

REVERSE TRANSCRIPTASE OF AN ONCORNAVIRUS-LIKE PARTICLE DERIVED FROM HUMAN HeLa CELLS

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Abstract

Oncornavirus-like particles have been observed and isolated from HeLa cells that contain a DNA polymerase activity with properties similar to RNA tumor virus reverse transcriptase. The virions have a density of 1.16–1.17 g/cm³ and contain nucleic acid species that sediment as RNA (1.68 g/ml) in a Cs₂SO₄ equilibrium density gradient. Antigenically the HeLa virus DNA polymerase is related to the Mason-Pfizer monkey virus DNA polymerase.

The presence of virus-like particles, morphologically similar to those of the known RNA tumor viruses, has been recently observed in HeLa cells (1). Further electron microscopical analysis revealed maturation of an intracellular A-type particle at the cell membrane releasing a particle with properties of both B- and C-type virus particles (2). Comparative studies suggested these virus particles to be morphologically similar to the Mason-Pfizer monkey virus (MP-MV).

From these results, it was of considerable interest to examine the biochemical and serological properties of these particles which is currently under investigation. It is the purpose of this communication to report some physical and biochemical properties of the HeLa virus and a particle-associated DNA polymerase activity.

Materials and methods

Unlabelled deoxyribonucleoside triphosphates, bovine pancreatic ribonuclease and oligodeoxythymidylate (10) – polyriboadenylate [oligo (dT) – poly (rA)] were products of Boehringer-Mannheim. Tritiated thymidine 5' – triphosphate (48 Ci/mmole) was purchased from New England Nuclear. Cesium sulfate was obtained from Merck and Nonidet-P40 was kindly supplied by Deutsche Shell Chemie Gesellschaft mbH. *Viruses*. HeLa virus was produced in culture from a line of HeLa cells originally obtained from Dr. W. A. K. Schmidt, Düsseldorf. Cells had been cultivated for three years in Homburg/Saar and one year in Berlin in Eagle's medium supplemented with 4–10 % heat-inactivated calf serum. Cultures were continually examined for contamination by *Mycoplasma salivarium* and were treated with anti-PPLO (Tylocine, Grand Island Biological Company).

Culture supernatants were collected every 24 hr and subjected to low speed centrifugations of 3000 x g for 15 min and 16,000 x g for 20 min before the material was pelleted by high speed centrifugation (90,000 x g, 60 min, 4 °C). The

pellets were then resuspended in TNE, pH 7.4 (0.01 M Tris, 0.1 M NaCl and 0.003 M EDTA), centrifuged 5 min at 2,000 rpm and the virus-containing supernatant was layered over a linear 20–70% (w/v) sucrose density gradient for equilibrium density centrifugation (15 hr, 4 °C, 23,000 rpm in a Spinco SW 25.1 rotor).

Mason-Pfizer monkey virus (MP-MV) was a gift of H. Daams, Amsterdam and avian myeloblastosis virus (AMV) was obtained by methods previously described (3) from the blood of chicks in the terminal stage of myeloblastic leukemia.

$^{32}\text{PO}_4$ -labelled virus was prepared by first washing HeLa cell cultures with Eagle-Dulbecco medium (Flow Laboratories) lacking phosphates (10 ml/dish). The cultures were then incubated for 20 hr at 37 °C in phosphate-free medium containing 100 $\mu\text{Ci}/\text{ml}$ of ^{32}P -orthophosphate (carrier free, Radiochemical Centre, Amersham, England) and 10 % dialyzed calf serum. $^{32}\text{PO}_4$ -labelled nucleic acid was extracted by the method of Kacian *et al.* (4) from virus banding in the region of density 1.15–1.18 g/cm³ of a sucrose equilibrium density gradient.

For DNA polymerase studies, a pool of the region of density 1.15–1.18 g/cm³ was made, diluted to 30 ml with TNE buffer (0.01 M Tris-HCL, pH 7.4, 0.1 M NaCl, 0.003 M EDTA) and centrifuged 60 min at 4° and 23,000 rpm in a Spinco SW 25.1 rotor. The concentrated pellet was resuspended in 0.5 ml of TNE buffer and stored in ice. Unless otherwise indicated this was the source of virus DNA polymerase.

Before use, the material was disrupted 15 min at 4 °C with Nonidet -P40 detergent (0.05 % for endogenous and 0.2 % for exogenous-templated reactions) and then standard reaction mixtures (100 μl) for DNA polymerase were prepared essentially as described previously. The specific activity of ^3H -TTP was 200–400 cpm/pmole for the synthetic RNA-DNA hybrid and DNA-templated reactions and 3,500 cpm/pmole for the endogenous DNA polymerase reactions.

Assays for neutralization of HeLa DNA polymerase activity by anti-MP-MV DNA polymerase IgG were carried out as described previously (5). Purified IgG fractions from normal rabbit serum (obtained prior to immunization) and rabbit anti-Mason-Pfizer monkey virus DNA polymerase serum were kindly supplied by Dr. A. YANIV, Institute of Cancer Research, Columbia University, New York, N. Y.

RESULTS

Density of the virions and detection of DNA polymerase activity.

Culture fluids of the HeLa cells were collected daily and the virus was concentrated and purified by sucrose equilibrium density centrifugation as described in Methods. Individual gradient fractions were then tested for DNA polymerase activity using the synthetic RNA-DNA hybrid, poly (rA)-oligo (dT). Figure 1 illustrates the presence of DNA polymerizing activity at a density of 1.17 g/cm³, characteristic of RNA tumor viruses. Examination of the gradient fractions by electron microscopy revealed the presence of virus-like structures only in the region of density 1.17 g/cm³, corresponding to the DNA polymerase activity.

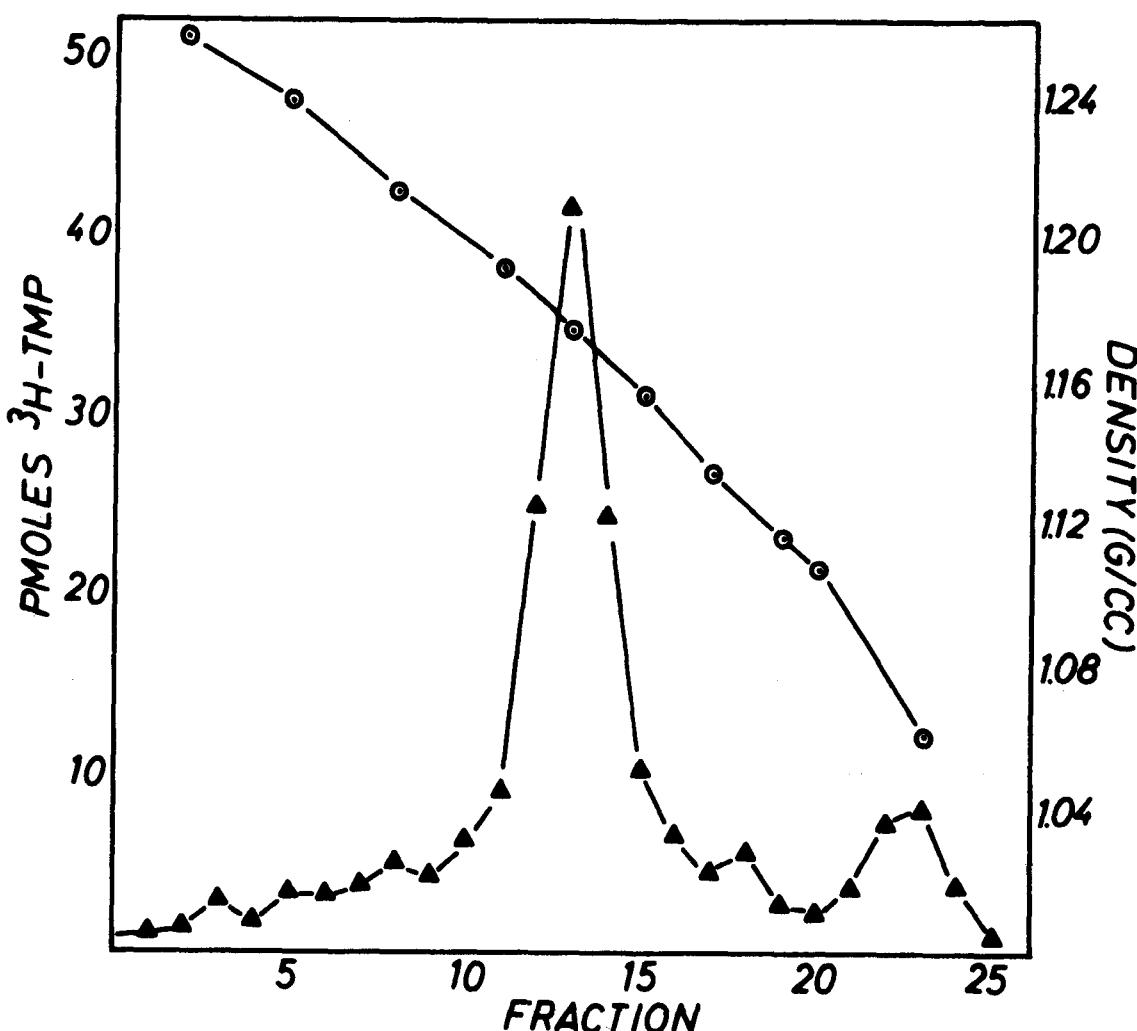


Fig. 1: Sucrose equilibrium density gradient centrifugation of HeLa virus and detection of DNA polymerase activity. HeLa virus was concentrated and subjected to sucrose gradient centrifugation as described in Methods. A 10 μ l aliquot of gradient fractions was added to standard DNA polymerase reaction mixtures containing synthetic (dT)₁₀-poly(rA) as the primertemplate and 3 H-TTP as the labelled substrate.

Conditions	Activity (pmoles 3 H-TMP)
Complete	0.31
Minus Mg ⁺⁺	0.001
Minus Mg ⁺⁺ , plus Mn ⁺⁺	0.005
Minus dATP	0.006
Minus dATP, dCTP	0.003
Minus Nonidet-P40	0.02

Table 1. Requirements of HeLa virus DNA polymerase.

Standard DNA polymerase assays were performed with omissions and additions as indicated. 3 H-TTP was the labelled substrate (specific activity, 3,500 cpm/pmole) and 10 μ l of density gradient purified virus suspension was used as the source of endogenous DNA polymerase activity.

Properties of DNA polymerase activity.

Response of the DNA polymerase to its resident nucleic acid (endogenous reaction), activated DNA and poly (rA)-oligo(dT) is illustrated in Figure 2. The kinetics of incorporation of ^3H -TMP into acidprecipitable product was linear in all cases for more than 90 min. As illustrated in Figure 3, the rate of the endogenous reaction was proportional to the amount of virus suspension added to the reaction mixture. Also pretreatment of the detergent-disrupted virus with pancreatic ribonuclease (50 $\mu\text{g}/\text{ml}$) completely inhibits incorporation of radioactive substrate into DNA product (Fig. 3), suggesting RNA as the instructive agent in this reaction.

Chemical requirements of the endogenous reaction are shown in Table 1. A nonionic detergent (Nonidet-P40) pretreatment is necessary for expression of polymerizing activity as is the presence of all four deoxyriboside triphosphates. Magnesium ions are required and manganese ions do not substitute as the divalent action.

Further evidence that the endogenous reaction is instructed by RNA is shown in Figure 4. A simultaneous detection assay (6) designed to indicate the presence of RNA-instructed DNA polymerase and high molecular weight (60–70S) viral RNA template was performed. Our results indicate DNA product sedimenting with species with estimated sedimentation coefficients of 20–30S. Analysis of the nucleic acid content of the HeLa virus could explain these results.

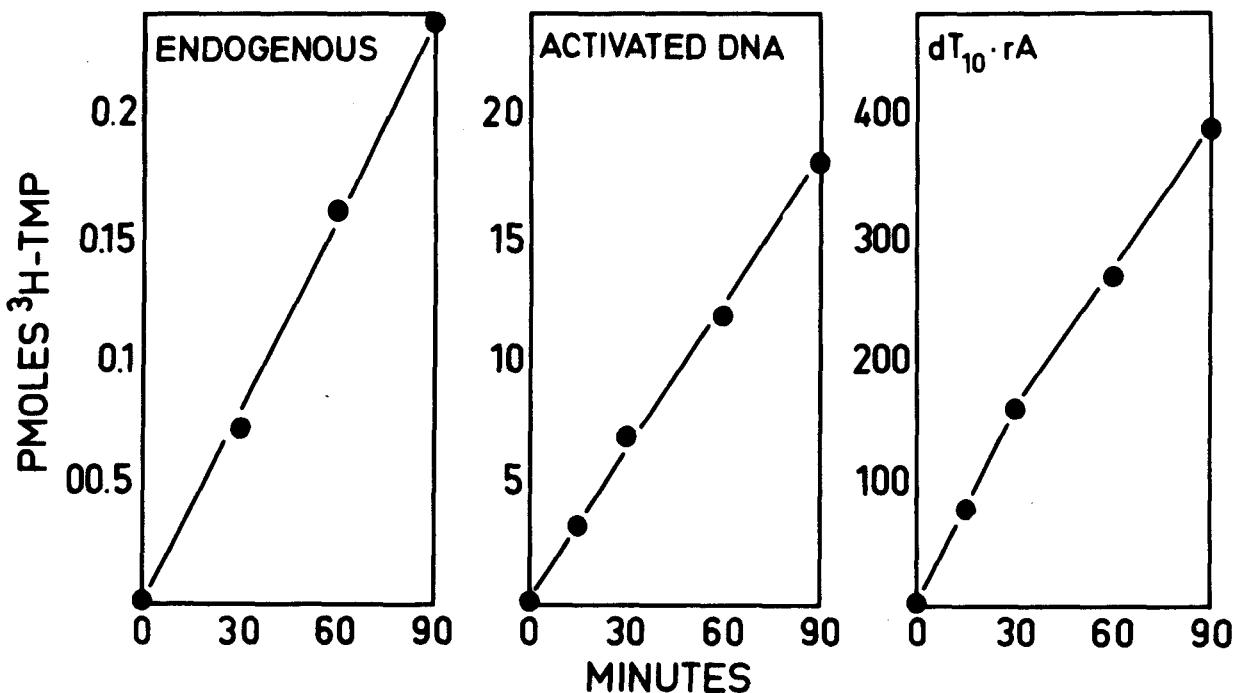


Fig. 2: Response of HeLa virus DNA polymerase to various nucleic acid templates. Standard DNA polymerase reaction mixtures (0.4 ml) were prepared and 100 μl aliquots were removed at the indicated time points. Activated calf thymus DNA, prepared by the method of Aposhian and Kornberg (12), was added at a concentration of 15 $\mu\text{g}/\text{ml}$, while $(\text{dT})_{10}$ -poly (rA) was added at a concentration of 5 $\mu\text{g}/\text{ml}$.

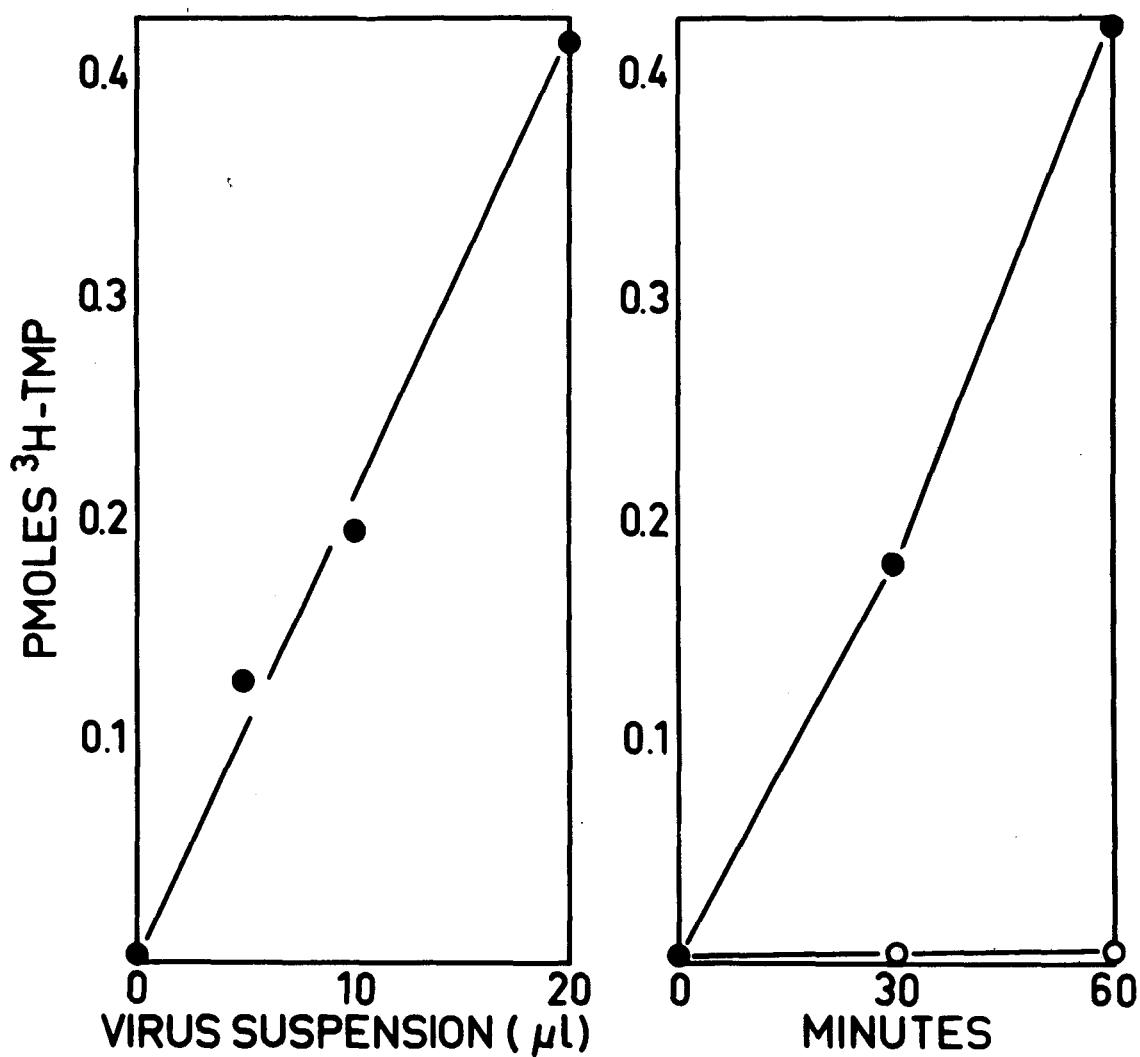


Fig. 3: Response of HeLa virus DNA polymerase activity to increasing virus concentration and to ribonuclease treatment. Standard endogenous DNA polymerase reaction mixtures were prepared with increasing amounts of virus suspension added (left figure). For sensitivity to ribonuclease (right figure), two reaction mixtures (0.3 ml) were prepared. To one, pancreatic ribonuclease (50 $\mu\text{g}/\text{ml}$ final concentration) was added, while to the other an equal volume of 0.01 M Tris, pH 8.2 was added. Both mixtures containing 30 μl of virus suspension were then disrupted 15 min at room temperature with 0.05 % Nonidet-P40. After addition of Mg^{++} and the deoxriboside triphosphates, the mixtures were incubated at 37 °C, removing 100 μl aliquots at 0, 30 and 60 min. The radioactivity was analyzed as previously described in Methods. The control is designated by the closed circles and the open circles represent the reaction containing ribonuclease.

Characterization of the viral nucleic acid.

Following labelling of HeLa cell cultures with $^{32}\text{PO}_4$ and purification of the virus through the sucrose density gradient step, nucleic acid was extracted from the density region of 1.16–1.17 g/cm³ and further analyzed by velocity gradient centrifugation. Figure 5A shows a profile containing $^{32}\text{PO}_4$ -labelled nucleic acid with sedimentation coefficients of 30S, 18S and 4–8S. Analysis by Cs_2SO_4 equilibrium

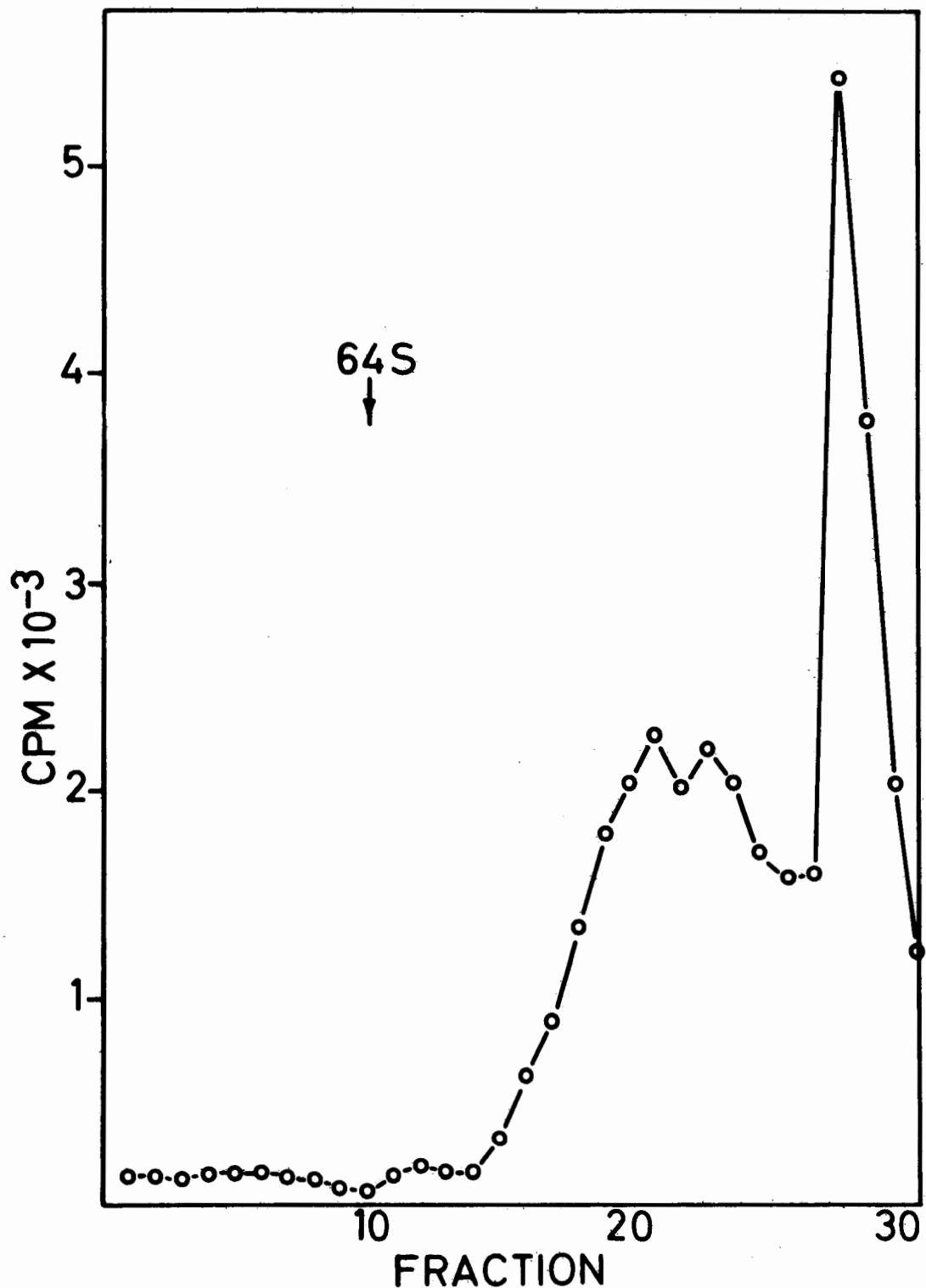


Fig. 4: Simultaneous detection of RNA-directed DNA polymerase activity and viral RNA. Following disruption of 0.1 ml of the peak activity fraction from Fig. 1 for 30 min in ice with 0.2 % Nonidet-P40 and 10 mM dithiothreitol, a standard endogenous DNA polymerase assay mixture (0.5 ml) was prepared containing $^3\text{H-TTP}$ as the labeled substrate with a specific acti-

vity of 1.9×10^4 cpm/pmole. After 30 min at 37°, the reaction was terminated with the addition of sodium dodecyl sulfate to a final concentration of 0.5 %, and an equal volume of phenol (pH 7) containing 8-hydroxyquinoline (0.1 g per 100 ml) was added. After shaking 3 min at room temperature, the mixture was centrifuged 5 min at 3,000 rpm and 20 °C. The aqueous phase was layered over a preformed linear gradient of glycerol (10–30 %) and centrifuged 90 min at 50,000 rpm, and 4 °C in a Spinco SW 50.1 rotor. Gradient fractions were collected and processed as described in Fig. 5A. AMV RNA used as an external marker being centrifuged under identical conditions.

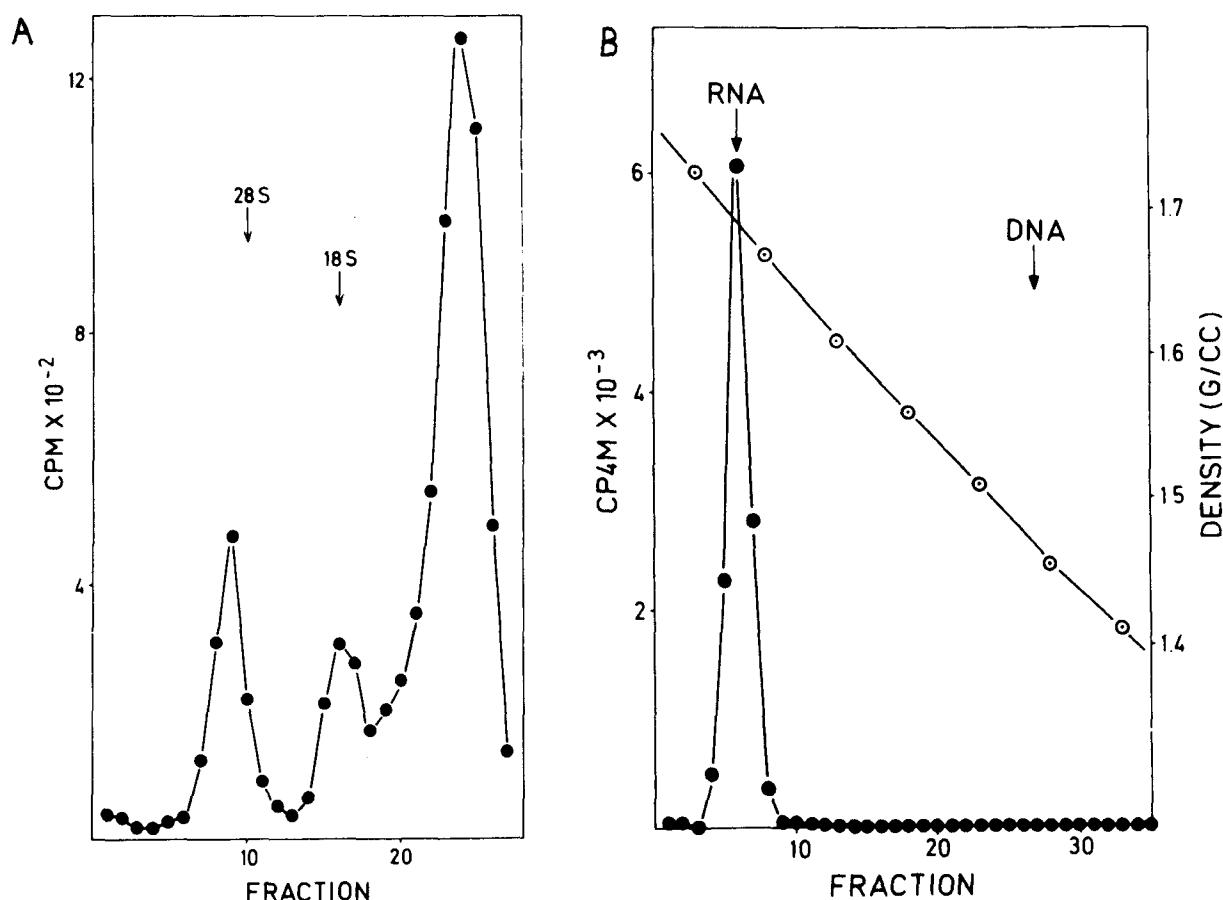


Fig. 5: (A) Sedimentation analysis of nucleic acid from HeLa virus. $^{32}\text{PO}_4$ -labelled nucleic acid was extracted from the 1.15–1.18 g/cm³ region of a sucrose equilibrium density gradient as described in Methods. The nucleic acid solution was layered over a 5 ml preformed 10–30 % glycerol gradient and centrifuged 3.5 h at 50,000 rpm and 4 °C in a Spinco SW 50.1 rotor. 12 drop fractions were collected from the bottom of the tube, 20 μl aliquots were precipitated with trichloroacetic acid and the radioactivity determined as previously described (KACIAN et al., 1971). ^3H -uridine-labeled 28S and 18S ribosomal RNAs from chick embryo fibroblasts were included as markers. (B) Cs_2SO_4 equilibrium density gradient centrifugation of ^{32}P -HeLa 30S nucleic acid. An aliquot containing 4,000 cpm was added to 2 ml of TNE buffer, pH 7.4, mixed with an equal volume of saturated Cs_2SO_4 , and centrifuged at 31,000 rpm in a Spinco SW 39 rotor for 60 h at 20 °C. Fractions were collected and processed for acid-precipitable radioactivity as described above (Fig. 5A). (^3H)-uridine-labeled 28S ribosomal RNA and 30 μg of calf thymus DNA were added as markers.

density gradient centrifugation demonstrated that each size specie had the characteristic properties of RNA. Figure 5B illustrated the density properties of the 30S specie.

Antigenic properties of HeLa virus DNA polymerase.

A technique useful for comparative studies of primate RNA tumor viruses involves serological characterization of the viral DNA polymerases and has been described (7). Because of the morphological similarities of HeLa virus with MP-MV, it was of interest to compare the antigenic properties of the MP-MV DNA polymerase and the HeLa virus DNA polymerase.

Using isolated IgG fraction from antisera produced against partially purified MP-MV DNA polymerase, our data demonstrates that MP-MV DNA polymerase and HeLa virus DNA polymerase are both inhibited to an equivalent extent by the anti-MP-MV DNA polymerase IgG. AMV DNA polymerase activity is not affected by this same IgG fraction (Fig. 6).

Discussion

Some physical and biochemical properties of a RNA tumor virus-like particle from HeLa cell culture supernatants have been described. Equilibrium density centrifugation in sucrose of this concentrated material revealed a DNA polymerase activity (Fig. 1) and the presence of RNA tumor virus-like particles at 1.17 g/cm³, characteristic of all known RNA tumor viruses.

Analysis of the endogenous DNA polymerase activity demonstrated a requirement for Mg⁺⁺ as the divalent action and the four deoxyriboside triphosphates as substrates (Table 1). Sensitivity of this reaction to pancreatic ribonuclease suggested RNA as the instructive agent for synthesis of DNA (Fig. 3).

Investigation of the endogenous DNA polymerase reaction by the simultaneous detection assay (Fig. 4) supports the presence of RNA-instructed DNA polymerase, although no 60–70S RNA specie was observed. Rather, DNA product sedimented with molecular weight species similar to those found to be present as RNA in the virions (Fig. 5). The significance of these species is not understood. However, they may represent sub-unit RNA structures known to be products of 60–70S tumor virus RNA after heat or dimethylsulfoxide treatment (8, 9).

Identity of this DNA polymerase with the R-DNA polymerase from HeLa cells (10) is unlikely, in that their primer-template, ion and temperature requirements are quite different.

In a further attempt to relate biochemical properties of this virus and its DNA polymerase activity with the RNA tumor viruses and particularly with the morphologically similar MP-MV that contains a reverse transcriptase activity (11), antigenic properties of the DNA polymerase activity of the two viruses were compared. The results indicate that both DNA polymerase activities were inhibited to an equivalent extent by anti-MP-MV DNA polymerase IgG (Fig. 6) suggesting a serological relationship between the two enzymes. AMV DNA polymerase was unaffected by the anti-MP-MV DNA polymerase IgG.

Therefore, we believe we are observing a virus containing a DNA polymerase activity characteristic of the RNA tumor viruses, particularly the MP-MV. However, to establish that we are, indeed, dealing with a functionally enzymatic viral reverse transcriptase will require back-hybridization of RNA-instructed DNA product to its homologous RNA template.

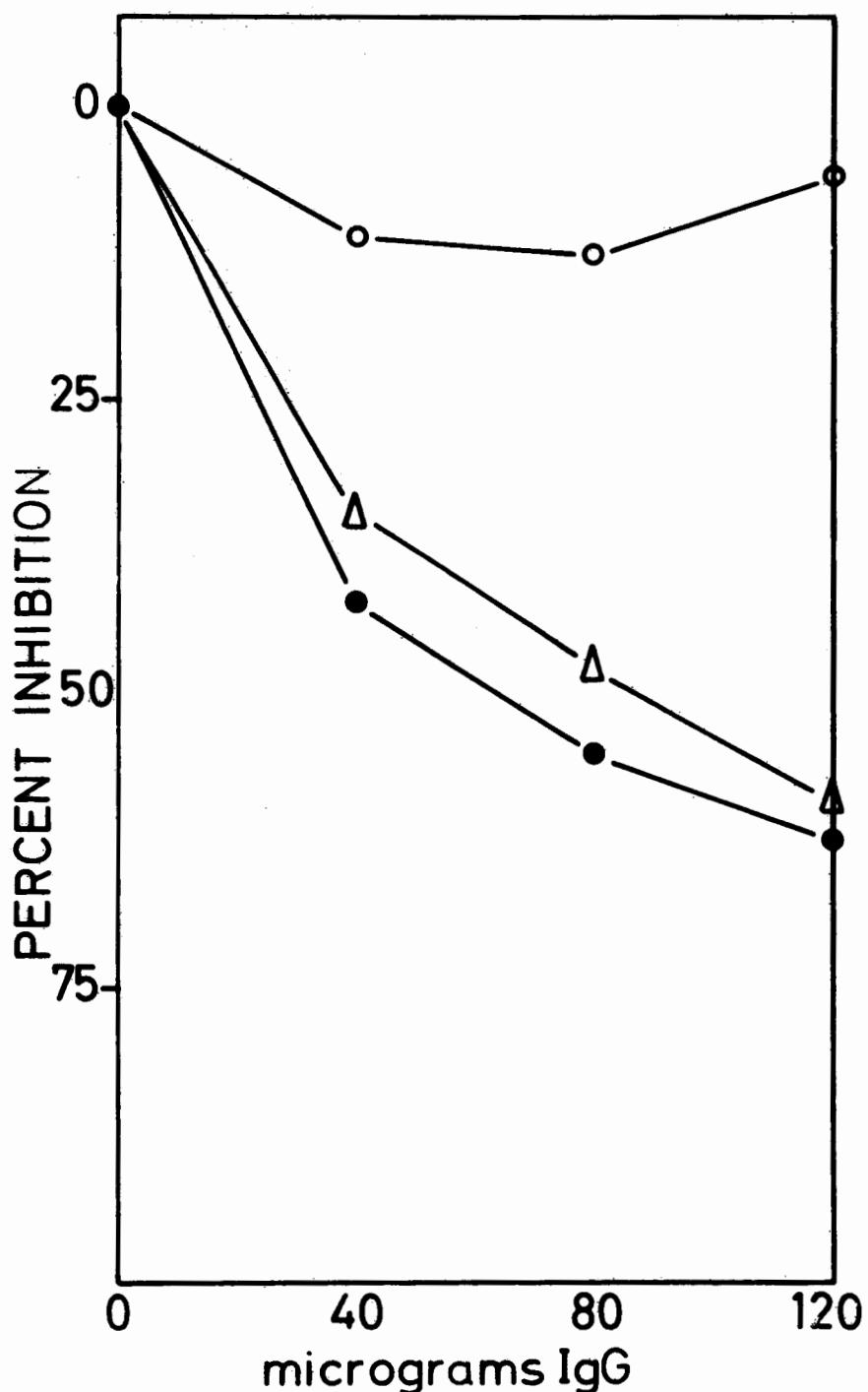


Fig. 6: Neutralization of HeLa DNA polymerase activity by a purified IgG fraction from anti-MP-MV DNA polymerase serum. Neutralization assays were carried out essentially as previously described (WATSON et al., 1972). Equivalent amounts (amount of virus suspension necessary to incorporate 1 pmole of $^{3}\text{H-TMP}/\text{min}$ with oligo(dT)-poly(rA) as primer-template) of detergent-disrupted HeLa virus, MP-MV and AMV were incubated with increasing amounts of anti-MP-MV DNA polymerase IgG and purified normal rabbit IgG as control. The results are expressed as percent inhibition by anti-Mp-MV DNA polymerase IgG compared to the same amount of control normal rabbit IgG. Neutralization of DNA polymerase from HeLa virus (closed circles), MP-MV (open triangles), AMV (open circles) is demonstrated.

As HeLa cells were derived from a human cervical carcinoma, the origin of this virus and the relationship of its genetic information to primates and their viruses are of particular interest. These studies are presently in progress.

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