$ has been shown to be required (Accola *et al.*, 1999).

c. *vpu*

The *v*iral *p*rotein *u* gene is present in HIV-1 and SIV_{CPZ}, located 3' to *tat* and *rev*. HIV-2 and other lentiviruses lack this gene.

Virus strains deficient in *vpu* can replicate in CD4+ T-cell lines and in both primary T-lymphocytes and macrophages. However, these viruses are several fold less efficient at releasing virions into the extracellular medium compared with wild type viruses (Klimkait *et al.*, 1990, Terwilliger *et al.*, 1989). These studies demonstrated that functional Vpu was necessary to release particles from the plasma membrane of

infected cells. Vpu has been demonstrated to cause the rapid degradation of the CD4 receptor in the endoplasmic reticulum (Willey *et al.,* 1992, Vincent *et al.*, 1993) when cells were expressing all three proteins. This activity is sequence specific, requiring the anchor and cytoplasmic domain of CD4. It is also specific to the ER. However, the mechanism of Vpu dependent CD4 degradation remains unclear.

d. *nef*

The *nef* gene (previously described as 3'*orf* or *orf* B) is unique to primate lentiviruses and is located at the 3' end of the viral genome, where it overlaps part of the 3' long terminal repeat (Figure 1.9). This gene is expressed early in the viral replication cycle, with reports of Nef specific mRNA constituting approximately 80% of the viral mRNA present in infected cells at early time points after infection (Robert-Guroff *et al.*, 1990). The *nef* gene encodes a protein product ranging from 206 amino acids in HIV-1 to 265 amino acids in HIV-2 and SIV, with a molecular mass between 27-35 kDa (Harris, 1996). The high degree of conservation of this gene in HIV and SIV suggests an critical role in the viral life cycle. However, contrary to the current opinion for an essential role of *nef* in the viral life cycle, it was initially described to have a negative influence on viral replication, hence the term *ne*gative *f*actor.

The Nef protein is cotranslationally modified at the N terminus by the addition of a 14 carbon saturated fatty acid; myristate; Met-Gly-X-X-X-Ser/Thr (Allan *et al.*, 1985). Studies have shown that the addition of this group is required for the membrane association of some viral and cellular proteins, such as the MARCKS protein (Li & Aderrem, 1992) and the Src family of protein tyrosine kinases (Resh, 1994). The myristate is covalently linked to the N terminal glycine in a reaction catalysed by N-myristyl transferase. Myristoylation is required for localisation of Nef on the

cytoplasmic face of the plasma membrane in infected cells (Yu & Felsted 1992). It has been described to be essential for the interaction of Nef with a subset of cellular membrane–associated proteins designated p280, p32 and p28 according to molecular weight. It was suggested that one or more of these proteins could represent a membrane target for Nef. However p97, a protein present in soluble and membrane-associated cell fractions was found to bind *nef* independently of myristoylation suggesting that for Nef the particular interaction with the N terminus of Nef is not required (Harris & Coates 1993). Myristoylation has also been described to be essential for the interaction of HIV-1 Nef with actin, forming a high-molecular-mass complex (Fackler *et al.,* 1997).

In order to investigate if myristoylation is important for interactions between Nef and cellular processes, Harris (1995) generated Nef as an N-terminal fusion with GST and expressed this protein in the baculovirus system. These myristoylated Nef fusion proteins interacted with Lck, a member of the Src family of tyrosine kinases (section 1.9.1e) and β-COP, a component of non-clathrin coated vesicles. These interactions were demonstrated to be dependent on the presence of the myristate moiety as a non-myristoylated Nef fusion protein did not bind to β-COP. This suggests that as well as a mechanism for targeting Nef to membrane structures, myristoylation is also important in mediating interactions between Nef and other cellular proteins, therefore contributing to the ability of Nef to perturb signal transduction pathways.

Nef has been reported to be detected in HIV-1 virions which lack an active viral protease (PR), whilst smaller amounts of intact Nef were present in wild-type virion preparations. In these instances, a smaller Nef with an apparent molecular mass of 18 kDa was observed.

Myristoylation was observed to be crucial for the association of Nef with virions. A truncated Nef, containing only the N-terminal 86 amino acids including the myristate moiety was found to be incorporated as effectively into virions as full length Nef, suggesting that cell membrane association of Nef through myristoylation may be sufficient for the incorporation of Nef into the virion particles. (Bukovsky *et al.*, 1997). Welker and colleagues (1998) defined the signal at the N terminus required for virion incorporation as a bipartite membrane targeting signal, consisting of a covalently attached myristic acid and a cluster of positive charges in the N terminus of the protein. This signal was found to be reminiscent of a Src homology region 4 (SH4), which mediates plasma membrane targeting of protein tyrosine kinases of the Src family (Resh, 1994). The ability of Nef and N-terminally altered Nef proteins to bind to the membrane correlated with their incorporation into virus particles and was independent of other viral proteins.

1.8. Structural and Biochemical Features of Nef

The Nef protein of HIV-1 is 206 amino acids, whilst that of SIV and HIV-2 is approximately 250 amino acids in length. As previously mentioned, it is a myristoylated protein which is important for association with cellular membranes.

The three dimensional structure of HIV-1 Nef, coupled with the SH3 domain of Fyn or Hck tyrosine kinases has been resolved (section 1.12e) (Arold *et al.*, 1997, Lee *et al.*, 1996). Furthermore, the structure of Nef alone and bound to a peptide from the CD4 cytoplasmic tail has been determined by nuclear magnetic resonance (NMR) (Grzesiek *et al.*, 1996a, Grzesiek *et al.*, 1996b). These studies showed that the HIV-1 Nef core consists of a type II polyproline helix (amino acids 70-77), which is the main binding site for the Src family kinases. Also present are two alpha helices (aa 81-120),

a four stranded anti-parallel beta-sheet (aa 121-186) and two additional alpha helices (aa 187-203). Residues 60-71 and 149-180 form flexible solvent-exposed loops. The three proximal helices of the Nef core domain (aa 70-120) form a cavity which is potentially accessible to drugs. Binding to this crevice could disrupt interactions between Nef and Src family kinases (Grzesiek *et al.*, 1996a, Grzesiek *et al.*, 1996b).

The NMR structure of a peptide corresponding to the n-terminal domain of Nef has also been resolved. This region was found to form a well-ordered alpha-helix from residues 6-22, with the N and C terminal regions having a less ordered structure (Barnham *et al.*, 1997).

1.9. Early Studies of *nef*

The *nef* gene was initially described as exhibiting homology with the nucleotide binding sites of GTP binding proteins (Guy *et al.,* 1987). Data indicated that *E. coli* derived Nef could bind GTP and autophosphorylate, suggesting that it had homology with regions in the Ras family of proteins involved in GTP-binding. This implicated a role for *nef* in interfering with pathways involved in control of cellular functions. Subsequent experimentation failed to reproduce these findings and it was suggested that the results may have been due to contamination with a bacterial GTP-binding activity (Harris *et al.,* 1992).

In addition, early studies of *nef* described it to be dispensable for viral replication *in vitro* (Fisher *et al.,* 1986). A series of mutants of the biologically active molecular clone HIV pHXB2D containing deletions of 55, 109, 85 and 100 base pairs in the *nef* gene, as well as deletions encompassing the last 14 bases of the envelope gene, the initiation codon for *nef* and the first 159 or 182 base pairs of *nef*, were transfected into the human T-lymphoid cell line H9. The ability of these mutants to produce virions

indicated that the various deletions within the *nef* gene had no effect on the ability of HIV-1 (then known as HTLV-III) to replicate *in vitro*.

The pHXB2 plasmid was used in further studies where deletions were introduced into the *nef* gene (Terwilliger *et al.,* 1986) removing 11 or 50 amino acids from the 3' *nef* open reading frame. After transfection of these mutants into the Jurkat-*tat* III and the C8166-45 cell lines, cell number, cell-free reverse transcriptase activity, HTLV-III specific surface immunofluorescence and syncytium formation were investigated. Provirus containing deletions within the *nef* gene exhibited a cytopathic effect. Although it was concluded that *nef* was dispensable for replication and the cytopathic ability of the virus, the high degree of conservation of the *nef* gene indicated an important role in the life cycle of the virus. It was therefore suggested that this gene may be required for growth in cell lines other than those used in these experiments, or that the function of this gene may be one which was not measured in the assays described.

By contrast, Luciw *et al.* (1987) used molecularly cloned provirus of HIV San Francisco isolate 2 (HIV_{SF2}) containing mutations in three regions of the *nef* gene (then known as 3' *orf*), to transfect the human lymphoid T cell line HuT78. They demonstrated that the virus produced was cytopathic, with titres five fold greater in comparison to wild type virus. A greater level of viral protein was also detected in mutant virus infected cells, with increased levels of both proviral and unintegrated forms of DNA being produced in lymphoid cell cultures. It was suggested that these mutants were exhibiting increased replication ability, possibly through interaction with the LTR region.

Initial studies of the effect of Nef therefore suggested that mutation of *nef* led to more efficient viral replication, implicating a nonessential role in the viral life cycle,

although this was to be disputed and proved incorrect in subsequent investigations. (Fisher *et al.,* 1987, Terwilliger *et al.,* 1986, Luciw *et al.,* 1987).

Two reports appeared simultaneously in 1989 that disputed data demonstrating that deletions within *nef* resulted in an increase in viral infectivity *in vitro*. Hammes *et al.* (1989) used two different *nef* expression vectors, as well as a control vector containing a frameshift mutation in the *nef* coding sequence. This group showed an inability of Nef proteins to inhibit transcriptional activity of the LTR in a variety of cell types, including primary human T lymphocytes, Jurkat or YT-1 leukaemic T cells, U-937 promonocytic cells and nonlymphoid COS cells, over a broad dose range. No differences were exhibited by the proviral clones containing wild type or mutated *nef* genes, indicating the lack of a Nef-mediated negative effect on *in vitro* viral replication.

This result was supported by a parallel study using two isogenic HIV-1 (HIV-1-W13) strains, one of which lacked *nef* expression (Kim *et al.,* 1989). Comparison of viral entry, measurement of unintegrated HIV DNA after infection of H9 or primary T cells and assays to measure reverse transcription, RNA expression and virus production in a number of different cell lines were performed. No obvious differences were observed between the wild type and the *nef* deficient virus strains. By contrast, it was observed that the presence of *nef* conferred a growth advantage to the virus, an effect that was most apparent in primary T cells. This data was therefore consistent with speculation that the high degree of conservation of the *nef* gene implicates an important role in the viral life cycle.

It is likely that early experiments performed which concluded that *nef* was dispensable for viral replication may have been due to the restricted number of cell lines used, or due to conditions during experimentation which did not allow the true effect of *nef* to

be exerted. Indeed, Terwilliger *et al.* (1986) concluded that although within their experiments *nef* was proven to be dispensable for replication, that due to the high degree of conservation of this gene that it must have an important role in the viral life cycle.

1.10. Genetic & Molecular Analysis of *nef* Using the Macaque Model

The progress from infection to disease in cynomolgus and rhesus macaques infected with SIVmac is usually 1-4 years. However, infection with virus harbouring deletions within specific regions has been shown to result in low viral loads and animals remaining asymptomatic for longer periods of time. At the nucleotide level SIV , HIV-1 and HIV-2 exhibit an overall homology of approximately 40%, with SIV and HIV-2 having a homology of approximately 70% (Chakrabarti *et al.,* 1987), suggestive of a common origin for these viruses. This could implicate cross species transmission of SIV into humans, similar to that of the sooty mangabeys to macaques. The macaque therefore represents a model in which viruses can be tested to determine which genetic modifications attenuate pathogenesis *in vivo*.

Studies were performed using the SIV model to determine if deletions within specific regions of the *nef* gene correlated with an attenuated phenotype *in vivo*. Deletions were introduced into key regions of the *nef* gene and the subsequent effect of these mutations studied in the pathogenic outcome of disease.

Kestler *et al.* (1991) used the infectious molecular clone SIVmac239 (Reiger & Desrosiers, 1990) to investigate the importance of the *nef* gene for virus replication *in vivo*. SIVmac239 contains a premature stop codon at the 93rd codon of *nef*. This codon was mutated by oligonucleotide-directed site specific mutagenesis to create SIVmac239/*nef*-open. The same method was used to introduce a 182 bp deletion in

nef. These three forms of virus, SIVmac239/*nef*-stop, SIVmac239/*nef*-open and SIVmac239/*nef*-deletion were used to determine the requirements of the *nef* gene both *in vivo* and *in vitro*.

In vitro kinetics in CEMx174 cells, in primary cultures of rhesus monkey peripheral blood lymphocytes (PBLs) and in primary rhesus monkey alveolar macrophage cultures revealed no differences between the three types of virus, consistent with previous reports for HIV-1 (Hammes *et al.*, 1989, Kim *et al.*, 1989).

Studies were extended to rhesus macaques. Five animals were infected with SIVmac239/*nef*-stop, seven with SIVmac239/*nef*-open and six with SIVmac239/*nef*-deletion. Although all animals became infected, virus recovery from animals inoculated with the SIVmac239/*nef*-deletion proved increasingly difficult with time. The *nef* gene from two animals infected with SIVmac239/*nef*-stop was amplified and cloned. These clones were obtained from one animal at the time of death and from others at various time points during the asymptomatic stage of persistent infection. All clones revealed that the stop signal at the 93rd codon had reverted to a coding codon. This would imply that there is a strong selective pressure for the virus to maintain an open, functional form of the *nef* gene, thereby suggesting that *nef* is performing a critical function for the virus life cycle *in vivo*.

The virus load in rhesus macaques that received the SIVmac239/*nef*-deleted virus was observed to be at least 100-fold lower than those in rhesus macaques that received the other two viruses. The p27 *gag* protein in the cell-free plasma was also found to be below the level of detection (approximately 0.05 ng/ml). Therefore, whilst the presence of the 182 bp deletion had no effect on the ability of the virus to replicate *in vitro*, *in vivo* the virus exhibited a much lower virus load. This indicates that the presence of a fully functional *nef* gene is an important factor for the progression to

AIDS in rhesus macaques. This is in agreement with the effect of the reversion of the stop signal at the 93rd codon to a coding codon.

SIVmac251 is a swarm of SIV which can infect and cause an AIDS-like syndrome in rhesus and cynomolgus macaques. A stock of SIVmac251 virus was used to infect rhesus macaque number 32H (Cranage *et al.,* 1990). A low passage pool, designated 11/88 stock was subsequently prepared from virus re-isolated from this monkey. This swarm of virus, designated SIVmac32H, derived from the 11/88 stock has been selected as a virus challenge for EU co-ordinated projects for the development of an AIDS vaccine and has also been used following inactivation with formaldehyde as an immunogen in macaque vaccination trials. The attenuated clone SIVmac32H(C8) has also been selected for a subsequent European study. The wild-type clone SIVmac32H(J5) has also been used as a challenge virus in a number of UK studies.

Rud and colleagues used SIVmac32H (Rud *et al.,* 1994) to study the effects of specific deletions within the *nef* gene on the pathogenic outcome of infection. The molecular clone SIVmac32H(J5) (hereafter referred to as J5) has no premature stop codons within any of its genes and is derived from SIVmac32H. This clone encodes a full length Nef protein. The SIVmac32H(C8) (hereafter referred to as C8) clone contains a naturally occurring 12 base pair deletion within the *nef* gene which results in an in-frame deletion of four amino acids, DMYL, at positions 143-146. Clone C8 has two additional amino acid changes, at amino acid 191, Arg>Lys (R191K), and amino acid 249, Thr>Ala (T249A). The location of these changes in a region of the *nef* gene that overlaps with the 5' end of the U3 region of the 3' LTR. There are further nucleotide changes within the 3' LTR which are not known to be associated

with any critical motifs. The position of these changes in the 3' LTR, however, are not in any of the major transcriptional control elements of the 3' LTR.

Electroporation of C8 and J5 clones into C8166 cells revealed no replicative advantage or disadvantage to be associated with the defect in the *nef* gene of clone C8 (Rud *et al.*, 1994), an observation that is consistent with reports of Kim *et al.* (1989) and Hammes *et al.* (1989) for HIV-1.

However, data generated using *in vivo* models yielded significantly different results, which relate to the difference in the *nef* gene between the two viruses (Rud *et al.*, 1994). Pairs of Chinese rhesus macaques were infected with equivalent titres of virus derived from J5 or C8 and blood samples taken at regular intervals. PBMCs were isolated and co-cultivated with freshly passaged C8166 cells. Virus load and the time to appearance of a cytopathic effect was measured. It was found that virus could be isolated from macaques infected with the C8 virus only during the first 20 weeks post infection, whilst from the macaques infected with J5, virus could be isolated for at least 40 weeks. Virus was recovered from various tissues of both J5 infected macaques and from one of the two C8 infected macaques. To rule out the possibility that Chinese macaques may be more resistant to AIDS-related pathogenesis, the virus pool of J5 was titrated in Indian rhesus macaques. Seven of the eight macaques became infected, with one animal being sacrificed due to its rapidly deteriorating condition. Post mortem examination showed several pathological abnormalities within this animal consistent with SIV infection. This data highlights the difference in the pathogenic outcome of the wild type J5 and the attenuated C8 virus.

Cynomolgus macaques were also found to be susceptible to infection by J5 and C8, as determined by virus isolation and PCR analysis. However, whilst the condition of one of the J5 infected animals declined rapidly until it had to be sacrificed at nine months

post infection, the C8 infected cynomolgus macaques which had been infected for the same duration of time did not exhibit any signs of disease. As observed in the Chinese rhesus macaques, all of the cynomolgus macaques infected with C8 became intermittently positive for virus isolation after approximately 6 months of infection (Rud *et al.,* 1994, Almond *et al.*, 1995). The observation that such a small deletion can significantly affect the virus's ability to achieve its full pathogenic potential *in vivo* leads one to speculate on the potential functions of the region in the J5 *nef* gene, which is deleted in C8 *in vivo*. A comparison of the phenotypes that C8 and J5 virus exhibit in *in vitro* assays would enable a detailed study of the effect subtle differences in *nef* can have on some of the critical functions of the virus (section 1.10).

Serendipitous observations by Whatmore *et al.* (1995), using the molecular clone SIVmacC8, has enabled the dissection of Nef function to be taken further. Seven Indian rhesus macaques were each infected with SIVmacC8 by intravenous inoculation. Whilst replication of the virus was consistent with C8 virus in six out of seven animals, one animal, 45R, was found be persistently positive for virus isolation. The proportion of virus infected-PBMC in 45R, as determined by serial dilution, was found to be at least 583-fold greater than the other animals, suggesting that a virus with a more virulent phenotype than that of the attenuated SIVmacC8 had developed in the animal coincident with higher levels of virus replication The anti-SIV p27 antibody titre in this animal was initially higher than the mean titre for the rest of the group but fell after 17 weeks of infection to levels much lower than that of the other animals. At 12 weeks post infection a significant decline in CD4/CD8 ratio was observed in animal 45R and by 58 weeks had reached just 28% of the preinfection value. The clinical condition of this animal rapidly deteriorated, consistent with the development of an AIDS-like syndrome. At 63 weeks post infection lymphocyte

depletion from lymph nodes and spleen was observed, as well as colitis, small intestinal cryptosporidiosis and *Pneumocystis carinii* pneumonia (Whatmore *et al.*, 1995).

Analysis of the complete nucleotide sequence of the *nef*/3' LTR region of PBMC associated provirus at regular intervals revealed that at 62 weeks after infection at post mortem, the 12 base pair region deleted in the SIVmacC8 virus had been repaired with the amino acid sequence EKIL. This sequence motif continued to evolve, such that at 25 weeks the sequence was EIYL and by 45 weeks it had evolved to DIYL, identical to the equivalent motif in the pathogenic clone SIVmac239 and only one amino acid different from the original wild type sequence of DMYL. The remaining nucleotide changes within SIVmacC8 remained stable. Interestingly, at week 45, when the gene sequence had evolved to DIYL, the SIVmacC8 provirus used a different aspartic acid codon (GAT) than that used in SIVmacJ5 (GAC). This would suggest that there is a pressure for the virus to restore the amino acid sequence of Nef, rather than to restore the specific nucleotide sequence. This supports the idea that the attenuation of C8 is through the disruption of Nef rather than a U3 specific sequence. Therefore, the reversion to virulence of the virus was attributable only to the effect the mutations had on the *nef* gene and was not due to effects on the 3' LTR. This is consistent with previous observations which concluded that the function of the *nef*/3' LTR region is as a *nef* coding sequence, as mutation of the U3 region has limited effect on the ability of the virus to replicate or virulence *in vivo* (Ilyinskii *et al.*, 1994).

It therefore appeared that the repair of this 'attenuating' region within SIVmacC8 was directly responsible for the progression to disease in animal 45R, indicating that this region may be vital to the virus for achieving its full pathogenic potential *in vivo*. It is

possible that the attenuation within C8 results in a Nef protein that is not as stable as wild type Nef and this could contribute to the observed decrease in pathogenicity. Certainly, the stability of C8 Nef has been demonstrated with by infecting cells with vaccinia derived C8 and J5 Nef virus proteins and pulse labelling with 35^S methionine over a specific period of time. It was observed that the rate of turnover of the C8 proteins was greater than that of J5 protein, but only by a marginal difference (A. Gallimore, PhD thesis, personal communication).

The study by Whatmore *et al.* (1995) is consistent with the previous report (Kestler *et al.,* 1991) which showed that progression of infection in rhesus macaques occurred when SIVmac239/*nef*-stop, a clone containing a stop signal at the 93^{rd} codon within *nef*, exhibited reversion of the stop signal to a coding codon. Whilst these observations are intriguing, these studies still demonstrate that C8 is remarkably stable in the remainder of infected animals.

1.11. Study of *nef* in Viral Pathogenesis Using the Mouse Model

Mouse models provide an alternative *in vivo* model for the study of *nef* and AIDS pathogenesis. The SCID-hu mouse is an *in vivo* model which allows the measurement of HIV pathogenesis in the absence of a host immune response. As a result of this the pathogenicity of the virus can be observed without the interference of a normal immunological response. The SCID-hu mouse model is constructed by the surgical implantation of human fetal liver and thymus under the murine kidney capsule of severe combined immunodeficient (SCID) mice (McCune *et al.*, 1988, Namikawa *et al.*, 1999). These tissues form a conjoint (Thy/Liv) organ, which histologically resembles a normal human thymus. Infection of this organ with HIV-1 induces a similar disease pathology as that observed in humans (Papiernik *et al.*, 1992,

Rosenzweig *et al.*, 1993). The depletion of CD4 cells observed in the SCID-hu mouse system correlates with a high viral load.

Jamieson and colleagues (1994) showed that HIV-1 strains with deletions or frameshift mutations in *nef* were attenuated in their replicative and pathogenic potential using the SCID-hu mouse model. This was confirmed by Aldrovandi & Zack (1995), who looked at the deletions of individual accessory genes on the pathogenicity of HIV-1_{NL4-3} in the SCID-hu mouse model. The *nef*-minus strains were observed to exhibit a significantly attenuated phenotype. This was observed as decreased levels of thymocyte depletion and proviral load in Thy/Liv implants infected with 100 IU of virus, decreased pathology in biopsies taken at 3 weeks post inoculation and decreased virus detected by flow cytometry and quantitative PCR. However, when a ten-fold increase in the levels of the *nef*-minus virus was introduced to the system, the virus replicated to high levels *in vivo*. This data suggests that in absence of a complete immune response, the deletion of *nef* in HIV-1_{NL4-3} results in a less virulent phenotype than that of wild type HIV-1_{NL4-3}.

Studies utilising transgenic mice expressing the whole coding sequences of HIV-1 under the regulatory sequences of the human CD4 (CD4C) gene, with viral expression occuring in the same subsets of cells normally found to be infected in HIV-1 have also been performed. These mice develop a severe AIDS-like disease similar to that observed in humans infected with HIV-1 (Hanna *et al.*, 1998a & b). Simard and colleagues (2002) investigated disease progression of SIV using a similar transgenic mouse model. Transgenic mice were constructed expressing the SIVmac239 *nef* gene in the natural target cells of the virus, under the control of the human CD4 gene promoter (CD4C). These mice developed a severe AIDS-like disease, similar to that observed when HIV-1 *nef* was expressed in transgenic mice and exhibited a disease

progression similar to that of simian AIDS. Whilst the mouse model represents an important *in vivo* system within which to study HIV-1, this system does present limitations for study, primarily from the point of view that within HIV-1 infected mice, every CD4+ cell expresses protein, compared to the macaque model, where only a proportion of the CD4+ cells express the viral protein. This should therefore be taken into consideration if choosing an animal model within which to study disease pathogenesis.

1.12. Clinical Evidence for the Importance of *nef*

Rapid Progressors (RPs) of HIV infection are defined as developing AIDS within three years of infection and are estimated to constitute 3% of HIV infected individuals. By contrast, approximately 5-15% of patients will remain asymptomatic for more than 12 years before any clinical signs of disease. These long term non progressors are distinct from the RPs in that their viral loads remain low, CD4 levels are normal and virus isolation proves difficult. The reasons for the differences in progression rates are unclear but could reflect the genotype of the virus or initial immune response to the presence of virus, which if sufficiently strong to suppress viral replication could result in viral loads remaining low. It is also possible that some other unknown factor(s) within the host can prevent viral replication.

The study of a variety of cohorts of long term non progressors has proved important for understanding HIV pathogenesis and the mechanisms underlying disease development, both from the perspective of the virus and the host (Learmont *et al.*, 1992, Deacon *et al.*, 1995, Kirchhoff *et al.*, 1995). In certain cohorts long term non progression has been associated with virus apparently attenuated by encoding a defective *nef* gene. Such clinical data obtained from long term non progressors

represents an invaluable insight into the effect attenuations within the *nef* gene can have on the replication of the virus *in vivo*, in terms of the viral load, CD4 counts and the ability to reisolate virus from such individuals.

The Sydney Bloodbank Cohort consists of a group of 6 patients infected with HIV-1 by a single donor over a period of 2 years (Learmont *et al.,* 1992). It was found that, between approximately 6-10 years after infection, 5 of the 6 recipients and the donor remained clinically free of symptoms, with normal CD4 levels and no evidence of virus through detection of p24 antigen production. The sixth subject died soon after HIV-1 infection was diagnosed from unrelated causes. HIV-1 was only isolated from one recipient. This isolate was unable to induce syncytia in a co-culture assay using SupT1 cells and exhibited a reduced host range *in vitro*. This group of patients was of a broad age range and an equal proportion of the sexes and there were no other obvious common host factors. The possibility of a host immune response being responsible for the delay to progression to AIDS was excluded and it was thought more likely to be due to the transmission of a non pathogenic strain of the virus.

A report in 1995 by Deacon *et al.* (1995) revealed the possible cause for the slow progression exhibited by this cohort. At this time the patients still exhibited normal levels of CD4 and had not developed any AIDS-defining symptoms or HIV-related symptoms, or received any form of anti-retroviral chemotherapy.

HIV was isolated from 3 of the recipient members of the cohort and the ability of the viruses to replicate was compared to that of reference isolates HIV-1_{NL43} and HIV-1_{ADA}. All three virus isolates were replication competent. However, two replicated very poorly in comparison to the third isolate, which in turn revealed a prolonged replication time in comparison to HIV-1_{NL43} and HIV-1_{ADA}. Subsequently, a region of the HIV-1 DNA, including the whole of the *nef* gene and the U3 region of the long

terminal repeat (LTR) was amplified by double or triple-nested PCR (Deacon *et al.*, 1995). The sizes of amplified DNA obtained from members of the cohort compared to the HIV-1_{NL43} control virus were found to be consistently between 160-430 bp smaller and the regions harbouring the deletions were found to be within the *nef*/LTR region. The original donor yielded an amplified fragment of approximately 550 bp in length, which, with comparison to full length virus indicated a deletion of approximately 290 bp. Subsequent sequence analysis of the amplified fragments from the *nef* gene-LTR region of the recipients revealed deletions of various lengths; some from the portion of the *nef* gene that does not overlap with the U3 LTR region, others from at least one part of the *nef*-LTR overlap region. It was therefore concluded that the *nef* or LTR defects were responsible for the attenuated course of infection observed in these patients.

Kirchhoff *et al.* (1995) described a group of haemophiliacs exposed to HIV-1 through transfusion of contaminated Factor VIII before 1983 and subsequently diagnosed as HIV positive. Analysis of DNA prepared from peripheral blood mononuclear cells (PBMCs) from a 44 year old male, known as patient 1, revealed a deletion of 118 base pairs in the *nef* gene. Recovery of HIV-1 from this patient proved difficult, with antigen capture assays for p24 proving negative, and serum and plasma samples taken in 1985 and 1994, respectively, proving negative by Western blotting for antibodies to Nef. This patient also exhibited a low viral DNA burden, approximately 1 viral DNA copy per 100,000 PBMC by HIV-1 *gag* PCR, which is approximately 10-3,500 times lower than levels observed in patients with parallel progressive or slowly progressing infection. However, five other patients from this group who also appeared to have non progressive HIV-1 infection revealed a prevalence of full length *nef* sequence, as well as the presence of Nef reactive antibodies.

It was assumed that patient 1 was originally infected with a *nef* defective strain of HIV-1, although such information is not available as the patient was infected before the advent of blood screening in 1983. This report therefore supported the idea that an intact *nef* gene is required for the virus to achieve its full pathogenic potential *in vivo*. However, one must appreciate the role of additional host factors contributing to long term non progression, such as the maintenance of a strong initial immune response to the virus.

More recently Greenough & Desrosiers (1999) described that patient 1 had subsequently exhibited declining CD4 T-lymphocyte levels, observed initially in 1997, although the plasma viral RNA levels have remained low and attempts to culture virus have remained unsuccessful. Therefore, it may be that this patient, having been through a long asymptomatic phase (which may have been due to defective *nef* gene), may now have progressed into the first stages of disease development.

A study by Huang *et al.* (1995a) revealed results that did not agree with those previously described. This group performed a study on 10 patients who lacked any clinical symptoms and exhibited normal stable CD4+ T-cell counts, despite being HIV positive for between 12-15 years. Sequences of *nef* were isolated and analysed by nested PCR and DNA sequencing. No obvious deletions were apparent within the *nef* gene, with the majority (91.1%) of sequences containing an intact *nef* open reading frame. There were no obvious differences between consensus *nef* sequences derived from patients with AIDS and those from this group of 10 long term non progressors. In this study the presence of strong HIV-1 neutralising antibodies and virus-suppressive CD8+ T-cell activity suggested that a vigorous immune response is involved in maintaining low levels of virus (Huang *et al.*, 1995a).

These observations were taken further by replacing the *nef* gene of an infectious molecular clone, HIV-1_{HXB2}, with the *nef* gene derived from the above group of 10 patients (Nef-HIV-1_{HXB2}) (Huang *et al.,* 1995a & b). A *nef* gene from a separate patient with AIDS was also replaced into the HXB2 clone as a control (Nef+HIV-1_{HXB2}). The Nef+ HIV-1_{HXB2} replicated more efficiently than Nef- HIV-1_{HXB2} in resting PBMCs and mitogen pre-activated PBMC. A comparison of *in vitro* growth kinetics of these viruses carrying *nef* genes derived from long term non progressors with that of the virus containing *nef* derived from a patient with AIDS revealed no obvious differences.

Parallel studies reported by Michael *et al.* (1995a) showed no obvious correlation between the structure or function of the *nef* genes and the rates of disease progression amongst nine patients of the San Francisco Men's Health Study. A subsequent study was carried out by the same group (Michael *et al.,* 1995b) of a patient infected with HIV in 1981 by blood from an HIV+ blood donor, whereby the blood donor and two other recipients of the same blood transfusion had all died of AIDS, whilst this patient remained healthy. No gross deletions were observed in the *nef* gene of this patient. By contrast, there were obvious deletions in *vif*, *vpr*, *vpu* and *tat*. It was concluded that this patient may have been infected with an attenuated component of a virus pool. The defects in a variety of accessory genes within this patient suggests that this was contributing to the lack of progression to AIDS. However, due to the absence of changes within *nef*, this gene cannot have contributed to the attenuated phenotype of virus in this patient.

Therefore, although in some cases infection with HIV defective in regions of the *nef* gene may account for increased length of the asymtopmatic phase prior to overt disease progression, one cannot rule out other important factors. These include the

hosts' initial immune response, virus which is defective in regions other than *nef* and the ability of the host to somehow suppress the ability of the virus to replicate *in vivo*. However, the *nef* gene remains of considerable interest due to the effect deletions within this regulatory gene can have on disease outcome. Further study of the *nef* gene could contribute to our understanding of the behaviour of the virus *in vivo*.

The combination of clinical data and results from infection studies should further our understanding of these viruses and help in the development of novel therapeutic or preventative strategies. However, in order to study the viral genetics of HIV one needs to be able to manipulate experimental conditions and this is not possible with patients. Experimental study requires an animal model where the virus can be manipulated to observe the outcome, with the hope of understanding the underlying mechanisms of the properties of viruses in long term non progressors. The SIV macaque model, as described in section 1.9 represents such a model.

1.13. Functions of *nef*

The effect of attenuations within specific regions of the immunodeficiency viruses are not restricted to the *nef* gene, as has been widely described within the literature. However, due to the difference in pathogenicity between the clones SIVmacC8 and SIVmacJ5, significant interest lies in studying how such subtle differences within this gene can have such an effect on the viruses' ability to propagate itself *in vivo*. The Nef protein has been described to have wide and diverse functions *in vitro* and it is possible that the deleted region within SIVmacC8 may contribute to one or more of these functions. Disruption of this region would prevent the virus from achieving its full pathogenic potential.

Since there is little evidence that the Nef protein interacts directly with other viral proteins, components of the infected cell which interact with or are influenced by Nef have been described extensively. In order to fully understand the function of Nef, it will be important to identify and characterise potential target molecules in the infected cell with which it may interact. An understanding of the interactions of Nef may pave the way for new therapeutics against HIV. Described are some of the functions of Nef and the benefit each of these functions may provide to the virus *in vivo*.

a. Interaction with a Cellular Serine Kinase

Sawai *et al.* (1994) demonstrated that HIV-1 SF2 Nef expressed either in the form of a CD8-Nef fusion protein, or native Nef protein in T-cell lines, specifically interacted with a cellular serine kinase. Two proteins, of molecular mass 62 kDa and 72 kDa (subsequently named p62 and p72 respectively) were found to co-immunoprecipitate with Nef and to be phosphorylated in *in vitro* kinase assays. Inhibitors of protein kinase A and protein kinase C failed to inhibit this Nef-associated activity. A serine kinase was implicated as these proteins were phosphorylated solely on serine residues. The region of Nef between amino acids 45-127 was revealed to be important for this Nef associated kinase (NAK) interaction and truncations within this region resulted in decreased kinase activity. There are several amino acid motifs in this region which are conserved throughout HIV-1, HIV-2 and SIV Nef, which in turn implicates them in this function. In particular, mutations of Arg>Lys at positions 109 and 110 of HIV-1SF2 Nef produced a stable protein which exhibited no kinase binding activity. Further investigation with a myristoylation defective mutant (a mutation of Gly>Ala at amino acid 2), again disrupted kinase binding activity. This indicates the

importance of the targeting of Nef to intracellular membranes as a requirement for its association with a cellular kinase activity (Sawai *et al.,* 1995).

Expression of Nef in the baculovirus system, as the N-terminal portion of a GST fusion molecule, has supported the notion that myristoylation is required for binding to cellular proteins. Proteins identified were both soluble and membrane associated in cytoplasmic extracts of the Jurkat human T cell line. Mutations which prevented myristoylation prevented these interactions (Harris & Coates 1993).

Antibodies directed against a p21 associated kinase (PAK) were found to immunoprecipitate the p62 and p72 proteins in an *in vitro* kinase assay. PAKs are highly conserved ubiquitous serine kinases important for the transduction of external signals to the nucleus. Nef from SIV or HIV-1 infected cells has been shown to associate with PAK in infected lymphoid cells (Sawai *et al.,* 1996).

A proline rich region, PXXP (positions 104-107), previously identified to have homology to the consensus sequence required as a ligand for the SH3 domains in members of the Src family of non-receptor protein tyrosine kinases, is present in the Nef protein. *In vitro* kinase assays showed that alanine substitution of the prolines at positions 104 and 107 disrupted the ability of the Nef to associate with NAK. Positions 106, 108, 109 and 122 were also shown to be essential for Nef association with NAK (Khan *et al.,* 1998) since mutations at these sites rendered the Nef defective for the activation of PAK.

Subsequent *in vivo* data using seven juvenile rhesus macaques infected with SIVmac239, where the proline residues at positions 104 and 107 had been substituted for alanines, revealed that five of these animals showed a reversion to the PXXP motif, as well as restoration of the NAK positive phenotype. These observations demonstrate a strong selection for a functional SH3 ligand *in vivo*, which is associated

with the ability of Nef to interact with NAK. This reversion was also associated with the progression of virus pathogenesis, with two of the animals developing fatal simian AIDS. However, findings by Lang *et al.* (1997) contradict this data. This group similarly used SIVmac239 (Reiger & Desrosiers 1990) to investigate the importance of the PXXP motif *in vivo*. Changes from PXXP to AXXA at positions 104 and 107 abolished the ability of Nef to associate with the PAK-related serine/threonine kinase. Whilst single nucleotide changes in the *nef* gene have been shown to rapidly revert to wild-type within two weeks of infection (Kestler *et al.,* 1991) there was only a slight selective pressure for the reversion of this double mutant. At the same time, mutation of this region did not seem to attenuate the virus, as the two rhesus macaques infected with the AXXA-Nef variant showed very weak humoral responses, rapid disease progression, high viral load and high p27 plasma antigenaemia. This data suggests association of SIV Nef with serine/threonine kinases is dispensable for high virus load and disease progression in macaques. However, the failure of infection with even wild-type SIV to induce a strong antibody response may be an indication that the two macaques used in the study by Lang *et al.* (1997) were particularly susceptible to SIV infection. Therefore, these results may not be representative of the majority of animals challenged with this virus.

An association with a cellular serine kinase therefore appears to benefit the virus in some way, as observed by the strong selective pressure for the maintenance of the PXXP motif essential for an interaction with the NAK (Khan *et al.,* 1998). Further investigation is required to be able to identify the exact mechanisms of viral replication or pathogenesis that this interaction is affecting.

b. Downregulation of CD4

The CD4 receptor is the primary receptor for SIV, HIV-1 and HIV-2 and an integral component of the T cell receptor (TCR) complex on MHC-class II restricted T-cells. A decrease in the number of T cells carrying this marker is a clinical hallmark of the immune system dysfunction characteristic of AIDS.

HIV-1 and SIV Nef have been described to downregulate cell surface CD4 expression, by inducing endocytosis of the CD4 receptor and its subsequent degradation in lysosomes. The TCR on CD4 positive cells recognises specific antigens carried by the major histocompatibility complex class II (MHC-II) molecules on antigen presenting cells and it is the extracellular domain of CD4 which binds to MHC-II (Doyle & Strominger 1987). This results in activation of an associated tyrosine kinase, $p56^{lck}$ (Rudd *et al.,* 1988) and the internalisation of CD4 (Acres *et al.,* 1986). The $p56^{lck}$ phosphorylates the ζ chain of the TCR and may also recruit another kinase, ZAP-70 (Strauss & Weiss 1992). This activates the T cell cascade, the end product of which is interleukin-2 (IL-2) production and cell proliferation (Altman *et al.,* 1990). Interference with T cell function would disrupt an important cellular cascade, promote survival of the virus in the host and benefit replication. The precise role and mechanism of CD4 downregulation, however, remains to be defined.

Downregulation of CD4 by Nef is independent of species type (Garcia *et al.,* 1993). This function of Nef was observed in murine NIH 3T3 cells transfected with retroviral vectors containing the HIV-1 *nef* gene, compared with control cells transfected with empty vector, using a variety of laboratory adapted strains of HIV-1. Thus HIV-1 Nef does not require human specific factors for this effect. Furthermore, human CD4+ cells with a truncated CD4 cytoplasmic domain were unaffected by Nef (Garcia *et al.,* 1993). The conservation of the amino acid sequence within the cytoplasmic domain of

the CD4 receptor between different species (human and murine) suggests that this region may be important for downregulation. The specificity of CD4 downregulation by Nef was shown in a study (Aiken *et al.,* 1994) whereby the surface levels of CD4 in CEM T lymphoid cells infected with wild type HIV-1 strains revealed a more rapid degree of CD4 downregulation compared with cells infected with strains mutated in *nef.*

Myristoylation has been described to be a requirement for Nef induced CD4 downregulation (Harris & Neil 1994), suggesting *nef* needs to be associated with cell membranes to exert this effect. This requirement has been assessed using the baculovirus system. Sf9 cells were co-infected with AcCD4 and other baculoviruses expressing Nef-GST, a non-myristoylated derivative (AcNef(m-)GST), myristoylated GST (AcmyrGST) or non-myristoylated GST. Fractions from lysates of these cells were immunoblotted with antibodies against GST or CD4. It was found that CD4 could be detected only when co-expressed with Nef-GST, revealing a requirement for both myristate and Nef amino acid sequences (Harris & Neil 1994).

The 20 amino acid long membrane proximal region of the cytoplasmic domain of CD4 has been described to be essential for downregulation (Aiken *et al.,* 1994). Furthermore, it has been shown that the mutation of two consecutive leucines at positions 413 and 414 to alanines within the cytoplasmic domain, generating the molecule $CD4_{LL}414_{AA}$, resulted in the complete absence of Nef mediated downregulation. This observation was confirmed by mutation of the leucine at position 414 to an alanine alone. This dileucine motif is also present in the cytoplasmic regions of CD3 γ and δ chains, where it has been described to function as an endocytic and lysosomal targeting signal (Letourner & Klausner 1992), illustrating the importance of this motif in the endocytic pathway.

The downregulation of CD4 by Nef occurs via a mechanism distinct from that of phorbol ester induced downregulation. This was demonstrated by mutation of CD4 at the serines at positions 408 and 415 to alanine and leucine respectively, and the methionine at position 407 being replaced by a threonine. All of these changes were tolerated by Nef, suggesting that Nef downregulates CD4 by an alternative mechanism which remains to be defined.

Various mechanisms for Nef induced downregulation of CD4 have been postulated. The most feasible is that Nef is acting as a connector between CD4 and the cell sorting machinery. Using chimeric integral membrane proteins consisting of extracellular and transmembrane regions of CD4 or CD8 and the membrane associated domain of HIV-1 Nef, Mangasarian *et al.* (1997) showed that Nef was able to act in *cis* as a connector between CD4 and the cell sorting machinery. This was dependent upon the presence of the dileucine motif within CD4, as previously described (Aiken *et al.*, 1994).

c. Interactions with Adaptor Proteins

Further studies to identify key components of the endocytic pathway that interact with Nef to downregulate CD4 have been performed. Clathrin coated pits (CCPs) are plasma membrane invaginated domains with clathrin on their cytoplasmic side. They play a key role in the endocytosis of membrane associated receptors, through the recognition of specific motifs in the cytoplasmic tails of the receptors.

The implication that Nef induces CD4 downregulation through interaction with members of the endocytic pathway has led to studies observing interactions between Nef and CCPs. Evidence for CD4 internalisation by Nef, by increasing the association of CD4 with CCPs has been documented (Foti *et al.*, 1997). It was observed that Nef

triggered the formation of cell surface CCP, whilst at the same time inducing CD4 endocytosis, by mediating the generation of CD4-enriched CCP. This was exhibited by tracing surface CD4 molecules with ^{125}I-anti-CD4 and labelling transferrin receptors (Tf-R) with anti-Tf-R antibodies as controls. When Nef was present, the number of CCP per cell containing only CD4 was increased three fold, whilst the effect on the CCP incorporating the transferrin receptor was only increased by approximately 7%. Furthermore, mutation of the dileucine motif at positions 413 and 414 in CD4 to alanines completely abolished the ability of Nef to induce CD4 downregulation. Nef induced the formation of CCP only in the presence of wild type CD4. Therefore, to generate CCP with the ability to internalise CD4 an intact receptor cytoplasmic tail is required.

Adaptor proteins (APs) mediate protein sorting and are present in the cell. In order for CCPs to assemble at the plasma membrane and at the trans-golgi network (TGN), an interaction is required between clathrin and these adaptor proteins (Pearse & Robinson 1990). Adaptor Protein-1 and Adaptor Protein-2 (AP-1 and AP-2 respectively) act at the TGN and plasma membrane respectively. They are heterotetramers consisting of two large subunits of approximately 100 kDa (γ and β' for TGN APs, α and β for plasma membrane APs), a medium chain of approximately 50 kDa (μ1 or AP47 for AP-1, μ2 or AP50 for AP-2) and a small chain of approximately 20 kDa (σ1 and σ2) (Figure 1.10). As well as mediating the formation of CCPs, they also recruit receptors that carry internalisation signals recognisable by the CCPs in their cytoplasmic tail.

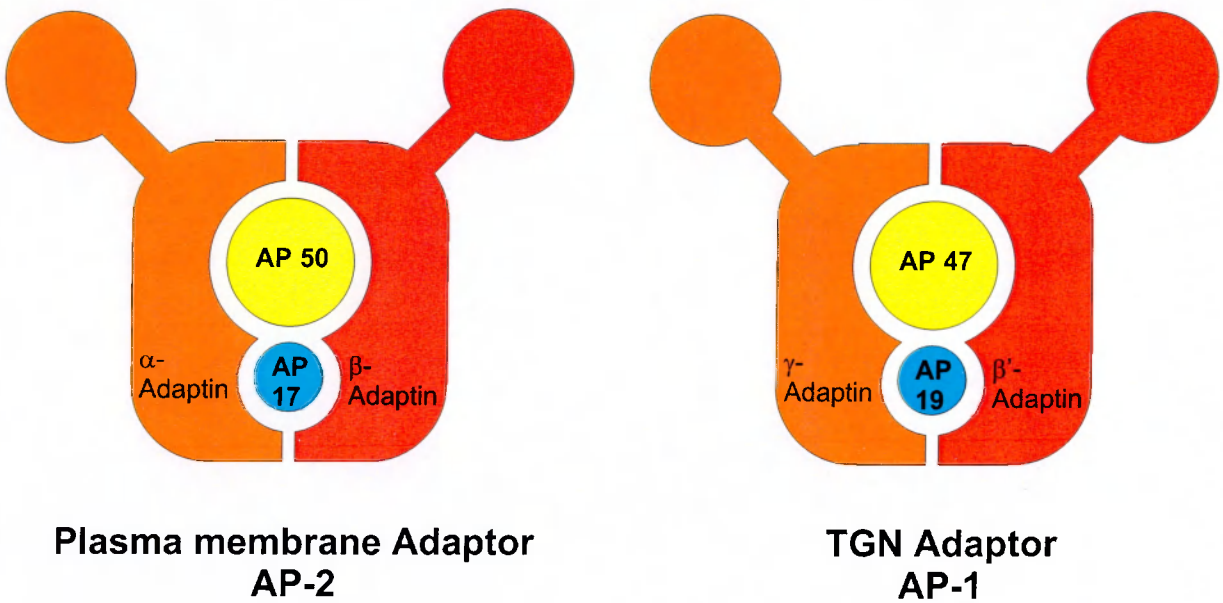


Figure 1.10. The Adaptor protein complexes. AP-1, the trans-golgi network (TGN) adaptor, AP-2, the plama membrane adaptor. They are involved in mediating protein sorting, by interacting with clathrin coated pits (CCPs) in order for the CCPs to assemble at the plasma membrane. They are heterotetramers consisting of two large subunits of approximately 100 kDa (γ and β for the TGNs APs, α and β for the plasma membrane APs), a medium chain of approximately 50 kDa (μ1 or AP47 for AP-1, μ2 or AP50 for AP-2) and a small chain of approximately 20 kDa (σ1 or AP 19 for AP-1, σ2 or AP17 for AP-2).

Adapted from: Page LJ & Robinson MS (1995). Targeting signals and subunit interactions in coated vesicle adaptor complexes. *J Cell Biol.* **131**(3): 619-30.

based motifs within the cytoplasmic domain of integral membrane proteins (Ohno *et al.*, 1995). The signal is YXXϕ, whereby Y is a tyrosine, X is any residue and ϕ is an amino acid with a bulky hydrophobic side chain and is present in a variety of membrane proteins (Trowbridge *et al.*, 1993). The medium chains have been postulated to act as signal-binding components of the clathrin-dependent sorting pathway (Ohno *et al.*, 1995).

Nef accelerates the endocytosis of CD4 by interaction with CCPs. The medium chain of adaptor complexes recognise the cytoplasmic tail of some receptors. Therefore the likelihood of a possible interaction between Nef and the medium chain of the adaptor proteins has been investigated.

Chimeric proteins consisting of the GAL 4 activation domain (see section 4.1) with either full length or truncated (amino acids 121-435) murine μ2 adaptin, or full length adaptin (AP47), were constructed. These were used as targets for an interaction through the use of yeast-2-hybrid technology with fusion proteins of GAL 4 DNA binding domain comprising HIV-1_{R7}, HIV-1_{LA1}, HIV-2_{ROD} or SIVmac239 Nef used as baits (Piguet *et al.*, 1998). A strong and specific interaction was detected with full length and truncated μ2 with HIV-2 and SIV Nef. A weaker interaction was detected with AP47, whilst HIV-1 exhibited no interaction with any of the target proteins.

The N-terminus of HIV-2_{ROD} and SIVmac239 Nef contains amino acid residues reminiscent of the endocytic signal, YXXϕ, as previously described. Mutation of the tyrosines to glycines at positions 39 of HIV-2_{ROD} Nef and 28 and 39 of SIV Nef abolished the interaction between these Nef proteins and the μ2 adaptins. These results were further confirmed with Nef proteins fused to GST (Piguet *et al.*, 1998).

However, it was also suggested that Nef may be downregulating the CD4 receptor by an additional unknown mechanism to that described, due to the low levels of CD4 downregulation that were consistently observed.

Interactions of SIV Nef with the AP-2 complex have been further demonstrated to be dependent on the presence of sequences within the N-terminus of SIV Nef (Piguet *et al.,* 1998), whilst HIV-1 Nef interacts with components of the AP-2 complex via sequences within its C-terminus (Bresnahan *et al.,* 1998). These have been confirmed by studies using SIVmac239-Nef, where co-localisation of SIVmac239-Nef GFP was observed with the β and α subunits of AP-2 (Greenberg *et al.*, 1997). This co-localisation was mediated by an N-proximal targeting element on SIVmac239 Nef, between tyrosine Y29 and glycine G38, and an N-distal element between glutamine Q58 and aspartic acid D88, as determined by examining the effect of a range of mutations and substitutions on the cellular distribution of the SIVmac239-GFP fusion protein relative to AP-2. Disruption of residues 64-67 in the N-distal AP-2 interacting element of SIVmac239-Nef also affected CD4 downregulation, whilst disruption of the sequences within the N-proximal region had no effect, suggesting that these two regions have different functional roles (Greenberg *et al.*, 1997).

CD4+ cells are involved in co-ordinating an efficient immune response, therefore downregulating the receptor favours viral replication, resulting in survival and spread of the virus both *in vivo* and *in vitro*. Removal of cell surface CD4 would impair the normal T cell activation pathway and could minimise the toxic effects of envelope and superinfection by a second virus (Weller *et al.,* 1980).

Downregulation of the CD4 receptor is a complex process dependent upon many factors. Further investigation of the interaction between Nef and CD4, and members

of the endocytic pathway may lead to a better understanding of this effect of Nef and identify possible targets for therapeutic intervention.

d. Downregulation of MHC Class I Molecules

Major histocompatibility complex (MHC) restricted cytotoxic T lymphocytes (CTL) contribute to the cellular immune response mounted against virus infections in humans. They are effector T cells which following recognition of viral protein antigens, displayed in context of MHC class I, can mediate the killing of virus infected cells. Viral antigens are processed within the infected cell into short peptide fragments, transported to the cell surface and presented in association with MHC molecules. HIV-1 specific CTLs have been detected in HIV-1 infected patients at different clinical stages of infection and isolated from different sites. HIV-1 infection may perturb MHC-class I molecule expression, affecting the ability of the host immune system to respond.

The Nef protein of HIV-1, HIV-2 and SIV downregulates the expression of MHC Class I molecules (Schwartz *et al.,* 1996), preventing the recognition and lysis of infected cells by CTLs. Infection of human CD4+ peripheral blood lymphocyte (PBL) T cell lines, CEM, HT and U937 cell lines with HIV-1 revealed a decrease in the expression of MHC class I molecules on the cell surface. Further studies using promonocytic U937 cells chronically infected with HIV-1 showed a decrease in the expression of MHC class I heavy chain at the cell surface, compared with non-infected cells (Schwartz *et al.,* 1996). The cell surface expression of MHC class I molecules was unchanged in the presence of a *nef* HIV-1 mutant in U937 cells, indicating that Nef stimulates the degradation of MHC class I molecules, similar to CD4. The ability of ammonium chloride, an inhibitor of lysosomal degradation to

restore the half life of the MHC class I molecules localised this effect to the lysosomal compartment.

Further attempts to identify the elements within the MHC class I molecules responsive to Nef by construction of mutants with deletions within the HLA-A2 (human leukocyte antigen) gene of the MHC-I molecules also revealed the cytoplasmic domain to be important (Le Gall *et al.,* 1998). The sequence YSQA (between residues 320 and 323) was observed, similar to that of the prototypic tyrosine based signal found in many membrane proteins that are endocytosed (section 1.9.1c). Mutational analysis also revealed a requirement for Tyr-320 for Nef induced downregulation. Expression of HLA-B molecules was also modulated by Nef, an effect which was observed in HeLa cells and CEM cells, whilst HLA-C molecules were unaffected. The absence of a prototypic tyrosine based motif within the HLA-C molecule could account for this.

In the presence of Nef, MHC-I molecules were also found to co-localise with clathrin and AP complexes. HeLa cells were transfected with vectors containing HLA-B7, HLA-A2 WT and HLA-A2-Y320A (HLA-A2 gene with tyrosine 320 mutated to an alanine) in the presence and absence of Nef. Intense cell surface staining was observed for HLA-B7, HLA-A2 WT and A2-Y320A in the absence of Nef, which decreased when Nef was present, with the exception of HLA-A2-Y320A which remained unaffected.

Furthermore, HeLa cells transfected with HLA-B7 in the presence of Nef showed that MHC-I colocalised with clathrin and γ-adaptin, at the Golgi and plasma membrane, whilst in the absence of Nef the MHC-1 molecules were found at the cell surface. Nef appears to be mediating the recognition of tyrosine based motifs in the MHC-1 by adaptor complexes.

By downregulating the cell surface expression of MHC-I, Nef can protect cells against lysis by CTLs (Collins *et al.,* 1998). The modulation of MHC-I expression can result in target cells becoming increasingly sensitive to NK lysis (Brutkiewicz & Welsh 1995). HLA-C molecules are not affected by Nef and can protect against NK lysis. It has been speculated that in the presence of Nef, *in vivo* infected cells exhibit limited recognition by CTLs without increased lysis by NK cells. Nef-induced escape of CTLs and NK could contribute to the survival of HIV (Le Gall *et al.,* 1998).

CTLs have been implicated as playing a critical role in the control of HIV replication. Most HIV positive individuals exhibit a strong CTL response against HIV, which is mediated by CD8+ MHC class I restricted lymphocytes. The appearance of HIV-specific CTLs is associated with the initial control of viraemia. However, during disease progression, the fall in CD4+ T cells is associated with a decrease in CD8+ T cell numbers and a reduction in virus specific CTL activity. A decrease in CD4+ and CD8+ cells is also associated with HIV-associated programmed cell death (apoptosis). This apoptotic event can be triggered by the binding of a variety of cell surface receptors to their ligands. Fas, a member of the TNF / nerve growth factor receptor superfamily and Fas ligand (FasL) appear to play an important role in the apoptotic process in HIV.

The mechanisms of protection induced by attenuated SIV (SIVmacC8) have been proposed to involve the induction of a viral-specific CTL response (Xu *et al.,* 1997). Although CTL responses were elicited from SIVmacJ5 infected animals, at 3 months post infection there was a striking difference in the responses of the animals to challenge with C8 and J5. All C8 infected animals showed multiple virus specific CTL responses to *nef, gag / pol, env,* RT, *rev* and / or *tat.* By contrast, no detectable

virus specific CTL activity was observed in PBMC of J5 infected animals. The degree of apoptosis in each set of infected animals was investigated. Spontaneous apoptosis was found to be higher in J5 infected macaques, therefore implying that CD8+ T cells from J5 infected animals are more vulnerable to apoptosis than those from C8 infected animals. The J5 infected animals showed a higher level of Fas expression than C8 and a greater degree of FasL upregulation compared with C8.

The strong CTL activity observed in the C8 protected animals correlated with a lower frequency of apoptosis of CD4+ and CD8+ T cells. It was suggested that the failure of C8 infected CD4+ cells to upregulate FasL expression avoided apoptosis and could allow the generation of an efficient antiviral response. By contrast, the J5 infected CD4+ cells upregulated FasL expression, which induced apoptosis of SIV-specific lymphocytes, including CTLs expressing Fas. This resulted in an increase in virus pathogenicity and subsequent disease progression. Therefore, the upregulation of FasL represents a mechanism by which the virus can evade the immune response, by interfering with the development of an effective CTL response (Xu *et al.,* 1997).

e. Interactions with Proteins containing SH3 Domains

Src Homology 3 (SH3) domains are present in a variety of proteins involved in intracellular signalling and cytoskeletal organisation. The Src family of protein tyrosine kinases includes 9 members; Src, Lck, Fyn, Yes, Blk, Fgr, Lyn, Hck and Yrc. A short proline rich motif acts as a ligand for SH3 domains and a minimal PXXP consensus sequence has been identified as a critical determinant for SH3 binding.

Nef possesses an SH3 binding motif between amino acids 69-78, a domain which is well conserved amongst *nef* isolates. These residues are repeated in every third

position of the polypeptide, occurring in HIV-1 as a tetraproline motif, whilst HIV-2 and SIV Nef have three such repeated prolines.

The interactions between Nef and members of the Src family kinases were investigated by generating a Nef peptide containing amino acids 65-82 of HIV-1_{NL4-3}. This contains the tetraproline PXXP motif as a fusion with GST (Saksela *et al.,* 1995). Specific binding to Hck and Lyn SH3 domains was observed, whilst there was no binding to the SH3 domains of Lck and Fyn. These observations confirmed that a minimal PXXP motif is required within Nef in order to interact with proteins containing SH3 domains. Furthermore, due to the selectivity observed, these interactions appear to be highly specific.

Mutational analysis of full length HIV Nef protein, expressed as GST fusions, revealed an absolute requirement for the two internal prolines at positions 72 and 75. Mutation to alanine abolished the binding of Hck SH3, confirming this interaction was indeed mediated by the PXXP motif. The binding of Lyn SH3 appeared to be dependant upon the arginine residue at position 71 and was significantly weaker than that observed for Hck. Further investigation revealed the presence of a single PXXP motif in the C terminus of Nef, disruption of which significantly affected the ability of Nef to interact with Hck, even in the presence of an intact N terminal PXXP motif. The binding to Lck was affected to a much less degree than that of Hck, suggesting that in this interaction the N terminal PXXP motif was the more critical factor. Mutation of the PXXP motif does not impair the ability of Nef to downregulate CD4. Furthermore, viruses containing intact *nef* genes grew significantly better than those containing mutations within the PXXP motif in PBMCs, revealing a requirement of intact PXXP for Nef-mediated enhancement of HIV replication (Saksela *et al.,* 1995). The interaction with proteins containing SH3 domains is advantageous, as the virus

has evolved to exploit SH3 mediated cellular processes to enhance its replication capacity, as well as perturbing signal transduction pathways and preventing normal cell function.

f. Interaction with the ζ Chain of the T Cell Receptor Complex

The T cell antigen receptor (TCR) is a complex multichain structure. It consists of an antigen-binding heterodimer (Ti α/β) and five invariant chains, (CD3 γ, δ, ε, ζ_2). The CD3 chains can be divided further into γ, δ, ε and ζ chains. Expression of each of these chains is required for efficient surface expression of the TCR (Figure 1.11).

The Nef protein alters T cell receptor signalling in T cells. TCR signals resulting from antigen recognition events are involved in the regulation of all the major aspects of T lymphocyte function. The interaction of Nef with the TCR could have important implications for AIDS pathogenesis. Natural HIV-1 and SIV Nef proteins have been demonstrated to block a subset of signals emanating from the TCR in human CD4+ Jurkat T cell line, as was measured by a block to the induction of expression of the early activation marker CD69 by anti-CD3 monoclonal antibody (Iafrate *et al.*, 1997). Mutations in the core region of HIV-1 Nef significantly affected the ability of Nef to block CD3 signalling, particularly the mutation of P72 and P75 in the PXXP motif to alanine and D86 for alanine. These three amino acid residues form part of the SH3 binding surface of Nef and are required for the interaction with the Src family SH3 domains (Lee *et al.*, 1996) (section 1.9.1e).

Using a chimeric molecule, consisting of the extracellular and transmembrane domains of the ζ chain, it has been demonstrated that transducing signals can be elicited that are indistinguishable from those generated by the intact TCR (Irving &

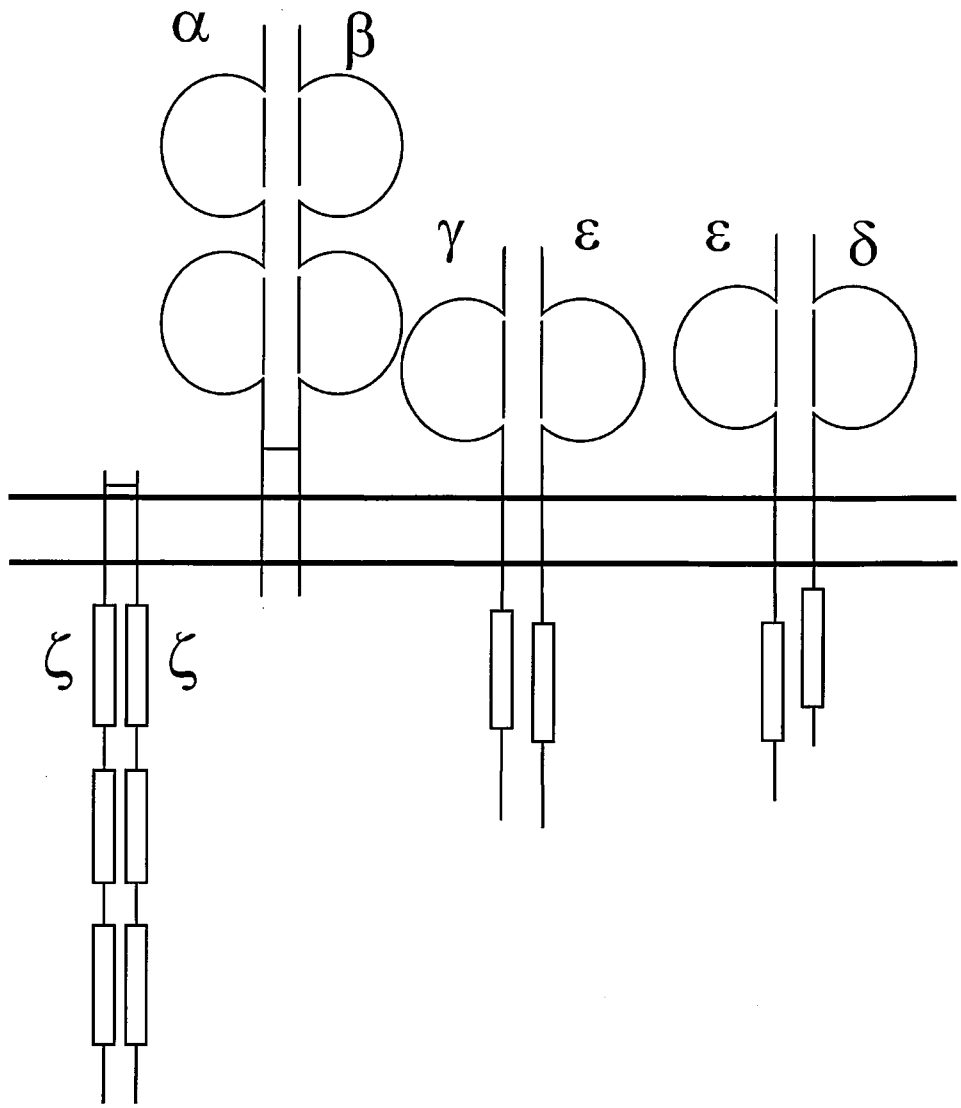


Figure 1.11. The oligomeric T cell receptor (TCR). The TCR consists of six genes, all of which are required for efficient plasma membrane expression. The α and β chains form the ligand binding subunit involved in recognition of an antigenic peptide bound to a major histocompatibility (MHC) molecule. The CD3ζ chains are responsible for signal transduction. The antigenic recognition motif (ARAM), within these chains (depicted as rectangles) is responsible for the signal transduction capability of these chains.

Adapted from: Weiss A (1993). T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell* **73**(2): 209-12

Weiss 1991). This data implies that the ζ chain is an essential component of the TCR for signal transduction.

Specific binding of Nef to TCRζ has been observed. Using deleted forms of SIVmac239 Nef protein and yeast-2-hybrid analysis Howe *et al.* (1998) showed the binding of full length Nef to TCRζ for SIVmac, SIVsm and HIV-2. Through the use of a series of amino and carboxy terminal deletion mutants of SIVmac239, the central core region (aa 98 to 235) of Nef was identified to be responsible for this binding. A parallel study by Bell *et al.* (1998) confirmed this interaction. Interactions of TCRζ with J5 and C8 Nef were investigated using yeast-2-hybrid technology. J5 was found to interact with three clones which expressed the cytoplasmic chain of TCRζ, as did SIVmac239. C8, however was incapable of binding to the ζ chain. This was confirmed by the observation that when TCRζ was co-immunoprecipitated with J5 and C8 Nef in the JJK T-cell line, J5 exhibited the highest efficiency of co-immunoprecipitation. Furthermore, J5 and SIVmac239 Nef proteins downmodulated the cell surface expression of the TCR-CD3 complex, whilst C8 had no effect.

The ability of these viruses to disrupt T cell function could provide a selective advantage for the virus by reducing the ability of the host to mount an efficient immune response. Furthermore, Nef could have an effect on a range of T cell responses, which could in turn have a direct effect on the viral life cycle *in vivo*, such as functional activation and programmed cell death.

1.14. Summary

The *nef* genes of SIV, HIV-1 and HIV-2 can exert multiple that have been widely documented both *in vivo* and *in vitro*. The ability of Nef to exploit a variety of host mechanisms in order to maintain efficient viral replication *in vivo* reveals the highly

adaptable nature of the immunodeficiency viruses and makes the treatment and prevention of these viruses a difficult task. The *nef* gene is therefore an important part of the viral genome to study in order to further our understanding of the behaviour of the virus *in vivo* and develop a novel strategy against the virus.

1.15. Objectives

Numerous *in vitro* properties of Nef have been outlined, as well as the role it has *in vivo* through use of the SIV model of infection and the study of asymptomatic patients. However, it still remains unclear which of the numerous properties ascribed to Nef is responsible for the dramatic effect it has on viral replication *in vivo*.

By using the SIV model system the biological properties of Nef can be investigated. Through the study of different SIV clones which differ only in sequences within the *nef* gene it is possible, by using *in vitro* based assays, to relate *in vivo* and *in vitro* biological properties to the differences that exist between the clones.

The aim of this project is to examine the functional differences that exist between clones of SIV differing only in the *nef* gene and attempt to relate the results to the differences observed in the pathogenic outcome of these viruses *in vivo*.

This will be achieved using a range of molecular clones that differ only in their *nef* sequence (see section 3.1). The experiments will involve:

- Antigenic studies of SIV *nef* clones expressing wild type and attenuated phenotypes in macaques.
- The ability of SIV *nef* clones, expressing wild type and attenuated phenotypes in macaques to interact with members of the signal transduction pathway

2. Materials & Methods

2.1. Methods used in antigenic studies

2.1.1. Polymerase chain reaction (PCR)

PCR reactions contained 1.2 mM magnesium chloride, 10 μM dNTPs, 0.5 μM of each primer, 2.5 units Ampli Taq Gold™ polymerase (Perkin-Elmer, Roche, UK), template DNA and buffer supplied with the enzyme in a final volume of 100 μl. Primers had been designed to incorporate *Bam*HI and *Eco*RI restriction sites to facilitate cloning. The reaction was incubated in a thermal cycler (Hybaid, UK) at 94°C for 10 minutes followed by 40 cycles of 94°C for 1 minute, 55°C for 2 minutes and 72°C for 3 minutes. This was followed by a 10 minute incubation at 72°C and the reaction cooled to 4°C.

2.1.2. Analytical gel electrophoresis

Agarose gels were prepared in 1 x TAE buffer and electrophoresis performed using the Horizontal gel electrophoresis system, model H4, BRL, USA.

DNA samples (10 μl) were added to 3 μl 5x sample buffer and run on a gel against a Φx174 and/or λ*Hind*III molecular weight marker at 150V for approximately 1.5 hours. Following electrophoresis, gels were stained using 1 μg/ml ethidium bromide (in water) for 10 minutes and destained in water for 15 minutes. DNA was visualised by transillumination and the result recorded photographically.

2.1.3. Southern blotting

To transfer nucleic acids from an agarose gel onto a membrane for detection by radio-labelled probes, gels were immersed with gentle agitation in denaturing solution for

20 minutes (to denature double stranded DNA) followed by neutralising solution for 1 hour (to neutralise the DNA).

A sheet of stiff plastic was placed over a tray and a piece of blotting paper folded and laid on top, ensuring the blotting paper hung over the edges and into the tray. Approximately 500 ml of 6 x SSC was poured over the blotting paper and into the tray, ensuring the overhanging edges of the blotting paper were immersed in the buffer. The denatured gel was placed on top of the blotting paper, reverse side up (to ensure optimum transfer of DNA) and a piece of nylon membrane (cut to the size of the gel) placed on top of the gel. An additional three pieces of blotting paper were laid on top and wetted with 6 x SSC. A stack of absorbant paper towels was placed on top and weighed down with a flat sheet of plastic and heavy weights. This was left overnight at room temperature to allow adequate transfer of nucleic acids.

The following day the membrane was moistened with 6 x SSC and the nucleic acids were cross-linked onto the nylon membrane by irradiation with ultraviolet light (UV Stratalinker^{TM} 2400, Stratagene, UK).

2.1.4. Labelling of probes

To detect a specific DNA sequence by hybridisation, oligonucleotides were end-labelled with γ^{32}P ATP, as follows.

To a microfuge tube, 2.5 µl γ^{32}P ATP (4000 Ci/mmol), 1 µl 5 µM oligonucleotide, 2.5 µl 10 x One-Phor-All buffer (Pharmacia, UK) and 1 µl T4 polynucleotide kinase were added and the volume made up to 25 µl with water. The contents were mixed by briefly pulsing in a microfuge. Contents were incubated for 10 minutes at 37°C followed by 10 minutes at 65°C.

2.1.5. Hybridisation of DNA

Membranes were incubated for approximately 45 minutes at 42°C with approximately 20 ml hybridisation buffer. Hybridisation of a specific oligonucleotide to membrane bound DNA sequences was achieved by overnight incubation at 42°C (Hybridisation oven, Hybaid, UK) of a $\gamma^{32}P$ labelled oligonucleotide with the membrane in the presence of the hybridisation buffer.

Following hybridisation, the membrane was washed three times in 6 x SSC at room temperature and exposed to X-ray film with an intensifying screen at –70°C for an appropriate period of time.

2.1.6. Purification of PCR products

The volume of each PCR reaction was made up to 200 µl with filtered water. This was extracted twice with phenol and the DNA (aqueous layer) was transferred to a fresh microfuge tube. This was further extracted with chloroform and the aqueous layer transferred to a fresh microfuge tube.

Selective precipitation of DNA molecules was achieved by the addition of a half volume of propan-2-ol and a one tenth volume of 5 M sodium perchlorate followed by incubation on ice for 5 minutes. This was centrifuged at 10,000 x *g* for 10 minutes, the supernatant was removed and the pellet allowed to dry for 5 minutes. The DNA pellet was resuspended in 96 µl filtered water. DNA (10µl) was analysed by agarose gel electrophoresis (section 2.2.3) and the results recorded photographically.

2.1.7. Restriction endonuclease digestion of PCR and plasmid DNA

Endonuclease reactions were performed as follows (using GibcoBRL products). For PCR amplified DNA, 86 µl PCR DNA, 20 units *Bam*HI, 20 units *Eco*RI and 10 µl

REact^R 3 buffer (Gibco BRL, UK) were added to a microfuge tube and incubated at 37°C for 2 hours. For plasmid DNA, 1 μg DNA, 5 units *Bam*HI, 5 units *Eco*RI, 2 μl REact^R 3 buffer (Gibco BRL, UK) and 15 μl water were added to a microfuge tube and incubated at 37°C for 2 hours.

Endonuclease digested PCR DNA (5 μl) and plasmid DNA (1 μl) were analysed by agarose gel electrophoresis (section 2.2.3) to ensure complete digestion and the result recorded photographically.

2.1.8. Purification of restriction endonuclease digested DNA

Restricted DNA was purified as described previously (section 2.2.6) and resuspended in 50 μl water. The purified DNA was analysed by gel electrophoresis (section 2.2.3) to check for recovery and the result recorded photographically.

2.1.9. Ligation of PCR DNA into plasmid

Ligation reactions contained approximately 0.25 μl restricted plasmid DNA, 1 μl, 3 μl or 9 μl of restricted purified PCR DNA (as described above), reaction buffer and 1 unit T4 DNA ligase (Gibco BRL, UK) in a final volume of 20 μl. Control reactions were also included, from which insert DNA, or insert DNA and DNA ligase were absent. The reactions were incubated at 16°C overnight.

2.1.10. Transformation of bacteria

A glycerol stock (stored at -70°C) of *E. coli* XL-1 Blue cells (Stratagene, UK) was used to inoculate 10 ml of LB broth and grown overnight at 37°C at 220 rpm in an orbital incubator (Gallenkamp). The overnight culture was centrifuged at 4°C/20,000 x *g* for 5 minutes, resuspended in half the original volume using cold 100 mM

calcium chloride and incubated on ice for 30 minutes. Cells were re-centrifuged at 4°C/20,000 x *g* for 5 minutes and resuspended in one-tenth of the original volume using cold 100 mM calcium chloride.

Bacteria were transformed by adding 10 µl of ligation product to 200 µl competent bacterial cells, chilling on ice for 30 minutes, heat shocking at 42°C for 90 seconds and incubating at 37°C with 300µl of LB broth for 2 hours. Transformed bacteria were plated onto LBamp plates and grown overnight at 37°C to select for transformants.

2.1.11. Replica plating

A grid of squares measuring approximately 1 cm x 1 cm was drawn onto the base of LBamp plates. Single bacterial colonies which had grown on the selective media were picked using a sterile loop and streaked diagonally across each square. These were incubated at 37°C overnight.

Transfer of bacterial colonies onto charged nylon hybridisation membranes (Whatman, UK) was performed by placing the membrane on top of the bacterial colonies and incubating at room temperature for 5 minutes. The bacterial colonies that had been transferred to the membrane were denatured by incubating in denaturing solution for 3 minutes, neutralised by incubating in neutralising solution for 5 minutes and hybridised with a $\gamma^{32}P$ probe specific for the DNA as described (section 2.2.4 & 2.2.5). Autoradiography was performed by exposing membranes to X-ray film at approximately -70°C for an appropriate period of time with an intensifying screen to confirm the presence of the appropriate DNA in each replica colony.

2.1.12. Preparation of DNA from transfected *E. coli*

Replica colonies identified as being positive by hybridisation were used to inoculate 3ml LBamp broth and incubated overnight at 37°C at 220 rpm in the orbital incubator. Approximately 1 ml of the overnight culture was added to 1 ml 50% glycerol in LB and stored at -70°C.

a. Small scale preparation of DNA

Small scale preparation of plasmid DNA from 2 mls of overnight culture was performed using a commercial mini-prep kit (Promega Wizard™ minipreps DNA purification system). This kit utilises a modified alkaline lysis method (Birnboim & Doly (1979), Ish-Horowicz & Burke (1981)) which is followed by the binding of the plasmid DNA to resin under low salt and pH conditions. All other impurities are removed by a medium salt wash. The plasmid DNA is eluted in a high salt buffer and concentrated and desalted by isopropanol precipitation.

DNA was resuspended in 50 µl TE buffer. The DNA was analysed for the presence of the appropriate DNA by restriction endonuclease analysis using *Bam*HI and *Eco*RI followed by agarose gel electrophoresis.

b. Large scale preparation of DNA

A glycerol stock identified as positive for the appropriate insert DNA was used to inoculate 5 ml of LBamp broth which was grown at 220 rpm at 37°C for approximately 7 hours. This was diluted into 500 ml LBamp broth and grown overnight at 220 rpm at 37°C.

A large scale preparation was performed using a commercial maxi-prep kit (Promega Wizard™ maxipreps DNA purification system). This method utilises the same

approach as the small scale preparation. Following isopropanol precipitation, DNA was eluted in 1.5 ml TE buffer. The concentration of DNA was determined using a spectrophotometer at 260 nm. Typically, a concentration of between 0.5-1.0 mg/ml was obtained, assuming 1.0 OD_{260} is equal to 50 μg/ml.

DNA was analysed for the presence of the appropriate insert DNA by restriction endonuclease analysis using *Bam*HI and *Eco*RI. The restricted DNA was electrophoresed and the result recorded photographically.

2.1.13. Production of recombinant fusion protein

a. Intitial procedure

Glycerol stocks of bacteria identified as containing the relevant DNA insert (encoding a GST-Nef fusion protein) by small and large scale preparations of DNA were used to inoculate 100 ml cultures of LBamp broth and grown at 37°C overnight at 220 rpm in an orbital incubator. This culture was then added to 900 ml LBamp broth and grown to an optical density of 0.3 at 595 nm. Recombinant protein production was induced by the addition of 1 ml 100 mM IPTG and cultures were grown for a further 4 hours. Cells were centrifuged at 4°C/20,000 x *g* for 15 minutes and the pellet resuspended in 3 ml PBS. Cells were sonicated at 5 microns for 4 minutes and centrifuged at 4°C/20,000 x *g* for 15 minutes to obtain the final protein supernatant.

b. Modified procedure

Glycerol stocks of recombinant bacteria containing the relevant DNA inserts were used to inoculate 3 ml cultures of LBamp broth which were grown at 37°C overnight at 220 rpm in an orbital incubator. Approximately 30 μl of this culture was inoculated into 3 ml LBamp and grown to an optical density of 0.3 at 595 nm. Recombinant

protein production was induced by the addition of 30 µl 100 mM IPTG and cultures were grown for a further 1 hour. Cells were centrifuged at 4°C/20,000 x *g* for 15 minutes and permeabilised by freeze-thawing using dry ice in two short bursts.

Cell pellets from each of the 3 ml cultures were resuspended in 3 ml ice cold PBS containing a predissolved commercially available protease inhibitor cocktail tablet (Roche, UK). Cells were sonicated on ice at 5 microns in 2 x 2 minute bursts and further permeabilised using 1% Triton X-100 with gentle agitation at 4°C for 1 hour. Cells were centrifuged at 4°C/20,000 x *g* for 15 minutes to obtain the final protein supernatant.

2.1.14. Preparation of samples for SDS-PAGE analysis

Samples were taken periodically during the course of protein production (1 hour after IPTG induction, after freeze thawing, after sonication and after permeabilisation using Triton X-100) for analysis by SDS-PAGE. Cells from 100 µl of culture were centrifuged and resuspended in 100 µl filtered water; 100 µl of 2 x SDS-PAGE sample buffer was added and the samples boiled for 5 minutes.

2.1.15. SDS –PAGE

The electrophoresis apparatus was set up according to manufacturer's specifications (BIO-RAD, Protean® II, xi cell, UK) (Table overleaf).

	Separating gel 12.5%	Stacking gel 3.75%
Acrylamide	9.4 ml	1.5 ml
1 M Tris HCl pH 8.8	7.5 ml	-
1 M Tris-HCl pH 6.8	-	2.5 ml
Filtered water	12.74 ml	15.7 ml
10% SDS	300 µl	200 µl
Temed	30 µl	50 µl
10% ammonium persulphate	60 µl	100 µl

Table 2.1.1. Recipe for the preparation of SDS-PAGE gels

Acrylamide solutions were prepared according to Table 2.2.1. The separating gel was poured between the glass plates and allowed to set. The stacking gel was then poured, the combs for the wells inserted and the gels allowed to set. In the apparatus, two gels were run in parallel. When set, gels were transferred to the central segment of the SDS-PAGE tank. Running buffer (800 ml) was added to the base of the tank and 350 ml added in between the two gels, to allow the flow of current. Approximately 25 µl of protein samples were loaded against a 20 µl protein ladder and the samples run at 150V for 5 hours.

2.1.16. Semi-dry electrophoration transfer

To transfer proteins onto nitrocellulose membranes for detection by Western blot, 3 pieces of blotting paper cut to the size of the SDS-PAGE gels were soaked in blotting buffer and placed on the semi-dry electrophorator base (Sartorius, UK). The gel was

laid down and a piece of Hybond–C Extra nitrocellulose membrane (Amersham Life Science, UK) laid on top for transfer of the protein. An additional three pieces of blotting paper moistened in buffer were laid on top and the voltage set to 4 mA/cm^2 of gel surface area. Transfer of protein from the gel to the nitrocellulose membrane was allowed to occur for 45 minutes.

Following transfer, membranes were blocked overnight at room temperature with gentle agitation in PBS containing 5% (w/v) milk powder/1% (v/v) Tween-20.

2.1.17. Western blotting

Membranes were washed in PBS containing 1% (v/v) Tween-20 for 5 x 5 minutes with gentle agitation at room temperature prior to incubation with either the primary monoclonal or polyclonal antibody at the recommended dilution in PBS containing 5% (w/v) milk powder/1% (v/v) Tween-20 for 1 hour at room temperature.

After antibody binding, membranes were washed further in PBS containing 1% (v/v) Tween-20 for 5 x 5 minutes with gentle agitation. Membranes were then incubated with the appropriate secondary antibody at the recommended dilution in PBS containing 5% (w/v) milk powder/1% (v/v) Tween-20 for 1 hour at room temperature. Membranes were washed 5 x 5 minutes in PBS containing 1% (v/v) Tween-20 with gentle agitation at room temperature. Western blots were developed by incubating for 1 minute at room temperature with equal volumes of Solutions I & II (ECL™ Western Blotting detection system, Amersham, UK), exposing to X-ray film for approximately 1 minute (can be adjusted to improve exposure) and developing.

2.1.18. Binding of recombinant protein to Glutathione Sepharose 4B (GS4B) beads

Approximately 2 ml samples of recombinant GST-Nef fusion protein (prepared as described in section 2.2.13) were added to 400 µl of Glutathione Sepharose 4B beads (GS4B, Pharmacia Biotech, UK) prepared according to manufacturer's specifications. The GST-Nef fusion protein was allowed to adsorb to the GS4B beads via the GST tag for 30 minutes using gentle agitation at 4°C. The suspension was centrifuged and the supernatant removed. The pelleted sepharose was washed 3 times with 1 ml PBS. Approximately 200 µl of elution buffer was added to each sample and the suspension incubated at 4°C with gentle agitation for 30 mins. The suspension was centrifuged and the supernatant, containing the Nef protein, was collected.

Before elution, samples of GST-Nef protein bound to GS4B beads were prepared for SDS-PAGE analysis as previously described (section 2.2.14). Samples of purified Nef protein, after elution, were used to coat an ELISA plate (section 2.2.20).

2.1.19. ELISA using glutathione coated plates

A 3-fold dilution of GST and GST-Nef protein in PBS containing 5% (w/v) milk powder/1% (v/v) Tween-20 was dispensed into the wells of Reacti-Bind™ Glutathione coated 96 well plates (Pierce & Warriner, UK) and incubated at room temperature for 1 hour. Plates were washed manually 5 times using PBS containing 1% (v/v) Tween-20 and incubated with a 1:20 dilution of an appropriate anti-Nef Mab in PBS containing 5% (w/v) milk powder/1% (v/v) Tween-20 for 1 hour at room temperature. Anti-GST antibody was also used, at a dilution of 1:2000.

Plates were washed extensively with PBS containing 1% (v/v) Tween-20 to ensure removal of the primary antibody. They were incubated with a 1:200 dilution of

secondary anti-mouse HRP antibody (for anti-Nef) or anti-rabbit HRP antibody (for anti-GST antibody) for 1 hour at room temperature and finally washed with PBS containing 1% (v/v) Tween-20.

ELISA substrate was added and the reaction allowed to proceed for 15 minutes. The chromogenic reaction was stopped by the addition of 25 µl sulphuric acid. The absorbance was determined spectrophotometrically at 450nm on an ELISA plate reader (Coulter Microplate Reader). The absorbance readings were analysed graphically and statistically using Microsoft Excel 97. Absorbance readings were entered into a Lotus 123 package using an in-house procedure (NIBSC, UK). End-point titres were calculated by extrapolating the linear portion of the absorbance (OD)–log dilution curve. The end-point was defined as the dilution at which the extrapolated linear portion is equal to the cut-off readings.

2.1.20. Coating of ELISA plates with recombinant GST-Nef protein

The concentration of the protein, obtained as described in section 2.2.13, was determined using a commercial protein assay kit according to manufacturer's recommendations (BIO-RAD, UK).

The recombinant GST-Nef fusion protein was diluted in filtered water and 50 µl added to each well of a labelled ELISA plate. The recombinant protein was dried onto the plate overnight at 37°C. The plate was blocked by addition of 200 µl 5% (w/v) milk powder/1% (v/v) Tween-20 for 30 minutes and washed 5 times with PBS containing 1% (v/v) Tween-20. The plate was incubated with antibodies according to the method described for the glutathione coated plates (section 2.2.19).

2.2. Methods used in yeast studies

2.2.1. Large scale preparation of plasmid DNA

A glycerol stock of *E. coli* strain DH5α (Stratagene, UK) transformed with plasmid (pASI-CYHII/*Bss*HII.I) DNA was streaked onto an LBamp plate. This plate was incubated overnight at 37°C in an incubator (Gallenkamp).

A single colony was inoculated into 3ml LBamp broth and grown at 37°C at 220 rpm for 7 hours in a bench top incubator (New Brunswick Sciences). Approximately 200 µl of this culture was used to inoculate 200 ml LBamp broth and grown at 37°C overnight at 220 rpm in the bench top incubator.

The overnight culture was used in a large scale preparation of plasmid DNA using a commercial maxi-prep kit (QIAGEN QIAfilter Plasmid Maxi Prep kit). This protocol is based on a modified alkaline lysis method (Birnboim & Doly (1979), Ish-Horowicz & Burke (1981)) followed by the binding of plasmid DNA to resin under low salt and pH conditions. All other RNA, proteins, dyes and low-molecular weight impurities are removed by a medium salt wash. The plasmid DNA is eluted in a high salt buffer and concentrated and desalted by isopropanol precipitation.

The DNA pellet obtained was dissolved in 1.5 ml TE (pH 8.0) and the concentration calculated using a spectrophotometer at 260 nm, assuming an OD_{260} of 1.0 is equal to 50 µg/ml. Typically, a concentration of between 0.5-1.0 mg/ml was obtained.

2.2.2. Sequential restriction endonuclease digestion of plasmid DNA

Restriction endonuclease digestion was performed using 1 µg plasmid DNA, 2 units *Sal*I, 10 x buffer B (Roche, UK) in a final 50 µl volume, incubated at 37°C for 1 hour.

The plasmid DNA was then isolated using a commercial nucleotide removal kit (QIAgen QIAquick Nucleotide Removal kit). This kit utilises a silica membrane, to which the DNA adsorbs in the presence of high salt, whilst all other contaminants pass through the column provided. The final DNA was eluted in 45 µl water.

The plasmid DNA was subsequently digested using 2 units *Bam*HI, 10 x buffer B (Boehringer Mannheim) in a total volume of 50 µl. This was incubated at 37°C for 1 hour.

2.2.3. Dephosphorylation of plasmid DNA

The digested plasmid DNA (50 µl) was dephosphorylated by incubation with 1 µl calf intestinal phosphatase at 37°C for 1 hour.

2.2.4. Preparative gel electrophoresis

Agarose gels were prepared in 1 x TBE buffer (containing 5 µg/ml ethidium bromide) and electrophoresis performed using the Horizontal® 58 gel electrophoresis system (Gibco BRL). Sample (9 µl) was added to 1 µl 10 x DNA gel loading buffer and run on a gel against a 1 kb DNA ladder at 100 V for 1 hour. DNA was visualised by transillumination and the result recorded photographically.

2.2.5. Extraction of plasmid DNA from agarose gels

A slice of gel containing the plasmid DNA was excised carefully using a sterile scalpel and the DNA extracted from the gel using a commercial kit (QIAgen QIAquick Gel Extraction Kit). This kit utilises the same principle as the nucleotide removal kit. The DNA was eluted in 30 µl sterile water.

2.2.6. Transfection of pGEX-4T3-*nef* DNA into *E. coli* DH5 α cells

Approximately 1 ng of pGEX-4T3-*nef* maxi-prep DNA (section 2.2.12b) was used to transform competent *E. coli* DH5α cells. Competent *E. coli* DH5α cells were removed from storage at –80°C and thawed on ice for 10 minutes. Cells (40 µl) were added to 1 ng DNA. As a control, pUC19 plasmid DNA (1 ng) was added to 40 µl DH5α cells. Mixtures were chilled on ice for 30 minutes and heat shocked at 42°C for 1 minute. Samples were further incubated on ice for 2 minutes prior to addition of 900 µl LB broth and incubated for 1 hour at 37°C at 225 rpm in the bench top incubator. A sample of the cells (200 µl) was plated onto LBamp plates and incubated at 37°C overnight.

2.2.7. Preparation of pGEX-4T3-*nef* DNA

Six single colonies (transformants) from each different pGEX-4T3-*nef* clone were picked, inoculated into 6 ml LBamp broth and grown overnight at 37°C at 220 rpm in the bench top incubator.

a. Midi scale preparation of DNA

Four of the pGEX-4T3-*nef* overnight cultures were used in a midi scale preparation of DNA performed using a commercial kit (QIAgen Tip 20 kit). This is based on a modified alkaline lysis method (Birnboim & Doly (1979), Ish-Horowicz & Burke, (1981)) followed by the adsorption of DNA onto silica in the presence of high salt. The final DNA was eluted in 50 µl TE (pH 8.0).

b. Small scale preparation of DNA

Four of the pGEX-4T3-*nef* cultures were used in a small scale preparation of DNA performed using a commercial kit (QIAGEN QIAprep Spin miniprep kit). This kit is based on the same principle as the QIAgen Tip 20 kit. The final DNA obtained was eluted in 50 µl TE (pH 8.0).

c. Restriction endonuclease digestion of DNA

The DNA obtained from midi and small scale preparations was restriction endonuclease digested sequentially using *Bam*HI and *Sal*I as described (section 2.2.2). The digested DNA was subjected to agarose gel electrophoresis and the DNA excised and extracted as described (section 2.2.5).

2.2.8. Ligation of *nef* DNA into plasmid DNA

Ligations reactions contained 5 µl restricted plasmid vector DNA (section 2.2.2), 12 µl restricted purified *nef* DNA (section 2.2.7c), reaction buffer and 1 unit DNA ligase (Sigma, UK) in a final volume of 20 µl. Control reactions were included, from which either insert DNA, insert DNA and DNA ligase, or plasmid DNA were absent. The reactions were incubated at 16°C overnight.

2.2.9. Transfection of *nef* DNA into plasmid DNA

A 5 µl portion of the ligation mix was transfected as described (section 2.2.6) into competent *E. coli* DH5α cells. Cells were plated onto LB plates and incubated overnight at 37°C.

2.2.10. Preparation of pASI-CYHII/*Bss*HII.I-*nef* DNA

a. Small scale preparation

For each transformed clone, six single colonies of *nef* DNA ligated into plasmid vector DNA (pASI/CYHII-*Bss*HII.I-*nef*) were inoculated into 3 ml cultures of LBamp broth and grown overnight at 37°C at 220 rpm, in the bench top incubator.

An overnight culture (1 ml) was added to 1 ml 50% glycerol and stored at –80°C. A small scale DNA preparation of the remainder of the culture was performed as described (section 2.2.7b). The DNA obtained was digested sequentially with restriction endonucleases *Sal*I and *Bam*HI (section 2.2.2), gel electrophoresed (section 2.2.4) and visualised by transillumination to detect for the presence of the appropriate DNA insert.

b. Large scale preparation of DNA

A positive glycerol stock (for each pASI/CYHII-*Bss*HII.I-*nef* clone) identified by restriction endonuclease analysis of small scale preparations of DNA was used to streak LBamp plates and incubated overnight at 37°C to generate single colonies. A single colony was picked and inoculated into 5 ml LBamp broth and grown overnight at 37°C at 220 rpm for approximately 7 hours in a bench top incubator. Approximately 100 µl of this culture was inoculated into 100 ml LBamp broth and grown overnight at 37°C at 220 rpm in the bench top incubator. A large scale DNA preparation was performed using a maxi-prep commercial kit as described (section 2.2.1). The concentration of DNA was determined at 260 nm using a spectrophotometer.

2.2.11. Restriction endonuclease analysis of large scale DNA preparations

Maxi prep DNA (1 μg) was sequentially digested using restriction endonucleases *Sal*I and *Bam*HI as described (section 2.2.2). The presence of the appropriate DNA insert was determined by analytical gel electrophoresis and transillumination and the result recorded photographically.

2.2.12. Sequencing of pASI-CYHII/*Bss*HII.I-*nef* constructs

Sequencing was performed using 1 μg of DNA, 15 pmol primer and 1.5 μl DMSO in a final 30 μl reaction volume and utilising BigDye terminator chemistry. Cycle sequencing protocol was performed according to manufacturer's recommendations (Applied-Biosystems, CA, USA) for a 96-well block PCR machine. The cycle parameters were adapted for templates of approximately 9 kb and above. Neat BigDye terminator mix was used in each reaction. The cycle consisted of denaturation at 95°C for 5 minutes and then 30 cycles of annealing at 55°C for 15 seconds, extension at 64°C for 4 minutes and denaturation at 95°C for 30 seconds. Samples were stored at 15°C.

Equipment used were PE Biosystems ABI Prism 377 sequencers and ABI Prism 3700 Capillary sequencers. Sequences were analysed using Lasergene 99 software.

2.2.13. Double transformation of Y190

A plate of YEPD agar (supplemented with adenine and glucose) was streaked with a glycerol stock of Y190, wrapped with parafilm (Parafilm 'M' laboratory film, American Can™, Chicago) to prevent drying and incubated at 30°C for 3-4 days. A single colony was picked and inoculated into 10 ml YEPD media (supplemented with adenine and glucose) and grown overnight at 200 rpm at 30°C in the bench top

incubator. Approximately 1 ml of overnight culture was added to 100 ml fresh, pre-warmed YEPD media (supplemented with adenine and glucose) to an OD of approximately 0.2 at 600nm. This was then grown at 30°C at 200 rpm in the bench top incubator for approximately four hours. When the OD at 600nm was between 0.6 and 1.0 the cells were transferred into 2 x 50 ml Falcon tubes and centrifuged at 800 x *g* at room temperature for 2 minutes. The pellet was washed twice by resuspension in 20 ml water and centrifuged. The cells were resuspended in 20 ml LiAc/TE solution and centrifuged again. The pellet obtained was resuspended in 1 ml LiAc/TE solution, transferred to a microfuge tube and centrifuged at 7000 x *g* for 2 minutes. For every 100 ml of original culture the resulting pellet was resuspended in 1 ml LiAc/TE solution.

In sterile microfuge tubes, 1 μg of binding domain (BD) DNA and 1 μg of the appropriate activation domain (AD) DNA (section 4.1) were added to 5 μl of 10 mg/ml salmon sperm carrier DNA. Approximately 50 μl of cell suspension and 300 μl of 40% PEG/LiAc/TE solution were then added. The samples were mixed by gentle tapping and incubated at 30°C for 30 minutes at 150 rpm in the bench top incubator. Tubes were transferred to 42°C for 20 minutes. Cells were pelleted at 7000 x *g* for 2 minutes, the final pellet resuspended in 100 μl filtered water and spread onto minimal media agar plates (supplemented with the appropriate nutrients, but lacking tryptophan and leucine). Plates were wrapped in parafilm and incubated at 30°C for approximately 4 days, with periodical inspection for signs of colony growth.

2.2.14. Liquid assay (adapted from Harshman *et al.*, 1988)

Single yeast colonies transformed with BD DNA and AD DNA were picked in quadruplicate from selection plates and inoculated into 5 ml minimal media (supplemented with the appropriate nutrients, but lacking leucine and tryptophan), and grown overnight at 30°C at 200 rpm in the bench top incubator. Overnight cultures were added to fresh minimal medium (supplemented with the appropriate nutrients but lacking tryptophan and leucine) to an OD of approximately 0.2 at 600 nm and grown for a further four hours until the OD was between 0.6-0.8 at 600 nm. One millilitre of the culture was centrifuged for 1 minute at 6000 x *g* and the cells resuspended in 200 µl lysis buffer (see section 2.3). Cells were permeabilised by two rounds of plunging into liquid nitrogen and thawing at 37°C. Samples were placed on ice and 100 µl of the sample (V) was added to tubes containing 600 µl Z-buffer/ONPG/β-mercaptoethanol. These were vortexed and transferred to a 37°C water bath. The development of yellow colour was observed and reactions stopped by the addition of 250 µl 1M sodium carbonate. Tubes were centrifuged for 1 minute and the absorbance of the supernatant read at A_{420}. In order to normalise these readings, 1 ml water was added to a cuvette, 20 µl of the sample added and the absorbance read at A_{600}. The values obtained were substituted into the equation;

$$U = 1000 \times A420 / [(A600/W)V(To-T_1)]$$

Where A420 = absorbance at 420 nm

A600 = absorbance at 600 nm

T_1 = Time in

T_0 = Time out

W = 20 µl

V = 100 µl

Graphical representation and statistical analysis of values obtained were performed using a Microsoft Excel worksheet.

2.2.15. β-galactosidase assay

The remaining colonies were lifted onto filter discs and permeabilised by plunging into liquid nitrogen and thawing at room temperature twice. Discs were placed in a tray and 2 ml of 100 mg/ml X-gal/DMF/β-mercaptoethanol applied to the underside of each filter disc which were covered and incubated at room temperature in a fume hood for approximately 4 hours to allow colour to develop. The filters were dried overnight.

For a kinetic assay, filter discs were incubated with X-gal/DMF/β-mercaptoethanol solution for 1 hour, 2 hours or 6 hours and then dried overnight before inspection.

2.2.16. Protein production

Vector DNA (pASI/CYHII-*Bss*HII.I) and plasmids containing the appropriate DNA insert (pASI/CYHII-*Bss*HII.I-*nef*) were transfected into Y190 cells as described in section 2.2.13. One single colony was picked and inoculated into 10 ml of WHAUL media (supplemented with the appropriate nutrients but lacking tryptophan). This was grown overnight at 30°C at 220 rpm in the bench top incubator. The following day this was subcultured in fresh WHAUL media (containing the appropriate nutrients but lacking tryptophan) to an OD of 0.2 at 600 nm and grown for a further 4 hours until the OD was approximately 0.6 at 600 nm. The cultures were centrifuged at 4°C/2000 x *g* and the pelleted cells resuspended in 20 ml filtered water. Cells were centrifuged at 4°C/2000 x *g* for 3 minutes and the pellet resuspended in 300 µl 10% TCA. Glass

beads (Sigma, UK) were added to the level of the meniscus and the tubes vortexed for 5 minutes to lyse the yeast. A Pasteur pippette was used to transfer the suspension to a fresh microfuge tube, avoiding the beads. This was then centrifuged at 10,000 x *g* for 20 minutes and the supernatant discarded. The pellet was resuspended in 100 µl SDS-PAGE sample buffer containing β-mercaptoethanol and the sample heated at 100°C for 5 minutes. The pH was adjusted to neutral by adding 1 µl volumes of 5 M sodium hydroxide. The sample was centrifuged at 10,000 x *g* for 5 minutes, the supernatant removed and the samples incubated at 100 °C for 10 minutes. Samples were ready for analysis by SDS-PAGE.

2.2.17. SDS-PAGE

A 4-20% Tris-Glycine precast gradient gel was used (Novex, UK) for sodium dodecyl sulphate polyacrylamide gel electrophoresis of protein samples. Gels were set into the tank (Novex, UK) and 100 ml of Tris-Glycine SDS running buffer (Novex, UK) added between the two plates and to the base of the tank. Approximately 15 µl of protein samples were loaded against 3 µl protein marker and the gels run at 120V for 1.5 hours.

2.2.18. Semi-dry electroblotting

Before transfer, an Immobilon^{TM}-P membrane (Miilipore, MA) was soaked in 100% methanol for 1 minute and dipped briefly in SDS-PAGE running buffer. Semi-dry electroblotting (Ancos semi-dry electroblotter, model A, Denmark) was performed at 100 mA per gel, as described (section 2.2.16). After transfer membranes were blocked overnight in 3% (w/v) non fat milk powder in PBS (pH 7.4).

2.2.19. Western blotting

Western blotting was performed as previously described (section 2.2.17). The antibodies were added at the recommended dilutions in 3% (w/v) non fat milk powder/1% (v/v) Tween-20 in PBS (pH 7.4).

3. Antigenic studies of SIV *nef* clones which express wild type and attenuated phenotypes in macaques

3.1. Introduction

There is strong evidence that a functional Nef protein of human and simian immunodeficiency viruses is a critical component in determining the rate of progression to disease. Disruption of the SIV *nef* gene alone is sufficient to attenuate infection significantly (Kestler *et al.,* 1992, Rud *et al.*, 1994). Repair of attenuating mutations *in vivo* also appears to be responsible for the reversion to a wild type pathogenic phenotype (Whatmore *et al.,* 1995). Furthermore, clinical studies of HIV-1 positive patients have identified a proportion of long term non progressors (LTNPs) from whom only viruses attenuated in their *nef* genes have been isolated (Kirchhoff *et al.,* 1995, Learmont *et al.,* 1992). These attenuations appear to be responsible for these patients remaining clinically healthy.

The observation that small, yet stable genetic mutations in SIV *nef* are sufficient to disrupt key functions of the virus responsible for conferring a pathogenic phenotype could provide an invaluable tool for elucidating further information on functions of *nef in vitro*. It is now important to characterise the critical properties of *nef* that correlate with pathogenic phenotype *in vivo*.

Very little information exists on the structure of SIV Nef, whilst the crystal structure of HIV-1 Nef has been resolved (Lee *et al.,* 1996). Nevertheless through the use of monoclonal antibodies, it is possible to investigate subtle changes in the structure and conformation of novel SIV Nef variants. This can reveal potentially important information regarding key regions of the Nef protein which are required for optimal viral replication *in vivo*. As a result it may be possible to make predictions regarding the structure of the SIV Nef protein.

Previously, the structure of viral proteins from HIV envelope have been successfully characterised using monoclonal antibodies. Moore *et al.* (1993) extensively studied the structure of the HIV Envelope (Env) protein using monoclonal antibodies directed against Env proteins containing a series of deletions to elucidate key amino acid sequences important for antibody binding. Furthermore, as part of a program to design vaccines, Moore *et al.* (1994) identified which regions of the HIV Env protein are exposed on the surface, by investigating antibody binding to the SU subunit. Many of the complexities of the conformational structure predicted from these studies were proven once the crystal structure was resolved (Kwong *et al.,* 1998).

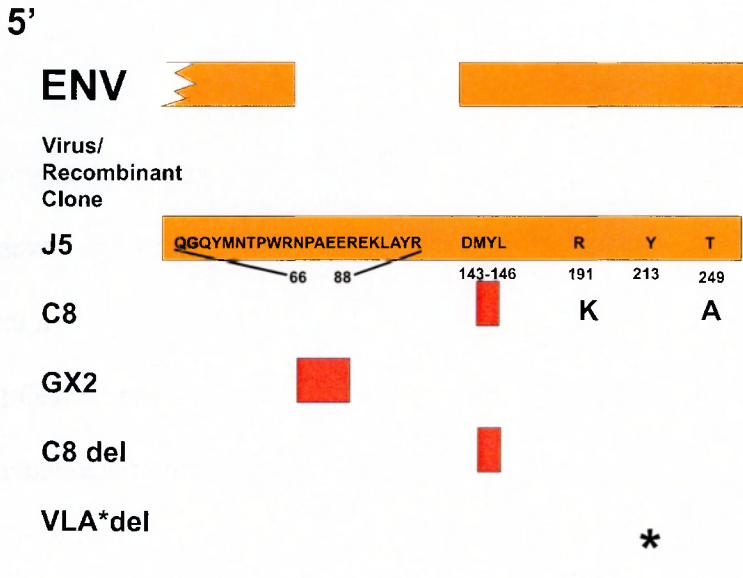
To facilitate a comparable study of the structure of SIV Nef, mutant clones of SIV have been constructed that vary only in the *nef* gene (A. Jenkins, NIBSC). These *nef* sequences are derived from wild type J5, attenuated C8, or from passage of these viruses *in vitro* or *in vivo* (Figure 3.1.1 & 3.1.2). The various deletions and/or substitutions within these parental clones are known to have a dramatic effect on the pathogenic outcome of the viral infection (Rud *et al.,* 1994, Almond *et al.,* 1995, Whatmore *et al.,* 1995). However, in addition it is necessary to have a panel of monoclonal antibodies such as those described by Arnold *et al.,* (1999). These were generated in response to the immunisation of mice with wild type SIV Nef protein, were characterised by Western blot and their specificity determined based on their reactivity in an ELISA assay using recombinant proteins and synthetic overlapping peptides (Table 3.1.1). The MAbs were divided into five separate groups (I-V) which were confirmed through competition analysis using the recombinant baculovirus J5 Nef as antigen, with all antibodies within the groups showing good reciprocal competition, with the exception of group I (Table 3.1.2). Two distinct epitopes were

Figure 3.1.1. A schematic representation of SIV *nef* clones harbouring attenuations within the *nef* gene and the effect of these attenuations on mean virus load at 2 weeks post infection, as the number of infected PBMCs/10^6. Results are the mean of a total of four animals within each group. Results are indicative of one scheduled experimental group. Animals used were cynomolgus macaques *(macaca fascicularis)*. Expermental results were taken at 2 weeks post infection only.

Wild type J5 carries a full length *nef* gene and has a virus load of 560 infected PBMCs/10^6. By contrast, the attenuated clones have a virus load that is decreased dramatically.

The red boxes denote deletions within the amino acid sequence of the clones. The asterix denotes a stop codon at amino acid 213 of VLA*del.

Courtesy of: N. Almond, Division of Retrovirology, NIBSC, personal communication.



Mean virus load at 2 weeks pi. (No. of inf'd PBMC/10^6)
2.75 +/- 0.87
0.75 +/- 0.5
1.125 +/- 0.48
1.125 +/- 0.75
0.8 +/- 0.57

Figure 3.1.2. The 45R revertant clones. Recombinant virus was grown for less than 28 days and propagated on CEMx174 cells. Reisolated virus was derived by co-culture of PBMCs taken from macaque 45R with C8166 cells and Facscan analysis performed. Sequence data was derived by PCR of the DNA in the cell pellet used in Facscan analysis.

Whatmore *et al.* (1995) used rhesus macaques for their studies, from which the reisolated virus was taken.

The deduced amino acid sequence of *nef* of virus taken from PBMCs at various time points after infection with SIVmacC8 is shown. The virus was observed to revert back to a nearly wild type sequence at 41 weeks post infection. The ability of two Nef specific monoclonal antibodies, KK70 and KK75 to bind to reisolated virus (from macaque 45R) and recombinant virus (generated by site directed mutagenesis) was investigated, by Facscan analysis.

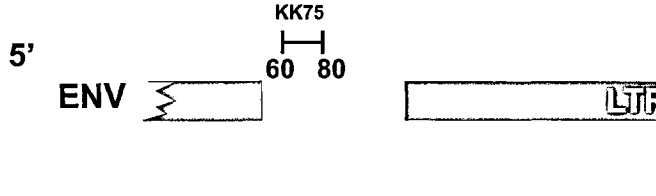
A difference in monoclonal antibody binding between the two viruses was detected at 25 weeks post infection. A spontaneous nucleotide change at position 191 was observed, to an aspartic acid (R191E), in the reisolated virus. Due to the coincidence of this position being mutated in C8 to a lysine residue (R191K) and that the mutation of R191E appeared to disrupt monoclonal antibody binding by Facscan analysis attention was focused upon this position as having an important role in protein conformation.

Red box denotes a deletion within the *nef* gene, p/i; post infection.

The linear epitope for Nef specific monoclonal antibody KK75, is shown between *env* and the 3' LTR. The epitope for KK70 has not been defined.

45R revertant sequence data adapted from: Whatmore AM, Cook N, Hall GA, Sharpe S, Rud EW, Cranage MP (1995). Repair and evolution of *nef in vivo* modulates simian immunodeficiency virus virulence. *J. Virol.* **69** (8): 5117-23.

Recombinant virus and Facscan analysis: Courtesy of N. Almond, Division of Retrovirology, NIBSC, personal communication.



		Reisolated virus		Recombinant virus	
		KK 70	KK 75	KK 70	KK75
J5	DIYL	+	+	+	+
	143-146				
C8	K (191) A (249)	-	+	-	+
45 R Revertant Sequences: 17 Wks (p/i)	EKIL	-	+	-	+
25 Wks (p/i)	EIYL	+	+	-	+
41 Wks (p/i)	DIYL	+	+	+	+

	ELISA end-point titre against indicated antigen					
					EVA777 Nef Peptides	
Group	**MAb**	**J5 Nef**	**C8 Nef**	**HIS-Nef**	**Peptide 7 (60-80)**	**Peptide 8 (70-90)**
I	KK71	3.3	2.8	-	-	-
	KK76	3.7	3.0	-	-	-
II	KK78	3.3	2.2	3.3	-	-
	KK79	3.0	1.9	2.8	-	-
	KK80	3.9	2.8	3.5	-	-
	KK81	3.4	2.3	3.2	-	-
III	KK70	3.2	-	2.1	-	-
	KK74	3.4	-	2.1	-	-
IV	KK73	4.4	3.8	3.5	5.7	-
	KK75	3.8	3.7	3.6	4.9	-
V	KK77	4.5	4.1	3.7	-	6.3

Table 3.1.1. The reactivity of monoclonal antibodies with recombinant Nef proteins and synthetic peptides.

J5 and C8 Nef proteins were derived from recombinant baculovirus, the HIS-Nef protein was purified from recombinant *E. coli* expressing BK-28 Nef and the EVA777 Nef peptides were based on the sequence of SIVmac32H. Recombinant proteins were used at 1 μg/ml and peptides were used at 2.5 μg/ml. All antigens were prepared in distilled water. Results are expressed as $\log_{10}$ end-point titres. -, ELISA end point <1.5.

Adapted from: Arnold C, Jenkins A, Almond N, Stott EJ, Kent KA (1999). Monoclonal antibodies recognise at least five epitopes on the SIV Nef protein and identify an *in vitro*-induced mutation. *AIDS Res. Hum. Ret.* **15**(12): 1087-1097

		Maximum percent inhibition by indicated unlabeled Mab										
				Grp II				Grp III		Grp IV		Grp V
Grp	**Biotinylated MAb**	**Grp Ia (KK71)**	**Grp Ib (KK76)**	**KK78**	**KK79**	**KK80**	**KK81**	**KK70**	**KK74**	**KK73**	**KK75**	**KK73**
Ia	KK71	100	0	100	90	100	60	0	0	0	0	0
Ib	KK76	0	90	50	50	40	40	0	0	0	0	0
II	KK81	100	100	100	100	100	100	100	70	0	0	0
III	KK70	0	0	0	0	0	0	90	90	0	0	0
	KK74	60	60	<10	<10	0	0	80	90	<10	<10	<10
IV	KK75	0	0	0	0	0	0	0	0	90	90	90
V	KK77	0	0	0	0	0	0	0	0	90	90	90

Table 3.1.2. The specificity of monoclonal antibodies based on competition analysis. Results are shown as the maximum percent inhibition of biotinylated antibody in the presence of unlabeled competitor. Group I to V are those defined by the reactivity of the MAbs with recombinant Nef proteins and synthetic peptides.

Adapted from: Arnold C, Jenkins A, Almond N, Stott EJ, Kent KA (1999). Monoclonal antibodies recognise at least five epitopes on the SIV Nef protein and identify an *in vitro*-induced mutation. *AIDS Res. Hum. Ret.* **15**(12): 1087-1097

defined, using overlapping synthetic peptides, spanning amino acids 60-80 (group IV) and 70-90 (group V). The other epitopes recognised by the remaining groups of monoclonal antibodies were thought to be partially conformational due to their inability to react with any of the linear peptides (Arnold *et al.,* 1999).

As summarised in Figures 3.1.1 and 3.1.2 wild type J5 virus exhibits binding by KK70 and KK75 and a virulent *in vivo* phenotype, where the viral load is 560 infected cells per 10^6 PBMC. By contrast, C8 is not recognised by KK70 and has an attenuated *in vivo* phenotype, with a virus load 100-fold lower. This would suggest KK70 recognised a conformational epitope which was disrupted by the attenuation in C8 (Arnold *et al.*, 1999).

The conformational structure of the KK70 epitope was confirmed when binding of KK70 was disrupted in the clone VLA*del (Arnold *et al.*, 1999). This clone was constructed by site directed mutagenesis based upon reproducible changes in the *nef* gene sequence following passage of SIVmacJ5 on C8166 cells. In two independent experiments reported by Arnold *et al.,* (1999), a premature stop codon was generated at amino acid 213, by a G>A change at nucleotide 9713, disrupting the ability of monoclonal antibody KK70 to bind. SIVmacJ5 virus with this reconstructed truncated Nef protein also exhibits an attenuated phenotype *in vivo* (Figure 3.1.1).

Clone C8del is a molecular clone derivative of C8 and carries only the four amino acid deletion between amino acids 143-146, with no additional nucleotide changes relative to J5. This deletion is sufficient to disrupt binding of KK70 by Facscan analysis of virus infected cells, indicating that amino acids 143-146 are an important part of, or are in close proximity to the conformational epitope that KK70 recognises (N. Almond, personal communication).

Clone GX2 was isolated from macaque I227 one month after challenge with an uncloned stock of virus known as the 11/88 stock of SIVmac32H. Relative to wild type J5 virus it contains a naturally occurring deletion between amino acids 62-83, which lies directly within the linear peptide sequence recognised by group IV and V monoclonal antibodies. This abolishes KK75 binding, but not that of KK70 (N. Almond, personal communication). The effect of the attenuation reduces average $\log_{10}$ virus titre to 13 infected cells per 10^6 PBMC.

Additional clones of SIV Nef have been isolated from a rhesus macaque (45R) following infection with the attenuated C8 virus (Whatmore *et al.,* 1995). In this study a total of seven rhesus macaques were infected with the C8 virus (see section 1.9). Six of the animals remained healthy whilst the condition of one animal (45R) declined rapidly until it was sacrificed at 58 weeks post infection. Analysis of the nucleotide sequence of the *nef*/3' LTR region of PBMC associated provirus revealed that over a 41 week period the sequence had evolved to near wild type, and in doing so the virus appeared to have regained the highly pathogenic disease characteristics of a wild type J5 phenotype (Figure 3.1.2, Whatmore *et al.,* 1995). A flow cytometry based comparison of virus from macaque 45R re-isolated by co-culture on C8166 cells and of recombinant viruses generated by site directed mutagenesis in a J5 backbone revealed differences in the binding of monoclonal antibodies KK70 and KK75 (Figure 3.1.2) (K. Kent, A. Jenkins, C. Arnold, N. Almond, personal communication).

At 41 weeks post infection a discrepancy was revealed between re-isolated virus and recombinant virus. Whilst the re-isolated virus bound to KK70 and KK75 the recombinant virus, expressing the same motif, did not bind to KK70. The reasons for this discrepant pattern of antibody binding are unclear. However, as there are additional sequence differences between J5 and the reisolated EIYL variant, it is

possible that one or more of these changes in the *nef* gene of the re-isolated virus co-operate with the repair sequence EIYL at amino acids 143-146 and reconstruct the conformational epitope recognised by KK70.

Analysis of these sequences (Figure 3.1.3) revealed a mutation at position 191 (R>E). This amino acid location is intriguing since it is the location of one of the two other coding variations in the Nef protein that differentiate C8 and J5 (Figure 3.1.2).

The aims of this study were two fold. Firstly, to develop a quantitative method for characterisation of the binding of multiple recombinant SIV Nef proteins, derived from the clones described by Whatmore *et al.* (1995), to a panel of monoclonal antibodies (Arnold *et al.*, 1999). This assay required extensive experimentation to select a method where Nef protein could be expressed in a form that was suitable for use in this assay. The final assay format utilised glutathione-S-transferase tagged Nef protein, expressed by a bacterial system, in a 96-well ELISA-based format. The second aspect of the study was to apply this novel method to determine whether the mutation of amino acid R191E of J5 Nef would complement the amino acid motif EIYL at positions 143-146 and restore the conformational epitope recognised by KK70. The sequences of these SIV Nef proteins used in this study are shown in appendix 1, Figure 7.1.

```
            1                                                    50
Recom-EIYL  ------------------------ ------------------------
Reiso-EIYL  ------------------------ ------------------------
 Consensus  MGGAISRRRSKSAGDLRQRLLRARG ETYGRLLGEVEDGYSQSLGGLDKGL

            51                                                   100
Recom-EIYL  ------------------------ ------------------------
Reiso-EIYL  ------------------------ ------------n-----n-----
 Consensus  SSLSCEGQKYNQGQYMNTPWRNPAE EREKLAYRKQNMDDVDEEDDDLVGV

            101                                                  150
Recom-EIYL  ------------------------ ------------------------
Reiso-EIYL  ----------------i-i------ ------------------------
 Consensus  PVMPRVPLRTMSYKLAVDMSHFIKE KGGLEGIYYSARRHRILEIYLEKEE

            151                                                  200
Recom-EIYL  ------------------------ ------------------------
Reiso-EIYL  ------------------------ ---------------e----h----
 Consensus  GIIPDWQDYTSGPGIRYPKTFGWLW KLVPVNVSDEAQEDERHYLMQPAQT

            201                                                  250
Recom-EIYL  ------------------------ ------------------------
Reiso-EIYL  ------------------------ ----------------------a-
 Consensus  SKWDDPWGEVLAWKFDPTLAYTYEA YVRYPEEFGSKSGLPEEEVRRRLTA

            251       263
Recom-EIYL  ------------
Reiso-EIYL  ------------
 Consensus  RGLLNMADKKETR
```

Figure 3.1.3. An alignment of the nucleotide sequence of reisolated virus (reiso-EIYL) from macaque 45R at 41 weeks post infection with SIVmacC8, with virus generated by site directed mutagenesis (recom-EIYL) and wild type J5 virus. Dashes indicate 100% identity to wild type SIV J5 Nef. The R191E mutation is highlighted.

3.2. Results

3.2.1. Analysis of *nef* DNA

PCR amplification of *nef* DNA templates GX2, C8del, VLA*del, J5(EIYL), J5(EIYL+R>E), J5(R>E), J5(EKIL) and J5(DIYL) (Table 3.2.1) generated products of approximately 800 base pairs when analysed by electrophoresis through a 1.75% (w/v) agarose gel (Figures 3.2.1a & 3.2.1b). A smaller band of approximately 730bp was obtained from the amplification of GX2. This was consistent with the 66 bp deletion within this clone (see section 3.1) compared with wild-type SIV J5.

Hybridisation with a *nef* specific probe γ^{32}P-SN9763C confirmed that the PCR products were derived from the SIV *nef* gene. The PCR product derived from the *nef* construct VLA*del did not hybridise in the presence of 20% (v/v) formamide at 42°C, presumably due to the deletion of a proportion of the sequence complementary to the radiolabelled probe. However, hybridisation of the probe in the absence of formaldehyde and at 29°C was found to permit hybridisation of the probe to this clone (data not shown).

The PCR-derived DNA was phenol/chloroform extracted and digested with the restriction enzymes *Eco*R1 and *Bam*H1. The digested DNAs were subjected to electrophoresis on a 1.75% (w/v) agarose gel to confirm that the DNAs were present (data not shown). PCR products were ligated into plasmid pGEX-4T3 overnight at 16°C and transfected into *E. coli* strain XL-1 Blue. A number of recombinant clones were identified for each PCR product by colony hybridisation.

The identification of recombinant *nef* inserts in selected colonies for each construct was confirmed by restriction endonuclease analysis of a small scale preparation of

Construct	Attenuation/Mutation
J5	Wild type full length Nef
C8	Deleted between aa 143-146; R191K and T249A substitutions
C8del	Deleted between aa 143-146
VLA*del	Mutation identified in J5 virus that lost the ability to bind to KK70 following passage on C8166 cells (Arnold *et al.*, 1999). Is truncated by a premature stop codon at aa 213
GX2	66 nucleotide deletion between aa 66-88
J5(EIYL)	Four amino acid substitution with sequence EIYL between aa 143-146
J5(EIYL+R>E)	Four amino acid substitution with sequence EIYL between aa 143-146 and R191E substitution
J5(R>E)	R191E substitution in a wild type backbone

Table 3.2.1. A list of the constructs used in the study and their various attenuations and deletions. All constructs, with the exception of GX2, were generated by the site directed mutagenesis of the wild type J5 clone. GX2 was generated from an M13 clone containing an SIV *nef* insert derived by PCR from the blood of macaque I225 one month after challenge with the 11/88 pool of SIVmac251.

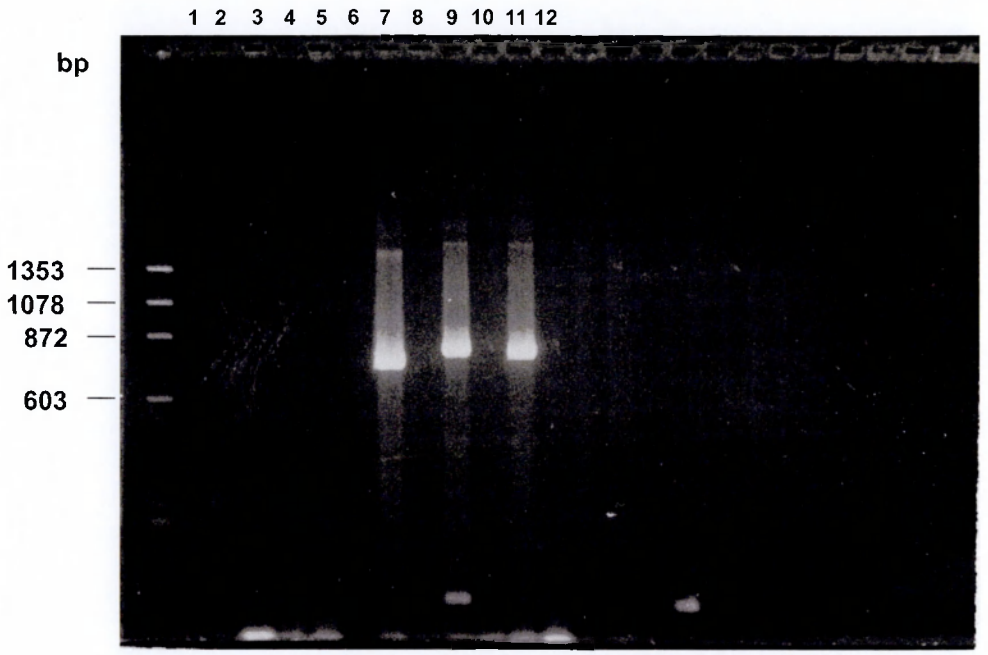


Figure 3.2.1a. Agarose gel electrophoresis of products following amplification of SIV *nef* sequences by polymerase chain reaction. Products were resolved on a 1.75% (w/v) agarose gel and visualised by staining with 1 μg/ml ethidium bromide and UV transillumination. Lanes 1-6, 8, 10, 12; negative water controls, lane 7; product of SIVmacGX2, lane 9; product of SIVmacC8del and lane 11; product of SIVmacVLA*del. The electrophoretic mobility of PCR products are compared with φX174 DNA restricted with *Hae*III (unmarked left hand lane). Bands of specified sizes in base pairs are marked.

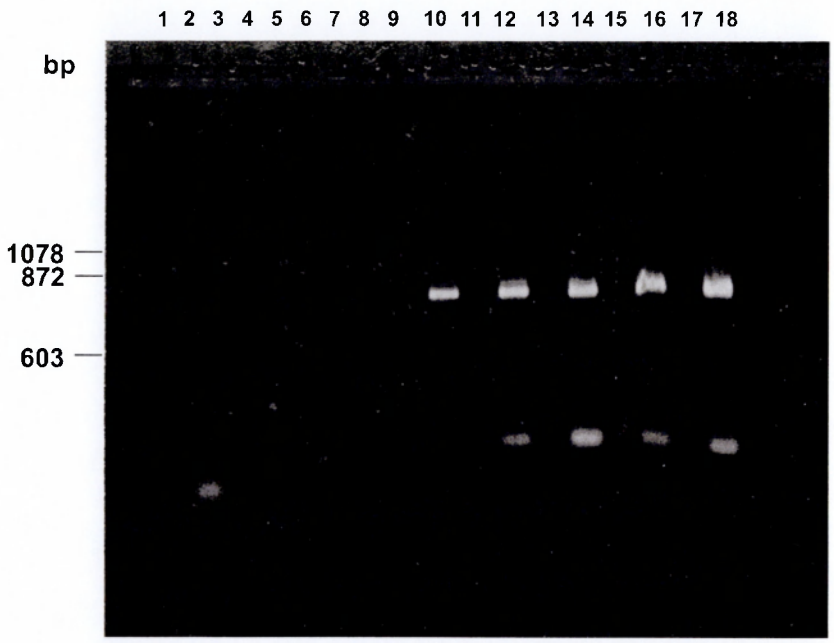


Figure 3.2.1b. Agarose gel electrophoresis of products following amplification of SIV *nef* sequence by polymerase chain reaction. Conditions as stated for Figure 3.3.1a. Lanes 1-6, 8, 10, 12, 14 and 16; negative water controls, lane 7; product of SIVmacC8del, lane 9; product of J5(EKIL), lane 11; product of J5(EIYL); lane 13; product of J5(EIYL+R>E), lane 15, product of J5(R>E), lane 17; product of J5(DIYL), lane 18; product of SIVmacJ5 (positive control). The electrophoretic mobility of PCR products are compared with φX174 DNA restricted with *Hae*III (unmarked left hand lane). Bands of specified sizes in base pairs are marked. The products towards the bottom of lanes 2, 12, 14, 16 & 18 are thought to be residual primer product.

DNA from 3 clones randomly selected for each construct (data not shown). One clone of each construct was selected for a larger scale DNA preparation and the presence of the appropriate *nef* DNA confirmed by restriction endonuclease analysis and gel electrophoresis (Figure 3.2.2).

3.2.2. Western blot analysis of GST-Nef recombinant protein

Triton X-100 soluble fractions of IPTG induced *E. coli* XL-1 Blue cultures transformed with the recombinant pGEX-4T3 plasmid containing the selected SIV *nef* genes were cleared by high speed centrifugation. Expression of the recombinant GST-Nef proteins for each construct was analysed by SDS-PAGE and immunoblotting using Nef specific monoclonal antibodies, KK70 or KK75. The analysis of selected clones is presented in Figure 3.2.3.

Recombinant fusion protein of the expected RMM of between 53-66 kDa was detected, reflecting the addition of GST (RMM 26 kDa) and Nef (RMM 27-35 kDa), for preliminary preparations of GST-GX2, GST-C8del and GST-VLA*del fusion proteins when Western blotting was performed with KK70 or KK75. However a significant proportion of the recombinant protein detected was degraded into multiple bands ranging from 30-60 kDa. Furthermore, in the absence of IPTG induction, the bacteria still expressed and degraded the fusion protein. Interestingly, for GST-GX2 fusion protein, there appears to be more protein produced when the bacteria were not induced by IPTG (Figure 3.2.3a, lane 6) compared to those that were induced by IPTG. This indicates a very high basal level of protein expression. On some occasions, little protein was obtained, evident by the difficulty of detection even by immunoblotting (data not shown).

Figure 3.2.2. Restriction endonuclease analysis of large scale preparation of pGEX-4T3-*nef* DNA, using *Bam*HI and *Eco*RI, run on a 1% agarose gel (w/v) and visualised by staining with 1 μg/μl ethidium bromide and UV transillumination. Lane 1; pGEX-4T3 undigested, lane 2; GX2 undigested, lane 3; C8del undigested, lane 4; VLA*del undigested, lane 5; J5(EIYL) undigested, lane 6; J5(EIYL+R>E) undigested, lane 7; J5(R>E) undigested, lane 8; J5(EKIL) undigested, lane 9; GX2 digested, lane 10; C8del digested, lane 11; VLA*del digested, lane 12; J5(EIYL) digested, lane 13; J5(EIYL+R>E) digested, lane 14; J5(R>E) digested, lane 15; J5(EKIL) digested. The electrophoretic mobility of the DNA is compared with φX174 DNA restricted with *Hae*III and λ DNA restricted with *Hind*III (unmarked left hand lane). Bands of specified sizes in base pairs are marked.

The appropriate *nef* gene appears to be present in all clones at approximately 800 bpwith the exception of GX2 (lane 9) that runs slightly faster due to the 66 nucleotide deletion within it. A band corresponding to *nef* at 800 bp in J5(EKIL) is absent. Attempts to re-clone this construct proved unsuccessful.

Figure 3.2.3a. Western blot analysis of recombinant GST-GX2 fusion protein, produced in *E. coli* strain XL-1 Blue, using a Nef specific monclonal antibody KK70. Lane 1; GST-J5 protein, produced by IPTG induction, lanes 2-5; GST-GX2 protein, produced by IPTG induction, lane 6; GST-GX2 protein produced in the absence of IPTG induction. A high basal level of protein expression is observed when IPTG is not added to the system, suggesting the bacteria are switching on the promoter in the absence of IPTG to produce protein. Bands corresponding to GST-J5 and GST-GX2 are present at approximately 60 kDa, reflecting the addition of GST (26 kDa) and Nef (27-35 kDa). Breakdown products are visible between 30-60 kDa. The GST-J5 protein, in this Western blot, appears relatively stable, exhibiting no breakdown.

Figure 3.2.3b. Western blot analysis of recombinant GST-C8del fusion protein, produced in *E. coli* strain XL-1 Blue, using a Nef specific monoclonal antibody KK75. Lane 1; GST-J5 protein, produced by IPTG induction, lanes 2-6; GST-C8del protein, produced by IPTG induction. Bands corresponding to GST-J5 and GST-C8 del are present at approximately 60 kDa, reflecting the addition of GST (26 kda) and Nef (27-35 kDa). In this Western blot, both GST-J5 and GST-C8del proteins exhibit breakdown products, at 30-60 kDa

Figure 3.2.3c. Western blot analysis of recombinant GST-VLA*del fusion protein, produced in *E. coli* strain XL-1 Blue, using a Nef specific monoclonal antibody KK75. Lane 1; GST-J5 protein, produced by IPTG induction, lanes 2-6; GST-VLA*del protein, produced by IPTG induction. Lanes 7 & 8; wild type GST negative control. Bands corresponding to GST-VLA*del and GST-J5 are present at approximately 60 kDa, reflecting the addition of GST (26 kDa) and Nef (27-35 kDa). Breakdown products are visible between 30-60 kDa. On some occasions it proved difficult to isolate protein, as it was present in very small amounts (lane 3-6).

Overall, it is observed that the level of binding of the monoclonal antibodies to the various GST-Nef fusion proteins on each Western blot differs considerably. This is most likely due to the difference in the renaturation of the various recombinant GST-Nef fusion proteins on the blots after preparation.

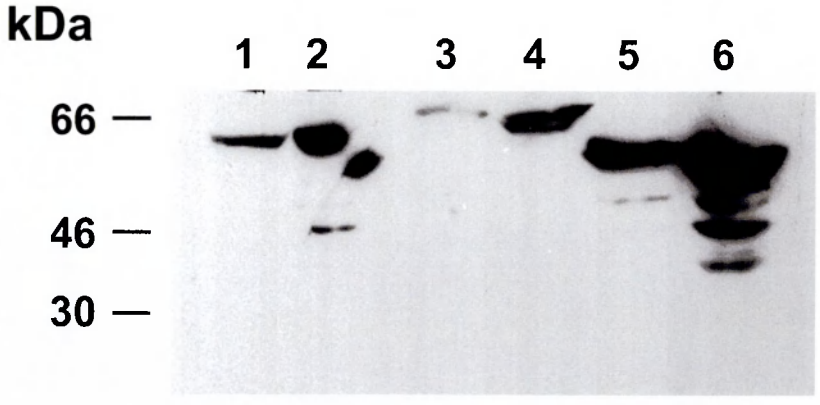


Figure 3.2.3a

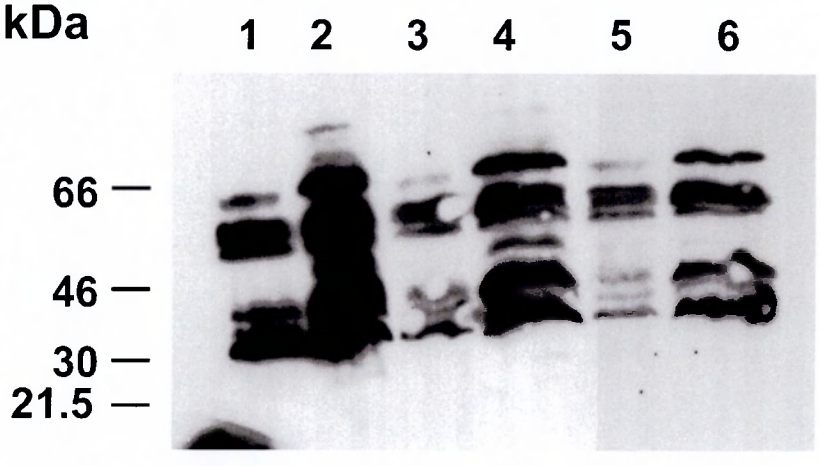


Figure 3.2.3b

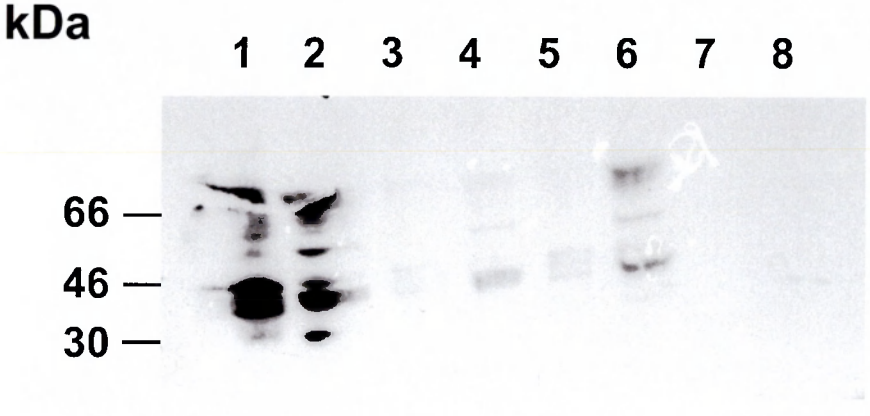


Figure 3.2.3c

To address the problem of degradation of GST Nef fusion proteins a number of modifications were introduced into the protocol. These included:

i. Use of the *E. coli* BL21 strain, which is deficient in *lon* and *ompT* genes encoding for bacterial proteases (Pharmacia Biotech, UK). The use of a strain carrying mutations which eliminate the production of proteases would be expected to reduce protein degradation.
ii. The addition of a protease inhibitor tablet (Roche, UK) containing a 'cocktail' mixture of protease inhibitors to cultures prior to preparation of bacterial lysates.
iii. The preparation of recombinant protein from small culture volumes (approximately 3 ml). Due to a high basal level of protein production, expression and breakdown of recombinant protein otherwise occurs over the extended periods of culture growth. By reducing the volume of overnight culture, the volume that is subcultured in fresh media the next day is also reduced, leading to a reduction in the amount of potentially degraded protein that is used.
iv. Optimisation of the growth period required for protein production after IPTG induction. Cultures were set up and samples were taken every hour for a total of 4 hours after induction. These were taken through the protein production protocol and analysed by Western blot to determine which time period was optimum for growth. It was observed that 1 hour after IPTG induction yielded optimal levels of full length fusion proteins (data not shown).
v. Freeze thawing with dry ice to ensure complete cell lysis, allowing release of all the recombinant GST-Nef protein into the supernatant for purification.
vi. Optimisation of sonication time period, to ensure complete lysis of bacterial cells. Samples were sonicated for 1 minute, 2 minutes, 3 minutes and 4 minutes

respectively, prior to analysis by SDS-PAGE and Western blotting. The optimum time period for sonication was determined to be 4 minutes. This was delivered as two bursts of 2 minutes of sonication, performed on ice to minimise heating of cultures and denaturation.

vii. Growth of samples at room temperature. This would reduce the rate of growth and protein synthesis, therefore minimising protein misfolding and aggregation.

Initially, the *E. coli* strain XL-1 Blue was replaced with *E. coli* strain BL21. This was observed to have a marked increased effect on protein production (Figure 3.3.4). However protein was still being degraded as was evident by the presence of multiple bands for each recombinant GST-Nef fusion protein although they were not as strong as observed in the *E. coli* XL-1 Blue strain. The combined effect of the remaining modifications produced protein of a higher yield, higher quality and increased stability (Figure 3.3.5) when analysing crude GST-Nef protein supernatant and supernatant that had been purified onto glutathione sepharose 4B beads (section 2.2.18). This was evident by the presence of a single band at the predicted molecular mass of between 53-66 kDa (Figure 3.3.5) when Western blotting was performed with a Nef specific monoclonal antibody, KK75, for GST-J5 and GST-C8. For each construct, the bands representing protein were very strong, indicating a good yield of recombinant protein. In contrast, an additional study that evaluated the quality of protein produced when the bacterial cultures were incubated at room temperature did not improve the quality of the protein (data not shown). All GST Nef protein samples used subsequently were made by the final modified protocol (section 2.2.12b).

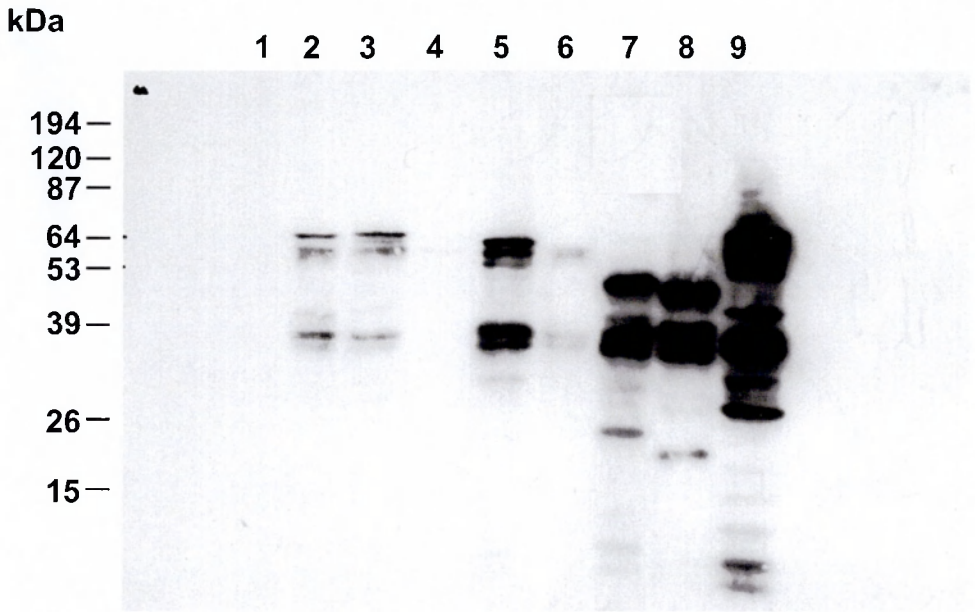


Figure 3.2.4. Western blot analysis of recombinant GST-Nef fusion protein, produced in *E. coli* strain BL21, using a Nef specific monoclonal antibody KK75. Lane 1; wild type GST negative control, lane 2; GST-C8, lane 3; GST-J5, lane 4; GST-GX2, lane 5; GST-C8del, lane 6; GST-VLA*del, lane 7; GST-J5(EIYL), lane 8; GST-J5(EIYL+R>E), lane 9; GST-J5(R>E). GST-J5(EIYL) and GST-J5(EIYL+R>E) are present at approximately 45 kDa, which is smaller than the RMM of approximately 60 kDa for the addition of GST (26 kDa) and Nef (27-35 kDa). Breakdown products, although still present appear to be minimised by the use of *E. coli* BL21, a strain which is deficient in bacterial proteases.

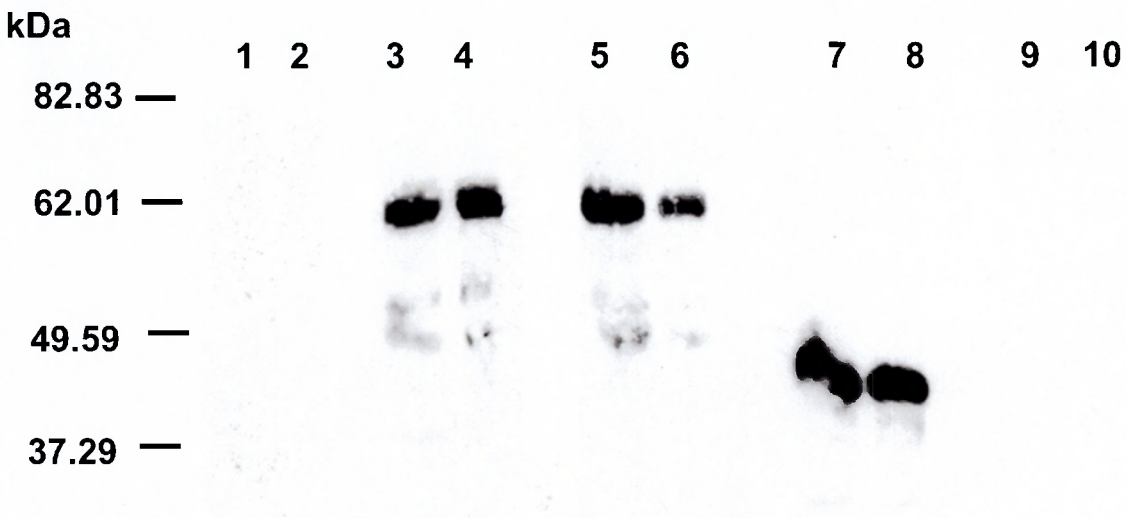


Figure 3.2.5. Western blot analysis of recombinant GST-Nef fusion protein produced in *E. coli* strain BL21 after incorporation of the modifications described in section 3.3.2, using a Nef specific monoclonal antibody KK75. GST Nef fusion proteins were prepared either as crude supernatants after Triton X100 solubilisation (section 2.2.13b) or purified onto glutathione sepharose 4B beads (section 2.2.18) and then prepared for SDS-PAGE Lane 1; wild type GST supernatant (negative control), lane 2; wild type GST bound to GS4B beads (negative control), lane 3; GST-J5 supernatant, lane 4; GST-J5 bound to GS4B beads, lane 5; GST-C8 supernatant, lane 6; GST-C8 bound to GS4B beads, lane 7; GST-J5(EIYL) supernatant, lane 8; GST-J5(EIYL) bound to GS4B beads, lane 9; GST-GX2 supernatant, lane 10; GST-GX2 bound to GS4B beads. The wild type GST is negative as expected as Western blotting was performed with a Nef specific monoclonal antibody. GST-J5 and GST-C8 are present at the expected RMM of approximately 60 kDa. GST-J5(EIYL) is present at the smaller RMM of approximately 45 kDa. GST-GX2 is not visible, as this protein contains a deletion within the linear epitope that KK75 recognises.

KK75 did not bind to recombinant GST-GX2, whilst it bound to the remainder of the GST-Nef constructs. This was expected due to the deletion within the GX2 Nef protein which encompasses the linear epitope recognised by KK75.

Protein expression from control bacterial cell lysates expressing only GST were confirmed to be negative when probed with KK75 (Figure 3.2.5).

In spite of the technical improvements, GST-J5(EIYL) and GST-J5(EIYL+R>E) recombinant proteins appeared reproducibly to be a smaller size (approximately 45 kDa) than that predicted or observed for the remainder of the GST-Nef fusion protein constructs (Figures 3.2.4 & 3.2.5).

Western blotting was performed with rabbit anti-GST polyclonal antibody to confirm the expression of the recombinant proteins as GST fusions. A product for each recombinant GST-Nef protein was observed at a relative molecular mass of 53-60 kDa (Figure 3.2.6). A breakdown product, for GST was also observed at approximately 26 kDa. Control samples of GST supernatant and GST purified onto GS4B beads also yielded a product of approximately 26 kDa. Again, GST-J5(EIYL) appears at a smaller size molecular weight of approximately 45 kDa.

3.2.3. Analysis of binding of anti-Nef monoclonal antibodies to GST-J5 Nef and GST-C8 Nef proteins by ELISA

The ability of SIV Nef alleles, expressed in bacterial cells as GST-fusion proteins, to bind selected anti-Nef MAbs was evaluated in a semi-quantitative 96 well microplate assay. Undiluted or three-fold step wise dilution of crude recombinant bacterial cell lysates (identified and characterised as described) were allowed to bind to glutathione coated plates and end point titres of binding by individual anti-SIV Nef monoclonal antibodies were determined. In each assay, the end point titre of antibody binding to

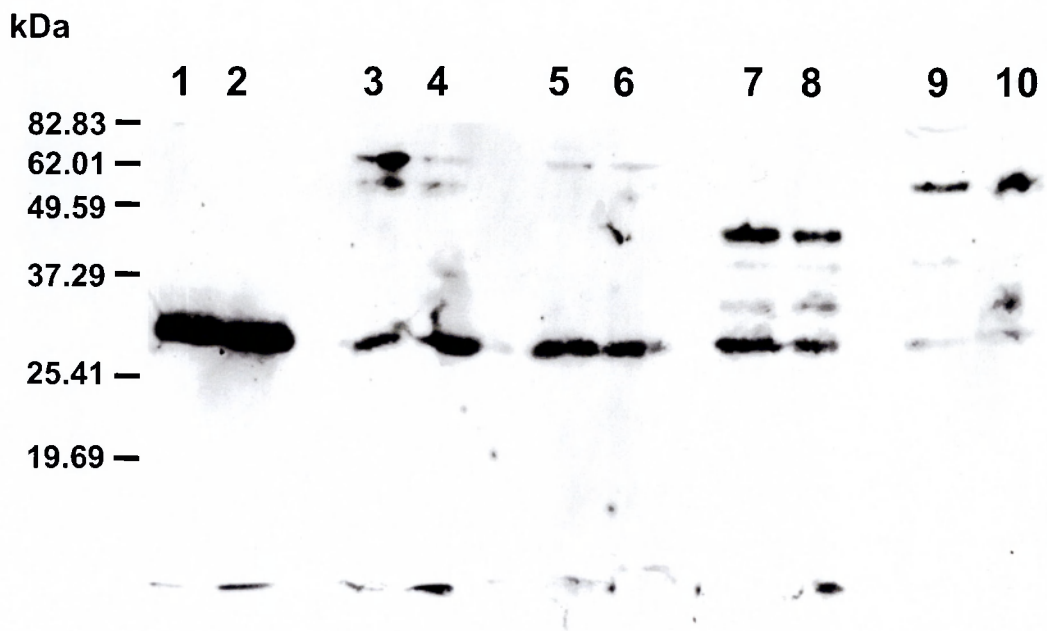


Figure 3.2.6. Western blot analysis of recombinant GST-Nef fusion protein produced in *E. Coli* BL21 after incorporation of the modifications described in section 3.3.2, using an anti-GST polyclonal antibody. GST-Nef fusion proteins were prepared either as crude supernatants after Triton X100 solubilisation (section 2.2.13b) or purified onto glutathione sepharose 4B (section 2.2.18) and then prepared for SDS-PAGE.
Lane 1; wild type GST supernatant, lane 2; wild type GST bound to GS4B beads, lane 3; GST-J5 supernatant, lane 4; GST-J5 bound to GS4B beads, lane 5; GST-C8 supernatant, lane 6; GST-C8 bound to GS4B beads, lane 7; GST-J5(EIYL) supernatant, lane 8; GST J5(EIYL) bound to GS4B beads, lane 9; GST-GX2 supernatant, lane 10; GST-GX2 bound to GS4B beads. Wild type GST is present at the predicted RMM of 26 kDa. For GST-J5, GST-C8 and GST-GX2, a band corresponding to GST-Nef is present at approximately 60 kDa, with breakdown product of GST present at approximately 26 kDa. GST-J5(EIYL) is present at the smaller RMM of 45 kDa as observed in Figure 3.3.5.

the Nef protein variant was compared to that of parental wild-type virus J5 and attenuated C8 fusion proteins and wild type GST protein alone. The results of initial studies using only GST, GST-J5 and GST-C8 are presented in Table 3.2.1. Only the anti-GST polyclonal antibody bound to the GST bacterial recombinant protein. None of the *nef* specific monoclonal antibodies bound to GST protein as predicted. All representatives from each group of monoclonal antibodies bound to wild type GST-J5 recombinant protein (Table 3.3.1). By contrast, GST-C8 recombinant Nef bacterial protein only bound anti-GST and MAbs KK75 and KK77, representing competition groups IV and V respectively (Table 3.2.1).

3.2.4. Analysis of binding of anti-Nef monoclonal antibodies to GST-SIV Nef protein by ELISA

GST-VLA*del bound representative anti-SIV Nef MAbs of competition groups IV and V only. The pattern of binding was similar to that of GST-C8 (Table 3.2.2). By contrast, GST-GX2 recognised representative monoclonal antibodies of competition groups I, II & III, but not groups IV and V that are known to recognise linear peptides between amino acids 60-80 and 70-90 respectively of the SIV Nef protein (Table 3.2.3).

GST-J5(EIYL) bound representatives of groups IV and V only, whereas GST-J5(R>E) bound representatives of each group of monoclonal antibodies in a pattern comparable to that observed for GST-J5 (Tables 3.2.4 & 3.2.5 respectively). GST-J5(EIYL+R>E) bound representatives of groups IV and V only (Table 3.2.6). None of the representative MAbs from groups I, II & III bound to this protein, as observed for GST-C8. This observation was also consistent for GST-VLA*del (Table 3.2.7).

Ab	GST-SIV Nef fusion protein		
	GST recombinant protein	**GST-J5 recombinant protein**	**GST-C8 recombinant protein**
α-GST	2.24	2.12	2.07
KK76 (I)	<0.47	1.6	<0.47
KK79 (II)	<0.47	2.12	<0.47
KK80 (II)	<0.47	2.08	<0.47
KK70 (III)	<0.47	2.32	<0.47
KK75 (IV)	<0.47	2.35	2.35
KK77 (V)	<0.47	1.68	1.21

Table 3.2.2. The ELISA endpoint titres (log_{10}) of the ability of representatives from the five groups of anti-*nef* monoclonal antibodies and α-GST polyclonal antibody to bind to recombinant GST, J5-GST and C8-GST Nef fusion proteins. The binding of the recombinant proteins to the α-GST polyclonal antibody was used as an estimate at the amount of protein that was present in each preparation. However, the amount of protein was not accurately quantified as crude bacterial lysates of the recombinant proteins were used in these experiments.

The cut-off value was calculated as the mean of the negative values, + 3 standard deviations. A result equal to or less than this value is comparable to background values and is therefore negligible.

Fusion Protein	Antibody						
	α-GST	KK76 (I)	KK79 (II)	KK80 (II)	KK70 (III)	KK75 (IV)	KK77 (V)
GST	1.64	<0.47	<0.47	<0.47	<0.47	<0.47	<0.47
J5	0.71	1.49	1.08	1.97	1.14	1.71	1.46
C8	1.22	<0.47	<0.47	<0.47	<0.47	1.84	1.66
GX2	1.25	1.69	1.46	1.84	1.43	<0.47	<0.47

Table 3.2.3. ELISA endpoint titres (log_{10}) of the ability of representatives from each of the groups of anti-Nef monoclonal antibodies to bind to GST-GX2. The cut-off value was calculated as the mean of the negative values, + 3 standard deviations. A result equal to or less than this value is comparable to background values and is therefore negligible.

Fusion protein	Antibody						
	α-GST	KK76 (I)	KK79 (II)	KK80 (II)	KK70 (III)	KK75 (IV)	KK77 (V)
GST	3.35	<0.47	<0.47	<0.47	<0.47	<0.47	<0.47
J5	2.39	2.26	2.33	2.07	3.29	1.93	2.57
C8	2.55	<0.47	<0.47	<0.47	<0.47	1.83	2.18
J5 (EIYL)	2.55	<0.47	<0.47	<0.47	<0.47	2.85	2.39

Table 3.2.4. ELISA endpoint titres (log_{10}) of the ability of representatives from each of the groups of anti-Nef monoclonal antibodies to bind to GST-J5(EIYL). The cut-off value was calculated as the mean of the negative values, + 3 standard deviations. A result equal to or less than this value is comparable to background values and is therefore negligible.

Fusion protein	Antibody						
	α-GST	KK76 (I)	KK79 (II)	KK80 (II)	KK70 (III)	KK75 (IV)	KK77 (V)
GST	2.54	<0.47	<0.47	<0.47	<0.47	<0.47	<0.47
J5	2.22	1.03	2.39	1.66	2.02	2.67	1.94
C8	2.21	<0.47	<0.47	<0.47	<0.47	2.67	1.88
J5+ (R>E)	2.49	1.68	2.77	2.2	2.73	3.46	2.45

Table 3.2.5. ELISA endpoint titres (log_{10}) of the ability of representatives from each of the groups of anti-Nef monoclonal antibodies to bind to GST-J5+(R>E). The cut-off value was calculated as the mean of the negative values, + 3 standard deviations. A result equal to or less than this value is comparable to background values and is therefore negligible.

Fusion protein	Antibody						
	α-GST	KK76 (I)	KK79 (II)	KK80 (II)	KK70 (III)	KK75 (IV)	KK77 (V)
GST	2.24	<0.47	<0.47	<0.47	<0.47	<0.47	<0.47
J5	2.12	1.6	2.33	2.07	1.93	2.35	1.68
C8	2.07	<0.47	<0.47	<0.47	<0.47	1.84	1.21
J5+ (EIYL R>E)	2.1	<0.47	<0.47	<0.47	<0.47	2.01	1.44

Table 3.2.6. ELISA endpoint titres (log_{10}) of the ability of representatives from each of the groups of anti-Nef monoclonal antibodies to bind to GST-J5(EIYL+R>E). The cut-off value was calculated as the mean of the negative values, + 3 standard deviations. A result equal to or less than this value is comparable to background values and is therefore negligible.

Fusion protein	Antibody						
	α-GST	KK76 (I)	KK79 (II)	KK80 (II)	KK70 (III)	KK75 (IV)	KK77 (V)
GST	0.8	<0.47	<0.47	<0.47	<0.47	<0.47	<0.47
J5	1.14	1.94	2.46	2.22	2.1	2.6	2.49
C8	0.96	<0.47	<0.47	<0.47	<0.47	2.73	2.04
VLA* del	0.76	<0.47	<0.47	<0.47	<0.47	2.19	1.63

Table 3.3.7. ELISA endpoint titres (log_{10}) of the ability of representatives from each of the groups of anti-Nef monoclonal antibodies to bind to GST-VLA*del. The cut-off value was calculated as the mean of the negative values, + 3 standard deviations. A result equal to or less than this value is comparable to background values and is therefore negligible.

3.2.5. Binding of Group I & II monoclonal antibodies to GST-C8 and GST-C8del by ELISA

During these studies, a low level of binding was detected for GST-C8 and GST-C8del by representatives of the group I and II monoclonal antibodies. Previously no binding of GST-C8 to either of these groups had been observed as GST-C8 was included as a control on every plate. In order to eliminate the possibility of contamination between batches of protein samples new samples were generated. New glycerol stocks were also derived by re-cloning bacterial cultures used to produce the recombinant protein, to eliminate the possibility that the original glycerol stock may have become contaminated. Low levels of binding were still observed, which were approximately 2-3 times greater than that for background binding.

These observations were in contradiction to previous experimental results which had shown consistently that these MAbs did not recognise GST-C8 recombinant fusion protein. However, the results agree with previous work by Arnold *et al.* (1999) which demonstrated binding by the group I and group II MAbs to GST-C8 Nef derived from recombinant baculovirus.

To investigate this discrepancy further, recombinant GST-C8 and GST-C8del fusion protein were affinity purified from bacterial lysates, quantified (Protein Assay Kit II, Bio-Rad, UK) and used as antigen in an ELISA.

The plates were coated with 200 μg/ml of recombinant fusion protein and a three fold dilution series performed to detect levels of binding. Levels of KK75 binding to GST-C8 and GST-C8del detected were comparable to the low levels obtained using the glutathione coated plates.

When a maximal concentration of approximately 1600 μg/ml of protein was used to coat the plates no significant levels of binding by the group I & II monoclonal

antibodies to GST-C8 or GST-C8del was detected. Low levels of binding appeared to be non specific as there was no obvious pattern of binding that reflected the dilution of the coating protein (Figures 3.2.7 & 3.2.8).

Figure 3.2.7. Analysis by ELISA of binding by representative monoclonal antibodies, KK76 (group I), KK79 (group II) and KK80) (group II) to recombinant GST-C8 Nef protein expressed in *E. coli* BL21 cells.

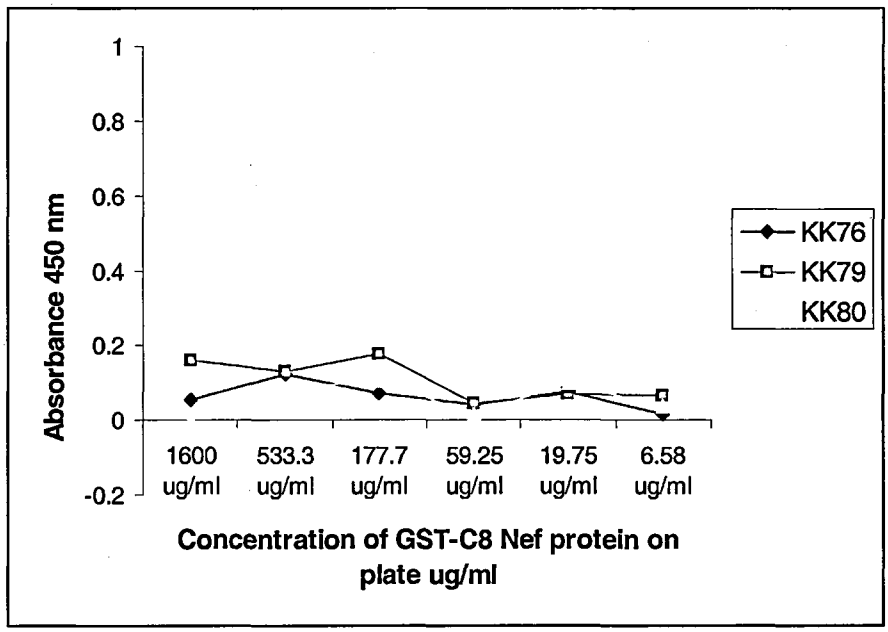
Recombinant protein was batch purified by using Glutathione Sepharose 4B beads, quantified and used to coat a 96 well plate.

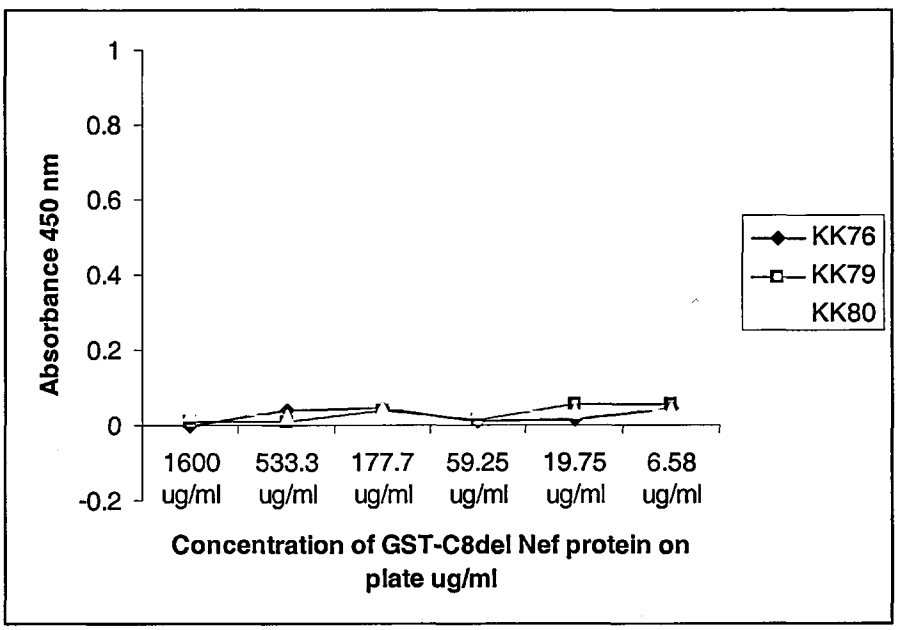
The level of binding observed, even at the highest concentration of 1600 μg/ml is comparable to that of the baseline levels of antibody binding in the absence of Nef protein. Therefore, the ability of the group I, II & III monoclonal antibodies to bind to C8 was not reproducible in this system.

Figure 3.2.8. Analysis by ELISA of binding by representative monoclonal antibodies, KK76 (group I), KK79 (group II) and KK80) (group II) to recombinant GST-C8del Nef protein expressed in *E. coli* BL21 cells.

Recombinant protein was batch purified by using Glutathione Sepharose 4B beads, quantified and used to coat a 96 well plate.

The level of binding observed, even at the highest concentration of 1600 μg/ml is comparable to that of the baseline levels of antibody binding in the absence of Nef protein. Therefore, the ability of the group I, II & III monoclonal antibodies to bind to C8del was not reproducible in this system.


1
0.8
0.6
0.4
0.2
0
-0.2
Absorbance 450 nm
1600 ug/ml
533.3 ug/ml
177.7 ug/ml
59.25 ug/ml
19.75 ug/ml
6.58 ug/ml
Concentration of GST-C8 Nef protein on plate ug/ml
KK76
KK79
KK80


1
0.8
0.6
0.4
0.2
0
-0.2
Absorbance 450 nm
1600 ug/ml
533.3 ug/ml
177.7 ug/ml
59.25 ug/ml
19.75 ug/ml
6.58 ug/ml
Concentration of GST-C8del Nef protein on plate ug/ml
KK76
KK79
KK80

3.3. Discussion

The *nef* gene of human and simian immunodeficiency viruses is believed to be a critical component in the pathogenesis of infection. However, the mechanism by which *nef* carries out this process remains poorly understood.

Whilst the crystal structure of HIV-1 Nef has been resolved (Lee *et al.,* 1996, Freund *et al.,* 1994) relatively little information exists concerning the structure of the SIV Nef protein. By studying the antigenic structure of Nef it is hoped to gain an insight into the key regions of the protein that may play important roles in viral pathogenesis. The aim of this study of mutant SIV Nef proteins, differing only in a few base pairs that are known to confer significant changes to *in vivo* phenotypes (N. Almond, personal communication) was to utilise an immunological approach to determine whether the pattern of monoclonal antibody binding can predict the underlying protein structure.

This study revealed that there are two antigenically distinct regions of SIV Nef, characterised by clones GX2 and C8 respectively, that attenuate the virus if either is disrupted. This study went on to analyse the effect on antigenic structure of repairing the four amino acid deletion in the C8 Nef protein in a manner similar to that observed *in vivo* (Whatmore *et al.*, 1995). The repair of the amino acid sequence DMYL between amino acids 143-146 with the repair sequence EIYL alone was observed to be insufficient to repair the epitope in Nef recognised by the monoclonal antibody KK70. Furthermore, the results indicate that substitution of the amino acid at position 191 (R191E) does not complement the EIYL motif to restore this epitope.

In order to evaluate the different *nef* alleles a relatively stable prokaryotic expression system was developed. Each *nef* allele was expressed as a C-terminal fusion protein

with the stabilising protein partner glutathione-S-transferase in the vector pGEX-4T3. It was important to prove with key *nef* alleles that results obtained in this relatively novel system were in agreement with previously reported data (Arnold *et al.,* 1999).

J5 exhibited the highest level of binding to representatives of all groups of Nef specific monoclonal antibodies. This would suggest that the full length recombinant fusion protein is stable in comparison with those that carry deletions and/or point substitutions. Furthermore, even conformation dependent epitopes within J5 were observed to be intact, which could result in more optimal expression as there are no disruptions within or surrounding important antigenic sites that could affect their presentation.

Arnold *et al.* (1999) (Table 3.1.1) previously demonstrated binding by the group I & II monoclonal antibodies to C8 Nef-GST baculovirus derived recombinant protein. The level of binding was approximately one log lower than that observed for J5 Nef-GST baculovirus derived recombinant protein. This was consistent for group IV and V monoclonal antibodies, which recognise linear epitopes. It is possible, that the deletion in C8 affects the protein folding and presentation of the epitope that the MAbs are recognising, resulting in a lower level of binding than that observed for wild type J5 protein in the baculovirus system.

Initially, no binding of the group I & II MAbs to GST-C8 was observed. However, during investigation of the binding of GST-C8del to the panel of MAbs, a low level of binding to the group I & II MAbs by GST-C8 and GST-C8del was detected. Investigation of this by production and purification of recombinant GST-C8 and GST-C8del fusion protein and coating of ELISA plates at high concentrations failed to demonstrate significant levels of binding by the group I & II MAbs to GST-C8 and GST-C8del. The binding observed by group I & II MAbs to GST-C8 and GST-C8del

may be a true result, representing low levels of binding by the MAbs when high concentrations of protein are present. However, when the protein is titrated to lower concentrations binding by the MAbs is no longer observed (Figure 3.2.7). In retrospect, it would have been valuable to repeat these experiments with purified GST-J5 Nef protein on a separate plate to assess the degree of binding by the MAbs to the wild type protein as a positive control, to which the results for GST-C8 and GST-C8del could be compared. Also, binding by KK75 could have been used as an additional control. Analysis of the GST-C8 and GST-C8del by SDS-PAGE in this instance was not deemed necessary due to the sensitivity of the plate assay overall being greater than that of SDS-PAGE.

It is possible that the mutations in GST-C8 and C8del could be affecting the epitopes recognised by the group I & II MAbs even if the regions attenuated do not directly form part of the epitopes. The influence of surrounding amino acid residues on antibody binding has been documented for studies of HIV gp120, whereby substitutions outside a linear sequence within a specific domain of gp120 either abolished or significantly reduced the binding of MAbs specific for that domain (Moore *et al.*, 1993).

The differences in the observations from this study and those published by Arnold *et al.* (1999) may be attributable to the different expression systems employed. Arnold *et al.* (1999) used the baculovirus system to express proteins, whilst we utilised a prokaryotic approach. One of the drawbacks of utilising a prokaryotic system is the formation of inclusion bodies as the result of overexpression of the recombinant protein of interest. If the proteins being overexpressed are not completely soluble then they may fold incorrectly and this could conceal antigenic sites. The formation of inclusion bodies does not occur in the baculovirus system, therefore the proteins

produced retain their full biological activity. Where on occasion low levels of binding were observed, this may have been due to the protein folding properly or closer to its correct conformation, resulting in the expression of antigenic sites for detection. Nevertheless, there was no direct evidence within this study that any of the recombinant fusion proteins produced formed inclusion bodies. Furthermore, the production of these recombinant proteins as a fusion with the C terminus of GST should facilitate the production of fusion proteins which are soluble in aqueous solutions (Smith & Johnson 1988).

The baculovirus system has the capacity to perform post translational modifications on the recombinant proteins produced, whilst the prokaryotic system does not. Nef is myristoylated at its N terminus by the addition of a myristoyl group (Allan *et al.,* 1985). It is possible that the myristate moiety may affect the ability of the group I and II monoclonal antibodies to bind, possibly by altering the conformation of the protein, which may make antigenic sites more accessible. This could be investigated by producing a non-myristoylated recombinant SIV Nef fusion protein using the baculovirus system and myristoylated SIV Nef fusion protein within the prokaryotic system. By analysing the ability of the different groups of monoclonal antibodies to bind, the effect of mysristoylation on antigenic structure could be determined. If the pattern of binding was identical to that observed previously then it could be concluded that the myristoyl group within this system has no effect on protein conformation. The production of HIV-1 Nef recombinant protein in bacterial cells which is myristoylated by co-expression with n-myristoyltransferase has been successful (M.Harris, personal communication). It has also been described that a non-myristoylated HIV-1 Nef has a different structure to that of a myristoylated Nef, in that it has an extended conformation (Geyer *et al.,* 1999).

The use of the bacterial expression system for the production of SIV Nef proteins appears to be robust enough for use in epitope mapping studies. SIV Nef, when expressed as a C-terminal fusion partner with glutathione-S-transferase is particularly valuable. HIV-1 and SIV Nef are myristoylated at the amino terminus when expressed in eukaryotic cells (Harris & Coates 1993, Harris & Neil 1994). This myristoylation is essential for association of a Nef protein with the plasma membrane, but whether this is a direct association of the myristoyl with the membrane, or a result of conformational changes that occur at amino terminus of the Nef protein following myristoylation remains to be determined (Harris & Coates 1993, Harris & Neil 1994). Nevertheless, it has been demonstrated by others (Azad *et al.*, 1994), that bacterially expressed recombinant HIV-1 Nef protein can be transfected into mammalian cells and function in a predictable manner indicating that it is capable of folding sufficiently to mediate selected functions, regardless of the myristoylation state. Our confidence of the predictive value of this bacterially expressed Nef protein, however, is based upon parallel data obtained from binding of antibodies to recombinant bacterial protein, as well as recombinant viruses that express the same set of Nef alleles. The equivalent of antibody binding in both systems for a number of alleles provides good supporting evidence for the predictive value of this approach of the antigenic structure for novel epitopes such as J5(EIYL) and J5(EIYL+R>E).

Initially problems were encountered in the production of bacterially expressed recombinant SIV Nef that did exhibit evidence of degradation during expression on subsequent purification. The production of recombinant SIV Nef protein in the presence and absence of IPTG, suggests the bacteria were able to drive expression of the promoter and as a consequence, expression of the GST-*nef* genes without the need for induction. Unless the promoter is tightly regulated, growing large cultures of

bacteria transformed with toxic genes for extended periods or high density will frequently lead to the synthesis of the protein and its subsequent degradation.

This effect was controlled by using smaller culture volumes for protein production. Less protein degradation was observed when 30 µl volumes of 3 ml overnight cultures were used to inoculate fresh media (3 ml) prior to induction for 1 hour. The reason for this may be that if the bacteria have been synthesising and degrading protein (during the course of the overnight incubation), only a small volume of this is used to grow a fresh culture. By harvesting after two hours of growth (1 hour before and 1 hour after IPTG induction), it minimised the extent of protein degradation. In retrospect, the use of a large culture of bacteria inoculated from glycerol stocks but harvested after just a total of 2 hours of growth may have produced protein of a reasonable quality which was not degraded due to the shorter incubation period. This approach could be adopted for future studies.

The use of a vector containing a more tightly regulated promoter designed for the production of toxic proteins may also be of benefit. The use of the T7 promoter for this purpose has been described extensively (Brown & Campbell 1993, Doherty *et al.*, 1993). The over expression of the *E. coli* primase enzyme, required for DNA replication of *E. coli* has been performed successfully using the T7 promoter (Godson 1991). By cloning the *dnaG* gene after various modifications into the T7 polymerase-transcribed expression vector pET-3d, the production of this primase was tightly regulated and cells which contained the constructed plasmid were able to express 30% of the cellular protein as primase. It would be a possibility to investigate the use of the T7 promoter for production of these SIV Nef proteins, as tight control of the expression of the recombinant proteins the potential problems of toxicity of SIV Nef to the bacteria could be minimised.

In spite of the modifications still introduced to minimise protein degradation a certain degree of protein breakdown was still observed for clones GST-J5(EIYL) and GST-J5(EIYL+R>E). On SDS-PAGE gels, these recombinant proteins appear at a consistently smaller size than that predicted for the GST-Nef proteins (between 40-45 kDa). This degradation is occurring at the carboxy terminus of the protein as Western blotting performed with KK75, which recognises a linear peptide sequence at the amino terminus of SIV Nef, showed evidence of binding to the recombinant proteins. It is possible that the amino acid substitutions in these two constructs are particularly toxic to the bacteria. Proteolytic enzymes are known to remove abnormal proteins from bacterial systems, including those with mutations caused by amino acid substitution (Goldberg *et al.,* 1986). Therefore, despite attempts to minimise degradation, protein will still be broken down. The DNA sequence of these proteins has been confirmed to be intact as these sequences were subcloned into the yeast episomal vector and sequencing performed to confirm the correct sequence for each construct (Chapter 4). Therefore the truncation of these clones must be occurring once the proteins are synthesised.

It has been described during synthesis of recombinant proteins, that *E. coli* shows a bias in its utilisation of codons between genes. This bias differs between *E. coli* and mammalian cells, suggesting that a mammalian gene expressed in *E. coli* will have an inappropriate distribution of codons. If bacteria encounter codons for a sequence for which they are not biased, a longer time is taken to synthesise the amino acids, which, as a result, leaves the protein open to proteolytic cleavage. Optimisation of codon usage will prevent this. Codon optimisation of specific genes has been documented to improve the level of expression of recombinant protein in *E. coli* (Hale & Thompson 1998). Optimisation of the codons for the sequence J5(EIYL), therefore, could result

in more efficient synthesis of the recombinant proteins and reduce its susceptibility to breakdown.

Since breakdown of recombinant SIV Nef was restricted to selected alleles it would be of interest to use mass spectrometry to elucidate the exact region at which these recombinant proteins are being degraded. It would be expected that a specific region of these proteins is particularly unstable, resulting in the proteins being degraded until the minimal stable domain of the protein is encountered. This stable domain could be determined and it would be interesting to observe if wild type Nef protein, grown in culture for an extended period of time such as 12 hours, would breakdown to the same size as J5(EIYL) and J5(EIYL+R>E) and thus determine whether these sizes do indeed represent a stable domain of the protein.

The data obtained from this study is in general agreement with previously unpublished studies from the Division of Retrovirology at NIBSC, in which the selective repair mutants of SIV Nef (Whatmore *et al.*, 1995) have been re-introduced into the parental molecular clone SIVmacJ5 and the binding of anti-SIV Nef monoclonal antibodies analysed by flow cytometry. It suggests that, in spite of the technical difficulties encountered in the production of recombinant bacterial Nef fusion protein, due its relative instability, the protein produced is capable of binding glutathione and is folded in a conformation that is indistinguishable from native viral proteins. Thus, the approach developed and applied in this study may be suitable for exploring further the effects of sequence changes in Nef on epitopes that appear to be critically associated with a pathogenic phenotype to the virus. The development of this relatively rapid method to map the structurally important epitopes of SIV Nef will facilitate research to elucidate the key activities of Nef that are essential for its functions *in vivo*.

The mapping of protein structures with monoclonal antibodies has been proven to be a valuable technique employed in the study of other HIV-1 proteins. John Moore and colleagues (1993a, 1994) used monoclonal antibodies to investigate the structure of the HIV envelope glycoprotein before the X-ray crystal structure of gp 120 was resolved (Kwong *et al.*, 1998). Moore *et al.* (1993) performed studies using monoclonal antibodies directed against various domains of the HIV gp 120 molecule to predict its conformation. Monoclonal antibodies that were dependent on a specific domain of gp 120 were found to be sensitive to changes within this region, indicating that their ability to bind was dependent upon the native structure of the protein. Substitutions outside the epitopes could also significantly affect the ability of monoclonal antibodies to bind, demonstrating the importance of neighbouring amino acid sequences on the structure of important epitopes. Some amino acid substitutions also increased the binding of one particular antibody, possibly by improving the accessibility of the epitope.

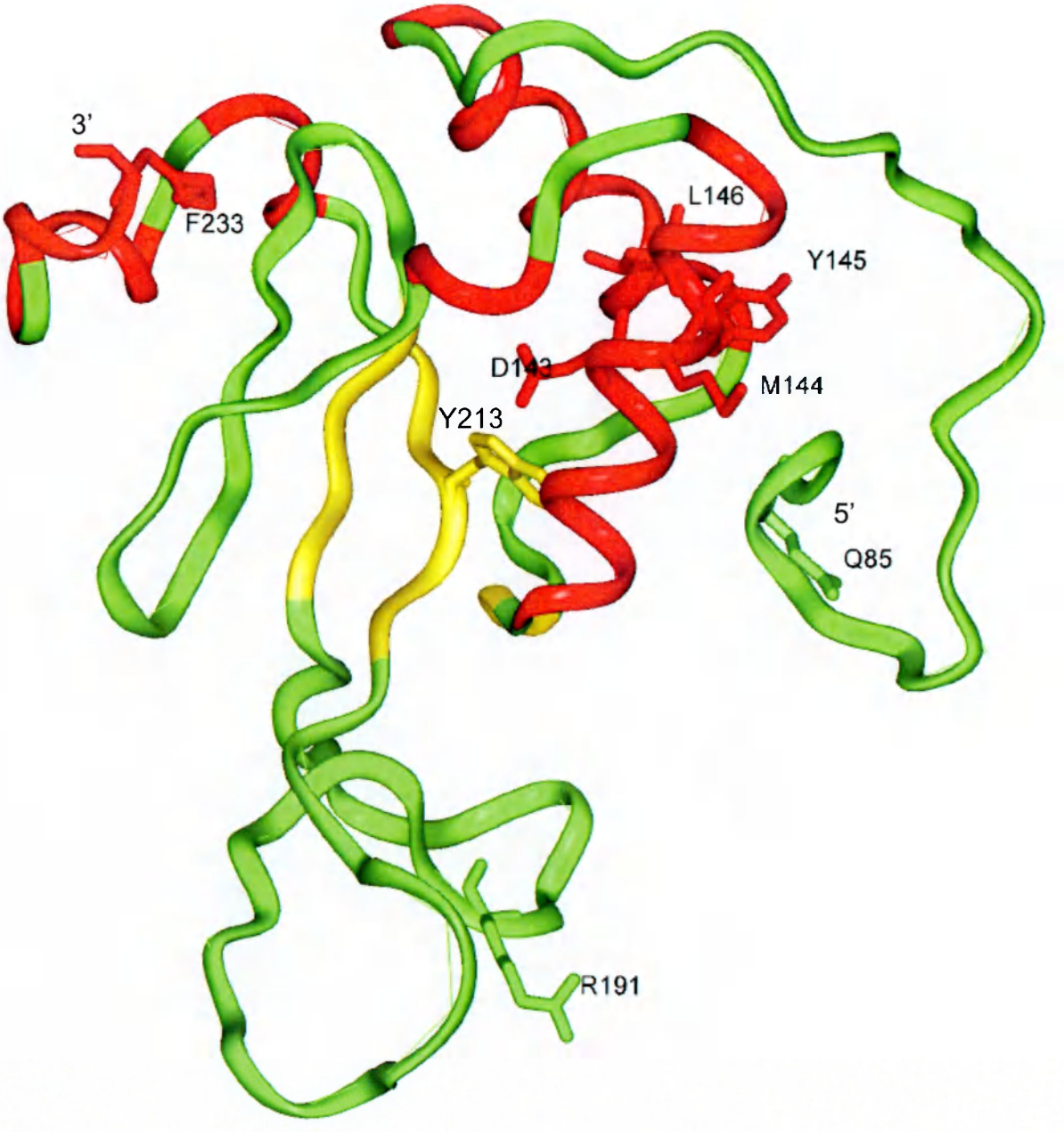
X-ray crystallography has now proven that the HIV-1 envelope is heavily dependent upon conformation to yield a functional protein (Kwong *et al.*, 1998). From the information obtained by direct structural analysis it was revealed that many of the features of envelope were accurately predicted from the antibody mapping studies performed a number of years previously by Moore and colleagues. We should, therefore, move forward and evaluate the implications of the binding data on the potential roles of Nef in bringing about the high level replication of the virus *in vivo*.

It would be valuable to have some structural information of SIV Nef to ascertain the relative distance between the two non-overlapping epitopes of SIV recognised by the two competition groups represented by KK70 and KK75. HIV-1 Nef consists of two

domains, a small N-terminal domain and a larger C-terminal domain. The N terminal domain contains a myristoylation motif and is highly susceptible to degradation. The C-terminal is highly conserved amongst isolates and forms a well-defined and relatively stable structure (Lee *et al.*, 1996). By superimposing and modelling the structure of the C-terminal portion of SIV Nef onto the crystal structure of HIV-1 Nef (Figure 3.3.1) we can test the predictions of the antibody data. It is clear that the deletion of four amino acids in the alpha helical domain, similar to the deletion of amino acids 143-146 in SIVmacC8, would have extensive effects on the structure of the carboxy terminus of the protein. Interestingly, position 213, truncated in VLA*del, is closely juxtaposed to the alpha helical region of SIV Nef. It is intriguing that the same epitope recognised by KK70 is disrupted if the Nef protein prematurely truncates at this region. By contrast, the region surrounding amino acid 191 would not be predicted to be closely associated with the epitope recognised by KK70, as our results indicate, as it is not predicted to be in close juxtaposition to the alpha helical region.

This study has shown that there are two regions of SIV Nef that are antigenically as well as genetically distinct. These result in attenuation of the virus in a broadly similar manner when they are deleted, as observed by previous *in vivo* experiments (Figure 3.1.1). This suggests that Nef may be mediating two or more distinct activities in order to facilitate high level replication of SIV. What these may be, remains to be determined. Furthermore, mutations that arise spontaneously are an intriguing phenomenon. The mutant clone VLA*del arose spontaneously in tissue culture, abrogating the ability of KK70 to bind and resulting in a reduced virus load *in vivo* (N. Almond, personal communication).

Figure 3.3.1. A protein homology model of SIV Nef based upon the known structure of the HIV-1 Nef protein. Due to poor homology, structural information between amino acids 85-233 only is available. Amino acids 143-146, the region which is deleted in C8 Nef, is highlighted. This region is postulated to form part of the conformational epitope recognised by monoclonal antibody KK70 and sits within an α-helical structure. The tyrosine at position 213, at which the Nef protein is truncated in VLA*del is located in close proximity to amino acids 143-146.
Figure computed and prepared by M. Forster, NIBSC.


3'
F233
L146
Y145
D143
M144
Y213
5'
Q85
R191

Clone GX2 arose spontaneously in a macaque infected with SIVmac32H one month after infection and exhibits a reduced viral load *in vivo*. Could it be that the virus evolves in such a way, *in vivo*, to escape detection by the immune system, by removing epitopes that are the target of effective anti-viral immunity. Although viral titres are reduced by the removal of selected regions of the protein, if it can lead to effective escape from the immune system, then it could result in the virus persisting in the host for longer. There is evidence that Nef can be the target of protective immunity since Nef-specific CTLs, *in vivo*, have been observed to confer protection. Immunisation with recombinant vaccinia virus expressing SIV Nef conferred partial protection against SIV challenge, with high levels of Nef-specific CTLs on the day of challenge correlated with a low virus load after challenge (Gallimore *et al.*, 1995). Moreover, in the study by O'Connor *et al.* (2002) evidence for CTL immune escape by selected sequence variation at regions closely flanking the epitopes recognised by KK70 and KK75 monoclonal antibodies was observed. Thus the fortuitous association of T cell and B cell epitopes in SIV Nef enables us to use monoclonal antibodies to study immunological pressure created by T cell immunity *in vivo*.

In the case of C8 and VLA*del these were viral variants that were selected following culture in C8166 cells (Rud *et al.*, 1992, Arnold *et al.*, 1999). Presumably the loss of the epitope recognised by KK70 is advantageous for the replication of SIV in this cell line and these epitopes may coincide with functionally important regions of the Nef protein that may be dispensable under certain conditions *in vitro* or *in vivo*. In that situation then it may be more valuable to try to elucidate functional changes that result from mutation of SIV Nef at these specific regions. This is the goal of the next chapter.

4. The ability of SIV *nef* clones which express wild type or attenuated pheotypes in macaques to interact with adaptor proteins, TCRζ and members of the Src family of protein tyrosine kinases

4.1. Introduction

Protein/protein interactions are an essential feature of most biological processes. Identification of critical interactions and definition of the epitopes involved may lead to a situation where it is possible to predict the influence that changes in protein sequences and/or structure may have upon biological processes. This is particularly important in the characterisation of the pathogenesis of viral infections. A better definition of the protein interactions that are essential for, or that facilitate virus replication may permit the rational selection and development of novel antiviral drugs.

Examples of interactions that may influence viral pathogenesis have been described in the context of human and simian immunodeficiency virus replication. The viral Tat and Rev proteins of these viruses bind to regions of the LTR and *env* mRNA respectively. However, host cell proteins that bind to the viral LTR region are required for effective viral transcription and replication, particularly in macrophages. Furthermore, viral structural proteins, Gag and Env are only able to assemble into viable structural particles by making appropriate interactions with host proteins (Zhang *et al.,* 1994). Most intriguing and perhaps least well understood are the protein interactions of Nef that have been demonstrated in the SIV macaque model to be essential for high level viral replication, observed prior to the induction of disease. The importance of Nef in pathogenesis has been confirmed with the identification of selected HIV infected long term non progressors in which a *nef* defective HIV-1 has established infection.

The Nef proteins of SIV, HIV-1 and HIV-2 have been demonstrated to interact with a number of cellular partners that may contribute to the influential role of this protein in pathogenesis. For example, sequence analysis has identified a PXXP motif that enables Nef to interact with the SH3 domain of members of the Src family of protein tyrosine kinases. Attempts to delete this motif from SIV isolates have led to rapid reversion indicating a strong selective pressure to maintain this peptide motif (Saksela *et al.*, 1995). This motif is well conserved amongst *nef* isolates, suggesting that this specific interaction is beneficial to the virus *in vivo*. Other studies have identified a dileucine motif ($L_{413}L_{414}$) within the cytoplasmic domain of Nef as essential for down regulation of cell surface CD4 (Aiken *et al.*, 1994). Down regulation of CD4 may be a mechanism by which the viruses can escape the immune system or avoid superinfection of cells.

Furthermore, the region between amino acids 45-127 has been described to be important for the association of Nef with a Nef associated kinase (NAK) (Sawai *et al.*, 1994). This region consists of several amino acid motifs that are conserved throughout HIV-1, HIV-2 and SIV Nef. The conservation of the sequence in this region of Nef indicates that interaction with a kinase may be beneficial for viral replication *in vivo*.

None of the interactions described above fully account for the effect brought about by the disruption of Nef through deletion of amino acids 143-146, as in the attenuated virus SIVmacC8 or amino acid 66-88 as in SIVmacGX2. This indicates that Nef may be involved in further protein interactions required for virus replication, or that these changes within Nef could have far reaching effects on the interactions previously documented.

A number of methods have been described for investigation of protein/protein interactions. One of them, the yeast-2-hybrid system utilises yeast in a genetic assay designed to detect protein/protein interactions *in vivo* (Fields and Song, 1989). This system can either be used to confirm interactions between two known proteins or alternatively to search a genomic cDNA library for proteins that interact with a particular target protein of interest. Furthermore, this assay can be used to define the critical domains that mediate an interaction and identify specific residues that are involved.

The yeast-2-hybrid system exploits the properties of the GAL4 protein of the yeast *Saccharomyces cerevisiae.* GAL4 is a transcriptional activator required for the expression of genes encoding enzymes of galactose utilisation (Johnston, 1987). It consists of two distinct domains, an N-terminal domain (the DNA binding domain) which binds to specific DNA sequences (UAS_G) and a C-terminal domain (the activating domain) which is required for transcriptional activation.

In order to use the yeast-2-hybrid system, two plasmids are cloned each with a selected protein fused to a domain from GAL4. The plasmids are designed to express either the GAL4 DNA binding domain fused to a protein designated 'X' or the GAL4 activating domain fused to a protein designated 'Y'. The two separate plasmids are introduced into a yeast strain. If there is an interaction between proteins X and Y the GAL4 domains are bought into contact with each other and transcription of a gene regulated by UAS_G takes place (Figure 4.1.1). The transformants can then be identified by the blue staining of colonies following the conversion of the chromagenic substrate X-gal by the galactosidase produced in cells where interactions occur. Alone, the native GAL4 protein, containing both domains can activate transcription when yeast cells are grown on galactosidase supplemented media.

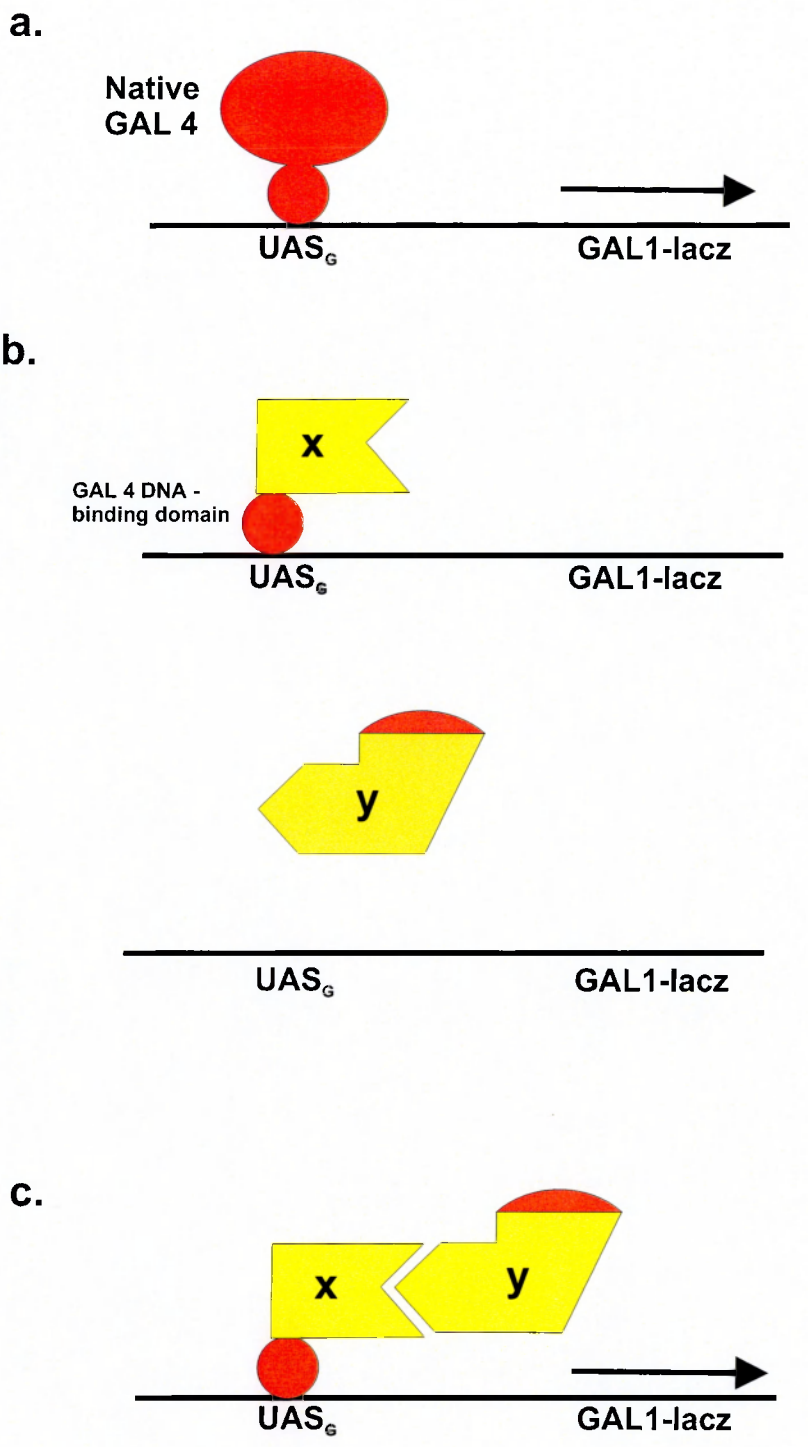


Figure 4.1.1. The yeast-2-hybrid system, an assay designed to detect protein-protein interactionswhich may occur *in vivo* by utilising the GAL4 protein of the yeast strain *Saccharamyces cerevisae*. a, The native GAL4 protein consists of both DNA binding and activating regions and induces GAL1-lacZ transcription. b, Hybrids consisting of either the DNA-binding domain or activating region cannot induce transcription alone. c, A protein-protein interaction between proteins X and Y brings the GAL4 domains into close proximity and results in transcriptional activity.

Adapted from: Fields S & Song O (1989). A novel genetic system to detect protein-protein interactions. *Nature* **340**: 245-246

However, although the N-terminal domain of GAL4 can bind to DNA it cannot activate transcription. The C-terminal DNA, containing the activating regions cannot activate transcription alone, as it is unable to bind to the specific DNA sequence UAS_G.

Within the system utilised in this study, the β-gal reporter gene in the pASI/CYHII-*Bss*HII.I plasmid is chromosomal and under the control of the GAL1 promoter (Appendix 2, Figure 8.3). GAL4 transcription factor activates the GAL1 promoter and drives the expression of the β-gal gene. Active GAL4 is reconstituted by a positive yeast-2-hybrid interaction bringing together the GAL4 activation domain and the GAL4 DNA binding domains present in the yeast-2-hybrid proteins.

The yeast-2-hybrid system has been applied in the analysis of protein interactions by selected SIV *nef* alleles, including SIVmacJ5 and SIVmacC8 (Bell *et al.*, 1998). These studies revealed differential binding of the two alleles of *nef* for selected proteins, including TCRζ. The interaction of wild type SIV Nef with TCRζ was also confirmed independently by Howe (*et al.*, 1998) using the *nef* allele of the wild type clone SIVmac239.

If the interaction with the TCRζ is important for viral pathogenesis it would be valuable to confirm whether the revertant SIV Nef proteins (J5(EIYL), J5(EIYL+R>E) and J5(R>E)) also interact with the protein. Furthermore, it may be possible to gain an insight into which of these sequences is responsible for restoring the ability of the SIV Nef protein to regain a wild type-like function and ultimately to enhance infection.

Previously, it was demonstrated that the premature truncation of Nef at amino acid 213 disrupts the binding of monoclonal antibody KK70, which indicates that a major

conformational structure has been disrupted (Chapter 3). It would be valuable to elucidate whether the disruption of the epitope recognised by KK70 disrupts the protein/protein interactions of Nef in a similar manner to that observed for deletion of amino acids 143-146 in C8.

The GX2 *nef* allele is disrupted in a genetically distinct region of the protein compared with the changes from wild type sequence observed in C8 Nef and is also associated with a similar level of virus replication as C8 (Chapter 3, Figure 3.1.1). It is possible that the GX2 Nef protein could display a discrete pattern of protein/protein interactions, that differs from the range of proteins shown to interact with C8 Nef. This hypothesis could be tested using yeast-2-hybrid technology.

The proteins selected to be tested for their ability to interact with SIV Nef in this study are listed below. In each case, they were selected on the basis of previous evidence that differential properties may exist between the J5 and C8 clones.

(i) The medium chains AP47 and AP50 of AP-1 and AP-2 respectively (section 1.10a), which play an important role in the transport of clathrin coated vesicles during endocytosis. These proteins have been described to interact with a specific class of sorting signal, containing tyrosine based motifs within the cytoplasmic domain of some membrane proteins (Ohno *et al.*, 1995). The N terminus of certain Nef proteins contain such endocytic motifs (Piguet *et al.*, 1998). Previous studies have described an interaction between either HIV-2_{ROD} Nef or SIVmac239 Nef protein with full length or truncated AP50 chain of the adaptor proteins (Piguet *et al.*, 1998). These interactions appeared to depend upon specific C-terminal sequences (Bresnahan *et al.*, 1998).

(ii) Lck is a member of the Src family of protein tyrosine kinases, which are involved in cellular signalling and cytoskeletal organisation. The ligand for these proteins is a minimal PXXP motif. Such a motif is well conserved amongst Nef isolates (section 1.11e). A variety of interactions have been described between various Nef isolates and proteins containing Src homology domains.

(iii) The ζ chain of the T cell receptor is an important component of the immune system. SIVmacJ5 and SIVmacC8 have previously been investigated for interactions with TCRζ. SIVmacJ5 was found to interact with TCRζ whilst SIVmacC8 did not. Two clones of TCRζ will be investigated, $TCR\zeta_{1.11}$, which expresses the entire cytoplasmic domain and $TCR\zeta_{2.21}$, which expresses only the C-terminal 56 amino acids (Bell *et al.,* 1998). The $TCR\zeta_{2.21}$ clone contains only the two distal immunoreceptor tyrosine-based activation motif (ITAM), whilst the $TCR\zeta_{1.11}$ clone contains all three ITAMs (Figure 4.1.2).

Due to previous studies describing interactions between various Nef isolates and the proteins mentioned above, it was reasoned that it would be intriguing to investigate the interactions between these proteins and the various SIV Nef proteins that have been described within this thesis. C8 and VLA*del are both proteins mutated in different ways, one by a premature stop codon, the other by a four amino acid deletion and other nucleotide substitutions. Both types of mutations, however, have an overall affect on the same α-helical region within the Nef protein with regards to shape. Studying the interactions of these SIV Nef proteins will provide insight into the effect a change in the overall shape of the Nef protein has on its various functions. Furthermore, with regards to the repair mutations (J5(EIYL), J5(EIYL+R>E) and J5(R>E)), it would be particularly interesting to see which of these interactions, if

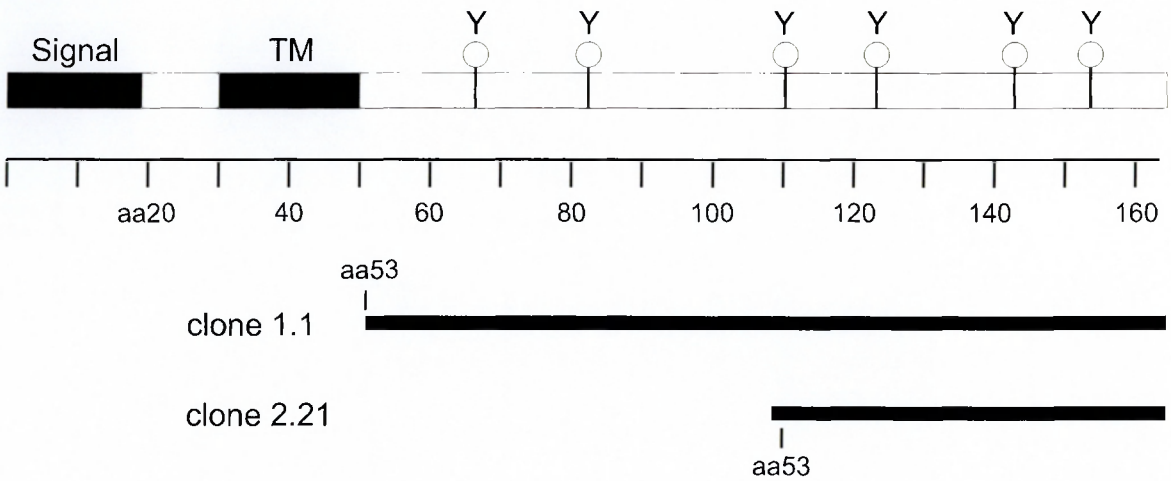


Figure 4.1.2. A schematic representation of the TCRζ. The tyrosines (Y) are indicated within the immunoreceptor tyrosine-based activation motifs (ITAMs). Regions of the TCRζ contained in cDNA clones used in the study are indicated by the black lines. TM; trans-membrane domain.

Adapted from: Bell I, Ashman C, Maughan J, Hooker E, Cook F, Reinhardt T (1998). Association of simian immunodeficiency virus Nef with the T-cell receptor (TCR) ζ chain leads to TCR down-modulation. *J. Gen. Virol.* **79**: 2717-2727

any, are regained as the virus evolves closer to a wild type sequence. This may provide an insight into the mechanism which drives the selective pressure for the virus to maintain a wild type sequence and increase our understanding of whether this is associated with the virus regaining certain functions, which may contribute to disease progression.

These proteins had been previously introduced into the pACTII plasmid (Molecular Immunology Unit, GlaxoSmithKline, Appendix 2) as a GAL4 activation domain fusion, whilst the SIV Nef proteins were introduced into the pASI/CYHII-*Bss*HII.I plasmid (Molecular Immunology Unit, GlaxoSmithKline, Appendix 2) as a GAL4 DNA binding domain fusion.

4.2. Results

4.2.1. Analysis of pASI/CYHII-*Bss*HII.I DNA

A large scale preparation of yeast episomal plasmid pASI/CYHII-*Bss*HII.I was performed using a commercial maxi-prep kit. The plasmid DNA was digested sequentially using restriction endonucleases *Bam*HI and *Sal*I and the digested DNA de-phosphorylated by incubation at 37°C with 1 µl calf intestinal phosphatase. Analytical gel electrophoresis on a 1.75% (w/v) agarose gel containing 5 µg/ml ethidium bromide confirmed the presence of the pASI-CYHII/*Bss*HII.I DNA at the correct size of 8.5 kb. The DNA fragments were excised from the gel and the plasmid DNA extracted using a commercial kit.

4.2.2. Analysis of *nef* DNA

Restriction endonuclease analysis of small and midi scale preparations of each of the various pGEX-4T3-*nef* clones revealed a product of approximately 800 bp corresponding to the expected size for SIV *nef*. Clone pGEX-4T3-GX2 was found to be smaller, as expected, due to the 66 nucleotide deletion within it. The various DNA fragments were excised out of gels, the DNA extracted using a commercial kit and ligated into the yeast episomal vector pASI-CYHII/*Bss*HII.I using restriction sites *Bam*HI and *Sal*I. Ligations were performed with multiple controls, to ensure that the clones being isolated were likely to be positive for the appropriate *nef* insert (section 2.2.8).

Each vector DNA, ligated with an appropriate *nef* DNA insert was used to transform *E. coli* DH5α cells. Plasmid pUC19 (at a concentration of 1 ng/µl) was used as a control to estimate the efficiency of transformation, which was calculated to be between 500-800 colony forming units per µg of control plasmid. This was sufficient

for our purposes. Six transformants for each SIV *nef* clone were picked for small scale preparation of DNA. Restriction endonuclease analysis, using *Bam*HI and *Sal*I, and electrophoresis on a 1% (w/v) agarose gel containing 5 μg/ml ethidium bromide, revealed the presence of each appropriate SIV *nef* gene at the expected size of 800 bp in the majority of clones. Clone GX2 gave a product which was slightly smaller due to the presence of a 66 nucleotide deletion within this gene (data not shown).

One positive transformant was picked for each SIV *nef* clone and the appropriate glycerol stock used to perform a large scale preparation of DNA. The presence of the appropriate *nef* gene was confirmed by restriction endonuclease digestion using *Bam*HI and *Sal*I (Figure 4.2.1) and electrophoresis on a 1% (w/v) agarose gel containing 5 μg/ml ethidium bromide.

4.2.3. Sequence analysis of pASI-CYHII/*Bss*HII.I-*nef* clones

All pASI-CYHII/*Bss*HII.I-*nef* clones were shown to be a match to the expected SIV Nef sequence except where the mutations had been introduced, where sequences were confirmed to be as expected.

4.2.4. Expression of Nef-BD fusion proteins

Protein was prepared from yeast cells transformed with the BD-Nef constructs. Protein preparations from cells transformed with pASI-CYHII/*Bss*HII.I vector alone or untransformed Y190 cells were prepared to serve as controls during Western blotting. Analysis of these samples on a 4-20% tris-glycine gradient gel (Novex, UK) followed by Western blotting and probing with anti-HA antibody which recognises the HA tag in the pASI-CYHII/*Bss*HII.I vector is presented in Figure 4.2.2. No binding to preparations of parental Y190 cells was detected, whereas the MAb binds

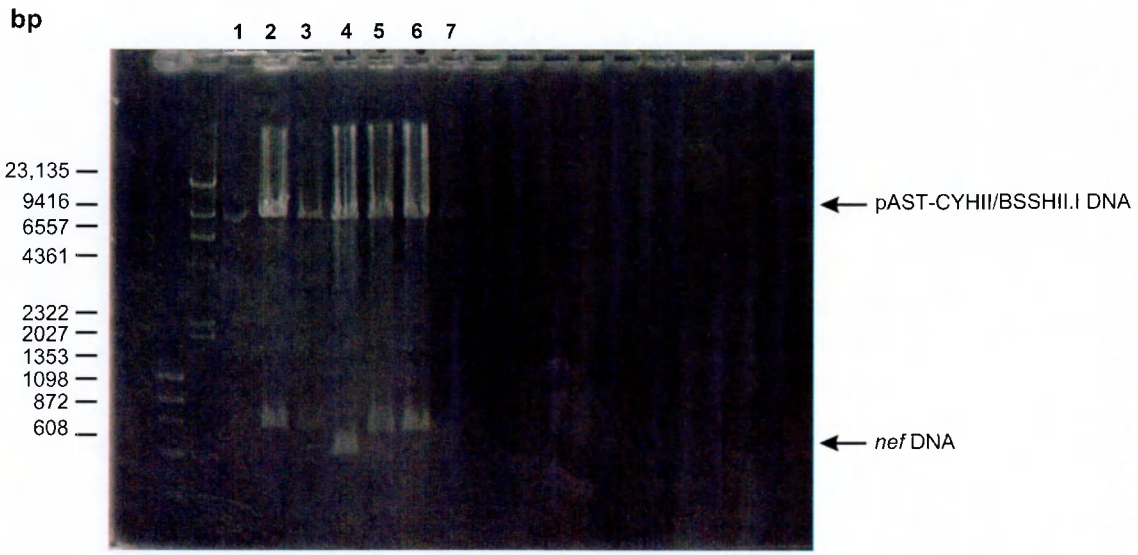


Figure 4.2.1. Restriction endonuclease analysis of large scale preparation of pASI-CYHII/*Bss*HII.I-*nef*DNA on a 1% agarose gel containing 5 μg/ml ethidium bromide. Lane 1; pASI-CYHII/*Bss*HII.I, lane 2; pASI/CYHII/*Bss*HII.I-J5, lane 3; pASI-CYHII/*Bss*HII.I-C8, lane 4; pASI-CYHII/*Bss*HII.I-GX2, lane 5; pASI-CYHII/*Bss*HII.I-VLA*del, lane 6; pASI-CYHII/*Bss*HII.I-J5(EIYL), lane 7; pASI-CYHII/*Bss*HII.I-J5(EIYL+R>E), lane 8; pASI-CYHII/*Bss*HII.I-J5(R>E). The size of the restriction fragments were compared with fragments of l λ DNA digested with HindIII and φX174 DNA digested with HaeIII.

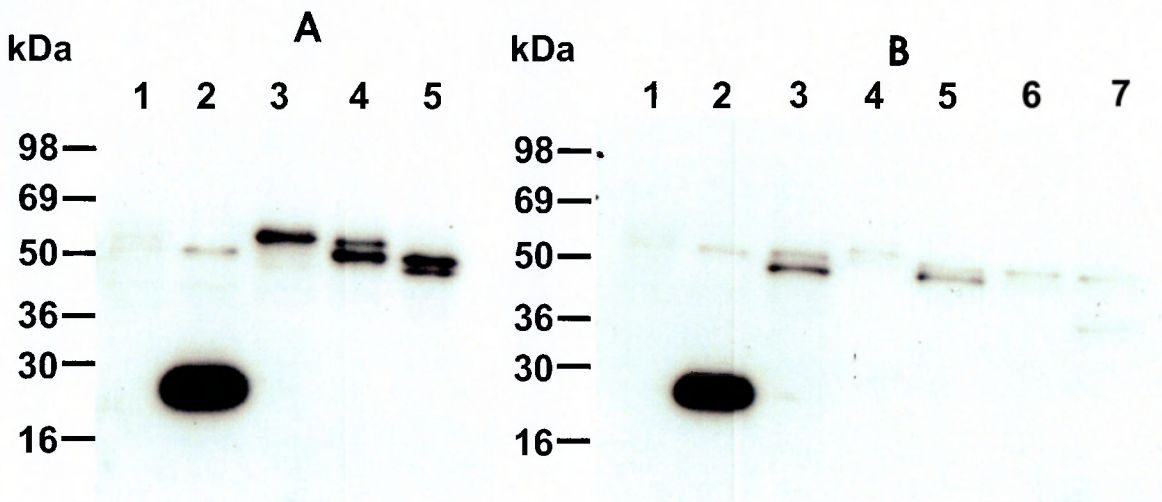


Figure 4.2.2. Western blot analysis of recombinant Nef-BD protein performed using anti-HA monoclonal antibody on a 4-20% Tris-Glycine gel (Novex, UK). Gel A: lane 1; control Y190 cells, lane 2; pASI-CYHII/*Bss*HII.I, lane 3; J5-Nef-BD, lane 4; C8-Nef-BD, lane 5; VLA*del-Nef-BD. Gel B: lane 1; control Y190 cells, lane 2; pASI-CYHII/*Bss*HII.I, lane 3; VLA*del-Nef-BD, lane 4; J5(R>E)-Nef-BD, lane 5; GX2-Nef-BD, lane 6; J5(EIYL+R>E)-Nef-BD, lane 7; J5(EIYL)-Nef-BD. The relative molecular mass of protein bands bound by the anti-HA antibody was calculated by comparison with protein standards (Gibco BRL, UK). Protein product corresponding to pASI-CYHII/*Bss*HII.I is present at the predicted size of between 16-20 kDa. An additional band, a 'doublet' is present at approximately 50 kDa. This is also present in the lanes corresponding to Nef-BD protein.

to pASI-CYHII/*Bss*HII.I vector protein at the expected weight of between 16-20 kDa. Of interest, a second faint band was detected of an RMM of 52 kDa. For each of the recombinant Nef constructs, a band of RMM 55 kDa would be predicted as the RMM of Nef is between 27-35 kDa. This was the case for J5 and C8 Nef fusion proteins. VLA*del Nef fusion protein appears to be smaller, presumably due to the truncation of the Nef protein. The remaining fusion proteins appeared to have an RMM of approximately 55 kDa, with the notable exception of J5(EIYL) which appeared to have an RMM of approximately 36 kDa. This confirms that the appropriate Nef protein was being expressed by each of the constructs.

Parallel analysis of these protein preparations by Western blotting and probing with the SIV Nef specific MAb KK75 is presented in Figure 4.2.3. No binding of this antibody was observed to protein preparations derived from the yeast cells or cells transformed with the parental plasmid pASI-CYHII/*Bss*HII.I as there are no *nef* sequences present. By contrast, J5-Nef-BD and C8-Nef-BD are present at the predicted relative molecular mass of 55 kDa, as is J5(R>E)-Nef-BD. Two preparations of VLA*del-Nef-BD are present at approximately 50 kDa. GX2-Nef-BD is not recognised by KK75 due to the amino terminus of this protein harbouring a deletion that encompasses the linear epitope recognised by KK75. Surprisingly, the J5(EIYL+R>E) and J5(EIYL) recombinant proteins appear at the smaller size of 50 and 40 kDa respectively.

The presence of the appropriate Nef protein, in each construct is confirmed as Western blotting was performed with a Nef specific MAb, KK75. However, J5(EIYL+R>E)-Nef-BD and J5(EIYL)-Nef-BD are not present at the predicted size, as observed for all other clones. The possible reasons for this are discussed in section 4.3.

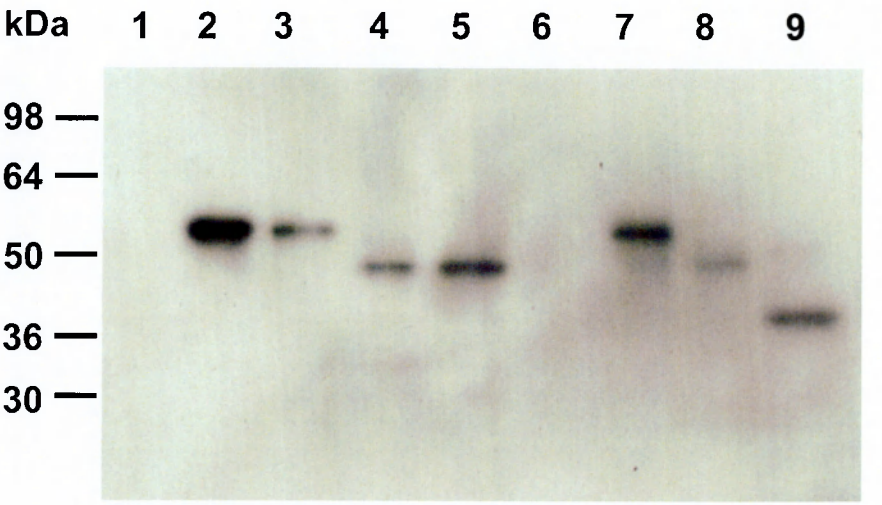


Figure 4.2.3. Western blot analysis of recombinant Nef-BD protein performed using KK75 on a 4-20% Tris-Glycine gel (Novex, UK). Lane 1; pASI-CYHII/*Bss*HII.I, lane 2; J5-Nef-BD, lane 3; C8-Nef-BD, lane 4; VLA*del-Nef-BD, lane 5; VLA*del-Nef-BD, lane 6; GX2-Nef-BD, lane 7; J5(R>E)-Nef-BD, lane 8; J5(EIYL)R>E-Nef-BD, lane 9; J5(EIYL)-Nef-BD. The relative molecular mass of protein bound by anti-Nef monoclonal antibody was calculated by comparison with a protein standards (Gibco BRL, UK). The doublet, previously observed in Figure 4.2.2 in the lanes corresponding to SIV Nef protein, is absent.

4.2.5. β-Galactosidase assays

a. Interaction of Nef-BD proteins with members of the endocytic pathway

An interaction between the two proteins is denoted by a +, indicating that the yeast colonies were blue. A negative interaction (denoted by -), indicates the colonies were white. Differences in the number of blue colonies were observed on different plates. This may be a result of different transfection efficiencies, or the potential toxicity of the proteins to the yeast they are being transformed with.

The interaction of the Nef-BD proteins with the parental activation domain plasmid pACTII-AD not containing any additional sequences was used as a control in every set of assays. No specific interactions of parental pASI-CYHII/*Bss*HII.I plasmid was observed with this plasmid.

A positive interaction was detected between pASI-CYHII/*Bss*HII.I expressing J5 Nef as a fusion partner with the BD and AP47-AD and AP50-AD. This was apparent by large numbers of blue colonies (Table 4.2.1). The interaction with pACTII-AD was negative, as expected. Intriguingly, the interaction with AP47-AD consistently revealed a high number of white colonies amongst those that had developed blue. Although these types of white breakthrough colonies were evident in a number of other transformations, they appeared in relatively small numbers in comparison with this specific interaction of J5-Nef-BD + AP47-AD where they constituted approximately 50% of the colonies grown. The number of blue colonies in the interaction of J5-Nef-BD with AP50-AD was greater than that observed for AP47-AD.

C8-Nef-BD exhibited no interaction with AP47-AD or AP50-AD, neither did clone VLA*del-Nef-BD.

Nef allele expressed in pASI-CYHII/*Bss*HII.I	Gene expressed in pACTII-AD		
	pACTII	**AP47**	**AP50**
pASI-CYHII/*Bss*HII.I	-	ND	ND
J5	-	+	+
C8	-	-	-
VLA*del	-	-	-
GX2	+	+	+
J5(EIYL)	-	+	+
J5(EIYL+R>E)	-	-	-
J5(R>E)	+	+	+

Table 4.2.1. The interaction of the Nef-BD DNAs with AP47-AD and AP50-AD. Data is representative of three independent experiments. The interaction of the various Nef-BD DNAs with the pACTII-AD was used as a negative control in each experiment, as well as that of the pASI-CYHII/*Bss*HII.I vector with pACTII-AD. - , negative, no blue colonies, + , positive, blue colonies present.

An interesting feature was observed for the interactions of GX2-Nef-BD and J5(R>E)-Nef-BD. Although these clones exhibited a strong interaction with AP47-AD and AP50-AD fusion proteins, interactions were also observed with pACTII-AD. This was observed reproducibly. A low level of interaction was observed between J5(EIYL) Nef-BD and pACTII-AD, although this was less than that observed for GX2-Nef-BD and J5(R>E)-Nef-BD (Table 4.2.1).

In order to investigate this observation further, a kinetic assay was performed to determine if there was any differential rate of β-galactosidase production in the +pACTII-AD interactions.

Double transformations of yeast Y190 either with J5-Nef-BD, J5(EIYL)-Nef-BD, GX2-Nef-BD or J5(R>E)-Nef-BD DNA, alongside one of pACTII-AD, AP47-AD or AP50-AD were performed. All three interactions were plated onto one plate, divided into three segments, in triplicate (Figures 4.2.4a, b & c). After lysis of the yeast colonies by liquid nitrogen the discs were incubated, with Z buffer/ONPG/β-mercaptoethanol (section 2.2) for a period of 1, 2 or 6 hours for colour development and allowed to dry.

The one hour incubation plates (Figure 4.2.4a) reveal a faint blue colour for the interaction of GX2-Nef-BD and J5(R>E)-Nef-BD with pACTII-AD. It appeared there were fewer blue colonies in the pACTII-AD interaction, by comparison to the AP47-AD and AP50-AD interactions. However, it was difficult to determine unequivocally whether the blue colonies present in the +AP47-AD and +AP50-AD interactions were indicators of true interactions or were partially due to trans-activation of pACTII-AD. By two hours of incubation (Figure 4.2.4b) it was not possible to distinguish between the pACTII-AD and the AP47-AD or AP50-AD interactions with GX2-Nef-BD and J5(R>E)-Nef-BD. This was consistent after 6 hours of development (Figure 4.2.4c).

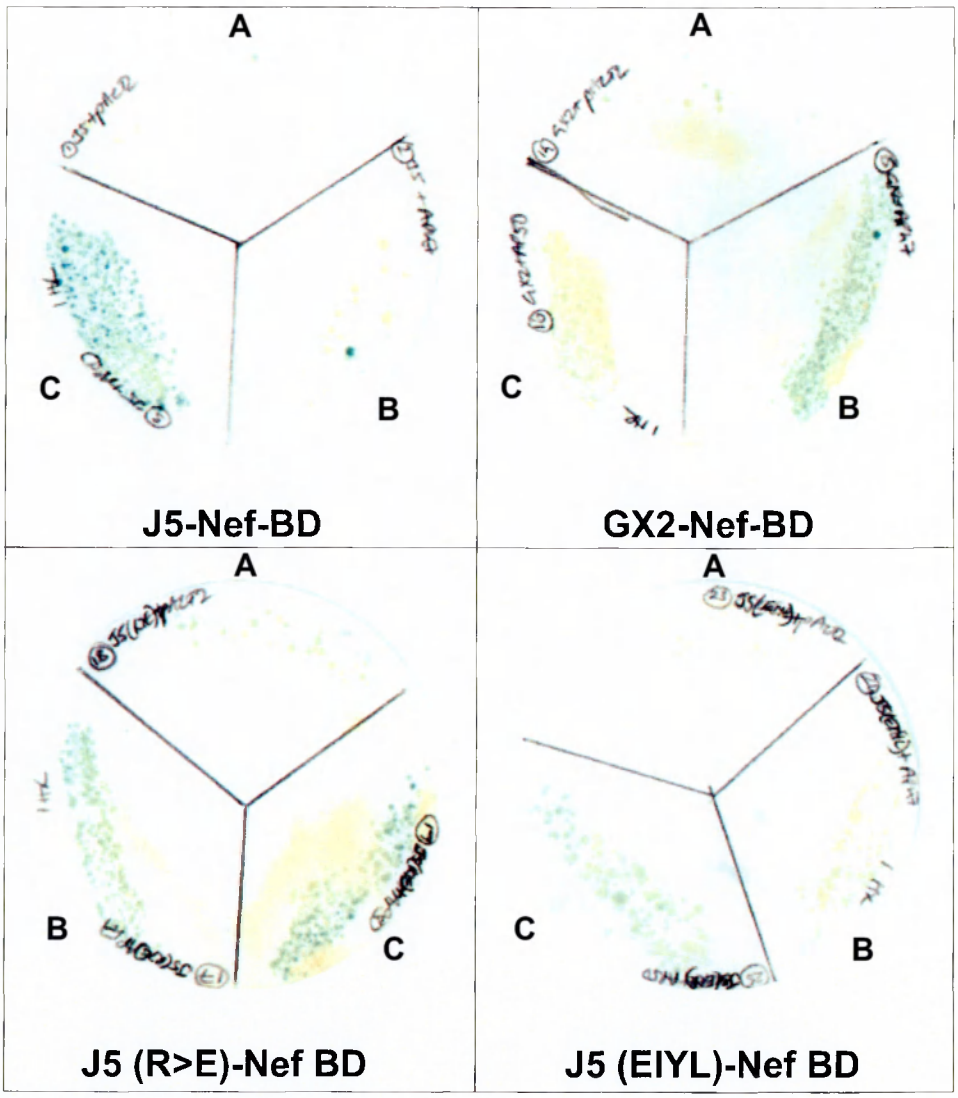


Figure 4.2.4a. β-galactosidase disc assay of the interactions of Nef-BD proteins with pACTII-AD (A), AP47-AD (B) and AP50-AD (C), after 1 hour incubation with the chromogenic substrate. A positive interaction is denoted by the production of β-galactosidase, which results in the colonies producing a blue colour after lysis and incubation with the chromogenic substrate. A negative interaction is denoted by the colonies remaining white. The interaction with pACTII-AD alone serves as a negative control.

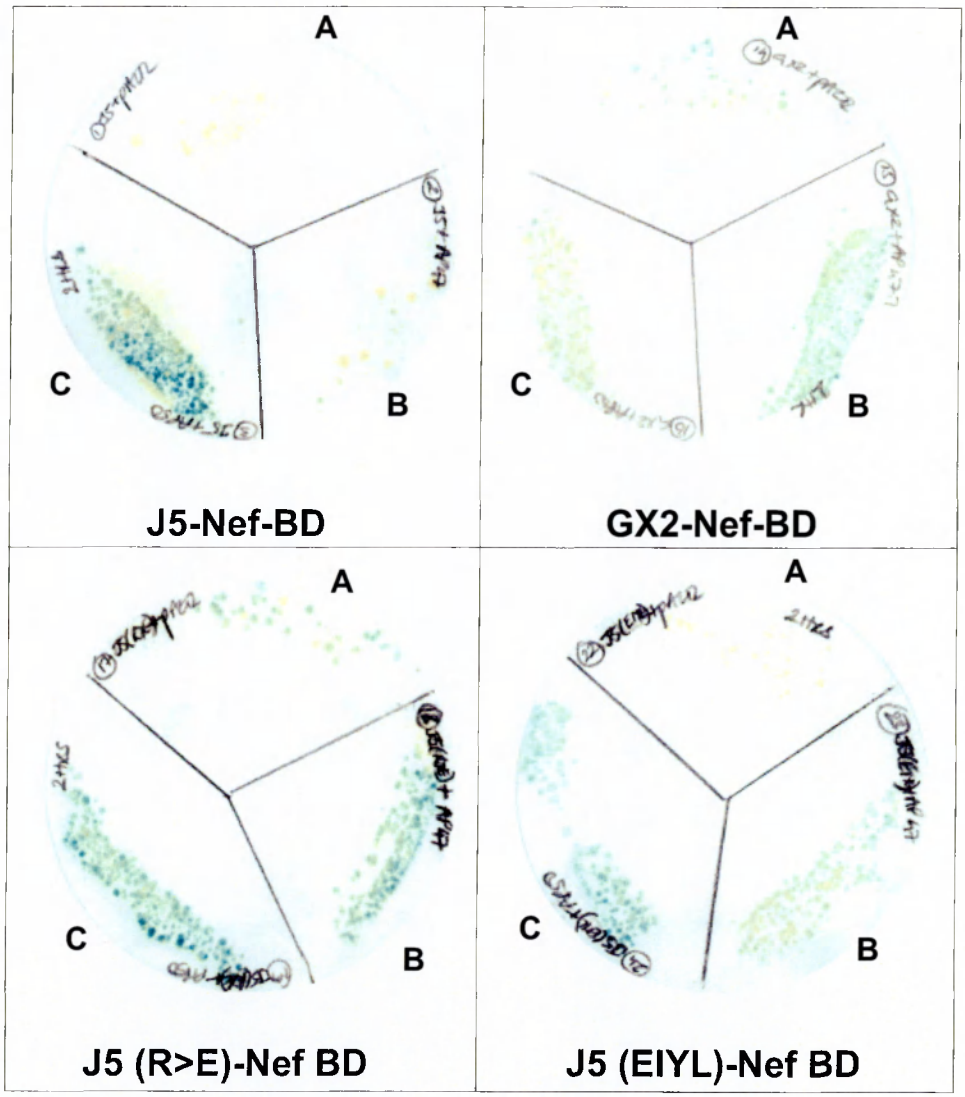


Figure 4.2.4b. β-galactosidase disc assay of the interactions of Nef-BD proteins with pACTII-AD (A), AP47-AD (B) and AP50-AD (C), after 2 hours incubation with the chromogenic substrate. A positive interaction is denoted by the production of β-galactosidase, which results in the colonies producing a blue colour after lysis and incubation with the chromogenic substrate. A negative interaction is denoted by the colonies remaining white. The interaction with pACTII-AD alone serves as a negative control.

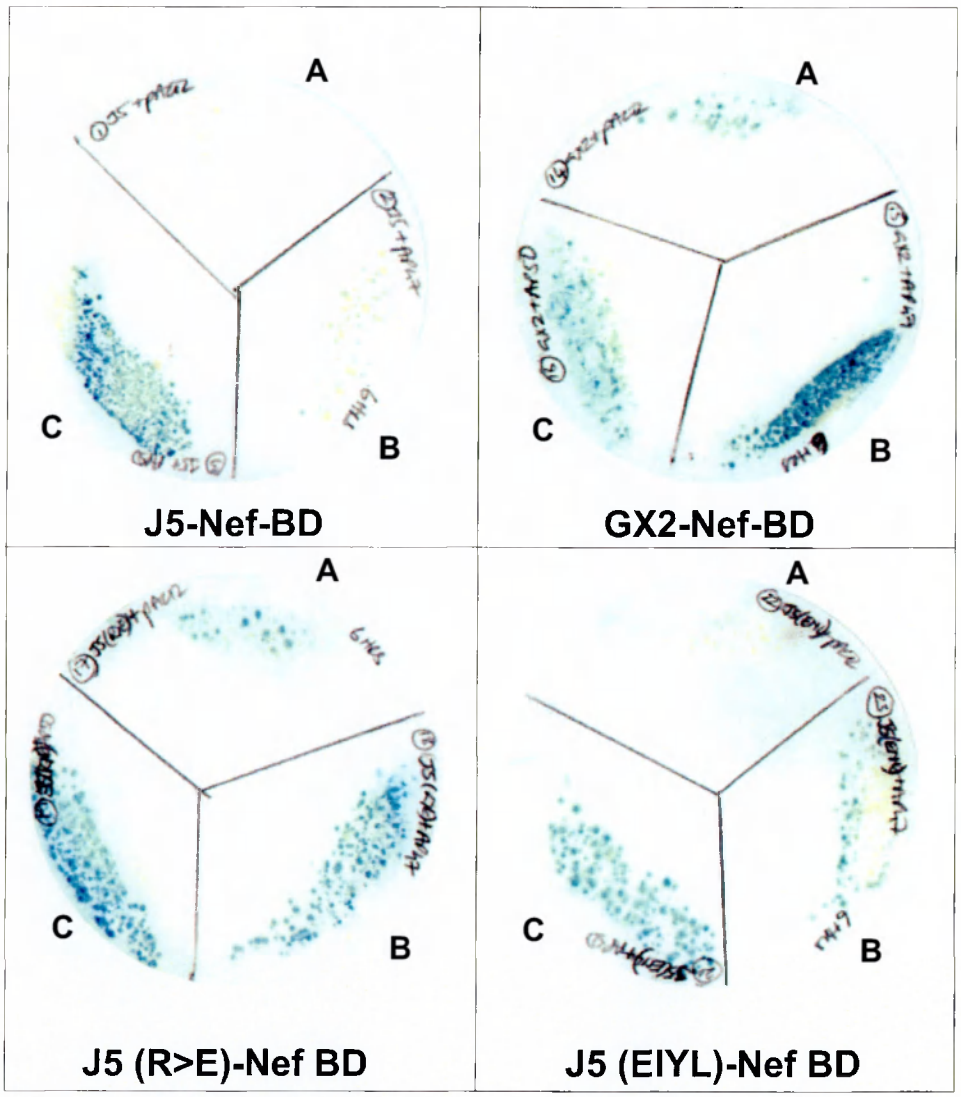


Figure 4.2.4c. β-galactosidase disc assay of the interactions of Nef-BD proteins with pACTII-AD (A), AP47-AD (B) and AP50-AD (C), after 6 hours incubation with the chromogenic substrate. A positive interaction is denoted by the production of β-galactosidase, which results in the colonies producing a blue colour after lysis and incubation with the chromogenic substrate. A negative interaction is denoted by the colonies remaining white. The interaction with pACTII-AD alone serves as a negative control.

Previously, some β-galactosidase production for the interaction of J5(EIYL)-Nef-BD with pACTII-AD had been observed. However, in this set of experiments, the interactions of J5-Nef-BD and J5(EIYL)-Nef-BD with pACTII-AD were negative. J5(EIYL)-Nef-BD revealed a positive interaction with AP47-AD and AP50-AD, that was comparable to that of J5-Nef-BD with AP47-AD and AP50-AD (Figure 4.2.5, Table 4.2.1). By contrast, J5(EIYL+R>E)-Nef-BD did not interact with AP47-AD and AP50-AD, as observed for C8-Nef-BD (Table 4.2.1, Figure 4.2.5).

b. Interaction of Nef-BD proteins with Lck, a member of the Src family of protein tyrosine kinases

An interesting phenomenon was observed for the interaction of Lck-SH3-AD protein with J5-Nef-BD, C8-Nef-BD and VLA*del-Nef-BD proteins. The number of colonies, in contrast to those of other interactions of the Nef-BD proteins, were very low, never being more than 10 and on some occasions as few as 2. Only on one occasion was a single positive colony observed, for J5-Nef-BD + Lck-SH3-AD, amongst negative white colonies. This made interpretation of this set of experiments difficult. The results of this set of experiments cannot be taken as an accurate reflection of this interaction (data not shown).

c. Interaction of Nef-BD proteins with TCRζ proteins

All of the Nef-BD clones were investigated for interactions with $TCR\zeta_{1.11}$ and $TCR\zeta_{2.21}$ (Table 4.2.2).

Wild type J5-Nef-BD interacted with both $TCR\zeta_{1.11}$ and $TCR\zeta_{2.21}$ clones. The attenuated C8-Nef-BD clone did not interact with either of the TCRζ clones, an observation which was consistent for VLA*del-Nef-BD.

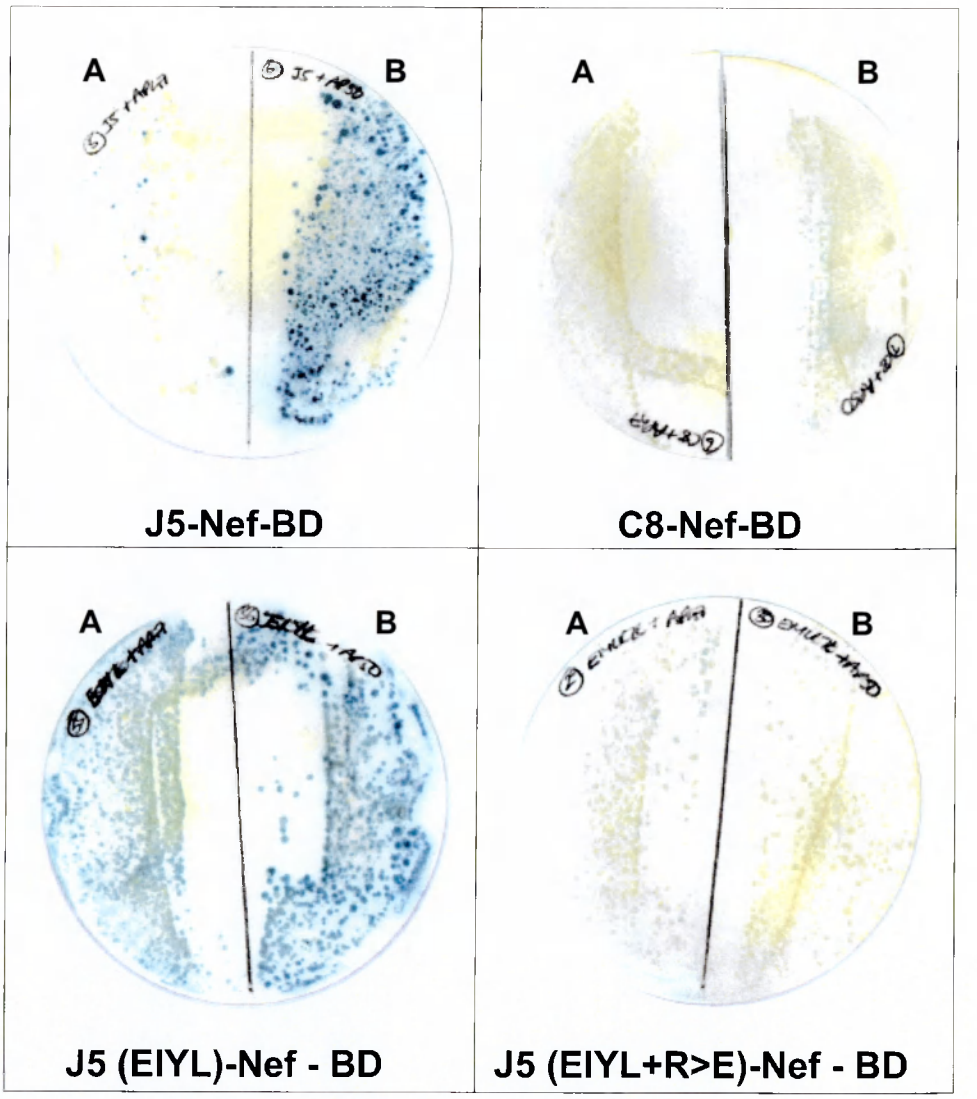


Figure 4.2.5. β-glalactosidase disc assay to show the interactions of J5-Nef-BD, C8-Nef-BD, J5(EIYL)-Nef-BD and J5(EIYL+R>E)-Nef-BD with AP47-AD (A) and AP50-AD (B). Repair of the deletion in C8 with the sequence EIYL repairs the ability to interact with the adaptor proteins, in a manner similar to that of J5. By contrast, when the point mutation R191E is presented in conjunction with the EIYL sequence, the interaction is disrupted. All proteins were tested for an interaction with pACTII-AD, as a negative control. In all instances, this was negative (data not shown).

Nef allele expressed in pASI-CYHII/*Bss*HII.I	Gene expressed in pACTII-AD		
	pACTII	**TCR$\zeta_{1.11}$**	**TCR$\zeta_{2.21}$**
pASI-CYHII/*Bss*HII.I	-	ND	ND
J5	-	+	+
C8	-	-	-
VLA*del	-	-	-
GX2	+	+	+
J5(EIYL)	-	+	+
J5(EIYL+R>E)	-	-	-
J5(R>E)	+	+	+

Table 4.2.2. The interaction of the Nef-BD DNAs with TCR$\zeta_{1.11}$-AD and TCR$\zeta_{2.21}$-AD. Data is representative of three independent experiments. The interaction of the various Nef-BD DNAs with the pACTII-AD was used as a negative control in each experiment, as well as that of the pASI-CYHII/*Bss*HII.I vector with pACTII-AD. - , negative, no blue colonies, + , positive, blue colonies present.

GX2-Nef-BD and J5(R>E)-Nef-BD were observed to cause trans-activation of pACTII-AD. A time course was not carried out for this set of experiments; instead the assay was observed during the course of incubation with the Z buffer/ONPG/β-mercaptoethanol to determine if there was any delay in β-galactosidase production for the interactions with pACTII-AD. Although a slight delay was observed, it was not long enough to determine whether the subsequent interactions with the TCRζ clones were true interactions and not partially or completely due to trans-activation of pACTII-AD.

J5(EIYL)-Nef-BD revealed a positive interaction with $TCR\zeta_{1.11}$ and $TCR\zeta_{2.21}$ that was comparable to that of J5-Nef-BD. J5(EIYL+R>E)-Nef-BD did not interact with any of the TCRζ clones, as observed for C8-Nef-BD (Table 4.2.2, Figure 4.2.6).

4.2.6. Liquid assay

To obtain more quantitative data for each of the interactions described, semi-quantitative liquid β-galactosidase assays were attempted. However, difficulty was experienced in growing yeast cultures to the appropriate density required for these experiments. The possible reasons for this are discussed in section 4.3.

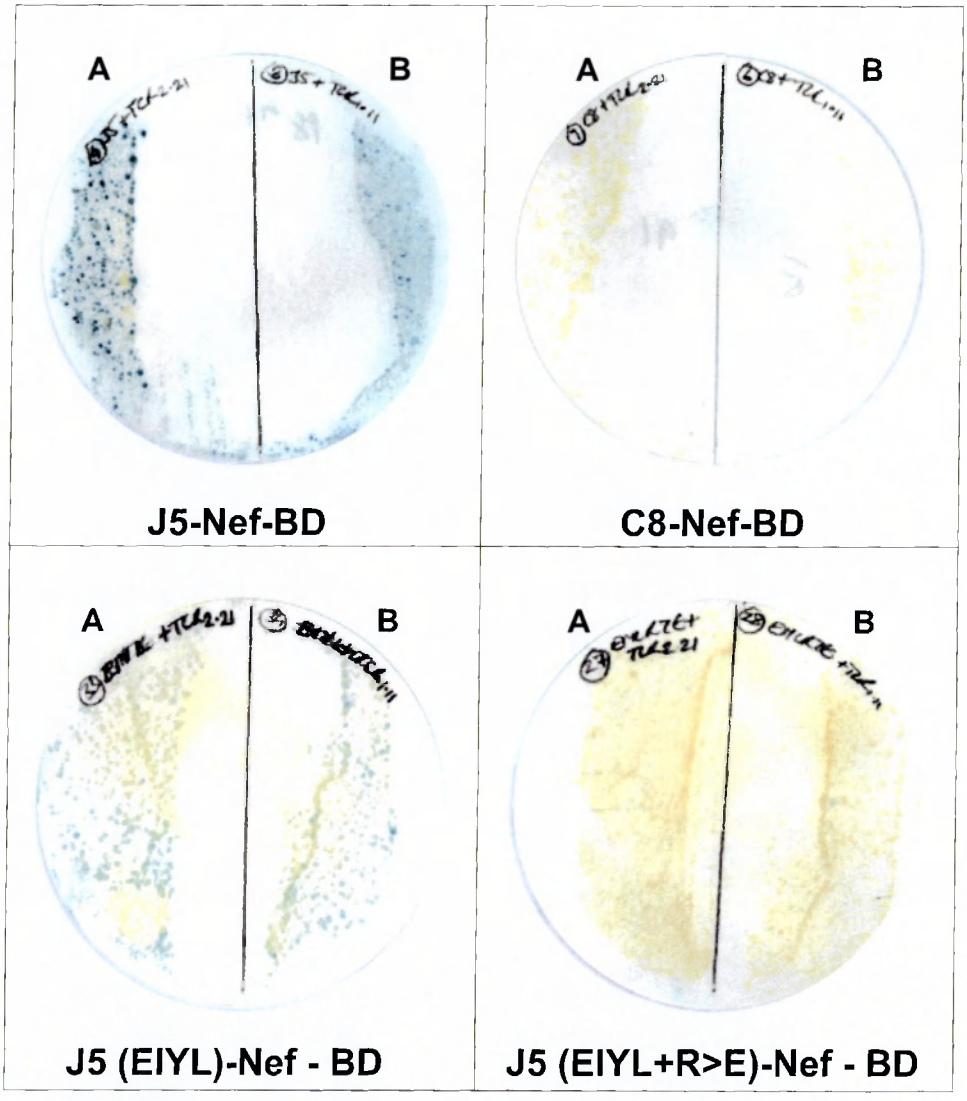


Figure 4.2.6. β-glalactosidase disc assay to show the interactions of J5-Nef-BD, C8-Nef-BD, J5(EIYL)-Nef-BD and J5(EIYL+R>E)-Nef-BD with TCR$\zeta_{2.21}$-AD (A) and TCR$\zeta_{1.11}$-AD (B). Repair of the deletion in C8 with the sequence EIYL repairs the ability to interact with the adaptor proteins, in a manner similar to that of J5. By contrast, when the point mutation R191E is presented in conjunction with the EIYL sequence, the interaction is disrupted. All proteins were tested for an interaction with pACTII-AD, as a negative control. In all instances, this was negative (data not shown).

4.3. Discussion

The ability of Nef to interact with a range of cellular partners represents a unique opportunity to subvert normal cell function to the benefit of the virus. The aim of this part of the project was to utilise yeast-2-hybrid technology to investigate the interaction of selected cellular components with novel SIV *nef* clones. The selected SIV *nef* clones had been characterised as having the ability to affect the level of virus replication observed either *in vitro* or *in vivo* (Whatmore *et al.*, 1995, Arnold *et al.*, 1999, N. Almond, personal communication). The aim was to identify the domains of Nef which mediate these interactions.

Protein analyses indicated that the SIV Nef proteins were being expressed as fusions with the GAL4 BD (Figure 4.2.2) as well as confirming that the proteins were indeed SIV Nef (Figure 4.2.3). Western blotting with the anti-HA MAb revealed the presence of an additional band at approximately 52 kDa in all of the protein preparations (Figure 4.2.2). This band was present in the control lane of vector only (Figure 4.2.2, Gels A & B, lanes 2), but absent when Western blotting was performed with Nef specific MAb KK75 (Figure 4.2.3). This is most likely to be a protein expressed by the transformed yeast that contains a peptide sequence similar to the HA tag. The exact identity of this protein is unknown although it has been observed previously (M. Morse, personal communication). However, as the control lane of the Y190 cells remained negative and no cross reactivity with the anti-Nef Mab was observed it was not considered that it was likely to have any adverse effects on the results obtained.

Whilst the majority of Nef-BD fusion constructs were expressed as relatively stable proteins of the predicted RMM, clones J5(EIYL)-Nef-BD and J5(EIYL+R>E)-Nef-

BD resolved to approximately 36-40 kDa and 48-50 kDa respectively. This result is remarkably similar to observations in the previous chapter where expression of GST-J5(EIYL) and GST-J5(EIYL+R>E) in a bacterial expression system also yielded proteins of distinct sizes which did not correlate with the predicted RMM of GST-SIV Nef. For GST-J5(EIYL) and GST-J5(EIYL+R>E), the proteins had relative molecular masses of 40-44 kDa (Figures 3.2.4 & 3.2.5), and 44 kDa (Figure 3.2.4) respectively. The reason for this reproducible aberrant RMM of these two Nef alleles is unclear. Substitution of the wild type sequence by these repair sequences could affect protein folding such that sites recognised by proteases are more accessible and the proteins are cleaved at specific sites into these smaller fragments. It is also possible, as discussed in section 3.2, that these recombinant proteins are particularly unstable and so are degraded by proteases until the minimal stable domain of the protein is encountered. However if these proteins of smaller RMM than predicted represented partially degraded proteins then the binding data becomes more significant, as regions of the protein which may influence the binding capacity of the Nef protein have potentially been altered.

The J5-Nef-BD and C8-Nef-BD clones had previously been tested in the yeast-2-hybrid system (Bell *et al.,* 1998). J5-Nef-BD had been observed to interact with the same TCRζ-AD and AP-AD clones that were utilised in these studies, allowing the interactions of J5-Nef-BD with each of the activation domain fusion proteins to be used as a positive control.

Wild type J5-Nef-BD protein was found to interact with AP47-AD, AP50-AD, $TCR\zeta_{1.11}$-AD and $TCR\zeta_{2.21}$-AD. These observations are in agreement with those previously published by Bell *et al.* (1998), and are consistent with observations for the

interaction of SIVmac239 Nef with AP47 and AP50 as demonstrated by Piguet *et al.* (1998) and Le Gall *et al.* (1998).

Interestingly, the interaction of J5-Nef-BD with AP47-AD consistently gave rise to a high number of white colonies, which appear to have lost one or both of the plasmids and have overcome the nutritional selection of the plates. In the case of the interaction between J5-Nef-BD + AP47-AD, colonies of this type constituted approximately 50% of those present. Although white colonies were detectable in other interactions, they were not so abundant. The reason for their prevalence in this particular interaction remains unclear.

A greater number of blue colonies were present following the interaction of J5-Nef-BD with AP50-AD than that of J5-Nef-BD with AP47-AD. Although parameters, including the transfection efficiency and the toxicity of the Nef protein to the yeast could be affecting the number of yeast colonies which are transformed with both proteins, these differences may also be attributable to differences in the ability of Nef to interact with these two proteins.

The adaptor proteins are present at different locations in the cell. AP47 attaches to the trans golgi network (TGN) and AP50 attaches to the plasma membrane. Nef is post translationally modified by the addition of a myristoyl group at its N terminus, which is essential for its association to the cytoplasmic face of the plasma membrane (Yu & Felsted, 1992). A myristoylated Nef may interact with AP50 to a greater extent than AP47 due to its tendency to localise to the plasma membrane. It is not known whether the Nef proteins produced in these experiments were myristoylated or not. However, the yeast strain *Saccharomyces cerevisiae* has been described to post translationally modify the amino terminus of the HIV-1 *gag* gene product by myristoylation

(Bathurst *et al.*, 1989). This has a direct effect on the cellular localisation of the *gag* precursors. HIV-1 Nef has also been described to be myristoylated in *Saccharomyces cerevisiae* (Macreadie *et al.*, 1993), although this was in a system where yeast transformants were grown in selective media containing myristic acid, which was not the case in the experiments described. Alternatively, subtle differences in the point of contact with Nef of these two proteins may make AP47-AD binding less stable.

SIV and HIV Nef have been described to downregulate the CD4 receptor possibly as a result of interacting with the cellular trafficking machinery. Nef directs CD4 to the endosome, by transport through clathrin coated pits (CCPs), a process which occurs primarily at the plasma membrane, and increases the rate of CD4 endocytosis. This process also occurs at the TGN, although to a lesser extent. This could also account for the differences observed in the ability of wild type J5 Nef to interact with AP47 and AP50, since, if the downregulation of CD4 by Nef involves the TGN to a lesser extent than the plasma membrane then Nef may interact with AP47 (which attaches to the TGN) less so than AP50 (which attaches to the plasma membrane).

The interaction of J5-Nef-BD with AP47-AD also correlates with observations that HIV-1 Nef induces the endocytosis of MHC class I complexes by internalisation in vesicles that accumulate at the TGN, sorting them into lysosomes and degrading them. This process involves AP-1, of which AP47 is the medium chain. These interactions allow Nef to reduce surface expression of CD4 receptors and MHC class I molecules and create an environment within which the virus can thrive.

The interaction of Nef with the adaptor proteins, (particularly with AP50), could serve as a bridge for Nef to associate with the plasma membrane and hence with TCRζ.

This is consistent with the observation that the Nef-BD proteins shown to associate with the TCRζ also associate with the adaptor proteins. It would be interesting to elucidate whether the interaction of Nef with TCRζ affects either of the two specific signal transduction pathways that are activated by the TCR upon stimulation. All the components of the TCR are required for efficient plasma membrane expression of the receptor (Weiss, 1993). By interacting with the ζ chain Nef could prevent the normal signal transduction through the T cell receptor, which could reduce the ability of the host to mount an effective immune response against the virus.

It appears that the cytoplasmic domain of the TCRζ is sufficient for the interaction with the various Nef proteins and the membrane proximal ITAM (present in $TCR\zeta_{1.11}$) is not required. ITAM motifs are required for interactions with Src homology 2 (SH2) domain containing proteins, such as ZAP70 kinase, involved in the T cell signalling pathway (Wang & Samelson, 1996). Previously, Bell *et al.* (1998) showed subtle differences between different *nef* alleles interacting with these TCRζ clones through the liquid assay, which was thought to be due to the presence of the different ITAM motifs. Within these experiments, this observation was not reproducible, due to the non-quantitative nature of the β-galactosidase assay.

Bell *et al.* (1998) reported that J5-Nef-BD did not interact with Hck–SH3, a member of the Src family of protein tyrosine kinases. In this study the interactions of SIV Nef with Lck-SH3 were investigated. Analysis of J5-Nef-BD, C8-Nef-BD and VLA*del-Nef-BD proved problematic, with very few colonies being generated by this interaction by comparison with the others described. The great reduction in the number of colonies suggests that the interaction of the Nef-BD proteins with Lck-SH3-AD may be toxic to the yeast, preventing their growth despite the availability of

nutrients. It is impossible to conclude whether any of the SIV-Nef-BD proteins investigated interact with Lck-SH3-AD. In future, it may be beneficial to repeat these experiments, using an inducible promoter such as that described by Macreadie (*et al.*, 1993) to regulate expression of toxic proteins in yeast cells, which could overcome the problems encountered in these experiments.

Previously, Harris *et al.* (1995) described an interaction between a baculovirus derived myristoylated HIV-1 Nef-GST fusion protein and baculovirus expressed Lck by taking lysate from Sf9 cells infected with Lck and incubating with myristoylated Nef-GST immobilised on glutathione agarose beads and subsequent analysis by immunoblotting. In this situation, where interactions of over expressed proteins may be toxic to the cell, the use of a purely biochemical method to look for interactions may also be advantageous.

By contrast to J5-Nef-BD, no specific interactions were observed between C8-Nef-BD and any of the cellular partners (Figures 4.2.5 & 4.2.6, Tables 4.2.1 & 4.2.2). This data is in agreement with that of Bell *et al.* (1998), who similarly observed no interaction of C8-Nef-BD with the TCRζ clones and the Hck-SH3-AD clone. However, Bell *et al.* (1998) showed, through the quantitative liquid assay, that C8-Nef-BD interacted with AP50-AD at a level approximately five fold less than that of J5-Nef-BD. A low level of β-galactosidase production observed for the interaction of C8-Nef-BD with AP47-AD and AP50-AD was revealed to be a basal level of production as it was comparable to the level of β-galactosidase produced when the yeast were transformed with pASI-CYHII/*Bss*HII.I vector and AP47-AD and AP50-AD (data not shown). It is possible that there is a low level of interaction of C8-Nef-BD with AP50-AD within this system that is detectable only through a quantitative assay. It is difficult to deduce whether C8 Nef may still downregulate CD4, despite

showing little or no interaction with the adaptor proteins in these experiments. Previously, Bell *et al.* (1998) failed to show any significant levels of CD4 downmodulation by C8 Nef. Therefore, attenuated SIV Nef proteins, exhibiting a reduced ability to interact with the adaptor proteins may also display an inability to down modulate cell surface levels of CD4.

VLA*del-Nef-BD revealed a pattern of interactions identical to that of C8-Nef-BD. Previously, VLA*del had been shown to exhibit an identical pattern of MAb binding to C8 (Chapter 3). The similarity of these observations can be explained by reference to the putative structure of SIV Nef (Figure 3.3.1). The truncation of VLA*del begins at position 213 which is in close proximity to the alpha helical region that is deleted in C8. The alpha helical region around which the attenuations in C8 and VLA*del are centralised may form a critical part of the Nef protein structure that is important for mediating cellular interactions and protein antigenicity. These sequence changes could also be affecting the overall folding of the protein, making the regions recognised by interacting partners or monoclonal antibodies inaccessible.

The ability of clones GX2-Nef-BD and J5(R>E)-Nef-BD to interact with the native activation domain of GAL4 when it is not fused to additional peptides/proteins makes analysis of the results gained for the interactions with AP47-AD, AP50-AD and the TCRζ-ADs difficult. This phenomenon was observed consistently for these two particular Nef-BD proteins, on multiple independent occasions. It is unclear as to why this non specific interaction is limited to these two mutant SIV Nef clones. Possibly, the overall shape of these clones exposes a region of the proteins that can bind to the GAL4 activation domain. Interestingly, the attenuations within these two clones are at

opposite ends of *nef* relative to each other. The 66 nucleotide deletion in GX2 is at the 5' end of *nef,* whilst the R191E mutation is at the 3' end. Little is known about the crystal structure of SIV Nef (Chapter 3). Homology mapping of the structure of SIV Nef based on HIV-1 Nef (M. Forster, personal communication, NIBSC) has predicted a putative structure of SIV Nef (Figure 3.3.1). This structure extends from amino acid 85-233 which does not include the deletion in GX2. Although position 191 is present in this putative structure, its location is in a region where relatively little spatial information is available. Therefore there is little or no relevant structural knowledge concerning the regions of these mutations that could help explain the mechanism of trans-activation to be mediated by the GX2 and J5(R>E) Nef proteins.

Attempts to further investigate the yeast-2-hybrid assay results using the semi-quantitative liquid assay failed. Problems were encountered at the preliminary stage of growing overnight cultures of the yeast transformed with the various Nef-BD and AD genes. Many of the cultures did not grow at all, or not to the appropriate density required to perform the liquid assay. This may be an issue of toxicity caused by the *nef* gene to the yeast. Whilst the yeast are growing on the selection media plates, it is possible that they may selectively delete regions of *nef* associated with toxicity, whilst retaining parts of the plasmid that confer the ability to grow on the selection media. By the time the yeast colonies are picked for the liquid assay they may be missing the parts of the DNA that are required for the yeast to grow in the selection media, or the regions of the gene that are required for the parts of the protein to interact. Problems associated with the toxicity of *nef* in the expression system used have been encountered previously (Chapter 3). For future studies, this should be taken into consideration when choosing an expression system within which to express SIV *nef.*

Interpretation of the yeast-2-hybrid data would be improved if information on the oligomeric states of Nef *in vivo* was known. If Nef forms dimers or trimers *in vivo* then the results from the β-galactosidase assays could be very revealing. It has been reported recently that the core domain of HIV-1 Nef forms stable homo-dimers and trimers in solution (Arold *et al.*, 2000). The non-specific trans-activation of GAL4-AD observed by clones GX2-Nef-BD and J5(R>E)-Nef BD could be a consequence of the structural status of the SIV Nef protein carrying these attenuations. Arold *et al.* (2000) investigated the accessibility of amino acids involved in interactions. It was observed that the configuration of the dimeric and trimeric states of the Nef core domain did not interfere with the ability to interact with the SH3 binding site, the protease cleavage site and the acidic cluster between the PXXP motif. However, Liu *et al.* (2000) reported that a mutation at position D123, in the HIV-1 Nef core domain (located on the oligomer interface) abrogated the ability of the protein to downregulate MHC class I resulting in enhanced viral infectivity and affecting the ability of Nef to form dimers. Thus, the oligomerisation of Nef could have an important effect on its ability to interact with other proteins. It is possible that, in GX2-Nef-BD and J5(R>E)-Nef-BD the attenuations are destabilising the oligomeric state of Nef and exposing surfaces that can interact with the GAL4-AD. In the absence of structural data on SIV Nef this is difficult to confirm or investigate further. The observation that repair of the last two amino acids to wild type sequence in J5(EIYL)-Nef-BD was sufficient to restore the interactions of Nef with AP47, AP50 and TCRζ is highly significant. This suggests the folding of the protein, when this repair sequence is present, is restored to its native structure. The observation that the pattern of interactions of J5(EIYL)-Nef-BD was similar to that for wild type J5 Nef-BD suggests that as the sequence within the initially attenuated virus (C8) reverts

back to wild type (J5(EIYL)), Nef regains the ability to exploit cellular processes, an observation which highlights the selective pressure for specific sequences within Nef to be intact. There are no premature stop codons within J5(EIYL) (Appendix 1, Figure 7.1). This is in agreement with the observations of Whatmore *et al.* (1995) who reported that repair of the deleted sequence in C8, (that one macaque (45R) was infected with), was associated with progression to disease and a wild type phenotype. Intriguingly, the repair sequence J5(EIYL) did not regain the ability to bind monoclonal antibody KK70 (Chapter 3). This indicates that whilst the binding of KK70 has been a useful surrogate marker for wild type Nef function, it may not be an absolute correlate.

By contrast, when the repair sequence, J5(EIYL+R>E)-Nef-BD is present the ability to interact with the cellular components is disrupted, an observation which is consistent with previous results (Chapter 3) where the binding of KK70 to this repair sequence was also disrupted. This focuses attention on the point mutation R191E as being a key factor in disrupting the interaction with cellular machinery. However, as previously discussed, the point mutation in J5(R>E) is one which caused trans-activation of the native GAL4-BD and therefore its interactions with the cellular components could not be determined.

The observation that the J5(EIYL) and J5(EIYL+R>E) proteins are smaller than the predicted size makes these results more intriguing. It may be that the combination of the repair sequence and the smaller size of the J5(EIYL) Nef protein contribute to exposure of the region that is involved in cellular interactions. Subsequently, the R191E mutation in J5(EIYL+R>E) may be obscuring this region.

The analysis of interactions between SIV Nef variants and components of the signal transduction pathway has proven illuminating particularly with regards to the various sequences across amino acids 143-146 which could potentially restore wild type function *in vivo*. These predictions require further investigation.

5. General Discussion

5. General Discussion

An intact *nef* gene is important for pathogenicity of HIV and SIV but the mechanisms by which it exerts effects on virus replication *in vivo* remain poorly understood. Molecular studies have identified a number of cellular activities by which the Nef protein may alter viral pathogenesis, either by modulating virus replication (either increasing or decreasing) or by facilitating escape from the immune system (Fackler & Baur 2002, Johnson & Desrosiers 2002, Collins & Baltimore 1999). However, it has been difficult to determine which of the interactions mediated by HIV-1 Nef are most critical *in vivo*. The work presented in this study applied a model system that permitted comparison of results generated from *in vitro* observations with results from experiments designed to investigate the *in vivo* replication of SIV. In particular these studies focused on the *in vitro* characterisation of a panel of five SIV *nef* clones whose influences on the *in vivo* replication of SIVmacJ5 have been characterised previously (Table 5.1, column 2) (Arnold *et al.*, 1999, N. Almond, personal communcation).

A further set of three *nef* alleles identified during the phenotypic reversion of attenuated SIVmacC8 to a virulent phenotype *in vivo* were also studied (Whatmore *et al.*, 1995). These mutants represented selected SIVmacC8 revertants isolated over 41 weeks post infection from macaque 45R. By 41 weeks post infection, the initially deleted four amino acid region in SIVmacC8 Nef had been repaired and evolved to the sequence DIYL. This sequence is very similar to the sequence of wild type SIVmacJ5 (DMYL) and the motif is identical to that in the pathogenic clone SIVmac239, associated with the ability of the virus to cause disease. These data

provide strong support to the proposal that the four amino acid region, amino acids 143-146, that is deleted in SIVmacC8, abrogates the capacity of Nef to mediate critical functions that result in the virus causing disease.

The purpose of these studies was to characterise the range of Nef alleles in order to identify if any correlations existed between selected *in vitro* biochemical properties and their defined *in vivo* phenotypes. Two types of analyses were performed. The first approach was to apply monoclonal antibodies to investigate the structure of SIV Nef. This required the development of a semi-quantitative assay to measure the capacity of selected specific MAbs to bind SIV Nef. The second approach was to apply yeast-2-hybrid analysis to probe interactions between the Nef variants and selected cellular components that have been observed to bind differentially between wild type and attenuated isolates of SIV Nef (Bell *et al.,* 1998, Howe *et al.,* 1998). Studies by other groups have been performed looking at related isolates of Nef in a variety of functional assays. The results of these and relevant data from other groups will be discussed in the context of data presented in this study.

A summary of the results obtained by the two analyses of SIV Nef described above is presented in table 5.1. It demonstrates clearly that there is a strong association between the antigenic structure of the carboxy terminus of Nef and the ability of the Nef protein to interact with TCRζ, AP47 and AP50 proteins. Thus, it confirms the importance of the four amino acid motif DMYL, that extends a proposed α-helical region encompassing surrounding amino acids 143-146, to create the structure necessary for Nef to bind KK70 and these specific cellular proteins. Nevertheless, the four amino acid motif itself is not directly involved in these interactions since the loss of the KK70 epitope by premature truncation of Nef at amino acid 213 (as in

Table 5.1. A summary of the results obtained for the panel of SIV Nef clones from *in vivo,* antigenic and yeast-2-hybrid analysis.

SD; standard deviation, +; a positive interaction / binding exhibited by the appropriate MAb, -; a negative interaction / no binding exhibited by the appropriate MAb, ND; not done, NI; non-interpretable.

In vivo data, courtesy of N. Almond (personal communication).

Nef Allele	Mean log_{10} virus load at 2 weeks p/i* +/- SD	Characterisation in vitro								
		Binding of monoclonal antibodies					Interaction assessed by yeast-2-hybrid analysis			
		I	II	III	IV	V	pACTII	AP47/AP50	SH3-Lck	TCRζ
J5	2.75 +/- 0.87	+	+	+	+	+	-	+	N/I	+
C8	0.75 +/- 0.5	-	-	-	+	+	-	-	N/I	-
C8del (Δ 143-146)	1.125 +/- 0.75	-	-	-	+	+	-	ND	ND	ND
VLA*del (Δ 213)	0.8 +/- 0.57	-	-	-	+	+	-	-	N/I	-
GX2	1.125 +/- 0.48	+	+	+	-	-	+	+	ND	+
J5(EIYL)	ND	-	-	-	+	+	-	+	ND	+
J5(EIYL +R>E)	ND	-	-	-	+	+	-	-	ND	-
J5(R>E)	ND	+	+	+	+	+	+	+	ND	+

* Number of SIV infected cells per 10^6 PBMC

VLA*del) also disrupts the binding of TCRζ, AP47 and AP50. Thus it would suggest that it is some aspect of the conformation of the carboxy terminus of Nef that is important in protein interactions rather than the specific amino acids delineated by these mutations.

Analysis of C8 revertant clones demonstrated that replacement of the deletion of amino acids 143-146 with the sequence EIYL disrupted the conformational dependent epitope recognised by KK70. Furthermore, the KK70 epitope was not reconstructed when the EIYL motif was expressed in the presence of an aspartic acid residue at amino acid 191 (R>E), as may have been predicted from the analysis of virus recovered from macaque 45R at 41 weeks post infection and maintained on C8166 cells for 28 days (A. Jenkins & C. Arnold, NIBSC, personal communication).

The results outlined in this thesis draw interesting parallels with those of Carl *et al.* (1999). This group utilised similar SIV Nef clones in their studies that contained the repair motifs that had been observed by Whatmore (*et al.*, 1995) during infection of these animals with SIVmacC8. However, these motifs were engineered into the SIVmac239 backbone. Their experimental system used stocks of virus (generated by transient transfection of 293T cells) to infect CEMx174 cells. Infection with wild type SIVmac239 produced much higher amounts of Nef when compared with production by SIVmac239ΔC8 and 1823ΔC8. This suggests that a fully intact *nef* gene is required for the efficient expression of virus. By contrast, all the Nef repair alleles, containing the various repair sequences, EKIL, EIYL, DMYL, 1820EKFLΔ10, 1823DIYL20 and 1823 DIYL42 were expressed efficiently. This was confirmed by Western blot analysis of 293T cells and is in agreement with data obtained from Western blot

analysis of SIV Nef-BD fusion protein expressed in yeast cells (section 4.2), whereby the level of expression of J5-Nef-BD and C8-Nef-BD revealed a marked difference between these two proteins. The consistency of these results between the two different groups using different expression systems suggests that the deletion of the four amino acid motif DMYL in C8 Nef affects the stability of the protein, possibly due to its position within the highly conserved core of the protein.

Another feature of the data presented by Carl *et al.* (1999) were the observations from the clone 1820EIYLΔ10 *nef*, which has a 30 bp deletion downstream removing amino acids 189-198 as well as the 12 bp mutation creating the EIYL motif. This clone was reported to be unable to downregulate CD4 expression. In that feature this result is similar to that generated for the repair sequence J5(EIYL+R>E) (section 4.2) which lost the ability to interact with the AP-47, AP-50, TCR$\zeta_{1.11}$ and TCR$\zeta_{2.21}$ clones, unlike clone J5(EIYL) which did interact with these proteins. From these two parallel sets of data, it appears that the mutation of sequences downstream of the 12 bp deletion sequence in C8 could be of importance in enabling important functions of Nef to be carried out. These downstream mutations could involve amino acid 191. However the analyses of the R191E mutant, when presented in a J5 backbone alone (J5(R>E)), were inconclusive. Although this mutant Nef did not have the ability to bind KK70 attempts to analyse interactions with selected cellular proteins were uninterpretable due to the non-specific trans-activation of the GAL4-AD by this clone, thus the potential role of amino acid 191 to be able to influence interactions remains unanswered. Nevertheless, the presence of this mutation appeared to influence the binding of MAb KK70 depending upon whether it was expressed in combination with EIYL or DMYL motifs in Nef.

It was disappointing that no valuable results were forthcoming from the analyses of Nef with members of the Src family of protein tyrosine kinases since it has been the subject of much investigation (Collette *et al.*, 1997, Coates *et al.*, 1997). The failure to gather any useful data appeared to be due to a technical difficulty, resulting from the biology of the system, as very few yeast cells were observed to survive the double transformation with Lck-SH3 and Nef. This is an intriguing observation and is suggestive that co-expression of these two proteins results in cellular toxicity not observed when either was expressed individually. This result makes it a very attractive topic to pursue within the system described within this thesis, with modifications as discussed in Chapter 4.3. However, others have investigated the interaction between these proteins. Arold *et al.* (1997) showed a high affinity binding of HIV-1 Nef with Hck-SH3 domain, which appeared to be dependant upon the isoleucine residue at position 96 in the RT loop of Hck. The SH3 domain of Lck, by contrast, has a serine at position 96 (Arold *et al.*, 1997). Furthermore, it was shown that if the arginine at position 96 of Fyn-SH3 was mutated to an isoleucine, this restored the interaction of the SH3 domain with Nef, showing that this amino acid may be important in mediating this interaction, possibly by affecting the overall protein shape that is presented for an interaction with Nef, or possibly forming part of the region that the Nef protein binds to.

Contradictory results have been obtained from studies to investigate the interaction between HIV-1 Nef and Lck *in vivo* and *in vitro*. Some groups have documented interactions between HIV-1 Nef and Lck (Cheng *et al.*, 1999, Collette *et al.*, 1996, Dutartre *et al.*, 1998, Greenway *et al.*, 1996) whilst others have not detected any interactions (Moarefi *et al.*, 1997, Saksela *et al.*, 1995). It is the PXXP motif at the N

terminus of HIV-1 Nef, consisting of amino acids 72-75 which appears to mediate the interactions with the SH3 domain of the src family tyrosine kinases. This region is critical for the interaction between HIV-1 Nef and Hck-SH3 (Briggs *et al.,* 1997, Cheng *et al.*, 1999, Collette *et al.*, 2000, Greenway *et al.*, 1999, Lee *et al.*, 1996). Some groups have found that the PXXP motif is required for an interaction with Lck (Collette *et al.*, 1996, Greenway *et al.*, 1996), whilst others have shown that it is dispensable (Baur *et al.,* 1997, Cheng *et al.,* 1999). The PXXP motif has also been demonstrated to be required for the interaction of SIV Nef with the Nef associated kinase (NAK) (Khan *et al.*, 1998, Manninen *et al.*, 1998). The NAK has since been identified as a p21 associated kinase (Renkema *et al.*, 1999), although opinions are divided as to whether it is PAK1 (Fackler *et al.*, 2000) or PAK2 (Renkema *et al.*, 1999, Arora *et al.*, 2000, Renkema *et al.*, 2001).

In vivo, experiments reported by Hanna and colleagues (2001) investigated the effects of mutating the HIV-1 Nef allele in the SH3 binding domain and expressing it in transgenic mice under the control of the CD4C gene. They also bred wild type transgenic mice on a Hck knockout background. It was found that these mice did not develop disease and the mutation in the Nef allele was the most likely cause of this. The absence of Hck had no effect on disease progression, therefore Hck was concluded to be dispensable for the development of AIDS-like disease in transgenic mice. However, the observation that disease progression was delayed in the *hck* knockout mice suggested that in the presence of Nef the level of Hck expressed may contribute to determining the rate of disease progression (normally Hck expression is restricted to cells of myeloid origin (Briggs *et al.*, 2002)). These results show that although within the system described in this thesis it was not possible to obtain any

results for the interaction of Nef with Lck, others have successfully demonstrated interactions with members of the Src family of tyrosine kinases.

The proline rich motif in Nef has been concluded to be central to the ability of Nef to induce cellular activation and to be required for the support of viral replication in resting cells (Saksela *et al.*, 1995, Fackler *et al.*, 1999). As discussed, the PXXP motif also contributes to the interaction between Nef and a NAK (Khan *et al.*, 1998, Manninen *et al.*, 1998), which is also required for the cellular activation of Nef, an increase in viral infectivity, production and disease progression (Sawai *et al.*, 1996). Time permitting, it would have been valuable to investigate interactions between the various Nef mutants with Lck-SH3, Hck-SH3 and NAK, as well as testing a further Nef construct, mutated in the PXXP motif. The various assay modifications discussed in Chapter 4 would have been incorporated to ensure that viable results were obtained. A mammalian two-hybrid system has also been described for the screening of Nef with cellular partners, such as Hck (Murakami *et al.*, 2002), which would be worth investigating as an alternative experimental system if the described yeast-2-hybrid system did not lead to viable results.

Use of the yeast-2-hybrid system could contribute towards understanding which of the interactions were dependant upon an intact PXXP motif, as well as identifying which of the novel mutants used in this study disrupt these interactions. Indeed, for the interaction of HIV-1 Nef with the NAK (Trono & Wang 1997, Sawai *et al.*, 1996) two arginines at the N terminus of helix α4 of Nef (R105 and R106) have been demonstrated to be required in co-immunoprecipitation and pull down assays (Sawai *et al.*, 1995, Fackler *et al*, 2000). This is particularly interesting given that the four amino acid deletion in C8 also sits within the equivalent α helical region of SIV Nef

(amino acids 143-146). Furthermore, considering the apparent importance of the PXXP motif in functional interactions between Nef and cellular proteins, it would also be useful to determine the effect of disrupting this motif on the ability of Nef to bind MAb KK70.

Of equal biological interest, but disappointing from the point of view of analysing Nef function, was the observation that two distinct Nef alleles GX2 and J5(R>E) exhibited binding activity with the parental activation domain plasmid. It is difficult to see how distinct changes in the amino and carboxy termini of the Nef protein result in binding to the GAL4 activation domain. This observation was confirmed on at least three independent occasions. Thus the results from these clones remain inconclusive. Hopefully it is possible that the application of alternative vectors to yeast-2-hybrid analyses may be more successful in reducing or removing this background binding activity (JF Allen, personal communication).

From the summary table (Table 5.1) the data accrued from the analyses of SIV Nef variants indicates that conservation of KK70 binding is necessary for the carboxy terminus of the protein to mediate its ability to support wild type virus replication kinetics *in vivo* (Table 5.1). In view of this observation it is surprising that mutation of amino acids 143-146 to an EIYL motif restored the interaction of Nef with AP47, AP50 and TCRζ in a manner indistinguishable from wild type J5 Nef in the absence of restoration of the epitope recognised by KK70 (Table 5.1). More intriguing were the observations that these interactions were lost when the EIYL sequence was present in conjunction with the R191E mutation. These observations raise the question of whether J5(EIYL) would express a wild type phenotype *in vivo*, and as a

result determine whether binding of TCRζ, AP47 and AP50 are critical functions for wild-type Nef activity.

The J5(EIYL) *nef* allele has been reconstructed into the SIVmacJ5 clone background and a virus stock prepared for *in vivo* studies (N. Almond, personal communication). Preliminary results indicate that this virus exhibits an attenuated phenotype *in vivo.* This suggests that the interactions between Nef and TCRζ, AP47 and AP50 whilst valuable to the virus are not critical *in vivo* and other properties may be more important. Nevertheless a more detailed examination of this preliminary data is necessary to determine whether J5(EIYL) is fully attenuated *in vivo* (N. Almond, personal communication).

The hypothesis that the interaction between SIV Nef and TCRζ is important and critical for Nef to exert its function *in vivo* would appear attractive. The TCRζ chains may prove important in elucidating the processes that could be subverted within an infected cell. Within this study, the cytoplasmic domain of the TCRζ was found to be sufficient for the interaction with the various Nef proteins, whilst the membrane proximal ITAM (present in $TCR\zeta_{1.11}$) was not required. It is well documented that the three ITAMs within the TCR are involved in specific signal transduction (Irving *et al.*, 1991), TCR/CD3 biogenesis (Love *et al.*, 1993, Shores *et al.*, 1994) and thymocyte selection (Shores *et al.,* 1997). It has been demonstrated that the interaction of J5 Nef with the TCRζ was not dependent upon a single ITAM motif, rather, J5 Nef was observed to interact with two separate domains of the TCRζ. One domain was between amino acids 52 and 86 and the other between amino acids 111 and 164, of which ITAMs 1 and 2 respectively are components (Schaefer *et al.*, 2000). Either of these interactions were found to be sufficient to lead to Nef-mediated

downmodulation of the TCR. This would be consistent with the results presented within this study, whereby the interaction of J5 Nef with TCRζ was unaffected by the absence of the most membrane proximal ITAM in the $TCR\zeta_{2.21}$ clone, indicating that this interaction was dependent upon the presence of ITAMs 1 and 2. In the context of the results presented within this thesis and those of Schaefer (*et al.,* 2000), it appears that overall, regions within both the TCRζ and Nef are important in mediating the interaction. This would suggest that the phenotype of the attenuated viruses observed *in vivo* could, in part, be due to an inability to interact with the TCRζ, hence preventing loss of immune function within the host. Although it is not clear how interaction with TCRζ may be beneficial for the virus, it is likely that it is the result of facilitating the replication of infected cells through the TCR rather than by reducing the capacity of bystander cells to deliver anti-viral immune responses. Nevertheless, there may be an alternative mechanism of inducing disease through the interactions between Nef and the TCR. One possibility is by the induction of apoptosis in uninfected bystander cells. For example, both Nef and the TCR have been described to be required for HIV-mediated upregulation of FasL (Johnson 1997, Depraetere & Goldstein 1997). HIV infected patients exhibit upregulation of Fas and FasL, as well as an increased susceptibility of CD4+ and CD8+ cells to Fas-mediated killing (Katsikis *et al.*, 1995). Both Nef and TCRζ are required for the upregulation of FasL by HIV. Furthermore, it has been demonstrated that, in Jurkat cells lacking TCR, binding of the Nef-associated serine kinase p62/NAK was disrupted (Xu *et al.*, 1999). As discussed previously, the PXXP motif within Nef, required for the interaction of Nef with members of the src family of tyrosine kinases, is also required for the interaction of Nef with p62/NAK. However, this study also demonstrates that

reconstitution of Jurkat cells with TCRζ ITAMs 2 and 3, restored binding of p62/NAK with Nef (Xu *et al.*, 1999). It would therefore be interesting to extend the studies described within this thesis and investigate whether Nef alleles interacted with the individual ITAMs of the TCRζ as well with the p62/NAK. This would allow investigation of the importance of the attenuated clones of SIV Nef in a wider molecular context and determine the specificity of Nef mediating broad molecular interactions. It would also determine which parts of the TCRζ was targeted by Nef.

The depletion of T cells observed during HIV infection is usually due to the effect of widespread apoptosis which is induced (Amiesen, 1992). Therefore, this is possibly a mechanism evolved by the virus in order to decrease the rate of apoptosis of infected cells. Several different apoptotic pathways have been described in HIV infection, such as Fas/FasL (Shearer 1998), TNF/TNFR (Herbein *et al.*, 1998) and TRAIL (TNF related apoptosis inducing ligand) and its receptors (Katsikis *et al.*, 1995).

The sophisticated interaction of Nef with TCRζ appears to bypass the requirement for TCR ligation by antigen, leading to upregulation of FasL. However, the upregulation of FasL by Nef is not the only mechanism by which the viral protein induces or accelerates apoptosis. It has been demonstrated that Nef directly interacts with p53 via amino acids 1-57 at the N terminus of Nef, which leads to the inhibition of p53 mediated apoptotic events (Greenway *et al.*, 2002). The ability of Nef to interfere with this apoptosis pathway, as well as that mediated by up-regulation of FasL, demonstrates clearly that certain interactions of Nef are dependant upon its subcellular localisation. Apoptosis is an important mechanism that can efficiently result in depletion of cells during HIV infection. Nef may contribute to two distinct

mechanisms that results in apoptosis – firstly, by up-regulation of FasL; secondly, by interacting with p53.

The interaction of Nef with the TCRζ highlights an important property of Nef. Simmons and colleagues (2001) investigated Nef action in putative T cell activation and virulence induction by monitoring transcriptional changes observed on large scale gene expression profiling (Simmons *et al.*, 2001). Nef was found to exert a widespread transcriptional program in T cells that was 97% identical to that of anti CD3 T cell activation. A comparison of the Nef expression profile with that obtained after T cell activation through CD3 revealed a strong identity. Fifteen transcription factors upregulated by Nef, which were capable of transactivating the LTR were identified, seven of which were inducibly expressed on T cell activation. This could represent a mechanism through which virion production is facilitated by Nef. Interestingly, it was observed that proteins that interact with Tat were induced, which suggests that Nef may indirectly facilitate the function of other HIV accessory genes. Comparing both transcription profiles it was observed that there was an overrepresentation of genes which influence HIV replication. Whilst the exact mechanism by which Nef can generate an expression profile similar to that of anti-CD3 T cell activation remains unknown, this observation is important in identifying avenues to elucidate key Nef functions.

The ability of Nef to alter the activation state of an infected cell has been described (Stevenson 1996). It has been demonstrated that adenovirus-mediated expression of Nef in primary macrophages was sufficient for chemokine induction and induced the chemotaxis of resting T lymphocytes, resulting in productive HIV-1 infection of the T lymphocytes (Swingler *et al.*, 1999). An additional, unidentified factor(s) secreted by

the Nef-expressing macrophages lead to the activation of the T lymphocytes, thereby creating an environment more permissive to the transmission of infection. This demonstrates how the virus has adapted the infection of macrophages as an indirect mechanism for increasing the transmission of infection.

A culture system which allows a direct comparison of the replication kinetics of an attenuated virus to a wild type virus would be valuable. To this end, Messmer *et al.* (2000) demonstrated that immature dendritic cells, when exposed to a Δ*nef* virus (with the *nef* gene deleted) and co-cultured with T cells, exhibited virus replication which was significantly lower than that observed for wild type virus. It was suggested that the immature dendritic cells are unable to support the replication of Δ*nef in vitro* which may contribute to the decreased pathogenicity of Δ*nef in vivo*. Indeed, it would be interesting to investigate if SIVmacC8 would exhibit an attenuated phenotype within this system described, especially as the deletion within it consists of only four amino acids. This could contribute to our understanding of the mechanisms underlying the attenuated phenotype observed *in vivo* for SIVmacC8.

Data from these studies did not determine how deletions in two genetically distinct regions of Nef could attenuate the *in vivo* viral replication to a similar degree. Both C8 and GX2 replicate in a similar manner *in vivo* (Chapter 3, Table 3.1.1) yet the studies presented have demonstrated that the two genetic mutations occur in antigenically distinct regions of the protein that disrupt distinct sets of protein interactions. One model to account for these observations is that Nef may exist in either a dimeric or a multimeric form in order to be functional and one of these genetic mutations may affect multimerisation (GX2, as evident by the ability to

transactivate native GAL4) and the other affects regions which interact with cellular components (C8). It is conceivable that one of the genetic mutations may affect multimerisation. Indeed it has been reported that HIV-1 Nef can form stable homo-dimers and trimers in solution (Arold *et al.*, 2000, section 4.4). Another potential model is that Nef acts as an adaptor protein that brings together specific cellular pathways or components resulting in enhanced viral replication. However, to prove this, further investigation into the interactions of Nef are required.

One property of Nef that was not investigated in the studies presented here was the downmodulation of cell surface MHC-1 molecules (Schwartz *et al.*, 1996, Le Gall *et al.*, 1998, Collins *et al.*, 1998). It had been demonstrated previously that there was little differential activity between J5 Nef and C8 Nef with regards to CD4 downmodulation (Bell *et al.*, 1998). However, selected Nef alleles have been observed to down-regulate MHC Class 1 and it has been ascertained that SIV and HIV Nef use different mechanisms in order to achieve this (Swigut *et al.*, 2000, section 1.2d). The downregulation of MHC-1 molecules may confer partial protection to cells from lysis by CTLs, which play an important role in viral load decline during primary infection and in determining the subsequent viral load set point. Furthermore, the downmodulation of MHC-1 by Nef may depend upon the rate or stage of disease progression. For example, it has been demonstrated that *nef* alleles derived from asymptomatic individuals efficiently downregulated the cell surface expression of MHC-1, whilst *nef* alleles obtained after progression to AIDS were unable to downregulate MHC-1 (Carl *et al.*, 2001). The ability of Nef to regulate interactions at specific points during disease progression, as well as that of being able to utilise more

than one mechanism of inducing apoptosis shows how sophisticated the level of interactions elicited by this protein are. The mechanism of downmodulation of MHC-1 by Nef suggests that in an immunocompetent host those properties of Nef which allow the virus to escape a CTL response are selected, such as MHC-1 downmodulation. Furthermore, Betts *et al.* (2001) used cytokine flow cytometry and overlapping peptide pools encompassing all of the HIV-1 genome to investigate the total T cell response to all potential epitopes in a group of 23 untreated HIV positive patients. High levels of HIV specific CD8+ and CD4+ T cell responses were detectable in nearly all 23 patients observed, demonstrating the importance of MHC-1 molecules and CTL responses in controlling infection. Therefore, if the virus is able to downmodulate MHC-1 molecules and consequently escape an immune response, it will be able to persist in the host and spread. It would be interesting, therefore, to investigate the levels of expression of TCRζ clones, MHC class 1 molecules and CD4 (given previous data on interactions with the APs) on cells which have been transfected with these proteins and the various Nef clones.

It was recently described that uncloned SIVmac239 variants expressing a truncated Nef (*tnef*) protein of approximately 25 kDa were pathogenic in infected rhesus macaques (Sawai *et al.*, 2000). These variants were observed in animals infected with recombinant SIVmac239 viruses containing a missense mutation of the Nef initiator methionine, combined with either a 152 bp deletion in the *nef*-unique region or an insertion of the interleukin-2 (IL-2) cDNA in place of the 152 bp deletion. Both of these resulted in a frameshift into the *nef* open reading frame (ORF). In reverted *tnef* alleles, the *nef* ATG initiation codon was restored and frameshift mutations were

repaired to restore contiguous, although truncated *nef* ORFs. The tNef proteins lacked approximately 50 amino acid long region overlapping the highly conserved core of the Nef protein. This deletion was reported to fall between elements which mediate the interactions of Nef with the adaptor proteins required for downregulation of CD4 and elements required for an interaction with PAK2 (Carl *et al.*, 2001, Lang *et al.*, 1997, Manninen *et al.*, 1998). Interestingly, the deletion in clone GX2, between amino acids 66-88, would also fall within the equivalent region in a SIVmac32H backbone. However, the DMYL sequence deleted in C8 falls outside of this region. Within this context it appeared surprising that a pathogenic phenotype was observed *in vivo*, as it has been observed that the deletion of 20 amino acids in GX2 is sufficient to confer an attenuated phenotype *in vivo* (Chapter 3, Figure 3.1.1). Also, it is surprising that a wild type phenotype was observed in the absence of a wide range of functions and interactions of the Nef protein. However, this is an example of the ability of the Nef protein to utilise a multitude of mechanisms and pathways, whereby, in the absence of specific interactions, the protein is still able to utilise alternative regions in order to subvert alternative pathways and ultimately to enhance pathogenesis.

Munch and colleagues (2002) took these studies further by reconstructing viruses containing the restored sequences. It was observed, by comparison to wild type Nef, the mutated Nef proteins did not have any effect on cell surface levels of CD4, CD28 and MHC-1 molecules. However, the mutated Nef was able to downregulate the cell surface expression of the T cell receptor in a manner indistinguishable from that of wild type Nef. No differences in growth kinetics between wild type and mutated *nef* alleles were observed *in vitro* in CEMx174 cells, however there was a marked difference between viral replication in rhesus PBMCs and herpes virus saimiri

transformed macaque T cell line 221, whereby the mutated *nef* alleles could not stimulate SIVmac239 replication. Six juvenile rhesus macaques infected with one of the mutated viruses were found to exhibit an attenuated phenotype. These results are partly consistent with the observations described within this thesis, whereby clone VLA*del and C8 (Chapter 3, Figure 3.1.1) did not interact with AP47 or AP50 and exhibited an attenuated phenotype *in vivo*, by comparison to wild type SIVmacJ5 infection. However, VLA*del and C8 also failed to interact with the TCRζ clones, whereas Munch and colleagues observed downregulation of the T cell receptor by the attenuated clones.

It is possible that the down modulation of the TCR:CD3 complex is not governed by the regions mutated in the Nef protein within the studies performed by Munch and colleagues, and that the attenuations of VLA*del and C8 are sufficient to prevent an interaction with the TCRζ clones within the yeast-2-hybrid system.. This also demonstrates, once again, that in the absence of specific functional interactions, the virus is able to utilise alternative pathways or interactions to exert a pathogenic phenotype *in vivo*.

The studies presented in this thesis, taken in the context of other concurrent research have paved the way for highlighting important areas of experimentation that could elucidate further information regarding the various molecular interactions of SIV Nef. Furthermore, they have demonstrated that the selective removal or disruption of selected regions of Nef can be sufficient to attenuate virus replication. If the critical functions mediated through specific regions of Nef can be interrupted, then it could identify novel targets for a therapeutic strategy against HIV. However, until key

functions of Nef can be identified this will prove difficult. Clearly, Nef augments virus replication through its interaction with cellular proteins to modify the environment within which it is located. The results from this study have provided valuable information as to the properties of SIV Nef that facilitate virus replication or enhance evasion of the immune system. Definition of any interactions that enhance the pathogenesis of HIV may allow a better focus of future resources to apply our knowledge to control virus infection and combat the global pandemic of AIDS by the development of more effective drugs and vaccines.

6. References

6. References

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7. Appendix 1: Sequences of SIV *nef* clones

Figure 7.1. Sequence alignment of SIV Nef clones used in the studies described. Highlighted are the attenuations or mutations within these clones. To summarise, C8, carries a four base pair deletion between amino acids 143-146 and two additional nucleotide changes relative to J5, C8del carries a four amino acid deletion between amino acids 143-146, J5(R>E) has a R>E mutation at amino acid 191, Nef 32H is the parental SIVmac32H from which J5 is derived, J5(EIYL) has the repair sequence EIYL between amino acids 143-146, EIYL+R>E has the repair sequence EIYL between amino acids 143-146 as well as the nucleotide change at amino acid 191 of R>E, GX2 carries a 66 nucleotide deletion at the N terminus, VLA*del is truncated prematurely by a stop codon at position 213, and the consensus wild type sequence J5.

```
          1                                                                                                   100
C8        ------------------------ ------------------------ ------------------------ ------------------------
C8del     ------------------------ ------------------------ ------------------------ ------------------------
J5(R>E)   ------------------------ ------------------------ ------------------------ ------------------------
Nef 32H   ------------------------ ------------------------ ------------------------ ------------------------
J5(EIYL)  ------------------------ ------------------------ ------------------------ ------------------------
(EIYL+R>E) ------------------------ ------------------------ ------------------------ ------------------------
GX2       ----------------- ------------s-------g--- ----------..............  ........----------------
VLA*del   ------------------------ ------------------------ ------------------------ ------------------------
Consensus MGGAISRRRSKSAGDLRQRLLRARG ETYGRLLGEVEDGYSQSLGGLDKGL SSLSCEGQKYNQGQYMNTPWRNPAE EREKLAYRKQNMDDVDEEDDDLVGV

          101                                                                                                 200
C8        ------------------------ ----------------....----- ------------------------ ---------------k---------
C8del     ------------------------ ----------------..*..----- ------------------------ ------------------------
J5(R>E)   ------------------------ ------------------------ ------------------------ ---------------e---------
Nef 32H   ------------------------ ------------------------ ------------------------ ------------------------
J5(EIYL)  ------------------------ -----------------ei------ ------------------------ ------------------------
(EIYL+R>E) ------------------------ -----------------ei------ ------------------------ ---------------e---------
GX2       ------------------------ ------------------------ ------------------------ ------------------------
VLA*del   ------------------------ ------------------------ ------------------------ ------------------------
Consensus PVMPRVPLRTMSYKLAVDMSHFIKE KGGLEGIYYSARRHRILDMYLEKEE GIIPDWQDYTSGPGIRYPKTFGWLW KLVPVNVSDEAQEDERHYLMQPAQT

          201                                                                  265
C8        ------------------------ -..--------------------- a--------------
C8del     ------------------------ -..--------------------- ---------------
J5(R>E)   ------------------------ -..--------------------- ---------------
Nef 32H   ------------------------ -..--------------------- ---------------
J5(EIYL)  ------------------------ -..--------------------- ---------------
(EIYL+R>E) ------------------------ -..--------------------- ---------------
GX2       ------------------------ -..-----------s-------- ----i------r---
VLA*del   ------------*lin*hmld-qks l--sqacqrkrlee-*-q-afltw- -r-k-a~~~~~~~~~
Consensus SKWDDPWGEVLAWKFDPTLAYTYEA YEAVRYPEEFGSKSGLPEEEVRRRL TARGLLNMADKKETR
```

8. Appendix 2: Plasmid maps

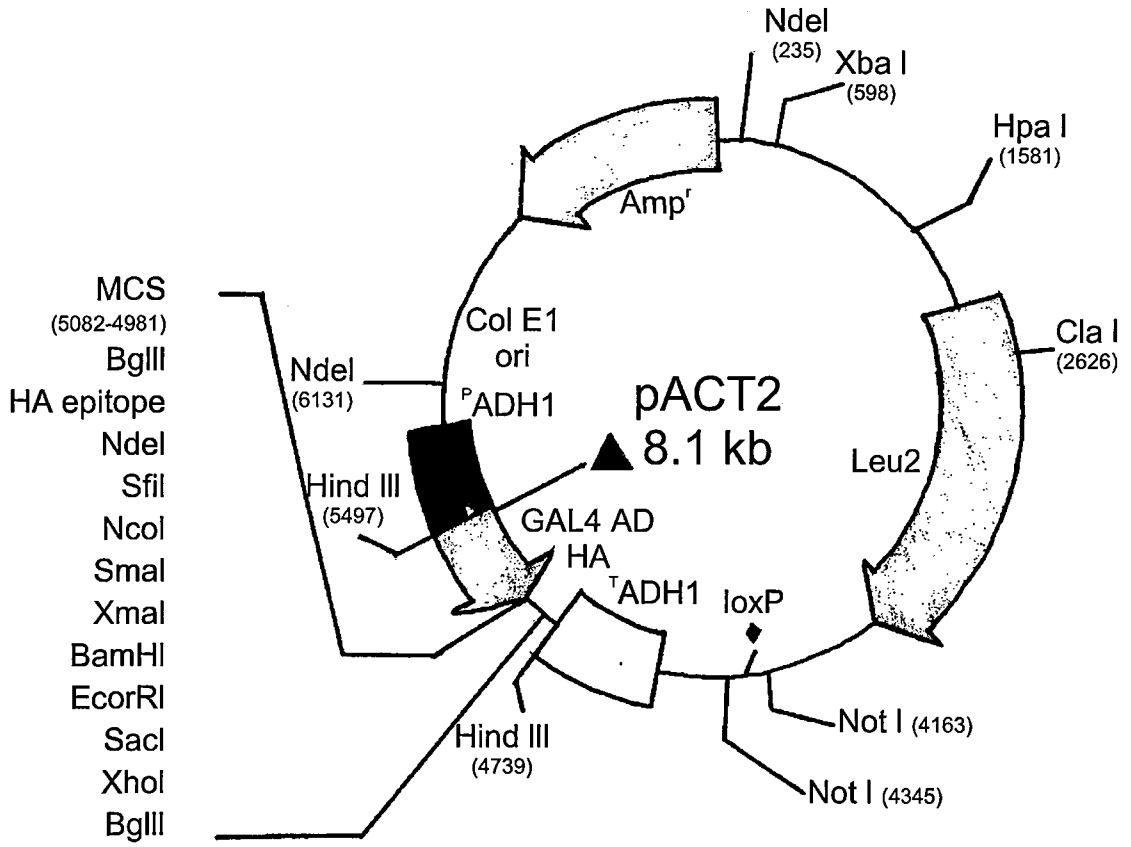


Figure 8.2. The map of plasmid pACTII (8.1 kb, Molecular Immunolonogy Unit, GSK). The multiple cloning sites are shown. The plasmid was made available with the genes for AP47, AP50, SH3-Lck, TCR$\zeta_{1.11}$ and TCR$\zeta_{2.21}$ already cloned, for use within the yeast-2-hybrid assay.

Leu2; the selection maker for the plasmid. Only yeast expressing the Leu2 gene can grow in media lacking leucine.

ADH1; alcohol dehydrogenase 1, the ADH promoter region, a constitutive gene promoter.

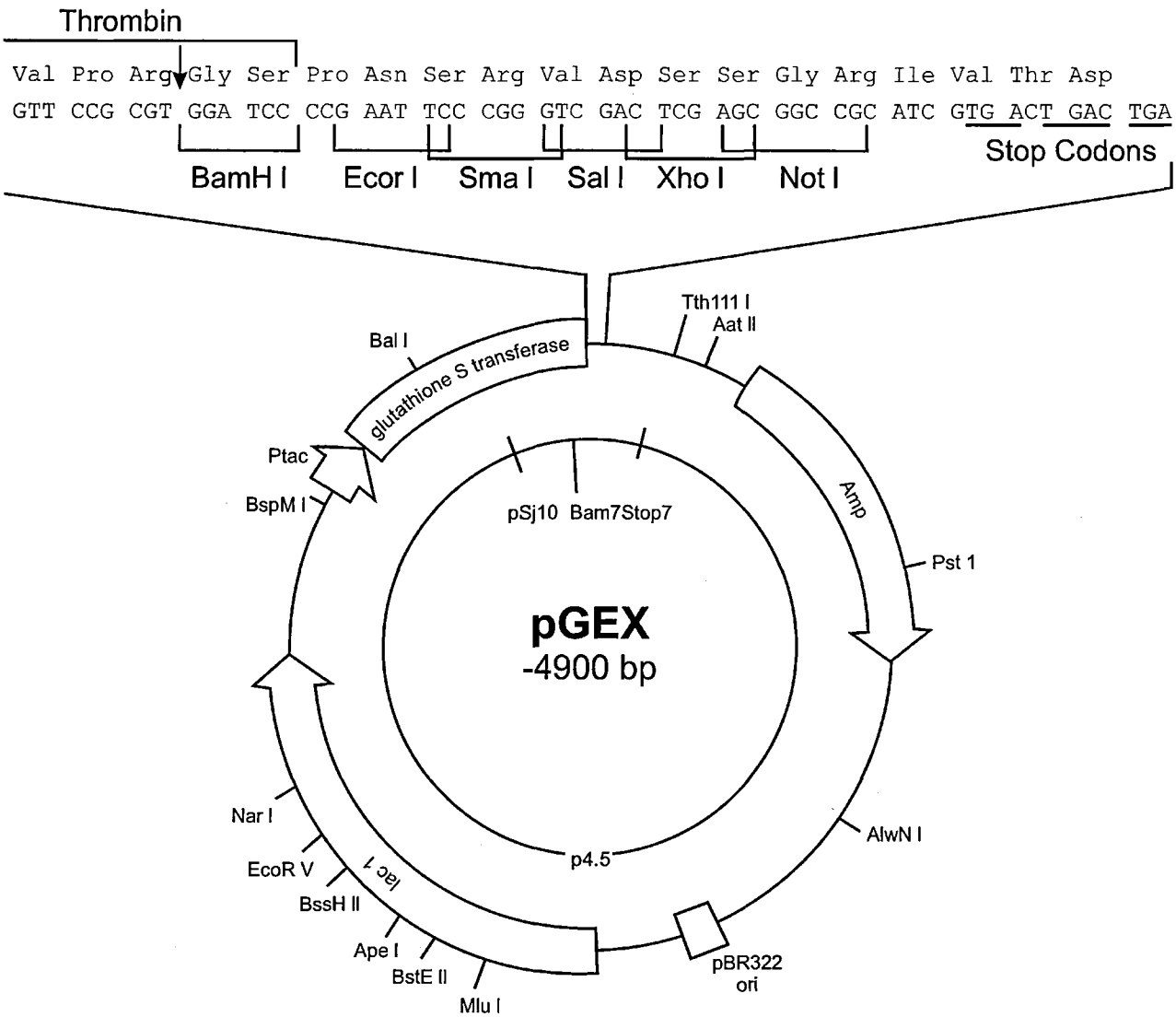
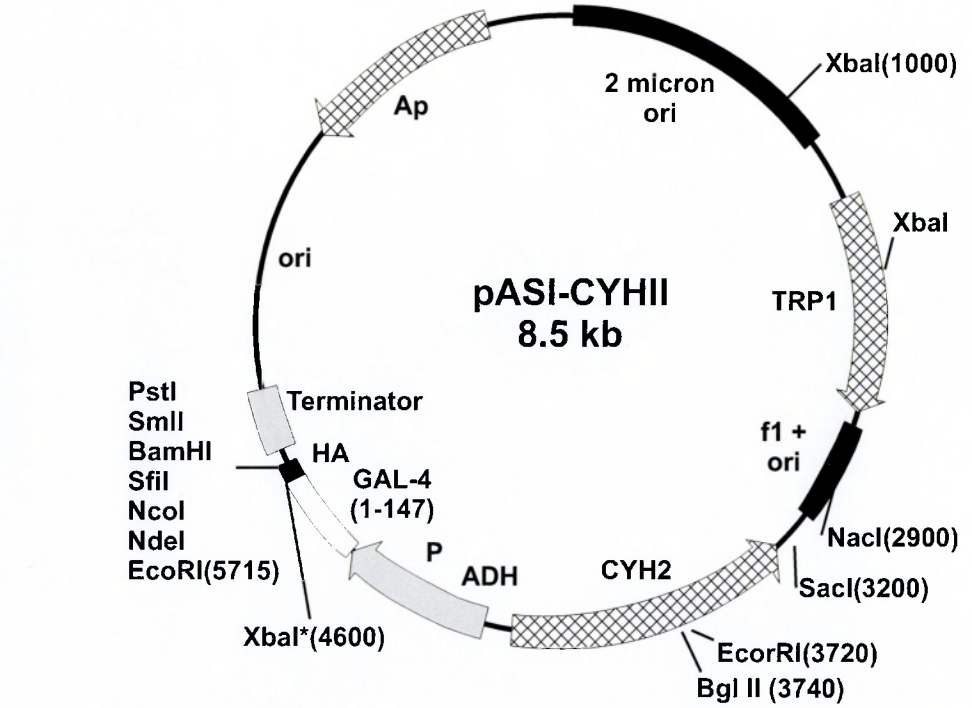


Figure 8.1. The map of plasmid pGEX-4T3 (4.9 kb, Pharmacia, UK), a GST fusion protein expression vector. The mutiple cloning sites are shown. For cloning of SIV *nef* mutants, restriction endonuclease sites *Bam*HI and *Eco*RI were used.




```
      NdeI        SfiI   NcoI                    BssHII          BamHI
5'    CAT-ATG-GCC-ATG-GAG-GCC-CCG-GCG-CGC-TCT-GGA-TCC-GTC-
      H   M    A   M   E   A   P   A   R   S   G   S   V

                  SalI
5'    -GAC-CTG-CAG-CCA-AGC-TAA-
       D   L   Q   P   S   stop
```

Figure 8.3: The map of plasmid pAS-CYHII. A modified *Bss*HII.I was introduced into the multiple cloning site to generate pASI-CYHII/*Bss*HII.I (M. Morse, Molecular Immunology Unit, GSK).

ADH; alcohol dehydrogenase, the ADH promoter region, a constitutive gene promoter.

CYH2; a cyclohexamide sesitivity gene. Allows for the selective loss of the pASI-CYHII plasmid if yeast are grown in cyclohaxamide. This is utilised if performing a yeast-2-hybrid library screen with episomal plasmids and the pACTII (activation domain) plasmid is extracted from the yeast – this allows selection for the loss of the binding domain of the pASI-CYHII plasmid.

9. Appendix 3: Recipes for Buffers and Media

9.1. Antigenic studies

Unless stated, water was purified using the Milli-Q plus Ultra-pure water system (Millipore). This is equivalent to using double distilled water.

Filtered water	milli Q water, filtered through a 0.75 μm filter unit
Phosphate buffered saline	10 g sodium chloride 0.25 g potassium chloride 1.44 g di-sodium hydrogen orthophosphate 0.25 g potassium dihydrogen orthophosphate water to 1 litre
Denaturing Solution	584 g sodium chloride 500 ml 10 M sodium hydroxide water to 10 litres
Neutralising Solution	605 g Trizma base 816 g sodium chloride 400 ml concentrated hydrochloric acid water to 10 litres adjust pH to 7.4
Standard Saline Citrate (SSC) (20x)	1753 g sodium chloride 882 g sodium citrate water to 10 litres
20% Hybridisation Buffer	10 ml formamide 12.5 ml 20 x SSC 2 ml 500 mM sodium phosphate

	1 ml 50 x Denhardt's 0.5 ml 10% (w/v) SDS 0.5 ml 100 mg/ml salmon sperm DNA water to 50 ml
Hybridisation buffer (low strigency incubations)	10 ml 5 x SSC 12.5 ml 20 x SSC 2 ml 500 mM sodium phosphate 1 ml 50 x Denhardt's 0.5 ml 10% (w/v) SDS 0.5 ml x 100 mg/ml salmon sperm water to 50 ml
10% SDS	10 g SDS water to 100 ml
50 x Denhardts	2 g Ficoll 2 g bovine serum albumin 2 g PVP-40 water to 200 ml
Luria-Bertram (LB) broth	25 g LB powder (Anachem, UK) per litre of water
Luria-Betram (LB) agar for plates	40 g LB agar powder (Anachem, UK) per litre of water
TAE Buffer for gel electrophoresis (50 x)	242 g Trizma base 57.1 ml glacial acetic acid 100 ml 0.5 M (EDTA) (pH 8.0) per litre of water

5 x sample buffer	1.25 g Ficoll 0.25 g SDS 0.5 ml 0.5 M EDTA 0.5 ml 5% bromophenol blue water to 5 ml
ELISA substrate	10 ml 0.1 M sodium acetate 1.4 ml 0.1 M citric acid 90 ml water 1 ml (10 mg/ml) 3, 3', 5, 5' TMB in DMSO
Equilibrated Phenol	Equilibrated with 1M Tris-HCl pH 7.6
10 mg/ml 3, 3', 5, 5' TMB in DMSO	10 mg 3, 3', 5, 5' TMB 1 ml DMSO
0.1 M sodium acetate	41.01 g sodium acetate water to 500 ml
0.1 M citric acid	2.1 g citric acid water to 100 ml
30% hydrogen peroxide	30 ml hydrogen peroxide water to 100 ml
100 mg/ml Ampicillin	5 g ampicillin water to 50 ml
1 M Tris-HCl pH 8.8	60.55 g Trizma base water to 500 ml adjust pH to 8.8

1 M Tris-HCl pH 6.8	60.55 g Trizma base water to 500 ml adjust pH to 6.8
10% ammonium persulphate	1 g ammonium persulphate water to 10 ml
100 mM calcium chloride	7.35 g calcium chloride water to 500 ml
50% glycerol in LB broth	50 ml glycerol 50 ml luria-bertram broth
100 mM IPTG	1 g IPTG 41.96 ml sterile filtered water
5% (w/v) milk /1% (v/v) Tween-20	5 g milk powder (Marvel) 1 ml Tween-20 100 ml PBS
10 mg/ml ethidium bromide	10 mg tablet ethidium bromide 1 ml water
5 M sodium perchlorate	61.2 g sodium perchlorate water to 100 ml
5% TCA	5 g TCA water to 100 ml
Running buffer	150 ml 10 x Tris Glycine 15 ml 10% (w/v) SDS 1335 ml filtered sterile water Reserve 350 ml of above Dilute the remainder with 800 ml water

Blotting buffer (for semi dry electrophoration)	250 ml 10 x Tris glycine 500 ml methanol water to 1 litre
Tris glycine	144 g glycine 30.27 g Trizma base water to 1 litre
SDS-PAGE sample buffer	48.4 g Trizma base 16.4 g sodium acetate 7.4 g EDTA water to 1 litre Adjust pH to 7.8
10 M sodium hydroxide	400 g sodium hydroxide water to 1 litre
500 mM sodium phosphate	69 g sodium phosphate (monobasic, monhydrate) water to 1 litre
1 M Tris-HCl pH 7.6	121 g Trizma base water to 1 litre pH to 7.6 using 11.6 M HCl

9.2. Yeast-2-Hybrid studies

Unless stated, water was purified using the Milli-Q plus Ultra-pure water system (Millipore). This is equivalent to using double distilled water.

All media, unless stated otherwise, was obtained from Sigma, UK.

Phosphate buffered saline	8 g sodium chloride 0.2 g potassium chloride 0.2 g potassium dihydrogen orthophosphate 1.15 g di-sodium hydrogen orthophosphate water to 1 litre adjust to pH 7.2
1X TBE	108 g tris 55 g boric acid 7.4 g EDTA water to 1 litre adjust to pH 8.0
Transfer buffer (for SDS-PAGE gels)	20% methanol 10% running buffer (Novex, UK)
YEPD agar	10 g yeast extract 20 g peptone 20 g glucose 20 g agar water to 1 litre
YEPD agar plates	270 ml YEPD agar 7.5 ml adenine

YEPD media	10 g yeast extract 20 g peptone 20 g glucose water to 1 litre
YEPD media (for overnight cultures)	170 ml YEPD media 10 ml 40% glucose 5 ml adenine
10X Yeast Nitrogen Base (YNB) without amino acids (GibcoBRL)	67 g YNB water to 1 litre
40% Glucose	400 g glucose water to 1 litre
-WHAUL dropout broth	1.1 g -WHAUL dropout powder water to 1 litre Adjust to pH 5.4
Histidine solution	2.4 g L-histidine water to 1 litre
Adenine solution	5 g L-adenine water to 1 litre
Uracil solution	2.4 g L-uracil water to 1 litre
Leucine solution	7.2 g L-leucine water to 1 litre

-WHAUL dropout agar	1.1 g -WHAUL dropout powder 23.53 g bacto agar Adjust to pH 5.4 water to approximately 220 ml
-WHAUL droput powder	1.2 g L-arginine 6 g L-aspartic acid 6 g L-glutamic acid 1.8 g L-lysine-HCl 1.2 g L-methionine 3 g L-phenylalanine 22.5 g L-serine 12 g L-threonine 1.8 g L-tyrosine 9 g L-valine
WHAUL selection plates	240 ml WHAUL agar 30 ml 10 x YNB 15 ml 40% (w/v) glucose 3 ml tryptophan 3 ml histidine 7.5 ml adenine 3 ml uracil 3 ml leucine 3 ml methionine
1 M Lithium acetate	102 g lithium acetate water to 1 litre
10X Tris EDTA (TE)	10 ml 100X TE (Sigma, UK) water to 100 ml
50% Polythylene Glycol (PEG) MW 3,350	50 g PEG water to 1 litre

LiAc/TE solution	10 ml lithium acetate 10 ml 10X TE 80 ml water
40% PEG/LiAc/TE	5 ml 1M lithium acetate 5 ml 10X TE 40 ml 50% PEG
Z buffer	16.1 g sodium phosphate (dibasic, heptahydrate) 5.5 g sodium phosphate (monobasic, monohydrate) 0.75 g potassium chloride 0.25 g magnesium sulphate (heptahydrate) water to 1 litre adjust to pH 7.0
100 mg/ml X-gal in DMF	100 mg X-gal 1 ml DMF
X-Gal/DMF/β-ME	100 mg/ml X-gal in DMF 100 ml Z-buffer 270 µl β-mercaptoethanol
Lysis buffer	0.1 M Tris-Cl pH 7.4 +/-0.05 (Sigma, UK) 0.05% Triton X-100 (final volume) water to 100 ml
Z buffer/ONPG/β-ME	50 ml Z-buffer 10 ml 4 mg/ml ONPG in 0.1M sodium phosphate pH 7.4 164.4 µl β-mercaptoethanol

4 mg/ml ONPG in 0.1 M sodium phosphate pH7.4	4 mg ONPG 1 ml 0.1M sodium phosphate pH7.4
1 M sodium carbonate	105.99 g sodium carbonate water to 1 litre
10% TCA	10 g TCA water to 100 ml
5 M sodium hydroxide	200 g sodium hydroxide water to 1 litre
0.1 M sodium phosphate pH 7.4	13.8 g sodium phosphate water to 1 litre adjust pH to 7.4

10. Appendix 4: Sources of Reagents

10. Appendix 4: Sources of Reagents

Reagent	Source
Ampli-Taq Gold™ polymerase	Perkin–Elmer (Roche)
dNTPs	Boehringer Mannheim
$MgCl_2$	Perkin-Elmer (Roche)
10 x PCR buffer	Perkin-Elmer (Roche)
ΦX174 DNA ladder	Gibco BRL
λHindIII DNA ladder	Gibco BRL
1 Kb DNA ladder	Gibco BRL
DNA gel loading buffer 10x	Eppendorf
$\gamma^{32}P$ ATP	ICN, UK
10x One-Phor-All buffer	Pharmacia, UK
T4 polynucleotide kinase	Gibco BRL
Glass microfibre filters	Whatman
X-ray film	Konica
T4 DNA ligase	Gibco BRL
5x ligation buffer	Gibco BRL
protein ladder (NIBSC)	Gibco BRL
protein ladder (GSK)	Gibco BRL
ELISA plates	Nunc
Alkaline phosphatase-calf intestinal	NE Biolabs
pUC19	Gibco BRL
Anti HA monoclonal antibody	Boehringer Mannheim
50 mM non fat milk powder (pH7.4)	Sigma

Salmon sperm DNA	Amersham USB
Ethidium bromide tablets	Amersham USB

11. Appendix 5: List of Primers

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For PCR purposes:

SN9075N

5' GTT CCG CGT GGA TCC ATG GGT GGA GCT AT TCC 3'

SN9866C

5'AAA GTC CCT GAA TTC TCA GCG AGT TTC CTT CTT 3'

Designed to incorporate *Bam*HI and *Eco*RI restriction sites, respectively.

For hybridisation purposes:

SN9763C

Synthesised by Gibco Life Technologies Ltd, UK.

For sequencing purposes:

NefF1:

5' CCA GTG ATG CCA CGA GTT 3'

NefR1:

5' AAC TCG TGG CAT CAC TGG 3'

Gal4BD Forward:

5' GAC AGC ATA GAA TAA GTG GCG 3'

Gal4BD Reverse:

5' CGT TTT AAA ACC TAA GAG TCA C 3'

Synthesised by Sigma-Genosys Ltd, UK.