Physical Mapping of HIV Reverse Transcriptase to the 5′ End of RNA Primers*

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Enzymatic analysis of RNA cleavage products has suggested that human immunodeficiency virus (HIV) reverse transcriptase (RT) binds to the 5′ end of RNAs that are recessed on a longer DNA template (RNA primers) yet binds to the 3′ end of DNA primers. One concern is that RT molecules bound at the 3′ end of RNA would not be easily detected because RT may not catalyze substantial RNA extension or cleavage when bound to the 3′ end. We used physical mapping to show that RT binds preferentially to the 5′ end of RNA primers. An HIV-RT that lacked RNase H activity (HIV-RT E478Q) was incubated with the RNA-DNA hybrid followed by the addition of Escherichia coli RNase H. RT protected a ~23-base region at the 5′ end of the RNA and 4 additional bases on the DNA strand. This footprint correlated well with the crystal structure of HIV-RT. No protection of the RNA 3′ end was observed, although when dNTPs were included, low levels of extension occurred, indicating that RT can bind this end. Wild-type HIV-RT cleaved the RNA and then extended a small portion of the cleaved fragments, suggesting that very small RNAs may be bound similar to DNA primers.

The conversion of the single-stranded RNA genome of retroviruses to a double-stranded DNA (provirus) requires several steps that are carried out by the multifunctional viral reverse transcriptase (RT). Enzymatic analysis indicates that the polymerase and RNase H active sites of HIV-RT are arranged such that they contact polymer substrates ~18 nucleotides apart (8–12). Crystal structures also show 3′-recessed termini of double-stranded DNA substrates bound at the presumed polymerase active site, suggesting that the RNase H site is associated with the substrate ~18 bases upstream (13, 14). In addition, results have shown that some RT-mediated cleavages, which were termed “polymerase-dependent cleavages,” could be advanced upon primer extension and remained a fixed distance from the extended 3′-primer terminus (10). Taken together, this work suggests that the positioning of the RNase H active site on the RNA-DNA hybrid is coordinated by the binding of the polymerase active site to the 3′-terminal nucleotide of a recessed DNA primer.

In the enzymatic assays cited above, the substrates were relatively short segments of DNA hybridized to longer segments of RNA such that the 3′ terminus of the DNA was recessed on the RNA strand. This is the general arrangement that exists during first-strand DNA synthesis. Clearly, under such conditions RT associates with the 3′ DNA terminus in a configuration that allows DNA extension from the 3′ DNA terminus. In contrast, several reports have indicated that HIV-RT associates preferentially with the 5′ end of an oligonucleotide RNA that is bound to a relatively long DNA template in a primer configuration (15–19). The orientation of RT on RNA primers seems contradictory because two RNA primers (tRNAs and the poly purine tract) must be used during provirus replication. Results with RNA primers are based on experiments that assessed the position of the enzyme by RNase H cleavage. Initial cleavages of the RNA primers were observed ~18–20 bases from the 5′ end of the RNA and were independent of the 3′ end. Cleavages or nucleotide additions consistent with RT binding at the 3′ end were not observed. Because the conclusions were based on enzymatic activity, it remains possible that binding to the 3′ end was not observed, because the enzyme cannot carry out efficient catalysis (either RNA cleavage or primer extension) when bound at this location. Consistent with this possibility is the low efficiency of the natural RNA primer for second-strand DNA synthesis (the poly purine tract) when compared with a DNA primer of the same sequence (20). Although the enzymatic results clearly indicate that the RT can bind to the 5′ end of an RNA primer, these other considerations leave in doubt if this is the preferred binding orientation. It is
possible that RT prefers to bind to the RNA primer at the 3’ end in an orientation favoring extension but can switch to a less favored position at the 5’ end. The lack of a catalytic event at the 3’ end would mean that the preferred binding position would go undetected.

One method that could be used to determine where RT binds to RNA primers would be to physically map the location of the enzyme using a protections type assay. However, *Escherichia coli* RNase H or other enzymes that could be used for the mapping would require divalent cation to cleave the regions of the hybrid not protected by RT. The RNase H activity of RT precludes the use of divalent cation, because this would activate the RNase H activity of RT, leading to cleavage of the protected region. To overcome this problem an RT enzyme that lacks RNase H activity would be required. Although many such mutants have been characterized, most are also defective for DNA polymerization, suggesting that they may not interact with primer-template-like wild-type enzyme. However, a particular mutation in a putative catalytic residue of RNase H, an aspartate-to-asparagine at position 478 (HIV-RT^Glu → Gln), seems to have no effect on polymerization (21). This mutant has polymerization activity that is essentially identical to the wild-type enzyme, indicating that it binds primer-template correctly. HIV-RT^Glu → Gln has been used extensively by others for DNA experiments requiring DNA polymerase activity in the absence of RNase H (22–25). Using HIV-RT^Glu → Gln we were able to physically map RT molecules to the 5’ end of RNA primers while molecules were not detected at the 3’ end. The results are consistent with HIV-RT binding preferentially to the 5’ end of RNA primers. The polyuridine tract and tRNA<sup>3’</sup>-<sup>5’</sup> likely represent structurally unique RNA primers that coordinate binding in a different orientation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant mutant (Glu<sup>378</sup> → Gln) lacking RNase H activity and wild-type HIV-1 reverse transcriptase were provided to us graciously by Dr. Stuart Le Grice representing the Center for AIDS Research at Case Western University. Properties of these enzymes have been described previously (21). Aliquots of HIV-RT were stored frozen at −70°C, and a fresh aliquot was used for each experiment. Klenow polymerase and E. coli RNase H were from Promega. Calf intestinal phosphatase, DNase I/RNase-free, T3 and T7 RNA polymerases, RNase T1, T4 RNA ligase, placental RNase inhibitor (RNasin), rNTPs, dNTPs, and all restriction enzymes were obtained from New England Biolabs. RNA markers including base hydrolysis ladders and RNA oligonucleotides were synthesized by Genosys, Inc. Radiolabeled compounds were from PerkinElmer Life Sciences.

**Production of RNA Transcripts**—Run-off transcription with T7 RNA polymerase was performed using the protocol of the manufacturers. Plasmid pBSM13<sup>+</sup> (Stratagene) was cleaved with BamHI or HindII, and T7 RNA polymerase was used to prepare run-off RNA transcripts 30 or 40 nucleotides in length, respectively. The RNAs were isolated from polyacrylamide gels and end-labeled with <sup>32</sup>P as described below. The transcription reactions were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. The recovered RNA was end-labeled using T4 polynucleotide kinase and [γ<sup>32</sup>P]<sup>ATP</sup> using manufacturer protocol and the supplied buffer. The material was run on a denaturing polyacrylamide gel, located by autoradiography, and recovered as described above.

**End-labeling of RNAs**—Gel-purified RNAs were dephosphorylated with calf intestinal phosphatase using manufacturer protocol and the supplied buffer. The reactions were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. The recovered RNA was end-labeled using T4 polynucleotide kinase and [γ<sup>32</sup>P]<sup>ATP</sup> using manufacturer protocol and the supplied buffer. The material was run on a denaturing polyacrylamide gel, located by autoradiography, and recovered as described above.

**End-labeling of RNA**—The 30-nucleotide RNA described above was labeled at the 3’ end using T4 RNA ligase and cytidine-3’,5’-biphosphate. The 3’,5’-biphosphate with a radiolabeled 5'-phosphate group was prepared and used to label RNA as described. After incubation the mixture was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. The recovered material was then treated with calf intestinal phosphatase as described above to remove the 3’-phosphate group from the terminal 3’,5’-biphosphate. The material was run on a denaturing polyacrylamide gel, located by autoradiography, and recovered as described above.

**RNA-DNA Hybridization**—The hybrids were prepared by mixing RNA and DNA at a 1:2 ratio of 3’ termini in buffer containing Tris-HCl (pH 8.0), 80 mM KCl, 1 mM dithiothreitol, and 0.1 mM EDTA (pH 8.0). The mixture was heated to 65°C for 5 min and then cooled to room temperature. The DNAs used were 100 (as illustrated in Fig. 1A), 80, 70, or 50 nucleotides in length as specified in the text and figure legends.

**RNase H Protections Assays**—Protection assays were performed by preincubating for 3 min at 37°C, HIV-RT (Glu<sup>378</sup> → Gln) (about 50 nM final concentration in reactions) and 5 nM (RNA portions) substrate (5’ or 3’ end-labeled on the RNA strand) in a volume of 10.5 μl containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 1 mM dithiothreitol (buffer A) plus 7 mM MgCl<sub>2</sub> and 12 mM KCl. Two microliters of E. coli RNase H (0.25 units/μl) in buffer A was then added, and the incubations were continued for the times indicated on the figure. The reactions were stopped with 12.5 μl of 2× gel loading buffer (90% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol, and 0.1% bromphenol blue). Samples were subjected to electrophoresis on 8% polyacrylamide denaturing gels, and autoradiography was performed.

**HIV-RT Primer Extension Assays**—Primer extension assays were performed by preincubating the primer-template as described above under “RNase H Protection Assays” except that RT was excluded from the preincubation step, and dNTPs (100 μM final concentration) were included. The reactions were initiated by the addition of wild-type or mutant HIV-RT (0.8 pm) or the Klenow fragment of E. coli polymerase I (2.5 units) in buffer A. Incubations at 37°C were continued for 20 min, and reactions were terminated and processed as described above. In some cases fully extended products were recovered from gels as described above and treated with 1 unit of DNase I (RNase-free) for 15 min at 37°C. Reactions were performed in the same buffer used in the transcription reactions in a final volume of 10 μl. Samples were stopped with 10 μl of 2× gel loading buffer and processed as described above except that a 12% polyacrylamide gel was used.

**Exonuclease I Protection Assays**—Protection assays with Exo I were performed as described under “RNase H Protection Assays” except that E. coli RNase H was replaced with Exo I. The amount of Exo I used is indicated in the Fig. 7 legend. After Exo I addition, the reactions were continued for 10 min at 37°C.

**Gel Electrophoresis**—Denaturing polyacrylamide gels (19:1 acrylamide/bisacrylamide) were prepared and subjected to electrophoresis as described (26). RNA markers including base hydrolysis ladders and RNA<sub>T<sub>1</sub> cleavage ladders were prepared as described previously (16).

**RESULTS**

**Rational for Substrate Construction**—Substrates were designed to determine the physical location of HIV-RT on RNA primers (Fig. 1A). The 30- or 40-nucleotide (shown) RNA molecules were end-labeled at the 5’ or 3’ end with <sup>32</sup>P and hybridized to an internal region of a relatively long complimentary DNA. HIV-RT<sup>Glu → Gln</sup> was incubated with the substrates and allowed to bind. E. coli RNase H was then added. If RT bound at the end of the RNA molecule with the label, then this chain should be protected from cleavage by E. coli RNase H, and an RNA cleavage product corresponding to the protected portion of the molecule would appear on an autoradiogram. Note that the lack of RNase H activity of HIV-RT<sup>Glu → Gln</sup> prevents this enzyme from cleaving the RNA.

**HIV-RT Protects ~23 Bases of the 5’-labeled RNA**—Shown in Fig. 2 is a protection assay using the substrate shown in Fig. 1A (40-base RNA bound to 100-base DNA) in which the RNA was labeled at the 5’ end with <sup>32</sup>P. After preincubation with RT, E. coli RNase H was added (as indicated), and the incubations were continued for the indicated time. In the absence of RT the RNA was digested to small (<10 nucleotides) fragments by RNase H as expected. When RT was included in reactions,
Mapping of HIV-RT on RNA primers

A

5’–(N)_{40}GACTCTAGAGATCCCGGGTACCGAGCTGAATTCGCC–(N)_{30}–3’ DNA
3’–CGUGAGGUCUGCAGCCCGUAAAGGGG–5’ RNA

B

5’–(N)_{15}GACTCTAGAGATCCCGGGTACCGAGCTCG–3’ DNA
3’–CGUGAGGUCUGCAGCCCGUAAAGGGG–5’ RNA

Fig. 1. Substrates used in experiments. Shown are schematic diagrams of the substrates used for specific assays. A, 40-nucleotide RNA hybridized to a 100-nucleotide DNA. This substrate and various derivatives were used in RNA mapping experiments as described in the text and figure legends. B, the 40-nucleotide RNA from A is shown bound to a 50-nucleotide DNA such that 9 bases of single-stranded RNA overhang at the 5’ end of the RNA. (N), the number (x) of unspecified nucleotide (N) at the ends of the molecules.

Fig. 2. RNase H protection assay with and RNA primer bound to a DNA template. Shown is an autoradiogram of a protection assay (performed as described under “Experimental Procedures”) with the substrate shown in Fig. 1A. The position of the 1^{32}P label on the RNA portion of the substrate is indicated on the schematic representation of the substrate (*). The presence or absence of various enzymes as well as the time of incubation with RNase H are indicated above each lane. The position of the protected RNA is also indicated. Size markers (in nucleotides) on the left side of the gel correspond to positions of guanosine residues. (performed as described under “Experimental Procedures.”) G, G ladder showing the RNA portion of the substrate digested with RNase T1; B, base hydrolysis ladder showing the RNA portion of the substrate subjected to base hydrolysis as described under “Experimental Procedures”; C, control showing the substrate without enzyme addition.

5’-derived RNA fragments were observed over the course of the reactions (1–16 min). Initially a group of fragments ranging between 23 and 28 nucleotides was observed, whereas at later time points the smaller of these predominated. The smallest protected fragments corresponded to a length of 23 nucleotides, and this was taken as the region protected by RT (see “Discussion”). The fact that protection was observed over the course of the reactions indicated that the interaction between RT and the 5’ end of the RNA was stable. Substrates were also designed to determine whether the position of the 3’ end (see Fig. 3) or the sequence of the RNA influenced RT association (data not shown). In all cases experiments yielded similar results with a protected region of the same size. The results indicate that RT associates with the 5’ end of the RNA primer irrespective of the position of the 3’ end or RNA sequence.

Protection of the 3’ End of the RNA Is Not Observed—Although the above experiments show that the 5’ end of the RNAs is protected, they do not rule out the possibility that the 3’ end is also protected by other RT molecules. For example, some RNA molecules could have RT at the 5’ end and others at the 3’ end. To test this, a 30-nucleotide RNA corresponding to the first 30 nucleotides from the 5’ end of the RNA shown in Fig. 1A was labeled at the 3’ end with 3’,5’-bisphosphate as described under “Experimental Procedures.” The results in the addition of a C residue to the 30-mer, making the molecule 31 nucleotides in length. This RNA was chosen because the addition of a C would allow the RNA to completely bind to the 100-base DNA used in the experiments because a G was at the next position in the DNA. After 3’-end-labeling the RNA was dephosphorylated to remove the 3’-phosphate group (see under “Experimental Procedures”), producing an authentic 3’-OH group. Presumably if RT bound to the 3’ end of the RNA in a configuration consistent with primer extension a region of 18 or more bases should be protected. This is drawn from the fact that the distance between the polymerase and RNase H active sites is about 18 nucleotides (see the Introduction). As shown in Fig. 3A, no products consistent with protection were observed even at very early points in the protection assay. Other experiments performed with lower levels of E. coli RNase H over short time points also yielded no clearly protected products (data not shown). These results indicated that the polymerase does not bind to the 3’ end of the molecule or at least that a stable interaction does not occur between RT and the 3’ end. For comparison, the same experiment was performed with this RNA labeled at the 5’ end (Fig. 3B). Protected products identical in size to those observed with the 40-nucleotide RNA used in Fig. 2 were observed. The results indicate that RT forms a stable association with the 5’ end of the RNA primer.

HIV-RT Can Bind to and Extend the 3’ End of an RNA Primer—Although 3’-end protection was not observed, it is still possible that RT can bind transiently to the 3’ end of the primer and perhaps extend the primer. To test this, the 5’-end-labeled 30-nucleotide RNA substrate bound to an 80-base DNA (a 5’-truncated version of the 100-nucleotide DNA shown in Fig. 1A) was used to perform an extension assay with HIV-RT^{Glu} → Gln, wild-type HIV-RT, and Klenow polymerase (Fig. 4). The substrate was incubated with the indicated enzyme and dNTPs as described under “Experimental Procedures.” Klenow is able to extend RNA primers with dNTPs and was used as a control. Both RT enzymes carried out some extension with RT^{Glu} → Gln, producing a very small amount of extension products, whereas the wild-type enzyme produced more. Note that these enzymes have essentially identical levels of activity on DNA-primed RNA or DNA templates (data not shown). The reason for the
HIV-RT could not be removed by DNase I. The results indicate that products likely include 1/1032–2 additional dNTPs at the 3’ end. The 30-nucleotide RNA is more extended than the 30-nucleotide RNA. The ability of wild-type RT to cleave the RNA is the apparent reason for the significantly greater level of extension observed in Fig. 4.

RT Has Affinity for the 5’ End of the RNA Even When It Is Not Hybridized to DNA—Although it is clear that RT associates with the 5’ end of an RNA primer, it is not clear what properties of the substrate direct this association. Other reports have suggested that the 5’ terminus of the RNA plays a key role in directing binding (17). Results showed that RT RNase H-directed cleavages occurred 18 bases 3’ of the 5’ end of an RNA that was bound to DNA. This approximate distance was maintained even if the 5’ end of the RNA was not bound to the DNA. Such a configuration produces a 3’-recessed DNA end and a single-stranded 5’ RNA overhang. The extent to which cleavage directed by the 5’ end of the RNA occurred depended on the length of the overhang requiring that it be less than the distance between the RNase H and polymerase active sites of HIV-RT (about 18 bases). A hybrid was configured to determine what region of such a substrate would be protected by RT binding (Fig. 1B). In this case a DNA molecule was bound to RNA such that the 3’-terminal nucleotide of the DNA was bound to the 10th base from the 5’ end of the RNA. This created a 9-base single-stranded region at the 5’ end of the RNA. A protection assay performed with this substrate is shown in Fig. 6. In this experiment two sets of protected fragments were observed. The longer set of about 30–34 bases corresponded to RT binding to the 3’-recessed end of the DNA and protecting about 21–24 bases back from the 3’ end. This is consistent with protection assays performed using DNA primers (25, 27). A second smaller set of protected fragments were 23–27 bases in length and corresponded to the polymerase binding to the 5’ end of the RNA despite the fact that this portion of the molecule was not bound to DNA. These results are consistent with the previous enzymatic analyses and suggest that RT has an affin-
ity for the 5′ end of the RNA. Note that when the 5′-RNA overhang was extended to 20 nucleotides, only protection corresponding to RT associating with the 3′ end of the DNA primer was observed (data not shown).

Approximately 4 Bases of Single-stranded DNA Are Protected in the Region in Front of the 5′ RNA Termini When the RNA Is Recessed on a Longer DNA—The above mapping assays indicated that ~23 bases of RNA were protected when RT bound to the 5′ end of an RNA primer. To determine the size of the complete footprint of HIV-RT, the region in front (toward the 3′-DNA terminus) of the 5′ end of the RNA was examined for protection. In this case this region consists of single-stranded DNA; therefore, a DNase had to be used for the footprint. Exo I proved most effective in this regard. This enzyme has a 3′–5′ exonuclease activity that initiates from a 3′-DNA terminus.

The 30-base RNA shown in Fig. 1A was hybridized to a 5′-32p end-labeled 70-base DNA (a 5′-truncated version of the 100-nucleotide DNA shown in Fig. 1A) as shown in Fig. 7. The configuration left 20 bases of single-stranded DNA after the 5′ end of the RNA. Assays were performed using the conditions of the mapping assay except that E. coli RNase H was replaced with Exo I. The figure shows an experiment with increasing levels of Exo I added to substrate that had been preincubated in the presence or absence of RT. In the absence of RT the DNA was shortened producing a major product that was 55 nucleotides in length. Lower levels of some smaller products were also observed with high levels of Exo I. Because the 55-base product was most prevalent even with high amounts of enzyme, this length was taken as the furthest that Exo I could easily proceed on the substrate. It corresponded to 5 bases upstream of the RNA-DNA hybrid region. The inability of Exo I to cleave upstream of the hybrid junction could have resulted from steric effects, because the leading edge of the enzyme abutted against the hybrid or a loss of substrate specificity as the enzyme interacted with the hybrid. In reactions including RT the length of the protected region increased with the most prominent protected DNA being 59 nucleotides long. Therefore in the presence of RT an additional 4 bases were protected. This suggests that RT covers 4 bases beyond the 5′ terminus of the RNA.

However, this assumes that the interaction between Exo I and RT or the RNA-DNA hybrid junction both result in Exo I terminating cleavage 5 bases upstream. It is not clear if the steric effects induced by these different molecules would necessarily be identical.

**DISCUSSION**

We have used physical mapping techniques to determine the location of HIV-RT on RNA molecules recessed on a longer DNA (RNA primer configuration). Previous enzymatic analyses...
have indicated that RT binds near the 5' end of the RNA on these substrates and generally recognizes the RNA as a substrate for RNase H cleavage rather than extension (15–19). The previous results are subject to other interpretations as noted in the Introduction, namely that RNA molecules bound at the 3' end of the RNA may not manifest an activity (either cleavage or polymerization) that could be detected easily. In this report we show that physical mapping techniques are consistent with the enzymatic results and that RT binds with high stability to the 5' end of the RNA primer. This mode of binding was independent of the position of the 3' end of the RNA and was also sequence-independent, at least with respect to the sequences tested. Results also indicated that RT can bind to and extend from the 3' end of the RNA (see Figs. 4 and 5), although this likely represents a low affinity binding mode because protection of the 3' end was not observed and the extension efficiency was very low.

Although it is not entirely clear what causes RT to associate with the 5' end of the RNA primer, it seems that the 5' RNA terminus plays some role. Others have shown that cleavages within an RNA-DNA hybrid region can be directed by the association of RT with the 5' end of the RNA, even if that end is not part of the hybrid region (17). The limitations on this direction seem to be that the 5'-RNA terminus must be within ~18 bases (the length between the polymerase and RNase H active sites of RT) of the hybrid region, although no substrates with 10–27-base overhangs were tested. Consistent with these findings, RNA fragments that corresponded to RT associating with the 5' end of a 9-base overhang region of RNA were observed in protection assays (Fig. 6). However, fragments corresponding to RT binding at the 3'-recessed DNA terminus on this substrate were also observed. The proportion of the different protected RNAs was approximately equal, suggesting that RT can bind at both positions with about equal affinity. Alternatively RT may preferably bind in one orientation and then occasionally switch to the second. Consistent with this hypothesis, the group of longer fragments corresponding to RT binding to the 3'-recessed DNA terminus decreased over time, whereas the level of shorter products corresponding to 5' RNA end-binding increased. The observed decrease could have resulted from RT molecules dissociating from the substrate and allowing access by E. coli RNase H as was observed with other substrates. It is also possible that some of the decrease was caused by these longer RNAs being further cleaved when RT moved to the 5' end of the RNA. This could explain the increase in shorter products over time. Further experimentation would be required to definitively determine how RT binds to these types of substrates.

It was not possible to determine the exact length of the substrate that was protected by RT. Because cleavage data shows that RT cleaved about 18 bases 3' of the 5' end of the RNA (15–19), presumably the polymerase domain would be bound at the 5' end, and the RNase H site would be positioned 18 bases “behind” the polymerase. Using this logic the results suggested that HIV-RT protects about 23 bases behind the polymerase active site on the primer strand and 4 bases in front of this site on the DNA template strand. Steric effects were invoked in determining protection in front of the polymerase site, because results without RT indicated that Exo I could only get within 5 bases of the RNA-DNA hybrid region. No steric inferences were used in estimating the 23-base protected region, although it is not known if cleavage by E. coli RNase H was limited as this enzyme abutted against RT. It is also not known if RT is completely stationary on RNA primer substrates or if the enzyme can slide to some extent. Sliding might allow bases that are normally protected to become transiently susceptible to cleavage. Note also that the 23-base length represents the shortest protected fragments observed, although some protection was observed as much as 3–4 bases beyond this length. Given these considerations an exact length of protected bases would be difficult to state using this approach. However, similar experiments used to map DNA primers hybridized to a DNA template suggested that a 23-base region behind the 3'-recessed primer terminus was protected on the template strand (25). The current proposed model for the binding of RT to an RNA primer suggests that the RNA primer strand is synonymous with the DNA template strand of a DNA-DNA primer-template (15–19). Therefore the previous experiments are essentially in agreement with those shown here. The authors of the latter work also found that a 7-base region in front of the 3'-DNA terminus was protected on the template strand. The 4-base region suggested here for an RNA primer is not inconsistent with this, because in current experiments these 4 bases would correspond to the primer strand in the latter work. It should also be noted that the 23-base distance is in good accord with the proposed crystal structure of HIV-RT (25).

The presented work confirms the findings of enzymatic analysis in showing that RT binds preferentially to the 5' end of RNA bound internally on a longer DNA template. The orientation of binding clearly positions RT to cleave the RNA rather than use it as a primer. In this regard, RNA fragments left associated with the DNA strand during minus-strand DNA synthesis would likely be cleaved away rather than be used to prime plus-strand DNA synthesis. This mode of binding may
have evolved to prevent spurious priming of second-strand DNA synthesis limiting priming of the plus strand to the specialized polypurine tract RNA primer. However, there was evidence suggesting that after cleavage of the relatively long RNA primers used in this work, a small proportion of the short cleavage products can be used to prime DNA synthesis (see Figs. 4 and 5 with wild-type RT). The RNA portion of products thus produced (corresponding to about 9 nucleotides (see “Results”)) is apparently resistant to RT RNase H activity. Priming of HIV synthesis by RNAs other than the polypurine tract has also been shown in vivo and in vitro system designed to mimic plus-strand DNA synthesis (28). Recent reports have shown that after the initial cleavage of an RNA primer 18 bases in from the 5′ end, a secondary cleavage is made about 9 bases from the 5′ end, resulting in two separate 9-base products (18, 19). From the results shown here it is likely that some small proportion of the 5′-derived 9-base products can be extended by RT. The likelihood of extension may depend on the sequence of the product. In our experiments the moderately G-C-rich nature of the 9 5′-ends (5′-GGGCCGAUU-3′) may have allowed these fragments to remain bound to the DNA long enough to be extended. It should also be noted that these experiments were performed in the absence of the viral nucleocapsid protein (NC), which has been shown to destabilize short hybrids (29–31). Further analysis of the conditions required for extension of random RNAs by HIV-RT will be required to determine whether extension of some nonspecific RNAs is likely to occur in the cell.

FIG. 7. Protection assay performed with Exo I. A, shown is an autoradiogram of a protection assay (performed as described under “Experimental Procedures”) with the substrate shown at the top of the figure (30-nucleotide RNA use in Fig. 3 hybridized to a 5′ 32P end-labeled 70-nucleotide DNA (see “Results”). Samples were incubated in the presence or absence of HIV-RT(Glu → Gin) (as indicated), after which various amounts of Exo I were added (from left to right, pairwise, not including lanes L and C, 0.3, 0.6, 1.3, 2.5, or 5 units), and the incubations were continued. Exo I will digest the unprotected DNA starting from the 3′ end. The sizes of prominent (see “Results”) cleavage products from lanes with or without RT(Glu → Gin) are indicated. C, starting material; L, starting material digested with 0.1 units of Exo I for 2 min to produce a ladder. B, map of the substrate including indicated positions of prominent Exo I cleavage products on the DNA strand.

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