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THE DIFFERENCE IN BUOYANT DENSITY BETWEEN VIRAL AND NORMAL POLYRIBOSOMES IN KREBS II CELLS

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1. Introduction

Viral polyribosomes in picornavirus-infected cells were shown to be larger in size than the polyribosomes in uninfected cells [1, 2]. If the only structural difference between the two types of polyribosomes is their size one should expect their buoyant density values to be identical. In this paper evidence of a small but definite difference in the buoyant density of normal and viral polyribosomes is presented.

2. Materials and methods

Mouse ascites carcinoma Krebs II cells were washed, suspended in Earle's saline at a concentration 5–10 × 10^6 per 1 ml, inoculated with encephalomyocarditis (EMC) virus at a multiplicity 10–20 PFU per cell and incubated at 2–4° for 14–16 hr. Actinomycin D was added (5 μg/ml), the suspension was heated up to 37° (this moment was considered to be the start of infection) and incubated at this temperature on a magnetic stirrer at low speed of rotation. The cells were labeled either with ^{3}H-uridine (3 μCi/ml, 0.3 Ci/mmole) from 2 to 5.5 hr post infection or during 2 min (5.5 hr post infection) with ^{14}C-chlorella hydrolysate (1 μCi/ml). In some experiments the mixture of ^{3}H-alanine (40 mCi/mmole) and ^{3}H-leucine (32 mCi/mmole) was used (0.5 μCi/ml each). At the end of incubation the suspension was poured into frozen and crushed Earle's saline. All subsequent procedures were performed at 0–2°. The cells were pelleted, resuspended in standard buffer (0.01 M triethanolamine-HCl pH 7.8; 0.01 M KCl; 0.003 M Mg acetate) and destroyed in a tight-fitting Dounce homogenizer. The supernatant after the centrifugation at 15,000 *g* for 20 min (cytoplasmic extract) was used for further analysis. Some portions of the extract were incubated for 10 min at 0° with pancreatic ribonuclease (RNase) (5 μg/ml) or with sodium ethylenediaminetetraacetate (EDTA) (0.02 M, pH 7.6. The extracts were layered on 15–30% (w/w) sucrose gradients containing 4% formaldehyde [3], the top of the gradient being 1 ml of 5% sucrose without formaldehyde. All sucrose solutions were made in standard buffer or, for EDTA-treated material, in standard buffer lacking Mg^{2+}. The gradients were centrifuged in a 3 × 23 rotor of a Superspeed-50 ultracentrifuge (MSE, England) at 25,000 rpm and 2° for 90 min. Eighteen to twenty fractions were collected from each tube. Distilled water, 2.5 ml, was added to 0.1 ml aliquots from every fraction. A_{260} was measured in a spectrophotometer, SP4A (USSR). Acid-insoluble material was precipitated with trichloroacetic acid (TCA) in the presence of 0.2 mg of casein, washed on nitrocellulose filters (mean pore size 0.7 μm) with TCA and ethanol, immersed into scintillation fluid (PPO 5 g, POPOP 0.3 g, toluene 1 l) and counted in Tricarb liquid scintillation spectrometer (Packard, USA). Chosen fractions from sucrose gradients were dialysed overnight against standard buffer containing 4% formaldehyde and analysed in preformed CsCl gradients [4] (rotor 3 × 5, 35,000 rpm, 4–6°, 16–18 hr). In some experiments differently labeled materials were mixed after dialysis so as to ensure 2–5 times excess

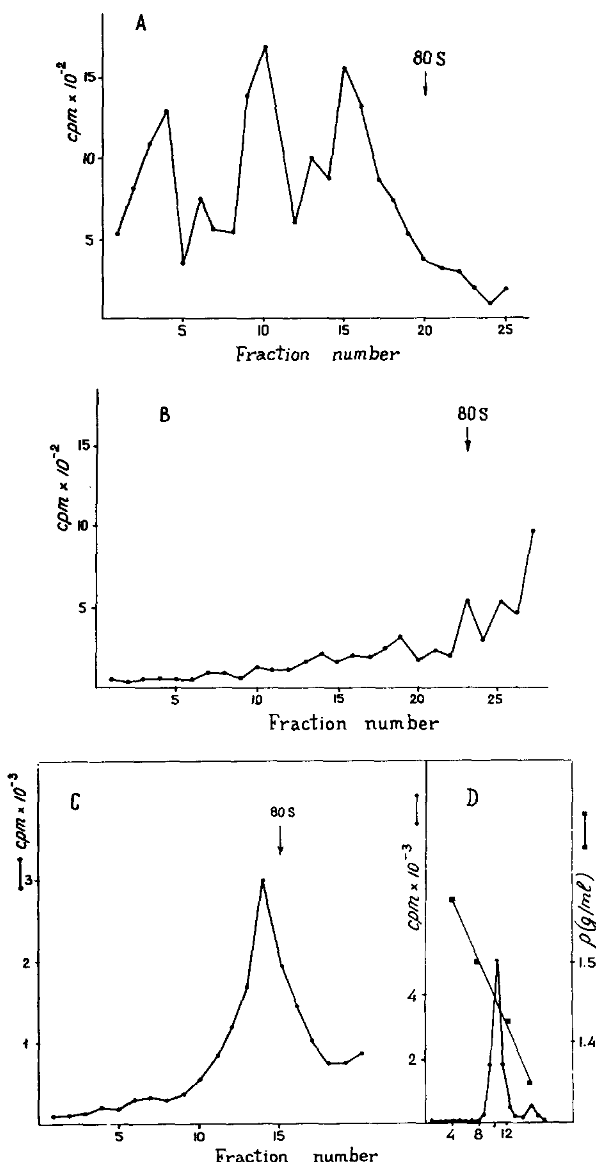

Fig. 1. Analysis of viral RNA-containing structures. Cytoplasmic extracts were analysed in sucrose gradients; A, no treatment; B, RNase treatment; C, EDTA treatment; D, fraction 14 from C was analysed in a CsCl gradient.

of ^{3}H-label over ^{14}C (in cpm). After centrifugation the bottom of the tube was punctured and 23–25 fractions were collected. Four drops from every 5th fraction were taken separately for refractometric measurement. The density of CsCl solution was determined from refractometric index [5] with a

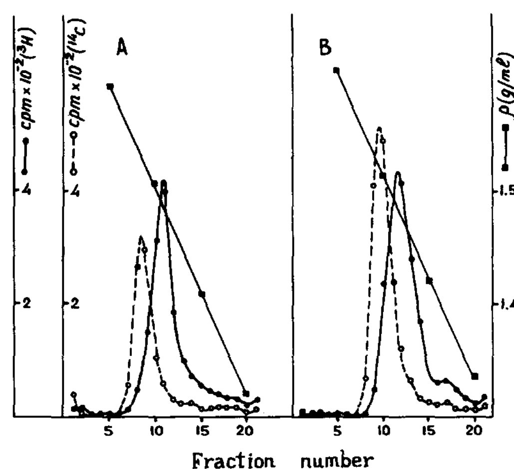

Fig. 2. The buoyant density (in CsCl) of virus-specific preribosomal structures (A) and free ribosomes (B) from deoxycholate-treated cytoplasmic extracts. The cytoplasmic extracts were treated with DOC in final concentration 0.5%.

correction for formaldehyde. A_{260} and radioactivity were determined as described above. When ^{14}C- and ^{3}H-labels were determined in the same sample the discrimination conditions were adjusted so less than 1% of ^{3}H-label was counted in the channel for ^{14}C and no more than 10% of ^{14}C-label were counted in the channel for ^{3}H. *Reagents*: actinomycin D (Reanal, Hungary). RNase (Calbiochem, USA). EDTA (Sigma, USA). Labeled precursors of RNA and protein (Isotop, USSR).

3. Results and discussion

3.1. *The identification of viral polyribosomes and the determination of their buoyant density in CsCl.*

In actinomycin D-treated uninfected Krebs II cells RNA synthesis was decreased by 96–98%, and in cytoplasmic extracts the amount of ^{3}H-uridine-labeled acid-insoluble material was negligible. For this reason the labeled RNA in the extracts of EMC-virus-infected cells was considered to be virus-specific. This RNA was distributed in a wide preribosomal zone after a fractionation of the cytoplasmic extract in a sucrose gradient (fig. 1A). Generally viral RNA-containing structures may include virions, viral poly-

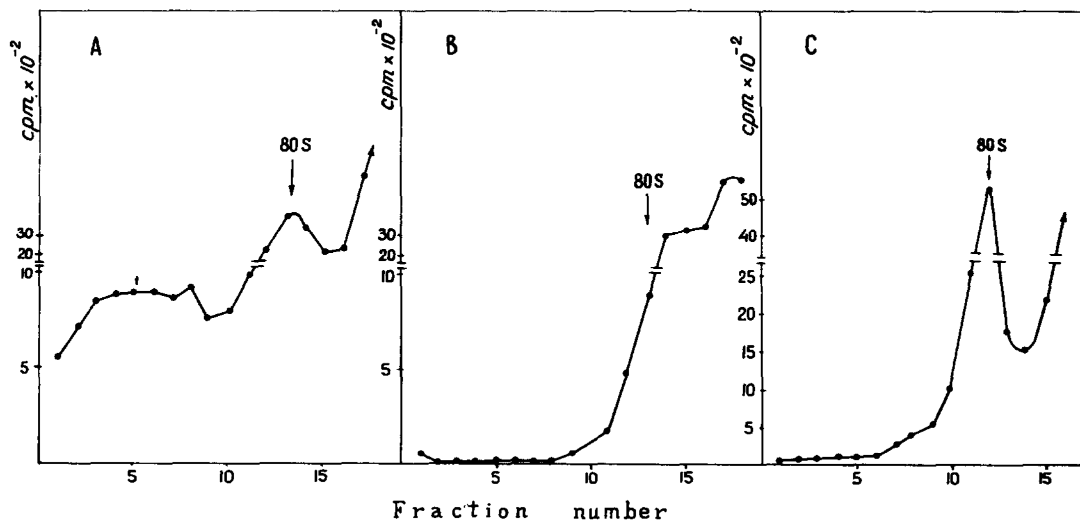

Fig. 3. Sedimentation analysis of cytoplasmic extracts after pulse-labeling of infected cells with ^{14}C-chlorella hydrolysate. A, no treatment; B, EDTA treatment; C, RNase treatment.

ribosomes, replicative complexes, etc. The data presented below indicate that the labeled preribosomal structures in EMC-virus infected Krebs II cells are represented mainly by viral polyribosomes.

The treatment of cytoplasmic extract with RNase leads to a considerable general loss of radioactivity and to a removal of the labeled material from the preribosomal zone (fig. 1B). This means that the preribosomal structures are not mature virions.

EDTA treatment leads to a displacement of the label to the 90–100 S zone (fig. 1C). A similar result had been obtained with poliovirus polyribosomes [6]; it had been shown that the 90 S component released from polyribosomes is a ribonucleoprotein. For this reason the material of the 90–100 S zone (fraction 14, fig. 1C) was analysed in a CsCl gradient. As can be seen from fig. 1D this material has a buoyant density of 1.44 g/ml which agrees well with similar data on the component released from poliovirus polyribosomes [6].

So viral RNA-containing structures resemble polyribosomes in their sedimentation behaviour and in their sensitivity to RNase and EDTA. Their buoyant density in CsCl after deoxycholate (DOC) treatment of the extracts is 1.57–1.58 g/ml (fig. 2A) which is characteristic of polyribosomes [7] and identical with the density of DOC-treated free ribosomes (fig. 2B).

To confirm the polyribosomal nature of viral RNA-containing preribosomal structures they were compared in buoyant density analysis with polyribosomes containing labeled nascent polypeptides.

EMC-virus-infected Krebs II cells were labeled for 2 min with ^{14}C-chlorella hydrolysate. The label in the preribosomal zone after such pulse-labeling is assumed to belong to nascent polypeptides [8]. The analysis of the extracts of pulse-labeled Krebs II cells has shown that radioactivity of the preribosomal zone (fig. 3A) may be displaced to the postribosomal zone after EDTA treatment (fig. 3B) and to the 80 S zone after RNase treatment (fig. 3C). This indicates that the structures containing labeled proteins are polyribosomes. When they were fractionated in a CsCl gradient together with viral RNA-containing structures the buoyant densities of both types of structures were shown to be identical (fig. 4A). It should be noted that the density of free ribosomes (80 S UV-absorbing material) is higher (fig. 4B). This is in agreement with the data obtained in other systems [9].

Two conclusions may be drawn from this part of our data: (1) Preribosomal structures containing viral RNA are represented predominantly by viral polyribosomes; (2) The buoyant density of polyribosomal material differs from that of 80 S ribosomes.

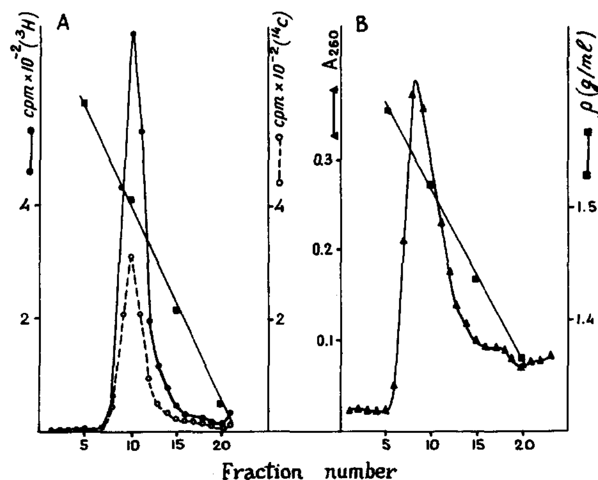

Fig. 4. The buoyant density of polyribosomal structures and of free ribosomes. EMC-virus-infected cells were incubated either with ^{3}H-uridine or with ^{14}C-chlorella hydrolysate, A, ^{3}H- and ^{14}C-preribosomal material mixed before fractionation in a CsCl gradient; B, 80 S ribosomes.

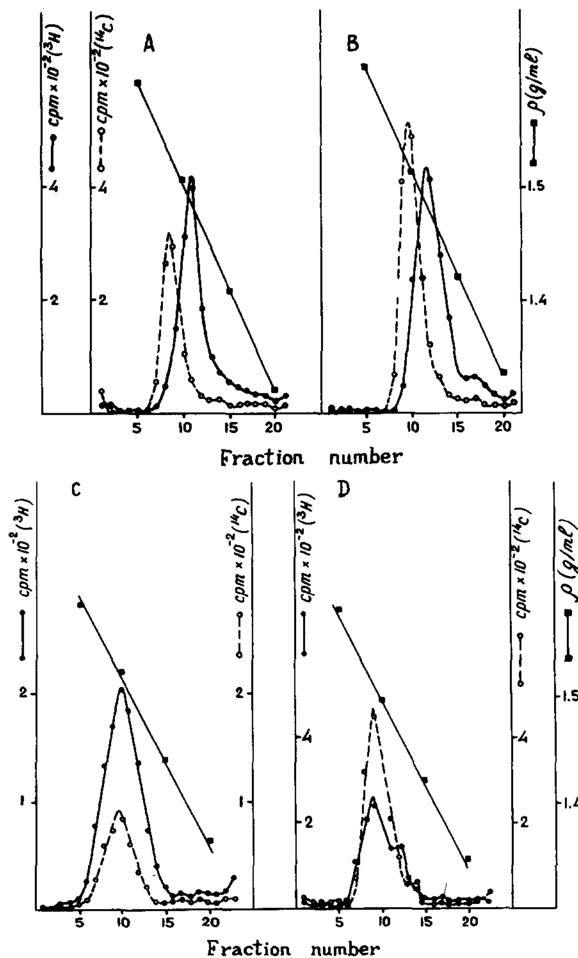

Fig. 5. A comparative buoyant density analysis of viral and normal polyribosomes and the effect of RNase on the buoyant density of both types of polyribosomes. EMC-virus-infected cells were incubated with either ^{3}H-uridine or labeled protein precursors (^{3}H-aminoacids or ^{14}C-chlorella hydrolysate). Uninfected cells were incubated with ^{3}H- or ^{14}C-protein precursors. RNase-treated and untreated extracts were fractionated in sucrose gradients. ^{3}H- and ^{14}C-labeled materials were mixed and analysed in CsCl gradients. A, polyribosomal material containing ^{3}H-labeled viral RNA was mixed with polyribosomal material from uninfected cells containing ^{14}C-labeled nascent proteins; B, polyribosomal material from infected cells containing ^{3}H-labeled nascent proteins was mixed with 80 S material from RNase-treated extract of infected cells containing ^{14}C-labeled nascent proteins; C, polyribosomal material from uninfected cells containing ^{14}C-labeled nascent proteins was mixed with 80 S material from RNase-treated extract of uninfected cells containing ^{3}H-labeled nascent proteins; D, 80 S material from RNase treated extracts containing ^{3}H-labeled nascent proteins (infected cells) and ^{14}C-labeled nascent proteins (uninfected cells).

3.2. *The buoyant density of viral and normal polyribosomes and the action of RNase*

The buoyant density of viral polyribosomes in CsCl in several determinations had a value of 1.49–1.50 g/ml, while that of normal polyribosomes (i.e. polyribosomes from the extracts of uninfected cells pulse labeled with ^{14}C-chlorella hydrolysate) was usually 1.51–1.52 g/ml. In order to compare directly the buoyant density of the two types of structures they were mixed and analysed together in the same gradient. The data presented in fig. 5A show that the buoyant density of viral polyribosomes is slightly lower than that of polyribosomes from uninfected cells.

It had been supposed [6, 10, 11] that mRNA in polyribosomes is associated with protein and that the lower density of polyribosomes as compared to monosomes is caused, at least in part, by this complex (mRNP) [9]. From this point of view the different buoyant densities of viral and normal polyribosomes may be a consequence of a different spacing of ribosomes along mRNP strand and of a greater amount of mRNP per ribosome in the viral polyribosome as compared to the normal one. If this is so one could expect that RNase digestion of free parts of mRNP would lead to the disappearance of the difference in the buoyant densities of the two types of polyribosomes.

The treatment of cytoplasmic extract with RNase leads to a displacement of the label (associated with nascent proteins) to the 80 S zone (fig. 3C). The buoyant density of the labeled material after such treatment increased if EMC-virus-infected cells were used (fig. 5B). No substantial increase in buoyant density was registered when RNase treatment was applied to extracts of uninfected cells (fig. 5C). When the 80 S materials from uninfected and infected cells (after RNase treatment of the extracts) were mixed and analysed in the same gradient no differences in the buoyant densities of the labeled structures could be detected (fig. 5D). It should be noted that RNase treatment slightly lowers the buoyant density of free 80 S ribosomes (perhaps because of partial loss of ribosomal RNA).

Three conclusions were drawn from this group of data: (1) The buoyant density of viral polyribosomes in EMC-virus-infected cells is lower than that of normal polyribosomes; (2) RNase treatment affects the buoyant density of the viral polyribosomes much more strongly than that of the normal polyribosomes; (3) The difference in the buoyant densities between the two types of polyribosomes is abolished by RNase treatment.

The basic experimental result of this work is the fact of lower buoyant density of viral polyribosomes in EMC-virus-infected cells as compared to normal polyribosomes. One of possible explanations of the fact may lie in the assumption of a different spacing of ribosomes along mRNP strand (in general terms, in a different amount of mRNP per ribosome). The data on the effect of RNase treatment upon the buoyant density of viral and normal polyribosomes are in agreement with this assumption. Some additional data comforming to this suggestion are presented in another report [12].

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