NOTE TO USERS

Characterization and functional analysis of the SIV untranslated region.

By
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ABSTRACT

The focus of this dissertation is twofold. The first section aims at elucidating and describing the functional role(s) of the 5' untranslated region of simian immunodeficiency virus (SIVmac239). This work focuses on several interrelated areas involving this important control locus of SIV. Specifically, the first section involves the determination of *cis*-acting structural elements and sequences involved in RNA encapsidation and regions impacting RNA dimerization. This work also elucidates the location of the KLD/DIS region within the SIV leader expanse. This work is novel and has contributed significantly to the overall understanding of this region and its role in RNA-protein interaction.

Although the second portion of this dissertation is somewhat arbitrarily divided, the general focus is on the characterization of novel SIV vaccine candidates.

The salient difference of the abovementioned variants is that they are devoid of all accessory genes. The missing functions have been replaced by comparable elements from other viruses.

One adjunct to this work, in conjunction with the above RNA packaging studies, was a discovery process for genetically stable attenuated variants suitable for evaluation as vaccine candidates in the macaque model system. One area of concern in live attenuated vaccine research is compensatory reversion and the inherent genetic instability of live-attenuated vaccine approaches. This latter topic is addressed in part in the second half of this dissertation. The final chapter specifically considers methodologies that might impair or offset viral compensatory mutagenesis. Interestingly, the region investigated in the first half of this thesis, .ie. SL1, is a determinant of retroviral variation, that has been found necessary for efficient reversion in conjunction with RT. The first chapter of this section summarizes this work and integrates it into the current body of literature in review format.

RÉSUMÉ

Le but de cette dissertation est double. La première section vise à élucider et décrire le(s) rôle(s) fonctionnel(s) de la région 5' non traduite du virus de l' mmunodeficience simienne (SIVmac239). Ce travail se concentre sur plusieurs domaines reliés entre eux, qui impliquent le contrôle locus important du SIV. Spécifiquement, la première section implique la détermination des éléments structurels *cis* et les séquences qui sont impliquées dans la capsidation de l'ARN, et les régions qui ont un impact sur la dimerization de l'ARN. Ce travail explique l'emplacement de la région KLD/DIS dans le "leader expanse" du SIV. Ce travail est novateur et a contribué de manière significative à une compréhension globale de cette région et de son rôle dans l'interaction de la protéine ARN

Malgré que la seconde portion de cette dissertation est en quelque sorte divisée de manière arbitraire, le but général est axé sur la caractérisation de nouveaux candidats de vaccin du SIV.

La différence saillante des variantes mentionnées ci-dessus est qu'elles sont dénudées de tout gène accessoire. Les fonctions manquantes ont été remplacées par des éléments comparables d'autres virus.

Un accessoire de ce travail, conjointement à l'étude de l'enveloppe d'ARN mentionnée ci-dessus, a été un processus de découverte des variantes atténuées génétiquement stables qui convient à l'évaluation de vaccins candidats dans des systèmes de modèles macaques. Un domaine d'intérêt dans la recherche des vaccins vivants atténués est la réversion compensatoire et l'instabilité génétique inhérente des approches de vaccins vivants atténués. Ce dernier sujet est abordé en partie dans la deuxième portion de cette dissertation. Le dernier chapitre en particulier examine les méthodes pour diminuer ou contrebalancer la mutagénèse virale compensatoire. Il est intéressant de constater que la région examinée dans la première partie de cette thèse, c'est-à-dire que le SL1 est une déterminante de la variation rétrovirale, a été nécessaire pour une réversion efficace conjointement à la TI. Le premier chapitre de cette section résume ce travail et l'intègre dans le corps actuel de la littérature sous forme de révision.

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Finally, I must thank my wife Berna, for being there when I needed her the most.

PREFACE

This Ph.D. thesis was written in accordance with the Guidelines Concerning Thesis Preparation from the Department of Graduate Studies and Research at McGill University. The experimental aspect of this thesis is presented in the format of complied original papers, exercising the option found in the guidelines which states:

"The candidate has the option, subject to the approval of the department, of including, as part of their thesis, copies of the text of a paper(s)... If this option is chosen, connecting texts, providing logical bridges between the different papers are mandatory... The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; ... since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

The contribution of co-authors to published articles, as well as the information from published articles which appear in this thesis, are found on the title page of each concerning chapter. The author's " contribution to original knowledge" and references cited appear at the end of the thesis.

Manuscripts, either submitted or published works incorporated into this thesis, in which the candidate was directly involved, are as follows:

Chapter 2: James B. Whitney, and Mark A. Wainberg. Attenuating viral evolution. To be submitted to ***AIDS reviews***, 2003.

Chapter 3: Yongjun Guan, **James B. Whitney**, Karidia Diallo, and Mark A. Wainberg. Leader sequences downstream of the primer-binding site are important for efficient replication of simian immunodefiency virus. ***J Virol.*** 2000; 74(19): 8854-60.

Chapter 4: James B. Whitney, Maureen Olivera, and Mark A. Wainberg. A Dual Role for Leader Sequences of Simian Immunodeficiency Virus in RNA packaging and Dimerization. Submitted to ***Virology***, September 2003.

Chapter 5: Yongjun Guan, **James B. Whitney**, Mervi Detorio, and Mark A. Wainberg. Construction and *in vitro* properties of a series of attenuated simian immunodeficiency viruses deleted of all accessory genes. ***J Virol.*** 2001; 75(9): 4056-67.

Chapter 6: James B. Whitney, Maureen Olivera, Mervi Detorio, Yongjun Guan, and Mark A. Wainberg. The M184V Mutation in Reverse Transcriptase Can Delay Reversion of Attenuated Variants of Simian Immunodeficiency Virus. ***J. Virol.*** 2002; 76(17): 8958-62.

Other manuscripts, either submitted or published works, not incorporated into this thesis, but in which the candidate was involved, are as follows:

James B. Whitney, Maureen Olivera,Yongjun Guan, and Mark A. Wainberg. Recovery of Fitness of an Attenuated Simian Immunodeficiency Virus through Compensation in Coding and Non-coding regions. . In preparation for ***Virology*** 2003.

Xiaofeng Guo, Jing Hsu, **James B. Whitney**, and Chen Liang. An important role for the spacer peptide p3, in the assembly of bovine immunodeficiency virus. Submitted to ***J. Virol.*** June 2003.

Chen Liang, Jing Hsu, **James B. Whitney**, Lawrence Kleiman, and Mark A. Wainberg 2003. A Structurally Disordered Region at the C-terminus of Capsid Plays Essential Roles in Multimerization and Membrane-Binding of the Gag Protein of Human Immunodeficiency Virus Type-1. ***J. Virol.*** 77(3): 1772-83.

James B. Whitney, and Mark A. Wainberg. 2003. Isoniazid, the Frontline of Resistance in *Mycobacterium Tuberculosis*. ***McGill Journal of Medicine.*** 2002 6(2):114-123.

Yongjun Guan, Karidia Diallo, **James B. Whitney**, Chen Liang, and Mark A. Wainberg. 2001. An Intact U5-leader Stem Is Important for Efficient Replication of Simian Immunodeficiency Virus. ***J Virol.*** **75** (23): 11924-9.

Yongjun Guan, Karidia Diallo, Mervi Detorio, **James B Whitney**, Chen Liang, and Mark A.Wainberg. 2001. Partial Restoration of Replication of Simian Immunodeficiency Virus by Point Mutations in Either the Dimerization Initiation Site (DIS) or Gag Region after Deletion Mutagenesis within the DIS. ***J Virol.*** **75** (23): 11920-3.

Yongjun Guan, **James B. Whitney**, Chen Liang, and Mark A. Wainberg. 2001. Novel live attenuated simian immunodeficiency virus constructs containing major deletions in leader RNA sequences. ***J. Virol.*** **75** (6): 2776-85.

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LIST OF ABBREVIATIONS

3TC	2'3'-dideoxy-3'-thiacytidine
AIDS	acquired immunodeficiency syndrome
ADCC	antibody dependent cell-mediated cytotoxicity
APC	antigen presenting cell
CD	cluster of differentiation
CMV	cytomegalovirus
CTE	constitutive transport element
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DIS	dimerization initiation site
DLS	dimerization localization signal
DNTP	deoxynucleotide triphosphate
DMEM	Dubeccos' modified essential media
dsDNA	double stranded deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
ELISA	enzyme-linked immnosorbent assay
Env	envelope
Gag	group specific antigen
GM-CSF	granulocyte macrophage-colony stimulating factor
gp	glycoprotein
HIV-1/HIV-2	human immunodeficiency virus type 1/ type 2
HLA	human leukocyte antigen (several)
IFN	interferon
Ig	immunoglobulin
IL-2	interleukin-2
kb	kilobase
kD	kilodalton
KLD	kissing-loop domain
LTNP	long-term non-progressor
LTR	long terminal repeat
Mab	monoclonal antibody
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK cell	natural killer cell
Nef	negative regulatory factor
NEO	neomycin G418
NRE	negative regulatory element
nt	nucleotide
ORF	open reading frame
PBMC	peripheral blood mononuclear cells

PBS	primer binding site
PHA	phytohemagglutinin
Pol	polymerase
Poly-A	polyadenylation
R	repeat region
Rev	regulator of virion expression
RPMI	Roswell Park Memorial Institute
RRE	Rev responsive element
RT	reverse transcriptase
RT-PCR	reverse transcriptase- polymerase chain reaction
SD	major splicing donor site
SDS	sodium dodecyl sulfate
SL	stem loop
SIV	simian immunodeficiency virus
SFV	simian foamy virus
SRV-1	simian retrovrus type 1
TAR	Tat-responsive element
Tat	transactivator
TNF	tumor necrosis factor
U3	unique 3' region
U5	unique 5' region
UTR	un-translated region
Vif	viral infectivity factor
Vpr	viral protein R
Vpu	viral protein U
Vpx	viral protein X

1.0 Introduction

1.0.1 Retroviridae

The family Retroviridae comprises a uniquely important group of infectious organisms. Their significance is underscored by the award of the Nobel Prize, on at least three separate occasions to scientists in this field (Rous 1966, Baltimore, 1970, Temin and Mizutani 1970, Bishop and Varmus 1989).

The family is divided into three broad groups oncovirinae, spumavirinae, and lentivirinae. More generally, retroviruses comprise a large and diverse group of enveloped, icosahedral, double-stranded, positive sense RNA viruses. Their diameter determined by transmission electrom microscopy, ranges from 100-140 nm. Their characteristic hallmarks include reverse transcription of viral RNA into proviral ds-DNA by the virally encoded reverse transcriptase (RT). Concurrent with reverse transcription is the migration of the retroviral pre-integration complex (PIC) to the nucleus. In the case of some genera, nuclear targeting occurs irrespective of whether the target cell is in an activated or quiescent state. This is followed by the integration of the proviral DNA into the host genome, mediated by the viral integrase (IN) protein.

Distinct extracellular morphogenesis and maturation characterize the final events of the retroviral life cycle, enabling nascent virions to initiate further rounds of infection.

All retroviruses encode and express three major domains, *gag*, *pol* and *env*. The *gag* gene encodes the viral structural proteins matrix (MA), capsid (CA), nucleocapsid (NC), and p6, and the CA/NC and NC/p6 junctions are delimited by p2 and p1 spacer peptides, respectively. The *pol* gene encodes the viral enzymes protease (PR), RT, and IN. RT possesses both polymerase and Rnase H functions. The *env* gene encodes the surface (SU) and transmembrane I envelope proteins.

Figure 1.1 The retroviral virion, general topological relationships between viral proteins and core placement is as shown in the mature state. All non-structural viral proteins are depicted in their known position within the virion. In frame, the retroviral morphogenesis pathway shown involves the extracellular maturation of SIVmac239 virus. A. Budding virions. B. Immature virus, with electron dense perihery. C. Mature virions with conical core. Electron micrographs contributed by J.B.W.

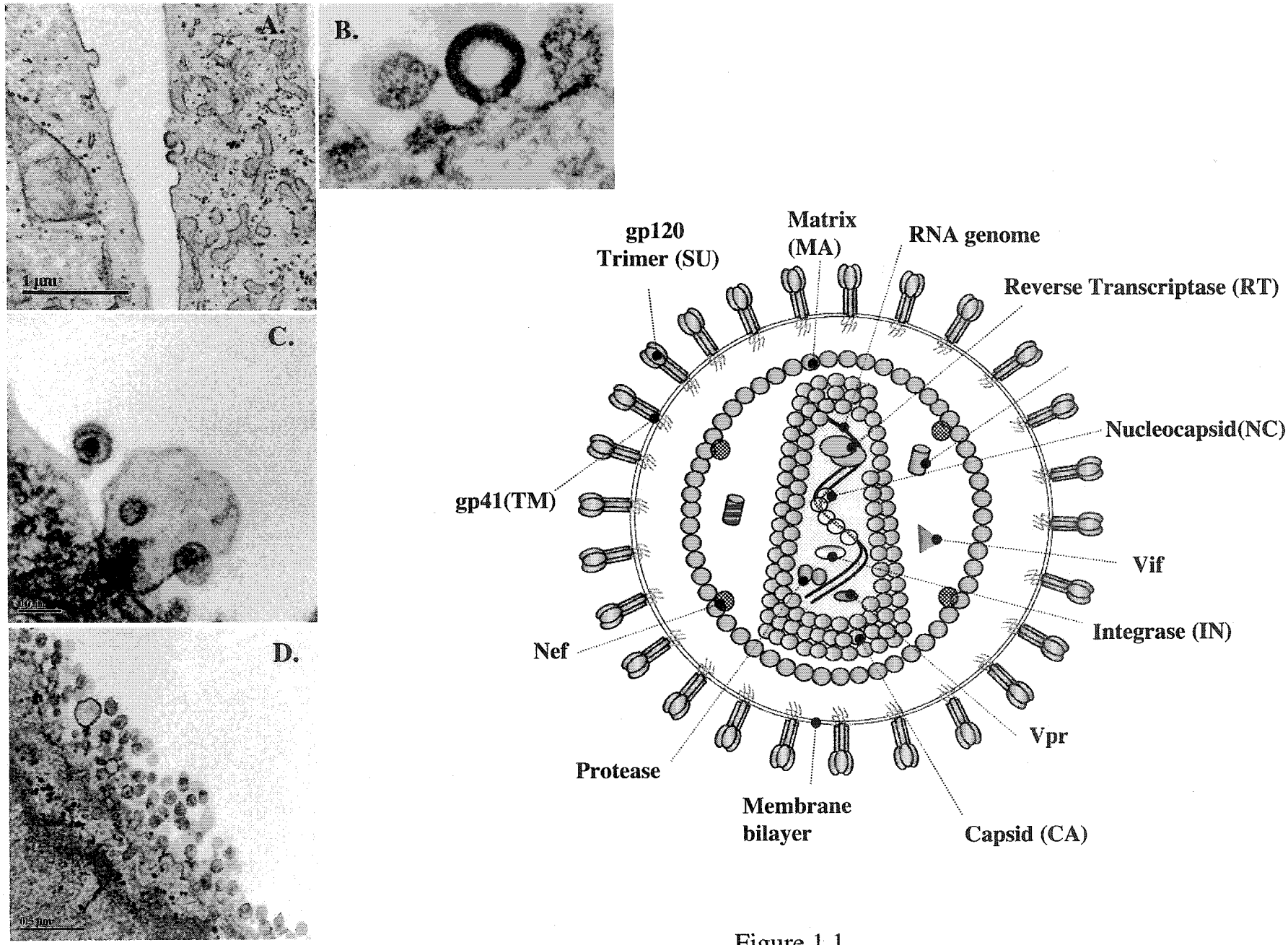

A.
B.
1 μm
C.
D.
gp120
Trimer (SU)
Matrix
(MA)
RNA genome
Reverse Transcriptase (RT)
Nucleocapsid(NC)
gp41(TM)
Vif
Integrase (IN)
Nef
Vpr
Protease
Membrane
bilayer
Capsid (CA)

Figure 1.1

Figure 1.2 Schematic overview of the HIV-1/SIV genome, with the position and functions of the various gene products and untranslated elements. Each genome encodes *gag*, *pol*, and *env*, as well as six additional open reading frames that include *tat, rev, nef, vif, vpr, vpr, vpu* (HIV-1, and SIVcpz) or *vpx* (SIVmac and SIVsmm). The vpx gene, is thought to have arisen as a result of intraspecies recombination between SIV smm and SIVagm (likely *C. sabaeus*, Sharp *et al.*, 1996).

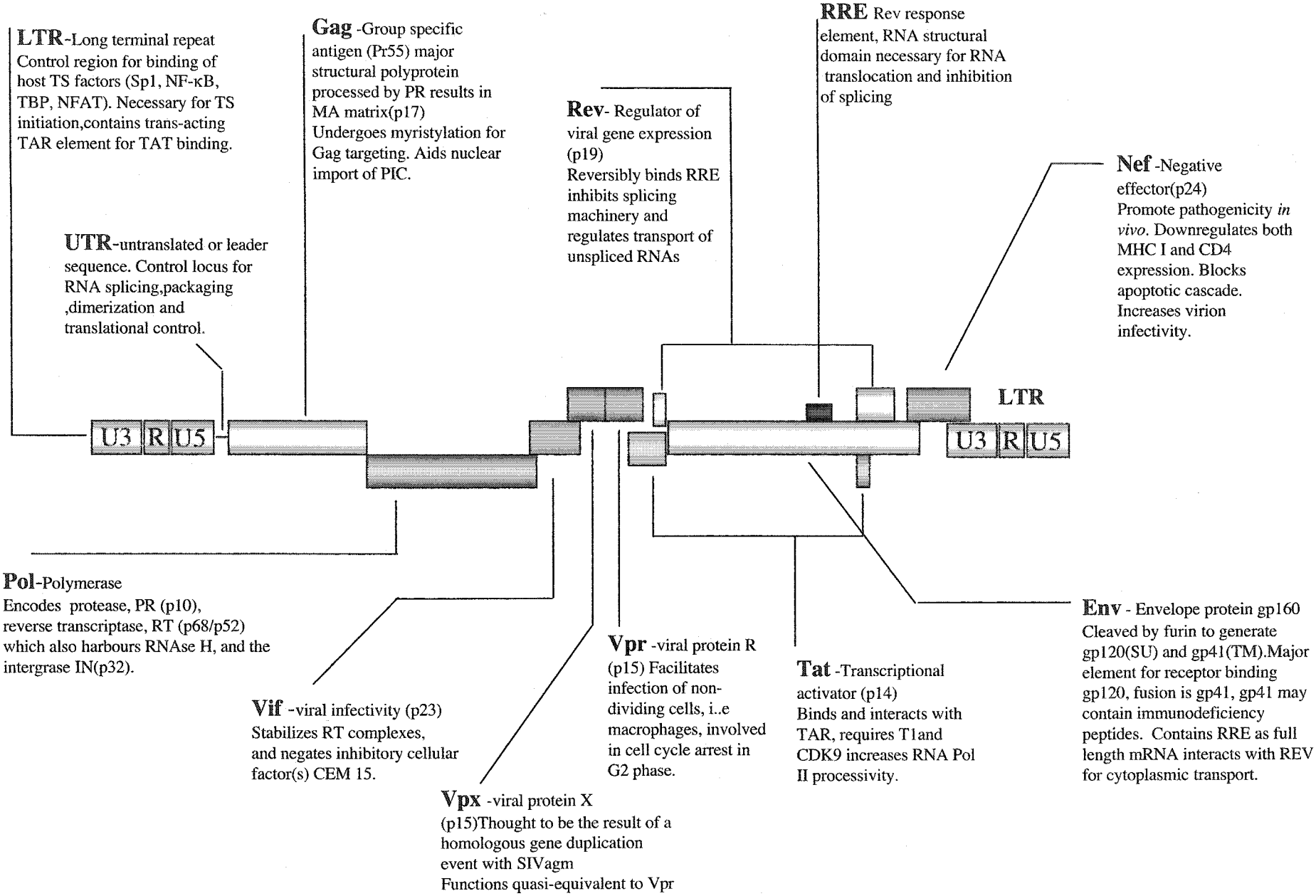

LTR-Long terminal repeat
Control region for binding of host TS factors (Sp1, NF-κB, TBP, NFAT). Necessary for TS initiation,contains trans-acting TAR element for TAT binding.
Gag -Group specific antigen (Pr55) major structural polyprotein processed by PR results in MA matrix(p17)
Undergoes myristylation for Gag targeting. Aids nuclear import of PIC.
RRE Rev response element, RNA structural domain necessary for RNA translocation and inhibition of splicing
Rev- Regulator of viral gene expression (p19)
Reversibly binds RRE inhibits splicing machinery and regulates transport of unspliced RNAs
Nef -Negative effector(p24)
Promote pathogenicity *in vivo*. Downregulates both MHC I and CD4 expression. Blocks apoptotic cascade. Increases virion infectivity.
UTR-untranslated or leader sequence. Control locus for RNA splicing,packaging ,dimerization and translational control.
U3 R U5
LTR
U3 R U5
Pol-Polymerase
Encodes protease, PR (p10), reverse transcriptase, RT (p68/p52) which also harbours RNAse H, and the intergrase IN(p32).
Vif -viral infectivity (p23)
Stabilizes RT complexes, and negates inhibitory cellular factor(s) CEM 15.
Vpx -viral protein X (p15)Thought to be the result of a homologous gene duplication event with SIVagm
Functions quasi-equivalent to Vpr
Vpr -viral protein R (p15) Facilitates infection of non-dividing cells, i..e macrophages, involved in cell cycle arrest in G2 phase.
Tat -Transcriptional activator (p14)
Binds and interacts with TAR, requires T1and CDK9 increases RNA Pol II processivity.
Env - Envelope protein gp160
Cleaved by furin to generate gp120(SU) and gp41(TM).Major element for receptor binding gp120, fusion is gp41, gp41 may contain immunodeficiency peptides. Contains RRE as full length mRNA interacts with REV for cytoplasmic transport.

Figure 1.2

Retroviruses have been broadly classified according to genomic organization and as to whether RNA transcription is simple or complex, based on the presence of additional open reading frames. Retroviruses can encode upto six genes labeled as accessory, in HIV-1 these include, *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu**, whose functions are in fact critical at differing stages of disease or within different physiological compartments (see Figure 2). Retroviruses may be further subdivided into seven genera based on genomic sequence similarity (Vogt 1997, HIV sequence compendium 2001). Five of these genera were formerly classified as oncoviridae because these viruses display transforming or oncogenic potential. Some of them contain oncogenes, that are typically of host cell origin, such as the *src gene* in Rous sarcoma virus, while others can induce tumors on the basis of their own genomes, e.g. Moloney leukemia virus (MLV), and human T cell leukemia virus (HTLV). Oncoviruses are further classified as simple retroviruses with the exception of HTLV and bovine leukemia virus (BLV). HTLV, BLV and the sub-genera lentivirinae and spumavirinae are all considered to be complex. All members of the foregoing categories exhibit alternative splicing capability.

The lentiviral sub-group has become an important cause of human disease. Infections by these viruses are characterized by a long subclinical period of latency followed by progressive disease that is correlated with increasing immunodeficiency. Lentiviruses can infect a wide range of animal species. Spumaviruses or foamy viruses can be considered to be orphan viruses, since they cause no known disease, yet they have the ability to cause cytopathicity in cell culture in the form of vacuolization, hence the "foamy" term. It is presumed that these viruses either co-evolved or evolved a symbiotic relationship with their hosts, due to their apparent lack of pathogenicity.

1.0.2 The Lentivirinae

The lentivirinae sub-classification describes those etiologic agents that cause disease in both human and non-human primates. However, they are also associated with bovine, equine, and feline immunodeficiencies, as well as caprine encephalies. The two major lentiviruses associated with human disease are HIV-1 and HIV-2, and the former are far more prevalent than the latter worldwide; perhaps reflecting greater virulence and facile transmission (Kong *et al.*, 1988). Currently, over 40 million persons are estimated to be

infected by HIV-1; although under-reporting of the disease burden in certain countries might mean that the estimated worldwide burden of HIV disease should in fact, be considerably higher.

1.0.3 HIV in human subjects

In 1981, several reports described epidemiologically localized clusters of patients who presented with an unusual immunodeficiency state that was later termed acquired immunodeficiency syndrome (AIDS). The disease was characterized by multiple opportunistic infections, Kaposi's sarcoma, lymphomas, and marked depletion of T cells bearing the $CD4^+$ receptor (Gottlieb *et al.*, 1981, Masur *et al.*, 1981). Initial risk factors for transmission included high-risk sexual behaviors that was predominant in homosexual men, sexual intercourse, intravenous drug use, injection of blood products and transfusions.

In 1983 and 1984, several groups, described a novel retrovirus that had been isolated from patients suffering from acute lymphadenopathy and immunodeficiency. This agent was variously termed lymphadenopathy-associated virus (LAV), human T cell leukemia virus III (HTLV-III) and AIDS-related virus (ARV) (Barré-Sinoussi *et al.*, 1983, Levy *et al.*, 1984, Montagnier *et al.*, 1984, Popovic *et al.*, 1984), and was later renamed human immunodeficiency virus type-1 (HIV-1). Strong epidemiologic evidence shows that HIV-1 is the causative agent of AIDS. However, a virus termed HIV-2 was subsequently isolated in West Africa; this virus shares approximately 40% sequence homology with HIV-1 but is phylogenetically closer to certain species of SIV (Clavel *et al.*, 1986). Both HIV-1 and HIV-2 were present in Africa at least 10 years prior to their emergence in North Americian populations (Saxinger *et al.*, 1985).

1.0.4 Natural history of SIV.

As stated, HIV seems to have arisen in Africa and the bulk of evidence suggests that multiple zoonotic transmissions have occurred, since at least 20 distinct primate lentiviruses, that infect distinct African primate species have been described (Hahn *et al.*, 2000, Letvin and Desrosiers, 1994 see Table 1). Interestingly, primate lentiviruses have not been observed to cause disease in their natural hosts (Feinberg *et al., 2002,* 2003).

Notwithstanding, the primate population of Africa represents a considerable viral reservoir with potential for transmission across species barriers.

1.0.5 Comparative phylogeny and genomic structure of primate lentiviruses.

The retroviral taxonomy of simian viruses is based on full-length genome sequences. To date, five major phylogenetic clusters have been described (Hahn *et al.*, 2000) These phylogenies (Retrovirus sequence compendium 2001, and Hahn *et al.*, 2000, Gao *et al.*, 1999) form host specific clusters, that are not geographically restricted, suggesting that natural host may have been infected over many years (see discussion below of SIVagm and SIVcpz, see also Fig. 3).

1.0.6 Origins of SIVcpz and HIV in Humans

Evidence of the origin of HIV-1 and HIV-2 are based on molecular epidemiologic and phylogenic studies in which the HIV infection of humans was found to result from at least 7 separate zoonotic transmissions. The initial founder virus for each subtype has been shown to result from a single zoonotic transmission event, but three and four such events appear responsible for infection in humans of HIV-1 and HIV-2 respectively (see Fig. 3A). Thus, in the case of HIV-1, this has resulted in the production and classification of three major groups, M (main) N (non-M/non-O) and O (outlyer), with the predominant subtype being M (Fig. 3B). Five major criteria support the hypothesis that HIV-1/HIV-2 initiated infection in humans as a result of (multiple) zoonotic transmission events involving SIV (Gao *et al., 1999).* These include:

i) similarities in genomic organization;

ii) phylogenetic relatedness;

iii) prevalence in the natural host;

iv) geographic coincidence of host habitat and cross-species transmission;

v) plausible routes of transmission.

Isolate	Common source	Species	Reference
SIV_{mac}	Rhesus monkey	*Macaca mulatta*	Kanki, Daniel *et al.*,1985
SIV_{cyn}	Cynomologous monkey	*Macaca fascicularis*	Daniel, Kestler *et al.*, 1988
SIV_{cpz}	Chimpanzee	*Pan troglodytes troglodytes*	Peeters *et al.*,1989, 92, Huet *et al.*, 1990
SIV_{mne}	Pig-tailed macaque	*Macaca nemestrina*	Benveniste *et al.*,1986
SIV_{smm}	Sooty mangabey monkey	*Cercocebus atys*	Fultz *et al.*,1986, Hirsch *et al* ., 1989
SIV_{agm}	African green monkey	*Cercopithecus* aethiops*	Ohta, Daniel *et al.*, 1988
		Chlorocebus pygerythrus	Allan *et al.*, 1990
		Chlorocebus sabaeus	Allan *et al.*, 1991
		Chlorocebus tantalus	Muller *et al.*, 1993

Table 1. Various SIV isolates and corresponding non-human primate reservoirs (adapted from N. Letvin and R. Desrosiers in Simian Immuondeficiency Virus, Adapted from Current Trends in Microbiology and Immunology 1994.) *genus name has been changed to *Chlorocebus* Groves, C.P. 1993. *Mammalian Species of the World: A Taxonomic and Geographic Reference*. 243-277 Smithsonian Press Wash. DC

Figure 1.3 (A.) Representations of potential cross-species transmissions for the HIV-1 virus from SIVcpz, and HIV-2 from SIVsmm. Arrows signify potential inter-species transmissions for HIV-1(I) and HIV-2 (II) respectively

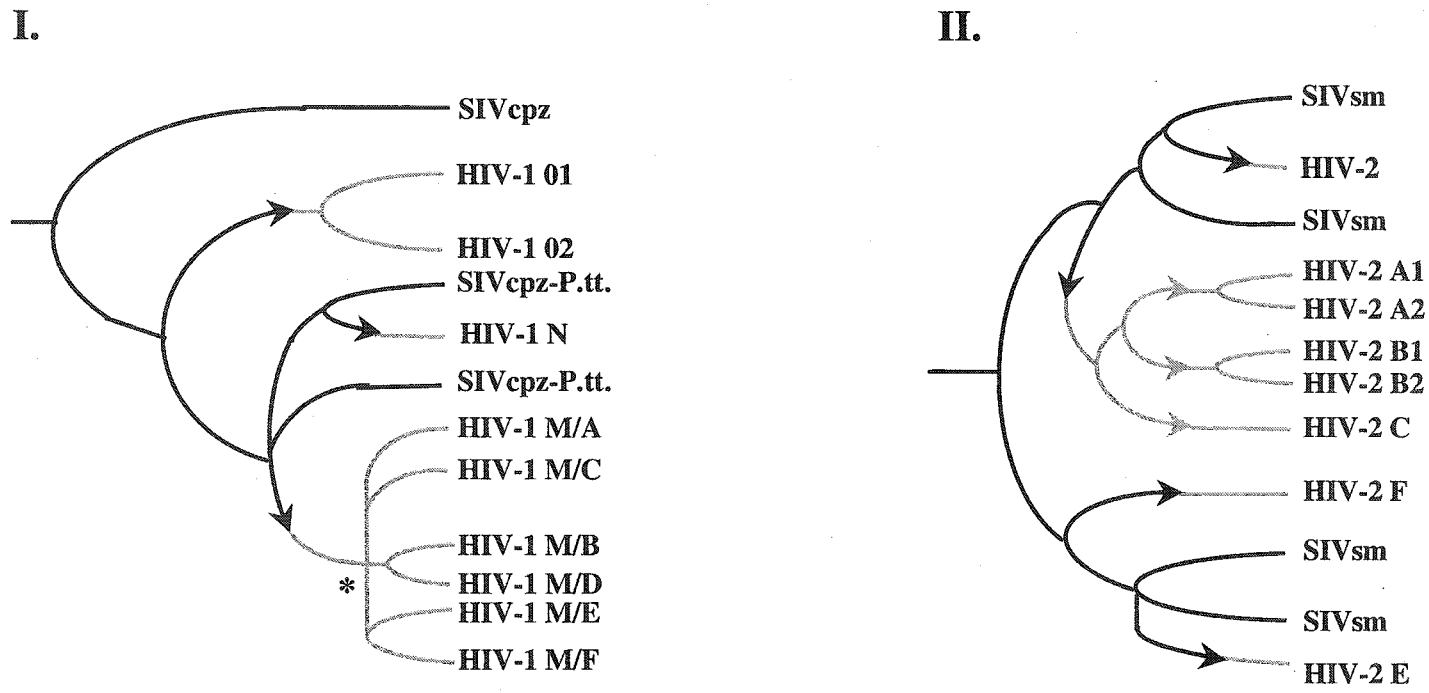

I.
SIVcpz
HIV-1 01
HIV-1 02
SIVcpz-P.tt.
HIV-1 N
SIVcpz-P.tt.
HIV-1 M/A
HIV-1 M/C
HIV-1 M/B
HIV-1 M/D
HIV-1 M/E
HIV-1 M/F
*
II.
SIVsm
HIV-2
SIVsm
HIV-2 A1
HIV-2 A2
HIV-2 B1
HIV-2 B2
HIV-2 C
HIV-2 F
SIVsm
SIVsm
HIV-2 E

Figure 1.3 (A)

Figure 1.3 (B.) Phylogentic analysis of the evolutionary relationships of various lentiviral genomes implicated in the current HIV epidemic. All of the above support chimpanzee populations as the reservoir for SIVcpz and subsequent introduction of HIV-1into humans. Similarly sooty mangabey (sm) monkey serve as the natural host for HIV-2. (Adapted from Gao *et al.*, 1999, Hahn *et al.*, 2000, Calef *et al.*, 2001).

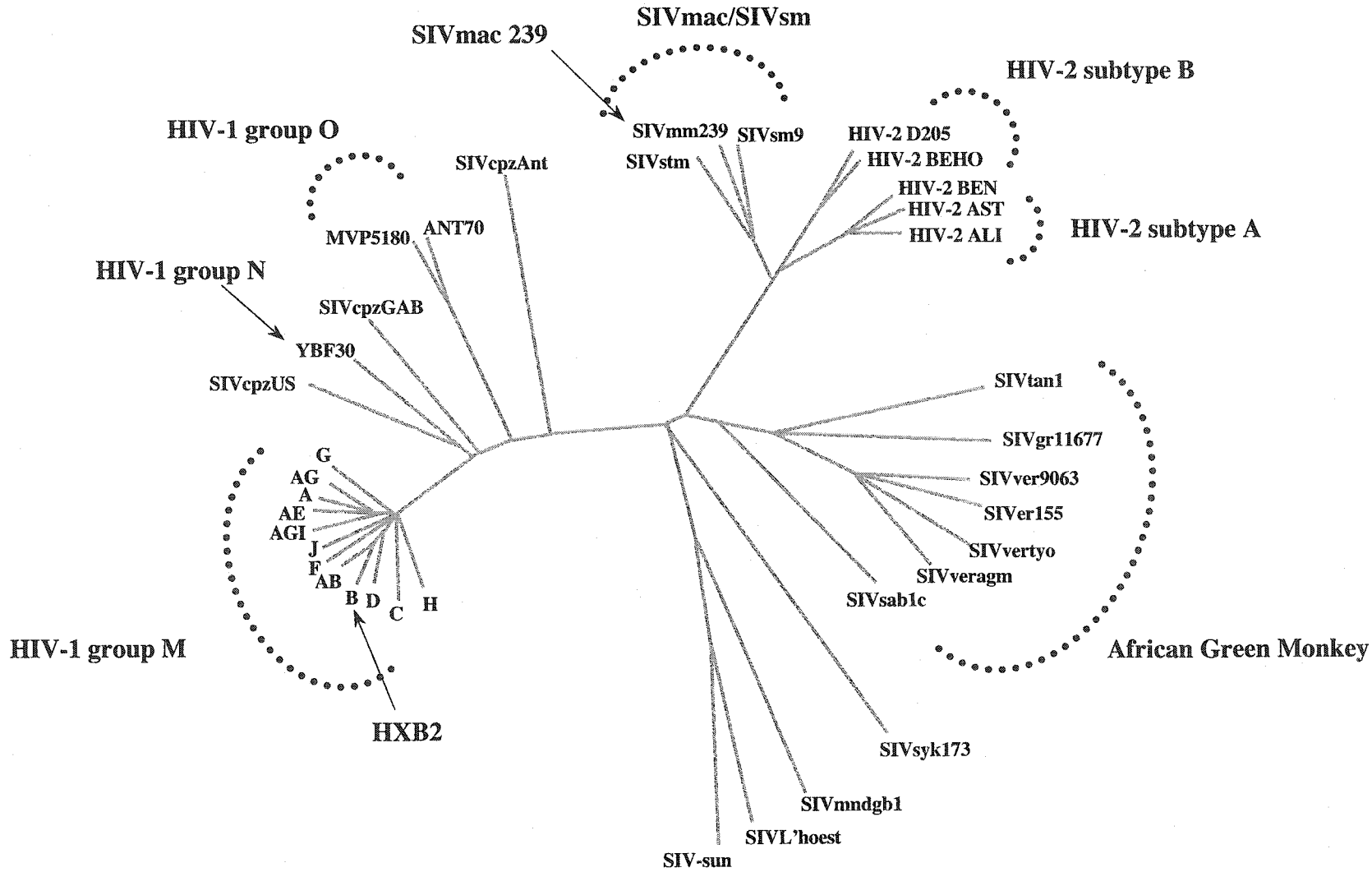

SIVmac/SIVsm
SIVmac 239
HIV-2 subtype B
HIV-1 group O
SIVmm239
SIVsm9
HIV-2 D205
HIV-2 BEHO
SIVcpzAnt
SIVstm
HIV-2 BEN
HIV-2 AST
HIV-2 ALI
HIV-2 subtype A
MVP5180
ANT70
HIV-1 group N
SIVcpzGAB
YBF30
SIVcpzUS
SIVtan1
SIVgr11677
SIVver9063
SIVer155
SIVvertyo
SIVveragm
SIVsab1c
G
AG
A
AE
AGI
J
F
AB
B
D
C
H
African Green Monkey
HIV-1 group M
HXB2
SIVsyk173
SIVmndgb1
SIVL'hoest
SIV-sun

Figure 1.3 (B)

Indeed, high prevalence rates of SIVsm infection have been observed among sooty mangabeys both in captivity (50-75%) and in the wild (40%) (Gao *et al.*, 1999, Hahn *et al.*, 2000). Both the particular strain that was isolated from primates (SIVsm) and HIV-2 appear to comprise a single, diverse group of lentiviruses that cannot be separated phylogenetically into discrete lineages. As well, these SIV isolates and HIV-2 show overlap in regard to original habitat and endemicity (transmission is facilitated by animal husbandry, and the use of these primates as food i.e., bushmeat.

Within the SIV lineage (SIVsm and SIVmac) is phylogenetic evidence of intraspecies infection, since the aquisiton of the *vpx* gene is the result of a non-homologous recombination event between an ancestral SIVsm and SIVagm (Sharp *et al.*, 1996). Studies aimed at identifying the natural host of HIV-1 have proven more difficult, although similarities had been shown in the genomic organization of a virus infecting chimpanzees (SIVcpz) with that of HIV-1 (Huet *et al.*, 2000). However, extensive genetic diversity was seen in the *vpu* genes of the two viruses. In addition, there appeared to be a significant phylogenetic distance between HIV-1 and SIV-cpz. Also, the incidence of SIVcpz infection appeared to be low in both wild and captive animals. Moreover, the natural habitat of the chimpanzee, *Pan troglodytes*, was localized to equatorial Africa and not to areas in which the HIV-1 epidemic was first thought to occur.

A chimpanzee imported to the US from Africa for purposes of breeding has shed light on these issues (Hahn *et al.*, 2000). This animal was found to have antibodies against HIV-1 and PCR amplification of retroviral sequences from spleen and lymph tissue revealed a simian virus of the same family as SIVcpz, rather than HIV-1. This so-termed SIVcpz-US strain was compared to previously known SIV-cpz isolates from Gabon (GAB1 and GAB2) and Antwerp (ANT). SIVcpz US was found to be divergent from the SIVcpz-ANT strain and pylogenetically closer to the GAB strains. To assess whether host-dependent evolution of SIV-cpz accounted for the diversity seen among these strains and if subspecies of SIV-cpz-infected chimpanzees could be detected, mitochondrial DNA analysis was performed, and revealed that two subspecies of chimpanzees, the central *Pan troglodytes troglodytes*, and the eastern *Pan troglodytes shweinfurthii*, might serve

as natural hosts for SIVcpz. Unfortunately, the prevelance of SIVcpz in natural populations was not decisively known.

The SIVcpz in these two subspecies appeared to be phylogenetically diverse. Further phylogenetic analyses of all known HIV-1 strains that infect man (groups M, N, and O) revealed that these HIV-1 strains were all related to the SIVcpz found in *Pan troglodytes troglodytes*, and that there was evidence for three independent introductions of SIVcpz into human populations (see Fig. 3).

Interestingly, sequence analyses demonstrated that HIV-1 group N is actually a recombinant mosaic of SIVcpz and HIV-1 sequences. The highly conserved sequences of SIV-cpz strains, and the analogous sequence in HIV-1 group N, indicate that the latter may be the most recently transmitted. Collectively, the evidence for cross-species transmission of SIVcpz to humans can be related to that for HIV-2 (Gao *et al*):

i) similar genomic organization (see Fig. 2)

ii) phylogenetic relatedness (I. HIV-1 group M, N, and O viruses and all SIV-cpz strains infecting Pan troglodytes troglodytes form a single phylogenetic lineage similar to that seen with HIV-2 and SIV-sm).

iii) prevalence in the natural host

iv) geographic coincidence of host habitat and viable cross-species transmission (Pan troglodytes troglodytes habitat coincides with areas of HIV-1 of group M, N, and O endemicity.)

v) plausible routes of transmission (Chimpanzees in these regions are hunted for food.)

Based on these lines of evidence, the African chimpanzee (*Pan troglodytes)* appears to be the natural host and reservoir for HIV-1. Recent prevalence data from five wild chimp communities (*Pan troglodytes schieinfurthi*), dispersed throughout Kibale and Gombe national parks, in east Africa has corroborated previous findings. However the results show that SIVcpz endemicity is non-uniform, with infections only found in some groups compared with much higher and uniform SIV infections in other nonhuman primates (Santiago *et al.*, 2003), perhaps implying that the transmission from smaller primates to chimpazees is poor. Recent data indicating that SIVcpz is in fact a hybrid, the result of multiple cross species transmissions and intrastrain recombinantion from smaller monkeys that are hunted by chimpanzees. These findings have corroborated previous

investigations in regard to transmission and provide an example of natural infections broadly comparable to the transmission of SIVcpz into human primates (Bailes *et al.*, 2003).

Research Objectives and Specific Aims

The overall objective of this project was to elucidate the functional characteristics of the SIVmac239 leader, with the first specific aim to focus on the *cis*-acting determinants regulating viral RNA encapsidation and RNA dimerization. The rationale for this was two-fold, first SIV has been somewhat understudied in comparison to other retroviruses. SIV is closer phylogenetically to HIV-2 and neither has been fully characterized in regard to *cis*-elements involved packaging or dimerization. Perhaps more important is the fact that SIV, in conjunction with the rhesus macaque is presently the best available model for investigations into HIV pathogenesis and vaccine development. Therefore, a thorough characterization and analysis of this important viral sub-class is necessary and overdue. The second major aim of this work was a discovery process in conjunction with the above studies for genetically stable attenuated variants suitable for further evaluation as vaccine candidates in the macaque model system. This latter topic is addressed in part in the second half of this dissertation, in two somewhat divergent papers. The first deal with the production of a simplified virus possessing only the cognate *gag, pol* and *env* genes as a potential vaccine candidate. The second paper addresses an issue important within the fields of HIV pathogenesis, vaccinology and antiretroviral therapy, that of viral plasticity and escape. The subject of viral reversion and evolution is broadly reviewed in the following chapter (chpt. 2).

Chapter 2:

Attenuating viral evolution

Abstract

Retroviruses, and particularly lentivirinae, are known to exhibit remarkable genomic pliancy, a capacity that has been attributed to one or more "error prone steps" in the viral replication cycle. Although perhaps true *de facto*, an increasing body of evidence indicates that such proclivity for error is anything but a result of viral imprudence. This is exemplified by shifts in both host and cellular tropism, an increasing incidence of circulating recombinant forms, and multiple examples of viral escapism. Each of the foregoing has broad implications in regard to disease progression, antiretroviral treatment, vaccine development and efforts to control and limit the ongoing HIV pandemic.

I. Introduction

II. Evolutionary dynamics in lentiviral infection.

III. Mechanisms of lentiviral diversity.

   a. The fidelity and processivity of HIV-1 RT in the context of ongoing viral replication.

   b. Recombination: mechanisms and implications based on the current model

   c. Role of viral factors in retroviral variation.

   d. Host driven viral selection and evolution.

IV. Attenuating viral evolution: theoretical considerations, and lessons from studies of drug resistance and live attenuated vaccines.

V. Future directions.

I. Introduction

Disease causing pathogens exhibit a wealth of strategies to evade the immune response of the host and promote diversification. This point is well illustrated by the adaptive potential and capacity for transmission across species barriers exhibited by influenza virus and *Mycobacterium tuberculosis*. However, no microorganism shows a greater propensity for niche adaptation than HIV-1 and the closely related lentiviruses of both human and non-human primates (HIV-2, SIV).

Multiple mechanisms facilitate lentiviral evasion of host immunity, these briefly include: The glyco-masking of the Env receptor from Ab neutralization, viral integration and latency, upregulation of Fas-ligand on $CD4^+$ T cells. The concomitant down-regulation of MHC class I ligands mediated by the viral Nef protein also obscures infected cells from immune surveillance. Likewise, as a consequence of viral infection, the central role of macrophage and $CD4^+$ T_h cells in the immune response is negated by the selective destruction of the this compartment.

This review focuses on viral and host factors that enable rapid adaptation that underlies viral escape from Ab neutralization and host CTL respsonse. These same mechanisms facilitate resistance to antiretroviral therapy, and underscore the obstacles to HIV vaccine development. Intense immune selection being the predominant force that drives viral diversity. At the same time, the enormous plasticity of the viral genome is the penultimate cause of immune capitulation.

II. Evolutionary Dynamics in Lentiviral infection.

The acute phase of pathogenic HIV or SIV infection is characterized by an exponential increase in plasma viral load (Little *et al.,* 1999, Nowak *et al.,* 1997). Despite the fact that viral titres are modulated during dynamic phases by a suppressive immunological response, viral replication is unrelenting throughout each stage of disease, including the protracted asymptomatic phase (Pantaleo *et al.*, 1993, Piatak *et al.*, 993), or in the setting of potent antiretroviral therapy (Ramratnam *et al.*, 2000, Hermankova *et al.*, 2001). This attribute provides one requisite for evolutionary change, and mathematical modeling has estimated that the rate of HIV virus production is on the order of 10^{10} virions/day (Ho *et*

al., 1995, Markowitz *et al.*, 2003, Perelson *et al.*, 1996; Wei *et al.*, 1995). Recent studies have further estimated the *in vivo* replicative capacity of HIV-1 to be 180 generations per year (Markowitz *et al.*, 2003). Approximation of the base pair substitution rate of currently circulating HIV-1 strains is .0024 substitutions per base per year, whereas the mammalian rate dwindles by comparison, being about six orders of magnitude lower (Korber *et al.*, 2000).

Quasispecies theory implicitly requires the above conditions and dictates that RNA viruses, by virtue of their plasticity and replication kinetics, are capable of exploring an enormous sequence space in relation to other organisms, resulting in a heterogeneous swarm in which all possible genome permutations are represented (Eigen 1971, Wright 1912). However, a more constrained viral fitness landscape is represented phenotypically, due to the need to conserve protein function, and by the imposed limits of genome size. Thus, the majority of mutations that are generated are deleterious, resulting in the production of sub-optimal phenotypes or non-infectious virions. Regardless, one important outcome of this diversity is the capacity for continuous interaction between the selective environment and the viral population.

This has serious implications for the selection of drug resistance-conferring mutations during antiretroviral therapy, necessitating high compliance and multi-drug regimens to yield a therapeutically useful lifespan for each drug. Likewise, the efficacy of the host's immune response is under constant challenge (Boulerice *et al.*, 1990, Coffin, 1995). It has been proposed that this may result in a broadening of immune recognition (Wain-Hobson *et al.*, 2001). However, in view of the large numbers of defective particles produced, the latter is more likely an immunological red herring.

Retroviral evolutionary theory, as proposed by Nowak, suggests that limited viral diversity, in early stages of infection, can result in a rapid and effective suppression of the virus. However, at some later stage, discrete changes occur within the virus population as a function of the immune response, such that expanding antigenic diversity, or breach of an antigenic diversity threshold occurs that is beyond the capability of the immune compartment to control. In this scenario, the steady-state virus load is a function of virus replication and is limited only by the number of available target cells. (Nowak *et al.*, 1990, Nowak *et al.*, 1996, Nowak *et al.*, 1996a, Nowak *et al.*, 1999).

III. Mechanisms of retroviral diversity; the dynamics of change

The lentivirinae sub-genera are known to exhibit remarkable genome pliancy, a capacity that has been attributed to one or more "error-prone steps" in the viral replication cycle. Collectively, it is agreed that genetic variation within infected individuals and comparable infections of primates, is high (Nowak *et al.*, 1997, Overbaugh *et al.*, 2000). An increasing body of evidence indicates that this is anything but a result of viral imprudence. Rather, the nature of lentiviral infection is manifested in viral shifts of host and cellular tropism, the increasing existence and transmission of circulating intra-subtype recombinants, and several examples of viral escapism. Several interrelated factors have been implicated to elicit genetic variation and these include reverse transcriptase infidelity and viral recombination. Perhaps less well established are the contributions from other viral and cellular factors that can also influence viral mutation rates.

a. The fidelity of HIV-1 RT in the context of ongoing viral replication:

Lentiviral RTs exhibit an infidelity that is several magnitudes higher than that of any equivalent polymerases involved in nucleic acid replication (Arts *et al.*, 1998, Williams and Loeb, 1992). The generation of viral quasispecies has been largely attributed to the lack within RT of a 3'-5' exonuclease activity combined with high rates of viral replication (Coffin 1995, Wei *et al.*, 1995). Although this lack of proofreading ability may partially account for the high error rate, other mechanisms are directly responsible for codon change (Roberts *et al.*, 1988, Yu and Goodman, 1992).

As mentioned, HIV-1 RT displays marked infidelity compared to host cellular polymerases and other retroviral RTs (Bebenek *et al.*, 1989, Ho *et al.*, 1995, Mansky 2000, Perelson *et al.*, 1996, Preston *et al.*, 1988, Roberts *et al.*, 1988, Takeuchi *et al.*, 1988, Wainberg *et al.*, 1996). The mutation rate of HIV-1 RT is approximately 10^{-4} per nucleotide per replication cycle and this is at least one order of magnitude higher than that of cellular polymerases (Bebenek *et al.*, 1993, Boyer *et al.*, 1992, Patel *et al.*, 1992, Preston *et al.*, 1988).

The combination of sequence and structural information has offered a partial explanation for the above observations. The catalytic domains of the RTs of HIV-1/HIV-2 and its simian counterpart, simian immunodeficiency virus (SIV), each encodes a highly conserved YMDD motif, one generally found in all RNA dependent polymerases (Poch *et al.*, 1989). Comparisons of the crystal structure of RT and its *E. coli* homolog (Klenow) implicate the polymerase active site of RT in diminished nucleotide discrimination and substrate incorporation, because it is the physically larger (Kohlstaedt *et al.*, 1993, Huang *et al.*, 1998). Secondly, structural analysis has indicated that the HIV-1 RT, complexed with a DNA duplex, is conformationally more fluid at the YMDD catalytic cleft and primer grip region than other RTs (Ding *et al.*, 1998).

Additional studies have ascribed a role for the enzyme target, as substrate discrimination by RT appears to be highly dependent on both the choice of template (i.e. DNA versus RNA), and sequence context. For example, homopolymeric sequences, particularly repetitive tracts of As and Ts, are statistically favored for nucleotide substitutions and/or insertional mutagenesis. These events result in frameshifting, presumably as a consequence of RT slippage on the primer template duplex (Bebenek *et al.*, 1989,1993). Moreover, highly structured sequences can commonly induce transcriptional pauses, highlighting the relationship between enzyme processivity and fidelity (Bebenek *et al*, 1993).

Substitutions may also be due to the insertion of the incorrect nucleotide followed by elongation from the site of the mispair (Yu and Goodman, 1992, Preston *et al.*, 1988). It should come as little surprise that HIV-1 RT incorporates and extends mispairs with a higher frequency (up to 50-fold higher) compared with mammalian DNA polymerases such as pol α (Preston *et al.*, 1988, Perrino *et al.*, 1989).

Different assay systems have been employed to study RNA dependent DNA polymerization (RDDP) and DNA dependent DNA polymerization (DDDP) fidelity *in vitro*. Not surprisingly, the use of different experimental conditions has generated conflicting results. Analytical studies contrasting the rate of base-substitutions using identical ϕX174 DNA and RNA templates have shown that the accuracy of DNA synthesis was as much as 20-fold reduced when utilizing an RNA template (Hübner *et al.*, 1992, Preston *et al.*, 1989, Perrino *et al.*, 1989). Conversely, other studies employing

equivalent DNA or RNA templates from the *lacZ* locus, showed little difference in fidelity during transcription of RNA templates (Boyer *et al.*, 1992, Ji and Loeb, 1992). These opposing results support the above notion that sequence context may play a role in enzyme fidelity.

Additionally, the HIV-1 RT is capable of introducing non-templated errors into RNA or DNA with a preference for the insertion of purines into the latter substrate (Patel *et al.*, 1994). Collectively, these findings point to an unequal contribution of the overall mutation rate during the RNA dependent versus DNA dependent polymerization steps of reverse transcription. This apparent difference has been clarified by recent studies showing that the relative contribution may be roughly equivalent when considering the involvement of viral and host cellular factors (Preston *et al., 2002*).

A consequence of the foregoing suppositions is that mutations are not equally distributed in the viral genome. For example, mutations in the variable domains of the *env* gene, particularly G → A transversions, termed hypermutations (Vartanian *et al.*, 1991), are more frequent than errors generated in the *gag* or *pol* genes, indicative of strong selective pressure on the former (Ji and Loeb, 1994, Vartanian *et al.*, 1994). Work by our group and by others indicates that *gag* is also pliant, albeit less so than *env*, and clinical studies have corroborated the potential for CTL escape at both these loci.

b. Recombination and its implications based on the current model.

Convincing theoretical and experimental data have indicated that HIV nucleotide diversity is a composite of high replication kinetics and enzyme infidelity (Coffin 1995). Moreover, the rate of non-synomous nucleotide incorporation in HIV is roughly six orders of magnitude higher than in the host genome, and at least three fold higher than the comparative mutation of DNA viruses. *A priori*, the number of nonsynomous mutations induced by RT necessitates an ability to rapidly combine mutations into various beneficial permutations, a mechanistic requirement that can only be efficiently accomplished through recombination (Temin 1991). This attribute, in conjunction with RT infidelity, represents the major source of retroviral diversity (Coffin 1995, Temin 1991).

When recombination was assessed under single cycle conditions the recombination frequency was found to be two to three recombinations per replication event or $2.4x10^{-4}$ bp per cycle; however this calculation conservatively excludes "recombination hotspots" (Jetzt *et al.,* 2000). Noteworthy is that lentiviral leader sequences are essential for viral recombination (Balakrishnan *et al.*, 2001). Events of reverse transcritption, genomic dimerization and recombination are linked and mutually dependent (Balakrishnan *et al.*, 2003).

Similarly, efficient reverse transcription is contingent on interactions between RT and discrete regions of the 5' retroviral genome. The latter includes the untranslated leader region, located between the primer binding site (PBS) and the major splice donor, but also involves other sequence regions that govern multiple aspects of viral replication (Ilyinski and Desrosiers *1998*). These include splicing of genomic RNA, the dimerization and packaging of viral genomic RNA, efficiency of $Pr55^{gag}$ processing, and regulation of gene expression (Rein 1994, Liang *et al.*, 1999).

During reverse transcription, recombination has been shown to occur frequently (Jetzt *et al.,* 2000), and RNA-RNA interaction while necessary, is insufficient, implying a role for other proteins that minimally include RT and NC (Balakrishnan *et al.,* 2001, Preston *et al.*, 2003). Recombination events follow the copy choice model (reviewed by Negroni and Buc *2002*) and are of critical importance during synthesis of minus-strand DNA (Anderson *et al.*, 2000). Since a large proportion of recombination events are a result of intermolecular interactions, this helps to explain the functional significance of a diploid retroviral genome. Temin had previously suggested that retroviruses exhibit features such as pseudo-diploidy and low proccessive rates to promote recombination (Temin 1989). However, this requirement is not absolute, since variants that contain only a single genome have been shown to be capable of undergoing reverse transcription, although they were compromised in regard to efficiency of initiation and/or template elongation (Berkhout and van Wamel 1996).

The studies mentioned above have shown that RT can also make non-templated additions to both RNA and DNA substrates and this may impact the accuracy of strand transfer reactions (Patel and Preston, 1994, Peliska and Benkovic, 1992).

Other viral protein activities that contribute to the efficiency of recombination include the viral nucleocapsid (NC) protein and the Rnase H activity of RT. The NC of HIV-1 exists in its final processed form as NCp7 (or the NCp8 equivalent in SIV) and is a multifunctional zinc-chelating protein that influences numerous stages of viral replication. Generally, NC functions as a nucleic acid chaperone catalyzing structural rearrangements of RNA and DNA into more thermodynamically favorable states (Gatte *et al.*, 1999). This is demonstrated by up to 10 fold increases in elongation through pausing regions, modeled to contain structural motifs, by way of NC-mediated relief of structural inhibition in the template (Ji *et al.*, 1996, Wu *et al.*, 1996). Two zinc-finger motifs within NC, as well as flanking basic amino acid residues proximal to the zinc fingers, have been shown to be important in this regard (Wu *et al*, 1996).

Direct interaction *in vitro* has been demonstrated between the NC and RT of HIV-1 and is associated with increases in enzyme processivity (Druillenec *et al.*, 1999, Lener *et al.*, 1998). NC also increases the efficiency of strand transfer reactions, a requirement not only for recombination but also for plus-strand DNA synthesis. Template-template hybridization between the (-) ssDNA strong-stop product and an acceptor template containing the 3'-R region, a structure that exhibits considerable secondary structure and that is involved in the first strand transfer of reverse transcription, was increased by 3000-fold in the presence of NC in cell-free reactions (You and McHenry, 1994). The potential for self-priming by the folding back of (-) ssDNA is an event witnessed in strand transfer reactions *in vitro*, this is likely artifactual since the latter products are largely absent *in vivo*. Moreover, these can be effectively blocked by the addition of NC to *in vitro* reactions (Guo *et al.*, 1997).

The RNase H of RT activity also plays an important role in liberating nascently transcribed DNA from the template duplex by virtue of its Rnase activity, making it available to the acceptor template during the course of strand transfers (Peliska *et al*, 1994). This has been shown to be a result of direct interactions between RT and NC (Cameron *et al.*, 1997, Peliska *et al.*, 1994).

Structural studies have shown that the structure of the HIV-1 RNase H domain is remarkably similar to that of *E. coli* RNase H1 (Davies *et al.*, 1991, Mizrahi *et al.*, 1989). In contrast to other retroviral RTs, the RNase H domain of HIV-1 RT has both

endonuclease and 3'-5' exonuclease capabilities (Furfine and Reardon, 1991, Schatz *et al.*, 1990). The temporal and spatial coordination of both the polymerase and RNase H activities may be critical for the synthesis of proviral DNA. The degradation of RNA in an RNA/DNA duplex appears to be sufficiently rapid such that polymerization and RNase H digestion can be seen as essentially synchronous (Gopalakrishnan *et al.*, 1992). RNase H activity is required to digest the genomic RNA template in the RNA/DNA heteroduplex, to generate poly-purine tract (PPT) primers for the initiation of plus-strand synthesis. Rnase H then subsequently removes the $tRNA^{Lys3}$ and PPT primers (Cirino *et al.*, 1995. Fuentes *et al.*, 1995, Huber and Richardson, 1990, Peliska and Benkovic, 1992).

In sum, most recombinants are the result of homologous copy choice recombination, although the reactions involved are complex and may incorporate multiple mechanisms. The end result is that rapid viral codon change occurs in response to immunological flux. Viral reversion in the aftermath of deletion mutagenesis provides a model system to assess viral response to stress. Our studies have shown that both RT and Gag work together with upstream UTR sequences in restoration of viral replication, and that viral recombination is necessary for efficient compensatory mutagenesis. Variants that lack recombination capability would be relegated to increasing their fitness in an iterative fashion, setting the scene for strong viral interference or error catastrophe. In contrast, recombination offers the ability to fix or recombine beneficial genomes rapidly. In the context of natural infection, HIV is also capable of superinfection (Gratton *et al.*, 2000), resulting in multiple independent integrations per infected cell (Wain-Hobson *et al.*, *2002*). Studies of murine systems have shown that recombination is causally linked with pathogenic outcome (Studinsky *et al.*, 1994).

Implicit proof of the impact of recombination at the population level is the phylogenic distribution of the numerous HIV clades. Indeed, at least three independent zoonotic transmissions of SIVcpz appear to be responsible for the current HIV-1 epidemic (Hahn *et al.*, 2000). Further phylogenetic evidence of interspecies crossover is SIVsmm that gave rise to the HIV-2 lineage. The acquisition of the *vpx* gene (found in SIVsmm and SIVmac) is the result of a non-homologous recombination event between an ancestral SIVsmm and SIVagm (Sharp *et al.*, 1996). Recent phylogenetic analysis corroborates the

foregoing notion and has further indicated that the SIVcpz founder strains implicated in the current HIV epidemic, are also the result of cross-species transmission of mosaic viruses from smaller primates that are hunted by chimpanzees (Bailes *et al.*, 2003). Since the purported introduction of the M subgroup of HIV-1 over 70 years ago, viral diversification has resulted in the production of more than 9 different clades. Moreover, the radial distribution of the viral phylogenetic tree, particularly for clade B isolates, implies an exponential rate of viral growth within their target population (Korber *et al.*, 2000, Peeters and Sharp 2000). The global distribution of HIV-1 genetic subtypes, particularly intra-subtype recombinants, or circulating recombinant forms (CRFs), is becoming an increasing concern. A particular lineage is classified as a CRF when related lineages are found in numerous epidemiologically or geographically unlinked individuals. The existence of CRFs and second-generation variants ($CRFs^2$), which result from the recombination of two CRFs further complicates the situation and underscores the genomic plasticity of retroviruses (Yang *et al.*, 2003). The role of CRFs may be more serious than previously realized.

For example, the ongoing epidemic in Thailand is the result of transmission of CRF of African origin. Similarly, a recent molecular epidemiologic survey of HIV-1 was conducted in Nigeria to determine the prevalence of subtype(s) for inclusion into candidate vaccines strains. All samples were initially screened by heteroduplex mobility assay (HMA). The majority of strains in this region, i.e. 54.3%, were initially identified as subtype A. Five of the latter samples were selected for full envelope sequencing that revealed that all samples were in fact intra-strain recombinants. Moreover, none of which were comparable to any CRF strain previously reported to be prevalent in the region (CRF-02AG) (Agwale *et al.*, 2002). Further evidence was reported in a recent global analysis that showed that the largest proportion of HIV-1 infections from the year 2000 was caused by subtype C strains (47.2%). However subtype A CRF02_AG was estimated to be the second leading cause of new infections, confirming the increasing role of HIV-1 CRFs in the pandemic. There is an increasing necessity for such information, particularly inherent differences in molecular attributes in regard to the of drug resistance profiles of different subtypes and development globally effective HIV vaccines (Osmanov *et al.*, 2002).

c. Role of viral factors in retroviral variation.

Several studies have indicated that the error rate of HIV-1 RT, as assessed *in vitro*, does not accurately reflect the mutation rate observed *in vivo* (Mansky and Temin, 1995). The mutation rate of HIV-1 was found to be 20-fold lower in tissue culture during single round replication assays than predicted by cell-free fidelity assays. Thus, the continuous high rate of viral replication is a major mechanism of exploring sequence space that is as critical as recombination or RT-induced codon change. These studies also argue convincingly for important roles for other factors of either viral or host origin that impact diversity and pathogenicity (Coffin 1995, Mansky and Temin, 1995).

Studies involving the viral protein R (Vpr) have also shed light on the contribution of viral factors, in addition to NC and RT, in moderating variation (Mansky *et al.*, 1996). The factor, encoded by the *vpr* gene, is a late viral product incorporated into virions (Cohen *et al.*, 1990); its packaging is mediated by interaction between sequences of Vpr and the carboxy terminus of $Pr55^{gag}$, localizing to the viral p6 protein (Paxton, *et al.*, 1993). Vpr encodes a nuclear localization signal (NLS) and its association with the viral core provides a means for the preintegration complex (PIC) to efficiently traverse the nuclear barrier of non-dividing cells such as macrophages (Heinzinger *et al.*, 1994, Popov *et al.*, 1998). Vpr has also been shown to induce cell cycle arrest at the G2 phase, by saturating the kinase activity of $p34^{cdc2}$-cyclin B, required to transit from the G2 to M phase (Re *et al.*, 1995, Rogel *et al.*, 1995).

As a means of limiting the incoporation of uracil into growing DNA strands during reverse transcription, most retroviruses either encode a dUTP pyrophosphatase (dUTPase) or package host-derived uracil DNA glycosylase UNG. The UNG family of proteins comprise a part of the base excision and repair pathway, whose normal cellular role is to remove erroneously incorporated uracil during DNA replication, since G-A transitions might otherwise result. The nuclear isoform, UNG-2, has been found to be incorporated into HIV-1 viral particles. However, they are surprisingly absent in the HIV-2/SIVmac lineage (Priet *et al.*, 2003). The Vpr protein of HIV-1 has been shown to bind to UDG, and correlates with the diminished mutation rates observed by Mansky (Mansky *et al.*, 2001). As well Vpr effects may be independent of other viral constituents since

Vpr can reduce UV-induced damage in plasmid vectors as well as HIV-1 vectors (Jowett *et al.*, 1999, Mansky 1996). This association with a DNA repair enzyme is intriguing, given that nonprimate lentiviruses typically encode a dUTPase, which like UDG, minimizes the misincorporation of uracil into DNA and is important for virus replication in primary nondividing macrophages, but not in dividing cells. The dependence upon Vpr for infection of nondividing macrophages appears to be determined by cell type and relate solely on its ability to interact with UDG. Similarly, members of the HIV-2/SIVsm classification encode both Vpr and the homologous related protein Vpx. It has been demonstrated that HIV-2/SIVsm Vpx is solely capable of sustaining infections of primate macrophages, independent of Vpr (Fletcher *et al.*, 1996). Curiously, a later report demonstrated that it was Vpr of the HIV-2/SIVsm group that associates with UDG, and not Vpx, implying that Vpx facilitates infection of macrophages by a mechanism independent of UDG (Sleigh *et al.*, 1998). It has been recently shown that Vpx is critical for up-regulation of HIV-2 replication in host target cells, by enhancing the import of the genome into the nucleus (Ueno *et al.*, 2003).

Similar to the foregoing, dUTPases are another class of enzyme, that are incorporated into non-primate lentiviruses, that can diminish cytoplasmic dUTP pools. The absence of this activity has been linked to large increases (up to 5 fold) in genome mutagenesis. Their absence from HIV-1 and other primate lentiviruses has led to the proposal that virus replication under conditions of low dUTPase activity may lead to increased mutagenesis and facile viral diversification (Elder *et al.*, 1992, Elder *et al.*, 1995, Klarmann *et al.*, 2003). Some reports suggest that HIV-1 or SIV do not encode a dUTPase, but that other viral and host cell factors may compensate for this activity. There is evidence that primary HIV-1 isolates may differ in their requirement for cellular factors necessary for reverse transcription and this difference may also be reflected in outbred hosts (Fouchier *et al.*, 1994).

Another crucial "accessory" factor is the viral infectivity factor (Vif) that has been shown to play an important role early in viral replication. Vif, expressed from a single-spliced mRNA is found in high concentrations within the cytoplasm, but also co-localizes with membrane-associated p6 domain of Gag, (Cullen, 1998). Vif does not appear to be directly involved in virion budding or release, but rather, determines the infectivity state

of nascent virions and phenotype is dependent on the producer cell type from which the particles are budding, since *vif* is dispensible in so called "permissive lines" (Gabuzda *et al.*, 1992). Moreover, this phenotype has also been shown to be species dependent, where Vif complementation is restricted to cells of the originating virus. Virions produced from Vif defective recombinants are capable of cell entry, but the generation of full-length proviral DNA is impaired (Simon and Malim, 1996), and this block correlates with impaired production of minus strand DNA synthesis, implying a role for Vif early in transcription (Goncalves *et al.*, 1996). Perhaps at the stage of initiation since it is known to be part of the pre-integration complex (PIC) although this point is unresolved as of yet. Recently, considerable progress has been made in unraveling the mechanistic details of Vif function. Importantly a cellular gene product, CEM15 or APOBEC3G has been identified that interacts directly with Vif (Sheeny *et al.*, 2003). CEM15/APOBEC3G has been identified as a cellular antiviral factor belonging to the cytosine deaminase family of RNA and DNA editing enzymes.

From a mechanistic perspective, it appears that Vif functions in non-permissive cellular environments to exclude CEM15/APOBEC3G from incorporation into assembling virions, which upon subsequent infection into a permissive or non-permissive enviroment, acts to disrupt the PIC in the process of reverse transcription. The prescribed role of the CEM15/APOBEC3G protein seems to agree with the observed hypermutagenesis of minus-strand DNA products in *vif* negative mutants (Lecossier *et al.*, 2003).

The abovementioned cellular factors, likely acting in concert, can negatively impact codon variation. There is likely additional mutagenic pressure stemming from fluctuations within the cellular dNTP pools as a result of different cell types, cell cycle variation, or as a result of nucleoside antiviral therapy all of which can similarly alter base substitution frequencies and incur mutagenesis, although a variety of different mechanisms may be responsible (Bebenek *et al.*, 1992, Echols and Goodman, 1991, Mansky *et al.*, 2002). Several studies have shown that the frequency of G → A hypermutation is increased in the presence of low intracellular dCTP concentrations (Martinez et al., 1994, Vartanian *et al.*, 1994). Thus, the cellular activation state can alter viral replication and incur replicative infidelity, an effect that can be magnified in the

presence of antiretroviral drugs. Gao *et al* has shown that certain drug combinations can result in disproportionate antiviral effects depending on the cellular activation state, i.e. both ddI and ddC can exert antiviral effect more favorably in resting cells as compared to activated cells; whereas AZT preferentially protects activated cells against HIV infection and may be due in part to blockage of the first strand transfer during reverse transcription. (Arts *et al.,* 1995, Gao *et al.*, 1993.)

d. Host driven viral selection and evolution

Similar to the foregoing viral constituents, fluctuations in the host environment can also alter quasispecies development. It is possible that both the strength and direction of immune selection are modulated throughout the disease course. (Yamaguchi and Gojobori, 1997). Virus populations are known to be relatively homogeneous early in infected individuals and similarly in primate model systems (Overbaugh *et al.*, 2000, van'tWout *et al.,* 1994, Nowak *et al.*, 1997). Sequential analysis of resident quasispecies from infected patients shows substantial variation over the course of infection (Cichutek *et al.,* 1992, Delwart *et al.,* 1997).

Typicallly, macrophage-tropic, non-syncytium-inducing, variants with a predilection for the CCR5 co-receptor predominate in the asymptomatic phase of infection. It is viruses of this phenotype that appear to be responsible for establishing infection in an individual exposed to heterologous HIV-1 variants.

Both sexual and parenteral transmission cases revealed a selective outgrowth in the recipient of the most macrophage-tropic variant(s) present in the donor. In three out of five vertical transmission cases, more than one highly macrophage-tropic virus variant was present in the child shortly after birth, suggestive of transmission of multiple variants, or multiple independent transmissions. Similarly results were seen in the analysis of primary infections, where homogeneous virus populations of M-tropic, variants were present prior to seroconversion, strengthening the association for the R5 co-receptor in transmission. As a consequence may exclude humoral mediated pressure for selecting macrophage-tropic variants. (van't Wout *et al.,* 1994).

However, for intermediate macrophage-tropic isolates replication was abrogated at the level of reverse transcription. Entry of highly macrophage-tropic isolates resulted in

efficient completion of the reverse transcription process, whereas entry of intermediate macrophage-tropic isolates did not. Experiments indicate that primary HIV-1 isolates may differ in their dependency on cellular factors required for reverse transcription, particulary in macrophages. Differences in the susceptibility of macrophages for *in vitro* HIV-1 infection also suggests that there is significant variation in the availability of these cellular factors from outbred individuals (Fouchier *et al.*, 1994).

At late stages of disease there is little noteworthy immune selection, this point can be supported by the static equilibrium in viral diversity (Wolinsky *et al.*, 1996) as well, opportunistic disease prevails at this stage signifying the decline of immunological functions. Furthermore, the temporal changes of co-receptors and cellular tropism correlate with fitness changes that in turn reflect pathogenesis. RT enzymes cloned from viral isolates recovered at late stages of primate infection, have been shown to exhibit increased polymerase fidelity in cell-free assays (Kimata *et al., 2001*). This gives credence to the notion that in the absence of immune driven selection HIV or SIV attempts to maintain the "status quo" in regard to codon diversity.

Increased replicative efficiency as a result of selection is a basic tenet of evolution, a point underscored by seemingly disparate areas of HIV research that include genetic reversion, drug resistance, and phenotype switches. The switch in co-receptor usage correlated with the change from NSI to SI phenotypes offers an opportunity for phenotype mixing and similar increases in recombination partners provides a powerful capability to overcome any conceivable environmental stress.

Both deterministic and stochastic models have been used to determine the contributions of selection (shifts) or the drift that dominates small populations. Certainly, this discounts the massive numbers of infected cells but if one considers the segregation into biologically distinct tissue compartments the potential for drift becomes clear. Each niche environment, or transference to a new host environment, offers a new series of constraints and bottlenecks enforced by selection, favoring the maintenance or extinction of variants of the highest relative fitness. Reports have shown that replication-competent virus within the pool of latently infected resting T cells is genetically distinct from the rebounding plasma virus after discontinuation of therapy in four of six, and five of eight patients studied (Chun *et al.*, 2000; Ho and Zhang, 2000). Therefore one cannot exclude

the pool of latently infected memory T cells from playing a role in viral pathogenesis since they provide a genetic archive, available at any point when the selective environment changes. As well, one cannot discount other potential reservoirs either new or not yet identified (Marras *et al.*, 2002).

There is an accumulating body of evidence seen in both human and primate cohorts that supports the importance of MHC class I directed CTL in providing immunological control and ameliorating disease outcome. Clinical data has also indicated that antiviral CTL activity is predominantly responsible for diminishment or maintenance of low viral loads at acute or chronic phases respectively. The specific contribution of CTL effector functions as protective correlates is still unclear. However the ability of HIV and related viruses to elude the host CTL is clear, although its prevalence in natural infections is still an open queston. However, in broad terms it is clear that this is a major obstacle to vaccine development. The underlying problem seems to be that due to the protracted immune response in the early acute phase of infection makes clearance impossible. Invariably at later stages, despite often vigorous and directed CTL responses against HIV-1 or SIV, there is inevitable failure to contain the viral evolution.

Certainly, better described is the capability of lentiviruses to change phenotypes to those capable of evading neutralizing antibodies. The determinants for receptor tropism are located in the V3 hypervariable region of gp120 and the presence or selection of discrete amino acid substitutions are sufficient to alter viral cell tropism (Cocchi *et al.*, 1996, Ivanoff *et al.*, 1992, Takeuchi *et al.*, 1991). During both the acute and chronic phases of infection amino acid variation of the Env domains can be as high as 30%, depending on the clade in question. Moreover, mutations in V3 of M-tropic viruses can produce viruses exhibiting dual-tropic phenotpes that can then utilize multiple co-receptors (Doranz *et al.*, 1996). It may be that both CTL and neutralizing Ab escape *in vivo* are more common than previously recognized. Of greater importance is the fact that comparable escape from antiretroviral drugs can be transmitted, as single and mutiple (MDR) forms (Goulder *et al.*, 2001, Langedijk *et al.*, 1995). The cost of the relentless race towards optimal viral fitness, may be exhaustion of the host immune compartment.

Attenuating viral evolution: theoretical support, and evidence from cases of drug resistance and live attenuated vaccines.

In sum, the mechanisms involved in retroviral variability are multipartite, however all abet viral pathogenesis. The fact that these aforementioned attributes underlie antiretroviral resistance and at least immune escape, if not immune capitulation, mandates a serious and directed research focus. The vast majority of progress in this area has been limited to theoretical models or the use of molecular epidemiological, and phylogenetic analysis. Unfortunately by comparison, applied basic work in this area has been rather limited (Crotty *et al.*, 2001, Whitney *et al.*, 2002).

Our group has studied the forced evolution (i.e. reversion) of viruses containing mutations in both the leader and RT regions. In fact, the case of reversion parallels that seen in studies of infected individuals and primate the progression, in essence reversion is a case of less fit variants ascending to an optimal replication peak i.e. a wild type phenotype, for each respective niche.

However, viruses resistant to the antiretroviral nucleoside 2', 3'-dideoxy-3' thiacytidine (3TC) harbor a single M184V substitution within the aforementioned motif (63). Convincing evidence has been shown for drugs that increase viral mutation rates (Crotty *et al.*, 2000, 2001) Thus, indicating that RNA viruses are replicating on an error threshold. The existence of an error threshold and the resulting Muller's ratcheting effect has not been shown conclusively in HIV, however its existence is supported by several experimental and theoretical studies with other RNA viruses.

Phenotypically the M184V mutation in both HIV and SIV is associated with high-level resistance to 3TC, and exhibits low-level cross-resistance to several other nucleosides (10,12,16) In HIV-1, it is well documented that the M184V mutation also confers a deficit in fitness that is most apparent in primary cell lines. The reasons for this are multifaceted and include the fact that RT enzymes containing M184V are associated with diminished proccessivity, diminished nucleotide primer unblocking, and diminished ability to initiate reverse transcription. This domain is common throughout the polymerase family of enzymes (Poch *et al.*, 1993)); perhaps indicative of purifying selection at this codon cluster, therefore it is not surprising that any non-synonymous changes affecting this region negatively impact viral fitness.

Particularly disadvantaged are those harbouring impaired dimerization in the background of decreased replicative efficiency, where even modest increases in RT fidelity may be particularly are less able to effect repair by way of compensatory mutagenesis. Similar results have been reported with HIV-1 viruses containing the M184V mutation in RT (29, 38). In SIV, a recent study showed *in vivo* that M184V containing viruses failed to revert to WT, and may have been initially impaired in ability to multiply to high titre. However, this replication deficit may have been corrected in these animals over time, with the appearance of a compensatory mutation (46). Here, we show in an *ex vivo* context that the reverse transcription of mutants containing both the M184V and DIS mutations was further compromised. Notable as well is the fact that reversions can occur through selection of alternate mutations to those in SD2 alone.

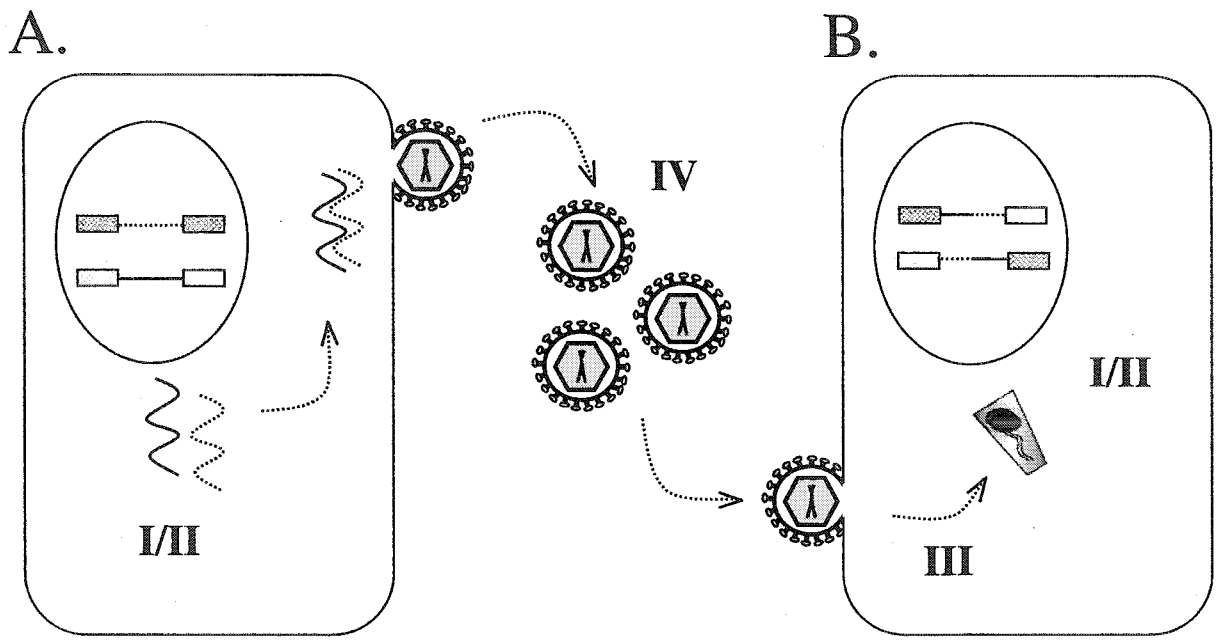
Attenuation of the various diversity generating mechanisms through small molecule inhibition or by way of directed poly-epitope vaccines is certainly a laudable, if not an immediately attainable goal. Likewise, is increasing evidence to support or rather drive viral muatgenesis to a point that is incompatible with genome integrity, i.e. error catastophe.

Future directions

Elucidating and understanding evolutionary mechanisms and their role in basic viral replication is clearly necessary for developing new, and optimizing current, antiviral therapies. A concerted effort to curtail genome diversity may offer additional targets to an already growing list, not only for HIV, but potentially be translated to other diseases that utilize antigen diversification and/or variation as a mechanism of persistance including, but not limited to HCV and Malaria.

Specifically, in regard to the HIV pandemic, these same attributes are of paramount importance in defining and developing an effacious vaccine. As the particular immune correlates, and the relevance of recombinant forms have defied precise definition. This has already shown itself to be a rather daunting task, where the best-case scenario available for comparison is that of the Influenza vaccines. Downtrodden by their low vaccine efficacy, the task is further complicated by Influenzas' constant antigenic drift and spurious shifts that necessitate its constant update. By comparison HIV, even by conservative estimates, shows nearly a 1000 fold greater adaptive potential.

In sum investigation into of the underlying mechanisms for genetic diversity could be translated to more effective vaccines, highly suppressive second-generation antiretrovirals, and better second line salvage therapies.



I. Anti-mutational Processes

Cellular DNA polymerases
RNA polymerase II
Excision repair system
Uracil DNA Glycosylase
RNA Editing?

II. Mutational Processes

RT infidelity
RNA polymerase II
Cellular DNA polymerases
Modulation of dNTP pools
Replication rate
RNA editing?

III. Genomic recombination

IV. Intra and inter-host selection

Figure 2.1

Chapter 3:

Leader Sequences Downstream of the Primer Binding Site are Important for Efficient Replication of Simian Immunodeficiency Virus.

This chapter is an adaptation from an original paper published in the *Journal Of Virology*. 2000; 74(19): 8854-60. Authorship is as follows; Yongjun Guan, James B. Whitney, Karidia Diallo, and Mark A. Wainberg. The contributions to this chapter, including manuscript preparation, are divided equally between Yongjun and myself. K.D. participated in the mutagenesis of the revertant clones.

3.1 Preface

This first chapter details a series of SIV deletion mutants and their impact on viral replication, gene expression, and RNA encapsidation. These mutants were constructed within the first 97 nt from downstream of the PBS upto and including SL1. Long-term serial passage of these constructs revealed the propensity of several mutants to regain wild type replication phenotypes. These revertant virus were then shown to posses second site compensatory mutations in several disparate regions of the genome. The biological relevance of these nonsynonomous point mutations was validated by site directed mutatgenesis and *ex vivo* replication kinetic studies. These compensatory mutations were shown to recover RNA encapsidation efficiency and partially restore proteolytic processing of $Pr55^{Gag}$.

3.2 Abstract

Simian immunodeficiency virus (SIV) infection of macaques is remarkably similar to that of human immunodeficiency virus (HIV-1) in humans, and the SIV/macaque system is a good model for AIDS research. We have constructed an SIV proviral DNA clone that is deleted of 97 nt, i.e. construct SD, at positions (+322-+418) immediately downstream of the primer binding site (PBS) of SIVmac239. When this construct was transfected into COS-7 cells, the resultant viral progeny were severely impaired in regard to ability to replicate in C8166 cells. Further deletion analysis showed that a virus termed SD1, containing a deletion of 23nt (+322-+344) was able to replicate with wild type kinetics, while viruses containing deletions of 21nt (+398-+418) (construct SD2), or 53nt (+345-+397) (construct SD3), displayed diminished capacity in this regard. Both the SD2 and SD3 viruses were also impaired in regard to ability to package viral RNA, while SD1 viruses were not. The SD and SD3 constructs did not revert to increased replication ability in C8166 cells over 6 months in culture. In contrast, long-term passage of the SD2 mutated virus resulted in a restoration of replication capacity, due to the appearance of four separate point mutations. Two of these substitutions were located in leader sequences of viral RNA within the PBS and the dimerization initiation site (DIS) while the other two were located within two distinct gag proteins, i.e. CA and p6. The biological relevance of three of these point mutations was confirmed by site -directed mutagenesis studies that showed that SD2 viruses containing each of these substitutions had regained a significant degree of viral replication capacity. Thus, leader sequences downstream of the PBS, especially the U5-leader stem and the DIS stem-loop, are important for SIV replication and for packaging of the viral genome.

3.3 Introduction

Simian immunodeficiency virus (SIV) and human immunodeficiency virus type 1 (HIV-1) belong to the primate lentivirus subfamily of retroviruses. They both possess at least six auxiliary genes and are considered complex retroviruses (Cullen 1991, 1992). SIV can induce an AIDS-like disease in certain monkeys such as rhesus macaques, and is an excellent animal model for the study of human HIV disease (Kestler *et al.*1990). The 5' untranslated leader sequences of HIV possess a number of functional domains, including elements for trans-activation of transcription, initiation of reverse transcription, packaging of viral RNA and integration of the proviral genome (Cobrinik *et al.*, 1990 Cobrinik *et al.*, 1988, Hu and Temin. 1990, Kao *et al.*, 1987, Lever *et al.*, 1989, Steffy and Wong-Staal, 1991). A 54nt leader sequence in HIV-1, located downstream of the primer binding site (PBS) and upstream of the dimerization initiation site (DIS), has been shown to be involved in efficient HIV-1 gene expression and virus replication (Lenz et al 1997, Li *et al* 1997, Liang *et al* 1999). SIVmac239 has 97nt sequences in this region, which is therefore much longer than that of HIV-1 (Regier and Desrosiers, 1990). The 5' untranslated leader sequence of the SIV RNA genome has little sequence similarity with that of HIV-1, but similar secondary structures have been predicted (Rizvi and Panganiban. 1993). SIV also shows certain unique features in the leader sequence such as an intron located in the 5' R-U5 region and an internal ribosome entry site found in the SIV leader sequence but not in HIV-1 (Ohlmann *et al.*, 2000, Viglianti *et al.*, 1992). We conducted studies to determine the role of the region located downstream of the PBS and upstream of the DIS in SIV, an area that is not well understood.

Using mutational analysis, we have shown that SIV mutants containing a 97nt deletion of these leader sequences is severely impaired in regard to both viral replication and packaging of viral RNA. A 23nt sequence within the 5' portion of this 97nt region had only minor effects on viral genomic RNA packaging and SIV replication in C8166 cells. However, the remaining 74 nt within this region played a significant role in viral genomic RNA packaging and replication in the aforementioned cell line.

3.4 Materials and Methods

3.4.1 Construction of deletion mutations

The two half-genome plasmids of SIVmac239, molecular clones, p239SpSp5' and p239SpE3', were obtained through the AIDS Research and Reference Reagent Program (Regier and Desrosiers, 1990). Nucleotide designations for SIVmac 239 are based on the published sequences, the transcription initiation site corresponds to +1. Table 1 shows primers used in our experiments. To obtain the full-length clone, the 5' cellular sequence was replaced with an Eco RI site and the 3' cellular sequence was replaced with a *Xho I* site by PCR-based methodology, using primers pSU3/pSPBS and pSU5-1/pSenf. A full-length clone was constructed by inserting the ligation product of the 5' *Eco RI-Sph I* fragment and the 3' *Sph I-XhoI* fragment into the *Eco RI-Xho I* site of a pSP73 vector. Deletion mutants were then constructed based on this full-length infectious clone, termed SIV/WT. We used PCR-based mutagenesis methods to generate deletions downstream of the PBS. Pfu polymerase was used to increase the fidelity of the PCR. All constructs were confirmed by sequencing. Figure 1 illustrates a graphic description of the mutants generated in regard to both sequence and tertiary structure. Briefly, the region between the *Nar I* and *Bam HI* sites in SIV/WT was replaced by PCR fragments to generate mutant constructs (primers pSD/pSgag1 were used for SD deletion, primers pSD1/pSgag1 were used for SD1). For construction of SD2 and SD3 deletion, PCR fragments (pSD2/pSgag1 for SD2, pSD3/pSgag1 for SD3) were purified and were then used as mega-primer paired with primer pSU5 to generate PCR fragments to replace the region between *Nar I* and *BamHI* sites in SIV/WT.

3.4.2 Cells and preparation of virus stocks

COS-7 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum. C8166 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. All media and sera were from GIBCO (Burlington, Ontario, Canada). Molecular constructs were purified using a Maxi Plasmid Kit (QIAGEN Inc. Mississauga, Ontario, Canada). COS-7 cells were transfected using these constructs with lipofectamine-plus reagent (GIBCO, Burlington, Ontario, Canada). Virus containing supernatant was harvested at 60 h after transfection and was clarified by centrifugation for 10 min at 4°C at 3,000 rpm in a Beckman GS-6R centrifuge. Viral stocks were stored in 0.5 or 1 ml aliquots at –70°C. The concentration of p27 antigen in these stocks was quantified using a Coulter SIV core antigen assay kit (Immunotech Inc. Westbrook, ME, U.S.A.).

3.4.3 Virus replication in C8166 cells

Viral stocks were thawed and treated with 100 U of DNase I in the presence of 10mM $MgCl_2$ at 37°C for 1 h to eliminate any residual contaminating plasmids from the transfection. Infection of C8166 cells was performed by incubating $1x10^6$ cells at 37°C for 2 h with an amount of virus equivalent to 10ng p27 antigen. Infected cells were then washed twice with phosphate-buffered saline (PBS) and incubated with fresh medium. Cells were split at a 1:3 ratio twice per week if they had grown to a sufficient level, otherwise the culture fluid was replaced with fresh medium. Supernatants were monitored for virus production by both reverse transcriptase (RT) assay and SIV core antigen capture assay (Immunotech Inc. Westbrook, ME, U.S.A.).

3.4.4 Detection of viral DNA

At various times post infection, C8166 cells were collected and washed with PBS. To ensure that no contaminating plasmid remained, fluid from the wash was routinely checked by PCR using SIV specific primers. Cellular DNA was isolated using a QIAamp DNA Mini kit(QIAGEN). DNA samples were analyzed by PCR using primers pSPBS-1 and Sg to amplify the deletion region between the PBS and the major splice donor site of SIV. PCR assays were performed with 0.1-1μg of sample DNA, 50mM Tris –HCl (pH

8.0), 50 mM KCl, 1.5 mM MgCl2, 2.5 U Taq polymerase, 0.2mM dNTPs, 10 pmol 32p-end-labelled reverse primer and 20 pmol of unlabelled forward primer and programmed as follows: 95°C at 3 min, 25 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1min, and 72°C for 10 min. Reactions were standardized by simultaneous amplification of a 567 bp DNA fragment of human β-actin gene as an internal control. Products were separated through 5% native polyacrylamide gels. Products derived from PCR using unlabelled primers were separated in agarose gels and extracted using a QIAEX II GEL Extraction Kit (QIAGEN). The purified DNA was used as template to confirm deletion mutations via sequencing.

3.4.5 Detection of viral proteins produced by transfected COS-7 cells

Expression of viral proteins by transfected COS-7 cells was determined using a Coulter SIV core antigen assay and western-blot. For the purpose of western-blot, nascent extracellular virions were precipitated by ultracentrifugation and used as protein samples. Western blotting was performed using SIVmac 251 antiserum by standard protocol (Manatias *et al.*, 1985).

3.4.6 Detection of RNA in virions by RT-PCR

To study packaging of viral genomic RNA, viral RNA was isolated using the QIAamp viral RNA mini kit (QIAGEN) from equivalent amounts of COS-7 cell-derived viral preparations based on levels of SIV p27 antigen. RNA samples were treated with RNase-free DNase I at 37°C for 30min to eliminate possible DNA contamination. DNase I was then inactivated by incubation at 75°C for 10min. The viral RNA samples were quantified by RT-PCR, using the Titan One Tube RT-PCR system (Boehringer Mannheim, Montreal, Quebec, Canada). The primer pairs sg1 and sg2 were used to amplify a 114-bp fragment representing full-length viral genome. The primer sg2 was radioactively labeled in order to visualize PCR products. Equivalent RNA samples, based on p27 antigen levels, were used as templates in a 18-cycle RT-PCR. The products were fractionated on 5% polyacrylamide gels and exposed to X-ray film. Relative amounts of products were quantified by molecular imaging (BIO-RAD Imaging). Levels of genomic

packaging were calculated on the basis of four different reactions, with wild type virus levels arbitrarily set at 1.0.

3.4.7 Site directed mutagenesis

For introduction of point mutations into the SD2 genome, the fragment between the *BamHI* and *Sph I* sites was subcloned into the pSP73 vector to generate a clone termed pSIV-BSp, and the fragment between the *Eco RI* and *Bam HI* sites was subcloned into the pSP73 vector to generate the clone termed pSIV-EB-SD2. The QuikChange™ site-directed mutagensis kit (Stratagene, la Jolla, CA, U.S.A.) was used to introduce the M2, CA1 and Mp6 point mutations into SD2 DNA, using procedures that have been previously described[21] utilizing the following primer pairs, i.e. M2-1 (5' ccaaccacgacgg agtggtgccagacggcgtgagg 3') and M2-2 (5'cctcacgccgtctggcaccactccgtcgtggttgg 3') for M2, CA1-1 (5'gctaacccagattgcaggctagtgctgaaggg 3') and CA1-2 (5'cccttcagcactagcctgcaa tctgggttagc 3') for CA1, Mp6-1 (5'gccttacaaggaggtgacaaaggatttgctgcacctc 3') and Mp6-2 (5'gaggtgcagcaaatcctttgtcacctccttgtaaggc 3') for Mp6. The *Eco RI-Bam HI* fragment was cloned back into the SD2 genome to generate the SD2-M2 clone; the *BamHI -Sph I* fragment was cloned into the SD2 genome to generate both the SD2-CA1 and SD2-Mp6 clones. To generate the M1 mutation, the fragment which was produced by PCR using primers PBS-M1 (5' tggcgcccgaacagggacttg 3') and pSgag1 (based on the SD2 template, see above) was inserted into the SD2 genome between the *Nar I* and *Bam HI* sites to yield the SD2-M1 clone. The presence of all point mutations was confirmed by direct sequencing.

3.5 Results

Sequences downstream of the PBS are important for SIV replication in C8166 cells. To investigate the role of leader sequences located downstream of the PBS in SIVmac239, we constructed deletion mutations in this region (Fig.1, A and B). First, a 97nt (+322-+418) deletion was introduced into the region immediately downstream of the PBS, i.e. construct SD; this construct abolished both the putative U5-leader stem and DIS stem-loop. Alternatively, three sub-deletions within this 97nt region were generated, termed SD1 (+322-+344), SD2 (+398-+418) and SD3 (+345-+397), respectively. SD1 retains a

stable U5-leader stem but is deleted of the small stem-loop within the U5-leader stem. SD2 is deleted of the left side half of the DIS stem-loop. Finally, SD3 retains the DIS stem-loop but is deleted of the U5-leader stem (Fig.1).

To investigate the replicative potential of these constructs, viral stock was thawed and treated with DNase I to eliminate any possible contaminating plasmids. Viruses containing 10 ng of p27 antigen were used to infect C8166 cells and culture fluids were monitored for virus replication by RT assay and by SIV p27 antigen capture assay. Figure 2 shows that each of the SD, SD2 and SD3 deletion mutants were significantly impaired in their ability to replicate in C8166 cells, while wild-type virus and one of the deletion mutants (SD1) replicated efficiently, as determined by levels of RT activity in culture fluids. The data in Table 2 also show that the SD1 construct yielded levels of p27 antigen similar to those of wild-type virus, while the SD, SD2 and SD3 deletion constructs were severely impaired in this regard.

We also measured levels of proviral DNA in these studies by PCR. The sequencing of PCR products indicated that the deletions were retained, even after replication over several passages (results not shown). Fig. 3A shows the PCR results of samples at 7 days post-infection, confirming that these deleted viruses were indeed able to infect C8166 cells, but that levels of proviral genomic DNA in regard to the SD, SD2 and SD3 viruses were diminished relative to wild-type virus (Fig. 3B).

3.5.1 The deletion mutations affect the packaging of viral genomic RNA

To investigate the potential mechanisms whereby virus replication was compromised, we determined levels of virus production by transfected COS-7 cells. Levels of extracellular SIV p27 antigen were quantified using the SIV p27 antigen capture assay. The results show that similar amounts of p27 were produced in each case (Table 3). We next analyzed viral proteins by Western blot, and the results also show that no significant differences were present in regard to viral protein production (Figure 4).

To determine the efficiency of packaging of the viral genome, RNA samples were isolated from equivalent amounts of SIV virus, based on p27 levels. A 114-bp fragment that represents the full-length, unspliced RNA genome was amplified and quantified by RT-PCR. The results of Figure 5 show that the SD1 deletion had no effect on

encapsidation of viral RNA, while the SD, SD2, and SD3 constructs resulted in diminution of RNA packaging by about 6-, 2-, and 3- fold, respectively. Therefore, sequences in each of SD2 and SD3 are likely involved in the packaging of the viral genome, while those in SD1 are not.

3.5.2 Long -term culture results in reversion of SD2 viruses

To investigate the possibility of reversion, we cultured the infected cells over longer periods, and did not find any sign of reversion of the SD and SD3 constructs over 6 months of passage. In contrast, modest amounts of RT activity in cultures infected by the SD2 viruses were present after 6 weeks. The supernatant fluids of the SD2 infection were then used to infect new C8166 cells, and viral culture fluids at peak levels of RT activity were again passaged onto new C8166 cells. After 4 passages (18 weeks), viral replication capacity was now similar to that of wild-type viruses (Figure 6). Proviral DNA of these reverted viruses was detected by PCR, and the region from the 5' LTR to the end of the gag gene was cloned. Six of these clones were sequenced and the results showed that the original deletion had been retained in each case but that four additional point mutations were also present. These four point mutations were located within the PBS (termed M1), the putative DIS loop (termed M2), the capsid protein (termed CA1) and the p6 protein (termed Mp6) of the gag gene (Figure 7). Each of these mutations is novel with the exception of M1, which has been observed in sequences of some wild-type viruses. The CA1 mutation involved a change of Lys-197 to Arg while the Mp6 substitution results in a change from Glu-49 to Lys. Neither the M1 nor M2 mutations involve amino acid substitutions, since both are located in non-coding areas of the viral genome. The M1 mutation (thymidine (T) to cytidine (C) at position 310) resulted in an alteration of the PBS, such that complementarity now existed with the 3' end of $tRNA^{Lys3}$ instead of the original $tRNA^{Lys5}$. The M2 substitution involved a change from adenosine (A) to guanosine (G) at position 423 that is located in the loop of the putative DIS stem-loop structure. RNA secondary structure analysis suggests that this point mutation cannot restore the destroyed DIS stem-loop structure in SD2 (data not shown).

In order to pursue the biological relevance of these various substitutions we performed site-directed mutagenesis to introduce each of these four point mutations into the SD2

genome. The resultant DNA clones termed SD2-M1, SD2-M2, SD2-CA1 and SD2-Mp6 were then transfected into COS-7 cells and the virus particles thereby recovered were assayed for viral replication capacity in C8166 cells. The results of Fig. 8 show that each of the constructs tested, with the exception of SD2-M1, was able to replicate more efficiently than SD2 in the C8166 cell line, although not as efficiently as wild-type virus. Thus, each of the M2, CA1 and Mp6 point mutations was able to partially compensate for the SD2 deletion, whereas the M1 substitution could not.

3.6 Discussion

Previous work has shown that leader sequences downstream of the PBS are important for HIV-1 gene expression and replication, but little about this subject is known in regard to SIV. In the present work, we have investigated this subject by constructing a series of mutated SIV clones containing deletions within a 97nt region immediate downstream of the PBS. The results show that mutants containing deletions in the entire 97nt region as well as two sub-regions were significantly impaired with respect to replication capacity in C8166 cells. A potential mechanism that may affect viral replication capacity in this context is that these sequences appear to be important for the packaging of the viral RNA genome. These results imply that both the U5-leader stem and the DIS stem-loop structures are important for SIV replication and for packaging of viral genomic RNA. Packaging determinants have not been completely described for any lentivirus, but interactions of multiple regions that are distributed widely within the HIV-1 genome have been proposed (Berkowitz *et al.,* 1995). The encapsidation of the HIV-1 viral genome is dependent on cis-acting RNA elements located around the major splice donor site and the core-packaging signal is composed of a series of stem-loops (Berkowitz *et al.,* 1996, Harrison *et al.,* 1998). It was originally thought that RNA sequences downstream of the major splice donor site were responsible for the specific packaging of viral genomic RNA in a manner that would exclude the packaging of spliced viral RNA species in the case of HIV-1. However, it has been reported that sequences upstream of the splice donor are also important for efficient packaging of HIV-1 viral genomic RNA (Berkhout *et al.,* 1996, Clever *et al.*, 1999, Liang *et al.,* 1998, McBride and Panganiban. 1997, Poeschla *et al.,* 1998). Similar results for HIV-2 have also been reported, but it was suggested that

sequences upstream of the major splice donor site were more important than those downstream for efficient encapsidation of HIV-2 RNA. Therefore, HIV-2 may use different mechanisms to select unspliced RNA for encapsidation (Kaye and Lever 1999 Kaye and Lever 1998, McCann and Lever. 1997 Poeschla *et al.,* 1998).

In regard to SIV RNA packaging determinants, only one study has reported that leader sequences upstream of the major splice donor site can be packaged into HIV-1 particles (Rizvi, and Panganiban 1993). Our results now show that sequences located downstream of the PBS and upstream of the major splice donor site, nt +345 to +418, are necessary for the efficient encapsidation of SIV genomic RNA, since deletions within this region have a detrimental effect on RNA packaging. This region includes half of the putative DIS and half of the putative U5-leader stem (Berkhout *et al.,* 1996, Rizvi and Panganiban. 1993). Therefore, these proposed structures likely serve a functional role in the encapsidation process. The fact that genomes with deletions of this entire region can still be packaged to some extent indicates that sequences in disparate regions may also play a role in encapsidation of SIV genomic RNA.

Deletions in this region that result in impaired replication may not only affect RNA packaging. Comparable work with HIV-1 has indicated that sequences in this region also affect HIV-1 gene expression and may affect Gag polyprotein processing (Lenz *et al.,* 1997, Liang *et al.,* 1999). Although our results show that these deletions do not have any significant effect on SIV protein expression in transfected COS-7 cells, further work is required to characterize whether these deletions can affect proviral DNA synthesis and gene expression in permissive cell lines.

Reversions of deleted mutated viruses have also been observed in similar studies on HIV-1, and point mutations within four distinct gag proteins were shown to contribute to the increased replication capacity of these viruses (Liang *et al.,* 1999). Our results reveal that two of our SIV constructs, i.e. SD and SD3, did not revert to increased replication ability in C8166 cells over 6 months in culture. In contrast, long-term passage of the SD2 mutated virus in these cells did result in a restoration of replication capacity, due to the appearance of four point mutations, M1, M2, CA1 and Mp6. Interestingly, two of these mutations were located in leader sequences that flank the deletion site, i.e. M1 and M2, and only two of these mutations were located in gag proteins, i.e. CA1 and Mp6. The M1

mutation was located within the PBS 87 nt upstream of the SD2 deletion while the M2 substitution was identified in the loop of the DIS only 3 nt downstream of the SD2 deletion. These findings imply that there may be important differences between SIV and HIV-1 in regard to mechanism(s) of RNA packaging and in regard to interactions between Gag proteins and leader sequences.

Site-directed mutagenesis studies have confirmed the biological relevance of each of these substitutions with the exception of M1. Since the M1 mutation has also been observed in both infections of wild-type and SD1 virus (data not shown), this substitution does not appear to be novel; rather, it may be a natural polymorphism involved in the binding of $tRNA^{Lys3}$, which is used more efficiently by SIV than $tRNA^{lys5}$ in human cells as a primer of reverse transcription (Das *et al.,* 1997). The M2 mutation was best able to rescue the SD2 deletion, but could not restore the putative DIS stem-loop structure. This implies that functions other than dimer formation may account for the partially restored replication capacity of SD2-M2 virus in C8166 cells. Further work is needed to determine how these point mutations are individually involved in restoration of viral replication of the SD2 deletion virus; such studies are in progress and also involve analyses of the M2, CA1 and Mp6 mutations in various combinations. While M2 alone was not capable of restoring the DIS stem-loop, it remains possible that a combination of M2 with other mutations not yet discovered could do this, while simultaneously enabling viral replication to resume with wild-type kinetics.

Fig. 3.1 Illustrations of deletion mutations and RNA secondary structure.

(A) Deletions are located between the arrows and their positions are shown relative to the transcription initiation site.

(B) Secondary structure of SIVmac 239 leader RNA model was predicted by free energy minimization (Zuker, 1989) and was adapted from published structures. All hairpin motifs are named after their putative function or after similar elements encoded by HIV-1. The following sequence motifs are highlighted: the polyadenylation signal at position 153, the PBS at position 303, the DIS palindrome at position 419, and the Gag start codon at position 534. The splice donor and acceptor sites in the R-U5 region (positions 60 and 204) are marked by a dotted arrow, while the major splice donor site at position 466 is marked by a solid arrow. The positions of deletion constructs are shown above the structure.

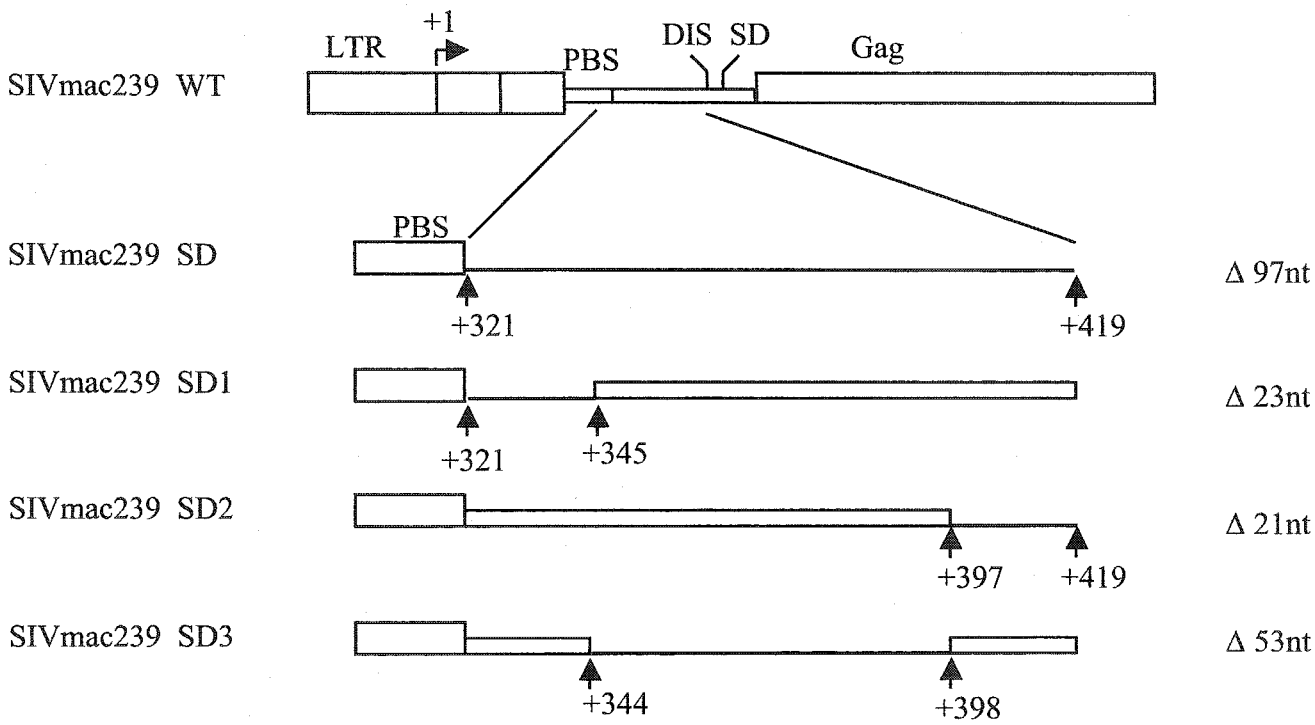
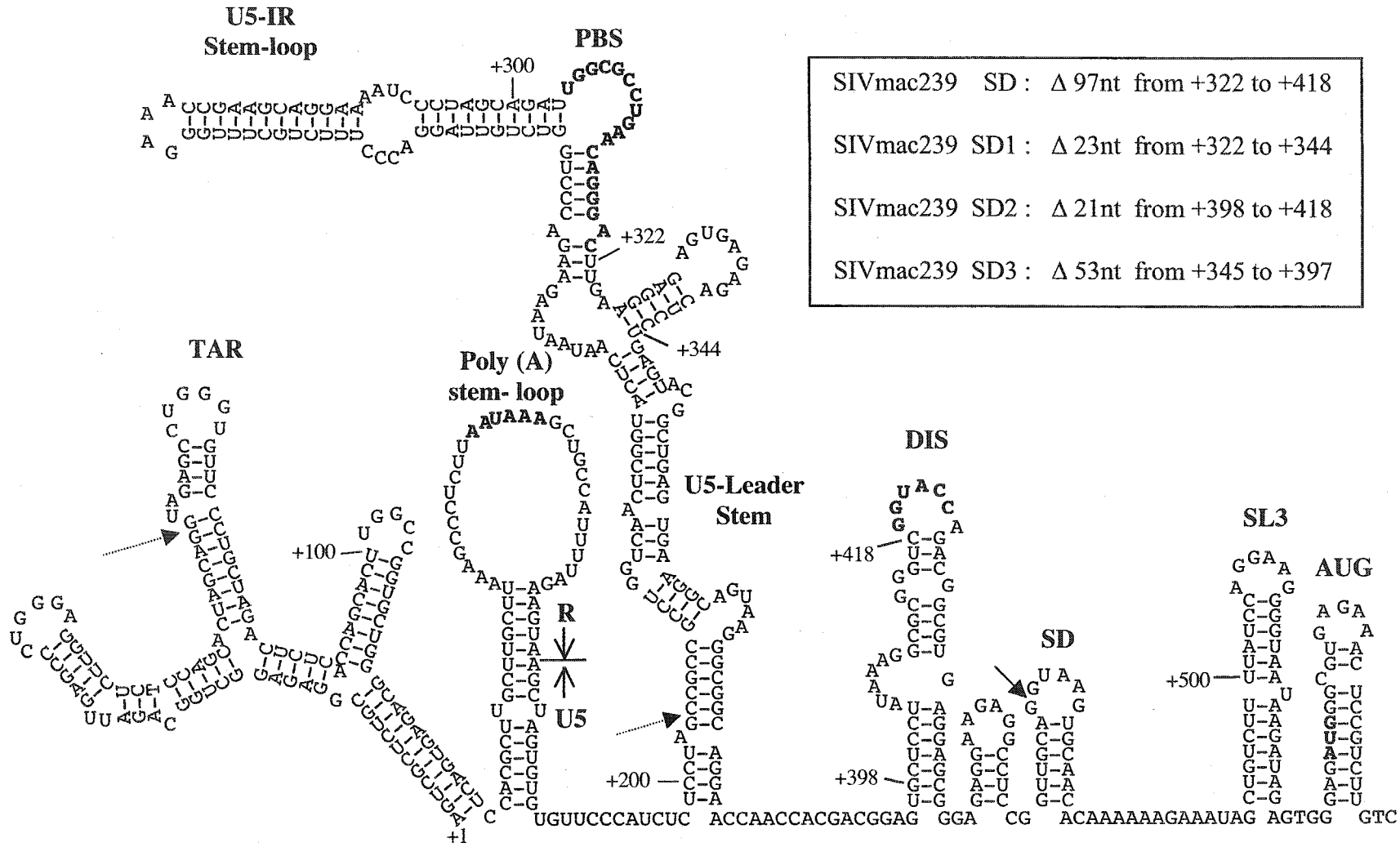

SIVmac239 WT
LTR
+1
PBS
DIS
SD
Gag
SIVmac239 SD
PBS
+321
+419
Δ 97nt
SIVmac239 SD1
+321
+345
Δ 23nt
SIVmac239 SD2
+397
+419
Δ 21nt
SIVmac239 SD3
+344
+398
Δ 53nt

Figure 3.1(A)



SIVmac239	SD :	Δ 97nt from +322 to +418
SIVmac239	SD1 :	Δ 23nt from +322 to +344
SIVmac239	SD2 :	Δ 21nt from +398 to +418
SIVmac239	SD3 :	Δ 53nt from +345 to +397

Figure 3.1(B)

Fig. 3.2 Growth curves of mutated viruses in C8166 cells

Equivalent amounts of virus from COS-7 transfected cells were used to infect C8166 cells based on levels of p27 antigen (10 ng per 10^6 cells). Viral replication was monitored by RT assay of culture fluids. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control.

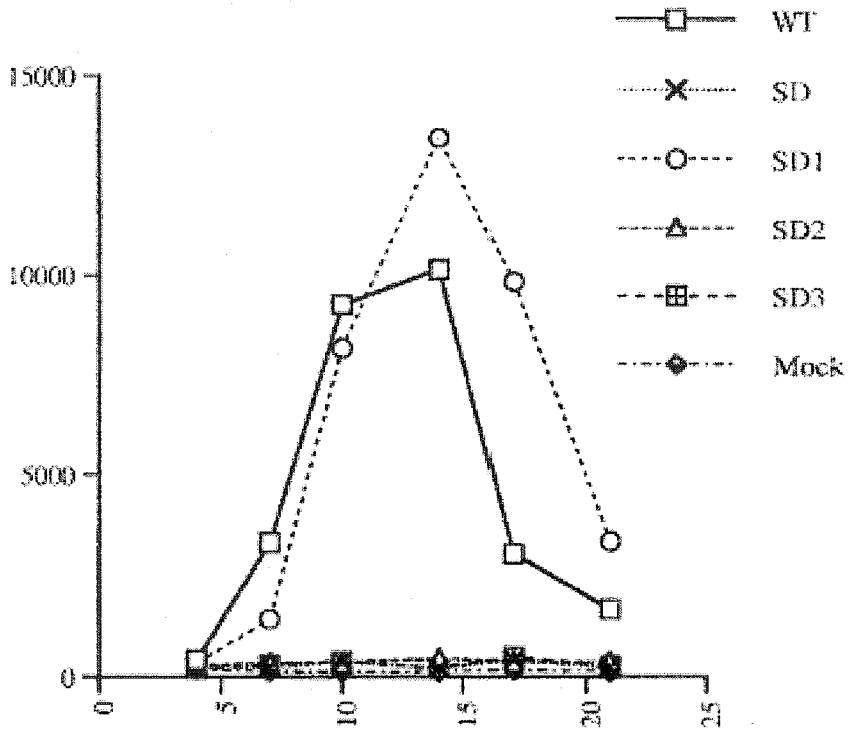

WT
SD
SD1
SD2
SD3
Mock
15000
10000
5000
0
0
5
10
15
20
25
Days after infection

Figure 3.2

Fig. 3.3 Detection of viral DNA.

A) Viruses derived from COS-7 cells were standardized on the basis of p27 and used to infect C8166 cells. Total cellular DNA was isolated from infected cells at 7 days after infection and subjected to PCR analysis with primers pSPBS-1 and sg, that specifically amplify a SIV cDNA fragment between the PBS and a site downstream of the DIS. The size of the PCR products vary based on the type of construct used and are 264 bp for wild-type virus (lane 1), 241 bp for SD1 deleted virus (lane 2), 243 bp for the SD2 deletion virus (lane 3), 211 bp for the SD3 deletion (lane 4) and 167 bp for the SD construct (lane 5). Primers amplifying a 587bp fragment of ß-actin were used as an internal control. Mock infection denotes inoculation of cells with heat-inactivated viruses (lane 6). A DNA marker of a 100 bp ladder is also shown (lane 7).

B) The intensity of each band was quantified by molecular imaging and the band intensity of viral DNA relative to cellular DNA for each sample is shown.

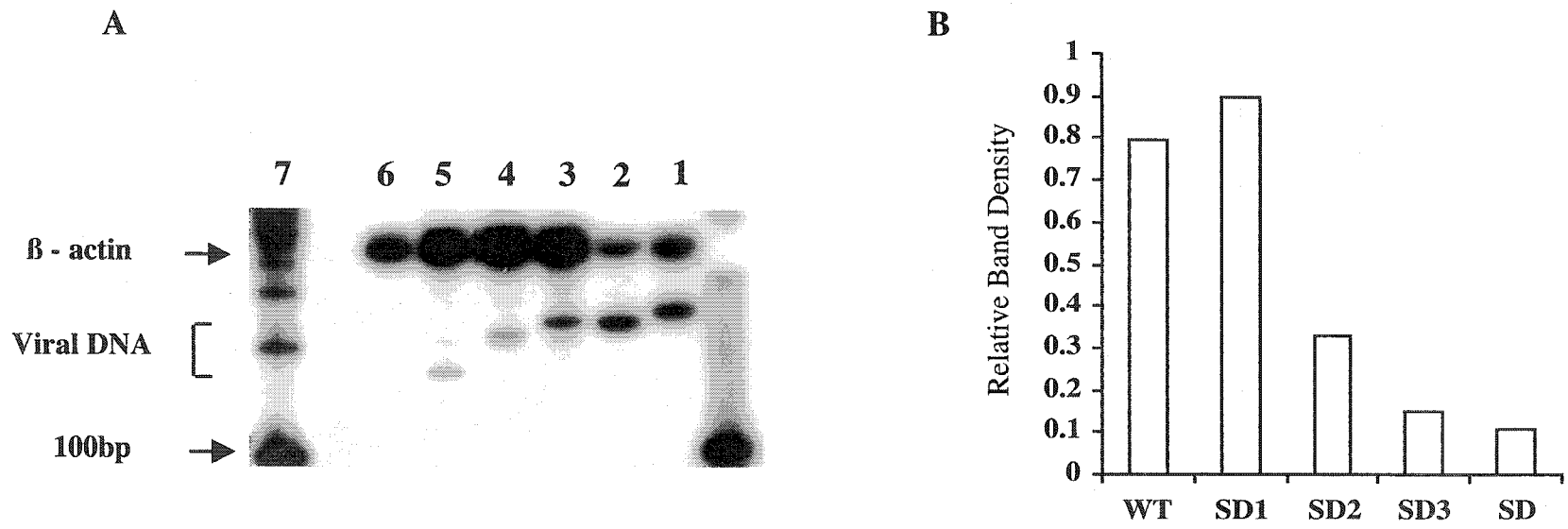

A
7
6
5
4
3
2
1
ß - actin
Viral DNA
100bp
B
Relative Band Density
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
SD1
SD2
SD3
SD

Figure 3.3

Fig. 3.4 Western blot to detect viruses derived from COS-7 cells.

Viruses were pelleted by ultracentrifugation at 60h after transfection and viral proteins were detected using SIV positive serum. The band indicating p27 protein is highlighted by the arrow.

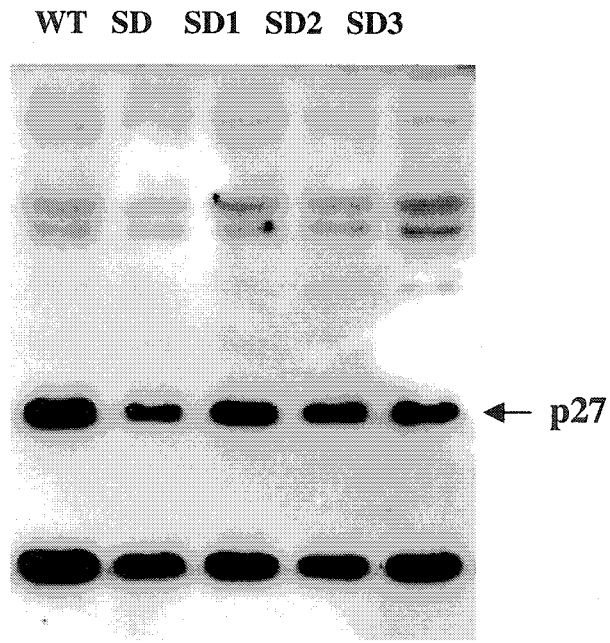


Figure 3.4

Fig. 3.5 Viral RNA packaging in wild type and mutated viruses.

Viral RNA was purified from virus stock derived from transfected COS-7 cells. Equivalent amounts of virus based on levels of p27 antigen were used as template. Quantitative RT-PCR was performed to detect full-length viral RNA genome in an 18-cycle PCR reaction. Relative amounts of a 114-bp DNA product were quantified by molecular imaging, with wild-type levels arbitrarily set at 1.0. Reactions run with RNA template, digested by Dnase-free RNase, served as a negative control for each sample to exclude any potential DNA contamination. Relative amounts of viral RNA that were packaged were determined on the basis of four different experiments.

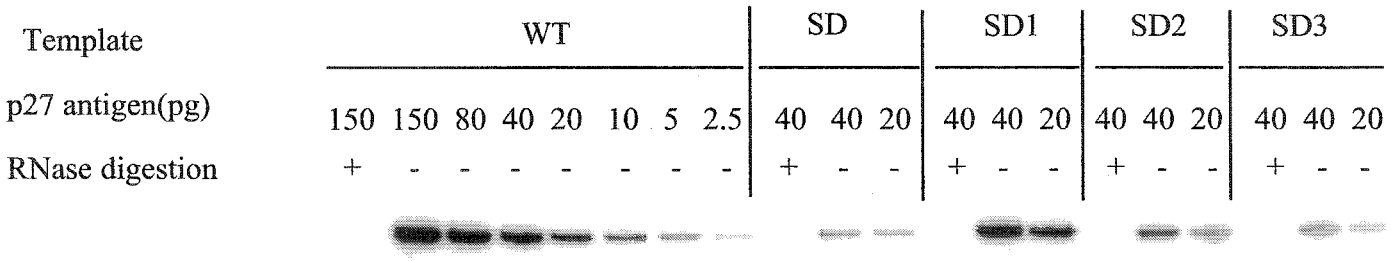

Template
WT
SD
SD1
SD2
SD3
p27 antigen(pg)
150 150 80 40 20 10 5 2.5
40 40 20
40 40 20
40 40 20
40 40 20
RNase digestion
+ - - - - - - -
+ - -
+ - -
+ - -
+ - -
Relative amount
1.0
0.17 ±0.03
1.11 ±0.16
0.44 ±0.03
0.34 ±0.04

Figure 3.5

Fig. 3.6 Reversion of the SD2 mutant after long term culture in CEMx174 cells. (A) Growth curves of viruses in long-term culture. Equivalent amounts of virus from COS-7 transfected cells were used to infect CEMx174 cells based on levels of p27 antigen (10 ng per 10^6 cells). Infected cells were cultured over protracted periods and culture fluids were monitored by RT assay. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control. (B) Growth curves of reverted SD2 viruses in C8166 cells.

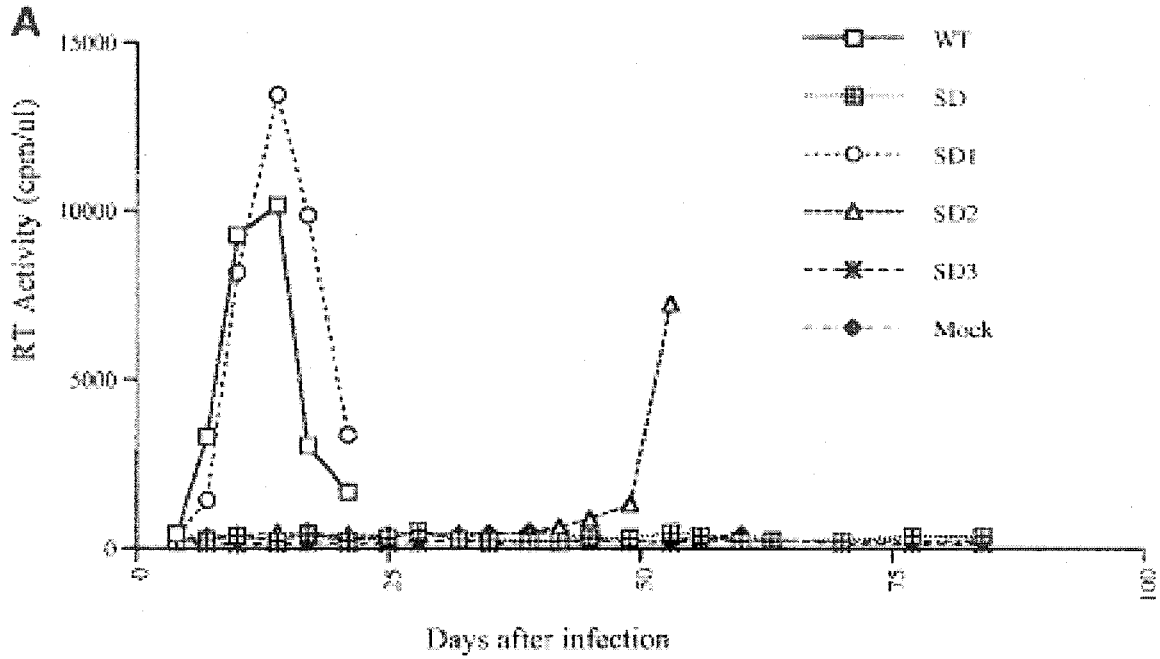

A
RT Activity (cpm/ul)
15000
10000
5000
0
0
25
50
75
100
Days after infection
WT
SD
SD1
SD2
SD3
Mock

Figure 3.6 (A)

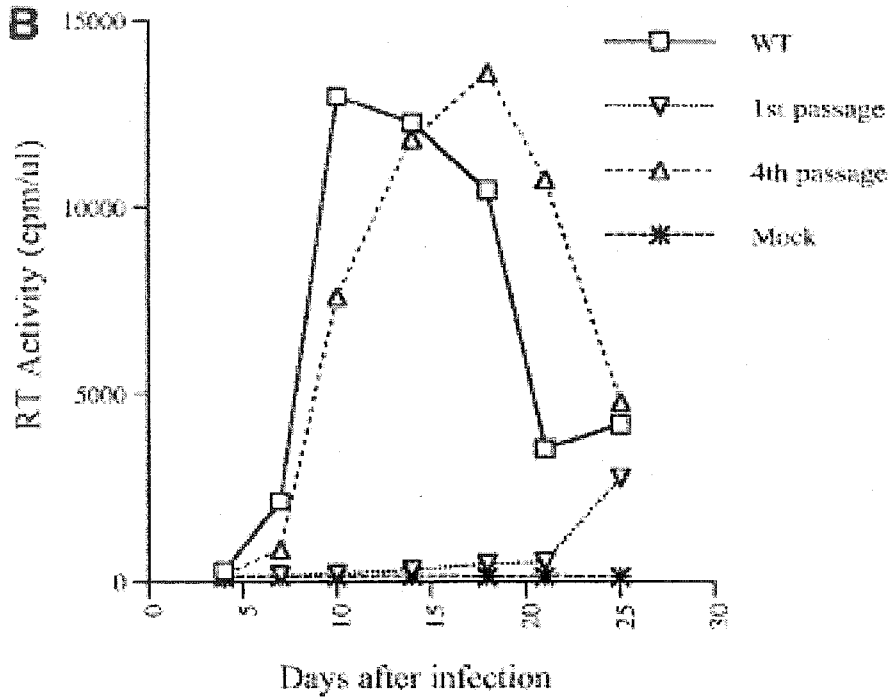

B
15000
10000
5000
0
RT Activity (cpm/ul)
0
5
10
15
20
25
30
Days after infection
WT
1st passage
4th passage
Mock

Figure 3.6 (B)

Fig. 3.7 Locations of the point mutations M1, M2, CA1 and Mp6 within the SIV genome, as indicated by asterisks. The substitutions observed are as follows: M1, T-+310 to C within the PBS; M2, A-+423 to G within the loop of the DIS; CA1, Lys-197 to Arg within CA; Mp6, Glu-49 to Lys within p6. Letters in bold indicate the original bases and amino acids as well as the mutations. The primer binding site (PBS) and the putative dimerization initiation site (DIS) are indicated. Sequences that were deleted in SD2 are underlined.

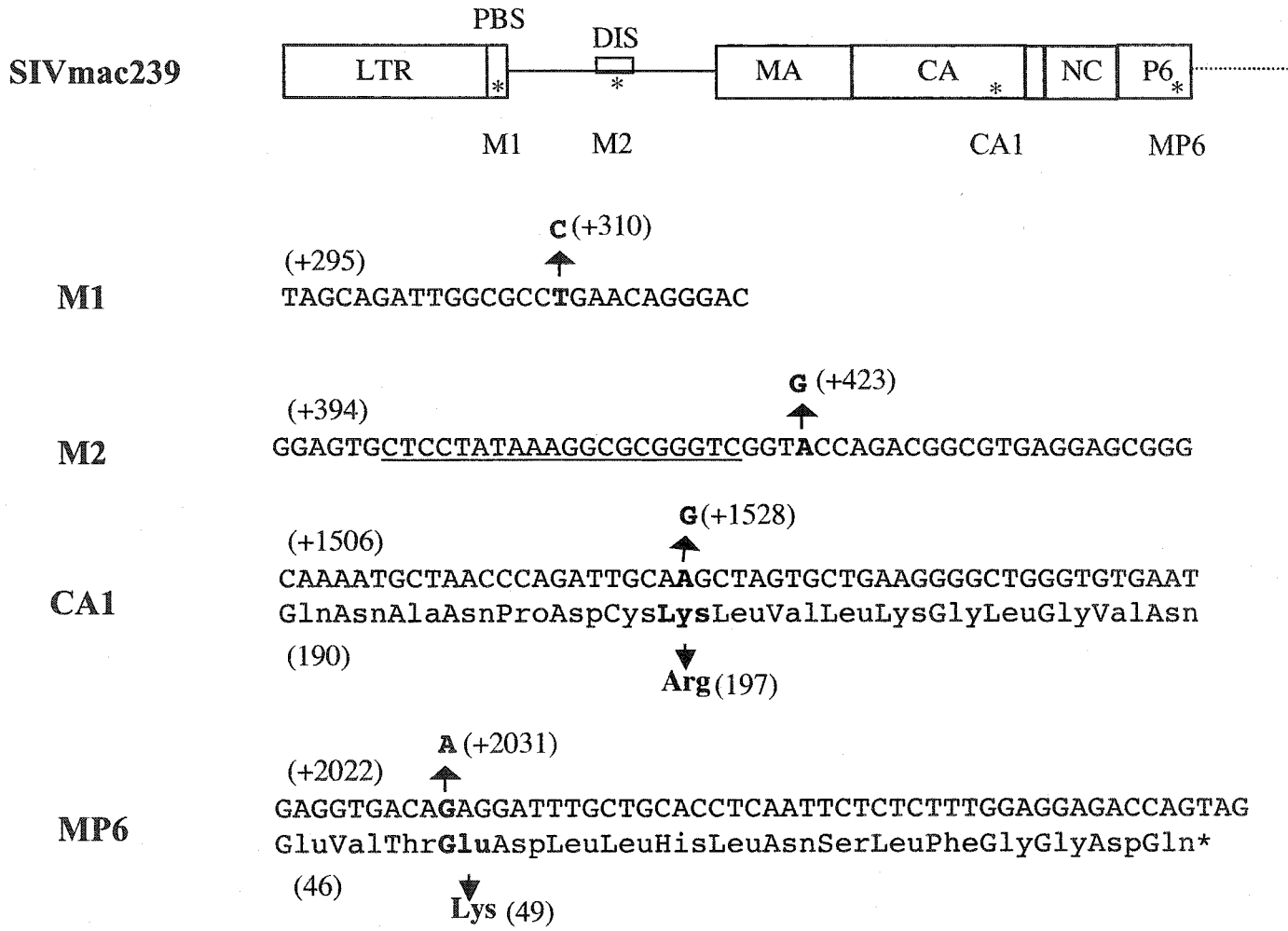

SIVmac239
PBS
DIS
LTR
MA
CA
NC
P6
M1
M2
CA1
MP6
C (+310)
(+295)
M1
TAGCAGATTGGCGCCTGAACAGGGAC
G (+423)
(+394)
M2
GGAGTGCTCCTATAAAGGCGCGGGTCGGTACCAGACGGCGTGAGGAGCGGG
G(+1528)
(+1506)
CA1
CAAAATGCTAACCCAGATTGCAAGCTAGTGCTGAAGGGGCTGGGTGTGAAT
GlnAsnAlaAsnProAspCysLysLeuValLeuLysGlyLeuGlyValAsn
(190)
Arg(197)
A (+2031)
(+2022)
MP6
GAGGTGACAGAGGATTTGCTGCACCTCAATTCTCTCTTTGGAGGAGACCAGTAG
GluValThrGluAspLeuLeuHisLeuAsnSerLeuPheGlyGlyAspGln*
(46)
Lys (49)

Figure 3.7

Fig. 3.8 Growth curves of reverted viruses in C8166 cells

Equivalent amounts of virus from COS-7 transfected cells were used to infect C8166 cells based on levels of p27 antigen(10 ng per 10^6 cells). Viral replication was monitored by RT assay of culture fluids. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control.

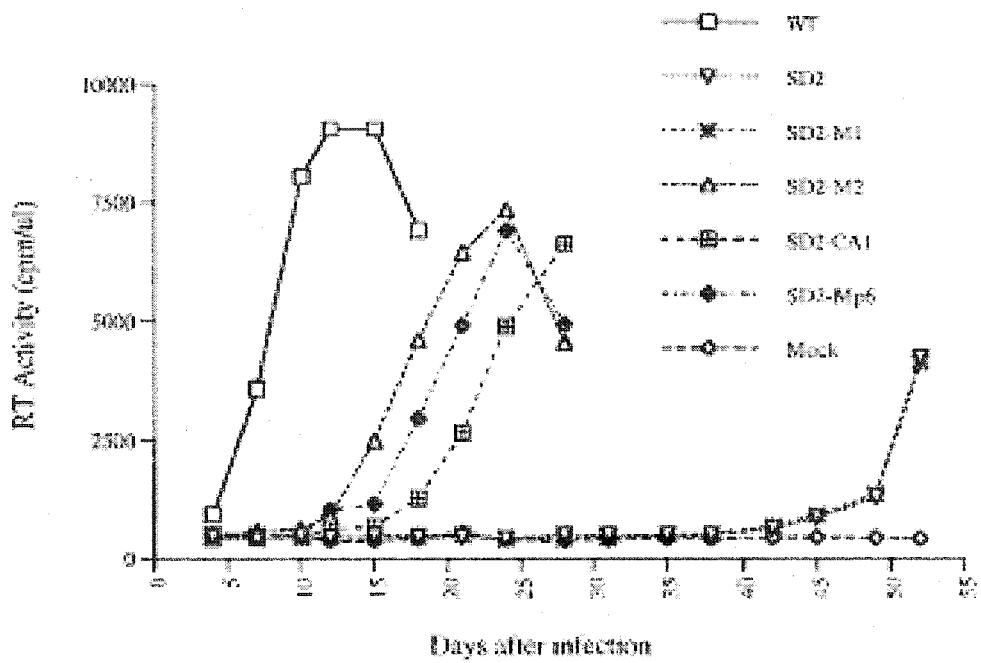

WT
SD2
SD2-M1
SD2-M2
SD2-CA1
SD3-Mp6
Mock
RT Activity (cpm/ml)
10000
7500
5000
2500
0
Days after infection

Figure 3.8

Table 3.1 Levels of p27 antigen expression in C8166 cells [a]

Viral construct	p27 concentration (ng/ml) at day				
	7	10	14	17	28
Wild-type	27.75	126.8	211.8	ND	ND
SD	0.20	0.12	0.17	0.17	0.05
SD1	29.05	125.0	179.2	151.4	ND
SD2	0.22	0.25	0.18	0.32	1.7
SD3	0.14	0.24	0.18	ND	0.06

[a] C8166 cells were infected with various viral constructs (10ng), and p27 levels in culture fluids were measured using a SIV p27 antigen capture assay. ND, not detected.

Table 3.3 Levels of p27 antigen expressed by transfected COS-7 cells

Virus	p27 (ng/ml)
Wild-type	46.8 ± 12.1
SD	46.1 ± 13.8
SD1	46.3 ± 13.5
SD2	43.1 ± 13.8
SD3	42.4 ± 13.8

Chapter 4

A Dual Role for Leader Sequences of Simian Immunodeficiency Virus in RNA packaging and Dimerization.

This chapter is an adaptation from an original paper submitted to *Virology*. 2003; Authorship is as follows: James B. Whitney, Maureen Olivera and, Mark A. Wainberg. All data present in this chapter were done by the candidate, with the exception of RT assays completed by M. Olivera. Assistance in the manuscript preparation was given by Dr. Wainberg.

4.1 Preface

The major focus of this chapter was to extend the findings of the previous chapter, in analysis of the downstream region, however this analysis included the foregoing region in regard to its role in RNA dimerization. The series of mutants removed various regions from the distal region of SL1 to the Kozak consensus of *gag*. The regions having the greatest impact on packaging also had comparably large negative impacts on dimerization and incurred larger deficits to infectivity. These result imply that RNA packaging and dimerization may be linked processes.

4.2 Abstract

We have constructed and analyzed a series of deletions in the simian immunodeficiency virus (SIVmac239) leader sequence from downstream of the primer binding site (PBS) to the initiation codon of *gag*, in order to determine the importance of these regions in each of packaging and dimerization of genomic RNA, viral replication, and Gag expression. Regions between stem loop 1(SL1) and Gag are important for the efficiency and specificity of viral genomic packaging. In contrast to previous results of *in vitro* studies, large deletions adjacent to the PBS did not impair RNA dimerization. Deletion of regions that included the 3' portion of SL1 significantly reduced both packaging of genomic RNA and disrupted RNA dimerization. Downstream regions between the splice donor and the Gag initiation site of SIV are also involved in RNA packaging but to a lesser extent than is true for equivalent sequences in HIV-1. These data suggest that the primary encapsidation determinant of SIV is SL1 and that downstream structures serve as secondary determinants in this regard. Each of the regions implicated in SIV packaging also plays a role in RNA dimerization, signifying a potential linkage between the two processes.

4.3 Introduction

The RNA genome in all retroviral lineages characterized thus far exists in the form of a linked duplex composed of two full-length RNA molecules. The exact nature of the linkage interaction has not been completely established, but is presumed to be facilitated at a post-transcriptional point by the presence of non-covalent interactions with the terminal palindromic loop of SL1 (a region which is termed the dimerization initiation site or DIS in the case of HIV-1). Considerable evidence from model structures of HIV-1 suggests that SL1 is sufficient for dimerization *in vitro*. Moreover, the rate of the dimerization *in vitro* is greatly enhanced by the presence of this stem-loop structure (Darlix *et al.*, 1990, 1992). However, SL1 has been shown to be partially dispensable *in vivo,* indicating that intravirion dimerization is likely facilitated by multiple or redundant contact points throughout the genome. In regard to human inmunodeficiency virus type 2 (HIV-2), there is disagreement as to the mechanisms that control dimerization and additional structures have been proposed for the *in vitro* determinants of dimerization in this case (Dirac *et al.*, 2001, 2002, Jossinet *et al.*, 2001, Lanchy *et al.*, 2002, 2003).

The encapsidation by retroviruses of two full-length copies of viral genomic RNA relies on viral RNA interactions with the Gag protein. This association is mediated by *cis*-acting functions that localize to specific secondary structures, located predominantly in the 5' untranslated region or leader sequence of the viral genome (Aldovini and Young 1990, Berkowitz *et al.*, 1996, Rein 1994). Although the selective incorporation of retroviral genomic RNA into nascent virions has been extensively studied in a variety of retroviral systems, the identification of all relevant packaging signals in complex retroviruses is still incomplete. This is largely due to the dispersion of these elements and, as well, the likelihood of functional redundancy among at least some of them (Berkowitz *et al.*, 1996, McBride *et al.*, 1996). These determinants also display strict dependence on spatial orientation and tertiary conformation (Dirac *et al.*, 2002, McBride *et al.*, 1997).

In the case of human immunodeficiency virus type 1(HIV-1), the major regions involved in encapsidation of viral RNA have been localized to stem loop 1 (SL1) and to regions downstream of the major splice donor (SD), which include those structures that make up stem loop (SL4) of the *gag*-coding region (Harrison *et al.*, 1998). Efficient encapsidation

in this case is dependent on both RNA structure and concentration. RNA interaction with multiple domains of the viral nucleocapsid (NC) protein, in the context of the Gag precursor, plays a key but not exclusive role in this process (Bacharach and Goff 1998, Kaye and Lever 1999, Shehu-Xhilaga *et al.*, 2002).

Similar secondary structures have been predicted for all leader regions of the 5' untranslated regions (UTRs) of simian immunodeficiency virus (SIV), HIV-2, and HIV-1, even though variations in sequence are evident, Indeed, HIV-1 can package genomic RNA of either HIV-2 and SIV (Kaye and Lever 1999, Rizvi and Panganiban 1993), even though the reverse is not true, pointing to important differences in the mechanism of selection of genomic RNA species (Arya and Gallo 1996, Dorman and Lever 2000, Kaye and Lever 1999, Kaye and Lever 1998). Several reports have localized the major *cis*-determinants of HIV-1 to be downstream of the major SD and have attributed packaging specificity to the p2 region of gag (Kaye and Lever 1998).

In HIV-2, the major packaging locus or Ψ core is located upstream of the SD (Browning *et al.*, 2001, Garzimo-Demo *et al.*, 1995, McCann and Lever1997, Negre *et al.*, 2002, Poeschla *et al.*, 1998). Here, as well, RNA-protein interactions with the NC domain of Gag appear to play a pivotal role in packaging, but the level of selection may be temporally associated with the translation of viral genomic RNA (Damgaard *et al.*, 1998, Griffin *et al.*, 2001, Gorelick *et al.*, 1999, Yovandich *et al.*, 2001).

Our group has described a region within the SIV leader located between the primer binding site (PBS) and the leftmost portion of SL1, and has shown that deletions within this region have a negative impact upon viral replication and RNA encapsidation (Guan *et al.*, 2000). However, no other regions within the SIV leader expanse were examined, and the importance of these other regions on SIV RNA dimerization has never been elucidated.

In the present study, we used non-denaturing northern analysis and scanned the first 95 nt downstream of the PBS in the SIVmac239 leader. We have extended our mutational analysis of the SIVmac239 leader sequence to include the region from the right side of the DIS stem loop to the AUG initiation codon of Gag, thereby including regions on either side of the major SD. We now provide evidence *in vivo,* that the putative DIS of SIV localizes downstream of the PBS and that RNA dimerization is controlled by

elements within SL1. We have identified several regions that impact on viral RNA packaging efficiency, specificity, and dimerization. We have also related deficits in packaging and genomic dimerization to overall viral infectivity and replication capacity, as well as to viral core morphology.

4.4 Materials and Methods

4.4.1 Construction of mutated provirus

We have used a PCR-based mutagenesis method and conventional cloning techniques to generate deletions; Pfu polymerase was used in all PCR cloning reactions. The full-length infectious clone of SIV, SIVmac239/WT was used as a template to construct all deletion mutants. Briefly, the region between the *Nar I* and *BamHI* sites in SIVmac239/WT was replaced by PCR fragments to generate mutant constructs (Figure 1). For construction of the PE1, PE2, and PE3 mutants, primer pairs PE1/pSgag, PE2/pSgag1, and PE3/pSgag1 (Table 1) were used to generate deletion fragments that were purified, digested with KpnI/BamHI, and ligated with a corresponding EcoRI/BamHI fragment from the WT vector. The resulting fragment was digested with EcoRI and BamHI and inserted into the WT SIVmac239 clone. For the mutants PE 4 to PE11, a first round PCR was performed using consecutive primers paired with the primer SU5. The resultant fragments were used as mega-primers paired with the Sgag1 primer to generate the required deletion fragments that were then used to replace the NarI/BamHI fragment from the WT clone. The validity of all constructs was confirmed by sequencing. Nucleotide designations are based on published sequences; the transcription initiation site corresponds to position +1.

4.4.2 Cell culture and preparation of virus stocks. Both 293T and COS-7 cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum. All media and sera were purchased from GIBCO Inc. (Burlington, Ontario, Canada). CEMx174 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Monkey peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy rhesus macaques (*Macaca Mulatta*), purchased from L.A.B. pre-clinical research international Inc., Montreal, Quebec. All primates were housed in accordance with accredited laboratory care standards. All donor

macaques were serologically negative for simian type-D retrovirus 1 (SRV-1), simian T-cell lymphotrophic virus type 1(STLV-1), and simian foamy virus (SFV-1).
Ficoll purified monkey peripheral blood mononuclear cells (PBMC) were phytohemagglutinin (PHA) stimulated for 3 days, then maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 20 U/ml IL-2. Molecular constructs were purified using a Maxi Plasmid Kit (QIAGEN Inc. Mississauga, Ontario, Canada). COS-7 cells were transfected using the above constructs with lipofectamine-plus reagent (GIBCO, Burlington, Ontario, Canada). Virus-containing culture supernatant was harvested at 48 h post-transfection and clarified by centrifugation for 30 min at 4°C at 3,000 rpm in a Beckman GS-6R centrifuge. Viral stocks were passed through a 0.2μm filter and stored in 0.5ml or 1 ml aliquots at –70°C. The concentration of p27 antigen in these stocks was quantified using a Coulter SIV core antigen ELISA assay (Immunotech Inc., Westbrook, ME, U.S.A.).

4.4.3 Virus replication in CEMx174 cells and macaque PBMCs. To initiate infection, viral stocks were thawed and treated with 100 U of DNase I in the presence of 10 mM $MgCl_2$ at 37°C for 0.5 h to eliminate any residual contaminating plasmid DNA prior to inoculation of cells. Infection of CEMx174 cells was performed by incubating 10^6 cells at 37°C for 2 h with 10 ng of virus p27 antigen equivalent. Infected cells were then washed twice with phosphate-buffered saline and resuspended in fresh supplemented RPMI-1640 medium. Cells were split at a 1:3 ratio twice per week. Supernatants were monitored for virus production by reverse transcriptase
(RT) assay.Virus infectivity ($TCID_{50}$) was determined by infection of CEMx174 cells and results were calculated by the method of Reed and Muensch (Dulbecco 1998).
Virus replication was also determined in rhesus PBMCs. Briefly, $4x10^6$ activated rhesus macaque PBMCs were infected with SIV stocks containing 10ng of p27 viral equivalent at 37°C for 2 hours; the cells were then washed extensively to remove any remaining virus. Cells were maintained in 2 ml of culture medium as described above, and fresh stimulated PBMCs were added to the cultures at weekly intervals. Virus production in culture fluids was monitored by both RT assay and SIV p27 antigen capture assay (Coulter Immunotech Inc. Westbrook, ME, U.S.A.).

4.4.4 Radio-immune precipitation assay (RIPA) of viral proteins.

Metabolic labelling of nascent viral proteins was accomplished by first transfecting either 293T cells or COS-7 cells with either wild type or mutant constructs. At 20 hours post-transfection, cells were serum starved at 37°C for 30 min in DMEM medium lacking methionine (Met) and cysteine (Cys). Radiolabeling was performed with a ^{35}S-Met and ^{35}S-Cys pulse, at a concentration of 100 μCi/ml of each radiolabelled amino acid, for 30 min at 37°C. Then, the cells were thoroughly washed with complete DMEM and cultured for 1 hr in fully supplemented media. Culture fluids were collected and clarified using a Beckman GS-6R bench centrifuge at 3,000 rpm for 30 min at 4°C. Viral particles were further purified through a 20% sucrose cushion at 40,000 rpm for 1 hour at 4°C using a SW41 rotor in a Beckman L8-M ultracentrifuge. Virus pellets were suspended in 2 x SDS-PAGE loading buffer, denatured, then fractionated by 12% SDS-polyacrylamide gel electrophoresis and exposed to X-ray film. The labeled cells were washed twice with cold phosphate-buffered saline and lysed in buffer containing 0.1% NP40. Cell lysates were incubated with MAb directed against SIV p27 epitopes at 4°C for 1hr, and the resultant Ag-Ab complexes were precipitated with protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Montreal, Quebec, Canada).

4.4.5 Viral RNA packaging analysis by RNA slot-blot and RT-PCR.

For slot blotting experiments, RNA was isolated from virus recovered from transient transfection assays, clarified, and subsequently purified through a 20% sucrose cushion. Virus pellets were resuspended in TE buffer and digested with DnaseI to remove potential plasmid contamination. Sample RNA was purified and then analysed as per Northern analysis described below, using a Scheicher and Schuell minifold II slot blotting system. Probes were prepared by digestion and purification of the NdeI-BstIIE fragment to assess total virion RNA content, using a BstIIE double digestion fragment for genomic RNA. Probes were labelled by nick translation following standard manufacturer's protocols (Roche, Indianapolis, IN, USA).

For RT-PCR analysis, viral RNA was isolated using the QIAamp viral RNA mini kit (QIAGEN Inc.) from equivalent amounts of COS-7 cell-derived viral preparations, based on levels of SIV p27 antigen. Purified RNA samples were treated with RNase-free DNase

I at 37°C for 30 min to eliminate possible DNA contamination. The DNase I was inactivated by incubation at 75°C for 10 min. The viral RNA samples were quantified by RT-PCR, using the Titan One Tube RT-PCR system (Boehringer-Mannheim Inc., Montreal, Quebec, Canada). Two sets of primer pairs, sg1/sg2 and sU3/sU3-1, were used in a multiplex reaction,. Relative amounts of products were quantified by molecular imaging (BIO-RAD, Toronto, Ontario, Canada). Levels of genomic RNA packaging were calculated on the basis of four replicate experiments with wild-type viral values arbitrarily set at 1.0.

4.4.6 Northern analysis of viral RNA.

Culture fluids from transfected COS-7 cells were collected and clarified using a Beckman GS-6R bench centrifuge at 3,000 rpm for 30 min at 4°C. Viral particles were further purified through a 20% sucrose cushion at 40,000 rpm for 1 hour at 4°C using a SW41 rotor in a Beckman L8-M ultracentrifuge. Viral pellets were dissolved in TE buffer and then in lysis buffer containing proteinase K(100 μg/ml) and yeast tRNA (100 μg/ml). Samples were incubated for 20 min at 37 °C, followed by two extractions in phenol: chloroform: isoamyl alcohol, then chloroform. Viral RNA was then precipitated, washed in 70% ethanol and stored at -80 °C until required, at which time samples were resuspended in TE buffer at 4 °C. Alternatively, for thermal stability assays, RNA preparations were suspended in TE containing 100 mM NaCl and heated at the temperatures described. RNA was then analysed by non-denaturing electrophoresis on 0.9% agarose gels in 1x TBE running buffer for 4 hrs at 4^0C. Products were subsequently denatured in 50 mM NaOH and equilibrated in 200mM Na-acetate. Following electrophoresis, RNA was blotted to Hybond-N nylon membranes by capillary blotting with 20x SSPE buffer. Membranes were baked for 2 hrs at 80 °C. Probes were prepared by digestion and purification of the NdeI-BstE III fragment from the WT plasmid. These were recovered and labelled by nick translation following standard protocols (Roche, Indianapolis, IN, USA).

The analysis of cellular RNA was accomplished by denaturing Northern analysis. Briefly, transfected COS-7 cells were washed twice with cold phosphate-buffered saline and lysed with NP-40 lysis buffer. The cellular RNA within lysates was incubated in the

presence of proteinase K (100 μg/ml), for 20 min at 37°C. RNA was further extracted in phenol/chloroform as described above. RNA was separated on 1% denaturing agarose gels containing 1x MOPS buffer and 18% formaldehyde was prepared according to Maniatis *et al.*, 1989. RNA samples were denatured in the presence of formamide and formaldehyde, prior to being loaded onto gels which were run at 100 V. Post-electrophoresis, the RNA was transferred onto nylon membranes by capillary blotting as described above. Prehybridization and hybridization were performed as for non-denaturing gels. Equivalent volumes of RNA were also run on 1% ethidium bromide (EtBr) stained gels as an internal control for total RNA and 28S and 18S ribosomal RNAs which are shown.

4.4.7 Electron Microscopic Analysis of Virion Morphology

Viral ultra-structure for the described mutant viruses was examined by transmission electron microscopy. Briefly, COS-7 cells transfected with wild-type or mutant SIV constructs were fixed 48 hours post-tranfection in 2.5% glutaraldehyde/phosphate buffered saline followed by a secondary fixation of lipids in 4% osmium tetroxide. Samples were routinely processed and serially dehydrated, and subsequently embedded in epon under vaccum. Thin-sectioned samples were stained with lead citrate and uranyl acetate and visualized at 80 KeV using a JEOL JEM-2000 FX transmission electron microscope equipped with a Gatan 792 Bioscan wide-angle 1024 x1024 byte multi-scan CCD camera.

4.5 Results

4.5.1 Replication in CEMx174 cells

Deletion of regions implicated in viral RNA packaging alters infectivity and viral spread. The CEMx174 hybrid line has been used extensively in our studies and provides a good system for standardized evaluation of SIV mutants. The results of Fig. 2A and B show that all mutants displayed some replication impairment as measured by RT assay. Specifically, the mutants PE1, PE2, and PE3, involving deletions upstream of the splice donor site (SD), showed severely impaired replication in the aforementioned cell line. Over 6 months of extended culture, both the PE1 and PE2 mutants failed to replicate

when monitored by RT assay, showing that deletions within this region may present an insurmountable barrier to recovery of productive viral replication. Although the PE3 mutant did show evidence of replication, this was delayed to approximately 50 days post-infection, prior to which results were consistently negative on the basis of RT assay. Conversely, both the PE4 and PE5 variants, involving deletions in nt regions +445-458 and nt +459-465, respectively, exhibited minor delays in viral replication. Interestingly, the impairment observed for the PE3 mutant, involving removal of the right side of the DIS stem (Δ+426-444), was considerably larger than that observed for previously described mutants (which involve similar disruptions to the left side of the DIS) in CEMx174 cells; these results are consistent with data on mutations in the SL1 of HIV-1 (Li *et al.*, 1997, Liang *et al.*, 1998).

In comparison to sequences proximal to the DIS, the elimination of sequences downstream of the splice donor (SD) site resulted in lesser delays in viral replication in the CEMx174 line. The most significant deficits in replication resulted from deletions that removed the entire sequence from just downstream of the SD to the gag initiation codon; i.e. PE6 (Δ+473-532) and to a lesser extent PE7 (Δ+481-531). Interestingly, smaller deletions spanning this region showed relatively insignificant alterations in replication kinetics, comparable to the replication of the PE 4 and 5 mutants. Of these intermediate deletions, only the PE9 (Δ +513-531) mutant showed more than a minor disruption in replication.

We also considered the infectivity of these mutants using the endpoint dilution method ($TCID_{50}$) in the CEMx174 cell line. The results of Fig. 2C show a diverse range of infectivities involving viruses that were deleted over the described nucleotide expanse. Deletions between nt +426 to +464 had the most dramatic effect on viral infectivity, resulting in an drop greater than 5 log for the mutants PE1, PE2 and PE3 compared with WT virus. By comparison, mutant PE4 showed modestly impaired viral spread, whereas PE5 was relatively unaffected, consistent with the cell culture results described above. In the region downstream of the SD, i.e. nt +473 to +532, the deletion mutants termed PE6 and PE7 were moderately diminished in replication capacity. Of the smaller deletions, PE

9 showed depressed infectivity while the others, in general, showed only minor differences in this regard.

4.5.2 Replication in PBMCs

We also evaluated the impact of these deletions on viral replication capacity in rhesus PBMCs by monitoring the production of SIV capsid protein (p27) in culture supernatants. The results of Fig.3A show that PE1 and PE2 were the most adversely affected, while PE3 was also significantly compromised. The PE4 and PE5 mutants were not significantly affected.

Impairments in regard to deletions downstream of the SD site were most apparent in mutants PE 6 and PE 7; the deficit seen in primary cells was similar to that observed in the CEM line (Fig. 3B). For the more minor deletions, PE8 - PE11, smaller alterations in viral replication were noted and similar data were obtained in both cell types.

4.5.3 Expression of Gag from mutant viruses impairs $Pr55^{gag}$ proccessing but not RNA encapsidation.

We next investigated the impact of our deletions on the expression and processing of Gag precursor proteins by pulse-chase labeling experiments in both COS-7 and 293T cells. Immunoprecipitation of solubilized viral proteins was accomplished with anti-p27 CA Mabs. The viral proteins recovered from cytoplasmic lysates were analyzed by SDS-PAGE. Total viral cytoplasmic proteins were quantified by densitometry, and, for each virus, the percentage of protein product was compared to total protein. The results show relatively comparable band representation in all cases compared to wild-type, indicating that the expression of Gag in these mutants (deletions within the nt region +426 to +464) was not limiting in terms of overall precursor availability (Fig.4 A and B). However, variations in efficiency of processing were seen in the case of the protein intermediate for the mutants between SL1 and SD. Similarly, in the downstream region, i.e. those mutations encompassing the SD to the gag initiation codon (nt +473 to +532), relevant constructs exhibited comparable levels of Gag expression, with some deviations seen in band representation of intermediate processing products (Fig. 4C and D).

^{35}S- labeled viral progeny recovered from culture supernatants were also analyzed via SDS-PAGE. Labeled protein bands representing the viral structural proteins included the Gag precursor Pr 55 (band 1), Gag intermediate p40 (band 3), immature p28 (band 6) and the mature capsid p27 (band 7). The remaining three bands have molecular masses of 43 kDa (band 2), 36 kDa (band 4), and 34 kDa (band 5), respectively, and are presumed to represent Gag intermediate proteins in immature viruses as shown in Fig. 4. Again, total viral proteins were quantified by densitometry. For each virus, the percentage of each band was compared to total values. The results indicate comparable band representation in all mutants as compared to WT (Fig. 4E through H). However, all mutants displayed a clear alteration in the mobility of the p43/40 and p36/34 intermediate processing products, as shown in Fig.4E and 4G for the mutants encompassing the nt regions +426-464 and +473-532, respectively.

4.5.4 RNA structural domains within nucleotide regions +426 to +465 and +473 to +532 regulate both the efficiency and specificity of SIV RNA packaging.

We have previously shown that regions of the SIV leader sequence, downstream of the PBS, are involved in the packaging of the RNA genome (Guan *et al* 2000, 2001b). To more fully evaluate this topic, we constructed a series of deletions that span the distal 3' leader region and assessed packaging efficiency through slot-blotting experiments. The results of Fig. 5A, indicate that the most dramatic reduction in packaging efficiency was seen with the constructs that disrupted regions near or including the DIS stem (i.e. mutants PE1, PE2, and PE 3). This effect was localized to the rightmost portion of SL1 removed in PE3 (Fig 5A. panel II). Furthermore, there was no apparent shortage of available RNA (Fig 5A, panel III), since each of the mutants incorporated significant amounts of spliced viral RNA (Fig 5A. panel I), and northern analysis showed comparable levels of intracellular spliced products for all mutants as compared to wt (Fig 6); this indicates a loss of ability to discriminate between full-length and genomic RNA. The elimination of large sequences downstream of the SD (i.e. PE6 and PE7) also resulted in impaired packaging, which was further localized to the distal SL encoding the SD and SL3. In the case of HIV-1, there is controversy as to whether the structures that make up

stem loop (SL4) of the *gag*-coding region are also involved in RNA encapsidation (Harrison *et al.*, 1998, McBride *et al.*, 1997).

Relative packaging efficiencies are shown as a percentage of WT values in Fig.5B and show that the amount of viral RNA incorporated into virions was generally conserved. These experiments also indicate that there is limited complementarily of other sites in the absence of the major SL1 determinant, and that these other sites acts in a roughly additive fashion.

In consideration of previous work by our group and others on this topic (Griffin et al., 2001, Guan *et al.*, 2001a), we also considered the impact of these deletions on the specificity of RNA encapsidation by quantifying and comparing genomic and total virion ratios in RNA slot-blotting experiments, shown in Fig. 5 B. These experiments were confirmed in a multiplex RT-PCR reaction (as described in Materials and Methods), and gave comparable results to RNA slot blotting.

We also considered intracellular viral RNA concentrations to ensure that packaging deficits were not the result of diminished cytoplasmic pools of viral RNA. Total RNA was recovered from transfected cellular lysates and normalized on the basis of p27 capsid Ag present in the lysate. Experiments used the same full-length genomic probe as in slot-blotting analysis (see Materials and Methods). The cytoplasmic viral RNA levels in each case did not substantially deviate from those observed with WT virus. Any minor deviations observed during replicate experiments were presumed to result from minor variations in transfection efficiency.

4.5.5 The *in vivo* SIV dimerization initiation site localizes to SL1.

In vitro work from other groups has implicated regions upstream of SL1 as capable of initiating dimerization. To resolve this issue, we studied three mutants from previous studies i.e. SD1, SD2, and SD3, that involve the removal of nucleotide regions +322 – 344, +398- 418, and +345-397, respectively (Guan *et al.*, 2000, Whitney *et al., 2002*). Of particular interest is the from nt +322 to +344, and purified RNA from this mutant showed no qualitative differences in RNA dimerization compared to wild-type virus in either its native form or as heat- denatured controls (Fig.7 A and B).

We also scanned the entire nucleotide region from downstream of the PBS to the start point of Gag translation. Fig.6A shows the relative contribution of nt sequences upstream of the major SD; in brief, disruption of the region encoding the rightmost side of the DIS stem impaired dimerization. Each of the PE 1- PE3 mutants showed no capacity for dimerization, and reductions in the presence of monomeric species were also observed, consistent with the packaging results. Several of the intermediate deletions (PE4 and PE5) were capable of limited dimer formation, indicating that the specific determinants that regulate this are located close to or within the SL1.

Similarly, analysis of regions downstream of the major SD showed that only the largest deletions, i.e. PE6 and PE 7, disrupted dimerization (Fig 6B). In contrast, smaller intermediate deletions within this region did not significantly alter this process, with the exception of PE9, which consistently showed a decrease in the amount of packaged dimer.

We also determined whether the mutants that did not impair dimerization might nonetheless affect the overall stability of the RNA duplex in a manner similar to HIV-1 for the 5' DLS and 3'DLS (Sakuragi and Panganiban 1997). Toward this end, thermal analysis of our mutants was accomplished using a gradient of temperatures (Fig. 7). The results indicate that only the PE4 mutant altered dimer stability, whereas all of the mutants located downstream of the SD retained melting profiles similar to wild-type, suggesting that regions on both sides of the major splice donor can contribute to genomic dimerization.

4.5.6 Virion Morphology

To characterize the effect of deletions within the leader regions on particle formation and viral maturation, COS–7 cells were transfected with WT and mutant constructs. Particles were fixed 48 hrs post transfection in 2.5% glutaraldehyde and processed as described in Materials and Methods. Examination of particles showed that the WT construct (Fig.8A), produced particles of typical morphology and dimensions (≈100 nm, average) and condensed conical cores were observed in >85% of particles with the remainder being of immature morphology. In contrast, neither mutant produced virus in significant quantities, nor with morphology comparable to WT. Most common were viruses

containing either condensed but pleomorphic cores or condensed cores that were laterally displaced (Fig. 8B PE3, Fig. 8C PE6).

4.7 Discussion

In the current study, we have introduced mutations into the leader sequence of SIVmac239 and have demonstrated that leader regions located on both sides of the major SD are necessary for efficient viral replication. Our data indicate that the major blocks to replication are a result of deletions that impair RNA packaging and dimerization, but are not limited to such, with ensuing effects on other viral processes.

RNA packaging

Packaging of viral RNA was determined by several independent methods, i.e. RNA slot-blotting, and RT-PCR. Our results indicate that the primary *cis*-encapsidation determinant is SL1, as this was associated with the greatest impairment of packaging compared to all other regions. Although flanking regions of SL1 may also be involved, this is unlikely since smaller deletions, proximal to SL1, had little impact on RNA packaging. Moreover, broad comparisons between HIV-1 and SIV showed that sequence-specific effects can be localized to SL1 and other studies have shown that SL1, and in particular the bulge on the 5'side stem of SL1, can play a critical role in packaging (Lawrence *et al., 2003*). The regions we have deleted resulted in dramatically larger deficits and also affected other functions, similar to HIV-1 (Liang *et al., 1998*). These mutants displayed severely diminished replication capacity in comparison to deletions that removed the left side of the DIS stem, harboring the bulge sequences in both SIV and HIV-1. The PE3 mutant showed an approximate 90% drop in packaged genomic RNA, but the comparative decrease in infectivity was more than100 fold lower (i.e. 5 logs).

We also investigated the role of the nt region downstream of the SD with respect to RNA packaging. This area appears to have a defined role in RNA encapsidation, albeit a secondary one relative to the upstream SL1. The most dramatic defects in packaging were observed when the entire terminal 3' leader sequence was deleted in PE6, or very nearly so in PE7. However, decreases in packaging specificity as a result of the largest deletions

can be equivalently ascribed to smaller mutations disrupting SL3 or the SD. Even large deletions in SIV failed to completely abrogate genome packaging, in contrast to murine viral elements (Berkowitz *et al.*, 1996). This suggests a multipartite arrangement in SIV or some level of redundancy in packaging elements (Berkowitz *et al.*, 1996, McBride and Panganiban 1997, Patel *et al.*, 2003). Our experiments indicate limited complementarily of other sites in the absence of the major SL1 determinant, and that these secondary sites act in a roughly additive fashion. Further RT-PCR experiments confirmed our slot-blotting data that show that structures within the deleted regions contribute to the specificity of genome incorporation. Potentially, several functional domains may act in concert and this notion is plausible, since region also functions in SIV as an internal ribosomal entry site (IRES) *in vitro* (Ohlmann *et al.*, 2000). In addition, our group has reported that insertion of a poliovirus IRES element upstream of the *env* reading frame increased overall encapsidation efficiency (Guan *et al.*, 2001a). This idea is also supported by studies on the canonical IRES of poliovirus, suggesting that common mechanisms may be employed in both cases (Johansen and Morrow 2000a, Johansen and Morrow 2000b, Martinez-Salas *et al.*, 2001, Nugent *et al.*, 1999). Furthermore, in the case of HIV-2, a co-translational mechanism of RNA packaging has been proposed, whereby RNA, in the appropriate conformation, encounters nascently translated Gag precursors within the ribosomal translation complex, thereby increasing the specificity of incorporation (Griffin *et al.*, 2001). Phylogenetically, HIV-2 is closely related to SIV, and therefore it is likely that a similar mechanism is employed here.

In our mutants, the increased incorporation of RNA subspecies indicates a loss of specificity for recognition of full-length viral transcripts. Conceivably, certain deletions may genetically unlink *cis*-encapsidation to a degree that also supports efficient *trans*-encapsidation. This would result in a commensurate loss of packaging specificity, but could be offset by an increase in overall encapsidation efficiency, i.e. counteracting *cis*-packaging defects, e.g. PE5, PE8. An additional possibility is that the high proportion of incorporated RNA subspecies (e.g. PE10, PE11) may yield a replicative advantage, either by maintaining core morphology, or in early replication though the action of independent

RNA products such as Tat. Other studies have indicated a structural role for RNA of viral or host origin in virion morphology (Muriaux *et al.*, 2001, Wang *et al*, 2002).

RNA Dimerization

In regard to RNA dimerization in SIV, non-denaturing Northern analysis of our mutants revealed that dimerization *in vivo* is associated with SL1 or the putative DIS. Deletion of regions downstream of the SD also disrupted dimerization but the nature of the mechanism(s) involved is less clear. In HIV-1, each of *ex vivo* and *in vivo* studies in conjunction with electron microscopy have shown that the genomic regions involved in dimerization are located immediately proximal to the major splice donor (Clever *et al.*, 1996, Hoglund *et al.*, 1997, Laughrea and Jette 1994, Skripkin *et al.*, 1994). The relative paucity of packaged RNA seen in all mutants that disrupted either dimerization or the SL1 indicates that the deleted regions may be integral to packaging.

We also analyzed our panel of mutants to determine if any of the regions under study facilitate the formation of a mature extended dimer/DLS complex (Darlix *et al.*, 1990, Fu and Rein, 1993). We found that the thermal stability of the mutant that deleted, what we termed the DLS (Fig. 1), was compromised (i.e. PE4), as it influenced the maturation state and stability of RNA extracted from viral supernatants. The function of this sequence may be to assist the transition from a kissing-loop (KL) dimer to an extended, more stable conformation (.i.e. a DLS like structure). This may be a result of pugga repeats found within this structure that coincide with similar motifs in other retroviruses. Whether the decreased replication kinetics of PE4 observed in cell cultures is due to this altered biophysical profile is unknown. The fact that PE 9 exhibits a comparable melting profile to the WT dimer (not shown), yet involves a similar replication delay to that observed with PE4, suggests that dimer integrity alone may not be a critical factor in replicative efficiency. In HIV-1, a region analogous to the proposed SIV IRES, downstream of the SD, has been purported to act as a dimer linkage structure or 3' DLS (Darlix *et al.*, 1990); it is thought that this region promotes dimer stability in the context of the mature virion.

We have also evaluated the impact of these mutations on protein expression and processing of $Pr55^{gag}$ and shown that these genomic ablations did not disrupt precursor expression or processing to the same degree as reported in HIV-1 (Shehu-Xhilaga *et al.*,

2001, Liang *et al., 2000*). Although differences in the mobility of intermediate processing products were seen in our analysis, this likely resulted from the altered RNA content and commensurate changes in protein conformation that may have affected accessibility of processing sites. The putative interaction of these RNA elements with their respective binding sites in $Pr55^{gag}$ may enable discrimination of genomic versus spliced RNA, resulting in specific incorporation. Of interest is also the benign effect of the PE3 mutation on $Pr55^{gag}$ processing, since a corresponding deletion on the left side of the DIS of HIV-1 impaired gag processing to a significantly greater extent (Guan *et al.,* 2001). In sum, the elimination of structures on either side of the SD in SIV yields a replication-impaired phenotype, which is partially attributable to a block in RNA encapsidation. As well, each of the more severely depressed replication phenotypes are associated with mutants containing only about 10% of WT genomic incorporation and no evidence of genomic dimers. These mutants displayed diminished viral spread and replication. Overall, virion morphology was disturbed by either core displacment or impaired preoteolytic maturation. Collectively, these data may reveal an apparent role of the elements that comprise the DIS/ kissing loop domain (KLD) at multiple points in the replicative cycle.

Ongoing studies within our laboratory involve the analysis of two revertants, PE3 and PE6, as a result of serial passage in CEMX174 cells. These experiments will shed light as to the specifics of RNA-protein interaction in the packaging and maturation process. In parallel with HIV-1, it is also probable that accessory packaging elements exist elsewhere within the untranslated leader region and possibly within other coding regions as well (Berkowitz *et al.,* 1995, Richardson *et al.,* 1993). For instance, the presence of a functional intron within the SIV/HIV-2 R-U5 region of the UTR, a novel element for lentiviruses, could afford an additional level of regulation of packaging of genomic length RNA (Poeschla *et al.*, Viglianti *et al.,* 1992). The presence of packaging determinants within coding regions has also been established for other retroviruses. Additional studies will help to resolve whether dimerization and packaging are linked in SIV/HIV-2 or whether they exist as independent functions.

Fig. 4.1 Proposed RNA secondary structure and position of nucleotide deletion mutations in SIV.

(A). Denotes the size and exact nt position of packaging element (PE) deletion mutations. All nt positions are relative to the transcriptional initiation site (1+) based on the sequence of the WT clone of SIVmac239.

(B). Secondary structure of SIVmac239 leader RNA, as predicted by free energy minimization, was adapted from published information (Guan *et al.*, 2000, Zuker 1989). All hairpin motifs are labeled according to their putative function and/or after comparable elements encoded by HIV-1/HIV-2 leader sequences. The following motifs are shown in bold type: the putative DIS palindrome at position +419, the splice donor (SD) at position +467, and the Gag initiation codon at position +535.

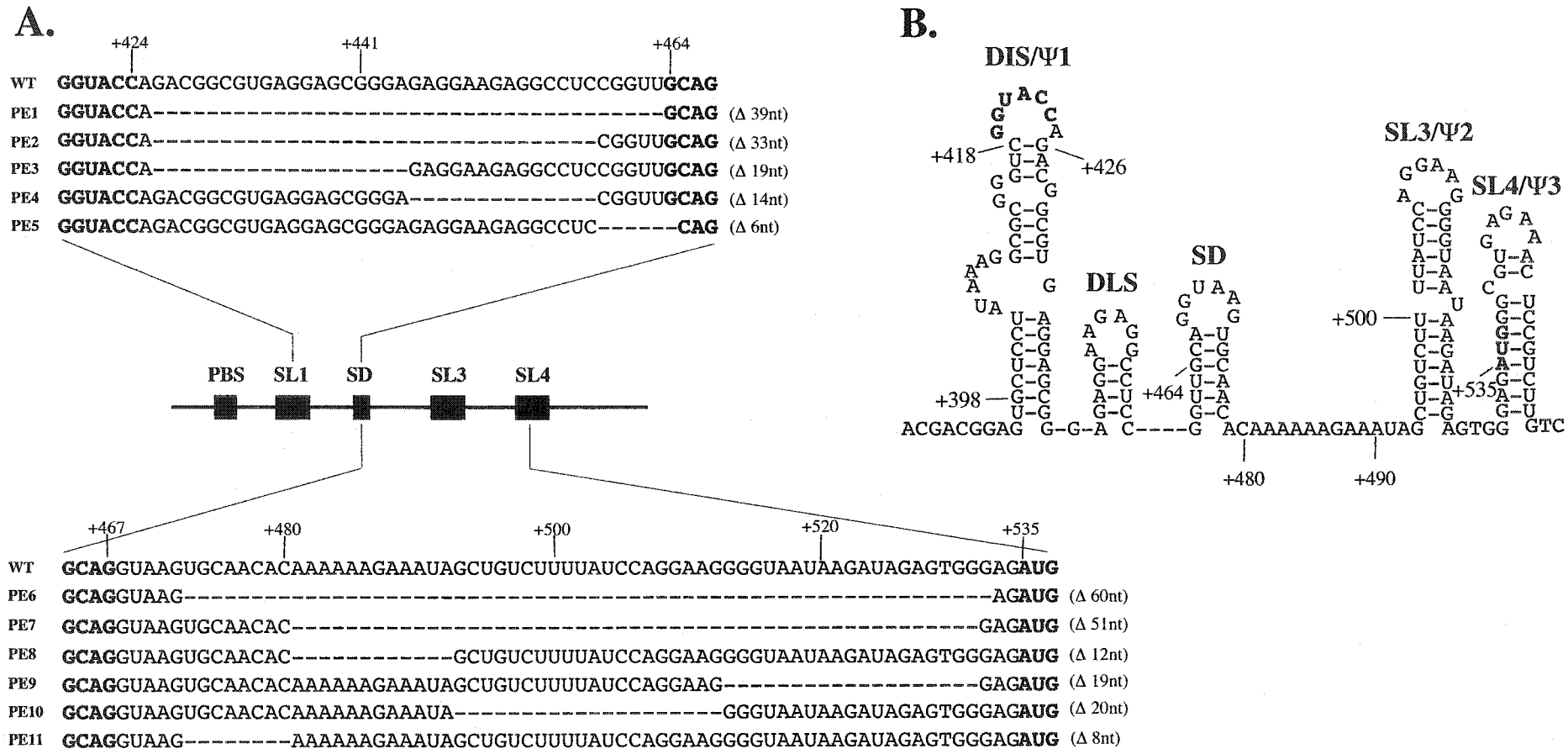

A.
+424
+441
+464
WT GGUACCAGACGGCGUGAGGAGCGGGAGAGGAAGAGGCCUCCGGUUGCAG
PE1 GGUACCA----------GCAG (Δ 39nt)
PE2 GGUACCA----------CGGUUGCAG (Δ 33nt)
PE3 GGUACCA----------GAGGAAGAGGCCUCCGGUUGCAG (Δ 19nt)
PE4 GGUACCAGACGGCGUGAGGAGCGGGA----------CGGUUGCAG (Δ 14nt)
PE5 GGUACCAGACGGCGUGAGGAGCGGGAGAGGAAGAGGCCUC------CAG (Δ 6nt)
PBS
SL1
SD
SL3
SL4
+467
+480
+500
+520
+535
WT GCAGGUAAGUGCAACACAAAAAAGAAAUAGCUGUCUUUUAUCCAGGAAGGGGUAAUAAGAUAGAGTGGGAGAUG
PE6 GCAGGUAAG----------AGAUG (Δ 60nt)
PE7 GCAGGUAAGUGCAACAC----------GAGAUG (Δ 51nt)
PE8 GCAGGUAAGUGCAACAC----------GCUGUCUUUUAUCCAGGAAGGGGUAAUAAGAUAGAGTGGGAGAUG (Δ 12nt)
PE9 GCAGGUAAGUGCAACACAAAAAAGAAAUAGCUGUCUUUUAUCCAGGAAG----------GAGAUG (Δ 19nt)
PE10 GCAGGUAAGUGCAACACAAAAAAGAAAUA----------GGGUAAUAAGAUAGAGTGGGAGAUG (Δ 20nt)
PE11 GCAGGUAAG---------AAAAAAGAAAUAGCUGUCUUUUAUCCAGGAAGGGGUAAUAAGAUAGAGTGGGAGAUG (Δ 8nt)
B.
DIS/Ψ1
+418
+426
DLS
SD
+398
+464
+480
+490
SL3/Ψ2
SL4/Ψ3
+500
+535
ACGACGGAG G-G-A C----G ACAAAAAAGAAAUAG AGTGG GTC

Figure 4.1

Fig. 4.2 Replication kinetics of various mutant constructs in CEMx174 cells. Cells were infected with 10ng viral equivalents and viral replication was monitored by RT assay of culture supernatants at multiple time points. Mock denotes infection with heat-denatured WT virus. All replication experiments were conducted in triplicate. (A) Representative growth curves of viruses deleted between the DIS and the SD (i.e. +426-464). (B) Replication of viruses deleted between the SD and the Gag AUG (+473-532). (C) $TCID_{50}$ analysis of viral infectivity, scale of ordinate is logarithmic.

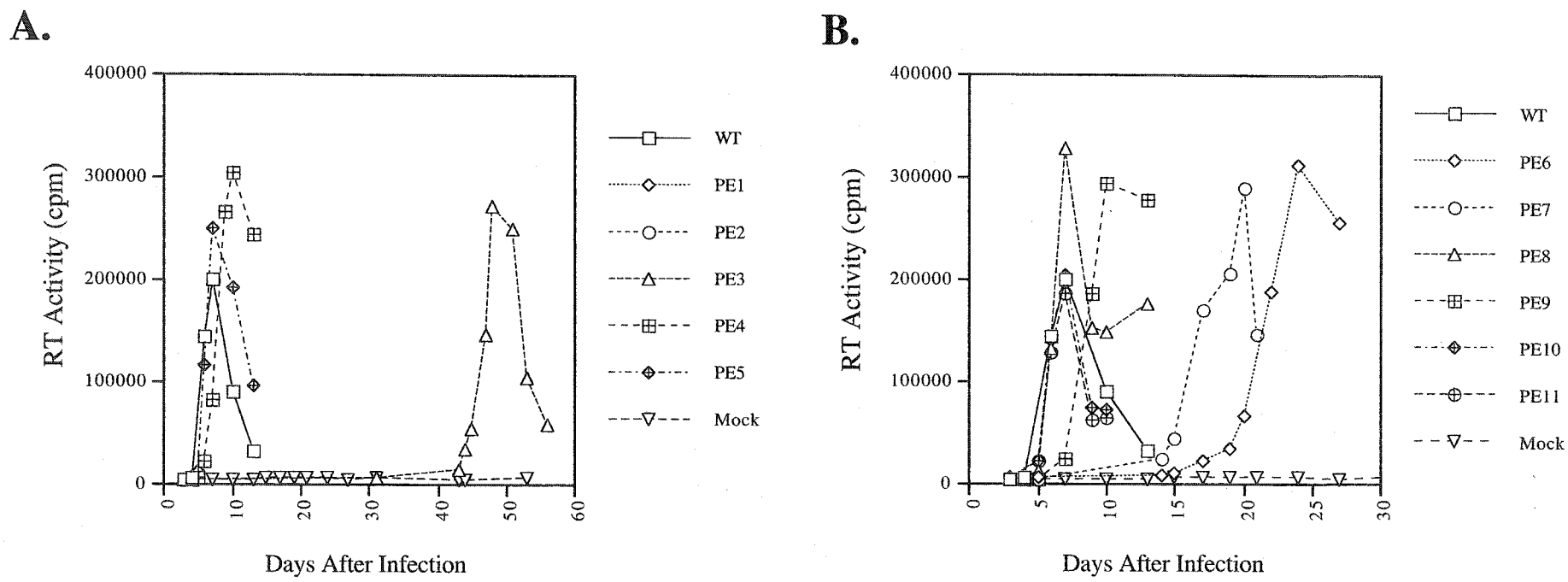

A.
B.
RT Activity (cpm)
400000
300000
200000
100000
0
0
10
20
30
40
50
60
Days After Infection
WT
PE1
PE2
PE3
PE4
PE5
Mock
RT Activity (cpm)
400000
300000
200000
100000
0
0
5
10
15
20
25
30
Days After Infection
WT
PE6
PE7
PE8
PE9
PE10
PE11
Mock

Figure 4.2

C.

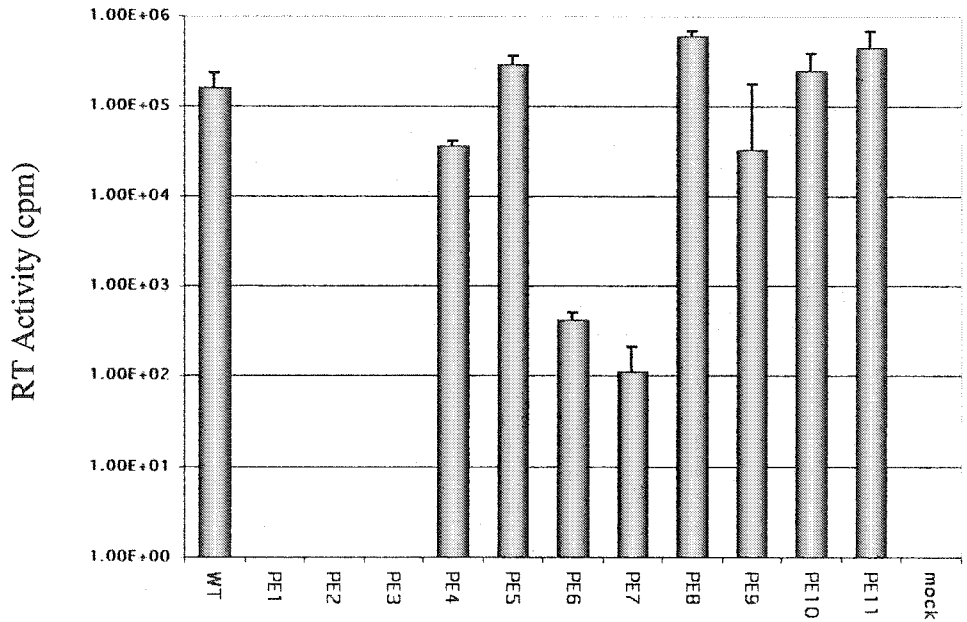

RT Activity (cpm)
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
WT
PE1
PE2
PE3
PE4
PE5
PE6
PE7
PE8
PE9
PE10
PE11
mock

Fig. 4.3 Replicative ability of viral constructs in primary lymphocytes.
Viral replication was assessed in activated rhesus PBMCs using viral inocula normalized on the basis of p27 Ag. Growth curves were determined by p27 Ag Elisa of culture supernatants taken at multiple time points. All p27Ag results are the average of duplicates. (A) Growth curves of variants deleted within the region +426-464. (B) Growth curves of variants deleted within the region +473-532. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control. Note that the scales of the ordinates are logarithmic. The dotted line representing 0.01 ng of p27/ml indicates the threshold of sensitivity of the assay.

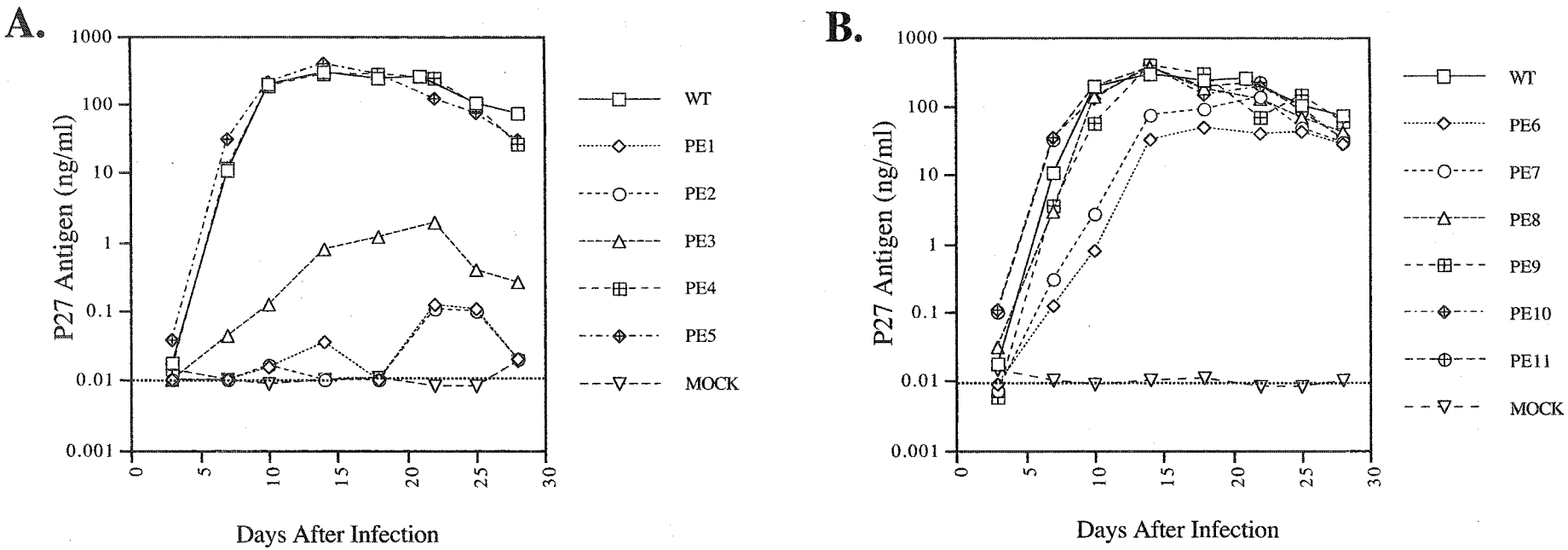

A.
1000
100
10
1
0.1
0.01
0.001
P27 Antigen (ng/ml)
0
5
10
15
20
25
30
Days After Infection
WT
PE1
PE2
PE3
PE4
PE5
MOCK
B.
1000
100
10
1
0.1
0.01
0.001
P27 Antigen (ng/ml)
0
5
10
15
20
25
30
Days After Infection
WT
PE6
PE7
PE8
PE9
PE10
PE11
MOCK

Figure 4.3

Fig. 4.4 Pulse labeling analysis of intracellular processing of the Gag precursor protein.

COS-7 cells transfected with either wild-type or mutated SIV constructs were pulse-labelled and viral proteins in the cellular lysate were immunoprecipitated with MAbs directed at p27 CA epitopes. Results shown are representative of three independent labelling experiments. The observed processed Gag products include Pr55, p43, p36, p28 and p27, and are shown in (A) and (C). The percentage of each viral protein, relative to total viral protein detected, was calculated using the NIH Image Program, and shown in graphs (B) and (D). The figures (E) and (G) show the pulse-labeling analysis of ^{35}S-labelled protein of purified viral progeny released from COS-7 cells. Again, protein bands were quantified by densitometry using the NIH Image Program. For each virus, the average percentage of each band versus total protein was calculated (see Fig. F and H). The results are representative of replicate experiments performed in parallel to those of Fig. 4 A and C.

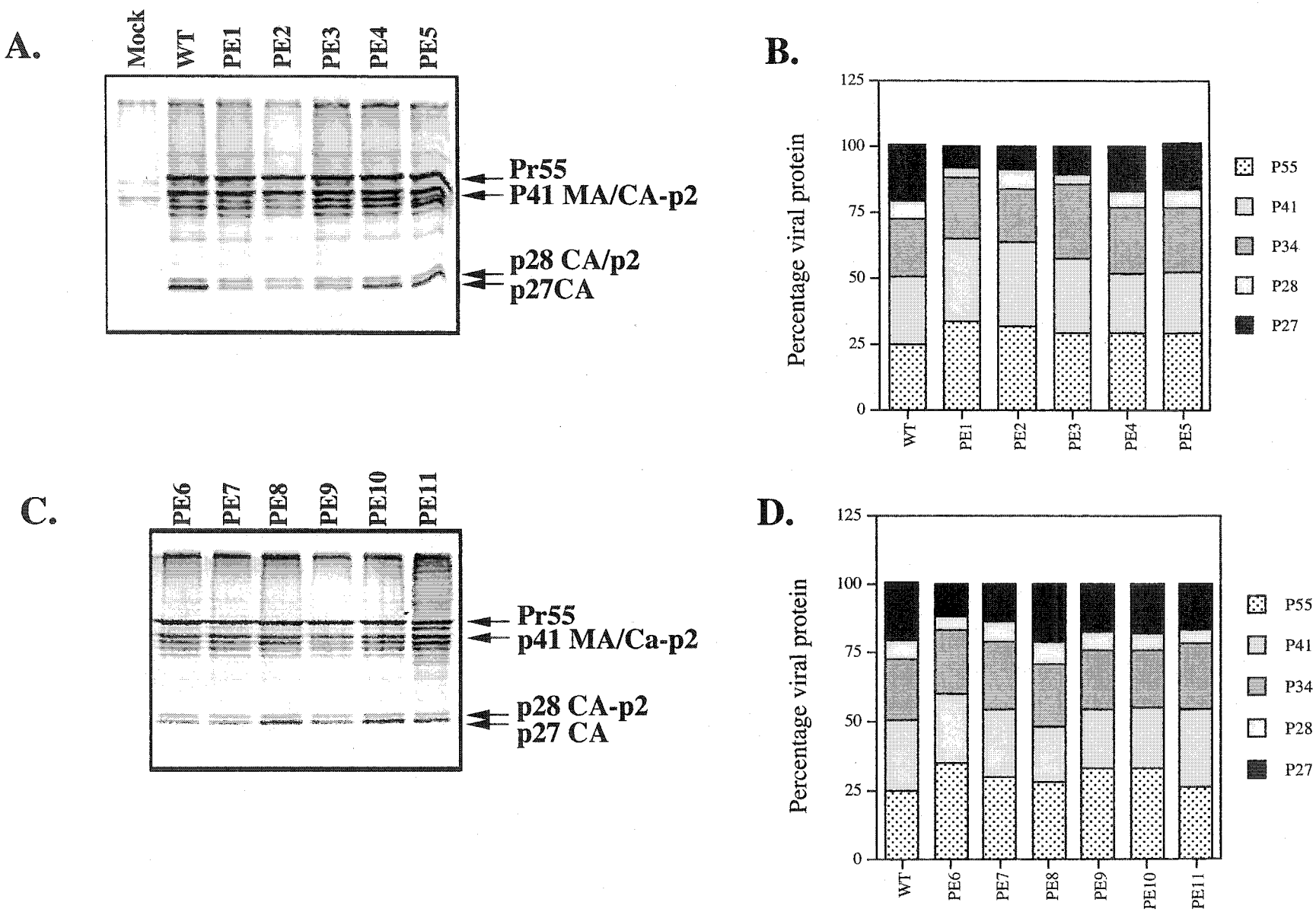
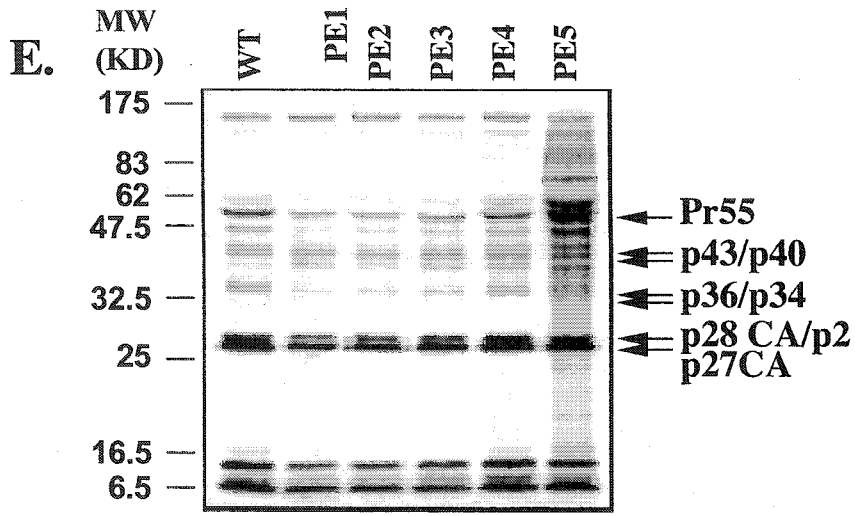
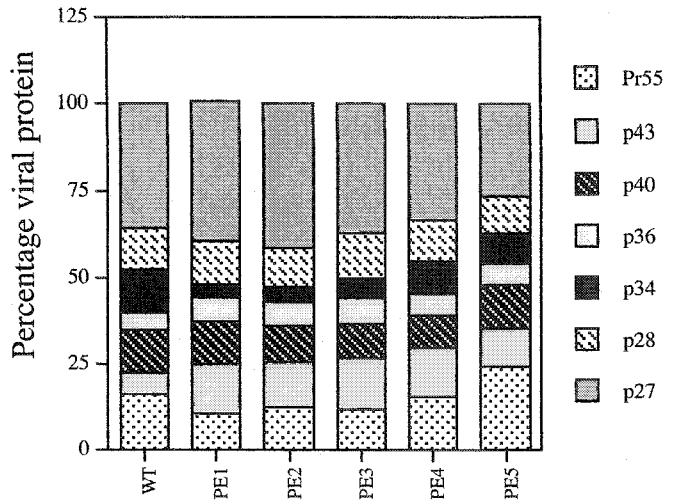
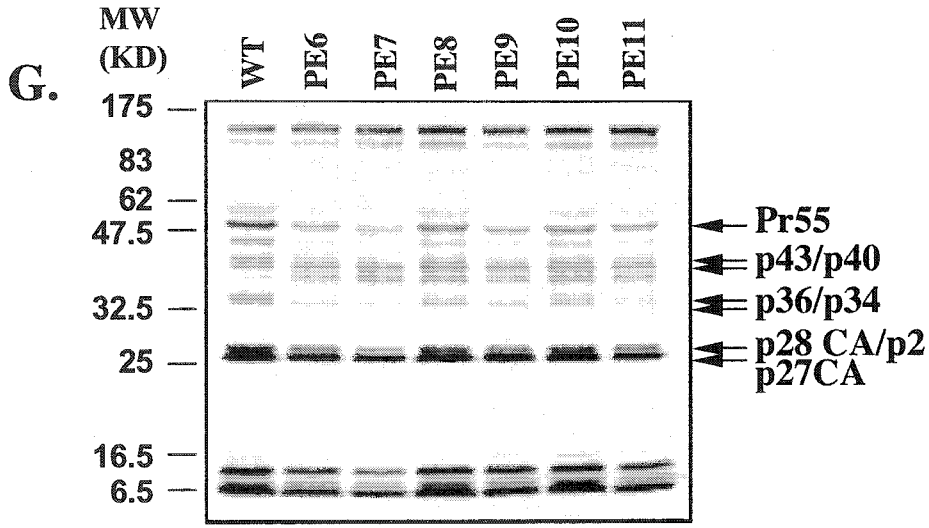

A.
Mock
WT
PE1
PE2
PE3
PE4
PE5
Pr55
P41 MA/CA-p2
p28 CA/p2
p27CA
B.
Percentage viral protein
125
100
75
50
25
0
WT
PE1
PE2
PE3
PE4
PE5
P55
P41
P34
P28
P27
C.
PE6
PE7
PE8
PE9
PE10
PE11
Pr55
p41 MA/Ca-p2
p28 CA-p2
p27 CA
D.
Percentage viral protein
125
100
75
50
25
0
WT
PE6
PE7
PE8
PE9
PE10
PE11
P55
P41
P34
P28
P27

Figure 4.4


E.
MW
(KD)
WT
PE1
PE2
PE3
PE4
PE5
175
83
62
47.5
32.5
25
16.5
6.5
Pr55
p43/p40
p36/p34
p28 CA/p2
p27CA


F.
Percentage viral protein
125
100
75
50
25
0
WT
PE1
PE2
PE3
PE4
PE5
Pr55
p43
p40
p36
p34
p28
p27


G.
MW
(KD)
WT
PE6
PE7
PE8
PE9
PE10
PE11
175
83
62
47.5
32.5
25
16.5
6.5
Pr55
p43/p40
p36/p34
p28 CA/p2
p27CA

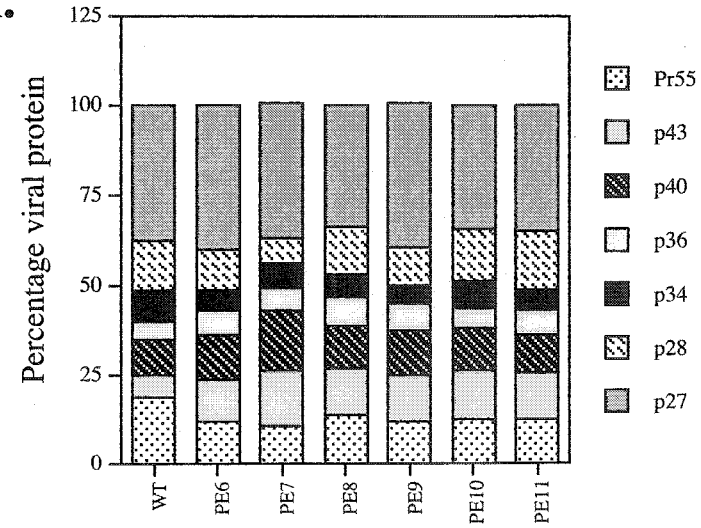

H.
Percentage viral protein
125
100
75
50
25
0
WT
PE6
PE7
PE8
PE9
PE10
PE11
Pr55
p43
p40
p36
p34
p28
p27

Fig. 4.5 Genomic RNA packaging of SIVmac239 viruses containing mutations in the leader region.

(A) Analysis of viral RNA packaging as assessed by slot-blotting was conducted in triplicate in COS-7 or 293T cells. Shown are results from COS-7 derived viral stocks that were normalized on the basis of p27 antigen. The five WT standards are serial 2:1 dilutions starting from 100ng p27 Ag, i.e. 100ng, 50ng, 25ng, 12.5ng, and 6.25ng. Each of the mutant samples is a serial 2:1 dilution based on 50ng p27 Ag, i.e. lane 1, 50ng, lane 2, 25ng. The addition of RNase to RNA samples as a negative control is denoted as (+) (lane 3). Relative amounts of products were quantified by molecular imaging using the NIH image program, with WT levels arbitrarily set at one. (B) RNA packaging of various mutant constructs as a percent average of WT virus. The bars of the first lane for each sample represents total incorporated viral RNA, the second bar for each lane represents incorporated full-length viral RNA.

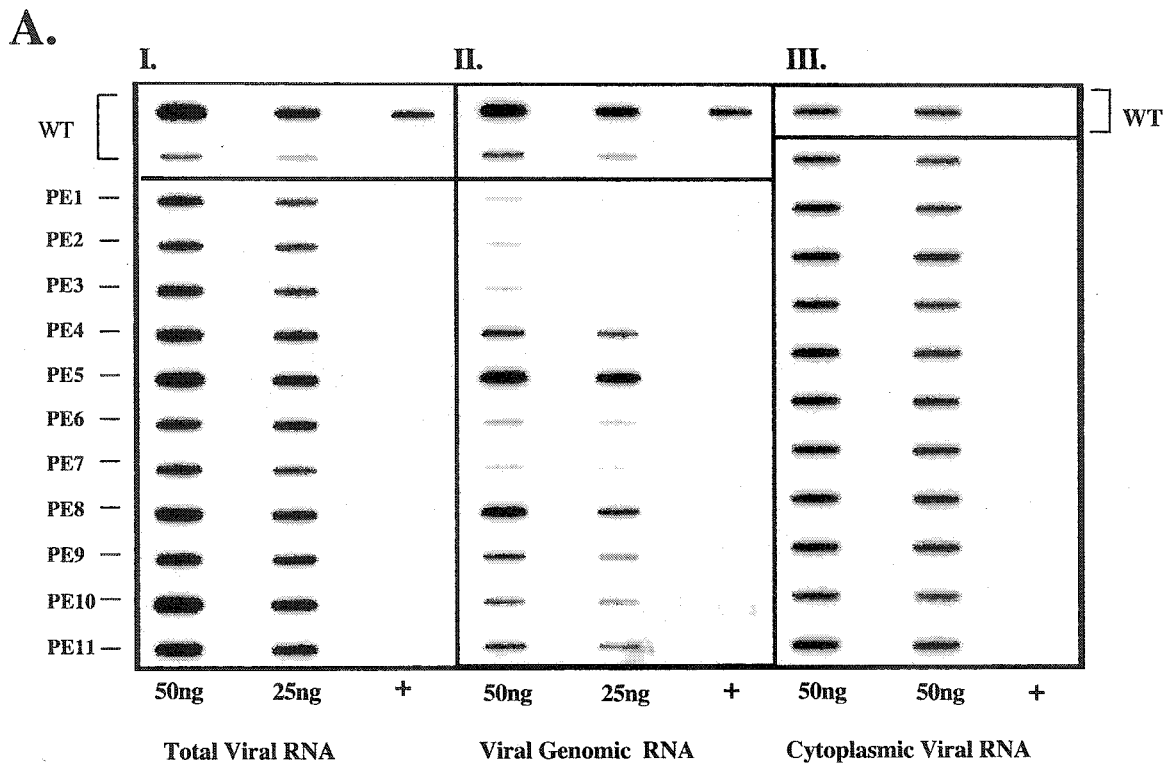
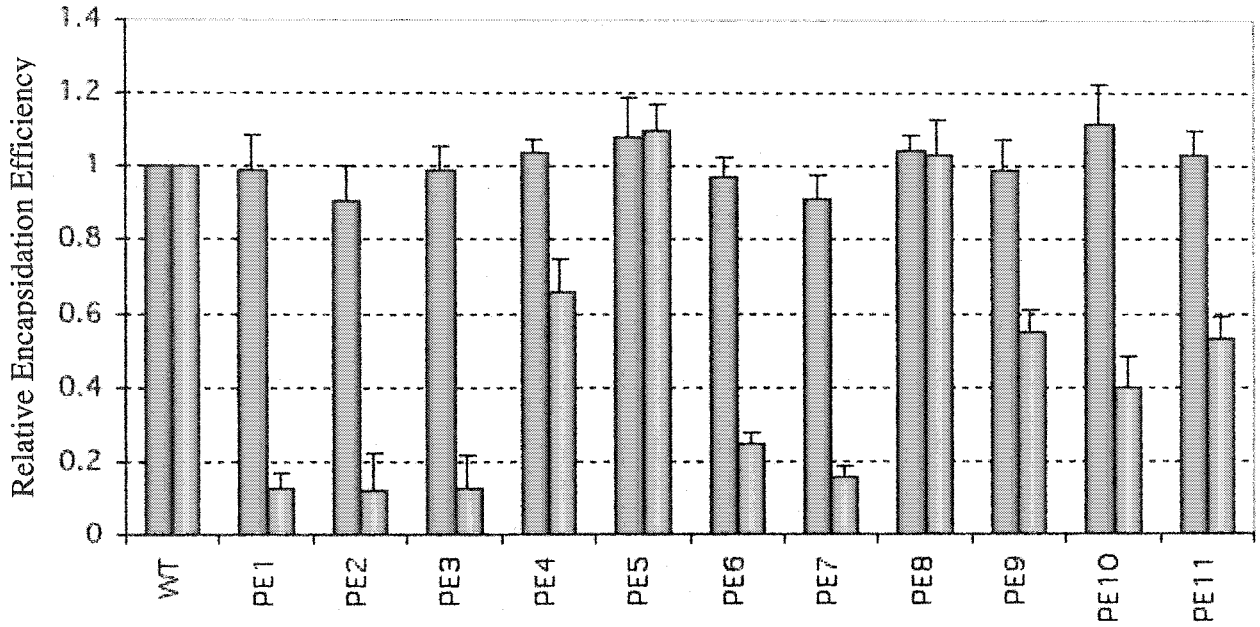

A.
I.
II.
III.
WT
WT
PE1
PE2
PE3
PE4
PE5
PE6
PE7
PE8
PE9
PE10
PE11
50ng
25ng
+
50ng
25ng
+
50ng
50ng
+
Total Viral RNA
Viral Genomic RNA
Cytoplasmic Viral RNA

Figure 4.5

B.



Ratio of viral genomic RNA to total viral RNA	1.18	0.18	0.20	0.16	0.61	0.93	0.33	0.22	0.85	0.57	0.30	0.46

Fig. 4.6 Denaturing Northern analysis of SIV RNA

(A.) Transfected COS-7 cells were first lysed in buffer containing NP-40. RNA was extracted from cell lysates using phenol/chloroform/isoamylalcohol and normalized by p27 Ag present in cellular lysates (160ng p27CA loaded per sample). As a negative control, RNA was recovered from COS-7 cells mock-transfected in parallel with wt and mutant constructs. All RNAs were subject to denaturing Northern analysis. The major RNA bands represent full-length (9.2K) and single and multiply spliced (4K and 2K respectively) viral RNA transcripts.

(B.) To serve as an internal control for cellular RNA integrity and loading equivalence, total RNA preparations were separated on 1% agarose gels and visualized by staining with EtBr (shown are ribosomal RNAs) (28S and 18S)

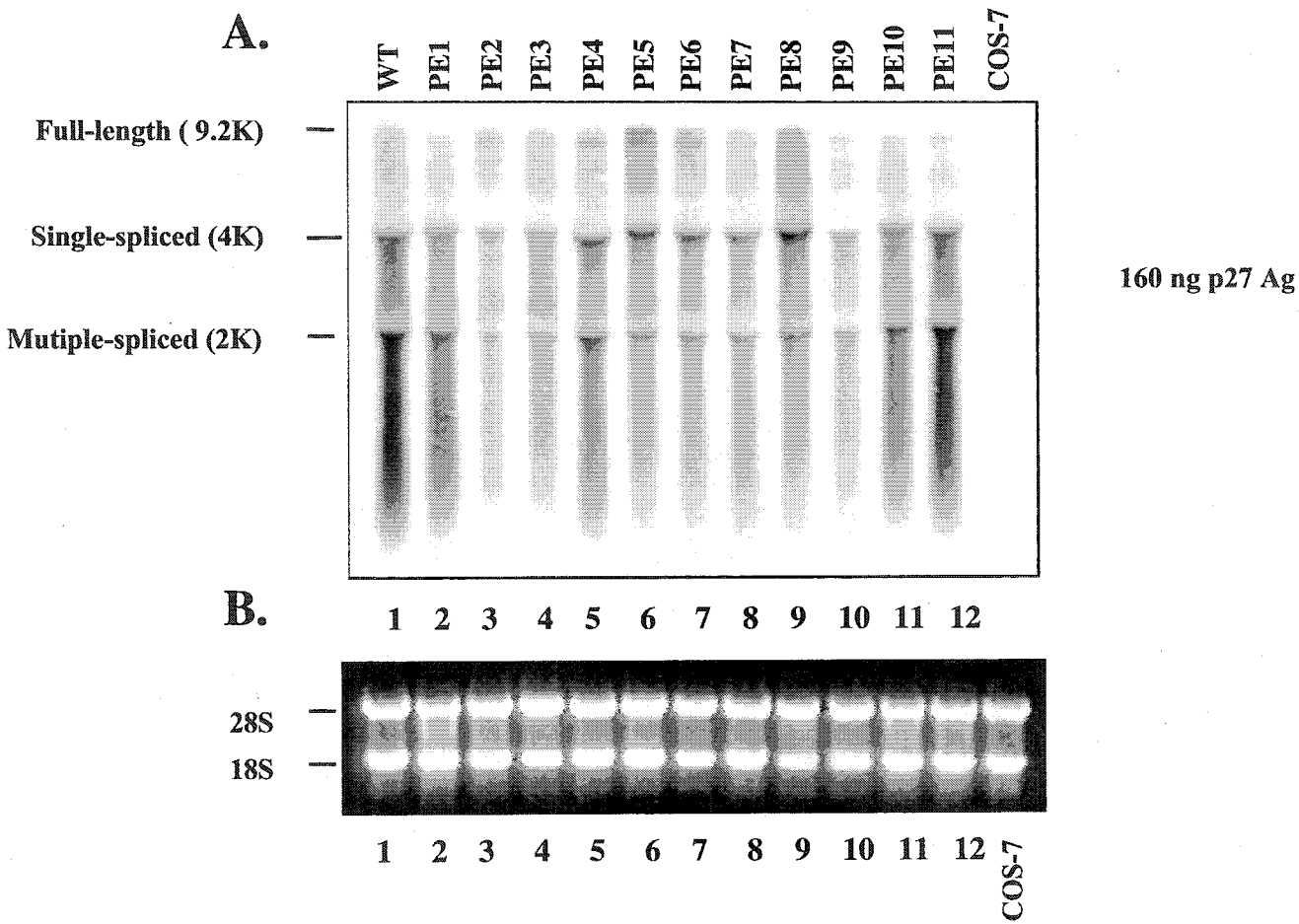

A.
WT
PE1
PE2
PE3
PE4
PE5
PE6
PE7
PE8
PE9
PE10
PE11
COS-7
Full-length (9.2K)
Single-spliced (4K)
Mutiple-spliced (2K)
160 ng p27 Ag
B.
1 2 3 4 5 6 7 8 9 10 11 12
28S
18S
1 2 3 4 5 6 7 8 9 10 11 12 COS-7

Figure 4.6

Fig. 4.7 Non-denaturing Northern blot analysis of SIV RNA

Genomic RNA was isolated from virus particles after transfection of COS-7 cells with WT or mutant plasmids. The relative mobilities of dimers (D) and monomers (M) in 0.90% agarose are indicated. Fig.7A depicts RNA preparations from mutants encompassing the region. 7B show RNA preparations from deletion mutants encompassing the region +426 - +465. Fig 7C shows RNA preparations from mutants encompassing regions +473 - +480. Fig. 7D Assessment of the thermal stabilities of WT and mutant RNA dimers. Extracted RNA from purified virions was subjected to non-denaturing Northern analysis in 0.90% agarose after 10 min of heating at assigned temperatures in the presence of 100 mM NaCl. Positions of dimers and monomers are as indicated.

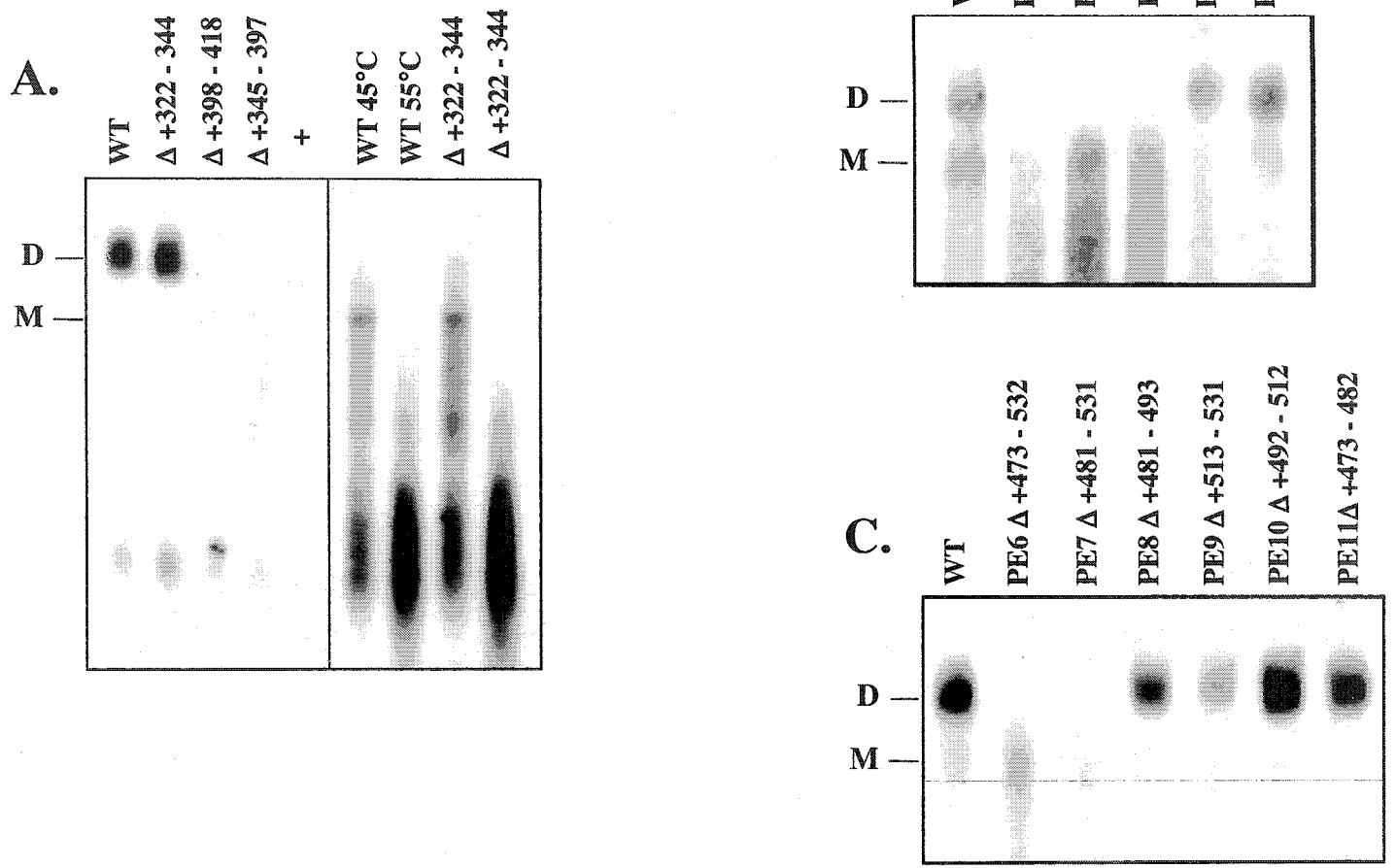

A.
WT
Δ +322 - 344
Δ +398 - 418
Δ +345 - 397
+
WT 45°C
WT 55°C
Δ +322 - 344
Δ +322 - 344
D
M
B.
WT
PE1 Δ +426 - 463
PE2 Δ +426 - 458
PE3 Δ +426 - 444
PE4 Δ +445 - 458
PE5 Δ +459 - 465
D
M
C.
WT
PE6 Δ +473 - 532
PE7 Δ +481 - 531
PE8 Δ +481 - 493
PE9 Δ +513 - 531
PE10 Δ +492 - 512
PE11Δ +473 - 482
D
M

Figure 4.7

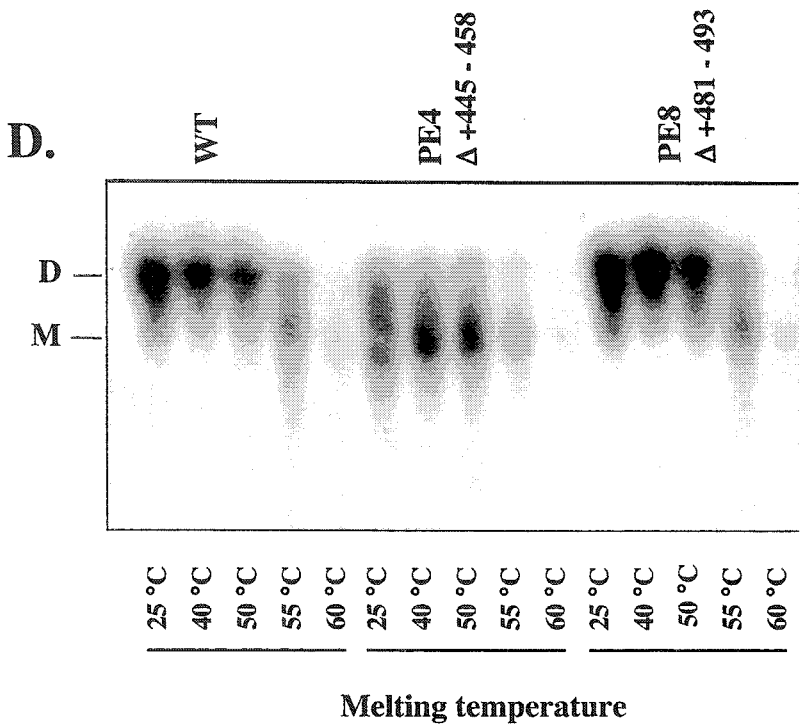

D.
WT
PE4
Δ +445 - 458
PE8
Δ +481 - 493
D
M
25 °C
40 °C
50 °C
55 °C
60 °C
25 °C
40 °C
50 °C
55 °C
60 °C
25 °C
40 °C
50 °C
55 °C
60 °C
Melting temperature

Fig. 4.8 Transmission EM (TEM) of wild-type and mutant viral particles

TEM of late (fixed 48hr post transfection), wt and mutant particles were assessed and scored from multiple sections. In panel A, the wt virus has typical size and core morphology. Panel B shows results with the PE3 mutant, i.e. diminished production of viral particles, with altered diameter and core morphology. Panel C shows the PE6 mutant; virus particle production is higher than with the upstream mutants; however particle condensation to a mature state is delayed. Bar size is .5μM in each panel.

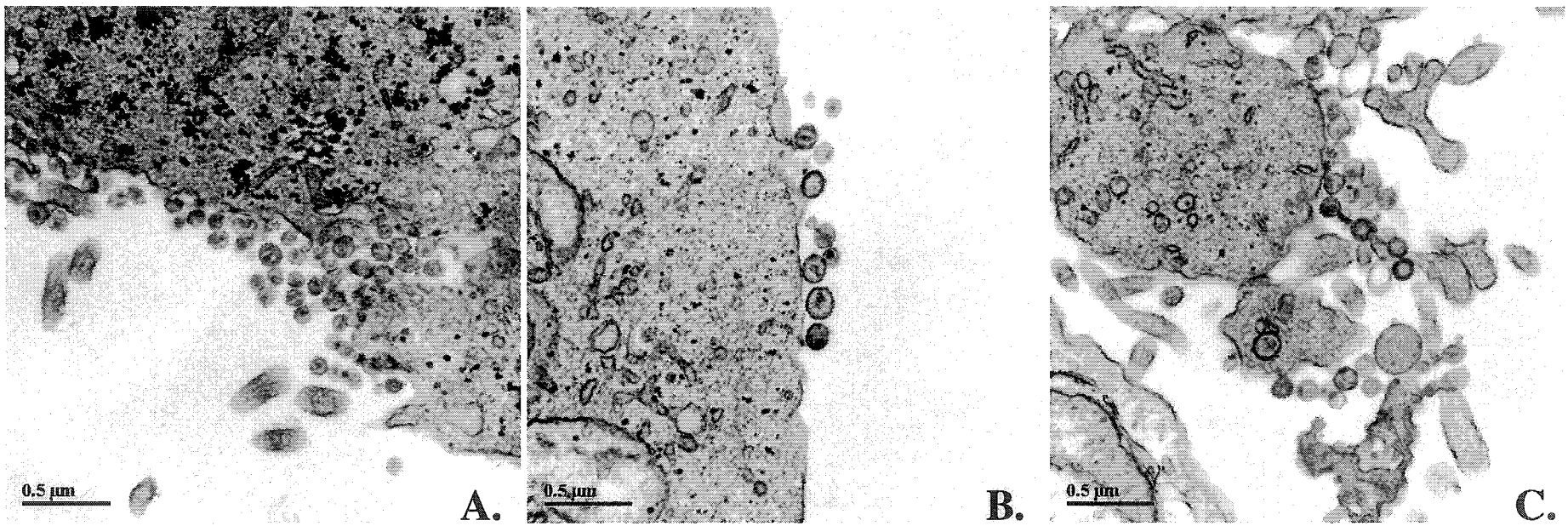


Figure 4.8

Chapter 5

Construction and In Vitro Properties of a Series of Attenuated Simian Immunodeficiency Viruses Deleted of All Accessory Genes

This chapter is an adaptation from an original paper published in the *Journal Of Virology*. 2000; 74(19): 8854-60. Authorship is as follows; Yongjun Guan, **James B. Whitney**, Mervi Detorio, and Mark A. Wainberg. The contributions to this chapter, including manuscript preparation, were divided equally between Yongjun and myself. MD contributed by conducting the p27 Ag ELISA.

5.1 Preface

This chapter involves the construction of a "simple", or at least simplified SIV, containing only the cognate *gag, pol* and *env*. The six accessory gene functions that were removed were replaced by quasi-equivalent functional elements from other viruses. For example, to recover the loss of Tat, we upregulated viral promoter efficiency by introducing a chimeric CMV promoter in the LTRs. Similarly a constitutive transport element from SRV-1 was used in place of the deleted Rev/RRE RNA transport element. Finallly the IRES from either polio virus or EMCV was used to increase translational efficiency of *env*.

5.2 Abstract

We have generated simplified simian immunodeficiency virus (SIV) constructs deleted of each of the *nef*, *vpr*, *vpx*, *vif*, *tat* and *rev* genes (Δ6 virus). To accomplish this, we began with the infectious molecular clone of SIV, i.e. SIVmac239, and replaced the deleted segments with three alternate elements: 1. a constitutive transport element (CTE) derived from simian retrovirus type 1 (SRV-1) to replace the Rev/ Rev responsive element (RRE) posttranscriptional regulation system; 2. a chimeric SIV long terminal repeat (LTR) containing a cytomegalavirus (CMV) promoter to augment transcription and virus production; 3. an internal ribosome entry site (IRES) upstream of the env gene to ensure expression of envelope proteins. This simplified construct (Δ6CCI) efficiently produced all viral structural proteins and mature virions possessed morphology typical of wild-type virus. It was also observed that deletion of the six accessory genes dramatically affected both the specificity and efficiency of packaging of SIV genomic RNA into virions. However, the presence of both the CTE and the chimeric CMV promoter increased the specificity of viral genomic RNA packaging, while the presence of the IRES augmented packaging efficiency. The Δ6CCI virus was extremely attenuated in replication capacity, yet retained infectiousness for CEMx174 and MT4 cells. We also generated constructs that retained either the rev gene or both the rev and vif genes, and showed that these viruses , when complemented by the CMV promoter, i.e. Δ5-CMV and Δ4-CMV, were able to replicate in MT4 cells with moderate and high-level efficiency, respectively. Long-term culture of each of these constructs over 6 months revealed no potential for reversion. We hope to shortly evaluate these simplified constructs in rhesus macaques to determine their long-term safety as well as ability to induce protective immune responsiveness as proviral DNA vaccines.

5.3 Introduction

A major advantage of an attenuated virus strategy for the development of a human immunodeficiency virus (HIV) vaccine might be the ability of live attenuated viruses to induce broad and persistent immunity. The existence of long-term non-progressors in regard to HIV-1 infection (Deacon *et al.*, 1995, Kirchhoff *et al.*, 1995, Mariani *et al.*, 1996, Michael *et al.*, 1995, Salvi *et al.*, 1998, Schwartz *et al.*, 1996, Yamada *et al.*, 2000) and of multiply exposed, uninfected individuals (Clerici *et al.*, 1999, Dyer *et al.*, 1999, Fowke *et al.*, 1999, Kaul *et al.*, 2000, Mazzoli *et al.*, 1997, Mazzoli *et al.*, 1999, Rowland-Jones *et al.*, 1998, Rowland-Jones *et al.*, 1999) suggests that naturally attenuated species might exist and play a protective role for at least a transient period. Similarly, inoculation of macaques with attenuated variants of simian immunodeficiency virus (SIV), containing deletions in nonessential genes, has yielded protection against subsequent challenge by virulent SIV strains (Almond *et al.*, 1995, Daniel *et al.*, 1992, Desrosiers *et al.*, 1998, Johnson *et al.*, 1998, Johnson *et al.*, 1999 Wyand *et al.*, 1999). However, important safety concerns have limited the application of these findings in HIV vaccine research, because even multiply-deleted SIV constructs, containing deletions in nef, vpr and the negative regulatory element (NRE), were pathogenic in both infant and adult macaques (Baba *et al.*, 1999, Gundlach *et al.*, 2000). Long-term human nonprogressors, known to be infected by nef-deleted variants of HIV, were shown to have falling CD4 counts and rising viral loads, accompanied by disease progression, over time (Greenough *et al.*, 1999, Learmont *et al.*, 1999). Therefore, live attenuated primate lentiviruses that contain deletions in nonessential genes may harbor residual potential for pathogenesis.

The SIV macaque model has proven invaluable (Burton and Moore 1998, Nathanson *et al.*, 1999), yet basic research on SIV has been limited in comparison with that performed with HIV. It is notable that all of the above mentioned, live attenuated SIVs, with the exception of those mutated in vif, have retained efficient replication capacity in permissive cell lines. Indeed, even SIVs defective in each of the NRE, nef, vpr, vpx and vif, i.e. Δ5 viruses, were able to replicate to high titers after long-term passage in CEMx174 cells (Gibbs *et al.*, 1994). This finding may account for the fact that even the highly attenuated Δ3 virus, containing deletions in nef, vpr, and the NRE, can cause

disease, since quickly replicating viruses probably retain considerable capacity for compensatory mutagenesis. In addition, all of these mutated viruses retained the two important regulatory genes, i.e. tat and rev, known to be essential for the efficient replication of both HIV and SIV. Indeed, the presence of tat is strongly linked to viral pathogenesis, and strong immune responses to both Tat and Rev are correlated with nonprogression (Van Baalen *et al.,* 1997, Zagury *et al.,* 1998). Both the Tat and Rev proteins may also have adverse effects on the host (Gallo, 1999, Mabrouk *et a.,* 1991, Rappaport *et al.,* 1999). Therefore, a safe, live-attenuated vaccine might even require that both *tat* and *rev* be deleted.

At the same time, research has shown that defective tat viruses can be partially corrected by the replacement of regulatory elements within the upstream part of the LTR by a stronger CMV promoter (Chang and Zhang. 1995). Replication-competent rev-negative viruses have also been independently generated, using a constitutive transport element (CTE) derived from simian retrovirus type 1 (SRV-1) to replace the Rev/RRE system (Von Gegerfelt *et al.,* 1999, Zolotukhin *et al.*, 1994). Therefore, it should be theoretically possible to construct a simplified SIV that is devoid of all accessory genes; the question that remains is whether such viruses will retain pathogenic potential.

Here we describe the construction and characterization of a series of such simplified SIVs, using the molecular SIVmac239 clone as an initial genome. We have generated a Δ6 virus that is deleted of each of nef, vpr, vpx, vif, tat, and rev through a series of large deletions. Select functional elements, such as a constitutive transport element (CTE), a chimeric SIV LTR containing a CMV promoter, and an internal ribosome entry site (IRES), have been introduced into our simplified SIV vectors to increase the production of viral structural proteins in the absence of accessory genes. These simplified SIV constructs can also form mature virions that possess wild-type morphology after transfection into COS-7 cells. Although the deletion of all viral accessory genes affected both the specificity and efficiency of viral genomic RNA packaging into virions, this defect was repaired by insertion of the CTE, the chimeric CMV promoter, and the IRES into the viral genome. With the help of the CMV promoter, a variant that retained the rev gene, i.e. Δ5-CMV, was able to persistently replicate in MT4 cells, while a variant that retained both the rev and vif genes, i.e. Δ4-CMV, was able to efficiently replicate in this

cell line. Most importantly, these simplified SIVs were extremely attenuated in replication capacity yet remained infectious in CEMx174 cells, with no evidence of reversion after 6 months in tissue culture. We next wish to evaluate the safety and protective efficacy of these constructs in rhesus macaques, while, at the same time, studying the *in vivo* replication capacity of these viruses to gain further insights into the specific roles of SIV accessory proteins as determinants of pathogenesis.

5.4 Materials and Methods

5.4.1 Generation of SIV constructs

The full-length infectious wild-type (WT) clone of SIV, i.e. SIVmac239/WT [20,25], was used to generate all of the constructs described in this paper (Fig 1). Both a PCR-based mutagenesis method and Pfu polymerase were employed to generate the deletions shown. In the case of the SIVmac239Δ5 mutant, two distinct regions were deleted. First, the sequences between the *SphI* and the *Bgl II* sites (positions 6702 to 4952) were replaced with the PCR fragments amplified by primers Svif (5'-GGCGCATGCGTCGACTCTGCTACCTCTCTA GCCTCTCCG-3') and Sintl (5'-CCCAGAATAGTGGCCTGATAGATAGTAGACACCTG TG-3), resulting in deletion of *vif*, *vpx*, *vpr* and *tat*. Second, the region between the *Sac I* and *Xho I* sites (positions 9482 to 10535) was replaced with the PCR fragments amplified by primers Senf-1 (5'-GGCGAGCTCACTCTCTTGTGATTGGCAATAGACATGTCTC-3') and SU5-1 (Guan et al 2000) to delete the nef gene. This SIVmac239Δ5 construct was then used to generate the SIVmac239Δ6 mutant by replacing the region between the *Sph I* and *HindIII* sites (positions 6702 to 7079) with the PCR fragments amplified by primers Senv-1 (5'-GGCGGCATGCATGGGGTGTCTTGGTAATCAGCTGCTTATCGCC-3') and Sen1 (5'-GC CATACATCCTCTATTGCCTG-3') to delete both the tat and rev genes. The SIVmac239Δ4 mutant , i.e. deleted of tat, nef, vpr, vpx but not rev or vif, was generated in similar fashion as SIVmac239Δ5, except that the region between the *Sph I* and *Sac I* sites (positions 6702 to 6011) was replaced with PCR fragments amplified by primers Svif-2 (5'-GGCGCATGCA TCATGCCAGTATTCCC-3') and Svif-4 (5'-CAAAGATTATGGAGGAGGAAAAGAGGT GG-3).

A constitutive transport element (CTE) was inserted into the region of the deleted nef gene in the SIVmac239Δ6 construct to generate SIVmac239Δ6-CTE. Toward this end, the CTE was amplified from the p72S240 plasmid (Zolotukhin et al 1994), using primers CTE-1 (5'-GGCGAGCTC ACTCTCTTGTGAATAGACCACCTCCCCTGCG-3') and CTE-2 (5'-GAGACATGTC TATTGCCAACAAATCCCTCGGAAGCTGCG-3'). The PCR products were then used as mega-primers paired with the primer SU5-1. The resulting PCR fragments were used to replace the region between the *SacI* and *XhoI* sites in SIVmac239Δ6.

For the construction of the chimeric LTR construct termed CMV-LTR, the cytomegalovirus (CMV) promoter was first amplified from the vector termed pIRES-EGFP (Clontech) using primers cmv-1 (5'-GGAAGGGATTTATTACAGTGCCGCGTTACATA ACTTACGG-3') and cmv-2 (5'-GAATACAGAGCGAAATGCAGTGCTTATATAGACC TCCCACCG-3'). The resulted CMV fragments were then used as mega-primers paired with the primer SU5-1 or the primer CTE-1 to generate fragments CMV-U5-1 and CTE-CMV, respectively, based on Δ6-CTE. The two fragments were combined and used as template to amplify the final fragment, CTE-CMV-LTR, using primers CTE-1 and SU5-1. The CTE-CMV-LTR fragment was then used to replace the region between the *Sac I* and *Xho I* sites (positions 9482 to 10535) in SIVmac239Δ6 to generate the intermediate plasmid SIVmac239 Δ6CTE3'CMV. A 5'-CMV-LTR fragment was generated by PCR based on CTE-CMV-LTR, using primers pSU3 and SU5-2 (5'-GTTCAGGCGCCAATCTGCTAGGGATTTTCCTGC TTCGG-3'). This fragment was then cloned into the *EcoRI-NarI* site of the SIVmac239 Δ6CTE3'CMV vector to generate the SIVmac239Δ6-CTE-CMV (Δ6CC) construct.

The SIVmac239Δ6-CTE-CMV-IRES (Δ6CCI) mutant was generated based on the Δ6CC construct. First, a *NcoI-HindIII* fragment was generated by PCR using primers Senv-Nco (5'-GTGCCATGGGGTGTCTTGGTAATCAGCTGCTTATCGCC-3') and Sen1. The IRES of the encephalomyocarditis virus (ECMV) was cut out from the pIRES-EGFP vector (Clontech) using *Sph I* and *Nco I* enzymes. Then, the ligation product of these two fragments was inserted into the *Sph I-HindIII* site (positions 6702 to 7079) of the Δ6CC vector to generate the Δ6CCI-E construct containing the IRES of EMCV. Another

construct containing the IRES of polio virus, Δ6CCI-P, was also generated using this same strategy, except that the *Sph I-Nco I* fragment of the IRES was produced by PCR from the plasmid pCDNA3-rLuc-polIRES-fLuc using primers polio-1 (5'-GCAGCATGCTCTGGGGTTGTTC CCACC-3') and polio-2 (5'-GCACCATGGCCGGATGG CCAATCCAA-3').

To construct the SIVmac239Δnef-CMV mutant (Fig 1B), the 5'CMV-LTR of Δ6CC was used to replace the *EcoRI-NarI* region of the wild-type vector. Then, the 3' LTR (SacI to XhoI, positions 9482-10535 in this wild-type vector) was replaced with a chimeric 3' CMV-LTR fragment that was generated in the same way as the CTE-CMV-LTR fragment described above, except that primer Senf-1 was used instead of CTE-1 and vector Δ6 was used as template for PCR instead of Δ6-CTE. To generate Δ5-CMV and Δ4-CMV, both the 5'LTR and 3'LTR were similarly replaced with the chimeric CMV-LTR. The Δ2-CMV construct was constructed by replacing the region between the Sph I and HindII sites (positions 6702 to 7079) of SIVmac239Δnef-CMV with the PCR fragments amplified by primers Srev-1 (5'GAAGCATGCTATAACTGATGATATTGTAAAAA GTGTTGC-3') and Sen1 (5'-GC CATACATCCTC TATTGCCTG-3') to introduce double stop codons in the first exon of tat without affecting the overlapping vpr and rev genes.

The SIVmac239Δ6-CTE-CMV-IRES-GFP (Δ6CCI-GFP) mutant (Fig 1C), containing the enhanced green fluorescence protein (EGFP) reporter gene downstream of the IRES, was generated based on the Δ6CC construct. The fragment produced by *SphI-SacI* in the envelope gene in the Δ6CC construct was replaced with the *SphI-SacI* fragment containing the IRES and the EGFP region from the pIRES-EGFP vector (Clontech) to generate the Δ6CCI-E-GFP construct (in which E designates ECMV), containing the IRES of ECMV. Alternatively, the ligation product of the polio-IRES (*SphI-NcoI*) and the *EGFP (NcoI-SacI)* was used to replace the *SphI-SacI* region in the Δ6CC vector to yield the Δ6CCI-P-GFP construct (in which P designates poliovirus), containing the IRES of poliovirus.

All constructs were sequenced to confirm the validity of all fragments derived by PCR. Nucleotide designations are based on published sequences (Kestler et al 1990).

5.4.2 Cells and preparation of virus stocks

COS-7 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum. CEMx174 and MT4 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Molecular constructs were purified using a Maxi Plasmid Kit (QIAGEN Inc. Mississauga, Ontario, Canada). COS-7 cells were transfected using these constructs with lipofectamine-plus reagent (GIBCO, Burlington, Ontario, Canada). Virus-containing culture fluids were harvested at 60 h after transfection and were clarified by centrifugation for 30 min at 4°C at 3,000 rpm in a Beckman GS-6R centrifuge. Viral stocks were passed through a 0.2μm filter and stored in 0.5 or 1 ml aliquots at –70°C. Levels of viral reverse transcriptase (RT) were determined as described [29] and levels of viral capsid antigen (CA), i.e. p27, were quantified by Coulter SIV core antigen assay kit (Immunotech Inc. Westbrook, ME).

5.4.3 Viral protein analysis by radiolabeling and immunoprecipitation

COS-7 cells were transfected with wild-type or mutant constructs. At 20 hours after transfection, cells were starved at 37°C for 30 min in DMEM without methionine (Met) and cysteine (Cys). Radiolabelling was performed with ^{35}S-Met and ^{35}S-Cys at a concentration of 100 μCi/ml for 30 min at 37°C. Then the cells were thoroughly washed with complete DMEM and cultured for 1 hour. Culture fluids were collected and clarified on a Beckman GS-6R bench centrifuge at 3,000 rpm for 30 min at 4°C. Viral particles were further purified through a 20% sucrose cushion at 40,000 rpm for 1 hour at 4°C using a SW41 rotor in a Beckman L8-M ultracentrifuge. Virus pellets were suspended in 1x SDS -PAGE loading buffer and boiled for 5 min, then fractionated by 12% SDS-polyacrylamide gel electrophoresis and exposed to X-ray film. The labeled cells were washed twice with cold PBS and lysed in buffer containing 0.1% NP40. Cell lysates were incubated with a MAb against SIV p27 at 4°C for 30 min and the resultant Ag-Ab complexes were precipitated by a 30 min incubation with protein A-Sepharose CL-4B (Amersham-Pharmacia Biotech, Montreal, Quebec, Canada). The recovered viral proteins were analyzed by 12% SDS-PAGE and exposed to X-ray film.

5.4.4 Virion Morphology

The morphology of the viruses produced by the various constructs described above was examined by transmission electron microscopy. Briefly, COS-7 cells transfected with wild-type constructs or the simplified SIV constructs were fixed after 40 hours with 2.5% glutaraldehyde followed by 4% osmium tetroxide. Thin-sectioned samples were stained with lead citrate and uranyl acetate and visualized using a JEOL 200 FX electron microscope as described (Smith and Gehle. 1969).

5.4.5 Packaging of viral genomic RNA

Viral RNA was isolated from equivalent amounts of COS-7 cell-derived viral preparations, based on levels of SIV p27 antigen, using the QIAamp viral RNA mini kit (QIAGEN). RNA samples were treated with RNase-free DNase I at 37°C for 30min to eliminate possible DNA contamination. DNase I was then inactivated by incubation at 75°C for 10min. The viral RNA samples were quantified by RT-PCR, using the Titan One Tube RT-PCR system (Boehringer-Mannheim, Montreal, Quebec, Canada) as described previously [20], except that two pairs of primers were employed in tandem. The primer pairs sg1(5'GAAGCATGTAGTATGGGCAG-3') and sg2 (5'GGCACTAATGGAGCTAAGAC CG-3') were used to amplify a 114-bp fragment representing the full-length viral genome. Another pair of primers, Senf-3 (5'-GGAAGATGGATA CTCGCAATCC-3') and SU3-3 (5'-GCACTGTAATAAATCCCTTCCAG-3'), was used to amplify a fragment between the end of the env gene and the beginning of the 3' U3 region, which represents total viral RNA. The size of the Senf-3-SU3-3 product is 317-bp in the case of the wild-type viral genome, 142-bp in the case of the Δ6 genome, and 315-bp for each of the other constructs. Relative amounts of product were quantified by molecular imaging (BIO-RAD, Toronto, Ontario, Canada).

5.4.6 Virus infection

Viral stocks were thawed and treated with 100 U of DNase I in the presence of 10mM $MgCl_2$ at 37°C for 1 h to eliminate any residual contaminating plasmids from the transfection. Infection of CEMx174 or MT4 cells was performed by incubating $1x10^6$

cells at 37°C for 2 h with an amount of virus equivalent to 10ng p27 antigen. Infected cells were then washed extensively with phosphate-buffered saline and resuspended in fresh medium. Cells were split at a 1:3 ratio twice per week if they had grown to a sufficient level, otherwise the culture fluid was replaced with fresh medium. Supernatants were monitored for virus production by SIV p27 antigen capture assay using the Coulter SIV core antigen assay kit. Virus replication was also performed in primary rhesus monkey PBMCs. $5x10^6$ activated PBMCs were infected with SIV stocks containing 10 ng of p27 at 37°C for 2 hours; the cells were then washed extensively to remove any remaining virus. Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 20 U/ml IL-2. Virus production in culture fluids was monitored by SIV p27 antigen capture assay using the Coulter SIV core antigen capture kit.

5.4. 7 Detection of viral DNA

At various times post infection, cells were collected and washed with PBS. Cellular DNA was isolated using a QIAamp DNA Mini kit (QIAGEN). DNA samples were analyzed by PCR using primers sg1 and sg2 to amplify a 114-bp fragment in the gag gene. PCR assays were performed with 0.1-1μg of sample DNA, 50mM Tris –HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 2.5 U Taq polymerase, 0.2mM dNTPs, 20 pmol of reverse primer and 20 pmol of forward primer and programmed as follows: 95°C for 3 min; 25 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1min; and 72°C for 10 min. Products were separated on 2% agarose gels. In the case of transient infections, cells were exposed to virus as described above, collected after 6 hours and washed extensively with PBS; negative infection controls for each construct were performed at 4°C using pre-cooled cells and viruses. Cellular DNA was isolated using a QIAamp DNA Mini kit (QIAGEN). DNA samples were analyzed by PCR as described above, except that the sg1 primer was ^{32}P-labeled and reactions were standardized by simultaneous amplification of a 567-bp DNA fragment of the human β-actin gene as an internal control as described previously (Guan *et al.*, 2000).

5.5 Results

5.5.1 Generation of simplified SIV constructs.

A series of SIV mutants containing deletions within various nonstructural genes was constructed as described in Materials and Methods and is shown in Figure 1A. In the case of all the mutants, both the *vpx* and *vpr* genes were completely deleted, while most of the *vif* gene was deleted, and only the first 21 amino acids of the *vif* gene remained. The *nef* gene was interrupted by deletion of the sequences from positions 9500 to 9674. In the case of the Δ5 construct, only one nonstructural gene, rev, was retained and the tat gene was inactivated by a large deletion that included the first 145-bp of the first *tat* exon. In all constructs that contained only structural genes (Δ6 series), both the tat and rev genes were inactivated by deletion of the first exons of tat and rev including their splice donor sites.

To compensate for removal of the rev/RRE post-transcriptional regulation system, a 173-bp CTE sequence of SRV-1 was inserted into the site of the nef deletion to form the construct termed Δ6-CTE. To increase the expression of viral genes in the absence of the tat gene, a CMV IE promoter (including its enhancer and TATA box) were inserted into the LTR U3 promoter region of SIV (a 473-bp fragment from its upstream sequence to the TATA box) to generate construct Δ6CTE-CMV(Δ6CC). To determine whether this chimeric LTR was functional, it was inserted into a wild-type construct containing the nef deletion. This construct, termed SIVmac239Δnef-CMV (Figure 1B), was transfected into COS-7 cells to produce viral stock. As shown in Figure 2, the Δnef-CMV virus replicated in similar fashion to wild-type virus in CEMx174 cells. This result confirms that the chimeric LTR with the CMV promoter can be used efficiently by SIV in the CEMx174 cell line.

The deletion of the upstream sequences of the env gene may inhibit the efficient translation of envelope proteins due to deletion of upstream sequences that include splice acceptor sites. Therefore, an IRES element was inserted between the gag-pol and envelope genes into the Δ6CCI construct to aid expression. To confirm that the IRES element was functional in the SIV construct, the envelope gene (from its ATG to the SacI site) was replaced with the EGFP reporter gene. The EGFP gene is in the same open reading frame as the env gene and this construct, termed Δ6CCI-GFP (Figure 1C), was

transfected into COS-7 cells. Both fluorescence microscopy (not shown) and fluorescein isothiocyanate (FITC)-gated fluorescence-activated cell sorting (FACS) confirmed the expression of the EGFP gene. The results of Figure 3 show that the IRES of poliovirus resulted in the highest degree of expression of GFP, i.e. 51%, whereas the ECMV IRES resulted in only 30% expression of GFP, while background levels were about 10%. Similar results were obtained in each of three separate experiments. Therefore, the variant of the Δ6CCI construct that was used in all other experiments was that which contained the polio IRES, i.e. Δ6CCI-P.

5.5. 2 Production of modified SIV

All of these simplified SIV constructs were transfected into COS-7 cells, and virus production in supernatants was detected by RT assay and p27 antigen quantification. As shown in Figure 4, viruses without tat (Δ5) were efficiently produced after transfection of COS-7 cell lines. Interestingly, mutants with deletions in all six nonstructural genes (Δ6) were produced at levels 100 times less than those of wild-type virus in the absence of additional elements. In contrast, the addition of the CTE (construct Δ6-CTE), the chimeric CMV promoter (construct Δ6CC), and the IRES (construct Δ6CCI), efficiently increased SIV production to levels comparable to those of wild-type virus.

We also analyzed viral protein production by radiolabeling and immuno-precipitation as described in Materials and Methods. Figure 5 presents the viral protein pattern of viruses produced during 1 hour by transfected COS-7 cells; the data show that the Δ5 construct was able to produce viral proteins efficiently, while the Δ6 virus was severely impaired in this regard. These findings are similar to those obtained through use of the p27 ELISA and RT assays (Figure 4). In contrast, viral proteins were efficiently produced with the help of each of the CTE (construct Δ6CTE), the chimeric CMV promoter (construct Δ6CC), and the IRES (construct Δ6CCI). However, these simplified viruses were devoid of some proteins such as those between bands p6 and p15; these may represent accessory proteins such as Vpr. Immunoprecipitation of viral proteins in cell lysates with MAbs against SIV p27 showed that Gag protein was efficiently expressed in these simplified constructs (although certainly not coprecipitated with Vpr). However, the processing of Gag precursor proteins was delayed, resulting in accumulation of Pr55 (Figure 6).

Immunoprecipitation of viral proteins in cell lysates with MAbs against SIV gp120 antigen showed that viral gp120 was efficiently expressed only in the case of constructs Δ5 and Δ6CCI, while the expression of gp120 protein in the Δ6, Δ6-CTE and Δ6CC constructs was diminished (Figure 6).

Viral production was also analyzed by electron microscopy. Figure 7 shows that these simplified viruses retained morphology typical of wild-type mature virions in the case of each of constructs Δ5, Δ6CTE, and Δ6CC, indicating that these viruses retained the ability to form structures that appear to have both envelopes and normally dense cores. The Δ6 viruses could not be analyzed by electron microscopy because of very low levels of particle formation and production.

5.5.3 The deletion of accessory genes affects both the specificity and efficiency of SIV genomic RNA packaging.

Inactivation of the rev gene may impair viral RNA packaging, because Rev regulates viral RNA export from the nucleus as well as the expression of structural proteins. Therefore, we investigated the extent to which our simplified viruses could package viral RNA by RT-PCR. For this purpose, two pairs of primers were used; one of these amplified total viral RNA while the other amplified only full-length genomic RNA. As shown in Figure 8, the Δ6 viruses were able to package viral genomic RNA only to an extent of about 20% of that of total viral RNA and of about 50% of viral genomic RNA packaged by wild-type viruses. These results indicate that both the efficiency and specificity of viral genomic RNA packaging were significantly diminished in the Δ6 virus that contained only viral structural genes. The insertion of the CTE and the CMV promoter increased the specificity but not the efficiency of viral genomic RNA packaging, since the Δ6CTE and Δ6CC constructs packaged viral genomic RNA with a specificity of 75% and 97%, respectively. Interestingly, the additional insertion of the IRES remarkably increased the efficiency of viral genomic RNA packaging (Figure 8, construct Δ6CCI). The simplified construct, Δ6CCI-P, was able to specifically package viral genomic RNA as efficiently as wild-type virus.

5.5.4 Infection of CEMx174 cells.

We next investigated the infectiousness and replication capacity of these simplified viruses. Virus stocks were used to infect CEMx174 cells as described above and culture fluids were monitored for viral replication by SIV p27 antigen capture assay. As shown in Fig. 9A, detectable amounts of viral antigen were only detected after 6 days in the case of Δ6CCI-P; the other simplified constructs showed no signs of replication in these studies. The positive p27 result for the Δ6CCI-P construct was seen with duplicate experiments. To further confirm this finding, proviral DNA was harvested from cells at various times after infection and subjected to PCR analysis. The data of Figure 9B show that infection by Δ6CCI-P virus of CEMx174 cells had indeed occurred. However, long-term culture of these infected cells over 6 months did not show any signs of reverted or more replication-competent viruses (data not shown). Thus, this simplified SIV is extremely attenuated in replication capacity yet can still infect CEMx174 cells.
To further investigate this subject, we also performed transient infections of CEMx174 cells alongside control experiments performed at 4°C. The PCR results of Fig. 9C show that the Δ6CCI-P virus was indeed able to infect CEMx174 cells but less well than wild-type virus. All infections performed and maintained at 4°C yielded negative results.

5.5.5 Continuous propagation of simplified SIVs in MT4 cells

In addition to the simplified construct Δ6CCI-P, we also generated five additional viruses constructs in which the Rev/RRE system or vif gene were maintained, i.e. Δ5CCI, Δ4, Δ4-CMV, Δ5-CMV and Δ2-CMV (See Materials and Methods, Fig.1). The results of their infections of CEMx174 cells are shown in Figure 10A. The Δ4 and Δ5 mutants did not show any sign of replication, while each of Δ5CCI, Δ4-CMV, Δ5-CMV and Δ2-CMV replicated with similarly impaired efficiency as the Δ6CCI-P construct (Fig. 9A). We further infected MT4 cells, that have been shown to be permissive for replication of either vif-negative or tat-negative SIVmac239 viruses. Remarkably, the viruses that contained the CMV promoter, i.e. Δ4-CMV and Δ2-CMV, showed efficient, although delayed, replication kinetics in MT4 cells. The Δ5-CMV viruses yielded persistent low-level replication in MT4 cells, while the Δ6CCI-P and Δ5CCI viruses showed similar results in MT4 as in CEMx174 cells (Figure 10B).

Cell-free viruses harvested after initial infection of MT4 cells were then passaged in this same cell line. As shown in Figure 10C, the Δ2-CMV, Δ4-CMV and Δ5-CMV viruses all displayed similar replication capacity as seen in the initial infections. These results demonstrate that these three simplified viruses are all stably attenuated in vitro.

5.5.6 Infection of monkey peripheral blood mononuclear cells (PBMCs)

We also performed experiments to investigate the infectiousness of our simplified viruses in monkey PBMCs, using protocols that have been previously described (Guan *et al.*, 2000). As shown in Fig 11A, only the Δ2-CMV and Δ4-CMV viruses displayed transient replication capacity in these cells, while the Δ5-CMV, Δ6CCI-P and Δ5CCI viruses showed no sign of replication. We further assessed the presence of viral DNA in monkey PBMCs by PCR using primers sg1 and sg2. The results of Fig 11B show that our simplified viruses were indeed able to infect monkey PBMCs, albeit at low efficiency.

5.6 Discussion

We have generated a series of simplified SIVmac constructs that are devoid of several or all accessory genes. One of these, termed Δ6CCI, with the help of each of a CTE, the CMV promoter and an IRES, can efficiently produce mature virions that package viral genomic RNA as well as do wild-type viruses. These viruses also retain the ability to infect target cells, yet are deficient in replication capacity. The Δ6CCI construct might be suitable for use as a DNA vaccine, because it causes expression of natural viral antigens that are exposed during infection.

Highly attenuated SIV mutants containing partial deletions in nonessential genes have elicited strong protection against pathogenic challenge (Almond *et al.*, 1995, Daniel *et al.*, 1992, Desrosiers *et al.*, 1998, Johnson and Desrosiers. 1998, Johnson *et al.*, 1999, Wyand *et al.*, 1999). Wide ranges of attenuation levels have been achieved in such experiments and protective efficiency was shown to be inversely proportional to the degree of attenuation (Johnson *et al.*, 1999). Prevailing opinion suggests that live attenuated strategies may fail if viruses are too attenuated (Ruprecht 1999). However, a highly attenuated SIV lacking nef, vpr, vpx and upstream sequences in U3 (SIVmac239Δ4) maintained ability to induce reasonable levels of protection against

vaginal challenge (Desrosiers *et al.*, 1998, Johnson *et al.*, 1999). A SIVmac239Δvif construct, which could only grow consistently on vif-complementing cells, was able to infect rhesus monkeys and to elicit persistent, albeit weak, immune responses (Johnson *et al.*, 1999). Thus, even severely attenuated viruses retain ability to induce protective immune responses, something that no other vaccine strategy has been shown to accomplish. We believe that it is worthwhile to further attenuate viruses such as SIV until they are devoid of disease-causing ability, and to then increase their capacity to elicit protective immunity through improved immunization protocols (Temin, 1993). As an example, a simplified bovine leukemia virus (BLV) has been successfully generated, and in vivo studies have shown that it is both immunogenic and safe, and can induce protective immune responses against wild-type viruses in rabbits (Boris-Lawrie and Temin. 1995, Boris-Lawrie *et al.*, 1997, Boris-Lawrie *et al.*, 2000). As shown here, we have constructed simplified forms of SIV that might now be studied in primate models. Toward this end, we eliminated all of the nonstructural genes of SIV through large deletions, and introduced three functional elements in their stead to restore viral production. First, a 173 bp CTE of SRV-1 was used to increase viral genomic RNA transportation, because this element has been shown to compensate for deficits of the Rev/RRE post-transcriptional regulation system (Tang *et al.*, 1997, Von Gegerfelt *et al.*, 1999, Zolotukhin *et al.*, 1994). These findings are confirmed by our insertion of the CTE into the Δ6 construct, resulting in increased expression of viral structural proteins (Δ6CTE, Figures 4,5).

Tat is essential for the replication of both HIV and SIV. We therefore employed a strong promoter, i.e. the CMV immediate early promoter, to increase the efficiency of transcription and to partially compensate for the deletion of tat, since previous works have shown the rationale for this approach (Chang, and Zhang. 1995). We found that a chimeric CMV-LTR, when introduced into the Δ6CTE construct, significantly increased the expression of viral structural genes (Δ6CC, Figures 4 and 5). Remarkably, this CMV-LTR can also drive the efficient replication of the Δ2-CMV, Δ4-CMV and Δ5-CMV mutants in MT4 cells (Fig10B, and C).

Although Vif has been shown to be essential for replication of both HIV-1 and SIV, several groups have suggested that this effect may be cell-type dependent. In the case of

CEMx174 cells, *vif*-deficient SIVmac239 mutants were able to establish productive infection (Zou and Luciw, 1996). Others, however, have suggested that replication of vif-mutated SIVmac239 viruses in CEMx174 cells was severely restricted. The Desrosiers group has shown that Δ5 (vif-) mutants replicated to high levels after prolonged culture in CEMx174 cells, while other vif- viruses displayed only low-level replication in this same cell line (Gibbs *et al.*, 1994). Our data are similar in that a construct that retained the vif gene, i.e. Δ5CCI, showed similar replication patterns in both CEMx174 and MT4 cells as did the vif-deficient Δ6CCI virus. Therefore, replication of a SIVmac239 mutant, that lacks vif, can occur, albeit with impairment, in CEMx174 cells.

Our Δ6CCI mutant is at least as attenuated as the Δ5(vif-) virus of the Desrosiers group, but the additional removal of both tat and rev, which are important in the pathogenesis of HIV-1(Gallo 1999, Mabrouk *et al.*, 1991, Rappaport *et al.*, 1991, Van Baalen *et al.*, 1997, Zagury *et al.*, 1998), may provide an extra margin of safety. It has been shown that vaccination with proviral DNA, that encodes intact but noninfectious viruses, may induce a protective immune response. Our Δ6CCI construct retains ability to produce all viral structural proteins, to form mature virions and to transiently infect target cells, while being severely impaired in regard to replication. These properties make it a good DNA vaccine candidate, since conformational epitopes that are exposed only during infection are believed to elicit cross-subtype immune responsiveness (LaCasse *et al.*, 1999 retracted).

In the case of HIV-1, *vif*-defective viruses have been shown to persistently replicate in primary macrophages and were able to enter PBMCs with the same efficiency as wild-type virus. The fact that *vif*-negative SIV could induce antibody response in macaques after a single injection suggests that these viruses were able to complete at least a single round of infection *in vivo*. Theoretically, our Δ6CCI construct should also retain this ability. At the same time, the deficiency of propagation of our Δ6CCI construct seen in primary cells might not compromise its utility as a proviral DNA vaccine.

Our large deletions had removed sequences between the *gag-pol* and *env* genes, including splice acceptor sites for *env*, therefore diminishing the translation of the latter gene (Figure 6). This was corrected by introduction of a functional poliovirus-derived IRES between the *gag-pol* and *env* genes; the result was that *env* gene expression was rendered

independent of splicing and dependent on the same mRNA as involved in expression of the gag-pol gene. The presence of the IRES in the Δ6CC construct significantly increased the expression of viral structural proteins and especially that of Env (Figure 6, Δ6CCI). Furthermore, the Δ6CCI construct produced viral particles with comparable efficiency to wild-type SIV constructs. However, this simplified SIV, that contained only viral structural genes, was extremely attenuated in replication capacity in CEMx174 cells (Figure 9).

Our simpler SIVs differ from other, partially deleted SIV constructs (Gibbs *et al.*, 1994) in that our viruses contain only structural genes. These simplified SIVs are live, but diminished in replication capacity and are presumably attenuated due to the deletion of nonstructural genes and the functional loss of these regulatory elements. Although few mechanistic studies have been performed on viruses of this type, it is known that HIV that was inactivated in regard to the *rev*/RRE system, by replacement of *rev* with a CTE, regained replication competence while displaying deficient processing of the Gag precursor protein Pr55 (Zolotukhin *et al.*, 1994). Similar results have now been observed with our simplified SIVs. However, this effect was not due solely to abrogation of the rev/RRE system, since our Δ5 construct, that retains rev/RRE, displayed similar patterns of deficiency (Fig.6). Furthermore, an even simpler construct, Δ6, was deficient in both specificity and efficiency of encapsidation of viral genomic RNA. We found that replacement of rev by the CTE element partially compensated for this impairment in specificity, and that introduction of a stronger promoter, i.e. CMV, even further increased the specificity of packaging. These results indicate that packaging of viral genomic RNA may require efficiency in regard to both transcription and transport of viral genomic RNA. The fact that insertion of an IRES, i.e. construct Δ6CCI, even further increased the efficiency of packaging also indicates that both the expression and length of viral genomic RNA may be key factors in this regard.

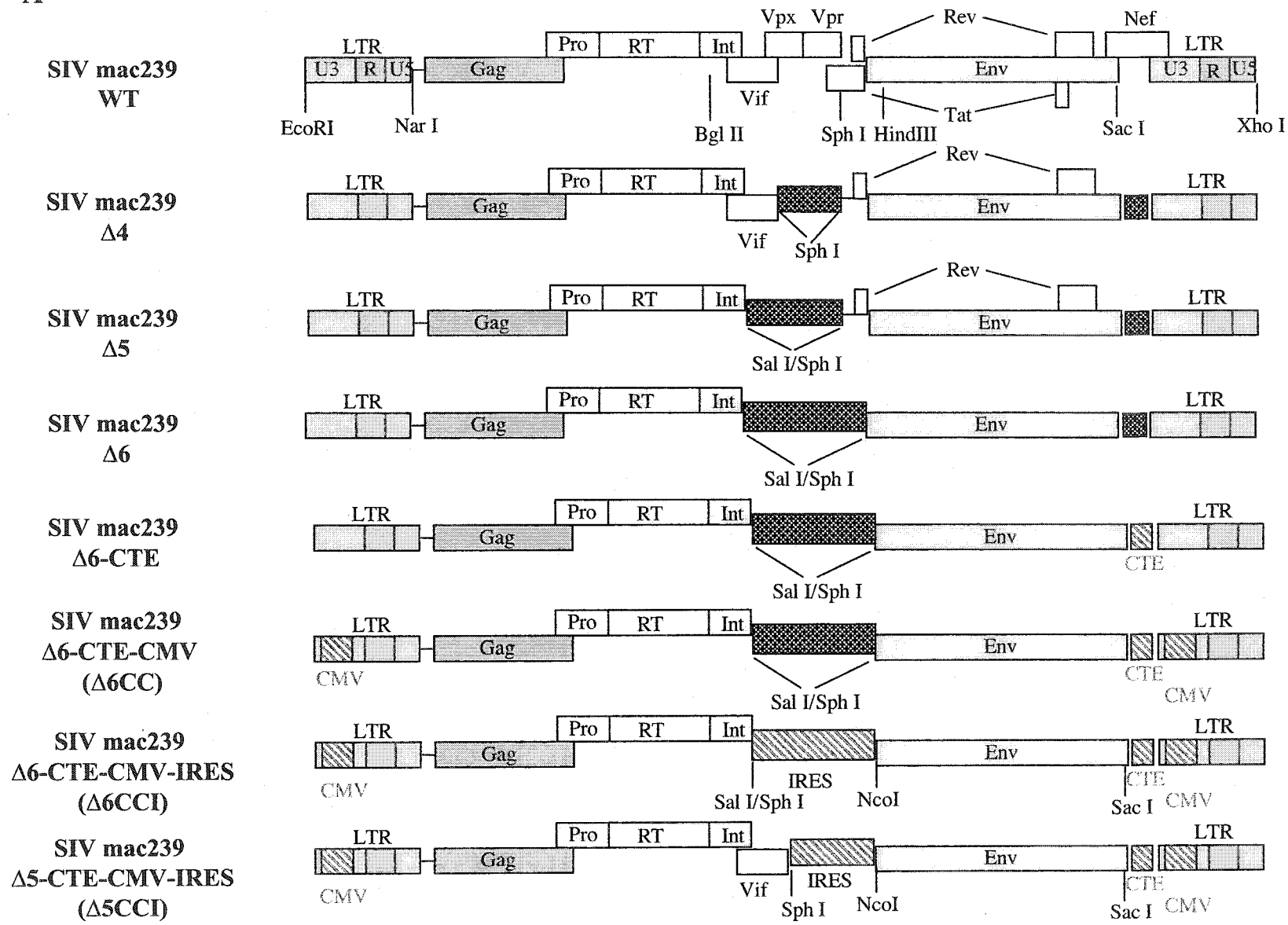
However, If these simplified viruses are to be used as live attenuated vaccines, further modifications may be required to improve their replication capacity in order to efficiently generate protective immunity (Johnson *et al.*, 1999). In this context, our simplified SIV constructs still contain certain trans-activated elements within the LTR, such as TAR sequences, that may affect SIV replication in the absence of Tat. Previous work has

shown that the strong CMV promoter might only partially correct for the absence of Tat protein (Chang and Zhang, 1995). Our results also show that the CMV promoter can only rescue our tat-negative viruses in MT4 but not in CEMx174 cells. We are planning to generate SIV constructs that contain LTRs of simpler retroviruses in order to have viruses for study that are fully independent of retroviral trans-activated factors (Boris-Lawrie and Temin. 1995, Butsch *et al.,* 1999, Temin, 1993). We next hope to evaluate the infectivity, safety, immunogenicity, and protective ability of our constructs in macaque monkeys by inoculation of viral DNA constructs.

Fig. 5.1 Schematic illustration of the SIV constructs generated.

All enzyme sites used in these constructs are indicated, and both deletions ( ) and alternative elements ( ) are shown. (A) Construction of simplified SIVs. All the mutants contain two deletions. In the case of SIVmac239 Δ4, one deletion involves the vpx, vpr and tat genes (from positions 6241 to 6702) while the other results in inactivation of nef (positions 9500 to 9674). The SIVmac239 Δ5 and Δ6 constructs are identical to Δ4, except that the first deletion was extended to also delete the vif gene(positions 5667 to 6702) and both the vif and rev genes (positions 5667 to 6859), respectively. In order to generate Δ6 CTE, a 173-bp CTE of SRV-1 was inserted into the Δ6 vector at the position of the nef deletion. The Δ6-CTE-CMV construct was derived from Δ6CTE by replacing both the 5' and 3' LTRs with a chimeric LTR containing the CMV IE promoter. The Δ6CTE-CMV-IRES (Δ6CCI) construct was generated by insertion of an IRES element immediately upstream of the env gene in the Δ6-CTE-CMV vector. The Δ6CCI constructs that contained either a poliovirus-derived IRES or a ECMV-derived IRES are designated Δ6CCI-P and Δ6CCI-E, respectively. The Δ5CCI construct is identical to Δ6CCI-P, except that the vif gene is retained. (B) Construction of SIV mutants that retain the rev gene. The SIVmac239Δnef-CMV construct contains both the chimeric CMV-LTR insert and the nef deletion, while Δ2-CMV contains additional mutations in the first exon of tat (that truncates the tat gene). Δ4-CMV and Δ5-CMV are identical to Δ4 and Δ5 except that both the 5' and 3' LTRs were replaced with the chimeric CMV-LTR. (C) Construction of the simplified SIV vector, SIVmac239Δ6-CTE-CMV-IRES-GFP (Δ6CCI-GFP), containing the EGFP reporter gene. Similar to the case of Δ6CCI, the Δ6CCI-GFP contained either a poliovirus-derived IRES or a ECMV-derived IRES are termed Δ6CCI-P-GFP and Δ6CCI-E-GFP, respectively.

A


SIV mac239 WT
LTR
U3
R
U5
Gag
Pro
RT
Int
Vpx
Vpr
Vif
Rev
Env
Nef
Tat
LTR
U3
R
U5
EcoRI
Nar I
Bgl II
Sph I
HindIII
Sac I
Xho I
SIV mac239 Δ4
Vif
Sph I
SIV mac239 Δ5
Sal I/Sph I
SIV mac239 Δ6
Sal I/Sph I
SIV mac239 Δ6-CTE
CTE
Sal I/Sph I
SIV mac239 Δ6-CTE-CMV (Δ6CC)
CMV
CTE
CMV
Sal I/Sph I
SIV mac239 Δ6-CTE-CMV-IRES (Δ6CCI)
CMV
IRES
Sal I/Sph I
NcoI
CTE
Sac I
CMV
SIV mac239 Δ5-CTE-CMV-IRES (Δ5CCI)
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Sph I
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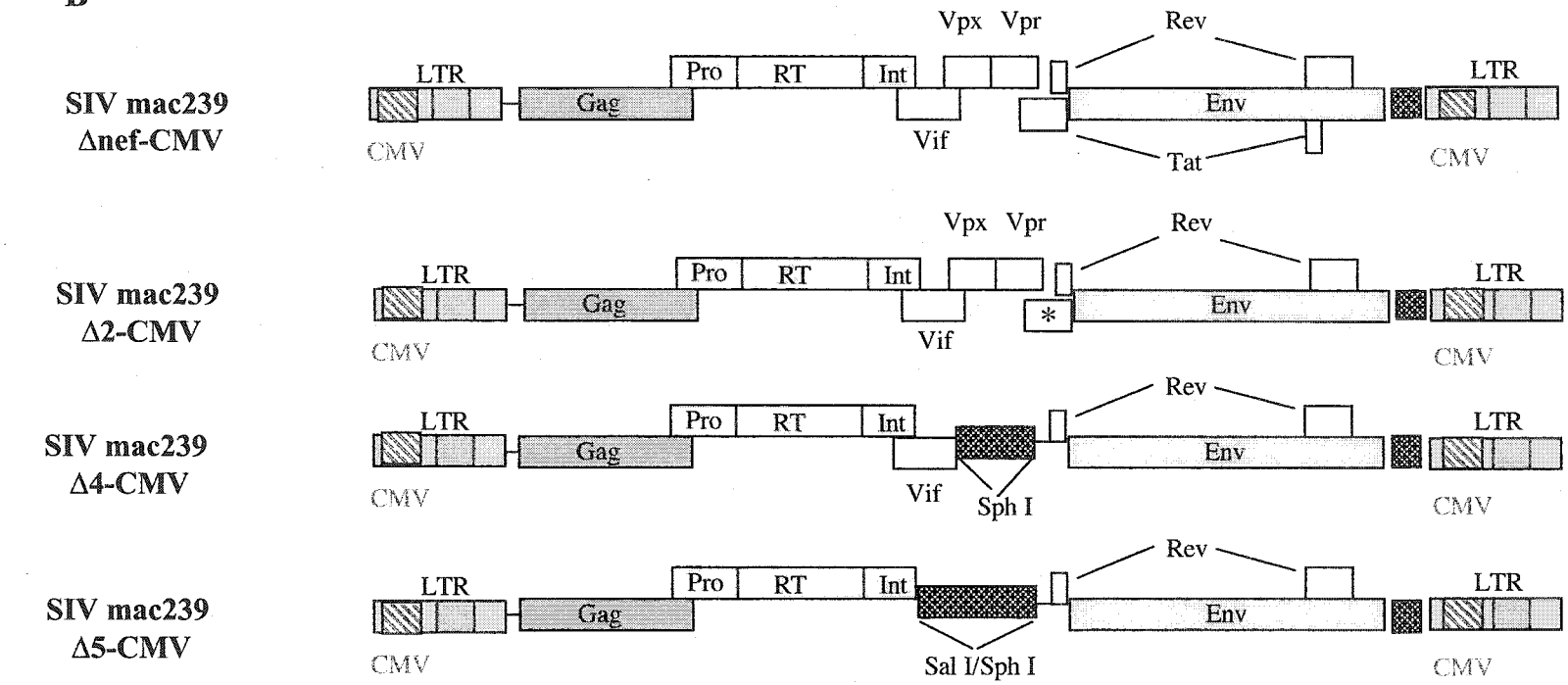
Sac I
CMV

Figure 5.1

B


SIV mac239
Δnef-CMV
Vpx Vpr
Rev
LTR
Pro
RT
Int
Gag
Env
LTR
CMV
Vif
Tat
CMV
SIV mac239
Δ2-CMV
Vpx Vpr
Rev
LTR
Pro
RT
Int
Gag
*
Env
LTR
CMV
Vif
CMV
SIV mac239
Δ4-CMV
Rev
LTR
Pro
RT
Int
Gag
Env
LTR
CMV
Vif
Sph I
CMV
SIV mac239
Δ5-CMV
Rev
LTR
Pro
RT
Int
Gag
Env
LTR
CMV
Sal I/Sph I
CMV

C

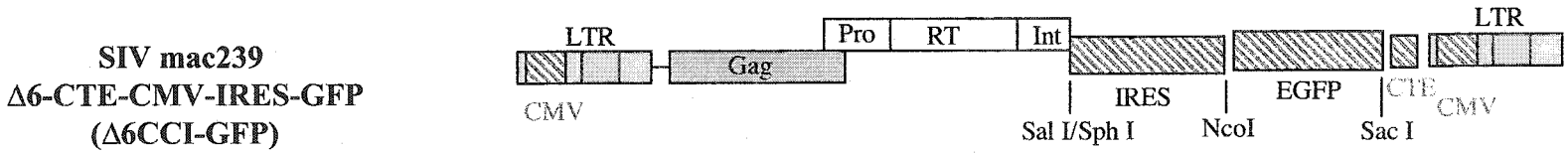

SIV mac239
Δ6-CTE-CMV-IRES-GFP
(Δ6CCI-GFP)
LTR
Pro
RT
Int
LTR
Gag
IRES
EGFP
CTE
CMV
CMV
Sal I/Sph I
NcoI
Sac I

Figure 5.1

Fig. 5.2 Growth curves of viruses containing a chimeric CMV LTR.

Equivalent amounts of viruses were used to infect CEMx174 cells. Viral replication was monitored by RT assay of culture fluids. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control.

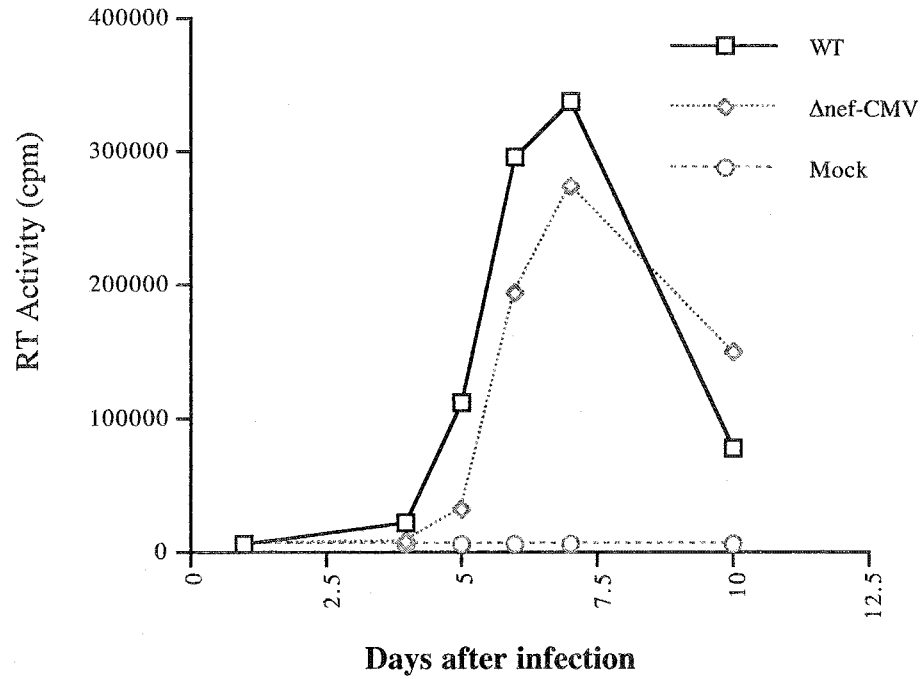

400000
300000
200000
100000
0
RT Activity (cpm)
0
2.5
5
7.5
10
12.5
Days after infection
WT
Δnef-CMV
Mock

Figure 5.2

Fig. 5.3 FITC-gated FACS analysis of simplified SIV vectors.

SIV-Δ6-CTE-CMV-IRES-GFP vectors containing the IRES of EMCV, i.e. construct Δ6CCI-E-GFP, or of poliovirus, i.e. construct Δ6CCI-P-GFP, were transfected into COS-7 and the expression of GFP was analyzed by flow cytometry. The percentage of GFP-positive cells is indicated in each of these graphs. The x axis designates cell number while the y axis refers to the fluorescence density of GFP. Mock denotes transfection of COS-7 cells by the Δ6CCI construct, which lacks the GFP gene, as a negative control; hence the background of fluorescence in these studies was about 10%.

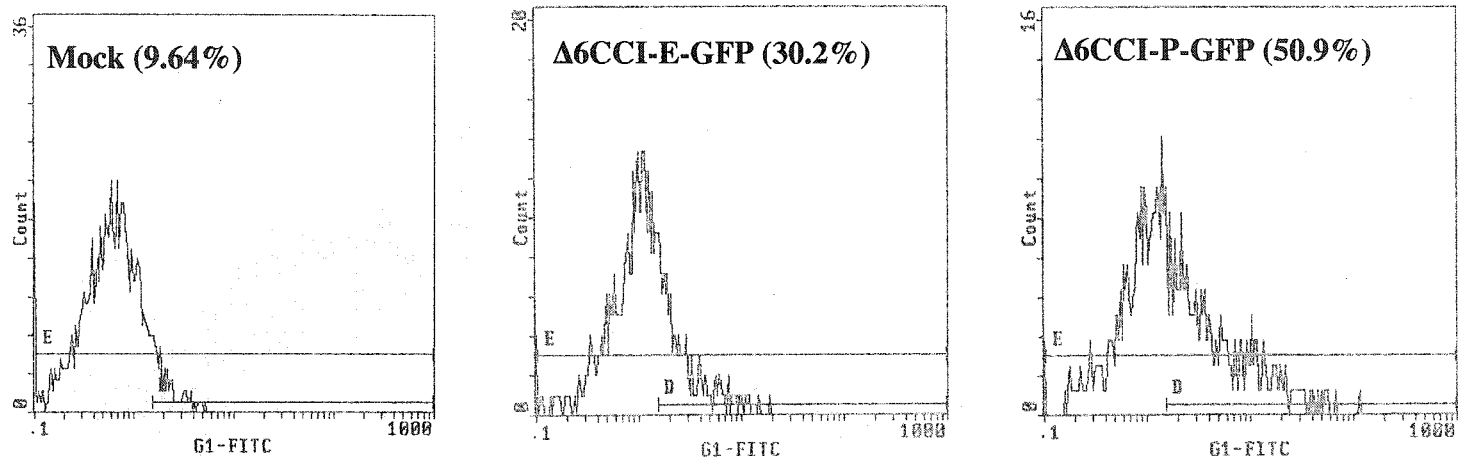
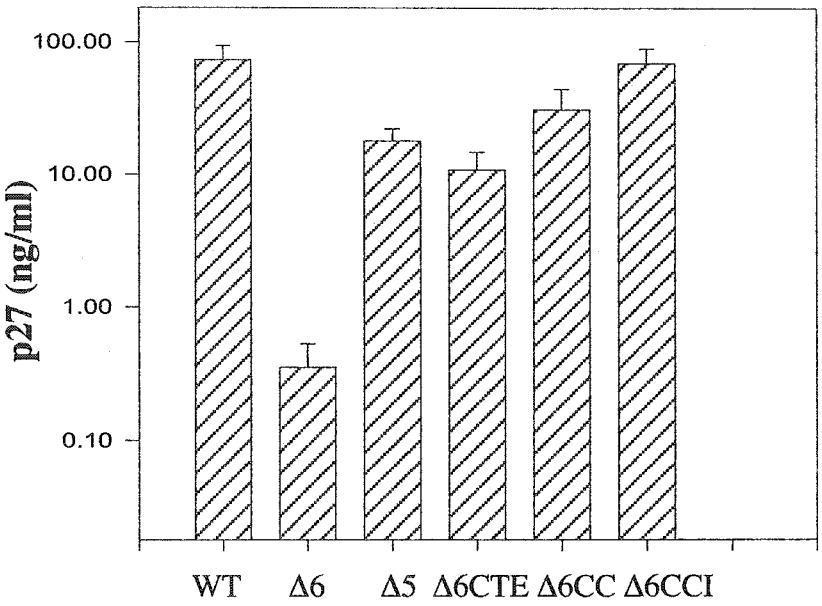


Figure 5.3

Fig. 5.4 Virus production following transfection of COS-7 cells.

SIV wild-type or mutant constructs were transfected into COS-7 cells. Levels of RT activity and SIV p27 antigen in culture fluids were quantified at 60 hours after transfection and plotted. Results were calculated on the basis of three independent transfections and are shown as averages ± standard deviation.


100.00
10.00
1.00
0.10
p27 (ng/ml)
WT
Δ6
Δ5
Δ6CTE
Δ6CC
Δ6CCI

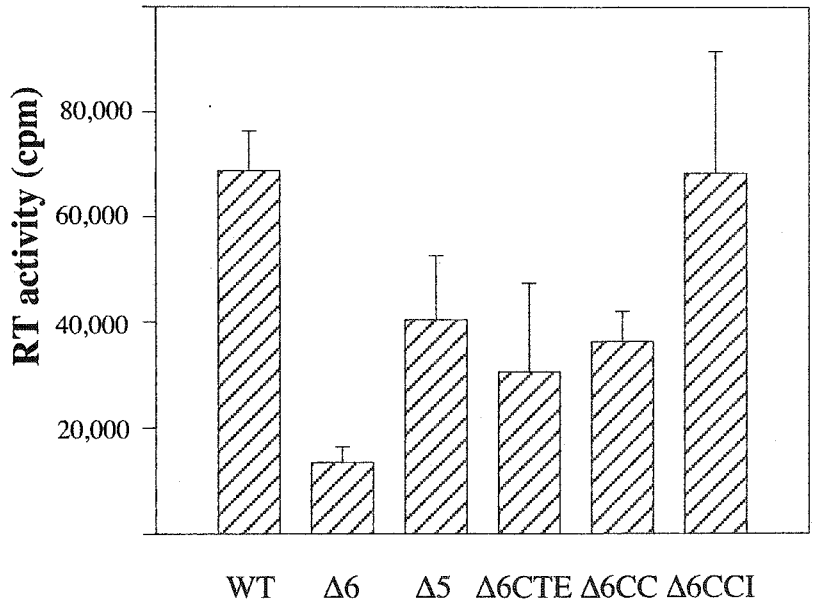

80,000
60,000
40,000
20,000
RT activity (cpm)
WT
Δ6
Δ5
Δ6CTE
Δ6CC
Δ6CCI

Figure 5. 4

Fig. 5.5 Protein patterns of viral particles

^{35}S-labeled viral progeny that were released during 1 hour by transfected COS-7 cells were purified at 24 hours after transfection. Proteins were analyzed by PAGE. Mock denotes transfection of COS-7 cells by vector pSP73, not containing any SIV genomic material, as a negative control.

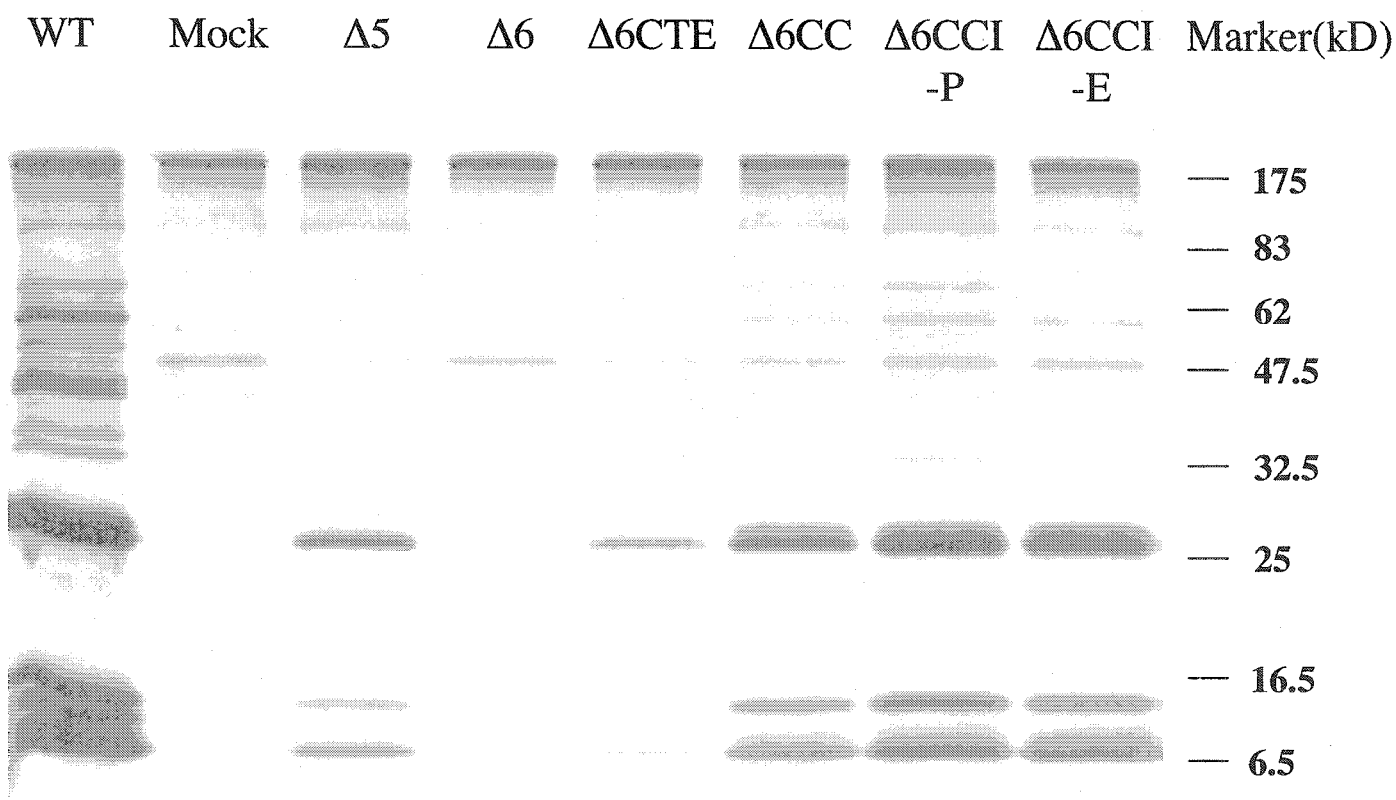

WT
Mock
Δ5
Δ6
Δ6CTE
Δ6CC
Δ6CCI -P
Δ6CCI -E
Marker(kD)
175
83
62
47.5
32.5
25
16.5
6.5

Figure 5.5

Fig. 5.6 Identification of SIV gp120 and p27 antigens

COS-7 cells transfected with wild type or mutated SIV constructs were radiolabelled with both ^{35}S-methione and ^{35}S-cysteine, and viral proteins in cell lysates were then immunoprecipitated with MAbs against SIV gp120 or p27 antigen. The bands of gp120 and Gag proteins are shown. Mock denotes transfection of cells by vector pSP73 as a negative control.

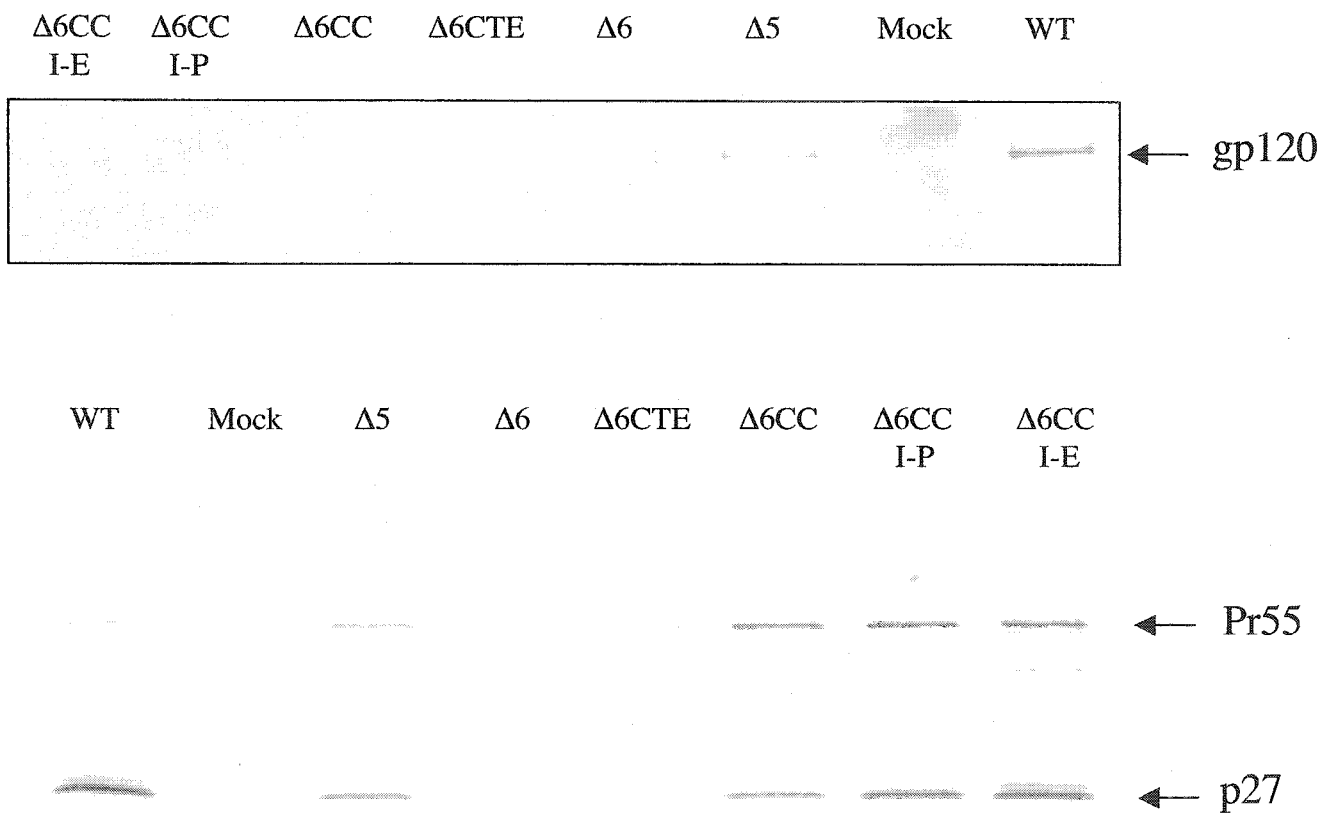

Δ6CC I-E
Δ6CC I-P
Δ6CC
Δ6CTE
Δ6
Δ5
Mock
WT
gp120
WT
Mock
Δ5
Δ6
Δ6CTE
Δ6CC
Δ6CC I-P
Δ6CC I-E
Pr55
p27

Figure 5.6

Fig. 5.7 Morphology of virions produced by wild type or simplified SIV constructs. COS-7 cells transfected with wild type or simplified SIV constructs were fixed, at 40-hr post transfection, then embedded, sectioned, and stained. Ultrastructure was visualized by a JEOL 200 FX electron microscope. The bar represents 100nm. Mature virions are indicated by the arrows.

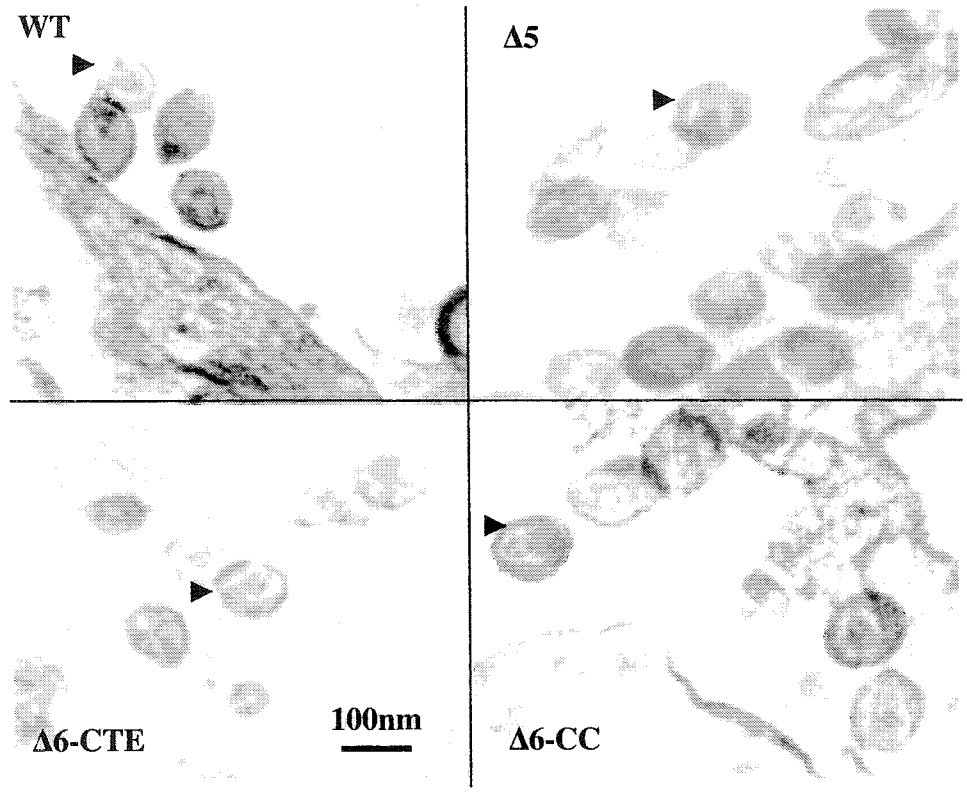
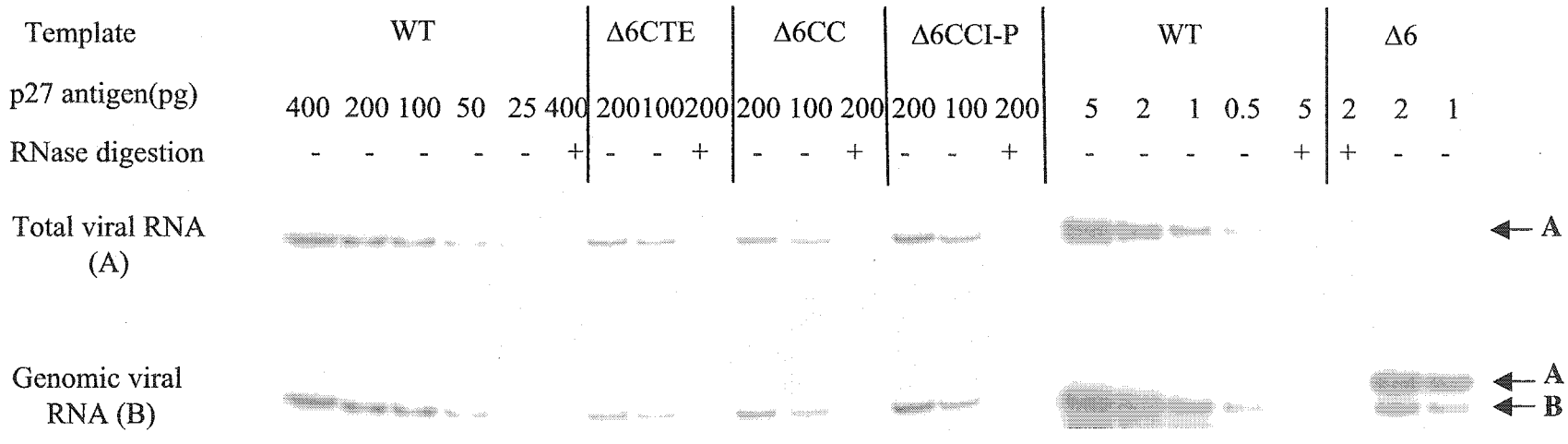


Figure 5.7

Fig. 5.8 Packaging of viral RNA as assessed by RT-PCR.

RNA was purified from viruses derived from transfected COS-7 cells. Equivalent amounts of virus, based on levels of p27 antigen, were used as template in quantitative RT-PCR to detect the presence of total viral RNA (A) and of the full-length viral RNA genome (B) in 18-cycle PCR reactions. Reactions run with RNA template that had been digested by Dnase-free RNase served as a negative control for each sample to exclude any potential DNA contamination. Relative amounts of a 114-bp DNA product representing full-length viral RNA (B) were quantified by molecular imaging, with wild-type levels arbitrarily set at 1.0, to determine the efficiency of genomic RNA packaging. The relative amounts of full-length viral RNA (B) to total viral RNA (A) in each sample were also quantified to determine the specificity of viral RNA packaging. The relative amounts of viral RNA that were packaged were determined on the basis of four different experiments and are shown as averages± standard deviation.



Relative amount of viral genomic RNA	1.0	0.46±0.12	0.54±0.08	1.09±0.10	1.0	0.52±0.10
Ratio of viral genomic RNA to total viral RNA	1.13±0.17	0.75±0.14	0.97±0.23	1.02±0.05	1.05±0.11	0.19±0.04

Figure 5.8

Fig. 5.9 Detection of viral DNA and p27 antigen after infection of CEMx174 cells. (A) Equivalent amounts of wild-type (WT) or modified viruses, based on p27 content, were used to infect CEMx174 cells. Viral replication was monitored by p27 antigen assay of culture fluids. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control. The dashed line, representing 0.01ng/ml p27, indicates the threshold sensitivity of the assay. (B). At various times post-infection, cellular DNA was analyzed by PCR using primers sg1 and sg2 to amplify a 114-bp fragment in the gag region (20). PCR products were separated on 2% agarose gels. Lane 1: infection by WT virus after 4 days; lane 2: infection by heat-inactivated WT virus after 4 days; lanes 3-6: infection by Δ6CCI-P virus at days 4, 7, 14 and 21, respectively, after infection; lane 7: infection by heat-inactivated Δ6CCI-P virus after 4 days. M designates 100bp ladder. (C) PCR analysis of viral DNA in transiently infected CEMx174 cells, as described in Materials and Methods. The 114-bp band of viral DNA and the 567-bp band of β-actin cellular DNA employed as an internal control are indicated. Infections performed and maintained at 4°C served as negative controls for each of the constructs.

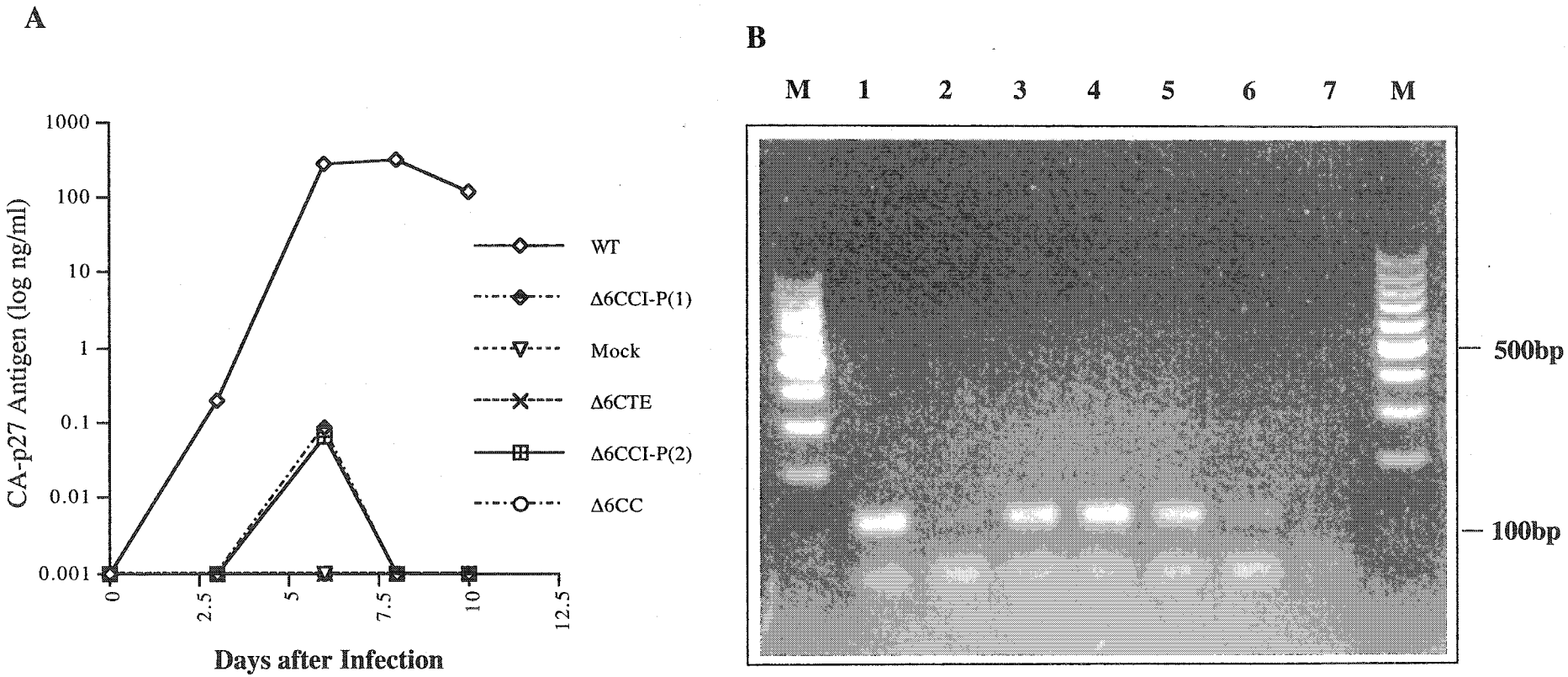

A
1000
100
10
1
0.1
0.01
0.001
CA-p27 Antigen (log ng/ml)
0
2.5
5
7.5
10
12.5
Days after Infection
WT
Δ6CCI-P(1)
Mock
Δ6CTE
Δ6CCI-P(2)
Δ6CC
B
M
1
2
3
4
5
6
7
M
500bp
100bp

Figure 5.9

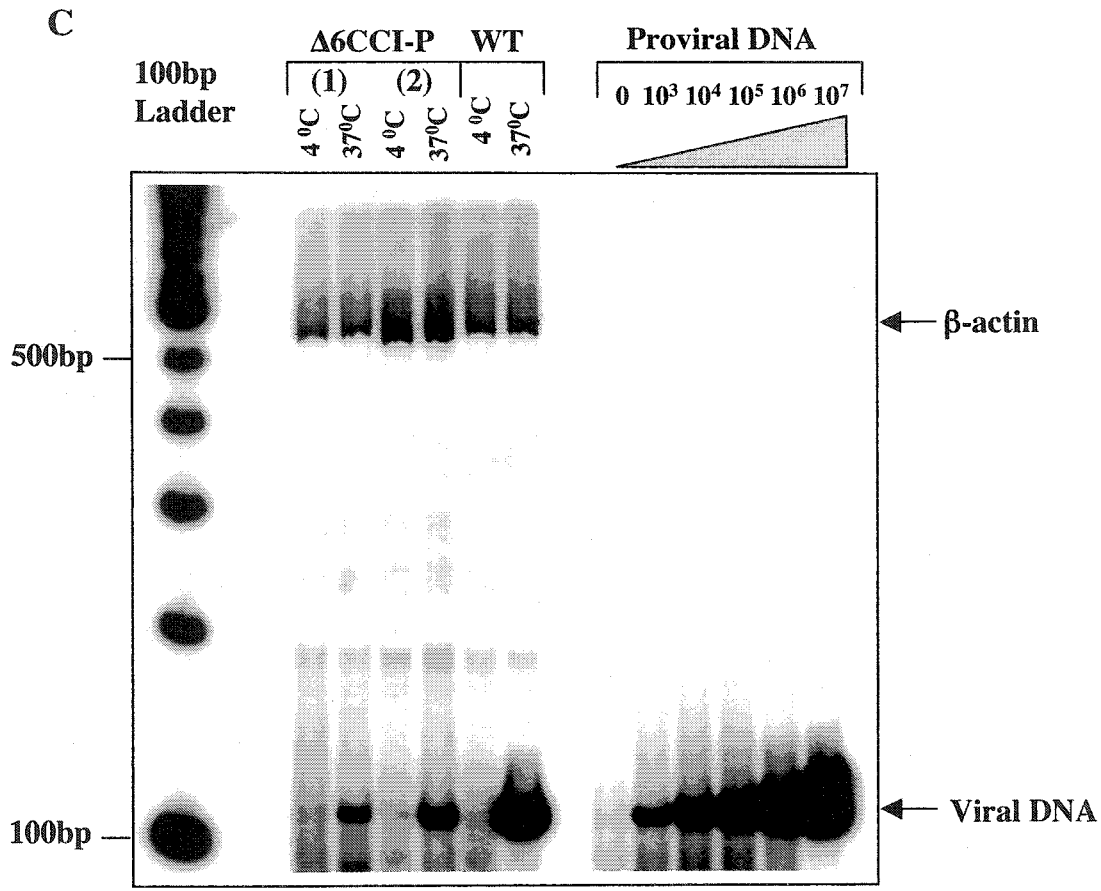

C
100bp
Ladder
Δ6CCI-P
(1)
(2)
WT
4 °C
37°C
4 °C
37°C
4 °C
37°C
Proviral DNA
0 10³ 10⁴ 10⁵ 10⁶ 10⁷
500bp
100bp
β-actin
Viral DNA

Figure 5.9

Fig. 5.10 Replication capacity of the Δ4 and Δ5 constructs.

Equivalent amounts of WT or modified viruses, based on p27 content, were used to infect both CEMx174 and MT4 cells. Viral replication was monitored by p27 antigen assay of culture fluids. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control. The dashed line, representing 0.01ng/ml p27, indicates the threshold sensitivity of the assay. (A) Growth curve in CEMx174 cells. (B) Growth curves in MT4 cells. (C) Growth curves of second passage MT4-derived viruses, i.e. from the experiment of Fig. 10B, in fresh MT4 cells.

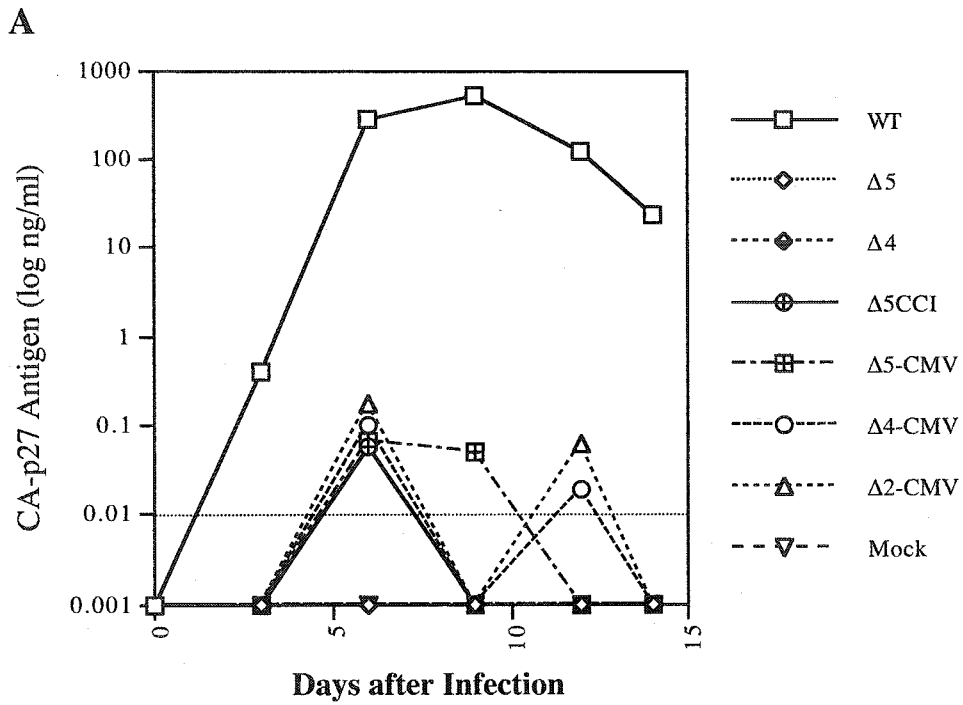

A
1000
100
10
1
0.1
0.01
0.001
CA-p27 Antigen (log ng/ml)
0
5
10
15
Days after Infection
WT
Δ5
Δ4
Δ5CCI
Δ5-CMV
Δ4-CMV
Δ2-CMV
Mock

Figure 5.10

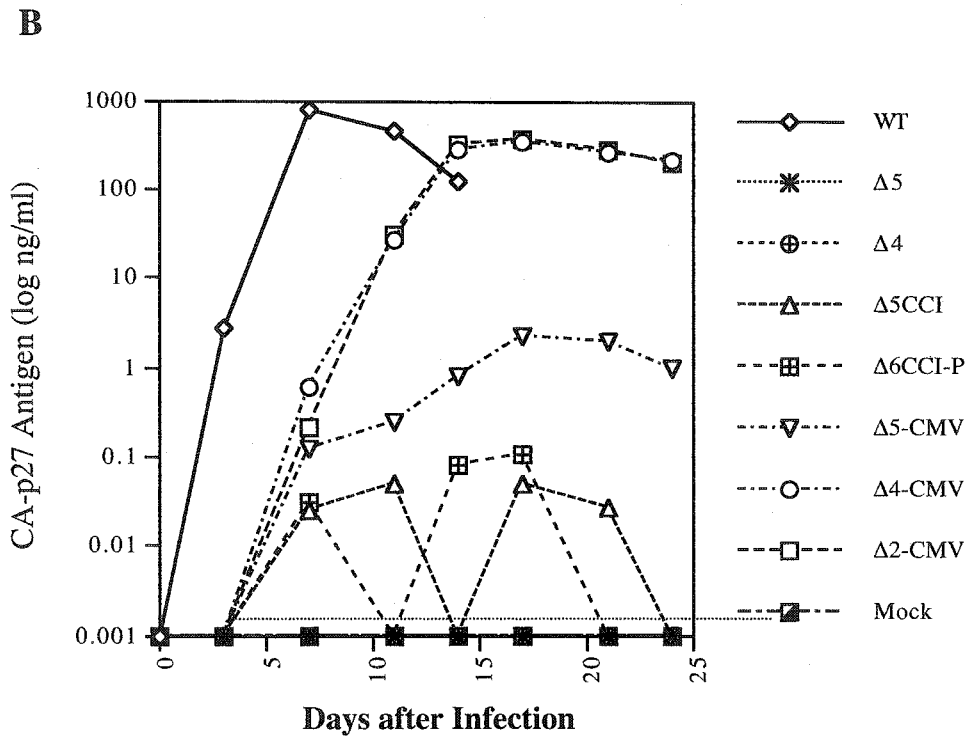

B
1000
100
10
1
0.1
0.01
0.001
CA-p27 Antigen (log ng/ml)
0
5
10
15
20
25
Days after Infection
WT
Δ5
Δ4
Δ5CCI
Δ6CCI-P
Δ5-CMV
Δ4-CMV
Δ2-CMV
Mock

Figure 5.10

C

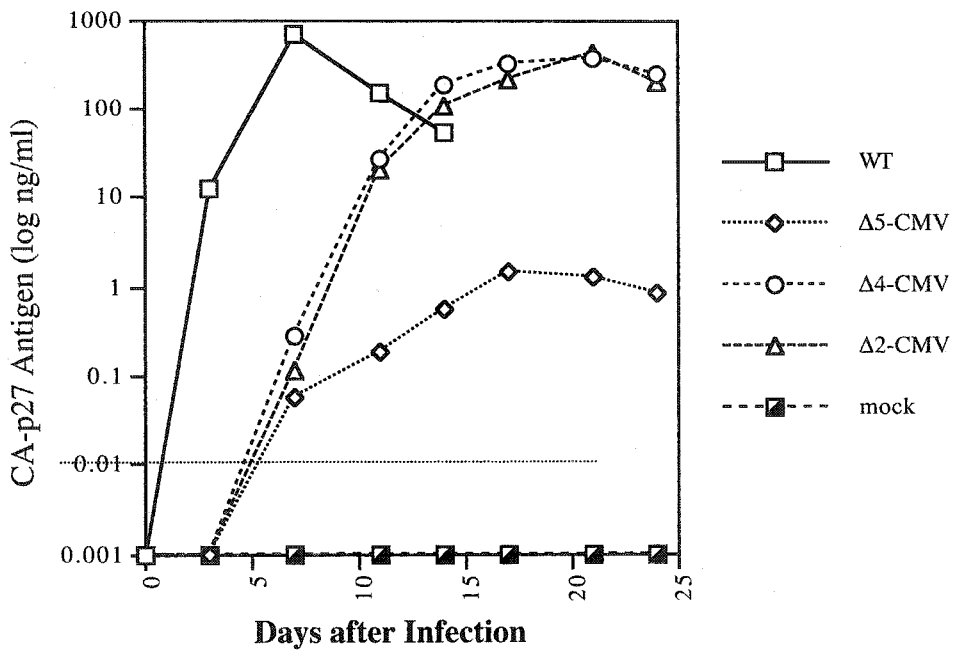

1000
100
10
1
0.1
0.01
0.001
CA-p27 Antigen (log ng/ml)
0
5
10
15
20
25
Days after Infection
WT
Δ5-CMV
Δ4-CMV
Δ2-CMV
mock

Figure 5.10

Fig. 5.11 Infection of Monkey PBMCs.

(A) Equivalent amounts of WT or modified viruses, based on p27 content, were used to infect monkey PBMCs. Viral replication was monitored by p27 antigen assay of culture fluids. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control. The dashed line, representing 0.01ng/ml p27, indicates the threshold sensitivity of the assay. (B) PCR analysis of viral DNA in transiently infected monkey PBMCs, as described in Materials and Methods. The 114-bp band of viral DNA is indicated. Infections performed and maintained at 4°C served as negative controls for each of the constructs.

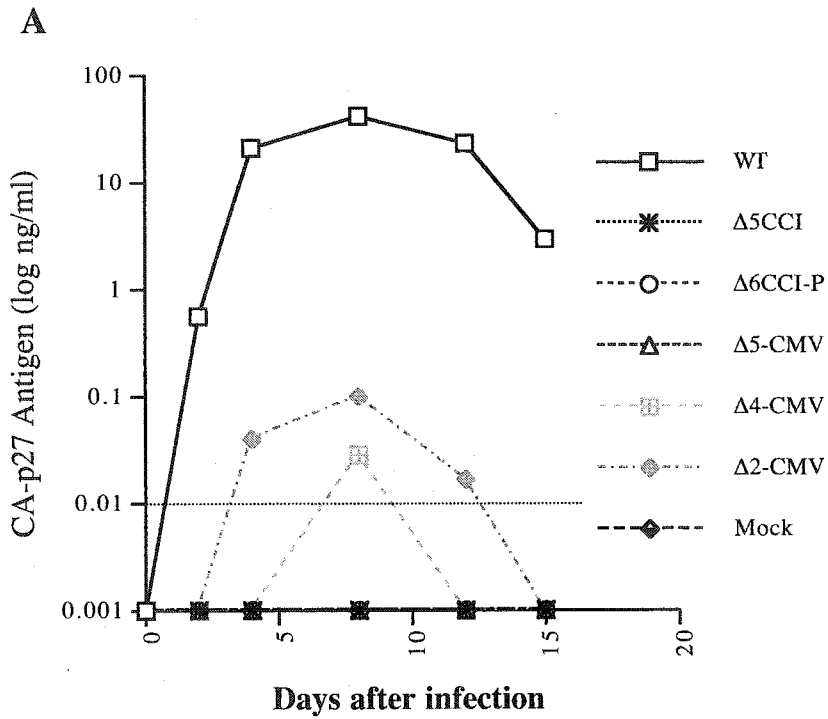

A
100
10
1
0.1
0.01
0.001
CA-p27 Antigen (log ng/ml)
0
5
10
15
20
Days after infection
WT
Δ5CCI
Δ6CCI-P
Δ5-CMV
Δ4-CMV
Δ2-CMV
Mock

Figure 5.11

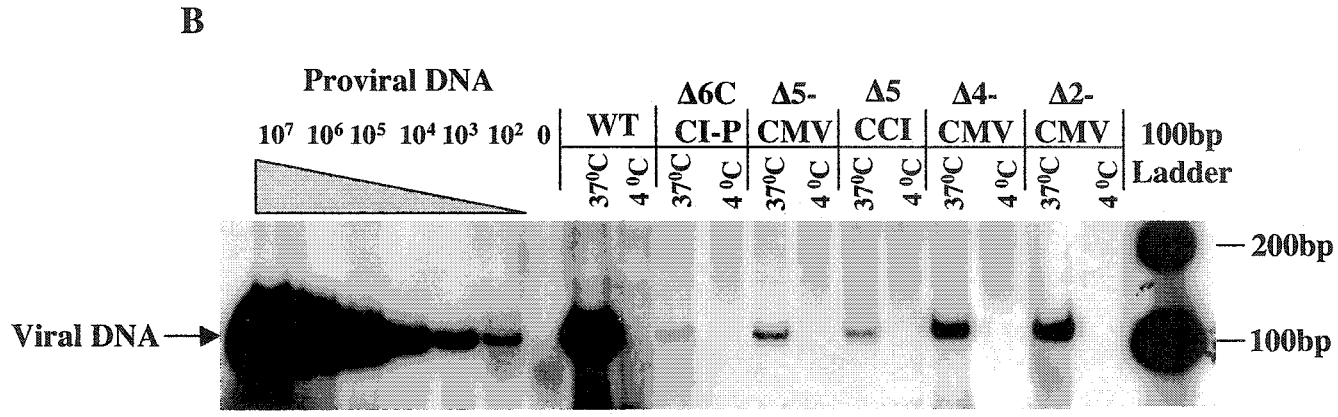

B
Proviral DNA
10⁷ 10⁶ 10⁵ 10⁴ 10³ 10² 0
WT
Δ6C CI-P
Δ5- CMV
Δ5 CCI
Δ4- CMV
Δ2- CMV
100bp Ladder
37⁰C
4 ⁰C
Viral DNA
200bp
100bp

Figure 5.11

Chapter 6

The M184V Mutation in Reverse Transcriptase Can Delay Reversion of Attenuated Variants of Simian Immunodeficiency Virus

This chapter is an adaptation from an original note published in the *Journal of Virology* 2002; 76(17): 8958-62. Authorship is as follows: **James B. Whitney**, Maureen Olivera, Mervi Detorio, Yongjun Guan, and Mark A. Wainberg. All experiments were completed by myself; manuscript preparation was with the assistance of Dr. Wainberg. Both M. Olivera, M. Detorio provided technical assistance with RT assays. We thank Y.G. for the contribution of the SD2 mutants utilized in these studies.

6.1 Preface

A major advantage of an attenuated virus strategy for the development of a human immunodeficiency virus (HIV) vaccine is the ability of live attenuated viruses to engender broad and persistent immunity. Numerous live attenuated candidates have been assessed in the preclinical SIV/macaque model, many of which employ deletions in the viral accessory genes. Non-human primates inoculated with certain attenuated variants of SIV have yielded protection against subsequent challenge by virulent SIV and SHIV strains. Lentiviruses are known to exhibit remarkable genomic pliancy, a capacity that has been attributed to one or more "error prone steps" in the viral replication cycle. Consequently major caveat to the live attenuated approach is one of safety due to the potential for compensatory reversion, and/or that incompletely attenuated viruses may harbor residual pathogenicity. Even multiply deleted SIV constructs, containing deletions in *nef*, *vpr* and the negative regulatory element (NRE), were shown to be pathogenic in both infant and adult macaques, indicating that these variants were merely replication impaired, not apathogenic. This may be analogous to some human long-term nonprogressors infected by nef-deleted variants of HIV, in whom a slowly increasing viral burden was accompanied by disease progression. Recently published longitudinal studies have further corroborated the pathogenic potential, particularly of the *nef* attenuated variants. In 1999 the Desrosier group, among others, published a paper regarding the protective efficacy of live attenuated vaccines as being inversely correlated with the attenuation level (Johnson *et al.*, 1999). Therefore attenuated for HIV vaccines capable of replicative vigor and capability of reversion should be viewed as proportionally equivalent. This final chapter, again building upon the findings of chapter 1, investigates potetial means of limiting the reversion capacity inherent in live attenuated vaccine designs.

6.2 Abstract

We have previously constructed a series of simian immunodeficiency virus (SIV) mutants containing deletions within a 97 nt region of the SIVmac239 untranslated region (UTR) or leader sequence. However, as is common with live attenuated viruses, several of the mutants exhibited a moderate propensity for reversion. Since the M184V mutation in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase is associated with diminished fitness as well as lamivudine resistance, we introduced this substitution into several of our deletion mutants to determine its effects on viral replication and compensatory reversion. Our results indicate that M184V impaired viral fitness in pair-wise comparisons of mutants that contained or lacked this substitution. We also observed that M184V significantly impaired the potential for both compensatory mutagenesis and reversion in these mutants both in cell lines and in PBMCs.

6.3 Introduction

Genetic variation in HIV-1 and other retroviral lineages has been associated with multiple factors that include viral recombination, reverse transcriptase infidelity, and both viral and cellular factors that can influence mutation rates (Goncalves *et al.*, 1996, Hu and Temin 1990, Mansky and Temin. 1995, Perelson *et al.*, 1996, Vartanian *et al.*, 1991). The generation of viral quasispecies as a result of reverse transcriptase infidelity is largely due to the lack within viral reverse transcriptase (RT) of a 3'-5' proofreading ability, combined with high rates of viral replication (Coffin 1995, Skalka and Goff 1993).

The catalytic domains of the RTs of HIV-1 and its simian counterpart, simian immunodeficiency virus (SIV), both include a highly conserved YMDD motif. This domain is common throughout the polymerase family of enzymes (Poch *et al.*, 1989), and mutations within this region are commonly lethal. However, viruses resistant to the antiretroviral nucleoside 2', 3'-dideoxy-3' thiacytidine (3TC) harbor a single M184V substitution within the aforementioned motif (Tisdale *et al.*, 1993). This same substitution in RT is associated with resistance to 3TC in the case of SIV (Cherry *et al.*, 1997).

In HIV-1, it is well documented that the M184V mutation also confers a deficit in fitness that is most apparent in primary cell lines (Back *et al.*, 1996). The reasons for this are multifaceted and include the fact that RT enzymes containing M184V are associated with diminished proccessivity (Back *et al.*, 1996, Back and Berkhout 1997, Newstein and Desrosiers 2001, Oude-Essink *et al.*, 1997, Quan *et al.*, 1998), diminished nucleotide primer unblocking (Gotte *et al.*, 2000), and diminished ability to initiate reverse transcription (Gotte *et al.*, 5th Int. Workshop on HIV Drug. Resist. Treat. Strat. 2001). These events are also modulated by intracellular dNTP substrate availability (Back and Berkhout 1997).

In the aftermath of deletion mutagenesis, leading to attenuation of replication, genetic variation requires passage through the constraints of an artificially produced bottleneck. Under these conditions, the spectrum of compensatory mutations is likely to be restricted (Rouzine and Coffin 1999, Rouzine *et al.*, 2001). In this study, we demonstrate that the M184V substitution can impair the viral capacity for reversion in the context of specific deletions within the 5' leader region of a series of attenuated SIVmac239 constructs. As

well, the presence of the M184V substitution may affect the process of compensatory mutagenesis in regard to codon change.

6.4 Results and Discussion

Several of our viral deletion mutants were previously shown to display moderate reversion kinetics over serial passage (Guan *et al.*, 2000, Guan *et al.*, 2001), i.e. constructs SD2, SD5, and SD6. The M184V mutation was introduced into the RTs of these constructs by site-directed mutagenesis of the pCRII vector containing 1.7-kb of the SIV RT coding region as described previously (Cherry *et al.*, 1997). The recovered M184V-containing RT fragment was then inserted between the *Nar I* and *BamHI* sites in the full length WT and mutant SIV proviral clones. All recombinant viruses were confirmed by sequencing.

After transfection of COS-7 cells with appropriate plasmid DNA using lipofectamine (GIBCO, Burlington, Ontario, Canada), viral supernatants were recovered and the concentration of p27 antigen in these stocks was quantified using a Coulter SIV core antigen assay kit (Immunotech Inc. Westbrook, ME, U.S.A.).

Viral Replication Assays. Viral inocula for each construct, equivalent to 10 ng p27 CA-antigen, were treated with DnaseI and used to infect CEMx174 cells. RT assays were used as a surrogate for viral replication and revealed that the presence of M184V together with the various deletions in the 5' leader resulted in an additional impairment in viral replication compared to when the M184V was not present. These results were observed consistently in replicate experiments, regardless which leader mutant was studied. The impairment for each mutant virus containing M184V was further amplified by the presence of 8μM 3TC, which further constrained viral replication by an additional 2-4 days (results not shown). This may have been due to additional selective pressure by 3TC to maintain the M184V mutation and prevent the outgrowth of revertant viruses.

To establish the potential for viral reversion over protracted periods, we performed serial passage or "forced evolution" of our mutant constructs using the CEMx174 cell line. Typically, aliquots of viral supernatants were taken at the observed peak of infection, and these samples were then used to infect fresh CEMx174 cells at doses equivalent to 10ng

viral p27 antigen. During each successive passage, viruses containing deletions in leader sequences plus M184V showed delays in growth kinetics and reduced replicative capacity as assessed by RT assay (results not shown).

Subsequent PCR and sequencing analysis at each passage of these M184V-containing variants indicated that the original leader sequence deletions were retained in all instances. As well, each of the three leader mutants that encoded the M184V mutation retained this mutation over at least three consecutive passages. However, both SD5-M184V and SD6-M184V had lost the M184V substitution by the fourth round of passage in the absence of 3TC. This sequence alteration was commensurate with a measurable increase in viral replication (see Fig 1, SD2-M184V and SD6-M184V shown only). In contrast, the SD2-M184V variant retained M184V until at least four passages. The latter variant also showed decreased RT activity and replication rates in comparison to either the SD5 or SD6 mutants at the same passage (SD2-M184V and SD6-M184V shown only). A similar number of clones from experiments performed under the pressure of 3TC were also sequenced and showed no loss of either the leader and/or M184V up to the same number of passages. As well, we carried out equivalent experiments with only the leader mutations under 3TC pressure and these cultures showed undetectable levels of viral replication, during two months of passage as assessed by RT assay.

To evaluate viral replication under more relevant cellular conditions, rhesus PBMCs were obtained from two healthy, SRV-1 negative rhesus macaques. These cells were stimulated with phytohemmaglutin (PHA) for 72 hr prior to infection as described (12). Infection and viral antigen production were assessed in complete RPMI-1640 culture media supplemented with 10% heat-inactivated fetal bovine serum and 20 U/ml IL-2 (GIBCO Inc. Burlington, Ontario, Canada) throughout the monitored period. Viral replication was assessed in culture in 10ml and 2ml total culture volume for both donors A and B, and fresh stimulated PBMCs were added to the cultures at weekly intervals. The results show that all the mutant viruses grew at diminished levels relative to WT (Fig. 2A and B). Furthermore, the addition of M184V to each of the SD2, SD5, and SD6 variants resulted in further reductions in p27 antigen production (SD2 and SD6 are shown).

To determine the propensity for phenotypic reversion in PBMCs, additional passages were performed using supernatants taken from the peak levels of viral p27 antigen production at 21 days after infection. These were used to infect fresh stimulated PBMCs from the same animal, i.e donor B. This second passage of mutant virus harbouring the M184V substitution exhibited near identical levels of antigen production to that seen during the first infection (not shown), suggesting that no increase in replication capacity had occurred in this case.

We also used viral supernatants collected from the peak of the first passage in monkey PBMCs, again from donor B, to infect fresh CEMx174 cells. The results show that increased viral replication had occurred in variants that lacked the M184V mutation (Fig 2C). In contrast, the M184V-harboring species showed only minor increases in replication capacity compared to replication of clonal infectious stock.

RNA dimerization. We also assessed the ability of the SD2 mutant virus to properly incorporate a mature RNA dimer. Non-denaturing northern analysis of purified RNA preparations had indicated that deletion of the +398 to +418 nt sequence in SD2 completely abrogated viral RNA dimerization. The additional presence of the M184V mutation together with the leader mutation appeared to outwardly affect RNA dimerization (unpublished data).

Those viruses that were continually passaged in the CEMx174 cell line were sequenced by PCR amplification of proviral DNA recovered from cells isolated at the peak of the fourth round of infection. Sequencing of the complete SD2-M184V UTR and *gag* regions showed numerous point mutations in all clones. Despite this variability, one point mutation was found in all six sequenced clones that corresponded to a G to A transition in MA, encoding a change from a threonine to isoleucine at residue 70 (T70I).

To assess the relevance of this mutation, site-directed mutatgenesis was performed with the SD2-M184V clone to produce the variant termed SD2-MA-M184V. Infectious inocula were produced in COS-7cells and were then used to infect CEMx174 cells in parallel with controls that included two previously described SD2 reversion mutants (Guan *et al.*, 2000). The compensatory mutations that restored the SD2 virus to replication competence had been shown to involve two distinct sets of mutations located within the putative DIS loop (A423G) and within several different Gag proteins, i.e., NC

(E18G and G31K) or CA (K197R) and p6 (E49K). These amino acid changes are responsible for restoration of viral RNA packaging and viral fitness (Guan *et al.*, 2001). A similar situation has been observed in the case of deletions within the SL1 region of HIV-1 (Liang *et al.*, 1999).

Figure 3A also shows that the T70I mutation in MA was sufficient to confer a replicative advantage in the context of SD2-M184V. This codon change had no observable effect on a WT virus (not shown). MA is known to be involved in targeting of both Gag and genomic RNA to the cell membrane and in the formation and stabilization of genomic RNA dimers (Burniston *et al.*, 1999, Garbitt *et al.*, 2001). The potential role of the T70I MA mutation in rescue of viral replication is also suggested by recent studies on a role for upstream leader sequences and the MA coding sequence in formation of an extended RNA pseudoknot structure. Extended interactions involving a region of MA and the *pol* open reading frame have also been reported (Paillart *et al.*, 2002, Purhoit *et al.*, 2001). Additional *in vitro* evidence has also supported a role for higher order structures in regulation of viral replication (Huthoff *et al.*, 2001).

To further explore the notion that reversion of SD2-M184V virus was impaired specifically as a result of M184V, we inserted this substitution into the two aforementioned SD2 reversion mutants, termed SD2-DIS-NC1+2-M184V and SD2-DIS-CA-SP6-M184V. Both these variants were impaired in replication ability in CEMx174 cells compared with equivalent constructs that lacked M184V (Fig. 3B).

In summary, the M184V mutation in RT impacts adversely on the replicative fitness of a number of SIV constructs. Furthermore, SIVs containing both the M184V and DIS mutations are less able to effect repair through compensatory mutagenesis than are viruses containing a wild-type RT. Our results also show that viral species that harbored both the M184V mutation and deletions in the region of the DIS displayed reduced replication capacity over multiple passages. Similar results have been reported with HIV-1 viruses containing the M184V mutation in RT (Inouye *et al.*, 1998, Li *et al.*, 1997). In non-human primate studies, M184V-containing SIV failed to revert to WT, and may have been initially impaired in ability to multiply to high titer. However, this replication deficit may have been corrected over time, as a consequence of a distinct compensatory mutation within RT (Newstein and Desrosiers 2001).

We should point out that attenuation of the SD2-M184V variant may be partly attributable to synergy between the 5' UTR and the Gag-Pol region in regard to both structure and function. HIV-1 RTs that harbour M184V suffer from diminished ability to initiate reverse transcription and to participate in the elongation phase of minus strand DNA synthesis (Berkhout *et al.,* 1998, Jones *et al.*, 1994). In addition, the UTRs of both HIV and SIV play key roles in RNA dimerization and strand transfer (Anderson *et al.*, 1998, Balakrishnan *et al.,* 2001, Jones *et al.*, 1994).

Finally, we have shown that both RT and UTR sequences are necessary for restoration of viral replication, and our experiments suggest that viral recombination is involved in the process of compensatory mutatgenesis. Those mutants presumed to lack this function in the process of reversion might be relegated to fixing adventitious mutations in an iterative fashion, likely imparting delays to restoration of a wild-type replication phenotype.

Fig. 6.1 Delay of phenotypic reversion of the SD2-M184V mutant after long-term culture in CEMx174 cells.

Growth curves of viruses over extended culture. Equivalent amounts of virus from COS-7 transfected cells were used to infect CEMx174 cells based on levels of p27 antigen (10 ng per 10^6 cells). Infected cells were grown over protracted periods and culture fluids were monitored by RT assay. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control. A representative example of the SD2-M184V and SD6-M184V variants at the fourth passage is shown (experiment performed three times with similar results).

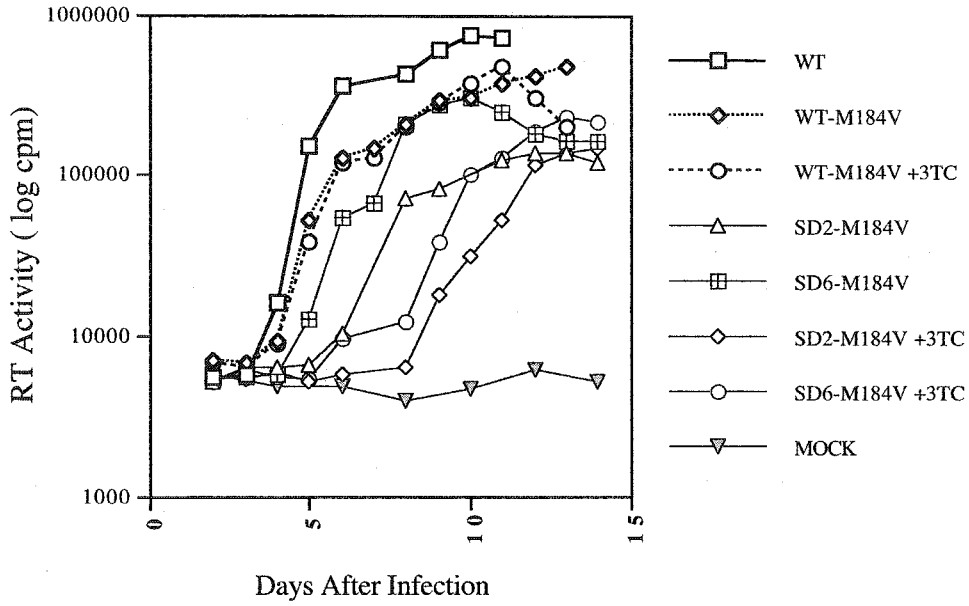

1000000
100000
10000
1000
RT Activity (log cpm)
0
5
10
15
Days After Infection
WT
WT-M184V
WT-M184V +3TC
SD2-M184V
SD6-M184V
SD2-M184V +3TC
SD6-M184V +3TC
MOCK

Figure 6.1

Fig. 6.2 Replication capacity of wild-type and mutated viruses in monkey PBMCs. Equivalent amounts of virus were used to infect rhesus macaque PBMCs based on levels of p27 antigen, typically 10 ng of WT or mutant virus per 4 x 10^6 PBMCs. Viral replication was monitored by determining levels of SIV p27 antigen by ELISA of culture fluids. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control. The dashed line representing 0.01ng/ml p27 indicates the threshold sensitivity of the assay. (A) Growth curves indicate antigen production in PBMCs from donor monkey A. (B) Growth curves in PBMCs from donor monkey B. p27 Ag results are depicted as the average of duplicates. (C) Second passage of mutated viruses in 10^6 CEMx174 cells using an inoculum of 10 pg p27 antigen derived from the infected PBMCs of monkey B. Viral replication was monitored by RT assay in culture fluids. Shown is a representative replication curve of experiments conducted in duplicate. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control.

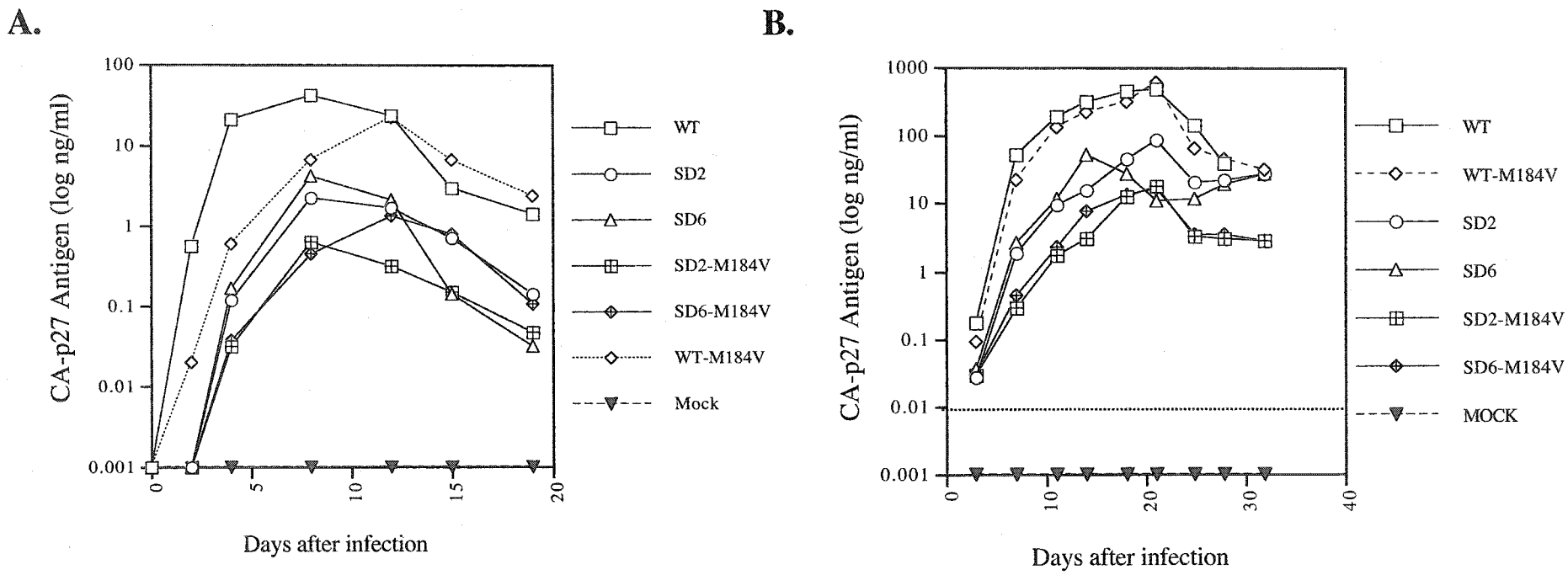

A.
B.
CA-p27 Antigen (log ng/ml)
Days after infection
WT
SD2
SD6
SD2-M184V
SD6-M184V
WT-M184V
Mock
MOCK

Figure 6.2

C.

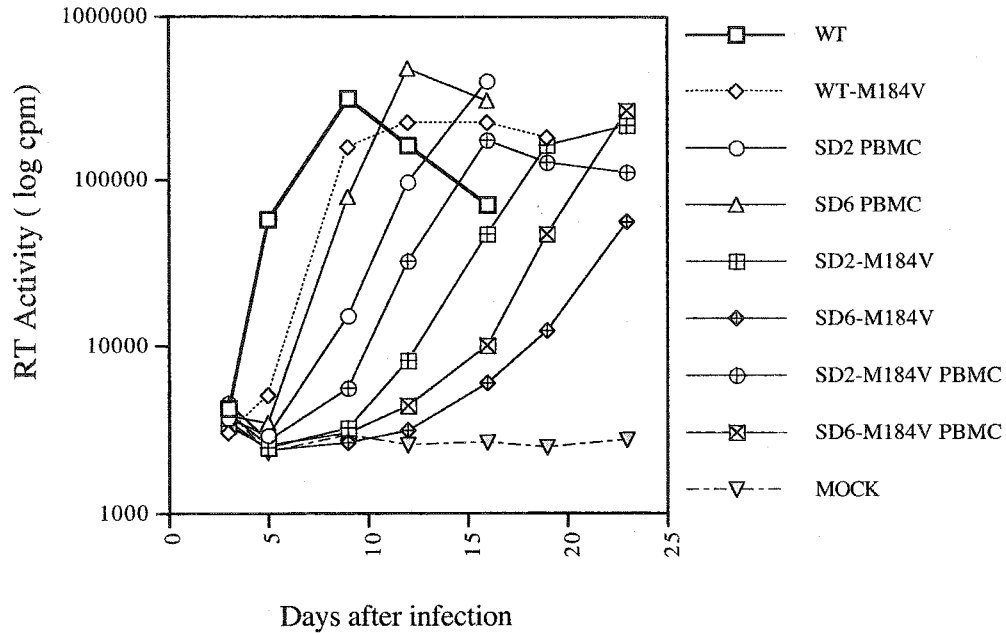

1000000
100000
10000
1000
RT Activity (log cpm)
0
5
10
15
20
25
Days after infection
WT
WT-M184V
SD2 PBMC
SD6 PBMC
SD2-M184V
SD6-M184V
SD2-M184V PBMC
SD6-M184V PBMC
MOCK

Fig. 6.3A. Reversion of the SD2-M184V variant after replication in CEMx174 cells. Growth curves of reverted viruses in CEMx174 cells. Equivalent amounts of virus from COS-7 transfected cells were used to infect CEMx174cells based on levels of p27 antigen (10 ng per 10^6 cells). Viral replication was monitored by RT assay of culture fluids. Shown is a representative growth curve of experiments conducted in triplicate. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control.

A.

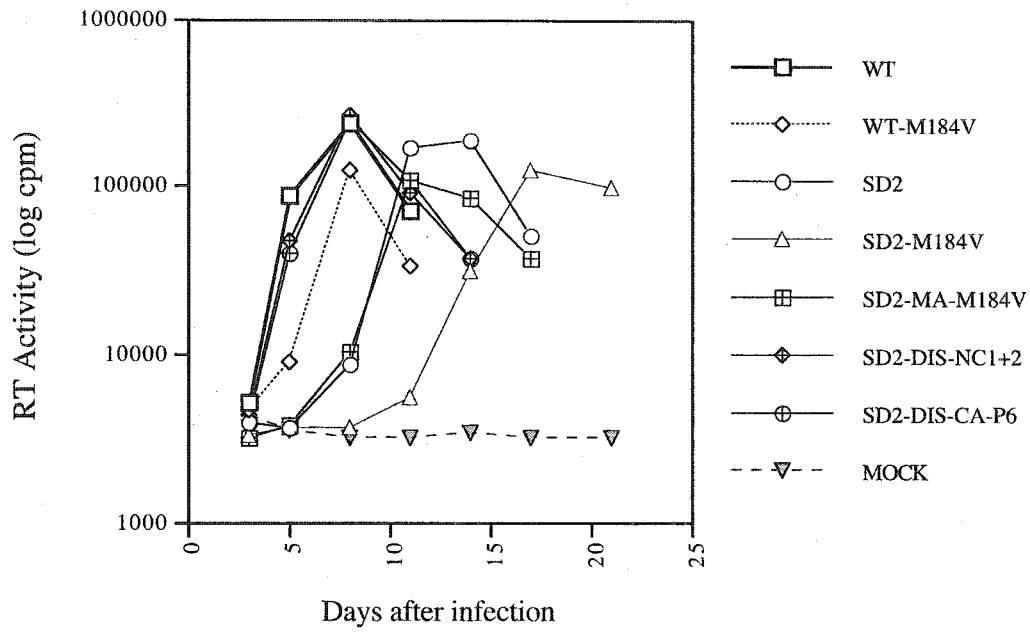

1000000
100000
10000
1000
RT Activity (log cpm)
0
5
10
15
20
25
Days after infection
WT
WT-M184V
SD2
SD2-M184V
SD2-MA-M184V
SD2-DIS-NC1+2
SD2-DIS-CA-P6
MOCK

Figure 6.3 (A)

Fig. 6.3b The M184V mutation restricts compensatory mutagenesis in the case of the SD2 variant

Viruses derived from COS-7 cells were standardized on the basis of p27 CA Ag and used to infect 10^6 CEMx174 cells. RT activity of culture fluids was used to monitor replication. Shown is a representative growth curve of experiments performed in triplicate. Mock infection denotes exposure of cells to heat-inactivated wild-type virus as a negative control.

B.

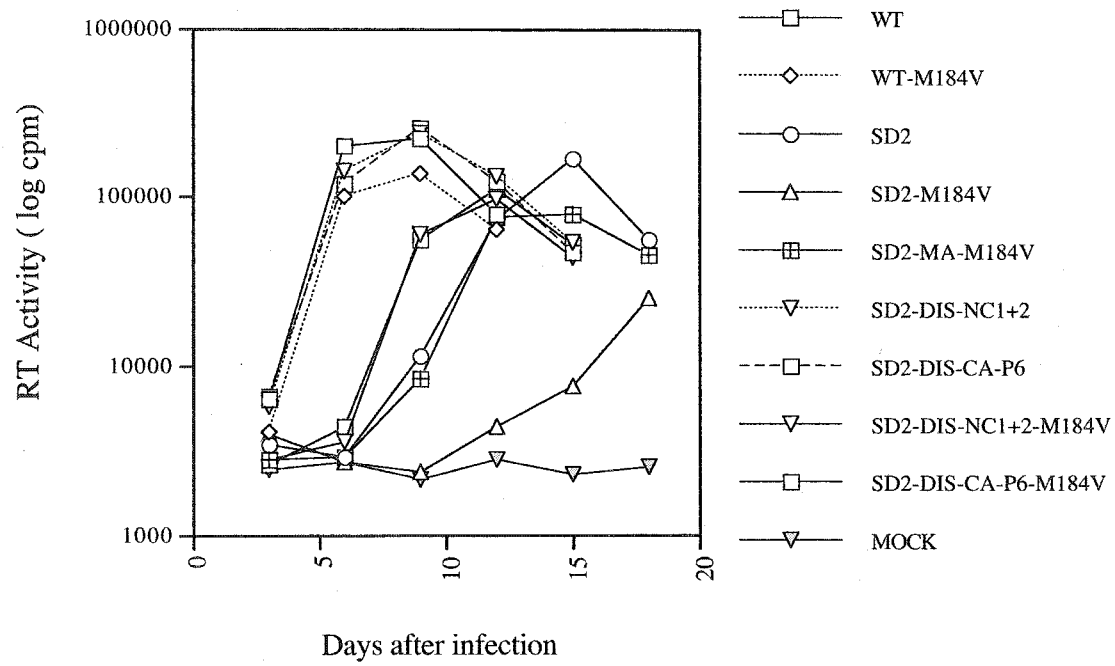

1000000
100000
10000
1000
RT Activity (log cpm)
0
5
10
15
20
Days after infection
WT
WT-M184V
SD2
SD2-M184V
SD2-MA-M184V
SD2-DIS-NC1+2
SD2-DIS-CA-P6
SD2-DIS-NC1+2-M184V
SD2-DIS-CA-P6-M184V
MOCK

Figure 6.3 (B)

7.0 General Discussion and Contribution to Original Knowledge:

The work presented herein is an adaptation of published work, manuscripts either submitted or to be submitted shortly to peer-reviewed journals. The following passages compile my novel contributions to the literature and scientific community. Since a reader of this thesis is likely to make their own conclusion in regard to the original contributions contained herein, therefore, I will be as succinct as possible.

Chapter 3

Simian immunodeficiency virus (SIV) infection of macaques is remarkably similar to that of human immunodeficiency virus (HIV-1) in humans, making the SIV/macaque system a valuable model. Despite its utility as a model system; comparatively little is known in regard to the molecular biology of SIV, particularly in regard to the role of the leader region in viral replication, gene expression, and RNA encapsidation.

We constructed multiple SIV proviral DNA clones within a 97 nt, expanse downstream of the primer binding site (PBS) of SIVmac239. We found that leader sequences downstream of the PBS, especially the U5-leader stem and the DIS stem-loop, are important for SIV replication and for packaging of the viral genome. Long-term serial passage of these constructs in C8166 cells revealed the propensity of several mutants to regain wild type replication phenotypes. One revertant in particular, termed SD2 was shown to possess second site compensatory mutations in disparate regions of the genome. The biological relevance of these nonsynonomous point mutations was validated by site directed mutagenesis and kinetic studies. These compensatory mutations were shown to recover RNA encapsidation efficiency, and *ex vivo* replication. This study implicates multiple *cis* and *trans* regions presumably acting in concert to package the viral genome.

Chapter 4

The major focus of this chapter was to extend the findings of the previous chapter, in analysis of the downstream region, however this analysis included the foregoing region in regard to its role in RNA dimerization. The series of mutants removed various regions from the distal region of SL1 to the Kozak consensus of *gag*. The regions having the greatest impact on packaging also had comparably large negative impacts on

dimerization and incurred larger deficits to infectivity, implying multiple functions of the region under investigation. Regions between stem loop 1(SL1) and Gag are important for the efficiency and specificity of viral genomic packaging. In contrast to previous results of *in vitro* studies, large deletions adjacent to the PBS did not impair RNA dimerization. Downstream regions between the splice donor and the Gag initiation site of SIV are also involved in RNA packaging but to a lesser extent than is true for equivalent sequences in HIV-1. These data suggest that the primary encapsidation determinant of SIV is SL1 and that downstream structures serve as secondary determinants in this regard. Each of the regions implicated in SIV packaging also plays a role in RNA dimerization, signifying a potential linkage between the two processes.

Chapter 6

The generation of a simplified simian immunodeficiency virus (SIV) constructs deleted of each of the *nef*, *vpr*, *vpx*, *vif*, *tat* and *rev* genes (Δ6 virus). To accomplish this, we began with the infectious molecular clone of SIV, i.e. SIVmac239, and replaced the deleted segments with three alternate elements. The Δ6CCI virus was extremely attenuated in replication capacity, yet retained infectiousness for CEMx174 and MT4 cells. Long-term culture of each of these constructs over 6 months revealed no potential for reversion. We are currently evaluating these simplified constructs in rhesus macaques to determine their long-term safety as well as ability to induce protective immune responsiveness as mucosal vaccines.

Chapter 7

In 1999 the Desrosier group published a paper regarding the protective efficacy of live attenuated vaccines inversely correlated with the attenuation level (Johnson *et al.*, 1999). Therefore, attenuated vaccines capable of eliciting protective responses also possessed replicative vigor and a proportionally equivalent capability of reversion. This final chapter, again building upon the findings of chapter 1, investigates potential means of limiting the reversion capacity inherent in attenuated vaccine designs.

We have previously constructed a series of simian immunodeficiency virus (SIV) mutants containing deletions within a 97 nt region of the SIVmac239 untranslated region

(UTR) or leader sequence. However, as is common with live attenuated viruses, several of the mutants exhibited a propensity for reversion. Our results indicate that the addition of M184V to these constructs impaired viral fitness in pair-wise comparisons of mutants that contained or lacked this substitution. We also observed that M184V significantly impaired the potential for both compensatory mutagenesis and reversion in these mutants both in cell lines and in PBMCs. We also observed a significant bias to the mutational outcome of serial passaged variants.

8.0 References

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