Control of Initiation of Viral Plus Strand DNA Synthesis by HIV Reverse Transcriptase*

(Received for publication, August 20, 1997, and in revised form, November 19, 1997)

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**This work was supported by National Institutes of Health Grant GM 49573 (to P. J. F. and R. A. B.) and in part by Core Grant CA 11198 to the University of Rochester Cancer Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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HIV reverse transcriptase catalyzes the conversion of retroviral single-stranded genomic RNA to double-stranded DNA following entry of the virus into the cytoplasm of the host cell. HIV reverse transcriptase (RT) degrades all of the non-polypurine tract oligomers; one of these, the polypurine tract, primes synthesis of a plus strand DNA. The viral reverse transcriptase (RT) degrades all of the non-polypurine tract oligomers. We show that unlike other DNA polymerases the retroviral RT can bind either end of an annealed RNA primer, the 5′-end for degradation and the 3′-end for synthesis. The competition between the two binding modes at any primer determines whether it will be extended or degraded. The 5′-end binding can be suppressed in at least two ways. The sequence of the primer can be such that a region at the 5′-end is unannealed or a DNA primer can be annealed just adjacent to the 5′-end of the RNA primer. This promotes binding of RT to the RNA 3′-end, allowing a primer that would normally be degraded to be extended. Implications for human immunodeficiency virus replication and antiviral therapy are discussed.

Reverse transcriptase (RT) catalyzes the conversion of retroviral single-stranded genomic RNA to double-stranded DNA following entry of the virus into the cytoplasm of the host cell. RT is a multifunctional enzyme that displays DNA polymerase activity on both RNA and DNA templates, RNase H, strand displacement, and strand transfer functions, which are all essential steps in viral replication (1). Minus strand DNA synthesis is initiated from a cellular tRNA primer packaged within the virion (for a review see Ref. 2). DNA synthesis is accompanied by the cleavage of genomic RNA by the RT-RNase H. A critical component of retroviral replication is the creation and extension of a polypurine-rich genomic RNA fragment, the polypurine tract (PPT), that primes plus strand DNA synthesis (3). According to the model of reverse transcription for viral replication, the proper utilization and precise removal of both minus strand and plus strand RNA primers are vital for the creation of the proper length terminal repeat ends required for proviral integration (4, 5).

Cuts are made in the HIV-1 genomic RNA during minus strand synthesis, while the polymerization active site of the RT is bound to the 3′-end of the growing DNA primer as shown in Fig. 1A (6–12). This has been termed the DNA polymerase-dependent mode of cleavage (6, 7). The polymerase and RNase H activities are not totally coupled during this process (13). RNA fragments of size ranging from 13 to 45 nts are left behind uncleaved and remain annealed to the newly synthesized minus strand DNA. We have shown that these can be removed by a rebinding of the RT to each RNA segment (9, 13). During this rebinding, positioning of the RT and subsequent cleavage is dictated by the 5′-end of the substrate RNA primer now reaccessed on the DNA template as shown in Fig. 1B. Initial cleavage occurred between 14 and 20 nts from the RNA 5′-end, a distance reminiscent of the spatial separation between the polymerase and RNase H active sites (9–11, 13–16). The dominant influence of the RNA 5′-end on RT positioning was confirmed by biochemical and mutational analyses (9, 17). Evidence for uncoupling of the polymerase and cleavage activities also comes from genetic studies conducted independently by Telesnitsky and Goff (18) using the Moloney murine leukemia system.

The availability of a free 3′-OH on all of these short RNA intermediates should have allowed them to serve as primers for RT-mediated plus strand DNA synthesis. However, experiments performed by us and others (19–29) verify that RNA segments with a large variety of sequences, nucleotide compositions, and lengths are ineffective as primers for RT-directed DNA synthesis. Only a PPT primer could be effectively extended. Furthermore, when a PPT sequence was present within a large segment of RNA, RT specifically degraded the adjoining segments and initiated nucleotide addition from the 3′-OH of the consensus PPT sequence (19–30). In vivo, synthesis initiated from any RNA primers from regions downstream of the PPT-U3 junction could not generate ends required for retroviral integration and would be disruptive to viral replication. In HIV and lentiviruses a second copy of the U3-PPT sequence, referred to as the central PPT (cPPT), is present 5′ to the U3-PPT within the integrase coding region of the pol gene (31–33). Synthesis from the cPPT appears to improve viral replication kinetics. Although mutations within the cPPT result in significantly delayed replication kinetics, proviral DNA formation competent for replication can still be completed (31). This contrasts with a mutation in U3-PPT that does not allow complete reverse transcription. Although there is evidence for multiple plus strand initiation sites in both avian sarcoma-leukosis virus (for a review see Refs. 34 and 35) and HIV (36), extension of RNA oligomers downstream from the U3-PPT has not been reported. Sequence analysis of intermediates in HIV replication revealed that plus strand synthesis initiated from the two PPT sequences was vastly higher than synthesis from
RNA primer 2 (41-mer) was prepared similarly from plasmid pBS1 required, 5 synthesized and gel-purified by Midland Certified Reagent Co. When realkaline phosphatase. A 19-nt PPT RNA (19, 20) was chemically synthesized using calf intestine phosphorylation of the RNA molecules was carried out using T4 polynucleotide kinase. RNA samples were gel-purified and quantitated by "shift-up" assays using labeled DNA primers of known concentrations as described previously (11). For some experiments, an internally labeled 41-mer RNA primer was utilized by inclusion of labeled rNTPs in the run-off transcription mixture.

**RNA-seq Determination—**Concentrations were determined according to Bradford (40) using bovine serum albumin as standard. RNA-DNA Hybridization—Annexin of RNA and DNA was performed in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 80 mM KCl. For annealing, the RNA and DNA components were added in equimolar amounts. Components were mixed, heated to 65°C for 10 min, and slowly cooled over a 90-min period.

**DNA Polymerase Assay**—Final reaction mixtures (12.5 μl) contained 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1.0 mM EDTA, 34 mM KCl, 6 mM MgCl₂, 50 μM all four dNTPs, 4 mM substrate, and HIV-1 RT. Reactions were performed at an approximate ratio of active RT molecules to substrate molecules of 1:2, unless otherwise stated. In all cases the enzyme was allowed to prebind to the substrate for 5 min at 37°C. The reaction was initiated by addition of dNTPs and MgCl₂, allowed to incubate for 15 min, and then terminated with 12.5 μl of 2× termination mixture. For time course analysis, a mixture of all components except dNTPs and MgCl₂ was prepared and allowed to react with RT at 37°C for 5 min. Subsequent to the initiation of the reaction by the addition of dNTPs and MgCl₂, 12.5-μl aliquots were drawn at varying intervals, and the reaction was terminated by adding those aliquots to an equal volume of the 2× termination mixture (90% formamide (v/v), 10 mM EDTA (pH 8.0), and 0.1% each of xylene cyanol and bromphenol blue). Eight-μl samples were then subjected to denaturing electrophoresis to resolve reaction products. Unless otherwise specified, both extension products and cleavage products resulting from the action of RT were monitored by employing labeled primers. The gels were then vacuum-dried and subjected to autoradiography by employing standard protocols (42). Exposures were carried out using Kodak XAR-5 or Biomax films unless otherwise specified.

**Preparation of Molecular Size Markers**—A base hydrolysis ladder was generated by treatment of 5′-labeled RNA with alkali according to standard procedures (9). MspI-digested fragments of plasmid pBR322 (Life Technologies, Inc.) that were labeled at the 5′-end were also run in each gel as size markers.

**Substrate Sequences**—The nucleotide sequences of templates and primers employed are as follows: RNA primer 1 (21-mer RNA), 5′-GG-GAACAAGGCUUCAUGCC; DNA primer 1 (21-mer DNA), deoxynucleotide sequence of the above and "dT" in place of "U"; RNA primer 2 (41-mer RNA), 5′-GGGGCAGAAUUGCCGCUACCAUGCAGCUAGG; DNA primer 2 (41-mer DNA), deoxynucleotide sequence of the above and "dT" in place of "U"; RNA primer 3 (PPT DNA primer), 5′-UUUUUAAGAAAGGGGGG; DNA primer 3 (PPT DNA primer), deoxynucleotide sequence of the above and "dT" in place of "U"; DNA template 1 (104 nt), 5′-TAGAGTCAGCCTGCAGGCATGCAAGCTTT-TGTCTTTGATGGGTTGCGGCGGCGCCTGTGATGTTAATATTCTCATGCGGCTGTCGTTTTTTAACAACGACTGACTGG (the underlined region binds to 21-mer primer); DNA template 2 (58 nt), 5′-GGGCGAUGGCCACGTAGAGGCAGCGTACCGAGCGTTCAGCC-GCCACGAGCTTAGAGGAGGTCCCGAATGCGCCACAG-GTATGTGCT (the underlined region binds to 41-mer primer); DNA template 3 (80 nt), 5′-TGTTGCTCTTCTTGGAGGAATTTAGCCCTTCAGTCCCTCCCCCTTTTCTTTTAAAGGGTCTAGATCTAGCTGCGTCTGTTG (the underlined region binds to PPT RNA primer); DNA template 4 (77 nt), 5′-TGATAGTCGGCTGACTGAGGACCTGACTAGGACTCCCGGCGAGTCGAGTCCTGACCCCTGACTCTGCATAGCTATATTACATATAGTG (the template allows the formation of a 20 nt 5′-un annealed region when the 41-mer RNA is bound); upstream blocking primer 1 (forms a nick, upstream of 41-mer primer), 5′-GGTCGGAGCCTTAGCTAGAGGAGGTCCCGAATGCGCCACAG-GTATGTGCT (the underlined region binds to 41-mer RNA; the upstream blocking primers 1, 4, and 5 bind adjacent to this region); DNA template 5 (58 nt), 5′-GGTCGGAGCCTTAGCTAGAGGAGGTCCCGAATGCGCCACAG-GTATGTGCT; DNA template 6 (forms a 1-nt gap, upstream of 41-mer primer), 5′-GTAATACATGCACCACTAG-TGGTAATACATAGCTAC; upstream blocking DNA primer 5 (forms a 4-nt gap, upstream of 41-mer primer), 5′-GAATACATGCACCACTAG-TGGTAATACATAGCTAC; upstream blocking DNA primer 6 (forms a 13-nt gap, upstream of 41-mer primer), 5′-GAATACATGCACCACTAG-TGGTAATACATAGCTAC.
FIG. 1. Modes of HIV-1 RT positioning for RNase H cleavage and synthesis. In mode A the 3′-end of the DNA directs RT positioning for optimal RNase H cleavage. This is the polymerase-dependent mode of action. In mode B the 5′-RNA end directs RT positioning for optimal RNase H cleavage. In both of these modes the RNase H active site is positioned on the RNA portion of the duplex. Mode C would be expected for synthesis to occur from any RNA primer. The letters P and H represent the polymerase and RNase H active sites, respectively. In this and the following figures, the RNA is represented by thin (hatched) lines, and the DNA is represented by thick (dark) lines.

PPT RNA) or DNA primer 3 (19-mer PPT DNA), respectively. The only difference between substrates B1 and B3 was that the template sequence was altered in B3 to allow for the formation of 20-nt long unannealed region at the 5′-end of RNA. Substrate D1 was formed by annealing RNA primer 2 (41-mer RNA) to DNA template 4. Substrate D3 was the same as D1, except that it had a short DNA primer (upstream blocking primer 1), annealed immediately upstream of the 41-mer RNA primer. Likewise, substrates A3 and C3 have short upstream DNA primers (upstream blocking primers 2 or 3) annealed immediately 5′ of 21-mer RNA or PPT RNA, respectively. In substrates D4 and D5, the upstream blocking DNA primers (upstream blocking primers 4 or 5) are placed to form a 1- or 4-nt gap, respectively, 5′ to the 41-mer RNA.

RESULTS

Hypothesis—The efficiency with which HIV-RT can extend the 3′-end of an RNA primer could depend on the binding distribution of the RT at the 3′-versus 5′-ends. We propose that the preferential association of RT molecules to the 5′-end of non-PPT RNA primers is responsible for their inability to support RT-directed DNA synthesis (Fig. 1, mode B). Blocking this preferred association to the 5′-end would allow binding of RT to the RNA 3′-end in a mode favorable for synthesis (Fig. 1, mode C). Such 5′-blocked RNA primers would be efficiently extended by the RT.

Plus Strand Priming by HIV-1 RT from Non-PPT RNA Primers Versus a PPT RNA Primer—We examined the ability of PPT versus non-PPT RNA primers to support plus strand synthesis by HIV-1 RT. Substrates A1, B1, C1, A2, B2, and C2 were employed to monitor DNA synthesis by HIV-1 RT. In these substrates either a 5′-labeled RNA primer 1 (non-PPT 21-mer), RNA primer 2 (non-PPT 41-mer), PPT RNA primer (19-mer) or, respectively, corresponding DNA primers of identical sequence (DNA primer 1, 2, and PPT DNA primer) were annealed to templates as indicated under “Methods.” The profiles of the reaction products on denaturing polyacrylamide electrophoresis gels are shown in Fig. 2. Priming efficiency was calculated from band intensities determined by densitometry measurements. The proportion of extension product to the total band intensity per each reaction was calculated. DNA synthesis products are either absent or present at a very low level (<0.1% efficiency) in reactions containing substrates A1 and B1 incubated with wt HIV-1 RT. This demonstrates the inability of the two non-PPT RNA primers to support synthesis by wt RT. Instead, the majority of observed products derive from cleavage dictated by RT positioning at the RNA 5′-end as shown by our model in Fig. 1B (Fig. 2, A and B, lane 1 of each). An initial cleavage about 18 nts from the 5′-end followed by subsequent digestion toward the 5′-end of the RNA result in a final product 9 nt long. In contrast, there is RT-mediated synthesis on substrate C1, PPT RNA (Fig. 2C, lane 1), as expected. As a control we have employed Sequenase™ (modified T7 DNA polymerase), an enzyme proficient in utilizing RNA primers of various sequence composition for DNA synthesis. Sequenase-mediated DNA synthesis is observed with substrates A1, B1, or C1 (Fig. 2, A–C, lane 3 of each). The priming efficiency obtained using Sequenase was about 40% or more for each of the RNA primers tested. This verifies the capacity of all of the substrates to undergo DNA synthesis. It illustrates that the absence of synthesis seen with wt HIV RT on substrates A1 and B1, with non-PPT RNA primers, is a result of the nature of RT interaction with these primer-templates. The existence of an intact RNA/DNA hybrid was further confirmed by the thorough susceptibility of the RNA segment of the duplex to cleavage by Escherichia coli RNase H (Fig. 2, A–C, lane 4 of each).

Furthermore, in substrates having a DNA rather than RNA primer (substrates A2, B2, and C2) HIV-RT carries out efficient DNA synthesis, making a similar amount of synthetic product as that observed in reactions containing Sequenase (Fig. 2, D–F, compare respective lanes). Although a low enzyme to substrate ratio seems to have caused a significant amount of pausing, priming efficiencies on DNA primers for RT and Sequenase-mediated synthesis were about equivalent. The values were found to be >80% for 41- and 21-mer non-PPT DNA primers and about 50% for the PPT DNA primer for all three enzyme preparations. This indicates that all tested DNA primers, of identical base composition to RNA primers employed here, support efficient RT-mediated plus strand DNA synthesis. Experiments with DNA primers also serve as controls to demonstrate that the specific activities of the different enzyme preparations are similar. This further demonstrates that differences in priming efficiencies seen on RNA primers are consequences of the properties of RT-nucleic acid interaction.

Inactivation of RNase H Activity Does Not Alter Primer Utilization—We considered an alternate possibility that non-PPT primers fail to prime because they are simply not available for synthesis since they undergo destruction by RT-RNase H. Another possibility is that wt RT did perform synthesis from RNA primers but returned to remove the RNA portion following extension. Calculation of priming efficiency in experiments involving labeled RNA primers using wt RT imposes a complication by the inherent ability of RT to return after synthesis to cleave the RNA segment. In the case of PPT RNA, extension followed by precise cleavage at the RNA/DNA junction would return the RNA primer to its original location on the gel. One way to resolve these issues is by using an RT mutant deficient in RNase H function.

Plus strand priming by an RNase H-defective RT point mutant was tested on all substrates. The RT-RNase H mutant behaved similarly to the wt RT with regard to priming on all substrates employed (Fig. 2, A–F, lane 2 in each). As with the wt HIV RT, the RNase H (−) RT did not support synthesis from both non-PPT RNA primers (21- and 41-mer RNA), substrates...
A1 and B1 (Fig. 2, A and B, lane 2). Generally the RNA primer remained intact, but this in itself did not allow it to prime synthesis any better. Priming efficiency was found to be <0.1%. Clearly, increasing the longevity of an undegraded non-PPT RNA oligomer is not sufficient for RT-mediated priming to occur from the 3'-end. From this observation we can infer that the nature of association of the RT to the nucleic acid is crucial for efficient priming to occur.

Comparable levels of priming efficiency were observed from PPT RNA using the RT- RNase H mutant (11.5%) as with the wt RT (9%) (Fig. 2, C and F, compare lanes 1 and 2 in each). This suggests that concentrations of wt RT used in the reaction were not sufficient to perform efficient cleavage at the junction of the extended PPT RNA.

It has been recently reported that an RNA primer rich in purines, when bound to a template DNA, assumes a structure that has a similarity to the major groove width of B-form DNA. This is different from the structure assumed by RNA primers of other sequence composition which have a narrow major groove width (38, 43, 44). Perhaps this structure of the PPT which has partial resemblance to B-form DNA favors greater RT association to its 3'-end. An intermediate level of synthesis from the PPT RNA, essentially between zero for non-PPT RNA to high levels for DNA primers, may be a reflection of intermediate structure of PPT RNA.

Blocking RT Association to the RNA 5'-End with an Unannealed Tail Promotes Utilization of Non-PPT RNAs for Synthesis—Experiments presented above and our previous studies (9, 10, 17) suggest that RT association to the non-PPT RNA 5'-end is dominant, preventing utilization of the RNA primer for synthesis. We next attempted to stop RT from binding the 5'-end of the RNA primer. Based on our hypothesis, we anticipated that this would give the RT molecules a greater opportunity to bind the 3'-end. Previously we found that a sufficiently long 5'-unannealed tail on the RNA portion of RNA/DNA primer-template does not bind RT, with a resultant lack of RNA 5'-end
FIG. 3. Blocking RT association to the RNA 5'-end favors plus strand synthesis from a non-PPT RNA. A schematic representation of substrates B1 (A) and B3 (B) is shown on the top. A representative 41-mer non-PPT RNA, radiolabeled internally (multiple asterisks) was bound to the template DNA such that it annealed either completely (substrate B1, A) or formed a 20-nt unannealed tail on the RNA 5'-end (substrate B3, B). A time course analysis of extension (polymerase/cleavage (RNase H) products formed by wild type HIV-1 RT was monitored at various intervals by following the assay procedure indicated under “Methods.” Lanes containing Sequenase and E. coli RNase H served as controls as in the previous experiment. Priming efficiencies from substrate B1 (A) were <0.1, <0.1, <0.1, <0.1, <0.1, <0.1, and 0.2% after 15°, 30°, 1°, 2°, 4°, 8°, 16°, and 32°, respectively, and from substrate B3 were <0.1, <0.1, <0.1, 0.1, 0.2, 1.2, 2.5, and 3.9%, respectively, in the same periods (B).

Directed cleavage (9). To test the effect of an unannealed tail, substrates B1 (Fig. 3A) and B3 (Fig. 3B) were employed. RT-mediated synthesis was monitored over a time course. In these substrates, the RNA primer 2 (41-mer non-PPT) was bound to alternative template DNAs such that it was either annealed completely, substrate B1 (Fig. 3A), or formed a 20-nt unannealed tail at its 5'-end, substrate B3 (Fig. 3B). As in the previous experiment, no noticeable RT-mediated synthesis was seen with the fully annealed primer (substrate B1), but cleavage products resulting from RT positioning from RNA 5'-end were clearly produced (Fig. 3A). In contrast, marked RT-mediated DNA synthesis was observed with the 5'-tailed primer (substrate B3, Fig. 3B). Priming efficiencies from tailed RNA primer over the time course was found to be <0.1, <0.1, 0.1, 0.2, 1.2, 2.5, and 3.9% after 15°, 30°, 1°, 2°, 4°, 8°, 16°, and 32°, respectively (Fig. 3B). This is in contrast to values of <0.1, <0.1, <0.1, <0.1, <0.1, <0.1, and 0.2% in similar time intervals when the 41-mer RNA was completely annealed to the template DNA (Fig. 3A). This remarkable result illustrates that prevention of 5'-end binding can activate RT-directed synthesis at the 3'-end.

Production of RNase H-directed cleavage products is suppressed on tailed substrate B3 (Fig. 3B). This observation is a good control that strongly suggests that the presence of an unannealed tail is preventing high affinity binding of the RT to the 5'-end of the RNA (Fig. 3B). We point out that there is a limit on tail length below which binding of the RT to the 5'-end is not blocked. An unannealed region of 10 nt does not pose a significant impediment for RT association to the RNA 5'-end. Consequently, synthesis is not favored with such a substrate (data not shown). Additional control reactions with E. coli RNase H and Sequenase were performed to verify the structure of the substrates (Fig. 3, A and B). The presence of an intact RNA/DNA duplex in substrates B1 and B3 is demonstrated by susceptibility of the hybrids to E. coli RNase H treatment (Fig. 3, A and B). Cleavage product around 24 nt in length (indicated by open arrow) following E. coli RNase H treatment demonstrates the presence of the 5'-unannealed tail in substrate B3 (Fig. 3B, lane marked E. coli RNase H). No differences in Sequenase-mediated synthesis were observed on substrates B1 (Fig. 3A) versus B3 (Fig. 3B) which had priming efficiencies of 45.6 and 41%, respectively, showing that irrespective of the structure of the 5'-end region, either primer-template is extendable. We previously demonstrated that an unannealed region of 26 nt or longer completely abolished RT binding to the RNA 5'-end and RNase H cleavage (9). This is the approximate distance spanned by the RT nucleic acid binding cleft (45). We anticipate that with substrates which totally block accessibility of RT to the RNA 5'-end, there could be even more efficient primer extension by RT. Our observations that 5'-tailed RNA structure facilitated enzyme association to the 3'-end for synthesis has relevance in minus strand initiation as well. Strikingly, tRNA when bound to genomic RNA also forms a similar 5'-tailed structure. Acquisition of tRNA usage as a primer for minus strand initiation by retroviruses may have simply been to limit non-productive association of RT on the RNA 5-end and to promote productive association to 3'-end for synthesis.

Blocking RT Association to the RNA 5'-End with an Upstream Primer Promotes Utilization of Non-PPT RNAs for Synthesis—In the above experiment tailed RNA disfavored RT binding to the RNA 5'-end. We sought to determine additional ways of blocking RT association to the RNA 5'-end. One approach that we expected to work was to employ RNA primers with a tag such as a biotin adduct on their 5'-end. The biotin could then be specifically bound by streptavidin to prevent subsequent association by RT when the proteins were added in sequence. However, generation of adducted RNAs posed several technical limitations. Consequently, we resorted to alternative means of preventing RT association to the RNA 5'-end. The first was to determine whether a DNA/RNA chimera primer with the DNA sequence on the 5'-end and non-PPT RNA sequence on the 3'-end would support RT-mediated synthesis. Here we detected RT-directed synthesis (data not shown). However, the possibility that helical structure of the chimera influenced RT binding could not be ruled out. So we wondered whether separation of the upstream DNA sequence from the RNA by a nick would inhibit favored RT association to the RNA 5'-end.

To explore this possibility we placed a DNA primer immediately upstream of the RNA primer. The ability of the 41- and 21-mer non-PPT RNA primers to support DNA synthesis was examined in the presence (substrate D3 and substrate A3) or absence (substrate D1 and substrate A1) of an upstream blocking DNA primer (Fig. 4, see illustration on top). The upstream blocking DNA primer was placed adjacent to the RNA segment so as to create a nick. The presence of the DNA primer upstream of the RNA primer resulted in an increase in RT-
mediated extension of the both non-PPT RNA primers, with the wild type RT. In this experiment, in the absence of blocking primer the priming efficiency by wt RT was found to be <0.1% and 0.5% for the 41- and 21-mer non-PPT RNA primers, respectively. However, placement of the upstream primer resulted in priming efficiencies of 3.3% (C, lane 1) and 4.6% (D, lane 1) for the 41- and 21-mer primers, respectively. RT-RNase H mutant synthesis on the 21-mer RNA primer with and without upstream blocking oligomer resulted in values of 0.5% (E, lane 1) and 40% (F, lane 1), respectively. Furthermore, in lanes containing wt RT, the increase in synthesis is accompanied by a noticeable reduction in secondary cleavage products of the 41- and 21-mer RNAs. This observation is consistent with a change in the mode of RT binding to the primer to favor synthesis at the 3' end and disfavor RNase H-directed degradation (Fig. 1 mode B versus C). It should be noted that placement of an upstream primer did not totally eliminate 5'-mediated RNase H cleavage. Control experiments show that Sequenase-mediated synthesis from the 41- and 21-mer RNA primers remained unaltered in the presence of an upstream DNA primer (Fig. 4, A and B, lane 1 in each).
2 in each; Fig. 4, E and F, lane 2 in each). When an identical set of reactions was performed with DNA instead of RNA primers, the presence or absence of the upstream primer did not alter the basal level of synthesis for wt RT, RNase H (minus) RT, or Sequenase (data not shown).

In the above experiments the presence of a nick between the upstream blocking DNA oligomer and RNA primer may have allowed RT association to the 3'-end of the upstream DNA in a polymerization mode which would block another RT molecule from binding the downstream RNA 5'-end. Under such circumstances, synthesis may have ensued from the DNA resulting in the displacement of the RNA. Displacement synthesis could not have been detected since the DNA was not labeled. However, the very fact that synthesis is facilitated from the RNA 3'-end argues against this possibility. Additionally, our previous observations suggest an apparent high affinity of RT for RNA 5'-ends even compared with DNA 3'-ends (14). Therefore, it appears that by keeping these two ends, 3'-DNA and 5'-RNA ends, in close proximity, RT association to either end is hindered thus promoting association of RT to the RNA 3'-end as shown in mode 2C.

Synthesis from a PPT RNA Is Enhanced by an Upstream Blocking Primer—It is evident from above that although PPT RNA is the natural primer, priming by wt RT or by the RNase H mutant was still 2–3-fold less efficient than synthesis performed by Sequenase. We considered whether blocking the PPT 5'-end would enhance synthesis by RT. In fact, placement of a blocking DNA primer upstream of annealed PPT-RNA (substrate C3, Fig. 5B, see illustration on top) resulted in an increase in RT-mediated synthesis from the PPT RNA but with minimal effects on Sequenase-mediated synthesis. The priming from the PPT RNA primer in the absence of a blocking primer was found to be 10, 14.8, and 36%, respectively, for wt RT, RT-RNase H mutant, and Sequenase (Fig. 5A, compare lanes 1 and 2 with Sequenase in lane 3). In contrast placement of an upstream blocking oligomer resulted in values of 24.9, 31.6, and 37% for wt RT, RT-RNase H mutant, and Sequenase, respectively (Fig. 5B, compare lanes 1 and 2 with Sequenase in lane 3). This demonstrates that 3' versus 5' binding competition is also responsible for lowering the efficiency of the PPT for synthesis. It further suggests that the 3'-end of the PPT RNA competes more effectively with its 5'-end for binding RT than do other RNAs.

Determining the Range of Effectiveness of the Upstream Primer—In the above experiments the blocking DNA segment was in very close proximity to the RNA primer, forming a nick with the downstream 41- and 21-mer RNA primers. We wondered whether the ability of the upstream DNA to block RT binding to RNA 5'-end would be altered if the separation between the two oligomers is greater. Presumably at some distance the RT would regain accessibility to the RNA 5'-end. To address this, we varied the position of annealing of the upstream blocking oligomer such that it would form either a 1- or 4-nt gap (substrates D4 or D5, Fig. 6, see illustration), rather than a nick with the 41-mer RNA primer (substrate D3). Results in Fig. 6 show that indeed the effectiveness of the upstream primer in promoting synthesis decreases as it is moved so as to produce a larger gap with the downstream primer (Fig. 6, compare lanes 1–4 for substrates D1, D3, D4, and D5, respectively). The synthesis efficiencies on substrates D1, D3, D4, and D5 were <0.1, 2.4, 1.8, and 1.2%, respectively. Thus the apparent effectiveness of the upstream primer at blocking binding of the RT did not change abruptly. Stimulation of synthesis was reversed moderately when the nick became a 1-nt gap and more with a 4-nt gap. Even though with the 4-nt gap the reversal was not complete, it is likely that with larger gap the reversal would have gone to completion. Qualitatively similar results were seen with the 21-mer RNA primer (data not shown). As controls for the above experiments, reactions were performed to test for efficiency of priming from equivalent DNA primers with upstream blocking DNA primers located at various relative positions. No differences in priming were evident (data not shown).

Overall, our results show that the preferred binding of the RT to the 5'-ends of most RNA primers favors primer degradation and is a major determinant of their inability to prime synthesis. Progressive inhibition of 5'-end binding allows more and more efficient synthesis.

**DISCUSSION**

Proviral DNA synthesis of HIV by reverse transcriptase is accompanied by genomic RNA degradation (for a review see Refs. 1 and 3). This process results in the generation of many RNA oligomers of different sizes and sequence compositions (13). Plus strand initiation, however, takes place from a single RNA oligomer, having the PPT sequence. One or more other purine-rich RNA primers also participate. All other RNA oligomers are not extended by the RT but instead are degraded by RT-RNase H. In this study we have explored the nature of RT-nucleic acid interactions that preclude the use of non-PPT RNA oligomers for priming. We show here that the competition for binding of RT to the 5' versus 3'-RNA ends ultimately determines whether an RNA segment will be destroyed or serve as primer for synthesis. When bound to the RNA 5'-end,
RT positions so as to degrade the RNA segment of the RNA/DNA hybrid. In this mode, the polymerase active site is not oriented in a way that allows it to catalyze synthesis from the RNA primer (Fig. 1B). When RT is bound to the RNA 3'-end, the DNA polymerase active site is positioned to perform synthesis, but the RT-RNase H is not oriented to carry out cleavage (Fig. 1C).

We demonstrate that the preferential functional association of RT to the RNA primer 5'-end can be blocked in at least two ways: (a) an unannealed region on the 5'-end of the RNA primer is not recognized by the RT; (b) a DNA oligomer immediately 5' to the RNA primer limits access of the RT to the RNA 5'-end. Disrupting RT association with the 5'-end of the RNA shifts the distribution of RT molecules to the RNA 3'-end. This favors synthesis from the RNA primers over cleavage. The very fact that blockage of 5'-end binding allows synthesis reveals a great deal about the inability of most RNA primers to allow synthesis. They could have been inert because the RT is simply incapable of catalyzing nucleotide addition to the 3'-end of the primer. This is clearly not the case. Instead the 3'-ends of RNA primers can support synthesis if RT is available for binding. Since our techniques specifically disrupt 5'-end binding, the RT had resided on the 5'-end. Evidently, most RNA primers are ineffective for synthesis because the 5'-end is sequestering all of the RT. The PPT RNA primer and DNA primers are effective for synthesis because their 3'-ends compete effectively with their 5'-ends for RT. Obviously, this could be because their 5'-ends bind RT more weakly than most RNA primers, or their 3'-ends bind RT more strongly, or both. This issue can be resolved by quantitative binding assays currently underway.

From our results we would predict that at higher enzyme concentrations once the preferred end of the RNA primer is saturated, the low affinity end will become populated by the enzyme. However, the lengths of RNA fragments left over following cleavage during the first round of synthesis are generally of sizes that can accommodate only one RT molecule (13). Therefore, RT association to the 5'-ends of remaining RNA segments effectively blocks any other RT molecule from binding to 3'-end of same RNA. Under these circumstances, the only way that the 3'-end could be utilized for synthesis would be by reducing the affinity of RT for the 5'-end until the 3'-end can effectively compete for binding the first and only RT. This assessment suggests why in vivo there are a substantial number of RTs present in the virion. Normally these RTs would associate with the many 5'-ends of all non-PPT RNA fragments remaining from minus strand synthesis, leading to their destruction in a mode shown in Fig. 1B. Only a few of the RTs would associate with PPT RNA primers for synthesis. We have also carried out studies using different retroviral RTs suggesting that the differential binding to 5'- and 3'-ends of RNA observed with HIV-1 RT appears to be conserved among retroviruses (data not shown).

Our observations also implicate the role of 5'-end RT positioning in the removal of PPT RNA primer. Since PPT RNA is the natural primer for plus strand initiation, RT association to its 3'-end is a must for synthesis (Fig. 1, mode C). However, in these studies we have demonstrated that even on PPT RNA there is RT association to the RNA 5'-end as represented in Fig. 1, mode B. Once the PPT RNA has served its role as a primer for DNA synthesis, it has to be precisely removed by a cleavage at the RNA/DNA junction. This removal is probably accomplished by RT repositioning to the PPT 5'-end. Interestingly, the length of PPT RNA is in the size range predicted for the spatial separation between the RNase H and polymerase active sites on the RT. The appropriate balance between binding of RTs to the 3'-end and 5'-end of the PPT RNA may have been imparted by the peculiar structure of PPT RNA/DNA duplex.

One of the crucial steps in viral replication is correctly positioned plus strand initiation. Initiation from any RNA oligomers downstream of the U3-PPT would disrupt the formation of correct long terminal repeat sequence, and the new strand would lack proper termini required for subsequent steps in productive viral infection. To circumvent this problem the virus has to have some way to avoid initiation from RNA oligomers downstream of the U3-PPT RNA. We show here that although RT could bind to either RNA 3'- or 5'-ends, on non-PPT RNAs there is a very strong bias to preferentially bind to the 5'-end (Fig. 1B). We believe that this bias prevents unintended disruptive extension of RNA primers downstream of the PPT. Previously Powell and Levin (22) showed that substitu-

**Fig. 6.** The effectiveness of the upstream blocking primer is dictated by its location with respect to non-PPT RNA primers. A schematic representation of substrates D1, D3, D4, and D5 is shown on the top. A representative 41-mer non-PPT RNA, 5'-radiolabeled, was bound to template DNA either alone (substrate D1, lanes 1 and 5–7) or with an upstream DNA primer such that it formed a nick (substrate D3, lane 2), 1-nt gap (substrate D4, lane 3), or 4-nt gap (substrate D5, lane 4) next to the RNA 5'-end. Extension (polymerase) or cleavage (RNase H) products formed by wild type HIV-1 RT (lanes 1–4) were monitored following the addition of dNTPs and MgCl₂. Lanes containing substrate D1 reacted with Sequenase (lane 5), or E. coli RNase H (lane 6), or unreacted substrate D1 alone (lane 7) served as controls as in previous experiments. The priming efficiencies on substrates D1, D3, D4, and D5 by wt RT were <0.1% (lane 1), 2.4% (lane 2), 1.8% (lane 3), and 1.2% (lane 4), respectively.
tion of 4 Cs for four of the Gs at the 3′-end of the PPT sequence completely abolished the priming function. Furthermore, they showed that a run of 6 G residues at the 3′-end of an RNA oligomer was capable of imparting plus strand priming function. The consequent absence of long runs of Gs to U3-PPT in the sequence of HIV (46) suggests that virus has evolved to safeguard from accidental detrimental initiation events.

The observation that a 5′-tail on an RNA primer favors RT binding and extension of the 3′-end is relevant to another important step in reverse transcription. The primer for initiation of minus strand synthesis in HIV is a cellular tRNA having a run of 6 G residues at the 3′-end complementary to the viral primer binding site. A similar mechanism of priming is employed by other retroviruses (for a review see Refs. 2 and 47). This differs from most other organisms, which use a short, fully complementary RNA transcript to prime DNA replication. The tRNA priming structure is essentially an RNA oligomer bound to an RNA template, with a large, folded unannealed region at its 5′-end. Our results suggest that retroviruses have evolved to utilize a partially complementary tRNA rather than a fully complementary RNA oligomer for minus strand initiation to improve priming efficiency. The structure should preclude unproductive RT association to the RNA 5′-end and favor productive RT binding to 3′-end for synthesis.

Our results show how to promote RT-directed plus strand DNA synthesis even from non-PPT RNA oligomers. This capacity has implications for developing gene therapy methods that can disrupt HIV infection. One approach would involve triggering of minus strand initiation using extendable RNA oligomers designed to bind downstream of the U3-PPT. Such RNA oligomers can be made to have characteristics of full-length tRNA that would facilitate packaging in the virion. We have recently employed a variant of Lys-3-tRNA that is highly inhibitory to HIV replication in cell culture (48). When expressed in infected cells, this variant is packaged in the virus and interferes with priming during subsequent infection. The results shown here suggest that RNA molecules shorter than tRNAs, more readily administered and less likely to interfere with the host cell, could be effective therapeutic agents.

Acknowledgments—We thank Dr. Stuart Le Grice from Case Western Reserve University for the generous gift of the RNase H-deficient HIV-1 RT (p66E478Q/p51) preparation. We thank Drs. Jasbir Sehra and Ziping Fu, Peter Gerondelis, and other laboratory members for helpful discussions.

REFERENCES

1. Telenitsky, A., and Goff, S. P. (1993) in Reverse Transcriptase (Skalka, A. M. and Goff, S. P., eds) pp. 49–83, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

2. Litvak, S. (1996) in Retroviral Reverse Transcriptase (Litvak, S., ed) pp. 83–114, Chapman and Hall, New York

3. Champoux, J. J. (1993) in Reverse Transcriptase (Skalka, A. M. and Goff, S. P., eds) pp. 103–117, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

4. Gilboa, G. M., Mitra, S. W., Goff, S., and Baltimore, D. (1979) Cell 18, 93–100

5. Whitcomb, J. M., and Hughes, S. H. (1992) Annu. Rev. Cell Biol. 8, 275–306

6. Gopalakrishnan, V., Peliska, J. A., and Benkovic, S. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 16763–16767

7. Furfine, E. S., and Reardon, J. E. (1991) J. Biol. Chem. 266, 406–412

8. Mizrahi, V. (1989) Biochemistry 28, 9088–9094

9. Palaniappan, C., Fuentes, G. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1996) Nucleic Acids Res. 23, 3901–3908

10. Defrancisco, J. D. (1997) Annu. Rev. Biochem. 66, 257–284

11. Defrancisco, J. D., and Goff, S. P. (1993) in Reverse Transcriptase (Skalka, A. M. and Goff, S. P., eds) pp. 49–83, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
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J. Biol. Chem. 1998, 273:3808-3816.
doi: 10.1074/jbc.273.7.3808

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