Ribonuclease-Sensitive Deoxyribonucleic Acid Polymerase Activity in Uninfected Rat Cells and Rat Cells Infected with Rous Sarcoma Virus

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Rat cells infected with the B77 strain of avian sarcoma virus [R(B77) cells] produced no virus-like particles but contained information for the production of infectious B77 virus. ³H-labeled deoxyribonucleic acid (DNA) product of the B77 virus endogenous DNA polymerase system was used to determine the relative amounts of B77 virus-specific ribonucleic acid (RNA) in B77 virus-infected chicken and R(B77) cells. R(B77) cells were found to contain much less B77 virus RNA than did B77 virus-infected chicken cells. Ribonuclease-sensitive DNA polymerase activity was present in high-speed pellet fractions from Nonidet extracts of B77 virus-infected rat cells. Similar preparations from some uninfected rat cells contained lesser amounts of a similar ribonuclease-sensitive DNA polymerase activity. The endogenous template for the DNA polymerase activity in high-speed pellet fractions from R(B77) cells was not related to B77 virus RNA or to RNA of a rat C-type virus. The DNA product of the endogenous DNA polymerase in high-speed pellet fractions of R(B77) cells hybridized to a small extent with RNA from the same fraction and to a similar extent with RNA from uninfected rat cells.

Some strains of Rous sarcoma virus (RSV) induce tumors in mammals (23) and transform mammalian cells in culture (1). In general, RSV-transformed mammalian cells produce no infectious RSV or RSV-like particles (8). However, infectious RSV can be recovered from RSV-infected mammalian cells after co-cultivation or fusion with chicken embryo fibroblasts (18). The reason for the failure of RSV-transformed mammalian cells to produce RSV is unknown.

Virions of ribonucleic acid (RNA) tumor viruses contain an endogenous RNA-directed deoxyribonucleic acid (DNA) polymerase system (2, 22). Particles containing a similar polymerase system have been isolated from RSV-producing chicken cells. The template for the endogenous DNA polymerase activity in these particles has been shown by nucleic acid hybridization experiments to be related to RSV RNA (5). These particles probably are precursors of the RSV virion core.

In the experiments described in this report, we attempted to isolate such RSV-related particles from RSV-infected mammalian cells. We found ribonuclease-sensitive DNA polymerase-containing particles in extracts of rat cells infected and transformed by the B77 strain of RSV. Similar

particles were found in extracts of some uninfected rat cells. The product DNA from the particles isolated from the rat cells infected with B77 virus did not hybridize with RNA from B77 virus. The product DNA from these particles did hybridize to a small extent with RNA from uninfected rat cells and to the same extent with RNA from rat cells infected with B77 virus.

MATERIALS AND METHODS

General experimental procedures were the same as those previously described for this laboratory (19, 20).

Viruses. B77 virus and the Schmidt-Ruppin strain of RSV were grown and purified as previously described (5). Rat C-type rousvirus was grown and purified from the rat mammary tumor cell line R-35 supplied by W. Ray Bryan and Muntaz Ahmed (4). Labeling of cell supernatant fluids with uridine-5-³H (New England Nuclear Corp., Boston, Mass.) was described previously (5).

Cells. B77 virus-infected chicken embryo fibroblasts were described previously (5). Rat embryo fibroblast cultures were prepared from 14- to 16-day Sprague-Dawley rat embryos (Holtzman Co., Madison, Wis.) and were grown in Eagle's medium with 20% tryptose phosphate broth (ET medium) containing 5% calf serum or 5% fetal bovine serum. The cultures were transferred every 10 to 14 days at 2×10^6 cells per 100-mm plastic culture dish (Falcon Plastic, Div. of

B-D Laboratories, Los Angeles, Calif.) and sometimes were stored frozen between transfers. Extracts were prepared from uninfected rat cells between the second and sixth tissue culture passage.

R(B77) cells are a line of rat embryo fibroblasts infected and transformed in culture by B77 virus (1, 14). They were maintained in culture by serial transfer every 5 days at 10^6 cells per 100-mm culture dish in ET medium containing 5% calf serum. Extracts were prepared from confluent cultures containing 10×10^6 to 15×10^6 cells per dish.

Cell extracts. Extracts were prepared from trypsinized, washed cell pellets as described previously (5). Cells were disrupted by treatment with 0.25% Nonidet P-40 (Shell Chemical Co., New York, N.Y.) and 0.4% dithiothreitol (Sigma Chemical Co., St. Louis, Mo.) in Tris-EDTA buffer [0.01 M tris(hydroxymethyl)-aminomethane, 0.001 M ethylenediaminetetraacetic acid; pH 7.5] containing 0.25 M sucrose. The disrupted cells were centrifuged at 8,000 rev/min ($6,000 \times g$) for 10 min in a Spinco SW 50.1 rotor, and the supernatant fluid was centrifuged at 45,000 rev/min ($190,000 \times g$) for 1 hr in an SW 50.1 rotor. The pellet from the high-speed centrifugation was resuspended in 1 ml of Tris-EDTA buffer containing 0.4% dithiothreitol per 2×10^8 original cells.

Polymerase reactions. The standard polymerase reaction of Temin and Mizutani (22) was used throughout. Reactions were carried out in a 0.125-ml volume containing 0.025 ml of sample and 2.5 μ Ci of 3 H-thymidine-5'-triphosphate (3 H-TTP; Schwarz BioResearch, Inc., Orangeburg, N.Y.) or 3 H-deoxycytidine-5'-triphosphate (3 H-dCTP; Schwarz BioResearch Inc.). In these assays, 1 pmole of 3 H-TTP incorporated corresponded to approximately 800 counts/min, and 1 pmole of 3 H-dCTP incorporated corresponded to approximately 1,900 counts/min. Trichloroacetic acid-insoluble radioactivity was determined at the times indicated. Samples were sometimes pretreated by incubating 0.025 ml of sample with 0.025 ml of ribonuclease A (100 μ g/ml; Worthington Biochemical Corp., Freehold, N.J.) or with 0.025 ml of water for 60 min at 0 C before addition to the reaction mixture. The ribonuclease was heated at 80 C for 10 min before use. Some reactions contained 5 μ g of calf thymus DNA (Worthington Biochemical Corp., Freehold, N.J.).

Labeled DNA product was prepared from standard polymerase reactions carried out in a total volume of 1.25 to 6.25 ml containing both 3 H-TTP and 3 H-dCTP. These reactions were divided into an appropriate number of tubes at 0.5 ml per tube, incubated for 1 hr at 40 C, and then pooled for nucleic acid extraction.

Nucleic acids. Extraction of nucleic acids from polymerase reactions and purified virus was carried out with diethyl pyrocarbonate (Baycovin; Bayer Ltd., Leverkusen, Germany) and sodium dodecyl sulfate (SDS) as described previously (5). RNA was prepared from high-speed pellet fractions of Nonidet-disrupted cells by the same procedure. RNA was extracted from cells by a modification of the procedure of Solymosy et al. (16). Washed cell pellets were resuspended in 1 ml of 0.05 M Tris, 0.005 M MgCl₂, and 1% SDS (pH 7.6) per 10^7 cells, and Baycovin was added to a final concentration of 0.5% (v/v). This

mixture was incubated at 40 C for 10 min, 10% dry NaCl was stirred in, and the mixture was incubated at 40 C for another 10 min. The disrupted cells were centrifuged at $20,000 \times g$ for 20 min, and the supernatant fluid was poured into 2 volumes of cold 100% ethanol. After 4 hr or longer at -20 C, the precipitated nucleic acids were collected by centrifugation at $3,000 \times g$ for 10 min, resuspended in 1 ml of 0.05 M Tris, 0.05 M MgCl₂, 0.005 M MnCl₂ (pH 7.5) per 5×10^7 cells, and 50 μ g of deoxyribonuclease I (electrophoretically purified, Worthington Biochemical Corp.) per ml was added. After incubation for 1 hr at room temperature, 0.005 ml of Baycovin per ml was added, the mixture was incubated at 40 C for 10 min, and the Baycovin was dissolved by swirling under vacuum. This mixture was dialyzed overnight against 500 volumes of standard saline citrate (SSC; 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). The RNA was again precipitated with cold ethanol, redissolved in $2 \times$ SSC, and clarified by centrifugation at $15,000 \times g$ for 10 min. Approximately 100 μ g of RNA per 10^7 cells was obtained by this procedure. The RNA preparations had an optical density (OD)_{260 nm}/OD_{280 nm} ratio of 2.0 to 2.1.

Nucleic acid hybridization. RNA-DNA hybridization reactions were carried out by a modification of the method previously described (5). Extracted nucleic acid preparations from large-scale polymerase reactions were brought to 0.2 N NaOH and 0.05 M Tris and were incubated overnight at room temperature. After incubation, a small amount of phenol red was added, and the preparation was neutralized with 0.2 N HCl. Samples containing approximately 3,000 counts/min of labeled DNA were distributed in screw-cap vials, and 5 μ liters of phenol and 100 μ g of yeast RNA (Worthington Biochemical Corp.) were added. Appropriate amounts of test RNA were added to each vial, and all vials were adjusted to the same volume (0.2 or 0.4 ml) with $2 \times$ SSC. Annealing was at 65 C for 4 hr or longer. The contents of each vial were then analyzed by equilibrium density gradient centrifugation in cesium sulfate gradients.

Density gradient centrifugation. Equilibrium density gradient centrifugation in sucrose gradients was described previously (5). Cesium sulfate equilibrium density gradient centrifugation was carried out by a slight modification of our previous procedure (5). In addition to the sample to be analyzed, each gradient contained 1.85 ml of saturated cesium sulfate and 100 μ g of calf thymus DNA in Tris-EDTA buffer in a total volume of 3.1 ml. The presence of formaldehyde, although it prevented precipitation of RNA in the gradients (11), was found to contribute to excessive variability in the recovery of labeled DNA from the gradients. For this reason, formaldehyde was not used in these gradients. All gradients were centrifuged at 32,000 rev/min for approximately 65 hr in a Spinco SW 50.1 rotor, and 20 to 25 fractions were collected from each gradient through a hole in the bottom of the tube. The refractive index of alternate fractions was measured, and trichloroacetic acid-insoluble radioactivity in a 100- μ liter portion of each fraction was determined.

The amount of hybridization between labeled DNA

and RNA was defined as the per cent of counts per minute which banded in such gradients at densities of greater than 1.53 g/ml. In control experiments in which ^3H -labeled DNA product of the endogenous RSV virion polymerase system was used, the greatest difference between hybridizations with RSV RNA and hybridizations with control RNA was consistently observed in the region of the gradient with densities greater than 1.53 g/ml.

In gradients with no chicken or rat cell RNA, as in Fig. 7C, the DNA-RNA hybrid appeared at densities less than the density of RNA in a broad band. In gradients containing a large amount of chicken or rat cell RNA, most of the hybridized DNA banded at about 1.68 g/ml coincidentally with sharp light-scattering bands, presumably of precipitated RNA. Thus, the banding of this material at a density typical of RNA is probably due to coprecipitation of RNA-DNA hybrids with RNA rather than to a difference in relative lengths of RNA and DNA molecules in the hybrid.

RESULTS

Absence of RSV-like particles in supernatant fluids of RSV-transformed rat cells. Most lines of RSV-transformed mammalian cells have been reported not to produce detectable RSV-like particles (8). To determine whether this was also true for R(B77) cells, the experiment shown in Fig. 1 was carried out. The supernatant media from ^3H -uridine-labeled cultures of R(B77) cells and B77 virus-infected chicken cells were mixed with unlabeled B77 virus, and virus was isolated by sucrose gradient centrifugation. Only the supernatant fluid from B77 virus-infected chicken cells contained labeled material banding with infectious virus. The R(B77) cells produced no virus detectable by this procedure. In addition, 200-fold-concentrated supernatant fluids from R(B77) cells were found to contain less than 2% of the endogenous or DNA-directed DNA polymerase activity of a similar concentrate from B77-infected chicken cells (Table 1). The small amount of these activities observed in the R(B77) cell supernatant fluid was probably not significant because of the large amount of cell debris in this preparation.

R(B77) cells also had no C-type particles when examined in electron micrographs (J. Perdue, *personal communication*). Concentrated supernatant fluids from R(B77) cells were also negative in tests of infectivity and of early interference with B77 virus (10a).

Although R(B77) cells produce no detectable RSV-like particles, they do contain the information for the production of RSV. Virus similar to B77 virus can be recovered readily from these cells by fusion with chicken embryo fibroblasts

FIG. 1. Lack of incorporation of ^3H -uridine into RSV-like particles in the supernatant fluid of B77 virus-infected rat cells. The medium on confluent cultures containing similar numbers of R(B77) (A) or B77 virus-infected chicken cells (B) in 60-mm petri dishes was changed to 5 ml of ET medium with 5% calf serum and uridine- ^3H (4 $\mu\text{Ci/ml}$). After 1 day, the medium was harvested, and an equal volume of unlabeled supernatant medium from B77 virus-infected chicken cells (A) or uninfected chicken cells (B) was added to each sample. Media were clarified by low-speed centrifugation and then centrifuged at 30,000 rev/min in a Spinco 30 rotor. Resuspended virus pellets were layered on a discontinuous gradient of 1 ml of 65% sucrose and 3 ml of 15% sucrose in Tris buffer and centrifuged at 30,000 rev/min in an SW 39 rotor. The interface was collected, diluted with buffer, layered on a linear 15 to 65% sucrose gradient, and centrifuged for 4 hr in an SW 50.1 rotor. Fractions (20 drops) were collected from the bottom of each tube. Samples of selected fractions were assayed for infectious virus (●) and radioactivity (○). Radioactivity was corrected to disintegrations per minute (dpm) by the use of an automatic external standard.

mediated by ultraviolet-irradiated Sendai virus (1; Coffin, unpublished data).

B77 virus RNA in R(B77) cells. R(B77) cells did not produce virus-like particles detectable by the above procedures but did contain the B77 virus genome. Other workers have reported that RNA of RSV (7) or murine leukemia virus (9) could be detected in infected cells by hybridization with the labeled DNA product of the endogenous virion DNA polymerase system. To determine whether significant B77 virus RNA could be detected in R(B77) cells, the following experiments were performed (Fig. 2 and 3).

TABLE 1. DNA polymerase activity in the supernatant medium from B77 virus-infected and uninfected chicken and rat cells^a

Cell type	Counts/min of ³ H-TTP incorporated ^b	
	With no added template	With 5 μ g of DNA per reaction
Uninfected chicken.....	0	0
B77 virus-infected chicken.....	11,000 ^c	42,000
R(B77).....	170	640
Uninfected rat.....	0	320

^a Medium which had been on cells for 5 days was clarified by low-speed centrifugation and centrifuged at 55,000 $\times$ g for 1 hr, and the pellet was suspended in 0.05 volume of Tris-EDTA buffer. All cultures contained 10×10^6 to 12×10^6 cells at the time the medium was harvested.

^b Samples (0.0125 ml) were disrupted with 0.25% Nonidet and 1% dithiothreitol and assayed in a standard polymerase assay.

^c Trichloroacetic acid-insoluble counts per minute at 1 hr minus trichloroacetic acid-insoluble counts per minute at 0 time.

³H-labeled DNA product from the endogenous B77 virion polymerase reaction was prepared and annealed with various amounts of uninfected chicken cell RNA (Fig. 2A), B77 virus RNA (Fig. 2B, C), B77 virus-infected chicken cell RNA (Fig. 2D-F), uninfected rat cell RNA (Fig. 2G), or R(B77) cell RNA (Fig. 2H). One reaction contained both R(B77) cell RNA and B77 virus RNA (Fig. 2I). The annealing mixtures were analyzed in Cs₂SO₄ gradients. Only a small amount of hybridization was observed between ³H-labeled B77 virion product DNA and R(B77) cell RNA. In repeated experiments of this type, greater hybridization of the B77 virus product with R(B77) cell RNA was frequently observed. However, the relative amounts of hybridization to B77 virus RNA, B77 virus-infected chicken cell RNA, and R(B77) cell RNA were always similar to those observed in Fig. 2. When, in similar experiments, the per cent of DNA banding at a density of greater than 1.53 g/ml was determined for each gradient and plotted against the amount of test RNA added, Fig. 3 was obtained. A maximum of about 45% hybridization was observed in this experiment with B77 virus RNA and B77 virus-infected chicken cell RNA. A maximum of about 15% of B77 virus DNA polymerase product hybridized to R(B77) cell RNA. In this experiment, the R(B77) RNA hybridized a maximum of 570 counts/min of B77 virus DNA product, compared to 100 counts/min banding in the hybrid region after

annealing with uninfected rat cell RNA. B77 virus-infected chicken cell RNA hybridized the product of the B77 virus endogenous DNA polymerase approximately 1% as efficiently per milligram of RNA as B77 virus RNA. Much less RNA hybridizing with B77 virion DNA product was observed when R(B77) cell RNA was used. It is not clear from these data whether the RNA hybridizable to B77 virion DNA product in R(B77) cells was quantitatively or qualitatively different from the B77 virus-infected chicken cell RNA which hybridized to B77 virion DNA product. In a control experiment (Fig. 2I), the presence of R(B77) RNA did not affect the hybridization between B77 virus RNA and ³H-labeled B77 virus product DNA.

DNA polymerase in extracts of R(B77) cells. Detergent extracts of RSV-infected cells have been shown to contain particles with endogenous RNA-directed and exogenous DNA-directed DNA polymerase activity (5). Nearly half of the DNA product of the endogenous reaction of these particles was complementary to RSV RNA in nucleic acid hybridization tests. To determine whether R(B77) cells contained such particles, the experiment shown in Table 2 was carried out. R(B77) and uninfected rat embryo fibroblast cells were disrupted with the nonionic detergent Nonidet. The extracts were subjected to low- and high-speed centrifugation, and all fractions obtained were assayed with no added template for DNA polymerase activity. As with RSV-infected chicken cells (5), the highest specific endogenous DNA polymerase activity in R(B77) cells was found in the 45,000-rev/min pellet fraction. Less activity was present in the 45,000-rev/min pellet fraction from uninfected rat cells (*see below*).

To determine whether the endogenous DNA polymerase activity observed in high-speed pellet fractions of extracts of R(B77) cells was RNA-directed, samples of a 45,000-rev/min pellet fraction of Nonidet-extracted R(B77) cells were treated with ribonuclease or left untreated and were assayed for DNA polymerase activity (Fig. 4). Pretreatment with ribonuclease destroyed most of the endogenous DNA polymerase activity of the R(B77) DNA polymerase. The endogenous DNA polymerase activity was also sensitive to pretreatment with ribonuclease in the presence of 0.1 M KCl (data not shown). These experiments suggest that the template for the endogenous DNA polymerase activity in high-speed pellet fractions from R(B77) cell extracts was single-stranded RNA.

This particulate fraction from R(B77) cells contained significant exogenous DNA-directed DNA polymerase activity in addition to the endogenous RNA-directed DNA polymerase activity. When calf thymus DNA was added to a

FIG. 2. Hybridization of the product of the endogenous B77 virion DNA polymerase system with B77 virion RNA and cell RNA. ^3H -labeled product DNA was prepared from a polymerase reaction by using the B77 virus endogenous DNA polymerase system. About 3,000 counts/min of this DNA was annealed for 4 hr at 65 C as follows. A, 90 μg of uninfected chicken cell RNA; B, 0.18 μg of B77 virus RNA plus 90 μg of uninfected chicken cell RNA; C, 0.88 μg of B77 virus RNA plus 90 μg of uninfected chicken cell RNA; D, 9 μg of B77 virus-infected chicken cell RNA plus 81 μg of uninfected chicken cell RNA; E, 46 μg of B77 virus-infected chicken cell RNA plus 44 μg of uninfected chicken cell RNA; F, 90 μg of B77 virus-infected chicken cell RNA; G, 80 μg of uninfected rat cell RNA; H, 80 μg of R(B77) cell RNA; I, 80 μg of R(B77) cell RNA plus 1.8 μg of B77 virus RNA. All reactions were analyzed by Cs_2SO_4 equilibrium density gradient centrifugation. Density in grams per milliliter (●) and trichloroacetic acid-insoluble radioactivity (○) were determined. Density of 1.53 g/ml in these gradients is marked with an arrow.

standard polymerase assay containing the 45,000-rev/min pellet fraction from R(B77) cells, a 10-fold increase in the amount of ^3H -TTP incorporation was observed (data not shown). This stimulation of DNA synthesis by DNA added to the R(B77) polymerase preparation was not affected by pretreatment with ribonuclease (data not shown). We did not observe stimulation of

the DNA polymerase activity of this material by added yeast RNA.

The endogenous and the DNA-directed DNA polymerase activities in the 45,000-rev/min pellet fraction from Nonidet extracts of RSV-infected rat cells were similar in several respects to the DNA polymerase activities in RSV virions. Both the RSV virion DNA polymerase activity (13,

FIG. 3. Hybridization of the product of the endogenous B77 virus DNA polymerase system with B77 virus RNA and cell RNA. Hybridization reactions (see Fig. 2) were carried out with ³H-labeled B77 virus product DNA and RNA extracted from B77 virus (○) or B77 virus-infected chicken cells (□) (A). The amount of RNA was adjusted to the same value in all reactions by addition of uninfected chicken cell RNA. Annealing was for 4 hr at 65 C. In another experiment (B), a similar ³H-labeled DNA product was annealed with B77 virus-infected chicken cell RNA (□) or R(B77) cell RNA (△). The amount of RNA was adjusted to a constant value with uninfected chicken cell RNA (□) or uninfected rat cell RNA (△). Annealing was for 23 hr at 65 C. All samples were analyzed by Cs₂SO₄ equilibrium density gradient centrifugation, and the per cent counts/minute banding at a density of greater than 1.53 g/ml was determined for each gradient.

17) and the R(B77) cell DNA polymerase activity were greater with native than with heat-denatured DNA templates (data not shown). DNA polymerase activities from both R(B77) cell extracts and RSV virions had similar divalent cation requirements and optima (data not shown). In standard polymerase assays with the 45,000-rev/min pellet fraction of R(B77) cells, Mn²⁺ could substitute for Mg²⁺ for the endogenous DNA polymerase activity but not for the DNA-directed activity. A similar phenomenon was also observed for RSV virion DNA polymerase activities (S. Mizutani, personal communication). In addition, the particulate R(B77) cell DNA polymerase and the RSV virion DNA polymerase activities were both inhibited by similar concentrations of sodium deoxycholate, and both were resistant to pretreatment with Nonidet concentrations of at least 4% (data not shown).

The product of the endogenous DNA polymerase reaction of the 45,000-rev/min pellet frac-

TABLE 2. Endogenous DNA polymerase activity in fractions of Nonidet-disrupted rat and R(B77) cells^a

Fraction	Counts/min of ³ H-TTP incorporated ^b	
	Rat embryo fibroblasts	R(B77) cells
Complete.....	80	300
8,000-rev/min supernatant fluid.....	0	390
8,000-rev/min pellet.....	340	480
45,000-rev/min supernatant fluid.....	0	810
45,000-rev/min pellet ^c	470	5,000

^a Extracts were made from equal numbers of uninfected rat embryo fibroblasts and R(B77) cells. Samples of each fraction were assayed in a standard polymerase assay.

^b Trichloroacetic acid-insoluble radioactivity determined at 1 hr minus trichloroacetic acid-insoluble radioactivity at 0 time.

^c Contained approximately 7% of the protein of the complete extract.

FIG. 4. Ribonuclease sensitivity of DNA polymerase activity from RSV-infected rat cells. A high-speed pellet fraction of R(B77) cells was treated with water (○) or 50 µg of ribonuclease per ml (●) for 1 hr at 0 C. DNA polymerase activity was determined in a standard assay with no additions. Samples were taken, and trichloroacetic acid-insoluble radioactivity was determined at the indicated times. A zero-time value of approximately 100 counts/min was usually observed in polymerase assays of cell or virus material.

tion of R(B77) cells was DNA according to its density in Cs₂SO₄ equilibrium density gradients (Fig. 7). Analysis of this product by hydroxyapatite column chromatography (12) implied a mixture of single-stranded and double-stranded

DNA. The latter was denatured to single-stranded DNA on heating at 100 C for 10 min and cooling on ice (data not shown).

Properties of R(B77) polymerase-containing particles. To determine the density of polymerase-containing particles from R(B77) cells, a preparation of R(B77) polymerase was centrifuged in 15 to 65% sucrose gradients after treatment with water (Fig. 5A) or ribonuclease (Fig. 5B). All fractions were assayed for DNA polymerase activity with and without added DNA. When the gradient with untreated material was assayed with no added template, a single peak, whose activity was wholly ribonuclease-sensitive, was found at a density of about 1.14 g/ml. When fractions from the same gradient were assayed with added DNA, a peak of polymerase activity was found at a density of about 1.12 g/ml, and some of the polymerase activity remained at the top of the gradient. A visible band of material, probably a membrane fraction, was present at 1.12 g/ml. When the preparation was treated with ribonuclease before centrifugation, no significant peak of endogenous polymerase activity was found, and all of the DNA-dependent DNA polymerase activity remained near the top of the gradient. A band of visible material was still seen at a density

of 1.12 g/ml. No DNA polymerase activity was found in either gradient near the virion core density of 1.25 g/ml. In another experiment, all fractions of a gradient similar to that shown in Fig. 5A were assayed for DNA polymerase activity with added yeast RNA. No increase in DNA polymerase activity over the activity with no added template was found anywhere in the gradient, including the top (data not shown). These results suggested that the RNA-directed and the DNA-directed DNA polymerase activities in these cells were present in two different RNA-containing particles.

To better separate these activities, velocity sedimentation in a sucrose density gradient was carried out with a high-speed pellet fraction of R(B77) cell extracts (Fig. 6). DNA polymerase activity was observed in two places in the gradient. A rapidly sedimenting fraction contained 60% of the endogenous DNA polymerase activity and 20% of the DNA-directed activity. A more slowly sedimenting fraction contained 40% of the endogenous polymerase activity and 80% of the DNA-directed activity. Most of the light-scattering material present in the extract sedimented to the bottom of the gradient. This result shows that the DNA polymerase activity present in the

FIG. 5. Sucrose density gradient centrifugation of DNA polymerase-containing material from RSV-infected rat cells. A high-speed pellet preparation from R(B77) cells was layered on 15 to 65% sucrose gradients after treatment with water (A) or 50 μ g of ribonuclease per ml (B). Gradients were centrifuged, and fractions were assayed for DNA polymerase activity in a 1-hr reaction after treatment with water (○), ribonuclease (●), or water and the addition of 5 μ g of calf thymus DNA per reaction (△). Samples (0.01 ml) were weighed to determine density in grams per milliliter (■). Arrows mark the location of a visible cloudy band of material in the gradients.

FIG. 6. Velocity sedimentation of polymerase-containing particles from RSV-infected rat cells. A high-speed pellet fraction from Nonidet-extracted R(B77) cells was layered on a 5 to 20% linear sucrose gradient and centrifuged for 1 hr at 10,000 rev/min in a Spinco SW 50.1 rotor. Fractions (20 drops) were collected from the bottom, and all fractions were assayed for DNA polymerase activity in a standard assay for 1 hr with no addition (○) or addition of 5 μ g of calf thymus DNA per reaction (△).

45,000-rev/min pellet fraction from Nonidet-extracted R(B77) cells can be fractionated into at least two components on the basis of sedimentation rates. One of these, with a larger *S* value, contained the bulk of the endogenous DNA polymerase activity and was stimulated about sixfold by the addition of DNA. The other of these, with a low *S* value, contained less endogenous DNA polymerase activity and was stimulated approximately 50-fold by the addition of DNA.

Nature of the template for the endogenous DNA polymerase activity from R(B77) cells. Previous reports have shown that the product of the endogenous DNA polymerase reaction in RSV virions (6) and in extracts of RSV-infected chicken cells (5) is complementary to RNA from virions of RSV. To determine whether the template for the endogenous DNA polymerase activity in extracts of RSV-infected rat cells was similar to RNA from virions of RSV, a standard polymerase reaction was carried out with a 45,000-rev/min pellet fraction from Nonidet-extracted R(B77) cells, and the labeled DNA extracted from this reaction was annealed with

B77 virus RNA (Fig. 7A) or yeast RNA (Fig. 7B). Parallel annealing mixtures contained labeled DNA from an endogenous B77 virus polymerase reaction and B77 virus RNA (Fig. 7C) or yeast RNA (Fig. 7D). Although the B77 virus DNA product hybridized with B77 virus RNA (Fig. 7C), no hybridization was observed between B77 virus RNA and the product of the endogenous DNA polymerase activity in the 45,000-rev/min pellet fraction of R(B77) cells (Fig. 7A). Thus, the RNA template for the endogenous DNA polymerase in R(B77) cells did not appear to be B77 virus RNA.

FIG. 7. Failure of the product of the endogenous DNA polymerase system from R(B77) cells to hybridize with B77 virus RNA. 3 H-labeled DNA product from the endogenous DNA polymerase reaction of the 45,000-rev/min pellet fraction of Nonidet-extracted R(B77) cells was prepared from a 1-hr reaction. DNA was annealed with 5 μ g of B77 virus RNA plus 100 μ g of yeast RNA (A) or with yeast RNA alone (B). 3 H-labeled DNA product was also extracted from a reaction of the endogenous B77 virion polymerase system and was annealed for 4 hr at 65°C with 5 μ g of B77 virus RNA plus yeast RNA (C) or yeast RNA alone (D). All samples were analyzed by Cs_2SO_4 equilibrium density gradient centrifugation. Density in grams per milliliter (●) and trichloroacetic acid-insoluble radioactivity (○) were determined for all fractions. Results similar to those in C and D were obtained under the same conditions with amounts of B77 virus product DNA similar to the amount of R(B77) product DNA used here.

Endogenous C-type RNA viruses (rousviruses) have been isolated from non-neoplastic and neoplastic cells of a number of species (4, 10, 15). R-35 virus is a rat endogenous C-type RNA virus (rousvirus) which is produced by a line of cells from a spontaneous rat mammary tumor (4). To determine whether the template for the particulate endogenous DNA polymerase from R(B77) cells was related to the RNA of R-35 virus, the following experiment was carried out. ³H-labeled endogenous DNA product (approximately 4,000 counts/min) from the 45,000-rev/min pellet fraction of Nonidet-disrupted R(B77) cells was annealed for 24 hr with 2 µg of RNA from the R-35 virion plus 100 µg of yeast RNA. When analyzed by Cs₂SO₄ equilibrium density gradient centrifugation, 2.7% counts/min banded at greater than 1.53 g/ml. The same R(B77) cell endogenous DNA product was annealed with 100 µg of yeast RNA alone. The per cent counts per minute banding at greater than 1.53 g/ml in a Cs₂SO₄ equilibrium density gradient was 3.4. Thus, the template for the endogenous DNA polymerase activity in R(B77) cell extracts was not related to the RNA of this endogenous rat virus. In addition, the product of the particulate endogenous DNA polymerase activity in R(B77) cell extracts did not hybridize to the virion RNA of murine sarcoma virus (Harvey), the only other mammalian RNA tumor virus used in this laboratory (data not shown).

Because the product of the particulate endogenous DNA polymerase activity in R(B77) cell extracts was not related to the RNA of these viruses, we decided to test whether the template for the R(B77) cell polymerase-containing particle was an RNA also present in uninfected rat cells. ³H-labeled product DNA from an endogenous DNA polymerase reaction of the 45,000-rev/min pellet fraction from R(B77) cells was annealed with RNA extracted from the 45,000-rev/min pellet fraction of Nonidet-disrupted uninfected rat cells. RNA from a similar fraction from R(B77) cells, B77 virus RNA, and yeast RNA were used as controls. The samples were annealed for 7 days at 65 C and analyzed in Cs₂SO₄ gradients (Table 3). Approximately 10% hybridization was observed with the RNA from the 45,000-rev/min pellet fractions of both R(B77) and uninfected rat cells. In contrast, only 1 to 2% hybridization was observed with either B77 virus RNA or yeast RNA. This result suggests that at least some of the RNA template of the endogenous DNA polymerase reaction of the 45,000-rev/min pellet fraction of R(B77) cell extracts was also present in similar fractions from uninfected rat cells.

TABLE 3. *Hybridization between the product of the endogenous DNA polymerase reaction in the 45,000-rev/min pellet fraction of R(B77) cells and RNA of B77 virus and uninfected and B77 virus-infected rat cells^a*

Type of RNA	Amt (µg)	Counts/min banding at > 1.53 g/ml ^b
Uninfected rat cell 45,000-rev/min pellet.....	50	180 (10)
R(B77) cell 45,000-rev/min pellet.....	50	130 (8)
B77 virus.....	7	35 (2)
Yeast.....	100	15 ₂ [†] (1)

^a Product of an endogeneous polymerase reaction from a 45,000-rev/min pellet fraction from Nonidet-extracted R(B77) cells was mixed with the indicated RNA species. Annealing was for 7 days at 65 C.

^b Hybridization mixtures were analyzed on Cs₂SO₄ equilibrium density gradients as described in the legend of Fig. 8. Values in parentheses represent per cent of total.

Properties of polymerase-containing particles from uninfected rat cells. Because these hybridization experiments indicated that the RNA template of the endogenous DNA polymerase activity in the particulate fraction from R(B77) cells was not related to the infecting B77 virus or to other rousviruses but was related to an RNA in uninfected rat cells, further characterization of the small amount of endogenous DNA polymerase activity in uninfected rat cells (Table 2) was carried out. Nonidet extracts were prepared as described above from a number of rat embryo fibroblast cultures prepared from different embryos, and the 45,000-rev/min pellet fractions obtained were assayed for DNA polymerase activity with no added template (Fig. 8). DNA polymerase activity of 100 to 900 counts/min of ³H-TTP incorporated per hr per 5 × 10⁶ cells (1.6 mg of protein) was found in approximately 70% of preparations from different embryos. This activity was always less in a standard assay than that of similar extracts of R(B77) cells, which varied from 900 to 7,000 counts/min of ³H-TTP incorporated per hr per 5 × 10⁶ cells (1.5 mg of protein). The presence and amount of this endogenous DNA polymerase activity in high-speed pellet fractions from uninfected rat cells were not correlated with the growth rate of the cells at the time the extracts were prepared (data not shown).

The endogenous DNA polymerase activity in the 45,000-rev/min pellet fraction from Nonidet-extracted uninfected rat cells, although low, was

FIG. 8. Endogenous and DNA-directed DNA polymerase activity in the 45,000-rev/min pellet fraction of uninfected rat cells. A high-speed pellet from Nonidet-extracted uninfected rat cells was assayed for DNA polymerase activity in a standard assay with the addition of 5 μ g of calf thymus DNA per reaction (Δ) or with no addition ($\circ$). Trichloroacetic acid-insoluble radioactivity was determined at the indicated times.

similar to the endogenous DNA polymerase activity from R(B77) cells in its sensitivity to pretreatment with ribonuclease (Table 4) and its requirement for the presence of all four deoxyribonucleoside triphosphates for full activity (Table 4). These results indicated that the endogenous template was RNA and that the reaction observed was neither terminal addition of thymidine monophosphate to a preexisting polymer nor random polymerization. In addition, the product of the endogenous DNA polymerase in the high-speed pellet fraction from uninfected rat cells was shown to be DNA by its buoyant density in an alkaline Cs_2SO_4 equilibrium density gradient (Fig. 9).

The 45,000-rev/min pellet fraction from uninfected rat cells also contained DNA-directed DNA polymerase activity (Fig. 8).

DISCUSSION

The experiments reported in this paper are relevant to the problems of the control of virus production in heterologous cells infected with RSV and the significance of RNA-directed DNA polymerase systems.

R(B77) cells, like most other RSV-infected mammalian cells, were found not to produce detectable RSV-like particles, although they contained the complete information for B77 virus. Therefore, we looked in R(B77) cells for RNA related to B77 virus by hybridization with the product of the endogenous DNA polymerase system in virions of B77 virus. We found that RNA from R(B77) cells hybridized with the product of the B77 virion DNA polymerase less

TABLE 4. Template and substrate requirements of the endogenous DNA polymerase activity in high-speed pellet fractions of uninfected rat cells^a

Reaction ^b	Pretreatment	Counts/min of ³ H-TTP incorporated
Expt A		
Complete	None	250 ^c
Without dATP	None	35 ^c
Without dCTP	None	20 ^c
Without dGTP	None	0 ^c
Expt B		
Complete	1 hr at 4 C	580 ^d
Complete	Plus 50 μ g of ribonuclease per ml, 1 hr at 4 C	0 ^d

^a The 45,000-rev/min pellet fractions were prepared from Nonidet-extracted uninfected rat cells and assayed for DNA polymerase activity with no added template under the indicated conditions.

^b Abbreviations: dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate.

^c Trichloroacetic acid-insoluble radioactivity at 10 min minus trichloroacetic acid-insoluble radioactivity at 0 time.

^d Trichloroacetic acid-insoluble radioactivity at 60 min minus trichloroacetic acid-insoluble radioactivity at 0 time.

than 8% as efficiently as RNA from B77 virus-producing chicken cells. This figure can only be approximate, as the maximum amount of hybridization observed with R(B77) cell RNA was less than that observed with RNA from B77 virus or from B77 virus-infected chicken cells. The lower maximum amount of hybridization with R(B77) cell RNA may represent a qualitative difference between the RNA of B77 virus-infected chicken cells and the RNA of R(B77) cells.

These conclusions apply only to those viral RNA sequences copied in the *in vitro* virion DNA polymerase reaction. As it appears that these sequences are a minority of the sequences of the viral RNA (6), the conclusions are limited to this minority RNA.

It is likely that the failure of R(B77) cells to produce B77 virus is related to this lower amount of viral-specific RNA. It is probable that the lower amount of RNA reflects a lower rate of transcription from the DNA provirus than in virus-producing cells, although a very much greater rate of breakdown cannot be excluded.

FIG. 9. Equilibrium density gradient centrifugation of the product of the endogenous DNA polymerase reaction of the 45,000-rev/min pellet fraction of uninfected rat cells. ^3H -labeled product DNA was prepared from the endogenous DNA polymerase reaction of the pellet fraction of Nonidet-extracted uninfected rat cells. The reaction was incubated for 1 hr before extraction. This product was centrifuged in an alkaline Cs_2SO_4 gradient at 32,000 rev/min for 65 hr, in an SW 50.1 rotor, and 15-drop fractions were collected. Density in grams per milliliter (●) and trichloroacetic acid-insoluble radioactivity (○) were determined.

RNA extracted from chicken cells producing B77 virus hybridized with the DNA product of the endogenous DNA polymerase of virions of B77 virus about 1% as efficiently per microgram as did B77 virion RNA. It cannot be concluded from these data that 1% of the cell RNA is virus RNA, as it is not known whether all portions of the RSV genome are represented equally in the *in vitro* product of the endogenous virion DNA polymerase reaction. It is also not known whether all portions of the RSV genome are equally present in RSV-infected cells or whether such cells contain, in addition, RNA complementary to virion RNA.

High-speed pellet fractions from Nonidet-disrupted RSV-infected chicken cells were previously shown to contain an endogenous DNA polymerase system. Although this endogenous DNA polymerase activity was ribonuclease-resistant, the template was shown by nucleic acid hybridization to be RNA related to RSV RNA (5).

High-speed pellet fractions of Nonidet-disrupted R(B77) cells contained particles with an endogenous ribonuclease-sensitive DNA polymerase system. The DNA polymerase activities of these particles had a number of properties in common with virion cores and the polymerase-

containing particles from RSV-infected chicken cells. The DNA polymerase-containing particles from R(B77) cells were similar to both RSV virion cores and RSV-infected chicken cell particles in their divalent ion requirements. Like virion cores, the DNA polymerase activity of these particles from R(B77) cells was sensitive to ribonuclease. The density of these R(B77) particles in equilibrium sucrose density gradients, however, was less than the density of both virion cores and DNA polymerase-containing particles from RSV-infected chicken cells.

Surprisingly, the product of the endogenous DNA polymerase activity in R(B77) cell particles did not hybridize with B77 virus RNA. Thus, although these R(B77) cells are known to contain the complete B77 virus genome, some B77 virus RNA, and a ribonuclease-sensitive DNA polymerase activity, the template for this DNA polymerase activity did not appear to be related to B77 virus RNA. In addition, it was found that the DNA product of this endogenous R(B77) cell DNA polymerase system did not hybridize to RNA from murine sarcoma virus or to RNA from the endogenous rat rousvirus produced by R-35 cells.

From these results, it appears that R(B77) cells do not contain particles with DNA polymerase and B77 virus RNA. In contrast, in RSV-infected chicken cells, an identical extraction procedure yielded an endogenous DNA polymerase activity, much of whose product was complementary to RSV RNA (5). High-speed pellet fractions from R(B77) cells do not appear to contain an inhibitor of the endogenous RSV RNA polymerase system. Mixing disrupted RSV virions with a high-speed pellet fraction from R(B77) cells resulted in no decrease in the endogenous viral DNA polymerase activity (*unpublished data*).

Although the ribonuclease-sensitive DNA polymerase activity observed in high-speed pellet fractions from R(B77) cells did not appear to be related to B77 virus, it was of interest to characterize this activity further because of the general question of the significance of RNA-directed DNA polymerases in cells.

To determine whether the template for the R(B77) cell particulate endogenous DNA polymerase activity could be detected in uninfected rat cells, nucleic acid hybridization experiments were performed. RNA extracted from 45,000-rev/min pellet fractions of both uninfected rat cells and R(B77) cells hybridized to a similar extent with the product of the endogenous DNA polymerase activity in the 45,000-rev/min pellet fraction of R(B77) cells. The amount of hybridization observed, although small, was significantly

greater than in control hybridizations with yeast RNA or with B77 virus RNA.

Much greater levels of hybridization were observed with endogenous RNA in experiments with the DNA product of the endogenous DNA polymerase in high-speed pellet fractions of RSV-infected chicken cells (5). There are two possible reasons for the failure to obtain a greater degree of hybridization between the template and product of the RNA-directed DNA polymerase system in R(B77) cell extracts. One is that the RNA template may not be homogenous, but a great many different sequences of RNA may be copied by the DNA polymerase. Under these circumstances, hybridization would be expected to proceed much more slowly than that which occurs between the product and template of the RSV virion DNA polymerase system, in which relatively few different sequences may be involved (3). The second possible reason for the low level of hybridization is that the enzymatic reactions involved in the endogenous DNA polymerase reaction of the R(B77) cell particles may favor the synthesis of DNA using as a template the DNA strand which is complementary to the template RNA. If this were the case, most of the DNA product would be identical in sequence to the endogenous RNA template and would not hybridize with it. Experiments are being performed to test these two possibilities.

These results suggested that the RNA-directed DNA polymerase-containing particles found in R(B77) cells may be related to those found in uninfected rat cells. Significant endogenous DNA polymerase activity was detectable in 70% of the 45,000-rev/min pellet fractions from Nonidet extracts of uninfected rat cells. The product of this endogenous polymerase activity was DNA, as determined by its density in alkaline Cs_2SO_4 centrifugation. This endogenous DNA polymerase reaction in the particulate fraction of uninfected rat cells was sensitive to pretreatment with ribonuclease, indicating an RNA template. The presence of all four deoxyribonucleoside triphosphates was required for the polymerase reaction. Thus, what is apparently an RNA-directed DNA polymerase-containing particle could be isolated from these uninfected cells. The larger amount of endogenous DNA polymerase activity in the R(B77) cells may be a secondary result of the viral transformation of these cells. Alternatively, it is possible that the enzyme involved may be virus-coded. The function of these RNA-directed DNA polymerase-containing particles in uninfected and B77 virus-infected rat cells is unclear. They do not appear to represent precursors of any RNA tumor virus related to those studied here. These particles may represent a chance

association between a cell DNA polymerase and cell RNA, or they may be related to an RNA-directed DNA polymerase involved in normal development (21). The finding of a particulate ribonuclease-sensitive DNA polymerase system in uninfected cells demonstrates the difficulty of concluding that particulate ribonuclease-sensitive DNA polymerase systems necessarily represent oncogenic viruses.

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LITERATURE CITED

- Altaner, C., and H. M. Temin. 1970. Carcinogenesis by RNA sarcoma viruses. XII. A quantitative study of infection of rat cells *in vitro* by avian sarcoma virus. *Virology* 40:113-134.
- Baltimore, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. *Nature (London)* 226:1209-1211.
- Britten, R. J., and D. E. Kohne. 1968. Repeated sequences in DNA. *Science* 161:529-540.
- Chopra, H., and D. J. Taylor. 1970. Virus particles in rat mammary tumors of varying origin. *J. Nat. Cancer Inst.* 44:1141-1147.
- Coffin, J. M., and H. M. Temin. 1971. Comparison of Rous sarcoma virus-specific deoxyribonucleic acid polymerases in virions of Rous sarcoma virus and in Rous sarcoma virus-infected chicken cells. *J. Virol.* 7:625-634.
- Duesberg, P. H., and E. Canaan. 1970. Complementarity between Rous sarcoma virus (RSV) RNA and the *in vitro*-synthesized DNA of the virus-associated DNA polymerase. *Virology* 42:783-788.
- Garapin, A. C., J. Leong, L. Fanshier, W. E. Levinson, and J. M. Bishop. 1971. Identification of virus-specific RNA in cells infected with Rous sarcoma virus. *Biochem. Biophys. Res. Commun.* 42:919-925.
- Gelderblom, H., H. Bauer, and H. Frank. 1970. Investigations on virus production in RSV mammalian tumors. *J. Gen. Virol.* 7:33-45.
- Green, M., H. Rokutanda, and M. Rokutanda. 1971. Virus specific RNA in cells transformed by RNA tumor viruses. *Nature New Biol. (London)* 230:229-232.
- Kajima, M., and M. Pollard. 1968. Wide distribution of leukaemia viruses in strains of laboratory mice. *Nature (London)* 212:188-189.
- Kotler, M. 1971. Interactions of avian sarcoma virus with rat embryo cells in culture. *J. Gen. Virol.* 12:199-206.
- Lozeron, H. A., and W. Szybalski. 1966. Suppression of RNA precipitation during Cs_2SO_4 density gradient centrifugation. *Biochem. Biophys. Res. Commun.* 23:612-618.
- Miyazawa, Y., and C. A. Thomas. 1965. Nucleotide composition of short segments of DNA molecules. *J. Mol. Biol.* 11:223-237.
- Mizutani, S., D. Boettiger, and H. M. Temin. 1970. DNA-dependent DNA polymerase and a DNA endonuclease in virions of Rous sarcoma virus. *Nature (London)* 228:424-427.
- Moore, E. G., and H. M. Temin. 1971. No correlation between conversion by RNA sarcoma viruses and increased agglutinability of cells. *Nature New Biol. (London)* 231:117-118.
- Nowinski, R. C., L. J. Old, E. A. Boyse, E. deHarven, and

- G. Geering. 1968. Group-specific viral antigens in the milk and tissues of mice naturally infected with mammary tumor virus or Gross leukemia virus. *Virology* **34**:617-629.
16. Solymosy, F., I. Fedorcsak, A. Gulyas, G. L. Farkas, and L. Ehrenberg. 1968. A new method based on the use of diethyl pyrocarbonate as a nuclease inhibitor for the extraction of undegraded nucleic acid from plant tissues. *Eur. J. Biochem.* **5**:520-527.
17. Spiegelman, S., A. Burny, M. R. Das, J. Keydar, J. Schlom, M. Travnicek, and K. Watson. 1970. DNA-directed DNA polymerase activity in oncogenic RNA viruses. *Nature (London)* **227**:1029-1031.
18. Svoboda, J., O. Machala, and I. Hlozanek. 1967. Influence of Sendai virus on RSV formation in mixed cultures of virogenic mammalian cells and chicken fibroblast. *Folia Biol. (Prague)* **13**:155-157.
19. Temin, H. M. 1967. Studies on carcinogenesis by avian sarcoma viruses. V. Requirement for new DNA synthesis and for cell division. *J. Cell. Physiol.* **69**:53-63.
20. Temin, H. M. 1968. Studies on carcinogenesis by avian sarcoma viruses. VIII. Glycolysis and cell multiplication. *Int. J. Cancer* **3**:273-282.
21. Temin, H. M. 1971. Guest editorial: the provirus hypothesis. *J. Nat. Cancer Inst.* **46**:III-VII.
22. Temin, H. M., and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature (London)* **226**:1211-1213.
23. Zilber, L. A. 1965. Pathogenicity and oncogenicity of Rous sarcoma virus for mammals. *Progr. Exp. Tumor Res.* **7**:1-48.