Polypurine Tract Primer Generation and Utilization by Moloney Murine Leukemia Virus Reverse Transcriptase*

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During reverse transcription, the RNase H activity of reverse transcriptase specifically cleaves the viral genome within the polypurine tract (PPT) to create the primer used for the initiation of plus-strand DNA synthesis and nonspecifically cleaves the viral genome to facilitate synthesis of plus-strand DNA. To understand how primer length and sequence affect generation and utilization of the PPT, we employed short hybrid substrates containing or lacking the PPT to evaluate cleavage, extension, and binding by reverse transcriptase. Substrates containing RNAs with the correct 3′ end for initiation of plus-strand synthesis were extended equally well by reverse transcriptase, but primer length affected susceptibility to RNase H cleavage. RNA substrates with 3′ ends extending beyond the plus-strand initiation site were extended poorly but were specifically cleaved to generate the correct 3′ end for initiation of plus-strand synthesis. Substrates containing RNAs lacking the PPT were cleaved nonspecifically and extended inefficiently. Specific cleavages to generate the plus-strand primer and 5′-end-directed cleavages were kinetically favored over cleavages that destroyed the PPT primer or degraded other short RNA fragments. The PPT was not intrinsically resistant to cleavage by the isolated RNase H domain, and the isolated polymerase domain extended RNA primers containing the PPT sequence irrespective of the primer 3′ end. These results provide insights into how reverse transcriptase generates and selectively utilizes the PPT primer for initiation of plus-strand DNA synthesis.

Moloney murine leukemia virus (M-MuLV) converts its single-stranded plus-sense RNA genome into a double-stranded DNA molecule through the replicative process termed reverse transcription (reviewed in Ref. 1). Minus-strand DNA synthesis initiates from a host cell-derived tRNA primer and extends through a unique sequence (U5) and a terminal repeat sequence (R) at the 5′ end of the genome before carrying out the first template jump to a second R sequence found at the 3′ end of the viral genome. As minus-sense DNA synthesis progresses, a purine-rich sequence in the RNA genome termed the polypurine tract (PPT) that lies immediately adjacent to a downstream unique sequence (U3) is copied and subsequently cleaved to generate the PPT primer. This primer is used to initiate plus-strand DNA synthesis, which extends through U3-R-U5 and continues after a second jump to the 5′ end of the DNA template. Displacement synthesis is required to create duplex DNA synthesis and formation of the redundant DNA ends called long terminal repeats (LTRs) (2).

The multifunctional enzyme that carries out this replicative process is the virally encoded reverse transcriptase (1). For M-MuLV, reverse transcriptase is a 75-kDa polypeptide that contains an amino-terminal RNA- and DNA-dependent DNA polymerase activity and a carboxyl-terminal RNase H activity that cleaves the RNA portion of an RNA/DNA hybrid. The RNase H and polymerase activities of reverse transcriptase are separable (3–5), but these domains carry out their functions in an interdependent manner (reviewed in Ref. 1). A truncated form of reverse transcriptase that is missing the RNase H domain results in a functional polymerase with decreased processivity (6, 7). An NH2-terminal deletion of reverse transcriptase lacking the polymerase domain retains an enzymatically active form of RNase H, but this domain has lost much of the specificity associated with the intact enzyme (8, 9).

Studies with human, murine, and avian retroviruses have indicated that the RNase H activity of reverse transcriptase can be classified into two distinct types (10–12). The polymerase-dependent activity of RNase H is spatially and temporally coordinated by association of the polymerase domain with a 3′ DNA primer terminus in association with an RNA template. Biochemical and structural studies have suggested that the spatial locations of DNA polymerase and RNase H active sites are approximately 15–20 bases apart on an RNA/DNA hybrid (10–24). Polymerase-dependent RNase H activity can take place during processive minus-strand synthesis but is not sufficient to degrade completely all of the template RNA (17, 25, 26).

The polymerase-independent type of RNase H activity occurs without concomitant DNA synthesis, is not positioned by a 3′ primer terminus, and generates fragments 15–20 nt long (10, 12, 13, 17, 21, 26–28). This activity can further degrade the RNA to generate cleavage products as short as 6–8 bases in size (10, 11, 13, 17, 20, 22, 29). The polymerase-independent activity of RNase H is positioned by the polymerase domain of reverse transcriptase binding at the 5′ end of an RNA annealed to a longer DNA (23, 28, 30–32), suggesting that this type of RNase H activity plays a major role in degrading the RNA genome following minus-strand synthesis.

In this study, we have evaluated parameters that influence the RNase H and polymerase activities of reverse transcriptase during the initiation of plus-strand synthesis. A series of RNAs containing all, some, or none of the PPT sequence were an-
nealed to longer DNAs to generate model hybrid substrates representing various primer-template possibilities that might occur at or near the beginning of plus-strand DNA synthesis. When these substrates were used to assay the polymerase-independent activity of RNase H, the sequence and length of the RNA dictated the cleavage pattern. When used as primers for extension, reverse transcriptase extended best those primers that contained the correct 3′ PPT primer terminus used for plus-strand DNA synthesis. These studies offer insights into how reverse transcriptase associates with sequences containing or lacking the PPT and extend our understanding of the initiation of plus-strand DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—Recombinant wild type M-MuLV reverse transcriptase, Sequenase version 2.0, and calf alkaline phosphatase were obtained from Amersham Pharmacia Biotech. Superscript (RTΔH), Superscript II (H·RT), and BamHI were purchased from Life Technologies, Inc. T4 polynucleotide kinase was obtained from both New England Biolabs and Life Technologies, Inc. The separate RNase H domain of M-MuLV reverse transcriptase (RTΔPol) was obtained as described previously (8).

**Oligonucleotides**—The cleavage site generating the PPT primer for plus-strand synthesis of M-MuLV is located between positions 7815 and 7816 on the plus-sense viral genome (33), and these two residues are designated positions 1 and +1, respectively, for the purposes of naming the oligonucleotides used in this study. Thus the name of a given oligonucleotide begins with an R or D designation for oligoribonucleotide or oligodeoxyribonucleotide, respectively, followed by the 5′ end position, a backslash, and the 3′ end position relative to the PPT cleavage site. Four 38-mer long oligodeoxyribonucleotides were used as the template strands as follows: D 5′/3′-39/TAAGCTAGCTTGCCA-

**Nucleic Acids Used to Generate Long RNA/DNA Hybrids—**Construction of bacteriophage M13LTR1 was similar to that of M13LTR2 (34) and will be described in detail elsewhere.2 The long DNA strand was generated by isolating the single-stranded insert from M13LTR1 as described previously for ssLTR2i (34) except that restriction enzyme digestion or lacking the PPT and extend our understanding of the initiation of plus-strand DNA synthesis.

**Kinetic Cleavage Analysis of Hybrid Oligonucleotide Substrates—**Hybrid oligonucleotide substrates (ranging in concentration from 0.625 to 225 nm) were incubated with 0.002 pmol (0.1 nm final concentration) of reverse transcriptase or RTΔPol in a 20-μl reaction containing RNase H cleavage buffer at 37 °C. At 1, 4, and 16 min, 2.5-μl aliquots were removed, added to 5 μl of formamide stop mix (80% deionized formamide, 1 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol), and samples were analyzed in a 20% sequencing gel.

**Kinetic Cleavage Analysis of Long Hybrid Substrates—**The long hybrid substrate was prepared by annealing 8.4 pmol of 5′-end-labeled 753-nt RNA with 7.6 pmol of long DNA in 200 mM KCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA for 45 min at 67 °C. Cleavage assays were carried out in 16-μl reactions containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 6 mM MgCl2, and 5 mM DTT with hybrid substrate concentrations ranging from 0.5 to 40 nm. Reactions were preincubated for 1 min at 37 °C, initiated by the addition of 1.6 fmol of reverse transcriptase (0.1 unit) and 1.0 μl of each primer/template. Reactions were treated with 150 μl of formamide and EDTA to a final concentration of 73.5% and 7.5 mM, respectively. Products were analyzed in a 20% sequencing gel and quantified by PhosphorImager analysis. Using data from two experiments, Kmax and Vmax values were determined using a Lineweaver-Burk plot as described above.

**Gel Shift Analyses**—2 fmol of duplex oligonucleotide substrates (0.1 pmol) were incubated with 0.2 to 64 nM of H·RT or reverse transcriptase in a 20-μl reaction containing 75 μM Tris-HCl, pH 8.0, 75 μM MgCl2, 7.5 mM DTT, and 150 μg per ml of BSA at 37 °C. After 15 min, 4 μl of 50% glycerol was added, and 10 μl of each sample was loaded on a 20% sequencing gel.

2 C. D. Kelleher and J. J. Champoux, submitted for publication.
enzyme concentration. Each experiment was performed two to eight
times.

**RESULTS**

Cleavage of Hybrid Oligonucleotide Substrates by RNase H Activity of Reverse Transcriptase—To create the PPT primer used for plus-strand DNA synthesis, the RNase H activity of reverse transcriptase cleaves the viral RNA hybridized to minus-strand DNA within the highly conserved PPT sequence between a G nucleotide and an A nucleotide (which we have designated as the −1 and +1 positions on the viral genome, respectively) (Fig. 1). (1). Some studies have shown that a short RNA fragment annealed to a longer DNA is recognized by reverse transcriptase as a substrate for degradation and not as a primer for DNA synthesis (28, 30–32). To understand better how reverse transcriptase might generate a PPT primer that escapes further RNase H degradation and facilitates plus-strand DNA synthesis, we initially tested whether length affected the recognition of PPT-containing RNA fragments as substrates for the RNase H activity of M-MuLV reverse transcriptase.

5′-End-labeled oligoribonucleotides containing the PPT sequence and ranging in length from 13 to 20 nt were each annealed to the 38-mer template D + 10′/−28 to generate partially duplex oligonucleotide substrates (Fig. 1). These substrates were incubated with a 10-fold molar excess of reverse transcriptase, and the resulting products were resolved in a 20% sequencing gel to assess qualitatively the extent of RNase H cleavages (Fig. 2). In all cases, control incubations showed no substrate degradation (Fig. 2, odd numbered lanes) when compared with the original 5′-end-labeled oligonucleotides (data not shown). Substrate R−18/−1 contained detectable amounts of smaller fragments ranging from 7 to 12 nt in size, but these fragments did not affect interpretation of the results (Fig. 2A, lanes 15 and 16). The two substrates that span the plus-strand initiation site, R−13/−7 and R−13/+3, were both cleaved to generate a primary product 13 nt in length with a 3′ end at position −1, corresponding to the PPT primer terminus (Fig. 2A, lanes 2 and 4, arrow). Minor cleavage products of higher molecular weight corresponding to a 15′- and a 14-mer for substrate R−13/+7 and a 14-mer for substrate R−13/+3 were also apparent.

Another set of RNA oligonucleotides had 3′ termini at position −1 but differed in the positions of their 5′ ends, extending from 13 nt (primer R−13/−1) to 20 nt (primer R−20/−1) upstream of the plus-strand initiation site (Fig. 1). When these RNAs were annealed to template D + 10′/−28, the resulting hybrid substrates exhibited a gradation of increasing cleavage as the RNA primer length increased (Fig. 2A, lanes 6, 8, 10, 12, 14, 16, and 18). Substrates with shorter RNAs such as R−13/−1 and R−14/−1 appeared more resistant to RNase H cleavages, whereas substrate R−20/−1 was cleaved the most extensively (Fig. 2A, lanes 6 and 8, respectively). A paucity of fragments smaller than 6 or 7 nt in length (data not shown) suggested that very little cleavage occurred toward the 5′ end of the primer. Most cleavages in the longer RNAs (R−17/−1, R−18/−1, R−20/−1) occurred in the stretch of G nucleotides found in the PPT just upstream of the plus-strand initiation site (see Fig. 1) and likely result from an RNA 5′-end-directed cleavage mechanism (28, 30) (see “Discussion”).

We next addressed whether PPT-containing substrate R−13/−1 was more resistant to RNase H cleavage because of its short length or because it exclusively contained the PPT sequence. The ability of RNase H to cleave this substrate was
compared with 13-mer oligoribonucleotides of different, non-PPT sequence that were positioned equivalently on appropriate 38-nt length template DNAs (see Fig. 1). As before, substrate R–13/−1 remained relatively resistant to the RNase H activity, but the other 13-mer duplex substrates that lacked PPT sequences were cleaved into fragments ranging from 7 to 11 nt in length (Fig. 2B, lanes 1–8).

RTΔPol Cleaves Substrates Irrespective of the PPT Sequence—Previously we reported that a form of reverse transcriptase that lacks the polymerase domain, termed RTΔPol in this study, contains an enzymatically active RNase H domain (8). To address whether the polymerase domain contributes to the specificity of reverse transcriptase for RNA primers derived from the PPT, we evaluated the ability of RTΔPol to cleave the duplex oligonucleotide substrates that contained PPT sequences.

Under identical conditions to those described above for reverse transcriptase, RTΔPol did not selectively produce the PPT primer from substrates R–13/+7 or R–13/+3 but instead cleaved at multiple sites upstream and downstream of the plus-strand initiation site as well (Fig. 3A, lanes 1–4). The absence of the polymerase domain in RTΔPol also rendered substrates with shorter primers susceptible to RNase H degradation (Fig. 3A, lanes 5–12). Notably, products ranging in size from 1 to 13 nt were apparent, indicating that cleavage could occur at every possible position throughout the PPT. RTΔPol also cleaved the 13-mer duplex substrates lacking PPT sequences more extensively than reverse transcriptase (Fig. 3B, lanes 3–8).

Kinetic Analysis of RNase H Cleavages by Reverse Transcriptase and RTΔPol—In the preceding experiments, we observed specific RNase H cleavages that generated the PPT primer as well as those that occurred within the PPT or in short 13-mers that lacked PPT sequences. We next asked if the specific cleavages required to generate the PPT primer were kinetically different from those associated with nonspecific RNase H cleavages. To examine the steady-state kinetics of RNase H activity, we incubated increasing amounts of different duplex substrates with rate-limiting amounts of enzyme in a standard Michaelis-Menten analysis.

Previous studies have indicated that the $K_m$ for HIV-1 reverse transcriptase association with RNA/DNA hybrids is approximately 5 to 10 nM (17, 37). To assay for specific cleavages that generate the PPT primer, we used 0.1 nM M-MuLV reverse transcriptase and 0.625 to 5.0 nM of the hybrid substrate containing R–13/+7. Aliquots were removed either before or at various times after the addition of reverse transcriptase and analyzed in a 20% sequencing gel (Fig. 4A). At each time point examined, the amount of substrate remained in vast excess to the amount of product observed. At the earliest time point for each concentration of substrate, the initial cleavage products were primarily 15-mers corresponding to R–13/+2 but also apparent were lower amounts of the 13-mer product R–13/−1, which has a correct 3' end for plus-strand initiation (Fig. 4A, lanes 2, 6, 10, and 14). A small amount of 14-mer product R–13/+1 was detected but was not considered in the kinetic analysis. Although we cannot exclude the possibility that the shorter 13-mer product was derived from the 15-mer, for the kinetic analyses we have assumed that the 13-mer is independently cleaved from the full-length substrate RNA.

To measure the kinetic constants, the velocity $V_o$ was calculated as the amount of product R–13/+2 or R–13/−1 produced per min as determined by a PhosphorImager analysis of the gel (see “Experimental Procedures”). By using the method of Lineweaver-Burk, the values of $1/V_o$ versus $1/[S]$ were plotted, and a linear regression line was drawn (Fig. 4B). The $V_{max}$ and $K_m$ values for each of these cleavage products were calculated as the intercepts of the line at the $1/V_o$ and $−1/[S]$ axes, respectively, and these values are presented in Table I. In repeated experiments, concentrations of substrate R–13/+7 ranging from 0.31 to 50 nM were tested for specific RNase H cleavages by reverse transcriptase. For the two major cleavage products, R–13/+2 and R–13/−1, the $K_m$ values were similar, at 0.79 and 0.63 nM, respectively. Consistent with the order of appearance of the two products (Fig. 4A), the $V_{max}$ for producing R–13/+2 was approximately two to three times greater than the corresponding value for the production of R–13/−1 (0.51 fmol/min versus 0.18 fmol/min). When reverse transcriptase was incubated with substrate R–13/+3 under identical condi-
tions, the $K_m$ values for the major product R–13/–1 was 1.44 nM with a $V_{max}$ value of 0.09 fmol/min. Both are within experimental error of the values for producing the same product from the longer R–13/–7 substrate (Table I).

By using high concentrations of reverse transcriptase, we detected numerous cleavage products for PPT-containing substrates of 17 nt or longer in length which are consistent with 5′-end-directed cleavage (Fig. 2A, lanes 13–18). When substrate R–20/–1 was incubated with 0.1 nM reverse transcriptase in the kinetic analysis, three primary products of 15, 16, and 17 nt in length were detected (data not shown). These products were also the major fragments evident after treating substrate R–20/–1 with a 10-fold molar excess of reverse transcriptase (Fig. 2A, lane 18). The $K_m$ values for the cleavages producing R–20/–4, R–20/–5, and R–20/–6 ranged from 9 to 12 nM, which are an order of magnitude greater than the values for specific cleavage by the enzyme to generate the plus-strand primer (Table I). Similarly, the $V_{max}$ values for these products ranged from 0.015 fmol/min to 0.025 fmol/min and are significantly lower than those that generated the PPT primer.

When the 13-mer non-PPT substrates R–58/–46, R–4/+4/+/6, and R–17/+29 were incubated with reverse transcriptase in identical kinetic assay conditions, we were unable to observe any cleavages using substrate concentrations as high as 225 nM (data not shown). However, high concentrations of reverse transcriptase generated very abundant products from the non-PPT 13-mer substrates (Fig. 2B) that, if produced, should have been apparent in our kinetic assay. We interpret the absence of such products to suggest that, for reverse transcriptase, either the $K_m$ for cleavage is greater than 225 nM or that the rate of cleavage is exceedingly slow for these substrates.

To ask how the polymerase domain might affect the kinetics of RNase H cleavages, we performed the same analyses with RTΔPol (data not shown). When up to 50 nM substrate R–13/+7 or substrate R–13/+3 was incubated with RTΔPol, no cleavage products were evident. Because high concentrations of RTΔPol produced a very equal distribution of cleavage products from substrates R–13/+7 and R–13/+3 (Fig. 3A, lanes 2 and 4), it is likely these same products were present in our kinetic assay but at a level beneath our limit of detection. However, when substrates R–58/–46 and R–20/–1 were treated with rate-limiting amounts of RTΔPol, low levels of cleavage products appeared that were identical to those observed after treatment with a 10-fold molar excess of RTΔPol (see Fig. 3B, lane 8, and Fig. 3A, lane 18, respectively). By using the most abundant product from each substrate to calculate the kinetic constants, we found that the $K_m$ values for RTΔPol were significantly higher than those observed for reverse transcriptase, whereas the $V_{max}$ values were comparable to the higher values observed for the intact enzyme (Table I).

**Kinetic Analysis of RNase H Cleavages on a Long RNA/DNA Hybrid**—To determine the kinetic constants for 5′-end-directed cleavage which did not occur in the PPT, we evaluated RNase H cleavages using a long RNA/DNA hybrid. A 753-nt long RNA containing the M-MuLV LTR and flanking sequences was synthesized, 5′-end-labeled, and annealed to a longer DNA to generate an RNA/DNA hybrid substrate (see “Experimental Procedures”). When this substrate was incubated with a rate-limiting amount of reverse transcriptase (0.1 nM) over a substrate concentration range of 0.5 to 40 nM, no large fragments resulting from internal cleavages on this substrate were observed, and the predominant product was a 16-nt long RNA amid several minor products (ranging from 8 to 20 nt in size; data not shown). These products resulted from 5′-end-directed cleavages, and a portion of a gel showing the major bands is shown in Fig. 5A. Using the 16-nt long species, a double-reciprocal plot of the amount of product generated as a function of the substrate concentration was generated (Fig. 5B). The $K_m$ was estimated to be 0.63 ± 0.11 nM and the $V_{max}$ was equal to 0.42 ± 0.17 fmol/min (Fig. 5B). These values approximate those obtained for the cleavage of substrates R–13/+7 and R–13/+3 by reverse transcriptase (Table I). Cleavage analysis with two other long RNA/DNA hybrids indicated that the 5′ ends of these hybrids were cleaved at a similar rate (data not shown), suggesting that 5′-end-directed cleavages occurring outside of the PPT sequences approximate those measured for generation of plus-strand primers (Fig. 4 and Table I).

**Differential Primer Utilization by H′ RT and RTΔH—**We next evaluated how the polymerase activity of reverse transcriptase extended RNA primers that contain or lack PPT sequences using an intact form of reverse transcriptase that has point mutations that destroy the RNase H activity (H′ RT) and a COOH-terminal deletion mutant of reverse transcriptase that contains only the polymerase domain (RTΔH). To compare the ability of these enzymes to extend the hybrid substrates, equal amounts of DNA polymerase activity were used for each extension assay.

### Table I

| Enzyme | Hybrid | Product | $K_m$ (nM) | $V_{max}$ (fmol/min) |
|--------|--------|---------|------------|---------------------|
| RT     | R–13/+7| R–13/+2  | 0.79 ± 0.11| 0.51 ± 0.06         |
|        | R–13/–1| R–13/–1  | 0.63 ± 0.42| 0.18 ± 0.07         |
|        | R–13/3 | R–13/–1  | 1.44 ± 0.44| 0.09 ± 0.07         |
|        | R–20/–1| R–20/–4  | 11.92 ± 2.45| 0.025 ± 0.006     |
|        | R–20/–5| 8.69 ± 2.64| 0.015 ± 0.003|
|        | R–20/–6| 11.15 ± 3.98| 0.017 ± 0.005|
| RTΔPol | R–58/–46| R–58/–50 | 85.64 ± 12.53| 0.38 ± 0.04         |
|        | R–20/–1 | R–20/–13 | 78.45 ± 2.19| 0.55 ± 0.36         |

**Fig. 5.** Kinetic analysis of RNase H cleavage by reverse transcriptase on a long RNA/DNA hybrid. A, a 5′-end-labeled 753-nt RNA was annealed to a longer template DNA, and 0.5–40 nM of this hybrid substrate was incubated with reverse transcriptase (+) or dilution buffer (−). Cleavage reactions were terminated at 1 min, and the products were separated in a 20% sequencing gel and visualized using a PhosphorImager. The data are taken from a representative gel showing fragments ranging from 13 to 20 nt in length, as determined by nuclease P1 digestion of the 5′-end-labeled RNA. The fragment lengths from the P1 nuclease ladder are indicated on the left. Arrow at right indicates the position at which the 16-mer cleavage product migrates. B, a Lineweaver-Burk plot of $1/[S]$ versus $1/V_5$ for the 16-mer product.
Duplex oligonucleotide substrates (Fig. 1) were incubated in the presence of dNTPs with H·RT or RTΔH for 15 min, and the resulting extension products were examined in a 20% sequencing gel. To measure the amount of extendable RNA in each hybrid preparation, the substrates were incubated with an excess of Sequenase under the same conditions (Fig. 6, lanes 25–32). The fraction of the primer extended by Sequenase corresponded well with the fraction of the RNA annealed to the template as assessed by native gel electrophoresis (data not shown). Irrespective of the primer 3’ end, the polymerase domain of reverse transcriptase alone (RTΔH) directed extension from all PPT-containing primers nearly as well as Sequenase under these conditions, (Fig. 6, compare lanes 9–13 with lanes 25–29).

The presence of the RNase H domain contributed some specificity to primer extension, as H·RT extended PPT-containing RNAs with 3’ ends at positions +7 or +3 2–5-fold less efficiently than RTΔH (Fig. 6, compare lanes 21 and 20 with 13 and 12) but extended primers that had the correct 3’ terminus for plus-strand initiation at a level comparable to RTΔH (Fig. 6, lanes 9–11 and 17–19). In general, primers that lacked the PPT sequence (R+4/+16, R+17/+29, and R–58/+46) were extended less efficiently under these conditions by both RTΔH and H·RT (Fig. 6, lanes 14–16 and 22–24), although RTΔH extended primers R+17/+29 and R–58/+46 2–3-fold better than H·RT (Fig. 6, lanes 15, 16, 23, and 24). In control experiments, DNA primers containing the same sequences as R+4/+16, R+17/+29 and R–58/+46 in duplex substrates were extended efficiently by all three polymerases (data not shown), demonstrating that the inability to extend these primers lies in the nature of the hybrids and not in the nucleotide sequence (see “Discussion”).

**DISCUSSION**

During processive minus-strand synthesis, the RNase H activity of reverse transcriptase begins degradation of the viral template to produce RNA fragments, some of which remain associated with the newly synthesized minus-strand DNA (17, 26, 30). These RNA fragments may (i) be further cleaved by the polymerase-independent activity of RNase H, (ii) used to prime DNA synthesis, or (iii) displaced by the polymerase during synthesis from upstream sites. Of the multiple RNA fragments derived from the original genome, reverse transcriptase cleaves the RNA and utilizes the primer derived from the PPT to initiate the synthesis of plus-strand DNA. This priming event precisely defines one of the ends of the linear duplex product of reverse transcription (42–44). Previous studies have shown that short RNAs hybridized to longer DNAs are suitable in vitro models for studying RNase H activity (23, 28, 30, 45) and that 15–18 nt of PPT sequence are sufficient to initiate plus-strand synthesis (45, 46). To understand better how reverse transcriptase selectively utilizes the PPT to initiate plus-strand synthesis, we have compared short RNA/DNA substrates containing or lacking PPT sequences in assays that measure RNase H cleavages, primer extension, and substrate binding.
Fig. 7. Analysis of substrate binding by H-RT using a gel shift assay. To assay the substrate binding specificity by H-RT, 0.1 nM substrate was incubated with the indicated concentrations of enzyme for 15 min and then analyzed in a non-denaturing 5% polyacrylamide gel at 4°C. Representative data from gels visualized by PhosphorImager analysis are presented. A, substrate R–13/+1 or substrate R+4/+16 was either untreated (lanes 1 and 9) or incubated with the indicated amount of H-RT (lanes 2–8 and 10–16). The positions of bound species are indicated with arrows. B, to determine the dissociation constant ($K_d$), the percentage of free substrate is plotted as a function of the concentration of H-RT (nM). Filled circles, substrate R–13/+1; filled triangles, substrate R+4/+16.

Table II

$K_d$ values for binding of duplex substrates to H-RT

| Substratea | Lengthb (nt) | $K_d$ (nM) |
|------------|-------------|-----------|
| R–13/+7    | 20          | 4.43 ± 1.14 |
| R–13/+3    | 16          | 4.17 ± 1.01 |
| D–13/–1    | 13          | 2.20 ± 0.18 |
| R–13/–1    | 13          | 5.05 ± 0.77 |
| R–14/–1    | 14          | 6.50 ± 0.85 |
| R–15/–1    | 15          | 2.90 ± 0.61 |
| R–16/–1    | 16          | 3.25 ± 0.71 |
| R–17/–1    | 17          | 8.82 ± 3.72 |
| R–18/–1    | 18          | 5.64 ± 2.28 |
| R–20/–1    | 20          | 7.22 ± 1.31 |
| R+4/+16    | 13          | 3.31 ± 1.06 |
| D+4/+16    | 13          | 3.75 ± 1.54 |

a Substrates correspond to the named RNA or DNA primer annealed to the appropriate template strand as described under “Experimental Procedures.”
b Refers to the length of duplex in the substrate.

Reverse Transcriptase Does Not Selectively Utilize PPT-containing Sequences Based Upon Affinity—Reverse transcriptase must bind a PPT-containing RNA/DNA hybrid in one orientation to create the PPT primer and in the opposite orientation to use the primer for the initiation of plus-strand synthesis (28). Because the active sites of the polymerase and RNase H domains are positioned to contact opposite strands on a duplex nucleic acid (14, 15), the use of the PPT sequence in a hybrid as a primer for synthesis or a substrate for cleavage is dependent upon how reverse transcriptase binds that hybrid. If reverse transcriptase binds with the polymerase domain oriented on the DNA strand and the RNase H active site associated with the RNA fragment, then RNA cleavage can occur. This binding orientation would allow both polymerase-dependent RNase H cleavages with the enzyme positioned by a 3' primer terminus, and polymerase-independent RNA 5'-end-directed cleavages, where positioning is determined by the 5' end of the RNA. Alternatively, if reverse transcriptase binds the hybrid such that the active site of the polymerase is associated with the 3' hydroxyl group of a recessed RNA primer terminus, then DNA synthesis can occur, but the RNase H domain sits inactive on the DNA strand (28, 30). When tested in our binding assay, reverse transcriptase shows no difference in affinity for the spectrum of hybrid substrates even though these substrates contain or lack the PPT and have different lengths of hybrid duplex. This finding reveals that reverse transcriptase does not selectively choose PPT-containing substrates for cleavage or extension at the level of binding and that the likelihood of catalytic activity in one orientation or the other depends on some structural feature of the substrate (see below).

Both Modes of Binding Occur On Short PPT-containing Substrates—Substrates containing primers from 13 to 20 nt in length that have the correct 3' terminus for plus-strand initiation are extended equally well by the RNase H-deficient form of reverse transcriptase, H-RT. Therefore these substrates are bound in the polymerase extension mode, and primer length does not appear to affect extension by reverse transcriptase. These same substrates are cleaved by the RNase H activity of reverse transcriptase in relation to primer length. Although shorter primers are more resistant to RNase H cleavages than the longer primers, it is likely that substrates with shorter primers are still bound at the RNA 5' end by reverse transcriptase in a cleavage orientation, but the primers are not sufficiently long to be cleaved by the RNase H domain. Because PPT-containing RNA primers of 16 nt or less are extended efficiently and not cleaved extensively by reverse transcriptase, we believe that such RNAs would function well as PPT primers in vivo.

Substrates containing PPT RNAs that are longer than 16 nt in length and have the correct 3' primer terminus for plus-strand initiation (R–17/–1, R–18/–1, and R–20/–1) can be degraded within the PPT sequence by reverse transcriptase. These “inappropriate cleavages” that would prevent correct plus-strand priming occur 15–18 nt from the 5' end of the RNA primer between the G nucleotide of the PPT, suggesting that these polymerase-independent RNase H cleavages are RNA 5'-end-directed. However, it is especially noteworthy that the $K_m$ for inappropriate cleavage of substrate R–20/–1 within the PPT is at least 10 times greater than the $K_m$ for cleavage of R–13/+7 or R–13/+3 to produce the plus-strand primer, and the corresponding $V_{max}$ value is on the order of 10 times slower. Thus during retroviral replication, proper cleavage within the PPT appears to be kinetically favored, and in this sense the PPT is relatively resistant to RNase H degradation as suggested previously (30).

Polymerase-independent RNase H Cleavages Are Not Determined Strictly by the 5' RNA End—RNA primers that share a
Polymerase Domain Protects PPT Sequence from RNase H Degradation—We tested the ability of the RNase H domain alone to cleave substrates containing or lacking the PPT. Although we had previously shown that the RNase H activity of reverse transcriptase has a specific activity that is 43 times the influence of the RNase H domain on primer extension. RTAH extends all PPT-containing primers, irrespective of whether the 3' end of the primer is located at the −1 position or at the +3 and +7 positions downstream of the plus-strand initiation site. By contrast, H-RT only poorly extends primers extending beyond the −1 position. These data suggest that the polymerase domain alone is sufficient to recognize the PPT as an initiation sequence for plus-strand synthesis but, without the RNase H domain, is not as discriminating with respect to primer selection. Thus the RNase H domain gives specificity to the polymerase domain for extension from the PPT primer. In addition, whereas the intact enzyme struggled or was unable to extend all non-PPT 13-mer RNAs, the polymerase domain alone extended two of the three non-PPT 13-mer primers although at a lower efficiency than PPT-containing primers.

Model for Plus-strand Priming by M-MuLV Reverse Transcriptase—The results presented here suggest a model for how the RNase H activity of reverse transcriptase is coordinated to both nonspecifically degrade genome RNA after minus-strand synthesis and specifically generate the PPT primer that is required for the initiation of plus-strand synthesis. Importantly, the kinetic parameters for cleavage in the PPT to produce the plus-strand primer indicate that this reaction is kinetically favored over most other cleavage events in the substrates tested. The only other major category of cleavage with comparably low \( K_m \) and high \( V_{max} \) values involved 5'-end-directed cleavages of the long RNA-DNA hybrid to produce predominantly a 16-mer product. These results are consistent with the view that processive 5'-end-directed cleavages that degrade the viral genome after first strand synthesis and cleavage within the PPT to generate the plus-strand primer represent the two kinds of cleavage events most important for the completion of reverse transcription during viral replication. Once the PPT has been cleaved at the correct site to produce the 3' end of the plus-strand primer, the ability of RNase H to cleave RNA-DNA hybrids at many sites and in both a polymerase-dependent and polymerase-independent fashion means that the 5' end of the PPT primer is not necessarily fixed. If cleavage occurs near the 5' end of the PPT to generate a plus-strand primer that is 13–16 nt in length, then the relative cleavage resistance of such a primer would facilitate utilization of the RNA as the plus-strand primer rather than being subjected to further degradation. If the 5' end were generated further upstream to yield RNAs 17–20 nt in length (or even longer), then we show that the 5'-end-directed mode of cleavage could destroy the option of plus-strand synthesis by causing cleavage in the 3' half of the PPT. However, based on kinetics of such cleavage events and the fact that the PPT primer is preferentially used for extension by the polymerase, such a scenario seems unlikely to present a problem for the virus during reverse transcription.

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