Thermal Effects on Reverse Transcription: Improvement of Accuracy and Processivity in cDNA Synthesis

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ABSTRACT

Reverse transcription, coupled with DNA amplification, has been widely used for molecular analysis of RNAs. Reverse transcriptases are retroviral DNA polymerases that can synthesize DNA from both RNA and DNA. In general, because of the lack of 3'→5' exonuclease activity in retroviral reverse transcriptases, the reverse transcription step is error prone. Mutations created during the reverse transcription step of cDNA synthesis or RT-PCR are delivered to the final products to be analyzed, interfering with accurate analysis of the RNAs. In addition, because reverse transcription uses RNA as a template, processive DNA synthesis by reverse transcriptase is frequently interrupted by secondary structures of the RNA templates, causing difficulties in full-length cDNA synthesis. Here, we report that an increase in reaction temperature greatly enhances both the accuracy and the processivity of reverse transcription catalyzed by murine leukemia virus (MuLV) and human immunodeficiency virus type 1 (HIV-1) reverse transcriptases.

INTRODUCTION

Molecular analysis of RNA molecules relies heavily on reverse transcription, which preserves molecular information of chemically unstable RNAs as stable DNAs that can be used in various analytical methodologies. The synthesized DNA molecules are often further amplified by PCR, which greatly enhances the efficiency of RNA analysis (7,9). RT-PCR has been widely used for various RNA analyses such as diagnostic detection (17–19,23), genotyping of various viral RNAs in infected samples (5,28), quantitation of gene expression (QRT-PCR) (1,6,7,24,25), and construction of cDNA libraries from various biological organisms (20).

Reverse transcription of RNA is catalyzed by retroviral DNA polymerases called reverse transcriptases (22). Unlike other DNA polymerases, reverse transcriptases synthesize DNA from both DNA and RNA templates. All retroviral reverse transcriptases, such as murine leukemia virus (MuLV) and human immunodeficiency virus type 1 (HIV-1), lack a 3'→5' proofreading exonuclease, which contributes to the accuracy of other DNA polymerases (26). For this reason, reverse transcription is highly error prone. Mutations created during the reverse transcription step of the RT-PCR are delivered to the final products to be analyzed; this interferes with the correct analysis of RNA molecules.

Another deleterious aspect of reverse transcription is its use of RNA as a template. RNA can form various types of secondary structures that inter-

fere with processive DNA synthesis (4,15,16). RNA molecules containing secondary structures, called pause sites, are difficult to extend to full-length DNA products. Indeed, accuracy and processivity of reverse transcription are key elements for the improvement of RNA analyses.

In this study, we report that an increase in temperature greatly enhances both the accuracy and the processivity of reverse transcription, with minimum impact on the DNA polymerase activity of reverse transcriptases. The mechanisms of thermal effects on the fidelity and the processivity of reverse transcriptases and the possible applications of this finding are discussed.

MATERIALS AND METHODS

Purification of MuLV and HIV-1 Reverse Transcriptase Proteins

Procedures for the purification of the hexahistidine-tagged MuLV reverse transcriptase were modified from our protocol, which was established for the purification of HIV-1 reverse transcriptase by Ni⁺⁺ chelation chromatography, which has been described previously (12). For expression of MuLV, we constructed a plasmid, pMuLVRT, that is a pET28 (Novagen, Madison, WI, USA) containing a full-length MuLV reverse transcriptase gene that was amplified from pMuLV (provided by Dr. M. Emerman, Fred Hutchinson Cancer Research Center, Seattle, WA, USA). The amplified MuLV reverse transcriptase

gene was inserted into pET28a at its *Nde*I and *Eco*RI sites. MuLV reverse transcriptase, expressed from pMuLV-RT, is fused at the N-terminus to six histidine residues. The buffers and reagents for the MuLV reverse transcriptase purification were obtained from Novagen. Expression of MuLV reverse transcriptase was induced by the addition of 0.1 mM isopropyl- β -D-thiogalactoside to 1000 mL log phase *E. coli* BL21 with pMuLV-RT and grown in 2 $\times$ YT to a A_{600} of 0.5 in the presence of kanamycin (30 μ g/mL). After incubation for 2 h, cells were harvested by centrifugation, and the pellets were resuspended and frozen at -70°C with 20 mL 1 $\times$ binding buffer and 200 μ g/mL lysozyme. Frozen cells were thawed and lysed on ice for approximately 2 h. The lysed cells were centrifuged at 27000 $\times$ g, and the supernatant solution was applied to a Ni⁺⁺-charged His Bind[®] column (5 mL). The resin was prepared by successive washes with deionized water, 1 $\times$ charge buffer, and 1 $\times$ binding buffer. All chromatographic steps were carried out at 4°C at a flow rate of 20 mL/h. Following the application of the crude supernatant solution, the column was washed with 1 $\times$ binding buffer and 1 $\times$ wash buffer. MuLV reverse transcriptase was eluted with 1 $\times$ elute buffer. Fractions containing purified MuLV reverse transcriptase were analyzed by electrophoresis in a 12% SDS polyacrylamide gel. Fractions containing MuLV reverse transcriptase were combined and dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, and 10% glycerol for 16 h. The dialyzed MuLV reverse transcriptase fraction was further dialyzed against the same buffer containing 1 mM dithiothreitol (DTT) for an additional 16 h. In this protocol, the purity of MuLV reverse transcriptase was at least 95%, estimated by the inspection of Coomassie[®] Blue-stained gels with previously purified reverse transcriptase proteins.

Assays for DNA Polymerase Activity of MuLV Reverse Transcriptase

RNA-dependent DNA polymerase (RDDP) activity of the purified reverse transcriptase enzyme was measured by a primer extension reaction (13). For

comparison, we also used two commercially available MuLV reverse transcriptases (New England Biolabs, Beverly, MA, and Roche Molecular Biochemicals, Indianapolis, IN, USA). Purified and purchased MuLV reverse transcriptase proteins were incubated for 3 min at 37°C, 45°C, 55°C, or 60°C in 20 μ L reaction mixture containing a 40-mer RNA template (5'-AAGCUUGGCUGCAGAAUAUUGCUAGC-GGGAAUUCGGCGCG-3'; 25 nM) annealed to a 17-mer (5'-CGCGCCGAATTCCCCGCT-3'; 10 nM) ³²P-labeled at the 5' end with T4 polynucleotide kinase (template:primer, 2.5:1), four dNTPs (250 μ M each), 25 mM Tris-HCl, pH 8.0, 100 mM KCl, 2 mM DTT, 5 mM MgCl₂, and 0.1 mg/mL bovine serum albumin. Reactions were terminated by the addition of 5 μ L 40 mM EDTA, 99% formamide. Reaction products were immediately denatured by incubation at 95°C for 3 min and analyzed by electrophoresis in 14% polyacrylamide-urea gels. RDDP activity was determined by the synthesis of 40-nucleotide-long fully extended products. Under this condition, the production of the fully extended primer was a linear function of the amount of enzyme and incubation time. We also used two purchased MuLV reverse transcriptases (from New England Biolabs and Roche Molecular Biochemicals) to compare the enzymatic activity of our MuLV reverse transcriptase.

Misincorporation Assay

Procedure for this assay has been previously described (13,14). The deoxyoligonucleotide template-primer was prepared by annealing a 40-mer RNA template (5'-AAGCUUGGCUGCAGAAUAUUGCUAGCGGGAAUUCGGCGCG-3') to a 17-mer matched primer (5'-CGCGCCGAATTCCCCGCT-3') or to a 16-mer G/T-mismatched primer (5'-CGCGCCGAATTCCCCGT-3') ³²P-labeled at the 5' end. The 17-mer matched primer was also annealed to a 63-mer DNA template (5'-TAA-TACGACTCACTATAGGGAGGAAGCTTGGCTGCAGAATATTGCTAGCGGGAATTCGGCGCG-3') for the reactions with the HIV-1 reverse transcriptase. Assay mixtures (20 μ L) con-

tained 10 nM template/primer, MuLV reverse transcriptase (15 or 25 nM), and three or four dNTPs (250 μ M each) in the same buffer as described above. Reactions were incubated at 37°C, 45°C, 55°C, or 60°C for 3 min and terminated by the addition of 5 μ L 40 mM EDTA, 99% formamide. HIV-1 reverse transcriptase (10 nM) was also used in this assay by RNA template encoding a 1.7-kb-long HIV-1 reverse transcriptase sequence, annealed to ³²P-labeled 3305 primer (14). This 1.7-kb HIV-1 RNA template was synthesized by T7 RNA polymerase-catalyzed transcription of pBK8 (12,14), which encodes the HIV-1 reverse transcriptase gene between the *Hind*III and *Eco*RI sites of pBlue-script[®] (+) (Stratagene, La Jolla, CA, USA). The transcribed RNA was purified by ethanol precipitation and annealed to a ³²P-labeled 21-mer (3305 reverse transcriptase primer) complementary to nucleotides encoding amino acids from 239 to 246 of HIV-1 reverse transcriptase. Otherwise, reaction conditions were the same as described above. The extension reactions were initiated by adding the trap mixture containing dNTPs (0.5 mM with final concentration 5 mM MgCl₂), a molar excess of polyA/oligodT (4 μ g/20 μ L), and heparin (10 μ g/20 μ L). The reactions were terminated by the use of 2 μ L stop solution after a 3-min incubation at 37°C or 55°C. Reaction products were immediately denatured by incubation at 95°C for 3 min and analyzed by electrophoresis in 14% polyacrylamide-urea gels. The amounts of the fully extended primer ("F" in Figure 1) and the primer extending beyond the first stop sites (Figures 1 and 2) were determined by the phosphorimager analysis of the gels. The fidelity changes (Figure 1) were determined as described (27). Our purified and purchased MuLV reverse transcriptases showed a similar level of misincorporation in this assay.

Kinetic Assay for Misinsertion Fidelity of MuLV Reverse Transcriptase

A modification of the steady state kinetic assay of Boosalis et al. (2) was used to determine the misinsertion frequencies of the reverse transcriptases at

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two different incubation temperatures. Reaction conditions were the same as described in the misincorporation assay (see above), except for the primer and the concentration of dNTPs. ^{32}P -labeled C primer (5'-CGCGCCGAATT-CCC-3') annealed to 40-mer RNA template (5'-AAGCUUGGUGCAGAAUAUUGCUAGCGGAAUUCGCGCG-3') was extended by MuLV reverse transcriptase with increased concentrations of the correct dCTP (2'-deoxycytidine 5'-triphosphate) and one incorrect TTP (2'-deoxythymidine 5'-triphosphate) at two different temperatures, 37°C and 55°C for 3 min. The amounts of reverse transcriptase and incubation time were adjusted to yield extension of 10%–35% of the total labeled primer at the highest dNTP concentrations. Products were resolved in 14% polyacrylamide-urea gels and quantitated by phosphor-imaging analysis. The k_{cat} and K_m values were determined from Hanes-Woolf plots (11). The misinsertion fidelity, f_{ins} , was calculated at each temperature from the k_{cat} and K_m values as specified (2).

Processivity Assay

The reaction condition for measuring processivity requires a single round of primer extension (14). MuLV reverse transcriptase protein (20 nM) was preincubated at 37°C or 55°C for 3 min with 5 nM of either poly(rA) (average size = 260 nucleotides) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) annealed to the ^{32}P -labeled 20-mer oligodT or 1.7-kb RNA template containing the coding sequence of the HIV-1 reverse transcriptase gene annealed to the ^{32}P -labeled 3305 reverse transcriptase primer (see Misincorporation Assay section) (14). Otherwise, reaction conditions were the same as described above. The extension reactions were initiated by adding the trap mixture containing dNTPs (0.5 mM with final concentration of 5 mM MgCl_2), a molar excess of poly(rA)/oligodT (4 $\mu\text{g}/20\text{ }\mu\text{L}$), and heparin (10 $\mu\text{g}/20\text{ }\mu\text{L}$). The extension reactions were terminated by 2 μL stop solution after a 3-min incubation at 37°C or 55°C. Under this condition, the primer was extended only once during the incubation, as confirmed by the same two control reactions with or

without the trap. The terminated processivity reaction and control reactions were analyzed by 8% urea-PAGE.

RESULTS

Thermal Effect on Accuracy of MuLV Reverse Transcriptase

To examine the effect of temperature on the accuracy of reverse transcription, we employed (i) purified MuLV reverse transcriptase, which is known to be a high-fidelity reverse transcriptase, and (ii) a misincorporation assay that can measure both the incorporation of incorrect dNTPs and the extension of

mismatched primer (21). First, the ^{32}P end-labeled matched primer (Figure 1A) annealed 40-mer RNA template was extended by reverse transcriptase in the presence of all four dNTPs or only three dNTPs. In the reactions with only three dNTPs, the incorrect dNTPs were incorporated at the sites where the deleted dNTPs should have been incorporated, called stop sites, and were extended by infidelity of reverse transcriptase. Therefore, in this misincorporation assay, the higher efficiency of primer extension beyond the stop sites (marked by "*" in Figure 1) reflected the lower fidelity of the reverse transcriptase protein (13,14,21). We performed the misincorporation assay with

Figure 1. Thermal effect on fidelity of MuLV reverse transcriptase. (A) Misincorporation assay with matched primer: the ^{32}P -labeled 17-mer matched primer ("S") annealed to a 40-mer RNA template was extended by MuLV reverse transcriptase protein (15 nM) at 37°C, 45°C, 55°C, and 60°C for 3 min in the presence of either all four dNTPs or only three complementary dNTPs (minus TTP and minus dCTP). As determined by amounts of the fully extended primer ("F") in all dNTPs, reverse transcriptase activity of MuLV reverse transcriptase was reduced to 65% at 55°C. The sites with "*" indicate the stop sites where the deleted dNTPs would be incorporated into the reactions with only three dNTPs. In the assay with matched primer and only three dNTPs (A), the higher efficiency of elongation of terminated primer beyond the stop sites reflected the lower fidelity of the reverse transcriptase protein assayed. (B) Extension of mismatched primer: the ^{32}P -labeled 16-mer G/T mismatched primer ["S (G/T)"] annealed to a 40-mer RNA template was extended by MuLV reverse transcriptase protein at 37°C, 45°C, and 55°C for 3 min. In this reaction, 2-fold higher concentrations (6 $\times$ and 2 $\times$) of MuLV reverse transcriptase were used at 55°C to compensate for the reduction of the reverse transcriptase activity at 55°C. The extension reactions with mismatched primer were performed in the presence of three dNTPs (minus dCTP), and the mismatched primer could be extended only up to the first stop site ["*" in (B)]. In the assay with mismatched primer (B), the higher efficiency of elongation of the mismatched primer reflected the lower fidelity of the reverse transcriptase protein. The reactions were analyzed by 14% denaturing gel electrophoresis. The DNA sequence of the first 12 nucleotides of the extended part of the primer is shown in (A).

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Table 1. Thermal Effect on Misinsertion Fidelity of MuLV Reverse Transcriptase

Temperature	Correct dNTP (dCTP)		Incorrect dNTP (TTP)		f_{ins}^a	Fold Change ^b
	Km (μM)	k_{cat} (s^{-1})	Km (μM)	k_{cat} ($\text{s}^{-1} \times 10^{-3}$)		
37°C	0.15	0.22	24	1.2	3.4×10^{-5}	×1
55°C	0.42	0.038	65 ^c	0.03	5.1×10^{-6}	×6.7

^aMisinsertion fidelity, $f_{\text{ins}} = (k_{\text{cat}}/\text{Km})$ with incorrect TTP/ $(k_{\text{cat}}/\text{Km})$ with correct dCTP (2).
^bFold difference = f_{ins} of 37°C/ f_{ins} of 55°C.
^cKm values of 55°C for incorrect TTP could not be determined accurately because of little misinsertion (approximately 5% of the primer), even with the highest MuLV reverse transcriptase protein concentration. The Km value at 55°C with TTP was calculated based on the difference of Km values observed with correct dCTP between 37°C and 55°C.

15 nM MuLV reverse transcriptase at four different temperatures: 37°C, 45°C, 55°C, and 60°C. As seen in Figure 1A with all of the dNTPs, the total amount of the fully extended product ("F" in Figure 1) was reduced to only 65% of normal when reactions were performed at 55°C rather than 37°C. Thus, MuLV reverse transcriptase remains highly active at high temperatures. However, as seen in the reactions lacking TTP or dCTP (Figure 1A), the amount of extended product beyond the first stop sites (* in Figure 1), which was determined by phosphor-imaging analysis of the gel, was greatly reduced at 55°C as compared to 37°C. Similar reduction of misincorporation was observed in reactions lacking dATP or dGTP (data not shown). To compensate for the slightly reduced reverse transcriptase activity at 55°C, we performed the misincorporation assay with a higher reverse transcriptase concentration (25 nM). Even with this compensated amount of reverse transcriptase, a much lower amount (5.3×) of the primer extension was observed at 55°C compared to 37°C (data not shown). We also used two commercially available MuLV reverse transcriptases in this assay, which showed similar levels of thermal effect on their fidelity (data not shown). These data indicate that an increase in temperature largely enhances the accuracy of DNA synthesis catalyzed by MuLV reverse transcriptase. It is likely that the improved accu-

racy of reverse transcription at a high temperature is caused by the melting of the 3' end of the primer.

Thermal Effect on Extension of Mismatched Primer by MuLV Reverse Transcriptase

The creation of a mutation during DNA synthesis consists of two sequential steps: (i) incorporation of incorrect dNTPs and (ii) extension of the mismatched primer generated by misincorporation. In the misincorporation assay shown in Figure 1A, the extension of primer beyond the stop sites measured the capability of the reverse transcriptase proteins both to misincorporate and to extend mismatched primer. Next, we examined the capability of MuLV reverse transcriptase only to extend the mismatched primer (i.e., the second step of mutation synthesis). In this study, we used a 16-mer mismatched primer containing a G/T mismatch at the 3' end of the primer that was annealed to a 40-mer RNA template. The primer extension reactions were performed with only three dNTPs (-dCTP in G/T-mismatched primer reactions). We used two different concentrations (3× and 1×) of MuLV reverse transcriptase at 37°C and 45°C, and 2-fold higher concentrations (6× and 2×) of MuLV reverse transcriptase were used at 55°C to compensate for the reduction of reverse transcriptase activity at 55°C. As seen in Figure 1B, MuLV

reverse transcriptase showed an approximately 4-fold lower level of extension of the G/T-mismatched primer at 55°C than at 37°C or 45°C, even with this compensated amount of reverse transcriptase. It is likely that the improved accuracy of reverse transcription at a high temperature could be caused by further melting of the unstable mismatched primer. However, Taq Pol (Applied Biosystems, Foster City, CA, USA), a thermo-stable DNA polymerase, can extend mismatched primer up to 70°C (10).

Thermal Effect on Misinsertion Kinetics of MuLV Reverse Transcriptase

Next, we employed a steady-state kinetic assay to measure the misinsertion fidelity (f_{ins}) of MuLV reverse transcriptase protein at 37°C and 55°C. The f_{ins} value was calculated by two kinetic parameters, k_{cat} and Km, which indicate the capability of DNA polymerases to incorporate dNTPs (25). It was reported that G→A mutation, which can be made by the misinsertion of TTP instead of the correct dCTP during the RNA-dependent DNA polymerization step, is a predominant mutation spectrum during reverse transcription (3). Therefore, we measured the misinsertion fidelity for the C (correct) to T (incorrect) mutation using an RNA template (Table 1). The k_{cat} and Km values of MuLV reverse transcriptase with correct versus incorrect nucleotides were measured at 37°C and 55°C by a gel-based analysis and Hanes-Woolf equation (11). As shown in Table 1, and consistent with the misincorporation assay data, MuLV reverse transcriptase showed at least 6.7 times lower misinsertion frequency at 55°C than 37°C. Catalysis (k_{cat}) for misinsertion was largely reduced at 55°C.

Thermal Effect on Fidelity of HIV-1 Reverse Transcriptase

HIV-1 reverse transcriptase is the most error-prone DNA polymerase among the known DNA polymerases (26). We tested whether the infidelity of HIV-1 reverse transcriptase is also affected by temperature changes. Both an RNA template encoding an HIV-1 viral

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genomic sequence and a 63-mer DNA template were used for this experiment. As seen in Figure 2A, the fidelity of HIV-1 reverse transcriptase with both RNA and DNA templates was largely enhanced at high temperatures with little loss of total reverse transcriptase activity (only a 20% reduction of HIV-1 reverse transcriptase activity at 55°C). Similar reduction of misincorporation was observed in reactions lacking dCTP, dATP, or dGTP (data not shown). Therefore, this observation supports the notion of a universal thermal effect on the fidelity of reverse transcription.

Thermal Effect on Processivity of Reverse Transcription

We tested whether an increase in temperature, which could remove secondary structures of RNA template, enhances the processivity of reverse transcription. For this test, we used two RNA templates: (i) heterogeneous RNA template encoding a (+) RNA strand sequence of the HIV-1 Pol gene and (ii) homogeneous poly(rA) template. The processivity assay requires a single round of primer extension; for this, a molar excess of cold poly(rA)/oligodT

and heparin were used to trap free reverse transcriptase molecules to prevent multiple rounds of reverse transcription initiation (14). As seen in Figure 3A, reverse transcription of the heterogeneous HIV RNA template generated multiple pause sites, showing a higher band intensity at 37°C (marked by “*” and arrows). As seen in the control reactions (+Trap) of Figure 3, pre-incubation with the trap before primer extension completely prevented primer extension, indicating that the trap successfully sequestered free reverse transcriptase molecules. In the reaction without the trap, the primer was extended further, indicating a multiple round of initiation (data not shown). These two controls indicate a single round of reverse transcription in the processivity assay with the trap. As predicted, at 55°C, the processivity of reverse transcriptase with the heterogeneous RNA template increased (Figure 3A). As seen in the reactions marked “Proc”, many of the pause sites disappeared at high temperatures. This could be the result of an increased processivity at high temperatures. However, some pause sites were still observed (marked by “*” in Figure 3). The processivity with homogeneous poly(rA) template was also largely enhanced at high temperatures (Figure 3B). This observation indicates that an increase in temperature enhances processive DNA synthesis by reverse transcriptase in both heterogeneous and homogeneous RNA templates.

DISCUSSION

Various molecular analyses of RNAs, such as diagnostic detection and genotyping of viral RNAs, and quantitation of gene expression and cDNA libraries require a conversion of RNA molecules to more stable DNA molecules. This conversion step is enzymatically catalyzed by reverse transcriptase. Synthesized DNA copies are further amplified to obtain detectable and useful amounts of DNA molecules. However, several intrinsic aspects of reverse transcriptases interfere with reliable and efficient DNA synthesis from RNA. First, lack of 3'→5' proofreading activity in retroviral reverse transcriptases limits faithful DNA synthesis (26).

Figure 2. Thermal effect on fidelity of HIV-1 reverse transcriptase. (A) The ^{32}P -labeled 21-mer matched 3305 primer (“S”) annealed to a 1.7-kb-long RNA template encoding positive strand of HIV-1 Pol sequence was extended by HIV-1 reverse transcriptase at 37°C, 50°C, 55°C, and 57°C for 3 min, as seen in the extension reactions with all four dNTPs or only three complementary dNTPs (minus TTP). (B) The ^{32}P -labeled 17-mer matched primer (“S”) annealed to a 63-mer DNA template was also extended by HIV-1 reverse transcriptase protein at 37°C, 50°C, and 55°C for 3 min, as seen in the extension reactions with all four dNTPs or only three complementary dNTPs (minus TTP). The first five (A) and four (B) stop sites were marked by “*.”

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Because mutations created during the reverse transcription step of RT-PCR are delivered to the final products to be analyzed, infidelity of reverse transcription is a major problem in RNA analysis. Second, RNA templates intrinsically form various types of secondary structures, which can stall translocation of the RT/dNTP/T-P ternary complex during reverse transcription (4,15). This limits the efficiency of full-length DNA synthesis. As observed in previous studies with other DNA polymerases, pause sites are mutational hot spots because of high mutation probability during the first dNTP incorporation (8).

The enhancement of accuracy and processivity of reverse transcription is essential to the reliable and efficient analysis of RNA. In this study, we demonstrated that accuracy and processivity of reverse transcription are greatly enhanced at high temperatures. It is possible that the thermal effect on the fidelity of reverse transcription could be because of instability of mismatches at the 3' end of the primer, which can be

generated by incorporation of incorrect dNTP (misinsertion). In other words, 3' mismatches can melt further, generating multiple mismatches at the 3' end of the primer. Most DNA polymerases, including reverse transcriptases, have a largely reduced capability to extend such mismatched primers. However,

the thermostable DNA polymerase, Taq Pol, can extend mispaired primers efficiently up to 70°C (10). Presumably, Taq Pol might have evolved the ability to extend the mismatched primers that will be constantly generated in extreme host environments (i.e., high temperature). Other factors that might be affect-

Figure 3. Processivity of MuLV reverse transcriptase proteins with (A) heterogeneous and (B) homogeneous RNA templates. (A) Heterogeneous RNA template encoding an HIV-1 Pol sequence, annealed to the 32 P-labeled 21-mer 3305 primer, and (B) Poly(rA) template annealed to the 32 P-labeled 21-mer oligodT were used in this processivity assay. Processivity (Proc): two template-primers (T/Ps) were first preincubated with MuLV reverse transcriptase proteins at 37°C or 55°C for 3 min, and then the extension reactions were initiated by adding trap mixture containing dNTPs, a molar excess of poly(rA)/oligodT, and heparin at 37°C or 55°C for 3 min. This condition allowed only a single round of primer extension by reverse transcriptase as confirmed by two control experiments (+ Trap and -Trap). +Trap: RNA T/Ps were first pre-mixed with the trap mixture, and the extension reactions were initiated by adding MuLV reverse transcriptase protein at 37°C or 55°C for 3 min. This condition blocked reverse transcriptase binding to the labeled T/Ps and extending the primer, supporting a single round of primer extension in the processivity reaction. In addition, RNA T/Ps were also pre-mixed with reverse transcriptase proteins, and then the extension reactions were initiated by only dNTPs without trap (-Trap control) at 37°C or 55°C for 3 min. This condition allowed multiple rounds of primer extension by reverse transcriptase proteins, generating more and much longer extended products (data not shown). All reactions were analyzed by 10% denaturing gel electrophoresis. The pause sites sensitive to temperature were marked by arrows, and the pause sites insensitive to temperature were marked by “*”.

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ed by temperature changes, such as structure and kinetics of reverse transcriptases, could also influence the accuracy of reverse transcription.

It has been demonstrated that various RNA template sequences forming secondary structure interfere with processive DNA synthesis from RNA molecules. For example, HIV-1 5' transactivation response region (TAR) can form a stable stem loop secondary structure (15). Several biochemical studies showed that this RNA secondary structure terminates DNA synthesis catalyzed by HIV-1 reverse transcriptase (4,15). Relatively local minor structures can also contribute to the termination of processive DNA synthesis. These secondary structures are stabilized by limited basepairing between nucleotide sequences encoded in RNA template, which can be easily resolved by an increase in temperature. The elimination of the pause sites and enhanced processivity at high temperatures support this prediction (Figure 3A). It is also possible that the equilibrium of dNTPs may become favorable for the processive DNA synthesis at high temperatures.

In summary, this study uses temperature as a biochemical element that can be immediately applied to various types of biological assays for RNA molecules, and the quality of these RNA analyses will be largely enhanced by the adoption of this biochemical element.

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