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Low Fidelity of Cell-Free DNA Synthesis by Reverse Transcriptase of Human Immunodeficiency Virus

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The fidelity of DNA synthesis by reverse transcriptases from human immunodeficiency virus and other retroviruses was compared by measuring the rates of misincorporation of dCMP in the place of TMP in cell-free DNA synthesis with polyadenylic acid as the template. The fidelity of human immunodeficiency virus reverse transcriptase was found to be about one-third of that of the reverse transcriptases of other retroviruses.

The genetic variability of human immunodeficiency virus (HIV) has been well established (1, 3, 7, 15). This property of HIV might affect viral pathogenesis and enable the virus to escape from host immune defenses. The rate of evolution of HIV is reported to be more than a million times that of most DNA genomes and possibly 10 times that of some other RNA viruses, including certain oncoviruses (5, 7, 8). However, the molecular mechanisms of the rapid changes of HIV genomes are unknown. Polymorphisms in nucleic acid sequences have been found to be widely distributed over the whole HIV genome, although variation in amino acid sequences is much higher in the envelope region than in the remainder of the genome (1, 3, 7). Some processes of genomic replication may participate in hypervariability of HIV genome. In this study, we examined the possibility that the reverse transcriptase (RT) of HIV replicates the genome with low accuracy by comparing the fidelities of DNA synthesis by RTs of HIV and other retroviruses by a misincorporation assay (12).

The purification of RT from two independent isolates of HIV, HTLV-III_B (13, 14) and HIV[GUN-1] (14), were described by Chandra et al. (4). Briefly, the enzymes were purified from the culture fluids of HTLV-III_B-infected MOLT-4 cells and HIV[GUN-1]-infected U-937 cells by column chromatography on DEAE-cellulose and phosphocellulose (4). The RT of avian myeloblastosis virus (AMV) and cloned RT from Moloney murine leukemia virus (Mo-MuLV) were obtained from Pharmacia and purified by the FPLC system. RT of Rous-associated virus-2 (RAV-2) was from Takara and was also purified by high-performance liquid chromatography. With these similar levels of purity, we assume that the polymerase activities analyzed reflect the RT enzymes and not contaminating polymerases.

The reaction mixture for the misincorporation assay contained RT, polyadenylic acid [poly(A)] as a template, oligo(dT) as a primer, [^{3}H]TTP as a correct substrate, and [α-^{32}P]dCTP as an incorrect substrate. The specific radioactivity of [α-^{32}P]dCTP was about 2,000 times that of [^{3}H]TTP to allow detection and quantitation of minute amounts of incorrect nucleotide incorporated. A sample (20 μl) of enzyme solution (50 mM Tris hydrochloride [pH 7.5], 1 mM dithiothreitol, 0.02% Triton X-100, 10% glycerol, ca. 20 U of RT per ml [1 U of RT catalyzes the incorporation of 1 nmol of TMP into acid-insoluble product in 10 min at 37°C with poly(A)-oligo(dT) as the template-primer]) was mixed with 80 μl of a solution containing 250 μg of bovine serum albumin per ml, 40 mM Tris hydrochloride (pH 7.8), 4 mM dithiothreitol, 48 mM KCl, 200 mM poly(A), 10 mM oligo$(dT)_{12-18}$, 10 mM $MgCl_2$ 31.3 μM TTP, 31.3 μM dCTP, ca. 3×10^9 cpm of [^{32}P]dCTP per ml, and 1.5×10^6 cpm of [^{3}H]TTP per ml. After incubation for 1 h at 37°C, the mixture was extracted with phenol and then chloroform, and nucleic acid was precipitated with ethanol and 1 μg of carrier tRNA. Free mononucleotides were removed by four precipitations with ethanol in 2 M ammonium acetate–0.1% sodium dodecyl sulfate–20 mM EDTA. After the purification, high-molecular-weight nucleic acid was analyzed. The reaction conditions used in these studies gave better than 80% of the maximal activity as determined by comparison with optimal conditions for each RT.

Analysis by alkaline agarose gel electrophoresis showed that most of the ^{32}P-labeled products were more than 150 nucleotides long (data not shown). The products were degraded to 3′-mononucleotides by digestion with micrococcal nuclease and then spleen phosphodiesterase (9) or to 5′-mononucleotides by digestion with nuclease P_1. The undigested products and the digests were separated by two-dimensional thin-layer chromatography (2D-TLC). 2D-TLC was carried out on avicel SF cellulose plates (Funakoshi) in isobutyric acid–0.5 M NH_4OH (7:3) in the first dimension and in isobutyl alcohol–HCl–H_2O (75:12.5:12.5) in the second dimension. Then the plates were autoradiographed. The results obtained with the RTs of AMV and HTLV-III_B are shown in Fig. 1. A TLC autoradiogram of the undigested product (Fig. 1, panels a and d) showed no evidence of contamination with free nucleotides. If 5′-[^{32}P]dCMP were misincorporated into newly synthesized polynucleotides, which should be complementary to poly(A), decomposition of the polynucleotides to 3′-nucleotides and 5′-nucleotides should give 3′-[^{32}P]TMP and 5′-[^{32}P]dCMP, respectively. A single spot corresponding to 3′-TMP was observed on the 2D-TLC chromatogram of 3′-nucleotides (Fig. 1, panels b and e). In each case, the spot of 3′-nucleotides was located slightly to the right and above the spot of the corresponding 5′-nucleotide (data not shown). On 2D-TLC chromatograms of 5′-nucleotides, 5′-dCMP was detected as expected, and unexpectedly, two radioactive spots that corresponded to 5′-TMP and 5′-dUMP were also seen (Fig. 1, panels c and f). For the following two reasons, we concluded that the 5′-[^{32}P]TMP and 5′-[^{32}P]dUMP incorporated into polynucleotide synthesized by RTs were derived from [α-^{32}P]TTP and [α-^{32}P]dUTP, respectively, present in commercial [α-

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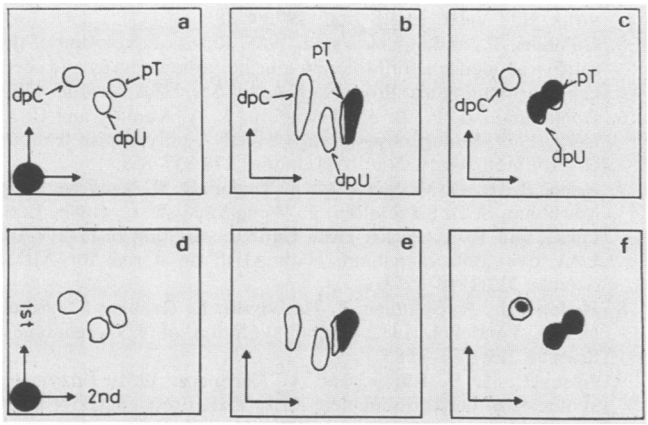

FIG. 1. 2D-TLC analysis of products of misincorporation by RTs of AMV and HTLV-III$_B$. Portions of the purified nucleic acid with authentic markers (5'-dCMP, 5'-TMP, and 5'-dUMP) were subjected to 2D-TLC before digestion (a and d), after digestion with micrococcal nuclease and then spleen phosphodiesterase (b and e) (9), or after digestion with nuclease P_1 (c and f). The spots of 5'-dCMP, 5'-TMP and 5'-dUMP were located under a UV lamp and are indicated as dpC, pT, and dpU, respectively. Results for the RTs of AMV (a–c) and HTLV-III$_B$ (d–f) are shown.

^{32}P]dCTP, although in 10^3 to 10^5 times smaller amounts than [α-^{32}P]dCTP. First, when [α-^{32}P]dCTP was purified by TLC on a cellulose thin-layer sheet (Merck) in isobutyric acid–0.5 M NH_4OH (5:3) before use in the misincorporation assay for RAV-2 RT, no spot of 5'-[^{32}P]TMP was detected and the spot of 5'-[^{32}P]dUMP was very faint in the nuclease P_1 digest of the product (data not shown). The 5'-[^{32}P]dUMP incorporated might be derived from [α-^{32}P]dUTP formed by deamination of [α-^{32}P]dCTP in acidic conditions during purification by TLC, because much more 5'-[^{32}P]dUMP was incorporated when [α-^{32}P]dCTP that had been exposed to the solvent for a long time was used (data not shown). Second, the incorporation rates of 5'-[^{32}P]TMP and 5'-[^{32}P]dUMP but not 5'-[^{32}P]dCMP varied markedly when different batches of [α-^{32}P]dCTP were used (data not shown). Thus, for accurate quantitation of misincorporation, we extracted the spots corresponding to 5'-dCMP and 5'-TMP from the TLC plates of 5'-nucleotides (Fig. 1, panels c and f), counted the radioactivities, and calculated the error rates. Nucleotides were extracted from the spots corresponding to 5'-dCMP and 5'-TMP with 50 μl of H_2O, and their counts of ^{32}P and ^{3}H, respectively, were counted in 5 ml of aquasol-2 (NEN). Then the error rate was calculated as follows: error rate = dCMP incorporated/TMP incorporated = (incorporated count of 5'-[^{32}P]dCMP/total counts of [^{32}P]dCTP in reaction mixture) × (total count of [^{3}H]TTP in reaction mixture/incorporated count of 5'-[^{3}H]TMP). Experiments were repeated three times with different batches of [α-^{32}P]dCTP (Table 1).

In another experiment, [α-^{32}P]TTP and [^{3}H]dCTP were used as correct and incorrect substrates, respectively, and the RTs of RAV-2 and HTLV-III$_B$ were used (Table 1, expt 4). In this experiment, the incorporation of 5'-[^{3}H]TMP and 5'-[^{3}H]dUMP was also observed (data not shown). The error rates of the RTs of RAV-2, AMV, and Mo-MuLV ranged from 1 in 60,000 to 1 in 152,000. On the other hand, the error rates of HIV RTs ranged from 1 in 17,400 to 1 in 38,300. These reproducible results indicated that the infidelity of HIV RTs was 2.5 to 5 times that of the other RTs.

The error rate of AMV RT was reported to be 1 in 700 when poly(A) was used as the template in misincorporation assays (2, 12) and 1 in 329 to 1 in 17,000 when φX174*am*3 DNA was used as a natural template in revertant assays (6, 10–12). The difference in these values for the error rate was probably due to the difference in the methods used. The sensitivity of the assay with the homopolynucleotide template may be affected by the purity of the deoxynucleotide substrate and the template. We detected contaminating radioactive TTP and dUTP in our dCTP preparation. Similar contamination probably affected the error rates determined in previous studies with poly(A) as the template, because the total counts incorporated into acid-insoluble products were considered to be the misincorporated counts (2), and so the error rates may have been overestimated. The error rates determined in our experiments might still have included a background error due to possible contamination of the poly(A) template with guanine or hypoxanthine residues. If so, the apparent error rates of RTs would have been over-

TABLE 1. Fidelity of DNA synthesis by RTs of RAV-2, AMV, Mo-MuLV, HTLV-III$_B$, and HIV[GUN-1]

RT	Expt	Incorporation of TMP (pmol)	Incorporation of dCMP (fmol)	Error rate	Relative infidelity[a]	Mean relative infidelity for group ± SD[b]
RAV-2	1	588	3.88	1/152,000	1	
	2	418	3.45	1/121,000	1	
	3	298	2.90	1/103,000	1	
	4[c]	375	5.18	1/72,400	1	
AMV	1	543	5.80	1/93,500	1.63	1.57 ± 0.18
	2	185	2.10	1/88,100	1.37	
	3	330	5.53	1/60,000	1.72	
Mo-MuLV	1	450	4.03	1/112,000	1.36	1.59 ± 0.27
	2	885	13.8	1/64,100	1.89	
	3	523	7.68	1/68,100	1.51	
HTLV-III$_B$	1	583	17.9	1/32,600	4.66	5.01 ± 0.31
	2	435	18.9	1/23,000	5.26	
	3	153	7.57	1/20,200	5.10	
	4[c]	79.3	4.55	1/17,400	4.16	
HIV[GUN-1]	1	27.8	0.725	1/38,300	3.97	3.98 ± 0.08
	2	41.8	1.39	1/30,000	4.03	
	3	31.5	1.21	1/26,100	3.95	

[a] Relative infidelity was calculated as error rate for each RT/error rate for RAV-2 RT.
[b] Values are means ± standard deviations for one to three experiments for each RT.
[c] [^{3}H]dCTP (7.1×10^8 cpm/ml) and [α-^{32}P]TTP (1.1×10^6 cpm/ml) were used instead of [α-^{32}P]dCTP and [^{3}H]TTP, respectively.

estimated. Therefore, the error rates determined so far, including ours, might all have involved background errors. If these backgrounds could be subtracted, the fidelities of the RTs of Mo-MuLV, AMV, RAV-2, and HIVs would be higher than observed in our experiments and reverse transcription by HIV RT would be relatively much more error prone than reverse transcription by other RTs.

To our knowledge, this is the first report of low fidelity of HIV RTs. The lower accuracy of reverse transcription of HIV than of other retroviruses might be responsible for the hypervariation of the HIV genome. Studies are required on the degree of fidelity of HIV RT in DNA synthesis with other homopolynucleotides or heteropolynucleotides as templates. Other processes in the HIV life cycle, such as synthesis of genomic RNA by RNA polymerase in the presence of transactivator proteins or other factors, may also participate in the hypervariability of the genome. We are now studying the fidelity of reverse transcription with heteropolynucleotides and the accuracy of genomic RNA synthesis.

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LITERATURE CITED

1. **Alizon, M., S. Wain-Hobson, L. Montagnier, and P. Sonigo.** 1986. Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients. Cell **46**:63–74.
2. **Battula, N., and L. A. Loeb.** 1974. The infidelity of avian myeloblastosis virus deoxyribonucleic acid polymerase in polynucleotide replication. J. Biol. Chem. **249**:4086–4093.
3. **Benn, S., R. Rutledge, T. Folks, J. Gold, L. Baker, J. McCormick, P. Feorino, P. Piot, T. Quinn, and M. Martin.** 1985. Genomic heterogeneity of AIDS retroviral isolates from North America and Zaire. Science **230**:949–951.
4. **Chandra, A., T. Gerber, and P. Chandra.** 1986. Biochemical heterogeneity of reverse transcriptase purified from the AIDS virus, HTLV-III. FEBS Lett. **197**:84–88.
5. **Gojobori, T., and S. Yokoyama.** 1985. Rates of evolution of the retroviral oncogene of Moloney murine sarcoma virus and of its cellular homologues. Proc. Natl. Acad. Sci. USA **82**:4198–4201.
6. **Gopinathan, K. P., L. A. Weymouth, T. A. Kunkel, and L. A. Loeb.** 1979. Mutagenesis *in vitro* by DNA polymerase from an RNA tumour virus. Nature (London) **278**:857–859.
7. **Hahn, B. H., G. M. Shaw, M. E. Taylor, R. R. Redfield, P. D. Markham, S. Z. Salahuddin, F. Wong-Staal, R. C. Gallo, E. S. Parks, and W. P. Parks.** 1986. Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS. Science **232**:1548–1553.
8. **Holland, J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. VandePol.** 1982. Rapid evolution of RNA genomes. Science **215**:1577–1585.
9. **Josse, J., A. D. Kaiser, and A. Kornberg.** 1961. Enzymatic synthesis of deoxyribonucleic acid. VIII. Frequencies of nearest neighbor base sequences in deoxyribonucleic acid. J. Biol. Chem. **236**:864–875.
10. **Kunkel, T. A., F. Eckstein, A. S. Mildvan, R. M. Koplitz, and L. A. Loeb.** 1981. Deoxynucleoside [1-thio]tri-phosphates prevent proofreading during *in vitro* DNA synthesis. Proc. Natl. Acad. Sci. USA **78**:6734–6738.
11. **Kunkel, T. A., R. M. Schaaper, R. A. Beckman, and L. A. Loeb.** 1981. On the fidelity of DNA replication. Effect of the next nucleotide on proofreading. J. Biol. Chem. **256**:9883–9889.
12. **Loeb, L. A., and T. A. Kunkel.** 1982. Fidelity of DNA synthesis. Annu. Rev. Biochem. **52**:429–457.
13. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224**:497–500.
14. **Takeuchi, Y., M. Inagaki, N. Kobayashi, and H. Hoshino.** 1987. Isolation of human immunodeficiency virus from a Japanese hemophilia B patient with AIDS. Jpn. J. Cancer Res. (Gann) **78**: 11–15.
15. **Weiss, R.** 1985. Human T-cell retroviruses, p. 405–485. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses: molecular biology of tumor viruses, 2nd ed., 2 supplements. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.