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Increased polymerase fidelity of the 3TC-resistant variants of HIV-1 reverse transcriptase

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) variants with resistance mutations in the reverse transcriptase (RT) gene appear during drug therapy with the nucleoside analogue 2′,3′-dideoxy-3′-thiacytidine (3TC). These 3TC-resistant RT variants have a single point mutation that changes the 184Met residue into either Val or Ile. Both codon 184 variants are frequently observed in 3TC-treated patients and can also be selected in cell culture infections. We demonstrated previously that the 184Ile and 184Val RT enzymes exhibit a processivity defect in *in vitro* assays, with 184Ile being the least processive enzyme [Met(wt) > Val > Ile]. In this study, we measured the polymerase fidelity of the wild-type (184Met) and 3TC-resistant RT enzymes (184Ile and 184Val) on DNA and RNA templates. Both virion-extracted and *Escherichia coli*-expressed recombinant RT enzymes were used to measure the nucleotide misinsertion and mispair extension efficiencies. The 3TC-resistant enzymes were more accurate than the wild-type RT protein in both type of assays. The order of accuracy observed for the codon 184 variants [Ile > Val > Met(wt)] may suggest an inverse correlation between the fidelity and processivity properties of these enzymes.

INTRODUCTION

Reverse transcriptase (RT) is the enzyme that copies the single-stranded RNA genome of retroviruses into double-stranded DNA. Studies with purified RT enzymes revealed an unusually high error rate in copying both DNA and RNA templates and the mutation rate in replication experiments ranges from 10^{-6} to 10^{-4} mutations per nucleotide per cycle through a host cell (1–4). The absence of 3′→5′ exonuclease activity, which is necessary for the removal of misincorporated nucleotides, has been suggested to be the major cause of this error-proneness (5,6). We previously studied the enzyme and virus replication properties of two RT variants of the human immunodeficiency virus type 1 (HIV-1) that are resistant to the nucleoside analogue 3TC (2′,3′-dideoxy-3′-thiacytidine, Lamivudine) (7). These 3TC-resistant RT variants have a single point mutation that changes residue 184Met within the catalytic core into either Val or Ile. Both codon 184 variants are frequently observed in 3TC-treated patients and in cell culture experiments (8–12). In some patients, the 184Ile variant is observed initially, followed by the outgrowth of the 184Val variant (12). This pattern can be explained by two separate effects: preferential mutation towards the Ile codon (13), and better enzyme properties of the 184Val variant (7). In cell culture, a third 3TC-resistant RT variant with the 184Thr substitution was obtained by a clonal selection protocol (13). This variant is not observed in patients, presumably because the 184Thr enzyme exhibits severely reduced polymerase activity.

The Met184 residue corresponds to the X residue of the YXDD motif that is conserved in the RT enzymes of all retroviral species (14–17). This motif is located within the catalytic core of the RT enzyme, and the 3-D crystal structure of HIV-1 RT places the 184Met in the turn of the connecting β-sheets 9 and 10 of the 2′-deoxyribonucleoside 5′-triphosphates (dNTP) binding pocket (18,19). This may suggest that residue 184 is critical for initiation of the β9-sheet conformation and it was proposed that the reduced activity of 3TC-resistant variants correlates with decreased stability of the β9-sheet (7). The carboxylic side chains of the two aspartic acid residues of the YMDD motif were proposed to coordinate two metal ions, which in turn coordinate the β- and γ-phosphates of the dNTP. Mutation of residue 184 may affect the interaction with the incoming dNTP in such a way that the 3TC drug is specifically rejected, thus explaining the resistance phenomenon. Interestingly, there is accumulating evidence that these RT variants exhibit functional defects. First, we and others showed that the 184Val and 184Ile RT enzymes exhibit a processivity defect in *in vitro* assays (7,20), with 184Ile being the least processive enzyme. Second, mutation of residue 184 has been reported to affect the polymerase fidelity with respect to nucleotide misincorporation (21–23). In particular, improved fidelity has been reported for the 184Val variant (22,23), which may preclude the development of additional drug-resistance mutations (22).

In this study, we measured the polymerase fidelity of the complete set of wild-type (184Met) and 3TC-resistant RT enzymes (184Ile, 184Val and 184Thr). We used both the virion-extracted RT enzymes and the *Escherichia coli*-expressed recombinant RT proteins. Because previous studies were performed exclusively with the 184Val RT variant in combination with DNA templates, we now tested the misinsertion fidelity of all 3TC-resistant enzymes on both RNA and DNA templates. Fidelity studies with the wild-type RT enzyme on RNA versus DNA templates have yielded controversial results, with either an increased or decreased error frequency on RNA templates, or no

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significant difference (24–27). In addition, the ability of these enzymes to extend a mismatched primer was assayed. The 3TC-resistant RT enzymes exhibited improved fidelity in both the misinsertion and mismatch extension assay, and a more pronounced increase in polymerase fidelity was measured for the 184Ile enzyme compared with the 184Val variant.

MATERIALS AND METHODS

Escherichia coli-expressed and virion-associated RT enzymes

The pGST-RT expression vector was constructed as follows. A 1.68 kb DNA fragment encoding the 66 kDa RT subunit was generated by PCR with the sense primer 5′-AGG.ATC.CCC.CAT.TAG.TCC.TAT.TGA.A and the antisense primer 5′-AGA.ATT.CTA.TAG.TAC.TTT.CCT.GAT.TCC (restriction sites underlined) on the HIV-1 pLAI plasmid as template. We used a standard PCR protocol (5 min 95°C; 35 cycles of 1 min 95°C, 1 min 55°C, 2 min 72°C and then 10 min 72°C and 10 min 4°C) with 100 ng of both primers and 1 ng HIV-1 pLAI. This fragment was cloned in the expression vector pGEX-2T (Pharmacia) in frame with the gluthatione *S*-transferase (GST) gene (28), with the *Bam*HI and *Eco*RI restriction sites provided by the PCR primers. The GST-RT66 fusion protein contains all amino acids (position 1–560, pLAI coordinates 2585–4265) of the mature 66 kDa subunit. This wild-type construct encodes Met (ATG) at codon position 184 of the RT gene. Mutant RT genes encoding 184Ile (ATA), 184Val (GTG) or 184Thr (ACG) were generated by site-directed mutagenesis on an RT subclone and subsequently introduced into the pGEX-2T-RT66 vector by exchange of an internal *Nsi*I fragment (pLAI position 2923–4087). All constructs were verified by sequence analysis on a 373 model DNA sequencer (Applied Biosystems, Perkin Elmer).

An overnight culture of *E.coli* DH5α transformed with the wild-type or mutant pGEX-2T-RT expression plasmid was diluted 1:10 and grown for 2 h at 37°C in brain heart infusion broth (Life technologies). Transcription and GST-RT protein expression was induced with 1 ml 10 mM isopropyl β-D-thiogalactoside (IPTG) per 100 ml culture for 4 h at 37°C. Bacterial cells were collected by centrifugation and resuspended in 1/100 of the original volume in NET-N buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl pH 8.0 and 0.5% Nonidet P-40). The bacteria were lysed by sonification and cellular debris was removed by centrifugation. Glutathione–agarose beads (Sigma, 25 µl 1:1 suspension in PBS) were added to 1 ml *E.coli* extract and incubated for 1 h at room temperature to allow the GST-RT66 fusion protein to bind to the matrix. The beads were collected by low speed centrifugation, and washed five times with phosphate buffered saline (PBS) to remove non-specifically bound proteins. Bound GST-RT66 fusion protein was eluted by addition of an equal volume of 10 mM reduced glutathione (ICN Biochemicals) in 50 mM Tris–HCl pH 8.0, and incubated for 10 min at room temperature. This step was repeated once and the combined solutions were brought to 50% glycerol and stored at –70°C. The RT protein concentration was determined by Coomassie brilliant blue staining of a 10% SDS–polyacrylamide gel. The RT preparations were found to be >90% pure (data not shown).

Wild-type and 3TC resistant virion-associated RT enzymes were released from the virions by detergent treatment (0.5% NP-40 final concentration) and corrected for CA-p24 levels as previously described (7).

Nucleotide misincorporation assay

An HIV-2 RNA transcript was synthesized by T7 RNA polymerase run-off transcription using the HIV-2 pUC 8 plasmid (29). This RNA was used as template in the read-through assays and contains the complete leader region and part of the *gag* gene [coordinates +1/+892 of the ROD isolate. Approximately 10 ng RNA template (0.003 pmol) was mixed with 20 ng complementary DNA-primer. The following oligodeoxyribonucleotides were used as primer: psi (5′-TCC.GTC.GTG.GTT.TGT.TCC.TGC-3′, complementary to nt 374–394 of the HIV-2 RNA) and the lys21 primer (5′-CAA.GTC.CCT.GTT.CGG.GCG.CCA-3′, complementary to region 303–323). The RNA/DNA duplex was formed in 12 µl annealing buffer (83 mM Tris–HCl pH 7.5, 125 mM KCl) by incubation at 85°C for 2 min, at 65°C for 10 min, and then slowly cooled to room temperature. For reverse transcription we added 6 µl 3× RT buffer (9 mM $MgCl_2$, 30 mM dithiothreitol, 150 µg/ml actinomycine D, 1.5 µM dCTP, 30 µM of two other dNTPs), 0.3 µl [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml), and RT enzyme. We used either the recombinant GST-RT enzymes (~5 ng per experiment) or the virion-extracted enzymes (the equivalent of 5 ng CA-p24 per experiment). The primer extension reaction was performed for 25 min at 42°C and was stopped by the addition of 1 µl 0.5 M EDTA, pH 8.0. After increasing the volume to 100 µl with H_2O, the cDNA products were precipitated at –20°C by addition of 10 µl 3 M sodium acetate (pH 5.2) and 250 µl 96% ethanol. The pellets were resuspended in formamide loading buffer, heated at 85°C for 3 min, and analyzed on a denaturing 6% polyacrylamide/7.1 M urea sequencing gel. cDNA products were quantitated by the PhosphorImager (Molecular Dynamics), corrected for the number of incorporated [α-^{32}P]dCTP molecules, and used to calculate the 'read-through frequency', which is a measure of the fidelity of the RT polymerase.

The misincorporation properties of the wild-type and mutant RT enzymes were also tested near the end of a DNA template (30). The DNA–DNA template/primer (T/P) was obtained by digestion of Bluescript SK+ plasmid with restriction enzyme *Xba*I. To 100 ng T/P duplex was added 12 µl annealing buffer (see above), 8 µl 3× RT buffer lacking actinomycine D (9 mM $MgCl_2$, 30 mM dithiothreitol, 1.5 µM dCTP, 30 µM of two other dNTPs) and 0.3 µl [α-^{32}P]dCTP and either 5 ng recombinant RT protein or virion RT enzyme (the equivalent of 15 ng CA-p24) as described above. The standard incubation was for 25 min at 42°C. In the 'all dNTP' assay, the annealed primer was first extended for 3 min at 42°C by addition of 3× RT buffer (see above, with 30 µM dATP/dTTP/dGTP, 1.5 µM dCTP and [α-^{32}P]dCTP). After addition of 1 µl dNTP mix (10 mM each dNTP), incubation was continued for 25 min at 42°C. The reaction was stopped and the DNA was precipitated as described above. The pellets were resuspended in 10 µl TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). To obtain DNA fragments that could be size-fractionated on a 6 % polyacrylamide/7.1 M urea gel, the DNA was digested with the *Hin*dIII restriction enzyme. The DNA was again precipitated and subsequently resuspended in formamide loading buffer. Primer extension products were quantitated by the PhosphorImager.

Site-specific nucleotide (mis)incorporation assay

The nucleotide misinsertion of the wild-type and the 3TC-resistant enzymes was analyzed in a standard misincorporation assay (31). An aliquot of 0.25 ng ^{32}P-end-labeled primer lys21 was purified

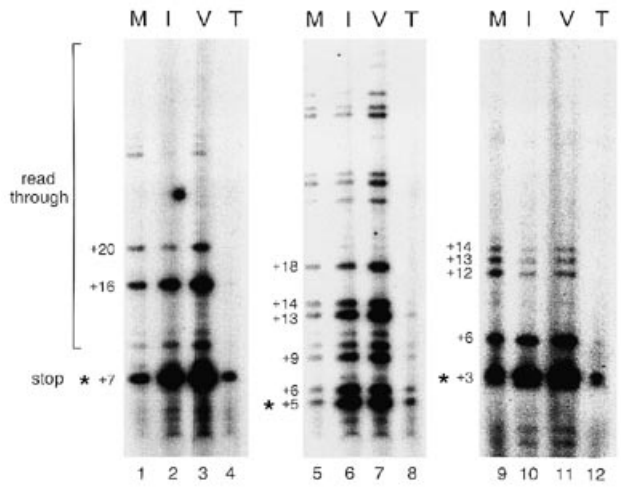

Figure 1. Nucleotide misincorporation assay on an HIV-2 RNA template with either the psi primer (lanes 1–8) or the lys21 primer (lanes 9–12). Lanes 1–4 represent the –dATP reaction, in which 30 µM dTTP, 30 µM dGTP, 1.5 µM dCTP and 0.3 µl [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml) were present. Lanes 5–12 represent the –dTTP reaction, in which the DNA primer was extended in the presence of 30 µM dATP, 30 µM dGTP, 1.5 µM dCTP and 0.3 µl [α-^{32}P]dCTP. *Escherichia coli*-expressed RT proteins were used as indicated above the lanes (M, 184Met; I, Ile; V, Val; T, Thr). The correctly terminated cDNA products are marked by asterisks. The terminated and extended cDNAs are indicated as 'stop' and 'read-through', respectively. Nucleotide incorporation was as follows (correct stop in bold): lanes 1–4, CGCCCT**T**$^{+7}$ACTGCCTTC^{+16}ACTC^{+20}AGCC; lanes 5–8, CGCC**C**$^{+5}$T^{+6}TAC^{+9}TGCC^{+13}T^{+14}TCAC^{+18}TCAGCC; lanes 9–12, AC**C**$^{+3}$TGC^{+6}TAGGGA^{+12}T^{+13}T^{+14}TTCC.

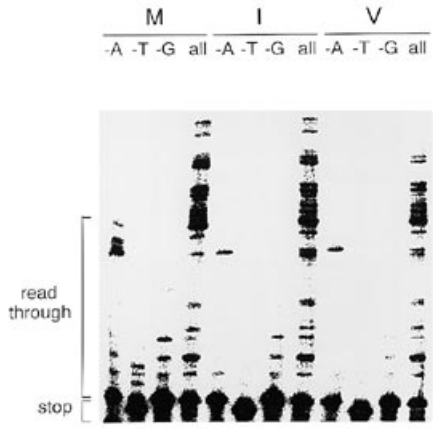

Figure 2. Nucleotide misincorporation assay on an HIV-2 RNA template with the lys21-primer. The virion-extracted RT enzymes 184Met, 184Ile and 184Val were used, as indicated above the lanes. The DNA primer was extended in the absence of one of the dNTPs (the missing dNTP is indicated above the lanes; e.g., –A is –dATP). The four dNTPs were added in the lanes marked 'all', resulting in the synthesis of extended cDNA forms. cDNAs of intermediary length represent pausing products.

on a 10% polyacrylamide/7.1 M urea gel and annealed to 20 ng synthetic DNA template (63mer) as described above. Reverse transcription was carried out with *E.coli*-expressed wild-type and mutant HIV-1 RT in 6 µl RT buffer (end concentration 3 mM $MgCl_2$, 10 mM dithiothreitol) and a single dNTP at 42°C during 3 min. With this T/P system, dCTP represents the correct nucleotide and dTTP the incorrect nucleotide, which were used to measure the V_{max} and K_m values of (mis)incorporation. The dCTP and dTTP concentrations used were 0, 0.04, 0.4, 4 and 40 µM. The reverse transcription products were ethanol-precipitated and analyzed on a 10% sequence gel as described for the read-through misincorporation assay.

Mismatch extension assay

Approximately 10 ng HIV-2 RNA template (0.003 pmol, see above) was annealed to 20 ng DNA primer 5′-GTC.CCT.GTT.CGG.GCG.CCN-3′, with N forming either a match (A) or mismatch (T, G, C) with the RNA template. The T/P duplex was formed and extended by HIV-1 RT as described above for the nucleotide misincorporation assay, in the absence of dGTP. In this experiment we used an equivalent of 5 ng CA-p24 as source of virion RT enzymes. Samples were processed and analyzed on a 6% sequence gel as described above.

RESULTS

Increased fidelity of 3TC-resistant RT enzymes on an RNA template

All three 3TC-resistant HIV-1 RT variants, 184Ile, 184Val and 184Thr, have been demonstrated to exhibit a processivity defect (7,13,20). Because there is some evidence that RT mutations may affect both processivity and fidelity properties (21,32,33), we tested the effect of the amino acid 184 variation on polymerase fidelity. In the first assay system, we tested the ability of the wild-type and mutant RT enzymes to continue polymerization in the absence of one of the dNTPs (34). We tested either *E.coli*-expressed (Fig. 1) or virion-extracted RT proteins (Fig. 2) on RNA–DNA template/primer duplexes. For instance, elongation of the psi primer used in Figure 1 (lanes 1–8) leads to synthesis of the cDNA sequence 5′-CGCCCTTA.... -3′. The dCTP was provided in this reaction as labeled dNTP and one expects incorporation of 7 nt in the –dATP reaction (lanes 1–4) and 5 nt in the –dTTP reaction (lanes 5–8). The correctly terminated cDNA products are indicated in Figures 1 and 2 by 'stop', and extended cDNA products are termed 'read-through'. The read-through efficiency was calculated as a relative measure of the infidelity of the RT polymerase by quantitating the correctly terminated and the read-through cDNA products, which were corrected for the number of incorporated [α-^{32}P]dCTP molecules (Table 1). The read-through efficiency of the 3TC-resistant RT enzymes was significantly lower than that of the wild-type RT protein, and 184Ile RT was more accurate in this assay than 184Val. This spectrum of polymerase fidelity was observed in several independent assays with both the *E.coli*-expressed and virion-extracted RT proteins. The order of polymerase fidelity [Ile > Val > Met(wt)] may indicate an inverse correlation with the processivity of these RT enzymes [Met(wt) > Val > Ile]. The fidelity of the 184Thr RT enzyme was difficult to measure because this variant demonstrated severely reduced polymerase activity, and we did not include this variant in the subsequent assays.

Increased fidelity of 3TC-resistant RT enzymes on a DNA template

To test the RT fidelity on a DNA template, we used a DNA–DNA template/primer combination that was obtained by digestion of the Bluescript SK+ plasmid with the restriction enzyme *Xba*I.

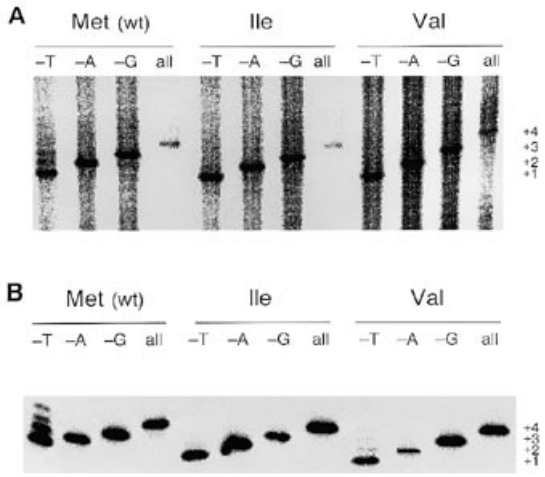

Figure 3. Nucleotide misincorporation properties of the virion-extracted (**A**) and *E.coli*-expressed (**B**) RT enzymes on a DNA–DNA T/P duplex. The reactions were performed in the absence of one dNTP as indicated above the lanes. In the lanes marked 'all', the four dNTPs were added. The reaction mixtures lacking either dTTP, dATP or dGTP are expected to yield products with an additional 1, 2 and 3 nt, respectively. The full-length product synthesized in the 'all dNTP' reactions contains an additional 4 nt.

This procedure generates a linear plasmid with 5′-CTAG-3′ protruding ends, which were filled in by the virion and recombinant RT enzymes in the absence of one of the dNTPs (Fig. 3). The first incorporated nucleotide was included in the form of [α-^{32}P]dCTP. The reaction mixtures lacking either dTTP, dATP or dGTP are expected to yield products with an additional 1, 2 and 3 nt, respectively. Consistent with the results on an RNA template, we measured considerable read-through synthesis with the wild-type RT enzyme in the –dTTP reaction. The read-through efficiency varied from 30 to 44% for the recombinant and virion 184Met enzyme, respectively (Table 2). More importantly, a reduced level of read-through DNA synthesis was measured for the 184Ile and 184Val RT enzymes. The fidelity of the recombinant 184Ile variant was significantly higher than 184Val, with 13 and 22% read-through efficiencies respectively (Fig. 3B). A similar trend was observed for the virion-extracted RT enzymes (Fig. 3A). However, we were unable to accurately measure the read-through efficiency of these samples because of high background signals. Thus, the results on the DNA template are very similar to the fidelity spectrum that was measured on an RNA template [Ile > Val > Met(wt)].

Table 1. Read-through efficiency of wild-type and 3TC-resistant RT variants on an RNA template

	Recombinant RT (%)			Virion RT (%)		
	–A[a]	–T[a]	–T[b]	–A[b]	–T[b]	–G[b]
184Met(wt)	24	54	17	13	23	5
184Ile	10	26	6	5	nd	2
184Val	13	45	7	2	nd	3
184Thr	nd	16	nd	nt	nt	nt

[a]T/P was vRNA with psi oligonucleotide.
[b]T/P was vRNA with lys21 oligonucleotide.
nd, not detectable; nt, not tested.

Table 2. Read-through efficiency of wild-type and 3TC-resistant RT variants on a DNA template[a]

	Recombinant RT (%)	Virion RT (%)
184Met(wt)	30	44
184Ile	13	nd
184Val	22	nd
184Thr	nd	nt

[a]T/P was *Xba*I-digested plasmid (–dTTP reaction).
nd, not detectable; nt, not tested.

It has been reported that the fidelity of HIV-1 RT improves when the enzyme reaches the end of a DNA template (30). In our assay system, we observed this effect in the –dATP and –dGTP reactions, which are predicted to stop cDNA synthesis respectively 2 and 1 nt from the 3′ end of the template. Indeed, when the template strand was short (1–3 nt beyond the duplex region), no nucleotide misincorporation was measured with any of the RT enzymes.

Site-specific (mis)incorporation of wild-type and mutant RT enzymes

The results presented above demonstrated increased fidelity of the 3TC-resistant RT enzymes as measured by read-through DNA synthesis. However, it can not formally be excluded that the processivity defects of the 3TC-resistant variants will result in reduced read-through polymerization. This possibility may seem unlikely because full-length DNA products were synthesized in reactions with all dNTPs (e.g., Fig. 3, lanes marked 'all'). To further rule out such processivity effects, we tested the fidelity of the RT enzymes in assays that measure the site-specific (mis)insertion of a single dNTP. An additional advantage of this assay over the read-through assay is that the identity of the misinserted nucleotide is known. We analyzed nucleotide misinsertion of the wild-type and 3TC-resistant enzymes in a standard misincorporation assay (21,22) with a DNA–DNA template/^{32}P-end-labeled primer duplex with dCTP as the correct nucleotide (Fig. 4A), and dTTP as an incorrect nucleotide (Fig. 4B). The Michaelis constant K_m and maximum velocity V_{max} for the correct and incorrect dNTP incorporation were determined from graphs, in which the rate of primer elongation (%/min) is plotted as a function of dNTP concentration (not shown). The ratios of the insertion efficiencies for the incorrect versus the correct nucleotides provide the frequency of misinsertion (f_{ins}); $f_{ins} = (V_{max}/K_m)^{mismatch} / (V_{max}/K_m)^{match}$ (Table 3). The results indicated that the 3TC-resistant mutants displayed a reduced frequency of misinsertion (lower f_{ins} values) compared to the wild-type RT enzyme and that 184Ile was more accurate than 184Val. This increase in fidelity for the 184Val and 184Ile variants is largely attributable to a decrease in K_m rather than a significant increase in V_{max}. It has been suggested that this is a more general characteristic of RT mutants with increased fidelity (24). In conclusion, the RT polymerase fidelity in the site-specific misinsertion assay was similar to that of the read-through assay [Ile > Val > Met(wt)].

Fidelity of the variant RT enzymes in mispair extension

The misincorporation of a nucleotide takes place in two steps: nucleotide misinsertion and elongation from the 3′-terminal mispair (35). The ability of HIV-1 RT to extend a mispaired primer terminus was examined by monitoring the addition of the next correct nucleotide onto a DNA primer with a 3′-terminal mismatch. Three mismatch primers (T, G or C opposite U in the

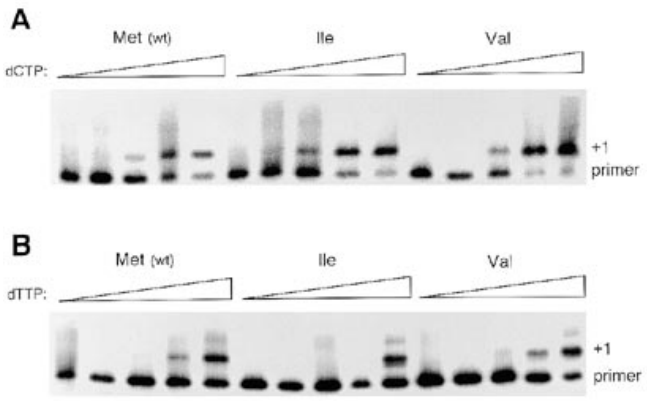

Figure 4. Misinsertion fidelity of *E.coli*-expressed RT proteins. A DNA–DNA template/^{32}P-end-labeled primer duplex was extended in the presence of a single dNTP. dCTP represents the correct nucleotide (**A**) and dTTP the incorrect nucleotide (**B**). Increasing amounts of dCTP and dTTP were used: 0, 0.04, 0.4, 4 and 40 µM. The positions of the primer and +1 extension product are indicated.

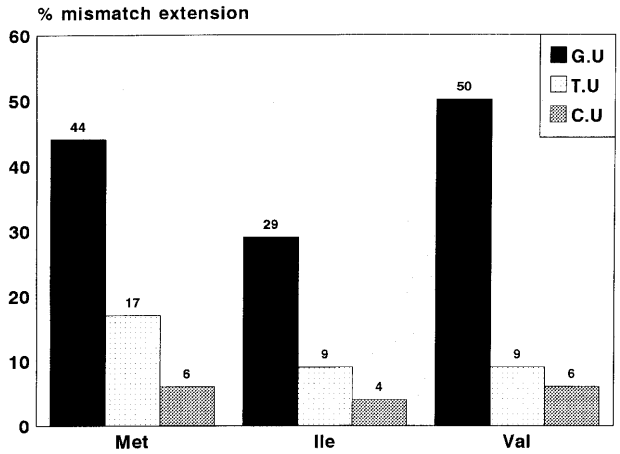

Figure 5. Mismatch extension capacity of the codon 184 RT variants. Three mismatch primers (3′ terminal T, G or C opposite U in the RNA template) and a control primer with a terminal A–U match were used to measure the extension efficiencies of the virion-extracted RT enzymes. The extension efficiency measured with the correctly paired primer was arbitrarily set at 100% for each RT enzyme.

RNA template) and a control primer with a terminal A–U match were used. We determined the relative efficiencies of extending each of the terminal mispairs, with the extension efficiency from the correctly paired terminus set at 100% for each RT enzyme (Fig. 5). With the recombinant 184Met enzyme, we measured up to 44% misincorporation efficiency with the transversion type G.U mismatch, but the two transition mispairs (T.U and C.U) were extended less efficiently (17 and 6%, respectively). This result is consistent with previous reports, in that a transition mispair (Pu.Py and Py.Pu) is extended more efficiently than a transversion mispair (Pu.Pu and Py.Py) (36). Most importantly, we measured a decrease of mispair extension for the 184Ile variant on the mismatched primers, in particular with the T.U mismatch. An intermediate phenotype was observed for the 184Val RT enzyme.

Table 3. The V_{max}, K_m and the efficiency of misinsertion (f_{ins}) for wild-type and 3TC-resistant RT variants

		V_{max} (%/min)	K_m (µM)	f_{ins}
184Met(wt)	G–C	20	1.7×10^{-3}	1.0[a]
184Ile	match	27	2.4×10^{-3}	1.0[a]
184Val		32	1.4×10^{-3}	1.0[a]
184Met (wt)	G·T	18	2.8	5.3×10^{-4}
184Ile	mismatch	14	18.6	6.5×10^{-5}
184Val		20	10.1	8.5×10^{-5}

[a]Arbitrarily set at 1.0 for each RT enzyme.

DISCUSSION

We determined the polymerization fidelity of 3TC-resistant variants of the HIV-1 RT enzyme on RNA and DNA templates. Both nucleotide misinsertion and mispair extension efficiencies were measured. First, we tested the ability of the wild-type (184Met) and mutant RT enzymes (184Ile, 184Val and 184Thr) to continue polymerization in the absence of one of the dNTPs, a reaction that scores both for a nucleotide misinsertion and the subsequent mispair extension. The read-through efficiency of the 3TC-resistant RT enzymes was significantly lower than that of the wild-type RT protein, and the 184Ile RT enzyme was more accurate than 184Val. Similar results were obtained with the recombinant and virion-derived forms of the RT enzyme. Full-length DNA products were synthesized in reactions with all dNTPs, indicating that the reduced read-through capacity of the 3TC-resistant RT variants is not caused by the processivity defect of these enzymes (7,20). We were unable to measure the fidelity of 184Thr because of the low activity of this enzyme. Second, we measured the RT fidelity in an assay that scores the misinsertion of a single dNTP. A similar ranking order of fidelity was observed [Ile > Val > Met(wt)]. Third, we also measured improved fidelity for the 184Ile variant in a mispair extension assay. Although only three RT mutants were tested, the initial results suggest that the fidelity [Ile > Val > Met(wt)] and processivity properties [Met(wt) > Val > Ile] of codon 184 HIV-1 RT mutants are inversely correlated. Obviously, this correlation may be different for other polymerases (37) and RT enzymes with mutations at other positions.

The 3-D crystal structure of HIV-1 RT places the 184Met in the turn of the connecting β-sheets 9 and 10 of the dNTP binding site (18,19). It is therefore likely that mutation of residue 184 does directly affect the interaction of RT with the incoming dNTP, and this may form the molecular basis of resistance against the 3TC nucleoside analogue. As shown in this paper, the 184 variants exhibit a broader fidelity increase that reduces the misinsertion of natural dNTPs and the extension of a mispaired primer terminus. Fidelity properties are determined not only by the precise configuration of the active site of the RT enzyme, but also by the position and conformation of the template-primer. For instance, nucleotide misincorporation was abolished when the template strand was 1–3 nt beyond the end of the primer strand (30, this study).

There is now convincing evidence that the 3TC-resistant RT variants exhibit improved fidelity in a variety of *in vitro* assays (20,22,23, this study). It has been suggested that this improved fidelity of codon 184 RT variants may interfere with the ability of 3TC-resistant HIV-1 variants to develop resistance to other drugs (22). However, there is recent evidence that the acquisition of

additional drug-resistance mutations is not delayed for 3TC-resistant viruses both in HIV-1 infected individuals and in cell culture experiments (38,39). Thus, there appears a discrepancy between the results of *in vitro* fidelity assays and virus replication studies. It is likely that the reverse transcription reaction within virion particles is much different from the *in vitro* assay. Consistent with this idea, the fidelity of the HIV-1 RT enzyme has been reported to differ significantly in these two experimental systems, which may suggest that viral co-factors are involved (40). For instance, high mispair extension efficiencies were reported within virion particles (41), and recent *in vitro* studies suggest that the virion NC protein is responsible for this effect (42).

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REFERENCES

1 Batschelet,E., Domingo,E. and Weismann,C. (1976) *Gene*, **1**, 27–32.
2 Leider,J.M., Palese,P. and Smith,F.I. (1988) *J. Virol.* **62**, 3084–3091.
3 Roberts,J.D., Bebenek,K. and Kunkel,T.A. (1988) *Science*, **242**, 1171–1173.
4 Pathak,V.K. and Temin,H.M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 6019–6023.
5 Varmus,H. and Swanstrom,R. (1984) In Weiss,R., Teich,N., Varmus,H. and Coffin,J. (eds), *RNA Tumor Viruses*. Cold Spring Harbor Laboratory Press, New York, Vol. 2, pp. 369–512.
6 Morrison,A., Bell,J.B., Kunkel,T.A. and Sugino,A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 9473–9477.
7 Back,N.K.T., Nijhuis,M., Keulen,W., Boucher,C.A.B., Oude Essink,B.B., van Kuilenburg,A.B.P., Van Gennip,A.H. and Berkhout,B. (1996) *EMBO J.* **15**, 4040–4049.
8 Boucher,C.A.B., Cammack,N., Schipper,P., Schuurman,R., Rouse,P.L. and Cameron,J.M. (1993) *Antim. Ag. Chemoth.* **37**, 2231–2234.
9 Gao,Q., Gu,Z., Parniak,M.A., Cameron,J., Cammack,N., Boucher,C.A.B. and Wainberg,M.A. (1993) *Antim. Ag. Chemoth.* **37**, 1390–1392.
10 Schinazi,R.F., Lloyd,R.M.Jr, Nguyen,M.-H., Cannon,D.L., McMillan,A., Ilksoy,N., Chu,C.K., Liotta,D.C., Bazmi,H.Z. and Mellors,J.W. (1993) *Antim. Ag. Chemoth.* **37**, 875–881.
11 Tisdale,M., Kemp,S.D., Parry,N.R. and Larder,B.A. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5653–5656.
12 Schuurman,R., Nijhuis,M., Van Leeuwen,R., Schipper,P., Collis,P., Danner,S.A., Mulder,J., Loveday,C., Christopherson,C., Kwok,S., Sninsky,J. and Boucher,C.A.B. (1995) *J. Infect. Dis.* **171**, 1411–1419.
13 Keulen,W., Back,N.K.T., van Wijk,A., Boucher,C.A.B. and Berkhout,B. (1997) *J. Virol.* **71**, 3346–3350.
14 Kamer,G. and Argos,P. (1984) *Nucleic Acids Res.* **18**, 7269–7283.
15 Argos,P. (1988) *Nucleic Acids Res.* **21**, 9909–9916.
16 Pelletier,H., Sawaya,M.R., Kumar,A., Wilson,S.H. and Kraut,J. (1994) *Science*, **264**, 1891–1903.
17 Sawaya,M.R., Pelletier,H., Kumar,A., Wilson,S.H. and Kraut,J. (1994) *Science*, **264**, 1930–1935.
18 Kohlstaedt,L.A., Wang,J., Friedman,J.M., Rice,P.A. and Steitz,T.A. (1992) *Science*, **256**, 1783–1790.
19 Jacobo-Molina,A., Ding,J., Nanni,R.G., Clark,A.D.C.,Jr, Lu,X., Tantillo,C., Williams,R.L., Kamer,G., Ferris,A.L., Clark,P., Hizi,A., *et al.* (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 6320–6324.
20 Boyer,P.L. and Hughes,S.H. (1995) *Antim. Ag. Chemoth.* **7**, 1624–1628.
21 Bakhanashvili,M., Avidan,O. and Hizi,A. (1996) *FEBS Lett.* **391**, 257–262.
22 Wainberg,M.A., Drosopoulos,W.C., Salomon,H., Hsu,M., Borkow,G., Parniak,M.A., Gu,Z., Song,Q., Manne,J., Islam,S., *et al.* (1996) *Science*, **271**, 1282–1285.
23 Pandey,V.N., Kaushik,N., Rege,N., Sarafianos,S.G., Yadav,P.N.S. and Modak,M.J. (1996) *Biochemistry* **35**, 2168–2179.
24 Bakhanashvili,M. and Hizi,A. (1992) *Biochemistry* **31**, 9393–9398.
25 Boyer,J.C., Bebenek,K. and Kunkel,T.A. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6919–6923.
26 Ji,J. and Loeb,L.A. (1992) *Biochemistry* **31**, 954–958.
27 Hubner,A., Kruhoffer,M., Grosse,F. and Krauss,G. (1992) *J. Mol. Biol.* **223**, 595–600.
28 Smith,D.B. and Johnson,K.S. (1988) *Gene*, **67**, 31–40.
29 Oude Essink,B.B., Das,A.T. and Berkhout,B. (1996) *J. Mol. Biol.* **264**, 243–254.
30 Boyer,P.L., Tantillo,C., Jacobo-Molina,A., Nanni,R.G., Ding,J., Arnold,E. and Hughes,S.H. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 4882–4886.
31 Mendelman,L.V., Boosalis,M.S., Petruska,J. and Goodman,M.F. (1989) *J. Biol. Chem.* **264**, 14415–14423.
32 Bebenek,K., Beard,W.A., Casas-Finet,J.R., Kim,H.-R., Darden,T.A., Wilson,S.H. and Kunkel,T.A. (1995) *J. Biol. Chem.* **270**, 19516–19523.
33 Martin-Hernandez,A., Domingo,E. and Menendez-Arias,L. (1996) *EMBO J.* **15**, 4434–4442.
34 Preston,B.D., Poiesz,B.J. and Loeb,L.A. (1988) *Science*, **242**, 1168–1171.
35 Perrino,F.W., Preston,B.D., Sandell,L.L. and Loeb,L.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8343–8347.
36 Mendelman,L.V., Petruska,J. and Goodman,M.F. (1990) *J. Biol. Chem.* **265**, 2338–2346.
37 Kornberg,A. and Baker,T.A. (1992) *DNA Replication*, 2nd edition. W.H.Freeman and Company, New York.
38 Keulen,W., Nijhuis,M., Schuurman,R., Berkhout,B. and Boucher,C.A.B. (1997) *Science*, **275**, 229.
39 Balzarini,J., Pelemans,H., Karlsson,A., De Clercq,E. and Kleim,J.-P. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 13152–13157.
40 Mansky,L.M. and Temin,H.M. (1995) *J. Virol.* **69**, 5087–5094.
41 Das,A.T. and Berkhout,B. (1995) *Nucleic Acids Res.* **23**, 1319–1326.
42 Lapadat-Tapolsky,M., Gabus,C., Rau,M. and Darlix,J.-L. (1997) *J. Mol. Biol.* **269**, 1–11.