The role of phenylalanine-119 of the reverse transcriptase of mouse mammary tumour virus in DNA synthesis, ribose selection and drug resistance

Michal ENTIN-MEER, Ziv SEVILYA and Amnon HIZI¹

Department of Cell Biology and Histology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

Phe-119 in the reverse transcriptase (RT) of mouse mammary tumour virus (MMTV) is homologous with Tyr-115 in HIV type 1 (HIV-1) RT and to Phe-155 in murine leukaemia virus (MLV) RT. By mutating these residues in HIV-1 and MLV RTs (which are strict DNA polymerases) the enzymes were shown to function also as RNA polymerases. Owing to the uniqueness of MMTV as a type B retrovirus, we have generated a Phe-119–Val mutant of MMTV RT to study the involvement of this residue in affecting the catalytic features of this RT. The data presented here show that the mutant MMTV RT can incorporate both deoxy-ribonucleosides and ribonucleosides while copying either RNA or DNA. In addition, this mutant RT shows resistance to nucleoside analogues and an enhanced fidelity of DNA synthesis;

all relative to the wild-type enzyme. The Phe-119–Val mutant is also different from the wild-type enzyme in its preference for most template primers tested and in its ability to synthesize DNA under non-processive and processive conditions. Overall, it is likely that the aromatic side chain of Phe-119 is located at the dNTP-binding site of MMTV RT and thus might be part of a putative ‘steric gate’ that prevents the incorporation of nucleoside triphosphates. Since the only three-dimensional structures of RTs published so far are those of HIV-1 and MLV, it is likely that MMTV RT folds quite similarly to these RTs.

Key words: nucleoside analogue, processivity and fidelity of DNA synthesis, reverse transcription, RNA synthesis, RNase H.

INTRODUCTION

Nucleic acid polymerases are classified traditionally as either RNA or DNA polymerases, depending on the ability of the enzyme to incorporate specifically either nucleoside triphosphates (rNTPs) or dNTPs into the nascent polymers [1]. However, three-dimensional studies have shown that the structures of RNA and DNA polymerases are quite similar, despite the low homology in their primary amino acid sequences. These observations have led to studies that have shown that reverse transcriptases (RTs), which are DNA polymerases, can function also as RNA polymerases after introducing a single mutation [2–4].

RTs are essential enzymes for replication of retroviruses. Replication starts with reverse transcription of the single-stranded viral RNA genome into double-stranded DNA, which is subsequently integrated into the chromosomal DNA of the host cell. The plus-strand RNA genome is copied by the RNA-dependent DNA polymerase (RDDP) activity of RT, producing an RNA–DNA hybrid. The ribonuclease H (RNase H) activity, which is an intrinsic part of RT, degrades specifically the viral RNA in the RNA–DNA heteroduplex. Finally, a double-stranded linear DNA is obtained by the synthesis of the second DNA strand, catalysed by the DNA-dependent DNA polymerase (DDDP) activity of the enzyme [5,6]. All RTs show relatively low specificity to their templates by being able to copy both RNA and DNA. However, the wild-type enzymes are highly specific in their capacity to synthesize only DNA and not RNA. Yet, it was shown [2–4] that mutant RTs of both murine leukaemia virus (MLV) and HIV type-1 (HIV-1) are capable of synthesizing RNA as well as DNA. In both cases, a single aromatic residue (Phe-155 and Tyr-115 in MLV and HIV-1 RT, respectively) was

replaced by the uncharged or hydrophobic residues glycine, valine or alanine. Based on the available crystal structures of both MLV and HIV-1 RTs, residues 155 and 115 in these two RTs, respectively, are located in the dNTP-binding sites of the proteins and interact directly with the deoxyribose of the incoming dNTP [2–4,7]. It was therefore suggested that Tyr-115 in HIV-1 RT and Phe-155 in MLV RT function by discriminating against incorporation of nucleotides with a 2′-hydroxyl group of the ribose into the nascent polynucleotide chain. Modifying these residues increases the flexibility of the RTs, allowing them to accommodate larger rNTPs as well as dNTPs. In addition, the fidelity and processivity of DNA synthesis of the Y115V and Y115A mutant HIV-1 RTs were also modified relative to wild-type enzyme [3,7–9].

In the present study we have extended these previous investigations to the RT of mouse mammary tumour virus (MMTV). MMTV is unique among retroviruses in its ability to replicate and function as a carcinogenic agent in mammary epithelial cells, resulting from transcriptional regulation by steroid hormones. Moreover, MMTV is the only infectious retrovirus with a distinct type-B morphology that is isolated both as an endogenous and exogenous virus [10–12]. We have expressed in bacteria an enzymically active wild-type RT from the BR6 milk-transmitted strain of MMTV [13]. Owing to the uniqueness of MMTV among retroviruses, it was of interest to study the effects of amino acid substitutions on the polymerase activities of MMTV RT, in comparison with the equivalent mutants of HIV-1 and MLV RTs. A sequence alignment has shown that Phe-119 in the MMTV *pol*-encoded protein is homologous with Tyr-115 in HIV-1 RT and to Phe-155 in MLV RT [14]. Therefore, Phe-119 in MMTV RT was changed to a valine residue. This mutant RT

Abbreviations used: DDDP, DNA-dependent DNA polymerase; ddGTP, 2′,3′-dideoxy-GTP; ddTTP, 2′,3′-dideoxy-TTP; HIV-1, HIV type 1; MLV, murine leukaemia virus; MMTV, mouse mammary tumour virus; RDDP, RNA-dependent DNA polymerase; RNase H, ribonuclease H; rNTP, nucleoside triphosphate; RT, reverse transcriptase.

¹ To whom correspondence should be addressed (e-mail ahizi@post.tau.ac.il).

was shown to incorporate both deoxyribonucleotides and ribonucleotides into the growing strand while copying either DNA or RNA. In addition, this mutant RT shows resistance to nucleoside analogues (compared with the wild-type enzyme) and has an enhanced fidelity of DNA synthesis. Moreover, the F119V RT also differs from the wild-type enzyme in its preference for most of the template primers tested. The data suggest that Phe-119 in MMTV RT has a cardinal role in DNA synthesis by MMTV RT.

EXPERIMENTAL

Construction of plasmids and protein purification

Wild-type and F119V MMTV RTs were expressed in *Escherichia coli* BL21(DE3) pLysS and purified from bacterial extracts as described previously for HIV-1 and HIV-2 RTs [15]. The RT coding sequence derived from the BR6 strain of MMTV [16] was previously cloned by us in pUC12N [13]. The numbering of the MMTV RT residues was according to the *pol* gene sequence, despite the fact that the recombinant RT has, in addition to the 576 *pol* gene-derived residues, an extra 27 *pro* gene-derived residues located at the N-terminus [13]. The F119V mutant RT-encoding DNA was generated by introducing a point mutation in nucleotide 355 of the wild-type RT-encoding gene by megaprimer PCR [17,18]. This mutation changes the TTT codon for Phe-119 of the MMTV RT to the valine GTT codon. DNAs encoding wild-type and F119V MMTV RTs were cloned into a pT5m expression vector, which was engineered to contain six consecutive in-frame histidine codons 5' to the polycloning site. The transformed *E. coli* BL21 (DE3) pLysS cells were induced to overexpress the RTs as described in [15]. The MMTV RTs were purified using two consecutive chromatographic columns, Ni²⁺-nitriloacetic acid-agarose and CM-Sephadex, as described previously in detail [13] with the following change: CM-Sephadex column was prewashed and equilibrated with sodium phosphate buffer at pH 6.5.

Assays for DNA polymerase activities

Enzymic reactions and kinetic studies were performed as described previously [19–21], using the following substrates. For RDDP, poly(rA)_n · oligo(dT)_{12–18} (with [³H]dTTP) or poly(rC)_n · oligo(dG)_{12–18} (with [³H]dGTP) was used. For DDDP assays, poly(dA)_n · oligo(dT)_{12–18} (with [³H]dTTP), poly(dC)_n · oligo(dG)_{12–18} (with [³H]dGTP) or herring sperm activated gapped DNA (prepared as described previously [19]) was used, with all four dNTPs. The final concentration of the synthetic templates was 5 µg/ml and that of the activated DNA was 30 µg/ml. In the kinetic assays performed with rNTPs, we used either α-³²P- or ³H-labelled rNTPs and the same template primers as described. In all other polymerization reactions, analysed by PAGE, as described below, we used 1 pmol of wild-type MMTV RT and 7 pmol of F119V mutant RT per reaction (to allow equal DDDP activities).

The processivity of DNA primer extension

Single-stranded circular ϕX174am3 DNA (New England Biolabs), used as a DNA template, was primed with an equimolar amount of a 15-mer oligonucleotide that hybridizes to nt 588–602 of ϕX174am3 DNA [22]. The sequence of this synthetic primer was 5'-AAAGCGAGGGTATCC-3'. The primer was 5'-end-labelled using [γ-³²P]ATP by T4 polynucleotide kinase, and annealed to the template as described in [22]. The extension

reactions were performed in a solution containing the final concentrations of 6.6 mM Tris/HCl, pH 8.0, 4 mM dithiothreitol, 6 mM MgCl₂, 24 µg/ml BSA and 30 µg/ml template primer. Wild-type and F119V MMTV RTs were preincubated with the template primer for 5 min at 30 °C. The reaction mixtures were divided into two, without or with a DNA trap of a large excess of activated DNA (at a final concentration of 0.6 mg/ml). All reactions were initiated immediately by adding the four dNTPs, each at a final concentration of 20 µM, followed by incubation for 30 min at 37 °C. Aliquots (10 µl) were removed into 10 µl of formamide dye mixture, denatured at 100 °C for 3 min and analysed by electrophoresis through 8% polyacrylamide/urea in Tris/borate and EDTA sequencing gels. The extension products were quantified and the amount of primer extended was calculated [23].

Gel assay for primer extension in the presence of rNTPs

The reactions were performed as described above for the processivity of DNA synthesis with the following modifications. No preincubation and no DNA trap additions were employed. The activities were assayed after the addition of dNTPs, with or without rNTPs, or with the addition of all four rNTPs (see Figure 2, below). The final concentration of each dNTP/rNTP was 20 µM. The products were analysed by electrophoresis through 12% polyacrylamide/urea sequencing gels.

Fidelity of DNA synthesis

Site-specific nucleotide misinsertion was assayed by dNTP incorporation opposite to the A residue at position 23 of a 50 nt synthetic template (with a sequence derived from nucleotides 565–614 of ϕX174am3 DNA; see Figure 3A, below). The labelled 15-residue primer was extended in the presence of increasing concentrations of either 0–1 µM of the correct dNTP (dTTP) or 0–1 mM each of the incorrect dNTPs (dATP, dCTP or dGTP). The primer elongation with UTP was measured with increasing concentrations of either 0–1 mM for wild-type MMTV RT or 0–1 µM for F119V RT.

Mispair extension was performed with four sets of 16-residue labelled primers, which were annealed to the same template used in the misinsertion assays, thus producing duplexes with 3'-terminal paired (A–T) or mismatched (A–C, A–G or A–A) primers. Elongation of those primers was assayed with increasing concentrations of dATP, as the only dNTP present (0–1 mM range for the mispaired A–A, A–C or A–G termini, or a 0–1 µM range for the A–T correct terminus).

All reactions, conducted in the same buffer described above for the processivity studies, were incubated at 37 °C for either 2 min (for the correct incorporation of dTTP or for the extension of the correct A–T pair), or for 5 min (for misincorporation or extension of preformed mismatched DNA termini). Kinetic reactions were performed with an approx. 10-fold molar excess of template primer over the enzyme to ensure steady-state kinetics in the linear range. All reaction products were analysed by electrophoresis through 14% polyacrylamide/urea sequencing gels.

Assays for RNase H activity

This activity was assayed by two methods. In the first, the release of [³H]AMP from [³H]poly(rA)_n annealed to poly(dT)_n (into the trichloroacetic acid-soluble fraction) was measured as described previously [19]. In the second method, we followed cleavage of the 5'-end-labelled 267 nt RNA synthesized from the pBLRA30 plasmid, annealed to oligomeric DNA [15]. The reaction mixtures

for these assays contained approx. 0.1 pmol of the [32 P]5'-end-labelled RNA transcript, which was annealed with 0.4 pmol of a 20-mer synthetic oligonucleotide DNA. The 3'-end of the DNA was complementary to the region 30 nt from the 5'-end of the RNA transcript. The reactions contained 50 ng of purified wild-type MMTV RT, F119V MMTV RT or HIV-1 RT and were performed, analysed and calculated as described in [15].

RESULTS AND DISCUSSION

Steady-state kinetic parameters for the DNA polymerase activities of the F119V and wild-type MMTV RTs

We have determined the kinetic values for dNTP incorporation into the nascent DNA strand with a variety of synthetic and natural template primers, commonly used with RTs, as described in detail in the Experimental section. The results presented in Table 1 show that the substrates used have a major impact on the differences between the K_m and k_{cat} values (and hence k_{cat}/K_m values) calculated for the RTs. On the other hand, the nature of the substrates copied by the RTs (RNA versus DNA) has a relatively low effect on these differences. Thus with poly(r/dA) $_n$ · oligo(dT) $_{12-18}$, the wild-type MMTV RT is 72–76-fold more efficient in DNA synthesis than the F119V mutant RT. Both the K_m and k_{cat} values are affected by the mutation, although with the template poly(rA) $_n$, the K_m value is the one mostly affected (with wild-type RT showing a value almost 50-

Table 1 Kinetic parameters of the wild-type and F119V mutant MMTV RTs calculated for dTTP and dGTP

The RDDP and DDDP activities of the wild-type or F119V mutant MMTV RTs were assayed with the template primers described with variable concentrations of either dTTP or dGTP, as described in the Experimental section. The reactions were performed for 10 min at 37 °C. The K_m and V_{max} values were calculated from the double reciprocal plots of the initial velocities of DNA synthesis as a function of substrate concentration. The plots were generated by linear regression analysis yielding regression coefficients larger than 0.98 (results not shown). The k_{cat} values were calculated from the V_{max} values divided by the amount (mol) of each enzyme present in the reactions. The K_m values are expressed in μ M and the k_{cat} values are expressed in s^{-1} . The k_{cat}/K_m values are expressed in $s^{-1} \cdot M^{-1}$. The values shown in the ratio column are the k_{cat}/K_m values of the wild-type RT divided by the comparative values of the F119V mutant RT.

	Wild type	F119V	Ratio
Poly(rA) $_n$ · oligo(dT) $_{12-18}$ (dTTP)			
K_m	5	247	
k_{cat}	0.37	0.24	
k_{cat}/K_m	74 000	972	76.1
Poly(dA) $_n$ · oligo(dT) $_{12-18}$ (dTTP)			
K_m	12	41.5	
k_{cat}	0.063	0.003	
k_{cat}/K_m	5250	72.3	72.6
Poly(rC) $_n$ · oligo(dG) $_{12-18}$ (dGTP)			
K_m	2.1	2.8	
k_{cat}	0.122	0.194	
k_{cat}/K_m	58 095	69 286	0.84
Poly(dC) $_n$ · oligo(dG) $_{12-18}$ (dGTP)			
K_m	2.8	5.5	
k_{cat}	0.28	0.411	
k_{cat}/K_m	100 000	74 545	1.3
Activated DNA (dTTP)			
K_m	1.8	4.7	
k_{cat}	0.04	0.015	
k_{cat}/K_m	22 222	3191	7.0
Activated DNA (dGTP)			
K_m	2.6	2.6	
k_{cat}	0.024	0.009	
k_{cat}/K_m	9231	3462	2.7

Figure 1 DNA primer extension and processivity exhibited by the wild-type and mutant F119V MMTV RTs

Reactions were performed with single-stranded circular ϕ X174am3 DNA as a template, which was annealed to a [32 P]5'-end-labelled 15-mer oligonucleotide. This primer hybridizes with nucleotides 588–602 of the ϕ X174am3 DNA. The sequence of the synthetic primer and the detailed experimental conditions are described in the Experimental section. The molar ratio between the DNA template and the primer was 2:1. Molecular-mass markers are *Hinf*I-cleaved double-stranded ϕ X174 DNA labelled with 32 P at their 5'-end by T4 polynucleotide kinase and [γ - 32 P]ATP. The lengths of the single-stranded DNA markers are indicated by arrowheads with the length of each in nt. —, DNA extensions performed in the absence of a DNA trap; +, extensions conducted in the presence of an excess of the unlabelled DNA trap.

fold lower than that of the mutant RT). With the poly(dA) $_n$ template, the K_m of wild-type RT is only 3.5-fold lower than that of the mutant RT, whereas its k_{cat} value is 23-fold higher than that of the F119V mutant. Unlike the poly(r/dA)-dependent DNA synthesis, there are almost no differences between the apparent kinetic values of the polymerase activities measured with poly(r/dC) $_n$ · oligo(dG) $_{12-18}$ for the two MMTV RT versions (since the ratios between the calculated k_{cat}/K_m values are close to 1). The DDDP activities of the two MMTV RT versions were also assayed using activated gapped DNA and all four dNTPs, and the kinetic parameters for dTTP and dGTP were determined (Table 1). The results show that, for both dNTPs, the wild-type RT is more active than the F119V mutant RT, although with dTTP the ratios between the k_{cat}/K_m values are somewhat higher than with dGTP. These results are generally

Figure 2 DNA synthesis in the presence of rNTPs

Single-stranded circular ϕ X174am3 DNA (annealed to the same [32 P]5'-end-labelled 15-mer primer described in Figure 1) was extended by either wild-type MMTV RT or F119V mutant RT under experimental conditions similar to those described in Figure 1. **(A)** The extensions were performed with either all four dNTPs or with three dNTPs and one rNTP, or with the same three dNTPs without the fourth rNTP. The final concentration of each dNTP or rNTP was 20 μ M. The lengths of the single-stranded DNA markers are indicated by arrowheads with their lengths in nt. The sequences of the first incorporated nucleotides are marked on the sides of the gel (along with the lengths of the products in parentheses). Bold letters with asterisks mark the first positions in which each of the four dNTPs (or rNTPs) is expected to be inserted (according to the sequence copied). F, All four dNTPs are present in the reaction. + rA, dCTP, dGTP, dTTP and rATP (lanes 3 and 12). - rA, dCTP, dGTP, dTTP (lanes 4 and 13). + rC, dGTP, dTTP, dATP and rCTP (lanes 5 and 14). - rC, dGTP, dTTP, dATP (lanes 6 and 15). + rG, dTTP, dATP, dCTP and rGTP (lanes 7 and 16). - rG, dTTP, dATP, dCTP (lanes 8 and 17). + U, dATP, dCTP, dGTP and UTP (lanes 9 and 18). - U, dATP, dCTP, dGTP (lanes 10 and 19). **(B)** The extensions were performed with either all four dNTPs or all four rNTPs. P, template primer with either wild-type or F119V RTs with no dNTPs or rNTPs present; F, all four dNTPs present; 4r, all 4 rNTPs present.

in agreement with the data presented for the comparable mutants of HIV-1 RT, Y115V and Y115A [4,7,24]. These mutants are less effective in their polymerase function relative to wild-type RT while utilizing several template primers [especially with poly(rA)_n · oligo(dT)₁₂₋₁₈]. On the other hand, the ability of F155V MLV RT to function as a DNA polymerase using the template primer poly(rA)_n · oligo(dT)₁₂₋₁₈ is quite similar to that of the wild-type MLV RT [2].

DNA synthesis under processive and non-processive conditions

Many enzymes are processive, i.e. they can bind to polymeric substrates and templates and perform a sequence of polymerization events without intervening dissociations from these substrates [25]. A fully processive event of RNA or DNA synthesis is defined as one in which the entire template strand is copied as a result of only one polymerase binding event [23,25]. Relative processivity measures what percentage of the DNA produced in the primer-extension reaction can be formed when only a single polymerization event is allowed (when no rebinding can occur) [23,25-27]. To study the effect of the Phe-119

substitution in MMTV RT, we conducted the experiment shown in Figure 1, in which we analysed the extent of DNA primer extension under processive and non-processive conditions (in comparison with wild-type RT). This was done by following the extension of the [32 P]5'-end-labelled 15-mer primer (using single-stranded ϕ X174am3 DNA as a substrate) in the absence and presence of a large excess of a trap of a 'quencher' unlabelled activated DNA. The DNA trap was added after preincubating the RTs with the labelled template primer and prior to the addition of the four unlabelled dNTPs. Therefore, the activated DNA is likely to trap the enzyme after the first event of dissociation from the labelled template primer, thus preventing further elongation of the labelled primer by re-associated RT molecules. As already shown for wild-type MMTV RT [13,26], in the absence of a DNA trap the labelled primer is elongated up to almost 700 nt with several strong pauses. The F119V mutant synthesizes shorter products (even without a DNA trap), indicating a higher pausing frequency, or a weaker 'persistence' in the elongation of the DNA (persistence being the ability to synthesize long DNA products in one round of synthesis without stopping or falling off the nascent DNA strand).

Table 2 Primer extension in the presence of three dNTPs and one rNTP or with all four rNTPs

The data presented in Figure 2 were quantified after scanning the autoradiogram. Synthesis was determined as follows: in all — columns the extension seen with only three dNTPs was calculated. This was done by calculating the percentage of the total amount (extended and unextended primers) that was elongated beyond the appropriate first position of the missing r/dNTP. These positions are marked in Figure 2(A) for each nucleotide with a bold letter and an asterisk. Similarly, the calculation performed in +rNTP columns was done from gel lanes of the reactions, which included, instead of the missing dNTP, the parallel rNTP. Net represents the difference between syntheses with the +rNTP after subtracting the corresponding — values without the rNTP. The difference in the net syntheses refers to the difference in the net values observed between the F119V mutant and the wild-type MMTV RTs.

		Extension (%)								
		rATP		rCTP		rGTP		UTP		
MMTV RT	Value calculated	+	—	+	—	+	—	+	—	4 × rNTP
Wild-type	Synthesis	32.8	26.6	50.8	40.4	24.1	23.0	42.1	41.3	2.1
	Net		6.2		10.4		1.1		0.8	
F119V	Synthesis	34.7	3.9	56.0	9.5	42.0	21.2	88.2	9.3	45.6
	Net		30.8		46.5		20.8		78.9	
Difference in net syntheses			24.6		36.1		19.7		78.1	43.5

The pattern of the primer extension exhibited by the wild-type RT in the presence of a DNA trap is almost indistinguishable from that without a trap, suggesting a considerable relative processivity [13,26]. The same conclusion can be reached from the data presented in Figure 1. In fact, despite producing shorter DNA products in the absence and presence of a DNA trap, the relative processivity of the F119V RT is about the same as that of the wild-type enzyme. In all, the data suggest that the F119V mutation of MMTV RT leads to a substantial increase in the pausing frequency (and thus reducing the 'persistence' of synthesis), probably without affecting the relative processivity substantially.

It was shown that the comparable mutant of HIV-1 RT (Y115V) is less processive than the wild-type RT [4]. However, in this study the processivity experiment was performed by following the extension only in the presence of a DNA trap. Therefore, Boyer et al. [4] could not discriminate between synthesis without a trap (what we define here as persistence) or with a trap. Consequently, we speculate that the Y115V HIV-1 RT, like the comparable MMTV mutant RT, would also produce short DNA products, had it been tested even without a trap (showing low persistence).

DNA or RNA synthesis in the presence of rNTPs and dNTPs

The major reason for constructing and studying the F119V mutant MMTV RT was to see whether it can serve also as an RNA polymerase by incorporating rNTPs into the growing DNA chain. In a primer-extension experiment (Figure 2) we used the same template primer as in the experiment in Figure 1. The extensions were performed with either all four dNTPs (marked F in Figure 2), or with only three dNTPs or with the same three dNTPs and one rNTP (Figure 2A). In addition, we have performed extensions of the same primer, using all four rNTPs (Figure 2B and Table 2). The differences in the extent of elongation in the presence of the appropriate rNTP and the comparable elongation without this rNTP (with only three dNTPs present) is a measure of the ability of the RT to incorporate this rNTP. The data (Figure 2A and Table 2) imply that the F119V MMTV RT is capable of incorporating each of the four rNTPs better than the wild-type enzyme. There is some low net incorporation by the wild-type RT of rATP and rCTP with hardly any significant incorporation of the other two rNTPs. On the other hand, the F119V mutant RT is efficient in

Table 3 Kinetic parameters of the wild-type and F119V mutant MMTV RTs calculated for rNTPs

The DDDP assays were performed with either three dNTPs and one rNTP or with all four rNTPs (with activated DNA as template primer) or with GTP or UTP alone in either the poly(r/dC)_n · oligo(dG)_{12–18} or poly(r/dA)_n · oligo(dT)_{12–18}-directed DNA synthesis, respectively. The reactions were carried out for 30 min, due to the low incorporation rate of rNTPs into the growing strand. The K_m and V_{max} values were calculated from double reciprocal plots of increasing concentrations of the studied rNTP (in the activated DNA-dependent reactions, all other three dNTPs or rNTPs were at fixed concentrations) against the initial velocities of DNA synthesis (results not shown). The plots were generated by linear regression analysis yielding regression coefficients greater than 0.98. The k_{cat} values were calculated from the V_{max} values divided by the amount (mol) of each enzyme present. The K_m values are expressed in μM , and the k_{cat} values are expressed in $s^{-1} \times 10^{-4}$. The k_{cat}/K_m values are expressed in $s^{-1} \cdot M^{-1}$. n.d., not detected due to an incorporation too low to be detected.

Template primer	rNTP	Wild-type RT			F119V RT		
		K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
Activated DNA (with three dNTPs + one rNTP)	ATP	90	0.15	0.17	50	3.4	6.8
	CTP	21.3	0.27	1.3	3.8	1.8	47.4
	GTP	8.5	0.094	1.1	4.9	1.3	26.5
	UTP	n.d.	n.d.	—	12	0.6	5
Activated DNA (with four rNTPs)	ATP	n.d.	n.d.	—	30	3.7	12.3
	CTP	94.9	0.2	0.21	5.8	1.1	19
	GTP	10.2	0.091	0.89	17	5.5	32.4
	UTP	n.d.	n.d.	—	13.9	1.7	12.2
Poly(rC) _n · oligo(dG) _{12–18}	GTP	n.d.	n.d.	—	19.8	1.04	5.3
Poly(dC) _n · oligo(dG) _{12–18}	GTP	n.d.	n.d.	—	20.3	6.5	32
Poly(rA) _n · oligo(dT) _{12–18}	UTP	n.d.	n.d.	—	n.d.	n.d.	—
Poly(dA) _n · oligo(dT) _{12–18}	UTP	n.d.	n.d.	—	n.d.	n.d.	—

incorporating all four rNTPs in the following order: UTP > rCTP > rATP > rGTP.

However, the primer-elongation pattern in the presence of only three dNTPs represents also the fidelity of DNA synthesis, because elongation beyond the position of the missing dNTP indicates misinsertion of one of the three other dNTPs present [28–31]. Therefore, a comparison of the lanes marked — in Table 2 indicates that the fidelity of DNA synthesis by the wild-type RT is substantially lower than that of the F119V mutant RT. This issue of fidelity of DNA synthesis was also studied separately by investigating the misinsertion and mispair extension properties

Figure 3 The pattern of misincorporation and mispair extension exhibited by wild-type and F119V MMTV RTs

(A) Nucleotide misinsertion activity. The synthetic 50 nt template was annealed to the same 5'-end-labelled primer used in the processivity experiment. The sequence of both template and primer are given in the Figure. The primer was extended by equal DNA polymerase activities of both RTs (determined by DDDP with activated DNA), either for 2 min in the presence of 1 μ M of the correct dNTP (dTTP), or for 5 min in the presence of 1 mM of UTP or each one of the incorrect dNTPs (dATP, dCTP and dGTP). (B) DNA mispair extension. [³²P]5'-End-labelled 16 nt primers were hybridized to the 50 nt template, producing duplexes with 3'-terminal preformed mismatches, where N at the 3'-end of each represents the incorrect nucleotide, A, C or G. The sets of duplexes performed are shown. The primers were extended, with equal DNA polymerase activities of both enzymes, for 2 min at 37 °C in the presence of 1 μ M dATP for elongation of the correct pair (A–T) or for 5 min with 1 mM dATP for elongation of the mismatched pairs.

Table 4 Kinetic parameters for the comparative site-specific misincorporation by wild-type and F119V MMTV RTs

In each set of kinetic experiments the ϕ X174am3 DNA annealed to [32 P]5'-end-labelled 15-mer primer (see Figure 3A) was incubated with wild-type or F119V RT in the presence of increasing concentrations of a single dNTP. The oligonucleotide products were analysed as described in the Experimental section. The apparent K_m and V_{max} values were determined from at least two independent experiments. To calculate the frequencies of misinsertions (F_{ins}) we employed a method used previously [22,26,27,34], as described in the text.

	Wild-type			F119V		
	K_m (μ M)	V_{max} (% $\cdot$ min $^{-1}$)	F_{ins}	K_m (μ M)	V_{max} (% $\cdot$ min $^{-1}$)	F_{ins}
dT	0.0035 $\pm$ 0.0003	12.6 $\pm$ 2	1	0.027 $\pm$ 0.012	22.3 $\pm$ 3	1
dA	23 $\pm$ 3	11.7 $\pm$ 3	1/7077	90 $\pm$ 15	1.48 $\pm$ 4	1/50225
dC	7.6 $\pm$ 2	12.9 $\pm$ 1	1/2120	85 $\pm$ 7	9.70 $\pm$ 2	1/7237
dG	63 $\pm$ 15	10.8 $\pm$ 2	1/21 000	127 $\pm$ 20	0.74 $\pm$ 0.2	1/141 747
U	288 $\pm$ 12	5.4 $\pm$ 0.1	1/192 000	3.2 $\pm$ 0.1	23.0 $\pm$ 1	1/115

of the enzymes (see below). The data (Figure 2B and Table 2) show that wild-type RT exhibits a very low level of RNA synthesis in the presence of all four rNTPs. There is a marginal extension of the primer by one nucleotide, incorporating a very low level of UTP (which amounts to an extension of only about 2% of the primer; Table 2). On the other hand, the F119V RT extends about 45% of the primer molecules by six nucleotides (Figure 2B and Table 2). Still, as mentioned above, it is obvious that this mutant RT functions much better as a DNA polymerase than as an RNA polymerase.

The experiments shown above followed the incorporation of rNTPs indirectly. To confirm these results by a direct measure of the extent of incorporating the labelled rNTPs and to study the steady-state kinetic parameters for these incorporations, we conducted the experiments summarized in Table 3 using the same template primers as employed in Table 1. It is apparent that the wild-type RT is hardly capable of incorporating rNTPs in the activated DNA-dependent reactions. On the other hand, the F119V RT can incorporate all rNTPs. A comparison of the kinetic parameters calculated shows that for rCTP incorporation both the K_m and k_{cat} values are affected by the mutation. In all other cases, where comparison with the wild-type RT is possible, the ratios between the polymerization efficiencies (k_{cat}/K_m) of the mutant RT and those of wild-type enzyme are attributed mostly to the differences in the k_{cat} values rather than to differences in K_m values. In addition, the kinetic parameters of the incorporation of a single labelled rNTP (UTP or rGTP) in both RDDP and DDDP reactions were studied with the appropriate synthetic template primers (see Table 3). We could not show any detectable amounts of incorporation by the wild-type RT of both rNTPs in poly(r/dC) $_n$ · oligo(dG) $_{12-18}$ - or poly(r/dA) $_n$ · oligo(dT) $_{12-18}$ -directed polymerization reactions. However, the F119V RT shows a significant incorporation of only rGTP in both RDDP and DDDP reactions with no detectable incorporation of UTP. This result is in line with the data presented in Table 1, showing that poly(rA) $_n$ and poly(dA) $_n$ are relatively poor templates for the F119V RT (even when tested for the incorporation of dTTP). Since we have shown that the F119V RT is still a much better DNA polymerase than an RNA polymerase (Table 1 and 3), it is not surprising that UTP is poorly incorporated. In all, the F119V MMTV RT is capable of transcribing DNA into RNA. In the case of poly(rC) $_n$ · oligo(dG) $_{12-18}$, this enzyme can also copy RNA.

The comparative fidelity of DNA synthesis of the mutant and wild-type MMTV RTs

The results presented above for the primer extension in the presence of three dNTPs and one rNTP (Figure 2A and Table 2)

suggest that the fidelity of DNA synthesis by the F119V mutant RT is higher than that of the wild-type enzyme. All our previous studies with a variety of retroviral RTs (including wild-type MMTV RT) have shown that these parameters of fidelity of DNA synthesis *in vitro* depend primarily on the sequences of the nucleic acids copied, rather than on whether DNA or RNA templates are copied [22,26,32,33]. Subsequently, in the present study we have analysed DNA templates as representing both DNA and RNA.

The formation of 3'-mispaired DNA

We have used an assay system that measures the standing-start reaction of 3'-end misinsertion [22,26,27,32–34] (see Figure 3A and the Experimental section). The results of the experiment (Figure 3A) show that the general pattern of the primer extension obtained with the mutant RT is quite different from that seen with the wild-type enzyme. Both RT versions can elongate the primer by one nucleotide in the presence of 1 μ M dTTP with no significant further extension. The highest extent of misincorporation by the two RT versions is observed with dCTP, forming C–A mispairs, which are further elongated only by the wild-type RT. With the other two incorrect dNTPs, dATP and dGTP, there is very little misincorporation by the mutant RT as opposed to the wild-type enzyme (see Figure 3A).

To measure the capacity to form 3'-end mispairs, separate sets of primer-extension reactions were carried out and analysed in the presence of increasing concentrations of a single dNTP, thereby determining the standing-start rate of synthesis of the correct pair versus the possible mispairs. A range of dNTP concentrations were used, always below 1 mM to obey steady-state kinetic conditions. The frequencies of misinsertions (F_{ins}) were calculated as described previously [22,26,27,34]:

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

where W denotes the incorrect nucleotide (dATP, dCTP dGTP or UTP) and R stands for dTTP.

The results shown in Table 4 indicate that the F_{ins} values for the mutant RT are substantially lower than those calculated for the wild-type enzyme. It is also apparent that this difference results from both higher K_m and lower V_{max} values of the F119V mutant relative to the wild-type RT. In addition, the fact that there is a significant difference in the K_m values calculated for the correct dTTP nucleotide (which is 7.7-fold higher for the mutant) also contributed to the observed differences in the calculated F_{ins} values. We have already shown above that the mutant RT can function much better than the wild-type MMTV RT as an RNA polymerase. Therefore, as could be expected, the extent of UTP

Table 5 Comparative parameters for the extension of 3'-end matched or preformed mismatched primer termini by wild-type and F119V MMTV RTs

Three mismatched and one matched template primers were incubated with wild-type or F119V RTs (see Figure 3B) in the presence of increasing concentrations of dATP. The apparent K_m and k_{cat} values were determined from at least two independent experiments performed as described in the Experimental section. The relative frequency F_{ext} values are ratios 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.

	Wild-type			F119V		
	K_m (μM)	V_{max} (% $\cdot$ min $^{-1}$)	F_{ext}	K_m (μM)	V_{max} (% $\cdot$ min $^{-1}$)	F_{ext}
A–T	0.0013 $\pm$ 0.0008	13.7 $\pm$ 4	1	0.016 $\pm$ 0.05	33.7 $\pm$ 4	1
A–A	16 $\pm$ 5	5.8 $\pm$ 2	1/29070	105 $\pm$ 20	2.3 $\pm$ 0.8	1/96155
A–C	4.5 $\pm$ 2	9.1 $\pm$ 1	1/5211	21 $\pm$ 3	11 $\pm$ 0.6	1/4021
A–G	35 $\pm$ 9	3.7 $\pm$ 1.2	1/99638	74 $\pm$ 10	4.0 $\pm$ 0.8	1/38965

insertion by the F119V mutant RT is apparently much higher than the incorporation by the wild-type enzyme. The quantitative analysis (Table 4) shows how massive the difference is in the apparent F_{ins} values (approx. 1670-fold higher for the mutant RT relative to the wild-type enzyme). The main contributor to this difference is the much lower K_m value, exhibited by the mutant RT relative to the wild-type enzyme, compounded by the big difference in the K_m of the correct dTTP.

Extension of preformed 3'-end mispaired DNA

Misinsertion by itself is not sufficient to create stable site-specific mutations, unless the terminally mispaired DNA is extended further, leading to the fixation of the mistaken sequence. Therefore, the efficiency of extending 3' preformed mismatched primers is an essential factor in determining the fidelity of DNA synthesis exhibited by different polymerases. The extension of preformed 3'-end mispaired 16-residue primers (A–A, A–C, A–G) was analysed during DNA polymerization in the presence of the next complementary dATP (as the only dNTP present; see Figure 3B and Table 5). Unlike the pattern of misinsertion, the differences between the mispair-extension capacities of the two MMTV RTs were found to be less obvious. With the A–C mispair, there is an elongation by the wild-type RT that generates products longer than those synthesized by the F119V mutant RT (despite the fact that the quantitative F_{ext} values, the relative frequencies of extension of the preformed mispairs, calculated for both are quite close). With the A–A mispair, the fidelity of the mutant RT is significantly higher, whereas the opposite is true for the A–G mispair.

Taken together, the F119V MMTV RT is apparently less error prone than the wild-type enzyme (see Figure 2A and Table 2), mainly due to its reduced capacity to misincorporate incorrect nucleotides (Figure 3A and Table 4). The fidelity of DNA synthesis was studied previously for Y115 mutants of HIV-1 RT [3,7–9]. In one study the fidelity of the Y115A mutant of HIV-1 RT appeared to be higher than that of the wild-type enzyme [7]. In another study (using a DNA forward mutagenesis method for analysing fidelity) it was shown that the Y115V mutant of HIV-1 RT is 3–4-fold less error prone than the wild-type enzyme [8]. However, two other studies done by another group showed the opposite, that the Y115W, Y115S and Y115A mutants have higher misinsertion and mispair extension efficiencies (namely a lower fidelity) than that of wild-type HIV-1 RT [3,9]. In all, our findings regarding the F119V mutant of MMTV RT are compatible with those reported for the Tyr-115 mutants of HIV-1 RT that show a higher fidelity relative to the wild-type enzyme [7,8].

Sensitivity to nucleoside analogues

The results presented here show that the F119V MMTV RT exhibits a reduction in the ability to discriminate between 2'-deoxyribose and ribose in incoming nucleotides. Therefore, it was of interest to determine whether this mutant RT can also discriminate between normal dNTPs and nucleoside analogues modified at the 3' position. The F119V mutant RT was found to be much more resistant than the wild-type enzyme to inhibition by 2',3'-dideoxy-TTP (ddTTP) and 2',3'-dideoxy-GTP (ddGTP) (Figure 4). The IC_{25} values (the inhibitor concentrations leading to an inhibition of 25% of the polymerase activity) of the wild-type MMTV RT to ddTTP and ddGTP were approx. 15 and 30 nM, respectively; whereas the equivalent values for the F119V mutant to those nucleoside analogues were much higher, above 2 μM for ddTTP and approx. 900 nM for ddGTP. In other words, the IC_{25} value of the mutant RT for ddTTP is more than 130-fold higher and that for ddGTP is about 30-fold higher than the equivalent values obtained with the wild-type enzyme.

The apparent resistance of the F119V MMTV RT to the nucleoside analogues modified at the 3' position on the ribose is similar to the behaviour reported for the comparable Y115V mutant of HIV-1 RT [4]. However, another study conducted with the same Y115V mutant concluded that although the ribose 3'-OH might be important for nucleoside binding, the side chain of residue 115 in HIV-1 RT is not critical for recognizing the 3'-OH of the ribose [3].

Since the F119V MMTV RT functions also as an RNA polymerase, it was of interest to test whether this enzyme is sensitive to cordycepin 5'-triphosphate, a nucleoside analogue of rATP (in which the 3'-OH is replaced by a 3'-H). This RNA chain terminator did not show any preferential inhibition of the mutant RT compared with the wild-type enzyme. In both cases, when activated DNA was used as a substrate for either RNA or DNA synthesis, inhibition was almost negligible (results not shown). Contrariwise, the Y115V mutant of HIV-1 RT showed some preferential sensitivity to cordycepin 5'-triphosphate relative to the wild-type RT [3].

The RNase H activity of F119V and wild-type MMTV RTs

All RTs studied so far, including MMTV RT, possess an RNase H activity. Sequence and structural analyses have revealed that the RNase H domain is located in the C-terminal region of RTs whereas the DNA polymerase domain is in the region close to the N-terminus. Still, there are mutual interactions between the domains of DNA polymerase and RNase H [5,6,14,15]. There-

Figure 4 Inhibition of DNA synthesis by chain terminators

(A) The effect of ddTTP on dTTP incorporation using poly(rA)_n · oligo(dT)₁₂₋₁₈ as a template primer. (B) The effect of ddGTP on dGTP incorporation with poly(rC)_n · oligo(dG)₁₂₋₁₈ as a template primer. The catalytic assays were performed as described in the Experimental section. Enzymic activities are shown as percentages of the control reaction performed under similar conditions with no inhibitor present.

fore, it was of interest to see whether the F119V mutation could affect also the RNase H activity of MMTV RT. The RNase H activity was assayed by two methods. In the first one the substrate used was a 267 nt-long 5'-end-labelled RNA that was annealed to a short complementary oligomeric DNA. The cleavage pattern provides information about the positioning of RT molecules on the nucleic acid substrate. The primary RNase H cleavage that was already shown by both HIV-1 and HIV-2 RTs centres around a position 17 or 18 nt from the 3'-end of the DNA in the heteroduplex (and produces a 47 nt-long RNA product), which corresponds to the molecular distance between the DNA polymerase and RNase H active sites in HIV RTs [15,35]. Then, the RTs perform a secondary RNase H cleavage at position 8 from the 3'-end of the primer, thus producing RNA fragments that are 38 nt long. Such secondary cleavages require repositioning of the enzyme on the RNA–DNA heteroduplex. The data presented in Figure 5 show that the pattern of RNA cleavage of both wild-type and F119V MMTV RTs resembles the pattern obtained with HIV-1 RT as well with HIV-2 RT [15]. This suggests that the distance in MMTV RT between the DNA polymerase and RNase H domains and the translocation that leads to the secondary cleavage are similar to those seen in HIV RTs. Quantitative analysis of the cleavage products shows that the RNase H activity of the F119V mutant RT is slightly lower

Figure 5 Cleavage pattern of the RNase H activity exhibited by wild-type and F119V MMTV RTs

Purified wild-type (WT) MMTV RT, F119V MMTV RT and HIV-1 RT (50 ng each) were incubated with the labelled RNase H substrate as described in the Experimental section for the indicated lengths of time. Wild-type HIV-1 RT was the heterodimer p66/p51 enzyme, expressed and purified as described in [15]. In the sample loaded in the left-most lane of the gel, no RT was present in the reaction mixture. After electrophoresis the gel was autoradiographed.

than that of the wild-type RT (showing a specific activity which is about 75 % of the wild-type RT).

In a second method employed to assess the RNase H activity, the release of radioactivity from the substrate [³H]poly(rA)_n · oligo(dT)_n was monitored. The specific RNase H activity of the F119V mutant RT was found to be about 60 % of the activity of the wild-type enzyme (results not shown). In all, the F119V mutation has no significant qualitative effect on the pattern of cleavage, with a small effect on the quantitative RNase H activity.

In summary, the data presented in this communication indicate that the F119 residue of MMTV RT is likely to be located in the nucleotide-binding pocket in the DNA polymerase domain of this enzyme, as are Tyr-115 and Phe-155 in the RTs of HIV-1 and MLV, respectively. Since the only crystal structures for RTs that have been resolved so far are for HIV-1 and MLV RTs [36,37], this assumption is based on several of the outlined catalytic features of the F119V MMTV RT described here (many of them similar to those reported for the comparable mutants of HIV-1 and MLV RTs). To sum up, these properties are as follows. First, the F119V MMTV RT, like Y115V HIV-1 RT and F155V MLV RT, can incorporate both ribonucleotides and deoxyribonucleotides into the nascent polynucleotide strand by copying either DNA or RNA (Figure 2 and Tables 2 and 3). It can

incorporate a mixture of rNTPs and dNTPs (while synthesizing a mixed RNA/DNA strand) as well as only rNTPs or dNTPs, thus synthesizing only RNA or DNA, respectively. Still, the mutant MMTV RT shows a strong preference for binding dNTPs and synthesizing DNA over utilization of rNTPs (Figure 2 and Tables 1 and 3). Second, like the Y115V HIV-1 RT, the F119V MMTV RT is significantly impaired in its polymerase activity with several template primers. These include poly(r/dA)_n · oligo(dT)_{12–18}, activated DNA (Table 1) and primed ϕ X174 single-stranded DNA (Figures 1 and 2). However, the templates poly(r/dC)_n are copied by the mutant RT as efficiently as by the wild-type MMTV enzyme. In this instance, the F119V mutant is closer to the F155V mutant of MLV RT that has shown a DNA polymerase activity similar to that of the wild-type MLV RT [2]. Third, the conversion of Phe-119 into a valine in MMTV RT affects substantially the ability of the enzyme to discriminate between nucleoside analogues (modified at the 3'-OH position of the ribose) and the natural dNTPs, hence reducing significantly the sensitivity of the polymerase to the chain terminators (Figure 4). The mutation-dependent drug resistance, shown here for the F119V MMTV RT, is in agreement with a study on the Y115V mutant HIV-1 RT [4] and disagrees with another study with the same HIV-1 RT mutant [3]. Fourth, the F119V MMTV RT possesses an apparent fidelity of DNA synthesis, which is higher than that of the wild-type enzyme (Figure 2A and Table 2) and is associated with a reduced capacity to incorporate incorrect dNTPs (Figure 3A and Table 4). The fidelity properties of the equivalent mutants of HIV-1 RT are probably affected as well, although there are some discrepancies, as mentioned above, regarding the trend of this effect [3,7–9].

Three-dimensional studies of MLV RT and HIV-1 RT have proposed that the phenyl rings of Phe-155 and Tyr-115, respectively, act directly as a putative 'steric gate' that allows the incorporation of dNTPs with a 2'-H group on the ribose and discriminates against the incorporation of rNTPs with the larger 2'-OH groups [2–4]. When smaller side chains replace the bulky aromatic side chains of these amino acids, this so-called gate opens and the larger rNTPs can be incorporated into the nascent polynucleotide chain. The ability of the mutant MMTV RT to incorporate rNTPs strongly suggests that Phe-119 is also located within a similar putative steric gate in MMTV RT and that by removing the aromatic ring the flexibility of the RT increases. However, this relatively simple explanation cannot account for other features of this mutant RT, such as the ability to discriminate better than the wild-type RT between the modified 3'-OH group of the ribose moiety in nucleoside analogues and unmodified nucleosides. In the absence of three-dimensional information on the structure of MMTV RT, we can only predict that the single mutation induces changes in the molecule that affect the enzyme beyond the mere opening of the steric gate.

Amino acid residues important for the function of steric gates (that are discriminating against rNTPs) were also implicated in other DNA polymerases, such as Glu-710 in *E. coli* DNA polymerase I [38], Glu-480 in T7 DNA polymerase and Glu-615 in *Taq* DNA polymerase [39,40]. Interestingly, in all these cases an acidic residue affects the gate, whereas in RTs an aromatic side chain probably controls the gate opening. The presence of the aromatic ring in residue 155 of MLV RT was shown to be critical for viral replication since it was shown that the MLV variant carrying the F155V mutation in its RT does not replicate [41]. In addition, the only mutation reported so far for position 115 in the RT of HIV-1 in virions (isolated from AIDS patients or from cell cultures) was the Y115F mutation [42]. Therefore, this conservative mutation is probably the only one allowed in

infectious HIV-1 virions, thus supporting the *in vitro* data that this mutation hardly affects HIV-1 RT activities [4,7]. It would be interesting to study whether the modification of Phe-119 to valine in the RT of MMTV virions will also have negative effects on viral growth.

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