ACCESSORY GENE COMPONENTS FOR AN HIV-1 SUBTYPE C VACCINE: FUNCTIONAL ANALYSIS OF MUTATED TAT, REV AND NEF ANTIGENS

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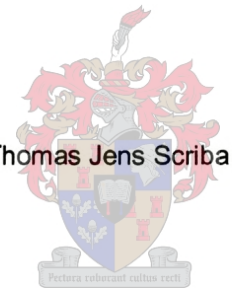

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Summary

HIV has attained a global distribution and the number of infected people reached an estimated 28.1 million in sub-Saharan Africa at the end of 2001. HIV-1 subtype C is overwhelmingly prevalent in Botswana and South Africa and to date no interventions have been successful enough to curb the rapid spread of the virus. A number of HIV-1 vaccine strategies are being developed, however the breadth and efficacy of such candidate vaccines, many of which are based on the HIV-1 structural genes *pol*, *gag* and *env*, have mostly been found to be inadequate.

The HIV-1 accessory genes are attractive components of HIV vaccines due to their role in viral pathogenesis, early expression and the high ratio of conserved CTL epitopes. Yet, because of undesirable properties questions regarding their safety as vaccine components are raised. In this study candidate *tat*, *rev* and *nef* mutants were assessed for efficient expression and inactivation of undesirable functionality.

Plasmid constructs that encode the South African HIV-1 subtype C consensus Tat, Rev and Nef proteins were constructed. The coding sequences of the genes were codon-optimised for optimum protein expression and these synthetic genes were constructed using overlapping 50-mer oligonucleotides. Furthermore, the proteins were mutated at previously described sites by PCR-based site-directed mutagenesis to render them inactive for their respective functions. Corresponding wild-type Tat, Rev and Nef constructs were also made from viral isolates that were least dissimilar to the respective consensus amino acid sequences. *In vitro* expression of the different constructs were assessed in 293 cells by Western blotting with polyclonal mouse sera, which were generated by DNA immunisation with one of the Tat, Rev and Nef constructs. The *trans*activation activity of Tat variants and Rev–mediated nuclear export activity of RRE-containing transcripts were studied in cotransfection experiments using reporter-gene-based assays while Nef functionality was assessed in a cotransfection assay with subsequent flow cytometric analysis of surface CD4 and MHC-I expression on 293 cells.

Sequence analysis of the South African HIV-1 subtype C consensus sequences of Tat, Rev and Nef revealed a high degree of similarity with a consensus sequence that was drawn up from a large number of viruses from southern Africa. These consensus sequences were also closer to individual viral isolate sequences than any individual sequences were, indicating that the use of a consensus sequence may serve to reduce genetic diversity between a vaccine and circulating viruses.

Expression levels of the sequence-modified *tat* and *nef* gene constructs were not significantly higher than the wild-type constructs, however, the codon-optimised *rev* mutant exhibited markedly higher expression than the wild-type *rev* construct. Immunoreactivity of the protein with the mouse sera demonstrates expression and immunogenicity of the Tat, Rev and Nef immunogens in mice. In the background of the subtype C Tat, a single C22 mutation was insufficient to inactivate LTR-dependent CAT expression in 293T and HeLa cells. Yet, this activity was significantly impaired using the single mutation, C37, or the double mutation, C22C37. Compared to the wild-type Rev, the function of the Rev with a double mutation, M5M10, was completely abrogated. Similarly, while the wild-type Nef and native, codon-optimised consensus Nef proteins mediated CD4 and MHC-I downregulation, CD4 downregulation was completely abrogated in one of the mutants, while both Nef mutants were entirely deficient for MHC-I downregulation.

These data demonstrate the high expression levels and impaired functionality of sequence-modified HIV-1 subtype C consensus Tat, Rev and Nef DNA immunogens that may be used as single-standing vaccine components or form part of a multi-component HIV-1 vaccine.

Opsomming

Sedert die eerste gevalle van MIV in die vroeë 1980's beskryf is het die virus wêreldwyd versprei en 'n beraamde 28.1 miljoen mense in sub-Sahara Afrika was teen die einde van 2001 geïnfekteer. MIV-1 subtipe C kom verreweg die meeste voor in Botswana en Suid-Afrika en tans is daar geen suksesvolle tussenkoms wat die vinnige verspreiding van die virus kan stuit nie. 'n Aantal MIV-1 subtipe C entstofstrategieë word tans ontwikkel maar die spektrum en effektiwiteit van sulke entstowwe, waarvan baie op die MIV strukturele gene *gag*, *pol* en *env* gebaseer is, is tans onvoldoende.

Die MIV-1 bykomstige gene is aantreklike entstofkomponente omdat hulle vroeg uitgedruk word, 'n belangrike rol in virale patogenese speel en omdat hulle 'n hoë verhouding van gekonserveerde sitotoksiese T-limfosiet (STL) epitope tot grootte besit. Vanweë hierdie gene se verskeie ongewenste eienskappe word vrae ten opsigte van hul veilige insluiting in enstofstrategieë geopper. Hierdie studie omskryf die evaluasie van kandidaat *tat*, *rev* en *nef* mutante vir doeltreffende proteïenuitdrukking en funksionele onaktiwiteit.

Plasmiedkonstrukte wat vir die Suid-Afrikaanse MIV-1 subtipe C konsensus Tat, Rev en Nef proteïene kodeer is saamgestel. Die koderingsvolgordes van die gene is geoptimiseer vir optimale uitdrukking en die sintetiese gene is van oorvleuelende 50-mer oligonukleotiede vervaardig. Deur van PKR-gebaseerde *site-directed* mutagenese gebruik te maak is hierdie proteïene gemuteer op posisies wat voorheen geïdentifiseer is. Ooreenstemmende wilde-tipe Tat, Rev en Nef konstrukte is gemaak vanaf virale isolate waarvan die aminosuurvolgordes die meeste ooreenstem met dié van die konsensusvolgorde. *In vitro* uitdrukking van die konstrukte in 293 selle is met behulp van immunoklad met poliklonale muissera bepaal. Die serum is gegenereer deur DNS immunisasie van muise met een elk van die Tat, Rev en Nef konstrukte. Die *trans*aktiverings-aktiwiteit van Tat variante en Rev bemiddelde uitvoer van RRE-besittende transkripte uit die nukleus is in verklikkergeen kotransfeksie-eksperimente bestudeer. Nef se funksionaliteit is deur kotransfeksie en die daaropvolgende vloeisitometriese analise van 293 selle se oppervlak-CD4 en MHC-I uitdrukking bestudeer.

Nukleotiedvolgorde-analise van die Suid-Afrikaanse MIV-1 subtipe C konsensus Tat, Rev en Nef proteïene toon 'n hoë vlak van ooreenkoms met 'n konsensusvolgorde wat afgelei is vanaf 'n groot aantal suider-Afrikaanse virusse. Hierdie

konsensusvolgordes is ook meer soortgelyk aan individuele virale isolate as enige individuele volgordes. Vanuit hierdie data kan afgelei word dat die gebruik van so 'n konsensusvolgorde die genetiese diversiteit tussen 'n entstof en sirkulerende virusse kan verminder.

Uitdrukkingsvlakke van die volgorde-geoptimiseerde *tat* en *nef* geenkonstrukte is nie merkbaar hoër as die van die wilde-tipe konstrukte nie. In teenstelling het die volgorde-geoptimiseerde *rev* mutant merkbaar hoër uitdrukkingsvlakke as die wilde-tipe getoon. Immunoreaktiwiteit van die proteïene met die muissera demonstreer dat die Tat, Rev en Nef proteïene uitgedruk word en immunogenies in muise is. 'n Enkele C22 mutasie in Tat is nie genoeg om LTR-afhanklike CAT uitdrukking in 293T en HeLa selle te inaktiveer nie. In teenstelling is hierdie aktiwiteit geïnhibeer vir Tat proteïene met die enkel mutasie C37 en die dubbel mutasie C22C37. In vergelyking met die funksionele aktiwiteit van die wilde-tipe Rev is dié van die Rev mutant M5M10 heeltemal geïnhibeer. Die wilde-tipe en geoptimiseerde, konsensus Nef proteïene het seloppervlak-CD4 en -MHC-I uitdrukking verlaag, maar hierdie effek van afregulering van CD4 uitdrukking was heeltemaal opgehef in een Nef mutant en van MHC-I uitdrukking in beide Nef mutante.

Hierdie data demonstreer die hoë uitdrukkingsvlakke en geïnhibeerde funksionaliteit van volgorde-gemodifiseerde MIV-1 subtipe C konsensus Tat, Rev en Nef DNS immunogene wat as enkelstaande enstof kan optree of deel kan uitmaak van 'n multi-komponent MIV-1 entstof.

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HIV-1_{JR-CSF} Nef Monoclonal Antibodies from Dr. Kai Krohn and Dr. Vladimir Ovod.

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List of Abbreviations

A	adenine
aa	amino acids
AIDS	acquired immunodeficiency syndrome
AP	adaptor complex
APC	allophycocyanin
APCs	antigen presenting cells
ARV	AIDS-related virus
ATV	active trypsin versene
bGH	bovine growth hormone
bp	base pairs
BSA	bovine serum albumin
ºC	degrees Celsius
C	cytosine
CA	California
Cat	catalogue
CAT	chloroamphenicol acetyl transferase
CCR5	CC chemokine receptor 5
CD	cluster of differentiation
CMV	cytomegalovirus
CTD	carboxyterminal domain
C-terminal	carboxy terminal
CTL	cytotoxic T lymphocyte
CXCR4	CX chemokine receptor 4
DC	dendritic cell
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
DNS	Afrikaans: deoksiribonukleïnsuur
EDTA	ethylene diaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
et al.	et alibi
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
G	guanine
HA	haemagglutinin

HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTLV	human T cell leukemia virus
Ig	immunoglobulin
IL	interleukin
IN	integrase
kb	kilo bases
kDa	kilo Dalton
L	litre
LTR	long terminal repeat
µg	microgram
µl	microlitres
M	molar
mA	milliamperes
MA	matrix
MHC-I	major histocompatability complex class I
MIV	Afrikaans: menslike immungebrekvirus
mg	milligram
ml	millilitres
mm	millimetres
M_r	molecular weight
mRNA	messenger RNA
MVA	modified vaccinia virus Ankara
N/A	not applicable
NAK	Nef-associated serine-threonine kinase
N/D	not determined
NES	nuclear export signal
NFAT	nuclear factor of activated T cells
NLS	nuclear localisation signal
nm	nanometres
No.	number
NSI	non-synsytium-inducing
N-terminal	amino terminal
OD	optical density
OPT	sequence optimised
ORF	open reading frame
pA	polyadenylation signal

PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-phycoerythrin
Pro	protease
RBD	RNA binding domain
RD	rhabdomyosarcoma
RE	restriction endonuclease
R_f	relative electrophoretic mobility
RNA	ribonucleic acid
rpm	rotations per minute
RRE	rev-responsive element
RSV	Rous sarcoma virus
RT	reverse transcriptase
sA	southern Africa
SA	splice acceptor
SD	splice donor
SDS	sodium dodecylsulphate
SV40	simian virus 40
T	thymine
TAK	Tat-associated kinase
TAR	transactivation-responsive element
TCR	T-cell receptor
TNFα	tumour necrosis factor alpha
TRRM	template ready reaction mixture
TSR	template suppression reagent
TV	Tygerberg Virology
U	enzyme units
UK	United Kingdom
VLP	virus-like particle
vs.	versus
WHO	World Health Organisation
wt	wild type
za	South Africa

Contents:

CHAPTER 1

1.1 Introduction and Literature Review

1.1.1 The history of HIV-1

In 1978, Robert Gallo *et al.* isolated the first human retrovirus from the lymphocytes of a leukemia patient and named it human T cell leukemia virus type I (HTLV-I) (Poiesz *et al.*, 1980). In the following years a second retrovirus, HTLV-II, was discovered and in 1981 a novel clinical syndrome characterised by the unexpected onset of immunodeficiency and opportunistic infection was recorded in homosexual men (Gottlieb *et al.*, 1981). It was termed acquired immunodeficiency syndrome (AIDS). Two years later, in 1983, Montagnier and Barré-Sinoussi were the first to isolate a retrovirus from an AIDS patient (Barre-Sinoussi *et al.*, 1983) and it was subsequently called human immunodeficiency virus type 1 (HIV-1) (Coffin *et al.*, 1986). HIV-2, the fourth human retrovirus, was isolated from immune-suppressed patients in west Africa (Brun-Vezinet *et al.*, 1985). Since these discoveries extensive research has been devoted to HIV and its origin. Genetic similarities with the simian immunodeficiency virus (SIV), which naturally infects a variety of African primates, led researchers to postulate that HIV originated in Africa where the close contact between monkeys and humans could have been responsible for the bridging between the species. Indeed, AIDS as a zoonosis has recently been demonstrated elegantly using phylogenetic analysis and this evidence indicates that the SIV counterparts of HIV-1 and HIV-2 were introduced into the human population on at least seven occasions (Hahn *et al.*, 2000). More specifically, phylogenetic data supported by geographic linkage reveal that HIV-2 originated from the transmission of SIVsm (Gao *et al.*, 1992), which naturally infects the sooty mangabey monkey whereas HIV-1 infection arose from the transmission of SIVcpz from chimpanzees to humans (Gao *et al.*, 1999). These zoonotic transmissions have been attributed to the direct contact between humans and infected sooty mangabey and chimpanzee blood and have been dated to an estimated 1930 (range 1910 to 1950) (Hahn *et al.*, 2000).

1.1.2 HIV-1 genetic diversity

Since the discovery of HIV, an ever-increasing effort to characterise strains from different global sources has generated substantial sequence data representing large numbers of viruses. Although HIV-2 is still primarily restricted to West Africa, HIV-1 has spread throughout Africa and some lineages have been dispersed around the globe (UNAIDS 2001). Phylogenetic analyses of the numerous HIV-1 strains have revealed three distinct monophyletic groups, which have been termed groups M (main) (Louwagie *et al.*, 1993), N (new or non-M, non-O) (Simon *et al.*, 1998a) and O (outlier) (Vanden Haesevelde *et al.*, 1994). Further analyses of the viruses that were classified as group M revealed that most sequences fell into a number of distinct clades, which led to the assignment of discrete subtypes (Louwagie *et al.*, 1993). Initially, five subtypes were identified, but extensive global characterisation has revealed at least nine to date, designated A to D, F to H, J and K, with a number of viruses that do not fall within these clades. The group M subtypes are approximately equidistantly related displaying amino acid sequence dissimilarities of 25 to 30% in their envelope proteins (Peeters and Sharp, 2000).

There are a number of factors that can collectively be held responsible for the extremely high genetic variability of HIV. The first one is that of the previously described multiple zoonotic transmissions from at least two different primate sources (Gao *et al.*, 1999; Gao *et al.*, 1992). Second, since the transmission into the human population, HIV has accumulated additional genetic diversity through the rapid turnover of virions in infected individuals (Ho *et al.*, 1995) during which the high error rate of the viral reverse transcriptase incorporates mutations in the course of synthesizing a deoxyribonucleic acid (DNA) copy of the ribonucleic acid (RNA) genome (Preston *et al.*, 1988; Roberts *et al.*, 1988). Furthermore, reverse transcriptase is highly recombinogenic, which facilitates genomic recombinations between genetically different viruses infecting a single individual to yield mosaic virus genomes (Hu and Temin, 1990). The first mosaic or recombinant virus MAL was first identified in Zaire in 1986 (Alizon *et al.*, 1986) and since then many recombinant viruses with different subtype combinations have been characterised. Recently, an intergroup recombinant virus was identified, which contained genomic segmental exchanges between group O and M (Takehisa *et al.*, 1999). Some of the intersubtype mosaic viruses are unique, however recombinant genomes with similar breakpoints and subtype representation have been identified in

apparently unlinked individuals (Carr *et al.*, 1998). The latter have been designated as circulating recombinant forms (CRFs) and play an important role in the global AIDS epidemic. A final factor which leads to the diversification of HIV is the pressure on the virus to mutate in such a way so as to evade various detrimental effects exerted by forces such as immune pressure (Borrow *et al.*, 1997; Price *et al.*, 1997). It has been demonstrated that strong immune responses, in the form of antibodies (McLain *et al.*, 2001; Ciurea *et al.*, 2000; Cheng-Mayer *et al.*, 1999) and cell mediated immunity (Barouch *et al.*, 2002; Goulder *et al.*, 2001; Phillips *et al.*, 2001; Allen *et al.*, 2000a; Price *et al.*, 1997), drive selection for randomly occurring mutants, which eventually may lead to fixation of previously subdominant quasispecies. Similar selective pressures, which drive viral escape from anti-HIV drugs that typically target viral enzymes, have emerged since the administering of antiretroviral treatment (Mayers, 1996). These factors all contribute to the extreme diversification of HIV, which leads to challenging implications in fields such as serological and molecular diagnostic testing, antiretroviral treatment and vaccine development (Peeters and Sharp, 2000).

1.1.3 The AIDS pandemic and its impact on South Africa

The global state of the AIDS pandemic has acquired enormous proportions and according to United Nations AIDS statistics, an estimated 40 million people worldwide were living with HIV or AIDS at the end of 2001 of which 28.1 million lived in sub-Saharan Africa (UNAIDS/WHO Report 2001). During the year 2001 alone, 3 million people died of AIDS related diseases and 5 million people were newly infected with the virus. Although AIDS is no longer one of the top 10 causes of death in the United States (Essex, 1998), it is one of the leading causes of death in Sub-Saharan Africa and developing countries in South America and East Asia (UNAIDS/WHO Report 2001). On a national level in South Africa, it was estimated that 24.8% of pregnant women were infected with HIV by the end of 2001. The KwaZulu-Natal province has the highest estimated prevalence at 33.5%, while the Western Cape has the lowest at 8.6% (Department of Health, 2001). The two confidence limits, from which the true HIV-1 prevalence estimates are estimated and the estimated true values for the nine provinces of South Africa are tabulated in **Table 1**. HIV/AIDS has also recently been shown to be the single leading cause of death in South Africa (Dorrington *et al.*, 2002). While the global distribution of

distribution of HIV was initially characterised by predomination of specific subtypes in certain geographical locations, such as subtype B in Europe and North America, an increasingly wider variety of subtypes are being documented on most continents (McCutchan, 2000).

Table 1: Provincial HIV prevalence estimates taken from antenatal clinic attendees at the end of 2001.

province	estimated prevalence (%)	confidence limits
KwaZulu-Natal	33.5	30.6 – 36.4
Mpumalanga	29.2	25.6 – 32.8
Gauteng	29.8	27.5 – 32.1
Free State	30.1	26.5 – 33.7
North West	25.2	21.9 – 28.6
Eastern Cape	21.7	19.0 – 24.4
Limpopo	14.5	12.2 – 16.9
Northern Cape	15.9	10.1 – 21.6
Western Cape	8.6	5.8 – 11.5
National	24.8	23.6 – 26.1

The true prevalence is estimated to fall within the two confidence limits. Source: National HIV and Syphilis sero-prevalence survey of women attending public antenatal clinics in South Africa – 2001 (HIV Seroprevalence Survey, 2001).

The statistics paint a desperate picture, which emphasizes the urgent need for control and more optimistically, the eradication of HIV. The development of antiretroviral treatment has had limited success even though immense attention has been devoted to this subject. A number of drugs aimed at inhibition of viral replication by targeting essential retroviral enzymes such as reverse transcriptase and viral protease have proven to be effective at curbing disease progression and reducing transmission (Harrington and Carpenter, 2000). However, the incredible mutation rate of HIV-1 results in quick acquisition of drug resistance mutations and combination therapies, which have to be altered regularly, are necessary to stem viral replication over extended periods of time. The extreme costs of such therapies and unavailability of sufficient funding in developing countries confine the availability of such drug regimes, which are often short, transient programmes, to a minority of infected people or patients with late-stage disease. The necessity for a preventative or therapeutic HIV-1 vaccine is blatant.

1.1.4 Structure of the HIV-1 virion and genome

The HIV-1 virion is an icosahedral structure as identified by high-resolution electron microscopy and consists of a membrane envelope, which is comprised of a host derived lipid membrane with interspersed glycoproteins gp120 (surface protein, SU) and the transmembrane protein gp41(TM) (**Figure 1**) (Gelderblom *et al.*, 1987a). The lipid bilayer is also studded with various host proteins such as major histocompatibility complex (MHC) class I and II, which are acquired during viral budding (Gelderblom *et al.*, 1987b). The membrane surrounds the viral matrix, which is made up of the myristylated structural protein p17 (MA). p17, along with p24, p9 and p6 are nucleocapsid proteins that are cleaved by the viral protease from a 55kD Gag precursor ($Pr55^{gag}$ coded for by the *gag* gene); see also 1.3.6. The phosphorylated p24 polypeptide forms the major component of the viral core, which is encapsulated by the viral matrix. The viral core contains two copies of single stranded genomic RNA molecules bound to p7 and associated with p9 as well as the preformed viral enzymes reverse transcriptase (RT), protease (Pro) and integrase (IN). The viral enzymes are coded for by the *pol* gene and are also derived by proteolytic cleavage of a precursor protein. The two precursor proteins $Pr55^{gag}$ and Pol are expressed from a single *gag-pol* mRNA which is translated in two different reading frames (Varmus, 1988).

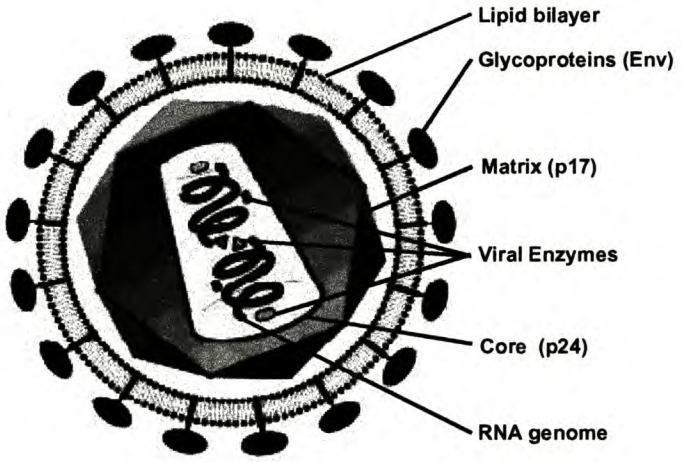


Figure 1: Graphical representation of the HIV virion structure.

In addition to the three genes *gag*, *pol* and *env* that are required for replication by all retroviruses, the more complex genome of the lentivirus HIV-1 encodes six additional genes (**Figure 2**). These additional genes are classified into two groups termed the regulatory (*tat* and *rev*) and accessory genes (*vif*, *vpr*, *vpu*

and *nef*) (Greene, 1991). Although the regulatory genes are essential, the accessory genes are dispensable for viral replication and the latter play an important role in pathogenesis and disease progression. Finally, the genome is flanked by regions of nucleotide sequence repeats termed the long terminal repeat (LTR). These repeat regions are involved in host integration of the HIV-1 genome and the 5'LTR acts as transcriptional promoter for viral gene expression.

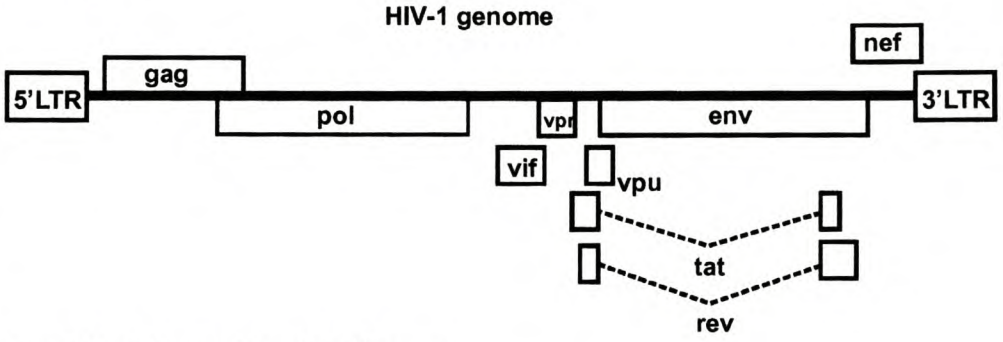


Figure 2: Graphical representation of the HIV-1 genome.

1.1.5 The replication cycle of HIV-1 and it's proteins

HIV-1 employs a very efficient utilisation of coding sequences by expressing the nine different gene products in a small genome of less than 10 kilobases (kb). This is accomplished by making use of a variety of splicing events and overlapping reading frames. In addition, HIV-1 also translates unconventional mRNAs that are incompletely or unspliced and thus retain introns, an event that is very unusual in eukaryotic cells (**Figure 3**) (Greene, 1991). This is because normally the splicing of mRNA must be completed before it can move from the nucleus to the cytoplasm to be translated. HIV gets around this by expressing the protein Rev, which functions as efficient exporter of unspliced or partially spliced viral mRNAs from the nucleus to the cytoplasm (Feinberg *et al.*, 1986). mRNAs that are completely spliced are exported to the cytoplasm by the cellular mRNA export pathways and therefore are Rev-independent.

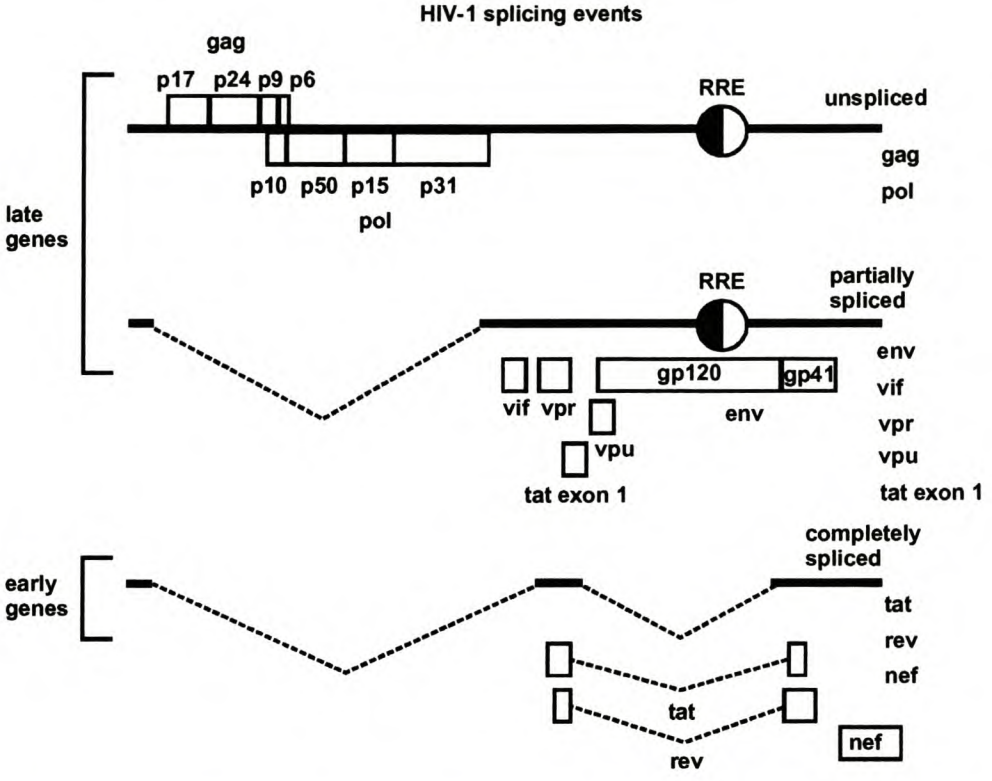


Figure 3: Splicing events of viral RNA transcripts of the HIV-1 genome. Unspliced and partially spliced transcripts contain a Rev-responsive element (RRE, depicted as a semi-filled circle), which renders the expression of the proteins they encode Rev-dependent and thus "late proteins". Expression of Tat, Rev and Nef is Rev-independent as these are translated from completely spliced mRNA, which is exported from the nucleus by cellular pathways.

Env

The envelope protein Env (gp160) is expressed from singly- or partially spliced mRNA (**Figure 3**). After synthesis in the endoplasmic reticulum (ER), Env migrates through the Golgi complex where it undergoes extensive glycosylation, which is required for functionality of the protein and infectivity of the virus (Capon and Ward, 1991). Subsequently, the gp160 is cleaved by a host cellular protease to generate gp120 and gp41. gp41 contains the transmembrane (TM) domain of Env, while gp120 is located on the surface of the virion through noncovalent interactions with gp41 (**Figure 1**). Viral entry into a host cell is mediated by interactions between specific domains of gp120 on the viral particle and the HIV-1 viral receptor molecule CD4 (Landau *et al.*, 1988). The structure of gp120 plays an important role in tropism of HIV-1 and the polypeptide has nine highly conserved intrachain disulphide bonds. Moreover, the enormous heterogeneity between HIV-1 isolates is largely

driven by five hypervariable regions in gp120 designated V1 through V5. One such region, the V3 loop, is not required for CD4 binding, but is a determinant of the preferential tropism of HIV-1 for either T lymphoid cell lines or macrophages (Hwang *et al.*, 1991). Interactions between sequence motifs in the V3 loop and the HIV-1 coreceptors CCR5 and CXCR4, which are part of the family of chemokine receptors, play a role in the susceptibility of cell types to infection (Deng *et al.*, 1996). Once attachment has occurred, viral entry through fusion of the viral and cellular membranes is mediated by the gp41 N-terminal fusogenic domain, allowing the delivery of the inner components of the virion into the cytoplasm of the now infected cell (Camerini and Seed, 1990).

Pol

The *pol* gene products protease (Pro), integrase (IN), RNase H and reverse transcriptase (RT) are expressed from a Gag-Pol fusion protein designated p160 which is generated through a ribosomal shift during p55 translation (Jacks *et al.*, 1988). This frameshift occurs at an approximate frequency of 5% of *gag* translation events, resulting in a 1:20 ratio of Gag-Pol to Gag precursor molecules. During maturation of the viral particle, the viral protease cleaves the Gag-Pol precursor to release the Pol polypeptide and subsequently cleaves the latter further to separate the protease (p10), reverse transcriptase (p50), RNase H (p15) and integrase (p31) proteins (**Figure 3**). Some of these cleavages are not very efficient and this results in uncleaved polypeptides such as the RNase H-linked RT polypeptide p65, which makes up approximately 50% of all RT protein (Jacks *et al.*, 1988). Generation of a double-stranded DNA copy of the viral RNA genome is the function of the HIV-1 polymerase (Pol), which predominantly occurs as a heterodimer consisting of p65 and p50. Pol has RNA-dependent and DNA-dependent polymerase activities. After viral entry, during the process of reverse transcription, reverse transcriptase generates a DNA copy using the viral RNA genome as template, which is subsequently removed from the first DNA strand by RNase H, allowing the synthesis of the complementary strand (Greene, 1991). This process requires the interaction of many *cis*-acting elements in the viral RNA such as the transactivation region (TAR), which contains the binding site for the HIV-1 transactivator of transcription Tat (Harrich *et al.*, 1996). The HIV-1 polymerase does not possess a proofreading activity, which results in error-

prone replication that is a major contributing factor to the enormous heterogeneity of HIV-1 (Preston *et al.*, 1988; Roberts *et al.*, 1988). The last enzyme that is cleaved from the Pol precursor is integrase (IN), which mediates insertion of the newly synthesized double-stranded DNA copy (proviral DNA) into the genomic DNA of the infected host cell (Bushman *et al.*, 1990). IN has three distinct functions which contribute to this process, the first of which is an exonuclease activity that trims two nucleotides from each 3' end of the viral DNA duplex. Thereafter, IN cleaves the host DNA at the integration site using it's double-stranded endonuclease activity and finally ligates the proviral DNA into the site by using its ligation activity (Bushman *et al.*, 1990).

Gag

The *gag* gene encodes the 55-kilodalton (kD) Gag precursor protein p55, which is expressed from unspliced viral mRNA and is thus also Rev-dependent. During translation a myristic acid is bound to the N-terminus of p55 (myristoylation), which effects its association with the cell membrane (Bryant and Ratner, 1990). Membrane-associated p55 recruits two copies of the viral genomic RNA as well as other cellular and viral proteins to initiate budding of a new viral particle from the cell. After budding, the viral protease, which was taken up into the immature virion during budding, cleaves the p55 polyprotein into four smaller proteins designated p17 (matrix, MA), p24 (capsid, CA), p9 (nucleocapsid, NC) and p6 (Gottlinger *et al.*, 1989). As described under the HIV-1 virion structure heading, p24 (CA) forms the conical core of viral particles and the majority of MA or p17 remains attached to the inner surface of the virion membrane to stabilize the particle. Some MA is engaged in deeper layers of the virion where it forms part of the complex that escorts viral DNA to the nucleus (Gallay *et al.*, 1995). p9 (NC) is responsible for recognition of the packaging signal, in the form of stem-loop structures, at the 5' end (5' LTR) of the HIV genomic RNA, to facilitate incorporation of RNA into the virion (Harrison and Lever, 1992). The last *gag* gene product p6 is used as a functional component in the Gag precursor protein p55 and is involved in the interaction between p55 and the accessory protein Vpr, leading to the incorporation of Vpr into the virion (Paxton *et al.*, 1993). In addition, the p6 region contains the "late domain", which mediates efficient release of budding virion from the infected cell.

1.1.6 The HIV-1 regulatory genes

Tat

In 1985 Sodroski *et al.* (1985b) showed that the HIV LTR has the ability to act as an inducible promoter by placing genes under the control of the LTR and noting expression levels of 200- to 300-fold higher in cells that had been previously infected with HIV. They postulated that this transactivation was due to the presence of a novel transcription factor, which they named Tat (transactivator of transcription). It was subsequently shown that the presence of Tat is associated with a dramatic increase in the levels of long transcripts, pointing to an involvement in transcription elongation (Kessler and Mathews, 1992; Ratnasabapathy *et al.*, 1990; Toohey and Jones, 1989; Kao *et al.*, 1987). Tat's exclusive function at the level of elongation was subsequently confirmed (Keen *et al.*, 1997; Graeble *et al.*, 1993; Marciniak and Sharp, 1991) and interestingly, it was noted that through interaction with other cellular factors, Tat is able to stimulate phosphorylation of the carboxyterminal domain (CTD) of RNA polymerase in elongation complexes to create a novel form of RNA polymerase called RNA polymerase II (Isel and Karn, 1999). The ability of Tat to stimulate transcription from viral LTRs has been demonstrated to be modulated by the direct binding of Tat to the transactivation region (TAR) in the 5'-LTR, which functions as a highly stable RNA stem-loop structure (Dingwall *et al.*, 1989). Transactivation activity of TAR-bound Tat is dependent on an association with cyclin T1, a subunit of the protein kinase complex TAK (Tat-associated kinase) (Fujinaga *et al.*, 1999; Herrmann and Rice, 1993).

HIV-1 Tat is a small nuclear protein of 72 to 101 amino acids (aa) in length that is encoded from two separate exons. Tat is normally expressed early from completely spliced mRNA (101 aa), but does exist in the form of the first exon only (72 aa) when it is expressed from partially spliced mRNA (**Figure 3**). The sequence of the protein has been subdivided into five distinct functional regions on the basis of residue composition (Kuppuswamy *et al.*, 1989): an N-terminal activation region (aa 1-21), a cysteine-rich domain (aa 22-37), a core region (aa 40-48), a basic domain (aa 49-72) and a glutamine-rich region, which makes up the second exon of Tat (aa 73-101). The basic region serves as a nuclear localization signal (Hauber *et al.*, 1989; Ruben *et al.*, 1989), while along with the core and glutamine-rich domains, it is involved in TAR-RNA binding (Roy *et al.*, 1990; Dingwall *et al.*, 1989;). The cysteine-rich domain

plays a vital role in Tat's transactivation function, correct protein folding and activity. A single cysteine at position 37 has been shown to be essential for homo-dimerization of Tat, and subsequently functional activity (Battaglia *et al.*, 1994). In addition, mutation in six of the seven cysteines in the cysteine-rich domain eliminates Tat function (Kuppuswamy *et al.*, 1989; Ruben *et al.*, 1989) and mutation of noncysteine residues within this region also reduces activity, in some cases drastically (Rice and Carlotti, 1990). It was further demonstrated that the cysteines are critical for the formation of disulphide bonds within Tat (Koken *et al.*, 1994; Frankel *et al.*, 1988). Recent studies have led to the belief that the cysteines are involved in metal ion binding (Bieniasz *et al.*, 1998; Garber *et al.*, 1998). Furthermore, Tat proteins with mutated cysteine residues (Tat_{22} and $Tat_{22/37}$) were shown to lack transactivation activity in Jurkat cells and these mutations effected inhibition of HIV reactivation from latency in Jurkat cells and primary PBMCs (Rossi *et al.*, 1997; Caputo *et al.*, 1996; Balboni *et al.*, 1993). Such Tat transdominant negative mutants have been extensively characterised and developed as potential prophylactic and/or therapeutic vaccines, showing very good immunogenicity and safety (Betti *et al.*, 2001; Caselli *et al.*, 1999;).

The second exon of Tat was initially thought to be functionally insignificant as the first exon was shown to be fully sufficient for transactivation (Battaglia *et al.*, 1994; Kuppuswamy *et al.*, 1989; Garcia *et al.*, 1988; Seigel *et al.*, 1986) and no apparent function could be targeted to the second exon. A very recent study by Jeang *et al.* revealed that the presence of two-exon Tat in infecting SIVmac239 and HIV (HXB2) viruses inversely correlated with high CD4 counts and correlated with viral loads. Furthermore, it was shown that whereas rhesus macaques infected with two-exon Tat progressed rapidly and died of AIDS, those infected with SIVmac239 containing a Tat with a premature stop mutation controlled viremia post-acutely. Interestingly, reversion of the viruses to the full-length Tat was observed in some animals. Similar associations and reversions were recorded in humans infected with the HIV-1 strain HXB2, which contains a pseudo-1exon HIV-1 Tat gene (Jeang *et al.*, 2002).

Tat is secreted from infected cells, taken up into uninfected cells and, in previously infected cells, initiates activation of latent HIV proviruses and new rounds of replication (Ensoli *et al.*, 1993; Helland *et al.*, 1991). Cells treated with Tat overproduce interferon-α (IFN-α) (Zagury *et al.*, 1998a), which, along with the direct action of Tat, increases expression of chemokine receptors

including the major receptors for HIV entry, CCR5 and CXCR4 (Secchiero *et al.*, 1999; Huang *et al.*, 1998; Li *et al.*, 1997) and thus rendering bystander cells more susceptible to infection. Moreover, Tat has been shown to be a potent immunosuppressant that stimulates apoptosis in T cells *in vitro*.

Yet, by inactivation through oxidation, it was demonstrated that Tat preserved its immunogenicity and is considered a good component of an antibody-based HIV-1 vaccine (Cohen *et al.*, 1999b; Gringeri *et al.*, 1998). Interestingly, Tat has been identified as a target for gene therapy. By transfer of a protein termed *antitat*, which sequesters Tat protein and blocks *tat* translation from mRNA, the function of the transactivator *in vivo* is inhibited (Li *et al.*, 2000). This therapeutic action was shown to be effective in inhibiting HIV-1 replication and may be a key alternative to antiretroviral therapy.

The immunogenicity of Tat has been receiving an increasing level of attention and the use of Tat as vaccine component is becoming increasingly popular. Allen *et al.* (Allen *et al.*, 2000a) showed that Tat is targeted frequently by CTL in acute HIV infection and may thus contribute considerably to the containment of initial viremia. Strong CTL responses to a number of defined epitopes within Tat have been identified in patients representing a wide range of HLA class I alleles (Addo *et al.*, 2001; Novitsky *et al.*, 2001; Allen *et al.*, 2000a; HIV Molecular Immunology, 2000). In addition, immunisation with Tat has been shown to elicit potent humoral and cellular immune responses in mice (Billaut-Mulot *et al.*, 2001; Cafaro *et al.*, 2001; Kjerrstrom *et al.*, 2001; Morris *et al.*, 2001a; Boykins *et al.*, 2000; Pauza *et al.*, 2000; Cafaro *et al.*, 1999; Caselli *et al.*, 1999; Gallo, 1999; Chenciner *et al.*, 1997; Hinkula *et al.*, 1997b) and non-human primates (Chenciner *et al.*, 1997).

A strong association has also been verified between anti-Tat immune responses and delayed disease progression to AIDS in a number of studies. Van Baalen *et al.* showed that rapidly progressing HIV-1 infected persons have less Tat-specific CTL compared to those that progressed slowly (1997). Moreover, high levels of anti-Tat antibodies were also correlated with non-progression (Fiorelli *et al.*, 2002; Demirhan *et al.*, 1999; Zagury *et al.*, 1998b) and slow disease progression to AIDS (Re *et al.*, 1995; Reiss *et al.*, 1990).

Finally, vaccination with chemically inactivated Tat toxoid was shown to attenuate replication and disease progression of the highly pathogenic SHIV89.6PD in rhesus macaques (Pauza *et al.*, 2000). Vaccination with HIV-1 biologically active Tat, has been demonstrated to control replication of SHIV89.6P to undetectable levels in 5 out of 7 macaques, slowing disease

progression for extended periods (Cafaro *et al.*, 2001; Cafaro *et al.*, 2000; Cafaro *et al.*, 1999). In addition, a Tat-Rev vaccine protected rhesus macaques from pathogenic SIV challenge (Osterhaus *et al.*, 1999).

Rev

The *rev* gene codes for the regulatory protein Rev, which as previously mentioned, is responsible for the transportation of unspliced or singly spliced viral mRNAs from the nucleus to the cytoplasm (Feinberg *et al.*, 1986). Thus, through the expression of the Rev protein, HIV possesses the ability to regulate the export and subsequent expression of intron-containing mRNAs. HIV Rev is a small, 18-kDa phosphoprotein, which localises within the nuclei and nucleoli (Malim *et al.*, 1989a). The protein consists of at least three functional domains that are responsible for its subcellular localisation in the nucleus, binding to RRE-RNA through a sequence-specific interaction and after multimerisation with other Rev molecules, interacts with cellular cofactors to shuttle between the nucleus and cytoplasm (Thomas *et al.*, 1998; Hope *et al.*, 1990b; Malim *et al.*, 1989a). The homomultimerisation domain is essential for oligomerisation of Rev and appears to be divided into two subdomains, which overlap on either side with the arginine-rich domain (Thomas *et al.*, 1998; Hope *et al.*, 1992; Zapp *et al.*, 1991). The arginine-rich domain mediates both RNA binding (RNA binding domain, RBD)(Kjems *et al.*, 1992; Cochrane *et al.*, 1990a) and nuclear/nucleolar localisation (Nuclear localisation signal, NLS) (Cochrane *et al.*, 1990b; Malim *et al.*, 1989a) and seems to be multimerisation dependent(Szilvay *et al.*, 1997). The nuclear export signal (NES) is also referred to as activation/effector domain and as suggested by the name is responsible for mediating export from the nucleus of a exportin-1 complexed Rev (Thomas *et al.*, 1998; Cole *et al.*, 1993; Hope *et al.*, 1992; Zapp *et al.*, 1991). Multimerisation of Rev appears to be very important for biological activity and it was demonstrated to be responsible for maintaining threshold levels of intracellular Rev. This Rev threshold must be overcome in order to establish productive HIV-1 infection and this mechanism was demonstrated to be responsible for regulation of viral latency (Pomerantz *et al.*, 1992; Pomerantz *et al.*, 1990). In addition, oligomerisation of Rev and subsequent binding to the RRE has been shown to be essential for RNA export and thus biological activity of Rev (Cole *et al.*, 1993; Hope *et al.*, 1992; Zapp *et al.*, 1991). Moreover, nuclear import has been shown to be mediated by a direct interaction with importin-β, a cellular transport protein, to which Rev

binds (Truant and Cullen, 1999). Following translocation into the nucleus, disassembly of the importin-β/Rev complex is induced and the arginine-rich domain becomes available to bind to the RRE (Pollard and Malim, 1998).

The Rev protein itself is expressed from a conventional, completely spliced mRNA and the expression of Rev-dependent proteins is reliant on the level of Rev in the cell (Felber *et al.*, 1990). This delay in expression of Rev-dependent genes accounts for the designation of such as late genes, whereas those translated from Rev-independent mRNAs are called early genes. Besides nuclear export Rev can have a number of effects on HIV-1 RNA that include increases in both stability and translation of HIV RNAs (Arrigo and Chen, 1991; Felber *et al.*, 1989). It has also been shown that the expression of Rev causes an increase in the half-life of HIV RNAs in the nucleus of a T-cell line infected with HIV (Malim and Cullen, 1993).

In early characterisation experiments in which a non-functional mutant Rev protein, RevM10, was cotransfected with wild-type Rev it was discovered that RevM10 could strongly inhibit wild-type Rev function in a *trans*dominant manner (Hope *et al.*, 1992; Hope *et al.*, 1990b; Malim *et al.*, 1989a). RevM10 was also shown to inhibit HIV-1 replication through RevM10-mediated prevention of functional Rev-mediated nuclear export of unspliced viral transcripts and subsequent inhibition of Gag-Pol and Env protein expression. This *trans*dominant-negative Rev has since been developed for gene therapy in HIV-1 infected individuals and was shown to be clinically safe in human trials (Ranga *et al.*, 1998; Woffendin *et al.*, 1996). RevM10-transduced T cells were also found to have prolonged survival and it was proposed that antiviral gene therapy with this *trans*dominant-negative Rev mutant may contribute to the reconstitution of immune function in AIDS.

CTL responses specific for a number of epitopes within Rev have been identified in HIV positive patients expressing a number of different HLA class I alleles (HIV Molecular Immunology, 2000; Addo *et al.*, 2001; van Baalen *et al.*, 1997) and Rev-specific CTL have been inversely correlated with rapid disease progression to AIDS (van Baalen *et al.*, 1997). Moreover, considerable success has been obtained by immunisation with Rev vaccines in various animal models (Calarota *et al.*, 1999; Osterhaus *et al.*, 1999; Boyer *et al.*, 1997a; Chenciner *et al.*, 1997; Hinkula *et al.*, 1997b; Kim *et al.*, 1997b; Wang *et al.*, 1993). Vaccination with a Tat-Rev immunogen was shown to protect rhesus macaques from pathogenic SIV challenge (Osterhaus *et al.*, 1999) and promising results have also been obtained from human trials in which Rev

was used as vaccine component along with Env (Boyer *et al.*, 2000; MacGregor *et al.*, 1998).

1.1.7 The HIV-1 accessory genes

Nef

The **ne**gative **f**actor (*nef*) gene is found in all primate immunodeficiency retroviruses and thus appears to play a vital role during natural infection. Nef is abundantly expressed during the early phase of HIV-1 gene expression to the degree that its mRNA represents 75% of the viral mRNA load of the cell (Klotman *et al.*, 1991; Guy *et al.*, 1987). The protein of around 206 amino acids is modified through means of myristic acid binding, through which it is anchored to host cellular membranes (Niederman *et al.*, 1993; Yu and Felsted, 1992; Kaminchik *et al.*, 1991; Allan *et al.*, 1985). The protein is also phosphorylated on serine and threonine residues, the function of which is as yet unclear (Yang and Gabuzda, 1999; Coates *et al.*, 1997). Nef is packaged into virions, where it is cleaved by the viral protease during virion maturation (Chen *et al.*, 1998; Pandori *et al.*, 1996; Welker *et al.*, 1996; Freund *et al.*, 1994). Studies in primates have shown that Nef is required for high viral loads and progression to AIDS disease (Kestler *et al.*, 1991). In addition, HIV-1 particles produced in the presence of Nef have been shown to be up to ten-fold more infectious than those produced in the absence of Nef (Miller *et al.*, 1994). The importance of Nef in HIV pathogenicity was further highlighted by studies in which attenuated forms of HIV-1 and HIV-2 with *nef* deletions were strongly associated with long term non-progression (Learmont *et al.*, 1999; Salvi *et al.*, 1998; Switzer *et al.*, 1998; Mariani *et al.*, 1996; Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995).

Nef has a number of distinct functional activities of which the downregulation of CD4 surface expression is perhaps the best understood. The protein mediates rapid endocytosis of cell surface CD4 molecules resulting in their degradation in lysosomes (Aiken *et al.*, 1994; Rhee and Marsh, 1994; Mariani and Skowronski, 1993; Garcia and Miller, 1991). Nef binding to CD4 is mediated by a number of motifs. The so-called CD4 binding site, stretching residues 57-59 binds the cytoplasmic tail of CD4 and internalisation of the bound CD4 is mediated by a dileucine motif (Mangasarian *et al.*, 1999;

Greenberg *et al.*, 1998a; Rossi *et al.*, 1996; Greenway *et al.*, 1995; Aiken *et al.*, 1994; Harris and Neil, 1994). To achieve internalisation Nef interacts directly with the AP-1 clathrin adaptor complex and recruits CD4 to the complex at the cell membrane after which degradation is effected through lysosomal targeting (Bresnahan *et al.*, 1998; Greenberg *et al.*, 1998a). This downregulation prevents superinfection and facilitates Env protein incorporation into virions by preventing interaction between Env and CD4 during virus budding (Lama *et al.*, 1999; Little *et al.*, 1994; Benson *et al.*, 1993). Further reports have shown that Nef-mediated CD4 downregulation stimulates HIV-1 production (Ross *et al.*, 1999) and enhances HIV-1 replication in activated T lymphocytes (Lundquist *et al.*, 2002).

A second activity of Nef, which is less well understood, is the downregulation of class I major histocompatability complex (MHC-I) by rapid internalisation and eventual degrading in the Golgi complex (Greenberg *et al.*, 1998b; Le Gall *et al.*, 1998; Schwartz *et al.*, 1996). This process serves to prevent the recognition and lysis of infected cells by cytotoxic T-lymphocytes, resulting in cytolytic evasion (Mangasarian *et al.*, 1999; Collins *et al.*, 1998). A tyrosine residue, which is located in the cytoplasmic tail of HLA-A and B, but not HLA-C, was found to be critical for this interaction with Nef (Le Gall *et al.*, 1998). In the background of this, it was found that unlike HLA-A and HLA-B, Nef does not downregulate HLA-C (Cohen *et al.*, 1999a). This distinction allows infected cells to escape from natural killer (NK) cells that would kill a cell completely devoid of surface MHC-I.

Thirdly, Nef disrupts T cell transduction and activation in both positive and negative contexts through a number of separate mechanisms. A recent study has demonstrated that Nef downregulates another T-cell receptor, namely CD28 (Swigut *et al.*, 2001). CD28 is a major co-stimulatory T-cell receptor that mediates efficient activation of T-cells and it is proposed that along with the downregulation of CD4 and/or CD3, this function disrupts antigen-specific signalling following productive antigen recognition (Swigut *et al.*, 2001). Moreover, Nef disrupts the T-cell receptor (TCR) machinery and signalling by interfering with the CD3-TCR complex on CD4 cells, thereby blocking events in the CD3-TCR signalling cascade (Iafrate *et al.*, 1997). In addition, Nef modulates additional effector and signalling pathways in T cells such as p21-activated kinase (PAK) and protein kinase C (PKC). Association of Nef with serine-threonine kinase Pak-2, a complex termed Nef-associated kinase (NAK) (Renkema *et al.*, 1999), has been shown to correlate with the ability of

Nef to enhance viral infectivity (Wiskerchen and Cheng-Mayer, 1996). Furthermore, Nef modulates calcium signalling (Baur *et al.*, 1994) and activates nuclear factor of activated T cells 1 (NFAT1) (Manninen *et al.*, 2001), which along with IL-2, is known to regulate a number of genes involved in T-cell function, such as the IL-4, tumour necrosis factor alpha (TNFα) and Fas ligand (FasL) genes (Rao *et al.*, 1997). Nef induction of FasL expression, which interacts with its receptor Fas (CD95) on neighbouring cells, induces apoptosis. However, Nef also associates with and inhibits apoptosis signal-regulating kinase 1 (ASK1), which leads to inhibition of Fas- and TNFα-mediated apoptosis in the infected cell (Geleziunas *et al.*, 2001). This function of Nef results in the killing of bystander cells, including HIV-1-specific CTLs, while inhibiting the same apoptotic signals in the infected cell to achieve immune escape. Although Nef-mediated cellular events have not been fully elucidated, these effects collectively support the model that Nef activates T-cells to provide an optimal environment for viral replication (Steffens and Hope, 2001).

Nef is the most immunogenic of the regulatory and accessory proteins. Anti-Nef antibodies have been identified in HIV-1 infected individuals and AIDS patients (Ayyavoo *et al.*, 2000; Gahéry-Ségard *et al.*, 2000; Mortara *et al.*, 1998). Very potent cytotoxic T lymphocyte responses targeting an increasingly large number of Nef epitopes have been recorded in humans representing a number of different population groups and expressing a wide range of HLA class I molecules (Nixon *et al.*, 1999; Ikeda-Moore *et al.*, 1998; Bauer *et al.*, 1997; Goulder *et al.*, 1997a; Goulder *et al.*, 1997b; Price *et al.*, 1997; Haas *et al.*, 1996a; Culmann-Penciolelli *et al.*, 1994; Culmann *et al.*, 1991; Koenig *et al.*, 1990). For the latest summary, refer to the HIV Immunology Database (HIV Molecular Immunology, 2000). In addition, Nef-induced T cell proliferative responses have been identified *in vitro* (Ayyavoo *et al.*, 2000; Gahéry-Ségard *et al.*, 2000). Nef as vaccine component has also generated promising humoral and cellular immune responses in mice and non-human primates (Billaut-Mulot *et al.*, 2001; Kjerrstrom *et al.*, 2001; Ayyavoo *et al.*, 2000; Hinkula *et al.*, 1997b).

Vif

Although initial studies suggested that the function of the HIV-1 **v**irion **i**nfectivity **f**actor (*vif*) gene was dispensable for virus infection of transformed T cell lines, it was found that this gene is required for HIV-1 infection of its primary target cells, CD4-bearing T lymphocytes (Ma *et al.*, 1994; Strebel *et al.*, 1987). In fact, the Vif protein appears make previously non-permissive cells tolerant to HIV-1 replication. This is thought to be brought about by Vif-mediated neutralisation of a cellular factor that inhibits HIV-1 replication and thus renders the cell non-permissive (Madani and Kabat, 1998; Simon *et al.*, 1998b). Vif is present in viral particles and associates with viral core structures (Liu *et al.*, 1995). More recent data has revealed that Vif also binds to intracellular genomic HIV-1 RNA (Khan *et al.*, 2001; Dettenhofer *et al.*, 2000; Zhang *et al.*, 2000) and may stabilize the reverse transcription complex (RT complex) to support reverse transcription (Dornadula *et al.*, 2000). These studies propose that Vif promotes efficient reverse transcription after virion entry through regulation of nucleic acid components within the viral core.

HIV-1 infected subjects produce Vif-specific antibodies which indicates that Vif is expressed and immunogenic and may thus play a role in vaccine development (Ayyavoo *et al.*, 1997a). CTL responses specific for epitopes within Vif have been identified in HIV positive patients (Altfeld *et al.*, 2001) and Vif vaccination has been shown to elicit significant immune responses in mice (Ayyavoo *et al.*, 2000; Ayyavoo *et al.*, 1997a).

Vpr

The **v**iral **p**rotein **R** (Vpr) consists of 96 amino acids and was initially thought to be as abundant in virions as Gag (Paxton *et al.*, 1993), but has recently been shown to be incorporated in much smaller amounts (Singh *et al.*, 2001; Muller *et al.*, 2000). The protein, which is expressed from incompletely spliced viral transcripts, has a number of functional activities which include contribution towards nuclear import of the HIV genome (Heinzinger *et al.*, 1994), induction of cell-cycle arrest, transactivation of viral and host cellular gene expression and regulation of viral replication kinetics. Nuclear import of the viral pre-integration complex is mediated by two putative nuclear localisation signals (NLS) (Sherman *et al.*, 2001; Kamata and Aida, 2000;). Virion associated Vpr is associated with the prevention of chronic HIV-1 infection (Hrimech *et al.*, 1999; Jenkins *et al.*, 1998). In addition, HIV-1 replication enhancement is achieved through Vpr-mediated inhibition of cell division (Rogel *et al.*, 1995). This is achieved through the accumulation of

cells arrested in the G2 phase of the cell cycle (Jowett *et al.*, 1995). Along with the cell cycle arrest, Vpr's effect on cell differentiation also contributes to the destruction of the immune system (Jenkins *et al.*, 1998) and suppresses immune activation (Ayyavoo *et al.*, 1997b). Notably, it was shown that Vpr may induce or abrogate apoptosis. Ayyavoo *et al.*, demonstrated that Vpr induces apoptosis in the absence of T-cell receptor (TCR)-mediated cell activation whereas Vpr works to inhibit the induction of apoptosis in TCR-triggered activated cells (Ayyavoo *et al.*, 1997b). In addition, Vpr has the ability to modulate transcription of HIV-1 LTR promoter activity and through synergistic interaction with the viral transactivator Tat, Vpr enhances transcription of the viral genome (Sawaya *et al.*, 2000). Although it was initially believed that most of these functions were mediated by a common pathway, recent evidence has shown that the cell-cycle arrest, induction of apoptosis and gene activation activities are regulated through more complex and divergent mechanisms (Zhu *et al.*, 2001). Interestingly, Vpr seems to possess the additional ability to target specific gene products into the virus-like particle (VLP) (Liu *et al.*, 1997; Wu *et al.*, 1996; Wu *et al.*, 1995) which makes Vpr a potentially popular sub-unit in particle-based HIV-1 vaccines.

Anti-Vpr antibodies have been shown to inhibit HIV replication potential, which may be a target for immune intervention (Levy *et al.*, 1995), and CTL responses specific for epitopes within Vpr have been identified in HIV positive individuals (Altfeld *et al.*, 2001).

Vpu

The **v**iral **p**rotein **u**nknown (*vpu*) gene and its protein product are unique to HIV-1. Vpu is not present in the virus particle but assembles as a homo-oligomer, which is phosphorylated and primarily localized in the internal membranes of the infected cell (Sato *et al.*, 1990). In infected cells, the HIV-1 envelope protein Env and the viral receptor CD4 form complexes within the endoplasmic reticulum, trapping both proteins within the ER compartment. This process impedes with virion assembly and formation and is overcome by Vpu-mediated liberation of Env protein through activation of the ubiquitin-mediated degradation of Env-complexed CD4 molecules (Willey *et al.*, 1992a; Willey *et al.*, 1992b). The cytoplasmic domain of the Vpu protein is responsible for this CD4 downregulation activity (Paul *et al.*, 1998; Schubert *et al.*, 1998;

Willey *et al.*, 1992b). The cytoplasmic domain of the Vpu protein is responsible for this CD4 downregulation activity (Paul *et al.*, 1998; Schubert *et al.*, 1998; Bour *et al.*, 1995; Schubert and Strebel, 1994). The transmembrane domain of Vpu is responsible for enhancement of virion release from the plasma membrane: in the absence of Vpu, large numbers of virions can be seen attached to the cell surface (Klimkait *et al.*, 1990). Recent evidence has targeted this Vpu-mediated mechanism of virion release to the formation of ion-conducting channels in the membrane (Ma *et al.*, 2002; Steffens and Hope, 2001). Additionally, Vpu mediates increased sensitivity of HIV-1 infected cells to FAS-mediated apoptosis, which may contribute to the short *in vivo* half-life of infected cells (Casella *et al.*, 1999).

1.1.8 HIV/AIDS vaccine strategies and the $CD8^+$ response

Introduction

Since the time of the ancient Greeks, people have realized that those who have survived smallpox, plague, yellow fever or a number of other infections, rarely contracted these diseases again. Successful artificial immunisation was started by Edward Jenner in the late eighteenth century by inoculation against smallpox with the related cowpox virus. Since then, vaccination with a pathogen, which is related to the one immunized against, derivatives of pathogens or attenuated and killed pathogens have been exceedingly successful in reducing disease, death and even in the case of smallpox, eradication of the pathogen (Prescott *et al.*, 1996). These successes led to the initial hope that an AIDS vaccine could be quickly developed and implemented. Yet the latent, non-curable nature of HIV infection has complicated this matter considerably. HIV manages to effectively hide from the immune system by integrating itself as proviral DNA in the genomic DNA of the infected cell.

First generation vaccine strategies

Early HIV-1 vaccine strategies, which progressed from developmental through clinical evaluation, were focused on generating neutralising antibodies with Envelope (Env)-based recombinant protein subunit vaccines (Kahn *et al.*, 1994; Haigwood *et al.*, 1992). These first generation vaccines elicited potent

elicited neutralising responses against primary isolates (Connor *et al.*, 1998; Mascola *et al.*, 1996; VanCott *et al.*, 1995). In addition, these protein-based vaccines were not able to elicit significant levels of $CD8^+$ CTL responses and failed to protect against infection in human phase I/II efficacy trials (Connor *et al.*, 1998). It was subsequently decided in June 1994 to terminate the proposed efficacy trials of two Env-based HIV vaccine candidates (Bolognesi and Matthews, 1998). It was clear that in addition to neutralisation, strong pathogen-specific cytotoxicity in the form of CTL responses were required.

Viral vectors

Due to these new challenges posed by HIV-1 infection, a number of novel vaccine delivery systems have been developed during the last decade. These include live viral and bacterial vector systems, which express genes that code for HIV antigens. Such delivery systems mimic natural microbial infections and thus allow synthesis of HIV antigens that are very effectively presented via MHC-I molecule pathways resulting in the priming of strong cellular responses. Popular systems employed currently include recombinant poxvirus vectors (Zavala *et al.*, 2001), modified vaccinia virus Ankara (MVA) (Sutter and Moss, 1992), avipoxviruses such as Canarypox virus (Taylor *et al.*, 1991) and Alphavirus-based vector vaccines such as Venezuelan equine encephalitis virus (VEE) (Caley *et al.*, 1999). While these delivery systems provide pathogen-like deliveries, clinical safety concerns for replication of these viruses within individuals, which may include immunocompromised persons, are relieved by the collective property of replication deficiency. Viral vector-based deliveries are well established, can elicit innate immune responses and have shown good cellular and humoral immunogenicity (Sutter and Haas, 2001). However, concerns are raised because of strong immune responses that may be raised against the antigenic vectors, many of which are derived from pathogens to which humans have been previously exposed.

DNA vaccines

Another attractive delivery system, which relies on the vaccination with plasmid vectors that encode the respective HIV-1 antigen under the control of strong regulatory elements such as the cytomegalovirus (CMV) immediate-early enhancer/promoter and bovine growth hormone (bGH) terminator. As

such constructs can be injected as naked plasmid nucleic acid, this system is termed DNA vaccine (Ulmer *et al.*, 1993). Initially DNA vaccines were thought to elicit cellular immune responses through one- or a combination of three antigen processing and presenting mechanisms: direct priming by somatic cells such as myocytes, direct transfection of professional antigen presenting cells (APCs) and cross-priming in which plasmid DNA transfects somatic and/or APC and the secreted protein is processed by untransfected dendritic cells (DCs) and presented to T cells (Gurunathan *et al.*, 2000). Early in the 1990's it was shown that muscle, which was directly injected with DNA, was able to express protein and induce antigen-specific $CD8^+$ CTL responses (Ulmer *et al.*, 1993). Yet it was not known whether protein-expressing myocytes could directly induce CTL responses or if the expressed protein was transferred to professional APCs (termed cross priming). Subsequent studies revealed that the responses are most likely raised through cross priming although myocytes may play a role in regulation of immune responses (Torres *et al.*, 1997). In addition, it has been demonstrated that CTL responses may be elicited through the direct transfection of small numbers of DCs. DNA vaccination can also be performed via the skin where somatic cells such as keratinocytes, fibroblasts and potent APCs such as Langerhans cells are found (Gurunathan *et al.*, 2000).

DNA vaccines have shown very promising results in terms of immune responses. Humoral responses specific for a large range of antigens including influenza NP and HA, hepatitis B surface antigen, bovine herpes virus glycoproteins and papillomavirus have been generated (Donnelly *et al.*, 1997) and HIV-1-specific antibodies raised by DNA vaccination were first shown by Wang *et al.* (1995; 1993;), Lu *et al.* (1995) and Okuda *et al.* (1995). DNA vaccines encoding a number of HIV and SIV antigens have been used in many vaccine studies and have proven to be effective in priming long-lived cellular and humoral responses in animals (Gurunathan *et al.*, 2000; Boyer *et al.*, 1997a; Boyer *et al.*, 1997b; Yasutomi *et al.*, 1996; Fuller and Haynes, 1994; Wang *et al.*, 1993). Furthermore, DNA vaccines have been shown to elicit immune responses and to be safe in a number of human trials (Calarota *et al.*, 2001; Boyer *et al.*, 2000; Calarota *et al.*, 1999; Calarota *et al.*, 1998; MacGregor *et al.*, 1998). A number of studies have also illustrated that immunisation with nucleic acids are able to protect animals after pathogenic challenge with a wide range of viral, bacterial and parasitic agents (Cafaro *et al.*, 2001; Barouch *et al.*, 2000; Boyer *et al.*, 1997b; Donnelly *et al.*, 1997;).

The importance of $CD8^+$ cytotoxic T lymphocyte responses in HIV-1 pathogenesis and disease progression has been demonstrated elegantly. HIV-1-specific CTL responses have been shown to control viral load during acute and asymptomatic infection (Kalams *et al.*, 1999; Musey *et al.*, 1997; Klein *et al.*, 1995; Moss *et al.*, 1995; Borrow *et al.*, 1994; Koup *et al.*, 1994) and are inversely correlated with disease progression (Ogg *et al.*, 1998). Moreover, strong and cross-reactive CTL responses have been associated with resistance to HIV-1 infection in highly exposed, seronegative commercial sex workers in Kenya and the Gambia (Kaul *et al.*, 2000; Rowland-Jones *et al.*, 1998; Rowland-Jones *et al.*, 1995).

The achievement of protection against heterologous HIV-1 infection by immunisation may only be reached through eliciting a combination of strong cellular and humoral responses (Mooij and Heeney, 2001; Morris *et al.*, 2001b; Hinkula *et al.*, 1997a). Although many vaccination strategies show much promise, the priming of strong humoral and cellular responses has been difficult to achieve with single delivery systems and thus multifaceted approaches such as prime-boost strategies are receiving increased interest (Mooij and Heeney, 2001; Sutter and Haas, 2001). Indeed, greatly improved immune responses have been obtained when DNA priming was followed by a protein boost in non-human primates (Barnett *et al.*, 1998; Barnett *et al.*, 1997) or MVA boost in non-human primates (Allen *et al.*, 2000b) as well as humans (Hanke and McMichael, 1999). In the same mould, increased durability and breadth of $CD8^+$ responses as well as markedly enhanced proliferative responses were recorded when a SIV *gag-env* DNA vaccine was boosted with the attenuated poxvirus-based vaccine NYVAC-SIV-*gag-env-pol* (Hel *et al.*, 2001).

Although such strong cellular and antibody responses are a necessity for a vaccine, certain limiting factors, which undermine the efficacy of these responses, remain. These include immune evasion and escape by the virus. Numerous studies have generated evidence that HIV evolves to escape both CTL responses (Barouch *et al.*, 2002; Allen *et al.*, 2000a; Pircher *et al.*, 1990) as well as CD4+ cell responses (Ciurea *et al.*, 2001). Furthermore, amino acid changes in the HIV-I envelope sequences permit viral escape from antibody neutralization (McLain *et al.*, 2001; Ciurea *et al.*, 2000; Cheng-Mayer *et al.*, 1999). In combination with immune evasion through such escape mechanisms, the high degree of viral diversity creates considerable problems for vaccine design and selection of a viral vaccine strain. A method to address

these issues is to increase the antigenic breadth of a vaccine in an attempt to increase the range of virus-specific responses. Thus, in addition to Gag, Pol and Env immunogens, vaccine strategies incorporating additional components such as Tat, Rev and Nef are being employed (Hanke *et al.*, 2002; Hel *et al.*, 2002; Kjerrstrom *et al.*, 2001; Tähtinen *et al.*, 2001; Ayyavoo *et al.*, 2000; Calarota *et al.*, 1999; Osterhaus *et al.*, 1999 Calarota *et al.*, 1998; Kim *et al.*, 1997b).

The inclusion of these early-expressed regulatory genes provide further attractive factors such as priming regulatory protein-specific CTL, which may kill infected cells prior to completion of viral replication and production of mature virions (Hel *et al.*, 2002; Addo *et al.*, 2001; Mooij and Heeney, 2001; van Baalen *et al.*, 1997). Multicomponent vaccines incorporating structural and regulatory genes would thus induce an array of immune responses that may kill infected cells at various stages of the viral replication cycle as well as recognize, bind and neutralize infectious virions.

Delivery method of multiple immunogens may be the next variable factor to consider as vaccination with multiple or excessively large plasmids may have inherent effects on protein expression and/or immunogenicity. Some studies have been conducted to elucidate the interactions of single and combined HIV-1 DNA vaccines and two studies have shown that the combination of up to five different gene constructs was viable (Kjerrstrom *et al.*, 2001; Hinkula *et al.*, 1997a;).

1.2 Aim of the Study

The principal aim of this study is to develop accessory gene components, which can be used in a multi-faceted DNA vaccine. To accomplish this a number of steps need to be achieved:

Firstly, expression plasmids encoding the wild-type HIV-1 subtype C accessory/regulatory proteins Tat, Rev and Nef have to be designed and constructed.

In parallel, codon-optimised expression plasmids coding for consensus, potentially non-functional, mutated Tat, Rev and Nef proteins have to be designed and constructed.

Verification of the resulting expression constructs will be done by sequencing and subsequently, *in vitro* expression and comparison of expression levels.

Finally, the proteins will be subjected to functional analysis to determine whether the mutations indeed render the proteins inactive for their respective activities.

1.3 Rationale for selection of specific accessory and regulatory genes as vaccine components

As described in the sections above, Tat, Rev and Nef make attractive vaccine components because they are expressed early in the viral life cycle, they are highly immunogenic and their importance and success as immunogens have been extensively highlighted in various models including humans. Significant amounts of data concerning the inactivation and functional analysis of these genes have been published, facilitating the development of non-functional immunogens from such proteins. A number of vaccines based on Tat, Rev and/or Nef have been approved and tested in human trials and these genes have been demonstrated to be suitable for inclusion into HIV-1 vaccines (Calarota *et al.*, 2001; Boyer *et al.*, 2000; Calarota *et al.*, 1999 Gringeri *et al.*, 1998; MacGregor *et al.*, 1998). In addition, these genes make up a significant portion of the open reading frames (ORF) of HIV-1 and they are well conserved in relation to some of the structural and enzymatic genes (Ayyavoo *et al.*, 2000; Ayyavoo *et al.*, 1997a). In a recent study, which was aimed at preparing for human Tat-based vaccine trials in Italy, Uganda and South Africa, it was shown that the functional and immunogenic regions in Tat from viruses representing a number of subtypes and geographic origins were highly conserved (Butto *et al.*, 2002). In addition, it was shown that Tat-specific antibody responses can be effective against infection with different HIV-1 subtypes (Fiorelli *et al.*, 2002).

It is well known that extracellular proteins do not ordinarily get taken up by cells and thus are not processed by the MHC-I pathway through which antigen is presented to stimulate $CD8^+$ CTL responses (Cresswell, 1994). Vaccine strategies that have focused on immunisation with recombinant Tat protein (Cafaro *et al.*, 2000; Cafaro *et al.*, 1999) and/or chemically inactivated Tat protein (Tat toxoid) (Gallo, 1999; Gringeri *et al.*, 1998; Zagury *et al.*, 1998b) have shown very good immunogenicity. As expected though, it was demonstrated that when the inoculated Tat was not biologically active, the

immunity elicited was predominantly humoral as biological activity is required for Tat uptake into cells and subsequent processing through the MHC-I pathway (Fanales-Belasio *et al.*, 2002; Cafaro *et al.*, 2000;). It is precisely a cell mediated $CD8^+$ response that is desired to achieve protection against intracellular pathogens such as HIV-1 (Kim *et al.*, 1997a). Moreover, as the Rev and Nef proteins are present only intracellularly, antibodies directed against Tat, Rev and Nef will not effect the killing of the cell prior to completion of viral replication. Thus the type of immunity aimed at by using these accessory genes as DNA vaccine immunogens is of the cell mediated type, which is adept at eradicating infected cells early.

The accessory proteins Vif, Vpr and Vpu were not selected for development as potential vaccine components for a number of reasons. Firstly, Vif, Vpr and Vpu are expressed from Rev-dependent, partially spliced transcripts and are thus not early genes. In addition, like Tat, Rev and Nef, these proteins possess intrinsic cytotoxic, immunosuppressive or undesirable activities, which would have to be eliminated through mutagenesis or deletion. Studies have demonstrated that Vpr plays a role in immune suppression, apoptosis and CD4 cell depletion in HIV-1 infected patients (Ayyavoo *et al.*, 1997b). More recent studies have shown that co-immunisation of Vpr with other immunogens compromises the antigen-specific immune responses as well as effector cell function (Ayyavoo *et al.*, 2002). This is thought to be achieved by Vpr-mediated interference with co-stimulatory molecule expression of APCs.

As revealed by its name, Vif (viral infectivity factor) plays a crucial role in viral infectivity and replication by possibly neutralizing a cellular factor that inhibits HIV-1 replication. Although Vif is immunogenic, the poorly understood role that it plays in viral replication (Janvier *et al.*, 2000) and limited amounts of data describing the inactivation of its intrinsic functions for it's safe incorporation as vaccine component, speak against Vif as vaccine component.

As reviewed above, Vpu enhances virion release and contributes to the downregulation of CD4 on the surface of infected cells. In addition, very little data describing the immunogenicity of Vpu is available and the few studies that produced such data reveal that Vpu is not particularly immunogenic.

The value of including a single HIV-1 gene as vaccine component may be measured in terms of the immunogenicity shown in terms of CTL epitope frequencies and response strength, immunogenicity in relation to protein size and the cost in terms of research and development into making it suitable and safe as well as immunogenic. Specifically when employing naked DNA

vaccination strategies, the immunogenicity of a vaccine component may be markedly reduced as a result of weak expression of the foreign gene that is introduced. This is observed because the codon usage of viral genes is such that transcribed messenger RNAs usually display instability and rapid turnover (Haas *et al.*, 1996b; Hentze, 1991). This may be overcome by changing the codon usage of a foreign DNA immunogen to that of highly expressed human genes (Haas *et al.*, 1996b), which requires expensive synthetic construction of such a component. When these factors are taken collectively, the pathogenic and/or undesirable functions and relatively low immunogenicity of Vif, Vpr and Vpu did not warrant their inclusion into this study.

CHAPTER 2: MATERIALS AND METHODS

2.1 Viral isolates and reagents

2.1.1 Isolation and characterisation of HIV-1 subtype C viral isolates

Blood samples from HIV-1 infected patients were obtained from the Infectious Diseases Clinic at the Tygerberg Hospital in the Western Cape during 1998 and 1999. Eighteen viruses were isolated from peripheral blood mononuclear cells (PBMCs) by co-cultivation with donor PBMCs at the Department of Medical Virology, Tygerberg and were allocated TV (Tygerberg Virology) numbers 001 through 019 (no TV011). These primary isolates were initially subtyped by serotyping as well as genotyping of the V3 region of the envelope gene and 15 were identified as HIV-1 subtype C. Further analysis revealed that these were all of the non-synsytium-inducing (NSI) phenotype and used CCR5 as their principal co-receptor (**Table 2**) (Treurnicht *et al.*, 2002).

Of the 15 patients infected with HIV-1 subtype C, all except one (TV019) presented with clinical symptoms such as pulmonary tuberculosis, oral candidiosis, diarrhoea, lymphadenopathy, and dermatitis. All patients were heterosexual (8 female, 7 male) with infection sources from various geographic regions (**Table 2**). These HIV-1 subtype C isolates were subsequently characterised by sequence analysis of the *gag* and *env* genes (Engelbrecht *et al.*, 2001) as well as the *vif*, *vpr* and *vpu* genes (Scriba *et al.*, 2001). These genomic regions were all shown to fall within the subtype C cluster using phylogenetic analysis. Recently, full-length genome analysis of isolates TV001, TV002 and TV012 was completed, confirming the non-recombinant phenotype of these subtype C viruses (zur Megede *et al.*, 2002).

Table 2 Demographic and clinical data of patients and viral isolates.

Patient	Sex	Sero-type*	Geno-type#	Clinical Symptoms	Geographic source of infection
TV001	M	C	C	Dermatitis	Cape Town
TV002	M	C	C	Oral Candidiosis	Cape Town
TV003	M	C	C	Pulmonary TB	Eastern Cape / Cape Town
TV004	F	C	C	TB adenitis	NA
TV005	F	C	C	Oral/tracheal Candidiosis	Cape Town
TV006	F	C	C	TB pleuritis	Cape Town
TV007	F	C	C	Dermatitis	Gauteng
TV008	F	C	C	Bladder infection	Zimbabwe
TV009	M	C	C	Pulmonary TB	Cape Town
TV010	F	C	C	Lymphadenopathy	Cape Town
TV012	M	C	C	Oral/nasopharyngeal Candidiosis, diarrhea	Cape Town
TV013	F	C	C	Pneumonia	Cape Town
TV014	M	C	C	Pulmonary TB	Namibia
TV015	M	NR	B	Wasting, stomach ulcers	Unknown
TV016	M	B/D	B	Loose stools	Unknown
TV017	M	B	B	Glandular fever, Candidiosis, wasting	Unknown
TV018	M	C	C	Pulmonary TB	Eastern Cape / Cape Town
TV019	F	C	C	Asymptomatic	Cape Town

M = male, F = female, C = coloured, B = black, W = white, NR = non reactive, NA = not available.

*determined with the V3 competitive peptide enzyme immunoassay as described by Engelbrecht *et al.* (1999)

based on partial sequencing of the gp120 region.

2.1.2 Sequence characterisation of *tat*, *rev* and *nef* genes from local South African HIV-1 subtype C viruses

The regulatory genes *tat* and *rev*, the accessory gene *nef* as well as the 5' long terminal repeat (5' LTR) of fourteen of these fifteen HIV-1 subtype C viruses were also sequenced. Similarly to the *gag*, *env*, *vif*, *vpr* and *vpu* genes, these regions were identified as subtype C by phylogenetic analysis and the *tat*, *rev* and *nef* genes were further characterised by analysis of their deduced amino acid sequences. It was found that thirteen of the fourteen isolates had the subtype C-specific Rev truncation. Some isolates had length variations in the second exon of Tat and the cysteine residue at position 31, which is highly conserved in non-C subtypes, yet absent in most subtype C Tat proteins was present in three isolates. Moreover, the *nef* genes were intact and only single residue variations were identified in the second glycine of the myristoylation signal (G_3) and motifs involved in CD4 and MHC-I downregulation. This work was written up as a sequence note manuscript for the journal *AIDS Research and Human Retroviruses* and was published in Volume 18, Number 2 in the January 20, 2002 issue (Scriba *et al.*, 2002). The manuscript is attached as **Appendix A**. Phylogenetic trees and amino acid sequences as well as the calculated consensus amino acid sequences for these proteins are included in the manuscript.

2.1.3 Selection of viral strains for wild-type constructs

Genetic distances between viral isolates and the consensus protein sequence calculated from the fourteen TV isolates, designated TV Cons, as well as the overall consensus sequence representing all reference subtype C isolates and designated C CONS (obtained from the Los Alamos HIV-1 Sequence Database, http://hiv-web.lanl.gov/content/hiv-db/mainpage.html) were calculated using the PROTDIST program (Phylip ver. 3.6, Felsenstein, J) with the Kimura distance matrix (Kimura, 1980) or the PAM distance matrix (Dayhoff *et al.*, 1978). All available reference subtype C Tat, Rev and Nef protein sequences from the HIV sequence database, all subtype C sequences from Botswana (including the 43 newly characterised ones (Novitsky *et al.*, 2002)), those from the four previously characterised full-length sequences from South Africa (van Harmelen *et al.*, 2001) and a number of consensus sequences (see **Table 3**) were included for construction of phylogenetic trees

using the Phylip programs SEQBOOT, PROTDIST, NEIGHBOR and CONSENSE (Phylip [phylogenetic inference package] ver. 3.6, Felsenstein, J). Manipulation of the trees was done in TREEVIEW. Based on phylogenetic clustering, calculated minimum genetic distance as well as comparison of amino acid sequences to the consensus, the isolate for which an individual gene resembled the consensus the most was chosen for construction of a wild-type expression plasmid for that gene. For amino acid sequences of the individual genes, refer to **Appendix C**. The isolates chosen for each gene are listed in **Table 4**.

Table3: Consensus sequences were constructed from viral sequences representing different geographic sources.

Name	Geographic origin	Viral isolates
TV Cons	South Africa	Tygerberg Virology (TV) isolates
sA Cons	southern Africa	all Botswana, TV and other South African isolates
Cons C	reference subtype C consensus (HIV sequence database)	all subtype C reference sequences available from HIV sequence database (consensus downloaded directly from HIV sequence database)
Compl Cons	global	all sequences in this column
India Cons	India	all Indian isolates

Table 4: Viral isolates chosen as templates for construction of wild-type expression plasmids.

Gene	Viral isolate(s)
tat	TV019
rev	TV010
nef	TV002

2.1.4 Plasmid vectors

Three eukaryotic expression vectors were used in this study.

pCMVKm2 employs the human cytomegalovirus (hCMV) immediate-early enhancer/promoter with its intron A and the bovine growth hormone (bGH) poly-adenine (pA) terminator (Chiron Corporation, Emeryville CA (Chapman *et al.*, 1991)). The plasmid incorporates the neomycin resistance gene, which confers antibiotic resistance to Kanamycin. The incorporation of the

immediate/early intron A allows for high level expression of glycoproteins and other proteins that may be difficult to express with other vector systems.

The pCR3.1 vector (Invitrogen, CA, USA) is similar in that it contains a CMV immediate/early promoter (without intron A) and bGH terminator, but contains the additional bacterial T7 promoter and the f1 origin for rescue of single stranded DNA. pCR3.1 contains the neomycin resistance gene under the control of the simian virus 40 (SV40) promoter and terminator, which confers resistance to gentamycin (G418) in mammalian cell culture. For selection in bacterial cells, pCR3.1 employs the beta-lactamase gene, which confers resistance to the antibiotic ampicillin. pCR3.1 is designed for simple and quick cloning of *Taq* polymerase-amplified PCR products with TA overhangs and unlike pCMVKm2 and pcDNA3.1, this vector is supplied in linear form with single thymidine overhangs.

The pcDNA3.1 vectors (Invitrogen, CA, USA) are designed for high-level, stable and transient expression in mammalian cells. These vectors are essentially very similar to pCR3.1, but whereas pCR3.1 employs the pUC origin for replication and maintenance of high copy numbers in E. coli, pcDNA3.1 vectors employ the ColE1 origin.

For technical data sheets of these plasmid vectors refer to **Appendix D**.

2.1.5 Cell lines

The cell lines used in this study as well as their characteristics are tabulated in **Table 5**. All were propagated in Dulbecco's Modified Eagles Medium (DMEM) containing heat-inactivated 10% fetal calf serum (FCS; Delta Bioproducts, Kempton Park, South Africa) and 100mg/L penicillin (Novo-Pen; Novo Nordisk (Pty) Ltd, Johannesburg, South Africa) and 100mg/L streptomycin (Novo-Strep; Novo Nordisk) at 5% CO_2, 95% humidity and 37°C.

Table 5: Cell lines and their properties used for the various experiments.

Name	Tissue	Properties	Source
293	Human embryonic kidney	established from primary embryonal human kidney cells transformed with sheared human adenovirus type 5 DNA. The E1A adenovirus gene is expressed in 293 cells and allows for very high levels of protein expression.	GibcoBRL, CA, USA Cat #:11631
293T	Human embryonic kidney	derived from the 293 cell line, transformed with SV40 large T-antigen. Allows for episomal replication of plasmids containing the SV40 origin and early promoter region.	donated by Ms. Hanna Veenstra Dept Medical Biochemistry, Tygerberg
HeLa	Human cervical adeno-carcinoma	HeLa cells are epithelial-like adherent cells that are commonly used in a wide range of investigations. They are transformed by the human papillomavirus 18 (HPV-18)	ATCC # CCL-2
RD	Human embryonal rhabdomyo-sarcoma	The RD cell line was derived from malignant embryonal rhabdomyo-sarcoma.	ATCC # CCL-136
COS-7	African green monkey kidney cells	This African green monkey kidney cell line was derived from simian virus 40 (SV40)-trans-formed CV-1 cells and expresses the SV40 large T antigen. It is commonly used in virology studies and assays requiring expression of SV40 large T antigen.	ATCC # CRL-1651

2.2 Construction of wild-type expression plasmids

2.2.1 Optimising start and stop codon contexts

While studying features of eukaryotic mRNAs, it was found that the nucleotide contexts of their translation initiation and termination codons affect their translational efficiency and thus the expression level of the encoded protein (Kozak, 1991). Subsequently, various studies have aimed at describing consensus initiator and terminator nucleotide contexts for start and stop codons from highly expressed eukaryotic mRNAs (Kochetov *et al.*, 1998; Pain, 1996; Kozak, 1991). Such optimal consensus sequences that allow for strong protein expression were defined as GCCACC**ATG**G for the initiation of

translation (start codon, ATG); and GCC**TAA**AG for the termination of translation (stop codon, TAA) (Kochetov *et al.*, 1998; Kozak, 1991). These optimised nucleotide contexts were incorporated into all expression plasmid constructs in this study, to enhance the translation efficiency and subsequent protein expression of the *tat*, *rev* and *nef* genes.

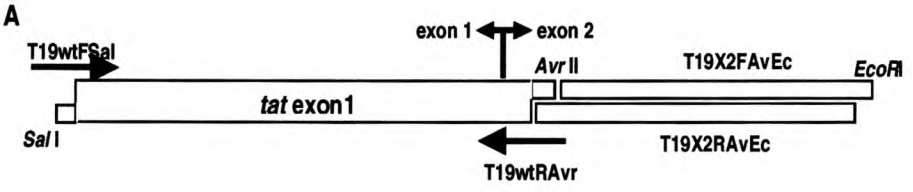
2.2.2 TAT

Tat is a small protein of 86 to 101 amino acid residues which is coded for by two separate exons, the first of which makes up the bulk of *tat's* coding sequence (approximately 72 amino acids). As the second exon is very small, in the case of TV019 only 90 basepairs, it may have been problematic to amplify and purify such a small fragment. As a consequence, it was decided to construct the second exon of TV019 *tat* by assembly of two complementary synthetic oligomers (T19X2FAvEc and T19X2RAvEc). The oligos (Operon Technologies, Qiagen, Alameda, CA, USA) were designed to incorporate the sticky end overhangs of the *Eco*RI restriction site behind the stop codon at the 3'-end of the *tat* coding region as well as the sticky end overhang of the *Avr*II restriction site at the 5'-end. The *Avr*II site naturally occurs within the coding sequence of the second exon of *tat* close to the 3'-end and could thus be used to ligate the synthetic second exon in frame with the PCR-amplified, restriction digested first exon to resemble the *tat* cDNA. **Figure 4A** illustrates the assembled *tat* coding region. The sequences of the two complementary oligomers and the primers used to amplify the first exon of *tat* are shown in **Figure 4B**. The forward PCR primer (T19wtFSal) incorporates the *Sal*I site and the reverse primer (T19wtRAvr) incorporates a short section (11 bp) encoding exon 2 up to, and including the *Avr*II restriction site.

The amplification reaction of *tat* exon one contained 10mM total dNTPs, 2.5U of *Pfu* Turbo polymerase (Stratagene, CA, USA), 25pmol of each oligo primer and 500ng of template DNA. The template was genomic DNA prepared from cultured PBMCs from HIV-1 infected patient TV019. The amplification reaction was performed by initial denaturing at 95°C for two minutes, followed by 23 cycles of denaturing at 95°C for 15 seconds, oligo annealing at 60°C for 30 seconds and elongation at 72°C for an initial 15 seconds and after that an additional 5 seconds per cycle. This was followed by 17 cycles of denaturing at 95°C for 15 seconds, oligo annealing at 60°C for 30 seconds and elongation at 72°C for 30 seconds. A final elongation step was at 72°C for 10 minutes.

The PCR product was electrophoresed through a 1% agarose gel in Tris-acetate EDTA buffer (40mM Tris-acetate, 1mM EDTA) and subsequently gel purified using the QIAquick Agarose Gel Extraction Kit (Qiagen, GmbH, Germany), following the manufacturers protocol. The purified DNA was digested with 10 units (U) each of *Sal*I and *Avr*II (Roche Diagnostics, GmbH, Germany) and thereafter purified of enzymes and buffer salts using the QIAquick PCR Purification Kit, following the manufacturers protocol. Assembly of the first exon amplicon (10ng) and second exon oligonucleotides (5ng) was done directly in a ligation reaction along with 25ng of the *Sal*I and *EcoR*I (all enzymes from Roche Diagnostics, GmbH, Germany unless otherwise stated) pre-digested pCMVKm2 plasmid vector using the Rapid Ligation Kit (Roche Diagnostics, GmbH, Germany), as directed by the manufacturers protocol (**Appendix E**).

A



B

primer	sequence
T19X2FAvEc	ctaggGGACCCGACAGGCTCAGAGGAATCGAAGAAGAAGGTGGA GAGCAAGACAGAGACAGATCCGTTCGACTAAg
T19X2RAvEc	aattcTTAGTCGAACGGATCTGTCTCTGTCTTGCTCTCCACCT TCTTCTTCGATTCCTCTGAGCCTGTCGGGTCCc
T19wtFSal	CTACGCgtcgacGCCACCATGGAGCCAGTAGATCCTAACC
T19wtRAvr	ATcctaggGTTCTGGGTAAGGGCTGCTTTGATATAGGATTTTGA TG

Figure 4: Construction of the wild-type *tat* gene of isolate TV019 by assembly of the amplified, restriction digested first exon fragment with synthetic oligomers representing the second exon.

A: Assembly of the fragments showing restriction sites and primer positions.

B: Sequences of the complementary oligomers making up the second exon (T19X2FAvEc and T19X2RAvEc) as well as the oligo primers used to amplify the first exon of tat (T19wtFSal and T19wtRAvr). The restriction overhangs (AvrII and EcoRI) and sites (SalI and AvrII) are indicated in small letters.

Two microlitres of ligation product was transformed into chemically competent DH5α cells (Gibco BRL, Rockville Md, USA) by heatshock at 42°C for 45 seconds, following the manufacturers protocol. The transformed cells were plated onto Luria Broth (LB; Pronadisa, Madrid, Spain) agar plates containing 50μg/ml kanamycin (Sigma-Aldrich, GmbH, Deisenhofen, Germany) and incubated overnight at 37°C.

The next day, single colonies were picked and inoculated into 5 ml LB media containing 50μg/ml kanamycin and these minicultures were incubated for 16 hours at 37°C with vigorous shaking. Miniprep DNA was prepared using the QIAprep Spin Miniprep Kit (Qiagen, GmbH, Germany), following the manufacturers protocol (**Appendix F**).

Positive clones were identified by restriction digestion of miniprep plasmid DNA with *Sal*I and *EcoR*I and subsequent confirmation of insert size by agarose gel electrophoresis. The plasmid insert was then verified for correct sequence by sequencing (see Section 2.5) and the resulting expression plasmid construct containing the wild-type *tat* coding sequence with optimised start and stop codon contexts was named pCMVKm2-Tat_{WT}.

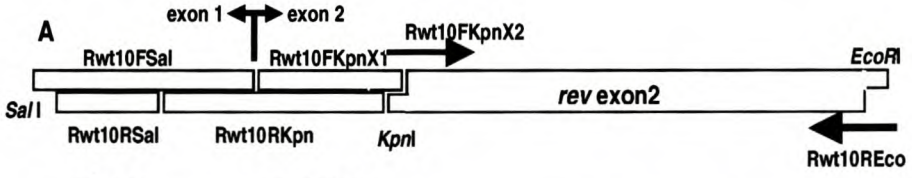
2.2.3 REV

Similarly to Tat, the Rev protein is encoded by a bicistronic gene. However, in this case the first exon (75 bp) of the 321 bp long TV010 *rev* is smaller than the second exon (246 bp), and again only the second exon could be PCR amplified from the primary lymphocyte culture extracted DNA of patient TV010. A *Kpn*I site, which naturally occurs towards the 3' end of the second exon of *rev* was incorporated into the forward PCR primer for amplification of the second exon. This site was used as ligation point for joining the two exons and because the length of the first exon (75 bp) added to the exon 2 section up to the *Kpn*I site was quite large (112 bp), it was broken up into a set of four overlapping oligonucleotides, which had to be assembled before they could be ligated along with the *Kpn*I- and *EcoR*I-digested second exon into the pCMVKm2 plasmid vector (**Figure 5**). The 3' end of these oligonucleotides were designed to incorporate the sticky end overhang of the *Sal*I site and the 5' end that of the *Kpn*I site.

The four oligonucleotides were added together and the pool was added to an assembly mixture composed of 1X *Pfu* reaction buffer (Roche Diagnostics, GmbH, Germany) at a final concentration of 5 ng/μl. This reaction mixture was

exposed to three denaturing-annealing cycles of 95°C for five seconds and 50°C for ten seconds respectively, followed by a final annealing step of 50°C for two minutes.

The second exon of *rev* was amplified from genomic DNA, prepared from primary lymphocyte culture cells. Identical PCR conditions, cycles and reaction mixtures to those used to amplify *tat* exon 1 were employed with the primer pair Rwt10FKpnX2 and Rwt10REco and the PCR product was gel purified and digested with *Sal*I and *EcoR*I as described before. Again, ligation of the two exons into the *Sal*I and *EcoR*I pre-digested pCMVKm2 plasmid vector, transformation and identification of positive clones were done as described for *tat*. Again, the plasmid insert was then verified for correct sequence by sequencing. The resulting expression plasmid containing the wild-type *rev* coding sequence with optimised start and stop codon contexts was designated pCMVKm2-Rev_{WT}.



B

primer	sequence
RwtF10Sal	tcgacGCCACCATGGCAGGAAGAAGCGGAGACAACGACGAAGCG
	CTTCTCCAAGCAGTAAGGATCATCAAAATCC
Rwt10FKpnX1	TATACCAAAGCAACCCCTACCCCAAGCCCGAGggtac
Rwt10RSal	GTCGTTGTCTCCGCTTCTTCCTGCCATGGTGGCg
Rwt10RKpn	cCTCGGGCTTGGGGTAGGGGTTGCTTTGGTATAGGATTTTGATG
	ATCCTTACTGCTTGGAGAAGCGCTTC
Rwt10FKpnX2	ATCTACggtaccCGACAGGCCCGGAAGAAT
Rwt10REco	CGACCGgaattcCTTTAAGGGCTTCCCACCCCCTCTGTAGT

Figure 5: Construction of the wild-type *rev* gene of isolate TV010 by assembly of the amplified, restriction digested second exon fragment with synthetic oligomers representing the first exon.

A: Illustration of the assembly of the fragments showing restriction sites, primer positions and the exon breakpoint.

B: Sequences of the complementary oligomers making up the first exon as well as the oligomer primers used to amplify the second exon of *rev* (Rwt10FKpnX2 and Rwt10REco). Restriction sites and overhangs are indicated by small letters.

2.2.4 NEF

The Nef protein is expressed from the monocystronic *nef* gene, which is approximately 620 bp in length, depending on the viral strain (Piguet and Trono, 1999). During the characterisation study in which the *tat*, *rev* and *nef* genes were sequenced, the *nef* genes were amplified from a 9kb HIV genomic PCR amplicon, which was PCR amplified from primary lymphocyte culture cell-extracted DNA previously (**Appendix C**) (Scriba *et al.*, 2002). The primer pair NefF and NefR (**Table 5**) was used to amplify the fragment of approximately 660bp, which contained the *nef* coding region along with short flanking sequences on either side. The amplification was performed as follows: two microlitres of 9kb PCR product template were added to the *nef* amplification reactions containing 40µM of each primer, 2.5mM $MgCl_2$, 10µM total dNTPs (Roche Diagnostics, GmbH, Germany) and 0.7U Expand High Fidelity polymerase in Expand High Fidelity PCR System buffer 1 (Roche GmbH, Mannheim, Germany). Thermal cycling was performed as follows: initial denaturing at 94°C for two minutes followed by 40 cycles of denaturing at 94°C for 90 seconds, primer annealing at 50°C for 90 seconds and elongation at 72°C for 3 minutes. A final elongation step at 72°C for 10 minutes was employed.

The PCR products of selected isolates were selected to be cloned and these were agarose gel-purified as described previously and cloned into the pCR3.1 bi-directional cloning vector by TA cloning as directed by the manufacturer (Invitrogen, CA, USA, see **Appendix D**). Transformation into Top10F' cells (Invitrogen, CA, USA), miniprep DNA preparation and identification of positive clones by *Hind*III and *Xho*I (Promega, Madison, WI, USA) restriction analysis were done as described previously. Orientation determination of the *nef* inserts was done by asymmetrical restriction analysis using the restriction endonucleases *Sal*I and *Xho*I (Promega, Madison, WI, USA) and the *nef* genes were verified by sequencing.

To construct a wild-type *nef* expression plasmid with optimised start and stop codon contexts, which could be used for expression level and functionality comparisons with codon-optimised *nef* constructs, the *nef* gene of TV002 (clone pCR-$Nef_{2.11}$) was selected and this clone was used as template for a PCR amplification using primer pair NwtFXho and NwtREco (**Table 5**). This PCR amplification was performed using identical reaction and cycling conditions as described for the amplification of the wild-type *tat* and *rev* genes. Similarly, cloning of the *nef* amplicon into the pCMVKm2 vector was done over

the *Xho*I and *EcoR*I restriction sites as described before and positive clones were identified by restriction analysis of miniprep DNA with *Xho*I and *EcoR*I. Positive clones were sequence verified and the resulting expression plasmid was designated pCMVKm2-Nef$_{WT}$.

Table 5: Oligomer primers used to amplify the wild-type *nef* genes. The *Xho*I and *EcoR*I sites incorporated into NefwtFXho and NefwtREco are indicated in small letters.

Primer	Sequence
NefF	CCTAGAAGAATAAGACAGGGCTT
NefR	CCTGGAACGCCCCAGTGG
NwtFXho	CTACGCctcgagGCCACCATGGGGGGCAAGTGGTCAAAAA
NwtREco	CGACCGgaattcTTTAGGCGCAGTCTTTGTAATACTCC

2.3 Construction of codon-optimised and mutant expression plasmids

2.3.1 Optimising codon-usage for eukaryotic expression

Although DNA vaccines have shown very promising results in terms of antigen delivery and presentation, one constraint, which affects the immunogenicity of plasmid vector delivered immunogens, is the poor expression of antigen because the codon usage of such foreign viral genes is most likely not optimal in eukaryotic organisms. Human mRNAs with elevated AU content percentages have been shown to display instability, increased turnover and generally low expression levels compared to those with higher GC content (Hentze, 1991). Moreover, in their study, Haas *et al.* (1996b) compared codon usage between the HIV-1 envelope gene gp120 and highly expressed human genes and their results are illustrated in **Figure 6**. As can be seen, they found a significant divergence between the codon prevalence of the coding regions in highly expressed human genes and the HIV-1 envelope gene. Similar codon usage patters are reported by Andre *et al.* (1998) for other HIV-1 genes. Upon subsequent replacement of native codons in gp120 with codons more prevalent in highly expressed human genes, a striking increase in expression was observed in eukaryotic cells (Andre *et al.*, 1998; Haas *et al.*, 1996b). A similar increase in expression was shown when the codons of the poorly expressed green fluorescent protein (GFP) from the jellyfish *Aequerea victoria* was changed in the same way as gp120 (Haas *et al.*, 1996b). Moreover, markedly increased expression levels were obtained by zur

Megede *et al.* (2000) by changing the codons of the HIV-1 protein Gag in a similar fashion.

Thus, in an attempt to potentially optimise expression in mammalian tissues over and above optimised start and stop codon contexts, the codon usage of the genes encoding the HIV-1 subtype C consensus Tat, Rev and Nef proteins was altered to that of highly expressed human genes as presented in **Figure 6** whenever it was permissible by the degeneracy of the genetic code.

		High	Env
Ala	C	**53**	27
	T	17	18
GC	A	13	**50**
	G	17	5
Arg	C	**37**	0
	T	7	4
CG	A	6	0
	G	21	0
	A	10	**88**
AG	G	18	8
Asn	C	**78**	30
AA	T	22	**70**
Asp	C	**75**	33
GA	T	25	**67**

		High	Env
Cys	C	**68**	16
TG	T	32	**84**
Gln	A	12	**55**
CA	G	**88**	45
Glu	A	25	**67**
GA	G	**75**	33
Gly	C	**50**	6
	T	12	13
GG	A	14	**53**
	G	24	28
His	C	**79**	25
CA	T	21	**75**
Ile	C	**77**	25
	T	18	31
AT	A	5	**44**

		High	Env
Leu	C	26	10
	T	5	7
CT	A	3	17
	G	**58**	17
	A	2	**30**
TT	G	6	20
Lys	A	18	**68**
AA	G	**82**	32
Pro	C	**48**	27
	T	19	14
CC	A	16	**55**
	G	17	5
Phe	C	**80**	26
TT	T	20	**74**

		High	Env
Ser	C	28	8
	T	13	8
TC	A	5	22
	G	9	0
	C	**34**	22
AG	T	10	**41**
Thr	C	**57**	20
	T	14	22
AC	A	14	**51**
	G	15	7
Tyr	C	**74**	8
TA	T	26	**92**
Val	C	25	12
	T	7	9
GT	A	5	**62**
	G	**64**	18

Figure 6: Codon usage of the HIV-1 gp120 coding region (Env) and that of highly expressed human genes (High). The third nucleotide for each amino acid codon is tabulated with its frequency for both systems inside each box. The frequencies of the individual codons for each amino acid are shown as a percentage value and the most prevalent codon is in boldface. Source: (Haas *et al.*, 1996b).

2.3.2 Selection of a representative vaccine strain

It is well known that the extreme diversity of HIV-1 makes vaccine development and especially selection of a candidate strain, clone or isolate for the development of a vaccine a major issue. Because a homologous vaccine is not possible, the next best approach may be using a consensus sequence, which is constructed from a panel of viral strains that represent the viral population in the local epidemic (Novitsky *et al.*, 2002). Such a consensus drawn up from subtype C sequences from South Africa may display a higher degree of homology to local circulating viruses than a single viral strain. Indeed, such an approach was recently proposed by Novitsky *et al.* (2002), who analysed consensus sequences that represent the entire subtype C sequences as well as a number of subpopulations including one for southern Africa. It was demonstrated that such approaches may be effective at overcoming the high genetic diversity and thus facilitate vaccine development.
Because the codon-optimised genes had to be constructed synthetically they did not necessarily have to be acquired from an existing physical template such as a viral isolate. The comparison of wild-type amino acid sequences with the respective consensus sequences (TV Cons) constructed from the 14 subtype C TV isolates revealed no sequence differences in the consensus that might account for loss of function or change in protein folding. It was thus decided to use the consensus amino acid sequences to design the codon-optimised *tat*, *rev* and *nef* constructs.

2.3.3 Design of codon-optimised gene sequences

Consensus amino acid sequences constructed from HIV-1 subtype C isolates from Tygerberg (Scriba *et al.*, 2002), designated TV Cons, were used as template for deriving the codon-optimised nucleotide sequences. The codons of the *tat*, *rev* and *nef* coding regions were changed to those more prevalent in highly expressed human genes by altering the codons to those described in **Figure 6** wherever it was permissible by the degeneracy of the genetic code to retain an unchanged protein sequence. This was done using the MacVector software program (Oxford Molecular Ltd., Oxford, UK).

2.3.4 Synthetic construction of DNA sequences

All codon-optimised gene sequences were constructed by PCR assembly of overlapping 50mer oligonucleotides. Briefly, the gene sequences were subdivided into 50mer single-stranded fragments, of which the positive and negative strand fragments overlap by 25 bases as illustrated in **Figure 7**. Oligos were synthesised by Operon Technologies (Qiagen, Alameda, CA, USA) and were purified by double polyacrylamide gel electrophoresis (PAGE).

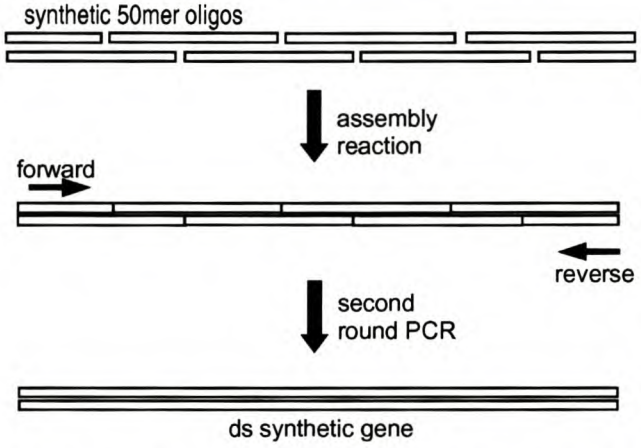


Figure 7: Assembly and amplification of overlapping 50mer oligonucleotides to construct the synthetic double-stranded gene sequences.

Oligonucleotides were reconstituted in nuclease-free water (Promega, Madison, WI) to a concentration of 1µg/µl and added together to create an oligo pool, which was diluted to a final concentration of 0.4µg/µl. Using serial dilutions, 2µg, 200ng and 20ng of pooled oligonucleotides were added to an assembly PCR reaction containing 0.2mM total dNTPs and 1.25U of *Pfu* Turbo polymerase (Roche Diagnostics, GmbH, Germany). The assembly reaction was performed by initial denaturing at 95°C for two minutes, followed by 55 cycles of denaturing at 95°C for 15 seconds, oligo annealing at 52°C for 30 seconds and elongation at 72°C for 30 seconds.

One and a half microlitres of the assembly product were transferred to a second amplification reaction containing 0.4mM total dNTPs, 20pmol forward and reverse primer (the first positive strand oligo and last negative strand oligo respectively) each and 1.25U of *Pfu* Turbo polymerase. The amplification was performed by initial denaturing at 95°C for two minutes, followed by 23 cycles of denaturing at 95°C for 15 seconds, oligo annealing at 60°C for 30 seconds and elongation at 72°C for 45 seconds. The entire volume of amplification

product was loaded onto a 1% agarose gel, electrophoresed and gel extracted as described before.

2.3.5 TAT

Designing non-functional Tat mutants

As reviewed in Section 1.1.6, the Tat protein consists of distinct functional regions, which include an N-terminal activation region, a cysteine-rich domain, a core region, a basic domain and a glutamine-rich region (Kuppuswamy *et al.*, 1989). Mutational analysis has identified a number of Tat inactivating mutations and it has been shown that the cysteine residues are critical for Tat function in several studies (Battaglia *et al.*, 1994; Kuppuswamy *et al.*, 1989; Ruben *et al.*, 1989). Specifically, Tat proteins with either a mutated cysteine at position 22 or double mutations at cysteine 22 (to glycine) and cysteine 37 (to serine) have been shown to be functionally inactive (Rossi *et al.*, 1997; Caputo *et al.*, 1996) and immunisation with such mutated Tat-encoding plasmid DNA induced humoral and cellular immune responses which recognised wild-type Tat (Caselli *et al.*, 1999). This study as well as one that followed further revealed that such mutated Tat proteins, when excreted, interfered in a competitive manner with the transactivating function of extracellular wild-type Tat, suggesting applications as therapeutic agents (Betti *et al.*, 2001; Caselli *et al.*, 1999).

In this study, we designed three different mutants (Tat_{C22}, Tat_{C37} and Tat_{C22C37}) and a native consensus Tat (Tat_{OPT}) (**Table 6**) that were tested for functional activity to identify a non-functional Tat with a maximum number of unmutated CTL epitopes within the conserved regions of Tat.

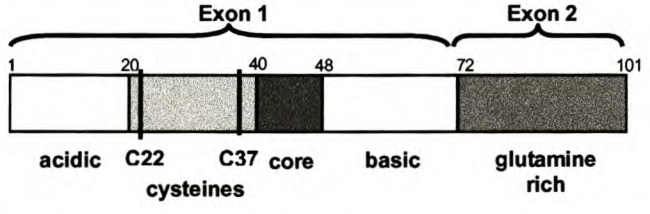


Figure 8: Diagrammatic representation of the 101-aa Tat protein showing the functional domains. The two critical cysteines to be mutated are indicated as black lines. Adapted from (Jeang, 1996).

Constructing the Tat_{optC22} expression vector

Because of the relatively small size of *tat* and *rev*, these genes were synthesised as a single polygene cassette. Thus the codon-optimised Tat_{C22} and Rev_{M5M10} (Section 2.3.6) genes were designed to incorporate the coding sequences of both genes in the same reading frame (**Figure 9**). An *Xma*I site divided the two coding regions, while the *Sal*I and *EcoR*I sites were incorporated upstream of the optimised start (*tat*) and downstream of the optimised stop (*rev*) codon contexts respectively. This Tat-Rev cassette was synthesised as described in section 2.3.4 and cloned into the pCMVKm2 plasmid vector over the *Sal*I and *EcoR*I sites. Ligation, transformation and selection of positive clones were done as described for the wild-type plasmid construction.

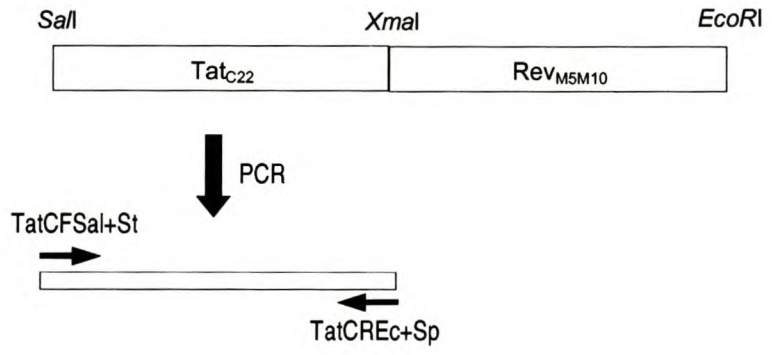


Figure 9: Diagrammatic representation of the synthetic Tat-Rev polygene cassette that was used to amplify the Tat_{C22} and Rev_{M5M10} genes (section 2.3.6).

The resulting plasmid construct was used as template for the amplification of the Tat_{C22} gene, using primer pair TatCFSal+St and TatCREc+Sp (**Table 7**). The latter was designed to introduce an optimised stop codon context with a *EcoR*I site downstream of the stop. PCR conditions and cycling using *Pfu* polymerase were done as described before. The resulting amplicon was gel purified, digested with *Sal*I and *EcoR*I, ligated into pCMVKm2 and transformed into DH5α cells. Preparation of miniprep DNA, identification of positive clones and sequence verification were all done as described before. The resulting expression construct was named pCMVKm2-Tat_{C22}.

Site-directed mutagenesis

Background

In order to construct plasmids expressing Tat with no mutations (native consensus Tat protein) or different mutations to C22, site-directed mutagenesis was used to induce single nucleotide changes. Various methods of site-directed mutagenesis have been established, most of which produce very efficient mutation frequencies of above 80% of colonies screened.

In this study, we decided to use a PCR-based oligonucleotide-directed mutagenesis system (QuickChange Site Directed Mutagenesis Kit, Stratagene, CA, USA) in which a pair of complementary oligonucleotide primers, which consist of the sequence with the changed nucleotide, anneal to the positive and negative strand of the plasmid DNA template at sites flanking the site to be mutated. The entire DNA plasmid is amplified by PCR, resulting in the newly synthesized DNA strands containing the nucleotide mutation. By digestion with the restriction enzyme *Dpn* I, which only cleaves methylated DNA templates, the plasmid template containing the original sequence is digested. Since the new DNA strands, which contain the induced mutation, have been synthesized *in vitro*, they are not methylated and are subsequently not digested and will therefore be the only functional, replicating plasmids upon transformation, lending antibiotic resistance to bacterial cells. This system, therefore, selects for bacterial cells, which have been transformed with the PCR amplified, mutated plasmid.

Construction of Tat_{OPT}, Tat_{C37} and Tat_{C22C37} by mutation of Tat_{C22}

The oligonucleotide primers used to induce mutations to construct the mutants Tat_{C37} and Tat_{C22C37} as well as the native Tat protein Tat_{OPT} are listed in **Table 6**, while the primer sequences are shown in **Table 7**. Mutagenesis PCR amplification was performed in a total reaction volume of 50µl, containing 0.125 µg of each primer, 10 mM total dNTPs, 1 U *Pfu* polymerase and 20 ng plasmid DNA template (pCMVKm2-Tat_{C22}). Amplification was performed by initial denaturing at 95ºC for two minutes, followed by 12 cycles of denaturing at 95ºC for 30 seconds, primer annealing at 60ºC for 1 minute and elongation at 68ºC for 13 minutes. A final elongation step at 68°C for 12 minutes completed the amplification. Following the amplification, 1 µl *Dpn*I (10 U) restriction endonuclease was added directly to the PCR reaction and incubated at 37°C for 90 minutes, after which 1 µl was transformed into XL1-

Blue supercompetent cells (Stratagene, CA, USA) by heatshock as described by the manufacturer. Miniprep DNA preparation and identification of positive transformants was performed as described previously.

Table 6: The four different *tat* variants. The mutations induced and primers that were used to do so are tabulated.

Construct	Mutations	Primers
Tat_{OPT}	native consensus protein	SiteTaCwtF, SiteTaCwtR
Tat_{C22}	Cys22Gly	synthesised directly
Tat_{C37}	Cys37Glu	SiteTaCwtF, SiteTaCwtR SiteTa37CF, SiteTa37CR
Tat_{C22C37}	Cys22Gly, Cys37Glu	SiteTa37CF, SiteTa37CR

Table 7: Sequences of the oligonucleotide primers for PCR amplification of the codon-optimised *tat* gene (TatCFSal+St and TatCREc+Sp) and those designed to induce site-directed mutations to construct *tat* variants. The restriction sites are indicated in small letters and changed bases (site-directed mutagenesis) are underlined.

Primer	Sequence	Mutation induced
TatCFSal+St	CTACGCgtcgacGCCACCATGGAGCCCGTGGAC CCC	PCR primer
TatCREc+Sp	CGACCGgaattcTTTAGTCGAAGGGGTCGGTCT CGGTCTTGCTCTCCA	PCR primer
SiteTaCwtF	GCAGCCAGCCCAAGACCGCCTGCAACAAGTGCT ACTGCAAG	Gly22Cys
SiteTaCwtR	CTTGCAGTAGCACTTGTTGCAGGCGGTCTTGGG CTGGCTGC	
SiteTa37CF	GCAGCTACCACTGCCTGGTGAGCTTCCAGACCA AGGGCCTG	
SiteTa37CR	CAGGCCCTTGGTCTGGAAGCTCACCAGGCAGTG GTAGCTGC	

Positive transformants were verified for correct sequence and the presence of mutations by DNA sequencing (see Section 2.4). The newly mutated, sequence verified tat variants were excised from the plasmid vector pCMVKm2 by digestion with SalI and EcoRI, re-ligated into the original pre-digested pCMVKm2 vector and transformed into DH5α cells as described previously. This subcloning step serves to eliminate potential mutations that may have arisen during PCR amplification within the plasmid vector sequence and could affect plasmid replication, antibiotic resistance or expression of the Tat proteins.

2.3.6 REV

Designing a non-functional Rev mutant

The functional and structural domains of HIV-1 Rev have been well studied and various critical positions and sequence motifs have been identified that are essential for a functional Rev protein (**Figure 10**). The amino-proximal arginine-rich domain spanning amino acids 35 to 50 has been shown to serve as nuclear localisation signal (NLS) and RNA binding domain (RBD) (Kjems *et al.*, 1992; Cochrane *et al.*, 1990b; Malim *et al.*, 1989b). The nuclear export signal (NES), which spans residues 75-93 presents as a leucine-rich domain (Fischer *et al.*, 1995; Wen *et al.*, 1995; Hope *et al.*, 1991; Malim *et al.*, 1991; Malim *et al.*, 1989a). Additional sites governing the multimerisation of Rev and its high-specificity binding to the RRE flank the NLS and RBD (Hope *et al.*, 1992; Zapp *et al.*, 1991). Although all domains are important for the nuclear export of unspliced or incompletely spliced viral mRNAs, a number of studies have identified single amino acid residues or short motifs that seem to abrogate Rev's functionality *in vitro* (Thomas *et al.*, 1998; Malim *et al.*, 1991; Hope *et al.*, 1990b; Hope *et al.*, 1990a; Mermer *et al.*, 1990; Malim *et al.*, 1989a).

Again, in an attempt not to excessively mutate the protein sequence of Rev in order to retain a maximum of potential cytotoxic T-lymphocyte (CTL) epitopes, we selected two well-defined Rev-inactivating mutations first described by Malim *et al.* (1989a), M5 (RR to DL at positions 38,39 in the arginine-rich RNA-binding NLS) and M10 (LE to DL at positions 78,79 in the activation domain of the NES) (**Figure 10**). Both mutations have been shown to be sufficient for functional inactivation and the M10 mutant has been shown to be a *trans*-dominant repressor of wild-type Rev function (Malim *et al.*, 1991; Hope

et al., 1990b; Malim *et al.*, 1989a). Hope *et al.* (1990b) also substituted $R_{38}R_{39}$ for GG, rendering Rev inactive, to confirm the importance of this site for functional Rev activity. Although the subtype C Rev proteins (consensus and wild-type) displayed residue differences in the M5 (RK) and M10 (IE) motifs to the subtype B proteins described in the literature (M5: RR; M10: LE), the amino acids (basic: Arginine (R), Lysine (K); nonpolar: Leucine (L), Isoleucine (I)) retain their physiochemical properties. Thus these mutations in the subtype C Rev_{M5M10} protein are M5: RK 38,39 DL; M10: IE 78,79 DL.

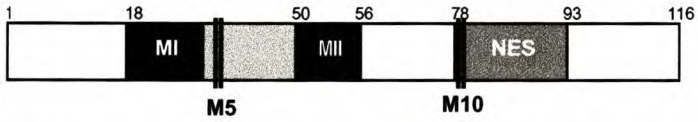


Figure 10: Diagrammatic representation of the 116-aa Rev protein showing the functional domains. MI and MII represent the multimerisation domains. The two double inactivating mutations M5 and M10 are indicated as black lines. Adapted from (Thomas *et al.*, 1998).

Constructing the Rev_{M5M10} expression vector

Similarly to Tat_{C22}, Rev_{M5M10} was PCR amplified from the synthetically constructed Tat-Rev polygene cassette (**Figure 11**) using the primer pair RoptFSal and RoptREco (**Table 8**). Primer RoptFSal was designed to incorporate a *Sal*I site upstream of an optimised start codon context in frame with the coding region of Rev_{M5M10}. The amplification, cloning and clone selection was done as described before, the inserts were sequence verified and the resulting expression plasmid was named pCMVKm2-Rev_{M5M10}.

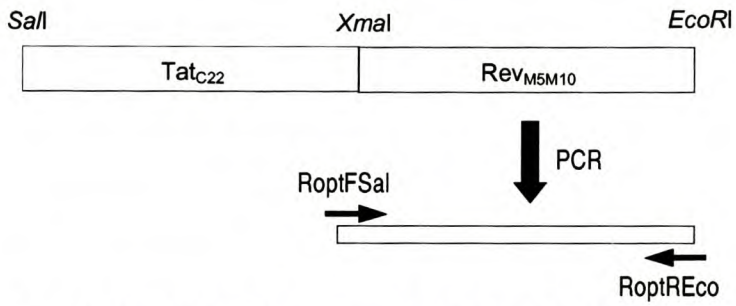


Figure 11: Amplification of the Rev_{M5M10} gene from the synthetic Tat-Rev cassette. The primers RoptFSal and RoptREco incorporated an optimised start context and *Sal*I site as well as an optimised stop context and *EcoR*I site respectively.

Table 8: Sequences of the oligonucleotide primers designed to amplify RevM5M10 from the Tat-Rev polygene cassette and to incorporate a SalI site and an optimised start codon context. The restriction sites are indicated in small letters.

Primer	Sequence
RoptFSal	CTACGCgtcgacGCCACCATGGCCGGCCGCAGCGGCGACAGC
RoptREco	CGACCGgaattcCTTTAGGCGCTGCCCACGCCCTCGGTGGT

2.3.7 NEF

Designing non-functional Nef mutants

A number of studies describe a range of mutations that partially or totally inactivate the different functionalities of Nef. Nef is myristoylated on glycine at position 2 and phosphorylated on serine and threonine residues (Niederman *et al.*, 1993). The Nef core (aa70-77) consists of a type II polyproline helix, which forms the main binding site for Src family kinases. Adjacently, stretching residues 81-120, is a region consisting of two alpha helices, followed by a four-stranded anti-parallel beta-sheet (aa121-186) and two additional alpha helices (aa187-203) (Grzesiek *et al.*, 1996). More recently it was shown that the N-terminal domain stretching from residue 6 through 22 forms another alpha-helix (Barnham *et al.*, 1997).

The myristoylation of Nef is essential for protein folding and association with the cell membrane and thus activity of most Nef functions including downregulation of CD4, MHC-I and a number of effects on T cell signalling (Lundquist *et al.*, 2002; Geleziunas *et al.*, 2001; Manninen *et al.*, 2001; Peng and Robert-Guroff, 2001; Aldrovandi *et al.*, 1998; Curtain *et al.*, 1998; Curtain *et al.*, 1997; Iafrate *et al.*, 1997; Goldsmith *et al.*, 1995; Sawai *et al.*, 1995; Aiken *et al.*, 1994; Niederman *et al.*, 1993;). Another study has identified a single inactivating mutation within the beta-sheet domains of Nef: the D123G mutation was reported to render Nef unable to downregulate CD4, MHC-I or enhance virion infectivity (Liu *et al.*, 2000). Another well-defined mutation that also falls within the beta-sheet domain is LL165AA, which disrupts the motif essential for CD4 down-regulation and interaction with AP-complexes (Lundquist *et al.*, 2002; Bresnahan *et al.*, 1998; Greenberg *et al.*, 1998a).

In order to minimize the elimination of important CTL epitopes, a minimum number of mutations within the Nef protein were desirable. Consequently we designed two different Nef mutants one in which the myristoylation signal (G2) and aspartate at position 124 (D124, corresponding to the described D123G mutation) were mutated ($Nef_{-myrD124}$), and one containing these two mutations as well the additional LL165AA mutation ($Nef_{-myrD124LLAA}$).

Constructing the Nef_{OPT}, $Nef_{-myrD124}$ and $Nef_{-myrD124LLAA}$ expression vectors

The different codon-optimised Nef variants along with the wild-type Nef construct are tabulated in **Table 9**. Firstly, the Nef gene containing a mutated myristoylation signal (G2A) and the single D124G mutation ($Nef_{-myrD124}$) was assembled and amplified from synthetic oligonucleotides. This was done using the oligonucleotide assembly amplification method as described for Tat and Rev. Similarly to Tat and Rev, this construct also incorporated optimised contexts for start and stop codons. The assembled $Nef_{-myrD124}$ gene was cloned into the pCMVKm2 plasmid vector over *Sal*I and *EcoR*I sites.

This construct was subsequently used as template to construct the two further variants. The additional LL165AA mutation was induced by PCR-based site-directed mutagenesis to yield a myristoylation-deficient Nef mutant with the D124G and LL165AA mutations designated $Nef_{-myrD124LLAA}$. The site-directed mutagenesis was performed as described for construction of the Tat variants using the primer pairs described in **Table 10**. The third Nef gene construct was generated to encode the native consensus Nef protein. To obtain this construct, the $Nef_{-myrD124}$ plasmid was again used as template for site-directed mutagenesis PCR reactions. Thus, at position 2, the G2A mutation was reversed by normal PCR using a forward primer (Nef+myrF, see **Table 10**), which incorporates a glycine codon at position 2. The PCR conditions and cycles were performed as described for construction of the wild-type *nef* constructs. Subsequently, to construct an optimised *nef* gene coding for the native protein sequence, the D124G mutation was reversed using primer pair NefG123DF and NefG123DR using the QuickChange Site-directed Mutagenesis Kit (Stratagene, CA, USA).

Table 9: The three different codon-optimised *nef* variants with the corresponding primers used to induce site-specific mutations.

Construct	Mutations	Primers
$Nef_{-myrD124}$	G2A, D124G	synthesized directly
$Nef_{-myrD124LLAA}$	G2A, D124G, LL165AA	NefLLAAFor & NefLLAARev
Nef_{OPT}	native consensus protein	Nef+myrF & PCMVRev, NefG123DF & NefG123DR

Table 10: Sequences of the oligonucleotide primers used to induce site-directed mutations to construct *nef* variants. Changed nucleotides are underlined.

Primer	Sequence
NefLLAAFor	AGGGCGAGAACAACTGCGCGGCGCACCCCATGAGCCAGC
NefLLAARev	GCTGGCTCATGGGGTGCGCCGCGCAGTTGTTCTCGCCCT
NefG123DF	CACCCAGGGCTTCTTCCCCGACTGGCAGAACTACACC
NefG123DR	GGTGTAGTTCTGCCAGTCGGGGAAGAAGCCCTGGGTG
Nef+myrF	CTACGAGTCGACGCCACCATGGGCGGCAAGTGGAGC
pCMVRev	GCTGGCAACTAGAAGGCACA

2.4 Constructing the human CD4 expression plasmid

The plasmid pBSKS(+)-hCD4 containing the cDNA of the human CD4 gene (hCD4) was a generous gift from Dr. Gillis Otten (Chiron Corporation, CA, USA). This construct was made by cloning the hCD4 gene into the pBluescript KS(+) (pBSKS(+); Stratagene, CA, USA) cloning vector over the *EcoRI* and *HindIII* restriction endonuclease sites.

The hCD4 fragment was excised from the pBSKS(+)-hCD4 vector by digestion with *EcoRI* and *HindIII* and ligated into the *HindIII*- and *EcoRI*-digested mammalian expression vector pcDNA3.1(-) (Invitrogen, Gröningen, The Netherlands) as described previously. Positive clones were identified by asymmetrical restriction analysis and functional expression of CD4 was determined by transfection into human embryonic liver cells (293), staining with anti-CD4 APC-conjugated antibody and flow cytometry as described under the methods for Nef functionality assays (**Section 2.7.3**).

2.5 Sequencing of expression plasmids

Sequencing reactions were carried out using the ABI Prism BigDye™ Terminator Cycle Sequencing kit (PE Applied Biosystems, CA, USA). Each reaction contained 0.35 - 1µg plasmid DNA and 5 pmoles primer in a reaction mixture containing either 4 µl Terminator Ready Reaction Mix (TRRM; a half reaction) or 8µl TRRM (a full reaction) in a total volume of 10 or 20 µl respectively. Sequencing primers used for the pCR3.1 construct were the T7 and BGHrev sequencing primers (Invitrogen, CA, USA) and are described in **Table 11**. Sequencing of the pCMVKm2 constructs was performed in the sequencing core at Chiron Corporation (Emeryville, CA, USA) using custom made in-house primers. Alternatively, the pCMVKm2 constructs made at the Department of Medical Virology, Tygerberg were sequenced using the primer pair pCMVFor and pCMVRev (**Table 11**).

The cycling reactions were performed using a program consisting of 25 cycles of template denaturing at 96°C for 10 seconds, annealing for 5 seconds at the respective primer annealing temperature (**Table 11**) and elongation at 60°C for 4 minutes. The finished products were immediately purified using the DyeEx Spin Kit following the manufacturer's protocol (Qiagen, GmbH, Germany). Purified extension products were vacuum dried after which they were resuspended in 25µl ABI Prism® Template Suppression Reagent (TSR, Perkin Elmer, CA, USA). Prior to loading samples into the 310 Genetic Analyzer (Perkin Elmer, CA, USA), the resuspended DNA was transferred to Genetic Analyzer tubes (ABI Prism™) and the DNA was denatured at 90°C for 5 minutes and immediately placed on ice. Analysis was done on an ABI Prism® 310 Genetic Analyzer (Applied Biosystems) using Performance Optimised Polymer 6, a 47 cm capillary, a polymer temperature of 50°C, injection time of 45 seconds and a gel run time of 50 minutes (Perkin Elmer, CA, USA).

Table 11: Sequences and annealing temperatures of the oligonucleotide primers used to sequence all constructs made at Tygerberg.

Primer	Sequence	Annealing temperature
T7forward	TAATACGACTCACTATAGGG	45ºC
BGHreverse	TAGAAGGCACAGTCGAGG	50ºC
pCMVFor	AGTCTGAGCAGTACTCGTTG	50°C
pCMVRev	GCTGGCAACTAGAAGGCACA	53°C

Sequence analysis

Overlapping sequences were assembled using the DNAMAN ver. 4.0 Package (Lynnon Biosoft), multiple alignments were performed with CLUSTALX ver. 1.81 (Thompson *et al.*, 1997; Higgins *et al.*, 1996) and translation into the deduced amino acid sequences and layout of multiple sequence alignments were performed with GENEDOC ver. 2.6.001 (Nicholas and Nicholas, 1997) and verified manually.

2.6 Generation of mouse sera by immunisation with naked plasmid DNA

Background

Because of very limited availability of accessory protein-specific antibodies representing any subtype and especially subtype C, alternative means to obtain the necessary reagents were required. It was thus decided to generate polyclonal antibody serum by immunising mice with the Tat, Rev and Nef expression constructs.

Preparation of plasmid DNA

Plasmid DNA was prepared using the Qiagen Endo-Free Plasmid Giga Kit (Qiagen GmBH, Germany, **Appendix G**). One litre of Luria Broth containing 50µg/ml Kanamycin was inoculated with 1ml of the respective starter culture and incubated at 37°C for 16 hours with vigorous shaking. The manufacturer's protocol was followed and the DNA was resuspended in nuclease-free water (Promega, MD, USA). DNA concentrations and purity were measured using a spectrophotometer and confirmed by agarose gel electrophoresis. To calculate the concentration and purity of a DNA solution, the optical densities of the solution at the wavelength of 260nm (OD_{260}) and 280nm (OD_{280}) were determined. Using the following two formulas, the concentrations and the purity of the plasmid DNA were calculated.

DNA concentration (µg/µl): [DNA] = OD_{260}/20 X dilution factor

plasmid DNA purity: purity factor = OD_{260}/OD_{280}

The DNA was diluted to 1 µg/µl for further use and only DNA with a purity factor within the range of 1.7 to 1.9 was suitable for immunisation.

Immunisation

Six to eight week old female CB6F1 mice (10 mice per group, n=10) were immunized with the respective plasmid DNA coding for the HIV-1 subtype C accessory/regulatory proteins Tat, Rev and Nef (**Table 11**). The dose of 75µg plasmid DNA in 100µl 0.9% sterile endotoxin-free saline solution (Sigma-Aldrich GmbH, Taufkirchen, Germany) per mouse was divided into two equal dosages, each of which was administered into either *tibia anterialis* muscles. Handling of the animals, immunisations and serum collection was performed by animal house technicians at the animal facility at Chiron Corporation. The mice were immunised twice, with a 4-week interval and sera were collected by cardiac puncture 2 weeks after the second immunisation and pooled for each immunogen group.

Table 11: Plasmid DNA constructs used to immunise female CB6F1 mice to generate HIV-1 subtype C Tat, Rev and Nef-specific polyclonal antibody sera.

Immunogen	Plasmid vector	Viral strain
Tat_{C22C37}	pCMV-Km2	TV consensus
Rev_{M5M10}	pCMV-Km2	TV consensus
$Nef_{-myrD124}$	pCMV-Km2	TV consensus

2.7 *In vitro* expression in human cells

2.7.1 DNA preparation

Plasmid DNA was prepared using either the QIAfilter Plasmid Maxi or Midi Kit (Qiagen GmBH, Germany, **Appendix H**). One hundred and fifty (Maxi) or fifty (Midi) millilitres of Luria Broth containing the appropriate selective pressure antibiotic (kanamycin or ampicillin; Ranbaxy, (Pty) Ltd, South Africa) at a concentration of 50µg/ml were inoculated at a volume ratio of 1:500 with the respective starter culture and incubated at 37°C for 16 hours with vigorous shaking. The manufacturer's protocol was followed to extract plasmid DNA and the DNA was resuspended in distilled water. DNA concentrations and

purity were measured by reading the absorbance at 260 and 280nm as described previously and confirmed by agarose gel electrophoresis. Plasmid DNA was diluted to 1µg/µl and had to fall within the purity factor range of 1.7 to 1.9.

2.7.2 *In vitro* expression assays

Maintenance of mammalian cells

To assess the expression of the accessory gene constructs, *in vitro* expression assays in mammalian cells were done. Human embryonic kidney (293, American Type Tissue Collection [ATCC, Atlanta, Ga] no. 45504 or National Institute of Health [NIH, Rockville, MD, USA] no. 103) cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, Rockville Md, USA) containing 10% fetal calf serum (FCS, Delta Bioproducts, Kempton Park, RSA), 100mg/L penicillin (Novo-Pen, Novo Nordisk (Pty) Ltd, Johannesburg, RSA) and 100mg/L streptomycin (Novo-Strep, Novo Nordisk (Pty) Ltd) (designated 10% DMEM) at 37°C, 95% humidity and 5% CO_2.

Transfections

When cells had grown to a confluence of approximately 70%, they were treated with 0.25% active trypsin versene (ATV; 2.5g/L trypsin, 0.38g/L EDTA, 0.4 g/L KCl, 8 g/L NaCl, 0.35 g/L $NaHCO_3$, 0.01g/L Phenol Red) until they detached from the flask sufficiently and washed once in 10% DMEM. Live cells stained with 0.4% trypan blue (Gibco, Invitrogen, CA, USA) were counted and cells were plated one day prior to transfection at a cell density of 1.4 X 10^6 cells per 35mm-diameter well (Corning, Schiphol-Rijk, The Netherlands) in 2ml 10% DMEM. For transfections, the 10% DMEM was replaced with serum-free medium (OPTImem I Reduced Serum Medium, Gibco, Rockville Md, USA). Cells were transfected with 2µg of plasmid DNA mixed with 10µl Mirus TransIT-LT1 polyamine transfection reagent (Panvera, Madison, WI, USA) in 100µl OPTImem per well. Transfected cells were incubated for five hours after which the OPTImem was replaced with 10% DMEM.

Cell lysate harvesting

Twenty-four hours posttransfection the cells were washed twice in sterile phosphate buffered saline (PBS) and then lysed with Reporter Gene Assay Lysis Buffer (Roche, Mannheim, Germany) following the manufacturers protocol (**Appendix I**). Cell lysates were frozen at –70°C or used immediately.

2.7.3 Western Blotting

Electrophoresis and transfer

Eighteen microlitres of 293-cell lysate or 10µl of Full-Range Rainbow Marker (Amersham-Pharmacia Biotech, Buckinghamshire, UK), added to 2.5µl NuPage Sample Reducing Agent and 6.5µl NuPage SDS Sample Buffer, were loaded on a NuPage Novex 4-12% BisTris SDS gel (Invitrogen, Groningen, The Netherlands). Electrophoresis was performed in NuPage MOPS SDS running buffer containing 0.1% NuPage Antioxidant at a constant current of 115mA until the running front of the sample buffer had reached the bottom of the gel. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane (0.2µm pore size, Invitrogen) in NuPage Transfer Buffer containing 10% Methanol at 150mA constant current for 60 minutes.

Blotting

Nitrocellulose membranes were rinsed in 1X PBS and blocking was performed at 4°C for 16 hours in 1X PBS containing 0.1% Tween and 0.3% Bovine Serum Albumin (BSA) Diluent/Blocking Solution (KPL, MD, USA). Blotting was performed in the above mentioned blocking solution containing 0.0025% 293 cell extract, prepared by lysis of untransfected 293 cells as described, as well as the previously prepared respective polyclonal mouse serum at a dilution of 1:250 (Section 2.5). As no positive protein control was available for Nef, these lysates were also blotted with subtype B (HIV-$1_{JR\text{-}CSF}$) Nef-specific monoclonal antibody (NIH AIDS Research and Reference Reagent Program) at a dilution of 1:1000. The secondary antibody in the same blocking solution containing 293 cell extract was horseradish peroxidase (HRP)-conjugated Goat anti-Mouse IgG (Zymed, San Francisco, USA) at a dilution of 1:10 000. The protein bands were visualized by chemo-luminescence using ECLPlus and

2.8 Functionality Assays

2.8.1 TAT

Background

To determine the degree to which Tat mutants are able to perform the function of transactivating the HIV-1 LTR, a transient reporter assay in which a reporter plasmid pLTR-CAT previously described by Peterlin *et al.* (1986) was cotransfected with *tat* variant plasmids as described by Kuppuswamy *et al.* 1989 and Neuveut *et al.*, 1996. The plasmid pLTR-CAT contains the reporter gene chloroamphenicol acetyl transferase (CAT) under the promoter control of the amended HIV-1 LTR and with the SV40 polyadenylation signal (pA). The HIV-1 5' LTR was amended by deletion of selected promoter elements to leave the HIV enhancer, Sp1 binding sites, TATA box and TAR sequences, rendering CAT expression from the pLTR-CAT plasmid virtually exclusively Tat dependent (Peterlin *et al.*, 1986). **Figure 12** depicts the pLTR-CAT reporter plasmid. Cotransfection of Tat with the pLTR-CAT reporter plasmid thus provides a good system in which the level of CAT expression gives a quantitative measure of the ability of that Tat protein to transactivate transcription from the HIV-1 LTR in a subgenomic transient system.

Functionality assay

RD, COS-7 and HeLa cells were plated one day prior to transfection at a density of 1 X 10^6 cells and 293T at 1.4 X 10^6 cells per 35mm well. Cells were cotransfected with 1µg *tat* plasmid DNA and 1µg pLTR-CAT reporter plasmid DNA (and 0.5µg pSV-βGal (Promega, Madison, WI) internal control vector for assays in HeLa cells) per 35mm well as described previously. Transfections were always done in duplicate wells. Twenty-four hours posttransfection the cells were washed with chilled PBS and lysed using 1X CAT ELISA lysis buffer as described by the manufacturer (Roche Diagnostics, GmbH, Germany). CAT enzyme levels in cell lysates were determined using a CAT ELISA and following the manufacturer's protocol (Roche Diagnostics, GmbH, Germany, **Appendix I**). Transfection efficiency was controlled for by assaying for β-galactosidase activity using the β-Galactosidase Enzyme Assay (Promega, Madison, WI), following the manufacturer's protocol (**Appendix K**).

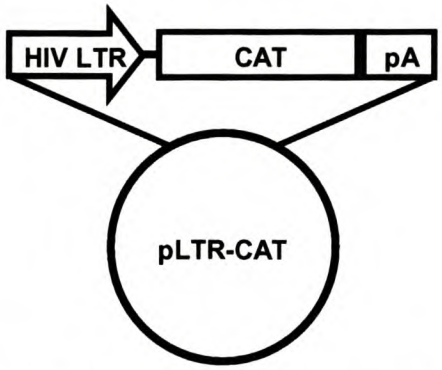


Figure 12: Diagrammatic representation of the pLTR-CAT reporter plasmid. The chloroamphenicol acetyl transferase (CAT) gene is under the promoter control of the Tat-dependent HIV-1 LTR and the simian virus 40 polyadenylation signal (pA).

2.8.2 REV

Background

Functional intactness of the Rev variants was determined by detecting the ability of the Rev proteins to export unspliced or partially spliced rev-responsive element (RRE)-coupled chloroamphenicol acetyl transferase (CAT) transcripts from the cell nucleus to the cytoplasm. This assay has been used extensively to determine the functional activity of HIV-1 Rev and was first described by Hope *et al.* (1990a). The reporter plasmid pDM128 was a kind donation from Prof. Thomas Hope (Chicago College of Medicine, University of Illinois, Chicago, IL, USA). The pDM128 plasmid contains the CAT gene, which was inserted into the truncated HIV-1 genome, upstream of the HIV-1 RRE (Hope *et al.*, 1990a). This entire transcription unit, which is flanked by splice donor and acceptor sites was inserted downstream of the simian virus 40 (SV40) promoter/enhancer and subcloned into the pUC18 plasmid to produce pDM128 (**Figure 13**). Cells transfected with pDM128 alone translate only fully spliced transcripts in the cytoplasm and thus yield only trace levels of CAT enzyme as the cellular splicing events have cleaved the CAT coding region from the promoter/enhancer elements. However, cotransfection with a functional Rev expression vector allows Rev-mediated nuclear export and subsequent expression of unspliced or partially spliced transcripts, increasing CAT levels 75-100 fold (Hope *et al.*, 1990a).

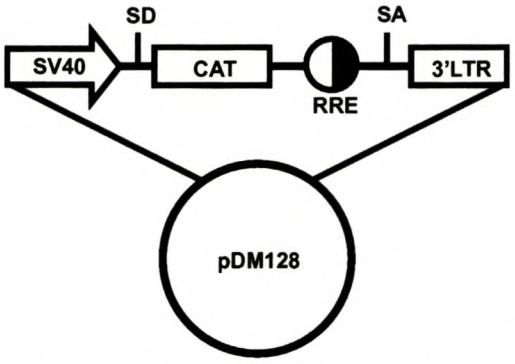


Figure 13: Diagrammatic representation of the pDM128 reporter plasmid. The rev-responsive element (RRE)-coupled transcription unit contains the CAT reporter gene under the control of the SV40 promoter and the HIV-1 transcription terminator, the 3'LTR. The splice donor (SD) and acceptor (SA) sites are indicated.

Functionality assay

The pSV-βGal reporter plasmid (Promega, Madison, WI), which expresses the enzyme β-galactosidase under the control of the Rous sarcoma virus (RSV) promoter, was used as internal transfection control. 293T cells were cotransfected with 0.5µg pSV-βGal, 1µg pDM128 and 1µg of the respective *rev* variants in duplicate 35mm wells as described before. Cell lysates were harvested 24-hours posttransfection and CAT expression was determined using a CAT ELISA as described before. Transfection efficiency was controlled for by assaying for β-galactosidase activity using the β-Galactosidase Enzyme Assay (Promega, Madison, WI), following the manufacturer's protocol. The assay was done is triplicate.

2.8.3 NEF

Background

As reviewed in chapter 1, the functional activity of Nef is multipronged and mediates several cellular events. Here, the CD4 and MHC-I down-regulation activities of Nef were determined using an assay previously described (Peng and Robert-Guroff, 2001; Goldsmith *et al.*, 1995). As 293 cells do not naturally express CD4 on their cell surface, cells were transfected with a CD4 expressing plasmid to determine whether a cotransfected Nef variant would be able to downregulate the level of surface CD4 expression.

Functionality assay

293 cells were cotransfected with 0.5µg of the human CD4 expressing plasmid pcDNA_hCD4 and 1µg Nef expression plasmid DNA per 35mm well as described previously. Forty-eight hours posttransfection, the cells were washed with chilled PBS and treated with 2% EDTA to detach the adherent cells from the plate surfaces. Subsequently, the cells were washed in PBS containing 0.25% BSA (FACS staining buffer, SB) and stained with allophycocyanin (APC)-conjugated anti-human CD4 antibodies and R-phycoerythrin (PE)-conjugated anti-HLA-A,B,C monoclonal antibody (BD Biosciences, San Diego, CA, USA) specific for MHC class I, which is naturally expressed on the surface of 293 cells. Analysis of PE (second fluorescence) and APC (fourth fluorescence) fluorescence was performed on 100 000 cells (events) gated by side scatter and forward scatter on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems).

CHAPTER 3: RESULTS

3.1 Design and construction of expression plasmids

Codon-optimisation and sequence analysis

The codon usage of the gene constructs encoding the HIV-1 subtype C consensus Tat, Rev and Nef proteins was optimised for high-level expression in mammalian tissues by altering it to that of highly expressed human genes as described by Haas *et al* (1996b) and Andre *et al.* (1998). Alignments of the nucleotide and amino acid sequences of all expression constructs are attached in **Appendix A**. The percent GC contents of the wild-type and codon-optimised *tat*, *rev* and *nef* genes are plotted along their coding sequences in **Figure 14**. As can be clearly distinguished, a marked increase in percent GC content was observed after codon optimisation across the entire coding regions of the three genes. Taken as a mean value for each reading frame, the percentage GC content was raised from an approximate level of 50% in the wild-type genes to approximately 70% for codon-optimised *tat*, *rev* and *nef* genes (**Table 12**). These levels are consistent with increased mRNA stability and translation efficiency (Haas *et al.*, 1996b; Hentze, 1991).

Table 12: Levels of %GC content for the wild-type and codon-optimised genes.

	% GC content		Difference
	wild type	codon optimised	
Tat	48%	67%	19%
Rev	52%	74%	22%
Nef	47%	68%	21%

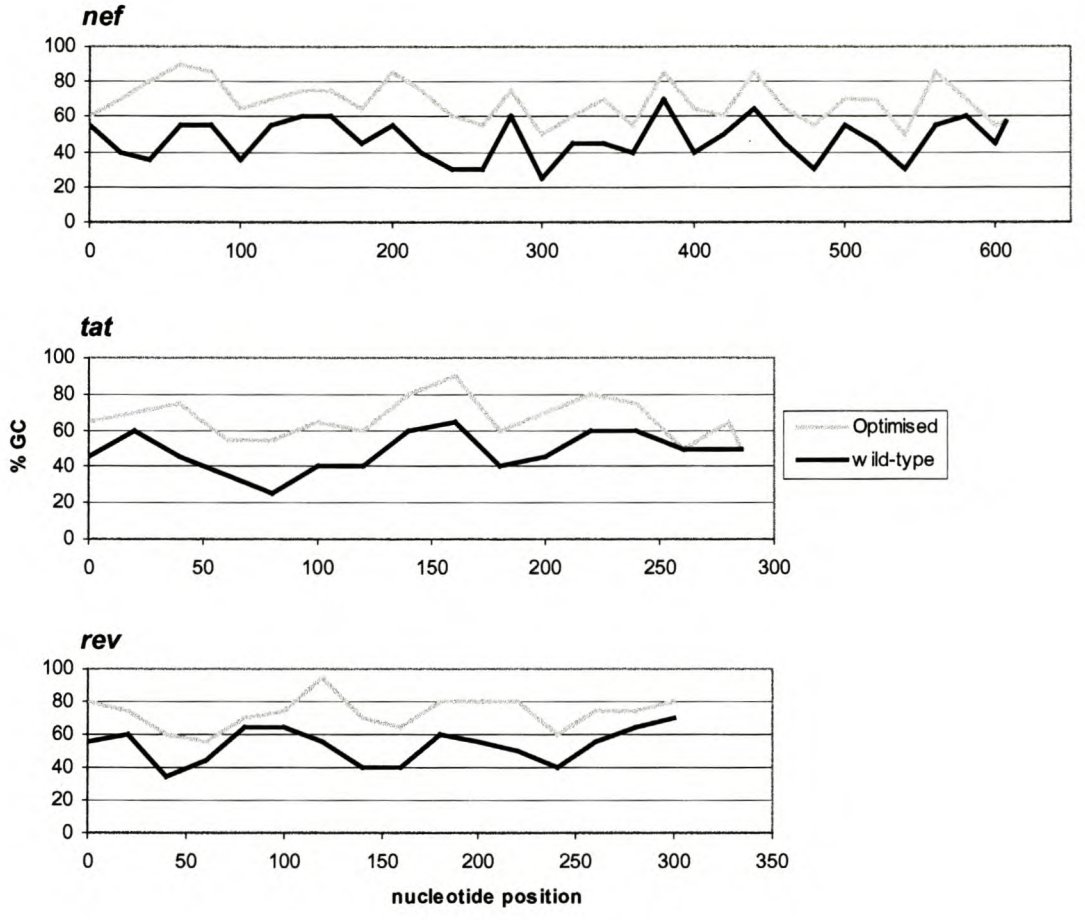


Figure 14: Comparison of GC content percentages between the wild-type (black) and codon optimised (grey) *nef*, *tat* and *rev* genes at a step size of 20 nucleotides along the coding sequences. Calculation of the percentages was done manually.

Tat, Rev and Nef amino acid distances between the fourteen HIV-1 subtype C isolates from Tygerberg Virology (TV) that were sequence characterised and the South African subtype C TV consensus (TV Cons) were calculated using two different protein distance models (Kimura and PAM) as described in Materials and Methods. The distances between each viral isolate and the consensus sequence were plotted for each protein in **Figure 15**. Although small differences were distinguishable between the two distance models, the amino acid distances calculated using the Kimura and PAM models between each isolate and the TV Cons correlated well. The viral isolate that was least dissimilar from the South African consensus (TV Cons) was selected as wild-type for the Tat (TV019) and Nef (TV002) constructs.

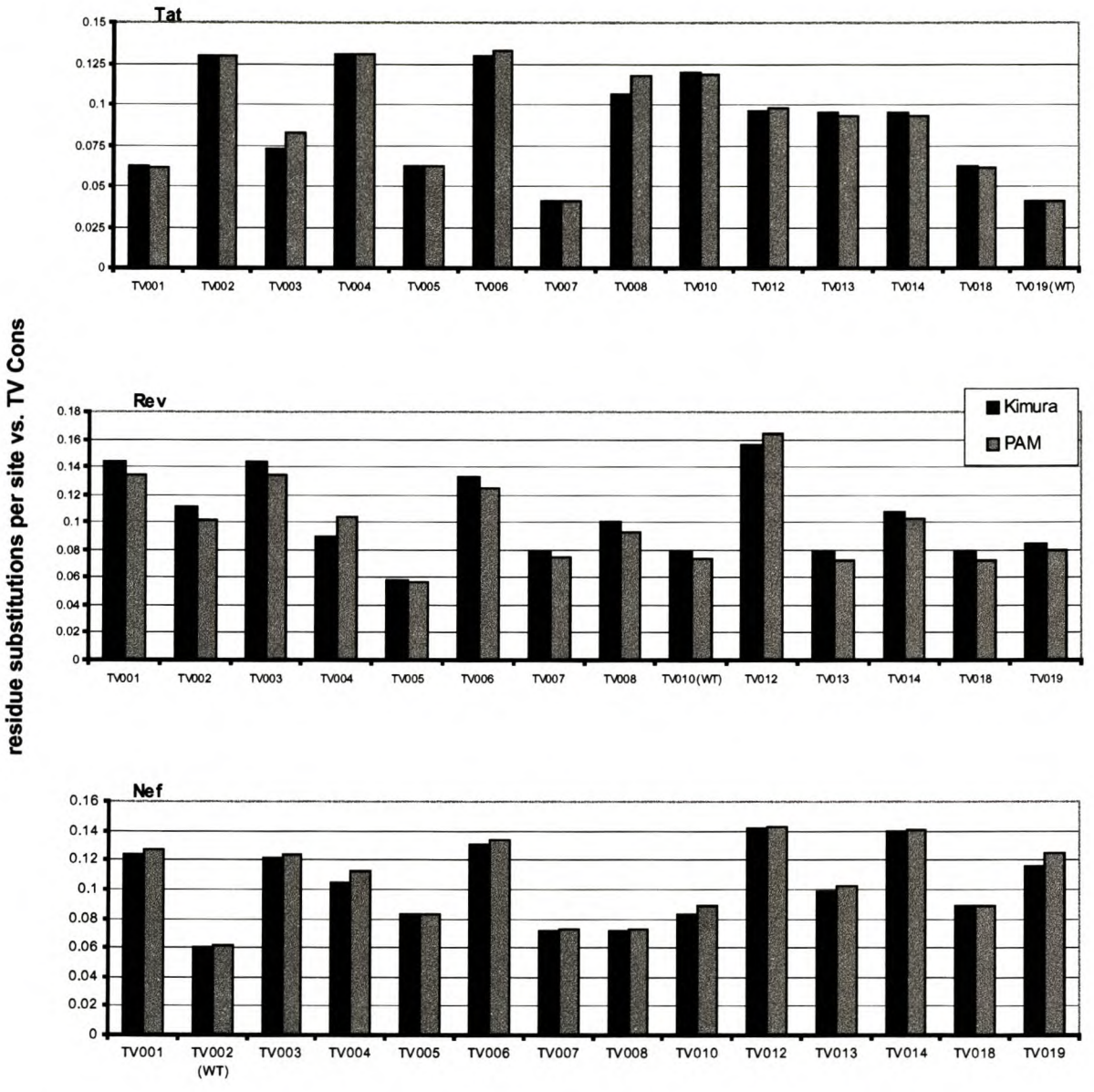
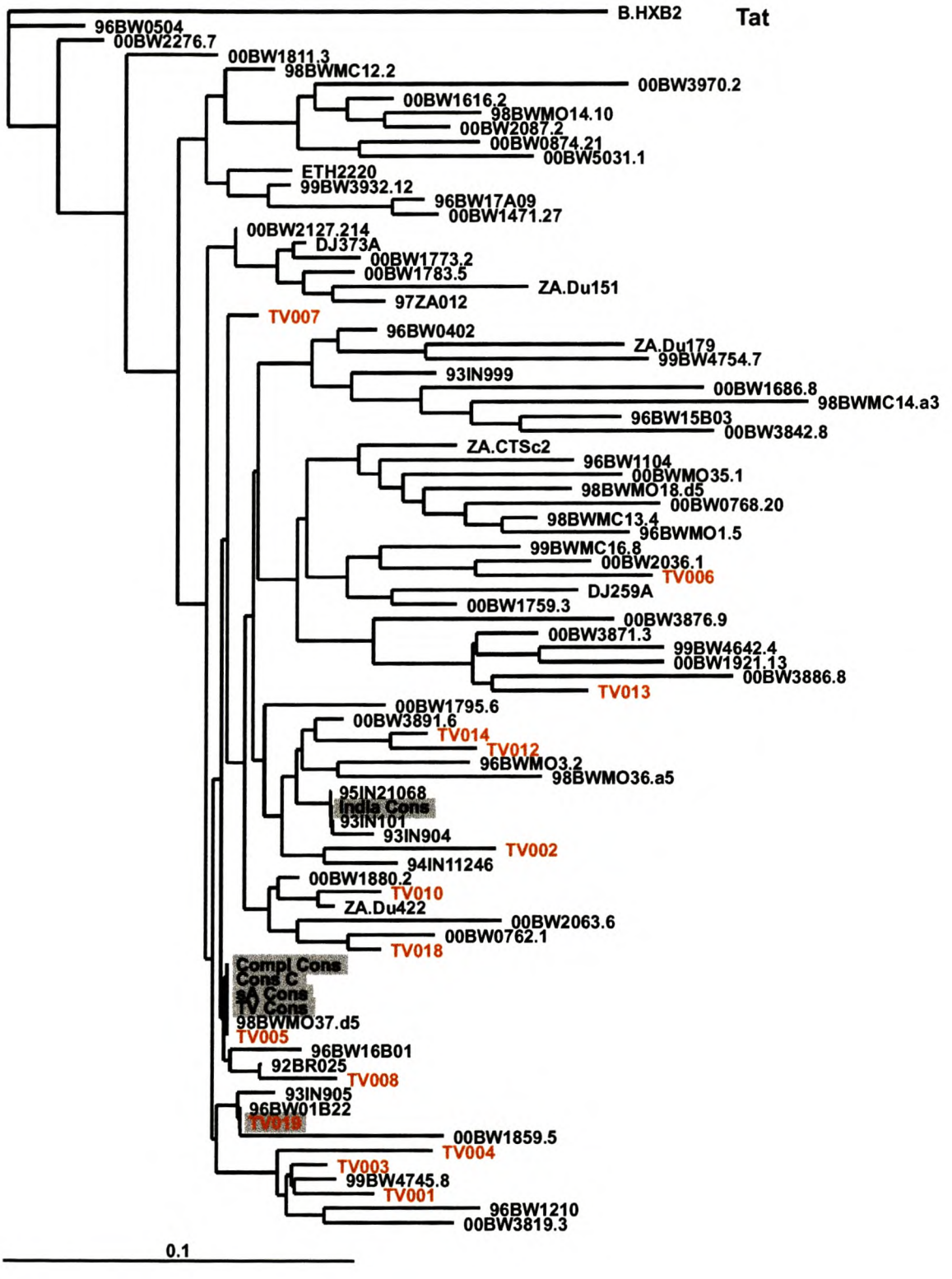
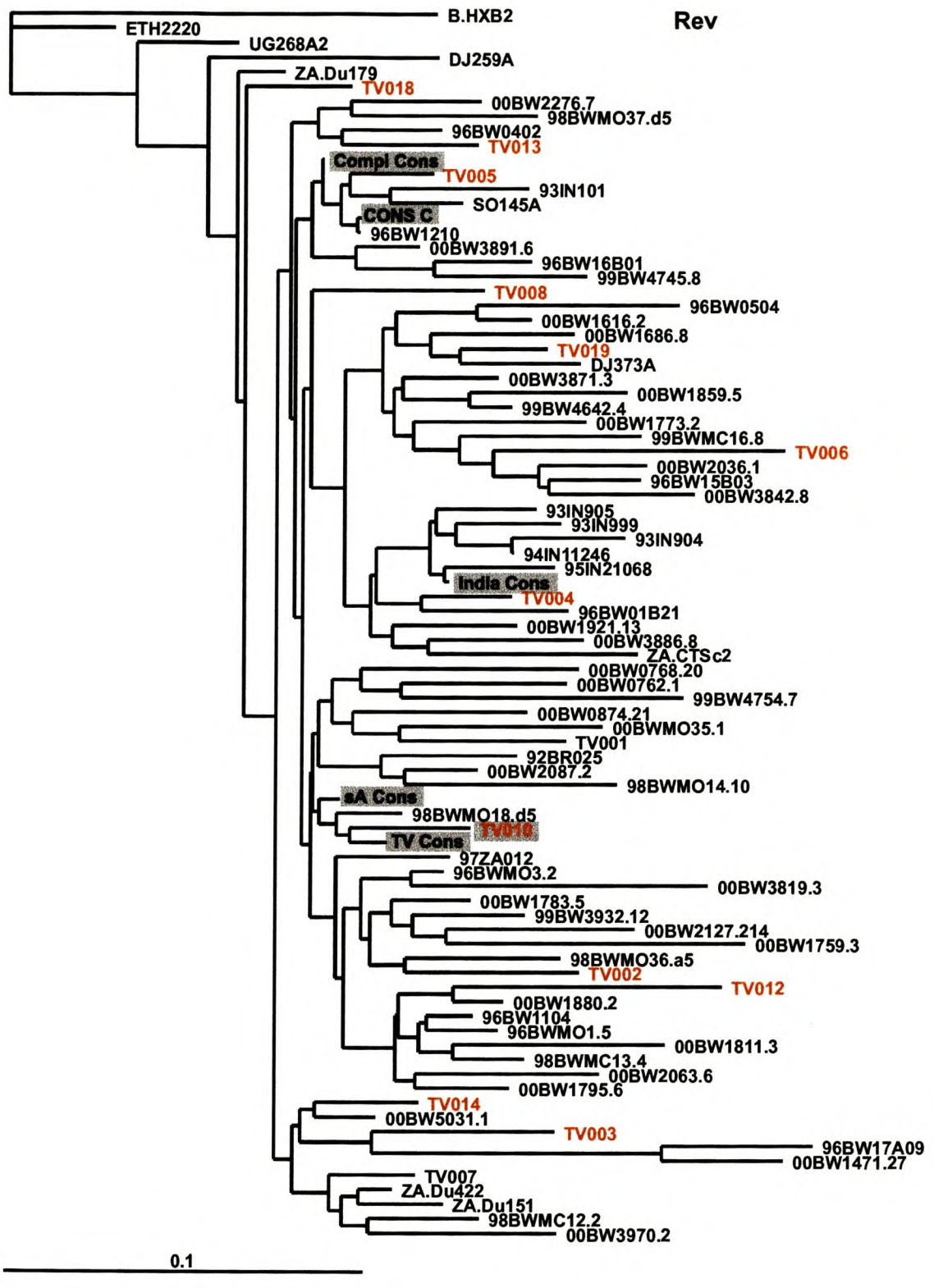


Figure 15: Amino acid distances between South African subtype C viral isolates from Tygerberg Virology (TV) and the consensus sequence drawn up from them (TV Cons) for the Tat, Rev and Nef proteins. Genetic distances were calculated using the PHYLIP program PROTDIST and the results obtained from the two distance models Kimura and PAM are compared. The TV isolate, which was selected for construction of the wild-type construct for the respective gene, is indicated with a "WT".

Although isolate TV005 displayed the least dissimilarity relative to TV Cons for its Rev protein, this isolate did not contain the characteristic subtype C Rev truncation (**Appendix C**; (Scriba *et al.*, 2002)) and was thus not selected as wild-type *rev* because it did not encode a conventional subtype C Rev protein. Several isolates including TV007, TV010, TV013 and TV018 displayed low dissimilarity to TV Cons and additional analyses had to be performed to differentiate between them in order to chose a wild-type candidate (see below).

Subtype B HXB2-rooted phylogenetic trees representing the evolutionary relationships of the Tat, Rev and Nef proteins between the South African subtype C TV isolates and all reference subtype C protein sequences in the HIV sequence database as well as additional sequences from Botswana, India, South Africa and other geographic origins were constructed (**Figure 16**). Five consensus sequences representing the TV isolates (TV Cons), southern African (Botswana and South Africa) sequences (sA Cons), Indian isolates (India Cons), the subtype C consensus available from the Los Alamos HIV sequence database (CONS C) as well as one representing all these subtype C sequences (Compl Cons) were drawn up as described in Materials and Methods and were also included in the trees. The Phylip PROTDIST program with the Kimura distance matrix (Kimura, 1980) was used to calculate genetic distances and the tree topologies were inferred using the Phylip package neighbor program. All three phylogenetic trees share the following properties: the TV isolates and other South African sequences were dispersed across the entire tree topologies; sequences from India (including the India Cons) formed monophyletic clusters with single exceptions; and non-Indian consensus sequences clustered closely together. Specifically, the TV Cons, sA Cons, Compl Cons and CONS C sequences clustered as monophyletic clades in the Tat and Nef trees. The Rev tree showed similar clustering of the TV Cons and sA Cons, while the Compl Cons and CONS C clustered from a different branch along with four viral isolate sequences. The consensus sequences fall closer to the "backbone" of the three trees than virtually any single sequence, demonstrating similarity to the so-called "ancestral sequence", which would represent the ancestor of the HIV-1 subtype C. The wild-type TV isolates fall relatively close to the TV Cons in the three trees and notably TV010 falls into the TV Cons and sA Cons cluster in the Rev tree, warranting its selection as wild-type Rev over the other similar isolates.





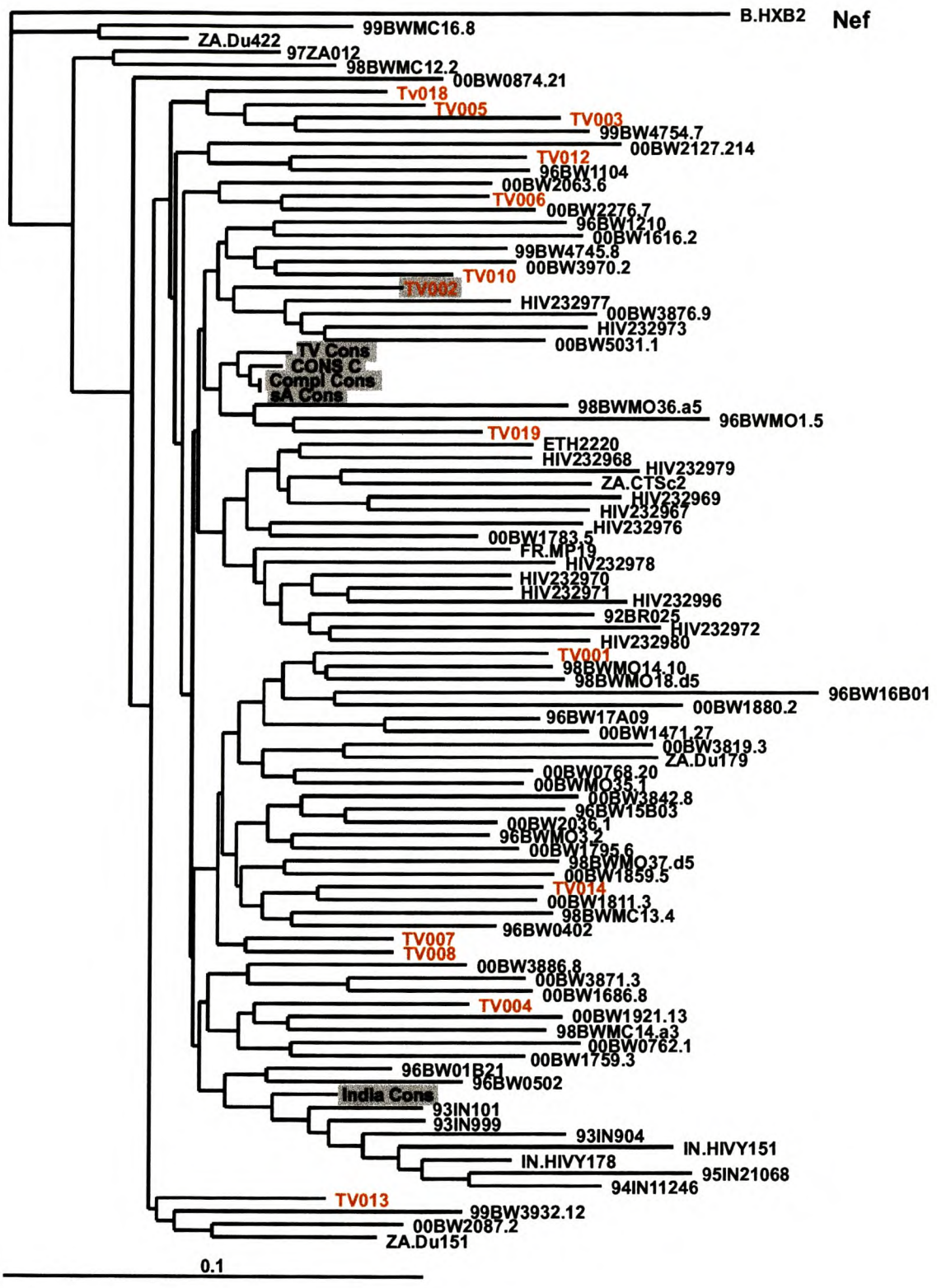


Figure 16: Phylogenetic trees constructed with Tat (page 65), Rev (page 66) and Nef (page 67) amino acid sequences representing viruses from Tygerberg Virology (TV), additional South African, Botswana, India and other geographic origins. The TV isolates are in red and the five consensus sequences and TV isolate selected for construction of the wild type plasmids are in boldface and boxed in grey. The trees are rooted with the subtype B reference sequence HXB2 and horizontal branch lengths represent genetic distance. Vertical branches are for clarity only and distance scales represent 0.1 residue replacements per site.

Of mention is the common phenomenon of short "backbone" branches compared to the terminal branches of the Tat, Rev and Nef trees. These short "backbones" accompany weak bootstrap support of the tree branching implying unreliable topologies. For this reason, only single bootstrap values represented significance and these were omitted from the phylograms as they were all towards the terminal branches of the trees. Nevertheless, the trees give a good representation of genetic distance between viral strain and consensus sequences and demonstrate the dispersed nature of Tat, Rev and Nef protein sequences from subtype C viruses originating from southern Africa.

The amino acid distances between the TV Cons and the four other consensus sequences as well as the isolate chosen as wild-type were determined using the Kimura and PAM distance models and are plotted in **Figure 17**. As supported by both models, the distance plots for Tat, Rev and Nef, which are represented as single stacked bars for each sequence, show that the stacked TV Cons, on which the Tat, Rev and Nef immunogens in this study are based, is least dissimilar to the consensus representing sequences from southern Africa (sA Cons). Significantly, these two consensus sequences differed by only single amino acid residues for Tat and Nef. The overall ascending order of dissimilarities of the different consensus sequences to the TV Cons were sA Cons < Compl Cons < CONS C < India Cons. Notably, the stacked distances representing the wild-type TV isolates, which were selected on the grounds of being the closest individual isolate sequence to the TV Cons, were only smaller than those of the India Cons, and markedly more distant than the sA Cons and Compl Cons sequences. Amino acid distances of the Tat and Nef proteins were quite similar while Rev displayed more distance and thus dissimilarity than Tat and Nef.

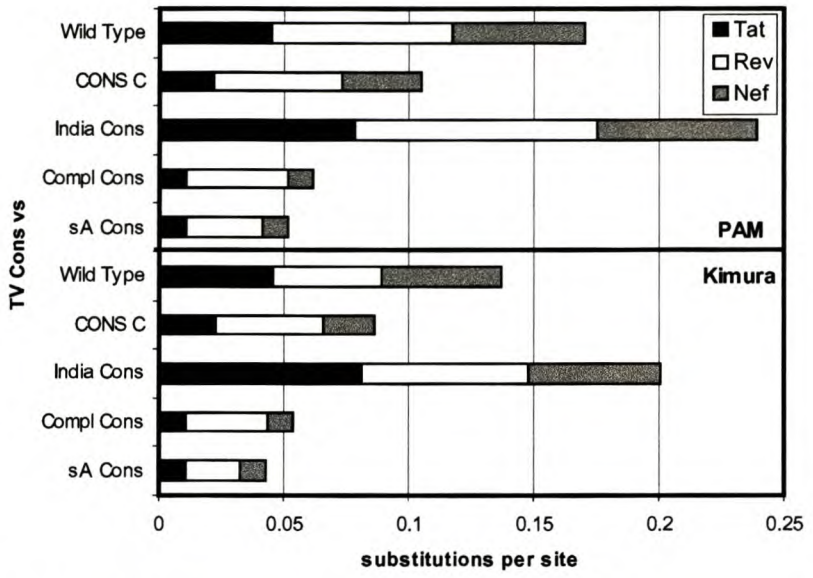


Figure 17: Stacked amino acid distances calculated with the Kimura and PAM distance models between the TV consensus (TV Cons), which was used for construction of the codon-optimised Tat, Rev and Nef variants, and four other consensus sequences as well as the respective isolates selected for the wild-type constructs. See above for description of various consensus sequences.

Figure 18 shows the percentage similarities of the Tat, Rev and Nef protein sequences of the fourteen individual TV isolates versus the TV Cons (red lines) and the respective viral isolate chosen as wild-type (black lines) versus the fourteen individual TV isolates along the alignments of the proteins. Although not dramatic, all three plots demonstrate that the TV Cons is on average more similar to individual protein sequences than the viral isolate sequence of the wild-type strains is. It should be noted that the wild-type sequences were chosen on the grounds of being most similar to the TV Cons which means that if other individual isolate sequences were compared as query sequence, possibly even less similarity would be displayed.

This phenomenon is more clearly illustrated when the amino acid distances of the consensus sequences TV Cons and sA Cons or the individual HIV-1 subtype C isolates from Tygerberg Virology (TV isolates) versus the panel of fourteen TV isolates were calculated for each protein with the Kimura distance matrix. The values of the distances between each sequence and the panel of 14 TV sequences were added together for Tat, Rev and Nef and are illustrated as stacked bars in **Figure 19**. As is clearly evident, the two consensus sequences TV Cons and sA Cons display markedly less sequence

dissimilarity to the fourteen TV isolates than any individual TV isolate does for Tat, Rev and Nef as stacked and individual bars.

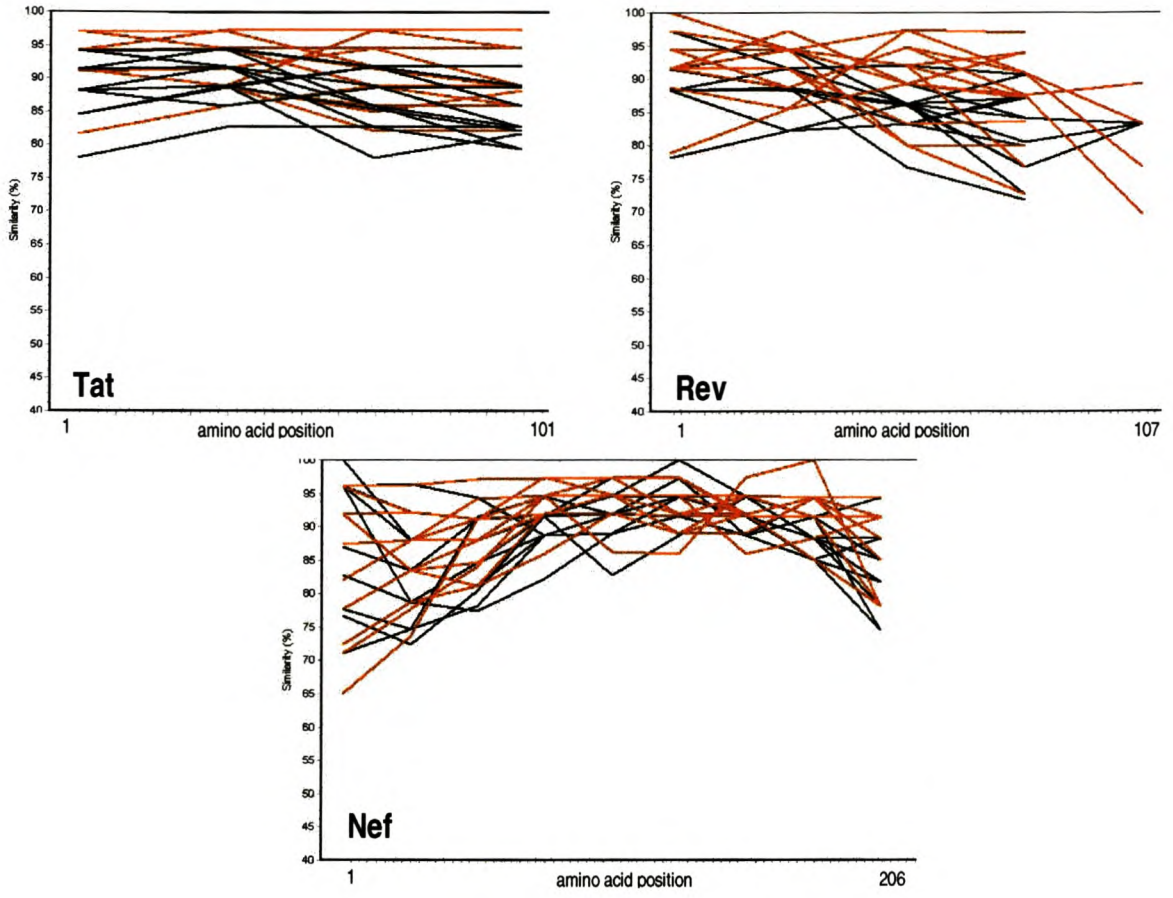


Figure 18: Similarity plots of Tat, Rev and Nef amino acid sequences in which the percentage similarity of the TV Cons versus TV isolates (red lines) and wild-type sequence (Tat: TV019. Rev: TV010, Nef: TV002) versus TV isolates (black lines) are compared. The program SIMPLOT was used with step sizes of 20 for Tat and Rev and 40 for Nef and a window size of 40. Gaps were excluded from the alignments.

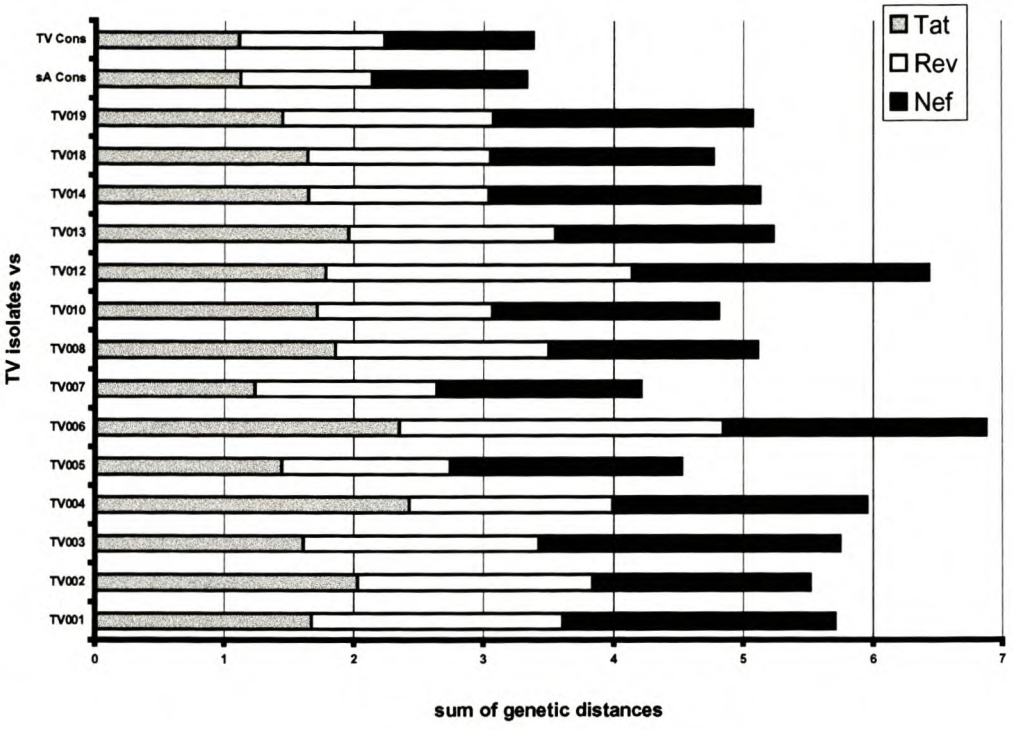


Figure 19: Stacked bar plot illustrating the sum of genetic distances between the Tat (grey bars), Rev (white bars) and Nef (black bars) amino acid sequences of the indicated isolate or consensus sequence and the panel of fourteen HIV-1 subtype C isolates from Tygerberg Virology (TV isolates). Amino acid distances were calculated with the Kimura distance matrix.

3.2 Expression of proteins

In vitro expression of all plasmid constructs in 293 cells was assessed by sodium dodecylsulphate (SDS) gel electrophoresis and Western blotting. Cells were transfected with 2µg plasmid DNA per 35mm well as described and cell lysates were harvested 24-hours posttransfection.

3.2.1 Tat

In vitro expressed Tat protein was detected by immunoblot analysis using mouse serum that was generated by immunisation of CB6F1 mice with plasmid DNA of construct pCMVKm2-Tat_{C22C37}. The polyclonal mouse serum was immunoreactive with single protein bands in Tat-transfected 293 cell lysates that were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE; **Figure 20**). No corresponding protein band was immunoreactive in

mock-transfected 293 cell lysates. A comparison of the expression levels of the four codon-optimised Tat variants is shown on blot A in **Figure 20**. As can be seen, the band intensities for the four Tat proteins are virtually identical, indicating very similar expression levels. In addition, the codon-optimised Tat proteins migrated at the same rate, which is consistent with these expression constructs encoding reading frames of identical length (101 amino acids). A comparison of expression levels of the codon-optimised *tat* constructs with the plasmid construct encoding the wild type Tat protein of subtype C isolate TV019 is shown on blot B in **Figure 20**. The Tat_{WT} (101 amino acids) band co-migrated at the same rate as the codon-optimised Tat proteins. Although it appears as if the wild-type Tat protein band is somewhat fainter than those of the codon-optimised Tat proteins as also exhibited on Blot C in **Figure 21**, the observed difference is not substantial enough to note a significant difference in expression levels.

To determine whether the mouse serum would be immunoreactive to a previously described Tat protein, cell lysates were electrophoresed along with Tat protein obtained from the NIH AIDS Research Reference and Reagent Program (**Figure 21**). Although this protein is encoded by the HIV-1 subtype B virus HXB2 (Ratner *et al.*, 1985), the subtype C Tat-specific serum was reactive to three separate, albeit somewhat smudged protein bands.

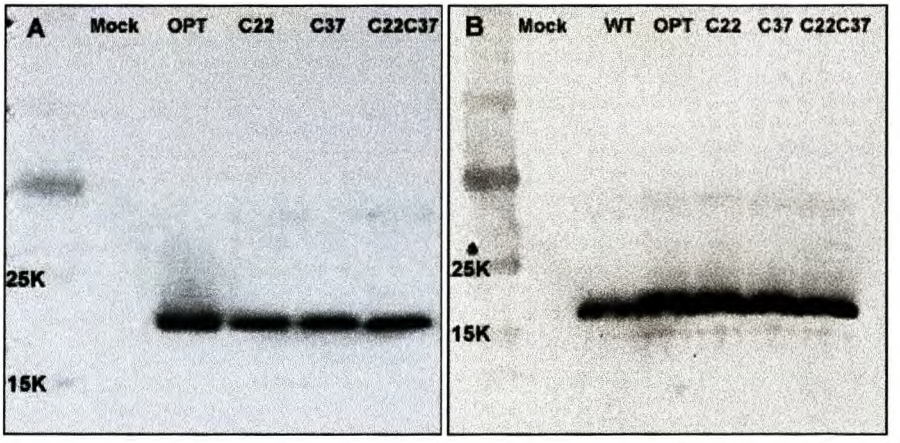
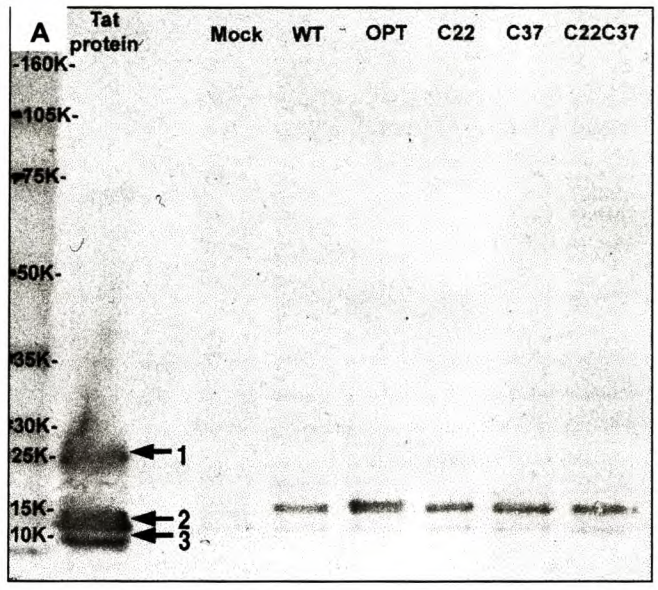
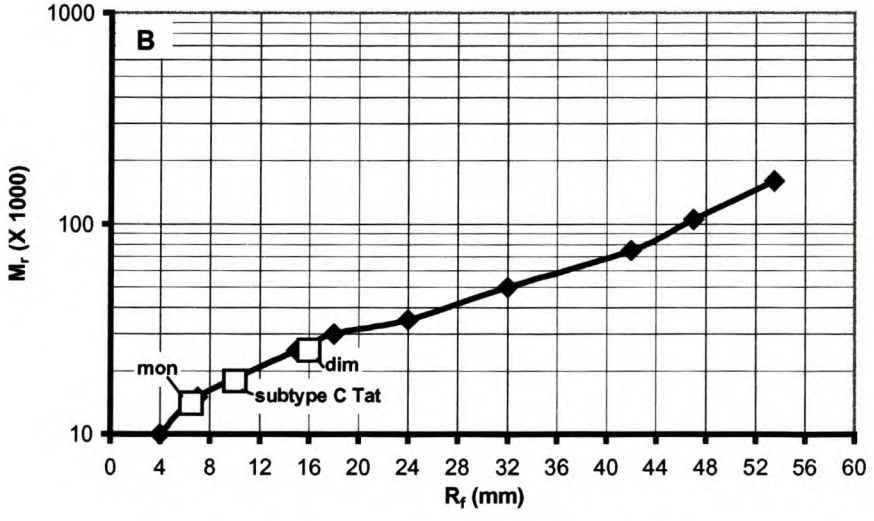


Figure 20: *In vitro* expression of Tat plasmid constructs and mock-transfected cells as detected by immunoblotting with Tat-immunised mouse serum. Blot A displays expression levels for the four codon-optimised Tat variants Tat_{OPT}, Tat_{C22}, Tat_{C37} and Tat_{C22C37}. Blot B shows the comparative expression level between the wild-type Tat construct Tat_{WT} and the codon-optimised Tat Variants.





C

	R_f (mm)	M_r X 10^3 (kDa)
Tat protein band (1) - dim	16.5	25
Tat protein band (2) - mon	5.5	14
Tat protein band (3)	co-migrated with 10kDa marker band	10
Subtype C Tat	8.5	17.8

Figure 21: Calculation of Tat protein size by plotting the molecular weight (M_r) versus the relative mobility (R_f) of the Full-Range Rainbow Molecular Weight Marker. (**A**) The Tat protein control yielded 3 bands indicated on the blot by the arrows marked 1 – 3. (**B**) The M_r of the different subtype C Tat variants were calculated from the distances migrated and are plotted on the standard curve as hollow squares (mon, monomer; dim, dimer; subtype C Tat variants; the Tat control band 2 comigrated with the 10kDa marker protein). (**C**) Tabulated R_f and M_r of the different protein bands.

Calculation of molecular weight of the Tat protein bands was performed as described in Current Protocols in Immunology (2000). The known log molecular weight (M_r) of the Full-Range Rainbow Molecular Weight Marker (Amersham Pharmacia Biotech, Buckinghamshire, UK) was plotted versus the relative electrophoretic mobility (R_f) as measured on Tat blot C (**Figure 21**). The R_f is defined as the difference between the distances migrated by the query protein and that of the electrophoretic front. The R_f of all Tat protein bands was plotted on the *x*-axis and interpolated onto the *y*-axis (□; **Figure 21**) to read off the corresponding molecular weights of the proteins. The approximate M_r (*y*-intercept) of the wild-type and codon optimised Tat proteins was thus calculated as 18 kDa, while the three control Tat protein bands were calculated to migrate at rates corresponding to approximately 25 kDa, 14 KDa and 10 kDa.

3.2.2 Rev

Similarly to Tat, expression levels of the codon-optimised and wild-type *rev* expression plasmids were determined by immunoblotting with pooled antibody serum raised in mice that were immunised with pCMVKm2Rev$_{M5M10}$ plasmid DNA. As a positive control, 4µg recombinant wild-type Rev protein, encoded by the subtype B HXB2 virus (Arya *et al.*, 1985) (Cat No. 1457, NIH AIDS Research and Reference Reagent Program, Rockville, MD), was analysed along with the 293 cells lysates. The mouse serum was immunoreactive to single bands in Rev$_{WT}$- and Rev$_{M5M10}$-transfected cell lysates (arrows) as well as the recombinant Rev control protein (**Figure 22**). No corresponding protein was reactive in the mock-transfected 293 cell lysates. *In vitro* expression levels of the codon-optimised, mutated Rev was markedly higher than the wild-type Rev as indicated by the greater band intensity of the Rev$_{M5M10}$ protein. Interestingly, the mutant Rev$_{M5M10}$ protein migrated more rapidly than the wild-type Rev, while the subtype B recombinant Rev protein, which was used as positive control migrated even slower than the two subtype C Rev proteins.

The molecular weights of the Rev proteins were determined by measuring the relative mobility of the Rev protein bands to calculate the M_r by plotting M_r and R_f of the High Range Molecular Weight Marker (Amersham Pharmacia Biotech, Buckinghamshire, UK) (**Figure 23**). Thus the codon-optimised, mutated Rev$_{M5M10}$ migrated at an R_f representing approximately 14.5 kDa while the wild-type Rev$_{wt}$ migrated slower than Rev$_{M5M10}$, to lie at an

approximate 17 kDa. The subtype B recombinant Rev control protein had an approximate molecular weight of 18kDa.

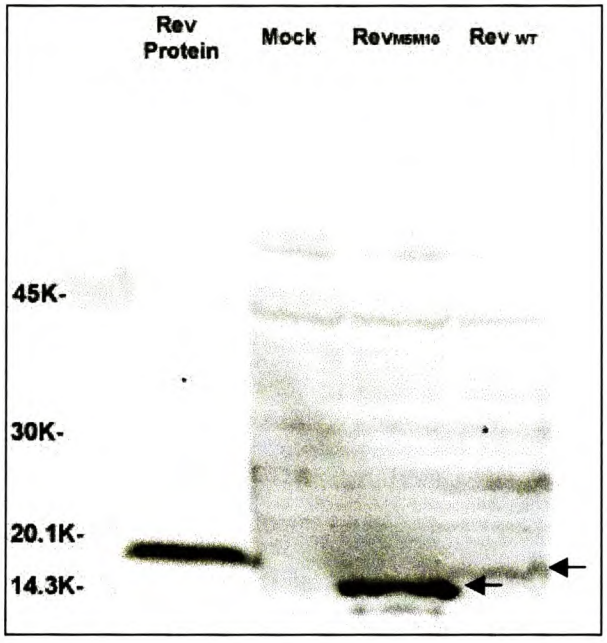
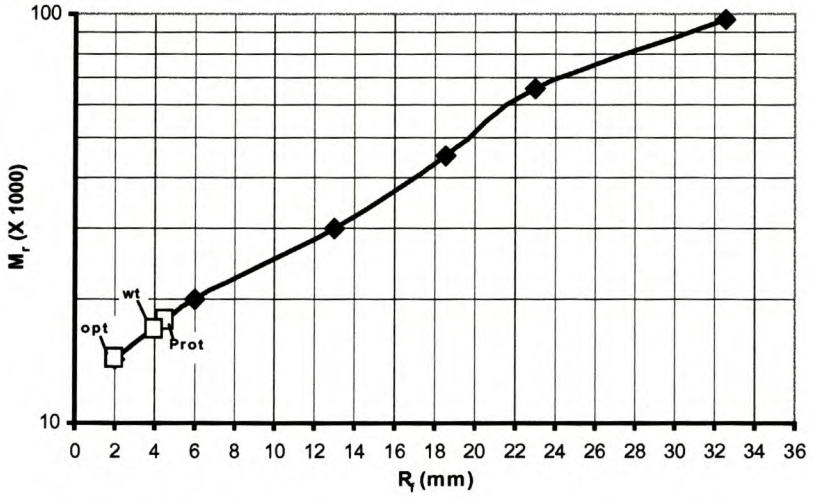


Figure 22: Immunoblot showing *in vitro* expression of the Rev expression constructs as well as mock-transfected cells. The Rev protein bands are indicated by the arrows.



	R_f (mm)	M_r X 10^3 (kDa)
Rev protein	4.5	18
Rev_{M5M10}	2	14.5
Rev_{WT}	4	17

Figure 23: Molecular weight determination of Rev proteins using a standard curve plotted from the relative mobility and molecular weight of the High-Range Rainbow Molecular Weight Marker proteins as detected in the Rev immunoblot (**Figure 22**). The interpolated relative mobility of the Rev proteins are also plotted (opt – Rev_{M5M10}, wt – Rev_{WT}, Prot – Control Rev Protein).

3.2.3 Nef

Similarly to Tat and Rev, Nef expression was assessed by SDS-PAGE and immunoblotting using subtype C Nef-specific mouse serum. As can be seen in **Figure 24 Blot A**, the mouse serum was not immunoreactive to any distinct protein bands in mock-transfected 293 cells. Reactivity was observed to proteins in cell lysates prepared from Nef_{WT}, Nef_{OPT}, $Nef_{myrD124}$ and $Nef_{myrD124LLAA}$-transfected 293 cells. As no positive protein control was available, the proteins were also blotted against a known subtype B ($HIV\text{-}1_{JR\text{-}CSF}$) Nef-specific monoclonal antibody (NIH AIDS Research and Reference Reagent Program, Rockville, MD) (**Blot B**), to which they were all immunoreactive. The codon-optimised *nef* genes appeared to express at somewhat higher levels than the wild-type *nef* gene from isolate TV002 as indicated by band intensities. Notably, the wild-type Nef protein migrated more rapidly than the mutant and native codon-optimised Nef proteins, which migrated somewhat faster than the 30kDa protein standard. All four *nef* constructs encoded proteins consisting of 208 amino acids (**Appendix A**). Molecular weight determination of the Nef proteins was done as described for the Tat and Rev proteins by plotting a standard curve with the known molecular weights of the Full-Range Rainbow Molecular Weight Marker (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the R_f of these proteins. The relative electrophoretic mobilities of the wild-type and codon-optimised Nef proteins were interpolated onto the standard curve (**Figure 25**) and the molecular weights were determined to be as follows: Nef_{WT} migrated to an approximate 26 kDa while the codon-optimised Nef proteins migrated to an approximate 28 kDa. The bands of the codon-optimised proteins were relatively thick and the R_f was read off from the estimated centre of the protein band.

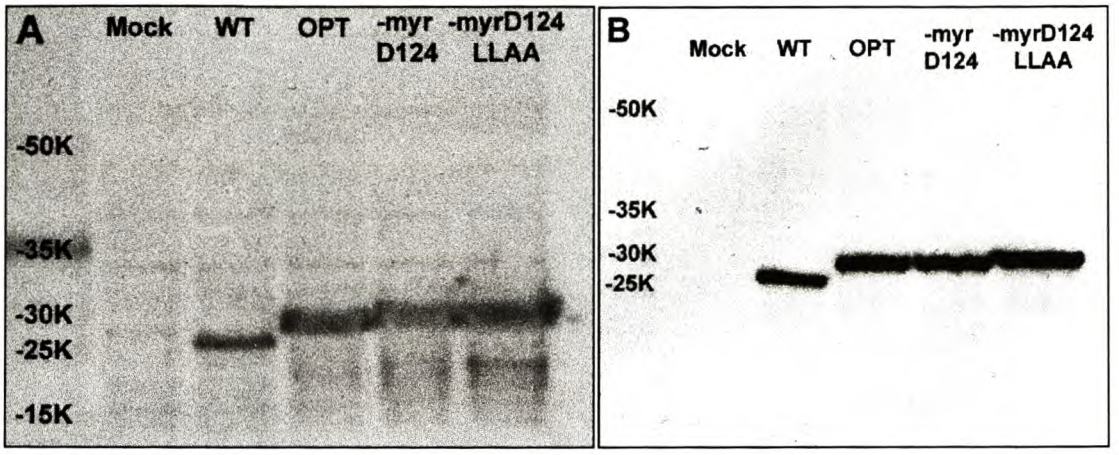
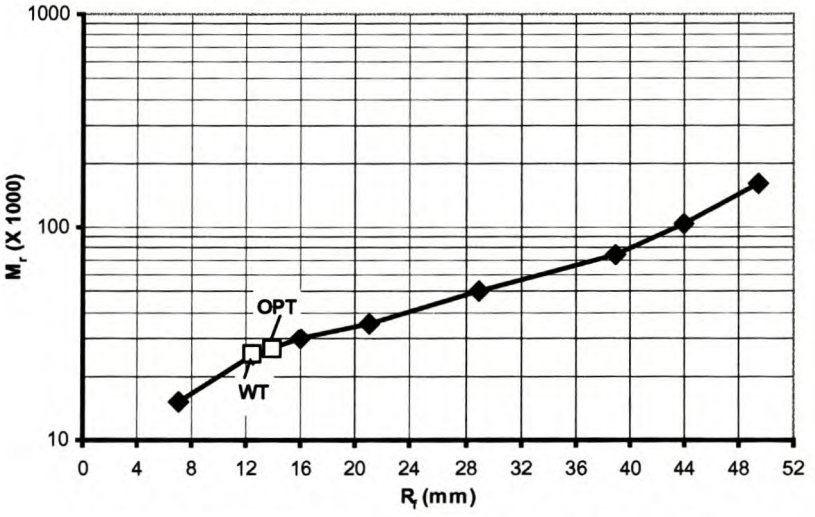


Figure 24: Immunoblots showing *in vitro* expression of the wild-type (WT) and codon optimised Nef expression constructs as well as mock-transfected cells. Proteins on Blot A were immunoreactive to the polyclonal mouse serum whereas blot B was blotted with a Nef-specific monoclonal antibody. The Full-Range Rainbow Molecular Weight Marker was included and the protein bands of the standards are demarcated with their molecular weights.



	R_f (mm)	M_r X 10^3 (kDa)
Nef_{WT}	12.5	26
codon-optimised Nef proteins	14	28

Figure 25: Calculation of the molecular weights of the wild-type and codon-optimised Nef proteins. The standard curve was plotted from the known molecular weights of proteins from the Full-Range Rainbow Molecular Weight Marker (Amersham Pharmacia Biotech, Buckinghamshire, UK) and their relative electrophoretic mobility (R_f). The interpolated R_f of the Nef_{WT} and Nef_{OPT} are shown as hollow squares.

3.3 Functionality Assays

3.3.1 Tat

The transactivation activities of the different Tat variants were assessed in an *in vitro* reporter assay previously described by Kuppuswamy *et al.* (1989). Tat variants were cotransfected with the pLTR-CAT plasmid, which encodes the reporter gene chloroamphenicol acetyl transferase (CAT) under the control of Tat-dependent promoter elements of the HIV-1 LTR (Peterlin *et al.*, 1986).

Preliminary experiments

A round of preliminary experiments, in which a limited number of *tat* plasmids were tested in various cell lines, was performed to compare the transactivation assay in these cell lines. CAT enzyme levels in cell lysates that were cotransfected with the functional Tat-expressing plasmid SF162-Tat (Tat from HIV-1 subtype B isolate SF162, Chiron Corporation) were set to 100% Tat activity for each cell line and CAT levels in the other cell lysates were calculated as a percentage of SF162-Tat activity. It was found that mock-transfected cells and cells transfected with the pLTR-CAT reporter plasmid only were unable to induce CAT expression in single experiments in RD, COS-7, 293T and HeLa cells (**Figure 26**). As expected, CAT expression was induced in all four cell lines when pLTR-CAT was cotransfected with the subtype B expressing Tat-expressing plasmid SF162-Tat. Cotransfection with the Tat_{C22} plasmid also induced strong CAT expression in COS-7, 293T and HeLa cells and at a lower level in RD cells, while Tat_{C22C37} seemed to be unable to induce transactivation of CAT expression in RD, 293T and HeLa cells (not done in COS-7 cells). Although only a single repeat was done of these preliminary experiments, the results served in showing that the four cell lines displayed similar systems in which comparable transactivation results seemed to be obtained. As these experiments were completed at Chiron Corporation in Emeryville, California and had to be reproducible in the laboratory at the Department of Medical Virology at Tygerberg in South Africa, availability of cell lines had to be considered and the Tat functionality experiments were thus done in 293T and HeLa cells.

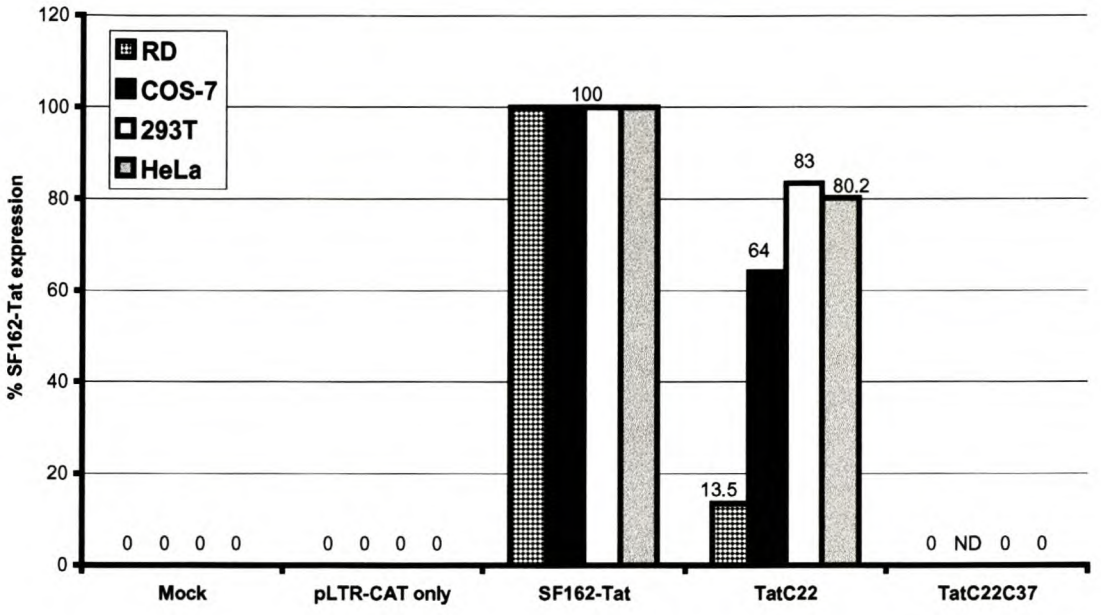
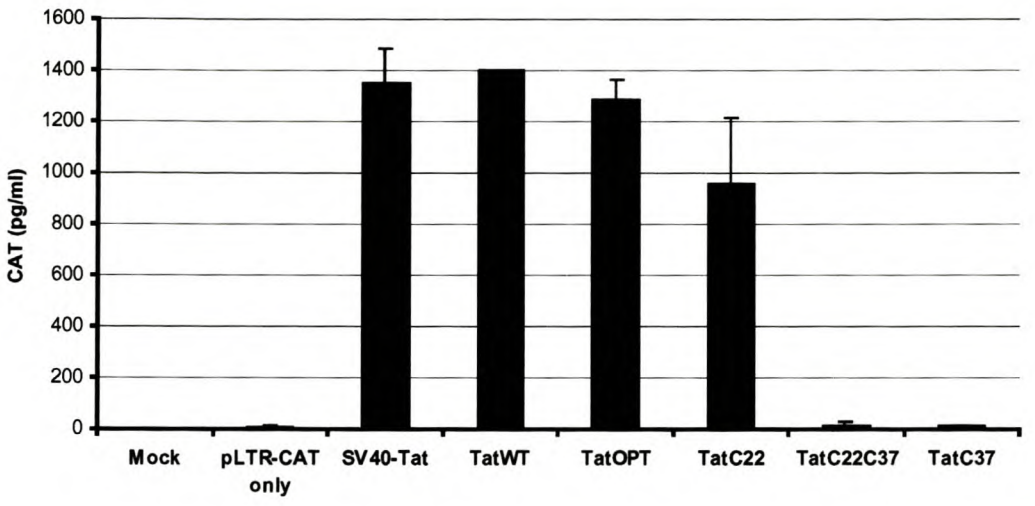


Figure 26: Preliminary Tat transactivation assays in RD, COS-7, 293T and HeLa cells. The CAT expression levels are presented on the *y*-axis as a percentage of that in cells that were transfected with SF162-Tat (set to 100%). ND, not determined.

Transactivation of Tat in 293T and HeLa cells

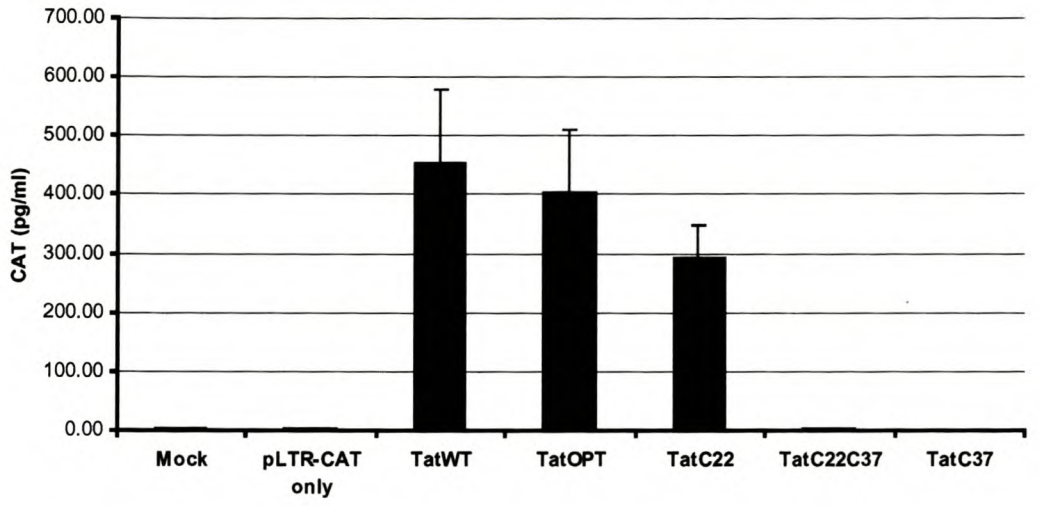
Subsequent Tat plasmid cotransfections with the pLTR-CAT reporter plasmid in 293T and HeLa cells were performed independently in triplicate and while those in 293T cells were not, the assays in HeLa cells were controlled by cotransfection with the internal transfection control plasmid pSV-βGal, a plasmid expressing the common reporter protein β-galactosidase. The raw data of CAT enzyme and β-galactosidase levels in HeLa cells as well as the normalisation of CAT enzyme levels are included as **Appendix B**. The results of these experiments in the two cell lines are presented separately in terms of CAT levels for each experimental repeat in **Figures 27** and **28** for 293T and HeLa respectively. In addition, the mean, standard deviation and standard error, the latter of which is also plotted on the graphs, are tabulated in both figures. The results in 293T and HeLa cells confirmed the preliminary data by showing that mock-transfected cells and cells transfected with the reporter plasmid alone showed no induction of CAT expression. Furthermore, an induction in excess of 230-fold was observed when pLTR-CAT was cotransfected with the plasmids Tat_{WT} and Tat_{OPT} in 293T and HeLa cells as well as SV40-Tat in 293T cells.



experiment 1	0.0	0.0	N/D	N/D	1443.0	1380.0	37.0	N/D
experiment 2	0.0	0.0	1173.5	N/D	1241.0	993.6	0.0	17.6
experiment 3	0.0	16.8	1520.0	1398.0	1286.8	320.0	15.2	13.5
mean	0.0	5.6	1346.8	1398.0	1323.6	897.9	17.4	15.6
standard deviation	0.0	9.7	245.0	N/A	105.9	536.5	18.6	2.9
standard error	0.0	5.6	141.5	N/A	61.2	309.7	10.7	1.7

Figure 27: Transactivation activity of the Tat constructs in 293T cells. The mean CAT enzyme concentrations along with the standard error for three independent experiments are plotted on the graph. The individual CAT enzyme levels, the mean, standard deviation and standard error are tabulated above. Tat_{WT} was assayed only once while only two repeats were done of SV40-Tat and Tat_{C37}. N/A, not applicable; N/D, not determined.

As indicated by the single preliminary repeats in 293T, HeLa, RD and COS-7 cells, cotransfection of pLTR-CAT with Tat_{C22} strongly induced transactivation of CAT expression by more than 160-fold in both 293T and HeLa cells, while cells cotransfected with the double mutant Tat_{C22C37} showed negligible induction of CAT expression in both cell lines. Moreover, negligible induction of CAT expression was also recorded in both cell lines by cotransfection with the plasmid encoding the singly mutated Tat protein Tat_{C37}.



experiment 1	5	5	613	608	408	5	ND
experiment 2	0	0	210.2	237.6	234.4	0	0
experiment 3	0	0	538.4	363.1	231.4	0	0
mean	1.7	1.7	453.9	402.9	291.3	1.7	0.0
standard deviation	2.9	2.9	214.3	188.4	101.1	2.9	0.0
standard error	1.7	1.7	123.7	108.8	58.4	1.7	0.0

Figure 28: Transactivation activity of the Tat constructs in HeLa cells. Again, the individual β-galactosidase normalised CAT enzyme levels, the mean, standard deviation and standard error are tabulated and the mean and standard error for the three independent experiments are plotted on the chart. For β-galactosidase raw data and normalisation of CAT enzyme values, see **Appendix B**. ND, not done.

These Tat functionality results are expressed as a percentage of native Tat (Tat_{OPT}, set to 100%) activity in **Figure 29**. As the difference in expression levels between the two cell lines is eliminated by presenting the data this way, the assays in 293T and HeLa cells could be compared directly. As already evident from the reported CAT expression levels, analogous transactivation activity levels between 293T and HeLa cells were recorded for all constructs, except SV40-Tat, which was not done in HeLa cells.

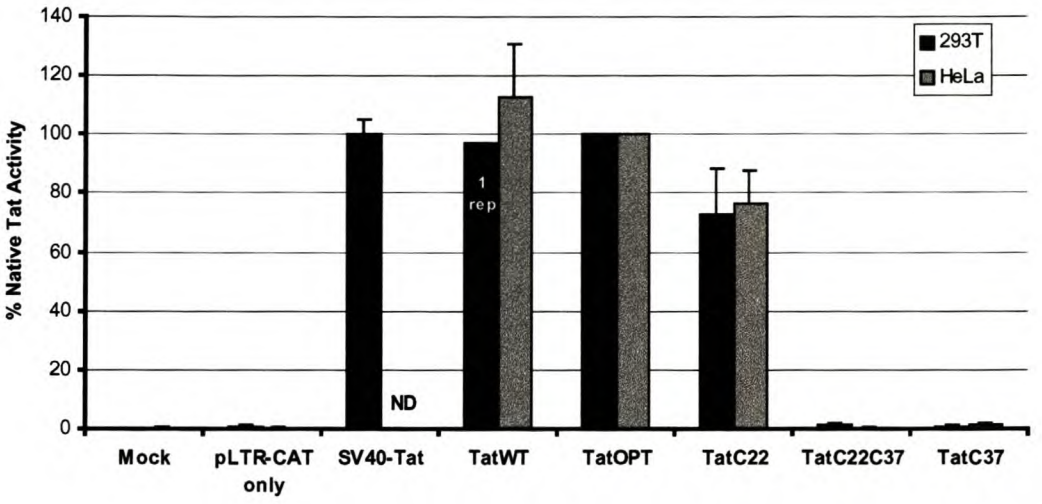
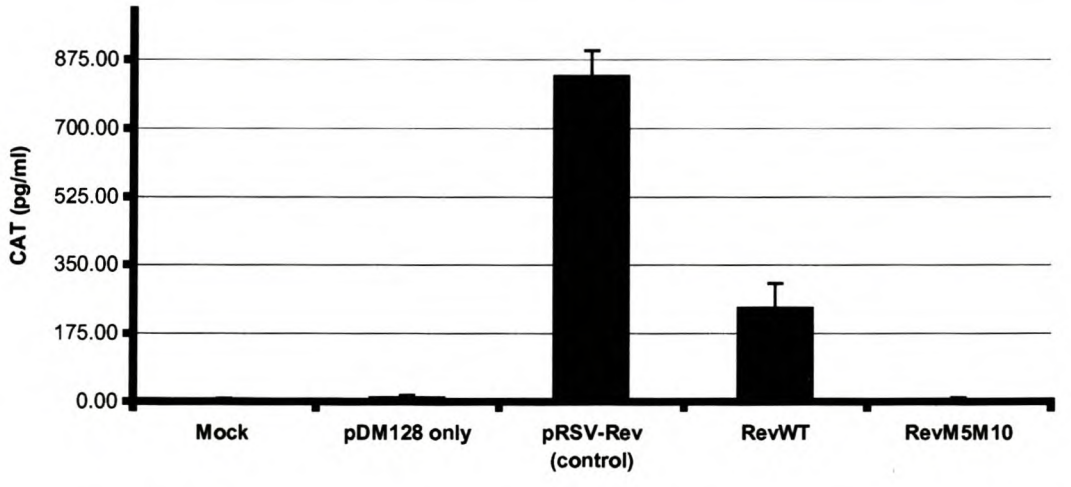


Figure 29: Mean transactivation activity of the Tat variants in 293T and HeLa (β-gactosidase normalised) cells as expressed in percent native (unmutated, codon-optimised Tat_{OPT}, set to 100%) Tat activity. Error bars represent the standard error for three independent experiments. Tat_{WT} was only assayed once in 293T cells (1 rep) and SV40-Tat was not tested in HeLa cells (ND).

3.3.2 Rev

The functional activity of the Rev variants was assessed by determining the level of the reporter enzyme chloroamphenicol acetyl transferase (CAT) in 293T cell lysates, which is indicative of the efficiency of RRE-coupled CAT transcripts to be exported from the nucleus by the Rev protein (first described by Hope *et al.* (1990a), who also kindly provided the pDM128 and pRSV-Rev plasmids). The β-galactosidase-normalised CAT levels from three independent experiments are tabulated and plotted in **Figure 30**. The raw data of CAT enzyme and β-galactosidase levels that were used to calculate these normalised CAT enzyme values are included as **Appendix B**. The mean, standard deviation and standard error (error bars on chart) for the three experiments are also tabulated in **Figure 30**. These values are also expressed as a percentage of wild-type Rev activity in **Figure 31**. The results show that mock-transfected 293T cells as well as cells transfected with the pDM128 reporter plasmid only yielded no more than basal CAT expression levels. Cell lysates of cells cotransfected with the subtype B Rev-expressing plasmid pRSV-Rev displayed strong CAT expression while the wild-type subtype C Rev-transfected cells yielded somewhat lower albeit noteworthy levels of CAT

(taken as 100% in **Figure 31** as no codon-optimised, native Rev plasmid was constructed). Taken from the mean and compared to cells transfected with pDM128 only, cotransfection with pRSV-Rev induced an increase in CAT expression greater than 85-fold while Rev_{WT} induced a 25-fold increase. In contrast, cells cotransfected with the codon-optimised, mutant Rev_{M5M10} plasmid displayed no induction of increased CAT levels in any of the three experiments. The significance of these results is highlighted by the small error bars seen for the three experiments when expressed as normalised CAT levels and especially when expressed as percentage of wild-type Rev activity.



experiment 1	5	5	897.6	329.4	5
experiment 2	8.5	23.5	N/D	116.8	13.5
experiment 3	0	0	767.7	273.7	0
mean	4.5	9.5	832.7	240	6.2
standard deviation	4.3	12.4	91.8	110.2	6.8
standard error	2.5	7.2	64.9	63.7	3.9

Figure 30: Rev nuclear export activity of RRE-coupled CAT mRNA as determined by CAT levels in 293T cells. Three independent repeats were performed and the CAT levels were normalised for the internal control β-galactosidase (for raw data and normalisation, see **Appendix B**). The CAT enzyme levels as well as the mean, standard deviation and standard error of the mean are tabulated above. The pRSV-Rev control plasmid was not included in one experiment repeat (N/D).

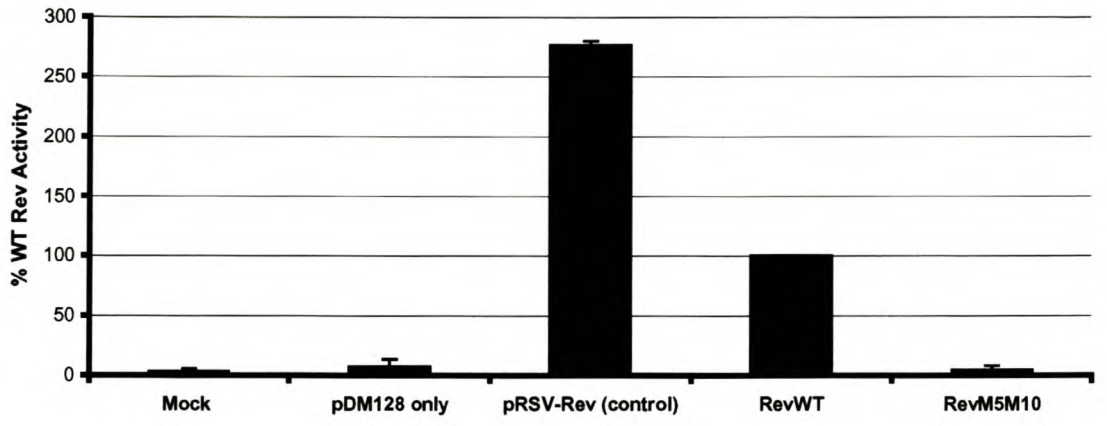


Figure 31: *In vitro* functional Rev activity expressed as percentage of wild-type activity, which was set to 100% (no codon-optimised, native Rev). The error bars represent standard errors for three independent experiments.

3.3.3 Nef

Functional activity of the Nef proteins was analysed by flow cytometric determination of CD4 and MHC-I expression on the surface of 293 cells 48-hours after cells were cotransfected with a CD4-expressing plasmid (pcDNA-hCD4) and the corresponding Nef-expressing vector. Cell surface CD4 was stained with APC coupled anti-CD4 antibodies, while MHC-I was stained with PE-coupled anti-HLA A,B,C antibodies. To select single 293 cells of specific size and composition, cells were gated on dot and density plots, which sort cells according to size on a forward scatter scale and according to granularity on a side scatter scale. The cells that were used for CD4 and MHC-I analysis fall within the gate demarcated R1 in **Figure 32**.

Because 293 cells do not naturally express CD4 on their cell surface, these cells had to be transfected with a CD4-expressing plasmid (pcDNA-hCD4) to study Nef-mediated CD4 downregulation. **Figure 33** shows the number of cells cotransfected with empty pCMVKm2 vector (vector), Nef_{OPT} or $Nef_{myrD124LLAA}$, showing CD4 APC fluorescence at intensities ranging from 0 to 10^4. By using these plots, cells that fluoresce at levels of 10^1 or higher (demarcated as M1) were set as positively CD4 expressing cells and could be measured as a fraction (percentage) of the total number of gated cells.

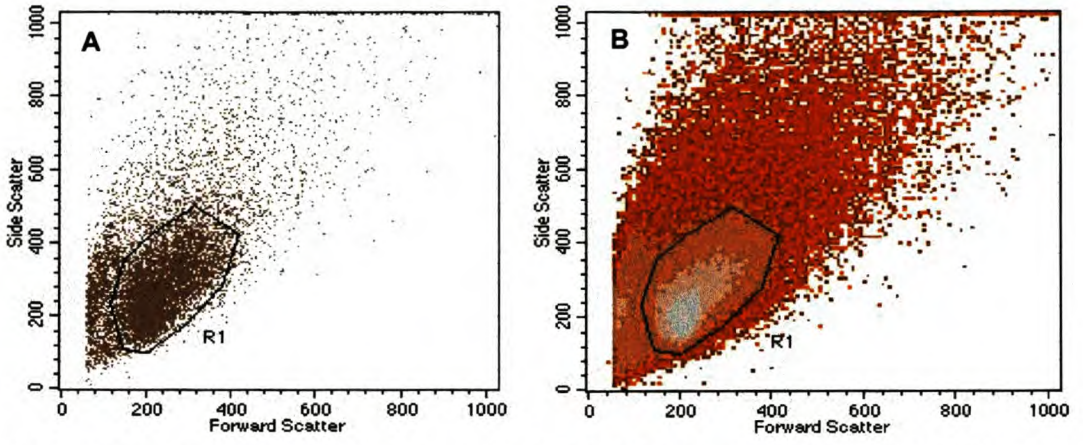


Figure 32: Flow cytometric analysis of 293 cells shown as dot plot (A, 10% of cells shown) and density plot (B). MHC-I PE and CD4 APC fluorescence was determined on cells gated (R1) by forward scatter and side scatter, which discriminate between cells on the grounds of size and granularity respectively.

Plot A in **Figure 33** illustrates the comparative CD4 expression profiles of cells cotransfected with empty vector (grey area) and cells transfected with the functional Nef-expressing plasmid Nef_{OPT} (area below/outlined in black). From the graph areas and data output it was determined that 34% of vector-transfected cells typically expressed CD4 while only 9.3% of Nef_{OPT}-transfected cells expressed CD4, indicating a marked Nef-mediated downregulation of cell surface CD4.

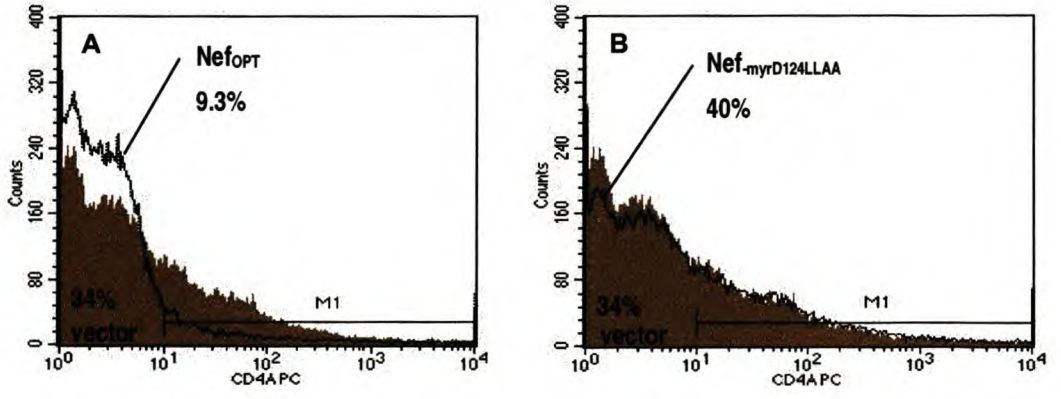
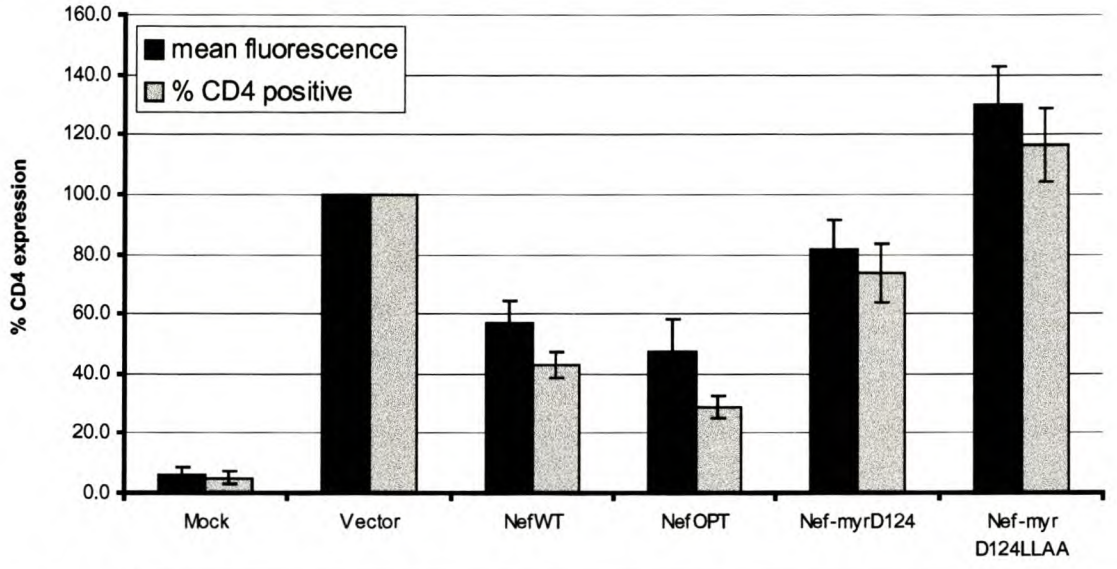


Figure 33: Flow cytometric determination of percentage CD4 expression on forward and side scatter-gated 293 cells. APC fluorescence above 1 log (10^1) was set as CD4 positive (demarcated as M1). Thirty-four percent of cells cotransfected with pCMVKm2 vector, shown in grey, were CD4 positive, while those cotransfected with Nef_{OPT} (A) and $Nef_{-myrD124LLAA}$ (B) displayed 9.3% and 40% CD4 expression respectively (below /outlined in black).

From plot B, it can be seen that typically 40% of cells cotransfected with the non-functional Nef variant $Nef_{-myrD124LLAA}$ expressed CD4, indicating no CD4

downregulation. These flow cytometric analyses were done for cells cotransfected with all Nef variants and the results of three independent experiments, expressed as percent CD4 expression of cells cotransfected with empty pCMVKm2 vector (set to 100%) are presented in **Figure 34**. To compare the way in which the flow cytometry data was recorded and interpreted, the CD4-APC mean fluorescence was also recorded and is included in **Figure 34** on the chart in black and the values for the three independent experiments in brackets in the table. Only base-level CD4 fluorescence was detected in mock-transfected cells and as expected, CD4 downregulation was observed in cells cotransfected with the functional Nef-encoding plasmids Nef_{WT} and Nef_{OPT}. Notably, Nef_{OPT}, which encodes the subtype C consensus (TV Cons) Nef protein, downregulated CD4 by up to 70%, while the wild-type Nef protein mediated a reduction in CD4 expression by approximately 50%. Both mutants exhibited abrogation of this activity compared to the unmutated Nef proteins. However, $Nef_{-myrD124}$ still appeared to mediate an approximate 20-30% downregulation, depending on the method of data readoff (% CD4 expression vs mean fluorescence). CD4 surface expression in $Nef_{-myrD124LLAA}$–transfected cells was comparable to that of cells transfected with vector, indicating complete abrogation of functional CD4 downregulation activity. Although not identical, the CD4 expression values that were recorded as percentage CD4 positive cells and those recorded as mean APC fluorescence were very comparable and exhibited the same results in terms of Nef_{WT} and Nef_{OPT} downregulating cell surface CD4 dramatically while $Nef_{-myrD124}$ downregulated CD4 expression to a small degree. Similarly, $Nef_{-myrD124LLAA}$ exhibited no CD4 downregulation activity. The significance of these data is validated by the small error bars.

Similarly to CD4 expression, the effects of the Nef variants on cell surface MHC-I expression were evaluated flow cytometrically on R1 gated cells. The PE-fluorescence of 293 cells transfected with 1µg/35mm well Nef_{WT}, $Nef_{-myrD124LLAA}$ or pCMVKm2 vector are compared in cell count histograms in **Figure 35**. As is clearly visible from the plots, no MHC-I downregulation could be distinguished in Nef_{WT}-transfected cells when compared to vector-transfected cells.



experiment 1	3.0 (1.9)	100.0 (100.0)	47.9 (48.6)	27.0 (28.3)	82.3 (79.9)	117.0 (146.9)
experiment 2	2.8 (5.7)	100.0 (100.0)	34.1 (52.7)	23.8 (46.3)	54.2 (65.7)	95.3 (105.6)
experiment 3	9.2 (10.8)	100.0 (100.0)	45.8 (70.7)	35.5 (67.1)	84.8 (99.1)	137.5 (137.7)
mean	5.0 (6.2)	100.0 (100.0)	42.6 (57.3)	28.8 (47.2)	73.8 (81.6)	116.6 (130.1)
standard deviation	3.7 (4.5)	0.0 (0)	7.4 (11.8)	6.1 (19.4)	17.0 (16.7)	21.1 (21.7)
standard error	2.1 (2.6)	0.0 (0)	4.3 (6.8)	3.5 (11.2)	9.8 (9.7)	12.2 (12.5)

Figure 34: Effect of Nef variants on cell surface CD4 expression expressed as percentage CD4 expression in Nef-transfected cells in relation to cells transfected with pCMVKm2 vector only (set to 100%). Percentages were determined either by recording the percentage CD4 positive cells (grey bars) or by recording mean APC-fluorescence (black bars). The percentages for three independent experiments for CD4 positive cells (and mean fluorescence) are tabulated along with their mean, standard deviation and standard error. The standard error is represented by error bars on the chart.

In addition, an apparent upregulation of MHC-I is exhibited for $Nef_{-myrD124LLAA}$-transfected cells. This finding is contrary to expected and previously reported results and further analysis of the flow cytometry data was employed. **Figure 36** illustrates dot plots of R1 gated cells as plotted by MHC-I PE fluorescence (*x*-axis) and CD4 APC fluorescence (*y*-axis). A CD4 APC fluorescence-independent cut-off, which selects only highly MHC-I PE fluorescing cells for analysis was introduced and the percentages of gated cells that fall above this cut-off were determined. As can be seen, the analysis of cells expressing high levels of surface MHC-I exhibited a small, but discernable difference between Nef variants in terms of the percentage of cells that fall within this region.

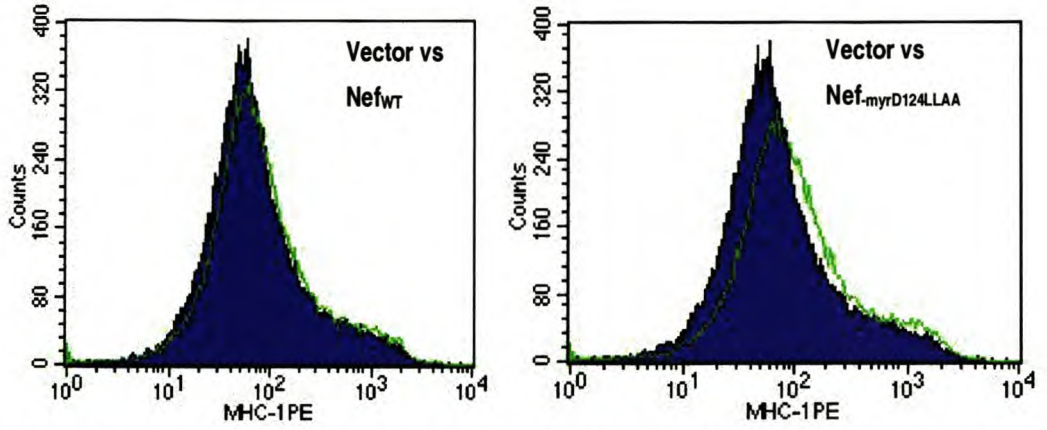


Figure 35: Comparative plots of PE-fluorescing cell counts representing cell surface MHC-I expression in Nef$_{WT}$ and Nef$_{myrD124LLAA}$-transfected cells (outlined in light grey) versus cells transfected with empty pCMVKm2 vector (colour-filled dark grey plots).

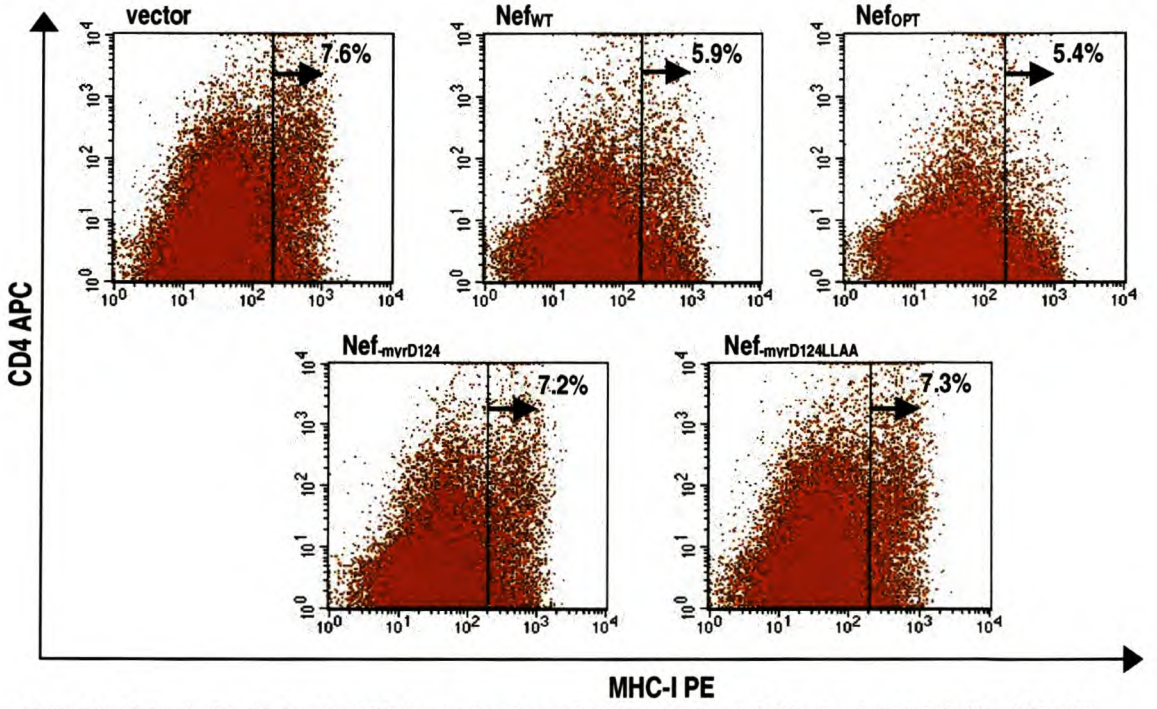
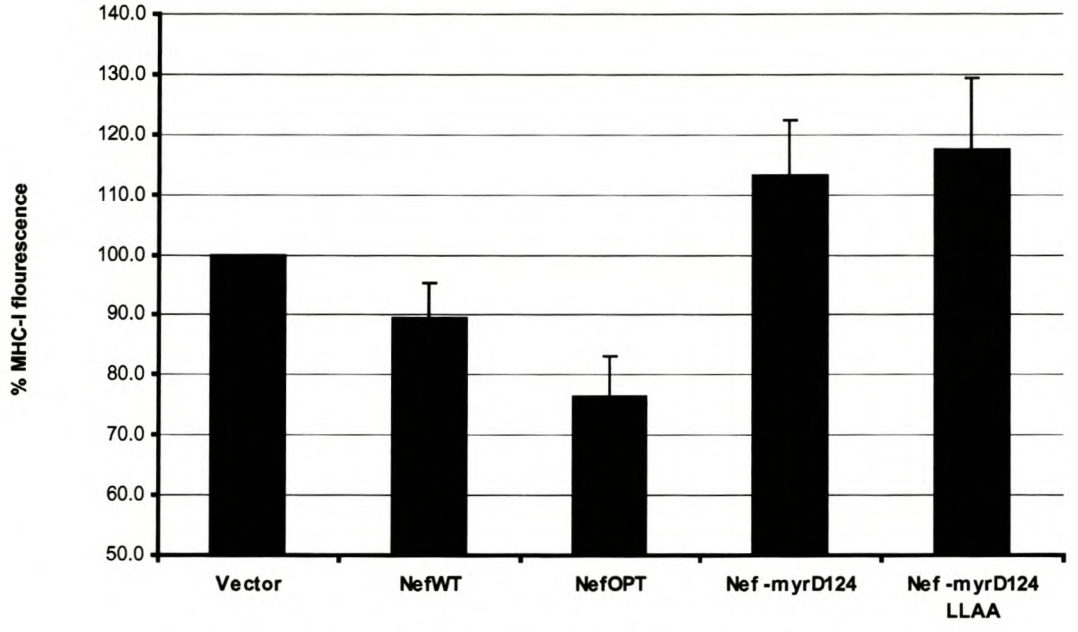


Figure 36: Determination of cell surface MHC-I expression. Forward and side scatter-gated 293 cells cotransfected with pcDNA-CD4 and Nef-expressing plasmids were plotted according to PE (*x*-axis) and APC (*y*-axis) fluorescence, indicating cell surface MHC-I and CD4 expression, respectively. Highly MHC-I PE- fluorescing cells with a minimum cut-off exceeding 10^2 (falling to the right of the line and indicated by the arrows) were analysed. The percent gated cells falling into this region are indicted in each dot plot.

These values are expressed for three independent experiments as a percentage of high intensity PE fluorescing vector-transfected cells in **Figure 37**. As is evident from comparing with vector-transfected cells, cotransfection with 1µg Nef$_{WT}$ and Nef$_{OPT}$ plasmid DNA per 35mm well effected a decrease in MHC-I PE

fluorescence, as also seen on the dot plots in **Figure 36**, indicating Nef-mediated MHC-I downregulation. Notably, MHC-I downregulation was stronger in cells cotransfected with Nef_{OPT} compared to Nef_{WT}-transfected cells. In comparison, cell surface MHC-I expression was restored to levels seen in vector-transfected cells when cells were transfected with either of the Nef mutants $Nef_{-myrD124}$ or $Nef_{-myrD124LLAA}$.



experiment 1	100.0	93.3	89.5	123.5	137.0
experiment 2	100.0	77.5	71.3	94.6	96.3
experiment 3	100.0	97.3	68.6	121.2	119.5
mean	100.0	89.4	76.5	113.1	117.6
standard deviation	0.0	10.5	11.4	16.1	20.4
standard error	0.0	6.1	6.6	9.3	11.8

Figure 37: Nef MHC-I downregulation as expressed as percentage vector-transfected 293 cells (set to 100%) exhibiting high-intensity PE-fluorescence. The values and mean for three independent experiments are shown along with the standard deviation and error, the latter of which is represented by the error bars on the chart.

CHAPTER 4: DISCUSSION

Tat, Rev and Nef make attractive vaccine components and have been developed as single-standing HIV-1 vaccines (Tat) (Cafaro *et al.*, 2001; Cafaro *et al.*, 2000; Pauza *et al.*, 2000; Cafaro *et al.*, 1999; Caselli *et al.*, 1999; Gallo, 1999; Gringeri *et al.*, 1998) as well as components in combination with structural genes (Boyer *et al.*, 2000; Hanke and McMichael, 1999; MacGregor *et al.*, 1998; Boyer *et al.*, 1997a; Boyer *et al.*, 1997b; Wang *et al.*, 1995) or as regulatory/accessory gene-cassette vaccines (Hel *et al.*, 2002; Calarota *et al.*, 2001; Tähtinen *et al.*, 2001; Ayyavoo *et al.*, 2000; Calarota *et al.*, 1999; Osterhaus *et al.*, 1999; Ayyavoo *et al.*, 1998; Calarota *et al.*, 1998; Kim *et al.*, 1997b). Because these genes are expressed early in the viral life cycle and expression of the structural genes is delayed until sufficient Rev is present for RRE-coupled transcript export, processing of Tat, Rev and Nef via the MHC-I pathway may lead to killing of the infected cell before the formation of new virions (Hel *et al.*, 2002; Addo *et al.*, 2001; Mooij and Heeney, 2001; van Baalen *et al.*, 1997). Numerous recent studies have highlighted the immunogenicity of these proteins and it has been shown that cellular and humoral immune responses to all three proteins inversely correlate with rapid disease progression (Fiorelli *et al.*, 2002; Demirhan *et al.*, 1999; Zagury *et al.*, 1998b; van Baalen *et al.*, 1997; Re *et al.*, 1995; Reiss *et al.*, 1990). Immunisation with these proteins in the form of DNA vaccines or protein immunogens has been shown to be effective and safe and a number of vaccines incorporating Tat, Rev and/or Nef have entered human trials and show promise (Hanke *et al.*, 2002; Calarota *et al.*, 2001; Boyer *et al.*, 2000; Calarota *et al.*, 1999; Gringeri *et al.*, 1998; MacGregor *et al.*, 1998). Immunisation with biologically active Tat protein has protected non-human primates after pathogenic SHIV challenge over an extended period of time and it has recently also been shown that the functional and immunogenic regions in Tat from viruses representing a number of subtypes and geographic origins were highly conserved (Butto *et al.*, 2002). In addition, it was shown that Tat-specific antibody responses can be effectively cross-reactive against infection with different HIV-1 subtypes (Fiorelli *et al.*, 2002). In a study in which numerous novel CTL epitopes in African individuals who were infected with HIV-1 subtype C were identified, a relatively equal density of CTL epitope distribution was observed for subtype C Gag, Tat, Rev and Nef when these were adjusted for protein size (Novitsky *et al.*, 2001). The study also identified

a number of immunodominant epitopes in Tat and Nef and a large number of subdominant epitopes in Tat, Rev and Nef and the authors propose that inclusion of such subdominant epitopes may help in providing protection after the emergence of escape variants. The development of Tat, Rev and Nef as vaccine components may provide important advantages such as increased breadth of immune responses with which Gag, Pol and Env immune responses may be reinforced.

Characterisation of the *tat*, *rev* and *nef* genes of HIV-1 subtype C from South Africa

This work was published recently as a sequence note in the journal AIDS Research and Human Retroviruses and the manuscript is attached as **Appendix C**. Phylogenetic analysis of the *nef* and 5'LTR sequences of 14 HIV-1 isolates from Tygerberg Virology (TV) identified these genomic regions as distinctly subtype C. This is supported by analysis of the deduced protein sequences of Tat, Rev and Nef, which revealed characteristically subtype C proteins (except Rev from TV005). When taken alongside the characterisation of the *env*, *gag* (Engelbrecht *et al.*, 2002) and accessory gene (Scriba *et al.*, 2002) sequences, the data support that the viral isolates are non-recombinant HIV-1 subtype C.

Sequence analysis of the wild-type and consensus sequences

Consensus protein sequences (TV Cons) were drawn up from the fourteen Tygerberg Virology (TV) Tat, Rev and Nef proteins and these were used as template for codon-optimisation to synthesise the immunogens that were used in this study. For comparative purposes, wild-type *tat*, *rev* and *nef* genes from those TV isolates that were least dissimilar in their deduced protein sequences to the TV Cons were cloned to construct expression plasmids named Tat_{WT} (TV019), Rev_{WT} (TV010) and Nef_{WT} (TV002). Four additional consensus sequences designated sA Cons (southern Africa i.e. South Africa and Botswana), Compl Cons (all subtype C Tat, Rev and Nef protein sequences used to draw up the phylogenetic trees), CONS C (the subtype C consensus from the HIV sequence database) and India Cons (drawn up from the subtype C isolates from India) were also included in the comparative sequence analyses. The results revealed that the collective amino acid distances of Tat, Rev and Nef of the consensus sequences were smallest

between the TV Cons and sA Cons. This similarity was further supported by the phylogenetic trees, which show that for the three proteins, the TV Cons and sA Cons were very close and fell within the same monophyletic clusters. Noteworthy was that again for all three proteins, the sA Cons was less dissimilar to the TV Cons than the wild-type proteins, which were chosen on the grounds of being the least dissimilar of the fourteen TV isolates. This was also confirmed by the phylogenetic trees which showed that the sA Cons and TV Cons were closer together and fell very close to the "backbones" of the trees, indicating a higher degree of homology to the taxa of the individual viral strains than virtually any individual viral strain. Moreover, similarity plots that compared the amino acid distances of the three proteins when calculated versus TV Cons or the wild-type TV isolates TV019 for Tat, TV010 for rev and TV002 for Nef were done. These plots did not illustrate a striking difference in amino acid distances due to step and window-size constraints of the Simplot program when using short protein sequences such as Tat and Rev. Thus, often 20-amino acid sequence steps would return the same distance output and this made it difficult to distinguish distance between sequences as the Tat sequences only generated four data points and the Rev sequences five data points for their entire reading frames. Nonetheless, when observed closely, distances were generally smaller between TV Cons and the TV isolates than the wild-type TV sequences and the TV isolates. This was more clearly evident when amino acid distances were expressed in stacked distance plots, in which the sum of amino acid distances of the TV Cons versus the fourteen TV isolates and each individual TV isolate versus the fourteen TV isolates were compared. The TV Cons (and sA Cons) were clearly found to be more similar to individual viral sequences than any one of the individual TV isolate sequences for Tat, Rev and Nef. Collectively, these results indicate that the use of a consensus sequence such as TV Cons as a vaccine candidate may contribute in reducing genetic variability between a vaccine strain and circulating viruses as also shown for other consensus sequences (Novitsky *et al.*, 2002; Brander and Goulder, 2001). Our results thus correspond with those described recently by Novitsky *et al.* (2002), who also showed more similarity between a southern African subtype C consensus and individual sequences than between an individual isolate sequence and a panel of individuals. Thus, a less heterogeneous vaccine may have direct implications on specificity of the vaccine induced immune responses and immunoreactivity with viruses in an epidemic of highly diverse subtype C viruses such as southern Africa. The fact that the TV Cons was only drawn up from fourteen viral strains rather than

a much larger number of viruses was a concern as the consensus may not have been representative of the actual population of circulating strains in South Africa or southern Africa. This concern was alleviated by the very high degree of similarity between the TV Cons and the sA Cons, the latter of which was drawn up from approximately 70 sequences (depending on the protein) from South Africa and Botswana. In terms of amino acid substitutions, the TV Cons and sA Cons only differed by single residues for Tat (1%) and Nef (0.5%) and four residues for Rev (3.7%). Thus, even though the TV Cons was drawn up from fourteen HIV-1 subtype C viruses isolated at the Department of Medical Virology, Tygerberg, Western Cape, these Tat, Rev and Nef consensus sequences were representative of the circulating viral strains in southern Africa. This also indicates the suitability of the TV Cons immunogens being suitable as subtype C vaccine components for southern Africa and not only South Africa. Taken together, these data demonstrate the suitability of the TV Cons amino acid sequences for candidate HIV-1 subtype C Tat, Rev and Nef vaccine immunogens.

Codon optimisation of the *tat*, *rev* and *nef* genes

In an attempt to increase the expression of proteins from the DNA plasmids encoding the HIV-1 subtype C consensus Tat, Rev and Nef proteins, the codon usage of these was changed to those of highly expressed human genes as described (zur Megede J. *et al.*, 2000; Andre *et al.*, 1998; Haas *et al.*, 1996b). The result was a marked increase in percentage GC content across the entire reading frames of all three genes and overall increases of approximately 20% per reading frame. Strong increases in expression levels in 293 cells up to 900-fold were recorded for genes such as GFP and HIV-1 Env and Gag when they were codon-optimised in similar manners (zur Megede J. *et al.*, 2000; Andre *et al.*, 1998; Haas *et al.*, 1996b). Although apparently minute differences in expression level could be distinguished between the wild-type and codon-optimised *tat* and *nef* plasmids, these results were not distinct enough to be significant. However, the codon-optimised Rev exhibited distinctly increased expression levels compared to that of the wild-type Rev. It must be noted that immunoblotting is not an accurate quantitative method with which protein levels can be determined and these results can thus not be expressed quantitatively like those that may be obtained from for example an ELISA with standard concentration curves. Nevertheless, markedly increased expression was apparent for Rev and possibly to small

degrees for Tat and Nef and these may result in increased immunogenicity, when used *in vivo* as immunogens, as more protein can be processed via the MHC-I antigen presentation pathways.

Biological characterisation of subtype C Tat proteins

The HIV-1 subtype C *tat* genes of viruses isolated at the Tygerberg Department of Medical Virology were notably conserved and although some length variation was observed, the deduced amino acid sequences showed low levels of residue variation (**see Appendix A**). Upon discovery, the *tat* gene was reported to encode a protein of 86 amino acid residues in length with a relative electrophoretic mobility corresponding to approximately 14kDa (Aldovini *et al.*, 1986; Wright *et al.*, 1986; Sodroski *et al.*, 1985a). This shortened Tat, which was encoded by the subtype B virus LAI/IIIB/ARV (from which HXB2 was derived) has been used for the majority of functionality studies under the heading of "full length Tat" (Jeang, 1996). However, most strains of HIV-1 encode a Tat protein that is 101 amino acid residues in length (Kuiken *et al.*, 2000; Jeang, 1996). Interestingly, the viral isolates and molecular clones used in these early studies incorporated a number of truncated reading frames, including that of *tat*, which terminated prematurely to express an 86 amino acid protein (Ratner *et al.*, 1985). Nevertheless, it was found that only the first exon of Tat, which normally consists of 72 residues, and more specifically the amino terminal 58 amino acids, were sufficient for transactivation activity (Battaglia *et al.*, 1994; Kuppuswamy *et al.*, 1989; Garcia *et al.*, 1988; Seigel *et al.*, 1986) and the C-terminal domain was considered unimportant.

The results from the present study indicate that the wild-type subtype C Tat protein derived from isolate TV019 as well as the codon-optimised consensus Tat variants which encode proteins of 101 amino acids in length migrate at a rate corresponding to approximately 18kDa on a denaturing SDS-PAGE gel. The C22G and C37S mutations in the Tat mutants did not have an effect on electrophoretic mobility. The subtype B Tat control protein, which was 86 amino acids in length, migrated at the expected 14kDa rate (Bohan *et al.*, 1992). Two additional bands were immunoreactive in the lane containing this Tat control protein, one of 25kDa and one of 10kDa. Although Aldovini *et al.* reported a similar band at 25kDa while characterising the Tat protein, they remarked that it was unlikely that a 25kDa dimer consisting of 14kDa subunits would be detected on a gel that was electrophoresed under denaturing

conditions (Aldovini *et al.*, 1986). Yet, it was shown later that the metal-linked Tat dimer was stable under SDS conditions and migrated as a 25kDa protein band on denaturing gels (Frankel *et al.*, 1988) and that such dimers form upon oxidation to which biologically active Tat is highly sensitive (Ensoli *et al.*, 1990; Viscidi *et al.*, 1989). The resuspension of the lyophilised protein in PBS buffer lacking reducing or chelating agents may have effected the oxidation and/or degradation of the protein resulting in the appearance of smeared bands and the additional 25kDa dimer and partial 10kDa products. Partial Tat fragments, which form by oxidation on SDS gels, have also been previously reported (Koken *et al.*, 1994; Frankel *et al.*, 1988).

Immunoblotting of Tat lysates with polyclonal mouse serum showed comparable reactivity of the polyclonal mouse serum for all subtype C Tat proteins irrespective of mutations. The subtype B Tat control protein was also immunoreactive, confirming the identity of the protein bands as indeed being HIV-1 Tat. Furthermore, these results point out that the Tat proteins were expressed and that Tat_{C22C37} was able to elicit substantial humoral immune responses in mice. It should be mentioned though that the dose of immunogen was very high (two immunisations of 75µg each) and that this can not be taken as indication of good immunogenicity.

Functional activity of the various Tat proteins was assessed by cotransfection of the Tat variants with the reporter plasmid pLTR-CAT, which was first described by Peterlin *et al.* (1986) and used in a number of Tat transactivation experiments, one of which first demonstrated the inactivity of Tat proteins mutated in the cysteine-rich domain (Kuppuswamy *et al.*, 1989). Although Kuppuswamy *et al.* performed these transactivation assays in HeLa cells, a comparison of the cotransfection assay in a number of cell lines was done to determine whether corresponding results could be obtained. Hence a round of preliminary experiments was performed in which a small number of Tat variants were tested for transactivation activity in COS-7, RD, HeLa and 293T cells. Contrary to a substantial amount of published data, we found the subtype C consensus Tat with a Cys22Gly substitution to be functionally active in terms of being able to transactivate the HIV-1 LTR. These results were subsequently confirmed in three independent experiments each in HeLa and 293T cells. However, in agreement with previously published reports, the single mutant Tat_{C37} and the double mutant Tat_{C22C37} were unable to transactivate CAT expression from the LTR. The importance of the cysteine-rich domain for the structure and function of Tat was shown soon after

discovery of the protein (Viscidi *et al.*, 1989; Frankel *et al.*, 1988) and a number of studies demonstrated that mutation of the cysteines at positions 22 and 37 as well as other positions rendered the Tat proteins unable to transactivate LTR-directed gene expression (Rice and Carlotti, 1990; Kuppuswamy *et al.*, 1989; Ruben *et al.*, 1989; Garcia *et al.*, 1988). It was also shown that a number of non-cysteine mutations in the cysteine-rich domain (residues 22 through 37) reduced Tat activity (Rice and Carlotti, 1990) and a study by Koken *et al.* indicated that the cysteines in this region are crucial for the formation of intermolecular disulphide bonds, which confer correct protein folding and functional activity (Koken *et al.*, 1994). These Tat mutants were subsequently analysed for additional functional effects and it was found that when Tat22 was constitutively expressed in Jurkat T cells, it was able to act as a *trans*dominant negative mutant by inhibiting HIV-1 replication as well as reactivation from latency (Caputo *et al.*, 1996). This role was also demonstrated in primary PBMCs and the authors suggested that it might be of therapeutic value (Rossi *et al.*, 1997). Additional studies revealed that the C22, C37 and C22/C37 mutations did not affect cellular localisation and that such mutant Tat proteins, when present extracellularly, were taken up and localised within cells in a manner similar to that of wild-type Tat (Betti *et al.*, 2001). Due to these findings, the authors proposed that these substituted cysteine residues do not drastically alter the conformation of Tat. However, mutations in this region did bring about a decrease in receptor binding affinity indicating a certain degree of conformational modification (Betti *et al.*, 2001).

Interestingly, while Tat_{WT} and Tat_{OPT} differed by four amino acid substitutions, both demonstrated virtually equal transactivation activity, showing that the subtype C consensus Tat protein (TV Cons), which presumably does not occur naturally, had full biological activity. It should be noted though that when broken down into its putative functional domains (Jeang *et al.*, 1999), the domains from Tat TV Cons were always identical to corresponding domains in individual viral isolates and thus the consensus did not contain any unnatural sequence combinations.

A possible reason for the finding that the subtype C consensus Tat_{C22} mutant was capable of *trans*activating the HIV-1 LTR is not clear. Because the assays used in this study were done as those described by Kuppuswamy *et al.* (1989), who also used the same reporter plasmid and performed the *trans*activation assays in HeLa cells, the possibility of different methodology can be excluded. The importance of cysteine residue conservation at position

22 in Tat has been well established. Cys22 is highly conserved among HIV-1 and also a large number of HIV-2 and SIV viruses (Kuiken *et al.*, 2000) and with the exception of our work, every previously published experiment has shown mutation of this residue to abrogate *trans*activation (Betti *et al.*, 2001; Caselli *et al.*, 1999; Rossi *et al.*, 1997; Caputo *et al.*, 1996; Balboni *et al.*, 1993; Rice and Carlotti, 1990; Kuppuswamy *et al.*, 1989; Ruben *et al.*, 1989; Garcia *et al.*, 1988). When dissected, only two disparities between these previous experiments and the Tat transactivation work described in this study can be recognized, and both implicate the Tat protein. Firstly, to my knowledge, all studies that concern functional dissection, mutation or structural analysis of Tat have been conducted on proteins encoded by HIV-1 subtype B viruses. While unlikely, the fact that the Tat proteins analysed here are from subtype C viruses may be accompanied by suspicions that the intrinsic structures and functions of Tat proteins from different HIV subtypes can differ. Indeed, it is well known that the long terminal repeat as well as a number of proteins including Rev, Vpu and the C-terminal of Pol are distinctly different in subtype C viruses when compared to other subtypes (Rodenburg *et al.*, 2001; Novitsky *et al.*, 1999; Gao *et al.*, 1998; Salminen *et al.*, 1996). It would thus not be unreasonable to speculate about the unexplored subtype factor in playing a possible role in a different structure or function of Tat. A second distinction between the work completed in this study and again, to my knowledge, all previous studies describing the Tat22 mutant is the factor of protein length. As a result of the premature termination site in the *tat* reading frame of the first laboratory HIV-1 strain, the majority of studies investigating the function and structure of Tat22 (and many other studies) were conducted on the 86 amino acid Tat protein and not the full-length 101 amino acid Tat that is seen predominantly in viruses (HIV Sequence database). As described before, early studies showed that the second exon was expendable for transactivation activity (Seigel *et al.*, 1986). A number of recent studies however, have demonstrated the second exon, which encompasses 29 amino acids in most strains, to enhance viral replication (Neuveut and Jeang, 1996) and pathogenesis (Jeang *et al.*, 2002) of HIV-1. The possibility exists that the full length Tat proteins used in this study may have not been inactivated for transactivation by mutation of C22 because of a counteracting function of the C-terminal second exon. Interestingly, a Tat mutant (Y26A) which was defective for transactivation activity and viral replication, regained activity when a second-site suppressor mutation (Y47N) was acquired (Verhoef and Berkhout, 1999). These data certainly provoke consideration of this concept,

but it remains unlikely that mutation of such a critical residue in such a critical domain of Tat can be suppressed by a longer C-terminal or a second-site suppressor mutation. These questions will remain unanswered until they are experimentally defined. Nonetheless, the aim of this study was to identify a non-functional Tat protein that may act as potential vaccine immunogen and this aim was most certainly achieved.

A note should be made about the experimental results obtained in the Tat functionality assays. Although the experiments were repeated independently in triplicate in both cell lines, a number of Tat protein variants were only assayed twice while Tat_{WT} was only assayed once in 293T cells. The reason for this is mostly due to the fact that the functionality assays were started without the full complement of Tat variants and these were included as they were constructed. SV40-Tat was only done in 293T cells as these assays were completed at Chiron Corporation in the USA and because the assays in HeLa cells were done in South Africa, this plasmid was not available. Nonetheless, the data obtained illustrates the feasibility of the assay in both cell lines as well as the validity of the results. However, it should be mentioned that for publication purposes, statistical significance can only be confirmed for all Tat proteins if these assays are done in triplicate.

Tat's role as immunogen has been discussed in the context of this study. The number of defined epitopes CTL epitopes in the HIV Immunology Database (2000) is expanding every year. Although most epitopes have been identified in HIV-1 subtype B infected individuals and some of these can be found in proteins from other subtypes due to protein sequence conservation, new studies are specifically targeting non-B subtypes for CTL mapping (Brander and Goulder, 2001; Novitsky *et al.*, 2001). Currently, only one subtype C-specific CTL epitope has been identified and it is indicated on the Tat_{OPT} protein sequence in **Figure 38**. It should be mentioned that a considerable amount of CTL epitopes that were defined in subtype B Tat proteins vary by a single residue and thus cannot be mapped on the subtype C consensus Tat protein. It can be expected though, that at least a fraction of these residue variations fall within non-anchor residue sites and that at least some of these will thus be cross-reactive to subtype C proteins.

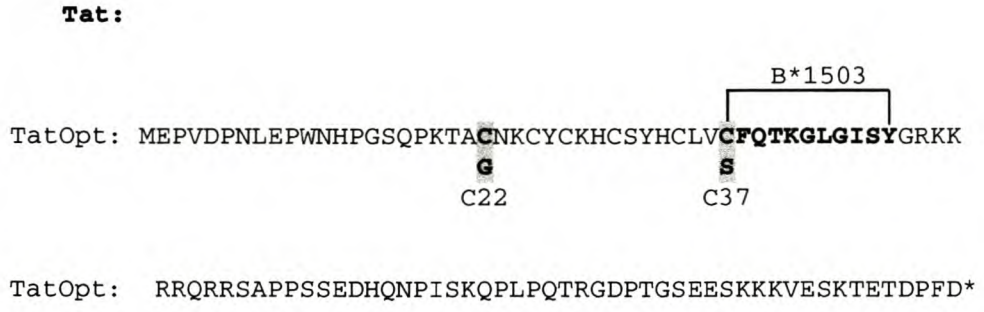


Figure 38: Sequences of the HIV-1 subtype C consensus (TV Cons) Tat immunogens constructed for this study. Amino acid substitutions in mutant proteins are indicated and named and the described subtype C-specific CTL epitope of known HLA restriction from the HIV Immunology Database (2000) is indicated.

Biological characterisation of subtype C Rev proteins

The molecular mass (M_r) of HIV-1 Rev is described quite inconsistently in the literature. Emerman (1998), Pollard (1998), Malim *et al.* (1989a; 1988) and Cochrane *et al.* (1989) report Rev to have a M_r of 18-19kDa, whereas it is reported as a 13kDa protein by Hope (1999). Our results show the recombinant subtype B Rev protein at the expected M_r of 18kDa, whereas the codon-optimised, mutated Rev_{M5M10} migrated to a distance calculated as approximately 14.5kDa. The wild-type Rev_{wt} migrated slower than Rev_{M5M10}, albeit more rapidly than the protein control, to lie at around 16.5kDa. The faster migration of the two subtype C Rev proteins relative to the subtype B recombinant Rev (116 amino acids) may be attributed to the characteristic Rev truncation (106 amino acids), which has also been reported in practically all subtype C Rev proteins (Novitsky *et al.*, 2002; Scriba *et al.*, 2002; Rodenburg *et al.*, 2001; van Harmelen *et al.*, 2001; Ndung'u *et al.*, 2000; Lole *et al.*, 1999; Novitsky *et al.*, 1999; Gao *et al.*, 1998; Salminen *et al.*, 1996). In addition, the increased migration of the codon-optimised, mutated Rev_{M5M10} relative to the wild-type Rev_{wt} was not entirely surprising as minor variations in the form of more rapid (Malim *et al.*, 1991; Mermer *et al.*, 1990; Malim *et al.*, 1989a) as well as decreased (Malim *et al.*, 1991; Hope *et al.*, 1990b; Malim *et al.*, 1989a) electrophoretic mobility have been reported for a number of Rev mutants. The cause of this mobility shift remains to be clarified, as the mutated proteins in these studies as well as the Rev_{M5M10} mutant described here were of the same length in amino acids as the wild-type proteins. A possible reason could be that mutation of the Rev protein may have effects on

posttranslational protein modification, which may lead to variable electrophoretic mobility. Rev is phosphorylated at one or more serine residues when expressed *in vivo* (Hauber *et al.*, 1988) and when compared to the sequence of Rev_{M5M10}, the wild-type Rev has three substitutions in which serine residues are lost while gaining a serine in one substitution (**Appendix A**). This is unlikely to affect electrophoretic mobility however, as posttranslational modification of Rev entails only phosphorylation and even though the M5 mutation (M10 had no effect) has been shown to dramatically reduce the level of phosphate incorporation into Rev, this effect was shown not to affect electrophoretic mobility (Malim *et al.*, 1989a; Hauber *et al.*, 1988). An additional possible cause could be variation in amino acid composition, which is suggested as possible cause for variable electrophoretic migration rates of Nef proteins (Kaminchik *et al.*, 1990). Rev_{WT} and Rev_{M5M10} differ by eleven amino acids (10%), a factor which may well affect migration rates of the proteins. Nonetheless, similarly to a number of previous studies, mutations in Rev effected a shift in mobility on SDS-PAGE gels, the basis of which remains speculative.

Comparison of *in vitro* protein expression levels from the codon-optimised, mutated and wild-type *rev* plasmids revealed a strong Rev_{M5M10} protein band compared to a faint Rev_{WT} band on immunoblots, indicating higher expression levels of the codon-optimised, consensus Rev_{M5M10} protein than the wild-type Rev_{WT}. Such differences have been reported for other codon-optimised HIV-1 genes (zur Megede J. *et al.*, 2000; Andre *et al.*, 1998; Haas *et al.*, 1996b). Although unlikely, it must be noted that the polyclonal mouse serum, which was generated by immunisation with the mutant Rev_{M5M10}, may display less efficient immunoreactivity to the wild-type Rev protein due to potentially altered structural conformations. If the reason that the two subtype C proteins migrate at different rates on a denaturing SDS-PAGE gel is as a result of possible differences in posttranslational modifications (Hope *et al.*, 1990b) or protein stability (Malim *et al.*, 1989a) this may also affect immunoreactivity. Interestingly, although the subtype B control Rev protein was immunoreactive, the subtype B Rev protein expressed by transfection of 293T cells (it showed strong functional activity) with the pRSV-Rev plasmid was not detected by immunoblotting with the subtype C-specific mouse serum.

Similarly to Tat, the immunoreactivity of the polyclonal mouse serum with the subtype C and B Rev proteins demonstrated the identity of the protein bands to be Rev and indicates protein expression and immunogenicity in mice.

The cotransfection assay for Rev activity that was first described by Hope *et al.* (1990b) and used extensively in other Rev functionality studies was altered in this study. Whereas the initial assays were performed in COS7 or CV1 cells, in this study 293T cells were cotransfected to assess Rev function. 293T cells, like the 293 cell line that they were derived from, strongly express transfection-introduced genes (Graham *et al.*, 1977) and although they may not exhibit cellular conditions conducive to all functional and biochemical studies, they have of late been increasingly used for studying such parameters in proteins including HIV-1 accessory/regulatory proteins (Lundquist *et al.*, 2002; Peng and Robert-Guroff, 2001; Craig *et al.*, 1998). Along these lines, the comparative preliminary Tat functionality assays, which were performed in RD, COS-7, HeLa and 293T cells, as well as the actual Tat functionality assays, which were repeatedly performed in 293T and HeLa cells, showed no demonstrable difference between the cell lines in terms of assay outcome or accuracy. In fact, due to the strong expression of the proteins in the 293T cells, a more definable difference could be distinguished between CAT levels exhibited. Although Rev functionality is distinct from that of Tat, these criteria were reason enough to perform the assays in 293T cells. Only basal CAT expression was observed in cells transfected with the reporter plasmid pDM128 only while co-transfection with the pRSV-Rev plasmid resulted in an 85-fold increase in CAT levels. This corresponds very well with the results obtained by Hope *et al.*, (1990) who recorded 75- to 100-fold increases in CAT enzyme activity when CV1 cells were cotransfected with pRSV-Rev and pDM128, further indicating the suitability of performing this assay in 293T cells.

While the wild-type subtype C Rev protein Rev_{WT} induced a marked 25-fold increase in CAT levels, this was abolished for the mutated Rev_{M5M10}. The small error bars obtained from three independent experiments indicate the repeatability and thus significance of these findings. The results indicate the subtype C Rev_{M5M10} protein containing the M5 (RK 38,39 DL; arginine-rich NLS) and M10 (IE 78,79 DL; NES) mutations to be unable to effect *in vitro* nuclear export of unspliced RRE-coupled transcripts in 293T cells and thus shows Rev_{M5M10} to be functionally inactive. It should be noted that this inactivity of Rev_{M5M10} was recorded in the background of the apparently stronger expressing, and thus more abundant Rev_{M5M10} protein compared to the Rev_{WT} protein. Possible explanations for the stronger activity of pRSV-Rev compared to Rev_{WT} may include abundance of Rev protein, a factor which was not clarified as pRSV-Rev was not detectable on the subtype C-specific

immunoblots (blots not shown). To my knowledge, no studies have investigated the functional effects of the subtype C-specific truncation in Rev, which truncates the protein by nine amino acids. However, two Rev mutants, which contained residue substitutions in the C-terminal nine residues (termed M13 and M14), were assessed for their ability to *trans*activate cytoplasmic expression of unspliced viral transcripts. Although both mutants were reported to have activity between 50% and 100% of wild-type Rev, M14 displayed less than 80% wild-type activity while M13 displayed less than 70% (Malim *et al.*, 1989a). Unfortunately no additional experiments were done to assess these mutants further and to attribute a decrease in Rev activity to these nine C-terminal residues, a truncated Rev will have to be compared directly with its wild-type form.

Further speculations could be made about the differences between the interactions of a subtype B and C Rev with the subtype B Rev-responsive element (RRE) present in the pDM128 plasmid. However, as these discussions are purely speculative and reach beyond the scopes of this study, these questions will remain unanswered.

The results obtained in this study are consistent with previous studies in which the M5 and M10 mutations have individually been shown to be sufficient for complete abrogation of functional Rev activity (Thomas *et al.*, 1998; Madore *et al.*, 1994; Hope *et al.*, 1992; Hope *et al.*, 1990b; Malim *et al.*, 1989a). Although our experiments demonstrate the functionality of Rev in terms of ability to transactivate cytoplasmic expression of unspliced pDM128 transcripts in transfected 293T cells, the extent to which the M5 and M10 mutations have been characterised reaches much further. M5 (also previously called MB2 by Hope *et al.* (1990a)) was shown to cause defects in nuclear localisation (Hope *et al.*, 1990a; Hope *et al.*, 1990b), phosphorylation of Rev (Malim *et al.*, 1989a) and multimerisation (Thomas *et al.*, 1998; Madore *et al.*, 1994) and abrogated functional activity in terms of being able to rescue *rev*-defective provirus and to transactivate cytoplasmic expression of unspliced HIV-1 transcripts (Malim *et al.*, 1989a) or unspliced pDM128 transcripts (Hope *et al.*, 1990a; Hope *et al.*, 1990b). Similarly, Rev proteins carrying the M10 mutation displayed defective multimerisation (Thomas *et al.*, 1998; Madore *et al.*, 1994) and were unable to rescue *rev*-defective provirus or transactivate cytoplasmic expression of unspliced HIV-1 transcripts (Malim *et al.*, 1989a) or unspliced pDM128 transcripts (Hope *et al.*, 1990b). In addition, cotransfection of M10 mutants with wild-type Rev was shown to inhibit wild-type Rev function in a

*trans*dominant manner (Hope *et al.*, 1990b; Malim *et al.*, 1989a) and to inhibit HIV-1 replication through the RevM10-mediated prevention of functional Rev-mediated nuclear export of unspliced viral transcripts and subsequent inhibition of Gag-Pol and Env protein expression (Hope *et al.*, 1992). This *trans*dominant-negative Rev has since been developed for gene therapy in HIV-1 infected individuals and was shown to be clinically safe in human trials (Ranga *et al.*, 1998; Woffendin *et al.*, 1996). Moreover, RevM10-transduced T cells were found to have prolonged survival and it was proposed that antiviral gene therapy with this *trans*dominant-negative Rev mutant may contribute to the reconstitution of immune function in AIDS. More recent studies demonstrated the emergence of RevM10-resistant HIV-1 variants and selection of such mutated viruses in a T-lymphoblastoid cell line constitutively expressing RevM10 (Hamm *et al.*, 1999). However, novel HIV-1-based retroviral vectors, which can transduce non-dividing cells were shown to reduce the infectivity of inhibition-escape virions and confer protection against HIV-1 replication to previously untransduced cells (Mautino *et al.*, 2001).

The inclusion of both M5 and M10 mutations into a potential HIV-1 subtype C Rev immunogen such as pCMVKm2-Rev_{M5M10} incorporates the collective abrogation of all of the abovementioned functions and may also mediate the *trans*dominant inhibition of wild-type Rev function. These characteristics are suggestive of a non-functional and thus potentially safe Rev immunogen, which, in addition to possibly eliciting HIV-1 subtype C Rev-specific immunity, may inhibit HIV-1 replication upon infection or lend a therapeutic value in HIV-1 infected individuals through the competitive *trans*dominant inhibition of functional Rev. When compared to a protein like Nef, very few CTL and T_h epitopes have been described in HIV-1 Rev (HIV Immunology Database (2000)) and those identified have been in individuals infected with predominantly HIV-1 subtype B. Thus no epitopes that correspond to subtype C Rev have been defined, although a study has identified subtype C Rev-specific responses in individuals from Botswana (Novitsky *et al.*, 2001). This makes it difficult to anticipate the immunological value of the pCMVKm2-Rev_{M5M10} as DNA immunogen. Yet as the sequences of the viruses from Botswana studied by Novitsky *et al.* (2001; 2002) were shown to be very similar to the ones from South Africa, the Rev_{M5M10} immunogen, which is based on the South African consensus sequence (TV Cons) may elicit anti-Rev responses similar to those detected in their study.

Biological characterisation of subtype C Nef proteins

Genetic characterisation of the *nef* genes of fourteen HIV-1 subtype C isolates from Tygerberg Virology (TV isolates) showed that these sequences did not cluster distinctly in phylogenetic trees, but rather clustered with subtype C *nef* sequences of global origin with the exception of *nef* sequences of Indian origin. Since the deduced Nef protein from isolate TV002 showed the least amino acid dissimilarity to the Nef TV Cons consensus sequence, which was drawn up from the fourteen TV isolates, this isolate was chosen as template for construction of the wild-type Nef construct. The Nef_{WT} and Nef_{OPT} as well as both mutant Nef plasmid constructs encoded proteins of 208 amino acids in length. However, the wild-type Nef protein, calculated to have a molecular weight of 26kDa, migrated at a distinctly more rapid rate than the codon-optimised Nef proteins, which migrated at a rate corresponding to 28kDa. The unmutated Nef proteins Nef_{WT} and Nef_{OPT} differ in terms of amino acid composition by nineteen residues, which accounts for 9% sequence dissimilarity (**Appendix A**). While it has been reported that differential posttranslational modification of proteins may account for variable electrophoretic mobilities, the myristoylation of Nef has been shown not to impact on mobility (Niederman *et al.*, 1993; Yu and Felsted, 1992; Kaminchik *et al.*, 1991). Because they contain intact myristoylation signals it is anticipated that both Nef_{WT} and Nef_{OPT} proteins will undergo myristoylation while both Nef mutants have had their myristoylation signals mutated (G2A) and will thus not be able to bind a myristic acid. The three codon-optimised consensus Nef proteins comigrated at indistinguishable rates, a result that is consistent with the previous results in that myristoylation did not affect electrophoretic mobility. Moreover, since phosphorylation of Nef proteins do not affect electrophoretic mobility (Coates *et al.*, 1997), posttranslational modification can be ruled out as basis for this phenomenon. A number of studies have described the expression of two different sized Nef proteins, usually 27kDa and 25kDa in size, from the same coding regions (Kaminchik *et al.*, 1991; Kaminchik *et al.*, 1990; Guy *et al.*, 1987; Franchini *et al.*, 1986). While Nef is cleaved by the viral protease (Chen *et al.*, 1998; Pandori *et al.*, 1996; Welker *et al.*, 1996; Freund *et al.*, 1994), the cleavage product typically comprises a molecular weight of 19kDa, eliminating proteolytic cleavage as possible cause for the variable Nef product. Thus the particular event in which the 27kDa and 25kDa Nef species were found was attributed to variable translation form the normal initiation codon as well as an additional ATG that occurs in a considerable fraction of *nef* reading frames around nucleotide

position 60 (Ratner and Niederman, 1995; Kaminchik *et al.*, 1991; Allan *et al.*, 1985). Since both the wild-type TV002 and the TV Cons consensus *nef* reading frames do not include this second initiation codon (**Appendix A**), this factor could also be eliminated as possible cause for such differential migration rates. Ultimately the more rapid electrophoretic mobility of Nef_{WT} in comparison to the native and mutated consensus Nef proteins may simply be due to variation in amino acid composition. Such a variable electrophoretic migration was previously observed for two subtype B Nef proteins from strains BH10 (28 kDa) and LAV1 (26 kDa), which were of equal length and only differed in amino acid composition (Kaminchik *et al.*, 1990).

In vitro expression levels of the Nef proteins could also be determined from the immunoblots. The four different Nef proteins were immunoreactive to anti-Nef polyclonal mouse serum as well as a subtype B Nef-specific monoclonal antibody, the latter confirming the identity of the proteins in the absence of a Nef protein control. The immunoreactivity of the Nef serum, which was raised by immunising mice with naked pCMVKm2-$Nef_{-myrD124}$ plasmid DNA, indicates that this protein was expressed in mice and elicited a substantial humoral immune response. While the codon-optimised, consensus Nef proteins were expressed at indistinguishable levels, the wild-type Nef protein appeared to be expressed at a somewhat lower level on both blots. Although not marked, this difference in expression level may be indicative of higher levels of protein expression due to codon-optimisation which is required for enhanced immunogenicity of DNA vaccine immunogens (zur Megede *et al.*, 2000; Andre *et al.*, 1998).

The Nef protein from HIV-1, HIV-2 and SIV is expressed abundantly in the early stages of viral gene expression and associates with host cellular membranes through its N-terminal myristoylation (Kaminchik *et al.*, 1991; Guy *et al.*, 1987). Along with a number of functions that contribute to the enhancement of virion infectivity and disease pathogenesis, Nef downregulates cell surface expression of MHC-I (Le Gall *et al.*, 1997; Schwartz *et al.*, 1996) and CD4 (Aiken *et al.*, 1994; Garcia and Miller, 1991; Guy *et al.*, 1987). Because these functional properties of Nef may raise safety concerns and result in poor immune responses, two Nef mutants containing a number of site-specific amino acid substitutions that have been shown to abrogate some of these functions were constructed. The first mutant, designated $Nef_{-myrD124}$, had a G2A substitution in order to mutate the myristoylation signal as well as a D124G substitution (described as D123G by

Liu *et al.* (2000), which was shown to abolish oligomerisation, downregulation of CD4 and MHC-I and decrease viral infectivity (Liu *et al.*, 2000). Myristoylation of Nef has been shown to be essential for membrane localisation (Niederman *et al.*, 1993) and thus biological function including viral pathogenicity (Aldrovandi *et al.*, 1998), downregulation of CD4 (Iafrate *et al.*, 1997; Goldsmith *et al.*, 1995; Aiken *et al.*, 1994) and MHC-I (Peng and Robert-Guroff, 2001), enhancement of viral infectivity (Goldsmith *et al.*, 1995) and replication (Lundquist *et al.*, 2002), activation of NFAT expression (Manninen *et al.*, 2001), disruption of T cell receptor (CD3) signalling and activation (Wang *et al.*, 2000; Iafrate *et al.*, 1997) and association with cellular serine kinases (Sawai *et al.*, 1995). Moreover, myristoylation of Nef has been demonstrated to convey a cytotoxic function to Nef (Curtain *et al.*, 1998; Curtain *et al.*, 1997).

The second mutant, designated $Nef_{-myrD124LLAA}$, contained the same G2A and D124G mutations as well as an additional, well described mutation in which the dileucine motif at position 165 (LL165AA) was substituted for alanines. While having no effect on MHC-I downregulation (Mangasarian *et al.*, 1999), mutation of this motif has been shown to completely abrogate Nef-mediated CD4 downregulation (Lundquist *et al.*, 2002; Swigut *et al.*, 2001; Mangasarian *et al.*, 1999; Bresnahan *et al.*, 1998; Craig *et al.*, 1998). In addition, LL165AA Nef mutants show defective viral replication (Lundquist *et al.*, 2002) and infectivity, disrupted Nef binding to the AP-1 clathrin adaptor (Bresnahan *et al.*, 1998) and CD28 downregulation (Swigut *et al.*, 2001).

The functional abilities of these Nef mutants as well as unmutated Nef proteins to downregulate cell surface CD4 and MHC-I were assessed in assays described previously (Peng and Robert-Guroff, 2001; Goldsmith *et al.*, 1995). The results indicate that both unmutated HIV-1 subtype C Nef proteins, Nef_{WT} and Nef_{OPT}, mediated downregulation of cell surface expression of CD4 and MHC-I, indicating functional activity. Like those for Tat, these results illustrate the biological activity of a consensus Nef protein, which does, in all probability, not occur naturally. Both Nef mutants were defective at downregulation of cell surface MHC-I expression, confirming that previously published functional analysis results apply to subtype C Nef proteins. However, in the background of cell surface CD4 downregulation, mutant $Nef_{-myrD124LLAA}$ displayed full abrogation of this function while mutant $Nef_{-myrD124}$, although not at full wild-type activity, showed CD4 downregulation. These results emphasize the importance of the dileucine motif for CD4

downregulation activity and demonstrate that the G2A and D123G mutations were not individually sufficient for complete abrogation of this Nef function. Furthermore, this finding corresponds with prior studies, which consistently showed minor levels (below 25%) of CD4 downregulation in G2A Nef mutants (Lundquist *et al.*, 2002; Goldsmith *et al.*, 1995).

It should be mentioned that while the Nef mutants displayed abrogated CD4 and MHC-I downregulation, the CD4 downregulation assay showed CD4 expression levels for $Nef_{-myrD124LLAA}$-transfected cells that exceeded vector-transfected cells (100%) and similarly the MHC-I downregulation assay showed MHC-I expression exceeding 100% for $Nef_{-myrD124}$- and $Nef_{-myrD124LLAA}$-transfected cells. Similar cell-surface expression levels that exceed vector-transfected cells, which are set as 100%, are reported in previously reported transient cotransfection Nef functionality experiments and are not discussed (Lundquist *et al.*, 2002; Manninen *et al.*, 2001; Peng and Robert-Guroff, 2001; Liu *et al.*, 2000; Mangasarian *et al.*, 1999; Goldsmith *et al.*, 1995). This study provides no possible explanation for such a phenomenon and ultimately the most probable rationalization would be to attribute this to an artefact of the transient cotransfection experiments.

With regard to the assay methodology that was employed to investigate Nef functionality, a number of points should be made. Even though the CD4 and MHC-I downregulation detected in this study were real, the methodology can be enhanced to improve confidence of the significance of the data obtained. In my opinion it is essential to include a control plasmid that expresses a protein that can be detected by flow cytometry. Due to the strong expression of MHC-I on the cell surfaces and the small percentage of cells that were transfected with Nef, it was difficult to demonstrate MHC-I downregulation when looking at the total cell population and only highly fluorescing cells showed the Nef-mediated effect. With such an internal transfection control, it is possible to analyse transfected cells only, a factor that may well have improved analysis of MHC-I molecule downregulation. It would thus be ideal to use a GFP-encoding plasmid for example, which constitutively expresses GFP in transfected cells that may be detected in the FITC-channel during flow cytometry. A second factor that can only improve the flow cytometric methodology is the inclusion of antibody-matched isotype controls for AP and APC in order to define the magnitude of background IgG binding to 293 cells.

Nonetheless, the flow cytometric assays served to identify CD4 and MHC-I downregulation in Nef transfected cells and also to identify mutant Nef immunogens that are defective for these functions.

Nef has been identified to be frequently targeted by CTL and T_h cellular immune responses and a very extensive array of epitopes that are restricted by all HLA types have and are being identified. Like Tat and Rev, Nef epitopes have nearly exclusively been identified in HIV-1 subtype B infected individuals and CTL epitopes elicited through vaccination have similarly been specific to the predominantly subtype B vaccines. Nonetheless, in addition to a handful of studies that have identified subtype C-Nef CTL epitopes, a considerable number of epitopes are conserved across subtypes and those listed in the HIV Immunology Database (2000) that are present in the TV Cons consensus Nef sequence that was used for construction of the Nef immunogens are shown in **Figure 39**. As can be seen, despite the mutations, which are indicated on the consensus, a number of epitope clusters are present on the subtype C Nef consensus sequence, a finding that was described recently for HIV-1 proteins (Yusim *et al.*, 2002). As most described CTL epitopes are specific for subtype B sequences, it can be postulated that only a fraction of subtype C-specific epitopes have been found to date either by experimental methods or interpolation of subtype B epitopes onto subtype C sequences in conserved regions. Thus, it is reasonable to expect that a significant number of subtype C-specific Tat, Rev and Nef CTL epitopes are still to be identified. Even so, **Figure 39** demonstrates the potential value of the pCMVKm2-Nef$_{myrD124LLAA}$ plasmid as DNA immunogen.

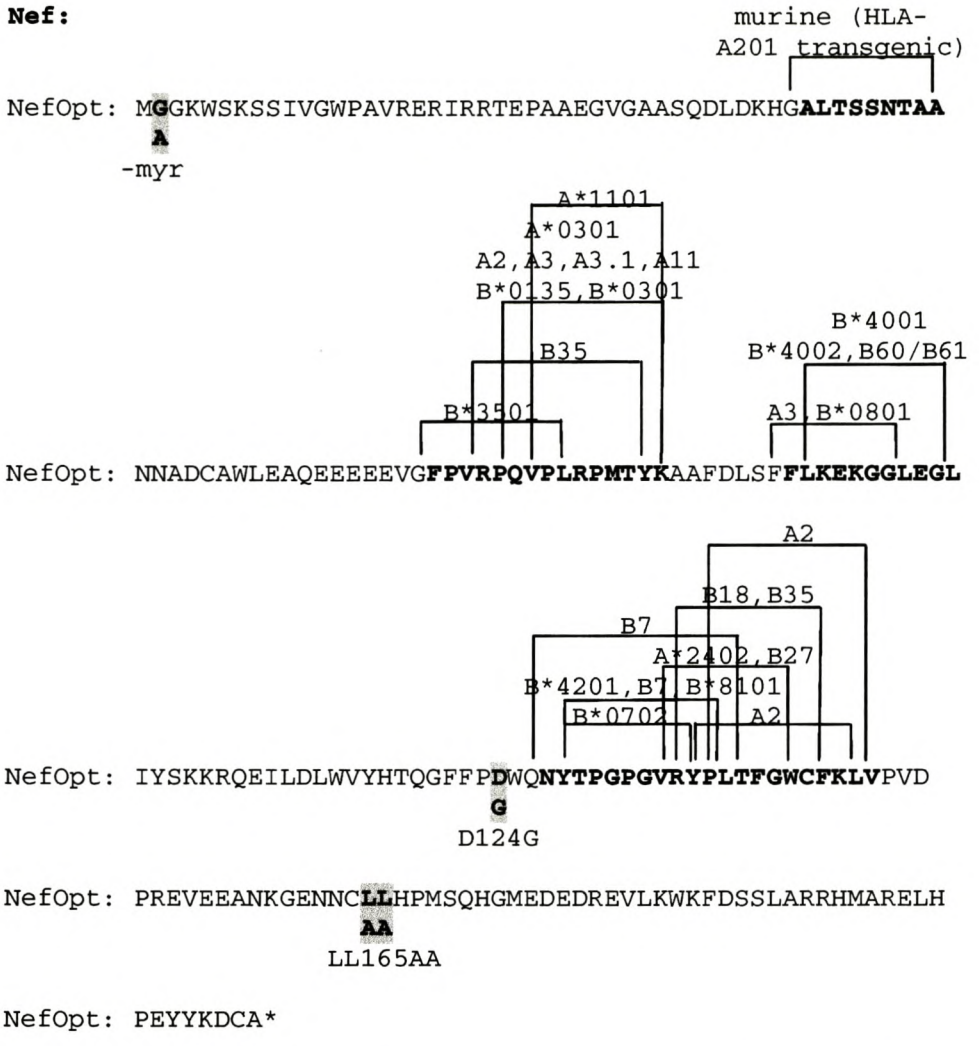


Figure 39: Sequences of the HIV-1 subtype C consensus (TV Cons) Nef immunogens constructed for this study. Amino acid substitutions in mutant proteins are indicated and named and all described CTL epitopes of known HLA restriction from the HIV Immunology Database (2000) are indicated.

CHAPTER 5: CONCLUSION

The continued screening and characterisation of currently circulating viral strains is of utmost importance, as an unnoticed change in circulating virus populations in terms of prevalence of subtypes and emergence of inter-subtype recombinant viruses may have detrimental consequences on vaccine design. Moreover, such screening is important to evaluate the conservation of genes that code for vaccine immunogens, providing breadth projections for immune responses. Finally, continued sequencing of HIV-1 genomic regions that are employed as immunogens in vaccine strategies will provide sequence data for the calculation of consensus proteins, providing means with which vaccine strains can be selected and with which the relevance of such selected vaccine strains can be assessed over time.

The collective aim of this study was to design, construct and experimentally identify plasmids that strongly express non-functional HIV-1 subtype C consensus Tat, Rev and Nef proteins that may serve as DNA vaccine immunogens. In fulfilment of the objectives, the plasmids pCMVKm2-Tat_{C22C37} and pCMVKm2-Tat_{C37}, pCMVKm2-Rev_{M5M10} and pCMVKm2-$Nef_{-myrD124LLAA}$ were shown to strongly express proteins that were found to be completely defective for their respective functional activities. Moreover, loss of activity was achieved without disruption or deletion of portions of these proteins that exceed two amino acid residues per site, a strategy employed to retain a maximum amount of conserved protein sequences that may contain immunodominant or subdominant CTL or T_h epitopes. Consensus protein sequences were used for construction of the candidate immunogens instead of individual viral strains or isolates and this approach was justified by the results that the consensus sequences for Tat, Rev and Nef displayed less genetic distance to viral strains than did any one of the fourteen subtype C isolates. The use of these consensus immunogens would thus serve to reduce genetic variation between vaccine-encoded immunogenic epitopes and those present in circulating viral populations. These potential immunogens may thus elicit broad immune responses specific for these early and abundantly expressed accessory/regulatory proteins that can potentially effect cytotoxic killing of HIV infected cells before the production and budding of new virions. Additionally, when employed in combination with the structural protein immunogens Gag, Pol and/or Env, the inclusion of these accessory gene

immunogens may effectively broaden the array of immune responses to achieve higher levels of viral suppression in several stages of viral replication.

The immunogenicity and thus potential value of these DNA vaccine constructs remain speculative until immunological studies can cast more light on this subject. It would be important to assess immune responses in mice that have been immunised with the Tat, Rev and Nef plasmids. Such a study will also allow a comparison of immunogenicity between wild-type and consensus, codon-optimised immunogens. Furthermore, these immunogens may be fused to form a single TatRevNef polygene cassette that may be more practical in terms of preparation and immunisation. The work contained in this study thus lays the foundation for extensive immunological studies to elucidate whether these Tat, Rev and Nef immunogens are of sufficient worth to warrant their inclusion into a HIV-1 subtype C vaccine.

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APPENDICES

Appendix A: Plasmid construct sequences

tat constructs: DNA sequence

```
                 SalI       start  20          *         40          *
TatOpt     : GTCGACGCCACCATGGAGCCCGTGGACCCCAACCTGGAGCCCTGGAACCA :  50
TatC22     : ..................................................... :  50
TatC37     : ..................................................... :  50
TatC22C37  : ..................................................... :  50
Tatwt      : ...................A..A..T..T.....A.................. :  50

                       60          *     C22           *         100
TatOpt     : CCCCGGCAGCCAGCCCAAGACCGCCTGCAACAAGTGCTACTGCAAGCACT : 100
TatC22     : ........................G..........................  : 100
TatC37     : ..................................................... : 100
TatC22C37  : ........................G..........................  : 100
Tatwt      : T..A..A..T.....T..A..T..T..T..T.CA.....T..T..AA.A.  : 100

                     *          C37        *         140         *
TatOpt     : GCAGCTACCACTGCCTGGTGTGCTTCCAGACCAAGGGCCTGGGCATCAGC : 150
TatC22     : ..................................................... : 150
TatC37     : ....................A.............................. : 150
TatC22C37  : ....................A.............................. : 150
Tatwt      : .T........T..T..A..T.....T.....A..A...T.A.....TTC.  : 150

                   160          *         180          *         200
TatOpt     : TACGGCCGCAAGAAGCGCCGCCAGCGCCGCAGCGCCCCCCCCAGCAGCGA : 200
TatC22     : ..................................................... : 200
TatC37     : ..................................................... : 200
TatC22C37  : ..................................................... : 200
Tatwt      : ..T...A.G........GA.A.....A..A.....T..T..A.....T..  : 200

                     *        220          *         240         *
TatOpt     : GGACCACCAGAACCCCATCAGCAAGCAGCCCCTGCCCCAGACCCGCGGCG : 250
TatC22     : ..................................................... : 250
TatC37     : ..................................................... : 250
TatC22C37  : ..................................................... : 250
Tatwt      : ...T..T..A..T..T..ATCA.........T.A...AGA....TA..G.  : 250

                   260          *         280          *         300
TatOpt     : ACCCCACCGGCAGCGAGGAGAGCAAGAAGAAGGTGGAGAGCAAGACCGAG : 300
TatC22     : ..................................................... : 300
TatC37     : ..................................................... : 300
TatC22C37  : ..................................................... : 300
Tatwt      : ....G..A...TCA.....ATCG.......................A...  : 300

                     *     stop  EcoRI
TatOpt     : ACCGACCCCTTCGACTAAAGAATTC :  325
TatC22     : ........................... :  325
TatC37     : ........................... :  325
TatC22C37  : ........................... :  325
Tatwt      : ..A..T..G.........-.......  :  324
```

Tat protein sequence

```
                     *         20          *         40          *
TatOpt     : MEPVDPNLEPWNHPGSQPKTACNKCYCKHCSYHCLVCFQTKGLGISYGRK :  50
TatC22     : ....................G.............................  :  50
TatC37     : ....................................S.............  :  50
TatC22C37  : ....................G.............S.............  :  50
Tatwt      : ......................T....K.....................  :  50
```

```
                    60          *         80          *        100
TatOpt     : KRRQRRSAPPSSEDHQNPISKQPLPQTRGDPTGSEESKKKVESKTETDPFD* : 101
TatC22     : ..................................................... : 101
TatC37     : ..................................................... : 101
TatC22C37  : ..................................................... : 101
Tatwt      : ......................R.L.......................... : 101
```

rev constructs: DNA sequence

```
            SalI       start                *         40         *
RevOpt    : GTCGACGCCACCATGGCCGGCCGCAGCGGCGACAGCGACGAGGCCCTGCT :  50
RevM5M10  : ..................................................... :  50
Revwt     : ................A..AA.A.....A....A......A..G..T.. :  50

                    60         *         80         *        100
RevOpt    : GCAGGCCGTGCGCATCATCAAGATCCTGTACCAGAGCAACCCCTACCCCA : 100
RevM5M10  : ..................................................... : 100
Revwt     : C..A..A..AA.G........A.....A.....A................. : 100

                   *        120      M5          140         *
RevOpt    : AGCCCGAGGGCACCCGCCAGGCCCGGAAGAACCGCCGCCGCCGCTGGCGC : 150
RevM5M10  : ......................GACCT..................... : 150
Revwt     : ..........T.....A.............T..AA.AA.AA.G...A.A : 150

                  160         *        180         *        200
RevOpt    : GCCCGCCAGCGCCAGATCCACAGCATCAGCGAGCGCATCCTGAGCACCTG : 200
RevM5M10  : ..................................................... : 200
Revwt     : ..AA.A...A.A........T.AG..T..T.....G..T..T.....T.. : 200

                   *        220         *        240      M10
RevOpt    : CCTGGGCCGCCCCGCCGAGCCCGTGCCCTTCCAGCTGCCCCCCATCGAGC : 250
RevM5M10  : ..........................................GA.CT.. : 250
Revwt     : ......A..AT.T.AG.....T.....T..T.T...A..A..G..T...A : 250

                  260         *        280         *        300
RevOpt    : GCCTGCACATCGACTGCAGCGAGAGCAGCGGCACCAGCGGCACCCAGCAG : 300
RevM5M10  : ..................................................... : 300
Revwt     : .A..T..T..T..T...........TG.T..A..TTCT..G..G...... : 300

                   *        320         *    stop  EcoRI
RevOpt    : AGCCAGGGCACCACCGAGGGCGTGGGCAGCGCCTAAAGGAATTC : 344
RevM5M10  : ..................................... : 344
Revwt     : TCT.....G..T..A.....G.....A...C.T..... : 344
```

Rev protein sequence

```
                  *        20         *        M5          *
RevOpt    : MAGRSGDSDEALLQAVRIIKILYQSNPYPKPEGTRQARRKNRRRRWRARQR :  50
RevM5M10  : ..................................DL.......... :  50
Revwt     : .......N..................................... :  50

                 60         *       M10         *       100
RevOpt    : QIHSISERILSTCLGRPAEPVPFQLPPIERLHIDCSESSGTSGTQQSQGT : 100
RevM5M10  : ........................DL..................... : 100
Revwt     : ...K...........SE.....L.............G.......... : 100


RevOpt    : TEGVGSA* : 107
RevM5M10  : ........ : 107
Revwt     : ......P. : 107
```

nef constructs: DNA sequence

```
                             -myr
                SalI      start|             *        40         *
NefOpt        : GTCGACGCCACCATGGGCGGCAAGTGGAGCAAGAGCAGCATCGTGGGCTG :  50
-myrD124      : ................C................................. :  50
-myrD124LLAA  : ................C................................. :  50
Nefwt         : .................G.........TCA..A.....T..AA.T..A.. :  50

                        60         *        80         *       100
NefOpt        : GCCCGCCGTGCGCGAGCGCATCCGCCGCACCGAGCCCGCCGCCGAGGGCG : 100
-myrD124      : .................................................. : 100
-myrD124LLAA  : .................................................. : 100
Nefwt         : ...T.AA..AA.A..AA.A...A.A..A..TAG.T.A..A..A.....A. : 100

                         *       120         *       140         *
NefOpt        : TGGGCGCCGCCAGCCAGGACCTGGACAAGCACGGCGCCCTGACCAGCAGC : 150
-myrD124      : .................................................. : 150
-myrD124LLAA  : .................................................. : 150
Nefwt         : .A..AT.A..GTCT..A...T.A..T..A.GT..G..A..T..A.C.... : 150

                       160         *       180         *       200
NefOpt        : AACACCGCCGCCAACAACGCCGACTGCGCCTGGCTGGAGGCCCAGGAGGA : 200
-myrD124      : .................................................. : 200
-myrD124LLAA  : .................................................. : 200
Nefwt         : .....A...CA..G...T..T..T...........A.A..G..A...... : 200

                         *       220         *       240         *
NefOpt        : GGAGGAGGAGGTGGGCTTCCCCGTGCGCCCCCAGGTGCCCCTGCGCCCCA : 250
-myrD124      : .................................................. : 250
-myrD124LLAA  : .................................................. : 250
Nefwt         : ...A..A..A..A.....T..A..CA.A..T.....A..TT.AA.A..A. : 250

                       260         *       280         *       300
NefOpt        : TGACCTACAAGGCCGCCTTCGACCTGAGCTTCTTCCTGAAGGAGAAGGGC : 300
-myrD124      : .................................................. : 300
-myrD124LLAA  : .................................................. : 300
Nefwt         : ....T..T..A..A..AA.A..T..C........TT.A..A..A.....G : 300

                         *       320         *       340         *
NefOpt        : GGCCTGGAGGGCCTGATCTACAGCAAGAAGCGCCAGGAGATCCTGGACCT : 350
-myrD124      : .................................................. : 350
-myrD124LLAA  : .................................................. : 350
Nefwt         : ..A.....A..GT.A..T...TC......AA.G..A.G......T..TT. : 350

                       360         *          D124G    *       400
NefOpt        : GTGGGTGTACCACACCCAGGGCTTCTTCCCCGACTGGCAGAACTACACCC : 400
-myrD124      : ................................G................. : 400
-myrD124LLAA  : ................................G................. : 400
Nefwt         : ......T..TA....A..A...........T..T.....A........A. : 400

                         *       420         *       440         *
NefOpt        : CCGGCCCCGGCGTGCGCTACCCCCTGACCTTCGGCTGGTGCTTCAAGCTG : 450
-myrD124      : .................................................. : 450
-myrD124LLAA  : .................................................. : 450
Nefwt         : .G..A..A..G..CA.A.TT..A........T..A..............A : 450

                       460         *       480         *       500
NefOpt        : GTGCCCGTGGACCCCCGCGAGGTGGAGGAGGCCAACAAGGGCGAGAACAA : 500
-myrD124      : .................................................. : 500
-myrD124LLAA  : .................................................. : 500
```

```
Nefwt          : .A...A..C.....A.AG..A..A..A........TG.A..A..A..... : 500

                    LL165AA        520          *        540          *
NefOpt         : CTGCCTGCTGCACCCCATGAGCCAGCACGGCATGGAGGACGAGGACCGCG : 550
-myrD124       : ..................................................... : 550
-myrD124LLAA   : ....GC.GC............................................ : 550
Nefwt          : ...TT.A..A.....T..........T..A........T..A...A.A.  : 550

                         560          *        580          *        600
NefOpt         : AGGTGCTGAAGTGGAAGTTCGACAGCAGCCTGGCCCGCCGCCACATGGCC : 600
-myrD124       : ..................................................... : 600
-myrD124LLAA   : ..................................................... : 600
Nefwt          : .A..AT.A.GA........T.....T.TG..A..A...A.A......... : 600

                       *        620          *      stop   EcoRI
NefOpt         : CGCGAGCTGCACCCCGAGTACTACAAGGACTGCGCCTAAAGAATTC     : 646
-myrD124       : ..................................................... : 646
-myrD124LLAA   : ..................................................... : 646
Nefwt          : ........A..T..G.....T.....A..................... : 646
```

Nef protein sequence

```
                -myr       *         20         *         40         *
NefOpt          : MGGKWSKSSIVGWPAVRERIRRTEPAAEGVGAASQDLDKHGALTSSNTAA :  50
-myrD124        : .A.................................................. :  50
-myrD124LLAA    : .A.................................................. :  50
Nefwt           : ..........I...E........RS......S.......R....T....H :  50

                         60         *         80         *        100
NefOpt          : NNADCAWLEAQEEEEEVGFPVRPQVPLRPMTYKAAFDLSFFLKEKGGLEG : 100
-myrD124        : .................................................. : 100
-myrD124LLAA    : .................................................. : 100
Nefwt           : S.......K.........................I.............. : 100

                         *            D124G   *        140         *
NefOpt          : LIYSKKRQEILDLWVYHTQGFFPDWQNYTPGPGVRYPLTFGWCFKLVPVD : 150
-myrD124        : ......................G.......................... : 150
-myrD124LLAA    : ......................G.......................... : 150
Nefwt           : ........G.......N..................F..........E... : 150

                       160  LL165AA         180         *        200
NefOpt          : PREVEEANKGENNCLLHPMSQHGMEDEDREVLKWKFDSSLARRHMARELH : 200
-myrD124        : .................................................. : 200
-myrD124LLAA    : ..............AA.................................. : 200
Nefwt           : .Q......E.......................R.....M........... : 200


NefOpt          : PEYYKDCA* : 208
-myrD124        : ......... : 208
-myrD124LLAA    : ......... : 208
Nefwt           : ......... : 208
```

Appendix B: Raw data for β-galactosidase internal control and normalisation of CAT enzyme levels for Tat and Rev functionality assays.

Normalisation of CAT enzyme levels with the internal transfection control β-Galactosidase for three Tat functionality experiments done in HeLa cells. The raw CAT enzyme readings were multiplied with a normalisation factor, calculated from β-Galactosidase enzyme levels, to control for transfection efficiency.

	experiment 1				experiment 2				experiment 3			
	CAT raw data	β-Gal	factor	normal. CAT	CAT raw data	β-Gal	factor	normal. CAT	CAT raw data	β-Gal	factor	normal. CAT
Mock	5.0	0.92	1.00	**5.0**	0.0	1.12	1.00	**0.0**	0.0	1.17	1.00	**0.0**
pLTR-CAT	4.7	0.87	1.06	**5.0**	0.0	1.16	0.96	**0.0**	0.0	1.27	0.93	**0.0**
Tat_{WT}	712.9	1.07	0.86	**613.0**	203.6	1.09	1.03	**210.2**	537.0	1.17	1.00	**538.4**
Tat_{OPT}	826.1	1.25	0.74	**608.0**	213.8	1.01	1.11	**237.6**	340.5	1.10	1.07	**363.1**
Tat_{C22}	399.1	0.90	1.02	**408.0**	196.1	0.94	1.20	**234.4**	202.0	1.02	1.15	**231.4**
Tat_{C22C37}	5.1	0.94	0.98	**5.0**	0.0	0.98	1.15	**0.0**	0.0	1.06	1.11	**0.0**
Tat_{C37}	0.0	1.20	0.77	**0.0**	0.0	1.08	1.04	**0.0**	0.0	1.15	1.02	**0.0**

β-Gal, β-Galactosidase reading; factor, normalisation factor calculated from β-Galactosidase reading, mock was set as 1; normal. CAT, normalised CAT level.

Normalisation of CAT enzyme levels with the internal transfection control β-Galactosidase for three Rev functionality experiments done in 293T cells. The raw CAT enzyme readings were multiplied with a normalisation factor, calculated from β-Galactosidase enzyme levels, to control for transfection efficiency.

	experiment 1				experiment 2				experiment 3			
	CAT raw data	β-Gal	factor	normal . CAT	CAT raw data	β-Gal	factor	normal . CAT	CAT raw data	β-Gal	factor	normal . CAT
Mock	5.0	1.50	1.00	**5.0**	8.5	1.76	1.00	**8.5**	0.0	1.15	1.00	**0.0**
pDM128	6.4	1.91	0.79	**5.0**	25.0	1.87	0.94	**23.5**	0.0	1.26	0.91	**0.0**
pRSV-Rev	1119.0	1.87	0.80	**897.6**	ND	ND	ND	ND	731.2	1.20	1.05	**767.7**
Rev_{WT}	395.3	1.80	0.83	**329.4**	110.2	1.66	1.06	**116.8**	278.2	1.22	0.98	**273.7**
Rev_{M5M10}	5.8	1.74	0.86	**5.0**	13.2	1.72	1.02	**13.5**	0.0	1.33	0.92	**0.0**

β-Gal, β-Galactosidase reading; factor, normalisation factor calculated from β-Galactosidase reading, mock was set as 1; normal. CAT, normalised CAT level; ND, not done.

Appendix C: Characterisation of the South African HIV-1 subtype C complete 5' LTR, nef and regulatory genes

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Sequence Note

Characterization of the South African HIV Type 1 Subtype C Complete 5′ Long Terminal Repeat, *nef,* and Regulatory Genes

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) subtype C has become the major etiological agent in the global and especially African epidemic. To gain better understanding of the genetic diversity and rapid transmission of HIV-1 subtype C, we have characterized the complete 5′ long terminal repeat (LTR) region along with the regulatory genes *tat* and *rev* as well as the accessory gene *nef* of 14 South African HIV-1 subtype C isolates. Phylogenetic analysis revealed a subtype C 5′ LTR cluster, as well as subclustering of our *nef* sequences with various subtype C strains separate from the India and China subclusters. At least 3 NF-κB sites were present in the 5′ LTR of most isolates and 13 isolates had the subtype C-specific Rev truncation. Some length variation in exon 2 and the absence of a critical cysteine were found in Tat. Residue variation in the myristoylation signal and motifs involved in CD4 and MHC-I downregulation was recorded in our *nef* gene sequences.

THE HUMAN IMMUNODEFICIENCY VIRUS type 1 (HIV-1) subtype C epidemic is responsible for most HIV-1 infections worldwide and close to half of the HIV-1 infections in sub-Saharan Africa.[1] This epidemic has attained a rapid global distribution and, whereas transmission of other subtypes such as B seems to be on the decline, HIV-1 subtype C is spreading at alarming rates.[1,2] To date, the majority of genetic characterization studies of South African HIV-1 strains have been focused on the *env* and the *gag* genes, and thus little is known about the regulatory regions and genes of local subtype C strains. Specifically, the 5′ long terminal repeat (LTR) region as well as the *tat* and *rev* genes of South African subtype C strains remain to be comprehensively characterized. Thus analysis thereof may provide more data about the diversification of this efficiently transmitted subtype. In this study we sequenced and phylogenetically analyzed the *tat, rev,* and *nef* genes as well as the 5′ LTR of 14 previously described HIV-1 subtype C isolates from South Africa.[3,4]

The three genomic regions containing *tat* and *rev* exons 1 and 2 as well as *nef* were amplified in a nested polymerase chain reaction (PCR) from a 9-kb, nearly full-length fragment as described previously.[3,4] The 5′ LTR was amplified by single-step PCR from DNA prepared from primary isolates. Primer pairs NefF (5′-CCTAGAAGAATAAGACAGGGCTT-3′) and NefR (5′-CCTGGAACGCCCCAGTGG-3′); NewS1_1T/NewS1_1C (5′-GCAGGACGTCAAGCTTGGAAGGGTTA-ATTTACTC[T/C]AAGAA-3′) and S1_651Not (5′-CCT-TGGCCAGCGGCCGCCCCTGTTCGGGCGCCACTGCTA-GAGA-3′); TatX1F (5′-AATTGGGTGCCAGCATAGC-3′) and NACD (5′-CCATAATAGACTGTGA-CCCACAA-3′); and RevX2F (5′-TGCTGTGCTCTCTATAGTRA-3′) and RevX2R (5′-TCCTATCTGTTCCTTCAGCTA-3′) were used for the amplification of the ~660-bp fragment containing the *nef* gene, the ~640-bp 5′-LTR region, the ~570-bp fragment containing the *tat/rev* exon 1, and the ~350-bp fragment containing the *tat/rev* exon 2, respectively. 5′-LTR amplicons of

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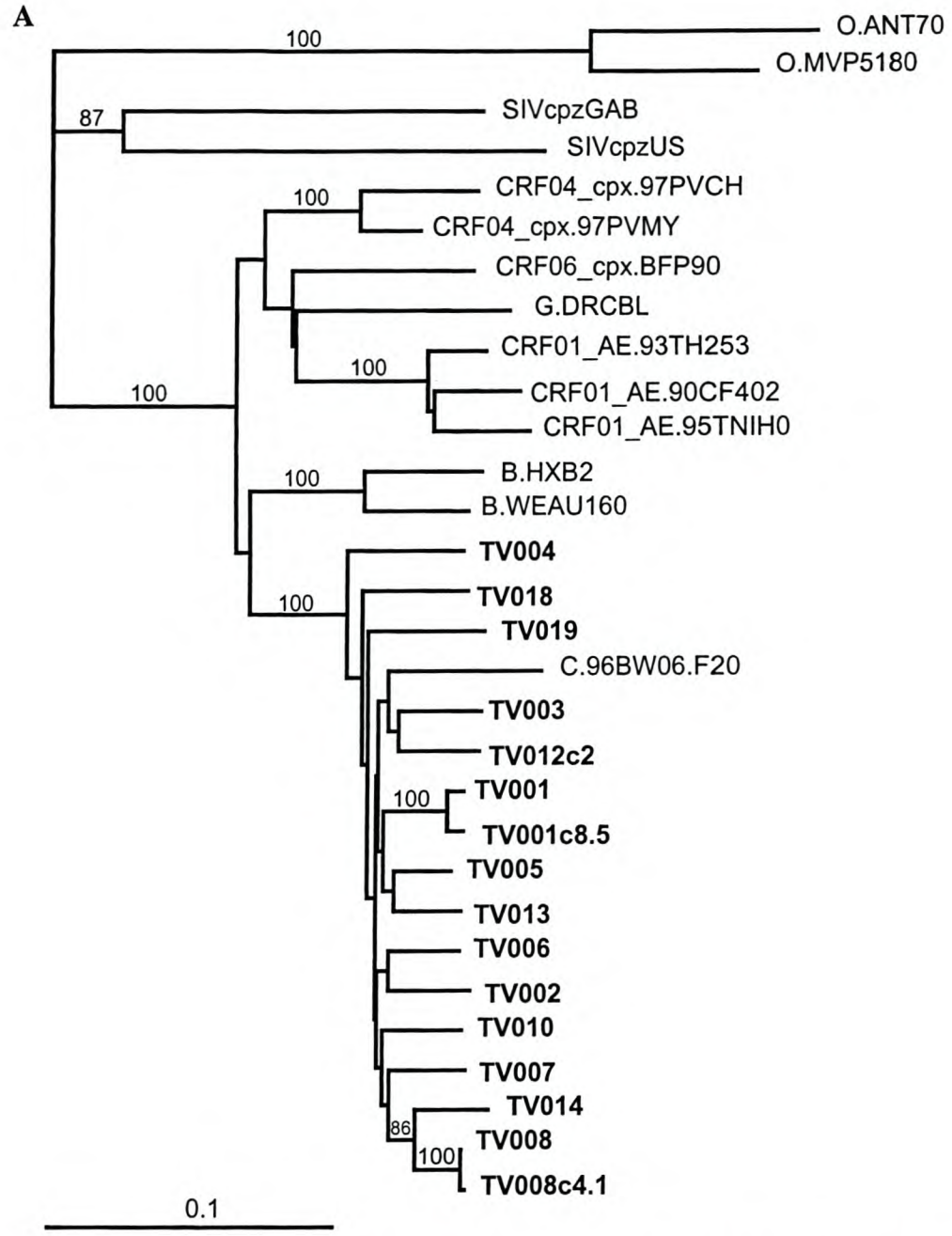

FIG. 1. Rooted trees depicting the evolutionary relationships among the 5′ LTR region (**A**) as well as the *nef* (**B**) nucleotide sequences from South African HIV-1 subtype C isolates. The Kimura two-parameter distance matrix and neighbor-joining method were used in the phylogeny calculations. The distance scale indicates 0.1 nucleotide replacements per site. Horizontal branch lengths are drawn to scale and the validity of the branching orders was estimated with 500 bootstrap replicates. The significant (above 85%) bootstrap values are indicated at the respective branch bases.

three isolates (TV001, TV004, and TV012) were cloned into the pGEM-11Zf(+) vector (Promega, Madison, WI) according to the manufacturer protocol and can be identified by their clone numbers. Sequencing reactions were carried out by using the ABI Prism BigDye terminator cycle sequencing protocol and sequencing was performed on a 310 genetic analyzer (PE Applied Biosystems, Foster City, CA). The PCR primers were used as direct sequencing primers with the addition of the LTRFor (5′-ACAAAGACTGCTGACACA-3′) and LTRRev (5′-TGTGTCAGCAGTCTTTGT-3′) primers for the 5′ LTR region. Sequences were verified manually and sequence assembly, multiple alignments, and translation into predicted amino acid sequences were performed as described.[3,4] Calculation of distance matrices, phylogeny inference, and construction of trees were performed with the PHYLIP software packages SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE. Rooting and manipulation of the trees were done in TREEVIEW.

The phylogenetic tree constructed with all full-length 5′ LTR

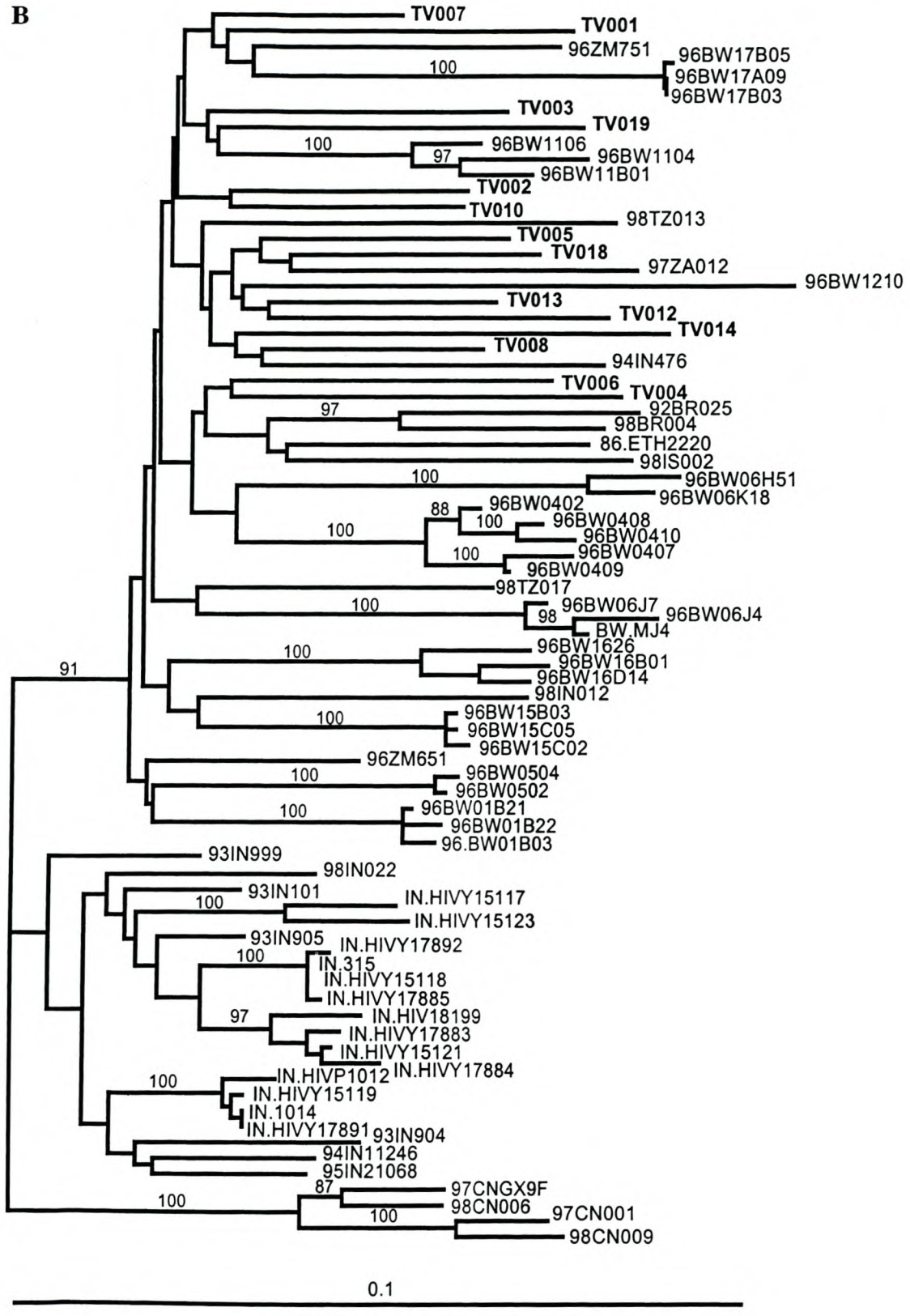

FIG. 1. *Continued.*

sequences from the Los Alamos database (Fig. 1A) shows a clustering of our isolates with the only available subtype C full-length 5′ LTR from Botswana (isolate 96BW06.F20). The tree constructed with all available subtype C *nef* coding sequences (approximately 625 bp in length) (Fig. 1B) shows the South African isolates including the recently described 97ZA012.1 (AF286227) from Durban,[5] subcluster with isolates from Botswana, Ethiopia, Brazil, Tanzania, and Zambia as well as one Israeli and two Indian isolates. This monophyletic subtype C clade is distinctly separate from the Chinese and Indian clades, as supported by statistically significant bootstrap values. These data thus display a lesser degree of diversity for the South African strains than was reported for the *gag* and *env* genes[4] as well as the genomic fragment encompassing the *vif, vpr,* and *vpu* genes.[3] In contrast with the *nef* sequence results, these previous studies showed a diversity within the South African isolates that virtually matches the diversity of the global C subtypes studied to date as a whole. However, this finding is expected because *nef* is a smaller, highly conserved coding region. Interestingly, two Indian sequences fall among the main monophyletic clade. Considering the distinct subclustering of the Indian isolates, which indicates that they share a common ancestor, this suggests that these two isolates may have originated in sub-Saharan Africa and migrated to or been transmitted in India through the frequent movement of people.

Nucleotide alignments of the full-length 5′ LTR sequences confirm the presence of all potential transcription factor-binding sites, with the third NF-κB site reported for HIV-1 subtype C sequences (Fig. 2[5a]).[6–8] All three sites in most of our isolates conform to the reported NF-κB sequence,[9] while NF-κB-like sites have been identified in TV008 [GGGGCG**G**TCC], TV012c2 [GG**A**ACTTTCC], and TV019 [GGGACTTTC**T**]. The C10T substitution seen in TV019 has previously been identified in another South African isolate, 98ZA445ZA.[8] In their previous report, Rodenburg *et al.*[5] described frequent deletions or mutations in the third NF-κB site, questioning the causal role of the third site in the explosive subtype C epidemic. In contrast, 12 of our isolates have 3 NF-κB sites and only TV008 and TV012 have two sites, with a third NF-κB-like site. Our data thus indicate that in the majority of South African isolates analyzed in this study, the presence of the three NF-κB sites may well confer a stronger promoter activity resulting in more rapid transmission. The Sp1-I, Sp1-II, and Sp1-III sites in the core promoter are conserved, with Sp1-III being the most variable and Sp1-II the most conserved. This contrasts with a report stating that Sp1-I is the more conserved element.[10] The guanine residue at position 4 of the Sp1 sites, imperative for maximizing Sp factor recruitment, is conserved for all our sequences.[10] A fourth Sp1 site (positions −433 to −441) discovered to be essential for negative-sense transcription in the HXB2 sequence (5′-TGGGAGTGA-3′) has up to three substitutions in our sequences.[11] The NF-AT sites show little divergence among our isolates, but differ substantially from the HXB2 sequence. A reported subtype C consensus in the core region of the NRE (CGCARACAYVTK) was almost perfectly conserved in all our sequences.[12] Johansson *et al.*[13] reported that the G/A CAGA motif within the above-mentioned sequence is diagnostic for subtype C strains. All but three of our isolates possess this motif: TV003 displays a G4C, TV010 a C2T, and TV019 a G4A substitution. The alanines at positions 5 and 9, as well as the guanine at position 10, of the TCF-1α site are conserved across our isolates and the reference strains, with the exception of subtype B and group O. TV019 has a C-to-T substitution in the bulge of the TAR region, which has been identified in subtype G, circulating recombinant forms, as well as SIV strains. The T-to-C nucleotide change at position 2 of the loop structure in the TAR element occurs in eight of our isolates.

The deduced Nef amino acid sequences (Fig. 3A) show previously described length variations, especially in the reported N-terminal variable region. Thirteen South African isolates have seemingly intact Nef open reading frames. The premature stop in the N-terminal of the protein sequence of isolate TV006 suggests that this isolate would start translation at the second initiation site.[14,15] This protein would lack the myristoylation signal and would thus, because of unsuccessful myristoylation, most probably not fold correctly or be directed to the membrane.[16] All isolates with the exception of TV005, TV006, TV012, and TV013 as well as reference strain 96BW12-10 from Botswana have conserved GGXXS motifs in their myristoylation signals. It has been described that GSXXS mutations are possibly associated with slow disease progression[17] and that deletion or substitution of the glycine residues, to which the myristylic acid is linked, prohibits myristoylation.[18] Other studies and sequences in the Los Alamos sequence database suggest sequence diversity is not uncommon among myristoylation signals.[19] Residue and length variations observed in our Nef proteins correlate with those in the other subtype C strains, suggesting relatedness. Most of the significant domains such as those involved in CD4 and MHC-I downregulation as well as altering of cell signaling are found to be well conserved in all sequences and variations correlate with results obtained in previous studies.[17,19–22] Furthermore, considering the presence of intact WL_{57} motifs, which allow for proteolytic cleavage, post-translational modification of Nef seems to be intact. None of the gene deletions associated with nonprogression or slow progression of HIV-1 disease have been found to be present in our isolates and, in fact, the disease progression of the 14 patients is not in any way abnormal.[3] However, some of the amino acid variations reported to be more frequent in progressors as opposed to nonprogressors[20] were present in our sequences as well as some subtype C reference sequences. Specifically, the A15, C163, and Q170 variations were found in most subtype C sequences. In addition, the N51 amino acid variation found to be more frequent in nonprogressors was predominant in subtype C.[20]

Whereas most South African isolates have Tat amino acid sequences of 101 residues in length, some length variation was found in 5 isolates (Fig. 3B). TV001 lacks the orthodox stop at position 102 and is terminated at the next position, whereas TV004, TV010, TV012, and TV018 terminate prematurely at position 100. Yet, all 14 isolates show intact open reading frames and, interestingly, this two-residue truncation in Tat has been identified in HIV-1 subtypes D, F, H, J, and K. Notably, the basic domain GRKKRRQRR (residues 48–56) as well as the highly conserved ESKKKVE motif are 100% conserved among all subtype C sequences except in TV012, which contains an EPKKKVE motif. The critical cysteine residues at positions 22, 25, 27, 30, 34, and 37 of all sequences are found to be 100% conserved. Yet at position 31, all isolates except

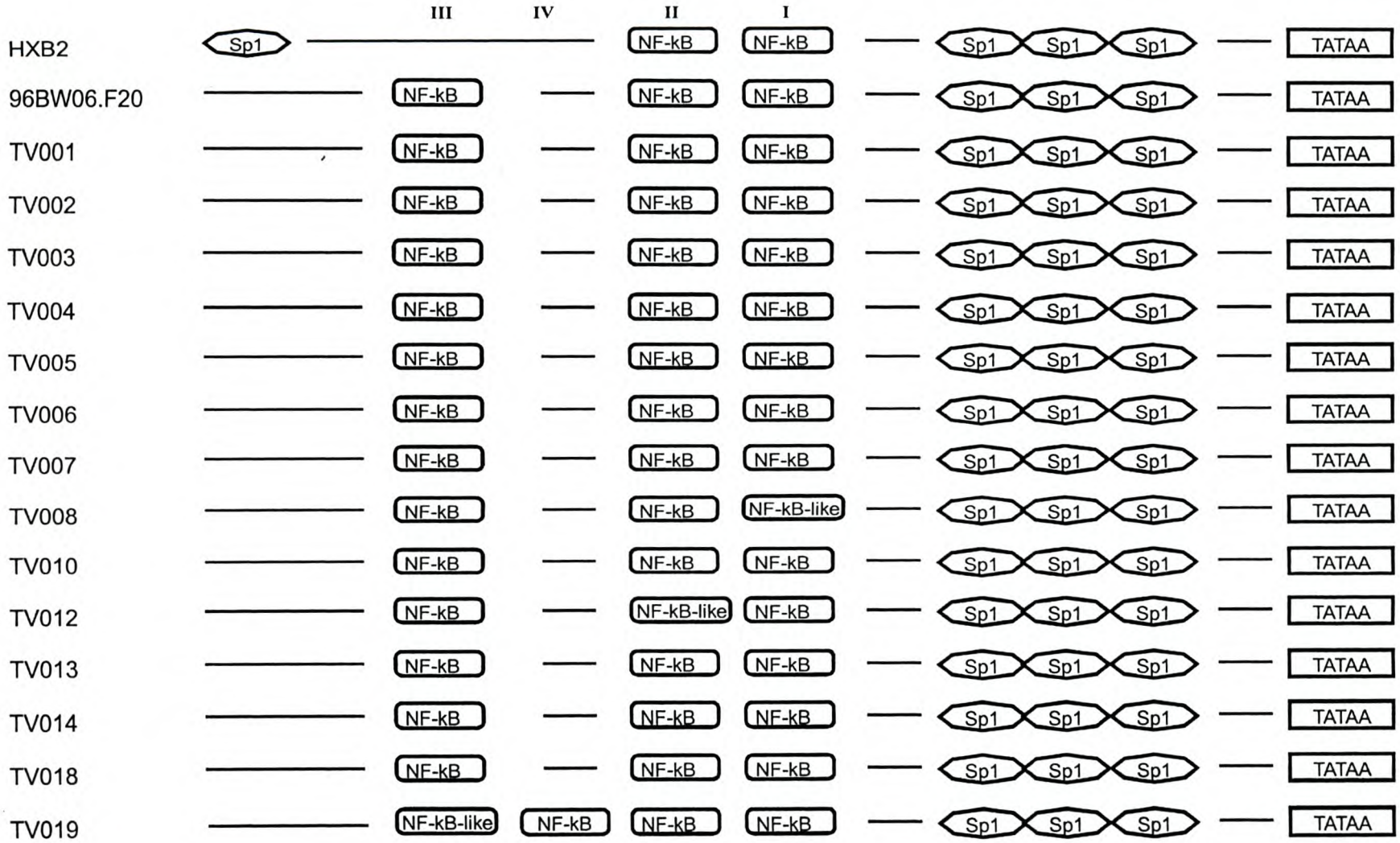

FIG. 2. Graphical representation of the nucleotide sequence alignment of the 5′ LTR from South African subtype C isolates along with the available subtype C strain from Botswana and HXB2. Regulatory elements are represented as blocks for clarity. For a detailed review of these elements refer to Pereira *et al.*[5a]

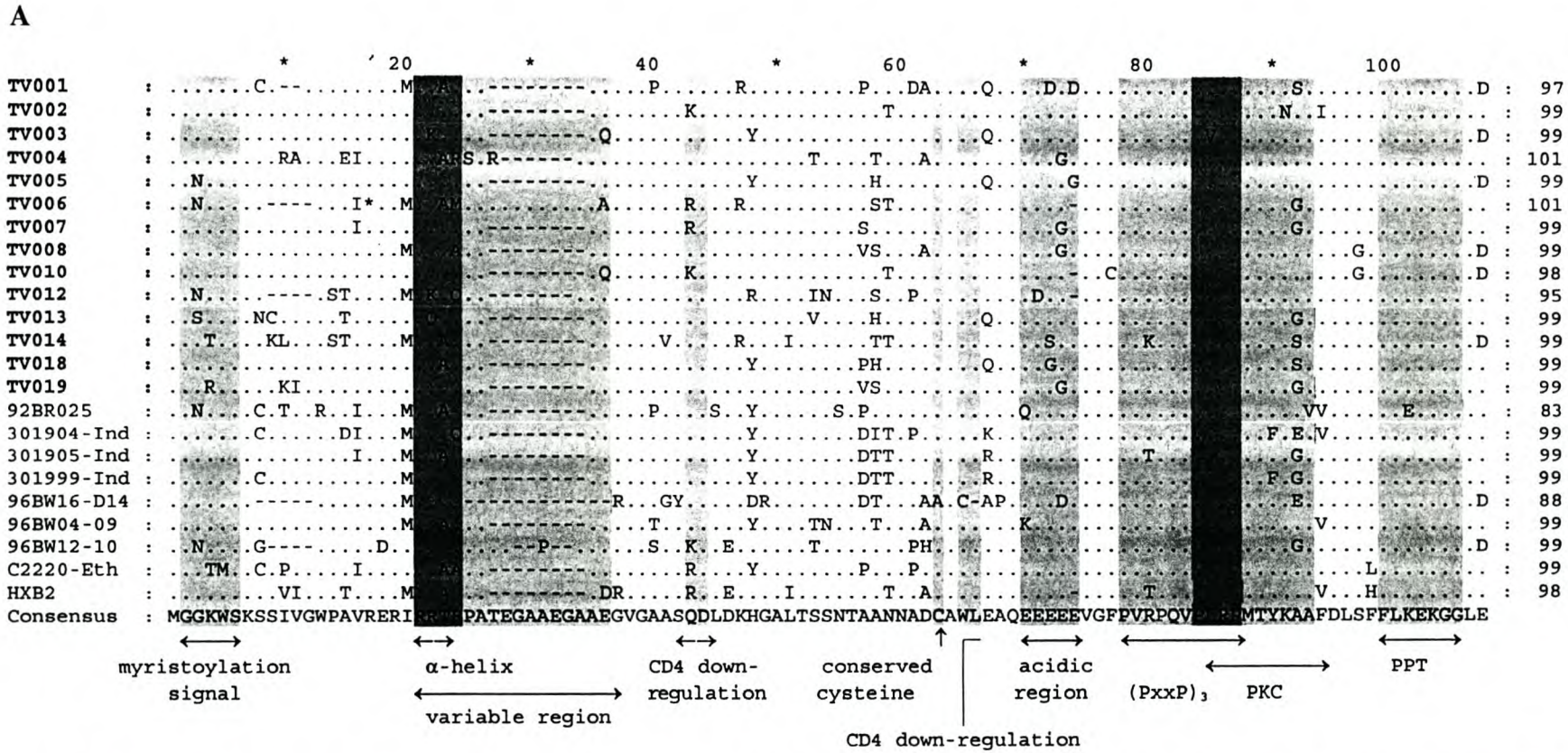

FIG. 3. Alignments of the deduced amino acid sequences of Nef (**A**), Tat (**B**), and Rev (**C**) from South African subtype C isolates (in boldface) in conjunction with some subtype C reference strains as well as HXB2. Dots represent identical residue sequences, dashes represent gaps, and asterisks represent stop codons. Significant protein domains and conserved motifs are shaded and labeled.

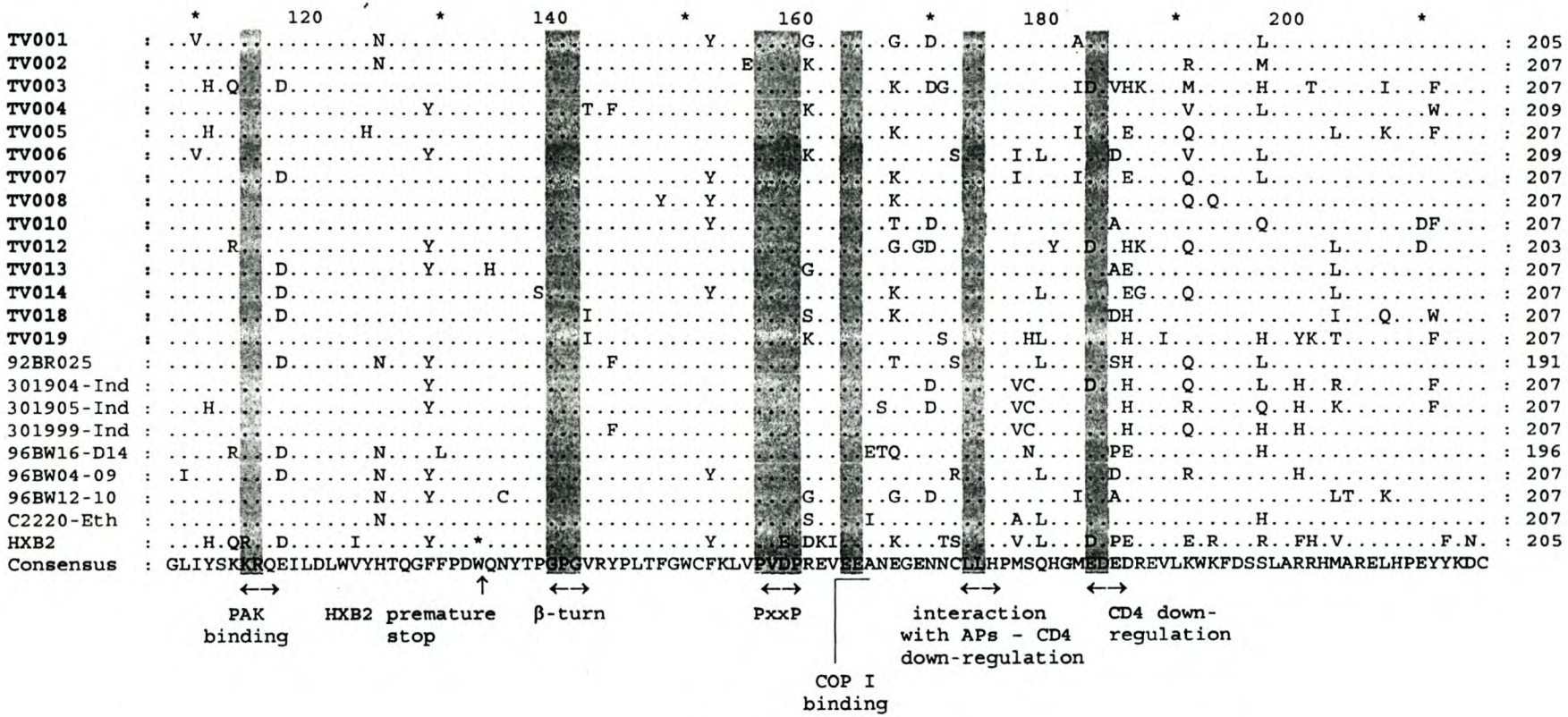

FIG. 3. *Continued.*

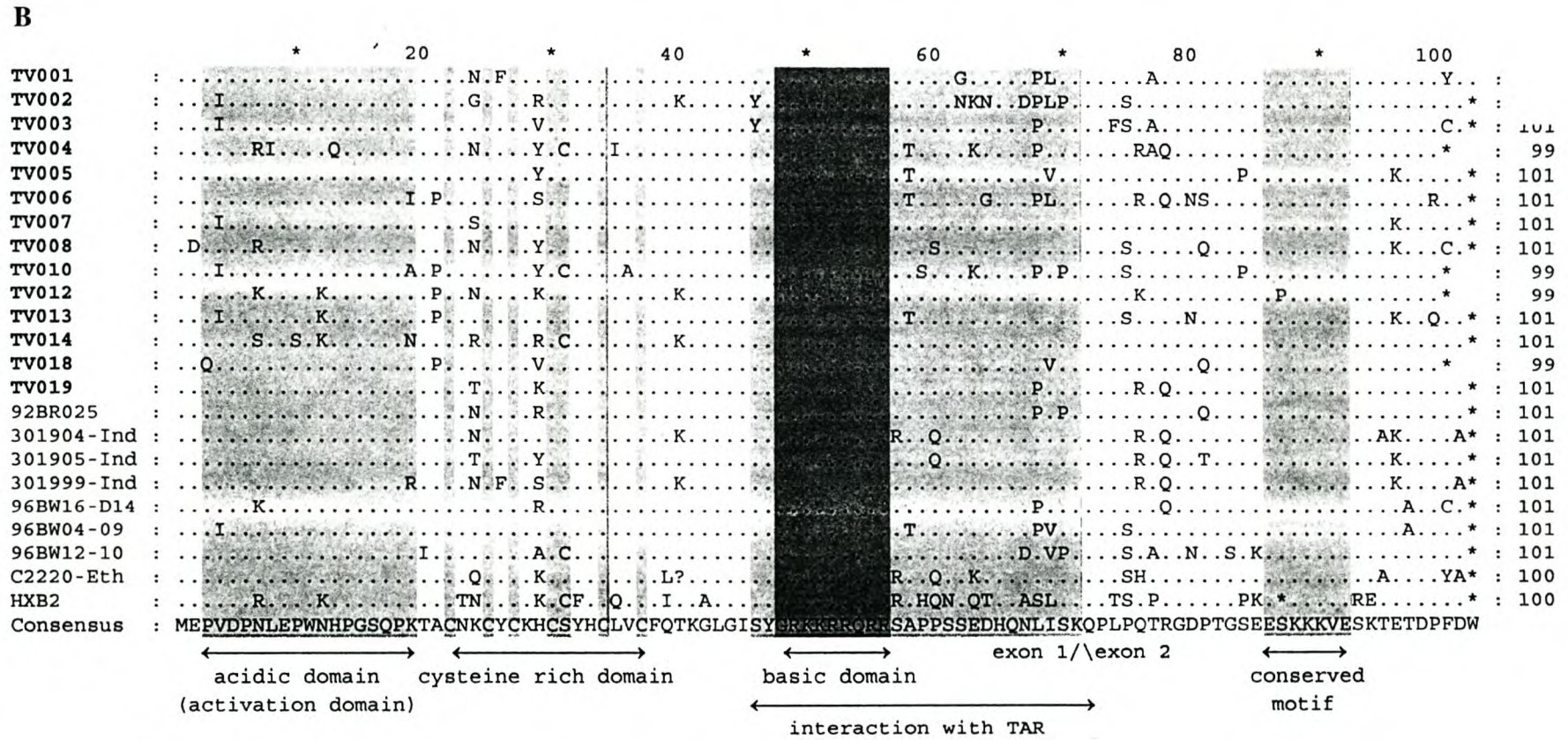

FIG. 3. *Continued.*

C

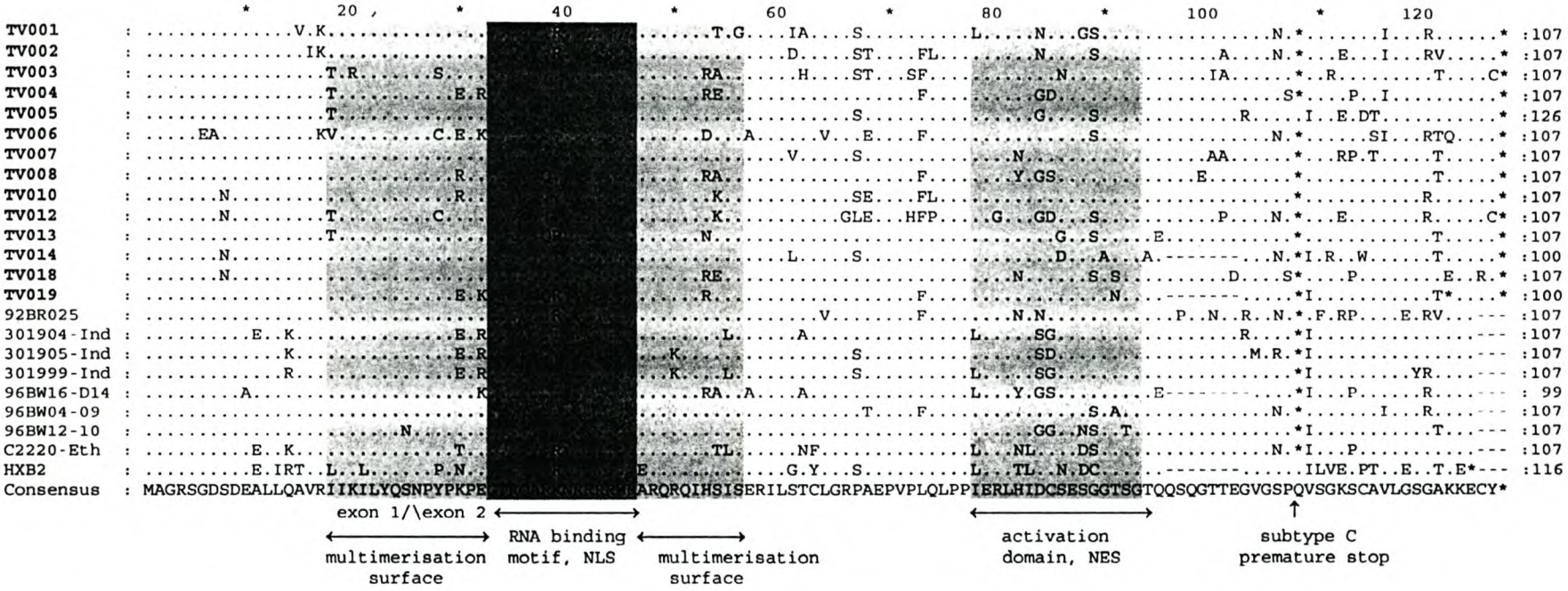

FIG. 3. *Continued.*

TV004, TV010, and TV014 have a putative serine residue instead of the prevalently conserved cysteine. Similarly to Tat, Rev shows well-conserved amino acid sequences with some variation in the C-terminal region and intact reading frames in all isolates (Fig. 3C). The truncation in exon 2 of Rev at position 108, which seems to be exclusive to HIV-1 subtype C strains, is found in our isolates. Notably, isolate TV005 does not have this subtype C-specific Rev truncation and thus has an Rev protein 126 amino acids in length. Isolates TV014 and TV019 have a seven-residue deletion directly following the nuclear export signal (NES), which is also present in one subtype C reference strain. In correlation with Tat and Nef, residue variations within both exons of Rev in our isolates are similar to those seen in the other subtype C strains.

As reported in the study of the *env* genes of these isolates,[4] no significant or prominent sequence variations in the Rev responsive element that may have implications on interaction with the Rev protein have been detected.

The rapid transmission of HIV-1, and in particular HIV-1 subtype C, has been the topic of extensive research to fathom the mechanisms involved. Yet, limited data concerning the subtype C epidemic in southern Africa make this a daunting task. Reports have suggested that the more rapid transmission of subtype C in relation to the other subtypes could be due to higher levels of LTR promoter activity resulting in upregulation of viral transcription.[6,23] This could be attributed to either the extra NF-κB-binding site and/or the distinctly different NRE sequence in the LTR of subtype C strains. The upregulation of transcription should thus be apparent in the isolates that show a conserved subtype C NRE region, especially TV019, which has three NF-κB sites as well as an additional NF-κB-like site. Gene expression should be efficient in all our isolates, as a result of the TATA box being well conserved. Moreover, stronger C-strain transcription may possibly be the combined effect of these unique features in the LTR along with the differences found in the cysteine-rich domain of subtype C Tat. Being the *trans*-activator of HIV-1 gene expression, mutations in the functional domains of Tat may well have significant effects on interaction with the promoter regions in the LTR. However, in their study, Jeang *et al.*[24] found that although a C31E substitution results in decreased *trans*-activation activity of Tat, a C31S substitution has no significant effect. In addition to the missing critical cysteines, the two-residue truncation in exon 2 of Tat in some isolates could possibly also contribute to variable interaction with the LTR. If the truncation of Rev confers enhancing effects on the efficiency of Rev-dependent transcript export and thus expression of late viral mRNAs and their proteins, this may point to a coevolution of the subtype C LTR, Rev, and Tat to constitute a transcriptional regulation pathway that functions at higher levels. Alternatively, the rapid transmission of subtype C could be a result of nonvirological factors and further studies will have to be performed to investigate the validity of these hypotheses. The local subtype C isolate that did not contain the signature truncation in Rev at position 108 (TV005) was the only isolate that was able to utilize the coreceptors CXCR4, CCR2b, CCR3, and CCR4 in addition to CCR5 in HOS-CD4 coreceptor transfectants. However, it was still nonsyncytium inducing in MT-2 cells and no changes in charge associated with the syncytium-inducing phenotype were present in the V3 loop area of the envelope protein.[25]

When taken alongside the characterization of the *env*, *gag*, and accessory gene sequences, these data show that the 14 isolates from Tygerberg Hospital are nonrecombinant subtype C isolates representative of the South African HIV-1 subtype C epidemic.

ACKNOWLEDGMENTS

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SEQUENCE DATA

GenBank accession numbers are as follows: *rev* exons 1 and 2, AY047230–AY047257; *tat* exons 1 and 2, AY047258–AY047285; *nef*, AY047286–AY047299; LTRs, AY047300–AY047315.

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Appendix D: Plasmid vectors

1. pCR3.1 (Invitrogen, CA, USA)
2. pcDNA3.1 (Invitrogen, CA, USA)

Eukaryotic TA Expression Kit
Bidirectional
Version J
053102
25-0053

Eukaryotic TA Expression Kit Bidirectional

Catalog nos. K3000-01, K3000-40

A Limited Label License covers this product (see Purchaser Notification). By use of this product, you accept the terms and conditions of the Limited Label License.

Invitrogen™
life technologies

www.invitrogen.com
tech_service@invitrogen.com

Introduction

Overview

Purpose

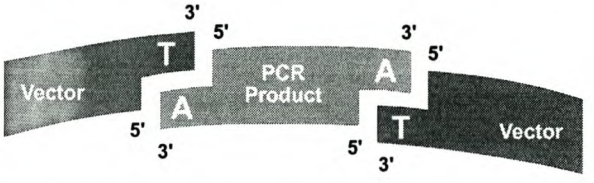
The Eukaryotic TA Expression Kit from Invitrogen provides a quick, one-step system to directly ligate a *Taq* polymerase-amplified PCR product into a mammalian expression vector. Once cloned, analyzed, and transfected, the PCR product can be directly tested for expression in mammalian cell lines.

How Eukaryotic TA Cloning® Works

Taq polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3′ ends of duplex molecules. The linearized vector supplied in this kit has single 3′ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Diagram

The diagram below shows the concept behind TA Cloning®.



Advantages

Using the Eukaryotic TA Expression Kit...

- Eliminates any enzymatic modifications of the PCR product.
- Does not require the use of PCR primers that contain restriction sites.
- Allows direct expression in mammalian cell lines without additional subcloning.

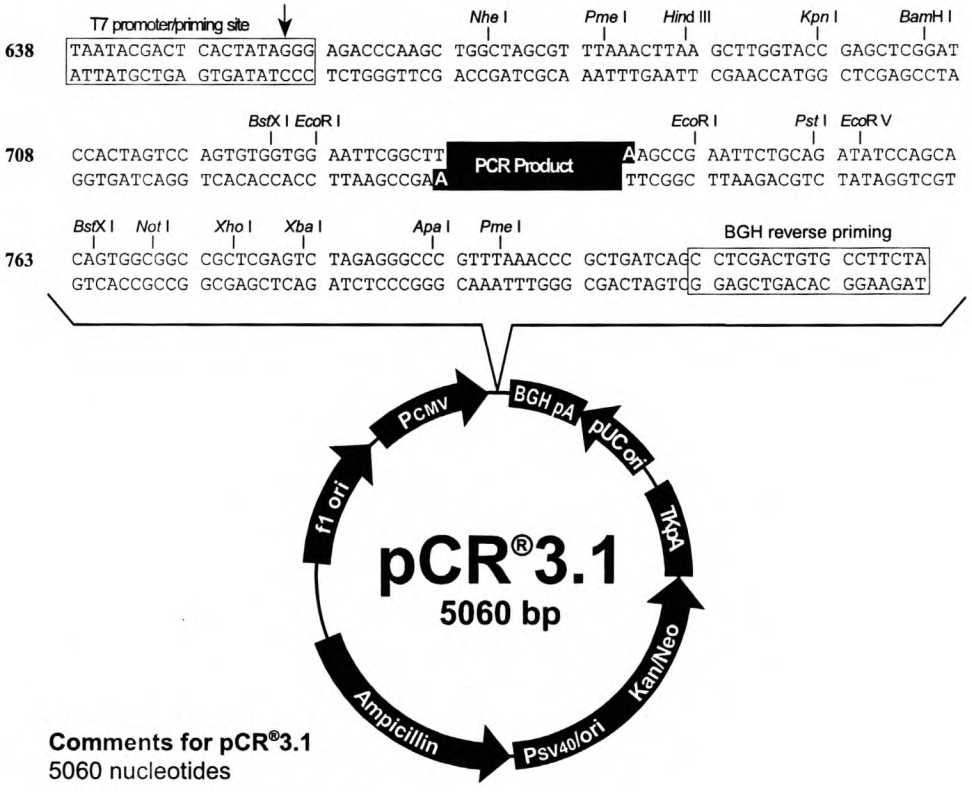
Note

Thermostable polymerases containing extensive 3′- to 5′-exonuclease activity, such as *Vent*™ and *Pfu*, do not leave 3′ A-overhangs. PCR products created using these enzymes can be cloned efficiently into TA Cloning® vectors after incubation with *Taq* polymerase (see page 15). Incubation with *Taq* polymerase adds 3′ A-overhangs to blunt ends to allow efficient cloning of blunt PCR products using TA Cloning® vectors.

The pCR®3.1 Vector

Figure of pCR®3.1

The figure below shows the map of pCR®3.1. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of T7 transcription. The complete sequence can be downloaded from our Web site (www.invitrogen.com) or requested from Technical Service (see page 24). Note that pCR®3.1 is supplied linearized at the TA Cloning® site.



Comments for pCR®3.1
5060 nucleotides

CMV promoter: bases 1-596
Putative transcriptional start: bases 620-625
T7 promoter/priming site: bases 638-657
Multiple cloning site: bases 670-801
TA Cloning® site: 737-738
BGH reverse priming site: bases 813-831
BGH polyadenylation site: bases 812-1026
pUC origin: bases 1116-1789
SV40 promoter and origin: bases 3194-3532 (complement)
Neomycin/kanamycin resistance gene (ORF): bases 2371-3159 (complement)
Thymidine kinase polyadenylation site: bases 1926-2196 (complement)
Ampicillin resistance gene (ORF): bases 3611-4471 (complement)
f1 origin: bases 4602-5058

Continued on next page

The pCR®3.1 Vector, continued

Features

The table below describes the features of pCR®3.1 (5060 bp)

Feature	Function
Cytomegalovirus (CMV) immediate-early promoter	High-level expression of cloned gene
T7 promoter and priming site	*In vivo* or *in vitro* transcription of sense RNA Sequencing of insert
TA Cloning® site	Insertion of *Taq* polymerase-generated PCR product
Polylinker	Unique restriction sites allow excision of PCR product, if desired
BGH reverse priming site	Sequencing of insert
Bovine growth hormone polyadenylation signal	mRNA stability and effective termination
SV40 early promoter	Expression of the kanamycin gene for G418 selection in mammalian cells
SV40 origin	Episomal replication in cells containing SV40 large T antigen
Neomycin/kanamycin resistance gene	Selection and generation of stable mammalian cell lines Selection and maintenance in *E. coli* **Note**: pCR®3.1 confers resistance to kanamycin by means of a cryptic promoter. The bacterial *kan* promoter was not subcloned into the vector.
Ampicillin resistance gene	Selection and maintenance in *E. coli*
pUC origin	Replication, maintenance, and high copy number in *E. coli*
f1 origin	Rescue of sense strand for mutagenesis and single-strand sequencing

Methods

Experimental Outline

Description

In order to clone into pCR®3.1, it is necessary to first generate the PCR product desired. Once your PCR product is synthesized, it is ligated into pCR®3.1 and transformed into One Shot® (TOP10F′) competent cells. The PCR product generated can ligate into the vector in either orientation. Individual recombinant plasmids need to be analyzed by restriction mapping for orientation. The recombinant plasmid with the correct orientation is purified for transfection into mammalian cell lines.

You should already have a mammalian cell line of choice and a protocol for transfection. References for transfection of mammalian cell lines are found in the **Reference** section (see page 29).

Experimental Process

The table below describes the general steps needed to clone your PCR product into pCR®3.1.

Step	Action	Page
1	Design your PCR primers to clone the DNA of interest	5
2	Amplify your PCR product	5
3	Ligate the PCR product into pCR®3.1	7
4	Transform the ligation mixture into TOP10F′ cells	9
5	Select transformants and analyze for insert and orientation	10
6	Isolate clean plasmid DNA for transfection into mammalian cells	11
7	Assay for expression of the PCR product	12

When using the Eukaryotic Expression Kit for the first time, it is recommended that you complete the control reactions for PCR, ligation, and transformation along with your sample. This will help you analyze any possible problems with your experiment.

Producing PCR Products

Design of PCR Primers

Design of the PCR primers is critical for expression of the desired gene of interest. pCR®3.1 is a nonfusion expression vector that does not contain an ATG initiation codon. If there is no initiating ATG codon or optimal sequences for translation initiation (Kozak sequences) in the DNA to be amplified, then these features need to be incorporated into your forward primer.

Kozak Sequence

In general, we recommend that you include a Kozak translation initiation sequence for proper initiation of translation in mammalian cells. An example of a Kozak consensus sequence is provided below. Note that other sequences are possible, but the A or G at position -3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is shown underlined.

A/G NN<u>ATG</u>**G**

Guidelines for PCR

Generally, 10-100 ng of DNA is sufficient to use as a template for PCR. If amplifying a pool of cDNA, the amount needed depends on the relative abundance of the message of interest in your mRNA population. For optimal ligation efficiencies, We recommend using no more than 30 cycles of amplification.

Amplifying Your Product

1. Set up the amplification reaction in a 50 µl volume as follows. For the DNA template, use 10 ng if you are using plasmid DNA and 100 ng if you are using genomic DNA.

DNA template	10-100 ng
10X PCR buffer	5 µl
dNTP Mix	0.5 µl
Primers	1 µM each
Taq DNA polymerase (1 unit/µl)	1 µl
Sterile water	bring volume to 50 µl
Total Volume	50 µl

2. Overlay the reaction with 70 µl of mineral oil.
3. Perform 20-30 amplification cycles with a final 7-10 minute extension step. The final 7-10 minute extension step is necessary to make sure all DNA is double-stranded with 3′ A-overhangs.
4. Remove 10 µl from each amplification reaction and analyze by gel electrophoresis in a 0.8-1.5% agarose gel. You should see a discrete band. If you do not see a discrete band, see the next page. Keep the amplification reaction on ice or store at -20°C.
5. Quantify the amount of DNA (µg/ml) by measuring against a known standard run on the same gel. Proceed to **Cloning into pCR®3.1.**

Continued on next page

Producing PCR Products, continued

Note

Smearing or multiple banding of product may necessitate gel purification. Gel purification may decrease ligation efficiency. It is recommended that your PCR reactions be optimized to avoid gel purification. Innis, *et al.* provides suggestions to optimize your PCR reaction. Invitrogen also offers the PCR Optimizer™ Kit (Catalog no. K1220-01) that incorporates some of the recommendations found in this reference.

Cloning into pCR®3.1

Before Starting

You should have a fresh solution of your PCR product that gives a discrete band on an agarose gel. There is usually no need to clean or purify the PCR product after the final amplification cycle.

Note

Since **there is no blue-white screening for the presence of inserts**, individual recombinant plasmids need to be analyzed by restriction analysis for the presence and orientation of insert. The T7 Forward and BGH Reverse primers can be used to sequence across an insert in the multiple cloning site to also confirm orientation.

For optimal ligation efficiencies, Invitrogen recommends using fresh (less than 1 day old) PCR products because the single 3′ A-overhangs on the PCR products may degrade over time, reducing ligation efficiency.

The pCR®3.1 vector is stable for 6 months from date of purchase if it has not been subjected to freeze-thaw cycles. Care must be taken when handling the linearized vector as loss of the 3′ T-overhangs will cause a blunt-end self-ligation of the vector and subsequent decrease in ligation efficiency with your product.

Cloning into pCR®3.1

1. Centrifuge one vial of pCR®3.1 briefly to collect all the liquid in the bottom of the vial.
2. Mark the date of first use on the vial, and if there is any vector remaining after the experiment, store at -20°C or -70°C.
3. Use the formula below to estimate the amount of PCR product needed to ligate with 60 ng (20 fmoles) of pCR®3.1 vector:

   $$\text{X ng PCR product} = \frac{\text{(Y bp PCR product) (60 ng pCR®3.1 vector)}}{\text{(size in bp of the pCR®3.1 vector: 5060)}}$$

   "X" ng is the amount of PCR product of "Y" base pairs to be ligated for a 1:1 (vector:insert) molar ratio. However, it is recommended that a 1:2 (vector:insert) ratio be used, so multiply "X" by 2 to get the amount needed for ligation.
4. Using the concentration previously determined for your PCR reaction, calculate the volume needed to give the amount determined in step 3. Use sterile water to dilute your PCR reaction if necessary.
5. For example, set up the ligation reaction as follows:

Fresh PCR product	X µl
10X Ligation Buffer	1 µl
pCR®3.1 vector (30 ng/µl)	2 µl
T4 DNA Ligase (4 units/µl)	1 µl
Sterile water	to a final volume of 10 µl

6. Incubate the ligation reaction at 15°C for a minimum of 4 hours (preferably overnight). Proceed to **Transformation**, page 9.
7. If you are unable to transform immediately, you can store your DNA at -20°C until ready for transformation.

Continued on next page

Cloning into pCR®3.1, continued

Note

In general, 0.5 to 1.0 µl of a typical PCR reaction with an average insert length will give a ratio of 1:2 (vector:insert). The ratio of 1:2 (vector:insert) gives the best efficiency of ligation. You may wish to do a second ligation reaction at a ratio of 1:3 (vector:insert) or 1:1 (vector:insert), if you are concerned about the accuracy of your DNA concentrations.

Ligation at higher or lower temperatures than 15°C may reduce the ligation efficiency.

Transformation

Before Starting

At this point you should have a ligation reaction with your insert ligated into pCR®3.1, and you are ready to transform into One Shot® cells (TOP10F′).

Using Competent Cells

Competent cells are highly sensitive to changes in temperature or mechanical lysis caused by pipetting. Therefore, be extremely gentle when working with competent cells.

Transformation should be started immediately following the thawing of the cells on ice. Stir gently with a pipette tip to mix reagents; do not pipette up and down. Do not hold the bottom of the tube in your fingertips as this warms the cells.

Remember to use sterile technique when handling and plating your transformations.

Use kanamycin to select transformants when PCR products are amplified from ampicillin-resistant plasmids. For example, the *lacZ* product amplified from the pUC18 Control DNA template included in the kit is a PCR product from an ampicillin-resistant plasmid. Selecting with kanamycin will prevent contamination of the transformation reaction by the original ampicillin-resistant plasmid (pUC18).

For Users of pCR®2.1

DO NOT use One Shot® INVαF′ competent cells as a substitute if you have these in your laboratory. Transformation efficiencies are 100-1000 fold less on kanamycin than TOP10F′. Also do not use TOP10F′ with pCR®2.1 unless you use IPTG along with X-Gal for blue-white screening.

Preliminary Steps

1. Equilibrate a water bath to 42°C. Thaw the vial of SOC medium and bring to room temperature.
2. Place an appropriate number of 10 cm diameter LB agar plates with either 25 µg/ml kanamycin or 50 µg/ml ampicillin in a 37°C incubator to remove excess moisture. Use two plates for each ligation/transformation.

Continued on next page

Transformation, continued

Procedure for Transformation

1. Centrifuge the vials containing the ligation reactions briefly and place them on ice.
2. Thaw on ice one 50 µl vial of frozen One Shot® TOP10F′ competent cells for each ligation/transformation.
3. Pipette 2 µl of each ligation reaction directly into the competent cells and mix by stirring gently with the pipette tip. **Do not pipette up and down.**
4. Incubate the vials on ice for 30 minutes. Store the remaining ligation mixtures at -20°C.
5. Heat shock for exactly 30 seconds in the 42°C water bath. Do not mix or shake.
6. Remove the vials from the 42°C water bath and immediately place on ice.
7. Add **250 µl** of SOC medium (at room temperature) to each tube.
8. Shake the vials at 37°C for 1 hour at 225 rpm in a shaking incubator.
9. Spread 10-50 µl from each transformation vial on separate, labeled LB agar plates containing either 25 µg/ml kanamycin or 50 µg/ml ampicillin. Make sure all the liquid is absorbed, then invert the plates and place them in a 37°C incubator overnight. **Note**: Plate two different volumes to ensure that at least one plate has well-spaced colonies. When plating small volumes (< 20 µl), add 20 µl SOC to ensure even spreading.
10. You should obtain 100-400 colonies per plate depending on the volume plated.

Restriction Analysis

1. Pick **at least** 10 colonies and inoculate into LB containing 50 µg/ml ampicillin or 25 µg/ml kanamycin.
2. Isolate plasmid DNA and analyze by restriction mapping or sequencing for the presence and orientation of the insert. The T7 Forward and BGH Reverse primers are included for this purpose. Resuspend primers in deionized water to the concentration desired. Note that not all T7 primers have the same sequence, so if you substitute primers, check the sequence carefully.
3. Once you have confirmed that you have the correct clone, be sure to prepare a glycerol stock for long-term storage and then proceed to **Transfection**, next page.

Troubleshooting

If you have problems obtaining a clone with the appropriate insert, review the control reactions described on pages 16-19. Perform the control reactions prior to contacting Technical Service (page 24).

Transfection

Introduction

Once you have confirmed that your insert is in the correct orientation for expression, you are ready to transfect your cell line of choice. We recommend that you include the positive control vector (pCR®3.1/CAT) and a mock transfection to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 µg, Catalog no. K1910-01), or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (*e.g.* HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells.

Methods for transfection include calcium, lipid-mediated, and electroporation. Invitrogen offers the Calcium Phosphate Transfection Kit for mammalian cell transfection and Lipofectamine™ 2000 Reagent to optimize lipid-mediated transfection.

Catalog No.	Description
K2780-01	Calcium Phosphate Transfection Kit
11668-027	Lipofectamine™ 2000 Reagent

Positive Control

pCR®3.1/CAT is provided as a positive control vector for mammalian cell transfection and expression and may be used to optimize transfection conditions for your cell line (see page 20). A successful transfection will result in CAT expression that can be easily assayed (see below).

Note: Resuspend the lyophilized plasmid in water and transform into TOP10F′ cells as described on page 10. Isolate plasmid DNA as described above.

Assay for CAT Activity

You may assay for CAT expression by a number of methods. A simple, one-vial procedure utilizing [^{14}C] or [^{3}H]acetyl CoA has been reported (Neumann *et al.*, 1987). For your convenience, Invitrogen offers the CAT Antiserum (Catalog no. R902-25). Other commercial kits are available for assaying CAT expression.

Continued on next page

Transfection, continued

Assay for Function

After you have transfected your pCR®3.1 construct into mammalian cells you need to assay for transient expression of your PCR product. After confirming expression using the method appropriate for your cell line and PCR product, you may elect to create stable cell lines.

Stable Cell Lines

pCR®3.1 confers resistance to the antibiotic G418. In general, we recommend that you perform a kill curve to determine the concentration of G418 that kills your host cell line. If you already know the concentration, you may proceed with transfection and selection, using your method of choice.

pcDNA3.1(+)
pcDNA3.1(-)

Catalog nos. V790-20 and V795-20, respectively

Version I
081401
28-0104

Invitrogen™
life technologies

www.invitrogen.com
tech_service@invitrogen.com

Methods

Overview

Introduction

pcDNA3.1(+) and pcDNA3.1(-) are 5.4 kb vectors derived from pcDNA3 and designed for high-level stable and transient expression in mammalian hosts. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. The vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells
- Multiple cloning sites in the forward (+) and reverse (-) orientations to facilitate cloning
- Neomycin resistance gene for selection of stable cell lines
- Episomal replication in cells lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS-1, COS-7)

The control plasmid, pcDNA3.1/CAT, is included for use as a positive control for transfection and expression in the cell line of choice.

Experimental Outline

Use the following outline to clone and express your gene of interest in pcDNA3.1.

1. Consult the multiple cloning sites described on pages 3-4 to design a strategy to clone your gene into pcDNA3.1.
2. Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants on LB plates containing 50 to 100 µg/ml ampicillin.
3. Analyze your transformants for the presence of insert by restriction digestion.
4. Select a transformant with the correct restriction pattern and use sequencing to confirm that your gene is cloned in the proper orientation.
5. Transfect your construct into the mammalian cell line of interest using your own method of choice. Generate a stable cell line, if desired.
6. Test for expression of your recombinant gene by western blot analysis or functional assay.

Cloning into pcDNA3.1

Introduction

Diagrams are provided on pages 3-4 to help you design a cloning strategy for ligating your gene of interest into pcDNA3.1. General considerations for cloning and transformation are listed below.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, please refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli Strain

Many *E. coli* strains are suitable for the propagation of this vector including TOP10F′, DH5α™-T1^R, and TOP10. We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A-deficient (*end*A).

For your convenience, TOP10F′ is available as chemically competent or electrocompetent cells from Invitrogen.

Item	Quantity	Catalog no.
One Shot® TOP10F′ (chemically competent cells)	21 x 50 µl	C3030-03
Electrocomp™ TOP10F′	5 x 80 µl	C665-55
Ultracomp™ TOP10F′ (chemically competent cells)	5 x 300 µl	C665-03

Transformation Method

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of pcDNA3.1

To propagate and maintain pcDNA3.1, we recommend resuspending the vector in 20 µl sterile water to make a 1 µg/µl stock solution. Store the stock solution at -20°C.

Use this stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10F′, DH5α™-T1^R, TOP10, or equivalent. Select transformants on LB plates containing 50 to 100 µg/ml ampicillin. Be sure to prepare a glycerol stock of your plasmid-containing *E. coli* strain for long-term storage (see page 5).

Cloning Considerations

pcDNA3.1(+) and pcDNA3.1(-) are nonfusion vectors. Your insert must contain a Kozak translation initiation sequence and an ATG start codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Please note that other sequences are possible (see references above), but the G or A at position -3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is shown underlined.

(G/A)NN<u>ATG</u>**G**

Your insert must also contain a stop codon for proper termination of your gene. Please note that the *Xba* I site contains an internal stop codon (TC<u>TAG</u>A).

continued on next page

Cloning into pcDNA3.1, continued

Multiple Cloning Site of pcDNA3.1(+)

Below is the multiple cloning site for pcDNA3.1(+). Restriction sites are labeled to indicate the cleavage site. The *Xba* I site contains an internal stop codon (TCTAGA). The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pcDNA3.1(+) is available for downloading from our web site (**www.invitrogen.com**) or from Technical Service (see page 13).** For a map and a description of the features of pcDNA3.1(+), please refer to the **Appendix**, pages 10-11.

enhancer region (3′ end)

```
689  CATTGACGTC AATGGGAGTT TGTTTTGGCA CCAAAATCAA CGGGACTTTC CAAAATGTCG
```

CAAT TATA

```
749  TAACAACTCC GCCCCATTGA CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT
```

3' end of hCMV ↓ putative transcriptional start

```
809  AAGCAGAGCT CTCTGGCTAA CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC
```

T7 promoter/primer binding site

Nhe I *Pme* I *Afl* II *Hin*d III *Asp*718 I *Kpn* I

```
869  GACTCACTAT AGGGAGACCC AAGCTGGCTA GCGTTTAAAC TTAAGCTTGG TACCGAGCTC
```

*Bam*H I *Bst*X I* *Eco*R I *Eco*R V *Bst*X I* *Not* I *Xho* I

```
929  GGATCCACTA GTCCAGTGTG GTGGAATTCT GCAGATATCC AGCACAGTGG CGGCCGCTCG
```

Xba I *Apa* I *Pme* I pcDNA3.1/BGH reverse priming site

```
989  AGTCTAGAGG GCCCGTTTAA ACCCGCTGAT CAGCCTCGAC TGTGCCTTCT AGTTGCCAGC

1049 CATCTGTTGT TTGCCCCTCC CCCGTGCCTT CCTTGACCCT GGAAGGTGCC ACTCCCACTG
```

BGH poly (A) site

```
1109 TCCTTTCCTA ATAAAATGAG GAAATTGCAT
```

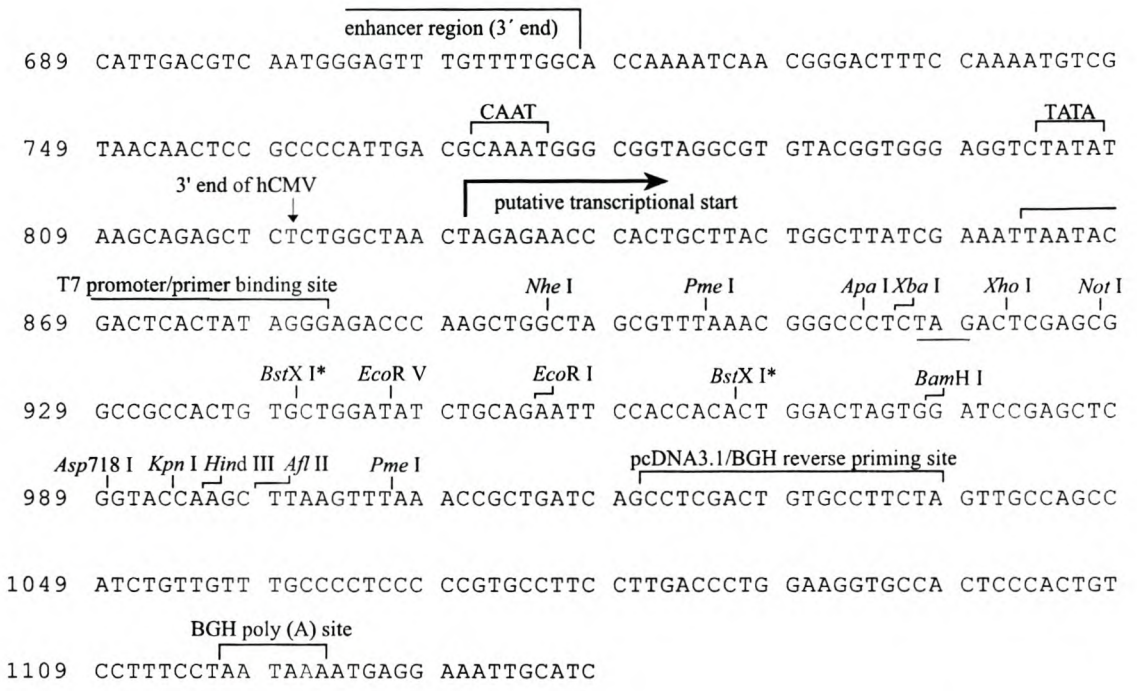
*Please note that there are two *Bst*X I sites in the polylinker.

continued on next page

Cloning into pcDNA3.1, continued

Multiple Cloning Site of pcDNA3.1(-)

Below is the multiple cloning site for pcDNA3.1(-). Restriction sites are labeled to indicate the cleavage site. The *Xba* I site contains an internal stop codon (TCTAGA). The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pcDNA3.1(-) is available for downloading from our web site (**www.invitrogen.com**) or from Technical Service (see page 13).** For a map and a description of the features of pcDNA3.1(-), please see the **Appendix**, pages 10-11.



*Please note that there are two *Bst*X I sites in the polylinker.

continued on next page

Cloning into pcDNA3.1, continued

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10F′, DH5α™-T1^R, TOP10) and select transformants on LB plates containing 50 to 100 µg/ml ampicillin. Select 10-20 clones and analyze for the presence and orientation of your insert.

We recommend that you sequence your construct with the T7 Promoter and BGH Reverse primers (Catalog nos. N560-02 and N575-02, respectively) to confirm that your gene is in the correct orientation for expression and contains an ATG and a stop codon. Please refer to the diagrams on pages 3-4 for the sequences and location of the priming sites. The primers are available separately from Invitrogen in 2 µg aliquots.

Preparing a Glycerol Stock

Once you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. You should keep a DNA stock of your plasmid at -20°C.

- Streak the original colony out on an LB plate containing 50 µg/ml ampicillin. Incubate the plate at 37°C overnight.
- Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml ampicillin.
- Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
- Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
- Store at -80°C.

Transfection

Introduction

Once you have verified that your gene is cloned in the correct orientation and contains an initiation ATG and a stop codon, you are ready to transfect your cell line of choice. We recommend that you include the positive control vector and a mock transfection (negative control) to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipids decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P. ™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01), or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (e.g. HeLa), please consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Invitrogen offers the Calcium Phosphate Transfection Kit (Catalog no. K2780-01) and a large selection of reagents for transfection. For more information, please refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 13).

Positive Control

pcDNA3.1/CAT is provided as a positive control vector for mammalian transfection and expression (see page 12) and may be used to optimize transfection conditions for your cell line. The gene encoding chloramphenicol acetyl transferase (CAT) is expressed in mammalian cells under the control of the CMV promoter. A successful transfection will result in CAT expression that can be easily assayed (see below).

Assay for CAT Protein

You may assay for CAT expression by ELISA assay, western blot analysis, fluorometric assay, or radioactive assay (Ausubel *et al.*, 1994; Neumann *et al.*, 1987). If you wish to detect CAT protein using western blot analysis, you may use the Anti-CAT Antiserum (Catalog no. R902-25) available from Invitrogen. Other kits to assay for CAT protein using ELISA assay are available from Roche Molecular Biochemicals (Catalog no. 1 363 727) and Molecular Probes (Catalog no. F-2900).

Creation of Stable Cell Lines

Introduction

The pcDNA3.1(+) and pcDNA3.1(-) vectors contain the neomycin resistance gene for selection of stable cell lines using neomycin (Geneticin®). We recommend that you test the sensitivity of your mammalian host cell to Geneticin® as natural resistance varies among cell lines. General information and guidelines are provided in this section for your convenience.

Geneticin® Selective Antibiotic

Geneticin® Selective Antibiotic blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression of the bacterial aminoglycoside phosphotransferase gene (APH), derived from Tn*5*, in mammalian cells results in detoxification of Geneticin® (Southern and Berg, 1982).

Geneticin® Selection Guidelines

Geneticin® Selective Antibiotic is available from Invitrogen (Catalog no. 10486-025). Use as follows:

- Prepare Geneticin® in a buffered solution (e.g. 100 mM HEPES, pH 7.3).
- Use 100 to 800 µg/ml of Geneticin® in complete medium.
- Calculate concentration based on the amount of active drug (check the lot label).
- Test varying concentrations of Geneticin® on your cell line to determine the concentration that kills your cells (see below). Cells differ in their susceptibility to Geneticin®.

Cells will divide once or twice in the presence of lethal doses of Geneticin®, so the effects of the drug take several days to become apparent. Complete selection can take up to 3 weeks of growth in selective media.

Determination of Antibiotic Sensitivity

To successfully generate a stable cell line expressing your gene of interest from pcDNA3.1, you need to determine the minimum concentration of Geneticin® required to kill your untransfected host cell line. We recommend that you test a range of concentrations to ensure that you determine the minimum concentration necessary for your host cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Geneticin® (0, 50, 100, 200, 400, 600, 800 µg/ml Geneticin®).
3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of Geneticin® that prevents growth within 2-3 weeks after addition of Geneticin®.

continued on next page

Creation of Stable Cell Lines, continued

Possible Sites for Linearization of pcDNA3.1(+)

Prior to transfection, we recommend that you linearize the pcDNA3.1(+) vector. Linearizing pcDNA3.1(+) will decrease the likelihood of the vector integrating into the genome in a way that disrupts the gene of interest or other elements required for expression in mammalian cells. The table below lists unique restriction sites that may be used to linearize your construct prior to transfection. **Other unique restriction sites are possible.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)	Location	Supplier
Bgl II	12	Upstream of CMV promoter	Invitrogen, Catalog no. 15213-028
Mfe I	161	Upstream of CMV promoter	New England Biolabs
*Bst*1107 I	3236	End of SV40 polyA	AGS*, Fermentas, Takara, Roche Mol. Biochemicals
*Eam*1105 I	4505	Ampicillin gene	AGS*, Fermentas, Takara
Pvu I	4875	Ampicillin gene	Invitrogen, Catalog no. 25420-019
Sca I	4985	Ampicillin gene	Invitrogen, Catalog no. 15436-017
Ssp I	5309	*bla* promoter	Invitrogen, Catalog no. 15458-011

*Angewandte Gentechnologie Systeme

Possible Sites for Linearization of pcDNA3.1(-)

The table below lists unique restriction sites that may be used to linearize your pcDNA3.1(-) construct prior to transfection. **Other unique restriction sites are possible.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)	Location	Supplier
Bgl II	12	Upstream of CMV promoter	Invitrogen, Catalog no. 15213-028
Mfe I	161	Upstream of CMV promoter	New England Biolabs
*Bst*1107 I	3235	End of SV40 polyA	AGS*, Fermentas, Takara, Roche Mol. Biochemicals
*Eam*1105 I	4504	Ampicillin gene	AGS*, Fermentas, Takara
Pvu I	4874	Ampicillin gene	Invitrogen, Catalog no. 25420-019
Sca I	4984	Ampicillin gene	Invitrogen, Catalog no. 15436-017
Ssp I	5308	*bla* promoter	Invitrogen, Catalog no. 15458-011

*Angewandte Gentechnologie Systeme

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Creation of Stable Cell Lines, continued

Selection of Stable Integrants

Once you have determined the appropriate Geneticin® concentration to use for selection in your host cell line, you can generate a stable cell line expressing your gene of interest.

1. Transfect your mammalian host cell line with your pcDNA3.1 construct using the desired protocol. Remember to include a plate of untransfected cells as a negative control and the pcDNA3.1/CAT plasmid as a positive control.
2. 24 hours after transfection, wash the cells and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing Geneticin® at the pre-determined concentration required for your cell line. Split the cells such that they are no more than 25% confluent.
4. Feed the cells with selective medium every 3-4 days until Geneticin®-resistant foci can be identified.
5. Pick and expand colonies in 96- or 48-well plates.

Appendix E: Rapid Ligation Kit (Roche)

For life science research only. Not for use in diagnostic procedures.
FOR *IN VITRO* USE ONLY.

Rapid DNA Ligation Kit

Cat. No. 1 635 379
Kit for 40 ligation reactions

Version 2, May 2002
Store at –15 to –25° C

1. Kit contents

Bottle/ Cap	Label	Content	Cat. No. (if available separately)
1	T4 DNA Ligation Buffer, 2x conc.	0.5 ml	
2	DNA Dilution Buffer, 5x conc.	0.5 ml	
3	T4 DNA Ligase	• 40 µl • [5 U/µl]	799 009

2. Product overview

Kit description
The Rapid DNA Ligation Kit enables ligation of sticky-end or blunt-end DNA fragments in just 5 min at 15-25° C. Depending on DNA concentration, either circular (low DNA concentration) or concatemeric (high DNA concentration) ligation products are formed. All necessary reagents required for ligation are provided. There is no need to prepare buffers or to add ATP or Mg^{2+}.

Application
The Rapid DNA Ligation Kit can be used for:
- cloning in plasmid vectors,
- cloning in phage vectors,
- linker ligation,
- recircularization of linear DNA

Note: Electroporation can be performed after ligation in combination with the High Pure Purification Kit.

Sample material
Dephosphorylated blunt- or sticky-end DNA.

Ligation time
5 min

Number of ligation reactions
40 ligation reactions (standard assay: 50 ng linearized and dephosphorylated vector DNA and 150 ng insert DNA).

Quality control
Each lot of the Rapid DNA Ligation Kit is tested under the above described procedures and the following results are guaranteed per µg vector DNA. Results are depending on the DNA purity. Purification by caesium chloride gradient is recommended:

Recircularization	pUC19/*Sma* I	pUC19/*Hind* III
Yield of transformed colonies	> $1x10^6$	> $1x10^6$

Insertion of an insert in plasmid vectors	pUC19/*Sma* I + 2100 bp insert	pUC19/*Hind* III + 2300 bp insert
Yield of white colonies after transformation into competent E. coli JM83 cells	> $1x10^5$	> $6x10^5$

Insertion of DNA into phage vectors	lambda gt 11 arms + insert/ *Eco* RI
Yield of white plaques	>$1x10^7$

Ligation and recutting
1 µg pUC19 DNA, digested with *Hind* III, is dephosphorylated with alkaline phosphatase and ligated with 1 µg of DNA molecular weight marker II* according to the standard protocol. After ethanol precipitation the ligation products are redigested with *Hind* III. Only the original digestion pattern was observed after separation on an agarose gel.

Kit storage/ stability
The unopened kit is stable at –15 to -25°C through the expiration date printed on the label.

Note: Repeated freezing and thawing should be avoided.

0502.1637 886⑦

Roche

3. Procedures and required material

3.1 Standard ligation reaction for DNA

Before you begin

In order to achieve optimal results, please consider the following:

Purification	The DNA to be ligated should be purified by phenol extraction and ethanol precipitation or use the Agarose Gel DNA Extraction Kit* (Cat. No. 1 696 505)
Dephosphorylation	For insertion of DNA into plasmid vectors the vector DNA should be dephosphorylated (except for recircularization) with alkaline phophatase* (Cat. No. 713 023)
Reaction volume	• Dissolve the DNA to be ligated in 1x conc. DNA dilution buffer (vial 2), in a total volume of 10 µl. • If the total volume of DNA solution in 1x DNA dilution buffer is greater than 10 µl, then the volume of all the other reagents in the reaction should be increased accordingly and the ligation reaction should be for 30 min.
Molar ratio	• The molar ratio of vector DNA to insert DNA in a total volume of 10 µl should be 1+3 (e. g. as described in the standard assay with approximately equal length of vector DNA and insert DNA, 50 ng linearized dephosphorylated vector DNA and 150 ng insert DNA). • When the vector DNA and insert DNA are not similar in lengths, other molar ratios 1+1, 1+2 are possible. • A molar ratio of 1+5 can be used for sticky-end ligations. ***Note:*** For blunt-ended DNA however, a decrease of transformed colonies was observed when a molar ratio of 1+5 was used.
Transformation	To avoid an inhibition by a surplus of DNA a maximum of 1/10 of the volume of the ligation reaction mixture should be used for the transformation assay.
Maximum amount of DNA	The maximum amount of DNA to be ligated in 5 min should not exceed 200 ng.
T4 DNA Ligase inactivation	T4 DNA ligase can be completely inactivated by a 10 min incubation at 65° C. This step should only be done if the ligation reaction mixture is used in experiments other than transformation assays. ***Note:*** Heat inactivation of the ligation reaction mixture before transformation leads to a drastic decrease (▷ factor 20) of transformed colonies.

Preparation of kit working solutions

Mix the 5x conc. DNA dilution buffer (vial 2) thoroughly and dilute with double dist. water to a final concentration of 1x.

Procedure

In the following table the standard protocol for the ligation reaction is described.

Step	Action
1	Dissolve vector DNA and insert DNA in thoroughly mixed and diluted **1 x conc. DNA Dilution Buffer** to a final volume of 10 µl in a sterile reaction vial.
2	• Mix thoroughly **T4 DNA Ligation Buffer** (vial 1). ***Note:*** It is absolutely necessary to mix the contents of vial 1 directly prior to use. • Add 10 µl **T4 DNA Ligation Buffer** (vial 1) to the reaction vial. • Mix thoroughly.
3	• Add 1 µl **T4 DNA Ligase** (vial 3). • Mix thoroughly.
4	Incubate for 5 min at 15-25°C.
5	The ligation reaction mixture can be used directly for the transformation of competent cells, or can be stored without heat inactivation at -15 to -25°C. ***Note***: Heat inactivation of the T4 DNA ligase drastically decreases the transformation efficiency.
6	The ligated DNA can be analyzed by agarose gel electrophoresis.

3.2 Electroporation of E.coli cells after recircularization

Additional required material and equipment

- High Pure PCR Product Purification Kit (Cat. No. 1 732 668)
- Electroporation unit, e.g. BioRad pulsar unit
- Electrocompetent cells

Preparation of working solutions

From the High Pure PCR Product Purification Kit:

Make sure that 40 ml for the 50 purification pack size and 200 ml ethanol p.a. for the 250 purification pack size have been added to the Wash Buffer (blue cap) prior to first use.

Caution

The Binding Buffer (green cap) contains guanidine-HCl, which is an irritant. Wear gloves and follow usual safety precautions when handling.

Procedure

Modified protocol for the purification of ligated DNA from the standard ligation reaction for use of the DNA in electroporation.

Note: The use of the High Pure PCR Product Purification Kit adds an additional 10-15 min to the overall time required to prepare electrocompetent reactions.

Step	Action
1	Add 100 µl **Binding Buffer** to the 20 µl ligation.
2	Pipet the sample into the upper reservoir of a combined Filter Tube-Collection Tube assembly.
3	Centrifuge 1 min at max. speed in a standard table top centrifuge.
4	• Discard the flowthrough. • Combine again the filter tube and the collection tube.
5	• Add 500 µl **Wash Buffer** (blue cap) to the upper reservoir. • Centrifuge 1 min at max. speed. ***Note:*** Make sure that the filter tube has no contact with the surface of the Wash Buffer flowthrough.
6	• Discard the flowthrough. • Combine again the filter tube and the collection tube. • Add 200 µl **Wash Buffer** (blue cap) to the upper reservoir. • Centrifuge 1 min at max. speed (13 000 rpm).
7	• Discard the flowthrough. • Combine again the filter tube and the collection tube. • Centrifuge 1 min at max. speed to remove residual Wash Buffer.
8	• Discard the collection tube. • Insert the filter tube in a clean 1.5 ml reaction tube.
9	• Add 100 µl **sterile double dist. water** to the upper reservoir, (pH approx. 7.4). • Centrifuge 1 min at max. speed
10	Recovered final volume is about 100 µl. 10 µl which represents 1/10 of the ligation reaction is used for electroporation.

Electroporation

- Electroporate 1/10 volume of the ligation reaction into electrocompetent cells using the BioRad pulsar unit and 0.2 cm cuvettes under the following conditions:

  2.5 kV, 25 MF, 200 ohm.

  (The specific electroporation conditions have to be evaluated for each strain.)
- Plate out a 1/20 volume of the electroporated cells.

Note: Colonies arising on each plate represent 0.5 ng of DNA.

3.3 Ligation reaction for insertion into phage-vectors (including linker ligation)

Before you begin

In order to achieve optimal results, please consider the following:

Purification	The DNA to be ligated should be purified by phenol extraction and ethanol precipitation or use the Agarose Gel DNA Extraction Kit*.
Dephosphorylation	For insertion of DNA in phage-vectors, the arms of the vector DNA should be dephosphorylated with alkaline phosphatase.
Reaction volume	If the total volume of DNA solution in 1 × DNA dilution buffer is greater than 10 µl, then the volume of all the other reagents in the reaction should be increased accordingly and the ligation reaction should be incubated for 30 min.
Ratio of vector arms to DNA	The ratio of vector arms to insert DNA should be approx. 8+1 in a total volume of 10 µl (e. g. 1000 ng DNA, lambda gt 11, Eco RI arms, dephosphorylated and 120 ng insert DNA).
T4 DNA Ligase inactivation	T4 DNA ligase can be completely inactivated by a 10 min incubation at 65°C. This step should only be done if the ligation reaction mixture is used in experiments other than packaging assays. ***Note:*** Heat inactivation of the ligation reaction mixture before packaging leads to a drastic decrease of plaques.

Procedure

Protocol for the ligation reaction for insertion into phage vectors.

Note: If an adaptor is necessary for the cloning strategy, add the required quantity to the DNA in a total volume of 10 µl.

Step	Action
1	Dissolve DNA (vector arms + insert + adaptor, if necessary) in **1 × conc. DNA Dilution Buffer** to a final volume of 10 µl in a sterile vial.
2	• Add 10 µl **T4 DNA Ligation Buffer** (2 × conc.) (vial 1). ***Note:*** It is absolutely necessary to thoroughly mix the contents of vial 1 directly prior to use. • Mix thoroughly.
3	• Add 1 µl **T4 DNA Ligase** (vial 3). • Mix thoroughly.
4	Incubate for 5 min at 15-25°C.
5	For each packaging reaction 4 µl of the product from the ligation reaction should be used. The ligation reaction mixture can be stored without heat inactivation at -15 to -25°C. ***Note:*** Heat inactivation of the T4 DNA ligase drastically decreases the packaging efficiency

4. Results

Analysis of results

The products from the DNA ligation reaction can be analyzed by agarose gel electrophoresis (1/2 of the ligation product for plasmid cloning experiments or 1/4 of the ligation product for phage cloning experiments), by the addition of 1/5 volume of gel loading buffer given below, e. g. 10 µl ligation product and 2 µl gel loading buffer.

Gel loading buffer:

1% SDS (w/v), 50 mM EDTA, 0,02% bromophenol blue (w/v), 50% glycerol (v/v), pH 7.5.

Note: For analysis of circular DNA on an agarose gel, a sufficient amount of DNA should be used for the DNA ligation reaction. The DNA concentration in the ligation reaction however, should never be more than 200 ng DNA in 20 µl ligation reaction mixture. (Raise the reaction volume, if necessary).

Recircularization

50 ng pUC19 DNA, digested with *Sma* I or *Hind* III respectively, were religated according to the standard protocol and transformed into competent [Hanahan method (3)] E. coli JM83 cells.

Yield of transformed colonies per mg of DNA:

pUC19 DNA undigested	pUC19/*Sma* I digest	pUC19/*Hind* III digest
2×10^7	7×10^6	8×10^6

Cloning of an insert into plasmid vectors

50 ng pUC19 DNA, digested with *Sma* I or *Hind* III, respectively, and dephosphorylated with alkaline phosphatase, were ligated with 150 ng blunt- or sticky-end insert DNA, respectively, according to the standard protocol. The inserts were purified by electrophoresis and isolated from the agarose gel by agarase* digestion.

Yield of white colonies per mg of vector DNA after transformation into competent E. coli JM83 cells.

pUC19 DNA undigested	pUC19/*Sma* I digest + 2100 bp insert DNA	pUC19/*Hind* III digest + 2300 bp insert DNA
2×10^7	6×10^5	1.3×10^6

Cloning of an insert into phage-vectors

1 µg of DNA, lambda gt 11, *Eco* RI arms, dephosphorylated, was ligated to 120 ng linearized pUC19 DNA, digested with either *Sma* I or *Eco* RI as insert (for the ligation of blunt ends, 20 ng of adaptor were added). After packaging into phages, E.coli Y1090 - cells were infected and plated onto agar plates.

Yield of white plaques:

lambda gt 11 vector control	lambda gt 11 arms + insert/*Sma* I + adaptor	lambda gt 11 arms + insert/*Eco* RI
1.6×10^9	4.9×10^4	1.2×10^8

5. Appendix

5.1 References

1 Hayashi, K. et al. (1986) Nucleic Acids Res. **14**, 7617-7631.
2 Sambrook, J..,Fritsch, E. F. & Maniatis, T (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, (NY): 1989, 2nd ed., Vol 1,2 and 3,p 561-563.
3 Hanahan, D. (1983) J. Mol. Biol. **166**, 557-580.

5.2 Related products

Kits

Product	Pack Size	Cat. No.
Agarose Gel DNA Extraction Kit	1 kit (max. 100 reactions)	1 696 505
cDNA Synthesis Kit	1 kit (for 25 µg RNA) 1 kit (for 50 µg RNA)	1 117 831 1 013 882
First Strand cDNA Synthesis Kit for RT-PCR (AMV)	1 kit	1 483 188
High Pure PCR Product Purification Kit	50 purif. 250 purif.	1 732 668 1 732 676

Single reagents

Product	Pack Size	Cat. No.
5-Bromo-4 chloro-3-indolyl-b-D-galactopyranoside (X-gal)	100 mg 250 mg 1 g 2.5 g	1 680 293 651 745 745 740 703 729
Agarase	100 U 500 U	1 417 215 1 417 223
Agarose MP	100 g 500 g	1 388 983 1 388 991
Alkaline Phosphatase	1000 U (1 U/µl) 1000 U (20 U/µl)	713 023 1 097 075
Alkaline Phosphatase Shrimp	1000 U	1 758 250
DNA MWM II	50 µg (1 A_{260} unit)	236 250
Isopropyl-b-D-thiogalactoside (IPTG)	1 g 5 g	724 815 1 411 446
Klenow Enzyme, labeling grade	100 U 500 U	1 008 404 1 008 412
Pwo DNA Polymerase	100 U 2x 250 U	1 644 947 1 644 955
T4 DNA Ligase	100 U [1 U/µl] 500 U [1 U/µl] 500 U [5 U/µl]	481 220 716 359 799 009
T4 RNA Ligase	500 U	1 449 478
Taq DNA Polymerase, 1 unit/ml	250 U 4x 250 U	1 647 679 1 647 687
Taq DNA Polymerase, 5 units/ml	100 U 500 U 4x 250 U 10x 250 U 20x 250 U	1 146 165 1 146 173 1 418 432 1 596 594 1 435 094

* available from Roche Applied Science (see also section 5.3)

How to contact Roche Applied Science

www.roche-applied-science.com

- to place your order
- find product information
- for answers to technical queries, or,
- contact your local sales representative.

www.roche-applied-science.com/pack-insert/1635379a.pdf

Roche Diagnostics GmbH
Roche Applied Science
Sandhofer Strasse 116
D-68305 Mannheim
Germany

Appendix F: QIAprep Spin Miniprep Kit (Qiagen)

Plasmid Mini Purification Protocol

This protocol is for Mini (up to 20 µg) preparations of high-copy plasmid DNA from cultures of *E. coli*. For cosmid and low-copy-number plasmid purification see recommendations on page 13. For purification of BACs, PACs, P1s, and purification of double-stranded M13 replicative form see recommendations on page 14.

Important notes before starting

- New users are strongly recommended to read Appendix A (page 21) at the end of this handbook before starting the procedure.
- To ensure high yields of pure DNA, use no more than 3 ml LB culture for high-copy-number plasmids (e.g., pUC, pBluescript®). For low-copy-number plasmids (e.g., pBR322), use no more than 10 ml LB culture and refer to the recommendations on page 13. We do not recommend the use of rich media such as TB or 2x YT for culture. When low-copy-number plasmids containing the ColE1 replication origin are prepared, the yield can be improved by amplification in the presence of chloramphenicol (34 mg/ml). They should then be treated as high-copy-number plasmids.
- Add the provided RNase A solution to Buffer P1 before use (spin down RNase A briefly before use). Buffer P1 should then be stored at 2–8°C and is stable for 6 months.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- Chill Buffer P3 at 4°C.
- Optional: To confirm purification or to identify a problem, samples may be taken at specific steps for analysis on an agarose gel. Appropriate samples and volumes are indicated in the protocol below.

Procedure

1. **Resuspend the bacterial pellet in 0.3 ml of Buffer P1.**

   Ensure that RNase A has been added to Buffer P1. The bacteria should be resuspended completely, leaving no cell clumps.

2. **Add 0.3 ml of Buffer P2, mix gently, and incubate at room temperature for 5 min.**

   After addition of Buffer P2, the solution should be mixed gently, but thoroughly, by inverting the tube 4–6 times. Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid any reaction between the NaOH and CO_2 in the air. If the buffer is left open for any length of time, it should be prepared fresh from stock solutions.

3. **Add 0.3 ml of chilled Buffer P3, mix immediately but gently, and incubate on ice for 5 min.**

   Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, the solution becomes cloudy and very viscous. To avoid localized potassium dodecyl sulfate precipitation, mix the solution gently, but thoroughly, immediately after addition of Buffer P3. Mix by inverting the tube 4–6 times.

4. **Centrifuge at maximum speed in a microcentrifuge for 10 min. Remove supernatant promptly.**

   Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed at maximum speed in 1.5 ml or 2 ml microcentrifuge tubes (e.g., 10,000–13,000 rpm in a microcentrifuge). Maximum speed corresponds to 14,000–18,000 x *g* for most microcentrifuges. After centrifugation, the supernatant should be clear. If the supernatant is not clear, a second, shorter centrifugation should be carried out to avoid applying any suspended or particulate material to the column. Suspended material (which causes the sample to appear turbid) will clog the column and reduce or eliminate flow.

   - Remove a 50 µl sample from the cleared lysate and save it for an analytical gel (sample 1).

5. **Equilibrate a QIAGEN-tip 20 by applying 1 ml Buffer QBT, and allow the column to empty by gravity flow.**

   Place QIAGEN-tips into a QIArack over the waste tray or use the tip holders provided with each kit (see "Setup of QIAGEN-tips" page 9). Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. The resin bed will retain some buffer and will not readily dry out. QIAGEN-tips can therefore be left unattended. Do not force out the remaining buffer.

6. **Apply the supernatant from step 4 to the QIAGEN-tip 20 and allow it to enter the resin by gravity flow.**

   The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be recentrifuged before loading to prevent clogging of the QIAGEN-tip.

   - Remove a 50 µl sample of the flow-through and save for an analytical gel sample 2).

7. **Wash the QIAGEN-tip 20 with 4 x 1 ml Buffer QC.**

   Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first 2 ml is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second 2 ml ensures complete removal of contaminants, and will also ensure consistent results in sequencing. (The second 2 ml is particularly necessary when large culture volumes or bacterial strains producing large amounts of carbohydrate are used.) It is particularly important not to force out residual wash buffers. Traces of wash buffer will not affect the elution step.

- Remove a 50 µl sample of the combined wash fractions and save for an analytical gel (sample 3).

8. **Elute DNA with 0.8 ml Buffer QF.**

   Place the upper part of a QIArack over the lower rack fitted with clean 1.5 ml or 2 ml microcentrifuge tubes and collect the samples in the tubes. Alternatively, use the tip holders provided. Flow begins when Buffer QF is added. Drain the QIAGEN-tip by allowing it to empty by gravity flow.

   - Remove a 50 µl sample of the eluate and save for an analytical gel (sample 4).

9. **Precipitate DNA with 0.7 volumes (0.56 ml per 0.8 ml of elution volume) of room-temperature isopropanol. Centrifuge immediately at ≥10,000 rpm for 30 min in a microcentrifuge, and carefully decant the supernatant.**

   Precipitation of DNA with isopropanol should be carried out with all solutions equilibrated to room temperature in order to minimize salt precipitation. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be easily located.

10. **Wash DNA with 1 ml of 70% ethanol, air-dry for 5 min, and redissolve in a suitable volume of buffer.**

    The DNA pellet should be washed briefly in 70% ethanol, and then recentrifuged. The 70% ethanol serves to remove precipitated salt, as well as to replace isopropanol with the more volatile ethanol, making the DNA easier to redissolve. A second wash with room-temperature 70% ethanol may improve results in more sensitive applications, such as transfection. After careful and complete removal of ethanol, the pellet should be air-dried briefly (approximately 5 min) before redissolving in an appropriate volume of TE buffer. Overdrying the pellet will make the DNA difficult to redissolve. Resuspend the DNA pellet by rinsing the walls to recover all the DNA. Pipetting the DNA up and down to promote resuspension may cause shearing, and should be avoided. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry and quantitative analysis on an agarose gel.

Analytical gel (optional): to analyze the purification procedure as shown in Figure 2 (Appendix A, page 27), precipitate samples 1–4 (from steps 4–8) with 35 µl of isopropanol. Rinse the pellets with 70% ethanol, drain well, air-dry, and resuspend in 10 µl of TE, pH 8.0. Use 2 µl of each for analysis on a 1% agarose gel (1).

Appendix G: Endofree Plasmid Giga Kit (Qiagen)

EndoFree Plasmid Mega and Giga Protocol

This protocol is designed for preparation of up to 2.5 mg of high- or low-copy plasmid and cosmid DNA using the EndoFree Plasmid Mega Kit, or up to 10 mg of high-copy plasmid DNA using the EndoFree Plasmid Giga Kit. Endotoxin-free DNA will improve transfection into sensitive eukaryotic cells and is essential for gene therapy research. For background information on endotoxins, see pages 68–70. (Please note: the EndoFree Plasmid Giga Kit is not recommended for low-copy plasmids or cosmids).

Low-copy plasmids which have been amplified in the presence of chloramphenicol should be treated as high-copy plasmids when choosing the appropriate culture volume.

Maximum recommended culture volumes*

	EndoFree Mega	EndoFree Giga
High-copy number plasmids	500 ml LB culture (1.5 g pellet wet weight)†	2.5 liters LB culture (7.5 g pellet wet weight)†
Low-copy number plasmids	2.5 liters LB culture (7.5 g pellet wet weight)†	Not recommended for low-copy plasmids or cosmids‡

* For high-copy plasmids, expected yields are 1.5–2.5 mg for the EndoFree Plasmid Mega Kit and 7.5–10 mg for the EndoFree Plasmid Giga Kit. For low-copy plasmids, expected yields are 0.5–2.5 mg for the EndoFree Plasmid Mega Kit. The EndoFree Plasmid Giga Kit is not recommended for low-copy plasmid preparations.

† On average, a healthy 1-liter shaker culture yields a pellet with a wet weight of approximately 3 g. When working with fermentation cultures, however, the pellet wet weight may be significantly higher. Therefore, when using fermentation cultures please refer to the pellet wet weight instead of the recommended culture volumes.

‡ Due to the large culture volume required for preparation of low-copy plasmid and cosmid DNA and the limited capacity of the QIAfilter Mega-Giga Cartridge, the EndoFree Plasmid Mega Kit is a better choice than the EndoFree Plasmid Giga Kit for purification of low-copy plasmids and cosmids.

Important notes before starting

- New users are strongly advised to read the "General Considerations for Optimal Results" section on pages 60–70 before starting the procedure.
- Use endotoxin-free or pyrogen-free plastic pipet tips and tubes for elution and subsequent steps (step 15 onwards). Endotoxin-free or pyrogen-free plasticware can be obtained from many common vendors. Please check with your current supplier to obtain recommendations. Alternatively, glass tubes may be used if they are baked overnight at 180°C to destroy endotoxins.

- The QIAfilter Mega-Giga Cartridge is designed for use with a 1 liter, 45 mm-neck, vacuum-resistant glass bottle (e.g., Schott, cat. no. 2181054 or Corning, cat. no. 1395-1L). **Note: Bottles are not included in the kit and must be supplied by the user.** The cartridge operates with any vacuum source (e.g., a house vacuum, vacuum pump, or water aspirator) that generates vacuum pressures between –200 and –600 millibars (–150 and –450 mm Hg). The vacuum pressure is measured as differential pressure between the inside of the bottle and the atmosphere (1013 millibars or 760 mm Hg). Vacuum recommendations are given in negative units to indicate the required reduction in pressure with respect to the atmosphere.
- To avoid the possibility of implosion do not use plastic/glass bottles or any other vessels that are not designed for use with a vacuum. Do not use plastic/glass bottles or any other vessels that are cracked or scratched. Wear safety glasses when working near a bottle under vacuum.
- Add the provided RNase A solution to Buffer P1 before use. Use one vial of RNase A (spin briefly before use) per bottle of Buffer P1, to give a final concentration of 100 µg/ml.
- To prepare endotoxin-free 70% ethanol, add 40 ml of 96–100% ethanol to the endotoxin-free H_2O supplied with the kit.
- Check Buffer P2 for SDS precipitation due to low storage temperatures and, if necessary, dissolve the SDS by warming to 37°C.
- Pre-chill Buffer P3 to 4°C.
- In contrast to the standard protocol, the lysate is not incubated on ice after addition of Buffer P3.
- **Optional:** remove samples at the steps indicated with the symbol ☞ in order to monitor the procedure on an analytical gel.

Procedure

1. **Pick a single colony from a selective plate and inoculate a starter culture of 5–10 ml LB medium containing the appropriate selective agent. Grow for ~8 h at 37°C with vigorous shaking (~300 rpm).**

   Use a flask with a volume of at least 4 times the volume of the culture.

2. **Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids inoculate 500 ml or 2.5 liters medium. For low-copy plasmids, inoculate 2.5 liters medium. Grow at 37°C for 12–16 h with vigorous shaking (~300 rpm).**

   Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of 3–4 x 10^9 cells per ml, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium (see page 65).

3. **Harvest the bacterial cells by centrifugation at 6000 x g for 15 min at 4°C.**

   6000 x g corresponds to 6000 rpm in Sorvall GSA or GS3 or Beckman® JA-10 rotors. Remove all traces of supernatant by inverting the open centrifuge bottle until all medium has been drained.

   ⊗ If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. **Screw the QIAfilter Mega-Giga Cartridge onto a 45 mm-neck glass bottle and connect it to a vacuum source.**

   Do not overtighten the QIAfilter Cartridge on the bottle neck, because the QIAfilter Cartridge plastic may crack.

5. **Resuspend the bacterial pellet in 50 ml or 125 ml Buffer P1.**

   Note: For efficient lysis it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. We recommend a 500 ml bottle for Mega preparations and a 1000 ml bottle for Giga preparations. Ensure that the RNase A has been added to Buffer P1. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

6. **Add 50 ml or 125 ml Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for 5 min.**

   Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO_2 in the air.

7. **Add 50 ml or 125 ml chilled Buffer P3, and mix immediately and thoroughly by inverting 4–6 times. Mix well until a white fluffy material has formed and the lysate is no longer viscous. Proceed directly to step 8. Do not incubate on ice.**

   Precipitation is enhanced by using chilled Buffer P3. After addition of Buffer P3, a fluffy white precipitate containing genomic DNA, proteins, cell debris, and SDS becomes visible. The lysate should be mixed well to reduce the viscosity and prevent clogging of the QIAfilter Cartridge.

8. **Pour the lysate into the QIAfilter Mega-Giga Cartridge and incubate at room temperature for 10 min.**

   Important: This 10 min incubation at room temperature is essential for optimal performance of the QIAfilter Mega-Giga Cartridge. Do not agitate the QIAfilter Cartridge during this time. A precipitate containing proteins, genomic DNA, and detergent will float and form a layer on top of the solution. This ensures convenient filtration without clogging.

9. **Switch on the vacuum source. After all liquid has been pulled through, switch off the vacuum source. Leave the QIAfilter Cartridge attached.**

10. **Add 50 ml (Mega and Giga) Buffer FWB to the QIAfilter Cartridge and gently stir the precipitate using a sterile spatula. Switch on the vacuum source until the liquid has been pulled through completely.**

    Gentle stirring of the precipitate enhances the flow of liquid through the filter unit. Take care not to disperse the precipitate, as this may result in carryover of cell debris and SDS, which will affect flow and binding characteristics of the QIAGEN column. The filtered lysate in the bottle contains the plasmid DNA.

    ☞ Remove a 120 µl or 75 µl sample from the cleared lysate and save for an analytical gel (sample 1) to determine whether growth and lysis conditions were optimal.

11. **Add 12.5 ml or 30 ml Buffer ER to the filtered lysate, mix by inverting the bottle approximately 10 times, and incubate on ice for 30 min.**

    After addition of Buffer ER the lysate appears turbid, but will become clear again during the incubation on ice.

12. **Equilibrate a QIAGEN-tip 2500 or QIAGEN-tip 10000 by applying 35 ml or 75 ml Buffer QBT and allow the column to empty by gravity flow.**

    Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

13. **Apply the filtered lysate from step 11 onto the QIAGEN-tip and allow it to enter the resin by gravity flow.**

    Due to the presence of Buffer ER the lysate may become turbid again, however this does not affect the performance of the procedure.

    ☞ Remove a 120 µl or 75 µl sample of the flow-through and save for an analytical gel (sample 2) in order to determine the efficiency of DNA binding to the QIAGEN Resin.

14. **Wash the QIAGEN-tip with a total of 200 ml or a total of 600 ml Buffer QC.**

    Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first half of the volume of wash buffer is enough to remove all contaminants in the majority of plasmid DNA preparations. The second half is particularly necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

    ☞ Remove a 160 µl or 120 µl sample of the eluate and save for an analytical gel (sample 3).

    Important: For all subsequent steps use endotoxin-free or pyrogen-free plasticware (e.g. new polypropylene centrifuge tubes) or pre-treated glassware.

15. **Elute DNA with 35 ml or 75 ml Buffer QN.**

    Drain the QIAGEN-tip by allowing it to empty by gravity flow. Use of polycarbonate centrifuge tubes for collection is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps.

☞ Remove a 22 µl or 15 µl sample of the eluate and save for an analytical gel (sample 4).

⊗ If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

16. **Precipitate DNA by adding 24.5 ml or 52.5 ml room-temperature isopropanol (0.7 volumes) to the eluted DNA. Mix, and centrifuge immediately at ≥15,000 x g for 30 min at 4°C. Carefully decant the supernatant.**

All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. A centrifugal force of 15,000 x g corresponds to 9,500 rpm in a Beckman JS-13 rotor and 11,000 rpm in a Sorvall SS-34 rotor. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5,000 x g for 60 min at 4°C. Isopropanol DNA pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be easily located. Isopropanol DNA pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

17. **Wash DNA pellet with 7 ml or 10 ml of endotoxin-free room-temperature 70% ethanol (add 40 ml of 96–100% ethanol to the endotoxin-free H_2O supplied with the kit) and centrifuge at ≥15,000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet.**

Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5,000 x g for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

18. **Air-dry the pellet for approximately 10–20 min, and redissolve the DNA in a suitable volume of endotoxin-free Buffer TE.**

Redissolve the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing, and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not dissolve easily in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry and quantitative analysis on an agarose gel.

Agarose gel analysis

We recommend removing and saving aliquots during the purification procedure (samples 1–4). If the plasmid DNA is of low yield or quality, the samples can be analyzed by agarose gel electrophoresis to determine at what stage of the purification procedure the problem occurred (see page 50).

Appendix H: QIAfilter Plasmid Maxi/Midi Kit (Qiagen)

QIAfilter Plasmid Midi and Maxi Protocol

This protocol is designed for preparation of up to 100 µg of high- or low-copy plasmid or cosmid DNA using the QIAfilter Plasmid Midi Kit, or up to 500 µg using the QIAfilter Plasmid Maxi Kit. In this protocol, QIAfilter Cartridges are used instead of conventional centrifugation to clear bacterial lysates. For purification of double-stranded M13 replicative-form DNA, we recommend using the protocol on page 49.

Low-copy plasmids which have been amplified in the presence of chloramphenicol should be treated as high-copy plasmids when choosing the appropriate culture volume.

Maximum recommended culture volumes*

	QIAfilter Midi	QIAfilter Maxi
High-copy plasmids	25 ml	100 ml
Low-copy plasmids†	50 ml	250 ml

* For high-copy plasmids, expected yields are 75–100 µg for the QIAfilter Plasmid Midi Kit and 300–500 µg for the QIAfilter Plasmid Maxi Kit. For low-copy plasmids, expected yields are 10–50 µg for the QIAfilter Plasmid Midi Kit and 50–250 µg for the QIAfilter Plasmid Maxi Kit using these culture volumes.

† The maximum recommended culture volumes apply to the capacity of the QIAfilter Midi and Maxi Cartridges. If higher yields of low-copy plasmids yields are desired, the lysates from two QIAfilter Midi Cartridges can be loaded onto one QIAGEN-tip 100, or the lysates from two QIAfilter Maxi Cartridges can be loaded onto one QIAGEN-tip 500.

Important notes before starting

- New users are strongly advised to read "General Considerations for Optimal Results" provided on pages 60–70 before starting the procedure.
- Add the provided RNase A solution to Buffer P1 before use. Use one vial of RNase A (spin down briefly before use) per bottle of Buffer P1, to give a final concentration of 100 µg/ml.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- Pre-chill Buffer P3 to 4°C.
- In contrast to the standard protocol, the lysate is not incubated on ice after addition of Buffer P3.
- **Optional:** remove samples at the steps indicated with the symbol ☞ in order to monitor the procedure on an analytical gel.

Procedure

1. **Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for ~8 h at 37°C with vigorous shaking (~300 rpm).**

   Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids inoculate 25 ml or 100 ml medium. For low-copy plasmids, inoculate 50 ml or 250 ml medium. Grow at 37°C for 12–16 h with vigorous shaking (~300 rpm).**

   Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 10^9 cells per ml, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium (see page 65).

3. **Harvest the bacterial cells by centrifugation at 6000 x g for 15 min at 4°C.**

   6000 x g corresponds to 6000 rpm in Sorvall GSA or GS3 or Beckman JA-10 rotors. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.

   ⊗ If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. **Resuspend the bacterial pellet in 4 ml or 10 ml Buffer P1.**

   For efficient lysis it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. **Add 4 ml or 10 ml Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for 5 min.**

   Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO_2 in the air.

   During the incubation prepare the QIAfilter Cartridge:

   Screw the cap onto the outlet nozzle of the QIAfilter Midi or QIAfilter Maxi Cartridge. Place the QIAfilter Cartridge in a convenient tube.

6. **Add 4 ml or 10 ml chilled Buffer P3 to the lysate, and mix immediately but gently by inverting 4–6 times. Proceed directly to step 7. Do not incubate the lysate on ice.**

   Precipitation is enhanced by using chilled Buffer P3. After addition of Buffer P3, a fluffy white precipitate containing genomic DNA, proteins, cell debris, and SDS becomes visible. The buffers must be mixed completely. If the mixture still appears viscous and brownish, more mixing is required to completely neutralize the solution. It is important to transfer the lysate into the QIAfilter Cartridge immediately in order to prevent later disruption of the precipitate layer.

7. **Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temperature for 10 min. Do not insert the plunger!**

   Important: This 10 min incubation at **room temperature** is essential for optimal performance of the QIAfilter Midi or QIAfilter Maxi Cartridge. Do not agitate the QIAfilter Cartridge during this time. A precipitate containing proteins, genomic DNA, and detergent will float and form a layer on top of the solution. This ensures convenient filtration without clogging. If, after the 10 min incubation, the precipitate has not floated to the top of the solution, carefully run a sterile pipet tip around the walls of the cartridge to dislodge it.

8. **Equilibrate a QIAGEN-tip 100 or QIAGEN-tip 500 by applying 4 ml or 10 ml Buffer QBT and allow the column to empty by gravity flow.**

   Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

9. **Remove the cap from the QIAfilter outlet nozzle. Gently insert the plunger into the QIAfilter Midi or QIAfilter Maxi Cartridge and filter the cell lysate into the previously equilibrated QIAGEN-tip.**

   Filter until all of the lysate has passed through the QIAfilter Cartridge, but do not apply extreme force. Approximately 10 ml and 25 ml of the lysate are generally recovered after filtration.

   ☞ Remove a 240 µl or 120 µl sample of the filtered lysate and save for an analytical gel (sample 1) in order to determine whether growth and lysis conditions were optimal.

10. **Allow the cleared lysate to enter the resin by gravity flow.**

    ☞ Remove a 240 µl or 120 µl sample of the flow-through and save for an analytical gel (sample 2) in order to the efficiency of DNA binding to the QIAGEN Resin.

11. **Wash the QIAGEN-tip with 2 x 10 ml or 2 x 30 ml Buffer QC.**

    Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid preparations. The second wash is particularly necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

    ☞ Remove a 400 µl or 240 µl sample of the combined wash fractions and save for an analytical gel (sample 3).

12. **Elute DNA with 5 ml or 15 ml Buffer QF.**

    Collect the eluate in a 10 ml or 30 ml tube. Use of polycarbonate centrifuge tubes for collection is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps.

☞ Remove a 100 µl or 60 µl sample of the eluate and save for an analytical gel (sample 4).

⊗ If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. **Precipitate DNA by adding 3.5 ml or 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x g for 30 min at 4°C. Carefully decant the supernatant.**

    All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. A centrifugal force of 15,000 x g corresponds to 9,500 rpm in a Beckman JS-13 rotor and 11,000 rpm in a Sorvall SS-34 rotor. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5,000 x g for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. **Wash DNA pellet with 2 ml or 5 ml of room-temperature 70% ethanol and centrifuge at ≥15,000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet.**

    Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5,000 x g for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. **Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE, pH 8.0, or 10 mM Tris·Cl, pH 8.5).**

    Redissolve the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry and quantitative analysis on an agarose gel.

Agarose gel analysis

We recommend removing and saving aliquots during the purification procedure (samples 1–4). If the plasmid DNA is of low yield or quality, the samples can be analyzed by agarose gel electrophoresis to determine at what stage of the purification procedure the problem occurred (see page 50).

Appendix I: CAT ELISA (Roche)

3.2 Sample preparation

Cell lysis

Preparing the cell extracts using the lysis buffer offers the advantage that

- very mild conditions are used
- adherent cells do not need to be scraped from the culture dish
- samples are processed very rapidly
- thereby markedly facilitating CAT determinations in large scale experiments.

Another advantage of the lysis buffer is, that it is fully compatible with the β-Gal ELISA and the enzymatic reporter gene assays for luciferase and β-galactosidase (see related products). Therefore, the CAT ELISA could be combined with the a.m. determination methods in co-transfection experiments.

Additional buffers required

For the preparation of cells Phosphate-buffered saline (PBS), pre-cooled to 2–8°C is required.

Preparation of suspension cells

Please refer to the following table

Step	Action
1	Pellet the suspension cells at 250 × g for 10 min in a refrigerated centrifuge at 2–8°C.
2	Discard the supernatant.
3	Resuspend the cell pellet and wash the cells with 5 ml of pre-cooled **PBS** (2–8°C).
4	Repeat the centrifugation and wash steps two more times.

Preparation of adherent cells

Carefully remove culture medium and wash cells with 5 ml of pre-cooled PBS three times.

Preparation of cell extracts

Please refer to the following table.

Step	Action
1	After the last washing step, carefully remove PBS.
2	Add 1 ml of **Lysis buffer** (solution 8) to the cells to stand for 30 min at 15 to 25°C. ***Note***: 1 ml Lysis buffer is sufficient for the lysis of approx. 2×10^6 cells grown in suspension or for adherent cells grown in a 6 cm culture dish.
3	Cell extracts should be used immediately for the CAT ELISA or stored at −70°C. We recommend that the cell extracts be frozen in dry ice/ethanol before transferring the cell extracts for storage at −70°C. This rapid freezing by the dry ice/ethanol step avoids the degradation of CAT. ***Note:*** Prolonged storage at 2–8°C should be avoided. In order to stabilize the cell extracts protease inhibitors may be added.

continued on next page

General comments

All cytoplasmic and nucleoplasmic components, including CAT, will be extracted by the lysis buffer.

IF you use...	THEN...
adherent cells, nuclei, including DNA packed in chromatin, will remain attached to the vessel surface (16).	• Transfer 1 ml of cell extract (i.e. the supernatant) to a microfuge tube. The cell extract contains soluble components of the cell including the CAT enzyme. • Spin the cell extract in a microfuge at maximum speed for 10 min to remove any cellular debris. Centrifugation at 2–8°C is recommended.
suspension cells the extract also contains nuclei and other cellular structures that remain attached to the vessel surface with adherent cells.	• We recommend spinning suspension cells using a microfuge at maximum speed for 15 min. Alternatively, centrifuge cell extracts for 10 min at approx. 15 000 × g in a refrigerated centrifuge. • Remove the supernatant and take an aliquot of the supernatant for protein determination (see 3.3).

3.3 Protein determination

Introduction

Results have to be normalized with respect to protein concentration or cell number.

For protein determination use Roche Molecular Diagnostic's Protein Assay ESL or other copper-based protein assays e.g. according to Lowry (17).

- Be aware that higher detergent concentrations may interfere with determination method. Therefore, check for interference or correct the calibration curve by addition of an equal amount of detergent lysis buffer.
- Protein determination should be performed in the linear range of the calibration curve. If absorbance in the sample is in the nonlinear range, we recommend repeating the protein determination to obtain reliable results.
- Volumes of samples should be adjusted so that the absorbance of the sample falls within the linear range. Do not dilute the cell extracts before performing the protein determination.

Alternatively different methods for determination of cell numbers can be used for normalization e.g. measurement of metabolic activity by cleavage of the tetrazolium salt WST-1*.

Additional reagents required

WST-1, Cell proliferation Reagent (Cat. No. 1 644 807)

How to use WST-1 assay:

Please refer to the following table.

Step	Action
1	Perform cell culturing and transfection according to your standard protocol.
2	30–150 min before cell lysis, add **10% WST-1 reagent** to the cell medium.
3	Quantify conversion of WST-1 directly from an aliquot, using an ELISA reader.
4	Withdraw reagent/medium and lyse cells for reporter gene assay.
5	Normalize reporter results according to the absorbance of the WST-1 assay. Further information: http://biochem.roche.com/techserv/ttip0598.htm

3.4 Measurement of CAT

General recommendations

- The amount of sample material required in the assay depends on the level of expression. The type of promoter, type of expression (stable versus transient) and cell type all affect the amount of CAT produced. In general, it is recommended to start with 50 µg or 1×10^3 cells.
- Most of the available ELISA plate readers reach their absorbance maximum at approx. 2 to 2.5 absorbance units. Measurement of supernatants with high CAT concentrations therefore requires further dilution of the cell extracts with Sample buffer (solution 7).

Weak CAT expression

When testing vectors with weak CAT expression, incubate the microplate with the CAT-containing cell extract for 2 h at 37°C. This results in an increase in sensitivity by a factor of 1.5 to 2. Alternatively, the protein concentration used per well can be increased (e.g. from 50 µg/well to 150 µg/well).

Substrate enhancer

The use of the substrate enhancer with POD substrate ABTS (solution 5) approximately doubles the sensitivity of the assay and can be used following the regular ABTS (solution 4).

Step	Action
1	Removal of the POD substrate ABTS without substrate enhancer (solution 4), when the test is first performed under non-optimal conditions.
2	Washing of each well two times with **Washing buffer** (solution 6).
3	Incubate with **POD substrate ABTS with Substrate enhancer** (solution 5).
4	Using the same procedure, an incubation with any of the substrate buffers may be followed by an incubation with another POD substrate solution to adapt the sensitivity. ***Note:*** In any case, an additional substrate reaction can only be performed when the preceding incubation step has not been stopped, e.g. using H_2SO_4.

Non-linear calibration curves

Prolonged incubation of the samples with the peroxidase substrate ABTS (e.g. overnight at 2–8°C) can produce a non-linear calibration curve and is therefore only recommended for qualitative analysis of CAT expression.

Since the POD substrates (solutions 4, 5) are slightly colored, leave one well free in order to determine the blank (baseline) value. Add POD substrate to this well for use as a reference when measuring the MP modules in the ELISA reader. Most readers can be programmed to automatically subtract the reference (blank) value from the values of the other samples.

3.5 Preparation of CAT enzyme standards

Preparation of CAT enzyme working dilution

Add 40 µl CAT enzyme stock solution (solution 1) to 3.96 ml Sample buffer (solution 7) to obtain a CAT enzyme working dilution (final concentration approx. 1 ng/ml), sufficient to produce a calibration curve in duplicate.

Handling instructions

- The CAT enzyme standard dilutions should be prepared freshly before use and should not be stored.
- Prepare the standard dilution series in reaction tubes in 1:2 dilution steps as described in the table below.
- To obtain a calibration curve, we recommend using the five concentrations listed.
- 200 µl of each dilution is needed per well.
- To ensure that the measurements and the calibration curve are accurate, we recommend preparing two samples of each concentration for measurement.
- To avoid carryover of the higher conc. solution to the lower conc. samples, use a fresh pipette tip for each dilution step.
- Each dilution must be measured in duplicate.

Procedure

Preparation of CAT enzyme standards are used to produce a calibration curve for the CAT enzyme.

Step	CAT enzyme working dilution (approx. 1 ng/ml)	Add Sample buffer (solution 7)	Approximate CAT enzyme conc. (ng/ml)
0	0	1000 µl	0
1	1000 µl	0	1.0
2	500 µl of step 1	500 µl	0.5
3	500 µl of step 2	500 µl	0.25
4	500 µl of step 3	500 µl	0.125

Pipetting scheme for the microplate

Please refer to the following table.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl	Bl	P1	P1								
B	S0	S0										
C	S1	S1										
D	S2	S2										
E	S3	S3										
F	S4	S4										
G	P1	P1										
H	P2	P2									P39	P39

Legend:
Bl = blank (= POD substrate, solution 4 or 5)
S0-S4 = CAT standard dilutions
P1-P39 = samples 1-39

3.6 ELISA ASSAY

Handling instructions

Use only the microplate (MP) modules required for the particular experiment and place them in the frame in the correct orientation. (Correct fitting ensures a tight support of the MP modules). The MP modules are ready-to-use and need not to be rehydrated prior to addition of the samples.

Procedure

Important: Reagents should be fully equilibrated to room temperature (20–23°C) before starting the test. Reagents from kits with different lot numbers must not be used in one test series.

Step	Action
1	Pipette 200 µl of **CAT standard, working dilutions** or 200 µl **cell extracts** per well.
2	Cover the MP modules with a cover foil. Incubate for 1 h at 37°C.
3	Remove the solution. Rinse wells 5 times with 250 µl of **Washing buffer** (solution 6) for 30 s each and remove Washing buffer carefully.
4	Pipette 200 µl of **Anti-CAT-DIG working dilution** (solution 2a) per well Cover the MP modules with the cover foil. Incubate for 1 h at 37°C.
5	Remove the solution. Rinse wells 5 times with 250 µl of **Washing buffer** (solution 6) for 30 s each and remove washing buffer carefully.
6	Pipette 200 µl of **Anti-DIG-POD working dilution** (solution 3a) per well. Cover the MP modules with the cover foil Incubate for 1 h at 37°C.
7	Remove the solution. Rinse wells 5 times with 250 µl of **Washing buffer** (solution 6) for 30 s each and remove washing buffer carefully.
8	Pipette 200 µl of **POD substrate without** (solution 4) **or POD substrate with substrate enhancer** (solution 5) into each well. ***Note:*** Use the substrate enhancer only if the CAT concentration is low! Incubate at 15 to 25°C until color development (green color) is sufficient for photometric detection (10–40 min). Shaking of microplates at 250 rpm during incubation with substrate solution can be employed to shorten the incubation period, but is not essential. If shaking is not carried out, gently tap on the side of the microplate before measuring absorbance to ensure a homogeneous distribution of the colored reaction product.
9	Measure the absorbance of the samples at 405 nm (reference wavelength: approx. 490 nm) using a microplate (ELISA) reader (e.g. EAR 340 ATTC, SLT Lab Instruments).

Interpretation of results

- Upon completion of the experimental procedure, calculate the exact CAT concentration (ng/ml) of the calibration standards. Plot the absorbance values obtained on the y-axis against the lot specific standard concentrations on the x-axis. This results in a linear calibration curve (for an example, see fig. 2).
- CAT concentration of unknown samples can then be determined by plotting the observed absorbance values also on the y-axis, extrapolating to meet the calibration curve and reading the CAT enzyme concentration from the x-axis. To obtain reliable results, the absorbance values of the unknown sample should lie within the linear portion of the calibration curve.

Note: A separate calibration curve must be established for each series. We recommend that one experimental series be performed on one microplate. When more than one microplate is used in one series, a calibration must be carried out on each plate.

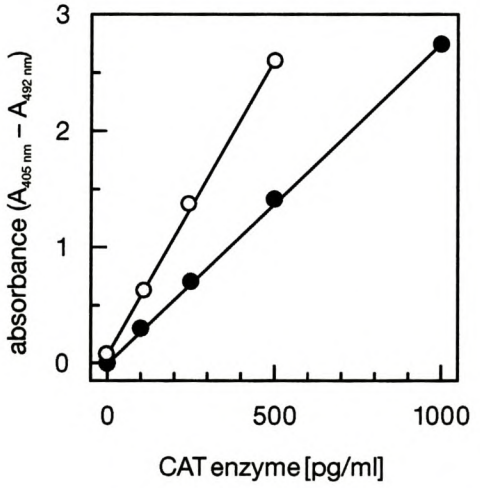

Fig. 2: A typical calibration curve using ABTS with (○) or without (●) substrate enhancer as peroxidase substrate.

Appendix J: ECLPlus (Amersham-Pharmacia Biotech)

ECL

Product specification

ECL Plus Western blotting reagent pack
RPN 2124

Before using this product, please read the instructions below for safe handling, storage and disposal.

Safety warnings and precautions

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

We recommend that this product and components are handled only by those persons who have been trained in laboratory techniques and that it used in accordance with the principles of good laboratory practice. As all chemicals should be considered as potentially hazardous, it is advisable when handling chemical reagents to wear suitable protective clothing, such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with the skin or eyes. In the case of contact with skin or eyes, wash immediately with water.

Description

This kit contains reagents for membrane blocking and secondary detection of membrane-bound mouse and rabbit primary antibodies. Detection reagents are not included, but the system is optimized for use with ECL Plus™ (1,2)

Contents of kit

Anti mouse IgG, peroxidase linked whole antibody (from sheep)	100µl
Anti rabbit IgG, peroxidase linked whole antibody (from donkey)	100µl
ECL™ Membrane blocking agent	5g

Detection of membrane bound primary antibodies

Prepare samples according to experimental requirements, following usual gel electrophoresis and western blotting techniques. We recommend use of Hybond™ ECL membrane (RPN2020D) or Hybond P PVDF membrane (RPN 2020P) for maximum sensitivity. To avoid membrane contamination or damage, always use forceps and wear gloves when handling membranes.

amersham pharmacia biotech

Protocol

1) Block the membrane

Non-specific binding sites are blocked by immersing the membrane in a 5%(w/v) solution of the blocking agent in PBS or TBS Tween™ (0.1% is usually sufficient) for 1 hour at room temperature on an orbital shaker.

2) Washing

PBS or TBS Tween (0.1%) is recommended as the wash buffer. Briefly rinse the membrane twice, then wash 3 times, once for 15 minutes and twice for 5 minutes at room temperature with fresh changes of wash buffer on an orbital shaker.

3) Dilution of the primary antibody

During the washing step, dilute the primary antibody. Incubate the washed membrane in the diluted antibody. 1 hour at room temperature is often sufficient, but this should be optimized for each antibody.

4) Washing

Wash the membrane as detailed above.

5) Incubation with the species specific peroxidase linked second antibody

These dilution factors are meant as a guide only, and are indicated as suitable for the system in which they were used. Users should optimize their own system to determine the dilution factors required. It is likely that when using ECL Plus, dilution factors may need to be greater than previously used with other detection systems.

- **For detection with ECL Plus when using Hybond ECL membrane**

We have found in our laboratories that dilutions of 1:25000 of the peroxidase linked anti mouse antibody is suitable to detect 3ng of actin protein with an exposure time of 5 minutes. We have also found that a dilution factor of 1:100000 of the peroxidase linked anti rabbit antibody is suitable for the detection of 6ng of beta galactosidase with an exposure time of 5 minutes.

- **For detection with ECL Plus when using Hybond PVDF membrane**

We have found in our laboratories that dilutions of 1:50000 of the peroxidase linked anti mouse antibody is suitable to detect 3ng of actin protein with an exposure time of 5 minutes. We have also found that a dilution factor of 1:200000 of the peroxidase linked anti rabbit antibody is suitable for the detection of 6ng of beta galactosidase with an exposure time of 5 minutes.

6) Washing

Wash the membrane as detailed above.

7) Detection
Follow the detection procedures as outlined in the protocol booklet provided with the detection reagents.

Notes
1) It is essential to optimize both primary and secondary antibodies for results with high signal and low background due to the extreme sensitivity of the detection system when using ECL and ECL Plus.
2) As a general rule, as large a volume of washing buffer as possible should be used each time. It may be necessary to adjust the blocking conditions for certain applications.
3) Do not use azide as a preservative for buffers to be used in immunodetection as it is an inhibitor of horseradish peroxidase.

Additional information

Reagent specifications

- **Peroxidase labelled anti species antibodies**

Horseradish peroxidase conjugated antibody is supplied in phosphate buffered saline (sodium phosphate 0.1M,NaCl 0.1M) pH7.5, containing 1%(w/v) bovine serum albumin and an anti microbial agent.
Store at 2-8°C, do not freeze. Under these conditions, the reagent is stable for 6 months.

- **ECL blocking reagent**

This reagent has been selected to give effective blocking of membranes in virtually all blotting contexts, if used as directed. Caution: Moisture will affect the solubility properties of the powder. Store the container at room temperature, ensuring that the lid is tightly closed.

References

(1) AKHAVEN-TAFTI, H. *et al., Clin. Chem.,* **41**, pp.1368-1369, 1995.
(2) AKHAVEN-TAFTI, H. *et al., Biolum. and Chemilum. Fundamentals and Applied Aspects,* pp.199-202, Chichester, 1994.

Related products

ECL Plus Western blotting detection reagents	
- sufficient for 3000cm^2 membrane	RPN 2133
- sufficient for 1000cm^2 membrane	RPN 2132
ECL Western blotting detection reagents	
- sufficient for 6000cm^2 membrane	RPN 2134
- sufficient for 4000cm^2 membrane	RPN 2106
- sufficient for 2000cm^2 membrane	RPN 2209
- sufficient for 1000cm^2 membrane	RPN 2109
ECL Western blotting analysis system	RPN 2108
For the detection of either mouse or rabbit membrane bound primary antibodies with ECL detection reagents. Sufficient for 1000cm^2 membrane	
ECL streptavidin-HRP and blocking reagent	RPN 2195
Mouse IgG, horseradish peroxidase linked whole antibody (from sheep)	NA 931
Rabbit IgG, horseradish peroxidase linked whole antibody (from donkey)	NA 934
Rat IgG, horseradish peroxidase linked whole antibody (from sheep)	NA 932
Human IgG, horseradish peroxidase linked whole antibody (from sheep)	NA 933
Mouse IgG, horseradish peroxidase linked F(Ab')$_2$ fragment (from sheep)	NA 9310
Rabbit IgG, horseradish peroxidase linked F(Ab')$_2$ fragment (from donkey)	NA 9340
Rat IgG, horseradish peroxidase linked F(Ab')$_2$ fragment (from sheep)	NA 9320
Human IgG, horseradish peroxidase linked F(Ab')$_2$ fragment (from sheep)	NA 9330
Streptavidin horseradish peroxidase conjugate	RPN 1231
Streptavidin biotinylated horseradish peroxidase complex	RPN 1051
ECL protein molecular weight markers	RPN 2107
Rainbow™ coloured protein molecular weight markers (molecular weight range 2350 – 46000)	RPN 755
Rainbow coloured protein molecular weight markers (molecular weight range 14300 - 200000)	RPN 756
Full Range Rainbow recombinant protein molecular weight markers (molecular weight range 10000 – 250000)	RPN 800
Hybond ECL nitrocellulose membrane	RPN 2020D
Hybond PVDF membrane	RPN 2020P
Hyperfilm™-ECL	RPN 2103

For details of sizes, availability and ordering information, please contact your local sales office.

http://www.apbiotech.com
Amersham Pharmacia Biotech UK Limited Amersham Place
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Amersham Pharmacia Biotech AB SE-751 84 Uppsala Sweden
Amersham Pharmacia Biotech Inc 800 Centennial Avenue PO Box 1327 Piscataway NJ08855 USA
Amersham Pharmacia Biotech Europe GmbH Munzinger Strasse 9 D-79111 Freiburg Germany

RPN2124PS/AB

Appendix K: β-Galactosidase Enzyme Assay Kit (Promega)

Promega

IV. β-Galactosidase Assays

A. Standard Assay

Preparation of a standard curve is optional. If a standard curve is desired, please see Section V.

1. Thaw the system components and mix each component well before use. Place the Assay 2X Buffer on ice. See Note 1.
2. It may be necessary to dilute the cell lysates in 1X Reporter Lysis Buffer. A 2:1 dilution of lysate to 1X Reporter Lysis Buffer (100µl of lysate plus 50µl of 1X Reporter Lysis Buffer) is a good starting dilution, but up to 150µl of cell lysate can be used per reaction. As a negative control, prepare the same dilution of a cell lysate made from cells that have not been transfected with the β-galactosidase gene.
3. Pipet 150µl of the appropriately diluted (or undiluted) cell lysates into labeled tubes.
4. Add 150µl of Assay 2X Buffer to each of the tubes.
5. Mix all samples by vortexing briefly.
6. Incubate the reactions at 37°C for 30 minutes or until a faint yellow color has developed. Color development continues for approximately 3 hours. If enzyme activity is low, samples may be incubated overnight if the reaction tubes are tightly capped.
7. Stop the reactions by adding 500µl of 1M Sodium Carbonate. Mix by vortexing briefly.
8. Read the absorbance at 420nm.

It is important to read the absorbance immediately after addition of 1M Sodium Carbonate (3).

B. 96-Well Plate Assay

This protocol is useful for testing numerous samples. The modified assay is performed directly in 96-well plates, and the absorbance of each sample is read using a plate reader. Preparation of a standard curve is optional. If a standard curve is desired, please see Section V.

1. Thaw the system components and mix each component well. Place the Assay 2X Buffer on ice. See Note 1.
2. It may be necessary to dilute the cell lysates in 1X Reporter Lysis Buffer. Mix 30µl of lysate with 20µl of 1X Reporter Lysis Buffer as a starting dilution, but up to 50µl of cell lysate can be used per reaction. As a negative control, prepare the same dilution of a cell lysate made from cells that have not been transfected with the β-galactosidase gene.
3. Pipet 50µl of the appropriately diluted (or undiluted) cell lysates into labeled wells of a 96-well plate.
4. Add 50µl of Assay 2X Buffer to each well of the 96-well plate.
5. Mix all samples by pipetting the well contents. Place a cover on the plate.
6. Incubate the plate at 37°C (an incubator works well) for 30 minutes or until a faint yellow color has developed. Color development continues for approximately 3 hours. **Due to the small sample volume, we do not recommend incubating the reactions overnight.**

Do not incubate reactions overnight.

7. Stop the reaction by adding 150µl of 1M Sodium Carbonate. Mix by pipetting the contents of each well. Avoid producing bubbles, which may interfere with absorbance readings; if present, bubbles may be removed by piercing with a fine gauge needle.
8. Read the absorbance of the samples at 420nm in a plate reader (see Note 5).

Notes:

1. If crystals are present in the 1M Sodium Carbonate, warm the solution to 37°C to dissolve the crystals and then leave it at room temperature. If a precipitate is present in the Assay 2X Buffer, warm briefly in a 37°C water bath to dissolve and then place the solution on ice.
2. The 96-well plate assay is configured for a plate that has a maximum well volume of approximately 300µl. For plates with different maximum well volumes, the reaction may be scaled up or down proportionally.
3. Plate readers generally perform best using plates that have flat-bottomed, optically clear wells.
4. The coatings applied to some 96-well plates may inhibit the β-galactosidase reaction. To test for this, perform identical reactions in a 96-well plate and in microcentrifuge tubes. Stop the reactions, pipet the tube reactions into the plate wells and read all samples in a plate reader. The absorbance values should be the same for both types of samples.
5. Some plate readers are limited in the number of wavelengths at which they can read. Although the peak absorbance of the reaction product is near 420nm, other wavelengths close to 420nm may be used to monitor the reaction. The greatest sensitivity is obtained with wavelengths of 410–430nm.

It is important to read the absorbance immediately after addition of 1M Sodium Carbonate (3).

V. Standard Curves

A. Preparation of Standard Curve for Standard Assays

If a standard curve is desired, use standards between 0 and 6.0 x 10^{-3} units of β-Galactosidase. Prepare the following dilution series in 1X Reporter Lysis Buffer immediately before use. Add 10µl of 1u/µl β-Galactosidase to 990µl of 1X Reporter Lysis Buffer and mix. Then add 10µl of this 1:100 dilution to 990µl of 1X Reporter Lysis Buffer and mix it to make a 1:10,000 stock solution. Using this stock, prepare 150µl of each β-Galactosidase standard per tube as described below.

β-Galactosidase Standard (milliunits)	Volume of 1:10,000 Stock	Volume of 1X Reporter Lysis Buffer
0	0µl	150µl
1.0	10µl	140µl
2.0	20µl	130µl
3.0	30µl	120µl
4.0	40µl	110µl
5.0	50µl	100µl
6.0	60µl	90µl

1. Follow the protocol described in Section IV.A, Steps 4–8.
2. Plot the absorbance at 420nm versus concentration of β-Galactosidase standards. An example of a standard curve prepared by the standard assay method is shown in Figure 1.

If a standard curve is used, prepare fresh enzyme dilutions each time the assay is performed.

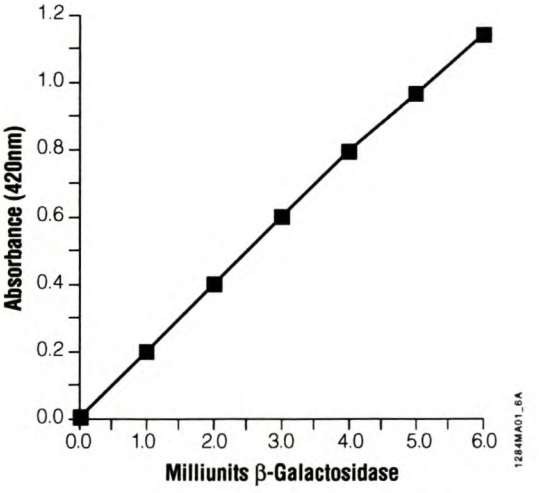

Figure 1. A sample standard curve for the standard assay. This standard curve was prepared as described in Section V.A. Samples were incubated for 30 minutes at 37°C.

B. Preparation of Standard Curve for 96-Well Plate Assays

If a standard curve is desired, use standards between 0 and 5.0×10^{-3} units of β-Galactosidase. Prepare the following dilution series in 1X Reporter Lysis Buffer immediately before use. Add 10µl of 1u/µl β-Galactosidase to 990µl of 1X Reporter Lysis Buffer and vortex. Then add 10µl of this 1:100 dilution to 990µl of 1X Reporter Lysis Buffer and vortex to make a 1:10,000 stock solution. Using this stock, prepare 50µl of each β-Galactosidase standard per well as described below.

β-Galactosidase Standard (milliunits)	Volume of 1:10,000 Stock	Volume of 1X Reporter Lysis Buffer
0	0µl	50µl
1.0	10µl	40µl
2.0	20µl	30µl
3.0	30µl	20µl
4.0	40µl	10µl
5.0	50µl	0µl

1. Follow the protocol described in Section IV.B, Steps 4–8.
2. Plot the absorbance at 420nm versus concentration of β-Galactosidase standards. An example of a standard curve prepared by the 96-well plate method is shown in Figure 2.

VI. In situ Staining of Cells for β-Galactosidase Activity

Cells transfected with the pSV-β-Galactosidase Control Vector and expressing β-galactosidase can be visualized by microscopy (4). The cells appear blue following fixation and incubation with the substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). For comparison, it is important to include control cells that have not been transfected with a β-galactosidase vector in order to visualize the level of background activity due to endogenous β-galactosidase or its isozymes. The following protocol is for use with a 60mm culture dish.

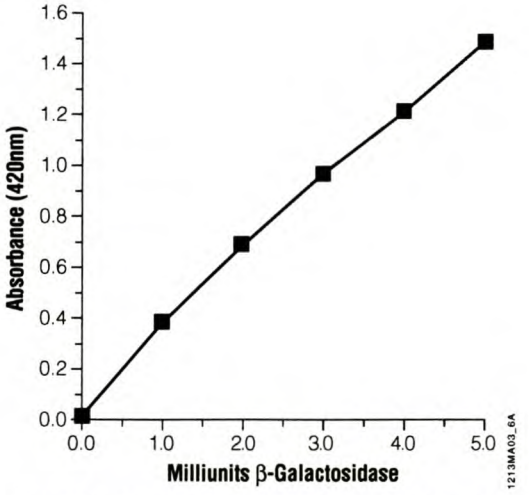

Figure 2. A sample of the standard curve for the 96-well assay. This standard curve was prepared as described in Section V.B. Samples were incubated for 30 minutes at 37°C.

If a standard curve is used, prepare fresh enzyme dilutions each time the assay is performed.

A. Staining Protocol

Materials to Be Supplied by the User
(Solution compositions are provided in Section VIII.A.)

- PBS 1X buffer (Mg^{2+}- and Ca^{2+}-free)
- glutaraldehyde solution
- X-Gal solution

1. Wash the cells twice with PBS 1X buffer. Remove all of the final wash buffer.
2. Fix the cells by adding 2ml of glutaraldehyde solution and incubating for 15 minutes.
3. Remove the glutaraldehyde solution and rinse gently 3 times with PBS 1X buffer. It is important to remove residual glutaraldehyde, which could inhibit β-galactosidase activity.
4. Add 1ml of X-Gal solution per plate of cells. Incubate the cells at 37°C between 1–16 hours until the cells are visibly stained. The exact incubation time must be optimized for each set of transfections.
5. Remove the X-Gal solution. Cover the cells with 1X PBS.
6. View the cells with a phase contrast or light microscope. To obtain a permanent record of the results, photograph in situ stained cells on the same day of the experiment.
7. For long-term storage (weeks to months) of in situ stained cells, store the cells under 70% glycerol at 4°C.

Caution: Glutaraldehyde is a carcinogen. Avoid contact with skin and avoid inhalation. Use in a fume hood and discard waste according to your institution's procedures.

VII. References

1. Rosenthal, N. (1987) Identification of regulatory elements of cloned genes with functional assays. *Meth. Enzymol.* **152**, 704–20.
2. Schenborn, E. and Goiffon, V. (1993) A new lysis buffer for luciferase, CAT and β-galactosidase reporter gene co-transfections. *Promega Notes* **41**, 11.