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DNA synthesis fidelity by the reverse transcriptase of the yeast retrotransposon Ty1

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ABSTRACT

The fidelity of the yeast retrotransposon Ty1 reverse transcriptase (RT) was determined by an assay based on gel electrophoresis. Steady-state kinetics analyses of deoxyribonucleotide (dNTP) incorporation at a defined primer-template site indicate that Ty1 RT misincorporates dNTP at a frequency of 0.45 $\times$ 10^{-5} for the A_t:A mispair in which dATP is misincorporated opposite a template A to 6.27 $\times$ 10^{-5} for the C_t:A mispair. The G_t:G and T_t:T mispairs are formed with very low efficiency. The fidelity parameters of Ty1 RT do not depend on whether RNA or DNA are copied. Relative to lentiviral RTs (HIV-1, HIV-2 or EIAV) Ty1 RT is ~10-fold less error prone. Our data also show that the Ty1 RT is able to recapitulate two error-generating mechanisms: extension of mismatches and non-templated addition of nucleotides at the end of a blunt-end primer-template.

INTRODUCTION

High mutation rate is a hallmark of retrovirus replication (1,2). This originates in the mechanism of genome replication by the viral-encoded reverse transcriptase (RT), which converts the genomic RNA of the virus to a double-stranded DNA. During this process, RT produces frequent replication errors. One accepted explanation of this inaccuracy is the lack of RT 3′→5′ exonuclease activity.

Numerous studies have been carried out *in vivo* and *in vitro* to address the role of retroviral RTs on the variability of the genome (3–7). In contrast, little is known about the replication fidelity of non-retroviral RTs. Recently we have expressed an enzymatically active form of recombinant Ty1 RT in *Escherichia coli* (8). The purified recombinant protein was shown to possess polymerase and RNase H activities characteristic of RTs. Here we have determined the fidelity of Ty1 RT by measuring the kinetics of misinsertion opposite A, T, G and C residues at a defined primer-template site. We find that insertion of A opposite a template C (C_t:A mismatch) is formed most efficiently whereas G_t:G and T_t:T mismatches are not detected. Surprisingly the G_t:A mismatch, which is generally not made very efficiently by other RTs, is made almost as efficiently as the A_t:C, G_t:T or T_t:G mismatches by the Ty1 RT. The susceptibility to error of Ty1 RT is comparable to that of AMV RT, which is as much as 10-fold less error-prone than the lentiviral HIV-1 and HIV-2 RTs. We also show that the recombinant Ty1 RT has the ability to continue primer elongation by extending some mismatches and to add non-templated nucleotides at the ends of blunt-ended DNA–DNA or DNA–RNA duplexes.

MATERIALS AND METHODS

DNA and RNA substrates, primer end-labeling, primer-template annealing

The following four 30mer oligodeoxyribonucleotides were used as templates. They differ in the underlined sequence: template A, 5′-TCTAATCCCTGAATAAACGTAGTTGATGCT-3′; template C, 5′-TCTAATCCTGACATAAACGTAGTTGATGCT-3′; template G, 5′-TCTAATCCACTGATAAACGTAGTTGATGCT-3′; template T, 5′-TCTAATCCGACTATAAACGTAGTTGATGCT-3′. The sequence of RNA template C was identical to that of DNA template C: 5′-UCUAAUCCUGACAUAAACGUAGUUGAUGCU-3′.

The 18mer (5′-AGCATCAACTACGTTTAT-3′) oligodeoxyribonucleotide primer (3 µg) was 5′-^{32}P-end-labeled using polynucleotide kinase (1 U) and [γ-^{32}P]ATP (50 µCi at 3000 Ci/mmol) in a reaction buffer (25 µl) containing 70 mM Tris–HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT.

The ^{32}P-labeled primer (13 nM) was annealed to the DNA (330 nM) or RNA template (330 nM) in annealing buffer (5 µl) containing 50 mM Tris–HCl pH 7.5, 50 mM NaCl by heating at 90°C for 2 min followed by cooling at 4°C for 2 min, 55°C for 30 min and room temperature for 10 min.

Titration of active RT

To evaluate the fraction of active enzyme in our RT preparations we used a method described by Kati *et al.* (9) based on the burst observed during steady-state incorporation of a single nucleotide. RT was preincubated for 4 min at 22°C with a known amount of primer-template in a reaction buffer containing 17 mM Tris–HCl pH 7.5, 17 mM NaCl and 20 mM $MgCl_2$. Reverse transcription was initiated by adding a single correct nucleotide at a final concentration of 200 nM. The reaction was carried out at 22°C for 15 s, 30 s, 45 s and 60 s, and terminated by adding an equal volume of formamide containing 200 mM EDTA. Quenched reactions were heated to 90°C for 2 min and products were quantitated by sequencing gel analysis (15% acrylamide, 8 M urea). The resulting time course of

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formation of extended primer as a function of time showed a burst of incorporation followed by a linear phase. The amplitude of the burst was taken as a measure of the concentration of active enzyme.

Purification of Ty1 RT

Ty1 RT was expressed in *E.coli* and purified as described previously (8).

Standing start assay

The standing start assay was performed as described by Goodman and co-workers (10–12). Reaction conditions were such that the total amount of primer extended during the reaction was <20% and the concentration of polymerase was small compared to the concentration of primer-template. Ty1 RT (0.8 nM of active enzyme) was incubated with 5′-^{32}P-end-labeled primer-template substrate (13 nM) and varying concentrations of wrong (0.5–1.5 mM) or right (20–60 nM) deoxyribonucleotide (dNTP) in a reaction mixture containing 17 mM Tris–HCl pH 7.5, 17 mM NaCl and 20 mM $MgCl_2$. Reactions were carried out at 22°C for 2, 4 and 8 min for the wrong dNTPs and 0.5, 2 and 4 min for the right dNTPs. The reactions were treated and analyzed as above. The data were quantitated with a Bio-Imaging Analyzer BAS 2000 (Fuji). The observed rate of deoxynucleotide incorporation plotted as a function of dNTP concentration obeys the Michaelis–Menten equation:

$$V_{obs} = (V_{max}\,[\mathrm{dNTP}])/(K_m + [\mathrm{dNTP}]) \quad \mathbf{1}$$

In the method developed by Goodman and co-workers (10–12), V_{obs} was determined using Equation 2:

$$V_{obs} = (I_e/I_u)/(1/t) \quad \mathbf{2}$$

where t is the reaction time, and I_e and I_u are the intensities of extended and unextended primer, respectively. The apparent V_{max} and K_m values for dNTP incorporation was calculated from the linear Lineweaver–Burk plot of $1/V_{obs}$ versus 1/[dNTP]. The apparent V_{max} and K_m values for insertion of the wrong (w) and right (r) nucleotides were used to calculate the frequency of misincorporation as follows:

$$f_{inc} = (V_{max}/K_m)^{w}_{app}/(V_{max}/K_m)^{r}_{app}$$

Terminal transferase activity assay

Blunt-end addition of dNTP was determined by incubating the dNTP (500 µM) in the presence of 20 mM $MgCl_2$, 12.5 mM Tris–HCl pH 7, 12.5 mM NaCl, 35 nM 40 bp ^{32}P-labeled DNA duplex (Fig. 3) and 0.8 nM Ty1 RT. At 2, 10 and 30 min, reaction samples were withdrawn and treated as above. The concentration of the extended product was plotted as a function of time. The initial slopes were used to calculate the rate constants.

RESULTS

Insertion fidelity is the capacity of a polymerase to incorporate a correctly base paired versus an incorrectly base paired nucleotide at the 3′-end of a nascent DNA strand. To determine the fidelity of Ty1 RT we used an assay based on gel electrophoresis described by Goodman and co-workers (10–12). RT-catalyzed reactions were performed with 5′-^{32}P-labeled primer annealed to unlabeled template. RT was incubated with the primer-template substrate and various concentrations of the right or wrong deoxyribonucleotide. Reactions were quenched and the unextended primers along with extended primers were resolved with polyacrylamide gel electrophoresis. An example of misincorporation and mispair extension ability of Ty1 RT using RNA and DNA templates C is shown in Figure 1. A qualitative inspection of the gel autoradiograms shows that Ty1 RT readily incorporates mismatches and has the ability to continue primer elongation by extending some mismatches. In a control experiment we have verified that no addition to a single-stranded primer was observed (data not shown) indicating that a duplex DNA substrate is required for nucleotide addition to take place. The patterns of incorporation are identical for the RNA and DNA templates. For the longer incubation times, addition of 12 nt to the primer 3′-terminus are clearly visible on the autoradiograms shown in Figure 1. Moreover the 8 min products are extended by 1 additional nt, which appears to be added without a matching or mismatching template residue. Such non-templated base addition at the end of blunt-end DNA has been observed for other RTs and DNA polymerases (13–15). The results presented below (see Fig. 3) show that Ty1 RT can indeed add extra bases beyond template ends *in vitro*. Pause bands are visible at primer-template sites containing mispairs (C_t:A1, A_t:A2, C_t:A5 and A_t:A8). The bands visible at some Watson–Crick base pairs (T_t:A4, U_t:A4, T_t:A7 or U_t:A7) imply that a rapid correct incorporation of A opposite T or U is followed by slower formation of the next

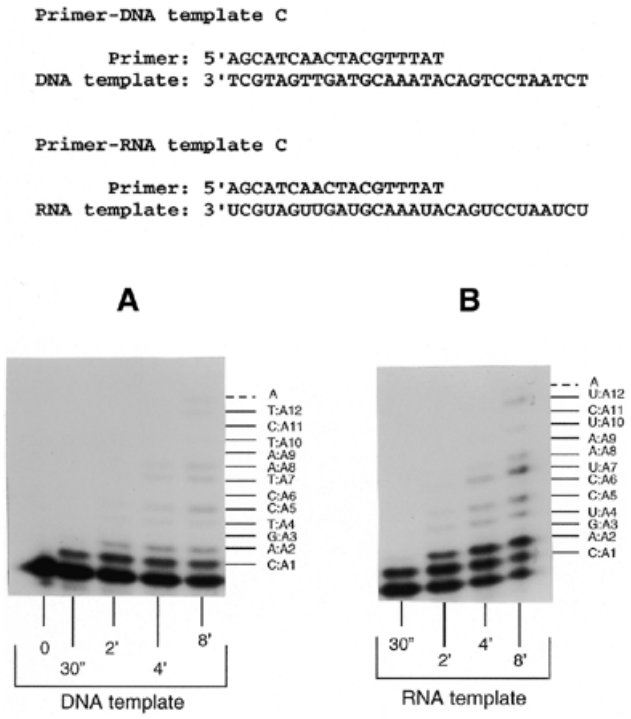

Figure 1. Gel autoradiograms showing Ty1 RT misincorporation and mispair extension ability on RNA or DNA templates C. The 5′-^{32}P-end-labeled primer was annealed to DNA or RNA template C, preincubated with Ty1 RT and mixed with dATP (1.5 mM) in a reaction buffer containing 17 mM Tris–HCl pH 7.5, 17 mM NaCl, 20 mM $MgCl_2$ to start the reaction. The reactions were quenched at the indicated times and analyzed by polyacrylamide gel electrophoresis.

Table 1. Kinetic parameters for misincorporation by Ty1 RT with DNA template A, C, G and T

	K_m (μM)	V_{max} (%/min)	f_{inc}
DNA template A			
dATP	441	3.58	0.45×10^{-5}
dTTP	0.018	32.32	1
dCTP	414	7.67	1.02×10^{-5}
dGTP	487	7.80	0.88×10^{-5}
DNA template T			
dATP	0.035	23.12	1
dTTP	ND	ND	ND
dCTP	442	2.38	0.82×10^{-5}
dGTP	689	4.72	1.04×10^{-5}
DNA template C			
dATP	138	9.69	6.27×10^{-5}
dTTP	271	9.00	2.96×10^{-5}
dCTP	411	10.37	2.25×10^{-5}
dGTP	0.023	25.53	1
DNA template G			
dATP	374	5.50	0.75×10^{-5}
dTTP	668	17.95	1.38×10^{-5}
dCTP	0.02	42.09	1
dGTP	ND	ND	ND

ND, not determined.

mispair (C_t:A5 or A_t:A8). Conversely, the absence of a visible pause band at a mispair site (G_t:A3, C_t:A6, A_t:A9 or C_t:A11) illustrates the ease with which Ty1 RT can elongate this mispair. Thus, we conclude that Ty1 RT, like other RTs, has little difficulty in forming mismatches and exhibits relaxed specificity for elongation of some mispairs.

Insertion kinetics

The quantitative assay described by Goodman and co-workers (10–12) was used to measure insertion kinetics at defined DNA or RNA template sites (see Materials and Methods). The V_{max} and K_m steady-state parameters for incorporation of wrong and right dNTP opposite a template residue were measured using the standing-start assay wherein the target-template residue immediately follows the 3′-terminal end of the primer (Fig. 2). The frequency of misincorporation of a wrong compared to the right dNTP, f_{inc}, was calculated as indicated in the Materials and Methods.

Four oligodeoxyribonucleotides (DNA templates A, C, G and T) and one oligoribonucleotide (RNA template C) were used as templates to measure fidelity of dNTP incorporation opposite A, C, G and T. DNA and RNA template C have the sequence of the plus strand of the Ty1-H3 element extending from position 1732 to position 1761 (16). The other templates differ only in the sequence of four target residues that immediately follow the 3′-end of the primer; this minimizes the influence of 5′ nearest neighbor effects on kinetics components (4,5,17). Fidelity of dNTP incorporation was measured by annealing an 18 nt primer to the 3′-end of templates A, C, G or T. In DNA and RNA templates C, the target-template residue, which immediately follows the 3′-end of the primer, is a C. In DNA templates G, T and A the target-template residues are G, T and A, respectively. Figure 2 shows the dCTP, dATP, dTTP and dGTP incorporation pattern opposite a template G residue. Values of f_{inc} on the four DNA templates and on RNA template C (Tables 1 and 2) range from 0.45×10^{-5} for misincorporation of A opposite a template A to 6.27×10^{-5} for misincorporation of A opposite a template C. The least favored mismatches are the homologous mispairs T_t:T and G_t:G, the f_{inc} value of these mispairs could not be accurately determined because the slopes were too low to yield significant values. Using the same steady-state kinetics assay, misincorporation frequencies have been found to vary from 10^{-4} to 10^{-7} for a variety of RTs (3–7). The lentiviral RTs (HIV-1, HIV-2 or EIAV) are the most inaccurate and are about ten times as error-prone as AMV RT. For the different RTs studied so far, the order of susceptibility to error is as follows: lentivirus RTs > MMTV RT > AMV RT > MLV RT. The fidelity of Ty1 RT is comparable to that of AMV RT, as illustrated in Tables 1 and 3, where the values of f_{inc} obtained on DNA template G with Ty1 and AMV RTs are compared.

The misincorporation rate depends on the type of mispair formed. For several RTs it has been shown that A_t:C mispairs are formed much more easily than A_t:G or A_t:A mispairs. HIV-1 or AMV RTs, for example, form A_t:C mispairs with ~10-fold higher efficiency than A_t:G mispairs. Misinsertion of T opposite template G is generally much easier than A or G opposite G. The same pattern of misinsertion (A_t:C > A_t:G > A_t:A or G_t:T

Table 2. Kinetic parameters for misincorporation by Ty1 RT with RNA template C

RNA template C	K_m (μM)	V_{max} (%/min)	f_{inc}
dATP	467	20.75	1.67×10^{-5}
dTTP	436	13.26	1.14×10^{-5}
dCTP	630	14.12	0.84×10^{-5}
dGTP	0.024	64.71	1

Table 3. Kinetic parameters for misincorporation by AMV RT with DNA template G

DNA template G	*K*m (μM)	V_{max} (%/min)	f_{inc}
dATP	897	6.03	0.6×10^{-5}
dTTP	550	14.7	2.39×10^{-5}
dCTP	0.055	61.27	1
dGTP	ND	ND	ND

ND, not determined.

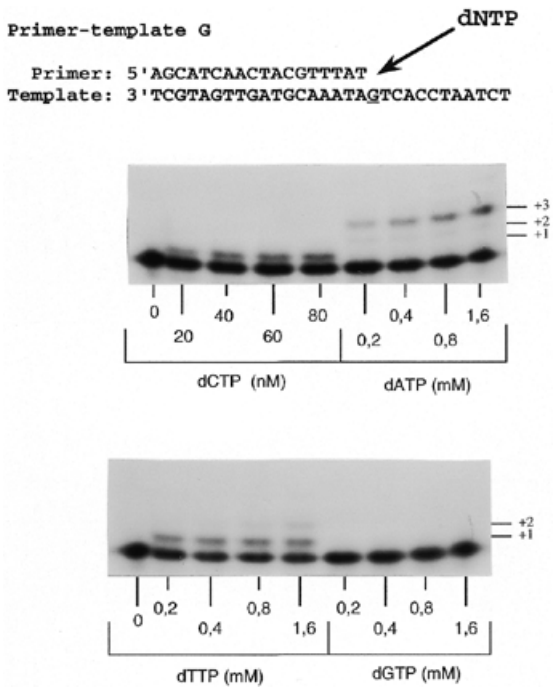

Figure 2. Fidelity of Ty1 RT opposite a template G residue. Ty1 RT (0.8 nM of active enzyme) was incubated with 5′-^{32}P-end-labeled primer-template substrate (13 nM) and varying concentrations of wrong (0.5–1.5 mM) or right (20–60 nM) dNTP in a reaction mixture containing 17 mM Tris–HCl pH 7.5, 17 mM NaCl, 20 mM $MgCl_2$. Reactions were carried out at 22°C for 5 min for the wrong dNTPs and for 1 min for the right dNTPs. The quenched reactions were analyzed by polyacrylamide gel electrophoresis.

> G_t:A >> G_t:G) is exhibited by Ty1 RT but there is only a 2-fold difference of misincorporation efficiency of A_t:C versus A_t:G and the G_t:A mispair is formed about as efficiently as G_t:T. Thus, although Ty1 RT discriminates well against incorrectly base paired nucleotides (the f_{inc} values are in the range of 10^{-5} to 10^{-6}), it does not discriminate very well between different incorrectly base paired nucleotides (with the exception of T_t:T and G_t:G mispairs, which are formed with very low efficiency).

Since RT must copy RNA and DNA templates to convert the plus strand genomic RNA into double-stranded preintegrative DNA, it was important to check whether there were differences in the fidelity of replication with either template. As shown in Tables 1 and 2, the affinity of Ty1 RT is higher for the RNA template. However, comparison of the frequencies of misincorporation of Ty1 RT on RNA and DNA templates C indicates that there are equal error rates during copying of DNA or RNA templates. This result is in agreement with previous studies showing that the fidelity parameters of retroviral RTs did not depend on whether DNA or RNA were copied (5–7). Figure 1 also illustrates that the pattern of incorporation of dATP is identical for RNA and DNA templates C.

Correlation of the insertion kinetics results with *in vivo* studies

Replication infidelity during a single cycle of Ty1 retrotransposition was studied by Gabriel *et al.* (18) by sequencing the entire 5967 bases of 29 independent Ty1 transposition events. A total of 13 substitutions were identified, corresponding to an average mutation rate of 2.54×10^{-5} per bp per replication cycle. Distribution of the substitutions was non-random along the Ty1 sequence. Five of the 13 substitutions were clustered near the U5-pbs border (between positions 240 and 333 of the Ty1-H3 sequence). It was proposed that this mutational hot spot could be accounted for by terminal non-templated base addition, which would form a terminally mismatched region that RT must extend past to complete synthesis of the 5′ LTR minus strand. Our biochemical *in vitro* data showing that Ty1 RT is able to add non-templated bases at the end of blunt-ended duplexes and to extend past mismatches (Figs 1 and 3) support the likelihood of this model.

Eight of the substitutions found in the Ty1 sequence between positions 654 and 5305 resulted in C→A, G→C and A→C transversions and one G→A transition. Four of the eight

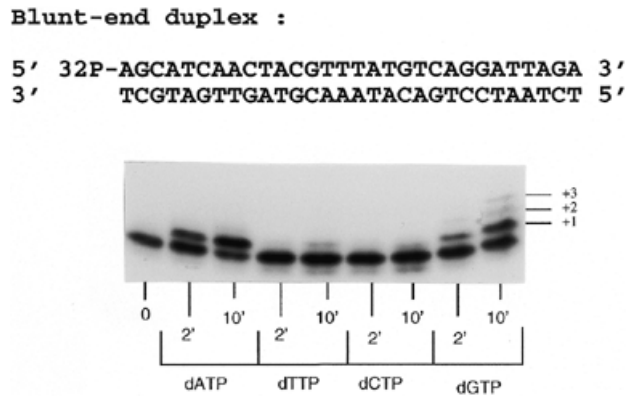

Figure 3. Blunt-end addition products synthesized by Ty1 RT. Lane 0 shows the unextended labeled 30mer oligodeoxyribonucleotide. The products synthesized in the presence of individual dNTPs for 2 and 10 min are shown in lanes dATP, dTTP, dCTP and dGTP.

substitutions are C→A transitions, which could have occurred by a C_t:T mispair during reverse transcription of plus strand genomic RNA or a G_t:A mismatch during plus strand DNA synthesis. As shown in Tables 1 and 2, C_t:T mispairs are formed with slightly greater efficiency ($f_{inc} = 1.14 \times 10^{-5}$ with RNA template C and 2.96×10^{-5} with DNA template C) than G_t:A mispair ($f_{inc} = 0.75 \times 10^{-5}$). The small difference in misincorporation efficiency of C_t:A versus G_t:A does not allow us to conclude whether the mismatch leading to the C→A transition occurred during minus or plus strand synthesis. The A→C transversion would imply an A_t:G mismatch during minus strand synthesis or a T_t:C mismatch during plus strand synthesis and the G→A transition would imply G_t:T or C_t:A mismatches. Here again the difference of misincorporation efficiency of A_t:G ($f_{inc} = 0.88 \times 10^{-5}$) versus C_t:T ($f_{inc} = 0.82 \times 10^{-5}$) or G_t:T ($f_{inc} = 1.38 \times 10^{-5}$) versus C_t:A ($f_{inc} = 6.27 \times 10^{-5}$) are small. In contrast, in the case of the G→C transversion, which would imply a G_t:G mispair during minus strand synthesis or a C_t:C mispair during plus strand synthesis, we can speculate that the mismatch leading to the G→C transversion occurred during plus strand synthesis since we have not been able to detect the formation of the G_t:G mispair in our *in vitro* study whereas the C_t:C mispair is formed at a frequency $f_{inc} = 2.25 \times 10^{-5}$.

Terminal transferase activity of Ty1 RT

The *in vivo* data and the results shown in Figure 1 suggest that Ty1 RT is able to add non-templated bases at the end of blunt-ended duplexes. To characterize non-templated nucleotide addition carried out by Ty1 RT, we used a blunt-ended 30 bp duplex (Fig. 3) in which one of the strands had been 5′-end-labeled with ^{32}P. Figure 3 shows the result of an experiment in which Ty1 RT was incubated with the blunt-ended duplex with each dNTP for 2–10 min. Ty1 RT had a preference for addition of non-templated A. dTTP and dCTP were not used efficiently whereas >1 nt was added in the presence of dGTP. The rate constants for non-templated addition of A, G, T and C were 14.9, 9.7, 3.3 and 2.6%/min, respectively. In comparison, the V_{max} for template-directed addition of A, G, T and C opposite T, C, A and G to form Watson–Crick base pairs were 23.1, 25.5, 32.3 and 42.1%/min (Table 1). Thus, non-templated addition of A and G occurs with about half the efficiency of template-directed incorporation of the right nucleotide. Non-templated addition of T and C is ~10-fold less efficient than their template-directed incorporation.

It is of interest that AMV RT (13) also efficiently uses dATP and to a lesser extent dGTP to carry out addition of nucleotides beyond template ends; as is the case for Ty1, pyrimidine precursors are used much less efficiently by AMV RT. Similarly, HIV-1 RT (14) shows a preference for addition of dAMP > dGMP > dTTP > dCTP in a ratio of 1:0.5:0.2:0.07.

By examining the end structure of the Ty1 replication intermediates synthesized in VLPs, Mules *et al.* (19,20) have demonstrated that Ty1 RT adds non-templated bases at template ends *in vivo*. 3′ Non-templated base addition was observed with both RNA and DNA template during Ty1 replication. Non-templated A residues were most common at all 3′-ends. Our *in vitro* results are in keeping with these results in that we observed blunt-end addition of nucleotides with both RNA and DNA templates (Fig. 1A and B) and a preference for addition of non-templated A.

DISCUSSION

RTs of retroviruses and retrotransposons are known to show poor fidelity during replication. Mutations resulting from errors during reverse transcription provide the basis for sequence diversity and rapid adaptation to changing environment. Several pathways to make mistakes have been described. The most common error is the misincorporation of a non-complementary nucleotide leading to a single base substitution. Slippage of the two strands of DNA at repetitive sequences leads to deletion or addition of one or more nucleotides. Replication errors involving strand misalignment can also lead to frameshift errors or base substitutions. By studying the replication infidelity during a single cycle of Ty1 retrotransposition Gabriel *et al.* (18) observed base substitutions exclusively; no frameshift, deletion or insertion were observed. This prompted us to determine the fidelity of recombinant Ty1 RT *in vitro* by measuring the kinetics of misincorporation at a defined primer-template site. Using a steady-state kinetics assay we find Ty1 RT to have an error rate of 0.45×10^{-5}–6.27×10^{-5} depending on the misincorporated nucleotide. Relative to other RTs, Ty1 RT has a fidelity comparable to that of AMV RT and it is ~10-fold less error-prone than lentiviral RTs. Our data also show that the recombinant Ty1 RT is able to recapitulate two error-generating mechanisms, i.e extension of mismatches and non-templated addition of nucleotides at the ends of blunt-ended DNA–DNA or DNA–RNA duplexes.

It has been suggested that the fidelity of polymerases could depend in part on the flexibility of the dNTP-binding pocket of the enzyme and on base pair geometry (21–23). A flexible pocket may be tolerant of distorsion of the DNA and would result in low fidelity of the polymerase whereas a rigid pocket is expected to be more sensitive to the geometry of the base pair between the incoming dNTP and the template base. In all RTs the amino acid residues involved in the formation of the putative dNTP-binding pocket include a highly conserved YXDD motif (X = M, A, L or V). Ty1 has the related FVDD sequence. Experimental evidence (22,24–26) suggests that the presence of a V residue at the X position results in a reduced error rate over polymerases containing M, A or L. It would be

interesting to compare the high resolution structure of Ty1 RT with the structure of more error-prone RTs in order to determine the impact of the V residue on the flexibility of the dNTP-binding pocket and to understand the structural basis of the higher fidelity of Ty1 RT.

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