Increased thermostability and fidelity of DNA synthesis of wild-type and mutant HIV-1 group O reverse transcriptases

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Running title: Thermostable HIV-1 reverse transcriptases

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ABSTRACT

Reverse transcription coupled with DNA amplification has become a well-established and powerful molecular technique for studying ribonucleic acids. However, the efficiency of those reactions is largely dependent on the molecular properties of currently used reverse transcriptases (RTs). Engineered and natural RT variants with improved thermostability and fidelity of DNA synthesis should be of great utility in the amplification of RNA targets. In this study, we demonstrate that the wild-type HIV-1 group O (O_WT) RT shows increased thermostability in comparison with Moloney murine leukemia virus RT and a prototypic HIV-1 group M:subtype B (BH10_WT) RT, while rendering higher yields in reverse transcription-polymerase chain reactions (RT-PCR), that included a cDNA synthesis step performed at a high temperature range (57 – 69°C). In addition, the O_WT RT showed 2.5-fold increased accuracy in M13 *lacZa* forward mutation assays in comparison with the BH10_WT RT. Unlike the BH10_WT enzyme, O_WT RT showed a very low error rate for frameshifts. Mutational hot spots induced by O_WT RT occurred at nucleotide runs, suggesting a dislocation-mediated mechanism for the generation of base substitutions. In HIV-1 group O RT, substituting Ile75 for Val rendered an enzyme which was 1.9 and 4.7 times more faithful than O_WT RT and BH10_WT RTs, respectively, in forward mutation assays. The mutant RT also showed increased misinsertion and mispair extension fidelity in kinetic assays. However, its mutational spectrum was similar to that obtained with the wild-type group O polymerase. V75I caused a loss of efficiency of RT-PCR amplifications at 65°C and 68°C in comparison with O_WT RT. However, a double mutant devoid of RNase H activity (V75I/E478Q) was found to reverse transcribe at temperatures as high as 68°C, while maintaining the increased accuracy of the V75I mutant.

Introduction

In retroviruses, the reverse transcriptase (RT) is the enzyme responsible for the replication of the viral genome. Retroviral RTs are multifunctional enzymes with RNA- and DNA-dependent DNA polymerase and endonuclease (RNase H) activities that catalyze the synthesis of proviral DNA using the viral RNA as template.¹

RTs are extensively used in recombinant DNA technology to synthesize cDNA from messenger RNA (mRNA). The viral polymerases of avian myeloblastosis virus (AMV) and Moloney murine leukemia virus (MLV) are the most frequently used RTs in many research applications, including diagnostic detection, cDNA cloning, end-point and real-time reverse transcription-polymerase chain reaction (RT-PCR) and microarray analysis. The AMV RT is a heterodimer composed of 95-KDa (β) and 63-KDa (α) subunits, whereas the MLV RT is 76-KDa monomeric protein (reviews^{2,3}). AMV RT shows increased stability and higher efficiency of cDNA synthesis *in vitro* in the temperature range of 42 – 52°C.^{4,5} However, the higher RNase H activity of AMV RT limits its efficiency while synthesizing cDNA from long mRNAs. Mutations introduced into AMV RT and MLV RT that eliminated RNase H catalytic activity enhanced thermal stability (by up to 8°C) and increased the yields of full-length cDNA in polymerization reactions.⁴

The human immunodeficiency virus type 1 (HIV-1) RT functions as an asymmetric heterodimer composed of 66-KDa (p66) and 51-KDa (p51) subunits. Both subunits have the same amino acid sequence, but p51 lacks the 120 residues of the C-terminal region of p66 that define the RNase H domain of the enzyme. The catalytic triad of the RNase H domain consists of Asp443, Glu478 and Asp498. Mutations at a single residue of the triad (e.g. E478Q) render the RNase H inactive.^{6,7} The DNA polymerase activity of the HIV-1 RT heterodimer resides within the 66-KDa subunit. In cDNA synthesis reactions, HIV-1 RT performs as well as MLV RT and AMV RT at 37°C and 42°C, but retains a significantly higher fraction of its activity at

50°C (<http://www.ambion.com/techlib/tn/111/12.html>). However, its use as a tool to copy mRNA has been limited by its relatively high error rate.⁸⁻¹¹ Previous reports have identified mutations in the HIV-1 RT that by themselves could increase the accuracy of the enzyme. Examples are F61A,¹² K65R,¹³ L74V,¹³⁻¹⁵ V75I,¹⁶ D76V,¹⁷ R78A,¹⁸ V148I,¹⁹ Q151N^{20,21} and M184I.²²⁻²⁴ These mutations were all introduced in the RT-coding region of wild-type strains classified as belonging to group M subtype B. However, there are no data on their thermal stabilities or their efficiency in RT-PCR reactions.

HIV-1 is characterized by extensive genetic heterogeneity. Due to this variability, HIV-1 variants are classified into three major phylogenetic groups: group M (main), group O (outlier) and group N (non-M/non-O).²⁵ HIV-1 group O infection is endemic to Cameroon and neighbouring countries in West Central Africa.²⁶ HIV-1 group O shares approximately 65% nucleotide sequence identity with other groups of HIV-1.²⁷ Amino acid sequence differences between the RTs of group M (subtype B) and group O HIV-1 variants can represent as much as 21% of the entire sequence.²⁸ Previously, we demonstrated that wild-type HIV-1 group O RT (O_WT RT) had increased stability in the presence of urea, in comparison with prototypic wild-type (WT) HIV-1 group M subtype B RT obtained from the BH10 strain (BH10_WT RT).²⁹

In this study, we report on the thermal stability and fidelity of DNA synthesis of O_WT RT. Thermal stabilities of O_WT, BH10_WT and MLV RTs have been compared by measuring the remaining RNA-dependent DNA polymerase activity after incubating the enzymes with template-primer at temperatures in the range of 37°C – 60°C. Moreover, RT efficiencies were assessed by using a two-step RT-PCR assay, where the cDNA synthesis reaction was carried out at different temperatures in the range of 42–70°C. The fidelity of DNA synthesis of O_WT RT has been determined by gel-based pre-steady-state kinetic assays and using the M13mp2 *lacZα* forward mutation assay, and compared with BH10_WT RT. In addition, we evaluated the effects of two amino acid substitutions, V75I and/or E478Q that

contributed to increase the RT's accuracy and its thermal stability in RT-PCRs, respectively. Our results support the utility of HIV-1 group O RT variants in the amplification of RNA targets.

Results

Thermal stability of HIV-1 RTs

High-level expression of heterodimeric variants of O_WT RT was achieved by using a previously described p66RTB plasmid,³⁰ containing the RT-coding region of the HIV-1 group O strain ESP49, which was coexpressed with the viral protease to generate functionally active RT heterodimers. Typical recoveries of 3-5 mg per liter of bacterial culture were >100-fold higher than those reported with other constructs,²⁹ and provided sufficient material for pre-steady-state kinetics and other applications described in this study.

The thermal stabilities of O_WT RT, BH10_WT RT and a commercially available MLV RT variant were compared by measuring the remaining RNA-dependent DNA polymerase activity after a 5-min incubation at different temperatures in the presence of template-primer ([Fig. 1](#)). BH10_WT and O_WT RTs showed increased activity in comparison with MLV RT after treatment at temperatures above 47°C. After heat-treatment at 52°C, both HIV-1 RTs retained 35-45% of their activity at 37°C, while the MLV RT activity remained below 10%. However, the O_WT RT was the only enzyme that showed significant activity after treatment at 54 or 56°C (approximately 33 and 14% of its maximum activity, respectively).

Temperature dependence of the efficiency of the RT-PCR reaction

RT performance at different temperatures was evaluated by using a two-step RT-PCR method including an initial step of cDNA synthesis at a fixed temperature. The amplification of a 1.2 Kb fragment of tubulin from mouse liver total RNA is shown in [Fig. 2](#). Maximum yields under our assay conditions were obtained with RT concentrations (active sites) in the range of 50 –

150 nM ([Fig. 3](#)). Both O_WT and BH10_WT RTs showed increased efficiency at higher temperatures in comparison with the MLV RT. Tubulin transcripts were not detected with MLV RT when the cDNA synthesis reactions were carried out at 57°C. However both HIV-1 RTs were able to synthesize cDNA at temperatures as high as 64°C. Evidence of the higher stability of O_WT RT was obtained from amplifications from reverse transcription reactions carried out at 64-69°C.

The improved performance of O_WT RT at higher temperatures was also confirmed by the amplification of actin transcripts of 0.5 and 0.95 Kb ([Fig. 4](#)). Efficient amplification was observed with O_WT RT when the cDNA synthesis reaction was carried out at 68°C. In contrast, amplified DNA remained undetectable in BH10_WT RT-catalyzed reactions at 68°C, and was significantly lower at 65°C when compared with O_WT RT-catalyzed reactions.

HIV-1 group O RTs carrying the amino acid substitutions V75I or E478Q showed decreased thermal stability in comparison with the O_WT RT, although a small amount of amplified DNA was detected at 68°C with both enzymes ([Fig. 4](#)). However, when the cDNA synthesis reactions were carried out at 65°C, the amount of amplified DNA (short or long fragments of actin DNA) were similar for both mutants and the BH10_WT RT. O_V75I RT appears to be somewhat less stable than O_E478Q RT, although the combination of both mutations (i.e. O_V75I/E478Q) rendered an enzyme with increased stability, albeit showing a slightly poorer performance than the O_WT RT in two-step RT-PCR reactions.

E478Q abrogates RNase H activity, thereby favoring RNA stability in the reaction. RNase H activity assays carried out with mutant and WT RTs showed that in the sequence context of HIV-1 group O RT, the enzymes containing the E478Q mutation were devoid of RNase H activity ([Fig. 5](#)). Both O_WT and O_V75I RTs had similar RNase H specific activities. Cleavage patterns were similar for all of the HIV-1 RTs containing an active RNase H (primary cleavages appeared at position +16 and in a lesser extent at position +15). In

contrast, MLV RT displayed a slightly different pattern, with major cleavages at position +18. Similar RNase H cleavage patterns have been previously reported for HIV-1 and MLV RTs.^{31,32} However, a comprehensive study using a large collection of RNA templates has demonstrated that the cleavage patterns of both RTs can be different depending on the RNA used in the assay, since the specificity of the 5'-end-directed cleavage by retroviral RNases H results from a combination of factors, including nucleotide sequence, permissible distance, and accessibility to the RNA 5'-end.³³

The amount of amplified actin cDNA in reactions carried out with mutants O_E478Q and O_V75I/E478Q was reduced in comparison with their O_WT and O_V75I counterparts. Although the substitution of Gln478 for Glu could affect template-primer binding, all of the enzymes were able to produce cDNA even in the presence of a small amount (e.g. 25 ng) of RNA ([Fig. 6](#)).

Misinsertion and mispair extension fidelity of O_WT and O_V75I RTs

Primer extension assays using deoxynucleotide templates and biased deoxyribonucleoside-triphosphate (dNTP) pools showed that both O_WT and BH10_WT RTs were able to synthesize longer DNA products than the mutant O_V75I RT, in the absence of a complementary dNTP (data not shown). These results were taken as preliminary evidence suggesting that O_V75I RT was more faithful than O_WT and BH10_WT RTs.

Misinsertion and mispair extension fidelity assays were then used to estimate the accuracy of DNA polymerization of HIV-1 RTs. Pre-steady-state kinetic parameters for the incorporation of nucleotides at the 3' end of the primer were obtained with a 31/21-mer heteropolymeric DNA-DNA template-primer. Misinsertion fidelity assays involved kinetics measurements for the incorporation of a correct (T) and an incorrect (C, G or A) nucleotide at the 3' end of the primer. Kinetic parameters, k_{pol} and K_d , and catalytic efficiencies (k_{pol}/K_d) of

misincorporation are given in [Table 1](#). All tested RTs misinserted 2'-deoxycytidine 5'-triphosphate (dCTP) and 2'-deoxyguanosine 5'-triphosphate (dGTP) with the highest efficiency, whereas 2'-deoxyadenosine 5'-triphosphate (dATP) was incorporated at a very low catalytic rate. O_WT and O_V75I RTs showed a 3- to 5-fold higher catalytic efficiency (k_{pol}/K_d) of incorporation of dCTP in comparison with dGTP. In contrast, BH10_WT RT showed a higher efficiency of incorporation of dGTP, which resulted in a 10-fold decrease in misinsertion fidelity for G opposite A. Differences between BH10_WT and O_WT RTs were non significant for dCTP *versus* 2'-deoxythymidine 5'-triphosphate (dTTP) and dATP *versus* dTTP incorporation. The amino acid substitution V75I increased the RT's accuracy in the sequence context of HIV-1 group O RT, by reducing the misinsertion efficiency of dATP *versus* dTTP and in a lesser extent dCTP *versus* dTTP.

The kinetics of mispair extension were studied for correctly matched base pairs (G:C) and for the mismatches G:T, G:G and G:A, using the template primer 31T/21P and its corresponding derivatives having primers 21PT, 21PG and 21PA, respectively. In all cases, we measured the incorporation of a correct T opposite A at the 3' end of the primer. The results are shown in [Table 2](#). As expected, all RTs showed higher mismatched extension ratios for G:T mispairs than for G:G or G:A mispairs. The differences between BH10_WT and O_WT RTs were relatively small, except for G:T mispair extensions. However, the mutant O_V75I RT showed large reductions in mismatch extension ratios with all tested mispairs. The increased mispair extension fidelity of O_V75I RT compared with the two other enzymes appears to be a consequence of its reduced rate of dTTP incorporation (k_{pol}) on mismatched template-primers, as well as a moderate increase in the nucleotide binding affinity (K_d) in the presence of a 3' end mispair. For most misinsertions and mispairs, BH10_WT and O_WT RTs showed similar nucleotide specificity ([Fig. 7](#)). However, the mutant O_V75I RT revealed itself as the most accurate for all tested nucleotide misincorporations and mispair extensions.

M13mp2 *lacZ* forward mutation assays

The mutation frequencies of O_WT and O_V75I RTs were determined with an M13-based forward mutation assay³⁴ and compared with those obtained with the BH10_WT RT.

Mutations generated when the RT copies the gapped region of the *lacZ* gene in M13mp2 can be scored by the number of plaques with altered color phenotype (pale blue or colorless) in a specific indicator strain. The mutant frequency is then calculated as the ratio of mutant to total plaques. The results shown in [Table 3](#) revealed that the O_WT RT was 2.5-fold more faithful than the BH10_WT RT. The mutant O_V75I RT showed 1.9- and 4.7-fold increased fidelity in comparison with O_WT RT and BH10_WT RT, respectively. Interestingly, after screening a smaller number of plaques (~5,400), we obtained a mutation frequency of 38.9×10^{-4} for the double mutant O_V75I/E478Q (data not shown), a value which was similar to the one obtained for the O_V75I RT.

DNA sequence analysis of the phage mutants generated by the individual RTs revealed significant differences in the distribution and specificity of the mutations induced ([Fig. 8](#)).

Mutations generated by O_WT and O_V75I RTs were distributed throughout the target *lacZ* sequence with significant clustering at three major hot spots located next to runs of Ts, Gs and Cs, at positions -36 to -34, +88 to +90 and +166 to +168, respectively. The mutational spectrum of both HIV-1 group O RTs was very similar, but quite different to that obtained with the BH10_WT RT ([Table 4](#)). HIV-1 group O RTs generated only base substitution mutations ($P < 0.0001$ for both enzymes *versus* BH10_WT RT). However, the BH10_WT RT generated base substitution mutations (63 %), as well as a significant proportion of frameshift mutations (37 %). Frameshift mutations detected included one-nucleotide insertions (17 %) and one-nucleotide deletions (78 %), all of them located at nucleotide runs. Interestingly, base substitution hot spots at positions +87 and +165 were not detected in assays carried out with BH10_WT RT.

Discussion

Coupled RT-PCR amplification is the most sensitive technique for RNA detection and quantitation currently available. The reduced efficiency of the reverse transcription reaction at the high temperatures required for melting the RNA secondary structures, together with the low fidelity of DNA synthesis of RTs and DNA-dependent DNA polymerases used in the reaction interfere with reliable and efficient DNA synthesis from RNA. Thermal stability of MLV RT has been markedly improved by eliminating its RNase H activity.³⁵⁻³⁷ As a result, the temperatures of the reverse transcription reaction could be increased from 37 – 45°C to 50 – 60°C.³⁶ In this study, we have demonstrated that these higher temperatures are optimal for reverse transcription reactions catalyzed by either BH10_WT RT or O_WT RT. Furthermore, we were able to detect activity at temperatures as high as 64°C for BH10_WT RT and 68°C for O_WT RT in RT-PCR amplifications of actin and tubulin RNAs. Despite being moderate, this increase could still be important to alleviate the problem of pausing by facilitating the unwinding of a fraction of RNA duplexes.

The increased stability of the HIV-1 RTs could be related to the stabilizing effect of the template-primer, as shown for MLV and AMV RTs.⁴ This hypothesis is supported by the low dissociation constants of HIV-1 RT to different template-primers (<10 nM),^{38,39} and by the increased thermostability of an MLV RT containing mutations that promote tighter binding to the template-primer.⁴⁰

Despite showing remarkable thermostability, HIV-1 RTs (group M:subtype B) are about 10-fold less accurate than MLV and AMV RTs, as determined in base substitution and forward mutation assays.^{8,9,41,42} The higher inaccuracy of HIV-1 RT has been attributed to its strong tendency to produce -1 frameshifts which can represent as much as 60% of the total number of errors found in these assays, while these errors represent 30% or less than 15%, in reactions carried out with MLV RT and AMV RT, respectively.^{8,9,43} Interestingly, our results show that

the HIV-1 group O wild-type RT is about 2-fold more accurate than the subtype B BH10_WT enzyme in M13 *lacZ* α forward mutation assays. In addition, we did not detect any deletions or insertions when the mutant spectrum of the O_WT RT was analyzed. All identified errors made by O_WT RT were base substitutions that occurred mainly at the vicinity of nucleotide runs, in a sequence context that could favor template-primer slippage.⁴⁴ Based on the local nucleotide sequences at the three DNA-templated substitution hot spots, we suggest that these errors result from a dislocation mechanism, wherein a substitution is initiated by template-primer slippage.⁴⁴ The absence of frameshift error hot spots suggests that misaligned intermediates are formed and/or used more frequently by the O_WT RT than by the BH10_WT RT.

Fidelity measurements based on kinetics of nucleotide incorporation showed that all HIV-1 RTs were proficient in extending mismatched template-primers, particularly G:T mispairs. Differences between O_WT and BH10_WT RTs were relatively small, although we observed that the incorporation rate (k_{pol}) of dTTP at the 3' end of a G:T mispair was about three times higher for the group O enzyme ([Fig. 7](#)). Although extension of a G:T mispair is required for the generation of T $\rightarrow$ C substitutions at the mutational hot spot located at position -36, other factors including the local sequence context might be also responsible for the observed differences. Thus, the mutant O_V75I RT shows similar incorporation rates with G:T mispairs in comparison with the BH10_WT RT, despite having a rather different mutational spectrum in *lacZ*.

The substitution of Ile for Val75 in the HIV-1 group O RT led to a two-fold reduction in the error rate determined by the forward mutation assay. The increased fidelity of O_V75I RT was also confirmed by the reduced misinsertion ratios of C and A opposite T, and by its lower mispair extension efficiencies on all tested mismatches in comparison with the O_WT RT. These results were consistent with those previously reported for V75I in the sequence context

of a subtype B BH10 RT.¹⁶ Although the similar effects of V75I on the fidelity of RTs derived from highly divergent HIV-1 strains are not unexpected, there are examples of amino acid substitutions introduced at equivalent positions of HIV-1 and HIV-2 RTs that do not have the same impact on fidelity of DNA synthesis. For example, in HIV-1 RT, L74V reduces the efficiency of misinsertion and mispair extension on several mismatches,¹⁴ but it does not affect misinsertion and mispair extension ratios when introduced into HIV-2 RT.⁴⁵ Similar differences have been reported for E89G that produced a significant increase in the fidelity of HIV-1 RT, as measured in gel-based kinetic assays,^{14,46,47} but had a minimal effect when introduced in HIV-2 RT.⁴⁵

The V75I substitution in the HIV-1 group O RT rendered an enzyme that was 4.5-fold more faithful than the prototype HIV-1 BH10 RT. Despite being more error-prone than MLV RT, its fidelity in RT-PCR amplifications could be probably increased by adding 3'-5' exonucleases.⁴⁸ In addition, it has been suggested that higher temperatures enhance the accuracy and processivity of MLV and HIV-1 RTs,⁴⁹ although these effects may not occur with all mutants and enzymes.⁵⁰ In any case, reliable assessments of the fidelity of currently available RTs, using RNA templates and high temperatures should be performed in order to validate the assumptions on the superiority of MLV and AMV RTs over HIV-1 RTs in RT-PCR amplification reactions.

O_V75I RT was found to be less thermostable than the O_WT RT and showed reduced activity at 65°C. RT-PCR amplification products were hardly detectable with O_V75I RT when the cDNA synthesis reaction was carried out at 68°C. Despite this reduction in thermal stability, under our assay conditions, the O_V75I RT was at least as efficient as the BH10_WT RT in detecting actin RNA. Eliminating RNase H activity by introducing the E478Q mutation did not result in a measurable increase of stability of the O_WT RT. However, it improved the efficiency of RT-PCR amplifications carried out with the V75I mutant. The double-mutant

O_V75I/E478Q RT, which was almost as faithful as the O_V75I RT, was able to reverse transcribe at temperatures as high as 68°C and reveals itself as an enzyme with an excellent potential for development into a useful tool for mRNA detection.

Materials and Methods

HIV-1 RT expression and purification

Recombinant heterodimeric HIV-1 RT (strain BH10) was expressed and purified using a modified version of plasmid p66RTB, as previously described.^{30,51} For high-level expression of wild-type HIV-1 group O (O_WT) RT, an insert derived from cleavage with EcoRI and XhoI of plasmid pRT6, and containing the RT-coding sequence of the ESP49 variant,²⁹ was cloned at the corresponding sites of p66RTB(SS).⁵¹ By using this system, p66 subunits carrying a His₆ tag at their C-terminus were co-expressed with the HIV-1 protease using the *E. coli* XL1 Blue strain. The resulting p66/p51 heterodimers were purified to homogeneity by ionic exchange on cellulose phosphate P11 (Whatman), followed by affinity chromatography on Ni²⁺-nitriloacetic-agarose (ProBondTM resin, Invitrogen).³⁰ Purity of enzymes was assessed by SDS-polyacrylamide gel electrophoresis. RT concentrations were determined spectrophotometrically by assuming a molar extinction coefficient at 280 nm of $2.6 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. RT active site titration was carried out as previously described.⁵²

Mutagenesis

Site-directed mutagenesis was carried out with the Quik-Change Site-Directed Mutagenesis kit (Stratagene) by following the manufacturer's instructions, using plasmid p66RTB(O_WT) as template and the following mutagenic primers: 5'-

AAGTGGAGAAAGCTGATAGACTTTAGGGAATTAAAC-3' and 5'-

GTTTAATTCCCTAAAGTCTATCAGCTTTCTCCACTT-3' for V75I, and 5'-

CCAATCAAAAGGCTCAATTAATGGCAG-3' y 5'-

CTGCCATTAATTGAGCCTTTTGATTGG-3' for E478Q. After mutagenesis, the entire RT-coding regions were sequenced, and if correct, used for RT expression and purification.

DNA polymerase activity assays

Assays were carried out in 50 mM Tris-HCl (pH 8.0), 20 mM NaCl, 10 mM MgCl₂, 8 mM dithiothreitol, 50 μM [³H]dTTP (6 – 8 μCi/ml; 120 – 160 Ci/mol) (Perkin Elmer), and 1 μM template-primer (concentration expressed as 3'-hydroxyl primer termini). Template-primer solutions of poly(rA)/oligo(dT)₁₆ were prepared as previously described.²⁸ Reactions (30 μl) were initiated by the addition of 0.2 – 1 pmol of enzyme, incubated at 37°C for 0 – 10 min, and terminated by adding 20 μl of 0.5 M EDTA. The amount of polymerized deoxynucleotide was determined by acid-insoluble precipitation.⁵³ Thermostability of RTs was assessed by measuring the RNA-dependent DNA polymerase activity after pre-incubating the enzyme with the template primer for 5 min at different temperatures in the range of 37°C – 60°C.

Two-step RT-PCR assays

The RNA amplification efficiency of retroviral RTs was determined by using a two-step RT-PCR assay. Reverse transcription reactions (20 μl) were carried out in 50 mM Tris-HCl (pH 8.3), containing 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 1 U/μl of RNase inhibitor (RNasin® Plus, Promega), 500 μM each of dATP, dCTP, dGTP, and dTTP, 5 μM oligo(dT)₁₆, 50 ng/μl of mouse liver RNA (Stratagene), and the corresponding RT at 150 nM (active sites). MLV RT was obtained from Promega. RNA template and oligo(dT)₁₆ were annealed by preincubation at 68°C for 3 min, followed by cooling at room temperature. All cDNA synthesis reactions were incubated at different temperatures (within a 37-70°C range) for 60 min, and then stopped by heating at 92°C for 10 min. Two microliters of each cDNA synthesis reaction were used in a 50-μl PCR containing 200 μM each dNTP, 200 ng each PCR primer, and 1.25

units of GoTaq® DNA polymerase (Promega) or 1.75 units of Expand High Fidelity DNA polymerase mix (Roche) in each enzyme's respective buffer. The PCR primers used in these assays were: 5'-CCTAGGCACCAGGGTGTGAT-3' (ACT1), 5'-CGGTCAGGATCTTCATGAGG-3' (ACT2), 5'-CGTACTCCTGCTTGCTGATCC-3' (ACT3), 5'-CTTCAGTGAGACAGGAGCTG-3' (TUB1), and 5'-CCACAGAATCCACACCAACC-3' (TUB2). Cycling conditions for all targets were as follows: an incubation at 95°C for 2 min; 30 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min; and a final incubation at 72°C for 10 min.

RNase H assays

A 31 nucleotide RNA (31T-RNA; 5'-UUUUUUUUUAGGAUACAUAUGGUUAAAGUAU-3') was labeled at its 5'-terminus with [γ -³²P]ATP (Perkin Elmer) and T4 polynucleotide kinase (Promega) and purified with a mini Quick Spin™ column (Roche). The ³²P-labeled RNA was then annealed to a 21-nucleotide DNA primer (21P; 5'-ATACTTTAACCATATGTATCC-3') in 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl. The primer was added in excess over the template, and the template-primer was prepared at a final concentration of 500 nM. Samples were incubated at 95°C for 4 min, and then cooled slowly to room temperature.

RNase H assays were carried out for 0 to 8 min at 37°C with 100 nM RT (active sites concentration), in 40 μ l of 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, and 50 nM ³²P-labeled template-primer (31T-RNA/21P). Reactions were initiated by adding the enzyme (1 μ l of a concentrated solution) and MgCl₂. Aliquots were removed after defined incubation periods, quenched with sample loading buffer [10 mM EDTA in 90% formamide containing 3 mg/ml xylene cyanol FF and 3 mg/ml bromophenol blue], and analyzed by denaturing polyacrylamide gel electrophoresis, as previously described.⁵¹

Pre-steady-state kinetic assays

The DNA-dependent DNA replication fidelities of BH10_WT, O_WT and mutant O_V75I RTs were studied by pre-steady-state kinetics. Kinetic parameters for the incorporation of correct or incorrect nucleotides were determined as previously described,^{16,52} using 5'-³²P-labeled 21P and 31T (5'- TTTTTTTTTTAGGATACATATGGTTAAAGTAT-3'), as primer and template, respectively. For mispair extension fidelity assays,⁵⁴ three additional primers were used: 21PT, 21PG and 21PA. All of them were identical with 21P, but had T, G, or A (instead of C) at their 3' terminus. Misincorporation and mispair extension reactions were carried out in 50 mM Tris-HCl (pH 8.0), containing 50 mM KCl and 12-24 mM MgCl₂. The concentration of template-primer 31T/21P in the reaction was 100 nM, and the RT was supplied at 50 nM (active sites), except for reactions involving the incorporation of incorrect dNTPs and mispair extensions (i.e., the incorporation of dCTP, dGTP and dATP on 31T/21P, or the extension of G:T, G:G and G:A mispairs). In these cases, the enzyme was supplied at 120 nM to eliminate the influence of the enzyme turnover rate (k_{ss}), which interferes in the measurements of low incorporation rates.

M13mp2 *lacZα* forward mutation assays

Mutation frequencies for wild-type and mutant HIV-1 RTs were determined as previously described.^{16,34} Briefly, M13mp2 DNA containing a single-stranded gap of 361 nucleotides (50 ng) was incubated with RT (100 nM) and the four dNTPs (250 μM each) in 10 μl of 25 mM Tris-HCl (pH 8.0) buffer containing 100 mM KCl, 2 mM dithiothreitol and 4 mM MgCl₂. Reactions were incubated for 30 min at 37°C and then stopped by adding 1 μl of 60 mM EDTA. The extended gapped DNAs were transformed to *E. coli* MC1061 cells, and the transformed cells were plated to M9 plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-1-thio-β-D-galactopyranoside with CSH50 lawn cells. The mutant frequency was determined as the ratio of mutant (pale blue and clear) plaques to mutant plus wild-type (dark blue) plaques as described.³⁴ Silent mutations are not detected in this assay. However,

M13mp2 *lacZ* α forward mutation assays score for phenotypically detectable nucleotide changes at 125 different sites, as well as additions or deletions of one nucleotide at 199 different positions,³⁴ thereby providing a fidelity assessment based on a relatively large number of mutational target sites.

Mutant plaques were picked from the plates and stored in 0.5 ml of 0.9% saline at -20°C. All mutants identified in the initial screen were confirmed by picking, resuspending and replating with an equivalent amount of wild-type phage as described above. Single mutant plaques were taken and multiplied in *E. coli* NR9099. Mutations were identified by DNA sequence analysis of the *lacZ* α gene in the replicative form DNA, using an automatic fluorescent sequencer (Macrogen Inc., Seoul, Korea).

Error frequencies were calculated as described by Bebenek and Kunkel.³⁴ Briefly, the mutational frequency is corrected by subtracting background mutational frequency. Specific error rates were derived by multiplying the corrected overall error frequency with the percentage of all mutations represented by the particular class of mutations (e.g. base substitutions). This number is divided by 0.6 (the likelihood of expression of the newly synthesized strand in *E. coli*), then divided by the total number of sites where this class of mutations can be detected. Comparative statistical analysis of hotspot error rates and the proportion of mutations of a particular class were assessed using Fisher's exact test.

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References

1. Telesnitsky, A. & Goff, S. P. (1997). Reverse transcriptase and the generation of retroviral DNA. In *Retroviruses* (Coffin, J. M., Hughes, S. H. & Varmus, H. E., eds), pp. 121-160, Cold Spring Harbor Laboratory Press, Plainview, NY.
2. Côté, M. L. & Roth, M. J. (2008). Murine leukemia virus reverse transcriptase: Structural comparison with HIV-1 reverse transcriptase. *Virus Res.*, **134**, 186-202.
3. Hizi, A. & Herschhorn, A. (2008). Retroviral reverse transcriptases (other than those of HIV-1 and murine leukemia virus): A comparison of their molecular and biochemical properties. *Virus Res.*, **134**, 186-202.
4. Gerard, G. F., Potter, R. J., Smith, M. D., Rosenthal, K., Dhariwal, G., Lee, J. & Chatterjee, D. K. (2002). The role of template-primer in protection of reverse transcriptase from thermal inactivation. *Nucleic Acids Res.*, **30**, 3118-3129.
5. Yasukawa, K., Nemoto, D. & Inouye, K. (2008). Comparison of the thermal stabilities of reverse transcriptases from avian myeloblastosis virus and Moloney murine leukaemia virus. *J. Biolchem. (Tokyo)*, **143**, 261-268.
6. Cirino, N. M., Cameron, C. E., Smith, J. S., Rausch, J. W., Roth, M. J., Benkovic, S. J. & Le Grice, S. F. J. (1995). Divalent cation modulation of the ribonuclease functions of human immunodeficiency virus reverse transcriptase. *Biochemistry*, **34**, 9936-9943.
7. Smith, C. M., Smith, J. S. & Roth, M. J. (1999). RNase H requirements for the second strand transfer reaction of human immunodeficiency virus type 1 reverse transcription. *J. Virol.*, **73**, 6573-6581.
8. Roberts, J. D., Bebenek, K. & Kunkel, T. A. (1988). The accuracy of reverse transcriptase from HIV-1. *Science*, **242**, 1171-1173.

9. Roberts, J. D., Preston, B. D., Johnston, L. A., Soni, A., Loeb, L. A. & Kunkel, T. A. (1989). Fidelity of two retroviral reverse transcriptases during DNA-dependent DNA synthesis in vitro. *Mol. Cell. Biol.*, **9**, 469-476.
10. Menéndez-Arias, L. (2002). Molecular basis of fidelity of DNA synthesis and nucleotide specificity of retroviral reverse transcriptases. *Prog. Nucleic Acid Res. Mol. Biol.*, **71**, 91-147.
11. Rezende, L. F. & Prasad, V. R. (2004). Nucleoside-analog resistance mutations in HIV-1 reverse transcriptase and their influence on polymerase fidelity and viral mutation rates. *Int. J. Biochem. Cell Biol.*, **36**, 1716-1734.
12. Fisher, T. S. & Prasad, V. R. (2002). Substitutions of Phe⁶¹ located in the vicinity of template 5'-overhang influence polymerase fidelity and nucleoside analog sensitivity of HIV-1 reverse transcriptase. *J. Biol. Chem.*, **277**, 22345-22352.
13. Shah, F. S., Curr, K. A., Hamburgh, M. E., Parniak, M., Mitsuya, H., Arnez, J. G. & Prasad, V. R. (2000). Differential influence of nucleoside analog-resistance mutations K65R and L74V on the overall mutation rate and error specificity of human immunodeficiency virus type 1 reverse transcriptase. *J. Biol. Chem.*, **275**, 27037-27044.
14. Rubinek, T., Bakhanashvili, M., Taube, R., Avidan, O. & Hizi, A. (1997). The fidelity of 3' misinsertion and mispair extension during DNA synthesis exhibited by two drug-resistant mutants of the reverse transcriptase of human immunodeficiency virus type 1 with Leu74→Val and Glu89→Gly. *Eur. J. Biochem.*, **247**, 238-247.
15. Jonckheere, H., De Clercq, E. & Anné, J. (2000). Fidelity analysis of HIV-1 reverse transcriptase mutants with an altered amino-acid sequence at residues Leu74, Glu89, Tyr115, Tyr183 and Met184. *Eur. J. Biochem.*, **267**, 2658-2665.

16. Matamoros, T., Kim, B. & Menéndez-Arias, L. (2008). Mechanistic insights into the role of Val75 of HIV-1 reverse transcriptase in misinsertion and mispair extension fidelity of DNA synthesis. *J. Mol. Biol.*, **375**, 1234-1248.
17. Kim, B., Hathaway, T. R. & Loeb, L. A. (1998). Fidelity of mutant HIV-1 reverse transcriptases: interaction with the single-stranded template influences the accuracy of DNA synthesis. *Biochemistry*, **37**, 5831-5839.
18. Kim, B., Ayran, J. C., Sagar, S. G., Adman, E. T., Fuller, S. M., Tran, N. H. & Horrigan, J. (1999). New human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) mutants with increased fidelity of DNA synthesis. *J. Biol. Chem.* **274**, 27666-27673.
19. Weiss, K. K., Chen, R., Skasko, M., Reynolds, H. M., Lee, K., Bambara, R. A., Mansky, L. M. & Kim, B. (2004). A role for dNTP binding of human immunodeficiency virus type 1 reverse transcriptase in viral mutagenesis. *Biochemistry*, **43**, 4490-4500.
20. Weiss, K. K., Isaacs, S. J., Tran, N. H., Adman, E. T. & Kim, B. (2000). Molecular architecture of the mutagenic active site of human immunodeficiency virus type 1 reverse transcriptase: roles of the β 8- α E loop in fidelity, processivity, and substrate interactions. *Biochemistry*, **39**, 10684-10694.
21. Weiss, K. K., Bambara, R. A., Kim, B. (2002). Mechanistic role of residue Gln¹⁵¹ in error prone DNA synthesis by human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). Pre-steady state kinetic study of the Q151N HIV-1 RT mutant with increased fidelity. *J. Biol. Chem.*, **277**, 22662-22669.
22. Hsu, M., Inouye, P., Rezende, L., Richard, N., Li, Z., Prasad, V. R. & Wainberg, M. A. (1997). Higher fidelity of RNA-dependent DNA mispair extension by M184V drug-resistant than wild-type reverse transcriptase of human immunodeficiency virus type 1. *Nucleic Acids Res.*, **25**, 4532-4536.

23. Oude Essink, B. B., Back, N. K. T. & Berkhout, B. (1997). Increased polymerase fidelity of the 3TC-resistant variants of HIV-1 reverse transcriptase. *Nucleic Acids Res.*, **25**, 3212-3217.
24. Rezende, L. F., Drosopoulos, W. C. & Prasad, V. R. (1998). The influence of 3TC resistance mutation M184I on the fidelity and error specificity of human immunodeficiency virus type 1 reverse transcriptase. *Nucleic Acids Res.*, **26**, 3066-3072.
25. Buonaguro, L., Tornesello, M. L. & Buonaguro, F. M. (2007). Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: Pathogenic and therapeutic implications. *J. Virol.*, **81**, 10209-10219.
26. Archer, J. & Robertson, D. L. (2007). Understanding the diversification of HIV-1 groups M and O. *AIDS*, **21**, 1693-1700.
27. Gürtler, L. G., Hauser, P. H., Eberle, J., von Brunn, A., Knapp, S., Zekeng, L., Tsague, J. M. & Kaptué, L. (1994). A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. *J. Virol.*, **68**, 1581-1585.
28. Quiñones-Mateu, M. E., Soriano, V., Domingo, E. & Menéndez-Arias, L. (1997). Characterization of the reverse transcriptase of a human immunodeficiency virus type 1 group O isolate. *Virology*, **236**, 364-373.
29. Menéndez-Arias, L., Abraha, A., Quiñones-Mateu, M. E., Mas, A., Camarasa, M.-J. & Arts, E. J. (2001). Functional characterization of chimeric reverse transcriptases with polypeptide subunits of highly divergent HIV-1 group M and O strains. *J. Biol. Chem.*, **276**, 27470-27479.
30. Boretto, J., Longhi, S., Navarro, J.-M., Selmi, B., Sire, J. & Canard, B. (2001). An integrated system to study multiply substituted human immunodeficiency virus type 1 reverse transcriptase. *Anal. Biochem.*, **292**, 139-147.

31. Gao, H.-Q., Sarafianos, S. G., Arnold, E. & Hughes, S. H. (1999). Similarities and differences in the RNase H activities of human immunodeficiency virus type 1 reverse transcriptase and Moloney murine leukemia virus reverse transcriptase. *J. Mol. Biol.*, **294**, 1097–1113.
32. Avidan, O., Loya, S., Tönjes, R. R., Sevilya, Z. & Hizi, A. (2003). Expression and characterization of a recombinant novel reverse transcriptase of a porcine endogenous retrovirus. *Virology*, **307**, 341–357.
33. Schultz, S. J., Zhang, M. & Champoux, J. J. (2006). Sequence, distance, and accessibility are determinants of 5'-end-directed cleavages by retroviral RNases H. *J. Biol. Chem.*, **281**, 1943–1955.
34. Bebenek, K. & Kunkel, T. A. (1995). Analyzing fidelity of DNA polymerases. *Methods Enzymol.*, **262**, 217–232.
35. Kotewicz, M. L., Sampson, C. M., D'Alessio, J. M. & Gerard, G. F. (1988). Isolation of cloned Moloney murine leukemia virus reverse transcriptase lacking ribonuclease H activity. *Nucleic Acids Res.*, **16**, 265-277.
36. Gerard, G. F., Schmidt, B. J., Kotewicz, M. L. & Campbell, J. H. (1992). cDNA synthesis by Moloney murine leukemia virus RNase H-minus reverse transcriptase possessing full DNA polymerase activity. *Focus*, **14**, 91-93.
37. Gerard, G. F., Fox, D. K., Nathan, M. & D'Alessio, J. M. (1997). Reverse transcriptase. The use of cloned Moloney murine leukemia virus reverse transcriptase to synthesize DNA from RNA. *Mol. Biotechnol.*, **8**, 61-77.
38. Beard, W. A. & Wilson, S. H. (1993). Kinetic analysis of template-primer interactions with recombinant forms of HIV-1 reverse transcriptase. *Biochemistry*, **32**, 9745-9753.

39. Menéndez-Arias, L. (1998). Studies on the effects of truncating α -helix E' of p66 human immunodeficiency virus type 1 reverse transcriptase on template-primer binding and fidelity of DNA synthesis. *Biochemistry*, **37**, 16636–16644.
40. Arezi, B. & Hogrefe, H. (2009). Novel mutations in Moloney murine leukemia virus reverse transcriptase increase thermostability through tighter binding to template-primer. *Nucleic Acids Res.*, **37**, 473-481.
41. Boyer, J. C., Bebenek, K. & Kunkel, T. A. (1992). Unequal human immunodeficiency virus type 1 reverse transcriptase error rates with RNA and DNA templates. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 6919-6923.
42. Bebenek, K., Abbotts, J., Roberts, J. D., Wilson, S. H. & Kunkel, T. A. (1989). Specificity and mechanism of error-prone replication by human immunodeficiency virus-1 reverse transcriptase. *J. Biol. Chem.*, **264**, 16948-16956.
43. Bebenek, K. & Kunkel, T. A. (1993). The fidelity of retroviral reverse transcriptases. In *Reverse transcriptases* (Skalka, A. M. & Goff, S. P., eds), pp. 85-102, Cold Spring Harbor Laboratory Press, Plainview, NY.
44. Kunkel, T. A. & Bebenek, K. (2000). DNA replication fidelity. *Annu. Rev. Biochem.*, **69**, 497-529.
45. Taube, R., Avidan, O. & Hizi, A. (1997). The fidelity of misinsertion and mispair extension throughout DNA synthesis exhibited by mutants of the reverse transcriptase of human immunodeficiency virus type 2 resistant to nucleoside analogs. *Eur. J. Biochem.*, **250**, 106-114.
46. Drosopoulos, W. C. & Prasad, V. R. (1998). Increased misincorporation fidelity observed for nucleoside analog resistance mutations M184V and E89G in human immunodeficiency

- virus type 1 reverse transcriptase does not correlate with the overall error rate measured in vitro. *J. Virol.*, **72**, 4224-4230.
47. Hamburg, M. E., Drosopoulos, W. C. & Prasad, V. R. (1998). The influence of 3TC-resistance mutations E89G and M184V in the human immunodeficiency virus reverse transcriptase on mispair extension efficiency. *Nucleic Acids Res.*, **26**, 4389-4394.
 48. Arezi, B. & Hogrefe, H. H. (2007). *Escherichia coli* DNA polymerase III ϵ subunit increases Moloney murine leukemia virus reverse transcriptase fidelity and accuracy of RT-PCR procedures. *Anal. Biochem.*, **360**, 84-91.
 49. Malboeuf, C. M., Isaacs, S. J., Tran, N. H. & Kim, B. (2001). Thermal effects on reverse transcription: Improvement of accuracy and processivity in cDNA synthesis. *Biotechniques*, **30**, 1074-1084.
 50. Fiala, K. A., Sherrer, S. M., Brown, J. A. & Suo, Z. (2008). Mechanistic consequences of temperature on DNA polymerization catalyzed by a Y-family DNA polymerase. *Nucleic Acids Res.*, **36**, 1990-2001.
 51. Matamoros, T., Deval, J., Guerreiro, C., Mulard, L., Canard, B. & Menéndez-Arias, L. (2005). Suppression of multidrug-resistant HIV-1 reverse transcriptase primer unblocking activity by α -phosphate-modified thymidine analogues. *J. Mol. Biol.*, **349**, 451-463.
 52. Kati, W. M., Johnson, K. A., Jerva, L. F. & Anderson, K. S. (1992) Mechanism and fidelity of HIV reverse transcriptase. *J. Biol. Chem.*, **267**, 25988-25997.
 53. Martín-Hernández, A. M., Gutiérrez-Rivas, M., Domingo, E. & Menéndez-Arias, L. (1997). Mismatch extension fidelity of human immunodeficiency virus type 1 reverse transcriptases with amino acid substitutions affecting Tyr115. *Nucleic Acids Res.*, **25**, 1383-1389.

54. Zinnen, S., Hsieh, J.-C. & Modrich, P. (1994). Misincorporation and mispaired primer extension by human immunodeficiency virus reverse transcriptase. *J. Biol. Chem.*, **269**, 24195–24202.

LEGENDS TO FIGURES

Fig. 1. Thermal stability of MLV, BH10_WT and O_WT RTs. RNA-dependent DNA polymerization reactions were carried out at 37°C in the presence of 1 μ M poly(rA)/oligo(dT)₁₆ and 50 μ M [³H]dTTP. Relative dTTP incorporation rates were measured after pre-incubating the enzymes with the template-primer for 5 min at the indicated temperatures. For each RT, percent activity was normalized relative to the value obtained after pre-incubating the enzymes at 37°C (100%). The nucleotide incorporation rates obtained after pre-incubating the enzymes at 37°C were $1.16 \pm 0.4 \text{ s}^{-1}$ for MLV RT, $1.17 \pm 0.4 \text{ s}^{-1}$ for BH10_WT RT, and $0.72 \pm 0.28 \text{ s}^{-1}$ for O_WT RT.

Fig. 2. Effect of temperature on cDNA synthesis and two-step RT-PCR. All cDNA synthesis reactions were carried out for 60 min at the indicated temperatures with 1 μ g of mouse liver RNA, in the presence of RT at 150 nM (active enzyme concentration). Reactions were stopped by heating at 92°C for 10 min. Two microliters of the cDNA synthesis reaction and 1.75 units of Expand High Fidelity DNA polymerase mix were used in all amplifications. The 1.2-Kb fragment of tubulin DNA was amplified with primers TUB1 and TUB2. O stands for wild-type HIV-1 group O RT; B for wild-type HIV-1 group M subtype B (strain BH10) RT; and M, MLV RT. A control reaction is shown on lane c. Molecular weight markers are a HindIII digest of phage Φ 29 DNA.

Fig. 3. Effect of the RT concentration on the efficiency of the RT-PCR reaction. All cDNA synthesis reactions were carried out for 60 min at 47°C with 1 µg of mouse liver RNA, in the presence of RT at 5, 10, 20, 50, 150, 250 and 500 nM (active enzyme concentrations). Reactions were stopped by heating at 92°C for 10 min. Two microliters of the cDNA synthesis reactions and 1.25 units of GoTaq® DNA polymerase were used in all amplifications. The 1.2-Kb fragment of tubulin DNA was amplified with primers TUB1 and TUB2. Molecular weight markers are a HindIII digest of phage Φ29 DNA. C stands for control reactions carried out in the absence of primers.

Fig. 4. Effect of temperature on actin cDNA synthesis and two-step RT-PCR. All cDNA synthesis reactions were carried out for 60 min at the indicated temperatures with 1 µg of mouse liver RNA. RTs were supplied at 150 nM (active enzyme concentration). Reactions were stopped by heating at 92°C for 10 min. Two microliters of the cDNA synthesis reaction and 1.25 units of GoTaq® DNA polymerase were used in all amplifications. The 0.5-Kb fragment of actin DNA was amplified with primers ACT1 and ACT2 (left panels), while the 0.9-Kb fragment was amplified with ACT1 and ACT3 (right panels). RTs used in the cDNA synthesis reaction were: O_WT (lane 1), BH10_WT (lane 2), mutants O_V75I (lane 3), O_E478Q (lane 4) and O_V75I/E478Q (lane 5), and MLV (lane 6). Control reactions are shown on lane c. Molecular weight markers are a HindIII digest of phage Φ29 DNA.

Fig. 5. RNase H activity of wild-type and mutant RTs. Cleavage of a [³²P]RNA/DNA substrate (50 nM) containing a 31-nt template RNA and a 21-nt primer was carried out at 37°C in the presence of the corresponding RT at 100 nM (active enzyme concentration). Time points in the experiments were 0, 0.25, 1, 2, 4 and 8 min, respectively. The template-primer sequences

are shown below. Arrows indicate the cleavage sites at positions +16 and +18, rendering template products of 26 and 28 nucleotides respectively.

Fig. 6. Influence of the RNA concentration on the efficiency of cDNA synthesis and PCR amplification in reactions carried out with HIV-1 RTs bearing the E478Q mutation. All cDNA synthesis reactions were carried out for 60 min at 47°C with different amounts of mouse liver total RNA. RTs were supplied at 150 nM (active enzyme concentration). Reactions were stopped by heating at 92°C for 10 min. Two microliters of the cDNA synthesis reactions and 1.75 units of Expand High Fidelity DNA polymerase mix were used in all amplifications. (A) The 0.5-Kb fragment of actin DNA was amplified with primers ACT1 and ACT2. From left to right, the amounts of RNA used in the assays carried out with O_WT and O_E478Q RTs were 100 ng, 75 ng, 50 ng and 25 ng. (B) The 0.9-Kb fragment was amplified with ACT1 and ACT3. From left to right, the amounts of RNA used in the assays carried out with mutants O_V75I and O_V75I/E478Q RTs were 1 µg, 100 ng, 75 ng, 50 ng, 25 ng and 10 ng. Control reactions are shown on lane c. Molecular weight markers are a HindIII digest of phage Φ29 DNA.

Fig. 7. Comparison of the fidelity of DNA synthesis of BH10_WT RT, O_WT RT and the mutant O_V75I RT, as determined by pre-steady-state kinetics. (A) Misincorporation efficiencies for the dCTP, dGTP and dATP *versus* the correct nucleotide (dTTP). (B) Mispair extension efficiencies for G:T, G:G and G:A mismatches.

Fig. 8. Spectrum of mutations induced by BH10_WT RT and HIV-1 group O RTs (O_WT and mutant O_V75I). Base substitutions are indicated by the letter corresponding to the new base (in blue) above the template sequence of the *lacZα* target. Open upright red triangles below the

target sequence represent insertions of one nucleotide. Inverted red triangles indicate deletions of one nucleotide. An inverted green triangle represents a deletion of 94 nucleotides.

Abbreviations and footnotes

Abbreviations used: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; mRNA, messenger RNA; AMV, avian myeloblastosis virus; MLV, murine leukemia virus; RT-PCR, reverse transcription-polymerase chain reaction; dNTP, deoxyribonucleoside-triphosphate; dCTP, 2'-deoxycytidine 5'-triphosphate; dGTP, 2'-deoxyguanosine 5'-triphosphate; dATP, 2'-deoxyadenosine 5'-triphosphate; dTTP, 2'-deoxythymidine 5'-triphosphate.

Table 1. Pre-steady-state kinetic parameters for misincorporation

Enzyme	Nucleotide	k_{pol} (s ⁻¹)	K_d (μM)	k_{pol}/K_d (μM ⁻¹ s ⁻¹)	Misinsertion ratio f_{ins}^a
BH10_WT ^b	dTTP	11.6 ± 0.5	13.4 ± 1.9	0.863 ± 0.126	
	dCTP	0.291 ± 0.052	7645 ± 2959	(3.80 ± 1.62) × 10 ⁻⁵	4.40 × 10 ⁻⁵
	dGTP	0.289 ± 0.032	3535 ± 1099	(8.40 ± 2.84) × 10 ⁻⁵	9.73 × 10 ⁻⁵
	dATP	0.0024 ± 0.0002	559 ± 191	(4.29 ± 1.50) × 10 ⁻⁶	4.99 × 10 ⁻⁶
O_WT	dTTP	14.7 ± 1.0	11.8 ± 2.9	1.25 ± 0.32	
	dCTP	0.724 ± 0.104	7652 ± 2083	(9.46 ± 2.91) × 10 ⁻⁵	7.57 × 10 ⁻⁵
	dGTP	0.151 ± 0.027	11730 ± 3750	(1.29 ± 0.47) × 10 ⁻⁵	1.03 × 10 ⁻⁵
	dATP	0.0073 ± 0.0002	1485 ± 150	(4.92 ± 2.17) × 10 ⁻⁶	3.94 × 10 ⁻⁶
O_V75I	dTTP	13.9 ± 1.2	14.6 ± 4.3	0.955 ± 0.295	
	dCTP	0.268 ± 0.046	11023 ± 3792	(2.43 ± 0.93) × 10 ⁻⁵	2.54 × 10 ⁻⁵
	dGTP	0.063 ± 0.009	7481 ± 2307	(0.837 ± 0.287) × 10 ⁻⁵	0.87 × 10 ⁻⁵
	dATP	0.0022 ± 0.0002	2308 ± 720	(0.953 ± 0.310) × 10 ⁻⁶	1.00 × 10 ⁻⁶

The template-primer 31T/21P was used as the substrate. Data shown are the mean values ± standard deviation of a representative experiment. Each of the assays was performed independently at least twice.

^a $f_{\text{ins}} = [k_{\text{pol}}(\text{incorrect})/K_d(\text{incorrect})]/[k_{\text{pol}}(\text{correct})/K_d(\text{correct})]$, where incorrect nucleotides were dCTP, dGTP or dATP, while the correct nucleotide was dTTP.

^b Reported values for BH10_WT RT were taken from Matamoros *et al.*¹⁶.

Table 2

Table 2. Pre-steady-state kinetic parameters for mispair extension

Enzyme	Base pair at the 3' end ^a	k_{pol} (s ⁻¹)	K_{d} (μM)	$k_{\text{pol}}/K_{\text{d}}$ (μM ⁻¹ s ⁻¹)	Mismatch extension ratio f_{ext} ^b
BH10_WT ^c	G:C	11.6 ± 0.5	13.4 ± 1.9	0.863 ± 0.126	
	G:T	2.54 ± 0.13	2628 ± 252	(9.66 ± 1.04) × 10 ⁻⁴	1.12 × 10 ⁻³
	G:G	0.266 ± 0.020	1014 ± 188	(2.62 ± 0.52) × 10 ⁻⁴	3.05 × 10 ⁻⁴
	G:A	0.0158 ± 0.0009	5110 ± 755	(3.09 ± 0.49) × 10 ⁻⁶	3.59 × 10 ⁻⁶
O_WT	G:C	14.7 ± 1.0	11.8 ± 2.9	1.25 ± 0.32	
	G:T	7.6 ± 0.9	2638 ± 785	(2.88 ± 0.92) × 10 ⁻³	2.30 × 10 ⁻³
	G:G	0.559 ± 0.071	1132 ± 316	(4.94 ± 1.51) × 10 ⁻⁴	4.01 × 10 ⁻⁴
	G:A	0.021 ± 0.003	7817 ± 2334	(2.71 ± 0.89) × 10 ⁻⁶	2.17 × 10 ⁻⁶
O_V75I	G:C	13.95 ± 1.22	14.60 ± 4.33	0.955 ± 0.295	
	G:T	2.76 ± 0.21	4400 ± 789	(6.27 ± 1.22) × 10 ⁻⁴	6.56 × 10 ⁻⁴
	G:G	0.322 ± 0.007	2426 ± 178	(1.33 ± 0.10) × 10 ⁻⁴	1.39 × 10 ⁻⁴
	G:A	0.0068 ± 0.0008	12110 ± 2536	(5.60 ± 0.01) × 10 ⁻⁷	5.90 × 10 ⁻⁷

The template-primer 31T/21P was used as the substrate. Data shown are the mean values ± standard deviation of a representative experiment. Each of the assays was performed independently at least twice.

^a The first base corresponds to the template and the second to the primer.

^b $f_{\text{ext}} = [k_{\text{pol}}(\text{mismatched})/K_{\text{d}}(\text{mismatched})]/[k_{\text{pol}}(\text{matched})/K_{\text{d}}(\text{matched})]$.

^c Reported values for BH10_WT RT were taken from Matamoros *et al.*¹⁶.

Table 3. Accuracy of RT variants in M13mp2 *lacZα* forward mutation assays.

Enzyme	Total plaques	Mutant plaques	Mutant frequency ($\times 10^{-4}$)	Fidelity (fold increase)
BH10_WT ^a	6736	139	206.3	-
O_WT	7579	63	83.1	2.5
O_V75I	9894	43	43.4	4.7

As a control, nucleotide sequencing of the entire 361-base pair *lacZα* gene obtained from M13mp2 DNA isolated from 5 mutant colonies of each DNA synthesis reaction was performed. All colonies contained single mutations within the *lacZ* target that produced gene inactivation. All numbers were corrected for background mutation frequency of 5×10^{-4} . Four to six gap-filling reactions were performed for each enzyme in this study.

^a Reported values for BH10_WT RT were taken from Matamoros *et al.*¹⁶.

Table 4. Summary of error rates for BH10_WT RT, O_WT RT and the mutant O_V75I RT, for various classes of mutations.

Mutation type	BH10_WT		O_WT		O_V75I	
	No. of errors	Error rate	No. of errors	Error rate	No. of errors	Error rate
All classes	49	1/7369	49	1/18267	47	1/34976
Base substitutions	31	1/5760	49	1/9025	47	1/17281
	(20/11) ^a		(25/24)		(22/25)	
Frameshifts	18	1/11745	0	ND	0	ND
		(1/6270) ^b				

ND, not determined

^a For each enzyme, the ratio of transitions *versus* transversions found in the assays is indicated between parenthesis.

^b All frameshifts generated by BH10_WT RT were located at nucleotide runs, and the error rate for frameshifts in runs is shown between parenthesis.

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Figure 5

Figure 6
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Figure 7

SUPPLEMENTARY MATERIALS

MATERIALS AND METHODS

Extension of primers in the absence of one dNTP

The template-primer M54/3TRP (**S1**) was used to determine the extent of misincorporation in the absence of one dNTP. Synthetic DNA oligonucleotides M54 (5'-CCCATACAAA-AGGAAACATGGGAAACATGGTGGACAGAGTATTGGCAAGCCACA-3') and 3TRP (5'-TGTGGCTTGCCAATACTCTGT-C-3') were obtained from Life Technologies. Prior to the elongation reaction, 3TRP was labelled at its 5' terminus with [γ - 32 P]ATP and T4 polynucleotide kinase, and annealed to the template (M54) in a solution containing 150 mM NaCl and 150 mM magnesium acetate (**S2**). Then, the template-primer was diluted 10-fold in 500 mM Hepes (pH 7.0), containing 150 mM NaCl and 150 mM magnesium acetate. Assays were carried out for 0 to 60 min at 37°C with 12 nM RT, in 10 μ l of 50 mM Hepes (pH 7.0), 15 mM NaCl, 15 mM magnesium acetate, 130 mM KCH₃COO, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol, and dNTPs at a concentration of 500 μ M each (*: dGTP, dATP, dCTP, dTTP; -G: dATP, dCTP, dTTP; -A: dGTP, dCTP, dTTP; -T: dGTP, dATP, dCTP; and -C: dGTP, dATP, dTTP). The template-primer concentration in the assay was 30 nM. At the end of the incubation period, the reactions were stopped by adding 4 μ l of stop solution (10 mM EDTA in 90% formamide containing 3 mg/ml xylene cyanol FF and 3 mg/ml bromophenol blue) and incubated at 90° C for 10 min. Reaction products were separated in denaturing 20% polyacrylamide/8 M urea gels, and visualized by phosphorimaging with a BAS 1500 scanner (Fuji).

References:

- S1.-** Olivares,I., Gutiérrez-Rivas,M., López-Galíndez,C. and Menéndez-Arias,L. (2004) Tryptophan scanning mutagenesis of aromatic residues within the polymerase domain of HIV-1 reverse transcriptase: Critical role of Phe-130 for p51 function and second-site revertant restoring viral replication capacity. *Virology*, **324**, 400–411.
- S2.-** Martín-Hernández,A.M., Gutiérrez-Rivas,M., Domingo,E. and Menéndez-Arias,L. (1997) Mismatch extension fidelity of human immunodeficiency virus type 1 reverse transcriptases with amino acid substitutions affecting Tyr115. *Nucleic Acids Res.*, **25**, 1383–1389.

Figure S1. Primer extension by wild-type and mutant RTs in assays containing a heteropolymeric DNA template-primer (M54/3TRP) and lacking a complementary dNTP. Reactions were incubated for 15 and 60 min. Lanes marked with an asterisk indicate that all four nucleotides were included in the dNTP mix. The lanes marked with -G, -A, -C and -T represent the missing nucleotide from the dNTP mix in the respective set of experiments. P and F indicate the positions of the unextended primer and the fully-extended products, respectively.