The 7S RNA Common to Oncornaviruses and Normal Cells is Associated with Polyribosomes

(L-cells/RNA fingerprinting/gel electrophoresis/dimethylsulfoxide)

T. A. WALKER*, N. R. PACE*, R. L. ERIKSON†, E. ERIKSON†, AND F. BEHR†

* National Jewish Hospital and Research Center and Departments of *Biophysics and Genetics, and † Pathology, University of Colorado Medical Center, Denver, Colo. 80206

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ABSTRACT The 7S RNA species first demonstrated in avian and murine oncornaviruses and later in normal, uninfected cells is found associated in part with cellular polyribosomes. A molar ratio of 7S RNA to 5S ribosomal RNA of 0.05 indicates that there is approximately one mole of 7S RNA per mole of messenger RNA. Dissociation of polyribosomes with dimethylsulfoxide results in a marked decrease in the sedimentation rate of the 7S RNA. The dimethylsulfoxide-induced dissociation of polyribosomes and the concomitant movement of the 7S RNA from the polyribosome region into lighter regions of a sucrose gradient are both inhibited by cycloheximide, indicating that the 7S RNA is indeed associated with polyribosomes and not with a ribonucleoprotein particle sedimenting with polyribosomes.

The avian oncornaviruses contain a variety of RNA components, including the 65S genome complex (1) and discrete populations of low-molecular-weight species designated 4 S (2), 5 S (3), and 7 S (4). The virion 4S RNA is structurally (3, 5, 6) and functionally (2, 7) similar to that of the host, and the 5S RNA apparently is identical in nucleotide sequence to the host 5S ribosomal RNA (3). The function of these small RNA species remains enigmatic.

The viral 7S RNA, first described from oncornaviruses by Bishop and his colleagues (4), occurs in virus preparations in molar concentrations approximately equivalent to the viral genome subunits. It also was established that the 7S RNA isolated from Rous sarcoma virus does not anneal with the viral 65S genome RNA (4). The 7S RNA, therefore, appeared to be derived from host rather than viral genetic information. Subsequently, Erikson *et al.* (8) reported that 7S RNA[†], identical in structure to that associated with the avian oncornaviruses, is present at low concentration in normal, uninfected chick cells. The presence of this RNA molecule in normal cells implies that it plays a role in normal metabolic processes. In this communication we report that the 7S RNA common to uninfected cells and to oncornaviruses is located in the cytoplasm, at least in part associated with polyribosomes. This finding suggests that the 7S RNA in some fashion participates in the translation process.

Abbreviations: NaDodSO₄, sodium dodecyl sulfate; Me₂SO, dimethylsulfoxide; MAK, methylated albumin-kieselguhr.

† Present address: 3M Company, St. Paul, Minn.

[‡] It must be noted that this 7S RNA molecule is distinct from the "7S" ribosomal RNA described by Pene *et al.* (9). This latter molecule actually is only about 5.5S in size, and it is an integral component of eukaryotic ribosomes.

MATERIALS AND METHODS

Growth and Labeling of Cells. Mouse embryo fibroblasts (L-929 strain) and HeLa S₃ cells were grown in suspension in Eagle's minimal essential medium supplemented with 5% calf serum. To isotopically label total cellular RNA, we suspended cells at a density of 4×10^6 cells per ml in minimal essential medium modified to contain 1 mM orthophosphate, supplemented with 10% (v/v) calf serum and 10–50 μ Ci/ml of H₃³²PO₄.

Fractionation of Cells. After 24–48 hr of incubation, cells (about 2×10^6) were collected by centrifugation, washed once in phosphate-buffered saline, and suspended at a concentration of 1 ml of packed cells per 4 ml of 50 mM Tris·HCl (pH 7.5), 25 mM NaCl, 5 mM MgCl₂. Cells were lysed by five strokes with a Potter homogenizer followed by addition of 1 volume of Tris·HCl–NaCl–MgCl₂ containing 1% Nonidet P-40 (Shell Chemical Co.). After 5 min at 0°, a postmitochondrial supernatant fraction (S-17) was prepared by centrifugation of the lysate at $17,000 \times g$ for 10 min (10). Before homogenization, a sample was removed from the cell suspension for extraction of RNA as described below.

Purification of Polyribosomes. Two milliliters of the S-17 fraction were layered over 4 ml of 1 M sucrose in Tris·HCl–NaCl–MgCl₂ and centrifuged at 50,000 rpm in a Spinco SW50.1 rotor for 1 hr at 4°. The upper 3 ml of the supernatant were removed and saved for RNA extraction (see below). The polyribosome pellets were rinsed with cold water and stored frozen at –80°.

Extraction of RNA. Before RNA extraction all samples, with the exception of intact cells, were adjusted to contain 0.5% sodium dodecyl sulfate (NaDodSO₄) and 10 mM EDTA. Samples were deproteinized by two to three extractions at 0–5° with water-saturated phenol containing 0.1% 8-quinolinol. Total RNA was precipitated from 70% ethanol.

Methylated Albumin–Kieselguhr (MAK) Column Chromatography. Purified RNA was dissolved in 2 ml of 20 mM Tris·HCl (pH 7.2), 0.3 M NaCl and adsorbed onto a 1- to 4-g column of MAK, constructed as detailed by Mandel and Hershey (11). Columns were washed with 2 column volumes of the equilibration buffer and the low-molecular-weight RNA species, including 7S RNA, were eluted with 20 mM Tris·HCl (pH 7.2), 0.75 M NaCl and recovered by precipitation with ethanol.

FIG. 1. Polyacrylamide gel electrophoresis of low-molecular-weight RNA of L-cells. (A) Total cytoplasmic low-molecular-weight RNA. Electrophoresis was performed on 8% polyacrylamide gels for 5 hr at 12 mA per gel (12). Insert shows the 7S and C components plotted on an expanded scale. (B) Autoradiograph of a two-dimensional electrophoretic separation of the ribonuclease T1 digestion products of 7S RNA derived from L-cells. The material in the 7S peak (A) was eluted from the gel, further purified by gradient elution from MAK, digested completely with ribonuclease T1, and analyzed by two-dimensional electrophoresis (13).

Polyacrylamide Gel Electrophoresis. Electrophoresis of RNA in 8% (w/w) polyacrylamide gels containing NaDodSO₄ was done as described (12).

Fingerprint Analysis. L-cell 7S RNA, purified by electrophoresis through and elution from an 8% acrylamide gel, followed by adsorption to and gradient elution from MAK, was concentrated by ethanol precipitation and digested with T1 ribonuclease. The resulting oligonucleotides were resolved by two-dimensional electrophoresis, all as detailed by Sanger *et al.* (13).

RESULTS

The 7S RNA of Mammalian Cells. Bishop and his colleagues originally recovered the 7S RNA molecule from oncornaviruses, but they did not observe it in either normal or virus-infected cells (4). The difficulty in finding the 7S molecule in cells probably can be attributed to its rarity, and to obscuring by fragments of high-molecular-weight rRNA. To minimize these difficulties we separate the low- and high-molecular-weight RNA components by MAK column chromatography immediately after purification of bulk, cellular RNA, as detailed in *Materials and Methods*. As illustrated in Fig. 1A, the low-molecular-weight RNA components recovered from mouse L-cells are similar to those described for avian cells (8). Three discrete RNA components are present in addition to tRNA and 5S rRNA. These are designated in Fig. 1A as 7S, C, and D. RNA components C and D are derived from the cell nucleus (14); they are not present in cytoplasmic extracts (see below). The 7S RNA recovered in this sort of experiment is not unique to murine or avian tissue culture lines; we have recovered 7S RNA from the human cell lines HeLa (see below) and WI38 (L. Fink, unpublished), and from liver, spleen, kidney, and brains of mice injected intravenously with [^{32}P]orthophosphate (M. L. Sogin, unpublished).

The ^{32}P -containing component designated as 7S RNA in fact is RNA; it is digested by T1 ribonuclease and unique collections of oligonucleotides result. We have previously reported approximate T1 oligonucleotide catalogs for the 7S RNA isolated from L-cell virion (8). Fig. 1B is an autoradiograph of the two-dimensional electrophoretic array of T1 RNase digestion products of L-cell 7S RNA. This fingerprint is identical to that of the 7S RNA isolated from L-cell virions (8). The recovery of radioactivity among the various oligonucleotides, as well as the electrophoretic mobility of 7S RNA in polyacrylamide gels, suggest that the molecule is about 280 nucleotides in length.

The Intracellular Location of 7S RNA. In pursuing the function of the 7S RNA, we first partitioned cells into nuclear and cytoplasmic fractions (15) and examined their relative 7S RNA contents by gel electrophoresis. The molecule could not be detected among the nuclear components; rather, it was recovered with the cytoplasmic fraction, in postmitochondrial supernatants. Moreover, we found that a substantial fraction of the cellular 7S RNA may be pelleted with the cytoplasmic polyribosomal aggregates. The contents of a postmitochondrial supernatant from ^{32}P -labeled L-cells were separated by sedimentation through 1 M sucrose into a "polyribosome supernatant" and a "polyribosome pellet," as described in *Materials and Methods*, and the 7S RNA contents of the total cytoplasmic RNA and the two fractions were then determined by gel electrophoresis, as displayed in Fig. 2. The partitioning of 7S RNA among the various subcellular fractions may be derived from comparisons of summed radioactivity of the various low-molecular-weight components in the 8% gels. The 7S RNA:tRNA molar ratio in the postmitochondrial supernatant (Fig. 2A) is 0.004, whereas that of the polyribosome supernatant (Fig. 2B) is 0.002. Since little tRNA sediments with the polyribosome fraction (Fig. 2C), the difference in these ratios provides a measure of "free" and

FIG. 2. Distribution of 7S RNA among various subcellular fractions. A postmitochondrial supernatant was prepared as described in *Materials and Methods*. A sample of the supernatant was extracted with phenol, and the remainder was resolved into a polyribosomal pellet and supernatant fraction by centrifugation through 1 M sucrose as described (28). The pellet and supernatant were each extracted with phenol-NaDodSO₄, and the resulting low-molecular-weight RNAs were then analyzed by electrophoresis on 8% acrylamide gels. The *inserts* show the 7S region of each profile plotted on an expanded scale. (A) Whole cell; (B) polyribosome supernatant; (C) polyribosome pellet.

"associated" 7S RNA; about 50% of the cellular 7S RNA is not recovered in the polyribosome supernatant. It is evident from Fig. 2C, however, that the remainder of the 7S RNA is recovered in the polyribosome pellet. Similarly the 7S RNA: 5S rRNA molar ratio of whole cells (Fig. 1A) is 0.056, while that of the polyribosome pellet is 0.026. About 90% of the 5S rRNA is sedimented (the 5S rRNA:tRNA molar ratio of the polyribosome supernatant is only 0.1 that of whole cells); therefore, about half of the cellular 7S RNA is associated with the polyribosome pellet.

To define more rigorously the association of the 7S RNA with the translation apparatus, we resolved ³²P-labeled L-

cell polyribosomes, prepared as described above, by sedimentation through a linear sucrose gradient, as described in the legend to Fig. 3. Fractions corresponding to the polyribosome region (Fig. 3A) were pooled, and the low-molecular-weight RNA content was determined by gel electrophoresis

FIG. 3. Cosedimentation of 7S RNA and polyribosomes. (A) Polyribosomes were removed from an L-cell postmitochondrial supernatant by centrifugation through 1 M sucrose-50 mM Tris·HCl (pH 7.5)-25 mM NaCl-5 mM MgCl₂. The polysome pellet was then dissolved in Tris·HCl-NaCl-MgCl₂ and centrifuged on a preformed linear 15-30% sucrose density gradient at 45,000 rpm in a Spinco SW 50.1 rotor for 35 min at 4°. Fractions of 0.1 ml were collected from the bottom of the tube and monitored for their absorbance at 260 nm. (B) Fractions constituting the polyribosomal region of the gradient illustrated in A were pooled and extracted with phenol-NaDodSO₄. The low-molecular-weight RNA was analyzed by electrophoresis on 8% acrylamide gels.

FIG. 4 (left). Me₂SO-induced dissociation of 7S RNA from HeLa cell polyribosomes. A culture of ³²P-labeled HeLa cells was concentrated by centrifugation to a density of 4 × 10⁶ cells per ml and divided into two subcultures. One culture was incubated at 37° for 15 min in the presence of 100 μg/ml of cycloheximide. Both cultures then received Me₂SO to a final concentration of 12% (v/v) and were incubated for an additional 20 min. The cultures were chilled and centrifuged, and the cell pellet was resuspended in 50 mM Tris·HCl (pH 7.5)-25 mM NaCl-5 mM MgCl₂. Cells were lysed by addition of NP-40 to 0.5%. After removal of nuclei by low-speed centrifugation, the cytoplasmic contents were resolved by centrifugation through 10-ml convex exponential 10-25% (w/v) sucrose density gradients (20) supported by a 1.5-ml cushion of 75% sucrose and buffered with Tris·HCl-NaCl-MgCl₂. The gradients were centrifuged at 41,000 rpm in a Spinco SW 41 rotor for 2.5 hr at 2°. Fractions of 0.3 ml were collected, and 30-μl samples were assayed for ³²P radioactivity. (●), Me₂SO plus cycloheximide; (○), Me₂SO alone.

FIG. 5 (right). Sedimentation of HeLa cell polyribosomes after incubation with ³²P-labeled 7S RNA of HeLa cells. A postmitochondrial supernatant (S-17), prepared from a culture of unlabeled HeLa cells, was mixed with approximately 35,000 cpm of purified HeLa cell 7S [³²P]RNA. After 5 min at 0°, the S-17 supernatant was fractionated by centrifugation through convex exponential sucrose gradients as described in the *legend* to Fig. 4.

TABLE 1. Distribution of 7S RNA after sucrose gradient centrifugation of Me_2SO -dissociated polyribosomes

	Cycloheximide + Me_2SO			Me_2SO		
	A	Pool B	C	A	Pool B	C
Σ cpm 7S	1,912	789	1,227	675	1,156	2,526
Σ cpm 5S	53,913	16,744	43,456	9,891	20,900	81,212
% 7S*	49	20	31	15	27	58
% 5S*	47	15	38	9	19	72
Molar 7S/5S†	0.015	0.020	0.012	0.029	0.023	0.013

* The amount of 7S or 5S RNA in the indicated pools is expressed as a percentage of the total 7S or 5S RNA in the three pools.

† Molar ratios were computed on the assumption that 7S and 5S RNA contain, respectively, 280 and 120 nucleotide residues.

(Fig. 3B). It is evident that 7S RNA is present among the polyribosomes, but the 7S RNA:5S rRNA molar ratio in Fig. 3B is 0.015, whereas that of the initial polyribosome pellet was 0.026. The balance of 7S RNA was recovered at the top of the sucrose gradient, presumably indicating that about half of the 7S RNA was disjoined from the particulate fraction during the purification schedule. Therefore, the measure of "free" and "associated" 7S RNA obtained in this experiment and in that of Fig. 2 may not reflect the true intracellular distribution of the molecule; the association of at least some 7S RNA with the polyribosome fractions may be quite tenuous.

Sedimentation of the 7S RNA with polyribosomes does not prove its association with the translation apparatus. It also could be a component of some other cytoplasmic particle of unknown character. It is predictable, however, that if 7S RNA indeed is bound in some fashion to polyribosomes, then their dissociation by appropriate inhibitors of protein synthesis should concomitantly lead to loss of 7S RNA from the "polyribosome" fractions of sucrose gradients. We have tested the ability of several inhibitors of protein synthesis, including puromycin (16), pactamycin (17) and dimethylsulfoxide (Me_2SO) (18) to dissociate polyribosomes *in vivo* and found the latter to have the most profound effects. Treatment of cells with 12% (v/v) Me_2SO leads to disruption of 80–90% of the polyribosomal aggregates within 20 mins. In concurrence with the results of Saborio and Koch (18), we find that the Me_2SO -induced breakdown of polyribosomes is inhibited by simultaneous treatment of cells with cycloheximide, an inhibitor of translocation (19).

In testing for the release of 7S RNA from polyribosomes during their disaggregation, we used HeLa cells to generalize the association of 7S RNA with the translation apparatus. Cells were treated with either 12% Me_2SO plus 100 μg of cycloheximide per ml (the control), or 12% Me_2SO (the experimental culture). After 20 min of incubation, the post-mitochondrial supernatants from the two cultures were centrifuged through 10–25% (w/v) exponential sucrose gradients constructed over a 75% sucrose pad (20), as described in the legend to Fig. 4. It is apparent that substantial dissociation of polyribosomes, with accumulation of 80S monosomes, occurs in the presence of Me_2SO (Fig. 4) and that this dissociation is retarded by cycloheximide. The various pooled fractions (Fig. 4) were analyzed by gel electrophoresis. Pool A represents total polyribosomes, pool B the 100–200S region of the gradient, and pool C contains 80S monosomes. Before purification of RNA, a constant amount of [^3H]RNA was introduced into each of the pools so that the quantities of

RNA components in the various polyacrylamide gels could be normalized to unit recovery on the basis of ^3H applied to the gels. The normalized quantities of ^{32}P -labeled 7S RNA and 5S rRNA and their relative distributions among the various pools from the sucrose gradients are summarized in Table 1.

The important fact to derive from Table 1 is that loss of 7S RNA from the polyribosome region of the sucrose gradients (pool A) almost quantitatively parallels dissociation of the polyribosomes by Me_2SO . Distribution of 5S rRNA provides a measure of the distribution of ribosomes in the gradients; 47% of the 5S rRNA is found in polyribosomes in the control culture (Me_2SO plus cycloheximide), whereas only 9% remains after incubation with Me_2SO alone. Thus, about 80% of the polyribosomes are disaggregated by the treatment. Similarly, in the control culture 49% of the 7S RNA is found among the polyribosomes, while after Me_2SO treatment only 15% resides there; 70% of the 7S RNA is lost from the region of the gradient containing polyribosomes during their dissociation. Therefore, 7S RNA must be associated with the translation apparatus and not some other unknown, particulate component of the cell cytoplasm that fortuitously sediments with the polyribosomes.

The amounts of 7S RNA recovered in the 80S and larger regions (the sum of pools A, B, and C) of both sucrose gradients shown in Fig. 4 are about the same, as are the quantities of 5S rRNA recovered; the molar ratio 7S RNA:5S rRNA for the 80S and larger regions of the control (Me_2SO plus cycloheximide) sucrose gradient (Fig. 4) is 0.015 and that of the experimental (Me_2SO alone) gradient is 0.016. Therefore, disaggregation of polyribosomes does not result in dissociation of 7S RNA from particles; rather, as polyribosomes (pool A) decay, 7S RNA accumulates in pool B and, predominantly, in pool C (monosomes).

7S RNA Does Not Readily Adhere to Ribosomes. It is conceivable that the association of 7S RNA with the translation apparatus is spurious. This might result from interaction of the anionic polynucleotide with cationic sites on the surface of the ribosome, although this sort of interaction would appear unlikely in view of the low (and expected) levels of tRNA found among the polyribosomal aggregates. Nevertheless, 7S RNA could be inherently adhesive toward ribosomes. This we have tested by introducing purified 7S [^{32}P]RNA into an unlabeled lysate of HeLa cells, and resolving the mixture on a sucrose gradient. The results and details of the experiment are presented in Fig. 5. It is apparent that little of the added 7S RNA sediments with particles; the very small

amount (about 1%) of added 7S RNA appearing in the polyribosome and monosome regions of the gradient may represent functional aggregation. We propose that the 7S RNA molecule is a minor and novel component of the protein-synthesizing machinery of eukaryotic cells. Whether it is associated with all or only a fraction of the polyribosomal aggregates remains to be determined.

DISCUSSION

In summary, these experiments provide evidence that a substantial fraction of the 7S RNA previously shown to be associated with avian and murine oncornaviruses and with normal, uninfected cells fractionates by sedimentation with the cellular translation apparatus and, therefore, presumably is a component of it. We cannot yet define the nature of the association of 7S RNA with the protein-synthesizing machinery, but the quantitative relationships of the 7S RNA with other components of the polyribosomes, notably of mRNA, are of interest. The molar quantities of 7S RNA that we normally recover from cells are about 0.05–0.06 that of 5S rRNA, and, therefore, of total ribosomes. The mass fraction of RNA within the translation apparatus that is ascribable to mRNA is variously estimated at 2.2 (16)–5.5 (21) % of total RNA. The size of the average mRNA molecule is on the order of 20–30S (21, 22), corresponding in molecular weight to 1 to 2×10^6 or, on the average, about 60–70% the mass of rRNA in one ribosome. Therefore, the mRNA:ribosome mole ratio in mammalian cells is very roughly 0.05–0.07, which is about equivalent to the cytoplasmic 7S RNA:ribosome mole ratio (0.05–0.06). Although we routinely recover only about 30–50% of the 7S RNA with sedimented polyribosomes, this may reflect a lower estimate of the fraction of 7S RNA that is bound to the translation apparatus in the cell. As was pointed out in *Results*, the association of 7S RNA with the polyribosomes apparently is rather tenuous, and so a substantial fraction of the 7S RNA might be disjoined from the complexes during their isolation. On the basis of the association of 7S RNA with the translation apparatus and its approximate quantitative correspondence with mRNA, we are tendering the hypothesis that 7S RNA interacts with mRNA in some fashion.

One interesting facet of this 7S RNA species is the rationale underlying its specific inclusion within oncornavirions. If the 7S RNA proves to be an essential component of the eukaryotic translation apparatus, in association with the mRNA, then its presence within virions in molar quantities equivalent to the viral genome subunits might be accounted for if the genome subunits serve as mRNA shortly before virus assembly and the associated 7S RNA is enclosed within the mature virion. Alternatively, the genome subunits may serve as mRNA soon after infection, but may be incapable of scavenging the host 7S RNA. Therefore, a preformed (within the virion) complex between 7S RNA and the viral genome would be required for productive association with the cellular ribosomes. There is no evidence at this time that the genome

components of the oncornaviruses are translated immediately after infection, but it is clear that RNA equivalent in structure to the genome appears in the polyribosomes during the later stages of the infection process (23–25). Furthermore, the RNA subunits of the viral genome certainly are capable of being translated, at least *in vitro* (26), and they do contain polyadenylate sequences at their 3' termini (27).

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