Substrate Requirements for Secondary Cleavage by HIV-1 Reverse Transcriptase RNase H*

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During and after minus-strand DNA synthesis, human immunodeficiency virus 1 (HIV-1) reverse transcriptase (RT) degrades the RNA genome. To remove RNA left after polymerization, the RT aligns to cut 18 nucleotides in from the 5’ RNA end. The enzyme then repositions, making a secondary cut 8 nucleotides from the RNA 5’ end. Transfer of the minus strong stop DNA during viral replication requires cleavage of template RNA. Removal of the terminal RNA segment is a special case because the RNA-DNA hybrid forms a blunt end, shown previously to resist cleavage when tested in vitro. We show here that the structure of the substrate extending beyond the RNA 5’ end is an important determinant of cleavage efficiency. A short single-stranded DNA extension greatly stimulated the secondary cleavage. Annealing an RNA segment to the DNA extension was even more stimulatory. Recessing the RNA from a blunt end by even one nucleotide caused the RT to reorient its binding, preventing secondary cleavage. The presence of the cap at the 5’ end of the viral RNA did not improve the efficiency of secondary cleavage. However, NC protein greatly facilitated the secondary cut on the blunt-ended substrate, suggesting that NC compensates for the unfavorable substrate structure.

HIV-1 reverse transcriptase is required for the conversion of the viral RNA genome to double-stranded DNA. This multifunctional enzyme has RNA- and DNA-dependent DNA polymerase, strand displacement, strand transfer, and RNase H activities. The RNase H activity degrades the RNA genome during and after synthesis of the first or minus DNA strand (1). The RNase H is used to clear the new minus DNA strand of the genomic RNA fragments, in preparation for minus-strand transfer and synthesis of the plus DNA strand. RNase H activity is required for the generation of the polypurine tract primers that initiate plus-strand synthesis, and for the removal of the minus- and plus-strand primers (Refs. 1 and 2 and reference therein).

HIV-1 RT is an asymmetric heterodimer comprising the p66 and p51 subunits (3–5). The structure of the p66 subunit is analogous to that of a right hand with the palm, thumb, fingers, and connection subdomains and an RNase H subdomain. The polymerase active site is located near the amino terminus of the p66, whereas the RNase H active site is near the carboxyl terminus. Biochemical as well as structural analyses show the spatial distance between the two active sites to be about 18–19 nucleotides in length when RT is bound to a duplex substrate (5–10). The active residues of the polymerase domain, Asp-185, Asp-186, and Asp-110, reside within the palm subdomain (5). The fingers, palm, and thumb subdomains of the p66 participate in substrate binding (8, 10). The p51 subunit, a proteolytic product of the p66, folds in a different conformation and does not contain any catalytic sites (5, 8, 10, 11). This subunit primarily serves a structural role in stabilizing the p66 subunit as well as positioning the RNase H subdomain and the tRNA (12–14). The active site residues of the RNase H domain include Asp-443, Asp-498, and Glu-478 (15, 16). The structure of the RNase H subdomain of HIV-1 RT resembles that of Escherichia coli RNase H except that RT lacks the helix C motif, which in E. coli RNase H is important for binding substrate (17, 18). The RNase H subdomain expressed independently is inactive since it cannot bind the RNA/DNA hybrid without the thumb and connection domains (19, 20).

During minus-strand synthesis, RT is positioned on the substrate with its polymerase active site at the 3’ terminus of the DNA primer. This places the RNase H active site 18 nt away on the RNA template (21, 22), allowing RT to make endonucleolytic cuts within the RNA as the RNA/DNA substrate is created. This is the polymerase-dependent mode of RNase H cleavage (6, 23). The RNase H activity is not strictly coupled with the polymerase function. It is distributive, periodically cleaving the RNA to leave fragments (24). Because a virus contains 50–100 copies of RT and only two RTs can be involved in synthesis at any given time, the residual RTs are available to rebind and degrade the fragmented genome. This mode of cleavage is referred to as the polymerase-independent RNase H and occurs in the absence of synthesis.

We have previously characterized the polymerase-independent RNase H activity (25–28). Although the orientation of RT on the RNA/DNA hybrid is the same as during polymerization, the recessed 5’ end of the RNA fragment directs the positioning (Fig. 1). Binding of RT to the RNA/DNA duplex at the RNA fragment 5’ end places the RNase H active site 18 nt into the RNA, where the enzyme makes a primary cut. RT then positions 8–9 nt toward the 5’ end of the RNA to make an 8–9-nt

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1 The abbreviations used are: HIV, human immunodeficiency virus; RT, reverse transcriptase; nt, nucleotide(s); NC, nucleocapsid.
**Polymerase Independent Mode RNase H (5’ Directed RNase H)**

![Diagram](image)

**Fig. 1. Mechanism of polymerase-independent RNase H cleavage.** The schematic shows the positioning of the RT for primary and secondary cuts during the polymerase-independent mode of RNase H activity. The gray lines represent DNA, and the black lines represent RNA. The rectangle represents RT with the indents corresponding to the polymerase (P) and the RNase H (H) active sites. The sizes of cleavage products are indicated by the numbered brackets and arrows.

secondary product or backward to the 3’ end of the RNA, creating a 5-nt product (27, 28). Ultimately the RNA is degraded into small fragments that dissociate or are displaced during plus-strand synthesis. Interestingly, the primary, secondary, and 5-nt cuts are independent of each other, each with a characteristic rate (27).

Blunt end substrates in which the RNA 5’ end and the DNA 3’ end are flush display a diminished rate of secondary cleavage compared with substrates in which the RNA segment is recessed on the DNA (6, 27, 29–31). These results suggest that RT requires a 3’ DNA extension to bind appropriately for secondary cleavage. Significantly, a blunt end intermediate is created during reverse transcription, when RT synthesizes to the 5’ end of the RNA genome to create the minus strong stop DNA. Presumably the virus has evolved a mechanism to effectively remove this RNA. Previous work suggests a role for the viral nucleocapsid (NC) protein in degradation of blunt-ended RNA-DNA hybrids. Peliska et al. (32) demonstrate that the presence of NC enhanced the rate of cleavage of the 5’-terminal RNA segment of a blunt-ended RNA-DNA hybrid. They proposed that NC interacts with RT to tether the enzyme to the substrate for more effective cleavage. Although these studies were done before our understanding of primary and secondary cuts, they are useful in providing insights into RNase H mechanism. We were therefore interested in reexamining secondary cuts, as they are useful in providing insights into RNase H mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified HIV reverse transcriptase (40,000 units/mg) was generously provided by Genetics Institute (Cambridge, MA). Oligonucleotides were purchased from Integrated DNA Technologies, Inc (Coralville, IA). The 18-nt RNA was purchased from Oligos Etc. (Wilsonville, OR). Accl, calf intestine phosphatase, dNTPs, rNTPs, DNase I, and polynucleotide kinase were purchased from Roche Molecular Biochemicals. The 32P isotope was purchased from PerkinElmer Life Sciences.

**NC—**HIV-1 Ncp7 (72 amino acids) was prepared by solid phase chemical synthesis as described by de Rocquigny et al. (33). HIV-1 Ncp7 (55 amino acids) was generously provided by Dr. Robert J. Gorelick. NC stocks were resuspended in NC dilution buffer containing 50 mM Tris-HCl (pH 7.5) and 5 mM dithiothreitol and stored in 5–10-μl aliquots at −80°C until use. For RNase H assays done in the presence of NC, the protein was added (3.5 nt/NC) to the reaction and incubated at 37°C for 5 min before the addition of RT. For control reactions without NC, an equal volume of NC dilution buffer was added instead.

**Generation of in Vitro Transcribed RNA—**All RNAs were generated by in vitro run-off transcription using the Ambion T7-MEGASHortscript kit (Austin, TX) as per the manufacturer’s protocol. The 41-nt RNA was generated from Accl-linearized pBSM13+ plasmid as previously described (26). The 28-nt RNA corresponding to the viral sequence was transcribed from a synthetic double-stranded DNA fragment containing the T7 promoter. The template comprised the sequence 5’-CACATCGG TAAACGACTCACATAGGGAGCTCTCGTTAGAGGAATGATT-3’, and its complimentary strand. The capped 28-nt transcript was generated using the Ambion mMessage mMachine RNA kit. To generate internally labeled RNAs, [γ-32P]CTP was included in the transcription reaction.

**Substrate Preparation—**The 41- and 28-nt RNAs were first dephosphorylated by calf intestine phosphatase and 5’-end labeled using [γ-32P]ATP (6000 (222 TBq) Ci/mmol) and polynucleotide kinase as previously described (26). All RNAs used in the study were PAGE-purified and resuspended in 10 mM Tris-HCl, 1 mM EDTA buffer (pH 8.0). RNAs were quantitated by using Ribogreen assay supplied by Molecular Probes (Eugene, OR). To generate hybrid substrates, the labeled RNA was annealed to the appropriate DNA template at a ratio of 1:3 in 50 mM Tris-HCl (pH 8.0), 80 mM KCl, and 1 mM dithiothreitol.

**RNase H Assays—**Reactions were performed as described previously (26). Briefly, reactions contained 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1.0 mM EDTA, 34 mM KCl, 4 mM substrate, and 32 nM HIV-1 RT. The reaction mixture was incubated for 2 min at 37°C to allow prebinding of RT to substrate. Reactions were then started by the addition of MgCl2 at 6 mM final concentration and incubated at 37°C. Reactions were terminated at the appropriate time with a 2× termination dye (10 mM EDTA (pH 8.0), 90% formamide (v/v), and 0.1% xylene cyanol and bromphenol blue). Control reactions did not contain RT and were incubated at 37°C for a period equaling the longest experimental time point. RNA ladders were created by base hydrolysis and RNase T1 digestion. Samples were subjected to 10% (15% for 28-nt RNA) denaturing polyacrylamide gel electrophoresis, visualized using a Molecular Dynamics PhosphorImager, and analyzed using ImageQuant software (version 1.2).

**RESULTS**

Synthesis of minus-strand DNA to the 5’ end of the RNA template results in a blunt-ended substrate in which the DNA 3’ end is flush with the RNA 5’ end. Previously studies show that RNase H cleavage at such blunt ends is very inefficient (27). We hypothesized that the inefficiency in processing the 5’-most RNA segment is because the blunt end RNA-DNA substrate lacks structural features needed for positioning of the enzyme for secondary cuts. We therefore set out to define the necessary structural requirements and to determine whether the other reaction components present in vivo compensate for the missing structures.

**Influence of DNA 3’ Extensions on Secondary Cleavages—**We have previously shown that a blunt end substrate in which the RNA 5’ end flush with the DNA 3’ end sustains a particularly slow secondary cleavage compared with an RNA that is recessed on the DNA template (27). Because this suggested that the DNA 3’ extension stimulates secondary cleavage, we pro-
FIG. 2. Secondary cleavage on substrates with different lengths of DNA 3' extension. A, sequences of the substrates used. The RNAs are in **bold**. In all substrates the 41-nt RNA was 5' end-labeled. B and C, time course of RNase H assays using recessed RNA, substrate 1 (B), and blunt end RNA, substrate 2 (C). D–G, time course of RNase H assays using substrates 3–6 containing 1-nt (D), 3-nt (E), 5-nt (F), or 7-nt (G) DNA overhangs. A schematic of the substrate is indicated above each panel. The star indicates the position of the 32P label, whereas X denotes the length of the overhang. Time points are indicated above each lane. Lane C corresponds to a control reaction without RT. Sizes of the starting material and cleavage products are indicated. Sizes were determined from an RNA ladder created by base hydrolysis and RNase T1 digestion.
posed to determine the minimum length of extension necessary to affect the cleavage (Fig. 2, D–G). Substrates with 3' end DNA extensions of 1, 3, 5, or 7 nt were generated by annealing the 5' end-labeled 41-nt RNA to a 51, 53, 55, or 57-nt DNA template (Fig. 2A, substrates 3, 4, 5, and 6). The recessed (Fig. 2A, substrate 1) and blunt end substrates (Fig. 2A, substrate 2) were used as controls. These substrates contained a 41-nt RNA annealed to a 77-nt DNA to create the recessed substrate in which the DNA 3' end extends 21 nt beyond the RNA 5' end or to a 50-nt DNA to produce a substrate with the DNA 3' terminus flush with the RNA 5' end. Equal amounts of each substrate were subjected to RNase H cleavage. As previously shown (27, 28), the control substrate having the 21-nt 3' DNA extension sustained an efficient secondary cut (Fig. 2B). The 8-nucleotide secondary product appeared within 15 s and became the major labeled product over the measured time course. The primary product is created more rapidly (28) but did not accumulate to a high level in this experiment because this 18-nt fragment was processed efficiently into the secondary product. The 15-nt product was observed as a minor product, as shown before (27, 28). By 16 min the majority of the 41-nt RNA was cleaved into the secondary product. In comparing cleavage patterns, processing of the blunt-ended substrate over time was distinctly different, although the same size products were generated: the 18-nt primary, 8-nt secondary, and the 15-nt product (Fig. 2C). As previously shown (27), although the 18-nt product was efficiently formed, it was poorly processed to the 8-nt secondary product. Instead some accumulation of the 15-nt product was observed with time. Unlike with the recessed substrate, the secondary cut product with the blunt end substrate appeared in detectable quantities only by 2 min and accumulated at a slower rate. A reasonable explanation is that the RT cannot move readily on the blunt end substrate to the appropriate position for the secondary cleavage. Instead, most enzymes move 3 nt to make the 15-nt product. Over a longer time course, enzymes ultimately make the secondary product.
When the template had a 1-nt DNA 3’ extension, the overall cleavage product profile was similar to that of the blunt end substrate (compare Fig. 2D to Fig. 2C). The secondary cut was inefficient and not detectable until 2 min. At 16 min, the amount of 8-nt product formed was only 25% of that observed with the 21-nt overhang substrate. Extending the overhang to 3 nt noticeably enhanced the efficiency of the secondary cut (Fig. 2E). Here the 8-nt secondary product was detectable at the 1-min time point, a 2× faster rate than observed with the blunt end and 1-nt DNA overhang substrates. At 16 min, the secondary cut was about 40% of that observed with the 21-nt overhang substrate (compare Fig. 2E to Fig. 2C). Increasing the overhang to 5 and 7 nt further enhanced the secondary cut efficiency (compare Fig. 2, F and 2G, to Fig. 2C). The 8-nt product was detectable as early as 15 s and was about 50% of that formed by the 21-nt overhang substrate at 16 min. Although the cleavage efficiency was enhanced, it was still not as high as that observed with the 21-nt overhang (Fig. 2B). Because DNA 5’ overhangs do not affect primary and secondary cleavages at the RNA 5’ end (data not shown), differences in this region of the substrate could not explain the low efficiency of secondary cuts observed on substrates 2–6. Clearly the process of cleavage enhancement is gradual with the length of extension, suggesting that the longer 3’ DNA extensions allow for the most optimal contacts with RT that are relevant to the secondary cut.

The Efficiency of Secondary Cleavage on 5’ RNA Extensions—Because reduced 3’ DNA extension inhibited secondary cleavage, we determined the effect of continuing this structural change beyond the blunt configuration into what might be considered a negative DNA extension (Fig. 3). RT normally encounters such a substrate configuration during minus-strand synthesis, where the extending primer terminus is re-cessed on the RNA template. To test the effect of RNA 5’ overhangs on secondary cleavages, the 5’ end-labeled 41-nt RNA was annealed to a 49-, 45-, 43-, or 41-nt DNA template to create hybrid substrates with 1-, 5-, 7-, or 9-nt 5’ RNA single-strand extensions. The configuration at the RNA 3’/DNA 5’ end was kept the same for these substrates (Fig. 3A, substrates 7, 8, 9, and 10). Equal amounts of each substrate were subjected to RNase H, and the profiles of cleavage products were compared with that of the blunt end hybrid substrate (compare Figs. 3, B–E, to Fig. 2C). Surprisingly, a 1-nucleotide RNA 5’ extension eliminated the 8-nt product (Fig. 3B). The 15-nt product was still produced but with noticeably lower efficiency. More interestingly, efficiency of the 18-nt primary product formation was also reduced, with the product accumulating only at the later time points. Instead, a 19-nt product was generated with high efficiency, suggesting that on this substrate most RTs use the DNA 3’ end rather than the RNA 5’ end for initial alignment. The 19-nt product was then the result of a primary cleavage in the polymerization-dependent binding mode for RNase H cleavage.

As the RNA 5’ end extension was further increased, the products of cleavage were consistent with DNA 3’ end-directed alignment of the RT (Fig. 3). The 18-nt product, expected from RNA 5’ end alignment, was very minor, suggesting that binding to generate this cut is unstable. The 15-nt product also was formed with less efficiency as the RNA overhang length increased. RNase H cleavages on the 5- and 5-nt RNA overhang substrates produced 21- and 23-nt primary cleavage products that accumulated with time, expected from 3’ DNA end positioning (Fig. 3C and data not shown). As with the 1-nt overhang substrate, very few of these products sustained secondary cuts, suggesting that short RNA 5’ overhangs restrict the realignment of RT for efficient secondary cleavage.
primer, the major secondary product was 8 nt in length (Fig. 2B). The 7- and 9-nt products occurred to a much lesser extent than the 8-nt product. When an RNA oligomer was annealed upstream of the 41-nt RNA (Fig. 4), products shorter than 8 nt appeared and accumulated. These might be made directly or result from additional cleavage of the 8-nt product. A reasonable explanation is that the double-stranded hybrid is a particularly good substrate for RT binding compared with the DNA single strand extension. This allowed RT to stably translocate even further from the primary cut site and to make very short products.

Influence of the 5' End Cap on the Formation of the Secondary Cut—As discussed earlier, synthesis of the minus-strand strong stop DNA results in the formation of a blunt-ended hybrid substrate. Presumably efficient execution of both primary and secondary cuts is necessary for exposure of the strong stop DNA 3’/H11032 terminus for minus-strand transfer. Therefore, RT must be able to cleave a blunt end substrate during reverse transcription. Because the viral genome is a messenger RNA that contains a 5’/H11032 end m7Gppp cap, we examined whether the presence of the cap structure makes a blunt end substrate more receptive to secondary cleavage.

We synthesized a 28-nt RNA corresponding to the 5’ terminus of the HIV-1 genome (Fig. 5). To control the positions of labeling and to facilitate annealing, base substitutions were made mostly near the 3’ end of the sequence (see “Experimental Procedures”). The RNA oligomer was transcribed in vitro with and without the 5’ cap. Because the cap structure prevents 5’-end labeling, the RNAs were internally labeled at positions 5, 7, and 9 from the 5’ end. To distinguish cleavage products containing the RNA 5’ end, an additional control substrate was generated in which the uncapped RNA was 5’ end-labeled. We first used the 5’ end-labeled and internally labeled uncapped RNAs to create recessed and blunt end hybrid substrates (Fig. 5A, substrate 12 and 13). The RNA was annealed to a 62-nt DNA template to create a hybrid with a 28-nt DNA extension or to a 34-nt DNA template to create the blunt end hybrid. The substrates labeled by either method yielded a similar pattern of cleavage (Fig. 5, B–E). A 19-nt product was the first to appear and resulted from the primary cut. The 7–8-nt product resulted from the secondary cut. A 13-nt product was also detected. The difference in the size of the cleavage products here from those seen with the 41-nt RNA substrates probably reflects the difference in sequence between the two substrates. Additional products 1–2 nt in length were made with the internally labeled RNA and are evidence of wobbling of the enzyme during secondary cuts. As expected, the difference in labeling did not affect the cleavage efficiency.

With the recessed RNA, both 5’ end-labeled (Fig. 5B) and internally labeled (Fig. 5C) secondary cut products appeared within 15 s. However, with the blunt end RNA, secondary cleavage products were not evident until 1 min even though the overall RNase H efficiency was similar to that of the recessed RNA (Fig. 5, D and E).

Next we determined whether the presence of the cap influenced the specificity or rate of secondary cleavage (Fig. 6).
Recessed (Fig. 6, A and B) and blunt end (Fig. 6, C and D) hybrid substrates were compared. When resolved by gel electrophoresis, both the starting RNA strand and 5′ end cleavage products (equal to or longer than 8 nt) containing the cap structure were identified. The diamond at the 5′ end of the RNA represents the cap structure. All RNAs were internally labeled at positions 5, 7, and 9, as indicated by the stars. A and B, time course of RNase H assays with substrates containing the capped (A) and uncapped (B) recessed RNA (substrate 12). C and D, time course of the RNase H assays with substrates containing the uncapped (C) and capped (D) blunt-ended RNA (substrate 13).

**Fig. 6.** Influence of the 5′ end cap on the formation of the secondary cut. The description of the figure is the same as in Fig. 2. Substrates 12 and 13, described in Fig. 5A, were generated using capped or uncapped 28-nt RNA. The diamond at the 5′ end of the RNA represents the cap structure. All RNAs were internally labeled at positions 5, 7, and 9, as indicated by the stars. A and B, time course of RNase H assays with substrates containing the uncapped (A) and capped (B) recessed RNA (substrate 12). C and D, time course of the RNase H assays with substrates containing the uncapped (C) and capped (D) blunt-ended RNA (substrate 13).

Recessed (Fig. 6, A and B) and blunt end (Fig. 6, C and D) hybrid substrates were compared. When resolved by gel electrophoresis, both the starting RNA strand and 5′ end cleavage products (equal to or longer than 8 nt) containing the cap structure were identified. The diamond at the 5′ end of the RNA represents the cap structure. All RNAs were internally labeled at positions 5, 7, and 9, as indicated by the stars. A and B, time course of RNase H assays with substrates containing the uncapped (A) and capped (B) recessed RNA (substrate 12). C and D, time course of the RNase H assays with substrates containing the uncapped (C) and capped (D) blunt-ended RNA (substrate 13).

**Fig. 7.** Effect of NC on RNase H cleavage of recessed RNA. Substrate 1 containing the recessed 5′ end-labeled 41-nt RNA was used. A schematic of the substrate is shown above the panel. A, time course of RNase H assays in the absence (left) and presence (right) of NC. B and C, quantitation of the data from A showing the effect of NC on the overall RNase H (B) and secondary cut (C) efficiency. To determine cleavage efficiency, the amount of the specific cleavage product was calculated as the percentage of the total starting material. The reaction with NC is shown in gray, whereas the reaction without NC is in black.
structure migrated slightly more slowly than the corresponding length un capped RNA and its products. Segments smaller than 8 nt, derived from either the capped or uncapped RNA, migrated to equivalent positions because they resulted from internal cuts and did not contain the cap structure. The capped RNA had similar RNase H cleavage speci city and efficiency as the uncapped RNA, indicating that the 5′ end cap structure did not have a detectable effect on the rate or specificity of cleavage.

**Influence of the NC Protein on the Secondary Cut—Peliska and co-workers (32) show that NC enhanced cleavage of a blunt-ended RNA-DNA hybrid substrate with a sequence derived from a 3′ end region of the HIV genome. The NC particularly stimulated generation of cleavage products 7–9 nt in length. This prompted us to examine more closely the effect of NC on both specificity and efficiency of RT RNase H cleavages on various RNA/DNA hybrid substrates. The 72-amino acid form of HIV-1 NC protein was used for this study. We first determined whether NC enhanced cleavage of the recessed 41-nt RNA substrate. NC had no significant effect on the specificity of cleavage (Fig. 7A). However, the presence of NC only slightly increased both the overall cleavage efficiency and the secondary cut efficiency by about 10% (Fig. 7B) and 25% (Fig. 7C), respectively.

We then tested the effect of NC on the cleavage of the blunt end 41-mer RNA. Again, NC did not change the cleavage speci city (Fig. 8A). It moderately increased the overall cleavage efficiency by about 20–35% (Fig. 8B). However, the presence of NC had a striking effect on the secondary cut efficiency, especially at earlier time points. In the absence of NC, secondary cut products appeared only after 1 min. However, the 8-nt product arose within 15 s in the presence of NC. NC increased the secondary cut efficiency by 4–7-fold (Fig. 8C). These results suggest that the main effect of NC on blunt end substrates is to stabilize RT binding for the secondary cut.

Finally, we tested the effect of NC on cleavage of uncapped and capped 28-mer blunt end HIV RNA used previously (Fig. 9). Once again, NC did not change the cleavage specificity (data not shown). For both uncapped and capped RNA substrates, NC increased the cleavage rate. The overall cleavage was increased by modest a 10–20%. However, the secondary cut rate was increased by a factor of 2–3 (Fig. 9, A–D). Again, these data indicate that the 5′ cap structure does not affect the RNase H cleavage even in the presence of NC. In addition, the 55-amino acid form of HIV-1 NC was also tested for the experiments discussed above, and similar results were obtained (data not shown).

**DISCUSSION**

We have previously examined the mechanism of the polymerization-independent mode of RNase H activity of the HIV RT (25–28). This mode is proposed to be employed by the RT to remove segments of RNA left annealed to the newly made minus-strand DNA. RT was shown to align to the 5′ end of the RNA and to rapidly make a primary cut about 18 nt into the RNA (Fig 1) (25, 26, 28). The enzyme then repositions to make a secondary cut approximately in the middle of the 18-nt segment, producing segments small enough for rapid dissociation. Previously, we have shown that chemical modifications of the substrate that prevents the primary cut still allows the secondary cut, indicating that the cuts are not linked in an obligatory fashion (27).

In this study, we have further examined the structural features of the substrate that allow efficient 5′ RNA-directed RNase H activity. We show that a blunt-ended RNA/DNA hybrid makes appropriate contacts with the RT to sustain efficient primary cleavage. However, this substrate lacks struc-
tures that allow effective positioning for secondary cleavage. A critical parameter is the length of template DNA extension, which greatly influences the binding orientation of the RT and, consequently, the position and rate of RNase H-directed secondary cleavages. Thus, when the substrate contains a DNA 3’ extension, secondary cuts are made with very high efficiency at a rate of \(5.5 \times 10^{-4}\) nM/ms (28). In contrast, on blunt-ended substrates RT appears to be able to move effectively to make a 15-nt product, but the rate at which this enzyme can position for the secondary cut is very slow. Interestingly, this result shows that a DNA extension as short as 3 nucleotides distinctly enhances the rate of secondary cleavage. Greater extensions lead to further enhancement. When the DNA 3’ end is recessed on the RNA, the RNase H cleavages indicate two binding orientations of RT on the substrate, the RNA 5’ end-directed orientation and the DNA 3’ end-directed orientation. The latter orientation is one where, in the presence of dNTPs, RT can prime synthesis. When the DNA is recessed on the RNA by several nucleotides, RNase H cuts are formed by RT predominantly positioning in the polymerizing mode. In the absence of synthesis, after making the primary cut, RT translocates to generate secondary cuts that further fragment the RNA at the DNA primer terminus. The mechanism involved is probably related to that employed by the RT to make primary and secondary cuts during a pause in synthesis. Remarkably, as little as a single nucleotide DNA recess allows only a small minority of the RTs to engage in 5’ RNA end-directed cleavage, indicating that the natural positioning of the polymerase via the DNA primer terminus is the highest affinity binding orientation on such duplexes.

After the primary cleavage, RT translocates 5’ of this site to create subsequent cuts that further fragment the primary product. Apparently, such translocations are more efficiently supported on double-stranded regions than on single-stranded extensions. The presence of an immediately upstream RNA primer annealed to the DNA template not only causes an additional improvement in the rate of secondary cleavage but allows the RNA to be cut into even smaller pieces. Studies with E. coli RNase H show that the smallest products created are ~6 nt, suggesting that anything smaller cannot bind DNA with enough stability for RNase H recognition. This suggests that the normal 8-nt product made on a recessed RNA annealed to a DNA template is a manifestation of the distance that the RT can move from the primary cut site while still remaining bound. Presumably, as RT moves further onto the single strand DNA extension, binding stability is reduced. In the presence of an upstream RNA, RT slides further 5’ to create cuts 2–5 nt from the 5’ end. Such a mechanism can be thought to occur during the normal degradation of the genomic RNA, where adjacent RNA fragments enhance the overall efficiency of RNA removal.

An important step in the conversion of the viral single-stranded RNA to double-stranded DNA is the synthesis of minus strong stop DNA. Synthesis of this DNA is initiated at an internal position on the viral RNA and is extended to the 5’ end of the genome. Degradation of the RNA frees the cDNA, allowing the minus strong stop DNA to anneal to complemen-
tary sequences near the 3' end of the viral RNA, thereby facilitating minus strand stop transfer. However, the RNA-DNA hybrid at the 5' end of the viral RNA should be resistant to cleavage because the fragment of RNA lacks the 3' DNA extension. We considered that additional factors present during viral replication might improve cleavage efficiency. One such factor is the message cap present at the 5' end of the natural HIV genome. However, examination of the cleavage of the RNA/DNA hybrid containing sequences from the 5' end of the HIV genome revealed that the presence of the message cap had little effect on the specificity or rate of cleavage.

Analyzing cleavage of a blunt-ended RNA-DNA hybrid having the sequence from the 3' end of the HIV genome, Peliska and Benkovic (32) observe that RNase H was enhanced in the presence of NC. Their model envisions that NC interacts both with RT and the RNA/DNA duplex to facilitate delivery of the substrate into the RNase H site, thereby enhancing the overall efficiency of cleavage. Expanding on their finding, we show that the presence of NC specifically enhances the secondary cleavage. This is a general effect that occurred with both the 41 nt RNA blunt hybrid substrate and the viral sequence of the minus strong stop DNA-RNA hybrid. Furthermore, the enhancement also occurred on capped viral RNA hybrid.

The NC-promoted increase in blunt end secondary cut efficiency on the 28 nt viral RNA was not as great as that observed on the 41 nt RNA substrate. This difference may derive from substrate length rather than sequences. In support of this, hybrid substrates containing 54- and 71 nt RNA segments corresponding to the 5' end of the HIV-1 genome showed a similar increase in secondary cleavage as observed with the 41-mer non-viral sequence in the presence of NC. The exact mechanism of how NC facilitates these cleavages is not clear. Possibly, NC increases RT binding on the substrate, particularly the sub-optimal binding during positioning for the secondary cut on a blunt-ended substrate, thereby preventing RT dissociation. Alternatively, NC may alter the structure of the RNA-DNA hybrid so that the substrate is more susceptible to RNase H cleavage. In this case a transient interaction of the RT at the secondary cut site would be more productive for cleavage. The greatly increased secondary cut on the blunt-ended substrate should further enhance the NC-facilitated strand annealing of the strong stop DNA 3' end and acceptor RNA, thereby increasing the overall efficiency of strand transfer.

Additionally, NC has been proposed to be necessary to prevent the minus strand strong stop DNA, which terminates in the very stable TAR hairpin, from folding back on itself and forming dead end synthesis products (34, 35). Such fold-back products if made in vivo would interfere with strand transfer, causing premature termination of reverse transcription.

Several previous studies have reported on secondary cleavages at the blunt end of the viral RNA. Studies from Levin and co-workers (36) show that the final product of donor RNA 5' end degradation during minus-strand transfer is 8 nt long in the absence of NC. This suggests that the secondary cut is linked to the transfer reaction. Our results agree with those of Guo et al. (36) and Peliska and Benkovic (31) that RNase H cuts at the 5' end of this substrate result in products ~8 nt in length. Hughes and co-workers (37, 38) report a somewhat different observation while following RNA cleavage during minus strand strong stop DNA synthesis. In the absence or presence of NC, they observe the smallest cleavage product from the RNA 5' end to be 14 nt long. The authors also report that the 14 nt fragment is readily dissociated from the DNA, whereas NC keeps this fragment annealed. Although differing from our observations and those reported by others (31, 37–39), these results emphasize that the terminal RNA might not be cleaved at the same rate as internal RNA segments.

A number of previous reports examine the degradation of the RNA genome by HIV RT into small fragments and the role of the RNase H-directed secondary cut (6, 29–32). Secondary cleavage is observed during both polymerization-dependent RNase H activity and the polymerization-independent RNase H activity. When the RT is aligned to the polymerase active site, primary and secondary cleavages can be readily detected in the absence of dNTPs. Several groups have determined amino acid residues of HIV-1 RT that are important for the formation of the secondary cut. Studies have examined properties of mutant RTs with altered residues within the primer and template binding domains. Mutations Y232A (40, 41) and Y181C (42) enhance the binding of RT to the secondary cut site. The Y232A mutation in the primer grip region is thought to enhance binding to the DNA primer strand, thereby increasing secondary cut formation. The Y181C mutation is located close to the polymerase active site residues and may influence substrate contacts near this site. The contacts that this mutation may enhance within RT are further away from the RNase H active site than the primer grip and may require longer strand extensions for stable protein-substrate interaction.

In this study we examined factors that facilitate secondary cuts during RNase H cleavage. Results with the RNA/DNA duplexes containing various lengths of 3' DNA or 5' RNA overhangs indicate that RT utilizes the single-stranded extensions for movement and repositioning for efficient secondary cleavage. In blunt-ended substrates, which lack this structural feature, secondary cleavages became less efficient. This prompted us to examine whether additional factors facilitate secondary cuts on the blunt-ended replication intermediate. The cap structure did not alter the cleavage efficiency. However, the viral NC protein enabled the enzyme to bind in a manner allowing efficient secondary cuts while also enhancing the overall RNase H activity.

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REFERENCES

1. Champoux, 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
2. Telemisitsky, A., and Goff, A. P. (1993) in Reverse Transcriptase (Skalka, A. M., and Goff, S. P., eds) pp. 49–83, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
3. di Marzo Veronese, F., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlán, S., Gallus, R. C., and Sarangadharn, M. G. (1986) Science 231, 1289–1291
4. Lightfoote, M. M., Coligan, J. E., Fau, A. S., Martin, M. A., and Venkataraman, S. (1986) J. Virol. 60, 771–775
5. Jacobo-Molina, A., and Arnold, E. (1991) Biochemistry 30, 6531–6536
6. Gopalakrishnan, V., Peliska, A., and Benkovic, S. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10763–10767
7. Gotte, M., Maier, G., Gross, H. J., and Heumann, H. (1998) J. Biol. Chem. 273, 10129–10146
8. Gopalakrishnan, V., Peliska, A., and Benkovic, S. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6320–6326
9. Pullen, K. A., Ichimoto, L. K., and Champoux, J. J. (1993) J. Virol. 66, 367–373
10. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Stett, T. A. (1992) Science 256, 1783–1789
11. Hsu, Y., Ding, J., Tan, X., Moore, L. R., and Benkovic, S. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 90, 5830–5834
12. Peters, G. G., and Hu, J. (1980) J. Virol. 35, 31–40
13. Peters, G. G., and Hu, J. (1980) J. Virol. 36, 692–700
14. Cameron, C. E., Ghosh, M., Le Grice, S. F., and Benkovic, S. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6700–6705
15. Kanaya, S., Kuhara, M., Inoue, H., Ohta, E., and Ichihara, N. (1991) J. Biol. Chem. 265, 4615–4621
16. Mizrahi, V., Ustsd, M. T., Harington, A., and Dudding, L. (1990) Nucleic Acids Res. 18, 5539–5543
17. Katayanagi, M., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S.,...
Ikehara, M., Matsuzaki, T., and Morikawa, K. (1990) *Nature* **347**, 306–309
19. Yang, W., Hendrickson, W. A., Crouch, R. J., and Satow, Y. (1990) *Science* **249**, 1398–1405
20. Smith, J. S., and Roth, M. J. (1993) *J. Virol.* **67**, 4037–4049
21. Smith, J. S., Gritsman, K., and Roth, M. J. (1994) *J. Virol.* **68**, 5721–5729
22. Levin, J. G., Crouch, R. J., Post, K., Hu, S. C., McKelvin, D., Zweig, M., Court, D. L., and Gerwin, B. I. (1988) *J. Virol.* **62**, 4376–4380
23. Yang, W., Hendrickson, W. A., Crouch, R. J., and Satow, Y. (1990) *Science* **249**, 1398–1405
24. Smith, J. S., Gritsman, K., and Roth, M. J. (1994) *J. Virol.* **68**, 5721–5729
25. Levin, J. G., Crouch, R. J., Post, K., Hu, S. C., McKelvin, D., Zweig, M., Court, D. L., and Gerwin, B. I. (1988) *J. Virol.* **62**, 4376–4380
26. Wohrl, B. M., Tantillo, C., Arnold, E., and Le Grice, S. F. (1995) *Biochemistry* **34**, 5343–5356
27. Furfine, E. S., and Reardon, J. E. (1991) *J. Biol. Chem.* **266**, 406–412
28. DeStefano, J. J., Mallaber, L. M., Fay, P. J., and Bambara, R. A. (1994) *Nucleic Acids Res.* **22**, 3793–3800
29. DeStefano, J. J., Mallaber, L. M., Fay, P. J., and Bambara, R. A. (1993) *Nucleic Acids Res.* **21**, 4330–4338
30. Palaniappan, C., Fuentes, G. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1996) *J. Biol. Chem.* **271**, 2063–2070
31. Palaniappan, C., Fuentes, G. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1996) *J. Biol. Chem.* **271**, 2063–2070
32. Wisniewski, M., Balakrishnan, M., Palaniappan, C., Fay, P. J., and Bambara, R. A. (2000) *J. Biol. Chem.* **275**, 37664–37671
33. Wisniewski, M., Balakrishnan, M., Palaniappan, C., Fay, P. J., and Bambara, R. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 11978–11983
34. Fu, T. B., and Taylor, J. (1992) *J. Virol.* **66**, 4271–4278
35. Gbbara, S., Davis, W. R., Hupe, L., Hupe, D., and Peliska, J. A. (1999) *Biochemistry* **38**, 13070–13076
36. Peliska, J. A., and Benkovic, S. J. (1992) *Science* **258**, 1112–1118
37. Peliska, J. A., Balasubramanian, S., Giedroc, D. P., and Benkovic, S. J. (1994) *Biochemistry* **33**, 13817–13823
38. de Rocquigny, H., Ficheux, D., Gabus, C., Fournier-Zaluski, M. C., Darlix, J. L., and Roques, B. P. (1991) *Biochem. Biophys. Res. Commun.* **180**, 1010–1018
39. Guo, J., Wu, T., Anderson, J., Kane, B. F., Johnson, D. G., Gorelick, R. J., Henderson, L. E., and Levin, J. G. (2000) *J. Virol.* **74**, 8980–8988
40. Guo, J., Henderson, L. E., Bess, J., Kane, B., and Levin, J. G. (1997) *J. Virol.* **71**, 5178–5188
41. Guo, J., Wu, T., Bess, J., Henderson, L. E., and Levin, J. G. (1998) *J. Virol.* **72**, 6716–6724
42. Driscoll, M. D., Golinelli, M. P., and Hughes, S. H. (2000) *J. Virol.* **75**, 672–686
43. Gao, H. Q., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2001) *J. Virol.* **75**, 11874–11880
44. Ghosh, M., Howard, K. J., Cameron, C. E., Benkovic, S. J., Hughes, S. H., and Le Grice, S. F. (1995) *J. Biol. Chem.* **270**, 7068–7076
45. Guo, J., Wu, W., Yuan, Z. Y., Post, K., Crouch, R. J., and Levin, J. G. (1995) *Biochemistry* **34**, 5018–5029
46. Archer, R. H., Dykes, C., Gerondelis, P., Lloyd, A., Fay, P., Reichman, R. C., Bambara, R. A., and Demeter, L. M. (2000) *J. Virol.* **74**, 8390–8401