HIV-1 reverse transcriptase-associated RNase H cleaves RNA/RNA in arrested complexes: implications for the mechanism by which RNase H discriminates beween RNA/RNA and RNA/DNA

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Reverse transcription of human immunodeficiency virus type 1 (HIV-1) is primed by tRNA^{Lys3}, which forms an 18 base pair RNA homoduplex with its 3' terminus and the primer binding site (PBS) of the viral genome. Using an *in vitro* system mimicking initiation of minus strand DNA synthesis, we analyzed the mechanism by which HIV-1 reverse transcriptase (RT)-associated ribonuclease H (RNase H) distinguishes between RNA/DNA and RNA/RNA (dsRNA). tRNA^{Lys3} was hybridized to a PBS-containing RNA template and extended by addition of deoxynucleoside triphosphates (dNTPs). In the presence of all four dNTPs, initial cleavage of the RNA template occurred immediately downstream of the tRNA−DNA junction, reflecting RNase H specificity for RNA in a RNA/DNA hybrid. However, in the absence of DNA synthesis, or limiting this by chain termination, the PBS was cleaved at a constant distance of 18 nucleotides upstream of the nascent primer 3' terminus. The position of cleavage remained in register with the position of DNA synthesis arrest, indicating that hydrolysis of homoduplex RNA is spatialy co-ordinated with DNA synthesis. Kinetic studies comparing cleavage rates of an analogous DNA primer/PBS heteroduplex and the tRNA^{Lys3}/PBS homoduplex showed that while the former is cleaved as rapidly as RT polymerizes, the latter proceeds 30-fold slower. Although the RNase H domain hydrolyzes dsRNA when RT is artificially arrested, specificity for RNA/DNA hybrids is maintained when DNA is actively synthesized, since residency of the RNase H domain at a single base position is not long enough to allow significant cleavage on dsRNA.
Key words: AIDS/HIV-1/reverse transcriptase/RNase H/RNase H*

Introduction

Conversion of the single-stranded retroviral RNA genome into pre-integrative double-stranded (ds) DNA requires the virus-encoded reverse transcriptase (RT), a multifunctional enzyme possessing polymerase activities on both RNA and DNA templates and ribonuclease H (RNase H) activity which degrades the RNA strand of RNA/DNA hybrids. RT of HIV-1 is a heterodimeric enzyme of 66 and 51 kDa subunits (p66 and p51). Both enzymatic activities reside in the p66 subunit, i.e. the RNase H domain constitutes the C-terminal and the DNA polymerase domain the N-terminal portion of the protein. Reverse transcription of HIV is initiated from human tRNA^{Lys3}, which primes synthesis of minus (−) strand DNA. The tRNA primer hybridizes with its 3' terminus to the complementary primer binding site (PBS), which is located near the 5' terminus of the viral RNA. As polymerization proceeds, RT-associated RNase H activity degrades RNA of the RNA/DNA replication intermediate, while the PBS of the initial RNA homoduplex is thought to remain intact (Varmus and Swanstrom, 1982; Telesnitsky and Goff, 1993).

Understanding the interplay between the DNA polymerase and RNase H active sites of RT has been facilitated by determination of the crystal structure of the HIV-1 enzyme complexed with nevirapine (Kohlstaedt et al., 1992) and the crystal structure of HIV-1 RT complexed with a 19 base template/18 base primer DNA homoduplex (Jacobo-Molina et al., 1993). In the RT−DNA co-crystal, 17–18 bp of dsDNA, adopting partly A- and partly B-conformation, is accommodated between both active sites (Jacobo-Molina et al., 1993). Assuming an all A-form duplex, a model of RT complexed with primer/template suggests that the distance between the active sites is 20 bp (Kohlstaedt et al., 1992). In support of the crystallographic data, recent biochemical studies indicate 18–19 bp between the positions of RNA hydrolysis and DNA polymerization (Wöhrl and Mölling, 1990; Gopalakrishnan et al., 1992, Kati et al., 1992), while Furfine and Reardon (1991) suggested a 15–16 bp distance between the two sites. Differences between biochemical measurements has been explained by the ability of HIV-1 RT-RNase H to cleave the template not only at a fixed distance with respect to the growing DNA strand, but also in the 3'→5' direction when the 3' terminus of the primer is not bound to the active site of polymerization (Gopalakrishnan et al., 1992; Kati et al. 1992). The corresponding RNase H activities in the two different binding modes have been designated polymerase-dependent and independent (Furfine and Reardon, 1991; Gopalakrishnan et al., 1992). It was suggested that RT-associated RNase H activity acts in the polymerase-dependent mode in spatial and temporal coordination, resulting in hydrolysis products of 18–20 bases, while in the polymerase-independent mode this coordination is lost, resulting in degradation products of down to seven bases (Schatz et al., 1990; Gopalakrishnan et al., 1992; Peliska and Benkovic, 1992).

Although RNase H activity and its interplay with the polymerase activity are well characterized, an unresolved problem is the mechanism by which RT-RNase H distinguishes between a RNA/DNA hybrid and the structurally similar RNA/RNA homoduplex. It has previously been reported that HIV-1 RT cleaves the PBS at two distinct positions when DNA synthesis was primed with *in vitro* synthesized tRNA^{Lys3} (Ben-Artzi *et al.*, 1992a). Subsequent reports by the same authors and others (Ben-Artzi *et al.*, 1992b; Hostomsky *et al.*, 1992) strongly indicated that this activity was due to *Escherichia coli* RNase III contamination. However, using *in situ* gel techniques in which proteins are renatured within an RNA-containing matrix, it was shown that HIV-1 (Ben-Artzi *et al.*, 1992b) and Moloney murine leukemia virus (M-MuLV) RT (Blain and Goff, 1993) cleave heteropolymeric dsRNA. Site-directed mutagenesis at residues thought to be involved in binding of the divalent metal ions required for RNase H activity reduces (M-MuLV RT^{D524N}) or eliminates (HIV-1 RT^{E478Q}) RNA cleavage activity on both RNA/DNA hybrids and dsRNA (Ben-Artzi *et al.*, 1992b; Blain and Goff, 1993). These observations provided strong evidence that hydrolysis of RNA/DNA hybrids or RNA/RNA duplexes is mediated by the same active site in the RNase H domain, but is in conflict with the accepted view that RT-associated RNase H is able to discriminate between such duplexes. In this report, we examined the RT-associated dsRNase activity directed against tRNA primer/template duplex and, more precisely, interplay between this and the polymerase activity of HIV-1 RT.

Results

In order to analyze the mechanism by which RT-associated RNase H distinguishes between RNA/RNA and RNA/DNA substrates, we designed an *in vitro* system closely resembling the initiation complex for (−) strand DNA synthesis. A 56 nt RNA fragment containing the PBS (designated PBS 1 in Figure 1A) was used as template. The 3′-terminus of tRNA^{Lys3} was hybridized with the RNA template over 18 complementary bases as described in Materials and methods. The resulting molecule contained a recessed 3′-terminus of tRNA^{Lys3} for initiation of DNA synthesis and 25 single-stranded template bases (Figure 1A). The rationale of our approach was to stepwise extend the 3′-terminus of the tRNA primer and analyze activity of the RNase H domain, the active site of which is positioned 18–20 bp upstream of the polymerization site (Gopalakrishnan *et al.*, 1992; Kohlstaedt *et al.*, 1992). The relative displacement of the two active sites along the primer/template duplex implies that during the first steps of DNA synthesis, the RNase H domain is positioned over the RNA/RNA homoduplex and only after ~18–20 synthesis steps will it encounter the junction between primer tRNA and the nascent DNA strand. Our aim was to characterize RNase activity of HIV-1 RT before and during transition from dsRNA into the RNA/DNA hybrid.

Analysis of limited tRNA primer extension

A study of reverse transcription is complicated by the fact that RT has two enzymatic activities on the same substrate; the polymerase domain copies the RNA template, while the RNase H domain subsequently degrades it. For direct comparison of DNA synthesis and RNA hydrolysis in the same experiment, the tRNA primer was labeled at its 5′-terminus and the template RNA at its 3′-terminus. In our assay, DNA synthesis was controlled by withholding particular dNTPs from the reverse transcription reaction and including corresponding ddNTPs. RT translocates in accordance with the extent of DNA synthesis and is arrested at a position (register) corresponding to the missing dNTP (Metzger *et al.*, 1993). We modified the viral template sequence downstream of base position +1 to facilitate arrest of complexes in a broader register. The authentic viral sequence allows arrest of complexes in registers 1, 2, 3 and 6 (Figure 4A, PBS 2), while the modified sequence yields stops at positions 1, 3, 9 and 18 (Figure 1A, PBS 1).

RNA/tRNA^{Lys3} and RT were incubated with dNTP/ddNTP mixes to produce complexes in the corresponding registers (Figure 1A) and analyzed by high resolution gel electrophoresis. Figure 1B depicts DNA synthesis from the 5′-end-labeled tRNA^{Lys3}, where homogeneous products of the expected size illustrate that chain termination is effective and specific (pre-annealing of template RNA and primer tRNA was performed in an excess of the latter, as described in Materials and methods; therefore, a portion of the labeled primer tRNA remains unextended). To control for contaminating ribonuclease activities such as *E.coli* RNase III (Hostomsky *et al.*, 1992), we compared activities in preparations of wild-type HIV-1 RT (Figure 1B, left) and the mutant p66$^{E→Q}$/p51 (Figure 1B, right) whose ability to degrade dsRNA and RNA/DNA is eliminated (Schatz *et al.*, 1991; Ben-Artzi *et al.*, 1992b).

Analysis of RNase activity during DNA synthesis

Since RT-RNase H can cleave the template RNA more than once, the hydrolysis profile of 3′-end-labeled template (e.g. Figure 2A, right panel) is biased toward upstream cleavage, while that of 5′-end-labeled template (e.g. Figure 2A, left panel) is biased towards those located furthest downstream. These patterns together provide a more complete picture of RNase H activity, which allowed us to distinguish between single endonucleolytic (only obtained from 3′-end-labeled template) and progressive multiple cleavages attributed to the 3′→5′ directional nuclease activity (Schatz *et al.*, 1990; Gopalakrishnan *et al.*, 1992).

To provide a basis for understanding Figures 1 and 2, we first discuss the scenario where RNase H activity is directed against its usual substrate, the template RNA of RNA/DNA hybrids. DNA synthesis on our model template in the presence of all four dNTPs yields a strong stop (run-off) primer elongation product of 25 bases (Figure 1B, lane 25). The pattern obtained with 3′-end-labeled template RNA in Figures 1B and 2A (left panel) shows that the dsRNA region between base positions −18 and +1, i.e. the PBS, remains uncleaved, while the region hybridized to the newly synthesized DNA is cleaved as expected from the known specificity of RNase H. As shown in Figures 1B and the 2A (left panel, lanes 25), initial cleavage in the 3′-end-labeled template RNA appeared between the first two bases after the transition to RNA/DNA (designated as base positions +1 and +2).
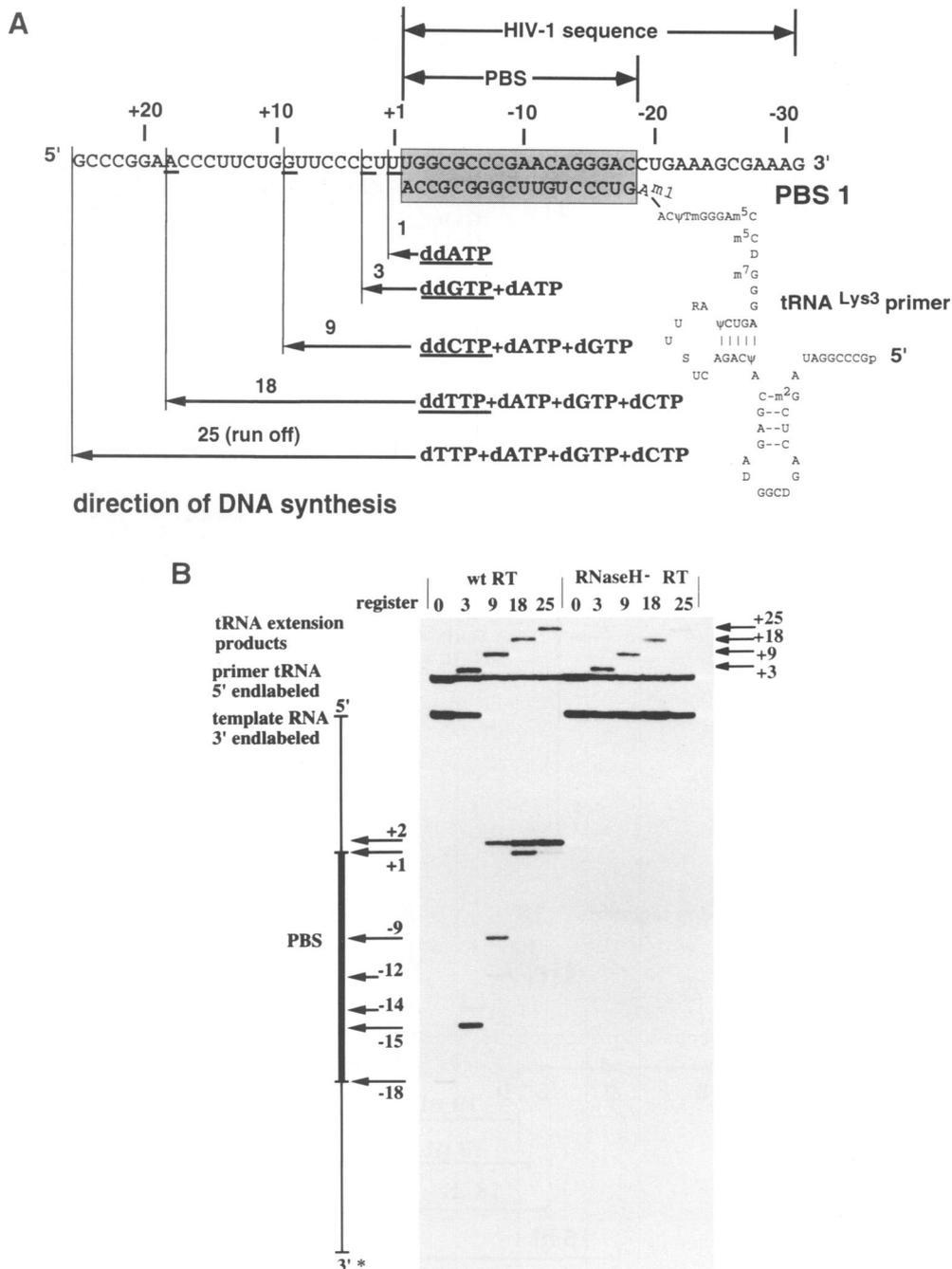
A

HIV-1 sequence

PBS

+20   +10   +1   −10   −20   −30

5′ GCCCGGAACCCUUCUGGUUCCCCUUUGGCGCCCGAACAGGGACCUGAAAGCGAAAG 3′

ACCGCGGGCUUGUCCCUG A $m1$ **PBS 1**

ACψTmGGGAm^{5}C

1 ←——— **ddATP**

3 ←——— **ddGTP+dATP**

9 ←——— **ddCTP+dATP+dGTP**   tRNA ^{Lys3} primer

18 ←——— **ddTTP+dATP+dGTP+dCTP**

25 (run off) ←——— **dTTP+dATP+dGTP+dCTP**

direction of DNA synthesis

B

	wt RT					RNaseH⁻ RT					
register	0	3	9	18	25	0	3	9	18	25	

tRNA extension products → +25, +18, +9

primer tRNA 5′ endlabeled → +3

template RNA 3′ endlabeled

+2
+1
PBS
−9
−12
−14
−15
−18

Fig. 1. (**A**) Primer/template (PBS 1) sequences for arresting complexes in specific registers. The sequence upstream of +1 containing the PBS is identical to that found in HIV-1 isolates. The sequence downstream of position +1 was chosen to arrest DNA synthesis at positions (registers) +1, +3, +9 and +18. After hybridization of the 3′-terminus of natural tRNA^{Lys3} to the PBS, forming an RNA homoduplex (shaded area), DNA synthesis was advanced into the indicated registers using nucleotide cocktails, including a chain terminating ddNTP. In the presence of all four dNTPs, a 25 nt extended tRNA was synthesized. (**B**) Analysis of DNA synthesis and RNase cleavage. Primer/template and substrate mixes were incubated with wild-type RT (left) or mutant, p66$^{E→Q}$/p51 (right), yielding complexes arrested in registers 3 (lane 3), 9 (lane 9) and 18 (lane 18). Register 0 (lane 0) represents the complex without dNTPs and register 25 (lane 25), the complex synthesizing a run-off cDNA. dNTP/ddNTP mixes for each register are depicted in Figure 1A. Nascent DNA is obtained as an elongation product of 5′-labeled tRNA^{Lys3}, while RNase cleavage products are visualized by 3′-end-labeling of the template. Arrows indicate DNA synthesis and RNase H cleavage products, which are depicted in the schematic drawing in Figure 2B. Shorter arrows at position −14 (lane 3) and position −12 (lane 9) represent minor cleavages, the intensities of which never exceed 10% with respect to the major cleavage products. The vertical line represents the RNA and the black bar the PBS.

Comparison with lane 25 in Figure 2A, right panel, in which the template is labeled at the 5′-end, shows that cleavage of the template RNA continues after position +1 as the polymerase domain translocates. The resulting fragments vary in size from 18 to 8 nt, in line with published results showing that RNase H activity cleaves the template in the 5′-direction in a polymerase-dependent mode, yielding fragments of 18 bases, as well as in the polymerase-independent mode, yielding fragments as small as seven bases (Schatz *et al.*, 1990; Gopalakrishnan
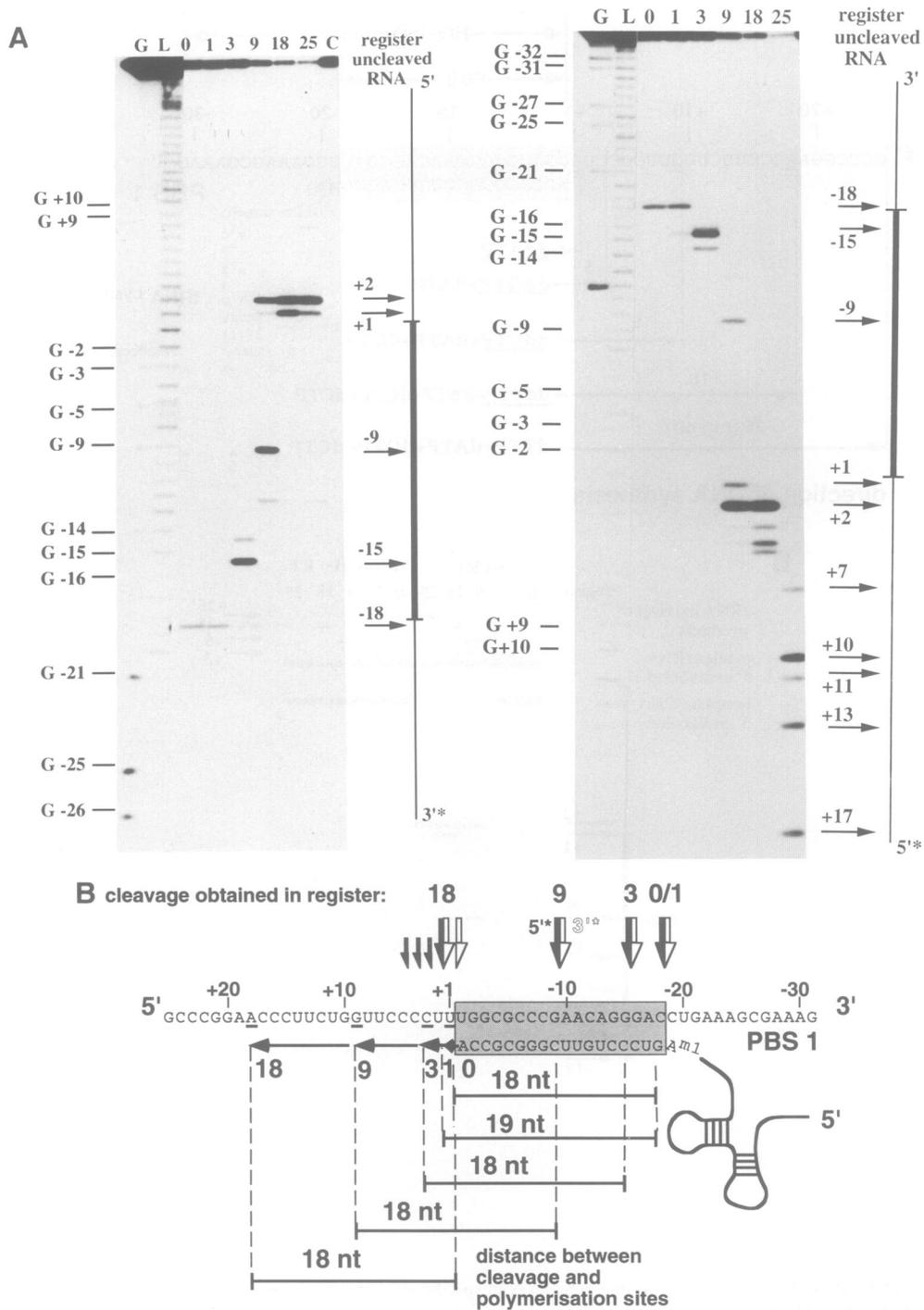
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Fig. 2. (A) Comparison of 3'- (left) and 5'-end-labeled (right) template cleavage products. Complexes in registers 0, 1, 3, 9, 18 and 25 were analyzed as described in Figure 1A and Materials and methods. Cleavage products are depicted in the corresponding lanes. Lane C is a control showing the template without incubation with RT, lane G is a G-specific length standard obtained by partial RNase T1 digestion and lane L is a sequence ladder obtained by partial alkaline hydrolysis. (B) Diagram of RNA cleavage positions after advancing DNA synthesis into registers 0, 1, 3, 9 and 18. Half arrows, filled and open, represent 5'- and 3'-derived cleavage products. The cleavage positions are obtained from Figure 2A, right and left panels respectively. Full arrows indicate the size of the synthesized DNA. Shaded area represents dsRNA.

et al., 1992; Peliska and Benkovic, 1992). Since polymerase-independent cleavages are in principle observed when the primer terminus is not in a proper position to allow synthesis, in the following we use the term 'synthesis-independent' in place of 'polymerase-independent' to stress functional uncoupling of this activity from the polymerization process.

Analysis of RNase activity within the PBS

If, however, wild-type RT is arrested before elongating past position +18 by incubation with an appropriate dNTP/ddNTP mix, cleavage is observed within the PBS at a distance strictly correlated with the extent of primer extension. This results in the cleavage pattern of 3'-end-labeled template depicted in Figures 1B and 2A, left panel.

Incubation of primer/template with RT in the absence of dNTPs (register 0; lane 0 in Figures 1B and 2A, left panel) results in a single cleavage at -18 within the template RNA, positioned directly upstream of the last PBS/tRNA primer base pair at a distance of 18 bp from the primer terminus. When tRNA^{Lys3} is extended by three or nine nucleotides (registers 3 and 9), cleavage at -15 and -9 within the 18 bp RNA homoduplex is again 18 nt upstream of the position of DNA polymerase arrest. The same cleavage distance is observed after addition of 18 nucleotides (register 18), at which point the template is hydrolyzed exactly at position $+1$, i.e. complementary to the junction between tRNA^{Lys3} and newly synthesized DNA. Additional cleavages within the RNA/DNA duplex (primarily at position $+2$) in registers 9 and 18 are thought to result from the synthesis-independent activity of RT and are discussed below.

To analyze whether hydrolysis results from a mechanism promoting single or multiple cleavage, the experiments described above were performed with a 5'-end-labeled template. The results are shown in Figure 2A, right panel, and Figure 2B depicts a schematic representation of the cleavage patterns derived from the 3'- as well as from the 5'-end-labeled templates. Cleavage within the PBS obtained by 3'- as well as by 5'-labeling is located at the same position, indicating that dsRNA-specific RNase activity cleaves solely endonucleolytically. This endonucleolytic cut is observed 18 bp upstream of the primer 3'-terminus, suggesting a strict correlation between progress of DNA synthesis and dsRNA-specific RNase activity. This pattern is reminiscent of the polymerization-dependent mode of RNase H.

The complex arrested in register 1 (lane 1 in Figure 2A) deviates from the 18 bp distance pattern, showing cleavage 19 bp upstream of the primer terminus, i.e. at the same position as in register 0. Studies are underway to test the hypothesis whether this finding is due to (i) RT$-$tRNA^{Lys3} interactions inhibiting translocation of the enzyme after incorporation of the first nucleoside or (ii) special structural features of the primer$-$template during the initial steps of DNA synthesis (Kohlstaedt and Steitz, 1992; Isel et al., 1993).

Parallel experiments with the RNase H-deficient RT mutant p66$^{E \rightarrow Q}$/p51 show that while it extends the primer as efficiently as wild-type enzyme, it does not hydrolyze RNA at any position, regardless of arrest (Figure 1B, right). The functional correlation of RNA hydrolysis and the extent of reverse transcription, together with the observation that the mutant lacks ribonuclease activity, excludes contaminating RNases from our assays. We can also exclude the possibility that the hydrolysis of homoduplex RNA is an artifact due to the use of chain terminating ddNTPs, since cleavage is also observed in their absence (register 0). We conclude then that the ability to cleave the RNA template is intrinsic to RT itself. In addition, the observation that both substrates are cleaved at the same distance with respect to the primer terminus strongly supports earlier suggestions that both activities are mediated by the same active site in the RNase H domain (Ben-Artzi et al., 1992b; Blain and Goff, 1993).

Analysis of RNase H activity at the tRNA$-$DNA junction

It is striking that after primer elongation by 9 or 18 nt, the template RNA is frequently cleaved opposite the junction between the tRNA primer and nascent DNA. The cleavage products in Figures 1B and 2A show prominent cleavage between the first and second RNA/DNA base pairs (position $+2$, and slight cleavage at position $+1$). Because these occur at a distance considerably less than 18 nt from the primer terminus, they must be attributed to the synthesis-independent cleavage mode.

The RNA/DNA hybrid in register 3 in the experiments of Figures 1 and 2 is not cleaved, indicating that it may be too small to be recognized by the RNase H domain alone. We have evidence that RNA hydrolysis in the vicinity of the junction requires a RNA/DNA duplex length of at least 7 nt (data not shown).

In order to exclude the possibility that the cleavage pattern at the tRNA$-$DNA junction is influenced by artificial sequences downstream of the $+1$ position in PBS 1, an RNA template (PBS 2) containing the wild-type viral sequence from position $+13$ to -29 was constructed and hybridized with a chimeric RNA$-$DNA primer consisting of nine ribonucleoside bases (positions -1 to -9) and of 14 deoxyribonucleoside bases (positions $+1$ to $+14$) (see Figure 3A). Elongation of this primer/template closely resembles the situation after synthesis of 14 nt (register 14). This substrate also allowed us to examine synthesis-independent activity in the absence of added nucleotides. Upon incubation of this primer/template substrate with HIV-1 RT, four cleavage products are evident (Figure 3B, lane 14), i.e. one 18 bp upstream of the primer terminus at position -4 and three at positions $+1$, $+2$ and $+4$. When the chimeric primer is extended by 1 nt, the upstream cleavage follows the extending primer terminus in a fixed distance of 18 nt, identifying it as the polymerase-dependent cleavage (lane 15). In contrast, cleavages in the vicinity of the junction are unaffected, regardless of whether full or limited DNA synthesis was allowed or even in the absence of dNTPs (registers 32, 15 and 14). These cleavages, schematically represented in Figure 3A, are the counterparts of the single synthesis-independent cleavage at $+2$ in the experiments of Figures 1 and 2. On templates PBS 1 and PBS 2, synthesis-independent cleavage occurs very near the RNA$-$DNA junction, suggesting that this region has a structure particularly well recognized by RNase H. The observation that the cleavage positions are not identical in the two substrates suggests, however, that this preferred structure is not entirely sequence-independent.

Analogously, when the tRNA^{Lys3} primer itself is removed during the process of second strand synthesis, the specific cleavage between the terminal CA (3') of tRNA^{Lys3} has also been shown to occur in both binding modes (Smith and Roth, 1993). Presumably, the conformation of the tRNA$-$DNA junction provides a particularly good target for RNase H activity, irrespective of whether the chimeric RNA$-$DNA structure is complexed with ($+$) strand RNA (initiation of reverse transcription) or ($+$) strand DNA (primer removal).

Efficiency of RNase H activity on homoduplex RNA and heteroduplex RNA/DNA

The observation that hydrolysis of dsRNA does not occur unless the translocated protein is artificially arrested clearly indicates that the rate of cleavage is slower than that of polymerization. In contrast, RNase H activity on RNA/
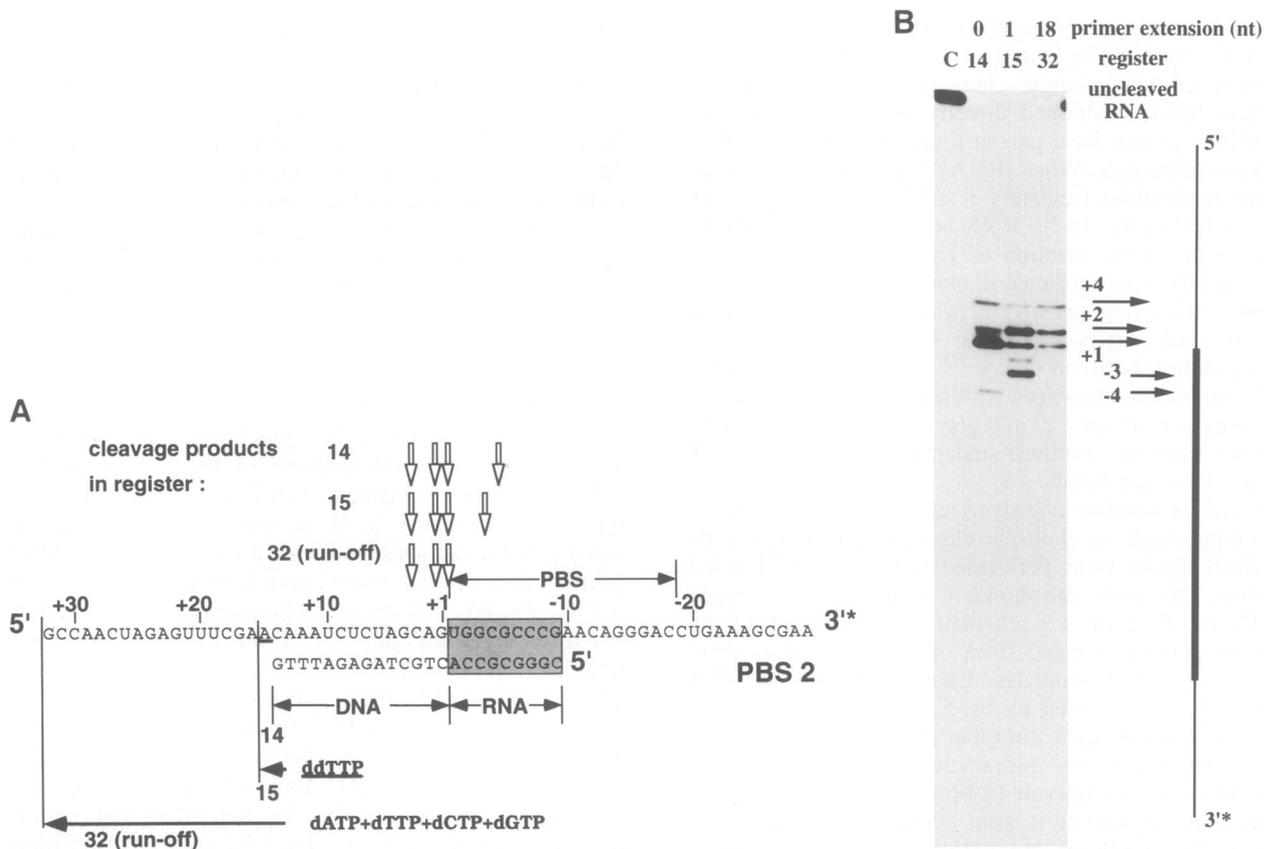
Fig. 3. (**A**) RNase cleavage at the RNA—DNA junction. Template RNA (PBS 2) containing the wild-type HIV-1 sequence from base position −25 to +13 was hybridized with a chimeric RNA—DNA fragment containing 14 base DNA and nine base RNA. This DNA–RNA/RNA primer/template was incubated with RT with and without dNTP mix, as depicted in the figure. RNase products without elongation (corresponding to register 14) and after elongation of the primer by one base (corresponding to register 15) and 18 bases (corresponding to register 32) are indicated by open arrows. The region containing dsRNA is shaded. (**B**) RNase products obtained with a template hybridized to the chimeric RNA—DNA primer. 3′-End-labeled primer/template was incubated with a dNTP/ddNTP mix and RT as described in Figure 3A and Materials and methods. Lane 18 shows the RNase products after elongation of the primer by 18 bases (run-off synthesis), lane 1 after elongation by one base, which corresponds to register 15, and lane 0 without elongation, corresponding to register 14. Lane C shows control uncleaved RNA.

DNA must be as fast or faster than the rate of polymerization, since the RNA is cleaved as soon as the RNase H domain has reached the hybrid of the RNA template and nascent DNA (Figure 1B, lane 25). To confirm this directly, we compared the rate of RNA hydrolysis when synthesis was primed with tRNA^{Lys3} or a related 24 nt DNA primer. The RNA template PBS 2, derived from the wild-type sequence, was hybridized to one of the primers and incubated with a dNTP/ddNTP cocktail permitting arrest of DNA synthesis in register 3 (Figure 4A). The polymerization/cleavage reaction was initiated by addition of RT and cleavage products were analyzed after incubation for 0.5–20 min. Figure 4B and C shows the patterns obtained with the 3′- and the 5′-labeled templates respectively. The left panels in each figure depict the tRNA-primed experiments and the right panels depict the DNA-primed experiments.

When synthesis is tRNA-primed, cleavage occurs after 5 min at position −15, 18 bp upstream of the DNA polymerase domain arrested in register 3. In agreement with the experiment of Figure 2A, the latter result is found on 3′- and 5′-end-labeled template RNA. When polymerization is DNA-primed, however, complete cleavage of the 3′-end-labeled template occurs after 30 s at positions −18 to −15

as RT polymerizes from registers 0 to 3. In addition, a synthesis-independent cleavage also appears immediately at −22. We suggest that cleavage at positions −17 to −15 is polymerase-dependent, as it does not occur in the absence of dNTPs (data not shown). The 3′-derived products in Figure 4B (right) show major cleavage at base position −18, indicating that RT-associated RNase H activity cleaves after binding and prior synthesis with high efficiency. Weak cleavage at positions −17 to −15 indicate that initial hydrolysis at position −18 was not completed and template RNA can also be cleaved during DNA synthesis (Gopalakrishnan *et al.*, 1992). Cleavages at positions −18 to −15 on the 5′-labeled template are compressed into nearly one band (Figure 4C, right); these fragments are further cleaved with time as a consequence of synthesis-independent RNase H activity.

Quantification of products at the different time intervals (Figure 4D) indicates that cleavage of dsRNA is at least 30 times slower than that of RNA/DNA, since tRNA-primed synthesis results in only 20% template cleavage after 20 min, while in DNA-primed synthesis the template is completely cleaved within 2 min (60% in a polymerase-dependent manner at positions −18 to −15 and 40% at position −22).
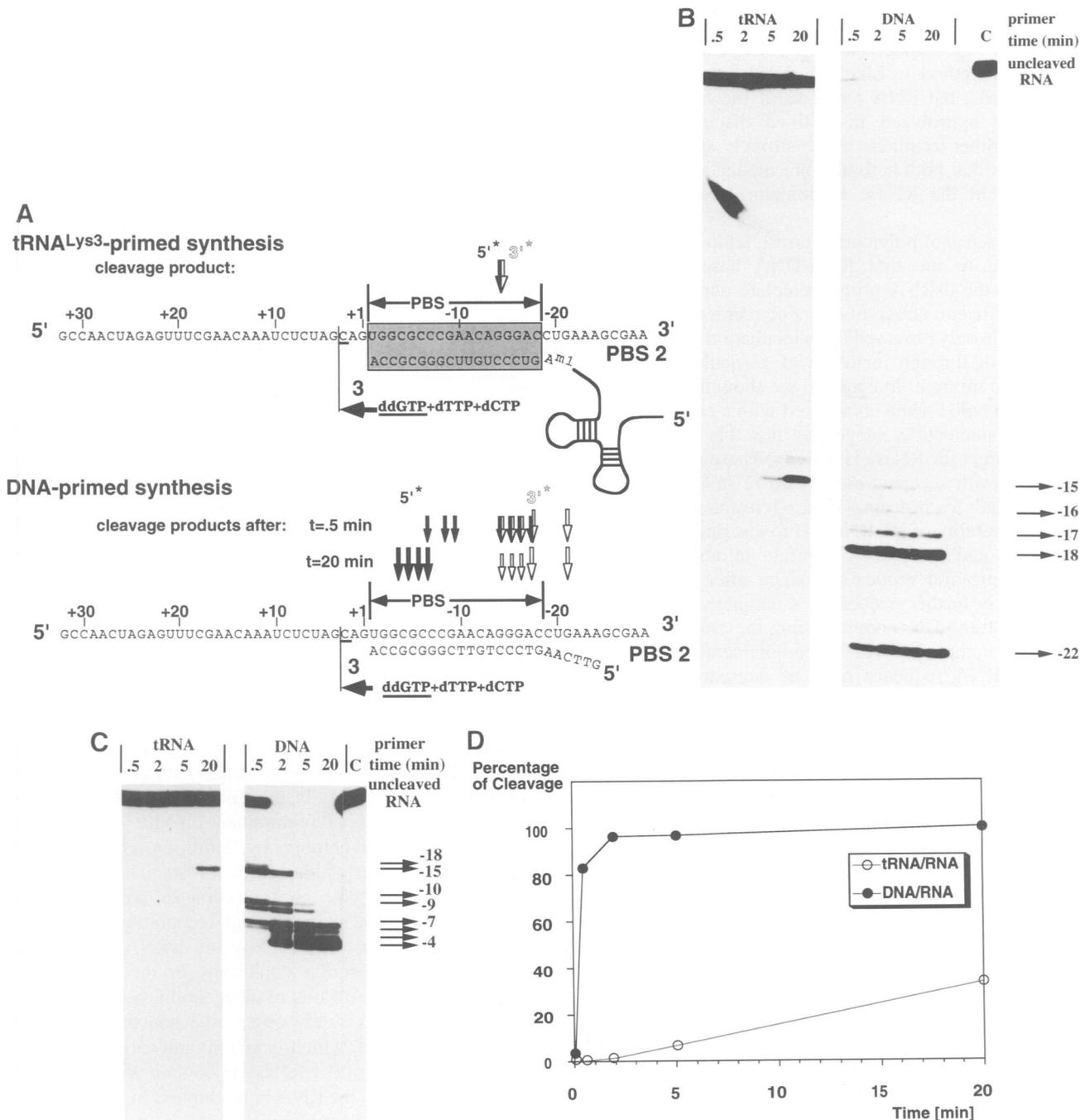
Fig. 4. (**A**) Template/primer variants for kinetic analysis of RNase activity on homo- and heteroduplex substrates. Template RNA was hybridized with either tRNA^Lys3 or a DNA fragment, forming a region of homoduplex RNA and heteroduplex RNA/DNA respectively. The sequence of the template RNA (PBS 2) in both experiments was identical. The primer was elongated by three bases using the nucleotide mix depicted. The sites of hydrolysis, determined by 5′- and 3′-end-labeling of the template RNA, are represented by filled and open arrows. (**B**) Kinetics of RNase activity on dsRNA (left panel) and RNA/DNA (right panel) using 3′-labeled template. Homoduplex primer/template RNA and heteroduplex primer/template RNA/DNA shown in Figure 4A were extended by three bases using a substrate mix as indicated. Hydrolysis products were analyzed at the indicated time points. Lane C shows uncleaved RNA as a reference. (**C**) Kinetics of RNase activity on dsRNA (left panel) and RNA/DNA (right panel) using 5′-labeled template. The conditions are as in Figure 4B. (**D**) Quantification of cleavage products. The amount of cleaved RNA, i.e. all RNase products, was determined at the time intervals indicated and normalized with the total amount of RNA template used for the experiment.

Discussion

The ability of retroviral RT to degrade heteropolymeric dsRNA has been demonstrated using *in situ* gel techniques (Ben-Artzi *et al.*, 1992b; Blain and Goff, 1993). In this study we have characterized this activity and its relationship to DNA polymerase activity using a template

modeled on the natural substrate for initiation of (−) strand DNA synthesis. After initiation of reverse transcription from a tRNA^Lys3 primer, polymerization was arrested at defined positions to determine the spatial arrangement of the DNA polymerase and RNase active centers. Although it was previously reported that HIV RT requires Mn^{2+} to cleave dsRNA (Ben-Artzi *et al.*, 1992b), we observed that

it can cleave the dsRNA primer/template duplex in the presence of Mg^{2+} under standard buffer conditions (Materials and methods). Like the RNA strand of RNA/DNA hybrids, the RNA template of the homoduplex is specifically hydrolyzed in a fixed distance of 18 nt from the primer terminus, which strongly supports earlier suggestions that both activities are mediated by the same active site in the RNase H domain (Ben-Artzi *et al.*, 1992b).

In the absence of polymerase arrest, template hydrolysis is localized to the first RNA/DNA base pair. RNase activity on the dsRNA primer/template duplex, however, requires efficient arrest of the polymerase, since RNA hydrolysis is only observed upon incubation in the absence of dNTPs or through inclusion of particular ddNTPs in the reaction mixture. In contrast, we show that the PBS is entirely degraded when complexed with a complementary DNA oligonucleotide, suggesting that this sequence is a common target for RNase H activity. These findings are in agreement with accepted models for reverse transcription (Varmus and Swanstrom, 1982; Telesnitsky and Goff, 1993). The ability of RT-RNase H to discriminate between RNA/RNA and RNA/DNA is in fact an intrinsic requirement of retroviral replication, since after initiation the PBS itself is further needed as a template for syntheses of its (−) strand DNA copy. During the process in which the PBS is complexed with its complementary DNA, this particular RNA sequence must be degraded in order to facilitate the second strand transfer. In support of this model, *in vivo* studies with proviral HIV-1 genomes containing mutations in the PBS strongly indicate that the (−) strand copy of the PBS must base pair with its (+) strand copy during the process of the second strand transfer to complete synthesis of the double-stranded pre-integrative DNA (Wakefield *et al.*, 1994).

The observation that dsRNA can, however, be cleaved when the enzyme is arrested on its primer/template provides new information concerning the mechanisms by which RT-RNase H distinguishes between RNA/RNA and RNA/DNA. While the rate of cleavage of RNA/DNA is equivalent to that of polymerization (Gopalakrishnan *et al.*, 1992), comparing the time dependence of RNA hydrolysis when the template RNA is hybridized with either tRNA or a DNA oligonucleotide primer indicates that RNase H-mediated cleavage of the RNA homoduplex is at least 30 times slower than that of RNA/DNA. We therefore conclude that the specificity of HIV-1 RT-RNase H for RNA/DNA hybrids is maintained kinetically, in that during polymerization the residence time of the RNase H domain at a single position on dsRNA is too short to permit cleavage. In contrast, on an RNA/DNA duplex the two activities can act co-ordinately, because the rate of RNA cleavage is as fast as or faster than polymerization (Figure 4B).

Although HIV-1 RT-RNase H can differentiate between the hybrid and the duplex, both appear to traverse the substrate binding groove between the active sites similarly. When RT is arrested, the RNA template is cleaved at a fixed distance of 18 bp from the primer terminus, independent of whether the duplex between the two active sites is entirely dsRNA, partly RNA/DNA and partly RNA/RNA or entirely RNA/DNA. This suggests that the duplex is bound similarly to the enzyme regardless of its precise

composition and that discrimination between RNA/DNA and dsRNA is accomplished locally near the RNase H active site, rather than globally through the general interaction of the duplex with RT. Recently, a basis for discrimination between RNA/DNA and dsRNA duplexes based on the NMR structure of an 8mer RNA/DNA hybrid has been proposed (Fedoroff *et al.*, 1993). Despite similarities in helical parameters of both duplexes, the width of the minor groove is narrower for the hybrid than for the RNA homoduplex, leading the authors to propose that *E.coli* RNase H is not able to cleave dsRNA because critical interactions cannot form with a duplex in a wide groove A-form conformation. Therefore, although *E.coli* RNase H does not display discernible activity against dsRNA, the relative substrate discrimination mechanisms of RT-RNase H and *E.coli* RNase H may not in fact be substantially different, both being mediated by local structural differences between dsRNA and RNA/DNA, as predicted for the *E.coli* enzyme. For the RT-associated RNase H activity, such local differences in structure may then be necessary, but not sufficient, for substrate discrimination in the context of reverse transcription. In contrast to *E.coli* RNase H, the retroviral enzyme must accept dsRNA as a substrate in order to initiate reverse transcription. Consequently, polymerization to rapidly remove the RNase H domain from the dsRNA target can be seen as an additional requirement for retrovirus-associated RNase H substrate discrimination.

Cleavage of dsRNA by RT, originally designated RNase D activity (Ben-Artzi *et al.*, 1992a), has been re-designated RNase H*, in the case of HIV RT partly on the basis of its reported requirement for Mn^{2+} (Hostomsky *et al.*, 1994). Star activity, by definition, is assigned to restriction enzymes which, under non-standard conditions, such as high enzyme concentration or replacement of Mg^{2+} with Mn^{2+}, show relaxed recognition specificity. Although we show that activity on dsRNA is observed under standard reaction conditions in the presence of Mg^{2+}, we agree with this re-designation, because hydrolysis of dsRNA is solely observed when the enzyme is artificially arrested, which is tantamount to vastly raising the concentration of the RNase H domain with respect to a particular site on the RNA homoduplex. In light of these findings, we find it unlikely that cleavage of dsRNA is relevant to the HIV life cycle, but a consequence of the artificially high local concentration of dsRNA with respect to the RNase H active site mediated by the arrest of the physically linked polymerase domain. Consistent with this interpretation is the observation that RT cleaves dsRNA exclusively endonucleolytically at a fixed distance of 18 bp from the primer terminus, while the RNA of RNA/DNA hybrids can be cleaved down to fragments of 7 bp by the synthesis-independent activity (Schatz *et al.*, 1990; Gopalakrishnan *et al.*, 1992; Peliska and Benkovic, 1992). Consequently, a tight interaction between the 3'-end of the primer terminus and the DNA polymerase active site is apparently an essential requirement for cleavage of dsRNA. In this regard it is interesting to note that while the separately expressed RNase H domain of the murine RT exhibits wild-type RNase activity on RNA/DNA

substrates, it is impaired for RNA/RNA nuclease activity (Blain and Goff, 1993).

Materials and methods

Materials

Heterodimeric HIV-1 RT and the RNase H-deficient enzyme were prepared by metal chelate and ion exchange chromatography from *E.coli* strains M15::pDM1.1::pRT6H-PR (Le Grice and Grüninger-Leitch, 1990) and M15::pDM1.1::pRT6H$^{E\rightarrow Q}$-PR respectively (Schatz *et al.*, 1990), as described previously (Le Grice and Grüninger-Leitch, 1990). The final purification step was chromatography on a sizing column to eliminate RNase contamination. RT was stored in liquid nitrogen in 50 mM Tris−HCl, pH 7.8, 500 mM NaCl, 1 mM DDT and 5% glycerol. The concentration of RT was determined by A_{280} measurements using an absorption coefficient of 1.4.

RNA templates were synthesized by *in vitro* transcription with T7 RNA polymerase and electrophoretically purified using 6% polyacrylamide−7 M urea gel containing 50 mM Tris−borate buffer, pH 8.0, 1 mM EDTA. RNA was visualized by UV shadowing at 254 nm and eluted from excised gel slices in a buffer containing 0.5 M ammonium acetate, pH 6.5, 0.1% SDS. Homogeneity of the purified 3′-end-labeled RNA was ensured by partial digestion using RNase T1. tRNA^{Lys3} with the anticodon SUU (S = 5-methoxycarbonyl-2-thiouridine) was purified from rabbit liver to at least 95% homogeneity as previously described (Raba *et al.*, 1979). DNA and chimeric DNA−RNA primers were chemically synthesized using the phosporamidite method and were purified as described above. Concentrations of nucleic acids were determined spectrophotometrically. The absorption coefficient was calculated from base composition.

End-labeling of nucleic acid components

RNA transcripts or tRNA were dephosphorylated with calf intestine phosphatase (Boehringer Mannheim) and 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Boehringer Mannheim) according to the manufacturer's specifications. 3′-end-labeling with [^{32}P]pCp and T4 RNA ligase (Pharmacia) was carried out as described (Bruce and Uhlenbeck, 1978).

Primer/template hybridization

RNA template was hybridized with a 1.5 molar excess of primer (tRNA^{Lys3}, chimeric DNA−RNA or DNA oligonucleotide). A mixture of 5.5 μM RNA template and 8.3 μM tRNA^{Lys3} in a buffer containing 50 mM Tris−HCl, pH 7.8, 100 mM NaCl, 6 mM MgCl$_2$ was heated to 90°C for 1 min followed by incubation at 58°C for 5 min and cooling for 60 min to room temperature. The formation of an 18 bp RNA homoduplex was checked by accessibility studies using 1,10-bis(orthophenanthroline)−Cu(I), a reagent which specifically cleaves the single-stranded regions of RNA (Murakawa *et al.*, 1989). Complete hybridization of the labeled RNA template was confirmed by analyzing aliquots of the pre-annealed duplexes on non-denaturing 4% polyacrylamide gels containing 20 mM Tris−HCl, pH 7.8, 2 mM MgCl$_2$. The RNA/DNA hybrid and the chimeric DNA−RNA primer/RNA template were prepared analogously.

Enzyme assay

RT preparations were dialyzed against 50 mM Tris−HCl, pH 7.8, 50 mM NaCl, 1 mM dithiothreitol. Enzyme reaction (in a total volume of 10 μl) was initiated by addition of HIV-1 RT (final concentration 700 nM) to pre-annealed primer/template (230 nM) and the dNTP/ddNTP cocktail (10 μM dNTPs and 100 μM ddNTP). Concentrations and ratios of RT to primer/template were optimized for maximum complex formation and yield of cleavage products. Non-denaturing gel electrophoresis (data not shown) showed that the yield of complex is optimal at a 3-fold excess of RT. This ratio was used after we ascertained that a change in the ratio had little or no effect on the characteristic features of the RNase H and RNase H* pattern. A concentration of 6 mM Mg^{2+} was found to be the optimal compromise to obtain a good yield of DNA synthesis and RNase cleavage activity. If Mg^{2+} was replaced by Mn^{2+} the characteristic features of the RNase H and RNase H* pattern did not change.

The assay containing RT, primer/template and nucleosides was incubated at 37°C for 15 min in a buffer containing 50 mM Tris−HCl, pH 7.8, 50 mM NaCl, 6 mM MgCl$_2$. The reaction was stopped by addition of 20 mM EDTA and subsequent precipitation with ethanol. The reaction

products were analyzed on 12% sequencing gels. Fragment sizes were assigned using alkaline- and G-ladders obtained by partial digestion of the end-labeled RNA with Na$_2$CO$_3$/NaHCO$_3$, pH 9.5, and ribonuclease T1 respectively. RNase products were quantified using a PhosphorImager.

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