RNA/DNA hybrids in human immunodeficiency virus (HIV) replication are cleaved by HIV-1 reverse transcriptase (RT) RNase H in locations determined by hybrid structure. Minus strand DNA synthesis is accompanied by cleavage of template viral RNA directed by RT positioned at the growing 3' DNA end. Some RNA remains as oligomers annealed to the new DNA strand and is cut by RTs positioned at the 5' RNA ends. We constructed substrates to test the hypothesis that internal helix structure, rather than strand end structure, drives the RT to position at 3' DNA and 5' RNA ends. On substrates with an RNA primer recessed on a DNA template, the 5' end of the RNA had a dominant role in the determination of RNase H cleavage positions. If the 5' end region of the RNA could not anneal, cleavage would not occur. Nevertheless, we obtained evidence that helix structure promotes the binding of RT to the end of the helical region closest to the 5' RNA/3' DNA end. When a DNA primer recessed on an RNA template had a 3' unannealed region, cleavage occurred, with RT positioned solely by helical structure at the 5' RNA/3' DNA end of the annealed region of the hybrid. Using substrates having RNA primers annealed to circular DNA templates, we showed that cleavage can be independent of the presence of a DNA 3' end and is directed by the 5' RNA end. Overall, the results suggest that the RT initially binds an internal region of the hybrid and then is driven in the direction to encounter a 3' DNA or 5' RNA end, where it is positioned for catalysis by the strand end. The requirement for two modes of RNA cleavage in viral replication and the unexpected requirement for the 5' RNA end structure are discussed.

Human immunodeficiency virus reverse transcriptase (RT) catalyzes many essential steps required for viral replication. RT has been shown to possess DNA-dependent and RNA-dependent DNA polymerase activities, RNase H activity, strand transfer and strand displacement activities, all of which are essential to complete the process of conversion of single stranded viral RNA genome to double-stranded proviral DNA (reviewed by Goff (1990)). Because the RT is vital to the life cycle of the virus, it has been the target of chemotherapy. Biochemical properties of the RT are being examined in many laboratories in an effort to obtain information needed to design more effective antiviral drugs.

The native enzyme is a heterodimer of 66- and 51-kDa subunits (diMarzo Veronese et al., 1986; Lightfoot et al., 1986). The amino-terminal region of each subunit harbors a polymerase domain, and the carboxyl terminus of only the larger subunit harbors the RNase H domain (J. ohnson et al., 1986). The absolute requirement of RT RNase H for retroviral replication has been established by mutational analysis (Repaske et al., 1989; Tanese and Goff, 1988). RNase H activity has been shown to catalyze the necessary destruction of the plus strand RNA and the RNA primer, and the generation and removal of the polypurinic tract plus strand primer (for a review, see Champaux (1993) and Telenitsky and Goff (1993)). RNase H activity has also been demonstrated to be required for the strand transfer reactions needed in the replication cycle (Cirino et al., 1995; DeStefano et al., 1992; DeStefano et al., 1994b; Ghosh et al., 1995; Peliska and Benkovic, 1992).

Several studies have shed light on the mechanism by which the RT uses its RNase H activity for the removal of the plus strand RNA during and after minus strand DNA synthesis (DeStefano et al., 1991a, 1993, 1994a; Schatz et al., 1990; Gopalakrishnan et al., 1992; Fu and Taylor, 1992; Furfine and Reardon, 1991; Huber et al., 1989; Krug and Berger, 1989; Wöhr and Modling, 1990). We and others have employed substrates that resemble intermediates of minus strand DNA synthesis. Results show that the RT makes initial cleavages of template RNA about 14–18 nucleotides upstream of the 3' hydroxyl of a DNA primer (DeStefano et al., 1991b; Gopalakrishnan et al., 1992; Furfine and Reardon, 1991; Ghosh et al., 1995; Schatz et al., 1990; Zhan et al., 1994). These are followed by additional cleavages in the direction of the primer terminus. Crystallographic studies show that the polymerase and RNase H active sites of the RT are separated by a distance of about 20 nucleotides (I. acobo-Molina et al., 1993; Kahlstedt et al., 1992), suggesting that interaction of the polymerase active site with the primer template determines the positioning of the RNase H active site.

RNase H activity that accompanies minus strand DNA synthesis directed from the growing 3' hydroxyl of the DNA primer was termed "polymerase-dependent" (Furfine and Reardon, 1991; Peliska and Benkovic, 1992). A "polymerase-independent" mode of RNase H activity has been implicated for cleavages not mediated by the 3' OH of the DNA primer (Furfine and Reardon, 1991; Peliska and Benkovic, 1992). Biochemical analyses indicate that polymerase-dependent RNase H activity does not completely degrade the plus strand RNA during minus strand DNA synthesis, but produces RNA oligomers, many of which are long enough to remain bound to the newly synthe...
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Methods

Generation of RNA Templates—Two RNA templates, 142 and 189 nucleotides in length, were generated by run-off transcription by T7 RNase polymerase from plasmids pBS(S) and pBS(+), respectively, following digestion by BstNI. A 41-nucleotide-long RNA substrate was also generated by a similar procedure following restriction digestion of plasmid pBS(+), respectively, followed by digestion by BstNI. RNA samples were purified by gel electrophoresis, subsequently eluted, and quantitated by gel "shift-up" assays using primers of known concentrations as described previously (DeStefano et al., 1993; Palaniappan et al., 1995).

Construction of Plasmids pCP1 and pCP2—A deletion of either 9 or 27 nucleotides immediately following the T7 promoter region in the parent phagemid pBS(+) was accomplished by oligonucleotide-directed mutagenesis (Kunkel, 1985; Kunkel et al., 1987), and the resulting variants were referred to as pCP1 and pCP2, respectively. Construction of these phagemids allowed us to anneal the 189-mer RNA generated from the parent plasmid to the single-stranded circular pCP1 and pCP2 DNA with 9- and 27-nucleotide tails (Fig. 2).

RNA-NAs Hybridization—Annealing of RNA and DNA was performed in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 80 mM KCl. Components were mixed, heated to 60°C for 10 min, and slow cooled over a 90-min period. Excess primer was removed by gel filtration spin chromatography. Hybrid were analyzed by native polyacrylamide or agarose gel electrophoresis to ensure the absence of excess unannealed primers. In some cases, hybrids were cut from the gels to be used in experiments. Escherichia coli RNase H was used when required to establish the presence of RNA-DNA hybrids.

Preparation of Single-stranded Circular DNA—The presence of an A site of replication in parent plasmid pBS(+), as well as the constructs pBS(S), pCP1, and pCP2, enabled us to prepare single-stranded plasmid DNA by adapting the procedure from the Promega application guide.

RNase H Assays—Final reaction mixture (25 μl) contained 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1.0 mM EDTA, 34 mM KCl, 6 mM MgCl₂, 2 mM substrate, and varying amounts of HIV-1 RT. In all cases the enzyme was allowed to prebind to the substrate for 5 min at 37°C. The reaction was initiated with MgCl₂, allowed to incubate for 15 min and terminated with 25 μl of 2 × termination mix. For time course analysis, a mixture of all components except MgCl₂ was prepared. Subsequent to the initiation of reaction with MgCl₂ at 37°C, 25-μl aliquots were drawn at varying time intervals and quenched by adding an equal volume of the 2 × termination mixture (90% formamide (v/v), 10 mM EDTA (pH 8.0), and 0.1% each of xylene cyanole and bromphenol blue). Samples of 8 μl were then subjected to denaturing gel electrophoresis to resolve reaction products. The gels were then vacuum dried and subjected to autoradiography employing standard protocols (Sambrook et al., 1989).

Preparation of Molecular Markers—RNA molecular markers were made either by alkaline treatment of 5′-labeled RNA substrates to generate a nuclease hydrolysis ladder or RNase T1 treatment to generate a G-ladder as described in the protocols supplied with the Pharmacia RNA sequencing kit. HindIII-digested fragments of plasmid pBR322 (Life Technologies, Inc.), which were labeled at the 5′ end, were also run in each gel as additional size markers.

Substrate Sequences—The nucleotide sequences of the 189- and 142-nucleotide RNAs generated from pBS(+) are as follows: 5′-GGAAUUCUGACCU CUGUACCCG GAGAUCCUCA GAGGGCGGCG GAGCGGCUAG AAGCUUUGUG UCCCGGAUGU GGGUACUAG UCUGUUGACAC AACAAUGCAC GCGGAAACAU AAGUGACCA GCCU and 5′-GGCGGUGA AAGCUUUGUG UCCCGGAUGU GGGUACUAG UCUGUUGACAC AACAAUGCAC GCGGAAACAU AAAGUGACCA GCCU.

EXPERIMENTAL PROCEDURES

Materials

Recombinant HIV-RT in its native form was graciously provided to us by the Genetics Institute (Cambridge, MA). This heterodimer RT has a specific activity of approximately 40,000 units/mg. One unit of RT is defined as the amount required to incorporate 1 nmol of dTTP into poly(A) oligo(dT) template in 10 min at 37°C. Aliquots of RT were stored frozen at −70°C, and fresh aliquots were used for each experiment. T4 polynucleotide kinase was from U.S. Biochemical Corp. DNase I, dNTPs, alkaline phosphatase, rNTPs, RNase inhibitor, T7 RNA polymerase, and quick spin gel filtration columns were purchased from Boehringer Mannheim. Radio nucleotides were from DuPont NEN. Plasmids pBS(+) and pBS(−) have been previously described (DeStefano et al., 1992).

In this paper we investigate the possibility that the structure of the RNA-DNA hybrid region of the substrate, rather than the termini of the RNA or DNA strands, is the ultimate determinant of RT positioning. We propose that the RT could first position at the 5′ end of the RNA fixed the 3′ end of the DNA, and then slide to the nearest catalytic site on the RT. The distance suggests that the separation between the catalytic sites on the RT is determining the positioning of the RNA-DNA hybrid region of the substrate, rather than the 5′ end of RNA. We propose that the RT could first position at the 5′ end of the RNA fixed the 3′ end of the DNA, and then slide to the nearest catalytic site on the RT. The distance suggests that the separation between the catalytic sites on the RT is determining the positioning of the RNA-DNA hybrid region of the substrate, rather than the 5′ end of RNA.
The plasmid pCP1 lacks a 9-nucleotide sequence 5’-GGGCGAATT present in the plasmid pBS downstream from the T7 promoter. The plasmid pCP2 lacks 5’-GGGCGAATTCGAGCTCGGTAC-CCGGGG present in the plasmid pBS downstream from the T7 promoter.

DNA primer 2 is 5’-GTGTGGAATTGTGAGCGGAT; DNA primer 3 is 5’-GTGTGGAATTGTGAGCGGATGGTCCG. DNA template 1 is 5’-ATGCTCTAGAGGATCCCCGGGTACCGATGCAGTTCATAGCTGTT; DNA template 2 is 5’-ATGCTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGCCCGCTGTT.

RESULTS

Hypothesis for the Mechanism of RT Positioning for RNase H Cleavage—The position of primary RNase H-directed cleavage of short RNA primers on longer DNA templates may be determined by the tendency of the RT to bind to a region of RNA/DNA hybrid, nearest to the 3’ end of the DNA strand. If so, the location of the 5’ end of the RNA strand would not determine the binding position of the RT.

The 5’ End of a Recessed RNA Primer on DNA Template Determines RT-RNase H Cleavage—A 41-mer RNA transcript (primer 1) was generated from plasmid pBS+ downstream from the T7 promoter.

The plasmid pCP1 lacks a 9-nucleotide sequence 5’-GGGCGAATT present in the plasmid pBS+ downstream from the T7 promoter. The plasmid pCP2 lacks 5’-GGGCGAATTCGAGCTCGGTAC-CCGGGG present in the plasmid pBS+ downstream from the T7 promoter.

DNA primer 2 is 5’-GTGTGGAATTGTGAGCGGAT; DNA primer 3 is 5’-GTGTGGAATTGTGAGCGGATGGTCCG. DNA template 1 is 5’-ATGCTCTAGAGGATCCCCGGGTACCGATGCAGTTCATAGCTGTT; DNA template 2 is 5’-ATGCTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGCCCGCTGTT.

Substrate A, when subjected to HIV-RT RNase H-mediated cleavages, underwent both primary and secondary cleavages at the previously observed positions of 18 and 10 nucleotides measured from the RNA 5’ end as previously observed (DeStefano et al., 1993) and as proposed in Fig. 1, model C. The anticipated primary and secondary cleavage product lengths with substrate A should be about 18 and 10 nucleotides measured from the RNA 5’ end, respectively, as proposed in Fig. 1, model A. Comparison of cleavage products from substrate A and substrate B, with varying amounts of RT ranging from 0.2 to 8 units after a 15-min incubation period are presented in Fig. 3.

Substrate A, when subjected to HIV-RT RNase H-mediated cleavages, underwent both primary and secondary cleavages at the previously observed positions of 18 and 10 nucleotides, respectively, measured from the 5’ terminus of RNA. Since with substrate A both the primary and the secondary cleavages were complete in the 15-min reaction time, the predominant products observable were 9–10 nucleotides long (Fig. 3, right panel, lanes 9–12). However, with substrate B the only products that appeared were cleaved 18 nucleotides from the 5’ end of RNA (Fig. 3, left panel, lanes 3–6). There were no other observable products even after long autoradiographic expo-
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**Fig. 3.** HIV-1 RT RNase H-mediated cleavage on substrate A versus B. A schematic representation of substrates A (right panel) and B (left panel) is shown at the top. The asterisk at the 5′ end of RNA indicates the radiolabel. Filled and open arrows represent points of initial and secondary cleavages, respectively. The numbers above the arrows represent the size of the labeled products. An X on the arrows represents the absence of the cleavage. The left panel comprising lanes 1–6 shows reaction products from substrate B, and the right panel comprising lanes 7–12 shows the reaction products from substrate A. The presence or absence of E. coli RNase H is indicated by + or −. The concentration of RT is indicated above each lane. The numbers on the left indicate the product length of RNA determined by an RNA ladder prepared as under “Experimental Procedures.”

Cleavage directed from the point of hybridization should have yielded products 28 and 20 nucleotides in length, but these were totally absent. Quantitation of primer 1 using PhosphorImager™ analysis showed that the amount of cleavage at any RT concentration was about the same in substrates A versus B (Fig. 3, compare the disappearance of the 41-mer in lanes 3–6 with lanes 9–12, respectively). Therefore, differences observed in product distribution did not result from fundamental differences in the sensitivity of the two substrates to cleavage. Contrary to our anticipation that the point of hybridization determines or influences the positional cleavage by RT, the products obtained from gel electrophoresis analysis indicate that the 5′ terminus of the RNA is a primary determinant. Influence of the 5′ end predominates even if it is not annealed!

Cleavage of substrate B also consisted of only primary products (Fig. 3, compare amounts of 18-nucleotide products). This was probably because, following the initial cleavage at about 18 nucleotides from the 5′ end of the RNA on substrate B, the cleaved product had such a short region of complementarity with the template that it dissociated before secondary cleavage could occur.

E. coli RNase H was employed to demonstrate the presence of RNA/DNA hybrid in substrates A and B (Fig. 3, lanes 1 and 7). E. coli RNase H cleavage does not follow the cleavage pattern of HIV-1 RT. Additionally, the presence of a product of 28 nucleotides in length following E. coli RNase H treatment of substrate B demonstrates that the tail region of substrate B does not undergo cleavage, and therefore is unannealed (Fig. 3, left panel, lane 1).

**Fig. 4.** Time course of RNase H cleavage on substrate A versus B. A schematic representation of substrates A (right panel) and B (left panel) is shown at the top. The time course RNase H reactions were performed as described under “Experimental Procedures.” Sampling times are indicated above each lane. All other labels are as described in the legend to Fig. 3.

16 nucleotides in length following E. coli RNase H treatment of substrate B demonstrates that the tail region of substrate B does not undergo cleavage, and therefore is unannealed (Fig. 3, left panel, lane 1).

Kinetics of RT-RNase H Cleavage on Substrate A Versus B—Substrates A and B were cleaved over a time course to compare the rates of the reactions (Fig. 4). Initial cleavage of primer 1 was evident in both substrates by 15 s. The appearance of secondary cleavage products of substrate A, with a concomitant disappearance of primary products, was evident starting at about 1 min (Fig. 4, right panel, lanes marked 1 min to 16 min). However, consistent with above results, secondary products from substrate B were not observable (Fig. 4, left panel, lanes marked 0.25 min to 16 min). In general, irrespective of the tail structure of substrate B, both substrates were cleaved with nearly identical kinetics.

**Helical Structure Influences RT-RNase H Cleavage Specificity When a DNA Primer Is Recessed on Long RNA Template—** The surprising observation that an unannealed 5′ RNA end could determine RNase H cleavage specificity prompted us to question whether an unannealed 3′ DNA end could have a similar influence on mode B cleavage (Fig. 1). The template used was a 142-mer RNA labeled at the 5′ end (template 3). A 20-nucleotide-long DNA (primer 3) was annealed with perfect complementarity to template 3 to generate substrate C (Fig. 2). Substrate D was generated by annealing a 26-nucleotide long DNA (primer 4) to template 3. Substrate D was identical to substrate C, except that the DNA primer, although annealed to the same 20-nucleotide region as primer 3, also had a 6-nucleotide-long unannealed 3′ tail. Primary and secondary RNA cleavage products resulting from the action of RT on substrate C were anticipated to be about 102 and 94 nucleotides long, respectively. These products would result from the expected
cleavages measured from the 3' end of the recessed DNA primer as in our previous studies (DeStefano et al., 1991b). With substrate D, if the point of hybridization determined the position of cleavage, the same length RNA products should be observed. However, if the DNA 3' end positioned the RT for cleavage, products 6 nucleotides shorter (96 and 88) should be seen.

Cleavage of substrates C and D over a range of RT concentrations is shown in Fig. 5. After 15 min, both substrates C and D sustained some cuts even at the lowest RT concentration tested, and both appeared similarly sensitive to cleavage as the concentration was raised. With both substrates, however, the predominant products observed were the secondary cleavage products at 94 nucleotides. There were also some minor residual primary cleavage products visible with substrate C (Fig. 5, left panel, lanes 1–5). Importantly, the appearance of cleavage products of same sizes with substrates C and D suggests that the enzyme measures the distance from the point of hybridization, indicating that helical structure does influence cleavage position on hybrids with recessed DNA oligomers.

Kinetics of RT-RNase H Cleavage on Substrate C Versus D—Kinetics of cleavage of substrates C and D was performed to determine whether the cleavage process proceeded in normal fashion from primary to secondary positions (Fig. 6). With substrate C, the primary cleavage is evident as early as 15 s (Fig. 6, left panel, lanes marked 0.25 min and above). Over the reaction period most of the primary cleavage products disappear with a simultaneous increase in secondary cleavage products. PhosphorImager quantitation reveals that about 90% of RNA sustained cleavages as early as 15 s (Fig. 6, left panel, compare 0' time point with 0.25 min). Very few secondary products are seen up to about 1 min into the reaction period. Secondary cleavage products continued to accumulate and reached maximum levels at 16 min. With substrate D, the same progression of cleavages, first to the 102-mer and then the 94-mer, is evident but over a shorter time frame (Fig. 6). The kinetic profile further supports the importance of the hybrid region in positioning the RT. It also shows that the presence of the 3' unannealed tail on this substrate actually improves the ability of the primer-template to act as a substrate.

RT-RNase H Cleavage Occurs in the Absence of a Free DNA Terminus—Results so far suggested that the 5' terminus of recessed RNA on a RNA/DNA hybrid plays an essential role in positioning of RT-directed cleavages. Although we anticipated 3' DNA terminus would have a similar influence when recessed on RNA, the above experiments yielded surprising results emphasizing the importance of hybrid helical structure. We next determined whether the 3' DNA end had any influence on cleavage of recessed RNAs, such as providing a site for RT loading as in mode C (Fig. 1). The experiment involved measuring cleavage specificity of hybrid substrates lacking a DNA 3' terminus, a structure obtained by using a circular DNA. Single-stranded circular RNAs from plasmids pBS- and pCP1 were prepared as described under “Experimental Procedures,” and annealed to the 5'-labeled 189-mer RNA to generate substrates E and F (see Fig. 2). The 189-mer RNA annealed completely to its circular SS pBS- DNA template in substrate E, but had a 9-nucleotide tail at its 5' end when bound to pCP1 resulting in substrate F. Both substrates were cleaved efficiently by RT (Fig. 7), demonstrating that the 3' end of the DNA is not needed for entry or positioning for cleavage. As before, cleavage occurred at the expected distances, 18 and 9 nucleotides from the 5' end of the RNA, whether that end was annealed or not. The low level of secondary cleavage with the tailed RNA could again be attributed to dissociation of the RNA product after primary cleavage reduced its annealed region. The characteristic RT RNase H cleavages observed on substrate E, wherein there is no free 3' DNA end, demonstrates the existence of "polymerase-independent mode" of RNase H cleavages as originally proposed (Furfine and Reardon, 1991; Peliska and Benkovic, 1992). The only difference in nuclease action between the two substrates was that the tailed substrate in this case required a higher level of enzyme for the same amount of cleavage (Fig. 7, compare lanes 1–3 with 7–9, respectively).

A Sufficiently Large Unannealed 5' Tail on an RNA Primer Disrupts RT-RNase H Cleavage—Since the unhybridized 5' tail in substrate F was only 9 nucleotides long, there was a possibility that the transient hybridization during the course of the reaction promoted by an annealing activity of RT held down the non-complementary region against the template, allowing the RT to measure from the 5' end to fix the position of cleavage. We reasoned that a substrate in which the tail at the 5' end of RNA exceeds the normal 18-nucleotide spanning distance from 5' end to the cleavage site, would not support terminus-directed cleavages. To test this we employed substrate G, in which the 189-mer RNA primer was annealed to a circular template, such that it had a 27-nucleotide-long 5'-unannealed tail (Fig. 2). Cleavage of substrates E and G by various concentrations of RT RNase H were compared (Fig. 8). Even at the highest enzyme concentrations tested, the 189-mer RNA in substrate G underwent virtually no cleavage (Fig. 8, right panel).
panel, lanes 8–12). However, as before, the 189-mer RNA in substrate E was cleaved effectively even at relatively low RT concentrations (Fig. 8, left panel, lanes 1–5). The presence of hybrid RNA/DNA substrates was established both by treatment with E. coli RNase H (Fig. 8, lanes 7 and 14) and by a separate native gel electrophoresis (data not shown). The insensitivity of substrate G clearly demonstrates that a sufficiently long 5' unannealed tail prevents the 5' RNA end from exerting a necessary influence on the positioning of the RT for cleavage. The result is a clear demonstration of the inability of helical structure alone to direct the cleavage reaction.

Helical Structure Has a Secondary Influence on RT-RNase H Cleavage of an RNA Primer Recessed on a DNA Template—We considered the possibility that although a 5' RNA end is required for cleavage of an RNA primer on a DNA template, the helical structure still has an influence on positioning of the RT. To test this, we constructed a substrate with an RNA primer having an unannealed loop (Fig. 2, substrate H). Starting at the 5' end, the RNA primer had a complementary region of 9 nucleotides, followed by a noncomplementary region of 40 nucleotides before complementation resumed and continued to the 3' end. Remarkably, the presence of the 9 5' end complementary nucleotides promoted the formation of primary and secondary cleavage products 18 and 9 nucleotides in length (Fig. 9, lanes 1, 2, and 4). These appear to derive from positioning of the polymerase active site at the 3' side of the loop, and then measurement of the usual 18 nucleotide distance to the RNase H site. This indicates that the beginning of the RNA/DNA hybrid structure was the determinant of the position of cleavage. These observations present a complicated picture. The results show that cleavage directed from the point of hybridization is possible but can only be facilitated by the presence of a hybridized 5' RNA terminus. They emphasize the essential role of the 5' RNA end, but also a potentially important role for helical structure.

DISCUSSION

We have examined the role of RNA/DNA hybrid structure versus the location of the ends of the RNA and DNA strands in the determination of HIV-RT-mediated RNase H cleavage specificity. In previous work, RT positioning either at the 3' end of a DNA primer on an RNA template, or the 5' end of an RNA primer on a DNA template was found to be important for the
RNA cleavage specificity (DeStefano et al., 1991a, 1994a; Gopalakrishnan et al., 1992; Ghosh et al., 1995; Schatz et al., 1990; Zhan et al., 1994). Since RT has to extend the DNA primer during minus strand DNA synthesis, it is easy to envision RT binding to the 3′ end of the DNA strand, and thereby mediating RNA cleavage of the template RNA at a distance determined by the spatial separation of active sites. It is more difficult to imagine why the 5′ RNA end influences the RT positioning on substrates with recessed RNA primers on DNA templates.

As a unified explanation for these observations, we proposed an alternative model whereby the structure of the hybrid influences or determines RT RNase H cleavages as proposed in Fig. 1, model C. In this model the RT binds the RNA/DNA hybrid region as close as possible to the 3′ end of the DNA strand and the 5′ end of the RNA strand. This, of course, is the same end of the duplex in either case. However, if the DNA is recessed on the RNA, the RT would contact the DNA 3′ end, whereas if the RNA is recessed on the DNA the RT would contact the RNA 5′ end. The model also suggests initial binding to the 3′ DNA end as a means for the RT to load onto the substrate.

We originally anticipated that the helical structure of the substrate would have a dominant role in the positioning of the RT when it was binding over the 5′ end of a recessed RNA. To our surprise, the 5′ RNA end has a predominant influence on positioning the RT. This was revealed by comparison of substrates having an RNA primer annealed completely to the template DNA, with substrates having an unannealed 5′ RNA (Fig. 3). For unannealed regions up to 10 nucleotides long, cleavage continued to be measured from the 5′ RNA end. There was no apparent influence of the hybrid region. When the unannealed region was 27 nucleotides long, cleavage was inhibited (Fig. 8). This suggested that the RT could “hold down” a short unannealed region to make the measurement, but was frustrated by a longer region.

By careful primer-template design, however, we could still observe a positioning effect of the helical region. The substrate in question had an RNA primer with an internal unannealed loop. The DNA template was circular to eliminate possible influence of the 3′ end of the DNA template. In this case, the patterns of cleavages indicated that some RTs positioned at the RNA 5′ ends, but others bound the hybrid adjacent to the loop. This latter positioning suggests that the RT could sense the helix orientation, and tried to position as close as possible to the 5′ RNA and 3′ DNA ends. However, it could not pass the unannealed region and stopped just next to it. The results of this experiment imply that the RT might bind the hybrid at any point and move in the direction of the 5′ RNA and 3′ DNA ends. However, we do not have direct experimental evidence for a movement process. Also, the means by which the RT downstream of the loop can sense the annealed 5′ RNA end is a mystery.

An additional surprising observation was that short unannealed 3′ end regions of a DNA primer on a RNA template relinquished their influence on RT positioning. In this case, the helical region dominated the binding location of the RT. In fact, use of circular DNA templates indicates that the presence of a DNA end on the substrate is not a requirement for RT binding or positioning for RNase H activity. This result demonstrates that our mode C model is overly complicated. The RT does not need to first bind a DNA 3′ end and then move to the hybrid region. It can bind and position itself through the use of the helix, and the 5′ RNA end.

When an RNA primer with a long 5′ tail was annealed to a circular DNA template, no cleavage occurred. This shows that in the absence of a DNA 3′ end, an RNA end, either annealed or with a sufficiently short unannealed region, is necessary for cleavage. Although on our template with a looped primer (substrate H) we observed cleavage indicative of positioning by helix structure, cleavage was possible only in the presence of an annealed 5′ RNA end. This shows a role of hybrid structure in the determination of cleavage specificity, but clearly a critical determinant is the 5′ annealed RNA end.

Overall, we have found that the positions of HIV-RT RNase H cleavage of RNA/DNA hybrid structures present during viral replication are determined by the location of strand ends, and by the helical structure of the hybrid. Cleavage of RNA on a substrate with a recessed DNA primer on an RNA template is directed by the binding of the polymerase active site to the DNA 3′ end. This is the normal binding configuration of the RT during minus strand viral synthesis. Results presented here show that in the absence of a fully annealed DNA 3′ end the RT still positions on the hybrid as close as possible to the DNA 3′ end. This suggests that the hybrid structure influences the RT to preferentially bind, and possibly move to, the correct position for minus strand synthesis and accompanying RNA template cleavage.

The RT is also expected to cleave short RNAs left annealed to the newly synthesized minus strand. A surprising finding was...
that the 5' ends of short RNAs annealed to DNA are a major determinant of the positioning of the RT for such cleavage. We also detected an influence of the helical structure to position the RT as close as possible to the 5' ends of these RNA primers.

Possibly, in both cases, the role of the helix is to direct a movement of bound RT to the appropriate strand for catalysis. The reason for the high specificity of cleavage is to direct a movement of bound RT to the appropriate RNA primer. It may simply have evolved to be the means by which short RNA primers are recognized for binding and cleavage.

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