Interaction of HIV Reverse Transcriptase with Structures Mimicking Recombination Intermediates*

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Aarti Raja‡ and Jeffrey J. DeStefano§
From the Department of Cell Biology and Molecular Genetics, University of Maryland College Park, College Park, Maryland 20742

Interactions between human immunodeficiency virus (HIV) reverse transcriptase (RT) and structures mimicking intermediates proposed to occur during recombination (strand transfer) were investigated. One mechanism proposed for strand transfer is strand exchange in which a homologous RNA (acceptor) “invades” a donor RNA:DNA duplex (replication intermediate) on which DNA synthesis is occurring. The acceptor displaces the donor of the duplex and binds to the DNA. During exchange a transient trimeric structure forms. A model structure was designed with a replication intermediate to which an acceptor RNA was bound. The acceptor was bound to the 5′-end of the DNA over a 54-base region, whereas the donor associated with the DNA 3′-end over a 28-base region. The dimeric constituents of the trimer (acceptor RNA:DNA and donor RNA:DNA) were also constructed. The acceptor RNA:DNA formed a branched structure in this case. Results showed that RT could cleave the RNA portion of all the structures examined. Association with junction substrates was less stable as determined by off-rates. On the trimer, RT cleaved both RNAs but showed a clear preference for cleaving the donor RNA region. This preference was accentuated by HIV nucleocapsid protein (NC). Results suggest that during recombination RT generally associates with the donor-RNA portion of the trimer and the acceptor RNA is protected but not immune from cleavage. The partial protection likely allows the acceptor RNA to more easily complete strand exchange and shield this RNA to provide a means to salvage replication if the DNA were to dissociate from the cleaved donor RNA.

Strand transfer is an essential step in retroviral replication and has been shown to occur from terminal regions and internal regions of RNA templates. Minus strand DNA strand transfer is proposed to potentially occur by two different mechanisms (1). In one, DNA being synthesized on an RNA template (referred to as the donor in this case) dissociates from the RNA and binds to a homologous region on a second RNA (referred to as the acceptor) where synthesis continues. A second mechanism proposes that the acceptor RNA actively participates in the displacement of the DNA strand by associating with the DNA and dislodging the donor. Because retroviruses are diploid, the donor and acceptor RNAs would represent the two genome copies in the capsid. Results from previous experiments suggest that viral nucleocapsid protein (NC) stimulates the binding of a complementary RNA acceptor template to the DNA displacing the donor RNA in the process (2). Consistent with the second mechanism above, this implies that strand transfer could proceed through a trimeric complex consisting of the nascent DNA bound to both the RNA template it was being made on (donor) and a second homologous RNA (acceptor). Such a structure has been shown to occur during DNA transfer in vitro (2, 3). Models developed in vivo (4) and in vitro (5, 6) also indicate the likely existence of a trimeric intermediate as one of the mechanisms used for strand transfer during recombination. Little is known about what activities RT manifests on such a structure. The binding and orientation of RT to such a structure would become important, because both the RNA templates are accessible to the enzyme. The fate of the acceptor template is important, because degradation of the acceptor prior to transfer would eliminate a potential pathway to continue DNA synthesis if the nascent DNA were to dissociate from the donor template.

In this report, experiments were designed to characterize the activity of RT on strand transfer intermediates with the goal of determining if RT has a preference for associating with the donor or acceptor RNA on a trimeric strand transfer intermediate and if the RNase H activity of RT cleaves these RNAs. Three separate templates were designed to mimic the entire strand transfer intermediate (trimeric structure) or various parts of this structure. One template consisted of the donor RNA bound to the DNA (donor RNA:DNA), which mimics the “replication intermediate.” The second template consisted of the acceptor RNA bound to the DNA such that the bases at the 3′-end of the DNA are not hybridized to the acceptor (acceptor RNA:DNA). This mimics the binding of the acceptor RNA to the DNA of the replication intermediate. The third structure, the strand transfer intermediate, contains both the donor and acceptor RNAs bound to DNA. Previous results have indicated that RT is anchored to nucleic acid substrates by a 3′ recessed DNA terminus or a 5′ recessed RNA terminus (7–13). With this in mind, RT would be expected to bind preferentially to the donor RNA of the trimer, because the 3′-end of the DNA strand is recessed on the donor RNA. In contrast, there is no 3′ recessed DNA end on the acceptor RNA and the 5′-end of the acceptor is not associated with the DNA suggesting that RT would not efficiently cleave the acceptor RNA. Results showed that RT cleaved both RNAs of the trimer but with a clear

‡ Present address: Dept. of Cancer Immunology and AIDS, Dana-Farber Cancer Inst. and the Dept. of Pathology, Division of AIDS, Harvard Medical School, Boston, MA 02115.
§ To whom correspondence should be addressed: Dept. of Cell Biology and Molecular Genetics, University of Maryland, Bldg. 231, College Park, MD 20742. Tel.: 301-405-5449; Fax: 301-314-9489; E-mail: jd146@umail.umd.edu.

† The abbreviations used are: NC, nucleocapsid protein; RT, reverse transcriptase; HIV, human immunodeficiency virus; RNase H, ribonuclease H, DTT, dithiothreitol.
preference for cleaving the donor RNA region. In the presence of HIV nucleocapsid protein (NC), this preference was even stronger. The findings imply that during recombination RT generally associates with the donor-RNA portion of the trimeric recombination intermediate and the acceptor RNA is protected but not immune from cleavage. The partial protection likely allows the acceptor RNA to more easily complete strand exchange and shield this RNA to provide a means to salvage replication if the DNA were to dissociate from the cleaved donor RNA.

EXPERIMENTAL PROCEDURES

Materials—Wild-type recombinant HIV-RT was graciously provided to us by Genetics Institute (Cambridge, MA). Aliquots of the enzyme were stored at −70 °C, and a fresh aliquot was used for each experiment. T4 polynucleotide kinase was from United States Biochemical. HIV-1 nucleocapsid protein (NC) was obtained from Enzyco (Denver, CO). SP6, T3 RNA polymerase, calf intestinal alkaline phosphatase, and rNTPs were obtained from Roche Molecular Biochemicals. Promega. Deoxyoligonucleotides were synthesized by Genosys Inc. (The Woodlands, TX). Proteinase K was obtained from Sigma. All other chemicals were from Fisher Scientific. Radiolabeled compounds were from PerkinElmer Life Sciences.

Construction of Substrates—Run-off transcription was performed according to the instructions of the enzyme manufacturer. The donor RNA (5’-GGGAAACAAAAUUGCCUCAUAGUGAGUCGUAAUC-AUACUGG) was made from H11032 cleavage of pBSM13(Δ(14)), and T3 RNA polymerase was used to prepare run-off transcripts 262 nucleotides in length. For the acceptor RNA (5’-GAUAUCACUGGUAAUCUAGGUAAUCUACAAGGUAAUCUAGGUAAUCUAGG), the vector plasmid pGEM-11ZF(+) was cleaved with BamHI, and plasmid pBSM13 was cleaved with EcoRI and HindIII. All termini were filled in with Klenow polymerase. The EcoRI/HindIII insert on pBSM13 was isolated and ligated into the plasmid vector pGEM-11ZF+ and transformed into E. coli XL1 blue cells. Blue white screening was used to isolate white colonies that contained the pGEM-11ZF+ plasmid with the insert. The plasmid with the insert was isolated and used to obtain the acceptor RNA. The plasmid was cleaved with PstI, and SPS RNA polymerase was used to prepare run-off transcripts 262 nucleotides in length. Reactions for both the donor and acceptor RNA were extracted with phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and precipitated with ethanol. The acceptor RNA was further cleaved to bases 94 of length. For the acceptor RNA (5’-GAAUACUCAAGCUUAU- AAUCACUGG) was made from

RNase H Cleavage Assays—In the standard RNase H cleavage assay, 5 μl of either the heteroduplex or the trimeric substrate was incubated in a volume of 10.5 μl containing the following components at their final concentrations: 50 μl Tris-HCl (pH 8.0), 80 μl KC1, 7.1 μM MgCl2, 0.2 μl/μl RNasin, 1 mM DTT, 0.1 mM EDTA (pH 8.0), and 2 μl Amp at 37 °C. Reactions were initiated with the addition of 2 units (0.4 pmol) or in some 0.2 unit (0.04 pmol) of HIV-RT in a 2-μl solution containing 50 μl Tris-HCl (pH 8.0), 1 mM DTT, and 80 μl KC1. The eluate was centrifuged and filtered as described above and used directly for experiments.

Quantification of Nucleic Acids—Donor and acceptor RNA templates were quantified spectrophotometrically by measuring the absorbance. The molecular weight of the RNA was used to determine the molar concentrations of the RNA templates. The amount of radiolabeled heteroduplex and trimeric substrates recovered from native gels was determined by specific activity. Quantification of RNase H cleavage products was accomplished by scanning the dried polyacrylamide gels with a phosphorimaging device (Bio-Rad, GS 525).

Results

HIV-1 nucleocapsid protein (NC) was obtained from Enzyco (Denver, CO), and SP6 RNA polymerase was used to prepare run-off transcripts 262 nucleotides in length. Two other DNAs were also used in the construction of dimeric substrates, a second 93-base DNA (5’-GAGGATCAGCTT-GCATGTCCTAGGCGTACCTCGTAGAGGATCCCGGGTGACCGCT-GAATTGGCCAGTGAATTGTAATACGACTCACTATA) was also radiola-
Fig. 1. Structure of substrates. Shown are schematic diagrams of the trimer and its constituent dimeric parts (acceptor RNA-DNA and donor RNA-DNA) as well as other substrates derived from the acceptor RNA-DNA (acceptor RNA-DNA (recessed 3′-DNA)) and acceptor RNA-DNA (+10 hybrid). The positioning and orientation of HIV-RT on the donor RNA-DNA is also shown. Numbers indicate the lengths of the various regions in nucleotides. Asterisks indicate positions of the 32P label.

containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 10 mM KCl, 0.2 unit/μl RNasin, 0.1 mM EDTA (pH 8), and 5 mM AMP at 37°C. Two microliters of a solution containing 2.5 μg of poly(rA)-oligo(dT20) trap in the above buffer was added to the reaction and allowed to incubate further. At various times after trap addition, 2 μl of a solution containing 37.5 mM MgCl2 in the above buffer was added to initiate the cleavage reaction, and samples were incubated for 5 min. The reactions were terminated by the addition of 12.5 μl of 2× gel loading buffer, and electrophoresis was performed as described above. For the time zero point, trap and MgCl2 were added at the same time. Determinations of koff values were performed by fitting the data for the relative amount of cleaved RNA versus time to an equation for exponential decay (f(x) = ae−bx), where a = 1, f(x) = y (relative cleavage), x = time, and b = koff value. In these experiments a slight modification was made. The value at 10 s was set equal to the starting relative value of 1 rather than the time 0 value. This allowed a significantly better fit of the data to the equation. This is probably due to the addition of MgCl2 along with the trap for time 0. Divalent cation greatly stabilizes binding of RT to the substrate (16). Because HIV-RT likely exists in a closed (tight binding) and open (weak binding) conformation (17–19), those bound in open conformation may dissociate in the presence of trap and absence of Mg2+. This would result in a disproportionately high cleavage amount at the time 0 point as was observed.

Gel Electrophoresis—Denaturing 8% polyacrylamide gels (19:1 acrylamide:bisacrylamide), containing 7 % urea, and native 6% polyacrylamide gels (29:1 acrylamide:bisacrylamide) were prepared and subjected to electrophoresis as described (15).

RESULTS

Construction of Substrates—The substrates used for RNase H cleavage assays were used under “Experimental Procedures” and are depicted schematically in each autoradiogram. Five different structures were used. The main structure from which the others were derived is referred to as the “trimer” (Fig. 1). It consisted of three nucleic acid strands, the donor and acceptor RNAs, and a DNA strand. The donor RNA and acceptor RNA were transcribed in vitro and radiolabeled at the 5′-end with 32P. The DNA oligonucleotide was also 5′-end-labeled. The donor RNA was 50 nucleotides in length, acceptor RNA approximately (see “Experimental Procedures”) 99 nucleotides in length, and the DNA 93 nucleotides in length. The acceptor RNA-DNA structure was designed such that a hybrid region of 54 bases formed between the 3′-end of the RNA and an internal portion of the DNA that started 30 bases from the 3′-end of the DNA. The donor RNA was designed such that a hybrid region of 28 bases formed between the 3′-ends of the DNA and donor. This design essentially left one unhybridized base (29th base from the 3′-DNA-end) between the donor and acceptor RNAs. There were also 10 bases at the 5′-end of the DNA that were not hybridized to RNA. Note that the 28-base hybrid formed between the donor RNA and the DNA is longer than the presumed 18 bases between the polymerase and RNase H active sites of HIV-RT. However, it is still within the realm of what is likely to exist in vivo. Because RNase H activity is generally only about one-tenth the polymerase activity (20), hybrid regions between the nascent DNA and genomic RNA are likely to range from as long as about 30 bases down to sizes too small to remain stably associated (21, 22). In addition to the timer, dimeric constituents consisting of the acceptor (acceptor RNA-DNA) or donor (donor RNA-DNA) bound to the DNA were also examined. Finally, two additional structures based on the acceptor RNA-DNA were also used. In one the hybrid region between the acceptor and DNA was increased to 64 bases by moving the junction 10 bases toward the 3′-end of the DNA (terminated acceptor RNA-DNA (+10 hybrid)). In the second the DNA was shortened by 29 bases at the 3′-end to produce a 64-nucleotide DNA. The resulting structure (terminated acceptor RNA-DNA (recessed 3′-DNA)) eliminated the branch point of the original acceptor RNA-DNA structure and produced a recessed 3′-DNA terminus.

To ensure that the appropriate structures were used, it was important to determine that the DNA remained associated with the RNA for both the duplexes and the trimeric structures. For all preparations of substrates obtained, this was verified by examination of the recovered material by native gel electrophoresis. As can be seen from Fig. 2, the various complexes and single-stranded nucleic acids migrated to unique positions on the native gel. This allowed recovery of pure material and confirmation of the integrity of the isolated complexes. It should be noted that the acceptor RNA-DNA and trimer structures represent “model substrates” of intermediates occurring during strand transfer. An authentic trimeric transfer intermediate would contain an acceptor RNA that was complementary to the 3′-end of the DNA. Various conditions were used in an attempt to isolate “authentic” structures by first forming donor RNA-DNA, then adding acceptor RNA that
was complementary to the complete DNA of the donor RNA-DNA. In all cases an acceptor RNA-DNA structure devoid of donor RNA was resolved on the native gels. Regardless, the model substrates that were produced should yield valuable information concerning the recognition of strand transfer intermediates by RT and the mechanism of RNase H cleavage on the substrates.

Cleavage of RNA in Heteroduplex Structures—RNase H cleavage assays done on heteroduplex structures provide information about the binding site of RT. When the donor RNA was bound to the DNA in the heteroduplex (donor RNA-DNA), results were as expected given the distance between the polymerase and RNase H active sites on RT and the 3’-5’ directional cleavage activity of RT (data not shown) (7, 8, 21, 22). The enzyme initially cleaved the RNA ~18 bases behind the 3’-end of the DNA, and the larger initial cleavage products were subsequently chased into smaller products over time by the 3’-5’ nuclease activity of the RT. This results in a shortening of the hybrid region between the DNA and RNA and eventually can cause dissociation of the nucleic acids.

In the heteroduplex structure mimicking the binding of the acceptor RNA to the DNA, cleavage of the acceptor RNA was observed (Fig. 3, acceptor RNA-DNA). This was in contrast to what had been reported previously. Work by others using RNA bound to single-stranded circular DNA suggested that RT could cleave structures with the 5’-end of the RNA not bound to the DNA. However, this was providing that the unbound 5’-RNA end was less than 12 nucleotides in length (11). Based on these results alone it would be predicted that the acceptor RNA-DNA duplex would be resistant to cleavage, because the acceptor RNA has an unbound 5’-end that is 45 nucleotides away from the hybrid region (see “Discussion” also). Despite this results indicated that the RNA was cleaved. The product sizes at the early time points were diverse indicating that RT was initially bound to the structure such that the RNase H active site contacted the RNA at several positions. The shortest (and most prominent) RNA observed corresponded to cleavage 15 bases behind the junction of the RNA-DNA hybrid (60-nucleotide product). Products longer than 60 nucleotides were processed over time to produce more of the 60-base product, whereas small amounts of 56-nucleotide and shorter products were observed later in the reaction. Assays in which E. coli RNase H was added after RT digestion indicated that the 60-nucleotide RNA was still bound to the DNA (data not shown). Overall results suggested that, after somewhat random (although with a preference for sites near junction) initial cleavages, subsequent cleavages proceed toward the RNA-DNA junction. Proceeding at the junction to produce a small unstable hybrid region appeared to be slow.

To compare the cleavage of the acceptor RNA-DNA substrate to cleavage of a more standard substrate with a 3’ recessed DNA termini, a 64-nucleotide DNA that bound the RNA and terminated at the end of the hybrid region was constructed (acceptor RNA-DNA (recessed 3’-DNA)). The DNA was identical to the first 64 bases at the 5’-end of the 93 base DNA used in the acceptor RNA-DNA substrate but was missing the 29 bases at the 3’-end. This configuration should direct RT cleavage ~18 bases behind the DNA 3’ terminus (~18 position). Results of a cleavage assay are shown in Fig. 3 (acceptor RNA-DNA (recessed 3’-DNA)). Early time points revealed initial cleavages 19 (64-nucleotide product) and 16 (61-nucleotide product) bases behind the 3’-end. There were also some non-specific cleavages that produced longer products but the ~19 and ~16 cleavages were clearly the most prominent. At later points cleavages at ~11 (56 bases) were observed as the levels of the larger products decreased. The results indicated that cleavage on the standard substrate with a recessed 3’ terminus leads to the expected results with a large proportion of initial cleavages occurring from RT molecules bound at the 3’ terminal. Subsequent 3’-5’ processing results in many of these products being processed to smaller products that likely destabilize the RNA-DNA hybrid. In contrast, on the acceptor RNA-DNA the RNA-DNA junction only loosely directed cleavage and processing was partially inhibited (see above).

To determine how the position of the junction affected cleavage a substrate was constructed in which the position of the junction was advanced by 10 nucleotides toward the 3’-end of the DNA. This produced an acceptor RNA-DNA substrate with a 64-base as opposed to a 54-base hybrid region. Results with this substrate are shown in Fig. 3 (acceptor RNA-DNA (+10 hybrid)). At early time points, as with the original acceptor RNA-DNA, an array of products were seen with the distribution shifted toward products produced by cleavages nearer the junction. Prominent products 56 (~21 relative to the junction), 54 (~19), 50 (~15), 44 (~9), and 43 (~8) nucleotides were observed. Most products were processed to the 44- and 43-base
products at latter time points. This was in contrast to the original acceptor RNA-DNA where processing to smaller products was slowed. Results indicate the junction structure does not directly inhibit processing to shorter products.

In the above experiments cleavages were carried out under conditions that allowed multiple binding and rebinding events between RT and the substrates. This makes it difficult to determine if the cleavages occurring even at early time points were initial or secondary cleavages. To more clearly determine the initial cleavage positions trap assays were performed with the substrates (Fig. 4). In these assays RT is preincubated with the substrate then reactions are initiated by addition of MgCl₂ and poly(rA)-oligo(dT) trap. The trap sequesters enzymes that dissociate from the substrate thus limiting cleavage to a single binding event (21). Because RT can catalyze both primary (initial) and secondary cuts in one binding event, the technique does not show only initial cleavage positions, however, secondary cuts occur slowly, so at early time points primary cleavages represent the majority of the observed products (7, 10, 12, 13).

A trap control assay in which RT was preincubated with trap was performed for each substrate (lanes E). A low level of cleavage was observed in these lanes in comparison to the starting material (lane F, shown for acceptor RNA-DNA only) indicating that the trap was not 100% effective. However, the level of trap read-through was very low and did not interfere with interpretations of the results. For the substrate with the recessed 3’ termini (acceptor RNA-DNA (recessed 3’-DNA)) the −19 and −16 (64 and 61 nucleotides, respectively) products identified above were clearly the dominant cuts at the 2- and 5-s time points, in agreement with initial cleavage being directed by the position of the 3’ recessed end. The preference was not as striking on the acceptor RNA-DNA substrates, however, cleavage at the −15 (60 nucleotide product) position relative to the junction was most prominent on the original acceptor RNA-DNA. On the acceptor RNA-DNA (+10 hybrid), cleavages at −21 (56 bases) and −15 (50 bases) were most prominent. This suggests that RT has some preference to bind and cleave near the junction.

The stability with which RT bound to the junction versus substrate with recessed 3’ terminus was also investigated. To eliminate possible sequence-specific effects, the acceptor RNA-DNA and the acceptor RNA-DNA (recessed 3’-DNA) rather than the donor RNA-DNA were used. A modified version of the trap assay was used to measure the rate of dissociation (kₐff) of RT from the substrate. For these experiments the KCl concentration was reduced to 10 molar to decrease the rate of dissociation. Using 80 molar KCl RT dissociates rapidly in the absence of MgCl₂ (16). Divalent cation must be omitted in the preincubation phase of the assay, because it would stimulate cleavage of the substrate. For this assay, RT was preincubated with substrate then a supplement containing poly(rA)-oligo(dT) trap was added. Incubation was continued for a fixed time then a supplement containing MgCl₂ was added to initiate cleavage and incubations were continued for 2 min. During the incubation with trap RT molecules that dissociate from the substrate are sequestered. The greater the time between trap and divalent cation addition the more dissociation occurs. This results in a progressive decrease in cleavage that can be used to determine the half-life of the complex (see “Experimental Procedures”). Fig. 5 (A and B) shows the results of a typical kₐff experiment. Clearly dissociation is considerably slower for acceptor RNA-DNA (recessed 3’-DNA). This indicates that the presence of a recessed 3’-DNA terminus increases the stability of RT binding relative to the junction substrate. Values for kₐff were 0.015 ± 0.003 and 0.008 ± 0.001 (average of three experiments ± S.D.) for acceptor RNA-DNA and acceptor RNA-DNA (recessed 3’-DNA), respectively, indicating that RT dissociated about twice as quickly from the substrate with no 3’ recessed terminus.

Cleavage of RNA in the Trimer Structure—RNase H cleavage assays performed on the trimer structure indicated that both the donor RNA and the acceptor RNA were cleaved (Fig. 6). The design of the trimeric structure allowed for the identification of cleavage product derived from both donor and acceptor RNAs in the same reactions. Cleavage products from the acceptor were longer than the donor RNA allowing resolution in indi-
Fig. 4. Autoradiogram of time-course cleavage of the acceptor RNA-DNA, acceptor RNA-DNA (recessed 3'-DNA), and acceptor RNA-DNA (+10 hybrid) complexes in a trap assay. Isolated complexes (as indicated), 5'-end-labeled on the acceptor RNA, were incubated with 2 units of HIV-RT as described under “Experimental Procedures” for the trap assay. After RT pre-binding, reactions were initiated with MgCl₂ in the presence of the poly(rA)-oligo(dT) trap. Digestion was continued for the indicated time in seconds. Lanes D, complete digests of the substrates with 5 units of RT for 20 min in the absence of trap; lanes E, trap control in which RT was pre-mixed with the trap before addition to the substrate and incubated for 80 s; lane F, control incubated in the absence of RT. Other labels were as indicated in Fig. 3.

Fig. 5. A and B, off-rate \( (k_{\text{off}}) \) determination for acceptor RNA-DNA and acceptor RNA-DNA (recessed 3'-DNA) complexes. Isolated complexes (as indicated), 5'-end-labeled on the acceptor RNA, were incubated with 2 units of HIV-RT as described under “Experimental Procedures” for the “Off-rate \( (k_{\text{off}}) \) determinations.” Panel A shows an autoradiogram from a typical experiment. After RT pre-binding, poly(rA)-oligo(dT) trap was added and the incubations continued for the time indicated. Digestion was then initiated by the addition of MgCl₂, and reactions were continued for 5 min. Positions of the full-length and cleaved acceptor RNA are indicated. Lane A, control incubated without enzyme; lane B, trap control in which RT was pre-mixed with the trap before addition to the substrate and incubated for 100 s; lanes C, complete digest of the material with 5 units of RT for 20 min in the absence of trap. Other markings are as indicated in Fig. 3. Panel B shows a plot of relative cleavage versus time derived from the experiment in A. Values are relative to the 10-s time point in the assay, which was set equal to 1 (see “Experimental Procedures”). The \( k_{\text{off}} \) values from this experiment were 0.018 and 0.009 s⁻¹ for acceptor-DNA and acceptor-DNA (recessed 3'), respectively. Overall values of 0.015 ± 0.003 and 0.008 ± 0.001 s⁻¹ (average of three experiments ± S.D.) were determined for acceptor RNA-DNA and acceptor RNA/DNA (recessed 3'-DNA), respectively.
age products in Fig. 6 indicated that RT bound each RNA in the same manner as it bound the RNAs in the duplex structures (see above). Note that the amount of enzyme used in the assay shown in Fig. 6 was low in comparison to cleavages with the substrates above (0.1 unit per reaction as opposed to 2 units in the assays for the other substrates). This accounts for the slower rate of cleavage observed and allows a more careful determination of the rate of cleavage of the individual RNAs. The results showed that the 50-base donor RNA was cleaved faster than the 99-base acceptor (see Fig. 10 below). This suggests that RT associates more frequently with the region of the trimer that has a recessed 3' terminus. This is despite the fact that this region contains only about one-half as much RNA-DNA hybrid in comparison to the region bound to the acceptor.

A second experiment was performed to measure the off-rate of RT from the two RNAs in the trimer. The experiment was performed as described above with 10 mM KCl in the reactions. Consistent with the more rapid dissociation of RT from the branched versus 3' recessed substrates above, RT dissociated more rapidly from the acceptor region of the trimer (Fig. 7, A and B, k_{off} equals 0.005 and 0.014 for donor and acceptor regions, respectively). Taken together, results with the trimer indicate that RT associates with both regions of the trimer but binding is more stable to the donor region. The observed cleavages of both RNAs in the trimer could occur in many ways. A single enzyme molecule could cleave both RNAs in succession without dissociating from the trimer. There could be a preference as to which RNA is bound first, or it may be random. Alternatively, RT may bind without preference to either RNA on the trimer and then dissociate after cleaving that RNA. If both RNAs were bound with nearly equal preference, then equal levels of donor and acceptor RNA cleavage would be observed throughout the reaction. Finally, two enzyme molecules could bind each trimer molecule in a cooperative fashion, one on the donor and a second on the acceptor. The latter mechanism is unlikely, because results have shown that RT does not bind cooperatively to substrates. However, at high enzyme concentrations it is possible that more than one RT molecule could be bound to a single trimer simply due to a substantially greater number of enzyme versus substrate molecules. This could lead to cleavage of both RNAs even if RT

\[ A. \text{Raja and J. J. DeStefano, unpublished data.} \]

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**Fig. 6.** Autoradiogram of time-course cleavage of the trimer. Isolated trimer, 5'-end-labeled on all three nucleic acid strands to approximately the same specific activity, was incubated with 0.2 unit of HIV-RT as described under “Experimental Procedures.” Digestion was continued for 0, 15, or 30 s, or 1, 2, 4, 8, 12, or 16 min. Positions of the starting material and cleaved acceptor or donor RNAs are indicated. A schematic diagram of the trimer substrate is shown at the top of the figure along with approximate positions of prominent early cleavage products (arrows). Arrow designations are as described in Fig. 3.

**Fig. 7.** A and B, off-rate (k_{off}) determination for the trimer. Isolated trimer, 5'-end-labeled on all three nucleic acid strands to approximately the same specific activity, was incubated with 2 units of HIV-RT as described under “Experimental Procedures” for the off-rate (k_{off}) determinations. Panel A shows an autoradiogram from a typical experiment. After RT pre-binding, poly(rA)-oligo(dT) trap was added, and the incubations continued for the time indicated. Digestion was then initiated by the addition of MgCl_2, and reactions were continued for 5 min. Lane A, control incubated without enzyme; lane B, trap control in which RT was pre-mixed with the trap before addition to the substrate and incubated for 100 s; lane C, complete digest of the material with 5 units of RT for 20 min in the absence of trap. Other markings are as indicated in Fig. 6. Panel B shows a plot of relative cleavage versus time derived from the experiment in A. Values are relative to the 10-s time point in the assay, which was set equal to 1 (see “Experimental Procedures”). The k_{off} values from this experiment were 0.014 and 0.005 s^{-1} for the acceptor and donor RNAs, respectively. The experiment was repeated with and yielded similar results.
preferred one RNA to the other. To eliminate this possibility reactions were performed with a limiting amount of enzyme under conditions allowing only a single binding event (trap reaction) between the enzyme and the substrate as described above. In trap assays it was observed that both the donor and acceptor RNAs of the trimer were cleaved even at low enzyme concentrations when only a fraction of the total substrate was cleaved (Fig. 8). The fact that only a small fraction of the substrate was cleaved at low enzyme amounts implies that the reactions were not overloaded with enzyme, such that more than one molecule was bound to the substrate. This conclusion assumes that RT is not binding cooperatively. Therefore, these results suggest that either one enzyme molecule cleaves both RNAs in succession or RT binds the RNAs randomly and dissociates after cleavage. In other words, at any given time during the reaction, RT may be bound to the donor RNA of one trimer molecule and acceptor RNA on another. After cleaving the RNA that is bound, RT dissociates without cleaving the second RNA.

In an attempt to differentiate between these possibilities, a time course trap reaction was performed (Fig. 9, A and B). In this experiment reactions were terminated at time points ranging from 2 to 160 s. Both RNAs were cleaved even at the earliest time point with more cleavage of donor versus acceptor. Both RNAs showed an increase in cleavage between 2 and 10 s and no further significant increase (Fig. 9B). This suggests that cleavage is due to individual enzyme molecules bound to either RNA and not to a single molecule cleaving the RNAs in succession (see “Discussion”). If this mechanism is correct, given that the level of donor cleavage was greater than for acceptor, then the implication is that RT associates better with the donor portion of the trimer as was noted above.

**Effect of NC on Cleavage of RNA in the Trimer Complex**—It has been well documented that NC affects the stability and formation of hybrid structures and can stimulate strand transfer by promoting the formation of a trimeric intermediate (see Introduction). Therefore, it is possible that NC would affect the binding and orientation of RT to the trimer complex. To determine if NC influenced RT binding to the trimer, RNase H assays were performed in the presence of NC. Two different amounts of NC were used corresponding to one molecule of NC for every seven nucleotides of nucleic acid (a final concentration...
showed that the two RNAs involved in a recombination event that proceeds through the formation of a trimer are recognized differently by RT. RT binds the acceptor RNA, especially in the presence of NC, relatively poorly. Binding and cleavage of the donor RNA is clearly favored. The partial protection of the acceptor as well as cleavage of the donor may serve to promote more effective strand transfer as well as provide a salvage pathway for replication should the DNA and donor RNA dissociate due to RT RNase H cleavage.

HIV-RT bound both the donor and acceptor RNAs of the trimer while showing a preference for associating with the donor region (Figs. 6–10). Cleavage of the acceptor was not anticipated because other experiments indicated that the 5′-end of an RNA molecule must be bound or in close proximity to the DNA to observe RT-directed RNA cleavage (11). Results showed that cleavage can be independent of the presence of a DNA 3′-end and is directed by the 5′-RNA end providing the unbound 5′-RNA end was short (less than 12 bases). In our experiments cleavage was clearly not directed by the 5′-RNA terminus, because it was too far away (45 bases from the RNA-DNA hybrid). In fact initial cleavages were only “loosely” directed by the position of the junction suggesting that RT binds the junction substrate with some tendency to bind nearer the junction (Figs. 3 and 4). The apparent contradiction between these and the previous results may be due to the difference in the substrates used. Palaniappan et al. (11) used a substrate in which RNA primers were annealed to large circular DNA templates. The presence of DNA termini on the substrates used in our experiments may have promoted the association of RT with the acceptor. A second possibility is that the difference in the size of the DNA templates used in the experiment affected binding. The circular DNAs employed by Palaniappan et al. were several thousand nucleotides long. This could have resulted in binding competition for RT, because the RNA-DNA hybrid regions were relatively small. It has been shown that RT has some affinity for single-stranded nucleic acid (24, 25). In the absence of a bound 5′-RNA end the single-stranded region of the substrate could have out-competed the hybrid region for RT binding. This argument is strengthened by the fact the binding to the junctioned acceptor RNA-DNA was less stable than binding to a comparable substrate with a recessed 3′-end (Fig. 5). Finally, it is possible that the particular substrate used in our experiments allowed cleavage due to an undefined sequence or structural property of the substrate. This seems unlikely because another substrate with a similar configuration but unrelated sequence was also cleaved by RT (data not shown).

As was noted in the results, cleavage of both the donor and acceptor RNA in a trimeric complex could occur in several ways. The results presented here support a mechanism where an RT molecule binds a given substrate on either the donor or acceptor with a preference for binding donor. After cleavage of the bound RNA, RT most likely dissociates without cleaving the second RNA. Several lines of evidence support this mechanism: 1) Results indicate that RT shows no cooperativity in binding substrate. This indicates that two RTs would not associate with a single substrate under conditions in which the concentration of enzyme is limiting. Under such conditions cleavage of both RNAs was still observed in a trap assay (Fig. 8). Therefore, cleavage must have resulted from a single RT molecule cleaving both RNAs in succession or to the mechanism noted above. 2) In trap assays that limited binding to a single event, cleavage of both RNAs was observed at early time points, and the extent of cleavage did not increase after a short time (Fig. 9). This makes it unlikely that cleavage occurred by one RT molecule cleaving both RNAs in succession. If this were
the case an increase in the level of cleavage over time should have been observed for one of the RNAs. There was an initial increase between 2 and 10 s in the trap reactions but no further increase. Others have shown that it takes as long as 10 s for single RT molecules to catalyze an initial cleavage event on an RNA-DNA hybrid, although most cleavages occur within a few seconds. Following initial cleavages RT remains associated with the RNA-DNA hybrid for up to several minutes catalyzing further cleavages of the initial product in a 3’-5’ direction (21, 22). Given that no significant increase in total cleavage products was observed beyond 10 s in trap assays, it is unlikely that RT molecules cleaved one RNA then migrated to and cleaved the second RNA. If this occurred it would have to have taken place within the first 10 s of the trap reactions. Because the previous results indicate that RT remains associated with the RNA potentially for several minutes, cleavage of two RNAs in succession within 10 s seems highly unlikely.

Because these data support non-successive cleavage of the two RNAs this implies that RT must have some preference for binding to the donor over the acceptor RNA on the trimer. A greater proportion of donor versus acceptor RNA was cleaved in the trap assay indicating that RT associated preferentially with the donor (Fig. 9). In addition, cleavage in assays with low amounts of enzyme showed the donor RNA of the trimer being degraded more rapidly than the acceptor (Fig. 10). The $k_{off}$ values from experiments with the trimer (Fig. 7) as well as dimeric substrates (Fig. 5) support this notion, because RT bound more stably to substrates with 3’ recessed termini, consistent with the role of these termini in stabilizing binding (26).

However, the tendency to cleave acceptor or donor in the trimeric substrate likely depends on the overall affinity ($K_a$) of RT for that particular region rather than the off-rate. Given that the rate of cleavage by RT is several times greater than the off-rate (20), RT will presumably cleave either RNA nearly 100% of the time that the enzyme binds to it. Therefore, RT tends to associate more frequently with donor RNA rather than the acceptor in the trimer. Whether this is due to binding stability or to the overall configuration of the trimer is not clear.

Cleavage in the presence of NC showed the same trend as experiments without NC. Cleavage of both RNAs occurred with faster cleavage of the donor RNA. The level of cleavage was slightly reduced on the donor RNA by NC, whereas cleavage on the acceptor was significantly inhibited (Fig. 10). This decrease likely resulted from NC competing with RT for binding to the substrate as has been suggested by others (2, 27). The greater inhibition of acceptor cleavage may result from RT binding this region of the substrate more weakly as indicated above. The weaker binding may make it more difficult to out-compete NC for binding sites.

The nature of the trimeric substrate used in this work did not allow a direct comparison to what might occur on an authentic strand transfer intermediate. As was noted under “Results,” the acceptor RNA 5’-end would be complimentary to the 3’-end of the DNA in the authentic intermediate. The effect of the complementarity would possibly be to further promote dissociation of the donor RNA from the intermediate. Other experiments using a replication intermediate similar to the donor RNA-DNA duplex showed that an acceptor RNA could displace the donor in the presence of NC (see the introduction and Ref. 2). This occurred in the absence of RT, indicating that no RNase H activity was required for the strand exchange. However, at the same time, the current work clearly shows that the acceptor would not be immune to degradation during this process. Extensive degradation before completion of strand exchange could result in dissociation of the acceptor from the trimer complex. But the acceptor could still potentially bind the nascent DNA strand if the donor dissociated, because the complementary regions near the 3’-ends of the acceptor RNA and DNA would be preserved, but shortened. In this regard NC may play two important roles in the exchange process, promotion of the association of the acceptor and DNA as well as limiting RNase H cleavage (see Fig. 10), which would favor exchange over acceptor dissociation. 

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Additions and Corrections

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Interaction of HIV reverse transcriptase with structures mimicking recombination intermediates.

Aarti Raja and Jeffrey J. DeStefano

Pages 10106 and 10107: Figs. 3 and 4 were reversed. The legends were in the correct order but accompanying the wrong figure. The correct figures and legends are shown below.

**Fig. 3.** Autoradiogram of time-course cleavage of the acceptor RNA/DNA, acceptor RNA/DNA (recessed 3'-DNA), and acceptor RNA/DNA (+10 hybrid) complexes. Isolated complexes (as indicated), 5'-end-labeled on the acceptor RNA, were incubated for the indicated time with 2 units of HIV-RT as described under “Experimental Procedures.” Digestion times were 0 or 15 s, or 1, 2, 4, 8, or 16 min, as indicated. Size markers on the left and right are in nucleotides and were based on the top of the figure above the lanes with that substrate along with approximate positions of prominent early cleavage products (arrows). The numbers above the arrows refer to the number of nucleotides behind either the junction (acceptor RNA/DNA and acceptor RNA/DNA (+10 hybrid)) or 3' recessed terminus (acceptor RNA/DNA (recessed 3'-DNA)) that the cleavage occurred. Refer to Fig. 1 for a more detailed drawing of the substrates. Lanes A, partial digestion of template RNA with RNase T1; lanes B, partial base hydrolysis of the template RNA for 15 s; lanes C, partial base hydrolysis of the template RNA for 1 min.

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Autoradiogram of time-course cleavage of the acceptor RNA-DNA, acceptor RNA-DNA (recessed 3′-DNA), and acceptor RNA-DNA (+10 hybrid) complexes in a trap assay. Isolated complexes (as indicated), 5′-end-labeled on the acceptor RNA, were incubated with 2 units of HIV-RT as described under “Experimental Procedures” for the trap assay. After RT pre-binding, reactions were initiated with MgCl₂ in the presence of the poly(rA)-oligo(dT) trap. Digestion was continued for the indicated time in seconds. Lanes D, complete digests of the substrates with 5 units of RT for 20 min in the absence of trap; lanes E, trap control in which RT was pre-mixed with the trap before addition to the substrate and incubated for 80 s; lane F, control incubated in the absence of RT. Other labels were as indicated in Fig. 3.