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RETROVIRAL MUTATION RATES AND REVERSE TRANSCRIPTASE FIDELITY

Evguenia S. Svarovskaia [1], Sara R. Cheslock [1,2], Wen-Hui Zhang [1], Wei-Shau Hu [1], and Vinay K. Pathak [1]

[1] HIV Drug Resistance Program, CCR, NCI-Frederick, Frederick, Maryland 21702. [2] Department of Microbiology and Immunology, West Virginia University, Morgantown, WV 26506

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1. ABSTRACT

Genetic variation in retroviral populations provides a mechanism for retroviruses to escape host immune responses and develop resistance to all known antiretroviral drugs. Retroviruses, like all RNA viruses, exhibit a high mutation rate. Polymerization errors during DNA synthesis by reverse transcriptase, which lacks a proofreading activity, is a major mechanism for generating genetic variation within retroviral populations. In this review, we summarize our current understanding of the processes that contribute to the generation of mutations in retroviruses. An overview of *in vivo* and *in vitro* studies of retroviral mutation rates determined by various fidelity assays is provided. Extensive mutational analyses of RTs are beginning to elucidate the relationship between structural determinants of RTs and fidelity of DNA synthesis. Recently, it was observed that the Y586F mutation in MLV RT results in a dramatic increase in the mutation rate in the vicinity of adenine-thymie tracts (AAAA, TTTT, and AATT), which are associated with bends in DNA. These results indicate that the template-primer duplex is a component of the polymerase active site and its structure can influence nucleotide selectivity and the mutation rate. Additionally, the results also suggest that the Y586 residue and the RNase H primer grip are structural determinants of RT that have evolved to attenuate the effects of unusual conformations of the template-primer duplex, such as bends in DNA, on fidelity of DNA synthesis.

2. IMPORTANCE OF RETROVIRAL GENETIC VARIATION

All retroviral populations exhibit tremendous genetic variation that allows them to adapt to changes in their environment. Genetic variation has been documented extensively in populations of human immunodeficiency virus type 1 (HIV-1) (1-3). This genetic adaptability has significant consequences for the evolution of HIV-1 and other retroviruses, their impact on human health, and the ability of human societies to deal with the epidemic of acquired immunodeficiency syndrome (AIDS).

The genetic variation found in HIV-1 populations has allowed the virus to adapt by expanding its host range; for example, HIV-1 can switch from using the CCR5 coreceptor to using the CXCR4 coreceptor (4-6). Genetic variation is evident in the numerous HIV-1 clades that now infect various human populations and cause AIDS (7). The genetic variation between as well as within individual clades is a significant obstacle to the successful development of an anti-HIV-1 vaccine (8, 9). For HIV-1-infected patients in the Western world, perhaps the most significant consequence of HIV-1 genetic diversity is the rapid development of resistance to antiretroviral drugs. Drug-resistant variants have arisen to over 116 antiretroviral agents that have been tested in the clinic or in the laboratory (10). Because of extensive genetic variation, it is expected that in response to any new antiretroviral agent, drug-resistant HIV-1 variants will emerge.

Genetic variation in retroviral populations is a consequence of the viral mutation rate (11-15, 16), recombination rate (17-21), rate of replication (1, 3), size of the viral population (22), and selective forces (2). In addition to a high rate of mutation, retroviruses also exhibit a high rate of recombination, which further increases variation in the viral population. Because the rates of viral mutation and recombination are considered as rates per cycle of replication, the number of replication cycles that occur per unit of time is an important factor that influences genetic variation in the viral population. The size of the viral population is also an important factor, because it sets an upper limit to the number of variants that can exist in the population at any given time. Of course, selective forces determine which viral variants will survive and contribute to the virion of the next generation (2).

In this review, we will outline the various mechanisms that affect the retroviral mutation rate. We will also summarize the current state of knowledge of the relationship between reverse transcriptase (RT) structure and its fidelity and discuss the influence of the template-primer duplex structure on the fidelity of reverse transcription.

3. HISTORIC DEVELOPMENTS IN UNDERSTANDING RETROVIRAL GENETIC VARIATION AND RT FIDELITY

Peyton Rous observed genetic variation associated with retroviruses soon after the discovery of the Rous sarcoma virus (RSV) (23). He observed that some chicken sarcomas induced by the ”filterable agent” were hemorrhagic, while others were composed of spherical rather than spindle-shaped cells. Although these variable characteristics could not be directly attributed to the viral genetic information, Duran-Reynals later found that certain variants of RSV, isolated from late-appearing tumors induced in with RSV, could infect ducks (24). Thus, genetic variants of RSV with different biological properties were identified.

Howard Temin developed a quantitative assay for RSV (25) and used it to perform a detailed study of variation associated with RSV (26). He noted that infection of chick embryo fibroblasts *in vitro* resulted in formation of foci with distinct morphological phenotypes. Clonal stocks of virus derived from a focus produced foci with the same morphology, indicating that the focus morphology was, at least in short-term cultures, a genetic characteristic of the virus.

Soon after the discovery and isolation of RT (27, 28), it was noted that this DNA polymerase exhibited a high error rate when it is used to copy homopolymeric RNA and DNA templates (29-32). In the first reported measurement of RT fidelity, the mutation rate of avian myeloblastosis virus (AMV) RT was estimated to be approximately 1 in 600 for dCMP incorporation on a poly A template (29). These observations led to the suggestion that under certain conditions of DNA polymerization, RT makes a significant number of errors that might play a role in spontaneous mutation (32). Later, Coffin et al. estimated the mutation rate of a specific nucleotide position to be approximately 10^{-4} mutations per replication cycle in a continuously passaged culture of RSV (33).

Gojobori and Yokoyama (34) compared the rate at which mutations accumulated in the retroviral v-*mos* oncogene in a Moloney murine sarcoma virus (MoMSV) and its cellular homolog, c-*mos*. They found that the rate of mutation accumulation in v-*mos* was approximately a million-fold higher than the rate of mutation accumulation in c-*mos* (1.3×10^{-3} vs. 1.7×10^{-9} per site per year, respectively). These results established that retroviruses evolve much more rapidly than mammalian organisms as well as DNA-containing microbes. However, their rates of evolution are similar to those of other RNA viruses (35). All RNA viruses, including retroviruses, appear to exhibit a high degree of variability and are described as a quasispecies, which is characterized as a large collection of genetically related but not identical genomes (36).

The development of sensitive genetic assays that measure either the reversion of a nonsense codon (37) or forward mutations that inactivate a reporter gene (38) has greatly facilitated measurements of the error rates of RTs (39). Additionally, accurate measurements of the *in vivo* retroviral mutation rates have been made possible by the development of retroviral vectors and packaging cell lines that allow the virus to undergo a single cycle of replication in a controlled manner (40, 41).

4. FACTORS THAT INFLUENCE RETROVIRAL MUTATION RATE

4.1. Reverse transcription

Mutations may be introduced into retroviral genomes during various steps in the viral life cycle (figure 1). After the viral RNA enters the cytoplasm of the target cell, it is first copied in an RNA-dependent DNA synthesis step to generate a minus-strand DNA, which is subsequently copied in a DNA-dependent DNA synthesis step to generate a double-stranded DNA form (42). RTs, which carry out these two polymerization steps, lack exonucleolytic proofreading activity and thus rely only on

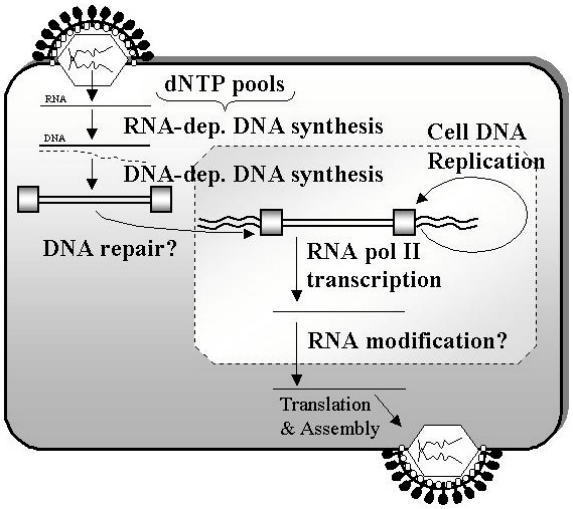

Figure 1. Retroviral replication cycle. Virion core containing two RNA copies of the retroviral genome enters the cytoplasm. RT copies Viral RNA (thin line) into minus-strand DNA (thick line) in a step labelled RNA-dependent DNA synthesis. For simplicity, only one RNA is shown. Next, RT copies minus-strand DNA into double-stranded DNA (thick lines) in a step labeled DNA-dependent DNA synthesis. The fidelity of both RNA- and DNA-dependent DNA synthesis may be influenced by intracellular dNTP pools. Polymerization errors that occur during DNA-dependent DNA synthesis form heteroduplexes that may be subjected to DNA repair. Integration of the viral DNA into the cell chromosome forms the provirus. Proviral DNA is replicated by mammalian DNA polymerases during each cell division. Viral RNA is synthesized by cellular RNA polymerase II, which uses the integrated provirus as a template. Translation and assembly of viral proteins is followed by formation of viral particles at the plasma membrane. Steps of retroviral replication cycle and factors that could influence the rate of viral mutation are indicated in bold type.

discrimination against the incorrect incoming nucleotide during polymerization to prevent errors. Consequently, polymerization errors could occur during either RNA-dependent or DNA-dependent DNA synthesis.

4.2. Other viral proteins

RT is not the only viral protein that influences the retroviral mutation rate. Several retroviruses encode a dUTPase that suppresses the incorporation of uracil into the viral genome and increases the fidelity of reverse transcription (43-48). Mutations in the HIV-1 accessory protein Vpr have been shown to influence the HIV-1 mutation rate by interacting with uracil DNA glycosylase and facilitating its incorporation into virion (49). Recent studies have shown that the nucleocapsid protein (NC) can enhance the rate of viral DNA synthesis in regions of the template containing secondary structure (50); the results also suggest that murine leukemia virus (MLV) NC could have a significant impact on the viral mutation rate.

4.3. Nucleotide pools, DNA repair, and mammalian DNA polymerases

In addition to viral proteins, the intracellular environment, mammalian polymerases, and nucleic acid modifying enzymes could potentially influence the retroviral mutation rates. Alteration of the intracellular nucleotide pools has been shown to increase the retroviral mutation rates (51).

Mutations that occur during DNA-dependent DNA synthesis result in the formation of heteroduplexes that could be potentially recognized by the host DNA repair enzymes and corrected in a strand-specific manner to influence the overall mutation rate. Recent studies have indicated that mismatches involving large loops can be efficiently repaired by the host repair system, which could affect the overall mutation rate (52). Mutations can also occur during replication of the integrated provirus through cell division. However, host cell DNA polymerases have mutation rates (10^{-9} to 10^{-12} mutations/basepairs (bp)/cycle) that are significantly lower than the mutation rates of RTs and their contribution to the retroviral mutation rates is probably negligible (53).

4.4. RNA transcription

Perhaps the most significant contribution to retroviral errors by a non-viral mechanism is polymerization errors during RNA polymerase II- mediated transcription. The mutation rate of RNA polymerase II has not been accurately determined, and thus its contribution to the retroviral mutation rate is unknown. Analysis of mutations that occur in a reporter gene inserted in the long terminal repeat (LTR) has indicated that approximately 1/3 of the mutations occur during the DNA-dependent DNA synthesis step of reverse transcription and the remaining 2/3 of the mutations occur during RNA transcription and the RNA-dependent DNA synthesis step of reverse transcription (54). These results provide an upper limit to the contribution of RNA polymerase II to retroviral genetic variation; assuming that the error rates of the three polymerization steps (RNA transcription, RNA-dependent DNA synthesis, and DNA-dependent DNA synthesis) are similar, the mutation rate of RNA polymerase II is around 0.5-1 x 10^{-5}mutations/bp/replication cycle. This estimate is at the lower end of the *in vitro* mutation rate measured for wheat-germ RNA polymerase II (10^{-3} to 10^{-5} mutations per/bp/cycle) (55).

Eukaryotic as well as bacterial RNA transcription processes appear to possess a proofreading ability, suggesting that RNA transcription may be more accurate than reverse transcription. Eukaryotic transcription factor SII and bacterial GreA and GreB proteins have been shown to stimulate the excision of misincorporated bases from transcribed RNA (56-58). A recent study suggested, however, that the rate of translational errors is significantly higher than that of transcriptional errors in yeast strains lacking SII, suggesting that any proofreading activity provided by SII is unlikely to be physiologically relevant (59).

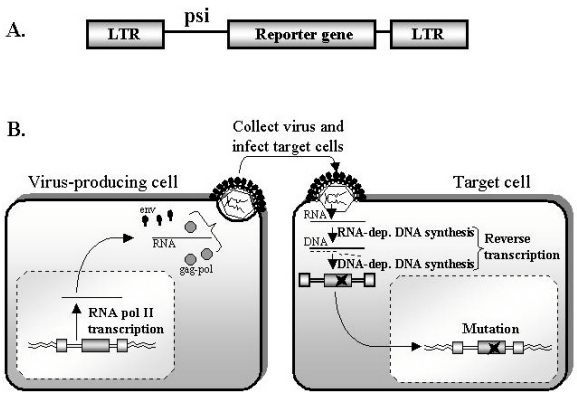

Figure 2. In vivo assays for measurement of retroviral mutation rates in a single replication cycle. A. Retroviral vector containing LTRs, packaging signal (psi), and a reporter gene is shown. Commonly used reporter genes are *lacZ*, *lacZa*, GFP, and HTK. B. A producer cell clone containing an integrated provirus derived from a retroviral vector is shown. Proviral DNA is transcribed by RNA polymerase II to generate viral RNA that is packaged into infectious viral particles. Virion produced from the producer cells are used to infect target cells. Upon infection, reverse transcription of the viral genomic RNA occurs and involves one cycle of RNA-dependent DNA synthesis and one cycle of DNA-dependent DNA synthesis. Mutations that inactivate the reporter gene during RNA transcription or reverse transcription can be identified phenotypically and characterized by DNA sequencing.

Slippage-induced frameshift errors induced by mammalian RNA polymerase II were analyzed by employing an apoB mutant allele containing a deletion of a single cytosine, creating a stretch of eight adenines. It was demonstrated that transcriptional slippage occurs with a frequency of 10% by the insertion of an extra adenine into the stretch of eight adenines (60). A similar transcriptional slippage frequency of 25-30% was also documented for the *Escherichia coli* (*E. coli*) RNA polymerase during elongation at stretches of ten or more adenines or thymines, which were detected by restoration of the proper reading frame of the bacterial beta-galactosidase (*lacZ*) reporter construct (61).

4.5. RNA modification

Another potential mammalian host cell mechanism that contributes to retroviral genetic variation is RNA modification. The host cell double-stranded RNA adenosine deaminase (dsRAD) can modify retroviral RNAs by deamination of adenosines to inosines, which ultimately result in A-to-G hypermutation of the viral genomes (54, 62, 63). However, the low frequency of A-to-G hypermutation in retroviral sequences suggests that RNA modification of retroviral genomes occurs rarely.

4.6. Antiviral nucleoside analogs

Treatment with the 3'-azido-3'-deoxythymidine (AZT) profoundly increases the retroviral mutation rate in an RT-dependent manner (64). The mutation rate of spleen necrosis virus (SNV) was increased seven- to tenfold in the presence of AZT while similar concentrations of AZT resulted in only a two- to threefold increase in the MLV mutation rate (64). The mutation rate of FIV was increased in the presence of AZT approximately threefold (65). Later, it was shown that AZT also increased the mutation rate of HIV-1 about eightfold (66). Interestingly, 2',3'-dideoxy-3'-thiacytidine (3TC) also increased the mutation rate modestly by threefold. The simple hypothesis that AZT competes with intracellular nucleosides for phosphorylation and thereby alters the intracellular nucleotide pools was not supported by experimental evidence (64). Thus, the mechanism by which AZT increases the mutation rate of RTs is unclear.

5. *IN VIVO* FIDELITY ASSAYS AND MUTATION RATES

5.1. *In vivo* fidelity assays

A generalized approach to measuring *in vivo* retroviral mutation rates is outlined in figure 2. A retroviral vector encoding a reporter gene is constructed. Typically, the product of a mutation reporter gene is easily identifiable phenotypically or can be selected. The *lacZ* or its truncated peptide (*lacZa*), herpes thymidine kinase gene (HTK), neomycin phosphotransferase gene (*neo*), and green fluorescent protein gene (GFP) have been used as mutation reporters. The retroviral vector encoding the reporter gene is introduced into a packaging cell line and the virus produced is used to infect target cells; the vector can complete one round of replication and integrate in the target cell genome to form a provirus. However, because the vector is unable to express any viral proteins, additional cycles of replication cannot occur. A single cycle of retroviral replication constitutes one cycle of RNA transcription by RNA polymerase II, one cycle of RNA-dependent DNA synthesis, and one cycle of DNA-dependent DNA synthesis.

The assays can be designed to detect the inactivation of the reporter gene (forward mutation assays) or to detect the reversion of an inactivating mutation introduced in the reporter gene (reversion assays) (13, 67). Because the forward mutation assays provide an average mutation rate of several hundred target nucleotides, the observed mutation rate is likely to be representative of the mutation rate of viral genes and sequences. In contrast, only one or a few nucleotide targets are monitored in the reversion assays; because retroviral mutation rates are highly sequence dependent and mutational hotspots and coldspots have been well-documented, the target nucleotides chosen in a reversion assay may or may not reflect the overall mutation rate.

The *in vivo* forward mutation rates represent the most reliable measurements of retroviral mutation rates because they are determinations of polymerization errors that occurred in the context of a replicating complex composed of all of the viral proteins under *in vivo* conditions of pH and nucleotide concentrations. However, the *in vivo* forward mutation assays also have their limitations. First, not all mutations in the reporter gene result in a detectable phenotypic change in the gene product; thus, estimates of the mutation rate depend on assumptions made about

Table 1. *In vivo* retroviral mutation rates

RT	Reporter gene	Mutation Rate (´ 10^{-5} mutations/bp /cycle)	Reference
HIV-1	*LacZa*	3.4[a]	14
SNV	*LacZa*	1.1	12, 13
SNV	*LacZa*	2.2	54
SNV	*Neo (reversion)*	500	71
SNV	*Neo (reversion)*	2.0	67
BLV	*LacZa*	0.5	16
HTLV-1	*LacZa*	0.7	15
RSV	viral genome	14[b]	114
Ty1	*Ty1*	2.5[c]	69
MLV	*LacZ*	2.5[d]	84, 85,104
MLV	*GFP*	2.1[e]	104
MLV	*HTK*	1.6[f]	86
MLV	*Neo (reversion)*	0.2	77

[a] Mutation rate was calculated based on the predetermined number of mutational targets for *lacZa* reporter gene (113 bp). [b] Presence of mutations in viral sequences was detected by denaturing gradient gel electrophoresis. [c] Mutation rate was calculated as follows: (13/510,777 = 2.5 x 10^{-5}) number of detected substitutions (13) was divided by number of replicated nucleotides (29 transpositions x 17,613 nt per replication cycle = 510,777 nt). [d] Mutation rate was calculated based on estimated number of mutational targets as 2/3 of the length of *lacZ* reporter gene. Mutation rate was determined as follows: (5.2% mutant frequency divided by number of mutational targets 2040 bp = 2.5 x 10^{-5}). [e] Mutation rate was calculated based on estimated number of mutational targets as 2/3 of the length of GFP reporter gene. Mutation rate was determined as follows: (1.0% mutant frequency divided by number of mutational targets 472 bp = 2.1 x 10^{-5}). [f] Mutation rate was determined only for the mutations that involved large genetic rearrangements. An approximate overall mutation rate is calculated by estimating the number of mutational targets as 2/3 of the length of HTK reporter gene (1747 of 2620 bp total) as follows: (8.8% mutant frequency divided by number of mutational targets 1747 bp = 5.0 x 10^{-5} mutations/bp/cycle).

the number of mutational targets present in the reporter gene that reliably result in a detectable mutant phenotype. Detailed information based on experimental data of mutational target sites is available for the *lacZa* gene (113 target sites for a total length of 280 nucleotides) and the measured rates are likely to be accurate (68). However, the numbers of mutational targets are not known for the complete *lacZ* gene, HTK, or GFP. A second potential limitation is that the mutation rates that are measured using reporter genes may not be representative of the viral genes and sequences. Because forward mutation rates represent an average of several hundred target sites, they are likely to be representative of viral sequences as well. The mutation rate of the Ty1 transposable element was measured by direct sequencing of 173,043 nucleotides and the rate was determined to be 2.5 x 10^{-5} mutations/bp/cycle (69). This result suggests that mutation rates based on direct sequencing of viral genomes are likely to be similar to those measured by using reporter gene-based assays. However, the possibility that viral sequences have evolved to minimize the impact of RT mutations cannot be ruled out. A third limitation is that the observed mutation rates represent the sum of the RT mutation rate and the RNA polymerase II mutation rate; thus, the RT mutation rate cannot be directly measured.

5.2. *In vivo* mutation rates

The *in vivo* forward mutation rates have been measured for SNV, HIV-1, bovine leukemia virus (BLV), human T cell leukemia virus type 1 (HTLV-1), MLV, RSV, and the Ty1 retroelement (table 1). With the exception of RSV, these mutation rates are very similar to each other and range from 0.5 x 10^{-5} mutations/bp/cycle for BLV to 3.4 x 10^{-5} mutations/bp/cycle for HIV-1. It is important to point out that the HIV-1 *in vivo* mutation rate is within twofold of the MLV and SNV mutation rates; even though HIV-1 appeared to be substantially more error-prone in some studies, its *in vivo* mutation rate is very similar to that of gammaretroviruses (11, 70). Most of the *in vivo* mutation rates observed to date are within a threefold range of an average of 1.5 X 10^{-5} mutations/bp/cycle. The only exception is the high mutation rate of RSV (14 X 10^{-5} mutations/bp/cycle), which was measured using viral sequences as a target and denaturing gradient gel electrophoresis. Because the method used to measure the RSV mutation rate was significantly different from the method used for other viruses, the rates may not be directly comparable.

The *in vivo* forward mutation rate of MLV was determined using several reporter genes (*lacZ*, GFP, and HTK) and the observed rates were similar, suggesting that sequence differences among reporter genes do not significantly affect fidelity and mutation rate estimates. There were exceptions, however, indicating that the method of measurement of the *in vivo* mutation rates could be important for obtaining an accurate result. For example, *in vivo* reversion assays using the *neo* reporter gene displayed two mutation rates for SNV RT that differ by 250-fold (67, 71).

6. *IN VITRO* FIDELITY ASSAYS AND MUTATION RATES

6.1. *In vitro* fidelity assays

In vitro assays can be used to measure the mutation rate of purified RT in the presence of nucleotide substrates and a template-primer complex. As discussed earlier, the *in vitro* assays can also be set up to measure the forward mutation rate of a reporter gene or reversion of a nonsense codon. A forward mutation assay in which the *lacZa* gene serves as a mutation reporter is frequently used to measure the mutation rates of purified RTs (11, 72-75). In this assay, a gapped-duplex DNA is generated from a genetically engineered single-stranded bacteriophage,

M13mp2; the gapped single-stranded DNA is copied by RT in the presence of dNTP substrates, and errors that occur during this synthesis are quantified by analyzing the phenotype of plaques generated by infection of host bacteria.

In misinsertion assays, a binary complex is formed between the RT and a template-primer (70, 76-78). Then, the ability to extend the primer in the presence of the correct and incorrect nucleotide substrate is determined. The efficiency of primer elongation is measured by quantitative gel electrophoresis; the data are analyzed using the Michaelis-Mention equation and the parameters K_{cat} and K_m are determined for the correct and incorrect nucleotide substrate. Misinsertion efficiency (F_{ins}) is defined as the ratio of K_{cat}/K_m for the incorrect nucleotide divided by the K_{cat}/K_m for the correct nucleotide. The rate of polymerization (K_{cat}) should be higher for the correct nucleotide than for the incorrect nucleotide; on the other hand, the affinity of RT for the correct nucleotide should be higher (lower K_m) than for the incorrect nucleotide.

In mismatch extension assays, template-primers in which the 3' terminus of the primer strand is correctly matched to the template or is mispaired are used (76-78). The kinetics of extending the mismatched primer are compared with the kinetics of extending the correctly matched primer. The mismatch extension ratio (F_{ext}) is K_{cat}/K_m for the mismatched primer divided by the K_{cat}/K_m of the correctly matched primer.

The *in vitro* fidelity assays have the advantage that they can be performed under defined conditions. RT fidelity can be measured on either the RNA or DNA template without the complication of errors introduced during RNA transcription. Misincorporation occurs through a series of steps that include discrimination of the correct and incorrect nucleotide substrate, the incorrect nucleotide binding to the substrate-binding site, phosphate bond formation, and extension of the mismatched nucleotide. *In vitro* assays have the potential to dissect and analyze these various steps in detail. However, like *in vivo* assays, *in vitro* assays also have limitations. First, the conditions of the assay such as pH, nucleotide concentrations, the nature and concentration of the divalent cation, and the nature of the template-primer can all significantly impact the observed mutation rate (79-83). Second, certain conditions such as the stability and structure of the template-primer complex and the ratio of RT to template-primer complex may impact the observed results. One concern about the misinsertion assays is the potential contribution of contaminating nucleotides to the primer extension that appears as misinsertion. Third, the limitations of a codon reversion assay also apply to these assays, because the misinsertion and mismatch extension rates at only one or a few target nucleotides, which may or may not represent the overall rate are measured. Finally, perhaps the most important drawback is that the potential influence on the mutation rate of other viral proteins, the structure of the reverse transcription complex, and other aspects of the intracellular environment such as the balance of endogenous nucleotide pools are not taken into account.

6.2. *In vitro* mutation rates

The *in vitro* forward mutation rate for HIV-1 RT has been determined using the *lacZa* reporter gene by several investigators (11, 72, 74, 75) (table 2). The reported mutation rates range from 5.3×10^{-5} mutations/bp/cycle to 59×10^{-5} mutations/bp/cycle. These mutation rates are up to 17-fold higher than the *in vivo* forward mutation rate determined using the same mutation reporter gene (11, 14). A comparison of the sites of mutations *in vivo* and *in vitro* indicates that the locations of the mutations as well as their rates vary widely between the *in vitro* and *in vivo* assays (14). Furthermore, a comparison of the mutational hotspots in the *lacZa* gene determined in three separate studies suggests that the sites and nature of mutations can be dependent on the conditions of the assay (72, 74, 75). These results have suggested that there are elements of the *in vivo* conditions that are missing from the *in vitro* assays and that these factors can greatly influence the fidelity of DNA synthesis.

Despite these caveats, the *in vitro* forward mutation assays for MLV RT have provided mutation rates that are similar to each other and to the *in vivo* forward mutation rates (72, 73, 84-86). A comparison of the *in vitro* forward mutation rates of SIV, AMV, and MLV RTs suggest that the mutation rates of these RTs are similar (72, 73, 75).

The mutation rates determined by the misinsertion and mismatch extension assays are quite variable, suggesting that they are highly dependent on the conditions of the assay. For HIV-1 RT, the range of misinsertions is approximately 2200-fold, varying from 0.02×10^{-5} mutations/bp/cycle to 44×10^{-5} mutations/bp/cycle (78, 87-89). The rates for HIV-1 RT mismatch extension vary from 10×10^{-5} mutations/bp/cycle to 590×10^{-5} mutations/bp/cycle (78, 90, 91). Varela-Echavarria et al. compared the MLV rate of mutation determined *in vivo* for a single nucleotide position (0.2×10^{-5} mutations/bp/cycle) with the rate of misinsertion for the identical nucleotide sequence (77). They found that the A-C mismatch occurs at a rate comparable to the *in vivo* mutation rate (0.4×10^{-5} mutations/bp/cycle) but the T-G mismatch occurs at a rate that is 30-fold higher (7×10^{-5} mutations/bp/cycle). Again, these results suggest that additional factors that improve the fidelity of reverse transcription are present in the infected cells that are absent from the *in vitro* assays.

7. SPECTRUM OF MUTATIONS AND THEIR RELATIVE FREQUENCIES

SNV, MLV, and HIV-1 RTs induce a similar broad spectrum of mutations during reverse transcription *in vivo* (12-14, 86, 92). Approximately 51–81% of the mutations characterized are substitution mutations. Among the substitution mutations, approximately 80% are transitions and 20% are transversions, and G-to-A transitions are generally the most frequent. About 10-25% of the mutations are frameshift mutations that occur in stretches of identical nucleotides; increasing the length of the stretches of nucleotides dramatically increases the

Table 2. *In vitro* retroviral mutation rates

RT	Assay – Reporter gene or nucleic acid template	Mutation Rate (´ 10^{-5} mutations/bp/cycle)	Reference
HIV-1	*In vitro*-FMA[a] – *lacZa*	59	11
HIV-1	*In vitro*-FMA – *lacZa*	16	74
HIV-1	*In vitro*-FMA – *lacZa*	17	72
HIV-1	*In vitro*-FMA – *lacZa* - RNA	14	72
HIV-1	*In vitro* FMA – *lacZa*	5.3	75
HIV-1	*In vitro* FMA – *EnvlacZa*[b]	1.8	75
HIV-1	*In vitro*-FMA – *HIV-1env*	19	115
HIV-1	*In vitro*-FMA – *HIV-1env*-RNA	20	115
HIV-1	Misinsertion – DNA	0.6 – 66.6	70
HIV-1	Misinsertion – DNA	4.4 - 16	78
HIV-1	Misinsertion – DNA	6 - 57	87
HIV-1	Misinsertion – DNA	1.4 - 28.9	89
HIV-1	Misinsertion – DNA	0.2 - 125	88
HIV-1	Misinsertion – RNA	15 - 67	78
HIV-1	Misinsertion – RNA	0.02 - 8.3	88
HIV-1	Mismatch extension – DNA	4.1 - 38.4	91
HIV-1	Mismatch extension – DNA	10 - 76.9	116
HIV-1	Mismatch extension – DNA	140 - 590	78
HIV-1	Mismatch extension – RNA	550 - 2700	78
BLV	Misinsertion – DNA	0.3 - 8.6	117
BLV	Mismatch extension – DNA	19.6 - 29	117
Ty1	Misinsertion – DNA	0.5 - 6.3	118
Ty1	Misinsertion – RNA	0.8 - 1.7	118
MLV	*In vitro* FMA – *lacZa*	3.3	73
MLV	*In vitro* FMA – *lacZa*	3.4	72
MLV	*In vitro* FMA – *lacZa* - RNA	2.7	72
MLV	*In vitro* FMA – *EnvlacZa*	1.9	75
MLV	Misinsertion – DNA	0.4 - 6.7	77
MLV	Mismatch extension – DNA	0.5 - 9.1	91
MLV	Mismatch extension – DNA	80 - 150	77
MLV	Mismatch extension – RNA	5.3 - 33.3	91
SIV	*In vitro* FMA – *EnvlacZa*	1.1	75
SIV	*In vitro* FMA – *lacZa*	2.9	75
SIV	Misinsertion – DNA	0.4	119
SIV	Misinsertion – RNA	2.7	119
AMV	*In vitro* FMA – *lacZa*	3.3	73
AMV	Misinsertion – DNA	0.3 - 3.8	70
AMV	Misinsertion – DNA	16 - 59	78
AMV	Misinsertion – DNA	0.3 - 4.7	120
AMV	Misinsertion – DNA	0.6 - 2.4	118
AMV	Misinsertion – RNA	3.2 - 30	78
AMV	Mismatch extension – DNA	56 - 5800	78
AMV	Mismatch extension – RNA	210 - 4500	78
AMV	Mismatch extension – DNA	0.3 - 23	120
HIV-2	Mismatch extension – DNA	0.5 - 34.4	106
HIV-2	Mismatch extension – DNA	2.9 - 55.5	91
HIV-2	Mismatch extension – RNA	8.3 - 71	91
MMTV[c]	Misinsertion – DNA	0.9 - 10.1	120
MMTV	Mismatch extension – DNA	0.3 - 11.2	120
EIAV[d]	Misinsertion – DNA	1.2 - 33.3	89
EIAV	Mismatch extension – DNA	2.8 - 25	89
EIAV	Mismatch extension – RNA	7 - 47	89

[a] FMA, forward mutation assay. [b] *EnvlacZa*, fusion product of the fragment of SIV envelope and *lacZa* (75). [c] MMTV, mouse mammary tumor virus. [d] EIAV, equine infectious anemia virus.

frequency of frameshifts, which are believed to occur through a slippage mechanism (12, 93, 94). The remaining 10-25% of the mutations occur by RT switching templates from one region of the template to another, which results in simple deletions and deletions with insertions; simple deletions occur through template switching events

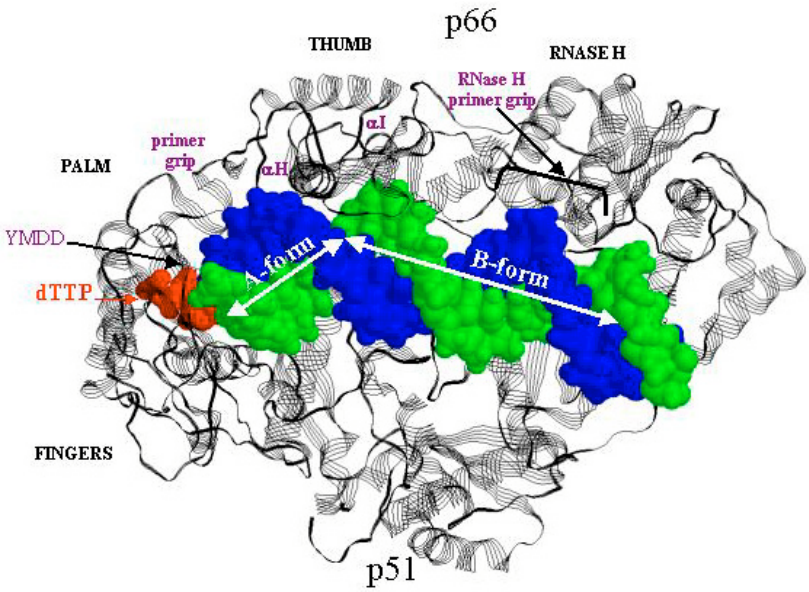

Figure 3. Structure of HIV-1 RT bound to a DNA-DNA template-primer complex. Representation of the HIV-1 RT bound to template-primer complex and incoming dTTP was generated based on RT structure described in reference (98) using the Ras-Mol program. The fingers, palm, thumb, and RNase H domain of the p66 subunit are indicated. The A-form and B-form DNAs of the template-primer complex are labeled. A 41-degree bend is present at the A-form/B-form junction.

involving short direct repeats at the deletion junctions, whereas deletions with insertions involve more complex template switching events. On rare occasions, G-to-A hypermutations are observed in which multiple substitutions occur within the same viral genome; the mechanism by which these mutations occur is unknown but may involve reverse transcription by highly error-prone polymerases or by biased nucleotide pools (13, 95). Other infrequent mutations involving duplications by RT template switching and A-to-G hypermutations by dsRAD have been reported (54).

8. STRUCTURAL DETERMINANTS OF RT THAT INFLUENCE FIDELITY

8.1. Structure of RT

The structure of RT is likely to be responsible for its low fidelity of DNA synthesis. As already mentioned, RT lacks the exonucleolytic proofreading activity that is a feature of most cellular DNA polymerases. It was hypothesized that because two template-switching events (called minus-strand transfer and plus-strand transfer) are necessary for the completion of reverse transcription, retroviral RTs evolved to possess low template affinity and low processivity (96). The template-switching property of RTs results in additional intramolecular and intermolecular template-switching events that lead to formation of deletions and recombination.

The structure of RT is likely to play an important role in its low template affinity and low processivity. Several crystal structures of HIV-1 RT have been determined, including cocrystals with nonnucleoside inhibitors, a DNA:DNA template-primer hybrid, an RNA:DNA hybrid, and a ternary complex with DNA and dTTP substrate (97-101). HIV-1 RT is a heterodimer composed of p66 and p51 subunits (figure 3). The p66 subunit possesses both polymerase and RNase H activities. The structure of RT is often compared to a right hand, and the various domains of RT are referred to as fingers, palm, thumb, connection, and RNase H. The p51 subunit lacks the RNase H domain and is folded in a different conformation.

A partial MLV RT crystal structure has been solved for the N-terminal segment of the protein containing the fingers and palm domains (102, 103). Despite a low primary sequence homology between HIV-1 and MLV RTs, the three-dimensional structures of the fingers and palm domains appear to be similar (102).

8.2. Structural determinants of *in vivo* fidelity

To date, few studies have analyzed the effects of mutations in RTs on the *in vivo* fidelity of reverse transcription (15, 66, 84, 85, 104) (table 3). Halvas et al. have performed extensive mutational analysis of MLV RT and determined the effects of the mutations on the *in vivo* fidelity of reverse transcription (84, 85). In the first study, mutational analysis of the V223 residue of the conserved YXDD catalytic site motif indicated that substitution with methionine, the residue found at the equivalent 184 position in HIV-1 RT, resulted in a 1.8-fold increase in the mutation rate. Mansky et al. made a similar observation that the opposite substitution in HIV-1 RT (M184V), which is associated with resistance to the antiviral drug 3TC, resulted in a 1.3-fold decrease in the mutation rate (66). Mansky and colleagues have also determined the effects of HIV-1 RT mutations that confer resistance to AZT on the

Table 3. Structural determinants of *in vivo* fidelity

Mutation	Description	Reporter gene	Relative mutant frequency	Reference
HIV-1				
M184V	YMDD/3TC[Ra]	*lacZa*	↓ 1.3X	66
T215Y	AZT[Rb]	*lacZa*	↑1.3X	66
M41L/ T215Y	AZT[R]	*lacZa*	↑3.3X	66
M41L/ D67N/ K70R/ T215Y	AZT[R]	*lacZa*	↑4.3X	66
HTLV-1				
M188A	YMDD	*lacZa*	↑ 2.6X	15
M188V	YMDD	*lacZa*	↑ 2.3X	15
MLV				
K103R	Fingers/dNTP binding	*lacZ*	No Change	84
T147A		*lacZ*	↑ 1.3 X	84
L151F		*lacZ*	↑ 2.4 X	84
K152A		*lacZ*	↑ 1.4 X	84
D153A	dNTP binding	*lacZ*	↑ 1.6 X	84
D153C	dNTP binding	*lacZ*	No Change	84
D153Q	dNTP binding	*lacZ*	No Change	84
D153S	dNTP binding	*lacZ*	No Change	84
A154S		*lacZ*	↑ 1.3 X	84
F155W	dNTP binding	*lacZ*	↑ 2.8 X	84
F155Y	dNTP binding	*lacZ*	No Change	84
F156L		*lacZ*	↑ 1.6 X	84
F156M		*lacZ*	↑ 1.7 X	84
F156W		*lacZ*	↓ 1.3 X	84
F156Y		*lacZ*	↓ 1.3 X	84
C157A		*lacZ*	↑ 1.3 X	84
R159A		*lacZ*	No Change	84
H161A		*lacZ*	↑1.2 X	84
Q190M	dNTP binding	*lacZ*	No Chg.	84
V223A	Palm/YVDD	*lacZ*	↑1.7 X	85
V223I	Palm/YVDD	*lacZ*	No Change	85
V223M	Palm/YVDD	*lacZ*	↑ 1.8 X	85
V223S	Palm/YVDD	*lacZ*	↑ 2.3 X	85
S526A	RNase H	*lacZ*	↑ 1.6 X	85
Y586F	RNase H	*lacZ*	↑ 5.4 X	104
		GFP	↑ 4.3 X	104
Y598V	RNase H	*lacZ*	No Change	85
R657S	RNase H	*lacZ*	↑1.4 X	85

[a] 3TC[R], mutation confers resistance to 3TC. [b] AZT[R], mutation confers resistance to AZT.

accuracy of DNA synthesis. In general, mutations that conferred resistance to AZT increased the mutation rate; the largest increase in the mutation rate, 4.3-fold, was observed with a quadruple mutant (M41L/D67N/K70R/T215Y) (66). Extensive mutational analysis of MLV RT dNTP-binding site residues was performed to determine their effects on fidelity (84). Substitution of F155, which contacts the base and ribose moiety of the substrate dNTP, with tryptophan increased the mutation rate 2.8-fold. Interestingly, substitution L151F, which is adjacent to the catalytic site residue D150, resulted in a 2.4-fold increase in the forward mutation rate.

Most single amino acid substitutions resulted in a less than 3-fold change in the *in vivo* forward mutation rate. The only exceptions were the triple and quadruple mutations that conferred resistance to AZT and increased the mutation rate 3.3- and 4.3-fold, respectively (66). Therefore, it was surprising that in a recent study a 5.4-fold increase was observed in the *in vivo* forward mutation rate of *lacZ* that resulted from a single amino acid substitution (Y586F) in the RNase H primer grip motif of MLV RT (104).

These studies have identified several different structural elements of RTs as important determinants that maintain the accuracy of DNA synthesis. Their overall influence on fidelity is to increase the *in vivo* mutation rate approximately two- to fivefold. The YXDD catalytic site motif, mutations that confer resistance to AZT, dNTP-

binding site, and the RNase H primer grip motif appear to influence the *in vivo* accuracy of reverse transcription (66, 84, 85, 104).

8.3. Structural determinants of *in vitro* fidelity

The effects of HIV-1 RT mutations on accuracy of DNA synthesis has been determined for several mutations by using an *in vitro* forward mutation assay in which the *lacZa* gene was used as the mutation reporter (table 4). In addition, mutational analyses of MLV and HIV-2 RTs were reported (105, 106). Of the mutations that have greater than twofold effects on fidelity, a majority increased the accuracy of DNA synthesis (9 of 12). Whether this bias reflects the nature of mutations that have been tested to date or the conditions of the assay, such as nucleotide concentrations or the use of only a DNA template, is unknown at this point. A cluster of four mutations in the fingers domain (F61A, K65R, D76V, and R78A) increased the accuracy of DNA synthesis 9- to 12-fold, and the L74V substitution increased the accuracy by 3.4-fold in one study. Two dNTP binding site mutants, Y115V and Q151N, increased fidelity 3.4- and 13-fold, respectively; in contrast, the Y115A substitution increased the mutation rate fourfold. Only two substitutions in the minor groove-binding tract, G262A and W266A, decreased the fidelity of DNA synthesis by three- to fourfold.

Misinsertion and mismatch extension assays have implicated the primer grip region of HIV-1 RT as being an important determinant of RT fidelity (107). Gutierrez-Rivas and Menendez-Arias made an interesting observation that the M230I primer grip mutation increased the rate of T-G misinsertions 16-fold, and prolonged passage of a virus containing this mutation resulted in the outgrowth of a revertant that possessed the M230I and Y115W mutations (108). The double mutant had a nearly wild-type efficiency of T-G misinsertions. This result indicated that the primer grip residue M230 and the dNTP-binding-site residue Y115 interacted with each other and the misinsertion defect of the M230I mutation was restored by the Y115W dNTP-binding-site mutation.

These *in vitro* studies have identified the fingers domain, the primer grip, and the minor groove-binding tract (alpha helix H) region of the thumb domain as important determinants of *in vitro* fidelity. In general, mutations in the fingers domain (F61, K65, L74, D76, and R78) appear to decrease the *in vitro* forward mutation rate; these results suggest that the wild-type residues at these positions in the fingers domain decrease the accuracy of DNA synthesis. Interestingly, the K65 residue in the fingers domain contacts the triphosphate moiety of the dNTP substrate and the K65R substitution increased the *in vitro* fidelity by eightfold. This result suggests that the K65R substitution increases nucleotide selectivity. Similarly, the Q151 residue contacts the base of the dNTP substrate and the Q151N substitution also increases the *in vitro* fidelity 13-fold.

Mutations of alpha helix H residues G262 and W266 that contact the template-primer in the minor groove increase the mutation rate (97, 98). Preliminary studies of mutations introduced at similar positions in MLV RT suggest that they also increase the mutation rate *in vivo* (Svarovskaia and Pathak, unpublished results). The alpha helix H of the thumb domain has been proposed to be an important component of a "helix clamp" that maintains contact with the template-primer complex during the translocation step of polymerization (109, 110). The G262 and W266 residues make sequence-independent contacts with the DNA primer 2 to 6 nt upstream of the 3' end of the primer (98, 111). These mutations were shown to decrease template affinity, processivity, frameshift fidelity, and the total amount of full-length DNA product generated.

8.4. The role of MLV RNase H primer grip and template-primer structure in fidelity

Recent analysis of the MLV RT Y586F mutant has provided novel insights into the structural features of the reverse transcription complex that are important for accuracy of DNA synthesis (104). The Y586 residue of MLV RT is part of a conserved DSXY motif that is present in most retroviral RNase H domains as well as *E. coli* RNase H. The MLV RT Y586 residue is equivalent to the HIV-1 Y501 residue, which is a component of the recently identified RNase H primer grip domain (99). One function of the RNase H primer grip domain and the Y501 residue, which contacts the DNA primer strand, is to position the template-primer near the RNase H active site and control RNase H cleavage specificity (112).

Zhang and colleagues determined the effect of the MLV Y586F mutation on the *in vivo* forward mutation rate. The presence of the Y586F substitution was associated with a 5.4-fold and a 4.3-fold increase in the forward mutation rates of the *lacZ* and GFP reporter genes, respectively. A summary of the characterization of the mutations induced in the GFP gene is shown in table 5. The results indicated that the frequency of substitution mutations increased approximately sixfold while the frequencies of frameshift mutations and other template switching mutations also increased about threefold.

Further analysis of the substitution mutations indicated that a large proportion of the substitutions induced by the Y586F mutation were clustered near adenine-thymine tracts (AAAA, TTTT, and AATT), which are known to induce bends in DNA (figure 4). The adenine-thymine tracts, also referred to as A-tracts, were present within 18-nt of 81% of the substitutions induced by the Y586F mutation. The high proportion of substitutions at these sites represented a 17.2-fold increase for substitutions near A-tracts in comparison to the wild-type RT (table 5).

These results indicated that the Y586F mutant is a mutator RT. What is the possible explanation for the strong correlation between the Y586F mutation and the increase in substitutions within 18 nt of A-tracts? Because the A-tract sequences are associated with bends in DNA, the

Table 4. Structural determinants of *in vitro* fidelity

Mutation	Description	Assay – Reporter gene	Relative mutant frequency	Reference
HIV-1				
F61A	Fingers	*In vitro* FMA – *lacZa*	↓11.7X	121
K65R	Fingers/dNTP binding/ddI^{R}	*In vitro* FMA – *lacZa*	↓8.1 X	122
R72A	Fingers	*In vitro* FMA – *lacZa*	↑1.6X	123
L74V	Fingers/ddI^{R}	*In vitro* FMA – *lacZa*	↓ 1.7 X	122
		In vitro FMA – *lacZa*	↓ 3.5X	124
D76V	Fingers	*In vitro* FMA – *lacZa*	↓8.8X	125
		Misinsertion	↓10X (A-C)	
R78A	Fingers	*In vitro* FMA – *lacZa*	↓8.9X	126
E89G	Fingers/foscarnet^{R}	*In vitro* FMA – *lacZa*	↓1.4X	127
		Mismatch extension	↑10X (G-A)	128
		In vitro FMA – *lacZa*	↓2X	124
		Misinsertion	↓16.8X (T-G)	129
E89G/ M184V	Fingers/3TC^{R}	*In vitro* FMA – *lacZa*	↑ 1.4 X	127
		Mismatch extension	↓ 1.3X (G-G)	128
			↑29X (G-A)	
Y115A	dNTP binding	*In vitro* FMA – *lacZa*	↑ 4X	124
		Misinsertion	↑ 4.6X (A-C)	130
			↑ 10X (A-G)	
		Mismatch extension	↑4.4X (A-C)	131
Y115V	dNTP binding	*In vitro* FMA – *lacZa*	↓ 3.4X	74
		Misinsertion	↑5.1X (A-G)	130
		Mismatch extension	↑ 4.6X (A-C)	131
		Mismatch extension	↑ 3125X (A-G)	130
Y115L	dNTP binding	Mismatch extension	↑13.5X (A-C)	131
Y115I	dNTP binding	Mismatch extension	↑3.8X (A-C)	131
Y115N	dNTP binding	Mismatch extension	↑ 17.8X (A-C)	131
Y115M	dNTP binding	Mismatch extension	↑ 3X (A-C)	131
Y115H	dNTP binding	Mismatch extension	↑ 8.1X (A-C)	131
Y115G	dNTP binding	Mismatch extension	↑ 34.9X (A-C)	131
		Mismatch extension	↑ 5416X (A-G)	130
Y115C	dNTP binding	Mismatch extension	↑ 10.5X (A-C)	131
Y115S	dNTP binding	Mismatch extension	↑25.3X (A-C)	131
Y115F	dNTP binding/ Abacavir	*In vitro* FMA – *lacZa*	↓ 1.6 X	74
		Mismatch extension	↑2.6X (A-C)	131
Y115W	dNTP binding	Misinsertion	↑3.5X (A-C)	108
		Mismatch extension	↑ 8.0X (A-C)	131
Q151M	dNTP binding/ Multi-drug^{R}	*In vitro* FMA – *lacZa*	↓1.2X	105, 132
		Misinsertion – RNA	↓ 6.5 X (C-A)	
A62V/ V75I/ F77L/ F116Y/ Q151M	Multi-drug^{R}	*In vitro* FMA – *lacZa*	↓ 1.7 X	132
Q151N	dNTP binding	*In vitro* FMA – *lacZa*	↓13 X	105, 133
		Misinsertion – RNA	↓8.3X to ↓26.5X	
K154A		*In vitro* FMA – *lacZa*	↓ 2.1X	134
F160Y	Palm	Misinsertion	↑1.9 – 2.1X	135
		Mismatch extension	↑1.1X (A-C)	135
F160W	Palm	Misinsertion	↑2.1X (A-C)	135
		Mispair Extension	↑2.6X (A-C)	135
Y183F	YMDD	*In vitro* FMA – *lacZa*	↑1.6X	124
		Misinsertion	↓1.1 – 1.4X	136
		Mismatch Extension	↓3.4 – 4.9X	136
M184V	YMDD/3TC^{R}	*In vitro* FMA – *lacZa*	↑ 1.2X	124
		In vitro FMA – *lacZa*	↓ 1.6X	127
		Mismatch Extension	↑ 3.5X (G-T)	128
		Mismatch Extension – RNA	↓48.6X (A-G)	137
		Misinsertion – RNA	↓6.5X (-A)	138
		Misinsertion	↓17.5X (C-T)	139-141
			↓ 3.6X (-C)	
			↓ 2.4X (C-T)	
M184I	YMDD/3TC^{R}	*In vitro* FMA – *lacZa*	↓4.0X	142, 143
		Misinsertion – RNA	↓2.6X (-A)	
		Mismatch extension – RNA	↓6.1X (A-C)	137
M184A	YMDD	Misinsertion	↑1.6X (-T)	140
M184L	YMDD	Mismatch extension	↓11.1X (A-C)	136
			↓ >26X (A-G)	
		Misinsertion	↓ >9X (T-G)	136
F227A	Primer grip	Misinsertion	↑5.5X (A-A)	107
W229A	Primer grip	Misinsertion	↓1.5X	107
M230I	Primer grip	Misinsertion	↑16X (T-G)	108
M230I/ Y115W	Primer grip/dNTP Binding –revertant	Misinsertion	↓1.3X (T-G)	108
G262A	Thumb/alpha H	*In vitro* FMA – *lacZa*	↑ 4 X	144
W266A	Thumb/alpha H	*In vitro* FMA – *lacZa*	↑ 3 X	144
R277A	Thumb/alpha I	*In vitro* FMA – *lacZa*	No Change	145
Q278A	Thumb/alpha I	*In vitro* FMA – *lacZa*	No Change	145
L279A	Thumb/alpha I	*In vitro* FMA – *lacZa*	No Change	145
C280A	Thumb/alpha I	*In vitro* FMA – *lacZa*	↑ 1.5 X	145
K281A	Thumb/alpha I	*In vitro* FMA – *lacZa*	No Change	145
L282A	Thumb/alpha I	*In vitro* FMA – *lacZa*	No Change	145
R284A	Thumb/alpha I	*In vitro* FMA – *lacZa*	No Change	145
G285A	Thumb/alpha I	*In vitro* FMA – *lacZa*	No Change	145
K287A	Thumb/alpha I	*In vitro* FMA – *lacZa*	No Change	145
HIV-2				
L74V	Fingers	Mismatch extension	↓1.1X	106
E89G	Fingers	Mismatch extension	↓6X (A-C)	106
S215Y		Mismatch extension	↓8.3X (A-C)	106
L74V/S215Y	Fingers	Mismatch extension	↓>13X	106
E89G/S215Y	Fingers	Mismatch extension	↓4.8X	106
MLV				
V223A	Palm/YVDD	Misinsertion – RNA	↑79.5X (T-C)	105
		Misinsertion – RNA	↑41.4X (T-C)	105

Table 5. Effect of Wild-type and Y586F Mutant RTs on Frequencies of Substitutions (data taken from (104))

Mutation type	Wild Type RT[a] Number of Mutants/ Total	Wild Type RT mutant frequency[b]	Y586F RT[a] Number of Mutants/ Total	Y586F RT mutant frequency[b]	Relative increase in mutant frequency[c]
Substitutions near A-Tracts[d]	7/51	0.15%	34/60	2.59%	17.2
Other substitutions[e]	19/51	0.39%	8/60	0.61%	1.6
All substitutions	26/51	0.54%	42/60	3.19%	5.9
Frameshifts and template switching mutations	25/51	0.51%	18/60	1.36%	2.7
All mutations	51/51	1.05%	60/60	4.55%	4.3

[a] Number of mutants containing GFP-inactivating mutations identified by DNA sequencing containing a particular type of mutation / total number of mutants containing GFP-inactivating mutations identified by DNA sequencing. [b] Mutant frequencies determined by multiplying the proportion of mutants sequenced by the overall mutant frequency (e.g., the mutant frequency for substitutions near A-tracts for the wild-type RT is (7/51) x 1.05% = 0.15%). [c] Fold increase in mutant frequency for the Y586F mutant relative to wild-type RT (e.g., the relative increase in the frequency of substitutions near A-tracts is 2.59% ÷ 0.15% = 17.2). [d] Substitution for which there was an A-tract (AAAA, TTTT, or AATT) within 18 nt of the mutation site. [e] Substitution for which an A-tract was not present within 18 nt of the mutation site.

Figure 4. Substitution mutations in GFP induced by the Y586F mutant RT are in regions containing A-tracts. The substituted nucleotides are shown in lower case letters and shaded. The A-tract sequences (AAAA, TTTT, and AATT) that are within 18-nt of the site of substitution are shown in bold type. Assuming that the A-tracts must be in contact with the RT to have an effect on accuracy of DNA synthesis, substitutions that occurred 5' of the A-tracts occurred during RNA-dependent DNA synthesis (A) and substitutions that occurred 3' of the A-tracts occurred during DNA-dependent DNA synthesis (B). Data taken from reference (104).

conformation of the template-primer complex appears to be a significant structural determinant of fidelity. It was hypothesized that the wild-type RT evolved to facilitate a proper conformation of the template-primer that is amenable to incorporation of the correct nucleotides at the polymerase active site. When wild-type RT encounters irregular template-primer conformations such as those induced by the presence of A-tracts, certain structural determinants of RT facilitate an alteration of the template-primer conformation that is necessary for fidelity of DNA synthesis. It was proposed that the Y586 residue and the RNase H primer grip region is a structural determinant of RT that is important for inducing a conformation of the template-primer duplex that is necessary for accuracy of DNA synthesis. When Y586 is substituted with F, it is no longer able to facilitate this template-primer conformation when A-tracts are present within 18 nt of the site of polymerization; as a result, the rate of substitutions is increased in the vicinity of A-tracts.

What is the nature of the template-primer conformation that is necessary for accurate DNA synthesis? The structures of the RNA:DNA and DNA:DNA hybrids in complex with HIV-1 RT have been determined (figure 3). These structures indicate that both hybrids possess A-form structure near the polymerase active site, a 41-degree bend, followed by B-form DNA near the RNase H active site. A-form conformation of the template-primer has been shown to be present near the active sites of other polymerases and is believed to contribute to fidelity by reducing the impact of sequence- dependent structural alterations on fidelity. In contrast, the presence of B-form DNA near polymerase active sites is associated with low fidelity and mutational hotspots (113). A-form DNA has a wider minor groove, which can provide more access for the RT to make contacts with the template-primer near the active site. Alterations in

the template-primer conformation can change the structure of the polymerase active site and have an impact on the ability of the polymerase to discriminate between correct and incorrect nucleotide substrates.

To summarize, these results have identified two important features of the reverse transcription complex that are important for accuracy of DNA synthesis. First, the conformation of the template-primer is an important determinant of fidelity; the A-form template-primer duplex that is present near the polymerase active site appears to be an important feature of the polymerase active site and perhaps is critical for accurate DNA synthesis. Second, the Y586 residue and the MLV RNase H primer grip are important structural elements that appear to be critical for maintaining a proper template-primer conformation near the polymerase active site, even when irregular template primer conformations such as A-tracts are encountered.

9. FUTURE DIRECTIONS

Genetic variation in HIV-1 populations has played a central role in our ability to deal with the AIDS epidemic by contributing to rapid emergence of drug resistance and escape from immune responses. A greater understanding of the mechanisms that contribute to RT fidelity could lead to novel antiviral strategies. In the future, it will be desirable to use a variety of experimental approaches to ascertain the retroviral mutation rates that will provide meaningful results that can be applied to a replicating HIV-1 virus in an infected patient. In this regard, it would be challenging but important to perform these experiments in cells that are natural targets for the viral infection. Another challenging task is to determine the contribution of RNA polymerase II-mediated RNA transcription to retroviral variation. The structural determinants of RT that are important for fidelity in vivo are now beginning to be elucidated. It will be necessary to take advantage of the available structural information on HIV-1 RT and apply it to the understanding of mechanisms of RT fidelity. Finally, the newly discovered role of the template-primer duplex structure and the RNase H primer grip domain in the fidelity of reverse transcription should be fully explored.

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Send correspondence to: Vinay K. Pathak, HIV Drug Resistance Program, National Cancer Institute, NCI-Frederick, Bldg. 535, Rm. 334, Frederick, MD 21702. Tel: 301-846-1710, Fax: 301-846-6013, E-mail: VPATHAK@ncifcrf.gov