JOURNAL OF VIROLOGY, Mar. 1968, p. 224-237
Copyright © 1968 American Society for Microbiology
Vol. 2, No. 3
Printed in U.S.A.

Production of Virus by Mammalian Cells Transformed by Rous Sarcoma and Murine Sarcoma Viruses

ARTRICE F. VALENTINE AND JOHN P. BADER
Viral Biology Branch and Chemistry Branch, National Cancer Institute, Bethesda, Maryland 20014

Received for publication 27 November 1967

Cultured cells of mammalian tumors induced by ribonucleic acid (RNA)-containing oncogenic viruses were examined for production of virus. The cell lines were established from tumors induced in rats and hamsters with either Rous sarcoma virus (Schmidt-Ruppin or Bryan strains) or murine sarcoma virus (Moloney strain). When culture fluids from each of the cell lines were examined for transforming activity or production of progeny virus, none of the cell lines was found to be infectious. However, electron microscopic examination of the various cell lines revealed the presence of particles in the rat cells transformed by either Rous sarcoma virus or murine sarcoma virus. These particles, morphologically similar to those associated with murine leukemias, were found both in the extracellular fluid concentrates and in whole-cell preparations. In the latter, they were seen budding from the cell membranes or lying in the intercellular spaces. No viruslike particles were seen in preparations from hamster tumors. Exposure of the rat cells to ^{3}H-uridine resulted in the appearance of labeled particles with densities in sucrose gradients typical of virus (1.16 g/ml.). RNA of high molecular weight was extracted from these particles, and double-labeling experiments showed that this RNA sedimented at the same rate as RNA extracted from Rous sarcoma virus. None of the hamster cell lines gave radioactive peaks in the virus density range, and no extractable high molecular weight RNA was found. These studies suggest that the murine sarcoma virus produces an infection analogous to certain "defective" strains of Rous sarcoma virus, in that particles produced by infected cells have a low efficiency of infection. The control of the host cell over the production and properties of the RNA-containing tumorigenic viruses is discussed.

The ribonucleic acid (RNA)-containing oncogenic viruses, which include the avian sarcoma-leukosis complex and murine leukemia viruses, appear generally to have similar structural and biochemical characteristics. They are about the same size, have similar morphology (7, 9, 12, 13) and are formed by a process of budding from the plasma membranes (7, 9, 13). Sedimentation in sucrose density gradients shows that virus particles in both complexes have a buoyant density between 1.15 and 1.18 g/ml, and the particles contain an RNA with a molecular weight of 10^{7} daltons (11, 18). A distinction can be drawn between the virus particles of the avian sarcoma-leukosis complex and the murine leukemia complex on the basis of antigenicity and of morphology of the "mature" type C particles. This morphological difference is expressed in the size of the viral nucleoid and its spatial relationship to the membranes of the virion.

Chick embryo cells infected with the "defective" Bryan strain of Rous sarcoma virus (RSV) require the addition of a second avian leukosis virus before infectious particles can be produced (15). The term "helper" virus has been used to describe the second or associated virus because it supplies information necessary for the synthesis of an envelope component which is required for viral infectivity (14). Chick embryo cells which contain the RSV genome, but no helper virus, have been called nonproducer (NP) cells (15). However, electron microscopic investigations (5, 10) showed that these NP cells do indeed produce particles that are morphologically identical to the mature infectious virion, and these particles contain RNA of a high molecular weight characteristic of viral RNA (19).

When mammalian cells are transformed by RSV, the resulting tumor cells contain the RSV genome, but infectious particles often are not released (1, 24, 26). This inability to produce the infectious particles in mammalian systems differs from systems where defective RSV strains are used, since these NP cells are obtained with both "defective" and "nondefective" strains of RSV. Biological evidence of the presence of the Rous genome in rat cells transformed by RSV has been reported by Svoboda (24), and later studies (22) demonstrated the presence of the RSV genome by its recovery after mixed cultivation of these transformed cells with chick embryo cells.

Recent experiments suggest that murine sarcoma virus (MSV) may be analogous to RSV. Certain hamster tumors induced with MSV produce no virus, but the viral genome can be transferred to mouse cells by mixed cultivation in vitro (16). Murine leukemia viruses may act as "helper" virus in the recovery of infectivity of MSV in mouse cells. Also, MSV cannot be distinguished morphologically from murine leukemia virus (8).

The ability of MSV and RSV to induce tumors in similar species afforded a means for comparison of the viral infections. Cell lines from hamster and rat tumors induced with these viruses were examined by a variety of techniques. The production of infectious particles was investigated, cells and cell-culture fluids were examined with an electron microscope and by radioactive labeling techniques, and RNA from viruslike particles was characterized. The results reveal basic similarities in MSV and RSV infections, and delineate a requirement for host function in the production of the RNA-containing tumor viruses.

MATERIALS AND METHODS

Nomenclature of cell lines. All rat lines are designated by R and all hamster lines by H, followed by either MSV for murine sarcoma virus or RSV for Rous sarcoma virus with the virus strain in parentheses. The original designation of each cell line follows the cell line description.

Experimental cell lines. The following cell lines were derived from tumors induced with either RSV or MSV: R-MSV, tumor induced in rat (BN strain) by MSV (Moloney), obtained from R. C. Y. Ting (R-MSB_1); R-RSV (SR), tumor induced in inbred Amsterdam rat by RSV (Schmidt-Ruppin), obtained from R. C. Y. Ting (R-R 1022); H-MSV, tumor induced in Syrian hamster by MSV obtained from R. Heubner (HT-1); H-RSV (SR), tumor induced in Syrian hamster by RSV (Schmidt-Ruppin strain), obtained from P. Sarma; H-RSV (BH), tumor induced in Syrian hamster by RSV (Bryan "high-titer" Strain), obtained from P. Sarma.

All of these cell lines contained the viral genome specified by the tumor-inducing virus RSV (SR), RSV (BH), or MSV. This was shown by the recovery of infectious virus after mixed cultivation of test cells with cells normally susceptible to the virus, as described by Šimkovič et al. (22). In the cases of RSV (BH) and MSV, addition of avian leukosis virus (RAV_1) or murine leukemia virus (Rauscher or Moloney), respectively, was required for recovery of infectious virus (16, 21).

Control cell lines. These virus-free cell lines were used to check fluids for transforming abilities or ability to cause the production of infectious virus particles. RE_1 was a serial line of rat embryo cells (derived in this laboratory) showing smooth monolayer growth; it was susceptible to MSV. BHK represents BHK 21/C1 13 line derived from baby hamster kidney by M. Stoker. CE (D) represents chick embryo cells susceptible to subgroup A and B avian leukosis viruses, obtained from R. M. Dougherty.

Cell cultures. All cells were propagated in Eagle's minimal essential medium containing 10% fetal bovine serum, 10% tryptose phosphate broth, and anbitiotics (penicillin, streptomycin, chlortetracycline, and nystatin). Cells were grown in plastic dishes (10 cm in diameter) in a CO_2 incubator at 39 C.

Virus. For comparative purposes, infectious virus was used in certain experiments. RSV, Schmidt-Ruppin strain (RSV-SR), was obtained from R. M. Dougherty, and a variant of the Bryan "high-titer" RSV (RSV-RAV_1) was obtained from H. Rubin. Both were propagated in chick embryo cultures as previously described (3). MSV-MLV, a mixture of sarcoma and murine leukemia virus was provided by J. B. Moloney and was propagated in rat embryo cells in a similar manner. Stock suspensions of murine leukemia virus were obtained from F. Rauscher and J. B. Moloney.

Assay for MSV. RE_1 cells were exposed to 0.2 ml of 10-fold dilutions of suspected MSV-containing fluids. After 30 min, growth medium was added and cultures were incubated at 38 C in a humidified CO_2 incubator. Microscopic foci of transformed cells were apparent within 4 days and were counted on the 6th day.

Concentration of particles. Cell-culture fluids were processed to concentrate possible extracellular virus particles. Growth medium was added to cell cultures, and the cultures were allowed to incubate for 12 hr. Culture fluids were centrifuged at $600 \times g$ to remove debris and floating cells, and then at $100{,}000 \times g$ for 1 hr to sediment virus. Pellets were then processed for electron microscopy.

Electron microscopy. Cells were rinsed in either tris(hydroxymethyl)aminomethane (Tris)-saline or physiological saline prior to fixation with either Dalton's chrome osmium (6) or 1.5% glutaraldehyde in cacodylate buffer (20). Fixation in glutaraldehyde was followed by postfixation in Dalton's chrome osmium. All fixations were carried out in the cold. After fixation, the plates were checked under an inverted microscope to make sure the cell layers were still intact. The cells were then scraped from the dishes and pelleted from the fixation or postfixation

fluid by centrifugation at approximately 3,400 rev/min for 5 min. These pellets were then stained with 0.5% uranyl acetate in 10% Formalin solution or in a 0.5% aqueous solution of uranyl acetate with sucrose, buffered to *p*H 4.9. In block staining periods varied from 4 hr to overnight. Dehydration was carried out by rinsing with a graded series of ethyl alcohol: 70, 90, 95, and 100%, followed by two 15-min rinses in propylene oxide. The pellets were then placed in a 1:1 mixture of propylene oxide and Epon-araldite embedding medium and kept overnight in a refrigerator. The following day, the pellets were cut into smaller pieces, drained, and flat-embedded in an Epon-araldite mixture (17). Polymerization was achieved by preliminary incubation at 56 C followed by incubation for at least 15 hr at 80 C. Sections were cut on an LKB ultratome with settings varying between 500 and 200 A, and were floated on distilled water prior to being picked up on 200 mesh, carboned, Formvar-coated grids. Sections were then doubly stained with alcoholic uranyl acetate for 15 min, followed by 15-min staining with lead citrate. The grids were examined in a Siemens Elmiskop I electron microscope equipped with a 50-μ objective aperture and an accelerating voltage of 80 kv.

Pellets obtained after high-speed centrifugation of culture fluids were fixed in either Dalton's chrome osmium or glutaraldehyde and were processed in the same manner described for cells.

Labeling of virus. Fluids were removed from cell cultures as cellular growth approached confluency. Fresh medium containing ^{3}H-uridine (100 μc in 5 ml of medium) was added, and 12 hr later culture fluids were removed and centrifuged at 600 $\times$ *g* for 10 min to remove floating cells. Purification of virus essentially followed the method of Robinson et al. (18). An equal volume of saturated ammonium sulfate was added, and the centrifuged precipitate was resuspended in 1 ml of Tris-EDTA (10 mM Tris plus 1 mM ethylenediaminitetraacetate, *p*H 7.4). The suspension was layered over 4.5 ml of a 15 to 55% (w/v) sucrose gradient (all sucrose solutions contained 0.1 M NaCl buffered to *p*H 7.4 with Tris) and centrifuged at 100,000 $\times$ *g* for 2 hr at 4 C in a swinging-bucket rotor. Gradients were fractionated by puncturing the bottom of the tube, and fractions were collected into 0.5 ml cold Tris-saline containing 0.5% bovine serum albumin. The total volume of each diluted fraction was approximately 0.8 ml. Samples were then taken for scintillation counting (10 μliters for the cells containing identifiable virus particles by electron microscopy; 100 μliters for cells containing no visible particles). The samples were added directly to vials containing 2,5-diphenyloxazole-1,4-bis-2-(5 phenyloxazolyl)-benzene scintillation mixture in toluene plus 30% methanol.

RNA extraction. Gradient fractions showing peaks of radioactivity in the area of 1.16 g/ml were pooled for each cell type, as were similar fractions from cell types showing no peaks in the gradients. The pools were diluted with an equal volume of water, 1 mg of yeast RNA was added as carrier, and Pronase (50 μg/ml) and sodium lauryl sulfate (0.5%) were added. After shaking the mixtures intermittently for 5 min, phenol was added, the mixture was again shaken and then centrifuged, and the aqueous layer was extracted again with phenol. The solution was made 2% in Na acetate, 2 volumes of cold ethyl alcohol were added, and, after allowing 10 min for association, the RNA precipitate was centrifuged to a pellet. After draining, the RNA was redissolved in 0.2 ml of Tris-EDTA and layered onto 5 to 20% sucrose gradients. The gradients were centrifuged at 4 C in a swinging-bucket rotor at 100,000 $\times$ *g* for 2 hr. Fractions were collected directly into scintillation vials containing methanol-toluene scintillation fluid, and then counted.

RESULTS

Infectivity studies. The infectivity of culture fluids from the experimental mammalian tumor cell lines was examined. Fluids were collected, centrifuged to remove floating cells, and then added to cultures of rat embryo (RE), chick embryo (CE), and baby hamster kidney (BHK) cells. In addition, cells exposed to infectious MSV-MLV or RSV, as well as uninfected cells, were used as controls. Test cultures were transferred twice at 3-day intervals and examined daily for a period of 9 days, for morphological transformation.

Cultures exposed to fluids from all of the experimental cell lines remained morphologically normal and were no different from uninfected controls. Under similar growth conditions, cultures of RE cells exposed to infectious MSV-MLV, and CE cells exposed to RSV, become grossly transformed. Transformation of RE cells by RSV was obvious, but not extensive, and the remaining cultures were similar to non-infected controls (Table 1). After the 9-day observation period, test cultures exposed to fluids from tumor cell cultures were processed for electron microscopy.

Experiments presented below demonstrated the presence of virus particles in R-MSV and R-RSV (SR) cultures. The failure to detect transformation in cells exposed to particle-containing culture fluids suggested that the particles may be infectious but nontransforming, a characteristic of avian and murine leukemia viruses. However, electron microscopic examination of the test cultures exposed to fluids from R-MSV and R-RSV (SR) cultures revealed no viruslike particles, indicating that viruses infectious for RE, CE, or BHK cells were absent from the tumor cells under investigation.

Electron microscopy. Presence of virus in the experimental cell lines, R-MSV, R-RSV (SR), H-MSV, H-RSV (SR), and H-RSV (BH), was determined with an electron microscope. No particles were detectable in ultrathin sections of the hamster tumor cells, and pellets obtained

TABLE 1. *Tests for infectivity of culture fluids from various tumor cells*[a]

Fluids from cell cultures	Test cells		
	CE	RE	BHK
R-MSV	0	0	0
R-RSV (SR)	0	0	0
H-MSV	0	0	0
H-RSV (SR)	0	0	0
H-RSV (BH)	0	0	0
R-MSV-MLV	0	+	0
C-RSV (SR)	+	+	0

[a] Fluids from cell cultures were centrifuged to remove floating cells, then added to sparse growing cultures of chick embryo (CE), rat embryo (RE), or hamster (BHK) cells. For controls, fluids from RE cells infected with MSV-MLV and CE cells infected with RSV (SR) were also added to test cells. Test cultures were transferred twice at 3-day intervals and examined daily for 9 days for morphological transformation. Positive (+) cultures became grossly transformed, negative (0) ones were no different from uninfected controls.

by high-speed centrifugation of culture fluids likewise contained nothing resembling virus particles. However, the rat lines transformed by both MSV and RSV showed virus particles budding from the plasma membrane (Fig. 1–9), and completed extracytoplasmic virus particles were abundant in culture fluid pellets. A summary of these observations is presented in Table 2. No particles were observed budding from intracytoplasmic vacuoles, although occasionally one or two particles were found within a vacuole in the cytoplasmic matrix (Fig. 5).

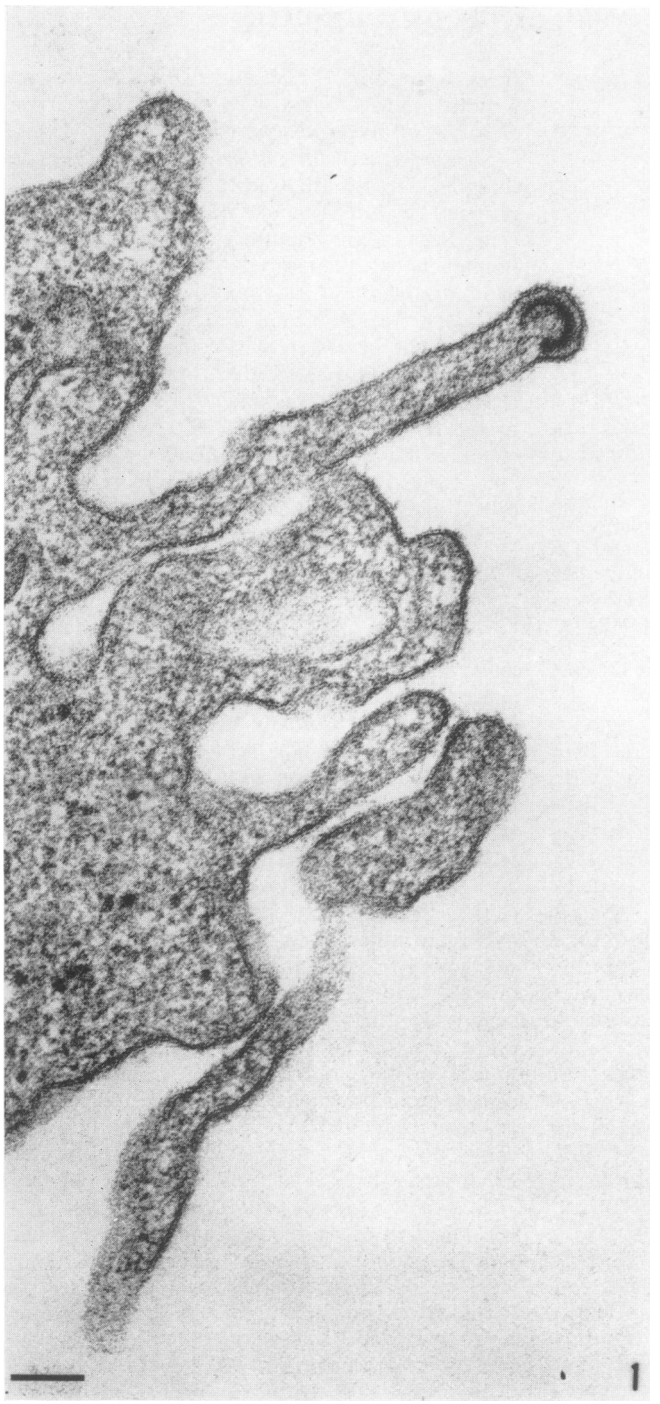
The viruses observed in the two cell lines were indistinguishable from each other (Fig. 1–9) and were similar to the viruses observed in the control rat embryo cells infected with the MSV-MLV mixture (Fig. 10–12). The particles found in cell culture fluids were typical "type C" particles (2, 7), having a diameter of about 100 mμ and being composed of an envelope, intermediate membrane, and nucleoids with electron-lucent (Fig. 4 and 8) or electron-dense (Fig. 6 and 9) centers. Most particles from both the RSV- and MSV-transformed rat cells contained nucleoids with electron-lucent centers, i.e., the "immature forms." Although the intermediate membrane was a feature of many of the observed particles from both R-MSV and R-RSV (SR) cultures (Fig. 2, 3, 8), this structural component of RNA-containing tumorigenic viruses was not readily distinguishable in all particles. Some "mature" forms containing condensed nucleoids were also seen. Such particles from both MSV and RSV cultures resembled murine leukosis virus particles more closely than avian viruses (Fig. 6, 9, 12, 14, 15).

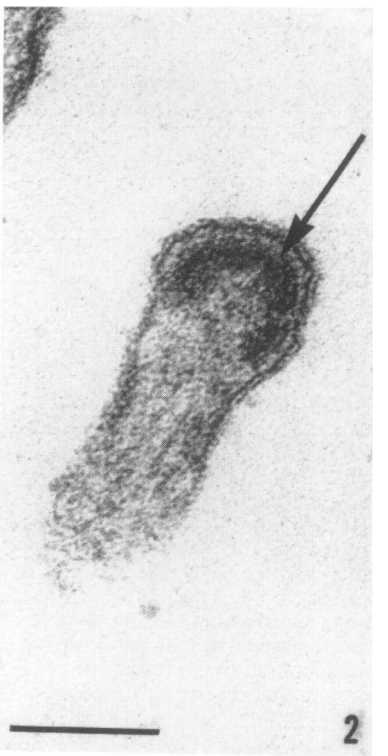
Superinfection with murine leukemia virus. The foregoing results showed production of virus particles by RSV- and MSV-transformed rat cells. Because these particles were found to be noninfectious, it appeared that these cells were analogous to the chick embryo cells transformed by "defective" RSV and that measurable infectivity might be restored by additional infection with a leukemia virus. Either Rauscher or Moloney leukemia virus was added to sparse cultures of the R-MSV or R-RSV (SR) cells. After incubation for 2 days, culture fluids were removed and assayed for transforming activity on RE cells. Fluids from leukemia virus-infected R-MSV cells produced foci of transformed cells which were identical to foci formed after infection with MSV (Table 3), whereas control R-MSV fluids were noninfectious, as before. No attempt was made to control the number of infecting leukemia virus particles. Therefore, the significance of finding more foci in Rauscher virus-infected versus Moloney virus-infected cultures cannot be assessed. The production of infectious MSV by superinfection with leukemia virus supports the hypothesis that the particles produced by R-MSV cells were indeed MSV particles which were incapable of reinfecting RE cells, and that these cells were analogous to the NP cells of the RSV-CE system (15).

On the other hand, no transforming activity was found in fluids from R-RSV (SR) cells co-infected with the leukemia viruses (Table 3), suggesting that the RSV genome cannot acquire the murine leukemia virus envelope required for infection of RE cells. Hamster tumor cells were also exposed to the leukemia viruses, but culture fluids taken subsequently failed to show any transforming activity in rat, hamster, or chicken cells.

Radioactive labeling of virus particles. The identification of virus particles in the R-MSV and R-RSV (SR) cultures by electron microscopy was not paralleled by infectivity of the culture fluids. It was important then to further characterize the particles to make sure that they had properties typical of the RNA-containing oncogenic viruses. To do this, an analysis of particle buoyant densities was made by centrifugation in sucrose density gradients, and labeled RNA from suspected virus-containing fractions was characterized by sedimentation studies. The radioactive labeling techniques provided a sensitive method for detection of virus particles as a check on the results of electron microscopy.

^{3}H-uridine was added to cultures of the hamster





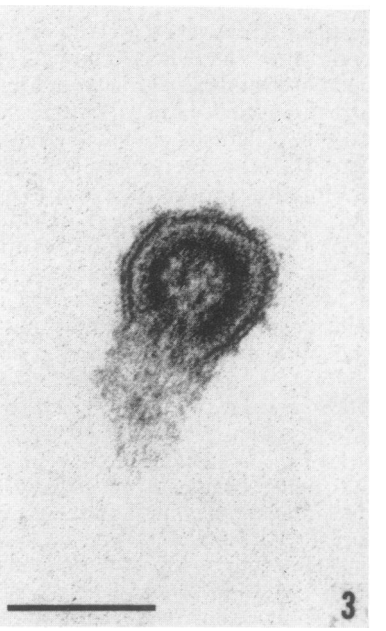
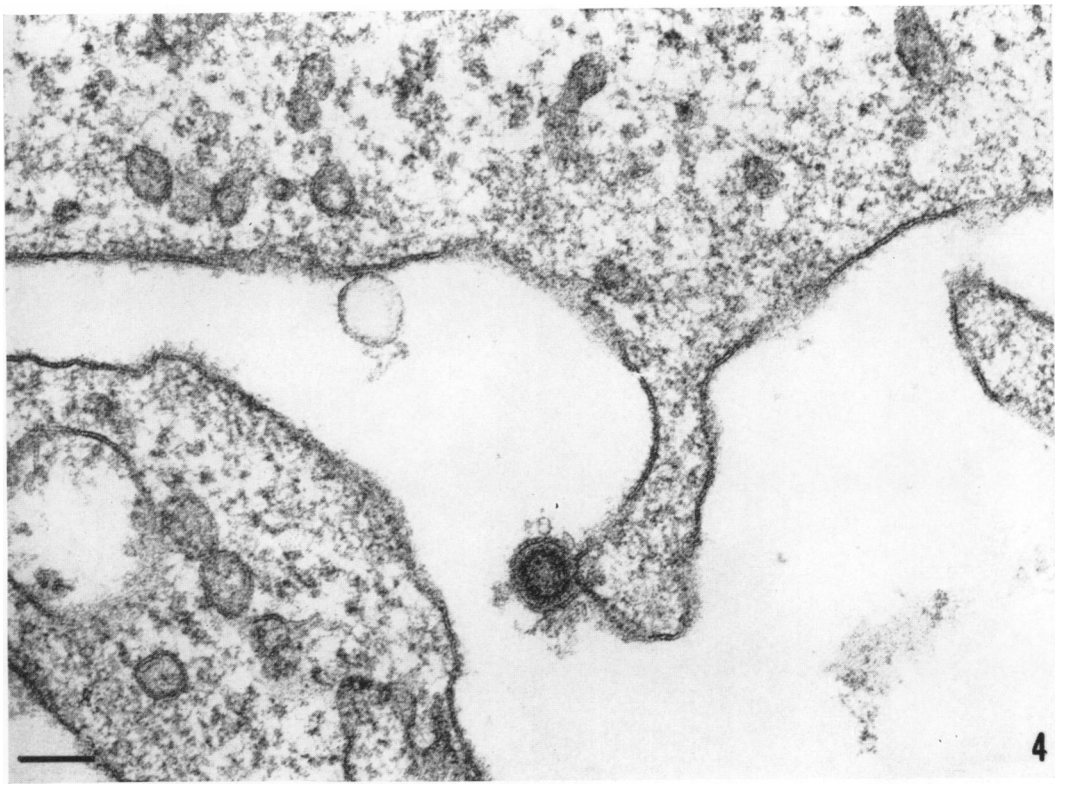
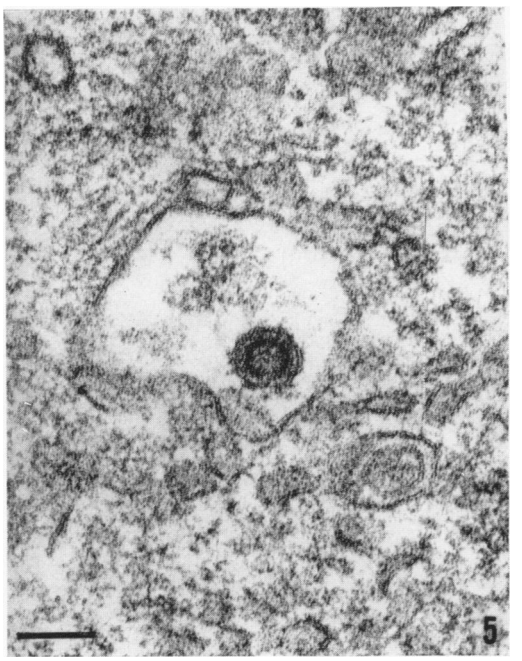


FIG. 1–3. *Budding particles representative of those observed in the rat tumor line infected with Rous sarcoma (Schmidt-Ruppin strain) and designated R-RSV (SR). (Glutaraldehyde fixation). All sections were doubly stained with alcoholic uranyl acetate and lead citrate. The magnification mark in each figure represents 0.1 μ.*

FIG. 1. *Virus particle budding from the end of an elongated protoplasmic stalk. Approximately 100,000 ×.*

FIG. 2 and 3. *Viral buds still attached to the plasma membrane of the cell but appearing as detached particles, because of the plane of sectioning. The arrow in Fig. 2 indicates the intermediate membrane of the viral particle. In Fig. 3, the intermediate membrane appears to be in closer association with the nucleoid. Approximately 200,000 ×.*





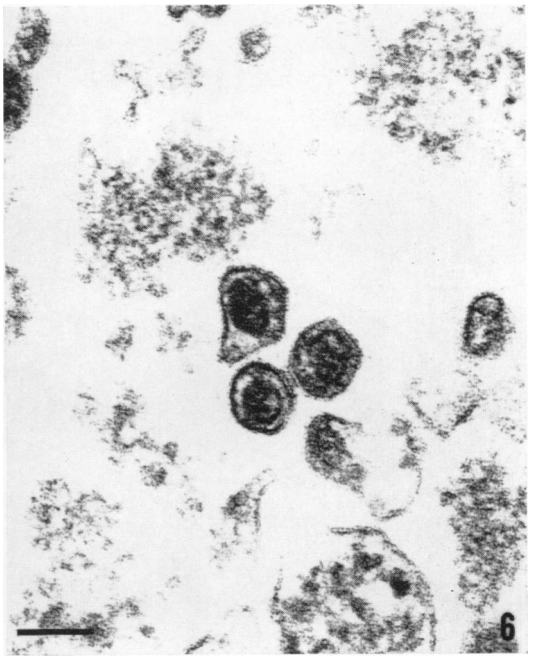


FIG. 4. *Virus particle which has completed budding from the plasma membrane. This extracytoplasmic particle is representative of the viruses with nucleoids with electron-lucent centers observed in the R-RSV (SR) line. Dalton's chrome osmium. Approximately 100,000 ×.*

FIG. 5. *Virus particle with a nucleoid with an electron-lucent center lying within a vacuole located in the cytoplasmic matrix of a cell from the R-RSV (SR) cell culture. Dalton's chrome osmium. Approximately 100,000 ×.*

FIG. 6. *Virus particles with nucleoids with electron-dense centers observed in a pellet derived from the concentration of fluids from the R-RSV (SR) cell cultures. The particles are identical to the "mature" type C virus particles associated with murine leukemias. Note the difference in these viruses and the "mature" viruses of the avian leukosis-sarcoma complex (Fig. 14 and 15). Glutaraldehyde fixation. Approximately 100,000 ×.*

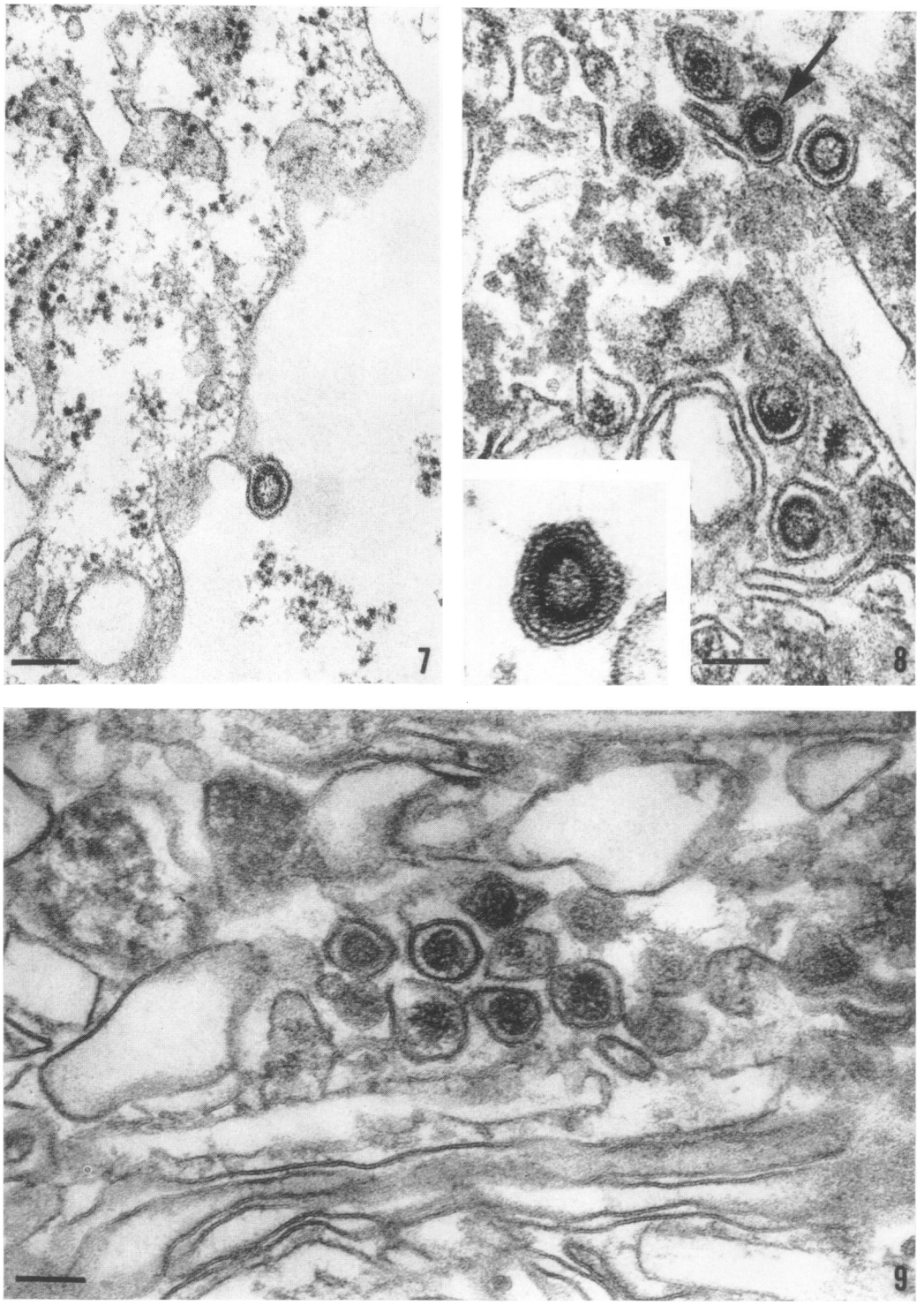


FIG. 7. *Virus particle budding from the plasma membrane of a rat cell infected with MSV (R-MSV). Glutaraldehyde fixation. Approximately 100,000 ×.*

FIG. 8. *Virus particles with nucleoids with electron lucent centers observed in a pellet obtained from extracellular fluids of the R-MSV line. The intermediate membrane designated by large arrow is seen as a distinct entity in this particular particle. A higher magnification of another particle in which the intermediate membrane is seen as a distinct entity is shown in inset. Dalton's chrome osmium. Approximately 100,000 and 200,000 ×, respectively.*

FIG. 9. *Virus particles with nucleoids with electron-dense centers observed in a pellet derived from the concentration of culture fluids of the R-MSV cell line and resembling the "mature" type C particles associated with murine leukemias. Dalton's chrome osmium. Approximately 100,000 ×.*

TABLE 2. *Summary of production of viruslike particles by cultured tumor cells*

Cell culture	Electron microscopy		^{3}H-uridine labeling
	Budding particles	Particles in culture-fluid concentrates	
R-MSV	+	+	+
R-RSV (SR)	+	+	+
H-MSV	0	0	0
H-RSV (SR)	0	0	0
H-RSV (BH)	0	0	0

and rat tumor cell lines. After incubation for 12 hr, culture fluids were concentrated by precipitation with ammonium sulfate and were analyzed by centrifugation in sucrose gradients. After removing the samples from the centrifuge, light-scattering bands at the appropriate position for virus were detected visually in the R-MSV and R-RSV (SR) samples. No bands were seen in gradients containing fluids from the hamster tumor cells.

The gradients were fractionated, and samples of the fractions were taken for scintillation counting. Distinct peaks of radioactivity were noted in samples derived from R-MSV cells (Fig. 16A) and R-RSV (SR) cells (Fig. 17A). The radioactivity was concentrated at a density of 1.16 g/ml, the expected density for avian or murine leukosis particles. RNA was extracted from the peak fractions, layered over 5 to 20% sucrose gradients, and sedimented by centrifugation at 100,000 $\times$ *g* for 2 hr. The resulting patterns of radioactivity (Fig. 16B and 17B) showed distinct peaks in the region of 65 to 70*S*, which is characteristic of viral RNA.

Although a portion containing 10 times as much fluid was analyzed from the hamster tumor samples, no distinct peaks of radioactivity were observed in H-MSV samples (Fig. 18A) or H-RSV (BH) samples (Fig. 19A), and no high molecular weight RNA could be extracted from fractions where virus might be expected (Fig. 18B and 19B). In several experiments, no radioactive band was found in H-RSV (SR) samples either, but in a single experiment (Fig. 20A) a small peak was noted at the appropriate density. However, no sedimenting RNA was extractable (Fig. 20B), and it was concluded that the radioactive peak was caused by something other than virus. The results of these experiments are compared with those of electron microscopy in Table 2.

Comparison with RNA from RSV-RAV$_1$. A closer approximation of the molecular size of the RNA from the R-MSV and R-RSV (SR) was obtained by a double-labeling experiment. Infectious RSV-RAV$_1$ was produced in chick embryo cells and labeled with $^{32}PO_4^{-3}$. Radioactive virus was purified by centrifugation in 15 to 55% sucrose gradients, and the virus bands were mixed with ^{3}H-uridine-labeled particles from R-MSV or R-RSV (SR). Viral RNA was extracted, layered over 5 to 20% sucrose gradients, and centrifuged. The resulting patterns (Fig. 21A and B) showed that the RNA preparations sedimented together, indicating that the sizes of the RNA from particles elicited by R-MSV or R-RSV (SR) were similar to that of RSV-RAV$_1$, about 10^7 daltons.

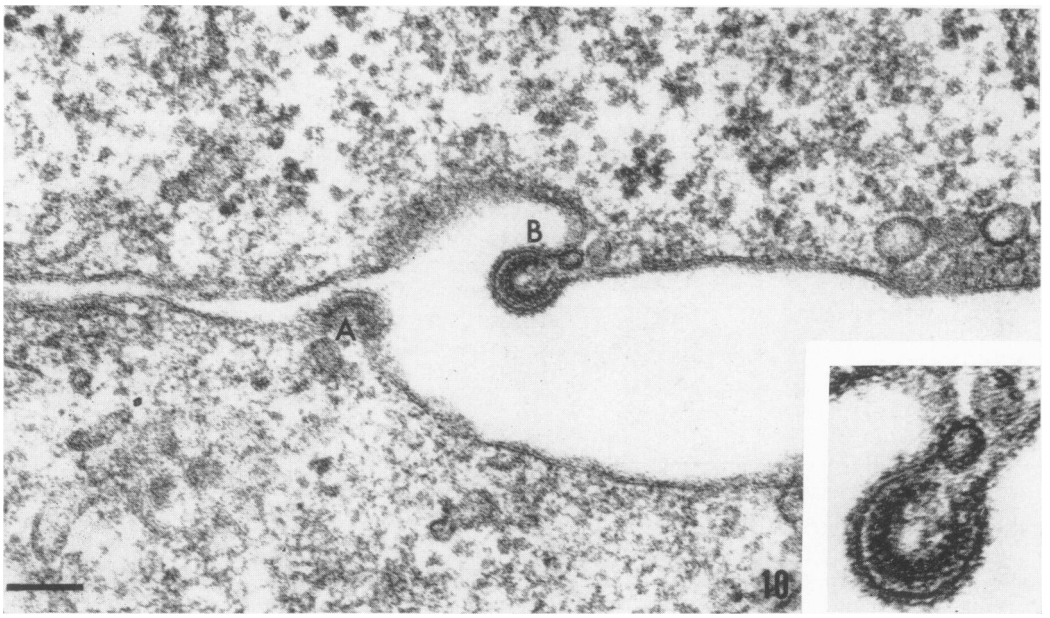
DISCUSSION

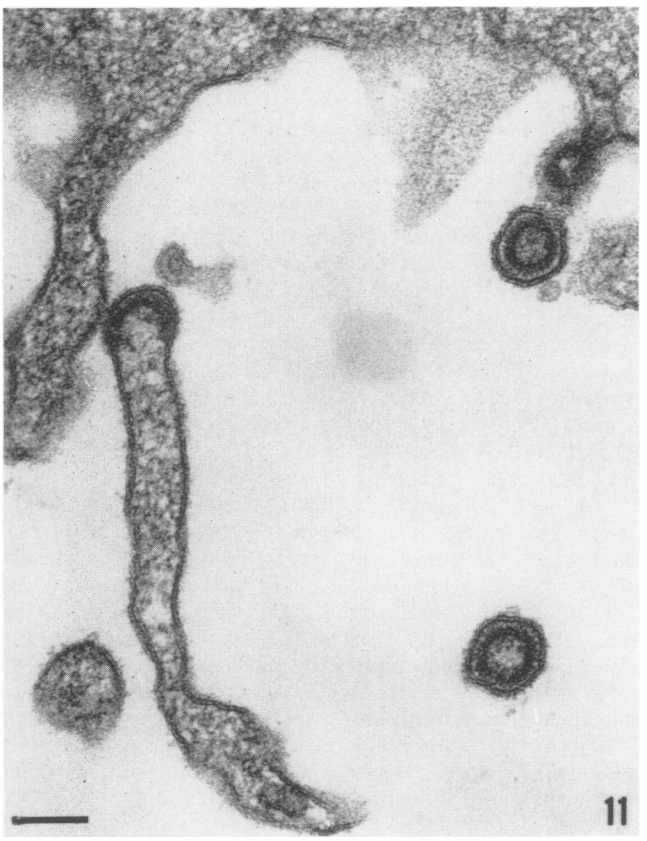
Tumors can be induced in mammals by RSV or MSV, and cells from these tumors can be cultivated in vitro. The presence of the viral genome in hamster and rat cell lines transformed by these viruses has been shown by in vivo and in vitro co-cultivation experiments. However, previous investigations revealed no morphologically detectable virus particles in these mammalian systems where infectious virus was not demonstrable.

Electron microscopy and radioactive labeling experiments provided complementary results which showed the presence of virus particles in rat cell lines infected with both RSV and MSV, and their absence in transformed hamster cell lines. The absence of particles from hamster cell lines, the presence of the noninfectious virus particles in the rat lines, and the similarity of the particles elaborated by R-RSV (SR) and R-MSV, all reflect the control of the host cell over the production of RNA-tumorigenic viruses.

Regardless of the initiating virus, the particles observed in the rat lines were identical to each other and indistinguishable from the "immature" and "mature" type C particle associated with murine leukemias. This lack of distinction between the virus particles in the R-RSV (SR) and R-MSV cultures suggests a factor specific to the host species which determine the final structural appearance of virus particles. The presence of particles in the rat cells and their absence in the hamster suggest that there is a genetically determined host factor that allows for the production of particles and that this factor is absent or suppressed in these hamster cells. Experiments showing a requirement for functioning cellular deoxyribonucleic acid in the late stages of virus production have been reported (4), and may be relevant to the observations described here.

The production of virus in these mammalian species is dependent upon the particular virus





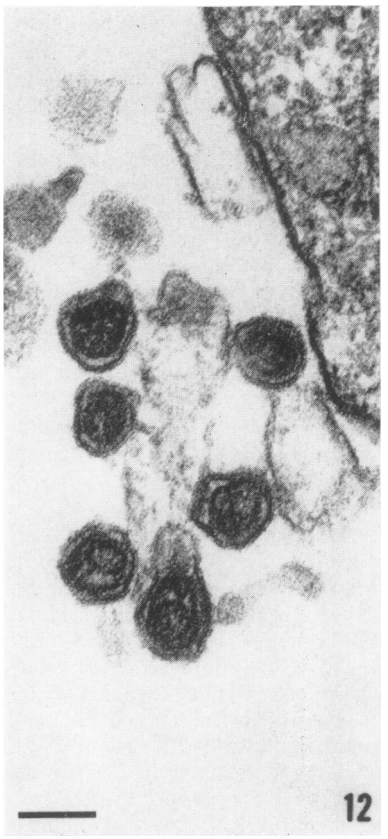
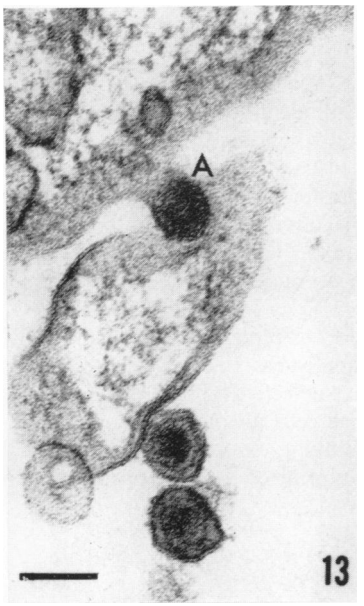
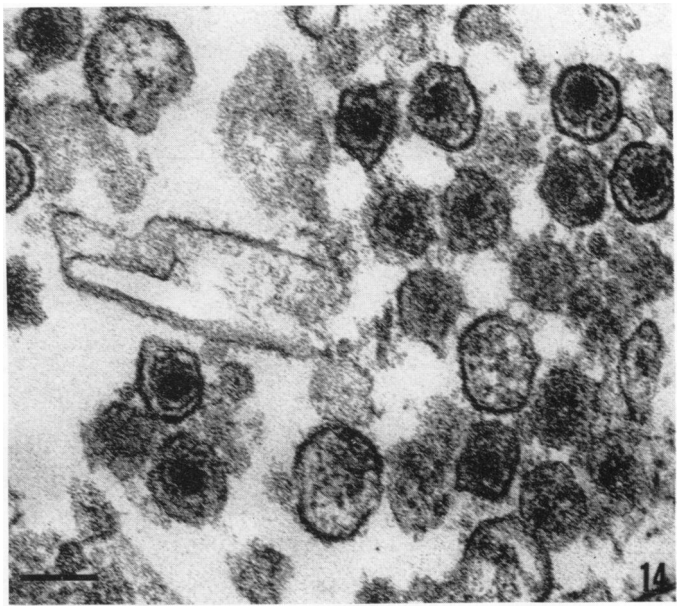


FIG. 10. *Virus particles are seen budding from the plasma membrane of cells in the control cell line R-MSV-MLV. (A) A particle in the earlier stages of budding. (B) A viral bud which has almost completed its formation from the plasma membrane. This particle is seen at a higher magnification in inset. The intermediate membrane of the particle is apparent. Dalton's chrome osmium. Approximately 100,000 and 200,000 ×, respectively.*

FIG. 11. *Budding particle and particles with nucleoids with electron lucent centers observed in the R-MSV-MLV cell line. These particles are representative of the "immature" particles of the murine leukemia complex. Dalton's chrome osmium. Approximately 100,000 ×.*

FIG. 12. *Viruses with nucleoids with electron-dense centers observed in the R-MSV-MLV cell line and representative of the "mature" type C particles of the murine leukemia complex. Dalton's chrome osmium. Approximately 100,000 ×.*





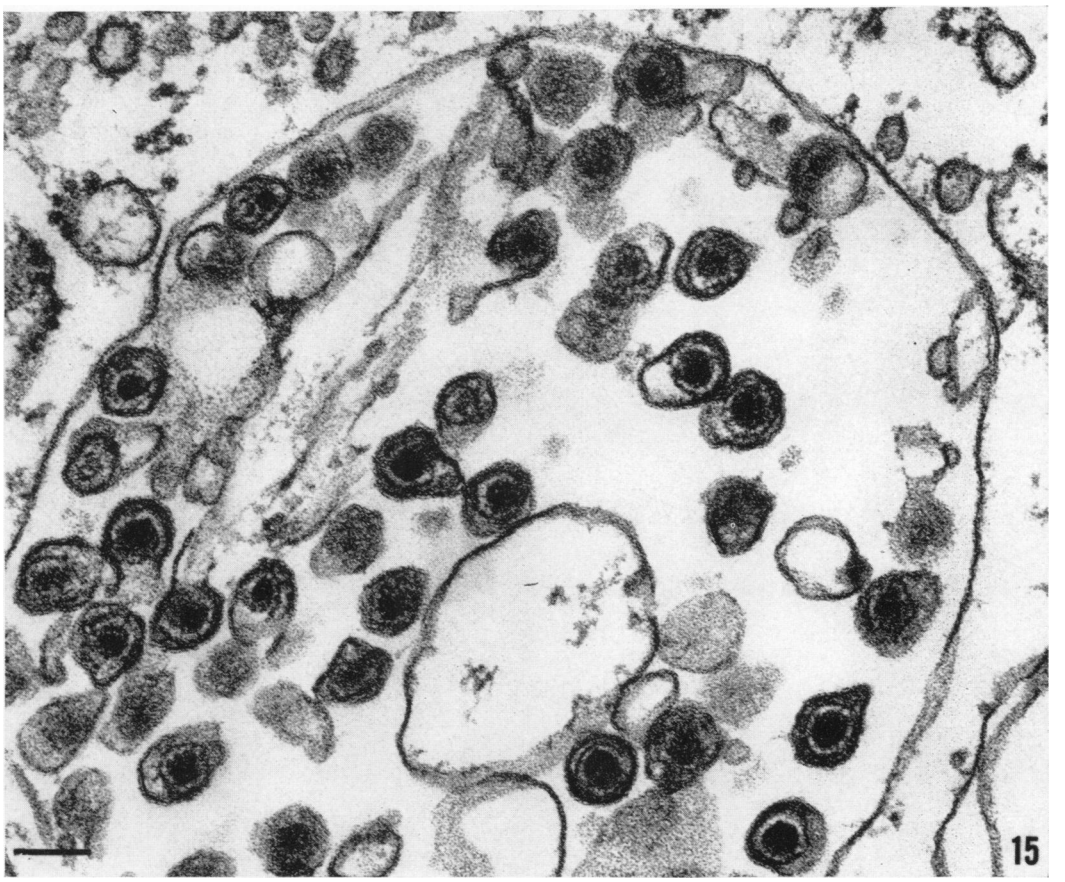


FIG. 13. *Virus particle budding from the plasma membrane of a chick embryo cell infected with avian leukosis virus (RAV_1) is seen at A. Dalton's chrome osmium. Approximately 100,000 ×.*

FIG. 14 and 15. *Virus particles typical of the "mature" type C virus of the avian leukosis-sarcoma complex. Particles from chick embryo cells infected with RSV (SR) are seen in Fig. 14. Figure 15 illustrates particles observed in chick embryo cells after infection with avian leukosis virus, RAV_1. Dalton's chrome osmium. Approximately 100,000 ×.*

TABLE 3. *Induction of MSV by murine leukemia virus*[a]

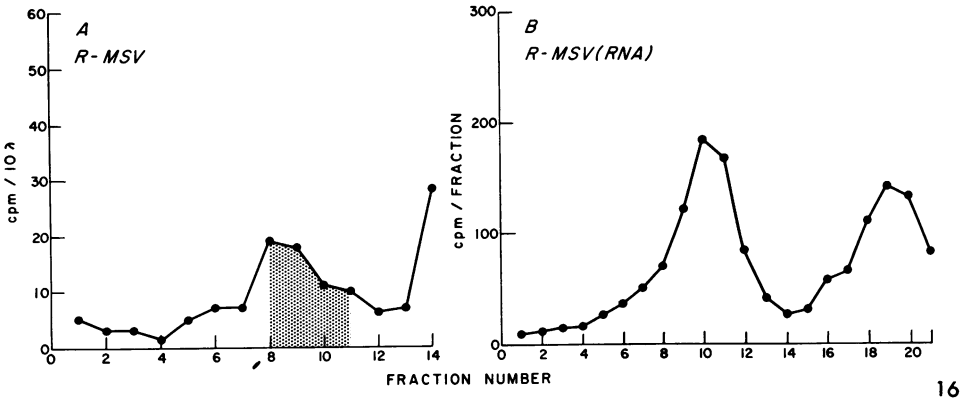
Cell culture	RLV	MLV
R-MSV	1,350[b]	36
R-RSV (SR)	0	0
H-MSV	0	0
H-RSV (SR)	0	0
H-RSV (BH)	0	0

[a] Cultured tumor cells were exposed to murine leukemia viruses (RLV or MLV) and after incubation for 2 days culture fluids were examined for transforming activity on rat embryo cells.

[b] Focus-forming units per culture.

strain, as well as unidentified host factors. In contrast to our negative findings in the hamster cells described here, virus production was detected in hamster cells transformed by RSV, Prague strain (25), and MSV, Harvey strain (23). Conversely, certain rat tumors induced by RSV (Prague strain) failed to produce any detectable particles (24).

The rat cells transformed by MSV and RSV appear to be analogous to chick embryo cells transformed by "defective" RSV. The particles extruded by these cells are noninfectious, as determined by the ability to induce morphological transformation typical of sarcoma viruses or to



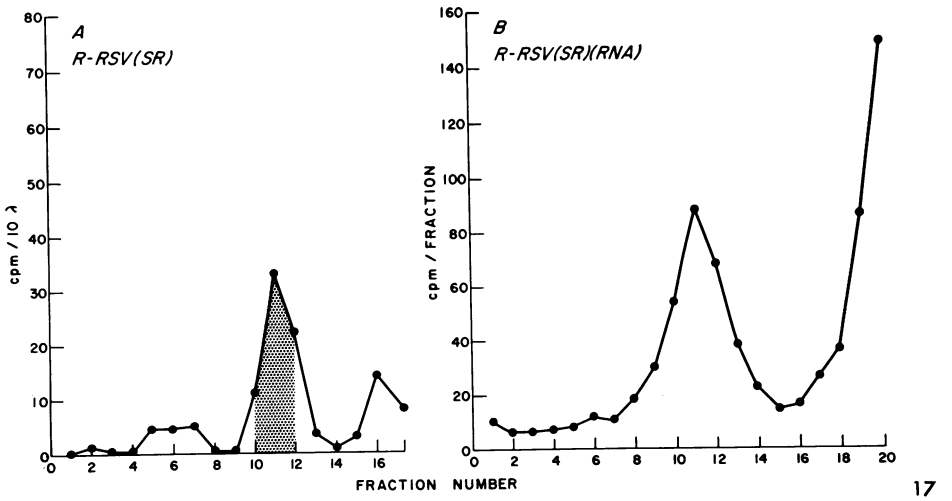
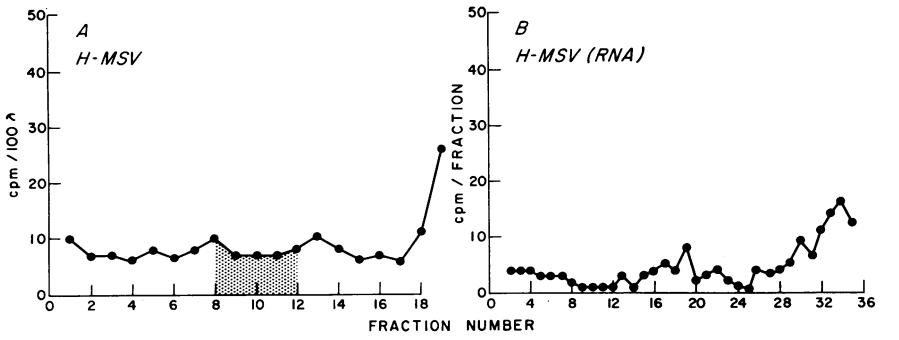
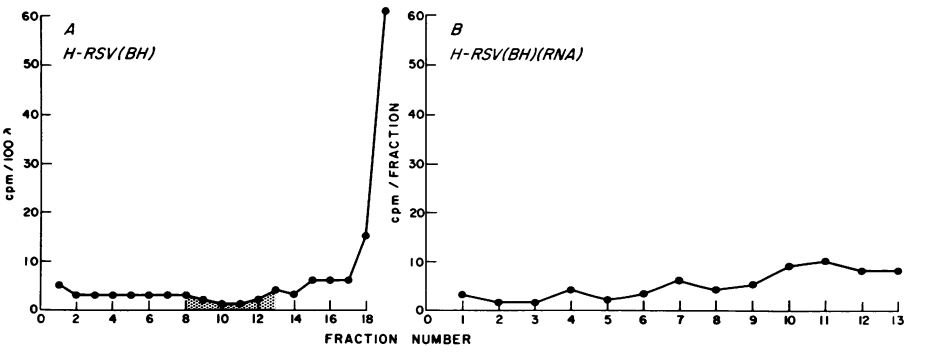


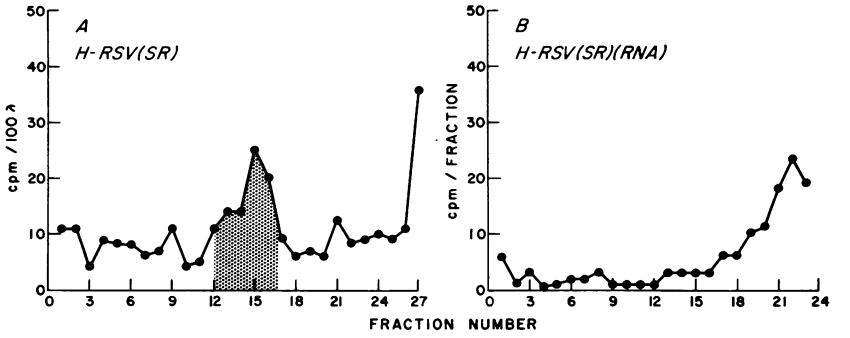
FIG. 16 and 17. *Density gradient centrifugation of possible virus-containing fluids. Rat cells, R-MSV, and R-RSV (SR) were exposed to* ^{3}*H-uridine (100 μc in 5 ml) for 12 hr. Culture fluids were processed (Materials and Methods) and centrifuged in sucrose gradients (15 to 55%, w/v) for 2 hr at 100,000 × g, 4 C. Gradients were fractionated and 10-μliter Samples were counted (A). RNA was extracted from the peak fractions (shaded areas), with the addition of 1 mg of carrier yeast RNA, and sedimented in 5 to 20% sucrose gradients for 2 hr (B).*



18



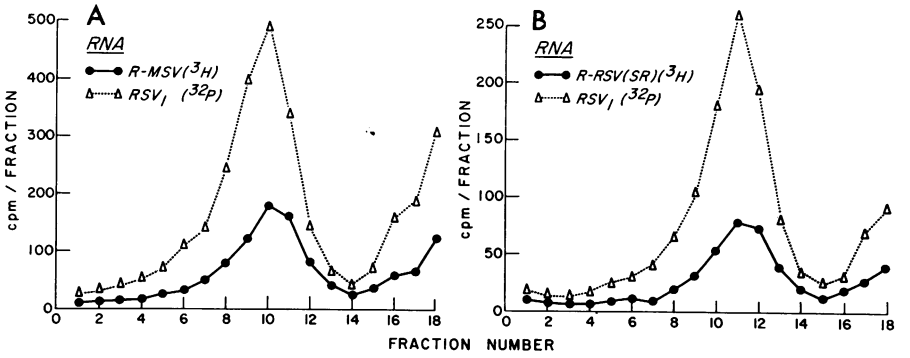
19



20

FIG. 18, 19, and 20. *Density gradient centrifugation of possible virus-containing fluids. Hamster cells, H-MSV, H-RSV (BH), and H-RSV (SR), were treated and processed as described for Fig. 16 and 17 and in Materials and Methods. Samples from fractions of buoyant density gradients (A) contained 100 μliters in this series. Samples in the density range 1.15 to 1.18 were taken for RNA extraction and sedimentation (B).*

reproduce morphological particles indicative of infection by progeny leukemia virus. Preliminary investigations in this laboratory have failed to detect murine leukemia antigen in R-MSV or R-RSV (SR) cultures, although control cells producing Rauscher leukemia virus contained antigen. Also, deliberate infection of R-MSV cells with leukemia virus resulted in production of infectious MSV. In a similar experiment with these R-MSV cells, R. C. Y. Ting obtained the same results (*personal communication*). On the basis of such results, it is concluded that the particles in the R-MSV cultures are most likely free from extraneous murine leukemia virus. This provides a favorable differential system for studies of the MSV. The virus, as described here, has



21

FIG. 21. *Comparison of RNA from observed particles in the R-MSV and R-RSV (SR) cell cultures with RNA from RSV-RAV$_1$ (RSV$_1$). Virus labeled with $^{32}PO_4^{-3}$ was prepared in CE cultures infected with RSV-RAV$_1$, and particles with ^{3}H-uridine in R-MSV and R-RSV (SR) cultures. Culture fluids were processed and banded at their buoyant density in 15 to 55% sucrose gradients. R-MSV and R-RSV (SR) particle were each mixed with ^{32}P-labeled RSV-RAV$_1$ particles. RNA was extracted and sedimented through 5 to 20% (w/v) sucrose gradients for 2 hr at 100,000 × g, 4 C.*

morphological and biochemical characteristics similar to the other viruses of the murine leukemia and avian leukosis-sarcoma complex.

The situation with the R-RSV line is not as clear as with the R-MSV line. Infectivity studies indicate that leukemia particles are absent, and no murine leukemia antigen was detectable. However, we were unable to recover infectious transforming particles after superinfection of R-RSV cells with murine leukemia viruses. The possibility of the observed particles being murine leukemia particles, while unlikely, has not been eliminated.

Viral RNA was demonstrated in the R-MSV and R-RSV (SR) particles by radioactive labeling, and there is little reason to doubt that these particles are viruses. Experiments involving manipulation of virus-particle-containing fluids (19), or the acquisition of cells susceptible to infection by these particles, should resolve the identity of the particles elaborated by these cells.

In summary, rat and hamster cell lines transformed with either RSV or MSV have provided systems for a comparative study of these two viruses. The rat lines, containing particles identified by both electron microscopy and biophysical techniques, appear to be analogous to NP cells infected with RSV, where virus particles which have a low efficiency of infection are generated. Results showing the presence of particles in the rat lines transformed with RSV and MSV and their absence in hamster lines transformed with either of the viruses bear on the requirements for specific host functions in the production of RNA-containing tumorigenic viruses. These virus-producing and -nonproducing cells described here may provide valuable systems for projected investigations into the nature of oncogenesis by the RNA-containing tumor viruses.

ACKNOWLEDGMENTS

We thank Benjamin F. Elliott, Jr., for technical assistance and Albert J. Dalton for helpful discussion of the electron micrographs.

LITERATURE CITED

1. AHLSTROM, C. G., AND N. FORSBY. 1962. Sarcomas in hamsters after injection with Rous chicken tumor material. J. Exptl. Med. **115**:839–850.
2. ANONYMOUS. 1966. Suggestions for the classification of oncogenic RNA viruses. J. Natl. Cancer Inst. **37**:395–397.
3. BADER, J. P. 1962. Production of interferon by chick embryo cells exposed to Rous sarcoma virus. Virology **16**:436–443.
4. BADER, J. P. 1966. Metabolic requirements for infection by Rous sarcoma virus. II. The participation of cellular DNA. Virology **29**:452–461.
5. COURINGTON, D., AND P. K. VOGT. 1967. Electron microscopy of chick fibroblasts infected by defective Rous sarcoma virus and its helper. J. Virol. **1**:400–414.
6. DALTON, A. J. 1955. A chrome-osmium fixative for electron microscopy. Anat. Record **121**:281.
7. DALTON, A. J. 1962. Micromorphology of murine tumor viruses and of affected cells. Federation Proc. **21**:936–937.
8. DALTON, A. J. 1966. An electron microscopic study of a virus induced murine sarcoma (Moloney). Natl. Cancer Inst. Monograph **22**:143–168.
9. DALTON, A. J., L. W. LAW, J. B. MOLONEY, AND R. A. MANAKER. 1961. An electron microscope study of a series of murine lymphoid neoplasms. J. Natl. Cancer Inst. **27**:747–791.

10. DOUGHERTY, R. M., AND H. S. DISTEFANO. 1965. Virus particles associated with "nonproducer" Rous sarcoma cells. Virology **27**:351–359.
11. DUESBURG, P. H., AND W. S. ROBINSON. 1966. Nucleic acid and proteins isolated from the Rauscher mouse leukemia virus (MLV). Proc. Natl. Acad. Sci. U.S. **55**:219–227.
12. HAGUENAU, F., AND J. W. BEARD. 1962. The avian sarcoma-leukosis complex; its biology and ultrastructure, p. 1–50. *In* A. J. Dalton and F. Haguenau (ed.), Tumors induced by viruses: ultrastructural studies, Academic Press, Inc., New York.
13. HAGUENAU, F., H. FEBVRE, AND J. ARNOULT. 1962. Mode de formation intra-cellularie du virus du sarcome de Rous. E'tude ultrastructure. J. Microscopie **1**:445–454.
14. HANAFUSA, H. 1964. Nature of the defectiveness of Rous sarcoma virus. Natl. Cancer Inst. Monograph **17**:543–556.
15. HANAFUSA, H., T. HANAFUSA, AND H. RUBIN. 1963. The defectiveness of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S. **49**:572–580.
16. HEUBNER, R. J., J. W. HARTLEY, W. P. ROWE, W. T. LANE, AND W. I. CAPPS. 1966. Rescue of the defective genome of Moloney sarcoma virus from a noninfectious hamster tumor and the production of pseudotype sarcoma viruses with various murine leukemia viruses. Proc. Natl. Acad. Sci. U.S. **56**:1164–1169.
17. MOLLENHAUER, H. H. 1964. Plastic embedding mixtures for use in electron microscopy. Stain Technol. **39**:111–114.
18. ROBINSON, W. S., A. PITKANEN, AND H. RUBIN. 1965. The nucleic acid of the Bryan strain of Rous sarcoma virus: Purification of the virus and isolation of the nucleic acid. Proc. Natl. Acad. Sci. U.S. **54**:137–144.
19. ROBINSON, W. S., H. L. ROBINSON, AND P. H. DUESBERG. 1967. Tumor virus RNA's. Proc. Natl. Acad. of Sci. U.S. **58**:825–834.
20. SABATINI, D. D., K. G. BENSCH, AND R. J. BARNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. **17**:19–58.
21. SARMA, P. S., W. VASS, AND R. J. HEUBNER. 1965. Evidence for the in vitro transfer of defective Rous sarcoma virus from hamster tumor cells to chick cells. Proc. Natl. Acad. Sci. U.S. **55**: 1435–1442.
22. ŠIMKOVIČ, D., J. SVOBODA, AND N. VALENTOVÁ. 1965. Induction of formation and release of infectious Rous virus by cells of rat tumor XC in vitro. Fol. Biol. (Prague) **11**:350–357.
23. SIMONS, P. J., R. H. BASSIN, AND J. J. HARVEY. 1967. Transformation of hamster embryo cells in vitro by murine sarcoma virus (Harvey). Proc. Soc. Exptl. Biol. Med. **125**:1242–1246.
24. SVOBODA, J., P. P. CHYLÉ, D. ŠIMKOVIČ, AND I. HILGERT. 1963. Demonstration of the absence of infectious Rous virus in rat tumor XC, whose structurally intact cells produce Rous sarcoma when transferred to chicks. Fol. Biol. (Prague) **9**:77–81.
25. SVOBODA, J., AND V. KLEMENT. 1963. Formation of delayed tumors in hamsters inoculated with Rous virus after birth and finding of infectious Rous virus in induced tumor P_1. Fol. Biol. (Prague) **9**:403–410.
26. VIGIER, P., AND L. MONTAGNIER. 1966. Persistence and action of the Rous sarcoma virus genome in transformed rodent cells, p. 156–175. *In* Y. Ito (ed.), Subviral carcinogenesis. "Nissha" Printing Co., Mibu, Nakagyo-Ku, Kyoto.