RIBONUCLEASE H ACTIVITY PRESENT IN PURIFIED DNA POLYMERASE FROM AVIAN MYELOBLASTOSIS VIRUS

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SUMMARY

The presence of ribonuclease H activity in the purified complex of DNA polymerase from avian myeloblastosis virus is described. Evidence includes co-chromatography of the two activities during all purification steps; the presence of ribonuclease H activity in the purified two-polypeptide complex of DNA polymerase; ion requirements for optimal activity of purified ribonuclease H are identical to those for the purified DNA polymerase; and monospecific antiserum against purified DNA polymerase neutralizes the ribonuclease H activity.

INTRODUCTION

It was recently demonstrated by Mölling, et al. (1) that avian myeloblastosis virus (AMV), a RNA tumor virus, contains a ribonuclease activity with the properties of ribonuclease H. This enzyme specifically degrades the RNA moiety of RNA·DNA hybrids (2,3). The activity appeared to be associated with the RNA-instructed DNA polymerase "reverse transcriptase" in some physical complex, since application of various fractionation techniques did not separate the respective enzymatic activities. However, as only partial purification of the activities was achieved, it was not possible to definitely conclude whether the ribonuclease H activity was an integral part of the viral DNA polymerase or separable upon further purification. Therefore, these studies were extended by examining purified AMV DNA polymerase for ribonuclease H activity. Enzymological and serological evidence is provided for the presence of ribonuclease H activity in the purified DNA polymerase complex.

MATERIAL AND METHODS

Unlabeled deoxyribonucleoside and ribonucleoside triphosphates, E. coli RNA polymerase, bovine pancreatic ribonuclease and oligodeoxythymidylate (12-18)·polyriboadenylate (oligo dT·poly rA) were products of Boehringer-Mannheim. Tritiated uridine 5'-triphosphate (36 Ci/mmole) and thymidine 5'-triphosphate (48 Ci/mmole) were purchased from New England Nuclear. Avian myeloblastosis virus was obtained from Dr. J.W. Beard, Duke University. fd DNA was a gift of Dr. Ch. Nüsslein, Max-Planck-Institute, Tübingen and M12 phage double-stranded ^{3}H-RNA was supplied by Dr. P.H. Hofschneider, Max-Planck-Institute, Munich.

The purification of AMV, extraction of virus RNA, isolation of the viral DNA polymerase and procedures for the DNA polymerase assay have been detailed elsewhere (4). Antisera against the two-polypeptide complex of AMV DNA polymerase was prepared by Watson, et al. (5). Purification of the immunoglobulin fraction (IgG) by G-200 Sephadex chromatography and assays for neutralization of DNA polymerase activity were also described (5). Protein was determined by the method of Lowry, et al. (6).

Preparation of synthetic RNA·DNA hybrid

Tritiated RNA·DNA hybrid was prepared with E. coli RNA polymerase and fd phage DNA as template using the reaction conditions of Burgess (7), with ^{3}H-UTP as the radiolabeled substrate (350 cpm/pmole). The reaction was terminated with the addition of sodium dodecyl sulfate (0.5%) and an equal volume of phenol solution (previously equilibrated with 0.01 M Tris, pH 8.2; 0.1 M NaCl; 0.001 M EDTA). After shaking 2 min at room temperature and centrifuging 5 min at 4000 rev/min

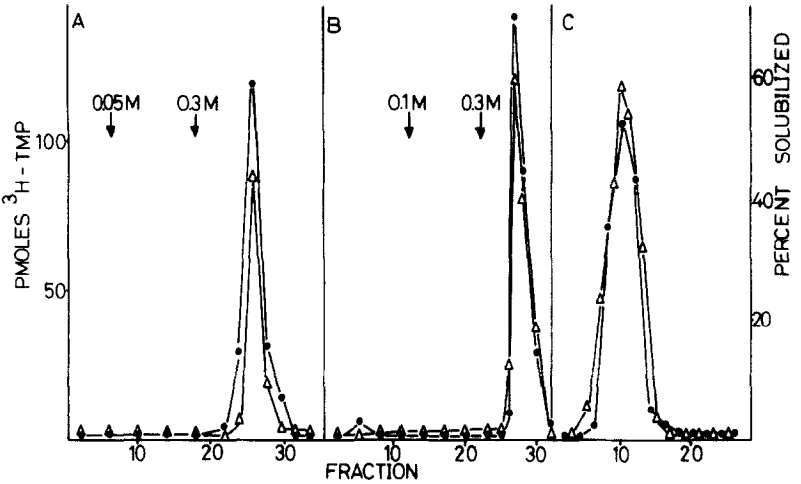
to separate the phases, the aqueous phase was chromatographed on a Sephadex G-25 (fine) column (0.9 x 55 cm) for removal of ribonucleoside triphosphates. The material eluting at the first void volume was pooled and precipitated in two volumes of ethanol and 0.1 volume of 4M NaCl at -20°. Following centrifugation, the RNA·DNA hybrid was resuspended in 10 mM Tris, pH 7.4 and stored at -20° in small aliquots. Treatment of the hybrid with bovine pancreatic ribonuclease (5 ug/ml, 15 min at 37°) resulted in 85-90% resistance; whereas, heat-denaturation of the hybrid (5 min, 80°) followed by quick cooling and ribonuclease treatment resulted in 98% sensitivity.

Assay for ribonuclease H activity

Standard reaction mixtures (100 µl) contained the following components: 50 mM Tris·HCl, pH 7.4; 8 mM $MgCl_2$; 100 mM KCl; 1 mM dithiothreitol; ^{3}H-RNA·DNA hybrid (2-4 x 10^3 cpm); and 0.5-1.0 ug of enzyme protein. After 15 min at 37°, the reaction was terminated at 0° with the addition of 0.5 ml trichloroacetic acid mixture (equal volume mixture of 100% trichloroacetic acid solution, saturated sodium orthophosphate and saturated sodium pyrophosphate). The acid-precipitable radioactivity was collected on nitrocellulose filters, dried, and the radioactivity determined in a liquid scintillation counter. Control reactions were terminated at zero time. Ribonuclease H activity is expressed as the percent ^{3}H-RNA from the RNA·DNA hybrid converted to a trichloroacetic acid-soluble form.

RESULTS

Since ribonuclease H and DNA polymerase activities from AMV were previously shown to be inseparable by various fractionation techniques (1), it was of initial interest to monitor ribonuclease H activity during purification of DNA polymerase.



(Fig. 1) Analysis of DNA polymerase and ribonuclease H activities during purification. DNA polymerase from AMV was purified and the enzyme activity monitored as previously described (4) utilizing oligo dT·poly rA (0.5 ug) as template-primer and ^{3}H-TTP as the labeled substrate (specific activity: 230 cpm/pmole). The ribonuclease H assay was performed as described in Methods. (A) DEAE-cellulose chromatography: chromatography was performed with potassium phosphate buffer, pH 7.2 at the indicated concentrations. One ul of the indicated fractions (2 ml) was tested for DNA polymerase activity and 10 ul was used for detection of ribonuclease H activity. (B) CM-Sephadex chromatography: chromatography was performed with potassium phosphate buffer, pH 8.0 at the indicated concentrations. One ul of the indicated fractions (1 ml) was tested for DNA polymerase activity and 10 ul was used for detection of ribonuclease H activity. (C) Glycerol gradient centrifugation: A pool of the activity from the CM-Sephadex column was concentrated by ammonium sulfate precipitation (50% of saturation) and layered over a preformed 10-30% glycerol gradient (5 ml) in 0.3 M potassium phosphate (pH 8.0), 1 mM dithiothreitol. After 22 hr at 50,000 rev/min in a Spinco SW 50.1 rotor (2°), fractions were collected from the bottom of the tube by needle puncture. One ul aliquots were taken for detection of DNA polymerase activity and 2 ul aliquots were used for the ribonuclease H assay. DNA polymerase activity is indicated by closed circles and the ribonuclease H activity by open triangles.

Following the procedure described by Kacian, et al. (4), both DNA polymerase and ribonuclease H activities were examined at each step of purification. Figure 1 summarizes the results. Throughout the fractionation, both activities co-chromatographed. Furthermore, sodium dodecyl sulfate-polyacrylamide gel elec-

trophoresis analysis of the enzymatic activity following the final step of velocity gradient centrifugation (Fig 1c), indicated that only the two-polypeptide complex previously described (4) was present. These results strongly suggest that the ribonuclease H activity resides in the DNA polymerase complex.

Employing the purified enzyme fraction from the final step of purification, properties of the DNA polymerase-associated ribonuclease H activity were examined. The ion requirements for optimal ribonuclease activity were found to be 8 mM Mg^{++}, 100 mM KCl and 1 mM dithiothreitol, with complete dependence on a divalent cation (Mg^{++}) and sulfhydryl-reducing agent (dithiothreitol) (Table 1). These conditions are identical to those for the purified DNA polymerase (4,8). Ribonuclease H activity was independent of the four deoxyribonucleoside triphosphates under the described assay conditions. Heat denaturation of RNA·DNA hybrid, which gives rise to single-stranded nucleic acid species, resulted in no degradation of RNA by ribonuclease H action (Table 1). To extend the specificity of ribonuclease H for RNA·DNA hybrid, M12 phage double-stranded RNA was added to a ribo-ribonuclease H assay and found to be completely resistant. Therefore, only the RNA of RNA·DNA hybrids is subject to nucleolytic attack. As shown in Figure 2, the rate of degradation of the RNA moiety of RNA·DNA hybrids was dependent upon enzyme concentration, and the extent of degradation was proportional to the time of incubation (Fig. 3).

Additional support for the presence of ribonuclease H activity in the DNA polymerase complex is provided by the neutralization of ribonuclease H activity with monospecific antiserum against the purified DNA polymerase (5). Figure 4 illustrates the equally effective manner in which increasing amounts

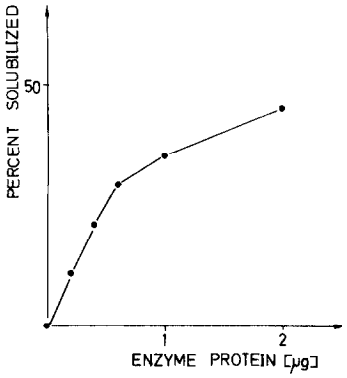


Fig. 2.

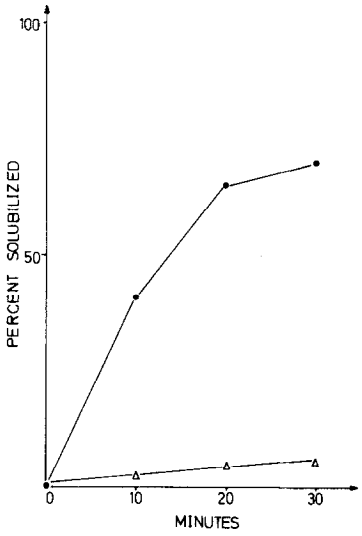


Fig. 3.

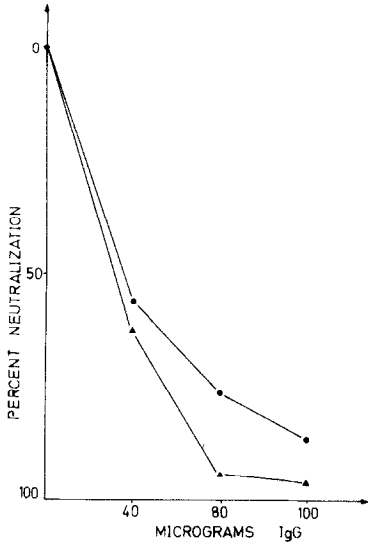


Fig. 4.

(Fig. 2) Response of ribonuclease H activity to increasing DNA polymerase protein. Standard ribonuclease H assay mixtures were incubated with increasing amounts of purified DNA polymerase protein. Ribonuclease H activity is expressed as the percent of ^{3}H-RNA converted to a trichloroacetic acid-soluble form.

(Fig. 3) Kinetics of ribonuclease H activity. Two standard ribonuclease H reaction mixtures (200 ul) were incubated at 37^{o} containing 2 ug of purified DNA polymerase, and 50 ul aliquots were removed at the indicated times, One mixture (closed circles) contained synthetic fd DNA·RNA hybrid and the control mixture (open triangles) contained heat-denatured fd DNA·RNA hybrid. The results are expressed as the percent ^{3}H-RNA solubilized.

of anti-DNA polymerase IgG protein inhibit the ribonuclease H and DNA polymerase activities.

DISCUSSION

The highly purified DNA polymerase complex from AMV has been shown to contain not only a DNA polymerizing capacity with various RNA and DNA templates (4), but also a nucleolytic activity previously reported to be present in a partially purified enzyme preparation (1).

Our results demonstrate that ribonuclease H activity co-chromatographs with the DNA polymerase activity at all stages of purification (Fig. 1). At the final step, analysis of the enzyme fraction by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed only the two-polypeptide complex, which was shown previously to represent the DNA polymerase (4). Keller and Crouch recently reported similar observations (9) when purifying the DNA polymerase essentially by the procedure of Kacian, et al. (4). Using slightly different fractionation techniques, Baltimore and Smoler report the presence of ribonuclease H activity with the DNA polymerase at their final step of purification (10), whereas Leis and Hurwitz find that the activities do not always coincide during their isolation procedure (personal communication).

Not only was it observed that the ribonuclease H and DNA

(Fig. 4) Neutralization of ribonuclease H activity by monospecific anti-AMV DNA polymerase IgG. Neutralization assays were performed as outlined previously (5). One ug of purified enzyme and increasing amounts of anti-AMV DNA polymerase IgG were added to the incubation mixtures. For determination of unneutralized DNA polymerase activity, oligo dT·poly rA (0.4 ug) was utilized as template and ^{3}H-TTP was the labeled substrate with a specific activity of 500 cpm/pmole. To determine neutralization of ribonuclease H activity, a standard assay was performed as described in Methods, following preincubation of enzyme and IgG protein.

Table 1. Properties of ribonuclease H activity in purified DNA polymerase complex

Reaction Conditions	Percent ^{3}H-RNA solubilized
Experiment 1	
Complete	64
-Mg^{++}	0
-Dithiothreitol	0
Experiment 2	
Plus RNA·DNA hybrid	55
Plus heat-denatured RNA·DNA hybrid	5
Plus double-stranded RNA	2

In Experiment 1, standard ribonuclease H reaction mixtures, containing 1 ug of enzyme protein, were prepared as described in Methods. In Experiment 2, the control reaction (plus RNA·DNA hybrid) was a standard ribonuclease H reaction. For heat denaturation, RNA·DNA hybrid was treated 3 min at 100° in a sealed ampule, with quick cooling, prior to addition to the assay mixture. ^{3}H-double-stranded M12 phage RNA (4×10^{3} cpm) was added as RNA·RNA duplex. This double-stranded RNA was 100% resistant to bovine pancreatic ribonuclease treatment (5 ug/ml, 15 min, 37°).

polymerase activities chromatographed together, but also the optimal ion requirements for purified ribonuclease H were found to be identical to those for the purified DNA polymerase activity. These results are in general agreement with those of others (9,10, Leis and Hurwitz, personal communication).

Perhaps, the most convincing evidence that the ribonuclease H activity resides in the same protein complex as the DNA polymerase comes from the neutralization of ribonuclease H with the IgG protein of antiserum against AMV DNA polymerase (Fig. 4). This antiserum was previously shown to be specific for DNA polymerase as there was no inhibition of DNA polymerase activity by antisera against the seven major virion components isolated by guanidine hydrochloride column chromatography. In

addition, the virion components did not react with the DNA polymerase antiserum in immunodiffusion tests (5).

Therefore, by biochemical and serological criteria, ribonuclease H activity has been demonstrated to be an integral part of the two-polypeptide complex of AMV DNA polymerase. Of obvious interest is to correlate the individual enzyme functions with the two polypeptides. This may contribute to elucidation of the virus replication mechanism.

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