Polypurine Tract Formation by Ty1 RNase H*

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To better understand the mechanism by which Ty1 RNase H creates the polypurine tract (PPT) primer, we have demonstrated the polymerase-dependent hydrolytic activity of Ty1 reverse transcriptase (RT) during minus-strand synthesis. Using RNase H and polymerase mutants of the recombinant Ty1 RT protein, we show that the two domains of Ty1 RT can act independently of one another. Our results indicate that RNA/DNA substrates containing a short RNA PPT, which serve as primers for plus-strand DNA synthesis, are relatively resistant to RNase H cleavage. RNA substrates with a correct 5’ end but with 3’ end extending beyond the plus-strand initiation site were cleaved specifically to generate the correct 3’ end of the PPT. Using long RNA/DNA duplexes containing the PPT, we show that Ty1 RT is able to make specific internal cleavages that could generate the plus-strand primer with correct 5’ and 3’ ends. Long RNA/DNA duplexes with mutations in the PPT or in a U-rich region upstream of the PPT, which abolish plus-strand initiation in vitro, were not cleaved specifically at the 5’ end of the PPT. Our work demonstrates that the in vitro enzyme can recapitulate key processes that control proper replication in vivo.

Reverse transcriptase (RT)1 of retroviruses and LTR retrotransposons is a multifunctional enzyme that possesses RNA-dependent and DNA-dependent DNA polymerase activities as well as RNase H activity that specifically degrades the RNA strand of RNA/DNA duplexes. RT converts the single-stranded plus-strand RNA genome of the retroelement into a double-stranded preintegrative DNA molecule that is subsequently integrated into the host genome (reviewed in Ref.1). Replication begins with the synthesis of minus-strand DNA, which is primed by a host cell-derived tRNA that binds to the primer binding site of the viral template RNA. Reverse transcription is initiated by extending the 3’ end of the primer, using the 5’ protruding end of the plus-strand genomic RNA as template. When RT reaches the 5’ end of the RNA template, the so called minus-strand strong-stop DNA (–sssDNA) is released and transferred to the 3’ end of the genomic RNA to allow continuation of minus-strand DNA synthesis. During minus-strand DNA synthesis, the template RNA strand of the newly formed RNA/DNA hybrid is degraded by the RT-associated RNase H activity (2–5). Using HIV-1 RT, two modes of RNase H activity can be distinguished: (i) a polymerase-dependent mode, which accompanies DNA synthesis and is positioned by the polymerase domain binding to the recessed nascent DNA 3’ end; and (ii) a polymerase-independent mode, which occurs without DNA synthesis but is oriented by the polymerase domain binding to the recessed 5’ end of RNA fragments paired to the DNA. A bimodal hydrolysis mechanism has also been demonstrated for the Saccharomyces cerevisiae retrotransposon Ty3 RT (6). The polymerase-dependent RNase H activity that takes place during minus-strand synthesis is not sufficient to completely degrade all of the template RNA (7, 8). One reason for this partial digestion is a greater rate of polymerization versus RNA template cleavage (7, 9). The fragments that remain bound to the minus-strand DNA must be digested further by the polymerase-dependent activity of RNase H. However, specific purine-rich fragments called polypurine tracts (PPT) are resistant to RNase H digestion and function as RNA primers for plus-strand DNA synthesis (2).

In all LTR-containing retroelements, a preferred PPT (the 3’ PPT) is located just upstream of the 3’ LTR boundary. Extension of this PPT by the polymerase activity of RT generates the plus-strand strong-stop DNA (+ssDNA). Additional initiation sites have been found in the middle of the genome of some retroviruses and of the yeast retrotransposon Ty1 (10–13). Cleavage at the 5’ and 3’ ends of the PPT must be specific to generate functional plus-strand primers. The precision of plus-strand initiation from the 3’ end of the PPT is essential because it defines the 5’ border of the upstream LTR, which contains cis-acting sequences required for integration of the preintegrative DNA into the host cell genome.

The mechanism by which RNase H creates the PPT primer is not well understood. Several reports in retroviruses have shown that the precise generation of a PPT primer is sequence-dependent, i.e. the integrity of some positions in the PPT is necessary for cleavage specificity (2). Structural studies suggest that a specific secondary structure of the hybrid RNA/DNA duplex could explain resistance of the PPT to RNase H digestion. Recently, a conserved U-rich sequence upstream of the PPT of several retroelements has been found to be important for their replication (14–18). Robson and Telesnitsky (18) have proposed that contacts between the DNA polymerase domain of RT and the U-rich sequence could contribute to the specificity of the RNase H cleavage at the PPT.

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1 The abbreviations used are: RT, reverse transcriptase; LTR, long terminal repeat; HIV, human immunodeficiency virus; PPT, polypurine tract; WT, wild type; nt, nucleotide(s); –ssDNA, minus-strand strong-stop DNA; +ssDNA, plus-strand strong-stop DNA; P-T, primer-template.
In this study we have used hybrid RNA/DNA substrates containing the PPT sequence to evaluate how recombinant Ty1 RT extends PPT-containing primers during initiation of plus-strand DNA synthesis and cleaves RNA/DNA duplexes near the site of initiation of plus-strand DNA synthesis. By working with a genetically tractable endogenous retroelement, we can gain insights into the mechanism by which RNase H activities of RTs generate a PPT primer.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Ty1 RT—The WT Ty1 recombinant RT (pEH Ty1 IN-RH-RH construct 2 described in Ref. 19) and mutant Ty1 RT versions derived from this construct contained short arrays of histidine residues at their amino termini and were expressed in Escherichia coli and purified by Ni

Polymerase source
Ty1 WT
Ty1 D468S
Ty1 D211E

Polymerase activity relative to WT
100
104
< 3.2

Polymerase and RNase H Activities Are Separable—We have demonstrated previously that WT recombinant Ty1 RT can synthesize DNA from a recessed primer as well as degrade RNA present in a RNA/DNA hybrid (19). Using a variety of mutant versions of Ty1 RT, we have shown that a mutation (D211E) in the active site of Ty1 RT cannot transspose, whereas a mutation in the active site of Ty1 RNase H, is still capable of polymerizing −ssDNA in vivo within virus-like particles but is blocked at the minus-strand transfer step and does not make +ssDNA (20). To examine the potential interactions of the two domains within RT, we generated mutant versions of the recombinant Ty1 RT protein, expressed them in E. coli, and purified them for in vitro study. As shown in Table I, the D468S RNase H mutant and the WT enzyme have approximately equal specific activity at incorporating dGTP in a homopolymer polymerization assay, whereas the D211E active site mutant is nearly devoid of polymerase activity. These same versions of the enzyme were then tested for their ability to cleave RNA/DNA hybrids. For this experiment, the RNA was a 28-mer sequence consisting of the Ty1 PPT, with flanking upstream sequence from the Ty1 3′ untranslated region and downstream sequence from the 3′ U3 segment (Fig. 1A). The RNA was 32P end-labeled so that cleavages could be visualized. The DNA portion consisted of a complementary oligonucleotide, which annealed either to a portion of (substrate B), or to the complete RNA (substrate C). As shown in Fig. 1B, Ty1 RNase H generates a pattern of cleavages that is distinct from E. coli RNase H (compare lanes 4–7 with lanes 10–13). As expected, the D468S mutant version lacks any detectable cleavage activity (lanes 14–19). On the other hand, the D211E mutant has a cleavage pattern identical to the WT enzyme, indicating that cleavage specificity is unrelated to the function of the polymerase domain (compare lanes 10–13 with lanes 22–25). Further, whereas the WT enzyme is able to make additional cleavages with substrate B, in the presence of dNTPs (lane 11), the D211E mutant cannot, indicating that these are polymerase-dependent cleavage events.

### Table I

| Polymerase activity of various recombinant Ty1 RTs | Homopolymer polymerase activity of various recombinant Ty1 RTs |
|--------------------------------------------------|---------------------------------------------------------------|
| Ty1 WT                                           | 100% activity equals 3.72 pmol of dGTP incorporated/μg of microgram enzyme/h. |
| Ty1 D468S                                        | 104 |
| Ty1 D211E                                        | < 3.2 |

**RESULTS AND DISCUSSION**

**Polymerase and RNase H Activities Are Separable**—We have demonstrated previously that WT recombinant Ty1 RT can synthesize DNA from a recessed primer as well as degrade RNA present in a RNA/DNA hybrid (19). Using a variety of mutant versions of Ty1 RT, we have shown that a mutation (D211E) in the active site of Ty1 RT cannot transspose, whereas a mutation in the active site of Ty1 RNase H, is still capable of polymerizing −ssDNA in vivo within virus-like particles but is blocked at the minus-strand transfer step and does not make +ssDNA (20). To examine the potential interactions of the two domains within RT, we generated mutant versions of the recombinant Ty1 RT protein, expressed them in E. coli, and purified them for in vitro study. As shown in Table I, the D468S RNase H mutant and the WT enzyme have approximately equal specific activity at incorporating dGTP in a homopolymer polymerization assay, whereas the D211E active site mutant is nearly devoid of polymerase activity. These same versions of the enzyme were then tested for their ability to cleave RNA/DNA hybrids. For this experiment, the RNA was a 28-mer sequence consisting of the Ty1 PPT, with flanking upstream sequence from the Ty1 3′ untranslated region and downstream sequence from the 3′ U3 segment (Fig. 1A). The RNA was 32P end-labeled so that cleavages could be visualized. The DNA portion consisted of a complementary oligonucleotide, which annealed either to a portion of (substrate B), or to the complete RNA (substrate C). As shown in Fig. 1B, Ty1 RNase H generates a pattern of cleavages that is distinct from E. coli RNase H (compare lanes 4–7 with lanes 10–13). As expected, the D468S mutant version lacks any detectable cleavage activity (lanes 14–19). On the other hand, the D211E mutant has a cleavage pattern identical to the WT enzyme, indicating that cleavage specificity is unrelated to the function of the polymerase domain (compare lanes 10–13 with lanes 22–25). Further, whereas the WT enzyme is able to make additional cleavages with substrate B, in the presence of dNTPs (lane 11), the D211E mutant cannot, indicating that these are polymerase-dependent cleavage events.
The PPT Primer of Ty1 Is Able to Initiate Plus-strand Synthesis in Vitro—In the yeast retrotransposon Ty1, plus-strand DNA synthesis is initiated at two sites located at the 5' boundary of the 3' long terminal repeat and near the middle of the pol gene in the integrase coding sequence (11, 21). The two plus-strand primers have an identical purine-rich sequence: ugguggua. We have tested the ability of recombinant Ty1 RT to use RNA primers containing this purine-rich sequence to support plus-strand synthesis in vitro. 32P-labeled RNA primers were annealed to a 61-nt-long minus-strand DNA template and extended in the presence of dNTPs with Ty1 recombinant RT. The sequences of the DNA template and of the −9/−1 and −9/+7 oligoribonucleotides are depicted. DNA versions of the two oligonucleotides were also used. C, the extension products were separated in a 12% denaturing polyacrylamide gel and analyzed by autoradiography. The first four lanes contain the extension products at 20 min, and the last four lanes contain the extension products of 40 min. Note that the RNA-containing products migrate differently than the corresponding DNA-containing products under these gel conditions.

To better understand the mechanism by which the RNase H activity of Ty1 RT creates the PPT primer, we have examined the polymerase-dependent hydrolytic activity of Ty1 RT during minus-strand synthesis. We have also evaluated the parameters that influence RNase H cleavage of the PPT-containing primers in a polymerase-independent manner by using model hybrid substrates obtained by annealing short PPT-containing RNA primers to longer minus-strand DNA molecules.

Polymerase-dependent RNase H Cleavage—We previously analyzed Ty1 RT polymerase-dependent RNase H cleavage using a 35-nucleotide RNA template/28-nt DNA primer as substrate (19). Our results suggested that the distance between the RNase H and polymerase active sites corresponded to the length of a 14-nt RNA/DNA heteroduplex. Here we anneal a 19-nt primer oligodeoxyribonucleotide, complementary to nt RT (data not shown). In a control experiment, we have found that the RNA primers were efficiently extended by T7 DNA polymerase (data not shown), demonstrating that the poor extension efficiency of the RNA primers is not because of a lower fraction of RNA annealed to the template. Our results are in line with observations that retroviral RTs also use PPT primers more efficiently than other RNA primers and that, in general, DNA primers are used more efficiently than RNA primers (22).

It is noteworthy that Schultz et al. (4) have shown that murine leukemia virus RT extends primers with 3' ends positioned beyond the PPT-U3 border inefficiently, in agreement with our observation that the −9/−1 RNA primer was extended more efficiently than the −9/+7 primer.
The 32P-labeled 61-nt RNA template and Ty1 PPT sequence. This generates a partially duplex PPT-arrows on the autoradiogram and along the RNA sequence. A weak extended (35 nt). 13 nt. of P-T, RT, dATP, dTTP, dGTP, and ddCTP; the primer was extended by amide gel and visualized by autoradiography. The cleavage products were separated in a 8% denaturing polyacrylamide gel and visualized by autoradiography. The solid arrow indicates an RNA with the correct PPT primer 3’-H11032 and again able to reach sequences outside the products were observed, because the RNase H active site was placed on a PPT sequence resistant to RNase H digestion. In the presence of dATP, dGTP, dTTP, and ddCTP, the primer was extended by 4 nt, 3’, incubation of P-T, RT, dATP, dTTP and ddGTP; the primer was extended by 13 nt, 4’, incubation of P-T, RT, and all four dNTPs: the primer was fully extended (35 nt). Lane L is a ladder obtained by alkaline hydrolysis of the RNA template. The major cleavage sites are indicated by solid arrows on the autoradiogram and along the RNA sequence. A weak cleavage site is indicated by an open arrow.

−10 to +9, with a 5’-end-labeled 61-nt RNA, which spans the Ty1 PPT sequence. This generates a partially duplex PPT-containing substrate (Fig. 3). When this duplex was incubated with RT, we observed major cleavage products between nt +4 and +5 and between nt −1 and −1 (Fig. 3, lane 1). It is noteworthy that RNase H cleavage between nt −1 and +1 generates an RNA with the correct PPT primer 3’-terminus. Two other cleavage products between nt +3 and +4 and between nt +2 and +3 were observed. Next we examined the effect of primer extension on RNase H cleavage. In the presence of dATP, dTTP, and ddGTP, RT extended the 3’-end of the primer by 4 nucleotides. The major RNase H cleavage site (Fig. 3, lane 2) remained at 14 nt from the elongated primer terminus, as would be expected if hydrolysis was directed by the polymerase active site binding at the 3’-terminus of the PPT. Cleavage to yield shorter products was not very efficient, probably because, after extension of the primer by 4 nt, the RNase H active site is placed on a PPT sequence resistant to RNase H digestion. In the presence of dATP, dGTP, dTTP, and ddCTP, the primer was lengthened by 13 nt. As before, a cleavage site was observed 14 nt from the fully advanced primer 3’-terminus between nt −9 and −10 (Fig. 3, lane 3). In addition, shorter products were observed, because the RNase H active site was again able to reach sequences outside the −9/−1 RNase H-resistant PPT primer region. Interestingly, the cleavage between nt −1 and +1 was still observed when the primer was lengthened by 13 nt. This cleavage site was also observed when the primer was extended to full length in the presence of all four dNTPs (Fig. 3, lane 4). This finding can be explained by the property of Ty1 RT to make a specific internal cut at this site. Indeed, Schultz et al. (5) have shown recently that murine leukemia virus RT is able to make specific internal cuts to generate the 3’-end of the PPT primer, and data of Powell and Levin (23) indicate that a HIV-1 PPT-containing RNA/DNA hybrid is specifically cleaved at the 3’-end of the PPT by HIV-1 RT. Further, using long PPT-containing RNA/DNA hybrids, we find that Ty1 RT is able to make a specific internal cleavage at the 3’-end of the Ty1 PPT sequence (see Figs. 1B and 7 below).

We next addressed whether RNA/DNA duplexes containing mutations in the PPT region were digested differently from the wild type substrate. For this purpose we used RNA templates with mutations in the PPT sequence and in the U-rich sequence upstream of the PPT (Fig. 4A). Both mutations have been shown to abolish plus-strand priming in vivo (11, 17). The 5’-end-labeled RNAs bearing the PPT or U-rich sequence mutations were annealed to 19-mer oligodeoxyribonucleotides with compensatory mutations to restore complementarity to nt −10 to +9 of the RNA template and were incubated with RT. As shown in Fig. 4B, the same sites were cleaved in the wild type and in the two mutant RNAs but differences in the intensity of cleavage at each site were observed. Of note, the band corresponding to the 3’-end of the PPT was the strongest in each case, and least influenced by the PPT or upstream mutations.

Weak bands on the autoradiogram shown in Fig. 3 indicate that some sites in the −9/−1 PPT primer region are not completely resistant to RNase H cleavage. Several weak cleavage sites are observed within the PPT sequence. The most visible of these weak cleavage sites is between nt −5 and −6 (open arrow in Fig. 3, lane 2). To test if this cleavage site is directed by the polymerase active site binding at the 3’-prime terminus, a 28-nt oligodeoxyribonucleotide primer was annealed to the RNA template so that the RNase H active site of RT was positioned between nt −5 and −6, i.e. 14 nt downstream of the polymerase active site bound at the 3’-prime terminus. After incubating this substrate with RT, a cleavage site between nt −5 and −6 was indeed observed for the wild type and mutant RNAs (Fig. 4C). The U-box mutant behaved similarly to the wild type. For the PPT mutant duplex, more extensive cleavage was observed and strong cuts occurred between nt −4 and −5 and −3 and −4 on each side of a G to C mutation in the PPT. This result is in line with the idea that mutations changing purines to pyrimidines within the −9/−1 PPT region increase the sensitivity of this sequence to RNase H hydrolysis.

**RNase H Cleavage of Short Oligoribonucleotides Hybridized to Minus-strand DNA**—To test RNase H cleavage of RNA primers annealed near the initiation site of plus-strand DNA synthesis, a series of short oligoribonucleotides containing the PPT sequence were hybridized to a 61-nt minus-strand DNA spanning the PPT region. The hybrid substrates were incubated with RT, and the digestion products were examined on a 20% polyacrylamide gel. The effect of length of the PPT-containing RNA primers on RNase H cleavage was evaluated using a set of oligoribonucleotides (−18/−1, −13/−1, −12/−1, −11/−1, −10/−1, −9/−1) which had their 3’-termini at position −1 but differed in the length extending upstream of the plus-strand initiation site. The result of this experiment is shown in Fig. 5. The shortest −9/−1 RNA primer remained mostly undigested. The other primers were cleaved into fragments of 8–10 nt. For the −18/−1, −12/−1, −11/−1, and −10/−1 primers, the RNase H cleavages occurred in the PPT upstream of the plus-strand initiation site (Fig. 5, A and B). This confirms the result shown above (Fig. 4) that RNase H is able to cleave RNA within the PPT sequence of arrested duplexes. The −18/−1 primer was cleaved into a 8-nt fragment, but, in this case, the cleavage site was outside of the PPT. These cleavages likely result from an RNA 5’-end-directed mechanism, which places the active site of Ty1 RT RNase H 8–10 nt from the 5’-end of the RNA. Our
results are consistent with those found for HIV-1 in similar arrested complexes (3–5).

We next tested how substrates spanning the plus-strand initiation site were cleaved. As shown in Fig. 5A, the two primers –9/+3 and –9/+7 that span the plus-strand initiation site were cleaved specifically to generate a product 9 nt in length with the correct PPT primer 3′/H11032/terminus at position 3′/H11002/1. A mutant –9/+3 primer with two G to C mutations in the PPT did not yield the 9-nt fragment with the correct PPT primer terminus (Fig. 5C, lane –9/+3 Mut PPT) but was digested at several sites, as was a non-PPT-containing substrate (Fig. 5C, lane –12/–1). In the experiment illustrated in Fig. 6, we have assessed the effect of mutations in U3 very close to the U3-PPT border on RNase H cleavage of –9/+7 primers. Three mutants were used. In MUT1, the wild type +1/+7 sequence uguuggga was changed to ugauga. In a previous report (17), we showed that this mutation had no effect on plus-strand priming in vivo. In MUT2, the +1/+7 sequence was changed to the sequence found 3′ of the inactive PPT3 sequence located in the middle of the Ty1 genome. In MUT3, the +1/+7 sequence was changed to the sequence found 3′ of the active central PPT2 (17). As shown in Fig. 6, the mutant primers were digested similarly to the wild type primer and generated a product 9 nt in length with a correct PPT 3′/H11032/terminus at position 3′/H11002/1. This indicates that the mutations in U3 do not affect the specificity of cleavage at the 3′/H11032/terminus of the PPT and is consistent with the observation of Champoux (2) that sequences downstream of the PPT have no detectable effect on priming specificity.

Internal RNase H Cleavage in Long RNA/DNA Hybrids—Results discussed above suggest that a specific internal cleavage might generate the correct 3′ end of the PPT primer at position –1 in the absence of positioning of the polymerase active site of RT by the 5′ end of an RNA or by the 3′ end of

![FIG. 4. RNase H cleavage of 61-nt wild type, U-box mutant, and PPT mutant RNAs (the mutations are underlined and indicated by bold letters in the RNA sequence; panel A). The 32P-labeled 61-nt RNA templates were annealed with 19-nt (B) or 28-nt (C) complementary DNA primers. The RNA/DNA heteroduplexes and the Ty1 recombinant RT were incubated for 6 min with Ty1 RT to induce RNase H cleavage. The cleavage products were separated in 8% (B) or 12% (C) denaturing polyacrylamide gel and visualized by autoradiography.](https://www.jbc.org/doi/10.1074/jbc.M102126200)

![FIG. 5. Cleavage of short RNA primers containing or lacking the PPT sequence. The oligoribonucleotides used to generate hybrid RNA/DNA duplexes for RNase H cleavage are shown above the complementary 61-nt minus-strand DNA. Arrows indicate the cleavage site deduced from the digestion patterns shown in panels A, B, and C. The 32P-labeled oligoribonucleotides annealed with the 61-nt minus-strand DNA were treated with Ty1 RT. Digestion products were separated in a 20% polyacrylamide gel and visualized by autoradiography. In panel A, the last five lanes contain the indicated substrates left untreated. In panels B and C, only the digested products are shown.](https://www.jbc.org/doi/10.1074/jbc.M102126200)
polyacrylamide gel because of their base composition. The sequences of the RNA/DNA duplexes are indicated. Digestion of all RNA/DNA duplexes by Ty1 RNase H generates the same product, a 9 nt fragment. The nondigested oligoribonucleotides are all 16 nt in length but migrate differently on the 20% polyacrylamide gel because of their base composition.

FIG. 6. Cleavage of short –9/+7 RNA primers with mutations in the +1/+7 sequence. The sequences of the RNA/DNA duplexes are indicated. Digestion of all RNA/DNA duplexes by Ty1 RNase H generates the same product, a 9 nt fragment. The nondigested oligoribonucleotides are all 16 nt in length but migrate differently on the 20% polyacrylamide gel because of their base composition.

newly synthesized DNA. We therefore examined the digestion pattern of three different RNA/DNA duplexes containing a 5′ end-labeled 61-nt RNA annealed to a complementary 61-nt DNA. One duplex contained the wild type PPT sequence; the two others had mutations in the PPT or in the U-box (the mutations shown in Fig. 4). Incubation of these duplexes with Ty1 RT generated several 5′ end-labeled fragments, 26–55 nt in size (Fig. 7). Because 5′ end-directed cleavage can generate fragments no longer than 8–10 nt, cleavage products longer than 10 nt represent internal cleavage of the 61-nt duplex. Cleavage of the wild type duplex generated fragments with 3′ ends at positions +1, −1, −2, −4, −10, −11, and −13 to −16. Notably, internal cleavages can generate the 3′ and 5′ ends of the PPT, i.e., the fragment with 3′ end at position −1 has the correct 3′ end of the PPT and cleavage generating a 3′ end at position −10 generates a downstream fragment with the correct 5′ end of the PPT primer. Thus, if internal cleavages that generate the 3′ and 5′ ends of the PPT occur on the same molecule, the resulting −9/−1 RNA primer could be used to initiate correct plus-strand synthesis. Alternatively, a fragment with only one internal cleavage generating the 5′ end of the PPT could be cleaved specifically at position −1 by the 5′ end-directed mechanism. In that case, plus-strand synthesis could also be initiated correctly from this primer. In the duplex containing the U-box mutation, there was still specificity for cleavage at position −1, whereas the PPT mutant was only weakly digested at positions +1 and −1. For both mutants, there were no cleavages to produce fragments with 3′ end at positions −10. The main cleavage produced a fragment with a 3′ end at position −14. Thus, in mutant duplexes, internal cleavages on the same molecule would not generate the correct −9/−1 primer, but rather generate longer primers extending beyond position −9. Based on data presented above (Fig. 5), these longer primers would be subjected to 5′ end-directed cleavage by RNase H and would be cleaved within the PPT sequence. We speculate that the inability of RT to properly recognize and/or extend primers with incorrect termini could explain in vivo plus-strand synthesis inhibition in PPT and U-box mutants.

CONCLUSION

To generate the primer for plus-strand DNA synthesis, the RNase H activity of retrovirus and retrotransposon RTs must make specific cleavages at the 3′ and 5′ ends of a RNA/DNA duplex. We have used model RNA/DNA duplexes spanning the PPT region of the Ty1 retrotransposon to investigate some of the parameters affecting Ty1 RNase H cleavage specificity. Our results indicate that the PPT sequence is relatively resistant to RNase H cleavage in vitro, although, in arrested complexes, when the RNase H active site interacts with residues within the PPT, hydrolysis of this sequence can occur. It has been suggested that the low efficiency of cleavage of the PPT stems from the structure of the purine-rich duplex, i.e., RNA/DNA duplexes with a narrow minor groove tend to be resistant to cleavage, whereas duplexes with a minor groove width of 9–10 Å are optimally digested by RNase H (24–28). Recently, a crystal structure of HIV-1 reverse transcriptase in complex with a polypurine tract RNA/DNA has been published by Sarafianos et al. (29). The most striking structural feature of the PPT bound to RT is a departure from Watson-Crick base pairing with a melting of the first 2 bp of the PPT. Sarafianos et al. (29) suggest that this “unzipping of the PPT” may play a role in the resistance of the PPT to RNase H. It remains to be seen if a similar unzipping of the PPT occurs in other retroelements.

In three instances we have observed specific cleavages at the...
5’ or 3’ end of the PPT. First, PPT RNA primers with correct 5’ end but with a 3’ extension spanning the plus-strand initiation site are cleaved specifically to produce a PPT primer with the correct 3’ end (Fig. 5). Second, in long RNA/DNA duplexes, specific internal cleavages were able to produce fragments with correct 3’ or 5’ ends (Figs. 1 and 7). Third, with long RNA and short DNA duplexes, in which the overlap includes the 3’ end of the PPT, correct 3’ end formation was observed (Fig. 1). It is striking that, in the wild type sequence, but not in two mutant sequences, the internal cleavage creating the correct 5’ end is one of the most efficient, whereas correct 3’ cleavage occurs in all settings. This suggests that some structural features of the wild type PPT RNA/DNA duplex are preferentially recognized and cleaved by the active site of RNase H. It is likely that neighboring sequences flanking the PPT play a role in the recognition. Indeed, in several retroelements, U-rich sequences immediately 5’ of the PPT have been found to be important for their replication (14–18). In the case of Ty1, we have shown that mutations within the U-rich sequence upstream of the 3’ PPT inhibit +ssDNA synthesis (17). By analogy to RNA/DNA −(5’rA:3’dT)n or DNA/DNA−(5’da:A:3’dT)n duplexes containing several As which form a rigid structure (26), it is possible that the U-rich RNA/DNA duplex−(5’rU:3’dA)n just upstream of the PPT sequence could also adopt a rigid conformation. We suggest that the junction between this rigid U-rich region and the PPT sequence is recognized and cleaved specifically by the RNase H.

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REFERENCES
1. Telesnitsky, A., and Goff, S. P. (1997) in Retroviruses (Coffin, J. M, Hughes, S. H., and Varmus, H. E., eds) pp. 121–160, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Champeux, J. J. (1993) in Reverse Transcriptase (Skalka, A. M., and Goff, S. P., eds) pp. 103–117, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
3. Wawrzkiewicz, M., Balakrishnan, M., Palanippan, C., Fay, P. J., and Bambara, R. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11978–11983
4. Schultz, S. J., Zhang, M., Kelleher, C. D., and Champeux, J. J. (1999) J. Biol. Chem. 274, 34547–34555
5. Schultz, S. J., Zhang, M., Kelleher, C. D., and Champeux, J. J. (2000) J. Biol. Chem. 275, 32299–32309
6. Rausch, J. W., Bona-Le Grice, M. K., Nymark-McMahon, M. H., Miller, J. T., and Le Grice, S. F. J. (2000) J. Biol. Chem. 275, 13879–13887
7. Kati, W. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992) J. Biol. Chem. 267, 25988–25997
8. DeStefano, J. J., Mallabar, L. M., Fay, P. J., and Bambara, R. A. (1994) Nucleic Acids Res. 22, 3793–3800
9. Götze, M., Fackler, S., Hermann, T., Perola, E., Cellai, L., Gross, H. J., Le Grice, S. F., and Heumann, H. (1995) EMBO J. 14, 833–841
10. Charneau, P., Alizon, M., and Clavel, F. (1992) J. Virol. 66, 2814–2820
11. Heyman, T., Agoutin, B., Friant, S., Wilhelm, F. X., and Wilhelm, M. L. (1995) J. Mol. Biol. 253, 291–303
12. Steter, S. R., Rausch, J. W., Guo, M. J., Burnham, J. P., Boone, L. R., Waring, M. J., and Le Grice, S. F. (1999) Biochemistry 38, 3656–3667
13. Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000) Cell 101, 173–185
14. Noud, R. J., Al-Kaff, E. S., Turner, D. S., and Covey, S. N. (1998) J. Biol. Chem. 273, 32568–32575
15. Robson, N. D., and Telesnitsky, A. (1999) J. Virol. 73, 948–957
16. Robson, N. D., and Telesnitsky, A. (2000) J. Virol. 74, 10293–10303
17. Wilhelm, M., Heyman, T., Boutabout, M., and Wilhelm, F. X. (1999) Nucleic Acids Res. 27, 4547–4552
18. Ilyinskii, P. O., and Desrosiers, R. C. (1998) EMBO J. 17, 3766–3774
19. Wilhelm, M., Boutabout, M., and Wilhelm, F. X. (2000) Biochem. J. 348, 337–342
20. Uzun, O., and Gabrieli, A. (2001) J. Virol. 75, 6337–6347
21. Pochart, P., Agoutin, B., Rousset, S., Chanet, R., Doroszkiewicz, V., and Heyman, T. (1998) Nucleic Acids Res. 26, 3513–3520
22. Fuentes, G. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1995) J. Biol. Chem. 270, 28169–28176
23. Powell, M. D., and Levin, J. G. (1996) J. Virol. 70, 5288–5296
24. Fedoroff, O., Salazar, M., and Reid, B. R. (1993) J. Mol. Biol. 233, 509–523
25. Fedoroff, O. Y., Ge, Y., and Reid, B. R. (1997) J. Mol. Biol. 269, 225–239
26. Han, G. W., Kopka, M. L., Cancio, D., Grzeskowiak, K., and Dickerson, R. E. (1997) J. Mol. Biol. 269, 811–826
27. Xiong, Y., and Sundaralingam, M. (1998) Structure 6, 1493–1501
28. Xiong, Y., and Sundaralingam, M. (2000) Nucleic Acids Res. 28, 2171–2176
29. Sarafianos, S. G., Das, K., Tantillo, C., Clark, A. D., Jr., Ding, J., Whitecomb, J. M., Boyer, D. L., Hughes, S. H., and Arnold, E. (2001) EMBO J. 20, 1449–1461
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