# STUDIES OF HIV-1 MUTAGENESIS DURING DRUG THERAPY AND THE MOLECULAR DETERMINANTS OF HIV-1 VARIATION

**DISSERTATION**

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## ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) is a member of the *lentivirus* genus of the *Retroviridae*. HIV-1 exists as a mixture of non-identical but closely related virions, known as “quasispecies”. Therefore, HIV-1 populations demonstrate high levels of genetic variation, which arise through mutation, recombination and selection. This dissertation extends studies of HIV-1 genetic variation in two areas: 1) the influence of antiretroviral drugs and drug-resistant reverse transcriptase (RT) variants on HIV-1 mutant frequencies, and 2) the role of the HIV-1 accessory protein Vpr and cellular DNA repair enzyme uracil-DNA glycosylase (UNG) interaction in HIV-1 mutagenesis,

In the first study, the influences of antiretroviral drugs and drug-resistant RT variants on HIV-1 mutant frequencies were tested. A new high-throughput assay system was developed in which the luciferase gene (*luc*) is used as a mutational target in order to more rapidly assess virus mutant frequencies. Using this system, I tested the following hypotheses: 1) All nucleoside RT inhibitors (NRTI’s) currently used in drug therapy increase virus mutant frequencies; 2) Non-nucleoside RT inhibitors (NNRTI’s) can increase virus mutant frequencies; 3) NNRTI-NNRTI drug combination can further increase virus mutant frequencies compared to individual drugs; 4) NRTI-NNRTI drug combination can increase

virus mutant frequencies higher than that observed in the presence of individual drugs; 5) High-level, drug-resistant RTs can influence virus mutant frequencies.

To better understand the mechanism(s) responsible for the influence of antiretroviral drugs on virus mutant frequencies, I tested the hypothesis that intracellular deoxynucleotide triphosphate (dNTP) levels are altered by antiretroviral drugs. Antiretroviral drugs were observed to alter intracellular dNTP concentrations, suggesting an association to increases in virus mutant frequencies. The HIV-1 mutant frequency was higher in quiescent PBMCs than in activated PBMCs, suggesting a connection between altered mutant frequencies and intracellular dNTP concentrations.

The second study analyzed Vpr-mediated UNG2 incorporation into virus particles. I tested the hypothesis that UNG2 incorporated into virus particles is catalytically active and may subsequently influence the reverse transcription process and viral replication. Several Vpr-UNG interaction-deficient mutants and Vpr-UNG fusion constructs were tested to demonstrate that 1) interaction between Vpr and UNG is necessary for the incorporation of UNG into virus particles and 2) Vpr-UNG fusion proteins do not eliminate UNG catalytic activity. To monitor UNG catalytic activity, a gel-based enzymatic activity assay was developed. I found that UNG activity could be recovered from virus particles only when the Vpr-UNG interaction was maintained. Moreover, the Vpr-UNG fusion proteins were efficiently incorporated into particles, indicating that these fusion proteins can be used to further study the role of UNG in HIV-1 replication.

Dedicated to my parents

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## PUBLICATIONS

1. **Chen, R.***, E. LeRouzic*, J. A. Kearney, S. Benichou, and L. M. Mansky. 2004. Vpr-mediated incorporation of UNG2 into HIV-1 particles is required to modulate the virus mutation rate and for replication in macrophages. J. Biol. Chem. **279**: 28419-25.
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3. Weiss, K. K.*, **R. Chen***, K. Lee, R. A. Bambara, L. M. Mansky, and B. Kim. 2004. A role for dNTP binding in HIV-1 mutagenesis. Biochemistry. **43**:4490-4500.
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5. **Chen, R.**, H. Wang, and L. M. Mansky. 2002. Roles of uracil-DNA glycosylase and dUTPase in virus replication. J. Gen. Virol. **83:**2339-2345.

## FIELDS OF STUDY

Major Field: Ohio State Biochemistry Graduate Program

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# CHAPTER 1

# INTRODUCTION

## 1.1 Significance of the dissertation topic

Retroviruses are RNA viruses that convert their RNA genome into double-stranded DNA by reverse transcription, which is subsequently integrated into the host chromosome to form the provirus. This process is catalyzed by the viral encoded enzyme reverse transcriptase (RT) (193). Therefore, the retroviral RT plays a pivotal role in the life cycle of the virus and is a major target for anti-HIV drug therapy.

High levels of genetic variation found in retrovirus populations arise through mutation, recombination and selection (30, 74, 130, 151, 152, 159). The low fidelity of HIV-1 RT and the abundance of recombination events during HIV-1 reverse transcription are responsible for the high mutation rate of HIV-1 RT and play an important role in generating diversity in HIV-1 populations. The most commonly detected mutations are base substitutions and frameshift mutations. It was observed that the *in vivo* mutation rate for HIV-1 was lower than the values predicated by cell-free systems (130). It has been proposed that the difference

between these measurements is due to several factors, including the association of viral or nonviral accessory proteins during reverse transcription, the influence of cellular mismatch repair mechanisms, and/or differences between the reverse transcriptase produced *in vivo* with that used *in vitro* (130). An HIV-1 accessory protein, viral protein R (Vpr), has been shown to influence HIV-1 mutant frequencies. HIV-1 Vpr is a 96 amino acid non-structural protein that is associated with virus particles and can accumulate at the nuclear envelope and in the nuclei of infected cells. Vpr was found to influence the mutation rate of HIV-1 (121). Moreover, the HIV-1 Vpr protein has been found to interact with several cellular proteins, including the DNA repair enzyme uracil-DNA glycosylase (UNG) (19). Vpr recruits the nuclear form of UNG (UNG2) into HIV-1 virus particles, and this recruitment is required for Vpr to modulate the *in vivo* mutation rate (129). Therefore, further analysis of the interaction between Vpr and UNG2 will provide additional evidence to support the hypothesis that the Vpr-dependent recruitment of UNG2 is critical for efficient HIV-1 replication. The focus of this dissertation is to analyze the correlation between UNG activities and Vpr-UNG2 interaction.

The continuous treatment of HIV-1 infection with combination drug therapy has significantly reduced morbidity and mortality (149, 157, 168). Combination antiretroviral therapy typically consists of at least two reverse transcriptase inhibitors as well as protease inhibitors. The risk for the emergence of drug-resistant HIV-1 increases when there is poor patient compliance to drug regimens, resulting in suboptimal therapy. Suboptimal drug therapy can lead to

drug resistance, which limits the clinical benefit of drug treatment and selects for new variant viruses with altered virulence and tropism (30, 100, 144). The highly error prone RT is thought to play a role in the generation of drug-resistant viruses. Drug-resistant viruses can reside in latently infected cells, which further complicates subsequent drug therapy during the life of the infected individual (55, 211). Antiretroviral drugs have been previously shown to influence the fidelity of retrovirus replication (87, 123, 126, 150). In addition, it has been found that drug-resistant RTs alone or in combination with antiretroviral drugs can influence the *in vivo* HIV-1 mutation rate, suggesting that when virus replication occurs in the presence of suboptimal concentrations of drug, drug-resistant virus is selected for and replication of drug-resistant virus in the presence of drug could further influence the virus mutation rate (126). To support this hypothesis, the work presented in this dissertation is focused on extending current knowledge of the influence of antiretroviral drugs and drug-resistant RT mutants on HIV-1 mutagenesis.

Data presented in Chapters 2 and 3 examine the influence of antiretroviral drugs and drug-resistant RT mutants on HIV-1 mutagenesis. Clinically used antiretroviral drugs were studied. A new high throughput assay system was developed to measure HIV-1 mutant frequencies. Several novel observations were reported: 1) non-nucleoside RT inhibitors (NNRTI's) could increase HIV-1 mutant frequencies; 2) an NNRTI-resistant mutant could influence HIV-1 mutant frequency; 3) the combination of NRTI and NNRTI in drug therapy could further increase HIV-1 mutagenesis; and 4) NNRTI-NNRTI drug combination led to

further increase in virus mutant frequency. A mechanism was studied to gain further insight into how antiretroviral drugs influence HIV-1 mutant frequencies.

Data presented in Chapter 4 characterize the interaction of HIV-1 accessory protein Vpr and cellular DNA repair enzyme UNG2. Vpr and UNG2 mutants, which were identified as Vpr-UNG binding-deficient mutants, were studied. The interaction of Vpr and UNG2 was required for the incorporation of UNG into virus particles and for the presence of UNG activity in virus particles. These data provide further support for the Vpr-UNG interaction being important in the HIV-1 replication cycle.

The first section of this introduction will provide general information on the retroviral genomic structure, the replication cycle of retroviruses, and HIV-1 RT. The second section will discuss HIV-1 genetic variability. The third section will focus on antiretroviral drug therapy and the emergence of drug-resistant RT mutants. The influence of antiretroviral drugs and drug-resistant RT mutants on HIV-1 mutagenesis will also be discussed. The final portion will detail current knowledge of how the HIV-1 accessory protein Vpr influences HIV-1 mutagenesis through interaction with cellular proteins.

## 1.2 Retroviral genomic structure and replication cycle

Retroviruses comprise a diverse family of animal viruses with RNA as their genetic material in virus particles and DNA as their genetic material in cells. Retroviruses were originally identified because of the oncogenic characteristics of various family members. Some of the best-studied retroviruses include Rous

sarcoma virus (RSV), mouse mammary tumor virus and murine leukemia viruses (MMTV and MLV). Other disease-causing retroviruses include human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV). Understanding the mechanisms by which retroviruses infect their hosts and manipulate cellular processes has had broad impact on the study of cellular growth control and carcinogenesis, on molecular genetics, and on biotechnology (203).

### *Retroviral genomic structure*

Retroviruses are enveloped RNA viruses, which are divided into two categories—simple and complex—based on their genomic structure (Figure 1.1). The retroviral virion contains two copies of linear, single-stranded RNA that is 7–12 kb in size, nonsegmented, and is of positive polarity (203). Like cellular mRNAs, viral RNA is modified with capping at the 5' end and polyadenylation at the 3' end. All retroviruses contain three major coding regions: *gag*, *pol* and *env* (Figure 1.1). The *gag* gene directs the synthesis of internal structural proteins of the virus that form the matrix (MA), the capsid (CA), and the nucleoprotein structures (NC); the *pol* gene encodes the reverse transcriptase (RT) and integrase (IN) enzymes; and the *env* gene encodes the surface (SU) glycoprotein and transmembrane (TM) proteins of the viral envelope, which form a complex that interacts specifically with cellular receptors. In addition to these three genes, a smaller coding domain present in all retroviruses is *pro*, which encodes the virion protease (PR). Protease is necessary for the cleavage of immature Gag-

Pol polyprotein to mature form. While simple retroviruses contain these elementary genes, complex retroviruses encode additional regulatory and accessory genes that are required for viral replication and involved in virus-host interactions (204).

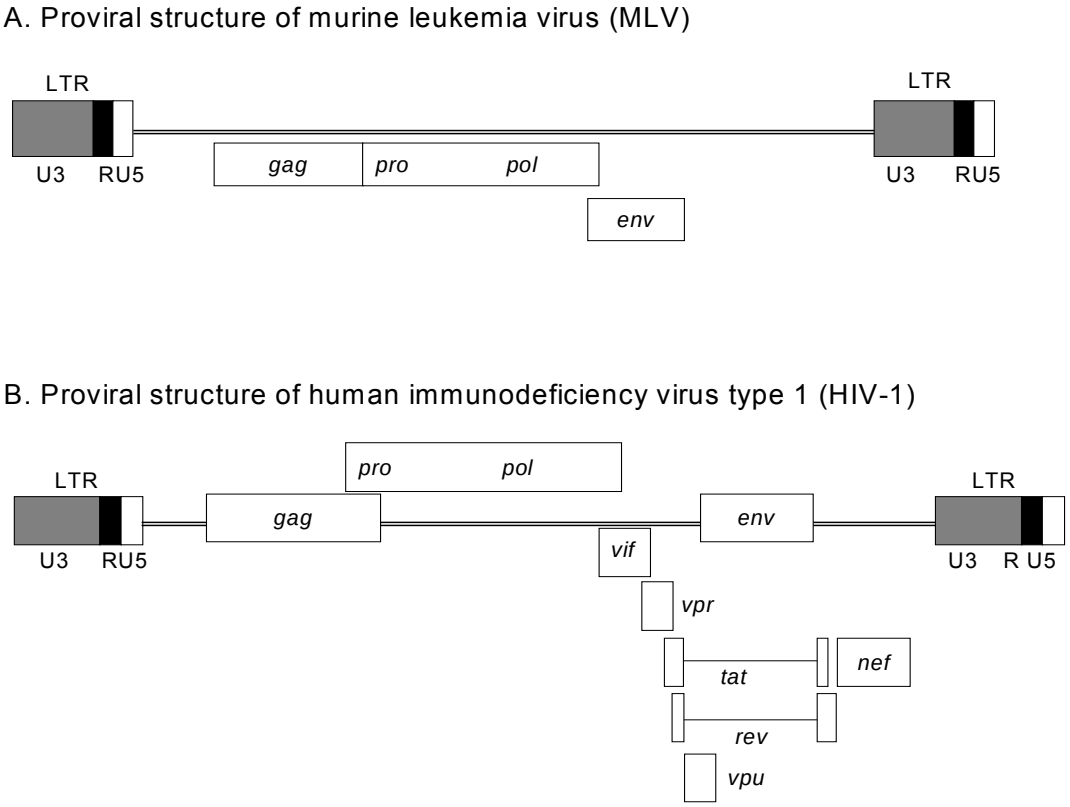



**Figure 1.1.** Genomic structures of simple and complex retrovirus. (A) A representative of simple retrovirus provirus. MLV contains three major coding regions, *gag, pol* and *env*. (B) A representative of complex retrovirus provirus. Besides the major coding regions, HIV-1 also contains two regulatory genes, *rev* and *tat*, and four accessory genes, *vif, vpr, vpu* and *nef*. LTR: long terminal repeats, which include U3, R, and U5. Adapted from (204).

### *Retroviral replication cycle*

The hallmark of a retrovirus is its replicative strategy, which includes the reverse transcription of virion RNA into linear, double-stranded DNA and its subsequent integration into the host cell genome as a provirus (Figure 1.2). Retrovirus replication is initiated by the attachment of Env protein to a specific receptor(s) on the target cell membrane. Subsequently, the viral envelope fuses with the plasma membrane, and the viral core enters cytoplasm. In the cytoplasm, the viral RNA genome is reverse transcribed by RT into a double-stranded DNA via a series of steps (discussed in more detail below). The double-stranded DNA is transported to the nucleus as part of a pre-integration complex (PIC) and integrated into the host chromosome by viral integrase. Integrated viral DNA is called provirus. After integration, host RNA polymerase II transcribes the provirus to generate full-length viral transcripts. A portion of the full-length transcripts undergoes splicing in the nucleus by the cellular splicing machinery. Then, spliced and unspliced RNA are exported to the cytoplasm. The cytoplasmic unspliced RNA serves either as a template for the synthesis of Gag and Gag-Pol polyprotein precursor or becomes encapsidated as progeny viral genomes. The spliced RNA acts as template for the synthesis of Env protein, and in complex viruses, the regulatory and accessory proteins. Following translation of Env protein in endoplasmic reticulum (ER), Env proteins are glycosylated at the Golgi apparatus, cleaved to form the SU and TM molecules and transported to the plasma membrane. The assembly of progeny virions takes place at the plasma membrane. To produce the infectious virion, two copies of unspliced

RNA are packaged into the newly assembled particles. The particle then buds from the cell and undergoes proteolytic maturation in which the viral protease cleaves the immature Gag-Pol polyprotein to produce a mature, infectious virion.

### *Reverse transcription*

Reverse transcription begins when the viral particle enters the cytoplasm of a target cell and proceeds in a series of steps that utilize several *cis*-acting elements in the viral genome. The viral RNA enters the cytoplasm as part of a nucleoprotein complex. Reverse transcription takes place in this nucleoprotein complex that includes RT, the RNA genome and other viral proteins. This process is dependent on the two distinct enzymatic activities of RT—polymerase and RNase H activities. The steps involved in the conversion of the RNA genome to DNA are described briefly below, and are depicted in Figure 1.3 (193).

1. Reverse transcription is initiated by using the 3’ end of a partially unfolded primer tRNA that is annealed to the primer-binding site (PBS) in the genomic RNA. Primer tRNA specificity is highly conserved within virus genera. Minus-strand DNA synthesis proceeds to the 5’ of the viral RNA genome, generating a RNA/DNA hybrid.
2. The RNA portion of the RNA/DNA is digested by the RNase H activity of RT, resulting a single-strand DNA product, known as minus-strand strong-stop DNA (-sssDNA).

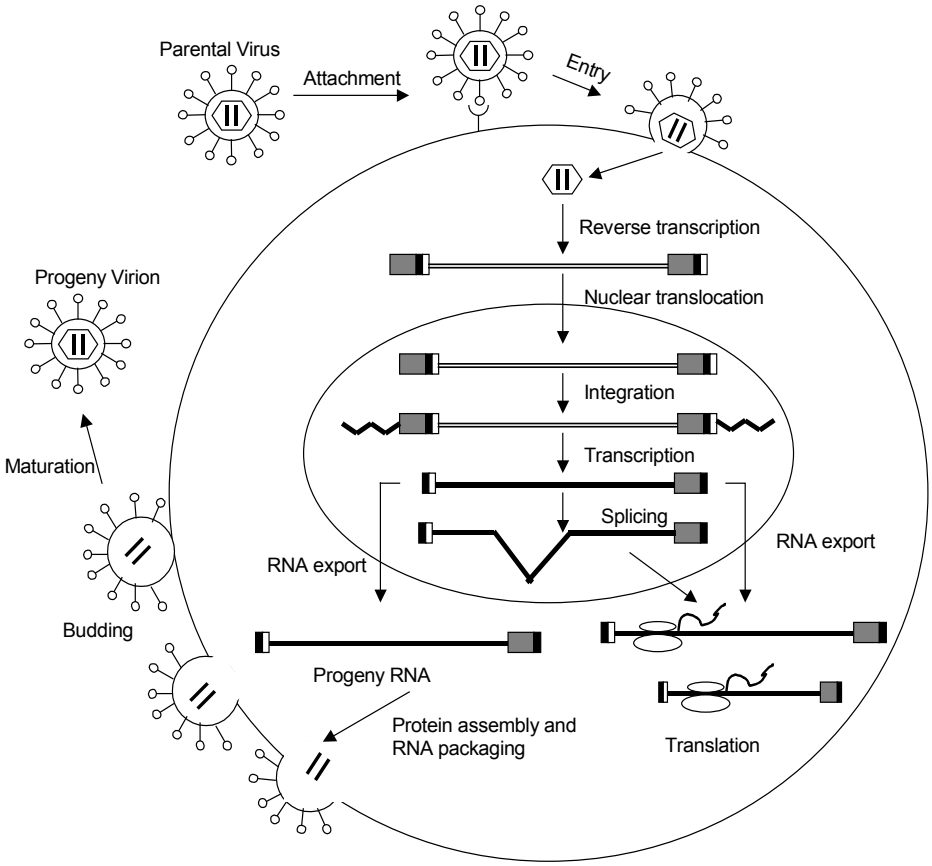



**Figure 1.2.** Schematic view of retroviral replication cycle. The major steps in a typical replication cycle are indicated. The parental virus attaches to a receptor on the surface of a susceptible cell leading to fusion, and the viral core enters cell. A double-stranded DNA copy of the RNA genome is generated by the reverse transcription process and is transported into the nucleus. The provirus is integrated into chromosomal DNA and is transcribed by host transcription machinery. Spliced and unspliced genome-length RNAs undergo nucleocytoplasmic export and the viral RNAs are translated by host cell ribosomes in cytoplasm. Virion proteins traffic to cell membrane and two unspliced RNAs are packaged into core particle. The progeny virus is released by a process of budding and subsequent maturation into infectious virus. Adapted from (203).

3. Continued minus-strand DNA synthesis requires the first strand transfer of –sssDNA to the 3’ end of viral genome. This transfer is mediated by identical sequences known as repeated (R) sequences, which are present at the 5’ and 3’ ends of the viral RNA genome. The newly synthesized region in -sssDNA complementary to the R sequence at the 5’ end of viral RNA genome anneals to the R sequence at the 3’ end of the viral RNA genome.
4. Following the first strand transfer, RNA-dependent DNA polymerization of the minus-strand DNA resumes using –sssDNA as a primer, accompanied by RNase H digestion of the RNA template strand.
5. Plus-strand DNA synthesis is initiated from an RNase H resistant polypurine tract (PPT) located within the viral genome. Lentiviruses differ from other retroviruses in that they also utilize a central PPT (cPPT) as a primer for plus strand DNA synthesis (57). Plus strand DNA synthesis proceeds to the end of the minus strand DNA template and stops after copying a part of the primer tRNA, generating a DNA called plus-strand strong-stop DNA (+sssDNA).
6. After RNase H removes the primer tRNA, a second strand transfer occurs allowing the PBS in +sssDNA to anneal to its complementary sequence within –sssDNA.
7. Plus and minus-strand DNA syntheses are then completed, resulting in a blunt-ended, double-stranded linear DNA with long terminal repeat (LTR) composed of U3, R and U5 at each end.

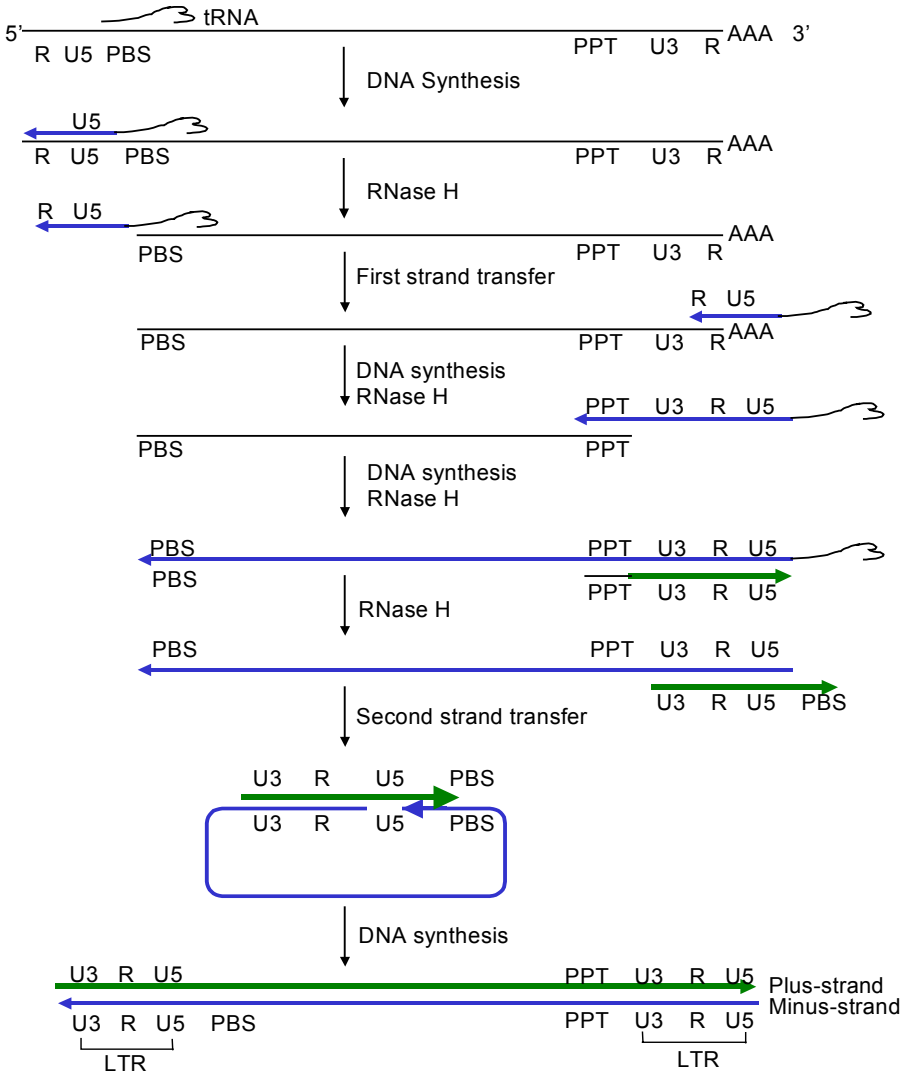



**Figure 1.3.** Process of reverse transcription of the retroviral genome. (*Black line*) RNA; (*blue color*) minus-strand DNAs; (*green color*) plus-strand DNA. See text for a description of this process. Adapted from (193).

## 1.3 Reverse transcriptase

An important stage in the replication of HIV is the conversion of its plus sense, single-stranded genomic RNA into double-stranded DNA (dsDNA), which is subsequently integrated into host cell chromosomes. The virally encoded RT, a multifunctional enzyme that has RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and RNase H activities, catalyzes this process. As a consequence, RT is an important target in the treatment of HIV-1 drug therapy not only because it is essential for HIV replication, but also because it is not required for normal host cell metabolism.

HIV-1 RT is matured by the viral encoded protease (PR) from a 165-kD Gag-Pol precursor polyprotein produced through ribosomal frameshifting. Virion-associated RT is a stable heterodimer of two subunits with relative molecular weight of 66 kD and 55 kD (p66 and p51, respectively). Amino acid sequence analysis demonstrates that these two subunits share a common amino terminus, suggesting that the smaller subunit is derived by processing of the 66-kD subunit (104). Genetic analysis of RT subunits has shown that the N-terminal 440 amino acids of p66 constitute the polymerase domain and the C-terminal 120 amino acids comprise the RNase H domain. The p51 subunit corresponds to the polymerase domain of the p66 subunit. Additionally, subunit-selective mutagenesis has demonstrated that both the DNA polymerase activity and RNase H activity reside within the p66 subunit. The p51 subunit is devoid of RNase H activity and does not contribute to the polymerase activity. Moreover,

mutagenesis studies of RT subunits suggest that the DNA polymerase domain and the RNase H domain in p66 interact with each other (104, 158).

The polymerase activity of HIV-1 RT shares several features with the cellular DNA polymerases in both structure and function. It incorporates deoxyribonucleoside triphosphates (dNTPs) by elongation of the primer 3'-OH terminus, forming 3'-5' phosphodiester bonds with the release of pyrophosphate. However, RT displays a relatively poor processivity in *in vitro* studies (11, 12) and does not have a 3'$\rightarrow$ 5' exonuclease activity capable of excising mispaired nucleotides. As a result, RT is more error-prone than cellular DNA polymerases (10). The RNase H activity of HIV-1 RT catalyzes the specific cleavage of the RNA of a RNA/DNA hybrid. RNase H has at least three functions in the reverse transcription process. First, it degrades the template RNA in the nascent RNA/minus-strand DNA hybrid, and facilitates the subsequent strand transfer. Second, RNase H generates plus-strand primers by cleaving the hybrid RNA at a specific site at the end of PPT. Third, it removes the minus-strand tRNA and the plus-strand primer, enabling synthesis of full length double-stranded DNA (26, 193). Although the RNase H activity can act independently of the polymerase activity, there is generally an interplay between the two active sites (158).

The first reported structure of HIV-1 RT was complexed with the non-nucleoside RT inhibitor, nevirapine (96, 166, 186). The structure of the polymerase domain of HIV-1 RT resembles a right hand. The polymerase domains of p66 and p51 each contain four subdomains, named fingers, palm, thumb and connection (Figure 1.4 and 1.5) (96). Although the structures of the

subdomains within p66 and p51 are similar, the relative arrangement of the four subdomains within the two subunits is different (96). The p66 palm subdomain contains the polymerase active site that is defined by a triad of aspartic acid residues at positions 110, 185 and 186. The Asp-185 and Asp-186 are in the YXDD motif, which is highly conserved among all the retroviruses, and the two aspartaic acid residues in this motif are found in all the RNA- and DNA-dependent polymerases (206). These amino acids bind the divalent cations that are required for catalysis. The regions surrounding the polymerase active site correspond to motifs that are conserved in polymerases. The polymerase active site of p66 has considerable structure similarity to that of the Klenow fragment of *E. coli* DNA polymerase I, T7 RNA polymerase, *Taq* polymerase, and a fragment of Moloney MLV RT (79). The finger, palm, and thumb of p66 appear to act together as a clamp that positions the template-primer relative to the polymerase active site. The 3'-OH of the primer terminus is close to the catalytic triad and is appropriately positioned for nucleophilic attack on the $\alpha$-phosphate of an incoming nucleoside triphosphate. The connection subdomains of p66 and p51, and the p51 thumb make up much of the "floor" of the template-primer binding cleft. Incoming dNTPs bind at a site that is located in the palm subdomain of the p66 subunit, adjacent to the 3' terminus of the primer strand. The catalytic triad of aspartic acids (Asp 110, Asp 185, and Asp 186) are located at this site (Figure 1.5) (192). Therefore, the interactions between RT and its substrates (i.e. the template-primer complex and dNTP) are important determinants of enzyme fidelity. The distance between the DNA polymerase and RNase H active sites is

17 or 18 nucleotides, which is consistent with the observed sites of cleavage of an RNA template, 15 to 18 bases from 3' end of the primer strand. Thus, the RNA/DNA hybrid product of synthesis lies between these two active sites with the 3' end of the template strand contacting the divalent metals of RNase H and the 3' terminus of the primer strand near the DD sequence. This suggests that the DNA polymerase and RNase H active sites can be simultaneously engaged on the primer-template (80, 96). Relative to its position in p66, the thumb of p51 is moved away from the palm and makes significant contacts with the RNase H domain. The connection subdomain of p51 is folded up onto the palm of p51 between the fingers and the thumb, so there is no template cleft in p51. The three aspartic acids that form the polymerase active site in p66 are buried in p51. Therefore, only one functional polymerase active site per p66/p51 heterodimer, which is consistent with genetic studies that only the large subunit contributes directly to the polymerase activity.

The RNase H domain of RT is similar to the RNase H present in both bacterial and eukaryotic cells. Comparison of the RNase H domain of HIV-1 RT with the *E. coli* enzyme suggests a catalytic role of four acidic residues, Asp-443, Glu-478, Asp-498, and Asp-549 of the retroviral enzyme. These residues bind to two divalent cations, which is similar to the two-metal-ion mechanism that has been proposed for polymerase. All four residues are conserved in all retroviral and bacterial RNase H sequences and three of four are essential for enzymatic activity. A short polypeptide loop (538-542) that contains the invariant His-539 interacts with a portion of the thumb of p51. The His-539-containing loop lies

near the two divalent metal ions at the catalytic center and can be involved in the RNase H activity (97).

## 1.4 Human immunodeficiency virus type 1 disease, variability and mutagenesis

### *HIV-1 distribution, grouping and tropism*

HIV-1 is a member of the *lentivirus* genus of the *Retroviridae*. Members of the lentivirus genus are responsible for a variety of neurological and immunological diseases, but are not directly implicated in any malignancies (31). The members of this family include human and simian immunodeficiency virus (HIV-1, HIV-2 and SIV), the more distantly related feline and bovine immunodeficiency viruses (FIV and BIV), equine infectious anemia virus (EIAV), and caprine arthritis-encephalits virus (CAEV). All lentiviruses are complex viruses.

HIV-1 is the major causative agent of the acquired immunodeficiency syndrome (AIDS), which was first identified in the early 1980's. Since its initial discovery, AIDS has become a major worldwide epidemic. AIDS is characterized by severe immmunosuppression, opportunistic infections and neoplasm caused by the depletion of the CD4+ T-lymphocyte population (54). According to the U.S. Centers for Disease Control and Prevention, as of December 2003, 40 million

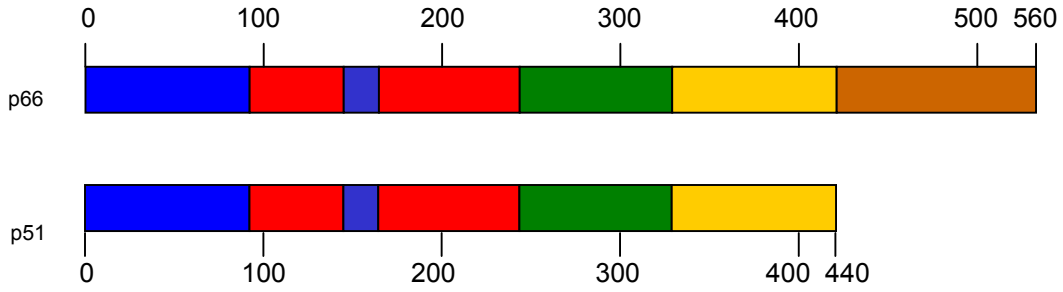



**Figure 1.4.** Cartoon overview of the subdomains of HIV-1 RT. Both subunits are shown. Fingers (blue), palm (red), thumb (green), and connection (yellow). The RNase H domain is part of the p66 subunit, is shown in light brown. Adapted From (193).

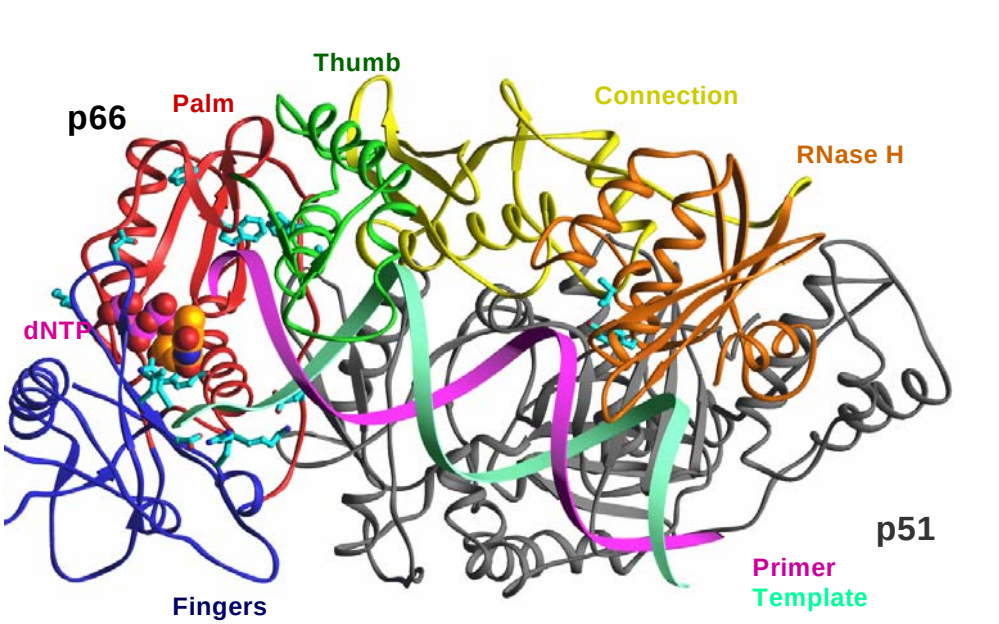



**Figure 1.5.** Ribbon structure of HIV-1 RT bound to DNA and dNTP substrates. The subdomains of p66 subunit are color-coded: Fingers (blue), palm (red), thumb (green), connection (yellow), and RNase H (brown). The p51 subunit is shown in gray. Figure courtesy of Kalyan Das and Eddy Arnold (Rutgers University).

people are estimated to be living with HIV/AIDS worldwide. During 2003, an estimated 5 million people acquired HIV, and AIDS caused the deaths of an estimated 3 million people (1). These staggering numbers indicate that an effective and affordable treatment needs to be developed if long-term control of the AIDS epidemic is to be achieved.

Of the two subtypes of HIV, HIV-1 is predominant and found throughout the world, whereas HIV-2 has been isolated primarily in West African countries such as Guinea Bissau, Ivory Coast, and Senegal, with some cases also identified in the Americas and western Europe (53). Considerable effort has been spent collecting and comparing nucleotide sequences of HIV isolates from around the world. Three divergent groups of HIV-1—group M (for “main”), group O (for “outlier”), and group N (for Non-M, Non-O or “new” group)—have been identified. Viruses of group M are responsible for the majority of infections worldwide, while group O is currently found in Cameroon, Gabon, and France. Group N has just one known isolate from Cameroon (53, 139). Group M can be divided into at least nine distinct subtypes or clades (A to D, F to H, J and K), as well as several circulating recombinant forms (115, 171). The different clades of HIV-1 are not distributed evenly throughout the world, i.e. clade B is found in North America and Europe and clade E predominates in northern Thailand. Like HIV-1, HIV-2 isolates exhibit genetic variability similar to what has been observed among HIV-1 isolates. Isolation of the virus and sequencing of the genome revealed that while the genetic organization of HIV-2 was highly similar to that of

HIV-1, the two viruses exhibited distant sequence similarity at the nucleotide level (139).

HIV-1 exhibits strong tropism for $CD4^+$ cells, including $CD4^+$ T lymphocytes and monocyte/macrophage-lineage cells. All HIV-1 infected cells use CD4 as the primary cell surface receptor (21). Although human CD4 is the major HIV-1 receptor, it is not sufficient for HIV-1 Env-mediated membrane fusion and virus entry. Members of the G-protein-coupled receptor superfamily of seven-transmembrane domain proteins have been identified as coreceptors for HIV-1 entry (16, 40, 41). The two most important coreceptors are CXCR4 and CCR5. Viruses that replicate in T-cell lines, but not macrophages or monocytes, are referred to as T-tropic, whereas viruses with the complementary specificity are referred to as M-tropic. The tropism of the virus has been shown to be a function of the coreceptor used: M-tropic viruses can use only CCR5 for entry; T-tropic viruses use CXCR4. Both T- and M-tropic isolates replicate in activated peripheral blood mononuclear cells (PBMC) (57).

### *HIV-1 variability and mutagenesis*

HIV-1 populations demonstrate high levels of variation, which is the consequence of the extensive replication of HIV-1 in infected individuals and the high mutation rate during reverse transcription (194). Therefore, HIV-1 exists as a mixture of nonidentical but closely related virions, known as “quasispecies” (45). This genetic diversity within the HIV-1 population manifests itself in the evolution of immune escape mutants, drug resistant mutants, and variants that

have the ability to alter their tropism. The genetic variation in HIV-1 populations depends on the mutation and recombination rates per replication cycle, the replication rate (replication cycle/time), and the selective forces that act on the population (31). Genetic errors can be introduced into the genome by either viral or cellular enzymes at any stage in the viral replication cycle. Virus encoded error-prone RT can make mistakes in copying viral RNA into DNA, including base-pair substitution, frameshift mutation, insertion, deletion, and recombination, which suggests a role for RT in generating retroviral diversity. Cellular enzymes that may play a role in introducing mutations into the viral genome include host RNA polymerase II, which does not have a proofreading function and is involved in the transcription of viral RNA, and host DNA polymerase that is involved in provirus replication. Although errors in replication provide the basis for diversity, the diversity seen in patients is the result of large numbers of virions present in the patient and the high rate of replication (30).

HIV-1 RT lacks a proofreading activity and exhibits poor fidelity when compared with host DNA polymerase in an *in vitro* system (10) (for review see (14)). In *in vitro* systems, synthetic polynucleotide, natural template (genetic assay), and kinetic assay were used to monitor the fidelity of RTs. It has been shown that the error rate depends on sequence context, “hot spots” where errors occur frequently, and templates. The overall fidelity of reverse transcription by HIV-1 RT is several fold higher with RNA-directed synthesis than with DNA-directed (2, 11, 12, 23). The base substitution error rate of HIV-1 RT is $1.6 \times 10^{-4}$ errors per base and the frameshift mutation rate is about $2.3 \times 10^{-4}$ errors per

base in the forward mutation assay (11). This rate would translate into an error rate in viruses of about one per genome in a reverse transcription cycle. Studies performed to measure the *in vivo* fidelity of HIV-1 RT indicate that the *in vivo* mutation rate of HIV-1 is lower than the values predicted by the *in vitro* system (130). The *in vivo* mutation rate for HIV-1 was determined to be $4 \times 10^{-5}$ mutations per target base pair per replication cycle, which correlated to about one mutation in every 3 new genomes produced. The most commonly detected mutations were base substitution mutations (G-to-A and C-to-T transition mutations) and frameshift mutations (130). The difference between the *in vivo* and *in vitro* mutation rate has been proposed to be due to several factors, including the association of viral or nonviral accessory proteins during reverse transcription, the influence of cellular mismatch repair mechanisms, and/or differences between the reverse transcriptase produced *in vivo* with that assayed *in vitro* (130). Moreover, a high frequency of mismatch extension during reverse transcription in a cell-free systems or in a single round of retrovirus replication is associated with various RTs, including HIV-1, SNV, avian myeblastosis virus (AMV), which suggests that the high HIV-1 misincorporation rate is not due to a higher frequency of incorporating incorrect nucleotides into the growing strand but rather to a higher frequency of extending these incorrect nucleotides after they are incorporated (155, 161, 212). Furthermore, it has been proposed that frameshift mutations and deletions in the SNV genome are due to the low processivity of RT. The low processivity is necessary for RT that participates in the strand transfer steps essential for viral replication (151, 152). A similar

suggestion was made by Bebenek et. al. on the basis of studies with purified HIV-1 RT (11).

Recombination is another remarkable feature associated with RT that can lead to the increased genetic variability. All retrovirus systems tested, including HIV-1, undergo recombination at a high rate (31, 74, 75, 194, 199). Frequent recombination allows distribution of mutations in the population, increases variation with in the population, and removes lethal mutations. Using a single round of replication to study recombination of SNV, it was shown that recombination occurs only following infection with heterozygous virions. This suggests that recombination (called homologous recombination) occurs between the two packaged RNAs during reverse transcription and that progeny may contain genetic information from both copies of RNA in the virion (75). Thus, It has been proposed that this high rate of recombination is a direct consequence of the requirement for transfer of the nascent strand at the reverse transcription growing point during retrovirus DNA synthesis (11, 194). Moreover, studies showed that recombination between nonhomologous sequences in the retroviral genome results in rearrangements and deletions (151). At a low frequency, recombination also occurs between viral sequences and nonhomologous host sequences, resulting in incorporation of host sequences into the viral genome (called transduction) (74). This process has given rise to naturally occurring highly oncogenic retroviruses, which contain an insertion of cellular protooncogene sequences (194). Therefore, as discussed above, if the tendency

of RT to switch templates is paired with its ability to extend mismatches, genetic recombination may be a significant source of replication errors.

Hypermutation, an accumulation of monotonously substituted bases within the viral genomes, which is thought to arise through the action of error-prone RT during reverse transcription, can also contribute to the mutation spectrum (18, 152). G-to-A hypermutated genomes have been described for retroviruses including HIV-1 (152, 198, 199). It has been shown that G-to-A transitions occur in both the GpG and GpA dinucleotide context. It has been proposed that transitions in GpG preferentially occur via base mispairing at the end of runs of G residues, whereas transitions within GpA may result from dislocation mutagenesis (11, 152, 198-200). However, some studies also showed that G-to-A hypermutations could not be attributed solely to HIV-1 RT (82). There are some studies that demonstrated that G-to-A hypermutations arise during reverse transcription as a result of monotonous substitution of dCTP by dTTP due to localized depletion of intracellular dCTP (133, 134, 200, 201). Moreover, aberrant RNA editing has been shown to play a role in the modulation of HIV-1 mutagenesis (22, 94). Recently, a cellular protein, APOBEC3G (also known as CEM15), was identified as a factor implicated in causing hypermutation. APOBEC3G is a member of the family of RNA editing enzymes that deaminate cytosines to uracil in DNA or mRNA (81). It has been reported that Δ*vif*-viruses produced in cells expressing APOBEC3G contain G-to-A hypermutations in newly synthesized plus-strand viral DNA, which suggests that APOBEC3G causes deamination of cytosines to uracils in minus-strand DNA during reverse

transcription. In addition, Vif expression in virus producer cells prevented the accumulation of G-to-A mutations (69, 106, 117, 215).

There is evidence suggesting that other proteins involved in reverse transcription may also influence retroviral genetic variation. Included are nucleocapsid (NC), deoxyuridine triphosphate nucleotidohydrolase (dUTPase), and the HIV-1 accessory protein Vpr. NC stimulates the annealing of $tRNA^{lys}$ primer and facilitates the annealing of complementary strands during strand transfer, and subsequently influences both the rate and efficiency of template switching (193). It has been shown that the HIV-1 accessory protein Vpr can influence mutation rate. Replication of *vpr*$^{-}$ HIV-1 led to a four-fold increase in the HIV-1 in vivo mutation rate, resulting primarily in an increase of G-to-A and C-to-T transition mutations (121) (discussed in more detail in section 1.6). dUTPase regulates the level of dUTP in cells and therefore influences the potential misincorporation of uracil into viral DNA. dUTPase is encoded and packaged into virions by non-primate lentiviruses, such as FIV, EIAV and CAEV. The replication of dUTPase-minus mutants is severely affected in non-dividing cells (i.e. primary macrophages) and the viral loads can be decreased 10- to 100-fold in comparison with wild-type virus. The frequency of G-to-A transition mutations in viral DNA increases during replication of dUTPase-minus CAEV and FIV and eventually leads to replication-defective proviruses. The decrease in virus production and replication is correlated with the misincorporation of uracil in viral DNA, which results from the increased dUTP pool (29). This suggests that

dUTPase increases the fidelity of retroviral replication by maintaining a low ratio of dUTP to dTTP in the cell.

Due to the notable fidelity of cellular DNA replication ($10^{-9}$ to $10^{-11}$ substitution per base pair) (42), it is unlikely that mutations occurring during cellular replication of the provirus contribute significantly to the high degree of HIV-1 diversity. The fidelity of RNA polymerase II is unknown. However, it has been demonstrated that the RNA polymerase from *E. coli* as well as T3 or T7 bacteriophage are highly accurate (23, 78, 83). Recently, the analysis of HIV-1 LTR mutations compared relative contribution of HIV-1 RT and cellular RNA Pol II. This study provides direct evidence that HIV-1 RT contributes significantly to HIV-1 mutagenesis and is likely to be the primary engine for HIV-1 mutagenesis (148).

## 1.5 Antiretroviral drug therapy, drug resistance, and increased HIV-1 mutagenesis

### *Antiretroviral drug therapy*

Since the discovery of zidovudine (AZT) as an effective antiretroviral agent against HIV-1, drug therapy has been used widely in the treatment of AIDS. All drugs that have been approved by FDA for clinical use in the treatment of HIV infections fall into one of the following three categories: 1) nucleoside reverse transcriptase inhibitors (NRTI's); 2) non-nucleoside reverse transcriptase inhibitors (NNRTI's); and 3) protease inhibitors (PI's). In addition to reverse

transcriptase and protease reactions, various other events in the HIV-1 replication cycle can be considered as potential targets for pharmacological intervention, including viral adsorption, viral entry, virus-cell fusion, viral assembly and disassembly, proviral DNA integration, and virus-specific transcriptional regulation (38, 49). In this chapter, I will primarily address RT inhibitors and protease inhibitors.

The HIV-1 RT is essential for viral replication. Therefore, it has become the most extensively studied viral target for pharmacological intervention. Two major classes of RT inhibitors have been developed and used in the clinic. The first class of RT inhibitors is composed of various nucleoside/nucleotide analogs (NRTI's/NtRTI's). A common characteristic of the NRTI's/NtRTI's is the lack of 3'-OH at the sugar moiety of the molecule. These compounds are metabolically activated within cells by phosphorylation to the corresponding 5'-triphosphate derivatives (for NRTI's) or 5'-diphosphate derivatives (for NtRTI's), which act as competitive inhibitors with respect to the dNTP substrates by directly interacting with the dNTP substrate binding site of HIV-1 RT. When NRTI's or NtRTI's are incorporated by RT in the growing template/primer, the lack of 3'-OH prevents further elongation of the primer with other dNTPs, resulting in chain termination and subsequent premature termination of the growing viral DNA (49, 102). This class of RT inhibitors currently licensed for treatment of HIV-1 includes the nucleoside analogs zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (D4T), lamivudine (3TC), abacavir (ABC), and nucleotide analog tenofovir (TDF) (66). The second major class of RT inhibitors is nonnucleoside

analogs, which is composed of compounds that are structurally diverse. These inhibitors are non-competitive with respect to both dNTP substrates and template/primer and all are specific for HIV-1 RT. They have little or no activity on other lentiviruses, including HIV-2 and SIV. Unlike the nucleoside analogs, the nonnucleoside inhibitors do not need to be metabolized in order to inhibit HIV-1 RT. Although they are structurally diverse compounds, they occupy the same binding site on the RT (49, 102). Crystallographic analyses of HIV-1 RT complexed with various NNRTI's demonstrated that these inhibitors bind allosterically to a hydrophobic pocket that is proximal to the polymerase active site. This NNRTI-specific binding pocket does not exist in unliganded RT but is created only when the inhibitor-RT complex is formed (51, 96, 166, 186). NNRTI's are not incorporated into viral DNA. Instead, they move the aspartic acid triad of the active site from its optimal position upon binding to HIV-1 RT, resulting in a distortion of the geometry of the substrate binding site and a decrease in the catalytic activity of the enzyme (187). Currently approved NNRTI's for clinic use are nevirapine (NPV), delavirdine (DLV), and efavirenz (EFV) (66).

In the replication cycle of HIV-1, immediately following budding of the retroviral particle at the host cell plasma membrane, the viral protease cleaves the Gag and Gag-Pol polyproteins to produce the mature viral core proteins and virus-specific, core-associated enzymes. These cleavages are required for the formation of infectious particles. HIV-1 protease is classified as an aspartic protease because of the conservation of two aspartic acid residues (Asp25 and

125) and the critical role of those residues in its catalytic mechanism. The potential of the HIV-1 PR as a therapeutic target was established by mutagenesis of these two aspartic acid residues in an infectious provirus. The mutant provirus directed the production of noninfectious immature viral particles that contained unprocessed Gag and Gag-Pol polyproteins. HIV-1 protease inhibitors act as competitive inhibitors of peptide substrate. The binding of protease inhibitors to PR active site prevents the cleavage of the Gag and Gag-Pol precursor polyproteins to the structural proteins and functional proteins, thus arresting maturation, and thereby, blocking infectivity of the nascent virions. Six compounds, including saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), and lopinavir (LPV), have been approved by FDA for the treatment of HIV-1 infection (38, 49, 66, 93).

The goal of antiretroviral therapy is to suppress HIV-1 replication to the greatest extent possible, that is, below the detectable limits of the most sensitive assays available. The emergence of drug-resistant virus is a major cause of failure of monotherapy, and results from genetic variation in the viral genome under selective pressure of a particular drug. As discussed in Section 1.4, because HIV-1 has a high replication rate, it has potential for extensive genomic variation. In addition, the HIV-1 RT does not have a proofreading function, which causes the reverse transcription process to be error prone. It is estimated that a single-point mutation may occur between $10^4$ and $10^5$ times per day (30). There is no currently available drug that is an effective inhibitor of HIV-1 replication to which the virus does not rapidly develop resistance when it is used in

monotherapy. Therefore, effective and durable suppression of viral replication needs the use of drug combinations. By doing this, the virus will not be allowed to replicate to acquire the new mutations that would eventually compromise therapy, which is one of the benefits that are observed when combination therapy is employed in HIV-1 treatment. Genetic variants of HIV-1 that confer resistance to individual antiviral drug may either exist in infected people prior to therapy so that exposure to a particular drug may select for these preexisting mutants or under selective drug pressure, HIV-1 replicates and acquires mutations de novo, becoming drug-resistant mutants that undergo rapid expansion. Mutations will continue to accumulate in the genome, unless the replication rate can be slowed substantially. Therefore, drug combinations that substantially reduce virus replication rates will decrease the selection pressure for resistant HIV-1 variants. The HIV-1 variants with resistance to one drug can be suppressed by the remaining drugs in the combination therapy (178, 196). In addition to prevent the emergence of drug-resistant virus, drug combinations can be additive or synergistic so that block HIV-1 replication more extensively than individual drugs. Some *in vitro* studies have shown that NRTI's can be synergistic with NRTI's (50, 142), NNRTI's (9, 167), and PI's (84). The other benefits of combination therapy include reduced toxicity and targeting different steps in viral replication cycle. Combination therapy that targets different sites may also prevent the breakthrough of viruses that have become resistant to particular drugs (196). Currently, potent antiretroviral therapy (ART), which includes NRTI's, NNRTI's

and PI's, has been used and drastically reduced the rate of HIV-1 and AIDS-related morbidity and mortality (157, 168).

### *Emergence of drug-resistant viruses*

Notwithstanding the increasing number of potent anti-HIV drugs, the major problem of antiretroviral therapy is the rapid emergence of resistance toward all known compounds if replication is not adequately suppressed by the drug therapy. The rapid emergence of resistance, which occurs after the administration of NRTI's, NNRTI's, as well as PI's, is a direct consequence of viral dynamics in the patients (30). Table 1.1 provides a list of known major mutations to current clinically used antiviral drugs.

Mutant viruses emerge in the presence of antiviral drugs whenever the mutations provide a selective advantage to the virus in the presence of drug. The development of drug resistance is caused by several factors. As mentioned earlier, drug-resistant virus may be generated by chance in the normal course of viral replication cycle, including 1) the drug-resistant mutations exist in infected people prior to therapy so that exposure to a particular drug may select for these preexisting mutants, 2) under selective drug pressure, HIV-1 replicates and acquires mutations de novo, becoming drug-resistant mutants that undergo rapid expansion. Any drug regimen that incompletely inhibits viral replication, such as suboptimal therapy, which is due to the lack of patient adherence to drug administration (8, 71), would enhance the probability of selecting drug-resistant virus.

Drug resistance to AZT after prolonged treatment was first reported by Larder *et. al*. (103), which was soon followed by the identification of HIV-1 resistant to other NRTI's. Although NRTI's compete with natural dNTP substrate to block the elongation of viral DNA synthesis, not all mutants that confer NRTI resistance map to the vicinity of the putative dNTP binding-site. Instead, some NRTI-resistant mutants are dispersed over the entire finger subdomain. For example, K65R, D67N, T69D, K70R and L74V are close to the dNTP binding-site, which folds over the triphosphate moiety of the incoming dNTP. Q151M is a key residue in the dNTP binding-pocket, which is present in the multiple-ddN-resistance (MDR) complex, and the 3TC-resistant mutation, M184V, lies within the dNTP binding-site and consequently interacts with the incoming dNTP or dNTP analogs. In contrast, M41L, L210W, T215F/Y, and K219Q/E are distant from the dNTP binding-site (76). This structurally puzzling aspect of NRTI resistance relates to the phenomenon that a resistance mutation for one drug can suppress a resistance mutation to a second inhibitor, such as the L74V mutation observed with ddI resistance can reverse the AZT-resistant effect of the T215Y mutation. The M184V/I mutation observed with 3TC resistance can restore the sensitivity to AZT-resistant virus strains (91). Unlike NRTI-resistant mutations, the amino acid changes that occur under NNRTI drug pressure in HIV-1 RT are within the hydrophobic NNRTI binding-site. Changes in single amino acids that arise from single-nucleotide substitution are often sufficient to confer high-level resistance to all NNRTI's, without a significant loss of fitness. As a result, drug-resistant viruses can appear quickly after the initiation of treatment

(141). Moreover, since different NNRTI's bind to the same pocket, the resistance to one NNRTI could result in the cross-resistance to other NNRTI's. Therefore, the clinical use of NNRTI's is limited if used in monotherapy. However, when used in combination with other classes of antiviral drugs, NNRTI's can exert potent anti-HIV-1 activity in patients. The mutant virus strains resistant to NNRTI's retain full sensitivity to the inhibitory action of NRTI's, and conversly, HIV-1 strains containing NRTI-resistant mutations in their RT are fully sensitive to NNRTI's. A number of NNRTI-specific mutations in the HIV-1 RT have been identified to sensitize HIV-1 to a number of well defined NRTI's and NNRTI's. For example, the Y181C mutation can reverse the phenotypic resistance of virus strains that contain typical AZT-resistant mutations by 30- to 35-fold (103).

Shortly after the introduction of HIV-1 PI's, various amino acid substitutions were reported to occur in the protease-encoding region of HIV-1 isolated from patients receiving HIV-1 PI's. In general, mutations in the protease gene, which are observed in patients receiving monotherapy of PI's, accumulate in a stepwise fashion and their appearance is associated with a loss of phenotypic susceptibility to the inhibitor. In contrast to some RT inhibitors, the acquisition of high-level resistance to PI's usually requires two or more amino acid substitutions (33). Primary resistance mutations are usually found in residues of the substrate-binding pocket and they are always accompanied by changes at other amino acids that are not within the active site, which are called secondary mutations. Such secondary mutations compensate for the loss of PR function by increasing either the stability or the activity of the enzyme. All

protease inhibitors studied can produce a common set of secondary mutations (i.e. changes at amino acid 10, 46, 54, 63 and 71). Among the substitutions known to be associated with resistance, some exist as naturally occurring genetic variants in the untreated population, such as variants of residue 10, 63 and 71 (33). Furthermore, increased resistance to PI's is often associated with mutations at the cleavage sites of the Gag precursor polyprotein. The resulting amino acid substitutions provide a better substrate for the PR and compensate for the loss of viral fitness caused by mutations in the PR-encoding region (17, 92). Moreover, cross-resistance between PI's is often observed as a result of similarities between the drug-resistance profiles of the inhibitors (92, 93, 141).

Although combination therapy can slow the emergence of drug-resistant mutations in HIV-1 infected patients, combination therapy with multiple drugs results in a different spectrum of mutations compared with monotherapy. For example, the simultaneous treatment with NRTI's can lead to the appearance of multiple-NRTI resistance (MDR) 151 complex, which includes substitutions at amino acids 62, 75, 77, 116, and 151 (182). In patients treated with AZT and other NRTI's, 69-70 insertion complex has been identify that contain an insertion of two amino acids (usually S-S/V/A/G/T) between position 69 and 70 of HIV-1 RT, together with the substitution T69S and AZT-related mutations, such as M41L, K70R, L210W, T215Y/F, and K219Q/E (209). The list of multiple-drug resistant mutations of NRTI's, NNRTI's and PI's is shown in Table 1.1.

**Table 1.1.** Amino acid substitutions in HIV-1 RT and PR that are associated with antiretroviral drug resistance. Data compiled from (6, 7, 93, 141, 154) and the international AIDS Society-USA (http://www.iasusa.org).

| Antiretroviral drug | Amino acid substitutions |
|---|---|
| **RT inhibitors** | |
| **Nucleoside RT inhibitors** | |
| AZT | M41L, E44D, D67N, K70R, V118I, L210W, T215Y/F, K 219Q/E |
| ddC | K65R, T69D, L74V, M184V |
| ddI | K65R, T69D, M184V |
| D4T | M41L, E44D, D67N, K70R, V75T, L210W, T215Y/F, K 219Q/E |
| 3TC | E44D, K65R, V118I, M184V/I |
| ABC | L65R, L74V, Y115F, M184V |
| Multiple NRTI's resistance (MDR) | |
| 151 complex | A62V, V75I, F77L, F116Y, Q151M |
| 69-70 insertion complex | M41L, A62V, Insertion of two S residues or a S and a V/A/G/T between T69 and K70, K70R, L210W, T215Y/F, K219Q/E |
| NRTI-associated mutations (NAM) | M41L, E44D, D67N, K70R, V118I, L210W, T215Y/F, K219Q/E, |
| **Nucleotide RT inhibitor** | |
| TDF | K65R |
| **Nonnucleoside RT inhibitors** | |
| NPV | K103N, V106M, V108I, Y181C/I, Y188L/C/H, G190A/S, Y318F |
| DLV | L100I, K103N, V106M, Y181C/I, Y188L, G190E, P236L, Y318F |
| EFV | L100I, K103N, V106M, V108I, Y181C/I, Y188L, G190S/A/T/Q, P225F, Y318F |
| Multiple NNRTI's resistance | L100I, K103N, V106A/M, Y181C/I, Y188L, G190S/A, M230L |
| **Protease inhibitors** | |
| SQV | L10I/R/V, G48V, I54V/L, A71V/T, G73S, V77I, V82A, I84V, L90M |
| RTV | L10I/R/V, K20M/R, V32I, L33F, M36I, M46I/L, I54V, A71V/T, G73S/A, V77I, V82A/F/T/S, I84V, L90M |
| IDV | L10I/R/V, K20M/R, L24I, V32I, L33F, M36I, M46I/L, I54V/L, A71V/T, V77I, V82A/F/T/S, I84V, L90M |
| NFV | L10F/I, D30N, M36I, M46I/L, A71V/T, V77I, V82A/F/T/S, I84V, N88D/S, L90M |
| APV | L10F/I/R/V, V32I, M46I/L, I47V, I50V, I54L/V/M, G73S, I84V, L90M |
| LPV | L10F/I/R/V, K20M/R, L24I, V32I, L33F, M36I, M46I/L, I41V/A, I50V, F53L, I54V/L/A/M/T/S, L63P, A71V, G73S, V82A/F/T/S, I84V, L90M |
| Multiple PI's resistance | L10F/I/R/V, V32I, I54V/L/M, I84V/A/C, L90M |

### *HIV-1 mutagenesis during antiretroviral drug therapy*

The failure of antiretroviral therapy often results from the emergence of HIV-1 drug resistance. The ability of drugs to influence retrovirus mutation rates was first observed by studying the influence of 5'-azacytidine on SNV mutation rates (150). Subsequent studies have shown that the mutation rate of retrovirus can be influenced by alteration of dNTP pools (87, 88, 150). The impact of drugs on HIV-1 mutation rates was first studied by testing how the NRTI's AZT and 3TC, as well as AZT- and 3TC-conferring resistance mutations, influence the HIV-1 mutation rate (122). These analyses used the *lacZ*$\alpha$ peptide gene as a mutation target, which has been used in previous mutation rate studies of HIV-1. AZT increased the HIV-1 mutation rate by 7.6-fold in a single round of replication, while 3TC increased the virus mutation rate by 3.4-fold (Table 1.2). AZT-resistant RT was also found to influence the mutation rate. In particular, HIV-1 replication with AZT-resistant RTs increased the mutation rate by as much as 4.3-fold, while replication of HIV-1 with a 3TC-resistant RT had no significant effect on the mutation rate (Table 1.2). It was observed that only high-level, AZT-resistant RT variants could influence the *in vivo* mutation rate (i.e., those containing the mutations M41L/T215Y and M41L/D67N/K70R/T215Y).

Further studies of drug resistant RTs has indicated that other amino acid residues in HIV RT associated with drug resistance can increase virus mutant frequencies when mutated. One example is the Y501F RT mutant, which leads to a 4-fold increase in virus mutant frequencies (125). The Y501 residue is located in the RNase H primer grip region of HIV RT and is associated with resistance to

N-(4-*tert*-Butylbenzoyl)-2-hydroxynaphthaldehyde hydrazone (BBNH), which is a potent inhibitor of RNase H activity (3).

Recent studies with other NRTI's (i.e., ddI, D4T, ABC and ddC) indicate that NRTI drug treatment may generally lead to increased virus mutant frequencies during HIV-1 replication (data will be presented in Chapter 2 and reference (120)). How NRTI's increase HIV-1 mutant frequencies is presently not known, but likely involves a similar mechanism. This is supported by the observation that virus mutant frequencies increase in an additive manner during virus replication in the presence of two NRTI's (i.e., AZT and 3TC, AZT and ddI, and 3TC and ddI) (120). These observations suggest that when virus replication occurs in the presence of suboptimal concentrations of drug, drug-resistant virus is selected for and that replication of drug-resistant virus in the presence of drug could further increase the virus mutation rate. To test this hypothesis, the combined effects of drug and drug-resistant virus were analyzed (127). It was found that the replication of AZT-resistant HIV-1 in the presence of AZT led to a multiplicative 24-fold increase in the virus mutant frequency to that observed with wild-type virus in the absence of drug (Table 1.2). This indicates that when drug failure occurs due to the evolution of drug resistance, replication of the drug-resistant virus in the presence of AZT significantly increases HIV-1 mutagenesis. In addition, it was observed that replication of an AZT/3TC dual-resistant virus in the presence of AZT and 3TC also led to a multiplicative 22.5-fold increase in mutant frequencies (Table 1.2). Thus, each of these drugs tested acted together with drug-resistant RT and increased virus mutant frequencies. Hypotheses

proposed to explain how NRTI's influence mutation rate include 1) NRTI's alter dNTP pool; 2) NRTI's are incorporated into plus-strand DNA and may result in discontinuous DNA synthesis of viral DNAs with proper ends that integrate with subsequent error-prone repair by host cell; and 3) NRTI's may bind non-catalytically to RT and cause a conformational change that influences enzyme fidelity (87).

Interestingly, other drugs that are non-RT, non-PR inhibitors used in conjunction with the AZT-resistant virus also led to a similar multiplicative increase in virus mutant frequencies (127). This indicates that when new drugs are added in drug therapy regimens they could also act with the drug-resistant virus to further increase virus mutant frequencies even though the drug-resistance phenotype is associated with another drug. For example, 3TC increased mutant frequencies of AZT-resistant virus to 13.6-fold compare to that with wild-type virus in the absence of drug (Table 1.2). Hydroxyurea, which is known to alter intracellular dNTP pools by inhibiting ribonucleotide reductase and results in a depletion of all dNTPs, is a well-documented drug used in HIV-1 treatment. AZT-resistant HIV-1 replication in the presence of hydroxyurea resulted in a 21.8-fold increase in mutant frequencies compared to that observed in the absence of drug (Table 1.2). Like hydroxyurea, thymidine has also been shown to alter intracellular dNTP pools and in addition has been shown to increase retrovirus mutation rates. AZT-resistant HIV-1 replication in the presence of thymidine increased mutant frequencies by 16.7 fold (Table 1.2). Thioguanine (an antileukemic agent that has been reported to inhibit RNase H

activity) has been shown to increase HIV-1 mutant frequencies by as much as 4-fold and to significantly alter mutant frequencies during virus replication with RTs containing mutations not associated with the drug (125). These data suggest that subsequent therapies could lead to increased HIV-1 mutagenesis even though the drug-resistant phenotype is not directed against the new drug(s) used in the drug therapy regimen.

| Drug | Drug-resistant RT | Mutant frequency increase (fold) |
|---|---|---|
| AZT | Wt RT | 7.6 |
| 3TC | Wt RT | 3.4 |
| Hydroxyurea | Wt RT | 4.5 |
| Thymidine | Wt RT | 7.0 |
| Thioguanine | Wt RT | 4.0 |
| ddI | Wt RT | 6.0 |
| -- | AZT-resistant RT | 4.3 |
| -- | 3TC-resistant RT | 1.0 |
| AZT | AZT-resistant RT | 24.0 |
| 3TC | AZT-resistant RT | 13.6 |
| AZT/3TC | AZT/3TC dual-resistant RT | 22.5 |
| Hydroxyurea | AZT-resistant RT | 21.8 |
| Thymidine | AZT-resistant RT | 16.7 |

**Table 1.2.** Drugs and drug-resistant reverse transcriptases that have been shown to increase HIV-1 mutant frequencies.

## 1.6 Role of Viral protein R and uracil-DNA glycosylase in virus replication

### *Viral protein R (Vpr)*

In addition to *gag, pol* and *env*, HIV-1 also contains accessory genes, *tat, rev, vpr, vpu, vif and nef*, which regulate and coordinate viral gene expression. One of these genes, *vpr*, could influence HIV-1 mutagenesis (121). Vpr is a 14 kD, 96-amino-acid-non-structural protein, which is associated with HIV-1 particles and accumulates at the nuclear envelope and in the nuclei of infected cells. Incorporation of Vpr into virus particles requires a direct interaction with the p6 region of the Gag polyprotein precursor (153). Vpr provides multiple functions for HIV-1 replication, including targeting of the preintegration complex (PIC) to the nucleus, induction of cell-cycle arrest in the $G_2$ phase of the cell cycle, transactivation of viral and cellular gene expression, induction of apoptosis, and modulation of viral replication kinetics (181, 188). Vpr is expressed in all primate lentiviruses (i.e. HIV-1, HIV-2 and SIV). The ability to replicate in non-dividing cells is a characteristic of the primate lentiviruses. While Vpr is not essential for the replication of HIV-1 in cultured cell lines or activiated T-cells, it is required for the efficient replication of the virus in non-dividing cells such as macrophages (34, 73).

An additional activity of Vpr is demonstrated by its ability to modulate HIV-1 mutation rate. Using a single round of replication system to measure *in vivo* forward mutation rate of HIV-1, it was shown that the mutation rate of *vpr*$^-$ HIV-1

was as much as 4-fold higher than that of the *vpr*$^{+}$ HIV-1. Moreover, a Vpr mutant with a single amino acid substitution, which led to Vpr not being efficiently incorporated into virus particles, was also found to have a mutant frequency similar to that of the *vpr*$^{-}$ HIV-1. When the *vpr*$^{-}$ HIV-1 was complemented *in trans* with a wild-type *vpr* expression vector, the mutant frequency of *vpr*$^{-}$ HIV-1 was found to be comparable to the *vpr*$^{+}$ parental virus. These data indicate that the *vpr* gene has a role in modulation of the HIV-1 mutation rate and that virion incorporation of Vpr coincides with the influence of Vpr on the mutation rate (121). The possible interpretations of these observations are: 1) Vpr directly interacts with RT to influence enzyme fidelity; and 2) Vpr interacts with other proteins that influence the accuracy of the reverse transcription process.

The HIV-1 Vpr protein has been found to interact with several cellular proteins, including two proteins involved in DNA repair process, the human homologue of the yeast RAD23 protein (HHR23A) and uracil-DNA glycosylase (UNG) (19, 63, 210). These associations were initially identified using yeast two-hybrid screens, and confirmed both *in vitro* and *ex vivo*. The HHR23A protein has been reported to associate with the xeroderma pigmentosum complementation group C (XPCC) protein, which functions in global nucleotide excision repair (135). It was previously suggested that HHR23A is a mediator of Vpr-induced cell cycle arrest based on the observation that overexpression of the full-length or truncated form of HHR23A prevented G2 arrest (210). However, recent studies have shown that the interaction of Vpr to HHR23A does not correlate with the ability of Vpr to cause cell cycle arrest and to influence *in vivo* mutation rate.

Moreover, the interaction of Vpr to HHR23A does not associate with the nuclear targeting of Vpr. These data indicate that the Vpr-HHR23A interaction is not required for multiple biological functions of Vpr (128). Unlike the HHR23A, it has been reported that the Vpr-UNG interaction influences the HIV-1 mutation rate. Using the model system developed to measure the *in vivo* forward mutation rate of HIV-1, recombinant virus containing mutant *vpr*, which does not interact with UNG, had a 4-fold increase in its mutation rate compared to recombinant virus containing wild-type *vpr* (129).

### *Uracil-DNA glycosylase (UNG)*

UNG is an enzyme involved in the base excision repair pathway and specifically removes the RNA base uracil from DNA. UNG was first discovered in *E. coli.* Subsequently, similar enzymatic activities have been demonstrated in numerous organisms, including yeast, plants, mammalian cells, and virus. Sequence analyses have shown a high degree of similarity among these UNGs, ranging from 40.3% (yeast) to 90% amino acid (mouse) identity relative to human UNG, which indicate UNGs are highly conserved in evolution (146). Structural analysis showed that these enzymes, in particular the active site, are highly conserved except for UNG from poxviruses (162). The human *ung* gene encodes two forms of UNG, UNG1 (mitochndrial form) and UNG2 (nuclear form), which are produced by alternative transcription start sites and alternative splicing (147). Analysis of UNG activity showed that UNG2 is far more abundant than UNG1 (184). The mRNAs for UNG1 and UNG2 encode 35 and 44 unique N-terminal

residues that are required for mitochondrial and nuclear translocation, respectively, whereas the C-terminal 269 amino acids are common to the two isoforms (147). Regulation of UNG activity is cell cycle dependent. It has been shown that UNG activity is higher in proliferating cells than in non-cycling cells. Furthermore, the induction of DNA synthesis in resting lymphocytes increases UNG activity several fold. The mRNA for UNG increases 8-12-fold late in G1 phase, whereas enzyme activity increases just prior to S-phase and reaches a maximum early in S-phase (185).

Within the same C-terminal sequence is the catalytic domain that contains a conserved DNA binding groove and a tight fitting uracil-binding pocket. UNGs have been shown to remove uracil from single-stranded and double-stranded DNA substrate (109, 146). The rate of uracil removal occurs almost twice as fast on single-stranded versus double-stranded DNA (108). In general, uracil is removed from U:A pair resulting from misincorporation of dUMP and from mutagenic U:G mispairs resulting from the deamination of cytosine. It is believed that the primary function of UNG is to remove uracil from U:G mispairs (59). Usually, the rate of removal of uracil is greater from U:G mispairs than from U:A pairs (44, 183). Interestingly, UNGs from human and bacteria remove uracils at different rates from different dsDNA sequence contexts, but at essentially similar rates from ssDNA. Consensus sequences for the “best” and “worst” removal are 5’ (A/T)UA(A/T) and (G/C)U(G/C/T), respectively (44). Uracil is not excised from RNA or RNA contained in a RNA/DNA hybrid; however, it has been reported that

UNG isolated from calf thymocyte nuclei was able to excise uracil residues from the DNA strand of a RNA/DNA hybrid (191).

UNG is encoded and expressed by DNA viruses of two main families, the *Herpesviridae* and the *Poxviridae*. The ability of UNG to influence virus replication of different herpesviruses has implied a role of viral UNG in the replication of virus in the host, particularly in non-dividing cells, where levels of cellular UNG are believed to be low (36, 163). Similarly, poxvirus-encoded UNG has been found to be associated with maintaining virus replication in cell culture (48, 145, 190).

### *Interaction of Vpr and UNG modulates HIV-1 mutation rate*

As mentioned earlier, the interaction between Vpr and UNG was identified using a yeast two-hybrid screen, and confirmed both *in vitro* and *ex vivo* in Vpr-expressing cells (19, 129). Moreover, the subsequent analysis of the interaction of Vpr to UNG demonstrated that UNG enzymatic activity was not diminished nor was the interaction disrupted by uracil-DNA glycosylase inhibitor (UGI) (19). Recently, it has been shown that cellular UNG is packaged into HIV-1 virus particle via the interaction with Vpr and the nuclear form of UNG (UNG2) is preferentially packaged into HIV-1 virus particles (129). The first evidence to show the correlation of Vpr-UNG2 interaction with HIV-1 mutation rate was to study a Vpr mutant, W54R. It has been reported that VprW54R leads to the same mutation phenotype observed during HIV-1 replication in the absence of Vpr. VprW54R has a phenotype that is comparable to wild type Vpr in its ability to

arrest cells in the G2/M phase of the cell cycle, to localize to the nucleus, and to be efficiently incorporated into HIV-1 particles (176). In contrast, the W54R substitution prevents Vpr from interacting with UNG2 and when *vpr*$^-$ HIV-1 was complemented *in trans* with VprW54R, there was a 4-fold increase in the over all mutation rate and the rate of G-to-A mutations. In addition, VprW54R does not allow for efficient packaging of UNG2 into HIV-1 particles. This indicates that the interaction and virion incorporation of Vpr and UNG2 into HIV-1 particles correlates to the influence of Vpr on the HIV-1 mutation rate. Subsequently, the other Vpr mutants were studied to support the hypothesis that Vpr-UNG2 interaction is involved in modulating the HIV-1 mutation rate. The two Vpr variants that were previously shown to bind poorly to UNG2 (176), VprH71R and VprH78R, were found to be efficiently incorporated into virus particles and increased the virus mutation rate in the same manner as VprW54R (124). In contrast, VprS79A, which interacted more efficiently with UNG2 than wild type Vpr (176), did not significantly influence HIV-1 mutation rate (124). These observations provide further evidence for the role of the interaction of Vpr with UNG2 modulating the HIV-1 mutation rate. Interestingly, when virus replicates with RT mutant and Vpr mutant, the ability of the RT mutant to influence virus mutation rate was counteracted by the Vpr effect. Moreover, when Y115A RT was used with VprW54R in the presence of AZT, the increase in mutation rate due to VprW54R was additive, whereas the Y115A RT mutant led to a multiplicative increase in the presence of AZT and wild type Vpr (124). These observations suggest that the mechanism used by Vpr to alter HIV-1 mutation

rate is distinct form the mechanism by which the RT mutants and antiretroviral drugs modulate fidelity.

It was recently reported that UNG was detected in HIV-1 virions in the absence of Vpr, requiring the presence of the viral integrase protein when Vpr is absent for UNG incorporation. Furthermore, integrase mediates the incorporation of the precursor form of UNG, which has little enzymatic activity, whereas Vpr mediates the incorporation of the nuclear form of UNG (129, 208). This suggests that integrase also contributes to UNG incorporation in the virions, and the interaction of UNG2 with Vpr is the major pathway for UNG incorporation into HIV-1 particles.

The observation that Vpr binding to UNG2 correlates with the *in vivo* mutation rate of HIV-1 implies a role for UNG2 in the accuracy of the reverse transcription process. Analysis of the mutation spectrum associated with HIV-1 shows the predominant base substitutions are G-to-A and C-to-T transition mutations. Uracil can occur in viral DNA either by misincorporation when dUTP levels are high or by cytosine deamination of dCMP. If cytosine deamination occurs in the minus-strand DNA and is not repaired, a G-to-A transition mutation would be present in the plus-strand DNA. Although the incorporation of dUTP instead of dTTP into DNA is not shown to be mutagenic, misincorporated dUTP could be cytotoxic as a result of alteration of sequences specific for binding of transcription factors, leading to alterations in gene expression (56, 202). Recently, it has been shown that miscorporation of dUTP can decrease the plus-strand viral DNA synthesis initiation and increase the non-PPT initiated DNA

synthesis, which results from the diminished PPT-specific RNase H activity and increased nonspecific cutting. This uracil-mediated aberrant initiation of plus-strand DNA synthesis may decrease viral DNA integration frequency, and subsequently decrease viral fitness (95). Therefore, lentiviruses need to contain enzymes that are predicted to limit the amount of uracil present in viral DNA, ensuring that critical protein/DNA interactions occur with the highest possible efficiency and fidelity. As discussed above and in Section 1.4, non-primate lentiviruses encode and package dUTPase into viral particles, whereas primate lentiviruses incorporate the cellular UNG2. When primate and non-primate lentiviruses lack UNG and dUTPase activity, respectively, they exhibit similar increase in G-to-A transition mutations (29). This suggests that lentiviruses have evolved different mechanisms to address the potential problem of uracil residues in viral DNA and to secure the integrity of its genetic material.

# CHAPTER 2

# BOTH NUCLEOSIDE AND NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS CAN INFLUENCE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) MUTANT FREQUENCIES

## 2.1 Abstract

The development of antiviral drug resistance is an important problem in the treatment of HIV-1 infection. Potent antiretroviral therapy (ART) is currently used for treatment, and typically consists of at least two reverse transcriptase (RT) inhibitors. We have previously reported that both drugs and drug-resistant RT mutants can increase virus mutant frequencies. To further assess the contributions of nucleoside RT inhibitors (NRTI's), non-nucleoside RT inhibitors (NNRTI's), and drug-resistant RTs to HIV mutagenesis, a new high-throughput assay system was developed. This assay system was designed to specifically detect frameshift mutations in the luciferase gene in a single virus replication cycle. Consistent with our previous observations of NRTI's, abacavir (ABC), stavudine (D4T), and zalcitabine (ddC) increased HIV-1 mutant frequencies, supporting the general hypothesis that the NRTI's currently used in antiviral drug therapy increase virus mutant frequencies. Interestingly, similar observations

were made with NNRTI's. This is the first report to show that NNRTI's can influence virus mutant frequencies. I also analyzed the influence of drug-resistant RT mutants from different subtypes on HIV-1 mutant frequencies. The results indicate that high-level, drug-resistant RT can significantly influence virus mutant frequencies. Moreover, combinations of drug and drug-resistant RTs led to significant changes in the virus mutant frequencies compared to virus replication of drug-resistant virus in the absence of drug or wild type virus in the presence of drug.

## 2.2 Introduction

Potent antiretroviral therapy (ART) of HIV-1 infection with antiretroviral drugs including nucleoside RT inhibitors (NRTI's), nonnucleoside RT inhibitors (NNRTI's) and protease inhibitors (PI's) has dramatically reduced the rate of HIV and AIDS-related morbidity and mortality (149, 157, 168). However, the lack of patient adherence to drug administration results in suboptimal therapy. Suboptimal drug therapy can lead to drug resistance, which limits the clinical benefit of drug treatment and selects for new variant viruses with altered virulence and tropism (30, 100, 144). The highly error prone reverse transcriptase (RT) is thought to play a role in the generation of retrovirus diversity.

The *in vivo* mutation rate for HIV-1 was previous determined to be $4 \times 10^{-5}$ mutations per target base pair per replication cycle (118, 130), which correlated to about one mutation in every 3 new genomes produced. Thus, viral genomes

with each possible mutation as well as many with double mutations are likely generated each day, allowing for the rapid selection and fixation of mutations that confer drug resistance occurs at a rapid rate (169, 175). These drug-resistant viruses can reside in latently infected cells, which further complicates subsequent drug therapy during the life of the infected individual (55, 211). Continued replication in the presence of drug will select for even greater levels of resistance and typically leads to cross-resistance to drugs of the same class (92, 174). Furthermore, the accumulation of drug-resistant mutations will reduce drug susceptibility and potency of antiretroviral therapy. Transmission of HIV-1 with reduced susceptibility to antiretroviral drugs may compromise the efficacy of drug therapy (62).

Antiretroviral drugs have been previously shown to influence the fidelity of retroviruses replication. First, a nucleoside analog, 5-azacytidine that is incorporated into RNA and inhibits protein synthesis, was found to increase the *in vivo* spleen necrosis virus (SNV) mutation rate by a factor of 13 (150). AZT was subsequently observed to increase the SNV mutation rate by 10-fold, while it increased the murine leukemia virus (MLV) mutation rate by a factor of 3 (87). The first study of the impact of drugs on HIV-1 mutant frequencies was investigating how the NRTI's 3'-azido-3'-deoxythymidine (zidovudine, AZT) and (-) 2', 3'-dideoxy-3'-thiacytidine (lamivudine, 3TC) influence HIV-1 mutant frequencies (123). These analyses used the *lacZ*$\alpha$ peptide gene as a mutational target that has been used in previous mutation rate studies of HIV-1. AZT increased the HIV-1 mutant frequency by 7.6-fold in a single round of replication,

while 3TC led to a 3.4-fold increase in virus mutant frequency. How NRTI's increase HIV-1 mutagenesis is presently not known, but likely all the NRTI's currently used in therapy have a similar mechanism to influence HIV-1 mutant frequencies. This is supported by the observation that HIV-1 mutant frequency increased in an additive manner during virus replication in the presence of two NRTI's (i.e. AZT and 3TC, AZT and ddI, and 3TC and ddI) (120).

AZT-resistant RT was also found to increase the mutant frequency by 4.3-fold, but the replication of HIV-1 with 3TC-resistant RT had no significant influence on the mutant frequency (123). It was observed that only high-level, AZT-resistant RT mutants could influence the *in vivo* mutant frequency, such as those containing mutations M41L/T215Y and M41L/D67N/K70R/T215Y. These observations suggested that when virus replication occurs in the presence of suboptimal concentrations of drug, drug-resistant virus is selected for and that replication of drug-resistant virus in the presence of drug could further increase the virus mutation rate. To test this hypothesis, the combined effects of drug and drug-resistant virus were investigated (126). It was observed that the replication of AZT-resistant virus in the presence of AZT led to a multiplicative 24-fold increase in the virus mutant frequency compared with that observed with wild-type virus in the absence of drug. In addition, it was found that replication of an AZT/3TC dual-resistant virus in the presence of both AZT and 3TC also led to a multiplicative 22.5-fold increase in the virus mutant frequency. These results indicated that when drug failure occurs due to the evolution of drug resistance,

replication of the drug-resistant virus in the presence of drug could significantly increase HIV-1 mutagenesis.

Further studies of drug-resistant RTs have indicated that other amino acid residues in HIV RT associated with drug resistance can also increase virus mutant frequencies when mutated. For example, the Y501F RT mutant, which is located in the RNase H primer grip region of HIV RT and is associated with resistance to N-(4-*tert*-Butylbenzoyl)-2-hydroxynaphthaldehyde hydrazone (BBNH) , led to a 4-fold increase in virus mutant frequencies (124).

Previous *in vitro* studies using purified HIV-1 RT showed that single base substitutions and single base frameshift mutations were predominant mutations in the HIV-1 mutational spectrum and were non-randomly distributed (11). Most of these mutations were found at mutational “hot spots”, which usually are homopolymeric runs. It was observed that many single base substitutions occurred at either the 5’ end or the 3’ end of homopolymeric runs, indicating many single base substitutions, as well as frameshift mutations, are initiated by template-primer slippage (11, 12). Consistent with these observations, the homopolymeric runs were found to be hot spots for SNV RT to initiate frameshift mutations (most common mutations were +1 and –1) in a single round of viral replication (25). The mutation rate for runs of T’s was the highest compared to rates for runs of A’s, C’s and G’s. Moreover, the analysis of the HIV-1 mutation rate in a single round of replication also demonstrated that both base substitutions and frameshift mutations were common mutations during HIV-1 reverse transcription; the most common frameshift mutations were +1 mutations

at a run of T's (130). Further study of mutations in HIV-1 provirus following treatment of antiretroviral drugs showed that the mutational spectrum of HIV-1 after drug treatment was comparable to the spectrum of mutants observed in the absence of drugs, indicating that the mechanisms by which mutations occurred were similar but that the rate had increased (123).

In order to extend our current knowledge for how antiretroviral drugs and drug-resistant RTs influence HIV-1 mutant frequencies, I first developed a new high-throughput assay system using the *luc* gene as a mutational target to measure HIV-1 mutant frequencies based on previous observations (11, 12, 25, 123, 130). Using this new assay system several issues were addressed. First, I analyzed whether specific mutations in HIV RT that conferred resistance to antiretroviral drugs could influence the rate of HIV-1 mutation. Second, I tested the hypothesis that NRTI's currently used in drug therapy could increase HIV-1 mutant frequencies. Third, I studied whether NNRTI's could influence HIV-1 mutant frequencies. Finally, I examined the combined effects of drug and drug-resistant RT on virus mutant frequencies. I found that high-level, drug-resistant RT mutants could significantly influence HIV-1 mutant frequencies. I also observed that drugs and drug-resistant RT mutants together could further influence HIV-1 mutagenesis. Furthermore, I found that NNRTI's had a similar influence on HIV-1 mutant frequencies compared to NRTI's.

## 2.3 Materials and Methods:

### *Retroviral vectors and expression plasmids*

HIV-SVLuc+$1_{bru2}$ was developed to measure the reversion mutation rate of HIV-1 using a mutated *luc* gene as reporter. This vector was designed to specifically detect frameshift mutations in the luciferase gene in a single virus replication cycle. Eight T residues were inserted after the start codon ATG of the *luc* gene by two-step PCR mutagenesis using pGL3 control vector as template. This insertion causes the complete loss of luciferase activity due to the loss of the open reading frame. A 1.5-Kb deletion within the open reading frame of *env* gene was made in HIV vector KP97, which was derived from pBRU2, from the *Sal*I site to the *Nhe*I site. This deletion was replaced with a 1.9-Kb *Xho*I to *Xba*I fragment from pGL3, which contains simian virus 40 (SV40) promoter and mutated *luc* gene (Figure 2.1A).

The HIV-1 *gag-pol* expression plasmid used was pSVgagpol-rre-MPMV, which has been described previously (129). This expression vector contains the SV40 promoter driving expression of HIV-1 *gag-pol* genes. The vector used for expression of vesicular stomatitis virus glycoprotein (VSV-G) envelope, pHCMV-G, was obtained from American Type Culture Collection (ATCC, Manassas, Va.). To be packaged into virus particles, HIV-SVLuc+$1_{bru2}$ was complemented *in trans* with HIV-1 *gag-pol* expression plasmid and pseudotyped with VSV-G envelope expression plasmid.

HIV-1 RT mutants (subtype B) analyzed in these experiments were constructed by introducing mutations coding for RT amino acid substitutions into pSVgagpol-rre-MPMV by site-directed mutagenesis (Stratagene, La Jolla, CA) following manufacturer's instruction.

The helper vectors containing HIV-1 subtype E RT variants were created by deletion of full-length subtype E clones (p93JP-NH3 and variants). The full-length subtype E clones, which contain either drug-sensitive RT or drug-resistant RT, were obtained from our collaborator, Dr. H. Sato (National Institute of Infectious Diseases, Tokyo, Japan). A 2.4-Kb deletion was made in each of the full-length subtype E clones between two *Avr*II sites. This deletion removed *vpr*, *tat* and *env* gene of subtype E, so these gene products will not compete with Vpr and Tat of HIV-SVLuc+$1_{bru2}$ vector to interfere with infection. The deleted vectors were then religated to obtain the subtype E helper vectors.

***Cell culture and Antiretroviral drugs***

The 293T and MAGI cell lines were obtained from ATCC. 293T cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) (GIBCO BRL, Gaithersburg, MD), supplemented with 10% of Fetal clone III serum (Hyclone, Logan, UT). MAGI cells were maintained in DMEM supplemented with 10% of Fetal clone III serum, G418 (0.2 mg/ml) and hygromycin (0.1 mg/ml). MAGI cells are indicator cells for HIV-1 infection, which contain HIV-1 LTR-driven *lacZ* gene. When MAGI cells are infected with HIV virus, HIV-1 Tat protein will transactivate HIV-1 LTR, and subsequently drive the

expression of *lacZ* gene. Virus-infected cells will be scored by staining MAGI cells with 5-bromo-4-chloro-3-indolyl-β-D-galacosidase actopyranoside (X-gal).

The NRTI drugs ABC, D4T, ddC, and NNRTI drugs EFV and NPV, were obtained from NIH AIDS Research Reagents Program (Bethesda, MD). 3TC was purchased from Sigma (St. Louis, MO).

***Transfections and infections***

The experimental protocol developed to obtain a single round of replication of HIV-1 vector is shown in Fig 2.1B, and described in detail elsewhere (130). Briefly, HIV-SVLuc+$1_{bru2}$ (9.5 μg) was co-transfected with pSVgagpol-rre-MPMV (9.5 μg) and VSV-G (1 μg) into 293T cells in a 100 mm petri dish using the calcium phosphate precipitation method. Viruses were harvested 48 hr posttransfection and concentrated using Centricon Plus-20 (Millipore, Billerica, MA) following manufacturer's instruction. To perform infection, concentrated viruses were mixed with Polybrene (8 μg/ml) (Sigma, St. Louis, MO) and the virus-polybrene mixture was used to infect two sets of $2.5 \times 10^5$ fresh MAGI target cells. After 24 hr, the medium was removed, cells were washed once with $1 \times$ PBS (GIBCO BRL), and fresh medium was added to the cells. The cells were incubated for an additional 48 hr under the same conditions. Supernatants were removed, and one set of cells was used to perform MAGI assay for determining the viral titer. The other set of cells was lysed and used for luciferase assay to detect the restored luciferase activity (described below, Figure 2.1B).

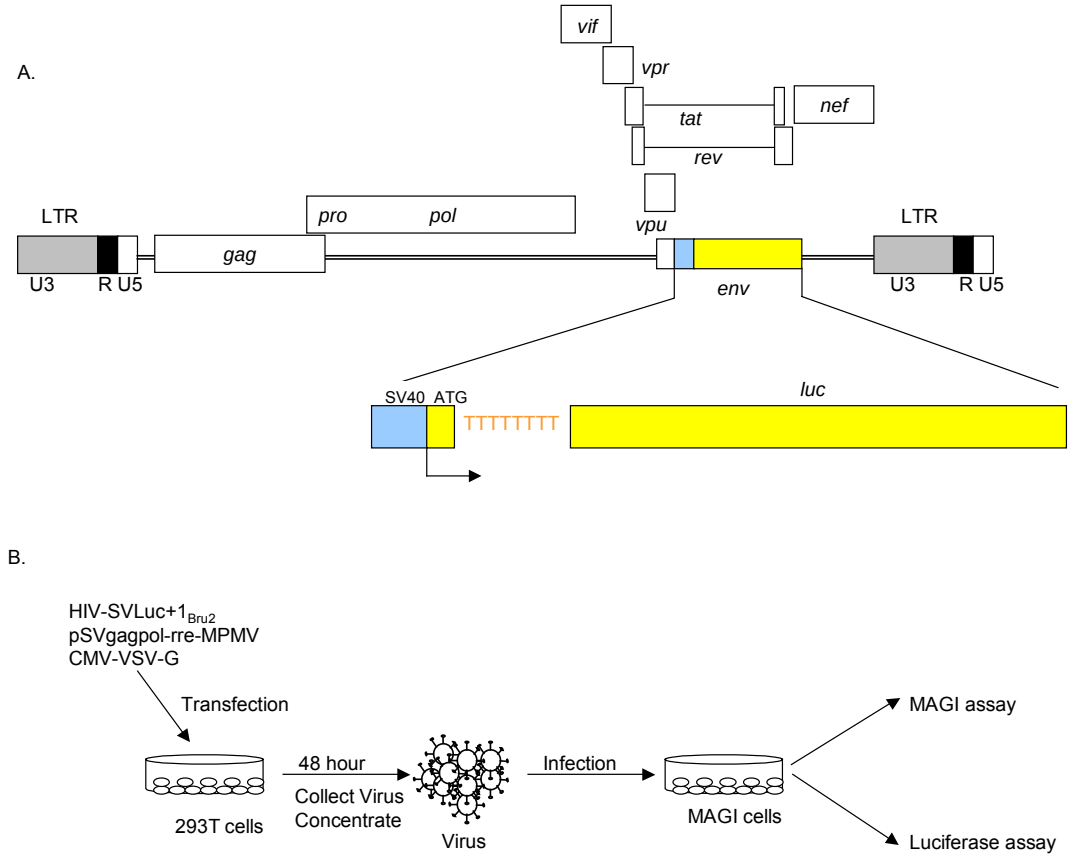



**Figure 2.1**. Assay system used for analysis of virus mutant frequencies. (A) Schematic representation of HIV-1 vector used to analyze HIV-1 mutant frequencies. The proviral DNA form of the vector is shown. The large rectangular boxes are long terminal repeats, which include U3 (gray), R (black) and U5 (white). The blue box is the simian virus 40 promoter (SV40). The yellow box is the *luc* gene. (B) Single round of replication assay for mutant frequencies. Virus producing cells were transfected with HIV-1 reporter vector and helper plasmids, and the produced viruses were concentrated and used to infect fresh MAGI cells. MAGI assay and luciferase assay were used to determine the reverse mutation frequencies.

### *MAGI Assay*

Viral titer was determined by MAGI assay as follows. After 72 hr of infection, the medium was removed, cells were washed once with 1 × PBS, and 2 ml of fixing solution (1% glutaraldehyde, 100 mM sodium phosphate (pH 7.0), 1 mM $MgCl_2$) was added. After a 20-min incubation, cells were washed once with 1 × PBS, and 2 ml of staining solution (10 mM sodium phosphate, 150 mM NaCl, 1 mM $MgCl_2$, 3.3 mM potassium ferro cyanide, 3.3 mM potassium ferric cyanide, and 200 μl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galacosidase actopyranoside [X-gal]) was added. Cells were incubated for 2 hr at 37°C. Staining was stopped by removing the staining solution, and the cells were thoroughly washed with PBS. Positive blue cells were counted, and the viral titer was determined.

### *Reversion mutation detection*

Mutations that restored luciferase activity were determined in MAGI cells, which were treated with or without antiretroviral drugs. Drug treatments were typically done by maintaining MAGI target cells in medium supplemented with drug at the $IC_{50}$ concentration from 2 hr prior to infection and until 24 hr after infection. Seventy-two hr post-infection, infected MAGI cells were counted using the trypan blue dye exclusion method, harvested and lysed in 1 × luciferase assay lysis buffer (25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N', N', N', N',-tetracetic acid, 10% glycerol, 1% Triton X-100). Luciferase activity was quantified using the Promega Luciferase Assay

System (Promega, Madison, WI). Mutant frequencies were calculated based on luciferase activity reading, viral titer and cell numbers. The relative mutant frequency of wild type viruses that were not treated with drug was defined as 1. The equation used to calculate the relative mutant frequencies (RMF) is:

$$RMF = \{[(Cell\#_{\text{no drug treatment}} / Cell\#_{\text{drug treated}}) \times \text{luciferase reading}_{\text{drug treated}}] / \text{luciferase reading}_{\text{no drug treatment}}\} \times (\text{Viral titer}_{\text{no drug treatment}} / \text{Viral titer}_{\text{drug treated}})$$

***Determination of 50% of inhibitory concentrations***

The MAGI assay was used to determine the $IC_{50}$ value for each drug. Briefly, $2.5 \times 10^5$ fresh MAGI target cells were treated with antiretroviral drug at different concentrations 2 hr prior to infection and continued until 24 hr postinfection. Virus-polybrene mixture was diluted to 1:1000 and used to infect MAGI target cells. Seventy-two hr postinfection, infected MAGI cells were stained with X-gal and positive blue cells were counted to determine viral titer at each concentration. The viral titer was plotted as a function of the drug concentration used, generating linear curves for all the drugs. $IC_{50}$ values for each drug were calculated based on the linear curves (Figure 2.3).

***Analysis of mutation spectrum***

Cellular DNA was prepared from $1 \times 10^5$ cells infected by wild type HIV-1 in the absence of drug. Following infection, the infected MAGI cells were washed once with 1X PBS, trypsinized and lysed in 500 μl of PCR lysis buffer (50mM KCl, 10mM Tris, pH 8.3, 1.8mM $MgCl_2$, 0.45% IGEPAL CA-630, 0.45% Tween 20). The cell lysate was treated with 3 μl of proteinase K (20 mg/ml) (Roche,

Indianapolis, IN) at 50°C for 1 hr. The 5'-end of the *luc* gene was amplified from cell lysate using nested PCR. The PCR products were then cloned into pCR2.1 TA cloning vector (Invitrogen, Calsbad, CA) following manufacturer's instruction. The plasmid DNAs were isolated and sequenced.

### *Statistical analysis*

To investigate the effects of antiretroviral drugs and mutations on mutant frequencies, we fit two related linear models to the normalized logarithms of the fold changes relative to control. Normalization was conducted using a standard protocol. Both models pooled data from all experiments and replicates to estimate effects for each drug and mutation. Both models also assume that assay variability is constant across all replicates and conditions, an assumption that is consistent with the data. Model 1 simply has fixed effects for all the treatments and estimable interactions. Model 2 further assumes that assay errors are well modeled as normal random variables. This second model differs from the first in that drug effects at standard doses are treated as random effects that are grouped into 2 varieties: NRTI's and NNRTI's (the model does not treat drug effects as from these groups if the dosage is not the $IC_{50}$). Thus model 2 is the same as the linear model except the coefficients for each class of drugs are assumed to come from a normal distribution with some overall mean and standard deviation. The parameters for model 2 are estimated with maximum likelihood (the EM algorithm is used to maximize the likelihood). Statistical analysis was done in collaboration with Dr. Cavan Reilly, University of Minnesota.

## 2.4 Results

### *Development of a new high throughput assay system to measure HIV-1 mutant frequencies*

The original HIV-1 vector used to measure HIV-1 mutant frequencies contains a *lacZ*$\alpha$ gene that is used as reporter gene (130). However, using this system is laborious and time-consuming. In order to more rapidly assess the ability of drugs and/or drug-resistant RTs to influence HIV-1 mutant frequencies, a new high throughput assay system was developed. This system was designed to be a reversion assay that would specifically detect frameshift mutations in the luciferase reporter gene in a single round of replication based on previous studies (11, 12, 25, 123, 130) (Figure 2.1). The HIV-1 vector developed contained a mutated *luc* gene, which was inserted into the *env* gene of HIV vector KP97 (derived from pBRU2). Specifically, eight T residues were inserted after start codon ATG of the *luc* gene (Figure 2.1A). To be packaged into virus particles, the vector was complemented *in trans* with an HIV-1 *gag-pol* expression plasmid and pseudotyped with a vesicular stomatitis virus glycoprotein (VSV-G) envelope expression plasmid. Vector virus produced from 293T cells was used to infect fresh MAGI target cells. Reversion mutations were detected by measuring restored luciferase activity in the infected MAGI cells, which were either untreated or treated with antiretroviral drug. The relative mutant frequency of virus that was replicated with wild-type RT and not treated with drug was defined as 1. The relative mutant frequencies of viruses treated

with antiretroviral drugs and of viruses replicated with RT variants were compared to this value (Materials and Methods).

Several approaches were used to validate this assay system. First, a control HIV-1 vector was constructed in which 2T residues were inserted after the ATG of the *luc* gene. A mutation hot spot has been defined as a homopolymeric run of 3 or more nucleotides (11, 25). Therefore, the reversion mutations that occurred in this 2T vector was expected to be much lower than that when the 8T vector was used. As expected, significantly lower reversion mutations were observed when the 2T vector was used (data not shown), providing indirect evidence that the observed reversion mutations occurred during the reverse transcription process. Second, analysis of the mutational spectrum at the hot spot revealed plus one, minus two and minus one frameshift mutations. In addition, a G-to-T base substitution was also observed (Table 2.1). This G-to-T mutation was located at the 3' end of the run of T's, indicating that this mutation was initiated by dislocation mutagenesis (12). The mutant frequency calculated from this analysis, $4.4 \times 10^{-3}$ mutations/cycle/base pair, is consistent with the previously determined HIV-1 frameshift mutation rate in a homopolymeic sequence, which is on the order of $10^{-3}$ (11, 130). Because only the +1 and –2 mutations can restore luciferase activity in this assay system, the relative mutant frequency 1 correlates to $2.2 \times 10^{-3}$ mutations/cycle/base pair. Based on the statistical analysis of data pooled from all control experiments, mutant frequencies are similar among all replicates, indicating that the calculated mutant frequency is representative.

| Mutational Class | Number of mutants | Mutant frequency ($\times 10^{-3}$ )[a] |
|---|---|---|
| +1 | 1/116 | 1.1 |
| -2 | 1/116 | 1.1 |
| -1 | 1/116 | 1.1 |
| G-to-T | 1/116 | 1.1 |
| Total | 4/116 | 4.4 |

**Table 2.1**. Analysis of mutational spectrum at the mutation hot spot. a: Mutant frequency is mutations/cycle/bp. The average luciferase reading of all control experiments (virus replication in the presence of wt RT) was $6.1 \times 10^2 \pm 35$.

Third, a control vector was constructed in which 9 T residues were inserted after start codon of the *luc* gene. This vector was designed to test whether the insertion of 3 amino acids would affect luciferase expression. The results revealed that the insertion of 3 amino acids did not significantly influence luciferase expression (data not shown). Fourth, I showed that the detection of luciferase activity caused by reversion mutations was in the linear detection range. As shown in Figure 2.2, when the cell lysate used in the luciferase assay was diluted, the reading of luciferase activity was in the linear detection range even at low levels of luciferase activity (Figure 2.2A). Moreover, when the infected cell number used in luciferase assay was increased, luciferase activity increased in a linear manner (Figure 2.2B), indicating the increase of luciferase activity is correlated to the increase in reversion mutations that occurred during HIV-1 replication.

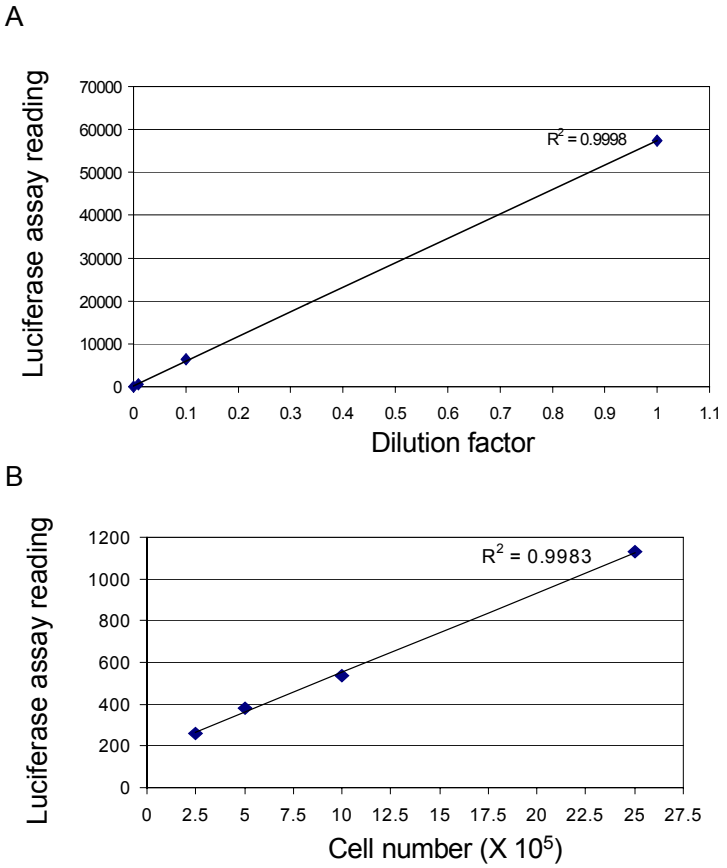



**Figure 2.2.** Detection range of luciferase activity. (A) MAGI cells were transfected with wild type Luciferase. After 48 hr, the transfected cells were lysed in luciferase assay lysis buffer, and cell lysates were diluted to 1, 1:10, 1:100, and 1:1000, and luciferase activity of each sample was determined. (B) Different numbers of MAGI cells were infected with viruses produced from cells transfected with HIV-1SVLuc+$1_{Bru2}$, pSVgagpol-rre-MPMV, and VSV-G. Cells were lysed in luciferase assay lysis buffer, and the luciferase activities were determined.

Finally, the influence of AZT and AZT-resistant RT on virus mutant frequencies was examined using this system. As shown in Table 2.2, AZT led to

a 2.6-fold increase in virus mutant frequency and an AZT-resistant RT increased virus mutant frequency by 2.2-fold. Replication of AZT-resistant virus in the presence of AZT led to a 6.5-fold increase in virus mutant frequencies. Moreover, it was reported that AZT increased the rate of +1 frameshift mutation at the run of T's in *lacZ*$\alpha$ gene by 2-fold (123). These data provide further evidence that this new assay system behaves in a predictable manner and follows the similar trends as previously published data using *lac*Z$\alpha$ as a mutation target (123, 126) (Table 2.2).

| Drug | RT variant | Ave. relative mutant frequency ± SD | |
|---|---|---|---|
| | | *luc*[a] | *lacZ*$\alpha$[b] |
| -- | Wt RT | 1 | 1 |
| AZT | Wt RT | 2.6 ± 0.4 | 7.6 |
| -- | AZT-resistant RT | 2.2 ± 0.5 | 4.3 |
| AZT | AZT-resistant RT | 6.5 ± 0.3 | 24.0 |

**Table 2.2.** The influence of AZT and AZT-resistant RT on HIV-1 mutant frequencies. a: data were obtained by using the new assay system in which luc gene was mutational target. b: previously reported results using *lac*Z$\alpha$ as mutational target (28). Data represent the average of two to three independent experiments. The average luciferase reading for virus replication in the presence of wt RT was $7.0 \times 10^2 \pm 45$.

***Determination of 50% of inhibitory concentrations of antiretroviral drugs***

In order to analyze the effects of antiretroviral drugs on virus mutant frequencies, the $IC_{50}$ values for each drug were first determined (Materials and

Methods, and Figure 2.3). The $IC_{50}$ values of each drug are summarized in Table 2.3. Typically, cells were treated at the $IC_{50}$ concentration of each drug to determine their influence on virus mutant frequencies.

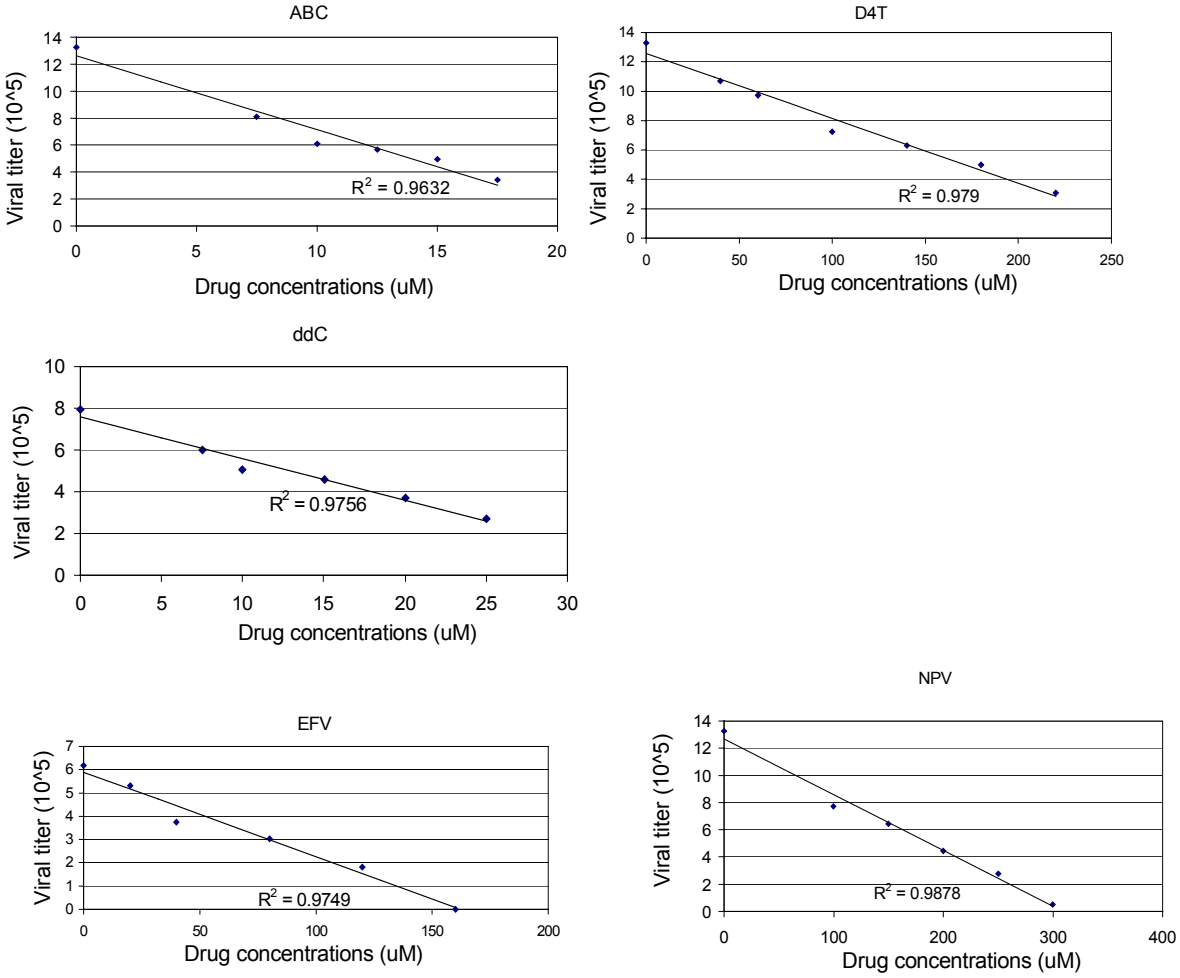



**Figure 2.3**. Linear curves used to determine $IC_{50}$ value of each drug. This is the representative of three independent experiments.

| | Drug | $IC_{50}$ |
|---|---|---|
| NNRTI | Efavirenz (EFV) | 80 μM |
| | Nevirpine (NPV) | 150 μM |
| NRTI | Abacavir (ABC) | 10 μM |
| | Stavudine (D4T) | 140 μM |
| | Zalcitadine (ddC) | 15 μM |
| | Lamivudine (3TC) | 1 μM[a] |

**Table 2.3.** Determined $IC_{50}$ of each drug. All values are determined based on three independent experiments. a: this value was determined in a previous study (123).

***Influence of drug-resistant RT mutants on HIV-1 mutant frequencies***

Previous studies showed that HIV-1 replication with AZT-resistant RTs increased the mutation rate by as much as 4.3-fold, while replication of HIV-1 with a 3TC-resistant RT had no significant effect on mutation rate (123). It was observed that only high-level, AZT-resistant RT variants could influence the in vivo mutation rate (i.e. those containing the mutations M41L/T215Y and M41L/D67N/K70R/T215Y) (123). Moreover, it was found that combined drugs and drug-resistant RTs could further increase virus mutant frequencies (123, 124, 126). To further examine if virus mutant frequencies are influenced by drug-resistant RT variants in the presence of drugs, I initially analyzed a series of drug-resistant RT mutants (4, 68, 77, 101, 154, 165, 195). The mutant enzyme V75T confers resistance to the drug stavudine (D4T); Y115F confers resistance

to abacavir (ABC); L74V/Y115F/M184V confers high-level resistance to ABC and mild resistance to zalcitadine (ddC); G190A, Y318F and K103N/Y318F confer resistance to the drugs efavirenz (EFV) and nevirapine (NPV); and K103N is the most frequently observed mutation in patients treated with drug combination therapy, which includes EFV (Table 2.4).

| Mutants | Drug-resistance | Mutation location |
|---|---|---|
| V75T | D4T | Between the template contact point and the dNTP binding site, finger subdomain |
| K103N | EFV + other drugs | Non-nucleoside inhibitor binding pocket, palm subdomain |
| Y115F | ABC | dNTP binding site, palm subdomain |
| G190A | EFV, NPV | Non-nucleoside inhibitor binding pocket, palm subdomain |
| Y318F | EFV, NPV | Non-nucleoside inhibitor binding pocket, thumb domain |
| K103N/Y318F | EFV, NPV | |
| L74V/Y115F/M184V | ABC, ddC | |

**Table 2.4.** Drug-resistant RTs analyzed.

V75T was previously observed as a novel resistant mutation in cell cultures treated with D4T. I observed that V75T slightly decreased HIV-1 mutant frequencies (0.7-fold) (Table 2.5). The amino acid residues that interact with the incoming dNTP and form the dNTP-binding site have been identified in structural studies. One substitution in the dNTP binding site, Y115A, has been previously

reported to decrease fidelity by a factor by 4 using the *lacZ*$\alpha$ gene (85). We previously observed that Y115A RT variant significantly increased (2.3-fold) virus mutant frequencies using one round of HIV-1 vector replication (124). Moreover, the Y115F and Y115V RT variants were found in *lacZ*$\alpha$ cell-free fidelity assays to have slightly lower error rates than that of wild type RT (24). In this study, I observed that Y115F RT mutant only increased virus mutant frequency by 1.2-fold, which is not significantly different from wild type RT (Table 2.5).

Cell culture selection of resistant mutants has shown that multiple mutations were required to create high levels of resistance to ABC. One triple mutant, L74V/Y115F/M184V, was isolated during an *in vitro* passage experiment. This triple mutant led to a 10-fold increase in $IC_{50}$ of ABC, while it caused a 4-fold decrease in susceptibility to ddC (195). I observed that the L74V/Y115F/M184V RT mutant led to a 1.4-fold increase in HIV-1 mutant frequency (Table 2.5).

Crystallographic analyses of HIV-1 RT and non-nucleoside RT inhibitor complexes have suggested that all NNRTI's occupy a hydrophobic binding pocket that is located in the palm subdomain of p66 and proximal to the polymerase active site (51, 166, 186, 187). NNRTI resistance is associated with mutations within the NNRTI binding pocket. In this study, I tested three single NNRTI-resistant RT mutants, K103N, G190A and Y318F, which all are located in the NNRTI binding pocket, and one double mutant, K103N/Y318F, to determine their influence on virus mutant frequencies. Clinically, the K103N mutation is the most frequently observed mutation in patients treated with efavirenz-containing therapies (4). The G190A mutation is also observed in patients treated with

NNRTI's (77). The Y318F mutation is also associated with a decrease in susceptibility to NNRTI's, and virus containing both Y318F and K103N have higher levels of drug resistance (68). As indicated in Table 2.5, three single RT mutants, K103N, G190A, and Y318F, influenced HIV-1 mutant frequencies by 1.1-fold, 1.0-fold and 0.7-fold, respectively; the K103N/Y318F mutant caused a 2-fold decrease of HIV-1 mutant frequency.

| | RT variant | Ave. relative mutant frequency ± SD |
|---|---|---|
| | Wt RT | 1 |
| NRTI-resistant variants | V75T | 0.7 ± 0.3 |
| | Y115F | 1.2 ± 0.1 |
| | L74V/Y115F/M184V | 1.4 ± 0.2* |
| NNRTI-resistant variants | K103N | 1.1 ± 0.3 |
| | G190A | 1.0 ± 0.04 |
| | Y318F | 0.7 ± 0.3 |
| | K103N/Y318F | 0.5 ± 0.07* |

**Table 2.5.** The influence of drug-resistant RTs on HIV-1 mutant frequencies. Data represent the average of three to six independent experiments. The average luciferase reading for virus replication in the presence of wt RT was 5.5 $\times 10^2 \pm 25$. *: Statistical analysis showed that these data are statistical significant ($p < 0.05$).

### *Influence of subtype E RT mutants on virus mutant frequencies*

HIV-1 is classified into groups and subtypes based on sequences within the *gag* and *env* genes. Three separate groups, M (main), O (outlier), and N (non-M, non-O) exist. The most prevalent strains belong to group M. Group M

also contains at least nine distinct subtypes or clades, (A to D, F to H, J and K), as well as several circulating recombinant forms (i.e. subtype E virus) (115, 171). Much of our current understanding of HIV-1 drug resistance is derived from the studies of subtype B virus, which is the major subtype that circulates in North America and Europe. However, other subtypes, such as A, C, and E, are rapidly expanding worldwide. These variants may differ in rates of transmission, ability to cause progression to AIDS, and drug resistance profiles compared to that of subtype B virus (52, 89). Recently, the analysis of drug resistance profiles of recombinant RT from subtypes E, B, and C demonstrated that each of these RTs possessed similar baseline sensitivity to NRTI's and NNRTI's (164). Therefore, in this study, I analyzed the influence of drug-resistant subtype E RT (ERT) mutants on HIV-1 mutant frequencies.

To assess the influence of ERT mutants on HIV-1 mutant frequencies, a subtype E molecular clone was used (provided by H. Sato, NIH, Japan). This molecular clone was constructed from the 93JP-NH1-virus isolate (p93JP-NH1). Based upon previous studies, RT mutants that confer multiple-drug resistance were introduced by site-directed mutagenesis (173). All ERT mutants, except ERT-mt1, have an insertion mutation that was observed in a virus from a patient treated with multiple NRTI's. The mutants and their drug resistance profiles are listed in Table 2.6. As shown in Table 2.7, the p93JP-NH1 RT influenced the virus mutant frequencies at a level comparable to wild type subtype B RT. None of the ERT mutants studied had a significant influence on HIV-1 mutant frequencies, even though some confer high levels of multi-nucleoside-analog

resistance (i.e. ERT mt-5, ERT mt-6 and ERT mt-7). We have previously shown that the high-level, AZT-resistant RT (i.e. M41L/D67N/K70R/T215Y) could increase the virus mutant frequency by 4.3 fold. In this study, the ERT mt-1 mutant, which contains AZT-resistant mutations of subtype B (M41L/L210W/T215Y) and confers mild resistance to AZT, did not influence the HIV-1 mutant frequency (Table 2.7), supporting our hypothesis that only high-level drug-resistant RT could influence HIV-1 mutant frequency.

| RT variants | Mutation | Drug-resistance |
|---|---|---|
| 93JP-NH1 | None | ND |
| ERT mt-1 | AZT resistance mutations of subtype B (M41L+L210W+T215Y) | AZT, mild |
| ERT mt-2 | Insertion | 3TC, weak |
| ERT mt-5 | Insertion + AZT resistance mutations | AZT (high), 3TC (mild), D4T (weak), ddI (weak) |
| ERT mt-6 | Insertion + AZT resistance mutations + T69I | AZT (high), 3TC (high), D4T (mild), ddI (mild), ddC (weak) |
| ERT mt-7 | Insertion + AZT resistance mutations + T69I and 8 mutations found in an in vivo virus | AZT (high), 3TC (high), D4T (mild), ddI (mild), ddC (weak) |

**Table 2.6.** The list of subtype E RT mutants and their drug resistance profile. a: the insertion is an 11-amino-acid insertion located between codons 67 and 68 in the β3-β4 loop coding region of the RT gene.

| RT variant | Ave. relative mutant frequency ± SD |
|---|---|
| WT RT (B) | 1 |
| p93JP-NH1 | 1.0 ± 0.2 |
| ERT mt-1[a] | 0.9 ± 0.06 |
| ERT mt-2 | 0.9 ± 0.2 |
| ERT mt-5 | 0.9 ± 0.08 |
| ERT mt-6 | 0.9 ± 0.06 |
| ERT mt-7 | 0.7 ± 0.09 |

**Table 2.7.** The influence of subtype E RT mutants on HIV-1 mutant frequencies. Data represent the average of three independent experiments. a: this result is the average of six independent experiments. The average luciferase reading for virus replication in the presence of wt RT was $6.3 \times 10^2 \pm 38$.

***Influence of NRTIs and NRTI-resistant RT variants on virus mutant frequencies***

Previous studies using the *lacZ*$\alpha$ gene as a mutational target have indicated that NRTI's could increase virus mutant frequencies (120, 123, 126). AZT increased the HIV-1 mutation rate by 7.6-fold in a single round of replication, while 3TC increased the virus mutation rate by 3.4-fold (126). A dose-dependent relationship between increased drug concentration and increased virus mutant frequencies has been reported for AZT, 3TC and ddI (120, 126, 129). The maximum increase in virus mutant frequencies in the presence of ddI was 6-fold higher than the virus mutant frequency observed during replication in the absence of drug (120). To further investigate the impact of NRTI's on HIV-1

mutant frequencies, virus mutant frequencies were determined in the presence of drugs, such as ABC, D4T, ddC and 3TC, at $IC_{50}$ concentrations. Like other NRTI's previously studied, both ABC and D4T led to a 2.9-fold and 3.1-fold increase of virus mutant frequencies, respectively. Unexpectedly, dCTP analogs, ddC and 3TC, only increased HIV-1 mutant frequencies by 1.5-fold and 1.2-fold, respectively, at their $IC_{50}$ concentration (Table 2.8). It has been reported that the antiretroviral drugs increase retroviral mutation rate in a dose-depended manner (87, 88, 120, 123). Therefore, one explanation for this observation is that the treatment of target cells with $IC_{50}$ concentration of ddC and 3TC was not sufficient to significantly influence virus mutant frequencies. To further test this hypothesis, the $IC_{90}$ concentration of ddC and 3TC was used. When higher concentrations of ddC and 3TC were used, virus mutant frequencies increased by 2.2- and 2.1-fold, respectively (Figure 2.4), indicating that higher concentrations of ddC and 3TC are needed for these drugs to significantly influence HIV-1 mutant frequencies. It should be noted that based on this and previous data, even greater changes in virus mutant frequencies are predicted when drug concentrations greater than $IC_{50}$ are used.

It has been shown that both RT variants and drugs together could increase virus mutant frequencies (123, 126). To determine if virus mutant frequencies are influenced by the presence of drugs during virus replication with RT variants, the selected NRTI-resistant RT mutants (listed in Table 2.4) were used for virus replication in the presence of different NRTI's. Virus replication with the Y115F RT or the L74V/Y115F/M184V RT in the presence of ABC at its

$IC_{50}$ concentration did not significantly increase virus mutant frequencies compared to those observed during virus replication with the wild type RT in the presence of drug (Table 2.8). Interestingly, I also observed that HIV-1 replication with the L74V/Y115F/M184V RT mutant in the presence of ddC significantly influenced HIV-1 mutant frequencies compared to that observed during virus replication with the wild type RT in the presence of ddC (3.4-fold vs. 1.5-fold). Similarly, in the presence of D4T, V75T RT mutant also affected virus mutant frequencies compared to wild type RT (1.7-fold vs. 3.1-fold) (Table 2.8).

| Drug | RT variant | Ave. relative mutant frequency ± SD |
|---|---|---|
| -- | Wt RT | 1 |
| ABC | Wt RT | 2.9 ± 0.5* |
| ABC | Y115F | 3.1 ± 0.8 |
| ABC | L74V/Y115F/M184V | 3.9 ± 0.8 |
| D4T | Wt RT | 3.1 ± 0.7* |
| D4T | V75T | 1.7 ± 0.2 |
| ddC | Wt RT | 1.5 ± 0.2 |
| ddC | L74V/Y115F/M184V | 3.4 ± 0.8* |
| 3TC | Wt RT | 1.2 ± 0.2 |

**Table 2.8.** The influence of NRTI and NRTI-resistant RTs on HIV-1 mutant frequencies. Data represent the average of three to six independent experiments. The average luciferase reading for virus replication in the presence of wt RT was $5.8 \times 10^2 \pm 26$. *: Statistical analysis showed that these data are statistical significant ($p < 0.05$).

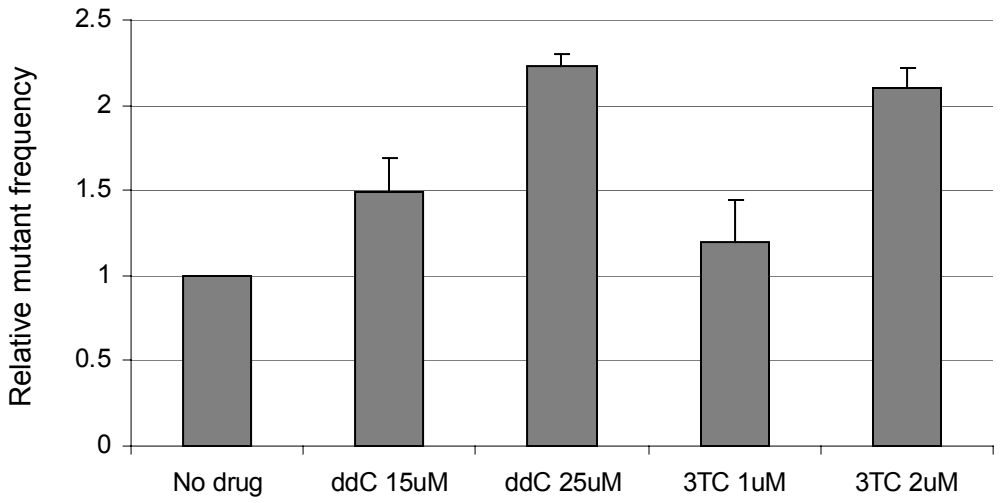



**Figure 2.4.** The increased drug concentrations of ddC and 3TC increased HIV-1 mutant frequencies. Data represent average of three independent experiments. The average luciferase reading for virus replication in the presence of wt RT was $6.0 \times 10^2 \pm 30$.

***Influence of NNRTIs and NNRTI-resistant RT variants on virus mutant frequencies***

NNRTI's inhibit reverse transcription by binding to a hydrophobic pocket that is proximal to the active site of HIV-1 RT (51, 96, 166, 170, 186, 187, 192). Three NNRTI's are currently used for treatment of HIV-1 as part of combination antiretroviral therapy (178). There is nothing known about how NNRTI's affect HIV-1 mutant frequencies. In this study, EFV and NPV and NNRTI-resistant RT variants were used to investigate how NNRTI's and NNRTI-resistant RT variants influence HIV-1 mutant frequencies. The results show that EFV and NPV increased HIV-1 mutation rate by 3.3 and 3.2-fold, respectively. This is the first

report of NNRTI's being able to influence (increase) HIV-1 mutant frequencies. This observation suggests that both NRTI's and NNRTI's have similar influence on HIV-1 mutant frequencies (Table 2.9).

To determine the effects of NNRTI-resistant mutants on the mutant frequencies of HIV-1 in the presence of NNRTI's, virus replication with NNRTI-resistant RT mutants was analyzed in the presence of these drugs. As shown in table 2.9, G190A and Y318F RT mutants did not influence HIV-1 mutant frequencies compared to those observed during virus replication with the wild type RT in the presence of drug. Similarly, the K103N/Y318F mutant also led to a 2-fold decrease in the virus mutant frequencies in the presence of drugs compared to those observed during virus replication with the wild type RT in the presence of drugs.

| Drug | RT variant | Ave. relative mutant frequency ± SD |
|---|---|---|
| -- | Wt RT | 1 |
| EFV | Wt RT | 3.3 ± 0.7* |
| EFV | G190A | 3.3 ± 0.6 |
| EFV | Y318F | 2.5 ± 0.8 |
| EFV | K103N/Y318F | 1.6 ± 0.6* |
| NPV | Wt RT | 3.2 ± 0.6* |
| NPV | G190A | 2.7 ± 0.5 |
| NPV | Y318F | 3.0 ± 0.8 |
| NPV | K103N/Y318F | 1.8 ± 0.5* |

**Table 2.9.** The influence of NNRTI and NNRTI-resistant RTs on HIV-1 mutant frequencies. Data represent the average of three to twelve independent experiments. The average luciferase reading for virus replication in the presence of wt RT $5.9 \times 10^2 \pm 32$. *: Statistical analysis showed that these data are statistical significant ($p < 0.05$).

***Statistical analysis of effects of RT inhibitors and drug-resistant RT mutants on HIV-1 mutant frequencies***

Using Model 1 to analyze our data, it showed that ABC, D4T, EFV, NPV, and L74V/Y115F/M184V were associated with an increase in mutant frequency that is statistically significant. The model also indicated that Y318F and K103N/Y318F were associated with a statistically significant decrease in mutant frequency. Moreover, this model identified that there is a marginally significant, negative interaction between D4T and V75T ($p=0.05$), and the main effect of V75T is just short of being negative and statistically significant ($p=0.06$). Overall, the effect of the combination of D4T and V75T is to increase the mutant frequency by a statistically significant amount, but by an amount that is less than what is expected given the individual effects of D4T and V75T. The use of model 2 led to basically the same conclusions, but provided more accurate estimates of fold changes relative to wild type virus due to the pooling of drug effects within drug classes. First, using this model, we found that both classes of drugs have a positive impact on the fold change of the virus mutant frequencies relative to control (with values of 1.89 for the NRTI's and 2.88 for the NNRTI's). This model found that the maximum likelihood estimate of the variance for the NNRTI group is zero, thus both NNRTI's behave the same in this set of experiments. Since this model smoothes drug effects within drug classes, the effect of ddC (15 μM treatment) became significant. Finally, now V75T has a significant negative main effect and the interaction between V75T and D4T is not significant. However, as

in the simpler model, the effect of the combination of D4T and V75T is to increase the mutant drug frequency by a statistically significant amount.

Given the large number of hypothesis tests being conducted here, there may be some concern about multiple hypothesis testing. To investigate this issue, we can use a Bonferroni correction (which here amounts to not rejecting the null unless that p-value is less than approximately 0.001). Of the statistically significant differences found with model 1, only 2 are not significant when we control the overall type I error rate using this correction: D4T and V75T combination and Y318F. For model 2 the effects that are not significant when using this correction are: Y318F and ddC (15 μM treatment). These data suggest that more experiments are needed for us to better assess how Y318F, V75T and D4T/V75T combination to influence HIV-1 mutant frequencies.
(Statistical analysis was done in collaboration with Dr. Cavan Reilly, University of Minnesota.)

## 2.5 Discussion

Previous studies using the *lacZ*$\alpha$ peptide gene as a mutational target have indicated that both antiretroviral drugs and drug-resistant RTs can increase HIV-1 mutant frequencies (123, 124, 126). In this study, I have analyzed virus mutant frequencies in a single round of replication with an HIV-1 vector containing the *luc* gene. This vector was designed to specifically detect frameshift mutations based on previous *in vitro* and *in vivo* studies (11, 12, 25, 123, 130). The

advantages of this new high-throughput assay system are: 1) it allows for the rapid assessment of the influence of drugs and drug-resistant RT variants on HIV-1 mutant frequencies; 2) the sensitivity of luciferase assay allows for detection of reversion mutations in relatively small sample sizes. The virus mutant frequencies measured are representative based on previous observations made of frameshift mutations created in the presence and absence of drugs or drug-resistant HIV-1 using *lacZ*$\alpha$ (26) and provide a good model for identifying mutations that occur during virus replication. However, in certain instances, this new assay may underestimate mutant frequency change because this HIV-1 vector was designed to only detect frameshift mutations and not the entire spectrum of mutations. One case in point is when I compared the influence of AZT and AZT-resistant RT mutant on virus mutant frequencies. In this comparison, AZT and AZT-resistant RT led to a lower mutant frequency using *luc* versus the *lacZ*$\alpha$ reporter (Table 2.2), indicating that there was an underestimate of the effects of drugs and drug-resistant RTs on virus mutant frequencies. Since our primary objective was to quickly assess the influence of drugs and drug-resistant RT mutations on HIV-1 mutant frequencies during virus replication, I took advantage of this new assay system. This system could be used to identify interesting experimental conditions that could subsequently be analyzed in greater detail using the *lac*Z$\alpha$ system.

A series of amino acid substitutions were created in RT to determine their influence on virus mutant frequencies. The amino acid residues were chosen based on their association with drug resistance in previous *in vitro* and *in vivo*

studies. It was reported that only high-level, AZT-resistant RT variants could influence the *in vivo* mutation rate (i.e. M41L/T215Y and M41L/D67N/K70R/T215Y). Therefore, we were interested in determining whether increased drug resistance generally correlates with an increase in virus mutant frequencies. All single mutants I studied did not have significant influence on HIV-1 mutant frequencies. An NNRTI-resistant RT mutant (i.e., K103N/Y318F) that confers higher drug resistance than each single RT mutant was observed to significantly decrease virus mutant frequencies by 2-fold. This observation is the first report to show that an NNRTI-resistant RT mutant can influence HIV-1 mutation. Moreover, I also observed that the L74V/Y115F/M184V RT mutant, which confers higher resistance to ABC than each individual mutant, significantly altered HIV-1 mutation. These data indicate that there may be a correlation between increased drug resistance and the ability of drug-resistant RTs to influence virus mutant frequencies.

Previous crystallographic studies showed that not all NRTI-resistant mutants map to the vicinity of the putative dNTP binding-site. Instead, some NRTI-resistant mutants are dispersed over the entire fingers subdomain (76). For example, Y115F and M184V are located in the dNTP binding-site of RT, whereas L74V and V75T contact the n+1 templating base. In this study, I observed that Y115F RT mutant led to slightly higher HIV-1 mutant frequency than that of wild type RT, while V75T RT mutant slightly decreased virus mutant frequency (Table 2.5). It is possible that RT mutants that are located in the dNTP binding-site could affect the rate of misincorporation and mismatch extension, and subsequently to

influence viral mutagenesis. Additionally, mutations that have been noted to contact the n+1 templating base could influence virus mutant frequencies by repositioning the active site in an unfavorable orientation for incorrect dNTP incorporation (192). It has been shown that the RT mutants V148I and Q151N, which are in direct contact with the incoming dNTP, increased RT fidelity 8.7- and 13-fold, respectively, as measured by the M13 *lacZ*$\alpha$ forward mutation assay (207). Using a cell-based pseudotyped HIV-1 mutation assay, it was found that the V148I and Q151N mutant viruses had 3.8- and 5.7-fold higher fidelities than wild-type viruses, respectively (207), suggesting that the molecular interaction between HIV-1 RT and the dNTP substrate contributes to viral mutagenesis. Moreover, another substitution in the dNTP binding site, Y115A, has been previously reported to decrease fidelity by a factor by 4 using the *lacZ*$\alpha$ gene (85). We previously observed that Y115A RT variant significantly increased (2.3-fold) virus mutant frequencies using one round of HIV-1 vector replication (124). These data suggest that HIV-1 mutant frequencies measured in a cell-based system are lower than that measured in a cell-free assay system. Therefore, use of a cell-free fidelity assay to study how V75T and L74V/Y115F/M184V influence HIV-1 RT fidelity might help to provide a greater understand of the role of interactions between HIV-1 RT and the dNTP substrate or templating base in viral mutagenesis. Unlike NRTI-resistant RTs, NNRTI-resistant RTs are located within the hydrophobic NNRTI-binding pocket, which could cause the conformational change of the NNRTI-binding pocket. Therefore, it is possible that NNRTI-resistant RT mutants could indirectly affect the conformation of the HIV-1

RT active site, and subsequently influence HIV-1 mutant frequencies. Biochemical studies of how NNRTI-resistant RT mutants to influence miscorporation, mismatch extension and processivity of RT would also help to understand how NNRTI-resistant RTs affect viral mutagenesis.

The analysis of drug-resistant subtype E RT (ERT) mutants on HIV-1 mutant frequencies showed that these RT mutants did not significantly influence HIV-1 mutant frequencies. These mutants all contain the insertion of 11 amino acids between codons 67 and 68 in the β3-β4 loop coding region of the RT gene, except ERT-mt1 (173). The insertion mutation alone did not confer drug resistance, but greatly increases drug resistance based upon preexisting drug resistance mutations under the selective pressure of NRTI's, suggesting that the emergence of this insertion mutation in patients treated with multiple antiretroviral drugs is an evolutionary survival strategy, which is under the strong selective pressure (173). Therefore, it is possible that the increased resistance to NRTI's of these ERT mutants that contain this insertion mutation is due to a fitness advantage and not due to an affect on RT fidelity.

The influence of ABC, D4T, ddC and 3TC on HIV-1 mutant frequencies was also investigated. ABC and D4T led to 2.9-fold and 3.1-fold increase in the virus mutant frequencies. However, the dCTP analogs ddC and 3TC had no effect on virus mutant frequencies at lower concentrations. Higher concentrations of both drugs were needed to significantly influence HIV-1 mutant frequencies. These data suggest that the NRTI's currently used in drug therapy can increase virus mutant frequencies. Since the HIV-1 vector used in this study was designed

to specifically detect frameshift mutations, I did not analyze the whole spectrum of mutations. Thus, it is possible that dCTP analogs could lead to a biased spectrum of mutations in HIV-1 (e.g., higher proportion of base substitutions) compared to other NRTI's. The proposed mechanisms of mutations that occur during reverse transcription are: 1) one-base frameshifts at template runs created by a strand slippage mechanism; 2) single-base substitutions generated by dislocation mutagenesis; 3) single-base substitutions by polymerase miscoding; and 4) one-base frameshifts at non-run sites, which may be initiated by base misinsertion (11). This may suggest that dCTP analogs have different effects on HIV-1 RT compared to other NRTI's, which could contribute to a biased mutation spectrum. We have previously reported that virus replication in the presence of 2 NRTI's (one of which is 3TC) increased HIV-1 mutant frequencies (120), suggesting that NRTI's influence HIV-1 mutant frequencies by a similar mechanism, but perhaps with a different mutational spectrum.

Previously, AZT and 3TC were found to increase virus mutant frequencies of HIV and other retroviruses (87, 123). Hypotheses proposed to explain the increased mutant frequency of AZT were: 1) AZT alters nucleotide pools; 2) AZT is incorporated into plus-strand DNA and may result in discontinuous DNA synthesis of viral DNAs with proper ends that integrate into the target cell DNA; subsequently, host cell may carry out error-prone DNA repair and increase the virus mutation rate; and 3) AZT may bind noncatalytically to RT and cause a conformational change that influences enzyme fidelity (87). AZT has been shown to affect nucleotide pools by competing with thymidine for the host cell enzyme

thymidylate kinase (TK) (58, 60). The pool imbalances result in increased levels of dCTP and decreased levels of dTTP and dGTP. In addition, the role of natural dNTP pool imbalances as a determinant of replication fidelity and specificity was shown in studies using *lacZ*$\alpha$ as a mutation target during phagemid replication (216). This suggests that the intracellular dNTP pool concentrations may influence both specificity and mutant frequency during retroviral replication as well. Although the influence of AZT on the SNV and MLV mutation rates has been shown to be due to a mechanism not involving alterations of dNTP pools (88), it is possible that this is not the case with HIV-1 (143, 200). The influence of antiretroviral drugs on intracellular dNTP pools is discussed in Chapter 3 of this dissertation.

I also observed that some drug-resistant RT mutants (i.e. V75T and L74V/Y115F/M184V) alone have no or very small effect on the virus mutant frequencies, while they could lead to larger effects on HIV-1 mutant frequencies compared to wild type RT in the presence of drugs. These data imply that the mechanisms responsible for the influence of virus mutant frequency due to RT and drugs are likely independent of each other. Therefore, it is likely that these two independent mechanisms acting together could have a significant influence on the virus mutant frequency, while the RT mutants alone had no or very small effect on HIV-1 mutation.

This is the first report of NNRTI's being able to increase HIV-1 mutant frequencies. Both EFV and NPV led to a 3.3-fold and 3.2-fold increase of virus mutant frequencies, respectively. These data suggest that NNRTI's have similar

effects on HIV-1 mutagenesis compared to NRTI's. The mechanism of how NNRTI's increase HIV-1 mutant frequencies is presently unknown. One hypothesis is that the conformational change caused by NNRTI's binding noncatalytically to RT may affect enzyme fidelity. Crystallographic studies have shown that NNRTI's cause a repositioning of the three-stranded β-sheet in the p66 subunit (containing the catalytic aspartic acid residues 110, 185 and 186), and there is a striking similarity between the actual conformations of the three-stranded β-sheet in the drug-bound p66 conformation and in the inactive p51 conformation (51). This suggests that the NNRTI's inhibit HIV-1 RT by locking the active catalytic site in an inactive conformation, reminiscent of the conformation observed in the inactive p51 subunit (51). This conformational change has a dramatic effect on the rate of the chemical step (transfer of dNMP to the end of primer molecule) of polymerization (170). Hence, the conformational change of the RT active site caused by NNRTI's could influence either nucleotide selectivity or RT processivity, which would lead to lower fidelity of NNRTI-bound RT compare to wild-type RT.

# CHAPTER 3

# INFLUENCE OF COMBINED DRUGS AND dNTP POOLS ON HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) MUTANT FREQUENCIES

## 3.1 Abstract

Combination therapy, comprising at least three anti-HIV drugs, has been used to treat AIDS or HIV-infected patients. To assess the impact of combination therapy on HIV-1 mutagenesis, the mutagenic outcome of combined drug treatment was determined. Significant increases in HIV-1 mutant frequencies were observed with combinations of NNRTI's as well as in NRTI-NNRTI combinations. I observed that the combinations of NNRTI's and NRTI-NNRTI led to higher HIV-1 mutant frequencies compared to that observed in the presence of individual drugs. This indicates that combinations of RT drugs can act together to increase HIV-1 mutant frequencies, which would have important implications for drug therapy regimens. How antiretroviral drugs influence HIV-1 mutant frequencies is unclear. It has been demonstrated that the imbalanced intracellular dNTP pools are associated with the increase of mutation rate during *in vitro* reverse transcription. In this study, I determined the effects of *in vivo* dNTP pool imbalances on HIV-1 mutant frequencies in a single round of

replication. The novel ribonucleotide reductase inhibitors, didox (DX) and trimidox (TX), increased HIV-1 mutant frequencies by 9.5- and 2.7-fold, respectively. Furthermore, virus replication in quiescent PBMCs led to higher mutant frequency than virus replication in both activated PBMCs and MAGI target cells. These results suggest that dNTP imbalances are associated with an increase in virus mutant frequencies. Therefore, we hypothesize that antiretroviral drugs increase HIV-1 mutant frequencies through induction of dNTP pool imbalances. To test this hypothesis, MAGI target cells were treated with antiretroviral drugs, and *in vivo* dNTP pools of MAGI cells were measured. Antiretroviral drugs induced dNTP pool imbalances, demonstrating that there is a correlation between increased HIV-1 mutant frequencies and alterations of dNTP pools during antiretroviral drug treatment.

## 3.2 Introduction

Retrovirus populations demonstrate high levels of variation (30, 194). The studies of human immunodeficiency virus type 1 (HIV-1) dynamics suggest that approximately $10^4$ to $10^5$ mutations arise at each nucleotide position every day in an HIV-1 infected patient (30). The emergence of drug-resistant virus, resulting from genetic variation in the viral genome under selective pressure of a particular drug, is a major cause of failure of monotherapy. There is no currently available drug that is an effective inhibitor of HIV-1 replication to which the virus does not rapidly develop resistance when it is used in monotherapy. It is likely that a certain threshold level of virus replication is required to generate appropriate

mutations that cause resistance to multiple drugs. Therefore, effective and durable suppression of viral replication needs the use of drug combinations. In addition to prevent the emergence of drug-resistant virus, drug combinations can be additive or synergistic so that blocks HIV-1 replication more extensively than individual drugs. Some *in vitro* studies have shown that nucleoside RT inhibitors (NRTI's) can be synergistic with NRTI's (50, 142), non-nucleoside RT inhibitors (NNRTI's) (9, 167), and protease inhibitors (PI's) (84). Combination therapy that targets different sites may also prevent the breakthrough of viruses that have become resistant to particular drugs (196). Currently, potent antiretroviral therapy (ART), comprising at least three anti-HIV drugs, has become the standard treatment of AIDS or HIV-infected patients (39, 157, 168).

Previously, it has been reported that the NRTI's, zidovudine (AZT) and lamivudine (3TC), increased HIV-1 mutant frequencies by 7.6-fold and 3.4-fold in a single round of replication, respectively (123). Moreover, AZT could increase the SNV mutation rate by 10-fold, whereas AZT increased the MLV mutation rate only 3-fold (87). Recent studies have demonstrated that two NRTI's (i.e. AZT and 3TC, AZT and ddI, and 3TC and ddI) can act together to increase HIV-1 mutation frequencies in an additive manner during virus replication (120, 126), suggesting that NRTI's use a similar mechanism to increase HIV-1 mutant frequencies. How NRTI's influence HIV-1 mutant frequencies is unknown. However, there are several potential mechanisms, including the alteration of intracellular deoxyribonucleoside triphosphates (dNTPs) concentrations.

It has been shown that the ability of some drugs to influence retrovirus mutation rate is associated with alteration of dNTP pools. Hydroxyurea (HU), a well documented drug used in HIV-1 treatment, is known to alter intracellular dNTPs by inhibiting ribonucleotide reductase, resulting in a depletion of dNTPs with the most significant reductions in the dATP pool (61, 143). Subsequent studies have shown that when HIV-1 replicates in the presence of HU, the virus mutant frequency increased by 4.5-fold (126). Like HU, thymidine (Thy), which has also been well documented to alter intracellular dNTP pools, increased HIV-1 mutant frequency by 7.0-fold (126). When SNV or MLV infected D17 target cells were treated with HU or Thy, the correlation between dNTP pools imbalances and increased mutation rates were also observed (88). These data demonstrate that dNTP pool imbalances are associated with an increase of *in vivo* retroviral mutant frequencies. Furthermore, studies of the influence of AZT on intracellular dNTP pools in some cell lines has shown that AZT can induce dNTP pool imbalances (58, 60). However, AZT treatment of D17 cells did not have influence on intracellular dNTP pools, suggesting that the influence of AZT treatment on the SNV and MLV mutation rates occurs by a mechanism not involving alterations in dNTP pools (88). The effect of NRTI's on nucleotide pools has not been extensively studied in different cell lines or in primary lymphocytes and macrophages, it is plausible that these drugs could influence dNTP pools in particular cell types.

There is a substantial body of *in vitro* and *in vivo* data demonstrating that imbalanced dNTP pools correlate with the fidelity of DNA replication (15, 99, 112,

216). For example, the frameshift fidelity of DNA synthesis is affected by perturbation in the relative concentrations of dNTPs present in a polymerization reaction (15). It has been known for some time that the process of reverse transcription is particularly sensitive to the type of host cell, which has been demonstrated to reflect differences in the intracellular dNTP pools (61, 200, 213). Previous studies have shown that G-to-A hypermutation of the HIV-1 genome is associated with the transient depletion of intracellular dCTP concentration (133, 134, 200, 201). In addition, the perturbation of dNTP pools can either restrict or enhance HIV-1 replication (143). The study of 3TC-resistant variants demonstrated that the processivity defect of 3TC-resistant RT is predominant at limiting dNTP levels (5).

In an effort to understand the influence of dNTP pool imbalances on *in vivo* HIV-1 mutant frequencies and to test our hypothesis that antiretroviral drugs increase HIV-1 mutant frequencies by altering intracellular dNTP pools, I first examined the effects of two novel ribonucleotide reductase inhibitors (RRI's, didox and trimidox) on HIV-1 mutant frequencies in MAGI cells. I also compared the virus mutant frequencies when HIV-1 replicates in different cell types (i.e. quiescent PBMCs vs. activated PBMCs vs. MAGI cells). The results suggest that the increased HIV-1 mutant frequencies are associated with dNTP pool imbalances. Moreover, we also observed that drug treatments consisting of NRTI's, NNRTI's and RRI's could decrease intracellular dNTP concentrations.

In this study, I also analyzed the impact of various drug combinations on HIV-1 mutant frequencies. It was observed that virus replication in the presence

of NNRTI's combination led to higher mutant frequencies than that observed in the presence of each individual drug. Virus replication in the presence of an NRTI and an NNRTI combination also further increased virus mutant frequencies. This is the first report to show that NRTI and NNRTI together can further influence HIV-1 mutant frequencies. This indicates that combination drug therapy increases HIV-1 mutant frequencies, which could have important implication for long-term administration of anti-HIV chemotherapy.

## 3.3 Materials and Methods

### *Retroviral vector, expression plasmids, and antiretroviral drugs*

All the retroviral vector and expression plasmids used in this study have been described in Chapter 2. EFV, NPV, and D4T were obtained from NIH AIDS Research Reagents Program (Bethesda, MD). 3TC was purchased from Sigma (St. Louis, MO). Didox and trimidox were obtained from Dr. Howard Elford (Molecules for Health, Inc., Richmond, VA).

### *Cell culture, transfections and infections*

The 293T and MAGI cell lines were obtained from ATCC. 293T cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) (GIBCO BRL, Gaithersburg, MD), supplemented with 10% of Fetal clone III serum (Hyclone. Logan, UT). MAGI cells were maintained in DMEM

supplemented with 10% of Fetal clone III serum in the presence of G418 (0.2 mg/ml) and hygromycin (0.1 mg/ml).

The HIV-1 vector, HIV-SVLuc+1$_{bru2}$ (9.5 μg), was co-transfected with pSVgagpol-rre-MPMV (9.5 μg) and VSV-G (1 μg) into 293T cells in a 100 mm petri dish using the calcium phosphate precipitation method. Viruses were harvested 48 hr posttransfection and concentrated using Centricon Plus-20 (Millipore, Billerica, MA) following manufacturer's instruction. To perform infection, concentrated viruses were mixed with Polybrene (8 μg/ml) (Sigma, St. Louis, MO) and virus-polybrene mixture was used to infect $2.5 \times 10^5$ fresh MAGI target cells. After 24 hr, the medium was removed, cells were washed once with $1 \times$ PBS (GIBCO BRL), and the fresh medium was added to the cells. Seventy-two hr postinfection, MAGI assay was performed to determine the viral titer and a luciferase assay was used to detect the restored luciferase activity (detail is described in Chapter 2 Materials and Methods).

***Isolation and infection of primary peripheral blood lymphocytes (PBMCs)***

PBMCs from HIV-1 seronegative donors were isolated by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ). PBMCs were incubated in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% Fetal clone III serum in the presence or absence of 0.5μg/ml phytohemagglutinin (PHA) (Sigma, St. Louis, MO). Subsequently, the cells were infected with virus-

polybrene mixture described above. After 24 hr, cells were washed once with 1 × PBS, and grown in 10% Fetal clone III RPMI 1640 containing 20U/ml human recombinant IL-2 (Sigma, St. Louis, MO). The cells were incubated for additional 48 hr under same conditions. Then, cells were lysed in 1 × lysis buffer of luciferase assay. Luciferase activity was measured by using the Promega Luciferase Assay System (Promega, Madison, WI).

### *Analysis of HIV-1 mutant frequencies*

The protocol for analyzing HIV-1 mutant frequencies has been described in Chapter 2. Briefly, the relative mutant frequencies were calculated based on the reversion mutation that was detected by the restored luciferase activity in the MAGI cells, viral titer and cell numbers. The relative mutant frequency of wild type viruses that was not treated with drug was defined as 1. The relative mutant frequencies of drug-treated viruses were compared to this value.

### *Determination of inhibitory concentrations*

MAGI assay was used to determine the $IC_{50}$ of individual drugs and drug combinations. Briefly, $2.5 \times 10^5$ fresh MAGI target cells were treated with individual drugs or different combinations of antiretroviral drugs 2 hr prior to infection and continued until 24 hr after infection. Concentrated virus-polybrene mixture was diluted to 1:1000 and used to infect MAGI target cells. Seventy-two hr postinfection, infected MAGI cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galacosidase actopyranoside (X-gal) and positive blue cells were

counted to determine viral titer at each concentration of individual drugs or at different drug combinations. The viral titer was plotted as a function of the drug concentration used, generating linear curves for all the individual drugs. $IC_{50}$ values for each individual drug were calculated based on the linear curves. Drug combinations, which could inhibit 50% or higher of viral infectivity, were used in the following experiments (Figure 3.1 and 3.2A).

***dNTP extraction from cells***

dNTPs were extracted from MAGI cells treated with antiretroviral drugs. For each measurement, $2 \times 10^6$ cells per 100 mm petri dish were plated the day before the drug treatment. The cells were incubated with antiretroviral drugs for 2 hr and 24 hr. The cells were then harvested, counted, and extracted in 100 μl of ice-cold 60% methanol. Samples were vortexed vigorously to lyse the cells and then heated at 95$^{o}$C for 3 minutes, prior to centrifugation at 12,000$\times$ g for 30 s. The supernatants were collected and completely dried under vacuum, using a SpeedVac (Savant, NY) with medium heat. The dried pellets were subsequently resuspended in dNTP buffer (50 mM Tris-Cl, pH 8.0 and 10 mM $MgCl_2$; 25 μl for $1 \times 10^6$ cells). Two microliter of the extracted dNTP samples were used for each single nucleotide incorporation reaction.

***Single nucleotide incorporation assay***

Four different 19-mer DNA templates containing sequence variations (N) at the 5' end nucleotide (5'-NTGGCGCCCGAACAGGGAC -3') were individually

annealed to an 18-mer DNA primer (5'-GTCCCTGTTCGGGCGCCA-3'), $^{32}$P-labeled at its 5' end (template:primer, 4:1). The primer (160 pmole in 80μl) was labeled using 40 units of T4 polynucleotide kinase (New England BioLabs, Inc., Beverly, MA) with 80 μCi α-$^{32}$P-ATP (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min at 37°C. An additional 40 units of T4 polynucleotide kinase was added for an additional 30 min period of labeling. After heat inactivation (95°C, 10 min), the labeled primer was annealed with each of the four 19-mer templates (160 pmole) with addition of 10 x STE (100 mM NaCl and 5 mM EDTA for 1x) in a final volume of 50 μl. The template-primer mixture was incubated for 10 min at 95°C, 5 min at 55°C, 5 min at 22°C and on ice until used.

The template/primer pairs (T/Ps) were extended using RT proteins in a standard dNTP assay reaction. Each assay reaction (20 μl) contained 0.2 pmole T/P (10nM, primer concentration), 2 μl appropriate dNTPs (Amersham Pharmacia Biotech, Piscataway, NJ) or extracted cellular dNTPs, 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, 5 μM $(dT)_{20}$, 0.1 mg/ml bovine serum albumin (New England BioLabs, MA). Reactions were initiated by addition of excess RT proteins (60 nM) in relation to [dNTP] (0.2~6.4 nM or 4~128 fmole in 20 μl), and incubated at 37°C for 5 min. Reactions were terminated with 10 μl of 40 mM EDTA, 99% formamide. Reaction products were immediately denatured by incubating at 95°C for 5 min and 4 μl of each 30 μl final reaction mixture was quantitated by PhosphorImager analysis (PerkinElmer, MA) of 14% polyacrylamide-urea denaturing gels.

### *Standard curves for single nucleotide incorporation*

The gels obtained from the incorporation assay were subjected to PhosphorImager analysis. The percent of primer extension in each reaction was calculated by determining the ratios of extended (19-mer) versus total (extended [19-mer] + unextended [18-mer]) primers. Each signal for the extended products was normalized using the background signal in the control reactions incubated without RT. The calculated percentage primer extension was plotted from triplicate reactions, as a function of the dNTP quantity used, generating standard curves for all four dNTPs (Figure 3.4). The percentage primer extension determined in triplicate reactions containing extracted cellular dNTPs was then extrapolated to the standard curves in order to determine the dNTP contents present in the cellular samples.

### *Statistical analysis*

The models of statistical analysis used in this study have been described in Chapter 2. Statistical analysis was done in collaboration with Dr. Cavan Reilly, University of Minnesota.

## 3.3 Results

### *Determination of inhibitory concentration for each drug combination*

In order to analyze the influences of drug combinations on mutant frequencies of HIV-1, various combinations of individual drugs for each drug combination were used to determine viral titers. The concentration of each individual drug was chosen based on previous observations (Chapter 2). The inhibitory concentrations of drug combinations were determined based on viral titers (Figure 3.1). The concentrations of individual drugs used in each combination in the following experiments are summarized in Table 3.1.

| Drug combination | Inhibitory concentrations |
|---|---|
| Efavirenz (EFV) and Stavidine (D4T) | EFV 80 μM and D4T 140 μM[a] |
| Efavirenz (EFV) and Lamivudine (3TC) | EFV 80 μM and 3TC 1 μM[b] |
| Efavirenz (EFV) and Nevirpine (NPV) | EFV 40 μM and NPV 100 μM[c] |

**Table 3.1.** Inhibitory effect of each drug combination. Data represent the average of three independent experiments. a: this combination of drugs inhibits 90% of HIV-1 replication capacity; b: this combination of drugs inhibits 70% of HIV-1 replication capacity; c: this combination of drugs inhibits 50% of HIV-1 replication capacity.

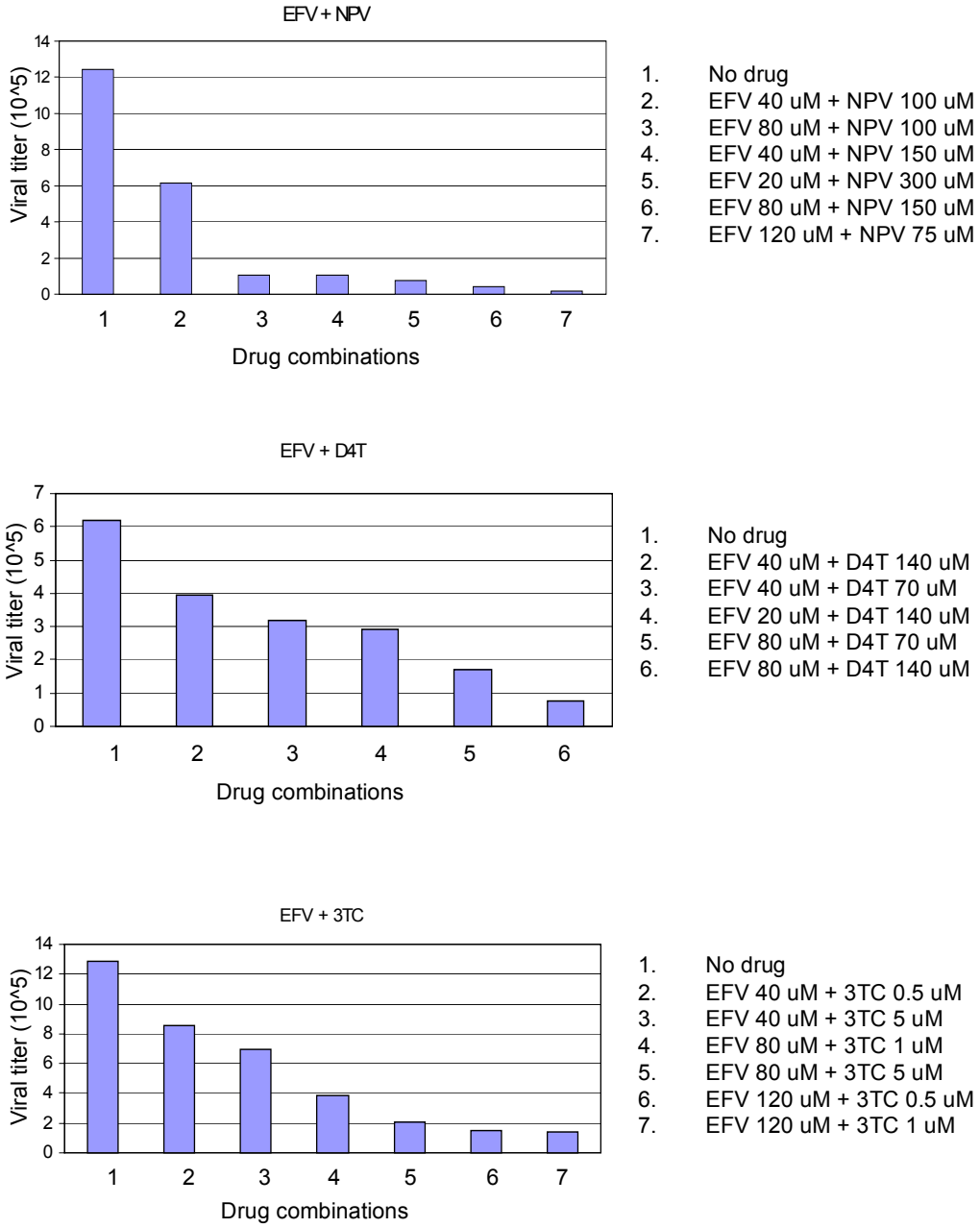



**Figure 3.1**. Determined viral titers at different drug combinations. The concentrations of individual drugs used in the following experiments were chosen based on the effects of the individual drugs and combined drugs on virus infectivity. This is the representative of three independent experiments.

### *The effects of combined drugs and HIV-1 RT variants on virus mutant frequencies*

Potent antiretroviral therapy (ART) regimens include drugs from two of the three classes of antiretroviral drugs (NRTI's, NNRTI's, and PI's). There are four 2-NRTI combinations typically used in ART, i.e., AZT plus 3TC, AZT plus ddI, D4T plus ddI and D4T plus 3TC (178). It has been reported that an additive increase in virus mutant frequencies was observed when virus replicated in the presence of NRTI combinations (i.e. AZT plus ddI, AZT plus 3TC and 3TC plus ddI) (120). In this study, various combinations of drugs (NNRTI plus NNRTI, NNRTI plus NRTI) were studied for their ability to act together to influence HIV-1 mutant frequencies during virus replication. First, the drug combination of EFV plus NPV (NNRTI plus NNRTI) was tested. Based upon the effects of the individual drugs and combined drugs on virus infectivity, an EFV concentration of 40 μM was used along with a NPV concentration of 100 μM. For each drug alone, these concentrations inhibited 25% of virus replication. It was observed that EFV and NPV together led to an increase of the virus mutant frequency by 5.8-fold, while virus replication in the presence of EFV or NPV alone led to 1.4-fold and 2.6-fold increase in the virus mutant frequencies, respectively (Table 3.2). The statistical analysis showed that EFV and NPV together significantly increased virus mutant frequencies compared to that observed for each drug alone.

| Drug | RT variant | Ave. relative mutant frequency ± SD |
|---|---|---|
| -- | Wt RT | 1 |
| -- | Y318F | 0.7 ± 0.3 |
| EFV 40 μM | Wt RT | 1.4 ± 0.2 |
| NPV 100 μM | Wt RT | 2.6 ± 0.1 |
| EFV 40 μM + NPV 100 μM | Wt RT | 5.8 ± 0.9 |
| EFV 40 μM | Y318F | 1.4 ± 0.2 |
| NPV 100 μM | Y318F | 2.7 ± 0.3 |
| EFV 40 μM + NPV 100 μM | Y318F | 4.9 ± 0.7 |

**Table 3.2.** The influence of EFV and NPV combination and drug-resistant RT on HIV-1 mutant frequencies. Data represent the average of three to twelve independent experiments. The average luciferase reading for virus replication in the presence of wt RT was $5.7 \times 10^2 \pm 33$.

Next, the clinically used drug combinations of NNRTI and NRTI were analyzed, which included EFV plus D4T and EFV plus 3TC. The 80 μM concentration of EFV was used in combinations with 140 μM of D4T or 1 μM of 3TC. These drugs act together inhibited the virus replication by 90% and 70%, respectively, while for each individual drug, these concentrations led to 50% decrease of viral titers. As shown in Table 3.3, EFV and D4T together could increase HIV-1 mutation rate by 8.3-fold, while virus replication in the presence of EFV or D4T alone led to 3.3-fold and 3.1-fold increase in the virus mutant frequencies, respectively. The statistical analysis indicated that EFV and D4T combination is associated with a statistically significant increase in HIV-1 mutant

frequencies compared to that observed for each drug alone. This indicates that NNRTI and NRTI act together could further increase the HIV-1 mutant frequency. Unexpectedly, the virus mutant frequency was only increased by 3.7-fold when virus replicated in the presence of both EFV and 3TC, which is similar to the virus mutant frequency caused by EFV alone (Table 3.3). This is because 3TC did not have significant influence on virus mutant frequency at lower concentration.

Since during the course of antiviral therapy there are HIV-1-infected individuals harboring viruses with drug resistance mutations, the effects of drug-resistant mutants and combined drugs on HIV-1 mutant frequencies were analyzed. It has been shown that the K103N mutant emerged in the patients treated with efavirenz-containing therapy (4), and the Y318F mutant was associated with a decrease in susceptibility to all NNRTI's (68). Therefore, the abilities of K103N and Y318F to affect the virus mutant frequencies in the presence of combined drugs were tested. In the presence of combined drugs, the K103N mutant did not have significant influence on virus mutant frequencies compared with the mutant frequency observed for virus replication with wild type RT in the presence of drugs (Table 3.3). Furthermore, the replication of the Y318F RT mutant in the presence of both EFV and NPV did not have significant influence on HIV-1 mutant frequencies compared to the replication of wild type RT in the presence of both EFV and NPV (Table 3.2).

| Drug | RT variant | Ave. relative mutant frequency ± SD |
|---|---|---|
| -- | Wt RT | 1 |
| EFV 80 μM | Wt RT | 3.3 ± 0.7 |
| D4T 140 μM | Wt RT | 3.1 ± 0.7 |
| 3TC 1 μM | Wt RT | 1.2 ± 0.2 |
| EFV 80 μM + D4T 140 μM | Wt RT | 8.3 ± 0.2 |
| EFV 80 μM + 3TC 1 μM | Wt RT | 3.7 ± 0.7 |
| -- | K103N | 1.1 ± 0.3 |
| EFV 80 μM | K103N | 3.5 ± 0.8 |
| D4T 140 μM | K103N | 2.5 ± 0.6 |
| 3TC 1 μM | K103N | 1.1 ± 0.6 |
| EFV 80 μM + D4T 140 μM | K103N | 8.7 ± 1.2 |
| EFV 80 μM + 3TC 1 μM | K103N | 3.5 ± 0.1 |

**Table 3.3.** The effects of combinations of NRTI and NNRTI and drug-resistant RT on HIV-1 mutant frequencies. Data represents the average of three to twelve independent experiments. The average luciferase reading for virus replication in the presence of wt RT was $5.9 \times 10^2 \pm 34$.

### *Ribonucleotide reductase inhibitors, Didox and Trimidox, increased HIV-1 mutant frequencies*

Previous studies showed that HU and Thy, which are well documented to alter intracellular dNTP pools, could increase HIV-1 mutant frequencies (126). To extend this study, two new ribonucleotide reductase inhibitors (RRI's), didox (DX) and trimidox (TX), were used to study whether the alteration of dNTP pools influence HIV-1 mutant frequencies. It has been shown that DX and TX have

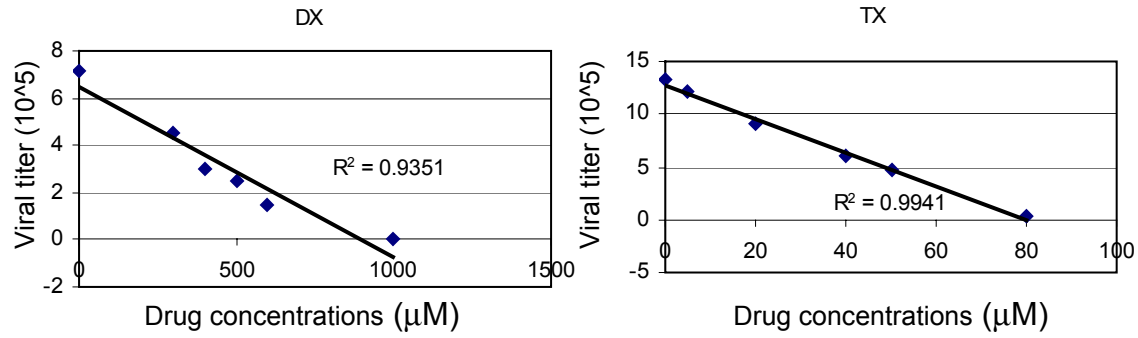
several advantages over HU. First, these two compounds inhibit enzyme activity of ribonuleotide reductase more effectively than HU (46). Second, these two compounds have been shown to have antitumor activity in various mouse tumor models (47). Third, both DX and TX are less toxic to the hematopoietic system of mice than HU (138). Recently, using murine AIDS model system showed that DX and TX had anti-retroviral activity both alone or in combination with ddI (136, 137).

To determine whether treatment of cells with DX and TX would increase virus mutant frequencies, the 50% inhibitory concentrations of both DX and TX were first determined based on viral titers (Figure 3.2A and B). DX and TX treatment was next tested to see if they could increase virus mutant frequencies. As indicated in Figure 3.2C, treatment of MAGI cells with 350 μM of DX or 40 μM of TX significantly increased virus mutant frequencies by 9.5-fold and 2.7-fold, respectively, suggesting that the increased error rate of reverse transcription may be due to the alteration of dNTP pools.

### *Mutant frequencies in quiescent and activated PBMCs*

Primary blood lymphocytes and macrophages are the natural target cells for HIV. In earlier studies, it was found that the dNTP levels in quiescent PBMCs were significantly lower than in the stimulated PBMCs (61). At these low dNTP concentrations, HIV-1 viral DNA synthesis is slower and less efficient in quiescent PBMCs than in activated PBMCs. Furthermore, HU treatment of

A




B

| Drug | $IC_{50}$ |
|---|---|
| Didox (DX) | 350 μM |
| Trimidox (TX) | 40 μM |

C

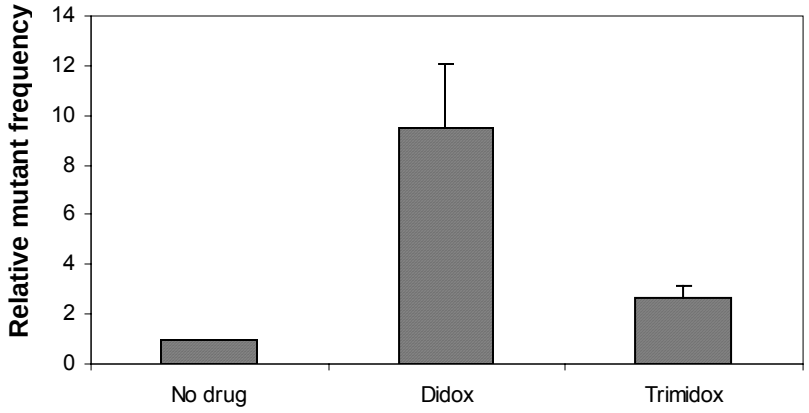



**Figure 3.2.** Effects of didox and trimidox on HIV-1 mutant frequencies. (A) The linear curves used to calculate $IC_{50}$ values of didox and trimidox. This is the representative of three independent experiments. (B) $IC_{50}$ concentration of didox and trimidox. Data represent the average of three independent experiments. (C) Both didox and trimtdox increased HIV-1 mutant frequencies. Both drugs were added at the $IC_{50}$ concentrations 2 hr prior to infection and continuing 24 hr after infection. Data represent the average of three independent experiments. The average luciferase reading for virus replication in the presence of wt RT was $6.3 \times 10^2 \pm 29$.

activated PBMCs decreased the dNTP levels and the DNA synthesis rate to levels comparable to quiescent PBMCs (61). Thus, we hypothesized that low levels of dNTPs in quiescent PBMCs could increase HIV-1 mutant frequencies. To test this hypothesis, the virus mutant frequencies were measured in both quiescent PBMCs and activated PBMCs. As shown in Figure 3.3, the virus replicated in quiescent PBMCs led to a 3.3-fold increase of mutant frequency compared to that observed in MAGI target cells, whereas the virus replicated in activated PBMCs had a mutant frequency comparable to that observed in MAGI target cells. This result is consistent with previously observed two to three-fold increase of G-to-A substitutions in resting PBMCs (201).

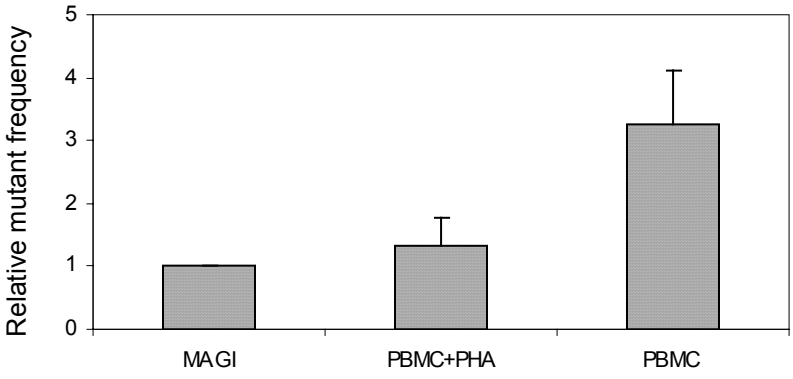



**Figure 3.3.** The replication of virus in quiescent PBMCs and activated PBMCs. PBMCs were cultured in the presence or absence of PHA 24 hr prior to infection. PBMCs were then infected with virus. The virus mutant frequencies were calculated based on restored luciferase activity detected in both PHA-stimulated PBMCs and quiescent PBMCs. Data represent the average of three independent experiments. The average luciferase reading for virus replication in the presence of wt RT was $6.4 \times 10^2 \pm 43$.

### *Antiretroviral drug treatments alter the intracellular dNTP concentrations in MAGI target cells*

To test the hypothesis that antiretroviral drugs increase HIV-1 mutant frequencies by altering intracellular dNTP pools, dNTP pools from MAGI target cells treated with antiretroviral drugs were measured. The concentration of each drug was indicated in Figure 3.5. Following incubation for 2 hr or 24 hr in the absence or the presence of drugs, cells were trypsinized and centrifuged, and dNTPs were extracted as previously described. Single nucleotide incorporation assay was used to measure the dNTP pools (Materials and Methods). The percent of primer extension in each reaction was calculated by determining the ratios of extended (19-mer) versus total (extended [19-mer] + unextended [18-mer]) primers. Each signal for the extended products was normalized using the background signal in the control reactions incubated without RT. The calculated percentage primer extension was plotted from triplicate reactions, as a function of the dNTP quantity used, generating standard curves for all four dNTPs. The representative standard curve of this HIV-1 RT-based dNTP assay is shown in Figure 3.4. The dNTP concentrations of cells incubated in the absence of drugs were defined as 100%. The changes of dNTP pools of drug-treated cells were compared with this value. The different incubation time were chosen to represent different stages in the infection protocol. The 2 hr time point was measured to determine whether dNTP pool concentrations were altered after MAGI cells were exposed to drug for 2 hr prior to infection, which could determine whether dNTP pools were altered at the time of virus infection. The 24 hr time point was chosen

because reverse transcription is expected to be completed during this period. This time point also was used to determine whether drug treatments for longer period further affected reverse transcription accuracy and intracellular dNTP pools.

The intracellular dNTP concentrations of MAGI cells treated with NRTI's were shown in Figure 3.5A and B. After 2 hr of each drug treatment, all NRTI's decreased intracellular dNTP concentrations, but with different magnitude. As expected, both ABC and D4T caused a higher reduction of dNTP concentrations than did ddC at lower concentration. However, we also observed that 1 μM of 3TC led to the highest decrease of dNTP concentrations among all the NRTI's, which is unexpected because I observed that 1 μM of 3TC did not have significant influence on virus mutant frequencies. When the concentrations of ddC and 3TC increased, further decreases in dNTP concentrations were observed. Furthermore, after 24 hr of each drug treatment, the dNTP concentrations were further decreased. These results indicate that NRTI's decreased the intracellular dNTP concentrations after 2 hr treatment, suggesting that the intracellular dNTP pools were altered at the time of infection and initiation of reverse transcription. The further decrease of intracellular dNTP concentrations during 24-hr treatment indicates that NRTI's altered dNTP pools throughout the time period in which reverse transcription occurred.

The intracellular concentrations of dNTPs were also measured after MAGI target cells were maintained in medium supplemented with 80 μM EFV or 150 μM NPV for 2 hr and 24 hr. The effects of NNRTI's treatment on intracellular

dNTP concentrations in MAGI cells are shown in Figure 3.5C. After 2 hr of EFV treatment, the dNTP concentrations were reduced to between not detectable level (dGTP) and 16% (dCTP), relative to control, while NPV treatment led to the reduction of concentrations of all dNTPs to about 30%, except dCTP, relative to control. Similar to what was observed for NRTI's treatment, the 24 hr treatments of EFV and NPV further decreased intracellular dNTP pools, indicating EFV and NPV altered dNTP concentrations prior to virus infection and initiation of reverse transcription and this effect persisted throughout the reverse transcription process. The implication of this observation will be discussed in the next section.

Finally, the effects of RRI's, DX, TX, and HU, on intracellular dNTP concentrations were analyzed. As shown in Figure 3.5D, after 2 hr treatment, DX led to a modest increase of dNTP level, while TX did not alter intracellular dNTP concentrations. Consistent with previous results (61, 88), HU reduced concentrations of dNTPs. When cells were maintained in the medium supplemented with DX, TX, or HU for 24 hr, the intracellular dNTP concentrations of cells treated with DX and TX were significantly reduced, but the dNTP levels of cells treated with HU were only modestly reduced compared with 2 hr treatment. These results indicate that the intracellular dNTP pools of the cells treated with DX and TX were not changed prior to virus infection. Thus, reverse transcription initiated at relative normal intracellular dNTP levels in the DX- or TX-treated cells. When the cells were continuously incubated with DX and TX, the intracellular dNTP concentrations started decreasing and reverse transcription continued in the altered dNTP pools.

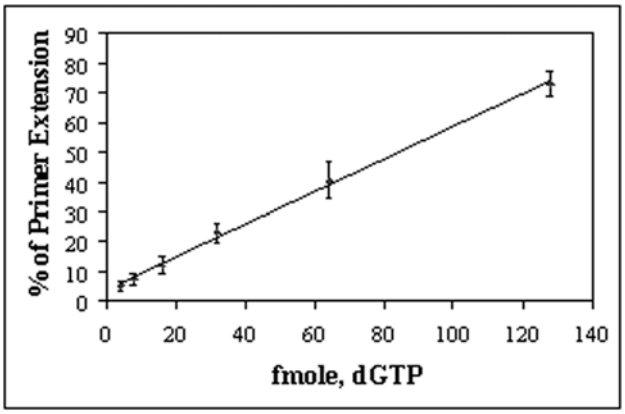



**Figure. 3.4.** Standard curve for the incorporation of dGTP onto the dGTP-specific template/primer pair. The percent primer extension in each reaction was plotted after background normalization (see Materials and Methods). Each data point was calculated from three independent reactions; error bars denote the standard deviation from the mean. (Data courtesy of Dr. T. Diamond and Dr. B. Kim, University of Rochester).

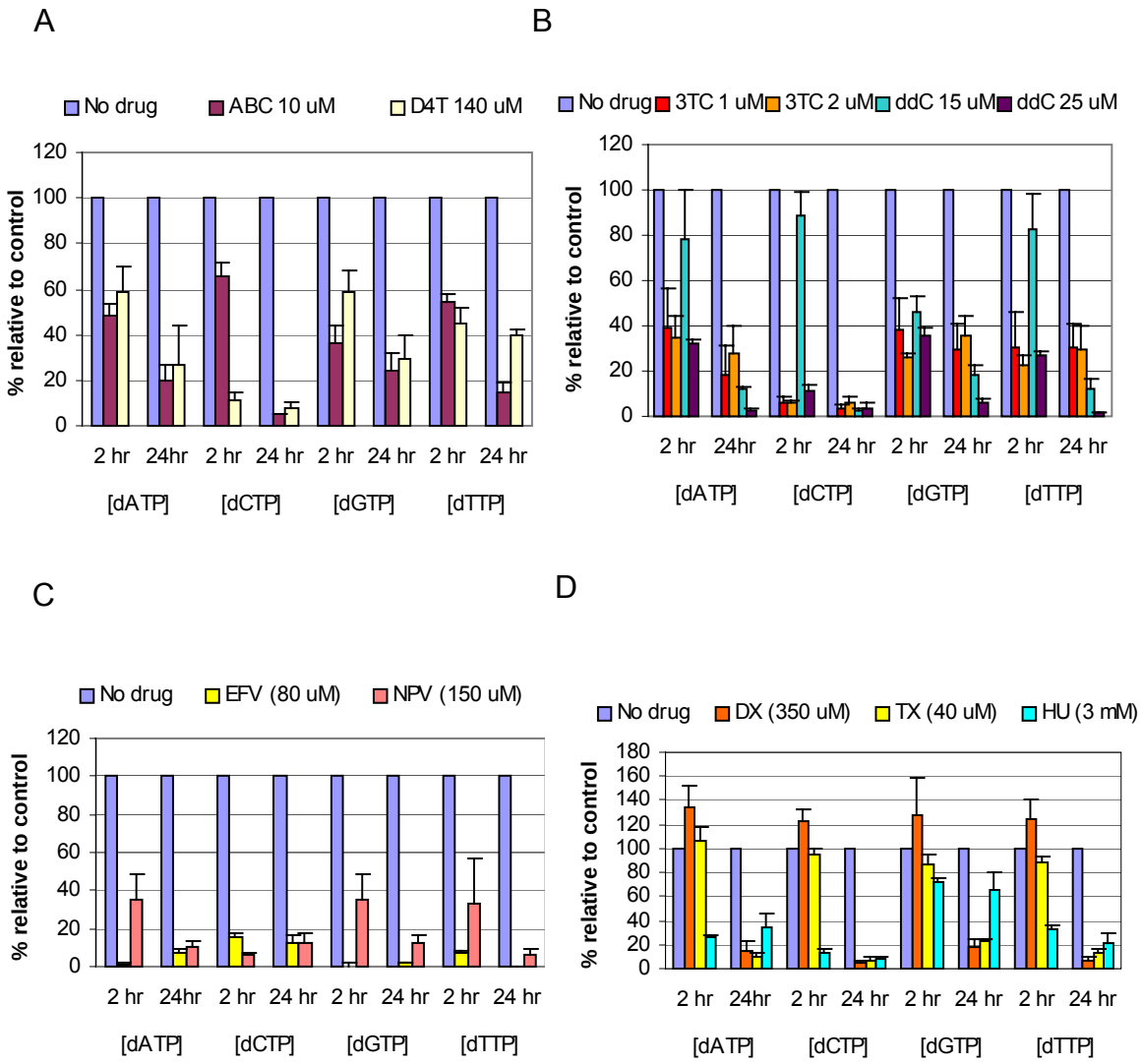



**Figure 3.5.** Effects of NRTI's (A and B), NNRTI's (C), and RRI's (D) on intracellular dNTP pools in MAGI cells. dNTPs were extracted as described in Materials and Methods and measured by using single nucleotide incorporation assay. The dNTP concentrations of cells without drug treatment were normalized to 100%. (Data courtesy of Dr. T. Diamond and Dr. B. Kim, University of Rochester)

## 3.5 Discussion

Previous work has indicated that many of the currently used antiretroviral drugs (i.e. RT inhibitors) can increase HIV-1 mutant frequencies (Chapter 2). The purpose of this study was to analyze the potential interplay of antiretroviral drugs and their combined effects on HIV-1 mutant frequencies. The effects of combined drugs (NNRTI plus NNRTI or NNRTI plus NRTI) on HIV-1 mutant frequencies were analyzed. In general, the virus mutant frequencies observed in the presence of combined drugs were significantly increased compared to that observed in the presence of individual drugs. Two NNRTI's, EFV and NPV, together led to an increase of the virus mutant frequency by 5.8-fold, while virus replication in the presence of EFV or NPV alone led to a 1.4-fold and 2.6-fold increase in virus mutant frequencies, respectively. This suggests that these two NNRTI's can act together to further increase HIV-1 mutant frequencies. The clinically used drug combinations of NNRTI plus NRTI, which included EFV plus D4T or EFV plus 3TC, were also examined in this study. I observed that EFV and D4T together could increase HIV-1 mutant frequencies by 8.3-fold, while virus replication in the presence of EFV or D4T alone led to 3.3-fold and 3.1-fold increases, respectively. This is the first report which shows that an NRTI-NNRTI combination together could further increase HIV-1 mutant frequencies. Given that potent antiretroviral therapy typically includes two or three RT inhibitors, the combined effects of RT inhibitors on HIV-1 mutant frequencies may be clinically relevant.

The mechanisms for how antiretroviral drugs increase virus mutant frequencies are still unknown. One plausible mechanism is that the increased virus mutant frequency phenotype observed in the presence of antiretroviral drugs is due to the alteration of intracellular dNTP pools (88). Restriction of host cellular dNTP synthesis has been proposed as a general strategy for inhibiting the rate of HIV-1 replication. For example, hydroxyurea can reduce the intracellular dNTP levels and has been demonstrated to inhibit HIV-1 DNA synthesis and viral replication (61, 113, 114). Virus mutant frequencies were also increased when virus replication occurred in the presence of hydroxyurea (126). In this study, I investigated whether dNTP pool imbalances are associated with an increased virus mutant frequency and tested the hypothesis that antiretroviral drugs increase virus mutant frequencies by altering intracellular dNTP levels. First, virus mutant frequencies were analyzed in infected cells in which the dNTP levels were manipulated by ribonucleotide reductase inhibitors. Second, cell-type-specific effects on virus mutant frequencies were observed. Finally, in collaboration with Dr. B. Kim (University of Rochester), we determined the effects of antiretroviral drugs on intracellular dNTP pools in MAGI cells. The experiments described in this chapter indicate the correlation between the alteration of intracellular dNTP pools and increases in HIV-1 mutant frequencies.

Like the well-documented anti-HIV-1 drug HU, DX and TX are ribonucleotide reductase inhibitors. Both DX and TX increased HIV-1 mutant frequencies and caused an alteration of intracellular dNTP pools. Moreover, I also demonstrated that when virus replication occurred in primary cells that have

imbalanced dNTP levels (i.e. quiescent PBMCs), an increase in virus mutant frequencies was observed, but not in activated PBMCs, which do not have dNTP pool imbalances.

In agreement with previous reports (61, 88), dNTP concentrations were reduced after 2 hr of HU treatment. It was observed, by comparing the 2 hr and 24 hr treatments, the continuous 24 hr HU treatment could lead to a moderate decrease in dNTP concentrations. Unlike HU, both DX and TX did not have significant effects on dNTP pools after 2 hr treatments. Following 24 hr treatments, both the DX and TX treatments led to dramatic decreases of dNTP concentrations compared to that of the HU treatment. Additionally, DX and TX altered the concentrations of all dNTPs to a very similar level. These data indicate that although ribonucleotide reductase inhibitors could increase virus mutant frequencies, they may have different effects on intracellular dNTP pools. Although DX and TX did not have significant effects on individual dNTP concentrations after 2 hr treatment, it is possible that both drugs could alter the ratios between dNTPs, and subsequently influences HIV-1 mutant frequencies. In order to understand how DX and TX to influence intracellular dNTP pools, and at which time point during reverse transcription process they start altering dNTP concentrations, one possible experiment is that to measure dNTP concentrations at different time points, such as at 4 hr, 6 hr, 8 hr and 10 hr.

In chapter 2, I reported that the NRTI's, which are currently used in drug therapy, could increase HIV-1 mutant frequencies, but that higher concentrations of ddC and 3TC were needed to influence HIV-1 mutant frequencies (Chapter 2).

Based on these results and a previous report (120), we hypothesized that NRTI's increase HIV-1 mutant frequencies by a similar mechanism. As shown in this study, NRTI treatment of MAGI cells decreased dNTP pools. These results provide evidence to support our hypothesis that the ability of NRTI's to increase HIV-1 mutant frequencies correlates with alterations in intracellular dNTP pools. In addition, the levels of dNTPs in ABC- and D4T-treated cells were lower than that in cells treated with 15 μM ddC. This result may provide one plausible explanation for why lower concentration of ddC did not influence HIV-1 mutant frequencies. Furthermore, we observed that higher concentrations of ddC and 3TC could lead to larger reductions of dNTP pools than that observed with lower drug concentrations. These data are in agreement with my observation that higher concentrations of ddC and 3TC were needed to significantly influence HIV-1 mutant frequencies. However, I also observed that treatment of cells with 1 μM of 3TC led to the highest decrease in intracellular dNTP pools, suggesting that 3TC could lead to a biased spectrum of mutations in HIV-1.

The other class of RT inhibitors, the NNRTI's, were also reported to increase HIV-1 mutant frequencies (Chapter 2). NNRTI's are structurally different from natural dNTPs, and they inhibit RT activity by binding to a hydrophobic pocket adjacent to the RT active site. Therefore, we hypothesized that the conformational change caused by the noncatalytic binding of NNRTI's to RT may affect HIV-1 mutant frequencies. Surprisingly, we observed that NNRTI's dramatically decreased intracellular dNTP pools (larger than that observed with the NRTI's). How NNRTI's influence HIV-1 mutant frequencies is unknown.

Based on our observation that EFV and NPV could reduce intracellular dNTP concentrations, we could suggest that NNRTI's could influence virus mutagenesis by altering host cell dNTP levels. However, we cannot exclude other possible mechanisms. We observed that EFV and NPV had a very similar influence on HIV-1 mutant frequencies (3.3- and 3.2-fold, respectively), as well as the results in this study which showed that NPV treatment induced less extensive reductions in dNTP pools, one could speculate that the influence of NNRTI's on virus mutant frequencies involves more than one mechanism.

How antiretroviral drugs influence intracellular dNTP levels is presently unclear. We have hypothesized that NRTI's could alter intracellular dNTP pools by competing with natural dNTPs for cellular dNTP biosynthesis machinery. It is known that the 5'- triphosphate derivatives of NRTI's are the active inhibitors of HIV-1 RT. NRTI's use cellular enzymes and follow different metabolic pathways to be converted to their active derivatives. For example, the thymidine analogs AZT and D4T are converted to their 5'-monophosphate derivatives by thymidine kinase. In contrast, dCTP analogs, such as ddC and 3TC make use of dCyd kinase (6) (Figure 3.6). Therefore, it is possible that NRTI's can alter intracellular dNTP pools by competing with natural dNTPs for a cellular enzyme. It has been demonstrated that AZTMP could be considered as a substrate-inhibitor of thymidylate kinase (TK), which correlates well with the observation that cells incubated with AZT have reduced intracellular levels of dTTP. (60) Thus, to understand how other NRTI's affect dNTP pools, studies of the cellular enzymes that are involved in dNTP pool regulation should be a topic of future studies. In

addition, it is possible that NNRTI's could act as non-competitive inhibitors of cellular enzymes that are responsible for natural dNTP biosynthesis. For instance, it is possible that NNRTI's could inactivate ribonucleotide reductase, and thereby deplete intracellular dNTP pools. The data reported in this study suggest that NRTI's and NNRTI's may have a similar mechanism that affects HIV-1 mutant frequencies.

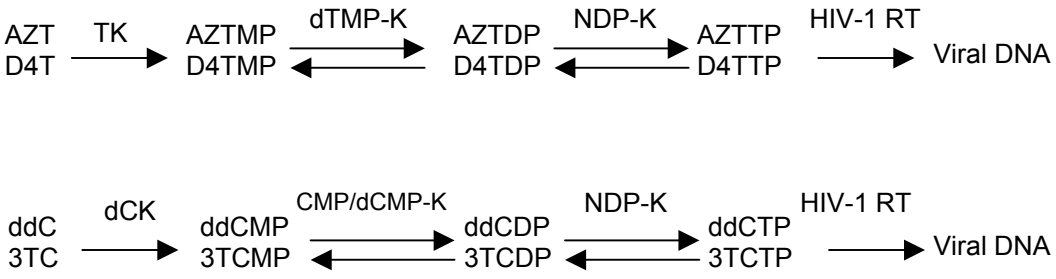



**Figure 3.6.** Metabolism of AZT, D4T, ddC and 3TC. TK: thymidine kinase; dTMP-K: thymidylate (dTMP) kinase; NDP-K: nucleoside diphosphate (NDP) kinase; dCK: deoxycytidine kinase; CMP/dCMP-K: CMP/dCMP kinase.

Taken together, the data presented in this chapter provide three lines of evidence in supporting our hypothesis that the influence of antiretroviral drugs on HIV-1 mutant frequencies correlates with an alteration of intracellular dNTP pools. First, when HIV-1 infected MAGI cells were treated with ribonucleotide reductase inhibitors (i.e. DX and TX), virus mutant frequencies increased by 9.5-fold and 2.7-fold, respectively, and this provides an evidence to show that

increased mutant frequencies may be due to the alteration of intracellular dNTP pools. Second, when virus replicates in quiescent PBMCs that have imbalanced dNTP pools, virus mutant frequencies are higher than that observed in activated PBMCs, which have more balanced dNTP pools. Third, when cells treated with NRTI's and NNRTI's, the intracellular dNTP concentrations were also altered. Although these observations indicate that altered HIV-1 mutant frequencies correlate with imbalanced dNTP pools, other possibilities exist for why there are changes in the mutant frequencies. Therefore, additional studies are needed to further elucidate the mechanisms used by both NRTI's and NNRTI's to influence HIV-1 mutant frequencies.

Substrate dNTP pool imbalances have been known to alter the frequency of base substitution mutations. It has been shown that dNTP pool imbalances induce mutations *in vivo* by promoting nucleotide misincorporation during DNA replication (99). The increased probability of extending a mismatched primer termini is thought to influence the rate of nucleotide misincorporation and the probability of a specific mispair forming is likely to be determined by the relative levels of dNTPs (99). Therefore, it has been suggested that the mutational spectrum and base substitution rates of retroviruses are affected by dNTP pool imbalances in the cells that they infect (88). Moreover, using purified HIV-1 RT has shown that the errors induced by a dNTP pool bias could be either frameshift or base substitution mutations (15). These observations suggest that base substitution mutations are caused by misincorporation of excess dNTPs, while frameshift mutations are likely to result from the low processivity of HIV-1 RT.

Previous studies have demonstrated that processivity of HIV-1 RT polymerization is influenced by dNTP concentrations, and in some instances, processivity correlates with frameshift fidelity (5, 11). Thus, it is possible that dNTP pool imbalances promote pausing by RT, and subsequently alter processivity in a manner that changes the probability of template-primer slippage.

The results of this study suggest that treatment of patients with combinations of RT inhibitors or ribonucleotide reductase inhibitors may result in an increase in the rate of mutations in the HIV-1 genome, which in the context of therapy could increase the odds of the selection of drug-resistant viruses and the evolution of drug resistance. In addition, these high mutant frequencies could extinguish virus replication by error catastrophe (lethal mutagenesis) (119). Based upon the analysis of HIV-1 mutant frequencies, 30-fold or higher increases in virus mutant frequencies would be necessary in order to extinguish virus replication (126). Therefore, combination therapy that could cause a progressive accumulation of mutations and ultimately lead to a drastic reduction in virus replication and fitness may be an effective way to manage HIV-1 infection.

# CHAPTER 4

# ANALYSIS OF URACIL-DNA GLYCOSYLASE INCORPORATION INTO HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) VIRUS PARTICLES

## 4.1 Abstract

Uracil-DNA glycosylase (UNG) was previously shown to be incorporated into HIV-1 particles via interaction with the accessory protein Vpr. UNG functions as a DNA repair enzyme in base excision repair by removing the RNA base uracil from DNA. Previous work has shown that the Vpr variant VprW54R could be efficiently incorporated into HIV-1 particles but prevented virion incorporation of UNG (176). This lack of UNG virion incorporation resulted in a 4-fold increase in the virus mutation rate, which is equivalent to that observed in Vpr-minus HIV-1. In this study, I further analyzed the ability of the Vpr-UNG interaction to influence UNG incorporation into HIV-1 particles by monitoring UNG enzymatic activity from virus particles. I tested various Vpr and UNG mutants to assess whether UNG activity could be detected in HIV-1 particles and observed that Vpr mutants which did not interact with UNG led to a lack of UNG activity in virus particles. Similarly, mutants of UNG that did not interact with Vpr failed to be

incorporated into virus particles. These data help to provide further support for the Vpr-UNG interaction having an important role in the HIV-1 life cycle.

## 4.2 Introduction

Human immunodeficiency virus type 1 (HIV-1) Vpr is a 14-kDa, 96 amino acid non-structural protein that is associated with virus particles and can accumulate at the nuclear envelope and in the nuclei of infected cells (32, 105, 116, 153). The incorporation of Vpr into particles requires a direct interaction with the p6 region of the Gag polyprotein precursor (177). Vpr also appears to be a viral protein that provides multiple functions for HIV replication. First, expression of Vpr alters cell cycle progression by arresting cells in the G2 phase (72, 73, 86, 172). Second, viruses with intact *vpr* gene cause cell cycle arrest, and can lead to apoptosis (172). Third, Vpr is required for the efficient replication of the virus in non-dividing cells (34, 73). This requirement is related, at least in part, to its role in the nuclear translocation of the preintegration complex (PIC) containing HIV-1 DNA. Vpr possesses an affinity for the components of the nuclear pore complex (NPC) (105), and it has been proposed that Vpr may facilitate the nuclear translocation of the PIC across the nuclear envelope (105).

The HIV-1 Vpr protein has been found to interact with several cellular proteins, including uracil-DNA glycoslyase (UNG) (19), an enzyme involved in the base excision repair pathway and specifically removes the RNA base uracil from DNA. UNG, the first DNA glycosylase to be discovered (108), is encoded by nearly all eukaryotes and prokaryotes. The structure of the enzyme, especially

the active site, is highly conserved except for UNG from poxviruses (162). Uracil can occur in DNA either by misincorporation of dUTP (creating U:A pair) when dUTP pool is high, or by cytosine deamination (creating mutagenic U:G mispair) (reviewed in (29)). UNGs have been shown to remove uracil from single-stranded and double-stranded DNA substrates (44, 109). Uracil is not excised from RNA or RNA contained in a RNA/DNA hybrid; however, it has been reported that UNG isolated from calf thymocyte nuclei was able to excise uracil residues from the DNA strand of a RNA/DNA hybrid (191).

The human *ung* gene encodes two forms of UNG, UNG1 (mitochondria form) and UNG2 (nuclear form), which are generated by alternative transcription start sites and alternative splicing (147). The yeast two-hybrid assay and other assays have shown that Vpr interacts with UNG and that the WXXF motif in UNG was likely involved in the Vpr-UNG interaction (19, 129). The HIV-1 integrase has also been shown participate in the recruitment of UNG (160, 208), but the Vpr-mediated pathway appears to be the main pathway involved in the recruitment of Vpr into virus particles (121, 129). In this study, the UNG activity from HIV-1 particles was detected using a gel-based UNG assay. I observed that UNG2 remained catalytically active after it was incorporated into virus particles. Moreover, UNG2 could be incorporated into viral particles independently of Vpr by expressing UNG2 as a fusion protein at the C-terminal extremity of Vpr, and that the fusion proteins was enzymatically active. These data provide support for the Vpr-UNG interaction being important in the HIV-1 life cycle.

## 4.3 Materials and Methods

### *Oligonucleotide and enzymes*

DNA oligonucleotide used was obtained from Sigma-Genosys (Woodlands, TX). The length of the oligonucleotide chosen was based on previous studies of UNG activity with DNA oligonucleotide substrates (98). The DNA oligonucleotide, TU13 (5'-TTTTTTTTTTTTUTTTTTTTTTTTT-3'), was used as a model substrate for UNG activity.

The purified *E. coli* UNG and the *Bacillus subtilis* bacteriophage PBS2 uracil-DNA glycosylase inhibitor (UGI) were obtained from New England BioLabs, Inc. (Beverly, MA).

### *Uracil-DNA glycosylase assay*

Assays of UNG activity using the single-stranded 25 bp DNA oligonucleotide substrate TU13 were performed in UNG reaction buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol (pH 8.0)) at 37°C for 1 hr. AP sites were cleaved by adding one-half volume of 0.5 M NaOH and one-half volume of 30 mM EDTA and then boiling for 30 min (205). After the cleavage of the AP site, samples were applied to a non-denaturing 20% polyacrylamide gel with electrophoresis at 60 V for 3.5 hr. Gels were stained with SYBR Gold (Molecular Probes, Eugene, OR), and nucleic acids were visualized with an ultraviolet transilluminator.

### *Assay for uracil-DNA glycosylase inhibitor (UGI) activity*

Single-stranded DNA substrate was incubated in UNG reaction buffer with UNG and UGI at 37°C for 1 hr. The reaction products were treated with one-half volume of 0.5 M NaOH and one-half volume of 30 mM EDTA and then boiling for 30 min. Samples were applied to a non-denaturing 20% polyacrylamide gel with electrophoresis at 60 V for 3.5 hr. Gels were stained with SYBR Gold and nucleic acids were visualized with an ultraviolet transilluminator.

### *Retroviral vectors and expression plasmids*

The HIV-1 *gag-pol* expression plasmid, pSVgagpol-rre-MPMV, was used as packaging plasmid, and has been described previously (Chapter 2 and (129)). The vectors used for expression of Vpr (wild type or mutants), UNG2 (wild type or mutants), *E. coli* UNG (wild type or mutants), and VprW54R-UNG2 fusion were constructed in plasmid pAS1B (Gift from Dr. Serge Benichou, INSERM, Paris).

### *Cell culture and Vpr-UNG packaging assay*

The 293T cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA), and maintained in Dulbecco's Modified Eagle's minimum essential medium (DMEM) (GIBCO BRL, Gaithersburg, MD) supplemented with 10% of Fetal clone III serum (Hyclone, Logan, UT). In order to detect the incorporation of UNG2 and its activity in virus particles, a Vpr and UNG packaging assay was developed in which Vpr and UNG2 were expressed in

trans and incorporated into virions. The HIV-1-based packaging vector pSVgagpol-rre-MPMV contains gag and pol gene, which is necessary for formation of virus particles. For analysis of Vpr-dependent incorporation of UNG2, cells were transfected with pSVgagpol-rre-MPMV, pAS1B VprWT and pAS1B UNG2 (wt or mutant) (10 μg of each plasmid) in a 100 mm petri dish using the calcium phosphate precipitation method. Cell culture supernatants were collected 48 h after transfection and filtered through 0.2 μm-pore-size filters (Millipore, Billerica, MA). Virions were collected by ultracentrifugation for 1 hr at 40,000 × g and resuspended in RIPA buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 5mM EDTA, 0.5% deoxycholate, 0.1% SDS and 1% IGEPAL). The protein concentration of viral lysate was measured (Bio-rad, CA). UNG assay was performed to detect UNG activity in the virus lysate (Figure 4.2A).

## 4.4 Results

### *Detection of UNG enzymatic activity in HIV-1 particles*

In order to detect UNG activity from HIV-1 virions, a gel-based UNG assay was used, which could indirectly detect UNG activity. First, purified *E. coli* UNG and synthetic DNA oligonucleotide were used to develop this gel-based UNG assay. The DNA oligonucleotide, TU13, was used as a substrate because single-stranded DNA is a known substrate for the enzyme. When the single-stranded DNA was treated with UNG, UNG recognized uracil and released uracil base from DNA to create an abasic (AP) site. Alkaline and heat treatment hydrolyzes

the phophodiester backbone at the AP site in the DNA and results in the creation of two, 12 bp DNA products. As shown in Figure 4.1, the UNG treatment led to the conversion of substrate to product (lane 2). In order to ensure that UNG activity was responsible for the conversion of substrate to product, an UNG assay was done in the presence of uracil-DNA glycosylase inhibitor (UGI) of the *Bacillus subtilis* bacteriophage PBS2, which inhibits UNG to excise uracil residues from DNA substrate. The presence of UGI prevented the accumulation of the 12 bp products (Figure 4.1, lane 3). This indicates that the creation of product from substrate was specifically due to UNG activity. Moreover, the treatment of single-stranded DNA with NaOH and boiling in the absence of UNG treatment did not result in the creation of 12 bp products (data not shown), indicating that hydrolysis itself could not break the phosphodiester backbone in the absence of uracil removal. Therefore, UNG treatment, which creates an AP site, is necessary for the conversion of the 25 bp substrate to the 12 bp products.

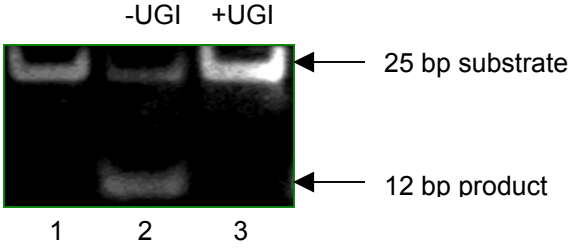



**Figure 4.1**. UNG utilization of a single-stranded DNA substrate. Lane 1: 25 bp DNA oligonucleotide; lane 2: 25 bp DNA oligonucleotide was treated with 1 unit of UNG at 37°C for 1 hr; lane 3: 25 bp DNA oligonucleotide was treated with 1 unit of UNG and 1 unit of UGI at 37°C for 1 hr. In both experiments, 1 μg of DNA oligonucleotide was used as a substrate for UNG activity. AP sites were cleaved by adding 0.5 M NaOH and then boiling for 30 min. After the cleavage of the AP site, samples were applied to a non-denaturing 20% polyacrylamide gel.

Next, UNG activity from purified HIV-1 virions was determined by transiently transfecting 293T cells with an HIV-1 vector with wild type Vpr alone or in combination with UNG expression plasmids. HIV-1 virions were collected 48 hr later, centrifuged and used in the UNG activity assay using the TU13 single-stranded DNA as substrate. In the presence of UNG, the uracil residue was excised and left the deoxyribose phosphate backbone. Alkaline and heating treatment could destroy the backbone at that position, resulting in 12 bp products (Figure 4.2A). As shown in Figure 4.2B, the expression of Vpr alone in virus producing cells led to UNG enzymatic activity from virions (lane 4). This indicates that the fully active endogenous UNG2 was incorporated into HIV-1 virus particles, but the expression of Vpr in combination with UNG2 led to the detection of a higher level of UNG activity in virions (lane 5 compare to lane 4). To verify that the activity detected in virions is related to a specific recruitment of UNG2, UGI was used in the UNG enzymatic assay. No activity was detected when the assays were performed in the presence of UGI (lane 2 and 3), indicating that Vpr-mediated recruitment of UNG2 allowed for the recovery of catalytically active enzyme from HIV-1 virions.

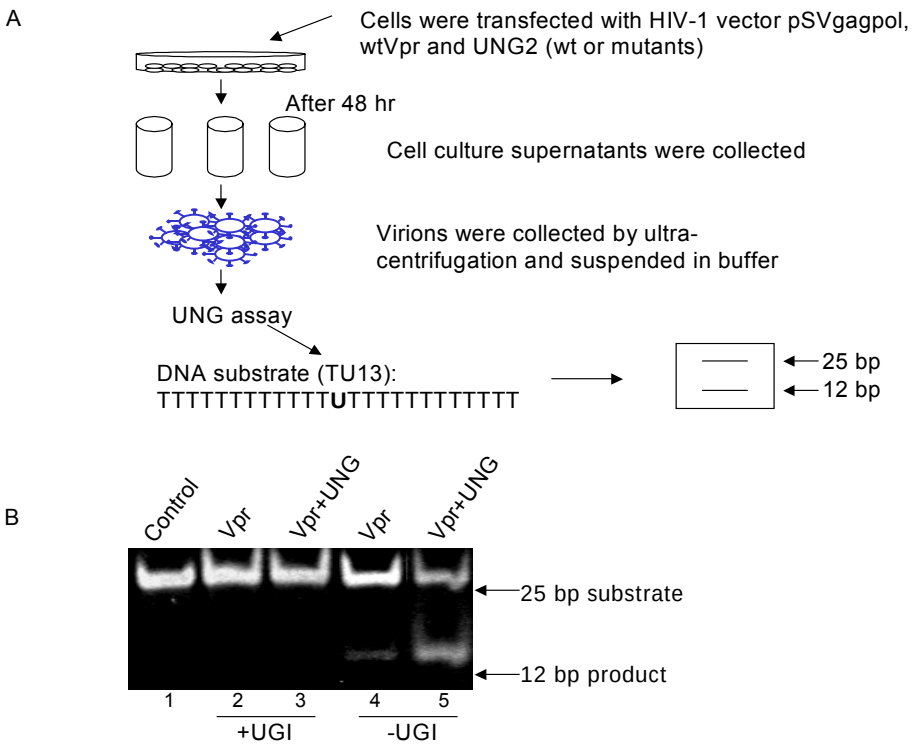



**Figure 4.2.** Recovery of virion-incorporated UNG with catalytic activity. (A) Schematic outline of Vpr-UNG packaging assay. Virions produced from cells expressing wild type Vpr alone or in combination with wild type UNG were collected from cell supernatants and prepared. Assays of UNG activity were performed using TU13 as DNA substrate, resulting in 12 bp products that can be visualized on a nondenaturing polyacrylamide gel. (B) Analysis of UNG activity in HIV-1 particles in the presence and absence of UGI. Lane 1: 25 bp substrate; lane 2 and 3: UNG assay was performed in the presence of UGI; lane 4 and 5: UNG assay was performed in the absence of UGI.

To further investigate the correlation between UNG activity in virus particles and Vpr-UNG interaction, various Vpr and UNG2 mutants were studied (Table 4.1). Previous studies have established a correlation between the Vpr and UNG2 interaction and the influence on the HIV-1 mutation rate (121, 124, 129).

In particular, the VprW54R mutant, which failed to bind UNG in a yeast two-hybrid assay, also failed to recruit UNG2 into HIV-1 virions, and was not be able to complement a *vpr* null mutant HIV-1 in the mutation rate assay (129). Moreover, the other two Vpr mutants, H71R and H78R that were previously shown to bind poorly to UNG2 (176), were found to be efficiently incorporated into virus particles and increased the virus mutation rate in the same manner as VprW54R (124). Therefore, I tested whether the UNG activity could be detected in virus particles when virus-producing cells express these two mutants in combination with wild type UNG2. As expected, UNG activity was detected in virus particles when virus-producing cells were co-transfected with wild type Vpr and wild type UNG2 (Figure 4.3A, lane 5), but no UNG activities were detected in virions when virus-producing cells express Vpr mutants that fail to bind to UNG2 in combination with wild type UNG2 (Figure 4.3A, lane 2-4). Virion incorporation of wild type Vpr and Vpr mutants was analyzed by Western blotting. The wild type Vpr and Vpr mutants were expressed in cells and subsequently incorporated into HIV-1 virus particles at the same level (data not shown).

Since it has been reported that Vpr binding to UNG2 is related to the presence of a WXXF motif found within the C-terminal part of UNG2 (a.a. 231-234) (20), I analyzed UNG activities when wild type Vpr and WXXF motif mutants were expressed in virus-producing cells. In a previous study, mutation at the position 234, F234G, lost its ability to interact with Vpr, whereas the mutant W231G, lost more than 60% of the binding with Vpr (20). Therefore, the F234G mutant and the W231A/F234G mutant were studied. The Western blotting

analysis showed that these mutants were expressed in cells at the same level as wild type UNG2 (data not shown). Moreover, the UNG activity of these mutants were detected using previously described UNG assay (109). The results showed that, for these mutants, UNG activity remained at the same level as wild type UNG2 (data not shown). As shown in Figure 4.3B, no UNG activity was detected when the wild type Vpr expressed in combination with UNG2 mutants (lane 3 and 4), indicating the UNG activity detected in virions is related to the Vpr and UNG2 interaction, and the WXXF motif is a molecular determinant of Vpr and UNG interaction.

| Mutant | Function |
|---|---|
| Vpr H71R | Poorly bind to UNG |
| Vpr H78R | Poorly bind to UNG |
| UNG2 F234G | Mutation in WXXF motif; lost Vpr-UNG interaction |
| UNG2 W231A/F234G | Mutations in WXXF motif; lost Vpr-UNG interaction |
| *E. coli* UNG F144G | Equivalent to UNG2F234G |
| *E. coli* UNG W141A/F144G | Equivalent to UDG2W231A/F234G |

**Table 4.1.** Mutations analyzed in this study, which lost the ability to interact with Vpr or UNG.

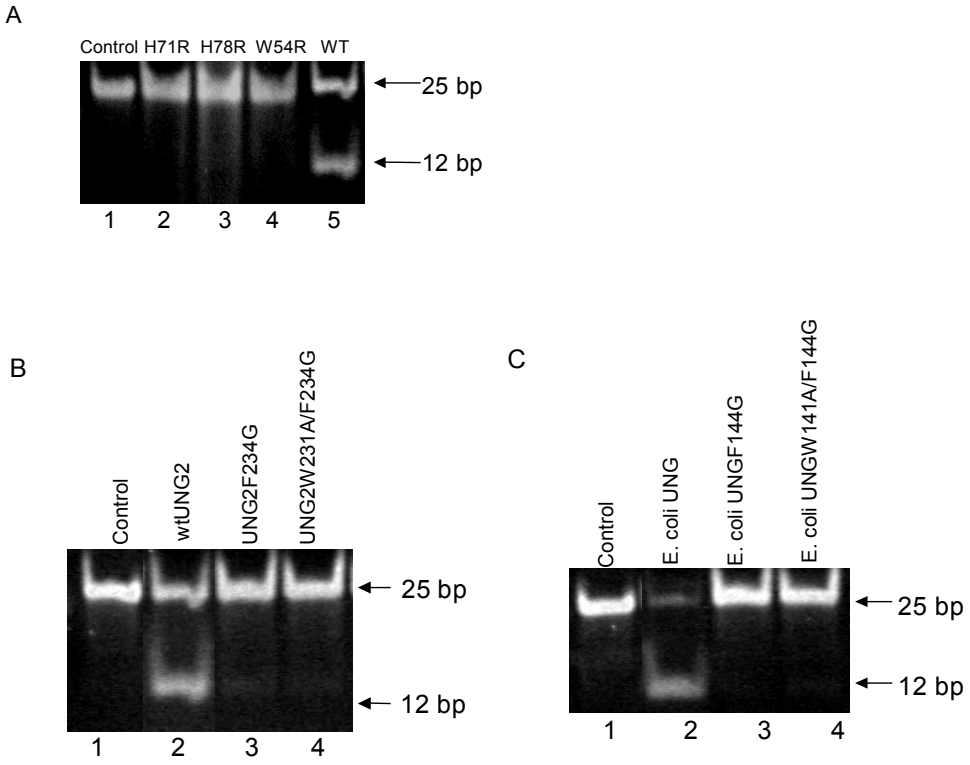



**Figure 4.3.** The lack of Vpr and UNG2 interaction led to undetectable UNG activity in virions. (A) The expression of Vpr or Vpr mutants in combination with human wtUNG2. Lane 1: 25 bp substrate; lane 2: VprH71R; lane 3: VprH78R; lane 4: VprW54R; lane 5: wtVpr. (B) The expression of Vpr in combination with human wtUNG2 or UNG2 mutants. Lane 1: 25 bp substrate; lane 2: wtUNG2; lane 3: UNG2F234G; lane 5: UNG2W231A/F234G. (C) The expression of Vpr in combination with *E. coli* wtUNG and mutants. Lane 1: 25 bp substrate; lane 2: *E. coli* wtUNG; lane 3: *E. coli* UNGF144G; lane 4: *E. coli* UNGW141A/F144G.

Sequence analysis showed that UNG is a highly conserved protein among different species, as is the WXXF motif (Figure 4.4). Based on this observation, I analyzed *E. coli* UNG mutants (gift from Dr. S. Benichou, INSERM, Paris), which

have the same amino acid substitution as UNG2 mutants in the WXXF motif. As shown in Figure 4.3C, wild type *E. coli* UNG was incorporated into virus particles and retained the UNG activity, but no UNG activities were detected from virions produced from cells co-expressing Vpr and a mutated *E. coli* UNG that did not interact with Vpr. The Western blotting analysis showed that these *E. coli* mutants were expressed in cells at the same level as wild type *E. coli* UNG (data not shown). Furthermore, these mutants retained UNG activity at the same level as wild type *E. coli* UNG when they expressed in *E. coli* (data not shown). These data provide further evidence that WXXF motif is essential for Vpr and UNG interaction. Together, these observations indicate that Vpr and UNG2 interaction is required for the presence of UNG activity in virus particles, and that the WXXF motif of UNG2 is directly involved in binding to Vpr.

| | | | |
|---|---|---|---|
| Human (219-247) | RAHQANSHKERG | **W**EQ**F** | TDAVVSWLNQNSN |
| *E. coli* (129-157) | RAGQAHSEASLG | **W**ET**F** | TDKVISLINQHRE |
| Yeast (228-256) | RAHNANSESKHG | **W**ET**F** | TKRVVQLLIQDRE |

**Figure 4.4.** Alignment of UNG from different organisms. The conserved WXXF motif is boxed.

### *Incorporation of Vpr-UNG fusion proteins into HIV-1 particles*

To gain further insight into the functional role of the incorporated UNG, UNG2 was fused to the C-terminus of VprW54R. The VprW54R mutant cannot interact with UNG2, but it is efficiently incorporated into virions. The virion

incorporation of the VprW54R-UNG2 fusion was analyzed using the packaging assay in which the fusion was expressed *in trans* in virus-producing cells. Cells and virion-assoicated VprW54R-UNG2 fusions were then assayed by Western blotting. The VprW54R-UNG2 fusion, as well as the wild type Vpr-UNG2 fusion used as a control, was well expressed in virus producing cells, and both fusions were detected from virions purified from the supernatant of transfected cells (data not shown). The enzymatic activity of the VprW54R-UNG2 fusion was analyzed in the presence or the absence of UGI using the UNG assay. The presence of UGI inhibited the accumulation of 12 bp products (Figure 4.5, compare land 2 and lane 3), suggesting that the VprW54R-UNG2 fusion was catalytically active when it was incorporated into virions. These results indicate that the VprW54R-UNG2 fusion protein is efficiently incorporated into HIV-1 particles and retains UNG enzymatic activity. Therefore, the VprW54R-UNG2 fusion protein can selectively incorporate overexpressed UNG. This approach presents a valuable tool for elucidating the role of UNG in the HIV-1 life cycle.

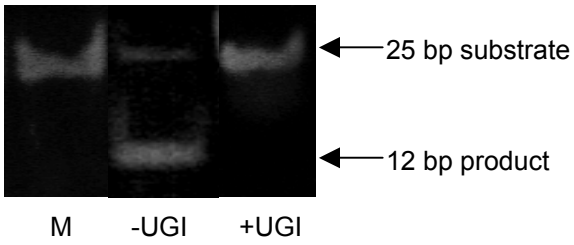



**Figure 4.5.** UNG activity from the VprW54R-UNG2 fusions incorporated into virions. Virions produced from cells expressing VprW54R-UNG2 was collected from cell supernatants and prepared. The UNG activity was detected in the presence or absence of UGI.

## 4.5 Discussion

The cellular DNA repair enzyme, UNG, is known to associate with HIV-1 Vpr and modulate the *in vivo* mutation rate (129). In addition, the Vpr-UNG interaction led to the incorporation of UNG into HIV-1 virus particles. This study has shown that catalytically active UNG can be recovered from HIV-1 particles.

In the development of the UNG assay I noticed that complete conversion of substrate to product was typically not observed in experiments where it was expected. There are several possibilities that could lead to this observation. First, the different NaOH treatments led to comparable amount of product, suggesting that NaOH hydrolysis of the phosphodiester backbone at the AP site was inefficient. Second, it is possible that the DNA substrate used in this assay was in excess, so UNG could not remove uracils from all DNA substrates. It has been shown that the rate of uracil removal occurs almost twice as fast on single-stranded versus double-stranded DNA (108); therefore, 1 unit of UNG (1 unit is defined as the amount of enzyme needed to catalyze the release of 60 pmol of uracil per minute from double-stranded DNA) can remove $7.2 \times 10^3$ pmol of uracil per hour from single-stranded, uracil-containing DNA. Based upon this, 1 μg of single-stranded substrate would create $1.4 \times 10^5$ pmol of uracil and would take about 20 hrs for 1 unit of UNG to completely convert substrate to product. Therefore, in these assays, the 1 hr reaction period would not be enough time for complete conversion of substrate to product. Third, it is also possible that UNG could not continue to remove uracils from DNA substrates when free uracil accumulates in reaction mixture and acts as a competitive inhibitor. Fourth, the

structure of the single-stranded DNA substrate may prevent UNG from accessing uracil residues; therefore, leading to inefficient excision of uracil residues. Overall, one or several of these possibilities might explain why I never saw complete conversion of substrate to product. In addition, because the UNG activity was detected based on the conversion of substrate to product, this gel-based assay may not be as sensitive as previously described UNG assays that measure the release of uracil residues from single-stranded DNA substrates (109). Since the purpose of this study was to qualitatively detect UNG activity in virus particles, this gel-based assay system could be used for this purpose.

Previous studies using the yeast two-hybrid screen or the peptide phage display method have shown that the W54 residue of HIV-1 Vpr is critical in maintaining the interaction of Vpr with UNG2, and that the WXXF motif at C-terminus of UNG2 is directly involved in Vpr-binding. The correlation between incorporation of UNG2 into virus particles and the HIV-1 mutation rate has been previously demonstrated. When *vpr*-deficient HIV-1 was complemented *in trans* with VprW54R, there was a 4-fold increase in the HIV-1 mutation rate (121). The observations made in my study provide further support that the Vpr-dependent incorporation of UNG2 into HIV-1 particles is important in the HIV-1 life cycle.

Using the UNG catalytic activity assay that I developed, UNG2 was found to remain catalytically active after it was incorporated into virus particles. Substitutions of the H71 or H78 residues of the Vpr C-terminus have been shown to abolish the interaction of Vpr and UNG2 and increase the HIV-1 mutation rate like that seen with VprW54R (124). When either the VprH71R or the VprH78R

mutant was expressed in combination with wild-type UNG2 in virus-producing cells, no UNG activity was detected in purified virions, whereas both mutants were efficiently incorporated into virions. Moreover, no UNG activity was detected in virus particles when *trans* complemented with wild type Vpr and one of the WXXF motif mutants of UNG2, indicating that the WXXF motif is involved in the binding of UNG2 to Vpr. To further confirm this observation, the mutations were analyzed with *E. coli* UNG. As expected, no UNG activity was detected in virions due to the lack of the Vpr-UNG interaction. These observations indicate that the UNG enzymatic activity detected in virus particles is related to the direct interaction that takes place in virus-producing cells between Vpr and UNG.

The W54 residue of HIV-1 Vpr is crucial for both binding to UNG2 and then its recruitment into virus particles (129, 176). However, W54 does not participate in the interaction of Vpr with the viral Gag precursor in virus producing cells, which allows for the incorporation of Vpr into virions (129, 177). We therefore took advantage of the VprW54R mutant to generate a Vpr-UNG2 fusion protein that could allow for an evaluation of the specific role(s) of UNG2 recruitment into virus particles on the early steps of HIV-1 infection. The VprW54R-UNG2 fusion was efficiently incorporated into virions, and enzymatic assays done with purified virions indicated that the UNG2 fused to VprW54R was still catalytically active. This approach may provide a valuable tool for future studies directed at whether UNG2 enzymatic activity is involved in the HIV-1 life cycle.

The ability to replicate in non-dividing cells is a characteristic of the primate lentiviruses. HIV-1 Vpr is known to facilitate nuclear import of the viral DNA in non-dividing cells (181). Recently published data showing that the virion incorporation of UNG2 via Vpr also contributes to the ability of HIV-1 to replicate in primary macrophages suggests another critical role of Vpr during the viral life cycle (27). This implies that UNG2 is a cellular factor that plays an important role in the early steps of the HIV-1 replication cycle. Recently, it has been reported that the misincorporation of uracil into minus strand viral DNA affects the initiation of the plus-strand DNA synthesis *in vitro* (95), suggesting that UNG2 is likely recruited into HIV-1 particles to subsequently minimize the accumulation of uracil into the newly synthesized proviral DNA. Similarly, most non-primate lentiviruses, such as FIV, CAEV, and EIAV, encode and package a dUTP pyrophosphatase (dUTPase) into virus particles to maintain a low level of dUTP (for review, see (29)). Replication of FIV, CAEV, and EIAV in the absence of dUTPase results in severely diminished replication in non-dividing cells (i.e. primary macrophages) (107, 197). These results indicate that uracil misincorporation in viral DNA strands during reverse transcription is deleterious for the ongoing steps of the virus life cycle and the presence of a viral dUTPase or a cellular UNG will prevent these detrimental effects on the replication of non-primate and primate lentiviruses in macrophages, respectively.

In conclusion, this report suggests a direct role of virion-incorporated UNG in the HIV-1 life cycle. Therefore, the interaction between Vpr and UNG2 could represent an attractive target for antiretroviral intervention.

# CHAPTER 5

# DISCUSSION AND PERSPECTIVES

## 5.1 Antiretroviral therapy and HIV-1 mutagenesis

A new high through assay system, which uses *luc* gene as the mutational target, was developed during the course of my dissertation research to study the influences of antiretroviral drugs and drug-resistant RT mutants on HIV-1 mutagenesis. The advantages of this new assay system are: 1) it allows me to quickly assess the influence of drugs and drug-resistant RT mutants on HIV-1 mutant frequencies compared to the old system using *lacZ*$\alpha$ as mutational target under different experimental conditions; 2) the sensitivity of luciferase assay allows for detection of reversion mutations in a small sample size. Using this new system, I analyzed the influence of individual drugs, drug-resistant RT mutants and combined drugs on HIV-1 mutant frequencies. Several observations were presented in this dissertation. First, I observed that NNRTI's could increase HIV-1 mutant frequencies. When two NNRTI's act together, they further increased HIV-1 mutant frequencies. Second, an NNRTI-resistant mutant, K103N/Y318F, was reported to decrease virus mutant frequency by 2-fold. Third, I also observed that some drug-resistant RT mutants had significant influence on HIV-1

mutagenesis in the presence of drugs, while these RT mutants alone only have small or no effect on HIV-1 mutant frequencies. Finally, I demonstrated that the combination of NRTI and NNRTI could lead to higher virus mutant frequencies than each individual drug.

The mechanisms responsible for antiretroviral drugs to influence HIV-1 mutagenesis are unknown. The proposed possible mechanisms include: 1) NRTI's can alter intracellular dNTP pools; 2) NRTI's can be incorporated into plus-strand of viral DNA and may result in discontinuous DNA synthesis of viral DNAs with proper ends that integrated into the target cell DNA; subsequently, host cell may carry out error-prone DNA repair and increase the virus mutation rate; and 3) NNRTI's may bind noncatalytically to HIV-1 RT and cause a conformational change that influences enzyme fidelity.

It has been well documented that dNTP pool imbalances are mutagenic to cells and correlate with the fidelity of DNA replication (15, 99, 112, 216). The data presented in Chapter 3 first demonstrated that the treatment of virus target cells with ribonucleotide reductase inhibitors, which could decrease intracellular dNTP pools, led to the increase of HIV-1 mutant frequencies. Moreover, when virus replicated in the dNTP pools imbalanced quiescent PBMCs, the virus mutant frequency is higher than that observed in dNTP pools balanced activated PBMCs. These data indicate that the increase of HIV-1 mutant frequency correlates with the alteration of intracellular dNTP pools. Previous study showed that AZT treatment of D17 target cells of SNV and MLV did not have influence on intracellular dNTP pools, suggesting that the influence of AZT treatment on the

SNV and MLV mutation rate by a mechanism not involved in alterations in dNTP pools (88). However, the data presented in this dissertation demonstrate that the NRTI treatments of MAGI target cells decreased intracellular dNTP pools, suggesting that there is a correlation between increased HIV-1 mutant frequencies and alteration of dNTP pools during NRTI treatments. Similarly, AZT has been shown to alter intracellular dNTP pools in some cell lines at high concentrations (58, 60). In order to gain further insight into the mechanism of how NRTI's influence HIV-1 mutant frequencies by altering intracellular dNTP pools, the analysis of the effects of NRTI's on intracellular dNTP pools in primary lymphocytes and macrophages would be an appropriate experiment since intracellular dNTP pools vary greatly between cell types. This will be more biologically relevant because both primary lymphocytes and macrophages are primary HIV-1 targets. In addition to studying the effects of NRTI's on dNTP pools in primary lymphocytes and macrophages, another possible study could be to study how NRTI's alter intracellular dNTP pools. It is known that 5'-triphosphate derivatives of NRTI's are the active inhibitors of HIV-1 RT. NRTI's use cellular enzymes and follow different metabolic pathways when they are converted to their active derivatives. Furman et. al. (60) first demonstrated that AZTMP could be considered a substrate-inhibitor of thymidylate kinase (TK), which correlates with the observation that cells incubated with AZT have reduced intracellular levels of dTTP. Therefore, the study of how other NRTI's affect cellular enzymes that are involved in regulation of intracellular dNTP pools

should help lead to a better understanding of how NRTI's influence HIV-1 replication fidelity.

NNRTI's inhibit HIV-1 RT activity by binding to a hydrophobic pocket near the active site, but NNRIT's are not incorporated into viral DNA. Once NNRTI's bind to HIV-1 RT, the conformation of the RT active site has been changed by repositioning the three-stranded β-sheet in the p66 subunit (containing the catalytic aspartic acid residues 110, 185 and 186). This conformational change has a dramatic effect on the rate of the chemical step of polymerization (170). Therefore, we have hypothesized that NNRTI's and NRTI's use different mechanisms to influence HIV-1 mutant frequencies. While NRTI's alter intracellular dNTP pools, NNRTI's could lead to a conformational change of HIV-1 RT that will affect nucleotide selectivity or RT processivity. Unexpectedly, we observed that NNRTI's could also decrease intracellular dNTP concentrations. Based on the observations that EFV and NPV have very similar effects on HIV-1 mutant frequencies, though had different alterations of intracellular dNTP pools, we speculated that NNRTI's may use more than one mechanism to influence virus mutant frequencies. Thus, biochemical studies of dNTP selectivity and processivity of NNRTI-bound RT may provide an understanding of how NNRTI's influence virus mutant frequencies. Previous studies showed that imbalanced dNTP pools could decrease RT processivity and increase base substitutions, and subsequently decrease the fidelity of viral DNA synthesis (5, 13, 15, 199, 201). In addition, it is also plausible that NNRTI's can act as non-competitive inhibitors of cellular enzymes that are responsible for natural dNTP biosynthesis. Thus,

another possible study could be how NNRTI's inhibit dNTP biosynthesis enzymes, such as ribonucleotide reductase. To elucidate the mechanisms of how NRTI's and NNRTI's influence HIV-1 mutant frequencies and alter intracellular dNTP levels will help us to further understand the combined effects of NRTI's and NNRTI's on HIV-1 mutagenesis.

The emergence of drug-resistant virus is a major cause of monotherapy failure. When drug failure occurs due to the evolution of drug resistance, the replication of the drug-resistant virus in the presence of drugs could further influence HIV-1 mutagenesis (Chapter 2 and (126)). In order to suppress the viral replication, the current management of HIV-1 infection involves combinations of NRTI, NNRTI and PI that are changed over time when drug resistance occur. The data presented in this dissertation suggest that antiretroviral drugs can increase HIV-1 mutant frequencies. When they act together, HIV-1 mutant frequencies can be further increased. Not only do the increased mutant frequencies lead to a shift in drug resistance from low-level to high-level, but also could extinguish virus replication by error catastrophe (or lethal mutagenesis) (119). The high rate of HIV-1 mutagenesis, coupled with the finding that most HIV-1 virions in the blood appear to be nonviable (30), suggests that the HIV-1 genome is unable to tolerate many additional mutations without a loss of viability. An increase in mutation rate could mediate a lethal increase in the already high proportion of defective viruses. Based upon the previous analysis of HIV-1 mutant frequencies, 30-fold or higher increases in mutant frequencies would be necessary for extinguishing HIV-1 virus replication (126). Therefore, an

intentional increase in virus mutant frequencies would be an approach for the treatment of HIV-1 infection. This approach has been tested for antiviral treatment of RNA virus infections (43). RNA viruses replicate with an extremely high mutation rate. It has been predicted that RNA viruses exist close to the edge of error catastrophe and can be forced into error catastrophe by a moderate increase in mutation rate. The well-documented experiments of inhibition of RNA virus replication include the efficient extinction of poliovirus and lymphocytic choriomeningitis virus (LCMV) by using a ribonucleoside analog, Ribavirin, and 5-fluorouracil (FU), respectively (37, 64). Furthermore, the promutagenic nucleoside analog, 5-hydroxydeoxycytidine (5-OH-dC), which is incorporated into the viral genome during nucleic acid replication, and results in a progressive accumulation of mutations in the viral genome and ultimately leads to a drastic reduction in virus replication and fitness, has been used to extinguish HIV-1 replication (111). It has been shown that only a 2-fold increase in HIV-1 mutations resulted in viral lethality, suggesting a mechanism for the success of combined drug therapy for HIV-1. Therefore, the unintentional increase in HIV-1 mutagensis by NRTI's and NNRTI's could be used for improving the efficacy of drug therapy by selecting drug combinations that can increase HIV-1 mutagenesis and enhance the likelihood of lethal mutagensis without increase cytotoxicity.

## 5.2 Vpr-mediated UNG2 incorporation into HIV-1 particles

The HIV-1 accessory protein Vpr is essential for viral replication in non-dividing cells such as macrophages. The cellular DNA repair enzyme, UNG2, is known to associate with Vpr and modulate the *in vivo* mutation rate (129). Using an UNG catalytic activity assay, I demonstrated that UNG2 remained enzymatically active after it was incorporated into virus particles, which is strictly related to the direct interaction that takes place in virus-producing cells between Vpr and UNG2.

The specific role(s) of UNG2 recruitment into virus particles on the early steps of HIV-1 infection was analyzed next. We took advantage of the VprW54R mutant to generate a Vpr-UNG2 fusion protein that allows us to determine whether UNG enzymatic activity is involved in modulating HIV-1 mutagenesis and viral replication in *vpr*-defective cells. In collaboration with Dr. S. Benichou (INSERM, Paris), we demonstrated that the incorporation of UNG2 into virions is critical for efficient replication of HIV-1 in non-dividing cells such as macrophage (28). When the VprW54R mutation was introduced into infectious HIV-1 molecular clones, replication in monocyte-derived macrophages (MDMs) was significantly diminished, whereas virus replication in stimulated PBMCs was not altered. Moreover, the lack of UNG2 virion-incorporation during virus replication in macrophages further exacerbated HIV-1 mutant frequencies compared to that measured in actively dividing cells. While the UNG-binding deficient VprW54R variant increased the virus mutation rate by 4-fold, the VprW54R-UNG2 fusion protein was able to influence HIV-1 mutant frequencies in a manner equivalent to

that of wild type Vpr, indicating that Vpr and the virion-associated UNG2 are responsible for the modulation of the virus mutant frequency *in vivo*. Taken together, these data suggest that Vpr-mediated UNG2 incorporation in HIV-1 virus particles has greater biological relevance in non-dividing cells than in actively dividing cells.

Recently, studies have demonstrated an association between the cellular protein APOBEC3G (also known as CEM15) and the HIV-1 encoded Vif (viral infectivity factor) protein in non-permissive cells (179). HIV-1 Vif protein is essential for viral replication in some non-permissive cells, such as primary T cells, macrophages or some $CD4^+$ transformed T cell lines. Viruses deficient in *vif* from non-permissive cells display non-infectious phenotype, implying that APOBEC3G is a specific antiviral factor in non-permissive cells, whose antiviral activity was overcome by the presence of Vif. APOBEC3G is a member of the family of RNA editing enzymes that deaminate cytosines to uracil in DNA or mRNA (81). The members of this family (i.e. activation-induced deaminase (AID), APOBEC1, as well as APOBEC3G) could function as a DNA mutator in *E. coli* (70, 156). It has been reported that Δ*vif*-viruses produced from cells expressing APOBEC3G contain G-to-A hypermutations in newly synthesized plus-strand of viral DNA, which suggests an activity of APOBEC3G that results in the deamination of cytosines to uracils in minus-strand DNA during reverse transcription. In addition, Vif expression in virus producer cells prevented the accumulation of G-to-A mutations. These findings suggest that APOBEC3G is the critical component of innate defense mechanism for HIV-1, which may induce

either lethal mutagenesis or instability of the incoming nascent viral reverse transcripts (69, 106, 117, 215).

Vif can directly interact with human APOBEC3G that prevents the incorporation of human APOBEC3G into HIV-1 virions. Vif inhibits the packaging of APOBEC3G by two ways. First, Vif impairs APOBEC3G mRNA translation and reduces its rate of synthesis (131). Second, Vif induces ubiquitination and proteasomal degradation of APOBEC3G (35, 90, 110, 132, 140, 180, 189). However, the interaction between Vif and APOBEC3G is species-specific. HIV-1 Vif did not inhibit the incorporation of mouse and African green monkey (AGM) APOBEC3Gs into HIV-1 virions and the antiviral activity of these APOBEC3Gs was maintained (131). This indicates that the mouse and AGM APOBEC3Gs can be potent inhibitors of HIV-1 replication, even in the presence of Vif.

It is noteworthy that two HIV-1 accessory proteins, Vpr and Vif, act together to contribute in the fidelity of the viral DNA synthesis and viral replication, but using two independent pathways. Vpr binds DNA repair enzyme UNG2 to increase the fidelity of the viral DNA synthesis in macrophages, while Vif protein forms a complex with APOBEC3G to suppress the antiviral activity of APOBEC3G (Figure 5.1). However, when both UNG2 and APOBEC3G are incorporated into virus particles, they can act together to reduce viral replication. The possible mechanisms for the roles of UNG2 and APOBEC3G in HIV-1 replication are shown in Figure 5.1. When wild type viruses are produced from virus-producing cells, Vif expression excludes APOBEC3G from virus particles, whereas UNG2 is incorporated into virus particles via Vpr. During reverse

transcription, uracil residues can be incorporated into minus-strand DNA either by misincorporation of dUTP or by cytosine deamination. Virion-incorporated UNG2 will excise uracil residues from minus-strand DNA and plus-strand DNA synthesis starts (Figure 5.1A). When *vif*-deficient viruses are produced from virus-producing cells, both APOBEC3G and UNG2 will be incorporated into virus particles. APOBEC3G will induce massive cytosine deamination in minus-strand DNA. This massive cytosine deamination and the creation of uracil residues during minus-strand DNA synthesis can block virus replication in at least three different ways (Figure 5.1B). First, the uracil residues can be excised by virion-incorporated UNG2, resulting in degradation of DNA before integration. Alternatively, HIV-1 RT can incorporate dATPs opposite to those abasic sites caused by uracil-excision by “A-rule” to create G-to-A hypermutations during plus-strand DNA synthesis. Second, uracils in the minus-strand DNA can impair the initiation of plus-strand DNA synthesis during reverse transcription. Third, the deamination of cytosines in minus-strand DNA will cause massive G-to-A substitutions in plus-strand of viral DNA that results in amino-acid changes and aberrant stop signals in the encoded proteins and generally reduce viral fitness at every subsequent stage in HIV-1 replication if infectious virions are produced (65, 95). Thus, further analysis of the mutant frequency of *vpr* $^{-}$ and *vif* $^{-}$ virus will provide further insights into the potential interplay between UNG2 and APOBEC3G in HIV-1 mutagenesis and viral replication. A possible experiment would be to incorporate the VprW54R-UNG2 fusion protein into *vpr*- and *vif*-deficient virus particles and then measure mutant frequencies of *vpr*- and *vif*-

deficient virus containing VprW54R-UNG2 compared to that of *vpr*- and *vif*-deficient virus or wild type virus. Additionally, we could express mouse or AGM APOBEC3G in both *vpr* $^{-}$ and *vpr* $^{+}$ viruses producing cells, and then compare mutant frequencies of viruses produced from both *vpr* $^{-}$ and *vpr* $^{+}$ cells.

In conclusion, the second part of this dissertation research further demonstrated the requirement of Vpr-mediated UNG2 incorporation for efficient virus replication in macrophages. Moreover, recently studies suggest that the use of APOBEC3G by the non-permissive cell (i.e. primary T cells and macrophages) as an innate immune response to HIV-1 infection may lead to the development of new classes of antiretroviral drugs. Therefore, the therapeutic intervention can be envisioned by the development of new classes of antiviral drugs that exploit the properties of the interactions between viral proteins and cellular proteins.

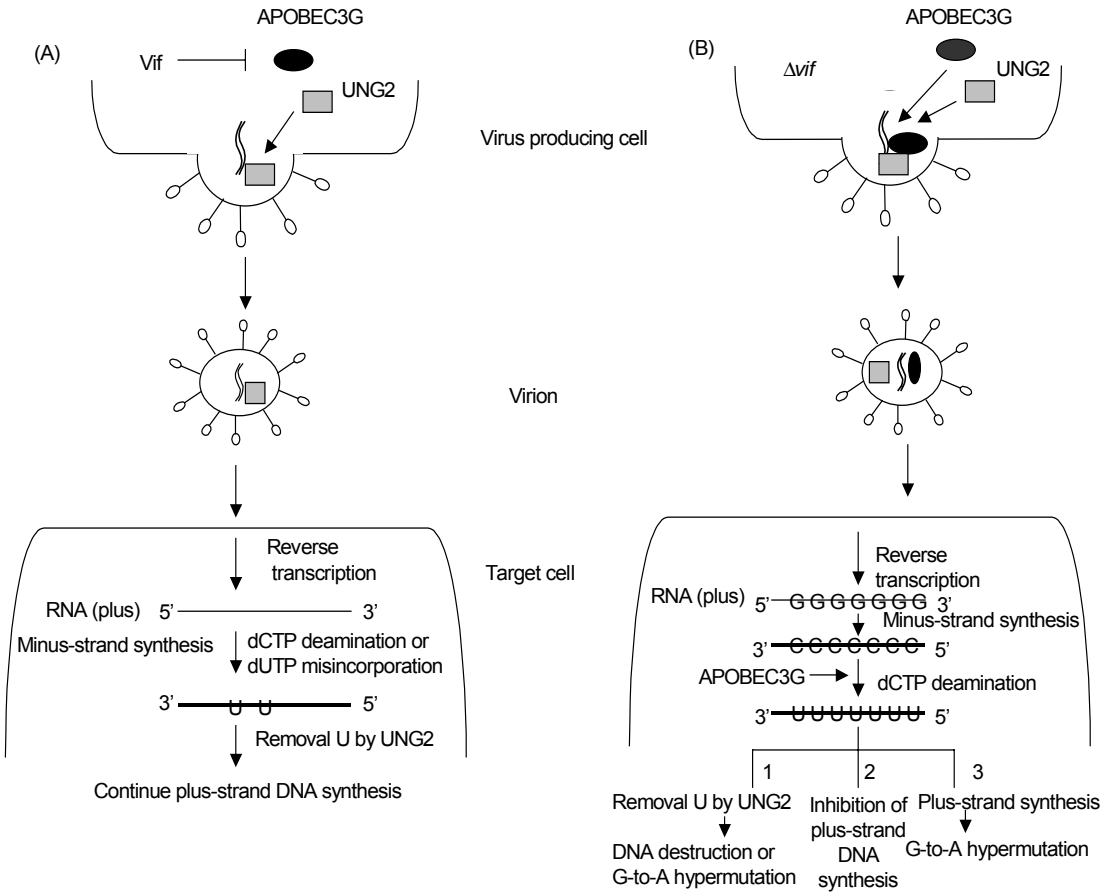



**Figure 5.1**. Possible mechanisms for the roles of UNG2 and APOBEC3G in HIV-1 replication. (A) Vif expression results in APOBEC3G being excluded from budding virions. The cellular DNA repair enzyme UNG2 is incorporated into the virus particle via Vpr. After viral infection, the minus-strand DNA (thick line) is synthesized using viral RNA (thin line) as template. The uracil residues can be incorporated into minus-strand DNA by either dCTP deamination or dUTP misincorporation. The virion-associated UNG2 will remove uracil residues from minus-strand DNA. The synthesis of plus-strand DNA will start. (B) In the absence of Vif, APOBEC3G is packaged into virions. UNG2 is also incorporated into virions via Vpr. After the virus enter new target cell, the reverse transcription starts. First, the minus-strand DNA (thick line) is synthesized using viral RNA (thin line) as template. The cytosine residues can be deaminated to uracils by APOBEC3G. The uracil containing minus-strand DNA will (1) be degraded after UNG2 excises uracil residues from minus-strand DNA; alternatively, dATPs will be incorporated into plus-strand DNA opposite to abasic sites by "A-rule", resulting in G-to-A hypermutations; (2) impair the initiation of plus-strand DNA synthesis; and (3) cause G-to-A hypermutation that will lead to lethal mutagenesis.

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