Volume 306, number 2,3, 151–156 FEBS 11289 July 1992

Fidelity of the reverse transcriptase of human immunodeficiency virus type 2

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Received 3 March 1992; revised version received 25 May 1992

The relatively low fidelity of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) was implicated as a major factor that contributes to the genetic variability of the virus. Extension of mismatched 3′ termini of the primer DNA was shown to be a major determinant of the infidelity of HIV-1 RT. Human immunodeficiency virus type 2 (HIV-2) also shows extensive genetic variations. Therefore, we have analyzed the fidelity of the DNA-dependent DNA polymerase activity of HIV-2 RT and compared it with those of RTs of HIV-1 and murine leukemia virus (MLV). Like other retroviral RTs, the HIV-2 RT was shown to lack a 3′→5′ exonuclease activity. The ability of HIV-2 RT to extend preformed 3′-terminal A:A, A:C and A:G mispairs was examined by quantitating the amount and length of extended primers. The results demonstrate a relatively efficient mispair extension by HIV-2 RT with a specificity of A:C>>A:A>A:G. The mispair extension appears to be affected mainly by the increase of apparent K_m values rather than by the change in V_{max} values. The relative extension frequencies from all mispairs with HIV-1 and HIV-2 RTs was 6- to 9-fold greater than that of MLV RT, suggesting that the HIV enzymes are substantially more error-prone than MLV RT.

Fidelity; DNA synthesis; HIV; Reverse transcriptase

1. INTRODUCTION

Human immunodeficiency viruses type 1 and type 2 (HIV-1 and HIV-2, respectively) represent a distinct group of lentiviruses associated with human acquired immunodeficiency syndrome (AIDS) [1,2]. HIV infection is characterized by the high genetic variability found in virus populations [3,4]. This genetic heterogeneity may be important in the pathogenesis of HIV and in the resistance of the virus to drug therapy. The calculated mutation rate during replication of the HIV genome is about a million times greater than that of eukaryotic DNA genomes [5]. Other retroviruses were also shown to exhibit a relatively high rate of mutagenesis, a phenomena attributed to the inaccuracy of the replication machinery that is unique to the retroviral life cycle [6]. It has been suggested that much of the genetic variability stems from the low fidelity of the reverse transcription step [4].

Reverse transcriptases (RTs) are multifunctional enzymes with RNA-dependent DNA polymerase, DNA-dependent DNA polymerase and ribonuclease H activities [7]. The DNA polymerase activities of all RTs studied so far exhibit no proofreading function, a well accepted explanation for their relatively high inaccuracy. Thus, RTs are capable of misincorporating inappropriate nucleotides into the synthesized DNA at frequencies in the range of 10^{-4} to 10^{-3}, and are therefore in sharp contrast to cellular DNA polymerases, that exert higher fidelity of DNA synthesis. Studies on purified viral or recombinant HIV-1 RT have revealed that HIV-1 RT is particularly error-prone, producing errors about ten times as frequently as RTs from avian myeloblastosis virus (AMV) and murine leukemia virus (MLV) [8–10]. This observation demonstrates that the lack of proofreading activity does not account for the exceptionally high infidelity of HIV-1 RT and that additional factors may contribute to its decreased fidelity. Indeed, recent kinetic analyses suggest that efficient extension from 3′-terminal mispairs is a major factor in the production of mutations by the enzyme [11]. Unexpectedly, the RT of simian immunodeficiency virus (SIV), an HIV-related retrovirus, with high genetic diversity [12], was reported recently to be 2- to 20-fold more accurate than HIV-1 RT [13].

HIV-2 exhibits genetic variability comparable to that found in HIV-1 strains isolated so far [14]. HIV-2 RT efficiently expressed in bacteria by us and purified to homogeneity [15]. The comparison of several catalytic properties of HIV-1 and HIV-2 RTs has indicated that, despite a high degree of amino acid sequence homology, some of their features are substantially different [16]. In addition, several novel compounds were reported to selectively inhibit only HIV-1 RT with no effect on HIV-2 RT [21,22]. Hence, it was of interest to examine the fidelity of HIV-2 RT in comparison to HIV-1 RT and the well-studied MLV RT, as representing both relatively low and high fidelity RTs, respectively.

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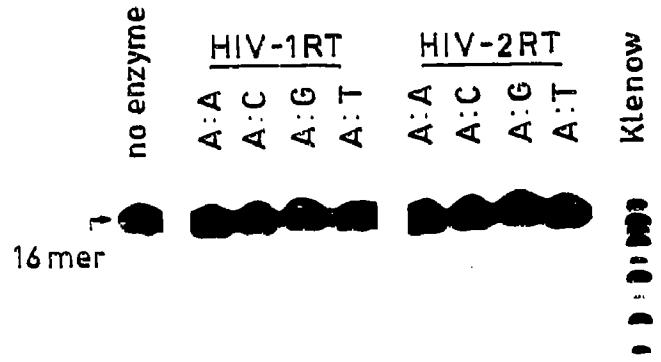

Fig. 1. Electrophoretic analysis of terminal mismatch excision. Reactions for terminal mismatched (A:A, A:C and A:G) or matched (A:T) excision by HIV-2 and HIV-1 RTs and Klenow fragment of *E. coli* DNA pol I were performed as described in section 2. Incubations were performed for 60 min at 37°C with the two HIV RTs or for 5 min with the Klenow fragment of *E. coli* DNA polymerase I used as a positive control. The position of the 16-mer primer is indicated by an arrow.

Since extension of mismatched 3′ DNA termini was found to be a major determinant of the infidelity of HIV-1 RT [11], we analyzed this parameter as representing the fidelity of DNA synthesis. The results presented herein demonstrate that HIV-2 RT, like HIV-1 RT extends mispaired termini efficiently.

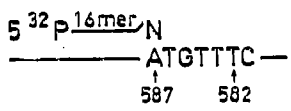

Chart 1. Primer-template used for analysis of terminal mismatch excision and for measuring extension kinetics for matched and mismatched primer 3′-terminal bases. The primer is 16 nucleotides long with 3′-terminal nucleotide N, representing A, C, G or T, and annealed to a complementary section of ϕ×174am^3 DNA template.

2. MATERIALS AND METHODS

2.1. *Enzymes*

All RTs used in this study were recombinant enzymes expressed by us in *E. coli* [15,19–21] and purified to homogeneity according to Clark et al. [22]. RTs used were 4000–5000 units per μg. One unit is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol dTMP into DNA in the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed reaction in 30 min at 37°C [20].

2.2. *Template primers*

The template single-stranded ϕ×174am^3 DNA was primed with a twofold molar excess of the 16-mer oligonucleotide, that hybridizes to the nucleotides at positions 587 to 602 of the ϕ×174am^3 DNA. Four versions of the 16 base primers were synthesized, all identical except for the 3′-terminal base (N) either an A,C,G or T (5′ AAAGC-GAGGGTATCCN 3′). The primers were 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and annealed to the template DNA as described [23].

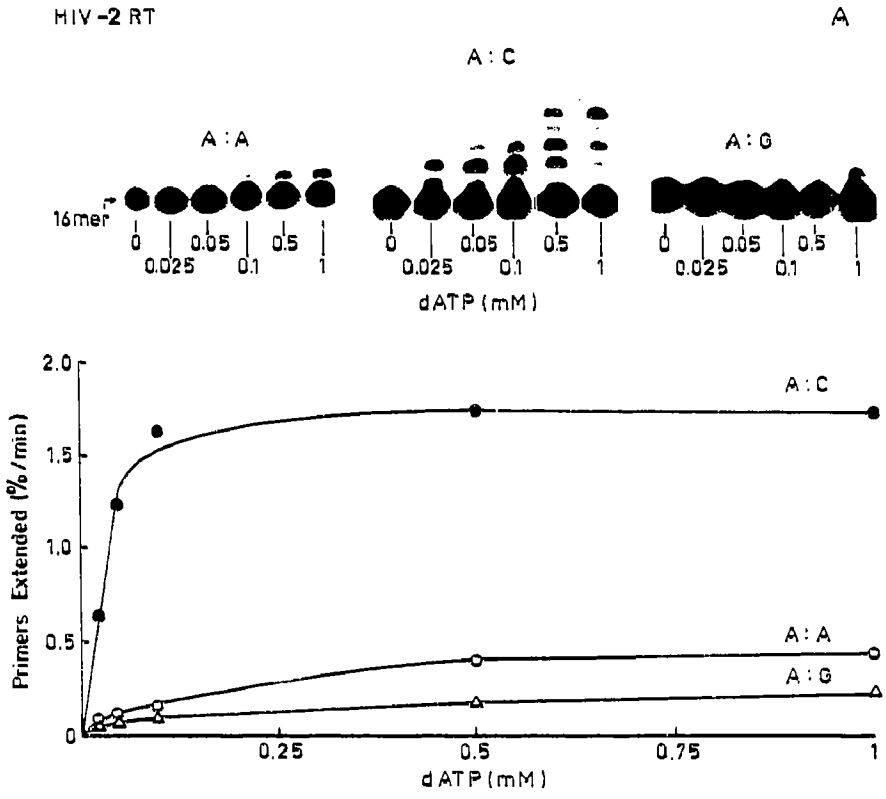

Fig. 2A.

2.3. *Electrophoretic analysis of terminal mismatch excision*

Four template-primers were designed, containing a 3'-terminal mispair (A:A, A:C or A:G) or the correct base pair (A:T), all opposite to nucleotide 587 of the ϕx174am^3 DNA (Chart 1). The 3'→5' exonucleolytic activity was measured as the removal of 3' terminal nucleotides from the 5' γ-^{32}P end-labeled oligonucleotide, determined by the increase of the mobility during polyacrylamide gel electrophoresis. The reactions were carried out in 25 μl contained 50 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 300 ng of mismatched DNA and 0.2 pmol of Klenow fragment of DNA polymerase I or 0.75

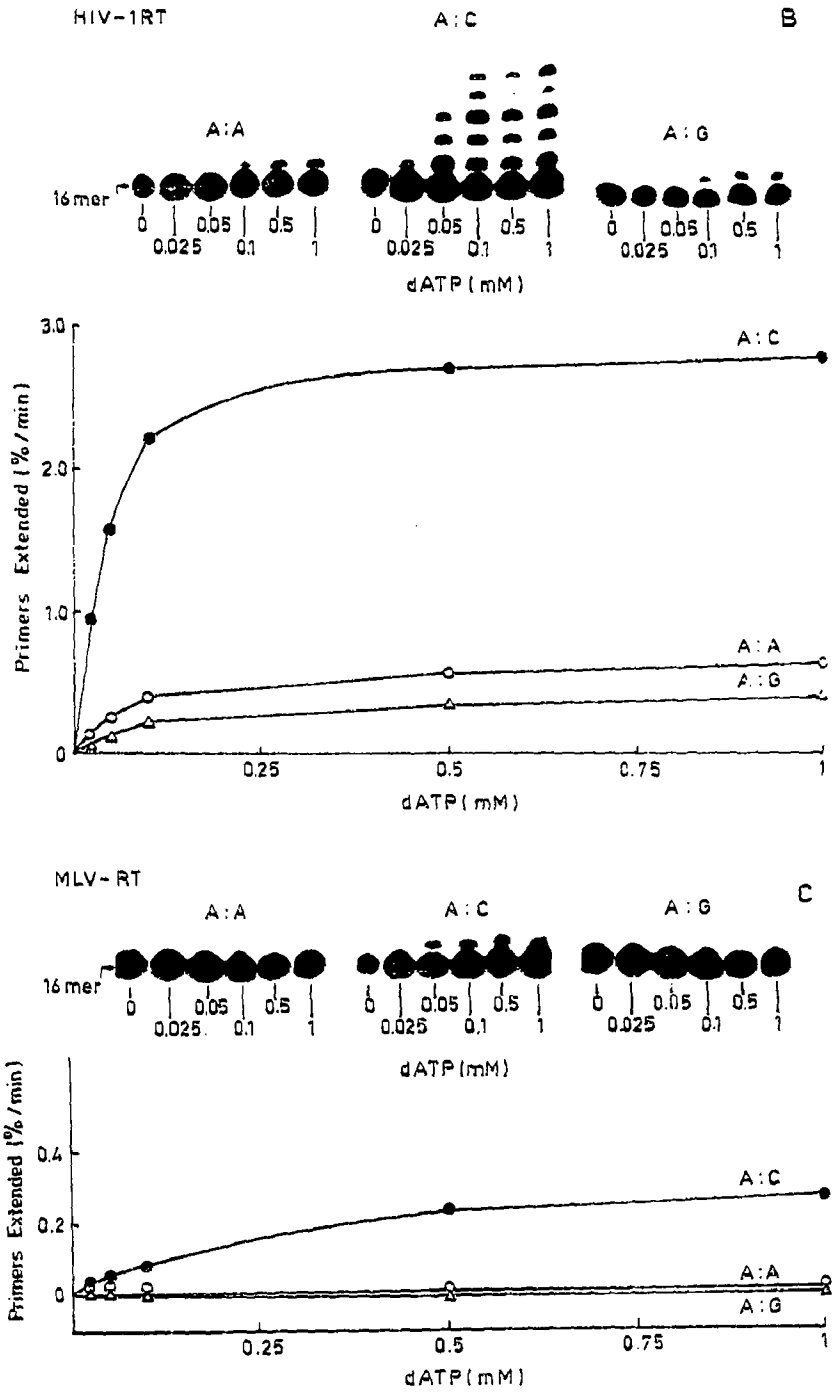

Fig. 2. Kinetics of mispair extension. Oligonucleotide primers (16-mer) were hybridized to ϕx174am^3 DNA (0.022 pmol) to produce the indicated 3' terminal mispairs at position 587 and extended for 10 min at 30°C with equal activities of either HIV-2 RT (A), HIV-1 RT (B) or MLV RT (C). Each reaction contained 0, 0.025, 0.05, 0.1; 0.5 or 1.0 mM dATP as the only deoxynucleoside triphophate substrate. PAGE analysis of the extended primers from A:A (○), A:C (●) and A:G (△) mispairs are shown above at left, center and right, respectively (A, B and C). The primer position (16-mer) is indicated by an arrow.

pmol of HIV-1 or HIV-2 RTs. Aliquots were removed into equal volumes of formamide dye mix. Electrophoretic analyses were performed in 20% polyacrylamide sequencing gels followed by autoradiography [9].

2.4. *DNA polymerization reaction*

The DNA polymerization reactions for HIV RTs contained 20 mM Tris-HCl (pH 7.5), 2 mM DTT, 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, primed DNA and increasing concentrations of dATP. For MLV RT, the reactions were carried out in the presence of 0.5 mM $MnCl_2$ substituting for $MgCl_2$. Reactions were incubated at 30°C for 10 min. Aliquots (5 μl) were removed into 5 μl of formamide dye mix, denatured at 100°C for 5 min, cooled on ice, analyzed by electrophoresis through 20% polyacrylamide gels and quantified by densitometric scanning of gel autoradiographs. Before measuring the kinetic constants for elongating the primers, a time-course study was done for each paired and mispaired terminus to determine the range of time during which the products accumulate linearly with time.

3. RESULTS AND DISCUSSION

Following misinsertion of a wrong nucleotide, DNA polymerase can either dissociate from the primer-template, excise the mispaired terminus if a proofreading exonuclease is associated with polymerase, or they can extend the mispaired nucleotides, resulting in transition or transversion mutations. To test the ability of HIV-2 RT to extend DNA from a mispaired terminus, as well as to confirm the absence of a 3′→5′ exonuclease activity, we prepared a series of templates with 16-mer primers using φx174am[3] DNA as template (see Chart 1 and section 2).

3.1. *Exonuclease activity*

We examined whether HIV-2 RT exhibits a 3′→5′ proofreading exonuclease activity with the four template primer substrates depicted in Chart 1. We have used HIV-1 RT as a negative control and the Klenow fragment of *E. coli* DNA polymerase I as a positive control. The excision of the terminal nucleotides occurs within 5 min with the Klenow fragment of *E. coli* DNA polymerase I (Fig. 1). In contrast, there is no change in the length of any of the oligonucleotide primers after an incubation up to 1 h with either HIV-2 RT or HIV-1 RT (Fig. 1). Terminal mismatch excision capability was analyzed in the absence of DNA polymerization since no dNTP were present in the reaction mixtures. Consistant with the notion that RTs are deficient in a 3′→5′ exonuclease activity, HIV-2 RT is found not exceptional, namely, uncapable of proofreading.

3.2. *Kinetic analysis of mispair extension*

The lack of exonuclease activity enables the analysis of the fidelity of the DNA polymerization activity of HIV-2 RT without the interference of any proofreading activity. It is obvious that misinsertion per se is not sufficient to create mutation by a DNA polymerase without the ability to extend the preformed mispairs. Several studies have suggested that HIV-1 RT is not more error-prone for the rates of misincorporation than MLT RT, AMV RT [24] or DNA polymerase α [11]. However, HIV-1 RT has a capacity to elongate the mispairs. Therefore, we have employed in the current study the mispair extension assay used for HIV-1 RT [11], as representing the fidelity of DNA synthesis. Moreover, the efficient elongation of the terminally-mismatched primers may involve additional misinsertions (see Chart 1). Thus, we have compared the ability of HIV-2 RT to extend DNA from various preformed

Table 1

Kinetics of mispair extension by HIV-2, HIV-1 and MLV reverse transcriptases

Oligonucleotide primers (16-mer) were hybridized to φx174am[3] DNA to generate the indicated 3′ termini. Extension reactions performed for 10 min at 30°C contained 0, 0.025, 0.05, 0.1, 0.5 and 1 mM dATP with either one of HIV-1 RT, HIV-2 RT or MLV RT (see Fig. 2). The percent of 16-mer extended by at least 1 nt was quantitated. The apparent K_m and V_{max} values ± S.E. and the relative extension frequencies were determined as described in the text. When the slopes were too low, the K_m and V_{max} values were not determined (ND).

	HIV-2 RT			HIV-1 RT			MLV RT		
Primer-template	K_m (μM)	V_{max} (%/min)	Relative extension frequency	K_m (μM)	V_{max} (%/min)	Relative extension frequency	K_m (μM)	V_{max} (%/min)	Relative extension frequency
___/A ___A –	170 ± 31	0.45 ± 0.035	1/27,000	80 ± 8.2	0.6 ± 0.041	1/22,000	ND	ND	1/180,000
___/C ___A –	45 ± 5.8	1.8 ± 0.056	1/1,800	46 ± 5.9	2.9 ± 0.062	1/2,600	280 ± 56	0.3 ± 0.04	1/11,000
___/G ___A –	120 ± 22	0.25 ± 0.018	1/34,000	68 ± 7.2	0.45 ± 0.033	1/24,000	ND	ND	1/200,000
___T ___A –	0.038 ± 0.0021	2.7 ± 0.19	1	0.026 ± 0.0031	4.2 ± 0.25	1	0.14 ± 0.011	1.7 ± 0.18	1

3'-mispaired termini with that of HIV-1 and MLV RTs (as representing relatively low and high fidelity RTs, respectively). The catalysis of the extension from preformed mispairs by the RTs was studied with four template-primers as described in Chart 1, by measuring primer elongation in a DNA-dependent DNA polymerization reaction. Under the conditions chosen the reaction was linear with respect to time (not shown). The kinetics of mispair extension as a function of increasing concentration of dATP, are shown in Fig. 2. Interestingly, both HIV RTs showed similar behaviors (Fig. 2A and B). Extensions from the A:A and A:G purine–purine mispairs by the two HIV RTs result in the production of only 17-mer oligonucleotides. However, the extension from the A:C purine–pyrimidine mispair led to the production of oligonucleotides from 17 to 22 nucleotides in length, indicating that the A:C mispair is extended more efficiently than the A:A and A:G mispairs. Thus, a greater proportion of 16-mer A:C mispaired primer is extended and the products are longer. It should be noted that the extension from the preformed A:C mispair up to a 22-mer involves the incorporation of two additional non-complementary dAMPs opposite the template guanidine and cytosine at positions 585 and 581, respectively (Chart 1). Therefore, the extension from an A:C mispair involves multiple misinsertions. In contrast to both HIV RTs, MLV RT exhibits extension only from the A:C mismatch as indicated by elongation of the 16-mer primer to 17 nucleotides (Fig. 2C).

The efficiency of extension is an essential component in determining whether the mismatched primer-termini are likely to be extended by the DNA polymerase. The apparent values of the kinetic constants, K_m and V_{max} for extension of each terminus, were calculated for both HIV RTs from the double-reciprocal (Lineweaver–Burk) plots of the initial catalytic velocities (percent of primer extended per min) versus the substrate dATP concentrations (not shown). However, with MLV RT, the slow rate of extension observed for all the mispairs made it difficult to quantitate accurately the K_m and V_{max} values. Subsequently, we had to use the slopes of V versus [S] plots (Fig. 2C) for approximation of extension kinetic values. It is apparent from Table I, that all three enzymes discriminate against extending A:A, A:C or A:G mispairs primarily by a large increase (1200- to 4500-fold) in apparent K_m values compared to A:T, with only a modest reduction (2- to 10-fold lower) in apparent V_{max} values. Therefore, K_m discrimination is predominantly the mechanism determining the mispair extension. The relative extension frequencies were defined as the ratios of apparent V_{max}/K_m values obtained with correct and incorrect base pairs at a given sequence on the template [25]. For both HIV RTs the rate of extension from the A:C mispair was over 12-fold greater than the rate from an A:A mispair and 16-fold greater than that from an A:G mispair. The relative extension frequency from all three mispairs A:A, A:C and A:G, exhibited by both HIV RTs was 6- to 9-fold greater than that of MLV RT, suggesting that the HIV enzymes are substantially more error-prone than MLV RT. The general trend in the order of mispair extension efficiency by HIV-2 RT is A:C>>A:A>A:G, and is similar to that of HIV-1 RT [11]. Therefore, HIV RTs catalyze more efficiently chain extension from transition mispair-A:C (purine–pyrimidine) than from transversion mispair A:A or A:G (purine–purine). It was recently reported that the mispair extension kinetics of *D. melanogaster* α DNA polymerase and that of AMV RT follow several general rules: (a) purine–pyrimidine mispairs are easily inserted and extended; (b) pyrimidine–pyrimidine and purine–purine mispairs are more difficult to extend than to insert [25]. Thus, our results are in an agreement with these suggested rules.

Acknowledgements: We are indebted to S. Loya and M. Shaharabany for helpful discussions. This research was supported by Grant NO RO1-AI27035 from the National Institute of Allergy and Infectious Diseases.

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