Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8343–8347, November 1989
Biochemistry

Extension of mismatched 3′ termini of DNA is a major determinant of the infidelity of human immunodeficiency virus type 1 reverse transcriptase

(AIDS/mispair extension/fidelity/mutagenesis/DNA polymerase α)

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Communicated by Aaron J. Shatkin, August 7, 1989 (received for review July 10, 1989)

ABSTRACT **The unusually high error rate of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) suggests that polymerization errors by this enzyme contribute to the genetic variability of the AIDS virus. We have analyzed the mechanism for HIV-1 RT infidelity by studying two distinct steps that might lead to base substitution mutations: nucleotide misinsertions and elongation from 3′-terminal DNA mispairs. Our results indicate that the capacity of HIV-1 RT to polymerize nucleotides onto mispaired termini is a major factor in the production of mutations by this enzyme. When a noncomplementary dAMP was inserted opposite a template adenine by HIV-1 RT, the nascent 3′-terminal A·A mispair was readily extended by subsequent incorporation of the next complementary nucleotide. The frequencies of nucleotide addition onto 3′-terminal A·A, A·C, and A·G mispairs were determined by quantitating the amount of extended primers with a gel electrophoresis assay and by measuring mutagenesis after hybridization of mismatched primers opposite an amber mutation in bacteriophage φX174 DNA. The mispair extension frequencies are ≈50-fold higher by HIV-1 RT than by the mammalian replicative enzyme DNA polymerase α.**

The genetic heterogeneity of human immunodeficiency virus type 1 (HIV-1) may be important in the pathogenesis of AIDS and in its resistance to therapy. This genetic variability is documented by differences in restriction enzyme cleavage patterns and in nucleotide sequences among isolates from different individuals and among clones obtained from the same individual (1–3). The rate of evolution of HIV-1 has been estimated to be a million times greater than that of eukaryotic genomes (2). Studies on purified HIV-1 reverse transcriptase (RT) have revealed an unusually high error rate in copying DNA or RNA templates, equal to or greater than those of RTs from other retroviruses (4–7). The frequency of base-substitution errors during DNA polymerization *in vitro* is ≈1/5000 and adequate to account for the high mutation rate of the virus.

The mechanisms responsible for incorporation of noncomplementary nucleotides by HIV-1 RT are unknown. Like other retroviral polymerases, HIV-1 RT has no 3′→5′ exonuclease to remove misincorporated nucleotides (5, 8). Thus, single base-substitution errors could occur during DNA polymerization by a simple two-step mechanism. (*i*) HIV-1 RT would misinsert a noncomplementary base into the nascent DNA by catalyzing phosphodiester linkage to the 3′ terminus. (*ii*) HIV-1 RT would append complementary nucleotides to this 3′-terminal mismatch to stably incorporate the mismatched nucleotide into the nascent DNA. This mechanism of base-substitution mutagenesis requires both a high frequency of nucleotide misinsertion and efficient mispair extension by HIV-1 RT.

In this work we show that efficient extension from 3′-terminal mispairs is a major determinant of the infidelity of HIV-1 RT. Using a gel electrophoresis assay, we show that a nucleotide misinserted by HIV-1 RT is efficiently extended by addition of the next correct nucleotide. In a kinetic assay (9) and in the φX174 *am3* reversion assay (10), we quantitate elongation from A·A, A·C, and A·G mispairs to determine the contribution of mispair extension to mutagenesis. The high efficiency of mispair extension by HIV-1 RT contrasts sharply with the low frequency of mispair extension displayed by DNA polymerase α (DNA Pol-α).

MATERIALS AND METHODS

Primed DNA Templates. Single-stranded φX174 *am3* DNA was primed at a 2:1 molar ratio (primer-to-template) with a 16-base oligonucleotide, the 3′ terminus of which hybridized to position 587 (*am3*) (11). The 30-base oligonucleotide template, identical to nucleotides (nt) 581–610 of φX174 *am3* DNA, was primed at a 2:1 molar ratio with a 15-base oligonucleotide to produce a correctly paired 3′ terminus at position 588. Primers were labeled with ^{32}P at the 5′ position and hybridized as described (12, 13) (see Fig. 1*A*). For designation of base pairs, the first base refers to the template base.

Enzymes and Reactions. Cloned HIV-1 RT was a gift of S. H. Wilson (National Institutes of Health). DNA Pol-α was immunoaffinity-purified from calf thymus as the polymerase–primase complex, as described (14), and was devoid of detectible exonuclease or endonuclease activity. The DNA polymerization reactions contained 20 mM Tris·HCl (pH 7.5), 2 mM dithiothreitol, 10 mM $MgCl_2$, bovine serum albumin at 0.1 mg/ml, primed DNA template at 2.2 nM of 3′ termini, and dNTPs as indicated. For kinetic analysis (Fig. 2; Table 1), HIV-1 RT or DNA Pol-α activity was 0.011 unit/μl; one unit catalyzes the incorporation of 1 pmol of [α-^{32}P]dTMP into 0.022 pmol of 15-mer-primed φX174 *am3* DNA in 30 min at 30°C with all four dNTPs at 50 μM. For φX174 *am3* reversion analysis and gel analysis of A·A mispair formation and extension (Fig. 1; Table 2), the concentration of polymerase was 0.11 unit/μl. For gel analysis, reactions were eluted through 0.5-ml Sephadex G-100 columns to remove unincorporated deoxynucleoside triphosphates (15), fractionated on 15% polyacrylamide sequencing gels, visualized by autoradiography, and quantified by densitometric scanning.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; DNA Pol-α, DNA polymerase α; nt, nucleotide(s).

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ϕX174 *am3* Reversion Analysis. Reaction products (0.055 pmol of copied ϕX174 *am3* DNA) were transfected into *Escherichia coli* KT-1 spheroplasts, and reversion frequencies were determined by plating on *E. coli* HF4714 (Sup^+) and HF4704 (Sup^-) to determine total and revertant progeny phage, respectively (10, 16).

RESULTS

Formation of and Extension from an A·A Mispair by HIV-1 RT. Base substitution mutagenesis by a DNA polymerase may require both incorporation of a noncomplementary nucleotide and subsequent extension. Catalysis of these two steps by HIV-1 RT was studied by measuring elongation of a primer in a DNA polymerization reaction containing only a single deoxynucleoside triphosphate that is noncomplementary to the first template nucleotide but complementary to the second (Fig. 1). A 15-mer oligonucleotide primer was hybridized to a 30-mer oligonucleotide template to produce a DNA duplex that contained a correctly paired 3′ primer terminus located 1 nt from the template target site (template adenine). This template 30-mer has the same sequence as nt 581–610 of the positive strand of bacteriophage ϕX174 *am3* DNA. The sequence at the target site is identical to the sequence at the amber3 codon; the template adenine corresponds to the adenine in the middle position of the amber codon, position 587 of the ϕX174 genome (Fig. 1*A*). An A·A mispair is produced by HIV-1 RT upon incubation of the primer–template in the presence of dATP as the only deoxynucleoside triphosphate. Formation of the A·A mispair is indicated by elongation of up to 30% of the 15-mer primer to oligonucleotide products of 16 or more nucleotides (Fig. 1*B*). When the same reaction was done with DNA Pol-α, up to 40% of the 15-mer primer was elongated (Fig. 1*C*). These results suggest that the A·A mispair can be produced with similar frequencies by HIV-1 RT and DNA Pol-α.

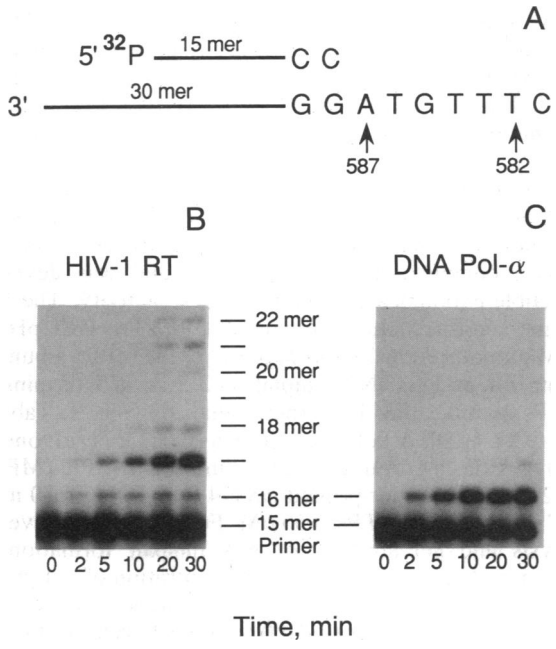

FIG. 1. Formation of and extension from an A·A mispair. Two DNA polymerization reactions (70 μl) were prepared, as described, each containing the primed oligonucleotide template (*A*) and 1 mM dATP. Equal amounts (final reaction concentration, 0.11 unit/μl) of either HIV-1 RT (*B*) or DNA Pol-α (*C*) activity were added, and the reaction was incubated at 30°C. Samples (10 μl) were removed before polymerase addition (t_0) and at the indicated times. The positions of the primer and extended oligonucleotides are indicated.

Extension from the A·A mispair by HIV-1 RT is indicated by the ladder of radioactive bands in Fig. 1*B* corresponding to oligomers >16 nt. The next template nucleotide after the target adenine is a thymine. Primers extended to 17-mer could result if misinsertion of dAMP opposite adenine at position 587 was followed by correct insertion of a second dAMP opposite thymine at position 586. Greater than 95% of the primers extended by HIV-1 RT after 30 min were detected at or beyond the 17-mer position, presumably reflecting extension from the A·A mispair by addition of a second dAMP opposite the template thymine at position 586 (Fig. 1*B*). This result contrasts with that seen with DNA Pol-α; <5% of the primers containing a misinserted adenine were further extended (Fig. 1*C*). To confirm that elongation of the primer by HIV-1 RT was due to addition of dAMP and not to addition of a contaminant nucleotide, the 17-mer oligonucleotide was isolated from the polyacrylamide gel and sequenced using the Maxam and Gilbert procedure (17). Base-specific chemical cleavage of the 17-mer oligonucleotide showed that at least 90% of the 16-mer oligonucleotide was produced from dAMP addition (data not shown).

The ability of HIV-1 RT to both misinsert dAMP and to extend mispairs is further indicated by detection of oligonucleotide products corresponding to 18-mer, 19-mer, 20-mer, 21-mer, and 22-mer (Fig. 1*B*, 10–30 min). The 18-mer product presumably results from a second misinsertion of dAMP opposite the template guanine (position 585). Extension of the G·A mispair by correct dAMP insertion results in production of the 19-mer, 20-mer, and 21-mer. Finally, the 22-mer would result from another dAMP misinsertion opposite template cytosine at position 581. In contrast, DNA Pol-α extended <5% of the mismatched primers, and multiple misinsertions were not detected (Fig. 1*C*, 10–30 min).

Kinetic Analysis of Mispair Extension. To measure the efficiency of mispair extension by HIV-1 RT, we prepared a series of templates with 16-mer primers that formed 3′-terminal A·A, A·C, or A·G mismatches at the amber3 site (position 587) of ϕX174 *am3* DNA. Extension from preformed mispairs by addition of the next complementary nucleotide was detected by an increase in the length of the oligonucleotide primer to 17 nt or greater. The ability of HIV-1 RT to extend each of the three mispairs under steady-state kinetic conditions is illustrated in Fig. 2*A*. Extension from the A·A and A·G purine–purine mispairs is indicated by the addition of a single nucleotide onto the 16-mer primer to produce the 17-mer oligonucleotide. Extension from the A·C purine–pyrimidine mispair is shown by elongation of the 16-mer primer to produce oligonucleotide products from 17 to 21 nt in length. The 18-mer product presumably resulted from misinsertion of dAMP opposite the template guanine at position 585 (refer to Fig. 1*A* for the template sequence). Subsequent extension from the G·A mispair by HIV-1 RT involves incorporation of complementary dAMPs opposite template thymines at positions 584–582, resulting in 19-mer, 20-mer, and 21-mer products. Thus, the A·C mispair is extended more efficiently than the A·A or A·G mispairs—i.e., a greater proportion of the mispaired primer is extended, and the products are longer. Extension from all three mispairs showed typical saturation kinetics.

Mispair extension by HIV-1 RT from the amber3 site of ϕX174 *am3* DNA is predominantly governed by the K_m for addition of the next correct nucleotide (Table 1). The apparent K_m values for nucleotide addition onto the A·A, A·C, and A·G mispairs were 1000- to 3000-fold greater than for nucleotide addition onto the correctly paired A·T terminus. Differences in the apparent V_{max} values for extension from correct and incorrect primer termini are not as large. Apparent V_{max} values for extension from the A·A and A·G purine–purine mispairs were 6- and 15-fold lower than from the correctly paired A·T terminus. There was only a 2-fold

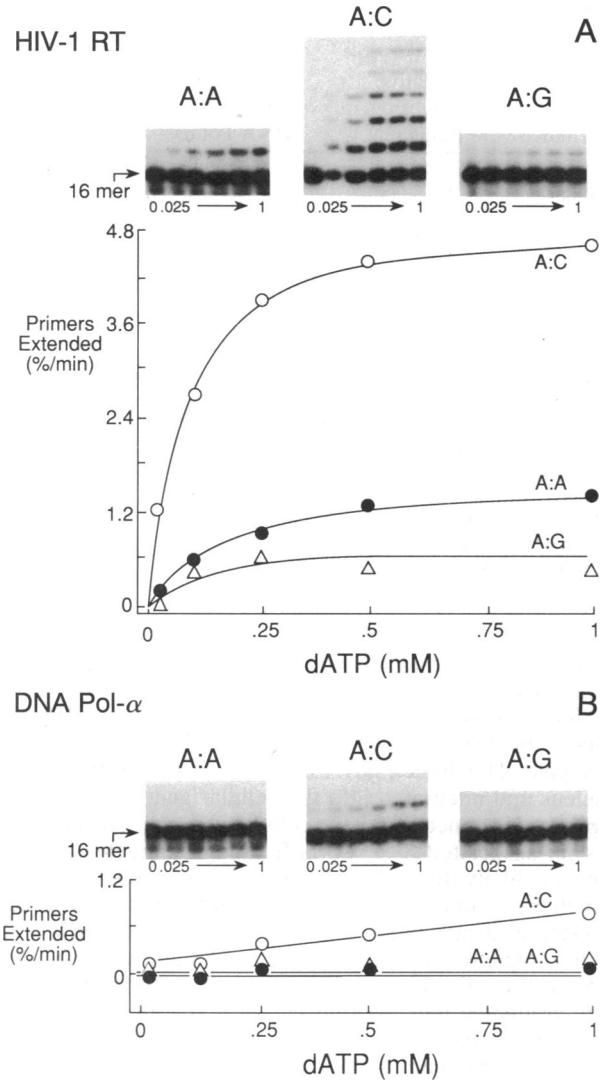

FIG. 2. Kinetics of mispair extension by HIV-1 RT and DNA Pol-α. Oligonucleotide primers were hybridized to φX174 *am3* DNA (0.022 pmol) to produce the indicated 3′-terminal mispairs at the amber3 site (position 587) and extended (10 min, 30°C) with equal activities of either HIV-1 RT (*A*) or DNA Pol-α (*B*) (final reaction concentration, 0.011 unit/μl). Each reaction contained 0, 0.025, 0.1, 0.25, 0.5, or 1.0 mM dATP as the only deoxynucleoside triphosphate substrate. PAGE analysis of the products synthesized from primers with 3′-terminal A:A (●), A:C (○), and A:G (△) mispairs are shown above at left, center, and right, respectively (*A* and *B*).

difference between the apparent V_{max} values for extension from the A·C purine–pyrimidine mispair and a complementary A·T terminus. Estimates of substrate efficiencies [i.e., V_{max}/K_m (19)] indicate that the A·C mispair was extended ≈8-fold more readily than the A·A mispair and ≈16-fold more readily than the A·G mispair (Table 1).

The kinetics of mispair extension by HIV-1 RT was contrasted to mispair extension by DNA Pol-α. To compare HIV-1 RT to DNA Pol-α we used the same amount of enzyme activity as measured on φX174 *am3* DNA at the amber3 site by the incorporation of dNMP using a correctly paired 3′ terminus (see *Materials and Methods*). Both HIV-1 RT and DNA Pol-α yielded similar K_m and V_{max} values for site-specific extension from the correctly paired A·T terminus. However, HIV-1 RT extended the A·A, A·C, and A·G mispairs much more efficiently than DNA Pol-α (Fig. 2). In fact, mispair extension was detected from all three mispairs by HIV-1 RT but only from the A·C mispair by DNA Pol-α, as indicated by elongation of the 16-mer primer to 17 nt (Fig. 2*B*). The relative extension frequency from an A·C mispair was ≈25-fold greater by HIV-1 RT than by DNA Pol-α (Table 1).

To compare the extension frequencies from A·A and A·G mispairs by HIV-1 RT and DNA Pol-α, the amounts of enzyme activity were increased 5-fold, and reaction times were increased from 10 to 60 min (Fig. 3). Extension from the A·A mispair by HIV-1 RT approached 100% at a concentration of dATP >0.025 mM. Extension from the A·A mispair by HIV-1 RT was greater than from the A·G mispair; both purine–purine mispairs were extended more efficiently by HIV-1 RT than by DNA Pol-α. The relative extension frequencies by HIV-1 RT from the A·A and A·G mispairs were >26- and >55-fold the frequencies by DNA Pol-α (Table 1).

Formation of a second mispair (G·A) at position 585 by HIV-1 RT was clearly influenced by the nature of the mispair at position 587. This was apparent by comparing the oligomers produced by HIV-1 RT during elongation from the A·A and A·G mispairs in Fig. 3 to that from the A·C mispair in Fig. 2*A*. Oligonucleotide products 18 nt in length and greater indicated that HIV-1 RT misinserted dAMP opposite the template guanine at position 585 more frequently when the purine–pyrimidine A·C mispair was present at position 587 than when either of the purine–purine mispairs were present. Thus, the nature of the mispair 2 nt upstream at position 587 influenced further misinsertion by HIV-1 RT at position 585.

Increased Mutagenesis by Mispair Extension. To quantitate the effect of mispair extension on mutagenesis, we studied mispair extension by HIV-1 RT using the φX174 *am3* reversion assay. The A·A, A·C, and A·G 3′ terminal mispairs and the A·T correctly paired 3′ terminus were produced at position 587 of the amber3 codon by hybridizing oligonucleotides to φX174 *am3* DNA (11). The primer–templates were copied *in vitro* with HIV-1 RT, and extension from mispairs was determined from the frequency of reversion of φX174 *am3* DNA to wild type upon transfection into spheroplasts. In this assay, all base substitutions at position 587 yield wild-type phage (10). Because unextended mispairs are efficiently hydrolyzed by endogenous *E. coli* exonucleases before elongation by endogenous DNA polymerases, the reversion frequency is indicative of the efficiency of mispair extension during the *in vitro* reaction (11). Reversion frequencies from 80- to 4400-fold above background were obtained with HIV-1 RT, indicating that all three mispairs were efficiently extended to produce base substitution mutations at the amber3 codon (Table 2).

Extension of mispairs depends on the concentration of nucleotide substrates in the reaction. That HIV-1 RT extended mispairs more efficiently than DNA Pol-α was apparent whether deoxynucleoside triphosphates were 10 or 1000 μM. This difference was particularly evident at 10 μM dNTPs; for HIV-1 RT, the reversion frequencies for the A·A, A·C, and A·G mispairs were 150-, 1700-, and 72-fold above background, respectively. For DNA Pol-α, reversion frequencies were, at most, 4-fold above background. Reversion frequencies for all three mispairs were increased for both HIV-1 RT and DNA Pol-α when dNTPs were increased to 1000 μM. However, the reversion frequencies indicated that extension from A·A mispair was 41-fold greater and extension from the A·G mispair was 93-fold greater for HIV-1 RT than for DNA Pol-α. As with the kinetic analysis, the reversion analysis shows the frequency of mispair extension by HIV-1 RT and DNA Pol-α to be A·C >> A·A > A·G.

DISCUSSION

Recent studies have shown that HIV-1 RT produces single-base substitution errors *in vitro* at high frequency in both

Table 1. Kinetics of mispair extension by HIV-1 RT

Primer–template	K_m, μM	V_{max}, %/min	Relative extension frequency	Ratio of extensions (HIV-1 RT/ DNA Pol-α)
____/A ____A____	190 ± 24	1.6 ± 0.064	1/18,000	>55*
____/C ____A____	77 ± 8.7	4.8 ± 0.13	1/2400	≈25
____/G ____A____	160 ± 80	0.66 ± 0.099	1/38,000	>26*
____T ____A____	0.068 ± 0.0084	9.9 ± 0.42	1	1

φX174 *am3* DNA was primed with 16-base oligonucleotide primers to generate the indicated 3′ termini. Extension reactions (10 min, 30°C) contained 0, 0.022 pmol of primer–template; 0, 0.025, 0.1, 0.25, 0.5, or 1.0 mM of dATP; and HIV-1 RT or DNA Pol-α (see Fig. 2). Percent of 16-mer extended by at least 1 nt was quantitated as described. The apparent K_m and V_{max} values ± SE for HIV-1 RT were determined by the method of Wilkinson (18). Relative extension frequencies for HIV-1 RT are ratios of the rate constant (V_{max}/K_m) for the mispair divided by the corresponding rate constant for the paired A·T terminus. Relative extension frequencies for DNA Pol-α were $<1/10^6$ for A·A, ≈1/60,000 for A·C, and $<1/10^6$ for A·G. Extension from A·T by DNA Pol-α yielded a K_m of 0.25 ± 0.042 μM and V_{max} of 9.5 ± 0.42%/min.
*Estimated from the data in Figs. 2 and 3.

reversion and forward mutation assays (4–6). In φX174 *am3* and *am16* reversion assays, HIV-1 RT is ≈100-fold more error-prone than the mammalian replicative DNA Pol-α (4, 6, 20). These genetic assays detect *in vitro* DNA synthesis errors only when mismatched nucleotides are stably integrated into duplex DNA at the amber codon of the relevant target genes. Mismatched nucleotides might be produced either directly by insertion of a noncomplementary nucleotide or by a transient primer–template slippage mechanism (21, 22). In either case, mispaired 3′ termini must be extended to yield mutations. The experiments reported here indicate that the rates of misinsertion of dAMP at the amber3 site are not dramatically different for HIV-1 RT and DNA Pol-α. However, HIV-1 RT extends mispaired termini with at least a 50-fold greater efficiency than does DNA Pol-α. These data show that the higher error rate of HIV-1 RT can be attributed to efficient extension of mispaired nucleotides.

The biological role of HIV-1 RT may require that mispairs be extended efficiently. Conversion of the retroviral single-stranded RNA genome to linear duplex DNA occurs in the cytoplasm (23). The HIV-1 RT first polymerizes deoxynucleoside triphosphates directed by the RNA genome as template to produce an RNA·DNA hybrid. Upon removal of the RNA strand by the inherent RNase H activity, HIV-1 RT copies the DNA strand to produce a linear duplex DNA molecule. The duplex DNA is then transported into the cell nucleus and integrated into the cellular genome, where it is replicated in concert with the cellular genome. The HIV-1 RT has no associated 3′→5′ proofreading exonuclease, and it seems unlikely that cellular proofreading exonucleases function in the cytoplasm with the HIV-1 RT to remove mispairs. Therefore, if nucleotide misinsertion occurs by HIV-1 RT, then complete formation of the viral duplex DNA would require the subsequent addition of correctly paired bases, thus producing mutations in the viral genome. Despite this mutagenic potential, the alternative—i.e., failure to extend a mispair—could lead to DNA chain termination and abortive viral replication. This mechanism of hypermutability may provide a means for the retrovirus to avoid immunologic surveillance by the host.

The rapid evolution of HIV-1 has made it difficult to develop effective vaccines and to design nucleoside analogs

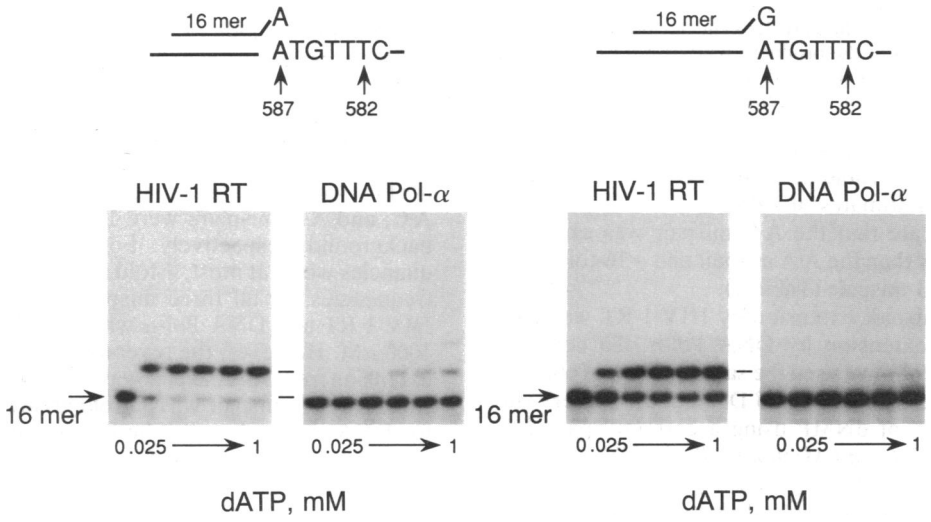

FIG. 3. Extension of A·A and A·G mispairs by excess HIV-1 RT or DNA Pol-α. Oligonucleotide primers (16-mer) were hybridized to φX174 *am3* DNA (0.022 pmol) to produce a A·A or A·G 3′-terminal mispair at the amber3 site (position 587). Incubation was for 60 min at 30°C with 5 times the amount of either HIV-1 RT or DNA Pol-α used in Fig. 2. Each reaction contained 0, 0.025, 0.1, 0.25, 0.5, or 1.0 mM dATP as the only deoxynucleoside triphosphate substrate. The primer position (16-mer) is indicated by arrows.

Table 2. Mutagenesis by mispair extension by HIV-1 RT and DNA Pol-α

Primer–template	Reversion frequency, $\times 10^{-6}$				
		HIV-1 RT		DNA Pol-α	
	No polymerase	10 μM dNTP	1000 μM dNTP	10 μM dNTP	1000 μM dNTP
_/A —A—	4	600	7700	5	190
_/C —A—	3	5100	11,000	11	7600
_/G —A—	6	430	3800	9	41
T —A—	2	4	8	2	6

The φX174 *am3* DNA (0.055 pmol) was primed with a 16-base oligonucleotide primer to produce the indicated 3′ terminus at the amber site (position 587). The primed DNA was copied by HIV-1 RT or DNA Pol-α in reactions containing all four dNTPs (10 μM or 1000 μM) for 5 min at 30°C. The copied DNA was transfected into *E. coli* spheroplasts and plated on both amber Sup^+ and Sup^- strains to determine reversion frequencies. Background reversion frequency of unprimed φX174 *am3* DNA was 3×10^{-6}, and this value was not subtracted from the given values.

for drug therapy. Reduced sensitivity of HIV-1 to 3′-azido-3′-deoxythymidine (AZT) has been shown in virus isolates from patients treated with this drug for only 6 mo (24). It remains to be determined whether this resistance to AZT is due to mutations in the viral RT.

The efficient extension of mispairs by HIV-1 RT, relative to the cellular DNA polymerases α and δ, might be exploited in designing nucleoside analogs that lethally mutagenize HIV-1 during its initial replicative stages. The concept is that HIV-1 exhibits an exceptionally high mutation frequency and that a further increase in mutations could overload the capacity of this small genome to produce viable progeny (4). Analogs could be designed for efficient insertion and extension by HIV-1 RT. Efficient extension from the incorporated nucleoside analog by HIV-1 RT and not by cellular DNA polymerases could reduce the potential for introducing mutations into the cellular genome. A simple *in vitro* gel assay could be used to initially screen analogs and predict whether these might be effective against HIV-1 replication.

We thank Kris Carroll for help in preparing the figures. This work was supported, in part, by National Institutes of Health Grants T32-CA-09437 (F.W.P.), CA-07263-03 (B.D.P.), AG00057 (L.L.S.), and R35-CA-39903 (L.A.L.) and by a Henry Rutgers Fellowship (Rutgers University) to B.D.P.

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