Fidelity of HIV-1 Reverse Transcriptase Copying RNA in Vitro†

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ABSTRACT: The genomic hypervariation of human immunodeficiency virus 1 (HIV-1) could result from misincorporations by the viral reverse transcriptase. We developed an assay for reverse transcriptase fidelity during RNA-dependent as well as DNA-dependent DNA polymerization in vitro. A *lacZα* RNA fragment transcribed by T3 RNA polymerase was used to mimic first-strand reverse transcription. The corresponding DNA template was used to examine errors by reverse transcriptase during second-strand DNA synthesis. With both templates, the mutations introduced by reverse transcriptase were identified by their mutant phenotypes in an M13 *lacZα*-complementation assay. We found that the reverse transcriptase from human immunodeficiency virus 1 (HIV-1 RT) was less accurate than the reverse transcriptase from Moloney murine leukemia virus (MLV RT) or the Klenow fragment of *Escherichia coli* DNA polymerase I (Pol I) on either RNA or DNA templates. The frequency of misincorporation by HIV-1 RT was 1 in 6900 nucleotides polymerized on the RNA template and 1 in 5900 on the DNA template. The error rates of MLV RT and Pol I on the RNA template were less than 1 in 28 000 and 37 000, respectively. The most frequent mutations produced by HIV-1 RT copying the RNA template were C → T transitions and G → T transversions resulting from misincorporation of dAMP.

An understanding of human immunodeficiency virus (HIV-1)[1] genome hypermutability may be crucial for the prevention and treatment of AIDS (Coffin, 1986; Fauci, 1988; McCune, 1991). The hypermutability of HIV-1 is so great that a nucleotide sequence change occurs in the *env* or *gag* genes essentially every replication cycle (Goodenow et al., 1989). This hypermutability could be a major impediment to the development of effective vaccines against the viral coat protein and to the design of nucleotide analogues that disrupt viral replication.

A major source of HIV-1 genomic instability is thought to be its reverse transcriptase (HIV-1 RT). This enzyme converts the RNA genome into double-stranded DNA in the host cell cytoplasm. There are three steps in this process: (1) HIV-1 RT copies the RNA genome to produce the initial minus-strand DNA product; (2) HIV-1 RT, with its RNase H-like activity, subsequently hydrolyzes the RNA strand; (3) HIV-1 RT then copies the DNA, yielding a double-stranded DNA copy of the viral genome. Other processes that may also contribute to its hypervariability include replication of the integrated viral DNA by host DNA polymerases, production of viral RNA by host RNA polymerase II, and recombination between multiple viral DNA copies within infected cells (Steinhauer & Holland, 1987; Hu & Temin, 1990).

Studies using several systems have estimated the error rate in vitro of HIV-1 RT copying a DNA template to be about 1 misincorporation per 5000 nucleotides polymerized (Preston et al., 1988; Roberts et al., 1988; Takeuchi et al., 1988; Bebenek et al., 1989; Weber & Grosse, 1989). In principle, this frequency of misincorporation is sufficient to account for the mutation rate observed in vivo. However, mismatches produced by HIV-1 RT copying a DNA template may not be the major source of HIV-1 genomic variation as these errors could be excised by the host postreplication mismatch repair system (Glazer et al., 1987; Stephenson & Karran, 1989). Furthermore, the mutational specificity of HIV-1 RT on a DNA template in vitro is not consistent with that observed in the viral genome in vivo, suggesting that other DNA metabolic processes determine HIV-1's genomic variability or that phenotypic selection is an overriding factor (Ricchetti & Buc, 1990; Vartanian et al., 1991).

Misincorporation by HIV-1 RT could occur during first-strand DNA synthesis on the RNA template. However, the fidelity of HIV-1 RT in copying heteropolymeric RNA templates is not known. Virtually all studies on the fidelity of reverse transcriptase copying RNA in vitro have used ribohomopolymers rather than native RNA as templates (Battula & Loeb, 1974; Takeuchi et al., 1988).

In this study, we present an assay for measuring the fidelity of reverse transcription. The assay detects a wide spectrum of mutations in a portion of the *lacZα* gene. We find that HIV-1 RT is more error-prone than either MLV RT or Pol I. Its high error rate while copying RNA templates supports the hypothesis that reverse transcription by HIV-1 RT could be a major factor in the mutability of its viral genome. The forward mutation assay used here can be readily adopted for measuring the fidelity of RNA polymerases as well as other reverse transcriptases.

Materials and Methods

Bacteria and Bacteriophage. Escherichia coli MC1061 [*hsdR, mcrB, araD, 139Δ-(araABC-leu), 7679ΔlacX74, galU, galK, rpsL, thi*] was the transformation host. An F′ derivative of strain CSH50 [Δ(*pro-lac*) *thi, ara, strA/F′* (*proAB, IacIa-zΔM15*)] was used as an indicator for the transfected *E. coli*. Both MC1061 and CSH50 were provided by T. A. Kunkel (NIEHS). *E. coli* CJ236 (*ung-1, dut-1, relA1, spoT1,*

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[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; dNTP, deoxyribonucleoside triphosphate; rNTP, ribonucleoside triphosphate; BSA, bovine serum albumin; HIV-1 RT, reverse transcriptase from human immunodeficiency virus 1; MLV RT, reverse transcriptase from Moloney murine leukemia virus.

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thi-1, pCJ105), provided by B. Bachman (Yale University), was used to produce uracil-containing single-stranded M13mp2 DNA.

Enzymes and Chemicals. HIV-1 RT was purified as previously described (Preston et al., 1988). A clone of HIV-1 RT from an M13mp18 kindly provided by S. Wilson (NIH) was inserted into the *tac*-driven expression vector pKH223-3 (Pharmacia LKB Biotechnology, Inc.). HIV-1 RT was expressed in *E. coli* upon IPTG induction (Huber et al., 1989; K. C. Cheng, unpublished data). Purification of the Klenow or large fragment of polymerase I (Pol I) from *E. coli* CJ155 was as described by Joyce and Grindley (1983). MLV RT, T7 DNA polymerase, *Hae*III endonuclease, and RNase H were from U.S. Biochemical Corp. *Sca*I endonuclease and T4 polynucleotide kinase were from New England BioLabs, and bovine pancreatic RNase A was from Sigma Chemical Co. RNase-free DNase, T3 RNA polymerase, and ribonuclease inhibitor (RNasin) were from Promega. pBluescript SK+ plasmid was from Stratagene. The deoxyoligonucleotide primer (TTC GCT ATT ACG CCA GCT) used for extension reactions was synthesized by Operon Technologies, Inc. dNTPs were from Sigma and rNTPs from Promega. [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham Corp.

RNA Template Preparation. The *lacZ*α RNA template for first-strand DNA synthesis by reverse transcriptase was produced by T3 RNA polymerase in vitro. The transcription reactions in a volume of 50 μL contained 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 500 μM each of the four rNTPs, 3 μg of linearized pBluescript SK+ plasmid DNA, 30 units of T3 RNA polymerase, and 2 units of RNasin. Incubation was at 37 °C for 60 min, and thereafter, 5 units of RNase-free DNase was added and incubation continued for an additional 60 min. A total of 10% of the RNA products were analyzed by electrophoresis through 1% agarose gel; the remainder was extracted with phenol, phenol/chloroform, and chloroform twice and precipitated with ethanol. The purified RNA was then dissolved in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0).

Primer Extension. Oligonucleotide primers were 5′-end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase as described (Maniatis et al., 1982). Unincorporated nucleotides were removed by centrifugation through a 1-mL Sephadex G-50 column. The labeled primers were annealed to DNA or RNA templates as described (Williams et al., 1990). The extension reactions in 50 μL contain 0.5 μg of RNA or DNA, 60 mM Tris-HCl (pH 8.2), 1.0 mM dithiothreitol, 7 mM $MgCl_2$, 0.5 mM EDTA, 10 mM KCl, 100 μM each of the four dNTPs, and 0.1 unit of RNasin. Upon addition of HIV-1 RT (20 units), MLV RT (20 units), or Pol I (3 units), the reactions were incubated at 37 °C for 30 min. In the reactions with RNA templates, the RNA was hydrolyzed by the addition of 20 μg/mL RNase A and 0.5 unit of RNase H, followed by incubation at 37 °C for 30 min. The extended DNA was then annealed to M13mp2 plus-strand DNA at a 1:2 molar ratio and digested with 1 unit of *Hae*III at 37 °C for 60 min. In the reaction with DNA templates, *Hae*III was added immediately after the extension reaction. The *Hae*III digestion fragments were resolved by electrophoresis through 8% polyacrylamide/6 M urea gel and then visualized by exposure to Kodak XAR film at room temperature. The 98-base *Hae*III digestion product was excised and extracted from the gel by shaking in 100 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 300 mM NaCl overnight at room temperature. The solubilized DNA was further purified by centrifugation through 1-mL Sephadex G-50 columns and dried.

Identification of Mutations. The 98-base DNA fragment was annealed to M13mp2 uracil-containing DNA at a 3:1 molar ratio in a total volume of 10 μL. T7 DNA polymerase (0.1 unit) was added, and the final reaction contained 88 mM Tris-HCl (pH 7.5), 6.7 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 250 μM each of the four dNTPs. Incubation was at 37 °C for 30 min. *E. coli* MC1061 cells were prepared for transformation as follows: An overnight culture in 2xYT was diluted 1:100 with the same (1.6% Difco Bacto tryptone, 1% Difco yeast extract, and 0.5% NaCl) and incubated until growth was in log phase. Thereafter, at 4 °C, cells were centrifuged and resuspended in 25% of original volume of distilled H_2O three times, then resuspended in 1.5% of original volume of 12% glycerol, and stored at −70 °C. A total of 80 μL of *E. coli* MC1061 cells were electroporated in the presence of 1 μL of DNA solution at 2 kV, 25 μF, and 400 Ω using a Gene-pulser electroporator (Bio-Rad Laboratories). The transformed cells were plated with *E. coli* CSH50 cells. Light blue and colorless plaques were scored (Kunkel, 1985; Takeshelashvili et al., 1991), and their mutated DNA was amplified and sequenced (Sanger et al., 1977). The fidelity assay was repeated at least twice, and identical treatments agreed within 15%.

Results

The assay used here to determine the fidelity of RNA-directed DNA synthesis is depicted in Figure 1. The *lacZ*α gene in the pBluescript SK+ plasmid was transcribed in vitro by T3 RNA polymerase. The RNA product was hybridized to a 5′-^{32}P-labeled deoxyribonucleotide 18mer and reverse-transcribed to yield a DNA copy. The RNA–DNA hybrid was digested with RNase H, and the resulting single-stranded DNA product was annealed to M13mp2 plus-strand DNA and digested with *Hae*III. The expected 98-base product was isolated and annealed to M13mp2 uracil-containing plus-strand DNA and extended by T7 DNA polymerase. After electroporating *E. coli* MC1061 in the presence of the double-stranded product, *lacZ*α mutants were identified by plaque color. Since the uracil-containing strand is degraded in vivo, the predominant template for DNA replication here is the minus-strand DNA synthesized by the reverse transcriptase.

To compare the fidelity of reverse transcription to that of DNA synthesis, a parallel assay was carried out with a DNA template. A synthetic 18mer was hybridized to M13mp2 plus-strand DNA and extended by reverse transcriptase. After the product was digested with *Hae*III, the 98mer was isolated, annealed to uracil-containing M13mp2 DNA, extended, and transfected in the *lacZ*α-complementation assay.

A map of the pBluescript SK+ plasmid used as the template for transcription of the T3 RNA polymerase products is shown in Figure 2A. The plasmid contains the *lacZ*α gene under the control of the phage T3 promoter. The plasmid was first linearized with *Sca*I, and T3 RNA polymerase was added to transcribe *lacZ*α RNA. We omitted the purification and isolation steps to minimize potentially mutagenic damage to the RNA. As shown by agarose gel electrophoresis (Figure 2B), there is no visible 2.9-kbp plasmid DNA upon incubation with DNase. Due to the large amount of protein in the samples including *Sca*I, T3 RNA polymerase, and BSA, some RNA remained in the loading wells during electrophoresis. However, upon extraction with phenol and chloroform twice, only a single RNA band corresponding in size to double-stranded DNA of 600 bp is seen. There is no visible band upon RNase A digestion (data not shown).

The DNA polymerized by HIV-1 RT, MLV RT, or Pol I using RNA or DNA templates was digested with *Hae*III and

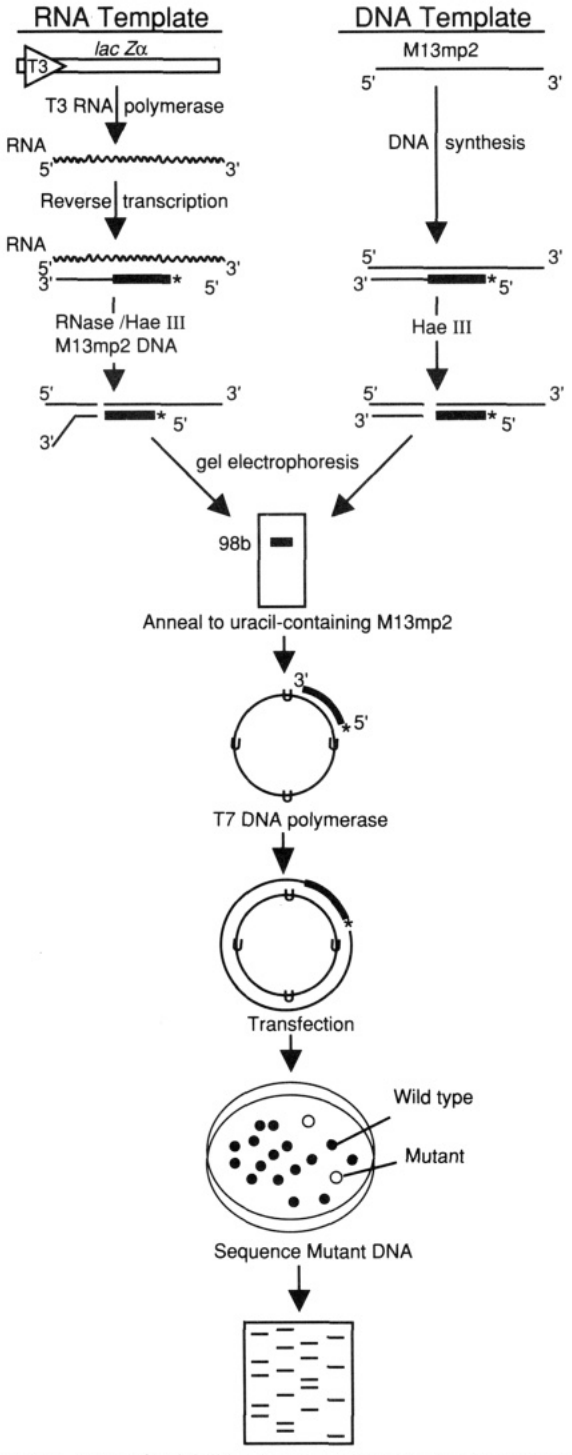

FIGURE 1: Assays for fidelity of nucleotide polymerization on RNA and DNA templates. The pBluescript SK+ plasmid contains the code for *lacZα*, except that the polylinker sequence has been inserted into the M13mp2 *Eco*RI site at position +58 from the *lacZα* transcriptional start site. The bold line represents the assay target at positions +64 to +142 from the M13mp2 *lacZα* transcriptional start site or at positions +510 to +608 from the pBluescript SK+ replication origin.

isolated by electrophoresis through polyacrylamide gel (see Figure 3). The major *Hae*III digestion product was a 98mer. DNA shorter than 98 bases resulted from pausing by the reverse transcriptase or DNA polymerase (Williams et al., 1990). Products longer than 98 bases resulted from either pausing or incomplete digestion. As seen in Figure 3, DNA polymerase I Klenow fragment (Pol I) can efficiently synthesize DNA from an RNA template, confirming an earlier observation by Loeb et al. (1974).

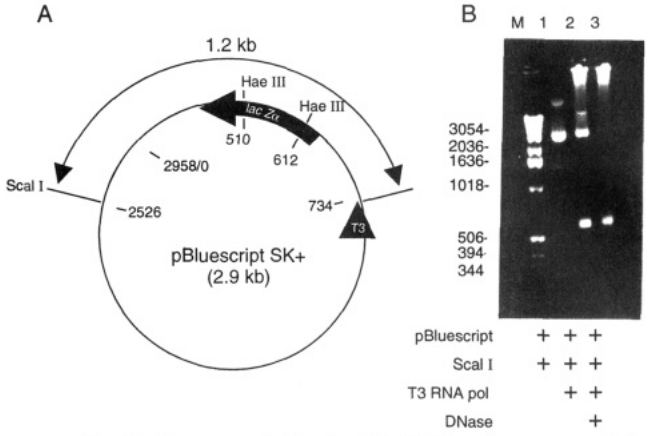

FIGURE 2: *lacZα* transcription by T3 RNA polymerase. (A) pBluescript SK+ map. This 2.9-kbp plasmid DNA contains the *lacZα* gene under phage T3 promoter control. The positions of the *Sca*I and *Hae*III restriction sites are numbered from the plasmid replication origin. (B) RNA transcript electrophoresed through 1% agarose gel: M, 1-kbp DNA ladder; 1, *Sca*I-digested pBluescript SK+ DNA (the 2.9-kbp DNA band corresponds to the linearized pBluescript SK+ DNA); 2, *lacZα* RNA transcribed by T3 RNA polymerase (the band with mobility corresponding to the 600-bp DNA marker represents the full-length RNA transcript); 3, digestion with DNase (upon incubation with DNase the 2.9-kbp plasmid DNA band is no longer visible).

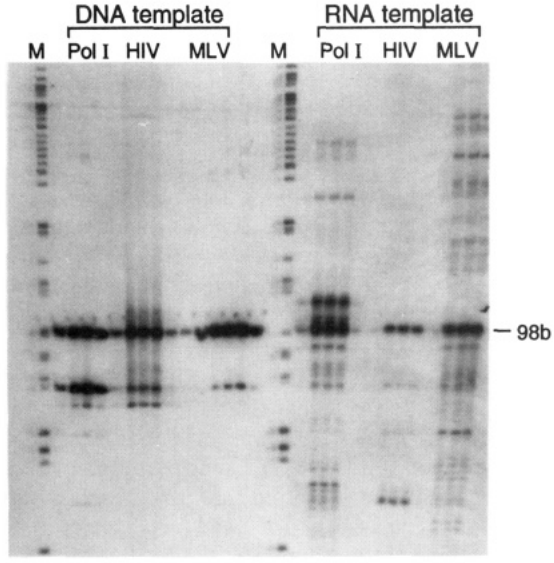

FIGURE 3: Isolation of extension products from polyacrylamide gels. Multiple aliquots of 5′-^{32}P-labeled oligonucleotide primer (5′-TTC GCT ATT ACG CCA GCT-3′) complementary to positions +142 to +159 from the M13mp2 *lacZα* transcriptional start site were annealed to either RNA or DNA templates and extended by HIV-1 RT, MLV RT, or the Klenow fragment of *E. coli* DNA polymerase I (Pol I). In the RNA reaction, RNA was degraded with RNase, and the newly synthesized DNA was annealed to the M13mp2 plus-strand DNA and digested with *Hae*III. In the DNA reaction, *Hae*III was added after the primer extension. The 98-base *Hae*III digestion fragment, corresponding to positions +64 to +162 on the *lacZα* gene, was identified by autoradiography after electrophoresis through 8% polyacrylamide gels. M represents the DNA sequences ladder used as a size marker.

The mutation frequencies resulting from reverse transcription or DNA polymerization are compared in Table I. Copying DNA, the most error-prone enzyme tested was HIV-1 RT, with an error rate of about 1 in 5900 nucleotides polymerized. This frequency is consistent with previous studies (Preston et al., 1988; Roberts et al., 1988; Takeuchi et al., 1988; Bebenek et al., 1989; Weber & Grosse, 1989). The error rate of HIV-1 RT copying RNA was about 1 in 6900 nucleotides, which is 4-fold greater than that observed with MLV

Table I: Mutation Frequency Induced by Different Polymerases

template	enzyme	total plaques	mutants	mutation frequency ($\times 10^{-4}$)	error rate[a]
DNA	Pol I	11703	14	12.0	1/29000
	MLV RT	10021	12	12.0	1/29000
	HIV RT	9508	45	47.3	1/5900
RNA	Pol I	9758	12	12.3	1/28000
	MLV RT	10888	11	10.1	1/37000
	HIV RT	34625	141	40.7	1/6900

[a] The error rate was calculated by subtracting the background mutant frequency of 3×10^{-4} from the observed frequency, divided by the target size of 80 bases, and the mutation detection factor of 0.35, which represents the fraction of possible nucleotide substitutions yielding detectable changes in *lacZα* gene expression. Pol I refers to the large or Klenow fragment of *E. coli* DNA polymerase I.

Table II: Single-Base Mutations Introduced by HIV-1 RT on RNA vs DNA Templates[a]

	mutation	mismatch	RNA template	DNA template
single-base substitutions	G → T	G·dATP	11	10
	C → T	C·dATP	15	4
	G → C	G·dGTP	5	2
	G → A	G·dTTP	0	13
	T → C	T·dGTP	1	12
	others		8	4
	total		40	45
single-base frame shift	deletion (%)		7	53

[a] The RNA template data are compiled from Figure 4, and the DNA template data are taken from Roberts et al. (1989).

RT or Pol I in the same experiments. Most of the errors observed with HIV-1 RT were not the result of transcription by T3 RNA polymerase, since in that case the same error rate would have been produced by MLV RT or Pol I.

The mutations induced by HIV-1 RT copying RNA template are depicted in Figure 4 and listed in Table II. These comprise three categories of mutations: single-base substitutions, −1 frame shifts, and multiple-base deletions. Forty of the forty-seven mutations sequenced were base substitutions; three were −1 frame shifts opposite four adjacent adenine residues; and four were multibase deletions, located near positions +84 and +113. Single-base substitutions were located at 21 different sites within the 80-base target. The overall base-substitution frequency of purines was the same as that of pyrimidines. The distribution of mutations is nonrandom; seven independent mutations were C → T transitions at position +129 and six were G → T transversions at position +89. The most frequent mutations were C → T transitions (15 of 40) and G → T transversions (11 of 40), which together constitute 65% of the base substitutions identified.

Discussion

We report here the fidelity of reverse transcription on a message RNA template. Previous attempts to measure the fidelity of RNA-dependent DNA polymerization have resorted to detection of the incorporation of radioactive deoxyribonucleotides that were noncomplementary to ribopolymer templates containing only one or two species of nucleotide residues (Battula & Loeb, 1974; Takeuchi et al., 1988). Due to limitation in the purity of labeled deoxyribonucleoside triphosphates and due to template-primer slippage with homopolymers during catalysis (Chang et al., 1972), it is difficult to equate the error rate on such systems with that which occurs on native RNA templates. Our M13 *lacZα* forward mutation

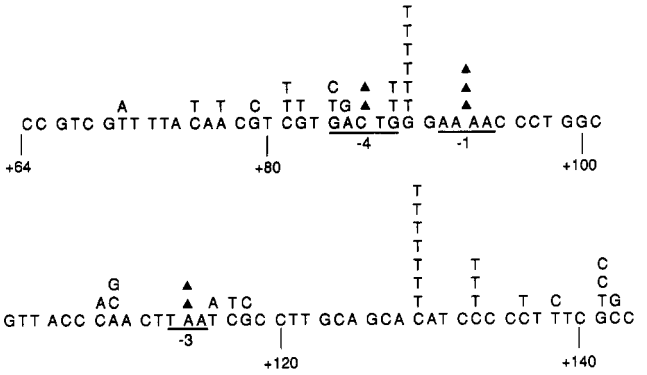

FIGURE 4: Spectrum of mutations induced by HIV-1 RT copying RNA. The 80-base target of the *lacZα* sense strand is shown. Numbering is from the *lacZα* transcriptional start site of M13mp2. Single-base substitutions are displayed above the wild-type sequence. Deletions are shown as ▲, a line below the sequence gives the presumed origin, and the number equals the deleted nucleotides.

assay avoids these problems and allows the detection of all types of base substitutions and frame-shift mutations. This assay provides a sensitive estimation for the fidelity of reverse transcriptase in copying either RNA or DNA.

We find that the error rate of HIV-1 RT copying RNA is comparable to the fidelity copying DNA, about 1 misincorporation in every 5000–7000 nucleotides polymerized. The high error rate of HIV-1 RT in vitro on both RNA and DNA templates suggests that it is at least partly responsible for the hypermutability of the AIDS virus. Although mismatches during viral plus-strand replication could be corrected by the host's mismatch repair enzymes, there is no known mechanism for correcting the mismatches on DNA–RNA hybrids. Misincorporations during viral minus-strand synthesis should be fixed immediately as a result of RNA degradation by the RNase H activity of HIV-1 RT.

Does the mutagenic specificity of HIV-1 RT copying the *lacZα* gene in vitro equate to the viral genome variation and evolution in vivo? Although the answer to this question is uncertain, one should keep in mind that our fidelity assay in vitro is influenced by selection for the expression of β-galactosidase; viral genomic evolution in vivo is mediated in part by enzymology, survivability, and infectivity. Recently, Vartanian et al. (1991) found that most mutations in the HIV-1 *env* gene after multiple passages on lymphocyte and monocyte cells are G → A transitions at GpA dinucleotides. Pathak and Temin (1990) also identified the G → A substitution as hypermutation in a single replication cycle for spleen necrosis virus (SNV) in vivo. So far, we find no evidence that this unusual specificity is a direct result of HIV-1 RT errors. The discrepancy between the in vitro findings with HIV-1 RT and the in vivo studies with viral sequence variation could be due to selective pressure for retroviral replication or the result of hot spots for G → A mutations within the retroviral genome.

Using the *lacZα* gene as a template in vitro, the error specificities of HIV-1 RT during reverse transcription and DNA replication are similar (see Table II). Both processes induced a high frequency of G → T transversions, 28% (11 of 40) during reverse transcription and 22% (10 of 45) during DNA replication, suggesting that G·dATP mismatches are frequently formed and extended regardless of whether the template contains a ribo- or deoxyriboguanosine residue. However, other aspects of the mutational specificities on the RNA template are different from those of the DNA template: (1) Frame-shift mutations were less frequently observed with RNA templates (7%) than with DNA templates (53%). (2) C → T transitions are the major misincorporations on RNA

templates while G → A transitions predominate on DNA templates. (3) T → C substitutions are less frequent on RNA than on DNA templates, suggesting that U·dGTP mismatches are less frequent and/or less easily extended than T·dGTP base pairing. The kinetics of mismatches by HIV-1 RT on DNA has been investigated (Preston et al., 1988; Perrino et al., 1989; Weber & Grosse, 1989), and these could be compared with those on homologous RNA templates. The overall mutagenic specificity of HIV-1 RT may reflect the distinct interactions of HIV-1 RT with specific bases on RNA versus DNA templates.

In summary, we measured the fidelity of HIV-1 RT in copying RNA templates and found that the HIV-1 RT is highly error-prone during reverse transcription. The high infidelity and nonrandom misincorporation introduced by HIV-1 RT in copying RNA template in vitro suggest that reverse transcriptase by human immunodeficiency virus 1 is a determinant for viral genome hypervariation.

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