JOURNAL OF VIROLOGY, May 1976, p. 745-756

Vol. 18, No. 2
Printed in U.S.A.

Intracisternal A Particles from FLOPC-1 BALB/c Myeloma: Presence of High-Molecular-Weight RNA and RNA-Dependent DNA Polymerase

R. G. KRUEGER

Laboratory of Molecular Oncology, Christ Hospital Institute of Medical Research, Cincinnati, Ohio 45219, and Departments of Internal Medicine and Microbiology, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267*

Received for publication 9 October 1975

Intracisternal A particles from the FLOPC-1 line of BALB/c myeloma have been shown to contain high-molecular-weight RNA (60 to 70S) that is sensitive to RNase, alkali degradation, and heat but resistant to Pronase treatment. The intracisternal A-particle RNA contains tracts of poly(A) approximately 180 nucleotides long. As shown in a reconstitution experiment, by antigenic analysis of A-particle preparations and the XC cytopathogenicity assay, the 70S RNA was not due to contamination by type C virus particles. The FLOPC-1 intracisternal A particles also possess an endogenous RNA-dependent DNA polymerase. The enzyme required Mn^{2+} or Mg^{2+}, dithiothreitol, detergent, and four deoxyribonucleoside triphosphates for maximum activity. Enzymatic activity was maximally stimulated by poly(rC)·oligo$(dG)_{12-18}$ and less with poly(rG)·oligo$(dC)_{10}$ or poly(rA)·oligo$(dT)_{12-18}$ as compared with synthetic DNA/DNA duplex templates such as poly(dA)·oligo $(dT)_{12-18}$. The enzyme can utilize the A-particle endogenous RNA as template as shown by analysis of the early and late DNA products of the endogenous reaction by $CsSO_4$ isopycnic gradient centrifugation and hybridization of purified 70S or 35S A-particle RNA with the purified complementary DNA product. Approximately 50% of the A-particle complementary DNA also hybridized with oncornavirus RNA.

Intracisternal A particles are present in a variety of normal and neoplastic mouse tissues (2-4, 6, 7, 11, 15, 28, 36, 39). They have also been observed in tumors of man (31), guinea pigs (22), gerbils (33), and rats (23). They consist of two concentric shells surrounding a relatively electron-lucent core, range in size from 70 to 100 nm in diameter, form by budding from membranes of the endoplasmic reticulum, and after budding remain localized in the cisternae. These particles have been considered in the classification of oncogenic RNA virus (26) because of their morphological resemblance to type C virus and the coincidence of their distribution with type B and C viruses in various murine tumors (9, 13, 30). However, intracisternal A particles show minimal antigenic relatedness to murine leukemia virus (MuLV) and, to date, no demonstrated biological activity (18).

Recent reports suggest that intracisternal A particles represent some expression of a viral genome since they contain high-molecular-weight RNA (60 to 70S) as the endogenous nucleic acid (27, 40), an endogenous RNA-dependent DNA polymerase (RDDP) (27, 41), and a group-specific structural protein of 70,000 molecular weight (18, 21).

In this report, evidence is presented demonstrating the existence of high-molecular-weight RNA (60 to 70S) associated with intracisternal A particles isolated from the FLOPC-1 line of BALB/c myeloma. This RNA, which is dissociated to smaller-molecular-weight fragments upon heating, can serve as template for an endogenous RDDP associated with the A particles. This enzyme, which requires Mn^{2+} as opposed to Mg^{2+}, dithiothreitol (DTT), and detergent for maximum activity, transcribes the synthetic template poly(rA)·oligo$(dT)_{12-18}$ better than poly(dA)·oligo$(dT)_{10}$ and synthesizes a DNA product that will hybridize back to A-particle RNA.

MATERIALS AND METHODS

Tumor and cells. The FLOPC-1 tumor, which originated in this laboratory (36), was maintained by subcutaneous transplantation of tissue fragments in BALB/c mice.

Isolation and purification of intracisternal A

particles. Preparation of purified intracisternal A particles from MOPC-21 and FLOPC-1 cells was described previously (17). Briefly, cytoplasmic extracts from tumor homogenates were centrifuged at 10,000 × *g* for 15 min at 4 C, and the pellet was suspended in Triton X-100 and incubated at 4 C for 30 min. The suspension was expressed through a 23-gauge needle 10 to 15 times, made 0.10 M potassium citrate, and centrifuged at 105,000 × *g* for 30 min at 4 C. The resulting pellet was suspended in 0.05 M potassium citrate (pH 7.2) containing 25% sucrose, layered over a cushion of 48% sucrose in 0.05 M potassium citrate, and centrifuged at 100,000 × *g* for 16 h. The resulting pellet was recycled over a discontinuous 25 to 48% sucrose gradient and finally subjected to isopycnic centrifugation on linear 33 to 68% sucrose gradients. The A particles banded at a density of 1.22 g/ml. The appearance of a typical gradient-purified A-particle preparation is shown in Fig. 1.

Intracisternal A particles, isotopically labeled with [^{3}H]uridine, were isolated from cultures of FLOPC-1 cells adapted to growth in vitro. Cultures of rapidly dividing cells (5 × 10^6/ml) were pulsed with 5 μCi of [^{3}H]uridine (43 Ci/mmol) per ml of medium for 18 h in a 15% CO_2-85% air environment at 37 C. The cells were pelleted by centrifugation at 500 × *g* for 10 min, and the A particles were isolated as described above.

Reconstitution experiment. Rapidly dividing cultures of either FLOPC-1 cells or normal rat kidney (NRK) cells productively infected with Kirsten MuLV (K-MuLV) were isotopically labeled with [^{3}H]uridine and [^{3}H]adenine, as described previously (35). Virus was purified by isopycnic sucrose gradient centrifugation as described previously (35). Approximately 20,000 counts/min of ^{3}H-labeled type C virus was added to 0.5 g of unlabeled FLOPC-1 cells. Radioactivity was monitored at every step of the A-particle purification procedure described above by solubilizing an aliquot in 1.0 ml of Biosolv-3 (Beckman) and then counting in 10 ml of TLA-BBS-3 (Beckman) as described below. Samples that were analyzed included: 10,000 × *g* pellet and supernatant; potassium citrate supernatant and pellet; 48% sucrose pellet and interface; 33 to 68% linear sucrose gradient fractions.

DNA polymerase assay. Details of the RDDP assay were described previously (35) except for minor variations used to optimize the assay conditions. Briefly, in a final volume of 100 μl, the assay mixture contained: Tris-hydrochloride, pH 8.1, 40 mM; NaCl, 40 μM; manganese acetate, 1 mM; DTT, 5 mM; and 5 to 20 μg of purified intracisternal A

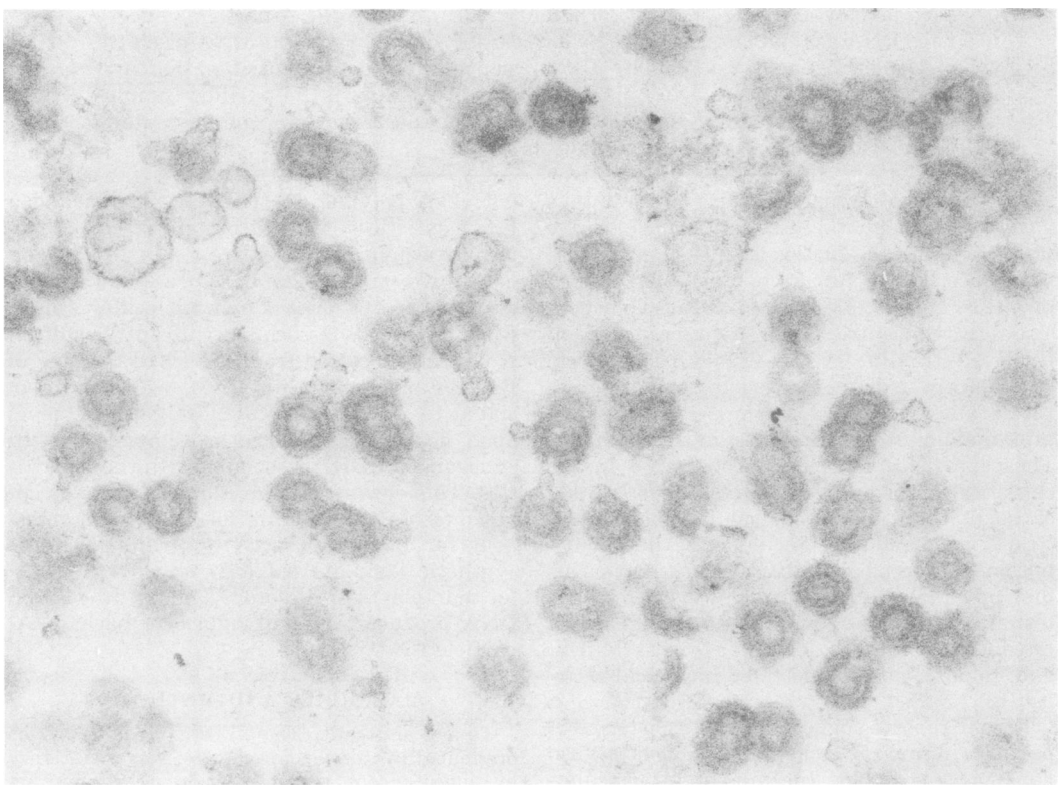

FIG. 1. *Electron microscope examination of a thin section of gradient-purified FLOPC-1 intracisternal A particles. Approximate magnification, ×100,000.*

particles treated with 0.1% Triton X-100 for 10 min at 4 C. For assaying the endogenous reaction, unlabeled dATP, dGTP, and dCTP at a final concentration of 0.1 mM each and 0.001 mM dTTP were added along with 2 μCi of [^{3}H]TTP (52.5 Ci/mmol), and the reaction was carried out at 30 or 37 C. For assays with synthetic templates, the reaction mixtures were as described for the endogenous reaction except that the deoxyribonucleoside triphosphates were deleted, 50 mM KCl was used instead of NaCl, a synthetic template (50 μg/ml) was added, and the reaction was incubated at 37 C. The reactions were terminated by the addition of 0.5 ml of 0.1 M sodium pyrophosphate and 0.5 ml of cold 25% trichloroacetic acid, and 200 μg of yeast RNA was then added. After 30 min at 4 C, the precipitates were collected on Gelman type A filters, dried, placed in 10 ml of TLA-toluene (Beckman Instruments Co., Fullerton, Calif.) scintillation mixture, and counted to 1% error in a Beckman LS-355 liquid scintillation counter.

All chemicals were obtained from Calbiochem, Los Angeles, Calif., and Sigma Chemical Co., St. Louis, Mo. Triton X-100 was obtained from Packard Instrument Co., Inc., Downers Grove, Ill. All isotopes were obtained from New England Nuclear Corp., Boston, Mass. The synthetic templates were obtained from Collaborative Research, Inc.

Purification and analysis of the DNA product of the endogenous RDDP reaction. The radioactive products from the endogenous RDDP reaction were adjusted to 0.3 M sodium acetate (pH 5.0) and purified by two phenol-sodium dodecyl sulfate (SDS) extractions and two ether extractions. The aqueous phase was applied to a G-50 sulfopropyl Sephadex column, and fractions eluting in the void volume were pooled and precpitated with ethanol-sodium chloride at −20 C. The DNA precpitates were dissolved in 0.3 M sodium acetate and centrifuged in isopycnic $CsCO_4$ gradients at 192,000 × g for 65 h at 4 C. The gradients were fractionated with an Isco model 640 gradient fractionator, and appropriate fractions were weighed for density determination before precipitation with cold 10% trichloroacetic acid. The trichloroacetic acid precipitates were collected on Gelman 25-mm type A glass-fiber filters, dried, placed in 10 ml of TLA-BBS-3 liquid scintillation cocktail, and counted in a Beckman LS-355 liquid scintillation counter to 1% error.

Hybridization reactions and analysis of the RNA-DNA hybrids with S-1 exonuclease. The [^{3}H]DNA product of the endogenous RDDP of intracisternal A particles was prepared, as described above, in the presence of 25 μg of actinomycin D per ml. The DNA product was chromatographed over G-50 Sephadex, and the material eluting in the void volume was adjusted to 0.3 N KOH and hydrolyzed at 100 C for 10 min. The sample was then neutralized with HCl and adjusted to 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate) and 50% formamide for hybridization. Purified intracisternal A-particle RNA (2 μg), 60 to 70S or 35S, was added to 200 μl of the hybridization mixture, and the reaction was incubated at 37 C for 20 h. Control samples were prepared in the same way with or without heterologous Qβ RNA at the same concentration. At the end of the reaction, the samples were adjusted to 25 mM sodium acetate (pH 4.5), 0.5 mM $ZnSO_4$, and 0.15 M NaCl, and 25 μl of purified S-1 exonuclease was added. The samples were incubated at 45 C for 60 min, and the reaction was terminated by addition of 10% trichloroacetic acid with 20 μg of yeast tRNA as carrier. The precipitates were collected on membrane filters (Millipore Corp., Bedford, Mass.), and the radioactivity was determined as described earlier.

S-1 exonuclease, which was purified from a crude enzyme preparation from *Aspergillus oryzae* by the method of Godson (10), was provided by Ronald Cox. One unit of enzyme will solubilize 5 μg of single-stranded DNA in 10 min at 45 C.

RNA extraction, purification, and analysis. RNA was isolated from gradient-purified A particles by a modification of the conventional SDS-phenol method described previously (35). Approximately 200 μg of A-particle protein equivalents, determined by the Lowry assay (20) using bovine serum albumin as the standard, was suspended in 0.5 ml of NET (0.1 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 0.001 M EDTA) containing 1% SDS, 1% DTT, and 1% diethyloxidiformate. The sample was incubated at 4 C for 5 min and made 0.05 M 2-mercaptoethanol, and an equal volume of redistilled phenol equilibrated with NET was added. The mixture was shaken by hand for 10 min, centrifuged at 30,000 × g for 10 min at 4 C, reextracted with phenol, and recentrifuged. The aqueous phase was made 0.2 M potassium acetate, and the RNA was precipitated either by the addition of 2 volumes of cold 95% ethanol at −20 C for 20 h or by the addition of magnesium phosphate as described by Dessev and Grancharov (5). The ethanol precipitate was suspended in NET, and the magnesium phosphate precipitate was resolubilized as described by Dessev and Grancharov (5).

The resolubilized RNA was analyzed by electrophoresis on acrylamide-agarose gels. The gels contained 1.5% acrylamide, 0.075% *N,N'*-methylenebisacrylamide, 0.5% agarose, and 0.1% SDS. Ammonium persulfate (0.05%) was added for polymerization. The electrophoresis buffer contained 36 mM Tris-hydrochloride, 30 mM NaH_2PO_4, and 1 mM EDTA. The gels were run at 10 V/cm and 3 mA/gel for 90 min. Gels of radioactive RNA were cut into 1-mm slices with a Mickle gel slicer, each slice was dissolved in 0.2 ml of 30% H_2O_2 at 70 C for 30 min, and 10 ml of BBS-III-TLA scintillation cocktail was added and counted in a Beckman LS-355 liquid scintillation counter to 1% error. Gels of unlabeled RNA were scanned in a Gilford 2400-S recording spectrophotometer fitted with a linear transport at 280 nm.

Poly(U)-Sepharose chromatography. Precipitated RNA was dissolved in 0.5 ml of 1% lauroyl sarcosine-0.03 M EDTA and applied to a 2-ml column of poly(U)-Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) previously equilibrated with CSB buffer (0.7 M NaCl, 50 mM Tris-hydrochloride, 10 mM EDTA, and 25% formamide [pH 7.5]) at room temperature. The column was washed with 3 bed volumes of CSB and then eluted with EB buffer (10 mM potassium phosphate, 10 mM EDTA, 0.1% lauroyl sarcosine in 90% formamide [pH 7.5]). Fractions of 0.5 ml were collected, and either the absorbance

at 260 nm or the amount of acid-precipitable radioactivity was determined.

The distribution and size of the poly(A) stretches in myeloma intracisternal A-particle RNA were analyzed by chromatography on poly(U)-Sepharose and by the temperature elution regimen described by Ihle et al. (14). Poly(A) preparations of various chain lengths (Miles) were chromatographed on, and eluted from, poly(U)-Sepharose columns to establish a standard curve. A plot of the log of the polymer length versus elution temperature was linear for poly(A) fragments of 25 to 90 nucleotides and comparable with the data of Ihle et al. (14) for fragments of 20 to 200 nucleotides.

Complement fixation assay and radioimmunoassay for gs antigens. Microtiter assays for MuLV antigen, using both a Fisher rat anti-MuLV-MSV serum (obtained from Roger Wilsnack, Huntingdon Laboratories) reactive towards the major group-specific (gs) antigen and rabbit anti-K-MuLV serum, and FLOPC-1 C-particle antigen (rabbit anti-FLOPC-1 C-particle serum) were carried out by a modification of the procedure described previously (16). Twofold dilutions (25 μl) of gradient-purified virus (50 μg/ml) were incubated with a 1:32 dilution (25 μl) of antiviral serum in the presence of 3 U of guinea pig complement for 1 h at 37 C and then for 24 h at 4 C in Cooke microtiter plates. The hemolytic system (50 μl), consisting of 5% sheep erythrocytes optimally sensitized with hemolysin, was added and the plates were incubated at 37 C for 1 h. The plates were centrifuged at 250 $\times$ g for 15 min, the supernatant was removed, and the absorbance at 540 nm (oxyhemoglobin) was determined. The data are expressed as the dilution of antigen (complement-fixing antigen units) giving $\leq$50% hemolysis. The radioimmunoassay for murine gs-1 antigen was performed under the direction of Wade P. Parks as in the assay described previously (24).

XC syncytium assay. The syncytium assay for type C viruses was carried out as described previously (34) using the XC rat tumor cell line.

RESULTS

Gradient purification of intracisternal A particles. Figure 2 shows the results of a representative isopycnic sucrose gradient purification of intracisternal A particles derived from the cell culture line of FLOPC-1 myeloma (radioactivity) and the in vivo FLOPC-1 tumor (absorbance). The A particles banded at a density of 1.22 g/ml, a result in agreement with that of the A particles isolated from the MOPC-104E myeloma (19). The isolates of purified myeloma intracisternal A particles were examined by electron microscopy, and the appearance of a representative preparation is shown in Fig. 1.

Antigenic and XC syncytium assay for type C virus. Kuff et al. (18) found that intracisternal A particles are antigenically distinct from murine leukemia, sarcoma, and mammary tumor viruses. However, FLOPC-1 A particles are antigenically related to FLOPC-1 type C particles, although this cross-reactivity is weak (unpublished data). Preparations of gradient-purified FLOPC-1 A particles, whether tested by complement fixation or radioimmunoassay, contain minimal amounts of MuLV gs or FLOPC-1 C-particle antigen (Table 1).

In the XC assay, gradient-purified or partially purified FLOPC-1 A particles did not induce the XC cells to form syncytium (34; Table 1). Thus, all evidence indicates that the results presented here were not due to contamination by exogenous type C virus.

Reconstitution experiment. The possibility that a trace contaminant of type C virus might have contributed to the results presented here was examined in the reconstitution experiment described above, in which [^{3}H]uridine-labeled FLOPC-1 type C particles or K-MuLV was added to 0.5 g of unlabeled FLOPC-1 cells and the standard A-particle isolation procedure was carried out. When aliquots of the various pellets and supernatants from the A-particle scheme were monitored for radioactivity, <0.01% of the radioactivity from labeled FLOPC-1 C particles was layered on the 33 to 68% linear sucrose gradient, and no radioactivity was detectable in the 1.22-g/ml region of the

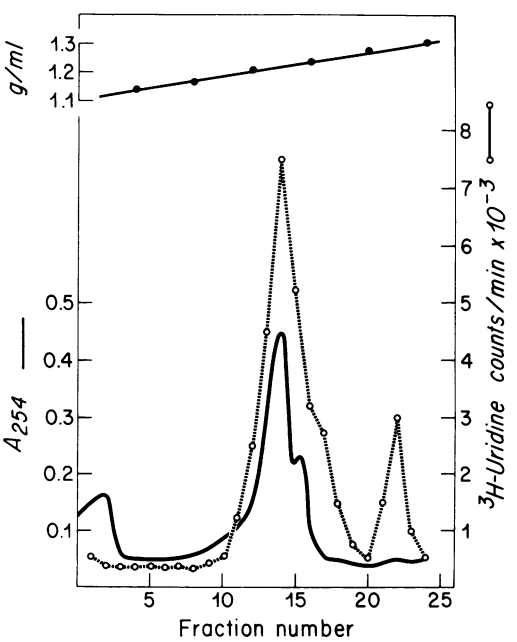

FIG. 2. *Isopycnic sucrose gradient centrifugation on FLOPC-1 intracisternal A particles. Particles isolated from FLOPC-1 tissue culture cells labeled with [^{3}H]uridine (○); particles isolated from in vivo FLOPC-1 tumors (——).*

TABLE 1. *Assays for type C viruses in gradient-purified FLOPC-1 intracisternal A particles*

Virus	Assay				
	Complement fixation[a]			Radioimmunoassay[b]	XC assay[c]
	Anti-MuLV-MSV	Anti-MuLV	Anti-FLOPC-C		
FLOPC-A	72	2	8	ND	5
FLOPC-C	128	64	512	9.6×10^3	728
K-MuLV	512	1,024	64	10^6	3,725

[a] Quantitative complement fixation carried out as described in the text. Data are complement-fixing antigen units.

[b] Radioimmunoassay performed by W. P. Parks. Data are expressed as nanograms of K-MuLV p30 protein per milligram of total protein in comparison with the K-MuLV standard. Viral protein per assay was: FLOPC-A particles, 44 μg; FLOPC-C particles, 6.2 μg; K-MuLV, 0.08 μg. ND, Not detectable.

[c] XC assay carried out as described in the text. Data are the average number of syncytia per chamber from duplicate samples in four assays after correction for the number of spontaneous XC syncytia (95/chamber). The number of syncytial forming units per milligram of viral protein was calculated as: FLOPC-A particles, 10^2; FLOPC-C particles, 8.23×10^5; K-MuLV, 3.81×10^6.

gradient (not shown). When the labeled type C particles were not put through the purification procedure but admixed with the A-particle preparation before isopycnic gradient centrifugation, both particles banded at their characteristic density with little or no cross-contamination (Fig. 3). This type of experiment, which is completely analogous to the results obtained by Yang and Wivel (40) in an identical experiment, reasonably eliminates type C virus contamination as an explanation for the source of the high-molecular-weight RNA and RDDP.

Intracisternal A-particle RNA. Analysis of labeled and unlabeled A-particle RNA by electrophoresis on SDS-acrylamide agarose gels is shown in Fig. 4. Purified RNA possessed high-molecular-weight species that migrated with the K-MuLV RNA marker (60 to 70S), with minor species migrating from 28 to 10S. After heating, the high-molecular-weight RNA was lost with the appearance of lower-molecular-weight structures of approximately 35, 20, and 18S plus a variety of even lower-molecular-weight species. This RNA was sensitive to pancreatic RNase and hydrolysis by KOH (not shown). The high-molecular-weight RNA of the FLOPC-1 intracisternal A particles, if similar to oncornavirus 70S RNA, appears to have many scissions as evidenced by the presence of the variety of low-molecular-weight structures after denaturation. Yang and Wivel (40) and Robertson et al. (27) showed previously that the intracisternal A particles from MOPC-104E and MOPC-460 myelomas possessed high-molecular-weight RNA.

The fact that the putative RNA was completely sensitive to RNase or 0.5 M KOH suggested that this material was indeed RNA. To assess whether the high-molecular-weight material was a ribonucleoprotein complex, the purified preparations were subjected to Pronase digestion (50 μg/ml) at 25 C for 60 min before electrophoresis on acrylamide-agarose gels.

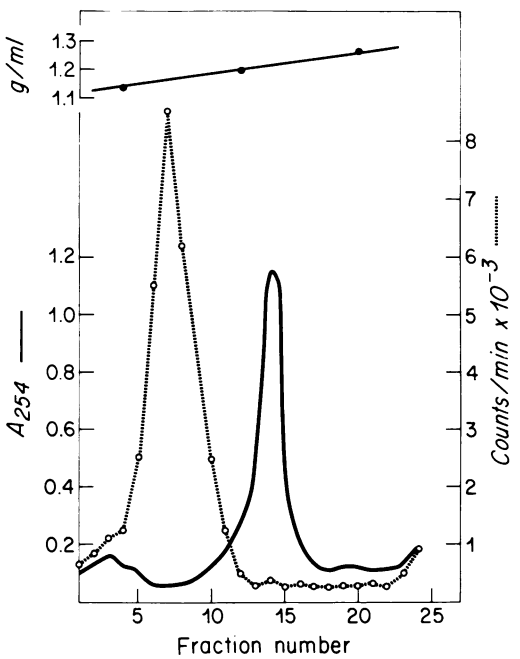

FIG. 3. *Isopycnic sucrose gradient centrifugation of [^{3}H]uridine-labeled FLOPC-1 C particles (○) and unlabeled FLOPC-1 intracisternal A particles. The labeled C particles (10,000 counts/min), isolated as described previously (35), were mixed with the A particles just before layering on the linear 33 to 68% sucrose gradient. The radioactivity in each gradient fraction was determined as described in the text. The density was determined by weighing a 100-μl sample from every fourth fraction.*

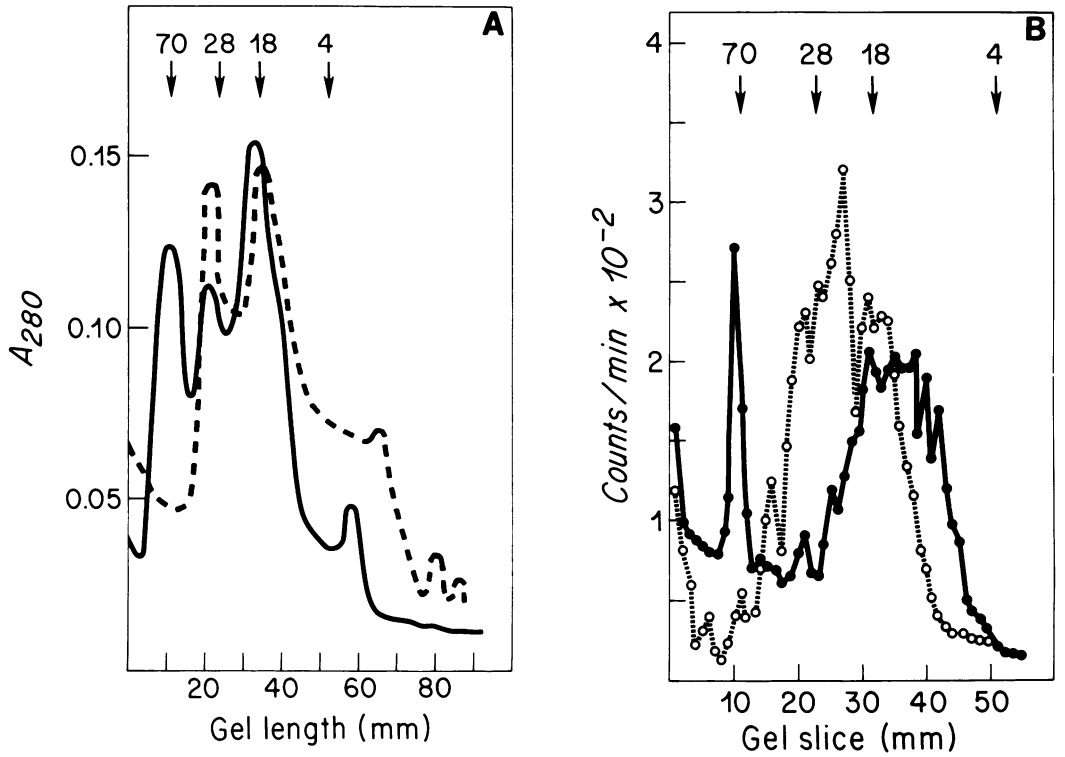

FIG. 4. *SDS-acrylamide gel electrophoresis of RNA from FLOPC-1 intracisternal A particles. Purified native and denatured RNA was subjected to electrophoresis on 1.5% acrylamide-SDS gels, and the gels were analyzed. (A) RNA from A particles isolated from FLOPC-1 in vivo tumors: (——) native RNA; (- - -) denatured RNA. (B) [^{3}H]uridine-labeled RNA from A particles isolated from FLOPC-1 tissue culture cells: (●) native RNA; (○) denatured RNA.*

The results showed that this treatment did not alter the radioactivity profile, suggesting that this was high-molecular-weight RNA (not shown).

Poly(U)-Sepharose chromatography. Generally, 50 to 83% of the RNA was bound to, and eluted from, a poly(U)-Sepharose column (Table 2). The low level of binding of some of the purified native A-particle RNA preparations suggests that it may be contaminated with rRNA, that some A-particle RNA lacks poly(A), or that the RNA is nicked so as to alter its binding to the poly(U) chains on the Sepharose. When 70*S* RNA was isolated on sucrose gradients, the amount bound increased to approximately 80%, in good agreement with the data of Ihle et al. (14) for MuLV. These results indicate that the high-molecular-weight A-particle RNA contains poly(A) regions and that some of these regions are probably not hydrogen bonded in the 60 to 70*S* form and available to hybridize with the poly(U) on the Sepharose. When the RNA preparation was heated at 80 C for 5 min and cooled slowly, the amount of RNA that bound to the column decreased to approximately 40% (Table 2). When the bound RNA was eluted from the column by increasing the temperature (14), approximately 80% of the high-molecular-weight and 60% of the dena-

TABLE 2. *Binding of RNA from FLOPC-1 intracisternal A particles to poly(U)-Sepharose*

Virus	RNA	Amt applied to column (counts/min)	% Bound[a]
K-MuLV	Native	20,000	62
	Native, 70*S*	20,000	82
	Denatured	20,000	41
FLOPC-1 A particle	Native	12,500	50
	Native, 70*S*	2,400	83
	Denatured	10,200	37

[a] The percentage of the viral RNA that bound to the column was assessed by the two different procedures described in the text. Data are for the procedure utilizing lauroyl sarcosine-formamide containing the binding and elution buffers. The amount of RNA bound to the column was that fraction eluting in the EB buffer. Approximately 20,000 counts/min of K-MuLV and 12,500 counts/min of A-particle RNA were applied to the column.

tured RNA eluted as a homogeneous peak at 40 to 45 C (not shown). This elution position corresponds to that of RNA with poly(A) regions approximately 180 nucleotides in length.

Association of an endogenous RDDP with FLOPC-1 intracisternal A particles. Figure 5 shows the association of endogenous DNA polymerase activity with purified intracisternal A particles banded by isopycnic sucrose gradient centrifugation. The absorbancy (254 nm) peak at 1.22 g/cm^3 contains a sharp peak of polymerase activity, and other enzyme activities were localized at 1.18 g/m^3, which may represent particles bound to excess membrane, and 1.28 g/cm^3, which may represent nucleocapsid cores. No polymerase activity was observed at 1.16 g/cm^3; hence, C particles were not contaminating the A-particle preparation. The possibility of C particles contaminating the intracisternal A-particle preparation was also ruled out by the immunological, biological, and reconstitution experiments described above. The coincident banding of endogenous polymerase activity with the bulk of the intracisternal A particles suggests that the RDDP activity is an intrinsic property of the particles.

Some of the requirements of the endogenous intracisternal A-particle polymerase are indicated in Table 3. This enzymatic activity shares many of the general characteristics common to the RDDP of oncornaviruses (32). The requirements for a maximum endogenous reaction include a divalent cation (either Mn^{2+} or Mg^{2+}; Table 4), a monovalent cation (Na^+ or K^+), DTT, and all four deoxynucleoside triphosphates. Elimination of dGTP, dCTP, and dATP, either singly or in combination, depressed the polymerase activity, indicating that the observed activity was not due to a terminal deoxynucleotidyl transferase. DTT and detergent treatment were required for maximum activity either for solubilization of

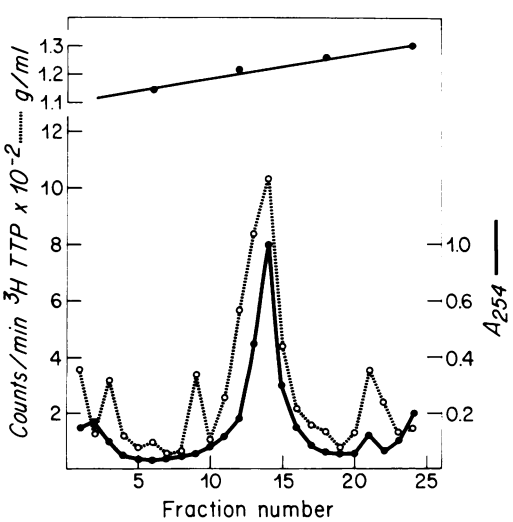

FIG. 5. *Analysis of the RDDP associated with FLOPC-1 intracisternal A particles on a linear sucrose gradient. Incorporation of [^{3}H]TTP in the endogenous reaction (○); absorbance at 254 nm (——).*

TABLE 3. *Requirements for endogenous RDDP activity associated with FLOPC-1 intracisternal A particles*[a]

Conditions	Incorporation of [^{3}H]TTP[b] (counts/min)	% Complete reaction
Complete	4,375	100
−Mn^{2+}	430	10
−KCl	2,680	50
−dATP	1,310	30
−dGTP	1,440	33
−dCTP	1,110	26
−dATP, dGTP, dCTP	340	7
−DTT	15	4
−Triton X-100 treatment	760	17
+Actinomycin D	2,110	48
+DNase	2,310	53
Preincubation with RNase	44	1

[a] Complete endogenous assay was as described in the text. Each assay contained 12 μg of A-particle protein. Preincubation with RNase (25 μg/ml) was carried out in 0.15 M NaCl at 37 C for 2 h.

[b] Incorporation of [^{3}H]TTP per 10 μg of A-particle protein in 30 min at 37 C.

TABLE 4. *Divalent cation requirements of the FLOPC-1 intracisternal A-particle RDDP*

Cation	Concn	Counts/min of [^{3}H]TTP incorporated per μg of viral protein at:[a]	
		30 C	37 C
Mn^{2+}	0	31.3	50.5
	1	*174.2*	*724.7*
	2	126.9	446.7
	3	113.7	396.2
	5	50.8	148.8
	7	25.2	86.4
	10	15.9	35.2
Mg^{2+}	2	50.0	50.5
	5	90.7	185.4
	7	*318.7*	279.2
	10	200.5	381.9
	12	85.2	*531.9*
	15	86.8	282.4

[a] Complete endogenous assay was as described in the text. The data are the average of duplicate experiments with two different viral preparations. The italicized values are the optimal concentrations of cation at each temperature.

the DNA polymerase, to increase accessibility of the endogenous RNA template to the substrates, or both.

The enzymatic activity was inhibited by preincubating gradient-purified particles in the presence of high salt (i.e., 0.15 M NaCl), indicating that the endogenous polymerase was probably dependent on the intrinsic RNA as template, consistent with the data reported for oncornavirus RDDP (32). The activity was partially inhibited by actinomycin D (25 μg/ml) and DNase, results consistent for the oncornavirus enzymes (Table 3).

The endogenous RDDP activity was stimulated maximally by Mn^{2+} rather than by Mg^{2+} (Table 4). At the optimal Mn^{2+} concentration (1 mM), approximately 1.5-fold greater incorporation was observed than for a broad range of Mg^{2+} concentrations. The preference of the FLOPC-1 intracisternal A-particle endogenous RDDP activity for Mn^{2+} is characterisitic of the polymerases of most oncornavirus (12, 29, 32) and other myeloma intracisternal A particles (27, 41). However, other workers (1, 38) have reported that the polymerase of intracisternal A particles from MOPC-104E preferred Mg^{2+} rather than Mn^{2+}, data consistent with the presence of a poly(dT) polymerase that resembles the cellular enzyme associated with murine tissue culture cells (8, 37).

The kinetics of the endogenous and poly(rA)·oligo(dT)-stimulated reaction for a typical intracisternal A-particle preparation are shown in Fig. 6. The endogenous reaction was linear for 20 to 30 min before it slowed, and the presence of actinomycin D (25 μg/ml) resulted in decreased incorporation of [^{3}H]TTP beyond 30 min. This effect suggests the presence of a DNA/RNA hybrid as a reaction intermediate. In the presence of poly(rA)·oligo(dT)$_{12-18}$, the initial rate of the RDDP activity was increased significantly.

Table 5 compares the RDDP activity of the FLOPC-1 intracisternal A particles, FLOPC-1 C particles, and K-MuLV with various synthetic templates. The intracisternal A-particle polymerase activity was stimulated to a greater degree by synthetic RNA/DNA hybrids as compared with the synthetic DNA/DNA duplexes, similar to the polymerase of the other two viruses. When the activities stimulated by poly(rA)·oligo(dT) and poly(dA)·oligo(dT) are compared, a ratio [poly(rA)·oligo(dT)/poly(dA)·oligo(dT)] of >9.0 was observed consistently, a characteristic of oncornavirus RDDP (32). FLOPC-1 A-particle polymerase was stimulated by poly(rC)·oligo(dG) to a greater extent than by poly(rA)·oligo(dT), whereas K-MuLV had more activity with poly(rA)·oligo(dT) than poly(rC)·oligo(dG) (Table 2), as was also true for another myeloma intracisternal A particle (41).

The nature of the DNA product, as analyzed on Cs_2SO_4 equilibrium density gradients, in the endogenous RDDP reaction differed depending on the duration of the reaction (Fig. 7). The [^{3}H]DNA synthesized early in the reaction (i.e., 5 min) was linked to endogenous RNA;

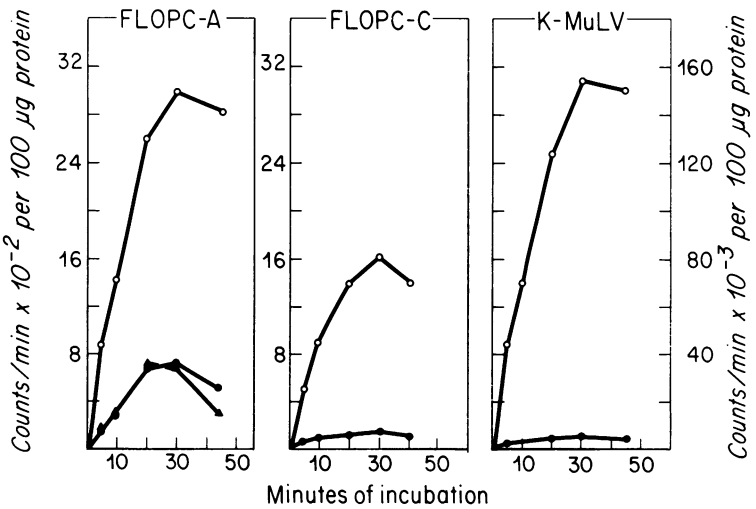

FIG. 6. *Kinetics of the RDDP of FLOPC-1 intracisternal A particles with (○) and without (●) exogenous synthetic templates. The time course of DNA synthesis in the presence of actinomycin D is also shown (▲). The kinetics of the RDDP activity for FLOPC-1 C particles and K-MuLV with (○) and without (●) exogenous synthetic templates is shown for comparative purposes. The synthetic template was poly(rA)·oligo(dT)*$_{12-18}$.

TABLE 5. *FLOPC-1 intracisternal A-particle, FLOPC-1 C-particle and K-MuLV RDDP: comparison of activity with various synthetic templates*[a]

Reaction conditions	Template added	% Endogenous reaction (×10²)		
		FLOPC-C particle	K-MuLV	A particles
Endogenous		1	1	1
Template stimulated	Poly(rA)·oligo(dT)[b]	23.09	193.8	3.91
	Poly(dA)·oligo(dT)[b]	2.23	3.71	0.13
	Poly(rC)·oligo(dG)[c]	6.91	96.50	10.69
	Poly(dC)·oligo(dG)[c]	0.91	2.35	1.41
	Poly(rG)·oligo(dC)[d]	2.72	6.09	4.77
	Poly(dG)·oligo(dC)[d]	0.68	2.93	1.18
	Poly(rU)·oligo(dA)[b]	3.06	7.21	0.20

[a] Reaction conditions were as described in the text. Virus samples were purified on isopycnic sucrose gradients. Data are calculated from the counts per minute of [³H]TTP incorporated per 10 μg of viral protein in a 30-min reaction at 37 C.

[b] Incorporation of [³H]dTTP.

[c] Incorporation of [³H]dGTP.

[d] Incorporation of [³H]dCTP.

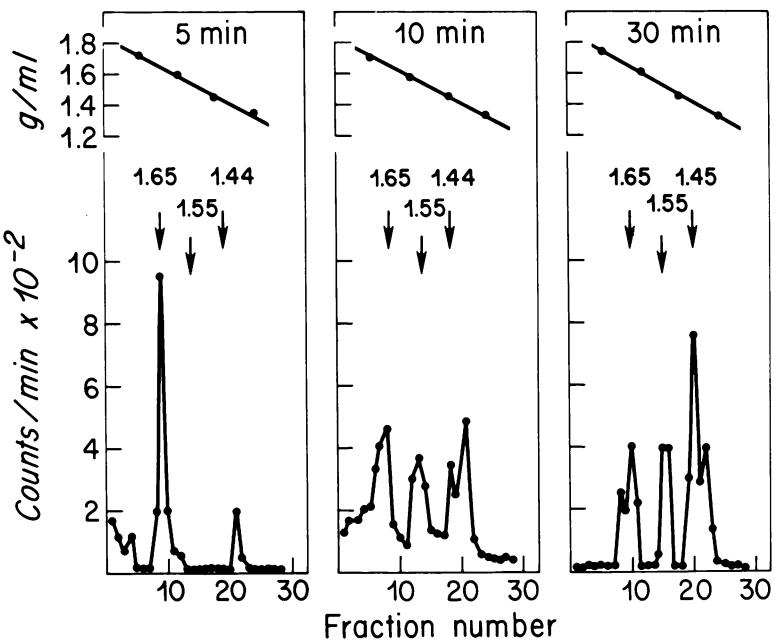

FIG. 7. *Analysis in $CsCO_4$ isopycnic gradients of the products of the endogenous reaction of FLOPC-1 intracisternal A-particle RDDP. A 1-mg amount of purified A particles was assayed for endogenous activity at 37 C in a final reaction volume of 0.5 ml. Samples (100 μl) were withdrawn at the indicated times, and the product was then purified and analyzed.*

depending on the proportion of the newly synthesized DNA to endogenous RNA, the product banded at an RNA density of 1.65 g/cm³ and at the DNA/RNA hybrid region from 1.50 to 1.55 g/cm³. Later in the reaction (i.e., 15 min), the [³H]DNA radioactivity shifted to the hybrid density, the density of single-stranded DNA (1.44 g/cm³) and double-stranded DNA (1.40 g/cm³). However, even late in the reaction (i.e., 30 min), some radioactivity was still associated with the endogenous RNA (1.65 g/cm³). This product was completely sensitive to DNase (not shown) and hybridized with the RNA intrinsic to the A particles (Table 6).

S-1 nuclease, which hydrolyzes the unhybridized region of single-stranded DNA in a DNA/RNA hybrid, was used to assess the extent of reaction of the [³H]DNA product of the endoge-

nous RDDP reaction to the A-particle RNA. Approximately 70% of the [^{3}H]DNA product hybridized with the high-molecular-weight RNA intrinsic to the A particle and 90% hybridized to denatured high-molecular-weight A-particle RNA. The [^{3}H]DNA product did not hybridize to a significant extent with heterologous Qβ RNA (Table 6) or BALB/c liver cellular RNA. The very low level of [^{3}H]DNA hybridized to liver RNA was probably due to the fact that the FLOPC-1 A-particle preparations were contaminated with low levels of rRNA.

A preliminary assessment of the genetic relationship of FLOPC-1 A particles to known murine oncornaviruses or FLOPC-1 C particles is shown in Table 7. The FLOPC-1 A-particle complementary DNA hybridized to a significant extent with the RNAs from FLOPC-1 C particles, K-MuLV, or K-MuLV-MSV. However, no significant difference in the extent of reaction was observed for the reaction of the A-particle complementary DNA and any of the heterologous viral RNAs. Although preliminary, the data suggest that the genome of the FLOPC-1 A particles shares homology with certain sequences with the genomes of type C viruses.

TABLE 6. *Analysis of the [^{3}H]DNA product of the endogenous FLOPC-1 intracisternal A-particle RDDP: hybridization with A-particle RNA*[a]

Condition	[^{3}H]DNA hybridized to RNA (counts/min per reaction)
[^{3}H]DNA input	4,025
[^{3}H]DNA + S-1 exonuclease	201
[^{3}H]DNA − native RNA + S-1 exonuclease	2,818
[^{3}H]DNA + denatured RNA + S-1 exonuclease	3,623
[^{3}H]DNA + Qβ RNA + S-1 exonuclease	242
[^{3}H]DNA + cellular rRNA + S-1 exonuclease	400

[a] All experimental conditions were described in the text. [^{3}H]DNA from an endogenous reaction incubated at 37 C for 30 min. Background radioactivity was 25 counts/min.

TABLE 7. *Hybridization of the [^{3}H]DNA product of the endogenous FLOPC-1 intracisternal A-particle RDDP with A-particle or C-type virus RNA*[a]

RNA	[^{3}H]DNA hybridized to RNA (counts/min per reaction)
None − input	6,040
FLOPC-1 A particle	5,496
FLOPC-1 C particle	3,080
K-MuLV	2,960
K-MuLV-MSV	3,019

[a] All experimental conditions were as described in the text. [^{3}H]DNA from the endogenous reaction incubated at 37 C for 30 min. Background radioactivity was 25 counts/min. The viral RNAs were denatured by heating at 80 C for 5 min before addition to the hybridization reaction.

DISCUSSION

The experiments described in this report demonstrate the presence of an RDDP and high-molecular-weight RNA associated with the intracisternal A particles of the FLOPC-1 line of BALB/c myeloma. These conclusions are in agreement with the reports of Yang and Wivel (40, 41) and Robertson et al. (27) for intracisternal A particles from other BALB/c myelomas.

The possibility that the results are artifacts due to contamination by C-particle nucleocapsid cores must be considered. Although some myeloma lines have been observed to produce C particles, especially when adapted to growth in cell culture (25), we have never observed C particles in a solid, subcutaneous FLOPC-1 tumor (34, 35; T. Warner and R. G. Krueger, manuscript in preparation), the tissue from which the intracisternal A particles were isolated and purified. Nevertheless, if some C particles had been present in this in vivo tumor, their nucleocapsid core ($\rho > 1.20$ g/cm^3) theoretically could have co-purified with the A particles and be the source of the high-molecular-weight RNA and the RDDP activity. However, the results of the immunochemical and biological assays, plus the reconstitution experiment described in this report, indicated that such contamination would be absolutely minimal. In addition, other lines of evidence suggest that the C particles are not a significant source of either the high-molecular-weight RNA or the RDDP activity. These include the nature of the RNA intrinsic to the FLOPC-1 C particle (35; R. G. Krueger, manuscript in preparation) plus the specific activity and template specificity of the C particle as compared with the A-particle DNA polymerase.

The RNA isolated from the FLOPC-1 A particles contained a major component of high-molecular-weight RNA, but minor components (28 to 5*S*) were also observed. The RNA contained tracts of poly(A) of approximately 180 nucleotides long. Thus, the presence of an RDDP plus high-molecular-weight poly(A)-containing RNA suggests a relationship between type A particles and oncornaviruses. Furthermore, the extent of hybridization of the [^{3}H]DNA product

of the endogenous A-particle RDDP to oncornavirus RNAs, as described here and previously (27), further substantiates the notion that these particles are related to tumor viruses. Yang and Wivel (40) compared the properties of intracisternal A particles and oncornaviruses and suggested that they arose from an incomplete expression of a viral genome rather than being an aberrant cellular constituent. Robertson et al. (27) suggested that A particles are defective sarcoma-like viruses that mistakenly bud into the cisternae of the rough endoplasmic reticulum instead of into the extracellular medium. It is also possible that A particles may be true viral entities with their own mode of action or spectrum of activity that play some, as yet unknown, role in the biology of tumor tissue. For example, this structure could be a prototype oncornavirus that lacks true oncogenic activity but which activity may be acquired in time (phenotypic and/or genotypic mixing), resulting in the formation of an ultrastructurally different virus known as the C particle.

Type A particles appear to possess many of the properties of known oncornaviruses, save for biological activity. It would appear important, therefore, that additional attempts be made to demonstrate some type of activity in either an in vitro or in vivo system for intracisternal A particles. If some type of activity were observed, then A particles would satisfy the essential requirements for a virus, and they would be removed from the realm of merely a biological curiosity.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants CA-13333 and CA-18936 from the National Cancer Institute.

The technical assistance of Randa Grisham, Betty Thorpe, and Jacqueline Proper is gratefully acknowledged.

LITERATURE CITED

1. **Bohn, E. W., and S. H. Wilson.** 1974. Studies on the activity of the A-particle-associated DNA polymerase. Cancer Res. 34:1977-1981.
2. **Dalton, A. J., and M. D. Felix.** 1956. The electron microscopy of normal and malignant cells. Ann. N.Y. Acad. Sci. 63:1117-1140.
3. **Dalton, A. J., M. Potter, and R. M. Merwin.** 1961. Some ultrastructural characteristics of a series of primary and transplanted plasma cell tumors of the mouse. J. Natl. Cancer Inst. 26:1221-1267.
4. **DeHarven, E., and C. Friend.** 1958. Electron microscopy study of a cell-free induced leukemia of the mouse. J. Biophys. Biochem. Cytol. 4:151-156.
5. **Dessev, G. N., and K. Grancharov.** 1973. Precipitation of RNA, DNA and nucleoprotein particles from very dilute solutions. Anal. Biochem. 53:269-271.
6. **Dmochowski, L., and C. E. Grey.** 1957. Electron microscopy of tumors of known and suspected viral etiology. Tex. Rep. Biol. Med. 15:704-756.
7. **Friedlander, M., and D. H. Moore.** 1956. Occurrence of bodies within endoplasmic reticulum of Ehrlich ascites tumor cells. Proc. Soc. Exp. Biol. Med. 92:828-831.
8. **Fry, M., and W. Weissbach.** 1973. Utilization of synthetic ribonucleic acid template by a new deoxyribonucleic acid polymerase from cultured murine cells. J. Biol. Chem. 248:2678-2683.
9. **Gardner, M. B., J. E. Officer, R. W. Rongey, J. D. Estes, H. C. Turner, and R. J. Hueber.** 1971. C-type RNA tumour virus genome expression in wild house mice. Nature (London) 232:617-620.
10. **Godson, G. N.** 1973. Action of the single-stranded DNA specific nuclease S_1 on double-stranded DNA. Biochim. Biophys. Acta 308:59-67.
11. **Granboulan, N., M. R. Riviere, and W. Bernhard.** 1960. Presence de particules d'aspect viral dans un sarcome Greffable de la souris provoque par le methylcholanthrene. Bull. Cancer 47:291-307.
12. **Green, M., M. Rokutanda, K. Fuzinaga, R. K. Ray, H. Rokutanda, and C. Gurgo.** 1970. Mechanism of carcinogenesis by RNA tumor viruses. I. An RNA-dependent DNA polymerase in murine sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. 67:385-393.
13. **Hall, W. T., J. W. Hartley, and K. Sanford.** 1968. Characteristics of and relationship between C-particles and intracisternal A-particles in clonal cell strains. J. Virol. 2:238-247.
14. **Ihle, J. N., K. L. Lee, and F. T. Kenney.** 1974. Fractionation of 34S ribonucleic acid subunits from oncornaviruses on polyuridylate-Sepharose columns. J. Biol. Chem. 249:38-42.
15. **Kakefuda, T., E. Roberts, and V. Suntzeff.** 1970. Electron microscopic study of methylcholanthrene-induced epidermal carcinogenesis in mice: mitochondrial dense bodies and intracisternal A-particles. Cancer Res. 30:1011-1019.
16. **Krueger, R. G., and G. C. Miller.** 1974. Relationship between the cellular and viral antigens of a BALB/c myeloma and murine leukemia virus. J. Natl. Cancer Inst. 53:997-1004.
17. **Krueger, R. G., W. H. Williams, and G. C. Miller.** 1974. Cellular and viral antigens of BALB/c myeloma. J. Natl. Cancer Inst. 52:1203-1210.
18. **Kuff, E. L., K. K. Lueders, H. L. Ozer, and N. A. Wivel.** 1972. Some structural and antigenic properties of intracisternal A-particles occurring in mouse tumors. Proc. Natl. Acad. Sci. U.S.A. 69:218-222.
19. **Kuff, E. L., N. A. Wivel, and K. K. Lueders.** 1968. The extraction of intracisternal A-particles from a mouse plasma-cell tumor. Cancer Res. 28:2137-2148.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
21. **Marciani, D. J., and E. L. Kuff.** 1973. Isolation and partial characterization of the internal structural proteins from murine intracisternal A-particles. Biochemistry 12:5075-5083.
22. **Nadel, E., W. Banfield, S. Burstein, and A. J. Tousimis.** 1967. Virus particles associated with strain 2 guinea pig leukemia (L2C/N-B). J. Natl. Cancer Inst. 38:979-981.
23. **Novikiff, A. J., and L. Biempica.** 1966. Cytochemical and electron microscopic examination of Morris 5123 and Reuber H-35 hepatomas after several years of transplantation. Gann Monogr. 1:65-87.
24. **Parks, W. P., and E. M. Scolnick.** 1972. Radioimmunoassay of mammalian type C viral proteins. Interspecies antigenic reactivities of the major internal polypeptide. Proc. Natl. Acad. Sci. U.S.A. 69:1766-1770.
25. **Potter, M.** 1972. Immunoglobulin producing tumors and myeloma proteins of mice. Physiol. Rev. 52:631-719.

26. **Provisional Committee for Nomenclature of Viruses.** 1966. Suggestions for classification of oncogenic RNA viruses. J. Natl. Cancer Inst. 37:395–397.
27. **Robertson, D. L., N. L. Baenziger, D. C. Dobbertin, and R. E. Thach.** 1975. Characterization of DNA polymerase and RNA associated with A type particles from murine myeloma cells. J. Virol. 15:407–415.
28. **Schubert, D., S. Humphreys, C. Barone, and M. Cohn.** 1969. *In vitro* differentiation of a mouse neuroblastoma. Proc. Natl. Acad. Sci. U.S.A. 64:316–323.
29. **Scolnick, E. M., E. Rands, S. A. Aaronson, and G. J. Todaro.** 1970. RNA-dependent DNA polymerase activity in five RNA viruses: divalent cation requirements. Proc. Natl. Acad. Sci. U.S.A. 67:1789–1796.
30. **Smith, G. H., H. B. Andervont, and T. B. Dunn.** 1970. Attempts to detect nodule-inducing virus in strain RIII mice. J. Natl. Cancer Inst. 44:657–671.
31. **Stewart, S. E., G. Kasnic, C. Draycott, and T. Ben.** 1972. Activation of viruses in human tumors by 5-iododeoxyuridine and dimethyl sulfoxide. Science 175:198–199.
32. **Temin, H. M., and D. Baltimore.** 1972. RNA directed DNA synthesis and RNA tumor viruses. Adv. Virus Res. 17:129–186.
33. **Tumilowicz, J. J., and J. J. Cholon.** 1971. Intracisternal type A-particles and properties of a continuous cell line originating from a gerbil fibroma. Proc. Soc. Exp. Biol. Med. 136:1107–1110.
34. **Volkman, L. E., and R. G. Krueger.** 1973. XC cell cytopathogenicity as an assay for murine myeloma C-type virus J. Natl. Cancer Inst. 51:1205–1210.
35. **Volkman, L. E., and R. G. Krueger.** 1973. Characterization of C-type particles produced by a tissue culture adapted murine myeloma. J. Virol. 12:1589–1597.
36. **Volkman, L. E., E. A. Smuckler, and R. G. Krueger.** 1971. Mouse myeloma: differentiation of neoplastic cells accompanied by an increase in intracellular virus. J. Natl. Cancer Inst. 46:953–962.
37. **Weissbach, A., S. Schlabach, B. Friedlander, and A. Bolden.** 1971. DNA polymerases from human cells. Nature (London) New Biol. 231:167–170.
38. **Wilson, S. H., E. W. Bohn, A. Matsukage, K. K. Lueders, and E. L. Kuff.** 1974. Studies on the relationship between deoxyribonucleic acid polymerase activity and intracisternal A-type particles in mouse myeloma. Biochemistry 13:1087–1094.
39. **Wivel, N. A., and G. H. Smith.** 1971. Distribution of intracisternal A-particles in a variety of normal and neoplastic tissues. Int. J. Cancer 7:167–175.
40. **Yang, S. S., and N. A. Wivel.** 1973. Analysis of high-molecular-weight ribonucleic acid associated with intracisternal A particles. J. Virol. 11:287–298.
41. **Yang, S. S., and N. A. Wivel.** 1974. Characterization of an endogenous RNA-dependent DNA polymerase associated with murine intracisternal A particles. J. Virol. 13:712–720.