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Characterization of the Steady-State Population of Messenger RNA and Its Poly(adenylic acid) Segment in Mammalian Cells†

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ABSTRACT: The poly(A) containing RNA of mouse sarcoma and rabbit reticulocyte polysomes was characterized using a method based on complex formation with radioactive poly(U). EDTA treatment of reticulocyte polysomes releases the poly(A) containing material as a homogeneous particle, while the same treatment applied to sarcoma polysomes releases a highly heterogeneous population of particles. The poly(A) segments obtained from steady-state sarcoma polysomal RNA exhibit a more heterogeneous sedimentation profile than the poly(A) from newly synthesized RNA. Their average sedimentation coefficient is also lower. The estimate of the average size of the steady-state poly(A) segment of sarcoma polysomal RNA, based on the observed sedimentation coefficient, is 130 residues, compared to the value of 170 residues for 2-hr labeled poly(A). The poly(A) of reticulocyte polysomal RNA sediments more slowly than the sarcoma poly(A), and appears to consist of two size classes of about 60 and 100 residues. The steady-state poly(A)-protein complex released from RNase-digested sarcoma polysomes is also more heterogeneous than the complex bearing newly synthesized poly(A); it also has an overall lower sedimentation coefficient. The reticulocyte poly(A)-protein complex is more homogeneous and sediments more slowly than the sarcoma steady-state complex. The characteristics of steady-state poly(A) appear to be a reflection of the aging process to which mRNA is subjected after its appearance in the cytoplasm.

Eukaryotic mRNA[1] is frequently detected by virtue of its selective labeling with radioactive precursors. Short periods of isotope incorporation primarily label mRNA present in the polysomes and other cytoplasmic particles (Penman *et al.,* 1963; Perry and Kelley, 1968; Knöchel and Tiedemann, 1972). Selective labeling of mRNA can also be achieved during longer incorporation periods in the presence of low levels of actinomycin D which prevents the synthesis of rRNA (Roberts and Newman, 1966; Penman *et al.,* 1968). Isotope incorporation studies have provided much information on the metabolism of newly synthesized mRNA, but this method is not well suited for the detection of changes which may occur during the life cycle of these molecules. For instance, it has been shown that the poly(A) segment of mammalian mRNA undergoes a gradual reduction in size after its appearance in the cytoplasm (Mendecki *et al.,* 1972; Brawerman, 1973; Sheiness and Darnell, 1973). The

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[1] Abbreviations used are: mRNP, messenger ribonucleoprotein; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); SDS, sodium dodecyl sulfate; EDTA, disodium ethylenediaminetetraacetate, mRNA, messenger ribonucleic acid.

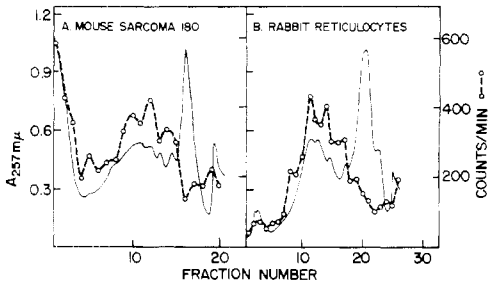

FIGURE 1: Zone sedimentation of sarcoma (A) and reticulocyte (B) polysomes. Two A_{260} units of polysomes was centrifuged at 45,000 rpm for 25 min in the Spinco SW50 rotor through 4.5-ml gradients containing a 0.5-ml cushion of 2 M sucrose at the bottom of the tube. (—) Absorbancy at 257 mμ. (- - -) Distribution of poly(A) as determined by [^{3}H]poly(U) assay.

steady-state population of poly(A) has been studied in mouse L cells labeled for long periods of time with ^{32}P and shown to be considerably more heterogeneous than newly synthesized poly(A) (Greenberg and Perry, 1972). Isotope incorporation methods are also unfavorable for detection of mRNA in mammalian reticulocytes, which are not active in transcription, or in other tissues which are not readily accessible to radioactive precursors.

In this report, we describe the characteristics of the steady-state population of mRNA and its poly(A) segment in an actively dividing and relatively unspecialized cell line, mouse sarcoma 180 ascites cells; and in a highly differentiated cell type which no longer synthesizes RNA, the rabbit reticulocyte. The poly(A) sequences were detected by complex formation with labeled poly(U) followed by removal of the excess poly(U) by RNase digestion. The size distributions of the poly(A) segments in the polysomal mRNA of the two cell types are compared. The poly(A)–protein complexes (Kwan and Brawerman, 1972) of sarcoma and reticulocyte polysomes were also characterized.

Experimental Section

Cell Lysate Preparation. Detailed procedures for the maintenance, labeling, and disruption of mouse sarcoma 180 ascites cells have been described elsewhere (Lee *et al.*, 1971a,b; Mendecki *et al.*, 1972). All cells were incubated in nutrient medium for 1 hr at 37° prior to disruption. Labeled cells were prepared by supplementation of the medium with 5 μCi/ml of [2,8-^{3}H]adenosine (32 Ci/mmol) or 1 μCi/ml of [8-^{14}C]adenine (60 Ci/mol, New England Nuclear, Boston, Mass.) in the presence of 0.04 μg/ml of actinomycin D to prevent labeling of rRNA (Penman *et al.*, 1968). After a 2-hr labeling period cells were lysed as described previously (Lee *et al.*, 1971a), and centrifuged at 1000*g* for 10 min to separate the nuclear fraction and the supernatant which was used for the preparation of polysomes.

Reticulocytes were obtained from the plasma of New Zealand white rabbits made anemic by daily injections of 1.2% phenylhydrazine hydrochloride (Housman *et al.*, 1970). The reticulocytes were washed three times in ice-cold saline solution (140 mM NaCl–5 mM KCl–1.5 mM $MgCl_2$) supplemented with 0.001% heparin and lysed by the addition of 1.5 volumes of 2 mM $MgCl_2$–1 mM 2-mercaptoethanol. The lysate was cleared twice by centrifugation at 10,000*g* for 15 min.

Polysome Preparation. Polysomes were prepared from the sarcoma and reticulocyte lysates by the Mg^{2+} precipitation method described previously (Mendecki *et al.*, 1972). The cleared lysates were supplemented with 0.1 volume of 0.3 M $MgCl_2$ and kept on ice for 30 min. The precipitated polysomes were recovered by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.6)–50 mM KCl–1 mM $MgCl_2$, and stored in liquid N_2.

RNA Extraction. Polysomal RNA was prepared by phenol extraction of polysomes in the presence of 0.1 M Tris-HCl (pH 9.0) and 0.5% SDS following the method of Brawerman *et al.* (1972). The aqueous phases were reextracted three times with fresh phenol and precipitated in the cold by the addition of 0.05 volume of 2 M NaCl and 2.5 volumes of 95% ethanol.

Zone Sedimentation. Zone sedimentations were carried out in the Spinco SW50 rotor (5-ml gradients) or the Spinco SW41 rotor (12-ml gradients) through 10–30% linear sucrose gradients in 50 mM Tris-HCl (pH 7.6)–50 mM KCl–1 mM $MgCl_2$, unless otherwise indicated. Gradients were collected, monitored for absorbance at 257 mμ, fractionated, and processed for measurements of acid-insoluble radioactivity as described previously (Lee *et al.*, 1971a).

Assay for Unlabeled Poly(A). The fractions obtained by zone sedimentation were assayed for poly(A) containing material as described by Kwan and Brawerman (1972). Aliquots were incubated with 5 nCi of [^{3}H]poly(U) (20 Ci/mmol; Miles Laboratories, Elkhart, Ind.) in 10 mM Tris-HCl (pH 7.6)–200 mM NaCl–5 mM $MgCl_2$ for 15 min at 25°. Pancreatic RNase A was added to a concentration of 0.25 μg/ml and the incubation was continued for 30 min. The incubation mixtures were chilled and processed for measurement of acid-insoluble radioactivity. In all cases, the amount of RNA used for the assay was maintained within a range where the extent of [^{3}H]poly(U) annealing was proportional to the amount of sample added. Blank values (assays without added RNA) were usually around 10–30 cpm as compared to assay values of 100–1000 cpm. Similar assays utilizing [^{3}H]poly(U) for the detection of unlabeled poly(A) have been developed by Gillespie *et al.* (1972) and Bishop *et al.* (1974).

Results

mRNA in Sarcoma and Reticulocyte Polysomes. The steady-state distribution of poly(A) containing RNA in sarcoma and reticulocyte polysomes (Figure 1) was found to be similar to that of newly synthesized polysomal mRNA studied by labeling with radioactive precursors (Brawerman, 1973). Disruption of sarcoma polysomes with EDTA led to the release of steady-state mRNA as a highly heterodisperse population of mRNP particles with sedimentation coefficients ranging from 25 S to more than 100 S (Figure 2). This distribution is similar to that observed with newly synthesized polysomal mRNA (Spirin *et al.*, 1964; Perry and Kelley, 1966; Infante and Nemer, 1968; Henshaw and Loebenstein, 1970; Kafatos, 1968; Lee *et al.*, 1971a; Lee and Brawerman, 1971). On the other hand, reticulocyte polysomes yielded the majority of the mRNP material as a 15S component after EDTA treatment (Figure 3). Huez *et al.* (1967) have shown that a similar component contains a 9S RNA coding for the globin chains. The difference in distribution of mRNP particles released from sarcoma and reticulocyte polysomes may not necessarily be a reflection of the degree of complexity of their respective mRNA populations. Rat liver mRNP particles exhibiting widely different sedimentation rates have been shown to contain RNA molecules of similar size (Lee and Brawerman, 1971; Olsnes, 1971).

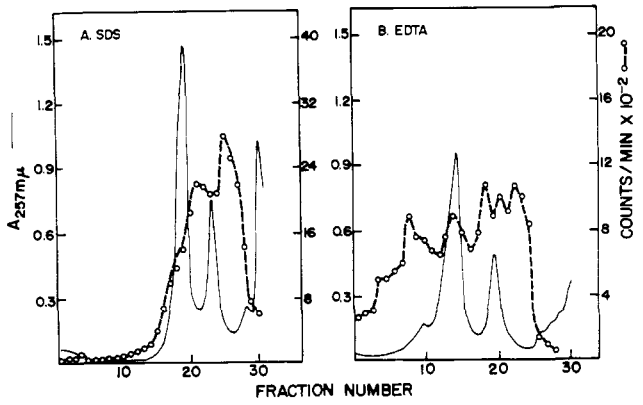

FIGURE 2: Sedimentation profiles of poly(A) containing material from sarcoma polysomes disrupted with SDS (A) or EDTA (B). Five A_{260} units of polysomes was brought to a concentration of either 0.5% SDS or 20 mM EDTA in 50 mM Tris-HCl (pH 7.6)-50 mM KCl-1 mM $MgCl_2$. The EDTA-treated sample was kept on ice for 10 min, while the SDS-treated sample was warmed to 37° for 1 min prior to centrifugation at 41,000 rpm for 3 hr in the Spinco SW41 rotor. For other details see Figure 1.

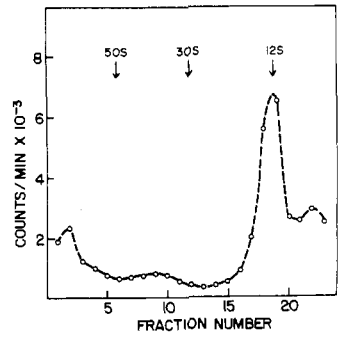

FIGURE 4: Zone sedimentation of the newly synthesized poly(A) protein complex from sarcoma polysomes. Polysomes labeled with [^{3}H]adenosine were incubated at 30° for 8 min with 1 μg/ml of RNase in 50 mM Tris-HCl (pH 7.6), 100 mM KCl, and 5 mM $MgCl_2$ prior to centrifugation at 41,000 rpm for 5 hr in the Spinco SW41 rotor. Fractions were incubated for 20 min at 30° with 1 μg/ml of RNase in 50 mM Tris-HCl (pH 7.6)-100 mM KCl-15 mM EDTA-5 mM $MgCl_2$ prior to measurement of acid-insoluble radioactivity. EDTA treated polysomes were run in a separate tube to provide markers for the 50S and 30S positions.

Deproteinization of sarcoma polysomes with SDS leads to the liberation of heterodisperse poly(A) containing RNA with far lower *s* values than those of EDTA-released mRNP (Figure 2A). The size distribution of this steady-state mRNA ranges from about 8 S to 35 S and is similar to that observed for newly synthesized mRNA from purified sarcoma polysomes (Lee *et al.*, 1971a).

Characteristics of the Poly(A)-Protein Complex from Sarcoma and Reticulocyte Polysomes. A complex containing poly(A) and protein components has been isolated from RNase-digested sarcoma polysomes (Kwan and Brawerman, 1972). The poly(A)-protein complex can be detected directly by incorporation of labeled adenosine, or indirectly through its capacity to form RNase-resistant structures with labeled poly(U). Figure 4 shows that the newly synthesized sarcoma poly(A)-protein complex sediments as a relatively homogeneous 12S component after RNase digestion of adenosine-labeled polysomes. The more slowly sedimenting component has been shown not to contain poly(A) (Kwan and Brawerman, 1972). On the other hand, assay of the total population of polysomal poly(A)-protein complexes with labeled poly(U) revealed a more heterogeneous distribution with components ranging in sedimentation coefficients from 9 S to 12 S (Figure 5A). The heterogeneous components represent the steady-state distribution of poly(A)-protein complexes and indicate that these complexes exist in a range of sizes or shapes within mature sarcoma polysomal mRNA.

A poly(A)-protein complex was also obtained by zonal centrifugation of RNase-digested reticulocyte polysomes (Figure 5B). The reticulocyte poly(A)-protein complex, which was detected by its interaction with labeled poly(U), showed a more homogeneous distribution than that of the steady-state sarcoma poly(A)-protein complexes, with a single peak sedimenting at about 8 S. The reticulocyte complex presumably represents an association of the poly(A) segment of the globin mRNA species with protein components.

Characteristics of the Sarcoma and Reticulocyte Poly(A) Segments. The poly(A) segments obtained from 2-hr labeled sarcoma polysomal RNA exhibited a sedimentation coefficient of 3.8 S when compared to *E. coli* tRNA (Figure 6A). Steady-state sarcoma poly(A) showed a

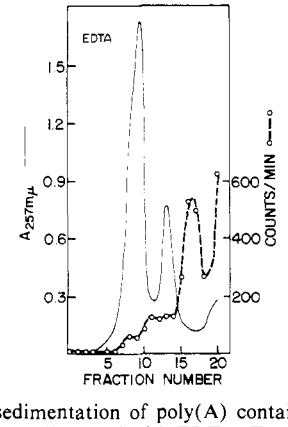

FIGURE 3: Zone sedimentation of poly(A) containing material from reticulocyte polysomes disrupted with EDTA. Two A_{260} units of polysomes was treated with EDTA as in Figure 2 and centrifuged at 45,000 rpm for 100 min in the Spinco SW50 rotor. See Figure 1 for other details.

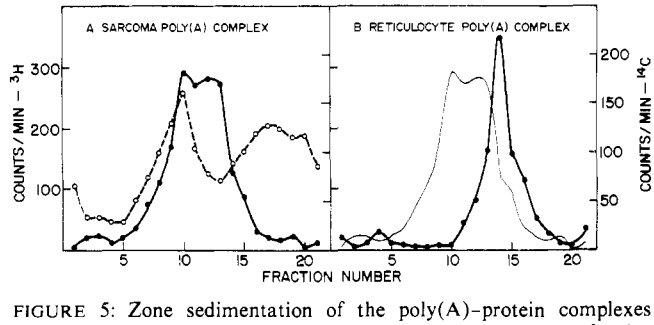

FIGURE 5: Zone sedimentation of the poly(A)-protein complexes from sarcoma and reticulocyte polysomes. Two A_{260} units of polysomes was digested with RNase as in Figure 4 and centrifuged through sucrose gradients in 50 mM Tris-HCl (pH 7.6)-100 mM KCl-1 mM $MgCl_2$ for 18 hr at 35,000 rpm in the Spinco SW50 rotor. (A) Comparison of sedimentation profiles of newly synthesized and steady-state poly(A) complex from sarcoma polysomes. Polysomes labeled with [^{14}C]adenine were used in order to permit comparison of newly synthesized (- - -) and total (—) poly(A) complex. Distribution of total poly(A) was determined by the [^{3}H]poly(U) assay on separate aliquots of each fraction. (B) Comparison of sedimentation profiles of steady-state poly(A)-protein complexes from reticulocyte and sarcoma polysomes. (—) Distribution of reticulocyte poly(A) as determined by [^{3}H]poly(U) assay. Sedimentation profile of steady-state sarcoma poly(A)-protein complex from Figure 5A is reproduced (light line) for comparison.

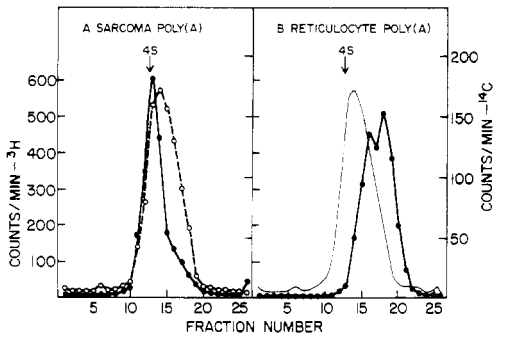

FIGURE 6: Zone sedimentation of poly(A) from sarcoma and reticulocyte polysomal RNA. Five A_{260} units of polysomal RNA was digested with RNase as in Figure 4. RNase was removed by phenol extraction in the presence of 0.1 M Tris-HCl (pH 9.0), phenol was removed from the aqueous phase by ether extraction, and ether was removed by an air current. The deproteinized RNA was centrifuged through sucrose gradients in 50 mM Tris-HCl (pH 7.6)–100 mM KCl–5 mM $MgCl_2$ for 40 hr at 40,000 rpm in the Spinco SW41 rotor. (A) Comparison of sedimentation profiles of newly synthesized poly(A) and steady-state poly(A) from sarcoma polysomal RNA. (—) [^{14}C]Adenine polysomal RNA used in order to permit comparison of newly synthesized and total poly(A); (- - -) distribution of total poly(A) as determined by [^{3}H]poly(U) assay on separate aliquots of each gradient fraction. (B) Comparison of sedimentation profiles of steady-state poly(A) from reticulocyte and sarcoma polysomes. Sedimentation profile of steady-state sarcoma poly(A) from Figure 6A is reproduced (light line) for comparison. Position of 4S marker was determined by centrifugation of *E. coli* tRNA in a separate tube.

broader sedimentation distribution which completely overlapped that of the newly synthesized poly(A) and also contained a high proportion of more slowly sedimenting material. The average sedimentation coefficient of the steady-state poly(A) was 3.4 S. The same sedimentation values of 3.8 S for short-term labeled poly(A) and 3.4 S for steady-state poly(A) were obtained in three independent sedimentation experiments. In Table I we show a comparison of the sedimentation coefficients and estimated molecular weights of steady-state and newly synthesized poly(A) chains. Approximate molecular weights were obtained from the data of Fresco and Doty (1957) relating poly(A) sizes to sedimentation coefficients. Sedimentation conditions in our experiments are different from those of Fresco and Doty and as such our values can only be taken as rough estimates. For instance, from a sedimentation coefficient of 3.8 S we calculate a chain length of 220 nucleotides for short-term labeled sarcoma poly(A); however, Mendecki *et al.* (1972) arrive at a value of 170 by comparison of the ratio of adenylic acid to adenosine residues released after alkaline hydrolysis. The data in Table I on the average chain lengths estimated for the steady-state poly(A) are consistent with the observation that the poly(A) segment is subjected to a gradual size reduction, presumably as a result of exonucleolytic cleavage, during the course of mRNA aging (Mendecki *et al.*, 1972; Brawerman, 1973; Sheiness and Darnell, 1973). They also indicate that the reduction is not indefinite, since a large proportion of the steady-state sarcoma mRNA molecules appear to contain a poly(A) segment of more than 100 nucleotide residues.

The sedimentation profile of reticulocyte poly(A) is shown in Figure 6B. The distribution of reticulocyte poly(A), presumably derived mainly from the globin mRNA species, is bimodal with components sedimenting at about 2.4 S and 3.0 S. A bimodal distribution was observed in three separate sedimentation experiments. The sedimentation cofficients of the reticulocyte poly(A) peaks correspond to poly(A) segments with chain lengths of approximately 60 and 100 nucleotide residues respectively (Table I). The size of the poly(A) segment of mammalian reticulocytes has been estimated to be in the range of 30–80 nucleotides by various investigators (Lim and Canellakis, 1970; Hunt, 1973; Gorski *et al.*, 1974). In mouse reticulocytes Mansbridge *et al.* (1974) have been able to fractionate poly(A) into two major components consisting of molecules composed of approximately 50 and 75 nucleotides. These latter segments may correspond to the two classes of rabbit globin poly(A) segments partially separated by zone sedimentation in the present study. There is no evidence supporting the possibility that these two classes of poly(A) segments are unique to the α and β globin mRNA species (Mansbridge *et al.*, 1974).

TABLE I: Relative Sedimentation Coefficients and Approximate Molecular Weights of Poly(A) Segments.

	Sarcoma		
	Short-term labeled	Steady State	Reticulocyte Total
s_w	3.8	3.4	3.0, 2.4
Approximate molecular weight $\times 10^4$	7.6	5.7	4.3, 2.5
Size (number of residues)			
I	220	170	130, 70
II	170	130	100, 60

[a] s_w calculated relative to 4S *E. coli* tRNA. Molecular weight and size (I) were obtained from the calibration curve of Fresco and Doty (1957). Sizes (II) were also calculated using a value of 170 residues for the short-term labeled sarcoma poly(A) (Mendecki *et al.*, 1972) and the slope relating molecular weight to S_w from the data of Fresco and Doty.

Discussion

This report describes the characterization of steady-state mRNA molecules and their poly(A) segments from mouse sarcoma and rabbit reticulocyte polysomes. Detection of these molecules is based on the ability of the poly(A) sequence to form stable base-paired complexes with labeled poly(U). The results suggest that a major change which occurs during the life cycle of mRNA is related to alterations in the size of its poly(A) segment. Steady-state sarcoma poly(A) sediments more slowly and with a greater degree of heterogeneity than newly synthesized poly(A). Greenberg and Perry (1972) have described a similar phenomenon in the steady-state poly(A) of mouse L cells labeled for long periods of time with ^{32}P. Considerable heterogeneity in the steady-state poly(A) of *Xenopus* tissue culture cells has also been reported (Rosbach and Ford, 1974). The reduced size of mature poly(A) is probably a consequence of gradual hydrolysis after its appearance in the cytoplasm (Brawerman, 1973; Sheiness and Darnell, 1973).

According to our results cleavage of the poly(A) sequence does not continue indefinitely as the mRNA ages in the cytoplasm. A terminal size appears to be reached. The final size of the poly(A) segment may vary between different cell types or different mRNA species. In sarcoma cells, which must contain a wide variety of different species of mRNA, the steady-state poly(A) is heterogeneous, with a

large proportion of the poly(A) segments being larger than 100 nucleotides. On the other hand, steady-state mRNA from reticulocytes, which are terminally differentiated cells, contain poly(A) segments which are shorter. The reticulocyte segments are likely to have already undergone cytoplasmic shortening, since Pemberton and Baglioni (1972) have shown that newly synthesized globin mRNA from duck erythroid cells contains a poly(A) segment of about 170 nucleotides. These results demonstrate that poly(A) segments of significant length are still present in the globin mRNA species of mammalian reticulocytes. Precise information concerning the terminal length of reticulocyte poly(A) segments cannot be provided by the present study since the minimal size of stable [^{3}H]poly(U):poly(A) complexes is unknown. It is clear, however, that the assay is adequate for detection of the bulk of the steady-state poly(A) segments of the sarcoma cells since segments smaller than those present in sarcoma are detected in reticulocyte polysomes.

Our experiments and those of Kwan and Brawerman (1972) suggest that the steady-state sarcoma poly(A)–protein complex in polysomes is on the average much smaller and considerably more heterogeneous than the newly synthesized complex. At present it is unknown whether these age-dependent modifications in the complex are solely related to continued erosion of the poly(A) moiety. Removal of protein components or alterations in their structure as the mRNA ages could also contribute to the reduced sedimentation coefficients characteristic of the steady-state complex. Loss of protein components from the complex could either be the cause or the result of age-dependent cleavage of the poly(A) moiety.

The reticulocyte poly(A)–protein complex was found to be rather homogeneous and sedimented more slowly than the sarcoma complex. While the slower sedimentation could be attributable to the smaller average size of the poly(A) segment, the relative homogeneity must be contrasted to the rather heterogeneous size distribution of the poly(A). Thus the unique size of the poly(A)–protein complex in globin mRNA could be related to the presence of distinct protein species as well as smaller poly(A) segments. This brings up the possibility that discrete classes of mRNA may be distinguished by particular protein components attached to the poly(A) sequence at their 3′ termini.

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