3066–3072 *Nucleic Acids Research, 1998, Vol. 26, No. 12*

The influence of 3TC resistance mutation M184I on the fidelity and error specificity of human immunodeficiency virus type 1 reverse transcriptase

Lisa F. Rezende, William C. Drosopoulos and Vinayaka R. Prasad*

Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

Received November 24, 1997; Revised and Accepted April 22, 1998

ABSTRACT

A common target for therapies against human immunodeficiency virus type 1 (HIV-1) is the viral reverse transcriptase (RT). Treatment with the widely used nucleoside analog (–)-2′,3′-deoxy-3′-thiacytidine (3TC) leads to the development of resistance-conferring mutations at residue M184 within the YMDD motif of RT. First, variants of HIV with the M184I substitution appear transiently, followed by viruses containing the M184V substitution, which persist and become the dominant variant for the duration of therapy. In the three-dimensional crystal structure of HIV-1 RT complexed with double-stranded DNA, the M184 residue lies in the vicinity of the primer terminus, near the incoming dNTP substrate. Recent studies have shown that 3TC resistance mutations, including M184I, increase the nucleotide insertion and mispair extension fidelity. Therefore, we have examined the effects of the M184I mutation on the overall polymerase fidelity of HIV-1 RT via an M13-based forward mutation assay. We found the overall error rate of the M184I variant of HIV-1 RT to be 1.7×10^{-5} per nucleotide. This represents a 4-fold increase in fidelity over wild-type HIV-1_{Hxb2} RT (7.0×10^{-5} per nucleotide) and a 2.5-fold increase in fidelity over the M184V variant (4.3×10^{-5} per nucleotide). Of the nucleoside analog resistance mutations studied using the forward assay, the M184I variant has shown the greatest increase in fidelity observed to date. Interestingly, the M184I variant RT displays significantly altered error specificity, both in terms of error rate at specific sites and in the overall ratio of substitution to frameshift mutations in the entire target.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infections are characterized by a high degree of genetic variation, resulting in the emergence of drug-resistant variants during treatment. A common target of anti-HIV-1 therapies is the reverse transcriptase (RT), which performs the DNA synthesis necessary to replicate the HIV-1 genome. To date, five nucleoside analogs which target RT have been approved for treating infected individuals. The development of resistance to each of these drugs is well documented (1,2).

The most potent single agent among the approved nucleoside analog inhibitors is (–)-2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine). However, in both cell culture and infected patients, resistance to 3TC develops rapidly. Resistant variants containing an M184I alteration in their RT sequence appear transiently and are replaced by those with the M184V substitution (3,4). The M184V alteration also confers a low level of resistance to ddC and ddI (5–7). These mutations are biochemically interesting because the M184 residue lies in the highly conserved YMDD motif in RT and in the three-dimensional crystal structure of HIV-1 RT complexed with double-stranded DNA the residue lies at the active site, near the incoming dNTP substrate (8,9).

Several studies suggest that nucleoside analog resistance can be associated with an increased RT fidelity. For example, it has been shown that recombinant RT enzymes containing the M184V mutation display a 2- to 45-fold increase in nucleotide insertion fidelity when compared with the wild-type (10,11). In addition, the multi-drug-resistant E89G variant of RT demonstrated a 2- to 17-fold increase in nucleoside insertion fidelity (12,13). Similarly, recent studies using M184I enzyme have shown an increase in both misinsertion and misextension fidelity (14,15). Since nucleoside analog resistance mutations enable RT to discriminate between a nucleoside analog inhibitor and its normal dNTP substrate, these results appear to suggest that such mutations may also allow RT to better discriminate between an incorrectly base paired dNTP and the Watson–Crick base paired dNTP substrate. In general, the assays employed in these studies were designed to measure misinsertion or misextension alone. One assay, the read-through assay, measured the net result of both misinsertion and mispair extension by omitting a single dNTP from the polymerization reaction, allowing quantitation of DNA products that were extended beyond the template site at which the missing dNTP was to be inserted. Thus, the studies cited above examined the individual contributions or the sum of two types of errors. However, a variety of processes contribute to overall polymerase fidelity, including misinsertion and extension of mispaired termini, as well as slippage-mediated errors (16). To assess the overall fidelity of the M184I variant of HIV-1 RT, we have employed an M13-based, gap filling assay which utilizes the *lacZα* gene as reporter (17,18). In results reported in this

*To whom correspondence should be addressed. Tel: +1 718 430 2517; Fax: +1 718 430 8976; Email: prasad@aecom.yu.edu

communication, we find that the M184I variant RT displays a 4-fold decrease in overall error rate when compared with the wild-type RT and a 2.5-fold decrease when compared with the M184V RT, suggesting that the M184I mutation confers increased overall fidelity upon RT. Additionally, the M184I variant generates fewer mutations at known wild-type RT hotspots and an altered pattern of frameshift mutations when compared with wild-type or M184V RTs.

MATERIALS AND METHODS

Phage and bacterial strains

The bacteriophage M13mp2 was used to generate the gapped duplex DNA substrate. M13 phage was grown in *Escherichia coli* strain NR9099 [Δ(*pro-lac*), *thi*, *ara*, *rec*A56/F′ (*pro*AB, *lac*IqZ-ΔM15)] for the production of both single-stranded phage DNA and double-stranded RF DNA. *Escherichia coli* strain MC1061 [*hsd*R, *hsd*M$^+$, *ara*D, Δ(*ara*, *leu*), Δ(*lac*IPOZY), *gal*U, *gal*K, *str*A] was used for the generation of mutant phage. *Escherichia coli* strain CSH50 [Δ(*pro-lac*), *thi*, *ara*, *str*A/F′ (*pro*AB, *lac*IqZ-ΔM15, traD36)] was the α-complementation strain used to visualize the phenotype of the mutant phage.

Enzymes

Wild-type HIV-1_{Hxb2} RT and enzymes containing the M184V and M184I mutations were bacterially expressed and purified. Phasmids (pIBI20) containing the cDNA clones of the *pol* gene from wild-type HIV-1_{Hxb2}, M184V and M184I (a kind gift of Dr Clyde Hutchison) (19) were used as the source of RT sequences. The cDNA encoding the RT sequence was PCR amplified using an upstream primer containing the recognition site for the restriction enzyme *Not*I and 27 nt at the beginning of RT and a downstream primer containing 25 nt at the end of the RT p66 sequence and the recognition site for *Bgl*II. PCR products were passed through a PCR magic column (Promega) and digested with *Not*I and *Bgl*II (New England Biolabs). The PCR products were subcloned into the high level expression vector pL6H-PROT (20), which contains a hexahistidine tag at the 3′-end of the cloning site and co-expresses the HIV-1 protease (PR), allowing the generation of heterodimeric RT.

Proteins were expressed in the M15 strain of *E.coli* containing the *lac* repressor plasmid pDMI.1. One liter cultures containing the RT/PR and *lac* repressor plasmids were grown to an A_{600} of 0.4 followed by addition of IPTG (Sigma) to a concentration of 1 mM and the cultures allowed to grow for 4 (M184V RT) or 6 h (wild-type HIV-1_{Hxb2} and M184I RT). Cells were lysed in 7 ml lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 20 mM imidazole, 1 mg/ml lysozyme and 100 µM PMSF) for 30 min at 4°C, then sonicated three times for 1 min each with 2 min cooling intervals and passed through a 21½ gauge needle five times. Lysates were spun down at 12 000 *g* at 4°C for 30 min. Supernatants were added to 125 µl NTA–Ni^{2+}–Sepharose beads (Qiagen) and allowed to bind for 30 min at 4°C. Beads were spun down at 2000 *g* for 2 min, then washed three times in 50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 20 mM imidazole and 100 µM PMSF. RT was batch-eluted by incubating the beads in 1 ml 50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 250 mM imidazole and 100 µM PMSF for 30 min at 4°C, then spinning for 2 min at 2000 *g*. Eluent was dialyzed against buffer D (50 mM Tris–HCl, pH 7.0, 25 mM sodium chloride, 1 mM EDTA and 5% glycerol) overnight in a Slide-a-Lyzer (Pierce). Dialyzed proteins were collected from the Slide-a-Lyzer (average volume 800 µl), added to 500 µl DEAE–Sepharose beads (Sigma) and allowed to incubate for 15 min at 4°C. The beads were spun down at 400 *g* for 5 min at 4°C and supernatants recovered. Supernatants were allowed to bind to 200 µl S-Sepharose (Sigma) for 1.5 h at 4°C, then centrifuged at 2000 *g* for 5 min. Beads were washed three times in buffer D, then proteins were eluted twice in 400 µl buffer D + 200 mM sodium chloride by incubating in the cold for 15 min. Supernatants were collected and dialyzed against storage buffer (50 mM Tris–HCl, pH 7.0, 25 mM sodium chloride, 1 mM EDTA and 50% glycerol) overnight. The protein was collected from the Slide-a-Lyzer, aliquoted and stored at –80°C.

The specific activities of the purified enzymes were found to be 310, 520 and 370 U/mg for the wild-type, M184V and M184I variant RTs respectively. One unit is defined as the amount of enzyme required to incorporate 1 nmol dTMP into DNA on poly(rA)·oligo(dT) template–primer at 37°C in 10 min. The enzyme preparations were shown to be DNase free in an incubation with ^{32}P-labeled DNA for 2 h under conditions that were used for gap filling reactions (data not shown).

Determination of forward mutation frequency

The gapped duplex M13mp2 DNA used as template–primer for DNA synthesis by RTs contained a gap of 361 nt, including the first 107 nt of *lacZ*α coding sequence and 121 nt of regulatory sequence. This substrate was prepared as previously described (18). DNA synthesis reactions by each of the purified RTs were performed in a 25 µl reaction containing 75 mM Tris–HCl, pH 8.0, 80 mM KCl, 6 mM $MgCl_2$, 10 mM DTT, 500 µM each dATP, dCTP, dGTP and dTTP (21) (Boehringer Mannheim), 59 ng gapped duplex DNA and 0.10 (wild-type, M184V) or 0.17 U (M184I) purified RT for 1 h. Reactions were stopped by adding EDTA to a concentration of 20 mM. Complete synthesis of the gapped region was confirmed by agarose gel electrophoresis (data not shown). Two to four independent gap filling reactions were performed for each enzyme in this study.

Products of the fill-in reactions were electroporated into *E.coli* strain MC1061. Reactions were electroporated in two to nine separate sets. After electroporation, the cells were allowed to recover for 10 min, then mixed with a log phase culture of *E.coli* strain CSH50. The mixed cells were overlaid, in top agar, on M9 plates containing 0.2 mM IPTG and 0.195 mM X-gal (Labscientific Inc, Livingston, NJ). Plates were incubated at 37°C for ~15 h before scoring for mutant plaques that did not display the dark blue color of the wild-type M13mp2. These included both clear plaques and three grades of reduced intensity blue plaques. Mutant plaques were picked from the plates and stored in 1 ml 0.9% NaCl at 4°C. Mutational frequencies were determined by dividing the number of confirmed mutants by the total number of plaques screened. Background mutation frequency was determined by electroporating unfilled gapped duplex DNA and scoring for mutants as above. All mutants identified in the initial screen were confirmed by picking, resuspending and replating alongside wild-type phage.

Sequencing of mutant templates

Single-stranded DNA was prepared from mutant plaques as described by Kunkel (18). Briefly, 50 µl plaque supernatant and 200 µl overnight culture of *E.coli* strain CSH50 were added to

1.8 ml 2× YT broth and incubated at 37°C for ~15 h. Culture supernatants were collected and phage precipitated with a quarter volume of 15% PEG, 2 mM NaCl for 6 min at 4°C. Phage were decoated by incubating with 50 µg proteinase K for 30 min, then adding a one-tenth volume of 5% CTAB (Sigma) in 0.5 mM NaCl for 10 min. The reaction was centrifuged at 12 000 *g* and the pellets were resuspended in 1.2 M NaCl. Single-stranded DNA was precipitated in 2.5 vol ethanol.

The phage DNA was sequenced using a Sequenase 2.0 DNA Sequencing kit (Amersham). Sequenced products were resolved on a 6% denaturing polyacrylamide gel using a Genomyx (Foster City, CA) DNA sequencer.

Calculation of error rates

Error rates were calculated as described by Kunkel (18). Briefly, the mutational frequency is corrected by subtracting background mutational frequency. The corrected mutational frequency is multiplied by the percentage of all mutations represented by the particular class of mutations (e.g. base substitutions). This number is divided by 0.6 (the likelihood of expression of the newly synthesized strand in *E.coli*), then divided by the total number of sites where this class of mutations can be detected.

Statistical analysis

Statistical differences between the proportion of mutations at specific sites and of the proportion of mutations of a particular class were assessed by Fisher's exact test, using the Statview statistical software package.

RESULTS

Determination of mutational frequency of wild-type, M184V and M184I RTs

The mutation frequencies of the wild-type HIV-1_{Hxb2} RT and its M184V and M184I variants were determined using a forward assay (18) in which recombinant purified RTs were employed to fill in a single-stranded gap in a duplex M13 DNA substrate. The gapped region of the substrate included 361 nt of *lacZ*α. As detailed in Materials and Methods, two to four independent fill-in reactions were performed with each RT and each fill-in reaction was electroporated in two to nine portions. Complete filling in of the substrate was assessed by agarose gel electrophoresis. All three RTs were capable of synthesizing DNA across the gap (data not shown).

The filled in phage DNAs were electroporated into a producer strain of *E.coli*, the phage released from transformants used to infect an α-complementation strain of *E.coli* and the resulting plaques screened for β-galactosidase activity. Approximately 30 000–52 000 plaques were screened for each enzyme. The mutation frequencies for the enzymes were determined by pooling data from each independent fill-in reaction and electroporation. The background-adjusted overall mutation frequencies for the wild-type, M184V and M184I RTs were determined to be 9.7×10^{-3}, 6.0×10^{-3} and 2.4×10^{-3} respectively (Table 1). The background mutation frequency was 1.5×10^{-3} (35 mutant plaques scored from 22 681 plaques screened). The mutation frequency of M184I was determined to be 2.5-fold lower than M184V and 4-fold lower than the wild-type RT.

Spectrum of mutations generated by M184I RT

To determine the spectrum of mutations generated by M184I and to compare this spectrum with those generated by the wild-type and M184V mutated RT, ~200 randomly selected phage mutants generated by the wild-type, M184V and M184I were sequenced. A comparison of the spectrum of mutations generated by the M184I and wild-type enzymes as well as that of the spectrum generated by M184I and M184V are presented in Figure 1.

Examination of the spectrum of mutations generated by the M184I mutant RT reveals several mutational hotspots. A hotspot in this study is defined as a site or a run of nucleotides with 10 or more mutations. Hotspots for base substitution errors are apparent at nucleotide positions –36 (T→C mutation), 88 (predominantly G→A), 89 (predominantly A→G) and 112 (predominantly T→C). As previously observed, these hotspots occur at runs of nucleotides (22). In addition to the mutations shown, two of the templates sequenced (each from separate gap filling reactions) contained large deletions. One deletion was 81 nt long, spanning positions 144–63. The second deletion was 78 nt long, beginning at the first nucleotide synthesized (position 145) and ending at position 67. A total of eight of the mutant phage generated by the M184I variant that were sequenced (4%) had mutations which involved more than one position.

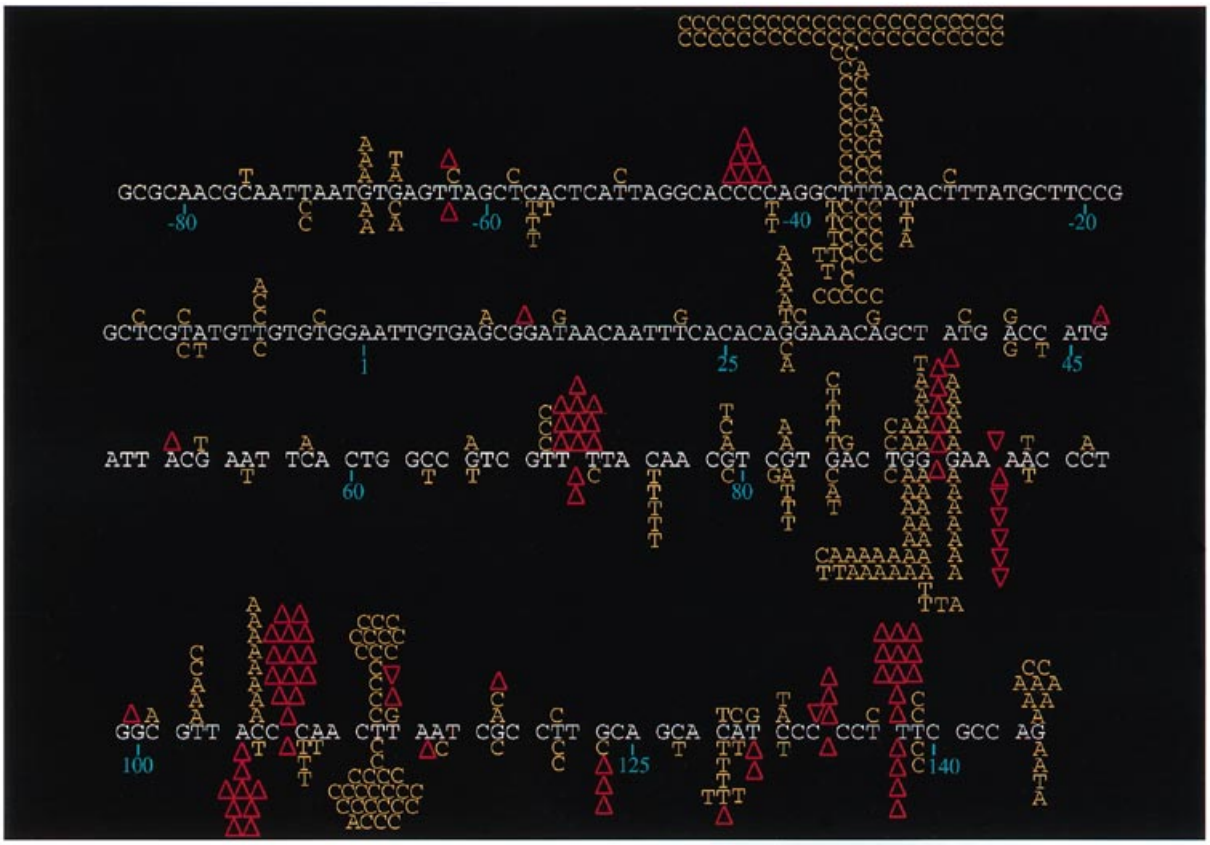
Table 1. Overall mutation frequencies of the three RTs

	Plaques screened[a]	Mutants[a]	Mutation frequency ($\times 10^{-3}$)[b]
Wild-type[c]	29 852	334	9.7
M184V[c]	36 097	268	5.9
M184I	52 046	203	2.4

[a]Numbers represent pooled totals from two independent fill-in reactions.
[b]These values were derived by subtracting the background mutation frequency (1.50×10^{-3}). Background mutation frequency was determined by electroporating gapped duplex DNA into the producer cells and screening the resulting plaques for mutants.
[c]Some of the data for the wild-type and M184V enzymes is from Drosopoulos and Prasad (27) and is included here for comparison.

Figure 1. (opposite) The spectrum of mutations generated by wild-type HIV-1_{Hxb2}, M184V and M184I RTs. The wild-type *lacZ*α sequence is shown with the nucleotide positions indicated below the sequence. (**A**) A comparison of the spectrum of mutations generated by the wild-type (above template sequence) and the M184I variant of HIV-1 RT (below template sequence). Base substitutions (in yellow) are noted directly above or below the *lacZ*α sequence and represent the nucleotide in the mutated template. Frameshift mutations (in red) are indicated with an upright triangle for single base deletions and an inverted triangle for single base insertions. Since it cannot be determined exactly which base in a run of nucleotides has been deleted or inserted, the frameshift events are indicated in the middle of the run. (**B**) A comparison of mutations generated by M184V (above template sequence) and M184I (below template sequence). In addition to the mutations shown here, two templates generated by the M184I variant and one template generated by the M184V variant contained large deletions (see text). Some of the sequence data from the wild-type HIV-1_{Hxb2} and M184V enzymes appeared in Drosopoulos and Prasad (27) and are provided here for comparison.

A


GCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCG
-80
-60
-40
-20
GCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCT ATG ACC ATG
1
25
45
ATT ACG AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT GAC TGG GAA AAC CCT
60
80
GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA CAT CCC CCT TTC GCC AG
100
125
140

B

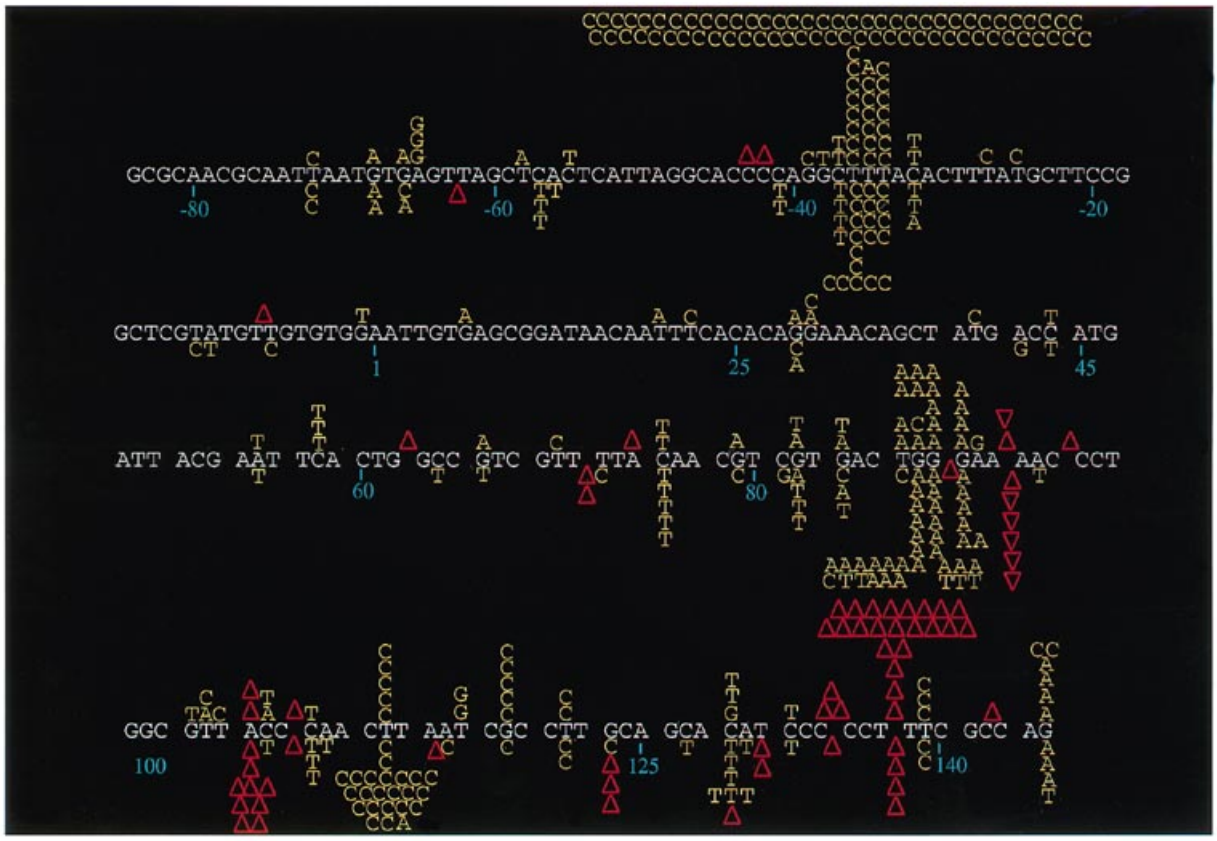

GCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCG
-80
-60
-40
-20
GCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCT ATG ACC ATG
1
25
45
ATT ACG AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT GAC TGG GAA AAC CCT
60
80
GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA CAT CCC CCT TTC GCC AG
100
125
140

Table 2. Comparison of error rates at specific hotspots

Position	M184I		Wild-type[a]		M184V[a]	
	Number of errors	Error rate	Number of errors	Error rate	Number of errors	Error rate
Base substitutions						
–36	11	1/4200	54[b]	1/270	76[b]	1/290
88	19	1/2400	2[b]	1/7400	2[b]	1/11 000
89	12	1/3800	6[d]	1/2500	9[c]	1/2500
112	22	1/2100	14[c]	1/1000	5[b]	1/4500
Frameshifts						
70–74	2	1/93 000	10	1/9000	0	CD
106–108	1	1/190 000	14[c]	1/4200	1	1/67 000
137–139	4	1/35 000	11	1/4000	23[b]	1/2900
All mutations	184	1/59 000	238	1/14 000	219	1/23 000

[a]Some of the data for the wild-type and M184V enzymes was reported in Drosopoulos and Prasad (27) and is included here for comparison.
[b]$P \leq 0.002$ by Fisher's exact test when compared with errors made by M184I enzyme.
[c]$P < 0.05$ by Fisher's exact test when compared with errors made by M184I enzyme.
[d]$P < 0.06$ by Fisher's exact test when compared with errors made by M184I enzyme.
CD, cannot be determined.

Table 3. Summary of error rate for various classes of mutations

Mutation type	M184I		Wild-type[a]		M184V[a]	
	Number of errors	Error rate	Number of errors	Error rate	Number of errors	Error rate
All classes	184	1/59 000	238	1/14 000	219	1/23 000
Frameshifts	32	1/210 000	60	1/36 000	38	1/83 000
At runs	17	1/200 000	57[b]	1/19 000	35	1/45 000
At non-runs	15	1/230 000	3[b]	1/370 000	3[b]	1/560 000
Base substitutions	150	1/32 000	178	1/8 800	180	1/13 000

[a]Some of the data for the wild-type and M184V enzymes was reported in Drosopoulos and Prasad (27) and is included here for comparison.
[b]$P < 0.002$ by Fisher's exact test when compared with errors made by M184I variant.

A comparison of the spectra of mutations generated by the M184I and wild-type RTs revealed several differences (summarized in Table 2). Both the M184I and wild-type enzymes show mutational hotspots for base substitutions at nucleotide positions –36 and 112. However, for M184I, the number of mutations at –36 is greatly decreased (11 mutations for M184I compared with 54 for the wild-type; $P < 0.0001$, Fisher's exact test) and the number of mutations generated at position 112 is increased (22 mutations for M184I compared with 14 for the wild-type; $P < 0.04$, Fisher's exact test). The wild-type enzyme makes fewer mutations at positions 88 ($P < 0.0001$, Fisher's exact test) and 89 ($P < 0.06$, Fisher's exact test). In addition to the base substitution hotspots, the wild-type enzyme has frameshift hotspots within the run of nucleotides at positions 70–74, 106–108 ($P < 0.003$, Fisher's exact test) and 137–139, where M184I does not generate many mutations. Differences are also apparent when comparing the spectrum of mutations generated by the M184I and M184V variants of HIV-1 RT. Unlike M184I RT, the M184V variant generates only two mutational hotspots, a base substitution hotspot at position –36 ($P < 0.0001$, Fisher's exact test) and a frameshift hotspot at the run of T residues between nt 137 and 139 ($P < 0.001$, Fisher's exact test).

Interestingly, the M184I variant shows a reduction in frameshift mutations when compared with the wild-type, as well as an increase in the number of mutations occurring outside runs of nucleotides. In the case of wild-type RT, 25% of the errors are single base insertions or deletions, while for the M184I variant, only 16% of the errors are in this class ($P < 0.06$, Fisher's exact test; Table 3). In addition to this difference, the M184I variant makes a significantly higher proportion of frameshift errors outside runs of nucleotides (46% versus 5% for the wild-type enzyme; $P < 0.0001$, Fisher's exact test; Fig. 2 and Table 3).

DISCUSSION

Several recent studies have shown that nucleoside analog resistance correlates with an increased fidelity of nucleotide insertion (10–14) and mispair extension (13–15). These studies examined only a single aspect of polymerase fidelity (nucleotide insertion or mispair extension). In addition, the assays were performed under conditions in which the proper nucleotide was absent and, therefore, do not measure fidelity under conditions in which a correct dNTP is selected from a pool of all dNTPs. HIV-1

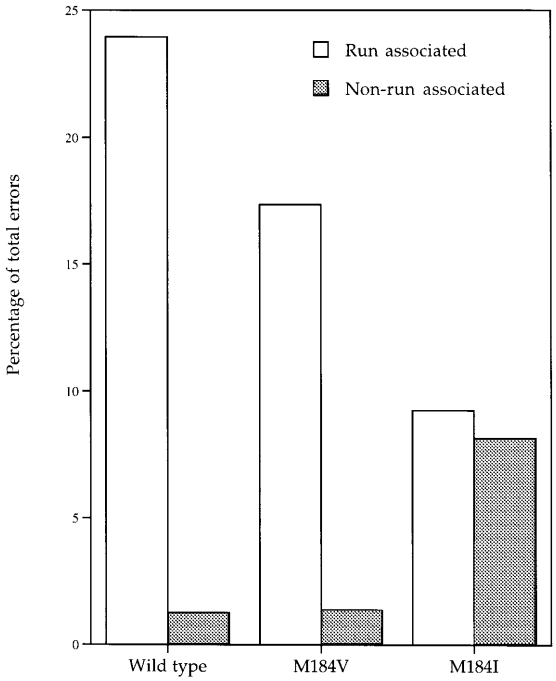


Figure 2. Bar graph illustrating the percentage of single base insertions and deletions occurring within (closed bars) or outside (open bars) runs of nucleotides as a percentage of the total mutations made by the wild-type, M184V and M184I RTs.

RT can make a number of errors, including base substitutions, mispair extension and multiple types of slippage-mediated frameshift errors (22). The M13-based forward mutation assay used here allows the detection of all classes of polymerase errors. We have used this assay to measure the polymerase fidelity of the 3TC-resistant M184I variant of HIV-1 RT. We have found that in this assay the overall error rate is 1.7×10^{-5} per nucleotide (1 in 59 000; Table 3), which represents a 4-fold decrease when compared with the wild-type (7.0×10^{-5} per nucleotide, 1 in 14 000) and a 2.5-fold decrease when compared with M184V (4.3×10^{-5} per nucleotide, 1 in 23 000) (Table 3).

The values we obtained for the overall mutation frequency and the spectrum of mutations generated by HIV-1_{HXB2} RT differ from previously published values for the wild-type enzyme. The difference in mutation frequencies [97×10^{-4} for HIV-1_{HXB2} in this work versus 800×10^{-4} for HIV-1_{NY5} (17) and 200×10^{-4} for HIV-1_{HXB2} previously (23,24)] can be attributed, in good part, to differences in the strains of HIV that served as the source of RT used in these experiments. The wild-type RT from the HIV-1_{NY5} strain has been shown to have an ~200-fold lower affinity for template–primer (25). This could lead to a lower processivity, which in turn contributes to the lower fidelity observed for this variant. When comparing the results of other laboratories (23,24) using the same assay with the RT from the HIV-1_{Hxb2} strain, the difference between our numbers and the previously published numbers is a mere 2-fold (see above). The differences in the mutation spectrum can also be attributed to the defect in template–primer binding of the HIV-1_{NY5}-derived RT. The major difference in error specificity is in the proportion of frameshift mutations generated. While the previous study with the HIV-1_{NY5} RT revealed ~50% frameshift mutations (22), the wild-type HIV-1 RT from three separate strains of HIV-1, HIV-1_{HXB2} (present study), HIV-1_{BH10} (26) and HIV-1_{NL4-3} (L.F.Rezende and V.R.Prasad, unpublished observations) generated only ~25% frameshift mutations.

The increase in overall fidelity of the M184I variant compared with the wild-type correlates well with previous observations. Using a DNA template, Oude Essink *et al*. have shown that M184I exhibits a 2.3-fold decrease in read-through efficiency, an 8-fold increase in nucleotide insertion fidelity (at a GT mismatch) and an ~1.5-fold increase in mispair extension fidelity (14). An increase in mispair extension fidelity (of 1.5- to 6.0-fold) has also been observed when an RNA template is used (14,15). Small increases in read-through (misincorporation) fidelity (1.7-fold) and mispair extension fidelity (1- to 1.7-fold) of M184I over M184V have also been observed (14). However, M184V showed a larger increase in mispair extension fidelity when an RNA template was used (15).

Alterations at the M184 residue have been shown to increase fidelity, decrease activity and decrease processivity (14,15,19,31,32). In all cases, the M184I variant displays larger deviations from the wild-type than the M184V variant. The mechanistic basis for these differences is currently unclear. Pandey and colleagues (11) modeled the ternary complex of HIV-1 RT with DNA and dNTP and used these models to investigate the effects of alterations at the 184 residue. The models suggest that the M184V alteration places its amino acid side chain in a conformation which may optimize the interaction between the side chain and the sugar base of the incoming dNTP (11). When amino acids with larger side chains were modeled (M184L and M184Y), a steric hindrance was observed between the side chain and the primer terminus, rendering the enzyme inactive (11). Unfortunately, a model for the M184I variant has not been described. However, based on the models reported by Pandey *et al*., one could speculate that its longer side chain interferes with the dNTP binding pocket (causing lower activity) and, still being able to allow favorable interactions with the sugar base, via an unknown mechanism, thus increases fidelity.

The M184I variant RT has shown the largest increase in polymerase fidelity yet observed using the M13-based forward mutation assay when comparing a nucleoside analog-resistant variant with the wild-type RT (26,27). The increase in fidelity is in relative agreement with previously published studies (14). This differs from the observed differences in fidelity when one compares gel-based fidelity assays and forward mutation assays for the M184V and E89G variants (27). For these two mutants, the forward mutation frequencies were very similar to those of wild-type HIV-1 RT. It is conceivable that the strong agreement between the gel-based fidelity studies and the forward mutation studies can be attributed to the relatively few frameshift errors made by the M184I variant. A single cycle infection study would be required to assess the effects of the M184I mutation on overall HIV-1 replication fidelity.

The M184I variant RT generates a spectrum of mutations along the *lacZ*α template which differs from wild-type and M184V RTs in the specificity of errors. One major distinction concerns the error frequencies at particular sites. Although M184I creates base substitution errors at hotspots known for wild-type HIV-1 RT (Fig. 1 and Table 2), the error frequencies are different. For example, the M184I variant makes significantly fewer errors at the common –36 hotspot, while making more errors at hotspots at positions 88 and 89 (Fig. 1B and Table 2). The second

characteristic feature of M184I is that it creates significantly more frameshift mutations outside homopolymeric runs (Fig. 2 and Table 3). In fact, M184I creates nearly equal numbers of frameshift mutations in runs and non-runs. Other RTs studied here, as well as those studied previously (22,28–30), show a strong bias towards run-associated frameshift errors. Whether such changes in the mutation specificity of M184I RT affect the viruses bearing this substitution and if the transient existence of this variant in patients is related to changes in error specificity is presently unclear.

The M184I mutation occurs early in patients receiving therapy with 3TC, preceding development of the M184V mutation (3,4). It has been suggested that the early emergence of the M184I variant in patients receiving 3TC reflects a mutational bias of HIV-1 RT (31). The eventual outgrowth of viruses harboring the M184V mutation in RT appears to be spurred on by the lower overall enzymatic activity of M184I-containing RTs (31,32) and a concomitant decreased viral fitness (31–33). It appears that the outgrowth of M184V viruses occurs despite an increase in polymerase fidelity for this RT, which is in accordance with the model put forth by Coffin (34), in which the high replication rate of HIV would allow for outgrowth of mutant virus despite small changes in fidelity.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service grants AI-30861 and AI40375 (to V.R.P.). L.F.R. and W.C.D. would like to acknowledge support from institutional training grants T32-GM07491 and T32-AI07501 respectively. The authors wish to thank Dr Clyde A.Hutchison III (University of North Carolina, Chapel Hill, NC) for providing plasmids of the mutant RT cDNA, Dr T.A.Kunkel (National Institute for Environmental Health Sciences) for being generous in providing the reagents for the M13 forward mutation assay, Dr Bradley Preston (University of Utah) for providing M13mp2 phage, Kenneth Curr for technical assistance, Dr Gloria Ho (this institution) for assistance with the statistical analysis and the Oligonucleotide Synthesis Facility of the Albert Einstein College of Medicine's Cancer Center for DNA oligonucleotides. Data in this paper are from a thesis to be submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University.

REFERENCES

1 Larder,B.A. (1993) In Skalka,A.M. and Goff,S.P. (eds), *Reverse Transcriptase*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 205–222.
2 Richman,D.D. (1992) *AIDS Res. Hum. Retroviruses*, **8**, 1065–1071.
3 Wainberg,M.A., Salomon,H., Gu,Z., Montaner,J.S., Cooley,T.P., McCaffrey,R., Ruedy,J., Hirst,H.M., Cammack,N. and Cameron,J. (1995) *AIDS*, **9**, 351–357.
4 Schuurman,R., Nijhuis,M., van Leeuwen,R., Schipper,P., de Jong,D., Collis,P., Danner,S.A., Mulder,J., Loveday,C. and Christopherson,C. (1995) *J. Infect. Dis.*, **171**, 1411–1419.
5 Winters,M., Shafer,R., Jellinger,R., Mamtora,G., Gingeras,T. and Merigan,T. (1997) *Antimicrobial Agents Chemother.*, **41**, 757–762.
6 Gao,Q., Gu,Z.X., Parniak,M.A., Li,X.G. and Wainberg,M.A. (1992) *J. Virol.*, **66**, 12–19.
7 Gao,Q., Gu,Z., Hiscott,J., Dionne,G. and Wainberg,M.A. (1993) *Antimicrobial Agents Chemother.*, **37**, 130–133.
8 Jacobo-Molina,A., Ding,J., Nanni,R.G., Clark,A.D.J., Lu,X., Tantillo,C., Williams,R.L., Kamer,G., Ferris,A.L., Clark,P. *et al.* (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 6320–6324.
9 Tantillo,C., Ding,J., Jacobo-Molina,A., Nanni,R.G., Boyer,P.L., Hughes,S.H., Pauwels,R., Andries,K., Janssen,P.A. and Arnold,E. (1994) *J. Mol. Biol.*, **243**, 369–387.
10 Wainberg,M.A., Drosopoulos,W.C., Salomon,H., Hsu,M., Borkow,G., Parniak,M., Gu,Z., Song,Q., Manne,J., Islam,S. *et al.* (1996) *Science*, **271**, 1282–1285.
11 Pandey,V.N., Kaushik,N., Rege,N., Sarafianos,S.G., Yadav,P.N.S. and Modak,M.J. (1996) *Biochemistry*, **35**, 2168–2179.
12 Drosopoulos,W.C. and Prasad,V.R. (1996) *J. Virol.*, **70**, 4834–4838.
13 Rubinek,T., Bakhanashvili,M. and Hizi,A. (1997) *Eur. J. Biochem.*, **247**, 238–247.
14 Oude Essink,B.B., Back,N.K.T. and Berkhout,B. (1997) *Nucleic Acids Res.*, **25**, 3212–3217.
15 Hsu,M., Inouye,P., Rezende,L., Richard,N., Li,Z., Prasad,V.R. and Wainberg,M.A. (1997) *Nucleic Acids Res.*, **25**, 4532–4536.
16 Bebenek,K., Abbotts,J., Wilson,S.H. and Kunkel,T.A. (1993) *J. Biol. Chem.*, **268**, 10324–10334.
17 Roberts,J.D., Bebenek,K. and Kunkel,T.A. (1988) *Science*, **242**, 1171–1173.
18 Bebenek,K. and Kunkel,T.A. (1995) *Methods Enzymol.*, **262**, 217–232.
19 Chao,S.F., Chan,V.L., Juranka,P., Kaplan,A.H., Swanstrom,R. and Hutchison,C.A., (1995) *Nucleic Acids Res.*, **23**, 803–810.
20 Kew,Y., Olsen,L., Japour,A. and Prasad,V.R. (1998) *J. Biol. Chem.*, **273**, 7529–7537.
21 Roberts,J.D., Preston,B.D., Johnston,L.A., Soni,A., Loeb,L.A. and Kunkel,T.A. (1989) *Mol. Cell. Biol.*, **9**, 469–476.
22 Bebenek,K., Abbotts,J., Roberts,J.D., Wilson,S.H. and Kunkel,T.A. (1989) *J. Biol. Chem.*, **264**, 16948–16956.
23 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.
24 Beard,W.A., Minnick,D.T., Wade,C.L., Prasad,R., Won,R.L., Kumar,A., Kunkel,T.A. and Wilson,S.H. (1996) *J. Biol. Chem.*, **271**, 12213–12220.
25 Jaju,M., Beard,W.A. and Wilson,S.H. (1995) *J. Biol. Chem.*, **270**, 9740–9747.
26 Rezende,L.F., Curr,K., Ueno,T., Mitsuya,H. and Prasad,V.R. (1998) *J. Virol.*, **72**, 2890–2895.
27 Drosopoulos,W.C. and Prasad,V.R. (1998) *J. Virol.*, **72**, 4224–4230.
28 Mansky,L.M. and Temin,M. (1995) *J. Virol.*, **69**, 5087–5094.
29 Mansky,L.M. (1996) *Virology*, **222**, 391–400.
30 Eckert,K.A. and Kunkel,T.A. (1993) *Nucleic Acids Res.*, **21**, 5212–5220.
31 Keulen,W., Back,N.T., van Wijk,A., Boucher,C.A.B. and Berkhout,B. (1997) *J. Virol.*, **71**, 3346–3350.
32 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.
33 Larder,B.A., Kemp,S.D. and Harrigan,P.R. (1995) *Science*, **269**, 696–699.
34 Coffin,J.M. (1995) *Science*, **267**, 483–489.