Reverse Transcriptase-Containing Particles Induced in Rous Sarcoma Virus-Transformed Rat Cells by Arginine Deprivation

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Incubation of rat cells transformed by Rous sarcoma virus (RSV) in an arginine-deficient medium resulted in accumulation of particles in the culture medium. Such particles did not appear when the transformed rat cells were incubated in a complete medium nor in the medium of primary rat cells which were incubated either in arginine-deficient or complete media. The particles which were released from the arginine-deprived transformed rat cells resemble C-type particles in their properties. These particles band in sucrose gradients at a density of 1.16 g/ml and contain 35S ribonucleic acid (RNA) molecules and a reverse transcriptase activity. Analysis of the cytoplasm of transformed and primary rat cells, deprived and undeprived of arginine, revealed the presence of reverse transcriptase-containing particles which banded in sucrose gradients at a density of 1.14 g/ml. These particles differed from the particles released into the medium by the arginine-deprived RSV-transformed rat cells. The deoxyribonucleic acid (DNA) molecules synthesized *in vitro* by the reverse transcriptase present in the particles isolated from the medium of arginine-deprived cells hybridized to RSV RNA, whereas the DNA synthesized by the cell-bound enzyme had no homology to RSV RNA.

Rat embryo fibroblasts, transformed *in vitro* by Rous sarcoma virus (RSV) do not synthesize virions although they contain RSV genomes (1, 6). The latent viral genomes can be activated by cocultivation or fusion of the transformed cells with permissive cells (1, 8).

Temin postulated (11, 12) that the integration of the viral deoxyribonucleic acid (DNA), synthesized by the virion-associated reverse transcriptase (2, 9, 10, 13), into the host genome is an essential step in the transformation process. Thus, the rescue of the viral genome might be initiated by the induction of the provirus present in the transformed cells. It was previously demonstrated that arginine deprivation of Burkitt's lymphoblasts (7) resulted in the induction of the synthesis of the herpes-type DNA genomes (EB virions) present in the lymphoblasts (3, 14). Therefore, the effect of arginine deprivation on the RSV-transformed rat cells was studied.

Recently, Coffin and Temin (5) demonstrated that the homogenized RSV-transformed rat cells contain particles which band in sucrose gradients at a density of 1.13 to 1.14 g/ml and contain a ribonucleic acid (RNA)-directed DNA polymerase (reverse transcriptase). These particles are not released into the medium. The RNA tem-

plate of this enzyme is not related, by molecular hybridization, to the RNA of the transforming virus or rat C-type virus.

In the present study, we demonstrate that incubation of the RSV-transformed rat cells in an arginine-deficient medium results in the release to the medium of particles which resemble C-type virions in their characteristics. These particles differed from the cell-associated particles described by Coffin and Temin (5).

MATERIALS AND METHODS

Cell cultures. Primary cultures of fibroblasts were prepared from 10- to 12-day-old White Leghorn chicken embryos and from 14-day-old Sprague Dawley rat embryos. The cells were grown in Eagle's minimal essential medium (GIBCO, Grand Island, N.Y.) with 20% tryptose phosphate broth (Difco Lab., Detroit, Mich.) and 5% calf serum. Cells were grown in 60-mm or 100-mm dishes (NUNC, Denmark) or in glass Roux bottles. Secondary cultures were prepared by transfer of 2×10^6 cells to plastic dishes (100 mm diameter) or to milk dilution bottles and 20×10^6 cells to Roux bottles.

Treatment of transformed rat cells (R[B77]). R(B77) is a cell line originated from rat embryo fibroblasts transformed *in vitro* by RSV (B77 strain) (1, 8). Cell cultures in Roux bottles, each containing $6 \times$

10^7 to 8×10^7 cells, were incubated in Eagle's medium without arginine, serum, and tryptose phosphate. The medium of the arginine-deprived cultures was harvested after 5 days and stored at -70°C .

Virus purification. Stocks of RSV (B77) were prepared by suspending freshly trypsinized chicken embryonic cells (about 10^8 cells) in 5 ml of medium containing 10^6 focus-forming units/ml. The suspension was incubated for 45 min at 37°C and then diluted with fresh medium, and 10^7 cells were seeded to each plastic plate (100 mm diameter) and incubated at 37°C . Four days later, the cells were trypsinized, diluted in fresh medium, and sedimented by centrifugation (3 min at $800 \times g$). The cells were transferred to gelatin (0.1%) coated plastic plates to ensure cell attachment and seeded at a concentration of 2×10^6 cells/plate. After 2 days, when transformation was apparent, the virus-containing culture medium was harvested twice a day. The virus-containing medium was frozen at -70°C immediately after collection. The pooled culture fluids were centrifuged for 10 min at 8,000 rev/min by using a no. 19 rotor of the Beckman preparative ultracentrifuge. The supernatant fluid was centrifuged for 150 min at 19,000 rev/min in the same rotor to sediment the virions. The virus-containing pellets were suspended in approximately 0.01 to 0.03 of the starting volume in Eagle's medium containing 5% calf serum. The virus concentrates were loaded on linear sucrose gradients (15–65%, w/v) made in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0) containing 0.001 M ethylenediaminetetraacetic acid (EDTA) and centrifuged for 180 min at 45,000 rev/min in an SW50.1 rotor. The band of purified virions was collected and diluted with buffer, and the virus was sedimented by centrifugation for 60 min at 45,000 rev/min in a Beckman no. 50 Ti rotor. The pellets were suspended in Tris-EDTA buffer containing 0.5% Nonidet P-40 and 0.2% dithiothreitol and kept frozen at -70°C until assayed for reverse transcriptase activity.

Isolation of particles from cells. Extracts were prepared from transformed and primary rat cells as described by Coffin and Temin (4, 5). The high-speed pellets were suspended in Tris-EDTA buffer containing 0.4% dithiothreitol (Calbiochem, Los Angeles, Calif.) and banded by centrifugation in sucrose gradients under conditions used for virus banding.

Labeling of cells. The medium of transformed cultures of chicken embryo fibroblasts or rat transformed cells was changed to 7 ml/plate (100 mm diameter) of Eagle's medium containing 1 or 2 μCi of uridine-5- ^3H (13.8 Ci/mmol, Nuclear Research Center, Negev, Israel) per ml or 0.1 μCi of uridine-2- ^{14}C (62 mCi/mmol, Radiochemical Center, Amersham, England) per ml. The radioactively labeled virions were isolated by the above mentioned technique.

Polymerase assay. The standard polymerase assay of Temin and Mizutani (13) was used. Reactions were started by adding 0.1 ml of standard reaction mixture containing 2.5 μCi of ^3H -thymidine-5'-triphosphate (16.8 Ci/mmol, Schwarz BioResearch Inc.) with 10 nmoles of the other three unlabeled deoxynucleoside triphosphates, 7.5 nmoles of adenosine triphos-

phate, 10 mmoles MgCl_2 , 20 mmoles of KCl, 2.5 μg of phosphoenol-pyruvate, 10 μg of pyruvate kinase (Sigma Chemical Company, St. Louis, Mo.), and 0.025 ml of enzyme preparation. Samples were also incubated in the presence of ribonuclease (Worthington Biochemical Corp. Freehold, N.J.). Duplicate samples of 0.025 ml each were taken at the indicated time, into 5 ml of cold 10% trichloroacetic acid. The radioactive precipitates were counted by the Packard Tri-Carb liquid scintillation counter.

Nucleic acids. RNA was extracted from purified B77 virus, from particles isolated from the medium of arginine-deprived cells, and from cell-associated particles. The RNA was extracted by treatment with 5% Sarkosyl in the presence of 2% diethyl pyrocarbonate (Baycovin; Bayer, Leverkusen, Germany). The RNA was gently loaded on sucrose gradients (10–30% [w/v] made in 0.01 M Tris-hydrochloride, 0.001 M EDTA, 0.2 M NaCl, pH 7.4) together with 20 μg of HeLa cell RNA as a carrier and centrifuged in an SW50.1 rotor for 100 min at 45,000 rev/min at 4°C . Fractions were collected, and the trichloroacetic acid-precipitable radioactivity was determined.

The products of the viral polymerase reactions, after 15 min of incubation at 39°C , were extracted and analyzed by centrifugation in sucrose gradients to isolate the 70S RNA-DNA hybrid (10).

Nucleic acids for hybridization tests. Three large-scale polymerase reactions were set up and incubated for 60 min: (i) DNA synthesis by the reverse transcriptase in the C-type particles isolated from arginine-deprived R(B77) cells; (ii) DNA synthesis by the reverse transcriptase present in the pellets from R(B77) cells; and (iii) DNA synthesis by RSV-associated reverse transcriptase. The *in vitro* synthesized labeled DNA was extracted with phenol-cresol (10), poured into cold 100% ethanol (1 volume per 2.5 volumes) and was left overnight at -20°C . The nucleic acids were sedimented by centrifugation for 20 min at 20,000 rev/min and suspended in $0.1 \times \text{SSC}$.

RNA was extracted from purified preparations of RSV (B77) and from *Escherichia coli* B cells by the same extraction procedure. After sedimentation from ethanol, each RNA preparation was suspended in $2 \times \text{SSC}$.

Nucleic acid hybridization tests. The labeled DNA which was extracted from each polymerase reaction was brought to 0.2 N NaOH, boiled for 5 min, and rapidly cooled. A small amount of phenol red was added, and the mixture was neutralized with 0.2 N HCl.

To the samples of labeled DNA (containing approximately 5,000 counts/min) 5 μl of phenol and 60 μg of RSV RNA or *E. coli* RNA were added. Annealing was performed by incubation of the DNA-RNA mixtures in $2 \times \text{SSC}$ at 65°C for 5.5 hr.

The samples were then analyzed by centrifugation in Cs_2SO_4 gradients. The Cs_2SO_4 solution was prepared in 0.05 M EDTA-0.001 M Tris (pH 7.8), and the mixture was adjusted to the density of 1.52 g/ml and centrifuged for 62 hr in a Spinco 50 Ti rotor at 45,000 rev/min at 22°C .

Fractions were collected dropwise, and the radio-

activity was determined in each fraction. The density of selected fractions was determined by weighing a 100- μ l sample in a Mettler balance.

RESULTS

Isolation of particles from the medium of arginine-deprived cells. The R(B77) cells and cultured embryonic rat fibroblasts were incubated in complete and in arginine-deficient media in the presence of ^3H -uridine. The medium was removed 5 days later, and the particulate material in each medium preparation was deposited by centrifugation. The pellet were suspended and analyzed by centrifugation in sucrose gradients with B77 virions as a marker. It was found (Fig. 1) that particles labeled with ^3H -uridine were obtained

FIG. 1. Isolation of RNA-containing particles released from R(B77) cells and uninfected rat fibroblasts. Transformed and uninfected rat cells were incubated in the presence of ^3H -uridine, and the particles present in the culture medium were collected and analyzed by centrifugation in sucrose gradients. A, R(B77) transformed cells deprived of arginine; B, R(B77) grown in complete Eagle's medium; C, uninfected rat cells deprived of arginine; D, uninfected rat cells in complete medium; E, B77 virions labeled with ^3H -uridine used as a marker. The particles present in the culture fluids were centrifuged in a linear sucrose gradient (15–65%, w/v) (see Material and Methods). Fractions were collected from the bottom of each tube, and the density (measured in a refractometer) and radioactivity in each fraction were determined.

FIG. 2. RNA-directed DNA polymerase activity associated with the particles released from arginine-deprived and undeprived R(B77) cells and uninfected rat cells. Unlabeled particles released from the rat cells were isolated from the culture medium by centrifugation in sucrose gradients (15–65%, w/v). The gradients were collected, and each fraction was treated with Nonidet P-40 and divided into two portions, one of which was left untreated (●) and the other was treated with 50 μg of ribonuclease per ml (○) and was incubated for 10 min at 37 C. Each fraction was assayed for the incorporation of ^3H -thymidine monophosphate into DNA. Particles were obtained from arginine-deprived R(B77) cells (A), from undeprived R(B77) cells (B), from arginine-deprived uninfected rat cells (C), and from undeprived uninfected rat cells (D). Purified B77 virions were similarly centrifuged in a sucrose gradient (E). The density of various fractions was determined.

only from the medium of the arginine-deficient R(B77) cells (Fig. 1A). These particles banded in the gradient at a density of 1.16 g/ml and resembled in this respect Rous sarcoma virions (Fig. 1E). These particles also have a morphology of C-type particles when viewed in the electron microscope. Such particles were not found in the medium of undeprived R(B77) cells (Fig. 1B) or arginine-deprived cultured embryonic rat fibroblasts (Fig. 1C). Nevertheless, particles which were labeled with ^3H -uridine and banded in the sucrose gradient at a density of 1.135 to 1.140 g/ml were found in the medium of undeprived R(B77) cells (Fig. 1B) and in rat embryonic fibro-

blasts (Fig. 1C and D). These might represent ribosomes released from disrupted cells. In the primary embryonic rat cells, radioactive RNA also banded at the top of the sucrose gradient (Fig. 1C and D).

Properties of the particles isolated from the medium of arginine-deprived cells. The existence of a reverse transcriptase activity in the particles band (2, 13) was determined by assaying the enzyme activity in each fraction of the sucrose gradient (Fig. 2). It was found that the particles which were isolated from the medium of arginine-deprived R(B77) cells (Fig. 2A), which banded at a density of 1.16 g/ml, incorporated ^3H -thymidine monophosphate into DNA, and the incorporation was completely inhibited by ribonuclease treatment. This result is taken to indicate that the particles isolated from the medium of arginine-deprived R(B77) cells contain a reverse transcriptase activity. In this respect the R(B77) particles resembled Rous sarcoma virions (Fig. 2E). A second band of DNA polymerase activity was observed at a density of 1.12 g/ml, but this enzymatic DNA synthesis was not sensitive to

ribonuclease treatment, thus indicating that the enzyme might be a DNA-dependent DNA polymerase. Reverse transcriptase activity was not found in the medium of R(B77) cells incubated in complete medium (Fig. 2B) or in arginine-deprived (Fig. 2C) and undeprived (Fig. 2D) embryonic rat cells.

Analysis of the radioactive RNA present in the particles isolated from the medium of arginine-deprived transformed rat cells is presented in Fig. 3A. It was found that 35S RNA molecules can be reproducibly isolated from the particles which band in the sucrose gradients at a density of 1.16 g/ml. Under similar conditions the RNA extracted from B77 virions was found to have a sedimentation coefficient of 70S and resemble the RSV RNA genome (Fig. 3A). Studies on the kinetics of DNA synthesis by the reverse transcriptase present in these particles (Fig. 3B) revealed that (i) DNA synthesis continued for 80 min before it stopped and (ii) the synthesis of DNA was reduced, but not completely inhibited, by ribonuclease treatment.

To characterize further the reverse transcriptase

FIG. 3. Characterization of the particles isolated from the medium of arginine-deprived R(B77) cells. A, sedimentation in a sucrose gradient of ^3H -RNA extracted from the particles released into the medium of arginine-deprived cells ($\bullet$). ^{14}C -RNA extracted from B77 virions was centrifuged, and RSV RNA was used as a marker ($\circ$). B, Kinetics of DNA synthesis by the RNA-directed DNA polymerase present in the particles released from arginine-deprived cells. The particles were disrupted with Nonidet P-40 (see Material and Methods), one-half ($\circ$) was incubated with ribonuclease (50 $\mu\text{g}/\text{ml}$), while the rest was similarly incubated untreated ($\bullet$) for 10 min at 37 C. The four nucleoside triphosphates were added to the disrupted virions and incubated for 2 hr. Samples were removed at different time intervals to determine the extent of DNA synthesis.

present in the particles isolated from the medium of arginine-deprived R(B77) cells, the particles were incubated *in vitro* with the four nucleoside triphosphates, and the RNA genomes on which DNA molecules were synthesized were extracted and isolated by centrifugation in sucrose gradients (10). It was found (Fig. 4A) that part of the radioactively labeled DNA molecules, synthesized *in vitro* by the reverse transcriptase present in the particles, had a sedimentation coefficient of 70S, similar to the hybrid molecules formed by the reverse transcriptase of RSV (Fig. 4B). This finding suggested that the RNA present in the particles, isolated from the medium of arginine-deprived R(B77) cells, is indeed 70S and that the 35S molecules presented in Fig. 3A could be subunits of the RNA genomes.

Characterization of the particles isolated from the arginine-deficient medium by the molecular hybridization technique. To determine the nature of the RNA genomes present in the particles which were released from arginine-deprived R(B77) cells, we studied the homology to RSV (B77) RNA using the nucleic acid hybridization technique. The RNA-DNA hybrids were resolved by centrifugation in Cs_2SO_4 gradients. The results presented in Fig. 5A demonstrate that the DNA synthesized by the reverse transcriptase present in the particles, which were obtained from the medium of arginine-deprived R(B77), hybridized with RSV (B77) RNA. No hybrid molecules were obtained when *E. coli* RNA was employed. The hybrid was identical to the hybrid formed by the RSV DNA and RNA (Fig. 5C). No homology was found between the DNA synthesized by the reverse transcriptase of the cytoplasmic pellet and RSV RNA (Fig. 5B). These results proved that the genomes present in the particles released by arginine-deficient cells are identical to the RNA genomes of the virus which had transformed the rat cells.

Characterization of particles containing RNA and reverse transcriptase activity isolated from R(B77) cells and rat fibroblasts. R(B77) cells and rat fibroblasts were labeled with ^3H -uridine and incubated for 5 days in arginine-deficient or complete medium. The cells were treated with Nonidet P-40, and the high-speed pellet was centrifuged in sucrose gradients under conditions similar to those used for the isolation of B77 virions. It was found (Fig. 6) that ^3H -uridine-labeled particles, which banded in the sucrose gradients at a density of 1.14 g/ml, could be isolated from the R(B77) cells and the rat fibroblasts, either deprived or undeprived of arginine.

The high-speed pellet obtained from arginine-

FIG. 4. Characterization of the DNA synthesized on the RNA template present in particles from the medium of arginine-deprived cells and the particles isolated from the cells. Particles were isolated from the medium and from the cells after arginine deprivation. The particles were treated with Nonidet P-40 and incubated for 15 min under conditions which permitted DNA synthesis. Particles were disrupted with Sarkosyl to release the RNA molecules which served as template for the reverse transcriptase (RNA-DNA hybrids). The RNA was centrifuged in sucrose gradients under conditions for the isolation of 70S RNA molecules. A, The sedimentation profile of DNA synthesized by the enzyme present in the particles released into the medium by arginine-deprived R(B77) cells (●) as well as DNA synthesized by the enzyme obtained from the particles (density 1.14 g/ml) isolated from R(B77) cells-cytoplasm (○); B, sedimentation of nascent DNA molecules synthesized by the reverse transcriptase of B77 virions, used as a marker. Part of the nascent DNA molecules (●) sediment in the sucrose gradient together with the viral 70S genomes.

deprived R(B77) cells was found to contain an RNA-directed DNA polymerase activity (Fig. 7A). This enzyme was found to be associated with the particles obtained from such cells and banded at a density of 1.14 g/ml (Fig. 7B). Also, a DNA polymerase activity was found at a den-

FIG. 5. Characterization of the particles released from arginine-deprived cells by the nucleic acids hybridization technique. A, The homology between the DNA synthesized by the reverse transcriptase present in the particles released from arginine-deprived cells and RSV RNA was studied by hybridization. The RNA-DNA hybrid molecules were found to band at 1.58 g/ml. As a control, the labeled DNA was hybridized with purified *E. coli* RNA; no hybrid molecules were found (not shown). B, Hybridization between the DNA synthesized *in vitro* by the reverse transcriptase associated with particles present in the R(B77) cells. No hybrid molecules were noted also when hybridized to *E. coli* DNA (not shown). C, Hybridization between DNA synthesized by RSV reverse transcriptase and the homologous RNA. Hybrid molecules were found at a density of 1.58 g/ml. No hybrids were noted when incubated with *E. coli* RNA (not shown). (●) Radioactivity; (---●) density.

FIG. 6. Isolation of RNA-containing particles from R(B77) cells and uninfected rat cells. Cytoplasmic extracts were prepared from ^3H -uridine-labeled R(B77) cells deprived of arginine (A), R(B77) cells incubated in complete medium (B), uninfected rat cells incubated in arginine-deficient medium (C), and uninfected rat cells grown in complete medium (D). The extracts were centrifuged to sediment particles which were then re-centrifuged in sucrose density gradients. The radioactivity in each fraction (●) and the density along the gradients (---●) were determined.

sity of 1.12 g/ml (Fig. 7B). It was found (Fig. 7B) that DNA synthesis by the particles in the band with a mean density of 1.14 g/ml was not stimulated by the addition of DNA to the reaction, whereas DNA synthesis by the enzyme present at the density of 1.12 g/ml was markedly enhanced. The RNA-directed DNA synthesis associated with the particles with a density of 1.14 g/ml requires for its activity either Mn^{2+} (1.0 mM optimal concentration) or Mg^{2+} (10 mM optimal concentration) (not shown) and resembles in this property the reverse transcriptase of RSV (2, 13). These results confirm the findings of Coffin and Temin (5). Similarly, it was found (Fig. 5B) that no homology could be demonstrable between the DNA synthesized *in vitro* by the cell-associated reverse transcriptase and RSV RNA.

The results shown in Fig. 4 and 5 distinguish between the RNA-directed DNA polymerases present in R(B77) cells and the reverse transcriptase in the particles released to the medium; the former enzyme did not synthesize DNA on a 70S RNA template (as does the latter enzyme and the polymerase of B77 virions) and its DNA product did not hybridize to RSV RNA.

DISCUSSION

The results of the present study demonstrate that R(B77) cells, which were transformed by RSV but do not produce virions, release particles similar to the C-type after incubation in an arginine-deficient medium. Such particles were not revealed in the medium of arginine-deprived normal rat fibroblasts nor in R(B77) cells incu-

FIG. 7. Further characterization of particles isolated from arginine-deprived rat cells. A, Kinetics of DNA synthesis by the RNA-directed DNA polymerase present in the cytoplasm of R(B77) cells. High-speed pellet was prepared from 10^8 cells and dissolved in 50 μl of Nonidet P-40 containing buffer. Part of the preparation was treated with ribonuclease (50 $\mu\text{g}/\text{ml}$) for 10 min at 37 C (○), while the rest was left untreated (●). The activity of the RNA-directed DNA polymerase in the preparations was studied by incubation *in vitro*. B, Sucrose density gradient centrifugation of DNA polymerase-containing fractions obtained from arginine-deprived R(B77) cells. The high-speed pellet, prepared from 10^8 cells was centrifuged in a sucrose gradient (15–65%, w/v) for 3 hr at 45,000 rev/min in a Beckman SW50.1 rotor. Each fraction was divided into two portions and assayed for RNA polymerase activity in the absence (●) or presence (○) of exogenous DNA (5 μg of calf thymus DNA per fractions). The figures 1.14 and 1.12 represent the density of the fractions (g/ml).

bated in complete medium. The particles isolated from the medium of arginine-deprived R(B77) cells resemble C-type particles in their density (1.16 g/ml), the molecular size of the RNA, the presence of an RNA-directed DNA polymerase, and the formation of a 70S RNA-DNA hybrid during DNA synthesis *in vitro*. Hybridization experiments revealed a homology between the genomes which were rescued from R(B77) cells and the genomes which transformed the cells. It is of interest that arginine deprivation of human leukemic cells also resulted in the release to the medium of C-type particles (*submitted for publication*).

Coffin and Temin (5) demonstrated that RSV-transformed rat R(B77) cells contain particles which band in sucrose gradients at a density of 1.14 g/ml. These particles have an RNA-dependent DNA polymerase and RNA which do not hybridize to the DNA synthesized by the transforming RSV B77 nor with murine sarcoma virus. The results of the hybridization tests also demonstrate that the RNA template for the cell-associated reverse transcriptase is not RSV RNA. The nature of the template is under investigation.

The particles released from the arginine-deprived R(B77) cells differ in their properties from the particles isolated from the cytoplasm of R(B77) cells, although both types of particles

contain RNA and a reverse transcriptase activity. The presence of cell-associated particles in normal rat cells might suggest that these particles are constitutive components of cells. The induction of the synthesis and release of C-type particles might be an independent phenomenon which is related to the presence in the transformed cells of Oncorna viral genomes. We assume that arginine deprivation, which affects the cellular metabolism, as reported in Burkitt's lymphoblasts (3, 14), results in the synthesis of C-type particles. The mechanism of this phenomenon is not yet known.

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