Analysis of Plus-strand Primer Selection, Removal, and Reutilization by Retroviral Reverse Transcriptases*

Received for publication, January 4, 2000, and in revised form, July 21, 2000
Published, JBC Papers in Press, July 26, 2000, DOI 10.1074/jbc.M00021200

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The ability of reverse transcriptase to generate, extend, and remove the primer derived from the polypurine tract (PPT) is vital for reverse transcription, since this process determines one of the ends required for integration of the viral DNA. Based on the ability of the RNase H activity of Moloney murine leukemia virus reverse transcriptase to cleave a long RNA/DNA hybrid containing the PPT, it appears that cleavages that could generate the plus-strand primer can occur by an internal cleavage mechanism without any positioning by an RNA 5′-end, and such cleavages may serve to minimize cleavage events within the PPT itself. If the PPT were to be cleaved inappropriately just upstream of the normal plus-strand origin site, the resulting 3′-ends would not be extended by reverse transcriptase. Extension of the PPT primer by at least 2 nucleotides is sufficient for recognition and correct cleavage by RNase H at the RNA-DNA junction to remove the primer. Specific removal of the PPT primer after polymerase extension deviates from the general observation that primer removal occurs by cleavage one nucleotide away from the RNA-DNA junction and suggests that the same PPT specificity determinants responsible for generation of the PPT primer also direct PPT primer removal. Once the PPT primer has been extended and removed from the nascent plus-strand DNA, reinitiation at the resulting plus-strand primer terminus does not occur, providing a mechanism to prevent the repeated initiation of plus strands.

Reverse transcriptase converts the single-stranded retroviral RNA genome into the linear double-stranded DNA that integrates into the chromosome of a host cell (1, 2). The reverse transcriptase of Moloney murine leukemia virus (MMLV) is a 75-kDa protein that contains an NH₂-terminal DNA- and RNA-dependent DNA polymerase activity and a COOH-terminal RNase H activity. Although the polymerase and RNase H activities are functionally separable (3–6), the polymerase and RNase H domains function in an interdependent manner (3, 7–10). The polymerase activity extends both RNA and DNA primers, although efficient extension from RNA primers appears limited to the host cell-derived tRNA primer used for minus-strand DNA synthesis and the primer used for plus-sense DNA synthesis that is derived from the polypurine tract (PPT) sequence in the viral genome (reviewed in Refs. 2 and 11) (12–20). The RNase H activity acts primarily as an endonuclease, hydrolyzing the RNA in an RNA/DNA hybrid to produce 3′-hydroxyl and 5′-phosphate ends (11, 21, 22). Cleavage by the RNase H activity of reverse transcriptase specifically generates the PPT primer during the process of reverse transcription (15, 23–26). RNase H is also responsible for removing the tRNA and PPT primers from the nascent DNA strands after they have been extended and for general degradation of the viral genome after minus-strand DNA synthesis (reviewed in Ref. 11) (12, 15, 24, 27–30).

Previous studies have indicated that two different modes of RNase H activity can be distinguished (18, 31–33). The polymerase-dependent mode is directed by the polymerase domain binding to a recessed DNA 3′-end in an RNA/DNA hybrid. RNase H cleavages are located 15–20 bases from the 3′ terminus of the DNA and can occur up to 8 nt away from the 3′-end of the DNA (13, 18, 21, 30–41). This form of RNase H activity accompanies minus-strand synthesis but is not sufficient to leave the newly synthesized minus-strand completely free of RNA (6, 36, 42). The polymerase-independent mode of RNase H activity occurs without DNA synthesis and is not coordinated by a DNA 3′ primer terminus but instead is positioned by the polymerase domain binding to the recessed 5′-end of an RNA hybridized to a longer DNA (6, 31–33, 36, 37, 43, 44). This 5′-end-directed activity produces fragments approximately 15–20 nucleotides long, and through additional degradation can generate fragments as short as 6–9 nt (18, 21, 30, 32, 33, 36, 45, 46). The polymerase-independent form of RNase H activity most likely participates in degradation of the template RNA after minus-strand synthesis (13, 17, 19, 20, 44). However, little is known about the relative importance of these two modes of RNase H cleavage in the generation of the plus-strand primer.

In a recent report, we used model hybrid substrates to characterize the production and extension of RNA primers derived from the PPT by MMLV reverse transcriptase (10). We observed that 5′-end-directed cleavages could occur within the otherwise RNase H-resistant PPT region in RNA primers that extend more than 15 nt upstream of the plus-strand start site. This observation suggests that the position of cleavages immediately upstream of the PPT might affect the accuracy of plus-strand primer generation. To extend these studies, we have characterized the RNase H cleavage sites both upstream and downstream of the PPT region that occur without RNA 5′-end.
positioning and evaluating the utilization of PPT primers with 3'-ends upstream of the normal initiation site for plus-strand DNA synthesis. Also, the effects of primer length on the efficiency of PPT primer removal and the consequences of downstream DNA on the utilization of the PPT primer have been examined.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—Recombinant wild-type MMLV reverse transcriptase and Sequenase version 2.0 (T7 DNA polymerase) were obtained from Amersham Pharmacia Biotech. Superscript (RT)1 and Superscript II (H-RT) were obtained from Life Technologies, Inc. RT transcriptase was purchased from Worthington. The production and characterization of the RNase H domain of MMLV reverse transcriptase (RTαPol) were described previously (8). T4 polynucleotide kinase and T4 DNA polymerase were obtained from New England Biolabs.

**Oligonucleotides**—Oligonucleotides are designated with an R for oligonucleotide or D for oligodeoxynucleotide followed by the coordinates of their 5'- and 3'-ends positions relative to the cleavage site generating the PPT primer for initiation of plus-strand DNA synthesis. This cleavage, defined as between positions 1 and +1 in this study, occurs between nucleotides 7815 and 7816 on the MMLV genome (47). The sequences and positions relative to the PPT cleavage site of RNA primer and downstream DNA transcriptase are presented in Fig. 2. Template strand oligonucleotides are as follows: D occurs between nucleotides 7815 and 7816 on the MMLV genome (47).

**Cleavage Analysis of Long RNA/DNA Hybrids**—To prepare hybrid substrates containing 5'-end-labeled complementary 807-nt single-stranded DNA derived and surrounding sequences, representing plus-strand positions 7756–8380 was gel-isolated, 5'-end-labeled, and annealed as described previously (10). The resulting RNA has a single MMLV LTR positioning and evaluated the utilization of PPT primers with 3'-ends upstream of the normal initiation site for plus-strand DNA synthesis. Also, the effects of primer length on the efficiency of PPT primer removal and the consequences of downstream DNA on the utilization of the PPT primer have been examined.

**Cleavage Analysis of RNA Oligonucleotide Primers**—Oligonucleotides (105 pmol) and phosphatase-treated 753-nt RNA (6 pmol) were 5'-end-labeled in 20-μl reactions using T4 polynucleotide kinase and 20–30 μM [γ-32P]ATP (NEN Life Science Products) essentially as described previously (10). For 5'-end phosphorylation, 1 nmol of D +1/35 was incubated with 1 μM ATP and T4 polynucleotide kinase under kinase reaction conditions and recovered by ethanol precipitation in the presence of 2 μl 3 M NaOAc.

**Preparation of Long RNA/DNA Hybrids**—To generate a 753-nt RNA containing the PPT cleavage site 68-nt downstream of the RNA 5'-end, BamHI-linearized plasmid pGEMLTR1 (46) was transcribed in vitro as described previously (10). The resulting RNA has a single MMLV LTR and surrounding sequences, representing plus-strand positions 7756–8330 joined to positions 69–231 and 11 nt of vector sequence at the 5'-end (47). This RNA was gel-isolated, 5'-end-labeled, and annealed to a 2-fold excess of complementary 807-nt single-stranded DNA derived from M13LTR1 (46) in 200 μM KCl, 10 μM Tris-HCl, pH 7.5, 1 μM EDTA for 45 min at 67 °C (10).

**Cleavage Analysis of Long RNA/DNA Hybrids**—Cleavage assays were carried out in 20-μl reactions containing 50 μM Tris-HCl, pH 8.3, 50 μM KCl, 6 μM MgCl2, 5 μM DTT, and 10 μM hybrid substrate. Following a 1-min preincubation at 37 °C, cleavage reactions were initiated with either 10 pmol of MMLV reverse transcriptase (50:1 enzyme/substrate ratio) or 2 pmol of MMLV reverse transcriptase (10:1) or 64 pmol of RTαPol and incubated at 37 °C. At the indicated times, 8-μl aliquots were terminated in 2.5 μl of 95% formamide and 10 mM EDTA. Products were separated in denaturing 20% polyacrylamide gels. Cleavage and extension products were visualized by PhosphorImager analysis, cleavage and extension products were quantified using ImageQuant software. Individual gel lanes were quantified as rectagular objects (5-pixel width) by area quantitation using the peak finder method. Automatic base-line parameters were suitable for quantification of individual peaks of interest was quantified as the percentage of the total area of all of the identified peaks.

**Generation and Polymerase Extension of RNAs with 3'-Ends Upstream of Plus-Strand Initiation Site**—0.4 pmol of hybrid substrate containing primer R–20/–1 was incubated with 4 pmol of MMLV reverse transcriptase in 80 μl of RNase H cleavage buffer at 37 °C for 15 min, and the reactions were stopped by adding EDTA to a final concentration of 10 mM. Cleaved hybrid substrates were precipitated in 0.3 μl sodium acetate, pH 5.2, with 2 μg of glycerol in 70% ethanol and resuspended in TE (10 μl Tris-HCl, pH 8.0, 1 mM EDTA). One-fourth of the recovered hybrids were treated with 3.9 units of T7 DNA Polymerase or 1 pmol of H-RT in a 20-μl reaction containing RT buffer (50 μM dNTPs, 500 μM MMLV LTR, 500 μM unlabeled primers), labeling mix (200 μM dATP, 200 μM dTTP, 200 μM dGTP, and 0.165 μM [α-32P]dGTP) at 37 °C for 15 min. ddCTP was used to eliminate non-templated addition in run-off extensions. Extension reactions were stopped by the addition of 2.2 μl of 0.1 mM EDTA, and 10 μl of each sample was treated with 0.3 μl NaOH at 65 °C for 45 min and neutralized with acetic acid. Samples were recovered by ethanol precipitation, resuspended in TE, and analyzed by denaturing gel electrophoresis as described above.

**Cleavage Analysis of RNA Primers Extended with Labeled DNA**—0.5 pmol of hybrid substrate containing unlabeled primers R–15/–1, R–17/–1, or R–20/–1 was labeled by run-off extension with 100 units of RTαPol in a 20-μl reaction containing RT buffer and labeling mix at 37 °C for 60 min, and products were precipitated with 70% ethanol, 2 μl 3 M NaOAc, and 2 μg of glycerol. After resuspension in TE, one-fifth of each extended hybrid substrate (0.1 pmol) was incubated with 1 pmol of MMLV reverse transcriptase in a 20-μl reaction containing RNase H cleavage buffer at 37 °C for 15 min. Cleavage reactions were stopped with 2.2 μl of 0.1 mM EDTA, and one-half of each sample was treated with alkali before analysis as described above.

**Cleavage Analysis of RNA Primers after Extension**—Hybrid substrates containing 5'-end-labeled primers R–15/–1, R–17/–1, or R–20/–1 were extended with 100 units of RTαPol, and 5'-end-labeled primer R+1/+17 or R+13/+29 was extended with 3.9 units of T7 DNA polymerase in 20-μl reactions containing RT buffer and 200 μM dNTPs at 37 °C for 60 min, and products were precipitated with 70% ethanol, 2 μl 3 M NaOAc, and 2 μg of glycerol. After resuspension in TE, one-fifth of each extended hybrid substrate (0.1 pmol) was incubated with 1 pmol of MMLV reverse transcriptase in 20-μl reactions containing RNase H cleavage buffer at 37 °C for 15 min, and products were analyzed as described above.

**Primer Removal after Limited Extension**—To prepare short extension substrates, 0.5 pmol of hybrid substrate containing 5'-end-labeled primer R–15/–1 was extended with 100 units of RTαPol in a 20-μl reaction containing 500 μM dATP and 0.5 pmol of dGTP (+1 extension product); 200 μM dATP (+2 extension product), 200 μM dGTP (+3 extension product); 200 μM dCTP, 200 μM dTTP, 500 μM dGTP (+4 extension product); or 200 μM dATP, 200 μM dCTP, 500 μM dGTP, 500 μM dTTP (+10 extension product). After incubating 30 min for the +2 extension products or 60 min for all other extension products, extended primers were gel-purified as described above, renaturated to template D–10/+28, and incubated with 0.5 pmol of MMLV reverse transcriptase or RTαPol in 10-μl reactions containing RNase H cleavage buffer for the indicated times at 37 °C. The products were analyzed as described above.

**Extension Analysis of PPT-containing Primers with Downstream DNA**—0.1 pmol of hybrid substrates containing 5'-end-labeled primer with or without downstream D–15/–35 was incubated with an equimolar concentration (0.1 pmol, 1.54 units), a 100-fold molar excess (1 pmol, 15.4 units), or a 100-fold molar excess (10 pmol, 154 units) of H-RT or with equal polymerase activity units (15.4 units) of H-RT, MMLV reverse transcriptase, or HIV-1 reverse transcriptase. As controls, 3.9 units of T7 DNA polymerase or 0.5 units of T4 DNA polymerase were used. Reactions were carried out in a 20-μl volume containing RT buffer and 200 μM dNTPs for 15 min at 37 °C. The products were analyzed as described above.
Plus-strand Primer Selection, Removal, and Reutilization

FIG. 1. Sites cleaved by MMLV reverse transcriptase on a long RNA/DNA hybrid containing the PPT. A, 5′-end-labeled 753-nt RNA containing sequence from the MMLV LTR (dotted line) was annealed to a longer DNA (solid line) to generate an RNA/DNA hybrid containing an internal PPT. B, the RNA/DNA hybrid was incubated with a 10-fold (lanes 5 and 6) or 50-fold (lanes 3 and 4) excess of MMLV RT or a 300-fold excess of RTΔPol (lanes 7 and 8) for 15 or 30 s as indicated. As a control, the hybrid was incubated without enzyme for 30 s (mock, lane 1). The products were separated in a denaturing 20% polyacrylamide gel and visualized using a PhosphorImager. Nuclease P1 digestion of the 5′-end-labeled RNA (P1) is shown in lane 2. Sizes of selected fragments (nt) and the position of cleavage for initiation of plus-strand DNA synthesis (PPT) are indicated at the left. C, for all samples in B, identical aliquots were run for a longer time on the same gel to facilitate analysis and are presented in identical order (lanes 1–8). D, the relevant sequence of the plus-sense MMLV genome (positions 7770–7822 (47)) for identical cleavage products found in both B and C (bracketed) is shown. The cleavage sites (arrows) and the positions of resulting 3′-ends relative to the start site of plus-strand DNA synthesis (positive and negative numbers) are indicated; the vertical line demarcates the PPT.

RESULTS

Preferred RNase H Cleavage Sites in a Long PPT-containing RNA/DNA Hybrid—Previously, the 5′-ends of RNAs positioned 15–20 nt upstream of the plus-strand start site at +1 were found to direct RNase H cleavages within the PPT in addition to the cleavage that generates the plus-strand primer (10). Here, we wanted to test whether specific cleavages that might generate the plus-strand primer at position +1 could occur in the absence of any proximal 5′-end positioning. RNase H specificity was examined in a cleavage assay that utilized a long hybrid substrate with a 5′-end-labeled 753-nt RNA containing the MMLV PPT and surrounding sequences. In this substrate, cleavage to generate the 3′-end of the plus-strand primer would occur between nt 68 and 69 from the 5′-end of the RNA (Fig. 1A). Since only cleavage products retaining the original 5′-end of the substrate would be observed, we could discriminate whether 5′-end-directed cleavages predominated or whether internal cleavages around the PPT region could be detected as well.

Treatment of this substrate with excess MMLV reverse transcriptase generated numerous fragments containing the original 5′-end that were not dispersed evenly throughout the RNA but rather were clustered in specific regions (Fig. 1B, lanes 3–6). 5′-End-directed cleavages generated multiple fragments smaller than 17 nt in length; together, the 13-, 15-, and 16-nt fragments constituted 20% of the total product at 15 s with an enzyme/substrate ratio of 50:1 (Fig. 1B, lane 3). Those fragments longer than 22 nt, comprising −67% of the products, could not have arisen by a 5′-end-directed mechanism and therefore represented internal cleavages in this substrate. Notably, the PPT was resistant to cleavage. Internal cleavage sites around the PPT were precisely mapped by longer electrophoresis of identical samples (Fig. 1C) and are shown on the adjacent sequence with small or large arrows, indicating band intensities (Fig. 1D). In addition to the cleavage that generated the 3′-end of the plus-strand RNA primer at the +1-position, these fragments had 3′-ends at nucleotide positions −35, −23, −22, −14, +1, +2, and +5 (Fig. 1, B and C, lanes 3–6; Fig. 1D). The most abundant fragments had 3′-ends at positions −23 and −1 and represented 7.6 and 7%, respectively, of the total product (Fig. 1, B and C, lane 3). Although this assay did not measure subsequent 5′-end-directed cleavages on the substrate due to the location of label, these data demonstrated that cleavages consistent with generation of the plus-strand primer can occur independent of 5′-end positioning. With lower ratios of enzyme to substrate, additional internal cleavages were observed, but 5′-end-directed cleavages still predominated, as would be predicted, since 5′-end-directed cleavages are kinetically favored over internal cleavages on a long RNA/DNA hybrid (10).
To investigate whether the cleavage pattern exhibited by reverse transcriptase required the polymerase domain, the same RNA/DNA substrate was incubated with the isolated RNase H domain, RTαPol. Although this form of the enzyme exhibited some limited specificity, including cleavages to produce fragments with 3'-ends mapping to positions −14 and +5, there was no specificity for cleaving at the plus-strand origin. Moreover no fragments were produced by 5'-end-directed cleavages (Fig. 1, B and C, lanes 7 and 8).

5'-End-directed Cleavage of PPT-containing RNAs Generates 3'-Ends That Are Not Extended Efficiently by H^− RT—The preceding data suggested that the RNase H activity of reverse transcriptase might generate long PPT-containing RNAs with the correct 3'-end for plus-strand priming at −1 and with a 5'-end at position −22 or −21. Such RNAs could serve as substrates for polymerase extension or for 5'-end-directed cleavages. Because 5'-end-directed cleavages of such PPT-containing primers produced RNAs with 3'-ends upstream of the −1-position (10) that might lead to aberrant plus-strand priming, we tested whether these ends could serve as primers for reverse transcriptase.

A long PPT-containing RNA that extended from position −20 to −1 (R−20−1; Fig. 2) and had been characterized previously (10) was chosen for this analysis. The 20-mer RNA oligonucleotide was annealed to the 38-mer template D+10/−28, and the resulting hybrids were incubated with MMLV reverse transcriptase in the absence of dNTPs to allow 5'-end-directed cleavages to occur. Similar to our previous findings, when the R−20−1 substrate was 5'-end-labeled, the cleavage products ranged from 7 to 18 nt in length (Fig. 3A, lane 2). The most prominent of these fragments had 3'-ends at positions −3 to −8 upstream of the initiation site for plus-strand DNA synthesis (see Fig. 2). Based upon the T<sub>m</sub> calculations (49, 50), those fragments 15 nt or longer are predicted to remain annealed to template DNA and, in principle, serve as primers for DNA synthesis. Thus, unlabeled hybrid substrates incubated with or without reverse transcriptase under the conditions described for Fig. 3A were tested for polymerase extension using either the RNase H-deficient form of MMLV reverse transcriptase (H^− RT) or as a control T7 DNA polymerase.

In the presence of unincubated hybrid substrate and dNTPs, including [α-<sup>32</sup>P]dGTP, both enzymes generated a 30-nt product consisting of the 20-mer RNA primer with 10 nt of labeled DNA (Fig. 3B, lanes 3 and 5). As expected, after treating these extension products with alkali to remove the RNA, only the 10-nt DNA extension product remained (Fig. 3B, lanes 4 and 6). The reverse transcriptase-cleaved hybrid substrate was purified and incubated with H^− RT, the 30-nt RNA/DNA product was again observed, but the amount of extension product was reduced relative to the extension from the same RNA using T7 DNA polymerase, suggesting that there had been a reduction in the number of primer ends available to reverse transcriptase (Fig. 3B, compare lanes 7 and 9). The positions of RNA 3'-ends that had been extended in the cleaved hybrid substrates were revealed by treating extension products with alkali. T7 DNA polymerase was capable of extending several of the RNAs with 3'-ends upstream of the −1-position, since 50% of the products were longer than 10-mers with lengths ranging from 12 to 17 nt that resulted from extension of RNA primers with 3'-ends at positions −3 to −8 (Fig. 3B, lane 8). In contrast, H^− RT very inefficiently extended PPT-containing primers with 3'-ends upstream of the −1-position, since only 3.4% of alkali-treated extension products were larger than 10 nt (Fig. 3B, lane 10). There was no carryover of active reverse transcriptase from the original cleavage reactions, since no labeled products were seen when cleaved hybrid substrate was incubated with dNTPs in the absence of T7 DNA polymerase or H^− RT (Fig. 3B, lanes 1 and 2).

Removal of Extended PPT Primers Is Affected by Primer Length—Since all lengths of PPT-containing primers with the correct 3'-end for plus-strand priming (−1-position) were readily extended by the polymerase activity of reverse transcriptase (10), we asked if primer length affected removal of an extended PPT primer. To generate the substrates for this analysis, unlabeled primers R−15/−1, R−17/−1, or R−20/−1 (which share the same 3'-end at position −1 but differ in 5'-end position; Fig. 2) were annealed separately to template D+10/−28 and extended by 10 deoxynucleotides in the presence of [α-<sup>32</sup>P]dGTP using a form of MMLV reverse transcriptase deleted for the RNase H domain (RTΔH) (Fig. 4, lanes 1, 7, and 13). When the extended hybrid substrates were subsequently incubated with MMLV reverse transcriptase in the absence of dNTPs to evaluate primer removal, the extended substrate was rapidly cleaved at the RNA-DNA junction to produce a prominent 10-mer DNA (see below) and a second band of slower mobility, which increased in proportion to the length of extended primer (Fig. 4, lanes 1–4, 7–10, and 13–16). Alkali treatment of the products present at 30 min had no effect on the mobility of 10-mer DNA, confirming that this species had no ribonucleotides remaining at its 5'-end (Fig. 4, lanes 5, 11, and 17). However, the slower migrating species did shift after alkali treatment to the position of a 10-mer with a 5'-hydroxyl (Fig. 4, lanes 5, 11, and 17). The mobility of the 5'-hydroxyl-containing 10-mer was confirmed by treating the original extended substrate with alkali (Fig. 4, lanes 6, 12, and 18). This result revealed that the slower migrating species contained an alkali-sensitive 5'-ribonucleotide G derived from cleavage one nucleotide away from the RNA-DNA junction (between posi-
FIG. 3. 5’-End-directed cleavage of primer R–20/–1 by MMLV reverse transcriptase and extension assay for RNAs with 3’-ends upstream of the plus-strand initiation site. A, a hybrid substrate containing 5’-end-labeled primer R–20/–1 was incubated without enzyme (lane 1) or with MMLV reverse transcriptase for 15 min (lane 2). Sizes of selected fragments in nt are indicated at the right. B, T7 DNA polymerase (lanes 3, 4, 7, and 8) or H+ RT (lanes 5, 6, 9, and 10) was tested for the ability to extend 3’-ends in hybrid substrate R–20/–1 (unclaved; lanes 3–6) or hybrid substrate R–20/–1 previously incubated with reverse transcriptase (cleaved; lanes 1, 2, and 7–10). One-half of the extension products were treated with alkali to remove ribonucleotides (even-numbered lanes, +). In lanes 1 and 2, reverse transcriptase-cleaved substrates were incubated without additional enzyme in the presence of dNTPs. For both A and B, the products were analyzed as described in the legend to Fig. 1, and the substrates are diagrammed above.

![Diagram](image)

FIG. 4. Removal of extended PPT primers by MMLV reverse transcriptase. Hybrid substrates labeled by DNA extension and containing primers R–15/–1 (lanes 1–6), R–17/–1 (lanes 7–12), or R–20/–1 (lanes 13–18) were incubated without (lanes 1, 6, 7, 12, 13, and 18) or with MMLV reverse transcriptase (lanes 2–5, 8–11, and 14–17) for 1, 5, or 30 min as indicated. Products were treated with alkali prior to analysis (lanes 5, 6, 11, 12, 17, and 18) or analyzed without alkali treatment (lanes 1–4, 7–10, and 13–16) as described in the legend to Fig. 1. Substrates are diagrammed above the appropriate lanes.

![Diagram](image)

Polymerase-extended RNA Primers Are Cleaved Differently than Unextended RNA Primers—Unlike the case with the extended hybrid R–20/–1 (Fig. 4, lanes 14–17), a hybrid containing unextended primer R–20/–1 was not cleaved between the penultimate and last ribonucleotides at the 3’-end to generate a 19-mer RNA product (Fig. 3A, lane 2). Therefore, we next tested whether the RNase H activity of reverse transcriptase might cleave the polymerase-extended RNA primers differently at the –2/–1-position than the unextended counterparts. Hybrid substrates containing 5’-end-labeled PPT primers R–15/–1, R–17/–1, or R–20/–1 were either extended or left unextended and then incubated with MMLV reverse transcriptase (Fig. 5, lanes 1–12). In each case, cleavage between the last and penultimate ribonucleotides increased when the primer was extended (bands marked by asterisks in Fig. 5, lanes 2, 4, 6, 8, 10, and 12). Cleavage at the –2/–1-position generated 6.4, 7.1, and 12.2% of the total products for unextended hybrid substrates R–15/–1, R–17/–1, and R–20/–1 (Fig. 5, lanes 2, 6, and 10), as compared with values of 15.4, 25.8, and 6.0% for the extended hybrid substrates R–15/–1, R–17/–1, and R–20/–1 (Fig. 5, lanes 2, 6, and 10). This analysis revealed that extended hybrid substrates R–15/–1, R–17/–1, and R–20/–1 had more than a 2-fold and up to a 5-fold increase in the RNA cleavage product 1 nucleotide short of the full-length primer. This effect was limited to cleavage at the –2/–1-position in extended substrates, since other bands were not
observed to increase, with the exception of products generated by specific cleavage of extended hybrid substrates at the RNA-DNA junction.

To address whether an increase in cleavage at the −2′−1-position was intrinsic to the RNase H domain or depended on the presence of the polymerase domain, the same hybrid substrates were treated with RTΔPol (Fig. 5, lanes 13–24). As anticipated based upon earlier findings (8, 9), the isolated RNase H domain lacked specificity for PPT primer removal at the RNA-DNA junction. In addition, the RNase H cleavage patterns were identical between extended and unextended substrates, indicating that the RNase H domain showed no preference to cleave between positions −1 and −2 of an extended primer.

To investigate if cleavage between the last two ribonucleotides of an extended primer was limited to PPT primers or was intrinsic to cleavage of extended RNA primers irrespective of sequence, similar cleavage analysis of extended versus unextended primers lacking the PPT sequence was performed. Two non-PPT RNA primers of 17 nt in length that correspond to sequences downstream of the PPT (R+1/−17 and R+13/−29; Fig. 2) were compared with R−1/−17 (Fig. 6). Consistent with data in Fig. 5, a 3-fold increase in the amount of product resulting from cleavage at the −2′−1-position was observed for extended versus unextended R−17/−1 hybrid (Fig. 6, lanes 4 and 2, respectively). However, cleavage at this position was dramatically higher for the non-PPT extended hybrid substrates. When treated with reverse transcriptase, bands representing cleavage at the −2′−1-position represented 11.4 and 26.7% of the total products for extended hybrid substrates with primers R+1/−17 and R+13/−29, whereas the corresponding values were 0.1 and 0.8% of the total products for the unextended counterparts (Fig. 6, lanes 8, 12, 6, and 10, respectively; see asterisks). This result constituted a 1−2-order of magnitude increase in the 16-mer product resulting from cleavage 1 ribonucleotide away from the RNA-DNA junction for extended primers R+1/−17 and R+13/−29. In addition, no cleavage occurred at the RNA-DNA junction of the extended non-PPT primers (Fig. 6, compare lanes 5–12). Notably, similar experiments with two unextended versus extended non-PPT 13-mer RNAs revealed a 1-order of magnitude increase in cleavage of extended RNAs at the −2′−1-position (data not shown).

Recognition of the PPT Primer-DNA Junction—To better define how the RNA-DNA junction in an extended PPT primer is recognized by the RNase H activity of reverse transcriptase, we tested how much DNA extension is required for cleavage at the junction in the absence of DNA synthesis. As substrates, hybrids containing 5′-end-labeled primer R−15/−1 were first extended by 1, 2, 3, 4, or 10 nt and gel-isolated, and then these substrates or the original unextended substrate was incubated with MMLV reverse transcriptase in a cleavage assay.

The unextended hybrid substrate was relatively resistant to RNase H cleavage (Fig. 7, lanes 1–4) as described previously (10). Extension by 2, 3, or 4 nt allowed cleavage at the RNA-DNA junction in a manner similar to that of the fully extended +10 nt control (Fig. 7, lanes 9–24). These data indicated that the addition of as few as 2 deoxynucleotides to a 15-mer PPT primer presented an RNA-DNA junction recognized and cleaved by the RNase H activity of reverse transcriptase. In contrast, reverse transcriptase did not recognize the RNA-DNA junction when the 15-mer had been extended by a single nucleotide but rather cleaved at the −2′−1-position to generate a 14-mer product (Fig. 7, lanes 5–8). This result suggested that the addition of 1 deoxynucleotide at the end of primer R−15/−1 was sufficient to substantially increase cleavage of this substrate but that cleavage specificity was transferred from the RNA-DNA junction to between the penultimate and last ribonucleotides. When similar short extended substrates were incubated with RTΔPol, no cleavage occurred at the RNA-DNA junction (data not shown), and the cleavage pattern was identical to that observed previously (Fig. 5, lanes 13–16).

Effects of Downstream DNA on PPT Primer Extension—After
extension of the PPT primer by reverse transcriptase, cleavage at the RNA-DNA junction leaves a nick between the RNA primer and the nascent DNA chain. Using oligonucleotides to construct substrates that model this nicked structure, we tested the effects of downstream nontemplate DNA on the capacity of reverse transcriptase to reutilize the PPT primer. Thus, the 5'-end-labeled primer R$_{215}$/$21$ was annealed to 61-mer template D$_{133}$/$228$ with or without the 35-mer downstream DNA, D$_{11}$/$135$ (Fig. 2). These substrates were tested for extension of the upstream RNA primer using H$^-$ RT at a 1:1, 10:1, or 100:1 molar ratio of enzyme to substrate. As controls, extensions were performed with T7 DNA polymerase, which can strand-displace, and T4 DNA polymerase, which cannot efficiently displace downstream DNA (51). In the absence of downstream DNA, all three enzymes extended the PPT RNA primer (Fig. 8a, lanes 1–6). The slower mobility of the extended products for T7 DNA polymerase and for the two higher concentrations of H$^-$ RT as compared with the products for extension of the upstream RNA primer using H$^-$ RT at a 1:1, 10:1, or 100:1 molar ratio of enzyme to substrate.
DNA PPT primers at a nick with downstream DNA by H-RT. A, primer-templates (0.1 pmol) containing 5'-end-labeled RNA primer R-15'/-1 without (lanes 1–6) or with downstream DNA D+1+/35 (lanes 7–12) were extended using H-RT (H-) at an enzyme/substrate ratio of 1:1 (lanes 4 and 10), 10:1 (lanes 5 and 11), or 100:1 (lanes 6 and 12). As controls, extensions were performed with T7 DNA polymerase (T7; lanes 2 and 8) or T4 DNA polymerase (T4; lanes 3 and 9). B, identical to A except that the primer-templates contained 5'-end-labeled DNA primer D-15'–1. Products were analyzed as described in the legend to Fig. 1 except that products in B were separated in a denaturing 15% polyacrylamide gel. For A and B, substrates are diagrammed above the appropriate lanes.

We next tested how the intact MMLV and HIV-1 reverse transcriptases compared with H-RT in extension of primers R-15'/-1 and R-20'/-1 at a nick (Fig. 9). In the absence of downstream DNA, the primers were extended, but fewer products were observed for the wild type enzymes due to cleavage of the extended RNA by the RNase H activities (Fig. 9, lanes 2–4 and 10–12), some of which must have occurred at the RNA-DNA junction as described above. In the presence of downstream DNA, MMLV and HIV-1 reverse transcriptases generated low but detectable levels of extended products using primer R-20'/-1 and even lower amounts of extension products using primer R-15'/-1 (Fig. 9, lanes 15 and 16 and lanes 7 and 8, respectively). Thus, the PPT 20-mer initiated some displacement synthesis better than the PPT 15-mer, but over-all the presence of a downstream oligonucleotide dramatically reduced the extension efficiency. Since complete extension by displacement synthesis through the downstream 35-mer DNA generates a product that is identical to that produced by extension on the single-stranded template, the paucity of extension products in lanes 7 and 8 of Fig. 9 as compared with lanes 3 and 4 cannot be simply due to RNase H removal of the primers in the former case. Thus, similar to MMLV H-R, the wild type enzymes are unable to efficiently extend the PPT primer in the presence of nontemplate downstream DNA.

When substrates containing 5'-end-labeled primer R-15'/-1 or R-20'/-1 without or with downstream DNA were incubated with reverse transcriptase in a cleavage assay, the presence of the downstream oligonucleotide had essentially no effect on the cleavage pattern (data not shown). This result indicated that the preference to cleave between the penultimate and last ribonucleotide in an extended RNA primer as described above required the covalent bond between the RNA primer and DNA.

**DISCUSSION**

Although numerous studies have suggested that generation of the PPT primer is sequence-dependent and highly precise (reviewed in Ref. 11), the underlying mechanisms dictating PPT primer generation and utilization remain less defined. The following discussion considers several questions relevant to PPT primer selection, removal, and reutilization in relation to the dual enzymatic activities of reverse transcriptase.

**Generation of the 3' End of the PPT Primer**—Polymerase-dependent RNase H activity could, in principle, produce the PPT primer concomitant with minus-strand synthesis when the PPT is first copied into an RNA/DNA hybrid. However, the following observations suggest that a polymerase-independent mode of RNase H cleavage could generate the PPT after minus-strand synthesis has extended through the PPT region. First, the rate of polymerization is greater than the rate of RNA template cleavage ([52], and the polymerase-dependent RNase H activity does not completely degrade the RNA in a nonviral hybrid substrate as the RNA template is copied ([6], [42]). In addition, RNase H activity occurs preferentially at polymerase pause sites during RNA-templated DNA synthesis ([53], [54]), but little pausing occurs during minus-strand DNA synthesis through the PPT region ([13], [55]).

By using a long 5'-end-labeled RNA containing the PPT in a hybrid, we found that internal cleavages can occur without positioning by an RNA 5'-end. Because the cleavage producing the 3'-end of the PPT primer was observed without any cleavages upstream of the PPT, RNase H specificity to generate the plus-strand primer was retained in the absence of an RNA 5'-end. It is likely that the PPT sequence alone is sufficient to direct this internal cleavage event ([14], [56], [57]). Thus, the 5'-end-directed cleavage mechanism of RNase H ([10], [19], [20]) is not necessarily required in the reaction that generates the 3'-end of...
the plus-strand primer. From the results presented here, we cannot exclude the possibility than an alternate pathway for generating the plus-strand primer involves successive 5'-end-directed cleavages.

The 5'-End of the PPT Primer—It is possible that the internal cleavage sites mapped here for MMLV in the region upstream of the PPT reflect the preferred sites used during reverse transcription in vivo, and it is intriguing to consider the relationship between such cleavages and plus-strand priming. If, in addition to the cleavage that generates the 3'-end of the plus-strand primer, internal cleavage produces a 3'-end on the same RNA molecule at position −22 (or −23) without cleavage to produce the 3'-end at position −14, then a primer with a length of 22 nt would be generated. There are two possible outcomes from this combination of cleavages based on a competition between utilization of the resulting 22-mer as a primer by reverse transcriptase and 5'-end-directed cleavage of the RNA by RNase H. If the 22-mer were extended by reverse transcriptase, then plus strands would be initiated correctly and primer removal would be nearly normal, although some nascent plus strands would retain one or more extra ribonucleotides on their 5'-ends. If only one or two extra ribonucleotides remained on the linear product of reverse transcription, previous results suggest that integration would be unaffected (58). The observation that a small fraction of plus strands retained 4–6 5'-ribonucleotides in the MMLV endogenous reaction (24) or the HIV-1 in vitro reaction (16) might be explained by this scenario of aberrant plus strand primers and that plus strand initiation would be seriously impaired. We believe that a more likely scenario is that, in addition to the PPT primer cleavage, internal cleavages invariably occur on the same RNA molecule to produce 3'-ends at both the −14-position and the −22 (or −23)-position to produce an 8–9-nucleotide gap. In this case, there is only one outcome; the RNase H-resistant 13-mer PPT primer would be used efficiently to correctly initiate plus-DNA strands (10). The lengths of the residual RNA primer on plus strands observed previously for avian sarcoma virus, MMLV, and HIV-1 (15, 16, 23, 24, 26) are also consistent with this possibility. Since cleavage analysis of 5'-end-labeled RNA can only detect the cleavage site nearest the 5'-end of any given molecule, the data presented here do not address whether multiple cleavages occur within an RNA and, if they do, whether they are linked in any way. Experiments are under way to further define both the temporal sequence of such cleavages and the incidence of two or more cleavages within a given RNA molecule.

Recognition of the PPT RNA-DNA Junction—In a recent study using HIV-1 reverse transcriptase (59), the shortest extension product that was tested and found sufficient for cleavage at the RNA-DNA junction contained an extension of three deoxynucleotides. Here we show that cleavage at the RNA-DNA junction in an extended PPT primer can occur with only two deoxynucleotides added to the primer 3'-end. Since MMLV reverse transcriptase does not exhibit strong pause sites during plus-strand synthesis immediately downstream of the PPT primer (this work, and see Ref. 10), it seems unlikely that a PPT primer with just 2 deoxynucleotides is available for cleav-
age by the RNase H activity of reverse transcriptase. However, if reverse transcriptase were to dissociate from the nascent extension product as early as after the addition of 2 nucleotides, RNase H cleavage at the RNA-DNA junction would force plus-strand synthesis to reinitiate.

**RNase H Cleavage Specificity for RNA Primer Removal**—It appears that the RNase H activity of MMLV and HIV-1 reverse transcriptases generally prefers to cleave an RNA that has been extended with deoxyribonucleotides between the penultimate and last ribonucleotide of the RNA primer rather than at the RNA-DNA junction. This preference is exhibited in removal of the tRNA primer (28–30, 48) and the extended non-PPT substrates in this study. Interestingly, the bias against cleavage at the RNA-DNA junction and the capacity to cleave an extended RNA primer between the penultimate and last ribonucleotides are retained in the isolated RNase H domain of MMLV reverse transcriptase, although the preference for cutting at this site is not retained (this work, and see Ref. 8). Notably, the presence of a single deoxyribonucleotide on the 3'-end of an otherwise resistant 15-mer PPT-containing RNA was sufficient to promote cleavage between the penultimate and last ribonucleotide of this substrate. Thus, in the absence of other specificity determinants (see below), the retroviral RNase H strongly prefers to cleave between two ribonucleotide residues in an RNA chain, and this preference dictates that primer removal generally leaves a single ribonucleotide on the 5'-end of the DNA.

In addition to specifically generating the PPT primer 5'- and 3'-ends (23, 26), the RNase H activity of avian retroviral reverse transcriptases removes both the extended tRNA and PPT primers by cleaving precisely at the RNA-DNA junction (12, 27). In contrast, the only RNA-DNA junction that is cleaved by the RNase H activity of the murine and human retroviral reverse transcriptases is that generated by extension of the PPT primer (this work, and see Refs. 13, 15, 59, and 60), where RNase H recognizes the same site that had been cleaved previously in the RNA strand (between positions −1 and +1) to generate the 3'-end of the PPT primer. Thus, the natural tendency of the RNase H to cleave 1 ribonucleotide away from an RNA-DNA junction can be mitigated by the specificity that is directed by the PPT, which apparently forms a unique structure different from other RNA/DNA hybrids (14, 61–63). Interestingly, when PPT-containing RNA primers are 15 nt or longer, then 5'-directed cleavage competes with PPT-directed cleavage at the RNA-DNA junction to generate once again the default mode, which leaves 1 ribonucleotide on the 5'-end of some of DNA molecules. However, it should be noted that incomplete removal of PPT primers is not reflected in the sequence of circle junctions or double-stranded DNA products of reverse transcription for MMLV and HIV-1 (64–71), suggesting that, in vivo, the shorter PPT-containing primers are most often used for plus-strand DNA synthesis.

**Reutilization of PPT Primers**—The DNA polymerase activities of reverse transcriptases are able to initiate synthesis from a DNA primer at a nick and carry out displacement of the non-template strand concomitantly with primer extension (43, 51, 72–77). We show here that this property of the polymerase also applies to extension of a DNA version of the PPT primer. However, we found that reverse transcriptase is essentially unable to extend the PPT RNA primer when there is a non-template DNA strand downstream of the nick. Based upon the footprint of MMLV reverse transcriptase (78), our duplex substrate was of sufficient length to eliminate the possibility that the lack of extension was due to reverse transcriptase preferentially binding the 3'-end of the downstream DNA and physically blocking access to the 3'-end of the PPT primer. Furthermore, this possibility is considered unlikely because the DNA primer was extended with the same length of downstream DNA. While HIV-1 reverse transcriptase has been reported to reutilize the PPT primer, extension from a nick occurred when a 12-mer downstream DNA was terminated with a deoxyribonucleotide at the 3'-end and did not occur efficiently when the 12-mer downstream DNA could be extended (59).

**Does Extension of the PPT Primer Require a Gap?**—When the PPT primer is first generated by an internal cleavage event, the resulting 3'-end is followed by downstream RNA that could influence the efficiency of primer extension, particularly given the observation that reverse transcriptase cannot extend the PPT primer when there is abutting DNA downstream. In the absence of 5'-directed cleavages, we observed preferred RNase H cleavage sites downstream of the PPT primer 3'-end at positions +1, +2, and +5. Cleavages at these sites as well as at the site that generates the PPT primer on the same molecule would generate a gap of up to 5 bases between the 3'-end of the PPT primer and the 5'-end of the downstream RNA fragment. We speculate that such a gap may greatly facilitate initiation from the 3'-end of the PPT RNA primer, as the capacity of a gap to facilitate extension from DNA primers has been previously (51, 76).

**Influence of Polymerase Domain on RNase H Specificity**—It is interesting to consider the influence of the polymerase domain on the specificity of the RNase H domain. The isolated RNase H subdomain of MMLV reverse transcriptase retains activity but does not specifically remove either the tRNA or PPT primer or generate the PPT primer from plus-sense RNA (this work, and see Refs. 8 and 9). Thus, for MMLV, the polymerase domain is required for all of the specificity functions exhibited by the RNase H domain of reverse transcriptase during reverse transcription. These conclusions differ from results reported with an isolated RNase H domain of HIV-1 (29, 64, 79, 80), where specificity in tRNA primer removal was observed. However, it is noteworthy that the purified RNase H domain in these studies requires a vector-derived six-histidine amino acid tag and the nonphysiological cation Mn2+ for activity, while the isolated RNase H domain of MMLV does not require an N-terminal amino acid tag and is active using Mg2+ or Mn2+ (8, 9).

**REFERENCES**

1. Skalka, A. M., and Goff, S. P. (eds) (1993) Reverse Transcriptase, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
2. Arts, E. J., and LeGrice, S. F. J. (1998) Prog. Nucleic Acids Res. Mol. Biol. 58, 339–393.
3. Tanese, N., Telesnitsky, A., and Goff, S. P. (1991) J. Virol. 65, 4387–4397.
4. Levin, J. G., Crouch, R. J., Post, K., Hu, S. C., McKelvin, D., Zweig, M., Court, D. L., and Gerwin, B. I. (1988) J. Virol. 62, 4376–4380.
5. Kotewicz, M. L., Sampson, C. M., D'Alessio, J. M., and Gerard, G. F. (1988)
