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Relationships Between Intracisternal Type A and Extracellular Oncornavirus-Like Particles Produced in Murine MOPC-460 Myeloma Cells

DONALD L. ROBERTSON, PETER YAU, DARRELL C. DOBBERTIN, T. KEVIN SWEENEY, SIGRID S. THACH, THERESE BRENDLER, AND ROBERT E. THACH*

Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri 63110

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Oncornavirus-like particles of the "A" (both intracisternal and intracytoplasmic) and "B" or "C" (extracellular) types are produced by murine MOPC-460 myeloma cells. This communication describes a comparative study on intracisternal A and extracellular particles. Both types of particles contain an RNA-dependent DNA polymerase activity, traces of 35*S* and 70*S* RNA in addition to larger amounts of degraded RNA, and proteins of approximately 76,000 and 45,000 daltons. The 76,000-dalton proteins from intracisternal A and extracellular particles have the same cyanogen bromide peptides. Hybridization kinetic analysis indicates that the RNAs in the two particles are identical or very closely related and share partial homology with Moloney leukemia virus RNA. In contrast, the particles appear to have little or no relationship to murine mammary tumor virus as judged by several different criteria. Electron microscope studies indicate that the extracellular particles arise from the budding of core components through the plasma membrane. These results suggest that the intracisternal A and extracellular oncornavirus-like particles produced by MOPC-460 cells are closely related.

Murine plasmacytoma (myeloma) tumors contain intracisternal type A particles which bud into the rough endoplasmic reticulum (21). Type A particles have also been found in many other types of transformed cells, as well as in normal cells (8, 21, 37). When these particles are purified from disrupted tumor cells, they are not infectious (21). Their role, if any, in the transformation of tumor cells is not known.

Type A particles resemble morphologically the cores of immature types B and C particles (22), but their biochemical similarity to these oncornaviruses has only recently been confirmed. Two research groups have reported that intracisternal A particles contain the characteristic reverse transcriptase and traces of 70*S* and 35*S* RNA which are common elements in all oncornaviruses (23, 32, 38, 39). Moreover, when complementary DNA (cDNA) was prepared using the A particle reverse transcriptase, 40 to 50% of this cDNA hybridized to Harvey and Moloney sarcoma virus 70*S* RNAs (23). These results suggest a definite relationship between the myeloma-associated A particles and other murine oncornaviruses.

Several research groups have noted that lines of myeloma cells which are adapted to growth in culture can produce type B or C extracellular particles (33, 34). However, the relationship of these particles to intracisternal type A particles was not studied. To determine whether these extracellular particles might contain the A particle genetic information, we have isolated a tissue culture cell line from MOPC-460 cells. Electron microscope examination has shown that these cells produce both intracisternal and intracytoplasmic A particles as well as extracellular particles which bud through the plasma membrane and leave the cell. The extracellular particles have been characterized morphologically and biochemically. Our results indicate that they contain many, if not all, of the protein and nucleic acid components found in intracisternal type A particles. A preliminary report of some of our data has been presented elsewhere (D. L. Robertson, N. L. Baenziger, D. C. Dobbertin, and R. E. Thach, Fed. Proc. **34**:527, 1975).

MATERIALS AND METHODS

Reagents and viruses. Triton X-100, sucrose (grade I, RNase free), actinomycin D, RNase A, DNase I, deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP), dithioerythritol, and bovine

serum albumin were all purchased from Sigma Chemical Co.; [^{3}H]dTTP was from New England Nuclear or Amersham/Searle; cyanogen bromide (CNBr), acrylamide, and bis-acrylamide were from Eastman Kodak Co.; poly(A) was from Miles Laboratories, Inc.; mouse mammary tumor virus was a generous gift of R. Gillette of Meloy Laboratories, Springfield, Va. Moloney murine leukemia virus (Mo-MuLV) was purchased from Electronucleonics Laboratories, Inc. (lot no. 19-29-5).

Preparation of A particles. Intracisternal A particles were prepared from the membrane fraction from solid tumors, which was prepared and sheared as previously described (23). The sheared membranes were diluted with TNM buffer [0.02 M Tris-hydrochloride (pH 7.4)–0.15 M NaCl–1 mM $Mg(OAc)_2$] and centrifuged for 30 min at 35,000 rpm in a Spinco type 42 rotor. The pellet was resuspended in TNM buffer with a Dounce homogenizer, and the gelatinous material was removed by filtration through glass wool. The filtrate was then sheared five times through a 25-gauge needle before clarification by centrifugation at 10,000 × *g* for 10 min. The supernatant was layered over a 48% sucrose cushion containing TNM buffer and centrifuged for 18 h in a Spinco SW27 rotor at 25,000 rpm. (The A particles at this stage of purification banded at 1.22 g/cm^3; little or no material banded at 1.29 g/cm^3, which is the buoyant density of intracytoplasmic A particles. The lack of contamination by intracytoplasmic A particles is due to the fact that most of the latter are present in the original homogenate in the form of large aggregates which are pelleted with the nuclear fraction.) The pellet, which contains crude intracisternal A particles, was suspended in 0.05 M potassium citrate (pH 7.2) and 0.5% Triton X-100 and then sheared five times through a 25-gauge needle. These particles were centrifuged through a 48% sucrose cushion containing potassium citrate in an SW56 rotor at 56,000 rpm for 60 min. The particles were resuspended, layered on top of 85% glycerol with TNM buffer, and centrifuged at 56,000 rpm in an SW56 rotor for 3 h. This pellet was resuspended, layered on top of a linear 30 to 69% (wt/wt) sucrose gradient containing TNM buffer, and centrifuged for 18 h in an SW27.1 rotor at 25,000 rpm. The purified intracisternal A particles banded at 1.28 to 1.30 g/cm^3, and the peak fractions were stored in liquid nitrogen.

Cells. MOPC-460 solid tumors were obtained from Ralph Graff and Ernest Simms (of the Jewish Hospital and the Department of Microbiology, respectively, Washington University School of Medicine, St. Louis, Mo.). Two-week-old solid tumors were dissected from BALB/c mice and kept in sterile ice-cold phosphate-buffered saline (Grand Island Biological Co.). Necrotic tissues and fat bodies were trimmed away, and tumors were minced into small pieces with scissors, homogenized gently in a Dounce homogenizer with a loose-fitting pestle, washed twice in ice-cold phosphate-buffered saline, and centrifuged at 800 × *g* for 5 min. About 1 × 10^7 cells in phosphate-buffered saline were injected intraperitoneally into BALB/c mice. Ascites tumors appeared in about 10 days, and these cells were then adapted to tissue culture by suspension in L-15 (Leibovitz) medium containing 10% fetal calf serum (K. C. Biologicals, Inc.). After a brief adaptive period, the cells grew with a doubling time of 40 to 48 h. They retained neoplasticity and the ability to produce the characteristic MOPC-460 immunoglobulin after over 9 months of continuous culture. Although the 460TC line was not cloned, electron microscope examination revealed morphologic homogeneity of the cells and, in particular, that cell sections containing budding extracellular particles also contain both intracisternal and intracytoplasmic A particles. Typically, less than 7% of the cells are dead as judged by trypan blue exclusion.

Preparation of extracellular particles. The cell-free used media was centrifuged in a type 15 rotor at 15,000 rpm for 4 h to pellet the extracellular particles. These particles were resuspended in TNE buffer (0.01 M Tris-hydrochloride [pH 7.4]–0.1 M NaCl–1 mM EDTA), layered on a step gradient consisting of 30% and 42% (wt/wt) sucrose in TNE buffer, and centrifuged for 3 h at 27,000 rpm in a Spinco SW27 rotor. The light-scattering band at the 42% interface was collected, diluted two to three times with TNE buffer, and pelleted in an SW27.1 rotor for 40 min at 27,000 rpm. The resulting pellet was resuspended, layered on a linear 25 to 54% (wt/wt) sucrose gradient, and centrifuged in a Spinco SW27.1 rotor at 27,000 rpm for 8 h. The gradient was fractionated and monitored for protein concentration, reverse transcriptase activities, and density. The peak fractions were kept frozen in liquid nitrogen.

Polymerase activity. Endogenous polymerase activity was assayed using the following reaction mixture in a final volume of 50 μl: Tris-hydrochloride (pH 8.3), 50 mM; $Mg(OAc)_2$, 10 mM; KCl, 0.2 M; dithioerythritol, 6 mM; Triton X-100, 0.1%; glycerol, 19% (vol/vol); dATP, dCTP, and dGTP, each at 0.5 mM; [^{3}H]dTTP (1,000 counts/min per pmol), 0.3 mM; and A particles (300 μg of protein per ml) or extracellular particles (200 μg of protein per ml). This same reaction mixture was used to assay exogenous polymerase activity, except dATP, dCTP, and dGTP were omitted and 0.460 mg of poly(A)·oligo$(dT)_{9,10}$ (10:1 mass ratio) per ml was added to the mixture. Actinomycin D and oligo$(dT)_{9,10}$, when used in endogenous assay, were at 50 μg/ml and 0.2 μg/ml, respectively. The above assay mixtures were preincubated without triphosphates for 10 min on ice, and then the reaction was initiated by the addition of triphosphates. Incubation time was for 60 min at 37 C. Incorporation of [^{3}H]dTTP was determined by adsorption on DEAE filters (Whatman DE81 chromatography paper) as previously described (23).

Preparation of ^{3}H-labeled cDNA reaction product. The endogenous reaction mixture (usually 2 ml) was made 1% in sodium dodecyl sulfate (SDS) and incubated an additional 10 min at 37 C. The mixture was then extracted two times with an equal volume of phenol, washed three times with ether, and applied directly to a Sephadex G-50 column (65 by 1 cm) containing TNE buffer (pH 8.0) with 0.2% SDS. Fractions were collected and monitored for radioactivity in the void volume, and the peak fractions were pooled. Carrier tRNA (200 μg/ml), 0.1 volume of 20% NaOAc (pH 5.4), and 2.5 volumes of absolute ethanol were added. After standing overnight at −20 C, the

nucleic acid was pelleted at 20,000 × g for 20 min, dissolved in 0.4 N NaOH, boiled for 10 min, and neutralized with HCl. The ^{3}H-labeled cDNA was then ethanol precipitated as above. The final pellet was dissolved in sterile TNE buffer and kept frozen at −20 C.

Extraction of RNA. Purified particles or virions were made 1% in SDS, extracted twice with an equal volume of phenol, washed three times with ether, and precipitated by the addition of 0.1 volume of 20% NaOAc (pH 5.4) and 2.5 volumes of absolute ethanol. After standing at −20 C overnight, the RNA was collected by centrifugation at 20,000 × g for 20 min. RNA was dissolved in sterile TNE buffer (pH 7.4) and kept frozen at −20 C. High-molecular-weight RNA (70*S* and 35*S*) isolated from sucrose gradients and virion RNA at a concentration less than 100 μg/ml was precipitated by the addition of $Mg(OAc)_2$ to 3 mM and 4 volumes of absolute ethanol. After standing at −20 C overnight, this RNA was recovered by centrifugation in a Spinco SW56 rotor at 50,000 rpm overnight. RNA was dissolved as above and stored at −20 C.

Hybridizations. Alkali-treated ^{3}H-labeled cDNA prepared from actinomycin D-containing reactions was used in all hybridization experiments. These were performed at 65 C in 0.6 M NaCl–25 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA–0.1% SDS. Portions (50 μl) were overlaid with mineral oil. To assay for hybridized DNA, the mixture was diluted with 0.5 ml S1 nuclease buffer (30 mM NaOAc [pH 4.5]–0.2 M NaCl–0.3 mM $ZnSO_4$), and 20 μg of denatured cold DNA per ml and 800 units of S1 nuclease per ml were added. After a 30-min incubation at 48 C, 150 μg of carrier tRNA was added, followed by an equal volume of 20% trichloroacetic acid. The precipitate was collected by filtration and counted. S1 nuclease was prepared by the method of Sutton (29) and kept frozen in 25% glycerol in liquid nitrogen. All calculated C_rt values have been adjusted to the standard Na^+ concentration (0.12 M sodium phosphate) according to Britten and Smith (6) and Sullivan et al. (28).

Electron microscopy. Virus particles were adsorbed to thin carbon films (supported by thick reticulated carbon films mounted on copper grids), positively stained with 2% uranyl acetate in 30% ethanol, rinsed with 30% ethanol, and dried. Negatively stained particles were stained in 1% uranyl acetate and were not subsequently rinsed. For thin sections, cells were fixed in glutaraldehyde and stained in 1% osmium tetroxide, followed by 2% uranyl acetate. The samples were prepared using the modified Spurr embedding medium (27). Sections were cut using a Sorvall-Porter Blum Ultra-Microtome MT-1.

Polyacrylamide gel electrophoresis. Polyacrylamide slab gels (7.5 to 20%) with a 4% stacking gel were used by the method of Laemmli (14). Electrophoresis was for 5 h at 110 V. Gels were fixed and stained with Coomassie brilliant blue in 25% methanol-10% acetic acid and destained in 7% acetic acid. CNBr peptides were subjected to electrophoresis in an acid-urea-SDS-polyacrylamide gel by the method of Swank and Munkres (30). The CNBr peptides were prepared by cutting the respective protein bands from a 7.5 to 20% SDS-polyacrylamide gel, which were added to 1 ml of 70% formic acid containing 2% CNBr. After 24 h at room temperature, the sample was diluted five times with water, lyophilized, and prepared for gel electrophoresis. Electrophoresis was for 18 h at 75 V in a 15% polyacrylamide slab gel.

Preparation of [^{14}C]glucosamine-labeled extracellular peptides and glycoproteins. Particles were prepared by adding 200 μCi of [^{14}C]glucosamine (52 mCi/mmol, Amersham/Searle Corp.) to 100 ml of 460TC cells in L-15 medium. After an 18-h incubation at 37 C, the cells were pelleted by low-speed centrifugation, and the extracellular particles were then pelleted in a Spinco type 42 rotor at 35,000 rpm for 60 min. The pellet was resuspended in TNE Buffer, layered over a step gradient consisting of 30% and 42% sucrose in TNE buffer, and centrifuged for 2 h at 40,000 rpm in a Spinco SW56 rotor. The light-scattering band at the concentration interface was collected by pipette, diluted, and centrifuged again using the same step gradient. Fractions were collected dropwise by puncturing the bottom of the tube and were monitored for radioactivity. The peak fraction was precipitated using 10% trichloroacetic acid with cold A particle protein added as carrier. The trichloroacetic acid pellet was washed with cold acetone, dissolved in electrophoresis sample buffer, and subjected to electrophoresis in an SDS-polyacrylamide gel. The gel was autoradiographed using the fluorography technique developed by Bonner and Laskey (4). The developed X-ray film was then scanned using a Joyce-Loebl optical densitometer.

RESULTS

Our initial studies on the virus-like particles found in MOPC-460 myeloma cells involved the characterization of intracisternal type A particles isolated from solid tumors (23). The tissue culture line derived from these tumors (designated 460TC) also contains these particles (Fig. 1a). In addition to intracisternal type A particles, the cells also contained intracytoplasmic A particles (Figure 1b). Intracytoplasmic A particles have been detected in several mouse plasmacytomas, but they have been assumed to represent cores of murine mammary tumor virions (MuMTV) and therefore to be unrelated to the intracisternal particles (10).

In addition to the intracellular A particles, extracellular particles can also be observed budding through the plasma membrane of 460TC cells. Figure 1c shows a series of electron micrographs depicting these particles at various stages of budding. In most cases the core of the nascent particle appears to be completely formed at an early stage in the budding process and resembles an intracytoplasmic A particle. Other workers have noted this phenomenon and have cited it as evidence supporting the view that the latter are MuMTV core precursors (10). However, in a few cases (approximately 5% of

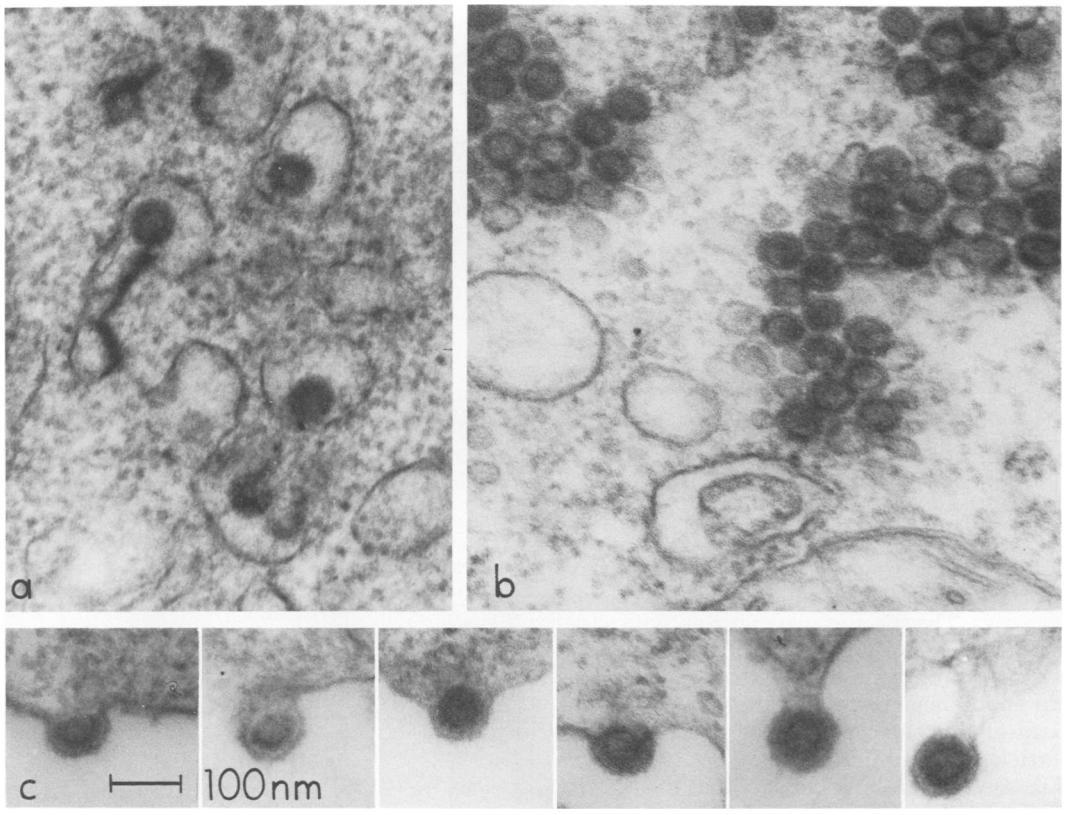

FIG. 1. *Electron micrographs of the virus-like particles associated with MOPC-460 tissue culture cells. (a) Intracisternal A particles; (b) intracytoplasmic A particles; (c) extracellular particles at various stages of budding through the plasma membrane. Bar = 100 nm.*

all budding particles), typical type C precursor structures are seen which contain only partially formed cores (Fig. 1c; see also Fig. 13 in Dalton and Potter, reference 10). This observation suggests an alternative interpretation, namely that the 460TC extracellular particles may always bud in basically a type C process, but that core formation may be rapid relative to detachment of the virion from the cell surface, thereby giving rise to structures which are immature type B in appearance. Other researchers have also observed particles which appear to bud like MuMTV but which resemble more closely the type C viruses when they are examined by other criteria (9, 12). It is also possible, however, that the rare cases of type C budding which we observe are due to unrelated murine type C viruses which are induced in the tissue culture cells. In view of these considerations, we feel that the morphological features of the budding particles are not reliable criteria for identification.

More direct evidence of morphologic dissimilarity between 460TC extracellular particles and the type B MuMTV was obtained from electron microscope examination of purified particles. MuMTV, when negatively stained, reveals characteristic "spikes" on the surface of the outer membrane (Fig. 2a). However, both the 460TC extracellular particles (Fig. 2b) and Mo-MuLV (Fig. 2c) are devoid of these spikes. Moreover, positively stained 460TC particles show characteristically type C morphology for the most part, although occasionally type B structures with eccentric, condensed nucleoids can be seen (Fig. 2d). We conclude that, although 460TC extracellular particles are morphologically similar to MuMTV in some respects, they are quite dissimilar in others. As will be shown below, the biochemical evidence is unambiguous in relating 460TC particles to other type C oncornaviruses.

Extracellular 460TC particles were purified by repeated banding in sucrose density gradients. Figure 3 shows a typical banding profile for purified particles. There is coincident banding of both protein and RNA-dependent DNA polymerase activities, with peaks at a density of

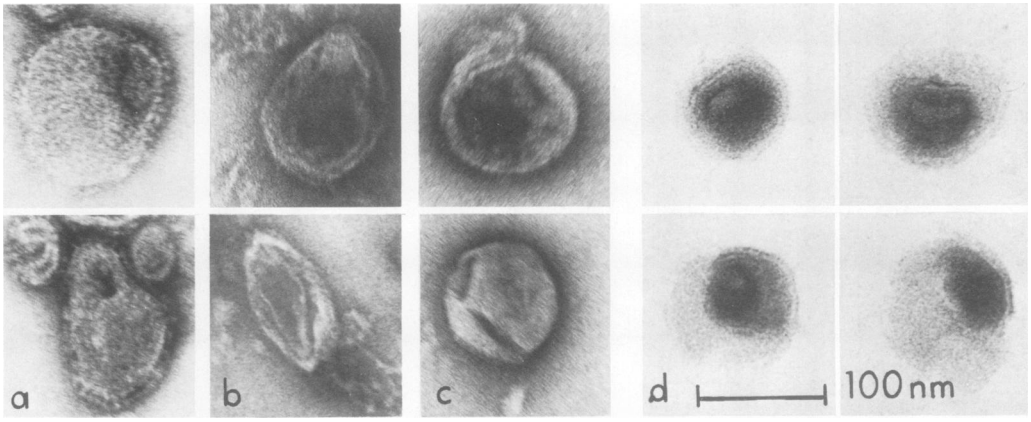

FIG. 2. *Electron micrographs of 460TC extracellular particles, MuMTV and Mo-MuLV virions. (a) MuMTV virions, negatively stained; (b) 460TC particles, negatively stained; (c) Mo-MuLV virions, negatively stained; (d) 460TC particles, positively stained. Bar = 100 nm.*

1.17 g/cm^3. The purified 460TC particles have both exogenous and endogenous polymerase activities. The specific enzymatic activities (defined as picomoles of [^{3}H]dTTP incorporated per milligram of protein) with both exogenous and endogenous templates are the same for the extracellular particles and the intracisternal A particles isolated from solid MOPC-460 tumors (Fig. 4). These results indicate that the activity present in A particles is not due to contamination by small amounts of extracellular particle cores, as will be discussed below.

To better characterize the extracellular particles and to compare them further to the intracisternal A particles, proteins from both types of particles were analyzed electrophoretically in an SDS-polyacrylamide gel. Figure 5 shows optical density scans of typical gels stained for protein with Coomassie brilliant blue. The extracellular particles contain proteins with apparent molecular weights of 76,000, 45,000, and 30,000 (hereafter referred to as p76, p45, and p30, respectively) in addition to lower-molecular-weight proteins ranging in size from 10,000 to 15,000. Both the p30 and p45 bands coelectrophoresed with the corresponding p30 and gp45 proteins of Mo-MuLV, as determined in separate experiments and depicted by arrows in the top panel of Fig. 5. The gp69/71 protein band of Mo-MuLV clearly migrated ahead of the p76 of the myeloma extracellular particles. The electrophoretic profile of proteins from intracisternal A particles is also shown in Fig. 5. The most prominent peaks migrate at 76,000, 70,000, and 45,000 daltons. Evidence will be presented below that the first of these is very similar or identical to the p76 present in extracellular particles.

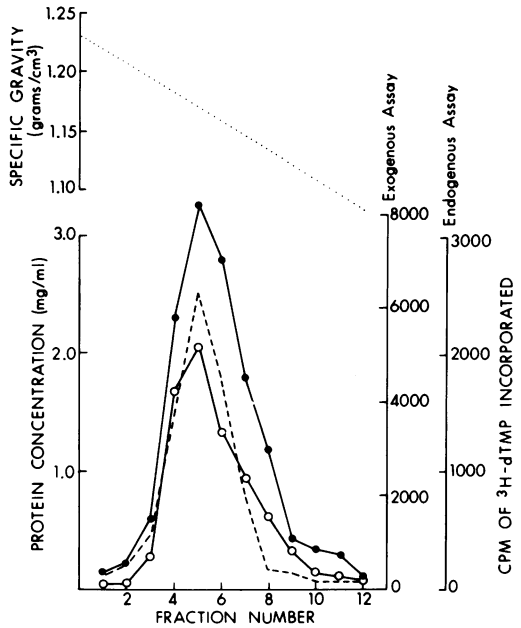

FIG. 3. *Sucrose gradient banding profile from the final purification step for extracellular particles from 460TC cells. Protein concentration (---) as determined by the biuret method, endogenous polymerase activity (○), exogenous polymerase activity (●), and specific gravity (·····) are shown. Details for the assay of polymerase activities are given in Materials and Methods.*

The proteins from both the 460TC extracellular particles and intracisternal A particles are quite different from the MuMTV proteins shown in the bottom panel of Fig. 5. (In our hands, the electrophoretic mobilities of the MuMTV proteins are virtually identical to those reported by Teramoto et al., reference 31).

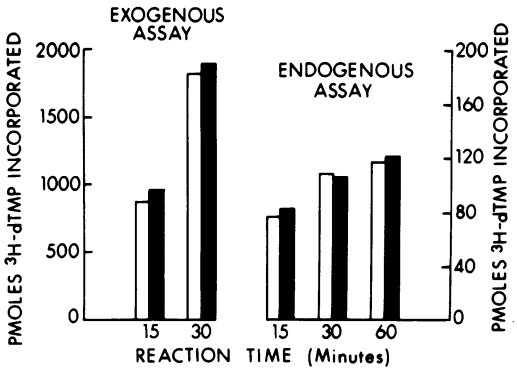

FIG. 4. *Comparison of the specific activities of exogenous and endogenous DNA polymerase activities associated with the extracellular (open bars) and intracisternal A (closed bars) particles at indicated reaction times. Specific activity is expressed as picomoles of [³H]dTMP incorporated per milligram of protein; zero time values are subtracted from all data.*

Moreover, Wade Parks (of the National Cancer Institute, Bethesda, Md.) has analyzed a sample of our extracellular particles immunologically and has found cross-reactivity with MuLV p30 antigen but not with MuMTV p14 antigen (personal communication). These results, in combination with nucleic acid hybridization studies described below, indicate that the MOPC-460TC extracellular particles are not closely related to MuMTV.

The most striking differences between the protein profiles of the extracellular 460TC particles and the intracisternal A particles are the virtual absence of the p30 protein and the presence of a major protein at 70,000 daltons (p70) in the A particles. In many respects the profile of the A particle proteins is similar to those previously reported by Marciani and Kuff (16) and Wivel et al. (36). The major structural protein found by these workers was estimated to be 73,000 daltons; minor protein bands were also seen at 30,000, 46,000, and 80,000 daltons.

When the proteins from MOPC-104E A particles (generously provided by E. L. Kuff and K. K. Lueders) were subjected to electrophoresis simultaneously next to those from MOPC-460 intracisternal A particles, it was clear that the 460 p76 and p70 polypeptides coelectrophoresed with the 104E p80 and p73 polypeptides, respectively. Moreover, when MOPC-460 intracisternal A particles were tested for cross-reactivity with antiserum made against the MOPC-140E 73,000-dalton protein, a small extent of reaction was seen (K. Lueders, personal communication). Thus it seems likely that the 460 p70 may be similar or identical to the 104E p73 and, by inference, the same may be true of p76 (from 460) and p80 (from 104E). The most significant difference between the results of the Kuff and Wivel groups and those reported here would seem to be the relative amounts of p70 (or p73) and p76 (or p80) found in A particles. This discrepancy may be due to minor differences in preparative procedures and hence may not be terribly significant.

The p76 protein found in both extracellular particles and A particles appears to be characteristic of these particles; we therefore deemed it important to confirm that this protein is really the same in both types of particles. As a preliminary step, we have verified that p76 is unique to the particles under study and is not a normal rough endoplasmic reticulum protein. To this end, a mouse liver membrane fraction was prepared in a manner identical to the preparation of A particles from myeloma tumor membranes. Cytoplasmic membranes were sheared in the absence of detergent and banded in a sucrose gradient. When the 1.22 g/cm³ region from the gradient was analyzed by SDS-polyacrylamide gel electrophoresis, no protein corresponding to p76 was found. To compare the p76 proteins from extracellular and intracisternal A particles, these proteins were extracted from SDS-acrylamide gels and treated with CNBr. The CNBr-cleaved peptides were reelectrophoresed in an acid-urea-SDS-polyacrylamide gel. Figure 6 shows an optical density tracing of the stained gel. The peptides derived from both of the p76 proteins appear to be very similar or identical, confirming that the intracisternal A and extracellular particles do in fact contain an identical protein.

To detect the presence of glycoproteins in the extracellular particles, 460TC cells were grown in the presence of ^{14}C-labeled glucosamine as described in Materials and Methods, and particles were isolated. A large amount of radioactivity co-purified with the particles and banded in a single peak at about 1.13 to 1.18 g/cm³ in the final sucrose gradient step. When protein from the labeled particles was extracted and analyzed by polyacrylamide gel electrophoresis, only two labeled glycoprotein bands were detected (Fig. 7). The major band migrates with an apparent molecular weight of 110,000 to 120,000, whereas the minor band, which appears to be heterogeneous, runs between 75,000 and 90,000. Little or no radioactivity is seen in the 69,000- to 71,000-dalton region, where the typical MuLV large glycoprotein should be found. Neither of the bands was labeled when [^{14}C]fucose was added to the cell culture medium. These results suggest that the extracellular particles contain an atypical

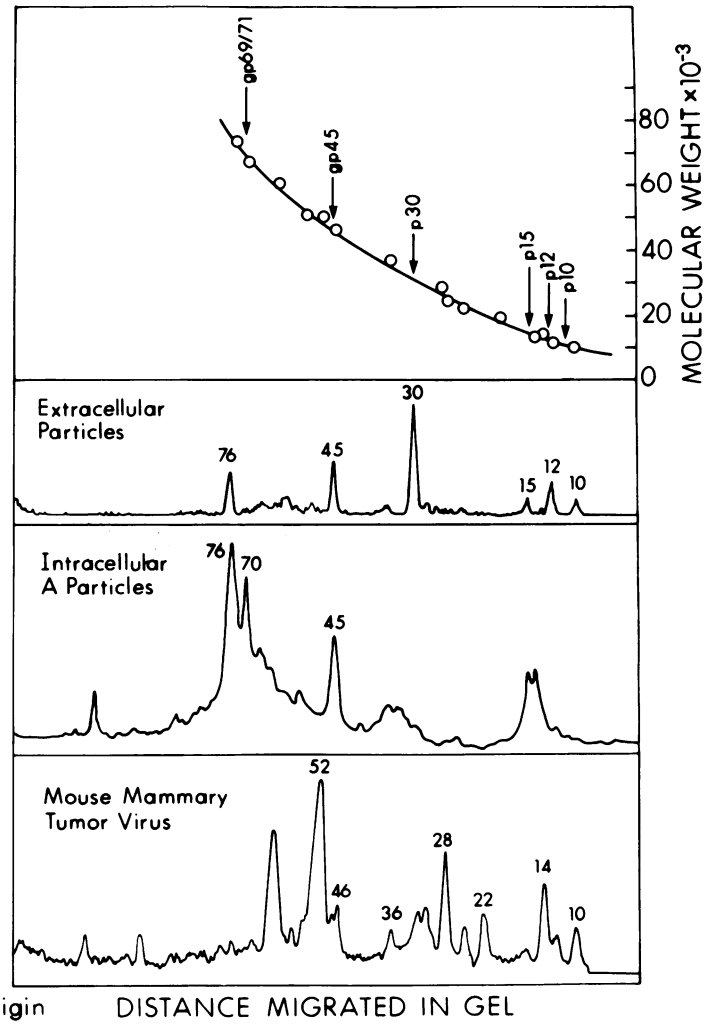

FIG. 5. *SDS-polyacrylamide gel electrophoresis of proteins from 460TC extracellular particles, MOPC-460 solid tumor intracellular (intracisternal) A particles, and mouse mammary tumor virus are depicted as optical density tracings from the stained gels. Position of proteins from Mo-MuLV are depicted by the labeled arrows in the top panel, which also contains the molecular weight calibration curve. Proteins used for molecular weight standards include: bovine serum albumin, 67,000; DNase I, 31,000; RNase A, 13,700; human fibrinogen (3 proteins), 73,000, 60,000, and 50,000; mouse mammary tumor virus, 52,000, 46,000, 36,000, 28,000, 22,000, 14,000, 10,000; and avian myeloblastosis virus, 24,000, 19,000, 11,000.*

glycoprotein which may represent an uncleaved precursor to the commonly found gp 69,000/71,000 (18).

As a control, the proteins in the endoplasmic reticulum fraction from [^{14}C]glucosamine-labeled cells were also analyzed by gel electrophoresis. Some 20 to 30 labeled bands were seen, including the two present in the extracellular particles. The results support (but do not prove) the view that the latter are in fact virion-specific glycoproteins.

The 460TC extracellular particles contain RNA, which can be purified by extraction with phenol and SDS. When this RNA is sedimented in a sucrose gradient, the sedimentation profile is almost identical to the A particle RNA profile previously reported (23) (Fig. 8). The major species of RNA sediments at about 10*S*-15*S*, with small amounts in the 70*S* and 35*S* regions of the gradient. Similar results were obtained with [^{3}H]uridine-labeled RNA prepared from particles harvested 4 h after the label was added to the medium (data not shown). To compare the extracellular particle RNA to that from intracisternal A particles more precisely, hybridization kinetic analyses were performed

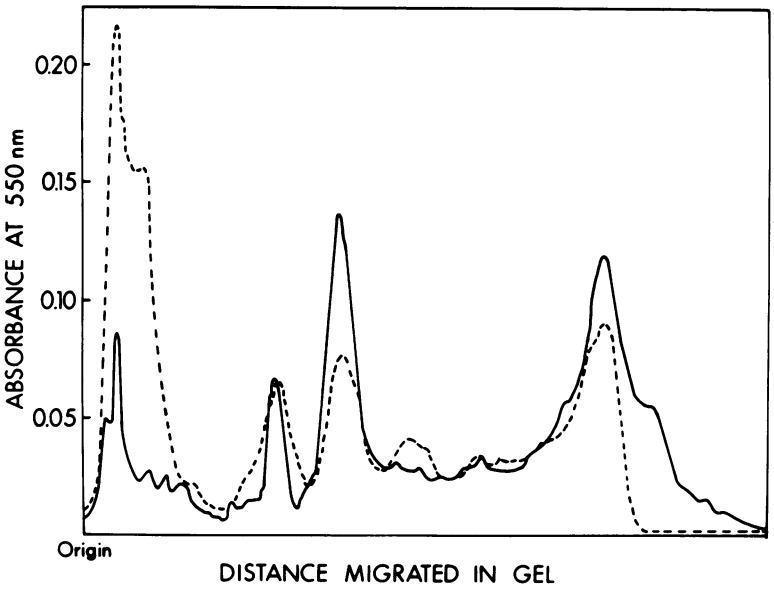

FIG. 6. *Acid-urea-SDS-polyacrylamide gel electrophoresis of CNBr peptides from the 76,000-dalton protein isolated from extracellular particles (---) and intracisternal A particles (——). See Materials and Methods for preparation of CNBr peptides.*

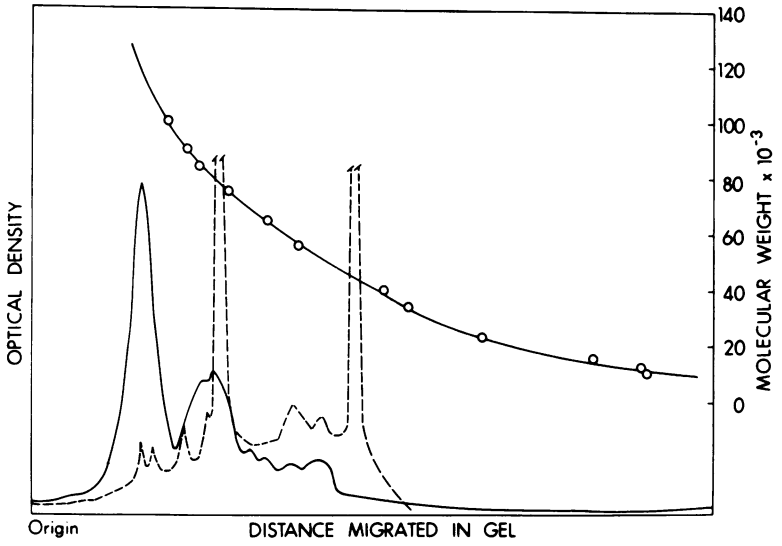

FIG. 7. *SDS-polyacrylamide gel electrophoresis of [^{14}C]glucosamine-labeled proteins from 460TC extracellular particles. The glycoprotein profile (——) is a Joyce-Loebl densitometric tracing of an autoradiographed X-ray film. An optical density tracing of a Coomassie blue-stained gel of the extracellular particle proteins is also included (- - -) which shows the positions of p76 and p45. The molecular weight curve (O) was determined using encephalomyocarditis virus protein precursors as molecular weight standards.*

using RNA and cDNA from both types of particles. cDNA was prepared using the endogenous reverse transcriptase reaction. Figure 9a shows the results using A particle cDNA as probe. The hybridization of this cDNA to both RNAs follows similar kinetics, although the final level of hybridization with A particle RNA is about 90%, whereas with extracellular particle RNA it is only about 75 to 80%. This difference in final levels of hybridization is apparently due to the transcription of a small amount of cellular rRNA which contaminates A particles (32). Thus, when purified 70*S* A particle RNA is used in the hybridization experi-

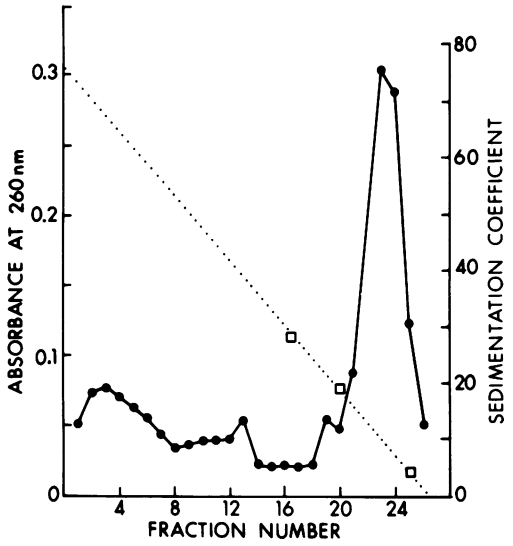

FIG. 8. *Rate zonal sedimentation of extracellular particle RNA (●) through a 5 to 30% sucrose gradient containing TNE buffer (pH 7.2) plus 0.5% SDS. Centrifugation was for 60 min at 53,000 rpm at 20 C in a Spinco SW56 rotor. Fractions were collected and monitored for absorption at 260 nm. RNA markers (28S, 18S, and 4S) were run in a parallel gradient (□).*

ments instead of whole, unfractionated A particle RNA, the final level of hybridization is identical to that with extracellular particle RNA (Fig. 9a). In contrast, when purified A particle 35*S* RNA is used in the hybridization analysis, the final level of hybridization is again about 90%, suggesting that the 35*S* RNA is contaminated with a partially transcribed species which is absent from the 70*S* RNA. Yang and Wivel (39) found similar differences in final hybridization levels with their A particle 70*S* and 35*S* RNAs during hybridization experiments using A particle cDNA. Thus it seems likely that the slight differences in hybridization levels can be accounted for by known contaminants and that the extra- and intracellular particle RNAs are otherwise completely homologous. This conclusion is supported by the results of the hybridization experiments using cDNA prepared from extracellular particles (Fig. 9b). The rates and final levels of hybridization obtained with RNAs from intra- and extracellular particles are now identical, although final levels are only about 60% in each case. The reason for the comparatively low extents of hybridization is not known. In any event, the kinetics of hybridization are similar for both RNAs, suggesting that they may be largely or completely homologous.

The hybridization of A particle cDNA with Mo-MuLV and MuMTV RNAs is shown in Fig. 9c. There is a small amount of sequence homology with MuMTV (about 15% hybridization, which may be due to the presence of a MuLV contaminant in the MuMTV preparation; Wade Parks, personal communication), but about 30% homology exists with Mo-MuLV. This is consistent with our earlier results which showed considerable homology between intracisternal A RNA and RNA from a Mo-MuLV-MuSV virus complex (23). The $C_rt_{1/2}$ values for all our hybridization experiments appear to be about 3×10^{-2} mol/s per liter, which is similar to that reported for other oncornaviruses (3).

The small size of the majority of the extracel-

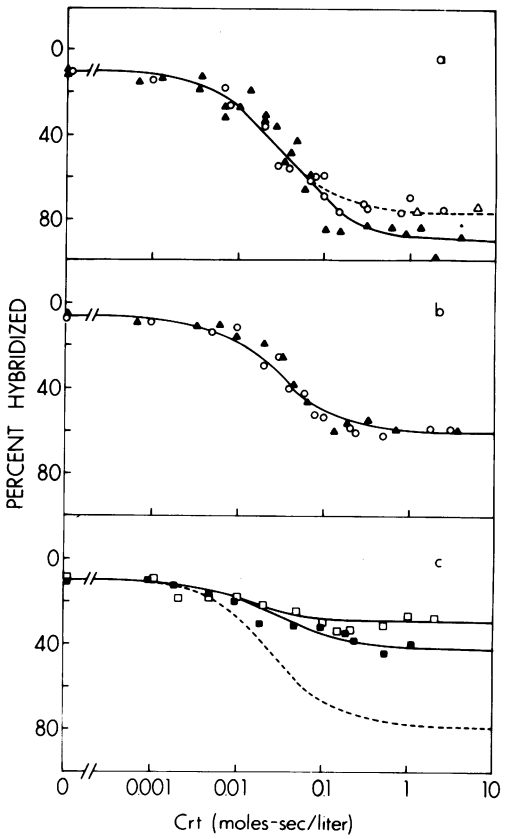

FIG. 9. *Hybridization kinetic analysis of RNAs from intracellular and extracellular particles. cDNA from intracisternal A particles was used as probe for experiments shown in (a) and (c); cDNA from extracellular particles was used in (b). RNAs used were: extracellular particle RNA (○), unfractionated A particle RNA (▲), 70S fraction of A particle RNA (△), mouse mammary tumor virus RNA (□), and Mo-MuLV RNA (■). The dashed line in (c) shows the hybridization curve for 70S A particle RNA reproduced from (a). Details for hybridization experiments are given in Materials and Methods. The second-order rate curves indicated by solid and dashed lines were calculated from formulas of Britten et al. (5).*

lular particle RNA is probably due to degradation of higher-molecular-weight species of RNA (35S-70S) originally present. This conclusion is supported by the fact that both species of RNA hybridize to cDNA with the same kinetics (unpublished data). In addition, the nearly identical $C_rt_{1/2}$ values for extracellular and intracisternal A particle RNAs would suggest that A particle low-molecular-weight RNA (10S-15S) is also a degradation product. (Since the low-molecular-weight RNA of A particles accounts for greater than 90% of the unfractionated RNA, similar C_rt values could not be obtained for hybridization experiments unless both RNAs were identical.) This is supported by the fact that, when the A particle RNA which originally sedimented at 35S is subsequently treated with either dimethyl sulfoxide prior to resedimentation or is resedimented in a formamide-sucrose gradient, most of the RNA sediments as low-molecular-weight RNA, indicating extensive nicking (unpublished data).

It is important to consider to what extent our results might be affected by contamination by other types of oncornaviruses. We are very concerned about this problem and have initiated a search for such contaminant production by 460TC cells, in collaboration with J. Hartley and W. Rowe (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Preliminary results have indicated that two infectious MuLV-type viruses are produced in small amounts, one B tropic (11) and the other xenotropic (15). We are presently looking to see if either of these viruses can cause the production of type A particles in their new hosts, whether they share extensive nucleic acid sequence homology with the major component of the MOPC-460 type A particles, and whether they contain the proteins which seem to be characteristic of these particles (p76, p70). It seems possible that the production of one or both of these oncornaviruses is induced during adaptation to tissue culture of the MOPC-460 cells, since no type C particles can be seen budding from solid tumor cells by electron microscopy. Moreover, the production of these oncornaviruses may actually be responsible for the appearance of A particle proteins (e.g., p76) and nucleic acid in extracellular particles. This could be due to phenotypic mixing of components of the various virus species or to complementation by induced oncornaviral enzymatic activities which may not be coded for by the A particle genome. However, it seems clear that the great majority of the 460TC extracellular particles are not typical oncornaviruses, since they lack both gp 69/71 and intact 35S RNA. In addition, it is evident that the majority of the transcribable and hybridizable RNA in these extracellular particles is closely related to A particle RNA and is not due to a contaminating unrelated oncornavirus, since the $C_rt_{1/2}$ values for the various hybridizations shown in Fig. 9 are all very similar and quite low. From the same type of considerations it seems highly unlikely that the transcribable and hybridizable RNA present in A particles is due to contamination by cores of an unrelated type C oncornavirus. Further support for this view is derived from the fact that the specific activity of the reverse transcriptase in A particles is very similar to that in extracellular particles. Similar conclusions about the absence of type C particle cores in A particle preparations have been reached by other workers using a variety of biochemical and immunological techniques (13, 23, 35, 38).

DISCUSSION

The tissue culture line derived from MOPC-460 ascites tumor cells contains intracellular particles present as both intracisternal and intracytoplasmic A particles. In addition, extracellular particles are produced by budding through the plasma membrane. The extracellular particles are closely related to the intracisternal A particles, as judged by our finding that both types of particles contain (i) RNA-dependent DNA polymerases of identical specific activities, (ii) traces of 70S and 35S RNA with larger amounts of degraded RNA, (iii) virtually complete nucleic acid sequence homology, and (iv) proteins with apparent molecular weights of 76,000 and 45,000. It was shown by analysis of CNBr peptides that the 76,000-dalton proteins from the two types of particles are identical or very similar. These results suggest that the intracisternal A particles and the extracellular particles produced by MOPC-460 myeloma cells may represent alternate forms of the same oncornavirus-like entity. It seems likely that this conclusion will extend to the intracytoplasmic A particles as well. It is possible that some of these particles are precursors of others, although it seems equally likely that each may be produced by an independent pathway. The putative oncornavirus represented by these particles is genetically related to other type C viruses. It shares about 30% sequence homology with Mo-MuLV, considerably less with Rauscher or AKR MuLV's (21), and still less with MuMTV. It is possible that the extracellular particles contain only some components in common with the A particles and derive other components (and/or nonvirion enzymes) from a

second oncornavirus which is induced in the cultured cells but not in the solid tumors. This could account for the presence of p30 in the extracellular particles and their ability to bud through the plasma membrane. Indeed, as noted above, J. Hartley and W. Rowe have found that the 460TC cells grown in our laboratory do in fact produce very low levels of at least one other type of MuLV. In any case, it seems possible that the genetic information which specifies MOPC-460 type A particles, and at least a portion of the 460TC extracellular particles, may represent the degenerate evolutionary remnant of an ancient viral integration event, as has been recently proposed by Baltimore (2). Indeed, the presence of unusually large proteins in both types of particles suggests that the ability to process proteins post-translationally may have been impaired or lost.

The intracytoplasmic A particles present in MOPC-460 cells have not yet been extensively characterized, although preliminary experiments in our laboratory indicate that they contain the same proteins as the intracisternal A particles. Electron microscope analysis suggests that most extracellular particles arise from the budding of preformed intracytoplasmic A particles through the plasma membrane, similar to MuMTV. This implies a precursor-product relationship between the two types of particles. Although we are presently investigating this possibility, we have no direct evidence that this relationship actually exists. An alternative explanation for the apparent budding of preformed intracytoplasmic A particles is that core formation of particles destined for budding may actually occur adjacent to the plasma membrane as it does for type C viruses, and for intracisternal A's at the rough endoplasmic reticulum membranes; however, this process may be very fast relative to the formation and subsequent release of the completed virion. This would result in the majority of budding particles having essentially completed cores which would be indistinguishable from intracytoplasmic A's. Indeed, the observation that most, if not all, intracisternal A type particles do not complete the budding process but remain attached to the rough endoplasmic reticulum membrane is consistent with the view that budding and release may be very slow relative to core formation in these systems (20). Further characterization of intracytoplasmic A particles and their relationship to intracisternal A and extracellular particles is currently in progress.

It is clear that none of the particles produced in MOPC-460 cells is closely related to MuMTV. This conclusion is supported by electrophoretic characterization of proteins, by hybridization analysis of nucleic acids, and by the immunological studies performed by W. Parks.

Although A particles have occasionally been found in normal cells (8, 37), they occur most often in transformed cells. Their role in the transformation of these cells is not known, however. In some transformed cells, A particles have been observed as the only virus-like particle (transplantable Leydig cell tumors and ML^+ leukemias of DBA mice [19]; murine myeloma tumors [21]). In other A particle-containing cells extracellular viruses are also produced. These include Mason-Pfizer monkey virus (7, 13), MuMTV (26), L1210 leukemia-associated virus (9), MuLV's (34), and a MuLV-related type C virus (33). In some of these cells electron microscope evidence suggests that preformed A particles can bud through the plasma membrane, thereby giving rise to the extracellular particles (MuMTV, Mason-Pfizer monkey virus, and L1210 leukemia-associated virus). On the basis of this type of evidence the intracytoplasmic A particles found in MuMTV-producing cells have been generally thought to be precursors of mature MuMTV. The results of Smith and Lee (26) and of Sarkar and Dion (24) tend to support this view. Similarly, two reports (C. Degiuli, S. Kawai, S. Dales, and H. Hanafusa, Abstr. 3rd Int. Congr. Virol., p. 195, 1975; 17) indicate that chicken embryo fibroblast cells infected with certain strains of Rous sarcoma virus contain intracytoplasmic A particles, and these particles contain the Rous sarcoma virus group-specific antigens. Thus the involvement of type A particles in the replication of other oncornaviruses is becoming clearer.

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ADDENDUM

After this manuscript was submitted, F. Wong-Staal, M. S. Reitz, Jr., C. D. Trainor, and R. C. Gallo (J. Virol. **16**:887–896, 1975) also reported the presence of reverse transcriptase activity and high-molecular-weight RNA in intracisternal type A particles.

LITERATURE CITED

1. **August, J. T., D. P. Bolognesi, E. Fleissner, R. V. Gilden, and R. C. Nowinski.** 1974. A proposed nomenclature for the virion proteins of oncogenic RNA viruses. Virology **60**:595–601.

2. **Baltimore, D.** 1974. Tumor viruses: 1974. Cold Spring Harbor Symp. Quant. Biol. **39**:1187-1200.
3. **Baluda, M. A., M. Shoyab, P. D. Markham, R. M. Evans, and W. N. Drohan.** 1974. Characterization of avian myeloblastosis virus genome by molecular hybridization. Cold Spring Harbor Symp. Quant. Biol. **39**:869-874.
4. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83-88.
5. **Britten, R. J., D. E. Graham, and B. R. Neufeld.** 1974. Analysis of repeating DNA sequences by reassociation, p. 363-418. *In* L. Grossman and K. Moldane, (ed.), Methods in enzymology, vol. 29. Academic Press Inc., New York.
6. **Britten, R. J., and J. Smith.** 1970. A bovine genome. Carnegie Inst. Washington Yearb. **68**:378-386.
7. **Bukrinskaya, A. G., G. C. Miller, E. N. Lebedera, L. V. Agaphonova, N. N. Masurenko, and K. W. Illyin.** 1974. Properties of intracytoplasmic A particles isolated from oncornavirus-producing human cells. J. Virol. **14**:924-933.
8. **Calarco, P. C., and D. Szollosi.** 1973. Intracisternal A-particles in ova and preimplantation stages of the mouse. Nature (London) New Biol. **243**:91-93.
9. **Chopra, H. D., G. Schidlovsky, and T. McBride.** 1967. Electron microscopic studies of virus associated with L_{1210} leukemia, p. 104-105. *In* C. J. Arceneaux (ed.), Proc. 25th Anniversary Meeting, Electron Microscope Society of America. Claitor's Book Store, Baton Rouge, La.
10. **Dalton, A. J., and M. Potter.** 1968. Electron microscopic study of the mammary tumor agent in plasma cell tumors. J. Natl. Cancer Inst. **40**:1375-1385.
11. **Hartley, J. W., W. P. Rowe, and R. J. Huebner.** 1970. Host-range restrictions of murine leukemia viruses in mouse embryo cell cultures. J. Virol. **5**:221-225.
12. **Kramarsky, B., N. H. Sarkar, and D. H. Moore.** 1971. Ultrastructural comparison of a virus from Rhesus-monkey mammary carcinoma with four oncogenic RNA viruses. Proc. Natl. Acad. Sci. U.S.A. **68**:1603-1607.
13. **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.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of head of Bacteriophage T4. Nature (London) **227**:680-685.
15. **Levy, J. A.** 1973. Xenotropic viruses: murine leukemia viruses associated with NIH Swiss, NZB, and other mouse strains. Science **182**:1151-1153.
16. **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.
17. **Maul, G. G., and L. J. Lawandowski.** 1975. Detection of type A oncornavirus-like structures in chicken embryo cells after infection with Rous sarcoma virus. J. Virol. **16**:1071-1074.
18. **Naso, R. B., L. J. Arcement, and R. B. Arlinghaus.** 1975. Biosynthesis of Rauscher leukemia viral proteins. Cell **4**:31-36.
19. **Nowinski, R., N. Sarkar, L. Old, D. H. Moore, D. Scheer, and J. Hilgers.** 1971. Characteristics of the structural components of the mouse mammary tumor virus. II. Viral proteins and antigens. Virology **46**:21-38.
20. **Perk, K., and J. E. Dahlberg.** 1974. Murine intracisternal A type particles fail to separate from the membrane of the endoplasmic reticulum. J. Virol. **14**:1304-1306.
21. **Potter, M.** 1972. Immunoglobulin-producing tumors and myeloma proteins of mice. Physiol. Rev. **52**:631-719.
22. **Provisional Committee for Nomenclature of Viruses.** 1966. Suggestions for the classification of oncogenic RNA viruses. J. Natl. Cancer Inst. **37**:395-397.
23. **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.
24. **Sarkar, N. H., and A. S. Dion.** 1975. Polypeptides of the mouse mammary tumor virus. I. Characterization of two group-specific antigens. Virology **64**:471-491.
25. **Sarkar, N. H., D. H. Moore, B. Kramarsky, and H. C. Chopra.** 1973. Oncornaviruses. 3. The mammary tumor virus, p. 307-321. *In* A. J. Dalton and F. Haguenau (ed.), Ultrastructure of animal viruses and bacteriophages: an atlas. Academic Press Inc., New York.
26. **Smith, G. H., and B. K. Lee.** 1975. Mouse mammary tumor virus polypeptide precursors in intracytoplasmic A particles. J. Natl. Cancer Inst. **55**:493-496.
27. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastructure Res. **26**:31-43.
28. **Sullivan, D., R. Palacios, J. Stavnezer, J. M. Taylor, A. J. Faras, M. L. Kiely, N. M. Summers, J. M. Bishop, and R. T. Schimke.** 1973. Synthesis of a deoxyribonucleic sequence complementary to ovalbumin messenger ribonucleic acid and quantification of ovalbumin genes. J. Biol. Chem. **248**:7530-7539.
29. **Sutton, W. D.** 1971. A crude nuclease preparation suitable for use in DNA reassociation experiments. Biochim. Biophys. Acta **240**:522-531.
30. **Swank, R. T., and K. D. Munkres.** 1971. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gels with sodium dodecyl sulfate. Anal. Biochem. **39**:462-477.
31. **Teramoto, Y. A., M. J. Puentes, L. J. T. Young, and R. D. Cardiff.** 1974. Structure of the mouse mammary tumor virus: polypeptides and glycoproteins. J. Virol. **13**:411-418.
32. **Thach, R. E., D. L. Robertson, N. L. Baenziger, and D. C. Dobbertin.** 1974. Reverse transcriptase associated with A-type particles from murine myeloma cells. Cold Spring Harbor Symp. Quant. Biol. **39**:963-968.
33. **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.
34. **Watson, J., P. Ralph, S. Sarkar, and M. Cohn.** 1970. Leukemia viruses associated with mouse myeloma cells. Proc. Natl. Acad. Sci. U.S.A. **66**:344-351.
35. **Wilson, S. H., and E. L. Kuff.** 1972. A novel DNA polymerase activity found in association with intracisternal A-type particles. Proc. Natl. Acad. Sci. U.S.A. **69**:1531-1536.
36. **Wivel, N. A., K. K. Lueders, and E. L. Kuff.** 1973. Structural organization of murine intracisternal A particles. J. Virol. **11**:329-334.
37. **Wivel, N. A., and G. A. Smith.** 1971. Distribution of intracisternal A-particles in a variety of normal and neoplastic mouse tissues. Int. J. Cancer **7**:167-175.
38. **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.
39. **Yang, S. S., and N. A. Wivel.** 1974. Characterization of an endogenous DNA polymerase associated with murine intracisternal A particles. J. Virol. **13**:712-720.