Fidelity of DNA Synthesis Exhibited *in Vitro* by the Reverse Transcriptase of the Lentivirus Equine Infectious Anemia Virus†

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ABSTRACT: The lentivirus equine infectious anemia virus (EIAV) shows high genetic variations. To gain insight into the relative contribution of the reverse transcription process to the EIAV mutation rate, the accuracy of DNA synthesis catalyzed *in vitro* by the reverse transcriptase (RT) of EIAV was determined. Since the RT of EIAV shows a relatively high sequence homology with other lentiviral RTs, most notable being the RTs of human immunodeficiency viruses (HIVs), type 1 and type 2, it was of interest to study the fidelity of EIAV RT as part of an investigation of the structure–function relationship in lentiviral RTs. Like other RTs, EIAV RT was found to lack a 3′→ 5′ exonuclease activity. The fidelity of EIAV RT was analyzed by studying two distinct steps that lead to base substitution mutations: nucleotide misinsertions and elongation from 3′-terminal DNA mispairs. Analysis of misincorporation rates opposite the template adenine residue in native ϕx174am3 DNA showed that EIAV RT catalyzes nucleotide mismatches with a specificity of A:C >> A:G > A:A. Interestingly, the same order of specificity was also detected during mispair extension with three templates tested (i.e., ϕx174am3 DNA, rRNA, and synthetic oligo DNA). The mispair extension efficiency and mispair formation appear to be affected mainly by the increase in apparent K_m values, rather than by the change in V_{max} values. Furthermore, EIAV RT exhibits similar mispair extension efficiencies with both RNA and DNA templates with identical surrounding sequences. However, dissimilarities were detected in mispair extension frequencies with two DNAs which have different sequences, thus emphasizing the importance of the sequences copied. The fact that EIAV RT is as error-prone as the two HIV RTs further strengthens the correlation between the reduced fidelity of RT and the genomic heterogeneity observed among strains of various lentiviruses. The data also suggest that an important component of retroviral genetic variability may be attributable to the efficient mismatch extension during the copying of both RNA and DNA templates. In all, as with other RTs studied so far, it is apparent that the fidelity of DNA synthesis exhibited by EIAV RT is also enzyme-dependent and sequence-related.

Equine infectious anemia virus (EIAV)[1] is the causative agent of equine infectious anemia (Cheevers & McGuire, 1985). EIAV is a member of the lentivirus subfamily of retroviruses (Gonda et al., 1978), which also includes the human immunodeficiency viruses, the etiological agents of human AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1983). These viruses have been demonstrated to possess similar genomic organizations and relatively high sequence homology (Stephens et al., 1986; Rushlow et al., 1986). EIAV and both HIV-1 and HIV-2 share common structural and biological features, including replication in cells of the immune system, establishment of persistent infections, and high mutation rates (Montelaro et al., 1984; Salinovich et al., 1986). Genomic mutations alter the structure and antigenic properties of EIAV and both HIVs, which correlate with the emergence of envelope gene variants. This particular feature explains the ability of these viruses to evade the immune defenses in naturally infected hosts (Hussain et al., 1987; Payne et al., 1987). The elusive nature of HIVs and EIAV reflects the unique life cycle of all retroviruses that possess mechanisms for the rapid generation of variant viruses.

Retroviral mutation rates are probably a reflection of the accuracies of three polymerization events: (1) reverse transcription of viral RNA into the double-stranded viral DNA by retroviral RT; (2) replication of the proviral DNA (after being integrated into the host chromosome) by cellular DNA polymerases; and (3) DNA-templated plus-strand RNA synthesis by cellular RNA polymerase II. Studies of reverse transcriptases show that these essential virus-encoded DNA polymerases are relatively error-prone and, therefore, are very likely to be major determinants in affecting the retroviral mutation rates (Echols & Goodman, 1991; Williams & Loeb, 1992). The RTs of retroviruses play a key role in the early stages of their life cycle by catalyzing the conversion of the viral single-stranded RNA into the proviral double-stranded DNA (Goff, 1990). RTs are multifunctional enzymes: (a) they synthesize minus-strand DNA from genomic RNA template by the RNA-dependent DNA polymerase function; (b) they hydrolyze the RNA by RT-associated RNase H activity; (c) they synthesize the second DNA plus-strand from the DNA minus-strand by the DNA-dependent DNA polymerase function of the enzyme.

EIAV RT exhibits homology with the RTs of other lentiviruses, particularly with those of HIV-1 and HIV-2. The comparison of the RT coding regions of the genomes of EIAV and the two HIVs reveals a sequence identity at the amino acid level of 41.9% with HIV-1 RT and 40.6% with HIV-2 RT (Myers et al., 1992). A recent report has suggested that the kinetic properties of EIAV RT are similar to those of HIV-1 RT (Thomas & Furman, 1991). For example, both enzymes have the same divalent cation requirements and

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[1] Abbreviations: EIAV, equine infectious anemia virus; HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; RT, reverse transcriptase; MLV, murine leukemia virus; AMV, avian myeloblastosis virus; rRNA, ribosomal RNA; dNTP, deoxynucleoside triphosphate; PAGE, polyacrylamide gel electrophoresis; AIDS, acquired immunodeficiency syndrome.

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similar K_m values for 2′-deoxynucleoside 5′-triphosphates with the synthetic template–primers tested. In addition, both RTs are inhibited to similar extents by most, but not all, deoxynucleoside triphosphate analogs tested. These data indicate that the EIAV RT, in addition to the HIV-1 and HIV-2 RTs, may be a good model for studying structure–function relationships of lentiviral RTs. However, we have found recently that there are substantial differences in several properties between EIAV and HIV-1 RTs (Rubinek and Hizi, unpublished experiments). These differences indicate that the functional similarity of these two proteins, albeit significant, is not absolute. Hence, it is important to investigate whether the predicted homology between the RTs of EIAV and the two HIVs is also reflected in their accuracy of DNA synthesis.

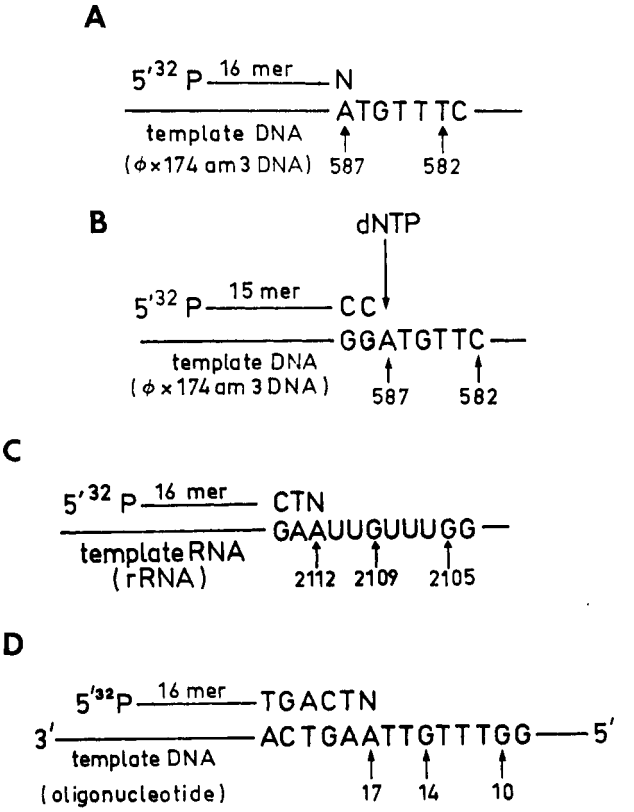
Several studies have indicated that HIV-1 RT has a low fidelity of DNA synthesis during the copying of DNA and RNA templates *in vitro* (Hubner et al., 1992; Ji & Loeb, 1992; Perrino et al., 1989; Preston et al., 1988; Ricchetti & Buc, 1990; Roberts et al., 1988). Our recent studies have found that HIV-2 RT, similar to HIV-1 RT, also exhibits a low accuracy of DNA synthesis with both templates, probably due its ability to efficiently elongate mismatched 3′-termini of primer DNA (Bakhanashvili & Hizi, 1992a,b). Furthermore, we have found that the fidelity of DNA synthesis with HIV-1 and HIV-2 RTs, as well as MLV RT, mainly depends on the sequences of polynucleotides copied by every given RT (Bakhanashvili & Hizi, 1993). In the current study, we have employed a kinetic assay that can be applied to any site selected on natural or synthetic template–primers (Boosalis et al., 1987). This assay has been utilized to measure both nucleotide misinsertion and mispair extension kinetics. The results presented herein indicate that EIAV RT is as error-prone as two HIV RTs during DNA synthesis with both RNA and DNA templates. The data further strengthen the possibility that lentiviral genetic variability may be correlated to the low fidelity of RT during both the RNA→DNA and DNA→DNA replication steps.

MATERIALS AND METHODS

Enzymes. The reverse transcriptase of HIV-1 used in this study is the recombinant enzyme expressed by us in *Escherichia coli* (Hizi et al., 1988) and purified to homogeneity according to Clark et al. (1990). EIAV RT was also expressed by us recently in bacteria and purified by similar techniques (Shaharabany & Hizi, unpublished experiments). The specific activities of the enzymes were 4000–16000 units/μg. One unit is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of dTMP into DNA in the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed reaction in 30 min at 37 °C. The Klenow fragment of *E. coli* DNA polymerase I and T4 polynucleotide kinase were purchased from USB.

Template–Primers. The different template–primer substrates used for measuring the fidelity of EIAV RT with RNA and DNA templates are depicted in Chart I. For terminal nucleotide excision and for the extension of DNA from a mispaired terminus, the native template, single-stranded ϕx174am3 DNA, was primed with the 16-mer oligonucleotide that hybridizes to the nucleotides at positions 587–602 of the ϕx174am3 DNA. Four versions of the 16-base primers were synthesized separately. They are identical except for the 3′-terminal nucleotide (N), which contains either an A, C, G, or T (5′-AAAGCGAGGGTATCCN-3′) (Chart IA). For kinetic analysis of site-specific nucleotide misincorporations, the template ϕx174am3 DNA was primed with a 15-mer

Chart I: Primer–Templates Used for Analysis of Terminal Mismatch Excision and for Measuring the Fidelity of Insertion and Extension Using RNA and DNA Templates



oligonucleotide (which hybridizes to the nucleotides at positions 588–602), producing a correctly paired 3′-terminus at position 588. The sequence of the primer is 5′-AAAGCGAGGGTATCC-3′ (Chart IB). The accuracy of the RNA-dependent DNA polymerization was studied using rRNA as a native substrate (a mixture of 16S and 23S *E. coli* ribosomal RNA), primed with 16-mer oligonucleotide primers, that hybridizes to the nucleotides at positions 2112–2127 of the 16S rRNA. The sequence of the primers is 5′-ATTTCACATCTGACTN-3′ (N = A, C, G, or T) (Chart IC). Mispair extension kinetics was also studied with a synthetic DNA substrate. The 34-base oligonucleotide template is identical to nucleotides 2096–2129 of *E. coli* 16S RNA (5′-ACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCC-3′). This DNA was primed with the 16-mer oligonucleotide primers (utilized with rRNA template) that hybridize to the nucleotides at positions 17–32 of the DNA oligonucleotide template (Chart ID). The primers were labeled at the 5′-end with T4 polynucleotide kinase and [γ-^{32}P]ATP and annealed to the template RNA or DNA as described (Bakhanashvili & Hizi, 1992a,b).

Electrophoretic Analysis of Terminal Nucleotide Excision. The 3′→5′ exonucleolytic activity was measured as the removal of 3′-terminal nucleotides (correct or incorrect) from the 5′-[γ-^{32}P] end-labeled oligonucleotide, determined by the increase in the mobility during the gel electrophoresis. The reactions were carried out in 25 μL each, containing 50 mM Tris·HCl (pH 7.8), 2 mM dithiothreitol, 10 mM $MgCl_2$, 300 ng of mismatched DNA, and 0.75 pmol of EIAV RT or HIV-1 RT or 0.2 pmol of the Klenow fragment of *E. coli* DNA polymerase I (serving as a positive control). Aliquots were removed into equal volumes of formamide dye mix. Electrophoretic analyses were performed in 20% polyacrylamide sequencing gels followed by autoradiography (Bakhanashvili & Hizi, 1992a).

Kinetic Analysis of Site-Specific Nucleotide Misincorporation. The template–primer substrate depicted in Chart IB was utilized to measure rates of dNTP incorporation opposite the A residue at position 587 of ϕx174am3 DNA. Reactions for kinetic analysis contained 20 mM Tris·HCl (pH 7.5), 2 mM DTT, 10 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, [γ-^{32}P]-end-labeled 15-mer oligonucleotide-primed ϕx174am3 DNA, and increasing concentrations of single dNTP. Reactions were incubated at 30 °C for 2 min for correct insertion and 5 min for misinsertion. Reaction products were analyzed by electrophoresis through 20% polyacrylamide sequencing gels and quantitated by densitometric scanning of the gel autoradiographs (Boosalis et al., 1987).

DNA Polymerization Reaction with Both DNA and RNA Templates. The procedures for the kinetic analysis of mispair extension with native DNA and RNA templates as well as with synthetic DNA template have been described in detail previously (Bakhanashvili & Hizi, 1992a,b). The template–primer substrates used for DNA polymerization reactions are shown in Chart IA,C,D. Before the kinetic constants for elongating the primers were measured, a time–course study was done for each paired and mispaired terminus to determine the time range during which the products accumulate linearly with time.

RESULTS

Analysis of the 3′→5′ Exonuclease Activity of EIAV RT. DNA polymerases, following a misinsertion of the wrong nucleotide, can either excise the mispaired terminus (if a proofreading exonuclease is associated with enzyme), continue chain elongation beyond the mismatch, or alternatively, abort the DNA synthesis by dissociating from the template–primer. All RTs studied so far have no associated 3′→5′ exonuclease activity. To confirm this general statement, we have examined whether EIAV RT also lacks 3′→5′ exonuclease proofreading activity. This was analyzed with native ϕx174am3 DNA annealed to either of the four primers depicted in Chart IA. Terminal nucleotide excision capability (either matched or mismatched) was analyzed in the absence of DNA synthesis since no dNTPs were present in the reaction mixtures. Efficient excision of the terminal nucleotides was found to occur within 5 min when the Klenow fragment of *E. coli* DNA polymerase I was used as a positive control (Figure 1). Conversely, in the case of EIAV RT, there was no change in the lengths of all four oligonucleotide primers analyzed after an incubation of up to 1 h. Similar results were obtained with HIV-1 RT used as a negative control. Thus, the data are consistent with the notion that RTs are deficient in 3′→5′ exonuclease activity; EIAV RT is found to be not exceptional, namely, it is incapable of proofreading.

Mispair Formation by EIAV and HIV-1 RTs. The lack of proofreading activity enables the analysis of the fidelity of DNA polymerization activity without the interference of any exonucleolytic activities. Two reactions indicative of the fidelity of DNA synthesis at defined template sites have been investigated. The first is the nucleotide misinsertion and the second is the extension of nascent mismatched primer termini. To characterize the fidelity of misinsertion by EIAV RT, we have employed an assay that measures site-specific nucleotide misincorporations. The rates of nucleotide insertion (A, C, G, or T) were examined opposite the template A residue at position 587 of native ϕx174am3 DNA primed with 5′-^{32}P end-labeled nucleotide oligomer (Chart IB). Four separate reactions were carried out, each using a single dNTP to measure the rate of synthesis of the correct pair and three possible mispairs. The quantitation of the primer extension bands resolved by PAGE provides a clear indication of the ability of EIAV RT to insert wrong nucleotides. To evaluate the nucleotide insertion fidelity, we have measured the ratios of gel band intensities, estimated by densitometry, and determined the extension rates as a function of dNTP concentrations for both EIAV and HIV-1 RTs (Figure 2). It is apparent that a misincorporation with incorrect nucleotides was obtained at much higher nucleotide concentrations than the correct insertion with dTTP. Following the addition of the correct nucleotide (dTTP) or of higher concentrations of the incorrect nucleotides (dATP, dCTP, or dGTP), the production of extended oligomers (16 nucleotides and longer) increased with the time of incubation (2- and 5-min incubation periods for correct and incorrect nucleotides, respectively). The apparent K_m and V_{max} kinetic values for each dNTP calculated from the double- reciprocal plots of the initial velocities versus the substrate concentrations (Lineweaver–Burk curves; data not shown) are summarized in Table I. The K_m value, calculated for correct insertion of dTTP opposite the A at position 587, is about 1750–6000-fold lower with EIAV RT and about 1700–10100-fold lower with HIV-1 RT compared to those values obtained for the insertion of incorrect dNTPs with both enzymes. In contrast, the apparent ratios of V_{max} values calculated for the insertion of correct to incorrect nucleotides are less than 1.7–13-fold for EIAV RT and 2–7-fold for HIV-1 RT. The ratio of the insertion efficiencies for wrong (W) versus right (R) deoxynucleotides indicates the misinsertion frequency (f):

$$f = \frac{(V_{max}/K_m)^W}{(V_{max}/K_m)^R}$$

The nucleotide insertion fidelity of a DNA polymerase is defined as the reciprocal value of f. It is evident from Table I that EIAV RT forms the A:C mispair with 26-fold greater efficiency than the A:A mispair and with 16-fold higher efficiency than the A:G mispair. Consequently, the specificity of mismatch formation by EIAV RT opposite the template A residue is A:T >> A:C >> A:G > A:A. Similarly, HIV-1 RT forms the A:C mispair 20-fold more efficiently than the A:A mispair and 12-fold more efficiently than the A:G mispair. Thus, the fidelity of nucleotide insertion by EIAV and HIV-1 RTs appears to be similar. In parallel, we have analyzed the extent of misinsertion by MLV RT with the same template–

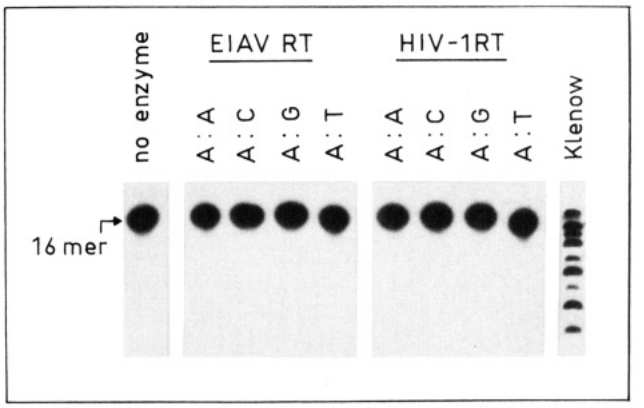


FIGURE 1: Electrophoretic analysis of terminal mismatch excision. Reactions for terminal mismatched (A:A, A:C, and A:G) or matched (A:T) excision by EIAV and HIV-1 RTs and the Klenow fragment of *E. coli* DNA polymerase I were performed with primer–templates (see Chart IA) as described in Materials and Methods. The position of the 16-mer primer is indicated by an arrow. The direction of the electrophoresis is from top to bottom.

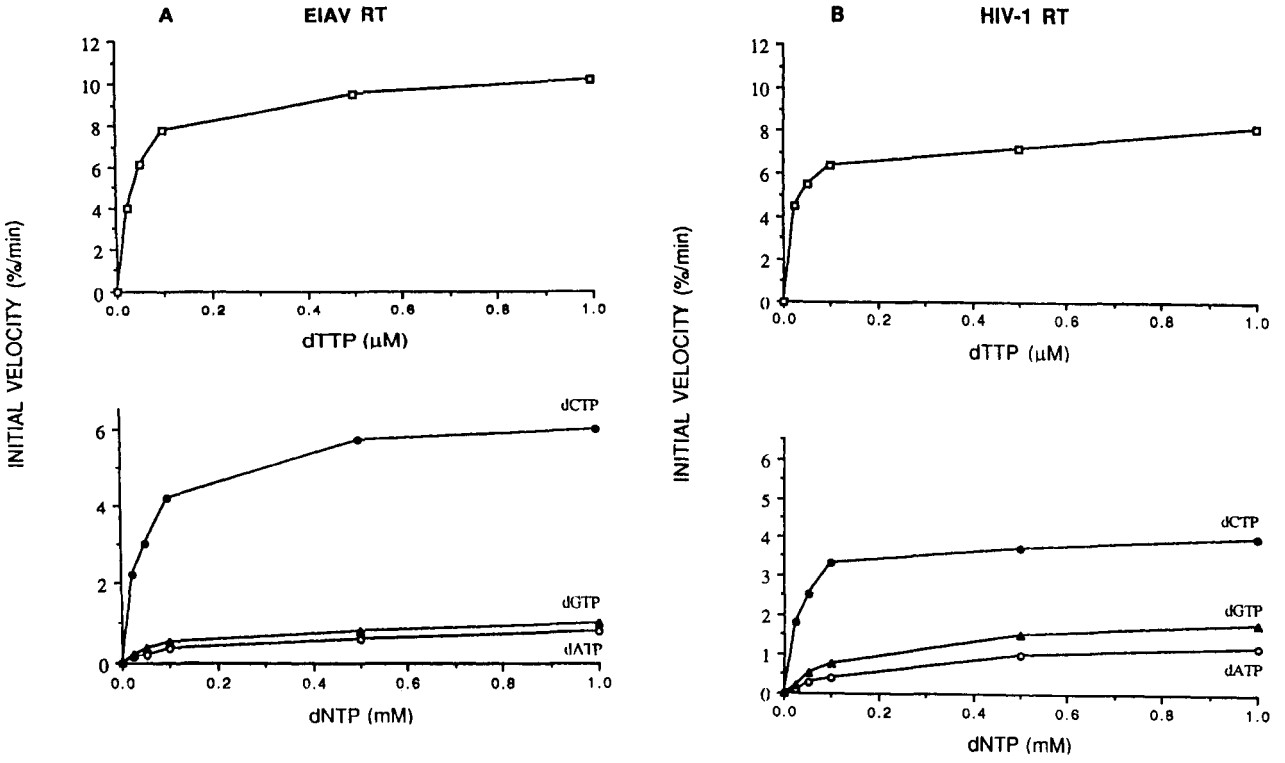


FIGURE 2: Kinetic assay for site-specific nucleotide misincorporation. 5'-end- labeled 15-mer oligonucleotide primer annealed φx174am3 DNA (see Chart IB) was incubated at 30 °C in the presence of increasing concentrations of single dNTP with EIAV RT (A) or HIV-1 RT (B). Product oligonucleotides were analyzed by PAGE and quantified as described in Materials and Methods.

Table I: Kinetic Parameters for Site-Specific Misincorporation by EIAV and HIV-1 Reverse Transcriptases[a]

	EIAV RT			HIV-1 RT		
dNTP	K_m (μM)	V_{max} (%/min)	relative insertion frequency	K_m (μM)	V_{max} (%/min)	relative insertion frequency
T	0.032 ± 0.02	10.5 ± 2.5	1	0.018 ± 0.01	8.3 ± 2.3	1
C	56 ± 4.2	6.2 ± 2.2	1/3000	30 ± 3.8	4.0 ± 2.0	1/3460
G	145 ± 48	1.0 ± 0.2	1/47600	160 ± 49	1.8 ± 0.4	1/41000
A	190 ± 50	0.8 ± 0.2	1/78000	180 ± 55	1.2 ± 0.3	1/69170

[a] Apparent K_m and V_{max} values were determined from Figure 2 as described in the text. The relative insertion frequencies were calculated from the V_{max}/K_m ratios and are expressed relative to dTTP.

primers. We could not detect any significant misinsertions with either dCTP, dATP, or dGTP (data not shown).

Mispair Extension Kinetics of DNA Synthesis by EIAV RT. The efficient extension of mismatched 3'-termini in DNA was found to be a major determinant of the low accuracy of both HIV-1 and HIV-2 RTs (Bakhanashvili & Hizi, 1992a; Perrino et al., 1989). In the experiments designed to measure mismatch extension kinetics, EIAV RT extends an annealed primer with a preformed mismatch at the 3'-terminus. The catalysis of the extension from preformed mispairs as a function of increasing concentrations of dATP was studied by measuring primer elongation in the DNA- or RNA-dependent DNA polymerization reactions with the template–primers, as described in Chart IA,C,D.

(1) Mispair Extension Kinetics with Native φx174am3 DNA Template. The extension from the preformed mispairs with native φx174am3 DNA was detected by an increase in the length of the oligonucleotide primers to 17 nucleotides or greater. The results illustrated in Figure 3 indicate that the extensions from the A:A and A:G purine–purine mispairs by EIAV RT result in the production of only 17-mer oligonucleotides. However, the extension from the A:C purine–pyrimidine mispair led to the production of oligonucleotides from 17 to 22 nucleotides in length. Extension from the A:C mispair up to a 22-mer involves the incorporation of two additional noncomplementary dAMPs opposite the template guanine and cytosine at positions 585 and 581, respectively. Thus, a greater proportion of 16-mer A:C mispaired primer is extended, and the products are longer, following multiple misinsertions.

The efficiency of mispair extension is an essential component in determining whether the mismatched primer termini are likely to be extended by the DNA polymerase. The apparent values of the kinetic constants, K_m and V_{max}, for extension of each terminus were calculated from the double-reciprocal plots as described above for the misinsertion. It is apparent from Table II that the discrimination of EIAV RT against extension from A:A, A:C, or A:G mispairs can be attributed to relatively large K_m differences governing the extension of matched versus mismatched base pairs with much smaller differences in V_{max} ratios (Table II). K_m values for the extensions of all three mispairs are 2000–5000-fold greater than the value to extend the A:T mispair. In contrast, differences of less than 2–7-fold are found when comparing the relative V_{max} values obtained with matched versus mismatched 3'-termini of primers. Consequently, K_m discrimination is predominantly the mechanism determining the mispair extension. The relative extension frequencies were defined as the ratios of

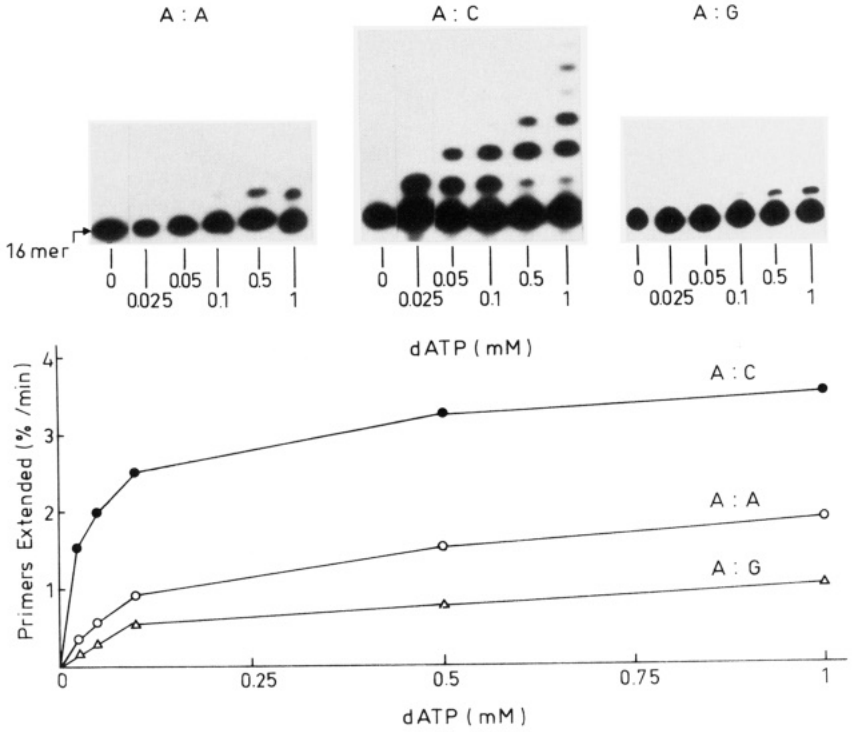


FIGURE 3: Kinetics of mispair extension by EIAV RT with ϕx174am3 DNA template. The 16-mer oligonucleotide primers were hybridized to ϕx174am3 DNA to produce the indicated 3′-terminal mispairs at position 587 (see Chart IA) and extended for 15 min with EIAV RT. Each reaction contained 0, 0.025, 0.05, 0.1, 0.5, or 1.0 mM dATP as the only deoxynucleotide triphosphate substrate. Polyacrylamide gel electrophoretic analyses of the extended primers from A:A (○), A:C (●), and A:G (△) mispairs are shown in the upper panel at left, center, and right, respectively. The primer position is indicated by an arrow. The quantitation of the primers extended was plotted in the lower panel after scanning the autoradiograms.

Table II: Kinetics of Mispair Extension by EIAV RT with ϕx174am3 DNA, rRNA, and oligo DNA Templates[a]

	ϕx174am3 DNA			rRNA			oligo DNA		
primer–template	K_m (μM)	V_{max} (%/min)	relative extension frequencies	K_m (μM)	V_{max} (%/min)	relative extension frequencies	K_m (μM)	V_{max} (%/min)	relative extension frequencies
A A–	100 ± 35	2.0 ± 0.7	1/17500	65 ± 4	3.2 ± 0.6	1/6500	71 ± 5	3.8 ± 0.8	1/5140
C A–	40 ± 6	3.6 ± 6	1/3900	34 ± 5	5.2 ± 1.5	1/2100	40 ± 4	4.6 ± 1.2	1/2400
G A–	100 ± 32	1.0 ± 0.2	1/35000	80 ± 14	1.8 ± 0.8	1/14200	130 ± 40	2.4 ± 0.6	1/14900
T A–	0.02 ± 0.001	7.0 ± 2.3	1	0.025 ± 0.002	8.0 ± 2	1	0.04 ± 0.02	11 ± 2	1

[a] Oligonucleotide primers (16-mer) were hybridized to the appropriate templates to generate the indicated 3′-termini. The apparent K_m and V_{max} values were determined from Figure 3 for ϕx174am3 DNA, from Figure 4 for rRNA, and from Figure 5 for oligo DNA template, as described in the text. The relative extension frequencies 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.

apparent V_{max}/K_m values obtained with correct and incorrect base pairs at a given sequence on the template. The rate of extension from the A:C mispair was 4.5-fold greater than the rate from an A:A mispair and 9-fold greater than that from an A:G mispair. Therefore, EIAV RT most efficiently catalyzes chain extension from the A:C purine–pyrimidine mispair.

(2) Mispair Extension Kinetics with Native rRNA Template. Since RT utilizes both RNA and DNA as templates, it is important to examine whether the polymerization errors would occur during RNA-dependent DNA polymerization as well. To evaluate the mispair extension efficiency by EIAV RT during DNA synthesis with native RNA template, we have prepared a series of templates with 16-mer primers that formed the 3′-terminal mismatches A:A, A:C, or A:G at position 2112 of 16S rRNA (see Chart IC). The ability of EIAV RT to extend each of the three mispairs under steady-state kinetic conditions is illustrated in Figure 4.

Extension from the A:C purine–pyrimidine mispair results in the production of oligonucleotides from 17 to 23 nucleotides in length. The 19-mer product presumably results from misinsertion of dAMP opposite the template guanine at position 2109 of rRNA template. Subsequent extension from the newly formed G:A mispair involves incorporation of complementary dAMPs opposite the template uridine at positions 2108–2106, leading to synthesis of 20–22-mer products. The 23-mer product probably results from an additional misinsertion of dAMP opposite the template guanine at position 2105. In all, the A:C and A:A mispairs are extended more efficiently than the A:G mispair. Moreover, it is evident

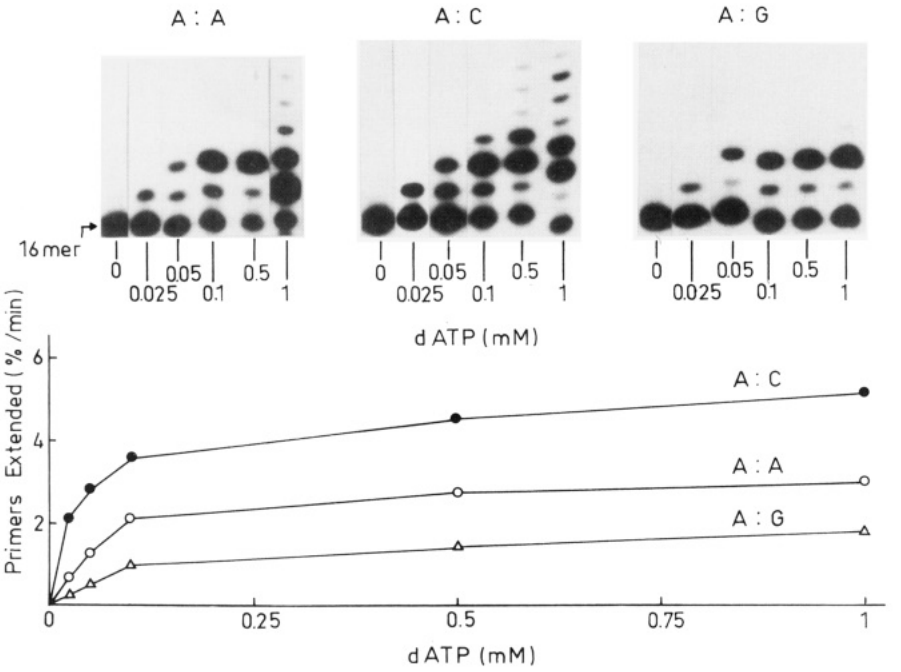


FIGURE 4: Kinetics of mispair extension by EIAV RT with rRNA template. The 16-mer oligonucleotide primers were hybridized to 16S rRNA to produce the indicated 3′-terminal mispairs at position 2112 (see Chart IC) and extended for 15 min with EIAV RT. The experiments were conducted and analyzed as described in Figure 3.

that EIAV RT displays a different pattern of extension and catalyzes the mispair extension with native rRNA more efficiently than with ϕx174am3 DNA template (Figures 3 and 4).

The kinetic parameters K_m and V_{max} were calculated as described above for the DNA template, and the results are summarized in Table II. The extension of mispairs was manifested through the differences in apparent K_m values. In addition, estimates of substrate efficiencies in this assay (i.e., V_{max}/K_m) indicated that for the RNA template the rate of extension from the A:C mispair was 3-fold greater than the rate from an A:A mispair and 7-fold greater than that from an A:G mispair. Comparative analysis reveals that the relative extension frequencies obtained with all three mispairs during the DNA polymerization with rRNA are higher than those observed with ϕx174am3 DNA template.

(3) Mispair Extension Kinetics with Oligonucleotide DNA Template. The sequences chosen for the previous analyses with two templates, ϕx174am3 DNA and rRNA, were similar but not identical (see Chart IB,C), although the preformed mispairs were identical. Consequently, the observed dissimilarities in the primer extension studies with these two templates might be due to the different polynucleotide sequences copied in the two systems or to the dissimilarity in the nature of the templates. This issue was resolved by estimating the importance of the surrounding base sequence on the fidelity of extension using a synthetic DNA template with a sequence identical to that of the rRNA, previously copied into DNA (see Chart ID). The results presented in Figure 5 indicate a pattern of mispair extension which is similar to that observed with the rRNA template and is substantially more efficient than that observed with the ϕx174am3 DNA template. Furthermore, it is evident from Table II that the mispair extension efficiencies with rRNA and oligonucleotide DNA templates (sharing identical sequences) are similar. It is evident, therefore, that EIAV RT behaves in a manner similar to that of HIV-1, HIV-2, and MLV RTs (Bakhanashvili & Hizi, 1993), namely, the sequences copied rather than the nature of the template determine the accuracy of DNA synthesis.

DISCUSSION

EIAV RT exhibits homology with the RTs of other lentiviruses, including the RTs of HIV-1 and HIV-2. In a comparison of the RTs of EIAV and the two HIVs, the amino acid sequence identity was calculated to be approximately 41%. Such a similarity positions EIAV RT, in addition to HIV-1 and HIV-2 RTs, as another good candidate for examining the structure–function relationships in lentiviral reverse transcriptases. In contrast to the extensive amount of information available on HIV-1 RT and the more recent information on HIV-2 RT, little is known about the fidelity of DNA synthesis exhibited by EIAV RT. De Vico et al. (1991), using a misincorporation assay in which poly(rC)·oligo(dG) served as a synthetic template–primer, indicated that EIAV RT might be as error-prone during DNA synthesis as HIV-1 RT. However, no thorough studies have been performed so far on EIAV RT. The availability of large quantities of the highly purified and enzymatically active recombinant EIAV RT facilitated our study on the fidelity of this enzyme.

Generally, the fidelity of DNA polymerases is an outcome of the combination of the nucleotide insertion, extension, and exonucleolytic editing steps. Retroviral RTs are 3′→5′-exonuclease-deficient enzymes. Thus, the base substitution mutations during reverse transcription could presumably arise from two sequential polymerization events at the target site. The first is incorporation of a noncomplementary nucleotide to generate a mispair at the 3′-terminus of the nascent strand. The second step is extension of the terminal mispair. We have shown (Figure 1) that EIAV RT is no exception to all RTs studied so far, namely, it is devoid of any 3′→5′ exonuclease activity. Hence, the lack of exonuclease activity in EIAV RT enables direct measurements of the fidelity of the DNA polymerization activity without the interference of proofreading activity. In order to explore the contribution of

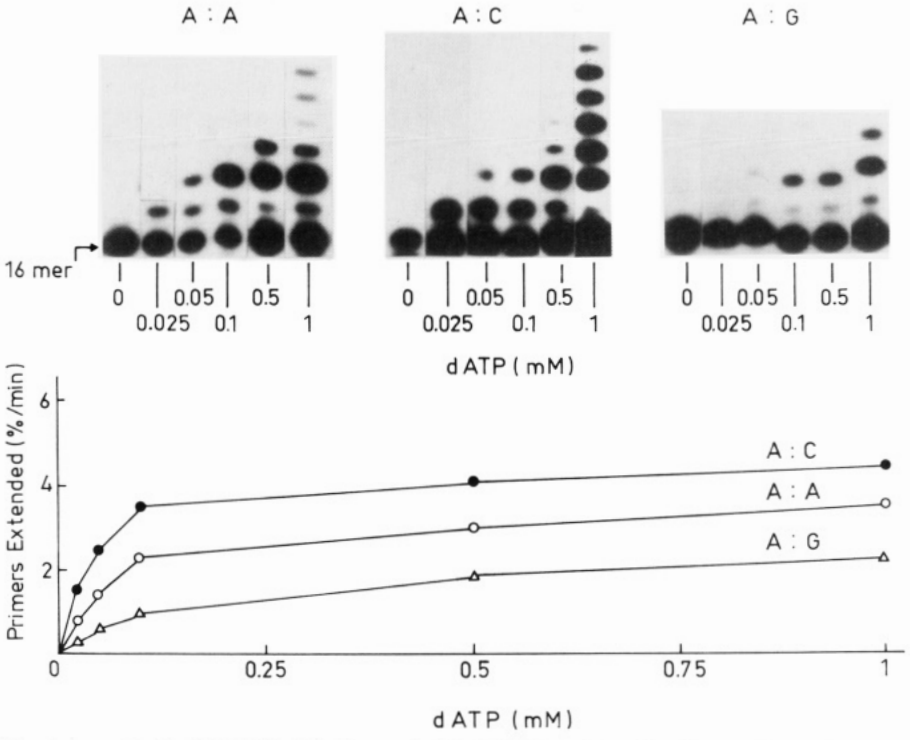


FIGURE 5: Kinetics of mispair extension by EIAV RT with oligonucleotide DNA template. The 16-mer oligonucleotide primers were hybridized to 34-mer oligonucleotide DNA template to produce the indicated 3′-terminal mispairs at position 17 (see Chart ID) and extended for 15 min with EIAV RT. The experiments were conducted and analyzed as described in Figure 3.

EIAV RT to the generation of mutations, we have measured the relative efficiencies of both mispair formation and extension with defined template–primers by steady-state kinetic methods. Using the gel assay, we have compared the kinetics of the correct insertion of T and the misinsertion of A, C, or G opposite the template A site at position 587 in ϕx174am3 DNA (Figure 2). The misinsertion frequencies observed with EIAV RT are as follows: 1/3000 for A:C, 1/47 600 for A:G, and 1/78 000 for A:A (Table I). The specificity of mismatch formation by EIAV RT is A:T >> A:C > A:G > A:A. The mispair formation appears to be affected mainly by the increase in apparent K_m values, rather than by the change in V_{max} values. In all, the misinsertion frequencies observed for EIAV RT are similar to those detected with HIV-1 RT.

It was found that the major difference between HIV RTs and other retroviral RTs on the one hand, or between HIV RTs and eukaryotic DNA pol α on the other hand, is the ability of both HIV RTs to elongate efficiently mismatched 3′-termini of DNA primers (Bakhanashvili & Hizi, 1992a; Perrino et al., 1989). The current data with EIAV RT demonstrate that this enzyme can also catalyze efficient mismatch extensions. We have analyzed the fidelity of DNA synthesis with both RNA and DNA templates. The results with ϕx174am3 DNA template indicate that EIAV RT extends preformed mispairs with greater efficiency, particularly the A:C mispair. This mismatch was extended about 4.5-fold more efficiently than the A:A mispair and 9-fold more efficiently than the A:G mispair (Table II). In all, the kinetic measurements of mispair extension seem to parallel those calculated for the insertion data. First, major discrimination can be attributed to the relatively large K_m differences (2000–5000-fold) governing the extension of matched versus mismatched base pairs with ϕx174am3 DNA template, with much smaller differences in V_{max} values. Second, the most efficient extension was detected from the same A:C mispair, which was formed most efficiently in the misincorporation reactions. This preference for the formation of the A:C mispair, as well as the preferential subsequent extension from this mispair, is typical of EIAV RT and both HIV-1 and HIV-2 RTs.

Reverse transcriptases exhibit a dual template specificity: they are capable of copying either RNA or DNA into complementary DNA. Therefore, we have also determined the ability of the EIAV RT to extend preformed 3′-terminal mispairs using rRNA as a template. The results demonstrate that EIAV RT exhibits efficient extensions from 3′-terminal mispairs with a specificity of A:C > A:A > A:G (Table II). Comparative analysis reveals that the relative extension frequencies obtained with all three mispairs during copying of the rRNA template are higher than those observed during copying of the ϕx174am3 DNA template. The sequences of both templates were similar but not identical. Consequently, in order to assess the relative importance of the sequence context versus the nature of the template, we analyzed the mispair extension efficiencies with oligonucleotide DNA sequences identical to those of the rRNA sequences copied. The results presented in Table II indicate that the mispair extension frequencies during DNA synthesis with both rRNA and oligo DNA templates (with the same surrounding sequences) are similar for EIAV RT. The comparative data obtained with RNA versus DNA templates are rather heterogeneous. Unequal HIV-1 RT error rates were detected with RNA and DNA templates. Hubner et al. (1992) conclude that the synthesis with an RNA template is less accurate than with a DNA template, whereas Boyer et al. (1992) suggest the opposite, namely, that the fidelity is higher with RNA than with DNA template. In contrast, other groups have found that the fidelity of DNA synthesis of HIV-1 RT with both types of templates is comparable (Ji & Loeb, 1992; Yu & Goodman, 1992). It is possible that these disparities result from the differences in experimental strategies or in the sequences used in the various experiments. The data obtained with EIAV RT are in accordance with our earlier observations with both HIV RTs and MLV RT (Bakhanashvili & Hizi, 1993) and further demonstrate that there are equal error rates

during both RNA→DNA and DNA→DNA replication.

The studies on two different DNA sequences revealed that the relative mispair extension frequencies observed with the ϕx174am3 DNA sequence by EIAV RT are significantly lower than those observed with the same mispairs with oligo DNA template (Table II). These dissimilarities may be explained by the differences in the nucleotide sequence copied (Chart IA,C). The major influences on mispair extension seem to involve residues within 2 or 3 bases on either side of the mismatch. In all, as with other RTs studied so far (Bakhanashvili & Hizi, 1993; Ricchetti & Buc, 1990), it is apparent that the fidelity of DNA synthesis exhibited by EIAV RT is also enzyme-dependent and sequence-related.

The studies on the fidelity of various DNA polymerases enable us to formulate a set of rules that describe misinsertion and mispair extension reactions. The handling of mismatched base pairs in the same way by all polymerases may reflect some important structural features of the DNA that elicit a common response from these enzymes. Conversely, differences between polymerases may be interpreted as the specific enzyme–template interactions peculiar to each polymerase. DNA polymerase-catalyzed misinsertion and mismatch extension reactions depend on several factors: the type of the mispair, the influence of surrounding sequences of the template, and the response of the polymerase to the altered geometry of the mispair. It was recently suggested that, on the basis of the misinsertion of dATP opposite a template A, DNA polymerases could be divided into two classes (Joyce et al., 1992). One class of enzymes, comprising Klenow fragment and DNA polymerase α, has a very strong preference for the formation of the A:A mispair; the other group, comprising DNA polymerase III and all reverse transcriptases hitherto studied, discriminates very strongly against this A:A mispair. The results obtained with EIAV RT demonstrates that the specificity of mismatch formation opposite the template A residue is A:T >> A:C >> A:G > A:A; i.e., this enzyme discriminates very strongly against the formation of the A:A mispair. Hence, EIAV RT belongs to the second class of DNA polymerases.

Furthermore, for the mispair extension reactions with DNA templates, the general rules are as follows: (a) purine–pyrimidine mispairs are easily inserted and extended; (b) pyrimidine–pyrimidine and purine–purine mispairs are more difficult to extend than to insert (Mendelman et al., 1990). The data on mispair extension frequencies obtained with EIAV RT demonstrate that this enzyme shares common patterns with other polymerases examined, e.g., Drosophila DNA polymerase α and AMV RT (Mendelman et al., 1990), calf thymus DNA polymerase α (Perrino & Loeb, 1989), MLV RT and both HIV-1 and HIV-2 RTs (Bakhanashvili & Hizi, 1992a; Perrino et al., 1989). A quantitative assessment of mispair extension by EIAV RT shows that purine–pyrimidine mispair is more efficiently extended than the purine–purine mispairs, i.e., A:C > A:A > A:G. It should be emphasized that the same trend in the order of mispair extension efficiency was detected with rRNA template by HIV-1, HIV-2, and MLV RTs (Bakhanashvili & Hizi, 1992b) and EIAV RT (Table II), suggesting that the same rules are relevant with RNA template as well. There is a strong correlation between reaction rates and the nature of the mispair being formed or extended. The observed order of preference for mispair formation and extension can be explained in geometrical terms as proposed by Sloane et al. (1988). The efficient formation and extension of purine–pyrimidine mispairs can be explained by the geometric selection principle, because the wobble pairs are the closest to Watson–Crick geometry (e.g., A:C, T:G). Thus, the general trend in the order of mispair extension efficiency may reflect enzyme-specific interactions with the individual mispair structures.

The comparative data for the retroviral RT-mediated relative frequencies of misinsertion or relative abilities to extend nascent mismatches during DNA synthesis indicate the following order: both HIV RTs >> AMV RT > MLV RT (Bakhanashvili & Hizi, 1992a,b; Preston et al., 1988; Ricchetti & Buc, 1990; Yu & Goodman, 1992). Such dissimilarities are expected, since these enzymes have different amino acid sequences, and may reflect some differences in the molecular structures of RTs and their interactions with substrates. The fact that EIAV RT is as error-prone as both HIV RTs positions this enzyme in a less accurate subclass of retroviral RTs. Moreover, the observed results with EIAV RT further strengthen the correlation between the reduced fidelity of RT, as determined *in vitro*, and the high genomic diversity observed among strains of various lentiviruses. Further studies on the fidelity of DNA synthesis of other lentiviral RTs will indicate whether the relatively low fidelity found so far for HIV-1, HIV-2, and EIAV RTs represents a general phenomenon showed by the RTs of all members of the lentiviral subfamily of retroviruses.

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REFERENCES

Bakhanashvili, M., & Hizi, A. (1992a) *FEBS Lett. 306*, 151–156.

Bakhanashvili, M., & Hizi, A. (1992b) *Biochemistry 31*, 9393–9398.

Bakhanashvili, M., & Hizi, A. (1993) *FEBS Lett. 319*, 201–205.

Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, T., Chamaret, S., Rouzioux, C., Rozenbaum, W., & Montagnier, L. (1983) *Science 220*, 868–870.

Boosalis, M. S., Petruska, J., & Goodman, M. F. (1987) *J. Biol. Chem. 262*, 14689–14696.

Boyer, J. C., Bebenek, K., & Kunkel, T. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6919–6923.

Cheevers, W. P., & McGuire, T. C. (1985) *Rev. Infect. Dis. 7*, 83–88.

Clark, P. K., Ferris, A. L., Miller, D. A., Hizi, A., Kim, K. W., Deringer-Boyer, S. M., Mellini, M. L., Clark, A. D., Jr., Arnold, G. F., Lebherg, W. B., III, Arnold, E., Muschik, G. M., & Hughes, S. H. (1990) *AIDS Res. Hum. Retroviruses 6*, 753–764.

De Vico, A., Montelaro, R. C., Gallo, R. C., & Sarngadharan, M. G. (1991) *Virology 185*, 387–394.

Echols, H., & Goodman, M. F. (1991) *Annu. Rev. Biochem. 60*, 477–511.

Gallo, R. C., Sarin, P. S., Gellman, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, S., Sidhu, G. D., Stahl, R. E., Zolla-Razner, S., Leibowitch, J., & Popovich, M. (1983) *Science 220*, 865–868.

Goff, S. P. (1990) *J. AIDS 3*, 817–831.

Gonda, M. A., Charman, H. P., Walker, J. L., & Coggins, L. (1978) *Am. J. Vet. Res. 39*, 731–740.

Hizi, A., McGill, C., & Hughes, S. H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1218–1222.

Hubner, A., Kruhoffer, M., Grosse, F., & Krauss, G. (1992) *J. Mol. Biol. 223*, 595–600.

Hussain, K. A., Issel, C. J., Schnorr, K. L., Rwambo, P. M., & Montelaro, R. C. (1987) *J. Virol. 61*, 2956–2961.

Ji, J., & Loeb, L. A. (1992) *Biochemistry 31*, 954–958.

Joyce, C. M., Sun, X. C., & Grindley, N. D. F. (1992) *J. Biol. Chem. 267*, 24485–24500.
Mendelman, L. V., Petruska, J., & Goodman, M. F. (1990) *J. Biol. Chem. 265*, 2338–2346.
Montelaro, R. C., Parech, B., Orrego, A., & Issel, C. G. (1984) *J. Biol. Chem. 259*, 10539–10544.
Myers, G., Berzofsky, J. A., Rabsen, A. B., Smith, T. F., & Wong-Staal, F. (1992) *Human Retroviruses and AIDS*, Los Alamos National Laboratory, Los Alamos, NM.
Payne, S. L., Fang, F. D., Liu, C. P., Dhruva, B. R., Rwambo, P. M., Issel, C. J., & Montelaro, R. C. (1987) *Virology 161*, 321–331.
Perrino, F. W., & Loeb, L. A. (1989) *J. Biol. Chem. 264*, 2898–2905.
Perrino, F. W., Preston, B. D., Sandell, L. L., & Loeb, L. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8343–8347.
Preston, B. D., Poiesz, B. J., & Loeb, L. A. (1988) *Science 242*, 1168–1171.
Ricchetti, M., & Buc, H. (1990) *EMBO J. 9*, 1583–1593.
Roberts, J. D., Bebenek, K., & Kunkel, T. A. (1988) *Science 242*, 1171–1173.
Rushlow, K., Olsen, K., Stiegler, G., Payne, S. L., Montelaro, R. C., & Issel, C. J. (1986) *Virology 155*, 309–321.
Salinowich, O., Payne, S. L., Montelaro, R. C., Hussain, K. A., Issel, C. J., & Schnorr, K. L. (1986) *J. Virol. 57*, 71–80.
Sloane, D. L., Goodman, M. F., & Echols, H. (1988) *Nucleic Acids Res. 16*, 66465–6475.
Stephens, R. M., Casey, J. W., & Rice, N. R. (1986) *Science 231*, 589–594.
Thomas, D. A., & Furman, P. A. A (1991) *Biochem. Biophys. Res. Commun. 180*, 1365–1371.
Williams, K. J., & Loeb, L. A. (1992) *Curr. Top. Microbiol & Immunol. 176*, 165–180.
Yu, H., & Goodman, F. M. (1992) *J. Biol. Chem. 264*, 10888–10896.