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Virus-Specific Messenger RNA on Free and Membrane-Bound Polyribosomes from Cells Infected with Rauscher Leukemia Virus

(hybridization/complementary DNA)

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ABSTRACT **Cells infected by Rauscher leukemia virus synthesize virus-specific RNA which can be detected by hybridization to the single-stranded DNA copy of the viral RNA. Evidence is provided that virus-specific RNA is present in free and membrane-bound polyribosomes of these cells. The relative content of virus-specific RNA, as measured by hybridization, is 6–10 times less on free polyribosomes than on membrane-bound polyribosomes. The messenger RNA associated with both classes of polyribosomes was characterized by density gradient centrifugation. In addition to a major RNA species identified as 36S RNA, at least 2 minor components in the 14S and 21S region have also been found. There is a striking difference in the distribution of these RNA species between free and membrane-bound polyribosomes.**

In cells infected with oncornaviruses, virus-specific RNA sequences could be detected by hybridization of cellular RNA with the single-stranded [^{3}H]DNA copy (cDNA) of the viral RNA (1–5). No indications have been found that in these cells RNA species complementary to viral RNA are present (4). This leads to the conclusion that the virus-specific mRNA molecules present on polyribosomes of oncornavirus-infected cells are complementary to the cDNA. The present paper describes the finding of such viral-specific RNA sequences on free and membrane-bound polyribosomes of Rauscher virus-infected cells. The virus-specific RNA associated with the polyribosomes can be resolved in different size classes. The relevance of these results for viral-specific protein synthesis is discussed.

MATERIALS AND METHODS

Materials. [^{3}H]dGTP (specific activity 13.4 Ci/mmol) and [^{3}H]dCTP (specific activity 7.6 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, England. *Aspergillus oryzae* S1 nuclease was purified from amylase type IV-A (Sigma Chemical Co.) by chromatography on a DEAE–cellulose column (6).

Cells and Virus. The JLS-V9 cell line, derived from bone marrow cells of BALB/c mice (7), infected with and producing Rauscher leukemia virus (R-MuLV), was grown in roller bottles in Eagle's minimal essential medium completed with 10% calf serum.

Abbreviations: R-MuLV, Rauscher leukemia virus; cDNA, synthetic single-stranded DNA copy of the viral RNA; TMK buffer, 50 mM Tris·HCl, pH 7.8–80 mM KCl–5 mM $Mg(OAc)_2$; TNE buffer, 10 mM Tris·HCl, pH 7.4–100 mM NaCl–1 mM EDTA.

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Cell Fractionation and Isolation of Polyribosomes. In order to obtain a maximal yield of polyribosomes, fresh growth medium and cycloheximide (0.1 μg/ml) were added 180 and 20 min, respectively, before the cells were harvested (8–10). After trypsinization, the cells were quickly chilled to 0° and washed with isotonic medium (35 mM Tris·HCl, pH 7.5–146 mM NaCl). The cells were suspended in hypotonic medium containing 10 mM Tris-HCl, pH 7.4–15 mM KCl–1.5 mM magnesium acetate–2 mM dithiothreitol, and after 15 min of swelling, were disrupted by 20 strokes of a Dounce homogenizer (B pestle). The broken cells were centrifuged at 700 × *g* for 10 min to remove nuclei and cell debris. All media used in further operation steps contained 100 μg/ml of an RNase inhibitor from bovine eye-lens purified on DEAE-cellulose (kindly provided by Mr. W. van de Broek of our laboratory). A flotation method was used for the isolation of membrane-bound polyribosomes. The postnuclear supernatant was made 2.2 M in sucrose by the addition of 90% (w/v) sucrose and transferred to tubes of the SW 27 rotor of the Beckman preparative centrifuge. Eight milliliters of 1.8 M sucrose in TMK buffer (50 mM Tris·HCl, pH 7.8–80 mM KCl–5 mM magnesium acetate) and 8 ml of 1 M sucrose in TMK buffer were layered on top of the 2.2 M sucrose cushion. Centrifugation was performed at 25,000 rpm for 180 min. The membrane layer on top of the 1.8 M sucrose was removed and solubilized by addition of Nonidet P-40–deoxycholate (1:1) to a final concentration of 0.5%. The released polyribosomes were further purified by centrifugation through a layer of 4 ml of 2 M sucrose in TMK buffer. Centrifugation was at 25,000 rpm for 18–24 hr in a SW 27 rotor. For the isolation of free polyribosomes, the postnuclear supernatant was recentrifuged at 13,000 g for 5 min. The supernatant fraction was layered on a discontinuous sucrose gradient consisting of 8 ml of 1 M sucrose in TMK buffer on top of 4 ml of 2 M sucrose in TMK buffer. Centrifugation was at 25,000 rpm for 18–24 hr. A total polyribosomal extract was isolated essentially as described (9). Cells were lysed in the presence of 2% Nonidet P-40 and high salt (50 mM Tris·HCl, pH 8.5–225 mM KCl–8 mM magnesium acetate–2 mM dithiothreitol) by 10 strokes with a Dounce homogenizer. Further isolation steps were performed as described for free polyribosomes.

Polyribosomal pellets were resuspended in TMK buffer, layered on 20–30.7% (w/v) isokinetic sucrose gradients in TMK buffer and centrifuged at 5° in a SB 283 rotor of an IEC centrifuge at 41,000 rpm for 60 min. To each gradient fraction, 20 μg of *Escherichia coli* tRNA was added as carrier and the polyribosomes were precipitated with ethanol. Mes-

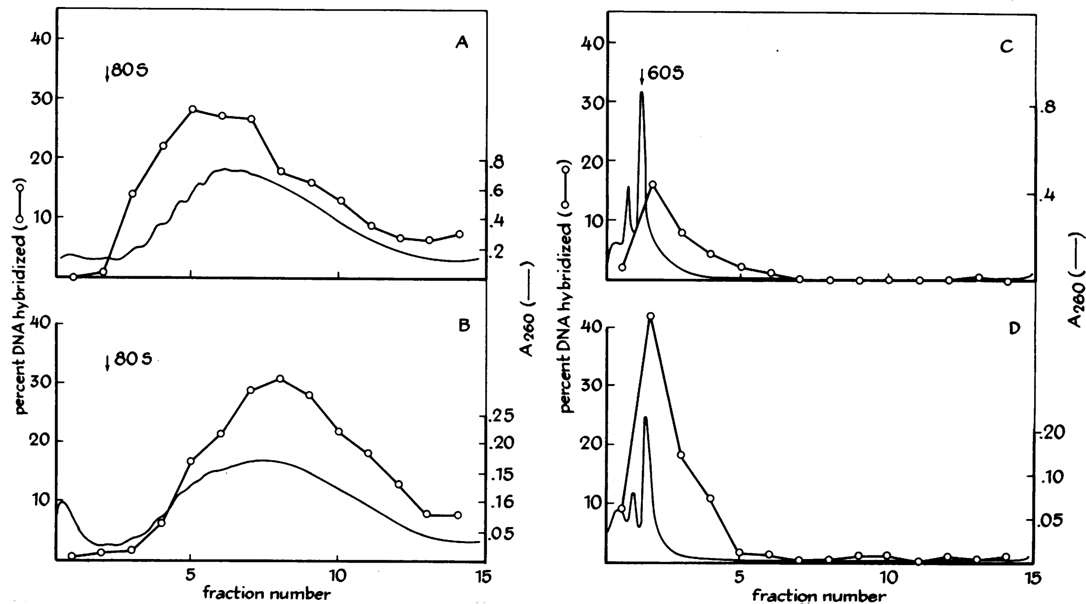

FIG. 1. Virus-specific RNA in free and membrane-bound polyribosomes of R-MuLV infected cells. Free and membrane-bound polyribosomes and ribonucleoprotein particles released by EDTA treatment of these polyribosomes were analyzed in 20–30.7% (w/v) isokinetic sucrose gradients. Fractions of 0.8 ml were collected, and the polyribosomes were precipitated with ethanol. After extraction of the RNA, aliquots of each fraction were hybridized with R-MuLV cDNA. (*A*) Free polyribosomes; (*B*) membrane-bound polyribosomes; (*C*) free polyribosomes dissociated by EDTA; (*D*) membrane-bound polyribosomes dissociated by EDTA.

senger ribonucleoprotein was released from polyribosomes, resuspended in TMK buffer, by the addition of 15 mM EDTA and analyzed in isokinetic sucrose gradients mentioned above but without magnesium.

Extraction and Analysis of Polyribosomal RNA. Polyribosomal RNA was extracted with phenol–chloroform as described (11). The extracted RNA was solubilized in TNE buffer (10 mM Tris·HCl pH 7.4–100 mM NaCl–1 mM EDTA) and 0.5% sodium dodecyl sulfate, layered on 15–30.2% (w/v) isokinetic glycerol gradients in TNE buffers and centrifuged at 5° in a SB 283 rotor at 41,000 rpm for 9 hr.

Synthesis of Virus-Specific [^{3}H]DNA. The synthesis of DNA *in vitro* with R-MuLV treated with Nonidet P-40, in the presence of 100 μg of actinomycin D was essentially as described (12). [^{3}H] dGTP (13.4 Ci/mmole) and [^{3}H] dCTP (7.6 Ci/mmole) were used at a concentration of 7.5 μM. cDNA was purified as described (12). The specific activity of the cDNA was 7500 cpm per ng.

Hybridization. Polyribosomal RNA was annealed with cDNA (1000 cpm) in 10-μl capillary tubes in a medium consisting of 0.2 M phosphate buffer, pH 7.0, and 0.5% sodium dodecyl sulfate at 65° (13).

The extent of hybridization was measured by digestion with *Aspergillus oryzae* S1 nuclease in 1 ml of digestion buffer (1 mM $ZnSO_4$, 100 mM sodium acetate, pH 4.5, and 10 μg/ml of denatured calf-thymus DNA) for 30 min at 45° (13). Acid-precipitable radioactivity was collected on Millipore filters and counted in a Packard Tri-carb Liquid Scintillation Spectrometer.

RESULTS

Virus-Specific RNA in Free and Membrane-Bound Polyribosomes of Cells Infected with R-MuLV. For our studies, the availability of pure fractions of both free and membrane-bound polyribosomes was a prerequisite. Contamination of free polyribosomes with membrane-bound polyribosomes is prevented by centrifugation of a 13,000 × g supernatant of the cell lysate through a 2 M sucrose layer. For the isolation of membrane-bound polyribosomes, a flotation technique was chosen to eliminate any contamination with free polyribosomes. Vecchio *et al.* (14) have shown that contamination of the polyribosomes larger than 150 S with viral core particles can be excluded. The absorbance profiles of both classes of ribosomes after centrifugation in isokinetic sucrose gradients are shown in Fig. 1, *A* and *B*. From these gradients, fractions were collected and the RNA was extracted. The content of virus-specific RNA in each fraction was determined by hybridization with cDNA. In order to obtain the same extent of hybridization with RNA from free polyribosomes, as much as six times the amount of membrane-bound polyribosomal RNA had to be used. This result indicates that a considerably higher amount of virus-specific mRNA is associated with membrane-bound polyribosomes. Hybridization of cDNA occurs with RNA from free and membrane-bound polyribosomes of all size classes and almost follows the optical profile of the ribosomes. The highest absolute amount of RNA that hybridizes to cDNA is associated with polysomes sedimenting at about 350 S. However, when hybridization is carried out with the same amount of RNA from each polyribosomal fraction, maximal hybridization (the highest relative amount) is found in polyribosomes sedimenting between 450 and 550 S, corresponding to 13–18 monomers per message (not shown).

An analysis similar to that carried out with free and membrane-bound polyribosomes was also performed after EDTA treatment. In this control experiment, no virus-specific RNA is recovered from the original polysomal regions of the gradient, indicating that the virus-specific RNA was indeed associated with polysomes (Fig. 1, *C* and *D*).

Characterization of Virus-Specific mRNA Associated with Polyribosomes. In order to identify the virus-specific RNA species associated with polyribosomes, the extracted RNA was analyzed in isokinetic glycerol gradients and the separate fractions were hybridized with cDNA. A mixture of free and membrane-bound polyribosomes was isolated by cell lysis in the presence of 0.5% Nonidet P-40 and high salt. Fig. 2 *A* illustrates that virus-specific RNA from this total polyribosomal extract is rather heterogeneous in size, with peaks in the 14, 21, and 36S region. The same RNA species are present in the separated free and membrane-bound polyribosomes (Fig. 2, *B* and *C*). However, the relative amounts are different for both classes of polyribosomes.

The presence of similar virus-specific mRNA species on free and membrane-bound polyribosomes allows several explanations: (*1*) Viral mRNA from either type of polyribosomes may code for different polypeptides despite the similarity of the S-values in both populations. (*2*) The same viral polypeptides are synthesized on both types of polyribosome. (*3*) Polypeptide synthesis starts on free polyribosomes. After the nascent chains have been elongated to a certain length, the polyribosomes become membrane-bound. Explanation 1 is unlikely since virus-specific RNA from both classes of ribosomes hybridizes to 100% of the cDNA. In order to get some evidence for one of the other possibilities, RNA from free and membrane-bound polyribosomes of different gradient fractions was isolated and analyzed in glycerol gradients. In all size classes of free polyribosomes the 36S RNA is found in addition to 14S and 21S RNA (Fig. 3, *A*, *B*, and *C*). A quite different picture is obtained from the membrane-bound polyribosomes (Fig. 3, *D*, *E*, and *F*). In these polyribosomes of up to 6 monomers, almost no 36S RNA can be detected. The amount of 36S RNA as compared to 14S and 21S RNA increases with the size class of the polyribosomes. If the different RNA species in membrane-bound polyribosomes are representative for the situation *in vivo*, these results suggest that viral polypeptide synthesis with the 36S message may start on free polyribosomes which, thereafter, become attached to membranes. Our results do not allow any conclusion concerning the mechanism operative for the 14S and 21S RNA species.

The results presented were not significantly affected if the pretreatment of the cells with 0.1 μg/ml of cycloheximide was omitted.

Relative Amount of Virus-Specific mRNA in Polyribosomes. By comparison of the C_rt required to hybridize 50% of cDNA to viral polyribosomal RNA, the relative amounts of virus-specific sequences contained in polyribosomal RNA can be computed (Table 1). The results indicate that about 0.2% of the total polyribosomal RNA is virus-specific. The relative amount of viral-specific RNA in membrane-bound polyribosomes is 5 to 10 times higher than in free polyribosomal RNA.

DISCUSSION

Most cell fractionation procedures described for the isolation of free and membrane-bound polyribosomes (from tissue culture cells) have the disadvantage of giving low yields. A substantial proportion of polyribosomes is removed together with nuclei and cell debris at 750 × *g*. This means that actually no quantitative comparison should be made between free and membrane-bound polyribosomes. From the JLSV-9 cell line, only 5 to 10% of the polyribosomes could be recovered as membrane-bound polyribosomes. An isolation

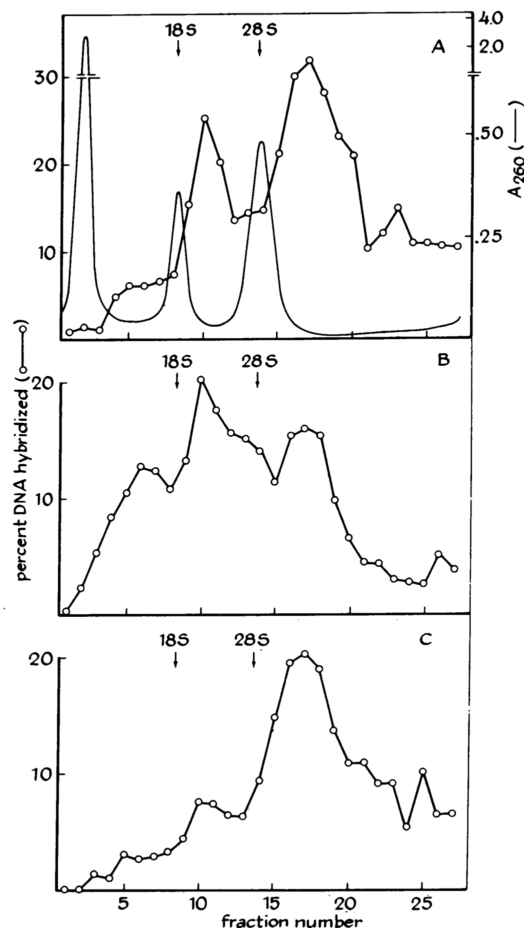

FIG. 2. Virus-specific mRNA associated with polyribosomes. RNA was extracted from free, membrane-bound, and total polyribosomes. The extracted RNA was dissolved in TNE buffer and 0.5% sodium dodecyl sulfate and analyzed on 15–30.2% (w/v) isokinetic glycerol gradients. Fractions of 0.4 ml were collected and precipitated with ethanol after addition of 20 μg of *E. coli* tRNA as carrier. The relative amount of virus-specific RNA was determined by hybridization with R-MuLV cDNA. (*A*) RNA from total polyribosomes; (*B*) RNA from membrane-bound polyribosomes; (*C*) RNA from free polyribosomes.

TABLE 1. *Virus-specific RNA in polyribosomes*

RNA	Half-C_rt (mole-sec/liter)	Virus-specific RNA (%)
60S R-MuLV	1.58×10^{-2}	100
Free polyribosomes	$3.16 \times 10^{1}(3.98 \times 10^{1})$*	0.05 (0.04)*
Membrane-bound polyribosomes	5.63 (3.98)*	0.30 (0.40)*
Total polyribosomes	7.95	0.20

RNA was isolated from free, membrane-bound, and total polyribosomes. Different concentrations of each RNA sample were hybridized with 800 cpm of cDNA for 20 hr. The percentage of DNA hybridized was plotted in a semilogarithmic graph against the product of RNA concentration × time (C_rt). The proportion of virus-specific sequences in the polyribosomal RNA was calculated by comparison of C_rt values at which 50% of the maximal hybridization is obtained with the half-C_rt of the viral RNA.

* RNA isolated from polyribosomes of cells pretreated with 0.1 μg/ml of cycloheximide.

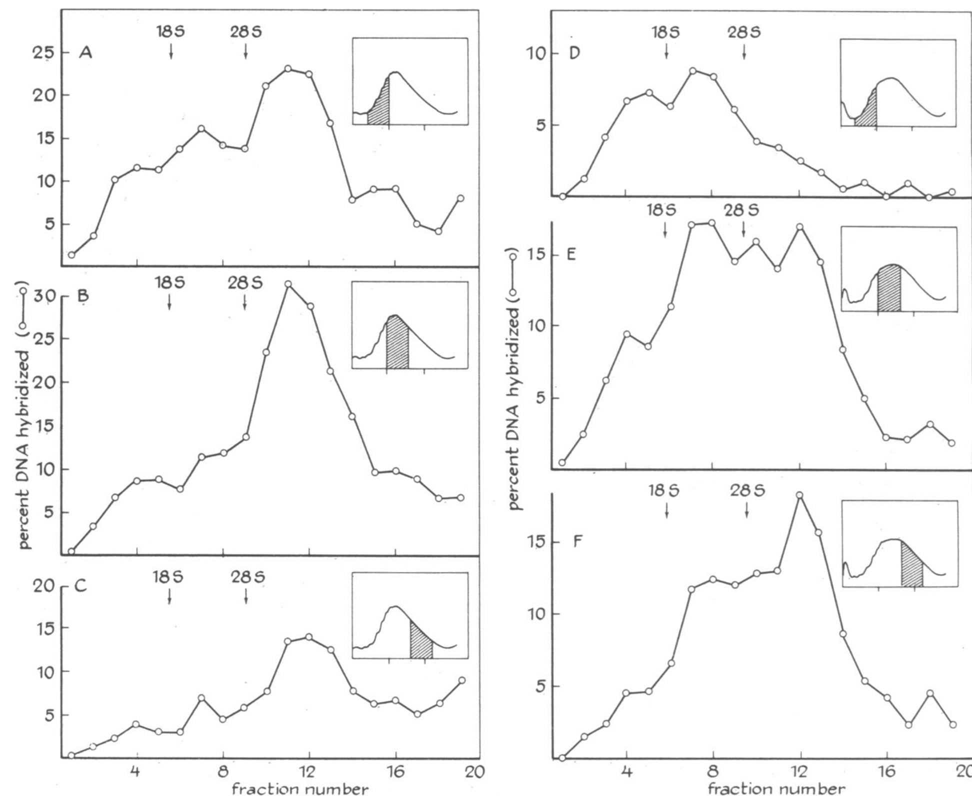

FIG. 3. Virus-specific RNA associated with polyribosomes of different size classes. The sedimentation profiles of isolated free and membrane-bound polyribosomes in 20–30.7% (w/v) isokinetic sucrose gradients are depicted in the *insets*. Fractions from these gradients were pooled as indicated by the *hatched areas*. Polyribosomes were precipitated with ethanol, and the RNA was extracted. The extracted RNA was analyzed in 15–30.2% (w/v) isokinetic glycerol gradients. Fractions of 0.6 ml were collected and precipitated with ethanol after addition of 20 μg of *E. coli* tRNA as carrier. The relative amount of virus-specific RNA in each fraction was determined by hybridization with R-MuLV cDNA.

procedure that may give higher yields has recently been described for the preparation of membrane-bound polyribosomes from rat liver (15). However, the results obtained after cell lysis in the presence of nonionic detergent and high salt indicate that we have isolated a representative sample of membrane-bound polyribosomes.

While this work was in progress, Vecchio *et al.* (14) described the presence of virus-specific mRNA and nascent polypeptides in polyribosomes of cells replicating the Moloney strain of murine sarcoma-leukemia virus. Virus-specific RNA was detected on free and membrane-bound polyribosomes. Polyribosomes bound to membranes contained between 4 and 5 times more virus-specific RNA than free polyribosomes. These data are in agreement with our results. A further analysis in our studies revealed that a rather heterogeneous population of virus-specific RNA molecules is associated with the two types of polyribosomes. Three size classes of RNA are resolved in the glycerol gradients with an estimated molecular weight of about 0.4×10^6 (14S), 1×10^6 (21S) and 3×10^6 (36S). If the 60S RNA species found in intact viral particles would represent a functioning messenger, one might expect to find this species associated with ribosomes. However, no 60S RNA could be detected in any polysomal fraction. The existence of other virus-specific RNA species, especially in the 27S region, that are not resolved in our gradients cannot be excluded.

It remains important to find out whether the RNA population sedimenting slower than 36 S consists of intact virus-specific mRNA or is a degradation product of the 36S mRNA. Three possibilities to explain degradation of the 36S mRNA may be considered. (*1*) The 36S mRNA is degraded during phenol extraction. (*2*) Polyribosomes are broken down by nucleases, during the isolation procedure. (*3*) Degradation of the 36S mRNA associated with functioning polyribosomes occurs *in vivo*.

If the first assumption were true, the extent of degradation should be the same in each polyribosomal fraction. The results presented show that this is not the case (Figs. 2 and 3). If the second or third possibility were correct, one has to assume that functioning polyribosomes are degraded at a limited number of weak points in the 36S mRNA. The assumption that the 14 and 21S RNA species are degradation products obtained by nuclease nicks in the polyribosomal-associated 36S mRNA, would be in contradiction to the following observations. Distinct virus-specific RNA species in the range from 10 to 40 S are found in cells infected with Rous sarcoma virus, even if these cells are disrupted in the presence of phenol–sodium dodecyl sulfate (2). Moreover, immature Rous sarcoma virus-Prague contains, instead of 68S RNA, a rather heterogeneous RNA component with a sedimentation coefficient of 60S (16). In addition, 36S RNA and RNA species sedimenting between 14 and 36S are present. The latter RNAs are virtually absent in mature virus. Our results and the experimental evidence mentioned suggest that RNA molecules between 10 and 30S found in the cytoplasm of cells infected by oncornaviruses may be assembled to a 36S or 60–70S RNA molecule. At least part of the assembly of these RNA molecules may occur at the plasma membrane in virus budding

particles. If the RNA species associated with both classes of polyribosomes are representative for the situation *in vivo*, the presence of virus-specific 36S mRNA in free and membrane-bound polyribosomes, together with the finding that this mRNA is almost absent in membrane-bound polyribosomes up to 230 S, suggests that the virus polypeptide synthesis directed by this mRNA starts on free polyribosomes. As soon as the nascent polypeptide chain reaches a certain length, binding to membranes occurs. That such a mechanism is operative has been proposed by different authors (14, 17, 18). Furthermore a large virus-specific polypeptide is precipitated by an antibody to disrupted avian myeloblastosis virus from the cytoplasm of cells infected with this virus (19). This polypeptide, with a molecular weight of 76,000, is a precursor of at least two major virus polypeptides. A virus-specific precursor polypeptide of the same size can be detected in cells infected with R-MuLV (unpublished results). Presumably this polypeptide is associated with membranous structures, since the recovery is strongly dependent upon the treatment of the cells with detergents.

Note Added in Proof. After this paper was submitted for publication, an article by Fan *et al.* [(1973) *J. Mol. Biol.* **80,** 93–117, came to our attention. They reported the presence of virus-specific 35S RNA in addition to 20–30S RNA in polyribosomes of mouse cells infected with Moloney murine leukemia virus.

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