Inhibition by RNA of RNase H Activity Associated with Reverse Transcriptase in Rauscher Murine Leukemia Virus Cores

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We reported earlier that core preparations of Rauscher murine leukemia virus, when separated on an isopycnic sucrose gradient, did not contain detectable levels of RNase H activity, while retaining high levels of reverse transcriptase activity. We reexamined this phenomenon, and the earlier observation was found to be reproducible. However, when doubly banded preparations of viral cores were solubilized and reverse transcriptase was isolated by ion-exchange chromatography, a coincident peak of a nuclease activity with the specificity of RNase H was observed, which indicated that RNase H was selectively inhibited in the core fractions. By direct activity measurements using the purified reverse transcriptase-RNase H from cores, this endogenous inhibitor has been identified as the viral RNA. Viral 70S RNA strongly inhibited RNase H activity purified either from whole virions or from prefractionated cores. Other RNAs tested that had inhibitory effects were yeast tRNA, polyadenylic acid, and polyguanylic acid. Polyuridylic acid and polyadenylic acid were moderately inhibitory, and polycytidylic acid did not inhibit the RNase H. A rabbit anti-reverse transcriptase immunoglobulin G inhibited both the reverse transcriptase and RNase H activities of the enzyme purified from cores. These data provide a rational explanation for the failure to detect RNase H activity in core preparations of Rauscher murine leukemia virus. Furthermore, these data are consistent with the idea that the RNase H and reverse transcriptase activities purified from cores reside on the same protein molecule. Possible biological implications of the observed inhibition of RNase H by RNA is discussed.

RNA tumor viruses contain a RNase activity which specifically hydrolyzes the RNA strands of RNA-DNA hybrids (1, 7-9, 14). In the case of avian viruses, this enzyme has been conclusively shown to be an associated activity of the reverse transcriptase molecule (1, 8, 14, 18). Some confusion still exists, however, on the relation between RNase H and reverse transcriptase molecules in the mammalian retroviruses. More than 90% of the total RNase H activity in mammalian type C viruses is separated from the reverse transcriptase by ion-exchange and other chromatography procedures (5, 21). In these viruses the proportion of the total RNase H activity that is copurified with the reverse transcriptase by conventional techniques is very small, and substrates of high specific radioactivity are required to detect it (21). There was a possibility that this minor portion of the nuclease activity copurifying with the reverse transcriptase was an adventitious contaminant. Selective inhibition of reverse transcriptase activity with pyri-

doxal phosphate, which had no effect on RNase H activity present in the same fraction (10), and a similar selective inhibition by DNA of RNase H activity, with no inhibitory effect on reverse transcriptase activity, had also been reported (11). In addition, we reported earlier that core structures of Rauscher murine leukemia virus (R-MuLV), isolated by isopycnic centrifugation on sucrose gradients, had no detectable RNase H activity but had high levels of reverse transcriptase activity (21). These observations are consistent with the idea that the two enzyme activities are present in two different protein molecules. Selective inhibition of one or the other activity could also be explained as mechanistic independence of two active sites on a single protein, as has been observed in the case of the RNase H-reverse transcriptase molecule of avian myeloblastosis virus (AMV) (2). Therefore, we reexamined the virion cores of R-MuLV to ascertain whether the absence of detectable RNase H activity in these fractions was a result

of some selective inhibition. Our results presented here show that there in fact was a selective inhibition of RNase H in the core fraction and that the reverse transcriptase isolated from the cores does indeed contain RNase H activity.

MATERIALS AND METHODS

Virus and chemicals. R-MuLV was grown in JLS-V10 cells and was obtained from Frederick Cancer Research Center. Tritiated nucleotides were obtained from Schwarz/Mann or New England Nuclear. RNA·DNA hybrid substrates [^3H](A) $_n$ ·(dT) $_m$ (15,300 cpm/pmol) and ϕX174 DNA·[^3H]RNA (14,800 cpm/pmol), [^3H](A) $_n$ (3,700 cpm/pmol), and [^3H](dT) $_n$ ·(A) $_m$ (16,000 cpm/pmol) were synthesized enzymatically as described earlier (17, 21). Nonradioactive homopolymeric nucleotides were purchased from Miles Laboratories. Rat liver tRNA was obtained from Grand Island Biological Co. R-MuLV 70S RNA was extracted from purified virus as described earlier (20), with the modifications that LiCl was substituted for NaCl and 0.2% sodium dodecyl sulfate was used in the glycerol gradient buffer.

Enzyme assays. Reverse transcriptase activity was assayed as previously described (21) using (dT) $_{-15}$ ·(A) $_n$ as primer-template in a 50- μl final volume unless otherwise specified. Incorporation of [^3H]-dTTP during 30 min of incubation at 37°C was measured. RNase H activity was assayed by measuring the amount of radioactivity in the RNA·DNA hybrid substrate that was rendered acid soluble at the end of a 45-min incubation at 37°C. The assay system in a 100- μl final volume contained: 50 mM Tris-hydrochloride (pH 7.5), 1 mM dithiothreitol, 1 mM MnCl $_2$, 50 mM KCl, 10,000 to 80,000 cpm of [^3H](A) $_n$ ·(dT) $_m$ or ϕX174 DNA·[^3H]RNA, and the enzyme. The reaction was stopped by adding 50 μl of a 5-mg/ml solution of yeast tRNA and 200 μl of 10% trichloroacetic acid. After 10 min in ice, the mixture was centrifuged, and a 200- μl sample of the supernatant was counted for radioactivity in the NCS system as described before (17).

Preparation and fractionation of R-MuLV cores. Sucrose gradient-purified R-MuLV (13.7 ml, 14.4 mg of protein) was pelleted by centrifugation for 45 min at 25,000 rpm in an SW27 rotor. The supernatant was removed, and the virus pellet was suspended in 1 ml of 10 mM Tris-hydrochloride buffer (pH 7.9), containing 1 mM EDTA and 1 mM dithiothreitol, and treated in ice for 15 min with 200 μl of a 3% solution of Sterox SL (Monsanto Chemicals) in the same buffer. The mixture was layered on a 35-ml sucrose gradient (20 to 60%, wt/wt) and centrifuged at 25,000 rpm for 14 h in a Spinco SW27 rotor. Fractions were collected from the bottom of the tube, and aliquots were assayed for reverse transcriptase and RNase H activities. Density was determined on every fifth fraction by refractive index measurement. Fractions comprising virion cores that banded at densities above 1.2 g/cm 3 were pooled and diluted to 20% sucrose with 10 mM Tris-hydrochloride containing 1 mM dithiothreitol and 1 mM EDTA. The core fractions were further purified by a second density banding on a 25 to 60% (wt/wt)

sucrose gradient. Material thus purified was used for isolation of reverse transcriptase.

Extraction and purification of reverse transcriptase from purified viral cores. The core fractions purified by two successive sucrose density gradient centrifugations were pooled and treated for 30 min with 0.5 M NaCl and 0.5% Triton X-100 at 4°C. The extract was diluted with an equal volume of Buffer A (10 mM Tris-hydrochloride, pH 7.9, containing 1 mM dithiothreitol, 1 mM EDTA, and 20% glycerol) and chromatographed on a 5-ml column of DEAE-cellulose previously equilibrated with Buffer A containing 0.25 M NaCl. The column was subsequently washed with the equilibration medium. Fractions of 1 ml were collected and assayed for reverse transcriptase and RNase H activities, and those that contained the bulk of the enzyme activities were used for the next step. A 1-ml column of phosphocellulose was prepared and equilibrated with Buffer A. Active fractions from the DEAE-cellulose column were diluted fivefold with Buffer A and applied to this new column at a rate of 15 ml/h. To avoid loss of enzyme activity during standing in dilute solution, the DEAE-cellulose column fractions were individually diluted, one fraction at a time, just prior to applying to the phosphocellulose column, rather than pooling them together and diluting at once. The column was thoroughly washed with buffer until all the unbound proteins were removed, and then it was developed with a 20-ml linear gradient between 0.05 M and 0.7 M NaCl in Buffer A. Approximately 1-ml fractions were collected, and aliquots were assayed for RNase H and reverse transcriptase activities. NaCl concentrations were determined by conductance measurement. Peak fractions comprising the bulk of the enzyme activities were pooled and used for the studies described in this paper.

RESULTS

Absence of RNase H activity in rebanded cores of R-MuLV. The reverse transcriptase of retroviruses is located in the core fraction (3, 6). Therefore, we rationalized that the purified core structures should be the most useful subviral material for studying the question of whether RNase H and reverse transcriptase are parts of the same molecule. The core structures are less likely to be contaminated by cellular proteins than are whole virions. In an earlier communication we had analyzed the cores of R-MuLV on a sucrose gradient and found no measurable RNase H activity in such fractions (21). On the other hand, although most of the RNase H activity of the virus was separable from the reverse transcriptase when chromatographed on a phosphocellulose column, the reverse transcriptase purified from whole-virus lysates nonetheless contained measurable amounts of RNase H activity (21). To clarify this apparent discrepancy, we performed the following experiments.

Core structures of R-MuLV were prepared by mildly treating a concentrated suspension of

purified viral particles with 0.5% Sterox-SL. These core particles were fractionated from intact virions and solubilized viral components by isopycnic centrifugation on a 20 to 60% sucrose gradient. The gradient was fractionated and analyzed for reverse transcriptase and RNase H activities. As reported earlier, the fractions representing the viral cores had very high reverse transcriptase activity but no significant RNase H activity. The interpretation of the experiments to follow would depend a great deal on the quality of the core preparations. Therefore, we pooled the core fractions with densities above 1.2 g/cm^3 , appropriately diluted them to reduce the sucrose concentration to 20%, and then subjected them to a second isopycnic banding through a sucrose gradient. The analysis of the fractions collected from the second gradient is shown in Fig. 1. Once again, the core fractions banding at a density of about 1.2 g/cm^3 showed only reverse transcriptase activity and no RNase H activity. These fractions were pooled and used as described in the following section.

Purification of reverse transcriptase from R-MuLV cores. As reported earlier (21), sucrose was not a selective inhibitor of RNase H, being equally inhibitory to both reverse transcriptase and RNase H. Further, dialysis of the

core fractions did not cause the appearance of RNase H activity. To establish whether the apparent absence of RNase H activity in the core fraction signified a true separation of RNase H and reverse transcriptase activities or was caused by a hitherto-unknown selective inhibition of RNase H by a component in these fractions, cores were extracted with Triton X-100 and NaCl, and the extract was used as the starting material for the purification of reverse transcriptase. Nucleic acids were removed by chromatography of the core extract on a DEAE-cellulose column at 0.25 M NaCl . The unbound fraction eluting from the column contained, as expected, the reverse transcriptase activity; but more significantly, the fraction also contained RNase H activity, indicating that the DEAE-cellulose step removed an inhibitor of the RNase H activity that was present in the core fraction. The relationship between the two enzyme activities was analyzed by a further chromatographic fractionation on a phosphocellulose column. Figure 2 shows that both the enzyme activities coeluted from the column when the column was developed with a linear salt gradient. The ratio of the two activities was essentially similar all across the peak. No substantial difference has been found between this result and those ob-

FIG. 1. Profile of reverse transcriptase and RNase H activities of density-banded R-MuLV cores. Core structures were prepared from R-MuLV (14.4 mg of protein) by treatment of the virions with 0.5% Sterox SL and fractionating the disrupted particles by banding on a 20 to 60% sucrose gradient as described in the text. The core fractions were pooled and rebanded on a second sucrose gradient (25 to 60%). Fractions were collected and analyzed for reverse transcriptase activity (●—●), RNase H activity (▲--▲), and density (*—*), as described in the text.

FIG. 2. Profiles of core-derived reverse transcriptase and RNase H activities on phosphocellulose chromatography. The rebanded core structures were extracted and the extract was chromatographed on DEAE-cellulose as described in the text. The nucleic acid-free fractions from DEAE-cellulose containing the bulk of reverse transcriptase and RNase H were chromatographed as described in the text. (●—●) Reverse transcriptase activity; (Δ—Δ) RNase H activity; (■—■) NaCl concentration. There was no significant RNase H or reverse transcriptase activity in the flow-through or buffer wash fractions.

tained using extracts of whole virus, except that in the latter case an additional large peak of RNase H activity free of reverse transcriptase is found in the phosphocellulose flow-through fractions (21). Similar substantial separation of RNase H activities free of reverse transcriptase has also been described upon chromatography on Sephadex G-100 (5).

To minimize loss of enzyme activities during purification, the purification steps, starting with the extraction of the cores, were performed as a continuous procedure without freezing of samples between steps or allowing samples to remain in dilute solutions for an extended time.

Characterization of the RNase H activity associated with reverse transcriptase. To examine whether the nuclease activity that copurified with reverse transcriptase on the phosphocellulose column was a true RNase H, we examined its ability to utilize [³H](A)_n·(dT)_m, φX174 DNA·[³H]RNA, [³H](A)_n, and [³H](dT)_n·(A)_m as substrates. The enzyme specifically hydrolyzed the RNA strands of the hybrid substrates φX174 DNA·[³H]RNA and [³H](A)_n·(dT)_m. There was no solubilization of the DNA strand of the latter substrate, evidenced by a complete precipitability of radioactivity in [³H](dT)_n·(A)_m after incubation with the enzyme. Further, [³H](A)_n was not a substrate in

the absence of (dT)_m. These results were similar to those that we observed for the RNase H activity associated with the reverse transcriptase when purified from whole virus (21).

Further characterization of the RNase H and reverse transcriptase activities was performed with an antibody to R-MuLV reverse transcriptase (16). The enzyme was preincubated at 4°C overnight with increasing amounts of immunoglobulin G (IgG) (up to 60 μg) from sera of a rabbit immunized against purified R-MuLV reverse transcriptase. In the incubations with the lower amounts of immune IgG, additional normal rabbit IgG was added to raise the total IgG concentration to 60 μg. After 12 h, the preincubated samples were assayed for residual reverse transcriptase and RNase H activities by supplementing the remaining components of the respective assay systems as described above, with one modification, namely, that the final volume for both assays was 100 μl. The results obtained are shown in Fig. 3. The antiserum inhibited both of the enzyme activities almost identically. As a control, the effect of the above antiserum on the RNase H and DNA polymerase activities of avian myeloblastosis virus reverse transcriptase was examined, and we observed no inhibition (data not shown). Similar results have been described for the RNase H associated with R-

FIG. 3. Inhibition of reverse transcriptase and RNase H activities by antibody to R-MuLV reverse transcriptase. Phosphocellulose column-purified reverse transcriptase (10 μ l) from R-MuLV cores was preincubated at 4°C in two triplicate sets with 5 μ l of bovine serum albumin (100 mg/ml in 1.2 M KCl) and 10 μ l of a solution containing a total of 60 μ g of rabbit IgG composed of normal rabbit IgG and 0, 7.5, 15, 30, or 60 μ g of anti-R-MuLV reverse transcriptase (16). After 12 h, one triplicate set (A) was assayed for residual reverse transcriptase activity (30 min, 37°C), and the other triplicate set (B) was assayed for residual RNase H activity (45 min, 37°C). In both cases, the assay components were added in a total volume of 75 μ l. Activities were expressed as percentage of the activity obtained with 60 μ g of normal rabbit IgG.

MuLV reverse transcriptase purified by nucleic acid affinity chromatography (12).

Inhibition of core-derived RNase H activity by natural and synthetic RNA. The results described thus far indicate that the RNase H activity present in isolated core fractions is inhibited by a nondialyzable component present in the same fractions. Since the activity was measurable after chromatography on DEAE-cellulose designed to remove nucleic acids from the extract, the logical choice for the inhibitor of RNase H was viral RNA. This is further indicated by the finding that polydeoxyribonu-

cleotides that work as templates for the reverse transcriptase activity inhibit the associated RNase H activity (11). The hypothesis was directly tested by measuring the effect of isolated viral 70S RNA on the RNase H activity associated with the reverse transcriptase purified from R-MuLV core structures, as described in the preceding sections. As shown in Fig. 4A, viral RNA had a strong inhibitory effect on the core-derived RNase H activity. Fifty percent inhibition of the enzyme activity was obtained at about 0.4 μ g of the RNA. A nonviral RNA (rat liver tRNA) also had a similar inhibitory effect (Fig. 4B). The inhibitory effect was not limited to natural RNAs. In fact, the strongest inhibition of the RNase H activity was observed with the synthetic homopolymer, polyguanylic acid [poly(G)] (67% inhibition at 60 ng) (Fig. 4C). These inhibition studies were performed using [3 H](A) $_n$ ·(dT) $_m$ as the enzyme substrate. Similar results were also obtained using the heteropolymeric hybrid ϕ X174 DNA·[3 H]RNA as the substrate (data not shown). In the latter system, polyadenylic acid [poly(A)] and polyuridylic acid [poly(U)] were moderately inhibitory, giving 65 to 57% inhibition at 2 μ g per assay, and polycytidylic acid [poly(C)] did not cause significant inhibition (Fig. 5). Although the inhibitory effects of poly(A) and poly(U) could not be measured using [3 H](A) $_n$ ·(dT) $_m$ as a substrate for obvious reasons, the effect of poly(C) was verified using this substrate and was again found to be noninhibitory (data not shown).

To verify whether the inhibition by RNA was unique to RNase H activity purified from isolated core fractions, we purified the reverse transcriptase of R-MuLV starting with a total virus lysate, using ion-exchange and nucleic acid affinity chromatography, and studied the effect of RNA on the RNase H activity of this preparation. The results obtained were identical to those observed with the enzyme purified from isolated cores (data not shown). A similar analysis was done on the RNase H activity associated with the reverse transcriptase purified from simian sarcoma virus (SSV) (data not shown). Once again, there was no qualitative difference obtained between the properties of the R-MuLV enzyme and the SSV enzyme. There was RNase H activity associated with purified SSV reverse transcriptase, and this activity was inhibited by natural (SSV 70S RNA and rat liver tRNA) and synthetic [poly(G), poly(A), and poly(U)] RNA. As in the case of R-MuLV RNase H, the SSV enzyme was inhibited the most by poly(G).

DISCUSSION

Data presented here confirm our earlier find-

FIG. 4. Inhibition of R-MuLV core RNase H by natural and synthetic RNAs. RNase H activity associated with the reverse transcriptase purified from R-MuLV cores was assayed in the presence of indicated concentrations of R-MuLV 70S RNA (A), rat liver tRNA (B), or poly(G) (C). [^3H](A) $_n$ ·(dT) $_m$ was used as the substrate. Inhibited activities are expressed as percentage of activity obtained in the absence of any RNA.

ing that R-MuLV cores, when isolated by a density gradient, show no detectable RNase H activity. Although this was earlier interpreted to mean possible separation of RNase H and reverse transcriptase activities, additional experimental evidence given here proved that the absence of RNase H activity in the core fractions was in fact due to a selective inhibition of this activity by the viral RNA. The nuclease activity was clearly measurable when the RNA was removed from extracts of viral cores by a chromatographic step using DEAE-cellulose. On subsequent phosphocellulose chromatography, the reverse transcriptase and RNase H activities eluted together as a coincident peak. In this respect, there was no difference between the results using whole-virus lysates and the extract of purified virus cores, except that there was no significant amount of RNase H activity in the unbound flow-through fraction from the phosphocellulose column. By substrate specificity determinations we have confirmed that the nuclease that copurified with reverse transcriptase from phosphocellulose was a true RNase H. The data presented here are consistent with the idea that RNase H and reverse transcriptase are two activities present on the same protein molecule.

The RNase H activity of the core-derived enzyme purified through the phosphocellulose step was inhibited by a rabbit antibody to purified R-MuLV reverse transcriptase to the same extent as the associated reverse transcriptase activity, similar to the reported finding with the reverse transcriptase-RNase H enzyme purified from a total viral lysate of R-MuLV (12). It is interesting that, unlike the results with antibody prepared in our laboratory (16), an earlier study using an antiserum to R-MuLV reverse tran-

scriptase (21) showed no inhibition of the RNase H activity associated with reverse transcriptase purified by ion-exchange chromatography of whole-virus lysates, but strong inhibition of the reverse transcriptase activity. The reasons for these differential effects on the two enzyme activities of the reverse transcriptase molecule between the two antisera are not clear. It is significant that conformational differences in the immunizing antigen are known to cause differential immune response. For instance, an antiserum raised against the α form of AMV reverse transcriptase inhibited both the DNA polymerase and RNase H activities of the α form, but inhibited only the DNA polymerase and not the RNase H activity of the $\alpha\beta$ form; conversely, an antiserum raised against the $\alpha\beta$ form of the AMV enzyme inhibited both the nuclease and DNA polymerase activities of the $\alpha\beta$ form, but only the DNA polymerase and not the RNase H activity of the α form (15).

The direct evidence that the viral RNA-mediated inhibition of the RNase H was the cause for the apparent absence of RNase H activity in the isolated core fraction was obtained by studying the effect of viral and other RNAs on RNase H purified from isolated viral cores. Data presented in Fig. 4 and 5 showed that, irrespective of the substrate used for measurement, the enzyme was generally inhibited by a variety of natural and synthetic RNAs. Although viral RNA was a strong inhibitor (Fig. 4A), poly(G) was by far the most potent inhibitor of the reverse transcriptase-associated RNase H. The RNase H activity associated with a highly purified preparation of SSV reverse transcriptase was also similarly inhibited by RNA, suggesting that this property may be general for mamma-

FIG. 5. Inhibition of R-MuLV core RNase H by synthetic homopolymer RNAs. Experimental details are the same as in Fig. 4, except that the substrate used was ϕ X174 DNA- $[3\text{H}]\text{RNA}$, and the effector nucleic acids examined were poly(A) (A), poly(U) (B), and poly(C) (C).

lian RNA tumor viruses. There is an apparent inconsistency in this interpretation: If viral cores do not exhibit RNase H activities because of inhibition by viral RNA present in the same fraction, how could one detect RNase H activity in unfractionated total viral lysates, which also contain RNA? The answer is evident from the finding that when a total viral lysate is chromatographed on phosphocellulose, a peak of RNase H activity free of reverse transcriptase is ob-

tained in the unbound flow-through fractions beside the RNase H associated with the reverse transcriptase that binds to phosphocellulose (21). When extracts of viral cores are chromatographed on phosphocellulose, only the RNase H associated with reverse transcriptase is found. The RNase H that is separable from reverse transcriptase probably accounts for the activity measured in virus lysates. This interpretation is consistent with the reported finding that the reverse transcriptase-free RNase H is not inhibited by RNA (S. L. Marcus and M. J. Modak, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S76, p. 225). Although direct evidence to show that this second RNase H is a viral protein is not available, several published reports (4, 5, 12, 21) indicate that this reverse transcriptase-free RNase H constitutes the major RNase H activity in mammalian retroviruses. Serologically, this activity seems to be unrelated to the reverse transcriptase-associated RNase H (4, 12).

The precise biological role of RNase H in general and the viral RNase H in particular is still a subject of speculation (9, 13, 17). Since the life cycle of RNA tumor virus infection involves synthesis of a double-stranded DNA provirus from the RNA of the infecting virus, and since one of the early intermediates in this process is an RNA-DNA hybrid generated by the transcription of the viral RNA by reverse transcriptase (19), both viral and cellular RNase H may have obligatory functions in viral replication. The RNA inhibition of the RNase H activity might be part of the physiological control mechanisms operating in the infected cell. Thus, during early phases of infection when the concentration of viral RNA should presumably be high, the RNase H activity should stay inhibited and the enzyme should function mostly in the synthetic mode. When most of the RNA is converted to hybrid structures, then the degradative function may become operative, resulting in the removal of RNA strands. DNA polymerase can then synthesize the second DNA strand.

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