

HIGH RESOLUTION TWO-DIMENSIONAL ELECTROPHORESIS OF PROTEINS BOUND TO HETEROGENEOUS NUCLEAR RNA

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1. Introduction

In eucaryotic cells, several intermediate steps exist in messenger RNA biogenesis between its transcription from the DNA in the nucleus and its utilization in the cytoplasm. This process is defined as mRNA maturation. It includes addition of the poly (A) sequences in the 3'-end; capping and methylation and possible cleavage of RNA precursors to shorter functional messengers. The fact that large portions of newly synthesized messenger-like RNA molecules (HnRNA) are degraded within the nucleus without ever reaching the cytoplasm suggests the existence of additional steps in regulation of messenger biogenesis which could consist in a selection mechanism for transport to the cytoplasm of some pieces of the processed RNA molecules or of some species of them [1].

Biochemical and electron microscopy studies have shown that messenger RNA during its life span in the nucleus is associated with protein(s) to form ribonucleoprotein structures (RNP) [2–10]. It has been reported that the RNA structures have a polysome-like constitution consisting of 30–50 S monomers connected by a RNA chain [9]. Conflicting results have been presented as to the protein constitution of these structures. According to Georgiev et al. [11] few and even a single protein is the constituent of the 30 S particles termed informofers. It is however more likely that the proteins associated with HnRNA are much more heterogenous as shown by several groups [7,10,12] as well as from the data of the present work.

Abbreviations: HnRNA heterogenous nuclear RNA, RNP ribonucleoprotein particles, HnRNP HnRNA–protein complex.

The study of these proteins and the overall structure of HnRNP could be very important for understanding the maturation step of messenger biogenesis and the mechanism of messenger transport from the nucleus to the cytoplasm.

This report concerns the isolation of HnRNP structures from monkey CV1 cells grown in culture and the characterization of their proteins using a powerful technique for protein identification, that of two-dimensional gel electrophoresis [13].

2. Materials and methods

Monkey kidney cells (CV1 cell line) were grown in monolayer cultures with Eagle minimal essential medium containing 10% tryptose and 10% fetal calf serum. Isotopic labeling with [³H]-uridine and [³⁵S] methionine were performed in a reduced volume of medium per plate at 37°C for various times. The plates were placed on ice and all further operations were performed at 0°C. Cells were scraped, washed in Tris–Dulbecco buffer (NaCl 0.14 M, KCl 5 mM, Na₂HPO₄ 0.7 mM Tris–HCl 25 mM, pH 7.4) and resuspended in reticulocyte standard buffer 10 mM Tris, 10 mM NaCl, 1.5 mM MgCl₂) to swell for 10 min at 0°C. The cells were broken with a Dounce homogenizer and nuclei were washed three times in reticulocyte standard buffer and HnRNP were prepared as indicated by Peterson [10]. Washed nuclei were disrupted in reticulocyte standard buffer by a brief sonication (5 S at 40 W in a Branson B12 Sonifier). The sonicate was centrifuged for 15 min at 4000 × g through a 30% sucrose cushion containing 10 mM Tris,

0.15 M NaCl to sediment nucleoli and nuclear membranes. The material remaining on top of the sucrose was removed, made 10 mM in EDTA, and layered on a 15–30% linear sucrose gradient and centrifuged for 2.5 h at 41 000 rev/min in the SW 41 Spinco rotor.

Portions of the gradients were pooled and the RNP particles were collected by high-speed centrifugation (16 h at 50 000 rev/min. For SDS–polyacrylamide gel electrophoresis the particles in the pellet were dissolved in sample buffer (3% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris, pH 7.0) boiled for 3 min at 100°C and run in a slab-gel apparatus in 15% acrylamide gels.

The two-dimensional gel electrophoresis were performed according to the description of O'Farrel [13]. Electrofocusing was performed with a mixture of ampholines of 1.6%, at pH 5–7 and 0.4%, at pH 3.5–10. After equilibration of isoelectric focusing gels for 2 h in 2.3% SDS, 62.5 mM Tris–HCl and 5% 2-mercaptoethanol, they were applied to the second-dimension SDS–slab-gel of 15% acrylamide 0.175 M bisacrylamide. The gels were stained, destained, dipped in dimethylsulfoxide containing PPO (2,5-diphenyl-oxazole), dried and autoradiograms were prepared by exposure to RP/S Kodak X-ray film at –70°C [14,15].

3. Results and discussion

Monkey cells were labeled with [³H]uridine and [³⁵S]methionine in order to label the RNA and protein

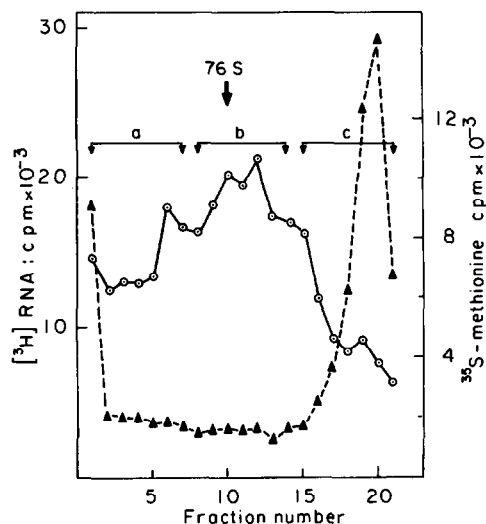


Fig.2. SDS–polyacrylamide gel electrophoresis of HnRNP particle proteins. (e) Fractions in the middle of the gradient in fig.1 were pooled and their protein content analysed on 15% acrylamide slab gel. (b) Ribosomal proteins (c) A preparation of histones. (d) Proteins from the pellet of the sucrose gradient (chromatin fraction). (f) Myosin and actin. (a,g) Bovine serum albumin and lysosyme.

Fig.1. Sucrose gradient centrifugation of HnRNP structures. 1.5 × 10⁷ CV1 cells were labeled for 1 h with [³⁵S]methionine (50 μCi/plate), the last 30 min [³H]uridine was added at 200 μCi/plate. Cell extract was prepared as indicated in Materials and methods and subjected to sucrose gradient sedimentation. 0.5 ml fractions were collected and an aliquot precipitated with trichloroacetic acid and counted. (—○—) ³H cpm, (—▲—) ³⁵S cpm. The position of 76 S single ribosomes was determined by centrifuging in parallel a ribosome preparation. Arrows demonstrate the 3 portions of the gradient which are pooled, centrifuged and subjected to protein analysis. Right side: top of the gradient.

moieties of the HnRNP. The size distribution of HnRNP structures isolated as described in Materials and methods and fractionated by zonal sedimentation is shown in fig.1. The totality of the chromatin solubilized during the sonication is found in the pellet of the sucrose gradient. The average size of the HnRNP isolated was approximately 75 S.

The RNP particles were concentrated and analysed for their protein constituents as described in Materials and methods. Figure 2 shows the pattern of polypeptides obtained after slab-gel electrophoresis of material from different pooled fractions of gradient similar to this described in fig.1 and from the pelleted chromatin fraction. A series of standards are run together in the same gel in order to calibrate the molecular weight distribution. The following conclusions can be drawn from the gel pattern.

(i) A complex pattern of polypeptides is associated with HnRNP particles with mol.wt. ranging from 32 000–200 000. The more prominent polypeptides are however in the range of 32 000–55 000 (slot e).

(ii) In agreement with the observation of Peterson [10], the RNP fractions of the gradients are not contaminated with chromatin proteins including histones. These later proteins sediment in the pellet of the gradient as shown by the pattern on slot d.

(iii) The different pattern of ribosomal proteins (slot b) demonstrate the absence of contamination of HnRNP with cytoplasmic material.

In order to better characterize the protein components of HnRNP structures, we have further applied a more powerful technique, the two-dimensional gel electrophoresis. Figure 3 shows the complex polypeptide complement of HnRNP particles as

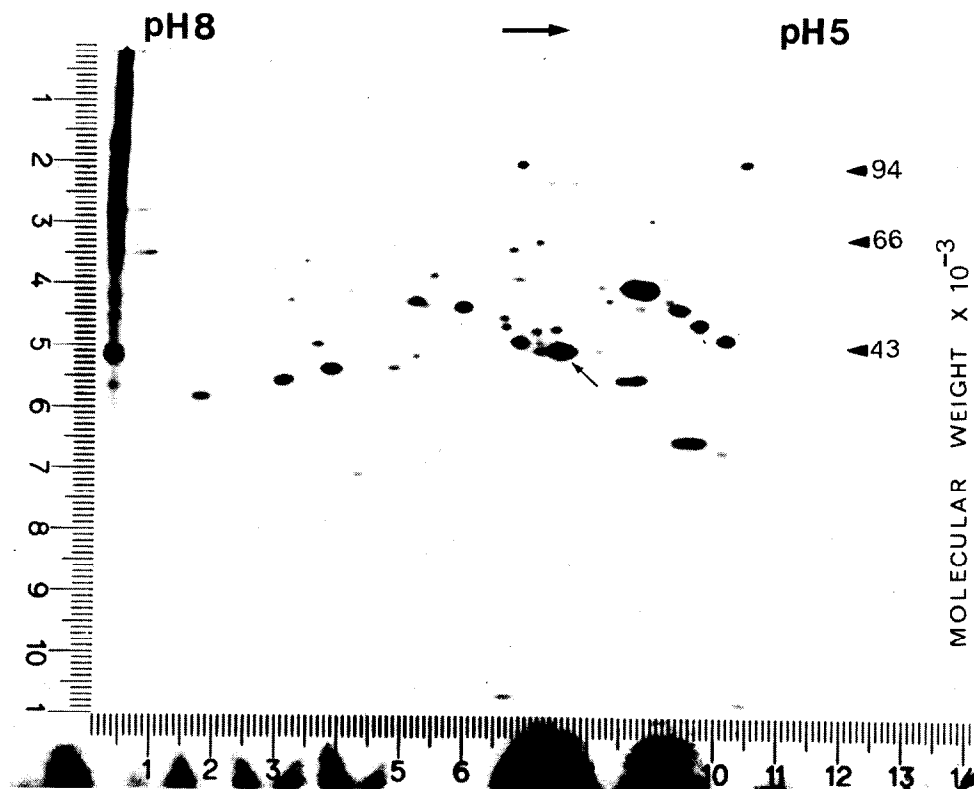


Fig.3. Autoradiogram of two-dimensional gel of HnRNP polypeptides. Cells were labeled for 12 h with [35 S]methionine (20 μ Ci/plate) and the last 30 min with [3 H]uridine 200 μ Ci/plate. HnRNP particles, fraction b in fig.1, were pelleted, digested with a mixture of deoxyribonuclease (2 mg/ml) and ribonuclease (1 mg/ml) made 9 M in urea and analysed [13]. Molecular weight standards were run in separate gels and the positions of their molecular weights are indicated in the fig. Arrow indicates the position of actin, pH range 5–8.

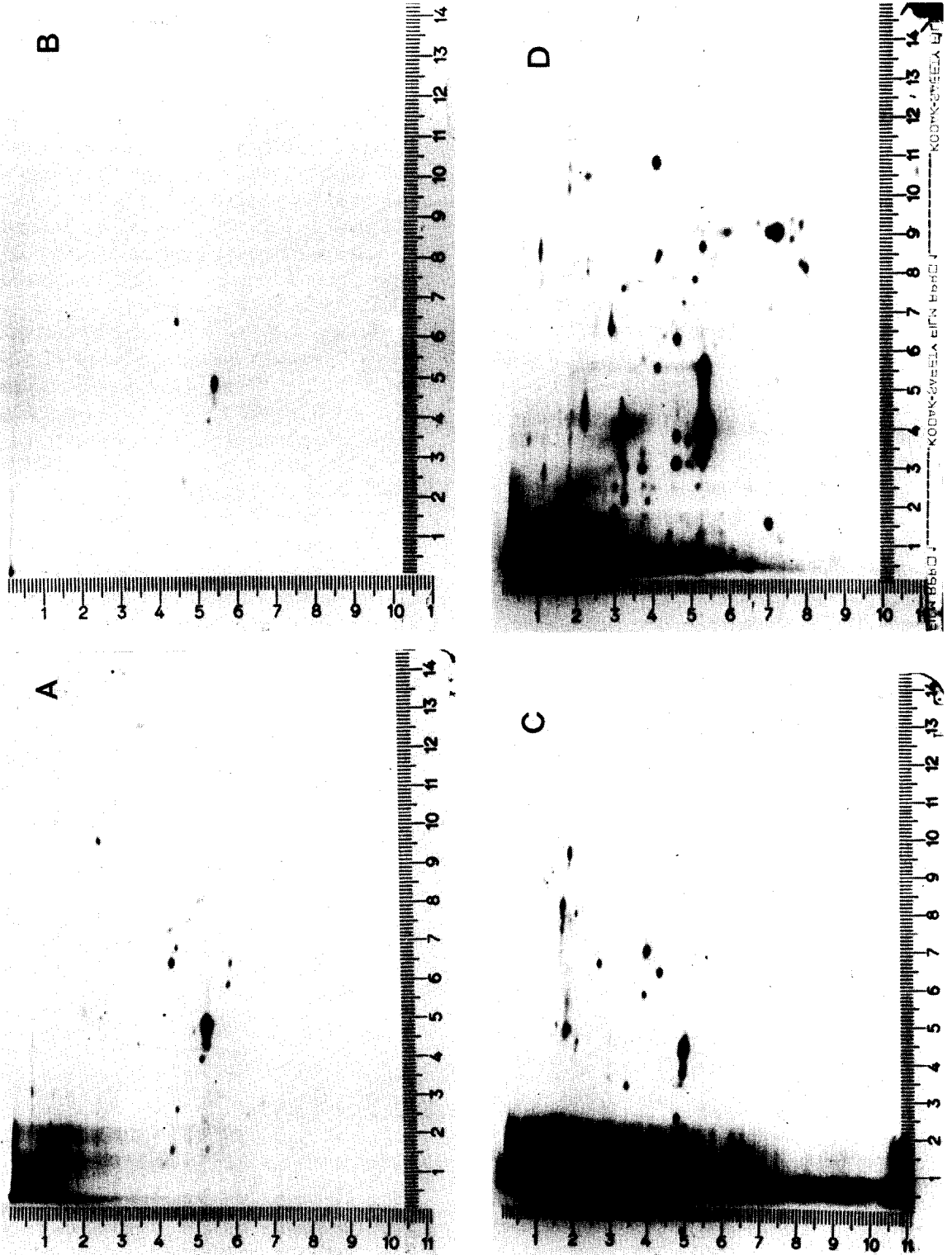


Fig.4

resolved by this technique. It is obvious that a number of spots are nearly of the same molecular weight and they cannot be resolved by the one-dimensional gel.

The presence of double spots (coordinates, e.g., 5.5/8.8, 6.6/9.7) with identical molecular weight but different charge positions suggests possible partial modifications. Evidence for phosphorylation of several HnRNP proteins in this molecular weight range was presented by Gallinaro-Matringe et al. [16].

Figure 4 shows the map of labeled proteins from HnRNP structures, chromatin and the nucleosol. To distinguish HnRNP polypeptides from free proteins which could cosediment with HnRNP, the following experiment was performed. A sample of crude HnRNP taken from the 30% sucrose supernatant was treated with pancreatic ribonuclease (0.2 γ /ml) and subjected to a 15–30% sucrose gradient sedimentation (see fig.1). The fractions corresponding to the middle of the gradient were concentrated and analysed (fig.4B). The fact that most of the polypeptides disappear after ribonuclease treatment shows that the proteins in plate A are indeed associated with HnRNA. However, two prominent spots whose positions correspond to those of actin and tubulin are still present in the autoradiograms demonstrating that these proteins are present as high molecular weight polymers that cosediment with HnRNP structures.

In agreement with the analysis by one-dimension, the more prominent HnRNP proteins comprise 10–12 spots in the mol.wt. range of 30 000–50 000 with isoelectric points between 5 and 7. Another dozen proteins seem to constitute a lower abundance group. It is possible that several basic proteins remain on the origin of the isoelectric-focusing gel. Further experiments are necessary to determine whether there are several classes of particles with different proteins, or

whether different HnRNP contain some common proteins (the major class) and some variable proteins (the minor class). Analysis of proteins associated with viral RNP is in progress in an attempt to clarify this question.

From comparison of the pattern of spots in fig.4 A, C and D, it is obvious that HnRNP proteins constitute a distinct class different from the proteins of chromatin and nucleosol fractions. In the case of chromatin, the basic proteins and the histones were not completely resolved at the pH used for the isoelectric-focusing. The present two-dimensional analysis of the different nuclear proteins relate different sets of proteins with distinct compartments in the nucleus. Many spots of HnRNP particles are not present in the nucleosol fractions, we can assume that there is not a large free pool for these proteins in a non aggregated form in the nucleosol.

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Fig.4. Autoradiograms of two-dimensional gels of HnRNP, chromosomal and nucleosol proteins. Cells were labeled as indicated in fig.1. HnRNP proteins (fraction b in fig.1) were prepared for electrophoresis as indicated in fig.3. Chromatin proteins correspond to the pellet of the gradient and were prepared for electrofocusing according to Peterson and McConkey [17]. Proteins were dissolved in 10 M urea 0.1% SDS, 1 mM Tris, pH 7.4 0.03 M lysine and 2.5 mM ZnCl₂. They were digested with 70 units of S1 nuclease for 5 min at 45°C, made 2% in NP40, 2% in ampholines, 5% in 2-mercaptoethanol and loaded onto the gel. Nucleosol proteins corresponding to the 4 last fractions of the gradient (fig.1) were dialysed against 10⁻² M Tris 10⁻³ M EDTA concentrated by evaporation and dissolved in lysis buffer (9.5 M urea, 2% NP 40, 2% ampholines, 5% 2-mercaptoethanol). Plate A HnRNP proteins, plate B HnRNP protein digested with ribonuclease before gradient centrifugation (see text), plate C chromatin proteins, plate D nucleosol protein. The first-dimension isoelectric-focusing gels were loaded with 20 000 cpm [³⁵S] methionine for HnRNP and nucleosol 30 000 cpm for chromatin. pH range 5–7.

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