Characterization of Intracellular Ribonucleic Acid Specific for the Murine Sarcoma-Leukemia Virus Complex

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Virus particles were continuously produced by a cell line (78A1) of rat embryo fibroblasts that had been transformed by the murine sarcoma-leukemia virus complex. Since most of the mature virions were found in the extracellular fluid and were not cell-associated, a measurable quantity of viral ribonucleic acid (RNA) could not be extracted from these cells. Cycloheximide, a protein inhibitor, was successfully used to accumulate viral RNA within the cells. This ribonuclease-sensitive RNA, with a sedimentation coefficient of 71S, had the same base composition as the high molecular weight RNA ($S_{20,w} = 71$) isolated from purified virions released by the transformed cells.

The Moloney isolate (13) of murine sarcoma virus (MSV) contains the Moloney murine leukemia virus (MLV) that acts as a "helper" for the defective MSV (10). Recently, Bernard et al. (2) described a cell line, 78A1, obtained after transformation of Wistar rat embryo fibroblasts by the MSV-MLV complex. The 78A1 cells were shown to be transplantable in 10-day-old rats and to produce both MSV and MLV (2).

The physical and chemical nature of the different strains of murine leukemia viruses and their nucleic acid has been well-characterized (1, 6, 8, 14). However, the previous reports are concerned with nucleic acid characterizations carried out on purified extracellular virus preparations only.

This study is concerned with the physical and chemical nature of the MSV-MLV complex in both its intracellular and extracellular state. Cycloheximide was used to block viral protein synthesis so that a pool of viral-specific nucleic acid could be accumulated and retained within the infected cells.

MATERIALS AND METHODS

Cell cultures and media. The 78A1 line of chronically infected transformed rat embryonic cells was used (2). This line was kindly supplied by M. Boiron and C. Bernard, Inst. de Recherches sur les Maladies du Sang, University of Paris, France. It was obtained in the 84th passage and was maintained in serial passage

by subculturing every 72 hr. The majority of the experiments were performed with cells 11 to 21 passages after receipt. Cells were grown in Eagle's medium with 10% calf serum and 0.075% NaHCO_3 (for cells grown in stoppered bottles) or 0.225% NaHCO_3 (for cells in petri dishes in a 5% CO_2 atmosphere).

As a control, normal rat (Sprague-Dawley strain) embryo fibroblasts in passages 3 to 14 were utilized. The growth medium was the same as for the 78A1 cells.

Buffers, reagents, and enzymes. The composition and sources were as follows: TNE buffer consisted of tris(hydroxymethyl)aminomethane (Tris)-chloride, 10^{-2} M (pH 7.4); NaCl, 10^{-1} M; and disodium ethylenediamine-tetraacetate (EDTA), 10^{-3} M. TNM buffer consisted of Tris-chloride, 10^{-2} M (pH 7.4); NaCl, 10^{-1} M; and MgCl_2 , 10^{-3} M. Polyvinyl sulfate (200 $\mu\text{g}/\text{ml}$) was obtained from General Biochemicals Corp., Chagrin Falls, Ohio. Cycloheximide was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Bovine pancreatic deoxyribonuclease (ribonuclease-free) and ribonuclease were obtained from Worthington Biochemical Corp., Freehold, N.J. $\text{Na}_2\text{H}^{32}\text{PO}_4$ (500 mc/mole) was obtained from the New England Nuclear Corp., Boston, Mass. ^3H -uridine (8 c/mole) and ^{14}C -leucine (170 mc/mole) were obtained from Schwarz Bio Research Inc., Orangeburg, N.Y. Sodium dodecyl sulfate (SDS) was prepared as a 10% solution in water or as a 40% solution in ethyl alcohol. Toluene-based scintillation fluids contained 1 liter of toluene and 5 g of 2,5-diphenyloxazole.

Growth curve experiments. Approximately 10^6 cells of the 78A1 line were seeded in 60-mm plastic petri dishes, containing 5 ml of growth medium, and were incubated at 37 C. The concentration of extracellular

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virus particles and of total virus particles per culture was determined in each of two replicate cultures harvested at 12-hr intervals. Cell counts were performed on trypsinized cell suspensions. Extracellular virus was that present in the supernatant fluid obtained by low-speed centrifugation ($1000 \times g$ for 10 min) of culture fluids. For total virus, the cells were lysed by three cycles of freezing and thawing in the culture fluid, and then the preparation was clarified by low-speed centrifugation. Both types of virus preparations were stored at -90°C until assayed by electron microscopy for particle concentration.

Electron microscopy. Virus particle quantitation was carried out on negatively stained preparations using the sedimentation-pseudoreplication procedure (12, 15).

Preparation of radioactive virus. Approximately 10^7 cells were seeded in 16-oz (473 ml) flat bottles containing 20 ml of the growth medium supplemented with 0.5 mc of $\text{Na}_2\text{H}^{32}\text{PO}_4$ or 0.2 mc of ^3H -uridine. The supernatant fluid containing the virus was collected every 24 hr for 3 days with replacement of fresh radioactive medium at each interval. These fractions were pooled, and the particles were purified with a purified preparation of nonradioactive virus particles as carrier.

Virus purification. Approximately 2 liters of supernatant fluid harvested from 48- to 72-hr-old 78A1 cultures was concentrated to 100 ml with Carbowax (20 M). The virions were purified in two steps. (i) The concentrated crude virus suspension was placed on top of a double-layer cushion of sucrose solution (8 ml of 70% sucrose and 10 ml of 30% sucrose in TNE buffer) and centrifuged at $107,000 \times g$ for 1.5 hr at 4°C in a Spinco SW25.2 rotor. This resulted in the appearance of three distinct bands. Electron micrographs of a portion from each band revealed that the heaviest band contained mostly ribosomal particles and cell debris along with some trapped virus particles; the middle band contained a relatively pure population of MSV-MLV particles (Fig. 1). The top band consisted of proteinaceous material of cellular origin.

To increase the concentration of pure virions, the medium above the sucrose layers was removed and replaced with more of the virus-containing medium. After centrifugation, the middle virus band was carefully removed.

(ii) The virus was further purified by centrifugation at $231,000 \times g$ for 3 hr in a linear 15 to 60% sucrose gradient (in TNE; Spinco SW50 rotor). Fractions were collected from the bottom of the tube and analyzed for optical density, radioactivity, particle counts, and buoyant density.

Fluids from the control rat embryo fibroblast cultures failed to contain any particles similar to the ones in the 78A1 cultures.

Extraction of viral RNA. Sterile conditions were used to isolate the nucleic acid. The purified virus preparation (with a density of 1.16 g/cm^3 in sucrose) was diluted threefold with TNE; polyvinyl sulfate ($2 \mu\text{g/ml}$) and SDS (to make 0.5%) were added to the virus suspension. After incubation for 3 to 5 min at room temperature, the mixture was depro-

teinized with phenol saturated with TNE buffer by gently shaking for 5 to 10 min at 0 to 4°C ; it was then centrifuged at $12,000 \times g$ for 5 min. The aqueous layer was removed, and the deproteinization procedure was repeated twice. The nucleic acid in the aqueous layer was precipitated with two volumes of 90% ethyl alcohol (containing 0.01 M sodium acetate buffer, pH 5.1), was allowed to stand for at least 1 hr at -40°C , and was centrifuged at $12,100 \times g$ for 10 min. This precipitate was then suspended in TNE buffer, and the precipitation procedure was repeated.

Extraction of intracellular RNA. Cellular RNA was isolated from monolayers in 16-oz (473 ml) bottles containing about 10^7 cells. After removing the culture fluids, the monolayers were incubated for 5 min at room temperature with 2 ml of a solution containing SDS (1.0%) and polyvinyl sulfate ($2 \mu\text{g/ml}$) in TNE buffer. Deproteinization of the resulting cell lysate and initial precipitation of the nucleic acid in the aqueous layer were performed as described above. The precipitate was then dissolved in TNM buffer, deoxyribonuclease ($20 \mu\text{g/ml}$) was added, and the solution was incubated at 37°C for 30 min. This mixture was then deproteinized once in cold phenol, and the RNA was extracted from the aqueous layer as described above.

Velocity sedimentation of RNA in sucrose gradients. The RNA preparation dissolved in 0.2 ml of TNE buffer was layered on top of 5.0 ml of a linear sucrose gradient (5 to 30% in TNE) and was centrifuged for 2.25 hr at $150,000 \times g$ in a Spinco SW50 rotor at 5°C . After centrifugation, 15-drop fractions were collected from the bottom of each gradient with a Buchler piercing unit.

Ribonuclease treatment of 71S RNA. Fractions from the above centrifugation containing high molecular weight RNA ($S_{20,w} = 71$) were pooled and diluted twofold with TNM buffer. The RNA was precipitated with two volumes of cold ethyl alcohol at -40°C . (Approximately 50% of the intracellular RNA is lost during this precipitation.) The precipitate was dissolved in TNM buffer to a concentration of 3,000 counts per min per 0.5 ml for extracellular RNA and 1,500 counts per min per 0.5 ml for intracellular RNA. The solutions were incubated for 0.5 hr at 37°C in the presence of ribonuclease ($50 \mu\text{g/ml}$). Bovine serum albumin ($500 \mu\text{g}$) was then added, and the contents were precipitated with an equal volume of cold 10% trichloroacetic acid. After chilling in an ice bath for 10 min, the contents were filtered through a membrane filter ($0.45 \mu\text{m}$, 25 mm; Millipore Corp., Bedford, Mass.), washed, and dried; the radioactivity was then measured in a Beckman LS 250 liquid scintillation counter.

Determination of base composition. ^{32}P -labeled 71S RNA (4,000 counts/min) and 0.2 mg of yeast RNA (added as carrier) were digested in 0.02 ml of 1 N KOH for 20 hr at 37°C . The mixture was spotted on Whatman 3MM paper strips, and the ribonucleotides were separated by high voltage electrophoresis for 3.5 hr at 960 v (11). The ultraviolet light-absorbing spots on the paper were cut out and placed in liquid scintillation vials containing toluene-based scintillation fluid, and radioactivity was determined.

FIG. 1. Electron micrograph of negatively stained (phosphotungstic acid) particles obtained at a density of 1.16 g/cm^3 after sucrose density gradient centrifugation of MSV-MLV. $\times 150,000$.

RESULTS

Growth curve of 78A1 cells and MSV-MLV. The production of total and extracellular virus by the 78A1 cells was monitored by electron microscopy during a cell growth curve experiment. There was an initial 12-hr lag in cell growth after trypsinization (Fig. 2). It was only at the end of this period that most of the particles were

cell-bound, and only 10% of the total virus particles were found in the extracellular fluid. This was followed by a pronounced virus release (from 6×10^8 to 1×10^{10} particles) into the supernatant fluid during the next 12 hr. A steady state of cell division and virus release occurred for the next 36 hr. During this period of exponential cell growth, as well as during the following

FIG. 2. Growth pattern of MSV-MLV in 78A1 cells. Approximately 10^6 cells were seeded in 60-mm plastic petri dishes and incubated at 37°C. Cell counts (*) and counts of extracellular virus particles (●) and of total virus particles (▲) were determined.

24 hr when cell replication had ceased, most of the virus particles were found in the extracellular fluid.

Nucleic acid from ^{32}P -labeled virus. Equilibrium density gradient experiments revealed that the virus particles had a buoyant density of 1.16 g/cm^3 in sucrose. RNA isolated from purified ^{32}P -labeled virus was analyzed by velocity sedimentation with tritium-labeled ribosomal RNA (28S) as a sedimentation marker. The viral RNA had two distinct sedimenting components in 0.11 M salt solution, a faster sedimenting component with a sedimentation coefficient of 71S and a slower sedimenting component in the 4 to 6S region.

Accumulation of 71S RNA in 78A1 cells. Preliminary experiments indicated a very low level of 71S RNA within the 78A1 cells. To obtain a measurable quantity of intracellular viral-specific RNA during the viral growth cycle, it is usually necessary to suppress cellular RNA synthesis with an inhibitor, such as actinomycin D. However, actinomycin D inhibits viral RNA synthesis as well as cellular RNA synthesis in the murine leukemia system (1, 5). It was then postulated that through the use of a protein inhibitor, protein synthesis might be inhibited

to such an extent that the synthesized viral RNA would not be encapsidated, and thereby it might accumulate in the cell. Preliminary experiments, based on the incorporation of ^{14}C -leucine into protein in the presence of various concentrations of cycloheximide, indicated that $50 \mu\text{g}$ of cycloheximide per ml was effective in inhibiting protein synthesis greater than 95%. To determine whether cycloheximide treatment could result in accumulation of viral RNA within the 78A1 cells, experiments were first conducted to establish the effect of the inhibitor on total protein and total RNA synthesis at various periods of time during cell replication. Monolayer cultures (20 hr) were labeled with ^{14}C -leucine and ^3H -uridine. At indicated time intervals thereafter, cycloheximide ($50 \mu\text{g/ml}$) was added to each set of two cultures, and incubation continued for an additional 1 hr. Two more sets of cultures served as controls to measure radioactivity just before and after cycloheximide treatment (Fig. 3). In the absence of cycloheximide, both protein and RNA synthesis proceeded at the expected rate throughout the 15 hr after addition of the labeled precursors. However, the addition of cycloheximide for 1 hr to cultures that had been prelabeled for 1, 4, 6, 12, or 14 hr resulted in an inhibition of both ^{14}C and ^3H incorporation compared to the control cultures that had not been exposed to cycloheximide. On the other hand, when the cells were prelabeled for 8 to 10 hr and then treated with cycloheximide for 1 hr, protein synthesis was inhibited, but the amount of intracellular RNA was in excess of that found in the untreated cells. Although this phenomenon is now unexplained, this differential effect of cycloheximide during a narrow time range was a consistent finding.

The amount of intracellular 71S viral RNA found in cycloheximide-treated cultures was then measured. Cultures (20 hr) were labeled for 10 hr with ^{32}P and then were incubated for 1 hr with cycloheximide ($50 \mu\text{g/ml}$). RNA was extracted from the cells. ^{32}P -labeled RNA preparations from 78A1 cells treated with cycloheximide, from untreated cells and from purified extracellular virions, were analyzed in parallel by velocity sedimentation (Fig. 4). Cycloheximide treatment resulted in a four- to fivefold increase of intracellular 71S RNA. Similar treatment of the control normal rat embryo fibroblasts yielded negative results.

Ribonuclease treatment of 71S RNA. Fractions 3, 4, and 5 (Fig. 4), representing 71S RNA from velocity sedimentation runs, were pooled and treated with bovine pancreatic ribonuclease. More

FIG. 3. Effect of cycloheximide on protein and RNA synthesis of 78A1 cells. Approximately 5×10^5 cells were seeded in 2.5 ml of growth medium in 35-mm petri dishes and incubated for 20 hr at 37 C. At this time, (which constituted the 0 hr), the medium was removed and replaced with fresh medium containing 0.5 μC of ^{14}C -leucine per ml and 0.2 μC of ^3H -uridine per ml. At indicated time intervals, cycloheximide (50 $\mu\text{g}/\text{ml}$) was added, and the cells were incubated for 1 hr. Cells not treated with cycloheximide served as controls. For determination of ^{14}C and ^3H incorporation, the cells were washed three times in TNE containing 10^{-2} M NaN_3 and were frozen at -40 C . Upon thawing, the cells were suspended in 5 ml of TNM buffer in test tubes, lysed by rapid freezing and thawing three times, and deoxyribonuclease (20 $\mu\text{g}/\text{ml}$) was added to each tube. After 30 min at 37 C, SDS was added to make 0.3% at room temperature. The resulting lysate was precipitated with an equal volume of cold 10% trichloroacetic acid, washed onto a membrane filter (0.45 μm , 25 mm), and dried; the radioactivity was then measured. (A) Incorporation of ^{14}C -leucine into control 78A1 cells (●) and into 78A1 cells treated with cycloheximide for 1 hr (○---○) at the indicated times. (B) Incorporation of ^3H -uridine into control 78A1 cells (●) and into 78A1 cells treated with cycloheximide for 1 hr (○---○) at the indicated times.

than 99% of both the extracellular and intracellular RNA was ribonuclease-sensitive.

Base composition of 71S intracellular RNA and MSV-MLV 71S RNA. Radioactive RNA preparations from purified virions and from cycloheximide-treated 78A1 cells were centrifuged separately at $150,000 \times g$. Fractions 3, 4, and 5 (Fig. 4), representing the 71S RNA, were pooled, and yeast RNA was added as carrier. This mixture was then diluted in TNE buffer, precipitated with ethyl alcohol, and the precipitate was processed for base composition analysis. The base composition of the intracellular 71S RNA was almost identical to that of the RNA extracted from purified virions (Table 1).

DISCUSSION

It has previously been shown by Bernard et al. (2) that chronically infected 78A1 cells continuously release infectious MSV and MLV. Studies with the electron microscope could not distinguish between the two types of virions. The particles released by the 78A1 cells (Fig. 1) were spheroid with distinct outer envelopes and internal nucleoids. The physical and chemical

properties of the purified virions and of their nucleic acid were similar to those reported by a number of investigators studying murine leukemia viruses (1, 6, 8, 14).

In view of the fact that MSV-MLV particles were released immediately upon maturation, very little viral RNA was found within the 78A1 cells. Actinomycin D was not utilized in this study because it markedly inhibits RNA synthesis of the murine leukemia viruses (1, 5). The protein inhibitor cycloheximide that inhibits protein synthesis in L cells (7) and in a variety of mammalian cells (9) was used to accumulate and retain RNA within the 78A1 cells. This inhibitor has been successfully used to accumulate RNA in reovirus-infected cells (16).

In these experiments, it was observed that $1.77 \times 10^{-4}\text{ M}$ cycloheximide (50 $\mu\text{g}/\text{ml}$) effectively inhibited protein synthesis. In addition, cycloheximide applied after a 10-hr pulse with ^3H -uridine resulted in a net retention of RNA within the 78A1 cells. Upon sucrose density gradient centrifugation, it was observed that the amount of intracellular 71S RNA had increased four- to fivefold. Base composition analysis and

FIG. 4. Velocity sedimentation of ^{32}P RNA from cycloheximide-treated 78A1 cells. Approximately 10^7 cells were incubated in 16-oz (473 ml) flat bottles for 20 hr, and the medium was replaced with fresh growth medium containing $\text{Na}_2\text{H}^{32}\text{PO}_4$ (100 $\mu\text{C}/\text{ml}$). After 10 hr at 37 C, cycloheximide (50 $\mu\text{g}/\text{ml}$) was added, and the cell monolayers were incubated for an additional 1 hr. The cells were then washed with TNE buffer containing 10^{-2} M NaN_3 , the intracellular RNA was extracted and purified, and was characterized by velocity sedimentation in a 5 to 30% sucrose gradient. The values obtained with RNA from cycloheximide-treated (●) and untreated (□) 78A1 cells and RNA from extracellular MSV-MLV (△) are given.

TABLE 1. Base compositions^a of 71S RNA from purified MSV-MLV and from cycloheximide-treated 78A1 cells

Nucleotide	MSV-MLV RNA	78A1 cell RNA
Cytidylic acid . . .	26.3 $\pm$ 0.2	25.7 $\pm$ 0.5
Adenylic acid . . .	25.1 $\pm$ 0.1	24.6 $\pm$ 0.4
Guanylic acid . . .	25.4 $\pm$ 0.3	26.9 $\pm$ 0.8
Uridylic acid . . .	23.2 $\pm$ 0.2	22.8 $\pm$ 0.7

^a Three analyses were carried out on each preparation.

ribonuclease sensitivity experiments revealed that the intracellular 71S RNA was identical to the RNA extracted from purified virions.

The possible mechanism by which cycloheximide inhibits protein synthesis remains unclear. Three possible explanations have been proposed (9): (i) cycloheximide inhibits the completion of nascent polypeptide chains; (ii) it inhibits the

release of the completed chains from the polyribosomes; (iii) it affects the steady-state balance between the polyribosomes and single ribosomes. Whatever the mechanism, cycloheximide treatment in this study resulted in a net accumulation of RNA, most likely by inhibiting protein synthesis at the level of translation and, therefore, preventing encapsidation of viral nucleic acid.

Preliminary experiments indicate that the 71S viral RNA hybridizes specifically with its complement in the 78A1 cells but not with control rat embryo fibroblast RNA. Experiments to localize the site of synthesis of this RNA molecule within the 78A1 cells are now in progress.

The findings obtained thus far with the 78A1 cell system may offer a model for similar studies with lymphoid cell lines derived from patients with leukemia (3, 4). The detection of similar high molecular weight RNA in such cells or circulating peripheral blood leukocytes obtained from patients with leukemia may shed additional light on the possible viral etiology of human leukemia.

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