Nucleic Acid and Proteins Isolated from a Strain of Murine Sarcoma Virus (MSV-0) (35731)

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In 1966, Perk and Moloney (1) described the induction of rhabdomyosarcomas in mice after injection of high doses of mouse derived MLV. The virus isolated from these tumors became known as the murine sarcoma virus (Moloney).

Ting has induced an epithelial neoplasm (MSB-1) in a strain of BN rats by injection of murine sarcoma virus (Moloney) (2). The MSB-1 tumor was found to be free from leukemia viruses (3) and was found to produce type C particles by Valentine and Bader (7). The virus produced by MSB-1 tumor was designated as MSV-0. The biological and serological properties of MSV-0 were studied by Ting (3), who showed that MSV-0 transformed adult rat kidney cells in the absence of leukemia viruses (3). The virus can easily be harvested from MSB-1 tissue culture fluids since a chronic virus infection exists in this system.

Recently the viral RNA and proteins of several of the RNA tumor viruses have been isolated and characterized. In 1965, Robinson *et al.* (4) reported the isolation of the nucleic acid of Rous sarcoma virus. Isolation of the nucleic acid of avian myeloblastosis was also reported by Robinson and Baluda in 1965 (5). In 1966, Duesberg and Robinson (6) characterized the nucleic acid and proteins from the Rauscher mouse leukemia virus. Valentine and Bader, in 1968, reported isolation of the RNAs of Rous sarcoma and murine sarcoma viruses (7).

This paper describes the isolation and characterization of the virual nucleic acid and proteins of the murine sarcoma virus MSV-0.

Materials and Methods. Cell culture and growth media. MSB-1 cells were kindly provided by Dr. R. C. Ting of the Laboratory of Biology, National Cancer Institute. The cells were grown in Eagle's medium No. 2 with 10% fetal bovine serum, 10% tryptose phosphate broth, glutamine, and antibiotics (penicillin, streptomycin, and Mycostatin). For the protein labeling experiments, Eagle's medium was used with 10% fetal bovine serum containing 1/100 of the normal amino acid concentration. The protein labeling control experiment was carried out using BN rat embryo cell tissue cultures, purchased from Microbiological Associates of Bethesda, Md. For the protein labeling experiment the cells were grown in leucine-free Eagle's medium with 3% fetal bovine serum. All tissue cultures were grown in plastic tissue culture flasks (75 cm^2; 250 ml) in a CO_2 incubator at 37°.

Labeling of the virus with ^{3}H-uridine and ^{14}C amino acid mixture. Media were decanted from cell cultures as the cells approached a confluent monolayer. Ten ml of medium containing 200 μCi of ^{3}H-uridine (20.0 mCi/mmole) or 20 μCi of a ^{14}C-labeled amino acid mixture (52 mCi/mA) was added to each flask. The cells were then incubated for 18 hr at 37° in a CO_2 incubator. The media containing the virus particles were collected and centrifuged at 1000 rpm in a refrigerated centrifuge for 10 min to remove suspended cells and cellular debris.

Virus purification. The virus concentration and purification was performed in two steps as described by Duesberg and Robinson (6).

Extraction of ^{3}H-uridine labeled RNA. The virus containing fraction was dialyzed in 1 liter of the cold buffer (0.1 *M* NaCl; 0.01 *M*

Tris, pH 7.2; 0.001 *M* EDTA) and, to the resultant 1 ml solution, sodium dodecyl sulfate (SDS) was added to 0.25%. Chick embryo fibroblast ribosomal RNA (500 μg) was added as a carrier. The mixture was shaken and extracted with 5 ml of buffer saturated phenol and centrifuged at 2000 rpm for 10 min. The aqueous phase was again extracted with phenol. The phenol was then extracted with several volumes of cold ether (—20°). Nitrogen was bubbled through the final aqueous solution and 0.3 ml of the solution containing the viral RNA was layered onto a 6–30% (w/v) sucrose gradient (in 0.01 *M* Tris–HCl, pH 7.4; 0.1 *M* NaCl; 0.001 *M* EDTA buffer) in a 5.4-ml polyallomer ultracentrifuge tube and was centrifuged at 39,000 rpm for 2 hr. Fractions of 0.15 ml were collected onto filter papers. The filter papers were dried and then washed with 6 vol of 1.8% perchloric acid followed by cold (—20°) absolute ethanol and ether washes. The dried filter papers were placed in vials containing Spectrafluortoluene scintillation fluid and were counted in a Packard Tricarb liquid scintillation counter.

Ribonuclease treatment of ^{3}H-uridine labeled cellular and viral RNA. Cell suspensions of MSB-1 were labeled with ^{3}H-uridine and incubated for 18 hr in a 37° CO_2 incubator. The cells were harvested by centrifugating at 1000 rpm for 10 min. The supernatant medium was used for isolation of viral RNA, as described above. The cells were washed twice with the standard buffer solution and the RNA was extracted by the SDS-phenol method at room temperature as described by Friedman (8). Cellular and viral RNA solutions were treated with pancreatic ribonuclease (1.0 μg/ml, 0.1 *M* NaCl) at 37° for 10 min. The solutions were then sedimented in a 6 to 30% sucrose gradient at 39,000 rpm for 2 hr. Fractions were collected and analyzed for radioactivity as described above.

Isolation of the viral proteins for acrylamide gel electrophoresis. The viral proteins were solubilized as described by Summers *et al.* (9). Virus containing fractions were acidified with 1/10 vol of glacial acetic acid and then made 0.5 *M* in urea and 1% in sodium dodecyl sulfate (SDS). The solution then was incubated at 37° for 1 hr and dialyzed for 18 hr at 24° against 2000 ml of 0.01 *M* sodium phosphate buffer, pH 7.1, containing 0.1% SDS, 0.5 *M* urea, and 0.1% 2-mercaptoethanol. After dialysis, the protein solutions were mixed with 1/6 vol of 60% sucrose (total volume of 0.3 ml) and layered on to 10% acrylamide gels. The electrophoresis was performed on 6-cm vertical gel columns at 5 mA per gel for 4 hr at 25°. The gels were sliced, fixed, stained, and dehydrated; and autoradiography of the gels was performed according to the method of Fairbanks *et al.* (10), by exposing films to gels for 7 to 14 days. The autoradiograms were scanned in a Joyce-Loebl microdensitometer. Longer incubation did not increase the number of lines recovered on the film.

Inhibition of ^{3}H-uridine incorporation by interferon. Rat interferon was prepared by infecting primary rat embryo fibroblast cultures with Chikungunya virus. After 24 hr, tissue culture fluids were harvested and treated with acid (to pH 2). After overnight incubation at 4°, the preparation was neutralized. The interferon titer of this preparation was 600 units/ml when tested against vesicular stomatitis virus in a virus growth inhibition assay.

MSB-1 cell monolayers were exposed to this rat interferon in 1:3 and 1:10 dilutions of the original solution. After 8 hr of incubation at 37° in a CO_2 incubator, 200 μCi of ^{3}H-uridine was added to each flask, and after labeling for 18 hr, the virus was purified as described above.

Control experiment for viral protein labeling. BN rat embryo cells were grown for 24 hr in the presence of ^{14}C-labeled L-leucine in the concentration of 20 μCi/tissue culture flask. The cells were grown under conditions described above. MSB-1 cells were grown under identical conditions except for labeling with ^{3}H-L-leucine in the concentration of 200 μCi/tissue culture flask. Virus harvesting, concentration, and purification were carried out in the standard way described above.

Reagents. ^{3}H-Uridine (20.0 mCi/mmole) was purchased from Schwarz Bioresearch, Inc., Orangeburg, New York. The purified

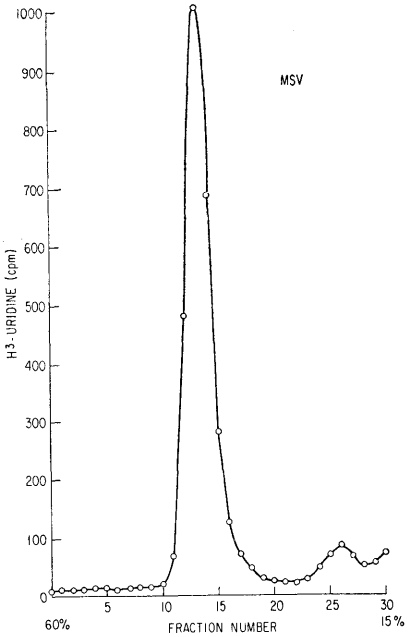

FIG. 1. Density gradient centrifugation of purified ^{3}H-uridine labeled MSV (0):0.5 ml of concentrated ^{3}H-uridine labeled virus solution in 0.01 *M* Tris–HCl (pH 7.1), 0.1 *M* NaCl, and 0.001 *M* EDTA containing buffer was layered on top of a 15–60% (w/v) sucrose gradient made up in the same buffer. Sedimentation was performed at 300,000*g* for 90 min using the SW 65 rotor in a Beckman Model L2-65B preparative ultracentrifuge. Fractions (0.16 ml) were collected by puncturing the bottom of the tube.

^{14}C-amino acid mixture (52 mCi/m atom of carbon) was obtained from Amersham/Searle; ^{3}H-L-leucine (58 Ci/mmole) and ^{14}C-L-leucine (263 mCi/mmole) from New England Nuclear Corp.

Partially purified rat interferon was donated by Mr. C. Buckler, National Institutes of Health.

Pancreatic ribonuclease was purchased from the Worthington Biochemical Corporation of Freehold, New Jersey.

Results. Purified virus. Results of the density gradient centrifugation of the purified MSV-0 in a linear sucrose gradient are shown in Fig. 1. The virus banded in a 37° sucrose layer. The buoyant density of the virus was therefore 1.16 g/ml.

Inhibition of ^{3}H-uridine incorporation by interferon. MSV was purified from cells treated with rat interferon. The acid precipitable radioactive counts present in the purified virus from control and interferon treated cells were compared (Fig. 2). In the tissue cultures treated with 1:3 dilution of interferon, the incorporation of ^{3}H-uridine was reduced to about 35% of the control. In the cells treated with a 1:10 interferon dilution a depression of the counts to 60% of the control was observed. The slowly sedimenting material in the gradient from cells treated with a 1:3 dilution of interferon (Fig. 2) was not consistently observed and we are uncertain of its significance.

Viral nucleic acid. Viral RNA was extracted by the SDS-phenol method, with 500 μg of chick embryo fibroblast RNA as carrier. The sedimentation rates were compared with those of ^{32}P-labeled Semliki Forest virus

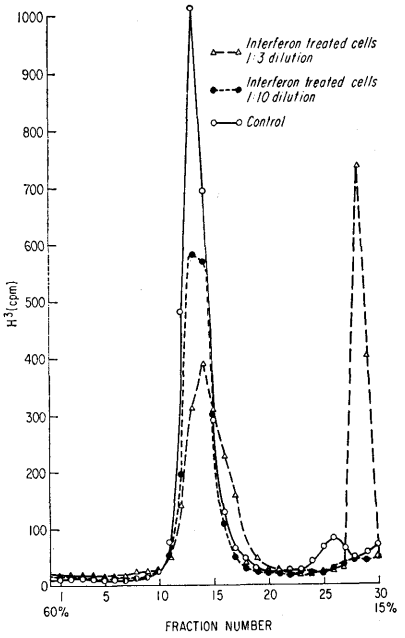

FIG. 2. Inhibition of ^{3}H-uridine incorporation by rat interferon. Cells were incubated with 1:3 (200 units/ml) and 1:10 (60 units/ml) dilutions of rat interferon solution (600 units/ml). After 8 hr of incubation, 200 μCi of ^{3}H-uridine were added to each of the interferon-treated and control tissue cultures, and incubation was continued (at 37° in a CO_2 incubator) for another 18 hr. The media were then analyzed for viral radioactive counts after purification and sucrose gradient ultracentrifugation as described in Methods and Fig. 1.

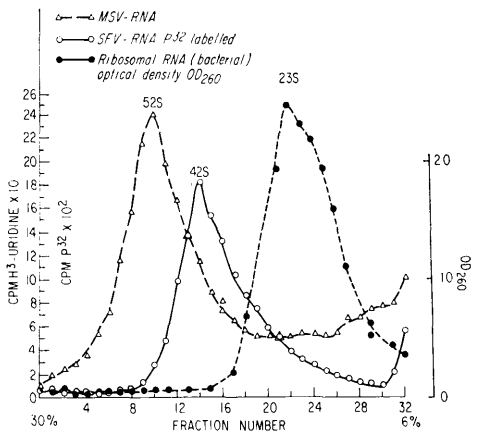

FIG. 3. MSV-RNA ^{3}H-uridine labeled: Velocity sedimentation of MSV-RNA in 6–30% linear sucrose density gradient. RNA was extracted (see Materials and Methods) and centrifuged at 100,000g for 2 hr. Gradients were fractionated and counted after perchloric acid precipitation. ^{32}P-labeled Semliki Forest virus RNA (42S) and bacterial ribosomal RNA (23S) were used as markers. MSV-RNA labeled with tritium was extracted in the presence of 500 μg of chick embryo fibroblast ribosomal RNA.

(SFV) RNA (42S) and bacterial ribosomal RNA (23S) (Fig. 3). The single peak of the MSV-RNA had a sedimentation value of 52S when compared with RNAs of SFV (42S) and ribosomal RNA (23S). The slight increase in counts toward the top of the gradient probably represents partially degraded RNA.

In a separate experiment, MSV-RNA was heated at 80° for 2.5 min and sucrose gradient sedimentation was performed (Fig. 4). The sedimentation constants were calculated by comparison with known values for chick embryo fibroblast ribosomal RNA (4S, 16S, and 28S). The unheated (control) MSV-RNA showed one sharp peak in the region of 64S, with a second peak at the top of the gradient, probably representing degraded RNA. The heated MSV-RNA showed no counts in the 64S region, but a peak was present in the 32S region with a trail of high counts to the top of the gradient.

Ribonuclease sensitivity of MSV (0) RNA and RNA extracted from MSB-1 cultures. Pancreatic ribonuclease treatment of the phenol extracted RNA resulted in no appreciable acid precipitable radioactive counts after ultracentrifugation in a 6 to 30% sucrose gradient (see Methods). Similarly when ^{3}H-uridine labeled MSB-1 RNA was exposed to pancreatic ribonuclease, no evidence for a double-stranded ribonuclease-resistant RNA species could be demonstrated.

Proteins of MSV (0). The proteins were extracted and solubilized from the purified virus fractions and electrophoresis was performed in 10% polyacrylamide gels which were sliced and stained with Coomassie blue. A photograph of the stained gel slice is shown in Fig. 5. There were six distinctly separated bands observed. The fastest migrating protein was labeled 1, the slowest 6. In microdensitometer tracing of a radio-autogram of the dried, sliced gel (Fig. 6), there were again six distinct peaks observed. These corresponded to the stained bands.

Control experiment to exclude possibility of nonviral protein labeling. MSB-1 cells were originally derived from thymectomized BN rats injected with murine sarcoma virus (Moloney). Inbred BN strain rat embryo cells were cultivated in tissue culture and

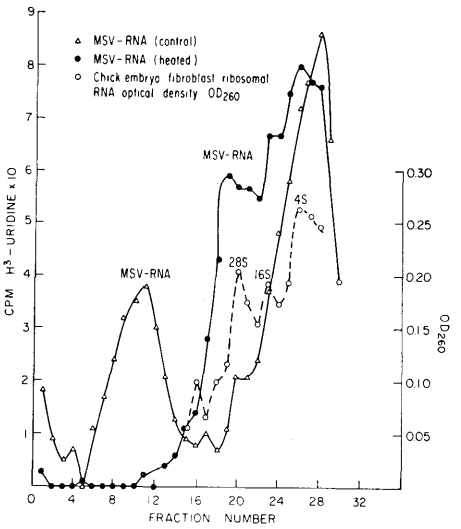

FIG. 4. MSV-RNA ^{3}H-uridine labeled: Velocity sedimentation in 6–30% sucrose gradient of untreated and heated MSV-RNA. Equal volumes (0.2 ml) of MSV-RNA, heat-treated MSV-RNA (incubated at 80° for 2.5 min) and chick embryo fibroblast ribosomal RNA were sedimented on a 6–30% sucrose gradient (w/v).

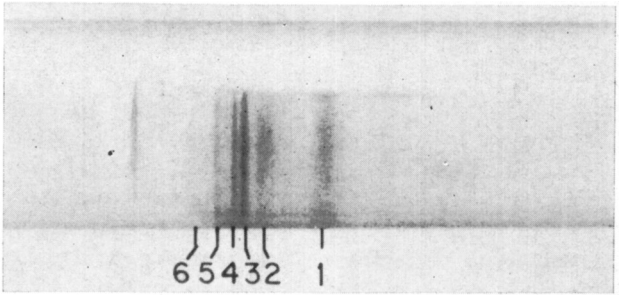

FIG. 5. Polyacrylamide gel electrophoresis pattern of the structural proteins of SMV (0). A photograph of the sliced and Coomassie brilliant blue stained gel is shown. MSB-1 cell cultures were labeled with ^{14}C-amino acid mixture containing 20 μCi/tissue culture. The cells were then incubated for 18 hr. The virus was purified as described in Materials and Methods. Proteins were extracted from the purified virus and analyzed as described in the text. The cathodal end is to the left.

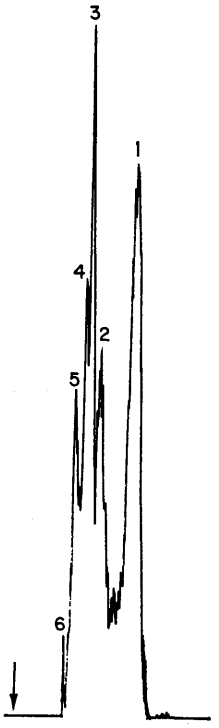

FIG. 6. Polyacrylamide gel electrophoresis of structural proteins of MSV (0). Tissue cultures of MSB-1 cells were incubated with 20 μCi/ml of ^{14}C-amino acid mixture (sp act 52 mCi/mA). The labeled virus was purified, and the viral proteins were extracted and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. Microdensitometer tracings were prepared from the radioautograms of the dried, sliced gels. A photograph of the original densitometer tracing is shown. The arrow indicates the cathodal end of the gel. Figs. 5 and 6 are taken from the same specimen.

were used as a control cell line to exclude the possibility of nonviral protein labeling. MSB-1 cell cultures were labeled with ^{3}H-leucine and BN rat embryo cell cultures were labeled with ^{14}C-leucine. Both sets of cultures were treated identically, as described in Methods. The tissue culture media were harvested after 24 hr of labeling and the virus isolation and purification procedure were carried out the same way as described before. Density gradient ultracentrifugation was performed in a linear sucrose gradient. The MSB-1 cell line supernatant showed a sharp increase in ^{3}H counts, similar to that depicted in Fig. 1. In contrast the BN rat embryo cell line supernatant showed no increase in counts over background throughout the sucrose gradient.

Discussion. The buoyant density of the virus was 1.16 g/ml. This value is in agreement with values previously reported for murine sarcoma viruses (7).

The rat interferon-treated cells showed a decreased ability to incorporate tritiated uridine into virus. This suggested an inhibition of virus production in the interferon-treated cells. The inhibition appeared to be dose related since treatment with the high concentration interferon resulted in greater inhibition. However, these results should be interpreted cautiously since the interferon used was not purified.

The sedimentation constant of 52S obtained in our experiment (Fig. 3) is lower than the reported 62S to 74S range for most

RNA tumor viruses. Our 52S value was obtained by using 42S Semliki Forest virus RNA as a reference value. When using a 45S reference value for the Semliki Forest virus RNA (11), an S value of 62 is obtained for our MSV-RNA. In our experiment of heating of the MSV-RNA (Fig. 4), chick embryo fibroblast ribosomal RNAs were used as reference values. Based on those, the MSV-RNA has an S value of 64S for the unheated and, of 32S for the heated RNA. The heated RNA appears, however, to be heterologous and the effect of the heat may be to induce a number of breaks in the molecule. Based on the formula of Spirin for correlation of S value with molecular weight (12), values of 10×10^6, 6.3×10^6, and 3×10^6 were obtained for S values of 64S, 52S, and 32S, respectively. Alternatively, differences in RNA configuration could account for the different sedimentation values obtained.

The changing of the S value of MSV-RNA with melting suggests a similarity in the secondary structure of MSV-RNA to RSV-RNA, since a similar 62S to 36S change was observed by Duesberg on heating the Rous Sarcoma Virus RNA (13).

Treatment of the MSV-RNA and RNA extracted from the MSB-1 cells with pancreatic ribonuclease showed no ribonuclease-resistant RNA species. This observation argues against a hypothetical double-stranded intermediary form of RNA in the MSB-1 cells and confirms the single-stranded nature of the MSV-RNA.

Duesberg *et al.* (14) reported three viral proteins in the Rous sarcoma virus and two viral proteins in the Rauscher mouse leukemia virus (6). Recently Bolognesi and Bauer (15) reported the isolation of four proteins from avian RNA tumor virus. Our results suggest that there are at least six proteins associated with MSV (0). The lack of ^{14}C-leucine incorporation in the control experiment makes the likelihood of cellular protein contamination very unlikely. The recent demonstration of an RNA-dependent DNA polymerase system in the virions of RNA tumor viruses by Temin and Mizutani (16) and by Baltimore (17) make the findings of six protein bands in MSV (0) virions significant.

Summary. Murine sarcoma virus (MSV-0, Moloney) was grown in tissue cultures of transformed rat cells (MSB-1). The virus was harvested from the medium and purified by sucrose density gradient ultracentrifugation. The incorporation of ^{3}H-uridine was shown to be reduced in virus preparation from cells treated with a crude rat interferon preparation, when compared to controls. The viral nucleic acid was extracted and characterized by sucrose density ultracentrifugation. A sedimentation value of 52S or 64S was obtained, depending on the reference values used. Upon heating to 80° the viral RNA dissociated into a major 32S and minor smaller S value species, suggesting that the larger RNA is either an aggregate of smaller 32S units, or that modifications had taken place in the secondary structure of the RNA. Pancreatic ribonuclease treatment disclosed no ribonuclease-resistant RNA in the virus or in the MSB cells. The proteins were extracted from purified MSV (0), solubilized, and separated by polyacrylamide gel electrophoresis. Six proteins were identified.

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1. Perk, K., and Moloney, J. R., J. Nat. Cancer Inst. **37**, 581 (1966).

2. Ting, R. C., Virology **28**, 783 (1968).

3. Ting, R. C., Proc. Soc. Exp. Biol. Med. **126**, 778 (1968).

4. Robinson, W. S., Pitkanen, A., and Rubin, H., Proc. Nat. Acad. Sci. U.S.A. **54**, 137 (1965).

5. Robinson, W. S., and Baluda, M. A., Proc. Nat. Acad. Sci. U.S.A. **55**, 1686 (1965).

6. Duesberg, P. H., and Robinson, W. S., Proc. Nat. Acad. Sci. U.S.A. **55**, 219 (1966).

7. Valentine, A. F., and Bader, J. P., J. Virol. **2**, 224 (1968).

8. Friedman, R. J., J. Virol. **2**, 547 (1968).

9. Summers, D. F., Maizel, J. V., and Darnell, J. E., Proc. Nat. Acad. Sci. U.S.A. **54**, 505 (1965).

10. Fairbanks, G., Levinthal, C., and Reeder, R. H., Biochem. Biophys. Res. Commun. **20**, 393 (1965).

11. Sonnabend, V. A., Martin, E. M., and Mecs, E., Nature (London) **213**, 365 (1967).

12. Spirin, A. S., Biochemistry (USSR) **26**, 511 (1961).

13. Duesberg, P. H., Proc. Nat. Acad. Sci. U.S.A. **60**, 1511 (1968).

14. Duesberg, P. H., Robinson, H. L., and Robinson, W. S., Virology **36**, 73 (1968).

15. Bolognesi, D. P., and Bauer, H., Virology **42**, 1097 (1970).

16. Temin, H. M., and Mizutani, S., Nature (London) **226**, 1211 (1970).

17. Baltimore, D., Nature (London) **226**, 1209 (1970).

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