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Intrinsic DNA synthesis fidelity of xenotropic murine leukemia virus-related virus reverse transcriptase

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Abstract

Although recent reports have provided strong evidence to suggest that xenotropic murine leukemia virus-related virus (XMRV) is unlikely to be the causative agent of prostate cancer and chronic fatigue syndrome, this recombinant retrovirus can nonetheless infect human cells *in vitro* and induce a chronic infection in macaques. We have determined the accuracy of DNA synthesis of the reverse transcriptases (RTs) of XMRV and Moloney murine leukemia virus (MoMLV) using a combination of pre-steady-state kinetics of nucleotide incorporation and an M13mp2-based forward mutation assay. The obtained results have been compared with those previously reported for the human immunodeficiency virus type 1 BH10 strain (HIV-1_{BH10}) RT. MoMLV and XMRV RTs were 13.9 and 110 times less efficient (as determined by k_{pol}/K_d) than the HIV-1_{BH10} RT in incorporating correct nucleotides. Misinsertion and mispair extension kinetic studies demonstrated that MoMLV RT was more accurate than the HIV-1_{BH10} RT. In comparison with MoMLV RT, the XMRV enzyme showed decreased mispair extension fidelity and was less faithful when misincorporating C or A opposite A. However, the XMRV RT showed stronger selectivity against G in misinsertion fidelity assays. Forward mutation assays revealed that XMRV and MoMLV RTs had similar accuracy of DNA-dependent DNA synthesis, but were >13 times more faithful than the HIV-1_{BH10} enzyme. The mutational spectra of XMRV and MoMLV RTs were similar in having a relatively higher proportion of frameshifts and transversions, compared with the HIV-1_{BH10} RT. However, the XMRV polymerase was less prone to introduce large deletions and one-nucleotide insertions.

Keywords

XMRV; reverse transcriptase; fidelity; DNA polymerase; retroviruses

Introduction

Xenotropic murine leukemia virus-related virus (XMRV) is a recombinant gammaretrovirus originally detected in human prostate cancer tumors [1], and later associated with myalgic encephalomyelitis or chronic fatigue syndrome, a disabling condition characterized by long-lasting debilitating physical and mental fatigue [2]. However, its relevance as a human pathogen remained controversial since many reports failed to confirm the presence of XMRV in multiple cohorts of prostate cancer or chronic fatigue syndrome patients or in

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healthy donors (for reviews see refs. [3,4]). Several studies suggested that XMRV found in human tissues could be the result of DNA contamination from laboratory cell lines or mouse DNA [5–8]. The lack of diversity among XMRV sequences supposedly isolated from different individuals [7,9] was also difficult to reconcile with a genuine process of infectious transmission. The nucleotide sequences of XMRV isolated from patients [1,2] were almost identical to those obtained from virus infecting 22Rv1, a common prostate cancer cell line [10]. This cell line was developed between 1992 and 1999 by serial passage of a human prostate xenograft (CWR22) in nude mice. In an elegant study, Paprotka *et al.* showed that XMRV was not present in the original CWR22 tumor, but would be the likely result of a recombination event between the two endogenous murine leukemia viruses (MLVs) [11]. More recently, large collaborative studies found no evidence of murine-like gammaretroviruses in patients with chronic fatigue syndrome, after careful re-examination of blood samples previously identified as XMRV-positive [9,12].

Although XMRV is unlikely to be a human pathogen, this recombinant retrovirus can infect *in vitro* human blood cells [13], as well as primary neuronal cells [14]. Moreover, intravenous inoculation of macaques with XMRV leads to persistent chronic disseminated infection [15]. A deeper knowledge of gammaretrovirus replication is thus relevant for a better understanding of retroviral infection, particularly in the context of potential risks associated with xenotransplantation of tissues and organs.

The DNA polymerase domain of XMRV reverse transcriptase (RT) shares 97.5% amino acid sequence identity, with its equivalent of Moloney MLV (MoMLV) RT (Fig. 1). However, amino acid differences between both enzymes accumulate at their C-terminal region that includes the RNase H domain. The DNA polymerase and the RNase H domains of XMRV RT derive from two different endogenous murine retroviruses, with the recombination site located within the nucleotide sequence encoding for amino acid residues 431 to 455 in the RT [11]. The structural and biochemical properties of the MoMLV RT have been extensively studied [16,17]. In comparison with MoMLV RT, the XMRV enzyme was less efficient in DNA synthesis and in unblocking chain-terminated primers, and showed lower processivity [18]. Estimates on the accuracy of DNA synthesis catalyzed by XMRV RT have been limited to qualitative assessments of primer extension products obtained in the absence of one dNTP, and comparing incorporation efficiencies for a single nucleotide mispair (dATP vs. the correct dTTP). These assays demonstrated a higher intrinsic fidelity for XMRV RT [18]. However, the mutation rates of XMRV and an amphotropic MLV were similar when assessed in cell culture by measuring the frequency of *lacZ* inactivation [18].

In our study, we have performed an extensive analysis of the fidelity of DNA-directed DNA synthesis of XMRV RT by using enzymatic assays (i.e. misincorporation and mispair extension fidelity measurements with various base pairs). In addition, we measured error rates for this enzyme in M13mp2-based *lacZ* α forward mutation assays. The kinetic assays showed differences between XMRV and MoMLV RTs in their selectivity between correct and incorrect nucleotides, as well as in their ability to extend mispaired 3' termini. However, our results do not reveal significant differences in the overall fidelity between XMRV and MoMLV RTs, as determined in forward mutation assays. Mutational patterns generated by XMRV and MoMLV RTs were different in relation to the nature and location of hot spots and their tendency to introduce frameshift mutations at homopolymeric sequences.

Results

Nucleotide incorporation kinetics

XMRV and MoMLV RTs were purified to homogeneity by a combination of metal affinity and ion exchange chromatography. In both cases, the enzymes were obtained as a single polypeptide chain containing a non-removable His₆ tag at their N terminus. Both enzymes were judged to be pure by SDS-polyacrylamide gel electrophoresis with M_r values of around 75,000–79,000 (Fig. 2A). Their catalytic efficiency of nucleotide incorporation was determined by transient kinetics, and compared with previous values obtained in the same conditions with HIV-1_{BH10} RT [19]. Pre-steady-state burst experiments for the incorporation of a correct nucleotide (dTTP) on the DNA-DNA template-primer 31T/21P were carried out in the presence of dTTP (at concentrations in the range of 5 to 150 μ M). Nucleotide incorporation rates (k_{obs}) were plotted against the corresponding dTTP concentration to obtain the kinetic parameters k_{pol} and K_d . As shown in Fig. 2B, both gammaretroviral RTs had similar K_d s, but their catalytic rates of nucleotide incorporation were different. The k_{pol} of XMRV RT was about 8 times lower than the k_{pol} obtained with the MoMLV RT. When compared with human immunodeficiency virus type 1 BH10 strain (HIV-1_{BH10}) RT, the XMRV enzyme showed >100-fold reduced catalytic efficiency (measured as k_{pol}/K_d) (Table 1).

Fidelity of DNA synthesis as determined by pre-steady-state kinetics

Misinsertion and mispair extension fidelity assays were used to estimate the accuracy of DNA polymerization catalyzed by XMRV and MoMLV RTs, and the results were compared with those obtained with the previously studied HIV-1_{BH10} enzyme [19]. The kinetics of misincorporation of C, G or A opposite A into the heteroduplex 31T/21P were determined under single turnover conditions. Misinsertion kinetic parameters (k_{pol} and K_d) are given in Table 1, and compared with those obtained in reactions carried out with the correct nucleotide, dTTP. The largest differences between RTs related to their k_{pol} values, which were around 100 times lower for gammaretroviral RTs than for the HIV-1_{BH10} enzyme. However, these effects were less pronounced when misinsertion ratios were compared. The MoMLV RT was more accurate than the HIV-1 RT for all tested misinsertions. In the case of XMRV RT, we observed differences in fidelity that affected specific mispairs. Thus, XMRV RT showed a very low misinsertion ratio for the incorporation of G opposite A ($f_{\text{ins}} = 4.52 \times 10^{-6}$) as compared to the HIV-1 enzyme ($f_{\text{ins}} = 9.73 \times 10^{-5}$). However, differences between both RTs were relatively small for the two other misinsertions (i.e. C or A opposite A). Overall, XMRV RT was found to be less accurate than the MoMLV RT, except for misincorporating G instead of T.

The kinetics of mispair extension were determined by measuring the incorporation of dTTP at the 3' end of the primer, using 31nt template/21nt primer duplexes containing mismatched termini (i.e. G:T, G:G or G:A). The results are shown in Table 2. The highest mismatched extension ratios (f_{ext}) were obtained for the G:T mispair. Their values were in the range of 0.24 to 2.12×10^{-3} . XMRV and HIV-1_{BH10} RTs showed similar efficiencies of G:T mispair extension. However, MoMLV RT showed 4.8-fold increased accuracy in comparison with the HIV-1_{BH10} RT. Interestingly, the increased accuracy of MoMLV RT in mispair extension assays was also observed with the template-primer having a G:G mismatch. Its mispair extension ratio was 1.72×10^{-5} , which is considerably less than the values reported for XMRV and HIV-1_{BH10} RTs. Extension of G:A mispairs was rather inefficient with all tested enzymes with f_{ext} values in the range of 0.36 to 1.78×10^{-5} . The XMRV RT was 3.5–5 times more efficient in extending G:A mispairs in comparison with the RTs of MoMLV and HIV-1_{BH10}.

Differences between XMRV and HIV-1_{BH10} RTs were relatively small, except for G:A mismatch extension and misincorporation of G opposite A. However, both enzymes showed decreased misinsertion and mispair extension fidelity in comparison with the MoMLV RT. Discrimination against C or A opposite A was 3.4 times and 13.0 times more efficient in reactions catalyzed by MoMLV RT compared with XMRV RT, while this enzyme showed about 8- to 9-fold increased efficiency in extending G:T and G:G mispairs.

M13mp2 *lacZ* forward mutation assays

Mutation frequencies of XMRV RT and the MoMLV RT used in the kinetic assays described above were determined with the M13-based forward mutation assay [20], and compared with those previously reported for the HIV-1_{BH10} RT and a commercially available MoMLV RT [19,21]. In these assays, a gapped M13mp2 DNA is used as substrate of the polymerization reaction. The mutational target for errors made by the RT is the wild-type *lacZ* α -complementation sequence which is used as template in the gap-filling reaction. Errors made by the RT may result in a decrease in α -complementation and can be detected as plaques with an altered color phenotype (pale blue or colorless) in a specific indicator strain. Silent mutations are not detected in these assays. Mutant frequencies are calculated as the ratio of mutant to total plaques. MoMLV RTs obtained from different sources showed similar mutant frequencies in these assays, and were about 15-fold more accurate than the HIV-1_{BH10} RT (Table 3). Interestingly, the XMRV RT showed a mutant frequency of 0.157%, similar to that determined for MoMLV RTs obtained from two different sources.

The mutational specificity of XMRV RT was determined after sequencing the *lacZ* mutants generated in the forward mutation assays. The mutational spectrum reveals an accumulation of errors at six major hot spots, located at positions -9, +100, +104, +129, +134 and +148 (Fig. 3). Four of these are represented by a high frequency of one-nucleotide deletions (i.e. at positions -9, +100, +104, and +134) while the two others involve C→T and G→T mutations. Only one of the observed hot spots occurs in a run of identical nucleotides (a run of Cs around position +134), suggesting that template-primer slippage may not be a large contributor to variability induced by the XMRV RT. The error distribution obtained with XMRV enzyme differed from published mutational spectra of HIV-1_{BH10} and MoMLV RTs [21,24]. These two enzymes generate fewer hot spots that occur at different locations in comparison with the XMRV RT. Only the sequence around positions +131/+136 emerges as a mutational hot spot for both gammaretroviral RTs. However, the types of errors found at this location are different in both enzymes. While XMRV RT introduced one-nucleotide deletions within the run of Cs, the MoMLV enzyme produced one-nucleotide insertions affecting the adjacent T (located at position +131) [21].

As in the case of the MoMLV RT, the XMRV enzyme had a relatively high propensity to introduce frameshift mutations. Frameshifts represented around 60% of all errors generated by those two enzymes. However, this figure was reduced to 36.7% in the case of HIV-1_{BH10} RT (Table 4). Compared with the HIV-1_{BH10} RT, the XMRV RT error rates were reduced 17.9 times for base substitutions and 8.2 times for frameshifts. These values were similar to those obtained with MoMLV RT. Both gammaretroviral RTs showed a higher tendency to produce transversions, while HIV-1_{BH10} RT was prone to generate transitions. Frameshift errors produced by the HIV-1_{BH10} RT accumulated at homopolymeric sequences. On the other hand, insertions and deletions produced by MoMLV RT were evenly distributed between runs and non-runs of nucleotides, and frameshift errors generated by XMRV RT were less frequent in nucleotide runs. For XMRV RT, the overall -1 base frameshift error rate in homopolymeric template sequences was around 1/247,400. This figure was about 2.5 times lower than the corresponding value obtained with the MoMLV RT (Table 4). Further differences between XMRV and MoMLV RTs were related to the distribution and nature of insertions and deletions. More than 30% of the errors found in the mutational spectrum of

MoMLV RT were large deletions or one-nucleotide insertions [21]. However, these types of errors were not detected in our assays with the XMRV RT.

Discussion

The association between XMRV and either prostate cancer or chronic fatigue has been hotly debated until Paprotka *et al.* provided compelling evidence on the recombinant origin of this novel gammaretrovirus [11], and multilaboratory studies revealed serious problems that affected the reproducibility of the assays, at least in the blood of patients with chronic fatigue syndrome [12]. These issues aside, other studies indicate that XMRV may infect human cells and establish a persistent infection in primates [13–15]. Gammaretroviruses could still pose a risk for human health as potential contaminants of biological products, and in particular interventions (i.e. xenotransplantation). In this context, studies on fundamental mechanisms of DNA synthesis, including fidelity of their retroviral RTs might be relevant to understand the biology of retroviruses and provide novel tools in biotechnology.

Patient-derived XMRV is identical to virus infecting the prostate cancer cell line CWR22Rv1, originating from two endogenous proviruses (PreXMRV-1 and PreXMRV-2) during passaging the prostate tumor in mice [11]. The 5' end of the XMRV genome derives from PreXMRV-2, while the 3' end derives from PreXMRV-1. One recombination site is located in the *pol* gene, at the RT-coding region (between nucleotides encoding residues 431 to 455 of XMRV RT). Therefore, the DNA polymerase domain of XMRV RT is almost identical to that of MoMLV RT (>97.5% sequence identity), while the differences between both enzymes are mostly concentrated at the RNase H domain (Fig. 1). Compared with MoMLV RT, XMRV RT showed an 8-fold reduced catalytic efficiency of DNA polymerization, which was a consequence of its low k_{pol} . Our results are broadly consistent with those recently reported by Ndongwe *et al.* [18], who also found that both gammaretroviral RTs had diminished DNA polymerase activity in comparison with the HIV-1_{BH10} RT. In their study, authors used pre-steady-state kinetics to compare the intrinsic fidelities of XMRV, MoMLV and HIV-1 RTs. However, their analysis was limited to the misincorporation of T opposite T on a heteropolymeric 100/18mer DNA-DNA template-primer. Their reported misinsertion ratios (f_{ins}) for the RTs of XMRV, MoMLV and HIV-1 were 1.76×10^{-3} , 7.32×10^{-3} , and 4×10^{-2} , respectively [18]. These results together with qualitative assessments of fidelity based on primer extension reactions using three dNTPs led authors to conclude that XMRV RT was more faithful than the MoMLV polymerase, while both enzymes showed increased accuracy relative to HIV-1 RT.

In our analysis, we demonstrate that MoMLV RT is more accurate than HIV-1_{BH10} RT in discriminating C, G or A *versus* T (Table 1). In addition, we found that the HIV-1 enzyme shows a higher efficiency in extending G:T and G:G mismatches (Table 2). Since pre-steady-state kinetics analyses of fidelity require relatively high concentrations of MLV RT, due to its low catalytic efficiency of DNA polymerization, there is only one additional report comparing MoMLV and HIV-1 RTs using transient kinetics [23]. In this study, MoMLV RT was found to be 50 times less efficient than HIV-1 RT in incorporating a correct dCTP on an RNA/DNA template-primer. Our results for the MoMLV RT were broadly in agreement with previous reports showing its higher accuracy, in misinsertion and mispair extension assays carried out with DNA-DNA template-primers under steady-state conditions [25–27], as well as with the results obtained by Skasko *et al.*, who showed that the MoMLV enzyme had a 3.6-fold increased ability to discriminate between G and C, and increased fidelity of G:T mispair extension [23]. Our data were also consistent with those studies in showing that misinsertion ratios were in the range of 10^{-4} to 10^{-7} , lower than those reported for XMRV and MoMLV RTs by Ndongwe *et al* [18]. XMRV and HIV-1_{BH10} RTs showed similar mispair extension efficiencies but were less faithful than MoMLV RT in these assays (Table

2). However, the XMRV enzyme showed remarkable selectivity against dGTP when the template base was A. The fixation of a mutation involves misincorporation followed by mispair extension. Therefore, discrimination at the nucleotide incorporation step might be critical to determine the fidelity of XMRV RT. Nevertheless, kinetic data are strongly dependent on the sequence and the template-primer used [25], and in this scenario, forward mutation assays may provide a better estimate of fidelity differences between different RTs, since they can screen for mutations in a relatively large number of sequence contexts.

The mutant frequency obtained for XMRV RT was similar to previous estimates for other oncoretroviral RTs, such as MLV RT or avian myeloblastosis virus RT [28,29; reviewed in ref. 30]. XMRV and MoMLV RTs were 13.1–15.4 times more accurate than the HIV-1_{BH10} enzyme. HIV-1_{BH10} RT and other phylogenetically close HIV-1 RTs are prone to introduce frameshifts, particularly at homopolymeric nucleotide runs. More than 90% of the one-nucleotide insertions or deletions generated by the HIV-1 RT occurred in homopolymeric sequences, likely originating from utilization of misaligned template-primers [19,31–33]. On the other hand, gammaretroviral RTs showed increased frameshift accuracy and a stronger tendency to introduced transversions. Compared with XMRV RT, the MoMLV enzyme showed a stronger tendency to introduce large deletions and one-nucleotide insertions [21]. Other significant differences between XMRV and MoMLV RTs were found in the distribution of frameshifts (i.e. one-nucleotide deletions) at run and non-run template sequences. XMRV RT showed 4.4-fold increased frameshift fidelity while copying homopolymeric *versus* heteropolymeric sequences, although similar error rates were obtained for MoMLV RT while copying both types of sequences. Interestingly, the distribution of frameshift errors at runs *versus* non-runs of nucleotides in the mutational spectrum of XMRV RT was similar to that previously reported for avian myeloblastosis virus RT [29]. These results suggest that unlike HIV-1 RT, template-primer slippage may play a secondary role in generating frameshifts during DNA synthesis catalyzed by XMRV RT.

As expected from the accuracy of their RTs in forward mutation assays, both XMRV and MoMLV showed similar mutation rates in single-cycle of replication assays carried out *ex vivo* [18], although their mutational spectra have not been determined. Unlike in the case of HIV-1, there is a good correlation between the intrinsic error rates of MoMLV RT and the mutation rates obtained with the virus, with estimates ranging from 2×10^{-6} to 2×10^{-5} [34,35]. Sequence analysis of XMRV obtained from prostate cancer tumors revealed extremely low variability [1,2] that was difficult to reconcile with the error-prone nature of retroviral replication. Our study demonstrates that the accuracy of XMRV RT is not unusually high but similar to that reported for other oncoretroviral RTs.

Finally, our data also suggest a role for the connection/RNase H domain in modulating fidelity of DNA synthesis. The differences in the catalytic efficiencies of nucleotide incorporation between XMRV and MoMLV RTs could result from altered template-primer binding. In agreement with this proposal, it has been shown that XMRV RT has lower DNA binding affinity and processivity in comparison with the MoMLV RT [18]. Although both RTs share identical amino acid sequences in their DNA polymerase motifs A, B, C, D and E (relevant for dNTP binding and catalysis), they differ at their connection/RNase H domains (Fig. 1). Sequence differences at those regions could affect polymerization by interfering with the positioning of the DNA at the polymerase active site, thereby influencing slippage and template switching. Further studies are warranted to elucidate the contribution of RT connection/RNase H domains to polymerization accuracy.

Materials and Methods

Expression and purification of RTs

XMRV RT derived from clone VP62 (GenBank accession number DQ399707)[1] was expressed in *E. coli* as a single polypeptide chain of 75.9 kDa containing a non-removable His₆ tag, and then purified by a combination of immobilized metal affinity and ion exchange chromatography [36]. Recombinant HIV-1_{BH10} RT was expressed and purified as previously described [37,38]. This enzyme was coexpressed with HIV-1 protease in *E. coli* XL1 Blue to obtain the p66/p51 heterodimer, which was later purified by ionic exchange followed by immobilized metal affinity chromatography on Ni²⁺-nitriloacetic acid-agarose. In this enzyme, the His₆ tag was introduced at the C-terminal end of p66.

MoMLV RT was overexpressed in *E. coli* BL21 using a plasmid (pMuLVRT) encoding the full-length enzyme with a His₆ N-terminal extension [22]. Plasmid pMuLV RT was kindly provided by Dr. Baek Kim (University of Rochester Medical Center, Rochester, NY, USA). The RT sequence encoded within pMuLVRT is identical to the one shown in Fig. 1, except for His8 which was replaced by Tyr. Bacterial cells obtained from 3 liters of culture were harvested by centrifugation. The obtained pellet was resuspended in 20 ml of 40 mM Tris-HCl (pH 8.0) buffer, containing 10% saccharose, 1 mM phenylmethyl sulfonyl fluoride, 2 mM benzamide and 1 mg/ml lysozyme and left on ice for 15 min. The resuspended pellet was then mixed with 20 ml of a solution containing 0.8% (v/v) NP-40, 20 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride and 2 mM benzamidine, and left at 4°C for another 15 min before adding NaCl to a final concentration of 0.5 M. Lysed cells were sonicated and centrifuged twice at 15,000xg for 15 min. The supernatant was diluted 7-fold in 40 mM Tris-HCl (pH 7.9) buffer containing 0.5 M NaCl and applied to a Ni²⁺-charged ProBondTM resin column (Invitrogen). After extensive washing, RT was eluted with a 0 – 1.0 M imidazole gradient. RT-containing fractions were combined and applied to a 3-ml Q-Sepharose column (GE Healthcare), previously equilibrated in 40 mM Tris-HCl (pH 7.9) buffer containing 0.5 M NaCl. Enzyme was recovered in the non-binding fraction and dialyzed against 50 mM Tris-HCl (pH 7.0) buffer containing 25 mM NaCl, 1 mM EDTA, 1 mM DTT and 10% glycerol. Purified and concentrated RTs were stored at –20°C in dialysis buffer containing 10% glycerol (HIV-1_{BH10} and MoMLV RTs) or 50% glycerol (XMRV RT). Purity of enzymes was assessed by SDS-polyacrylamide gel electrophoresis.

Active site titration

Enzymes were routinely quantified by active site titration before biochemical studies [39]. Briefly, individual RTs were incubated with 30 nM DNA-DNA template primer [D38 (5'-GGGTCCTTTCTTACCTGCAAGAATGTATAGCCCTACCA-3')/25PGA (5'-TGGTAGGGCTATACATTCTTGCAGG-3')] for 10 min at 37°C, in 10 µl of 100 mM Hepes (pH 7.0) buffer, containing 30 mM NaCl, 30 mM magnesium acetate, 130 mM potassium acetate, 1 mM DTT and 5% (w/v) polyethylene glycol. After adding 10 µl of the same buffer containing 1 mM dTTP, 4 µl aliquots were removed and quenched at 10, 20, 30 and 40 s with sample loading buffer [10 mM EDTA in 90% formamide containing xylene cyanol FF (3 mg/ml) and bromophenol blue (3 mg/ml)]. Products were analyzed by 20% denaturing sequencing gel electrophoresis and quantified by phosphorimaging. For each reaction, the percentage of elongated primer was plotted against the incubation times and the data were fit to a linear equation. Since the concentration of template-primer is well above the *K_d* under our assay conditions [40,41], the active RT concentration was calculated from the y-intercept that represents the amount of RT bound to template-primer at time zero. Optimal RT concentrations in these experiments are usually in the range of 2.5 to 5 nM.

Misinsertion and mispair extension fidelity of DNA synthesis

Fidelity estimates were obtained from transient kinetics experiments carried out in 50 mM Tris-HCl buffer (pH 8.0), containing 50 mM KCl and 12 mM MgCl₂ and a variable concentration of nucleotide [19,42]. Oligonucleotides 21P (5'-ATACTTTAACCATATGTATCC-3') and 31T (5'-TTTTTTTTTAGGATACATATGGTTAAAGTAT-3') were used as primer and template, respectively. Before annealing to the template, the primer was labeled at its 5' terminus with [γ -³²P]ATP (Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs). Correct nucleotide (dTTP) incorporation reactions were performed under single turnover conditions with 100 nM (active sites) RT and 100 nM template-primer. At appropriate times, reactions were quenched with EDTA (0.3 M final concentration). Products were analyzed by denaturing polyacrylamide gel electrophoresis and quantified by phosphorimaging.

Mismatched ³²P-labeled primers (i.e. 21PT, 21PG and 21PA) annealed to the 31-mer DNA template (31T) were used to determine G:T, G:G and G:A mispair extension efficiencies. In these assays, the relevant kinetic parameters were obtained for the incorporation of dTTP. Reactions involving misincorporation of dCTP, dGTP or dATP (instead of the correct dTTP) using 31T/21P, or extension of G:T, G:G or G:A mispairs, were conducted with an excess of RT (120 nM) over template-primer (100 nM). These conditions were chosen to eliminate the influence of the enzyme turnover rate (k_{ss}), which interferes in the measurements of low incorporation rates. Misincorporation reactions carried out with dGTP and dATP were carried out in the presence of 18 mM MgCl₂ to ensure that enough free Mg²⁺ was available to bind the catalytic site A in the DNA polymerase domain of the RT [42]. Pre-steady-state kinetic data were fitted to the burst equation:

$$[P] = A \times [1 - \exp(-k_{obs} \times t)] + k_{ss} \times t$$

where A is the amplitude of the burst, k_{obs} is the apparent kinetic constant of formation of the phosphodiester bond, and k_{ss} is the kinetic constant of the steady-state linear phase. The dependence of k_{obs} on dNTP concentration is described by the equation:

$$k_{obs} = k_{pol} \times [dNTP] / (K_d + [dNTP])$$

where K_d and k_{pol} are the equilibrium constant and the catalytic rate constant of the dNTP for RT, respectively. K_d and k_{pol} were determined from curve-fitting using Sigma Plot.

M13mp2 *lacZ* forward mutation assay

The assay was performed essentially as previously described [20]. Briefly, the single-stranded and replicative forms of the M13mp2 DNA were obtained from phage grown in the *E. coli* strain NR9099. The replicative form was digested with PvuII to obtain a 6.8-kb double-stranded DNA that was denatured and annealed to the single-stranded M13 DNA to obtain a gapped duplex DNA substrate. This DNA has a gap of 407 nucleotides (positions -216 to +191) and lacks the *lacZ* α -complementation target sequence in one of the strands. Gap-filling reactions (10 μ l) were carried out in 25 mM Tris-HCl (pH 8.0) buffer, containing 100 mM KCl, 2 mM DTT, 4 mM MgCl₂, 250 μ M of each dNTP, 5 μ g/ml gapped duplex DNA and 100 nM RT [24]. After 30-min incubation at 37°C, reactions were stopped by adding EDTA to a final concentration of 15 mM. The obtained products were electroporated into *E. coli* MC1061 host cells. After a brief recovery period (10 min), transformants were plated onto M9 medium containing 0.195 mM 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 0.2 mM isopropyl-1-thio- β -D-galactopyranoside, and a lawn of the α -complementation strain of *E. coli*, CSH50. Mutant plaques were picked and the phage

double-stranded DNA replicative form isolated. Phenotypes of mutant plaques were confirmed by nucleotide sequencing using primer 5'-GCTTGCTGCAACTCTCTCAG-3' (Macrogen Inc., Seoul, South Korea). At least five fill-in reactions were performed for each enzyme and the nucleotide sequence of the entire gap region was determined for all mutant plaques. Mutant frequencies and specific error rates were obtained as previously described [21].

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Abbreviations

XMRV	xenotropic murine leukemia virus-related virus
MLV	murine leukemia virus
RT	reverse transcriptase
MoMLV	Moloney MLV
dTTP	2'-deoxythymidine 5'-triphosphate
HIV-1	human immunodeficiency virus type 1
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dNTP	deoxyribonucleoside-triphosphate
DTT	dithiothreitol

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Fig. 1.

Amino acid sequence comparison of XMRV, MoMLV and HIV-1 RTs. Sequence alignments of gammaretroviral and HIV-1 RTs are based on sequence and structural motifs and were taken from Coté & Roth [16]. Amino acid differences between XMRV and MoMLV RTs are underlined and indicated with asterisks. In the HIV-1 RT sequence, underlined amino acids represent catalytic residues in the DNA polymerase domain (Asp110, Asp185 and Asp186) and the strictly conserved D-E-D motif in the RNase H domain (Asp443, Glu478 and Asp498). Conserved motifs A – E in the DNA polymerase domain are also indicated. The arrow shows the cleavage site rendering p51 in HIV-1 RT. Nucleotide sequences deposited in GenBank with accession numbers DQ399707 (XMRV), J02255 (MoMLV) and M15654 (HIV-1_{BH10}) were used to obtain the RT sequences shown in the alignment.

Fig. 2.

Analysis of purified XMRV and MoMLV RTs and kinetic parameters of DNA polymerization. (A) SDS-polyacrylamide gel electrophoresis of purified RTs. Molecular weight markers and their masses were: phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa). (B) Nucleotide concentration dependence for the incorporation of dTTP into the 31T/21P duplex DNA. The continuous line represents the best fit of the data to the Michaelis-Menten equation. The obtained k_{pol} values were $0.19 \pm 0.02 \text{ s}^{-1}$ for XMRV RT and $1.55 \pm 0.17 \text{ s}^{-1}$ for MoMLV RT. The K_d values of XMRV RT and MoLV RT were $24.1 \pm 4.3 \text{ μM}$ and $24.9 \pm 7.1 \text{ μM}$, respectively.

Fig. 3. Spectrum of mutations induced by XMRV RT. Single-nucleotide substitutions are indicated by the letter corresponding to the new base (in blue) above the template sequence of the *lacZa* target. Inverted closed red triangles indicate deletions of one nucleotide.

Table 1

Pre-steady-state kinetic parameters for misincorporation.

Enzyme	Nucleotide	k_{pol} (s ⁻¹)	K_d (μM)	k_{pol}/K_d (μM ⁻¹ ·s ⁻¹)	Misinsertion ratio (f_{ins}) ^a
XMRV RT	dTTP	0.19 ± 0.02	24.1 ± 4.3	(7.83 ± 1.71) × 10 ⁻³	
	dCTP	(2.34 ± 0.24) × 10 ⁻³	3800 ± 1181	(6.17 ± 2.02) × 10 ⁻⁷	(7.88 ± 3.10) × 10 ⁻⁵ (0.56)
	dGTP	(8.61 ± 0.97) × 10 ⁻⁵	2433 ± 199	(3.54 ± 0.49) × 10 ⁻⁸	(4.52 ± 1.17) × 10 ⁻⁶ (21.5)
	dATP	(4.16 ± 0.17) × 10 ⁻⁴	2914 ± 832	(1.42 ± 0.41) × 10 ⁻⁷	(1.81 ± 0.66) × 10 ⁻⁵ (0.27)
MoMLV RT	dTTP	1.55 ± 0.17	24.9 ± 7.1	(6.21 ± 1.88) × 10 ⁻²	
	dCTP	(2.08 ± 0.26) × 10 ⁻³	1466 ± 789	(1.42 ± 0.78) × 10 ⁻⁶	(2.29 ± 1.43) × 10 ⁻⁵ (1.9)
	dGTP	(8.30 ± 0.83) × 10 ⁻⁴	1135 ± 325	(7.33 ± 2.22) × 10 ⁻⁷	(1.18 ± 0.51) × 10 ⁻⁵ (8.2)
	dATP	(4.39 ± 0.69) × 10 ⁻⁴	4960 ± 2842	(8.87 ± 5.27) × 10 ⁻⁸	(1.42 ± 0.95) × 10 ⁻⁶ (3.5)
HIV-1 _{BH10} RT ^b	dTTP	11.6 ± 0.5	13.4 ± 1.9	0.863 ± 0.126	
	dCTP	0.291 ± 0.052	7645 ± 2959	(3.80 ± 1.62) × 10 ⁻⁵	(4.40 ± 1.98) × 10 ⁻⁵
	dGTP	0.289 ± 0.032	3435 ± 1099	(8.40 ± 2.84) × 10 ⁻⁵	(9.73 ± 3.58) × 10 ⁻⁵
	dATP	(2.40 ± 0.2) × 10 ⁻³	559 ± 191	(4.29 ± 1.50) × 10 ⁻⁶	(4.97 ± 1.88) × 10 ⁻⁶

The template-primer 31T/21P was used as the substrate. Data shown are the mean values ± standard deviation of a representative experiment. Each of the assays was performed independently at least three times.

^a $f_{ins} = [k_{pol}(\text{incorrect})/K_d(\text{incorrect})]/[k_{pol}(\text{correct})/K_d(\text{correct})]$, where incorrect nucleotides were dCTP, dGTP or dATP, while the correct nucleotide was dTTP. Numbers in bold and between parentheses represent the relative increase of misinsertion fidelity relative to the HIV-1_{BH10} RT.

^b Reported values for HIV-1_{BH10} RT were taken from Matamoros *et al.* [19].

Table 2

Pre-steady-state kinetic parameters for mispair extension.

Enzyme	Base pair at the 3' end ^a	k_{pol} (s ⁻¹)	K_d (μM)	k_{po}/K_d (μM ⁻¹ ·s ⁻¹)	Mispair extension ratio (f_{ext}) ^b
XMRV RT	G:C	0.19 ± 0.02	24.1 ± 4.3	(7.83 ± 1.71) × 10 ⁻³	
	G:T	(4.99 ± 0.26) × 10 ⁻²	3012 ± 418	(1.66 ± 0.25) × 10 ⁻⁵	(2.12 ± 0.56) × 10 ⁻³ (0.53)
	G:G	(3.35 ± 0.30) × 10 ⁻³	3084 ± 714	(1.09 ± 0.27) × 10 ⁻⁶	(1.39 ± 0.46) × 10 ⁻⁴ (2.2)
	G:A	(4.27 ± 0.21) × 10 ⁻⁴	3058 ± 557	(1.39 ± 0.26) × 10 ⁻⁷	(1.78 ± 0.51) × 10 ⁻⁵ (0.20)
MoMLV RT	G:C	1.55 ± 0.17	24.9 ± 7.1	(6.21 ± 1.88) × 10 ⁻²	
	G:T	(6.41 ± 0.38) × 10 ⁻²	4378 ± 502	(1.46 ± 0.19) × 10 ⁻⁵	(2.35 ± 0.77) × 10 ⁻⁴ (4.8)
	G:G	(4.84 ± 0.60) × 10 ⁻³	4535 ± 1291	(1.07 ± 0.33) × 10 ⁻⁶	(1.72 ± 0.74) × 10 ⁻⁵ (17.7)
	G:A	(8.21 ± 0.56) × 10 ⁻⁴	2816 ± 454	(2.92 ± 0.51) × 10 ⁻⁷	(4.70 ± 1.64) × 10 ⁻⁶ (0.76)
HIV-1 _{BH10} RT ^c	G:C	11.6 ± 0.5	13.4 ± 1.9	0.863 ± 0.126	
	G:T	2.54 ± 0.13	2628 ± 252	(9.66 ± 1.04) × 10 ⁻⁴	(1.12 ± 0.20) × 10 ⁻³
	G:G	0.266 ± 0.020	1014 ± 188	(2.62 ± 0.52) × 10 ⁻⁴	(3.04 ± 0.75) × 10 ⁻⁴
	G:A	0.0158 ± 0.0009	5110 ± 755	(3.09 ± 0.49) × 10 ⁻⁶	(3.58 ± 0.77) × 10 ⁻⁶

The template-primer 31T/21P was used as the substrate. Data shown are the mean values ± standard deviation of a representative experiment. Each of the assays was performed independently at least three times.

^aThe first base corresponds to the template and the second to the primer.

^b $f_{\text{ext}} = [k_{\text{po}}(\text{mismatched})/K_d(\text{mismatched})]/[k_{\text{po}}(\text{matched})/K_d(\text{matched})]$. Numbers in bold and between parentheses represent the relative increase of mispair extension fidelity relative to the HIV-1_{BH10} RT.

^cReported values for HIV-1_{BH10} RT were taken from Matamoros *et al.* [19].

Table 3

Accuracy of RT variants in M13mp2 *lacZ* α forward mutation assays.

Enzyme	Total plaques	Mutant plaques	Mutant frequency
XMRV RT	52196	82	0.00157 (13.1)
MoMLV RT (a)	29648	40	0.00135 (15.3)
MoMLV RT (b)	52207	70	0.00134 (15.4)
HIV-1 _{BH10} RT	6736	139	0.0206 (1)

Reported background frequencies in this assay (around 6×10^{-4}) [19,20] are in most cases a consequence of M13mp2 DNA rearrangements that result in the loss of the *lacZ* gene. Phage DNA was obtained from all mutant plaques and the sequence of the reporter gene was determined in all cases. No *lacZ* mutations were identified after sequencing phage DNA from more than 20,000 plaques obtained from 2–3 *E. coli* electroporations carried out with gapped M13mp2 DNA substrate.

Four to six gap-filling reactions were performed for each enzyme. MoMLV RTs were obtained from Promega Corp. (a) or expressed and purified using plasmid pMULVRT [22,23] (b). MoMLV RT (Promega) data were taken from Barrioluengo *et al* [21]. Reported values for HIV-1_{BH10} RT were taken from Matamoros *et al* [19]. Numbers between parentheses represent the fold-increase in accuracy, relative to HIV-1_{BH10} RT.

Table 4

Summary of error rates for RTs, for various classes of mutations.

Mutation type	XMRV RT		MoMLV RT ^a		HIV-1 _{BH10} RT ^b	
	No. of errors	Error rate	No. of errors	Error rate	No. of errors	Error rate
All classes	86	1/86305 (11.7)	49	1/86040 (11.7)	49	1/7369
Base substitutions	38	1/103018 (17.9)	19	1/117032 (20.3)	31	1/5760
Transitions	10 (26.3%)		3 (15.8%)		20 (64.5%)	
Transversions	28 (73.7%)		16 (84.2%)		11 (35.5%)	
Frameshifts	48	1/96563(8.2)	30	1/87758(7.5)	18	1/11745
Insertions	0 (0%)		8 (26.7%)		3 (16.7%)	
Deletions	48 (100%)		22 (73.3%)		15 (83.3%)	
At runs	10	1/247409 (39.6)	14	1/100380 (16.1)	18	1/6253
At non-runs	38	1/56866	16	1/76714	0	<1/98207

^aMoMLV RT data were taken from Barrioluengo *et al* [21].

^bHIV-1_{BH10} RT values were taken from Álvarez *et al* [24].

Numbers between parentheses represent the fold-increase in the accuracy of the RT (relative to the HIV-1_{BH10} RT).