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Comparison of Second-Strand Transfer Requirements and RNase H Cleavages Catalyzed by Human Immunodeficiency Virus Type 1 Reverse Transcriptase (RT) and E478Q RT

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Truncated tRNA-DNA mimics were examined in an in vitro assay for second-strand transfer during human immunodeficiency virus type 1 (HIV-1) reverse transcription. Strand transfer in this system requires the progressive degradation of the RNA within the 18-mer tRNA-DNA (plus-strand strong stop DNA) intermediate to products approximately 8 nucleotides in length. The ability of the truncated substrates to substitute for directional processing by RNase H or reverse transcriptase (RT) was examined. Using wild-type HIV-1 RT, substrates which truncated the 5′ end of the tRNA primer by 6, 9, and 12 nucleotides (Δ6, Δ9, and Δ12, respectively) were recognized by RNase H and resulted in strand transfer. An overlap of 5 nucleotides between the acceptor and newly synthesized DNA template was sufficient for strand transfer. The mutant RT, E478Q correctly catalyzed the initial cleavage of the 18-mer tRNA-DNA mimic in the presence of Mn^{2+}; however, no directional processing was observed. In contrast, no RNase H activity was observed with the Δ6, Δ9, and Δ12 substrates with E478Q RT in this strand transfer assay. However, when complemented with *Escherichia coli* RNase H, E478Q RT supported strand transfer with the truncated substrates. E478Q RT did cleave the truncated forms of the substrates, Δ6, Δ9, and Δ12, in a polymerase-independent assay. The size requirements of the substrates which were cleaved by the polymerase-independent RNase H activity of E478Q RT are defined.

Reverse transcription is a multistep process that is carried out by one virus-encoded enzyme, reverse transcriptase (RT). RT is a multifunctional enzyme which possesses RNA-dependent and DNA-dependent polymerase activities and RNase H activity. RNase H functions to remove RNA when it is present in an RNA-DNA hybrid (for a review, see reference 2).

In the process of reverse transcription, the viral RNA is converted to double-stranded DNA, which is subsequently integrated into the host genome. Human immunodeficiency virus type 1 (HIV-1) reverse transcription is initiated (3, 25–27, 33) using the cellular $tRNA^{Lys,3}$ as a primer (28, 35, 36). The first 18 3′ nucleotides of the tRNA primer are complementary to the primer binding site (PBS) sequence on the viral genome. Elongation of minus-strand synthesis pauses at the 5′ terminus of the viral RNA (31), completion of which requires a strand transfer, referred to as minus-strand or first-strand transfer. Plus-strand synthesis is initiated at the polypurine tract and continues through cDNA synthesis of the first 18 nucleotides of the tRNA primer (2, 4, 7). The second-strand transfer requires removal of the tRNA primer (4, 46, 50, 58), which allows an acceptor PBS molecule to enter, and subsequent completion of viral DNA synthesis.

HIV-1 RT consists of a heterodimer of two subunits, p66 and p51 (14, 34). The p66 subunit consists of the polymerase and the RNase H domains; the p51 subunit lacks the RNase H domain. Based on resemblance of the crystal structure to a right hand (1, 30), the subunits have been further divided into subdomains: palm, finger, thumb, connection, and RNase H. Mutagenesis studies have identified interactions between the polymerase and RNase H domains. Mutations within the thumb subdomain and the primer grip and deletions in the p51 C terminus decrease RNase H activity (9, 18, 21, 44).

During the viral life cycle, RNase H functions to degrade the viral genome, generate and remove the polypurine tract primer, and remove the tRNA primer. Removal of the tRNA primer has been extensively characterized for HIV-1 RT, Moloney murine leukemia virus RT, and an isolated HIV-1 RNase H domain (48, 49, 59). RNase H activity has been classified as either polymerase dependent or polymerase independent (2). The polymerization active site is spatially separated from the RNase H active site; polymerase-dependent RNase H activity results in RNase H cleavages which lag approximately 18 to 20 nucleotides behind the site of polymerization (55, 56). The catalytic residues of the RNase H active site are Asp 443, Glu 478, and Asp 498 (13, 17, 47). The requirements of RNase H activity during HIV-1 reverse transcription have been further characterized through the analysis of an RNase H-defective mutant, E478Q RT. This RNase H mutant possesses only Mn^{2+}-dependent RNase H activity. Additionally, it is capable of only a single endoribonucleolytic cleavage and lacks the ability to further degrade the tRNA primer (10).

Previously, we showed that RNase H activity is required for the HIV-1 second-strand transfer (50). More specifically, a single endoribonucleolytic cleavage is not sufficient to allow release of the tRNA primer. Rather, subsequent RNase H degradation must be carried out by RT or through complementation with *Escherichia coli* RNase H. We have now investigated the ability of HIV-1 RT and a mutant enzyme, E478Q RT, to support strand transfer with substrates possessing truncations in the 5′ portion of the tRNA primer. These substrates have the potential to substitute for RNase H-catalyzed directional processing, due to their decreased melting temperatures (T_m). If properly cleaved by E478Q RT, the truncated substrates could potentially support strand transfer without

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complementation by *E. coli* RNase H. In polymerase-independent assays, the truncated substrates were recognized and cleaved by E478Q RT. However, the results indicate a differential recognition by wild-type (WT) RT and E478Q RT on the truncated substrates in second-strand transfer reactions. Utilizing an in vitro strand transfer assay, we have shown that 5 nucleotides of overlap between the newly synthesized DNA strand and the acceptor molecule is sufficient for strand transfer.

MATERIALS AND METHODS

Enzymes and nucleotides. [γ-^{32}P]ATP was purchased from ICN. T4 polynucleotide kinase was purchased from New England Biolabs. RNasin was purchased from Promega. Klenow Exonuclease (−) was purchased from Boehringer Mannheim. HIV-1 RT was obtained either from Jeffrey Culp and Christine Debouch, Department of Protein Biochemistry, SmithKline Beecham Pharmaceuticals (37), or from Stuart Le Grice. The two HIV-1 RT preparations displayed equivalent specificity in the tRNA removal assay, indicating that variations in the expression and purification schemes did not result in altered biochemical properties (data not shown). The presence of the histidine tag on the HIV-1 RT has been previously shown not to influence catalytic functions (32). HIV-1 RT E478Q mutant was obtained from Stuart F. Le Grice and the AIDS repository (contributor, Stuart F. Le Grice). HIV-1-isolated RNase H (NY427) was purified from *E. coli* containing plasmid pET-NY427 (52). *E. coli* RNase H was purchased from Gibco BRL.

Oligonucleotides. The RNA-DNA hybrid oligonucleotides (RNA is indicated in bold) 17-mer (5′ **GUUCGGGCGCCA**CTGCT 3′), 14-mer (5′ **CGGGCGCCA** CTGCT 3′), 11-mer (5′ **GCGCCA**CTGCT 3′), 17-mer (DNA) (5′ AGCAGTG GCGCCCGAAC 3′), 14-mer (DNA) (5′ AGCAGTGGCGCCCG 3′), 11-mer (DNA) (5′ AGCAGTGGCGC 3′), HTD-1 (5′ GTGTGGAAAATCTCTAGCA GTGGCGCCCCGAACAGGGA 3′), 17080 (5′ ATCTCTAGCAGTGGCGCC CGAACAGGGAC 3′), and 17081 (5′ GAAAATCTCTAGCAGTGGCGCCC GAACAGGGAC 3′) were synthesized by Integrated DNA technologies. Oligonucleotides 5785 (5′ CCCTCAGCCCTTTTAGTCAGTGTGG 3′), 5786 (5′ CCCTTTTAGTCAGTGTGGAAAATCTCTAGCAGTGGCGCCCGAAC AGGGACCTGAAAGCGA 3′), 5331 (5′ AGCAGTGGCGCCCGAACGCGG GGCTTGTCCCT 3′), and 5580 (5′ TTTCGCTTTCAGGTCCCTGTTCGGGC GCCA 3′) were synthesized by the University of Medicine and Dentistry of New Jersey.

Strand transfer substrate preparation. The truncated RNA-DNA hybrid strand transfer substrates were prepared as follows. Portions (20 pmol) of the 17-mer, 14-mer, and 11-mer RNA-DNA hybrids were 5′-end labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The radiolabeled RNA-DNA oligonucleotides were isolated utilizing G-25 spin columns (Boehringer Mannheim). The labeled substrates were annealed to 40 pmol of oligonucleotide 5786 in a 25-μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 8 mM $MgCl_2$, and 2 mM dithiothreitol (DTT). The substrates were extended using Exonuclease (−) Klenow, isolated on gels, and eluted overnight as previously described (50). The product sizes for the input 17-mer, 14-mer, and 11-mer RNA-DNA hybrids are 44-mer, 41-mer, and 38-mer, respectively. The substrates were then annealed to oligonucleotide 5785 (referred to as 26-mer). This oligonucleotide was also 5′-end labeled in the same manner as described for the RNA-DNA hybrids.

Strand transfer reactions. Reactions were performed as previously described (50). Briefly, the annealed RNA-DNA hybrid substrates were incubated with either HIV-1 RT or E478Q RT. The strand transfer reactions were performed in a 20-μl reaction mixture containing approximately 4 pmol of substrate (substrate refers to the 50-mer RNA-DNA oligonucleotide annealed to primer 5785), 4 pmol of acceptor (oligonucleotide 5580), and 2 pmol of either HIV-1 RT or E478Q RT in a reaction buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 8 mM $MgCl_2$ or 8 mM $MnCl_2$, 2 mM DTT, and 0.25 μM each deoxynucleoside triphosphate (dNTP) (dATP, dCTP, dGTP, and TTP). Reactions were initiated upon the addition of enzyme and performed at 37°C. Aliquots (2.5 μl) were removed at the indicated time points. The reaction products were analyzed on a 15% polyacrylamide denaturing gel and exposed to autoradiography film.

In reactions in which multiple enzymes were used, such as *E. coli* RNase H, 1 pmol of the additional enzyme was added after the 12-min time point.

RNase H cleavage assays. Reactions were performed as previously described (49). Briefly, approximately 4 pmol of the indicated RNA-DNA hybrid substrate was incubated with either 1 pmol of HIV-1 RT or E478Q RT. RNase H cleavage substrates were prepared in the same manner as described for the strand transfer substrates. The annealing templates used for extension templates were oligonucleotides 5786, HTD-1, 17081, 17080, and 5531 for substrates B to F (see Fig. 6), respectively. These newly extended substrates were gel isolated and eluted as previously described (50). The substrates were again annealed to their corresponding extension templates. The reaction mixture (20 μl) contained 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 8 mM $MnCl_2$, and 2 mM DTT. Time course reactions were performed, and 3-μl aliquots were removed at 0, 2, 5, 15, and 30 min. Samples were analyzed on a 20% polyacrylamide denaturing gel and exposed to autoradiography film.

RESULTS

Strand transfer assays with truncated substrates. The second-strand transfer is a vital intermediary step in the synthesis of full-length double-stranded retroviral DNA. An in vitro assay has been developed to analyze the second-strand transfer reaction during HIV-1 reverse transcription (50). In this assay, model substrates were constructed which mimic the intermediate formed during plus-strand strong stop synthesis in which the first 18 nucleotides of the tRNA primer are reverse transcribed (Fig. 1, step 2). In the removal of the tRNA, the initial cleavage occurs at the penultimate nucleotide at the 3′ end of the tRNA (step 3) (51, 53). Previous studies have indicated that the tRNA needs to undergo further degradation for strand-transfer to proceed (50). Once the tRNA is removed, the acceptor molecule can enter and a 70-mer strand transfer product results (step 4).

Previous analysis indicated the RNase H-defective mutant, E478Q RT, was capable of performing only a single endoribonucleolytic cleavage in removing the tRNA primer (10, 50). Using our model system, this cleavage yields a 17-mer RNase H cleavage product, which was not sufficient for strand transfer to proceed (50). Therefore, it was reasoned that decreasing the size of the RNA component would lower the T_m of the RNA-DNA hybrid and could compensate for the lack of directional processing by E478Q RT. Model substrates were constructed that truncated the 5′ terminus of the RNA by 6, 9, or 12 nucleotides and were named Δ6, Δ9, and Δ12, respectively (Fig. 1). These truncated substrates were specifically recognized and cleaved at the expected position by both WT HIV-1 RT and an isolated HIV-1 RNase H domain (49).

Figure 1 summarizes the second-strand transfer assay with substrates possessing truncations in the 5′ RNA portion. Step 1 represents the input substrates for the Δ6, Δ9, and Δ12 constructs, in which the RNA-DNA oligonucleotides are hybridized with plus-strand primer 5785. In the presence of dNTPs and WT HIV-1 RT, polymerization occurs (step 2) and the full-length plus-strand product is synthesized. In this complex, the RT is paused with the terminus of the nucleic acid substrate bound in the polymerase active site. Once the RNA-DNA hybrid is formed, RNase H can cleave the RNA primer (step 3). Using a full-length 18-mer RNA, at the completion of plus-strand synthesis, the E478Q RNase H active site is optimally positioned for the single endoribonucleolytic cleavage to occur through a polymerase-dependent mechanism. However, with the truncated substrates, a polymerase-independent RNase H cleavage would be required to remove the RNA, since in a polymerase-dependent assay, the RNase H active site would be positioned within a double-stranded DNA region. Once the RNA primer is removed, the acceptor molecule enters and a 70-mer strand transfer product is produced (step 4). Decreasing the size of the RNA template affects the overlap between the newly synthesized DNA strand and the acceptor molecule. As the truncation increases, the amount of overlap between the acceptor and newly synthesized strand decreases. This assay therefore allows the determination of the minimum overlap sequence required for strand transfer to occur.

HIV-1 RT assayed with truncated substrates. The truncated substrates (Δ6, Δ9, and Δ12) were assayed with WT HIV-1 RT (Fig. 2) in the presence of Mg^{2+}, dNTPs, and acceptor molecule. Reactions performed in the presence of Mn^{2+} yielded equivalent results (data not shown). Figure 2 represents time courses from 0 to 30 min. Lanes 1 to 6 represent HIV-1 strand

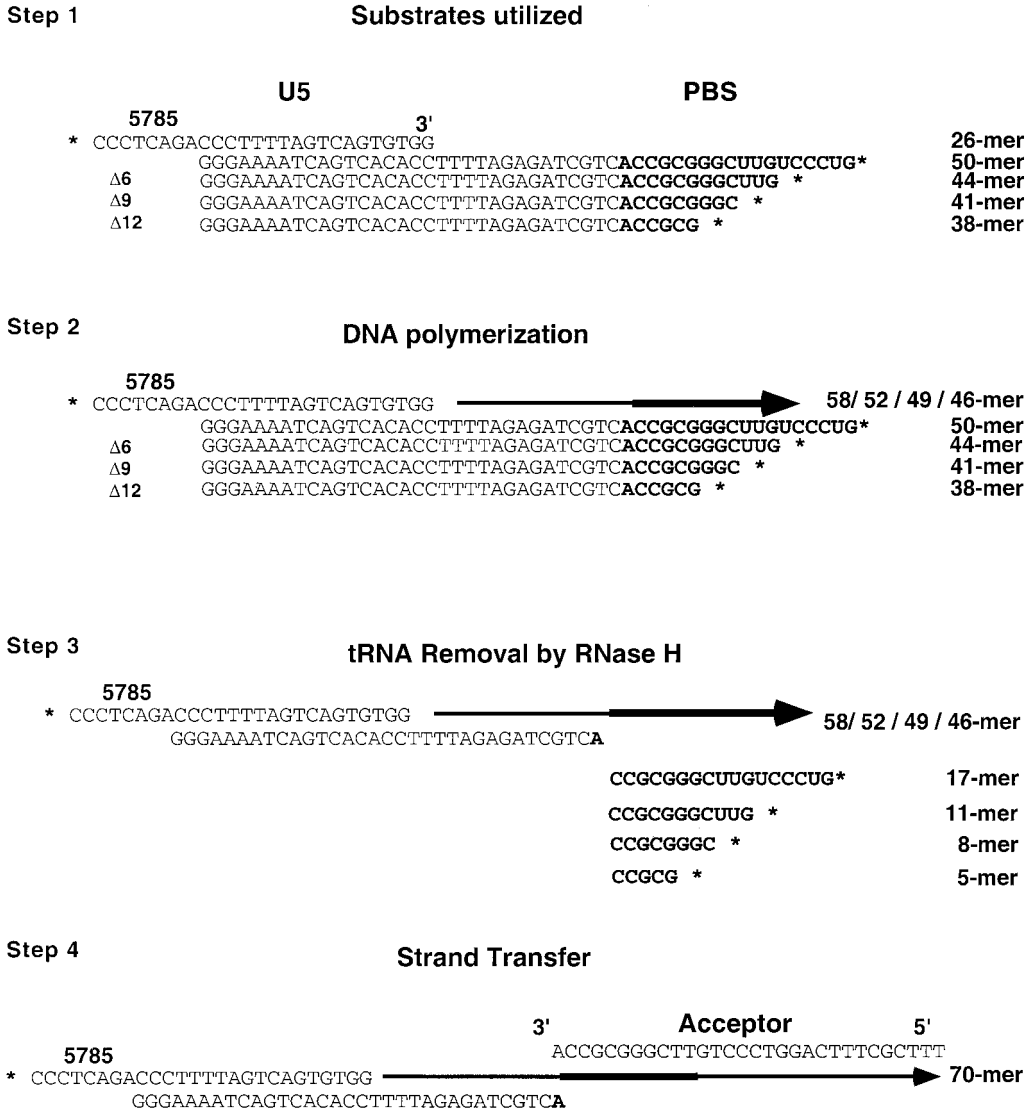


FIG. 1. Second-strand transfer assay with model substrates. This illustrates the model strand transfer assay with the truncated substrate possessing only 12 (Δ6), 9 (Δ9), and 6 (Δ12) positions of the RNA sequence. Step 1 illustrates the input substrate for each truncated substrate, along with their respective input RNA-DNA sizes. Step 2 illustrates the polymerization reaction which can occur in the presence of RT and dNTPs and the size of the polymerization product for each substrate, 52-mer (Δ6), 49-mer (Δ9), and 46-mer (Δ12). DNA polymerization creates the RNA-DNA hybrid, which is a substrate for the RNase H domain (step 3). Once the RNA has been removed between the terminal ribo-A and ribo-C, the acceptor molecule can enter and produce a strand transfer product, 70-mer (step 4). In each step, the RNA portion is indicated in bold and the 5′ radiolabel is indicated by an asterisk. The size of the strand transfer product (70-mer) would be the same for each truncated substrate.

transfer reactions with the Δ6 construct. The plus-strand oligonucleotide, 5785, was quickly extended from a 26-mer to the 52-mer product. The RNA portion of the RNA-DNA hybrid was degraded by the RNase H domain, and a strand transfer product (70-mer) was produced. Similarly, HIV-1 RT was capable of polymerization, RNase H activity, and strand transfer on the Δ9 substrate (lanes 7 to 12). The Δ12 construct was also assayed with HIV-1 RT (lanes 13 to 18). This construct had the largest deletion, possessing only 6 nucleotides of the tRNA primer. Therefore, there was only 5 bp of overlap between the acceptor and the newly synthesized DNA strand to support strand transfer. This substrate also successfully produced a strand transfer product (70-mer), albeit at lower efficiency. This result indicated that a 5-nucleotide overlap between the newly synthesized DNA strand and the acceptor strand is sufficient to support strand transfer.

E478Q RT assayed with the truncated substrates. The truncation substrates were assayed with E478Q RT in the same manner as for HIV-1 RT, except that reactions were performed in the presence of Mn^{2+}. Previous analysis indicated that the addition of Mn^{2+} to the WT HIV-1 RT does not change the RNase H or strand transfer properties of the enzyme, allowing direct comparison of the WT and E478Q mutant RT (50). It had been postulated that the single E478Q RNase H cleavage on the Δ9 and Δ12 substrates would release 8-mer and 5-mer RNA species, respectively. The reaction temperature is above the T_m of the products and would allow dissociation of the RNA. The T_m of the Δ6 construct, at ap-

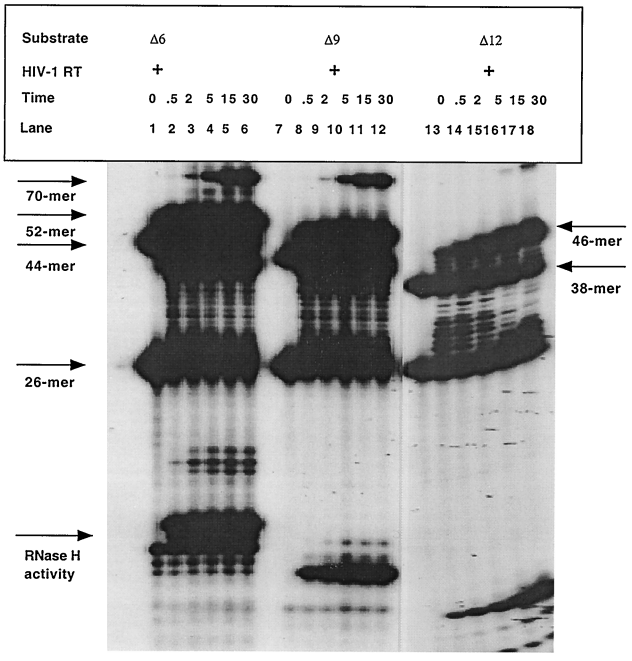


FIG. 2. Truncated substrates assayed with HIV-1 RT. Reactions were performed as described in Materials and Methods. Lanes 1 to 6, 7 to 12, and 13 to 18 represent HIV-1 RT incubated with the Δ6, Δ9, and Δ12 constructs, respectively. Time points are indicated above each lane in minutes. Strand transfer products (70-mer), DNA primer (26-mer), and RNase H products are indicated by arrows. Input substrates for the Δ6, Δ9, and Δ12 constructs are 44-mer, 41-mer, and 38-mer, respectively. Initial RNase H cleavage products for the Δ6, Δ9, and Δ12 constructs are 11-mer, 8-mer, and 5-mer, respectively.

proximately 42°C, may be too high for dissociation to occur. In previous studies, strand transfer correlated with the appearance of 8-mer RNA products (42, 50). Figure 3, lanes 1 to 6, lanes 7 to 12, and lanes 13 to 18 represent E478Q RT assayed with the truncations Δ6, Δ9, and Δ12 respectively. For all of the constructs, extension products were observed, indicative of complete synthesis. Interestingly, no RNase H cleavage products were observed for any of the constructs assayed in the presence of E478Q RT. Reactions performed for up to 40 min and/or in the presence of both divalent cations did not yield RNase H cleavage products (data not shown). A band was observed with the Δ6 construct (lanes 2 to 6). However, this band was also present in the absence of enzyme (lane 1) and is therefore not an RNase H cleavage product. These results were surprising because previously, E478Q RT was capable of producing an RNase H cleavage product on the intact full-length substrate (50). Without any RNase H activity, E478Q RT was unable to proceed with strand transfer. It was therefore possible that E478Q RT was not capable of catalyzing the polymerase-independent RNase H cleavages required for strand transfer in this modified assay.

Complementation of HIV-1 RT and E478Q RT with *E. coli* RNase H. To further characterize the defects of E478Q RT with regard to its RNase H cleavage, it was necessary to determine that this mutated enzyme could perform strand transfer under these modified conditions with the truncated substrates. Therefore, both HIV-1 RT and E478Q RT were complemented with *E. coli* RNase H in a strand transfer reaction. The reactions were performed in the presence of Mg^{2+}, dNTPs, and acceptor; *E. coli* RNase H was added after the 12-min time point for both enzymes. Under these conditions, both the WT HIV-1 RT and *E. coli* RNase H were active and their products could be distinguished. The WT HIV-1 RT products (Fig. 4A, lanes 3, 9, and 15) were similar to those identified in Fig. 2 (lanes 3, 9, and 15). Addition of *E. coli* RNase H after 12 min resulted in much more extensive RNase H degradation (Fig. 4, lanes 4, 10, and 16, indicated by vertical arrows). For E478Q RT, the visible cleavage activity can only be a result of *E. coli* RNase H since the reactions were performed in Mg^{2+} alone. The results of time course analyses using HIV-1 RT and E478Q RT are shown in Fig. 4A and B, respectively. Complementation of HIV-1 RT with *E. coli* RNase H was similar to the results (lanes 1 to 6, lanes 7 to 12, and lanes 13 to 18) with HIV-1 RT alone. A 70-mer strand transfer product was produced in each case. With HIV-1 RT, only a single RNase H cleavage product was observed for the Δ12 construct (Fig. 2). However, addition of *E. coli* RNase H yielded RNA products as small as a diribonucleotide (Fig. 4A, lane 16).

Complementation of E478Q RT with *E. coli* RNase H on the Δ6, Δ9, and Δ12 substrates (Fig. 4B, lanes 1 to 6, lanes 7 to 12, and lanes 13 to 18, respectively) did indeed result in the 70-mer strand transfer product. These results confirmed the requirement of RNase H activity for strand transfer to take place. These experiments also reinforce the finding with HIV-1

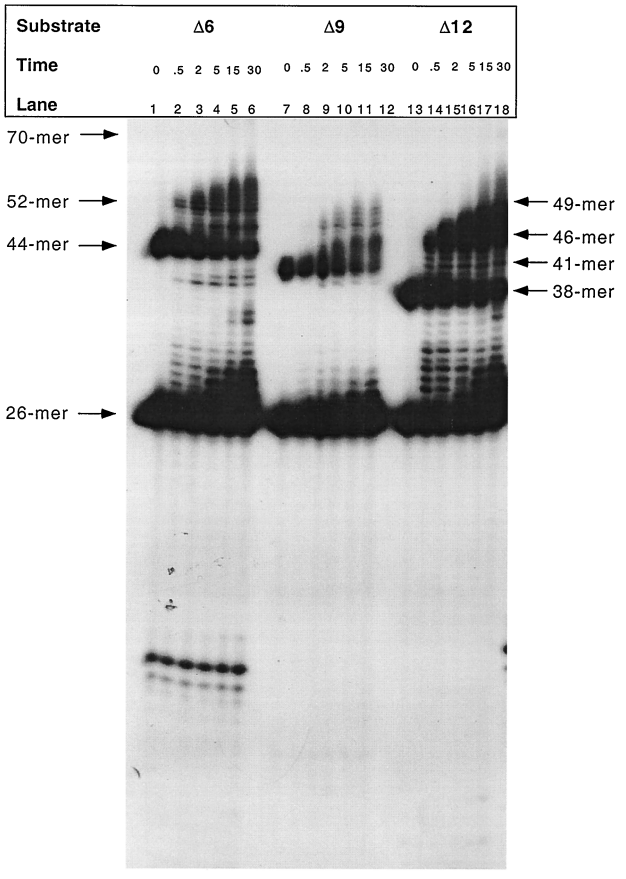


FIG. 3. Truncated substrates assayed with E478Q RT. Reactions were performed as described in Materials and Methods. Lanes 1 to 6, 7 to 12, and 13 to 18 represent E478Q RT assayed with the Δ6, Δ9, and Δ12 constructs, respectively. Time points are indicated above each lane in minutes. Strand transfer products (70-mer), DNA primer (26-mer), and RNase H products are indicated by arrows. Input substrates for the Δ6, Δ9, and Δ12 constructs are 44-mer, 41-mer, and 38-mer, respectively. Initial RNase H cleavage products for the Δ6, Δ9, and Δ12 constructs are 11-mer, 8-mer, and 5-mer, respectively.

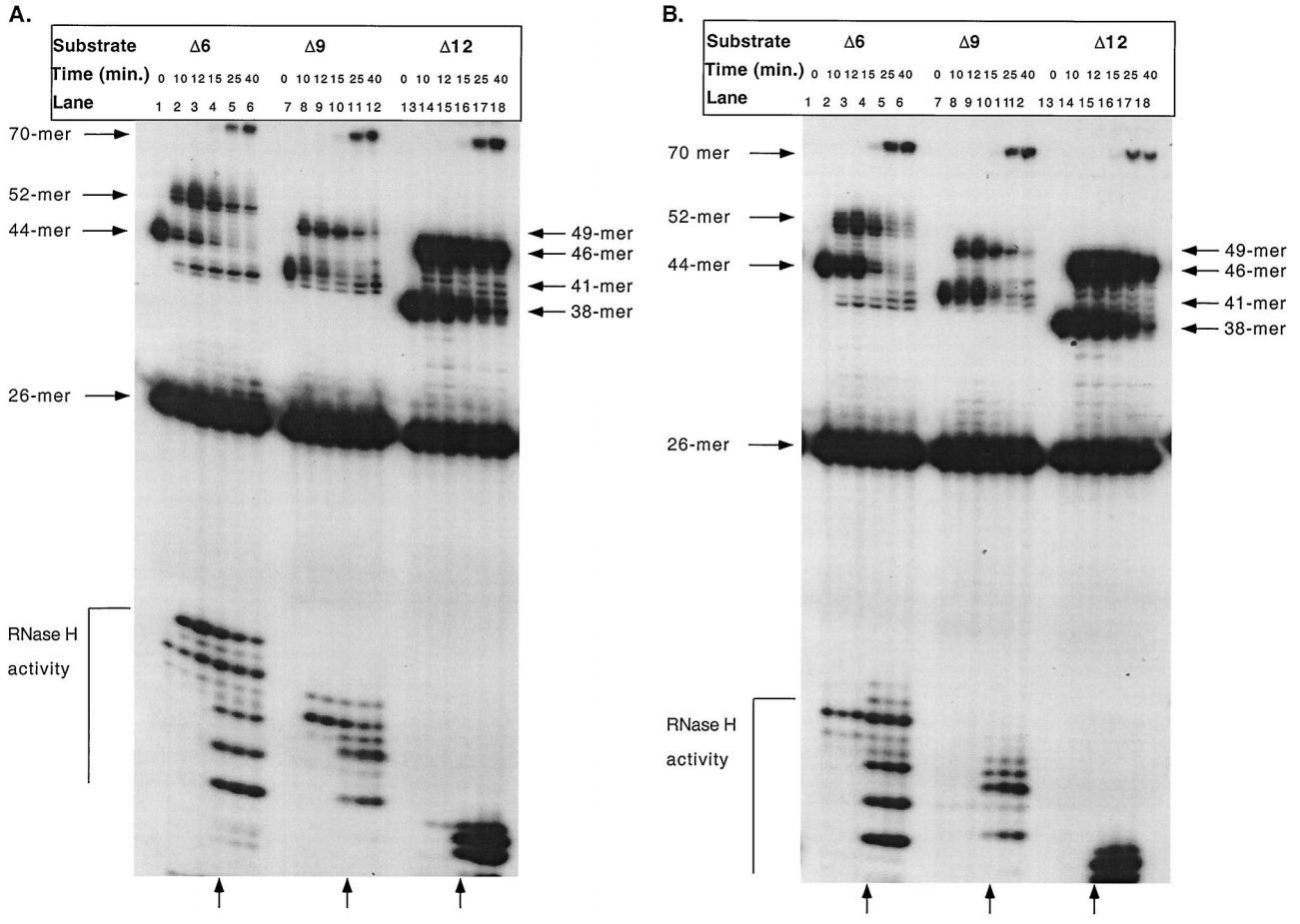


FIG. 4. (A) Complementation of HIV-1 RT with *E. coli* RNase H. Reactions were performed as described in Materials and Methods. Input RNA-DNA, DNA primer, and RNase H cleavage products are indicated by arrows. Reactions were allowed to proceed for 12 min in the presence of Mg^{2+}, and then of *E. coli* RNase H was added (indicated by the vertical arrows). Time points are indicated above each lane in minutes. (B) Complementation of E478Q RT with *E. coli* RNase H. Reactions were performed as described for panel A. Time points are indicated above each lane in minutes. Input RNA-DNA, DNA primer, and RNase H cleavage products are indicated by arrows.

RT that the 5-base overlap between donor and acceptor was sufficient for strand transfer to proceed.

E478Q RT in a polymerase-independent assay. Previous characterization of E478Q indicated that it lacks directional RNase H processing (10) during polymerization. However, the question remained whether E478Q RT can perform any polymerase-independent RNase H cleavages. To test this, a series of short, truncated substrates were generated (Fig. 5A). If RNase H cleavage occurred at the initial (−1) position (indicated by the arrow) (51, 53), substrate binding could not be dictated by the positioning of the 3′OH in the polymerase active site. These RNA substrates contained the same 5′-terminal truncations as the RNAs in the strand transfer assay. Additionally, these substrates truncated the DNA in the RNA-DNA oligonucleotide to 5 nucleotides (Fig. 5A). The RNA-DNA strands were hybridized to DNA oligonucleotides 17, 14, and 11 nucleotides in length, yielding blunt-ended substrates.

Figure 5B illustrates these substrates assayed with HIV-1 RT or E478Q RT. Figure 5B, lanes 1 to 5, 6 to 10, and 11 to 15 represent HIV-1 RT assayed with the truncated substrates. For the 17-mer (lanes 1 to 5), 14-mer (lanes 6 to 10), and 11-mer (lanes 11 to 15) substrates, the correct cleavage products at the −1 position were observed, as indicated by the arrows. These cleavage events have been extensively characterized for HIV-1 RT and an isolated HIV-1 RNase H domain with a related substrate containing a complementary oligonucleotide carrying the entire 18-nucleotide PBS sequence (49). The 17-mer, 14-mer, and 11-mer substrates assayed with E478Q RT are shown in Fig. 5B, lanes 16 to 20, 21 to 25, and 26 to 30, respectively. E478Q RT was capable of cleaving the 17-mer and 14-mer constructs at the predicted −1 position. In contrast to WT RT, E478Q RT did not cleave the 11-mer construct. These results indicate that on defined substrates, E478Q RT is capable of polymerase-independent cleavages.

RNase H cleavage analysis of the truncated DNA substrates. E478Q RT-RNase H recognized and cleaved the blunt 17-mer and 14-mer RNA-DNA hybrid substrates (Fig. 5B) but was inactive on the equivalent 52-mer (Δ6) and 49-mer (Δ9), RNA-DNA hybrid resulting after polymerization (Fig. 3). Two key differences between these substrates are the size of the DNA-DNA hybrid segment and the position of a 3′OH group with respect to the RNA-DNA hybrid. A large DNA substrate may lock the 3′OH within the polymerase active site, whereas a small substrate may have sufficient flexibility to permit binding of the substrate into the RNase H active site.

To address this, a series of substrates were constructed which varied the length of the double-stranded (ds) U5 DNA associated with the tRNA-DNA mimic (Fig. 6A). The sizes of

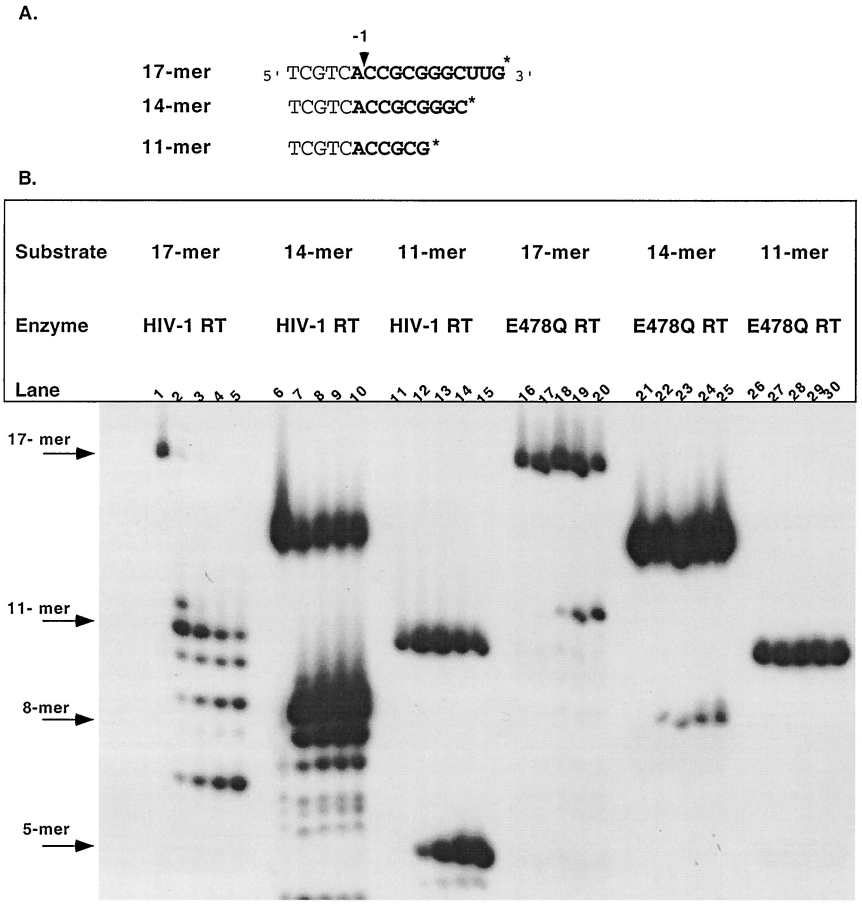


FIG. 5. E478Q RT assayed in polymerase-independent assay. (A) The substrates utilized are illustrated. Substrates were labeled at the 5′ termini with [γ-^{32}P]ATP and prepared as described in Materials and Methods. An asterisk indicates the radiolabel. The RNA portion is indicated in bold. (B) HIV-1 RT assayed with 17-mer (lanes 1 to 5), 14-mer (lanes 6 to 10), 11-mer (lanes 11 to 15); E478Q RT assayed with 17-mer (lanes 16 to 20), 14-mer (lanes 21 to 25), and 11-mer (lanes 26 to 30). Time points are 0, 2, 5, 15, and 30 min for each set of lanes. Reactions were performed as described in Materials and Methods.

the ds DNAs ranged from those that were cleaved, (17-mer; 5-mer DNA plus 12-mer RNA) to those not recognized by E478Q (substrate B; 32-mer DNA plus 12-mer RNA). E478Q RT was capable of recognizing and cleaving a 50-mer RNA-DNA hybrid substrate containing the intact 18-mer RNA portion plus a 32-mer of DNA (substrate A). However, when the RNA portion of the substrate was truncated by 6 nucleotides (substrate B), RNase H cleavage was inhibited. Substrates were constructed in which the DNA-DNA (ds DNA portion) size varied but the size of the RNA-DNA hybrid remained constant (12-mer RNA). Substrates C, D, E, and F possessed 20, 14, 11, and 7 nucleotides, respectively, of ds DNA. The 17-mer (5-mer DNA plus 12-mer RNA) and 14-mer substrates (5-mer DNA plus 9-mer RNA) were shown to be cleaved by E478Q RT (see above) (Fig. 5B).

HIV-1 RT and E478Q RT were assayed for their abilities to cleave these substrates in a polymerase-independent assay (Fig. 6B and C, respectively). For HIV-1 RT, all of the newly constructed substrates (substrates B to F) were cleaved, indicating that the substrates were intact RNA-DNA hybrids (Fig. 6B). Since all of the substrates contained 12 nucleotides of RNA, the RNase H cleavage sites were identical and an 11-mer RNA cleavage product was observed for each substrate. In contrast, with E478Q RT (Fig. 6C), the only substrate that was efficiently cleaved was substrate F, which possessed 7 nucleotides in the DNA-DNA portion and 12 nucleotides in the RNA-DNA hybrid portion of the substrate. The total size of this substrate was 19-mer (7-mer DNA plus 12-mer RNA), which approached that required for polymerase-dependent RNase H cleavages. This was only 2 nucleotides larger than the 17-mer substrate previously cleaved by E478Q RT (Fig. 5B). This result indicates that E478Q RT can perform polymerase-independent RNase H cleavages only on defined small substrates. It is of interest that these substrates are equal to or less than the length defined between the polymerase and RNase H active sites.

DISCUSSION

Two different modes of RNase H activity have been characterized for HIV-1 RT: polymerase dependent and polymerase independent (20). Polymerase-dependent RNase H cleavages are those that occur while the polymerase domain is actively synthesizing. The RNase H active site lags approximately 18 to 20 nucleotides behind the polymerase active site, which has been characterized through footprinting analysis for HIV-1 RT and Moloney murine leukemia virus RT (55, 57). On the plus-strand strong stop tRNA-DNA, tRNA removal occurs while the RT is paused trying to use the modified tRNA residue as template. Although the polymerase is not actively synthesizing, the initial RNase H cleavage can be viewed as polymerase dependent since the nucleic acid substrate remains bound in

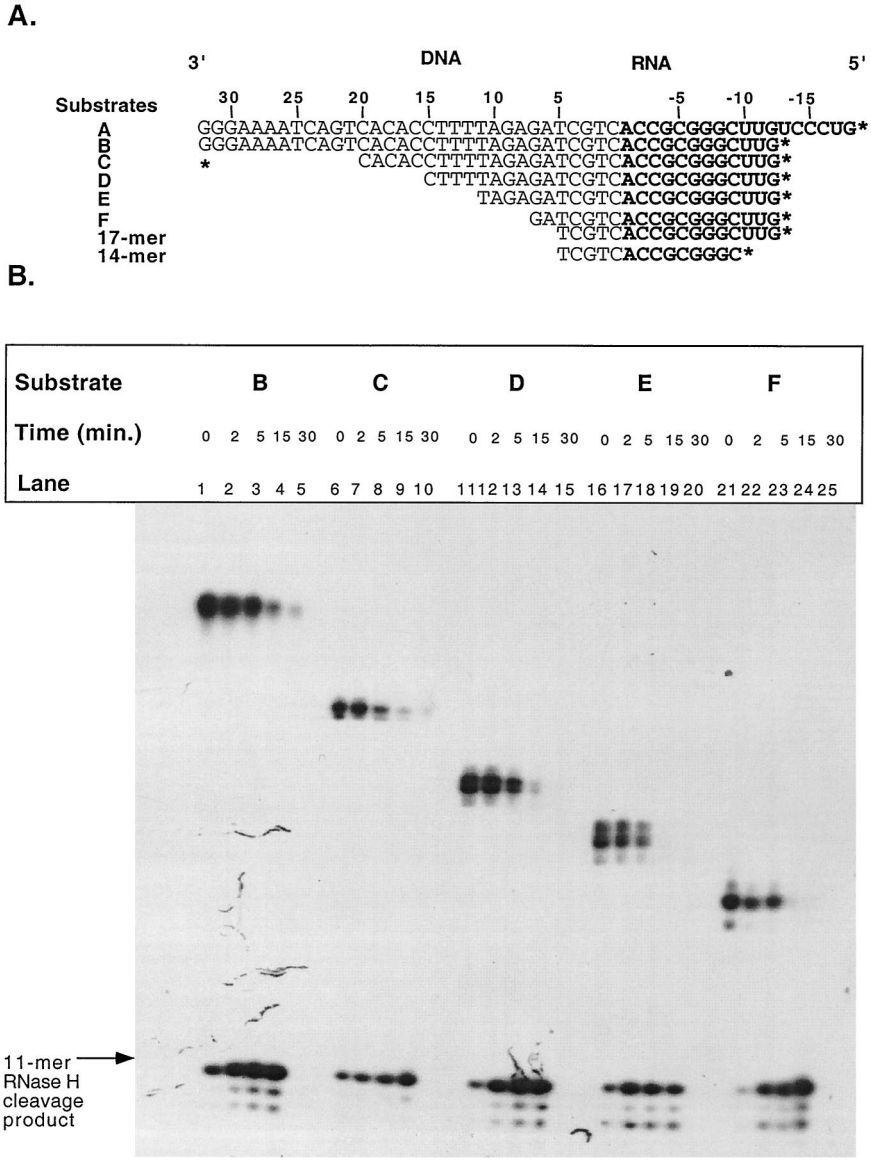


FIG. 6. RNase H cleavage analysis of truncated DNA substrates. (A) The substrates utilized are illustrated and are labeled A through F. The RNA portions are indicated in bold, and an asterisk indicates the radiolabel. The substrates were prepared as described in Materials and Methods. (B) Substrates B to F assayed with HIV-1 RT. Reactions were performed as described in Materials and Methods. Time course reactions are shown, and time points are indicated above each lane, along with the substrate utilized. The RNase H cleavage product is designated and indicated by an arrow. (C) Substrates B to F assayed with E478Q RT. Reactions were performed as described in Materials and Methods. Time course reactions are shown, and time points are indicated above each lane, along with the substrate utilized. The RNase H cleavage product is designated and indicated by an arrow.

the polymerase active site. The initial RNase H cleavage occurs at the penultimate nucleotide of the tRNA (53). Polymerase-independent cleavages are less well understood and may represent different types of cleavages. These cleavages result in the production of smaller products (22) and have been defined as "directional processing of the RNA primer" (10). Although secondary to the initial cleavage during reverse transcription, these events are required for the ultimate release of the RNA primer. Mutants with defects in the ability to perform this function are unable to perform strand transfer (10, 21, 23, 50). Polymerase-independent RNase H cleavages require a second binding event by RT (23). This "rebinding" event may require a change between the type of conformation required for active DNA synthesis and polymerase-dependent RNase H activity.

We have developed an in vitro assay which requires polymerase-independent RNase H activity during the second-strand transfer reaction. Previously, we utilized substrates which mimic the U5/PBS border, resulting from plus-strand strong stop DNA synthesis. The original substrates possessed either an 18-mer RNA oligonucleotide or the intact $tRNA^{Lys,3}$ (50). With those substrates, the RNase H activity we observed was most probably a combination of polymerase-dependent and -independent cleavages. The substrates utilized in this study have truncations in the 5′ RNA portion and have a maximum length of 12 ribonucleotides. Therefore, cleavage of these substrates would require polymerase-independent RNase H activity, due to the suboptimal distance between the polymerase and RNase H active sites.

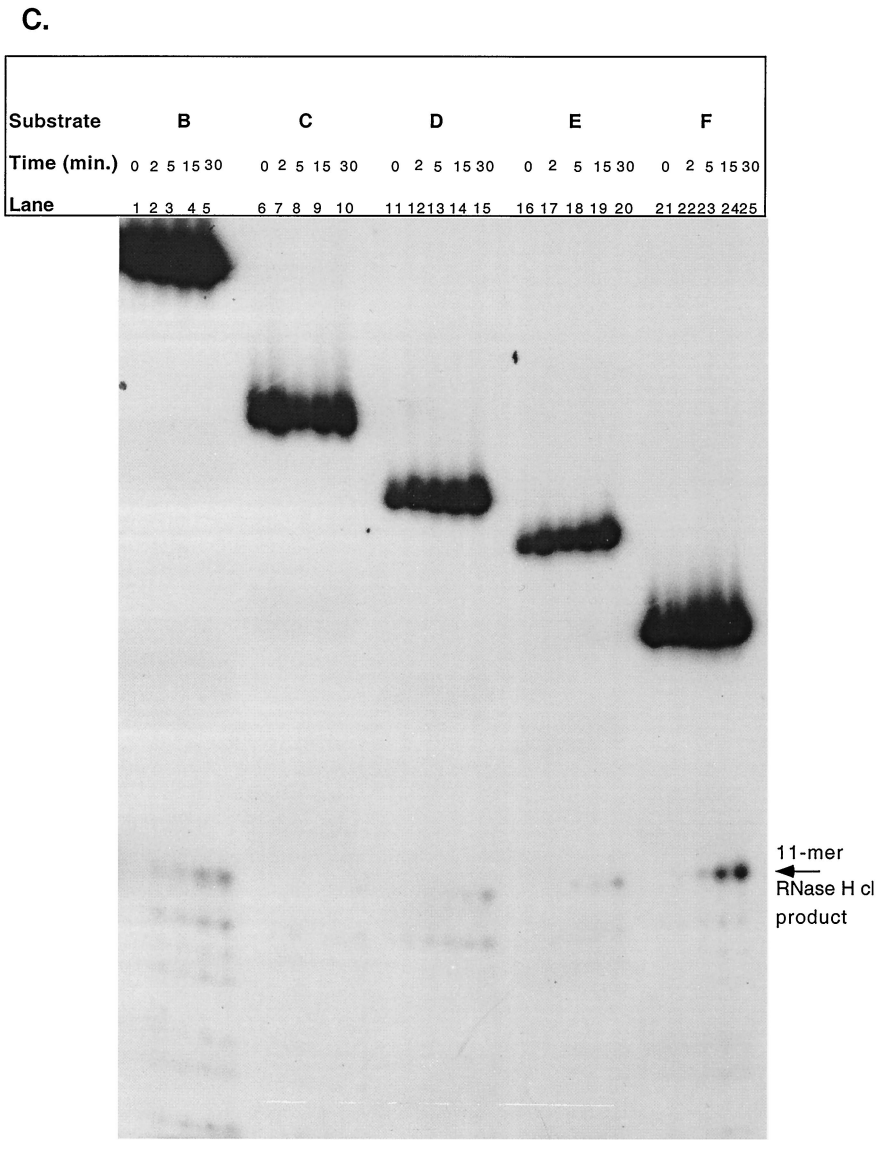
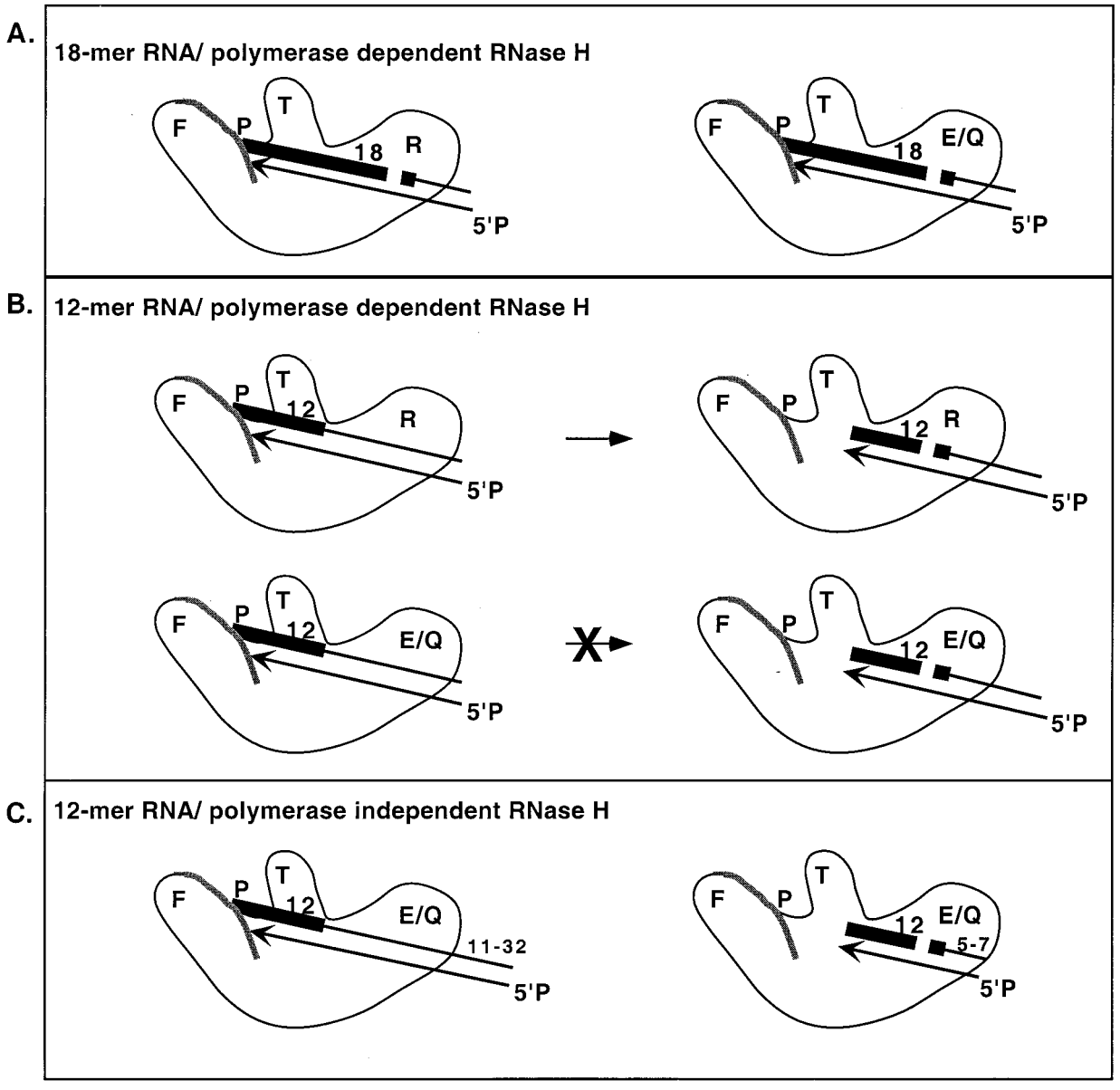
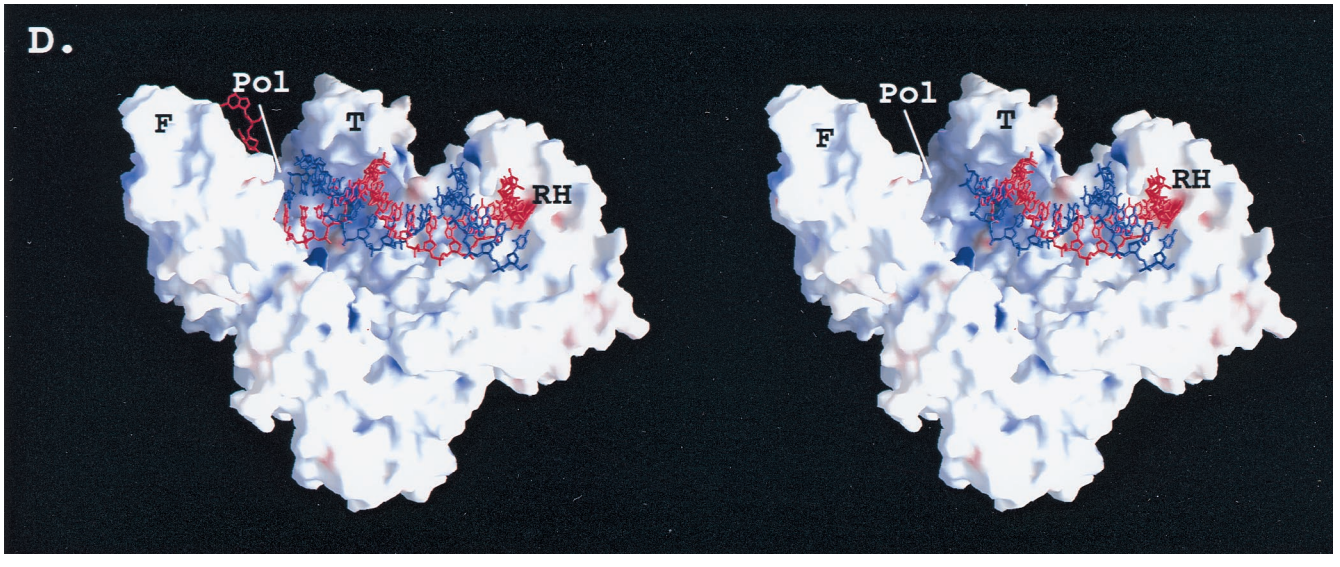


FIG. 6—*Continued*.

A model illustrating the possible binding conformations adopted by WT RT and E478Q RT is shown in Fig. 7. This model is based on the crystal structure of a covalently trapped catalytic complex of RT with DNA (24) (Fig. 7D, left panel). In this complex, the ds DNA spans 23 nucleotides and approximates the 12-mer RNA plus 11-mer DNA substrates used in the present study. In Fig. 7D, left panel, the substrate is positioned in the polymerase active site (labeled Pol). In Fig. 7D, right panel, the substrate was modified such that a potential RNA strand (red) is within the RNase H active site (RH) and extended 12 nucleotides toward the polymerase active site. This substrate lacks the stabilizing contacts within the thumb domain. The extension of the substrate exiting the RNase H domain was not modeled due to limited structural data on these contacts. Figure 7A to C utilize a schematic of this molecular model to summarize the results presented in this study. On a full-length model substrate (50-mer: 32-mer DNA plus 18-mer RNA) (Fig. 7A), both E478Q RT and WT HIV-1 RT bind in a polymerase-dependent conformation and produce the well-characterized −1 RNase H cleavage product (depicted as a nick within the RNA [thick bar]) (50). The size of the RNA portion of the substrate allows correct positioning of the RNase H active site near the RNA-DNA junction. This RNase H cleavage event is driven by the polymerase domain and is guided by the 5′ phosphate of the RNA and the 3′ OH of the DNA (39).

Figure 7B represents the truncated substrate (44-mer: 32-mer DNA plus 12-mer RNA). This substrate is suboptimal for a polymerase-dependent mode of binding due to its truncation in the 5′ portion of the RNA. Despite this, the WT RT maintains its RNase H cleavages on these substrates. This implies that it is capable of the release and rebinding of these substrates (23). E478Q RT, which is compromised in the RNase H domain, has regained the single endoribonucleolytic cleavage ability only in the presence of Mn^{2+} (10). E478Q RT binds in the same polymerase-dependent orientation; however, RNase H cleavage never occurs because the RNase H active site is never positioned near the RNA-DNA junction on this sub-


A.
18-mer RNA/ polymerase dependent RNase H
F
P
T
18
R
5'P
F
P
T
18
E/Q
5'P
B.
12-mer RNA/ polymerase dependent RNase H
F
P
T
12
R
5'P
F
P
T
12
R
5'P
F
P
T
12
E/Q
5'P
F
P
T
12
E/Q
5'P
C.
12-mer RNA/ polymerase independent RNase H
F
P
T
12
E/Q
11-32
5'P
F
P
T
12
E/Q
5-7
5'P


D.
Pol
F
T
RH
Pol
F
T
RH

strate. It appears that E478Q in the presence of Mn^{2+} has not maintained the ability to communicate the release and subsequent rebinding to these large substrates, which possess a larger portion of ds DNA than the smaller substrates do. Alternatively, catalysis for the polymerase-independent cleavages may be slower than dissociation on these substrates (12). In the polymerase-dependent reaction catalyzed by E478Q, the RNA-DNA hybrid remains accessible to further degradation by exogenously added *E. coli* RNase H. This indirectly implies that the enzyme is released from the substrate. Complementation with *E. coli* RNase H is sufficient for subsequent strand transfer catalyzed by E478Q RT.

When presented with a substrate truncated in the DNA portion as well as in the RNA portion (19-mer: 7-mer DNA plus 12-mer RNA), E478Q RT was capable of performing an RNase H cleavage. This substrate (Fig. 7C) is small enough that it is not locked into the polymerase active site. Therefore, a polymerase-independent or RNase H active site-driven cleavage may occur. HIV-1 RT preferentially binds a heteroduplex RNA-DNA region with a greater affinity than it binds ds DNA (12), which may be directing the RNase H cleavage. However, once the DNA portion was increased to 11 nucleotides (23-mer: 11-mer DNA plus 12-mer RNA), the substrate was again locked into the polymerase active site and no RNase H activity was observed. This indicates that the overall substrate size must be smaller than the distance between the polymerase and RNase H active sites (55, 57) in order for E478Q RT to perform these smaller polymerase-independent RNase H cleavages. The E478Q mutation may alter the ability of the enzyme to translocate across larger substrates to position itself correctly for RNase H cleavage, whereas a smaller substrate can slide into the RNase H active site.

Several factors have been identified which contribute to the positioning of the template. These include the position of the primer 3′OH, the position of the 5′ phosphate of the RNA, and the minor groove binding tract within the thumb subdomain. We propose that with substrates longer than 19 nucleotides, a polymerase-dependent conformation dominates, whereas with the smaller substrates, binding to either the polymerase or RNase H active sites may occur. This may be a combination of the high affinity of the polymerase domain for the termini of the substrate and a result of the defect in the RNase H domain of E478Q RT. With E478Q, subsequent rebinding to the substrate may remain driven solely by the recognition of the termini of the substrate. Rebinding (23) would occur in the same location and would never position the RNA-DNA substrate in a conformation required for a polymerase-independent cleavage. It is therefore surprising that the smaller substrates are capable of binding E478Q within the RNase H domain. This implies that there may be a stabilizing effect of having a substrate exit the RNase H domain. Reports have suggested that substrate binding by the RNase H domain contributes to processive DNA synthesis (8).

Studies have been performed which show that the 5′ end of the RNA sequence can be responsible for directing its cleavage by HIV-1 RNase H (39, 40). Those studies suggested that RT is capable of binding the substrate in two different ways: either based on the 3′ end of the DNA strand or based on the 5′ end of the RNA strand. The binding was dependent on the substrate structure and on whether the DNA or RNA ends were recessed. When binding was driven by the position of the RNA 5′ phosphate, RNase H cleavage still occurred 18 nucleotides upstream, maintaining the optimal spatial distance between the two active sites. However, with the substrates utilized in our study, binding of the 5′ phosphate within the polymerase active site would position the RNase H active site within the ds DNA region. WT RT is able to overcome this obstacle; therefore, this cannot be the dominant feature, since cleavage at the penultimate nucleotide within the RNA was consistently observed.

Another contributing factor may be the role of the p66 thumb subdomain in positioning the RNA-DNA substrate. In the crystal structure of HIV-1 RT bound with nonnucleoside inhibitor, the position of the thumb differs from that of the inhibitor free enzyme (11, 15, 16, 45). The α-helix H within the thumb acts as a minor groove binding tract (5, 6). This helix plays a role in the binding of primer-template complexes. The RT mutant W266A (mutated within α-helix H) lost the ability to position the 3′OH/5′ phosphate within the polymerase active site and resulted in imprecise removal of the polypurine tract primer (43). Molecular modeling of a truncated substrate in the RNase H active site precluded the interaction of a 12-base extension with the minor groove binding tract (Fig. 7D, right). Flexibility of the thumb and/or perturbation of the size of the major and minor groove of the substrate could alter these interactions. Cooperativity of the thumb with either the polymerase or RNase H active sites could therefore be a discriminating factor for the positioning of the tRNA-DNA substrate.

We propose two different binding conformations for HIV-1 RT in removing the tRNA, a polymerase-dependent binding mode and a polymerase-independent binding mode. Differential conformations for endonuclease and exonuclease RNase H activities were previously described (60). Another study demonstrated that in the switch from initiation to elongation, the RT must dissociate before efficient and processive elongation can occur (31). That study also concluded that the length of the synthesized DNA affects this switch, since it would change the proximity of the RNA/DNA junction on the primer strand relative to α-helix H. The truncated substrates utilized in our present study would place the RNase H active site within the U5 DNA portion once synthesis is complete; therefore, dissociation would have to occur for any RNase H cleavage to occur. HIV-1 RT has no defects in its ability to perform this switch and to cleave truncated substrate intermediates. However, the E478Q RT was unable to carry out these cleavages on substrates that were identical in size.

The present study has also determined that an overlap of 5

FIG. 7. Model of WT RT and E478Q RT polymerase-dependent and polymerase-independent RNase H activities. The possible binding orientations for HIV-1 RT and E478Q RT are shown. (A to C) WT RT and E478Q RT are shown positioned on the various substrates, 50-mer substrate (32-mer DNA, 18-mer RNA) (A), 44-mer substrate (32-mer DNA, 12-mer RNA) (B), and 19-mer (7-mer DNA, 12-mer RNA) (C). The RNA portion is indicated by the thick line, and the DNA is indicated by the solid black line. The position of RNase H cleavage is indicated by a nick in the RNA strand. The WT and E478Q RT can be distinguished by the presence of an R (WT) or E/Q (E478Q RT) in the RNase H domain. Additionally, the thumb and polymerase domains are indicated by T and P, respectively. The 5′ phosphate is indicated, as well as the size of the RNA on each model substrate. (D) Models of substrates bound in the polymerase active site (left) and the RNase H active site (right) in HIV-1 RT. The electrostatic potential mapped on the molecular surface rendering of the HIV-1 RT (GRASP [38]) is shown with the template-primer as bound in the structure reported by Huang et al. (24) (1rtd), and substrates are shown as stick models. Positively charged amino acids are shown in blue, and negatively charged amino acids are shown in red. In the right-hand panel, the substrate found in the 1rtd structure has been truncated to include only 12 bp of template-primer extending from the RNase H active site. This truncated substrate makes very limited interactions with the thumb. T, thumb; F, fingers; RH, RNase H active site; Pol, polymerase active site.

nucleotides between the acceptor DNA and the newly synthesized plus strand is sufficient for either the WT RT or E478Q mutant enzyme to perform the plus-strand transfer. This region of overlap includes the 3′OH of the primer. Deletions of acceptor molecules which destroy the base pairing with the primer terminus block plus-strand transfer in vitro and in vivo (4, 54). Our results are consistent with in vivo results in which maintenance of 5 nucleotides of the PBS adjacent to U5 plus complementarity of only 3 bp at the site of polymerization was sufficient for extension of plus-strand DNA during the second template transfer (54). This result is also in agreement with the occurrence of retroviral recombination events. In vivo, low levels of strand transfer events that yield deletions have been characterized with minimal overlap (41). Studies have shown that there is an average of 1 aberrant strand transfer event per replication cycle (29).

Interestingly, WT HIV-1 RT was capable of performing specific RNase H cleavages on all of the substrates utilized. Truncation of the DNA or RNA portion did not affect the RNase H cleavage site. Rather, all of the substrates produced the identical −1 cleavage product. This may be due to the strong structural recognition of the tRNA mimic or to an alteration of the binding conformation which allows specific RNase H cleavages to occur. Previously, we have demonstrated that sequences within the first nine positions of the tRNA were important for cleavage and recognition by an isolated RNase H domain (49). These studies support the concept that the structure defined by the tRNA sequence possesses strong intrinsic signals that lead to its precise cleavage between the terminal ribo-A and ribo-C. This cleavage occurs even when a large portion of the $tRNA^{Lys,3}$ sequence has been deleted.

We have shown that many factors play a role in influencing RNase H activity. The in vitro system developed allows the biochemical analysis of a minimal system. In vivo, replication occurs within reverse transcription complexes (19) in the presence of additional viral proteins, including the nucleocapsid, which can influence these reactions. The use of an enzyme with defects in the RNase H domain provides insight into the requirements for polymerase-independent RNase H cleavages.

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