The EMBO Journal vol.9 no.4 pp.1171–1176, 1990

HIV-1 RT-associated ribonuclease H displays both endonuclease and 3′→5′ exonuclease activity

Octavian Schatz, Jan Mous and Stuart F.J.Le Grice

Central Research Units, F.Hoffmann-LaRoche Ltd, CH-4002 Basel, Switzerland

Communicated by M.Steinmetz

We have analysed the mechanism of ribonuclease H (RNaseH) induced cleavage of a defined RNA–DNA hybrid by human immuno-deficiency virus (HIV-1) reverse transcriptase (RT). An *in vitro* transcribed RNA labelled at the 3′ end was hybridized to a pentadecameric DNA oligonucleotide complementary to an internal region of the RNA. Upon incubation of this RNA–DNA hybrid with recombinant p66 or p66/p51 HIV-1 reverse transcriptase, RT-RNaseH mediated cleavage is observed at most nucleotides within the short hybridized stretch, resulting in a spectrum of RNA fragments extending from the 3′ label to this region and differing in length by one nucleotide. The same RNA, this time labelled at the 5′ end, yields only one or two major cleavage products corresponding to RNA species extending from the 5′ label to the middle of the hybridized region. Such a result can be explained by the action of both endonuclease and 3′→5′ exonuclease activities inherent to the C-terminal domain of p66 RT. To investigate how RNaseH cleavage is coupled to reverse transcription, a combination of deoxynucleoside triphosphates was used which allowed controlled extension of the primer DNA. Concomitantly with the elongation of the oligonucleotide primer, RNaseH cleavage proceeds towards the 5′ end of the RNA with identical increments, suggesting a simultaneous action of both activities.

Key words: HIV-1/ reverse transcriptase/RNaseH cleavage

Introduction

By definition, ribonuclease H (RNaseH) is an enzyme that cleaves the RNA moiety of an RNA–DNA hybrid. Although it can be found in almost any living cell, conceivably reflecting its importance in vital cellular processes, a direct physiological role for RNaseH could not so far be conclusively demonstrated. It has been suggested that the enzyme is somehow involved in DNA replication because it is maximally expressed during DNA synthesis and in dividing cells (Crouch and Dirksen, 1982). Recently, it has also been shown that RNaseH is associated with renaturase activity required for the displacement of newly transcribed RNA strands, implying yet another function for cellular RNaseH (Kane, 1988). By far better understood is the role of RNaseH in retroviruses.

Retroviral polymerases comprise a C-terminal RNaseH domain, characterized by deletion experiments (Hansen *et al.*, 1988; Kotewicz *et al.*, 1988), linker insertion (Tanese and Goff, 1988), proteolytic fragmentation (Lai and Verma, 1978) and point mutagenesis (Repaske *et al.*, 1989; Schatz *et al.*, 1989). This inherent RNaseH function is vital for the viral replication and cannot be substituted by cellular RNaseH. Both in Moloney murine leukaemia virus (MoMuLV) and HIV-1, proviral DNAs carrying point mutations at highly conserved sites in their RNaseH coding regions fail to give rise to progeny virus when they are reintroduced into susceptible host cells (Repaske *et al.*, 1989; Schatz *et al.*, 1990).

RNaseH-function is probably needed at several stages in reverse transcription of viral genomic RNA, some of which require exquisite specificity. After initiation of minus strand DNA synthesis from a host tRNA primer, the RNA template is probably removed by RNaseH in order to allow hybridization of the 3′ end of the second RNA strand of the dimeric viral genome and completion of minus strand DNA synthesis (Panganiban and Fiore, 1988). Most of the RNA template hybridized to the (−) strand DNA is then degraded. A short polypurine tract (ppt) carrying a 3′ hydroxyl group remains uncleaved and serves as a specific primer in the initiation of (+) strand synthesis (Mitra *et al.*, 1982; Champoux *et al.*, 1984). To generate a perfect double stranded DNA, uninterrupted by ribonucleotides, both the tRNA primer and the (+) strand primer have to be removed from the relevant DNA strands. Such primers were found to be released intact (Omer and Faras, 1982; Champoux *et al.*, 1984), thus suggesting an endonucleolytic mechanism of cleavage for retroviral RNaseH.

On the other hand, contrary evidence has been presented. On the basis of the observed processivity of retroviral RNaseH and their apparent inability to cleave certain circular RNA–DNA hybrids, it was argued that these enzymes are exonucleases (Keller and Crouch, 1972; Leis *et al.*, 1973; Mölling, 1974; Verma, 1975; Hansen *et al.*, 1988). The finding that the end products of HIV-1 RT-RNaseH are primarily mono-, di- and trinucleotides may also be considered as supporting the idea of an exonucleolytic mechanism (Starnes and Cheng, 1989). However, a recent study investigating RNaseH mediated cleavage of a variety of synthetic substrates (Krug and Berger, 1989) has unequivocally demonstrated that both HIV-1 and avian myoblastosis virus (AMV) RNaseH are endonucleases. Using a defined template/primer system and high resolution gel electrophoresis of the cleavage products, we show here that HIV-1 RT-RNaseH (as well as that of *Escherichia coli*) contains both endo- and exonucleolytic activities.

Results

HIV-1 RT-RNaseH endonucleolytically cuts the RNA moiety of a RNA–DNA hybrid

As a result of asymmetric proteolytic processing by HIV-1 protease (PR) at an internal site in RT, HIV-1 reverse transcriptase preparations obtained from virions comprise

© Oxford University Press

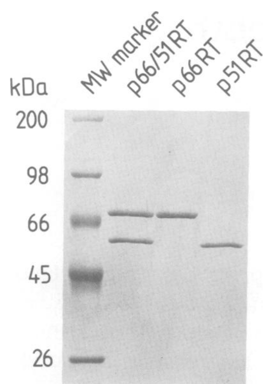

Fig. 1. Analysis of recombinant p66, p51 and p66/p51 HIV-1 RT preparations by SDS−PAGE. A 10% SDS−polyacrylamide gel was loaded with the indicated RT preparations. The amount of protein applied was 0.5 μg each for p66 and p51 RT and 1.0 μg for the p66/51 heterodimer. All preparations were estimated from scanning microdensitometry of the stained gel to be at least 90% pure.

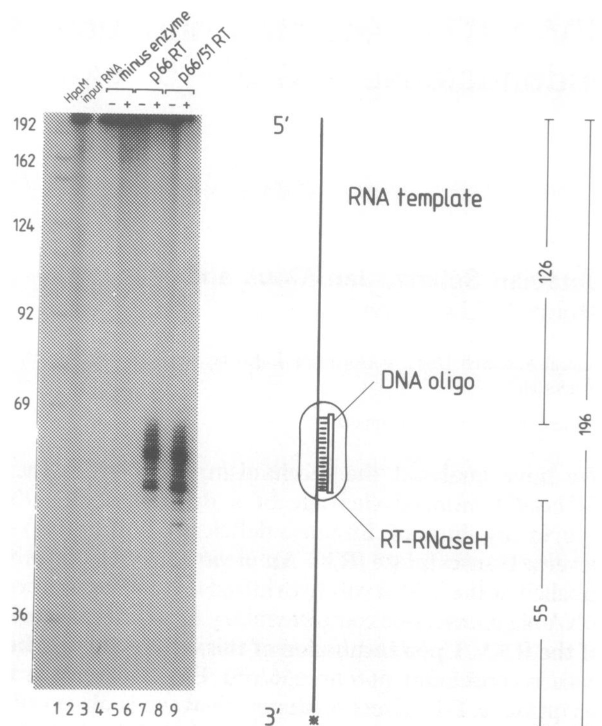

Fig. 2. RNaseH hydrolysis of 3′ labelled, hybrid containing RNA. The reaction products of incubations of different preparations of HIV-1 RT were displayed on a 8% sequencing gel. The nature of the samples is indicated above the lanes. (+) and (−) denote incubations that were carried out in the presence or absence of the complementary DNA oligonucleotide respectively. The diagram on the right shows the lengths of the corresponding RNA species. A 55 nucleotide RNA is generated by cleavage at the 3′ end of the hybrid. The 126 nucleotide RNA fragment extending from the 5′ end of the hybrid to the 5′ end of the RNA cannot be detected with 3′ labelled RNA. Mol. wt markers (HpaM) were radiolabelled *Hpa*II fragments of plasmid pBR322.

an equimolar mixture of 66 and 51 kd polypeptides that are colinear at their N-termini (di Marzo-Veronese *et al.*, 1986; Lightfoote *et al.*, 1986). An analogous distribution of the p66 and p51 polypeptides is observed when active HIV-1 protease is co-expressed with reverse transcriptase in a bacterial expression system (Mous *et al.*, 1988; Le Grice and Grüninger-Leitch, 1989). The p66/51 complex displays a higher specific RT activity than either polypeptide alone, implying that the active form of the enzyme is probably a heterodimer. This notion is also supported by the fact that both polypeptides copurify by metal chelate affinity chromatography even when only one of them carries a polyhistidine affinity label (Le Grice and Grüninger-Leitch, 1989). While it is clear that the formation of a heterodimer somehow enhances RT activity, the exact contribution of the 51 kd polypeptide (which itself has very low RT activity) in this or any other function of HIV-1 reverse transcriptase remains obscure.

To evaluate the contributions to RNaseH function made by the different polypeptides we analysed purified preparations of the p66, p51 and p66/p51 polypeptides. p51 RT, which lacks the C-terminal RNaseH domain, was not expected to have any RNaseH activity, but was nevertheless included as a convenient negative control. Although similar studies have been performed with p51 (Larder *et al.*, 1987; Hizi *et al.*, 1988), these polypeptides have a C-terminus inconsistent with that which we (Le Grice *et al.*, 1989) and others (Mizrahi *et al.*, 1989) have established as arising from HIV-1 protease mediated cleavage of p66 RT, allowing the formal possibility that lack of activity in p51 might be a consequence of an altered C-terminus. Additionally, we wished to investigate the possibility that p51 RT might modulate RNaseH activity of the larger polypeptide. RT p66, p51 and p66/p51 preparations used in these analyses were purified to >90% homogeneity (Figure 1), and shown to be free of contaminating DNase and RNase activity. Previously, we had shown that these preparations (except the p51 RT) exhibit RNaseH activity, both in filter assays and *in situ* gel analyses (Schatz *et al.*, 1989).

To examine the mechanism of RNaseH cleavage by HIV-1 RT in more detail, we designed an *in vitro* experiment in which the fate of a defined substrate could be monitored precisely in the course of the reaction. A short pentadecameric DNA oligonucleotide, complementary to an internal region of a 3′ labelled RNA, forms a limited RNA−DNA hybrid that is substrate for RT-RNaseH. This system permits ready identification of the resulting cleavage products as their lengths correspond to the distance from the point of cleavage to the terminal label. As shown in Figure 2, incubation of this hybrid with both p66 and p66/p51 RT-RNaseH results in the generation of a ladder of discrete bands, spaced one nucleotide apart, corresponding to RNA fragments terminating within the short region hybridized to the DNA oligonucleotide (Figure 2, lanes 7 and 9). No cleavage products were observed when either enzyme or oligonucleotide was omitted from the reaction (Figure 2, lanes 4 and 5 respectively). Two additional details are striking. (i) The distribution of cleavage products created by RT-RNaseH is not even, but rather there is a preference for cleavage at some nucleotides. This might reflect a characteristic attribute of the enzyme since RNaseH from *E.coli*, although it cuts the RNA at the same positions, exhibits a different bias (cf. lanes 5 and 12 of Figure 3A). (ii) The efficiency of RNaseH cleavage at the terminal 5′ nucleotides of the hybridized RNA seems to be significantly diminished, as bands corresponding to these RNA fragments become progressively fainter. Alternatively, this observation might also be explained by rebinding of RT-RNaseH after a first cut, as long as the residual hybrid remains stable. A second cut might then produce one of the predominant

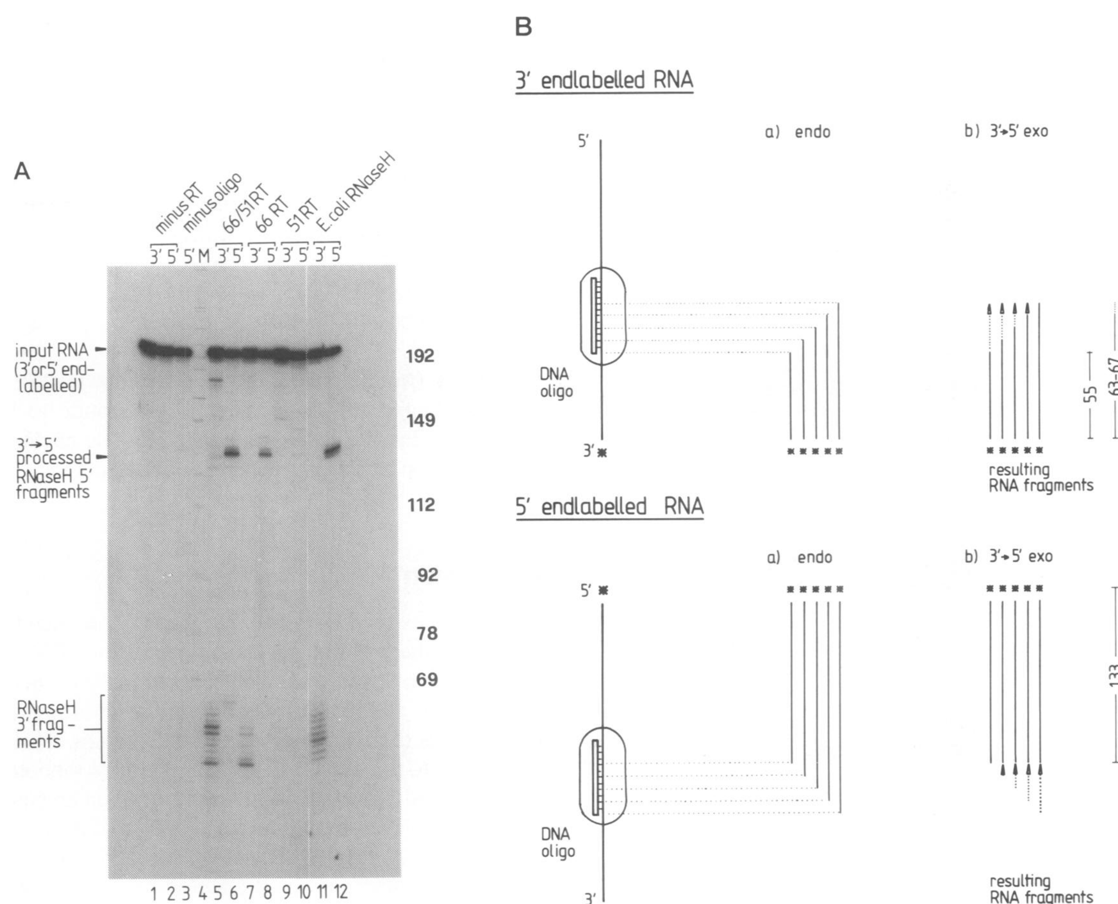

Fig. 3. (**A**) Comparison of RNaseH cleavage of 3′ and 5′ labelled RNAs. The autoradiogram shows the reaction products of the RNaseH preparations indicated above the lanes with either a 3′ or a 5′ labelled RNA substrate (denoted 3′ or 5′ respectively). M denotes *Hpa*II fragments of pBR322 as mol. wt standard. (**B**) Proposed model of endo/exo action of HIV-1 RT associated RNaseH. The schematic drawing illustrates the generation of the observed RNaseH induced RNA fragments by an endo- and an exonucleolytic step. The bars on the right show the lengths of the observed 3′ or 5′ labelled RNA fragments. See text for detailed explanation.

shorter RNA species. The two faint lower bands visible in Figure 2, lane 9 most probably do not result from cleavage within the RNA–DNA hybrid as there is little possibility for formation of a hybrid of reasonable stability at this position.

Endonucleolytic cleavage of the RNA–DNA hybrid is accompanied by a 3′→5′ exonucleolytic degradation inherent to RT-RNaseH

The observation of discrete bands corresponding to RNA fragments terminating within the RNA–DNA hybrid strongly argues in favour of an endonucleolytic mode of action for RT-RNaseH. To confirm this notion, we repeated the experiment with the same template/primer system, with the exception that the RNA component was labelled at its 5′ terminus. Analogous cleavage products of this RNaseH reaction would have presented strong evidence for an exclusively endonucleolytic mechanism for RNaseH. To our surprise, we noticed only two major cleavage products, matching RNA fragments extending from the 5′ end of the RNA to the middle of the hybridized region (seven nucleotides from the 5′ end of the hybrid) with both p66/51 and p66 HIV-1 RT-RNaseH (Figure 3A, lanes 6 and 8). The total amount of processed material seems to be roughly equivalent for both types of label (cf. lanes 5 and 6 or 7 and 8 respectively). In the case of RNaseH from *E.coli* (lane 12), slightly shorter RNA fragments are also observed that map nearer the 5′ end of the hybridized region. As expected, p51 RT (lanes 9 and 10) did not produce any of the RNaseH induced cuts, although there are some very faint bands that may result from a residual 'nicking activity'. The fact that RNaseH products can be seen with either type of labelled RNA clearly demonstrates, in agreement with previous results (Krug and Berger, 1989), that an endonucleolytic cleavage is involved. Unexpectedly, however, the observed cleavage patterns in the two experiments (summarized in Figure 3B) do not match. Why are there only one or two prominent bands present in the RNaseH digestion of 5′ labelled RNAs, whereas RNaseH digestion of 3′ labelled RNAs yields a spectrum of RNA fragments? Evidently, the missing nucleotides in between the different RNA species must have been removed, either by a secondary cleavage event (which cannot be detected with the 3′ label) or by the action of a processive 3′→5′ exonuclease. Since the final cleavage products of HIV-1 RT-associated RNaseH have been shown to consist primarily of mono-, di- and trinucleotides (Starnes and Cheng, 1989), most likely this secondary reaction proceeds exonucleolytically.

Reverse transcription and RNaseH hydrolysis proceed simultaneously

Finally, experiments were designed to determine how RNaseH cleavage is coupled to reverse transcription. To this end, we included a set of three different deoxynucleoside

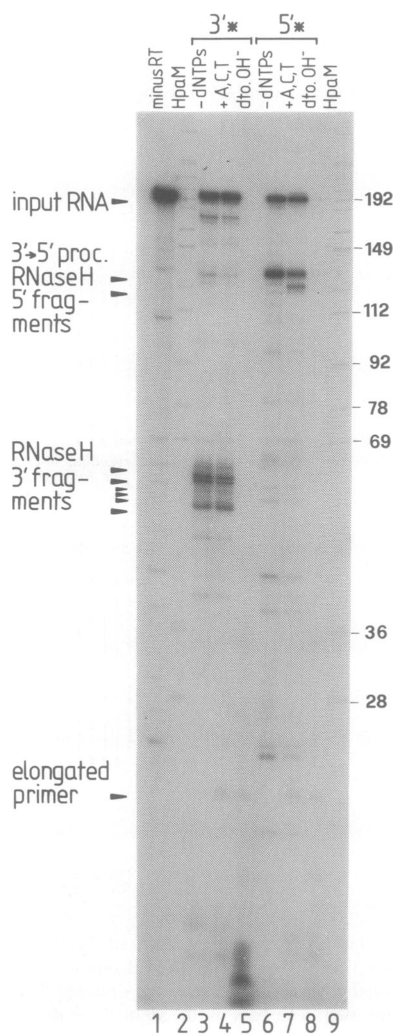

Fig. 4. RT-RNaseH sliding. RNaseH reactions were carried out in the presence or absence respectively of deoxynucleoside triphosphates, as indicated above the lanes. 3'* and 5'* refer to 3' and 5' labelled RNA substrates. OH^- in lanes 5 and 8 denotes an alkaline hydrolysis of the reactions shown in lanes 4 and 7 respectively. Minus RT, incubations without RT-RNaseH. HpaM, *Hpa*II fragments of pBR322 as mol. wt markers.

triphosphates (dATP, dCTP and dTTP/[α-^{32}P]dTTP) in the reaction. Since the sequence of the RNA template to be copied reads 3' UAUGGGUCC 5', this should allow the polymerizing activity of RT to elongate the DNA primer by seven nucleotides and terminate at a position where the first C residue is encountered. Extension of the DNA primer has the simultaneous effect of increasing the length of the RNA–DNA hybrid, allowing the possibility of analysing how the template RNA was reduced in size (by RT-RNaseH) as the primer DNA was extended (by the RNA dependent DNA polymerase activity of RT). The results of this approach are presented in Figure 4. To verify that indeed seven nucleotides had been added to the DNA primer, an aliquot of the reactions was subjected to alkaline hydrolysis to leave only the elongated DNA primer, labelled to low specific activity with [α-^{32}P]dTTP (evident as faint bands in lanes 5 and 8). The new, smaller 5' labelled RNaseH induced template RNA fragment that results (lane 7) is shortened by approximately the same increment (Figure 4, lanes 6 and 7), providing a strong argument for a simultaneous action of both the polymerase and RNaseH domains. In an analogous manner, new RNA fragments shortened by one or three nucleotides respectively, are seen when only dATP or dATP+dTTP are included in the reaction (not shown). The pattern of 3' labelled RNA fragments does not change upon cDNA synthesis, supporting our notion of simultaneous action of the exoribonuclease and polymerase functions of RT (only the primary cuts can be detected with the 3' label). That only a small portion of the RNA fragments is converted into the smaller 126 nucleotide species may be explained by the inherent propensity of HIV-1 RT-RNaseH to terminate cDNA synthesis after the first nucleotide (Majumdar *et al.*, 1988). Since dTTP was used as a label, and this is the second nucleotide to be incorporated, primer molecules elongated by one nucleotide only are not radioactive and therefore undetectable.

Discussion

We have presented evidence that the RT-associated RNaseH from both the p66 and p66/p51 form of the HIV-1 enzyme has both an endo- and an exonucleolytic activity. A hypothetical mechanism describing the coordinated action of these activities is given schematically in Figure 5. According to this model, RT-RNaseH binds randomly to the RNA–DNA hybrid and cleaves the RNA in an endonucleolytic fashion, thereby producing a ladder of RNA fragments (visible with 3' labelled RNAs). From this entry point, the RNA is then degraded by the action of a processive 3'→5' exonuclease which stops about seven nucleotides from the end of the hybrid. In the absence of dNTPs, the RT-RNaseH, once it has reached the end of the RNA–DNA hybrid, probably dissociates quite rapidly (attempts to demonstrate protection from RNase digestion by either p66/p51, p66 or p51 RT in this region have all been unsuccessful). This conclusion would be in good agreement with data by Huber *et al.* (1989) who noted that preformed complexes between HIV-1 RT-RNaseH and poly(rA)·oligo(dT) dissociate with an apparent half-life of < 120 s.

One possible interpretation of the fact that the observed end point of RNaseH cleavage does not coincide with the 5' end of the RNA–DNA hybrid could be that the polymerase and the RNaseH domain of RT-RNaseH are spatially arranged in such a way that the N-terminal polymerase domain points towards the 5' end of the RNA where cDNA synthesis would start and the C-terminal RNaseH domain faces the reverse side, as schematically represented in Figure 5. When the RT-RNaseH has migrated to the end of the hybridized region, the terminal nucleotides cannot be efficiently hydrolysed because the polymerase domain is covering these nucleotides and the RNaseH domain is still 'lagging behind'. This can be seen both with the 3' labelled RNA where only very few cuts are detectable near the 5' end of the hybrid (whereas there is substantial cleavage at the 3' end of the hybrid) and, even more clearly, with the 5' labelled RNA where the observed endpoint of RNaseH cleavage is about seven nucleotides from the 5' end of the hybrid. This distance remains invariant when the RT-RNaseH is allowed to synthesize a short stretch of cDNA. Similar distances were observed also with different primer/template systems, raising the interesting question which is the minimal number of consecutive base paired

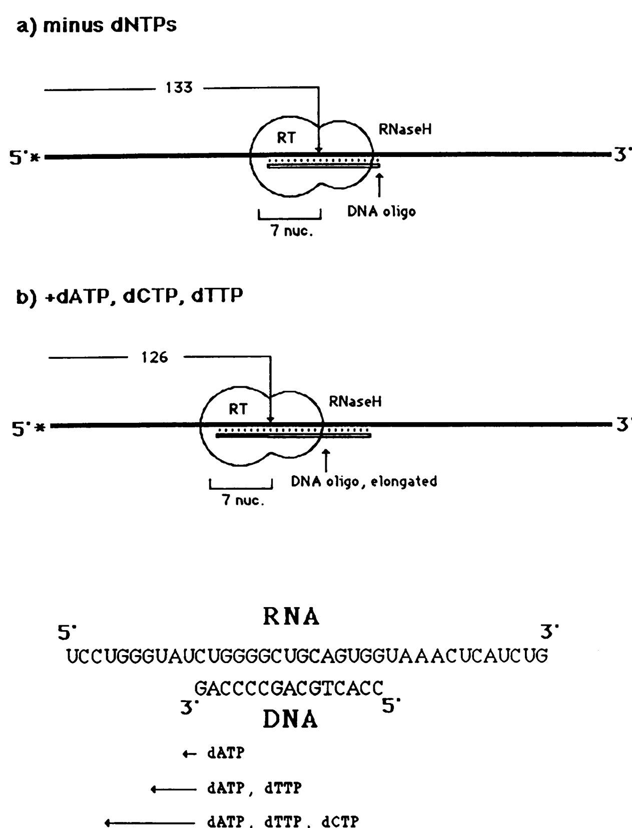

Fig. 5. Model of RT-RNaseH sliding. (**a**) Putative orientation of the RT-RNaseH on the RNA–DNA substrate with the polymerase domain pointing towards the start of cDNA synthesis and behind this, the C-terminal RNaseH domain. The arrows point to the observed endpoint of RNaseH cleavage, and numbers indicate the length of the corresponding RNA fragments. (**b**) Upon addition of dATP, dCTP and dTTP a seven nucleotide portion of cDNA is synthesized. Concomitantly, the endpoint of RNaseH cleavage moves the same distance. The nucleotides towards the 5′ end of the hybrid are proposed to be covered by the polymerase domain. The lower illustration of the figure shows the portion of the template RNA to which the complementary DNA oligonucleotide was hybridized, and the dNTP conditions used to generate discretely elongated molecules.

nucleotides that will be recognized and cleaved by RT-RNaseH.

In principle, another interpretation is also conceivable: the apparent gap of seven nucleotides could simply be due to the instability of smaller RNA–DNA hybrids. After having received a first cut, the remaining RNA–DNA hybrid might dissociate, thus making the RNA unavailable for further degradation. However, this is unlikely, since RNaseH from *E.coli* produces fragments that are cleaved nearer to the end of the hybrid (Figure 3, lane 12). In addition, one would not expect such a prominent stop in this case since the stability of the hybrid should decrease gradually with decreasing length. This proposed mode of endo/exo RNaseH action should be compatible with the requirements of a processive polymerase. In this manner, cDNA synthesis and RNaseH hydrolysis of the template RNA can proceed at the same time.

It is not clear from these experiments, how the endo- and exonucleolytic activities are organized within the p66 polypeptide; for example, are both functions in the same or separate domains of the 15 kd, RNaseH containing C-terminus of p66 RT? Preliminary data suggest that the endo- and exonucleolytic function might be differentially affected by point mutations in the C-terminal domain. Another open question is the role of the p51 polypeptide which itself has little RT and practically no RNaseH activity in an isolated form. There is also no significant qualitative difference in the RNaseH hydrolysis pattern between the p66 and the p66/51 RT, as both enzyme preparations yield virtually identical cleavage products. In this context, it would be interesting to see how a processing site mutant of HIV-1 RT-RNaseH that shows no detectable defect in either reverse transcriptase, RNaseH or tRNA binding (Barat *et al.*, 1989) activity would behave *in vivo*, i.e. could unprocessed p66 RT perform all the necessary steps required for successful replication and ultimately lead to viable progeny virus? Such an approach may well be suited to reveal new and as yet unexpected aspects of this surprisingly versatile enzyme. In this context, the role of the 15 kd, C-terminal RT domain freed from p66 by protease cleavage also requires elucidation. Data from Hansen *et al.* (1988), as well as preliminary experiments in our laboratory with a purified preparation of this recombinant 15 kd polypeptide, have demonstrated a weak non-processive RNaseH activity which might also play a role in the virus replication cycle.

Materials and methods

Plasmid constructions and oligonucleotides

Plasmid p6MAP was constructed by ligating a filled 249 bp *Bam*HI fragment, coding for an 80 amino acid portion of HIV-1 gp41 (Certa *et al.*, 1986) into the blunt ended *Hin*dIII site of pSP64 (Pharmacia). Linearization at a *Hin*dIII site within the 249 bp fragment and transcription with SP6 RNA polymerase yields a transcript of 195 nucleotides. The pentadecameric DNA oligonucleotide complementary to bases 127–141 of the SP6 generated RNA (3′ GACCCCGACGTCACC 5′) was synthesized according to standard phosphoramidite protocols (Bannwarth and Iaiza, 1986). The expression plasmids p6HRT and p6HRT-PROT have been described (Le Grice and Grüninger-Leitch, 1989). Plasmid p6HRT51 was obtained by cleaving p6HRT with *Asp*718 and *Hin*dIII which deletes the coding sequences for the 3′ terminal portion of RT. The deleted original sequence was restored up to the codon for Phe440 (after which a TAA stop codon was engineered) by ligating matching adapter oligos into these sites. The resulting plasmid p6HRT51 should express authentic p51 RT, as Phe440 was deduced to be the C-terminal amino acid of the p51 polypeptide of HIV-1 RT both by total amino acid analysis (our unpublished data) and by C-terminal protein sequencing of viral p51 RT (Mizrahi *et al.*, 1989).

Expression and purification of HIV-1 RT-RNaseH

Purification of the poly-histidine affinity labelled p66 or p66/51 RTs as well as the *E.coli* expression system in general have been described earlier (Le Grice and Grüninger-Leitch, 1989; Certa *et al.*, 1986; Hochuli *et al.*, 1988). The same protocols were used for the purification of p51 RT which also carries a poly-histidine affinity label. All protein preparations were dialysed against 50% glycerol/storage buffer (50 mM Tris-Cl, pH 7.0, 25 mM NaCl, 1 mM EDTA, 0.5 mM DTT) and kept at −20°C. *E.coli* RNaseH was from Boehringer (Mannheim). RT preparations were checked before use by incubation with radiolabelled tRNA to ensure that no contaminating ribonucleases were present. Likewise, incubation of RT with supercoiled plasmid DNA failed to generate open circular molecules, indicating the absence of DNase contamination.

Preparation of 5′ and 3′ labelled RNA by in vitro transcription with SP6 RNA polymerase

Run off transcription of *Hin*dIII cut p6MAP by SP6 RNA polymerase was performed essentially as described (Green *et al.*, 1983), except using 400 μM of each dNTP in a 100 μl reaction. For the preparation of uniformly labelled transcripts a 20:1 mixture of UTP and [α-^{35}S]UTP (final conc. 50 μM, 20 μl reactions) was used. Reactions were stopped by addition of 100 μl TE + 300 mM Na-acetate and phenol/chloroform extraction. Samples were precipitated with 2 volumes of ethanol, washed with 70% ethanol, dried, dissolved in 5–10 μl of formamide loading buffer (90% formamide, 1 × TBE, 0.1% each xylene cyanol and bromophenol blue) and electro-

phoresed on a 5% TBE–urea–polyacrylamide gel. By comigration of the labelled transcript in adjacent lanes, the band containing the 195 nucleotide template RNA was identified and excised from the gel. The RNA was eluted in 400 μl of HD buffer (750 mM NH_4-acetate, pH 6.0, 10 mM Mg-acetate, 0.1 mM EDTA, 0.1% SDS), extracted three times with phenol/chloroform and precipitated with 2 vol of ethanol. After washing with 70% ethanol and drying, the samples were resuspended in H_2O and quantitated by $OD_{260\ nm}$ of the solution. 5 pmol of the RNA were dephosphorylated with 0.5 U calf intestine phosphatase (Boehringer, Mannheim) at 50°C for 30 min in a 20 μl reaction. After phenol extraction and ethanol precipitation, the RNA was kinased with 25 μCi of [γ-^{32}P]-ATP and 5 U of T4 polynucleotide kinase (Pharmacia) for 30 min at 50°C and purified as above. 3′ labelling with 5′-[^{32}P]p*Cp was carried out as described (England *et al.*, 1980). The sp. act. of both 3′ and 5′ labelled RNAs was ~10^5 cpm/pmol.

Oligonucleotide-induced RNaseH cleavage

Pre-mixes with ingredients common to all reactions contained the following (per 4 μl reaction): 0.4 pmol of the RNA (~40 000 c.p.m. either 3′ or 5′ end labelled), 4 pmol complementary DNA oligonucleotide, 2.5 μg tRNA carrier, 1 μl 4 × RT buffer (200 mM Tris-Cl, pH 8.0, 200 mM NaCl, 8 mM $MgCl_2$, 8 mM DTT). Pre-mixes were briefly heated to 65°C and cooled down slowly to 37°C to facilitate hybridization. To start the reaction, 1 μl of enzyme dilution (150 ng/μl for the HIV-1 RT-RNaseH preparations, 3 ng/μl in the case of *E.coli* RNaseH) and, in RT sliding reactions, 1 μl of nucleotide mix (400 μM each of the indicated nucleotides and 5 μCi [α-^{32}P]dTTP) were added. The reactions were incubated for 5 min at 37°C and stopped by addition of an equal volume of formamide loading buffer and heating to 95°C for 1 min after which 2 μl of the samples was loaded on 5% acrylamide–7 M urea (0.4 mm) sequencing gels. Following electrophoresis, the gels were dried and exposed overnight without intensifying screens.

Acknowledgements

We wish to thank W.Bannwarth for oligonucleotide synthesis, F.Grüninger-Leitch for purified p66 and p66/51 RT and D.Stüber for expression vectors.

References

Bannwarth,W. and Iaiza,P. (1986) *DNA*, **5**, 413–419.
Barat,C., Lullien,V., Schatz,O., Keith,G., Nugeyre,M.T., Grüninger-Leitch,F., Barré-Sinoussi,F., Le Grice,S.F.J. and Darlix,J.L. (1989) *EMBO J.*, **8**, 3279–3285.
Certa,U., Bannwarth,W., Stüber,D., Gentz,R., Lanzer,M., Le Grice,S.F.J., Guillot,F., Wendler,I., Hunsmann,G., Bujard,H. and Mous,J. (1986) *EMBO J.*, **5**, 3051–3056.
Champoux,J.J., Gilboa,E. and Baltimore,D. (1984) *J. Virol.*, **49**, 686–691.
Crouch,R.J. and Dirksen,M.L. (1982) In Linn,S.M. and Roberts,R.J. (eds), *Nucleases*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 211–241.
Di Marzo-Veronese,F., Copeland,T.D., DeVico,A.L., Rahman,R., Oroszlan,S., Gallo,R.C. and Sarangadharan,M.G. (1986) *Science*, **231**, 1289–1291.
England,T.E., Bruce,A.G. and Uhlenbeck,O.C. (1980) *Methods Enzymol.*, **65**, 65–74.
Green,M.R., Maniatis,T. and Melton,D.A. (1983) *Cell*, **32**, 681–694.
Hansen,J., Schulze,T., Mellert,W. and Mölling,K. (1988) *EMBO J.*, **7**, 239–243.
Hizi,A., McGill,C. and Hughes,S. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1218–1222.
Hochuli,E., Bannwarth,W., Doebeli,H., Gentz,R. and Stueber,D. (1988) *Bio/Technology*, **6**, 1321–1325.
Huber,H.E., McCoy,J.M., Seehra,J.S. and Richardson,C.C. (1989) *J. Biol. Chem.*, **264**, 4669–4678.
Kane,C.M. (1988) *Biochemistry*, **27**, 3187–3196.
Keller,W. and Crouch,R. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 3360–3364.
Kotewicz,M.L., Sampson,C.M., d'Alessio,J.M. and Gerard,G.F. (1988) *Nucleic Acids Res.*, **16**, 265–277.
Krug,M.S. and Berger,S.L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 3539–3543.
Lai,M.-H. and Verma,I.M. (1978) *J. Virol.*, **25**, 652–663.
Larder,B., Purifoy,D., Powell,K. and Darby,G. (1987) *EMBO J.*, **6**, 3133–3137.
Le Grice,S.F.J. and Grüninger-Leitch,F. (1989) *Eur. J. Biochem.*, in press.
Le Grice,S.F.J., Mills,J., Ette,R. and Mous,J. (1989) *J. Biol. Chem.*, **264**, 14902–14908.
Leis,J.P., Berkower,I. and Hurwitz,J. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 466–470.
Lightfoote,M.M., Coligan,J.E., Folks,T.M., Fauci,A.S., Martin,M.A. and Venkatesan,S. (1986) *J. Virol.*, **60**, 771–775.
Majumdar,C., Abbotts,J., Broder,S. and Wilson,S.H. (1988) *J. Biol. Chem.*, **263**, 15657–15665.
Mitra,S.W., Goff,S., Gilboa,E. and Baltimore,D. (1982) *J. Biol. Chem.*, **257**, 5983–5986.
Mizrahi,V., Lazarus,G., Miles,L., Meyers,C. and DeBouck,C. (1989) *Arch. Biochem. Biophys.*, **273**, 347–358.
Mölling,K. (1974) *Virology*, **62**, 46–59.
Mous,J., Heimer,E.P. and Le Grice,S.F.J. (1988) *J. Virol.*, **62**, 1433–1436.
Omer,C.A. and Faras,A.J. (1982) *Cell*, **30**, 797–805.
Panganiban,A.T. and Fiore,D. (1988) *Science*, **241**, 1064–1069.
Repaske,R., Hartley,J.W., Karlick,M.F., O'Neill,R.R. and Austin,J.B. (1989) *J. Virol.*, **63**, 1460–1464.
Schatz,O., Cromme,F., Grüninger-Leitch,F. and LeGrice,S.F.J. (1989) *FEBS Lett.*, **257**, 311–314.
Schatz,O., Cromme,F.V., Naas,T., Lindemann,D., Mous,J. and Le Grice,S.F.J. (1990) In Papas,T.S. (ed.), *Gene Regulation and AIDS*. Portfolio Publishing Company, Houston.
Starnes,M.C. and Cheng,Y.-C. (1989) *J. Biol. Chem.*, **264**, 7073–7077.
Tanese,N. and Goff,S.P. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1777–1781.
Verma,I.M. (1975) *J. Virol.*, **15**, 843–854.

Received November 28, 1989; revised January 3, 1990