Volume 319, number 1,2, 201–205 FEBS 12222 March 1993

The fidelity of the reverse transcriptases of human immunodeficiency viruses and murine leukemia virus, exhibited by the mispair extension frequencies, is sequence dependent and enzyme related

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Received 21 December 1992; revised version received 29 January 1993

Sequence variations in HIV-1 and HIV-2 probably result in part from inaccurate DNA synthesis by viral reverse transcriptases (RTs). We have studied in vitro the fidelity of both the DNA- and RNA-dependent DNA polymerization functions of the two HIV RTs, as compared to that of murine leukemia virus (MLV) RT. The two HIV RTs were less accurate than MLV RT. The mispair extension frequencies observed previously with ribosomal RNA (rRNA) template were higher than those detected with ϕX174am3 DNA template with all three RTs. In the current study we have investigated whether the nature of the copied nucleic acid (RNA vs. DNA) or the template nucleotide sequences affect the accuracy of DNA synthesis. We have analyzed the fidelity of DNA synthesis with DNA sequences identical to those of the rRNA sequences previously employed for reverse transcription. The results indicate that the fidelity of DNA synthesis depends mainly on the nucleotide sequences copied by every given RT. Yet, fidelity of DNA synthesis depends not only on the sequences copied but also on the nature of the enzymes per se. It is possible that these factors are major contributors to the high mutation rates of the two human immunodeficiency viruses.

Fidelity; DNA synthesis; Human immunodeficiency virus; Reverse transcriptase

1. INTRODUCTION

Human immunodeficiency viruses type 1 and type 2 (HIV-1 and HIV-2), the etiological agents responsible for acquired immunodeficiency syndrome (AIDS) [1–3], exhibit an extensive genomic heterogeneity [4–6]. Recent evidence has implicated the viral-coded enzyme reverse transcriptase (RT) as a primary factor responsible for the high frequency of mutation leading to heterogeneity, since HIV RT exhibits a relatively low fidelity of DNA synthesis [7–11]. The rapid emergence of HIV mutants represents a formidable challenge to the development of broad spectrum vaccines and anti-HIV drugs [12,13]. Almost all anti-HIV drugs used so far for the treatment of AIDS patients are inhibitors of HIV RT. Yet, prolonged exposures to these drugs leads to the emergence of virions harboring resistant RT molecules with full catalytic activities [14,15].

The RT of HIV and other retroviruses is responsible for conversion of the viral RNA to double-stranded DNA [16,17]. RT is a multifunctional enzyme. First, it synthesizes the minus-strand DNA from the RNA template, then the RT copies this DNA into double-stranded DNA and degrades the RNA template through its RNAse H activity [18,19]. It has been demonstrated that HIV-1 and HIV-2 RTs have a decreased fidelity relative to other retroviral RTs while copying DNA templates [7–11], due to their ability to elongate efficiently mismatched 3′ termini of DNA [20]. Furthermore, we have recently suggested the possibility that cysteine residues of both HIV RTs might be involved in the fidelity of the DNA-dependent DNA synthesis [21]. Since RT utilizes both RNA and DNA as templates, it is important to examine whether the high error rates are also observed while copying RNA templates. Nevertheless, the data obtained with RNA templates are rather heterogenous. Unequal HIV-1 RT error-rates were detected with RNA and DNA templates. Hubner et al. conclude that the synthesis with an RNA template is less accurate than with a DNA template [22], whereas Boyer et al. suggest the opposite, namely that the fidelity is higher with RNA than with DNA templates [23]. In contrast, other groups have found that the fidelity of DNA synthesis of HIV-1 RT with both templates is comparable [24,25].

We have recently studied the ability of HIV-1, HIV-2 and MLV RTs to extend preformed mismatched 3′ termini using ribosomal RNA (rRNA) as a template. The results indicate that, as with ϕx174am3 DNA template, both HIV RTs are less accurate than MLV RT [26]. However, these observations also suggest that the mispair extension frequencies found during the RNA-dependent DNA synthesis are significantly higher than those detected for the DNA-dependent DNA synthesis, catalyzed by each given RT. It should be emphasized, nonetheless, that the RNA-dependent DNA synthesis was performed with a rRNA template [26], whereas the DNA-dependent DNA synthesis with a ϕx174am3

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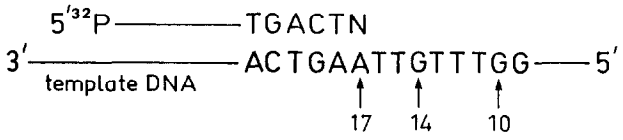


Fig. 1. Primer–template used for measuring extension kinetics for matched and mismatched primer 3′ terminal bases. The primer is 16 nucleotides long with 3′ terminal nucleotide N, representing A, C, G or T and annealed to 34 mer oligonucleotide template. This template has the same sequence as the sequence spanning nucleotides 2,096–2,129 of *E coli* 16 S rRNA.

DNA template [11]. The specific sequences chosen for the analyses with the two templates were similar but not identical, although the preformed mispairs were identical. Consequently, we have suggested that the dissimilarities observed in the primer extension studies between these two reactions might be due to the different polynucleotide sequences copied. It may be, as well, that the nature of the template (RNA vs. DNA) is the main factor affecting the extension frequency.

In the current study we have specifically addressed ourselves to this issue by analyzing the fidelity of the DNA-dependent DNA synthesis using a synthetic DNA template with a sequence identical to that of the rRNA previously reverse transcribed [26]. The results presented in this communication indicate a pattern of mispair extension which is similar to that observed with the rRNA template and is substantially more efficient than that observed with the ϕx174am3 DNA template. It is conceivable that the mispair extension frequency may depend upon the source of RT. Yet for a given RT, the template sequence has a major effect, which is independent of whether the template is RNA or DNA.

2. MATERIALS AND METHODS

2.1. *Enzymes*

All RTs used in this study were recombinant enzymes expressed by us in *E. coli* [27–29] and purified to homogeneity according to Clark et al. [30]. The specific activities of the different RTs used were 4,000–5,000 U 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 under our standard assay conditions [19].

2.2. *Template-primers*

The template was a 34-base oligonucleotide (5′-ACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCC-3′) identical to nucleotides 2,096–2,129 of *E. coli* 16 S rRNA. This template was primed with a 2-fold molar excess of the 16mer oligonucleotide that hybridizes to the nucleotides at positions 17–32 of the template (Fig. 1). Four versions of the 16 base primers were synthesized separately. All are identical except for the 3′ terminal base (N) which contains either an A, C, G or T. The sequence of these primers is 5′-ATTTCACATCTGACTN-3′. We verified with each primer that more than 85% was annealed to the template oligonucleotide DNA (not shown). The primers were 5′ end labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP and hybridized as described [11].

2.3. *DNA polymerization reaction*

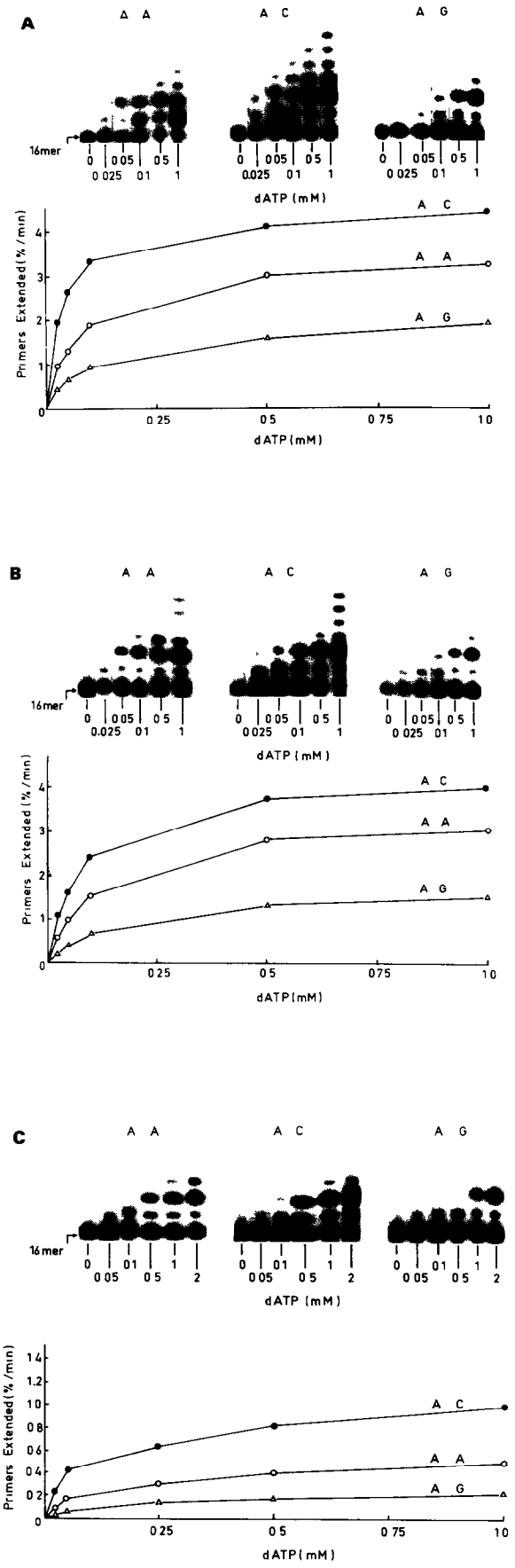
The polymerization reactions for HIV RTs and MLV RT were performed as described [11].

3. RESULTS AND DISCUSSION

The fidelity of DNA polymerases is an outcome of a variety of factors such as the sequences copied, the nature of the DNA polymerase, proofreading activities and the involvement of other proteins [31]. Our recent studies have demonstrated that HIV-1, HIV-2 and MLV RTs exhibit higher mispair extension efficiencies in copying rRNA rather than in copying ϕx174m3 DNA [26]. In the current study we have examined the fidelity of the DNA-dependent DNA synthesis with DNA sequences identical to those of the rRNA sequences previously used for reverse transcription [26] (employing the four template–primer substrates as described in Materials and Methods and in Fig. 1). The catalysis of the extension from the preformed mispairs by the RTs was studied by measuring primer elongation using dATP as the only deoxynucleotide triphosphate. Under the conditions employed (see Materials and Methods) the reaction was linear with respect to time (not shown).

The extension from the preformed mispairs was detected by an increase in the length of the oligonucleotide primers to 17 nucleotides or greater. The ability of HIV-1, HIV-2 and MLV RTs to extend each of the three mispairs under steady-state kinetic conditions is illustrated in Fig. 2. Extension from the A:A mispair is shown by elongation of the 16mer primer producing products from 17 to 22 nucleotides in length. The 19mer product presumably results from misinsertion of dAMP opposite the template guanine at position 14 (see Fig. 1). The subsequent extension from the newly formed G:A mispair involves incorporation of complementary dAMPs opposite the template thymidines at positions 13–11, leading to synthesis of 20–22mer products. The extension from the A:G mispair results in the production of the 17mer, 18mer and 19mer products. The extension from the A:C mispair results in the production of oligonucleotides from 17 to 23 nucleotides in length. The 23mer product probably results from an additional misinsertion of dAMP opposite the template guanine at position 10 (indicating that the extension from an A:C mispair involves multiple misinsertions). Thus, the A:C and A:A mispairs are extended more efficiently than the A:G mispair, i.e. a greater proportion of the mispaired primer is extended and the products are longer.

DNA polymerases might exhibit different binding affinities for each type of mispair, resulting in different efficiencies of mispair extensions. To evaluate the mispair extension efficiency, we have measured the ratios of gel band intensities (estimated by densitometry) and have determined the extension rates (V = percent of primer extended per min) as a function of dATP concen-



trations for every given mispair. The apparent K_m and V_{max} values for the extension of each terminus were calculated from the double-reciprocal plots of the initial velocities vs. the substrate concentrations (Lineweaver–Burk curves). It is evident from Table I that both HIV RTs, as well as MLV RT, discriminate against extension of A:A, A:C or A:G mispairs, primarily by a large increase (of 500 to 1,900-fold) in apparent K_m values compared to that of paired A:T termini (with only a slight reduction of about 2–13-fold in apparent V_{max} values). Hence, these results indicate that K_m discrimination is predominantly the mechanism determining the mispair extension. Estimates of substrate extension efficiencies (i.e. V_{max}/K_m) [32] for both HIV RTs indicate that the A:C mispair was extended by 1.8–2.6-fold more efficient than the A:A mispair and 6–8-fold more efficient than the A:G mispair (Table II). The relative extension frequencies by MLV RT from all three mispairs were 2–3-fold lower than those by HIV-1 RT or HIV-2 RT.

This study allows a direct comparison of preformed mispair extension frequencies obtained with identical DNA and RNA sequences for HIV RTs in comparison with MLV RT. All three preformed A:A, A:C and A:G mispairs were extended efficiently regardless the nature of template. It is apparent from Table II that the mispair extension frequencies on RNA and DNA templates with identical sequences for every given RT studied are similar. Thus, our results are in agreement with the observations of Yu and Goodman [25].

The comparative data presented in Table II suggest that the relative mispair extension frequencies observed with all three RTs are significantly higher with the synthetic DNA or native rRNA sequences than those observed previously with the particular sequence of native ϕx174am3 DNA template copied (i.e. up to more than 20-fold as in the case of A:A mispair with MLV RT). However, both HIV RTs were 2–3-fold less accurate than MLV RT with either the synthetic DNA or rRNA templates. This difference between HIV RTs and MLV RT becomes 4–8-fold when analyzed with ϕx174am3 DNA as a substrate. Consequently, the outlined dissimilarities in mispair extension frequencies may be ex-

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Fig. 2. Kinetics of mispair extension by HIV-1, HIV-2 and MLV RTs. The 16mer oligonucleotide primers were hybridized to a 34mer oligonucleotide template to produce the indicated 3′ terminal mispairs at position 17 as described in Materials and Methods. The primers were extended with HIV-1 RT (A) and HIV-2 RT (B) for 15 min at 30°C in the presence of 0, 0.025, 0.05, 0.1, 0.5 or 1.0 mM dATP as the only deoxynucleotide triphosphate substrate. The reaction with MLV RT (C) contained 0, 0.05, 0.1, 0.5, 1.0 or 2.0 mM dATP. Polyacrylamide gel electrophoretic analysis of the extended primers from A:A (○), A:C (●) and A:G (△) mispairs are shown in the upper panel at left, center and right, respectively. The primer position is indicated by an arrow. The quantitation of the primers extended were plotted in the lower panel after scanning the autoradiograms.

Table I

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

Primer–template	HIV-1 RT		HIV-2 RT		MLV RT	
	K_m (μM)	V_{max} (%/min)	K_m (μM)	V_{max} (%/min)	K_m (μM)	V_{max} (%/min)
A / A	76 ± 6.1	3.3 ± 0.1	100 ± 11	3.2 ± 0.09	370 ± 65	0.5 ± 0.081
C / A–	40 ± 4.9	4.4 ± 0.13	74 ± 7.9	4.3 ± 0.14	280 ± 51	1.0 ± 0.058
G / A–	140 ± 25	2.0 ± 0.08	150 ± 22	1.5 ± 0.065	360 ± 61	0.23 ± 0.039
T / A–	0.080 ± 0.0045	10 ± 0.45	0.078 ± 0.0041	7.2 ± 0.0032	0.26 ± 0.022	3 ± 0.011

A 16mer oligonucleotide primer was hybridized to a 34 mer oligonucleotide template to produce a DNA duplex with indicated 3′ termini. Extension reactions performed for 15 min, with either HIV-1 or HIV-2 RT at 30°C and containing 0, 0.025, 0.05, 0.1, 0.5 or 1.0 mM dATP (see Fig. 2A and B). Extension reactions performed for 20 min with MLV RT contained 0, 0.05, 0.1, 0.5, 1.0 or 2.0 mM dATP (see Fig. 2C). The percent of 16mer extended was quantitated as described.

plained by the difference in the nucleotide sequences copied.

Sequence-dependent differences in the utilization of templates by DNA polymerases are a well-known phenomenon [33]. The comparison of HIV-1 RT to RTs from avian and murine retroviruses reveals that the kinetic parameters of nucleotide misincorporation at a particular base are also sequence dependent, affected usually by the two bases 3′ in the template [34]. A strong sequence dependence of mispair formations by HIV-1 RT has been reported, employing an M13-based forward mutation assay for the DNA-dependent DNA synthesis [8]. A recent computer analysis of nucleotide sequences of HIV-1 isolates revealed the importance of purine and pyrimidine content of local nucleotide sequences (six bases long) for the evolution of the HIV-1. It was suggested that certain local sequences of 6mer are excessively frequent in the hypervariable regions, and the error spectra of these cyclic sets differ from each other [35]. Thus, the differences in extension frequencies between two DNA sequences with the same preformed mismatches may result from dissimilarities in the purine/pyrimidine content of the sequences analyzed, indicating that the base composition of sequences are of the utmost importance in affecting the error production during DNA synthesis by RTs.

Table II

Comparative relative extension frequencies by HIV-1, HIV-2 and MLV RTs with various primer-templates

Primer–template	HIV-1 RT			HIV-2 RT			MLV RT		
	Oligo[a] DNA	rRNA[b]	ϕx174[c] DNA	Oligo DNA	rRNA	ϕx174 DNA	Oligo DNA	rRNA	ϕx174 DNA
A / A–	1/2,900	1/3,600	1/22,000	1/2,900	1/2,800	1/27,000	1/8,500	1/9,000	1/180,000
C / A–	1/1,100	1/1,300	1/2,600	1/1,600	1/1,400	1/1,800	1/3,200	1/3,000	1/11,000
G / A–	1/8,800	1/10,000	1/24,000	1/9,200	1/12,000	1/34,000	1/18,000	1/19,000	1/200,000

The relative extension frequencies are the ratios of the rate constants (V_{max}/K_m) for the given mispair divided by the corresponding ratios of rate constants calculated from the paired A:T terminus.

[a] The relative extension frequencies were calculated from the data presented in Table I.

[b] The data was taken from our previous study with native rRNA template [26]. The template–primer was 5′-N–AUUGUUUGG-.

[c] The data was taken from our previous study with ϕX174am[3] DNA template [11]. The template–primer was 5′-N–ATGTTTC-

Acknowledgements. We are indebted to Dr. S. Hughes for the supply of purified RTs. This research was supported by a grant (No. RO1-AI27035) from the National Institute of Allergy and Infectious Diseases.

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