The influence of 3TC-resistance mutations E89G and M184V in the human immunodeficiency virus reverse transcriptase on mispair extension efficiency

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ABSTRACT

Two nucleoside analog resistance mutations in HIV-1 reverse transcriptase (RT), E89G and M184V, were previously shown to increase the dNTP insertion fidelity of HIV-1 RT. However, forward mutation assays using a lacZα reporter gene have revealed a lack of impact on the overall error rate of these variants. In an effort to investigate the basis for this discrepancy, we have examined whether the increases in misinsertion fidelity observed for E89G and M184V RTs are accompanied by an increase in mispair extension fidelity. The relative efficiencies with which the wild type, E89G, M184V and M184V/E89G HIV-1 RTs extend model template–primer duplexes containing 3'-OH terminal mismatches were measured. The calculated efficiencies of mispair extension (fext) were, in general, not significantly decreased from the wild type HIV-1 RT. In fact, the efficiency of extension from one of the mispaired primer–template duplexes was significantly increased for two of the mutants tested. These results suggest that amino acid substitutions that increase the fidelity of dNTP insertion do not necessarily increase misextension fidelity, and that the decreased misextension fidelity may counterbalance the increases in misinsertion fidelity observed for E89G and M184V RTs.

INTRODUCTION

The reverse transcriptase (RT) of human immunodeficiency virus (HIV) is a key player in the replication of the viral genome. In comparison to other viral and host DNA polymerases, HIV-1 RT is highly error prone (1–3) due to a lack of proof-reading exonuclease function, a low processivity (4) and an ability to promote slippage-mediated copying errors at a high rate (5). Mutations which confer resistance to nucleoside analog inhibitors of RT can affect the incorporation of a misinserted dNTP into the viral genome (6–10). One of these, M184V, appears in patients receiving 3TC therapy (11), confers up to a 1000-fold resistance to 3TC (12) and cross-resistance to ddI and ddC (13). The other mutation, E89G, originally identified via an in situ colony screening assay (14,15), confers resistance to several ddNTP analogs and has been shown to coexist with the M184V mutation in resistant variants of HIV-1 that were 3TC-selected in vitro (12). Mutations that significantly decrease the overall mutation rate of HIV could be of great value in suppressing the generation of drug-resistant and immune escape variants of HIV. This prompted the measurement of the overall mutation rate of these variant RTs via a forward mutation assay (16). However, despite a significant (2–45-fold) increase observed in assays measuring dNTP insertion fidelity, the overall mutation rates for both of these variants were unaltered (17). This suggested an increase (or a lack of decrease) in the efficiency at which other types of errors are generated by these mutant RTs. Therefore, we wished to investigate the degree of fidelity of these variant RTs with respect to a second aspect of error-formation, the mispair extension.

The incorporation of a misinserted dNTP into the viral genome depends not only on the efficiency of insertion of the incorrect dNTP (misinsertion), but also on other polymerase-mediated events. The extension of the initial mispair via insertion of the next correct base onto the growing primer (misextension) is a key step that must occur after misinsertion in order for the substitution to appear in the final product. Thus, in order to assess the influence of an RT mutation on error rate, one needs to measure the efficiency of misextension. In this report, we have analyzed the effects of the E89G and M184V mutations on the efficiency of misextension during DNA-dependent DNA synthesis (DDDP) by HIV-1 RT using a modified version of the gel shift assay previously developed by Goodman and co-workers (18,19). The assay involves measuring the ability of various recombinant purified RTs to insert the correct base on preformed, mispaired primer termini. Contrary to their positive effect on dNTP insertion fidelity, the E89G and M184V mutations, separately or together, did not significantly decrease the efficiency of mispair extension by HIV-1 RT. Therefore, the increased dNTP insertion fidelity of these variant RTs is not necessarily accompanied by an increased primer extension fidelity.

MATERIALS AND METHODS

HIV-1 RTs

The construction of the HIV-1 RT expression vector with the E89G substitution has been described previously (20). The
M184V mutation was introduced from the plasmid pE66M (gift of Clyde Hutchison, University of North Carolina at Chapel Hill; 21) into the overexpression plasmid pRT6H-PROT by polymerase chain reaction (PCR) (22). The E89G/M184V double mutant was generated by replacing the EcoRV-BglII fragment, encompassing the M184 codon, of pRT6H-PROT-E89G with that from pRT6H-PROT-M184V. All of the expression constructs encode the production of the heterodimeric RT. Purification of the wild type and the three mutant HIV-1 RTs preparations of wild type, E89G, M184V and E89G/M184V RTs were in a bound state. A set of reactions was carried out at 37°C increasing concentrations of dTTP, for an empirically determined reaction time (3–30 min), allowing a maximal conversion of 89 U/mg, respectively [1 U is defined as 1 nmol dTTP incorporated into poly(rA)–oligo(dT) in 10 min at 37°C].

Template–primers and dNTPs

DNA oligonucleotides were used as template and primers. The template DNA oligonucleotide was a 45mer corresponding to a region around the primer binding site of the HIV-1 genome. All primers were 28mers, of which one was complementary to the template position, yielding a ‘running finish’ product which is extended at least two bases further than unextended primer. Extended and unextended primers are resolved by denaturing polyacrylamide gel electrophoresis.

Mispair extension reactions

End-labeled 5'-32P-DNA primers with or without 3' terminal mismatches were employed to determine rates at which mispaired primers are extended by HIV-1 RT, via insertion of a dTTP opposite template base A (Fig. 1). The oligodeoxynucleotides used as template and primers were described as previously (8). The primers were end-labeled with 32P using T4 polynucleotide kinase (40 U) (New England Biolabs) and [γ-32P]ATP (3000 Ci/mmol). The template-primer duplexes were annealed by combining 108 pmol 32P-end-labeled primer with 148 pmol template (a template:primer ratio of 1:1.4) in 50 mM Tris–HCl pH 8.0, 50 µg/ml acetylated BSA, 2 mM β-mercaptoethanol (total volume 1 ml), heating to 100°C for 3 min and allowing the mixture to slowly cool to room temperature over 2–3 h.

Reactions (Fig. 2) were performed using purified recombinant wild type or mutant RTs (E89G, M184V and E89G/M184V) in the presence of varying concentrations of dTTP, the first base to be inserted during extension, and an excess of dTTP, to generate the running finish product (see below). The misextension reactions, performed according to procedures described by Goodman and colleagues (18), were initiated by combining equal volumes (5 µl) of enzyme:template–primer solution and dNTP:salts solution (dTTP at a concentration of 0–2 mM and dGTP at 40 µM in 160 mM KCl, 100 mM Tris–HCl pH 8.0, 20 mM dithiothreitol and 12 mM MgCl2). The enzyme:template–primer solution contained purified RT at enzyme concentrations ranging from 2.39 to 23.9 µM corresponding to enzyme:template–primer molar ratios of 1:25–1:2.5, respectively. In general, an excess of template–primer was used to ensure that all enzyme molecules were in a bound state. A set of reactions was carried out at 37°C with increasing concentrations of dTTP, for an empirically determined reaction time (3–30 min), allowing a maximal conversion of ~20% of primer to extended products. Reactions were terminated by the addition of 20 µl of stop solution (95% formamide, 20 mM EDTA). Six to ten microliters of boiled terminated reactions were loaded on to a 14% polyacrylamide gel and electrophoresed for 1.75–2.5 h at 30 W. Gels were then autoradiographed within 1.75–2.5 h at 30 W.
where \( V_{rel} \) is the initial relative velocity of extension, \( IT \) is the intensity of bands corresponding to extension products, \( IT-N \) is the intensity of bands corresponding to unextended primer molecules, and \( t \) is reaction time. Subsequent to plotting \( V_{rel} \) against dNTP concentrations, the initial slope of the curve was used to obtain accurate \( V_{max}, V_{rel} \) and \( K_m \) values via the use of Cleland’s FORTRAN program (24). The enzyme concentration was taken into account when calculating \( V_{max} \) since variable amounts of enzyme were used, depending upon differences in the rates of extension past different mispairs. The efficiency of mispair extension, \( f_{ext} \), for each of the four template–primer pairs (Fig. 1), was then derived using the following equation (23):

\[
f_{ext} = \frac{V_{max}/K_m}{mispair} / \frac{V_{max}/K_m}{correct \ pair}
\]

**RESULTS**

We employed a modified version of the gel mobility shift assay developed by Goodman and co-workers for the measurement of mispair extension efficiencies of HIV-1 RT variants (Fig. 2) using a preformed mismatch at the primer terminus (18). In this modified procedure, termed a ‘running finish’ mispair extension assay, two dNTPs are included. The first dNTP, which is required to extend the mispaired terminus via insertion opposite the next template base (+1 position) to which it is complementary, is present at varying concentrations. A second dNTP, that is complementary to the template base at +2 position, is present in excess at a constant concentration in the reactions (Fig. 1). Thus, any DNA product formed by the proper extension of the mispaired primer terminus will, with a high probability, be extended by at least one more base, allowing better resolution of unextended and extended primers (Figs 2 and 3).

To obtain highly accurate values, we used excess template–primer, a reaction time selected so that the conversion of the mispaired primer terminus will, with a high probability, be extended by at least one more base, allowing better resolution of unextended and extended primers (Figs 2 and 3).

To obtain highly accurate values, we used excess template–primer, a reaction time selected so that the conversion of the end-labeled primers did not proceed beyond 20%, and autoradiography within the linear response range of the film. The \( V_{max}, \) \( r_{ol} \) and \( K_m \) values were determined using a minimum of 4 points (except one case, in which 3 points were used) that corresponded to the initial portion of the Lineweaver–Burke plot and excluding the points that displayed dNTP substrate inhibition. Each mispair extension reaction was performed in triplicate, and standard deviations calculated.

The calculated \( f_{ext} \) values reveal that the wild type and the three mutants extend maximally from G–T mispairs and, in doing so, are \( 10–100 \) times less efficient than when extending from the correct pair (G–C) (Table 1). This is compatible with the fact that G–T basepairs are the most stable among non-Watson–Crick base pairs. The wild type and the two single mutants display a trend in their \( f_{ext} \): G–T > G–G > G–A, while the double mutant (E89G/M184V) shows relatively uniform levels of \( f_{ext} \) for all mispairs. Interestingly, changes in \( f_{ext} \) displayed by any mutant enzyme on the mispairs studied tended to be minimal when compared to the corresponding values determined for the wildtype RT. The only notable exception was the efficiency of extension from G–A mispairs by the E89G and the double mutant RTs, which increased by 10- and 29-fold, respectively, over wild type (Table 1).

In general, the efficiency of mispair extension was not significantly decreased for any of the mutant RTs compared to wild type. In fact, the misextension efficiency was lower only for E89G and the double mutant when extending from G–G and G–T mispairs (Table 1).

**DISCUSSION**

Biochemical analysis of the effect of the E89G and M184V mutations on the fidelity of dNTP insertion had previously revealed that these alterations decrease the efficiency of misinsertion (6–10). The residues altered by both of these mutations contact the template–primer duplex and thereby influence the geometry of the dNTP-binding pocket (25,26). The two mutations share many features in common: both residues contact template–primer, alterations at both residues confer resistance to multiple dideoxynucleoside analogs and both result in an increase in the misinsertion fidelity of HIV-1 RT (6–10). The M184V alteration is of particular significance since, in the wild type RT, residue 184 contacts the 3′-OH terminus of the primer. Although both of these mutations confer an increased misinsertion fidelity on HIV-1 RT, measurement of the overall error rate indicated a lack of increase in fidelity (17). Because misinsertion must be followed by mispair extension in order to misincorporate a nucleotide during viral DNA synthesis, increases or decreases in mispair extension efficiency will also affect the overall mutation rate. Therefore, it was of interest to determine whether these amino acid alterations affect mispair extension.

Interestingly, the patterns of mispair extension efficiencies by the three mutants are more similar to those of the wild type HIV-1 RT than they are different. For example, the pyrimidine–purine mispair (G–T) was more efficiently extended than purine–purine mispairs (G–G, G–A), as seen by other investigators for wild type RT (9). Additionally, the two mutations studied here did not significantly decrease the ability of RT to extend past the mispaired primer termini. It appears from our results that the M184V alteration led to little, if any, change in the mispair extension efficiencies of HIV-1 RT for the types of mispairs tested here: 2.0- (G–A), 1.8- (G–G) and 3.6-fold (G–T) increase in \( f_{ext} \) over wild type HIV-1 RT. For two of the three mispairs tested (G–G and G–T), the other two mutants, E89G and E89G/M184V, displayed small decreases in mispair extension efficiencies. There was a large increase in \( f_{ext} \) for E89G (10-fold) and E89G/M184V (29-fold) RTs on G–A mispairs. It is possible that such large changes could serve to counterbalance the decreased efficiency of specific misinsertions observed (9). These studies indicate that E89G and M184V mutations do not have a global effect on RT fidelity, but rather specifically increase the dNTP insertion fidelity. Similar observations were made by Mendelman et al. (23) who found that rates of misextension and misinsertion do not correlate in a comparison of pol α and AMV RT.

The mispair extension assay has been used by many laboratories to measure the fidelity of nucleoside analog-resistant variants of HIV-1 RT (7,9,10,27). However, it appears that the results obtained vary depending on the template (RNA or DNA), the nature of the mismatch, the sequence context and the RT that is being used for the study. For example, studies by Rubinek et al. show that there was a 7–66-fold decrease in \( f_{ext} \) for E89G when extending A–C and A–A mispairs, using two templates with differing sequences (9). Similarly, Hsu et al., in an RNA-dependent DNA synthesis reaction, reported significant decreases in the efficiencies of extension from A–G (49-fold), A–C (16-fold) and A–A (3-fold) mispairs by M184V RT compared to wild type enzyme (27). These observed increases in the misextension
Figure 3. Autoradiograms of 5′-end-labeled primer extension products. (A) Extension of correctly-paired (G–C) primer termini by wild type, M184V, E89G and E89G/M184V RTs. Bands, resolved here by 14% urea–PAGE, correspond to unextended primer (intense, lower band) and primers extended by two bases (lighter, upper band). The concentration of the variable dNTP, dTTP, is indicated. Note that dTTP concentrations >8 µM did not display linear kinetics and were not employed in kinetic analyses. Below, a schematic of the template–primer used is shown, with the +1 template position indicated in bold. (B) Extension of G–G-mispaired primer termini by wild type, M184V, E89G and E89G/M184V RTs. The intense band corresponds to unextended primer. The three bands of weaker intensity seen at higher dTTP concentrations correspond to primers extended up to template positions C, G and A (at +2, +3 and +4), respectively. As before, points corresponding to dNTP concentrations >1000 µM (which did not display linear kinetics) were not used for $K_m$ and $V_{max}$ measurements. The mispaired terminus is shown schematically at the bottom.

fidelity parallel the increase in dNTP insertion fidelity reported (7–10). If the increases in primer extension fidelity observed with different template sequences by many laboratories for M184V and E89G indeed apply to all mispair combinations, one could expect a cumulative effect of the increased fidelities of dNTP insertion and primer extension, perhaps decreasing the overall mutation rate of the variant viruses containing these substitutions. However, the overall error rates of E89G and M184V RTs were...
Table 1. The mispair extension efficiencies ($f_{ext}$) of wild type, E89G, M184V and E89G/M184V RTs

<table>
<thead>
<tr>
<th>5' terminus</th>
<th>RT</th>
<th>$bK_m$ (µM)</th>
<th>$V_{max,rel}$ (%/min)</th>
<th>$V_{max,rel}/K_m$</th>
<th>$f_{ext}$</th>
<th>$\Delta f_{ext}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.C</td>
<td>Wild type</td>
<td>2.14 ± 1.4</td>
<td>14300 ± 6600</td>
<td>6680</td>
<td>1</td>
<td>1</td>
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<tr>
<td></td>
<td>E89G</td>
<td>3.53 ± 1.38</td>
<td>1410 ± 480</td>
<td>399</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M184V</td>
<td>3.96 ± 0.55</td>
<td>6380 ± 118</td>
<td>1600</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>E89G/M184V</td>
<td>5.44 ± 0.52</td>
<td>1390 ± 266</td>
<td>256</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>G.A</td>
<td>Wild type</td>
<td>653 ± 450</td>
<td>628 ± 129</td>
<td>0.96</td>
<td>0.00014</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>E89G</td>
<td>289 ± 65</td>
<td>165 ± 23</td>
<td>0.57</td>
<td>0.0014</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>M184V</td>
<td>613 ± 340</td>
<td>164 ± 44</td>
<td>0.27</td>
<td>0.00016</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>E89G/M184V</td>
<td>280 ± 168</td>
<td>294 ± 30</td>
<td>1.05</td>
<td>0.0040</td>
<td>29</td>
</tr>
<tr>
<td>G.G</td>
<td>Wild type</td>
<td>311 ± 26</td>
<td>5210 ± 1180</td>
<td>16.7</td>
<td>0.0025</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>E89G</td>
<td>2900 ± 35</td>
<td>1750 ± 148</td>
<td>0.60</td>
<td>0.0015</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>M184V</td>
<td>490 ± 351</td>
<td>3530 ± 655</td>
<td>7.20</td>
<td>0.0045</td>
<td>1.8</td>
</tr>
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<td></td>
<td>E89G/M184V</td>
<td>1940 ± 35</td>
<td>1020 ± 28</td>
<td>0.53</td>
<td>0.0020</td>
<td>0.8</td>
</tr>
<tr>
<td>G.T</td>
<td>Wild type</td>
<td>95 ± 22</td>
<td>12300 ± 1920</td>
<td>135</td>
<td>0.020</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>E89G</td>
<td>473 ± 50</td>
<td>3250 ± 35</td>
<td>6.87</td>
<td>0.017</td>
<td>0.85</td>
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<tr>
<td></td>
<td>M184V</td>
<td>38 ± 12</td>
<td>4300 ± 931</td>
<td>113</td>
<td>0.071</td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>E89G/M184V</td>
<td>524 ± 320</td>
<td>1040 ± 303</td>
<td>1.98</td>
<td>0.008</td>
<td>0.40</td>
</tr>
</tbody>
</table>

- Template base is shown first, followed by the base at primer terminus.
- Represents the mean of three determinations ± standard deviations.
- Generated by using the mean $V_{max}$ and $K_m$ values.
- Derived from the $V_{max}$ and $K_m$ values (see text for equation).
- Ratio of mutant to wild type RT $f_{ext}$ values for a given mispair.

unaltered in forward mutation assays (17). Some laboratories have reported minimal changes in the efficiency of mispair extension by M184V RT. Results reported by Oude Essink et al. show very small increases in the efficiency of extension from G–U mispairs between wild type and M184V RT (10). Similarly, when Pandey et al. studied the effect of the M184V mutation on the efficiency of extension from A–A, A–G, A–C or T–T mispairs, they found a slight decrease in the efficiency of extension by M184V RT (at 0-, 3-, 0- and 1.4-fold, respectively) (7). In agreement with these observations, our results do not show a significant decrease in mispair extension efficiency during DDP (for any of the variant RTs studied) for mispairs involving a template G base, but rather an actual increase in the efficiency of extension for one subset of the results. An increase in the fidelity of both dNTP insertion and extension of some mispairs could be counterbalanced by a decrease in fidelity or lack of change with respect to other types of errors. Thus it is likely that a lack of change in the overall fidelity of the variant RTs bearing the E89G, M184V or both mutations, may result from increases in some types of errors combined with decreases in misinsertion efficiency.

Based on our results, it appears that E89G and M184V variants of HIV-1 RT do not display an increased misextension fidelity compared to wild type. The effects of these mutations on different aspects of error formation by RT must be taken into account prior to assessing their overall impact on in vitro polymerase fidelity.

availability of HIV vectors containing reporter genes (28) should help analyze the impact of RT mutations on the overall mutation rate during a single cycle of virus replication. It appears that prior to establishing a relationship between polymerase fidelity and viral mutation rate, the influence of drug resistance mutations on various types of polymerase errors (e.g., fidelity of dNTP insertion, mispair extension, slippage-mediated errors) should be quantified.

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**REFERENCES**