

Influence of naturally occurring insertions in the fingers subdomain of human immunodeficiency virus type 1 reverse transcriptase on polymerase fidelity and mutation frequencies *in vitro*

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The fingers subdomain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is a hotspot for nucleoside analogue resistance mutations. Some multi-nucleoside analogue-resistant variants contain a T69S substitution along with dipeptide insertions between residues 69 and 70. This set of mutations usually co-exists with classic zidovudine-resistance mutations (e.g. M41L and T215Y) or an A62V mutation and confers resistance to multiple nucleoside analogue inhibitors. As insertions lie in the vicinity of the dNTP-binding pocket, their influence on RT fidelity was investigated. Commonly occurring insertion mutations were selected, i.e. T69S-AG, T69S-SG and T69S-SS alone, in combination with 3'-azido-2',3'-deoxythymidine-resistance mutations M41L, L210W, R211K, L214F, T215Y (LAG_{AZ} and LSG_{AZ}) or with an alternate set where A62V substitution replaces M41L (VAG_{AZ}, VSG_{AZ} and VSS_{AZ}). Using a *lacZ*α gapped duplex substrate, the forward mutation frequencies of recombinant wild-type and mutant RTs bearing each of the above sets of mutations were measured. All of the mutants displayed significant decreases in mutation frequencies. Whereas the dipeptide insertions alone showed the least decrease (4.0- to 7.5-fold), the VAG series showed an intermediate reduction (5.0- to 11.4-fold) and the LAG set showed the largest reduction in mutation frequencies (15.3- and 16.3-fold for LAG_{AZ} and LSG_{AZ}, respectively). Single dNTP exclusion assays for mutants LSG_{AZ} and LAG_{AZ} confirmed their large reduction in misincorporation efficiencies. The increased *in vitro* fidelity was not due to excision of the incorrect nucleotide via ATP-dependent removal. There was also no direct correlation between increased fidelity and template–primer affinity, suggesting a change in the active site that is conducive to better discrimination during dNTP insertion.

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INTRODUCTION

Although highly active antiretroviral therapy (HAART) effectively suppresses virus load in patients infected with *Human immunodeficiency virus 1* (HIV-1) (Richman, 2001), resistance continues to be a problem. The primary targets of HAART include the viral reverse transcriptase (RT), which replicates the viral genome, and protease, which is essential to virion morphogenesis. Currently, there are two classes of approved anti-RT drugs, including nucleoside (NRTIs) and

non-nucleoside RT inhibitors (NNRTIs) (Richman, 2001). HIV can circumvent the immune system or HAART via escape mutants, a direct result of virus variation. Factors contributing to variation include rapid replication, a high mutation rate and recombination, which are all characteristic of HIV-1. RT contributes to the high mutation rate via its intrinsically low fidelity (Preston *et al.*, 1988; Roberts *et al.*, 1988), which facilitates the emergence of drug resistance.

Drug resistance mutations in HIV-1 are predominantly amino acid substitutions, but deletions and insertions are also observed. Resistance to 3'-azido-2',3'-deoxythymidine (AZT, Zidovudine) is associated with up to six mutations

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(M41L, D67N, K70R, L210W, T215F/Y and K219Q) (Larder *et al.*, 1989; Larder & Kemp, 1989; Richman, 2001). Resistance to (–)-2',3'-dideoxy-3'-thiacytidine (3TC, lamivudine) is via M184V (Gao *et al.*, 1993) and L74V, the primary ddI-resistance mutation (St Clair *et al.*, 1991). Other substitutions, such as Q151M and associated mutations (A62V, V75L, F77L and F116Y) (Shirasaka *et al.*, 1993, 1995) or the K65R mutation (Gu *et al.*, 1994, 1995), confer multi-dideoxynucleoside analogue resistance (MDR). Insertions (Winters *et al.*, 1998) and/or deletions (Winters *et al.*, 2000) within the fingers subdomain of HIV-1 RT have been reported to occur in combination with AZT-resistance mutations (M41L, L210W, R211K, L214F and T215Y) and/or MDR mutations (A62V) (Larder *et al.*, 1999) and were shown to confer resistance to multiple NRTIs. Insertions ranging from 1 to 16 aa residues occur between residues 67 and 70 within the β 3– β 4 loop, with most lying between codons 69 and 70 and invariably associated with a T69S substitution (Larder *et al.*, 1999; Winters *et al.*, 1998).

Most NRTI-resistance mutations, as in the case of the 3TC-resistance mutation M184V, alter the active site geometry causing the dNTP to be selectively incorporated over the nucleoside analogue (Sarafianos *et al.*, 1999). The AZT-resistance mutations, however, influence AZT triphosphate (AZTTP) susceptibility at a step subsequent to its insertion. A primer terminated with AZT monophosphate (AZTMP) can be subjected to a phosphorolytic reaction (Arion *et al.*, 1998; Meyer *et al.*, 1998, 1999) causing preferential removal of AZTMP from the primer terminus. The mechanism of AZT resistance involves the use of ATP or PPi as pyrophosphate donor, which facilitates AZTMP removal from the blocked primer. The product of the phosphorolytic reaction is a dinucleotide tetraphosphate when ATP acts as the phosphate acceptor or an AZTTP in the case of pyrophosphate (Meyer *et al.*, 1999). It is hypothesized that a pocket located near the dNTP-binding site can accommodate an ATP molecule that, when bound, will allow AZTMP removal from the primer terminus, creating a 3'-OH, thus allowing further elongation (Boyer *et al.*, 2001).

A ternary structure of HIV-1 RT, complexed with dsDNA primer–template and its dNTP substrate (Huang *et al.*, 1998) shows the fingers subdomain moving towards the active site making up a portion of the dNTP-binding pocket. This structure provides a basis for the observation that the β 3– β 4 loop is a hotspot for nucleoside analogue resistance mutations and highlights its role in dNTP selection and insertion fidelity. Residues within the fingers subdomain interact with both template overhang and the incoming dNTP (Huang *et al.*, 1998). Since the template overhang (templating base) itself forms a part of the dNTP-binding pocket, the direct interaction of selected residues in the β 3– β 4 loop with both the template and dNTP suggests a role for these residues in RT fidelity and dNTP selection. This notion is corroborated by reports from our laboratory and those of others showing that substitutions in the β 3– β 4 hairpin can significantly decrease the HIV-1 RT mutation rate (Fisher &

Prasad, 2002; Kim *et al.*, 1998, 1999; Shah *et al.*, 2000). We reported that a K65R mutation leads to an ~8-fold reduction in the forward mutation rate (Shah *et al.*, 2000). Kim *et al.* (1998, 1999) showed that D76V and R78A mutants each display a 9-fold reduction in the forward mutation rate. More recently, we have shown that an F61A substitution decreases the overall mutation rate of HIV-1 RT by approximately 12-fold (Fisher & Prasad, 2002).

Therefore, it was examined whether multi-NRTI-resistant RTs containing insertion mutations affect RT fidelity using RTs with three different dipeptide insertions by themselves (T69S-AG, T69S-SG and T69S-SS) or in combination with other mutations. It is reported here that RTs containing the T69S amino acid substitution combined with insertions between residues 69 and 70, in the presence or absence of the resistant background mutations, generally decrease HIV-1 RT mutation frequencies. For some of the mutants (e.g. LSG_{AZ} and LAG_{AZ}), this represents the largest decrease reported so far in the mutation rate of naturally occurring variants of HIV-1 RT.

METHODS

Bacterial strains and plasmids. *Escherichia coli* strain DH5 α F'⁺ [F'⁺, ϕ 80lacZ Δ M15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, (r_K^- , m_K^+), *supE44*, *relA1*, *deoR*, Δ (*lacZYA-argF*), U169] was used for expression of pRT, pRT- Δ BsmBI and p6HRT51 constructs. *E. coli* strain NR9099 [Δ (*pro-lac*), *thi*, *ara*, *recA56/F'* (*proAB*, *lacI^q* Δ M15)] was used for preparation of both the single-stranded and replicative form M13 DNAs. *E. coli* strain MC1061 [*hsdR*, *hsdM*⁺, *araD*, Δ (*ara*, *leu*), Δ (*lacIPOZY*), *galU*, *galK*, *strA*] was used to electroporate the products of fill-in reaction to generate phage and strain CSH-50 [Δ (*pro-lac*), *thi*, *ara*, *strA/F'* (*proAB*, *lacI^q* Δ M15, *traD36*)] was used as the α -complementation strain (Bebenek & Kunkel, 1995).

Generation of HIV-1 RT mutants. Mutant RTs described in this report were created via cassette mutagenesis using a modified pRT plasmid (Le Grice & Gruninger-Leitch, 1990) (a kind gift of S. Le Grice, NCI, Frederick, MD, USA). Substitutions at residue 69 (T69S) and the insertions were created in the intermediate vector, pRT β 3– β 4 Δ BsmBI, by cassette mutagenesis (Boyer *et al.*, 1992). Adapters containing codons 61–80 with the desired insertions or insertions in conjunction with the A62V mutation were employed to build mutations. pRT Δ BsmBI was also used separately to create a pRT210–215 intermediate and double-stranded adapters containing mutations L210W, R211K, L214F and T215Y were cloned into sites created by *BsmBI* digestion. The A62V, T69S-AG (or SG, SS) and L210W/R211K/L214F/T215Y RT mutants were created by combining the 5' and 3' fragments of RT sequences from the respective mutants. Another intermediate, pRT-W24, which lacked residues 21–43, was used to create the constructs M41L/T69S-AG/L210W/R211K/L214F/T215Y and M41L/T69S-SG/L210W/R211K/L214F/T215Y. The RT plasmids were transformed into *E. coli* host DH5 α F'⁺ and expressed and purified as described previously (Fisher *et al.*, 2002; Kew *et al.*, 1994). Table 1 shows all of the RTs used in this study.

Determining the kinetic constants, K_m and V_{max} . Kinetic constants K_{mTTP} and V_{max} were determined as described previously (Pandey *et al.*, 1996). Reactions were carried out in 50 μ l containing 50 mM Tris/HCl (pH 7.8), 60 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mg BSA ml⁻¹, 0.1–50 μ M TTP, 25 ng RT (except wild-type, where 10 ng was used) and 100 nM template–primer [poly(rA)–oligo(dT)]. Reactions, in triplicate, were at 37 °C

Table 1. The mutations present in each RT variant and the abbreviations used

RT mutations	Abbreviation used
None	WT _{HXB2}
T69S-AG	AG
T69S-SG	SG
T69S-SS	SS
A62V, T69S-AG, L210W, R211K, L214F, T215Y	VAG _{AZ}
A62V, T69S-SG, L210W, R211K, L214F, T215Y	VSG _{AZ}
A62V, T69S-SS, L210W, R211K, L214F, T215Y	VSS _{AZ}
M41L, T69S-AG, L210W, R211K, L214F, T215Y	LAG _{AZ}
M41L, T69S-SG, L210W, R211K, L214F, T215Y	LSG _{AZ}

for 5 min and quenched with the addition of ice-cold 5% TCA. DNA synthesis was measured by incorporation of [α -³²P]ITP as described previously (Pandey *et al.*, 1996). K_m and V_{max} were obtained from Michaelis–Menten plots of TTP concentrations using Enzyme Kinetics version 1.11 (Trinity Software).

Single dNTP exclusion assay. A 5'-³²P-labelled 28 mer DNA primer, PBS-A (5'-CGCTTTCAGGTCCTGTTTCGGGCGCCAC-3') was annealed to a 55 mer oligonucleotide template VP-229 (5'-TTT-AGTCAGTGTGGAAAATCTCTAGCAGTGGGCGCCCGAACAGGG-ACCTGAAAGCG-3') at a template to primer molar ratio of 5:1. Each reaction was carried out in a 10 μ l volume containing 80 mM KCl, 50 mM Tris/HCl (pH 8.0), 6 mM MgCl₂, 10 mM DTT, 0.1 mg BSA ml⁻¹, 250 μ M of three or four dNTPs and 10 nM template–primer (DNA–DNA). Reactions were incubated for 1.2 min each at 37 °C and terminated with 30 μ l stop solution (95% formamide, 20 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol). Three different RT concentrations were used at 1 \times , 2 \times and 4 \times , respectively, for the wild-type and the two mutants (LAG_{AZ} and LSG_{AZ}) in reactions containing either all four or only three dNTPs. Protein concentrations in the reaction mixtures at 1 \times input for these mutants were 0.2, 0.7 and 0.2 nM, respectively. Reaction products were separated on a 10% denaturing PAGE. Gels were dried and autoradiographed. Radiolabelled products were analysed by phosphorimaging and bands were quantified using ImageQuant. Misincorporation efficiencies were calculated as the ratio of the sum of the intensities of bands above the barrier band to the sum of bands above it for reactions missing each of the dNTPs (see Fig. 1a). Mean values of efficiencies obtained from all reactions allowed us to generate overall misincorporation efficiencies for each mutant enzyme (see Fig. 2).

Forward mutation assay. Mutation frequencies of RT mutants were measured as described previously (Drosopoulos & Prasad, 1998; Rezende *et al.*, 1998). The overall mutation frequency was determined by dividing the number of mutants by the total number of plaques minus background frequencies of 1×10^{-3} for the wild-type, SG and SS RTs and 2.4×10^{-3} for the RTs AG, VAG_{AZ}, VSG_{AZ}, VSS_{AZ}, LAG_{AZ} and LSG_{AZ} (the two sets of RTs were used to fill in two different preparations of gapped duplex substrates and hence two separate background frequencies).

Removal of an AZTMP-terminated primer via excision. The oligonucleotide primer L33 (5'-CTACTAGTTTTCTCCATCTAGAGC-ATACCAGAA-3') was 5'-labelled with [γ -³²P]ATP by T4 polynucleotide kinase. After purification, the labelled primer was annealed in excess (5:1 template to primer) to the template WL50 (5'-GAGTGCTG-AGGTCTTCATTCTGGTATCGTCTAGATGGAGAAAATAGTAG-3') and precipitated by the addition of 3 M sodium acetate and ethanol.

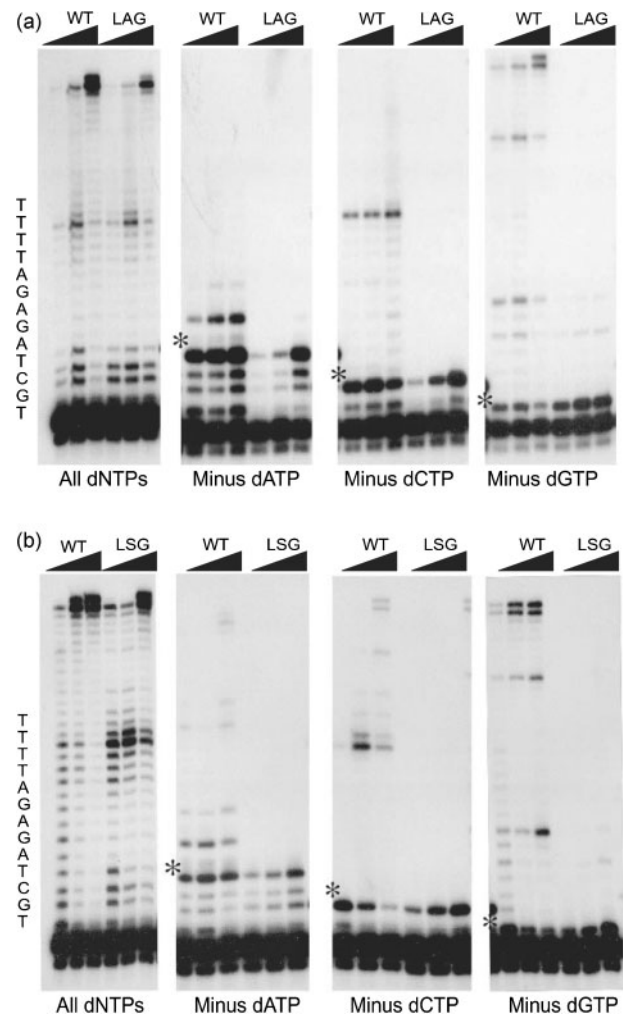


Fig. 1. Single dNTP exclusion assay to measure misincorporation efficiency of wild-type and two mutant RTs. Misincorporation efficiencies during DNA-dependent DNA synthesis of (a) LAG_{AZ} and (b) LSG_{AZ} RTs each in comparison with that of wild-type HIV-1 RT are shown. Gels on the left side of each panel show products from reactions performed in the presence of all dNTPs as a control for equivalent activity inputs of enzyme. Remaining gels each represent reactions carried out in the absence of one of four dNTPs (minus dATP, minus dCTP or minus dGTP) as indicated at the bottom of each gel. The sequence of the DNA product synthesized is indicated on the left of each panel. The position of the first misinsertion required for continued synthesis in the absence of the indicated dNTP is shown by an asterisk to the left of each gel.

The primer terminus was blocked as previously described (Boyer *et al.*, 2002). Briefly, the template–primer was resuspended in 50 μ l 25 mM Tris/HCl (pH 8.0), 75 mM KCl, 8.0 mM MgCl₂, 2.0 mM DTT, 100 μ g BSA ml⁻¹, 10.0 mM CHAPS and 10.0 μ M 3'-azido 3'-deoxythymidine 5'-triphosphate (AZTTP) (Moravek Biochemicals). Wild-type HIV-1 RT (8.55 fmol) was added to labelled template–primer and reactions were allowed to proceed at 37 °C for 60 min and then stopped by phenol/chloroform extraction. Samples were precipitated by addition of 1 vol. 2-propanol, followed by ethanol precipitation. The blocked template–primer was then

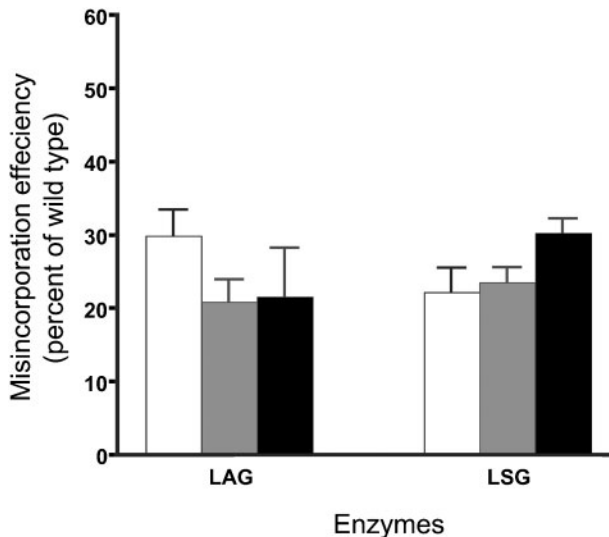
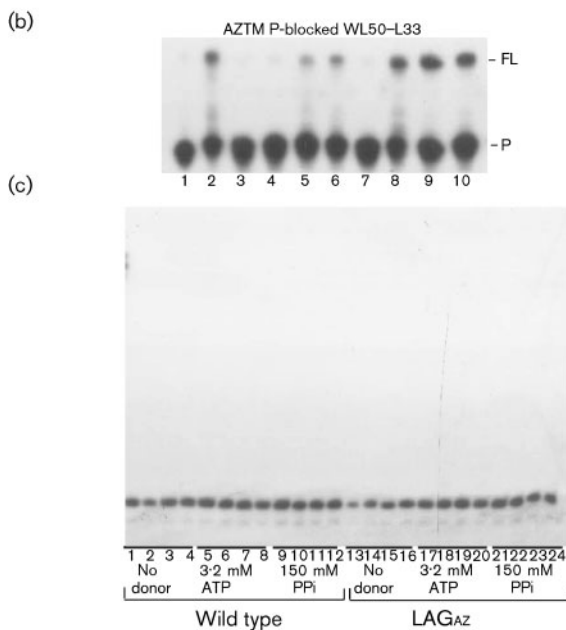
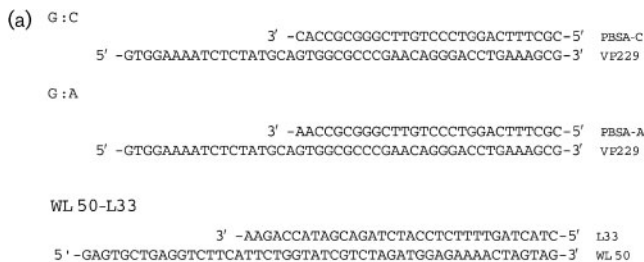


Fig. 2. Quantitative plot of extension products in dNTP exclusion assay. Relative misincorporation efficiencies for each enzyme were calculated from gels such as those shown in Fig. 1 as described in Methods. Misincorporation efficiencies in reactions missing each dNTP are presented as percentages of those calculated for wild-type HIV-1 RT. White bars, minus dATP; grey bars, minus dCTP; black bars, minus dGTP.



resuspended in 25 mM Tris/HCl (pH 8.0), 75 mM KCl, 16.0 mM MgCl₂, 2.0 mM DTT, 100 μg BSA ml⁻¹ and 10.0 mM CHAPS. The dNTP concentrations were as stated in the legend to Fig. 3.

The wild-type and mutant RTs were assayed for primer unblocking as described previously (Boyer *et al.*, 2001). The reaction mix contained 16 mM MgCl₂ instead of 8 mM to ensure that ATP or sodium pyrophosphate did not bind all of the magnesium ions. Reaction mixtures contained approximately 0.25 nM template-primer, 200 nM wild-type or the LAG_{AZ} mutant RT, and increasing concentrations of ATP (1.6–5.0 mM). Reactions were incubated for 10 min at 37 °C and then heated to 72 °C for an additional 10 min to heat-inactivate the RT. Extension reactions were carried out by the addition of 100 μM dNTPs and 0.1 U *Taq* polymerase (Roche) for 5 min at 72 °C. A control reaction, containing the WL50-L33 template-primer, with 100 μM dNTPs and 0.001 U *Taq* polymerase was performed to ensure that *Taq* could extend from a non-terminated primer. A control reaction to ensure that the RT was denatured at 72 °C was performed as described below. Reactions were stopped by the addition of phenol/chloroform and precipitated with ethanol. Products were separated on a 10% denaturing polyacrylamide gel and autoradiographed.

Removal of a mispaired dNMP by a pyrophosphorolysis reaction. Two primers, PBSA-C and PBSA-A (to generate G:C paired and G:A mispaired template-primer pairs; see Fig. 3a) were end-labelled with T4 DNA kinase and [γ -³²P]dATP and purified using a nucleotide removal kit (Qiagen). Primers were annealed separately to the template oligonucleotide VP229 (see Fig. 3a) by adding 108 pmol primer and 148 pmol template in annealing buffer [50 mM Tris/HCl (pH 8.0), 10 mM DTT, 30 mM KCl], heating to

Fig. 3. Excision of AZTMP or mispaired dNTP. (a) Sequences of oligonucleotides used in the study including the properly paired G:C template-primer, the template-primer with mispaired G:A terminus and the template oligonucleotide WL50 annealed to primer L33 with an AZTMP residue at the 3' terminus (WL50-L33). (b) Relative efficiencies of primer unblocking by wild-type and LAG_{AZ} mutant RTs using WL50-L33 template-primer. Lanes: 1, template-primer alone; 2, 0.001 U *Taq* only on template-primer without AZTMP blocking; 3–6, wild-type HIV-1 RT; 7–10, LAG_{AZ} mutant RT at increasing concentrations of ATP (0.00, 1.6, 3.2 and 5.0 mM, respectively). Positions of primer (P) and fully extended (FL) products are indicated on the right. (c) LAG_{AZ} RT mutant does not excise mismatched nucleotides by primer unblocking. Autoradiogram shows the lack of formation of products when a mismatched template-primer [G:A template primer shown in (a)] was used to measure the mispaired nucleotide excision ability of the wild-type and mutant RTs. Lanes 1–12 show reactions with wild-type RT and lanes 13–24 show reactions with LAG_{AZ} RT mutant. Lanes 5–8 and 17–20 represent reactions containing 3.2 mM ATP, whereas lanes 9–12 and 21–24 represent reactions containing 150 μM PPi. Control reactions with G:A template-primer alone (lanes 1 and 13), G:A template-primer and RT alone (lanes 2 and 14) or G:A template-primer and dNTP alone (lanes 3 and 15) are shown. All other reactions contain the G:A template-primer along with wild-type or LAG_{AZ} RT mutant as indicated above with the following modifications. Lanes: 4, 5, 9, 16, 17 and 21, 500 μM each of the four dNTPs; 6, 10, 18 and 22, 500 μM each of dCTP and TTP; 7, 11, 19 and 23, 500 μM each of dATP, dCTP and dGTP and 2 mM TTP; and 8, 12, 20 and 24, 2 mM TTP.

80 °C for 1 min and allowing slowly to cool to room temperature. Several reaction conditions were used to determine if the mutant RTs were able to catalyse the removal of misinserted dNTP. These included the use of both PPI and ATP as acceptors, as well as use of an increased level of TTP to force a slippage-mediated dNTP insertion. The template–primer (50 nM) and RT (200 nM) were incubated in a reaction volume of 10 µl with each phosphate acceptor separately (PPI or ATP) at 37 °C for 5 min. The reaction mixes were then heated to 72 °C for 10 min in order to denature the RT. Reactions were placed on ice for 5 min and 1.2 µl 10 × *Taq* reaction buffer was added with 0.1 U *Taq* polymerase (Roche) to all samples. Finally, 5 µl dNTP solution was added to each reaction to achieve the final concentration, as indicated in Fig. 3(c), and the mixtures were incubated at 72 °C for 10 min. Reactions were stopped by the addition of 90 % formamide, 20 mM EDTA (pH 8.0) and 0.1 % bromophenol blue, and electrophoresed on a 15 % denaturing polyacrylamide gel followed by autoradiography.

Determining the equilibrium dissociation constants (K_d). The dissociation constants (K_d) of the enzyme (E)-TP binary complexes of wild-type HIV-1 RT and its mutant derivatives were determined as described previously (Sharma *et al.*, 2003). The percentage of the labelled TP bound to the enzyme (E-TP binary complex) versus enzyme concentrations was plotted and the dissociation constant, $K_{d[\text{DNA}]}$ was determined as the enzyme concentration at which half-maximal DNA binding occurred.

RESULTS

Generation of RTs with insertion mutations and their characterization

Eight heterodimeric RT variants were created with mutations in p66 only (Table 1). Of these, three contained just the T69S substitution with each of the three most frequently observed dipeptide insertions: AG, SG and SS. The second set contained T69S-AG or T69S-SG in combination with mutations most commonly observed in AZT-resistant viruses (M41L, L210W, R211K, L214F and T215Y) and were called LAG_{AZ} or LSG_{AZ}, respectively. In the third set, each of the mutations T69S-AG, T69S-SG and T69S-SS were combined with a set including AZT-resistance mutations (L210W, R211K, L214F and T215Y) and the MDR mutation, A62V (identified here as VAG_{AZ}, VSG_{AZ} and VSS_{AZ}, respectively).

Effects of insertion mutations, with or without the accompanying resistance mutations, on the kinetic properties of HIV-1 RT were determined initially. The $K_{m\text{TTP}}$ and V_{max} values were measured on a homopolymeric RNA template annealed to a DNA primer [poly(rA):oligo(dT)]. The K_m and V_{max} values of the mutants showed a range of variation (Table 2). Catalytic efficiencies (V_{max}/K_m) of most mutants were 27–99 % of wild-type efficiency; however, one mutant (SS) showed a 2.2-fold increase over the wild-type.

Forward mutation frequencies of mutant RTs

Next, the effects of insertion mutations in the fingers subdomain of RT on the overall mutation rate of HIV-1 RT were determined. A previously described forward mutation assay was used (Bebenek & Kunkel, 1995; Drosopoulos &

Table 2. Kinetic properties of fingers subdomain insertion mutants of HIV-1 RT

Incorporation assays were performed as described in the text.

Enzyme	$K_{m\text{TTP}}$ (µM ± SD)	V_{max} (± SD)*	V_{max}/K_m	Mutant/ wild-type
WT	2.96 ± 1.27	168.0 ± 7.2	56.7	1.00
AG	6.20 ± 1.11	187.2 ± 6.0	30.2	0.53
SG	6.80 ± 2.95	242.4 ± 31.2	35.6	0.62
SS	3.20 ± 0.36	396.0 ± 84	123.7	2.18
VAG _{AZ}	7.90 ± 2.77	343.2 ± 52.5	43.4	0.76
VSG _{AZ}	3.10 ± 1.71	175.2 ± 12	56.5	0.99
VSS _{AZ}	22.50 ± 1.62	340.8 ± 2.4	15.1	0.27
LAG _{AZ}	3.60 ± 0.64	60.0 ± 4.8	16.7	0.29
LSG _{AZ}	7.90 ± 0.33	259.2 ± 12	32.8	0.58

* V_{max} is expressed as pmol µg⁻¹ min⁻¹.

Prasad, 1998). In this assay, recombinant wild-type and mutant RTs were used for filling an M13-gapped DNA duplex across the *lacZα* gene, followed by introducing the filled DNA duplex circles into indicator bacterial strain to facilitate scoring plaques that display a mutant phenotype. The ratio of mutant plaques scored to total plaques (minus the background frequency) gives the mutation frequency. Table 3 shows the results of the mutants tested in the forward mutation assay. Our results show that each mutant RT displayed a decreased mutation frequency compared with wild-type RT. The most impressive decrease in mutation rate was seen with the LAG_{AZ} and LSG_{AZ} RT mutants (15.3- and 16.3-fold, respectively). These are the highest decreases in mutation rate reported to date for a naturally

Table 3. Forward mutation frequencies of wild-type and mutant RTs

Enzyme	Corrected frequency*	Fold decrease†
WT _{HXB2}	9.70×10^{-3}	1.0
AG	2.0×10^{-3}	4.8
SG	1.2×10^{-3}	7.5
SS	2.4×10^{-3}	4.0
VAG _{AZ}	8.5×10^{-4}	11.4
VSG _{AZ}	1.9×10^{-3}	5.0
VSS _{AZ}	1.1×10^{-3}	8.8
LAG _{AZ}	6.3×10^{-4}	15.3
LSG _{AZ}	5.9×10^{-4}	16.3

*Obtained by subtracting the background mutation frequency of 1×10^{-3} for the wild-type, SG and SS RTs and 2.4×10^{-3} for the RTs AG, VAG_{AZ}, VSG_{AZ}, VSS_{AZ}, LAG_{AZ} and LSG_{AZ}. Background frequency is determined by electroporating the unfilled gap and scoring for mutants.

†Relative to the mutation frequency of wild-type RT.

occurring RT variant. The enzyme VAG_{AZ} also displayed a large decrease in its mutation frequency (11.4-fold), whereas VSS_{AZ} and SG each displayed mutation frequencies (8.8- and 7.5-fold reduction from the wild-type, respectively) that were similar to that observed previously for the multi-drug resistant K65R mutant RT (8-fold) (Shah *et al.*, 2000). The mutant RTs VSG_{AZ}, AG and SS all exhibited a moderate decrease in their overall mutation frequency (4- to 5-fold reduction). It appears that the presence of the AZT-resistance mutations generally decreased the mutation frequencies and the presence of the M41L mutation, in combination with the AZT-resistance mutations, had the largest effect.

Reductions in mutation frequencies are partly due to decreased efficiency of dNTP misincorporation

Previous studies have shown that forward mutation rates often do not directly correlate with misincorporation efficiencies (Drosopoulos & Prasad, 1998). This is due to the fact that forward mutation rates measure not only misincorporation, but also frameshifting, deletions and insertion errors. To determine whether decreases in forward mutation frequencies correlate with decreases in base substitutions, it was important to study misincorporation efficiency. For this, two of the mutants with largest reductions in mutation frequencies, namely LAG_{AZ} and LSG_{AZ}, which displayed 15.3- and 16.3-fold reduction in mutation frequency, respectively, were selected. The ability of the wild-type and each of these two mutant RTs to misinsert and misextend a DNA template–primer in the presence of three of the four dNTPs was determined in a ‘single dNTP exclusion’ assay (Shah *et al.*, 2000). In this assay, RT is allowed to copy a DNA template using a 5'-end-labelled primer in the presence of four combinations of three dNTPs each missing dATP, dCTP and dGTP, respectively (reactions missing dTTP displayed little synthesis and were therefore omitted). When RT reaches a template base for which the complementary dNTP is missing, continued polymerization requires both dNTP misinsertion and mispair extension activities. For a polymerase lacking proofreading function such as RT, the resulting products yield a gross estimate of the degree of dNTP insertion and mispair extension fidelity. The wild-type HIV-1 RT displayed many products beyond this ‘barrier’ site, indicating that both misinsertion and mispair extension occur at a high efficiency. Fig. 1 shows the extension products resulting from increasing concentrations of wild-type and two of the mutant RTs showing the greatest reductions in mutation rates. As control, each of the enzymes assayed were also tested in the presence of all dNTPs to ensure equivalent enzyme inputs for each reaction and allow for accurate comparison. The mutant enzymes LAG_{AZ} and LSG_{AZ} displayed the most dramatic effect on misincorporation fidelity (Fig. 1 and Fig. 2). Lesser amounts of products were extended past the barrier site in all of the single dNTP exclusion reactions. Although the other enzymes also displayed reduced misincorporation efficiencies (data not

shown), these two mutants displayed the highest fidelity. There was a good correlation between the two assays.

Increase in fidelity is not due to primer rescue

It has been shown that RTs containing AZT-resistance mutations are capable of removing the terminal nucleotide from primers terminated with AZTMP (Meyer *et al.*, 1999). A pyrophosphorolysis reaction, using ATP (Meyer *et al.*, 1999) or PPI (Arion *et al.*, 1998) as the phosphate donor, allows continued extension of the AZTMP-blocked primer. We wished to test whether the increase in misinsertion fidelity for RT mutants was due to the removal of incorrect dNTP by a mechanism similar to that of primer rescue. The mutant RT with the greatest decrease in misincorporation and mutation frequency (LAG_{AZ}) was chosen to test this hypothesis. The ability of the mutant RT to remove an AZTMP-terminated primer was tested to ensure that the RT variant contained excision activity. As expected, the wild-type RT was able to excise the AZTMP and the LAG_{AZ} mutant RT displayed a much higher degree of excision (Fig. 3b, lanes 3–6 vs 7–10). It was tested whether the mutant RT would excise a mismatched 3'-primer terminal dAMP (opposite template dGTP) in the presence of ATP or PPI phosphate donors (Fig. 3c). A 5'-end-labelled template–primer was incubated with RT in the presence or absence of a phosphate donor (150 µM PPI or 3.5 µM ATP) and then the RT was heat-inactivated. Extension of the primer was initiated by addition of dNTPs and *Taq* polymerase. A Watson–Crick base-paired template–primer should be extended to full-length by *Taq* in all reactions, whether or not the phosphate donor is present. Reactions with mispaired template–primer, however, will only produce a full-length product if RT is able to use the phosphate donor and remove the terminal mispaired dNMP to produce a Watson–Crick base-paired terminus, which can then be extended by *Taq*. Reaction conditions conducive to the excision by RT (in the presence of necessary acceptors) were employed. No evidence of removal of the incorrect dAMP from the primer terminus was observed under any of the conditions tested (Fig. 3c). Control reactions with the mispaired G:A template primers could be extended by Klenow enzyme, which contains proofreading activity (data not shown). Thus, the increase in fidelity observed is not via a proofreading-like activity seen in AZT-resistant variants of HIV-1 RT.

Measuring equilibrium dissociation constants

In the absence of a true proofreading-like activity, decreased efficiency of misincorporation and decreased mutation frequency of the mutant RTs could result from a change in the dNTP-binding pocket to allow better discrimination against non-Watson–Crick base-paired dNTPs. Alternatively, it is also possible to score higher fidelity in the above assays due to a general decrease in the affinity of the mutant RT to the template–primer, which is accentuated following misinsertion events. Therefore, the equilibrium dissociation constants (K_d) of the wild-type and the mutant RTs on a DNA

template–primer were measured. The results of the binding assay for all of the RTs are summarized in Table 4. The equilibrium dissociation constants of the mutant RTs showed that most of them displayed reduced affinity to template–primer (1.3- to 7.4-fold reduction in affinity). Mutants AG, SG and LSG_{AZ} showed milder effects (1.5-, 1.9- and 1.3-fold, respectively), whereas VAG_{AZ} and LAG_{AZ} had the greatest reductions in affinity (7.4- and 5.1-fold, respectively). Mutants SS, VSG_{AZ} and VSS_{AZ} had moderate effects (3.6-, 2.8- and 2.8-fold, respectively). However, there was no correlation between template–primer affinity and misincorporation efficiency. For example, AG, SG and SS all showed small 4.0- to 7.5-fold decrease in mutation rate with little change in template–primer affinity, while the mutant with an intermediate increase in fidelity (VAG_{AZ}, 11.4-fold reduction in mutation) showed a large reduction (7.4-fold) in template–primer affinity. Furthermore, mutants LAG_{AZ} and LSG_{AZ} both showed a >15-fold reduction in forward mutation frequency over the wild-type. Of these, LAG_{AZ} showed a 5.1-fold reduction in template–primer affinity, whereas LSG_{AZ} showed little change. Thus, the increased fidelity observed for these mutants does not appear to be due to a general reduction in affinity to template–primer.

DISCUSSION

Variant HIV isolates containing dipeptide insertions in the β_3 – β_4 hairpin loop of RT display both a wider range (for many nucleoside analogues) and an increased level of resistance to nucleoside analogues (Larder *et al.*, 1999; Winters *et al.*, 1998). The increased resistance to AZT is known to be due to a higher efficiency of primer unblocking caused by dipeptide insertions. Changes to the geometry of the dNTP-binding pocket, as a result of dipeptide insertion and the T69S substitution, could affect the fidelity of DNA

synthesis. Our results show that the T69S and the dipeptide insertions alone can increase the fidelity of RT, whereas the presence of the AZT-resistance mutations and/or the MDR mutation A62V can enhance the degree of RT fidelity to a level not observed previously. It is important to note that recombinant RT mutants bearing the AZT-resistance mutations (D67N, K70R, T215Y and K219Q) by themselves do not affect mutation rate in the forward mutation assay (Lacey *et al.*, 1992). Furthermore, an insertion of 15 aa residues between positions 67 and 68 led to increases in processivity (Kew *et al.*, 1998), but little change in mutation rates (Rezende *et al.*, 2001). Thus, it appears that the increase in fidelity observed in these mutants is primarily due to a specific effect of T69S substitution in combination with the dipeptide insertions, which is further enhanced by the AZT-resistance mutations or the A62V mutation.

Results of the forward mutation assay are striking in that all mutants tested showed decreases in mutation frequencies. Mutants containing the M41L substitution showed the largest decrease (15- and 16-fold over the wild-type for LAG_{AZ} and LSG_{AZ}, respectively) compared with the other mutants studied here. Mutant VAG_{AZ} also showed a large decrease in mutation frequency (11-fold reduced over the wild-type), whereas the remaining mutants showed moderate- to low-level decreases (5- to 8-fold compared with wild-type HIV-1 RT). Our data strengthen the argument that dipeptide insertions combined with nucleoside analogue resistance mutations influence the frequency at which mutations occur during reverse transcription.

The forward mutation assay measures all types of errors. Thus, the decrease in mutation frequencies observed may have resulted from reductions only in frameshifting or other slippage-mediated events without really affecting nucleotide insertion fidelity. Results of the ‘single nucleotide exclusion’ assay confirm that each of the mutant RTs displayed a decreased efficiency of misincorporation. Misincorporation requires both the insertion of the wrong dNTP (dNTP misinsertion) and extension of the mispaired primer terminus. This assay allows one to quantify the outcome of changes in both of these events together rather than individually. Although the results do not reveal specific changes in dNTP insertion or primer extension, it is clear from the misincorporation data that the mutations profoundly affect RT fidelity. Our results suggest that dipeptide insertions alone decrease the incorporation of an incorrect base to a growing primer (data not shown). The combination of insertions (SS, SG or AG) with AZT-resistance mutations and the multi-dideoxynucleoside resistance mutation A62V further decreased misincorporation efficiency (data not shown), with the greatest decrease observed for those with the M41L mutation (LAG_{AZ} and LSG_{AZ}) (Fig. 1). The misincorporation fidelity of several mutants of HIV-1 RT was previously assessed using this assay (D76A, R78A, F61A and K65R) (Fisher & Prasad, 2002; Kim *et al.*, 1998, 1999; Shah *et al.*, 2000). A comparison of the levels of increases reported showed that LAG_{AZ} and LSG_{AZ} are

Table 4. Equilibrium dissociation constants of wild-type and mutant RTs using a DNA template–primer

Enzyme	K_d (nM)*	Ratio of mutant to wild-type
WT _{HXB2}	14.15 ± 0.15	1.0
AG	20.70 ± 0.3	1.5
SG	27.25 ± 0.82	1.9
SS	51.10 ± 1.5	3.6
VAG _{AZ}	105.40 ± 2.0	7.4
VSG _{AZ}	39.79 ± 1.2	2.8
VSS _{AZ}	39.77 ± 1.8	2.8
LAG _{AZ}	72.04 ± 1.5	5.1
LSG _{AZ}	18.51 ± 0.5	1.3

*The $K_{d[\text{DNA}]}$ in the binary complex for wild-type HIV-1 RT and its individual mutants were determined by a gel mobility shift assay using a 33 mer DNA template/5'-³²P-labelled dideoxy-terminated 21 mer primer. The degree of template–primer associated in the binary and ternary complexes (%) was quantified as described in Methods.

among those displaying the highest increases in fidelity (Fig. 1 and Fig. 2).

It has been previously shown that mutant RTs containing insertions within the fingers subdomain, albeit with a plethora of background mutations, had the ability to excise an AZTMP-terminated primer much more efficiently than wild-type RT (Lennerstrand *et al.*, 2001; Mas *et al.*, 2000). A second study, in which many of the same RT mutants assayed in this study were used in excision assays (AG, SG, SS, LAG_{AZ} and LSG_{AZ}) showed that RT variants with SG or AG (but not SS) insertions have a 3- to 4-fold increase in their ability to unblock a terminated primer (Meyer *et al.*, 2003). Furthermore, the M41L, L210W, R211K, L214F and T215Y mutations associated with the insertions (LAG_{AZ} and LSG_{AZ}) had increased unblocking activity compared with the wild-type (Meyer *et al.*, 2003). Thus, it appears that the primary and secondary mutations that lie outside the active site of the RT enzyme bring about alterations in the side-chains of amino acid residues that are proposed to interact with a phosphate acceptor (i.e. ATP), enhancing excision of the AZTMP from the terminated primer through a pyrophosphorolytic reaction (Meyer *et al.*, 2003). It was conceivable that a similar process could occur in the case of a mismatched primer terminus, wherein a misinserted base could be removed in the presence of a phosphate acceptor (ATP or PPi). This was ruled out as a possible mechanism to increase the misincorporation fidelity in these mutants. LAG_{AZ}, the RT variant with the largest reduction in mutation rate, was chosen to test our hypothesis. In our hands, the LAG_{AZ} mutant RT displayed about 30% of wild-type activity on both RNA and DNA templates (data not shown). In agreement with the literature (Boyer *et al.*, 2002; Meyer *et al.*, 1999), the LAG_{AZ} RT variant had an elevated ability to excise an AZTMP-terminated primer in the presence of ATP (Fig. 3). Using either ATP or PPi as the acceptor at several different dNTP concentrations, no excision of misinserted bases was detected for the wild-type or LAG_{AZ} mutant RTs (Fig. 3c, lanes 5–12 and 17–24, respectively). Thus, our results show that the increased fidelity observed in the LAG_{AZ} RT mutant is not due to a phosphorolytic removal of mispaired base from the primer terminus.

An alternative explanation to account for the observed decrease in misincorporation efficiencies or mutation frequencies could be that mutant RTs bind to the template-primer with decreased affinity. Dissociation of the polymerase at the site of misinsertion would lead to elimination of that product from being counted as an error in the forward mutation rate assay as only the full-length products would result in a viable plaque. Similarly, RT dissociation soon after misinsertion would yield no products well beyond the site of misinsertion. Mutants with the T69S substitution and insertions displayed a range of reductions in affinity to the DNA template-primer (1.3- to 7.4-fold). However, there was no correlation between reduced affinity and decreased mutation frequencies or misincorporation efficiencies. In fact, LAG_{AZ} and LSG_{AZ}, which both had large but

similar reductions in mutation frequencies and misincorporation efficiencies, displayed divergent template-primer affinities – the former had a 5.1-fold decrease, whereas the latter had a slight decrease from that of the wild-type (1.3-fold).

Mansky & Temin (1995) developed an assay that is designed to measure mutation rates during virus replication. Although the overall mutation rate measured by this assay is lower than that observed *in vitro*, the trends in mutation frequencies obtained by this assay (Mansky *et al.*, 2003) for various nucleoside analogue resistance mutations such as M184V (Drosopoulos & Prasad, 1998), E89G (Drosopoulos & Prasad, 1998), K65R (Shah *et al.*, 2000), D76V (Kim *et al.*, 1999) and R78A (Kim *et al.*, 1999) were similar to those observed *in vitro*. Interestingly, however, Mansky & Bernard (2000) found that AZT-resistance mutations M41L/D67N/K70R/T215Y in HIV RT led to increased mutation frequencies. The mechanism by which these mutations increase the mutation rate is unclear. Based on our results, it is predicted that, in the context of dipeptide insertions, a virus replication-based assay would detect a reduction in mutation rates. As observed for mutations such as K65R or R78A, which showed 8- to 9-fold reduction in the *in vitro* assays (Shah *et al.*, Kim *et al.*), but a mere ~3-fold reduction in the virus replication-based assay (Mansky *et al.*, 2003), it is likely that the reduction observed using the virus replication-based assay may not be commensurate with the large reductions observed in the *in vitro* measurements reported here.

To date, the largest decreases in overall mutation rate have been associated with mutations located within the $\beta 3$ – $\beta 4$ fingers subdomain (Fisher & Prasad, 2002; Kim *et al.*, 1998, 1999; Shah *et al.*, 2000). Because this region of the fingers makes multiple contacts with both the dNTP and DNA substrates, mutations associated with fingers have a direct influence on the overall geometry of the RT active site. Our study suggests that insertions found in the fingers subdomain of RT may further change the geometry of the active site, thus acting as determinants of fidelity within RT.

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