Electrophoretic Analysis of the RNA from a Mouse Leukemia Virus

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SUMMARY

The RNA of mouse erythroblastosis virus was analyzed by electrophoresis in 2% acrylamide:0.5% agarose gels. An established mouse erythroblastosis virus-releasing mouse cell line was labeled with uridine-3H for 24 hr. Mouse erythroblastosis virus was purified from culture fluids by high-speed clarification, concentration through a 5% sucrose barrier, and centrifugation through a discontinuous sucrose gradient. Viral RNA was extracted by treatment of purified virions with 0.5% sodium dodecyl sulfate or phenol:sodium dodecyl sulfate. RNA profiles from virus of 24-hr culture fluids contained a sharp 70 S RNA peak and a minor 4 S RNA band. Aging of the virus in spent growth medium revealed considerable degradation of the 70 S viral RNA, in contrast to virus incubation in Tris buffer. The major differences in the electrophoretic chromatogam of RNA from 2- and 24-hr culture fluids suggest the presence of nucleases in the growth medium.

INTRODUCTION

Oncornaviruses contain a major, single-stranded 60 to 70 S RNA with an estimated molecular weight of 5 to 10 X 106 (9). The high-molecular-weight RNA is an aggregate that can be dissociated into several noncovalently linked 35 S subunits by treatment with heat, urea, or dimethyl sulfoxide (1, 6-8). In addition, heterogenous species of low-molecular-weight RNA's have repeatedly been isolated from avian and mammalian oncornaviruses (2-8, 16, 21, 24, 25). The low-molecular-weight RNA's appear to be intrinsic virion components and may represent RNA species of viral or cellular origin, degradation products of the high-molecular-weight RNA, or both.

We have previously shown (11, 22) that the RNA extracted from purified, RNase-treated MEV, a murine leukemia virus, consisted of a rapidly sedimenting 72 S species and slowly sedimenting 4 to 30 S components (25). The viral material was harvested at 24-hr intervals after labeling with uridine-3H and was purified by isopycnic banding in sucrose equilibrium gradients. However, Bishop et al. (3) have recently shown that small cell fragments cosediment with RSV in sucrose equilibrium gradients, and the low-molecular-weight MEV RNA may at least in part represent adventitious cellular material (1, 25). We have therefore reexamined the MEV RNA in an effort to exclude contamination of the virion preparation by cellular components. MEV was isolated by improved purification procedure, and the viral RNA was analyzed by polyacylamide gel electrophoresis. The presence of low-molecular-weight (4 S) RNA species was confirmed, although the relative proportion to the 70 S viral RNA was dependent on the exposure time of the virus to culture fluids and the method of RNA extraction.

MATERIALS AND METHODS

Cells. An established line of MEV-releasing C3H mouse embryo cells was grown at 37° in Earle’s modified Medium 199 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum, glutamine (2 mmoles/liter), sodium bicarbonate (40 mEq/liter), penicillin (100,000 units/liter), streptomycin (100,000 µg/liter), and polymyxin (123,000 units/liter) (25). Medium was changed every 2 days. Culture fluids were harvested from 670 sq cm Bellco roller vessels (Bellco Glass, Inc., Vineland, N. J.). The cultures were free of mycoplasma infection in repeated assays (20).

Radioactive Labeling and Purification of Virus. Nearly confluent cultures were exposed for 24 hr to 10 µCi/ml 3H-labeled uridine in 50 ml growth medium (specific activity, 33 Ci/m mole, Schwarz BioResearch, Inc., Orangeburg, N. Y.). Culture fluids were collected at 2- or 24-hr intervals after labeling, centrifuged at 20,000 X g for 20 min, and stored at -70°. Unless otherwise stated, all subsequent centrifugations were done in a Spinco Model L2 ultracentrifuge at 4°. The supernatant was spun through a 5% sucrose barrier in the Spinco type 30 rotor at 88,500 X g for 70 min (15). The pellets were suspended in 0.2 ml TNE, layered onto a discontinuous sucrose gradient (1 ml of 30%, 2 ml of 40%, and 2 ml of 50% sucrose in TNE), and spun in an SW-50L rotor at 221,800 X g for 2 hr. The 40 and 50% sucrose fractions were...
diluted with TNE, and virions were pelleted in a Spinco Model Ti50 rotor at 226,400 X g for 1 hr.

**Extraction of RNA.** For immediate extraction and analysis of the viral RNA, the pellets were resuspended in TNE (15 µl/gel). Twenty-five µl of 1% SDS and 10 µl of rRNA (22 µg/ml) were added. This mixture was incubated for 30 min at 25°, mixed with 25 µl of 40% sucrose (containing pyronin Y as tracking dye), and immediately layered onto 2% acrylamide:0.5% agarose gels. (Recent experiments indicate that no preincubation of the virus suspension is necessary.) Alternatively, a modification of the phenol:SDS method, as described by Bishop et al. (3), was used. An aliquot of the resuspended virion pellet was made 0.5% in SDS and 1% in 2-mercaptoethanol. Unlabeled cellular RNA, 100 µg, was added as carrier in a final volume of 2 ml TNE buffer. The extraction was carried out at room temperature by shaking with 4 ml phenol:m-cresol (10). Aqueous and phenol phases were separated by centrifuging at 1,000 X g for 5 min in an HL-8 rotor of a Sorvall RC-3 centrifuge. The 1st aqueous phase was added to 4 ml phenol:m-cresol and was reextracted, as was the 1st phenol phase by the addition of 1 ml TNE buffer. Extractions were repeated until no interface remained between the layers. RNA was precipitated from the combined aqueous phases by addition of 2 volumes 100% ethanol and by overnight storage at -20°. RNA was pelleted at 10,000 X g for 30 min at -20°, washed with 80% ethanol in TNE buffer, and dissolved in TNE buffer.

Marker RNA’s were obtained from adult rat liver. Finely minced tissue was suspended in 2 volumes of TNE buffer and homogenized with a large Dounce homogenizer. The suspension was filtered through 4 layers of cheesecloth, and debris was pelleted at 1,000 X g for 15 min. The suspension was made 3% in sodium-4-aminosalicylate and extracted with 2 volumes phenol:m-cresol according to the method of Kirby (10).

**Electrophoresis.** A stock solution of electrophoresis buffer for the preparation of polyacrylamide gels contained 0.4 Tris, 0.2 M sodium acetate, and 0.2 M EDTA adjusted to pH 7.4 with acetic acid. Glass tubes (0.6 cm inner diameter, 12 cm long) were cleaned with chromic acid, dried, and treated with a siliconizing agent (1% dimethyldichlorosilane in benzene (Bio-Rad Laboratories, Richmond, Calif.). Gels were prepared with acrylamide and bisacrylamide recrystallized from chloroform as described by Loening (12). Final concentrations were 2% acrylamide, 0.07% bisacrylamide, 0.05% N,N,N’,N’-tetramethylenediamine (Eastman Kodak Co., Rochester, N. Y.), 0.5% agrose (distributed by Bausch & Lomb, Inc., Rochester, N. Y.), 0.15% ammonium persulfate, and 0.2% SDS. The stock buffer was diluted 1:10 and made 0.2% in SDS. Electrophoresis was carried out at 5 ma/gel for 2.25 hr in a disc electrophoresis apparatus (Canalco Model 24; Canalco, Inc., Rockville, Md.).

Several methods were tried to produce gels of high optical quality because the location and intactness of the marker RNA were critical for estimating molecular weights and assessing the presence of RNase activity. The most effective “cleaning” method was to dialyze the gels against 1-fold electrophoresis buffer for 1 to 3 days prior to use. The gels were held in notched test tubes suspended in a 2-liter beaker. The gels were inserted into electrophoresis tubes, trimmed to the desired length, and held in place with presoaked dialysis tubing and a rubber band. This method produced gels of uniform baseline absorbance at 260 nm. The gels were scanned at 260 nm immediately after each run in a Gilford Model 240 spectrophotometer in a linear transport (Gilford Instrument Labs, Inc., Oberlin, Ohio).

After being scanned, the gels were stored at -70° overnight, thawed, and cut into 1-mm slices; 2 slices were placed in each Packard polyethylene scintillation vial. The radioactivity was eluted from the gels by 1 ml of a mixture of NCS tissue solubilizer (Amer sham/Searle Corp., Arlington Heights, Ill.) and ammonium hydroxide, 77:23, as described by Maitra et al. (13). The tightly capped vials were left at least 18 hr at room temperature. After hydrolysis, 5 ml of toluene scintillation fluid (4 g PPO; 200 mg POPOP/liter) were added. Occasionally, 0.1 ml of NCS was needed to ensure proper mixing of the sample and the scintillation fluid. This procedure routinely eluted 85 to 100% of the input radioactivity, with a counting efficiency of 30 to 40% for 3H. Samples were counted in the Packard Tri-Carb Model 3380 scintillation spectrometer. The A-B discriminators were set at 20 to 1000, and the amplification was set at 60%, which gave the optimal ratio of (sample dpm)^2/(background).

**RESULTS**

**Virus Purification.** The analysis of the viral RNA’s is complicated by the presence of cellular debris in the culture fluids from which the virus is isolated (1, 3, 6, 8, 22, 23, 25). Several methods of virus purification were tested in order to eliminate most of the cellular contaminants, if not all of them. The presently used 3-step procedure consisted of a high-speed clarification of virus-containing supernatants at 20,000 X g for 20 min, which sedimented whole cells, membrane fragments, and lysosomes (23). Virus from the clarified fluids was concentrated by centrifugation through a 5% sucrose barrier, and the resuspended pellets were layered on discontinuous sucrose gradients. The choice of velocity-type gradients was based on the well-known differences of sedimentation coefficients between oncornaviruses and cell fragments (16, 23). Peak fractions of radioactivity were concentrated as final purification step.

Discontinuous sucrose gradients consistently yielded broad peaks of 3H radioactivity in the 40%, 50%, and pellet sucrose gradient fractions (Chart 1). Approximately 30% of total radioactivity was recovered from each of the pelleted and 50% sucrose gradient fractions; another 10% of total radioactivity was present in the 40% sucrose gradient fraction. Pilot studies established that the material in the 40 and 50% fractions banded at buoyant densities of 1.16 to 1.18 g/cm^3 in linear 30 to 60% sucrose equilibrium gradients, whereas the pelleted fraction contained a peak of 3H radioactivity with buoyant densities of 1.14 to 1.16 g/cm^3 after centrifugation through 10 to 50% potassium citrate gradients. These buoyant densities are characteristic for murine leukemia virus, including MEV (14, 18, 22, 25). Thus, the 40%, 50%, and pellet fractions from the discontinuous sucrose gradients contained uridine-3H-labeled MEV, and each fraction was used separately to extract the viral RNA.
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Chart 1. Purification of MEV by centrifugation through discontinuous sucrose velocity gradients. Virus-containing culture fluids were harvested 2 hr after labeling and clarified at 20,000 X g for 20 min. Virus was pelleted from the supernatant at 85,500 X g for 70 min through a 5% sucrose cushion. The pellet was resuspended in 0.2 ml Tris buffer, layered on a 5-ml discontinuous sucrose gradient (1 ml 30% sucrose, 2 ml 40% sucrose, and 2 ml 50% sucrose) and centrifuged at 243,000 X g for 2 hr. Peak areas of the gradient approximating the 50 and 40% sucrose layers as well as the gradient pellet are identified. The numbers on the ordinate are expressed as 1/1000 of the absolute value.

RNA Profiles. We have experienced considerable variations in the recovery and relative electrophoretic mobilities of MEV RNA because of exogenous nucleases from disintegrating tissue culture cells, virions, and the fetal calf serum of the growth medium (unpublished data). For reduction of exposure to nucleases, the viral RNA was extracted in most experiments by treatment with SDS alone. This method is as effective as the conventional RNA extraction by phenol:SDS but requires fewer preparative steps (see "Materials and Methods"). Chart 2 compares the relative electrophoretic mobility of MEV RNA, which was prepared according to the procedures described in "Materials and Methods" from aliquots of the same culture fluid pool. The culture fluids were harvested 24 hr after a 24-hr labeling period, and aliquots from the 40%, 50%, and pelleted sucrose gradient fractions were extracted by SDS or phenol:SDS. For simplicity in presentation, only the electrophoretic chromatograms from the 50% sucrose fraction are shown in Chart 2. Large, skewed radioactive peaks migrated near the origin of the gels. The molecular weight of the major peak in each gel was 1 X 10^7, as estimated by the method of Peacock and Dingman (19). Further, the high-molecular-weight RNA's sedimented at 65 to 70 S in 15 to 30% sucrose velocity gradients (25). The high-molecular-weight RNA is considered to represent the viral genome of oncornaviruses (1-9, 16, 17, 21, 25) and will henceforth be referred to as "70 S viral RNA." A 2nd distinct peak with an estimated molecular weight of 8 X 10^6 was found near the 70 S RNA in the sample extracted by SDS alone (Chart 2A). Its absence from the RNA sample prepared by phenol:SDS may have been related to the shearing forces that are inherent in this method. Increasing radioactivity between the 28 S and 4 S marker RNA's in the RNA sample extracted by phenol:SDS suggested that the 2nd high-molecular-weight RNA peak was partially degraded following phenol:SDS extraction.

In addition to the 70 S viral RNA, rather large and heterodisperse uridine-3H bands migrated with the 4 S marker RNA in both electrophoretic chromatograms (Chart 2). The relative proportions of the 70 S to 4 S viral RNA's were calculated by 2 methods (3, 8) (Table 1). When the ratios of
70 S and 4 S RNA bands were computed from the total dpm in the gel, the 4 S RNA bands accounted for 8 to 23% of total radioactivity. This relatively high proportion was even more pronounced by computing the ratio of 4 S and 70 S RNA from the peak dpm alone (3). Accordingly, the viral RNA was resolved into 34 to 60% of 4 S RNA and 40 to 60% of 70 S RNA. Table 1 also shows that the approximate proportions of 70 S to 4 S RNA varied considerably among the sucrose gradient fractions used for viral RNA extraction. Viral RNA prepared from the 40% fraction contained the highest proportion, whereas RNA from the 50% fraction contained the lowest proportion of 4 S RNA. The significance of this finding is unclear, although it may reflect a heterogeneous virion population.

It should be emphasized that the 28 S, 18 S, and 4 S marker RNA were not degraded during SDS or phenol:SDS treatment of the viral pellet. The marker RNA's were added to the purified pellet prior to treatment with SDS or phenol:SDS, and every gel used in the present work was scanned for the intactness of the reference RNA's. In view of these findings, the heterodisperse viral RNA profiles from 24-hr culture fluids could not be attributed to RNA breakdown by exposure to nuclease during or after SDS or phenol:SDS extraction.

Since the heterogeneity of the viral RNA may be due to thermal degradation, we examined the RNA profiles from virion pools that remained at the temperature (37°) of the tissue culture fluid for a short period only. For this purpose, 2-hr culture fluids from several replicate cultures were pooled after the cells had been labeled for 24 hr. The viral RNA was prepared by SDS treatment of the 40%, 50%, and pelleted sucrose gradient fractions. Homogeneous peaks of 70 S RNA with estimated molecular weights of 8 to 10 x 10^6 were observed in all 3 electrophoretic chromatograms (Chart 3). Increased radioactivity also migrated to positions near the 4 S marker RNA’s. The relative proportions of these 4 S viral RNA’s to the 70 S viral RNA ranged from 6 to 12% of total radioactivity and from 8 to 22% of peak dpm (Table 1). Virions from the 40% sucrose gradient fraction again contained the highest proportion of 4 S to 70 S viral RNA’s (12 and 22%, respectively). Radioactive peaks were not observed near the 28 S and 18 S marker RNA’s, which would be expected if the viral preparations had been contaminated by adventitious rRNA’s. Moreover, there was no increased radioactivity near the 7 S RNA region. A distinct 7 S viral RNA has recently been described for RSV by Bishop et al. (3).

**Aging of Virus Samples.** Gradient-purified virion samples were kept at the temperature of tissue cultures (37°) for various times in order to determine whether the differences in RNA profiles between 2- and 24-hr culture fluids are related to the temperature, the culture medium, or both. The experiments were designed so that cells labeled for 24-hr culture fluids harvest were collected at 2-hr intervals for up to 3 days. The fluids were centrifuged at 20,000 x g for 20 min and stored at -70°. The total collection was thawed at once, mixed, and divided into Pools A and B. Pool A was used to purify virions that were resuspended in 120 μl TNE buffer and incubated for 12 or 24 hr at 37°. Culture fluid Pool B was divided into aliquots and used as incubation medium (“spent growth medium”). A control aliquot was held in spent growth medium for 24 hr at 4°; the other aliquots were kept in spent growth medium for 12 and 24 hr at 37°. The RNA was extracted by treatment with SDS and was subjected to electrophoresis as described.

Incubation of purified virions in TNE buffer for 12 or 24 hr at 37° did not significantly degrade the 70 S RNA (Chart 4). The estimated molecular weight of the 70 S RNA ranged from 7.6 to 9 x 10^6. The base of the 70 S RNA peak was somewhat wider after 12 or 24 hr of incubation than in the 0-hr control sample, thus suggesting minor degradation. Broad radioactive bands of low-molecular-weight viral RNA’s migrated even further than the 4 S marker RNA’s, but their relative proportions to the 70 S RNA did not exceed 10%. Additional experiments did not reveal degradation of the 70 S RNA after purified virions were incubated in TNE buffer for 2, 4, or 8 hr at 37°. Chart 5 presents the results of virus incubation in spent growth medium. Approximately 70 to 90% of the expected total radioactivity was lost after the virus was kept in spent growth medium for 12 or 24 hr. Moreover, both 70 S RNA peaks were broader than the RNA peak of the control incubation (24 hr at 4°). The small radioactive bands in the 4 S marker RNA region did not exceed 10% of total radioactivity, unlike the large, heterodisperse peaks shown in Chart 2. Fresh growth medium likewise degraded naked, viral RNA within minutes (to be published). The profound effects of spent or fresh growth medium on the electrophoretic mobilities of the viral RNA could also be demonstrated by incubating marker RNA’s. Whereas incubation in TNE buffer for 8 hr at 37° had no effects on the marker RNA profiles, the
18 S and 18 S species were completely degraded to 4 S RNA by exposure to spent growth medium for as little as 5 min.

**DISCUSSION**

The present work was undertaken to determine whether the previously described low-molecular-weight RNA species of MEV represents thermal degradation products of the high-molecular-weight (70 S) RNA, adventitious rRNA's, or both (25). For this purpose, MEV-releasing mouse cells were labeled for 24 hr, and culture fluids were collected 2 or 24 hr later. The electrophoretic mobilities of the RNA from purified virions of the 2 culture fluid samples were compared. One major difficulty in the interpretation of the results is the heterogeneity of the virion population examined. Tissue culture fluids are known to contain immature and mature type C particles, partially damaged virions, and viral aggregates, which cannot be entirely separated by presently available purification procedures (18, 22). For elimination of cellular debris and RNases from the culture fluids, a 3-step viral purification protocol was used, and marker RNA's were included in each experiment.

The heterodisperse RNA profiles from virions of the 24-hr culture fluid samples included large, skewed peaks of high-molecular-weight RNA as well as considerable radioactivity in the regions of the low-molecular-weight marker RNA's (Chart 2). This finding was not unexpected from previously published reports on RNA profiles of oncornaviruses (2–6, 8, 9, 21, 25). The exact size of the 70 S RNA cannot be accurately determined under the present experimental conditions, since this RNA is known to be an aggregate of 35 S subunits, and nicks in the RNA may be masked by hydrogen bonds (1, 3, 4, 6, 7). In contrast to RNA profiles from virions of 24-hr culture fluids, those from 2-hr fluid samples consisted of a sharp 70 S RNA peak and a minor band of radioactivity near the 4 S marker RNA (Chart 3). No peaks of radioactivity were detected near the 28 S or 18 S marker regions in several electrophoretic chromatograms of the 2-hr fluid material, which would be expected if rRNA's from cellular debris or vesicles had contaminated the purified viral pellet. Bader and Steck (1) have postulated that long labeling periods, such as those used in the present work, favor the inclusion of cellular RNA's into the virion either as an intrinsic component or as adventitious material. The electrophoretic chromatograms of Chart 3 appear to negate this conclusion. However, the origin of the low-molecular-weight MEV RNA band has not been resolved in the present work.

4 S RNA's with transfer-like properties have been best characterized in avian oncornaviruses (3, 8, 24). Erikson and Erikson (7) have recently shown that approximately 2.5 to 3% of 4 S RNA is released during heat dissociation of the high-molecular-weight (65 S) RNA from avian myeloblastosis virus, RSV, and murine sarcoma leukemia viruses. The base ratios of this transfer-like 4 S RNA differ from those of the 35 S subunits, thus suggesting that the 4 S RNA is not a degradation product of the high-molecular-weight RNA. Moreover, radioautographs of oligonucleotides showed...
similarities to the 4 S RNA's from host cells and from virions. The relationship of the 4 S RNA band to these transfer-like RNA's is presently unknown.

In an effort to reproduce the degradation of 70 S viral RNA in vitro, virions from 2-hr culture fluid pools were incubated for up to 24 hr in TNE buffer or growth medium at 37° (Charts 4 and 5). Whereas TNE buffer did not degrade the viral RNA, considerable radioactivity was lost from the 70 S region when virions were exposed to fresh or spent growth medium for 12 or 24 hr. With shorter periods of incubation (3 hr) in growth medium, 70 S RNA from Rauscher leukemia virus was not degraded unless treated with dimethyl sulfoxide or other agents that denature RNA by breaking hydrogen bonds (1). In the present work, degradation was likewise not observed unless the virions were incubated with growth medium for 12 hr. These results suggest the presence of nucleases in fresh and spent growth medium. We concluded that the cumulative effects of nucleases in the growth medium, rather than thermal denaturation alone, accounted for the heterodisperse RNA profiles observed in virions from 24-hr fluid harvests. Further studies on these nucleases will be published separately.

ACKNOWLEDGMENTS

We are indebted to Dr. T. L. Steck for suggestions on direct virus solubilization and other helpful discussion. Also gratefully acknowledged is the patient technical assistance of Mrs. T. Raineri and Mrs. J. Berry.

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