Misincorporation by HIV-1 Reverse Transcriptase Promotes Recombination via Strand Transfer Synthesis*

(Received for publication, January 22, 1996, and in revised form, June 19, 1996)

Chockalingam Palaniappan‡, Michele Wisniewski‡, Weimin Wu‡, Philip J. Fay‡‡, and Robert A. Bambara‡¶

From the Departments of ‡Biochemistry and §Medicine, and the ¶Cancer Center, University of Rochester, Rochester, New York 14642

Genome heterogeneity in retroviruses derives from poor fidelity of the reverse transcriptase (RT) and recombination via RT-catalyzed strand transfer synthesis. RTs lack proofreading ability, and they proficiently extend primers with mismatched termini. Recombination reactions carried out in vitro are accompanied by a high frequency of base substitution errors, suggesting a relationship. Here we present evidence that misincorporation during RNA-directed DNA synthesis promotes strand transfer recombination. Experiments involved measurement of DNA synthesis, RNase H-directed cleavage, and strand transfer synthesis from preformed mismatched primers on RNA templates by human immunodeficiency virus (HIV) RT in vitro. A significant pause in synthesis occurred from a G(primer), A(template) mismatch compared to the synthesis from a correctly paired (T, A) primer. The misincorporation-induced pause allowed an unusually large area of RT-RNase H-directed cleavage of the template RNA beneath the primer. Strand transfer to an acceptor molecule with sequence identical to the template RNA was about 50% more efficient than if the primer had had a correctly paired terminus. Overall transfer was measured over a large region of homology. Assuming that enhanced transfer occurs primarily at the site of the mismatch, the actual increase in transfer at that site must have been 1–2 orders of magnitude. Inclusion of a different acceptor molecule with complete complementarity to the originally mismatched 3’ primer terminus resulted in an additional 2-fold increase in strand transfer efficiency. Overall, these results suggest the mechanism by which misincorporation during minus strand DNA synthesis in retroviral replication would promote high frequency recombination.

Heterogeneity of the retroviral genome is in part attributed to the error-prone nature of the replicative enzyme reverse transcriptase (RT)† (Coffin, 1986; Katz and Skalka, 1990). RT catalyzes the conversion of a single-stranded RNA genome into a double-stranded proviral genome. The RT is a multifunctional enzyme having RNA- and DNA-dependent DNA polymerase, RNase H, strand displacement, and strand transfer activities (Goff, 1990). The role of RT in the generation of retroviral genome variation has been documented by a number of studies both in vivo and in vitro (Ji and Loeb, 1994; Ji et al., 1994; Patel and Preston, 1994; Perrino et al., 1988; Preston et al., 1988; Pulisinski and Temin, 1994; Ricchetti and Buc, 1990; Yu and Goodman, 1992). The lack of an associated 3’ to 5’ exonuclease for proofreading, and the propensity of RT to extend a mismatched primer on a DNA or an RNA template, have been considered sources of variation in the retroviral genome (Roberts et al., 1988). Recombination resulting from transfer of the growing primer between internal positions of one or more retroviral genomes appears to be another major source of variation (Temin, 1993). Base substitution, frameshift, deletion, and deletion with insertion errors have been widely observed in analysis both in vivo (Hahn, 1986; Klarman et al., 1993; Pathak and Temin, 1990a, 1990b; Pulisinski and Temin, 1994) and in vitro (Bakhanashvili and Hizi, 1993; Creighton et al., 1992; Mendelman et al., 1990; Perrino et al., 1989; Vartanian et al., 1991; Wu et al., 1995; Yu and Goodman, 1992; Zinnen et al., 1994). Base substitution and frameshift errors have been proposed to result from either direct misincorporation or a primer slippage mechanism (Bebenek et al., 1989; Roberts et al., 1989). Deletion and deletion with insertion errors were postulated to occur by aberrant RT-mediated strand transfer from a mispaired primer terminus (Temin, 1993). In studies performed in vitro using HIV-RT, a high degree of base substitution errors were found to accompany recombination (Peliska and Benkovic, 1992, 1994; Patel and Preston, 1994; Wu et al., 1995, 1996). Such errors were suggested to derive from polymerase-catalyzed, non-template-directed nucleotide addition (Peliska and Benkovic, 1992, 1994).

Fidelity of HIV-1 RT is 1–2 orders of magnitude lower than that of eukaryotic polymerases. It is also less accurate than other retroviral RTs (Mansky and Temin, 1995). Clearly, the difficulties encountered with the development of effective vaccine treatment or chemotherapy against HIV infection are primarily due to the rapid change of the genome resulting in variants.

While RT can extend a mispaired primer terminus, the relative efficiencies of extension from purine-pyrimidine (A:C or C:A) or purine-purine (A:A, A:G, and G:A) mismatches have been found to vary significantly (Yu and Goodman, 1992). While a limited number of investigations have addressed the characteristics of polymerization from mismatched primers on RNA templates, none have examined the action of the RNase H when RT encounters a mismatched terminus.

We have proposed that there is a sequential relationship among sequence-dependent pausing, RNase H degradation of the template, and efficiency of transfer to a homologous acceptor template resulting in recombination (DeStefano et al., 1992, 1994).
1994a, 1994b). We demonstrated a positive correlation between the specific positions of RT pausing on a donor template, and recombinational strand transfer from those positions (DeStefano et al., 1992, 1994a; Wu et al., 1995, 1996). Elimination of a pause site without altering the local sequence caused reduction in strand transfer from the surrounding several nucleotides (Wu et al., 1995). Additionally, we observed that strand transfer was error-prone. It is often accompanied by base substitution and frameshift errors (Wu et al., 1995, 1996). Furthermore, it appears that upon adding a wrong nucleotide, the RT pauses before further synthesis (Bebenek and Kunkel, 1993). From these previous findings, we hypothesized that misincorporation-related RT pausing during minus strand DNA synthesis allows the accompanying RNase H activity to degrade the template RNA beneath the growing primer to a greater extent than during normal synthesis as presented in Fig. 1, panel B. This should encourage the growing primer to free itself and reposition to another region on the same template, or to an acceptor template as shown in Fig. 1, panel C. Alternatively, an acceptor template with a region of complementarity to the primer could potentially invade the complex as shown in Fig. 1, panel D. Both situations would promote recombination.

We previously developed a system to measure strand transfer recombination in vitro (DeStefano et al., 1992). It involves primer extension on a template designated the donor, in the presence of a homologous but different length template designated the acceptor (DeStefano et al., 1992). With this system, the efficiency of recombination, and the structure of recombination products can be measured (DeStefano et al., 1992; Wu et al., 1995). In the current report, we examined the effect of misincorporation induced pausing on RT RNase H activity, and the consequences of that altered RNase H activity on strand transfer.

**EXPERIMENTAL PROCEDURES**

**Materials**

Recombinant HIV-RT in its native form was graciously provided to us by Genetics Institute (Cambridge, MA). This heterodimer RT has a specific activity of approximately 40,000 units/mg. One unit of RT is defined as the amount required to incorporate 1 nmol of dTTP into poly(A)-oligod(T) in 10 min at 37°C. This amount corresponds to approximately 214 fmol of purified RT. About 250 μg of the supplied lyophilized RT sample was dissolved in 500 μl of enzyme dilution buffer. Aliquots of the RT were stored frozen at −70°C, and a fresh aliquot was used for each experiment. Analysis of this RT sample by SDS-polyacrylamide gel electrophoresis followed by staining with silver revealed an essentially homogeneous preparation containing only the p51 and p66 subunits at stoichiometric levels. The preparation was free of detectable contaminating nucleases (data not shown). T4 polynucleotide kinase was from U. S. Biochemical Corp. DNase I, dNTPs, alkaline phosphatase, rNTPs, RNase inhibitor, T7 RNA polymerase, and quick spin gel filtration columns were purchased from Boehringer Mannheim. T4 RNA ligase was purchased from New England Biolabs. Radiolabeled nucleotides were from DuPont NEN. Plasmids pBS- (+) and pBS- (Δ) have been described previously (DeStefano et al., 1992).

**Methods**

**Generation of RNA Templates**—RNA templates 1 (142-mer), 2 (189-mer), and 3 (189-mer) were generated by run-off transcription by T7 RNA polymerase from plasmids pBS- (+), pBS- (+), and pCP3, respectively, following digestion by BstNI. Following DNase I digestion, the samples were gel-purified and isolated upon visualization by UV shadowing as described previously (Palaniappan et al., 1995). Dephosphorylation of RNA molecules was carried out using calf intestine alkaline phosphatase. When required, 5'-end labeling was carried out with [γ-32P]ATP (3000 Ci/mmol) in the presence of T4 polynucleotide kinase. 3'-end labeling of the RNA was performed by using T4 RNA ligase and 32P-cp. RNA samples were quantitated by “shift-up” assays using labeled primers of known concentrations as described previously (DeStefano et al., 1993, 1995; Palaniappan et al., 1995).
Misincorporation Promotes Strand Transfer by HIV-1 RT

RESULTS

We postulate that a misincorporation during synthesis of minus strand DNA would cause HIV RT to pause. This pause would lead to enhanced RNase H-directed cleavage of the template near the pause site, as illustrated in Fig. 1, panel B. This cleavage should then promote recombination by RT-mediated strand transfer as proposed by our model in Fig. 1 (panels C and D).

RNA-dependent DNA Polymerase Activity of HIV-1 RT at a Mismatched Primer Terminus—In order to examine the consequences of a misincorporation during minus strand DNA synthesis, we employed an RNA template annealed with DNA primers having or lacking a 3' terminal mismatch. The template RNA was a 142-mer transcript prepared as described under “Methods.” Substrates A and B were made by annealing the 142-mer RNA template 1 to the 30-mer DNA primers 1 and 2, respectively. Substrates C and D were generated by annealing the 40 mer DNA template 1 to the DNA primers 1 and 2, respectively. The numbers above the substrates represent the length in nucleotides. The region of homology shared with the donor RNA template 1 is marked by a rectangle on the acceptor RNA template 2 (acceptor 2). RNA template 3 (acceptor 3) is different from acceptor 2 only by the presence of C instead of an A at position 136 from the 5' end. The complete nucleotide sequences of the templates, acceptors and primers are given under “Methods.” In this and the following figures, RNA and DNA are represented by thin lines and thick lines, respectively.

FIG. 1. Proposed model for the mechanism by which misincorporation by RT can cause recombination via strand transfer synthesis. Panel A represents normal synthesis. Panel B represents the situation following the addition of a wrong nucleotide by RT. Panels C and D represent the two pathways leading to recombination. The letters P and H refer to polymerase and RNase H active sites, respectively.

GUAAA GCCU.

RNA template 3 (acceptor 3) has the same RNA sequence as template 2, except that a C is substituted for the A in boldface type.

The DNA components are as follows: DNA template 1, 5'-TGGATTATCCTGGACCAACATCCACAG; DNA primer 1, 5'-CTCGTATGTTGTGGAGTTGAGCGAG; DNA primer 2, 5'-CTCGTATGTTGTGGAGTTGAGCGAG.

FIG. 2. Substrates used in this study. Substrates A and B were made by annealing the 142-mer RNA template 1 to the 30-mer DNA primers 1 and 2, respectively. Substrates C and D were generated by annealing the 40 mer DNA template 1 to the DNA primers 1 and 2, respectively. The numbers above the substrates represent the length in nucleotides. The region of homology shared with the donor RNA template 1 is marked by a rectangle on the acceptor RNA template 2 (acceptor 2). RNA template 3 (acceptor 3) is different from acceptor 2 only by the presence of C instead of an A at position 136 from the 5' end. The complete nucleotide sequences of the templates, acceptors and primers are given under “Methods.” In this and the following figures, RNA and DNA are represented by thin lines and thick lines, respectively.

Plate-directed full-length product (Fig. 3A). The band intensities of all extended products from each terminus were quantitated using PhosphorImager analysis. We first measured DNA extension over a range of enzyme concentrations for a 15-min time period (Fig. 3A). At all enzyme concentrations examined, the overall production of extended products from the mismatched terminus was about 30% of that from the complementary terminus.

In order to detect whether there is a misincorporation induced pause in DNA synthesis by HIV-1 RT as proposed in Fig. 1 (panel B), we performed a comparative time course of synthesis on substrates A and B. Results of the analysis are presented in Fig. 3B. A significant delay in synthesis was observed with substrate B compared to synthesis from substrate A (Fig. 3B, compare products in lanes 0–16 min).

Consequences of a Misinsertion at the Primer Terminus on HIV-1 RT RNase H Activity—The above results demonstrate that the RT pauses after misincorporation on our experimental substrate. Previous results have shown that the affinity of the RT for the mispaired primer and matched primers are similar (Yu and Goodman, 1992), suggesting that the RT remains bound after generation of the mispair. Under those circumstances RT-RNase H would still be active.

Use of paired versus mispaired primers offered us the opportunity to determine whether misincorporation-induced pausing alters the efficiency of RNase H cleavage near the mismatched site as proposed by the model. We employed substrates A (TrA) and B (GrA) again, but this time 5' labeled the 142-mer template RNA instead of the DNA primers. We determined the fate of the template RNA in the presence or absence of DNA synthesis. A time-course analysis of RNase H cleavage in the absence or presence of dNTPs is presented in Fig. 4 (A and B, respectively). Without dNTPs, cleavage by RT RNase H generates labeled primary and secondary cleavage products about 102 and 94 nucleotides long. The initial endonucleolytic cleavage position is at a distance slightly smaller than the approximate 20-nucleotide spatial separation of DNA polymerase and RNase H active sites, as determined by x-ray crystallographic studies (Jacobo-Molina et al., 1993; Kohlstaedt et al., 1992). Secondary cleavage products accumulate during the reaction. These are shorter than the primary products by an additional
8 nucleotides. They result from the previously demonstrated processive 3' to 5' directional nucleolytic activity of RT-RNase H (DeStefano et al., 1993; Palaniappan et al., 1996; Schatz et al., 1990). The rate of formation of the secondary cleavage products is faster in substrate B than in A (Fig. 4A, compare lanes 0–16 min). It is evident that the presence of a mismatched primer terminus facilitates the processive RT RNase H directional cleavage activity.

To determine the effect of misincorporation on RT-RNase H activity during minus strand DNA synthesis, we analyzed the products of RNase H cleavage of template RNA in the presence of dNTPs. The degradation of the 142-mer template RNA was examined with substrates A and B (Fig. 4B). An evident difference between the distribution of products with the two substrates is the accumulation of 5' labeled products of lengths 20 nucleotides and below during the course of the reaction with the fully annealed substrate A (Fig. 4B, left panel). These products probably originate from cleavages that occur when the
enzyme has reached the 5’ end of RNA templates. The products about 20 nucleotides long are reminiscent of the cleavage site distance dictated by the spatial separation of the RT active sites. That is, the positioning of the polymerase active site at the very last nucleotide of a fully extended primer would place the RNase H active site 14–20 nucleotides from the 5’ end of the RNA. The formation of smaller length products of sizes around 9–14 is most likely mediated by the additional 3’ to 5’ directional nucleolytic activity. Several additional intermediate products, which were present at the early time points but disappeared during the course of the reaction, are apparently derived from cleavages determined by the normal sequence-dependent pausing of RT.

With the mismatched substrate B, virtually all of the early cleavages are limited to those making 94- and 102-nucleotide labeled products. These are products expected from cleavages made by RT delayed at the mismatched terminus. Since there is a delay in DNA synthesis from the mismatched primer, the cleavage products that would normally be seen with the fully annealed primer begin to appear only later in the reaction. The products that are 94 and 102 nucleotides long are absent in reactions with the fully annealed substrate A. It is very likely that the polymerase moves from that position so quickly that cleavage occurs on relatively few templates. Alternatively, cleavages are made at that position, but subsequent cleavages made closer to the 5’ end of the RNA destroy the 94- and 102-nucleotide products.

Quantitation of Cleavage Near the Mismatched Terminator during DNA Synthesis—In order to determine whether there is more cleavage during primer elongation on a mismatched versus fully complementary primer, we measured generation of cleavage products of 3’ end-labeled template RNA in substrates A and B (Fig. 5). During synthesis, cleavage is expected to begin on the template beneath the RT bound to the original length primer and progress toward the 5’ end of the RNA. The 3’ labeled products of template cleavage near the original 3’ end of the primer are not expected to be shortened by cleavages that occur during primer elongation. However, more extensive cleavage during the delay before synthesis on a mismatched primer could potentially shorten the 3’ products. When we looked at the distribution of more 3’ products, there were some small 3’ labeled segments, resulting from cuts in what should have been the single-stranded region of the RNA. These were possibly generated by the looping of the growing DNA primer to form an RNA/DNA hybrid near the 3’ end of the RNA, which would then be sensitive to RNase H-directed cleavage. To minimize such cleavage, which interferes with interpretation of results, we performed challenged reactions as described under “Methods.” These allow only synthesis and cleavage that can occur during a single interaction of the RT with the primer-template (Fig. 5). They involve binding the RT to the template, and then initiating the reaction by simultaneous addition of magnesium, dNTPs, and a trapping polymer that sequesters any RT molecules that dissociate from the primer-template. The 3’ cleavage products made in these reactions with substrate A were approximately 40–43 nucleotides long, indicating the expected cleavage at a distance of about 14–20 nucleotides from the original 3’ primer terminus. However, with substrate B, additional smaller length 3’ labeled RNA products of about 37–40 nucleotides were detected. This result, combined with the data from Fig. 4, demonstrates that the delay in synthesis that occurs at the position of the mismatch allows a wider swatch of cleavage of the template to occur before synthesis proceeds, than would occur if the primer had been fully annealed.

Nucleotide Extension from a Mispair Between a Primer and a DNA Template—The overall decrease in extension products obtained from a mismatched primer on an RNA template, compared to the synthesis from a fully annealed primer (Fig. 3), may reflect the difficulty with which the RT could use the mismatched terminus for synthesis. Alternatively, the increased cleavage of the template near the mismatched terminus allowed dissociation of most of the primers and templates so that synthesis could not occur. By employing a DNA template identical to the RNA template sequence, the inherent difficulty of extending a GA mismatch may be examined without the influence of template degradation by RNase H. We employed a shorter DNA template but with the same sequence as the 142-mer template RNA over the region of synthesis. Substrate D differed from C by the presence of a 3’ primer terminus G-A mismatch. Extension from the matched or mismatched primers on the DNA template should yield a 40-nucleotide, full-length template-directed product (Fig. 2). Time-course analysis of an experiment showing DNA extension on substrates C and D is presented in Fig. 6. PhosphorImager quantitation of products revealed that there was a overall reduction in extension from the G-A mismatched primer to about 42% of that with the complementary T-A primer. In addition, a pause in DNA synthesis similar to that observed in the RNA-dependent DNA synthesis was seen (compare Fig. 6 with Fig. 3B). This result suggests that the reduction in DNA synthesis from the G-A mismatch in the RNA template to about 30% of that with T-A primer is due, at least in part, to the inherent difficulty that the RT has with extending from the mismatch. An additional component may derive from degradation of the template.

Examination of Strand Transfer Efficiencies when the RT Encounters a Mispair 3’ Primer End—As proposed in Fig. 1 (panel B), results from the above experiments suggest that misincorporation during minus strand synthesis allows increased template degradation by RT RNase H. This scenario
would promote RT-mediated recombination via strand transfer as illustrated by Fig. 1 (panels C and D). To determine the effect of a mispaired primer terminus on recombination, we began by measuring transfer efficiency.

For this experiment, we measured DNA synthesis on a donor RNA template, and transfer of some of the primers to an acceptor RNA template using a procedure described earlier (DeStefano et al., 1992). The acceptor template has a region of homology with the donor template so that homologous recombination can occur. The donor and acceptor templates differ in length so that full-length primer extension either on the donor, or on the acceptor after transfer, will make products of distinct lengths. The efficiency of strand transfer is determined with the formula given under “Methods.”

In order to examine the influence of misincorporation on strand transfer efficiency, we employed substrates A and B for donor-mediated synthesis. We utilized RNA templates 2 and 3 as acceptor molecules. RNA acceptor 2 is different from acceptor 3 only by the presence of an A instead of C at position 136 from the 5’ end (see “Methods”). If transfer proceeded from the 3’ mispaired primer terminus in substrate B (G–A mismatch) to acceptor 3, a perfect complementarity (G–C match) of the primer with the acceptor would be made. On the other hand, transfer from the mispaired primer terminus of substrate B to acceptor 2 would still result in the same non-complementarity of the 3’ terminal nucleotide of the primer (G–A mismatch). The converse is true for transfer from the primer terminus in substrate A to acceptor templates 2 and 3, respectively. That is, the primer terminus would be matched on template 2 and mismatched on template 3. We have chosen the acceptor template such that the transfer from the primer terminus in substrate A or B would generate a donor template-directed full-length product 118 nucleotides long, and a full-length transfer product 166 nucleotides long. Strand transfer analysis was performed by titrating varying amounts of acceptors 2 and 3 in the DNA synthesis reaction (Fig. 7).

Transfer efficiencies were calculated using values obtained by PhosphorImager analysis of the gel represented in Fig. 7. Efficiencies of transfer from a mispaired primer terminus on substrate B to acceptor templates 3, restoring complementarity, or 2, retaining a mismatch, at 50 × acceptor concentration were 58% (Fig. 7, lane 21) and 31% (Fig. 7, lane 16), respectively. Transfer efficiencies from a correctly matched primer on substrate A to acceptor templates 3, producing a mismatch, or 2, correctly matched, were 19% (Fig. 7, lane 9) and 22% (Fig. 7, lane 4), respectively. Efficiencies at other acceptor concentrations are provided in the legend to Fig. 7. Overall, the size order of transfer efficiencies is as follows: transfer from substrate B to acceptor 3 > substrate B to acceptor 2 > substrate A to acceptor 2 > substrate A to acceptor 3. This order was maintained at acceptor concentrations in 10-, 50-, and 100-fold excess over the donor template-primer. There was some reduction in the efficiency of transfer at 100-fold excess acceptor, consistent with our previous reports (Rodríguez-Rodríguez et al., 1995). Transfer was low but detectable when acceptor was present in 2-fold excess or when added in equimolar ratio with donor template-primer.

The additional transfer product, measured when donor templates with a mismatch are compared to those without, results from the extra opportunity for transfer that can occur because of the mismatch. This extra transfer adds to the transfer that normally occurs during synthesis over the donor template. We assume that this extra component of transfer occurs at the mismatched site. This assumption is consistent with the concept that the mismatch allows greater template degradation, which should promote transfer. Since we have compared the
Misincorporation Promotes Strand Transfer by HIV-1 RT

transfer over a region of homology 118 nucleotides in length, the efficiency of transfer at the site of the mismatch in the above experiments must have been 10–100-fold greater than at the average site without a mismatch.

Results indicate that transfer is enhanced from a donor template with a mismatched primer irrespective of whether the primer forms a fully annealed structure or a mismatch on the acceptor template. In fact, transfer from a mismatched terminus is more efficient when full annealing can occur to the acceptor. However, there is clearly a stimulation of transfer that derives from the mismatch on the donor template even when the transferred primer is mismatched on the acceptor template. This latter situation would occur following the addition of an incorrect nucleotide by RT during minus strand DNA synthesis in vivo. In that case, the mismatched primer would have the opportunity to transfer to another retroviral genome, forming a 3′ terminal mismatch that would be extended on the acceptor genome. This would create a base substitution at the point of recombination.

**DISCUSSION**

Two reverse transcription-related phenomena, recombination and infidelity, are considered to be significant sources of retroviral genome variation (Temin, 1993). Based on results from previous studies, we postulated that misincorporation by the RT would stimulate recombination by the mechanism shown in Fig. 1. In this study we have examined the consequences of a specific misincorporation on recombination by RT during minus strand DNA synthesis in vitro using purified HIV-1 RT. Our results indicate that recombination via strand transfer can be promoted enormously at the sites of mismatch resulting from misincorporation by the RT.

Extension from a mispaired GrA (template) 3′ primer terminus by HIV-RT was found to be moderately inefficient, resulting in about 30% of the product yield compared to that from a perfectly complementary primer terminus. The inherent difficulty of extending a purine-purine mismatch, in combination with template degradation beneath the primer stimulated by pausing of the enzyme, may have contributed to the overall reduction in the yield of the extension product. We observed a similar delay in DNA synthesis from a mispaired primer compared to a correctly paired primer on both RNA and DNA templates. Since the DNA is not degraded, it appears that pausing is a major reason for the lower efficiency of synthesis. This pause in synthesis following a misincorporation has been proposed or observed by others (Temin, 1993; Fry and Loeb, 1992; Bebenek and Kunkel, 1993). After the pause, the RT continues extension of the terminus. In the case of most eukaryotic polymerases, the pausing following misincorporation allows time for proofreading by an associated exonuclease that removes the misinserted nucleotide. Because retroviral polymerases lack an associated proofreading function, it appears that they have evolved the capacity for facile extension past a mismatch terminus.

Since misincorporation causes a delay in synthesis, we anticipated that the delay would allow the RT to exhibit more RNase H-directed cleavage near the site of the mismatch than would normally have occurred had there been no misincorporation. The increased degradation of the template RNA should facilitate recombination, by transfer of the primer to another template. We have investigated the effects of a terminal GrA (primer-template) mismatch on RT RNase H activity and found that misincorporation does, in fact, promote enhanced RNase H cleavage. RT RNase H activity was analyzed either in the presence or absence of dNTPs (Fig. 4, A and B). Although the pattern of RNA cleavage beneath the bound mismatched primer was similar to that with the paired primer, an enhancement of 3′ to 5′ processive RNase H activity was evident in our time-course analysis. The effect is more clearly seen when RNA degradation products were analyzed during DNA synthesis. On a mismatched primer-template substrate, pausing resulted in extensive degradation of template beneath the unextended primer, although after reinitiation of primer elongation, cleavage occurred at sites similar to those on the substrate with correct primer terminus (Fig. 4B). Extensive cleavage of the template RNA beneath the mismatched primer was also established by employing a 3′ labeled RNA template (Fig. 5). The 3′ labeled RNase H cleavage product pattern on a substrate with a mispaired primer terminus is reminiscent of the relaxed association of RT to template-primer upon nevirapine binding, which we reported previously (Palaniappan et al., 1995). It is possible that a relaxation of the binding of the RT polymerase active site to the mispaired primer terminus results in “mooring” of RT. We have postulated mooring as a loose binding configuration that allows the RT to move enough during pausing to generate additional cleavage products.

We performed strand transfer experiments with two different acceptor molecules. Transfer from the mispaired substrate to acceptor 2 at the primer terminus would have generated the same GrA mismatch, whereas the transfer to acceptor 3 would result in the restoration of GrC complementarity. The reason for designing these two kinds of acceptor molecules was that, following misincorporation in vivo, the transfer could potentially take place to a completely homologous second RNA genome at the same region. Alternatively, the primer would transfer to a different region of either the template RNA or to another RNA genome by a partially homologous or nonhomologous process. In the first case it would result in the fixation of base substitution errors, and in the second case it could lead to deletion, and deletion with insertion errors. Our experiments demonstrate that transfer from a mispaired primer terminus is about 3 times greater when it occurs onto an acceptor that permits the restoration of perfect complementarity at the 3′ end of the primer. Transfer from a mispaired primer terminus to an acceptor that would still result in a similar mismatch was about 1.5-fold compared to the transfer efficiencies from correctly base-paired primer termini. Since the region of homology between the donor and acceptor templates spans a distance of 118 nucleotides, each transfer could have taken place at any of these positions. Even though the overall transfer efficiency in this region was enhanced about 50% by a mismatched primer, the transfer efficiency at the site of the mismatch is likely to have increased by 1–2 orders of magnitude to produce the observed change in transfer from the entire region. These results indicate that misincorporation promotes both homologous and nonhomologous recombination. Promotion of nonhomologous recombination by misincorporation could explain the observed high frequencies of aberrant strand transfer events observed in vivo (reviewed by Hu et al. (1993)).

Based on studies performed in vivo, Temin (1993) initially proposed that misincorporation by RT would hinder processive polymerization resulting in a pause. At such time the RT could continue to polymerize over the next template nucleotide or copy a nucleotide at a different location. He suggested that the observed variation in the retroviral genome due to phenomena such as deletion, deletion with insertion, homologous and nonhomologous recombination are consequences of aberrant strand transfer promoted by such pausing. However, no mechanistic studies had been done so far to support this notion. Our results provide a mechanism by which misincorporation-induced pausing during minus strand synthesis promotes RT-directed template cleavage by RNase H, and subsequent recombination. This concept links the processes of misincorporation...
and recombination as a concerted process that contributes to the evolution of retroviruses such as HIV.

Acknowledgments—We thank Drs. John McCoy and Jasbir Seehra of the Genetics Institute for the generous gift of HIV-1 RT. We also thank Gloria M. Fuentes and Lorna Rodriguez-Rodriguez for helpful discussions.

REFERENCES

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

Bebenek, K., and Kunkel, T. A. (1993) in *Reverse Transcriptase* (Skalka, A. M., and Goff, S. P., eds) p. 85, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Bebenek, K., Abbotts, J., Roberts, J. D., Wilson, S. H., and Kunkel, T. A. (1989) *Biochim. Biophys. Acta* **913**, 549–553

Bebenek, K., and Kunkel, T. A. (1993) in *Reverse Transcriptase* (Skalka, A. M., and Goff, S. P., eds) p. 85, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Coffin, J. M. (1986) *Cell* **45**, 1–4

Creighton, S., Huang, M.-M., Cai, H., Arnheim, N., and Goodman, M. F. (1992) *Science* **256**, 1783–1790

DeStefano, J. J., Wu, W., Palaniappan, C., Fay, P. J., and Bambara, R. A. (1996) *J. Biol. Chem.* **271**, 2063–2070

DeStefano, J. J., Mallabar, L. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1992) *J. Virol.* **66**, 6370–6378

DeStefano, J. J., Mallabar, L. M., Fay, P. J., and Bambara, R. A. (1993) *Nucleic Acids Res.* **21**, 4330–4338

DeStefano, J. J., Bambara, R. A., and Fay, P. J. (1994a) *J. Biol. Chem.* **269**, 101–104

DeStefano, J. J., Wu, W., Seehra, J., McCoy, J., Laston, D., Albone, E., Fay, P. J., and Bambara, R. A. (1994b) *Biochim. Biophys. Acta* **1219**, 380–388

Fry, M., and Loeb, L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 763–767

Hahn, B. H. (1986) *Science* **232**, 1548–1553

Hu, W. S., Pathak, V. K., and Temin, H. M. (1993) in *Reverse Transcriptase* (Skalka, A. M., and Goff, S. P., eds) pp. 251–274, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Jacobo-Molina, A., Ding, J., Nam, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Perris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6320–6324

Ji, J., and Loeb, L. A. (1994) *Virology* **199**, 323–330

Katz, R. A., and Skalka, A. M. (1990) *Annu. Rev. Genet.* **24**, 409–445

Klahrstra, D. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) *Science* **256**, 1783–1790

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 488–492

Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 488–492

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 488–492

Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382

Mansky, L. M., and Temin, H. M. (1995) *J. Virol.* **69**, 5087–5094

Mendelman, L. V., Petruska, J., and Goodman, M. F. (1990) *J. Biol. Chem.* **265**, 2338–2346

Palaniappan, C., Fay, P. J., and Bambara, R. A. (1995) *J. Biol. Chem.* **270**, 4861–4869

Palaniappan, C., Fuentes, G. M., Rodriguez-Rodriguez, L., Loeb, P. J., and Bambara, R. A. (1996) *J. Biol. Chem.* **271**, 2063–2070

Patel, P. H., and Preston, B. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 549–553

Patel, P. H., and Preston, B. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 549–553

Perrino, F. W., Preston, B. D., Sandell, L. L., and Loeb, L. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 8343–8347

Preston, B. D., Poiesz, B. J., and Loeb, L. A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 6320–6324

Ricchetti, M., and Buc, H. (1990) *EMBO J.* **9**, 1583–1593

Rodriguez-Rodriguez, L., Tsuchihashi, Z., Fuentes, G. M., Bambara, R. A., and Fay, P. J. (1995) *J. Biol. Chem.* **270**, 15065–15071

Sambrusk, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schutz, O., Mous, J., and Le Grice, S. F. J. (1990) *EMBO J.* **9**, 1171–1176

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schatz, O., Mous, J., and Le Grice, S. F. J. (1990) *EMBO J.* **9**, 1171–1176

Tasten, H. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6900–6903

Vartanian, J.-P., Meyerhans, B., Asjo, B., and Wain-Hobson, S. (1991) *J. Virol.* **65**, 1779–1788

Wu, W., Blumberg, B. M., Fay, P. J., and Bambara, R. A. (1995) *J. Biol. Chem.* **270**, 325–332

Wu, W., Palaniappan, C., Fay, P. J., and Bambara, R. A. (1996) *Nucleic Acids Res.* **24**, 1710–1718

Yu, H., and Goodman, M. F. (1992) *J. Virol.* **66**, 10888–10896

Zinzen, S., Haie, J.-C., and Modrich, P. (1994) *J. Biol. Chem.* **269**, 24195–24202
Misincorporation by HIV-1 Reverse Transcriptase Promotes Recombination via Strand Transfer Synthesis
Chockalingam Palaniappan, Michele Wisniewski, Weimin Wu, Philip J. Fay and Robert A. Bambara

J. Biol. Chem. 1996, 271:22331-22338.
doi: 10.1074/jbc.271.37.22331

Access the most updated version of this article at http://www.jbc.org/content/271/37/22331

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 29 of which can be accessed free at http://www.jbc.org/content/271/37/22331.full.html#ref-list-1