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Gina Marie Lenzi Date

The Interplay Between Reverse Transcription and SAMHD1 Degradation: Mechanistic Differences Between Reverse Transcriptases from HIV-1, HIV-2 and SIV Lentiviruses Able or Unable to Degrade SAMHD1

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Gina Marie Lenzi
B.S., University of Virginia, 2010

Advisor: Baek Kim, Ph.D.

An abstract of a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in the Graduate Division of Biological and Biomedical Science,
Molecular and Systems Pharmacology
2016

Abstract

The Interplay Between Reverse Transcription and SAMHD1 Degradation: Mechanistic Differences Between Reverse Transcriptases from HIV-1, HIV-2 and SIV Lentiviruses Able or Unable to Degrade SAMHD1

By Gina Marie Lenzi

Two distinct zoonoses, one from a SIV strain infecting chimpanzees and the other from a SIV strain infecting sooty mangabeys, have led to HIV-1 and HIV-2, respectively. Despite the difference in origin, both lentiviruses HIV-1 and HIV-2 are able to infect dividing CD4+ T cells and nondividing myeloid cells including macrophages and microglia. However, HIV-1 and HIV-2 display very distinct replication kinetics in nondividing myeloid cells such as macrophages. Nondividing cells remain in resting phase of the cell cycle and do not need to replicate their genome for upcoming divisions. An overexpressed enzyme, SAM domain and HD domain-containing protein 1 (SAMHD1), maintains low levels of dNTPs by hydrolyzing them into dNs in nondividing cells. Thus, one major difference between HIV-1 and HIV-2 is that HIV-2 is able to degrade SAMHD1 through its viral protein X (Vpx) and replicate under high cellular dNTP concentrations in nondividing macrophages. HIV-1 lacks Vpx and thus replicates under very limited dNTP conditions found in nondividing macrophages.

Previous research has shown that cellular environment may affect viral replication kinetics. Gammaretroviruses infect dividing cells with high dNTP concentrations but are unable to replicate in nondividing cells like HIV-1. Comparing these viral polymerases, the binding affinity for nucleotides is much tighter for HIV-1 reverse transcriptase (RT) versus gammaretroviral RT. This suggests that viral polymerase kinetics interplay with cell tropism.

Indeed, this work furthers that hypothesis by showing that RTs of multiple subtypes of HIV-1 reach maximum velocity at lower concentrations of dNTPs which enables them to remain highly active even in a low dNTP environment found in macrophages, compared to RTs of many Vpx coding lentiviruses. Mechanistically, RTs from Vpx encoding lentiviruses display similar binding affinities but lower incorporation rates particularly at pause sites, compared to RTs of Vpx noncoding HIV-1, supporting that faster rates of incorporation contribute to why the Vpx noncoding viral RTs show more efficient DNA synthesis at low dNTP concentration. We hypothesize that the RTs of the Vpx noncoding viruses have evolved to have faster rates of dNTP incorporation in order to overcome the SAMHD1-mediated dNTP dearth found in nondividing myeloid cells.

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Acknowledgements

First and foremost I would like to thank Dr. Baek Kim for accepting me into his lab, teaching me the techniques, but most importantly shaping the way I approach, analyze, and rationalize scientific problems. His encouragement and description of “easy”, next-step experiments have inspired me even when the PhD path seemed so daunting. Dr. Kim’s upbeat attitude and confidence in his students is unparalleled and has made the lab a wonderful place over the last few years. He is an excellent mentor, and I have learned a lot both scientifically and professionally from him.

I would also like to thank my committee members Dr. Chris Doering, Dr. John Hepler, and Dr. Raymond Schinazi for their many insightful suggestions, their time and effort mentoring, and their approachability. I feel very fortunate to have had a committee of such high caliber.

I also wish to thank some specific people who were instrumental to my training in the Kim lab. I would like to thank Michele Daly who introduced me to the Kim lab layout, made me laugh, and always was willing to stop what she was doing to help. Her expertise in tissue culture guided me through any primary tissue work I had to complete. Next I would like to thank Caitlin Shepard for her constant enthusiasm to help with any project- in or out of the lab. Finally I would like to thank Drs. Tatsuya Maehigashi and Robert Domaoal for their patience in teaching protein purification and enzyme kinetics. Both have been extremely supportive through out my PhD. To the other 10AM Kim lab meeting attendees, I greatly appreciate your insight, critique, and contribution to my presentations. I could not have asked for a better group of colleagues.

I would also like to thank the MSP program. I was so excited to come to Emory to be part of the MSP program in 2010 and I have never been disappointed. The combination of the professors, students and administration is truly something special and has made me feel at home since I started. Even with the numerous accolades of the PIs, the professors are approachable, easy-going, and always willing to help graduate students. I would like to thank Susan Hoffstadter for help initiating the student-run events and providing an open door with never-ending chocolates when necessary. Finally I would like to thank the other MSP students who are collegial but also amiable and have made the last 6 years the best of my life.

Lastly, I would like to thank my friends and family. Their support and encouragement started this path a long time ago with children's chemistry sets and wildlife walks by the river. I greatly appreciate the opportunities my parents provided for me, and I surely would not be writing this thesis without their investment in my education.

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List of Abbreviations

3TC	Lamivudine
AGS	Aicardi Goutières Syndrome
AIDS	Acquired immunodeficiency syndrome
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
AZT	Zidovudine
CA	Capsid protein
cART	Combination antiretroviral therapy
CCR3	C-C chemokine receptor type 3
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CDC	Center for Disease Control
CRF	Circulating recombinant forms
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor type 4
D4T	Stavudine
DC	Dendritic cell
ddI	Dideoxyinosine
ddC	Dideoxycytidine
DLV	Delaviridine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DRV	Drug resistant variant
dsDNA	Double-stranded DNA
EFV	Efavirenz
ESCRT	Endosomal sorting complexes required for transport
ETR	Etravirine
FDA	Food and drugs administration
FeLV	Feline leukemia virus
FTC	Emtricitabine
GPCR	G-protein coupled receptors
HAART	Highly active antiretroviral therapy
HD	Histidine-aspartic domain
HAND	HIV-associated neurocognitive disorders
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HTLV	Human T-cell leukemia virus
IN	Integrase
k_{cat}	Turnover number; the number of times each enzyme converts substrate to product per unit time
k_{conf}	Conformational change constant; together with k_{chem} makes k_{pol}
k_{chem}	Chemistry of incorporation constant; together with k_{conf} makes k_{pol}
K_D	Binding affinity between enzyme and T/P
K_d	Binding affinity between enzyme and substrate

K_m	Substrate concentration needed to achieve half-maximum enzyme velocity
k_{pol}	Rate of incorporation
k_{ss}	Steady-state rate constant
KS	Kaposi's sarcoma
LAV	Lymphadenopathy-associated virus
LTR	Long terminal repeat
MA	Matrix protein
MuLV	Murine leukemia virus
MHC-1	Major histocompatibility complex I
NC	Nucleocapsid
Nef	Negative regulatory factor
NLS	Nuclear localization sequence
NVP	Nevirapine
PBS	Primer binding site
PIC	Pre-integration complex
Pol II	RNA polymerase II
PR	Protease
Rev	Regulator of expression of virion proteins
RPV	Rilpivirine
RRE	Rev response element
RT	Reverse transcriptase
RTC	Reverse transcription complex
SAM domain	Sterile α motif domain
SAMHD1	SAM domain- and HD domain-containing protein 1
SIV	Simian immunodeficiency virus
SIVagm	African green monkey SIV
SIVgor	Gorilla SIV
SIVolc	Olive colobus SIV
SIVmus	Mustached monkey SIV
SIVrcm	Red capped mangabey SIV
SIVsmm	Sooty mangabey SIV
SIVwrc	Western red colobus SIV
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
T/P	Template/ primer
TAR	Transactivation response element
Tat	Transactivator of transcription
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpx	Viral protein X
Vpu	Viral protein U
UNAIDS	Joint United Nations Program on HIV/AIDS
WHO	World Health Organization

CHAPTER 1

Introduction

1.1 HIV-1 is the Causative Agent of AIDS

In 1981 doctors in San Francisco first noticed an increased prevalence of *Pneumocytis carinii,* a rare opportunistic infection that occurs in patients with compromised immune systems. The initial cases clustered around injection drug users and gay men who had no history of immune deficiency. Many of these initial pneumonia patients developed a rare skin cancer known as Kaposi's sarcoma (KS) (Friedman-Kien, 1981; Hymes et al., 1981). The Center for Disease Control (CDC) was alerted and initiated a task force to monitor the outbreak.

Unaware of the underlying cause, scientists struggled to name this emerging disease. They knew it was likely spread through blood or fluid contact, caused lymph nodes to swell, and decreased $CD4^+$ T cell counts (CDC, 1982). With the number of people afflicted rising, by 1982 the CDC settled on acquired immunodeficiency syndrome (AIDS) as the designation for this emerging epidemic without knowing the source of disease.

Building off his own work on human T-cell leukemia virus (HTLV), Dr. Robert Gallo in Bethesda, MD isolated a virus from an AIDS patient and developed a technique for growing viral isolates from AIDS patients in cell culture. He noted the morphology of the virus was similar to other HTLVs and so named the new virus HTLV-III (Gallo et al., 1984). At the same time, Dr. Luc Montagnier working in France isolated virus from the lymph nodes of an AIDS patient, and his group so named the virus lymphadenopathy-associated virus (LAV) (Barré-Sinoussi et al., 1983). Both discoveries were published back to back in *Science* in 1984. Although Montagnier's group showed the core proteins to be distinct from other HTLVs contradicting Dr. Gallo's original hypothesis, HTLV-

III and LAV turned out to be the same retrovirus, human immunodeficiency virus type-1 (HIV-1), and thought to be a causative agent of AIDS. Later in 1986, Dr. Montagnier's group isolated a virus from two West African patients with AIDS and found a morphologically similar but genetically distinct retrovirus known as HIV type 2 (HIV-2) (Clavel et al., 1986).

1.2 The HIV Pandemic

By the end of 1986 and following the initial classification of HIV-1 and HIV-2, more than 28,000 cases of AIDS had been reported with nearly 25,000 confirmed deaths in the US alone (UNAIDS, 2002). Twenty years later the CDC estimates that more than 1.2 million people are living with HIV-1 infection in the US. The World Health Organization (WHO) approximates that more than 37 million people worldwide are living with HIV-1 (WHO, 2015). In comparison to HIV-1, HIV-2 is less transmissible both sexually and perinatally and has a longer progression time to AIDS (Marlink et al., 1994). HIV-2 occurs mainly in West Africa and is thought to afflict between 1-2 million people with a small percentage (0.3-1%) dually infected with HIV-1 and HIV-2 (Landman et al., 2009).

With such devastating fatality rates, the first anti-HIV drug zidovudine (AZT), a nucleoside reverse transcriptase inhibitor (NRTI), was approved in record time by the Food and Drug Administration (FDA) and made available in 1987 (Fischl et al., 1987). Several similar NRTIs were approved but ultimately failed to suppress the virus (Shirasaka et al., 1995). However, in 1996 Hammer and colleagues published in the New England Journal of Medicine the substantial advantage of using 2 NRTIs with a protease inhibitor (Hammer et al., 1997). Combination therapy became the standard of care for HIV patients and is known

as highly active anti-retroviral therapy (HAART) or combination anti-retroviral therapy (cART) (Autran et al., 1997).

This therapy regimen dramatically improved the prognosis for HIV patients. The CDC studied four cohorts of patients longitudinally in the US and found that the death rate in their 1981-1987 cohort was 95.5% while the death rate in their 1996-2000 group was 22.6%. In addition there was a decrease in the number of new HIV cases in the US due to increased testing and education campaigns (CDC, 2002).

Globally the trend was stunted due to lack of resources and new therapies in lower income areas and third world countries. In 2002 Joint United Nations Program on HIV/AIDS (UNAIDS) reported that HIV/AIDS was by far the leading cause of death in sub-Saharan Africa. Of the 1.8 million deaths from HIV/AIDS in 2005, 1.3 million were estimated to be from sub-Saharan Africa (UNAIDS, 2006).

Since 2005, many global efforts have been implemented and effective at slowing the spread of HIV. With the impetus of a Political Declaration on HIV/AIDS, the global community mobilized nearly $23 billion to help low and middle income countries combat AIDS in 2015 (UNAIDS, 2015b). AIDS-related deaths have fallen 42% between 2005 and 2014 (Fig. 1.1). Of the nearly 37 million people globally living with HIV, UNAIDS now estimates that 15.8 million have access to antiretroviral therapy. In 2014, 73% of pregnant women living with HIV have access to antiretroviral medicines to prevent transmission of HIV to their babies. New HIV infections among children were reduced by 58% from 2000 to 2014 (UNAIDS, 2015a).

Despite the dramatic improvements in death rates, accessibility, and mother to child transmission, there are still 21 million people living with HIV without therapy (UNAIDS, 2015a). This group primarily resides in sub-Saharan Africa where it is extremely difficult for patients to receive continuous treatment and monitoring. Therefore, HIV remains a major health concern worldwide and UNAIDS estimates that $31.9 billion will be required for the AIDS response by 2020.

Figure 1.1

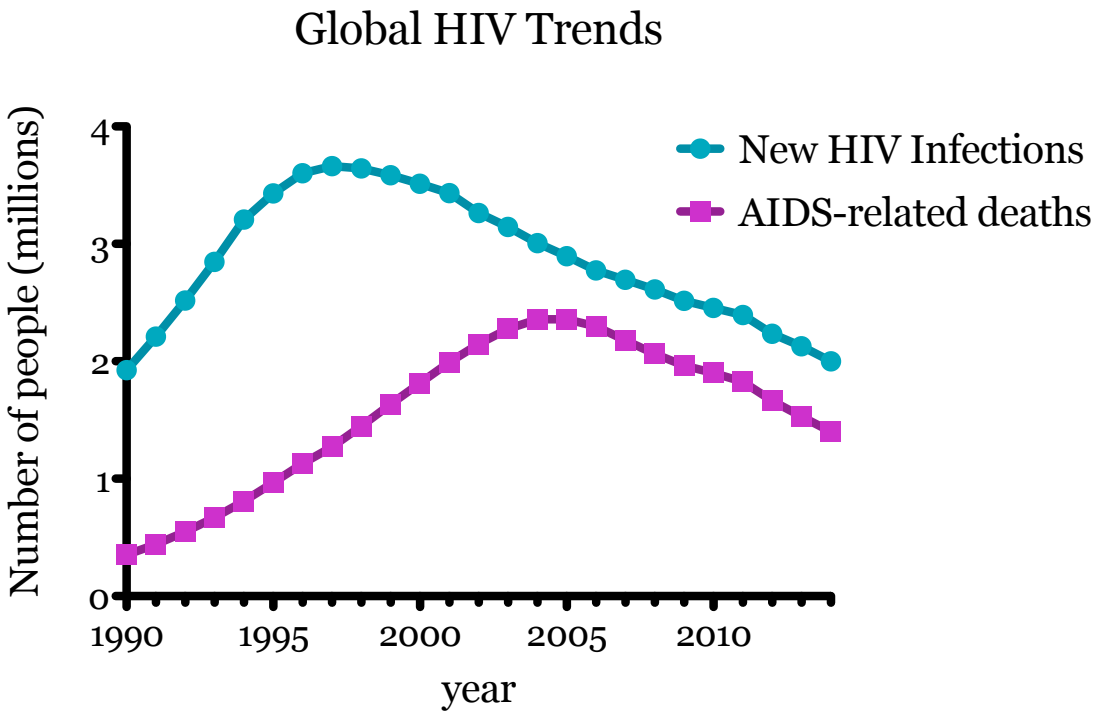


Figure 1.1 Global HIV trends over the last three decades. Global data for new HIV infections (teal) and deaths from AIDS (purple) are presented. New HIV infections peaked in 1997 while AIDS associated deaths peaked around 2005. Both have continued to decline with increase testing, monitoring, and access to HAART.

Note: Source of data is UNAIDS GAP Report 2014.

1.3 The Origins of HIV-1, HIV-2, and SIVs

The human viruses HIV-1 and HIV-2 causing AIDS are thought to have originated from multiple zoonotic transmission events from non-human primates. In a search to determine the origin and substantiate the zoonotic transmission from primates, researchers have used five different analyses: (i) genome organization; (ii) phylogenetic relatedness; (iii) frequency in the natural host; (iv) geographic access; and (v) potential transmission routes (Gao et al., 1999).

HIV-2 is genomically very similar to a simian immunodeficiency virus (SIV) found in wild sooty mangabeys (SIVsmm) (Figure 1.2A) (Hirsch et al., 1989). In addition HIV-2 and SIVsmm are closely related phylogenetically, and SIVsmm is prevalent in the sooty mangabeys inhabiting the forests of Littoral West Africa near the HIV-2 outbreak (Santiago et al., 2005). Sooty mangabeys are hunted for bush meat and retained as pets in areas of West Africa with the highest seroprevalence of HIV-2. Comparing the HIV-2 viral genomes found in humans with the sooty mangabey SIVsmm sequences, researchers propose that at least 8 independent transmission events occurred (Hahn et al., 2000). All five analyses strongly suggest that SIVsmm is the origin of HIV-2.

The origin of HIV-1 is less defined. Based on sequence similarity and genomic organization, HIV-1 is most comparable albeit not totally similar to an SIV found in chimpanzees (SIVcpz) (Gao et al., 1999; Keele et al., 2006; Korber et al., 2000). Hunting chimpanzee is common in west equatorial Africa, which would provide a likely transmission route to humans. Gao *et al.* suggest that multiple zoonotic transmissions from a subspecies of the common chimpanzee

(the central chimpanzee, *Pan troglodytes troglodytes*) (Figure 1.2B) are responsible for the HIV-1 epidemic. Each of the groups of HIV-1, M (major), N (non-M-non-O), and O (outlier), are likely caused by distinct zoonotic transmissions (Pasquier et al., 2001). In 2009, a new HIV-1 group emerged (Group P) which shows greater sequence similarity to an SIV from wild gorillas (SIVgor) than to SIVcpz (Plantier et al., 2009). A 2011 study in Cameroon confirmed the presence of HIV-1 Group P virus circulating in humans although it only accounted for 0.06% of HIV infections (Vallari et al., 2011).

SIVs are common in other African monkeys as well and have been isolated from more than 45 species of non-human primates (Boue et al., 2015). Unlike HIV-1 and HIV-2, SIV infections in their natural hosts appear in most cases to be non-pathogenic (Klatt et al., 2012). The zoonotic transmissions and phylogenetic differences between HIV-1, HIV-2, and SIV are depicted in Figure 1.2C.

Group M accounts for more than 90% of HIV/AIDS cases (Spira, 2003). Group M is further subdivided into subtypes, which are represented by a capital letter. With a high propensity to recombine as a coinfection in a single host, there are also designations for circulating recombinant forms (CRFs) of different subtypes. In the Americas, Europe, and Australia Subtype B is most common and derivatives of Subtype B are frequently used as lab adapted strains (Hemelaar, 2012). Subtype C is by far the most common accounting for more than 50% of HIV-1 infections and is the dominant form in Southern and Eastern Africa, India, and parts of China. Subtype A is the second most common subtype and most prevalent in Central Africa and North Asia (Arien et al., 2007). The visualization of the global distribution of HIV-1 subtypes is shown in Figure 1.3.

Figure 1.2

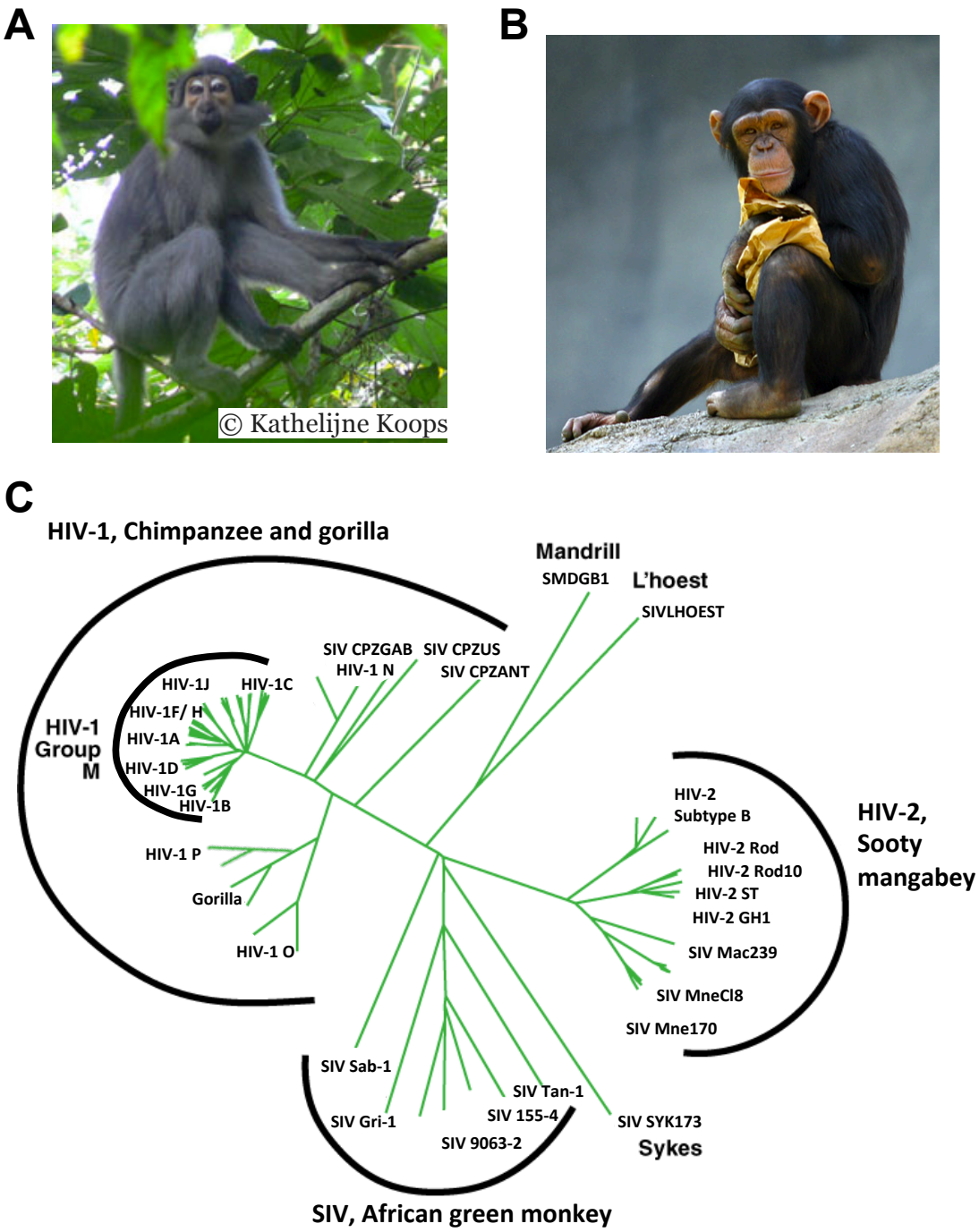


Figure 1.2 Non-human primates and phylogenetic tree of HIV-1, HIV-2 and SIVs. (A) A Sooty mangabey in the Nimba Mountains of Guinea, West

Africa. Reused with permission from Dr. Kathelijne Koops. **(B)** A chimpanzee in the Kibale National Park of Uganda. Photo by Ronald Woan and licensed under CC BY-NC. **(C)** A phylogenetic comparison based on full genome sequences organizing HIV-1, HIV-2, and SIV utilizing neighbor-joining methods. This figure was adapted from the Los Alamos National Laboratory.

Figure 1.3

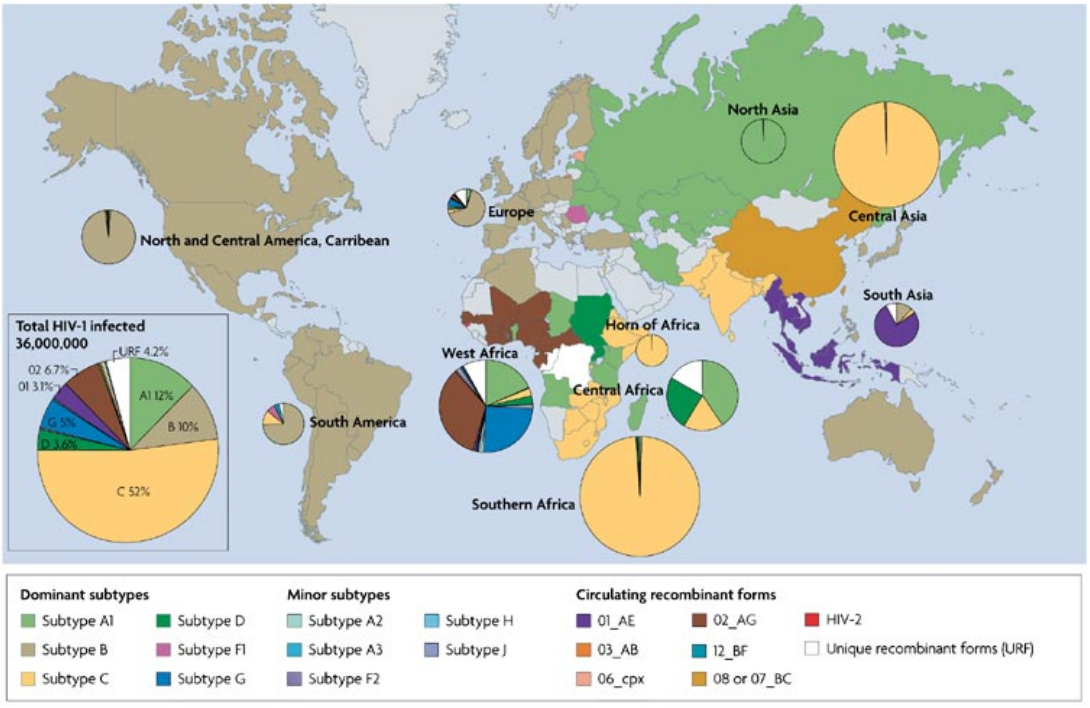


Figure 1.3 The global prevalence of HIV-1 by subtype. The frequency of each HIV-1 subtype form was estimated in each country based on published findings in 2007, and countries were color-coded based on the dominant HIV-1 Group M subtype. The pie charts depict the proportion of each subtype in that region with pie size proportional to the number of HIV-1 infected individuals in that region. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, 2007.

1.4 Retroviral Genome Organization

Lentivirus is a genus of the *Retroviradae* family and is typified by its long, slow (*lente-* is Latin for "slow") incubation period. HIV-1, HIV-2 and SIV are lentiviruses with two positive sense, single-stranded RNA (ssRNA) genomes compromised of about 10,000 nucleotides and 9 genes, which are processed into 19 proteins (Figure 1.4). The *gag*, *pol*, and *env* genes contain structural proteins. The other 6 genes are either accessory or regulatory. An illustration of the role of each of these viral proteins is depicted in Figure 1.5. Despite the conservation in genome organization, HIV-1/SIVcpz and HIV-2/SIVsmm only share about 40% sequence similarity at the amino acid level suggesting that they are evolutionary distant (Guyader et al., 1987).

A. Gag

Gag, or p54 protein, encodes four structural proteins required for recruitment of virion components, packaging, and budding of newly synthesized virions. These four proteins are matrix (MA), capsid (CA), nucleocapsid (NC), and p6.

MA is a 17 kDa protein which is formed from the N-terminus of the Gag polyprotein. After ribosomal synthesis, the N-terminal residues of MA are myristolyated, which is crucial for MA's interaction and targeting to the plasma membrane (Saad et al., 2006). MA is an important component of the pre-integration complex (PIC) and with its nuclear localization sequence (NLS), may abet nuclear import in nondividing cells (Gallay et al., 1995).

CA is a 24 kDa protein fused to the C-terminus of MA before processing (von Schwedler et al., 1998). CA protein forms the core structure of the virion and

recent X-ray diffraction and cryo-electron microscopy data has shown the pentameric and hexameric, conical shell that CA can form (Pornillos et al., 2009; Zhao et al., 2013). The CA shell encapsulates the two viral RNA genomic strands, reverse transcriptase (RT), and integrase (IN).

Figure 1.4

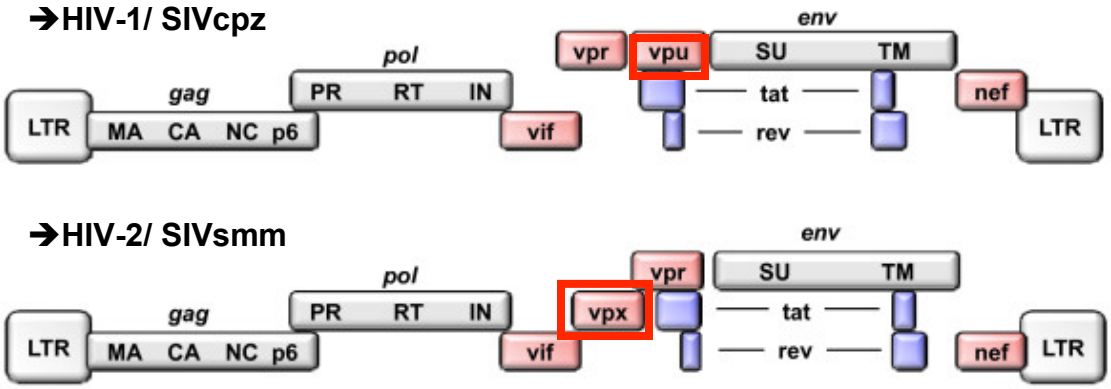


Figure 1.4 HIV-1 and HIV-2 genomic organization. The structural proteins are shown in grey, the regulatory genes in violet, and the accessory genes in pink. From left to right, the represented regions are: the 5' long tandem repeats (LTRs); the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 protein as part of the polyprotein gag; protease (PR), reverse transcriptase (RT), and integrase (IN) as part of the polyprotein pol; viral infectivity factor (Vif); viral proteins R, U, X (Vpr, Vpu or Vpx); the surface protein (SU) or transmembrane protein (TM) of *env* gene; the transactivator of transcription (tat); the regulator of expression of virion protein (rev); the negative regulatory factor (nef); and finally the 3' LTR. The two proteins, Vpu and Vpx, unique to HIV-1 and HIV-2, respectively, are boxed in red. This figure was adapted from BioMed Central with permission from the creator, Dr. Florence Margottin-Goguet.

Figure 1.5

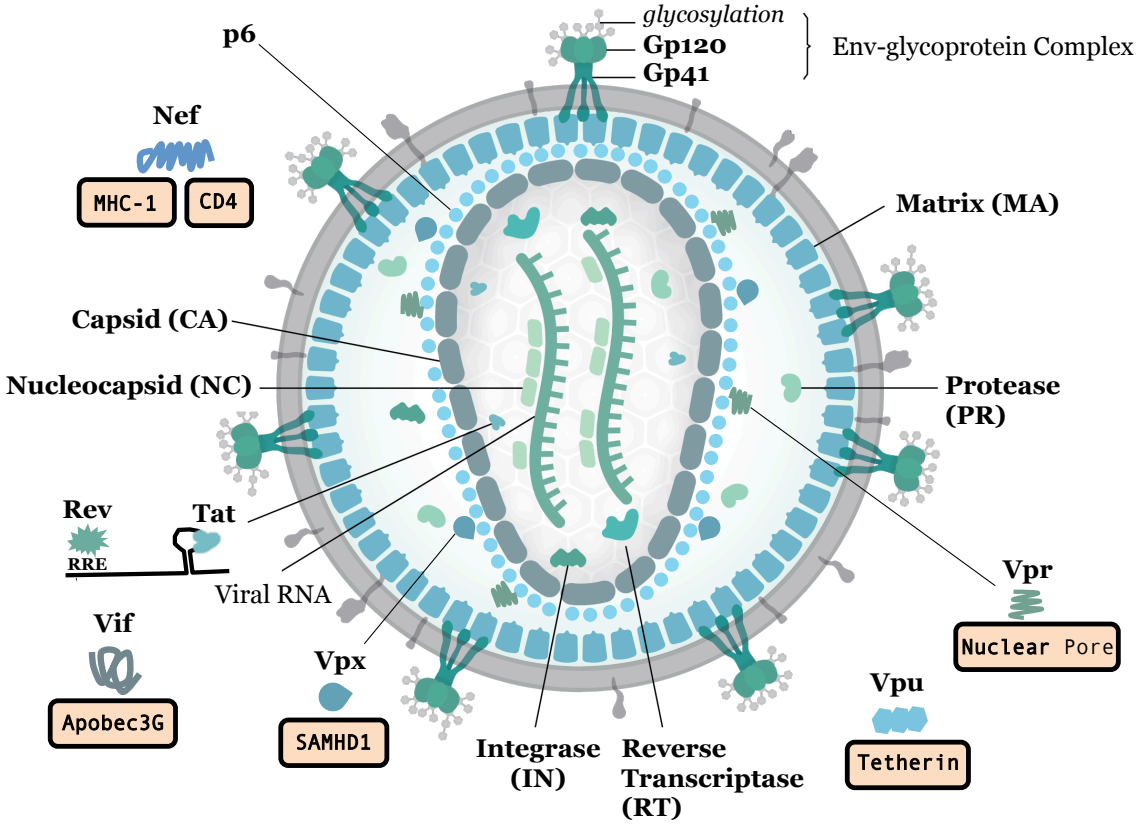


Figure 1.5 Diagram of the HIV virion. The viral proteins and their general location within the virion are depicted. Proteins shown outside the virion are likely produced during translation and not packaged within the virion. The orange boxes indicate host proteins with which the viral proteins interact. Vif, Vpx, and Vpu antagonize the host proteins, which would otherwise act as restriction factors. Nef downregulates MHC-1 and CD4 while Vpr facilitates nuclear shuttling by binding to the nuclear pore. The virion illustrates unique proteins from both HIV-1 (Vpu) and HIV-2 (Vpx). This figure by Thomas Splettstoesser was adapted with permission under a CC BY-SA 4.0 license.

NC is a small 7 kDa zinc finger protein which allows it to interact and bind to the RNA genome. After viral maturation, NC forms the viral nucleocapsid and recruits full length viral RNA to newly synthesized virions (Post et al., 2009). In addition NC has been shown to relax the secondary structure of RNA allowing increased RNase H activity by RT, enhanced strand transfer activity, and is required for the removal of 5' RNA fragments generated during the first (-) strong stop (Rodriguez et al., 1995).

p6 is the final protein at the N-terminus of the Gag polyprotein. It recruits viral accessory proteins Vpr and Vpx (only in SIVsmm derived viruses) to be packaged as well as endogenous cellular components to commence viral budding (Selig et al., 1999).

B. Pol

The translation of *pol* relies on a -1 frameshift by the ribosome at the junction between *gag* and *pol* (Dinman et al., 1991). This occurs in about 5-10% of translations and is due to a well-conserved pseudoknot in the RNA structure (Kontos et al., 2001). The fusion precursor protein is processed by viral protease (PR) into the gag polyprotein and the three proteins encoded by *pol*: PR, RT, and IN.

PR is an aspartic acid protease and is the first to be cleaved from the p160 pol polyprotein. PR functions as a homodimer and is only active at an acidic pH like the environment in the viral core (Davies, 1990). After budding, PR cleaves the polyproteins gag and pol into functional proteins like MA, CA, RT, and IN and thus is responsible for the maturation of the new virion (Kohl et al., 1988).

RT is a virally encoded polymerase responsible for the replication of the viral genome. RT is both an RNA- and DNA- dependent DNA polymerase and is thus able to transcribe the ssRNA genome into double-stranded DNA (dsDNA). RT functions as a heterodimer with p66 and p51 subunits in HIV-1 or p68 and p54 subunits in HIV-2 (Hizi et al., 1991). The two subunits are originally both larger (p66 or p68) but PR cleaves a 15 kDa (or 13 kDa, in HIV-2) fragment from the C-terminus creating a smaller subunit, which acts as a scaffold and joins with the larger subunit to make a heterodimer (Kohl et al., 1988). RT has additional activities, which will be discussed in depth in section 1.7.

IN is a 32 kDa protein with an N-terminal zinc-binding domain, a catalytic core domain, and a C-terminal DNA-binding domain. IN catalyzes 3' end processing and strand transfer, which allows for the integration of the newly synthesized dsDNA into the host genome (Bushman and Craigie, 1991; Fujiwara and Mizuuchi, 1988).

C. Env

The *env* gene is translated as a 160 kDa polyprotein known as gp160 but unlike the gag and pol polyprotein, Env is cleaved by a host protease, furin (Decroly et al., 1997). In HIV-1 the two cleaved products are gp120 (surface protein, SU, in Fig 1.4) and gp41 (transmembrane protein, TM, in Fig 1.4). In HIV-2 the size of the products is different and such the gp120 and gp41 are gp105 and gp36, respectively (Endres et al., 1996). These cleavage products are highly glycosylated. The gp41 anchors itself in the lipid bilayer of budding virions after trafficking through the Golgi apparatus. Gp120 migrates to the cell surface and later recruits gp41, which aids in attaching and fusing to target cells (Bahraoui et

al., 1992). Gp120 is responsible for viral entry due to a high affinity for CD4, a cell surface receptor on immune cells. Due to its exposure to the immune system, many neutralizing antibodies target gp120. This increased selection pressure forced on *env* creates a hypervariable sequence in order to avoid immune clearance (Rambaut et al., 2004).

D. Vif

Vif is a 23 kDa protein encoded downstream of the *pol* gene that antagonizes the host restriction factor apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G). APOBEC3G is a cytidine deaminase that catalyzes the deamination of cytidine to uridine in a single stranded DNA (ssDNA) substrate (Mangeat et al., 2003; Zhang et al., 2003). APOBEC3G exerts its antiviral effect by hypermutating the genome during transcription creating defective viral proteins during translation. Vif recruits APOBEC3G for ubiquitination and ultimately proteasomal degradation preventing errant genome editing (Yu et al., 2003).

E. Vpr

Vpr is a small, 14 kDa protein thought to induce G2 cell cycle arrest and perhaps to enhance viral genome transcription (Jowett et al., 1995). Vpr has a NLS that allows it to interact with the nuclear pore and makes it integral to the PIC (Di Marzio et al., 1995). This permits targeting of viral DNA to the nucleus and integration into the host genome even in nondividing cells where the nuclear membrane never dissociates. When leaving the host cell, Vpr is packaged into virions through interactions with p6 (Popov et al., 1998).

F. Vpx

Vpx is also a 14 kDa protein but only present in HIV-2 and some SIV strains. It also interacts with p6 in order to package into virions. Due to Vpr and Vpx similarities, researchers postulate that Vpx arose as a gene duplication of Vpr (Tristem et al., 1990). Recently it was discovered that Vpx also antagonizes a host restriction factor, sterile alpha motif domain and HD domain-containing protein 1 (SAMHD1) (Goldstone et al., 2011; Hrecka et al., 2011; Laguette et al., 2011). In nondividing cells, SAMHD1 hydrolyzes deoxyribonucleotides (dNTPs) into deoxyribonucleosides (dNs) maintaining low levels of dNTPs perhaps in order to prevent false starts of replication (Lahouassa et al., 2012). Vpx targets SAMHD1 to the E3 ubiquitin ligase complex for degradation (Ahn et al., 2012). Without SAMHD1, dNTP levels increase allowing for more efficient reverse transcription of the viral genome. Without Vpx, the kinetics of viral DNA synthesis for HIV-1 are delayed in nondividing cells.

G. Vpu

Vpu is specific to HIV-1 and is translated from the same bicistronic mRNA as Env through leaky scanning of the host ribosome (Strebel et al., 1988). Vpu counteracts another host restriction factor, tetherin, to allow for virion release from the plasma membrane (Neil et al., 2008). Tetherin is an integral membrane protein with its N-terminus in the cytoplasm, a transmembrane region, and its C-terminus anchored in the plasma membrane. As HIV-1 virion buds from the plasma membrane, tetherin can dimerize and prevent efficient release. Vpu, assisted by the host ubiquitin ligase complex, can target tetherin for proteasomal degradation (Douglas et al., 2009). Without Vpu, HIV-2 is able to obstruct

tetherin by using its Env to isolate tetherin in the perinuclear compartment precluding it from inhibiting viral release on the plasma membrane (Hauser et al., 2010).

H. Nef

Nef is a 27 kDa accessory protein encoded by a spliced mRNA present in both HIV-1 and HIV-2. Nef is able to downregulate CD4 through an interaction with the cytoplasmic tail of CD4 in order to prevent superinfection that often leads to cell death (Chaudhuri et al., 2007). In addition Nef can reroute and sequester major histocompatibility complex class I (MHC-1) molecules preventing antigen presentation and immune recognition by cytotoxic T cells (Lubben et al., 2007). Finally Nef is able to induce secretion of proinflammatory cytokines attracting new $CD4^+$ target cells to the infected cell (Dai and Stevenson, 2010).

I. Tat

Tat is expressed from a one exon or two exon mRNA and is required for viral gene expression (Harrich et al., 1997). Tat binds to the transactivation responsive element (TAR), a strong, well-conserved RNA hairpin located in the 5' LTR of the viral RNA, which is necessary for synthesis of full-length transcripts (Weeks et al., 1990). Tat can also be secreted and endocytosed by noninfected cells through ligand-receptor specific interactions causing autophagosome and lysosome fusion. Recent studies have shown that this dysregulation of critical intracellular components by Tat is associated with increased neurodegeneration in aging HIV patients (Fields et al., 2015).

J. Rev

Rev is a 13 kDa, regulatory protein translated early but required for late viral protein production. As an RNA binding protein, Rev utilizes an arginine-rich binding motif to interact with the rev response element (RRE) which is present on viral mRNA shuttling unspliced viral RNAs from the nucleus into the cytoplasm for translation (Najera et al., 1999; Van Ryk and Venkatesan, 1999).

1.5 The Retroviral Replication Cycle

The retroviral replication cycle is divided into two stages. The early stage involves viral entry, uncoating, reverse transcription and integration. The late stage encompasses transcription, translation, virion assembly, budding and maturation. Each step of the HIV-1 replication cycle will be described noting differences that occur in HIV-2. A depiction of the HIV-1 replication cycle is shown in Figure 1.6.

Figure 1.6

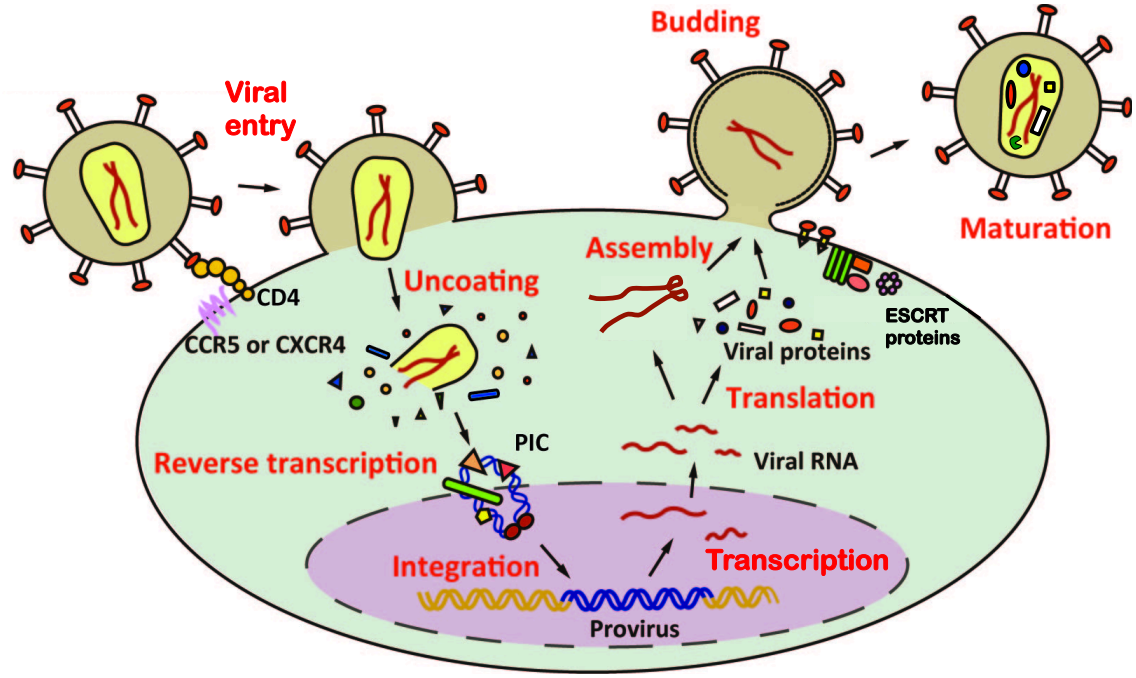


Figure 1.6 General steps in the retroviral replication cycle. The complete retrovirus replication cycle is shown starting on the left and proceeding to the right. The early stages consist of viral entry, uncoating, reverse transcription, and integration. The late stages include transcription, translation, assembly, budding, and maturation. This figure was adapted from *Viral Gene Therapy*. It was originally created by Drs. Suzuki and licensed under a CC BY-NC SA license.

A. Viral entry

Before HIV-1 can enter the cell, binding must first occur between the viral Env glycoprotein gp120, or gp105 in HIV-2, and the cell surface receptor CD4. Upon binding, a conformational change occurs in gp120 and gp105, which exposes a binding site for a cellular coreceptor. HIV-1 utilizes either C-X-C motif receptor 4 (CXCR4) on $CD4^+$ T cells or C-C motif receptor 5 (CCR5) on macrophages as a coreceptor (Choe et al., 1996; Deng et al., 1996; Kwong et al., 1998). Both CXCR4 and CCR5 are chemokine, G-protein coupled receptors (GPCRs) with seven transmembrane domains that respond to external cellular stimuli with internal signaling cascades (Berger et al., 1999). After both the CD4 and coreceptor are bound, the virion is drawn towards the target cell membrane. The hydrophobic N-terminal domain of the smaller Env protein, gp41 or gp36, forms a triple stranded, coil-coil structure that inserts into target cell membrane, and this allows fusion of viral lipid bilayer with the host cell membrane emptying the viral capsid into the cytoplasm (Kwong et al., 1998).

B. Uncoating

CA monomers hexamerize to form the conical-shaped, viral capsid. Based on cryo-electron microscopy data (Ganser-Pornillos et al., 2007), researchers know that the capsid is about 145 nm in length and 50 – 60 nm in diameter (Briggs et al., 2003). However, the nuclear pore is limited to molecules less than 39 nm in diameter and thus the viral capsid must disassemble in the cytoplasm before entering the nucleus (Pante and Kann, 2002). Where uncoating occurs in the cytoplasm is still disputed in the field. Researchers suggest three different possibilities: 1) uncoating occurs immediately following fusion, 2) uncoating

occurs simultaneously with reverse transcription, and 3) uncoating is delayed until attachment with the nuclear pore. These different possibilities for uncoating vary in timing, location, and stimuli, and the subtleties of these different models are not reflected in Figure 1.6.

The first possibility suggests that the capsid disassembles due to a change in environment entering the cytoplasm. CA assembly is driven by concentration and without the confinement of the viral core, CA hexamerization may break down (Dvorin and Malim, 2003; Mortuza et al., 2004). This model implies that CA is a structure delivering viral components to the cytoplasm of the target cell.

The second model proposes that capsid is intact when reverse transcription is initiated but begins to disassemble as reverse transcription continues. Gradual uncoating occurs due to cellular host protein interactions and molecular rearrangement during DNA synthesis (Fassati and Goff, 2001). Evidence for this theory is based on early immunofluorescent studies showing that CA interacts with the reverse transcription complex (RTC) and impairing capsid disassembly and completion of reverse transcription (Forshey et al., 2002; McDonald et al., 2002).

The final model suggests that the capsid remains intact until fusion with the nuclear pore. Reverse transcription would occur inside the viral capsid with dNTPs able to diffuse through the interring spaces of the hexameric, capsid structure (Dismuke and Aiken, 2006). Recent advances in real-time, direct visualization techniques demonstrate that capsid is associated with the RTC at the nuclear pore and some CA protein enters the nucleus (Chin et al., 2015; Hulme et al., 2015; Peng et al., 2014).

Despite the different theories on location, timing, and stimuli for capsid disassembly, the host restriction factor, the alpha isoform of tripartite motif-containing protein 5 (TRIM5α), is known to interact with cytoplasmic CA to prevent the uncoating process (Stremlau et al., 2004).

C. Reverse Transcription

Reverse transcription is the process in which the viral positive sense, ssRNA is converted into dsDNA (Figure 1.7). The process begins when host $tRNA^{lys3}$, which is complementary to the 5'-end sequence of the viral genome, binds to the primer binding site (PBS) (Barat et al., 1989). RT uses $tRNA^{lys3}$ as a primer and creates negative sense DNA through its RNA-dependent DNA polymerase activity until it reaches the first strong stop at the 5' end of the viral genome (Isel et al., 1995). Simultaneously while polymerizing the new DNA strand, RT is able to degrade the RNA strand associated through its RNase H activity (Peliska and Benkovic, 1992). This allows the newly synthesized ssDNA to anneal to a complementary region of the 3'-end of the viral genome. RT then transfers to the 3'-end to continue both synthesis of negative strand DNA synthesis and degradation of the RNA template (Gao et al., 2007). RT leaves only the polypurine tract (PPT) of the RNA template intact as the 15 base pair, AMP-GMP section is resistant to RNase H degradation. This also provides an RNA primer for RT to begin positive strand DNA synthesis using now its DNA-dependent DNA polymerase activity (Charneau et al., 1992). RT synthesizes DNA until it again reaches the 5'-end where $tRNA^{lys3}$ is bound creating the second strong stop DNA. Again employing its RNase H activity, RT degrades the tRNA primer allowing the PBS to anneal to the complementary region in the 3'-end

initiating the second strand transfer (Ben-Artzi et al., 1993). This circularizes the DNA and allows DNA synthesis to conclude creating the proviral dsDNA (Hu and Hughes, 2012).

Figure 1.7

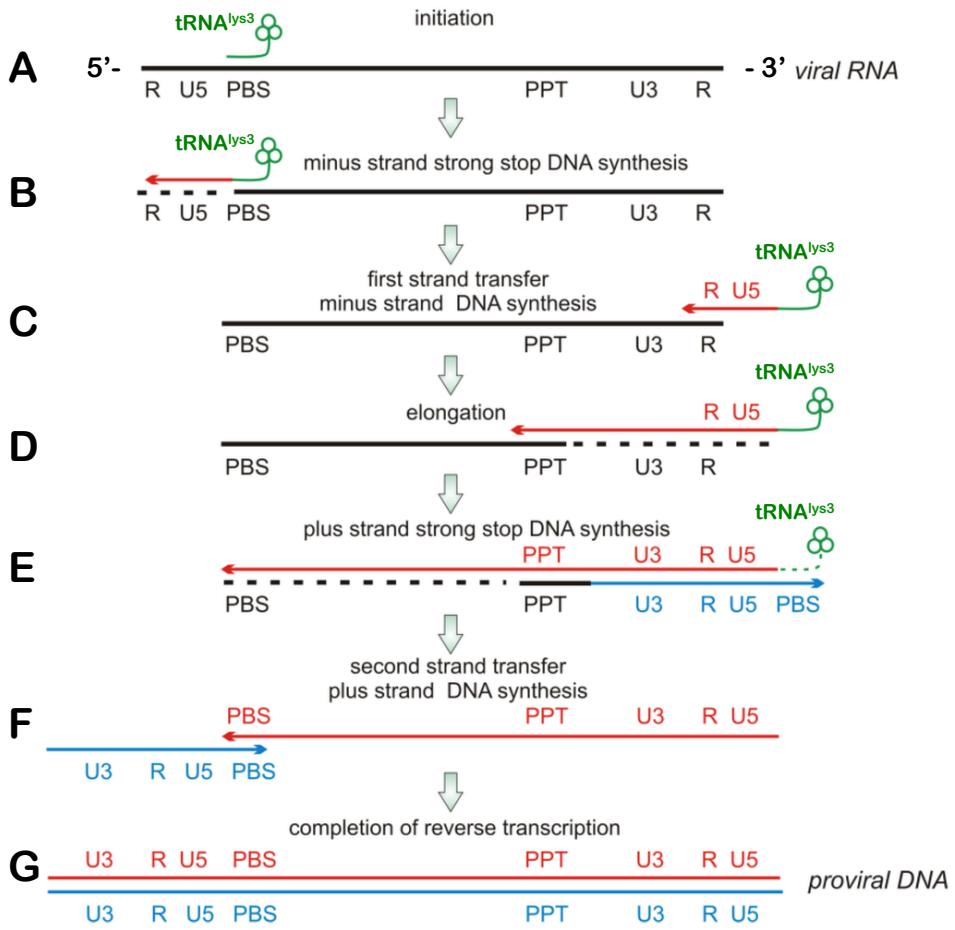


Figure 1.7 The synthesis of proviral DNA from viral RNA by RT. The viral ssRNA genome is shown in black. The host $tRNA^{lys3}$ (green) binds to the PBS and acts as a primer for RT to initiate minus strand DNA synthesis **(A)**. At the 5'-end in the R region, RT encounters its first strong stop. RT uses its RNase H activity to degrade the template RNA (dashed line) **(B)** and the first strand transfer event occurs **(C)**. RT continues DNA synthesis **(D)** and degradation of the RNA template (leaving the PPT) until it reached the second strong stop **(E)**. RT then goes through a second strand transfer to begin plus strand DNA from the PPT **(F)**. The completion of reverse transcription yields proviral DNA **(G)**. This

figure was adapted from *Biology* with permission from its creator, Dr. Michael Parniak, and licensed under a CC BY license.

D. Integration

After reverse transcription completes, the next step is transporting the PIC, which includes IN, MA, NC, p6, Vpr and the newly synthesized DNA into the nucleus. Although the exact mechanism of import is unknown, IN, MA, and Vpr have NLS, which suggests a functional role for their involvement inside the nucleus (Bukrinsky et al., 1993; Heinzinger et al., 1994). Moreover, recent research has shown that more than 10,000 Vpr molecules can be delivered into the cell nucleus within 45 minutes of pseudotyped, HIV-1 infection indicating a possible role in regulating nuclear activities of HIV (Desai et al., 2015). Inside the nucleus, the PIC targets relaxed, decondensed regions of the host genome for integration. IN removes two bases from the 3'-end of each LTR to expose the invariant CA dinucleotides and catalyzes the transesterification in which the free hydroxyl groups undergo a nucleophilic attack on the phosphodiester bond of host DNA (Engelman et al., 1991). The joining of the host and viral genomes leaves a two-nucleotide flap at the site of integration that is thought to be repaired by host DNA repair mechanisms (Brass et al., 2008). This completes the early stage of retroviral lifecycle.

E. Transcription and Translation

The late stage of the retroviral lifecycle relies mostly on host machinery and begins with transcription. The 5' LTR with its *cis*-acting elements acts as the viral promoter and recruits host RNA polymerase II (Pol II) to initiate transcription of short messages (Parada and Roeder, 1999). Tat is immediately transcribed and translated, and Tat protein binds to TAR hairpin to help Pol II produce full-length transcripts. The newly synthesized RNA can act as either

mRNA for viral proteins synthesis or RNA for new viral genomes. In the case of mRNA, host machinery is required for splicing, but viral Rev protein bound to the RRE is necessary to shuttle viral mRNA into the cytoplasm (Fischer et al., 1995). Once in the cytoplasm, the virus relies on the host ribosome via 5' cap independent initiation, internal ribosome entry sites, frameshifting, or leaky read though to produce viral proteins (Balvay et al., 2007). Polyproteins are further processed by viral or host proteases and undergo post-translational modifications similar to production of host proteins.

F. Virion Assembly, Budding, and Maturation

The synthesis of viable, viral particles from host cells that can spread to other susceptible cells is a defining characteristic of retroviruses. Virion production occurs in three stages: assembly, budding, and maturation.

Assembly occurs at the plasma membrane and is mediated by Gag and gag-pol polyprotein (Ono and Freed, 2001). Monomeric gag in the cytoplasm is folded into an autoinhibited conformation, which subsequently undergoes a conformational change to bind MA, NC-RNA, and other Gag molecules (Hatziioannou et al., 2005; Shkriabai et al., 2006). These biomolecular oligomers arrive at the plasma membrane and polymerize into a Gag-RNA complex (Zhou et al., 1994). The myristoylation of MA is essential for anchoring this complex into the host phospholipid bilayer (Spearman et al., 1997). The Env glycoproteins arrive later and independent of Gag. The two ssRNA, viral genomes dimerize though their 5' UTR creating a "kissing-loop" structure ensuring each virion packages two copies of its RNA genome (Dardel et al., 1998). p6 interacts with Vpr (and Vpx in HIV-2) to ensure packaging and a few copies of the accessory

proteins Vif and Nef are also included. Analysis of purified virions shows cellular constituents as well including $tRNA^{lys1,2,\ and\ 3}$, lysyl-tRNA synthetase, actin, ubiquitin, and many RNA-binding proteins although in some cases, their importance to the virion is unknown (Ott, 2008).

Budding of the immature virion refers to when the virion crosses the plasma membrane and obtains its lipid envelope. This process relies on the host endosomal sorting complex required for transport (ESCRT) and is endogenously used for vesicle formation and cytokinesis. The ESCRT pathway is intricate, multifaceted and involves more than 40 different protein-protein interactions (Sundquist and Krausslich, 2012). The Sundquist group at the University of Utah is responsible for much of what is known about the complexities of the ESCRT and how HIV hijacks the pathway.

Maturation is the final step in the production of a viable virion and relies on PR. PR dimerizes to form an active site with two aspartic acid residues that are utilized for peptide bond hydrolysis. The first PR is created by the transient dimerization two gag-pol molecules which self cleaves and then continues to cut at ten different sites in gag and gag-pol (Tang et al., 2008). After cleavage is complete, the mature virion will encapsulate functional MA, CA, NC, p6, PR, RT and IN ready to infect the next target cell (Ott, 2008).

1.6 HIV-1/ HIV-2 Pathogenesis

A. Transmission and Progression Towards AIDS

HIV-1 spreads through sexual contact, blood transfusions, contact with infected blood, sharing of needles, and vertically from mother to child.

Worldwide the principal mode of transmission is through heterosexual intercourse (Mastro et al., 1994; UNAIDS, 2015a). Pathogens are able to cross genital mucosal barrier through small lesions that often occur during intercourse. Macrophages, dendritic cells (DCs), and $CD4^+/CCR5^+$ memory T cells patrolling the mucosal surface are the first immune cells to encounter the virus (Ghosh et al., 2010; Greenhead et al., 2000; Porcheray et al., 2006). Macrophages secrete cytokines that attract T lymphocytes to the site of infection (Herbein et al., 2010; Swingler et al., 2003). Due to antigen presentation on MHC-II molecules of macrophages, $CD4^+$ T cells interact directly with macrophages and through the virological synapse/ cell-to-cell contact HIV-1 is transmitted (Groot et al., 2008). The robust production of HIV-1 in $CD4^+$ T cells is correlated with their depletion (Figure 1.8) (Ho et al., 1995). This process may take a few weeks and often patients are unaware of their infection during this time. At about six weeks when viral loads peak, the patient may experience lymph node swelling, rashes, and flu-like symptoms (Stevenson, 2003). This initial phase is the primary infection.

During the next stage deemed clinical latency, viral RNA levels plateau and $CD4^+$ T cell counts slightly recover. Importantly, viral replication is still occurring during clinical latency. It is estimated more than 1 billion virions are produced per day causing apoptosis of $CD4^+$ T cells (Perelson et al., 1996). $CD4^+$ T cells are rapidly regenerated so their cell counts only slightly decline over time. Cellular and humoral branches of the immune system attempt to control the infection with cytotoxic T lymphocytes (CTLs) recognizing specific viral epitopes and neutralizing antibodies preventing virion uptake. Unfortunately, immune host defenses are thwarted and the virus can evade the selective pressures by

mechanisms like mutagenesis and receptor downregulation (Allen et al., 2005; Chen et al., 1996). Clinical latency can persist for five to ten years depending upon an individual's genetics and immune system (Stevenson, 2003).

Interestingly, although transmission routes are the same, HIV-2 often remains in the clinically latent stage for longer than ten years. HIV-2 has lower transmission rates, lower viral RNA levels, lower mortality rate, and better immune control than HIV-1 (Azevedo-Pereira et al., 2005; Esteves et al., 2000). Neutralizing antibodies are more effective against the Env protein gp105 due to a more open structure with less glycosylation sites (Uchtenhagen et al., 2011). In cases of coinfection with HIV-1 and HIV-2, although it can depend on which virus is contracted first, HIV-1 tends to outcompete HIV-2 for disease progression (Kannangai et al., 2012).

The last stage of viral infection is marked by an exponential increase in viral load and the corresponding demise of $CD4^+$ T cells (Mellors et al., 1996). The immune system is unable to generate enough $CD4^+$ T cells to sustain itself and AIDS occurs when $CD4^+$ T cell counts fall below 200 cells per μL in plasma (a healthy person has between 500- 1200 cells/ μL) (Stevenson, 2003). Without a robust lymphoid system, the patient becomes immunocompromised and much more susceptible to opportunistic infections like *Pneumocystis carinii* pneumonia, *Mycobacterium tuberculosis*, as well as other viruses. These opportunistic infections are usually the cause of death (Chan et al., 1995; Seage et al., 2002).

Figure 1.8

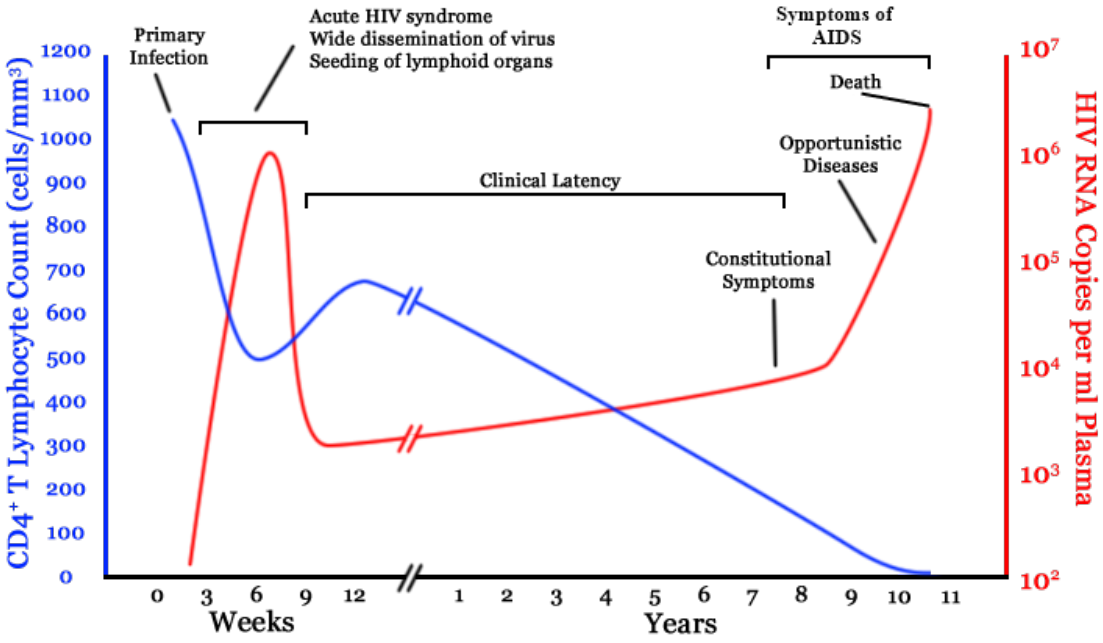


Figure 1.8 Typical course of HIV-1 infection. During the primary course of infection (left), HIV-1 (red) spreads through out the body. This leads to an abrupt decrease in $CD4^+$ T cells (blue) within the first weeks of infection and may cause to acute HIV syndrome. An immune response to HIV-1 ensues which decreases viral load and allows $CD4^+$ T cell numbers to recover during the period of clinical latency. Clinical latency may last for years during which $CD4^+$ T cell counts steadily decline. When $CD4^+$ T cells reach a critical low, opportunistic diseases overwhelm the depleted immune system and these diseases ultimately cause death (right). This figure by Juremia Oliveira was reproduced with permission under a CC BY-NC SA license and is adapted from *New England Journal of Medicine*.

B. Target Cells

HIV-1 cell type specificity is determined by several factors including receptor expression, cellular substrates, and host restriction factors. CD4, a glycoprotein expressed on the surface of T cells, monocytes, macrophages, and DCs, is the first determinant of HIV-1 infection (Lee et al., 1999). Secondly host cells must express a coreceptor- either CCR5 or CXCR4- which are chemokine activated GPCRs (Bleul et al., 1997). In order for a virus to enter a cell, the protein backbone of the Env protein gp120 must bind CD4. The binding causes a conformational change, which exposes a coreceptor binding site (Kwong et al., 1998). gp120's protein-backbone interaction with CD4 may explain the highly mutable nature of Env protein. Once the coreceptor binding site is exposed, gp41, the smaller Env protein, inserts itself into the cell plasma membrane and initiates lipid bilayer fusion with the target cell by binding either CCR5 or CXCR4 (Murakami and Freed, 2000).

i. Tropisms and Coreceptors

Early research from HIV patients found that HIV-1 isolates from the initial stage of infection grew slower in peripheral blood mononuclear cells (PBMCs) and failed to infect transformed cells while virus from late stage infection grew rapidly and could infect a wide variety of human cells (Asjo et al., 1986; Chengmayer et al., 1989). As cellular techniques improved, researchers were able to classify virus as either M-tropic or T-tropic. Although this classification was flawed because in culture M-tropic virus could infect $CD4^+$ T cells, it led scientists to the observation that the coreceptor usage on macrophage and $CD4^+$ T cells is typically different (Bleul et al., 1997). Macrophages, memory T-cells, and

immature dendritic cells characteristically express CCR5 and are thus infected by R5 strains. T cells typically express CXCR4 and are infected by X4 strains (Lee et al., 1999). Some HIV-1 strains are able to bind both coreceptors, infect both T cells and macrophages, and are known as X4R5. Follow up research has shown that R5 strains are generally found in early stages of infection and evolve to X4 or X4R5 over the course of infection (Connor et al., 1997; Jensen et al., 2003; Salazar-Gonzalez et al., 2009). The X4/ X4R5 replicate faster depleting $CD4^+$ T cells (Blaak et al., 1998). Summarizing the research and nomenclature on HIV-1 strains, in general the first stage of infection is with M-tropic, R5 variants that replicate slower. Through the course of infection the variants evolve to be more T-tropic, X4 strains that rapidly replicate and are able to deplete the $CD4^+$ T cell pool.

ii. Nucleotide Pools and SAMHD1

One of the unique characteristics of a lentivirus is its ability to infect nondividing cells like macrophages, DCs, and memory T cells. Previous research from the Kim lab has compared the dNTP concentrations in these nondividing but permissive cells to the concentrations found in rapidly dividing $CD4^+$ T cells. Diamond *et al*. showed that activated $CD4^+$ T cells contain 2-5 μM dNTPs while nondividing macrophages contain 40- 70 nM (Diamond et al., 2004). Follow up mass spectrometry data confirmed the 40-100 times concentration difference between $CD4^+$ T cells and macrophages (Kennedy et al., 2010). dNTP synthesis is dependent on S-phase of the cell cycle, and since $CD4^+$ T cells are actively dividing, they are constantly synthesizing DNA for new daughter cells and thus have abundant dNTPs (Bjursell and Skoog, 1980). Most macrophages are

terminally differentiated, locked in the G_0-phase and thus have a dearth of dNTPs.

Recently a cellular protein was discovered that maintains the low levels of dNTPs found in nondividing cells. SAM domain and HD domain-containing protein 1 (SAMHD1) is a cellular enzyme that exhibits phosphohydrolase activity converting deoxynucleotides into deoxynucleosides and triphosphates (Goldstone et al., 2011; Kim et al., 2012). SAMHD1 prevents false starts of replication and aberrant cell cycling in nondividing cells by limiting dNTP concentration but also restricts viral polymerase kinetics. SAMHD1 is regulated by an allosteric site that binds either dGTP or GTP creating a conformational change leading to tetramerization and allows dNTPs to bind in the active site (Ji et al., 2013; Zhu et al., 2013). Since GTP is abundant in the cell, SAMHD1 is likely not regulated by available activator (Amie et al., 2013a). Moreover the production of SAMHD1 is induced by interferon gamma and mutations in the enzyme cause the immune disorder Aicardi-Goutières Syndrome (AGS) (Dragin et al., 2013; Rice et al., 2009).

Post-translational modifications of SAMHD1 are able to negatively regulate SAMHD1. Researchers have shown that cyclinA2-CDK1/2 expressed during the S-phase of the cell cycle phosphorylate SAMHD1 at Thr-592 (Cribier et al., 2013; Pauls et al., 2014). The phosphorylation state inactivates the dNTPase activity of SAMHD1 increasing dNTP levels during replication (Yan et al., 2015).

Interestingly the additional protein in HIV-2 and some SIV strains, Vpx, is also able to negatively regulate SAMHD1 (Goldstone et al., 2011; Hrecka et al., 2011; Laguette et al., 2011). Vpx is packaged into the virion through its

interaction with p6 and immediately delivered into the host cell upon viral entry. Through its NLS, Vpx shuttles to the nucleus and binds SAMHD1 (Brandariz-Nunez et al., 2012; Guo et al., 2013; Hofmann et al., 2012). Vpx recruits DCAF1 to form a CUL4 E3 ubiqutin ligase which polyubiquitinates SAMHD1 leading to proteasomal degradation of SAMHD1 (Romani and Cohen, 2012). Interestingly in some strains of SIV lacking Vpx, Vpr is able to target SAMHD1 for degradation (Fregoso et al., 2013). Without SAMHD1, cellular dNTP concentrations increase and viral DNA synthesis is accelerated in nondividing cells for HIV-2 and SIV strains leading to a permissive infection (Figure 1.9, right) (Lim et al., 2012).

Interestingly it appears there is an evolutionary relationship between viral Vpx/ Vpr and host SAMHD1. Using multiple lineages, Fregoso *et al.* demonstrated that Vpx/ Vpr recognizes host SAMHD1 through different domains and that these interactions have been evolutionarily governed through virus-host selection (Figure 1.10). Nonetheless without Vpx, HIV-1 RT must synthesize viral DNA in a low dNTP environment. The concentration of dNTPs in nondividing cells is below the K_m (80-100 nM) and K_d (1-10 μM) of HIV-1 RT, which slows the kinetics of replication and leads to a restrictive infection in nondividing cells (Figure 1.9, left) (Kennedy et al., 2010).

Figure 1.9

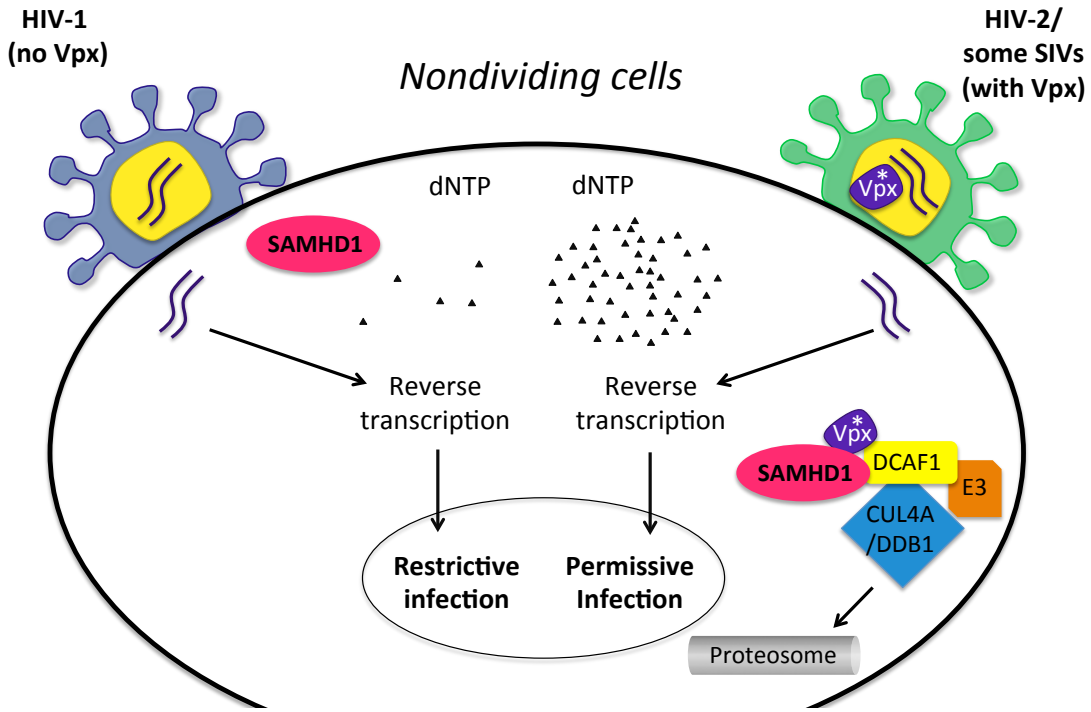


Figure 1.9 SAMHD1 restricts HIV-1 infection in nondividing cells by limiting reverse transcription. SAMHD1 is a host, dNTP triphosphohydrolase that degrades intracellular dNTPs into dNs and triphosphates. Expression of SAMHD1 in nondividing cells limits the dNTP pools restricting reverse transcription by HIV-1 (left). However, HIV-2 and SIV strains express the viral accessory protein, Vpx, which targets SAMHD1 for proteosomal degradation thus increasing the dNTP pools and leading to a permissive infection (right).

*Although the image displays Vpx, in some strains of SIV, Vpr is able to target SAMHD1 for degradation.

Figure 1.10

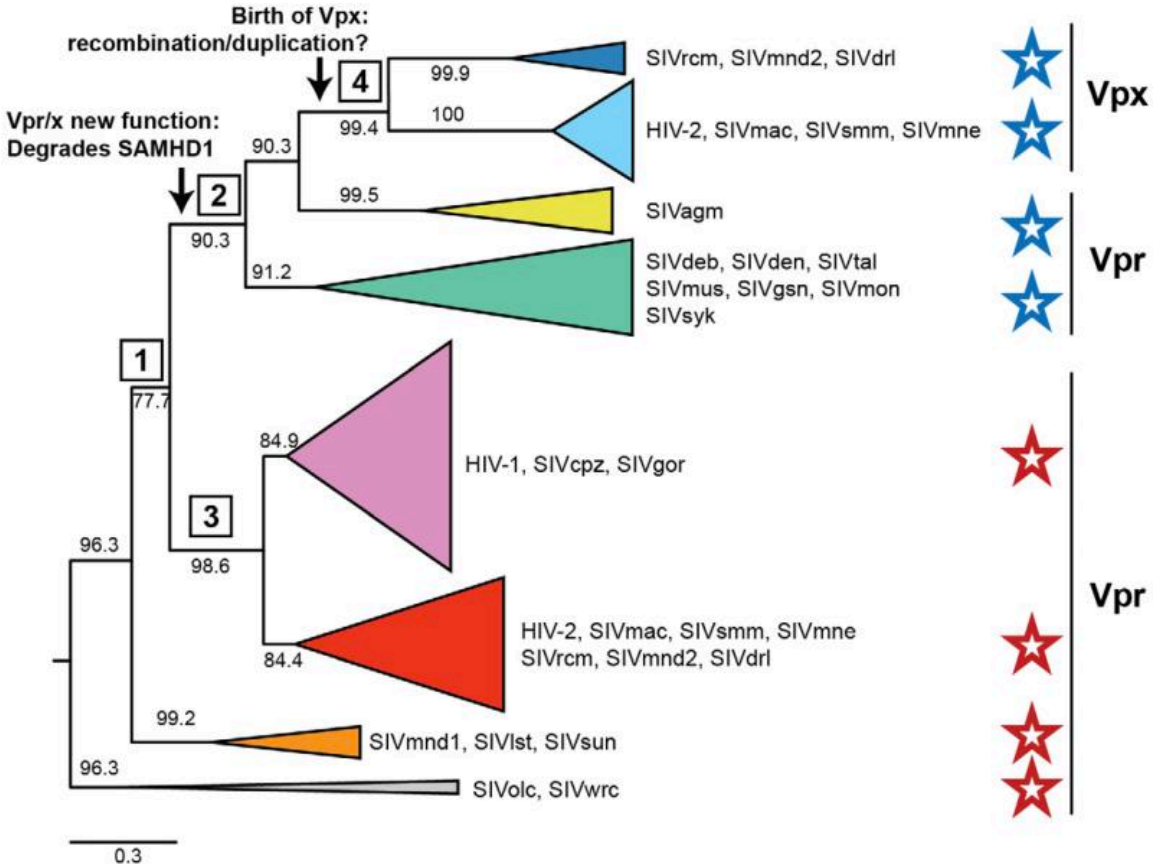


Figure 1.10 Evolution of the accessory proteins, Vpr and Vpx, in primate lentiviruses and their ability to degrade host SAMHD1 The phylogeny depicted is rooted to a common ancestor in SIV from olive colobus (SIVolc) and an SIV from Western red colobus (SIVwrc). The other strains were positioned based upon the flanking *pol* and *env*. Boxed numbers indicate nodes that infer ancestral traits. The accessory protein for each group is listed on the right with blue stars representing the ability to degrade SAMHD1 and red stars representing the inability to degrade SAMHD1. Reprinted with permission from Elsevier: Cell Host and Microbe, 2012.

C. Viral Latency

AZT was the first FDA approved drug for the treatment of HIV-1 in 1987. Since then six other nucleoside/ nucleotide reverse transcriptase inhibitors (NRTIs) have been approved along with multiple non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion, entry and integrase inhibitors as well as combination therapies (Dybul et al., 2002). Due to the ability of HIV-1 to rapidly mutate, combination therapy, known as HAART, is most effective at suppressing viral load, maintaining immune system function, reducing drug-resistant variants (DRVs) and preventing opportunistic infections (Autran et al., 1997). HIV-1 was once an acute death sentence but is now a manageable, chronic disease given proper combination therapy.

However, combination therapy is not a cure and the virus continues to replicate at low, sometimes undetectable levels during HAART therapy (Dornadula et al., 1999; Finzi et al., 1997; Furtado et al., 1999; Tobin et al., 2005; Wong et al., 1997). Low level viremia may occur due to ongoing viral replication from new DRVs or because of clonal outgrowth from long-lived HIV-1 infected cells. Considering the latter possibility, this suggests that nondividing cells have an essential role in the establishment of viral reservoir (Aquaro et al., 2002; Gavegnano et al., 2012; Koppensteiner et al., 2012). Three types of infected, nondividing cells will be discussed.

The absolute number of infected macrophages is low especially compared to the number of infected $CD4^+$ T cells, but due to their long-term survival, they play an important role in HIV latency (Aquaro et al., 2002). Infected macrophages are able to attract $CD4^+$ T cells through chemokines and virus

protein production in order to spread HIV to bystander $CD4^+$ T cells (Herbein et al., 2010). Moreover, HIV infection of microglia, resident macrophages of the brain and spinal cord, leads to alterations in neuronal metabolism and HIV-related encephalopathy (Williams and Hickey, 2002). This contributes to the development of a serious complication known as HIV-associated neurocognitive disorder (HAND), which can lead to delirium and dementia due to viral replication in the brain (Antinori et al., 2007; Boisse et al., 2008). Interestingly, individuals with a rare deletion in the CCR5 receptor preventing HIV-1 entry are unable to establish a viral reservoir. These patients are often resistant to HIV-1 infection and in the case of infection, do not progress to AIDS. This CCR5 polymorphism further highlights the importance of macrophages in both reservoirs and establishment of infection (Samson et al., 1996).

Resting $CD4^+$ T cells are also implicated in viral reservoirs and latency (Chun et al., 1998; Rong and Perelson, 2009). Although the virus enters the cell through CD4/CXCR4 receptors, after reverse transcription a block occurs and the cell enters a resting state (Zack et al., 1990). The provirus sits in the host genome for the life span of the cell and will only produce virions upon reactivation by cytokines (Unutmaz et al., 1999).

DCs play an important role in sustaining the viral reservoir as well. DCs are able to promote virion production through direct infection (Patterson and Knight, 1987). In addition DCs are able to engulf virions on the cell surface through DC-SIGN and infect neighboring T cells in *trans* through virological synapses (Geijtenbeek et al., 2000). Moreover follicular DCs (FDCs) can survive

for more than nine months (Burton et al., 2002). Reactivation and release of captured virions from all of these cell types is important to viral eradication.

Depiction of these viral reservoirs, their half-lives and their contribution to plasma viremia is shown in Figure 1.11.

Figure 1.11

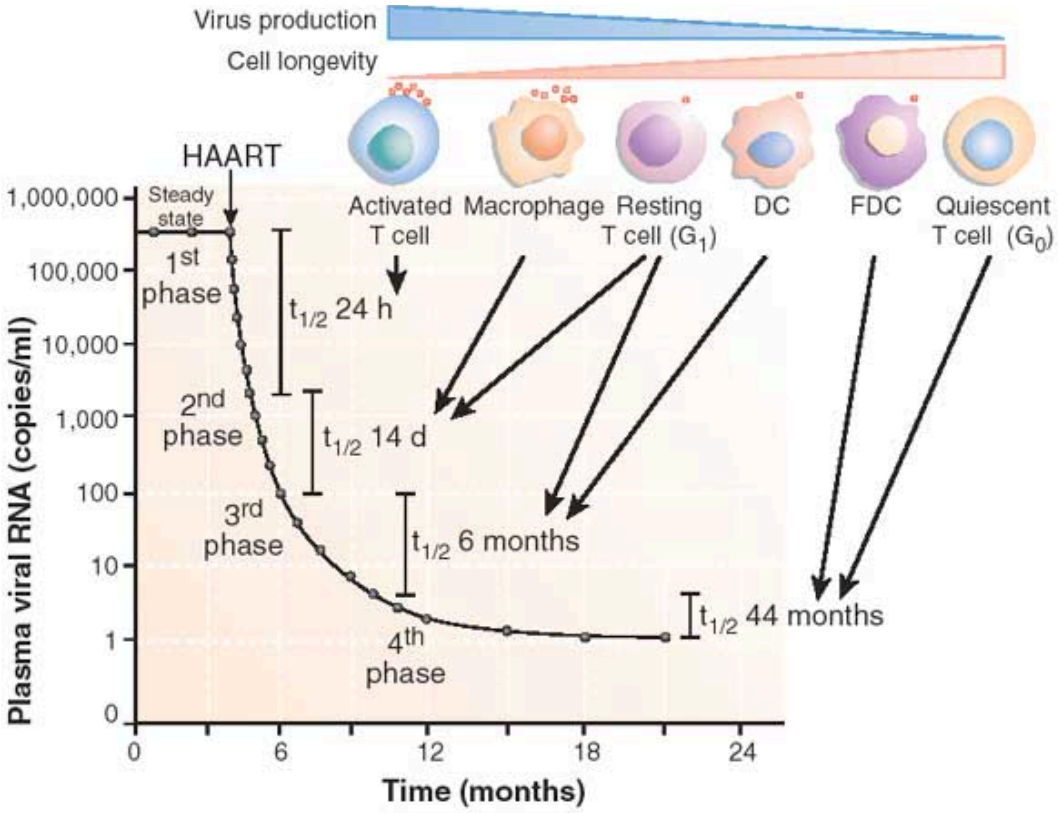


Figure 1.11 The viral reservoirs and their relative contribution to HIV-1 plasma viremia. Steady state levels of virus are produced until HAART therapy is initiated and plasma viral RNA begins to decrease. This decrease occurs in four phases due to the life span of various infected cell types. The first phase is inhibition of $CD4^+$ T cells. Then macrophages, resting T cells, and DCs are inhibited during the second and third phase. Finally FDCs and quiescent T cells may remain maintaining a low and sustained output of virus. Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine, 2003.

1.7 HIV-1 Reverse Transcriptase

A. Discovery of HIV-1 RT

During the scientific era from 1940-1960, researchers searched for the source of genetic information. They discovered that DNA served as the code that was transcribed to RNA and translated into proteins. These experiments formed a paradigm known as the "central dogma" (Crick, 1970). Given such a strong biological decree, it took mounting evidence from scientists studying RNA tumor viruses, now known as retroviruses, to show that there are exceptions to the central dogma.

Howard Temin was studying Rous sarcoma virus (RSV) and observed that the virus was able to transform healthy cells into cancerous cells. Due to the inherent instability of an RNA genome and the observation that viruses have new DNA, which hybridizes to RNA, Temin hypothesized that there must be an enzyme capable of synthesizing DNA from RNA. Using tritium labeled nucleotides and purified virions, Temin and his postdoctoral fellow Mizutani showed that RSV was able to incorporate dNTPs but not NTPs in a time and magnesium dependent manner (Temin and Mizutani, 1970).

At the same time Temin published his findings on RSV but working independently, David Baltimore performed a similar experiment using Rauscher murine leukemia virions. He too used a radioactive deoxynucleotide to show incorporation, which was dependent on magnesium and an RNA template (Baltimore, 1970).

With the back-to-back publications in Nature and the unearthing of RT, Temin and Baltimore discovered a natural exception to the central dogma for

which they won the Nobel Prize in 1975. The discovery and subsequent purification of RT has impacted both molecular biology and antiviral research.

B. Functions of RT

i. Viral replication

RT has three crucial functions in the replication cycle of HIV-1 and HIV-2 that are depicted in Figure 1.7. Firstly, RT acts as an RNA-dependent DNA polymerase synthesizing ssDNA from the viral RNA template where a $tRNA^{lys3}$ has bound as a primer. While synthesizing the new DNA, RT utilizes its RNase H activity to degrade the RNA template leaving only the PPT, which it will use as a primer to initiate synthesis of the second DNA strand. Having degraded the RNA, the second strand synthesis uses DNA as a template. This gives way to the third function of RT as a DNA-dependent DNA polymerase. The dsDNA product is able to integrate into the host chromosomes (Hu and Hughes, 2012).

ii. Source of mutagenesis

In addition to replicating the viral genome, RT contributes to viral diversity. RT has very low fidelity with an estimated mutation rate of 34 mutations/ million base pairs/ cycle (Mansky and Temin, 1995; Roberts et al., 1988). The viral enzyme lacks 3'-5' exonuclease proofreading activity which is employed by most polymerases to remove mismatched nucleotides before continuing extension (Bakhanashvili and Hizi, 1992). Thus RT is able to misincorporate nucleotides but also is unique in its ability to efficiently extend mismatches. Uncorrected mismatches may cause missense or nonsense mutations in coding regions of viral genes and lead to advantageous, neutral or

deleterious effects on the fitness of the virus. Thus the error prone nature of RT contributes to the rapid evolution and diversity of HIV-1 (Lloyd et al., 2014).

Another way RT contributes to viral diversity is through recombination. Recombination arises when RT is synthesizing DNA from an RNA strand and then switches to the other RNA strand to finish polymerizing (Hu and Temin, 1990; Peliska and Benkovic, 1992). Strong secondary structure like stem-loops and pseudoknots in the RNA promotes RT pausing which leads to increased RNase H activity and strand invasion by the second RNA strand (Purohit et al., 2007). When a single host cell is superinfected by two or more viral strains, recombination can significantly impact viral evolution (Dixit and Perelson, 2004). This phenomenon of recombination creates a CRF. Recombination is also a primary mechanism for development of sudden multidrug resistance (Gu et al., 1995; Levy et al., 2004; Moutouh et al., 1996).

C. RT as a drug target

i. NRTIs

Since RT is crucial to the viral replication cycle, contributes to viral diversity, and is distinct from our own polymerases, the enzyme is a valid drug target. The first antiviral approved to treat HIV-1 in 1987 was AZT and acts as a nucleoside reverse transcriptase inhibitor (NRTI). Originally developed as a cancer therapy, AZT is a thymidine analogue, which is converted to the triphosphate form by host cellular enzymes (Larder et al., 1989). RT incorporates AZT while synthesizing DNA, and the drug acts as a chain terminator inhibiting continued DNA synthesis and halting the viral replication cycle. At high doses, AZT is toxic likely due to its inhibition of mitochondrial polymerases (Lewis and

Dalakas, 1995). These adverse effects are reversible with discontinued use of AZT (Richman et al., 1987).

AZT was the first of many NRTIs to be developed as antivirals. NRTIs compete with the natural dNTP substrate and act as competitive inhibitors of DNA synthesis. NRTIs therefore share a similar structure to dNTPs with a nitrogenous base attached to a ribose sugar but lack a 3'-OH terminating transcription. Most NRTIs are administered in an unphosphorylated form and require phosphorylation by host cellular kinases (Munchpetersen et al., 1991). In general, the first phosphorylation event is the rate-limiting step in converting nucleosides to nucleoside triphosphates (Arner and Eriksson, 1995; Shewach et al., 1993).

The FDA has since approved 8 other NRTIs for the treatment of HIV-1, which in order of approval date are: didanosine (ddI), zalcitabine (ddC), stavudine (D4T), lamivudine (3TC), abacavir (ABC), tenofovir disoproxil (TDF), emtricitabine (FTC), and tenofovir alafenamide (TAF) (Cihlar and Ray, 2010). Tenofovir is an adenosine nucleotide given as a prodrug to increase absorption and decrease off target effects (Antoniou et al., 2003). Researchers recently observed that changing the prodrug formulation of tenofovir (disoproxil fumarate to alafenamide) increased antiviral potency allowing smaller doses and less adverse effects (Mills et al., 2015). The FDA approved a TAF-based treatment regimen for HIV-1 in November 2015.

ii. NNRTIs

A second class of drugs that target RT is known as non-nucleoside reverse transcriptase inhibitors (NNRTIs). As their name implies, these drugs are not

nucleoside analogues and thus do not compete with dNTP substrate. Instead these drugs bind RT in a hydrophobic pocket close to the active site in the palm subdomain of p66 causing a conformational change that blocks DNA polymerization (Kohlstaedt et al., 1992; Spence et al., 1995). Nevirapine (NVP), efavirenz (EFV), and delavirdine (DLV) are first generation NNRTIs approved from 1996- 1998 but they quickly developed similar resistance profiles (Bacheler et al., 2001). Thus second generation NNRTIs like etravirine (ETR) and rilpivirine (RPV) were developed to inhibit common resistance mutations (Lansdon et al., 2010). Interestingly NNRTIs are ineffective against HIV-2 due to amino acid differences in the palm subdomain while NRTIs, binding in the highly-conserved active site, are effective (Ren et al., 2002; Witvrouw et al., 1999).(El-Sadr et al., 2006)

iii. Resistance

Unfortunately HIV-1 has developed resistance to all FDA approved drugs (Tang and Shafer, 2012). RT introduces rapid variation to the viral genome, which leads to subsequent selection by the host. An accumulation of DRVs emerges which are selected for under the drug pressure (Hogg et al., 2006; Nadembega et al., 2006). Combination therapy (HAART) is an effective method at reducing DRVs but is not infallible. A substantial factor that contributes to DRVs is poor patient adherence (El-Sadr et al., 2006; Mills et al., 2006). During these “blips” in treatment, viral replication increases for a short amount of time giving the virus more sampling space to acquire a resistant mutation. As new mutations arise and drug regimens lose their efficacy, combination therapies

must be altered necessitating continued drug development for HIV-1 (Hammer et al., 2006).

D. Structural features of RT

Drug development against RT is aided by protein crystallization and X-ray diffraction that gives detailed snapshots of the molecular structure (Huang, 1998; Jacobomolina et al., 1993; Kohlstaedt et al., 1992). Biochemical and structural data shows that functional HIV RT is a heterodimer consisting of p66 and p51 subunits for HIV-1 and p68 and p54 subunits for HIV-2 (Hizi et al., 1991). Both subunits are synthesized from the same *pol* gene, but the p51 subunit undergoes additional cleavage of its p15 or p14 fragment in the case of HIV-1 or HIV-2, respectively, to form a heterodimer rather than homodimer (Hizi et al., 1991; Shehu-Xhilaga et al., 2001). The p66/p68 subunit has an N-terminal polymerase domain and a C-terminal RNase H domain and is responsible for the catalytic activities of the enzyme (Sarafianos et al., 2002). The p51/p54 subunit plays a structural role in orienting and stabilizing the larger subunit (Huang, 1998). The p66/p68 subunit resembles a right hand with a thumb, fingers, palm, connection and RNase H subdomains. The p51/p54 subunit also has the thumb, fingers, palm and connection subdomains but is folded into a slightly different structure and lacks the RNase H domain (Figure 1.12) (Kohlstaedt et al., 1992).

Figure 1.12

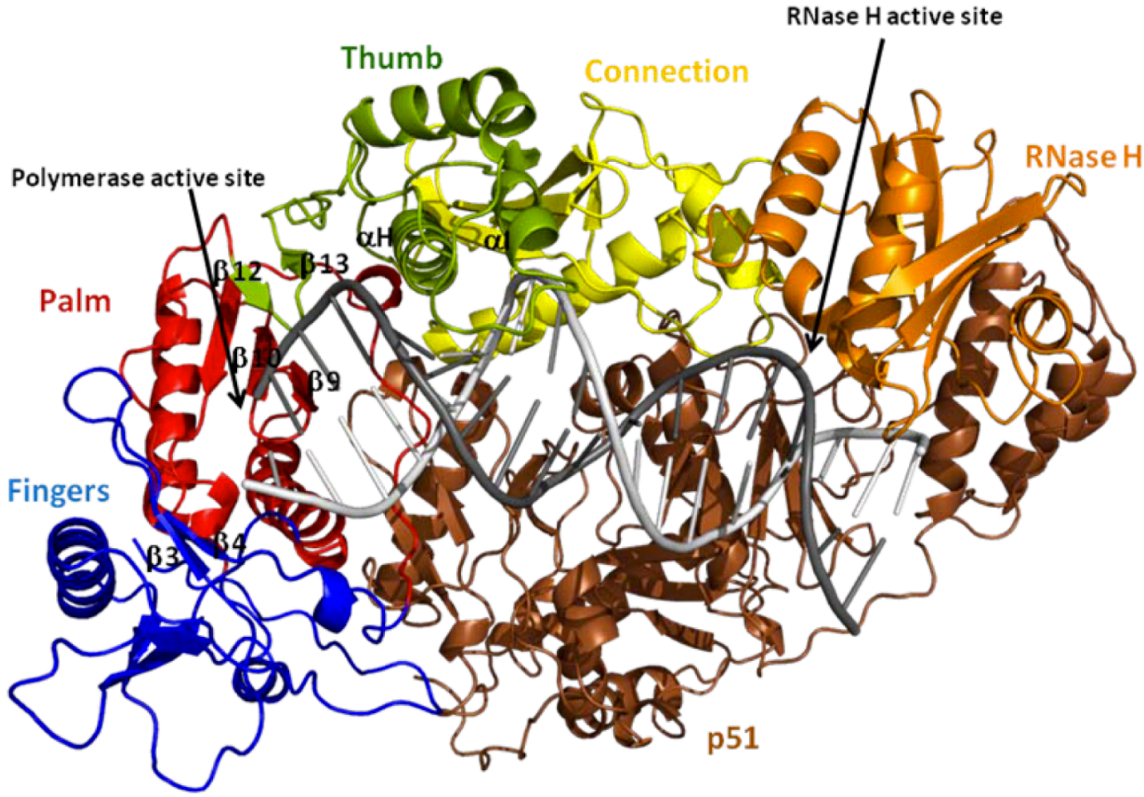


Figure 1.12 The structure of HIV-1 RT. The crystal structure of HIV-1 RT bound to dsDNA (grey) is shown (PDB: 2HMI). The p66 subunit resembles a closed right hand with the fingers (blue), palm (red), and thumb (green). The polymerase active site (YMDD) is denoted. The connection subdomain (yellow) links the polymerase domain to the RNase H domain (orange). The p51 subunit (brown) is a proteolytic cleavage product of p66 and the RNase H domain. The RNase H active site is denoted. Despite sequence similarity between p66 and p51, the tertiary structure of p51 is vastly different leading to a nonfunctional arrangement of catalytic residues. This figure was reprinted from *Viruses* and originally created by Dr. Stefan Sarafianos and licensed under a CC BY license.

The p66 subunit has both an active and inactive conformation. The four-helix bundle of the thumb subdomain lies over the active site and interacts with the fingers subdomain in the inactive confirmation. Once active, the p66 thumb domain moves away from the fingers subdomain revealing the catalytic site. The catalytic site is located in the palm subdomain, which is compromised of beta strands. The conformational change allows the thumb and fingers subdomains to act as a clamp holding the template ssRNA in the active site of the palm subdomain for DNA polymerization (Abbondanzieri et al., 2008; Sarafianos et al., 2002; Tuske et al., 2004).

Several regions of p66 are crucial for proper enzymatic activity. The active site itself is composed of the highly conserves residues YMDD which in combination with a third aspartate (D110) coordinate metal binding and aid in catalysis. dNTP binding residues include K65, R72, D113, A114, Y115, V148, and Q151 (Huang, 1998). Specifically, Y115's main chain –NH donates a hydrogen bond to the 3'-OH of the incoming dNTP and prevents incorporation of ribonucleotides by interfering with the 2'-OH . Tyrosines and in this case Y115 are often referred to as the steric gate and are well conserved among polymerase active sites (Cases-Gonzalez et al., 2000; Ray et al., 2002). The connection subdomain links the polymerase domain and RNase H domain. The active site residues for RNase H activity are D443, E478, D498, and D549 (Davies et al., 1991; Palaniappan et al., 1997). Despite the sequence and structural homology between HIV-1 and HIV-2 RTs, the RNase H activity of HIV-2 RT has been shown to be ten times less than the activity of HIV-1 RT (Bochner et al., 2008).

Although the functional activity of RT is dominated by the large subunit, p51/p54 is necessary for stabilizing and orienting p66/p68. Despite the sequence similarities to the large subunit, there are significant conformational differences in the small subunit. In p51/p54 subunit the fingers subdomain is oriented toward but farther from the palm subdomain (Jacobo-Molina and Arnold, 1991). The connection subdomain of the small subunit contacts the RNase H domain of the large subunit orienting the RNA template for degradation (Davies et al., 1991). The decrease in RNase H activity observed for HIV-2 RT is likely due to the allosteric effect of an amino acid change (proline to glutamine) in the small subunit (Bochner et al., 2008).

The homodimeric RT with two p66 or two p68 domains is still able to synthesize DNA and is actually the substrate for proteolytic cleavage to form the heterodimer. The connection subdomain is disordered or partially unfolded in one of the large subunits presenting PR with the cleavage site (Sluis-Cremer et al., 2004). Thus the question arises, why is one subunit cleaved? Of course it is more streamlined in a condensed retroviral genome to get two different proteins from the same gene but that fails to explain why cleavage occurs. Biochemical experiments comparing the heterodimer and homodimer suggest that the heterodimer is slightly more efficient in incorporating dNTPs, which may be advantageous replicating in a low dNTP environment (Kati et al., 1992; Marko et al., 2013). It is also possible that the cleaved p15/p14 fragment has an additional activity (Evans et al., 1994; Schulze et al., 1991).

E. Polymerization Reaction Pathway

i. Steady-state

Steady-state measurements of polymerases look at the incorporation of multiple nucleotides along a template using a fixed concentration of enzyme-substrate binary complex. Steady-state assays require multiple enzyme turnovers in order to turn a substantial amount of primer into full-length product and thus occur over longer time scales (Johnson, 1992). The steady-state rate, k_{cat}, is influenced mostly by the rate-limiting step; for RT, this step is the dissociation of enzyme from T/P. The steady-state affinity constant, K_m, is the concentration of substrate that yields half maximal velocity (V_{max}). K_m accounts for a complex mix of intervening steps between dNTP binding and enzyme dissociation. Thus it's more useful to compare the steady-state rate (k_{cat}) to the steady-state affinity (K_M). This ratio is called the catalytic efficiency (Fersht, 1985).

ii. Pre-steady-state

$$\mathrm{RT + T/P_N} \overset{K_D}{\leftrightarrow} \mathrm{RT{\bullet}T/P_N + dNTP} \overset{K_d}{\leftrightarrow} \mathrm{RT{\bullet}T/P_N{\bullet}dNTP} \overset{k_{conf}}{\leftrightarrow} \mathrm{RT^*{\bullet}T/P_N{\bullet}dNTP} \overset{k_{chem}}{\leftrightarrow} \mathrm{RT^*{\bullet}T/P_{N+1}{\bullet}PP_i} \overset{k_{ss}}{\leftrightarrow} \mathrm{RT + T/P_{N+1} + PP_i}$$

The reaction pathway depicted above represents the stepwise progression of the incorporation of a single dNTP (Joyce, 2010). The first step on the far left is the association of RT with the T/P (template/primer), and K_D denotes the affinity of RT for a given T/P. RT binding T/P (denoted with •) creates a binary complex and a conformational change, which now allows an incoming nucleotide to bind. The binding affinity of the incoming nucleotide is the K_d and is unique to each enzyme, T/P, and dNTP combination. RT bound to the T/P and dNTP is known as the ternary complex. Once dNTP is bound, RT must go through a

conformational change (denoted with a *, designated as k_{conf}) in order to bring its catalytic triad of aspartates and the dNTP in close proximity (Kati et al., 1992; Patel et al., 1991). The catalysis of the incoming nucleotide and release of pyrophosphate is the chemistry step of incorporation (k_{chem}). For RT, the forward reaction of k_{chem} is fast ensuring incorporation and not excision of the incoming nucleotide (Radzio and Sluis-Cremer, 2005). Together the conformational change and the chemistry of incorporation make up the maximum rate of incorporation (k_{pol}). The ratio of rate of incorporation (k_{pol}) to binding affinity of the incoming nucleotide (K_d) is known as the incorporation efficiency (Johnson, 1992). The steady-state rate constant (k_{ss}) is on the far right of which the rate-limiting step is the dissociation of RT from the extended T/P (Hsieh et al., 1993).

The development of a rapid-quench-flow instrument permits the individual measurement of each of these steps. The instrument is an out-of-the-box solution, which allows kinetic, single turnover assays for any polymerase. In addition, the experiments use small volumes conserving precious purified enzyme and can stop reactions at millisecond time points (Johnson, 2009). In comparison to steady-state kinetics, pre-steady-state measurements of polymerases look at the incorporation of a single nucleotide and using increasing concentrations of enzyme-substrate binary complex rather than fixed concentrations (Joyce, 2010).

Many wild type and mutant human and viral polymerases have been characterized utilizing this technique providing invaluable comparisons that may explain polymerase environment, function, and evolution (Ahn et al., 1997; Dahlberg and Benkovic, 1991; Johnson, 1995; Tissier et al., 2000). Pre-steady-

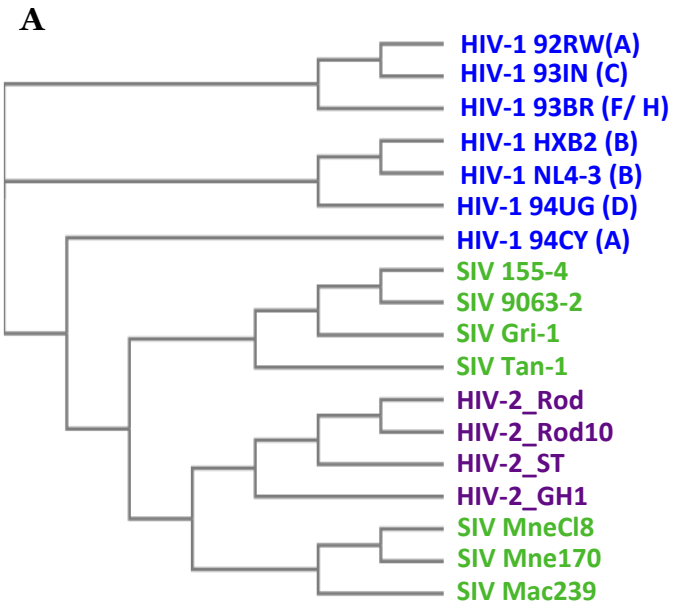
state kinetics has characterized HIV-1 RT, and generally the findings are assumed to apply to all HIV-1, HIV-2 and SIV polymerases (Hsieh et al., 1993; Radzio and Sluis-Cremer, 2005). Initial studies have suggested that there are substantial kinetic differences between HIV-1 and SIV RTs (Diamond et al., 2001; Post et al., 2003; Skasko et al., 2009).

F. Viral Polymerase Comparison

The RTs of HIV-1, HIV-2, and SIV have RNA- and DNA-dependent DNA polymerase capability as well as RNase H activity. However the RTs of HIV-1 and HIV-2/ SIV only share about 60-70% sequence similarity at the amino acid level. Comparing the entire genomic sequences of the two viruses gives similar sequence identity (Gao et al., 1998; Gao et al., 2001; Guyader et al., 1987; Hirsch et al., 1995). The RTs of HIV-2 bifurcates in its similarity to SIVs with one group sharing about 90% protein sequence similarity (SIVsmm lineages) and the other group sharing about 60% sequence similarity (SIVagm lineages). Within group analysis demonstrates that all HIV-1 RTs share about 90% sequence similarity. HIV-2 RT within group comparison is also 90%. Comparing all SIVs, two strong groups appear, the SIVsmm and SIVagm lineages, which share about 60% sequence similarity to each other but 90% with lineage (McWilliam et al., 2013). Of note, the regions involved with binding the incoming dNTP or in chemical catalysis are well conserved among the RTs studied (Huang, 1998). All RTs studied have the same catalytic triad of aspartates as well as the YMDD domain. A phylogenetic tree based on RT sequences and a chart summarizing RT sequence similarity is shown in Figure 1.13.

Comparing to other retroviral polymerases, the lentiviral HIV-1 RT has increased binding affinity but similar rates of conformational change and chemical catalysis compared with the gammaretroviral RT from murine leukemia virus (MuLV) (Skasko et al., 2005). The reduced dNTP binding affinity of MuLV RT (7-120 times lower than HIV-1 RT) results in its ability to synthesize DNA at low dNTP concentrations. This observation suggests that the kinetic characteristics of RT may influence the virus target cell type. MuLV replicates efficiently in dividing cells where dNTP concentrations are high but fails to productively infect nondividing cells where dNTP pools are kept low. Similar reductions in kinetic efficiency have been noted for other gammaretroviruses including feline leukemia virus (FeLV), which only replicate in fast dividing cancer cells (Operario et al., 2005).

Figure 1.13



B

	HIV-1	HIV-2	SIV
HIV-1	90%		
HIV-2	62%	90%	
SIV	61%	60, 87%	62, 91%

Figure 1.13 Phylogenetic comparison of immunodeficiency lentiviruses based on RT protein sequence. (A) The sequences of the p66 subunit are compared using a phylogenetic tree (McWilliam et al., 2013). HIV-1 RT sequences (blue) are grouped and the subtype is denoted in parentheses. The HIV-2 RT sequences (purple) are also grouped and most similar to the SIVsmm derived sequences. The split in the SIV group (green) displays the differences in SIVagms (155-4, 9063-2, Gri-1, Tan-1) and SIVsmm (MneCl8, Mne170, Mac239). **(B)** The percentage of amino acid similarity between and among the different RT groups is displayed.

1.8 Thesis Hypothesis

In this thesis the mechanistic differences among the RTs of human and simian immunodeficiency causing lentiviruses are compared. Since Vpx/ Vpr induces the degradation of SAMHD1 and increases the concentration of dNTPs in HIV-2 and some SIV lineages, it is hypothesized that these polymerases have adapted to transcribe in a high dNTP environment particularly in nondividing cells. Since HIV-1 is also able to replicate in nondividing cells and does not encode a protein to degrade SAMHD1, it is likely HIV-1 polymerases have evolved to be more efficient in a low dNTP environment. This thesis supports the hypothesis that kinetic properties of viral polymerases are mechanistically tied to dNTP availability in target cells, which is affected by viral accessory proteins.

CHAPTER 2

Kinetic Variations Between Reverse Transcriptases of Viral Protein X Coding and Noncoding Lentiviruses

This research was published in *Retrovirology*.

Lenzi GM, Domaoal RA, Kim DH, Schinazi RF, and Kim B.

Kinetic variations between reverse transcriptases of viral protein X coding and noncoding lentiviruses.

Retrovirology. 2014. Vol 11(111):30078-86

Gina Lenzi cloned, purified, and performed the experiments and wrote and edited the paper.

Robert Domaoal contributed training and materials for kinetic assays and assisted in data analysis.

Raymond Schinazi and Dong-Hyun Kim conceived the experiments.

Baek Kim conceived and designed the experiments and wrote the paper.

SUMMARY

Host SAM domain and HD domain-containing protein 1 (SAMHD1) suppresses reverse transcription kinetics of HIV-1 in nondividing cells such as macrophages by hydrolyzing and nearly depleting cellular dNTPs, which are the substrates of viral reverse transcriptase (RT). However, unlike HIV-1, HIV-2 and SIVsm encode viral protein X (Vpx), which counteracts the dNTPase activity of SAMHD1 and elevates dNTP concentration, allowing the viruses to replicate under abundant dNTP conditions even in nondividing cells. Here we tested whether RTs of these Vpx coding and noncoding lentiviruses display different enzyme kinetic profiles in response to dNTP concentrations. For this test, we characterized an extensive collection of RTs from 7 HIV-1 strains, 4 HIV-2 strains and 7 SIV strains, and determined their steady-state kinetic parameters. The K_m values of all HIV-1 RTs were consistently low and close to the low dNTP concentrations found in macrophages. However, the K_m values of SIV and HIV-2 RTs were not only higher than those of HIV-1 RTs but also varied significantly. However, the k_{cat} values of all eighteen lentiviral RTs were very similar. Our biochemical analysis supports the hypothesis that the enzymological properties, particularly, K_m values, of lentivirus RTs are mechanistically tied with the cellular dNTP availability in nondividing target cells, which is controlled by SAMHD1 and Vpx.

INTRODUCTION

Lentiviruses such as HIV-1, HIV-2 and SIV infect both activated/dividing $CD4^+$ T cells and various nondividing myeloid cell types including macrophages

and microglia during the course of their pathogenesis (Diamond et al., 2004; Jamburuthugoda et al., 2006). However, the kinetics of HIV-1 replication in these nondividing cells is significantly delayed, compared to activated $CD4^+$ T cells (Amie et al., 2013b; Diamond et al., 2004). Nondividing cells maintain lower dNTP concentrations than dividing cells that can activate cellular dNTP biosynthesis at S phase (Traut, 1994). Thus due to the limited dNTP availability, nondividing macrophage are suboptimal for supporting DNA synthesis of lentiviruses as compared to the activated and constantly dividing $CD4^+$ T cells (Diamond et al., 2004; Kennedy et al., 2010). Indeed, cellular dNTP concentrations are ~200 times lower in macrophage (20- 40 nM) than activated $CD4^+$ T cells (1- 16 μM) (Diamond et al., 2004). A series of recent studies showed that the host SAMHD1 protein has dNTP hydrolase and RNase activities and serves as a restriction factor that can delay the replication kinetics of lentiviruses (Ayinde et al., 2012; Berger et al., 2011; Planelles, 2012; Ryoo et al., 2014; St Gelais and Wu, 2011), and the dNTP hydrolase activity of SAMHD1 is responsible for the poor dNTP availability in the viral nondividing target cell types such as macrophages and DCs (Goldstone et al., 2011).

Interestingly, unlike HIV-1, HIV-2 replicates more rapidly in nondividing cells (Hollenbaugh et al., 2014). This phenotype is directly linked to a viral accessory protein, called Vpx, which is encoded by HIV-2 and many SIV strains (e.g. SIVmn, SIVsm, SIVmac, etc.) (Goujon et al., 2008; Sharova et al., 2008). Recent studies revealed that Vpx targets SAMHD1 for proteasomal degradation through the E3 ubiquitination pathway (Hrecka et al., 2011; Laguette et al., 2011), and the cellular depletion of SAMHD1 leads to elevated dNTP concentrations and

accelerated reverse transcription in macrophages, resting $CD4^+$ T cells, and DCs (Lahouassa et al., 2012). However, unlike HIV-2/ SIVsm which rapidly replicate under high dNTP concentration conditions even in the nondividing cells, the proviral DNA synthesis of HIV-1 lacking Vpx is kinetically restricted in the nondividing target cell types due to the limited dNTP pools established by SAMHD1 (Kim et al., 2012).

Cellular DNA polymerases replicate chromosomal DNAs at S phase where dNTP concentration is highly elevated due to the expression and activation of cellular dNTP biosynthesis enzymes such as ribonucleotide reductase and thymidine kinase (Traut, 1994). Other retroviruses such as the gammaretroviruses MuLV and FeLV exclusively replicate in dividing cells and therefore, like cellular DNA polymerases, these non-lentivirus RTs synthesize proviral DNAs only in high cellular dNTP environments (Operario et al., 2005; Skasko et al., 2005). Steady-state kinetic comparison between RTs of HIV-1 and MuLV demonstrated that the K_m value of HIV-1 RT is much lower than that of MuLV RT, and close to the dNTP concentrations found in macrophages (Diamond et al., 2004). Pre-steady state kinetic analysis confirmed that HIV-1 RT has 10-100 times tighter binding affinity to dNTP substrate than MuLV RT (Skasko et al., 2005). Finally, RT of feline immunodeficiency virus, which infects nondividing cells, also function more efficiently in low dNTP concentrations found in macrophages, compared to FeLV (Operario et al., 2005). These studies suggested that RTs might have evolved to function optimally at the dNTP concentrations found in their target cell types.

In this study, since Vpx-lacking HIV-1 replicates at extremely low dNTP concentration environments in macrophages, we tested whether RTs of HIV-1 strains display a higher affinity for dNTPs and low K_m values close to the dNTP concentrations found in macrophages as compared with RTs of Vpx-encoding lentiviruses such as HIV-2 and SIV where the selective pressure to function optimally at low dNTP concentrations is lifted by Vpx. For this study, we characterized 11 Vpx coding and 7 Vpx noncoding lentivirus RT enzymes by steady-state kinetic analysis. We found that RTs of HIV-1 clades display low K_m values and efficiently function at the low dNTP concentrations found in macrophages. In contrast, RTs from HIV-2 and SIV generally have higher K_m values suggesting that they may not function efficiently at low dNTP concentrations without the dNTP elevation by Vpx. Our data support the idea that the differences in steady-state kinetics for the different lentiviral RTs is mechanistically tied to the cellular dNTP concentrations at the time of proviral DNA synthesis in the target cell types.

EXPERIMENTAL PROCEDURES

Materials—E. coli DH5 (Invitrogen) was used for construction of plasmids and BL21 (Novagen, WI) for overexpression of RT enzymes. pET28a plasmids with RTs from SIVagm Sab-1, Tan-1, 9063-2, 155-4, Gri-1, SIVmac239, SIVMNE CL8, 170, HIV-1 NL4-3 and HIV-1 HXB2 were previously created (Skasko et al., 2009). The NIH AIDS Reagent Program generously offered the near-full length molecular clones for the different HIV-1 and HIV-2 subtypes. The RT genes were cloned from these molecular clones also into pET28a creating an N-terminus six

histidine tag with NdeI/ XhoI restriction enzymes. The restriction endonucleases were obtained from New England Biolabs.

Protein purification - Homodimeric (p66/ p66) RT enzymes were purified using a modification of our purification protocol for HIV-1 RT as described previously (Kim, 1997). *E. coli* BL21 with the RT expression plasmids described above was grown in Terrific Broth to log phase and expression of RTs was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were then harvested by centrifugation, and the pellets were resuspended and frozen (-70 °C) with 10 ml of 1x binding buffer and lysozyme (200 g/ml). All buffers and binding resin used in this work were purchased from Novagen (WI). Frozen cells were thawed and lysed on ice for 2 h. The lysed cells were centrifuged (27,000 g), and after addition of 10 ml of fresh 1x binding buffer the supernatant was applied to a charged 5-ml His Bind column (1 x 5 cm). All chromatographic steps were carried out at 4°C at a flow rate of 20 ml/h. Following application of the crude supernatant solution, the column was washed with 1x binding buffer (15 ml) and a mixture of 1x binding buffer and 1x wash buffer (7:3, 10 ml). RT enzymes were eluted with 1x elute buffer (20 ml); 90% of the recovered RT enzyme was released from the resin in the first 8 ml. Fractions containing purified RT enzymes were analyzed by electrophoresis on a 4-15% SDS-polyacrylamide stacking gel. Fractions containing RT were dialyzed against 1x dialysis buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 200 mM NaCl, 10% glycerol) for 16 h and 1x dialysis buffer with 1 mM DTT for 3 h. In this protocol the purity of the RT enzymes was typically greater than 95%.

dNTP incorporation Assay - The primer extension assay was modified from a previously described assay (Diamond et al., 2003). Briefly, an RNA template/ primer (T/P) was prepared by annealing a 40-mer RNA (5' – AAGCUUGGCUGCAGAAUAUUGCUAGCGGGAAUUCGGCGCG-3', IDT) to the 17-mer primer (5' – CGCGCCGAATTCC CGCT-3', template:primer ratio of 2.5:1) labeled with ^{32}P at the 5' -end by T4 polynucleotide kinase. The DNA template/ primer contained the same 17-mer primer annealed to a 40-mer DNA template 5' – AAGCTTGGCTGCAGAATATTGCTAGCGGGAATTCGGCGCG -3', IDT). The viral sequence used contained a 24-mer primer (5'—TCGGTCCCTGTTCGGGCGCCACT –3', IDT) annealed to a 48-mer DNA template (5'—CAGTGTGGAAAATCTCTAGCAGTGGCGCCCGAACAGGGACCTGAAAGC — 3', IDT). Assay mixtures (20 μl) contained 25 nM T/P, RT, and dNTPs as specified in each figure legend. Reaction mixtures were incubated at 37°C for 5 min and then terminated for analysis. These reaction conditions allow multiple rounds of primer extension. Products were resolved using 14% polyacrylamide-urea gels and visualized using a PharosFX (BioRad).

Data Analysis - The V_{max} and K_m values were determined by fitting the data to the Michaelis-Menten equation using nonlinear regression with Kaleidagraph (Synergy Software). k_{cat} was determined by dividing V_{max} by molar enzyme concentration. Values reported represent means and standard deviations. Two-tailed Student's t tests were used for the two group comparisons.

RESULTS

dNTP concentration effect on RNA-dependent DNA polymerization activity of RTs from 18 different lentiviruses. Due to the Vpx-mediated dNTP elevation in nondividing target cell types that normally harbor low dNTP pools established by host SAMHD1 protein, Vpx encoding lentiviruses such as HIV-2 and many SIV strains replicate in higher dNTP environments even in macrophages, while all HIV-1 strains lack Vpx and replicate under restricted dNTP pools in this nondividing cell type (Lahouassa et al., 2012). Therefore, we tested whether RTs from Vpx encoding and non-encoding lentiviruses display different dNTP concentration dependent RT activity profiles because these two groups of lentiviruses replicate in macrophages, but with significant dNTP availability differences. To test this, we cloned, overexpressed and purified RTs from 7 HIV-1 strains of various subtypes (A, B, C, D, F/H), 4 strains of HIV-2 and 7 strains of SIV. First we examined the effect of dNTP concentration on RNA-dependent DNA polymerization activity of these purified RT enzymes using a 40-mer RNA template (T) annealed to a 5'-^{32}P labeled 17-mer DNA primer (P, Figure 2.1A) at dNTP concentrations observed in activated/dividing $CD4^+$ T cells (1-16 mM, "T" in Figure 2.1B) and nondividing macrophages (20-40 nM, "M"). The primer was extended with the RT amount showing approximately 50% primer extension as estimated by the amounts of the unextend primer (P) and fully extended primer (F) in a 5 minute incubation at 37°C. As shown in some of representative RTs from each of HIV-1, HIV-2 and SIV RT groups (Figure 2.1B, other RT data not shown), all HIV-1 RTs (i.e. HIV-1 94CY in Figure 2.1B) were able to extend the primer efficiently even at low dNTP concentrations found in

macrophages, while many HIV-2 and SIV RT enzymes (i.e. HIV-2 ROD and SIV 9063-2 in Figure 2.1B) displayed reduced fully extended products at low dNTP concentrations found in macrophages. Significant pause sites (see "*" in Figure 2.1B), which are generated by the kinetic delay of dNTP incorporation, are more evident in HIV-2 and SIV RT enzymes, compared to HIV-1 RT enzyme. This initial qualitative analysis shown in Figure 2.1 suggests that RT enzymes from the three groups of lentiviruses (HIV-1, HIV-2 and SIV) have different RNA polymerase activity profiles, especially at low dNTP concentrations found in nondividing macrophages.

Figure 2.1

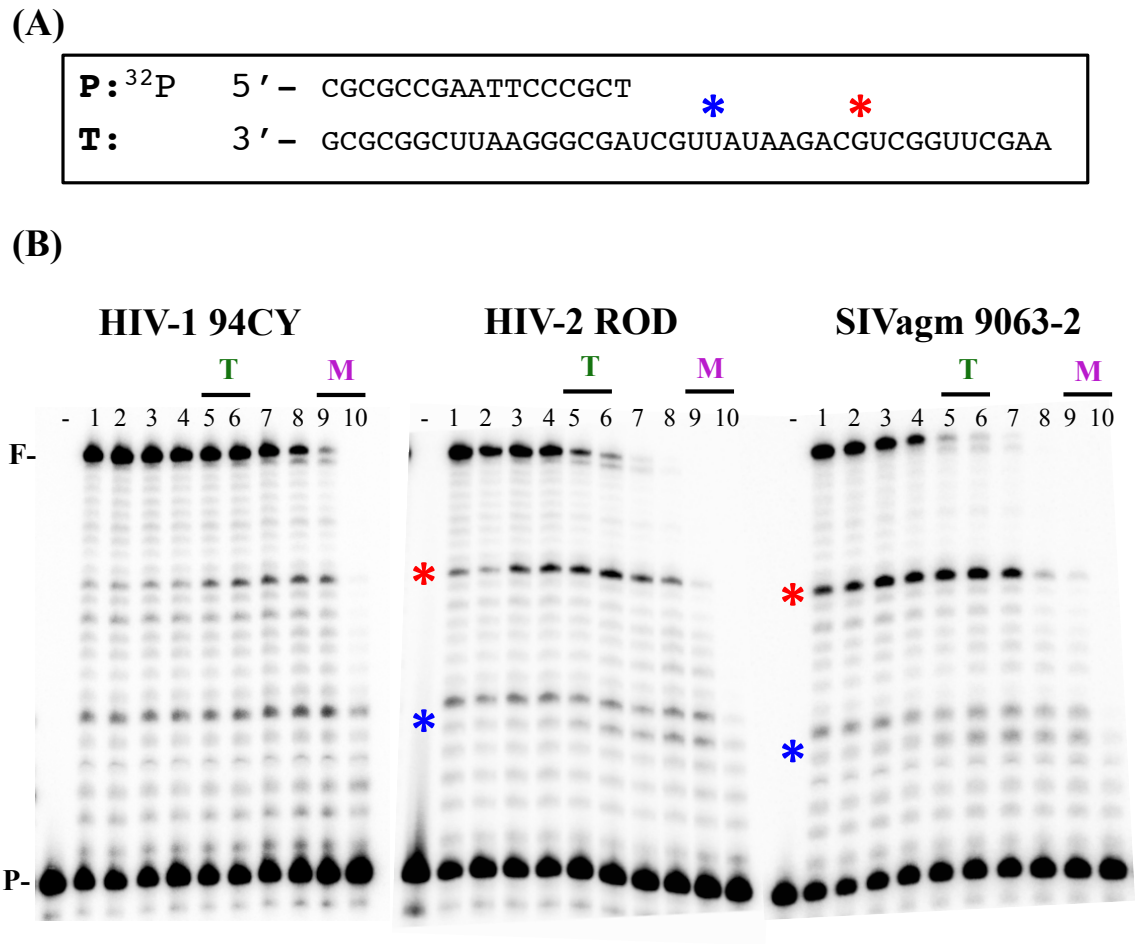


Figure 2.1: Effect of dNTP concentration on RNA-dependent DNA polymerization activity for lentiviral RTs. (A) 5' ^{32}P-labeled 17-mer primer (P) annealed to 40-mer RNA template. **(B)** The T/P was extended by 18 purified RT enzymes under the condition described in Experimental Procedures at different dNTP concentrations (lanes 1-10: 50 μM, 25 μM, 10 μM, 5 μM, 1 μM, 500 nM, 250 nM, 100, nM, 50 nM, 25 nM). RT enzyme amount used in this assay generated approximately 50% primer extension as determined by 40 bp fully

extended product (F) at the highest dNTP concentration (lane 1). Among 18 RT enzyme, the reactions with HIV-1 94CY, HIV-2 ROD, and SIVagm 9063-2 are shown in this figure, "*" indicates pause sites produced by kinetic delays of dNTP incorporations at lower dNTP concentrations. (-) no RT control. T: dNTP concentrations found in activated $CD4^+$ T cells, M: dNTP concentrations found in macrophages.

Determination of steady-state kinetic parameters of lentiviral RT enzymes. Next, in order to quantitatively and mechanistically differentiate the RT activity discrepancy among the 18 RT enzymes, we determined their steady-state K_m and k_{cat} values using the reaction condition described in Figure 2.1. As summarized in Figure 2.2A, we found that the average K_m value for RTs from HIV-1 strains tested were significantly lower than those from HIV-2 or SIV (0.183 μM vs 1.588 μM). This suggests that Vpx encoding HIV-2 and SIV have RTs that require higher concentrations of dNTPs to reach half maximal velocity as compared with RTs from HIV-1 strains. However, in contrast to the K_m value differences, we found that the there was no statistically significant difference in catalytic turnover (k_{cat}) among all of the 18 RT enzymes tested (Figure 2.2B). This suggests that the turnover of substrate per enzyme is well conserved and unaffected within lentiviruses regardless of Vpx. Next we determined and compared the overall steady-state catalytic efficiency (k_{cat}/ K_m) of these RT enzymes. Given that the k_{cat} values were nearly identical for all RTs tested and the K_m values were significantly lower for HIV-1, it was evident that the catalytic efficiency of HIV-1 RTs were significantly higher than RTs from HIV-2 and SIV which express Vpx (Figure 2.2C). This suggests that RTs from HIV-1 strains have higher overall enzyme efficiency indicating that they are more capable of synthesizing proviral DNA than RTs from HIV-2 or SIV, particularly at the low dNTP concentrations found in nondividing macrophages.

Figure 2.2

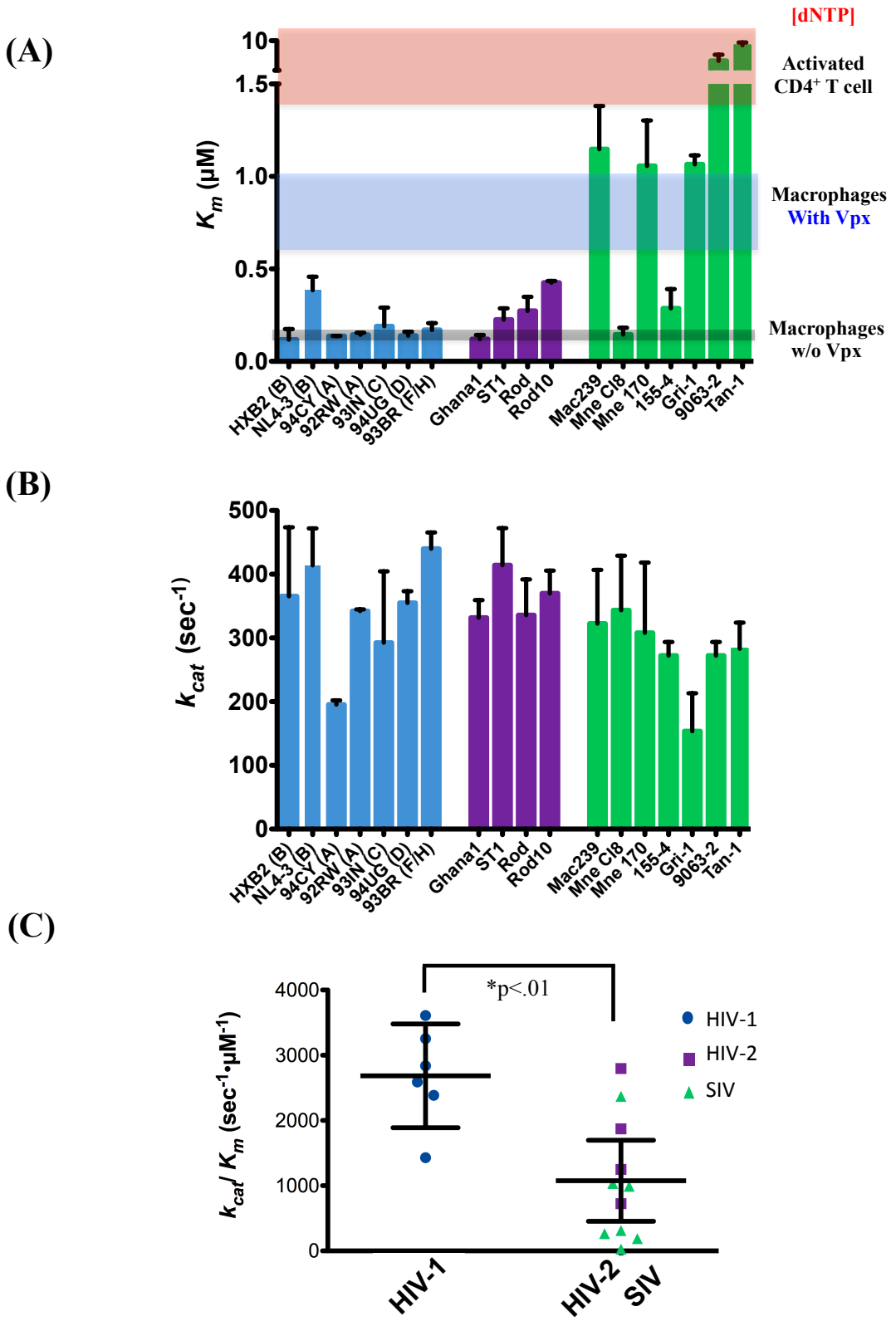

(A)
[dNTP]
Activated CD4+ T cell
Macrophages With Vpx
Macrophages w/o Vpx
Km (μM)
10
1.5
1.0
0.5
0.0
HXB2 (B)
NL4-3 (B)
94CY (A)
92RW (A)
93IN (C)
94UG (D)
93BR (F/H)
Ghana1
ST1
Rod
Rod10
Mac239
Mne CI8
Mne 170
155-4
Gri-1
9063-2
Tan-1
(B)
kcat (sec-1)
500
400
300
200
100
0
(C)
kcat / Km (sec-1·μM-1)
*p<.01
4000
3000
2000
1000
0
HIV-1
HIV-2
SIV

Figure 2.2: Comparison of the steady-state kinetic parameters for 18 lentiviral RT enzymes. The K_m (A) and k_{cat} (B) values of the 18 different RT enzymes (blue bars, HIV-1 RTs; purple bars, HIV-2 RTs; green bars, SIV RTs) were determined from the reactions described in Figure 2.1. dNTP concentrations found in macrophages (grey), activated $CD4^+$ T cells (pink), and macrophages exposed to Vpx (blue) were marked in (A) (Lahouassa et al., 2012). (C) The overall catalytic efficiency values (k_{cat} / K_m) were plotted with a 95% confidence interval and the efficiency difference between RTs of Vpx coding and noncoding viruses were compared.

RT activity comparison with DNA template and template encoding viral sequence. Next, we tested whether the observations shown in Figures 2.1 and 2.2 with RNA-dependent DNA polymerase activity of the RT enzyme are also common in their DNA-dependent DNA polymerase activity by employing a DNA template encoding the same sequence as the RNA template used in Figures 2.1 and 2.2. As shown in Figure 2.3A, HIV-1 94CY RT continues to extend at low dNTP concentrations as compared with SIVagm 9063-2 RT, which is consistent with the observation with the RNA template (Figure 2.1). Finally, we also tested whether the same discrepancy between HIV-1 RTs and other RTs can be observed in a template encoding a viral sequence: template encoding the primer binding site (PBS), which is one of the most conserved viral sequences among lentiviruses. As shown in Figure 2.3B, again, HIV-1 94CY RT enzymes are more capable of extending the primer, compared to SIV 9063-2 RT enzymes at the low macrophages dNTP concentrations (other RT data are not shown). Therefore, the data shown in Figure 2.3A and B support that HIV-1 RT enzymes are more efficient than Vpx-encoding lentivirus RT enzymes regardless of the types and sequences of template, particularly at low dNTP concentrations found in macrophages.

Figure 2.3

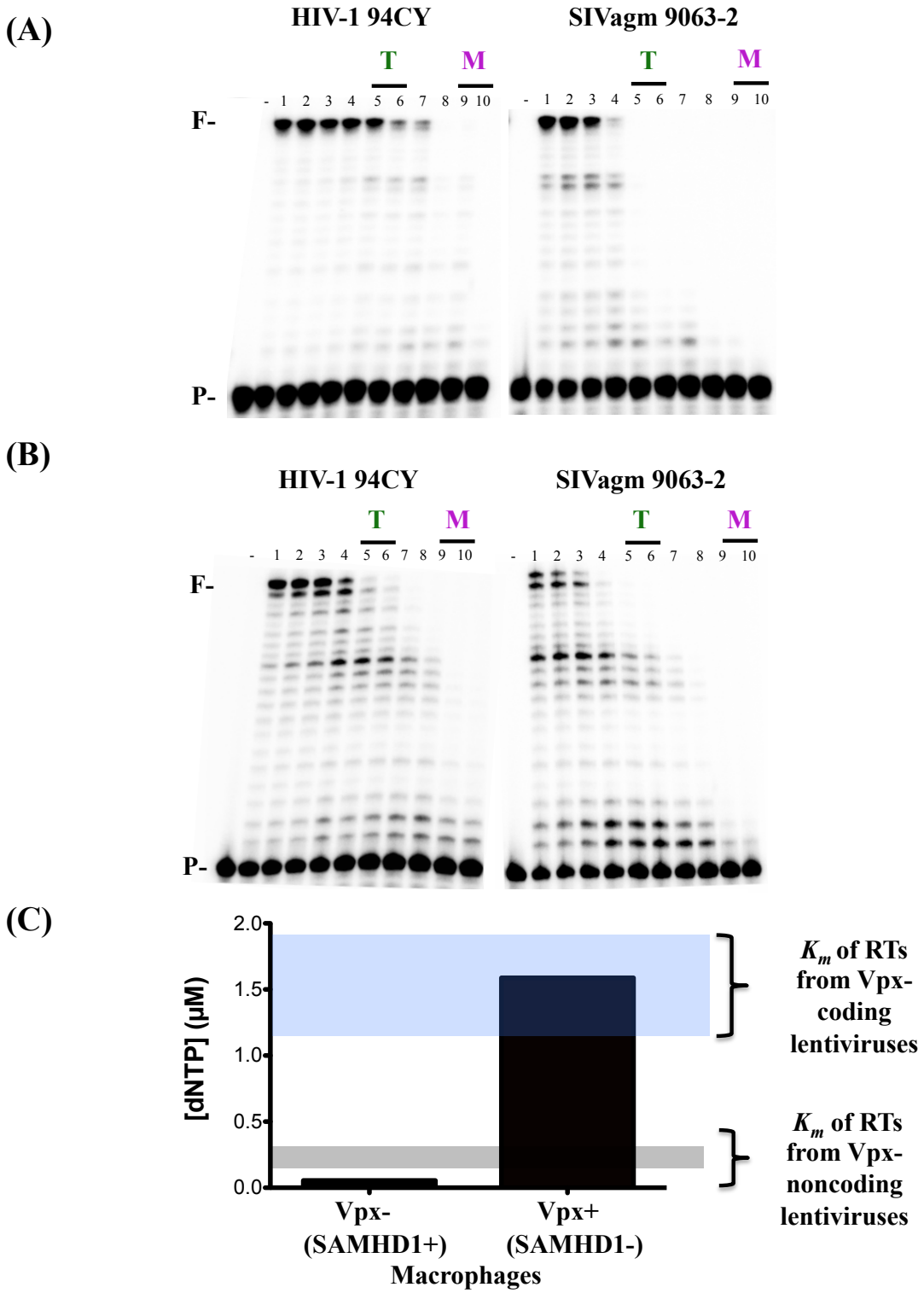

(A)
HIV-1 94CY
SIVagm 9063-2
T
M
- 1 2 3 4 5 6 7 8 9 10
F-
P-
(B)
HIV-1 94CY
SIVagm 9063-2
T
M
- 1 2 3 4 5 6 7 8 9 10
F-
P-
(C)
[dNTP] (μM)
2.0
1.5
1.0
0.5
0.0
Vpx-
(SAMHD1+)
Vpx+
(SAMHD1-)
Macrophages
K_m of RTs from Vpx-coding lentiviruses
K_m of RTs from Vpx-noncoding lentiviruses

Figure 2.3: Effect of dNTP concentration on DNA-dependent DNA polymerization activity for lentiviral RT. The primer extension reactions were conducted with the RT enzymes described except **(A)** 40-mer DNA template encoding the same sequence as the RNA template used in Figure 2.1 and **(B)** 48-mer DNA template encoding conserved HIV-1 PBS binding site under the same reaction condition described in Figure 2.1. **(C)** Scheme explaining potential mechanistic ties between K_m values of RT enzymes from lentiviruses encoding or non-encoding Vpx and cellular dNTP pools modulated by SAMHD1 and Vpx in macrophages.

DISCUSSION

Nondividing cells contain lower cellular dNTP concentrations than dividing cells because cellular dNTP biosynthesis is activated during the cell cycle particularly during S phase, where dNTPs are consumed for chromosomal DNA replication (Traut, 1994). Terminally differentiated/ nondividing macrophages, which permanently lack chromosomal DNA replication, harbor extremely low dNTP concentrations (Diamond et al., 2004), and host SAMHD1 protein, which is a dNTPase expressed at high levels specifically in nondividing cells, contributes to the low dNTP abundance in macrophages (Goldstone et al., 2011). Therefore, viruses that replicate and synthesize DNA in macrophages encounter the selective pressure generated from low dNTP availability during viral replication. We previously reported that HIV-1 RT has a uniquely low K_m value for dNTP substrates compared to RTs of other retroviruses that exclusively infect dividing cells such as oncoretroviruses (Operario et al., 2005; Skasko et al., 2005). It was postulated that this low K_m value and the ability to efficiently synthesize DNA at low dNTP concentrations could be an evolutionary outcome of the selective pressure of low dNTP abundance found in macrophages. However, other lentiviruses such as HIV-2 and many SIV strains overcome the low dNTP selective pressure by using another mechanism: they encode Vpx that counteracts the SAMHD1 mediated low dNTP availability by elevating dNTP level and enables these lentiviruses to replicate at high dNTP environments in the nondividing target cell types (Sharova et al., 2008). Therefore, we can predict that the Vpx containing lentiviruses might not have been constantly exposed to

the low dNTP selective pressure in macrophages and other nondividing target cells type such as DCs and resting $CD4^+$ T cells.

Indeed, when we conducted the most extensive enzyme kinetic analysis ever reported with 18 lentiviral RT enzymes, the data shows that the K_m values of the Vpx containing lentivirus RTs, particularly SIV RTs, significantly vary, unlike the K_m values of HIV-1 RT enzymes, which are consistently low and close to the low dNTP concentrations found in nondividing cells. This supports an idea that the interplay between high SAMHD1 and low dNTPs in nondividing cells creates a selective pressure for lentivirus RTs to maintain a low K_m. As illustrated in Figure 2.3C, lentiviruses expressing Vpx, which counteracts the role of SAMHD1 providing a high dNTP environment and removing the selective pressure, may have higher K_m values because they replicate in environments with higher substrate concentrations. However, unlike K_m values, k_{cat} values of all 18 RT enzyme are almost identical, supporting that lentiviruses did not evolve k_{cat} when encountering the vast dNTP concentration discrepancy between their dividing and nondividing target cells types (Figure 2.3C). Overall, this extensive enzymological study with a total of 18 lentivirus RT enzymes supports a close mechanistic tie between lentivirus RT kinetics and cellular dNTP availability which is regulated by the Vpx-SAMHD1 network in nondividing viral target cells.

CHAPTER 3

Mechanistic and Kinetic Differences Between Reverse Transcriptases of Vpx Coding and Noncoding Lentiviruses

This research was originally published in *Journal of Biological Chemistry*.

Lenzi GM, Domaoal RA, Kim DH, Schinazi RF, and Kim B.

Mechanistic and Kinetic Differences Between Reverse Transcriptases of Vpx Coding and Noncoding Lentiviruses.

Journal of Biological Chemistry. 2015. Vol 290(50):30078-86

Gina Lenzi purified the enzymes, performed the experiments, analyzed the data and wrote and edited the paper.

Robert Domaoal helped with the pre-steady-state experiments and contributed training and materials for kinetic assays.

Raymond Schinazi and Dong-Hyun Kim conceived the experiments.

Baek Kim conceived and designed the experiments and wrote the paper.

SUMMARY

Among lentiviruses, HIV-2 and many SIV strains replicate rapidly in nondividing macrophages, while HIV-1 replication in this cell type is kinetically delayed. The efficient replication capability of HIV-2/SIV in nondividing cell is induced by a unique, virally encoded accessory protein, Vpx, which proteasomally degrades the host antiviral restriction factor, SAM domain and HD domain containing protein 1 (SAMHD1). SAMHD1 is a dNTPase and kinetically suppresses the reverse transcription step of HIV-1 in macrophages by hydrolyzing and depleting cellular dNTPs. In contrast, Vpx, which is encoded by HIV-2/SIV, kinetically accelerates reverse transcription by counteracting SAMHD1 and then elevating cellular dNTP concentration in nondividing cells. Here, we conducted the pre-steady-state kinetic analysis of reverse transcriptases (RT) from two Vpx noncoding and two Vpx coding lentiviruses. At all three sites of the template tested, the two RTs of the Vpx noncoding viruses (HIV-1) displayed higher k_{pol} values than the RTs of the Vpx coding HIV-2/SIV while there was no significant difference in the K_d values of these two groups of RTs. When we employed viral RNA templates that induce RT pausing by their secondary structures, the HIV-1 RTs showed more efficient DNA synthesis through pause sites than the HIV-2/SIV RTs particularly at low dNTP concentrations found in macrophages. This kinetic study suggests that RTs of the Vpx noncoding HIV-1 may have evolved to execute a faster k_{pol} step, which includes the conformational changes and incorporation chemistry, to counteract the limited dNTP concentration found in nondividing cells and still promote efficient viral reverse transcription.

INTRODUCTION

Lentiviruses such as human immunodeficiency virus Type 1 (HIV-1), HIV Type 2 (HIV-2) and simian immunodeficiency viruses (SIV) replicate in both activated/ dividing $CD4^+$ T cells and terminally differentiated/nondividing myeloid cells such as macrophages and microglia while other retroviruses such as oncoretroviruses (i.e. murine leukemia virus, MuLV) replicate only in dividing cells (Operario et al., 2005; Skasko et al., 2005). A key metabolic difference between dividing and nondividing cells is the cellular deoxynucleotide triphosphate (dNTP) pool. Cellular dNTP biosynthesis is closely tied with cell cycle; the expression of various enzymes involved in dNTP biosynthesis is specifically activated at G1/S and S phases to support chromosomal DNA replication, which consumes cellular dNTPs (Bjursell and Skoog, 1980; Cohen et al., 1983). It is well established that cancer cells have higher dNTP concentrations than normal dividing cells due to cell cycle dysregulation (Jackson et al., 1980; Traut, 1994). Also it was postulated that nondividing cells including macrophages have lower dNTP concentrations than dividing cells due to lack of cell cycling and chromosomal DNA replication. However, the actual dNTP concentration of human primary macrophages was not available due to sensitivity limitations of available dNTP assays until we developed a highly sensitive method to determine the dNTP concentration in human primary macrophages (Diamond et al., 2004). Indeed, we reported that human primary monocyte-derived macrophages have 50-200 times lower dNTP concentrations (20-40 nM) than activated $CD4^+$ T cells (2-4 mM) (Diamond et al., 2004; Kennedy et al., 2010). Importantly, while HIV-1

replication and viral production are robust in activated $CD4^+$ T cells, its replication in nondividing macrophages is kinetically delayed (Collin and Gordon, 1994; O'Brien, 1994). Our studies demonstrate that the extremely low dNTP level found in macrophages mechanistically contributes to the delayed replication kinetics of HIV-1 in macrophages and nondividing cells.

Unlike HIV-1, HIV-2 and many SIV strains replicate rapidly even in macrophages, and this efficient replication capability of HIV-2/SIV in macrophages is engineered by a virally encoded accessory protein, called viral protein X (Vpx) (Marcon et al., 1991; Yu et al., 1991). Two groups independently reported that Vpx induces the fast replication kinetics in nondividing macrophages by proteosomally degrading a host myeloid specific anti-viral factor, SAM domain and HD domain containing protein 1 (SAMHD1) (Hrecka et al., 2011; Laguette et al., 2011). Later, SAMHD1 was reported to be a dNTPase that hydrolyzes dNTPs to dNs and triphosphates (Goldstone et al., 2011), and indeed, our study revealed that SAMHD1 restricts reverse transcription during HIV-1 replication in macrophages by depleting cellular dNTP, and that the Vpx-mediated SAMHD1 degradation enhances reverse transcription by elevating cellular dNTPs in nondividing macrophages (Lahouassa et al., 2012). This Vpx-mediated dNTP elevation also facilitates viral replication in other nondividing cell types including dendritic cells (St Gelais et al., 2012) and resting $CD4^+$ T cells (Baldauf et al., 2012). Basically, Vpx coding HIV-2/SIV replicate in an abundant dNTP condition even in nondividing cells by counteracting SAMHD1, while Vpx noncoding lentiviruses (HIV-1) always replicate under limited dNTP availability

in nondividing cells. This difference contributes to the delayed replication kinetics exhibited by HIV-1 in macrophages and other nondividing target cells.

We previously reported that HIV-1 RT very efficiently synthesizes DNA especially at low dNTP concentrations, compared to MuLV RT. Furthermore, the pre-steady-state kinetic data demonstrated that HIV-1 RT has a tighter dNTP binding affinity (K_d) than MuLV RT (Skasko et al., 2005). We suggested that the tight dNTP binding affinity of HIV-1 RT promotes synthesis of its proviral DNA in macrophages, which have a low dNTP concentration. Conversely, MuLV RT may not require tight dNTP binding nor DNA synthesis at low dNTP concentrations because MuLV does not infect nondividing cells such as macrophages. This study suggests that the enzyme kinetics of the RT contribute to the cell tropism (dividing vs. nondividing cells) of retroviruses. This idea was further supported by our finding that RT of a SIV clone that preferentially replicates in activated $CD4^+$ T cells where dNTP concentrations are high showed a reduced dNTP binding affinity, which results from a mutation (V148I), compared to a parental virus that preferentially infects macrophages (Diamond et al., 2001; Diamond et al., 2003).

Based on the findings that SAMHD1 mediates the dNTP depletion of macrophages, we reasoned that Vpx coding HIV-2/SIV replicate under increased dNTP conditions even in nondividing cells by counteracting SAMHD1. In constrast, Vpx noncoding lentiviruses (HIV-1) must replicate under limited dNTP availability in nondividing cells, which contributes to the delayed HIV-1

replication kinetics in the nondividing viral target cell types. Indeed, our study on the dNTP utilization efficiency of 7 different HIV-1 RTs (Vpx noncoding) and 11 different HIV-2/SIV (Vpx coding) RTs revealed that the Vpx noncoding viral RTs tested showed more efficient DNA synthesis at low dNTP concentrations, compared to the Vpx coding HIV-2/ SIV RTs (Lenzi et al., 2014), which supports the idea that Vpx and SAMHD1 can influence RT enzyme kinetics. Here we investigated the mechanistic differences between these two groups of RT enzymes using pre-steady-state kinetic analysis, which can separately determine the dNTP binding affinity (K_d) and the following conformational change/incorporation chemistry step (k_{pol} step). We observed that HIV-1 RT enzymes have faster k_{pol} step, but similar dNTP binding affinity, compared to the RTs tested from Vpx coding HIV-2/SIV.

EXPERIMENTAL PROCEDURES

RT Expression and Purification—The HIV-1 Ug, HIV-1 Cy, HIV-2 Rod and SIVagm 9063-2 RT clones were generously provided by the NIH AIDS Reagent Program and V. Hirsch (NIAID). The RT sequences were previously cloned into pET28a (Novagen), and the N-terminal hexahistidine-tagged-p66/p66 homodimer RTs were subsequently expressed in *E. coli* BL21 (DE3) pLysS (Stratagene) and purified as described previously (Weiss et al., 2002) with the following changes. Cleared lysate was applied to Ni-NTA His·Bind Superflow resin (Millipore) equilibrated with a binding buffer containing 40 mM Tris-HCl pH 7.5, 250 mM KCl, 20 mM imidazole, 5 mM $MgCl_2$, 10% glycerol and 5 mM beta mercaptoethernol (β-Me). The column was washed for 20CV with increased

KCl (1 M final), and finally, proteins were eluted with increased imidazole (300mM). Fractions containing $(his)_6$-p66 were combined, and further purified on HiPrep 16/60 Sephacryl S-200 HR (GE Healthcare) with a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM KCl, 20 % glycerol, 0.25 mM EDTA and 1 mM β-Me. The purity of the RT enzymes was typically greater than 95% as determined from SDS-PAGE. RT enzymes were flash frozen in liquid nitrogen and stored at -80°C until use.

Multiple dNTP Incorporation Assay - The primer extension assay was modified from a previously described assay (Diamond et al., 2004). Briefly, a template/ primer (T/P) was prepared by annealing a 5' [^{32}P]-labeled 17-mer DNA primer (5'– CGCGCCGAATTCCCGCT -3', IDT) to a 2.5 fold excess of 40-mer template RNA (5'– AAGCUUGGCUGCAGAAUAUUGCUAGCGGGAAUUC GGCGCG -3', IDT). Assay mixtures (20 μl) contained 10 nM T/P, RT, and dNTP at the concentrations specified in each figure legend. Reaction mixtures were incubated at 37°C for 5 min and then terminated for analysis. This reaction condition allows multiple rounds of primer extension and all measured enzyme activity was normalized for 50% extension at the highest dNTP concentration. Products were resolved using 14% polyacrylamide/ 8M urea gels and visualized using a PharosFX (BioRad).

Pre-steady-state Burst Experiments - Pre-steady-state burst experiments were performed using a RQF-3 rapid quench-flow apparatus (KinTek Corporation) to determine the active concentration of the purified RT enzymes. The T/ P was prepared as above and consisted of a 48-mer DNA template (5'-CGAGCTAAGCGCTTGACC GCAGAACATTGCTAGCGGGAATTCGGCGCG -3')

and a 21-mer primer (5'- CGCGCCGAAT TCCCGCTAGCA -3', template:primer ratio of 2.5:1). In this experiment 300 μM dATP and 10 mM $MgCl_2$ were rapidly mixed with RT (100 nM total protein) prebound to T/P (300 nM). All concentrations represent the final concentrations after mixing. The reactions were quenched at various time points with 0.3 M (final) EDTA. Products were then separated on a 20% polyacrylamide/ 8M urea gel, visualized using a PharosFX (BioRad), and quantified with Molecular Imager FX software (BioRad). Product formation was fit to the burst equation (1): $[Product] = A[1 - \exp(-k_{obs} \cdot t)] + (k_{ss} \cdot t)$ in which A is the amplitude of the burst, k_{obs} is the observed first-order burst rate constant, and k_{ss} is the linear steady-state rate constant (Kati et al., 1992).

Single-turnover experiments – Rapid chemical quench experiments were performed as previously described with a RQF-3 rapid quench-flow apparatus (Kintek Corporation) to examine the transient kinetics associated with incorporating a single nucleotide onto three different T/Ps (Kati et al., 1992; Kerr and Anderson, 1997). All reactions used the same 40-mer RNA template, but each annealed with different [^{32}P]-labeled DNA primer. Site 1 (no pause) used the 17-mer (5'– CGCGCCGAATTCCCGCT -3', IDT), Site 2 (unique pause) used a 22-mer 5'- CGCGCCGAATTCCCGCTAGCAA-3', and Site 3 (conserved pause) used a 30-mer 5'- CGCGCCGAATTCCCGCTAGCAATATTCTGC-3'. The reactions were carried out by rapid mixing of a solution containing the preincubated complex of 250 nM of the RT (active concentration) and 50 nM T/P with a solution of 10 mM $MgCl_2$ and varying concentrations of dNTP in the presence of 50 mM Tris-HCl pH 7.8 and 50 mM NaCl at 37 °C. Reactions were quenched, separated,

visualized and quantified as described above. Product formation was fit to a non-linear regression curve equation

(2): $k_{obs} = \frac{k_{pol}[dNTP]}{K_d + [dNTP]}$

where k_{obs} is the observed pre-steady-state burst rate, k_{pol} is the maximum rate of incorporation, and K_d is the equilibrium dissociation constant for the dNTP (Johnson, 1995; Kati et al., 1992; Reardon, 1992).

Generation of Viral RNA Templates - RNA templates previously used for strand transfer studies were generated as described previously (Hanson et al., 2005; Kim et al., 1997). Briefly, TAR and part of the pol gene were first PCR amplified using D3 as a template. The PCR product was then agarose gel purified using a Wizard SV Gel Clean-Up Kit (Promega). Finally, both RNAs were *in vitro* transcribed (IVT) with these PCR products as DNA templates usingMEGAshortscript kit (Ambion). IVT products were then gel purified by 10 % polyacrylamide/8M urea PAGE and UV shadowing.

Multiple dNTP Incorporation on Viral Template - The primer extension assays were performed similarly as described above, but using *in vitro* transcribed viral RNA (TAR and shortened pol gene) as RNA templates. [^{32}P]-labeled 20-mer DNA primers (5'– ACAGACGGGCACA CACTACT -3' and 5' – GACGCATGTG ACTGATATCC - 3' for TAR and pol RNA templates, respectively) were annealed onto these RNA templates to form each T/P pair. Assays were carried out as described above except enzyme activity was normalized by using

the same active concentration (200 nM).

RESULTS

dNTP concentration-dependent DNA synthesis efficiency of RTs from Vpx coding and noncoding lentiviruses. Since Vpx noncoding and coding lentiviruses are both able to replicate in macrophages with significant dNTP availability differences, we tested whether RTs from Vpx coding and noncoding lentiviruses display different concentration-dependent activity profiles. To test this, we cloned, overexpressed and purified homodimeric p66 RTs from 2 HIV-1 strains of different subtypes (A, D: Vpx noncoding), HIV-2 Rod and SIVagm 9063-2 (Vpx coding). First we examined the effect of dNTP concentration on the RNA-dependent DNA polymerization activity of these purified RT proteins using a 40-mer RNA template (T) annealed to a 5'-^{32}P labeled 17-mer DNA primer (P, Figure 3.1A) and varying dNTP concentrations observed in activated/dividing CD4^{+} T cells (1 mM, "T" in Figure 3.1B) and nondividing macrophages (50 nM, "M)". We initially determined the amount of the RT proteins showing approximately 50% primer extension at 1 mM dNTPs ("T": T cell concentration) as calculated by the ratio of unextend primer (P) to fully extended primer (F) in a 5 minute incubation at 37°C. Then, the same reactions were repeated with decreasing concentrations of dNTPs down to 50 nM ("M"). As shown (Figure 3.1B), the two HIV-1 RTs were able to generate the full length product (see arrow), even at low dNTP concentrations found in macrophages and this is consistent with our previous observations for other HIV-1 RT variants (Lenzi et al., 2014). The HIV-2 and SIV RTs also efficiently fully extended the primer in both T cell dNTP

concentration and the dNTP concentration found in macrophages treated with Vpx ("X: 500nM, (Lahouassa et al., 2012)). However, the HIV-2 and SIV RTs tested generated 5-10 times less fully extended product at low dNTP concentrations found in macrophages ("M") as compared with HIV-1 RTs. Pausing (see Sites 2 and 3, "*" in Figure 3.1A and 3.1B) is generated by the kinetic delay of dNTP incorporation and is more significant in the HIV-2 and SIV RT proteins compared to the HIV-1 RT proteins (unpaired t-test between RTs from Vpx noncoding and coding viruses, $p < 0.05$). This initial analysis shown in Figure 3.1 suggests that RTs from the these two groups of lentiviruses (Vpx noncoding and coding) have different DNA polymerase activity profiles, especially at low dNTP concentrations found in nondividing macrophages.

Figure 3.1

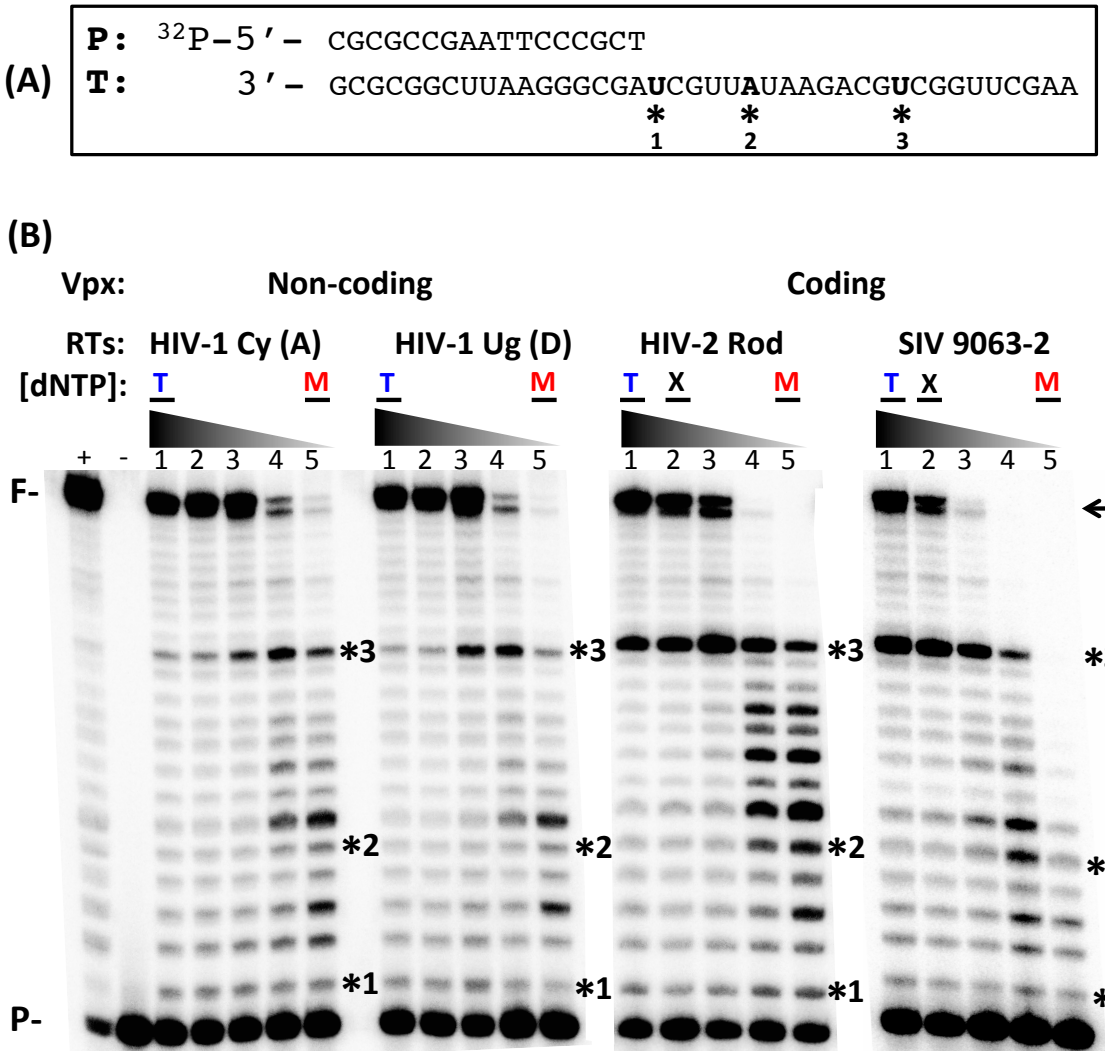


Figure 3.1: dNTP concentration-dependent DNA synthesis of RT proteins from Vpx coding and noncoding lentiviruses. (A) Template (T) and primer (P) used in this study. 5' ^{32}P-labeled 17-mer DNA primer was annealed to 40-mer RNA template. The three sites ("*") used for pre-steady-state analysis are indicated. **(B)** The T/P was extended by 4 purified RT proteins from either Vpx noncoding or coding lentiviruses under the conditions described in

Experimental Procedures at different dNTP concentrations (lanes 1–5: 1 μM, 500 nM, 200 nM, 100, nM, 50 nM). The RTs used from Vpx noncoding viruses were HIV-1 Cy (A) and HIV-1 Ug (D) and the RTs used from Vpx coding viruses were HIV-2 Rod and SIV 9063-2. RT activity used in this assay generated approximately 50% primer extension at the high dNTP concentration found in activated $CD4^+$ T cells ("T" and lane 1) as determined by the quantitation of the 40 bp fully extended product (F and ←). The three sites analyzed for the pre-steady-state kinetic study were also marked with "*". ($^+$): 50 μM dNTP positive control (–): no dNTP control. T: dNTP concentration found in activated $CD4^+$ T cells, M: dNTP concentration found in macrophages. X: dNTP concentration found in macrophages treated with Vpx (Lahouassa et al., 2012). F: Fully extended products. P: primer and unextended substrate.

Pre-steady-state kinetic analysis of the four RT enzymes at three different sites. In order to understand the mechanistic discrepancy in the DNA synthesis kinetics between the RTs from Vpx noncoding and coding lentiviruses, we sought to determine the dNTP binding affinity (K_d) and incorporation rate (k_{pol}) for each RT protein. To determine the active enzyme concentration of the four RT proteins, we first used pre-steady-state burst experiments (molar excess of T/P) with the T/P that does not induce RT pausing (see Methods and Materials). We observed typical burst kinetics for all four RT proteins (Figure 3.2A) followed by the slow steady state rate, giving a ratio of active protein ranging from 50-75%. This indicates that the mechanistic pathway was not changed for the enzymes tested and that a slow step following the chemistry is limiting the overall reaction pathway.

Using all four enzymes normalized for active concentration, we employed single turnover experiments in order to determine each enzyme's binding affinity (K_d) and incorporation rate (k_{pol}) at Site 1 of the T/P used in Figure 3.1. We used concentrations of dNTPs ranging from 1 to 100 μM and determined the rate of single nucleotide incorporation at each concentration. Those rates were plotted against the dNTP concentration in order to determine the maximum rate of incorporation and dNTP binding affinity. Figure 3.2B displays the binding curves for Site 1 for the four enzymes. As evident from the graph, the Vpx noncoding HIV-1 RTs plateau at a higher rate of incorporation compared with the Vpx coding HIV-2 Rod and SIV 9063-2. However the dNTP binding affinities for all four enzymes at Site 1 are not statistically different (Table 1). This data from Site 1 suggests that the RTs from HIV-1 may have higher rates of dNTP incorporation

but similar dNTP binding affinities to RTs from Vpx coding SIV and HIV-2 lentiviruses.

Figure 3.2

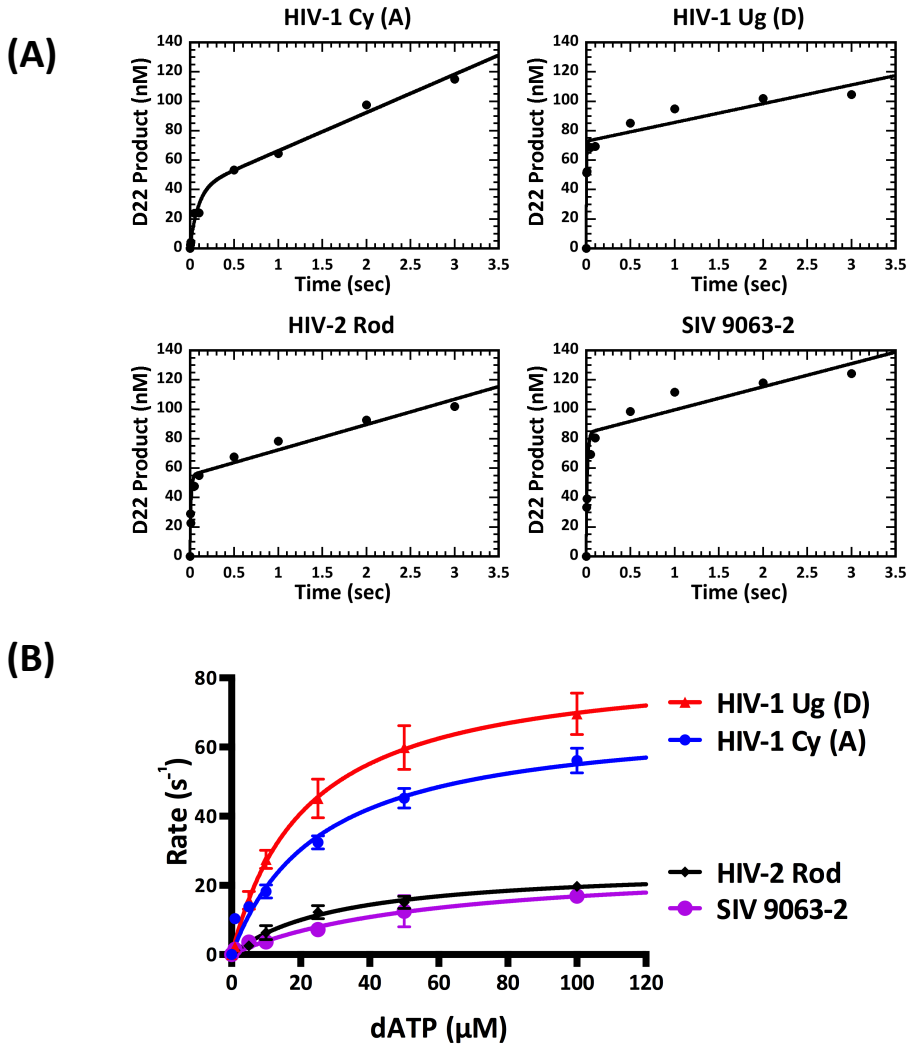


Figure 3.2: Active concentration determination and the pre-steady-state dATP incorporation kinetics of RT proteins from Vpx noncoding and coding lentiviruses at Site 1 of the 40-mer RNA template. (A) Pre-steady-state burst kinetics of incorporation of dATP onto the T/P described in Experimental procedures by the 4 RT proteins. The solid line represents a fit to a burst equation. Burst experiments were repeated 2-3 times for each enzyme, and a representative curve for each enzyme is shown. Percentages of the active

concentrations for HIV-1 Cy, HIV-1 Ug, HIV-2 Rod, and SIV 9063-2 are 40, 73, 55, and 84%, respectively. **(B)** Pre-steady-state incorporation rates of the four RT proteins at varying dATP concentration (1 μM to 100 μM) at Site 1 of the T/P described in Figure 3.1 were plotted. The fit to the data gave the following K_d (dNTP binding constant) and k_{pol} (maximum incorporation rate) values, respectively: HIV-1 Cy 30.9 μM and 67.2 sec^{-1}, HIV-1 Ug 28.4 μM and 86.6 sec^{-1}, HIV-2 Rod 30.4 μM and 29.6 sec^{-1}, and SIV 9063-2 40.0 μM and 22.3 sec^{-1} (see Table 3.1 for detail). Experiments were repeated 3-7 times for the four enzymes at Site 1, and the average is shown with error bars representing SEM.

Table 3.1

Site	RTs	k_{pol} (sec^{-1})	K_d (μM)	k_{pol}/K_d (sec^{-1} μM^{-1})
*1 No pause	**Vpx noncoding** HIV-1 Cy, HIV-1 Ug	79 ± 15 **(3.0x)***	29 ± 5.4 (0.7x) NS	2.7 ± 0.34 **(3.6x)****
	Vpx coding HIV-2 Rod, SIV 9063-2	27 ± 3.2	39 ± 8.4	0.76 ± 0.15
*2 Unique pause	**Vpx noncoding** HIV-1 Cy, HIV-1 Ug	290 ± 31 **(4.1x)*****	71 ± 11 (1.4x) NS	4.1 ± 0.26 **(3.2x)*****
	Vpx coding HIV-2 Rod, SIV 9063-2	69 ± 17	51 ± 7.8	1.3 ± 0.19
*3 Conserved pause	**Vpx noncoding** HIV-1 Cy, HIV-1 Ug	43 ± 4.5 **(2.0x)***	22 ± 4.6 (0.4x) NS	2.4 ± 0.52 **(4.8x)***
	Vpx coding HIV-2 Rod, SIV 9063-2	22 ± 5.1	50 ± 15	0.49 ± 0.10

Table 3.1: k_{pol}, K_d, k_{pol}/K_d values of Vpx noncoding and Vpx coding RTs at three different sites on the 40-mer RNA template. Fold changes between two groups of the RT enzymes indicated in parentheses. Statistical significance from an unpaired T-test is indicated as: NS not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Next, we repeated the experiments at two pause sites along the same template (Sites 2 and 3, Figure 3.1). Site 2 is a unique pause site where only HIV-2/SIV RTs showed pausing, whereas Site 3 is a common pause site where all four RTs experienced kinetic delays (Figure 3.1B). The entire K_d and k_{pol} values of the four RT proteins at the three different sites are shown in Table 1. When these values were compared, (Figure 3.3), the maximum rate of incorporation (k_{pol}, Figure 3.3A) is 2-4 fold higher for RTs from Vpx noncoding lentiviruses as compared to RTs from Vpx coding lentiviruses (Figure 3.3A). Indeed, there are no significant differences in dNTP binding affinity for the two classes of RTs at the all three sites tested (Figure 3.3B). When the overall dNTP incorporation efficiency, which is a ratio of incorporation rate to dNTP binding affinity (k_{pol}/ K_d), the overall dNTP incorporation efficiency is also 3-5 fold higher for the HIV-1 RTs compared with HIV-2/ SIV RTs at all three sites of the T/P tested (Figure 3.3C). Overall, these results suggest that the k_{pol} step, which includes two sequential sub-steps, 1) conformational change and 2) dNTP incorporation chemistry (Patel et al., 1991), is significantly faster in RTs from Vpx noncoding lentiviruses than compared with Vpx coding lentiviruses. Note that the conformational change step, which occurs after the dNTP binding and before incorporation chemistry, is a rate-limiting step for many DNA polymerases (Mizrahi et al., 1985; Patel et al., 1991).

Figure 3.3

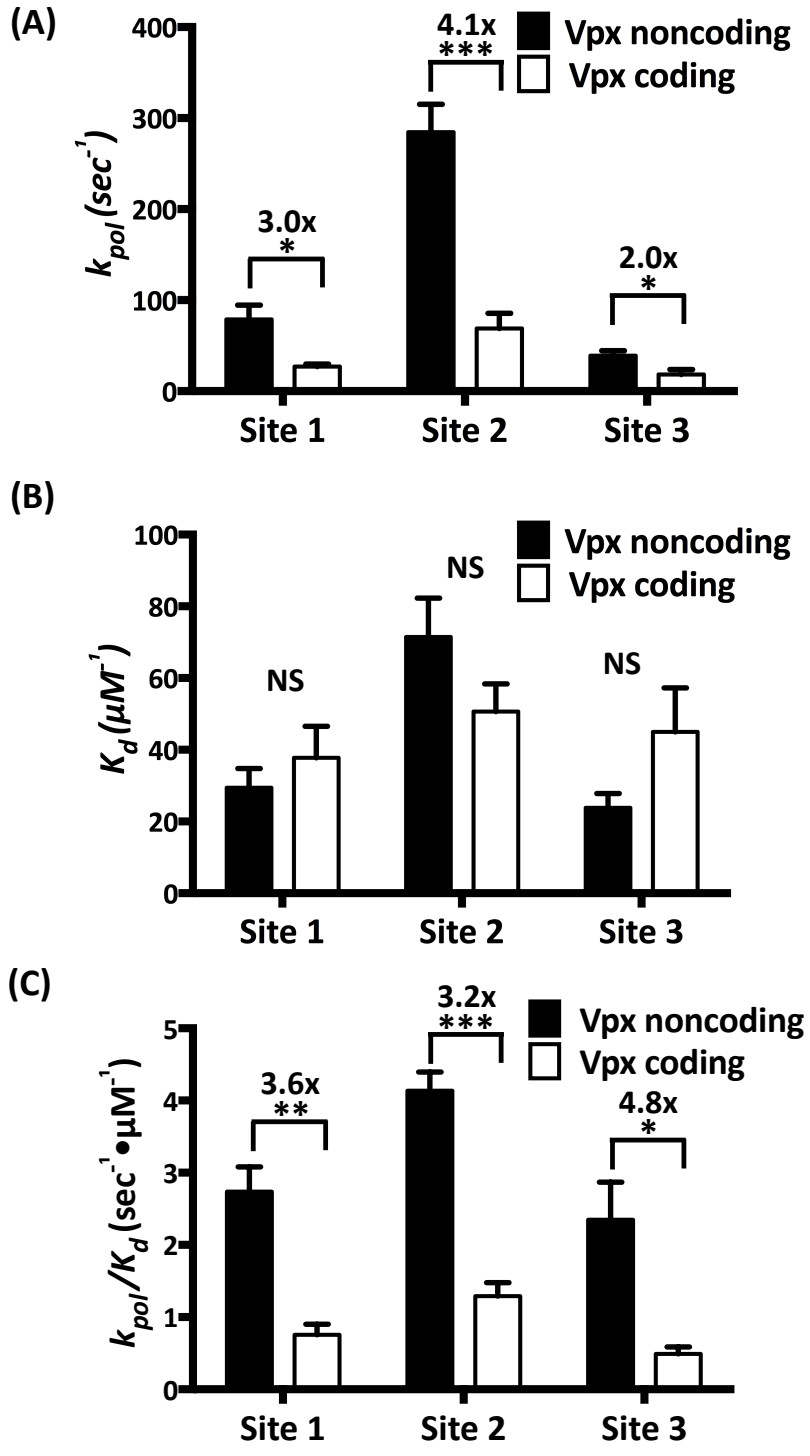


Figure 3.3: k_{pol}, K_d, k_{pol}/ K_d comparison of the four RT proteins at three different sites on the 40-mer RNA template. The maximum

incorporation rates (k_{pol}) **(A)**, the dNTP binding affinity (K_d) **(B)**, and the incorporation efficiency (k_{pol}/ K_d) **(C)** of RT protein from Vpx noncoding (black bars) and Vpx coding (open bars) lentiviruses at the three different sites described in Figure 1 were determined 3-7 times, and the average values are shown with error bars representing SEM. Fold changes are indicated by brackets above the bars and statistical significance from an unpaired T-test is indicated as: NS not significant, * $P< 0.05$, ** $P<0.01$, *** $P<0.001$.

dNTP-concentration dependent DNA synthesis with viral RNA templates harboring RNA structure-induced RT pause sites. RT-mediated RNA-dependent DNA synthesis kinetics are also affected by the secondary structure of RNA templates (Suo and Johnson, 1997). Basically, RT pauses at the bottom of the stem-loop structures found in RNA templates, leading to kinetic delay, particularly at short time points (Kim et al., 1997). This RT pausing is known to trigger RT strand transfer and recombination after the degradation of the RNA template by the RNase H activity of RT (Moumen et al., 2003; Purohit et al., 2007). To assess whether the two groups of lentiviral RTs also display different dNTP concentration-dependent DNA synthesis efficiency at the RNA structure induced pause sites, we performed the primer extension assay using long RNA templates that harbor strong secondary structures and induce RT pausing. First, we chose a sequence of the *pol* gene which is known to have multi-branched loops (Hanson et al., 2005; Wang et al., 2008) and the highly conserved 5' UTR TAR stem loop (Rosen et al., 1985) as illustrated in the top panels of Figure 3.4A and 3.4B (Zuker, 2003). When primers annealed to these long RNA templates were extended with the same amount of active RT enzyme (Figure 3.4A and 3.4B. bottom panels), we observed pause products at several key sites (see "*" in Figure 3.4) at or near the bottom of the stem-loop structures predicted in both RNA templates (Figure 3.4A and 3.4B), even at some high dNTP concentrations found in T cells (1 and 10 mM, "-" in Figure 3.4B). This RT pausing became more evident for all four RT proteins at low dNTP concentrations found in macrophages ("M") and macrophages treated with Vpx ("X"). However, it is clear that RTs from Vpx coding lentiviruses generated more incomplete short products

at the pause sites, particularly at low dNTP concentrations (see “X” and “M”), compared with RTs from noncoding lentiviruses (unpaired t-test between RTs from Vpx noncoding and coding viruses at macrophage conditions for both Pol and TAR, $p < 0.05$). These results indicate that RTs from Vpx coding SIV and HIV-2 experience more pausing and kinetic delay due to decreased dNTP concentrations than RTs from HIV-1 during reverse transcription of structured viral RNA templates.

Figure 3.4

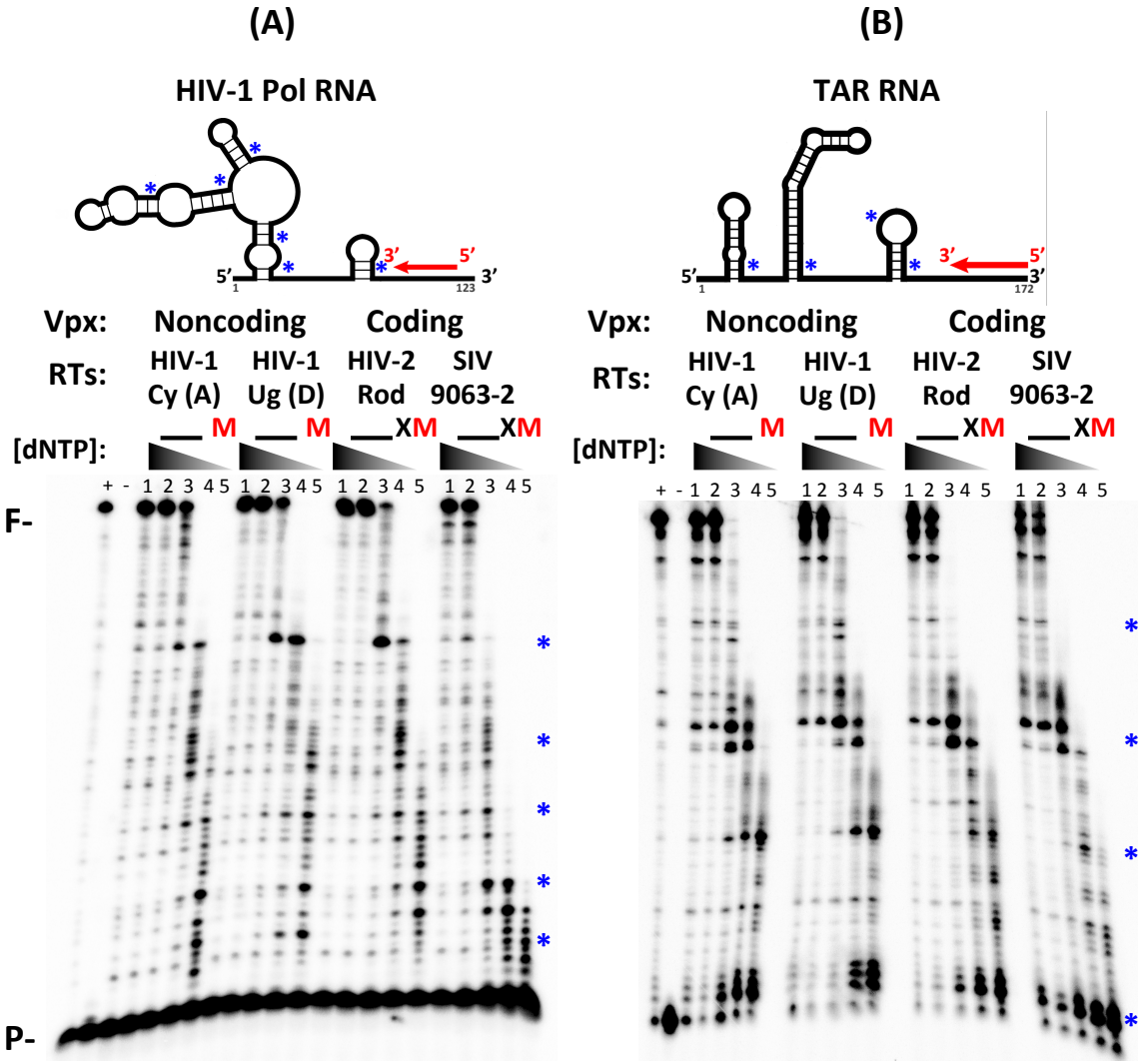


Figure 3.4: dNTP-concentration dependent RNA-dependent DNA synthesis of the four RT proteins with two long viral RNA templates. Schematic showing 5' ^{32}P-labeled 20-mer primer (P) annealed to HIV-1 pol (A, top) or TAR (B, top) RNA template. Template structure based on mfold prediction for lowest free energy (Zuker, 2003). The predicted bottom of each stem-loop structure in these RNA templates were marked by "*". The 5' ^{32}P-

labeled primers annealed to the pol or TAR RNAs (A and B) were extended by an equal active concentration of the 4 purified RT proteins at five different dNTP concentrations (lanes 1–5: 50 μM, 10 μM, 1 μM, 0.25 μM, 0.1 μM), which are close to the dNTP concentrations found in activated T cells ("___"), macrophages ("**M**"), macrophages treated with Vpx ("**X**"), respectively. "*" indicates pause sites produced by kinetic delays of dNTP incorporations at lower dNTP concentrations near the bottom of each stem loop structure predicted in the RNA templates. (+)100 μM dNTP positive control (–) no dNTP control. F: Fully extended products. P: primer and unextended substrate.

DISCUSSION

Cellular replicative DNA polymerases always operate at high dNTP concentrations found in dividing cells because they duplicate chromosomal DNA only during S phase of the cell cycle where dNTP biosynthesis is activated. Importantly, the steady state K_m values of many cellular replicative DNA polymerases are close to or above the cellular dNTP concentrations found in the dividing cells (Einolf and Guengerich, 2000; Martin et al., 1994), supporting the idea that there is evolutionary crosstalk between the enzyme kinetics of the cellular DNA polymerases and cellular dNTP concentrations.

In 2004, we reported that human primary macrophages harbor extremely low dNTP concentrations (20-40 nM), compared to activated $CD4^+$ T cells (1-10 μM) (Diamond et al., 2004), and in 2012, we reported that host SAMHD1 protein, which is a dNTPase, is responsible for the low dNTP concentrations found in macrophages (Lahouassa et al., 2012). Therefore, RTs of lentiviruses encounter two vastly different cellular dNTP environments in dividing (activated $CD4^+$ T cells) versus nondividing viral target cell types (macrophages, dendritic cells and resting $CD4^+$ T cells). Indeed, a series of our biochemical and virological studies suggested that lentiviral RTs might have evolved to efficiently synthesize DNA even at low dNTP concentrations in order to support viral reverse transcription in nondividing viral target cells (Jamburuthugoda et al., 2008; Lenzi et al., 2014; Van Cor-Hosmer et al., 2012). This was supported by the biochemical finding that RTs of gammaretroviruses or alphaviruses such as MuLV, feline leukemia virus and avian myeloblastosis virus, which replicate only

in dividing cells, synthesize DNA efficiently only at the high dNTP concentrations found in dividing cells (Operario et al., 2005; Skasko et al., 2005). Our pre-steady-state kinetic study revealed that HIV-1 RT has a higher dNTP binding affinity than MuLV RT (Skasko et al., 2005), supporting that lentiviral RTs may have evolved to bind dNTP tightly in order to support efficient reverse transcription at the low cellular dNTP concentration found in nondividing macrophages.

However, some Vpx coding lentiviruses replicate at higher cellular dNTP concentrations even in macrophages because Vpx elevates cellular dNTP concentration close to the dNTP concentration found in dividing cells (i.e. activated $CD4^+$ T cells) by counteracting the host SAMHD1 protein (Lahouassa et al., 2012). This led us to test whether Vpx coding and noncoding lentiviral RTs display different DNA synthesis efficiencies at low dNTP concentrations. Indeed, our previous steady-state kinetic analysis with RTs from 19 different lentiviruses revealed that the Vpx noncoding lentiviral RTs such as HIV-1 RTs have lower K_m values than the Vpx coding lentiviral RTs (HIV-2 and SIV RTs) (Lenzi et al., 2014).

Our pre-steady-state kinetic data with four different RTs at multiple sites supports that the polymerases from these Vpx coding and noncoding lentiviruses display different k_{pol} values, rather than K_d values. This finding was rather unexpected because the two SIV RT variants that we previously characterized (SIVmne CL8 and 170 RTs) showed different K_d values with similar k_{pol} values

(Diamond et al., 2001), and the T cell tropic SIVmne170 RT gained the V148I mutation near the active site that reduces its dNTP binding affinity (Diamond et al., 2003). This study led us to hypothesize that SIVmne 170 RT may have lost the tight dNTP binding affinity because this virus only infects activated CD4 T^+ cells where dNTP concentrations are high. Therefore, the pre-steady-state kinetic data from this study and previous studies support that both K_d and k_{pol} steps can vary among lentiviral RTs, and these distinct mechanistic variations may contribute to the cell tropism of lentiviruses (dividing vs. nondividing cells).

The K_d values, which represent the binding affinity to the incoming nucleotide, are the first reported for HIV-1 subtypes A and D, HIV-2, and SIV RTs. Comparing these values with previously reported pre-steady-state results, the binding affinities are slightly higher than those published for HIV-1 subtype B. Previous research has shown that using a homodimeric enzyme and RNA template has a weaker binding affinity compared with a heterodimeric enzyme and a DNA template (Kati et al., 1992; Marko et al., 2013). In addition to subtype differences, we hypothesize that the RNA template and p66 homodimers could contribute to the higher binding affinity.

Importantly, the k_{pol} values of DNA polymerases represent two sequential sub-steps following the dNTP binding to the active site (K_d step), 1) conformational change and 2) catalysis. Also, it is well established that the conformational change step, which occurs after dNTP binding and before incorporation chemistry, is a rate-limiting step during the overall dNTP

incorporation reaction (Mizrahi et al., 1985; Patel et al., 1991). Then, which of these two sub-steps (or both) varies between the tested polymerases from Vpx coding and noncoding viruses? To postulate on this question, we compared the sequences of the four RTs tested to HIV-1 HXB2 (B) in the fingers and palm domains that contain many residues important for DNA polymerization including dNTP binding and chemistry of DNA synthesis (i.e. metal binding). All residues known to be involved in dNTP binding (D113, A114, Y115, Q151, K65, R72) and metal binding/ catalysis (D110, Y183, M184, D185, D186) are conserved among these four RTs (Huang et al., 1998), indirectly supporting the similar K_d values for these four enzymes. V148 is also involved in dNTP binding and highly conserved, but our previous research has shown that the C148 of SIV 9063-2 has no effect on dNTP binding affinity (Skasko et al., 2009). While all key residues important for DNA synthesis are conserved among these four RTs, there are significant sequence variations throughout the proteins (Gao et al., 1998; Gao et al., 2001; Guyader et al., 1987; Hirsch et al., 1995), possibly implying that these sequence variations may affect the overall conformational change efficiency rather than the chemical catalysis step and may lead to efficiency differences in proviral DNA synthesis in the low cellular dNTP environments found in nondividing viral target cell types. Future studies will elucidate whether conformational change and/or chemical catalysis differ between RTs from Vpx noncoding and coding lentiviruses.

CHAPTER 4

General Discussion

4.1 Collective Results

HIV-1 infection of nondividing cells is less frequent than that of activated $CD4^+$ T cells but typically is associated with compartmentalization, reservoirs, and latency. Thus infected macrophages given their long half-lives (Figure 1.11) are critically important for the clinical course of HIV/ AIDS and often associated with HAND. Since these infected macrophages are nondividing cells, cell checkpoint signaling has not initiated dNTP biosynthesis and cellular enzymes like SAMHD1 maintain low concentrations of dNTPs. To counteract the dearth of dNTPs, HIV-2 and some SIV strains have an accessory protein, Vpx, to target SAMHD1 for degradation allowing the virus to replicate in a higher concentration of dNTPs. Strains of HIV-1 lack the ability to degrade SAMHD1 and thus must overcome the low dNTP environment in order to replicate. This thesis focuses on the relationship between viral polymerase efficiency and their dNTP replication environment as modulated by Vpx.

In Chapter 2, we presented enzymatic data from numerous reverse transcriptases from primate lentiviruses including 7 HIV-1 strains from different clades, four strains of HIV-2 and seven strains of SIV. The HIV-2 and SIV strains are all from Vpx-encoding viruses, a gene not encoded by HIV-1. Our work aimed at explaining how HIV-1 can infect nondividing cells, even in the absence of Vpx. Enzymes from viruses that encode Vpx are capable of generating an environment with much higher concentrations of dNTPs since SAMHD1 is targeted for degradation. Therefore these viral polymerases have Michaelis-Menten constants that are adapted to those substrate concentrations or more technically, have high K_m values. In contrast, HIV-1 does not encode Vpx and thus is unable to increase

dNTP levels in nondividing cells. Instead, HIV-1 RTs have evolved to have a higher affinity for substrate (i.e., lower K_m) and can therefore function efficiently at lower dNTP concentrations. We found that the catalytic turnover was fairly consistent among all enzymes suggesting that differences in catalytic efficiency between RTs from Vpx coding and non-coding lentiviruses are mainly due to differences in substrate affinity. These results were consistent for RNA-dependent and DNA-dependent DNA synthesis on generic and viral templates by all RTs tested.

In Chapter 3 we expanded on our steady-state findings to look at the pre-steady-state kinetics of nucleotide incorporation by RT enzymes derived from Vpx coding and non-coding lentiviruses. We showed that enzymes derived from Vpx non-coding viruses (HIV-1 RTs) have higher incorporation efficiency than enzymes from Vpx coding lentiviruses. Specifically, the rate of incorporation (k_{pol}) is faster for RTs from Vpx non-coding lentiviruses, while binding affinities (K_d) remain largely unchanged. These kinetic results were consistent for incorporation of different nucleotides at different sites along the same template. Moreover, these findings suggest that one aspect of the rate of incorporation- either the rate of conformational change required for catalysis and/or the chemistry of incorporation- increased while nucleotide binding is not affected. Overall, both of these studies helped to explain why RT enzymes of Vpx non-coding viruses can still engage in DNA synthesis at low dNTP concentrations found in nondividing cells.

4.2 Implications of Findings

This work furthered a very innovative theory that was proposed by the Kim Lab even before SAMHD1 was reported. Previous work from the Kim Lab showed that the K_m of HIV-1 was unusually low compared to other conventional gammaretroviruses (e.g. MuLV, FeLV), which infect only dividing cells (Operario et al., 2005; Skasko et al., 2005). In addition dNTP-binding mutants of RT, which have decreased incorporation efficiency, fail to infect macrophages (Diamond et al., 2004). Measurement of dNTP concentrations in macrophages demonstrated that their reduced permissivity resulted from substrate limitation during reverse transcription and could be overcome with addition of exogenous dNs (Goujon et al., 2013). Moreover, research from the Hizi and Loya labs compared the affinities of HIV-1 and HIV-2 RT for nucleotides and found a weaker affinity for HIV-2 RT compared with HIV-1 RT (Hizi et al., 1991). With the discovery of SAMHD1 and the role of Vpx in its degradation for HIV-2 and some SIV strains (Goldstone et al., 2011; Hrecka et al., 2011; Laguette et al., 2011), the present data not only confirms the earlier theory but also helps explain why certain lentiviruses have not evolved in the same direction and have, instead, “chosen” to incorporate Vpx.

Phylogenetic analyses of SIV genomes provide insight into the origins and divergence of the *vpx* gene. Analyzing the alignment of the surrounding regions of *vpx* suggests that *vpx* was acquired by both red-capped mangabey SIV (SIVrcm) and SIVsmm before their divergence rather than occurring by a more recent gene duplication or transfer event. This suggests that the SIVrcm stain that combined with mustached monkey SIV (SIVmus) to become SIVcpz to form

SIVcpz encoded a *vpx* gene between *vif* and *vpr* (Zhang et al., 2012). Thus the questions arise how and why would SIVcpz lose a viral antagonist of a host protein?

Genetically, it is likely that the *vpx* gene was deleted in its entirety rather than a recombination event between paralogous genes *vpr* and *vpx*. The phylogenetic analysis and sequence alignment of the 5' and 3' end of the *vpr* gene from SIVcpz do not correspond to regions of the *vpx* gene from SIVrcm supporting *vpx* deletion rather than a recombination (Lim et al., 2012).

This suggests that a selective pressure may have favored the loss of a poorly active gene (*vpx*) during the zoonotic transmission from old world monkeys to chimpanzees in order to restore or increase the function of an overlapping gene. Indeed, the upstream gene *vif* has developed unique features that allow for specific antagonism of host APOBEC3s. The C-terminal domain of Vif has a distinctive cullin box that is absent from HIV-2 and SIVs and allows for efficient degradation of host APOBEC3s (Barraud et al., 2008). Collectively, this may suggest that it was more critical to have the capabilities of Vif than Vpx in order for SIVcpz to efficiently replicate. Without Vpx, SAMHD1 is present in nondividing cells maintaining low levels of dNTPs. Thus, it follows that it was necessary for HIV-1 RTs to coevolve to increase their efficiency in order to counteract the loss of *vpx* (Fregoso et al., 2013).

This thesis not only reinforces the significance of the Vpx-SAMHD1 interface in the evolution of lentiviral interactions with Old World monkeys but also stresses the evolutionary plasticity and complexity of interactions between restriction factors and their lentiviral antagonists.

4.3 Limitations and Future Directions

Using multiple lentiviral strains, these studies suggest why RT enzymes of Vpx non-coding viruses are able to synthesize DNA at low dNTP concentrations found in nondividing cells such as macrophages. In fact in Chapter 2 we conducted the most extensive enzyme kinetic analysis ever reported comparing the steady-state kinetics of 18 lentiviral RT enzyme (Lenzi et al., 2014). To further these results and provide more evidence for the interplay between RT and Vpx, we could clone, overexpress, purify, and test RTs from additional Vpx non-coding strains such as HIV-1 CRFs as well as SIVcpz and SIVgor. In addition we could expand our study to look at the polymerases of other lentiviruses that are able to infect nondividing cells but unable to degrade SAMHD1 such as equine infectious anemia virus, caprine arthritis-encephalitis virus, and visna virus (Maury and Oaks, 2010; Pepin et al., 1998; Zink et al., 1990). We would expect that these RTs also possess high incorporation and catalytic efficiencies.

In Chapter 3 we used RTs from Vpx non-coding lentiviruses and two from Vpx coding lentiviruses to look at the incorporation efficiency at three different sites along the same template. We found that the significant difference in incorporation efficiency for HIV-1 RTs is due to increased rate of incorporation which involves two steps- the conformational change of the enzyme and chemistry of incorporation (Lenzi et al., 2015). Future experiments could elucidate whether it's the conformational change (k_{conf}) and/or the chemistry of incorporation (k_{chem}) that is responsible for the increased rate of incorporation (k_{pol}). Pulse-chase and elemental effect experiments performed with the KinTek Rapid Quench-Flow instrument would provide evidence for which of these two

steps- k_{conf} or k_{chem} - are affected. Previous research has shown that the incorporation rate of HIV-1 RT is not affected by using dATP-α-S as a substrate which suggests that the chemical step of polymerization (k_{chem}) is not rate limiting, at least for HXB2 RT (Radzio and Sluis-Cremer, 2005). Finally crystal structures of RTs from Vpx non-coding and coding lentiviruses in the apo form and ternary complex could give insight into structural rearrangements and key residues involved in dNTP incorporation.

In both Chapters we used titrations of dNTPs as a biochemical simulations of the dNTP environment found in dividing $CD4^+$ T cells and nondividing macrophages. However previous research in the Kim lab has shown that HIV-1 RT is able to incorporate ribonucleotide triphosphates (rNTPs) which are 100-1000 times more concentrated than dNTPs in dividing cells and 400-30,000 times more concentrated in macrophages (Kennedy et al., 2010). Under the heavily skewed dNTP/ rNTP ratio in macrophages, HIV-1 RT is able to misincorporate rNTPs and mismatch extend off of rNMPs after their incorporation. Future studies could use biochemical simulations that contain concentrations of both dNTPs and rNTPS in $CD4^+$ T cells and macrophages to test whether *in vitro* RTs from Vpx coding viruses can also misincorporate rNTPs and mismatch extend after their incorporation. Similarly, very little is known about the fidelity of RTs from Vpx coding lentiviruses and could be an area of future exploration.

The studies in these chapters were almost all biochemical experiments in order to determine disparities in kinetics between polymerases from Vpx coding and non-coding lentiviruses. We assume that an *in vitro* increase in catalytic and

incorporation efficiency for HIV-1 RTs in a low dNTP environment correlates with increased DNA synthesis and viral replication in macrophages. Although it was outside of the scope of these kinetic studies, future experiments could use lentiviruses expressing and not expressing Vpx to infect primary macrophages and $CD4^+$ T cells and look at differences in infectivity, viral titer and cell survival between the two classes of virus. Additionally lentiviral constructs could be made which exchange RTs from Vpx coding and non-coding lentiviruses to look at the dependence of HIV-2/ SIV RTs on Vpx to increase dNTP concentrations in order to replicate in macrophage but not $CD4^+$ T cells. Going further, animal models could be used to see if HIV-1 is more likely to infect and reside in nondividing cells of the brain (e.g. microglia, dendritic cells, astrocytes) leading to HAND than HIV-2/ SIVs due to their decrease in polymerase efficiency.

Finally these studies compared the efficiencies or RTs due to the presence or loss of Vpx. However we assume that all Vpx proteins are equally efficient at degrading SAMHD1 and increasing dNTP concentrations. Future studies could look at the kinetics of different lentiviral Vpx proteins and their ability to degrade their host SAMHD1 and increase dNTPs. It is possible that some of the RTs from Vpx coding lentiviruses with exceptionally low K_m values (Figure 2.2A; HIV-2 Ghana1, SIV Mne Cl8, SIV 155-4) have Vpx proteins that are less efficient at degrading SAMHD1 and increasing dNTPs. With poor functioning Vpx protein, these viruses may be unable to efficiently increase dNTP concentration forcing them to replicate in low dNTP environments found in macrophages.

4.4 Conclusion

This thesis investigated the kinetic differences among the RTs of human and simian immunodeficiency causing lentiviruses. HIV-2 and SIV strains encode an accessory protein, which induces the degradation of SAMHD1, increases the concentration of dNTPs, and allows for a permissive infection. Without Vpx, HIV-1 infected macrophages maintain a low dNTP environment, which acts as a selective pressure on RT to preserve a high steady-state affinity and incorporation rate. This thesis supports the theory that kinetic properties of viral polymerases are mechanistically tied to dNTP availability in target cells, which is affected by viral accessory proteins.

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