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Identification and characterization of HIV-specific RNase H by monoclonal antibody

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Human immune deficiency virus (HIV) replicates by conversion of the RNA genome into the double-stranded DNA provirus. The reverse transcriptase is not the only enzymatic function crucial in DNA-provirus synthesis. A viral-coded RNase H activity which specifically degrades RNA in RNA-DNA hybrids has been shown to be essential as well. Here we demonstrate that the HIV-reverse transcriptase which consists of a two-polypeptide complex, p66 and p51, copurifies with an RNase H activity which exhibits properties of a processive exonuclease. Only the p66 molecule, not p51, is active as polymerase as evidenced by activated gel analysis. p66 exhibits RNase H activity when precipitated as immune complex by a monoclonal antibody raised against a bacterially expressed carboxy-terminal portion of p66. The monoclonal antibody which does not interfere with enzyme activity also precipitates a second population of molecules with RNase H activity which is of low mol. wt, p15. This RNase H appears therefore to be derived from the carboxy terminus of p66 during processing to the p51 polypeptide. It exhibits low template-binding ability and is of a non-processing mode of action which may be due to the absence of the reverse transcriptase domain. These results lend experimental support to the hypothesis that the RNase H gene maps at the carboxy terminus of the reverse transcriptase. Since both RNase H populations are virus-coded they may be essential for retrovirus replication in general and useful targets for chemotherapeutic agents.

Key words: HIV-RNase H/two RNase H activities/copurification RT and RNase H/C-terminal localization/RNase H monoclonal antibody

Introduction

The structure of the human immune deficiency virus (HIV) genome is similar to that of most retroviruses with respect to the placement of the *gag*, *pol* and *env* genes. The HIV genome furthermore codes for several additional genes responsible for regulation of viral gene expression (Arya *et al.*, 1985; Sodroski *et al.*, 1985, 1986; Feinberg *et al.*, 1986). By analogy to other retroviruses expression of the *pol* gene probably occurs by the occasional translational frameshifting thereby fusing the *pol* reading frame with the upstream *gag* reading frame. The mammalian retroviral *pol* gene typically encodes three protein products that express several catalytic activities: (i) a protease that cleaves the viral *gag* and *gag-pol* precursor polyproteins; (ii) the viral RNA-dependent and DNA-dependent DNA polymerase (reverse transcriptase or RT); and (iii) an integrase/endonuclease that functions in the integration of viral DNA into the host cell genome. In the case of HIV all these functions are coded for by the *pol* gene. Three of the *pol*-encoded polypeptides have been identified in HIV virions so far, the two related forms of the RT, p66 and p51 that differ in size but share a common amino terminus (Di Marzo Veronese *et al.*, 1986; Lightfoote *et al.*, 1986), and a 34 kd protein, p34, that is the presumed integrase/endonuclease (Lightfoote *et al.*, 1986). The amino-terminal sequences of these three polypeptides have been determined, thus permitting their assignment to specific regions of the *pol* gene open reading frame. Like other mammalian retroviruses, the RT domain is encoded in the central portion, and the integrase/endonuclease is encoded in the 3′ portion of the *pol* gene open reading frame. Sequence comparisons show that the remaining 5′ portion of the HIV *pol* gene encodes a polypeptide that shares homology with the protease domain of other avian and mammalian retroviruses (Kramer *et al.*, 1986; Toh *et al.*, 1985).

An RNase H specific for degradation of RNA in RNA-DNA hybrids has not yet been identified in HIV particles. By analogy to the RNase H of avian and mammalian retroviruses, it should be an intrinsic property of the RT (Moelling *et al.*, 1971; Moelling, 1974). In a recent experiment we succeeded in protein expression of the HIV-*pol* gene in bacteria. The p66/p51 RT proteins arose by processing in these bacteria and exhibited enzymatic activity. The RT was purified by several column chromatography steps [DEAE-cellulose, phosphocellulose, poly(U) agarose] and subsequent glycerol density gradient centrifugation. It copurified with an RNase H activity during the final purification steps after removal of RNase H activity during phosphocellulose chromatography—which was presumably of bacterial origin (Hansen *et al.*, 1987). During these studies we noticed that a protein of ~15 kd arose during polymerase processing.

Based on experience with the bacterially expressed RT/RNase H we investigated these two enzyme activities in HIV lysates and describe for the first time the viral RNase H copurifying with the RT. We also discovered a low-mol.-wt RNase H, designated as p15. A monoclonal antibody raised against the carboxy-terminal portion of the p66 RT molecule does not interfere with RNase H activity and allows precipitation of active p66 and p15 RNase H enzyme activities immobilized in immune complexes. We characterized these two RNase H activities as a processive exonuclease and a non-processive nuclease, respectively. The p15 RNase H presumably arises by proteolytic processing from the p66 molecule. Assignment of RNase H to the carboxy-terminal portion of the RT supports the computer data of Johnson

et al. (1986) who analyzed the sequences of retroviral *pol* genes and detected sequence homology between the RNase H of *Escherichia coli* and the carboxy terminus of *pol*.

Results

Purification of RT and RNase H from HIV

Purified HIV was lysed by non-ionic detergent and processed for column chromatography by screening for RT and RNase H activities in parallel. Virus lysate was applied to a phosphocellulose (PC) column. While the *gag*-related proteins were washed off at 0.1 M salt, RT and RNase H were eluted from the column with 0.25 M salt. They contained p66 and p51 proteins and two other viral proteins as evidenced by immunoblotting (Figure 1C). A bulk of cellular RNase H elutes at 0.35 M salt. It is free of DNA polymerizing activity. Two fractions of the material eluted at 0.25 M were further passed over a poly(U) agarose column. The elution profiles of the RT and RNase H activities exhibit a single sharp peak shown in Figure 1A. Some RNase H activity was washed off this column at 0.1 M salt with no associated RT activity. These wash fractions were too dilute to allow detection of proteins either by staining or Western blotting. Both the eluted peak fraction number 12 and an aliquot of the wash fraction were further analyzed on two parallel glycerol density gradients (GDG). While the RT/RNase H complex sedimented as a heterodimer of p66 and p51 corresponding to about 120 kd mol. wt, the RNase H of the wash sedimented as a low-mol.-wt component in the top fractions of the GDG (Figure 1B). The RT/RNase H complex consisted of the two-polypeptide complex p66 and p51 during poly(U) Sepharose chromatography and GDG cen-

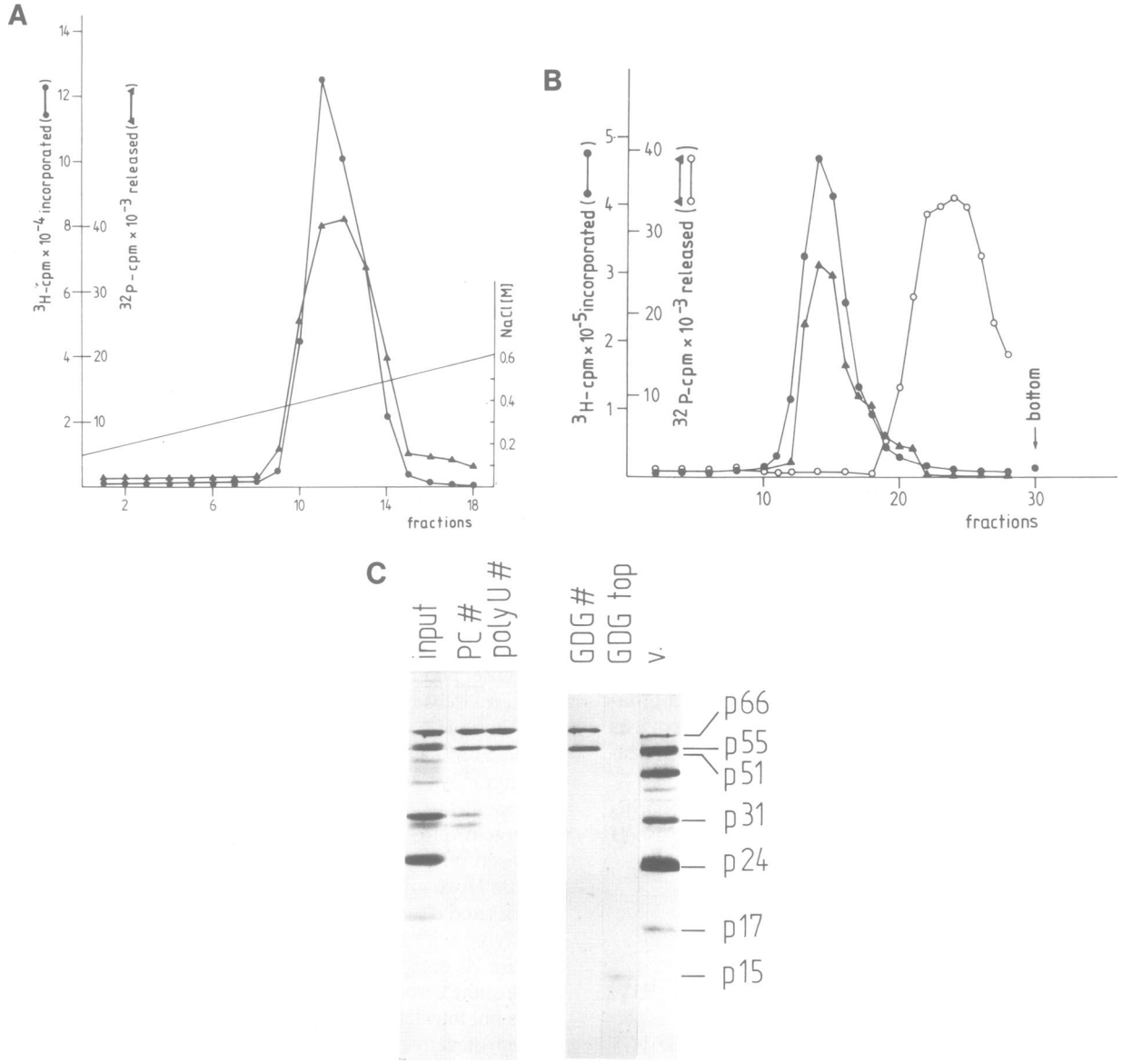

Fig. 1. (**A**) Elution profiles of HIV-associated RT (●) and RNase H (▲) activity from poly(U) agarose column. (**B**) RT and RNase H assays of GDG fractions. Two gradients were centrifuged in parallel, one of them was loaded with fraction 11 shown in (A) and the other one with 0.1 M flow-through of the poly(U) Sepharose column. RT (●), RNase H (▲) of fraction 11, and RNase H (○) of the flow-through, 'bottom' indicates the resuspended bottom of the tube. (**C**) Immunoblot of HIV-lysate input, eluted fraction 4 of the PC column, fraction 11 of poly(U) Sepharose, fractions 14 and 24 (indicated as 'top') from the GDG. Patient serum was used at a dilution of 1:500. A 12.5% gel was used. v = viral protein marker.

trifugation as evidenced in an immunoblot using a pool of heat-inactivated sera of patients with AIDS. The protein detected with the low-mol.-wt RNase H at the top of the GDG was of ~15 kd (Figure 1C).

Activated gel analysis sometimes allows detection of enzyme activities directly in polyacrylamide gels which contain template primers. Enzyme renaturation and infusion of radioactive deoxyribonucleotides leads to DNA synthesis only at sites where an active polymerase is located. This incorporation is visualized as a dark band on an X-ray film (Spanos *et al.*, 1981). Analysis of HIV lysate in such an experiment indicates that the p66 not the p51 polypeptide is the active polymerase molecule (Figure 2A).

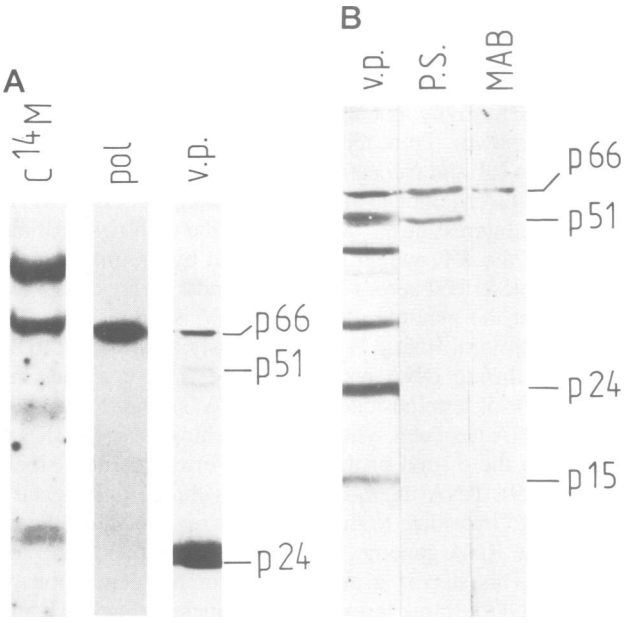

Fig. 2. (**A**) Activated gel analysis of RT from HIV-lysates (*pol*). ^{14}C M indicates a commercial radioactive marker (92, 68, 32 kd from top) which was run on the identical gel but cut off before infusion with radioactivity. Exposure time: 6 h for *pol*, 3 days for ^{14}C M. V.P. indicates HIV proteins (p66 and p24) from a parallel gel processed for immunoblotting. (**B**) Immunoblot of purified RT from HIV-lysates with patient sera (P.S.) and hybridoma supernatant (MAB). V.P. represents immunoblotted viral proteins from the identical gel.

Identification of two HIV-specific RNase Hs by a monoclonal antibody

We suspected the low-mol.-wt p15 RNase H to be of HIV-coded origin and to be cleaved off the C termius of the RT. To prove this point we cloned the *Kpn*I(left)/*Pvu*II *pol* fragment (nt 3863–4389) (Ratner *et al.*, 1985) into a bacterial expression vector and used the protein to immunize mice for production of monoclonal antibodies. One hybridoma supernatant reacted with p66 but not with p51 in a Western blot (Figure 2B MAB). Since this monoclonal also reacted with a protein expressed from the *Kpn*I(right)/*Sal*I DNA fragment (nt 4192–5820), the epitope recognized by the monoclonal antibody maps in the region between the *Kpn*I-(right) site and the RT C terminus (see drawing Figure 3A).

Using this hybridoma, an indirect immunoprecipitation of the two RNase H-containing fractions from the GDG was performed using protein A–Sepharose beads. This procedure immobilizes the enzymes in immune complexes, a procedure we have previously applied to protein kinase analysis (Moelling *et al.*, 1984). The monoclonal antibody does not interfere with RNase H activity and allows detection of RNase H activity in the immune complex-bound material from both regions of the GDG. No RNase H activity was detected in immune complexes prepared as controls with irrelevant myeloma supernatant from NS-1 cells. Degradation of the radioactively labeled hybrid was determined by subtracting precipitated radioactivity from the total radioactive hybrid input (corresponding to 100%) which corresponds to the released radioactivity. It increases with increasing input of immobilized enzyme. Two concentrations are shown (Table I). RNase H assays are linear only in a small range of enzyme concentrations around 50% of hybrid degradation.

Characterization of the two RNase H activities

Whether an enzyme is processive or non-processive is tested by the addition of excess substrate after the onset of the reaction. Using the peak fraction of the poly(U) Sepharose and the two GDG fractions, RNase H assays were performed by adding excess unlabeled hybrid to one of two parallel reactions 1 min after the beginning of the incubation time. The amount of radioactive hybrid was determined after 3, 8 and 16 min. While the poly(U) agarose and the peak fraction of the GDG did not exhibit significant changes in hybrid

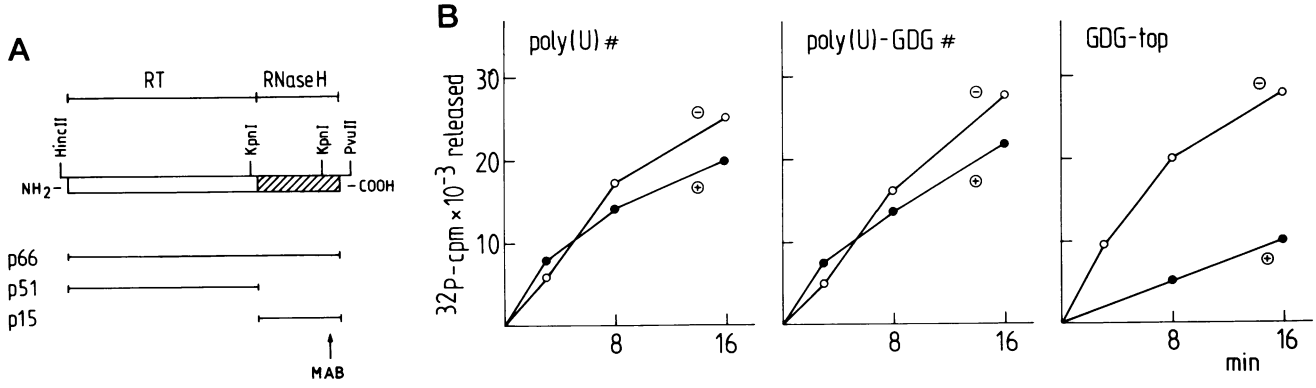

Fig. 3. (**A**) Schematic drawing of part of the HIV-polymerase gene (Ratner *et al.*, 1985) indicating the RT and the RNase H region. The *Kpn*I(left and right) and *Pvu*II restriction sites used for epitope mapping of the monoclonal antibody (MAB) are indicated. The recognition site of the MAB is indicated by an arrow. The dashed area defines the location of the RNase H. p66, p51 and p15 proteins are indicated by straight lines.
(**B**) Processing ability of the RNase H of the poly(U) agarose peak fraction, the GDG fraction 14 (indicated as polyU–GDG) and fraction 24 (GDG top) were tested without (⊖) and with (⊕) excess of competing hybrid added 1 min after the onset of the reaction.

Table I. Immunoprecipitation of RNase H by monoclonal antibody

Antibody		^{32}P c.p.m. released (%)	
		GDG no. 15	GDG no. 24
Hybridoma	10 μl	15 000 (24)	19 000 (28)
	30 μl	29 000 (43)	34 000 (51)
NS-1	10 μl	<300 (0)	<300 (0)
	30 μl	<300 (0)	<300 (0)
RS	10 μl	6000 (10)	10 000 (15)
	30 μl	13 000 (21)	19 000 (28)

Hybridoma supernatant was coupled to protein A–Sepharose beads and used to immunoprecipitate RNase H from the GDG fractions 15 and 24 (10 and 30 μl each, see Figure 1B). Both enzymes bound in immune complexes were pelleted, resuspended in RNase H assay buffer and tested in standard RNase H assays. The radioactivity precipitated after the reaction was subtracted from the amount of total hybrid input (66 500 c.p.m. corresponding to 100%) and is indicated as c.p.m. released. From this the percentage of degraded hybrid was calculated and is indicated in brackets. NS-1 represents myeloma supernatant used as control instead of hybridoma. RS: rabbit anti-p66 serum.

degradation after competition, indicating a processive nature, the RNase H present in the top fraction of the gradient exhibited reduction of hybrid degradation and is therefore of a non-processive or 'random' nature (Figure 3B).

In a preliminary analysis we determined that the purified p66-RNase H as well as the p15-RNase H do not act as endonucleases since they leave covalently closed circular plasmid DNA, which contains stretches of RNA, intact (Moelling, 1974) (data not shown). RNase H activities of mammalian cell origin are of higher mol. wt than p15 and endonucleases in character (Keller and Crouch, 1972; Roewenkamp and Sekeris, 1974; Cathala *et al.*, 1979; Sarngadharan *et al.*, 1975). Therefore it is unlikely that they contribute to the purified RNase H activities described here. The cellular RNase H is furthermore removed during phosphocellulose (PC) chromatography where it elutes at 0.35 M salt (data not shown). The p15-RNase H predominates in HIV lysates and exceeds the p66-RNase by 20-fold—a ratio which may depend on handling of the virus.

Discussion

This paper describes that HIV—like other known retroviruses—codes for an RNase H which copurifies with the RT (Moelling *et al.*, 1971; Moelling, 1974; Gerard, 1978). We have also recently been able to demonstrate the copurification of bacterially expressed HIV-RT with an RNase H (Hansen *et al.*, 1987). The RT/RNase H complex from HIV lysates as well as the bacterially expressed HIV-RT/RNase H can only be detected after extensive purification since RNase H activities of mammalian as well as of bacterial cell origin mask the viral activity. The viral RNase H can furthermore only be detected by use of a sensitive hybrid. A monoclonal antibody directed against a carboxy-terminal epitope of p66 allowed us to detect RNase H activity in the p66 polypeptide immobilized in an immune complex. The same monoclonal antibody also precipitates a low mol. wt protein from the top fractions of the GDG which exhibits RNase H activity in an immune complex as well. Taken together, these two observations suggest that the carboxy terminus of the p66 RT molecule codes for the RNase H and that processing of p66 creates the p51 subunit and also gives rise to the p15-RNase H. These observations are in agreement with a recent anlaysis by computer comparison of polymerase/RNase H genes in *E.coli* and other species which assigned a region of RNase H homology to the carboxy terminus of the retroviral RT (Johnson *et al.*, 1987). Johnson *et al.* also pointed out possible mis-interpretations of previous analyses which assigned the RNase H of avian and mammalian retroviruses to amino-terminal portions of *pol* (Lai and Verma, 1978; Grandgenett *et al.*, 1985).

Analysis of the purified HIV-RT by activated gel analysis revealed that only p66, not p51, is enzymatically active under these conditions (Figure 3A). This observation is supported by analysis of a bacterially expressed *pol* (*BglI*/*SalI*, Hansen *et al.*, 1987) with a *KpnI*/*KpnI* deletion. The product of this *pol* mutant was analyzed after column chromatography. It consists of a polypeptide of ~50 kd which is not only devoid of RNase H activity, but also lacks DNA polymerase activity (data not shown). Truncation of the RNase H therefore seems to ruin the RT and truncation of the RT results in an RNase H with a lower template affinity and a non-processive mode of action. Interestingly, we noted that the carboxy-terminal region of the RT, which is recognized by the monoclonal antibody described here, is one of the most highly conserved regions of the genome of various HIV isolates.

The retroviral RNase H has previously been shown to be essential during DNA provirus synthesis. It degrades the RNA at the 5′ terminus once the so-called strong-stop minus-strand DNA has been synthesized reaching from the tRNA primer to the 5′ end of the genome (Friedrich and Moelling, 1979). RNA degradation allows the complementary (c)DNA to hybridize to the redundant region, R, of the 3′ end of the RNA genome, sometimes defined as 'jump' of the RT. This step is a prerequisite for the generation of the viral LTRs (large terminal redundancies) (Friedrich and Moelling, 1979). The mode of action of the RNase H required for this step is that of a processive exonuclease (Keller and Crouch, 1972; Moelling, 1974, 1979). This specificity is different from other known RNase H activities in mammalian cells as well as in *E.coli* which have been characterized as endonucleases (Keller and Crouch, 1972; Roewenkamp and Sekeris, 1974; Cathala *et al.*, 1979; Sarngadharan *et al.*, 1975). The p66-associated RNase H with the mode of action of a processive exonuclease described here corresponds in character to the previously described retroviral RNase H.

The low-mol.-wt RNase H described here is non-processive or random. It may also play a role in DNA provirus synthesis which could require the action of an RNase H (at a polypurine tract) to allow the initiation of the so-called plus-strand DNA synthesis by the RT opposite to the tRNA primer site. Also, degradation of the template RNA into several distinct RNA pieces may be a consequence of the action of an RNase H. These two latter functions could not be performed by a processive RNase H. It rather appears possible that the non-processive RNase H of low mol. wt may perform one or both of these functions. In spite of the possibility that the p15-RNase H is generated by proteolytic processing, it may well be of biological relevance. To what extent proteolytic processing of retroviral proteins is essential for virus replication or accidental has not been fully elucidated. Proteolytic processing of *gag* precursor proteins is

certainly vital for virus budding. Processing of p66 to p51 until equal molar amounts of both polypeptides are created has been observed consistently. It may render higher stability to the complex. Therefore a low-mol.-wt RNase H may be essential for retroviral replication in general. We noted that this RNase H considerably exceeds the amount of p66-associated RNase H. Both RNase H populations should be analyzed for their biological functions and for inhibitory chemotherapeutic drugs which could prevent virus replication.

Materials and methods

Most of the procedures have been published in detail recently (Hansen *et al.*, 1987).

Purification of RT/RNase H

Purified HIV (~10 mg) was lysed in 1% Nonidet P-40 (NP-40), 1 mm dithiothreitol (DTT) and 0.1 mM NaCl. Before use it was diluted with buffer M (50 mM Tris–HCl, pH 7.0; 1 mM EDTA, pH 8; 1 mM DTT; 0.1% NP-40 and 10% glycerol) to a concentration of 1 mg/ml and applied to a PC column (1 × 2 cm). The column was washed with 30 ml buffer M plus 0.05 M NaCl, 30 ml of buffer M plus 0.1 M NaCl and eluted with buffer M plus 0.25 M NaCl (eight fractions, 1.5 ml each). Two fractions (3 ml total) were diluted 1:10 with buffer M and applied to a poly(U) agarose column (Pharmacia Co., Sweden) (1 × 1 cm). The column was washed with 20 ml of buffer M containing 0.1 M NaCl eluted with a salt gradient (2 × 10 ml) 0.1–0.6 M NaCl in buffer M. Samples of 1 ml per fraction were collected and tested for RT (5 μl) and RNase H (10 μl) activities. Then 500 μl of the 0.1 M NaCl flow through of the poly(U) agarose column and 500 μl of fraction 11 of the elution profile shown in Figure 1A were diluted in buffer M without glycerol to 0.8 ml and applied to a 15–35% GDG (10 ml total) and centrifuged in a SW41 rotor for 43 h at 35 000 r.p.m. at 4°C. Thirty fractions were collected and the bottom (b) resuspended. RT (5 μl) and RNase H (10 μl) were determined. A marker was cocentrifuged and was identified by gel electrophoresis and staining. The 92 kd protein appeared in fraction 16, the 66 kd protein in fraction 20, the 21/31 kd proteins in fraction 21 and the 21/14 kd proteins in fraction 24.

RT assay: samples of 100 μl contain 50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 0.02% NP-40, 1 μg poly(rA)·oligo$(dT)_{10}$, [^{3}H]TTP (5 μl, 50 Ci/mmol, 1mCi/ml), and 5 μl of enzyme. Incubation was for 60 min at 37°C, then the reaction was stopped [2 ml 10% trichloroacetic acid (TCA), 20 min, 4°C] and the acid precipitable material collected on filters (GF/C, Whatman) with 5 × 5 ml TCA wash procedures.

RNase H was analyzed using the same assay conditions except that hybrid was used as substrate. The hybrid was synthesized using single-stranded M13mp8 DNA (BRL Biochemicals, Bethesda, USA), [α-^{32}P]UTP (400 Ci/mmol) and *E.coli* RNA polymerase which results in a specific activity of about 10^6 c.p.m./pmol and 2×10^3 c.p.m./μl. 20 μl of hybrid and 10–20 μl of enzyme were used per assay. RNase H assays were incubated for 15 min at 37°C, TCA-precipitable activity determined as above and the radioactivity released from the hybrid determined by subtraction from the undigested hybrid control. Background variations of the assay never exceeded 500 c.p.m. Immunoblot was performed as described (Hansen *et al.*, 1987) using the diluted virus lysate input (30 μl), the eluted PC fraction 4 (50 μl), the poly(U) fraction 11 (50 μl), the GDG peak fraction 14 and fraction 24 (top) (50 μl) and a pool of patient sera (1:500 dilution).

Activated gel analysis of RT

Purified HIV-lysate (50 μg) was electrophoresed on a 10% polyacrylamide gel containing either 2 or 5 μg/ml of poly(rA)·oligo$(dT)_{10}$ in a total of 20 ml gel volume for RT activity analysis. After removal of sodium dodecylsulfate (SDS) the proteins were renatured and the slot containing the ^{14}C marker was sliced off, dried and exposed separately. The residual gel was incubated under RT assay conditions for 16 h at 37°C. 50 μCi of [α-^{32}P]TTP (3000 Ci/mol) in 50 ml buffer were used. Details of the procedure have been published elsewhere (Spanos *et al.*, 1981).

Immunoprecipitation of RNase H by monoclonal antibody

Hybridoma or NS-1 myeloma cell supernatant (30 ml each) was bound to protein A–Sepharose beads (Pharmacia Co., Sweden) precoated with anti-mouse IgG. Alternatively, rabbit antiserum against bacterially expressed purified p66 (RS) (5 ml) was bound to protein A–Sepharose beads. The bead-coupled IgGs were washed with phosphate-buffered saline (PBS) containing 0.1% NP-40 and resuspended in 1 ml each. These immunobeads were used to precipitate the RNase H from the peak and top fractions of the GDG (numbers 15 and 24, respectively) using two enzyme concentrations of each fraction (10 and 30 μl). The enzymes immobilized by the immunobeads were washed and used as an enzyme source in RNase H assays. The assay was performed by pelleting immobilized enzymes, washing them in PBS supplemented with 0.2% NP-40 (three times) and resuspending them in standard RNase H assay buffer. Hybrid was added and incubation was performed for 15 min at 37°C. The reaction was stopped by acid precipitation and the radioactivity remaining in the pellet determined. It represents the amount of undegraded hybrid and was subtracted from the total radioactive hybrid input which corresponded to 66 500 c.p.m. and was defined as 100%. The released radioactivity determined is listed in Table I and is also indicated in per cent. Variations of this assay never exceeded 1000 c.p.m. All assays were performed in duplicate.

Assay of processive nature

Standard RNase H assays were set up in duplicate using the poly(U) agarose fraction 11 (10 μl) and GDG fractions 14 and 25 (10 μl) as enzyme sources. To one of each pair of tubes 10 μl of unlabeled hybrid (corresponding to ~ 30-fold excess) was added 1 min after the start of the incubation. A pair of tubes with and without competing hybrid was stopped at 3, 8 and 16 min of incubation and analyzed for RNase H activity.

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