



Short communication

Comparative analysis of *in vitro* processivity of HIV-1 reverse transcriptases containing mutations 65R, 74V, 184V and 65R + 74VPrem L. Sharma^{a,b,*}, James H. Nettles^{a,b}, Anya Feldman^{a,b}, Kimberly Rapp^{a,b}, Raymond F. Schinazi^{a,b}^a Center for AIDS Research, Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, United States^b Department of Veterans Affairs, Decatur, GA 30033, USA

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ABSTRACT

While HIV-1 reverse transcriptase (RT) mutations of M to V at position 184 are commonly observed in the clinic, the double mutation of 65R + 74V is rarely seen. It has been demonstrated that rapid R → K reversion occurs at RT codon 65 during replication of HIV-1 in human peripheral blood mononuclear cells containing 65R + 74V mutations and that processivity of the RT is reduced relative to wild type. However, clinical studies show that M184V can be detected after several months of therapy interruption, suggesting more effective processivity. Herein, the *in vitro* RT processivity of genetically engineered M184V and double mutant 65R + 74V was compared. Virion-associated RTs of WT pNL4-3, K65R, L74V, M184V and 65R + 74V were used to perform RT processivity assays in the presence of trap, poly(rC)-oligo(dG). Both RTs with 184V and 65R + 74V mutations exhibited similar processivity when compared with each other and a significantly decreased processivity as compared to WT RT. Both mutant RTs synthesized shorter cDNA molecules (37–42 nt) relative to WT RT, which made longer (65–70 nt) cDNA molecules. Since these surprising biochemical results cannot explain the clinical phenotype, a hypothesis is presented to explain the discrepancy and suggest new approaches for future testing.

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During the past decade the effectiveness of highly active antiretroviral therapy in suppressing HIV load to undetectable levels has been demonstrated in several clinical trials. It has also become clear that most reverse transcriptase (RT) inhibitors are much more effective when given in combination than when given alone (Larder et al., 1995; Richman, 1996), although antagonism can be observed *in vitro* and in humans with nucleosides that use the same initial phosphorylation enzyme (Hernandez-Santiago et al., 2007). It is widely believed that such synergistic drug interaction arises from the fact that certain combinations of drug resistance mutations are particularly detrimental for the enzyme, which leads to a less fit virus as shown by our group and others for RT variants containing mutations K65R, L74V and M184V (Back et al., 1996; Sharma and Crumpacker, 1997, 1999; White et al., 2002; Sharma et al., 2004; Deval et al., 2004a,b).

In addition, certain mutations are mutually exclusive. For example passaging of HIV_{LAI} in the presence of nucleoside reverse transcriptase inhibitors (NRTI), β-D-dioxolane guanine (DXG) or DAPD (Amdoxovir®), results in the selection of K65R or L74V, but

both mutations were not selected together (Bazmi et al., 2000). Using human peripheral blood mononuclear (PBM) cell-based replication assays and genotype analysis, we demonstrated that the double mutant 65R + 74V in a pNL4-3 background is 'unstable' because a rapid 65R → K reversion occurs during replication of virus in PBM cells. We further showed that sub-optimal concentrations of human IL-2 slows down the replication of virus and 65R → K reversion. These studies provide evidence that 65R → K reversion is replication dependent and that RT mutations 65R and 74V are mutually exclusive (Sharma et al., 2004). Accordingly, the presence of 65R and 74V on the same viral genome mutations is rare in clinic (Wirten et al., 2005, and Stanford HIV drug resistance database, <http://hivdb.stanford.edu>). In contrast to the instability of 65R mutation in double mutant virus 65R + 74V, no reversion at codon 65 has been reported in double mutants 65R + 184V and 65R + 68S (Deval et al., 2004a; Røge et al., 2003). These observations indicate the significance of positioning of specific amino acid residues in 3D-RT structure in relation to the biological function of enzyme (Huang et al., 1998).

A biochemical mechanism to correlate attenuated replication capacity of a virus with altered RT function is the determination of *in vitro* RT processivity. However, controversial RT processivity results have been reported based upon the assay condition, choice of template/primer and the concentrations of dNTPs used in the reaction mixture (Arion et al., 1996; Back and Berkhout, 1997; Boyer et al., 1998; Sharma and Crumpacker, 1999; Deval et al., 2004b; Xu

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et al., 2009). Also, studies have demonstrated that replication defect by one mutation can be compensated by other distant mutation(s) leading to the generation of a virus which is replication competent (Olivares et al., 1999; Svarovskaia et al., 2008), and reduced enzyme activity due to single point mutation could be compensated by second-site mutation (Boyer et al., 1998; Harris et al., 1998). RT mutation M184V appears to be quite stable under therapy, however, upon discontinuation of lamivudine (3TC), 100% reversion to WT phenotype occurs after several months (Zaccarelli et al., 2003). The major goal of our study was to compare *in vitro* processivity of RTs containing a stable mutation M184V and an unstable double mutant 65R + 74V. We report herein a direct comparison of RTs containing 65R, 74V, 184V and 65R + 74V mutations in the same assay system.

Various point mutations were created in the background of proviral clone pNL4-3 (Adachi et al., 1986) by using pALTER⁻¹ mutagenesis system of Promega (Madison, WI) according to manufacturer's guidelines and our previously described protocols (Sharma et al., 1996, 2004; Nurpeisov et al., 2003; Sharma and Crumpacker, 1999). To eliminate the possibility of reversion during reverse transcription in mutant K65R + L74V virus, we used primary human embryonic kidney cells (293), which are non-permissive for HIV-1 infection. Viruses were produced using SuperFect[®] reagent of Qiagen (Valencia, CA) and manufacturer's guidelines. Cells were passed into 60 mm dishes 24 h prior to transfection in Dulbecco's Modified Eagles Medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS) and penicillin/streptomycin (complete medium). To generate virus, the complex containing 10 µg of DNA in 150 µL of serum-free medium and 30 µL of SuperFect reagent was incubated at room temperature for 10 min. One mL of complete DMEM was added dropwise onto 293 cells that were washed once with phosphate buffer saline (PBS). Cells were incubated at 37 °C in the presence of 5% CO₂ for 3 h. The remaining medium-complex was removed and the cells were washed with PBS (4 mL). Four mL of complete DMEM was added and dishes were incubated for 48–72 h. Culture supernatants were collected and centrifuged for 5 min at 833 × g ($g = 1.2$) to pellet any debris. The presence of specific mutation was confirmed by sequencing culture supernatant-derived HIV-1 RNA. One mL of each virus was centrifuged for 2 h at 15,000 rpm in a refrigerated centrifuge [Thermo Electron Corp., Marietta, OH; Model, MR 23i; Rotor, DRM6.M]. The pelleted virus was disrupted with 40 µL of lysis buffer containing NP40 and RT activity was determined using homopolymer template/primer [poly A-oligo d(T)] (Boehringer Mannheim, IN) and α -³²P dTTP (Morvaek Biochemicals Inc., Brea, CA) according to published protocols (Sharma and Crumpacker, 1999).

Previous, *in vitro* studies have shown that virion-associated protein Ncp7 (Li et al., 1996) and host proteins such as cellular topoisomerase I and p53 (Takahashi et al., 1995; Bakhanashvili, 2001) can interact with HIV RT and enhance RT activity and fidelity. Therefore, we used virion-associated RT lysates in all our assays. In order to compare *in vitro* processivity of various RTs, all assays were performed in the presence of 50-fold excess of trap [poly(rC).oligo(dG)]. The presence of trap ensures that processivity was measured during a single processive cycle.

Assays were performed as described elsewhere (Boyer and Hughes, 1995; Back et al., 1996; Sharma and Crumpacker, 1999). The reactions were terminated by placing the tubes in ice slurry and by adding an equal volume of buffered phenol. cDNA products were extracted by phenol:chloroform:isoamyl alcohol and the purified products were run on 6% polyacrylamide sequencing gel electrophoresis. The wet gels were exposed to autoradiography for 30 min to 2 h. The relative density of DNA bands was compared in autoradiograph using Intelligent Quantifier software (Bio Image Systems, Inc., Jackson, MI).

The visual examination of autoradiograph clearly shows that the entire panel of mutant RTs had decreased processivity in comparison to WT RT (Fig. 1A). Similar RT processivities were noted in three to five independent assays. In order to determine the relative synthesis of cDNA bands generated during processive reverse transcription, a set of six bands were highlighted and the density of each set was calculated. The quantification of cDNA bands revealed that while there is not a significant difference between shorter cDNA products (bands 1–6) generated by WT, 65R and 74 RTs, a significant difference ($p = 0.05$) among larger cDNA products (>6 bands) was evident (Fig. 1B). In contrast, 184V RT and 65R + 74V RT made fewer and shorter cDNA products throughout, suggesting a major processivity defect in these two RTs as compared to WT RT (Fig. 1B and C, Table 1).

A nonparametric (Kruskal-Wallis) test was performed to compare the first 6 band values for the five viruses. While no significant difference was observed ($p = 0.11$) between WT and 65R and WT and 74V viruses, a significant difference between WT and 65R + 74V ($p = 0.05$) and WT and 184V ($p = 0.05$) was observed. The student *t*-test indicated that DNA density decreased at a significantly higher rate for RTs containing 65R + 74V and 184V mutations as compared to WT RT, 65R and 74V RTs. The relative processivities for different RTs were, WT > 65R ≥ 74V > 65R + 74V ≥ 184V. Under the assay conditions, the maximum lengths for cDNA synthesized by WT, 65R, 74V, 65R + 74V and M184V RTs were 66, 60, 62, 42 and 37 nucleotides, respectively. The decreased processivity observed for L74V and M184V RTs were similar to those seen previously (Back et al., 1996; Sharma and Crumpacker, 1999). In a recent study, investigators demonstrated that under low dNTP concentration K65R RT exhibits lower activity in single-cycle processivity assay (Xu et al., 2009) which is in agreement with our observation of a decreased processivity of K65R compared to WT RT.

X-ray crystal structure coordinates for wild type HIV RT complexed with thymidine analogs were downloaded from the international protein data bank at <http://www.pdb.org> (Huang et al., 1998; Sarafianos et al., 1999, 2002). File 1rtp.pdb (Huang et al., 1998), a covalently trapped catalytic complex of HIV RT and DNA including thymidine triphosphate (TTP), was modified by addition of 3' hydroxyl to the primer residue. The entire system was protonated using Generalized Born/Volume Integral Formalism as implemented in MOE (v2007.09; CCG, Montreal). The protonated system including Mg²⁺ was typed for use in the Merck Molecular Force Field. Electrostatics were computed with a solvent dielectric of 80 and 15 Å cutoff. Steric interactions were calculated using a 12-6 Leonard-Jones potential. Systematic energy minimization and exploration of side chain rotomers associated with wild type and drug resistant mutants was performed as described previously (Hari et al., 2006). File 1n6q, wild type RT complexed with 3'-AZTMP terminated DNA in the pre-translocation or "N" site, was fit to the 1rtd reference coordinates using "Matchmaker" in Chimera (Pettersen et al., 2004) to align functional activity space. Best-aligning pairs of chains between reference and match structure were computed using the Needleman-Wunsch alignment algorithm with a BLOSUM-62 Matrix and gap extension penalty of 1. Possible matches were pruned until no pair exceeded 2.0 Å. The 3'-azido group was modified to OH for comparisons with 1rtd.pdb.

The wild type complex containing TTP as prepared in methods is shown in Fig. 2. Energetic analysis of possible rotomer interactions associated with mutations at positions 65 and 74 within the catalytic TTP complex revealed significant shifting of H-bonding patterns and template alignments similar to those suggested previously for the A nucleotide (Deval et al., 2004b). Although the 74V mutation does not have direct contact with the TTP it does provide template positioning for the incoming nucleotide. Repositioning of finger region, β4–β3, in response to the changing L74V caused shifting of R72 and K65. This shifting became more pronounced in the

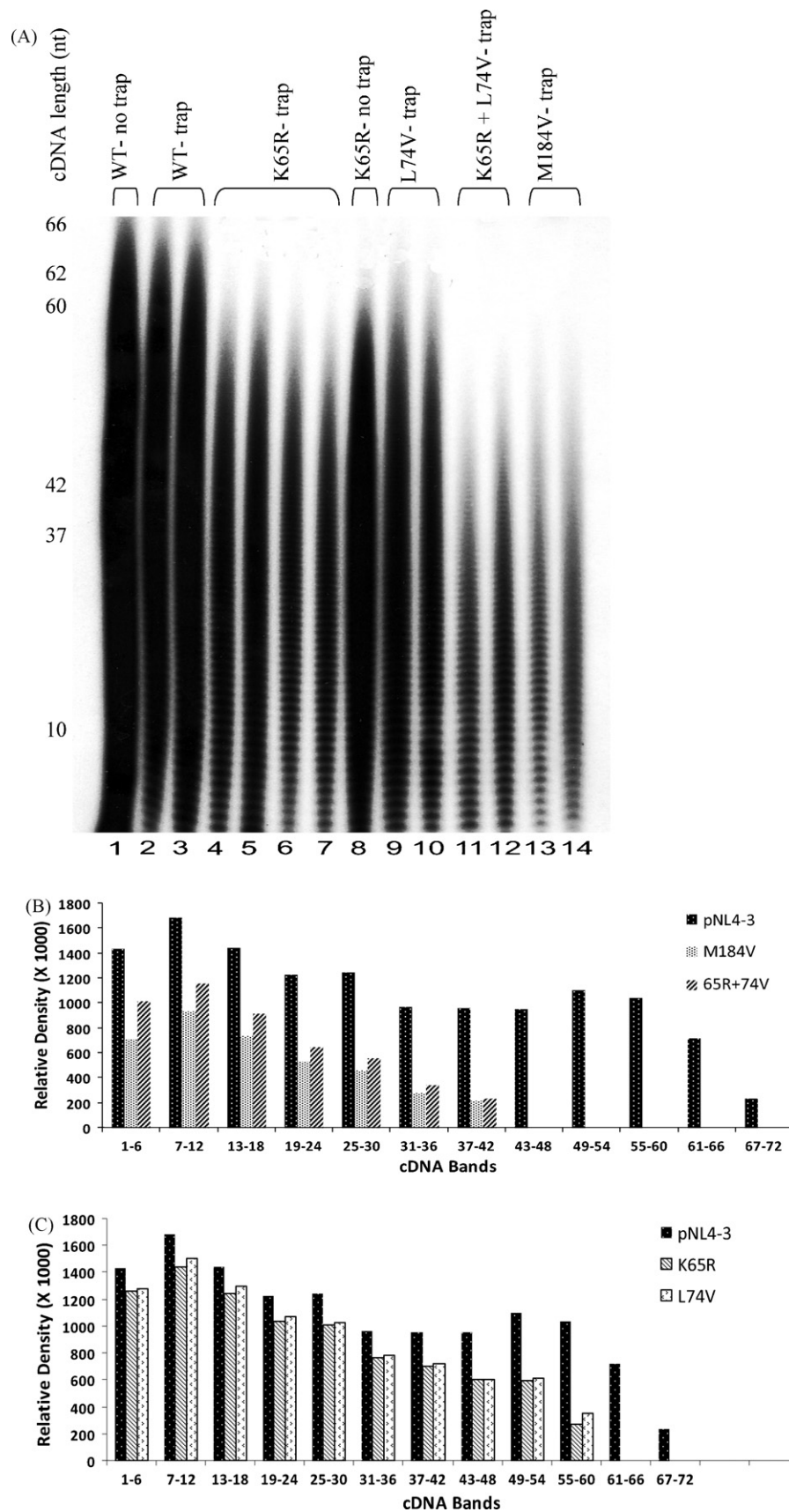


Fig. 1. Reduced processivity of RTs containing mutations 65R, 74V, 65R + 74V and 184V. The purified cDNA products were run on a 6% polyacrylamide vertical gel electrophoresis. The cDNA bands were visualized by autoradiography. The image contrast for whole gel was adjusted to visualize the relative cDNA densities among different lanes (A). Lanes: 1, WT (no trap); 2, 3, WT; 4–7, 65R; 8, 65R (no trap); 9, 10, 74V; 11, 12, 65R + 74V; 13, 14, 184V. Relative amounts of cDNA synthesized with WT and entire mutant panel were quantified by Intelligent Quantifier (Bio Image Systems, Inc.) software. The groups of 6 cDNA bands from bottom to the top were scanned for each lane and plotted against cDNA length (B and C).

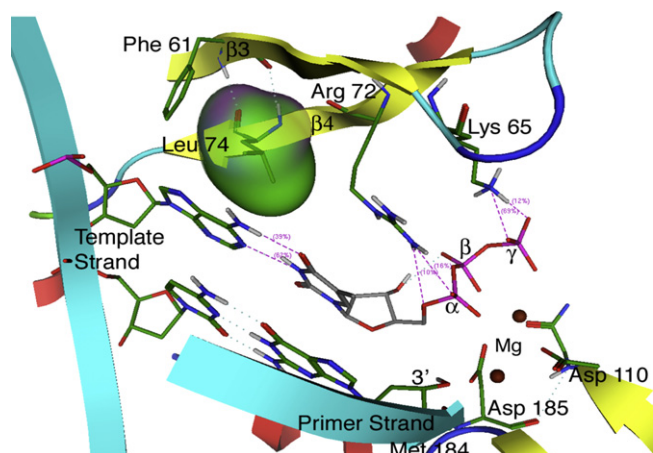


Fig. 2. Structural interactions of activated nucleotide. Positions of residues 65 and 74 as found in a covalently trapped catalytic complex of HIV-1 reverse transcriptase, 1rti.pdb (12), showing potential hydrogen bonds in magenta. Van der Waal surface around Leu74 shows simultaneous contact with aligning template strand residue and Phe 61 on adjacent strand β 3. Strand β 3 orientation is defined by position of Phe 61 and Lys 65.

65R+74V double mutant, suggesting that change in finger flexibility could be a contributor to the observed loss of processivity. Analysis of open HIV-RT compared to the closed form highlights the role of the 74 position as a hinge for finger flexibility. Fig. 3 illustrates the alignment of residue 74 on β 4 with residue F61 on β 3 during finger extensions that moves residue 65 10 Å out of the binding site. Through this extended secondary structure mechanism, the volume change associated with L74V is expected to alter absolute positioning of residue 65 as well as template positioning.

Residue 184 is located at the 3' end of the primer residue adjacent to the catalytic aspartic acids also shown in Fig. 3. This work, as well as previous crystallographic and modeling studies, suggests M184V most likely causes resistance to specific drugs due to increased steric hindrance by β -branched amino acids and can also cause repositioning of template-primer (Sarafianos et al., 1999; Deval et al., 2004a). Accordingly, structural changes due to mutations at 184 are not directly linked to residues on the β 3– β 4 finger through the protein.

Although each are clinically important, K65R and L74V rarely occur on the same virus genome (Winters et al., 1997; Wirden et

al., 2005; Henry et al., 2006), and Stanford HIV drug resistance databases (web site: <http://hivdb.stanford.edu>) confirm this rarity. Among 23 patients who received 1–2 years of 2',3'-dideoxyinosine (ddI) therapy, only one showed the presence of K65R and L74V on the same genome. Analysis of the Marseille database containing 8866 sequences from 3720 patients revealed the coexistence of K65R and L74V in only two clones (Henry et al., 2006). Investigators speculated on the compensatory role of second-site mutation(s) in these studies. Our analysis suggests that the structural proximity of these residues may underline the cause for this clinical observation. In contrast 3TC-selected M184V mutation (Schinazi et al., 1993; Tisdale et al., 1993) is prevalent during clinical trials with combination therapy where 3TC or FTC is one of the component of regimen (McColl et al., 2008). Also, no apparent reversion to wild type at mutated codon M184V has been detected during virus propagation in the absence of drug. Therefore, 184V could be termed as a 'stable' mutation *in vitro*. Based upon these observations, theoretically, it could be predicted that RT containing double mutation 65R and 74V would be significantly less processive than M184V RT. In contrast, we found similar *in vitro* processivity in terms of number and size of cDNA molecules generated with 'T' nucleotide using homopolymer template (Fig. 1A and C).

Comparing the nucleotide incorporation data from two previous studies with RTs containing 184V (Deval et al., 2004a) and 65R+74V (Deval et al., 2004b) demonstrated that the incorporation rate for nucleotides A, G and T was approximately similar for both RTs using a heteropolymer template. However, there was a 2-fold greater incorporation of 'C' nucleotide with 184V RT than the 65R+74V RT (Deval et al., 2004a,b). This raises a question about processivity of specific nucleotides and if homopolymer experiments were measuring the correct marker. Could it be that an assay of 'C' incorporation rather than "T" would better explain the clinical phenotype? It should be noted however, that in contrast to a decrease in processivity for M184V and K65R+L74V virion-associated RTs observed by us, no change in the processivity of 65R+74V RT was observed by Deval et al. (2004b). As we discussed earlier, these differences may be due to the differences in the assay system, RTs (virion-associated vs. purified) and/or traps used. Since our assay showed similar decrease in M184V RT as observed previously by Back et al. (1996), a direct comparison of 65R+74V and other mutant RTs was performed. Unfortunately, the homopolymer processivity assays may not be enough to distinguish stability of mutant M184V observed during clinical trials with the rare mutant

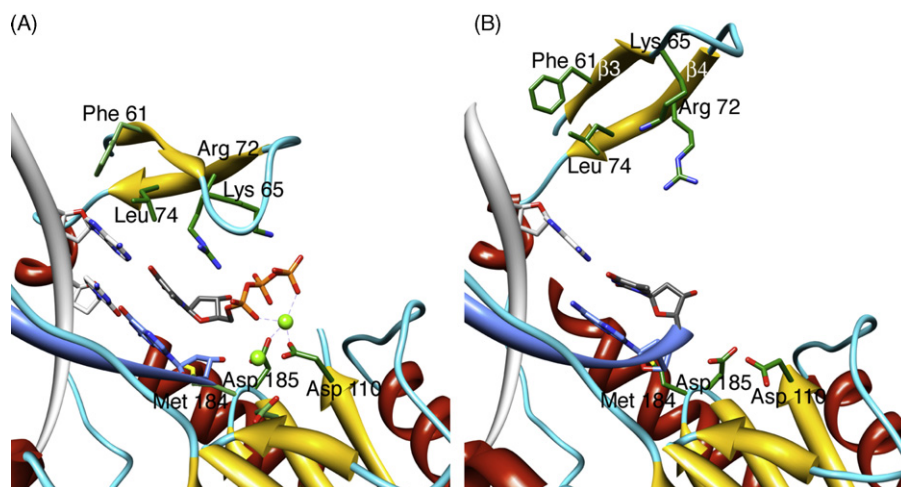


Fig. 3. Flexibility of β 3– β 4 fingers. Orientation of β 3 and β 4 varies significantly between (A) the catalytic complex, also shown in Fig. 2 (12) and (B) post incorporation complex derived from 1nq6.pdb by Sarafianos et al. (20). Torsional flexing of finger is centered at the interface of residues 61 and 74. Mutation of L74 to V would be predicted to change mobility vector of β 3 and directly impact positioning of residue 65 in the catalytic complex. Reduced rotational freedom of Arg may be responsible for its lessened activity relative to Lys at that position. Residue Met184 is shown positioned beneath the primer nucleotide.

65R + 74V. Since double mutant reverts back R → K at codon 65 rapidly in PBM cells (Sharma et al., 2004), a 2-fold difference in the incorporation of 'C' nucleotide between two RTs may or may not explain the debilitating effect on viral replication and 65R → K reversion in 65R + 74V mutant seen previously (Sharma et al., 2004).

These results show that the *in vitro* RT processivity of certain nucleotides may not always correlate with the replication kinetics of mutant viruses, but they can provide insights into the molecular mechanism. Processivity can depend upon the significant positioning of specific amino acid residues in p66 domain of polymerase (Huang et al., 1998). Since a major component of HAART, such as tenofovir disoproxil fumarate (TDF) can select for the RT mutation K65R while several nucleoside analogs (ddI, ddC, ABC) select for L74V (St. Clair et al., 1991; Schinazi et al., 2000), our observations that the 65R and 74V mutations are mutually exclusive and results in an RT with severely reduced enzyme activity, may be clinically relevant. This is part of a multistep effort to relate *in vitro* observations to *in vivo* findings. Analysis of incompatibility among different mutations can further advance our knowledge about drug-selected lethal combination(s).

In recent years, several investigators have addressed the issue of clinical benefits in relation to nucleoside analog mutations K65R, L74V and M184V. Analysis of clinical data on the risks and incidence of K65R and L74V mutations on virologic responses have revealed that if suboptimal HAART is used, infected persons with K65R experienced significantly higher rates of virologic suppression than did those with L74V mutation (Waters et al., 2008). Since the combination of mutations in the background of K65R may have a more positive impact on viral suppression than those with L74V, it will be difficult to rule out the role of other mutation(s) involved with viral suppression in this scenario. Appropriate assays for direct comparison of the replication kinetics and RT processivity of the viruses with K65R and L74V will be needed to understand the overall impact of multidrug resistance mutations in viral suppression. The role of M184V in viral suppression and overall clinical benefits has been studied by various investigators. Studies have clearly indicated that the presence of M184V during clinical trials lead to the maintenance of viral suppression and improved immunological profile even in the presence of 3TC or FTC (Miller et al., 2002; Petrella and Wainberg, 2002; Wainberg, 2004).

Our analysis explored whether processivity was dependent on similar structure constraints of RT for biological activities of this enzyme. As mentioned earlier, certain mutant RTs are less processive and make fewer and shorter cDNA products during *in vitro* RT processivity assays. It is conceivable that such RTs are inefficient in generating full-length cDNA products and will result in shorter double-stranded DNA during *in vivo* reverse transcription. Studies have revealed that viral attenuation is not always linked to *in vitro* RT processivity and fidelity. While M184V RT is associated with increased fidelity (Wainberg et al., 1996), L74V RT does not show

significant increase in fidelity (Shah et al., 2000). On the other hand K65R RT has largest fidelity increase reported so far for RT mutants (Shah et al., 2000).

In the case of a processivity defect due to M → V change at residue 184, previous structural studies have shown that polymerization by HIV RT involves the interaction between the minor groove of the template-primer and minor groove binding tract (MGBT) (Huang et al., 1998). These crystallographic studies have revealed that the direct interaction of DNA duplex with bases occurs in the minor groove at position n to n–3, the van der Waals contacts with P157 and M184 (base pair n) and with I94 (base pairs n–2 and n–3), and hydrogen bonds between Y183 and G(n–1) (12). I94 has been shown to be a part of significant structural element MGBT. On the basis of these structural studies, it was assumed that the mutation at codon 184 will result in an altered interaction with DNA duplex in the minor groove and indirectly may affect the translocation due to MGBT resulting in a decreased processivity (Back et al., 1996; Huang et al., 1998; Sharma and Crumpacker, 1999).

Amino acid K65 and L74 are part of the highly flexible β3–β4 linkage group in the fingers domain of the 66-kDa subunit of HIV-1 RT. The HIV RT crystal structure shows that the incoming triphosphate moiety is coordinated by K65 and R72, main-chain-NH groups of residues 113 and 114, and two Mg²⁺ ions. Further, Arg72 donates hydrogen bonds to the α-phosphate and the ε-amino group of K65 donates hydrogen bonds to the γ-phosphate. As a result of finger closure, both of these side chains move into a specific position. The closing down of the fingertips traps both the template strand due to the interaction of L74 with the templating base and the dNTP (Huang et al., 1998). Additionally, as illustrated in Fig. 2, L74 provides a positional interface for orientation of β3 relative to β4 that allows productive positioning of R72 for catalytic activity.

The dynamic nature of β3–β4 fingers is shown in Fig. 3 through aligned active sites before/after nucleotide incorporation and pyrophosphate release (Huang et al., 1998; Sarafianos et al., 2002). A change from L → V at 74 is likely to shift β3 orientation relative to β4 through direct interaction with Phe 61. In addition to template packing, this change can affect absolute positioning of both residues 65 and 72. Thus, the elucidation of interactions between residues 65 and 74 derived from comparative complexes of HIV RT suggests that the major influence due to changes K → R and L → V at residues 65 and 74, respectively, may occur during initiation. In contrast, M → V at 184 is thought to reduce binding of incoming nucleotides by shifting positioning of primer-template. Our analysis suggests that the interpretation of *in vitro* RT processivity assays may require additional related functional viral cell culture assays to assess the stability of altered RTs in relation to the level of *in vivo* attenuation and/or reversion of mutant viruses.

In this study, *in vitro* RT processivity of "T" nucleotides was used to compare "fitness" of the 65R + 74V double mutant relative to WT, M184V and K65R, L74V, single mutants. However, these *in*

Table 1
Summary of relative cDNA synthesis during processivity assays.

Group of cDNA bands	Median cDNA density (×10 ⁴)/(minimum/maximum)				
	pNL4-3	K65R	L74V	M184V	K65R + L74V
1–6	142.8 (132.6–153.3)	125.7 (117.6–134.1)	127.8 (114.6–141.3)	71.6 (62.6–79.7)	100.8 (100.4–101.7)
7–12	165.3 (158.7–172.7)	143.4 (143.5–144.1)	151.2 (135.4–164.8)	92.9 (84.1–101.3)	115.5 (111.0–120.3)
13–18	144.5 (143.4–145.2)	124.3 (121.6–127.6)	131.9 (117.4–142.3)	73.4 (68.1–78.9)	91.3 (86.8–95.1)
19–24	122.9 (121.8–123.4)	103.7 (100.6–106.0)	107.4 (91.2–114.5)	52.9 (49.6–55.6)	64.8 (60.6–68.4)
25–30	124.5 (122.3–126.4)	100.7 (99.0–102.1)	76.8 (69.2–87.1)	45.7 (43.2–48.3)	54.8 (50.2–59.9)
31–36	96.3 (96.1–96.5)	76.3 (76.2–76.7)	73.1 (62.9–81.5)	28.1 (26.8–29.6)	34.2 (29.1–38.2)
37–42	96.0 (94.7–97.0)	70.6 (70.5–70.6)	59.3 (52.5–68.1)	21.6 (21.1–22.0)	23.5 (20.2–26.1)
43–48	95.0 (93.5–96.8)	60.4 (60.0–60.8)	62.8 (51.4–71.7)	15.9 (15.5–16.3)	15.3 (14.1–16.5)

A group of 6 bands starting from the bottom of the gel (Fig. 1) were quantified and median values were calculated from 3 to 5 independent processivity assays. The ranges from the different scans are shown between the brackets. The largest cDNA fragments for M184V and K65R + L74V were between 37 and 42 nucleotides, therefore, the scan readings presented for bands 43–48 is background for these two mutant RTs.

vitro results do not correlate with clinical observations suggesting lower fitness for the double mutant. Our models suggest that the position of 65 and 74 within the linked finger region may cause disproportionate processing sensitivity for other nucleotide templates presenting other bases. Since *in vivo* processing requires many bases changes and combinations it may be necessary to systematically evaluate the limiting combination *in vitro*. Due to the location of residue 184 in the relatively stable floor of the active site, we expect it to be less sensitive to base changes. We are experimenting with modified assay conditions to test this hypothesis.

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