Identification of Mutations at Codon 184 of Simian Immunodeficiency Virus Reverse Transcriptase Which Confer Resistance to 3TC

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(iii) Abstract

SIV_{mac} variants resistant to 3TC were generated by *in vitro* passage with drug selection. C8166 human T-cells were infected with SIV_{mac}32H and passaged in tissue culture with increasing 3TC selective pressure. At 8 weeks (2.5uM 3TC) viral variants were capable of growth at >1000-fold the normal inhibitory concentration of 3TC, and at 24 weeks (750uM 3TC) were capable of growth at >4000-fold normal inhibitory concentration. RT-coding regions of 3TC-resistant variants were then PCR-amplified, cloned, and sequenced. All 2.5 uM clones sequenced contained a M184I (ATG->ATA) substitution, while all 750 uM clones contained a M184V (ATG->GTA) substitution. We introduced M184I and M184V mutations into SIV_{mac}239 by site-directed mutagenesis and analyzed the susceptibility of these mutants to several antiviral compounds. M184I and M184V mutants displayed elevated 3TC resistance (IC50's >500uM for both, versus 1.8uM for wild type SIV), thus substantiating the role of these mutations in SIV resistance to 3TC.

(iv) Résumé

Cellules de la ligne humaine C8166 étaient infectés avec le $VIS_{mac}32H$ et propagés en culture de tissu avec de la pression sélective exercée par le 3TC. Après huit semaines (2.5uM 3TC) les variants étaient capable de croissance à >1000-fois la concentration inhibitoire normale de 3TC. Après vingt-quatre semaines (750uM 3TC), les variants étaient capable de croissance à >4000-fois la normale. Séquences RT des variants résistantes à 3TC (2.5 et 750μM) étaient amplifiées par PCR, clonées, puis séquencées. Tous les clones resistants (2.5μM) sequencés avait un mutation M184I. Tous les clones resistants (750μM) sequencé avait un mutation M184V (ATG→GTA). Nous avons introduit des mutations M184I et M184V dans le $VIS_{mac}239$ par mutagenese dirigee. Les formes mutées ont démontrées un résistance élevée au 3TC (IC_{50}>500uM pour M184I et M184V, versus 1.8uM pour le type sauvage VIS). Ceci justifie le role qu'ont ces mutations auprès de la résistance qu'a le VIS au 3TC.

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List of abbreviations

3TC	: (-)-2',3'-dideoxy-3'-thiacytidine	NC	: nucleocapsid protein
AIDS	: acquired immunodeficiency syndrome	NLS	: nuclear localization signal
AZT	: 3'-azido-3'-deoxythymidine	nt	: nucleotide
CA	: capsid protein, p24	PCR	: polymerase chain reaction
ddC	: 2',3'-dideoxycytidine	PBS	: primer binding sequence
DDDP	: DNA-dependent DNA polymerization	PR	: protease
ddI	: 2',3'-dideoxyinosine	R	: repeat region
ddG	: 2',3'-dideoxyguanosine	RDDP	: RNA-dependent DNA polymerization
dNTP	: deoxynucleoside-5'-triphosphates	RNase H	: DNA/RNA-dependent ribonuclease
ds	: double-stranded	RRE	: *rev*-responsive element
gp	: glycoprotein	SDS-PAGE	: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
HIV	: human immunodeficiency virus	ss	: single-stranded
SIV	: simian immunodeficiency virus	SU	: surface glycoprotein
IC_{50}	: concentration required for 50% inhibition	TAR	: *trans*-activation response element
IN	: integrase	TM	: transmembrane glycoprotein
LTR	: long terminal repeat	U3	: 3' unique region
MA	: matrix protein	U5	: 5' unique region
		wt	: wild-type

Note: Abbreviations are defined at their first use. The standard one letter nomenclature is used for amino and nucleic acids.

Chapter 1. Literature Review

1.1 General Introduction

Human immunodeficiency viruses type 1 and 2 (HIV-1 and 2) are the etiological agents of acquired immunodeficiency syndrome (AIDS) in humans (Clavel et al., 1986; Popovic et al., 1984). As retroviruses, HIV-1 and 2 possess a genome composed of 2 ssRNA molecules which encode the *gag*, *pol*, and *env* gene products characteristic of the family retroviridae. However, HIV-1 and 2 share a number of unique biological and genetic properties which identify them with the retroviral subfamily lentivirinae and distinguish them from other human retroviruses, such as oncovirinae (HTLV-1 and 2) and spumavirinae (human foamy virus) (Simon et al., 1992). These include the induction of chronic debilitating disease following long-term persistent infection, the infection and destruction of $CD4^+$ helper lymphocytes, persistence in macrophages, and the presence of extra regulatory genes not found in other retroviruses (Simon et al., 1992; Levy, 1989).

The disease resulting from HIV-1 follows a largely predictable course. Primary infection is followed in 50 – 70% of individuals by an acute viral syndrome during which intense plasma viremia, a significant decrease in circulating $CD4^+$ T cells, and various mononucleosis-like symptoms are observed. This acute phase occurs approximately 3 to 6 weeks after the initial infection and is followed by an immune response mounted primarily within nodes of the

lymphatic system, where viral replication is heavily concentrated (Li et al., 1994; Roulston et al., 1995). Plasma viremia is suppressed, the number of circulating $CD4^+$ T cells rebounds, and as the acute syndrome resolves, an asymptomatic phase beings. Although this phase has often been described as 'latent', recent studies suggest that during this time (which can be 10 or more years) HIV-1 is actually replicating rapidly, and viremia is maintained in a steady state by a vigorous immune response. Virions are produced and cleared from circulation at a rate on the order of ~6×10^9 virions per day, while the net loss of $CD4^+$ lymphocytes has been estimated at ~2×10^9 cells per day (Ho et al., 1995; Wei et al., 1995; Pantaleo et al., 1993; Wain-Hobson, 1995). Patients become increasingly susceptible to opportunistic infections and malignancies once T-cell counts drop below 200 cells per mm^3 (Roulston et al., 1995).

Antiviral therapy versus HIV-1 can minimize HIV-1 replication and the related deterioration of immune function, thereby delaying the onset of AIDS. Although antiviral therapy may target numerous viral proteins and replication events, the drugs currently available are inhibitors of either HIV-1 reverse transcriptase or HIV-1 protease. There are two classes of HIV-1 reverse transcriptase inhibitors: nucleosidic and non-nucleosidic. Nucleosidic RT inhibitors (NRTI's), such as 3'-azido-3'-deoxythymidine (AZT), (-)-2', 3'-dideoxy-3'-thiacytidine (3TC), 2', 3'-dideoxyinosine (ddI), and 2', 3'-dideoxycytidine (ddC), each differ from their deoxyribonucleoside counterpart by their lack of 3' hydroxyl or 3' carbon in the ribose ring of the analogue. HIV-1 RT is unable to

link nucleosides to the 3' end of the analogues, but does incorporate them competitively into growing DNA strands, resulting in chain termination and blockage of a crucial and early step of HIV-1 replication. Although the effectiveness of NRTI's (particularly in combination with protease inhibitors) is well documented, the selection and emergence of HIV-1 variants resistant to therapeutics is frequently observed, and an outgrowth of drug resistant HIV-1 quasispecies may occur even in patients where viral load has been reduced to undetectable levels. Varying levels of resistance are encoded by HIV-RT mutations or combinations thereof, and specific mutations may also display altered phenotypic properties, such as reduced growth kinetics or increased fidelity of replication, as observed in the case of the HIV-1 RT mutation M184V (Wainberg et al., 1996). Detailed understanding of the replicative properties of these mutants and the patterns with which they emerge is essential for the design of effective treatment regimens.

There has been much scientific interest in the development of an animal model for HIV-disease and AIDS. Such a model would provide a means to study AIDS pathogenesis, vaccine strategies, and therapeutic interventions for many cytopathic retroviruses known to cause immune suppression. The simian immunodeficiency viruses (SIV's) have proven to be the most genetically similar to HIV. Further, there exist isolates of SIV which produce persistent infection in macaque monkeys (SIV_{mac}), with clinical and immunological changes closely resembling those of HIV-1 infected individuals. SIV_{mac} also induces AIDS in

macaques in a time frame suitable for laboratory investigation. This SIV/macaque model has been intensively developed, and currently constitutes the best available model to study AIDS. However, if the model is to be useful in studies of therapeutic intervention and drug resistance, there remains much to be done in determining precise similarities and differences between the SIV and HIV-1 systems. Of key importance for therapeutic studies is the question of whether targets of drug inhibition such as RT develop resistance mutations in similar patterns in the SIV and HIV-1 systems. While the SIV and HIV-1 RT's are largely conserved in structure and function, the locations, properties, and patterns of emergence of resistance-conferring mutations in SIV-RT have been largely uninvestigated until recently.

1.2 The HIV-1 Virion, Viral Genome, and Viral Proteins

1.2.1 The HIV-1 Virion

The HIV-1 virion is an icosahedral sphere with a diameter of approximately 110nm. The outer layer is a heavily glycosylated and sialated lipid bilayer membrane studded with envelope glycoproteins which appear ultrastructurally as electron dense spikes or knobs. The knobs, of which there are approximately 72 on newly budded virions and dramatically less on the mature virion, are each composed of a trimer of the transmembrane glycoproteins (TM, gp41) bound non-covalently to a trimer of surface glycoproteins (SU, gp120) on

the exterior. The matrix domain is the component of the viral capsid that lines the inner surface of the virus envelope. It is composed of matrix protein (MA, p17) monomers and appears to associate with both the envelope proteins and the capsid core. The capsid core is a bullet-shaped shell formed by the major capsid protein (CA, p24), containing two identical single-stranded (ss) RNA molecules which comprise the viral genome. The RNA molecules are associated at a discrete site near the 5' end (the dimer linkage site or DLS) and bound tenaciously by nucleocapsid protein (NC, p7) in a structured ribonucleoprotein complex (Skripkin et al., 1994). Protease (PR, p10), integrase (IN, p32), and reverse transcriptase (RT, p66/p51) enzymes are packaged within Gag-Pol precursors. Nef, Vif, host-derived $tRNA_{Lys}^{3}$ and cyclophilin molecules, and stoichiometrically significant amounts of Vpr are also present in HIV-1 virions (Thali et al., 1994; Barat et al., 1989; Liu et al., 1995). Substantial amounts of cellular surface antigens such as B_2-microglobulin and human lymphocyte antigen DR have also been reported for HIV-1 and other primate immunodeficiency viruses (Thali et al., 1994).

1.2.2 The Viral Genome

The HIV-1 provirus is about 9.7 kilobases long, including long terminal repeat (LTR) sequences at each end comprised of U5, R (repeat), and U3 regions. The LTR's contain the core promoter element and numerous elements for enhancement and modulation of transcription, as well as *cis*-acting elements

required for integration (Roulston et al., 1995; Vink et al., 1991; Du et al., 1997). Transcription initiation occurs in the 5'-LTR between the U3 and R regions, and termination occurs in the 3'-LTR where polyadenylation is specified by sequences at the junction of R and U5. A portion of Nef is also encoded within the 3' U3 region. Genes encoding all other viral proteins are located in the region between the LTR's, including the invariant retroviral *gag*, *pol*, and *env* genes, five auxilliary genes, and the 5' portion of *nef*, also an auxilliary gene. A difference from HIV-1 is evident in the genomes of HIV-2 and SIV in that no *vpu* gene is present, while a *vpr*-like gene not found in HIV-1 is encoded by the region between *pol* and *env* (designated *vpx*). The *vpx* gene of HIV-2/SIV was originally thought to have arisen from a gene duplication of *vpr* (or vice versa); however, new phylogenetic evidence suggests that the two genes arose from separate subfamilies of SIV altogether (Fletcher et al., 1996).

1.2.3 The Viral Proteins

Gene products encoded by the three genes common to all retroviruses, *gag*, *pol*, and *env* are synthesized by HIV-1 in the form of large polyprotein precursors. The mature viral proteins are produced upon proteolytic cleavage by PR, except in the case of Env gp160, which is cleaved by cellular proteases. Including the 6 auxilliary proteins, at least 15 functional gene products are encoded by HIV-1.

The *gag* (*g*roup *a*ntigen) gene encodes proteins which play a primarily structural role, forming the viral matrix and the capsid shell, and providing a framework for the RNA genome. Cleavage by viral protease of the $Pr55^{gag}$ polyprotein yields the mature matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. Pr55 is the only molecule required for assembly in HIV-1 and SIV (Jones and Stuart, 1996; Gheysen et al., 1989).

The matrix protein (MA, p17) is ~130 aa protein encoded by the amino terminal region of the Gag polyprotein which is important to both early and late stages of viral replication. A myristic acid moiety is added to the N-terminal glycine by cellular enzyme N-myristyl transferase co-translationally and following removal of the initiator methionine (Gonzalez et al., 1996; Veronese et al., 1988; Gottlinger et al., 1989). This modification, along with other signals primarily in the N-terminal part of MA, facilitates membrane targeting of the Gag and Gag-Pol precursors (Gonzalez and Affranchino, 1995; Veronese et al., 1988; Gottlinger et al., 1989; Bryant and Ratner, 1990). In HIV-1 the MA region is also responsible for recruiting of Env glycoproteins to the surface of host cells; a similar interaction has been reported for SIV, where a central hydrophobic alpha helix in the MA protein was found to be more critical then the N-terminal residues identified for Env incorporation in HIV-1 (Yu et al., 1992; Zhou et al., 1994; Gonzalez et al., 1996; Gonzalez and Affranchino, 1995; Freed and Martin, 1995; Dorfman et al., 1994). The HIV-1 MA protein also possesses a signal which interacts with nuclear membrane NLS receptors to facilitate active import of the

preintegration complex (Popov et al., 1998). In late viral replication, MA may also appears to bind genomic RNA for incorporation into HIV-1 virions, and it plays a key role in assembly of virions in both HIV-1 and SIV (Bukrinskaya et al., 1992; Luban and Goff, 1991; Laughrea et al., 1997). Remarkably, SIV MA has been shown to have intrinsic information for both self-assembly and release of virus-like particles, even in the absence of all other viral proteins (Gonzalez et al., 1996; Gonzalez et al., 1993).

Capsid protein (CA, p24), the unit which forms the virion core, is a largely hydrophobic ~240 aa protein encoded by the central region of the Gag polyprotein. The C-terminal third of CA contains the major homology region, which is conserved among retroviruses and involved in assembly (Jones and Stuart, 1996; Dorfman et al., 1994). Within these 80 aa there exists a Gag oligomerization domain (Gitti et al., 1996). NMR and x-ray crystallography studies reveal a dimeric structure for CA and a trimeric structure for MA in both SIV and HIV (Jones and Stuart, 1996; Gitti et al., 1996; Rao et al., 1995; Chazal et al., 1995). The predicted model where Gag polyproteins are linked dimerically through CA domains and trimerically through MA domains offers a simple mechanism to explain why only complete uncleaved gag precursors enter the assembly reaction (Jones and Stuart, 1996; Rao et al., 1995). Additionally, CA sequences in the N-terminal two-thirds of the molecule are essential for establishing proper capsid core morphology, and play a role in viral penetration or uncoating, or both (Gitti et al., 1996). Mutations in this region can also block

another essential function of HIV-1 CA, the binding of human cellular proline rotamase cyclophilin A (CypA), ~200 copies (ie. at a 1:1 ratio with Gag) of which are incorporated into each HIV-1 virion (Dorfman and Gottlinger, 1996; Luban et al., 1993; Braaten et al., 1996; Thali et al., 1994). The CypA binds at an exposed loop in the CA structure containing Pro^{90}, a residue which adopts kinetically trapped *cis* and *trans* conformations in multidimensional heteronuclear NMR studies (Gitti et al., 1996; Frankel, 1996). The binding of CypA is believed to catalyze interconversion between Pro^{90} loop structures, thereby facilitating viral uncoating (Gitti et al., 1996). SIV does not incorporate CypA or require it for replication, and while both HIV-1 and SIV Gag molecules bind cyclophilin B this molecule is targeted to the ER and appears to be dispensable for replication (Franke et al., 1994; Thali et al., 1994; and Braaten et al., 1996).

The nucleocapsid (NC, p15) protein is small, basic, hydrophilic, and has a strong affinity for nucleic acids. It is packaged within the capsid core (~2000 molecules per virion), where it surrounds and provides a substructure to the dimeric RNA genome. NCp15 is encoded by the carboxy terminal region of Gag and is cleaved by protease to yield four peptides: p7, p6, p2, and p1. p7 is a 70 aa protein with a nucleic acid binding zinc finger. It is required for the formation of infectious HIV-1 particles, and is functional in numerous viral activities. NCp7 promotes both dimerization and encapsidation of genomic RNA, it promotes annealing of primer tRNA to the PBS, it stimulates reverse transcription (likely linked to its reported promotion of strand transfer), and it protects genomic RNA

from attack by RNases (Reviewed in Darlix et al., 1995; Gotte et al., 1999). P6 binds Vpr for incorporation into virions; this interaction may also facilitate Vpr-associated nuclear import of the preintegration complex (De Rocquigny et al., 1997). P6 also plays an important role in particle maturation (Huang et al., 1995). The spacer peptide P2 is important for viral assembly and is thought to be involved in Gag-Gag interactions along with the C-terminal region of CA, and may be involved in Vif incorporation (Huvent et al., 1998). The function, if any, of the spacer peptide P1 is uncertain at this time.

The *pol* region gives rise to the key viral enzymatic functions, performed by protease (PR, p11), reverse transcriptase (RT, P66/51), integrase (IN, p32). Pol is translated only within the Gag-Pol fusion protein $Pr160^{gag\text{-}pol}$, which occurs only in the event of a minus ribosomal frameshift event. The frameshift is located in the carboxy-terminal NC region of *gag* at a frequency of about 5%, yielding a ~20:1 ratio of *gag* and *pol* gene products (Levin and Hatfield, 1993).

PR is an essential viral enzyme which processes Pr^{gag} and $Pr^{gag\text{-}pol}$ into functional structural proteins and enzymes. During early virion maturation, PR is excised from Gag-Pol precursors in an auto-proteolytic event which appears to follow a *trans* mechanism (Peng et al., 1989). Retroviral proteases belong to the ubiquitous aspartyl protease family. In the cases of HIV-1, HIV-2, and SIV, identical ~11 kDa protomers combine to form a symmetrical active dimer in which an Asp-Thr-Gly from each subunit contributes to the active site (Grant et

al., 1991; Wilderspin and Sugrue, 1994; Wlodawer et al., 1989). Hydrolysis of oligopeptide substrates is most likely catalysed by Asp-25-assisted attack of water upon the scissile bond with subsequent protonation by the second aspartate (Asp-25') group (Meek et al., 1990). A four stranded antiparallel beta-sheet which forms an important part of the substrate binding cleft is comprised of the amino- and carboxy-terminals of each subunit (Wlodawer et al., 1989; Lapatto et al., 1989; Miller et al., 1989). The SIV enzyme is more closely related to HIV-2 protease than that of HIV-1 (the amino acid homology of $SIV_{Mn}251$ and HIV-2 ROD protease is 89%, while those of HIV-1 and HIV-2 are only 45% identical) (Stebbins et al., 1997). However, as is generally observed among the proteins of HIV and SIV isolates, the secondary and tertiary structures are remarkably well conserved (Fitzgerald and Springer, 1991). Extensive similarities between the antiviral activities of HIV-1 protease inhibitors versus HIV-1 and SIV have been reported (Black et al., 1993; Black et al., 1996;). Furthermore, even though 3 out 13 major peptide binding residues in SIV PR differ from HIV-1 PR, SIV PR is capable of authentically processing HIV-1 $Pr55^{gag}$ *in vitro* (Miller et al., 1989; Grant et al., 1991). Interchangeability of the HIV and SIV proteins is not complete, however, as significant retardation has been observed in the processing of the p66/p51 cleavage site in HIV-1 reverse transcriptase (AETF*YCDG) by SIV PR (Grant et al., 1991). This difference was attributed to a reduced binding cleft subsite volume in SIV, determined by an altered conformation of exterior loop residues 78 – 85 (Stebbins et al. 1997).

Reverse transcriptase (RT, p66/p51) is an essential virion protein which catalyzes the conversion of genomic viral RNA into double stranded proviral DNA. RT is functional in the form of an assymmetric heterodimer of P66 and p51, which are alternatively processed forms of the RT protein found at a ~1:1 ratio in virions. The three major enzymatic functions of RT, RNA-dependent DNA polymerization, DNA-dependent DNA polymerization, and RNAse H activity, are attributed to the p66 subunit (Le Grice, 1993). An RNase D activity residing in p66 has also been observed, but its significance *in vivo* has not yet been determined (Le Grice, 1993). The p51 subunit is truncated near its C-terminus by protease, and appears to perform a largely structural function (Le Grice et al., 1995). The same region which is absent from p51 is responsible for the RNase H activity in p66. RT and the process of reverse transcription are discussed in section 1.4.

Integrase is the only protein known to be required for retroviral DNA integration into host chromosomal DNA. There is evidence for three functional domains in the 288 aa HIV-1 IN protein. The N-terminal 50 aa contain a Zn^{2+}-binding HHCC zinc finger motif which may play a role in the recognition and proper positioning of viral DNA (Vink et al., 1993; Mumm and Grandgenett, 1991). A central Mg^{2+}-binding domain (positions 50 – 194) contains a catalytic D, D(35)E motif and appears to encompass the complete active site of IN (Drelich et al., 1992; Vink and Plasterk, 1993; Kulkosky et al., 1992). The C-terminal domain (positions 195 – 288) exhibits specific binding to viral DNA ends and

nonspecific binding to other DNA sequences (Vink and Plasterk., 1993; Vink et al., 1991). HIV-1 integrase functions within the nucleus as a dimer or tetramer to perform a series of events (Engelman et al., 1993). IN removes two nucleotides from both proviral 3' ends, then cleaves host dsDNA to produce a staggered 5-nucleotide overhang. The newly generated 3'-OH proviral ends are coupled to host 5' phosphates in a one-step strand transfer (phosphoryl transfer) reaction (Engelman et al., 1991). Host cellular enzymes remove 2 unpaired proviral nucleotides and fill gaps left at the proviral 5' ends. Selection of target sites for integration is not completely random with regard to primary sequence or chromatin structure (Reviewed in Cragie, 1991).

The Env glycoproteins found within the virion membrane and on its surface are responsible for binding receptors on $CD4^+$ cells and mediating fusion. Env is initially translated as a gp160 precursor and is extensively glycosylated during translation. A signal peptide at the N-terminus is removed prior to cleavage in the Golgi of gp160 by the host endoprotease furin. The end products are the envelope surface glycoprotein (SU, gp120), containing more than 20 potential glycosylation sites, and the envelope transmembrane glycoprotein (TM, gp41), containing 4 potential glycosylation sites (Bour and Strebel, 1996).

SU glycoproteins form the spike structures which bind $CD4^+$ receptors on the host cell membrane. HIV-1 virions initially have about 72 such spikes which decrease in number by approximately 50% with an extended time in culture (Hart

et al., 1993). Interestingly, little or no such gp120 shedding is observed in the case of HIV-2 or SIV (Hockley et al., 1988; Palmer and Goldsmith, 1988).

The TM glycoprotein anchors the gp120 to the viral membrane and appears to play a complex role in mediating fusion of viral and cell membranes. A leucine zipper motif is found in an external N-terminus domain, which functions during fusion. A long cytoplasmic tail of over 150 amino acids is found in HIV-1, HIV-2 and SIV which appears also to be important for fusion and cell tropism (Johnston et al., 1993). A hydrophobic domain at the C-terminus of TM remains buried within the bilayer membrane, stabilizing the glycoprotein complex. Some evidence suggests that the shedding of gp120 spikes by HIV-1 may improve steric accessibility of the fusogenic amino terminus of the TM protein, thereby increasing viral infectivity (Hart et al., 1991; Sattentau and Moore, 1991). If this is correct, the lack of spike shedding by HIV-2 and SIV may partially explain their relatively modest virulence (Hart et al., 1993).

HIV-1 contains no less than six open reading frames in addition to *gag*, *pol* and *env*. Two of the proteins encoded by these auxilliary genes, Tat and Rev, are absolutely necessary for viral growth; in contrast, Nef, Vif, Vpr, and Vpu (or Vpx in HIV-2/SIV) are dispensable for growth in many vitro systems and are thus known as accessory proteins. Nevertheless, the high degree of conservation of these genes suggests a greater significance for the accessory proteins *in vivo*. Additionally, *in vivo* studies with SIV strains deficient in one or more of these

proteins confirm their importance in pathogenesis (Rud et al., 1994; Zou and Luciw, 1996)

Tat is a positive regulator of LTR-directed gene expression which acts in the early phase of the HIV-1 life cycle. It transactivates transcription by binding to a TAR RNA element located primarily in the R region of the LTR, and probably also to host cell proteins (Hauber et al., 1987; Muesing et al., 1987). One such TAR-interacting protein originating from human chromosome 12 has been described (Hart et al., 1995). The transactivating effect of Tat may be due to increased processivity of RNA polymerase, increased frequency of transcription initiation, or both (Calnan et al., 1991). SIV_{mac} and HIV-1 Tat proteins are functionally homologous but not interchangeable; this is likely related to the fact that SIV_{mac} TAR RNA is predicted to contain at least two stable RNA hairpin structures, while only one hairpin is predicted for HIV-1 TAR RNA (Berkhout and Jeang, 1989; Feng and Holland, 1988; Berkhout et al., 1989). Furthermore, a fraction of SIV mRNAs (as observed in SIV_{mac}–infected T-cells) splice an intron from their 5' ends which contains a portion of the TAR element (Viglianti et al., 1990). This alternative pre-mRNA splicing is not observed in HIV-1 and may affect several levels of gene regulation.

The Rev protein is responsible for the switch from early to late viral gene expression. Binding of Rev to its *cis*-acting Rev-responsive element (RRE), a complex stem-loop structure within the *env* gene of unspliced and singly spliced

RNAs, facilitates nuclear export and thus expression of these messages. Rev may accomplish this by binding to cellular cofactors involved in the export of RNAs from the nucleus to the cytoplasm (Fischer et al., 1995; Bogerd et al., 1995; Malim et al., 1989; Stutz and Rosbash, 1995). A leucine-rich activation domain in the carboxy-terminus is necessary for the transactivating activities of both HIV-1 and SIV_{mac} Rev (Berchtold et al., 1995), which are structurally and functionally homologous. However, the Rev genes of HIV-2, SIV_{mac}, and SIV_{agm} are largely unable to substitute for HIV-1 Rev, while HIV-1 Rev efficiently activates other viruses (Cullen and Garrett, 1992; Sakai et al., 1991).

The Nef protein down-regulates $CD4^+$ expression by causing endocytosis and lysosomal degradation of these receptors (Garcia and Miller, 1991). Other functions have been ascribed to Nef based on *in vitro* studies, including dowregulation of MHC 1 expression (Schwartz et al., 1996), enchancement of virion infectivity (Miller et al., 1994), stimulation of proviral DNA synthesis (Schwartz et al., 1995), activation of lymphocytes (Du et al., 1995), and upregulation of the FAS ligand (Aiken and Trono, 1995; Xu et al., 1997). At least some of these observations have been explained by the finding that HIV-1 and SIV Nef activate homologous members of the PAK family of protein kinases (reviewed in Trono and Wang, 1997; Sawai et al., 1997).

Vif acts during assembly to allow the formation of infectious virus particles. The sequences within both the N and C termini of Vif are required for

its activity. In absence of a *vif* gene, virions show abnormal particle assembly and reduced infectivity. The requirement for Vif is strictly dependent on the originator cell type (Trono, 1995; Zou and Luciw, 1996). Vif in both human and simian immunodeficiency viruses is packaged into virions (1 molecule of Vif per 20-30 molecules $p24^{gag}$) and associates with the core (Liu et al., 1995). A Vif-binding region in Pr55gag has been reported, which spans the C-terminal region of NC, the intermediate spacer P2 and the N-terminal half of P6 (Huvent et al., 1998) The mechanism of Vif action remains unknown (Huvent et al., 1998).

Vpr is a small (96 aa), highly conserved protein which is specifically incorporated into HIV-1 virions due to an interaction with $p6^{gag}$ (Paxton et al., 1993). HIV-1 Vpr prevents proliferation of infected cells by inducing a block in the G2/M phase of the cell cycle (He et al., 1995). This appears to halt the cell cycle at a point favorable to viral replication (Goh et al., 1998; Emerman, 1996). HIV-1 Vpr also facilitates the infection of non-dividing cells by regulating the nuclear import of the preintegration complex through an interaction with karyopherin-alpha, a cellular NLS receptor (Vodicka et al., 1998; Popov et al., 1998). This interaction may increase the affinity of karyopherin alpha for basic-type NLS's, like that of matrix protein (Popov et al., 1998). In HIV-2 and SIV (other than SIV_{cpz}) the two major functions of HIV-1 Vpr, cell cycle arrest and nuclear import, are encoded respectively by two separate genes, Vpr and Vpx (Fletcher et al., 1996). Vpr and Vpx both interact with $p6^{gag}$ and are incorporated

into virions in quantities comparable to the viral gag proteins (Wu et al., 1994; Kewel et al., 1996).

Vpu is a late viral gene product translated from a bicistronic mRNA which also contains the open reading frame for Env gp160. Vpu is an integral membrane phosphoprotein which associates primarily with the internal membranes of the cell in oligomeric form (Maldarelli et al. 1993). Vpu interacts with ER-retained CD4 molecules and triggers their degradation; this enhances intracellular transport and maturation of Env proteins, which complex with CD4 molecules in the ER (Willey et al., 1992; Bour et al., 1995). Vpu also stimulates the release of virions (Gottlinger et al., 1993). Although Vpu is a phosphoprotein, phosphorylation is not required for this activity. HIV-2 and SIV (other than SIV_{cpz}) do not encode a Vpu-like protein. However, HIV-2 envelope protein can complement the particle release enhancement function of Vpu (Bour and Strebel, 1996). SIV's relatively high release efficiency appears to be due more to intrinsic properties of Gag-Pol, although SIV envelope protein does exhibit modest Vpu-like effects (Bour and Strebel, 1996).

1.3 Overview of HIV – 1 Replication

1.3.1 The Early Phase of HIV-1 Replication

The events from viral attachment to proviral integration are referred to as the early phase of replication. CD4 is required by both HIV and SIV for attachment and fusion. For HIV-1, chemokine receptors CXCR4 (a.k.a. fusin) and CCR5 are the major coreceptors for entry into T-cell line adapted isolates and monocyte/macrophage adapted isolates, respectively (Feng et al., 1996; Harden et al., 1996; Ghorpade et al., 1998). Dualtropic HIV-1 can use either receptor, and other coreceptors may be required for entry into some cells, such as microglia (Ghorpade et al., 1998; Rabut et al., 1998). Binding of HIV-1 to the receptors causes a conformational change in the envelope proteins, allowing a portion of gp41 (fusion peptide) to enter the cell membrane. Fusion is known to be pH-independent, although there remains debate as to the precise mechanism(s) involved (Rabut et al., 1998; Ghorpade et al., 1998).

Upon membrane fusion, phosphorylation of the matrix protein permits host-cell membrane dissociation of the HIV-1 capsid core (Bukrynskaya et al., 1992). The cyclophilin A packaged in HIV-1 virions is thought to trigger conformational changes in CA protein, allowing capsid disassembly (Gitti et al., 1996). SIV virions do not contain cyclophilin A and this uncoating is completed in SIV by a different and unknown mechanism (Braaten et al., 1996). Reverse

transcription of the RNA genome produces double-stranded provirus for subsequent integration. This process in HIV-1 is primed by cellular $tRNA_{Lys}^{3}$ and some DNA synthesis is observed within virions prior to attachment (Arts et al., 1994; Barat et al., 1989). The proviral preintegration complex is then targeted to the nucleus for import, which occurs by a process of active transport. HIV-1 Vpr, MA and IN are each individually capable of inducing nuclear import to some extent, and for each protein interactions with components of the importin/karyopherin pathway have been described (Popov et al., 1998; Gallay et al., 1997; Vodicka et al., 1998). HIV-1 Vpr does not possess a canonical nuclear localization signal; however, it does localize to the nucleus and can upregulate MA-dependent nuclear import by increasing the affinity of karyopherin alpha for basic-type NLS's (Popov et al., 1998). Proviral DNA may remain in extrachromosal form but for efficient replication must be integrated into host chromosomal DNA by integrase (Goodarzi et al., 1997). Integration completes the early stage of replication.

1.3.2 The Late Phase of HIV/SIV Replication

The late phase HIV/SIV replication includes transcription and translation of viral mRNAs, followed by viral assembly, budding, and maturation. Proviral transcription is regulated primarily by the binding of cellular proteins and viral Tat to cis-acting elements of the 5′ LTR. Numerous elements which bind cellular factors for RNA pol II initiation are present in U3, such as a TATAA box, SP1

binding sites, and NF- kappa B elements (Roulston et al., 1995). The Tat gene product binds to the viral RNA R region within the TAR element, thus positively regulating transcription by either increasing the frequency of initiation or by increasing the stability of elongating RNA polymerase (Haseltine, 1991). Interactions between cellular factors and the TAR element may also be important for regulation of transcription and for initiation of reverse transcription (Hart et al., 1995). All viral RNA's are initiated at the 5′ LTR U3/R junction and terminated at the 3′ LTR R/U5 junction, followed by 7-methylguanosine capping and addition of poly-A tails.

In the early stage of viral RNA expression, only the multiply spliced RNAs encoding Tat, Rev, and Nef and are expressed. As Tat positively regulates transcription, Rev positively regulates the nucleocytoplasmic export of late (unspliced and singly-spliced) RNAs, which unlike the multiply-spliced early RNA molecules are not exported from the nucleus in absence of Rev (Emerman et al., 1989; Malim et al., 1989). Unspliced RNA's (encoding Gag-Pol and Gag, and also packaged in virions) and singly spliced RNA's (encoding Env, Vif, Vpr, and Vpu/Vpx) contain a Rev-responsive element to which Rev binds, allowing their nuclear export. The search for Rev export co-factors has yielded conflicting results, but co-factors may include the RIP/*Rab*, p32 and eIF-5A proteins in humans (Henderson and Percipalle, 1997; Bogerd et al., 1995; Stutz and Rosbash, 1995).

Assembly, budding, and maturation comprise the final steps of late-phase replication. During assembly, viral and cellular proteins become highly concentrated in regions from which virion budding will occur (Luban et al., 1993). Association of Gag and Gag-Pol polyproteins appears to drive the process and is dependent upon the N-terminal myristylation of these molecules (Gottlinger et al., 1991). The NC region of Gag and C-terminus of CA are critical for packaging of genomic RNA, as are numerous *cis* elements in the RNA: a major element (Ψ or PSI) between the 5′ splice donor site and the AUG *gag* initiation codon, an element in the 5′ end of *gag,* and a palindromic hairpin between the PBS and the splice donor site called SL1 (Luban and Goff, 1991; Paillart et al., 1996; Laughrea et al., 1997). *In vitro*, dimerization of HIV-1 RNA is also initiated by base-pairing and interstrand stacking of SL1 loops to form a kissing-loop complex (Paillart et al., 1996; Laughrea et al., 1997). Dimers are linked noncovalently in HIV-1 at a number of sites immediately upstream and downstream of the SD site, collectively known as the dimer linkage structure or DLS (Laughrea and Jette, 1994; Paillart et al., 1996; Skripkin et al., 1994). Dimerization negatively regulates *gag* and *gag-pol* translation and has been postulated to positively regulate RNA packaging (Paillart et al., 1996; Skripkin et al., 1994). Env glycoproteins are recruited to the budding site through interaction between Gag and Env gp41. Budding yields a virus coated with a host-derived lipid bilayer and studded with Env glycoproteins. Proteolytic maturation, initiated by protease during or after budding, cleaves Gag and Gag-Pol into their respective functional units. The maturation process is regulated similarly in both HIV and

SIV by different rates of cleavage at sites within the polyproteins, and thus cleavage products in both viruses are released in the same order (Henderson et al., 1990). NC protein is released early, and complexes extensively with genomic RNA to produce a condensed ribonucleoprotein conformation. Subsequently, cleavage between MA and CA releases CA protein from the membrane, followed by late release of the spacer peptide P1, which facilitates capsid condensation (Henderson et al., 1990). Upon completion of maturation, progeny virions are observed with a bullet shaped capsid core, thus signifying the end of late-phase replication.

1.4 HIV-1 Reverse Transcriptase and Reverse Transcription

1.4.1 HIV-1 Reverse Transcriptase

Reverse transcriptase possesses a DNA polymerase activity, which can utilize an RNA or DNA template, and an RNase H activity. These activities cooperate to convert the single-stranded viral RNA genome into double stranded DNA (Weiss et al., 1985; Varmus, 1988). In virions, HIV-1 RT is a heterodimer of two submits (of 66 and 51 kDa), the smaller of which is a proteolytic cleavage product of the larger. In the heterodimer the p66 subunit is responsible for the enzymatic activities of RT; p51 is lacking an RNase H domain and appears to contribute to the DNA polymerase activity in a largely structural manner (Hansen et al., 1988; Hizi et al., 1988; LeGrice et al., 1988). In the structure of the HIV-1 RT heterodimer, the p66 subunit resembles a right hand and the subunits have been named accordingly – fingers, thumb, and palm, along with a connection subdomain (Kohlstedt et al., 1992; Jacobo-Molina et al., 1993; Nanni et al., 1993). The fingers, thumb, and palm subdomain form the template binding cleft, while the fingers subdomain positions the extended template strand (Jacobo-Molina et al., 1993; Boyer et al., 1994b). The active site is located in the palm subdomain and contains a YMDD motif (positions 183 to 186) conserved at the active site of most retroviral RT's, including HIV-2, SIV, and HTLV-1. Asp-185 and Asp-186 with Asp-110 form a carboxylate triad essential for catalytic activity (Kohlstedt et al., 1992; Nanni et al., 1993). Key regions of palm secondary

structure including and proximal to the YMDD motif also hold a Mg^{2+} cation in position for catalysis. The thumb region is known to be involved in template-primer binding and like the palm region has been reported to influence fidelity of DNA synthesis (Beard et al., 1994). Although the same four subdomains are found in p51 and are observed to be similarly folded, the relative arrangement of the subdomains is substantially different. Consequently, p51 lacks a DNA binding groove and a polymerase active site (Boyer et al., 1994a). P51 may act as a platform of sorts for template nucleic acid, and interactions between p51 and the $tRNA_{Lys}^{3}$ primer have also been reported (Barat et al., 1989).

1.4.2 HIV-1 Reverse Transcription

The process of reverse transcription is usually complete within 4 – 6 hours of infection and takes place mainly in the cytoplasm, although some reverse transcription may occur within the virion (Haseltine, 1991; Lori et al., 1992; Arts et al., 1994). Reverse transcription is thought to be initiated by the specific binding of $tRNA_{Lys}^{3}$ to heterodimeric RT and possibly NC to form a complex on the primer binding sequence (PBS) in the viral RNA genome. The PBS is a short sequence found 3′ of the LTR U5 region which is complementary to sequences in the $tRNA_{Lys}^{3}$ molecule. Sequences flanking the PBS may also be important for initiation, e.g. a loop-loop interaction between the $tRNA_{Lys}^{3}$ anticodon and an adenosine-rich region upstream of the PBS may stabilize the initiation complex (Liang et al., 1997; Barat et al., 1989; Isel et al., 1996; Isel et al., 1993). RNA-

dependent DNA polymerase (RDDP) activity of RT is initiated from the 3′ - CAA end of annealed $tRNA_{Lys}^{3}$, and transcribes through U5 and R regions to the 5′ end of the viral RNA, producing minus (-) strong-stop DNA (Arts et al., 1995). RNase H digestion of the RNA occurs concomitantly with extension, facilitating transfer of newly synthesized DNA to an R region at the 3′ end. While this strand transfer reaction was initially thought to be exclusively intermolecular, one retroviral RNA is sufficient for DNA synthesis and there is currently no clear indication of the relative proportion of intra-to-inter molecular strand transfer (Hu and Temin, 1990; Pop, 1996; Peliska and Benkovic, 1992). Strand transfer may occur by a 'copy choice' (RT + new strand dissociate, new strand reanneals to an R region, reverse transcription continues) or by a strand displacement (initial template is actively displaced by a second template) mechanism. *In vivo* studies indicate that the first template switch acts through both mechanisms and may occur at any point along the R region (Pop, 1996; Luo and Taylor, 1990; Panganiban and Fiore, 1988; Peliska and Benkovic, 1992). Synthesis of (-) strand DNA continues through U3 and into the viral genome again with RNase digestion of template RNA. The exonuclease-like endonuclease activity of RNase H leaves short DNA/RNA hybrids, which are consistently observed at conserved polypurine tracts (PPT's) required for the priming of (+) strand DNA synthesis (Charneau and Alizon, 1992; Pullen et al., 1990). Polymerization occurs from PPT's just upstream of U3 and in the mid-genome region towards the 5′ end of the (-) strand template. A second strand transfer event then occurs in which the 3′ end of the (-) strand strong stop DNA is hybridized to the PBS sequence in (+)

strand DNA (Hu and Temin, 1990; Peliska and Benkovic, 1992). This strand transfer appears to be primarily intramolecular and relies upon the strand displacement activity of RT. Strand displacement allows completion of the reverse transcription process, as (-) and (+) strands use each other as templates to produce full-length double stranded DNA molecules. The synthesized DNA is longer than viral RNA, since the two strand transfers result in a duplicated LTR at either end.

1.4.3 Processivity of HIV-1 Reverse Transcriptase

Two modes of synthesis during *in vitro* DNA polymerization are possible for each residue of a given template. Distributive synthesis results from a polymerization arrest in which the polymerase dissociates from the template/primer. Reinitiation then occurs, allowing the reaction to proceed. In processive synthesis, polymerization is continuous and without dissociation. The processivity of a polymerase is defined as the number of nucleotides incorporated before the enzyme dissociates from a given template (Back et al., 1996). The extent to which an individual residue is replicated by processive synthesis, rather than distributive reinitiation of synthesis, is characteristic of the enzyme and each individual residue along the template sequence. Values for HIV-1 RT may differ from one residue to the next by more than 100-fold, and this effect is dependent on the context of surrounding sequences (Bebenek et al., 1989). HIV-1 RT processivity is also highly sensitive to template secondary structure, dNTP levels,

assay temperature, and the RT preparation used (Back et al., 1996; Goodman et al., 1993).

HIV-1 RT processivity *in vitro* varies from a few to >300 nt, with processivity values on different templates increasing in the order: poly (dA) < ds DNA < ss DNA < ss RNA < poly (rA). Processivity on RNA and DNA templates of random base composition is similar at about 50 – 100 nt (Huber et al., 1989). On the preferred template, poly (rA), synthesis is processive with an incorporation rate of 10 – 15 nt/s at 37°C (Majumbar et al., 1988). RT can catalyze limited strand-displacement synthesis on ds DNA of up to 50 nt. On RNA-DNA hybrids, significant DNA synthesis is only observed after the RNase H activity of RT degrades the RNA strand (Huber et al., 1989). Processive synthesis is also catalyzed by HIV-1 RT across multiple templates; this is thought to be one mechanism by which strand transfers proceed during *in vivo* proviral DNA synthesis (Pop, 1996; DeStefano et al., 1994).

The processivity of HIV-1 RT *in vitro* is thus relatively low among replicative polymerases (Kornberg and Baker, 1992). However, *in vivo* HIV-1 RT is likely to be part of a larger nucleoprotein complex. Such a complex may have an increased apparent processivity due to physical restraint of the polymerase or through interaction with host or viral proteins. *In vivo*, complete polymerization of both HIV-1 strands (20 000 nt) takes 4 – 8 hours; therefore a minimum estimate of the *in vivo* polymerization rate is 1 nt/s. The actual rate

may be significantly higher, since in this calculation no correction is made for the time of virus adsorption and penetration (Klarmann et al., 1993).

1.4.4 Fidelity of HIV-1 Reverse Transcriptase

HIV-1 exhibits extensive genomic heterogeneity. Three different replication systems in the viral life cycle contribute to this: proviral DNA synthesis by reverse transcriptase, replication of integrated proviral DNA by cellular DNA polymerases, and transcription of proviral DNA by RNA polymerase II. The contribution of HIV-1 RT to observed mutation rates is believed to be very significant, partly due its lack of 3' → 5' exonuclease proofreading activity. DNA is synthesized by HIV-1 RT with an error rate estimated at 1 per 10 000 nt incorporated (1 – 2 mutations per genome per replication cycle) (Roberts et al., 1989). The mutations observed are non-randomly distributed; sequences of viral isolates taken from a single patient at different times indicate mutation rate of 10^{-3} nt substitutions/site/year for the env gene and 10^{-4} nt substitutions/site/year for the gag gene (Bebenek et al., 1989). HIV-1 RT is 10-fold less accurate than comparable RT's from avian myeloblastosis virus (AMV) and murine leukemia virus (MLV) (Roberts et al., 1988, 1989; Bebenek et al., 1989). Since the RT's of both of these viruses lack 3' → 5' proofreading activity, it is clear that the error-prone nature of HIV-1 RT involves more than just the absence of a proof-reading activity (Bebenek et al., 1993).

HIV-1 RT infidelity results in base substitution mutations, which are the product of misincorporation (insertion of inappropriate nucleotides) and misextension (elongation past mismatches). RT infidelity also results in insertion and deletion errors, including frameshift mutations. *In vitro* studies have shed light on the relative distribution and mechanisms of these errors, particularly for the DDDP activity of HIV-1 RT, which has been more extensively studied. DNA synthesis by HIV-1 RT on an M13mp2 DNA template exhibited the following errors, in order of highest to lowest error rate: i) one-base frameshifts at homopolymeric runs, ii) single-base substitutions at the boundaries of homopolymeric runs, iii) single-base substitution due to polymerase miscoding, and iv) one-base frameshifts at non-run sites (Bebenek et al., 1989). The overall error rate for single-base frameshifts and single-base substitution was comparable, at 1/4300 nt and 1/5300 nt polymerized respectively. However, for both frameshift and substitution errors, there is a considerable discrepancy between error rates at homopolymeric runs and non-run template sites (e.g. –1 frameshift error in run vs. non-run template occur at rates of 1/2600 and 1/240 000 respectively). This discrepancy has given rise to the notion that certain homopolymeric runs act as mutational 'hot spots' which produce mutations usually by slippage-mediated mechanisms (Bebenek et al., 1989; Pathak and Temin, 1990). Pausing at such sites is correlated with mutagenesis, strand transfer, recombination, and (in the case of frameshift hot spots) termination (Bebenek et al., 1989, 1991; Klarmann et al., 1993; DeStefano et al., 1994). Polymerase pausing is frequent on runs of rG and rC during (-) strand synthesis

(RDDP) and on runs of dT and dA during (+) strand synthesis (Klarmann et al., 1993). The frequency of hot spot associated mutagenesis on RNA templates is reduced versus the frequency on DNA templates (4 – 34 fold lower for frameshift errors and 14 – 73 fold lower for base substitutions), suggesting that misaligned template-primers are formed and/or used less frequently on RNA templates (Ju and Loeb, 1992; Boyer et al., 1992a). This may partially account for the 5 – 10 fold lower error rate overall for (-) strand versus (+) strand synthesis (Bebenek et al., 1993). (-) strand (RDDP) errors may be more important for genomic heterogeneity however, since it is the (-) strand of the integrated provirus which is eventually transcribed; (+) strand (DDDP) errors may not be of relevance until cellular DNA replication or mismatch repair by cellular enzymes occurs.

1.4.5 Mutational Bias of HIV-1 Reverse Transcription

Substitution mutations of different kinds are generated by HIV-RT at different rates. Transitions (one purine or pyrimidine being substituted for another) occur at a higher frequency than transversions (a purine being substituted for a pyrimidine, or vice-versa) (Berkhout and Klaver, 1995). Among the various possible transitional substitutions, there is an order of preference for HIV-1 RT: G→A > T→C > A→G > C→T (Keulen et al., 1997). The variable frequency of different mutations results from an inherent mutational bias of RT, which is modulated by template/primer-dNTP interactions and intracellular conditions –

most notably the varying levels of available dNTP's (Bebenek et al., 1992, 1993; Vartanian et al., 1991; Boyer et al., 1992b).

The G→A transition is observed at certain locations in the genomes of HIV-1 and other retroviruses at an extremely high frequency; this phenomenon is referred to as G→A hypermutation (Vartanian et al., 1991; Pathak and Temin, 1990). From the *in vitro* observed rates for dTTP-rG misincorporation during (-) strand synthesis and dATP-dC misincorporation during (+) strand synthesis, we can calculate a predicted rate at which G→A substitutions would be observed in a single round of viral RNA replication. This predicted rate based on *in vitro* fidelity studies is at least 700-fold less than the rate actually observed for SNV (2×10^{-2}), for which the G→A hypermutation rate is thought to be less than for HIV-1 (Pathak and Temin, 1990; Vartanian et al., 1991). Thus, *in vitro* error rates do not appear to explain the hypermutation rates observed *in vivo*. Some theories have been proposed to account for this discrepancy. Since the G→A hypermutation occurs with high specificity at GpA dinucleotides, it appears that the modulating effect of template sequence on RT error and mutational bias may be exacerbated at GpA sites (Vartanian et al., 1991). Disproportionate dNTP ratios have also been observed to have an exaggerated effect in the case of G→A substitutions (Keulen et al., 1997; Martinez et al., 1994). The relative stability of rG-dT base pairs may also be conducive to the misincorporation and/or misextension of an inappropriate (-) strand dTTP. Finally, dislocation

mutagenesis (by –1 dislocations of the primer strand) has been proposed as the mechanism underlying G→A hypermutation (Kunkel and Alexander, 1986).

1.4.6 Resistance to HIV-1 Reverse Transcriptase Inhibitors

The mutation rate of sequences within the HIV-1 genome is remarkably high, due in large part to the infidelity of its reverse transcriptase. This mutation rate coupled with the equally remarkable rate of viral replication in infected individuals provides favourable circumstances for the production, selection, and outgrowth of drug-resistant viral variants. Resistance to nucleosidic reverse transcriptase inhibitors (NRTI's) such as 3TC, ddI, and ddC has been demonstrated for isolates obtained from patients (Smith et al., 1993; Wainberg et al., 1995). Variants resistant to more than one nucleoside analog are clinically isolated with increasing frequency. Such NRTI-resistant and multiply NRTI-resistant viruses can be readily generated by sequential *in vitro* passage in cell lines under NRTI selective pressure (Gao et al., 1992, 1994). Non-nucleosidic reverse transcriptase inhibitors (NNRTI's) generally select for highly resistant variants more rapidly than nucleoside analogs, and are rarely used in long term treatment for this reason.

Different NRTI's can produce significantly different patterns of drug resistance. Clinically and *in vitro*, AZT resistance is usually conferred by a series of five mutations in the RT gene, at codons 41, 67, 70, 215, and 219 (Gao et al.,

1994). However, recent reports that a 6-basepair insert in the RT gene of clinical isolates confers resistance to multiple nucleotide analogs (e.g. AZT, ddI, ddC) demonstrates that single amino acid alterations are not the only means by which NRTI resistance may arise (Winters et al., 1998; Wainberg, 1999). For 3TC, a single Met→Val mutation at codon 184 is sufficient to confer high-level (>1000 fold) resistance to 3TC as well as low level resistance to both ddI and ddC (Arts and Wainberg, 1996). Mutations at codon 65 (Lys→Arg) and 89 (Glu→Gly) have also been associated with 3TC resistance (Gu et al., 1994; Song et al., 1992). 3TC and AZT have nonetheless a common mechanism of inhibition which is shared by most triphosphorylated nucleotide analogs. They inhibit RT by i) competitive inhibition and ii) chain termination of elongating DNA, due to their lack of a 3' hydroxyl group (Arts and Wainberg, 1996).

Chapter 2: Identification of Mutations of Codon 184 of SIV Reverse Transcriptase Which Confer Resistance to 3TC

2.1 Abstract

With 3TC (lamivudine) now in widespread use in combination therapy for the treatment of HIV-1 infection, reports of clinical resistance are a growing concern. Mutations occurring at codon 184 in the highly conserved YMDD motif of RT are the primary source of 3TC resistance in HIV-1. M184I and M184V mutations confer high-level 3TC resistance (>1000 fold), with M184I variants appearing early, followed by outgrowth of M184V variants in HIV-1 *in vitro* and clinical studies. The M184V mutation also results in increased fidelity and processivity of the HIV-1 RT molecule, which may contribute to the sustained efficacy of 3TC/AZT dual therapy. To further assess the potential utility of SIV in models for antiviral therapy, we have investigated the molecular correlates of 3TC resistance in SIV-RT.

SIV variants resistant to 3TC were generated by *in vitro* passage and drug selection. C8166 human $CD4^+$ T-cells were infected with 200 $TCID_{50}$'s (50% tissue culture infectious doses) of either $SIV_{mac}32H$ (pJ5) or (pC8) viral stocks and passaged in tissue culture medium with increasing 3TC selective pressure. At 8 weeks (2.5 uM 3TC) viral variants of pJ5 and pC8 were capable of growth in the presence of > 500 uM 3TC (>1000 fold the normal inhibitory concentration),

and at 24 weeks (750 uM 3TC) were capable of growth in >2000 uM 3TC, (>4000 fold the normal inhibitory concentration). RT-coding regions (1.5 kb) of 3TC-resistant (2.5 and 750 uM) variants were amplified from genomic DNA of infected cells, cloned into the pCR™ 2.1 vector, and sequenced. 3TC sensitive variants were amplified from C8166 cells passaged in parallel without drug selective pressure. All (5) 3TC resistant (2.5 uM) clones sequenced contained a M184I (ATG->ATA) substitution, which was observed in none of five 3TC sensitive clones. All (8) 3TC resistant (750 uM) clones sequenced contained a M184V (ATG->GTA) substitution, while all (4) sensitive clones contained wild type M184 (ATG). Thus, M184I variants predominated in early passaging and were outgrown by M184V variants, as is observed in the case of HIV-1. A Cys508Tyr (TGC->TAC) substitution was also observed in four of eight 750 uM-resistant clones, while Thr296Ala (ACT->GCT) and Thr296Ser (ACT->TCT) substitutions were each observed in one 750 uM resistant clone. The significance of the 508 and 296 mutations for drug resistance remains uncertain. To confirm the role of the 184 mutations in 3TC resistance, we introduced M184I and M184V mutations into $SIV_{mac}239$ by site-directed mutagenesis and tested the mutant strains against wild type for susceptibility to a number of antiviral compounds. M184I and M184V mutants displayed high-level 3TC resistance (IC50's were >500 uM for both M184I and M184V viruses, versus 1.8 uM for wild type), thus substantiating the role of these mutations in SIV resistance to 3TC. The M184V mutation also appeared to modestly increase sensitivity to d4T (as did M184I), and decrease sensitivity to ddI. These studies confirm the biological relevance of

SIV-RT codon 184 mutations in 3TC resistance, and provide evidence that 184 substitutions in SIV may evolve in response to 3TC selection with a pattern similar to that observed in HIV-1.

2.2 Introduction

2.2.1 The 3TC-Resistance Mutations M184I and M184V

M184 is the X residue in the catalytically important YXDD motif found in all reverse transcriptases (Johnson et al., 1986). The two aspartate residues are part of a catalytic carboxylate triad (along with asp110) and are invariant among all the RNA dependent DNA and RNA polymerases. Enzymatic activity studies performed on a series of site-directed mutants demonstrated the significance of the YXDD residues in HIV-1 RT (Larder et al., 1987). The mutation Y183S resulted in a severe loss of RT activity (1.2% of the wild type) while D185H and D186N mutations eliminated RT activity completely. Mutation of the M184 residue has yielded varying results, from elimination of RT activity (M184G, M184P) to a scarcely detectable reduction (M184V) (Larder et al., 1987; Wakefield et al., 1992). The M184I and M184V mutations have been associated with high-level 3TC resistance, and M184V also confers resistance to ddI and ddC (Gao et al., 1993; Schinazi et al., 1993; Tisdale et al., 1993).

The recent publication of the crystal structure (3.2 angstrom) of a covalently trapped complex of HIV-1 RT with a DNA template:primer and incoming dNTP has answered many questions regarding the function of codon 184 (Huang et al., 1998). The methionine 184 side chain was observed to contact the sugar and base of the primer 3' nucleotide. Computer-assisted modeling

showed that introduction at 184 of an amino acid with a β-branched side chain (isoleucine or valine) would result in the same contacts with the primer, but with an additional contact to the dNTP sugar ring. Modeling of 3TC into the structure showed an enhanced interference between the Ile or Val side chain and 3TC; this resulted from the conformation of the oxathiolane ring in 3TC (Huang et al., 1998). These results are consistent with previous crystal structure data, and add significantly to the earlier understanding (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993; Beard et al., 1994; Tantillo et al., 1994).

M184I and M184V mutants of HIV-1 exhibit altered properties in addition to drug resistance which may be of relevance *in vivo*. The RT fidelities of M184I and M184V have been assessed (Rezende et al., 1998). In an M13-based forward mutation assay, overall error rates for DDDP were determined and the 184 mutations were found to have significant effects on fidelity (in order of increasing fidelity: Met, Val, Ile). The error rate for M184I was 1.7×10^{-5} per nucleotide, four times the fidelity for M184 (7.0×10^{-5} per nucleotide) and 2.5 times the fidelity of M184V (4.3×10^{-5} per nucleotide). These results are in good agreement with previous findings (Wainberg et al., 1996; Pandey et al., 1996).

The replication rate of HIV-1 may also be altered in 184 mutants. This variability is added to the already variable replication of HIV among different cell lines (Salomon et al., 1995). The M184V and M184I are generally observed to exhibit decreased replicativity, and this effect is more pronounced in primary cell

lines (Larder et al., 1995; Back et al., 1996). Reduced processivity of 184 mutants may partially account for this, and has been observed for both mutants (in order of increasing processivity: Met, Val, Ile) (Back et al., 1996). This effect is enhanced in conditions of low dNTP availability, and thus would be expected to vary with cell type and experimental conditions.

The mutations at codon 184 of the YMDD motif of HIV-1, in addition to conferring drug resistance, may thus lead to altered viral characteristics in the clinical context. While the increased fidelity of M184I and M184V mutants is not likely to attenuate the development of resistance to other drugs taken concomitantly, there is reason to believe that their increased fidelity could reduce variation in the *env* genes and thus reduce the likelihood that immunological 'escape mutants' will be generated (Wainberg et al., 1996). The decreased processivity and RT activity associated with M184I and M184V mutations may also have potential benefits for patients on 3TC regimens. The characteristics of 184 mutants in the YMDD of SIV reverse transcriptase have not yet been investigated. Such studies would facilitate assessment of the potential utility of SIV models in studies of antiviral chemotherapy and drug resistance.

2.3 Materials and Methods

2.3.1 Drugs

3TC (2', 3'-dideoxy-3'-thiocytidine was donated by BioChem Pharma Inc., Laval, Quebec. AZT (3'-azido-3'-deoxythymidine), was obtained from Glaxo-Wellcome Inc. (Research Triangle Park, North Carolina). ddI was obtained from Bristol-Myers Squibb (Wallingford, Connecticut). ddC was obtained from Sigma Chemical Co. (St. Louis, Missouri). D4T (2',3'-didehydro-2',3'-dideoxythymidine), nevirapine, indinavir, and saquinavir were gifts from Bristol-Myers Squibb, Boehringer-Ingelheim Inc. (Ridgefield, Connecticut), Merck Inc. (West Point, Pennsylvania), and Roche Inc. (Basel, Switzerland), respectively.

2.3.2 Cell Lines and Viruses

The C8166 cell line (European Collection of Animal Cell Cultures; Public Health Laboratory Service, London, United Kingdom) was used for all studies. C8166 cells are an HTLV-III – transformed, umbilical cord blood-derived human $CD4^+$ T-cell line (Salahuddin et al., 1983). C8166 cells were grown in RPMI 1640 medium (Gibco-BRL Laboratories, Mississauga, Ontario) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Toronto, Ontario), 2 mM L-glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin per

ml. The two viruses used for initial drug selection experiments, $SIV_{mac}32H$ (pJ5) and $SIV_{mac}32H$ (pC8), are full-length viruses cloned by molecular biology based techniques. These viruses were a gift of Dr. Erling Rud, Laboratory Center for Disease Control, Ottawa, Ontario. These viruses differ by a naturally occurring in-frame deletion of four amino acids and two conservative amino acid changes, all occurring in the Nef region of pC8 (Rud et al., 1994).

2.3.3 Selection for Resistance

10^6 C8166 cells were infected with 200 $TCID_{50}$'s of either the pJ5 or pC8 viral stocks for two hours. Cells were washed extensively following infection and were maintained in tissue culture medium in either the absence or the presence of subinhibitory concentrations of 3TC, starting at 0.01 μM. Media were changed twice weekly with each replacement containing an increased drug concentration, as follows: 0.01, 0.05, 0.2, 1, 2.5, 10, 25, 100, 250, 500, and 750 μM. From each passage 0.5 ml of culture fluids were used to infect fresh C8166 cells as described previously (Gao et al., 1993). Cultures were monitored for the presence of RT activity and the presence of cytopathic effect, as described previously (Gu et al., 1992). At 8 and 24 weeks (final 3TC concentration, 2.5 or 750 μM), IC_{50} (50% inhibitory concentration) values for 3TC were determined for viral variants of pJ5 and pC8 in assays of RT activity in culture fluids (Boulerice et al., 1990).

2.3.4 Cloning and Sequencing

The complete RT-coding regions of five 3TC-sensitive (passaged without 3TC addition for 8 weeks) and five 3TC-resistant (passaged with 3TC addition for 8 weeks, final concentration 2.5 μM) variants of each of pC8 and pJ5 were PCR-amplified from the genomic DNA of infected C8166 cells. Four 3TC-sensitive (24 week) and eight 3TC-resistant (24 week, 750 μM) pC8 variants were likewise amplified. In all cases, genomic DNA was prepared as follows: pelleted C8166 cells (~5 x 10^6) were resuspended in lysis buffer containing 0.5% sodium dodecyl sulfate and 1 mg of protease K per ml. After 6 hours at 37°C, the suspension was extracted 6 times with phenol chloroform (1:1) and twice with chloroform, precipitated with ethanol and sodium acetate (2.5 volumes, 1/10 volume). Two μg of DNA were subjected to 30 cycles of PCR amplification, using 2.5 units of AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut), 50 pmol of each primer SRT01 and SRT02 (Table 1), 0.2 μM of each of the four dNTP's, and buffers as recommended by the manufacturer in a volume of 100 ml. There were thirty cycles (denaturation, 94°C x 1 min; annealing, 58°C x 1 min 15s; and extension, 72°C x 1min) and a final extension at 72°C for 10 minutes.

The PCR products were cloned directly into T/A cloning vector pCR™ 2.1 (Strategene, La Jolla, California), according to manufacturer's instructions. RT sequences were determined by double-strand sequencing (dsDNA Cycle Sequencing System; Gibco BRL Laboratories) using a series of nine primers

designated SRT01 – SRT09, spaced roughly 200 bp apart in the RT-coding region (Table 1), according to manufacturer's instructions.

Oligonucleotide	Sequence (5' → 3')	SIVmac32H Coordinates
SRT01	GGGGATGTCTTTAAACC	3099-3115
SRT02	GCTGGCTCTATCTTTTCC	4817-4800
SRT03	GAACAAATGGAGAATGC	3321-3337
SRT04	GCAGAGCCAGGAAAGCG	3529-3545
SRT05	CTCTTGAATAGCATAGGG	3739-3756
SRT06	GGAGTATTAAATTGGGC	3901-3917
SRT07	GGTCTTATAAAATTCACCA	4127-4145
SRT08	GGCAGGTAACCTGGATACC	4331-4349
SRT09	CTACTAATCAACAAGCAG	4529-4546

Table 1. Oligonucleotides used for cloning and sequencing.

2.3.5 Site-Directed Mutagenesis

Site-directed mutagenesis was performed on a cloned SIV_{mac} virus to allow observation of the effect of M184I and M184V mutations on drug resistance. Mutations encoding M184I (ATA) and M184V (GTG) were introduced into the 5' half of $SIV_{mac}239$, p239SpSp5'. The $SIV_{mac}239$ virus is cloned as two halves due to the instability of the full-length plasmid in bacteria (Kestler et al., 1989; Regier and Desrosiers, 1990). A 1.5-kb fragment of p239SpSp5' (positions 4236 to 5732) of the RT coding region including codon 184 was amplified by PCR and T/A cloned into the pCR™ 2.1 vector; this was done to facilitate mutagenesis as

the resulting plasmid was considerably smaller than the original p239SpSp5' plasmid. PCR conditions were as described in 2.3.4 except that 50 pmol of SRT02 and SRT03 primers were used along with 250 ng of template DNA, and the annealing temperature was 54°C. Mutagenesis was then performed using the Quickchange mutagenesis kit (Strategene, La Jolla, California) with primers for M184I (5'CCTTAGTCCAGTAT (A/G) T (A/G) GATGACATCTTAA) and M184V (5'GCTATTAAGATCTCATC (T/C) A (T/C) ATACTGGACTAAGG), and was confirmed by sequencing. A 754-bp BstEII fragment containing codon 184 was then cloned back into p239SpSp5' by BstEII sites at positions 4496 and 5250. Correct orientation of the insert was confirmed by restriction digest, and presence of mutations at 184 was again confirmed by sequencing. Mutant and wild-type proviruses were regenerated by Sph1 digestion of mutant and wild-type p239SpSp5' prepared plasmid DNA and ligation of each to Sph1-digested p239SpE3', the plasmid containing the 3' half of the genome. Ligated DNA was then transfected into Cos-7 cells.

Cos-7 cells are a SV40-transformed African green monkey kidney cell line (CV-1) (Gerard and Glutzman, 1985). Cos-7 cells were grown at 37°C as a monolayer in DMEM medium (Dubbecco's modifed Eagles medium), containing 10% fetal calf serum, 100 U/ml penicillin G and 100 µg/ml streptomycin. Twenty four hours prior to transfection, exponentially growing cells were harvested by trypsinization and replated at a density of 1 x 10^6 cells per tissue culture dish (10 cm). Ligated DNA's were added to a total of 10 µg of DNA per dish. Proviral

DNA was suspended with $CaCl_2$ and added dropwise; the transfection was performed according to a published protocol (Jiang et al., 1992). Virus in culture fluids was harvested after 63 hours of incubation and clarified by centrifugation. Supernatant virus concentrations were determined by measuring RT activity in an *in vitro* scintillation-based assay (Boulerice et al., 1990). $SIV_{mac}239$ wild-type, M184V, and M184I viruses were then amplified in C8166 cells and viral stocks were produced. Proviral sequences were PCR-amplified from genomic DNA of infected C8166 cells and sequenced directly to confirm the presence of appropriate mutant or wild-type 184 codons. The mutant and wild type viral strains were tested for their susceptibility to numerous antiviral compounds. IC_{50}'s were determined on the basis of RT activity in culture fluids as before for the drugs 3TC, AZT, ddI, ddC, d4T, nevirapine, indinavir, and saquinavir.

2.4 Results

2.4.1 Selection of Resistant Variants of SIVmac32H (pC8) and (pJ5)

SIVmac32H (pC8) and (pJ5) viruses were passaged in tissue culture and selected for resistance with increasing concentrations of 3TC. IC_{50}'s for 3TC (concentration of 3TC required to inhibit RT activity of culture fluid virus by 50%) were determined at 0, 8, and 24 weeks. At 8 weeks (2.5 μM 3TC) variants of pC8 and pJ5 were capable of growth in the presence of greater than 500 μM 3TC, more than 1000-fold the usual inhibitory drug concentration. At 24 weeks (750 μM 3TC) variants of pC8 and pJ5 were capable of growth in the presence of greater than 2000 μM 3TC, more than 4000-fold the usual inhibitory drug concentration (Table 2).

Virus	No. of weeks in passage[a]	Highest concn (μM) of 3TC attained[b]	Mutations found by sequencing[c]	IC_{50} (μM) of 3TC[d]
$SIV_{mac}32H(pJ5)$	0	0		0.13 ± 0.04
	8	2.5	M184I	>500
	24	750	M184V	>2,000
$SIV_{mac}32H(pC8)$	0	0		0.45 ± 0.01
	8	2.5	M184I	>500
	24	750	M184V	>2,000

Table 2. Selection of SIV variants resistant to 3TC.

[a] All cultures were passaged twice weekly.
[b] Whenever cultures became positive for cytopathic effect and RT activity, the drug concentrations were gradually increased.
[c] Substitutions were detected by cloning and sequencing as described in the text.
[d] Results were calculated on the basis of RT activity in culture fluids. Data are means ± standard deviations for three replicate samples.

2.4.2 Sequencing of RT Regions of 3TC-Sensitive and 3TC-Resistant Variants of $SIV_{mac}32H$ (pC8) and (pJ5)

Complete RT coding regions of five 3TC-sensitive and 3TC-resistant (2.5 μM) variants of each of pC8 and pJ5 as well as four 3TC-sensitive and eight 3TC-resistant (750 μM) variants were PCR amplified from genomic DNA, cloned, and sequenced. Mutations resulting in amino acid substitutions were observed. All of the 3TC-resistant (2.5 μM) clones contained a methionine to isoleucine (ATG to ATA) substitution at codon 184, while none of the drug-sensitive variants contained this mutation. All eight 3TC-resistant (750 μM) clones contained a methionine to valine (ATG to GTA) substitution at codon 184, while all four sensitive variants contained the wild type codon (ATG) (Table 2). Among the 750 μM 3TC variants sequenced, several other nonsynonymous mutations were observed. A Cys→Tyr (TGC→TAC) codon 508 substitution was observed in four of the eight resistant clones. Two different codon 296 alterations were observed in resistant clones, one Thr→Ala (ACT→GCT) and one Thr→Ser (ACT→TCT). All other substitutions in resistant clones were observed singularly, including a Leu→Gln (CTG→CAG) alteration at codon 74. The observed amino acid substitutions for 24 week 3TC-sensitive and 3TC–resistant clones are summarized in Table 3.

	Amino Acid Residue																		
	35	**36**	**38**	**61**	**72**	**74**	**116**	**122**	**126**	**184**	**199**	**285**	**296**	**301**	**379**	**426**	**505**	**508**	**525**	**532**
	R	**E**	**C**	**F**	**R**	**L**	**F**	**E**	**Q**	**M**	**D**	**G**	**T**	**A**	**I**	**F**	**I**	**C**	**I**	**I**
Resistant Clones																				
1										V			A					Y		
2										V										
3						Q				V										V
4	K	K			K					V		E		V						
5				C						V									T	
6									R	V								Y		
7										V	G		S				T	Y		
8										V					V	L		Y		
Sensitive clones																				
1							S													
2			Y					K											T	
3																		Y		
4								K												

Table 3: Productive amino acid substitutions in the reverse transcriptase region of SIV viral isolates, passaged for 24 weeks in the presence (resistant clones) or in the absence (sensitive clones) of 3TC.

2.4.3 Determination of Sensitivity to 3TC and Other Antiviral Drugs for M184V, M184I, and Wild-Type $SIV_{mac}239$

IC_{50}'s for mutant and wild-type $SIV_{mac}239$ viruses were determined on the basis of RT activity in culture fluids as before, and mean values were calculated for three replicate samples. IC_{50}'s for 3TC were >500 μM for M184V and M184I, or >250-fold the value for wild-type virus, thus substantiating the role of these mutations in SIV resistance to 3TC (Table 4). In contrast, neither of the mutant viruses displayed resistance to AZT. The M184V substitution also appeared to confer a modest increase in the IC_{50} of ddI, while neither mutant virus displayed resistance to ddC. Both substitutions appeared to similarly increase sensitivity to d4T. SIV (like HIV-2) is not sensitive to NNRTI's, and all three strains tested here were similarly insensitive to nevirapine. In addition, while all three strains were susceptible to inhibition by the protease inhibitors indinavir and saquinavir, no statistically significant difference in their sensitivities were observed.

	IC50 (μM)[a]							
itation	3TC	AZT	ddI	ddC	d4T	nevirapine	indinavir	saquinavir
), ld	1.8 ± 0.6	0.37 ± 0.03	11.0 ± 0.9	1.0 ± 0.07	9.0 ± 0.87	>10	0.015 ± 0.008	0.006 ± 0.002
),	>500	0.24 ± 0.01	20.0 ± 1.1	0.6 ± 0.01	2.0 ± 0.09	>10	0.015 ± 0.006	0.003 ± 0.005
),	>500	0.40 ± 0.04	7.0 ± 0.4	2.0 ± 0.05	1.8 ± 0.06	>10	0.02 ± 0.001	0.0015 ± 0.007

Table 4. Susceptibility of M184V, M184I, and wild-type SIVmac239 to 3TC and other antiviral agents.

[a] Results were calculated on the basis of RT activity in culture fluids. Data are means ± standard deviations for three replicate samples.

2.5 Discussion

2.5.1 M184V and M184I Variants are Selected in Tissue Culture Passaging with 3TC.

No previous reports have formally associated mutations at codon 184 with SIV resistance to 3TC *in vivo*, in tissue culture selection, or by mutagenesis. The tissue culture selection studies with $SIV_{mac}32H$ pC8 and pJ5 described here document the observation of M184I and M184V mutations in SIV reverse transcriptase in response to 3TC *in vitro* drug selection. The level of resistance observed for these viruses was high (>1000-fold the normal inhibitory 3TC concentration), and comparable to that observed for HIV-1 (500- to 100-fold) (Gao et al., 1992; Schinazi et al., 1993; Larder et al., 1995). This similarity may prove advantageous for the use of SIV in models for antiviral chemotherapy; recently the homologous M183V and M183I mutations in FIV were observed to confer only 6- to 8-fold 3TC resistance (Smith et al., 1997). In addition, we observed a pattern of selection in which the M184I variants predominated in the $SIV_{mac}32H$ population prior to the selection and outgrowth of M184V variants; this is similar to the order of appearance of 3TC-resistant variants for HIV-1 *in vitro* studies and in isolates from 3TC-treated patients in longitudinal studies (Wainberg et al., 1995; Schuurman et al., 1995). These results are consistent with a model in which the Y*DD motif in SIV reverse transcriptase behaves during catalysis in a manner similar to that of HIV-1, and argues for a broader structural and mechanistic homology for the active sites of HIV and SIV reverse transcriptases than was previously known.

2.5.2 Other Alterations Observed in 750 μM-Resistant $SIV_{mac}32H$ (pC8) Clones

M184I and M184V were the only substitutions observed in all low-level (2.5 μM) and high level (750 μM) resistant clones, respectively. This provides strong evidence that these mutations are the primary agents of 3TC resistance in SIV. Nonetheless there is a possibility of contribution from other amino acid substitutions to the observed resistance. A C508Y substitution was observed in 4 out of 8 resistant clones. The relevance of this and other alterations is difficult to assess, since no crystal structure or NMR data have yet been published for SIV RT or HIV-2 RT. However, since the polymerase domain of SIV RT, like HIV-1 RT, extends only to the vicinity of amino acid 430, a direct contribution of this residue to 3TC resistance is not likely. Codon 508 in HIV-1, HIV-2, and SIV is found in a region of the RNase H domain which corresponds to the 'C-helix' motif conserved among RNase H sequences from Rous Sarcoma virus to E. coli (Telesnitsky et al., 1992; Prasad, 1993). Interestingly, deletion of 11 amino acids from this region in Mo-MLV (Moloney murine leukemia virus) diminished the transfer and elongation of minus strand DNA significantly while formation of minus strong stop DNA was unaffected. This suggested that the C-helix is required for a critical interaction between the DNA polymerase and RNase H domains, or for proper functioning of the polymerase. The deletion mutant was also defective in the processivity of DNA synthesis (Telesnitsky et al., 1992). While the significance of this mutation in SIV-RT is unclear, the perfect alignment of SIV amino acid sequences with those of HIV-1 and HIV-2 in this

region (e.g. residues 497-501 are DSQY for the consensus sequences of HIV-1, -2, and SIV) indicates that the 508 region in SIV may also be involved in C-helix-like function. If this is the case, the C508Y mutation may have been indirectly involved in the drug resistance observed. Another possibility is that the C508Y mutation had a compensatory effect on replication; for example, the M184V mutation may have diminished the processivity of RT, and the C508Y mutation may have ameliorated this loss of function via C-helix-like effects on processivity. The presence of a C508Y substitution in one of two 3TC-sensitive clones argues that the origin of this mutation may have been more trivial. Conversely, a I505C mutation in a single resistant clone favors the notion that mutations in this area may have conferred some advantage during tissue culture selection. Further studies will be required to assess any relevance of codon 508 and 505 mutations in 3TC resistance and/or polymerase function.

Only one other codon was observed to be mutated in more than one resistant clone. Thr296 was substituted by alanine in one clone and serine in another. In HIV-1, residue 296 is located in the thumb in a region of leucine repeats which is capable of assuming a helical conformation (aa 283-310) (Baillon et al., 1991). This region has been postulated to act as a leucine zipper, although mutagenesis studies have not provided evidence for a role of such a zipper in RT dimerization. Although there is considerable homology between HIV and SIV RT's, the leucine motif appears to be restricted to HIV-RT, and the secondary structure and function of this region of SIV is not clear (Le Grice,

1993). Another study of HIV-1 RT implicates the region including residue 296 in primer binding. A 15-nt poly $(dT)_{15}$ primer was found to contact a short polypeptide between residues 288 and 306 (Basu et al., 1991). As previously mentioned, codon 184 (wt, V, and I) contact the primer also, at the sugar and base of the primer 3' nucleotide. Interestingly, in the studies which originally identified M184V as encoding resistance to ddI and ddC, viruses selected *in vitro* by ddI selective pressure were repeatedly observed to possess both M184V and P294S mutations (Gu et al., 1992). The P294S mutation was not found to contribute to drug resistance, but a role as a compensatory mutation has not been ruled out. It is an intriguing possibility that mutations at codon 296 (or 505/508) could be playing a compensatory role for the loss of SIV RT function induced by M184V; if so, the homologous residues may play a compensatory role in 3TC-resistant HIV-1 RT. Any significance of codon 296 in SIV drug resistance or polymerase function awaits further investigation.

Only one of the codons found substituted (other than 184) has been previously associated in HIV, SIV, RT-SHIV, or FIV, with NRTI resistance. L74, which was substituted by glutamine in one resistant clone, has been demonstrated to confer resistance when substituted by valine to ddC (5 – 10 fold), ddI, and 1592U89 (St. Clair et al., 1991; Balzarini et al., 1995). L74 is located at the carboxyl end of the Beta 4 stretch in RT and contacts the extended template about three nucleotides beyond the active site (Boyer et al., 1994a). In L74 mutants, altered interactions with the template overhang influence the precise

geometry at the polymerase active site, leading to NRTI resistance (Tantillo et al., 1994). The L74V mutation also markedly diminishes the effect of a T215Y mutation in conferring AZT resistance, implying a close interaction of these residues in the active site of RT (St. Clair et al., 1991). An association between codon 74 mutations and 3TC-resistance has not yet been made for SIV-RT.

Although a role for mutations at codon 74 in 3TC resistance and/or polymerase function merits consideration, the low frequency at which this and all other mutations observed in a single clone were observed implies that these mutations were not strongly selected for in tissue culture. Furthermore, observed mutations may also be the result of errors made by Taq polymerase in the PCR amplification step required for production of clones. It is clear, however, that the predominant agents of 3TC resistance in our tissue culture passaging were the M184I and M184V mutations.

2.5.3 Confirmation by Site-Directed Mutagenesis of $SIV_{mac}239$ that M184I and M184V Mutations in SIV-RT Confer High-Level Resistance to 3TC

The observed IC_{50}'s of >500 μM for M184I and M184V $SIV_{mac}239$ mutants versus 1.8 +/- 0.6 μM for wild type confirm that high level resistance to 3TC is conferred by both of these mutations. The possibility of complication from non-184 mutations arising from other steps was minimized by the use of Pfu polymerase in the site-directed mutagenesis reaction (Pfu is a high-fidelity polymerase), and by minimizing the time over which viruses were maintained in

culture for IC_{50} determination. The IC_{50}'s observed for SIV were generally higher than those previously observed for HIV-1, but were consistent with those previously observed for SIV and FIV (Smith et al., 1997; Van Rompay et al., 1997).

The M184V substitution appeared to confer a modest increase in the IC_{50} for ddI but not in that for ddC. This mutation in HIV-1 confers a low-level (<5-fold) resistance to both ddI and ddC (Gao et al., 1992; Gao et al., 1993). M184I mutants in HIV-1 have been observed to retain susceptibility to ddI and ddC; susceptibility was retained for the M184I mutant of SIV also (Boucher et al., 1993). Interestingly, both 184 mutants displayed an increased (4- to 5-fold) sensitivity to d4T. Previous determination of Ki/Km ratios for 3TC have in contrast indicated no change in d4T sensitivity for M184V versus wild type HIV-1 (Uena and Mitsuya, 1997). SIV, like HIV-2, is insensitive to non-nucleosidic RT inhibitors, owing largely to the fact that four of five key binding-pocket residues are different from those observed in wild-type HIV-1 (residues 179, 181, 188, and 190), all of which are associated with HIV-1 NNRTI resistance (Tantillo et al., 1994). It is thus unsurprising that the mutants studied were as unsusceptible to nevirapine as the wild-type virus. For the protease inhibitors indinavir and saquinavir, a comparably high susceptibility was observed for mutant and wild-type virus. The susceptibility of SIV and HIV-2 to HIV-1 protease inhibitors is well-documented.

The IC_{50} values for AZT were of particular importance. The clinical effectiveness of AZT-3TC dual therapy is dependent upon a resensitization to AZT which is observed upon the acquisition of 3TC-resistance mutations in HIV-1 RT. When the M184V mutation arises in variants already resistant to AZT *in vitro*, phenotypic sensitivity to AZT is once again observed (Larder et al., 1995). The incompatibility in HIV-1 of simultaneous AZT-resistance mutations and 3TC-resistance at 184V with RT activity may thus explain the synergy of these two drugs in combination. In SIV, compatibility of codon 184 mutations with AZT-resistance has not previously been studied. The situation for SIV is somewhat different – in comparison with HIV-1, the reverse transcriptase of SIV is relatively resistant to AZT due to the pre-existence of codons in the wild type SIV-RT which encode decreased AZT susceptibility (I-75, I-118, E-123, V-135, V-202 and E-219) (Van Rompay et al., 1997). The homologous codons are found to be mutated in AZT-resistant and multiple-drug-resistant HIV-1 variants. Thus, the wild-type SIV-RT is said to possess a 'built-in' resistance to AZT. In HIV-1 RT, the Val^{184} AZT-resensitization observed in HIV-1 may be due to interactions between Val^{184} and AZT-resistance mutations; for example, a recombinant virus containing Tyr^{215} and Val^{184} showed a lower IC_{50} for AZT than that of Tyr^{215} (Larder et al., 1992). We were therefore interested in whether 184 mutants of SIV would exhibit an increased sensitivity to AZT, since 184 mutations may indirectly affect codons responsible for the built-in AZT-resistance of SIV-RT. A slight increase in AZT sensitivity of this nature may have occurred for the 184V mutant only; there was a modest reduction in the IC_{50} for AZT in the 184V mutant (0.24

+/- 0.01 μM) versus wild-type (0.37 +/- 0.03 μM). Overall, however, we found 3TC resistance at 184 was largely compatible with the built-in AZT resistance of SIV-RT. To date, the mutations which are the primary agents of SIV AZT-resistance (e.g. Q151M, which is also increasingly observed in HIV-1 isolates) have exhibited broad compatibility with mutations conferring resistance to additional RT inhibitors (Van Rompay et al., 1997). In consideration of this, an investigation of the compatibility of codon 184 mutations in SIV-RT with AZT-resistance mutations such as Q151M would be timely and of great interest toward the evaluation of the SIV model.

M184I and M184V high-level resistant variants of SIV were generated in response to tissue culture passaging with increasing selective pressure by 3TC. The order of appearance of these variants during *in vitro* 3TC-selection was the same as that observed for HIV-1. The resistance-conferring properties of M184I and M184V were confirmed by site-directed mutagenesis. These studies confirm the biological relevance of SIV-RT codon 184 mutations in 3TC resistance, and provide evidence that Met, Val, and Ile 184 in SIV may function in a manner mechanistically similar to their equivalents in the reverse transcriptase of HIV-1. These results further substantiate the utility of the SIV/macaque model for studies of antiviral chemotherapy and drug resistance.

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