Human Immunodeficiency Virus Reverse Transcriptase-associated RNase H Activity*

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Biochemical characteristics of the RNase H activity associated with immunoaffinity purified human immunodeficiency virus (HIV) reverse transcriptase (RT) were examined. Glyceral gradient centrifugation of HIV RT resulted in a single peak with RNase H, associated with RT activity, with an apparent molecular weight of 110,000. HIV RNase H exhibited a marked substrate preference for poly(dC)-poly(rG) compared to poly(dT)-poly(rA). It did not hydrolyze single-stranded RNA or the DNA component of DNA-RNA hybrids. Products of the HIV RT-associated RNase H reaction consisted primarily of monomers, dimers, and trimers with 3' OH groups. This reaction was Mg++ dependent, with greater than 90% of maximum activity at MgCl2 concentrations between 4 and 12 mM. The optimum KCl concentration for HIV RNase H was 50 mM, which was lower than that for HIV RT catalyzed polymerization with a poly(rA)-poly(dT) template. The optimum pH for HIV RNase H activity was between 8.0 and 8.5, in contrast to an optimum pH of 7.5 to 8.0 for HIV RT activity. The association of RNase H activity with the p66 component of HIV RT was demonstrated by activity gel analysis. These results indicate that HIV RT has an integral RNase H activity; however, some of its properties are different from those of RNase H associated with other retroviral RT's, and optimal assay conditions are different than those for HIV RT catalyzed DNA polymerization.

The human immunodeficiency virus (HIV) genome, like that of other retroviruses, contains three major genes, designated gag, pol, and env (1). The pol gene, thought to be expressed by fusion of gag and pol reading frames, codes for at least three distinct proteins. By analogy with similar viruses, these include a protease which is responsible for processing of precursor polypeptides, a reverse transcriptase (RT) which synthesizes a double-strand DNA copy of the viral ribonucleic acid genome, and an endonuclease/ligase (2). RT catalyzes several enzymatic reactions, including both RNA- and DNA-directed DNA polymerization (2, 3). In addition, retroviral RT typically catalyzes hydrolysis of the RNA component of DNA-RNA hybrids, known as RNase H activity (2).

HIV RT is composed of two polypeptides, p66 and p51, which share a common amino terminus (4, 5). p51 is derived from the proteolytic cleavage of p66, with release of a small polypeptide, p15 (6, 7). This processing is thought to result in production of a stable p66/p51 dimer, which may be the natural form of HIV RT (8). However, after separation of p66 and p51 by denaturing polyacrylamide gel electrophoresis, p66 can renature, with recovery of DNA- and RNA-dependent DNA polymerase activity, but recovery of p51 catalytic activity is very poor in comparison (3).

Amino acid sequence analysis of retroviral pol genes suggests that polymerase sequences are present within the amino-terminal portion of p66, while the carboxyl terminus is homologous with RNase H from Escherichia coli (9). The association of RNase H activity with HIV RT produced by expression of a recombinant gene in E. coli (10), and with RT from lysed virions (6), was recently reported. RNase H and RT activity co-purify during ion exchange and DNA affinity chromatography, as well as glyceral gradient centrifugation. In addition, proteolytic processing of p66 results in production of p51 and a small polypeptide (p15) that retains RNase H activity but lacks polymerase activity (6).

There are several possible functions of RNase H in proviral DNA synthesis. These include removal of 3' genome RNA in DNA-RNA after strong stop minus-strand DNA synthesis, removal of the rRNA primer at the 5' end of minus-strand DNA, and a role in generation of the primer for plus-strand DNA synthesis (2). In view of the importance of RNase H in the generation of proviral DNA, this enzyme activity is a possible chemotherapeutic target for inhibition of HIV replication. This article examines some of the biochemical characteristics of the RNase H activity associated with immunoaffinity purified HIV RT.

MATERIALS AND METHODS

Poly(dC), poly(dT), oligo(dT)12-18, poly(rC), (dG)12-18, poly(dC)12-18, poly(rA), (dT)18, E. coli RNA polymerase, phosphodiesterase I, Sephadex G-50, and unlabeled nucleotides were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Moloney murine leukemia virus (MMLV) RT, E. coli RNase H, T4 polynucleotide kinase, uracil and ultrapure reagents for polyacrylamide gel electrophoresis (PAGE) were obtained from Bethesda Research Laboratories (Gaithersburg, MD). Gel filtration molecular weight standards, bovine serum albumin (BSA), guaninyl-(3'5')-guanosine (GpG), and calf thymus DNA were acquired from Sigma. Avian myeloblastosis virus (AMV) RT and dithiothreitol (DTT) were purchased from Boehringer Mannheim. ACS scintillation mixture was obtained from Amerham Corp. Molecular weight markers for denaturing PAGE and a 4-chloro-1-naphthol containing horseradish peroxidase color development reagent were acquired from Bio-Rad. Biotinylated anti-human IgG and avidin D-horseradish peroxidase conjugate were

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The abbreviations used are: HIV, human immunodeficiency virus; RT, reverse transcriptase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin, AMV, avian myeloblastosis virus; DTT, dithiothreitol; GpG, guaninyl-(3'5')-guanosine; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MMLV, Moloney murine leukemia virus.
HIV Reverse Transcriptase-associated RNase H

0.05 or 0.5 units of HIV RNase H were incubated with poly(dC)-[32P]poly(rG) using standard reaction conditions for the time periods indicated in Fig. 2. Two 10-μl portions of each reaction were treated with 10 μl of 7% perchloric acid and processed for determination of acid-soluble radioactivity as described above. One 5-μl portion of each reaction was diluted with 20 μl of H2O and 12.5 μl of sequencing gel sample buffer (50 mM EDTA and 0.02% bromphenol blue in 88% formamide), 5 μl of an 80% acid-soluble reaction was further treated for 15 min with 0.1 unit of phosphodiesterase I at 37 °C, followed by addition of 44 μl of H2O and 25 μl of sequencing gel sample buffer. [5-32P]GMP was prepared by 5' phosphorylation of GpG with T4 polynucleotide kinase in the presence of [γ-32P]ATP as recommended by the kinase supplier. Part of the [5-32P]GMP was treated with phosphodiesterase I to produce [5-32P]GMP. Samples were boiled for 5 min and fractionated by electrophoresis on a 15% polyacrylamide-urea DNA sequencing gel as previously described (15), followed by autoradiography with Kodak X-Omat R film.

RNase H Activity Gel

A 10% polyacrylamide gel which contained SDS was prepared essentially as described by Laemmli (13), except for the addition of poly(dC)-[32P]poly(rG) (106 cpm) prior to polymerization. Enzyme samples for activity recovery were HIV RT (12 units), MMLV RT (200 units), and E. coli RNase H (10 units), each in a final volume of 40 μl which contained 5% glycerol, 2 mM EDTA, 1% SDS, 50 mM Tris-HCl, pH 7.5, 0.02% bromphenol blue, 1 mg/ml BSA, and 0.5 mM 2-mercaptoethanol. These were incubated for 5 min at 37 °C before application to the gel. One lane was reserved for protein standards and another for HIV RT transferred to nitrocellulose. These were mixed with standard sample buffer (13) and boiled before application to the gel. After electrophoresis, the lane with molecular weight standards was removed and stained with Coomassie Blue (14). The lane with HIV RT in standard sample buffer was transferred to nitrocellulose (18), and transferred proteins were stained by incubation with 2% nonfat dry milk and 4% used free-calf sarinum phosphate-buffered saline. Development was performed with a 4-chloro-1-naphthol-based reagent in the presence of H2O2 as recommended by the horseradish peroxidase substrate supplier.

The remainder of the gel, which contained enzymes for activity recovery, was shaken gently at room temperature for 1 h in 2 changes (1 liter each) of 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 20% glycerol, followed by 16 h in 2 changes (1 liter each) of the same buffer plus two changes except for 50 mM KC1 and 8 mM MgCl2. The gel was shaken for another 8 h in 50 mM Tris-HCl, pH 8.0, 50 mM KC1, 2 mM DTT, 8 mM MgCl2, in the absence of glycerol, followed by 16 h in the same buffer at 37 °C without shaking. This was followed by gentle shaking for 4 h in 4 (1 liter) changes of cold 5% trichloroacetic acid, 10 mM pyrophosphate. The gel was dried under vacuum and autoradiography was performed with Kodak X-Omat K film.

RESULTS

Glycerol Gradient Centrifugation—Immunofinity purified HIV RT was subjected to ultracentrifugation in a 10–30% glycerol gradient in the presence of 200 mM KC1, and fractions were analyzed for the presence of polymerase and RNase H activities (Fig. 1). A single peak of RNase H activity coincided with DNA polymerase activity measured with a poly(dC)-oligo(dG) template, and with RT activity measured with poly(rC)-oligo(dG) and poly(rA)-oligo(dT) templates.
FIG. 1. Glycerol gradient centrifugation of HIV RT. Ultracentrifugation of immunoaffinity purified HIV RT was performed as described under "Materials and Methods." Fractions of approximately 200 µl were collected from the top of the gradient and assayed for the presence of RNase H activity (●), or for polymerase activity with a poly(rA)-oligo(dT)18 (○), poly(rC)-oligo(dG)12-18 (△), or poly(dC)-oligo(dG)12-18 (▲) template. Positions of standard proteins are indicated with arrows: carbonic anhydrase (29,000), BSA (66,000), alcohol dehydrogenase (150,000), and β-amylase (200,000).

![Glycerol gradient centrifugation](image)

TABLE I

| Substrate preference of RNase H activity associated with HIV and AMV reverse transcriptase | HIV RT* | AMV RT* |
|------------------------------------------|---------|---------|
| DNA-[3H]RNA | 0.026 | 0.032 |
| DNA-[3H]RNA, heat-denatured | 0 | 0 |
| RNA-[3H]DNA | 0.004 | 0.056 |
| Poly(dT)-[3H]poly(rA) | 0.150 | 0.033 |

* Labeled substrate rendered acid-soluble (nanomole/h) per unit of RT. Nuclease activity with the indicated substrate was plotted as a function of RT activity, and the slope was determined by linear regression analysis. Numbers represent the average slope from at least two such experiments, each with at least four different enzyme concentrations. The lowest average correlation coefficient was 0.954.

The complex had an apparent molecular weight of approximately 110,000. When a similar experiment was conducted in the absence of KCl, a broad peak of polymerase and RNase H activity appeared in fractions 11–21, which corresponded to apparent molecular weights between 110,000 and 250,000 (data not shown).

Template Preference of HIV RNase H—HIV RT-associated RNase exhibited a marked substrate preference for poly(dC)-[3H]poly(rG) compared to poly(dT)-[3H]poly(rA), in contrast with AMV RT/RNase H which preferred the latter substrate (Table I). Intermediate activity was obtained with HIV RNase H and a mixed DNA-[3H]RNA hybrid. There was an absence of nonspecific nuclease activity in our preparation of HIV RT as demonstrated by the lack of degradation of single-stranded RNA, and an inability to hydrolyze [3H]DNA in a [3H]DNA-RNA hybrid.

Products of the HIV RNase H Reaction—HIV RT/RNase H was incubated with poly(dC)-[32P]poly(rG) under standard conditions, and the reaction products were identified after alkaline hydrolysis and fractionation on 7% polyacrylamide gel. Lanes a and b contain 5'-32P-labeled pGpG and GMP, respectively. Lane c is a control reaction, incubated for 40 min at 37°C in the absence of enzyme. Lanes d, e, and f are reactions with 0.05 units of HIV RNase H incubated for 20, 40, and 120 min, respectively. Lane g contains reaction products from a 120-min incubation with 0.5 units of HIV RNase H. The percentages of acid-soluble material in lanes d–f were 30, 54, 80, and 100%. Lane h contains one-half the amount of reaction products in lane f, treated with venom phosphodiesterase as described under "Materials and Methods."

![Products of the HIV RNase H reaction](image)

FIG. 2. Products of the HIV RNase H reaction. Products of the HIV RNase H reaction with a poly(dC)-[32P]poly(rG) substrate were analyzed as described under "Materials and Methods." Lanes a and b contain 5'-32P-labeled pGpG and GMP, respectively. Lane c is a control reaction, incubated for 40 min at 37°C in the absence of enzyme. Lanes d, e, and f are reactions with 0.05 units of HIV RNase H incubated for 20, 40, and 120 min, respectively. Lane g contains reaction products from a 120-min incubation with 0.5 units of HIV RNase H. The percentages of acid-soluble material in lanes d–f were 30, 54, 80, and 100%. Lane h contains one-half the amount of reaction products in lane f, treated with venom phosphodiesterase as described under "Materials and Methods."

![MgCl2 dependence of HIV RT and RNase H activities](image)

FIG. 3. MgCl2 dependence of HIV RT and RNase H activities. HIV RT (■) and RNase H (●) activities were measured using standard reaction conditions, except that MgCl2 was added at the indicated final concentrations. Data points represent the average values from two experiments, each performed in duplicate.
fold higher concentration of HIV RT was employed, 100% of the substrate was rendered acid soluble, and the products consisted almost exclusively of monomers, dimers, and trimers. Treatment of HIV RNase H reaction products with venom phosphodiesterase resulted in complete conversion to [5',-32P]GMP.

Divalent Cation Requirement—The HIV RNase H reaction with poly(dC)-[3H]poly(rG) as the substrate was absolutely dependent on the presence of divalent cation. MgCl2 was preferred to MnCl2 (not shown). Greater than 90% of the maximum RNase H activity was obtained at MgCl2 concentrations between 4 and 12 mM (Fig. 3). This is in contrast to the MgCl2 dependence of HIV RT activity measured with a poly(rA)- (dT)10 template, which exhibited a comparatively sharp MgCl2 optimum of 6-8 mM, and retained only 54% of maximum activity at a concentration of 12 mM.

Effect of Salt—The effect of KCl on RNase H activity and on polymerase activity with several different templates was examined (Fig. 4). KCl was added to various assay mixtures at 25 mM increments to 0.25 M. RT activity with a poly(rA)- (dT)10 template exhibited a maximum activation of about 2.3-fold at 75 mM KCl, and KCl concentrations greater than 175 mM resulted in less activity than observed in the absence of KCl. This was similar to the salt activation profile observed with a poly(rC)- (dT)10 template (not shown). In contrast, DNA polymerase activity with poly(dC)- (dG)12-18 retained 164% of control activity in the presence of 250 mM KCl. RNase H activity exhibited a salt profile different from that of polymerase activity with any of these templates. Maximum activation of 2.1-fold was obtained at 50 mM KCl, and the presence of KCl at concentrations greater than 100 mM was strongly inhibitory.

pH Optimum—HIV RT-associated RNase H had an alkaline pH optimum of 8.0-8.5, with either Tris or Hapes buffers (Fig. 5). In contrast, polymerase activity with a poly(rA)- (dT)10 template had a sharp pH optimum between 7.5 and 8.0, and retained about 60% of maximum activity at pH 8.5.

RNase H Activity Gel—The RNase H substrate poly(dC)- [32P]poly(rG) was incorporated into a polyacrylamide gel prior to polymerization. Purified HIV RT was denatured and subunits were separated during electrophoresis through this gel. One lane which contained HIV RT was transferred to nitrocellulose and immunostained with a monoclonal antibody against this enzyme (Fig. 6a). The remainder of the gel was washed extensively to allow protein renaturation, followed by incubation at 37°C under conditions optimal for detection of HIV RNase H activity. The gel was then dried, and autoradiography performed (Fig. 6b). RNase H activity was associated with the p66 subunit of HIV RT as demonstrated by the absence of radioactive substrate in the portion of the gel occupied by p66. A smaller band of nuclease activity which corresponded to a molecular weight of approximately 200,000 was also noted.

DISCUSSION

Immunoaffinity purified HIV RT possessed RNase H activity as well as DNA- and RNA-dependent DNA polymerase activity. All three activities co-migrated in a glycerol gradient during ultracentrifugation. We were unable to observe any small molecular weight RNAse H in glycerol gradient fractions or by activity gel analysis. RNase H activity was an integral part of HIV RT, since it was associated with the p66 subunit. A smaller amount of RNase H activity with an apparent molecular weight of 200,000 was also observed, which raises the interesting possibility that the gag-pol precursor could have RNase H activity; however, we have not ruled out the
possibility that the presence of high molecular weight RNase H could be due to enzyme aggregation. Others have observed concurrent production of p51 and a 15,000 RNase H during proteolytic cleavage of a carboxy-terminal fragment from p66 (6). Several factors may contribute to the absence of this activity in our preparation. The monoclonal antibody used for purification for p51 recognizes a determinant shared by p66 and p51, and would not be expected to recognize the carboxy-terminal portion of p66. Therefore, p15 should not be present unless it forms a complex with RT, or is produced after purification of RT by the action of a contaminating protease.

HIV RT-associated RNase H demonstrated characteristics of a true RNase H, since it did not degrade single-stranded RNA or the DNA portion of a DNA-RNA hybrid. Unlike AMV RT, it demonstrated a marked substrate preference for poly(dC)-[3H]poly(rG). In addition, we have confirmed the substrate potential of mixed DNA-[3H]RNA hybrids observed by others (6, 10). However, poly(dC)-[3H]poly(rG) is the preferred substrate for many applications due to higher activity, ease of separation, and homogeneity of the substrate.

Like AMV RT/RNase H, HIV RT-associated RNase H produced a series of oligomers; however, these products are primarily monomers, dimers, and trimers, while AMV RNase H products are primarily larger oligonucleotides (2, 17). Venom phosphodiesterase was able to convert HIV RNase H products to [5'-32P]GMP. Since this enzyme requires a substrate with a free 3'-hydroxyl group, HIV RNase H products, like those produced by other retroviral RNase Hs (2), are probably terminated with 5'-phosphate and 3'-hydroxy groups.

Several factors indicate that HIV RT contains distinct catalytically sites for polymerase and RNase H activity. Amino acid sequence analysis demonstrates homology with viral and bacterial polymerases within the amino-terminal portion of p66, and homology with other RNase Hs near the carboxyl terminus (9). Release of an active 15,000 RNase H which lacks polymerase activity during processing of p66 also supports this idea (6). Proteolytic fragments of AMV RT which possess RNase H activity but lack polymerase activity have also been reported (18). In spite of apparently distinct active sites for RNase H and polymerase, removal of the 15,000 fragment results in a 51,000 polypeptide that has greatly reduced RT activity. We previously reported that we were unable to detect polymerase activity associated with p51 by activity gel analysis (3); however, with highly concentrated enzyme, we are now able to detect a small amount of p51-associated polymerase activity. This is consistent with a recent report that p51 has 20-80-fold less RT activity than p66/ mg of protein (19).

RNase H activity catalyzed by p15 H exhibits a random mode of action, in contrast with RT-associated RNase H, which is processive (6). The change to a random mode of RNase H action after loss of the polymerase active site could be due to the loss of a nucleic acid binding domain. That this is the case for HIV p15 is supported by a site-directed mutagenesis study which indicates that the template binding site is present within the amino-terminal half of p66 (20).

Our results obtained with immunoaffinity purified HIV RT indicate several differences in optimum assay conditions for polymerase and RNase H activities. RNase H exhibits a broader MgCl2 optimum, lower KCl optimum, and prefers a more alkaline pH, than polymerase. For these reasons and those discussed above, it may be possible to discover selective inhibitors of HIV RNase H.

RNase H plays an important role in proviral DNA synthesis catalyzed by RT. Studies in progress may indicate whether or not this enzyme activity will be a useful target for inhibition of HIV replication. In addition, degradation of viral genomic or messenger RNA by RNase H could be an important mechanism of action of antiense oligonucleotides, a chemotherapeutic approach which is under active investigation in several laboratories. This article provides important basic information for investigation of these possibilities.

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