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Analysis of High-Molecular-Weight Ribonucleic Acid Associated with Intracisternal A Particles

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Intracisternal A particles, known primarily for their association with various tumors, have been shown to contain high-molecular-weight (HMW) ribonucleic acid (RNA) by velocity centrifugation, using linear glycerol gradients. This HMW RNA is sensitive to ribonuclease digestion and alkali treatment but is resistant to Pronase treatment. By a double-labeling experiment, HMW RNA was shown to be intrinsic to intracisternal A particles and not to have resulted from cytoplasmic polysomal RNA aggregation. By a reconstitution experiment, it was determined that the results were not due to C-type virus contamination. The synthesis of HMW RNA in intracisternal A particles is inhibited by actinomycin D and ethidium bromide. These observations emphasize that there are probably some taxonomic relationships between intracisternal A particles and oncogenic RNA viruses.

Intracisternal A particles are present in a variety of normal and neoplastic mouse tissues (6-9, 11, 13, 18, 29, 37; Abstr. Proc. Amer. Assoc. Cancer Res. 8:35). In addition they have been found in tumors of the gerbil (35), rat (22), guinea pig (21), and man (33). They consist of two concentric shells surrounding a relatively electron-lucent core, range in size from 70 to 100 nm in diameter, and form by budding from membranes of endoplasmic reticulum. After formation, they remain localized in the cisternae. These particles have previously been considered within a classification of oncogenic ribonucleic acid (RNA) viruses (26). Evidence in support for such a proposition is based primarily on some morphological resemblance of intracisternal A particles to cytoplasmic A particles and immature type C particles (26) and the coincidence of their distribution with B particles (mammary tumor virus) and C particles in various murine tumors (12, 14, 31). However, intracisternal A particles show little antigenic relatedness to extracellular type C particles and no demonstrated biological activity (19). Although it has been proposed that intracisternal A particles might represent, among other possibilities, biologically defective virus (19), certain properties characteristic of oncogenic RNA viruses, such as high-molecular-weight (HMW) RNA and reverse transcriptase have yet to be demonstrated. This communication describes the presence of HMW RNA (60-70*S*) and its synthesis in the presence of selected nucleic acid inhibitors. The presence of an endogenous RNA-dependent deoxyribonucleic acid (DNA) polymerase that is stimulated by natural template will be described in a separate publication.

MATERIALS AND METHODS

Cells. A continuous cell line (N-18) derived from a mouse neuroblastoma was kindly provided by John D. Minna (National Heart and Lung Institute) and was cultured in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal calf serum. All cultures were incubated in a 5% CO_2 atmosphere at 37 C and fed on alternate days.

A continuous cell line (JLS-V9) derived from bone marrow cells of a BALB/c mouse (5) was cultured in RPMI 1640 medium (GIBCO) supplemented with 15% fetal calf serum.

Strain A/He primary mouse embryo fibroblast cultures were prepared and cultured in RPMI 1640 medium supplemented with 15% fetal calf serum. Isotope labeling experiments were done when the cells were in their second passage.

Tumor. A mouse plasma cell tumor (MOPC-104E) was obtained from Michael Potter of the National Cancer Institute and was maintained by subcutaneous transplantation in this laboratory.

Radioactive labeling of cells and tumor. N-18 neuroblastoma cells were labeled for 48 hr with 10 μCi of ^{3}H-uridine (28.7 Ci/mmole) or 0.5 μCi of L-methionine-*methyl*-^{14}C (8 Ci/mmole) per ml of tissue culture medium. Each 75-cm^2 flask contained 20 ml of media, and the monolayers were essentially confluent at the time of harvest. All radioactive isotopes were obtained from New England Nuclear Corp.

The JLS-V9 cells were infected with 1 ml of Rauscher leukemia virus containing approximately 10^{10} particles (Electronucleonics Inc.), and 10 days post infection the cells were labeled for 24 hr with 10 μCi of ^{3}H-uridine per ml. The tissue culture medium was harvested, and the labeled virus was then concentrated and purified as described for avian myeloblastosis virus (3, 4, 24).

Eight days post tumor transplantation (MOPC-104E), female BALB/c mice were injected via the tail vein with 100 μCi of ^{3}H-uridine daily for a period of 5 days and then sacrificed.

Preparation of intracisternal A particles and other cellular components. N-18 neuroblastoma cells and strain A/He mouse embryo fibroblasts were washed at 4 C with phosphate-buffered saline (*p*H 7.2) and then resuspended in four volumes of a hypotonic buffer consisting of 10 mM Tris-hydrochloride (*p*H 7.4) and 1.5 mM $MgCl_2$. Then the cells were disrupted by vigorous expression through a 25-gauge hypodermic needle. Intracisternal A particles were isolated in the manner previously described (20). Briefly, cytoplasmic extracts were centrifuged at 10,000 $\times$ *g* for 10 min to yield a pelleted membrane fraction consisting of mitochondria, microsomes, and A particles. A particles were liberated from microsomal vesicles by shearing the resuspended membrane fraction through 19-, 22-, and 25-gauge needles in the presence of 0.2% Triton X-100. Such liberated particles were diluted with seven to nine volumes of 0.15 M potassium citrate and concentrated by sedimentation at 60,000 $\times$ *g* for 30 min; the resultant pellet was then resuspended, layered over a cushion of 48% (w/v) sucrose, and centrifuged at 420,000 $\times$ *g* for 2 hr. At this point the vast majority of the membranous material is removed from the sample, and the pellet below the 48% sucrose represents a concentrate of A particles. This pellet was resuspended and layered over a 33 to 68% (w/v) linear sucrose gradient, and the particles were banded isopycnically by centrifugation for 3 hr at 420,000 $\times$ *g*. For some experiments in which the amount of the starting preparation was quite small, partially purified A particles derived from the 48% sucrose pellet were used, and the second purification using the linear sucrose gradient was omitted.

To prepare polysome fractions from the N-18 neuroblastoma cells, the cells were disrupted as indicated above. The nuclei were sedimented by centrifugation at 700 $\times$ *g* for 10 min. The supernatant fluid was spun at 10,000 $\times$ *g* for 10 min to yield a membrane-mitochondrial fraction, and the supernatant fluid above the membrane pellet was centrifuged at 100,000 $\times$ *g* for 90 min to yield a polysome fraction.

Complement fixation assay and X-C plaque assay. The assays were kindly peformed under the direction of Janet W. Hartley. Microtiter assays for murine leukemia virus (MuLV) antigen were performed using both a pooled rat antiserum (MSV), broadly reactive towards the major group-specific (gs) antigen (16) and a guinea pig antiserum specific for gs-1 antigen (25). The concentration of protein in the samples tested ranged from 0.85 to 1.25 mg/ml as measured by the Lowry method. The plaque assay for MuLV was done as previously described, using the X-C rat tumor cell line (28).

Electron microscopy. The preparation of materials and the electron microscopy techniques have been described previously (20).

Nucleic acid inhibitors. N-18 neuroblastoma tissue culture cells were treated with 2.5 μg of actinomycin D per ml and 2.0 μg of ethidium bromide per ml (Calbiochem) in tissue culture medium. Thirty minutes after the addition of inhibitors, 10 μCi of ^{3}H-uridine per ml was added to the cultures. The cells were harvested approximately 48 hr later.

Protein determination. Protein concentrations of purified A particles were determined by the Lowry method as reported previously (20).

Avian myeloblastosis virus purification. Avian myeloblastosis virus (AMV) was kindly supplied by J. W. Beard and D. P. Bolognesi, and by Michael A. Chirigos of the Special Cancer Virus Program of the National Cancer Institute. Further purification of the virus was carried out by the methods described earlier (3, 4, 24). AMV materials banding at a density of 1.16 g/cm^3 were used for RNA extraction.

RNA extraction, purification, and analyses by glycerol velocity gradient centrifugation. RNA was extracted from purified or partially purified intracisternal A particles by a modification of the conventional sodium dodecyl sulfate (SDS)-phenol method at 0 to 2 C as described previously (2, 4, 24, 27). Approximately 125 to 250 μg of protein equivalents of A particles purified from about 2.5 to 5.0 g of cells or tumor tissues were suspended in 0.5 ml of STEM buffer containing Tris-hydrochloride, *p*H 7.2, 0.01 M; NaCl, 0.1 M; $MgCl_2$, 1.0 mM; ethylenediaminetetraacetic acid, 0.1 mM; mercaptoethanol, 1.0 mM; 2 mg of washed bentonite per ml (Fisher Scientific); and 50 μg of polyvinyl sulfate per ml or 0.1% diethyl oxydiformate (Eastman). The particles were lysed by adjustment to 1.5% SDS, and the labeled RNA was extracted with equal volumes of buffered phenol-creosol (55:7) twice. The aqueous phase was further purified with ether four times and then precipitated in 70% ethanol and 0.3 M NaCl overnight at -20 C. The labeled RNA precipitates were collected by centrifugation at 18,000 rev/min for 30 min and redissolved in 0.5 ml of STEM. The purified RNA was then layered over a neutral glycerol gradient of 10 to 35% (v/v) and centrifuged in an SW41 rotor at 40,000 rev/min for 2 to 3 hr along with standards of 70*S* (HMW) RNA from AMV and/or 18*S* ribosomal RNA and/or 4*S* phenylalanine-specific transfer RNA (Miles Laboratory and Boehringer). The resultant gradients were fractionated and analysed for density. Radioactivity of each fraction was determined by precipitating the labeled RNA with

20 μg of ribonuclease-free, carrier yeast-soluble ribonucleic acid (Calbiochem) at 0 C with 10% trichloroacetic acid; the precipitates were trapped on 0.45-μm pore size membrane filters (Millipore Corp.) and washed exhaustively with 5% trichloroacetic acid and then with ethanol. The filters were dried and counted in Liquiflor-toluene (New England Nuclear Corp.) by a liquid scintillation method.

RESULTS

Tritiated ribonucleoside labeling and electron microscopy of purified intracisternal A particles. Figure 1 shows the results of a representative sucrose gradient purification of intracisternal A particles derived from N-18 neuroblastoma cells labeled with all four tritiated ribonucleosides. Intracisternal A particles were recovered in the density region of 1.21 to 1.23 g/cm^3. This result is in agreement with an earlier observation in which A particles were isolated from murine plasma cell tumor MOPC-104E (20). The minor spike (gradient fraction 15) in the A particle peak probably represented a discontinuity in the sucrose gradient; such spike was not observed if the gradient was allowed to equilibrate for 12 hr prior to use. In the less dense region of the gradient (fractions 20 to 25), the two peaks represent ribosomal material (20), and one of them overlaps the A particle peak. Thus it is not surprising that there is some cytoplasmic RNA contamination of intracisternal A particles. The isolates of purified A particles used in these experiments were monitored by electron microscopy, and the appearance of a typical pooled sample is shown in Fig. 2. It is apparent that there has been a marked concentration of A particles at the expense of other cellular components. The purity of the various preparations was essentially constant.

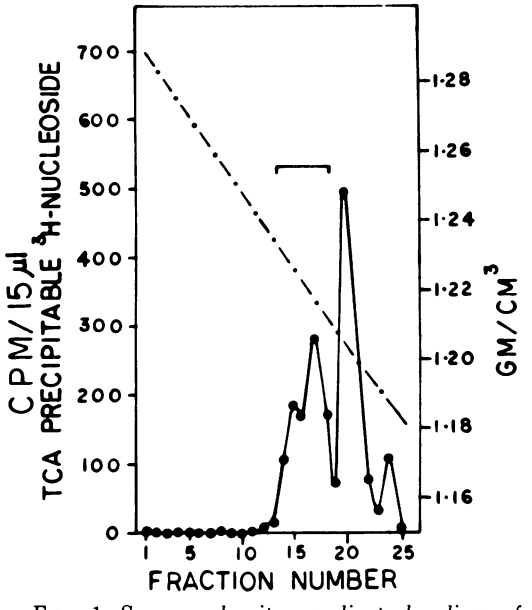

FIG. 1. *Sucrose density gradient banding of intracisternal A particles with all four* ^{3}*H-ribonucleosides. Radioactivity in counts per minute per 15-μliter sample is denoted by* —●—●—. *Density (in g/cm*^{3}*) is denoted by* - ● - ● -. *N-18 neuroblastoma cells were chronically labeled with all four tritiated ribonucleosides (10 Ci/mmole) at 10 μCi/ml of medium for 48 hr. Cells were harvested, and A particles were prepared as described in the text. A partially purified A-particle concentrate prepared from the first discontinuous sucrose gradient centrifugation was applied to a 33 to 68% linear sucrose gradient and spun at 420,000 × g for 3 hr. Resultant gradient was divided into 25 fractions and a 15-μliter sample from each was precipitated by 5% trichloroacetic acid along with 25 μg of bovine serum albumin. Density was determined by direct weighing of a 100-μliter sample from each fraction. A particles were recovered in the gradient region indicated by the bracket. The two lighter peaks represent ribosomal contaminants.*

Complement fixation and plaque assays for murine leukemia viruses. It has been previously established that intracisternal A particles are antigenically distinct from murine leukemia, sarcoma, and mammary tumor viruses (19). Purification of intracisternal A particles is not accompanied by a concentration of MuLV antigenic activity, even in those instances in which the MuLV gs antigen was demonstrated in the original tumor homogenate (*unpublished data*). When partially purified isolates of A particles from MOPC-104E and N-18 neuroblastoma cells were tested by complement fixation assay for MuLV gs antigens, the results indicated that, at most, negligible amounts of C particle antigen were present in the isolates derived from MOPC-104E tumor (Table 1). No MuLV group-specific antigens were found in isolates derived from N-18 neuroblastoma cells (Table 1). The miniscule amount of antigen together with the fact that C particles were never seen by electron microscopy in partially purified A particles suggested that contamination by exogenous virus could not account for the results presented here.

The X-C plaque assay for murine leukemia viruses, using the mixed culture cytopathogenicity reaction, was carried out by using the partially purified A particles. At the end of 7 days there were no plaques. Not only was there absence of biological activity, but there was no evidence of MuLV antigen production.

RNA associated with purified intracis-

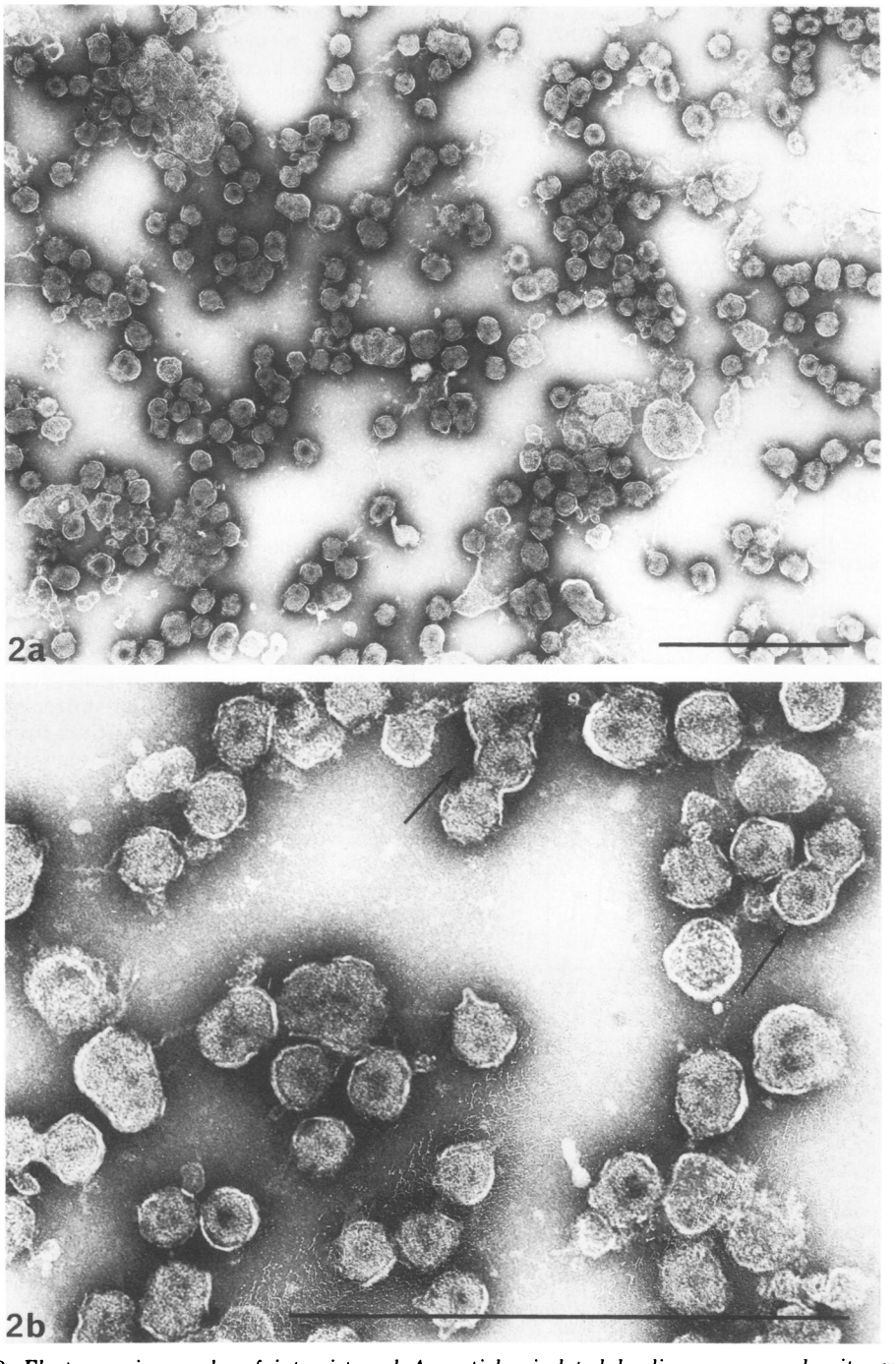

FIG. 2. *Electron micrographs of intracisternal A particles isolated by linear sucrose density gradient centrifugation. This material represents a sample from a pool of gradient fractions 13 through 18 (see Fig. 1). a, Negative stain preparation showing multiple clusters of A particles. There are scattered membranous structures representing remnants of microsomal vesicles. Scale line, 1 μm. Magnification, 25,500×. b, At higher magnification it is apparent that A particles exist as a heterogeneous population made up of single forms and elongated forms (arrows) consisting of multiple buds. Scale line, 1 μm. Magnification, 75,000×.*

ternal A particles from N-18 neuroblastoma cells and MOPC-104E plasma cell tumor. Sedimentation profiles of RNA labeled with ^{3}H-uridine and isolated from partially purified A particles derived from N-18 neuroblastoma cells and MOPC-104E plasma cell tumors are illustrated in Fig. 3A and 4, respectively. Purified ^{3}H-RNA from these A particle fractions possessed HMW species; one sedimented with the standard 62 to 70*S* (HMW) RNA from AMV, and the other component sedimented at the 60*S* area. Other observable components in the RNA extracted from the partially purified A particle fraction obtained from the N-18 neuroblastoma cells are 45*S*, 18*S* (with a shoulder overlapping the 28*S* and 35*S* areas), and a 4*S* RNA (Fig. 3A). Distinct peaks of 28*S*, 18*S*, and 4*S* RNA were also observed in the rest of the RNA components isolated from the partially purified A particle fractions obtained from MOPC-104E tumor (Fig. 4). An absorbency profile (260 nm) of RNA associated with intracisternal A particles derived from this mouse plasma cell tumor has been reported earlier (20). Except for the presence of HMW RNA and a quantitative difference in 4*S* RNA, there is essentially no change in the relative proportions of 18*S* and 28*S* RNA in this report and the previous one. With the use of a more sensitive technique, namely labeling with high-specific-activity, radioactive ribonucleosides, HMW RNA can be detected even though it is present in small quantities. It is probable

TABLE 1. *Complement fixation assays for C-type viral antigens in partially purified intracisternal A particles*

Source	Rat antiserum (MSV) (CF units)[a]	Guinea pig antiserum (gs-1 antigen) (CF units)[a]
Tumor		
MOPC-104E	<2	<2
N-18	Neg[b]	Neg
RLV	512[c]	NT[d]

[a] CF unit, Reciprocal of complement fixation titer.
[b] Neg, Negative.
[c] See reference 19; CF assay was carried out with a concentrate of partially purified virus.
[d] NT, Not tested.

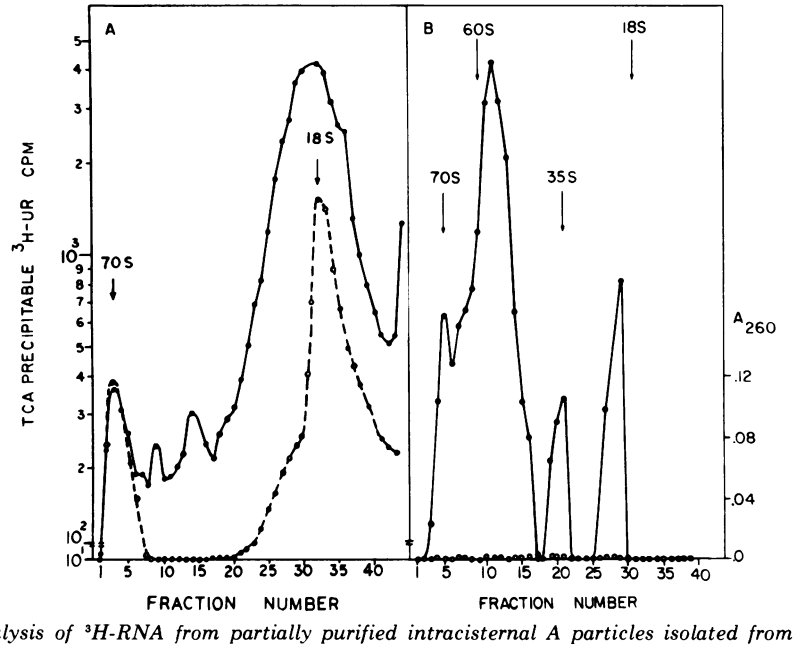

FIG. 3. *Analysis of ^{3}H-RNA from partially purified intracisternal A particles isolated from N-18 neuroblastoma cells by velocity linear glycerol gradient centrifugation. A, ^{3}H-RNA from A particles (—●—●) was analyzed in the same tube with 70S RNA from AMV and 18S ribosomal RNA from 3T3 cells (—O—O) as monitored by A_{260}. B, 70S RNA purified from intracisternal A particles was pooled by combining gradient fractions 2 to 6 from several experiments. This RNA was recycled through a second 10 to 35% linear glycerol gradient (—●—●). Ribonuclease-treated samples from each fraction are depicted (—O—O). Samples of 150 μliters from each fraction were digested with 20 μg of pancreatic ribonuclease per ml at 37 C for 2 hr prior to trichloroacetic acid precipitation.*

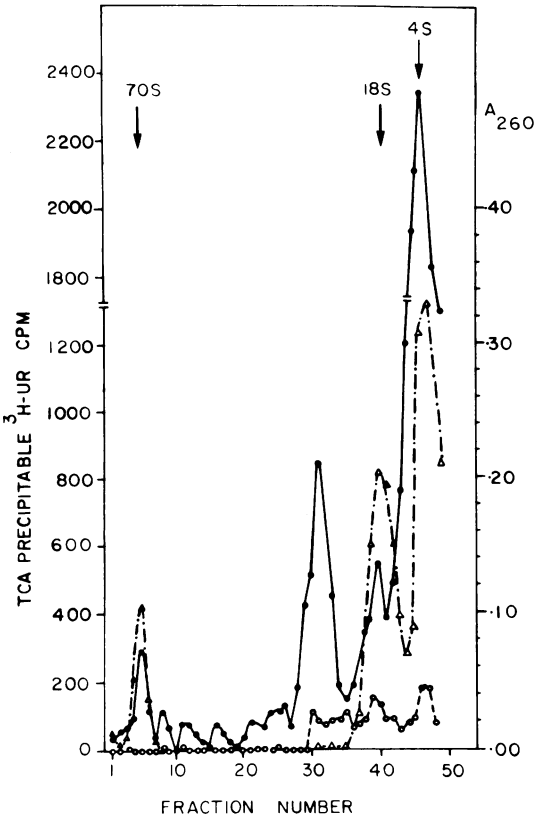

FIG. 4. *Analysis of ^{3}H-RNA from partially purified intracisternal A particles isolated from MOPC-104E plasma cell tumor. ^{3}H-RNA is depicted by —●—●—. Standards, 70S RNA from AMV and 18S ribosomal RNA from 3T3 cells and 4S phenylalanine-specific transfer RNA, are depicted by Δ···Δ···Δ and are monitored by A_{260} absorbency. Profile of radioactivity of samples treated with 0.5 N KOH is depicted by O - - O. Samples of 150 μliters from each fraction were incubated in alkali at 37 C overnight prior to preparation for radioactivity determination.*

that the 28*S* and 18*S* components represent cytoplasmic ribosomal RNA contaminants extrinsic to the A particles, but which co-fractionated with them since the trailing edge of the intracisternal A particle peak overlapped with the cytoplasmic ribonucleoprotein peak (Fig. 1). The 18*S* and 28*S* RNA associated with intracisternal A particles derived from N-18 neuroblastoma cells consistently appeared as a broad peak at the 18*S* area with a prominent shoulder at the 28*S* area. This is in contrast to the distinct peaks of 18*S* and 28*S* RNA associated with intracisternal A particles derived from MOPC-104E plasma cell tumor. Although there were experiment-to-experiment variations in the amount of 18*S* and 28*S* RNA present, this did not affect the quantitative yield of HMW RNA associated with intracisternal A particles from either N-18 neuroblastoma cells or MOPC-104E plasma cell tumor. Further efforts are being made to purify the labeled intracisternal A particle fractions free of ribosomal contaminants.

Pooled intracisternal A particles from fractions 13 to 18 (Fig. 1) were digested with 20 μg of crystalline pancreatic ribonuclease per ml (Worthington) at 25 C for 15 min prior to RNA extraction. Considerable loss of radioactivity (25%) in the 45*S*, 28*S*, and 18*S* ribosomal RNA areas, but little loss in the 4*S* RNA peak, was observed. Some degradation of the HMW RNA peak occurred with concurrent increases in the 35*S* and 60*S* areas of ^{3}H-RNA. In view of these results it should be noted that isolates of A particles consist of a heterogeneous population, intact particles, and fairly numerous elongated forms consisting of two to six buds (20). These buds are left with an open end as a result of being sheared free from membranes. If this particular form contains HMW RNA, then there is an excellent chance that it would be nicked by ribonuclease treatment.

All of the examined neuroblastoma lines have been found to contain A particles; thus it has not been possible to obtain a negative control from such tumor tissue. However, since the N-18 neuroblastoma cells are derived from strain A mice, we chose strain A/He mouse embryo fibroblasts as a related control and analyzed the RNA components from a 48% sucrose pellet preparation (which is equivalent to a partially purified A particle preparation). We found that, in the 48% sucrose pellet from A/He cells, which lack A particles, there was no observable HMW RNA (Fig. 5). This further suggests that the HMW RNA observed in partially purified intracisternal A particle preparations from N-18 neuroblastoma cells might indeed be intrinsic RNA.

Stability of 60 to 70S RNA from intracisternal A particles. Considerable degradation of the 60 to 70*S* ^{3}H-RNA peak was observed when the isolated HMW ^{3}H-RNA, pooled from fractions 2 to 6 in a number of experiments (Fig. 3A), was analyzed once more through a second glycerol gradient (Fig. 3B). Although the 70*S* peak remained, a broad peak of RNA in the 60*S* and 50*S* areas appeared. Two minor but distinct peaks were observed at the 35*S* and 18*S* regions. Earlier reports have implicated 35 to 37*S* RNA as a possible degradation product of the HMW RNA in Rous

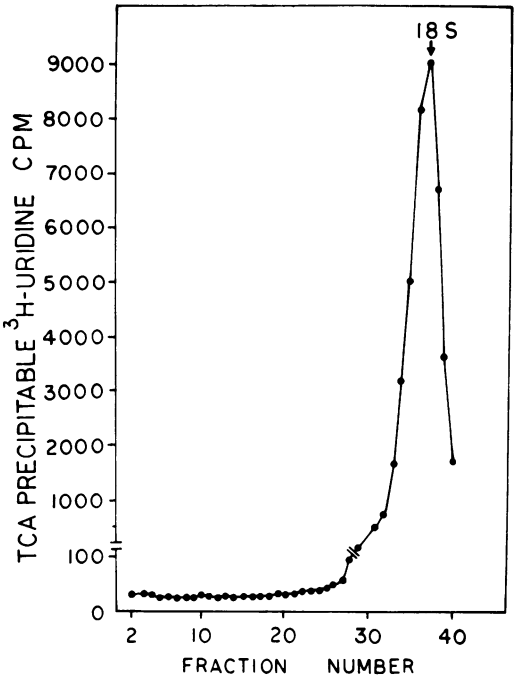

FIG. 5. *Analysis of ³H-RNA purified from 48% sucrose pellet isolated from A/He mouse embryo fibroblasts, which lack A particles. ³H-RNA is depicted (—●—●—). This analysis was carried out along with that in Fig. 3A and hence should be compared with the standard 70S RNA from AMV and 18S RNA from 3T3 cells as presented there.*

sarcoma virus (RSV) (10) and AMV (32) and of the 75S RNA in feline leukemia virus (17) as a result of heat or dimethyl sulfoxide treatment. Our 60 to 70S sample, on the other hand, was subjected to extensive dialysis against STEM at 4 C for the removal of excess glycerol prior to its reloading onto the second glycerol gradient. This observation suggests that the 60 to 70S RNA associated with intracisternal A particles is extremely susceptible to degradation during the process of its isolation. It has been established earlier that RNA accounted for 4.8 to 6.8% of A particles on a w/w basis (20). These studies did not employ isotope labeling, and the largest species of RNA had a molecular weight of only 29S. This could be explained by the present results documenting the instability of the HMW RNA from intracisternal A particles, especially in view of the strenuous purification procedures involved in their preparation. In our experimental procedures, all precautions against ribonuclease contamination and temperature inactivation were taken. Although the yield of such HMW RNA was extremely low (less than 0.2% in partially purified A particles, but approximately 5% in partially digested, ribonuclease-treated A particles), it seems unlikely that it was contributed by contaminant C particles when the immunological results and electron microscopy are considered.

Sensitivity to ribonuclease, alkali, and Pronase treatments. To establish that the tritium-labeled material isolated from the A particles was indeed RNA, samples from the glycerol gradient were treated with ribonuclease (Fig. 3B) or with 0.5 M KOH (Fig. 5) prior to trichloroacetic acid precipitation for radioactivity determination. All radioactivity was found sensitive to either ribonuclease or alkali treatment. This indicated that the ³H-uridine was incorporated into both the HMW RNA and other lower-molecular-weight species of intracisternal A particles from N-18 neuroblastoma cells and from MOPC-104E plasma cell tumor.

The possibility that the radioactivity located at the 70S peak might represent a ribonucleoprotein complex rather than RNA was examined. Samples for glycerol gradient analysis were treated with Pronase (50 μg/ml) at 25 C for 60 min prior to centrifugation and trichloroacetic acid precipitation for radioactivity determination. Results showed that Pronase digestion did not alter the profile of radioactivity at all, and suggested that the radioactivity associated with the 70S peak is indeed HMW RNA rather than a ribonucleoprotein complex.

Co-sedimentation of cytoplasmic polysomal RNA with intracisternal A particle RNA. To rule out the possibility that the 60 to 70S RNA of intracisternal A particles might have arisen from artifactual aggregates of cytoplasmic ribosomal RNA, we have resorted to double-labeling experiments. RNA samples, co-extracted and co-purified as described in Materials and Methods from (i) the polysome fraction of N-18 neuroblastoma cells labeled with [*methyl-*¹⁴*C*]methionine and from (ii) purified intracisternal A particles in the same cell line labeled with ³H-uridine, were analyzed together by neutral glycerol gradient. The resultant double-labeled RNA profiles are shown in Fig. 6. The ³H-RNA profile from purified intracisternal A particles showed a finite 70S peak coinciding with the standard HMW RNA from AMV. Some 60S RNA and an apparent 35S RNA peak were also observed. Such HMW RNA components were not seen in the ¹⁴C-polysomal RNA profile. The possibility that RNA aggregates would form in the presence of diethyl oxydiformate

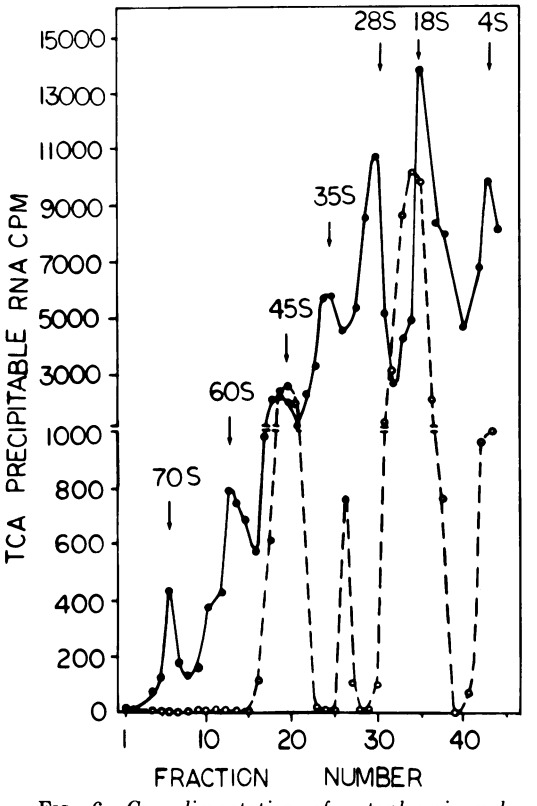

FIG. 6. *Co-sedimentation of cytoplasmic polysomal RNA with RNA purified from intracisternal A particles from N-18 neuroblastoma cells. Polysomal RNA profile, labeled in ^{14}C-methyl groups, is depicted by O - - O. RNA from intracisternal A particles, labeled with ^{3}H-uridine is depicted by —●—●—. Standards, 70S RNA from AMV, 18S ribosomal RNA from 3T3 cells and 4S phenylalanine-specific transfer RNA, as monitored by A_{260}, are indicated by arrows for clarity.*

was considered. However, the fact that the HMW RNA was observed only in the ^{3}H-RNA sample from intracisternal A particles but not seen in the ^{14}C-polysomal RNA, suggested that the HMW RNA did not arise from aggregation due to the use of this ribonuclease inhibitor. In other similar experiments, the omission of either diethyl oxydiformate or polyvinyl sulfate during RNA extraction yielded the same results. It should be noted that this particular A particle preparation also contained a great deal of cytoplasmic ribosomal RNA and 4S RNA contaminants as evident from the presence of ^{3}H-RNA peaks in the 28S, 18S, and 4S areas which coincided with the ^{14}C-RNA peaks from the polysomal preparation. From these results it is apparent that intracisternal A particles contain HMW RNA species which are distinct from those seen in polysomal materials.

Reconstitution experiment. The possibility that a trace contaminant of type C virus might have contributed to the presence of HMW RNA in the intracisternal A particle

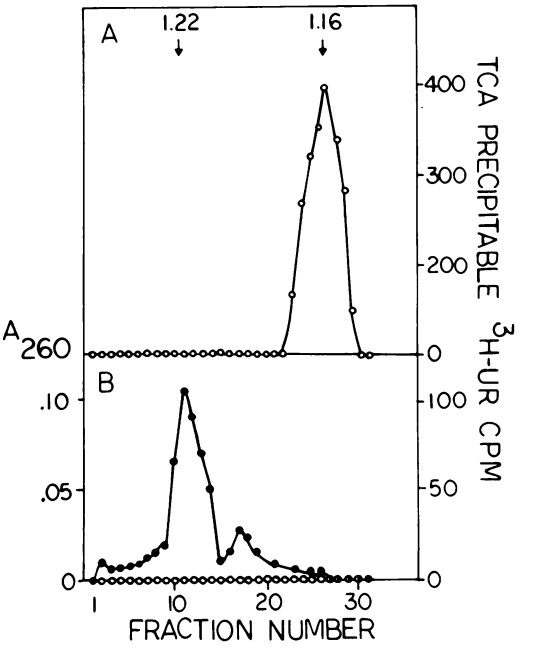

FIG. 7. *Elimination of ^{3}H-RLV during purification of intracisternal A particles in a reconstitution experiment. A, Gradient profile of purified RLV labeled with ^{3}H-uridine but not put through the purification scheme for intracisternal A particles. This represents a parallel analysis with the sample from B. B, Profile of ^{3}H-RLV and cold intracisternal A particles at the final step of purification in a linear sucrose density gradient centrifugation. Radioactivity profile of ^{3}H-RLV is depicted by —O—. Sedimentation profile of purified A particles by A_{260} is depicted by —●—. Density is determined by direct weighing of 100-μliter sample from every third fraction. ^{3}H-RLV was concentrated from tissue culture medium and purified by the methods described for AMV in the text. Approximately 7,000 counts/min of ^{3}H-RLV were added to 0.4 g of unlabeled N-18 neuroblastoma cells. Radioactivity was monitored throughout the purification of A particles (Materials and Methods) by taking sample and solubilizing it in 1.0 ml of Biosolv no. 3 (Beckman), and then counting it in 10 ml of Liquifluortoluene by a liquid scinctillation method (see Table 2). The purification procedure was modified slightly at one step: instead of concentrating the intracisternal A particles as a pellet at the bottom of the 48% sucrose cushion, they were collected as a band at the interface of 48% and 68% discontinuous sucrose cushions.*

preparation was examined by a reconstitution experiment as described in the legend of Fig. 7. It should be emphasized that type C virus has never been seen by electron microscopy in purified A particle preparations and not been detected by immunological techniques in N-18 neuroblastoma cells (Table 1). Although extremely low levels of type C viral antigen have been detected by immunological assays (Table 1) in the MOPC-104E plasma cell tumor, again no C particles or C particle nucleoids have been seen ultrastructurally. Table 2 summarizes the results of purification of A particles mixed with an excess of ^{3}H-labeled Rauscher murine leukemia virus (RLV), the latter being added prior to the initiation of the purification procedure. It is apparent that the greatest elimination of ^{3}H-RLV during the purification was accomplished during two steps. At the 10,000 × *g* centrifugation step all free particles such as type C virus remain in the supernatant fluid, whereas A-particles which are still bound to rough endoplasmic reticulum will sediment in the pellet. The step involving centrifugation of materials through the 48% sucrose cushion serves to remove the remainder of the counts. This result is predictable when it is considered that Triton treatment is used to free A particles from microsomal vesicles. Triton (0.2%) treatment of RLV liberates core-associated RNA and reverse transcriptase to the extent that they remain at the top of a sucrose or glycerol gradient with a density below 1.13 g/cm^3 (30; Yang and Gallo, *unpublished data*). However, freed A particles which have a density of 1.21 to 1.23 g/cm^3 (50 to 53% w/v sucrose) easily penetrated the 48% sucrose cushion and sedimented at the 48 to 68% sucrose interface. Since essentially all ^{3}H-RLV was eliminated prior to the further purification of A particles by the 33 to 68% linear sucrose gradient centrifugation, no counts were observable in the purified

TABLE 2. *Progressive elimination of ^{3}H-RLV during the purification of intracisternal A particles in a reconstitution experiment*

Purification steps	Total counts/min of ^{3}H-RLV		% of ^{3}H-RLV	
	Discard	Remain	Discard	Remain
Total homogenates (0.4 g N-18 cells + ^{3}H-RLV)		6,900		100
Loss due to shearing	270		4.0	
700 × *g* pellet	390		5.7	
700 × *g* supernatant		6,240		90.3
10,000 × *g* supernatant	4,784		69.3	
10,000 × *g* pellet		1,456		21.1
Potassium Citrate pellet	480		7.0	
Potassium Citrate supernatant		975		14.1
48% Sucrose	950		13.8	
48–68% Sucrose interface		0		0
33–68% Linear sucrose gradient analysis (fig. 7)		0		0

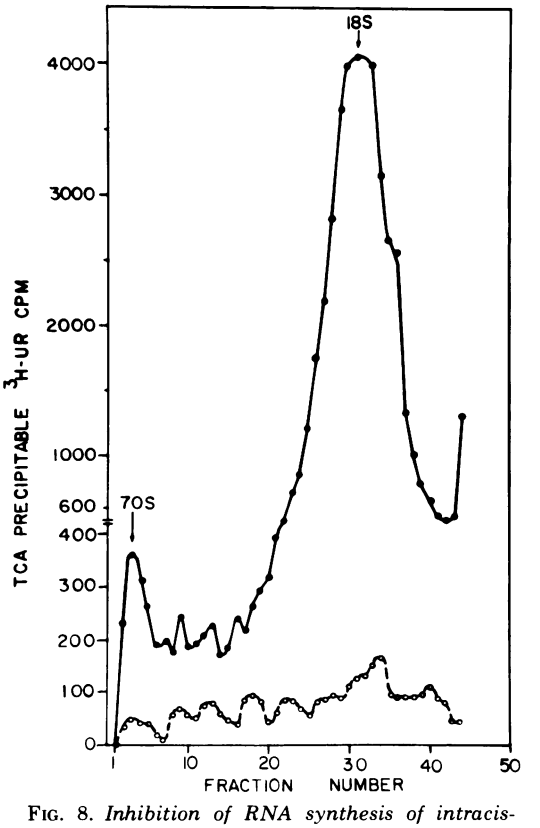

FIG. 8. *Inhibition of RNA synthesis of intracisternal A particles by actinomycin D and ethidium bromide in N-18 neuroblastoma cells. Profile of ^{3}H-RNA from A particles isolated from untreated N-18 neuroblastoma cells chronically labeled with ^{3}H-uridine is depicted by —●—. Profile of ^{3}H-RNA isolated from inhibitor-treated N-18 neuroblastoma cells chronically labeled with ^{3}H-uridine is depicted by - - O - -. Standard RNAs as monitored by A_{260} are indicated by arrows.*

A particle profile shown in Fig. 7. The absorbency profile (260 nm) of these gradient-purified A particles indicated that this preparation was relatively free of the usual ribosomal contaminants (compare with Fig. 1). In view of the clear-cut elimination of ^{3}H-RLV from intracisternal A particles during their purification, as demonstrated by this reconstitution experiment, it can be reasonably assumed that the HMW RNA associated with A particles is indeed not contributed by unrelated viruses.

Antibiotic inhibition of ^{3}H-uridine incorporation into RNA of intracisternal A particles. Fig. 8 shows the effect of actinomycin D and ethidium bromide on the incorporation of ^{3}H-uridine into RNA of intracisternal A particles. ^{3}H-RNA extracted from partially purified intracisternal A particles derived from N-18 neuroblastoma cells showed the typical HMW RNA with a minor 60*S* RNA peak and the 18*S* RNA peak. In a simultaneous analysis, partially purified intracisternal A particles derived from N-18 neuroblastoma cells cultured in the presence of actinomycin D and ethidium bromide showed little incorporation of ^{3}H-uridine into all species of RNA. Only a minor 18*S* RNA peak was present. In other experiments not shown here, actinomycin D alone inhibited the incroporation of ^{3}H-uridine into the 60 to 70*S* and 35*S* RNA components of intracisternal A particles. In these experiments the N-18 neuroblastoma cells showed little morphologic change by light microscopy. When the antibiotic-treated monolayers were stained with trypan blue, only 11% of the cells took up the stain as compared with the 3% in a companion control culture. This merely documents the fact that there is little difference between the membrane permeability of the control cells as compared with the treated cells. This observation is reinforced by the fact that ribosomal RNA synthesis was still continuing, albeit at a low level (Fig. 8). The inhibition of ^{3}H-uridine incorporation into HMW RNA of intracisternal A particles paralleled earlier observations on the replication of murine and avian sarcoma viruses, which were inhibited by actinomycin D (1, 34). Further investigations on the effects of antibiotics on the synthesis and turnover of intracisternal A particles are currently in progress.

DISCUSSION

A precise identification of intracisternal A particles is lacking. Morphological resemblance of such particles to known oncogenic RNA viruses and their concomitant distribution in various murine tumors containing such viruses encouraged their inclusion within a classification of oncogenic RNA viruses (26). However, the proposition that intracisternal A particles might be precursors of C-type particles has never been proven, and the fact is now established that they are antigenically distinct from murine leukemia and sarcoma viruses and mammary tumor viruses (19). It

TABLE 3. *Comparison of some properties of intracisternal A particles with those of oncogenic RNA viruses*

Properties of oncogenic RNA viruses (23)	Properties of intracisternal A particles
Common biochemical characteristics	
60–70*S* RNA as genetic materials	60–70*S* RNA present
Virus production inhibited by actinomycin D	HMW RNA production is inhibited by actinomycin D.
Reverse transcriptase	Reverse transcriptase activity has been described (36).
Distinctive characteristics	
Generally exhibit a spherical internal structure rather than an obviously cubic or helical symmetry	Inner shell of intracisternal A particles does not exhibit cubic or helical symmetry and there is no internal spherical component (20).
Density: 1.16 g/cm^3	1.22 g/cm^3 (20)
Extracellular	Intracellular
A small basic protein as the predominant gs antigen	Its own gs antigen associated with a major structural protein (or group of proteins); mol wt 70,000 (19).
Mammalian leukemia viruses share a gs component	No immunological relationship with MuLV or mammary tumor virus (19).

has been proposed that intracisternal A particles may have arisen from abnormal production or processing of a cellular constituent, or both; or, on the other hand, they could possibly represent biologically defective viruses since infectivity or oncogenicity has yet to be demonstrated (19). Results of this investigation force a reexamination of A particles, especially with regard to their taxonomic relationship with other oncogenic RNA viruses.

A recent classification of all biological and biochemical properties common to oncogenic RNA viruses (oncornaviruses) by Nowinski et al. (23) provides a pertinent guideline for the following evaluation of the similarities and dissimilarities between the oncogenic RNA viruses and intracisternal A particles. This is summarized in Table 3. In considering a significant number of properties jointly shared between intracisternal A particles and other known oncogenic RNA viruses, it is tempting to suggest that intracisternal A particles might have arisen from an incomplete expression of a viral genome rather than an aberrant cellular constituent. In essence, intracisternal A particles resemble defective type C RNA viruses such as RSV_0 (15) which are partially deficient in reverse transcriptase. Certainly the HMW RNA and the amount of an RNA-dependent DNA polymerase activity associated with the gradient purified intracisternal A particles were much lower than those observed with oncogenic type C viruses (*unpublished data*). Yet these results indicate that additional attempts to demonstrate biological activity are merited. If it could be shown that intracisternal A particles are infectious, then the identification as virus will be essentially achieved.

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