Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 1062-1067

PROTEIN COMPOSITION OF L-CELL MESSENGER RIBONUCLEOPROTEINS

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Received October 30, 1979

SUMMARY: The comparative protein composition of L-cell messenger ribonucleoproteins (mRNPs) was studied as a function of mRNP isolation methodology. Proteins with approximate molecular weights of 75,000, 50,000, 46,000 and 34,000 were common to mRNPs prepared by three methods: (1) isopycnic gradient centrifugation in Cs_2SO_4, (2) binding to poly(U)-glass fiber filters, or (3) oligo-deoxythmidylate cellulose chromatography.

It is well established that eukaryotic cytoplasmic mRNA[1] exists as a ribonucleoprotein complex. A review of mRNP[1] characterization has been published recently (1).

The functional significance of m-proteins[1] which bind to either the 5'- or 3'-terminus of mRNA is yet to be resolved (2-8). However, both positive and negative roles have been suggested for these proteins in the protein biosynthetic process (9-20). In this regard, for such studies, it is essential that reliable methods be employed during isolation and identification of mRNPs and their m-proteins. Thus, starting with a common source of cytoplasmic RNPs from L-cells we have evaluated different mRNP isolation methods by examining the qualitative and quantitative recovery of m-proteins from mRNP particles. These methods do not require crosslinking of RNP[1] particles.

METHODS

Reagents and their respective sources were: ultra-pure Tris[1] and sucrose from Schwartz/Mann; [5-^{3}H]uridine and (^{125}I) NaI from Amersham; "suprapur"

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[1]Abbreviations: mRNA, messenger ribonucleic acid; RNP, ribonucleoprotein; mRNP, messenger ribonucleoprotein; m-protein, proteins from mRNPs; poly(A), poly(riboadenylic acid); poly(U), poly(ribouridylic acid); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl) aminomethane; oligo(dT), oligo(deoxythymidylate); P-200, pellet from a 200,000 x g (average) centrifugation; Me_2SO, dimethyl sufoxide.

0006-291X/79/231062-06$01.00/0

cesium sulfate from E. Merck; poly(U)[1] and poly(A)[1] from Miles Labs; oligo(dT)[1] cellulose, Type 2 from Collaborative Research; GF/C glass filters from Whatman; and cell culture materials from Gibco. Composition of buffer solutions are: TSE 10/120/1, 10 mM Tris-Cl, pH 7.4, 120 mM NaCl, 1 mM EDTA-Na_2[1]; TSE 10/500/3, 10 mM Tris-Cl, pH 7.4, 500 mM NaCl, 3 mM EDTA-Na_2; TKE 10/500/10, 10 mM Tris-Cl, pH 7.4, 500 mM KCl, 10 mM EDTA-Na_2; TKEF 10/100/10/50, 10 mM Tris-Cl, pH 7.4, 100 mM KCl, 10 mM EDTA-Na_2, 50% formamide; TKMD (20/200/3/1, 20 mM Tris-Cl, pH 7.4, 200 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol.

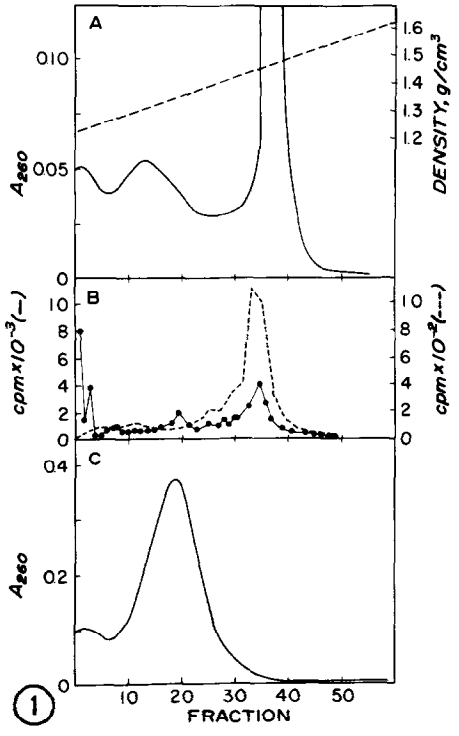
Preparation of 200,000 x g Cytoplasmic Pellet. Mouse fibroblast L-cells were grown as suspension cultures in Eagle's minimal essential medium. Approximately 75% of the [^{3}H]uridine is incorporated into heterogenous RNA, most of which contains poly(A), with the remainder to be found in small (approximately 4S) RNA (unpublished data). Lysis of L-cells, preparation of a 20,000 x g supernatant (S-20) and the 0.5 M KCl-washed 200,000 x g pellet (P-200)[1] was reported elsewhere (22, 27). Dissociation of mRNPs from polysomes was achieved by addition of 7 µmol EDTA, pH 7.0, per mg of ribosomes (23).

Isolation of mRNPs. Poly(U) was immobilized on Whatman GF/C glass filters (24). Approximately 30 A_{260} units of P-200 in TSE 10/120/1 buffer was treated with EDTA as described avove, rapidly diluted with 10-20 volumes of TSE 10/120/1 buffer and applied to a poly(U) filter at the rate of 1 ml/min at 4°. Each filter was washed with 300 ml of TSE 10/500/3 buffer and 20 ml TSE 10/120/1 buffer to remove unbound material. Elution of bound mRNP was accomplished by incubation of filters in 1 ml of water for 6 hr at 4°. The resultant eluate was lyophylized, iodinated and electrophoresed as described below. Extraction of total [^{3}H]uridine-labeled RNA from L-cells was performed by the method of Perry, et al. (29). Oligo(dT) cellulose chromatography of mRNPs is a modified procedure of Aviv and Leder (25). An EDTA-treated P-200 sample was diluted with 10 volumes of TKE 10/500/10 buffer. The sample (approximately 30 A_{260}/ml) was applied to a 0.5 cm diameter column containing 0.3 g oligo(dT) cellulose equilibrated in TKE 10/500/10 buffer. Unbound material was released with equilibration buffer and mRNPs were eluted with TKEF 10/100/10/50 buffer. Formamide in this buffer was deionized and treated with activated charcoal before use. The sample was precipitated overnight at -20° with 2.5 volumes of 95% ETOH prior to iodination. Cs_2SO_4 gradients were preformed from 1.30 and 1.65 g/cm^3 Cs_2SO_4 solutions made in 10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 1 mM EDTA-Na_2, 0.01% Triton X-100, and 15% Me_2SO[1] (26). Approximately 0.2 ml of EDTA-treated P-200 sample (6 A_{260} units) was centrifuged at 35,000 rpm for 24 hr at 25° in a Beckman SW 50.1 rotor. Fractions (0.1 ml) were collected and monitored for absorbance, Cl_3CCOOH-insoluble radioactivity and density using the formula (12.120 n - 15.166) g/cm3, where n is the refractive index (27). Gradient fractions were dialyzed and lyophylized prior to iodination.

Iodination, Electrophoresis and Autoradiography of m-Proteins. Messenger RNP samples isolated by the methods listed above were iodinated ($Na^{125}I$) in the presence of limiting chloramine-T (28). Iodinated mRNP samples were incubated at 100° for 5 min in 0.06 M Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol and 5% β-mercaptoethanol and electrophoresed in 1.25 mm thick 10% acrylamide gels.

RESULTS

Separation of L-cell mRNPs from ribosomes and soluble components was accomplished by centrifugation of a [^{3}H]P-200 sample in Cs_2SO_4 gradients. In Fig. 1A, the 1.36 g/cm^3 density peak appears to contain tRNA as indicated by banding free tRNA in an identical gradient (Fig. 1C). That the 1.47 g/cm^3 density peak in Fig. 1A contains mRNP particles is suggested by data presented



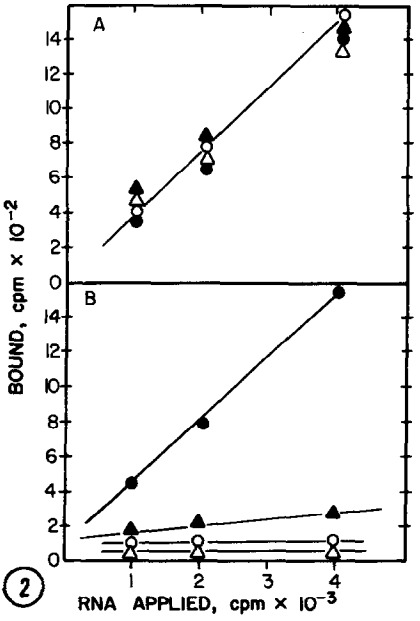


Fig. 1. Isopycnic Cs_2SO_4 centrifugation of L-cell P-200. A [^{3}H] P-200 fraction described in the Methods was centrifuged in preformed Cs_2SO_4 gradients. After centrifugation, gradient contents were monitored for (—) A_{260}, (●—●) radioactivity insoluble in cold 5% Cl_3CCOOH, (---) radioactivity binding to poly(U)-glass fiber filters (see Methods), and (— —) refractive index. Panel C: purified free tRNA. Gradient fractions were monitored for [^{3}H] uridine-labeled poly(A)-containing RNA by diluting in 100 volumes of TSE 10/120/1 buffer and application to poly(U)-glass filters, as described in Methods.

Fig. 2. Binding specificity of mRNPs and free mRNA to glass filters. EDTA-treated P-200 and phenol-extracted RNA was prepared from [^{3}H]uridine-labeled L-cells (see Methods). Samples of [^{3}H] P-200 (A) or [^{3}H] RNA (B) were added to poly(U)-glass fiber filters with binding measured as described in Methods. Where indicated, either poly(U) was omitted from the glass filters or excess poly(A) was added to P-200 and RNA samples prior to filtering. (o—o) — poly(U) — poly(A); (●—●) + poly(U), — poly(A); (Δ—Δ) — poly(U) + poly(A); (▲—▲) + poly(U), + poly(A).

in Fig. 1B. [^{3}H]Uridine-labeled Cl_3CCOOH-insoluble material is localized in this area of the gradient. Approximately 30% of the Cl_3CCOOH-precipitable radioactivity in the 1.38—1.47 g/cm^3 region could also be bound to glass fiber filters to which poly(U) had been immobilized (Fig. 1B). Proteins from the 1.43—1.47 g/cm^3 area banded with apparent molecular weights of 73,000, 50,000 and 34,000. Additional proteins observed are summarized in Table 1.

Table 1. Protein composition of mRNP particles from Fig. 3 polyacrylamide-SDS gel.

Isolation Method	Proteins (mol. wt. x 10^{-3})
Poly(U)-Glass Filter	74, 51, 34, 48, 46, 39, 32
Cs_2SO_4 Gradient	73, 50, 34, 67, 60, 47, 42, 32
Oligo(dT) Chromatography	77, 48, 33, 67, 45

Major proteins (underlined) are the more dense bands in the autoradiograph of Fig. 3.

Interaction of m-proteins with chromatography support materials has been considered a major factor in isolation of mRNP particles. This effect was also observed in the binding of L-cell mRNPs, but not mRNA, to poly(U) immobilized on glass fiber filters. Prior addition of excess poly(A) to an EDTA-treated P-200 fraction did not diminish subsequent binding of mRNPs to the filter (Fig. 2A). Binding of mRNPs to glass fiber filters devoid of immobilized poly(U) and to poly(U)-glass filters was equivalent. In contrast, free mRNA, phenol-extracted from the P-200 fraction, bound only to glass fiber filters to which poly(U) had been attached (Fig. 2B). Prior addition of excess free poly(A) to a deproteinated [^{3}H] RNA fraction inhibited binding to poly(U)-glass fiber filters. Binding of free [^{3}H] RNA to poly(U)-glass filters and [^{3}H] mRNPs to plain glass filters were equally efficient (~40%) processes (Fig. 2A, 2B). Analysis of the protein component of glass filter purified L-cell mRNPs by gel electrophoresis revealed 6 major proteins (Fig. 3B, Table 1).

Analysis of L-cell m-proteins was also performed with mRNPs isolated with oligo(dT)-cellulose. Autoradiographic patterns of electrophoresed m-protein were less reproducible with the oligo(dT)-cellulose chromatographed samples than was experienced with either Cs_2SO_4 gradient or poly(U)-glass filter preparations (Fig. 3D). However, L-cell m-protein species with molecular weights of 77,000, 48,000 and 33,000 were observed in a series of experiments.

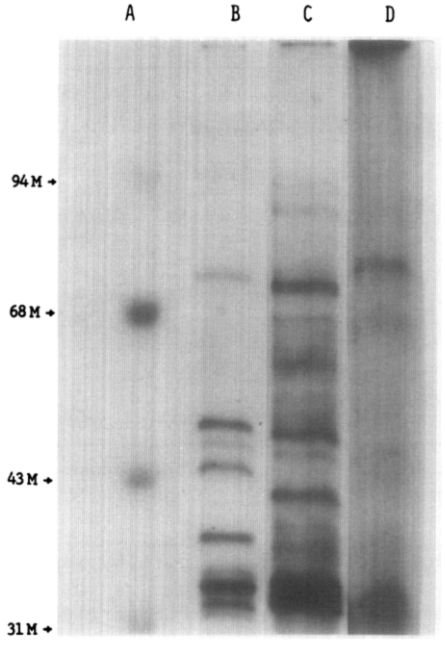


Fig. 3. Gel electrophoresis of L-cell m-proteins. A. Protein standards (molecular weight) were: phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), deoxyribonuclease I (31,000). B. A P-200 fraction (approximately 30 A_{260} units) was applied to poly(U) glass fiber filters (Whatman GF/C), eluted, iodinated (^{125}I), electrophoresed and autoradiographed. C. P-200 (6 A_{260} units) purified on isopycnic Cs_2SO_4 gradients and processed as above (see Methods). Gradient fractions from the 1.38—1.47 g/cm^3 region (see Fig. 2) were pooled for electrophoresis. D. A P-200 fraction (30 A_{260} units) was chromatographed on oligo(dT)-cellulose. Electrophoresis was carried out at 40 mA until the bromophenol blue dye front reached 13.5 ± 0.1 cm. The gel was fixed in 25% Cl_3CCOOH for 15 min, rinsed in H O for 10 min and dried on Whatman filter paper at 90° under vacuum. Autoradiography was performed with NS-5T No Screen Film (Kodak).

DISCUSSION

Labeling of m-proteins with radioactive iodine increased the sensitivity of protein detection and made it possible to observe major and minor proteins which could not be detected with protein staining techniques. Purification of L-cell mRNPs with the three methods resulted in m-protein patterns with some common features. As summarized in Table I, all three techniques yielded major protein bands of approximately 34,000, 50,000 and 75,000 molecular weight with the exception of the minor 48,000 molecular weight band in oligo(dT)-purified particles. The diversity of major and minor m-proteins shown in Fig. 3 and

Table 1 is considered to be evidence that mRNP isolation techniques are an important factor in recovery of individual m-proteins. Another explanation is that mRNP isolation methods result in particles with proteins which are either more or less accessible for iodination.

Acknowledgements

The authors are indebted to Dr. R. Perry for the L-cell line and to Deborah D. Moran for preparation of the typescript. This work was supported by National Institutes of Health Grant GM 24448.

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