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Human immunodeficiency virus type 1 reverse transcriptase: Spatial and temporal relationship between the polymerase and RNase H activities

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ABSTRACT **The spatial and temporal relationship between the polymerase and RNase H activities of human immunodeficiency virus type 1 reverse transcriptase has been examined by using a 40-mer RNA template and a series of DNA primers of lengths ranging from 15 to 40 nucleotides, hybridized to the RNA, as substrates. The experiments were executed in the absence and presence of heparin, an efficient trap to sequester any free or dissociated reverse transcriptase, thus facilitating the study of events associated with a single turnover of the enzyme. The results indicate a spatial separation of 18 or 19 nucleotides between the two sites. To examine the effect of concomitant polymerization on the RNase H activity, the substrate was doubly 5′ end labeled on the RNA and DNA. This enabled the study of RNase H activity as a function of polymerization in a single experiment, and the results in the absence and presence of heparin indicate a tight temporal coupling between the two activities.**

The virally encoded reverse transcriptases (RTs) are multifunctional enzymes possessing RNA- and DNA-dependent polymerase and RNase H activities (1, 2). The latter activity can cleave the RNA strand of an RNA·DNA hybrid (3). This hydrolytic processing is proposed to be required at several stages of viral genome replication, including removal of the RNA template after synthesis of the first strand of DNA (4), cleavage of the host tRNA from which first-strand DNA synthesis originates (5), generation of a specific oligopurine ribonucleotide primer from which the synthesis of second-strand DNA is initiated, and its subsequent removal (6–10). It has also been shown recently that for human immunodeficiency virus type 1 (HIV-1) RT, RNase H activity is essential for the occurrence of strand transfer during reverse transcription (11). The inherent RNase H is vital for viral replication and cannot be substituted with a cellular RNase H.

The HIV-1 RT is a heterodimer composed of 66- and 51-kDa subunits, p66 and p51 (12, 13). Mutational analysis has shown that the RNase H domain (p15) is confined to the C-terminal portion of the p66 polypeptide (14–16), whereas the polymerase domain resides at the N terminus (17). For HIV-1 RT, a functional interdependence of the two domains has been suggested, since linker insertion mutations at the N terminus of HIV-1 RT can affect RNase H activity, whereas such mutations at the C terminus can disrupt polymerization (15, 16, 18). The interdependence of the two domains, likewise, has recently been demonstrated: whereas the RNase H domain (p15) expressed in bacteria is inactive by itself, addition of the p51 polypeptide restores its enzymic activity (19). The p51 chain is inactive in DNA polymerization by itself (20) or in association with p66 (21) and is only active as a weakly associated dimer (22).

Presuming a close interdependence between the DNA polymerase and RNase H activities, the next question concerns the spatial relationship between the two active sites. According to the most commonly accepted model (23, 24), the N-terminal polymerase active site is positioned at the 3′ end of the DNA primer where cDNA synthesis would start and points toward the 5′ end of the RNA. The C-terminal RNase H domain faces the reverse side. The spatial separation in nucleotides between the two sites has been addressed earlier (23, 25–27) and the results vary from a length of 7 to 18 nucleotides. However more recent measurements (24, 28) have been interpreted in terms of a 15- or 16-base separation between the two sites.

In this paper we present evidence to show that the distance of separation between the two active sites in HIV-1 RT is approximately 18 or 19 nucleotides. The result has been obtained by using a series of sequence-defined RNA template-DNA primers as substrates for HIV-1 RT. The hydrolysis reactions were assayed in the presence of a heparin trap that effectively sequesters any enzyme released after the initial binding event, permitting determination of the extent of the nucleolytic cleavage catalyzed by the RNase H site during one encounter with the primer-template.

We have also examined the temporal relationship between the polymerase and RNase H activities by the use of a doubly radioactively 5′ end-labeled RNA template-DNA primer in the presence and absence of the heparin trap. The accumulated evidence indicates a concertedness or coupling between the two activities.

MATERIALS AND METHODS

Preparation of RNA Template. A 40-nucleotide, single-stranded RNA template was synthesized by run-off transcription with T7 RNA polymerase (29) and purified by electrophoresis through 20% acrylamide/8 M urea/1× TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) followed by its elution from the excised gel slice using 0.5 M ammonium acetate/1 mM EDTA at 37°C and precipitation by ethanol. The quantitation of RNA was facilitated by the inclusion of trace amounts of [α-^{32}P]UTP in the transcription reaction mixture. All reactions were performed using strict RNase-free conditions.

5′ ^{32}P-Labeled RNA Template. The RNA was first dephosphorylated using calf intestine alkaline phosphatase as described by United States Biochemical. The dephosphorylated RNA was 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase according to the United States Biochemical protocol. The radiolabeled RNA was purified as above.

DNA Oligonucleotide Primers. The DNA concentrations were estimated by measuring the absorbance at 260 nm using

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Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase.

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a calculated ε from the base composition. The primers were labeled as above at the 5′ terminus with [γ-^{32}P]ATP using T4 polynucleotide kinase.

RNA·DNA Hybridizations. To prepare RNA template-DNA primers with the RNA labeled at the 5′ end, a mixture of 200 nM RNA template/240 nM DNA primer/50 mM Tris·HCl, pH 8.0, was heated at 65°C for 1 min followed by slow cooling for 90 min to room temperature. To prepare RNA template-DNA primers with the DNA labeled at the 5′ end, a mixture of 200 nM DNA/240 nM RNA/50 mM Tris·HCl, pH 8.0, was similarly heated to 65°C followed by slow cooling. Small aliquots of the hybrids were then run on a 20% acrylamide/1× TBE nondenaturing gel to confirm complete hybridization. In the case of hybrids with the RNA and DNA labeled at the 5′ terminus, the independently 5′ end-labeled DNA and RNA were mixed in equimolar amounts and hybridized as above. The hybrid was diluted with an equal volume of loading dye (30% glycerol/0.25% bromophenol blue/0.25% xylene cyanol in water) and purified on a 20% acrylamide/1× TBE nondenaturing gel followed by elution from the excised gel and ethanol precipitation.

Enzyme Assays. All enzyme assays contained HIV-1 RT equimolar with 200 nM substrate, 7 mM $MgCl_2$, 0.1 mM dNTPs (when required) or 0.1 mM dNTPs and ddNTPs (when required), 2 mg of heparin trap per ml (when required), 50 mM Tris·HCl (pH 8.0), 75 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.1% Triton X-100. In the active site spatial relationship studies, the RNA template was 5′ end-labeled for RNase H activity determinations. For primer extension studies, the DNA was 5′ end-labeled in the template primer. In all cases the substrate was incubated with enzyme for 5 min and the reactions were initiated by the addition of Mg^{2+}, with or without the addition of dNTPs and heparin as required. In all cases the reaction volume was 50 μl; aliquots (5 μl) were removed from the reaction mixture at each time point and quenched by addition of 5 μl of 20 mM EDTA and 5 μl of loading dye. The samples were heated to 90°C for 30 s, cooled on ice, and electrophoresed through 20% acrylamide/8 M urea gels. The gel was exposed to a film

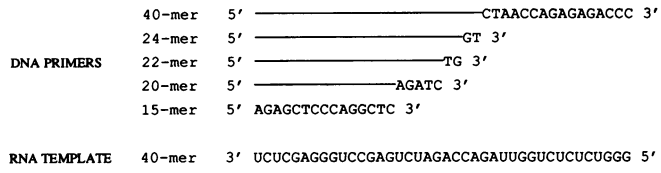

FIG. 1. Sequences of RNA template-DNA primers.

overnight. The concentration of heparin trap in the enzyme assays was fixed at 2 mg/ml on the basis of control pretrap experiments done by preincubation of substrate with enzyme and various concentrations of trap followed by initiation with Mg and dNTPs. The concentration of heparin at which the reaction was completely arrested under the given conditions was 2 mg/ml.

RNA Size Markers, Alkaline Ladders, and G Ladders. "Alkaline ladders" were prepared by treating the 5′ end-labeled RNA (excised and eluted from the gel) with sodium bicarbonate (0.5 M, pH 9.5). "G ladders" were prepared by digesting the 5′ labeled RNA fragments with ribonuclease T1, as described in the RNA sequencing kit (Pharmacia). The samples were diluted 1:1 with loading dye before electrophoresis. The RNA sequences of the 14- and 31-mer were established by the manufacturer's protocols (Pharmacia).

RESULTS

HIV-1 RT Polymerase and RNase H Site Separation. For studies concerning the spatial relationship between the HIV-1 RT polymerase and RNase H sites, a 5′ end-labeled 40-mer RNA run-off transcript derived from the first 40 bases of the HIV terminal repeat was used as substrate for RNase H cleavage. Sequence-specific DNA primers of lengths ranging from 15 to 40 bases (Fig. 1) were hybridized to the 3′ terminus of the RNA transcript. The corresponding primer extensions or polymerization reactions were followed by monitoring the ^{32}P label on the 5′ end of the DNA primer. Assays were also performed in the presence of heparin, an efficient trap added at the start of the reaction that sequesters

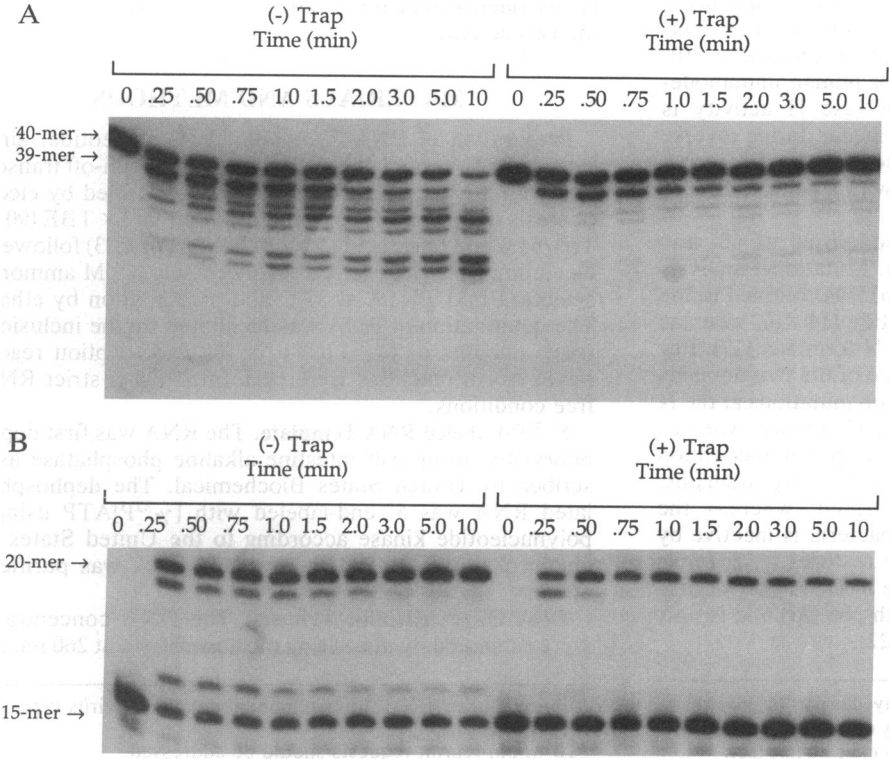

FIG. 2. Time-dependent hydrolytic cleavage of the 40/20 RNA·DNA duplex (5′ end-labeled RNA) by the RNase H activity (*A*) after extension of the 40/15 (5′ end-labeled DNA) by the polymerase activity of the HIV-1 RT (*B*). Experiments were run in the absence (−) and the presence (+) of heparin trap. All strand lengths are referred to the 5′ end label.

any enzyme that dissociates from the duplex. This permits the analysis of products resulting from a single binding event of HIV-1 RT to substrate.

The time course for cleavage of template/primer 40/15 (data not shown) produced three bands corresponding to cleavage at positions −36, −35, and −33 measured from the 5′ end of the RNA. The same reaction, in the presence of trap, left the RNA intact, implying that the enzyme dissociated from the substrate before any cleavage could take place.

The effect of DNA primer extension on the hydrolytic activity of the RNase H gave contrasting results. Since the sequence of the first 5 nucleotides encoded by the template is AGATC, various combinations of dNTPs and ddNTPs were used to terminate primer elongation after the first, second, fourth, and fifth base additions. Extension of the 3′ end of the primer by 1 or 2 nucleotides (using ddATP or dATP and ddGTP) provided RNase H cleavage bands identical to those obtained in the absence of polymerization. In the presence of trap no RNase H cleavage was observed upon addition of 1 or 2 nucleotides. When the primer was extended by 4 (dATP, dGTP, and ddTTP) or 5 (dATP, dGTP, dTTP, and ddCTP) bases producing 19- and 20-mer DNA strands (Fig. 2*B*), respectively, a strong band corresponding to a 39-mer RNA fragment appeared in experiments carried out in the presence or absence of trap (Fig. 2*A*). This result implied cleavage at a site that was 18 or 19 nucleotides upstream from the 3′-OH of the extended DNA-primer. Presuming that the polymerase site of the enzyme is preferentially bound to the 3′-OH of the DNA, this observation implicates an 18- or 19-base separation between the polymerase and RNase H sites. The RNase H activity in the absence of trap also generated a series of shorter fragments, probably arising from subsequent binding and cleavage events. In the presence of trap, their amount was <10%. Nevertheless, their presence indicates that HIV-1 RT is capable of more than one cleavage per enzyme binding event, suggesting a degree of processivity (30).

To verify our inferences, a preformed RNA·DNA 40/20 template-primer (Fig. 1) was subjected to RNase H hydrolysis in the absence of polymerization. The cleavage bands obtained in the absence and presence of trap (data not shown) were consistent with those obtained in Fig. 2*A*. In the reaction without trap, after an initial RNase H cleavage to give a 39-mer RNA within 30 s, the RNA is rapidly processed at longer times to generate shorter fragments. In the reaction with trap, a band corresponding to a 39-mer is predominant, once again implicating a 19-nucleotide separation between the two sites of the enzyme. It was also evident that under these conditions, the enzyme's RNase H activity did produce shorter fragments, beside the 39-mer, confirming its processivity. However, it is primarily the quantity and not the distribution of the 5′-derived cleavage products that changes in a comparison of trapped and nontrapped reactions. The level of products less than 19 nucleotides in length measured from the 3′ primer terminus increased considerably in the untrapped assay, at the expense of the larger cleavage products. This may be attributed to a 3′ → 5′ directional nuclease activity (23).

To ensure the observed distance in nucleotides between the DNA polymerase and the RNase H sites is indeed 18 or 19 and is independent of sequence, the reaction was repeated with template-primers 40/22, 24, and 40. For the 40/22 and 40/24 duplexes the major cleavage band corresponded to a 37- and 35-mer RNA fragment, respectively (data not shown); for the 40/40 duplex the major RNA fragments were 19- and 17-mers (Fig. 3), with all experiments carried out in the presence of a trap. These results are consistent with a 19-nucleotide separation between the two sites. We note that bands corresponding to RNA cleavage products less than a 15-mer are not found in reactions containing a trap, suggesting that a minimum duplex length of 15 base pairs (bp) is required for the enzyme's rate of cleavage to compete with its rate of dissociation from the duplex.

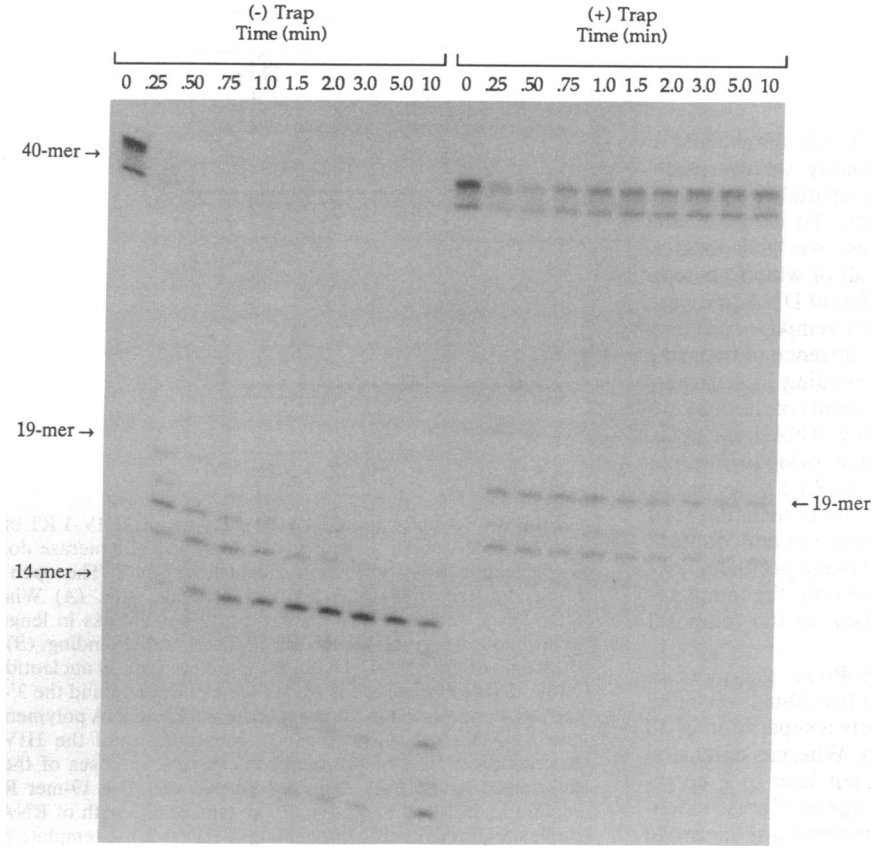

FIG. 3. Time-dependent hydrolytic cleavage of 40/40 RNA·DNA duplex by the RNase H activity of HIV-1 RT in the absence (−) and the presence (+) of heparin. The lowest band in the reaction without trap was assigned to a 14-mer by alkali digestion and sequence analysis. All other bands were assigned with reference to the 14-mer. The uppermost band is due to incompletely denatured duplex.

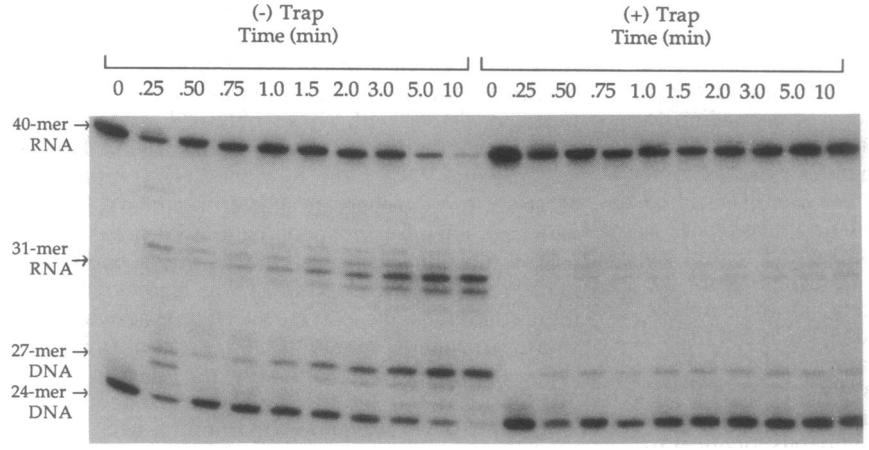

FIG. 4. Concomitant hydrolytic and polymerization activities of HIV-1 RT on doubly 5′ end-labeled 40/24 RNA–DNA template-primer in the absence (−) and the presence (+) of heparin.

Temporal Relationship Between Polymerase and RNase H Activities. To determine whether RNase H activity is coupled to transcription, the RNA–DNA substrate was doubly labeled with ^{32}P at the 5′-OH of the DNA and the RNA in the duplex. On the basis of the above results it was imperative that a template-primer duplex longer than 18 or 19 bases be used to obtain meaningful data. Therefore, a 40/24 mer was 5′ end-labeled on both strands and used as a substrate. The first 3 bases encoded by the RNA template are CTA, permitting elongation of the DNA primer by 3 bases. Extension of the DNA primer has the simultaneous effect of increasing the length of the RNA·DNA hybrid, allowing the possibility of analyzing how the template RNA is reduced in size (by RNase H) as the primer DNA is extended (by RT polymerase). The result of this experiment is shown in Fig. 4. The time-dependent appearance of bands corresponding to 33-, 32-, and 31-mers of RNA (18 bases behind the advancing primer terminus) correlates with the stepwise growth (25-, 26-, and 27-mers) of the extended DNA primer, in the trap-challenged and unchallenged reactions. This result provides a strong argument for the simultaneous action of the polymerase and RNase H activities.

DISCUSSION

The HIV-1 RT enzyme possesses a DNA polymerase and a RNase H activity (17). This bifunctionality on the same enzyme suggests an important coupling (spatial and temporal) between the two opposing activities. To estimate the spatial separation between the two sites, we have used a series of RNA template-DNA primers, all of which possess a common 40-mer RNA template but different DNA primers. The action of HIV-1 RT on the different template/primers was studied in either the presence or the absence of heparin, an efficient trap for free HIV-1 RT, providing information pertaining to single and multiple binding events of the enzyme with the different template/primers. Any RNA hydrolysis product that was normally further degraded owing to multiple binding and cleavage events could therefore be observed in the challenged reaction. The RNase H activity also has been studied in either the absence or the presence of concomitant polymerization; the former gives the cleavage products arising from the initial binding of the enzyme onto the template-primer, whereas the latter furnishes data on the temporal coupling between the two activities.

With the RNA–DNA template-primer 40/15, polymerase-catalyzed extension of the 15-mer primer to a 20-mer revealed from the onset of RNA cleavage products a separation of 18 or 19 bases between the two active sites. Whereas extension of the 40/15-mer by 1 or 2 bases did not lead to a corresponding change in the RNase H cleavage products, extension by 4 or 5 bases produced a predominant cleavage product at the 39 position of the RNA—i.e. 18 or 19 nucleotides upstream from the 3′ primer terminus. Further proof for this observation was obtained by studying the dNTP-independent cleavage patterns of template-primers 40/20, 40/22, and 40/40 in the presence of an enzyme trap. If the distance of separation between the two sites is indeed 18 or 19 nucleotides, one would expect to see a major RNA hydrolysis product 18 or 19 bases upstream from the 3′-OH

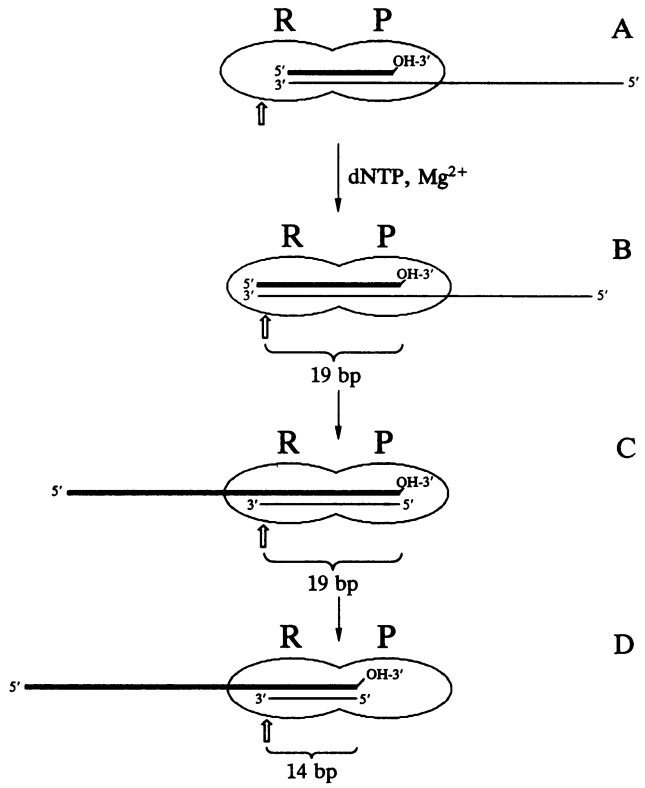

FIG. 5. Possible model for HIV-1 RT action. HIV-1 RT binds to a RNA·DNA duplex. R and P, RNase H and polymerase domains, respectively; heavy line, DNA; light line, RNA. The open arrow represents the probable RNase H cleavage site. (*A*) When the RNA·DNA duplex is less than 18 or 19 nucleotides in length, the hybrid does not reach the RNase H site on initial binding. (*B*) When the length of the RNA·DNA duplex is greater than 19 nucleotides, the initial RNase H cleavage is 18 or 19 nucleotides behind the 3′-OH of the DNA primer. (*C*) In the presence of dNTPs, DNA polymerization and RNA hydrolysis proceed concomitantly until the HIV-1 RT reaches the 5′ end of the template, leaving 19 bases of the RNA hybridized to the fully extended primer. (*D*) The 19-mer RNA is rapidly hydrolyzed to a 14-mer, the minimum length of RNA·DNA duplex required for efficient binding of HIV-1 RT to template-primer.

of the DNA primer. As expected, the major RNA fragments corresponded to a 39-mer for template-primer 40/20, 37-mer for 40/22, and 19-mer for 40/40, all consistent with RNAse site 18 or 19 bases upstream for the 3'-OH terminus of the primer.

It has been reported recently that the two active sites are separated by a length of 15 or 16 nucleotides measured relative to the 3'-OH (25, 26) of the primer. However, in that study, the first time point was at 5 min and the reaction was done in the absence of trap. Thus, any product corresponding to a 19-base cleavage might have been subjected to subsequent degradation by multiple turnovers. We observed that in the absence of trap, a RNA product corresponding to the first upstream 18- or 19-base cut is only visible at early times (0–30 s). It is rapidly degraded to a 14-base fragment, which is then processed further, in a slow reaction, until the RNA·DNA duplex undergoes spontaneous denaturation. The consistent appearance of a gel band corresponding to a 14- or 15-nucleotide fragment may indicate the minimum length of the hybrid essential for efficient binding of enzyme (34). Consequently, further processing of RNA·DNA duplexes where the RNA is less than 15 bases is slower and is also retarded by the decreasing thermal stability of the shorter duplexes. An 18- or 19-base distance between the two active sites in HIV-1 RT has been observed (27, 30) in a model system involving the RNA harboring the polypurine tract complexed to complementary synthetic DNA oligonucleotides and in measurements of the partially processive 3' → 5' endonuclease activity of HIV-1 RT RNase H.

In the viral system, the synthesis of the first strand, minus-strand DNA, is primed by a $tRNA^{Lys}$ of cellular origin hybridized to the viral genomic RNA at a complementary sequence of 18 nucleotides (31)—i.e., the primer binding site. It has also been shown (32) that in the processing of the RNA primer for plus-strand DNA synthesis by HIV-1 RT, the activity of RT cleaves the RNA strand into multiple fragments. However, only two primers are extended in the presence of nucleoside triphosphates, with the major RNA primer including the entire polypurine tract possessing the sequence 5'-UUUUAAAAGAAAAGGGGGG-3'. This sequence again is 19 bases in length. These collective observations may be more than coincidental given our result for the 18- or 19-nucleotide distance between the two active sites.

The doubly labeled RNA–DNA 40/24 template-primer permitted study of the temporal linkage between the polymerase and RNase H activities in the same reaction. The RNase H cleavage pattern was determined as a function of a polymerase-catalyzed extension of the 24-mer DNA primer by three nucleotides. As can be seen from Fig. 4, there is a one-to-one correspondence between the intensity patterns of the DNA polymerization and RNA hydrolysis bands, suggesting that reverse transcription and RNase H hydrolysis proceed simultaneously.

On the basis of the above results, the following model is proposed for HIV-1 RT action (Fig. 5). Processive polymerization is initiated on RNA·DNA duplexes where the RNA is less than 18 or 19 nucleotides but RNase H cleavage results only from multiple binding events. This is evident from the repression by trap of further hydrolytic RNA fragmentation. When the HIV-1 RT is bound on a template/primer longer than 18 or 19 bases, the polymerization and RNase H activities proceed concomitantly until polymerization is complete with the RNA cleavage products arising 18 or 19 bases distant from the advancing primer terminus. Finally, the enzyme, in multiple binding events, further rapidly degrades the remaining 18 or 19 bases of the duplex RNA to a 14-bp strand followed by a slower, polymerase-independent cleavage of the remaining fragments.

Note. A separation of 20 nucleotides between the RNase H and polymerase sites of HIV-1 RT has been inferred from a model of A form DNA–RNA template-primer bound to the x-ray crystallographic structure of the enzyme (33).

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