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Relationship between plus strand DNA synthesis and removal of downstream segments of RNA by human immunodeficiency virus, murine leukemia virus and avian myeloblastoma virus reverse transcriptases

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ABSTRACT

During retroviral reverse transcription the genomic RNA is degraded by the RNase H activity of reverse transcriptase (RT). Previous results suggest that after RNA-directed DNA synthesis, fragments of RNA remain annealed to the newly synthesized DNA [DeStefano *et al.* (1991) *J. Biol. Chem.* 266, 7423–7431]. These must be removed to allow synthesis of the second DNA strand. We measured the ability of HIV-, AMV- and MuLV-RT to coordinate DNA-dependent DNA synthesis and removal of downstream segments of RNA. The substrates employed were DNA templates having upstream DNA and downstream RNA primers. We found that none of the wild type RTs elongated the upstream DNA without simultaneous degradation of the RNA. Consistent with these results, HIV-, AMV- and MuLV-RT showed relatively higher affinity for RNA than for DNA oligonucleotides bound to a DNA template. Differences were observed in the RNA degradation and DNA extension patterns generated by the different RTs. AMV-RT degraded the RNA to segments 11–12 nt long, and readily elongated the upstream DNA to the end of the template. MuLV- and HIV-RT degraded the RNA primarily to segments 15–16 nt long. At low concentrations of the latter two RTs, the DNA primer stalled when it encountered the 5′-end of the RNA. In sufficient excess, all of the RTs elongated the upstream primer without stalling. Even though we were unable to detect displacement of the downstream RNA by the wild type RTs, MuLV- and HIV-RT lacking RNase H, were able to elongate the upstream DNA to the end of the template without degradation of the RNA. This suggests that degradation of downstream pieces of RNA is not absolutely required before synthesis of the plus strand DNA. The implications of these findings for viral replication are discussed.

INTRODUCTION

Members of the retrovirus family include viruses responsible for causing leukemia in mice (murine leukemia virus—MuLV), tumors in chicken (avian myeloblastosis virus—AMV), and acquired immunodeficiency syndrome (AIDS) in humans (human immunodeficiency virus—HIV). Retroviruses rely on reverse transcription to replicate (see 1 for a review). This process, which converts single stranded RNA into double stranded DNA, is carried out by the enzyme reverse transcriptase (RT). This enzyme is encoded by the *pol* gene and is carried inside the virion. In HIV the native enzyme is a heterodimer composed of 66 and 51 kDa subunits (2–3). In AMV the enzyme is also a heterodimer composed of 95 and 63 kDa subunits (4). In MuLV the native enzyme is a 80 kDa monomer (5). Despite the differences in structure, all RTs have common mechanisms of action. They are multifunctional enzymes capable of RNA-dependent polymerization, DNA-dependent polymerization, ribonuclease H (RNase H) activity, strand transfer, and strand displacement. The RNA-dependent polymerase activity is responsible for the synthesis of the minus strand of DNA. The RNase H activity degrades the genomic RNA, creates the primer for plus strand synthesis, and later removes the primers used for minus and plus strand synthesis. Synthesis of the second strand is catalyzed by the DNA-dependent polymerase activity of the RT. Additionally, RT catalyzes at least two strand transfer events and strand displacement synthesis to complete the process of reverse transcription.

The RNase H activity of RTs has been the subject of extensive studies. This activity has been classified as polymerase dependent RNase H and polymerase independent RNase H (6–7). Polymerase dependent RNase H cleavage advances upon primer extension and remains fixed at a distance from the 3′-OH terminus of the elongating DNA. Biochemical studies have shown that the polymerase dependent RNase H activity accompanying RNA-directed DNA synthesis is not sufficient to eliminate all of the template RNA (8–10). The amount of degradation that accompanied RNA-directed DNA synthesis differed among AMV-, MuLV- and HIV-RT (8). While HIV-RT and MuLV-RT generated small products, AMV-RT generated mostly large products. However, in all cases, some of the template RNA remained undigested. In a further study, the amount of degradation that accompanied RNA directed DNA synthesis by AMV- and HIV-RT was measured (11). Results showed that with HIV-RT ~20% of the template RNA remained annealed after one round of processive DNA synthesis, while with AMV-RT ~80% the template RNA remained annealed

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after one round of processive synthesis. In both cases, the template that remained annealed to the newly synthesized DNA was composed of oligoribonucleotides 13–49 nt long. These findings suggest that relatively long segments of RNA remain annealed to the first strand of DNA during reverse transcription. These segments could be displaced during second strand synthesis by the unwinding activity of the RT performing synthesis (12–16) or could be degraded by the polymerase independent RNase H activity of other RT molecules present in the virion. It is unlikely that these RNA segments would be removed by an unwinding activity since it has been shown that the strand displacement activity of RT is strictly associated with synthesis (14–15).

Here we examine the ability of AMV-, MuLV- and HIV-RT to process downstream segments of RNA, during synthesis from an upstream DNA primer. Wild type RTs degraded the RNA before synthesis beyond the RNA annealing point could occur. Our results suggest that excess RT molecules found in the virion degrade downstream segments of RNA before or during plus strand synthesis. However, as indicated by RNase H mutant RTs, degradation of the RNA is not an absolute pre-requisite for plus strand DNA synthesis.

MATERIALS

HIV-RT with native primary structure was provided by the Genetics Institute (Cambridge, MA). The enzyme had a specific activity of 40 000 U/mg. One unit (U) is defined as the amount required to incorporate 1 nmol of dTTP into nucleic acid product in 10 min at 37°C using poly(rA)–oligo(dT)$_{16}$ as template primer. The enzyme was divided into aliquots, stored at –70°C, and a fresh aliquot was used for each experiment. An HIV-RT lacking RNase H, HIV-1$_{HXB2}$ (p66$^{E>Q}$/p51), was obtained through the AIDS Research and Reference Reagent Program from Dr Stuart Le Grice. MuLV-RT was purchased from United States Biochemical Corp., and Superscript II was purchased from Bethesda Research Laboratories. AMV-RT, T4 polynucleotide kinase, T4 DNA polymerase, T3 RNA polymerase, RNase inhibitor, bovine pancreatic DNase I, calf intestinal phosphotase, *Acc*I, rNTPs, dNTPs, poly(rA)–oligo(dT)$_{16}$ and Quick Spin columns (G-25 Sephadex) were purchased from Boehringer Mannheim. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from New England Nuclear. The DNA oligonucleotides used as primers and templates were obtained from Genosys, Inc. All other chemicals were from Sigma Chemical Co.

METHODS

Preparation of the substrates

The substrates used are shown in Figure 1. The DNA oligonucleotides used as primer and template were chemically synthesized. The DNA primer was labeled at the 5′-end with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase, and purified through a G-25 Sephadex Quick Spin column. The RNA primer was made by run-off transcription as described in the Promega protocols and application guide (1991) using the T3 promoter of the pBS+ plasmid linearized with *Acc*I. To make the 5′-end-labeled RNA, the transcript was treated with calf intestinal phosphatase, and labeled with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase. The RNA primer was purified by electrophoresis through a 12% polyacrylamide gel containing 7 M urea. The labeled transcript was localized by autoradiography, and was excised and eluted using the crush and soak method (17).

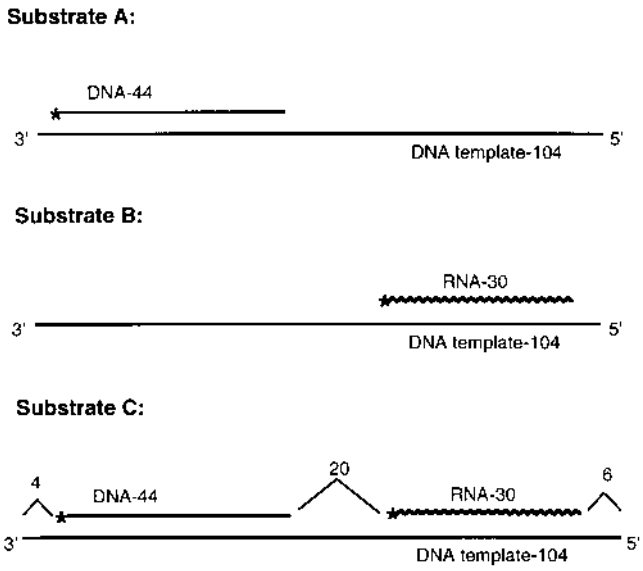


Figure 1. Substrates used in this study. The DNA template was the same for substrates A–C. The DNA and RNA primers were 5′-end-labeled. The hybrids were gel purified by native polyacrylamide gel electrophoresis. The sequences of the substrates are shown in the Methods section. The sizes and the spacing of the primers are indicated on substrate C. Full length extension of the DNA primer generates a 100 nt product. Extension of the DNA primer up to the RNA generates a 64 nt product.

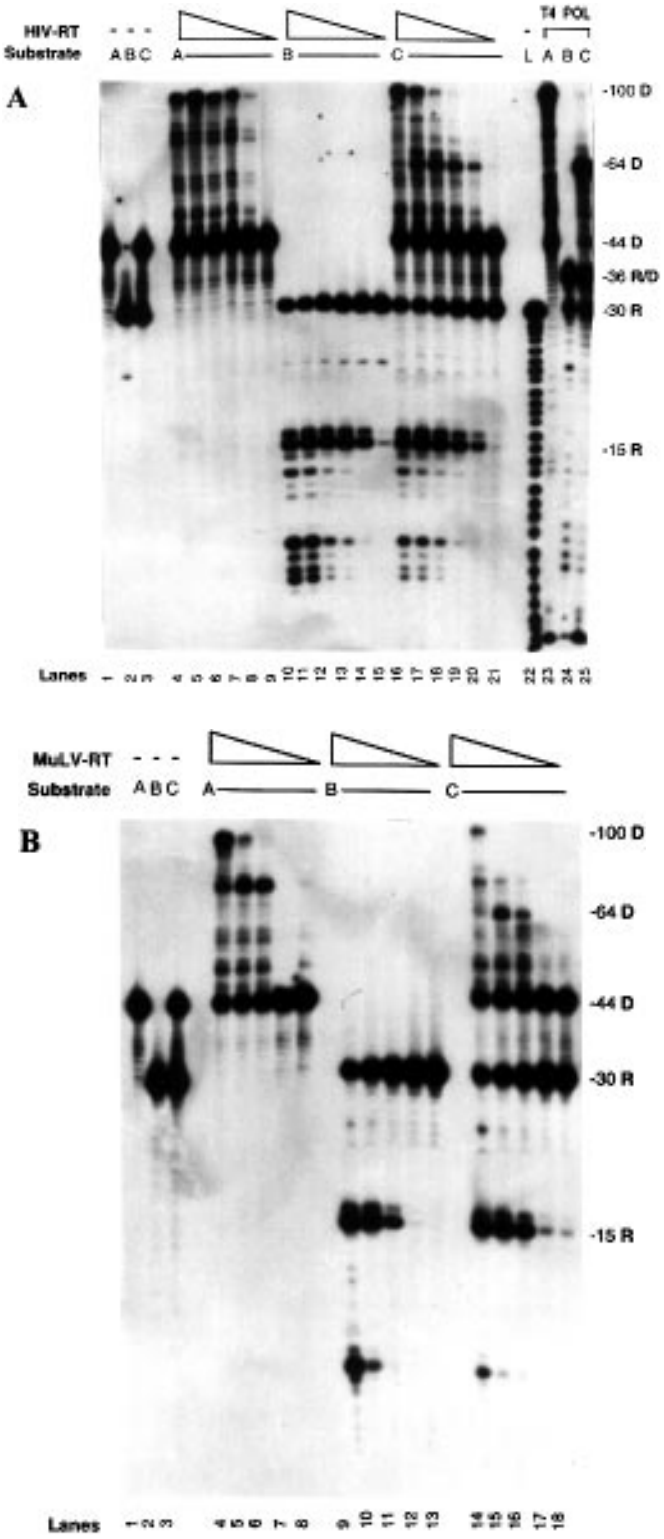
Each hybrid was prepared by mixing the primer(s) with the template in 50 mM Tris–HCl (pH 8.0), 0.1 mM EDTA and 80 mM KCl. The primer(s) were present at a 5:1 molar ratio over the template. The mixture was heated at 65°C for 10 min and then cooled slowly to room temperature. The hybrids were purified by electrophoresis on an 8% native polyacrylamide gel using the procedure described above.

Substrate sequences (5′→3′ direction):

Template:
TAGAGTCGACCTGCAGGCATGCAAGCTTTTGTTCCCCGAGGGTGTGGGGCCGG
TGGCGCCTGTTAGTTAATTCACTGGCCGTCGTTTTACAACGACGTGACTGG
DNA primer: TCACGTCGTTGTAAAACGACGGCCAGTGAATTAACTAACAGGCG
RNA primer: GGGAACAAAAGCUUGCAUGCCUGCAGGUCG

RT reactions

Approximately 1.5 nM of substrate was pre-incubated with the enzyme for 3 min in 50 mM Tris–HCl (pH 8.0), 80 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA and the reactions were started by the addition of 6 mM $MgCl_2$ and 50 µM of each deoxynucleoside triphosphate (dNTP). The reactions were performed in a final volume of 12.5 µl at 37°C for 15 min. The amount of enzyme used in each experiment is specified in each figure legend. The reactions were stopped by adding an equal volume of a 2× loading buffer [90% formamide (v/v), 10 mM EDTA (pH 8.0), 0.1% xylene cyanole, 0.1% bromophenol blue]. Trapped reactions were performed as described above except that 1 µg of poly(rA)–oligo(dT)$_{16}$ was added to start the reactions along with the $MgCl_2$ and the dNTPs. In the trap control reactions, the trap was added to the substrate before pre-incubation with RT.



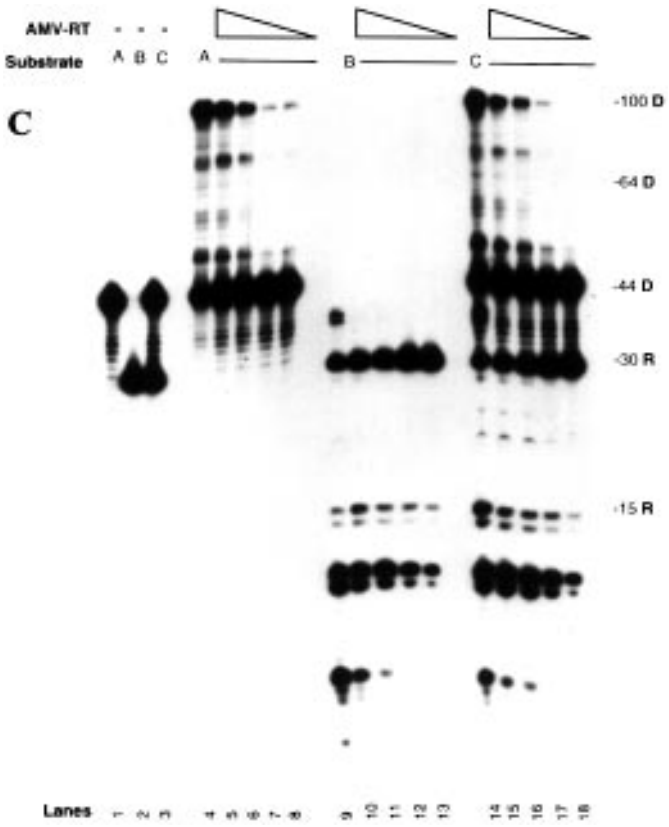


Figure 2. Titration of HIV-, MuLV- and AMV-RT with substrates A, B and C. (**A**) HIV-RT titration. Lanes 1–3 contain substrates A, B and C, respectively. Lanes 4–9 contain substrate A, lanes 10–15 contain substrate B, and lanes 16–21 contain substrate C after incubation with 0.2, 0.05, 0.01, 0.002, 0.0004 and 0.00008 U of HIV-RT under the reaction conditions described in the Methods section. Lane 22 contains a base hydrolysis ladder of the RNA. The base hydrolysis ladder was prepared using a protocol supplied with the Pharmacia RNA sequencing kit. Lanes 23–25 contain reactions of substrates A, B and C with T4 DNA polymerase. These reactions were carried out as described by the manufacturers. (**B**) MuLV-RT titration. Lanes 1–3 contain substrates A, B and C, respectively. Lanes 4–8 contain substrate A, lanes 9–13 contain substrate B, and lanes 14–18 contain substrate C after incubation with 20, 2, 0.2, 0.02 and 0.002 U of MuLV-RT. (**C**) Titration of AMV-RT with substrates A, B and C. Lanes 1–3 are substrate control lanes. Lanes 4–8 contain substrate A, lanes 9–13 contain substrate B, and lanes 14–18 contain substrate C after incubation with 2, 0.2, 0.02, 0.002 and 0.0002 U of AMV-RT. The products sizes are indicated in the figure; the letters 'D', 'R', and 'R/D' indicate whether the products are DNA, RNA or RNA that has been extended with DNA.

Gel electrophoresis

Samples were separated using 12% denaturing sequencing gels containing 7 M urea (17). The gels were dried and analyzed by autoradiography.

RESULTS

To determine the effect of a downstream segment of RNA on DNA-dependent DNA synthesis, we used substrates as shown in Figure 1. Substrate A had a DNA primer annealed to a template; substrate B had an RNA primer on the same template; and substrate C had both primers with the DNA annealed upstream of the RNA.

As a control, substrates A, B and C were incubated with T4 DNA polymerase (Fig. 2A, lanes 23–25). T4 DNA polymerase does not have RNase H activity, and cannot catalyze strand displacement. On substrate A, T4 DNA polymerase elongated the DNA to the end of the template at position 100. On substrate C,

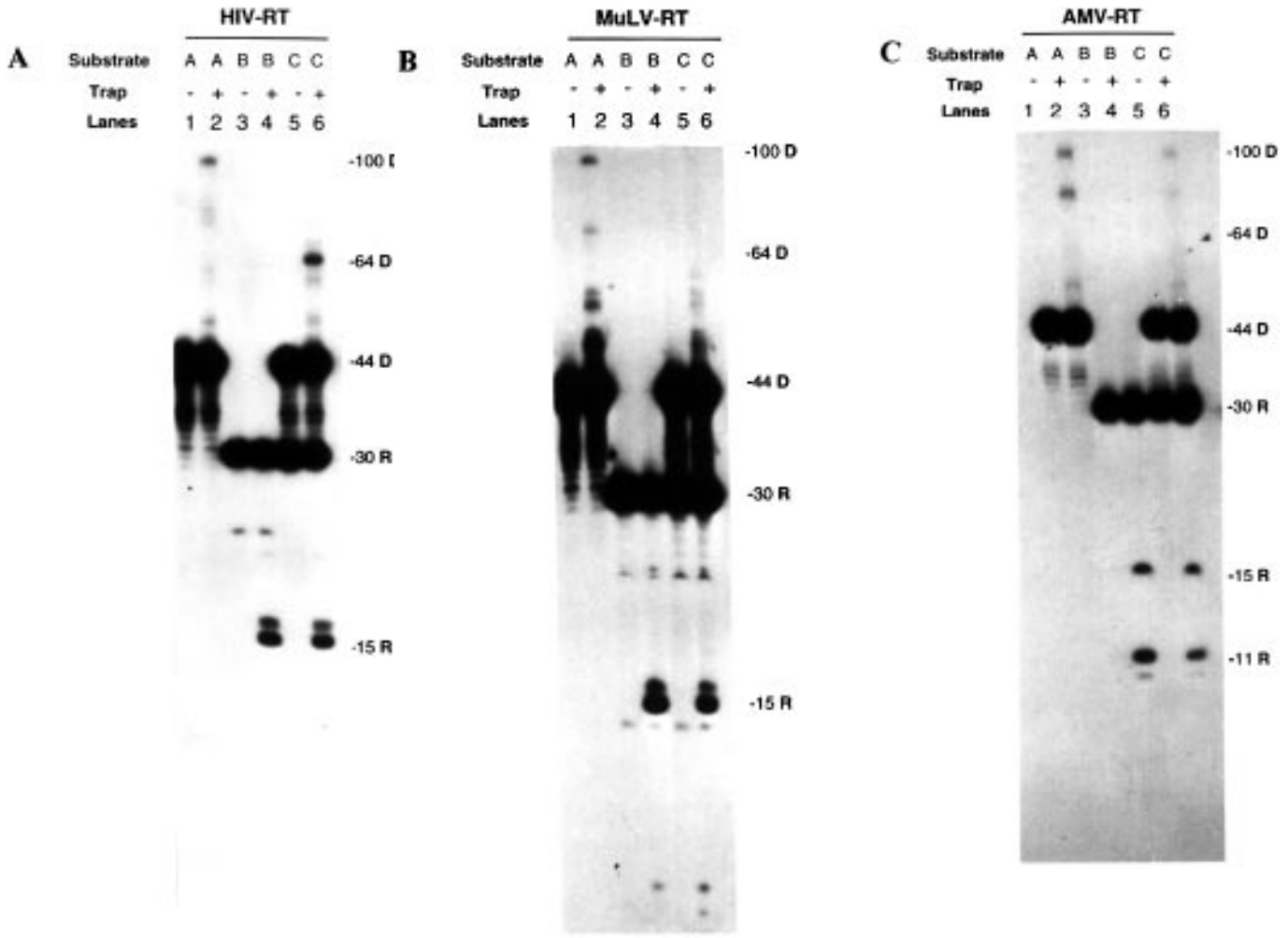


Figure 3. Reverse transcriptase reactions performed in the presence of trap. Trapped reactions contained 0.02 U of RT and were carried out as described in the Methods section. In each panel lanes 1 and 2 contain substrate A, lanes 3 and 4 contain substrate B, and lanes 5 and 6 contain substrate C. Lanes 1, 3 and 5 are trapped control reactions and lanes 2, 4 and 6 are trapped reactions. (**A**) HIV-RT reactions. (**B**) MuLV-RT reactions. (**C**) AMV-RT reactions. The product sizes are labeled as indicated in the legend of Figure 2.

T4 DNA polymerase elongated the DNA until it reached the RNA at position 64. This shows that all of the substrate C molecules contained a downstream segment of RNA. Using both substrates B and C, T4 DNA polymerase elongated the RNA to the end of the template at position 36.

The presence of a downstream segment of RNA can impede RT directed synthesis

Substrates A, B and C were incubated with decreasing concentrations of HIV-RT (Fig. 2A). Lanes 1–3 contain the substrates in the absence of enzyme, and show that at the start of the reactions the substrates were intact. Incubation of HIV-RT with substrate A (lanes 4–9) resulted in elongation of the DNA primer to the end of the template at position 100. All extension products became fainter with decreasing concentrations of HIV-RT, but no intense pauses were observed. No reaction occurred with any of the substrates at the lowest enzyme concentration. Incubation of HIV-RT with substrate B, having only an RNA primer, resulted in degradation of the RNA primarily to fragments ~15–16 nt long (lanes 10–15). This is consistent with the proposed model of RT binding to the 5′-end of recessed RNAs on DNA templates (18,19), and cleaving the RNA at a distance that corresponds to the spatial separation of the polymerase and RNase H domains of the RT (6,20–23). At high enzyme concentrations, the 15–16 nt long RNA degradation products were further cleaved to smaller segments. HIV-RT did not elongate the RNA primer.

Incubation of HIV-RT with substrate C (lanes 16–21) showed simultaneous elongation of the DNA and degradation of the RNA. In substrate C the presence of a downstream segment of RNA induced a strong pause at the point where the growing DNA encountered the RNA (position 64). Elongation of the DNA correlated with the degradation of the RNA fragment to segments <15 nt. These results suggest that HIV-RT waits until the RNA is degraded to pieces that will no longer stay annealed to the template before it can synthesize past the RNA. The polymerization and RNase H cleavage products made with substrate C decreased in concentration to approximately the same degree as the amount of RT was decreased. However at low enzyme concentrations the percentage of primers being extended was lower than the percentage of primers being degraded (lane 14; data not shown). These results

suggest that HIV-RT shows a preference for binding and acting on RNA primers.

Figure 2B shows incubation of substrates A, B and C with decreasing concentrations of MuLV-RT. Most results were similar to those with HIV-RT. The degradation products generated with MuLV-RT were primarily 15–16 nt long, and the presence of a downstream RNA induced a strong pause for synthesis. At low enzyme concentrations the percentage of primers being extended was lower than the percentage of primers being degraded (lanes 14–15; data not shown)

The same reactions were then performed with AMV-RT (Fig. 2C). Incubation of AMV-RT with substrate A (lanes 1–5) resulted in elongation of the DNA to the end of the template. The RNA primer in substrate B (lanes 6–10) was degraded to fragments ~11–12 nt long, a distinctly smaller size distribution than that produced by HIV- and MuLV-RT. Unlike with MuLV- and HIV-RT, at high concentrations AMV-RT is able to elongate the RNA primer (lane 6). Incubation of AMV-RT with substrate C resulted in a greater amount of degradation than extension products (lanes 11–15). At the lowest enzyme concentration used there were intense bands of degradation products but virtually no extension products (lane 15). Furthermore, there was no pause at the point where the elongating DNA encountered the RNA. This is an indication that the RNA is efficiently degraded to segments small enough to dissociate from the template, before the arrival of the elongating upstream DNA primer.

The experiments shown in Figure 2 were carried out at 80 mM KCl. Since it has been shown that lower KCl concentration enhances RNase H activity (20,24), we repeated these experiments at 32 mM KCl (data not shown). At 32 mM KCl there was a small increase in the amount of degradation products, but the polymerase and RNase H activities of the RTs still decreased at approximately the same rate. These findings show that the results observed here are not dependent on salt concentrations from 32–80 mM KCl.

Relative affinity of RT for DNA and RNA oligonucleotides bound to template DNA

In Figure 2 we observed that as the RT concentration was lowered, the polymerase activity of the RTs decreased to a greater degree than the RNase H activity. At low enzyme concentrations the percentage of primers being degraded was higher than the percentage of primers being extended. This increment was particularly marked for AMV-RT. To determine the relative affinities of these RTs for each of the primers on substrates A, B and C we performed the RT reactions in the presence of excess poly(rA)–oligo(dT)$_{16}$. Poly(rA)–oligo(dT)$_{16}$ is used as an enzyme trap to prevent the RT from returning to the primer-template after disassociation. The RT concentration used in the reactions was low enough so there would be less than one enzyme per substrate. Under these circumstances, we will observe the action of RTs only on the primers to which they were bound at the moment the reaction was initiated. Relative affinity, as used in this paper, is directly proportional to the RT activity observed upon binding to the substrate. We assume that each binding of the enzyme to the substrate results in the appearance of extension or degradation products. This is likely since the disassociation constant for RT is generally one to three orders of magnitude smaller than the rate constant for the RNase H and polymerase catalysis (25).

Figure 3A shows the HIV-RT reactions in the presence of excess poly(rA)–oligo(dT)$_{16}$. Lanes 1, 3 and 5 show control reactions in which the trap was pre-incubated with the substrate before addition of the RT. The lack of observed RT activity demonstrates that the trap is effective. Lanes 2, 4 and 6 show reactions in which HIV-RT was pre-incubated with the substrates, and then the trap was added with the magnesium and dNTPs used to start the reactions. Under these conditions HIV-RT elongated the DNA primer in substrate A to the end of the template (lane 2), and degraded the RNA in substrate B to segments ~15–16 nt long (lane 4). Using substrate C, HIV-RT elongated the upstream DNA up to position 64 and degraded the downstream RNA to segments 15–16 nt long (lane 6). It is remarkable that practically all of the extension products stalled when the growing DNA encountered the RNA. The percent of degraded RNA primers and extended DNA primers were quantitated using the PhosphorImager. We measured ~2-fold more degradation than extension products in these reactions (lane 6). These results show that HIV-RT has a relatively higher affinity for RNA than for DNA primers bound to a DNA template. However, the difference in affinity is not so high that only cleavage is observed.

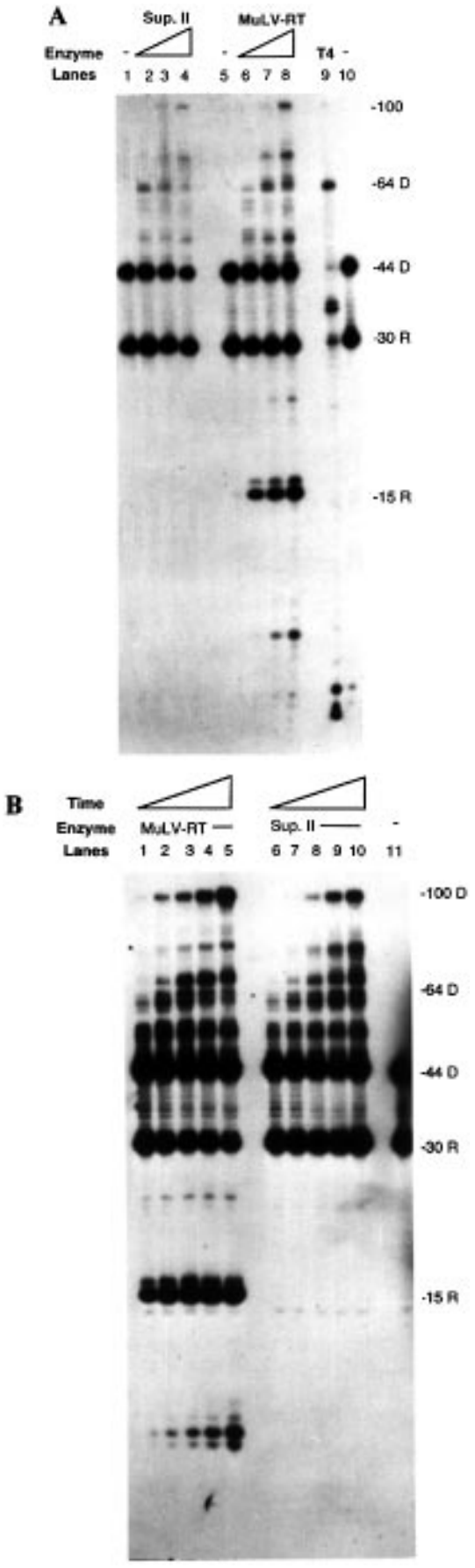
In trapped reactions the action of MuLV-RT on substrates A and B was similar to that of HIV-RT (Fig. 3B). A notable difference is that MuLV-RT formed very short DNA extension products on substrate C. These results may reflect the lower processivity of MuLV-RT when compared with HIV- and AMV-RT (20). The amount of degradation products was 2.2-fold greater than the amount of extension products (lane 6). These results show that MuLV-RT also has a relatively higher affinity for RNA versus DNA oligonucleotides bound to the same DNA template.

In the trapped reactions AMV-RT produced the characteristic RNA cleavage products from 11 to 16 nt long, and little DNA extension product (Fig. 3C). The amount of degradation products was 3-fold greater than the amount of extension products (lane 6). A distinct feature of AMV-RT is that some DNA primers were extended until the end of the template in the trapped reaction. This may be due to the more extensive degradation of the RNA observed with this enzyme, or to the higher processivity of AMV-RT compared with HIV- and MuLV-RT (8).

RNase H minus RTs are able to displace the downstream RNA during synthesis

Reverse transcriptases are efficient at displacing DNA (12–16). We expected that this strand displacement activity would also be active on RNA. We showed in Figure 3 that RTs have higher relative affinity for RNA oligonucleotides than for DNA oligonucleotides bound to template DNA. Therefore our inability to detect RNA strand displacement synthesis by RTs may be because the RNA primer gets degraded before displacement can be observed.

To determine whether this was the case, we tested the ability of Superscript II to displace RNA. Superscript II is an MuLV-RT multiple point mutant that lacks RNase H activity. We incubated substrate C with increasing concentrations of Superscript II (lanes 2–4) or MuLV-RT (lanes 6–8, Fig. 4A). Incubation of substrate C with T4 polymerase (lane 10) resulted in the formation of a 64 nt long product. Since T4 polymerase is unable to perform strand displacement, this result shows that all of the substrate C molecules contained a downstream segment of RNA. Superscript II was able to displace the RNA to synthesize full length DNA extension products. MuLV-RT also synthesized full length DNA extension products, but this activity was accompanied by the degradation of the downstream RNA. This observation suggests that wild type MuLV-RT also has the intrinsic ability to displace



RNA, but because its high affinity for RNA primers, and efficient RNase H activity, displacement cannot be observed.

Strand displacement slows RT directed synthesis (15–16), therefore degradation of the downstream RNA should accelerate the rate of synthesis through this region by the wild type MuLV-RT. Approximately the same amount of extension past the RNA was observed with decreasing concentrations of MuLV-RT and Superscript II (Fig. 4A). When these enzymes were incubated with substrate C in a time course reaction we observed faster kinetics of full length synthesis for MuLV-RT than for Superscript II (Fig. 4B). The relative concentration of the two RTs in the experiment shown in Figure 4B was adjusted such that both show equal rates of full length synthesis on substrate A, containing only a DNA primer (data not shown). This result suggests that even though the RNase H minus MuLV-RT can displace the RNA, the wild type enzyme has an advantage when it has to synthesize over these regions *in vivo*.

The experiments described here with MuLV-RT and Superscript II were repeated with HIV-RT and an RNase H minus HIV-RT (26) and similar results were obtained (data not shown).

In order to determine whether extension past the RNA using wild type RTs is a combination of degradation and displacement, enzyme titration experiments similar to those shown in Figure 2 were performed, and the reaction products were separated on native polyacrylamide gels (data not shown). Before the reactions were started only a faint band was observed at the position where the unannealed full length RNA migrates. As the amount of full length extension products increased we were unable to detect an increase in unannealed full length RNA, indicating that little or no displacement of undegraded RNA was taking place with the wild type RTs.

DISCUSSION

In retrovirus replication, synthesis of the first strand of DNA by the RT is accompanied by the degradation of the RNA genome. We and others have shown that the RNase H activity that accompanies RNA-directed DNA synthesis is not extensive enough to eliminate all of the template RNA (8–10). The segments of RNA that remain must be removed before or during synthesis of the second strand of DNA. The object of this study was to determine the fate of downstream segments of RNA *in vitro*, when synthesis from an upstream primer is taking place. Since reverse transcriptases are capable of efficient strand displacement, downstream segments of RNA might be displaced by the RT carrying out second strand synthesis. Alternatively, the fragments of RNA could be degraded by the excess RT molecules found in the virion, before or during plus strand synthesis.

To resolve this issue, we incubated AMV-, MuLV- and HIV-RT with a DNA template containing an upstream DNA primer and a downstream RNA primer. We found that HIV-, MuLV- and AMV-RT had a relatively higher affinity for RNA than for DNA

Figure 4. Extension of substrate C with Superscript II and MuLV-RT. (**A**) Substrate C was incubated with increasing concentrations of Superscript II (Sup. II) and MuLV-RT. Lanes 1, 4 and 10 are substrate control lanes. Lanes 2–4 and 6–8 contain 2, 0.2 and 0.02 U of Superscript II or MuLV-RT, respectively. Lane 9 contains a control reaction with T4 polymerase. (**B**) Time course reaction of Superscript II (Sup. II) and MuLV-RT extension. Lanes 1–5 contain 0.2 U of Superscript II and lanes 6–10 contain 0.2 U of MuLV-RT. The reactions were stopped at 0.5, 1, 2, 4 and 8 min, respectively. Lane 11 is a substrate control lane. The product sizes are labeled as indicated in the legend of Figure 2.

oligonucleotides bound to template DNA. This result agrees with a recent report by DeStefano (25) which shows that HIV-RT binds preferably to RNA–DNA versus DNA–DNA hybrid regions. Consistent with this higher affinity for RNA primers, incubation of HIV-, MuLV- and AMV-RT with the template that contained an upstream DNA and a downstream RNA primer resulted in the appearance of more degradation than extension products (Fig. 3). Furthermore, using the wild type RTs we never observed full length DNA synthesis without degradation of the downstream RNA. These results demonstrate that RNA fragments that remain annealed to the minus strand DNA are generally degraded before or during synthesis of the plus strand DNA.

During reverse transcription a variety of nucleic acid structures are created; RNA–RNA, RNA–DNA and DNA–DNA hybrids are present simultaneously. In order for genomic replication via reverse transcription to be successful, the RT must perform its multifunctional activities in an appropriate order. One of the ways in which the RT could achieve this, is to have an order of preference for binding and reacting with these structures. The fact that HIV-, MuLV- and AMV-RT have a relatively higher affinity for RNA primers suggests that most segments of RNA left over from first strand synthesis could be degraded before plus strand DNA synthesis begins. However, each virus particle contains an excess of RT molecules. It is likely that these RT molecules participate in the degradation of the RNA segments after initiation of plus strand synthesis. Therefore, an alternative possibility is that degradation of these RNA segments is not obligatory in order for an RT to initiate plus strand DNA synthesis, since the RNA can also be degraded during DNA synthesis by the other RT molecules present in the virion.

HIV- and MuLV-RT were unable to extend the downstream RNA primer, while AMV-RT extended this primer at very low efficiency (Fig. 2). RTs prefer to extend RNA primers with sequences very similar to that of the polypurine tracts, and are unable to extend RNA primers of other sequences (27,28). This preference of the RTs to specifically elongate the polypurine tract RNA primers may be responsible for the specific initiation of the plus strand DNA at these sites. If the RTs were able to elongate all of the RNA fragments that are left over after minus strand DNA synthesis, plus strand DNA synthesis would be fragmented, and there would be extensive strand displacement synthesis.

When we compared the amount of degradation that accompanied RNA-directed DNA synthesis we found that while HIV- and MuLV-RT generated RNA degradation products <13 nt, AMV-RT generated mostly large degradation products (8). The amount of RNA degradation products that are long enough to remain annealed to the templates was quantitated (11). With HIV-RT, 20% of the template RNA remained annealed after one round of processive DNA synthesis, while with AMV-RT in the same situation 80% the template RNA remained annealed. Here we found that when the RT is cleaving the residual RNA segments in a polymerase independent fashion, AMV-RT is much more efficient than HIV- and MuLV-RT at generating small cleavage products that will spontaneously disassociate from the template (Fig. 2). Therefore, the inefficiency with which AMV-RT degrades the template RNA in a polymerization dependent fashion, is compensated by its very effective polymerase independent RNase H activity.

Our experiments provide no indication that wild type RTs displace RNA. However RNase H minus MuLV- and HIV-RT were able to displace downstream segments of RNA. This result suggests that the wild type RTs also have the intrinsic capacity to displace RNA. Our inability to detect displacement of RNA fragments by the wild type RTs can be explained because RTs have high affinity for RNA oligonucleotides bound to template DNA. This presumably led to RNA degradation before we could observe strand displacement. In our experiments strand displacement of RNA by wild type RTs would only be detected if the RT molecules delayed degrading the RNA until the arrival of the elongating DNA primer. Since the RNase H minus RTs cannot degrade the RNA, their RNA displacement activity becomes unmasked. These results suggest that if the RNase H activity of wild type RTs cannot degrade a particular segment of RNA quickly enough, the segment could be displaced during plus strand synthesis.

At some RT concentrations the DNA primer extension paused at the 5′-end of the RNA. In excess RT, the upstream primer was elongated with no significant pause. This suggests that, *in vivo* at least one purpose for excess RTs in the virion is to degrade remaining plus strand RNA segments sufficiently quickly, before or during DNA primer elongation, such that elongation is not impeded.

In vivo reverse transcription occurs in the cytoplasm of the infected cell, inside a capsid structure, in the presence of nucleocapsid protein (NC) and other viral proteins. The degradation and elongation events presented in this paper could be affected by any of these components. The role of nucleocapsid protein in these reactions is currently under investigation.

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