RNA-DEPENDENT DNA POLYMERASE AND RIBONUCLEASE H FROM FRIEND VIRIONS

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1. Introduction

It has been shown that DNA polymerases of various RNA tumor viruses have Ribonuclease H (RNase H) activity associated with them [1–7], which specifically degrades the RNA strand of a RNA–DNA hybrid [8]. The two enzyme activities could not be separated by a variety of fractionation techniques, indicating that both activities might be an integral part of the viral enzyme. There are, however, conflicting reports as to whether or not RNase H is present in murine RNA tumor viruses [5,9].

In this report we briefly describe our results obtained with the murine Friend virus complex (spleen focus forming virus–lymphatic leukemia virus, SFFV–LLV). Both DNA polymerase and RNase H enzyme activities were present in Friend virions. As with the enzyme complexes from the other viruses the two activities could not be separated from one another. For every 70 nucleotides incorporated by the purified DNA polymerase one nucleotide is released by RNase H. This ratio remained constant throughout the purification.

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2. Materials and methods

Synthetic poly rA-$(dT)_{10}$ was purchased from Boehringer, Mannheim, Germany. Poly dT was obtained from Miles Laboratories. Radioactive nucleotide triphosphates were bought from Amersham, England.

2.1. *Virion isolation*

Friend virions were isolated from cell culture medium of a chronically-infected mouse spleen cell line FSD-1 [10] by ammonium sulphate precipitation at 50% saturation, followed by centrifugation through a linear sucrose gradient as described [11]. Material banding at a density of 1.16 g/cm^3 was used for isolation of DNA polymerase and RNase H. Enzyme assays: Poly rA- oligo dT directed DNA polymerizing activity was assayed for 15 min at 37°C in a 0.05 ml test volume containing the following reaction mixture: 20 mM Tris–HCl, pH 8.2; 5 mM dithiothreitol (DTT); 5 mM Mg-acetate; 0.5 mM Mn-acetate; 50 mM KCl; 0.02% Triton X-100; 100 μg/ml poly rA-$(dT)_{10}$; 10^{-5} M dTTP; [^{3}H]TTP (300 cpm/p mole). When activated calf thymus DNA was used as a template 0.1 mM each of dATP, dCTP, dGTP were added additionally and Mn-acetate was omitted. The amount of polymer formed was measured by binding to Whatman DEAE-cellulose (DE

81) paper discs [12]. The RNase H activity was assayed as described [1,8], except that 0.4 mM Mn-acetate and 5 mM DTT were used. Each reaction mixture contained 7 × 10^3 cpm synthetic [^{3}H] poly rA-poly dT hybrid.

Preparation of [^{3}H] -poly rA-poly dT: Synthetic poly dT-[^{3}H] -poly rA hybrid was prepared according to the methods of Grandgenett et al. [5] and Hausen et al. [8].

2.2. *DEAE- and phosphocellulose column chromatography*

DE 32 and P11 (Whatman) were equilibrated in: 0.05 M Tris–HCl, pH 8.0; 5 mM DTT; 0.02% Triton X-100 and 20% glycerol (v/v) (standard buffer). Samples were eluted from columns (0.9 × 10.0 cm) with 60 ml linear gradients from 0.05–0.5 M KCl in standard buffer. Glycerol gradient centrifugation: Conditions were used as described by Grandgenett et al. [6].

2.3. *SDS-polyacrylamide gel electrophoresis*

Microgel electrophoresis on polyacrylamide gradient gels (1–40%) was carried out following the procedure of Rüchel et al. [13].

3. Results

Spleen focus forming virus-transformed erythroleukemic FSD-1 cells [10] were stimulated with 1.5% dimethylsulfoxide (DMSO) for two days. The cell-free supernatant, the source of Friend virions, was prefractionated by ammonium sulphate precipitation and discontinuous sucrose gradient centrifugation [11]. The resulting crude virus preparation was further purified by sucrose density gradient centrifugation as shown in fig. 1. The DNA-polymerase activity was tested across the gradient by incorporation

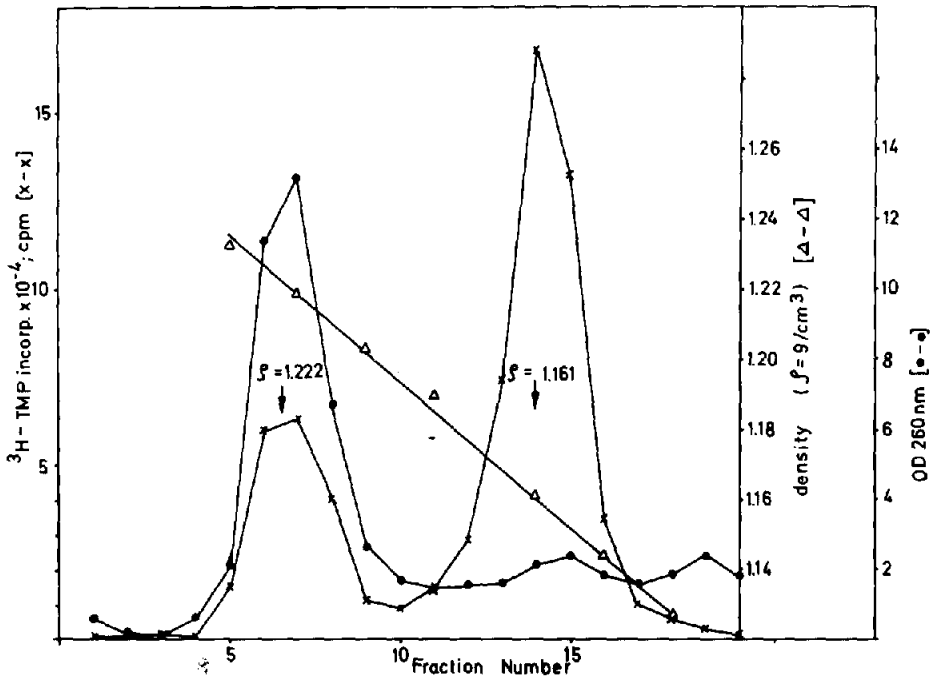

Fig. 1. Sucrose gradient centrifugation of a Friend virus preparation in a linear 20–60% gradient containing: 0.01 M Tris–HCl, pH 7.4; 0.05 M NaCl; 0.001 M EDTA. After 24 hr at 27 000 rpm in a Spinco SW 27 rotor at 4°C, fractions were collected by piercing the bottom of the tube. The OD_{260} was monitored (•——•). To destroy the virus particles, an aliquot of each fraction was incubated with 0.05 M Tris–HCl, pH 8.2, 0.01 M DDT and 0.3% of the detergent NP 40 at 37°C for 15 min followed by ultrasonication (Branson Sonifier, B 12, Danbury, Connecticut, microtip, maximal output, 3 times 15 sec at 0°C). Incorporation of ^{3}H- labelled TMP into polymer with poly rA-oligo dT was assayed using 10 μl aliquots as described in Materials and methods (x——x).

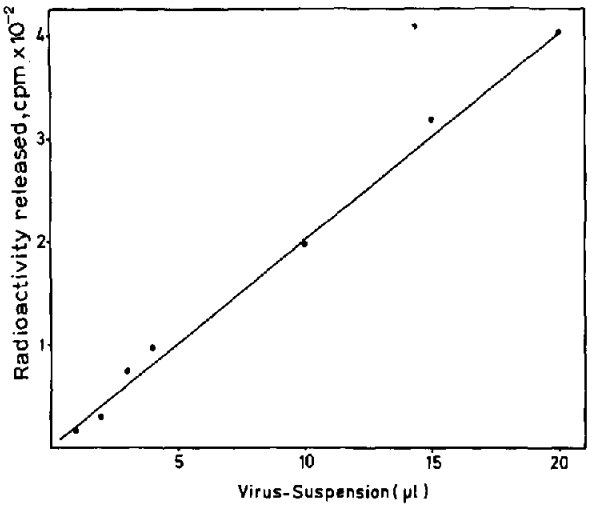

Fig. 2. RNase H activity in Friend virus. A purified virus suspension was disrupted as described in the legend of fig. 1. Increasing amounts of disrupted virus suspension were assayed containing the following ingredients in 0.1 ml: 0.1 M $(NH_4)_2SO_4$; 0.03 M Tris–HCl, pH 7.8; 0.4 mM Mn-acetate and [^{3}H]-poly rA-poly dT (7 × 10^3 cpm). After 10 min at 37°C, the reaction was stopped by the addition of 200 µg carrier RNA (yeast) and 10% ice-cold trichloroacetic acid final concentration. The released radioactivity was measured in the supernatant after centrifugation at 8 000 *g* for 5 min.

of [^{3}H]TMP into polymer with the synthetic template poly rA-oligo dT.

There are two peaks of activity in the density regions of 1.16 and 1.22 g/cm^3. The heavier peak with a density of 1.22 g/cm^3 is only observed after DMSO-stimulation. Its nature is unknown. FSD-1 cells grown in cell culture medium without DMSO produce only virus particles with a density of 1.16 g/cm^3. In the spleen focus forming assay [14] transforming activity can only be found in the density region of 1.16 g/cm^3. The material with a density of 1.16 g/cm^3 was therefore used for isolation of the enzyme activities.

The presence of RNase H activity in Friend virions is shown in fig. 2. Purified virus particles were disrup-

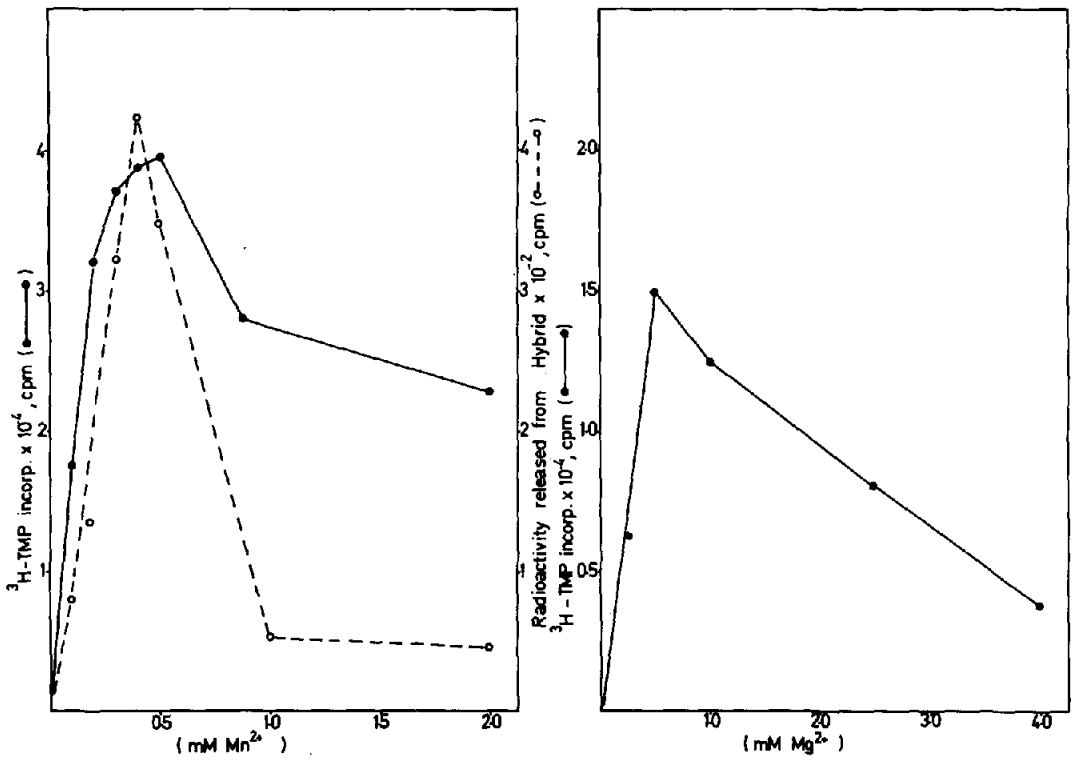

Fig. 3. Dependence of Friend virus DNA polymerase and RNase H activity on divalent cation concentrations. The experiment was carried out as described in Materials and methods with increasing concentrations of manganese (o——o) and magnesium (●——●).

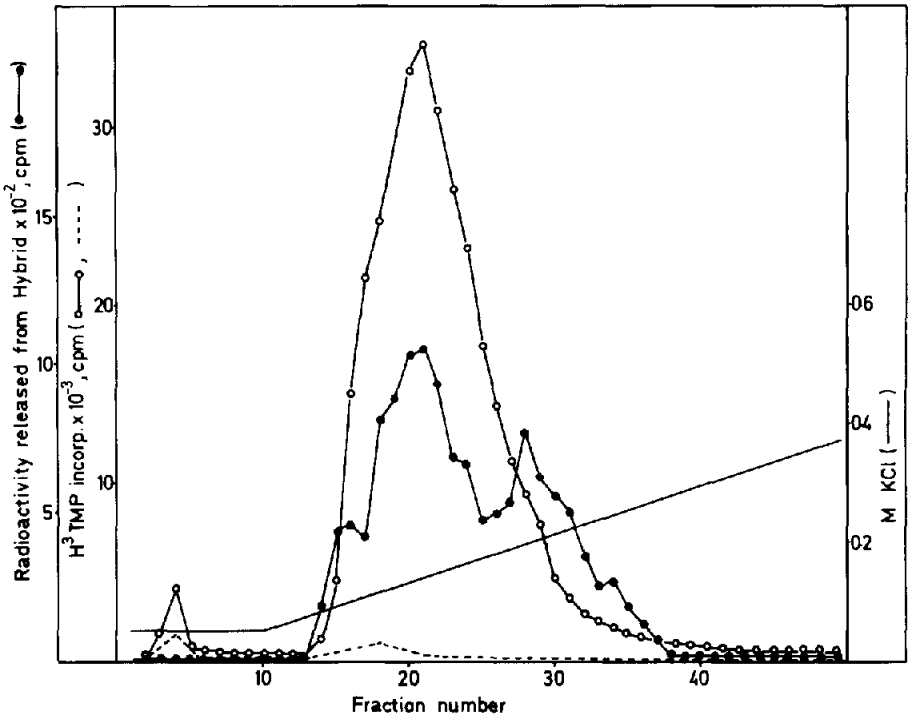

Fig. 4. Chromatography of the Friend virus enzymes on DEAE-cellulose. Aliquots of the fractions were assayed with poly rA-oligo dT (o——o) and activated calf thymus DNA (– – –) for DNA polymerase and for RNase H with [^{3}H] -poly rA-poly dT (●——●).

ted with the nonionic detergent Nonidet P40. Increasing amounts of the disrupted virus preparation were assayed. A linear relationship is observed between the amount of virus and the amount of radioactive nucleotides released from the RNA–DNA hybrid. When [^{3}H]-poly rA-poly dT was treated with pancreatic RNase (5 μg/ml, 15 min, 37°C) in an identical reaction mixture, no radioactivity was released. We conclude, therefore, that the released radioactivity is due to the action of RNase H present in the virions.

The optimum conditions for DNA polymerase and RNase H were investigated. We found a pH-optimum between pH 8.0–8.3. The monovalent cation optimum lies within a broad range of 20–200 mM. The enzyme activities show a strict requirement for divalent cations, however, as shown in fig. 3. For both enzyme reactions we obtained optimal reaction rates at 0.3–0.5 mM manganese and 5 mM magnesium concentrations. It should be noted, however, that when the assay mixture contains only magnesium ions only little RNase H activity can be detected. A complete dependence on divalent cations and sulfhydryl-reducing agent is observed.

Since DNA polymerase and RNase H of avian myeloblastosis virus (AMV) were shown to be inseparable [1–7], we have followed the RNase H activity during the purification of DNA polymerase. The purification procedure includes ammonium sulphate precipitation, DEAE- and phosphocellulose column chromatography, and glycerol gradient centrifugation. Partial purification has been reported [15]. To solubilize the enzymes, purified virus particles were treated with the detergent Nonidet P 40, followed by ultrasonication. The enzymes were precipitated with ammonium sulphate between 20% and 50% saturation. After dialysis against standard buffer the sample was adsorbed onto DEAE-cellulose and eluted with a linear 0.05–0.5 M KCl gradient in standard buffer. The two activities eluted at 0.15 M KCl, shown in fig. 4.

There is always a trailing shoulder of activities. The

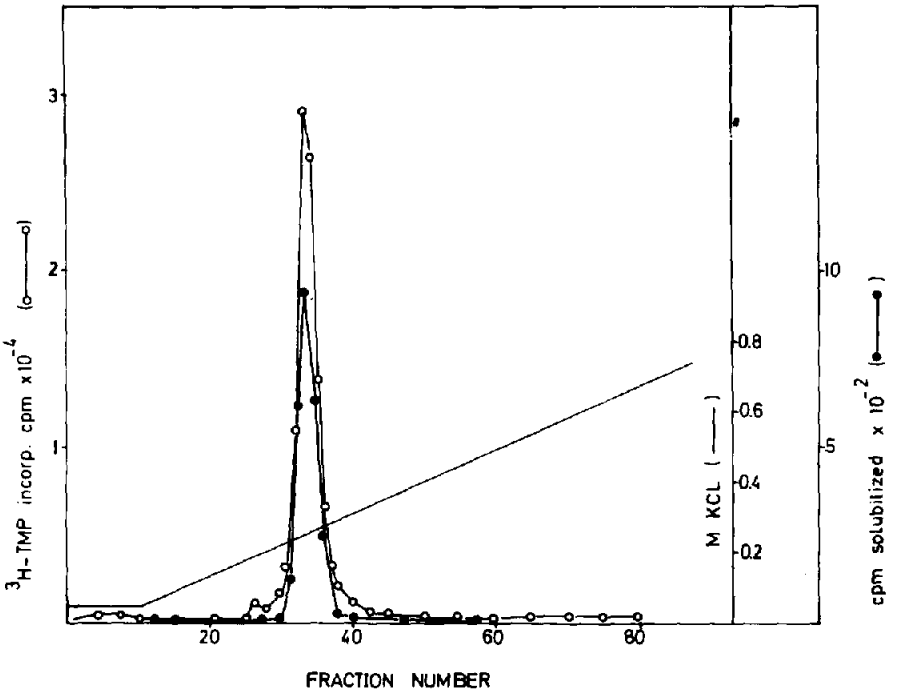

Fig. 5. Phosphocellulose column chromatography of Friend virus DNA polymerase. Aliquots of the fractions were assayed for DNA polymerase with poly rA-oligo dT (o——o) and for RNase H with [^{3}H] -poly rA-poly dT (•——•) as described in Materials and methods.

ratio of DNA polymerase to RNase H in the main peak is about 70:1. This ratio is changed in the trailing peak to 25:1. Chromatography on phosphocellulose of the main peak (fraction 16–25) from DEAE-cellulose column with a linear 0.05–0.8 M KCl salt gradient in standard buffer gave a symmetrical peak of DNA polymerase, which eluted at a salt concentration of 0.3 M KCl. The RNase H activity co-eluted at the same salt concentration (fig. 5).

Further purification by glycerol gradient centrifugation of the top fraction of the phosphocellulose peak is illustrated in fig. 6. The viral DNA polymera-

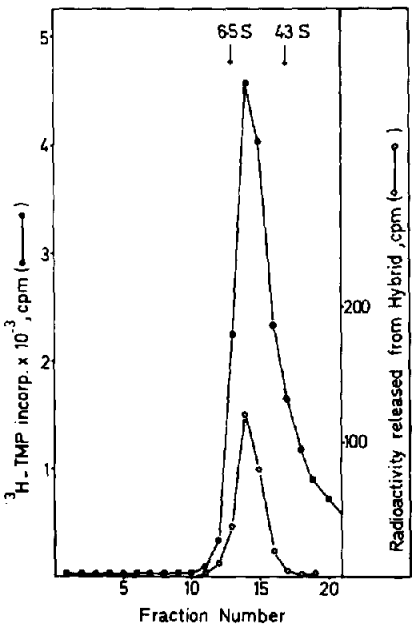

Fig. 6. Glycerol gradient centrifugation of the top fraction from the phosphocellulose column of fig. 5. The enzyme was centrifuged in a linear 20–40% glycerol gradient containing; 0.05 M Tris–HCl, pH 8.0; 0.35 M KCl; 1 mM DTT and 0.2% Triton X-100 for 15 hr at 60 000 rpm at 2°C in a SW 65 Spinco rotor. Gamma-globulin (6.5 S) and hemoglobin (4.3 S) were run in parallel tubes as markers. Samples were assayed for DNA polymerase with poly rA-oligo dT (•——•) and for RNase H with [^{3}H]-poly rA-poly dT (o——o).

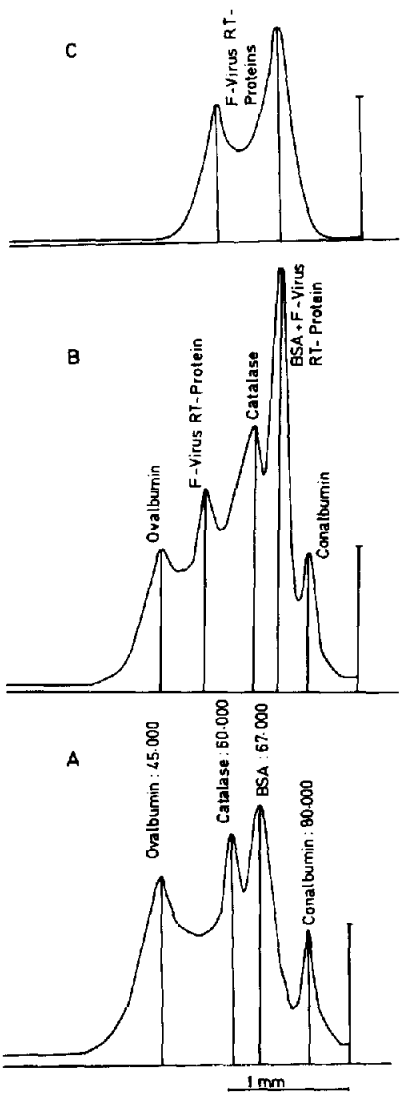

Fig. 7. Densitometric tracing of 1–40% polyacrylamide microgel gradients. Electrophoresis was carried out with the following discontinuous buffer system: Gel.buffer: 0.375 M Tris–H_2SO_4, pH 8.4; Running buffer: 0.05 M Tris–glycine, pH 8.4; 0.1% SDS. Prior to the run, samples and markers were treated for 1 min at 100°C with a solution containing 1% SDS and 0.5% β-mercaptoethanol, then applied to 5 μl gels. The gels were run for 60 min at 60 V/gel. (A) marker proteins, (B) marker proteins plus sample, (C) sample.

se-RNase H complex sedimented as a single component with an estimated sedimentation coefficient of 5.7 S. Based on this S value, using the formula (SW 1/SW 2) = (MW 1/MW 2) $^{2}/^{3}$ [16] and gamma-globulin as a standard (6.5 S, mol. wt. = 150 000), we calculate the molecular weight for Friend virus DNA polymerase to be 123 000.

Upon further analysis by SDS-microgel electrophoresis on polyacrylamide gradient gels with a discontinuous buffer system, the purified enzyme preparation from the glycerol gradient gave two protein bands with molecular weights of about 51 000 and 67 000. In fig. 7A a densitogram of a set of marker proteins is shown. In fig. 7B the same marker proteins are run together with the sample, wheras fig. 7C chows only the sample. As can be seen, one band is superimposed on bovine serum albumin, while the other shows up between the catalase and ovalbumin markers.

The enzyme after phosphocellulose column chromatography was practically free of single-stranded RNA degrading RNase. If purified 18 S ^{32}P-labelled RNA from FSD-1 cells was incubated for 15 min at 37°C with the enzyme, there was no detectable degradation as determined by radioautography after polyacrylamide gel electrophoresis. Furthermore, testing the enzyme under the same conditions as used for the RNase H assay, no degradation of [^{3}H]-poly U was found.

Based on published data [9] we find in the Friend virus complex 3–5 times less RNase H than in avian viruses (PR RSV-B, PR RSV-C), but 2–3 times more than in Kirsten murine sarcoma–leukemia viruses (Ki-MSV(MLV)). In the Friend virus preparations the ratio of DNA polymerase to RNase H is about 45:1. After ammonium sulphate precipitation between 20–50% saturation this ratio is changed to about 70:1 and then stays constant throughout the remaining purification steps. There also exists activity in the 55–90% ammonium sulphate fraction, but here the DNA polymerase to RNase H activity is 5:1. We ascribe this activity to a contaminant of cellular origin, but we have not further investigated this fraction.

The template specificity of the Friend virus DNA polymerase purified on phosphocellulose is shown in table 1. The most efficient template-primer is poly rA-$(dT)_{10}$. Poly rC-oligo dG is much less effective,

Table 1
Template specificity of Friend Virus DNA polymerase

Template	p moles [^{3}H]TMP incorp.
poly rA $(dT)_{10}$	72
$(dT)_{12-18}$	< 0.1
poly d(A-T)	0.94
poly dA- poly dT	< 0.1
oligo dA-oligo dT	< 0.1
poly rC- oligo dG	8.7
CT - DNA	< 0.1
act. CT - DNA	1.05
F. Virus RNA	0.21

Various polymers were used to assay the Friend virus DNA polymerase purified on phosphocellulose. The polymer concentrations were: poly rA $(dT)_{10}$, poly rC-oligo dG 100 μg/ml, dT_{12-18}: 20 μg/ml, poly d(A-T): 30 μg/ml, poly dA-poly dT: 100 μg/ml, oligo dA-oligo dT: 30 μg/ml, activated calf thymus DNA: 100 μg/ml, native calf thymus DNA: 100 μg/ml, Friend virus RNA: 100 μg/ml.

in agreement with data reported for DNA polymerase of Moloney leukemia virus (MLV) [17].

4. Discussion

Mölling et al. [1] have reported that DNA polymerase found in avian myeloblastosis virus (AMV) is associated with ribonuclease H. They proposed a model for the involvement of this nuclease in RNA directed DNA synthesis by the AMV DNA polymerase. Later reports [2–7] are in agreement with their data. There are, however, conflicting reports as to whether or not RNase H is also associated with murine RNA tumor virus DNA polymerase [5,9]. Our results show that the Friend virus complex contains both DNA polymerase and RNase H activity. Both enzyme activities have a very narrow optimal divalent cation requirement (0.3–0.5 mM Mn^{2+} and 5 mM Mg^{2+}), different from that of AMV. Furthermore, throughout the purification steps such as ammonium sulphate precipitation, DEAE- and phosphocellulose column chromatography and glycerol gradient centrifugation, the enzyme activities are not separable. In purified virus preparations the ratio of DNA polymerase to RNase H is about 45:1, but changes after ammonium sulphate precipitation to about 70:1. The ratio then stays constant during the remainder of the purification, indicating that there is no preferential loss of one activity. The results suggest that both enzyme activities reside in one enzyme complex. However, upon analysis on SDS-polyacrylamide gradient gels we find two polypeptide chains. We have no experimental evidence to prove that both enzyme activities reside in one polypeptide chain as reported by Grandgenett et al. [6] for the AMV enzyme, or whether the different activities are attributable to different chains. We are attempting to solve this question.

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