Deficiency of 60 to 70S RNA in Murine Leukemia Virus Particles Assembled in Cells Treated with Actinomycin D

JUDITH G. LEVIN, PHILIP M. GRIMLEY,¹ JANET M. RAMSEUR, AND IRENE K. BEREZESKY

*Laboratory of Molecular Genetics, National Institute of Child Health and Human Development and
Laboratory of Pathology, National Cancer Institute, National Institutes of Health,
Bethesda, Maryland 20014*

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Production of particles with the ultrastructural appearance of C-type virions persisted for at least 6 h in actinomycin D-treated cells infected with murine leukemia virus. This phenomenon occurred despite severe inhibition of viral RNA synthesis. Virus particles present in a 6-h harvest sedimented in sucrose gradients with the buoyant density characteristic of RNA tumor viruses (1.16 g/cm³) and exhibited high levels of reverse transcriptase activity in response to the exogenous template polyriboadenylic acid·oligo deoxythymidylic acid in the range of untreated controls. However, RNase-sensitive endogenous activity was only 1/3 the level found in controls. This observation correlated with a marked reduction in infectivity. Kinetic studies on the appearance of labeled RNA in banded virions revealed that within the first hour after addition of actinomycin D, particles contained 60 to 70S RNA and two low-molecular-weight RNA species corresponding to 8 and 4S RNA. After approximately 1 h of incubation with actinomycin D, 60 to 70S RNA could not be detected and 4S RNA was the predominant species. These findings suggest that murine leukemia virus particles assembled in the presence of actinomycin D are deficient in 60 to 70S viral RNA.

Actinomycin D treatment of cells infected with RNA tumor viruses causes a rapid cessation of viral RNA synthesis (2, 7, 14, 40) and a marked decrease in virus production, as determined by assay of focus-forming activity in culture fluids (2, 39, 44) or incorporation of [³H]uridine into purified virions (7, 14). Nevertheless, some data indicate that virus release and assembly must persist for a brief interval since 70S RNA continues to appear extracellularly for 1 or 2 h after addition of the drug (3, 6).

Our interest in the effect of actinomycin D on assembly of murine leukemia virus (MuLV) developed while we were studying group A arbovirus replication in murine cell lines chronically infected with MuLV (27). Quite unexpectedly we found that throughout the logarithmic phase of arbovirus growth C-type particles continued to bud from the same cell surfaces as preassembled arbovirus cores (22). Since arboviruses are routinely grown in the presence of actinomycin D (19, 26, 38), the ultrastructural observations suggested that terminal steps in the assembly and morphogenesis of murine C-type virus can occur for a longer period than

expected under conditions where tumor virus RNA synthesis is severely inhibited (2, 7, 14, 40).

The present study was initiated to investigate this phenomenon in MuLV-infected cells. Our approach was to monitor virus production by electron microscopy and by assay of reverse transcriptase activity (4, 41) in response to the exogenous template polyriboadenylic acid·oligo deoxythymidylic acid [poly rA·oligo dT₍₁₂₋₁₈₎] (1, 25, 37). The results indicated that particles with the typical morphology and sedimentation characteristics of C-type virus were released from actinomycin D-treated cells for periods up to at least 6 h. However, particles harvested after incubation for approximately 1 h were deficient in 60 to 70S RNA. A preliminary account of these findings has appeared (J. G. Levin et al., Fed. Proc. 33:1603, 1974).

MATERIALS AND METHODS

Materials. Actinomycin D was a gift from Merck, Sharp and Dohme Research Laboratories (Rahway, N.J.). dATP, dGTP, dCTP, and dithiothreitol were purchased from Calbiochem (Los Angeles, Calif.). [methyl-³H]TTP and *Escherichia coli* tRNA were obtained from Schwarz/Mann (Orangeburg, N.Y.).

¹ Present address: Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. 12201.

[5, 6-³H]uridine (40 to 50 Ci/mmol) and Triton X-100 were purchased from New England Nuclear Corp. (Boston, Mass.). Calf thymus DNA was purchased from Miles Laboratories, Inc. (Elkhart, Ind.), and poly rA-oligo dT₍₁₂₋₁₈₎ was obtained from Collaborative Research (Waltham, Mass.). Bovine pancreatic RNase A and DNase I were received from Worthington Biochemical Corp. (Freehold, N.J.). Trypsin-EDTA was obtained from Gray Industries, Inc. (Fort Lauderdale, Fla.).

Cell lines. JLS-V9 cells, chronically infected with Rauscher leukemia virus (45), were generously provided by Nelson Wivel, National Cancer Institute. A clone of AKR mouse embryo cells, initially leukemia virus-free (34), was the generous gift of Natalie Teich and Wallace Rowe, National Institute of Allergy and Infectious Diseases. Rowe et al. (34) have described conditions that lead to spontaneous activation of the virus-free AKR clones. In our experience, thawing of the frozen stock resulted in activation of C-type virus production. The JLS-V9 cells had a complement-fixation titer of 1:128 in a test (23) which detects the major MuLV group-specific antigen. When the AKR cell line was "virus-free," the test for virus antigen was negative (34); after spontaneous activation, the CF titer was 1:32. Procedures for propagating these cell lines have been described (27).

Concentration of extracellular virus from culture fluids. Subconfluent monolayers on 100-mm Falcon plastic petri plates (eight plates per set) were washed five times with 5-ml portions of medium lacking serum or as described below. Each plate then received 5 ml of medium containing 10% calf serum (JLS-V9) or 2.5% fetal bovine serum (AKR) and actinomycin D, as indicated. Incubation was carried out at 37°C for the times specified in the text. In the experiments with AKR cells, the cells were incubated for 1 to 2 min at room temperature in a solution containing 0.25% trypsin and 0.1% EDTA in order to remove any cell-associated virus (17) and were then washed five times, twice with 5-ml portions of McCoy medium containing 10% serum and three times with medium lacking serum. Control experiments showed that the amount of reverse transcriptase activity detected in the fluids was not appreciably affected by the additional treatment with trypsin-EDTA.

Cellular debris was removed from the culture fluids by centrifugation at $1,000 \times g$ for 20 min. The supernatants were stored at -70°C. After thawing, the fluids were again centrifuged at $1,000 \times g$ for 20 min and were then sedimented at $7,000 \times g$ for 45 min in a Sorvall SS-34 rotor. Virus was concentrated by sedimentation of the final supernatant for 110 min at $96,000 \times g$ in a Spinco SW27 rotor. The pellet was suspended in 1/80 of the original supernatant volume in 10 mM Tris buffer (pH 7.4) containing 50 mM KCl and 1 mM dithiothreitol. Small portions (0.1 to 0.2 ml) were stored in a liquid nitrogen freezer. Portions (10 μ liters) were used for assay of reverse transcriptase activity (see below).

Procedures for banding leukemia virus. Fluids from control or actinomycin D-treated cells (20 plates per set) were subjected to low- and intermediate-speed centrifugation, as described above. Virus was

precipitated from the final supernatant by adding an equal volume of saturated ammonium sulfate in TNE buffer (10 mM Tris [pH 7.4], 100 mM NaCl, and 1 mM EDTA). The pellet was resuspended in 3.5 ml of TNE buffer and layered on a linear 15 to 60% (wt/vol) sucrose gradient made up in the same buffer. Sedimentation was carried out for 16 h at $96,000 \times g$ in a Spinco SW27.1 rotor. One-milliliter fractions were collected. Portions (10 μ liters) were removed for assay of reverse transcriptase activity (see below). The density of the fractions was determined from the refractive index. The peak fractions from each gradient were pooled and resedimented at $96,000 \times g$ for 75 min. The pellets were suspended in 0.5 ml of TNE buffer containing 1 mM dithiothreitol.

Extraction of leukemia virus RNA from banded virions. The peak gradient fractions were pooled and extracted at room temperature with sodium dodecyl sulfate (1% final concentration), 1 volume of buffer-saturated phenol, and one volume of chloroform-isoamyl alcohol (24:1, vol/vol). The aqueous layer was reextracted with phenol and chloroform-isoamyl alcohol, and RNA was precipitated from the final aqueous layer with 2 volumes of absolute alcohol and carrier tRNA at a final concentration of 100 μ g/ml. The RNA was stored as an alcoholic suspension at -20°C, and portions were removed as needed.

Analysis of leukemia virus RNA by polyacrylamide gel electrophoresis. Electrophoresis was carried out on composite 1.8% polyacrylamide-0.5% agarose gels as described previously (26), with the following modifications. The gel buffer and electrophoresis buffer contained sodium acetate at final concentrations of 20 and 2 mM, respectively; and the runs were performed at a constant voltage (3 mA/gel) for 1.5 h with cold water circulating through the lower chamber of the electrophoresis apparatus. The gels were sliced and processed as described previously (26).

Assay of reverse transcriptase activity. Samples were assayed in 0.1-ml reactions essentially according to Parks et al. (30). Endogenous reactions contained 40 mM Tris (pH 8.0), 60 mM KCl, 2 mM dithiothreitol, 6 mM MgCl₂, 0.014% (vol/vol) Triton X-100, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dATP, 2×10^{-2} mM [³H]TTP (3,500 to 5,000 counts per min per pmol), and, where indicated, 5 μ g of "activated" calf thymus DNA (33). Reactions which measured the response to a synthetic RNA template contained Tris, KCl, dithiothreitol, and [³H]TTP as specified above; 0.2 mM manganese acetate; 0.1% (vol/vol) Triton X-100; and, where indicated, 0.02 absorbancy (A_{260}) unit of poly rA-oligo dT₍₁₂₋₁₈₎. After incubation for 1 h at 37°C, reactions were terminated by the addition of 2 ml of ice-cold 5% trichloroacetic acid containing 0.125 M sodium pyrophosphate. After 5 to 10 min at 4°C, the precipitates were collected on type HA membrane filters (0.45 μ m, 25-mm diameter; Millipore Corp.) and washed seven times with ice-cold 5% trichloroacetic acid. The filters were counted in 10 ml of a toluene-Liquifluor cocktail, and radioactivity was determined in a Packard liquid scintillation spectrometer.

To determine whether endogenous activity was

sensitive to RNase, reactions without [^3H]TTP were preincubated for 30 min at 25 C in the presence or absence of 5 μg of RNase. [^3H]TTP was then added and the complete reactions were incubated for 1 h at 37 C. The RNase was heated at 80 C for 10 min to inactivate any contaminating DNase.

Electron microscopy. Cell monolayers were detached from plastic petri dishes with a rubber policeman, and pellets were formed by sedimentation at $780 \times g$. After 12 to 48 h of fixation in cold 3% glutaraldehyde buffered with Sorensen phosphate (pH 7.4), the cells were washed in buffer overnight and treated with 1% OsO_4 for 1 h. Samples were embedded in epoxy resin, sectioned with an ultratome, and prepared for examination in a Hitachi HU 11E microscope as described previously (22). The ultrathin sections were stained with uranyl acetate and lead citrate.

For negative staining, single drops of fluid sample were placed on Formvar-coated copper mesh grids by means of a no. 25 needle. After 1 to 2 min, the drop was partially blotted with coarse filter paper, and a drop of 1% phosphotungstic acid (pH 5.2) was added and almost immediately blotted. The grid was then allowed to dry. The phosphotungstic acid solution was prepared as described by Huxley and Zubay (24).

RESULTS

Morphogenesis of C-type particles in murine cells treated with actinomycin D. Morphogenesis of C-type particles was studied initially in JLS-V9 cells chronically infected with Rauscher leukemia virus (45). At concentrations of actinomycin D up to 5 $\mu\text{g}/\text{ml}$, C-type particles were observed in the process of budding from cell surfaces for as long as 12 h after addition of the drug. During this period, the numbers of budding particles (30 to 90 per 100 cell sections) did not vary significantly from those found in controls. The immature budding particles which were detected in the presence of actinomycin D had an average diameter of 110 nm (Fig. 1). They consisted of an electron-lucent core surrounded by an intermediate ring and limiting envelope and could not be distinguished from particles formed under normal conditions. Mature extracellular virus was abundant and also appeared typical in morphology.

The ultrastructural observations with Rauscher leukemia virus-infected JLS-V9 cells were confirmed in similar experiments with endogenously infected, spontaneously activated AKR cells treated with actinomycin D (Fig. 2). In addition, these cells were pretreated with trypsin-EDTA (17) to eliminate the possibility that particles persisted from a time prior to addition to actinomycin D. Figures 1 and 2 illustrate that many of the particles were at-

tached to the cell surface merely by slender stalks and thus were obviously in the process of being released. Control experiments carried out when the AKR cells were still "virus-free" (see above) showed no microscopically detectable C-type virus for up to 24 h in the presence of actinomycin D.

Appearance of reverse transcriptase activity in extracellular fluids of cells treated with actinomycin D. Actinomycin D strongly inhibits cellular and viral RNA synthesis under the experimental conditions employed (2, 7, 14, 40). When AKR or JLS-V9 cells were labeled with [^3H]uridine in the presence of 1 μg of actinomycin D per ml, incorporation of radioactivity into virions banded in sucrose density gradients was reduced by 90%, and no newly formed 70S RNA could be detected. Cellular RNA synthesis was inhibited by 95% within 1 h after addition of actinomycin D at a concentration of 1 or 2 $\mu\text{g}/\text{ml}$. Thus, the electron microscopic evidence that C-type particles with a normal ultrastructural appearance were released from these actinomycin D-treated cells was of special interest and stimulated further investigation.

Since, in several other systems, production of C-type virus has been correlated with the presence of reverse transcriptase activity (4, 41) in culture fluids (1, 25, 37), measurement of this enzyme activity was selected as a probe for MuLV production in the present studies. Fluids were assayed for their response to poly rA-oligo dT₍₁₂₋₁₈₎ (5), a template which has been shown to be efficiently copied by viral reverse transcriptase (5, 21, 32, 33) and also for activity in the presence of "activated" DNA (calf thymus DNA briefly treated with DNase) (33). The assay was always performed in the linear range of enzyme concentration. All of the samples tested exhibited high levels of reverse transcriptase activity and the striking preference for the poly rA-oligo dT₍₁₂₋₁₈₎ template (Table 1) characteristic of viral reverse transcriptase enzymes (5, 21, 32, 33). In control experiments with non-producer AKR cells, the fluids gave virtually no response to poly rA-oligo dT₍₁₂₋₁₈₎ (3.7 pmol of [^3H]TMP incorporated per 10 μliters of concentrated fluids), whereas the response to "activated" DNA was somewhat greater (6.0 pmol incorporated).

Treatment of AKR cells for 6 h with 1 μg of actinomycin D per ml resulted in only a small reduction in the response to poly rA-oligo dT₍₁₂₋₁₈₎ (Table 1). Similar results were obtained with fluids from JLS-V9 cells, even when the concentration of actinomycin D in the medium was

FIG. 1. C-type particles budding from the surfaces of JLS-V9 cells chronically infected with Rauscher leukemia virus, 6 h after treatment with actinomycin D ($5 \mu\text{g/ml}$). (A) Immature budding particles; (B) mature extracellular and immature budding particles. $\times 60,000$.

FIG. 2. Budding of immature C-type particles from spontaneously activated AKR cells 6 h after addition of actinomycin D ($1 \mu\text{g/ml}$). Cells were washed with trypsin-EDTA prior to the beginning of the experiment, and zero time controls showed no budding particles. $\times 40,000$. (a) Higher magnification of budding C-type particle showing two inner shells and outer membrane. $\times 127,000$.

maintained at 5 $\mu\text{g/ml}$. Since the final wash fluids (zero time incubation) consistently showed little or no reverse transcriptase activity, the activity present at 6 h was attributed to virus particles released during the incubation period.

To determine whether the virus particles were released continuously throughout the incubation, the kinetics of appearance of reverse transcriptase activity was investigated. The results of one experiment with JLS-V9 cells are shown in Table 2. Samples were taken at 2- or 4-h intervals and were assayed by measuring the response to poly rA-oligo dT₍₁₂₋₁₈₎. The data were consistent with a constant rate of virus production up to 8 h. Between 8 and 12 h, cytotoxic effects of the drug began to appear

and, although virus particles were still produced, there was evidently a decline in the rate of release. At each of the time intervals tested, numerous budding C-type particles as described above (Fig. 1 and 2) were seen in thin sections examined by electron microscopy.

Since electron microscopy and the data of Tables 1 and 2 suggested that actinomycin D had little effect on production of the C-type particles, it was of interest to determine whether these particles had biological activity. The infectivity tests were graciously performed by Janet Hartley and Wallace Rowe using the XC plaque assay (35). The titers of samples collected after 6 h of incubation with actinomycin D were 50- to 60-fold lower than that of comparable controls, e.g., control, $10^{5.6}$ PFU/ml; actinomycin D, $10^{3.8}$ PFU/ml. This finding suggests that defective particles were present in the virus yield and agrees with previous reports demonstrating a loss in focus-forming activity after actinomycin D treatment of Rous sarcoma virus-infected chicken cells (2, 39, 44). Further characterization of MuLV particles released in the presence of actinomycin D is presented below.

Characterization of particles released from actinomycin D-treated cells. To confirm that the reverse transcriptase activity found in fluids of actinomycin D-treated cells was associated with C-type virus particles, the extracellular fluids were banded in equilibrium sucrose density gradients. Fractions were assayed for their response to poly rA-oligo dT₍₁₂₋₁₈₎ and were also examined by electron microscopy. The distribution of reverse transcriptase activity in the control and actinomycin D gradients was strikingly similar (Fig. 3). In both cases, most of the activity sedimented as a large peak with a buoyant density characteristic of the RNA tumor viruses (1.15 to 1.17 g/cm³) and, in accord with the data of Table 1, there were few quantitative differences. In addition, a minor peak of activity was consistently observed in the low-density fractions. When the material present in the major peak of each gradient was examined by a negative-staining technique (24), numerous C-type particles were detected. However, no intact virus particles could be seen in the fractions constituting the minor peak.

Further analysis of the material sedimenting in the major peak of each gradient was carried out after pooling the fractions and concentrating them by high-speed centrifugation. The resedimented material was negatively stained and reexamined in the electron microscope (Fig. 4). Numerous particles with the characteristic morphology of C-type virus (28, 46) were ob-

TABLE 1. Reverse transcriptase activity in extracellular fluids of AKR and JLS-V9 cells incubated for 6 h with actinomycin D

Cell line	Actinomycin D ($\mu\text{g/ml}$)	[³ H]TMP incorporated ^a (pmol)	
		+ Poly rA-oligo dT ₍₁₂₋₁₈₎ ^b	+ "Activated" DNA
AKR	None	753.6	12.9
	1.0	518.6	20.3
JLS-V9	None	686.2	27.0
	0.1	743.7	30.3
	1.0	554.9	50.3
	5.0	455.9	37.3

^a Ten-microliter portions of the concentrated fluids were assayed as described. The assay was performed in the linear range of enzyme concentration.

^b Incorporation in the absence of added template (0.1 to 0.2 pmol) was subtracted from each value.

TABLE 2. Kinetics of appearance of reverse transcriptase activity in fluids of JLS-V9 cells treated with actinomycin D^a

Time interval ^b (h)	[³ H]TMP incorporated ^c (pmol)
0-2	97.4
2-4	97.4
4-6	124.1
6-8	113.6
8-12	82.3

^a The concentration of actinomycin D in the growth medium was 1 $\mu\text{g/ml}$.

^b Fluids were collected at the indicated times, and prior to each addition of fresh medium the cells were washed five times.

^c Assays were performed in the presence of poly rA-oligo dT₍₁₂₋₁₈₎.

FIG. 3. Reverse transcriptase activity of banded virus from control and actinomycin D-treated AKR cells. Subconfluent monolayers of AKR cells were washed with trypsin-EDTA and were then incubated for 6 h with or without actinomycin D (1 μ g/ml). The extracellular fluids were collected and banded. Ten-microliter portions from each fraction were assayed for activity in response to poly rA $\cdot$ oligo dT₍₁₂₋₁₈₎ as described, except that the incubation was for 30 min at 37 C. The density of the fractions was determined from the refractive index. (A) Control; (B) actinomycin D. Symbols: $\bullet$, [3 H]TMP incorporated (picomoles); $\blacksquare$, density (grams per cubic centimeter).

served, and again there were no differences in the appearance of virus originating from control or actinomycin D-treated cells. Contamination with cellular material appeared minimal in both samples. On the basis of control experiments with serial dilutions of Rauscher leukemia virus of known particle concentration, it appeared that the control and actinomycin D samples contained comparable numbers of particles, at least within a 10-fold range.

The resedimented virus was also assayed for reverse transcriptase activity. Both the control and actinomycin D samples exhibited a marked preference for poly rA $\cdot$ oligo dT₍₁₂₋₁₈₎ compared with "activated" DNA (the response being 70- to 80-fold greater with the synthetic template). However, when endogenous activity was assayed, the activity of the actinomycin D sample was fivefold lower than that of the control (Table 3). This was true regardless of whether gradient-purified material (Table 3, line 1) or concentrated fluids (Table 3, line 3) was used as the source of virus. Table 3 also shows that the endogenous activities of both the control and actinomycin D samples were sensitive to RNase and, in the case of the gradient-purified material (compare lines 1 and 2), the reduction in activity was 90% (control sample) and 70% (actinomycin D sample), respectively.

Characterization of RNA. The data presented above indicate that particles released

from actinomycin D-treated cells have several characteristics usually associated with C-type virus, i.e., typical ultrastructure, buoyant density of 1.16 g/cm³ in equilibrium sucrose gradients, and high levels of reverse transcriptase activity in response to the exogenous template poly rA $\cdot$ oligo dT₍₁₂₋₁₈₎. The low level of endogenous activity detected in fluids or in banded particles (Table 3) is consistent with the previously noted loss in infectivity and provides evidence for a defect or deficiency in the RNA content of the particles.

To determine whether a high-molecular-weight RNA template was directing the endogenous activity of the actinomycin D sample, the product formed in the endogenous reaction (3 H-labeled DNA covalently linked to unlabeled RNA) was extracted and sedimented in a glycerol gradient by the method of Schlom and Spiegelman (36). A small proportion of the radioactivity was detected in the 60S region of the gradient (not shown), indicating that some high-molecular-weight RNA (60 to 70S) accumulated during the 6-h treatment with actinomycin D.

This result led us to consider that the 60 to 70S RNA could either be distributed evenly in small amounts in all of the particles or, alternatively, be present mainly in those particles assembled and released early in the incubation. To resolve this point, the kinetics of appearance of the 60 to 70S RNA was investigated. Cells were labeled with [3 H]uridine and then incubated in the presence or absence of actinomycin D. Fluids were collected at hourly intervals, and the RNA extracted from banded virions (density 1.15 to 1.17 g/cm³) was analyzed by polyacrylamide gel electrophoresis (Fig. 5). After only 1 h of treatment with actinomycin D (Fig. 5A), there was a considerable amount of 60 to 70S RNA and two distinct species of low-molecular-weight RNA, one with an estimated molecular weight of 1.1×10^5 (8S RNA), and the other with the mobility of marker 4S RNA. A similar pattern was obtained with the control sample. At 2 h traces of high-molecular-weight RNA were detected (not shown), but by 3 h after the addition of actinomycin D (Fig. 5B) only 4S RNA and small amounts of 8S RNA were observed. The relative amount of 4S RNA was almost double that found in control samples or in samples collected within the first hour of incubation with the drug (Fig. 5A). The small amounts of 28 and 18S RNA present at 3 h (Fig. 5B) were also observed in the 3-h control sample and probably represent some contamination with cellular material which is not removed by one cycle of gradient purification.

FIG. 4. Negatively stained C-type particles from pooled and resedimented fractions 5, 6, and 7 of the gradients shown in Fig. 3. (A) Control; (B) actinomycin D. In both samples, the majority of the particles are densely stained with phosphotungstic acid and consist of partially disrupted circular and hexagonal heads from 90 to 130 nm with tails of varying lengths. $\times 92,500$.

DISCUSSION

The present study demonstrates that, in cells chronically infected with MuLV, particles with the morphological (Fig. 1, 2, and 4) and biophysical (Fig. 3) characteristics of C-type virus continue to be released for at least 6 h in the presence of high concentrations of actinomycin D. The data are in accord with previous evidence for intracellular pools of virus-specific

TABLE 3. *Endogenous activity of AKR leukemia virus*

Source of virus	Reaction	[³ H]TMP incorporated (pmol)	
		Control	Actinomycin D ^a
Gradient fractions ^b	Complete	1.34	0.27
	Plus RNase	0.13	0.096
Concentrated fluids ^c	Complete	3.50	0.67
	Plus RNase	1.05	0.32

^a The concentration of actinomycin D in the growth medium was 1 µg/ml.

^b Fractions 5, 6, and 7 of the gradients shown in Fig. 3 were combined and resedimented as described, and 50-µl portions of the concentrated virus were assayed.

^c Forty-microliter portions of concentrated fluids from AKR cells incubated for 6 h in the presence or absence of actinomycin D.

macromolecules (3, 6) and suggest that these molecules can be utilized for terminal steps in assembly under conditions of severely restricted viral RNA synthesis.

At least two classes of MuLV particles with the same buoyant density appear to be present in the virus yield obtained after 6 h of incubation with actinomycin D: (i) standard C-type virions with 60 to 70S RNA, and (ii) defective particles deficient in 60 to 70S RNA but possessing other biochemical characteristics as well as the ultrastructural morphology usually associated with MuLV. On the basis of the kinetic data presented in Fig. 5, it seems likely that the standard particles are released during the first hour or so after addition of actinomycin D (3, 6), whereas after 1 or 2 h defective particles undoubtedly represent the major group in the virus population. The relatively high proportion of defective particles in a 6-h harvest would account for the low levels of infectivity and RNase-sensitive endogenous activity (Table 3) which are observed. The apparent disproportion in the residual levels of infectivity and endogenous activity may reflect differences in the sensitivities of the two assay procedures.

In accord with our present conclusions, we have found in recent experiments that particles released between 2 and 6 h of incubation with actinomycin D are indistinguishable from standard MuLV with respect to size and ultrastruc-

FIG. 5. Polyacrylamide gel electrophoresis of ³H-labeled RNA from AKR virions released 1 and 3 h after the addition of actinomycin D to the growth medium. Subconfluent monolayers of AKR cells were incubated for 1 h with 3 ml of medium containing 150 µCi of [³H]uridine per ml. Unlabeled medium was then added, and incubation was continued for another hour, at which time all virions released should have been labeled (3). The cells were washed with trypsin-EDTA and then divided into two sets (10 plates per set). One set received medium alone and the other set received medium containing 1 µg of actinomycin D per ml. Incubation was continued and the fluids were collected at hourly intervals. Virus particles were concentrated and banded in sucrose gradients, and RNA was extracted from the fractions sedimenting with a density of 1.15 to 1.17 g/cm³. Samples (50 µliters) were analyzed by polyacrylamide gel electrophoresis. Parallel gels were run with marker 28, 18, and 4S RNA from chicken embryo cells. Data are for RNA from samples collected 1 h (A) or 3 h (B) after the addition of actinomycin D.

tural morphology, buoyant density in sucrose gradients, and relative response to exogenous templates in the reverse transcriptase assay. Endogenous activity is extremely low or absent; infectivity has not yet been determined.

Interestingly, there is a biological precedent for the class of defective C-type particles which accumulate during treatment with actinomycin D. Certain clones of murine sarcoma-positive, leukemia-negative transformed 3T3 cells produce noninfectious virions which have a buoyant density of 1.16 g/cm³ in sucrose gradients and are identical in size and morphology to standard C-type virus (8), despite the fact that they are missing 60 to 70S RNA (31). Greater than 80% of the RNA contained in particles from one of the clones (C243) has a sedimentation value of 18S or less; 17% is 28S RNA, of which only 4% appears to have sequences capable of hybridizing with a DNA reverse transcript of 60 to 70S MuLV RNA (31). Thus, a large proportion of the virion RNA may be derived from the host.

As might be expected, the C243 particles have no endogenous reverse transcriptase activity; however, they do respond to the exogenous template poly rA·oligo dT₍₁₂₋₁₈₎ (B. I. Gerwin, personal communication). Similarly, immature Rous sarcoma virus particles, which contain mostly 35 (11) or 55S (13) RNA, have little (11) or no (13) endogenous reverse transcriptase activity but are capable of responding to an exogenous template such as poly rA·oligo dT₍₁₂₋₁₈₎ (11). The dissociation of endogenous and exogenous enzyme activities seen in these systems, as well as in our own experiments indicates that packaging of the reverse transcriptase enzyme within a C-type virion does not depend upon the presence of the 60 to 70S RNA template found in mature particles.

The finding that 4S RNA is the major RNA species present in banded fluids after 1 or 2 h of incubation with actinomycin D (Fig. 5) was also of interest. Most of the 4S RNA molecules normally found in C-type virions have the same characteristics as host cell tRNA, including similar nucleotide composition (16, 29) and amino acid acceptor activity (9, 12, 15, 42). Although the role of these RNA molecules in viral replication is still largely unknown, it has been suggested that some of the 4S RNA species could function as primers for the viral reverse transcriptase enzyme (10, 18, 43) or as linkers of subunit RNA in the 60 to 70S viral RNA complex (10). In addition, hybridization data indicate that the low-molecular-weight RNAs contained in Rous sarcoma virus have se-

quences homologous to the single-stranded DNA product synthesized by virions (20). Whether the low-molecular-weight RNA released from actinomycin D-treated cells has any virus-specific sequences or is comprised entirely of host cell RNA which is encapsidated during the budding process remains to be determined. Further information on this point may contribute to our understanding of the specificity required for the interaction between viral proteins and RNA species during the assembly of C-type particles at the cell surface.

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