Thermostable HIV-1 group O reverse transcriptase variants with the same fidelity as murine leukaemia virus reverse transcriptase

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INTRODUCTION

The HIV-1 RT (reverse transcriptase) is the enzyme responsible for the conversion of the viral genomic RNA into integration-competent double-stranded DNA [1]. The HIV-1 RT is a heterodimeric enzyme composed of subunits of 66 and 51 kDa, designated p66 and p51 respectively. It shares structural homology with other DNA polymerases, including common subdomains (i.e. fingers, palm and thumb) that in p66 form the nucleic-acid-binding cleft. Asp110, Asp185 and Asp186 define the RT DNA polymerase active site in the palm of p66, which also contains an RNase H domain located at its C-terminus [2].

Reverse transcription is error prone and contributes to the high genetic variability of HIV-1. Studies with purified HIV-1 RT have revealed an unusually high error rate while copying DNA or RNA templates (for reviews, see [3,4]). Although reported error rates for HIV-1 RT show relatively large variability, ranging from $6 \times 10^{-5}$ to $6.7 \times 10^{-4}$, it is widely assumed that oncoretroviral RTs (e.g. MLV (murine leukaemia virus) RT or avian myeloblastosis virus RT) are $\sim$10–15 times more faithful than lentiviral RTs, while showing higher efficiency in reverse transcription–PCR assays that included a cDNA synthesis step performed at a high temperature range (57–69°C) [25]. In forward-mutation assays, the WT HIV-1 group O RT showed 2.5-fold increased accuracy in comparison with the WT BH10 RT, and replacing Le$^\text{e}$ with a valine residue produced a small additional increase in fidelity [25]. In the present study, we have examined the effects of mutations K65R and R78A on the thermostability and fidelity of DNA synthesis of HIV-1 group O RTs in the presence or absence of V75I, while retaining significant DNA polymerase activity [11,16,26,27]. Our results show that mutations K65R and

Mutational studies with HIV-1 RT have shown that molecular determinants of nucleotide specificity and fidelity of DNA synthesis map within the p66 subunit, mostly at or in the vicinity of the dNTP-binding site (reviewed in [4]). Several amino acid substitutions in the HIV-1 RT (group M subtype B) have been shown to increase its intrinsic fidelity, as determined with the M13mp2 lacZ forward-mutation assay [9]. Examples are F61A [10], K65R [11], L74V [11–13], V75I [14], D76V [15], R78A [16], V148I [17], Q151N [18,19] and M184I [13,20–22].

Group O HIV-1 RTs differ in $\sim$21% of their amino acid sequence when compared with their homologous counterparts of subtype B, and contain amino acid substitutions that confer resistance to non-nucleoside RT inhibitors [23,24]. We have recently demonstrated that a WT (wild-type) HIV-1 group O RT variant (derived from the ESP49 clone) shows increased thermal stability in comparison with MLV RT and a prototypic HIV-1 group M subtype B RT (i.e. derived from the BH10 strain), while showing higher efficiency in reverse transcription–PCR assays that included a cDNA synthesis step performed at a high temperature range (57–69°C) [25]. In forward-mutation assays, the WT HIV-1 group O RT showed 2.5-fold increased accuracy in comparison with the WT BH10 RT, and replacing Le$^\text{e}$ with a valine residue produced a small additional increase in fidelity [25]. In the present study, we have examined the effects of mutations K65R and R78A on the thermostability and fidelity of DNA synthesis of HIV-1 group O RTs in the presence or absence of V75I. K65R and R78A were chosen as mutations that produced large increases in the fidelity of HIV-1 group M subtype B RTs, while retaining significant DNA polymerase activity [11,16,26,27]. Our results show that mutations K65R and

Abbreviations used: DTT, dithiothreitol; MLV, murine leukaemia virus; RT, reverse transcriptase; WT, wild-type.

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K65R/V75I do not affect the thermal stability of the enzyme, but increase its accuracy to similar levels as the MLV RT. Mechanistic insights into the role of both mutations in the fidelity of DNA synthesis were obtained from transient kinetic assays.

EXPERIMENTAL

Mutagenesis, expression and purification of recombinant RTs

Site-directed mutagenesis was carried out with the QuickChange® site-directed mutagenesis kit (Stratagene), as described in the manufacturer’s instructions and using the following mutagenic primers: 5′-CTTTGCTATATAAAAAAGGAAGTAGACTAAG-TGG-3′ and 5′-CCACTTGTACTTCTTCTTATAGCAAG-3′ for K65R, 5′-GCTGTTGAGACTTTTGCGGAAATTTAC-AAGAG-3′ and 5′-CTTTTGTTTTAATCCGCAAAGTCTACCAGC-3′ for R78A, and 5′-GCTGTGACATTTTGGCGAATTTAACAAAGAC-3′ and 5′-GGTCTTCTTTTTAATTTCCGGCAGTCTACAGC-3′ for the double mutant V75I/R78A. The plasmid p66RTB (O_WT) was used as a template in the mutagenesis reactions involving the specific primers for K65R, R78A and V75I/R78A [25]. The double mutant K65R/V75I was obtained with the K65R mutagenic primers and the template p66RTB plasmid containing the DNA that encodes the V75I mutant of HIV-1 group O RT [25]. After mutagenesis, the entire RT-coding regions were sequenced and, if correct, used for RT expression and purification.

Recombinant RTs were expressed and purified as previously described [25, 28, 29]. RTs were co-expressed with HIV-1 protease in Escherichia coli XL1 Blue to obtain p66/p51 heterodimers, which were later purified by ion exchange followed by affinity chromatography. The purity of the enzymes was assessed by SDS/PAGE. Enzymes were quantified by active-site titration before biochemical studies [30]. The MLV RT was obtained from Promega.

DNA polymerase activity assays

Assays were carried out in 50 mM Tris/HCl, pH 8.0, 20 mM NaCl, 10 mM MgCl₂, 8 mM DTT (dithiothreitol), 50 μM [³H]dTTP (6–8 μCi/ml; 120–160 Ci/mol) (PerkinElmer) and 1 μM template–primer [poly(rA)/oligo(dT)₁₆] (concentration expressed as 3 μM). For RT assays, 30 μl of enzyme and the template–primer for 5 min contained the enzyme and the template–primer for 5 min at different temperatures in the range 37–60°C containing the enzyme and the template–primer for 5 min at 37°C for 30 min and then stopped by adding 1 μl of 60 mM EDTA. Polymerization reactions were initiated by adding 30 μl of buffer containing [³H]dTTP. The final active RT concentrations in these assays were around 20 nM. At different times, aliquots (20 μl) were removed into 20 μl of 0.5 M EDTA and processed as described previously [31].

Reverse transcription–PCR assays

The effect of the temperature on the efficiency of the reverse transcription reaction catalysed by different RTs was determined by using a previously described two-step reverse transcription–PCR assay [25]. DNA amplifications were carried out with the Expand High Fidelity DNA polymerase mix (Roche). PCR primers used in these assays were: 5'-CCTAGGCACACGG-CAGAGG-3' (ACT1), 5'-CGTACTCTGCTTGTGATCC-3' (ACT3), 5'-CCTAGGAGAGAAGGCTG-3' (TUB1) and 5'-CCAGAGATCCACACACCC-3' (TUB2).

Pre-steady-state kinetic assays

Kinetic parameters for the incorporation of correct or incorrect nucleotides were determined as previously described [14, 25], using 5'-P-labelled 21P (5'-ATACCTTTAACCATTAGTATCC-3') and 31T (5'-TTTTTTTTAGTAGACTATGGTTAAAGTAT-3') as primer and template respectively. Three additional primers (21PT, 21PG and 21PA) that differ from 21P in having T, G or A (instead of C) at their 3’ terminus were used in mispair extension fidelity assays. Reactions were performed under single turnover conditions in a solution containing 50–100 nM (active sites) HIV-1 RT and a 100 nM concentration of template–primer 31T/21P, in RT buffer (50 mM Tris/HCl, pH 8.0, 50 mM KCl and 12–24 mM MgCl₂), and a variable concentration of nucleotide. Reactions involving the incorporation of incorrect nucleotides or mispair extension kinetics (i.e. incorporation of dCTP, dGTP or dATP on 31T/21P, or the extension of G:G or G:A mispairs) were conducted with an excess concentration of the enzyme (120 nM) over the template–primer duplex (100 nM). These conditions were chosen to eliminate the influence of the enzyme turnover rate (kᵥ), which interferes with the measurements of low incorporation rates.

M13mp2 lacZα forward-mutation assays

Gapped duplex M13mp2 DNA was prepared as described previously [9], and used as template–primer for DNA synthesis reactions using purified WT or mutant RTs. Gap-filling synthesis reactions were performed in a 10 μl reaction volume, containing 25 mM Tris/HCl, pH 8.0, 100 mM KCl, 2 mM DTT, 4 mM MgCl₂, 250 μM of each dNTP (dATP, dGTP, dCTP and dTTP), 5 μg/ml gapped duplex DNA and 100 nM RT [25]. The reactions were incubated at 37°C for 30 min and then stopped by adding 1 μl of 60 mM EDTA. Polymerization products were electrophorotyped into E. coli MC1061 host cells, and after a brief (10 min) recovery period, transformants were plated on to a bacterial indicator lawn (E. coli CSH50) in M9 plates containing 0.195 mM X-Gal (5-bromo-4-chloroindol-3-yl-β-D-galactopyranoside) and 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Mutant plaques were picked, their phenotype confirmed and the phage replicative-form DNA isolated for nucleotide sequencing using primer 5’-GCTTGTGCAGAATCTTCAGC-3’ (Macrogen).

Error frequencies were calculated as described previously [9]. At least ten fill-in reactions were performed for each enzyme. The nucleotide sequence of the entire gap region was determined for all mutant plaques. 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 was divided by 0.6 (the average probability of an error being expressed in the M13mp2 assay) [6] and by the total number of sites where this class of mutations could be detected (e.g. 125 for base substitutions and 148 for frameshifts).

RESULTS

Thermal stability of RTs

The residual RNA-dependent DNA polymerase activity of WT and mutant RTs obtained after pre-incubating the enzymes for...
Relative dTTP incorporation rates were measured after pre-incubating the enzymes with the template–primer for 5 min at the indicated temperatures. For each enzyme, percentage activity was normalized relative to the value obtained after pre-incubating the RTs at 37 °C (100%). Reported values for each enzyme were obtained from at least four independent experiments. The nucleotide incorporation rates obtained after pre-incubating the enzymes at 37 °C were 0.72 ± 0.28 s⁻¹ for O_WT RT, 0.92 ± 0.25 s⁻¹ for O_K65R RT, 0.61 ± 0.26 s⁻¹ for O_K65R/V75I RT, 0.45 ± 0.06 s⁻¹ for O_R78A RT, 0.25 ± 0.06 s⁻¹ for O_V75I/R78A RT, 0.37 ± 0.06 s⁻¹ for O_V75I RT, 1.17 ± 0.4 s⁻¹ for BH10_WT RT and 1.16 ± 0.4 s⁻¹ for MLV RT.

Figure 1  Thermal stability of RTs

RNA-dependent DNA polymerization reactions were carried out at 37 °C in the presence of 1 μM poly(rA)/oligo(dT)₆₈ and 50 μM [3H]dTTP. The active RT concentration in the assay was 15–20 nM. Relative dTTP incorporation rates were measured after pre-incubating the enzymes with the template–primer for 5 min at the indicated temperatures. For each enzyme, percentage activity was normalized relative to the value obtained after pre-incubating the RTs at 37 °C (100%). Reported values for each enzyme were obtained from at least four independent experiments. The nucleotide incorporation rates obtained after pre-incubating the enzymes at 37 °C were 0.72 ± 0.28 s⁻¹ for O_WT RT, 0.92 ± 0.25 s⁻¹ for O_K65R RT, 0.61 ± 0.26 s⁻¹ for O_K65R/V75I RT, 0.45 ± 0.06 s⁻¹ for O_R78A RT, 0.25 ± 0.06 s⁻¹ for O_V75I/R78A RT, 0.37 ± 0.06 s⁻¹ for O_V75I RT, 1.17 ± 0.4 s⁻¹ for BH10_WT RT and 1.16 ± 0.4 s⁻¹ for MLV RT.

Pre-steady-state kinetic analysis of thermostable HIV-1 group O RTs

Misinsertion and mispair extension fidelity assays were used to estimate the accuracy of DNA synthesis catalysed by HIV-1 group O RTs. The kinetic parameters [kcat (pol is polymerization) and Kd] for the incorporation of correct (dTTP) and incorrect nucleotides (dCTP, dGTP or dATP) are given in Table 1. For dTTP incorporation, the catalytic efficiencies (kcat/Kd) of studied RTs were in the range 0.55–1.82 μM⁻¹ s⁻¹, with O_R78A RT showing the lowest values. However, the kcat/Kd values for the incorporation of C, G or A by mutant O_K65R and O_K65R/V75I RTs were largely reduced.

The double mutant K65R/V75I showed the highest misinsertion fidelities for the incorporation of A or C opposite to A, although the differences with the single mutant K65R were relatively small (Table 1; see Supplementary Figure S2A at http://www.BiochemJ.org/bj/436/bj4360599add.htm). The kinetic analysis showed that K65R produces a larger reduction of the kcat for the incorporation of incorrect dNTPs in comparison with V75I. In comparison with the WT enzyme, the mutant O_R78A RT showed similar misinsertion fidelity for the incorporation of C, G or A by mutant O_K65R and O_K65R/V75I RTs were largely reduced.

The kinetics of mispair extension were determined by measuring the incorporation of a correct T opposite to A at the 3’ end of the primer, using template–primer duplexes containing matched (G:C) or mismatched (G:T, G:G or G:A) termini. The results are shown in Table 2. Mismatched extension ratios were in the range 0.24–3.95 × 10⁻³ for the G:T mispair, 0.18 × 10⁻⁴–3.95 × 10⁻⁴ for the G:G mispair and...
Figure 2  Effect of temperature on cDNA synthesis and two-step reverse transcription–PCR

The cDNA synthesis reactions were carried out for 60 min at the indicated temperatures, in a buffer containing 50 ng/μl of mouse liver RNA and 150 nM RT (active-site concentration). Reactions were stopped by heating at 92°C for 10 min. A 2 μl volume of the cDNA synthesis reaction and 1.75 units of Expand High Fidelity DNA polymerase mix were used in all amplifications. (A) Amplifications of a 0.9 kb fragment of actin DNA, obtained with primers ACT1 and ACT3. (B) Amplifications of a 1.2 kb fragment of tubulin DNA, obtained with primers TUB1 and TUB2. Lanes 1 and C show molecular-size markers (HindIII digest of phage κ-29 DNA) and a control reaction (carried out in the absence of RT) respectively. Lanes 2–9 show the result of two-step reverse transcription-PCRs that included a cDNA synthesis step, carried out with O_K65R RT (lane 2), O_K65R/V75I RT (lane 3), O_R78A RT (lane 4), O_V75I/R78A RT (lane 5), O_WT RT (lane 6), O_V75I RT (lane 7), BH10_WT RT (lane 8) and MLV RT (lane 9). Results are representative of three independent experiments.

lower than $2.2 \times 10^{-6}$ for the G:A mispair. By themselves, the mutations K65R and V75I produced a moderate increase in mispair extension fidelity, by rendering enzymes with 2.8–5.4-fold decreased mismatched extension ratios (Table 2 and Supplementary Figure S2B). However, the double mutant (O_K65R/V75I) showed 9.5- and 22.3-fold increases in mispair extension fidelity for G:T and G:G mismatches respectively, suggesting an additive effect of both mutations. In the case of G:T mismatches, the increased mispair extension fidelity of O_K65R/V75I RT can be attributed to a loss in nucleotide-binding affinity (a $K_d$ effect). However, in the case of G:G, the effects are due to the $k_{pol}$ reduction observed for the incorporation of T on the mismatched template–primer. Interestingly, the single mutant O_R78A RT was also highly accurate in mispair extension assays carried out with template–primers bearing G:T or G:G mismatches. As in the case of the O_K65R/V75I RT, discrimination efficiencies for G:T and G:G mispair extensions resulted from $K_d$ and $k_{pol}$ effects respectively.

M13mp2 lacZα forward-mutation assays

An M13mp2-based forward-mutation assay was used to analyse how amino acid substitutions in the HIV-1 group O RT could affect its intrinsic fidelity. Mutations generated when the RT copies the gapped region of the lacZ gene in M13mp2 can be scored by the number of plaques with an altered colour phenotype.
The template–primer 31T/21P was used as the substrate. Results shown are means ± S.D. Each of the assays was performed independently at least three times.

### Table 1  Pre-state kinetic parameters for misincorporation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Nucleotide</th>
<th>$k_{pol}$ (s⁻¹)</th>
<th>$K_i$ (μM)</th>
<th>$k_{pol}/K_i$ (μM⁻¹·s⁻¹)</th>
<th>Misinsertion ratio ($f_{mis}$)†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0_WT RT†</strong></td>
<td>dTTP</td>
<td>14.7 ± 1.0</td>
<td>11.8 ± 2.9</td>
<td>1.25 ± 0.32</td>
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<tr>
<td></td>
<td>dCTP</td>
<td>0.72 ± 0.10</td>
<td>7652 ± 2083</td>
<td>(9.5 ± 2.9) × 10⁻⁵</td>
<td>7.57 × 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>dGTP</td>
<td>0.15 ± 0.03</td>
<td>11730 ± 3750</td>
<td>(1.3 ± 0.5) × 10⁻⁵</td>
<td>1.03 × 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>dATP</td>
<td>(7.3 ± 0.2) × 10⁻³</td>
<td>1485 ± 150</td>
<td>(4.9 ± 2.2) × 10⁻⁶</td>
<td>3.94 × 10⁻⁶</td>
</tr>
<tr>
<td><strong>0_V75I RT†</strong></td>
<td>dTTP</td>
<td>13.9 ± 1.2</td>
<td>14.6 ± 4.3</td>
<td>0.96 ± 0.30</td>
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<tr>
<td></td>
<td>dCTP</td>
<td>0.27 ± 0.05</td>
<td>11029 ± 3792</td>
<td>(2.4 ± 0.9) × 10⁻⁵</td>
<td>2.54 × 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>dGTP</td>
<td>(6.3 ± 0.9) × 10⁻²</td>
<td>7481 ± 2307</td>
<td>(8.4 ± 2.9) × 10⁻⁶</td>
<td>8.76 × 10⁻⁶</td>
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<tr>
<td></td>
<td>dATP</td>
<td>(2.2 ± 0.2) × 10⁻³</td>
<td>2308 ± 720</td>
<td>(9.5 ± 3.1) × 10⁻⁷</td>
<td>9.98 × 10⁻⁷</td>
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<tr>
<td><strong>0_K65R RT</strong></td>
<td>dTTP</td>
<td>10.7 ± 0.9</td>
<td>5.8 ± 1.9</td>
<td>1.82 ± 0.60</td>
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</tr>
<tr>
<td></td>
<td>dCTP</td>
<td>0.17 ± 0.02</td>
<td>9559 ± 1908</td>
<td>(1.7 ± 0.4) × 10⁻⁵</td>
<td>9.51 × 10⁻⁶</td>
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<tr>
<td></td>
<td>dGTP</td>
<td>(3.1 ± 1.9) × 10⁻²</td>
<td>6452 ± 857</td>
<td>(4.8 ± 0.7) × 10⁻⁶</td>
<td>2.66 × 10⁻⁶</td>
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<tr>
<td></td>
<td>dATP</td>
<td>(4.8 ± 0.1) × 10⁻³</td>
<td>3680 ± 178</td>
<td>(1.3 ± 0.1) × 10⁻⁷</td>
<td>7.09 × 10⁻⁷</td>
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<tr>
<td><strong>0_K65R/V75I RT</strong></td>
<td>dTTP</td>
<td>12.7 ± 1.3</td>
<td>13.3 ± 3.7</td>
<td>0.95 ± 0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dCTP</td>
<td>(3.3 ± 0.3) × 10⁻³</td>
<td>5686 ± 905</td>
<td>(5.8 ± 1.1) × 10⁻⁶</td>
<td>6.06 × 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>dGTP</td>
<td>0.11 ± 0.02</td>
<td>13430 ± 4605</td>
<td>(8.3 ± 3.3) × 10⁻⁶</td>
<td>8.70 × 10⁻⁶</td>
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<td></td>
<td>dATP</td>
<td>(3.3 ± 0.2) × 10⁻³</td>
<td>5290 ± 553</td>
<td>(6.2 ± 0.7) × 10⁻⁷</td>
<td>6.52 × 10⁻⁷</td>
</tr>
<tr>
<td><strong>0_R78A RT</strong></td>
<td>dTTP</td>
<td>9.4 ± 1.1</td>
<td>17.2 ± 5.3</td>
<td>0.55 ± 0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dCTP</td>
<td>(1.6 ± 0.2) × 10⁻²</td>
<td>6939 ± 1740</td>
<td>(2.4 ± 0.7) × 10⁻⁶</td>
<td>4.27 × 10⁻⁶</td>
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<tr>
<td></td>
<td>dGTP</td>
<td>(6.2 ± 0.4) × 10⁻³</td>
<td>2313 ± 361</td>
<td>(2.7 ± 0.5) × 10⁻⁶</td>
<td>4.89 × 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>dATP</td>
<td>(1.9 ± 0.7) × 10⁻³</td>
<td>1122 ± 165</td>
<td>(1.7 ± 0.3) × 10⁻³</td>
<td>3.13 × 10⁻⁶</td>
</tr>
</tbody>
</table>

* $f_{mis} = (k_{pol}(incorrect)/K_i(incorrect))/(k_{pol}(correct)/K_i(correct))$, where incorrect nucleotides were dCTP, dGTP, or dATP, and the correct nucleotide was dTTP. Numbers in parentheses represent the relative increase of fidelity, as determined for each incorrect nucleotide as the ratio $f_{mis}(O\_WT\_RT)/f_{mis}(mutant)$.

†Reported values for O_WT RT and O_V75I RT were taken from [25].

(pale blue or colourless) in a specific indicator strain. Silent mutations are not detected in this assay. However, M13mp2 lacZα forward-mutation assays provide a fidelity assessment based on a relatively large number of mutational target sites [9]. In these assays, mutant RTs O_K65R, O_K65R/V75I and O_R78A were >9 times more faithful than the WT HIV-1 group O enzyme (Table 3). Moreover, their mutant frequencies were 1.5–2.3 times lower than those calculated for the MLV RT. Although V75I confers 1.7-fold increased fidelity when introduced in a WT HIV-1 group O RT sequence context, this amino acid substitution had no effect on the accuracy of the RT when the K65R mutation was present.
The mutational specificity of HIV-1 group O RTs bearing the amino acid substitutions K65R, K65R/V75I and R78A was determined after sequencing the lacZα mutants generated in the forward-mutation assays. Their mutational spectra (see Supplementary Figures S3–S5 at http://www.BiochemJ.org/bj/436/bj4360599add.htm) were compared with those obtained with WT HIV-1 group O RT and mutant V75I [25], as well as with the MLV RT (see Supplementary Figure S6 at http://www.BiochemJ.org/bj/436/bj4360599add.htm). Unlike the case of O_WT RT and mutant O_V75I RT, mutations generated by O_K65R, O_K65R/V75I and O_R78A RTs appeared to be scattered throughout the target lacZ sequence. A mutational hot spot located next to runs of Ts at positions 36 to 34, and involving mostly T→C substitutions was observed with mutant RTs O_K65R/V75I and O_R78A. The O_K65R RT showed different mutational hot spots, located at positions +66 (G→T substitutions), +148 (one-nucleotide deletions or G→T substitutions) and +151 (mostly G→C substitutions). Unlike the case of the WT enzyme (O_WT RT), frameshift errors represented 15.7–34.3 % of all errors, in the mutational spectra generated by the mutants K65R, K65R/V75I and R78A (Table 4). However, MLV RT had a higher propensity to introduce frameshift mutations, and showed frameshift error rates that were 3.5–11 times higher than those calculated for the three mutant HIV-1 group O RTs. These enzymes showed a remarkable tendency to generate one-nucleotide deletions, which in the case of the single mutants K65R and R78A were predominantly located at non-runs. In addition, mutant O_K65R RT and to a lesser extent O_R78A RT showed a stronger tendency to generate transversions instead of transitions, a property shared by the MLV RT.

**DISCUSSION**

Mutational studies carried out with HIV-1 group M subtype B RTs allowed the identification of amino acid substitutions that produce significant increases in fidelity of DNA synthesis [10–22]. However, most of those amino acid changes have a negative effect on the specific DNA polymerase activity. Thus substituting an alanine residue for Phe61 produces an 11.7-fold increase in fidelity [10] while decreasing strand-displacement DNA synthesis, processivity and template–primer binding [32,33]. On the other hand, mutant RTs with the amino acid substitutions V148I or Q151N showed 8.7–13.1-fold increased accuracy in comparison with the WT enzyme, although their catalytic efficiencies of dNTP incorporation were >23 times lower, as determined by using pre-steady-state kinetics [17,19]. Interestingly, K65R, V75I and R78A were previously identified as mutations that increased fidelity without impairing the DNA polymerase activity of HIV-1_R183 mutant frequencies were also reduced when two of those mutations (i.e. K65R and R78A) were introduced in the viral RT-coding region [34]. The interactions between the side chain of Lys65 and the γ-phosphate of the dNTP are important for the stabilization of the incoming nucleotide in the RT active site [35] (Figure 3). K65R confers resistance to dideoxynucleotide RT inhibitors and tenofovir, and this has been related to a reduction in the insertion rate (k

### Table 3 Accuracy of RT variants in M13mp2 lacZα forward-mutation assays

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Total number of plaques</th>
<th>Number of mutant plaques</th>
<th>Mutant frequency*</th>
<th>Fidelity relative to O_WT RT (fold change)</th>
<th>Fidelity relative to MLV RT (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O_WT RT</td>
<td>7579</td>
<td>63</td>
<td>0.00831</td>
<td>1</td>
<td>0.16</td>
</tr>
<tr>
<td>O_K65R RT</td>
<td>75474</td>
<td>58</td>
<td>0.00077</td>
<td>10.8</td>
<td>1</td>
</tr>
<tr>
<td>O_K65R/V75I RT</td>
<td>55657</td>
<td>50</td>
<td>0.00069</td>
<td>9.3</td>
<td>1.5</td>
</tr>
<tr>
<td>O_V75I RT</td>
<td>9894</td>
<td>47</td>
<td>0.00047</td>
<td>1.7</td>
<td>0.28</td>
</tr>
<tr>
<td>O_R78A RT</td>
<td>80664</td>
<td>48</td>
<td>0.00059</td>
<td>14.1</td>
<td>2.3</td>
</tr>
<tr>
<td>MLV RT</td>
<td>29446</td>
<td>40</td>
<td>0.00135</td>
<td>6.2</td>
<td>1</td>
</tr>
</tbody>
</table>

*Reported background frequencies in this assay (usually –6 × 10−4) [9,14] are in most cases a consequence of M13mp2 DNA rearrangements that result in the loss of the lacZα gene. In order to have a better estimate of the background due to errors introduced by E. coli polymerases while copying the lacZα sequence, phage DNA was obtained from all mutant plaques and the sequence of the reporter gene was determined in all cases. In control experiments involving a total of 22 189 plaques, no mutations in lacZα were identified after analysing the results obtained from 2–3 E. coli electroporations carried out with gapped M13mp2 DNA substrate.

†Reported values for O_WT RT and O_V75I RT were taken from [25].

### Table 4 Summary of error rates for RTs, for various classes of mutations

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>O_WT RT*</th>
<th>O_K65R RT</th>
<th>O_K65R/V75I RT</th>
<th>O_R78A RT</th>
<th>MLV RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of errors</td>
<td>Error rate</td>
<td>Number of errors</td>
<td>Error rate</td>
<td>Number of errors</td>
<td>Error rate</td>
</tr>
<tr>
<td>All classes</td>
<td>49</td>
<td>1/17166</td>
<td>67</td>
<td>1/160185 (9.3)†</td>
<td>50</td>
</tr>
<tr>
<td>Base substitutions</td>
<td>49</td>
<td>1/9053</td>
<td>44</td>
<td>1/128649 (14.2)</td>
<td>39</td>
</tr>
<tr>
<td>Transitions</td>
<td>25 (51 %)</td>
<td>8 (14.2 %)</td>
<td>25 (53.8 %)</td>
<td>18 (46.2 %)</td>
<td>19 (44.2 %)</td>
</tr>
<tr>
<td>Transversions</td>
<td>24 (49 %)</td>
<td>36 (81.8 %)</td>
<td>25 (53.8 %)</td>
<td>19 (44.2 %)</td>
<td>24 (55.8 %)</td>
</tr>
<tr>
<td>Frameshifts</td>
<td>0</td>
<td>&lt;1/541516</td>
<td>23</td>
<td>1/291395 (&lt;0.5)</td>
<td>11</td>
</tr>
<tr>
<td>Insertions</td>
<td>0</td>
<td>&lt;1/280371</td>
<td>21</td>
<td>1/248881 (8.7 %)</td>
<td>21</td>
</tr>
<tr>
<td>Deletions</td>
<td>0</td>
<td>&lt;1/280371</td>
<td>21</td>
<td>1/248881 (8.7 %)</td>
<td>21</td>
</tr>
</tbody>
</table>

*Reported values for O_WT RT were taken from [25].

Numbers between parentheses represent the accuracy of the RT for each mutation type (given as fold change of the error rate relative to the value obtained with O_WT RT).
Arg78 are shown in blue, magenta and orange respectively. (be a consequence of the structural constraint imposed on Arg72, sphere models. The incoming dNTP is shown in yellow. The side chains of Lys65, Val75 and co-ordinates from [35], PDB code 1RTD). The RT subunits p66 and p51 are represented by cyan consistent with the previously observed effects have been attributed in part to an incorrect positioning of (and Arg78 in the 66 kDa subunit of the enzyme dsDNA (double-stranded DNA) and dTTP showing the location of Lys65, Val75 and Arg78 in the 66 kDa subunit of HIV-1 RT interact with the template nucleotide at position + 1 (Figure 3). We have previously demonstrated that V75I produces a relatively modest increase in fidelity when introduced in HIV-1 RTs of groups M and O [14,25]. The mutational spectrum of the O_V75I RT was similar to that obtained with the WT enzyme [25]. Available evidence indicates that, when introduced in HIV-1 

![Image](43x440 to 287x721)

Figure 3 Crystal structures of the ternary complex of HIV-1 RT bound to dsDNA (double-stranded DNA) and dTTP showing the location of Lys65, Val75 and Arg78 in the 66 kDa subunit of the enzyme

(A) Ternary complex of HIV-1 (group M subtype B (isolate HXB2)) RT, dsDNA and dTTP (PDB co-ordinates from [35], PDB code 1RTD). The RT subunits p66 and p51 are represented by cyan and green ribbons respectively. The template and primer strands are shown as grey and white sphere models. The incoming dNTP is shown in yellow. The side chains of Lys65, Val75 and Arg78 are shown in blue, magenta and orange respectively. (B) Two views of the β3–β4 hairpin loop and the dNTP-binding site with the location of relevant residues.

of correct dNTPs on mismatched template–primers [39]. These effects have been attributed in part to an incorrect positioning of the 3' end of the mispaired primer relative to the dNTP-binding site [39]. Our results obtained with HIV-1 group O RTs are consistent with the previously observed k_cat effect. However, we have also observed a significant reduction (4–8-fold decrease) in nucleotide misinsertion efficiencies when the K65R substitution was present. The k_cat reduction produced by K65R could be a consequence of the structural constraint imposed on Arg72, a residue that interacts with the β-phosphate of the incoming dNTP, due to the formation of a stacking interaction between the guanidinium planes of Arg65 and Arg72 [40]. K65R exerts similar effects on the accuracy of HIV-1 group M subtype B and group O RTs, as determined in forward-mutation assays [11]. Furthermore, with both types of RTs, the K65R mutation produces a higher ratio of transversions versus transitions and significant alterations in the mutational spectra. However, the K65R mutant displays increased frameshift fidelity over the WT HIV-1_{HXB2} RT, with a strong propensity to introduce deletions at nucleotide runs [11]. In contrast, the O_K65R RT shows a higher proportion of frameshift errors compared with the WT enzyme and a marked tendency to generate one-nucleotide deletions at non-runs.

Val75 and Arg78 are located at the base of the β3–β4 hairpin loop (residues 56–77), a site containing several residues involved in interactions with the incoming dNTP that are important for drug resistance and fidelity of DNA synthesis ([35]; reviewed in [38]).

Both amino acids in the 66 kDa subunit of HIV-1 RT interact with the template nucleotide at position + 1 (Figure 3). We have previously demonstrated that V75I produces a relatively modest increase in fidelity when introduced in HIV-1 RTs of groups M and O [14,25]. The mutational spectrum of the O_V75I RT was similar to that obtained with the WT enzyme [25]. Available evidence indicates that, when introduced in HIV-1_{HXB2} RT, R78A produces a large increase of fidelity as determined in forward-mutation assays [16], but no information related to its mutational spectrum has been reported. Interestingly, the mutational spectrum of O_R78A RT shared similar hot-spot distributions (including a major hot spot at position ~34 to ~36), very similar ratios of transitions versus transversions and very low frameshift error rates with those of WT and mutant O_V75I RTs. The types and frequencies of mutations generated by the O_R78A RT were different from those obtained with the O_K65R RT that also showed a higher frameshift error rate than the O_R78A, O_V75I and WT RTs. The high fidelity of O_R78A RT is further confirmed by the results of our kinetic assays. This enzyme appears to be very inefficient in misincorporating C opposite A, as well as in extending G:T and G:G mispairs. Significant differences in the misincorporation ratios of A opposite A and G:T and G:A mispair extension efficiencies were found between O_K65R and O_R78A RTs. These results could justify in part the different mutational spectra obtained with both enzymes.

Substituting an alanine residue for Arg78 has a destabilizing effect on the RT. The large effects on thermal stability observed with mutants R78A and V75I/R78A could be the result of the loss of interactions (mostly hydrogen bonds) between the side chains of Arg78 and Asp76 that could affect the stability of the RT subunits. The specific RNA-dependent DNA polymerase activity at 37°C of the double mutant (O_V75I/R78A RT) was approximately three times lower than the activity shown by the WT enzyme. Both O_R78A and O_V75I/R78A showed largely reduced efficiency in reverse transcription–PCR reactions carried out at temperatures above 52°C, limiting their further development as high-fidelity thermostable RTs. V75I produced a small, but detectable, reduction in reverse transcription efficiency at high temperatures [25]; when K65R was present, these effects were almost undetectable.

The mutants K65R and K65R/V75I showed similar accuracy in the M13mp2 lacZa forward-mutation assays. However, the observed mutational spectra were different. O_K65R RT showed a stronger tendency to generate frameshifts and produced more transversions than transitions. However, error specificities changed when V75I was present. Thus the double mutant showed a mutational spectrum with the hot spots at positions ~34 to ~36 and +87 found with O_V75I RT [25], but absent from the mutational spectrum of O_K65R RT. In addition, the double mutant showed a stronger tendency to generate one-nucleotide deletions at nucleotide runs, in comparison with the O_K65R RT. These results argue in favour of a functional interaction (or epistatic effect) between K65R and V75I, and against a dominant effect of one of these two mutations.

Further evidence of this interaction has been obtained from gel-based fidelity assays. Previous kinetic studies showed that O_V75I RT increases both misinsertion and mispair extension fidelity [14,25]. Unlike in the case of O_K65R RT, nucleotide affinity loss (i.e. increased K_M for nucleotide incorporation on mismatched template–primers) had a significant effect on the reduced mispair extension efficiencies of O_V75I RT. The double mutant K65R/V75I showed increased mispair extension fidelity for G:T and G:G mismatches, in comparison with the single mutants K65R and V75I. The increased accuracy of the double mutant was largely dominated by a K_M effect in the case of G:T
mispair extension, whereas in the case of G-G mispairs both the \( k_{\text{c}} \) and \( k_{\text{e}} \) values were affected by the presence of K65R together with V75I. Interestingly, these effects on G:T and G:G mispair extension were also observed with the R78A mutant. Interactions between the tip of the \( \beta3-\beta4 \) hairpin loop (including Lys48\(^{68} \)) and the dNTP could be greatly affected by removal of the side chain of Arg3, which could have a strong influence on the conformation of the \( \beta3-\beta4 \) hairpin loop and its interactions with the template nucleotide at position +1.

In summary, we provide evidence that demonstrates that the fidelity of lentiviral RTs (i.e. HIV-1 RT) can be improved to the levels shown by the more faithful MLV RT, without altering the stability or the specific DNA polymerase activity of the enzyme. O_K65R, O_K65R/V75I and O_R78A RTs showed >10-fold increased accuracy for base substitutions in comparison with the WT enzyme. Base-substitution error rates were similar to those obtained with MLV RT. However, the MLV RT showed a higher error rate for frameshifts (e.g. >3 times higher than for K65R) and a stronger tendency to produce transversions versus transitions in forward-mutation assays. Overall error rates for MLV RT are also in good agreement with previous estimates obtained with the M13mp2 lacZa forward-mutation assay [5,6]. However, pre-steady-state kinetic analyses of fidelity using MLV RT were limited by its low catalytic efficiency in comparison with HIV-1 RTs [41] (results not shown), as well as by the requirement for high concentrations of enzyme. The higher fidelity of the MLV RT in comparison with the HIV-1 RT has been shown previously in gel-based assays using synthetic heteropolymeric template–primers [41–44]. Most of those studies have been carried out under steady-state conditions. Reported misinsertion and mispair extension ratios for MLV RT were ~2–8 times lower than those obtained with HIV-1 RT [42–44]. However, results were strongly dependent on the sequence and the template–primer used. Therefore, in this scenario, forward-mutation assays provide a more reliable estimate of fidelity differences between both enzymes.

Although the role of K65R in the acquisition of drug resistance in HIV-1 group O RT has not been studied in detail in the clinical setting, recent report estimates that ~10 % of the HIV-1 (group M subtype B) clinical isolates bearing the K65R mutation also contain V75I [45]. It remains to be determined whether the RTs found in vivo display high fidelity and if this property has any impact on viral evolution. In any case, the RTs described in the present study combine increased efficiency of reverse transcription at high temperatures with high fidelity, and should be of great utility in the amplification of RNA targets.

**AUTHOR CONTRIBUTION**

Verónica Barriolengo obtained the mutants, designed and performed the reverse transcription–PCR and fidelity assays, and analysed the results. Verónica Barriolengo and Mar Álvarez purified the enzymes and performed the DNA polymerase activity assays to assess the thermal stability of RTs. Daniela Barbieri purified the R78A mutant RT and determined its fidelity with the forward-mutation assay. Luís Menéndez-Arias conceived the idea, supervised the project and wrote the paper.

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SUPPLEMENTARY ONLINE DATA
Thermostable HIV-1 group O reverse transcriptase variants with the same fidelity as murine leukaemia virus reverse transcriptase

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Figure S1 Influence of the RNA concentration on the efficiency of cDNA synthesis and PCR amplification on reactions carried out with different RTs

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 50 nM (active-enzyme concentration). Reactions were stopped by heating at 92 °C for 10 min. A 2 μl volume of the cDNA synthesis reactions and 1.75 units of Expand High Fidelity DNA polymerase mix were used in all amplifications. The 0.9 kb fragment of actin DNA was amplified with primers ACT1 and ACT3. From left to right, the amounts of RNA in the assays were 1 μg, 100 ng, 75 ng, 50 ng, 25 ng and 10 ng. Control reactions carried out in the absence of RT are shown in lane C. Molecular-size markers shown on the left-hand side are a HindIII digest of phage Φ29 DNA. For each gel, results are representative of two independent experiments.
Figure S2  Effect of mutations on the fidelity of DNA synthesis, as determined by pre-steady-state kinetic assays

(A) Misincorporation efficiencies for dCTP, dGTP and dATP relative to the correct nucleotide (dTTP). (B) Mispair extension efficiencies for G:T, G:G and G:A mismatches relative to those having a correctly matched G:C base pair at the 3′ end of the primer.

Figure S3  Spectrum of mutations induced by O_K65R RT

Single-nucleotide substitutions are indicated by the letter corresponding to the new base (in blue) above the template sequence of the lacZα target. Open red triangles represent insertions of one nucleotide (duplication of the base where the triangle is positioned). Inverted closed red triangles indicate deletions of one nucleotide.
High-fidelity HIV-1 group O reverse transcriptases

Figure S4 Spectrum of mutations induced by O_K65R/V75I RT

Single-nucleotide substitutions are indicated by the letter corresponding to the new base (in blue) above the template sequence of the lacZα target. The open red triangle represents an insertion of one nucleotide (duplication of the base where the triangle is positioned). Inverted closed red triangles indicate deletions of one nucleotide. The inverted green triangle represents the deletion of two nucleotides (CG).

Figure S5 Spectrum of mutations induced by O_R78A RT

Single-nucleotide substitutions are indicated by the letter corresponding to the new base (in blue) above the template sequence of the lacZα target. The open red triangle represents an insertion of one nucleotide (duplication of the base where the triangle is positioned). Inverted closed red triangles indicate deletions of one nucleotide.
Figure S6  Spectrum of mutations induced by MLV RT

Single-nucleotide substitutions are indicated by the letter corresponding to the new base (in blue) above the template sequence of the lacZα target. Open red triangles represent insertions of one nucleotide (duplication of the base where the triangle is positioned). Inverted closed red triangles indicate deletions of one nucleotide. Inverted green triangles represent larger deletions. Numbers in parentheses indicate the length of the deletion. Plus or minus signs are used to indicate that the nucleotide below the triangle is the 5′ end or 3′ end of the deletion respectively.