A Procedure for the Isolation of Mammalian Messenger Ribonucleic Acid*

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ABSTRACT: The presence of a large poly(A) segment has been demonstrated in the heterogeneous rapidly labeled components of polysomal RNA from mammalian cells. Unique properties of poly(A) permit the isolation of this RNA species, which possesses various characteristics of mRNA. The poly(A) segment causes the transfer of these RNA molecules to the nonaqueous phase during phenol extraction in the presence of Tris-HCl (pH 7.6) and of polysomal proteins or methylated albumin. They are recovered by reextraction with pH 9.0 Tris buffer. The critical factor for this behavior appears to be the ionic concentration during phenol extraction. The poly(A) segment also causes the effective binding of the RNA molecules to nitrocellulose membrane filters (Millipore filters) at high ionic strength. RNA molecules lacking such a poly(A) sequence (such as rRNA) do not share these properties. The RNA adsorbed on Millipore from unlabeled mouse sarcoma 180 polysomal RNA shows the presence of ultraviolet-absorbing material with a 10–30S range of sedimentation values, together with some ribosomal RNA. With rabbit reticulocyte polysomal RNA, the material retained on Millipore consists of a very prominent 10S component, together with some heterogeneous material and a small amount of ribosomal components. The phenol fractionation procedure leads to preparations greatly enriched in poly(A)-containing RNA species, but is not as effective in removing rRNA.

Progress in the development of procedures for the separation of animal cell mRNA from rRNA has been hampered by the apparent lack of distinguishing physical features. The size distribution of mRNA molecules in most cells is such that their sedimentation values overlap those of the ribosomal components. Only in the case of reticulocytes, in which the 10S messenger for hemoglobin represents the major species, has it been possible to isolate this RNA component by zone sedimentation (Lockard and Lingrel, 1969). We wish to report on a procedure for the isolation of polysomal RNA molecules that appear to represent mRNA, based on the occurrence in these molecules of a large poly(A) sequence.

It has been known for some time that RNA fractions considerably enriched in components with a DNA-like base composition could be obtained from animal cells by differential phenol extraction. Treatment of rat liver or ascites cells with aqueous phenol at low temperature was shown to release preferentially rRNA, while reextraction at elevated temperature yielded RNA components with a nucleotide composition similar to that of DNA (Georgiev and Mantieva, 1962). The basis of the fractionation was thought to be the localization of the DNA-like RNA in the nucleus. A similar separation was obtained by sequential phenol extractions with neutral and alkaline Tris-HCl buffers (Brawerman *et al.*, 1963). In this latter case, the "DNA-like" RNA was observed to be particularly rich in adenylic acid. Subsequent studies with this technique showed that rat liver cytoplasm contained rapidly labeled, adenylate-rich, DNA-like RNA refractory to phenol extraction in the presence of neutral Tris buffer (Hadjivassiliou and Brawerman, 1967). Poly(A) of relatively large size was isolated from this material, thus accounting for high adenylic acid content (Hadjivassiliou and Brawerman, 1966).

The behavior of the cytoplasmic DNA-like RNA during phenol extraction can now be explained by the presence of a large poly(A) segment as integral part of the RNA molecules. Poly(A) has been shown to be associated with rapidly labeled polysomal RNA components of both mouse sarcoma 180 cells and HeLa cells (Lee *et al.*, 1971b; Edmonds *et al.*, 1971; Darnell *et al.*, 1971). The components that carry the poly(A) segment have a DNA-like base composition (Mendecki *et al.*, 1972) and a heterogeneous distribution of sedimentation values ranging from 10 S to 30 S. It is shown in this report that poly(A) possesses unusual binding characteristics which cause it to shift to the nonaqueous phase during phenol treatment in the presence of ribosomal proteins or methylated albumin. It will also bind to nitrocellulose membrane filters (Millipore filters) at high ionic strength (Lee *et al.*, 1971b). These characteristics are shared by the poly(A) segment associated with the presumed mRNA, and provide the basis for the present isolation procedures.

Experimental Section

Cell Incubations and Preparation of Polysomes. The detailed procedures for the manipulations involving mouse sarcoma 180 ascites cells have been described previously (Lee *et al.*, 1971a,b). The cells were incubated in complete medium for 1 hr prior to isolation of the polysomes. Labeled preparations were obtained by incubating the cells in the presence of 5 μCi/ml of uridine-*5-t* or adenosine-*5,8-t*$_2$, and 0.04 μg/ml of actinomycin D to prevent labeling of the rRNA (Penman *et al.*, 1968). Polysomes were prepared as described previously (Lee *et al.*, 1971a). Rabbit reticulocyte polysomes were prepared by the procedure of Allen and Schweet (1962). All polysomal pellets were suspended in 50 mM Tris-HCl (pH 7.6), 50 mM KCl, and 1 mM $MgCl_2$, and stored in liquid N_2.

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TABLE I: Phenol Fractionation of Sarcoma 180 Polysomes.[a]

RNA Fraction	Total RNA (μg)	Radioactivity (cpm)	Sp Radioactivity (cpm/μg of RNA)	Millipore-Bound Radioactivity (% of Total in Fraction)
pH 7.6 I	1068	45,000	43	2
II	84	9,500	113	4
pH 9.0 I	24	61,000	2540	135
II	14	18,250	1300	116

[a] Polysomes from uridine-labeled cells were subjected to sequential phenol extraction as described in the Experimental Section. Samples of each aqueous phase from which phenol had been removed were used for measurements of optical density at 260 mμ, acid-insoluble radioactivity and Millipore-binding material. I and II refer to first and second extraction at given pH.

TABLE II: Effect of Salts on Phenol Extraction of Labeled Sarcoma 180 Polysomes.

	Conditions			Amount Extracted	
	Tris-HCl (pH 7.6) (mM)	KCl (mM)	$MgCl_2$ (mM)	Cpm	% of Total Polysomal RNA
First extraction[a]				3020	92
	100			1790	55
	100	50	1	1485	45
	Tris-HCl (pH 9.0)	NaCl			
Reextraction[b]				605	18
	100			815	25
	100	100		425	13

[a] Samples (10 μl) of polysome suspensions from uridine-labeled cells diluted with 1 ml of indicated solutions, all containing 0.5% sodium dodecyl sulfate, and mixed with 1 ml of phenol for 10 min. Aqueous phases used for measurements of acid-insoluble radioactivity. [b] Polysome samples diluted in 100 mM Tris (pH 7.6), 50 mM KCl, 1 mM $MgCl_2$, and 0.5% sodium dodecyl sulfate, were treated with phenol, and nonaqueous residues were reextracted with 100 mM Tris (pH 7.6). Residues were next extracted twice with indicated solutions in 0.5% sodium dodecyl sulfate. Combined aqueous phases were used for radioactivity measurements.

RNA Extractions. The polysomes were diluted to concentrations lower than 100 A_{260} units per ml. All operations were carried out at 0–4°. For the sequential extractions with neutral and alkaline Tris buffers, the polysomes were diluted with 50 mM Tris (pH 7.6), 50 mM KCl, and 1 mM $MgCl_2$, 0.1 volume of 5% sodium dodecyl sulfate and of 1 M Tris (pH 7.6) were added, followed by 1 volume of water-saturated phenol. The mixture was shaken for about 5 min, centrifuged at 12,000*g* for 10 min, and the aqueous phase removed. One volume of 0.1 M Tris (pH 7.6) was added to the nonaqueous residue (interphase plus phenol phase), and the shaking and centrifuging were repeated. The second aqueous phase was combined with the first one. The nonaqueous residue was reextracted successively with equal volumes of 0.1 M Tris-HCl (pH 9.0) in 0.5% sodium dodecyl sulfate and of 0.1 M Tris (pH 9.0), and the two alkaline extracts were combined. For the direct extraction of the total polysomal RNA, the polysomes were diluted in H_2O, 0.1 vol of 5% sodium dodecyl sulfate and of 1 M Tris (pH 9.0) were added followed by 1 volume of phenol, and the extraction carried out as above. The nonaqueous residue was reextracted with 0.1 M Tris (pH 9.0), and the two aqueous phases were combined.

The combined aqueous phases were reextracted at least 3 times with fresh phenol. This served to remove residual protein as well as the remaining sodium dodecyl sulfate. The RNA was either precipitated from the aqueous phases with ethanol, or used directly after removal of the phenol. The precipitation was done by addition of 2.5 volumes of ethanol and 0.1 volume of 1 M NaCl, and storage overnight in the cold. The precipitate was collected by centrifugation and washed several times with 66% ethanol in 0.1 M NaCl. It was dissolved in water and stored at −20°. When no precipitation was required, the aqueous phases were extracted 4 to 5 times with ether, and the latter removed by a stream of air.

Adsorption on Millipore Filters. The RNA solutions were diluted at least 20-fold with 500 mM KCl, 10 mM Tris (pH 7.6), and 1 mM $MgCl_2$, and passed slowly through a Millipore filter presoaked in the same solution at a rate of approximately 0.5 ml/min. When unlabeled preparations were used, the RNA was applied at concentrations up to 0.2–0.3 mg/ml, and quantities of RNA as high as 60 μg were adsorbed on a single filter (see Table V). The maximum capacity of the filters was not determined.

The adsorbed material could be eluted with an ice-cold solution of 0.5% sodium dodecyl sulfate in 0.1 M Tris (pH 9.0). The filter was kept in 0.5–1 ml of this solution for about 30 min, with occasional shaking. Because of the residual KCl on the filters, a large amount of potassium dodecyl sulfate crystals appeared, but this did not interfere with the elution of the RNA.

Zone Centrifugations. These were carried out in the SW 41 Spinco rotor at 41,000 rpm in 13-ml linear 5–30% sucrose gradients supplemented with 10 mM KCl and 20 mM Tris (pH 7.6). The contents of the tubes were pumped from the bottom through a Uvicord uv detector (LKB, Stockholm).

Counting Procedures. The radioactivity measurements were done as described previously (Lee *et al.*, 1971a,b).

Results

Conditions for the Phenol Fractionation of Polysomal RNA from Mouse S-180 Cells. Phenol extraction of polysomes in the presence of Tris-HCl (pH 7.6) removes nearly all the rRNA into the aqueous phase, but less than half of the labeled nonrRNA components are recovered under these conditions (Table I). The remainder of the labeled RNA can be obtained by reextraction of the nonaqueous residue in the presence of pH 9 Tris. As previously reported, the latter material is capable of binding to Millipore filters in the presence of 0.5

TABLE III: Phenol Fractionation of Labeled Sarcoma 180 Polysomal RNA in the Presence of Methylated Albumin or Unlabeled Polysomes.[a]

RNA Prepn	Addn	Remaining in Aqueous Phase: Cpm	Remaining in Aqueous Phase: % of Total Polysomal RNA	Reextracted (cpm): H_2O	Reextracted (cpm): Tris (pH 9.0)
Poly(A)	None	700	96	30	
	Polysomes	20	3	280	280
	Methylated albumin	40	6	290	400
Polysomal RNA, pH 9.0	None	470	98	10	
	Polysomes	90	19	320	10
	Methylated albumin	170	35	290	30
Polysomal RNA, pH 7.6	Polysomes	380	68	120	
	Methylated albumin	450	80	110	

[a] Polysomal RNA fractions prepared by sequential phenol extraction of adenosine-labeled cells; tritiated poly(A) obtained from Miles Laboratories, Elkhart, Ind. Labeled RNA preparations mixed in 1 ml of 100 mM Tris (pH 7.6), 10 mM KCl, 0.2 mM $MgCl_2$, and 0.5% sodium dodecyl sulfate with either 10 A_{260} units of unlabeled polysomes or 200 μg of methylated albumin. Mixtures treated with equal volumes of phenol as in the Experimental Section; aqueous phases used for radioactivity measurements. Residues washed with 100 mM Tris (pH 7.6), 10 mM KCl, and 0.2 mM $MgCl_2$, then extracted twice with H_2O and once with 0.1 M Tris (pH 9.0). Values expressed as acid-insoluble radioactivity.

TABLE IV: Adsorption of Labeled Sarcoma 180 Polysomal RNA on Potassium Dodecyl Sulfate Crystals.[a]

RNA Fraction	Not Adsorbed: Cpm	Not Adsorbed: % of Total	Adsorbed (cpm): Extracted at pH 9	Adsorbed (cpm): Not Extracted
pH 7.6	312	66	76	88
pH 9.0	55	14	235	170

[a] Sodium dodecyl sulfate added to a concentration of 0.5% to RNA solutions in 100 mM Tris (pH 7.6), 500 mM KCl, and 1 mM $MgCl_2$, at 0°. Precipitates collected by centrifugation, and extracted with 0.1 M Tris (pH 9.0) in 50 mM KCl. For other details, see Table III.

M KCl, while the RNA obtained at pH 7.6 lacks this capacity (Lee *et al.*, 1971b). It was shown that the Millipore-binding property is provided by a large poly(A) segment present in the RNA molecules. Thus the phenol fractionation procedure separates polysomal RNA components with poly(A) from those lacking this sequence, and leads to labeled RNA preparations with relatively little contamination by ribosomal RNA. The efficiency of this separation, however, depends on the initial polysome concentration. Only dilute polysome suspensions lead to efficient removal of the rRNA at pH 7.6.

The above fractionation procedure is due to the failure of the poly(A)-containing RNA molecules to shift to the aqueous phase in the presence of pH 7.6 Tris. The behavior of these RNA molecules is highly dependent on the ionic strength of the water–phenol mixtures. It can be seen in Table II that the totality of the polysomal RNA is extracted at neutral pH when very little salt is present. Since Tris-HCl at pH 7.6 is nearly entirely in the ionic form, its effect on the extraction of the poly(A)-containing RNA can be accounted for by a rise in the ionic strength. The inclusion of KCl and $MgCl_2$ leads to a further decrease in the yield of labeled polysomal RNA. The influence of ionic strength is also evident during the reextraction of the nonaqueous residue after the usual pH 7.6 phenol treatment. A portion of the poly(A)–RNA is extracted with water without rise in pH (Table II). Tris buffer of pH 9.0 improves the efficiency of the extraction, but inclusion of NaCl lowers considerably the yield of pH 9 RNA. Although the overall yields of pH 9 RNA shown in Table II are relatively low (compare with Table I), the results are consistent with those of other experiments dealing with the effect of salts on polysomal RNA extraction.

It appears from the data in Table II that the presence of salt is essential to prevent the extraction of the poly(A)-containing RNA together with the rRNA, and that it must be avoided in the subsequent extractions to insure a good recovery of this poly(A)-RNA. The increase in pH, although not essential, appears to enhance the extraction of the latter material. The totality of the polysomal RNA could be obtained in the aqueous phase with sodium dodecyl sulfate–H_2O alone, but the ionic species normally present in polysomal suspensions would interfere with this procedure. Phenol extraction in the presence of sodium acetate (pH 5.2) at 60° also leads to the appearance of the poly(A)-containing molecules in the aqueous phase (Edmonds and Caramela, 1969; Mendecki *et al.*, 1972).

Binding of Polysomal RNA to Proteins during Phenol Extraction. The behavior of the poly(A)-containing RNA during phenol extraction suggested at first that these molecules might be normally associated with other components which would prevent their release into the aqueous phase under certain conditions. A reconstruction experiment, however, in which RNA fractions mixed with unlabeled polysomes were subjected to the phenol treatment, showed that preexisting RNA–protein complexes are not required for the retention of the poly(A)-containing molecules in the nonaqueous phase. As can be seen in Table III, the deproteinized RNA fraction containing poly(A) as well as the synthetic homopolymer were present in the nonaqueous phase after treatment with phenol at pH 7.6 in the presence of polysomes or even methylated albumin. The polysomal RNA fraction lacking poly(A) remained in the aqueous phase under these conditions. The synthetic poly(A) appeared to be more tightly bound to the denatured proteins, since it could not be entirely reextracted with water, in contrast to the behavior of the pH 9 RNA fraction.

It appears from the above results that the distribution of RNA molecules between aqueous and nonaqueous phases observed during the phenol treatment of polysomes is due to interaction of the poly(A) sequences with denatured proteins. While the presence of proteins was required for the transfer of the poly(A)-containing RNA to the nonaqueous phase under

TABLE V: Recoveries of Unlabeled Polysomal RNA Components from Mouse Sarcoma 180 Cells and Rabbit Reticulocytes after Phenol Fractionation and Millipore Filtration.

Material	Fractionation Procedure	Total RNA Used (mg)	RNA Recovd (mg)	% Yield
Polysomes, Sarcoma 180	Phenol fractionation,[a] pH 9 fraction	5.9	0.58	10
Total polysomal RNA, Sarcoma 180	Millipore binding, 0°	2.0	0.06	3
	18°	2.0	0.03	1.5
pH 9 RNA fraction,[a] Sarcoma 180	Millipore binding, 22°	0.5	0.04	8
Total polysomal RNA, Reticulocytes	Millipore binding, 22°	8.8	0.04	0.5

[a] Polysomes subjected to sequential phenol extraction (see Experimental Section). Data on RNA recovered refer to fraction obtained at pH 9. Same pH 9 RNA fraction used for Millipore binding. For other details, see Figures 1 and 2.

the usual ionic conditions, a transfer in the absence of proteins occurred when the KCl concentration was raised to 0.5 M. In this case, the poly(A) segment apparently became adsorbed to the crystals of potassium dodecyl sulfate. Such adsorption also occurred in the absence of phenol (Table IV).

Binding of Polysomal RNA to Millipore Filters. As shown previously (Lee *et al.*, 1971b), Millipore filtration provides an effective procedure for the removal of rRNA from the rapidly labeled polysomal components with the poly(A) segment. The potential of this technique as a preparative isolation procedure was examined with the use of unlabeled polysomal RNA. When the filtration was carried out at 0–4° using 2 mg of RNA from mouse sarcoma, about 60 μg of RNA was retained on the filter (Table V). The two ribosomal components were still present in the adsorbed RNA, but a substantial amount of heterogeneous uv-adsorbing material with a range of sedimentation values similar to those of the rapidly labeled RNA (10–30 S, see Lee *et al.*, 1971b) was also evident (Figure 1). Filtration at 18° instead of 0–4° reduced the yield of adsorbed material, mostly at the expense of the rRNA. In this case, the 18S ribosomal component was barely visible.

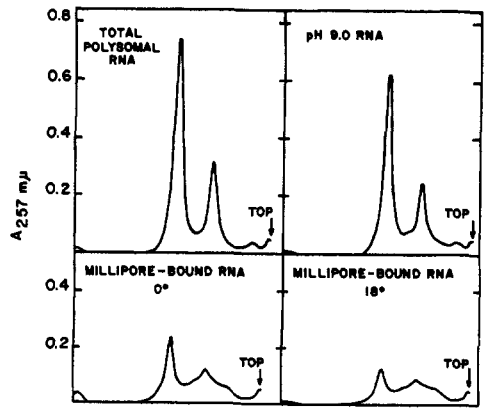

FIGURE 1: Zone sedimentation patterns of unlabeled sarcoma 180 RNA components obtained by phenol fractionation of polysomes and by adsorption on Millipore of total polysomal RNA. Polysomes subjected either to direct phenol extraction in pH 9 Tris buffer (total polysomal RNA) or to sequential extractions in pH 7.6 and pH 9.0 buffers. Samples of total polysomal RNA adsorbed on Millipore filters at either 0–4° or 18°, as described in the Experimental Section. For quantitative aspects, see Table V. Samples of all fractions subjected to zone centrifugation at 12° for 3.5 hr.

Filtration of 8.8 mg of rabbit reticulocyte polysomal RNA led to adsorption of only 40 μg. As with the mouse sarcoma material, some rRNA was still present. Instead of the heterogeneous material, however, a sharp 10S peak constituted the predominant nonribosomal species (Figure 2). There was also some material in the 15S zone, and probably some heterogeneous RNA underlying the 18S peak. The light material near the top of the tube does not appear to represent RNA, since it could also be obtained from blank filters.

The phenol fractionation procedure was less effective in removing rRNA from the unlabeled poly(A)-containing species. A fraction obtained by the sequential extraction procedure at pH 7.6 and pH 9.0, which represented 10% of the total polysomal RNA from sarcoma cells, still consisted primarily of rRNA. The heterogeneous RNA, however, was sufficiently enriched to become noticeable around the 18S ribosomal peak (Figure 1). Filtration of this material led to a considerably greater yield of adsorbed RNA (Table V), showing that the pH 9 fraction was enriched in poly(A)-containing species. The preparation obtained by the two-step procedure, however, showed the same extent of ribosomal contamination as that obtained by direct filtration of the total RNA (Figure 2).

Discussion

This report describes procedures for the separation of a species of polysomal RNA molecules that contain poly(A) from the rest of the polysomal RNA. These procedures rely on unique properties of poly(A). It is shown that this polymer

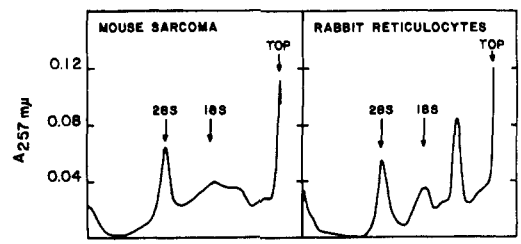

FIGURE 2: Zone sedimentation patterns of unlabeled mouse sarcoma 180 and rabbit reticulocyte polysomal RNA components adsorbed on Millipore filters. Total reticulocyte polysomal RNA and pH 9 RNA fraction from mouse sarcoma cells adsorbed on Millipore at 22°, as described in the Experimental Section. See Table V for quantitative aspects. Samples of eluted material centrifuged at 15° for 4 hr.

binds to denatured proteins under the conditions of phenol extraction in the presence of Tris-HCl (pH 7.6). It will also bind to crystals of potassium dodecyl sulfate and to Millipore filters in the presence of 0.5 M KCl. The Millipore-binding property of poly(A) is shared by single-stranded DNA (Nygaard and Hall, 1963). It is possible that it is due to a peculiar configuration common to the two types of molecules. Both polymers have been found to react with antibodies to single-stranded DNA (D. Stollar, personal communication). The binding of poly(A) to Millipore filters under conditions similar to those used for DNA–RNA hybridization studies (Nygaard and Hall, 1963, Gillespie and Spiegelman, 1965) indicates the possibility of a pitfall in this technique as applied to animal cell studies.

The capacity of poly(A) to bind to Millipore filters provides a convenient and reliable means for the isolation of RNA molecules that contain poly(A) sequences. The procedure described in the Experimental Section leads to apparently undegraded preparations nearly free of rRNA. Preparations entirely free of this material, however, could not be obtained. The behavior of poly(A) during phenol extraction could serve as the basis for another isolation procedure. Sequential phenol extraction of polysomes yields preparations considerably enriched in poly(A)-containing molecules, but still with rRNA as the predominant component. This latter procedure is potentially useful for the large-scale preparation of material enriched in mRNA, but it may not be effective with all cell types. Preliminary experiments with P3 mouse myeloma cells (kindly supplied by Dr. C. Baglioni) have led to the extraction at pH 7.6 of a large proportion of the poly(A)-containing polysomal RNA species.

The available evidence is consistent with the identification of the poly(A)-containing RNA molecules as mRNA. These molecules are labeled rapidly under conditions which prevent rRNA synthesis, and they constitute a heterogeneous population with a range of sedimentation values of 10–30 S (Lee *et al.*, 1971b). They also have a DNA-like base composition (Mendecki *et al.*,[1] 1972). The isolation of a 10S component from rabbit reticulocyte polysomal RNA by the Millipore-binding technique provides additional evidence for the identification of the poly(A)-containing molecules as mRNA. It has been shown that the 10S reticulocyte RNA isolated by zone centrifugation functions as a messenger for globin synthesis (Lockard and Lingrel, 1969). Since it is reasonable to assume that the 10S component retained on Millipore represents the same material as that used for the above study, our results provide evidence for the occurrence of poly(A) in the globin mRNA. The occurrence of adenylate-rich sequences in the reticulocyte 10S RNA has been suggested by the findings of Lim and Canellakis (1970). The reticulocyte polysomes also appear to contain additional mRNA species, as indicated by the sedimentation pattern of the material retained on Millipore.

The occurrence of poly(A) in mRNA appears to be a general characteristic of mammalian cells, since it has been detected in rat liver, mouse sarcoma cells, HeLa cells, and rabbit reticulocytes. It is possible that the occurrence of poly(A) is widespread among eucaryotic cells. Adenylate-rich RNA fractions refractory to phenol extraction have been detected in *Euglena gracilis* (Brawerman, 1963). It appears, therefore, that the Millipore-binding technique described in this report may constitute a rather general procedure for the isolation of eucaryotic mRNA. It is not known however, whether all mRNA species in animal cells could be obtained in this manner, since the occurrence of mRNA molecules without poly(A) remains a possibility.

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