Using hybrid RNA/DNA substrates containing the polypurine tract (PPT) plus-strand primer, we have examined the interaction between the Ty1 reverse transcriptase (RT) and the plus-strand initiation complex. We show here that, although the PPT sequence is relatively resistant to RNase H cleavage, it can be cleaved internally by the polymerase-independent RNase H activity of Ty1 RT. Alternatively, this PPT can be used to initiate plus-strand DNA synthesis. We demonstrate that cleavage at the PPT/DNA junction occurs only after at least 9 nucleotides are extended. Cleavage leaves a nick between the RNA primer and the nascent plus-strand DNA. We show that Ty1 RT has a strand displacement activity beyond a gap but that the PPT is not efficiently re-utilized in vitro for another round of DNA synthesis after a first plus-strand DNA has been synthesized and cleaved at the PPT/U3 junction.

During reverse transcription of the plus-strand RNA genome of long terminal repeat retroelements, the RNase H activity of reverse transcriptase (RT) degrades the genomic RNA while the polymerase activity synthesizes minus-strand DNA (Fig. 1) (1–7). For plus-strand DNA synthesis, RNase H generates the polypurine tract (PPT) primer and cleaves the minus-strand tRNA primer. The manner in which this enzyme carries out these multiple functions is not well understood, but two modes of RNase H activity can be distinguished as follows: (i) a polymerase-dependent mode that accompanies DNA synthesis and is positioned by the polymerase active site binding to the recessed nascent DNA 3’ end and (ii) a polymerase-independent mode that occurs without DNA synthesis. Wisniewski et al. (5–7) have suggested that the polymerase-independent mode of RNA cleavage by HIV-1 RT is oriented by the polymerase domain binding to the recessed 5’ end of RNA fragments paired to the DNA. Both modes of RNase H activity are necessary to remove the RNA after minus-strand DNA synthesis. During DNA synthesis, the RNA template is only partially digested because of a greater rate of polymerization versus RNA template cleavage (8–10). This implies that polymerase-dependent RNase H activity is not sufficient to completely degrade all of the template RNA. The fragments, which remain bound to the minus-strand DNA, must be digested further by the polymerase-independent activity of RNase H. Specific purine-rich fragments called PPTs are more resistant to RNase H digestion and function as RNA primers for plus-strand DNA synthesis (1, 2). Precise cleavage of the RNA at the PPT/U3 junction by RNase H and extension of the PPT primer by the polymerase activity of RT are critical for production of integration-competent elements. We previously showed that a recombinant Ty1 RT can recapitulate in vitro key processes that control proper replication of the Ty1 element in vivo (11). Using wild type RNA/DNA duplexes, we showed that the RNase H activity of Ty1 RT is able to digest the template RNA at the PPT/U3 border (i.e. between nt −1 and +1, see Fig. 2 for numbering) during elongation of minus-strand DNA. The polymerase-independent RNase H activity cleaves the RNA into fragments of 8–10 nt in size. In this paper, we show that the RNA can be further degraded to generate smaller cleavage products of 6 nt in length.

Cleavage of the PPT sequence between nt −1 and +1 generates the correct PPT 3’ terminus, which can be used to initiate plus-strand DNA synthesis. At some time during plus-strand DNA synthesis, the PPT primer must be precisely cleaved at the PPT/DNA junction. We demonstrate here that extension of at least 9 nt is necessary to allow cleavage of the PPT/DNA junction. This leaves a nick between the RNA primer and the nascent plus-strand DNA. Using model heteroduplexes that mimic this nicked structure, we tested whether the Ty1 enzyme is able to elongate the cleaved PPT primer in the presence of downstream DNA. Our results indicate that reinitiation at the nick is highly inefficient. This result cannot be explained by a lack of Ty1 RT strand displacement activity, because extension of the primer and displacement of the downstream DNA does take place when gaps are introduced between the PPT primer and the downstream DNA.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Ty1 RT—The wild-type Ty1 recombinant RT and the D468S RNase H active site mutant version (12) of the wild-type construct contained six histidine residues at their amino termini. They were expressed in Escherichia coli and purified by Ni²⁺-nitroloacetic acid-agarose (Qiagen) affinity chromatography as described previously (13).

RNA and DNA Oligonucleotides and RNA Labeling—The RNA oligonucleotides were chemically synthesized in our laboratory with a DNA-RNA Applied Biosystem Synthesizer (Model 392) or purchased from Thermo Hybaid, the Interactiva Division (Ulm, Germany). The DNA oligodeoxyribonucleotides were purchased from Thermo Hybaid. The chemically synthesized RNAs were labeled at their 5’ termini with ³²P using [γ-³²P]ATP and T4 polynucleotide kinase.

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Extension and Cleavage of the Ty1 Plus-strand Primer

**Results and Discussion**

Interaction between Ty1 RT and the Plus-strand Primer/Template Initiation Complex—In Ty1, the primer for plus-strand DNA synthesis is a 9-nt oligoribonucleotide corresponding to the Ty1 PPT oligoribonucleotide. A. the sequence of the DNA template and three RNA primers (−9/−1, −9/+3, and −9/+7) used in B. Ribonucleotides are shown in lowercase letters, and deoxyribonucleotides are shown in uppercase letters. The star at the 5′ end of the RNA primer represents the 32P label. B, the template hybridized to the −9/−1 primer shown in A was incubated with wild type Ty1 RT (RH +) for 0.5–8 min. C, the template hybridized to primer −9/−1, −9/+3, or −9/+7 was incubated with wild type Ty1 RT (RH +) or RNase H− (RH−) Ty1 RT for 10 min.

To prepare the internally labeled 9R/9D primer, the 9R primer was hybridized to the primer template RNA in a buffer containing 50 mM Tris-HCl, 1 mM MgCl2, 15 mM NaCl, and 8 mM β-mercaptoethanol. The mixture was heated at 90°C for 1 min and then incubated for 10 min at 70°C, 10 min at 50°C, and 10 min at room temperature. After the addition of the Ty1 RT sample buffer containing 25 mM EDTA, the mixture was incubated with Ty1 RT (11). However, we observed partial cleavage of the PPT primer beyond the 5′ end of the RNA. Although Wisniewski et al. (5–7) have proposed that ∼1 cleavage occurs rapidly with primers containing different 3′ extensions beyond +1, whereas a second cut between −3 and −4 occurs as well. Both cuts are absent when an RNase H minus (RH−) Ty1 RT is used (Fig. 2C). Wisniewski et al. (5–7) have proposed that similar cleavage events, observed using HIV-1 RT, represent a polymerase-independent mode of RNase H activity positioned by the RT binding at the 5′ end of the recessed RNA. Although we cannot determine from our data the positioning responsible for this cleavage event, it appears that the internal cleavage of the PPT does occur in our in vitro system.

In the polymerization mode, the polymerase domain of RT

**Fig. 1.** Simplified model for retroelement reverse transcription. Thin lines represent the template mRNA; thick lines with arrowheads represent the newly synthesized DNA. A, the minus-strand RNA primer anneals to the primer binding site (pbs) of the genomic RNA. B, the minus-strand strong-stop DNA is synthesized. The 5′ end of the genomic RNA is degraded by RNase H. C, minus-strand strong-stop DNA is translocated to the 3′ end of the genomic RNA. D, the translocated nascent minus-strand DNA is extended. The genomic RNA template is degraded and removed with the exception of the PPT fragments. E, plus-strand DNA synthesis is initiated from the PPT fragment. F, the nascent plus-strand strong-stop DNA is translocated to the 3′ end of the minus-strand DNA. G, reverse transcriptase completes DNA synthesis.

**Fig. 2.** Internal RNase H cleavage of the PPT primer. A, the sequence of the DNA template and three RNA primers (−9/−1, −9/+3, and −9/+7) used in B. Ribonucleotides are shown in lowercase letters, and deoxyribonucleotides are shown in uppercase letters. The star at the 5′ end of the RNA primer represents the 32P label. B, the template hybridized to the −9/−1 primer shown in A was incubated with wild type Ty1 RT (RH +) for 0.5–8 min. C, the template hybridized to primer −9/−1, −9/+3, or −9/+7 was incubated with wild type Ty1 RT (RH +) or RNase H− (RH−) Ty1 RT for 10 min.

three pyrimidines at positions −2, −5, and −9. To mimic the plus-strand Ty1 primer-template initiation complex, a 9-nt PPT oligoribonucleotide corresponding to the Ty1 PPT was annealed to a 35-nt DNA template containing flanking upstream sequences from the Ty1 untranslated region and downstream sequences from the Ty1 U3 region (Fig. 2A). We have previously shown that the 9-nt PPT primer is relatively resistant to RNase H cleavage when this hybrid substrate is incubated with Ty1 RT (11). However, we observed partial cleavage of the PPT primer 6 nt from the 5′ end of the primer between nt −3 and −4 (Fig. 2B). As shown in Fig. 2C, a −1/+1 cleavage occurs rapidly with primers containing different 3′ extensions beyond +1, whereas a second cut between −3 and −4 occurs as well. Both cuts are absent when an RNase H minus (RH−) Ty1 RT is used (Fig. 2C). Wisniewski et al. (5–7) have proposed that similar cleavage events, observed using HIV-1 RT, represent a polymerase-independent mode of RNase H activity positioned by the RT binding at the 5′ end of the recessed RNA. Although we cannot determine from our data the positioning responsible for this cleavage event, it appears that the internal cleavage of the PPT does occur in our in vitro system.

In the polymerization mode, the polymerase domain of RT
binds the RNA primer at its 3' end. Because 3' binding means progression of replication while internal cleavage could generate incorrect termini, it was of interest to analyze the relative efficiency of these two outcomes. We annealed a 48-nt template including 14 nt from the Ty1 untranslated region and 25 nt from the Ty1 U3 downstream region to a 9R/9D chimeric primer containing the nine RNA residues of the PPT and nine DNA residues (internally labeled with radioactive dGTP at positions +2, +5, and +6 as described under “Experimental Procedures”) complementary to 9 nt of the U3 sequence. This model substrate is a probable intermediate during plus-strand DNA replication. The primer-template was incubated with either wild type or RNase H− Ty1 RT in the presence of all four dNTPs (Fig. 3). With the RNase H− enzyme, the major elongation product was a 9R/25D band corresponding to the run-off species (Fig. 3, lanes 3 and 4). This product is not prominent, in line with previous data from our laboratories and others (for example, see Ref. 17) that RNA primers are not used efficiently in vitro. The pattern obtained with the wild type enzyme, which has both RNase H and polymerase activity, is more complex because elongation is superimposed upon primer removal and internal primer cleavage. In this case, no 9R/25D run-off band was observed (Fig. 3, lanes 1 and 2), indicating the marked propensity of cleavage of the primer RNA by RNase H. Instead, a 25D band is present corresponding to the run-off product with the primer removed. Two stronger low molecular weight bands (9D and 3R/9D) were also observed. These bands probably result from the polymerase-independent activity of Ty1 RT, which cleaves the chimeric 9R/9D primer at the PPT/U3 junction (9D band) and within the RNA moiety of the chimeric 9R/9D primer (3R/9D band). The 9D band could also be generated during elongation of plus-strand DNA by the polymerase-independent RNase H activity. Thus, there are multiple outcomes depending on the nature of the binding of RT to its substrate. The enzyme, which binds to the 3’ end of the primer, is able to initiate plus-strand synthesis. With the wild type enzyme, the elongated product is rapidly cleaved at the DNA/RNA interface (i.e. the PPT/U3 junction). A large proportion of the enzyme, however, prefers to cleave the RNA within the PPT sequence. In the case of Fig. 3, it appears that only ~8% of the product is elongated, whereas ~87% is cleaved. If this were to occur in vivo, it would limit the production of plus-strand strong-stop DNA.
Processing of the Initially Synthesized Plus-strand DNA—

Once the PPT is used as a primer, under what circumstances is it removed from the 5′ end of the nascent plus-strand? To follow the processing of the initially synthesized plus-strand DNA, a −9/−1 PPT primer was annealed to different length templates ranging from P/T7 to P/T16 (Fig. 4A) so that elongation products containing 7, 9, 12, 13, 14, and 16 oligodeoxynucleotides could be obtained after incubation of the primer-template with RT and dNTPs. The primer was not labeled but [α-32P]dGTP was included in the reaction mixture. This allowed visualization of the elongated primer and of the newly synthesized DNA released after cleavage by RNase H (Fig. 4B). Run-off bands of the expected length were obtained for all of the primer-templates used as well as bands with n + 1 residues added to the primer, attributable to the non-templated base addition by the terminal transferase activity of Ty1 RT (18). Bands containing n and n + 1 deoxyribonucleotides seen in Fig. 4B, lanes 2–6, correspond to cleaved newly synthesized DNA. Alternatively, these bands could correspond to the stoppage of extension, so we carried out a parallel experiment using an RNase H− RT. No bands containing n or n + 1 deoxyribonucleotides were observed with the RNase H− RT, confirming the identity of the cleaved band (for example, see Fig. 6 where no 16D band is observed in lane 2 when the primer is elongated by the RNase H− enzyme).

The length of the cleaved band indicates that cleavage by RNase H is precise and occurs exactly at the RNA/DNA junction. Cleaved products were observed with primer-templates P/T9 to P/T16 but not with P/T7, which allows the addition of 7 nt to the primer. This finding suggests that the RNA primer is efficiently cleaved from the growing plus-strand DNA only after 9 or more nucleotides have been added to the primer.

To confirm that the n + 1 bands represent non-templated base addition, we carried out extension with P/T16 in the presence or absence of dideoxynucleotides (Fig. 4B, lanes 6 and 7). In one reaction (lane 6), the four dNTPs were included in the nucleotide mixture, whereas in the second reaction (lane 7), ddCTP was included along with dATP, dTTP, and dGTP. A comparison of these two lanes shows that the run-off bands with n + 1 residues added to the primer and the cleaved fragment of DNA containing n + 1 residues were not observed in lane 7 because the last residue of the newly synthesized DNA is a dideoxynucleotide, which cannot be elongated by terminal transferase.

To confirm that cleavage does not occur after 7 nt have been added to the PPT primer, we compared elongation and cleavage on two primer-templates, P/T7 and P/T16, using wild type and RNase H− Ty1 RT (Fig. 5). In these experiments, the primer RNA is 5′ end-labeled. With P/T7, extended product accumulates with time and the pattern of elongation is the same regardless of the presence or absence of RNase H activity. For P/T16, the accumulation of product with time is observed in the absence of RNase H. In its presence, only a low and constant level of product is observed, consistent with an equilibrium between extension and cleavage of the labeled primer.

These results demonstrate that RNase H does not cleave a PPT substrate extended by only 7 bases. It also suggests that the wild type enzyme remains bound to the 3′ end of the elongated primer, blocking the cleavage site. Otherwise another molecule of RT could bind to the 5′ end of the primer and cleave at the PPT/U3 junction. Separate studies of Ty1 RT turnover after extending a model substrate indicate a marked propensity of this enzyme to remain bound to its extended substrate. In contrast, when 9–16 nt are extended, cleavage

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reaction product was then mixed with wild type enzyme. Both samples were then separated on a sequencing gel. As shown in Fig. 6, lane 3, after the addition of the wild type enzyme, 70% of the product synthesized by the RNase H− enzyme is cleaved by the RT-associated RNase H activity of the wild type enzyme. These data show that cleavage can indeed be accomplished by a RT molecule that is not involved in DNA synthesis and are consistent with results obtained with HIV-1 RT (19). This finding suggests a model in which a second RT molecule binds to the 5′ end of the extended primer and is possibly recruited to that location to remove the RNA primer when the growing strand of the newly synthesized DNA has been sufficiently extended. Although we do not know that a second RT molecule is involved in the in vitro cleavage of Ty1 replication intermediates within virus-like particles, 15–30 RT molecules are probably present within a virus-like particles and could take part in such reaction (20).

**Extension of the Cleaved PPT Primer at a Nick**—We have observed that initiation of plus-strand DNA synthesis is rapidly followed by the cleavage of the PPT primer between nt −1 and nt +1. This results in a nick between the primer and the nascent plus-strand DNA. We next asked whether this cleaved RNA could be re-utilized as a primer for a second round of plus-strand synthesis in the presence of downstream DNA. A model heteroduplex was generated in which a template DNA was annealed to the −9/−1 RNA primer with or without +1/+32 downstream DNA (Fig. 7A). These substrates were incubated with Ty1 RT in a reaction mixture containing [α-32P]dGTP. In the absence of downstream DNA, the primer is readily elongated (Fig. 7B). In contrast, the primer is very poorly extended in the presence of downstream DNA. The band indicated by a small arrow is a 32-nt downstream DNA that has been elongated by one radioactive dGTP by the terminal transferase activity of Ty1 RT. A lack of strand displacement activity of Ty1 RT cannot explain our observed result since extension and strand displacement do take place when gaps of 4 or 8 nt are introduced between the primer and the downstream DNA (Fig. 7B, lanes 4–6, large black and white arrow). Thus, it appears that the Ty1 RT requires a recessed primer end or at least a gap beyond the primer end to initiate polymerization.

Strand displacement activity of Ty1 RT was confirmed using a primer-template with a 28-nt gap between the primer and the downstream DNA. In this experiment, a 5′ end-labeled RNA primer was annealed to the template DNA with or without excess downstream DNA. We verified that all of the template DNA was bound to downstream DNA (data not shown). Polymerization was followed as a function of time (Fig. 8B). An examination of the time course reveals that in the presence of downstream DNA (Fig. 8B, lanes 5–7), a pause in elongation of the primer is observed at the position where RT encounters the downstream DNA. Once the downstream DNA has been displaced, a run-off band of the expected size is produced. This indicates that strand displacement can be efficient and occurs when RT is actively synthesizing a new strand of DNA.

**CONCLUSION**

One important function of the RNase H activity associated with retroviral or long terminal repeat retrotransposon reverse transcriptases is to create the PPT primers for plus-strand DNA synthesis. Using RNA/DNA duplexes containing the PPT, we previously showed that a recombinant Ty1 RT is able to make specific internal cleavages that could generate the plus-strand primer with correct 5′ and 3′ ends. The PPT is relatively resistant to RNase H cleavage in vitro, but we show here that it can be cleaved internally by the polymerase-independent
FIG. 7. Extension of the PPT primer in the presence of downstream DNA. A, sequence of the primer-template with or without downstream DNA. B, template DNA annealed to the 5'-RNA primer with or without a 32-nt downstream DNA was incubated for 4, 10, or 30 min with Ty1 RT in a reaction mixture containing [α-32P]dGTP. In the absence of downstream DNA, a run-off band is observed (lanes 1–3). The primer is very poorly extended in the presence of downstream DNA (lanes 3–5). The band indicated by an arrow is the 32-nt downstream DNA, which has been elongated by one radioactive dGTP by the terminal transferase activity of Ty1 RT. C, autoradiography of the reaction products obtained after 20-min incubation of the primer-template annealed to downstream DNA molecules of 32, 28, or 24 nt. Run-off bands are detected when gaps of 4 or 8 nt are introduced between the primer and the downstream DNA (large black and white arrow). The bands indicated by small arrows are the 32-, 28-, and 24-nt downstream DNA molecules that have been elongated by one radioactive dGTP by the terminal transferase activity of Ty1 RT.

FIG. 8. Strand displacement activity of Ty1 RT. A, sequence of the primer-template with or without downstream DNA. 5' end-labeling of the primer is indicated by a star. A 5-fold molar excess of template DNA was mixed with 5' end-labeled primer. Downstream DNA was present in 5-fold molar excess over template. This ensures that all of the template molecules are bound to the downstream DNA B, template DNA annealed to the 5' end-labeled DNA primer with or without downstream DNA was incubated for 0.5–20 min with Ty1 RT. A run-off band is observed for both substrates. In the presence of downstream DNA, a pausing site is observed.
RNase H activity of Ty1 RT. Similar in vitro cleavages have been observed with HIV-1 (5–7) and Ty3 RT (21) and have been attributed to positioning by the polymerase domain of RT binding at the 5' end of the recessed RNA.

In this work, we have further examined the interaction between Ty1 RT and the plus-strand initiation complex. We have also investigated the interplay between progress of plus-strand synthesis and primer removal. We show that Ty1 RT is able to bind a recessed RNA primer on a longer template molecule, leading to either 3' extension or primer cleavage. We see that subsequent cleavage of the extended product is efficient but that extension of the cleaved product is not observed. The lack of extension of the PPT in the presence of a nick suggests that these structures are not efficiently bound by RT in our in vitro system.

Plus-strand DNA synthesis is initiated by the binding of an RT molecule to the 3' end of the PPT. Concomitantly with DNA synthesis, the elongated product is cleaved at the PPT/U3 junction to remove the primer. We show that this occurs after nine or more DNA residues have been added to the primer. Our data suggest that this cleavage can be accomplished by an RT molecule that is not involved in DNA synthesis and that only after the enzyme bound to the growing DNA strand is far enough away can a second molecule bind and cleave the RNA/DNA junction. Alternatively, although we have previously shown that during minus-strand DNA synthesis the polymerase-dependent cleavage remains at a fixed distance of 14 nt from the 3' end of the elongated molecule, the distance between the polymerase and RNase H active sites may be more dynamic in the case of the PPT sequence, allowing RNase H cleavage after only the addition of 9 nt.

Finally, we have shown that, although Ty1 RT has a strand displacement activity, the PPT is not efficiently re-utilized in vitro for another round of DNA synthesis after a first strong-stop DNA synthesis, the PPT is re-used to initiate a second round of synthesis. It is possible that the nucleocapsid activity associated with the Gag-I (TyA1) protein (24) or other factors, which are not present in our in vitro experiments, are used in vivo to allow recycling of the PPT. This will be the subject of further investigations.

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