Isolation of a Nuclear Ribonucleoprotein Network that Contains Heterogeneous RNA and is Bound to the Nuclear Envelope†§

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ABSTRACT: Rapidly labeled polydispersed nuclear RNA is part of a ribonucleoprotein (RNP) network which in turn is tightly bound to the nuclear membrane. The membranous attachment, therefore, established a connection between chromatin and cytoplasm. The ultrastructure of the RNP network comprises fibrils and granules similar to those observed in intact nuclei. When bound to the nuclear membrane it has the composition of 63% protein, 14% RNA, 0.4% DNA, and 22.6% lipids. The proportion of lipids diminishes to 2.2% when nuclear membrane is not present. Chromatin, nucleoli, and ribosomes are minor contaminants since histones and ribosomal proteins are not detectable in polyacrylamide gel electrophoresis. Nuclear disruption at high pressure in a French pressure cell causes fragmentation of the RNP network into a series of polydispersed RNP particles. Fragmentation can be prevented by using mild pressure, or by disrupting nuclei with high salt buffer and digesting the dispersed chromatin with deoxyribonuclease. A RNP network, almost free of membrane, is also obtained if the nucleus is deprived of its envelope by treatment with Triton X-100. Since no polydispersed RNP particles are found following dissolution of the nuclear membrane, it is assumed that the particles are components of the RNP network whose fragmentation occurs as a consequence of two processes: (a) activation of nuclear nucleases and (b) shearing forces.

Ribonucleoprotein (RNP)[1] particles that contain putative mRNA were first described by Spirin (1966) in the cytoplasm, and by Georgiev and Samarina (1971) in the nucleus. Several researchers have reported that similar structures have been isolated from a variety of nuclei (Moulé and Chauveau, 1968; Parsons and McCarty, 1968; Lawford et al., 1967; Niessing and Sekeris, 1971; Köhler and Arends, 1968; Faiferman et al., 1970; Martin and McCarthy, 1972; Stévenin and Jacob, 1972; Albrecht and Van Zyl, 1973; Sommerville, 1973; Pederson, 1974). Since the protein moiety of this RNP cannot be displaced easily by foreign RNA (Spirin, 1969; Henshaw and Loebenstein, 1970), and the protein species are not merely random samples of nucleoplasmic proteins bound to any RNA (Faiferman et al., 1971a), it is assumed that RNP particles cannot be generated as a result of adventitious RNA–protein interactions. Nevertheless, the real nature, origin, and role of these particles as mRNA carriers still remain to be established.

From the nucleus, evidence was obtained indicating that these particles might be fragments of a more complex structure (Faiferman et al., 1971a). For example, when determination of the molecular weight was made through the method of sedimentation equilibrium instead of through sedimentation alone (Samarina et al., 1966), a larger value was obtained than that reported previously. This seems to be unique to these particles and strongly suggests that, contrary to the compact structure displayed by ribosomes and ribosomal subunits, the shape of the nuclear particles is very irregular and/or elongated. On the other hand, it was observed that in ascites tumor cells, as well as in yeast, the polysomal mRNA precursor is not as RNP particles but is bound to cytoplasmic membranes (Faiferman et al., 1971b; Shiokawa and Pogo, 1974). Therefore, it is possible that cytoplasmic membranes, in conjunction with the nuclear envelope, establish a linkage between chromatin and polysomes. If this is so, it may be assumed that mRNA and pre-mRNA do not exist in the form of nuclear particles but in a special structure that links genetic loci with the nuclear envelope.

In this paper, evidence is presented indicating that nuclear HnRNA appears in the form of a RNP network, which is tightly bound to the nuclear membrane. This is the first report on the extraction of this structure, which was first described in ultrastructural studies of nuclei obtained from a variety of systems (Swift, 1963; Shankar Narayan et al., 1967; Monneron and Bernhard, 1969). A preliminary report has been presented about this finding (Faiferman, 1973).

Materials and Methods

(*a*) *Isolation of Labeled Krebs Ascites Nuclei.* Ascites cells were obtained 5–7 days after inoculation of white mice. The cells were washed in saline solution (0.14 *M* NaCl), resuspended at a density of 2×10^6 cells/ml in Eagle's medium (Grand Island Biological Co.), and supplemented with amino acids as explained (Faiferman et al., 1971b). They were incubated at 37° for 30 min in the presence of 0.04 μg/ml of actinomycin D and 1 μg/ml of ethidium bromide; 5 μCi/ml of [5-^{3}H]uridine (specific activity 25 Ci/mmol; Schwarz/Mann) was added and the incubation continued for an additional 30 min. Then 0.6 mg/ml of nonradioactive uridine was added, and the cells were col-

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[1] Abbreviations used are: RNP, ribonucleoprotein, HnRNA, heterogeneous RNA; TMS, Tris-HCl–magnesium chloride–sucrose; HSB, high salt buffer.

lected and resuspended in a fresh medium containing 0.6 mg/ml of nonradioactive uridine. Finally, the cells were cultured in the chase medium for 3 hr. The cells were fractionated and the nuclei isolated (Faiferman et al., 1971b).

(*b*) *Isolation of Labeled Rat Liver Nuclei.* Adult male rats (250–300 g) were labeled by intraperitoneal injection of 250 μCi of [5-^{3}H]orotic acid/rat (specific activity 16 Ci/mmol; Schwarz/Mann). After a period of 30 min, the animals were sacrificed, and the rat liver nuclei were isolated (Pogo et al., 1967).

(*c*) *Nuclear Disruption in a French Pressure Cell and Nitrogen Cavitation Bomb.* A pellet of Krebs ascites or rat liver nuclei was resuspended in 5 volumes of a buffer solution containing 10 m*M* Tris-HCl (pH 7.5) (23°), 2.5 m*M* $MgCl_2$, and 0.25 *M* sucrose (TMS). Prior to disruption, rat liver cytoplasmic ribonuclease inhibitor was added at a concentration of 0.5% (weight of protein per volume of packed nuclei). This suspension was disrupted either in a French pressure cell or a nitrogen cavitation bomb, as explained in Figure 1 and Table I. The disrupted nuclei were then centrifuged at 3000*g* for 10 min. The pellet contained the bulk of the DNA, and the supernatant, the nucleoplasmic fraction, was further centrifuged in a 10–30% sucrose gradient (w/v) containing the components of the TMS buffer. The gradients were fractionated in an ISCO Gradient fractionator, equipped with a uv absorption recorder, 254 mμ, 1-cm path length (Faiferman et al., 1970). To each fraction was added about 5 ml of 10% cold trichloroacetic acid and acid-insoluble material collected in a glass fiber filter, Whatman GF/B, washed several times with 10% cold trichloroacetic acid, and the radioactivities were determined by counting in Triton–toluene solution (1:2; v/v) in a scintillation counter.

(*d*) *Nuclear Disruption in a High Salt Buffer Solution (HSB–DNase Method).* A modification of the method of Penman (1966) was used. A pellet of nuclei, isolated as described, was resuspended in 5 vol of a high salt buffer (HSB) solution, which contained 0.8 *M* NaCl, 8 m*M* $MgCl_2$, and 10 m*M* Tris-HCl buffer (pH 7.6). The dispersed chromatin was incubated with 150 μg/ml of DNase (Worthington Biochemical Corp.) at 37° for 2 min, then cooled in ice, and homogenized with four strokes in the Dounce homogenizer. This homogenization is essential to dissociate the nucleolus from the nuclear matrix. The unbroken nuclei were separated by centrifugation at 1000*g* for 7 min. The supernatant was further centrifuged in a 10–30% sucrose gradient, containing the HSB components, at 19,000 rpm for 15 min in the Spinco SW40 rotor. The gradient was fractionated in an ISCO sucrose gradient fractionator, and the upper 12 fractions were collected, pooled, and recentrifuged in a gradient that was similar but contained 2 *M* sucrose cushion, at 16,000 rpm for 16 hr in the Spinco SW27 rotor. The gradient was fractionated in an ISCO gradient fractionator.

(*e*) *Isolation and Characterization of the RNA Obtained with the HSB–DNase Method.* A modification of the procedure of Perry et al. (1972) was used, which resulted in the highest yield of RNA (Gross and Pogo, 1974). Prior to digestion of the dispersed chromatin, the solution containing the DNase was treated with iodoacetic acid (Zimmerman and Sandeen, 1966), to eliminate traces of RNase. The extracted RNA was analyzed either in a 15–30% sucrose gradient (w/v) containing 0.1 *M* sodium acetate buffer (pH 6.0), 0.1 *M* NaCl, and 1 m*M* EDTA, or in a dimethyl sulfoxide sucrose gradient (Shiokawa and Pogo, 1975). The gradients were fractionated in an ISCO gradient fractionator, as explained previously, the fractions were collected in vials, and the radioactivity was measured by counting in a Bray solution in a scintillation counter.

(*f*) *Isolation and Characterization of Proteins in Polyacrylamide Gel Electrophoresis.* (i) Soluble Nuclear Proteins. Rat liver nuclei were disrupted in the nitrogen cavitation bomb (1.2–1.4 $\times$ 10^3 psi); the nucleoplasmic fraction was obtained, centrifuged in a 10–30% sucrose gradient containing TMS buffer at 16,000 rpm in the Spinco SW27 rotor for 16 hr, and fractionated in an ISCO gradient fractionator. Fractions, where the upper uv absorption peak sedimented, were collected and pooled, and the proteins were precipitated with 10% cold trichloroacetic acid, which was resuspended in the appropriate buffer and processed (Faiferman et al., 1971a).

(ii) Proteins of 43S Rat Liver Nuclear Particles. Rat liver nuclei were disrupted in the French pressure cell at 15–20 $\times$ 10^3 psi, and the nucleoplasmic fraction that was obtained was divided into two aliquots. NaCl and $MgCl_2$ were added to one aliquot at final concentrations of 0.8 *M* and 8 m*M*, respectively. Then 150 μg/ml of DNase was added, incubated for 2 min at 37°, and cooled, and Triton X-100 was added at a final concentration of 1%. Both treated and nontreated aliquots were centrifuged on a 10–30% sucrose gradient and fractionated. The sucrose gradient, where the treated nucleoplasmic aliquot was centrifuged, contained 0.8 *M* NaCl and 8 m*M* $MgCl_2$. The fractions, where the 43S peak sedimented, were collected, pooled, and centrifuged at 65,000 rpm in the Spinco 65 rotor for 2.5 hr. The pellets of treated and nontreated 43S particles were processed, as described below.

(iii) Proteins of the RNP Network. A pellet of rat liver nuclei was resuspended in 40 volumes of TMS buffer supplemented with rat liver cytoplasmic supernatant (Faiferman et al., 1970). Triton X-100 was added at a final concentration of 1%; the suspension was centrifuged at 1000*g* for 7 min and washed three times with 40 volumes of TMS buffer. Finally, these washed nuclei were disrupted by the HSB–DNase method and centrifuged at 1000*g* for 7 min and Triton X-100 was added to the supernatant at a final concentration of 1%. The Triton-treated supernatant was then layered on a 10–30% sucrose gradient containing HSB and centrifuged at 16,000 rpm for 16 hr in the Spinco SW27 rotor. The pellet contained a highly purified RNP network which was processed as explained below.

The pellets of the treated and nontreated 43S particles (ii) and of the RNP network (iii) were resuspended in a solution containing 4 *M* urea and 2 *M* LiCl, and maintained at 4° for 20 hr. They were centrifuged at 65,000 rpm in the Spinco 65 rotor for 1 hr. The clear supernatant was collected, and cold trichloroacetic acid was added at a final concentration of 10%. The precipitants were collected by centrifugation and dissolved in the appropriate buffer (Faiferman et al., 1971a).

(iv) Histones. Duck erythrocyte histones, provided through the courtesy of Dr. V. G. Allfrey, were dissolved in distilled water, 1 *M* Tris-HCl buffer (pH 9.0) was added at a final concentration of 0.1 *M*, and 2-mercaptoethanol was added at a final concentration of 1%. Then the solution was heated at 50° for 45 min, diluted with the appropriate buffer, and processed.

All proteins were dissolved in the appropriate buffers and fractionated in acetic acid–urea or sodium dodecyl sulfate gel electrophoresis (Faiferman et al., 1971a).

(*g*) *Electron Microscopy.* The specimens were fixed for 2

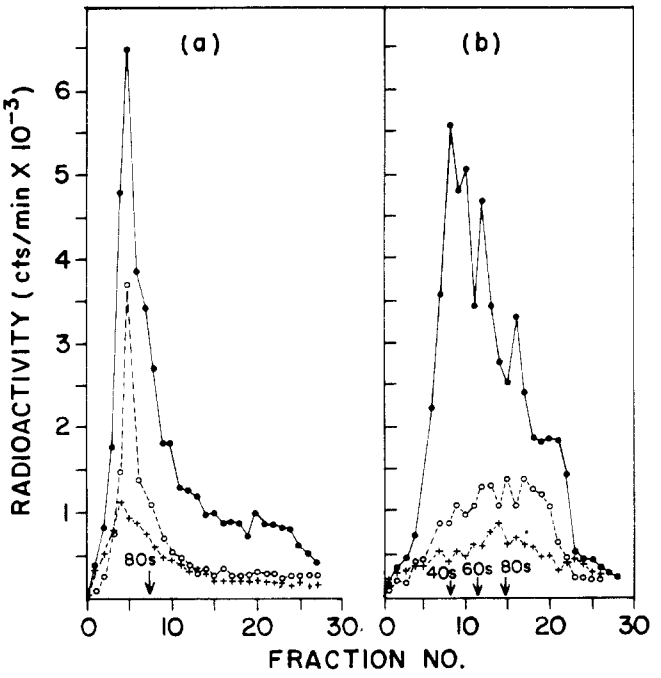

FIGURE 1: Effect of the pressure applied to nuclei on the release of RNP nuclear particles. Labeled ascites and rat liver nuclei were disrupted in a French pressure cell and nitrogen cavitation bomb. The nucleoplasms were centrifuged in a 10–30% sucrose gradient, as explained in the section on Materials and Methods. Ascites nucleoplasms were centrifuged at 40,000 rpm in the Spinco SW40 rotor for 1 hr (a) and rat liver nucleoplasms were centrifuged in the Spinco SW27 rotor at 16,000 rpm for 16 hr (b). (●—●) 15–20 × 10^3 psi and (○- -○) 3–4 × 10^3 psi in the French pressure cell; (×- -×) 1.2–1.4 × 10^3 psi in nitrogen cavitation bomb. Direction of the centrifugal force, from left to right.

hr at 4° in 2% glutaraldehyde and post-fixed for 1 hr at room temperature in a solution containing 1% OsO_4 and 0.1 *M* phosphate buffer. The pellets were dehydrated in a graded ethanol series and embedded in Epon. The blocks were sectioned and stained with uranyl acetate. Thin sections were examined under a Siemens Elmiskop I electron microscope.

(*h*) *Ribonuclease and Pronase Digestions of the RNP Network.* The membrane-bound RNP network was isolated as explained in (d). The material which sedimented on top of the 2 *M* sucrose cushion was collected and diluted with TMS buffer to bring the sucrose concentration down to 0.25 *M*. Then it was divided into three aliquots and incubated with the enzymes at 37° for 10 min as explained in Plate 3. Afterwards they were cooled in ice and centrifuged in the Spinco 65 rotor at 50,000 rpm for 30 min. The supernatant was decanted and the pellets were fixed, dehydrated, and embedded in Epon for electron microscopic examination.

(*i*) *Ribonuclease Inhibitor.* Rat liver cytoplasmic ribonuclease inhibitor was prepared according to Roth's (1958) method.

(*j*) *Chemical Analysis.* Protein was determined through modification of the biuret method of Crampton et al. (1959). RNA was determined through the method of Fleck and Munro (1962); DNA through the procedure of Burton (1953); and lipids through the technique of Folch et al. (1957).

Results

(*a*) *Effects of Different Methods of Nuclear Disruption on the Recovery of RNP Particles.* When rat liver or ascites nuclei are disrupted by compression and decompression at high pressure in a French pressure cell, the majority of the rapidly labeled RNA appears in nucleoplasm as RNP particles (Faiferman et al., 1970, 1971b). In order to determine whether there is a correlation between the degree of pressure used and the amount of particles recovered, nuclear disruptions by different mechanical procedures as well as by different degrees of pressure were explored. Since compression and decompression by a French pressure cell produce disruption mainly through shearing forces, a nitrogen cavitation bomb was introduced because it disrupts through a different mechanism at a very low pressure. This method consists of rapid decompression of a nitrogen pressurized cell. The absorption of nitrogen by nuclei during the pressure cycle results in nuclear expansion and rupture when the suspension is suddenly released to the atmospheric pressure. Consequently, complete nuclear disruption is produced at minimal pressure.

Table I: Amout of RNP Particles in the Nucleoplasm.[c]

	Krebs Ascites Nuclei[a]			Rat Liver Nuclei[a]		
	French Pressure Cell		Cavitation Bomb	French Pressure Cell		Cavitation Bomb
	a	*b*	*c*	*a*	*b*	*c*
Nucleoplasm	20.6[b]	6.6	4.7	4.8	2.0	1.5
Pellet	28.0	42.0	44.0	4.0	6.0	6.9

[a] *a* = 15–20 × 10^3 psi; *b* = 3–5 × 10^3 psi; *c* = 1.2–1.4 × 10^3 psi. [b] All values are × 10^4 cpm. [c] Krebs and rat liver nuclei were labeled, isolated, and disrupted as explained in Materials and Methods. The suspensions of disrupted nuclei were centrifuged at 3000*g* for 10 min. Radioactivities were determined in nucleoplasm, pellet, and intact nuclei as explained in Materials and Methods. The radioactivity in intact Krebs nuclei was 49 × 10^4 cpm and in intact rat liver nuclei was 9.6 × 10^4 cpm.

These experiments are shown in Figure 1a and b. A large number of RNP particles in nucleoplasm were recovered when vigorous shearing forces were used. Conversely, small amounts were recovered when nuclei were disrupted either by mild shearing force (French pressure cell at low pressure) or expansion and sudden decompression in the nitrogen cavitation bomb. It should be noted that similar results were obtained when mild procedure was used, through two procedures of disruption in nuclei isolated from different systems. Thus, small amounts of RNP particles were obtained when minimal pressure was exerted, either on ascites or on the nuclei of rat liver (Figure 1a and b).

In order to determine whether this poor recovery was due to degradation or to insufficient release of RNP particles, the distribution of rapidly labeled RNA was measured in nucleoplasm, as well as structures that sedimented rapidly after centrifugation for 10 min at low speed (Table I). It was observed that radioactive RNA, which did not appear in the nucleoplasm, was recovered in the sediment. Preliminary experiments indicated that when this sediment was resuspended in the same buffer used for disruption of the nuclei, and fractionated in a 10–30% sucrose gradient, containing a 2 *M* sucrose cushion, a substantial amount of labeled RNA sedimented at the top of this cushion. The remainder penetrated the 2 *M* sucrose layer and sedimented at the bottom of the tube. It is reasonable to assume that the material containing radioactive RNA, which sediment-

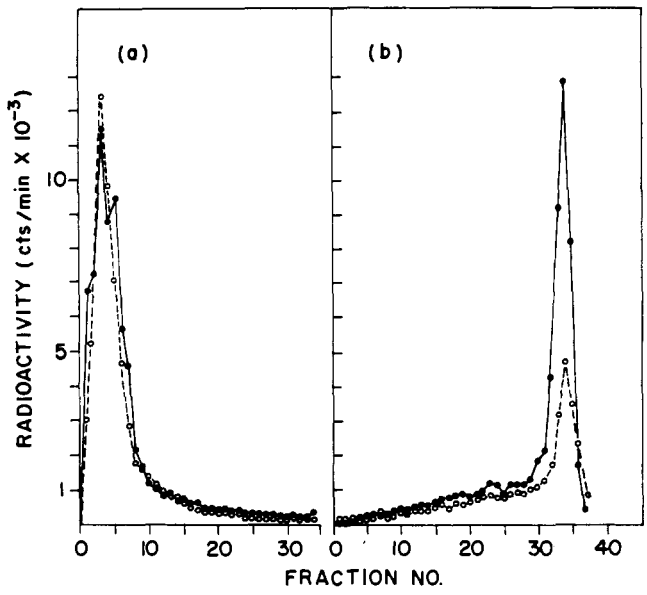

FIGURE 2: Sucrose gradient centrifugation of the nuclear structure that contains rapidly labeled RNA. Rat liver nuclei were labeled and prepared, as explained in the section on Materials and Methods, and divided in two aliquots. One was washed with TMS buffer containing 1% Triton X-100 and the other was used as a control. Both were disrupted by the HSB-DNase method and clarified by centrifugation at 1000*g* for 7 min, and the supernatants were layered on separate 10–30% sucrose gradients containing the HSB. They were centrifuged in the Spinco SW40 rotor at 19,000 rpm for 15 min (a). They were fractionated and aliquots used for the determination of radioactivities. Input of radioactivity was 78.1 × 10^3 for control and 66 × 10^3 for nuclei treated with Triton X-100; the amounts recovered in the pellet were 8 × 10^3 and 9 × 10^3, respectively. In (b) the upper 12 fractions of (a), where the radioactivities sedimented, were pooled and layered in identical sucrose gradients, but containing a 2 *M* sucrose cushion, and centrifuged at 16,000 rpm for 16 hr in the Spinco SW27 rotor. Input of radioactivity was 60 × 10^3 in both control and Triton-treated nuclei; the amounts recovered in the pellet were 1.8 × 10^3 and 36.0 × 10^3, respectively. For fractionation and determination of radioactivities see Materials and Methods. (●—●) Control and (O- -O) Triton-treated nuclei. Direction of the centrifugal force, from left to right.

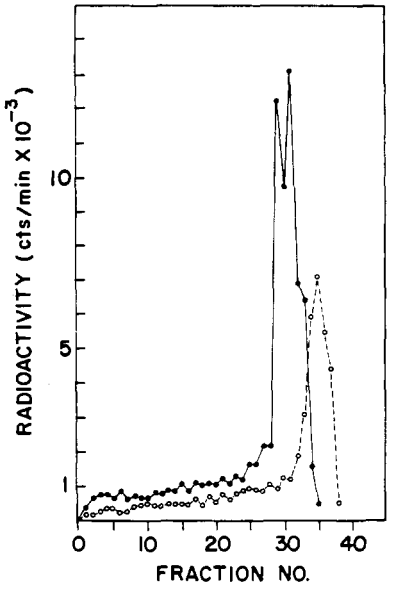

FIGURE 3: Effect of Triton X-100 on the structure that contains rapidly labeled RNA. Labeled rat liver nuclei were disrupted by the HSB-DNase method and after centrifugation at 1000*g* for 7 min, the supernatant was divided in two aliquots. To one aliquot was added Triton X-100 at a final concentration of 1%. The sample treated with detergent and the control sample were centrifuged in a 10–30% sucrose gradient containing the HSB, and made on top of a 2 *M* sucrose cushion. They were centrifuged for 16 hr at 16,00 rpm in the Spinco SW40 rotor. For fractionation and determination of radioactivities, see Materials and Methods. (●—●) Control and (O- -O) detergent-treated samples. Input of radioactivity 94 × 10^3 in both control sample and sample treated with Triton X-100; the amounts recovered in the pellet were 15 × 10^3 and 50 × 10^3, respectively. Direction of the centrifugal force, from left to right.

ed at the top of the sucrose cushion, was attached to the nuclear envelope. Therefore, it was decided to use a procedure that, although completely disruptive to the nuclei, preserves fragile nucleic acid–protein complexes, prevents aggregation of particles, and does not alter the nuclear envelope. Accordingly nuclei were treated with a solution containing high salt concentration. This method was employed initially by Mirsky and Pollister in 1942 for isolation of DNA–protein complexes and later on by Sibatani et al. (1962) for isolation and characterization of calf thymus nuclear Hn RNA species. To avoid the effect of shearing forces, the dispersed chromatin was not stirred vigorously but was digested partially by DNase treatment, a modification Penman (1966) introduced successfully for the fractionation of the nuclei of HeLa cells. However, in our experiments a low concentration of Mg^{2+} was used in order to prevent adventitious interaction of nuclear structures.

Although this procedure was applied to ascites cells, L cells, and rat liver tissue, the rat liver tissue was used mainly because a highly purified nuclear fraction could be obtained without employing sodium deoxycholate to strip the nuclei from cytoplasmic membranes (Pogo et al., 1966). As demonstrated previously (Moulé and Chauveau, 1968; Faiferman et al., 1971a; Stevenin and Jacob, 1972), the nuclear RNP particles were dissolved through the use of this detergent, rather than Triton X-100.

The majority of the rapidly labeled nuclear RNA sedimented in the upper regions of the gradient while very small (10–20%) sedimented at the bottom of the tube when a centrifugation was used to sediment nuclei (Vesco and Penman, 1968) (Figure 2a). Moreover, the sedimentation properties of the material containing rapidly labeled RNA were not altered when rat liver nuclei were stripped from the nuclear envelope, as a result of treatment with Triton X-100. As will be shown later, this treatment, which dissolves the majority of the lipids, preserves intact the remaining nuclear structures.

The material containing most of the rapidly labeled RNA was then recentrifuged for 16 hr in a gradient that was similar but contained a 2 *M* sucrose cushion. It is evident that provided the nucleus was not deprived of its envelope, the majority of the rapidly labeled RNA sedimented on top of the sucrose cushion (Figure 2b). Thus, if nuclei treated with Triton X-100 were disrupted through use of the HSB-DNase method, only 20–25% of the rapidly labeled RNA was retained by the sucrose cushion. Furthermore, if Triton X-100 treatment was given to disrupted nuclei, similar amounts of the rapidly labeled RNA were recovered from the 2 *M* sucrose cushion (Figure 3). These experiments indicated that most of the rapidly labeled RNA are associated with a structure that is tightly bound to the nuclear membrane. Moreover, and most important, upon dissolution of the nuclear envelope, the rapidly labeled RNA is not released as RNP particles, but in a structure that is so large and dense it penetrates the 2 *M* sucrose cushion and a significant amount sediments at the bottom of the tube (Figures 2b and 3).

Table II: DNA, RNA, Proteins, and Lipids in RNP Network, RNP Particles, and Total Nuclei.[a]

	DNA		RNA		Proteins		Lipids	
	MG[b]	%[c]	MG[b]	%[c]	MG[b]	%[c]	MG[b]	%[c]
Nuclei	106.5 (104.0–109.0)	31.9	14.5 (13.1–15.4)	4.3	186.5 (180.5–192.5)	55.8	26.5 (21.0–30.4)	8.0
Triton-treated nuclei	105.5 (102.0–107.0)	35.2	13.3 (13.0–13.5)	4.4	177.2 (172.0–185.0)	59.2	3.6 (3.4–3.8)	1.2
RNP network	0.049 (0.036–0.063)	0.78	0.99 (0.95–1.04)	17.0	4.69 (4.35–5.35)	80.0	0.12 (0.10–0.15)	2.2
43S RNP particles[d]		0.5		17.3		82.2		
Membrane-bound RNP network	0.056 (0.042–0.068)	0.4	2.02 (1.6–2.2)	14.0	9.0 (7.9–10.1)	62.8	3.3 (3.2–3.4)	22.8

[a] These represent amounts recovered from 100 g of rat liver tissue. Triton-treated and nontreated nuclei were disrupted by the HSB–DNase method and the RNP network was isolated as explained. For chemical analysis see Materials and Methods. In the RNP network 0.046% DNA, 6.8% RNA, 2.5% proteins, and 0.45% lipids of the intact nucleus are recovered. [b] Mean value of three to four different preparations; maximum and minimum values are in parentheses. [c] DNA + RNA + proteins + lipids = 100%. [d] Taken from Faiferman et al., 1970.

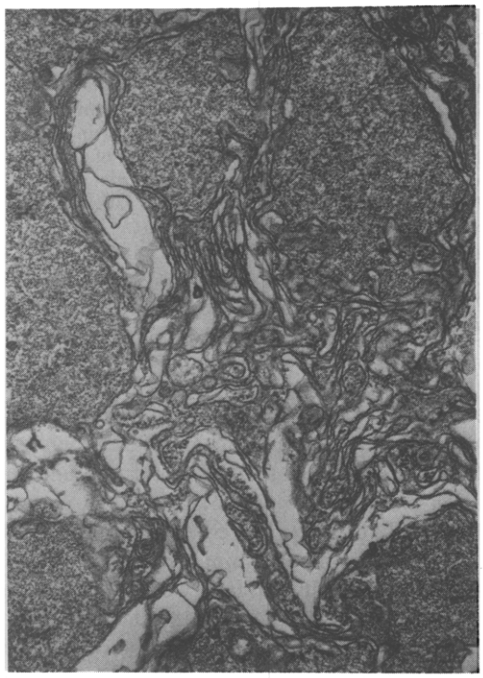

PLATE 1: Typical appearance of the structure that sedimented on top of the 2 *M* sucrose cushion when rat liver nuclei were disrupted with the HSB–DNase method and fractionated, as explained in Figure 2b. The nuclear membrane and a network of fibrils and particles are clearly observed; ×20,000.

(*b*) *Chemical Composition and Density of Nuclear Structure that Contains Rapidly Labeled RNA.* The chemical composition of the structure that contains rapidly labeled RNA is shown in Table II. As might be expected, when it was isolated from nuclei that were not treated with Triton X-100, lipids were a significant component. On the other hand, when it was obtained from nuclei treated with Triton X-100, its chemical composition was no different basically than that of the nuclear particles. The presence of a small amount of lipids is probably due to residual nuclear membrane. The insignificant amount of DNA, which is similar to that observed in the nucleoplasm of nuclei disrupted by the French pressure cell (Faiferman et al., 1970), proved that chromatin is absent.

It should be noted that Triton X-100 removes about 85% of the lipids; yet, the nucleus is very well preserved. This indicates that, contrary to the earlier assumption made by Blobel and Potter (1966) that this detergent removes solely the external nuclear membrane, it dissolves virtually the whole nuclear envelope. The absence of nuclear envelopes was recently demonstrated by electron microscopic examinations of rat liver nuclei treated with Triton X-100 (Aaronson and Blobel, 1974). Therefore the integrity of the nucleus depends upon structures other than the nuclear membrane.

A single uv and radioactive peak was observed at a density of 1.21 g/ml, when the structure that sediments on top of the 2 *M* sucrose cushion was sedimented to a state of equilibrium in a discontinuous sucrose gradient. This density was slightly greater than that reported for the nuclear envelope (Kashnig and Kasper, 1969). It reinforces the assumption that rapidly labeled RNA is a component of a structure that is tightly bound to nuclear membranes.

(*c*) *Electron Microscopic Study of Rat Liver Nuclear Structure that Contains Labeled RNA.* The ultrastructural appearance of the material that sedimented on top of the 2 *M* sucrose cushion is shown in Plate 1. The presence of membranes and a fibrogranular material are seen clearly. Since this structure was obtained from a highly purified nuclear fraction, it is assumed that these membranes are the inner and outer nuclear envelopes. The absence of DNA indicates that none of the fibrogranular material is chromatin.

The morphological characteristics of this material, after treatment of the nuclei with Triton X-100, are shown in Plate 2. As expected, the membranes can hardly be seen, and the fibrogranular mesh is very well preserved after this treatment. It is composed essentially of a network of fibrils and granules of different sizes and is similar to that originally observed by Shankar Narayan et al. (1967) in rat liver nuclei treated with low and high concentrations of sodium chloride. Although some distortion might be expected as a result of the treatments, its similarity to interchromatinic granules, perichromatinic granules, and perichromatinic fibrils, described by Monneron and Bernhard (1969) in EDTA-treated tissue (Plate 2), is remarkable. Enzymatic digestion of the membrane-bound RNP network is shown in Plate 3. Digestion with ribonuclease produces an enrichment in the membranous components and a marked diminution in the fibrogranular material. Nevertheless, some

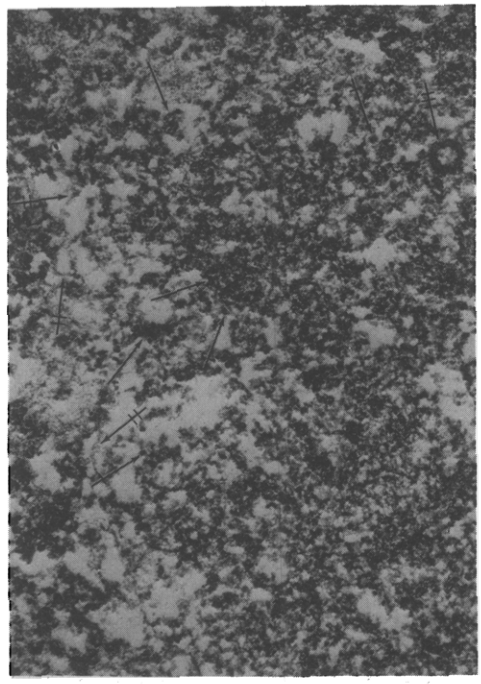

PLATE 2: Appearance of the RNP network after treatment of the structure shown in Plate 1 with Triton X-100. Note that few nuclear membranes are seen. The aggregation of small granules (→) seems to be similar to the interchromatinic granules of Monneron and Bernhard (1969); the large granules (+→) to the perichromatinic granules; and the fibrils (‖→) to the perichromatinic fibrils. The ring of aggregates of granules (‖→), observed consistently in several specimens, may be coiled bodies or nuclear bodies (Monneron and Bernhard, 1969); ×27,000.

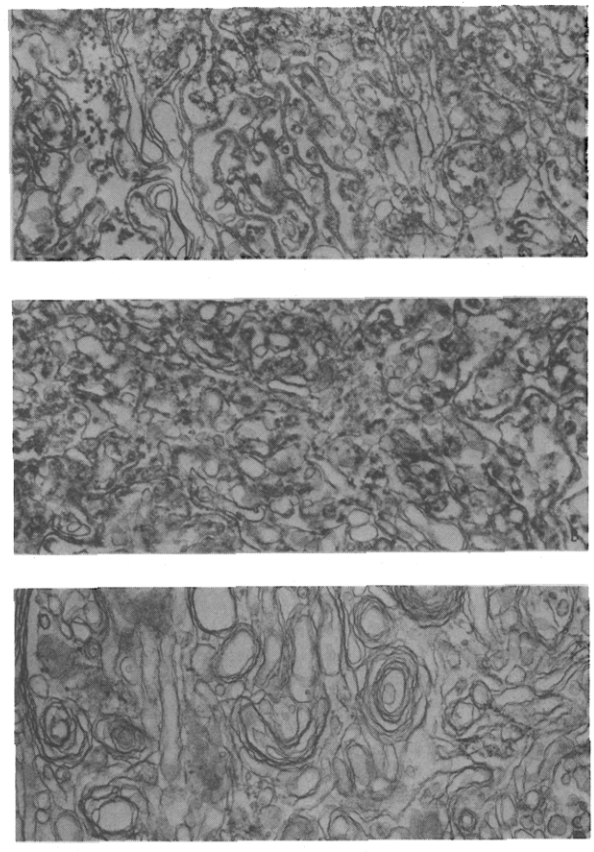

PLATE 3: Similar to Plate 1 but treated with 5 μg/ml (A) and 50 μg/ml (B) of ribonuclease A (Worthington Biochemical Corp.) and 100 μg/ml of Pronase (Calbiochem) followed by 50 μg/ml of ribonuclease A (C), see Materials and Methods. The disappearance of the RNP network is clearly observed by these treatments; ×20,000.

large granules (perichromatinic granules) and fibrils appear to be resistant to this digestion since no further diminution was observed when the amount of ribonuclease was increased by one order of magnitude (Plate 3a and b). However, an almost complete disappearance of the fibrogranular material was obtained when digestion with Pronase was made prior to digestion with ribonuclease (Plate 3c). This is similar to that observed by Monneron and Bernhard (1969) in intact tissue, and so far the best evidence of the RNP composition of this nuclear network. We have named this structure the nuclear RNP network.

(*d*) *Sedimentation Profile of RNA Isolated from RNP Network Bound to the Nuclear Envelope.* The radioactive profile and the uv profile of the RNA, extracted from the RNP network attached to the nuclear membrane, are shown in Figure 4a. A polydispersed radioactive profile was obtained with a sedimentation rate ranging from 10 S to 60 S, and four distinct uv peaks of 7S, 18S, 28S, and 45S RNA resulted.

The presence of 18S and 28S rRNA is attributed to ribosomes attached to the outer nuclear membrane and to the RNP network at the level of the nuclear pore, as well as the entrapment of ribosomal subunits in transit to the cytoplasm. The presence of a small amount of 45S rRNA precursor can be due to nucleolar fragments. The 4S to 7S uv peak corresponds to small molecular weight nuclear RNAs which have been studied extensively (Busch and Smetana, 1970). Current experiments indicate that the RNP network contains, like the RNP particles, stable small molecular weight RNAs species distinct from the nucleolus and nucleoplasm. These findings will be the subject of a subsequent paper.

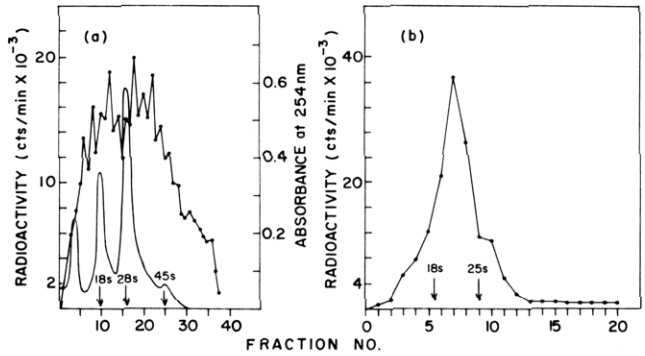

FIGURE 4: Sucrose gradient analysis of RNA. Labeled rat liver nuclei were disrupted by the HSB–DNase method and fractionated as explained in Figure 2b. The material that sedimented on top of the 2 *M* sucrose cushion was collected, diluted with HSB, centrifuged at 60,000 rpm in the Spinco SW 65 rotor for 1 hr, and the RNA extracted from the pellet as explained in Materials and Methods. The RNA was centrifuged on a 15–30% sucrose gradient at 25,000 rpm in the Spinco SW40 rotor for 16 hr (a). The RNA was centrifuged in dimethyl sulfoxide 0.5–20% sucrose gradients (w/v) at 45,000 rpm in the Spinco SW65 rotor for 17 hr (b) (Shiokawa and Pogo, 1974). In a parallel dimethyl sulfoxide sucrose gradient ^{14}C-labeled yeast rRNA was centrifuged and the two rRNA species were used in order to determine the sedimentation coefficient of the rat liver rapidly labeled RNA.

When labeled RNA in dimethyl sulfoxide–sucrose gradients was analyzed (Figure 4b), a polydispersed profile with sedimentation rates ranging from 10S to 30S (mean value, 21–22S) and a mean molecular weight of 1.0–1.3 × 10^6 were observed. Since none of the rRNA species is labeled, it is assumed that most of this rapidly labeled RNA is com-

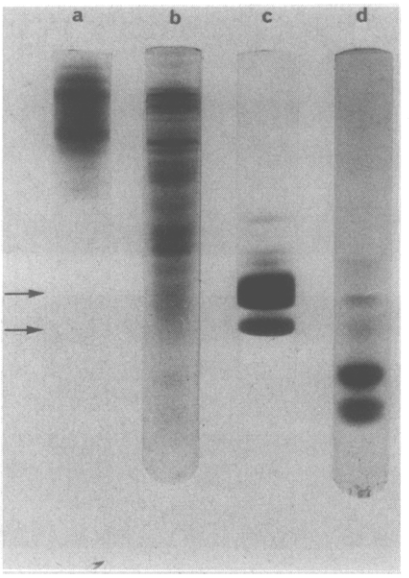

PLATE 4: Polyacrylamide gel electrophoretic analysis of proteins. The RNP network was obtained from rat liver nuclei treated with Triton X-100, disrupted by the HSB–DNase method, and fractionated in a sucrose gradient centrifugation, as explained in Materials and Methods. (a) Acetic acid–urea gel; (b) sodium dodecyl sulfate gel electrophoretic analysis; (c) acetic acid–urea gel; and (d) sodium dodecyl sulfate gel electrophoretic analysis of duck erythrocyte histones. The arrows indicate histone migration in an acetic acid–urea gel (compare a and c).

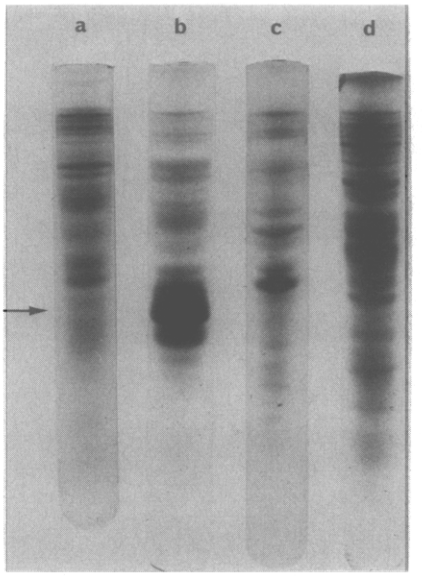

PLATE 5: Polyacrylamide sodium dodecyl sulfate gel electrophoretic analysis of proteins from: (a) RNP network, (b) nuclear particles, (c) nuclear particles treated with HSB–DNase method and Triton X-100, and (d) total soluble nuclear proteins. The arrow indicates proteins having 40,000 in molecular weight (Faiferman et al., 1971a).

posed of HnRNA. Evidently it seems to be well preserved, since the two rRNA species appear in the proportion expected (Figure 4a). However, the possibility should not be ruled out that polydispersed RNA might be more sensitive to degradation than rRNA.

(*e*) *Analysis of the Protein Moiety of the Rat Liver RNP Network.* As indicated previously, the RNP network obtained from the nucleus of rat liver, treated with Triton X-100, contains very little nuclear envelope; therefore, it was used regularly to study its protein species.

As seen in nucleoplasm and nuclear RNP particles (Faiferman et al., 1971a), these proteins also display a different number of protein bands when they are analyzed in acetic acid–urea and sodium dodecyl sulfate gel electrophoresis (Plate 4a and b). As expected, practically no bands with the mobility of histones were detected, which is consistent with the lack of DNA (Plate 4c and d). Finally, the fact that there are practically no protein bands in the middle and lower region of acetic acid–urea gel indicates that the structure contains very small amounts of ribosomes (Faiferman et al., 1971a) (Plate 4a).

In Plate 5, mobilities in sodium dodecyl sulfate gels of proteins obtained from the RNP network, nuclear particles, and soluble nucleoplasm are shown for purposes of comparison. There are similarities in the pattern between the majority of proteins obtained from the RNP network and those obtained from nuclear particles. However, in the RNP network there is almost no protein species whose molecular weight is approximately 40,000 (Plate 5a and b) (Faiferman et al., 1971a; Hamilton et al., 1973). In order to ascertain whether this was due to the procedure used to obtain the RNP network, rat liver nucleoplasm obtained through compression and decompression in a French pressure cell was treated with the HSB–DNase method and Triton X-100 (Plate 5c). It is evident that treated 43S particles are deficient in the protein species that the RNP network lacks. The pattern of soluble nucleoplasmic proteins is shown in Plate 5d. Although common bands may be observed between these proteins and those of the RNP network, the overall distribution is different, which indicates that nuclear RNA–protein interactions are quite specific.

Discussion

Studies of the ultrastructural organization of the nucleus produced the first evidence of the existence of a nuclear RNP network. When isolated rat liver nuclei are extracted with buffers containing high concentrations of NaCl, which removes most of the DNA (Shankar Narayan et al., 1967), or when thin sections of rat liver tissue are treated with EDTA (Monneron and Bernhard, 1969), a technique that bleaches the chromatin, a network of fibrils and granules is seen under the electron microscope. These have been designated as interchromatinic and perichromatinic granules and perichromatinic fibrils (Monneron and Bernhard, 1969). The fibrils seem to link the chromatin, the nucleoli, and the nuclear membrane.

This paper described the isolation of a nuclear membrane-bound RNP network which, in its morphology and sensitivity to ribonuclease and Pronase digestion, appears similar to that observed through ultrastructural analysis of the nucleus in intact tissue. This RNP network contains the majority of the HnRNA, some of which is presumed to belong to the pre-mRNA and mRNA species.

Although the HSB–DNase method completely disperses and fragments the chromatin, it does not detach the RNP network from the nuclear envelope. Only after treatment with Triton X-100 can the structure be disengaged from the nuclear membrane. It is improbable that this attachment is adventitious, due to the high salt and low Mg^{2+} concentration used, and due to the fact that connections between the RNP network and the nuclear envelope have been observed in intact nuclei (Shankar Narayan et al., 1967; Monneron and Bernhard, 1969). Moreover, since a mild disruption of the nuclei (cavitation bomb or French pressure cell at low pressure) does not liberate the RNP particles or the net-

work, it indicates that adventitious interactions are highly improbable.

The chemical composition and the protein species of this structure are similar to that reported for the nuclear particles (Faiferman et al., 1970, 1971a). The similarity is apparent mainly in proteins of high molecular weight but proteins with a molecular weight of approximately 40,000 seem to be exclusive components of the nuclear particles (Plate 5a and b). However, since these proteins are selectively removed from the nuclear particles when they are treated with the HSB–DNase method, it may be assumed that they constitute real components of the RNP network. Not withstanding, the morphological integrity of the RNP network is preserved without these proteins. In any case, until the biochemical properties of the 40,000 molecular weight proteins are known, the discrimination between spurious and real constituents cannot be clearly established.

Negligible fragments of chromatin and nucleolus are present in the RNP network. The absence of DNA and of histones is an indication that the former is an insignificant contaminant, while no detection of ribosomal proteins indicates the latter is also present in very small amounts. It is noteworthy that although rRNAs are major components of the RNP network, the ribosomal proteins are hardly observed (see Plate 4). A logical explanation of this contradiction is that proteins are the main structural component of the RNP network and small amounts of ribosomes are either attached to or entrapped into the network. The fact that Pronase digestion produces an almost complete disappearance of the fibrogranular material strongly supports this assumption (Plate 3c).

The product–precursor relationship between nuclear particles and the RNP network is supported by the following facts. (1) The amount of nuclear particles released into the nucleoplasm is proportionate directly to the intensity of pressure applied to the nuclei. (2) Nuclear particles are polydispersed structures, irregular and/or elongated in shape (Faiferman et al., 1971a). (3) Patterns of proteins extracted from the nuclear particles which were treated with the HSB–DNase method are similar to those extracted from the RNP network. (4) Similar small and high molecular weight RNAs were obtained from both structures (our unpublished results).

It has been recently reported that the nuclei of HeLa cells, disrupted by a method similar to the one described in this paper, release nuclear particles (Pederson, 1974). However, several technical problems are involved. One is that the sucrose gradient fractionation was carried out at low speed. Thus, when the nuclear RNP network of the rat liver was centrifuged at 10,000 rpm in the Spinco SW27 rotor for 16 hr, a large proportion of rapidly labeled RNA sedimented in the gradient, and the remainder on top of the 2 *M* sucrose cushion. Furthermore, in conformance with the results of the experimentation with HeLa cell nuclei, when the material that sedimented in the gradient was analyzed, DNA and histones were found. It is evident that this method of centrifugation fails to separate fragments of chromatin from fragments of the RNP network bound to the nuclear envelope. Pederson (1974) indicated that, at a higher speed, more than one-half of the rapidly labeled nuclear HeLa RNA sedimented as a pellet. It is evident that the absence of 2 *M* sucrose cushion in the sucrose gradient precluded the detection of HeLa RNP network bound to the nuclear membrane.

The nature and origin of nuclear and cytoplasmic informosomes, which function as carriers of mRNA, must be reexamined in view of the findings reported here. The pioneer work of Spirin (1966) has established that mRNA in the cytoplasm appears as RNP particles. Later, Georgiev and Samarina (1971) isolated similar RNP particles in the cell nucleus. Consequently, a simple model of mRNA transport has been currently accepted, i.e., mRNA transported as RNP particles and, as such, entering the polysomes. However, previous observations in our laboratory cast serious doubts on the validity of this model. Evidence from experiments made first in ascites tumor cells (Faiferman et al., 1971b, 1973), and later in yeast (Shiokawa and Pogo, 1974) indicates that transport of mRNA in these systems may occur through cytoplasmic membranes. In addition, recent experiments on the kinetics of polysomal formation in yeast strongly suggest that the entrance of mRNA into polysomes occurs at the level of the cytoplasmic membranes (Brañes and Pogo, 1975). In other words, polysomes are first formed on the membrane and then discharged into the soluble cytoplasm. Therefore, it is reasonable to assume that there may be some kind of structural continuity between the nuclear RNP network that is bound to the nuclear envelope and a similar structure that is bound to the cytoplasmic membranes.

All our findings lend support to the concept of Pitot and Shires (1973) that membranes are involved in the control of genetic expression in eukaryotes. This implies that their function extends beyond the simple secretion of proteins, as indicated in the pioneer work of Palade and Siekevitz (1956). Evidently, by virtue of holding the RNP network to the membranes, the membranes participate in this essential function.

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Surface Polypeptides of the Cultured Chinese Hamster Ovary Cell†

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ABSTRACT: The organization of the plasma membrane of logarithmically growing Chinese hamster ovary (CHO) suspension cells has been probed using surface label techniques in conjunction with subcellular fractionation and sodium dodecyl sulfate gel electrophoresis. Five components of apparent molecular weights 137,000, 121,000, 97,000, 67,000, and 57,000 have been shown to be exposed at the outer surface of the cell. These components fully meet the criteria of being (a) reactive with two or more surface label reagents, (b) enriched in a purified plasma membrane fraction, and (c) sensitive to proteolytic digestion of intact cells. Three other components of molecular weights 200,000, 44,000 and 30,000 are also reactive with certain surface label reagents, but fail to meet other criteria for cell surface components. Two polypeptides of molecular weights 180,000 and 37,000 are substantially enriched in the plasma membrane fraction, but are unreactive with surface label reagents. The organization of the CHO cell membrane and the applicability of surface label techniques to cultured cell systems are discussed.

Surface label techniques have yielded much information concerning the molecular architecture of the erythrocyte membrane (Juliano, 1973). In this experimental approach, the intact cells are treated with reagents which can covalently radiolabel polypeptides, but which are excluded by the membrane from the interior of the cell. Thus, only the polypeptides of the cell periphery are labeled and these may be discriminated from other membrane components (Juliano, 1973).

Several workers have applied surface label techniques to the analysis of the membranes of nucleated mammalian cells (Poduslo et al., 1972; Shin and Carraway, 1973; Kinzel and Mueller, 1973; Hunt and Brown, 1974; Huang et al., 1973; Gahmberg and Hakamori, 1973a,b), but few of them have seriously evaluated the pitfalls inherent in the application of these techniques to complex cells. We have previously delineated some of the problems of the surface label approach (Juliano, 1974; Juliano and Behar-Bannel-

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