Mutational studies of human immunodeficiency virus type 1 reverse transcriptase: the involvement of residues 183 and 184 in the fidelity of DNA synthesis

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Abstract The high error rates characteristic of human immunodeficiency virus type-1 reverse transcriptase (HIV-1 RT) are a presumptive source of the viral hypermutability that impedes prevention and therapy of acquired immunodeficiency syndrome (AIDS). We have analyzed two mutants of HIV-1 RT by conducting a comparative study of the accuracy of DNA synthesis. Each mutant bears a single amino acid substitution adjacent to the two aspartic acid residues at positions 185 and 186 in the highly conserved DNA polymerase active site. The first mutant, Met 184→Leu (M184L), displays a marked reduction in both misinsertion and mispair extension, suggesting a fidelity of DNA synthesis significantly higher than that of the wild-type HIV-1 RT. The second mutant, Tyr 183→Phe (Y183F), shows a decrease in mispair extension with no significant change in misincorporation. Thus, the overall pattern of error-proneness of DNA synthesis is: wild-type HIV-1 RT > Y183F > M184L. Taken together, it is possible that residues 183 and 184 contribute to the low fidelity of DNA synthesis characteristic of the reverse transcriptases of HIV-1, HIV-2 and possibly, of other lentiviruses. Our observations may bear on the nature of potential mutations responsible for resistance to the nucleoside analogs used in chemotherapy of AIDS.

Key words: HIV; Reverse transcriptase; Mutant; Fidelity; DNA synthesis

1. Introduction

Human immunodeficiency viruses type 1 and type 2 (HIV-1 and HIV-2) are the causative agents of AIDS [1,2]. These retroviruses exhibit extensive genetic heterogeneity that substantially affects viral pathogenesis and resistance to drug therapy. The ability of HIV to evade the immune defenses of the host is related mainly to the high mutation frequency in the viral genome. Likewise, resistance to drugs against the viral enzyme reverse transcriptase (RT) is attributable to mutations in the *pol* gene that lead to amino acid substitutions in RT [3,4]. Sequence changes observed in serial virus isolates from a single patient have led to the suggestion that the progression of HIV can be viewed as an exceptionally fast evolutionary process due to rapid rates of mutation [5,6]. It is assumed that base substitutions, caused by the viral RT, are the primary mechanisms for the genomic changes in HIV [7,8].

RTs of HIV and other retroviruses are responsible for copying the viral single-stranded RNA into double-stranded DNA. Unlike many cellular DNA polymerases, retroviral RTs are devoid of $3' \rightarrow 5'$ exonuclease proofreading activity [7]. However, the fidelity of both the RNA-dependent and DNA-dependent DNA polymerase activities of HIV-1 and HIV-2 RTs is up to 10-fold lower than that detected for other proofreading-deficient RTs, i.e., RTs of murine leukemia virus (MLV) and avian myeloblastosis virus (AMV) [9–14]. It has been proposed that efficient misinsertion and extension of mismatched 3'-termini of the nascent DNA are major determinants for the low fidelity of HIV-1 and HIV-2 RTs [11,15]. Both HIV-1 and HIV-2 belong to the lentivirus subfamily of retroviruses. We have recently analyzed another lentiviral RT, from equine infectious anemia virus (EIAV), and found a fidelity of DNA synthesis as low as that of both HIV RTs [16]. This supports the notion that RTs of lentiviruses are generally more error-prone in DNA synthesis than other retroviral RTs, in the following order: HIV RTs ≈ EIAV RT > AMV RT > MLV RT. For all RTs studied so far with both RNA and DNA templates, the fidelity of DNA synthesis is not only enzyme-related but also sequence-dependent [17,18].

Analysis at the molecular level of the fidelity of RT requires the identification of amino acid residues that are involved in maintaining insertional fidelity and mispair extension. We have already suggested that the low cysteine content in HIV-1 and HIV-2 RTs might be associated with the error-proneness of these enzymes [19]. Two mutants of HIV-1 RT (Tyr-181 → Ile and Tyr-188 → Leu) resistant to non-nucleoside inhibitors, exhibit a 3'-mispair extension frequency that is similar to that of wild-type RT [20]. In the current study we have analyzed the fidelity of DNA synthesis of two additional mutants of HIV-1 RT in which the modified amino acids, Tyr-183 and Met-184, are adjacent to the contiguous aspartic acid residues at positions 185 and 186 that are believed to be part of a highly conserved putative active site for the DNA polymerase activity [21,22]. We have elected to analyze mutants with conservative amino acid modifications (Tyr-183 → Phe and Met-184 → Leu). Both mutant RTs were already shown by us to have diminished sensitivities to nucleoside analogs (unpublished results). The fidelity studies conducted are all basically comparative in nature, using template-primers already used previously for comparing the fidelity of a variety of RTs [11–13,16,17,20]. The results suggest that both mutants studied have an enhanced fidelity of DNA synthesis relative to wild-type HIV-1 RT.

2. Materials and methods

2.1. Enzymes

The wild-type and mutant versions of the p66/p51 heterodimers of

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HIV-1 RT were recombinant enzymes of the BH-10 isolate of HIV-1 RT [23]. The purified proteins were kindly provided by Drs. S. Hughes and P. Boyer. The genomes encoding the mutant RTs were modified by the cassette mutagenesis technique [24] and the proteins including six consecutive histidine residues at their amino termini were expressed in bacteria and purified as described [25]. The specific activities of all RTs used were between 2500 and 5500 U/μg protein determined as described [26]. Recombinant MLV RT was purchased from USB. Equal activities of all enzymes (10 U) were used for the comparative experiments performed. All dNTPs used were of the highest purity available (purchased from Pharmacia).

2.2. Enzymatic assays

The template-primer substrates used for assessing 3'-mispair formation and the extension of preformed 3'-mispaired have been previously described [16]. The [^{32}P]5'-end labeled DNA products were resolved by polyacrylamide gel electrophoresis (PAGE) and quantitated after phosphoimaging the acrylamide gels, as outlined in detail [15,17]. The initial rates of primer extensions are expressed in percent of primers present in the reaction mixtures extended per minute.

3. Results

We examined the fidelity of recombinant mutant HIV-1 RTs which have single amino acid substitutions, Y183F and M184L, within the conserved sequence Tyr-Met-Asp-Asp at the putative active site for DNA polymerase activities. Similar amounts of DNA polymerase activities of mutant and wild-type HIV-1 RTs were used for the comparative fidelity studies. MLV RT was also analyzed since it represents a relatively high fidelity RT with misinsertion and mispair extension efficiencies substantially lower than lentiviral RTs [9,11]. All mutant and wild-type enzymes were, as expected, devoid of any 3' → 5' exonuclease activity, permitting direct analysis of the fidelity of DNA polymerization activity.

3.1. 3'-DNA misinsertion by the mutant and wild-type RTs

To characterize the fidelity of insertion, we employed an assay that measures site-specific nucleotide misinsertion [16]. Insertional fidelity of the two mutant enzymes was measured in comparison to wild-type HIV-1 RT and MLV RT, using a standing start template-primer. The template is a synthetic oligonucleotide, derived from sequence 565–614 of φ×174am3 DNA. We have analyzed the incorporation of either the correct nucleotide (dTTP) or the incorrect ones (dATP, dCTP or dGTP) opposite the template A residue
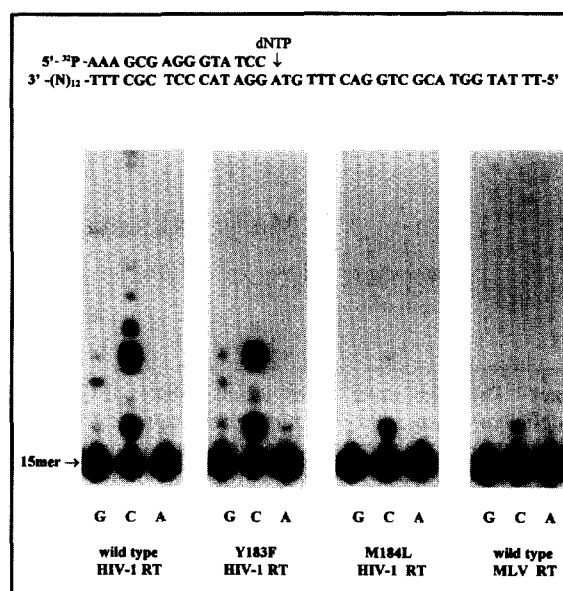

Fig. 1. Misinsertion by wild-type HIV-1 RT, Y183F and M184L mutants of HIV-1 RT, and MLV RT. The synthetic 50mer oligonucleotide (with sequences identical to nucleotides 565–614 in φ×174am3 DNA) was hybridized with 15mer [^{32}P]5'-end labelled primer. The primer was extended with equal DNA polymerase activities of all four RT versions used, in the presence of 1 mM of a single wrong dNTP (C, G or A) at 37°C, and products were analyzed by PAGE, as described in Section 2.

that corresponds to position 587 of the φ×174am3 DNA in this synthetic template. Each dNTP was at a fixed concentration of 1 mM, allowing the extension of the 15mer primer. The results of the primer extension assays, shown in Fig. 1, demonstrate that each enzyme analyzed exhibits a different pattern of primer extension. The possibility that misinsertion that is followed by mispair extension, are due to contaminating dNTPs is highly unlikely, since in such a case (using similar DNA polymerase activities and dNTP concentrations) all RT versions should behave similarly. These results also suggest that the pattern of elongation by the Y183F mutant is quite similar to that obtained with the wild-type counterpart. However, the M184L mutant hardly shows any significant misinsertion. The behaviour of this modified HIV-1 RT is

Table 1
Kinetic parameters for site-specific misinsertion by different RTs analyzed

dNTP	Wild-type HIV-1 RT			Y183F Mutant HIV-1 RT			M184L Mutant HIV-1 RT			Wild-type MLV RT		
	K_m (μM)	V_{max} (%/min)	F_{ins}	K_m (μM)	V_{max} (%/min)	F_{ins}	K_m (μM)	V_{max} (%/min)	F_{ins}	K_m (μM)	V_{max} (%/min)	F_{ins}
dTTP	0.02±0.002	25±0.8	1	0.017±0.003	23±2	1	0.05±0.005	14.9±0.3	1	0.2±0.02	10±0.15	1
dCTP	40±6	8.3±0.5	1/6000	50±3.5	9.8±0.4	1/6900	250±10	3.2±0.2	1/23,000	300±25	0.6±0.08	1/25,000
dGTP	125±21	4.8±0.6	1/32,550	130±8	3.9±0.3	1/45,000	N.D.	N.D.	<1/300,000	N.D.	N.D.	<1/300,000
dATP	180±20	3±0.5	1/75,000	114±6	2.3±0.1	1/67,000	N.D.	N.D.	<1/300,000	N.D.	N.D.	<1/300,000

The 15mer [^{32}P]5'-end labeled primer was hybridized to the 50mer template derived from sequence 565–614 of φ×174am3 DNA (see Fig. 1). The template primer was incubated with the RTs at 37°C in the presence of increasing concentrations of a single dNTP in each set of kinetic experiments. The product oligonucleotides were analyzed by PAGE as described in Section 2 and in the text.
The apparent K_m and V_{max} were determined from at least two independent experiments performed as described in Section 2 and in the text. The relative insertion frequency (F_{ins}) values were calculated from the V_{max}/K_m ratios and are expressed relative to dTTP. Some of the slopes for M184L mutant HIV-1 RT and MLV RT were too low to yield significant figures. Hence, the K_m and V_{max} values were not determined (N.D.). In other unrelated experiments we could detect (F_{ins}) values as low as 1/300,000. Therefore, we assume that in these specific cases where the K_m and V_{max} were N.D. The F_{ins} values are below 1/300,000.
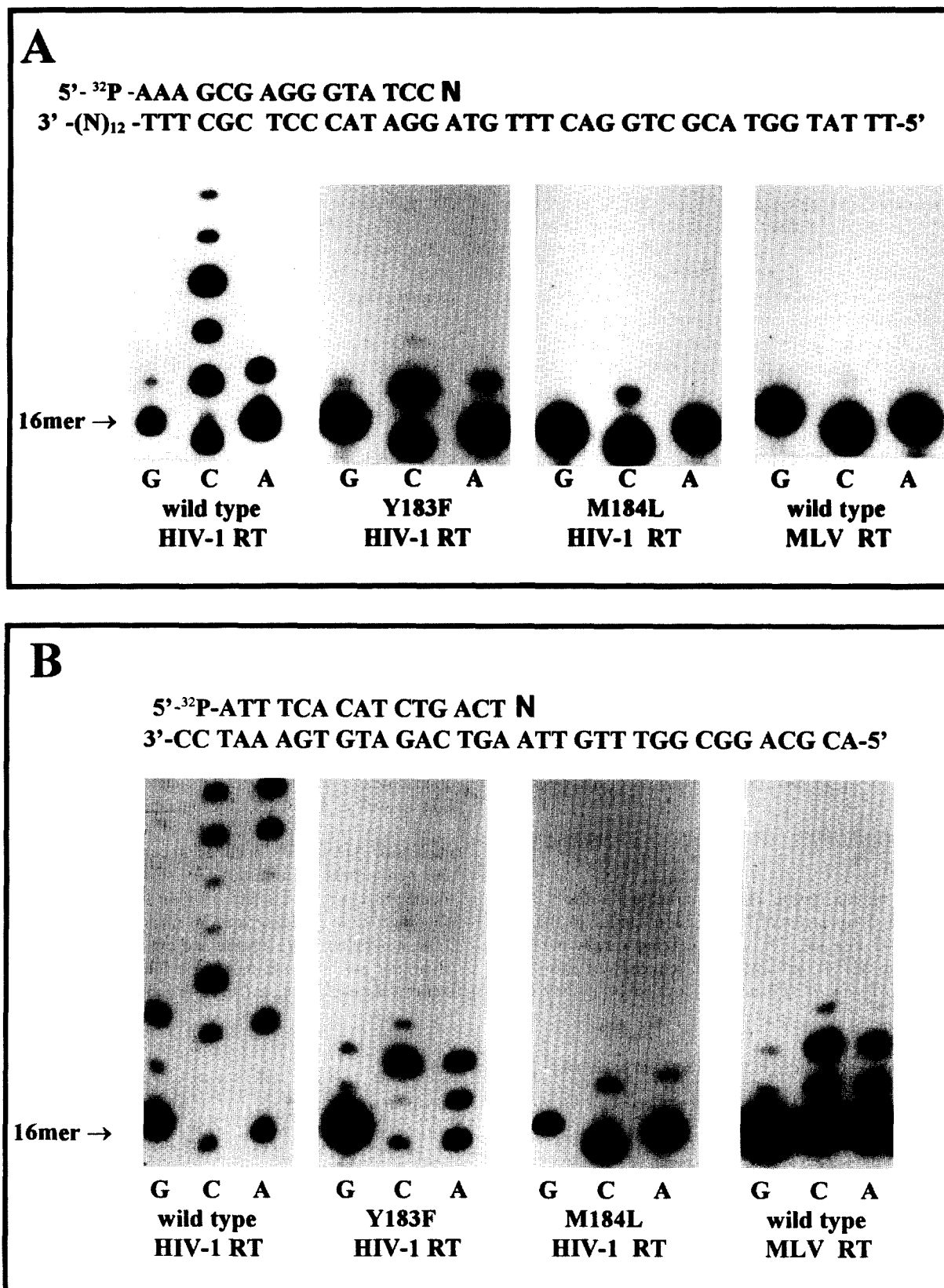
A

5'- ^{32}P -AAA GCG AGG GTA TCC **N**
3' -(N)$_{12}$ -TTT CGC TCC CAT AGG ATG TTT CAG GTC GCA TGG TAT TT-5'

16mer →

| G | C | A | G | C | A | G | C | A | G | C | A |

wild type HIV-1 RT   Y183F HIV-1 RT   M184L HIV-1 RT   wild type MLV RT

B

5'-^{32}P-ATT TCA CAT CTG ACT **N**
3'-CC TAA AGT GTA GAC TGA ATT GTT TGG CGG ACG CA-5'

16mer →

| G | C | A | G | C | A | G | C | A | G | C | A |

wild type HIV-1 RT   Y183F HIV-1 RT   M184L HIV-1 RT   wild type MLV RT

Fig. 2. Mispair extension exhibited by wild-type HIV-1 RT, Y183F and M184L mutants HIV-1 RT and MLV RT. The [^{32}P]5'-end labeled set of primers were hybridized to the appropriate templates producing duplexes with 3'-terminal mispairs, where the N at the 3' end of each represents the nucleotide A, C or G. The primers were extended by the different RT versions in the presence of a fixed concentration (1 mM) of dATP at 37°C. The reaction products were analyzed by PAGE. (A) A synthetic 50mer oligonucleotide, identical to the one used in the experiment described in Fig. 1, was hybridized to 16mer [^{32}P]5'-end labeled primer, as described. (B) The 34mer synthetic oligonucleotide template has a sequence similar to nucleotides 2096–2129 in *E. coli* 16S rRNA. The template was primed with [^{32}P]5'-end labeled 16mer oligonucleotide primers.

very similar in this respect to that of MLV RT. It is also clear that, only with wild-type and Y183F HIV-1 RTs, there are extensions of mispaired primers with dCTP and dGTP, generating products longer than 16mers (indicating that the initial event of misinsertion is followed by elongation). This reflects the ability to extend the mispairs, as is also demonstrated below in detail in separate experiments (Fig. 2 and Table 2). For example, in the case of wild-type HIV-1 RT (which has the lowest fidelity) in the presence of dCTP, the mispair C·A is elongated forming the mispair C·T, which is followed by the correct pair C·G. Further, a second C·T mispair was formed at a low efficiency with almost no subsequent extension beyond this point. This is compatible with the general rules already determined for misinsertion and extension by wild-type HIV-1 RT, namely, C·A mispairs are easily formed and extended and C·T mispairs are less efficiently formed and extended [14].

To further quantitate the frequency of misinsertion with all RTs studied, four sets of reactions were carried out, each using increasing concentrations of a single dNTP, thus measuring the rate of synthesis of the correct pair and the three possible mispairs. We measured the ratios of gel band intensities (of extended bands relative to unextended 15mers) and determined the overall misinsertion rates as a function of dNTP concentrations, as done previously [16]. The apparent K_m and V_{max} steady-state kinetic values for each dNTP were calculated from double-reciprocal plots of initial elongation velocity versus substrate concentrations (not shown) and are summarized in Table 1. As apparent for wild-type RT [16,19], misinsertion of the non-complementary nucleotides and subsequent extension is obtained at substantially higher nucleotide concentrations than complementary insertion of dTTP. The ratios of the insertion efficiencies for the wrong (W) versus right (R) nucleotides provide the frequency of misinsertion (F_{ins})

$$F_{ins} = \frac{(V_{max}/K_m)^W}{(V_{max}/K_m)^R}$$

The results, summarized in Table 1, indicate that the Y183F mutant exhibits frequencies of misinsertion similar to that of wild-type HIV-1 enzyme. The most striking results are obtained with the M184L mutant. The frequency of misinsertions with dATP and dGTP are too low to be quantitated

(and to the best of our estimates are lower than 1/300,000). The extent of A·C mispair formation is almost 4 times lower than with wild-type HIV-1 RT. Therefore, the M184L mutant is reminiscent of the MLV RT, which has very low misincorporation rates. It is interesting to note that the K_m for dTTP for wild-type MLV RT is higher by 10-fold than the corresponding value for wild-type HIV-1 RT.

3.2. Elongation of 3'-mispaired DNA by the mutant and wild-type RTs

The studied enzymes were compared for their ability to extend mismatched template-primers. Extension from preformed mispairs (A·A, A·C, and A·G) was studied by measuring primer elongation in DNA polymerization reactions with template-primers and the next complementary nucleotide dATP, as the only dNTP present, as done previously with various DNA polymerases [14–16,18,27–29]. Two DNA templates with different sequences were tested: (A) a synthetic oligomer corresponding to nucleotides to 565–614 in $\phi \times 174$am3 DNA (Fig. 2A); (B) a synthetic oligomer DNA with a base sequence identical to nucleotides 2096–2129 of *E. coli* 16S rRNA (Fig. 2B). The results indicate that both HIV-1 RT mutants extend purine-pyrimidine (A·C) and purine-purine (A·A, A·G) mismatches less efficiently than does the wild-type enzyme. The mispair extension capacity of these mutants are in several cases even lower than that of MLV RT. We have also examined the extent of mispair elongation with an RNA template, *E. coli* 16S rRNA, using the primers as those in (B). The mispair extension pattern obtained was identical to that shown in Fig. 2B (data not shown). A comparable pattern of reduction in the extension with all substrates used was observed with the mutants of HIV-1 RT. Consequently, we have elected to use only one DNA template for a further quantitative comparison of the mutant and wild-type HIV-1 RTs as well as MLV RT (the one shown in Fig. 2B).

We have studied the kinetics of adding the next correct nucleotide (dATP) onto either a correctly-matched or mismatched primer 3'-terminus [30]. Elongation from the preformed mispairs was detected by an increase in length of the oligonucleotide primers from 16 nucleotides to 17 or greater (data not shown). We determined extension rates (V = percent of primer extended per min) as a function of dATP concentrations for each mispair (A·A, A·G and A·C). Extension rates

Table 2
Kinetics of mispair extension from 3'-matched and mismatched primer termini

Paired or mispaired terminus	Wild-type HIV-1 RT			Y183F Mutant HIV-1 RT			M184L Mutant HIV-1 RT			Wild-type MLV RT		
	K_m (μM)	V_{max} (%/min)	F_{ext}	K_m (μM)	V_{max} (%/min)	F_{ext}	K_m (μM)	V_{max} (%/min)	F_{ext}	K_m (μM)	V_{max} (%/min)	F_{ext}
A·T	0.08 ± 0.002	14.3 ± 0.8	1	0.12 ± 0.02	16.6 ± 0.9	1	0.6 ± 0.02	5.5 ± 0.3	1	0.12 ± 0.02	14 ± 0.7	1
A·C	58 ± 4	9.5 ± 0.3	1/1000	220 ± 10	9.0 ± 0.5	1/3400	220 ± 12	0.25 ± 0.02	1/11,100	83 ± 3.6	3 ± 0.1	1/4600
A·G	160 ± 20	2.5 ± 0.2	1/11,400	350 ± 22	0.9 ± 0.06	1/54,000	N.D.	N.D.	$< 1/300,000$	260 ± 12	1.1 ± 0.08	1/39,400
A·A	61 ± 6	9.0 ± 0.4	1/1,200	280 ± 14	6.6 ± 0.6	1/5900	250 ± 12	0.14 ± 0.008	1/16,300	130 ± 8	2.5 ± 0.1	1/6100

The 16mer [³²P]5'-end labeled primers were hybridized to a 34mer oligonucleotide template (with a sequence similar to nucleotides 2096–2129 in *E. coli* rRNA) producing duplexes with a 3'-terminal paired (A·T) or mismatched (A·C, A·G or A·A) primers (see Fig. 2B). The different sets of template primers were incubated with the RT versions at 37°C in the presence of increasing concentrations of dATP. The products were analyzed as described in the text.

The apparent K_m and V_{max} values were calculated each from at least two independent experiments as described in Section 2. The relative extension frequency (F_{ext}) values are the ratio of the rate constants (V_{max}/K_m) for the mispair divided by the ratio of the corresponding constants for the paired A·T terminus. In the case of the A·G mispair of the M184L mutant the slopes were too low to give significant figures (as explained in the legend to Table 1).

from all mispairs were estimated from double-reciprocal plots of V versus [S] and the kinetic parameters are summarized in Table 2. The extension frequency is defined as the apparent V_{max}/K_m value and the frequency of relative extension frequency (F_{ext}) is the frequency with the wrong terminus divided by that obtained with the correctly-pair terminus. The values for Y183F mutant with all three mispairs are 3.4–4.9-fold lower than those of wild-type HIV-1 RT. Thus, the modification of Tyr-183, which is highly conserved among retroviral RTs, mostly decreases the mispair extension ability, with no significant effect on misinsertion. However, the comparative values of the mutant M184L are even lower — 10- to above 26-fold lower than those of the wild-type HIV-1 RT (Table 2). As in the case of misinsertion (Fig. 1 and Table 1), the M184L mutant displays a low ability of mispair extension, similar to the wild-type MLV RT.

4. Discussion

In all retroviral RTs studied so far the fidelity of DNA synthesis was found to be enzyme-dependent and sequence-related [16–18]. For each given RT there are differences in the efficiency of extension of 3'-mispaired DNA, depending on the sequences flanking the 3'-terminus. The comparative study presented in this paper indicates that in HIV-1 RT both Tyr-183 and Met-184 are probably involved in the low fidelity of DNA synthesis of the enzyme. In the case of the M184L mutant, there is a linkage between the decrease in the ability to introduce non complementary nucleotides at the 3'-end of the growing DNA strand and the reduction in the ability to extend a preformed 3'-mismatch. However, the Y183F mutant shows a substantial decrease in only mispair extension. Thus, the overall pattern of error-proneness of DNA synthesis is: wild-type RT > Y183F > M184L. With all RTs analyzed, the specificity of mismatch formation with the particular sequences studied is A·C > A·G≥A·A, and the specificity of mispair elongation is A·C≥A·A > A·G (Tables 1 and 2). Similar to other RTs and DNA polymerases, employing various template-primers [15,30,31] a strong correlation was observed between the reaction rates and the nature of the mispair being formed or extended. Our results are in line with the general rules already determined for DNA polymerases [14,15,30,31]. Thus, the purine-pyrimidine mispair (A·C) is the most easily formed and further extended with all RT versions in this study. As already seen earlier, the frequencies of both mispair formation and extension are affected within each given enzyme mainly by the increase in apparent K_m values (rather than by substantial changes in the V_{max} values) [11,12,16,17].

Recent studies of human DNA polymerase α suggest that the phenyl ring of Tyr-865 interacts with the nucleotide moiety of the incoming dNTP. This explains the critical role of this residue in the misinsertion fidelity of DNA synthesis [27]. Ser-867, which is involved in recognizing the 3'-OH terminus of the primer, plays a role in mispair extension with no apparent effect on misinsertion fidelity [28]. Parallel studies of the Klenow fragment of E. coli DNA polymerase I have revealed that the substitution of Tyr-766 leads to a substantial decrease in fidelity [29]. Reduced fidelity of DNA synthesis has also been observed with HIV-1 RT mutants in which Cys-38 and Cys-280 were replaced by serines [19]. It should be emphasized that all of the above-mentioned mutations result in a decrease in fidelity, while the mutant M184L and

Y183F of HIV-1 RT show the opposite — an increase in fidelity.

The three-dimensional structure of wild-type HIV-1 RT complexed with double-stranded DNA provides clues for the positioning Tyr-183 and Met-184 relative to the DNA polymerase active site [22,32,33]. The enzyme possesses a catalytic triad of three aspartic acid residues (at positions 110, 185 and 186) located in the 'palm' subdomain, which serves as the center of the catalytic site. These residues can bind divalent cations that are required for catalysis. The 3'-hydroxyl terminus of the primer is close to the catalytic Asp triad and is appropriately positioned for the nucleophilic attack on the α-phosphate of the incoming nucleoside triphosphate. The close proximity of Tyr-183 and Met-184 to the active site of the aspartic acid triad of HIV-1 RT can affect the fidelity of DNA synthesis. It is important to note that modifications of Met-184 in HIV-1 RT were reported to confer resistance to ddCTP, ddITP and oxathiolane-cytosine nucleotides [34–36]. We have found that both the M184L and Y183F mutants show also a significant reduction in sensitivity to ddNTPs (unpublished results). Residues 183 and 184 are located directly under the 3'-end of the primer strand [31]. It is not clear whether the mutations affect the positioning of the template-primer or whether Met-184 and Tyr-183 interact directly with the incoming dNTPs.

Amino acid sequence comparisons in the DNA polymerase domain of a variety of retroviral RTs reveals that the sequence Tyr-Met precedes the two Asp-Asp residues in the active sites of almost all RTs studied. The only exception is MLV RT, where the methionine is replaced by a valine residue [21]. Since the fidelity of DNA synthesis of MLV RT is substantially higher than those of HIV-1, of HIV-2 and EIAV RTs [16,17], it is tempting to speculate that this methionine residue contributes to the low fidelity of DNA synthesis of all lentiviral RTs. It is very likely that the fidelity of DNA synthesis by a given RT reflects complex interactions between multiple amino acid sequences that lead to overall structural differences, which ultimately affect the error-proneness of the DNA polymerase. The interactions of these sequences and their involvement in the catalytic mechanisms of RTs should explain in molecular terms the high mutation rates observed in HIV RTs. To attain a better understanding additional selective mutants of HIV-1 RT need to be similarly investigated. It is usually believed that a higher fidelity of DNA synthesis is advantageous to a given DNA polymerase. Yet, despite the fact that HIV-1 RT can potentially reach a higher fidelity by mutating methionine 184 and tyrosine 183, the enzyme in wild-type virions retains these residues under no selective pressure. This can strongly suggest that it is biologically beneficial for the virus to harbor a low fidelity RT for purposes such as allowing the virions to evade the immune system of the host.

While this work was near completion two very recent reports were published supporting the role of methionine 184 in the fidelity of HIV-1 RT. Wainberg et al. reported that an M184V substitution leads to an increase in fidelity of HIV-1 RT in vitro, which is accompanied by a decrease in rates of mutations in virions with such a mutated RT [37]. Pandey et al. reported that the mutation M184V results in an increase in the fidelity of DNA synthesis, whereas the mutant M184A retains the low fidelity [38].

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