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Comparison of Rous Sarcoma Virus-Specific Deoxyribonucleic Acid Polymerases in Virions of Rous Sarcoma Virus and in Rous Sarcoma Virus-Infected Chicken Cells

JOHN M. COFFIN AND HOWARD M. TEMIN
McArdle Laboratory, University of Wisconsin, Madison, Wisconsin 53706

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Labeled virions of Rous sarcoma virus (RSV) were disrupted with detergent and analyzed on equilibrium sucrose density gradients. A core fraction at a density of approximately 1.24 g/cc contained all of the ^{3}H-uridine label and about 30% of the ^{3}H-leucine label from the virions. Endogenous viral deoxyribonucleic acid (DNA) polymerase activity was only found in the same location. Additional ribonucleic acid (RNA)- and DNA-dependent DNA polymerase activities were found at the top of the gradients. RNA-dependent and DNA-dependent DNA polymerase activities were also found in RSV-converted chicken cells. Particles containing these activities were released from cells by detergent and were shown to contain viral RNA. These particles were analyzed on equilibrium sucrose density gradients and were found to have densities different from virion cores.

Virions of ribonucleic acid (RNA) tumor viruses have recently been shown to contain five enzyme activities: an RNA-dependent deoxyribonucleic acid (DNA) polymerase (3, 21), a DNA-dependent DNA polymerase (11, 16), a DNA endonuclease (11), a DNA exonuclease, and a DNA ligase (12). The presence of these enzymes supports the DNA provirus model of replication of RNA tumor viruses (17).

Treatment of various RNA tumor viruses with detergents releases a core particle of greater buoyant density than the complete virion (2, 6, 10). These core particles presumably contain the viral group-specific (gs) antigens (4).

In this communication we report experiments on the nature of the core of Rous sarcoma virus (RSV) virions and of particles in RSV-infected chicken cells which contain endogenous viral RNA-directed and exogenous DNA-directed DNA polymerase activity. A preliminary report of this work has been presented (20*a*).

MATERIALS AND METHODS

General experimental techniques and sources of virus were described previously (18, 19).

Cell cultures. Primary cultures of fibroblasts were prepared from 12-day-old white Leghorn chicken embryos (Sunnyside Hatchery Co., Oregon, Wis.) and grown in modified Eagle's medium with 20% Tryptose phosphate broth (ET medium) and 5% calf serum or 5% fetal calf serum. Secondary or later cultures of chicken cells were prepared at 5×10^5 cells per 60-mm plastic culture dish (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) or at 2×10^6 cells per 100-mm dish in ET medium with no serum. One day after preparation, cells were infected with Schmidt-Ruppin virus (SRV) or B77 virus at a multiplicity of approximately 0.2 to 0.5 focus-forming units per cell. After incubation at 37 C for 40 min, the virus inoculum was replaced with ET medium containing 5% fetal bovine serum. Two or three days after infection, the cultures were transferred to 100-mm dishes at 2×10^6 cells per dish. Except as otherwise described, extracts were made from the cultures at 10 to 14 days after infection.

Viruses. SRV and B77 virus were described previously (1). Stocks were prepared by infecting cultures of chicken embryo fibroblasts as described above and harvesting the culture fluids twice daily starting approximately 7 days after infection. Virus harvests were frozen at −70 C immediately after collection. Virus was concentrated and purified by centrifuging pooled culture fluids for 20 min at 8,000 rev/min in a Sorvall GSA rotor. The supernatant was centrifuged either 1 hr at 25,000 rev/min in a Spinco 30 rotor or 2 hr at 19,000 rev/min in a Spinco 19 rotor. The virus pellet was resuspended in approximately 2 ml of ET medium

with 5% calf serum per liter of starting material. The virus concentrates were layered on discontinuous gradients of 1 ml of 65% sucrose and 3 ml of 15% sucrose in tris(hydroxymethyl)aminomethane (Tris)-ethylenediaminetetraacetic acid (EDTA) buffer (0.01 M Tris-hydrochloride, *p*H 8.0, 0.001 M EDTA) and centrifuged 1 hr at 30,000 rev/min in a Spinco SW39 or SW50.1 rotor. The virus band was collected from the interface, diluted approximately 1:3 with Tris-EDTA buffer, layered on a continuous 15 to 65% sucrose gradient in Tris-EDTA buffer, and centrifuged 1 hr at 45,000 rev/min in a Spinco SW50.1 rotor. The band of purified virus was collected in a final volume of approximately 2 ml per liter of starting material, distributed into 0.1-ml samples, and frozen for future use. The virus preparation was sometimes treated with 200 μg of Pronase per ml for 10 min at 40 C immediately prior to the final centrifugation (11).

Labeling. The medium on chicken embryo fibroblast cultures infected 7 days previously with SRV was changed to 30 ml of Eagle's medium with 5% calf serum containing 5 μCi of uridine-5-^3H per ml (12 Ci/mM, New England Nuclear Corp., Boston, Mass.) or to 30 ml of leucine-free Eagle's medium containing 5 μCi of L-leucine-4,5-^3H per ml (58 Ci/mmole, Schwarz BioResearch, Inc., Orangeburg, N.Y.). One day later the culture fluids were removed, and virus was concentrated as described above to a final volume of approximately 1 ml and frozen in 0.1-ml samples.

Density gradient centrifugation. For preparation of virion cores, appropriate mixtures of labeled RSV and concentrated unlabeled RSV were disrupted by treatment with Tris-EDTA buffer, containing 0.25% Nonidet P-40 (Shell Chemical Co., New York, N.Y.) and 1% Dithiothreitol (DTT; Sigma Chemical Co., St. Louis, Mo.), for 5 min at 0 C and then layered on a linear sucrose gradient prepared from 70% (w/v) sucrose in deuterium oxide (Sigma Chemical Co.) and 30% sucrose in water. Both solutions contained Tris-EDTA buffer and 0.4% DTT. Centrifugation was for 2.5 hr at 45,000 rev/min in a Spinco SW50.1 rotor.

Extracts from RSV-infected chicken cells were centrifuged on similar gradients. All gradients were collected by taking 20-drop fractions through a hole in the bottom of the tube. Sedimented material was resuspended in 0.2 ml of Tris-EDTA buffer containing 0.4% DTT. Densities were determined by weighing 0.01-ml samples of selected fractions.

Extracted nucleic acid preparations were centrifuged in cesium sulfate density gradients. A 0.4-ml sample was mixed with 0.3 ml of 10× Tris-EDTA buffer and 0.6 ml of 0.5% formaldehyde and was incubated for 20 min at 25 C (H. Lozeron, *personal communication*). A 1.7-ml amount of saturated Cs_2SO_4 in water was added. Alkaline Cs_2SO_4 gradients were prepared in the same way except that the Tris-EDTA buffer was replaced with 0.15 ml of 2 N NaOH and 0.15 ml of 2 N KCl. Centrifugation was for 48 to 65 hr in a Spinco SW39 or SW50.1 rotor at 35,000 rev/min. Sixteen-drop fractions were then collected through a hole in the bottom of the tube. Densities were determined from refractive index measurements of selected fractions. Each fraction was precipitated with 10% trichloroacetic acid onto filters and washed twice with trichloroacetic acid and twice with 95% ethanol, and the dried filters were counted.

Cell extracts. Confluent cell cultures, as described above, were removed from the dish with the aid of trypsin and washed twice in ET medium containing 5% calf serum, and the cells were counted. The cell pellets were washed twice more with Tris-EDTA buffer containing 0.25 M sucrose. Cells were disrupted by suspending the pellets in 5 or 10 ml of Tris-EDTA buffer, *p*H 7.2, containing 0.25 M sucrose, 0.25% Nonidet, and 0.4% DTT and mixing with a pipette. Final recovery of polymerase activity was independent of cell concentration in this step up to more than 2×10^8 cells per ml of Nonidet solution. After 1 to 2 min at 0 C, nuclei and other large cell fragments were removed by centrifugation for 10 min at 8,000 rev/min in a Spinco SW50.1 rotor. If the Nonidet solution was at *p*H 8.0, the nuclear pellet formed a gel which could not be resuspended. The supernatant was centrifuged for 1 hr at 45,000 rev/min in an SW50.1 rotor, and the pellet was resuspended in Tris-EDTA buffer containing 0.4% DTT at a concentration of 2×10^8 original cells per ml. The resuspended pellet was frozen for later use. This fraction contained approximately 10% of the protein of the starting material.

Polymerase assays. The standard polymerase assay of Temin and Mizutani (21) was used throughout. Reactions were started by adding 0.1 ml of standard reaction mix containing 2.5 μCi of ^{3}H-thymidine-5′-triphosphate (^{3}H-TTP; 11.1 Ci/mM; Schwarz BioResearch, Inc.) or ^{3}H-deoxycytidine-5′-triphosphate (^{3}H-dCTP; 26.4 Ci/mM; Schwarz BioResearch, Inc.) with 10 nmoles of the other three unlabeled deoxynucleoside triphosphates to a 0.025-ml sample containing the indicated additions. Samples of 0.025 ml were spotted on filters either at the times indicated or at 1 hr. Filters were fixed in 10% trichloroacetic acid, washed with ethanol, and dried. In these assays 1 pmole of ^{3}H-TTP incorporated per 0.125 ml of reaction corresponds to approximately 800 counts per min, and 1 pmole of ^{3}H-dCTP incorporated corresponds to approximately 1,850 counts per min. Except where otherwise stated, all counts were corrected for background by subtracting trichloroacetic acid-insoluble radioactivity determined after 1-hr incubation for a parallel reaction with no added enzyme. Samples were sometimes pretreated by mixing with an equal volume of water or 100 μg of ribonuclease A per ml (Worthington Biochemical Corp., Freehold, N.J.) and incubating for 1 hr at 0 C prior to use. The ribonuclease was pretreated by heating at 80 C for 10 min. Reaction mixtures sometimes contained calf thymus DNA (Worthington Biochemical Corp.) or yeast RNA (Worthington Biochemical Corp.).

Nucleic acids. Nucleic acids were extracted from polymerase reactions after 30 or 60 min of incubation. A 25-μliter amount of 5% sodium dodecyl sulfate (SDS), 5 μliters of diethyl pyrocarbonate (Baycovin; Bayer Ltd., Leverkusen, Germany), and 25 μg of calf thymus DNA were added to 1.25 ml of reaction mixture. This mixture was incubated 10 min at 40 C and then swirled in a tube under vacuum until all the

Baycovin was dissolved. A 0.25-ml amount of 2 M KCl was then added, and the mixture was centrifuged at 12,000 × *g* for 20 min. The supernatant was poured into 3 ml of cold 100% ethanol and left at −20 C for 4 hr or more. Nucleic acids were sedimented by centrifugation at 3,000 × *g* for 10 min and resuspended in 0.9 ml of water. A 0.1-ml amount of 20 × standard saline citrate (SSC; 0.15 M NaCl and 0.015 M Na citrate, *p*H 7.0) was added, and the preparation was centrifuged at 12,000 × *g* for 10 min. The clear supernatant was stored frozen. Approximately 85% of the trichloroacetic acid-precipitable radioactivity of the reaction mixture was finally recovered by this procedure.

RNA was extracted from purified RSV preparations by the same procedure, except that no carrier DNA was added. Approximately 20 μg of viral RNA [as determined by optical density ($OD_{260\ nm}$)] was recovered per milligram of viral protein. The extracted nucleic acid preparation had an OD_{260}/OD_{208} ratio of approximately 2.1.

Annealing was performed by incubation in 2× SSC at 65 C for 5 to 6 hr with the addition of RSV RNA, yeast RNA, or nothing. Samples to which RNA was to be added were pretreated by overnight incubation with 0.2 N NaOH and neutralized by the addition of 0.2 N HCl. After annealing, samples were analyzed on Cs_2SO_4 gradients as described above.

Radioactivity determinations. Sucrose gradient samples were taken directly to Scintisol scintillation counting fluid (Isolab Inc., Akron, Ohio) in counting vials. Dried filters were counted under toluene and Liquifluor (New England Nuclear Corp.). All counting was done in Packard Tri-Carb liquid scintillation counters.

RESULTS

Density gradient centrifugation of disrupted RSV. Treatment of virions of RSV with nonionic detergent is necessary to obtain DNA polymerase activity (21). This result indicates that viral polymerases are present within the virus envelope, perhaps in the virion core. To determine whether the endogenous polymerase activity was located in a virion core particle, preparations of ^{3}H-uridine-labeled SRV were centrifuged on parallel sucrose-D_2O density gradients (Fig. 1) after no treatment (A), disruption by brief treatment with the detergent Nonidet (B), or disruption and treatment with ribonuclease (C). Another preparation was disrupted and centrifuged on a gradient containing 0.1 M KCl (D). Almost all of the labeled RNA of undisrupted virions banded at a density of 1.16 g/cc, as expected (14). The RNA of disrupted virions banded at a density of 1.22 to 1.23 g/cc, and the endogenous RNA-dependent DNA polymerase banded in the same position. Pretreatment with ribonuclease destroyed all endogenous polymerase activity in the gradient, and most of the ^{3}H-uridine label was then found at the bottom of the gradient. Centrifugation in a gradient containing 0.1 M KCl apparently destroyed the core structure. The labeled viral RNA was found spread throughout the gradient, and only a small amount of endogenous polymerase activity was found. This activity was located at the top of the gradient.

The gradients shown in Fig. 1 were centrifuged for 2.5 hr. Centrifugation of similar gradients for times up to 16 hr has also been performed. The viral core banded at approximately 1.24 g/cc, as above. The total endogenous polymerase activity in such gradients, however, was greatly reduced from the starting value, probably because of separation of the polymerase from the template.

To determine the distribution of proteins of disrupted RSV virions in such gradients, ^{3}H-leucine-labeled SRV was used in a similar experiment (Fig. 2). Whole virus (A) and detergent-treated virus (B) were centrifuged on sucrose-D_2O gradients. All of the label of undisrupted virus again banded at about 1.16 g/cc. Polymerase activity (determined after Nonidet treatment of each fraction) was found in the same position. When virions were disrupted prior to centrifugation, approximately 30% of the ^{3}H-leucine was found in a broad band at 1.20 to 1.24 g/cc with the remainder at the top of the gradient. Endogenous polymerase activity, as before, was in a band at 1.23 to 1.24 g/cc.

It was possible, in such gradients, that some polymerase activity dependent on exogenous template was present. To test this hypothesis, fractions from a gradient similar to that shown in Fig. 1 (B) were assayed for TTP incorporation with no added nucleic acid and with added RNA or DNA (Fig. 3). The exogenous template-stimulated DNA polymerase activity was found in two locations: one in a peak coincident with the endogenous activity and the viral RNA and the second very near the top of the gradient, where there was little endogenous polymerase activity. DNA-dependent and RNA-dependent DNA polymerase activities were not separated on these gradients.

The kinetics of incorporation of ^{3}H-TTP by different fractions from SRV virions is seen in Fig. 4. Disrupted SRV (A) and pooled fractions from the core peak (B) or the top (C) of a gradient similar to that shown in Fig. 3 were assayed for TTP incorporation in the presence of nothing, RNA, or DNA. In this experiment, polymerase activity of the complete virus was stimulated by DNA but not RNA. The core material was stimulated by both DNA and RNA, as well as having significant endogenous activity. Enzyme taken from the top of the gradient had no activity in the absence of added template and greater activity than the core material in the presence of RNA or

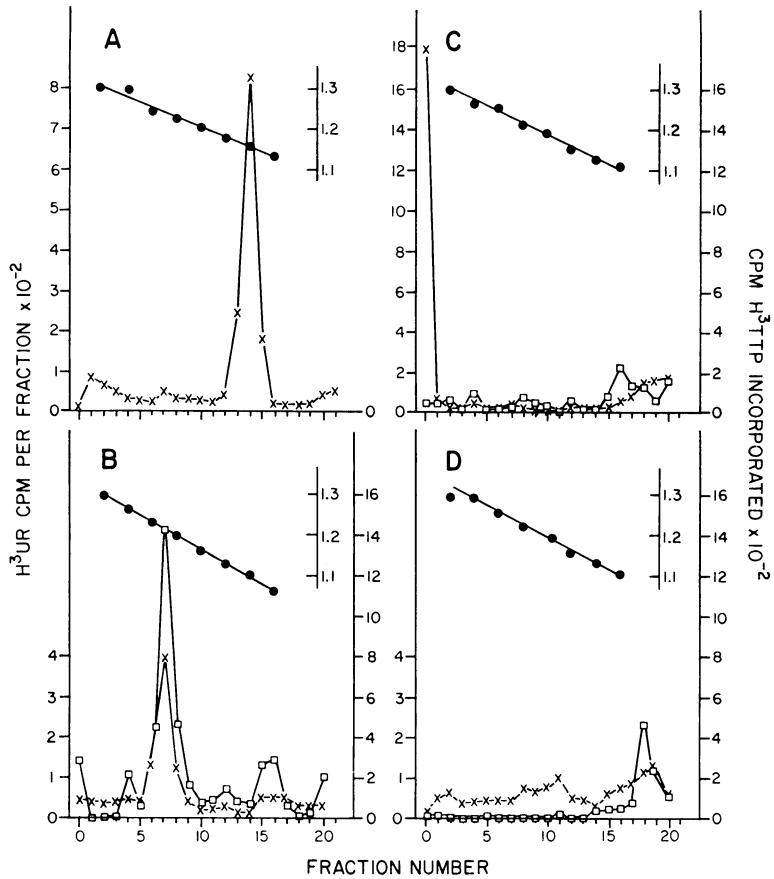

FIG. 1. *Sucrose density gradient centrifugation of ³H-uridine-labeled SRV. Mixtures of concentrated unlabeled and ³H-uridine-labeled SRV were layered on sucrose-D_2O gradients and centrifuged as described in Materials and Methods after treatment with buffer (A), Nonidet (B), and Nonidet and ribonuclease for 1 hr (C). A parallel sample was treated with Nonidet and layered on a similar gradient containing 0.1 M KCl (D). DNA polymerase activity (□) and ³H counts per minute (×) were determined for all fractions. The dilution of virus used in the polymerase assay was sufficient to reduce counts per minute of ³H from input viral ³H-uridine by a factor of at least 50. Samples of some fractions were weighed to determine density in grams per cubic centimeter (●).*

DNA. This result demonstrates that RNA-dependent and DNA-dependent DNA polymerase free of endogenous template can readily be prepared from RSV. Both these activities are then dependent on the addition of a template.

DNA polymerase activities in RSV-infected cells. Since treatment with the nonionic detergent Nonidet readily releases intact, polymerase containing cores from RSV virions and is also an effective agent for preparation of cytoplasmic extracts from cells (13), we decided to prepare an extract from RSV-infected chicken cells using a Nonidet treatment. Parallel preparations of uninfected and B77-infected chicken embryo fibroblasts were extracted with Nonidet and subjected to low- and high-speed centrifugations. The 45,000 rev/min pellet fractions obtained were assayed for DNA polymerase activity with no addition or with added DNA (Fig. 5). Significant activity, in the presence or absence of added DNA, was found only in the preparation from infected cells. The low-speed pellet and high-speed supernatant fractions had less than 10% as much polymerase activity (*data not shown*). Both the uninfected and the RSV-infected cells were growing rapidly at the time the extracts were prepared. Endogenous DNA polymerase activity from RSV-infected chicken cells was similar to polymerase activity from virions in its Mg^{2+} requirement and optimum (*data not shown*).

Unlike the virion endogenous DNA polymerase system, however, endogenous DNA polymerase activity from RSV-infected chicken cells was insensitive to treatment with ribonuclease (Fig. 6). In a control experiment, endogenous DNA polymerase activity in RSV virions re-

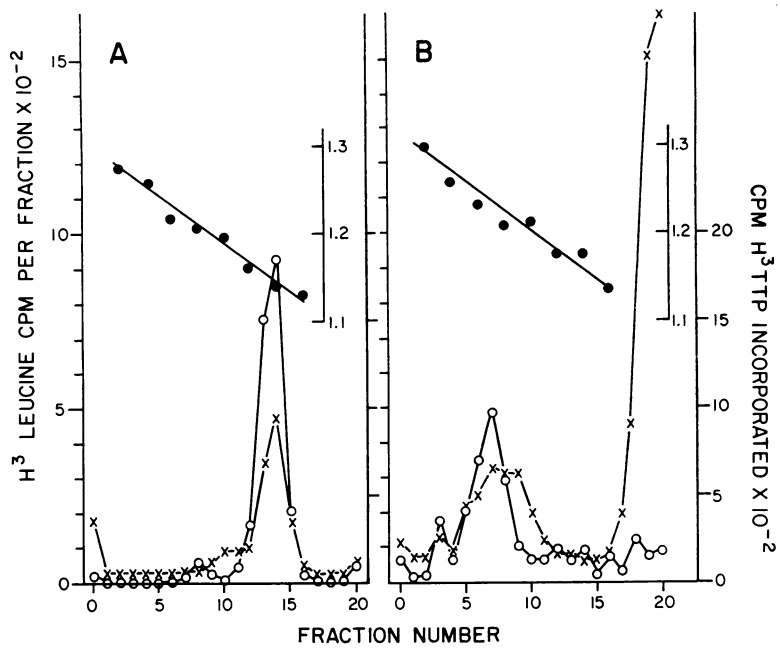

FIG. 2. *Sucrose density gradient centrifugation of ^{3}H-leucine-labeled SRV. Mixtures of ^{3}H-leucine-labeled SRV and concentrated unlabeled SRV were centrifuged on sucrose-D_2O gradients after treatment with buffer (A) or Nonidet (B). Polymerase activity* (○) *and ^{3}H counts per minute* (×) *were determined for all fractions. Density in grams per cubic centimeter was determined for selected fractions* (●).

tained its ribonuclease sensitivity when mixed with a similar extract. To determine whether the endogenous polymerase activity observed in the high-speed pellet fraction of RSV-infected chicken cells is RNA-directed, the following experiment was performed. A standard polymerase reaction containing ^{3}H-TTP was carried out with a 45,000 rev/min pellet fraction of an extract of SRV-infected chicken cells. The total nucleic acids from this reaction were extracted by a Baycovin-SDS procedure. The nucleic acids were either left untreated (Fig. 7A) or were annealed at 65 C for 5 hr (Fig. 7B), and then both samples were analyzed on cesium sulfate equilibrium density gradients. The majority of label in the untreated material banded at a density of 1.46 g/cc, typical of DNA. A small amount of label banded at a density of 1.65 g/cc, a typical density for RNA. This denser material was not consistently found in such gradients. When the same preparation was annealed prior to centrifugation, two peaks were observed: one with a density of 1.45 g/cc and one with a density of 1.55 g/cc. This result suggests that at least half of the labeled DNA hybridized with an RNA already present in the extract. This conclusion is supported by the observation that after centrifugation of the annealed nucleic acids in an alkaline gradient, the label banded only at 1.47 g/cc (Fig. 7C). This result suggests that a DNA-RNA hybrid was formed by annealing and was denatured by the alkali. These results indicate that the newly synthesized DNA was made by using an RNA template.

To determine if this RNA was related to the RNA of RSV, an endogenous DNA polymerase reaction was run with a high-speed pellet fraction from SRV-infected chicken cells, and the product DNA was extracted and annealed either with RNA extracted from SRV virions or with yeast RNA (Fig. 8). Prior to the annealing, the nucleic acid preparation was treated with 0.2 N NaOH overnight, to digest RNA and to denature the DNA, and then neutralized with 0.2 N HCl. The annealed samples were analyzed on Cs_2SO_4 gradients. When the labeled DNA was annealed with SRV RNA (Fig. 8A), 36% of the label banded at densities greater than 1.53 g/cc, suggesting DNA-RNA hybrid structures with varying proportions of RNA to DNA. The remainder of the label banded at 1.46 g/cc, typical of DNA. If the extracted labeled DNA was annealed with yeast RNA, only a single band of label at 1.45 g/cc was observed. Only 5% of the label was found at densities greater than 1.53 g/cc. These results show that a third of the DNA made in vitro by the endogenous DNA polymerase from SRV-infected chicken cells was complementary to RSV RNA.

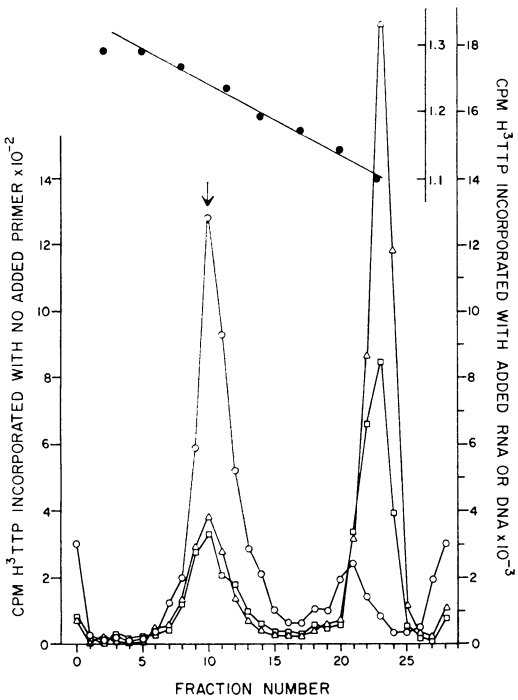

FIG. 3. *Sucrose density gradient centrifugation of disrupted RSV: endogenous and template-requiring DNA polymerase activity. Nonidet-treated SRV was centrifuged on a sucrose-D_2O gradient, and all fractions were assayed for DNA polymerase activity with no addition* (○) *or with addition of 5 μg of yeast RNA* (□) *or 0.5 μg of calf thymus DNA* (△). *The arrow marks the peak of counts of ^3H-uridine-disrupted SRV in the same gradient. Density in grams per cubic centimeter was also determined* (●).

Density of DNA polymerase containing particles from RSV-infected chicken cells. To determine the density of the particulate DNA polymerases found in RSV-infected chicken cells, the experiment shown in Fig. 9 was carried out. A polymerase preparation from SRV-infected chicken cells was centrifuged in a 30 to 70% sucrose-D_2O gradient (Fig. 9), and the fractions were assayed with no added template or with added DNA. A peak of DNA-dependent polymerase activity was found at a density of approximately 1.15 g/cc. Endogenous DNA polymerase activity was found at 1.24 g/cc and at the bottom of the gradient, indicating a particle of density greater than 1.30 g/cc. The DNA polymerase activity in the 1.25 g/cc peak and at the bottom of the gradient was stimulated approximately twofold by the addition of DNA. No stimulation of polymerase activity was observed in a similar gradient with the addition of RNA (*data not shown*). The pattern of endogenous DNA polymerase activity in sucrose gradients shown in Fig. 9 was the pattern most consistently observed with polymerase preparations from SRV-infected chicken cells. This pattern has also been found with extracts of B77-infected chicken cells. A few gradients with similar preparations, however, contained only one of the two bands of endogenous DNA polymerase activity.

DISCUSSION

Brief treatment with the nonionic detergent Nonidet disrupts RSV virions to release core particles with a buoyant density of 1.23 to 1.24 g/cc. These particles contain almost all of the

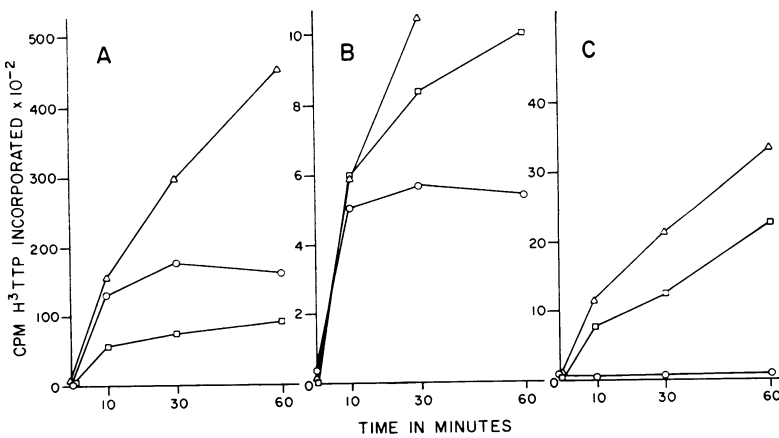

FIG. 4. *Kinetics of incorporation of ^3H thymidine-5'-triphosphate by different virus components. Standard polymerase assays were done with complete, Nonidet-treated SRV (A), pooled core fractions (B), and top fractions (C) from a gradient similar to that shown in Fig. 3. The core was from fractions 9 to 13, and the top was from fractions 21 to 24. Assays were done with no addition* (○), *or with addition of 5 μg of yeast RNA* (□) *or 0.5 μg of calf thymus DNA* (△). *Samples were taken at the indicated times and trichloroacetic acid-insoluble radioactivity determined.*

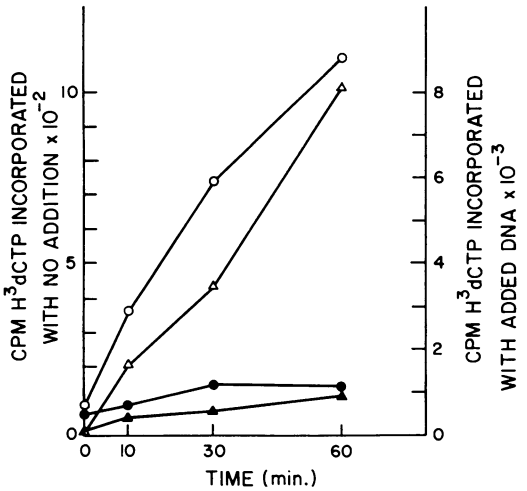

FIG. 5. *DNA polymerase activity in extracts of uninfected and RSV-infected chicken cells. Parallel cultures of chicken embryo fibroblasts were infected with B77 virus (open symbols) or left uninfected (closed symbols). Four days after infection, the medium on all cultures was replaced with ET medium containing 5% fetal bovine serum. Five days after infection, extracts were prepared with the use of Nonidet as described in Materials and Methods. The 45,000 rev/min pellet fractions obtained were assayed for DNA polymerase activity with no additions (○, ●) or with the addition of 5 μg of calf thymus DNA per reaction (△, ▲). Trichloroacetic acid-insoluble radioactivity was determined at the indicated times.*

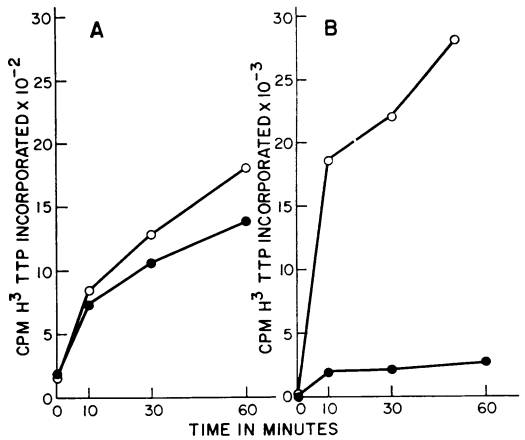

FIG. 6. *Effect of ribonuclease on polymerase preparations from RSV virions and from RSV-infected chicken cells. A high-speed pellet fraction of Nonidet extracted SRV-infected chicken cells (A) and a preparation of Nonidet-disrupted SRV (B) were incubated with water (○) or 50 μg of ribonuclease per ml (●) for 1 hr at 0 C. DNA polymerase activity was then determined for each sample in a standard assay with no additions. Samples were taken and trichloroacetic acid-insoluble radioactivity was determined at the indicated times.*

viral RNA and about 30% of the viral protein. Endogenous DNA polymerase activity bands with the virus RNA, both before and after disruption. A large amount of RNA-directed and DNA-directed DNA polymerase activity dissociates from the virion cores upon disruption. A similar result has been obtained by Gerwin et al. (8) with Triton X-100-disrupted Rauscher leukemia virus. This "soluble" polymerase is free of endogenous template although it is not free from other viral proteins. Properties of polymerase containing fractions from virions of RSV and from RSV-infected cells are summarized in Table 1.

The core from RSV virions is sensitive to both ribonuclease and 0.1 M KCl. The sensitivity of the core to salt may explain the difficulty of others in obtaining cores in high yield from virions of avian leukosis viruses (2, 9). Duesberg (6) reports a similar result and also that the core is more stable in very high salt, as in potassium tartrate gradients, than in 0.1 M salt. Treatment of disrupted virions with ribonuclease A apparently causes the separation of the RNA, in large pieces, from much of the core protein. Such ribonuclease-treated proteins have no endogenous DNA polymerase activity. The RNA after this treatment is either dissociated from the polymerase, or it is unsuitable for use as a template.

In a number of separate experiments, we have always found that although the RSV DNA polymerase in disrupted virions bands almost exactly with the viral RNA (as in Fig. 1B), it bands differently from the viral protein (Fig. 2B). These results indicate that the core peak seen in density gradients of ^{3}H-leucine-labeled disrupted RSV is not homogenous. Only the denser part contains the virus RNA and the polymerase activities. The lighter part of this peak may represent a significant fraction of "empty" cores free of viral RNA and associated polymerase. Further experiments are in progress to clarify this question.

DNA polymerases with properties similar to those found in intact virions can readily be extracted from virus producing RSV-infected cells. Endogenous (RNA-dependent) and exogenous DNA-dependent DNA polymerase activities were found in a particulate form in high-speed pellet fractions of detergent-disrupted cells.

The endogenous DNA polymerase activity extracted from these cells was insensitive to pretreatment with ribonuclease. However, analysis of the product of this endogenous DNA polymerase strongly suggests that it uses RNA as a template. Prior to annealing of the nucleic acids extracted from such a reaction, the majority of the labeled DNA banded at 1.46 g/cc in Cs_2SO_4 equilibrium density gradients. (A variable amount

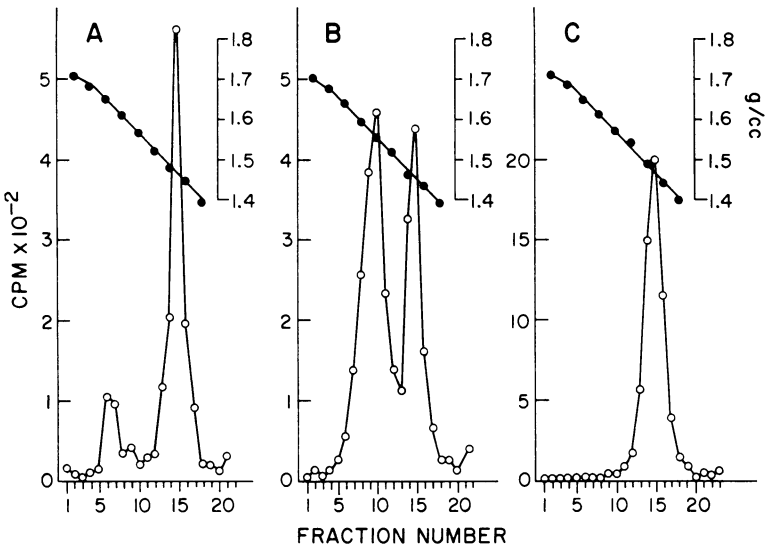

FIG. 7. *Equilibrium density gradient centrifugation of the product of the endogenous DNA polymerase in extracts of RSV-infected chicken cells. A DNA polymerase reaction was carried out with 1 ml of standard reaction mix plus 0.25 ml of a high-speed pellet fraction from Nonidet-extracted SRV-infected chicken cells. After 35 min of reaction, the nucleic acids were extracted with Baycovin and sodium dodecyl sulfate. Samples of the nucleic acid preparation were centrifuged in Cs_2SO_4 gradients after no treatment (A) or after annealing at 65 C for 5 hr (B). Another sample of the same preparation was annealed and then centrifuged in an alkaline Cs_2SO_4 gradient (C). Centrifugation was for 65 hr in a Spinco SW50.1 rotor at 35,000 rev/min. Density of selected fractions was determined from refractive indeexs (●). Trichloroacetic acid-insoluble radioactivity was measured for all fractions (○).*

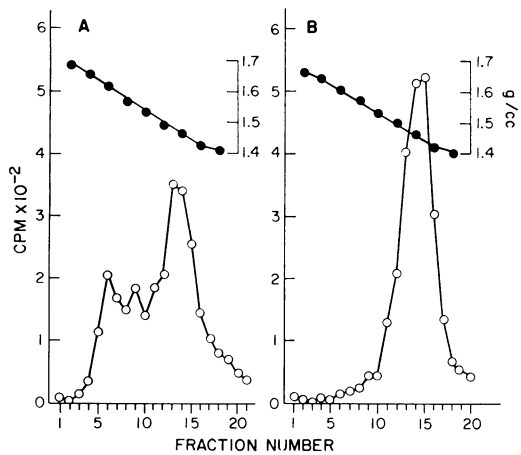

FIG. 8. *Hybridization of the product of the endogenous DNA polymerase in RSV-infected chicken cells with RSV RNA. A standard DNA polymerase reaction was carried out and extracted after 60 min as described in the legend to Fig. 7. Samples of the extracted nucleic acids were treated with 0.2 N NaOH and then neutralized with 0.2 N HCl. A 10-μg amount of RNA extracted from SRV (A) or yeast RNA (B) was added, and the samples were incubated for 5 hr at 65 C. Samples were then analyzed in neutral Cs_2SO_4 gradients. Centrifugation was for 60 hr at 35,000 rev/min in a Spinco SW50.1 rotor. Density of selected fractions was estimated from refractive indexes (●). Trichloroacetic acid-insoluble radioactivity was measured for all fractions (○).*

of label banding at 1.65 g/cc may represent a DNA-RNA hybrid intermediate in the reaction.) After annealing, approximately half of the labeled DNA banded at about 1.55 g/cc, the density of an RNA-DNA hybrid with roughly equal amounts of RNA and DNA. The denser band did not appear if the annealed product was analyzed in an alkaline gradient. This result shows that the polymerase-containing fraction extracted from RSV-infected chicken cells contained an RNA complementary to the DNA product, and therefore the DNA probably was synthesized by using the RNA as a template.

The DNA of the annealed material that banded at 1.45 g/cc in the neutral gradient may be the complement of the DNA which hybridized to the endogenous RNA and thus identical in sequence to the RNA. The formation of DNA strands complementary to the product of the endogenous DNA polymerase is expected from the presence of DNA-dependent DNA polymerase activity in the cell extract.

In addition to being complementary to an endogenous RNA, at least some of the DNA made in vitro by the polymerase containing particles from RSV-infected chicken cells was complementary to RSV RNA. This complementarity implies that some or all of the RNA template in these particles is specific for the infecting RSV. More than half of the labeled DNA from the endogenous polymerase system did not

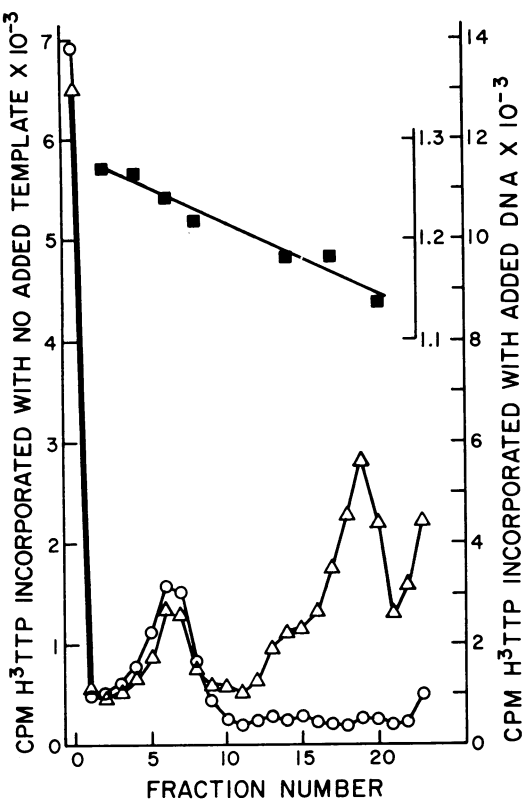

FIG. 9. *Sucrose density gradient centrifugation of polymerase containing material from RSV-infected chicken cells. A high-speed pellet preparation from SRV-infected chicken embryo fibroblasts was layered on a 30 to 70% sucrose-D_2O gradient and centrifuged as described in Materials and Methods. Each fraction was assayed for DNA polymerase activity with no addition* (○) *or with 5 μg of calf thymus DNA added per reaction* (△). *Samples of 0.01 ml were weighed to determine density in grams per cubic centimeter* (■).

change density upon annealing with RSV RNA. This may reflect some inefficiency in our hybridization system or may indicate that some of the RNA in these particles is not RSV-specific.

The particles with endogenous DNA polymerase activity extracted from RSV-infected chicken cells are most likely precursors of mature virions. These particles were further analyzed by equilibrium sucrose density gradient centrifugation. The material which banded at 1.24 g/cc in an equilibrium sucrose density gradient probably did not contain cores from disrupted whole virions, since whole virions should have been removed from the cells by the trypsin treatment followed by the multiple washes, and this material was not altered by pretreatment with ribonuclease either in polymerase activity or in density (*unpublished data*). This ribonuclease resistance may

TABLE 1. *Comparison of properties of polymerase-containing fractions from Rous sarcoma virus (RSV) virions and RSV-infected chicken cells*

Preparation	Approx. density (g/cc)	DNA polymerase activity		
		Endogenous (RNA-dependent)	Exogenous (RNA-dependent)	Exogenous (DNA-dependent)
Virion	1.16	+	○	+
Disrupted virion				
top	soluble	○	+	+
core	1.24	+	+	+
RSV-infected chicken cells	1.15	○	○	+
RSV-infected chicken cells	1.24	+	○	+
RSV-infected chicken cells	≥1.3	+	○	+

reflect some property of a core precursor which protects it from being degraded by cell enzymes prior to maturation.

Like RSV virion cores, this 1.24 g/cc material has both endogenous and DNA-directed DNA polymerase activity. The denser than 1.3 g/cc polymerase-containing particles in RSV-infected chicken cells may represent an earlier core precursor consisting of viral RNA with only a small amount of attached protein. This particle was also similar to the virion core in that its polymerase activity was stimulated by DNA.

A particle of density 1.15 g/cc with DNA polymerase activity was also obtained from RSV-infected chicken cells. This particle had no endogenous polymerase activity, but it had DNA-dependent DNA polymerase activity. It is not known whether this particle (1.15 g/cc) contains a polymerase which is also present in virions of RSV.

Other workers have obtained soluble DNA polymerases from tumor cells (7) and from normal cells (15) which can utilize RNA or synthetic ribohomopolymers as templates. The origin and biological function of these enzymes are not known. In chicken cells, the particulate DNA polymerase systems we have observed were found in RSV-infected cells and not in uninfected cells. These systems contained DNA polymerase activities and an endogenous RNA template and required only appropriate precursors to make DNA complementary to the RNA template. The abilty of these particles to make DNA in vitro by using an endogenous RNA template implies that they have the same biological role in vivo. Finding particles with similar abilities in cell extracts provides evidence of the presence of an enzyme

whose function is to make new DNA with an RNA template. The presence of such particles in certain normal cells would support the protovirus hypothesis (20, 20*a*). The presence of such particles in tumor cells might imply a viral etiology for natural tumors.

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