RAPID ISOLATION OF UNDEGRADED POLYSOMAL RNA WITHOUT PHENOL

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Received 17 March 1972
Original figures received 21 April 1972

1. Introduction

Recently we have described a method for the isolation of mRNA from polysomes using proteinase K to suppress ribonuclease action and to degrade ribosomal proteins [1]. In these experiments, the proteinase itself was finally removed by phenol treatment. We present now a simpler way of isolating mRNA avoiding the use of phenol, and leading to nearly complete yield of mRNA even in small samples.

2. Materials and methods

Proteinase K (chromatographically pure) and insoluble (CM cellulose-bound) proteinase K were kindly supplied by Dr. H. Lang (E. Merck, Darmstadt). Diethyl pyrocarbonate (DEP, 'Baycovin') was a gift of Farbenfabriken Bayer, Leverkusen. Nonidet P-40 (NP 40) was purchased from Shell Corp., sucrose (density grade, RNase-free) from E. Merck, Darmstadt.

HeLa S3 cells were propagated in suspension culture as described previously [2].

2.1. *Isolation of polysomes from pulse-labeled cells*

1 mCi ^{3}H-uridine (uridine-T-5, specific activity 30 Ci/mmole; The Radiochemical Centre, Amersham, England) was added to 1800 ml suspension culture (5×10^5 cells/ml) for 60 min. Cells were harvested after cooling and addition of cycloheximide (final conc. = 10^{-4} M) [2] by centrifugation. Cells (1×10^8/ml) were broken up in 'cell buffer' (containing per l 8 g NaCl, 0.2 g KCl, 0.1 g $MgCl_2 \cdot 6\,H_2O$, 0.2 g KH_2PO_4, 1.15 g $Na_2HPO_4 \cdot 12\,H_2O$, 0.1 g $CaCl_2 \cdot 2\,H_2O$, 28 mg cycloheximide) containing 0.5% NP-40 [3, 4] by 2–3 controlled strokes in a Dounce homogenizer. Nuclei were sedimented at 700 *g*. The supernatant was layered on 5 ml 0.25 M sucrose in TKMC buffer (0.05 M Tris-HCl, pH = 7.4; 0.1 M KCl; 0.01 M $MgCl_2$; 0.1 mM cycloheximide), and centrifuged at 10,000 *g* for 20 min. Polysomes were isolated from the post-mitochondrial supernatant by centrifugation through 1.85 M sucrose according to Kramer and Hilz [5]. The polysomes were stored at −30°.

2.2. *RNA extraction from polysomes*

Polysomes isolated as described above were suspended in 0.1 M sucrose in TKMC buffer at a conc. of 5–10 A_{260} units/ml and centrifuged at 2000 *g* to remove insoluble material. Aliquots of 13.8 A_{260} units in a final vol of 2.0 ml were taken for RNA extraction by one of the following methods:

2.2.1. 'Hot' phenol method

Polysomal RNA was extracted by the 'hot' phenol–SDS method of Penman [6], with re-extraction of the interphase. RNA was collected from the aqueous phase by ethanol precipitation.

2.2.2. 'Cold' phenol method

RNA was extracted at 25° as described under sect. 2.2.1 without intermitted heating.

2.2.3. DEP-'cold' phenol method

Prior to 'cold' phenol extraction polysomes were preincubated with 30 mM diethyl pyrocarbonate for 60 min at 25° according to [7].

2.2.4. Proteinase K-'cold' phenol method

The polysome suspension was incubated with 500

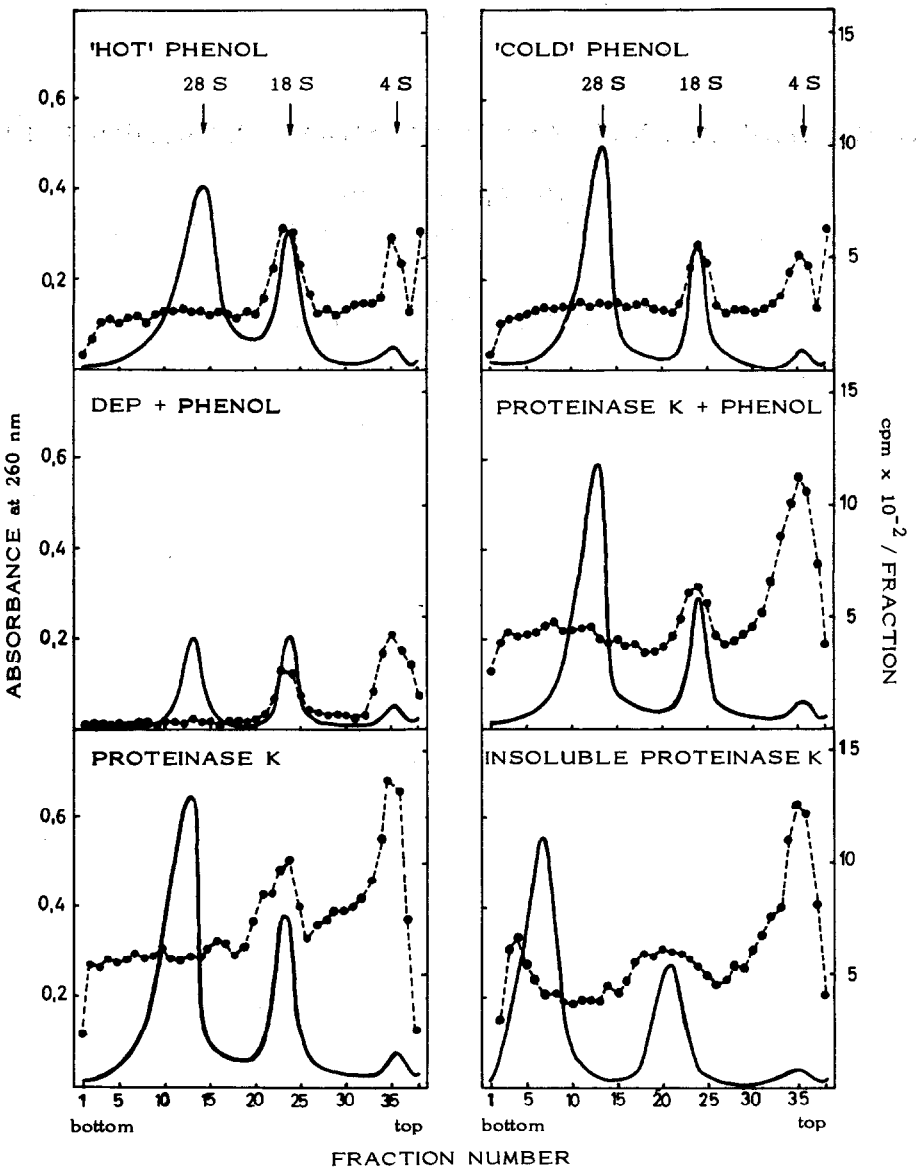

Fig. 1. Sucrose gradient analysis of polysomal RNA isolated by different methods. Polysomes were isolated from pulse-labeled HeLa S3 cells and RNA was extracted and analyzed as described under Methods.

μg/ml of soluble proteinase K at 25° for 60 min followed by 'cold' phenol–SDS extraction [1].

2.2.5. Proteinase K method

The polysome suspension was incubated with 500 μg/ml of soluble proteinase K at 25° for 60 min. After the addition of 100 μl of 2 M NaCl per ml, RNA could be isolated in two ways: either i) ethanol precipitation: 2.0 vol of absolute ethanol were added to 1 vol of digestion mixture, and the solution was kept at −30° for 10 hr, or the second way leading to the isolation of mRNA + rRNA and separation from

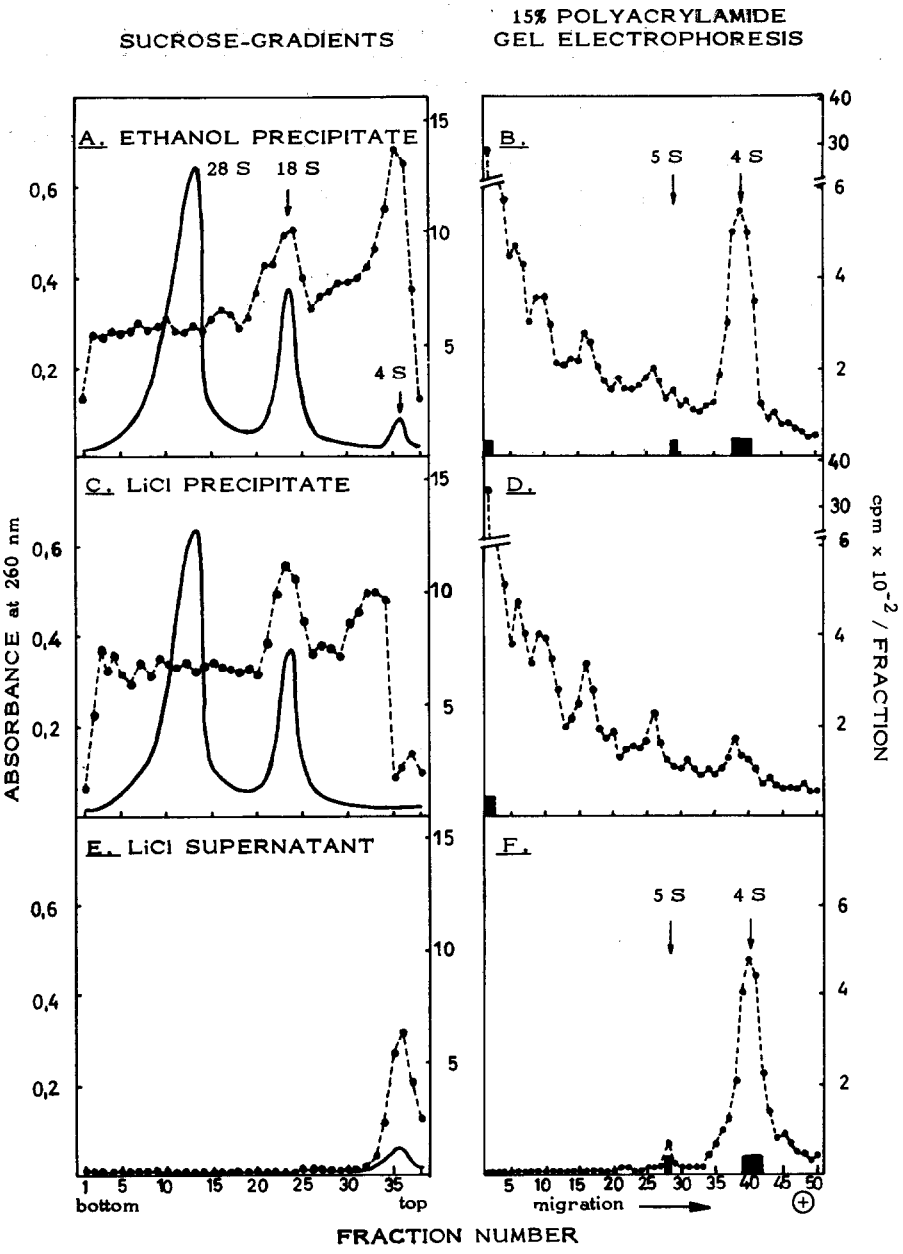

Fig. 2. Ethanol and LiCl precipitation of polysomal RNA. Analysis by sucrose gradient centrifugation and polyacrylamide gel electrophoresis. Cells were labeled and polysomes were isolated as described under Methods. RNA was collected after digestion of polysomal proteins with soluble proteinase K by ethanol and by LiCl precipitation. 5 S RNA and tRNA were precipitated from the LiCl supernatant by the addition of 2 vol of absolute ethanol. For details see Methods. Solid blocks = stained RNA bands.

Table 1
Yield and specific radioactivities of polysomal RNA isolated by different methods.

Isolation procedure	Yield A_{260} (%)	Yield cpm (%)	Specific radioactivity (cpm/A_{260} unit)
'Hot' phenol	52	38	21,900
'Cold' phenol	68	39	18,100
DEP + phenol	33	16	14,500
Proteinase K + phenol	67	61	27,800
Polymer-bound proteinase K	75	75	30,400
'Proteinase K'			
alcohol ppt.	94	92	29,300
LiCl ppt. + supernatant	99	96	29,200
LiCl ppt.	94	89	28,600
LiCl supernatant	5	7	41,800

13.8 A_{260} units and 416,000 cpm of the polysomal suspension were taken for RNA extraction in different methods (see Material and methods).

tRNA + 5 S RNA is ii) LiCl-preparation: By the addition of 1 vol of 3 M LiCl, the RNA was separated into a LiCl-soluble and an insoluble fraction [8] after standing at 0° for 10 hr. The 1.5 M LiCl-soluble RNA was precipitated from the supernatant by the addition of 2.0 vol of absolute ethanol. It contained the tRNA and the 5 S RNA, while most of proteinase K was left in the supernatant.

2.2.6. Insoluble proteinase K

Polysomes were incubated with shaking at 25° for 60 min with 2.5 mg/ml of proteinase K covalently linked to CM-cellulose. After incubation the insoluble proteinase K was centrifuged off at 2000 *g* for 10 min. RNA was precipitated from the supernatant by the addition of 100 μl 2 M NaCl and 2.0 vol of absolute ethanol.

In all cases, the precipitated RNA was dissolved in 1.0 ml of sterile (boiled) water. Aliquots were taken for sucrose gradient centrifugation analysis, for acrylamide gel electrophoresis, and for determination of recoveries (radioactivity and A_{260} units).

2.3. *Sucrose gradient analysis*

Isolated RNA was analyzed by sucrose gradient centrifugation on a linear gradient (5–20% sucrose (w/v) in 0.01 M Tris-HCl, pH = 7.4; 0.1 M NaCl; 0.001 M EDTA) for 6 hr at 40,000 rpm in a Spinco SW 40 rotor. Absorbance at 260 nm was monitored automatically (Hellma microflow cell, 5 mm lightpath), and fractions of 0.3 ml were collected. TCA-insoluble material was analyzed for radioactivity by the filter paper method [9].

2.4. *Gel electrophoresis*

Polyacrylamide gel electrophoresis was performed in an analytical apparatus according to conventional methods [10, 11]. To analyse low molecular weight RNA, 5–100 μg of RNA were applied to 15% polyacrylamide gels and run for 8 hr at 5 mA/gel. Staining procedure with methylene blue was performed according to Peacock [10]. The migration distance of the RNA bands was measured, and the gels were cut into 1.5 mm slices. The slices were dissolved in 200 μl of 30% H_2O_2 by incubation for 15 hr at 50°. 0.2 ml aliquots were used for radioactivity determination in 15 ml dioxane scintimix (E. Merck, Darmstadt).

2.5. *Determination of proteinase activity*

2 mg of 'Albumine Azure' (Calbiochem, Luzern, Switzerland) were suspended in 1.9 ml TKM buffer. After addition of 100 μl of proteinase-containing solution and incubation for 15 min at 30°, absorbance at 578 nm was determined in the supernatant [12].

2.6. *Final method for the isolation of polysomal RNA*

Polysomes were suspended in 0.1 M sucrose in TKMC buffer at a concentration of 2.5–50 A_{260} units/ml, followed by low speed centrifugation (2000 *g* for 5 min) to remove eventual aggregates. Proteinase

K (freshly dissolved in 0.1 M sucrose in TKMC buffer at a concentration of 10 mg/ml) was added to the polysomal suspension to a final concentration of 500 μg/ml. The suspension was incubated for 60 min at 25°. RNA was precipitated either by ethanol precipitation (2 vol, 8 hr at −30°), eventually reprecipitated to eliminate the bulk of proteinase K, or by LiCl precipitation (1 vol of 3 M LiCl, 8 hr at 0°) leaving the proteinase quantitatively in the supernatant (together with tRNA and 5 S RNA). When ethanol precipitated RNA species were separated in a sucrose gradient, proteinase remained in the top fractions.

3. Results

When HeLa S3 cells were exposed to a 60 min ^{3}H-uridine pulse, the bulk of radioactivity of the polysomal fraction represented heterogeneous mRNA [13]. Bound tRNA and 18 S rRNA species were also labeled to a significant degree [14]. When these polysomes were extracted by various methods, considerable differences in yield and purity of mRNA and rRNA became apparent (fig. 1). There is no significant difference between 'hot' and 'cold' phenol extraction (cf. fig. 1), while diethyl pyrocarbonate (DEP) treatment induced heavy destruction of all kinds of polysomal RNA except for tRNA [15]. Combination of (cold) phenol extraction with a prior digestion of polysomal proteins by proteinase K proved superior as has been shown previously [1]. Phenol had been used in these experiments to eliminate proteinase K. A still better yield of mRNA, however, was achieved when phenol extraction was completely avoided (fig. 1, 'Proteinase K'). This applied to the whole spectrum of heterogenous RNA including a peak fraction in the region of 4 S not identical with tRNA (cf. fig. 2), which appeared only when the proteins were degraded enzymatically. Proteinase K bound to an insoluble carrier was not able to digest the polysomal proteins at the same rate as soluble proteinase K. This is indicated by the higher S values of the peaks containing the 28 S and 18 S RNA, respectively. A quantitative comparison of the different methods is given in table 1. Proteinase K without phenol led to a nearly complete recovery of total RNA (A_{260}) and radioactivity, independent of the kind of the subsequent precipitation.

Elimination of proteinase K after digestion of polysomal proteins posed some problems. The enzyme was insoluble in 80% ethanol, and dissolved again on the addition of buffer to a conc. of 70% ethanol. However, in the presence of RNA, proteinase K was partially coprecipitated. It could not be removed quantitatively under these conditions even after several reprecipitations. When RNA was separated in a sucrose gradient, however, proteinase K remained in the top fractions. An alternative procedure for the final elimination of proteinase K used the precipitation of rRNA and mRNA with LiCl, leaving the enzyme quantitatively in the supernatant together with 5 S RNA and tRNA previously bound to the polysomes (cf. fig. 2).

Sucrose gradient analysis of RNA from proteinase digested polysomes (fig. 2A) yielded a peak fraction in the region of 4–10 S, which was composed of tRNA and other RNA species. Polyacrylamide gel (15%) electrophoresis (fig. 2B) showed a major peak at the tRNA position as well as RNA species < 10 S and < 5 S RNA, the bulk of the rapidly labeled RNA being excluded and remaining at the top of the gel. When LiCl precipitation of tRNA + mRNA was applied to the proteinase digest 5 S RNA and tRNA could be precipitated from the LiCl supernatant and separated by gel electrophoresis (fig. 2E and 2F).

Sucrose gradient analysis and gel electrophoresis of the LiCl precipitate (fig. 2C and 2D) revealed that the rest of the 4–10 S peak material had a size distribution well above 5 S RNA. The bulk of the RNA appeared at the top of the gel again.

4. Discussion

The introduction of proteinase K for the isolation of polysomal RNA [1] greatly improved the yield of undegraded mRNA. A further increase in yield to nearly completion could now be achieved when the final elimination of proteinase by phenol was omitted. A fraction of low molecular weight (< 10 S) RNA not identical with tRNA seemed to be lost in preference when proteins were removed by phenol extraction only. The method is applicable to samples containing low concentrations of RNA (0.25 mg/ml). LiCl precipitation of the proteinase digest combined two advantages. It separated out tRNA and 5 S RNA,

and eliminated at the same time the proteolytic enzyme.

When this method was applied to HeLa cells, mRNA species would be obtained with chain length clearly above 28 S. These mRNA molecules could code for proteins > 180,000 daltons.

Acknowledgements

We like to thank K. Klapproth for competent technical assistance, and G. Jarmers for propagating cell cultures. This work was supported by the Deutsche Forschungsgemeinschaft.

References

[1] U. Wiegers and H. Hilz, Biochem. Biophys. Res. Commun. 44 (1971) 513.
[2] C. Schlaeger, D. Hoffman and H. Hilz, Hoppe-Seyler's Z. Physiol. Chem. 350 (1969) 1017.
[3] T.W. Borun, M.D. Scharff and E. Robbins, Biochem. Biophys. Acta 149 (1967) 302.
[4] A.L.J. Gielkens, T.J.M. Berns and H. Bloemendal, European J. Biochem. 22 (1971) 478.
[5] G. Kramer and H. Hilz, Hoppe-Seyler's Z. Physiol. Chem. 352 (1971) 843.
[6] S. Penman, J. Mol. Biol. 17 (1965) 2.
[7] F. Solymosy, J. Federcsak, A. Gulyas, G.L. Farkas and L. Ehrenberg, European J. Biochem. 5 (1968) 520.
[8] D. Baltimore, J. Mol. Biol. 18 (1966) 421.
[9] J. Bollum, J. Biol. Chem. 234 (1959) 2733.
[10] A.C. Peacock, and C.W. Dingman, Biochemistry 6 (1967) 1818.
[11] D.H.L. Bishop, J.R. Claybrook and S. Spiegelmann, J. Mol. Biol. 26 (1967) 373.
[12] G.L. Moore, Anal. Biochem. 32 (1969) 122.
[13] S. Penman, C. Vesco and M. Penman, J. Mol. Biol. 34 (1968) 49.
[14] S. Penman, S.I. Smith and E. Holtzman, Science 154 (1967) 786.
[15] M. Denic, L. Ehrenberg, J. Federcsak and F. Solymosy, Acta Chem. Scand. 24 (1970) 3753.