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Characterization of Messenger Ribonucleoprotein and Messenger RNA from KB Cells

(eukaryotic cells/polysomes/molecular weight/density gradient centrifugation)

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ABSTRACT **Messenger ribonucleoprotein and mRNA from KB-cells were isolated under conditions designed to minimize nonspecific RNA-protein interaction and to minimize degradation by contaminating ribonucleases. A large fraction, 60–70%, of the messenger ribonucleoprotein from polysomes dissociated *in vitro* by either EDTA or puromycin sedimented faster than the large ribosome subunit. Messenger ribonucleoprotein particles with sedimentation coefficients up to 200 S were observed. Released mRNA was also large, with maximal molecular weights around 5×10^6.**

Efforts have been made to unravel the mechanisms of transcription, processing, and transport of mRNA from nucleus to cytoplasm in eukaryotic cells. The mRNA appears to be associated with proteins (1–4), although the function of these messenger ribonucleoprotein (mRNP) complexes is unknown. Since these complexes could be artifacts of the cell fractionation procedures, their characterization depends on elimination of nonspecific interactions between RNA and proteins during their preparation (5, 6); elimination of RNase activities in cell extracts is also important.

We have characterized the mRNP and mRNA from KB cells under conditions that minimized the presence of artifacts. We avoided nonspecific interactions between RNA and protein by preparing cytoplasmic extracts in isotonic buffer; subsequently, polysomes were prepared in either isotonic or hypertonic buffer (0.5 M KCl). mRNP was released from the polysomes in either of two ways, either by EDTA (3, 4) or by puromycin treatment *in vitro* in a buffer of high salt concentration (7). The sedimentation rate and buoyant density of such mRNP were determined. Degradation by ribonucleases from the cytoplasmic extract was avoided as follows: the RNA from the polysomes was released from attached proteins by treatment with Sarkosyl and Pronase; RNA size was determined by centrifugation in a sucrose density gradient and by electrophoresis on polyacrylamide gel in Sarkosyl-containing buffers at 4°C. Ribonucleases contaminating the reagents used were removed either by treatment with diethylpyrocarbonate (DEP) or by autoclaving.

Our results indicate that both mRNA and mRNP are significantly larger than has been described. Furthermore, the buoyant density determination of mRNP from polysomes dissociated as described above inferred that mRNP contains different classes of proteins.

Abbreviation: mRNP, messenger ribonucleoprotein.

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MATERIALS AND METHODS

Cell culture and radioactive labeling

KB cells (heteroploid cell line of human origin obtained from Flow Laboratories Ltd, Scotland) (8) were grown in Eagles' spinner medium (9) with double-strength amino acids. The medium contained penicillin and streptomycin and was supplemented with 7% calf serum. Tests (culturing on agar plates) for contaminating mycoplasma were negative. Cells were usually maintained at a density of $2–4 \times 10^5$ cells/ml. They were concentrated ten-fold and incubated with 0.05 μg/ml of actinomycin D for 15 min before radioactive labeling. This concentration of actinomycin D stops synthesis of ribosomal RNA without affecting mRNA or tRNA synthesis (10–13). The cells were labeled in the continued presence of the drug with either [5-^{3}H]uridine (New England Nuclear Corp., 25 Ci/mmol) or [^{14}C]uridine (New England Nuclear Corp., 0.5 Ci/mmol) as stated in the text. Manipulations until this stage were performed at 37°C. Incorporation of radioactive precursors was terminated by the addition of sufficient crushed, frozen balanced salt solution (see below) to lower the temperature of the culture to 0–4°C.

Solutions

Balanced Salt Solution. 0.17 M NaCl–34 mM KCl–10 mM Na_2HPO_4–10 mM KH_2PO_4 (pH 7.3).

Isotonic Buffer. 0.15 M NaCl–10 mM Tris·HCl (pH 7.8)–1.5 mM $MgCl_2$.

High-Salt Buffer. 0.5 M KCl–50 mM Tris·HCl (pH 7.8)–5 mM $MgCl_2$.

High Salt–EDTA Buffer. 0.25 M KCl–10 mM EDTA–10 mM Tris·HCl (pH 7.8).

Low Salt-EDTA Buffer. 10 mM NaCl–10 mM EDTA–10 mM Tris·HCl (pH 7.8).

Sarkosyl–Pronase Buffer. 50 mM Tris·HCl (pH 7.8)–0.1 M EDTA–0.5% (w/w) Sarkosyl–0.5 mg/ml Pronase.

Sarkosyl Buffer. 0.1 M NaCl–10 mM EDTA–10 mM Tris·HCl (pH 7.8)–0.5% (w/w) Sarkosyl.

Sarkosyl (lauryl-sarcosine) was obtained from Geigy and Pronase (RNase-free) was from Calbiochem. Stock solutions of 52% (w/w) sucrose containing appropriate amounts of $MgCl_2$ or NaCl or both were treated with diethylpyro-

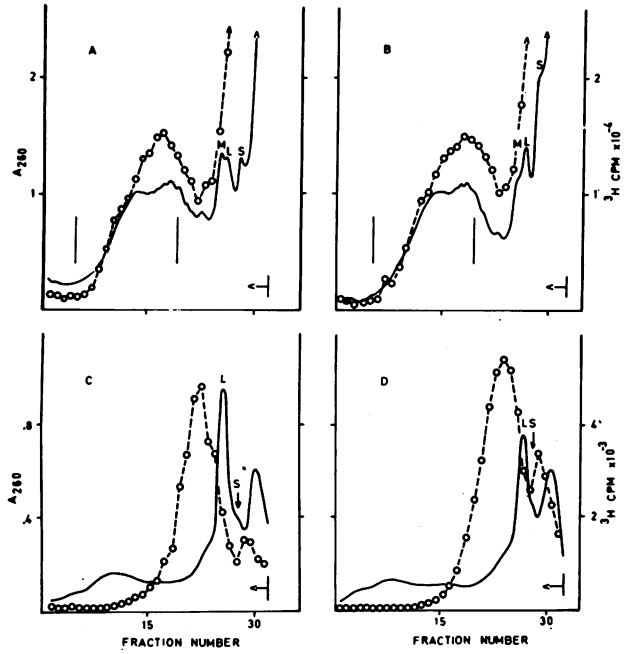

FIG. 1. Polysomes and mRNP analyzed by sucrose gradient centrifugation. Cells were labeled with [^{3}H]uridine (100 μCi/ml of medium) for 2 hr. Cytoplasm was prepared and centrifuged on 7–47% sucrose gradients in isotonic buffer (*A*) and high-salt buffer (*B*) for the preparation of isotonic and high-salt polysomes, respectively. Polysomes from pooled fractions (*within bars*) were dissociated with EDTA and again centrifuged on 7–47% sucrose gradients in low salt–EDTA buffer (*C*) and high salt–EDTA buffer (*D*) for the preparation of isotonic and high salt mRNP (EDTA), respectively. All gradients were centrifuged in a Spinco SW27 rotor for 3 hr at 26,000 rpm (4°C). Absorbance at 260 nm, ——; cpm in [^{3}H]RNA, O– – –O.

carbonate (Baycovin, Bayer Leverkusen, Germany) (0.3 ml/100 ml solution) at 37°C for 16 hr (14, 15). We removed the remaining traces of diethylpyrocarbonate by heating the stock solutions in a boiling-water bath for 10 min. Appropriate amounts of Tris·HCl (pH 7.8) or EDTA from separately autoclaved stock solutions were added to give the desired concentrations of sucrose.

Cell fractionation

The cells were centrifuged, washed once with cold balanced salt solution, and lysed in 2.3 ml (per 4–8 × 10^7 cells) of isotonic buffer containing 0.65% NP 40 (Nonidet P40, Shell Chemical Corp.) for 10 min at 0°C (16). The nuclei were sedimented at 1000 rpm (100 × *g*) for 3 min (supernatant CI), washed once with 1.15 ml of the lysis buffer, and centrifuged again (supernatant CII). The cytoplasm (CI + CII) was centrifuged at 10,000 rpm (10,000 × *g*) for 10 min to remove remaining nuclei and cell fragments, and the supernatant fluid was layered on a 7–47% sucrose gradient for the preparation of polysomes, as described in Fig. 1.

Analysis of polysomal mRNP and mRNA

mRNP particles were released from polysomes prepared in isotonic buffer with 0.02 M EDTA and from polysomes prepared in high-salt buffer with either 0.02 M EDTA or 1 mM puromycin. For dissociation of polysomes by puromycin *in vitro*, the samples were incubated for 20 min at 37°C without prior incubation at 0°C (17). This procedure gave a complete dissociation of the polysomes, whereas the original procedure (7) in our hands yielded only 50–70% dissociation. Dissociated polysomes from isotonic buffer were analyzed in sucrose gradients made in low salt–EDTA buffer; the high-salt polysomes were analyzed either in high-salt or high salt–EDTA buffer. All gradients were eluted through a Gilford recording spectrophotometer.

For buoyant density determination of mRNP, the samples were fixed with 6% glutaraldehyde and centrifuged in 5-ml preformed CsCl gradients made in isotonic buffer containing 0.8% Brij 35 (Pierce Chemical Co., Rockford, Ill.) (18). The gradients were usually centrifuged at 45,000 rpm for 15 hr in a Spinco SW 50.1 rotor. Gradients were fractionated by puncturing the bottom of the tubes and collecting fractions (12 drops) directly on Whatman 3 MM filter-paper discs. Density was determined by refractive index readings on every fifth fraction. To determine radioactivity, we treated the filter-paper discs with ice-cold 10% Cl_3CCOOH for 10 min, followed by 5% Cl_3CCOOH and 70% ethanol rinses. Dried discs were immersed in toluene-based scintillant and counted in a Packard Tri-Carb spectrometer.

We prepared polysomal RNA by incubating the pooled polysomal fractions in Sarkosyl–Pronase buffer for 3 min at 37°C. It was analyzed on sucrose gradients made in Sarkosyl buffer. Parallel samples were analyzed on 5-ml, 5–20% sucrose gradients in dimethylsulphoxide containing 0.01 M LiCl. Polysomal RNA was also analyzed by electrophoresis on polyacrylamide gel, as described by Peacock and Dingman (19) and modified by Öberg and Philipson (20). As a further modification, sodium dodecyl sulphate was replaced by Sarkosyl, and electrophoresis was performed at 4°C. The gels were sliced and analyzed for radioactivity (19).

RESULTS

Size distribution of polysomes and mRNP

The profiles of polysomes prepared in isotonic and high-salt buffers from cells labeled with [^{3}H]uridine are shown in Fig. 1, *A* and *B*. In isotonic buffer (Fig. 1*A*) the locations of

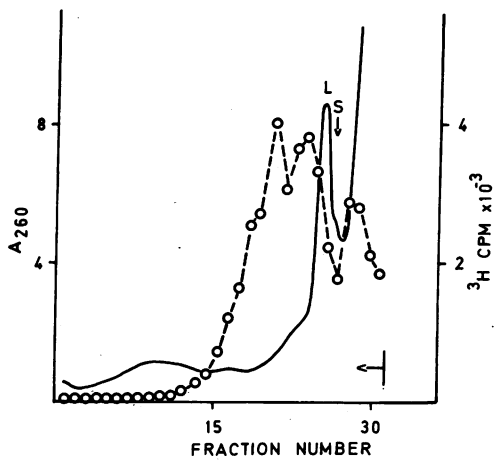

FIG. 2. Size distribution of mRNP from polysomes dissociated with puromycin. Polysomes from the pool described in Fig. 1*B* were dissociated with puromycin *in vitro* and centrifuged in parallel with the samples of Fig. 1, *C* and *D*, on a 7–47% sucrose gradient in high-salt buffer. Absorbance at 260 nm, ——; cpm in [^{3}H]RNA, O– – –O.

monosomes (M), and large (L) and small (S) ribosomal subunits are indicated by distinct peaks. In the high-salt buffer (Fig. 1*B*), the whole pattern is shifted somewhat to the right, and the monosomes are partially dissociated to subunits.

The pooled polysomes (*bars*, Fig. 1, *A* and *B*), were dissociated with EDTA and treated as described in the figure legend. A sample of the high-salt polysomes was dissociated with puromycin and analyzed in high-salt buffer in parallel (Fig. 2). Most of the absorbance was recovered in particles sedimenting as ribosomal subunits or more slowly, indicating complete dissociation of the polysomes (Fig. 1, *C* and *D*, and Fig. 2). Radioactivity was recovered in structures sedimenting between 5 and 200 S, with a major peak at 120 S. No labeled material was observed in the polysomal region. 60–70% of the labeled material sedimented faster than the large ribosomal subunit, whether polysomes prepared in isotonic or high-salt buffer were used to prepare mRNP, or whether EDTA or puromycin was used for dissociation.

Buoyant density of polysomes and mRNP

A comparative study of the buoyant density of mRNP from isotonic buffer and mRNP prepared in high-salt buffer was made with polysomes prepared in RNase-free media. Fig. 3

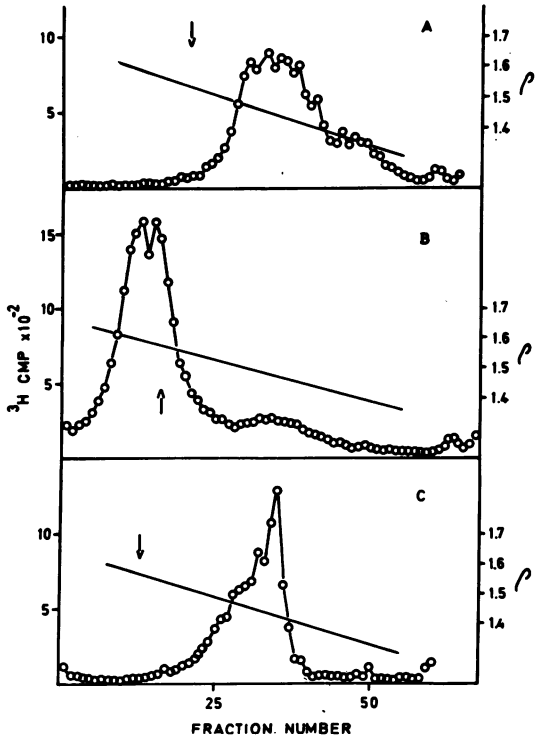

FIG. 3. Buoyant density of mRNP prepared in RNase-free media. Pooled polysomes (see Fig. 1, *A* and *B*) were dissociated with EDTA, fixed with glutaraldehyde and analyzed on CsCl gradients (see *Methods*). (*A*, *isotonic*-mRNP; *B*, *high-salt* mRNP.) *High-salt* polysomes (see Fig. 1 *B*) were also treated with puromycin for the preparation of mRNP (*puro*) (*C*), fixed with glutaraldehyde, and analyzed on a CsCl gradient. This sample was kept at 0°C for an additional 30 min after the incubation at 37°C (see *Methods*) to ensure complete dissociation before fixation. The gradients were centrifuged in a Spinco SW 50.1 rotor for 18 hr at 40,000 rpm (4°C).

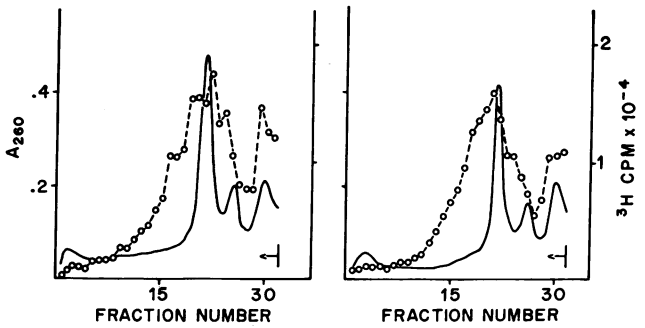

FIG. 4. Size distribution of polysomal RNA in sucrose gradients. Pooled polysomes (Fig. 1, *A* and *B*) were treated with Sarkosyl–Pronase buffer (see *Methods*) and layered on 15–30% sucrose gradients in 0.1 M NaCl–10 mM EDTA–10 mM Tris·HCl (pH 7.8)–0.5% (w/w) Sarkosyl. The gradients (*Left*, RNA from *isotonic* polysomes; *Right*, RNA from *high-salt* polysomes) were centrifuged at 4°C for 12 hr at 26,000 rpm in an SW 27 rotor. Absorbance at 260 nm, ——; ^{3}H-cpm, O– – –O.

shows that mRNP from isotonic buffer was distributed between 1.35 and 1.47 g/cm^3, which is a broader range than earlier reported (3, 13). The mRNP from high-salt buffer, derived by EDTA dissociation, banded at 1.57–1.60 g/cm^3, and thus contained far less protein than the mRNP from isotonic buffer. The mRNP from high-salt buffer, obtained by puromycin dissociation, had a density of 1.40–1.50 g/cm^3, with the most prominent peak at 1.43 g/cm^3. Similar preparations of mRNP from high-salt buffer were also examined by isopycnic banding in Cs_2SO_4 gradients (not shown). All the pulse-labeled RNA banded at the density of mRNP (1.45 g/cm^3); no free RNA was observed.

The size distribution of polysomal RNA

Pooled polysomes (see Fig. 1, *A* and *B*) were treated with Sarkosyl–Pronase buffer for 3 min at 37°C. The RNA released from the polysomes was analyzed on sucrose gradients at 4°C (Fig. 4). The absorbance was largely caused by ribosomal RNA and tRNA. The acid-insoluble radioactivity sedimented between 4 and 65 S, and about 60% sedimented faster than 28 S ribosomal RNA. The maximum molecular weight for the mRNA estimated from this analysis was between 5 and 10 × 10^6.

Fig. 5 shows a parallel analysis of the same polysomal RNA by electrophoresis on polyacrylamide gels. About 60% of the radioactivity migrated more slowly than 28 S ribosomal RNA. Thus, analysis of polysomal RNA from both isotonic and high-salt buffer by sucrose density gradients and gel electrophoresis showed that the majority of the mRNA was larger than 28 S ribosomal RNA.

Finally, polysomes were prepared (labeled and processed as those described in Fig. 1), treated with Sarkosyl–Pronase buffer as above, and analyzed on a sucrose gradient in dimethylsulfoxide. As shown in Fig. 6, the distribution of the labeled RNA in relation to the ribosomal RNA was similar to that obtained from the sucrose density gradient and gel electrophoretic analyses presented above.

DISCUSSION

The mRNP in eukaryotic cells appears to be associated with proteins in the form of ribonucleoprotein particles (2–4, 13,

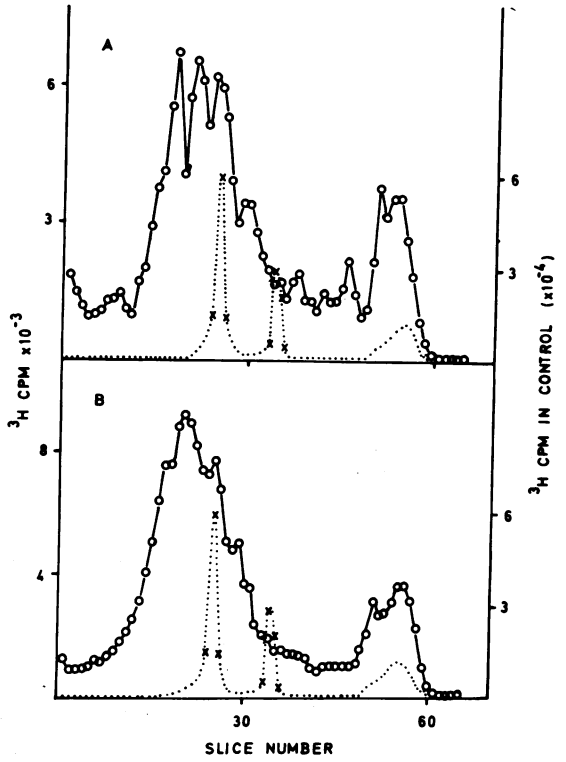

FIG. 5. Size distribution of polysomal RNA in polyacrylamide gels. Polysomal RNA from the same source as in Fig. 4 was analyzed by gel electrophoresis in polyacrylamide gels (see *Methods*). Cytoplasmic RNA from cells labeled overnight with [^{3}H]uridine (20 μCi/ml) was run as a control on a separate gel. ^{3}H-cpm in pulse-labeled polysomal RNA, O- - -O; cpm in control [^{3}H]RNA, $\times\cdots\times$. Migration, *left* to *right*.

21). Recent studies on mammalian cells point to distinctive features of these mRNP particles (13, 21, 22), but the biologic role of the proteins is still unclear.

The mRNP prepared from EDTA-dissociated polysomes has been reported to have *s*-values between 10 and 50 S (3, 4). Our results show that mRNP released from polysomes by either EDTA or puromycin can be much larger. In the experiment illustrated in Fig. 1, *A* and *B*, 60–70% of the mRNP sedimented faster than the large ribosomal subunit. The complete dissociation of the polysomes in this experiment was measured by recovery of the UV-absorbing material as ribosomal subunits and tRNA. The presence of RNA of large molecular weight in these particles may explain their rapid sedimentation, and we have no evidence for remaining ribosomes or subunits in the mRNP. The polysomes as prepared in this work were *not* unusually large.

It appears unlikely that a large part of the radioactive material sedimenting as polysomes is nuclear RNP particles (3, 12, 13, 16) released by nuclear leakage. All material labeled in the presence of actinomycin D was EDTA-sensitive (Fig. 1, *C* and *D*); banding of these polysomes in CsCl gave only one peak (not shown); and, after dissociation of polysomes prepared in isotonic buffer, all labeled material banded in CsCl at about the density (1.45 g/cm^3) characteristic of polysomal mRNP (3, 13). If prepared under isotonic conditions, the nuclear RNP particles band at a density of 1.61 g/cm^3 (unpublished observations).

From the density (1.45 g/cm^3) of mRNP from isotonic buffer (Fig. 3), a composition of 60% protein and 40% RNA could be calculated (24). The mRNP from polysomes prepared in high-salt buffer had a density of 1.58 g/cm^3 that indicated a composition of 40% protein and 60% RNA. This means that compared to the mRNP prepared in isotonic buffer, these particles had lost about 50% of their protein, suggesting that polysomal mRNP might contain at least two different groups of proteins: one which is easily removed by a high-salt wash, and a second that is more firmly bound. The polysomes remain intact in the high-salt environment (23, 24), and functional ribosomal subunits have been obtained under analogous conditions (25–27).

Puromycin-dissociation of polysomes prepared in high-salt buffer (Fig. 2) led to the appearance of mRNP of similar density as EDTA-dissociation of polysomes prepared in isotonic buffer. It should be stressed that we did not detect any free mRNA after dissociation of the polysomes prepared in high-salt buffer either with EDTA or puromycin (compare ref. 34).

It has been claimed that all rapidly-labeled RNA molecules found associated with polysomes of eukaryotic cells (by definition, mRNA) are smaller than 1.7 $\times$ 10^6 daltons (3, 12, 29). On the other hand, the pool of rapidly-labeled nuclear RNA, which contains precursors to cytoplasmic mRNA, contains molecules that are considerably larger (12, 28, 29). This observation has been interpreted to mean that mRNA, like ribosomal RNA and tRNA, is synthesized in the nucleus as large precursor molecules, which at some later stage (but before they become engaged in protein synthesis in the cytoplasm) are cleaved by specific mechanisms (28). This hypothesis was supported by studies of cells transformed by the DNA viruses, SV40 (30, 31) and adenovirus (32), where virus-specific RNA molecules as large as 2–4 $\times$ 10^6 daltons were identified in the nucleus, while their counterparts on the cytoplasmic polysomes all appeared smaller than 1.7 $\times$ 10^6 daltons. However, there is no direct evidence for the proposed cleavage mechanism.

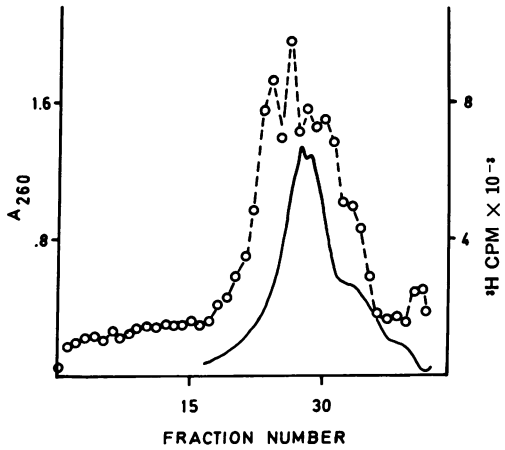

FIG. 6. Size distribution of polysomal RNA analyzed by sucrose gradient centrifugation in dimethylsulfoxide. Polysomes (labeled and prepared as in Fig. 1 *A*) were treated with Sarkosyl–Pronase buffer (see *Methods*) and layered on a 5-ml, 5–20% sucrose gradient in dimethylsulfoxide (containing 0.01 M LiCl), and centrifuged for 16 hr at 20,000 rpm in the Spinco SW 50.1 rotor (25°C). Absorbance at 260 nm, ——; cpm in [^{3}H]RNA, O- - -O.

Our data indicate that mRNA on the polysomes can be larger than 1.7×10^6 daltons (Figs. 4, 5, and 6). The best evidence, perhaps, for this conclusion comes from the analysis of polysomal RNA on sucrose gradients in dimethylsulfoxide. In this medium size comparisons between RNA molecules seem more reliable, since they are made under conditions where the RNA is denatured (33). Even under these conditions, 50% of the rapidly-labeled polysomal RNA sedimented faster than the 28 S ribosomal RNA. The maximum molecular weight of the mRNA, as estimated from our studies, was $5\text{–}10 \times 10^6$; this finding agrees well with the finding of mRNP structures sedimenting with a modal sedimentation coefficient of 120 S.

When RNA from polysomes that were treated with EDTA, Sarkosyl, and Pronase was analyzed by Cs_2SO_4 density gradient centrifugation after fixation with glutaraldehyde (unpublished data), the labeled material banded at a density of 1.63 g/cm^3 (phenol-extracted ribosomal RNA and mRNA markers banded at 1.69 and 1.63 g/cm^3, respectively), an observation that demonstrated that the RNA had been effectively freed from attached proteins. Polysomal RNA treated with Sarkosyl–Pronase buffer, deproteinized by extraction with phenol–chloroform–isoamylalcohol (4:1:0.002) (or with chloroform–isoamylalcohol alone) at 0°, and analyzed on sucrose gradients (unpublished) also displayed a size distribution of the labeled RNA similar to those in Fig. 4.

Our results indicate that the striking difference in molecular weight between polysomal mRNA and its alleged precursors in the nucleus may be fortuitous. If a posttranscriptional cleavage of the mRNA occurs, it may not be as extensive as previously believed. To clarify this point, nuclear RNA must be studied under similar conditions.

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1. Spirin, A. S. & Nemer, M. (1965) *Science* **150,** 214–217.
2. Spirin, A. S. (1966) in *Current Topics In Developmental Biology,* eds. Monroy, A. & Moscona, A. A. (Academic Press, New York), p. 1.
3. Perry, R. P. & Kelley, D. E. (1968) *J. Mol. Biol.* **35,** 37–59.
4. Henshaw, E. C. (1968) *J. Mol. Biol.* **36,** 401–411.
5. Girard, M. & Baltimore, D. (1966) *Proc. Nat. Acad. Sci. USA* **56,** 999–1002.
6. Baltimore, D. & Huang, A. (1970) *J. Mol. Biol.* **47,** 263–273.
7. Blobel, G. & Sabatini, D. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 390–394.
8. Waymouth, C., ed. (1970) *Advances in Tissue Culture* (Williams and Wilkins Co., Baltimore, Md.), Vol. 5, pp. 5 and 9.
9. Eagle, H. (1959) *Science* **130,** 432–437.
10. Perry, R. (1963) *Exp. Cell Res.* **29,** 400–406.
11. Roberts, W. D. & Newman, J. (1966) *J. Mol. Biol.* **20,** 63–73.
12. Penman, S., Vesco, C. & Penman, M. (1968) *J. Mol. Biol.* **34,** 49–69.
13. Spohr, G., Granboulan, N., Morel, C. & Scherrer, K. (1970) *Eur. J. Biochem.* **17,** 296–318.
14. Kivinen, A. (1965) *Suom. Kimistilehti B* **38,** 106–117.
15. Solymosy, F., Fedorcsák, I., Guylás, A., Farkas, G. L. & Ehrenberg, L. (1968) *Eur. J. Biochem.* **5,** 520–527.
16. Borun, R. W., Scharff, M. D. & Robbins, E. (1967) *Biochim. Biophys. Acta* **149,** 302–304.
17. Monneron, A., Liew, C. C. & Allfrey, V. G. (1971) *J. Mol. Biol.* **57,** 335–350.
18. Baltimore, D. & Huang, A. (1968) *Science* **162,** 572–574.
19. Peacock, A. C. & Dingman, C. W. (1968) *Biochemistry* **7,** 668–674.
20. Öberg, B. & Philipson, L. (1969) *Eur. J. Biochem.* **11,** 305–315.
21. Lebleu, B., Marbaix, G., Huez, G., Temmerman, F., Burny, A. & Chantrenne, H. (1971) *Eur. J. Biochem.* **19,** 264–269.
22. Schochetman, G. & Perry, R., *J. Mol. Biol.,* in press.
23. Zylber, E. & Penman, S. (1970) *Biochim. Biophys. Acta* **204,** 221–229.
24. Perry, R. & Kelley, D. (1966) *J. Mol. Biol.* **16,** 255–268.
25. Martin, T. E. & Wool, I. G. (1968) *Proc. Nat. Acad. Sci. USA* **60,** 569–574.
26. Martin, T. E., Rolleston, F. S., Low, R. B. & Wool, I. G. (1969) *J. Mol. Biol.* **43,** 135–149.
27. Falvey, A. K. & Staehelin, T. (1970) *J. Mol. Biol.* **53,** 1–19.
28. Darnell, J. E. (1968) *Bacteriol. Rev.* **32,** 262–290.
29. Warner, J., Soeiro, R., Birnboim, C. & Darnell, J. (1966) *J. Mol. Biol.* **19,** 349–361.
30. Lindberg, U. & Darnell, J. E. (1970) *Proc. Nat. Acad. Sci. USA* **65,** 1089–1096.
31. Tonegawa, S., Bernardini, W. A. & Dulbecco, R. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35,** 823–831.
32. Green, M., Parson, J. T., Pina, M., Fujinaga, K., Gaffier, H. & Landgraf-Leurs, I. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35,** 803–818.
33. Strauss, J. H., Kelley, R. B. & Sinsheimer, R. (1963) *Biopolymers* **6,** 793–807.
34. Blobel, G. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 832–835.