Analysis of Mutations Made during Active Synthesis or Extension of Mismatched Substrates Further Define the Mechanism of HIV-RT Mutagenesis†

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ABSTRACT: The effect of reverse transcriptase (RT) catalyzed mutations on continued extension of the nascent DNA chain was investigated. A system using the α-*lac* gene of *β*-galactosidase as template and two sets of conditions was used. In one, RT was allowed to reassociate with the primer-template after falling off, while in a second RT was sequestered after dissociating. In the first condition, subsequent extension of errors that may have initially caused enzyme dissociation can occur. In the second, such errors would not be extended. Fully extended products were assayed by α-complementation to assess mutation frequency. A lower frequency in the latter scenario implies that some errors caused the polymerase to dissociate. Allowing only a single binding event lowered the mutation frequency of the products by about 1/2 suggesting that approximately 1 in 2 errors terminated synthesis. In other experiments, when added to a primer-template with a terminal mismatch at the 3′ end, RT dissociated from the template about 50−90% of the time (depending on mismatch type) rather than extending. Running start reactions indicated that extension was more likely if an actively synthesizing RT made the mutation. RT RNase H cleavage analysis showed that 3′ mismatches weakened the association of RT with the primer-terminus. Taken together, these results suggest that an actively synthesizing RT enzyme that has just made a mistake is likely bound in a configuration that generally enhances extension of the mistake. This is in contrast to RTs that must bind to then extend mismatches. The importance of these findings with respect to the mechanism of mutagenesis is discussed.

The human immunodeficiency virus reverse transcriptase (HIV-RT)[1] is among the most error prone of all tested polymerases (*1*−*7*). Although the error rate of RT in the cell may be less than that of the purified enzyme, it is still high (*8*). Apparently this virus, like many others, tolerates a high error rate to enhance genetic diversity. A high level of genetic diversity coupled with ongoing replication in the infected host leads to the production of many viral mutants. The mutant viruses are important in circumventing the immune response and escaping drug treatment. High genetic diversity is a major reason for the difficulty encountered when treating HIV patients (*9*).

Although mutations can occur by physical or chemical damage of nucleic acids, most HIV errors result from the low intrinsic fidelity of RT. Catalysis and fixation of a mutation involves incorporation of an incorrect base followed by extension of the growing chain (for a review, see ref *10*). After incorporation, the incorrect base may be removed by an exonuclease proofreading activity or pyrophosphorolysis (essentially the reverse reaction of polymerization). In HIV-RT, the latter activity is nearly negligible under physiological conditions, although it may be important in some drug resistant RT mutants (*11*). Also, RTs do not possess proofreading exonuclease activity (*3*). Therefore, mutations are dependent on the rate of misinsertion and the ability of RT to extend the mismatched substrate. Several reports suggest that relative to other polymerases, HIV-RT is particularly adept at extending mismatched primer-templates (*12*−*16*). Despite this, these substrates are extended about 1000−20 000 times less efficiently than matched substrates, depending on the mismatch type. One potential consequence of this and the relatively low processivity (average number of bases incorporated in a single binding event between the polymerase and substrate) of RT (*17*−*19*) is that mismatches would cause RT to dissociate from the substrate. Re-binding would be required to fix the mutation into the nascent DNA strand.

Dissociation of RT from an RNA−DNA substrate like that occurring during minus strand DNA synthesis sets up a scenario where the nascent DNA could essentially have one of four fates: (1) rebinding of RT and subsequent extension; (2) dissociation of the DNA from the RNA substrate; (3) strand transfer of the DNA to a second copy of RNA leading to recombination; or (4) excision of the mismatch by a host repair mechanism and continued extension. Clearly, the first of these is the most likely. The second would create a dead end DNA product if the dissociated DNA did not rebind to the RNA. The frequency with which this occurs is largely unknown as the proportion of virions that abort synthesis of the provirus at various stages and the reasons for replication

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[1] Abbreviations: NC, nucleocapsid protein; RT, reverse transcriptase; HIV, human immunodeficiency virus; RNase H, ribonuclease H; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; X-gal, 5-bromo-4-chloro-3-indolyl-*β*-D-galactoside; IPTG, isopropyl-*β*-D-thiogalactopyranoside; BSA, bovine serum albumin; DTT, dithiothreitol; *E. coli*, *Escherichia coli.*

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failure are unclear. The third possibility has been suggested to occur based on the fact that in vitro strand transfer assays indicate that transfer often occurs at sites where DNA synthesis pauses (pause sites). Presumably, RT would be more likely to dissociate at these sites although it is not clear that dissociation is required for strand transfer. Also, HIV nucleocapsid protein (NC) is able to catalyze transfer from substrates designed to mimic retroviral replication intermediates in the absence of RT (*20*). It has been suggested that errors made by RT may induce pausing and/or dissociation of RT and that this could lead to strand transfer (*21*−*24*). The recombination products thus produced would contain errors. Since most recombination events appear to be accurate, it is clear that most transfers do not result from an error-induced process (*25*−*29*). However, it remains possible that a proportion does. The forth scenario, although potentially possible, has not been shown to occur.

Most of the above phenomena require that RT dissociate from the substrate, and since this enzyme has relatively low processivity this occurs frequently, even in the absence of polymerase errors. For RT base misincorporations to provoke the above phenomena, these mistakes would generally have to induce dissociation also. The mismatched primer-terminus that results from the error creates an additional hurdle that is not normally encountered in dissociation-rebinding. In these cases, RT must then extend the mismatched substrate. The low efficiency of this process could potentially serve to promote scenarios 2 and 3 noted above.

In this paper, the impact of misincorporations and mismatched primer termini on the dissociation of RT from the primer-template was investigated. In agreement with the literature, RT was able to quantitatively extend mismatched termini, but extension generally required several dissociation-rebinding events relative to matched substrates. In contrast, actively synthesizing RT continued synthesis after miscincorporations more frequently with about 50% (depending on mismatch type) of errors not resulting in dissociaiton. The results imply that an actively synthesizing RT enzyme that has just made a mistake is likely bound in a configuration that often allows extension of the mistake. This is in contrast to RTs that must bind to then extend a mismatch. These results are discussed in the context of the implications of RT errors on recombination and provirus synthesis.

MATERIALS AND METHODS

Materials. Recombinant HIV-RT, having properties described (*19*), was graciously provided to us by Genetics Institute (Cambridge, MA). This enzyme had a specific activity of approximately 40 000 U/mg (one unit of RT is defined as the amount required to incorporate 1 nmol of dTTP into nucleic acid product in 10 min at 37 °C using oligo(dT)-poly(rA) as primer-template). As we have previously reported, the enzyme preparations contained very low levels of single strand nuclease activity (*30*). We found that this activity could be inhibited by including 5 mM AMP in the assays. The AMP, at this concentration, did not affect the polymerase or RNase H activity of the RT (data not shown). HIV-RT insertion mutant FE103 was kindly provided by Dr. Vinyaka Prasad (Albert Einstein University) (*31*). RNase H minus HIV-RT with a glutamate to glutamine change at position 478 ($E^{478}>Q$) was a kind gift of Dr. Stuart Le Grice through the AIDS Research and Reference Reagent Program. This enzyme has been shown to lack RNase H activity while retaining essentially wild-type DNA polymerase activity (*32*−*34*). Aliquots of HIV-RT were stored frozen at −70 °C, and a fresh aliquot was used for each experiment. T7 RNA polymerase, calf intestinal phosphatase (CIP), T4 DNA ligase, Pwo polymerase, DNase I (RNase-free), RNase (DNase-free), rNTPs, and dNTPs were obtained from Roche. T4 polynucleotide kinase (PNK) and restriction enzymes were from New England Biolabs. Placental RNase inhibitor was from Promega. Sequenase and terminal deoxynucleotide transferase (TdT) were from United States Biochemical. Oligonucleotides were synthesized by Invitrogen. All other chemicals were from Fisher Scientific or Sigma Chemical Co. Radiolabeled compounds were from Amersham.

Preparation of RNAs for Fidelity and Mismatch Extension Assays. Runoff transcription with T3 or T7 RNA polymerase was performed using the manufacturer's protocol. For the fidelity assay, plasmid pBSM13+ (Stratagene) was cleaved with *Nde*I, and T3 RNA polymerase was used to prepare runoff transcripts 765 nucleotides in length. After transcription for 2 h, 5 units of DNase I (RNase-free) was added, and incubation was continued for 20 min. The reactions were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. Material was run through two successive Sephedex G-50 spin columns, and the amount of RNA was determined using a spectrophotometer. The length and purity of the RNA was evaluated by gel electrophoresis to ensure that it was full-length (data not shown). For extension of mismatched primer-templates, the RNA was prepared using T7 RNA polymerase and plasmid pBSΔMCS (*35*) cleaved with *Mva*I. Transcription produced a 142 nucleotide RNA. The RNA was processed and quantified as described above.

RNA−*DNA Hybridization*. For fidelity assays, the 765 nucleotide RNA was hybridized to a 40 base 5′-^{32}P-labeled DNA primer (5′-GAGTGCACCATATGCCATTCAGGCTACGCAACTGTTGGGA). Note that only the last 27 bases at the 3′ end of the primer bind to the RNA template. The remaining bases comprise an *Nde*I site used in the fidelity assay (see below). The primer bound to the RNA such that extension to the end of the template would produce a 256 nucleotide DNA product. Hybridization was accomplished by mixing primer and template at approximately a 3:1 ratio, respectively, of 3′ termini in buffer containing 50 mM Tris-HCl (pH = 8), 80 mM KCl, 1 mM DTT, and 0.1 mM EDTA (pH = 8). The mixture was heated to 65 °C for 5 min and then slowly cooled to room temperature. For mismatch extension assays, the 142 nucleotide RNA was hybridized to one of five DNA primers (5′-ACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAA-X_1X_2, X_1 = G (match), A, or T (mismatches), and X_2 is not present; or X_1 = G and X_2 = C (match) or T (mismatch)) as described above. For running start assays, a 45 nucleotide version of the above primer, lacking the six 3′ bases, was bound to the 142 nucleotide RNA. Extension of the primers to the end of the template resulted in a 61 nucleotide product.

DNA Synthesis Reactions with HIV-RT for Fidelity Determinations. Primer-RNA templates (765 nucleotide RNA, 5 nM final [template]) prepared as described above and HIV-RT (50 nM final concentration) were preincubated for 3 min

at 37 °C in 21 μL of buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 80 mM KCl, 5 mM AMP (pH = 7), 0.1 mM EDTA (pH = 8), 0.2 U/μL RNase inhibitor, and 0.1 μg/μL BSA. Reactions were initiated by adding 4 μL of a supplement containing $MgCl_2$ and dNTPs in the above buffer such that the final concentrations were 6 mM and 100 μM, respectively. In some reactions, the concentration of dATP or dCTP was reduced to 1 μM. In trap assays (see Results), 2.5 μg of poly(rA)-oligo$(dT)_{20}$ (8:1, w/w) was included in the supplement to sequester RT molecules that dissociated from the substrate (*36*). Reactions without trap were incubated for 60 min at 37 °C, while those with were incubated for 10 min. Although ideally the + and −trap reactions should be run for the same time period, a longer incubation in the presence of trap was avoided to limit trap read-through. Time course analysis of trapped reactions indicated that extension was complete by 10 min (data not shown). All reactions were stopped by addition of 25 μL of 2X formamide dye (90% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol, 0.1% bromophenol blue) containing 0.5 μg of RNase (DNase free). Samples were heated to 65 °C for 5 min, then 37 °C for 5 min to digest the RNA, and then for 2 min at 90 °C. Samples were electrophoresed on 8% denaturing polyacrylamide gels as described below. Wet gels were exposed to film, and products were excised and eluted by the crush and soak method (*37*). Some gels were dried and used for autoradiography.

Gel Electrophoresis. Denaturing 8 or 10% polyacrylamide sequencing gels (19:1 acrylamide/bis-acrylamide), containing 7 M urea, native 8% gels (29:1 acrylamide/bis-acrylamide), and 0.8% agarose gels were prepared and subjected to electrophoresis as described (*37*).

α-*lac-Based Fidelity Assay*. Products from reactions were excised and eluted from denaturing gels as described above. The eluted DNA was amplified by PCR using the following primers: 5′-GAGTGCACCATATGCCATTCAGGCTACG-CAACTGTTGGGA and 5′-GCTCGAATTCGCCCTAT-AGTGAGTC-3′. The first is identical to the primer used to prime the template, while the second overlaps the *Eco*RI site on the plasmid used to make the template RNA (*29*). Reactions were performed in 100 μL of 10 mM Tris-HCl (pH = 9 at 25 °C), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 200 μM dNTP, 100 pmol of each primer, and 5 U of Pwo polymerase. Thirty-five cycles of 94 °C (1 m), 50 °C (1 m), and then 72 °C (1 m) were performed. Products were extracted, precipitated, and double-digested for 1.5 h with 50 U each of *Eco*RI and *Nde*I in New England Biolabs optimal buffer for *Eco*RI. The samples were then electrophoresed on an 8% native polyacrylamide gel as described above. Products were located by UV shadowing, recovered as described above, and quantified by spectrophotometry. These products were ligated into plasmid pBSM13+, which had been previously cleaved with *Eco*RI and *Nde*I. The plasmid vector was prepared by cleaving 40 μg of pBSM13+ with 200 units each of *Eco*RI and *Nde*I for 2 h at 37 °C. Material was run on a 0.8% agarose gel with 0.5 μg/mL ethidium bromide. The large fragment from the cleavage was located, excised, and recovered by electro-elution into a dialysis bag followed by precipitation (*37*). The fragment was then treated with 5 U of calf intestinal phosphatase using the manufacturer's protocol. Material was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and recovered by ethanol precipitation. The vector (150 ng, approximately 0.075 pm) and insert (0.15 pm) were ligated using a Fast-Link DNA Ligation Kit (Epicenter Technologies). Five μL of the ligation was used to transform 50 μL of *E. coli* DH-5α competent cells (PGC Scientific). Transformed cells were plated with carbenicillin (100 μg/mL) in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG). Colonies were analyzed and scored as blue, faint blue, or white (*38*). For results presented as $X \pm Y$, X was an average value from at least three experiments, while Y is the standard deviation.

Mismatch Extension Assays. Matched or mismatched primer-templates (142 nucleotide RNA, 5 nM final [template]) prepared as described above and HIV-RT (approximately 50 nM final concentration) were preincubated for 3 min at 37 °C in 10.5 μL of buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 80 mM KCl, 5 mM AMP (pH = 7), 0.1 mM EDTA (pH = 8), 0.2 U/μL RNase inhibitor, and 0.1 μg/μL BSA. Reactions were initiated by adding 2 μL of a supplement containing $MgCl_2$ and dNTPs in the above buffer such that the final concentrations were 6 mM and 100 μM, respectively. In some assays, the concentration of KCl was reduced as indicated. In trap assays (see Results), 1.25 μg of poly(rA)-oligo$(dT)_{20}$ (8:1, w/w) was included in the supplement to sequester RT molecules that dissociated from the substrate. Reactions without trap were incubated for 30 min at 37 °C, while those with were incubated for 10 min. Longer incubations in the presence or absence of trap did not change the level of extended primers, and time course reactions showed that extension in the trapped reactions was completed by about 2 min after initiation (data not shown). All reactions were stopped by addition of 10 μL of 2X formamide dye. Samples were heated for 2 min at 90 °C and then electrophoresed on 10% denaturing polyacrylamide gels as described above. Gels were dried and used for autoradiography or quantified using a Phosphoimager (BioRad 525). For results presented as $X \pm Y$, X was an average value from at least three experiments, while Y is the standard deviation.

Running Start Assays. Reactions were performed as described above for the mismatch extension assays except that the 142 nucleotide RNA template was bound to a 45 nucleotide primer (see *RNA−DNA Hybridization*). Also, specific nucleotides were omitted from some assays as indicated in the figure legends. For results presented as $X \pm Y$, X was an average value from at least three experiments, while Y is the standard deviation.

Sequencing of Products from Running Start or Mismatch Extension Reactions. Extended products from running start or mismatch extension assays were sequenced using the RACE (Rapid amplification of cDNA ends) method (*39*). Three primers were used, 5′-GGCGAATTCAAGTCGAC-CTGTTTTTTTTTTTTTTTTTTT-3′ (primer 1), 5′-GGC-GAATTCAAGTCGACCTG-3′ (primer 2), and 5′-ACCA-TGATTACGCCAAGCTCGAAATTAACCCTCACTAA-AGGGAAC-3′ (primer 3). Primer 3, which was phosphorylated with T4 PNK at the 5′ end, was the same primer used to prime the running start reactions, while primer 2 is homologous to primer 1 for the first 20 nucleotides and contains an *Eco*RI restriction site. Extension products (extended to the end of the template in mismatch extension assays or beyond the misincorporation point for running start

assays) were excised from denaturing gels and recovered by the crush and soak method (*37*). The material was tailed at the 3′ end with A residues using terminal deoxynucleotide transferase (TdT). An aliquot of the tailed material was used in a PCR reaction using Pwo polymerase and the three primers described above in the supplied reaction buffer. An initial extension phase using primer 1 (10 pmol) was carried out at 72 °C. Primers 2 and 3 (100 pmol each) were then added, and reactions were continued for 35 cycles of 94 °C (40 s), 55 °C (1 min), and 72 °C (30 s). A final cycle was carried out for 5 min at 72 °C. For details, refer to ref *39*. Samples were extracted and precipitated and then cleaved with *Eco*RI (50 units). The cleaved PCR products were recovered on a 15% native acrylamide gel. Products appeared as a smear because of the TdT tailing procedure. Shorter products (with shorter tails) were excised and eluted as described above. The recovered material was cloned into vector pBSM13+ that was previous cleaved with *Eco*RI and *Hind*II and dephosphorylated with CIP. Ligations and transformations were carried out as described above for the fidelity assay. Colonies were picked, and mini-prep DNA was prepared using spin columns (Qiagen) according to the manufacturer's protocol. The insert DNA was sequenced using the primer 5′-GGAAACAGCTATGACCATGA-3′ and Sequenase according to the manufacturer's protocol.

RESULTS

System Used to Measure HIV-RT Fidelity and Assay Theory. The substrates used to analyze the fidelity of RT have been described previously (*29*). In these experiments, a small variation of the previous system was employed (see Materials and Methods). The error rate of RT during synthesis over the α-*lac*-based template can be measured after converting the fully extended single stranded DNA synthesis product to double stranded DNA by PCR (see ref *29* for an overview). Fidelity is then determined using blue-white colony screening with white or faint blue colonies scored as mutations and blue colonies as correctly synthesized. HIV-RT reactions were carried out in the absence or presence of excess poly(rA)-oligo(dT) trap. The trap sequesters enzyme molecules that dissociate from the substrate thus limiting synthesis to a single binding event between the enzyme and the substrate (termed processive DNA synthesis) (*36*). Since only fully extended DNA synthesis products are used to measure fidelity, mutations observed in assays performed with trap should represent only those that did not induce dissociation of the polymerase from the substrate. In the absence of trap, RT can rebind incomplete DNA synthesis products and complete extension. These products would include both errors produced during processive synthesis and those that induced dissociation (although the results do not rule out the possibility that RT remains associated with the mismatch in a dead-end complex (see Discussion)) as well as those produced after rebinding of RT. Assuming the error rate along the template does not change depending on where RT reinitiates synthesis (i.e., the error rate over a particular sequence will be the same whether RT is synthesizing over or initiates synthesis within that sequence (see Discussion)), an increase in mutations in reactions without trap would represent errors that had induced RT dissociation. The percent of mutations that induced dissociation can be calculated by dividing the difference between the colony mutation frequencies ((white + faint blue colonies)/(total (white + faint blue + blue)) of reactions without (−trap) or with (+trap) trap by the error rate of reactions without trap ((−trap) − (+trap)/(−trap − BKG)), then multiplying by 100. BKG denotes experimental background calculated as explained below. Note that this calculation assumes that all errors that induce dissociation can be subsequently extended to the end of the template upon RT rebinding. Results suggested that most errors would be subsequently extended (see below).

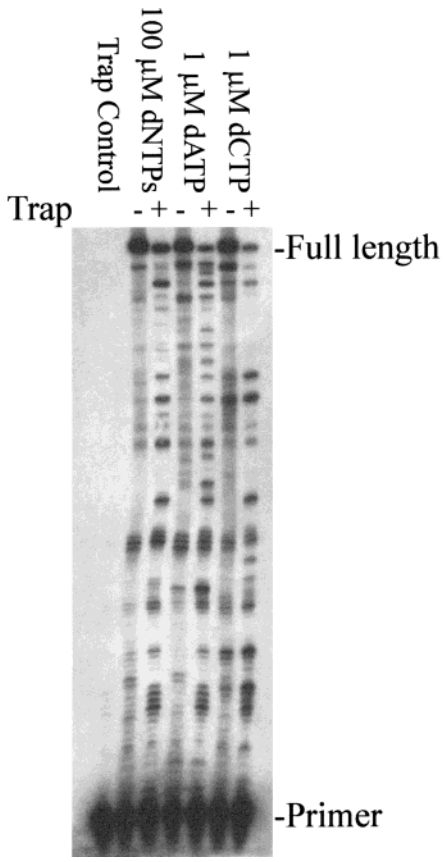

FIGURE 1: DNA synthesis on the α-lac RNA template. Shown is an autoradiogram of samples run on a 6% denaturing gel using the 40 nucleotide 5′-^{32}P end labeled DNA primer bound to the 765 nucleotide RNA template. Reactions were performed in the presence or absence of poly(rA)-oligo(dT) trap as indicated (see Materials and Methods). The Trap Control reaction was used to test the effectiveness of the trap. This reaction was performed by premixing the enzyme with the trap prior to addition to the substrate-containing reaction mix. The positions of the labeled primer and fully extended 256 nucleotide product are indicated. Assays were preformed with 100 μM of all four dNTPs or with 1 μM dATP or dCTP along with 100 μM of the other three dNTPs (as indicated).

DNA Synthesis in Fidelity Assays. Figure 1 shows an autoradiogram from a primer extension reaction performed as described under Materials and Methods with 100 μM of each dNTP or 1 μM dATP or 1 μM dCTP and 100 μM of the other three nucleotides. As expected, reactions with trap yielded significantly lower levels of full-length products than those without. There were also variations in the pause sites observed in reactions with and without trap. This presumably results from RT extending paused products by rebinding in the assays without trap. Also, there are clear differences in the profile depending on the nucleotide conditions with more

Table 1: Colony Mutation Frequencies and Dissociation Rates from Fidelity Assays with a RNA Template

experiment no.[a]	100 μM dNTPs[b]		%	1 μM dATP		%	1 μM dCTP		%
	−trap	+trap	dissociate[c]	−trap	+trap	dissociate	−trap	+trap	dissociate
1	0.024	0.014	63	0.106	0.093	13			
2	0.026	0.015	50	0.116	0.088	26	0.088	0.070	23
3	0.023	0.015	53	0.074	0.065	14	0.055	0.048	15
4	0.028	0.014	70	0.071	0.055	25	0.056	0.037	40
5	0.018	0.013	50	0.051	0.044	16			
6	0.022	0.016	43						
7	0.024	0.014	63						
8	0.032	0.021	46						

[a] Experiments were performed independently with at least 1000 colonies counted for each result. Refer to Materials and Methods for details. [b] Reactions with 1 μM dATP or 1 μM dCTP contained 100 μM of each of the other three dNTPs while those listed under 100 μM dNTPs contained 100 μM of each dNTP. [c] Value is the percent of RT molecules that dissociate from the template after making a mutation. The approximate background value for the colony mutation frequency was 0.008 (see Results). The calculation was (colony mutation frequency (−trap) − colony mutation frequency (+trap)/(colony mutation frequency (−trap) − BKG)) × 100. See Results for details of the calculation theory.

pausing evident in trap reactions with 1 μM dATP or dCTP. Again, this would be expected as inclusion of a single nucleotide at levels well below the enzyme K_m value would induce pausing at positions where that nucleotide is incorporated. Not all products are extended to the end of the template even in the absence of trap. This is typical with RT reactions (*18*, *19*). The validity of the fidelity calculations described above is not dependent on all products being extended in reactions without trap but that there is no strong bias against extending products with mismatched 3′ termini (see below). Products running in the region of fully extended DNAs were excised from the gels and used to determine the mutation rate for the reactions.

Colony Mutation Frequencies Indicate that Mutations Result in RT Dissociation Approximately 50% of the Time. Table 1 shows the colony mutation frequencies ((white + faint blue colonies)/total colonies) from experiments along with calculated rates of mutation-induced dissociation for each experiment (see below). Results in the absence of trap are similar to those found in previous experiments (*22*, *29*). The mutation rate per base copied can be estimated from these results as described previously (*40*). Clearly, reactions performed in the presence of trap showed lower frequencies than the corresponding reactions without trap. This indicates that some of the mutations resulted in dissociation of the polymerase. To estimate the proportion of times that RT dissociated after making a mistake, it was necessary to determine the assay background. The background in this case would include all errors except those made by RT. These include errors made by the PCR polymerase, errors resulting from the ligation and manipulation of DNA, and errors from T3 RNA polymerase. Since the RNA template cannot be used for PCR without conversion to DNA, it is not possible to directly determine the background. The upper limits of the background can be estimated by using an enzyme with high fidelity to produce a cDNA from the RNA template. An HIV-RT insertion mutant (FE103) reported to produce less frameshift errors than wild type was used for this (*31*). Results showed a mutant colony frequency of 0.013 ± 0.002 (three experiments ± standard deviation) for this enzyme. This result is similar to that obtained using the Klenow fragment of *E. coli* polymerase I (*29*) and is significantly lower than values for wild-type RT in the absence of trap indicating that a significant proportion of the errors observed in the assays with wild-type enzyme result from HIV-RT. Since the error rate of FE103 is clearly not 0, this value is an overestimate of the actual background. The lower boundary for the assays was estimated by performing PCR on plasmid DNA and using the isolated inserts in the fidelity assay. A value of 0.007 ± 0.002 (four experiments ± standard deviation) was obtained by this approach. This should include all errors in the assay except those made by T3 RNA polymerase during synthesis of the template. Estimates for T3 have been made by others and suggest that this enzyme makes less than 1 error in 37 000 base incorporations in α-*lac*-based assays (*41*). Using 35% as the proportion of errors detectable in the assay (*38*) and a target size in the α-*lac* region in our assay of about 127 bases, this corresponds to a mutant colony frequency of about 0.001 (127/37 000 × 0.35). Therefore, T3 RNA polymerase likely contributes very little to the error background in the assay. The estimated background value used in the calculations was 0.008, which is the sum of the values for the PCR reactions of the plasmid DNA, plus the small increment contributed by T3 RNA polymerase.

As can be seen in Table 1, there was some variation in the mutant colony frequencies between experiments as is typical in these types of experiments (*22*, *29*). Some of the variation is likely due to new templates and vector material being prepared for each set of experiments. However, the values for + and − trap reactions were clearly different as judged from p value analysis ($p = 0.001$ for experiments with 100 μM dNTPs). For reactions with 100 μM of each dNTP, nearly all the experiments showed similar trends with an approximately 50% decrease in mutation frequency in the presence of trap (after background correction). These results indicate approximately one in two RT molecules dissociated after a mutation. Because of the nonexact nature of the background estimate and to limitations noted above, the value of 50% is clearly only an estimate. Despite this, it is evident that mutations do not strongly influence RT to dissociate from the substrate nor are they insignificant with respect to this.

Other experiments were performed with limiting levels of dATP or dCTP (1 μM with 100 μM of each of the other 3 dNTPs). These conditioned significantly increased the mutation frequency as expected (Table 1). Because of this, the assay background levels become essentially insignificant. The calculated dissociation rates for RT in these cases were less than 50% averaging about 19 and 26% for limiting dATP and dCTP, respectively.

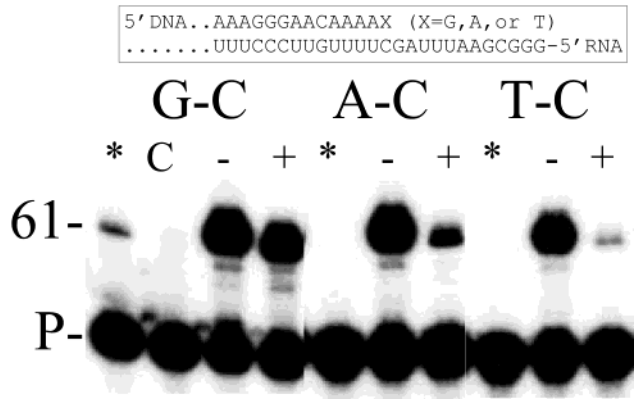

FIGURE 2: Extension of preformed matched or mismatched primer-templates with HIV-RT using 80 mM KCl. Shown is an autoradiogram of samples run on a 10% denaturing gel using 50 nucleotide 5′-^{32}P end-labeled DNA primers bound to the 142 nucleotide RNA. Three different primers were used. The primers contained different terminal 3′ bases (either G, A, or T) that formed a G−C match with the template or A−C and T−C mismatches (as indicated). A schematic of sequences in the region of the primer terminus is shown in the box at the top of the figure. Reactions were performed in the presence (+) or absence (−) of poly(rA)-oligo(dT) traps as indicated (see Materials and Methods). The lane labeled * was as described in Figure 1 for Trap Control. The lane labeled C had no enzyme added to the reaction. Extension of the primer to the end of the substrate produced a 61 nucleotide product (as indicated). Dried gels were quantified using a phosphoimager, and results are presented in the Results section.

Extension of Mismatched Primer-Templates Suggests that RT Generally Dissociates Rather than Extending the Mismatch. HIV-RT's propensity to extend mismatches is well-documented (see introductory paragraphs). The above experiments indicated that despite this, about 1/2 of the RT molecules dissociate from the substrate after catalyzing a mutation. In those assays, the RT molecule was actively synthesizing along the template. To determine how RT would cope with mismatched primer-templates upon rebinding to the substrate, primer-templates with terminal 3′ mismatches were used. Three types of substrates with 50 nucleotide 5′-^{32}P end-labeled primers bound to the same RNA were initially used (Figure 2). One had a correctly matched G−C at the 3′ terminus, while two others had mismatches (A−C or T−C). RT was allowed to pre-bind to each substrate, and primer extension was initiated in the presence or absence of trap. In the absence of trap, all three substrates showed a high level of extension indicating that the mismatch did not represent a barrier to extension given sufficient time and a high level of enzyme (lanes marked − trap). An alternative explanation is that the mismatch was excised prior to extension leading to extension of a correctly matched end. This could result from contaminating exonuclease activity in the enzyme preparation or pyrophosphorolysis (essentially the reverse of RT's incorporation activity) (*42*−*45*) or other chain unblocking activities that HIV-RT possesses (*46*). Of these, the first and last seem most likely as the conditions employed in the reactions should have prevented pyrophosphorolysis. To test this, fully extended products from the A−C, T−C, and T−G (see below) mismatch reactions performed in the absence of trap were cloned and sequenced (see Materials and Methods). For the A−C mismatch, 18 of 20 clones had retained the mismatched A residue, while two had lost it (*21*). The small proportion (1/10) that had lost the A residue could possibly be explained by a weak exonuclease or chain unblocking activity. In any case, the vast majority of synthesis resulted from extension of the mismatched substrate. For the T−C and T−G mismatches, 10 and 12 clones, respectively, were sequences. All had retained the mismatched base.

In contrast to extension without trap, in the presence of trap (lanes marked + trap in Figure 2) the G−C matched substrate was extended with much greater efficiency than the A−C and T−C mismatches. Relative to extension with wild-type RT in the presence of trap on the G−C match, the A−C mismatched and T−C mismatched substrates showed extension of 13 ± 2 and 5 ± 2%, respectively. Essentially, including trap in the reactions sets up a situation where the bound RT molecule will have only one chance to extend the primer. If it is assumed that the match is extended 100% of the time when RT binds and that RT binds the mismatched and matched substrates equally (see below), these results indicate that dissociation is much more likely than extension on the mismatched substrates.

Mismatched Substrates Are Extended More Efficiently with Lower Salt Concentrations. Extension of the matched and A−C mismatch substrates was further investigated using various salt concentrations. Two additional substrates were also used. Both consisted of 51 nucleotide DNA primers bound to the RNA used above. The primers were identical for the first 50 bases from the 5′ end to the G−C matched primer above. The 51st base was either a C (C−G matched) or T (T−G mismatch). Salt concentrations were 80 mM as in the experiments shown in Figure 2, or 40 or 10 mM. The lower salt stabilizes the binding of the RT to the primer-template resulting in a decrease in the enzyme off-rate (*47*). An autoradiogram from a typical experiment is shown in Figure 3. Using 40 mM KCl, the proportion of the A−C mismatch that was extended relative to the G−C match was 37 ± 2%, while with 10 mM KCl extension increased to 55 ± 4%. Note that relative extension at 80 mM KCl was 13 ± 2% (see above). Clearly, decreasing the salt concentration increases the likelihood of extension. This is probably because of the relatively slow rate of nucleotide addition for mismatches versus matched substrates being partially overcome by the slower off-rate for RT in low salt. The 51 nucleotide T−G mismatch was also extended less efficiently than the corresponding match. However, in this case the difference was much smaller with just over 50% extension relative to the match at 80 mM KCl and 100% at 10 mM (two independent determinations yielding essentially the same results). It was interesting that two substrates that are nearly identical except for one additional base would show such a large diversity.

Also shown in Figure 3 is the extension of the substrates with an RNase H minus version of HIV-RT (mutant $E^{478}>Q$ (see Materials and Methods), lanes marked R− in Figure 3). This enzyme was used to determine the maximum amount of primer that could be extended on each substrate. Since $E^{478}>Q$ has no RNase H activity, the possibility of cleavage-induced dissociation of the DNA and RNA is eliminated. About the same level of all three substrates was extended by $E^{478}>Q$, again showing that the mismatches do not present a barrier to primer extension in the presence of excess enzyme and given sufficient time. In comparison to the mutant, the level of extension by wild type RT was dependent

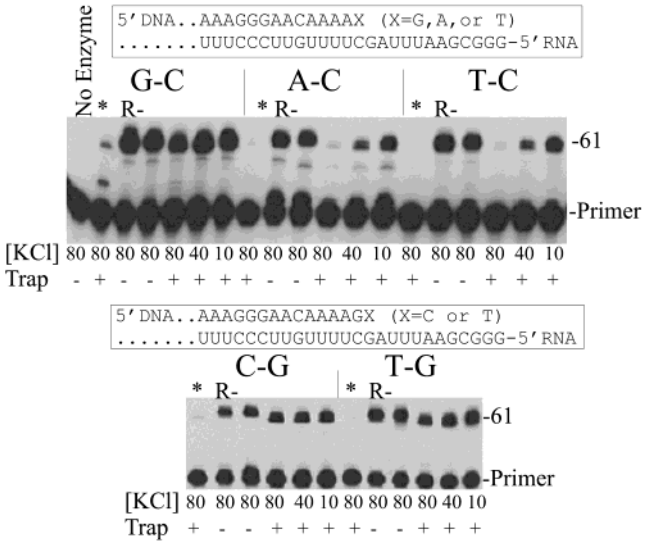

FIGURE 3: Extension of preformed matched or mismatched primer-templates with HIV-RT using different salt concentrations. Shown is an autoradiogram of samples run on a 10% denaturing gel using 50 (G−C, A−C, and T−C) or 51 (C−G or T−G) nucleotide 5′-^{32}P end-labeled DNA primers bound to the 142 nucleotide RNA. The 50 base primers were as described in Figure 2, while the 51 base primers were identical to the G−C primer for the first 50 nucleotides from the 5′ end, while the 51st nucleotide was either C (C−G match) or T (T−G mismatch). A schematic of sequences in the region of the primer terminus is shown in the box at the top of the figure. All reactions contained wild-type HIV-RT except those in lanes labeled R− that were performed with RNase H minus RT (HIV-RT$^{E478>Q}$, see Materials and Methods). Reactions were performed in the presence (+) or absence (−) of poly(rA)-oligo-(dT) traps as indicated. The concentration of KCl in the reactions in mM is indicated below each lane. Lanes labeled * were as described in Figure 1 for Trap Control. Extension of the primer to the end of the substrate produced a 61 nucleotide product (as indicated). Dried gels were quantified using a phosphoimager, and results are presented in the Results section.

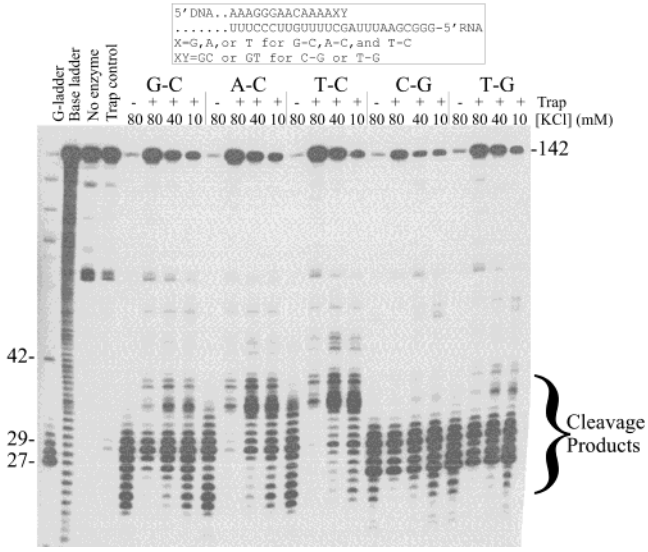

FIGURE 4: RNA cleavage with matched and mismatched preformed primer-templates. Shown is an autoradiogram of samples run on a 10% denaturing gel using 50 or 51 nucleotide DNA primers bound to 5′-^{32}P end labeled 142 nucleotide RNA. Five different primers were used as described in Figure 3. A schematic of sequences in the region of the primer terminus is shown in the box at the top of the figure. Reactions were performed in the presence (+) or absence (−) of poly(rA)-oligo(dT) trap as indicated (see Materials and Methods). The concentration of KCl in the reactions is indicated above each lane. The Trap Control was performed with the G−C substrate as described in Figure 1. Positions of cleavage products and the 142 nucleotide RNA template (142) are indicated. The G-ladder was produced by limited digestion the template RNA with RNase T1, while the Base ladder was produced by base hydrolysis of the template for 30 s at 65 °C in the presence of 100 mM NaOH. Quantification from phosphoimager analysis of experiments is reported in the Results section.

on the type of substrate used. With matched primer-template, wild type and RNase H mutant enzymes showed essentially no difference in extension (100 ± 6%), while a 15−20% reduction was observed with the mismatches (83 ± 5 and 80 ± 4% for the A−C and T−C mismatches, respectively). This result points out that mismatched termini, although generally extended by RT, sometimes are not. The small proportion of primers that are not extended may dissociate from the template because of RNase H activity. This activity is likely more pronounced on the mismatched substrates since the rate of extension on these substrates is much slower, and RNase H and polymerase activity are uncoupled (*7, 36*). The fact that the majority of primers are extended lends support to the assumption that most mutations that cause dissociation in the fidelity assay are ultimately extended by RT in the absence of trap (see above).

RNase H Cleavage Assays Show that RT Orients Differently on the Mismatched versus Matched Substrates. A possible explanation for the lower level of extension observed with the mismatches in the presence of trap is that RT simply binds relatively poorly to the mismatched primers. To test this, the RNA portion of the substrate was 5′ end-labeled with ^{32}P, and assays in the presence or absence of trap with various concentrations of KCl (as indicated) were performed (Figure 4). Essentially all of the RNA was cleaved in assays without trap (lanes labeled − trap) as expected. In assays with trap (lanes labeled + trap), the level of substrate cleaved was dependent on the type of substrate used and the salt concentration. Relative to the matched substrate, less RNA was cleaved with the mismatches at the corresponding salt concentration. For the 50 nucleotide A−C mismatch, using 80 mM KCl about 1/2 as much material was cleaved relative to the G−C match (50 ± 9%). At lower salt concentrations, the proportions increased to 88 ± 4 and 97 ± 6%, for 40 and 10 mM KCl, respectively. The T−C mismatch showed similar results. Assuming that RT cleaves the substrates equally (although this is not known) and that each binding event results in cleavage, these results indicate that RT binds somewhat less efficiently to the mismatches. On the basis of these numbers the above determinations of the level of extension in the presence of trap on the A−C and T−C mismatch, substrates can be corrected for the lower binding to the mismatches. Dividing the previously determined numbers by the proportional decrease in binding accomplishes this. Values of approximately 26% (13/0.5) and 10% (5/0.5) relative to the G−C match for the A−C and T−C mismatches, respectively, can be calculated. These results still clearly indicate that HIV-RT dissociates more times than not when attempting to extend this mismatch under the higher salt conditions. In contrast, the 51 nucleotide T−G mismatch was cleaved at approximately the same level as the C−G match at all the tested salt concentrations. Note that this substrate was also extended more efficiently than the other mismatches that were tested (see above).

Also notable in the RNase H cleavage assays was the change in the distribution of cleavage products with the matched versus mismatched substrates for the 50 base A−C and T−C mismatches. The most prominent cleavage product in trapped assays with matched substrate was about 29 nucleotides in length (Figure 4, determined by base hydrolysis and RNase T1 cleavage of the substrate RNA). This product corresponds to cleavage 18 bases behind the 3′ primer terminus (−18) and is consistent with the polymerase site of RT binding at the 3′ terminus (*48*−*52*). Other larger products corresponding to −22 (33 base product) −24 (35 base product) cleavages were also observed. These cleavages must occur when the polymerase site is not bound at the terminus. All these products are also visible in the lanes with mismatched substrates, but the proportions are significantly shifted. Relative to the −22 and −24 products, the −18 product is greatly reduced with the mismatches. In contrast, the 51 base T−G mismatch showed a cleavage profile nearly identical to the corresponding matched substrate. A small increase in larger cleavage products was also noted, but most products were consistent with cleavages from RT molecules bound at or near the 3′ DNA terminus. The result suggests that RT has more difficulty associating with the 3′ terminus of the primer if it is mismatched, but the type and/or context of the mismatch also plays a role.

Mutations Made in Running Start Reactions Are More Frequently Extended than Those on Mismatched Primer-Templates. To examine extension of the mismatches tested above during active synthesis by RT, a second form of the mismatch extension assay that more closely mimicked synthesis in the fidelity assay was employed. In these running start reactions, one of the four dNTPs is excluded forcing misincorporation at positions complementary to the excluded dNTP (*16*). The polymerase is allowed to start synthesis by including the nucleotide(s) following the 3′ primer terminus. Synthesis continues unimpeded until positions requiring the deleted nucleotide are reached. At this point, RT must misincorporate to continue synthesis. By measuring the level of nucleotide extension at the point where misinsertion must occur and comparing this to extensions beyond this point, the proportion of RTs that continue after misincorporation can be determined. In these experiments, the same template used in the mismatch extension assays was hybridized to a 5′-^{32}P end-labeled primer that was five bases shorter than those used for the mismatches (Figure 3). Four U residues followed the base on the template that is bound to the 3′ primer terminus (Figure 5). The next template base, C, was the base used to study mismatch extension in the above experiments for the A−C and T−C 50 base mismatch substrates. G, A, and then a U followed the C residue. Running-starts were performed by including 100 μM each of dATP, dCTP, and dTTP or dATP, dGTP, and dTTP. This approach forced mutations to occur at either the C or the G residues after the run of Us. Trap was included in reactions used to determine the proportion of RTs that dissociate after a mistake. When dCTP was excluded from the reactions, about 85−90% of the RT molecules that added a base at the misincorporation point proceeded through without dissociating from the substrate (data not shown). However, sequencing of the products indicated that about 1/2 of the read-through resulted from a C residue being added opposite the G. This must have resulted from a low-level contamination of one of the other nucleotides with dCTP. Therefore, it was not possible to accurately assess these experiments. In contrast, sequencing of the products from reactions without dGTP showed that only 1 of the 18 products sequenced had incorporated a G opposite the C. Ten had an A residue in this position (A−C mismatch) and seven had a T (T−C mismatch). Therefore, the running start assays without dGTP can be reliably used to evaluate extension of mismatches during active synthesis. Results from a typical assay are shown in Figure 5. The influence of the excluding dGTP was evident as a strong termination site was observed one base in front of the position requiring dGTP, even in reactions in the absence of trap. In the presence of trap, read-through increased as the concentration of salt decreased, although the level of products extended to the mutation point remained relatively constant. Results indicated that the percent of RTs that dissociated after misincorporation was 49 ± 2, 39 ± 2, and 34 ± 1% for reactions using 80, 40, and 10 mM KCl, respectively. Overall, the results indicate that actively synthesizing RTs dissociate less frequently than those attempting to extend a preformed mismatch. Comparing read-through in the running start reactions at 80 mM KCl to corrected values for extension of the A−C and T−C mismatches (26 and 10%, respectively) indicates that dissociation is about 45 (49−26%/49%) to 80% (49−10%/49%) more likely when extending the preformed mismatch.

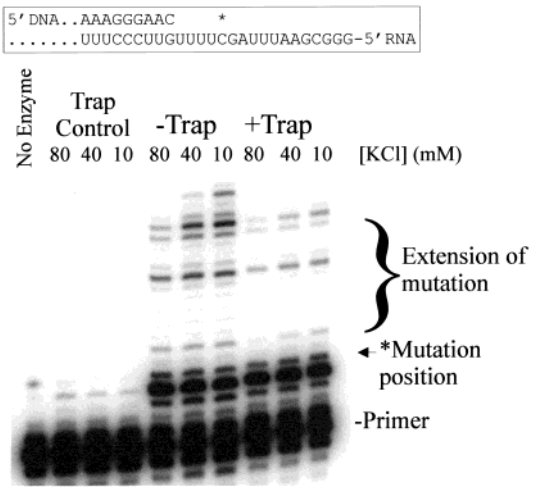

FIGURE 5: DNA extension in running start reactions with HIV-RT. Shown is an autoradiogram of samples run on a 10% denaturing gel using a 45 nucleotide 5′-^{32}P end-labeled DNA primer bound to the 142 nucleotide RNA. These reactions were performed as described under Running Start reactions in the Materials and Methods. A schematic of sequences in the region of the primer terminus is shown in the box at the top of the figure. An asterisk (*) is positioned over the C residue in the template where mutations are forced to occur in the reaction. Reactions contained 100 μM each of dATP, dCTP, and dTTP, while dGTP was omitted. Assays were performed in the presence (+Trap) or absence (−Trap) of poly(rA)-oligo(dT) trap at the indicated KCl concentration. Trap controls were performed as described in Figure 1. The migration position of products extended to the C residue on the template is indicated (*Mutation position), as are the primer and products extended beyond the mutation. Quantification from phosphoimager analysis of experiments is reported in the Results section.

DISCUSSION

HIV-RT Dissociates About 1/2 the Time After Misincorporations. In this report, the ability of HIV-RT to extend

primer-templates with 3′ terminal mismatches was examined. The goal was to determine if these mismatches result in dissociation of RT from the substrate. The released mismatched substrates could potentially be used to produce recombinants that contain mutations or lead to aborted replication (see introductory paragraphs). Results indicated that the fate of RT on mismatched substrates depended on whether the enzyme had actively synthesized the mismatch or was reassociating with a preformed mismatch and depended on the type and context of the mismatch. The fidelity assay essentially averages probabilities over several sequences. These assays indicated that RT dissociated about 1/2 the time after making a mistake when saturating concentrations of all four dNTPs were used (Table 1). Since the background in these assays can only be estimated and the calculation assumes that mutation that caused dissociation events in the absence of trap are ultimately extended to the end of the template (see Results), the calculated dissociation rate is clearly an estimate. However, while the running start type assay used only a single sequence region, the mutation-induced dissociation under the same assay conditions (80 mM KCl) was also about 50% (49 ± 2%). Given that HIV-RT can normally carry out processive synthesis (processivity is defined as the average number of nucleotides incorporated during a single binding event between the enzyme and the primer-template) for up to a few hundred bases (*17*), the 50% dissociation rate indicates that mutations are relatively potent terminators of synthesis. Even if the rate were closer to the 19−26% range (see Results) calculated in reactions with lowered levels of dATP and dCTP (see Table 1), this would still support the notion that mutations substantially increase the likelihood of RT dissociating.

The lower dissociation rates in reactions with limiting dATP or dCTP in comparison to those with 100 μM of each dNTP could have been due to relatively poor extension of mismatches even in the absence of trap; an effect brought on by having one of the dNTPs present at a concentration well below the enzyme's K_m value. In this case, the values for mutation frequency without trap would underestimate the actual level of mutations made since some may not have been extended to the end of the template. It should also be noted that using a limiting level of one dNTP skews the types of mutations that are occurring. For example, with limiting dATP mutations at Us or runs of U on the template would make up the majority. Reactions with saturating concentrations of all the dNTPs would produce a more random population of errors; thus, results are averaged for several types of mutations. Therefore, although the calculations are more precise because of mutation frequencies being well above background, the actual determined percent dissociation value is probably less meaningful. However, keeping in mind that the trap limits RT to a single binding event, the experiments with limiting nucleotide clearly show that mutations frequently do not result in dissociation. The mutation rates were much higher than those with 100 μM of each dNTP, and the background would have been the same for each type of experiment. Therefore, RT produced and extended many mutations in these assays without dissociating.

Running Start Assays Suggest that Actively Synthesizing RTs Dissociate Less Frequently than Those Rebinding on a Mismatched Substrate. Also notable was the clear difference between the levels of extension for the preformed mismatches versus the same mismatches in running start assays (about a 2-fold or greater difference as noted in the Results). The increased propensity to continue without dissociating in the latter may be due to the nature of the assays. To extend the preformed mismatches, the enzyme must bind to the mismatched substrate and then position the polymerase active site at the 3′ terminus and catalyze extension. In the running start and fidelity assays, a polymerase molecule that has just incorporated a mismatch would already be positioned at the terminus, poised for extension. This may favor extension in these assays. In support of this idea, RNase H cleavage assays indicated that RT's propensity to bind to the 3′ recessed terminus was much less pronounced on the 50 nucleotide A−C and T−C mismatches than on the corresponding G−C match substrate (see Figure 4). The greater proportion and amount of cleavage at positions inconsistent with RT associating with the 3′ end of the primer may have resulted from a decreased affinity for the mismatched relative to the matched end, an idea supported by the decrease in total cleavage in trapped assays with 80 mM KCl. In any regard, the results suggest that one reason for the low relative extension in assays with preformed mismatches is that a higher proportion of RT molecules are not bound in a position where extension can occur. For misincorporations occurring during active synthesis, the polymerase may be momentarily locked at such a position, increasing the likelihood of extension. In addition, in many cases an incorrect 3′ terminal base may not actually be part of a mismatch immediately after it is inserted. Many mutations are likely catalyzed through primer or template slippage mechanisms (*10*). This is especially true near runs of the same nucleotide. In these mechanisms, temporary misalignment of the primer and template allows the mutation to be added as a complementary base. Subsequent realignment yields the substitution, deletion, or insertion mutation. In such a mechanism, the polymerase would not actually be extending a mismatch, as was the case in the assays with preformed mismatches. Matched bases would be extended if extension occurred before realignment. It was not possible to test this in the running start assay since omission of the nucleotide following the run (dGTP in this case) would likely impact such a mechanism.

Curiously, the substrate with the 51 nucleotide primer and T−G mismatch behaved quite differently from the mismatches discussed above. Relative to the corresponding C−G matched substrate extension of preformed mismatch was 50% at 80 mM and essentially 100% at 10 mM KCl (Figure 3). This was a considerably higher level of extension in comparison to the A−C and T−C mismatches discussed above. Cleavage assays showed little difference in either the amount or the types of cleavage products that were generated from the T−G mismatch versus the C−G match (Figure 4). It appears that the polymerase domain of RT is able to bind to the mismatched terminus similarly to the corresponding match. This despite the fact that the primer used is only one base removed from the primers used to make the A−C and T−C mutations. One possible explanation is that this mismatch comes after a correctly matched G−C pair, while the A−C and T−C mismatches follow a run of four A−U pairs. The nucleotide run followed by a mismatch would make the 3′ terminal region less thermodynamically stable

and more slippery, thus possibly effecting RT's ability to recognize the terminus. The G−C pair following the A−U run may serve to clamp and stabilize the terminus.

The level of extension of mismatched termini in both preformed and running start assays was significantly affected by the concentration of KCl in the assays. This probably resulted from more stable binding of RT to the substrate when lower salt was used. In one experiment, the off-rates (k_{off}) for HIV-RT at 5 versus 80 mM KCl on a particular RNA−DNA primer-template differed by a factor of about 35 (0.012 versus 0.43 for 5 and 80 mM KCl, respectively) (*47*). Since the rate and efficiency of mismatch extension and incorrect nucleotide addition is significantly lower than the correct events, more stable binding between RT and the substrate is likely to enhance the incorrect events relative to the correct (*7*, *14*, *16*). With respect to what occurs in vivo, it is difficult to predict what the precise concentration of salts and other ions are in the cell. Also, it is not known if other factors might influence RT extension in the cell. Clearly, however, the 80 mM KCl concentration more closely mimics cellular salt levels than the 40 and 10 mM amounts used in some assays. It is therefore more likely to give an accurate picture of what occurs during synthesis in the cell.

Parameters that May Affect the Interpretation of Results from the α-lac-Based Fidelity Assay. As was noted in the Results, an occurrence that could have lead to misleading results in the fidelity assay was if RT extended mismatched primer-templates with relatively low efficiency. Since extension in the absence of trap is used in the assay to estimate the total error frequency (errors made during processive synthesis and those that initially resulted in RT dissociation), inability of RT to extend mismatches upon rebinding would lead to an underestimate of the total errors. This would in turn lead to an overestimate of the proportion of polymerase molecules that dissociate after a mistake. With regard to this possibility results with preformed mismatches suggested that, in the absence of trap, mismatches are extended only about 15−20% less frequently than matched substrates by wild-type RT. This suggests that some mutations may result in discontinued synthesis but, in a quantitative sense, given sufficient time, the majority are extended. It should be pointed out that there may be some sequence specificity to this process, and more substrates would have to be examined to generalize this finding.

A second occurrence that could lead to an incorrect estimate from the fidelity assay is the possibility that the error rate of HIV-RT varies depending on whether the polymerase is initiating synthesis or is actively traversing the template. If this were the case, the effect on the results would depend on whether the rate were higher or lower during reinitiation. Although this is largely unknown, there is clear evidence for elevated error rates at runs of the same nucleotide that are also pause sites (sequences on the template were DNA synthesis tends to slow or terminate) (*1*, *53*). It is not known whether the elevated error rates at these sites are due to an increased frequency of mutations during reinitiation. Presumably, reinitiation would be frequent because of the sites also being pause sites. If this were the case in the fidelity assays, the error rate in the absence of trap would be an overestimate of the total error, at least with respect to synthesis in the presence of trap. This would occur since the steps required for completing synthesis without trap would involve an additional error-prone step. The calculated results for the percent of RTs that dissociate after mistakes would in this case be lower than the actual amount. Clearly, this is speculative and awaits a determination of whether the RT error frequencies vary during reinitiation. In any regard, the overall effect on the calculations is likely to be small.

Dissociation of HIV-RT After a Misincorporation Is the Most Likely Explanation for the Observed Results, Although RT Remaining Bound to the Mismatched Termini in a Nonproductive Complex Cannot Be Ruled Out. It should also be noted that the assays used throughout this paper actually measure the loss of a product, either a loss of mutated products in the presence of trap in the fidelity assays or a loss of extended primers in the extension assays with mismatched substrates. Dissociation of the polymerase was not directly measured, although it was assumed that the mutations resulted in product loss by inducing dissociation. A second possibility is that RT simply remains bound to the mismatch in a nonproductive complex. Such a complex would be similar to the long-lived dead-end complexes formed by HIV-RT on dideoxynucleotide-terminated DNA:DNA primer:templates in the presence of the next correct dNTP (*54*). The complex would not necessarily be dead-end in the case of the mismatch since nothing is blocking further extension. Although stalled or dead-end type complexes cannot be ruled out, we strongly favor polymerase dissociation. First, the templates used in our experiments were RNA. A stalled RT molecule would extensively degrade the template in the time allowed, which would further favor dissociation of RT. Second, in the mismatch extension assays, when 80 mM KCl was used, the A−C and T−C mismatches were extended relatively poorly in the presence of trap (Figure 2). In contrast, longer extensions without trap resulted in levels of product comparable to the G−C matched substrate. RT dissociating in the presence of trap and rebinding being required to efficiently extend the mismatch most easily explains this result. Also, if long-lived dead-end type complexes were forming, extension in the trapped reaction should occur over an extended period as complexes are released to form product. However, results showed that these reactions were essentially complete within 2 min (data not shown, see Materials and Methods). A trivial explanation is that the long-lived complexes are truly dead-end and cannot be extended, but this would presumably block primer extension such that extension in reactions without trap should also be inhibited. It is possible that some RT molecules remain associated with the mismatch for a long period; however, the vast majority more likely dissociate.

Implications of the Results with Respect to Retrovirus Replication and Recombination. Overall, the results indicate that on RNA template about 1/2 the time HIV-RT fixes mutations after making them rather than dissociating. If dissociation occurs, then subsequent extension of the mismatch usually occurs but may require multiple binding events depending on the particular mismatch and context. The results with the limited sequences used did suggest that a small (about 15−20%) proportion of the substrates with mismatched 3′ primer termini are not ultimately extended on the RNA template. Presumably, these would be aborted or require strand transfer to a second homologous template (analogous to the second copy of the viral genome during replication) to be completed. The propensity for this to occur

is apparently reduced since the tendency for RT to fix mutations reduces the level of 3′ mismatched substrates. The results also offer a potential explanation for the apparent high fidelity of recombinant products (*25*−*29*) without reducing the possibility that RT mutations can promote recombination (*21*−*23*). Using an estimated base misincorporation frequency of 10^{-4} for HIV-RT (about one error per RNA genome copied) (*1*−*7*). If one in two errors resulted in RT dissociation, then the level of potential mutated recombinants would be about 0.5×10^{-4} during each genome copying. Most of these are probably subsequently extended on the original template or aborted without transfer. If 10% lead to erroneous recombination products, then $0.10 \times 0.5 \times 10^{-4}$ or 5×10^{-6} would be the level of such products. This corresponds to one error-induced recombination event per approximately 200 000 bases. The level of total recombination events in vivo for HIV-RT has been estimated to be 2.8 per replication cycle or about three per 10 000 bases (*28*). This would mean that only about one in 60 would have resulted from an RT error. The determination depends heavily on the fidelity of HIV-RT, which may be significantly higher than 10^{-4} in vivo (*8*). This scenario would suggest that the low fidelity of RT does not contribute significantly to the induction of recombination events at least on intact genomes. Other potential mechanisms for error-prone recombination such as transfer from damaged RNA molecules could be quantitatively more significant, although this awaits further examination (*55*−*57*).

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REFERENCES

1. Bebenek, K., Abbotts, J., Wilson, S. H., and Kunkel, T. A. (1993) *J. Biol. Chem. 268*, 10324−10334.
2. Boyer, J. C., Bebenek, K., and Kunkel, T. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6919−6923.
3. Preston, B. D., Poiesz, B. J., and Loeb, L. A. (1988) *Science 242*, 1168−1171.
4. Roberts, J. D., Bebenek, K., and Kunkel, T. A. (1988) *Science 242*, 1171−1173.
5. Takeuchi, Y., Nagumo, T., and Hoshino, H. (1988) *J. Virol. 62*, 3900−3902.
6. Weber, J., and Grosse, F. (1989) *Nucleic Acids Res. 17*, 1379−1393.
7. Kati, W. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992) *J. Biol. Chem. 267*, 25988−25997.
8. Mansky, L. M. a. H. M. T. (1995) *J. Virol. 69*, 5087−5094.
9. Coffin, J. M. (1995) *Science 267*, 483−489.
10. Kunkel, T. A., and Bebenek, K. (2000) *Annu. Rev. Biochem. 69*, 497−529.
11. Arion, D., Kaushik, N., McCormick, S., Borkow, G., and Parniak, M. A. (1998) *Biochemistry 37*, 15908−15917.
12. Pulsinelli, G. A., and Temin, H. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9490−9494.
13. Perrino, F. W., Preston, B. D., Sandell, L. L., and Loeb, L. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8343−8347.
14. Bakhanashvili, M., and Hizi, A. (1992) *Biochemistry 31*, 9393−9398.
15. Creighton, S., Huang, S. M., Cai, H., Arnheim, N., and Goodman, N. F. (1992) *J. Biol. Chem. 267*, 2633−2639.
16. Yu, H., and Goodman, M. F. (1992) *J. Biol. Chem. 267*, 10888−10896.
17. Goff, S. P., and Telesnitsky, A. (1997) in *Retroviruses* (Coffin, J. M., Hughes, S. H., and Varmus, H. E., Eds.) pp 121−160, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
18. DeStefano, J. J., Buiser, R. G., Mallaber, L. M., Fay, P. J., and Bambara, R. A. (1992) *Biochim. Biophys. Acta 1131*, 270−280.
19. Huber, H. E., McCoy, J. M., Seehra, J. S., and Richardson, C. C. (1989) *J. Biol. Chem. 264*, 4669−4678.
20. DeStefano, J. J. (1996) *J. Biol. Chem. 271*, 16350−16356.
21. Diaz, L., and DeStefano, J. J. (1996) *Nucleic Acids Res. 24*, 3086−3092.
22. Diaz, L., Cristofaro, J. V., and DeStefano, J. J. (2000) *Arch. Virol. 145*, 1117−1131.
23. Palaniappan, C., Wisniewski, M., Wu, W., Fay, P. J., and Bambara, R. A. (1996) *J. Biol. Chem. 271*, 22331−22338.
24. Temin, H. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6900−6903.
25. Zhang, J., and Temin, H. M. (1994) *J. Virol. 68*, 2409−2414.
26. Yin, P. D., Pathak, V. K., Rowan, A. E., Teufel, R. J., II, and Hu, W. S. (1997) *J. Virol. 71*, 2487−2494.
27. Yu, H., Jetzt, A. E., Ron, Y., Preston, B. D., and Dougherty, J. P. (1998) *J. Biol. Chem. 273*, 28384−28391.
28. Zhuang, J., Jetzt, A. E., Sun, G., Yu, H., Klarmann, G., Ron, Y., Preston, B. D., and Dougherty, J. P. (2002) *J. Virol. 76*, 11273−11282.
29. DeStefano, J., Ghosh, J., Prasad, B., and Raja, A. (1998) *J. Biol. Chem. 273*, 1483−1489.
30. DeStefano, J. J., Bambara, R. A., and Fay, P. J. (1994) *J. Biol. Chem. 269*, 161−168.
31. Rezende, L. F., Kew, Y., and Prasad, V. R. (2001) *J. Biomed. Sci. 8*, 197−205.
32. Schatz, O., Cromme, F. V., Gruninger-Leitch, F., and Le Grice, S. F. J. (1989) *FEBS Lett. 257*, 311−314.
33. Le Grice, S. F., Cameron, C. E., and Benkovic, S. J. (1995) *Methods Enzymol. 262*, 130−144.
34. Wohrl, B. M., Tantillo, C., Arnold, E., and Le Grice, S. F. (1995) *Biochemistry 34*, 5343−5356.
35. DeStefano, J. J., Mallaber, L. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1992) *J. Virol. 66*, 6370−6378.
36. DeStefano, J. J., Buiser, R. G., Mallaber, L. M., Myers, T. W., Bambara, R. A., and Fay, P. J. (1991) *J. Biol. Chem. 266*, 7423−7431.
37. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
38. Boyer, P. L., and Hughes, S. H. (1996) *Methods Enzymol. 275*, 538−555.
39. Davis, L. G., Kuehl, W. M., and Battey, J. F. (1994) in *Basic Methods in Molecular Biology*, pp 497−507, Appleton & Lange, Norwalk, CT.
40. Rodriguez-Wells, V., Plotch, S. J., and DeStefano, J. J. (2001) *Virus Res. 74*, 119−132.
41. Ji, J. P., and Loeb, L. A. (1992) *Biochemistry 31*, 954−958.
42. Reardon, J. E. (1993) *J. Biol. Chem. 268*, 8743−8751.
43. Hsieh, J. C., Zinnen, S., and Modrich, P. (1993) *J. Biol. Chem. 268*, 24607−24613.
44. Carroll, S. S., Geib, J., Olsen, D. B., Stahlhut, M., Shafer, J. A., and Kuo, L. C. (1994) *Biochemistry 33*, 2113−2120.
45. Arion, D., and Parniak, M. A. (1999) *Drug Resist. Update 2*, 91−95.
46. Meyer, P. R., Matsuura, S. E., So, A. G., and Scott, W. A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 13471−13476.
47. Cristofaro, J. V., Rausch, J. W., Le Grice, S. F., and DeStefano, J. J. (2002) *Biochemistry 41*, 10968−10975.
48. DeStefano, J. J., Buiser, R. G., Mallaber, L. M., Bambara, R. A., and Fay, P. J. (1991) *J. Biol. Chem. 266*, 24295−24301.
49. Gopalakrishnan, V., Peliska, J. A., and Benkovic, S. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10763−10767.
50. Furfine, E. S., and Reardon, J. E. (1991) *J. Biol. Chem. 266*, 406−412.
51. Fu, T. B., and Taylor, J. (1992) *J. Virol. 66*, 4271−4278.
52. Wohrl, B. M., and Moelling, K. (1990) *Biochemistry 29*, 10141−10147.
53. Ji, J., Hoffmann, J. S., and Loeb, L. (1994) *Nucleic Acids Res. 22*, 47−52.
54. Tong, W., Lu, C.-D., Sharma, S. K., Matsuura, S., So, A. G., and Scott, W. A. (1997) *Biochemistry 36*, 5749−5757.

55. Patel, P. H., and Preston, B. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 549−553.
56. Peliska, J. A., and Benkovic, S. J. (1994) *Biochemistry 33*, 3890−3895.
57. DeStefano, J. J., Raja, A., and Cristofaro, J. V. (2000) *Virology 276*, 7−15.

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