CONTROLLED LABELING AND RELEASE OF RIBONUCLEOPROTEIN PARTICLES IN "NUCLEAR COLUMNS"

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1. Introduction

As previously reported [1] a new method has been developed for the study of biochemical reactions in nuclei. The isolated subcellular particles are immobilized on the surface of membrane filter pieces which are then filled into columns ("nuclear columns"). The synthesis and release of compounds can be studied by passing selected media through the system.

Labeled RNA is continuously released at a fairly constant rate when the columns are perfused with a solution of labeled ribonucleoside triphosphates [1]. Compared with the conventional incubation in the test tube, the reaction proceeds for longer periods of time and yields greater amounts of product. The reaction can be interrupted and reinitiated by omission and addition of the reactants; it can be influenced by the addition of inhibitors and by changes in the flow rate and other variables [2].

In the following an analysis of the nuclear column effluent will be presented. Besides labeled RNA with a peak in the area of 4 S, labeled ribonucleoprotein particles with a peak in the area of 20–25 S are found in the effluent fractions. From the ribonucleoprotein particles with a buoyant density of 1.40–1.43 in CsCl, labeled RNA can be dissociated sedimenting in a zone with a peak at 10–12 S.

2. Methods

Nuclei from the livers of Sprague Dawley rats were isolated by the method of Widnell and Tata [3]. Ribonuclease inhibitor was added to all media. The crude ribonuclease inhibitor was prepared by a modification of the method of Roth [4]: 1 part of rat liver was homogenized in 3 parts (w/w) of sucrose A (0.25 M sucrose, 1 mM $MgCl_2$, 6 mM mercaptoethanol, pH 7.4). After centrifugation for 3 hr at 2.5×10^5 *g*, an equal volume of saturated ammonium sulfate was added to the clear supernatant. The precipitate was dialyzed against sucrose A.

The nuclei were attached to the membrane filter pieces and the columns were prepared as previously described [1].

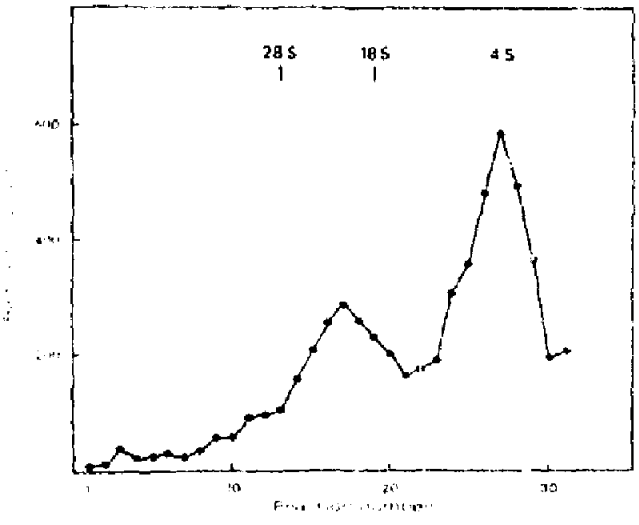

Fig. 1. Sucrose gradient analysis of the column effluent. The effluent of the nuclear column was layered on top of a 10–40% (w/w) sucrose gradient in 10 mM triethanolamine-HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$ and centrifuged at 2.6×10^5 *g* for 200 min in the Spinco SW 65-L-Ti rotor at 2°. Equivalents of the fractions were placed on filter paper pieces and the radioactivity of the acid-insoluble material was determined.

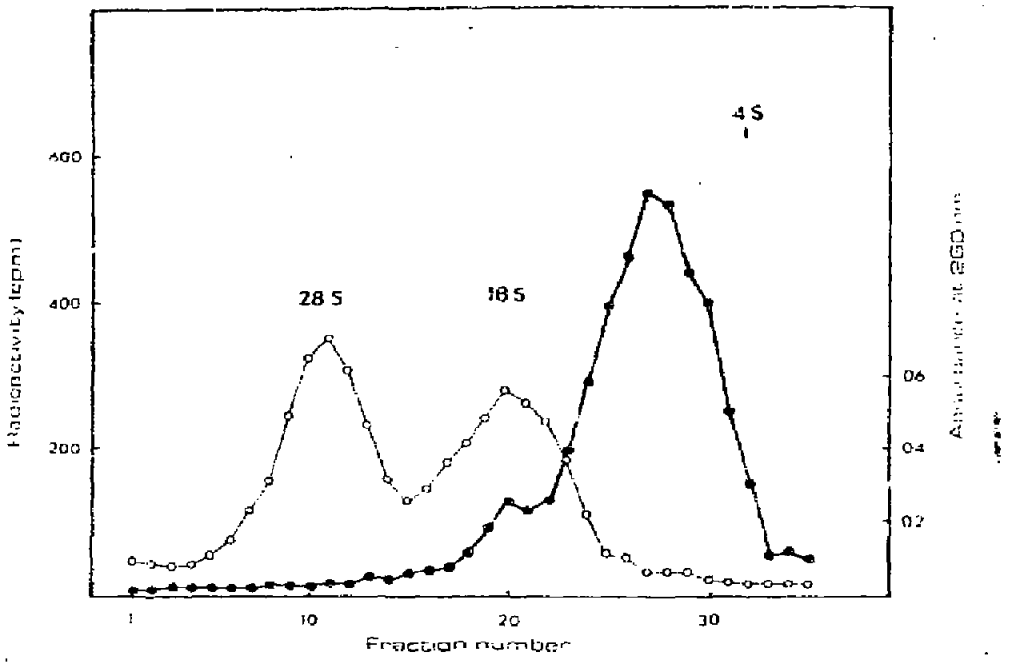

Fig. 2. Sedimentation pattern of RNA from the 22 S zone of the sucrose gradient. Fractions 14–21 of the gradient of fig. 1 were pooled. Carrier RNA and SDS to a final conc. of 1% were added and the RNA was precipitated with 2 vol ethanol. The material was centrifuged on a 5–20% sucrose gradient in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM NaEDTA, 0.25% SDS at 1.6×10^5 g for 175 min at 20° [7]. (○---○) Absorption at 260 nm. (●—●—●) Radioactivity of the TCA-insoluble material.

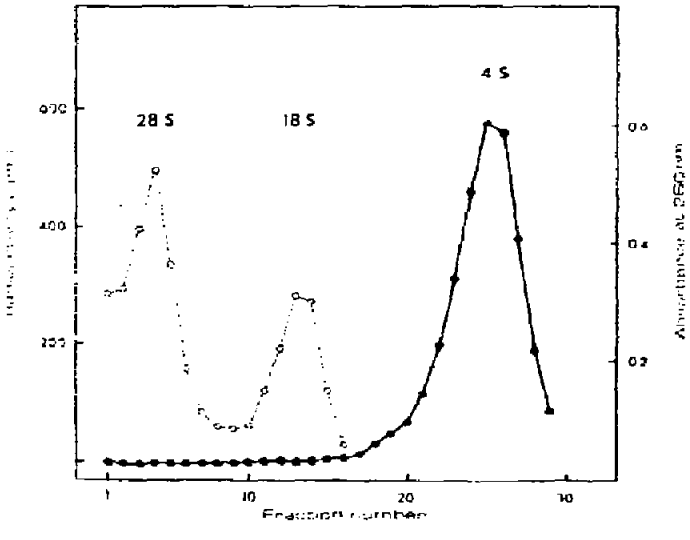

Fig. 3. Sedimentation pattern of RNA from the 4 S zone of the sucrose gradient. Fractions 22–29 of the gradient of fig. 1 were pooled. Other conditions were as stated for fig. 2, except that the sample was centrifuged at 1.6×10^5 g for 200 min.

The incubation medium contained 50 mM Tris-HCl (pH 8.1), 125 mM sucrose, 5 mM $MgCl_2$, 35 mM NaCl, 6 mM NaF, 5 mM dithiothreitol, 3 mM mercaptoethanol, 25 μM [^{3}H]UTP, 1.25 mM ATP, CTP and GTP, and ribonuclease inhibitor (equivalent to 30 g wet weight of the ammonium sulfate precipitate per ml medium).

2.1. *Incubations*

Nuclear columns with a bed volume of 30 μl were perfused at a rate of 20 μl/min. Initially, the column was washed with sucrose A at 0° with about 10 times the bed volume. Eight column volumes of incubation medium were then passed through. Finally, the reaction was initiated by raising the temperature to 30°. The effluent was collected and the rate of the reaction was followed by intermittant collection of effluent fractions on filter paper discs. The discs were washed once with 10% trichloroacetic acid (TCA), 1% pyrophosphate, 3 times with 5% TCA, 1% pyrophosphate, twice with ethanol, and once with ethanol:ether, 3:1 (v/v), dried and counted in toluene scintillator solution.

2.2. *CsCl buoyant density gradient centrifugation*

The pooled fractions of the sucrose gradient were adjusted to 4% final formaldehyde conc. and kept at 4° for 24 hr (Spirin et al. [5]). The sample was dialyzed against 10 mM triethanolamine (pH 7.5), 1 mM $MgCl_2$, 2% formaldehyde and 0.8% Brij-58 (Baltimore et al. [6]). After addition of CsCl a gradient was performed.

3. Results and discussion

The columns of immobilized nuclei were continuously perfused at a rate such that the bed volume was exchanged every 1.5 min. The medium contained the four ribonucleoside triphosphates, one being labeled, and a ribonuclease inhibitor fraction. The column was initially kept at 0°, then the reaction initiated by raising the temperature to 30°. As has been shown before [1], acid-insoluble radioactive material is continuously synthesized and released at a fairly constant rate. For the present analyses the effluent fractions were collected for 20 min, pooled and directly layered on top of a sucrose gradient.

Fig. 1 shows the sedimentation profile of the labeled acid-insoluble material. There is a broad zone with a peak at about 22 S and a peak in the area of 4 S.

For the RNA analysis, the fractions of the two main zones were separately pooled, precipitated with SDS-ethanol and recentrifuged in a sucrose gradient containing SDS. It can be seen in fig. 2 that the 22 S material of the first gradient yields RNA sedimenting in a broad zone with a peak at about 10 S. The sedimentation profile of the 4 S material, however, is practically unaffected by the presence of SDS (fig. 3).

The data suggest that the faster sedimenting material of the first gradient consists of ribonucleoprotein particles, whereas the material of the 4 S zone constitutes free RNA. This conclusion is supported by the observation that only the 22 S material is quantitatively retained on membrane filters.

In order to fix ribonucleoprotein complexes, fractions of the nuclear column effluent sedimenting between 15–30 S were pooled and treated with formaldehyde. CsCl equilibrium centrifugation revealed that the material banded at a density of 1.40–1.43 g/cm^3 (fig. 4).

It appears that the ribonucleoproteins are released

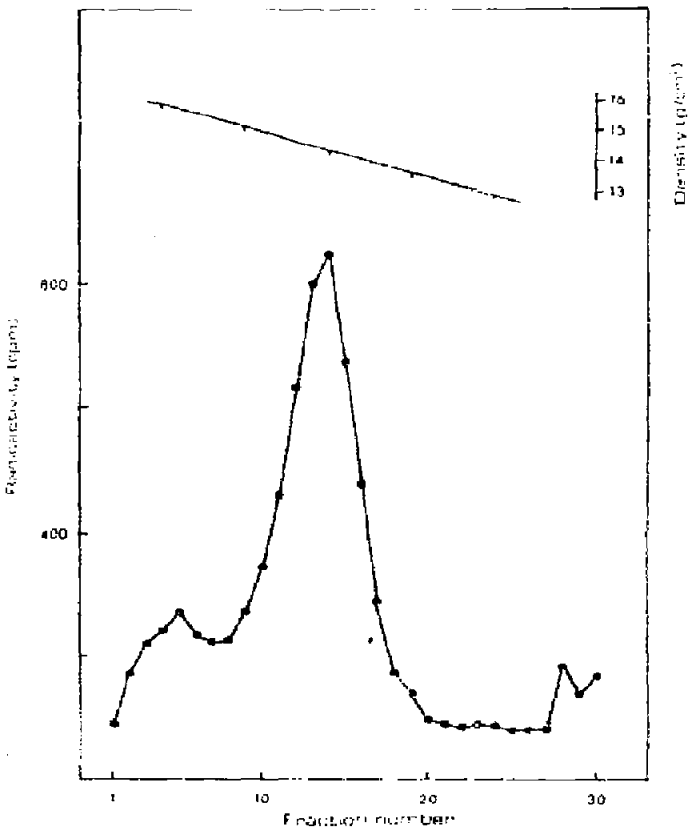

Fig. 4. Density distribution of the ribonucleoprotein particles. Fractions within the 15–30 S zone of a sucrose gradient of a column effluent were pooled and fixed with formaldehyde as described in Methods. A preformed CsCl gradient (1.25 – 1.6 g/cm^3) was centrifuged at 10^5 *g* for 18 hr at 4°. The acid-insoluble material of the fractions was collected on glass filter discs.

by the immobilized nuclei and are not formed by absorption of RNA to proteins present in the incubation medium [8, 9] as results similar to those shown are obtained when the ribonuclease inhibitor is omitted from the incubation medium.

When the effluent is subsequently collected over three periods of time of 12 min each, ribonucleoprotein particles are found in all fractions indicating that the material is gradually released.

Ishikawa et al. [10] reported that prelabeled messenger ribonucleoprotein complexes are gradually released by rat liver nuclei in an ATP dependent reaction. The characteristics of the present ribonucleoprotein particles so far determined correspond to those described by Spirin [11] in his definition of informo-

somes. However, further results must be obtained before the products formed in the continuous flow system can be fully characterized.

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