ATTACHMENT OF RIBOSOMES TO MEMBRANES DURING POLYSOME FORMATION IN MOUSE SARCOMA 180 CELLS

SE YONG LEE, VELIBOR KRSMANOVIC, and GEORGE BRAWERMAN

From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06501. Dr. Se Yong Lee's and Dr. George Brawerman's present address is the Department of Biochemistry-Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111

ABSTRACT

Addition of nutrients to starved mouse S-180 cells leads to rapid conversion of ribosomal monomers to polysomes. During this process, a portion of the ribosomes originally found in the 17,000 *g* (10 min centrifugation) supernatant of cell lysates becomes firmly attached to structures sedimenting at 500 *g* (5 min centrifugation). Electron microscopy of sections of the intact cells showed the change from randomly distributed ribosomal particles to clusters. Association with membranes also became evident. The material sedimenting at 500 *g* comprised nuclei enclosed in an extensive endoplasmic reticulum (ER) network. This fraction prepared from recovering cells showed numerous ribosome clusters associated with the ER network. The appearance of many of these clusters indicated that the ribosomal particles were not directly bound to the membranes. RNase treatment released about 40% of the attached ribosomes as monomers, and ethylenediaminetetraacetic acid released 60% as subunits. It is suggested that during polysome formation a portion of the ribosomes becomes attached to the membranes through the intermediary of messenger RNA.

INTRODUCTION

In animal cells, a portion of the ribosomes has been shown to be attached to membranes of the endoplasmic reticulum, while the rest is free in the cytoplasm. The proportion of bound ribosomes is high in differentiated cells active in synthesis of proteins for secretion (7, 8, 10). Studies of the mode of attachment of liver ribosomes have indicated that it is the large ribosomal subunit that is bound directly to the membrane (4, 11). HeLa cells have also been reported to have a significant proportion of ribosomes bound to membranes in the same manner, although no obvious secretory function is evident for these cells (1).

We present evidence for a form of polysome association with membranes in which the binding appears to be through the mRNA.[1] In the course of a study of polysome formation initiated by restoration of nutrients to starved S-180 cells (6), it was observed that a portion of the ribosomes becomes rapidly attached to membranes. With the present method used for cell lysis, an extensive endoplasmic reticulum network remained attached to the nuclei, and could be washed free of

[1] Abbreviations: mRNA, messenger RNA; RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid.

nonattached polysomes. This permitted the characterization of the bound polysome fraction both by biochemical studies and by electron microscopy.

EXPERIMENTAL

Tumor Cells

Mouse sarcoma 180 cells, kindly supplied by Dr. Sartorelli (Department of Pharmacology, Yale University), were maintained by weekly transfers into the peritoneal cavity of albino mice.

Incubations and Cell Fractionations

The detailed procedures have been described elsewhere (6). The cells, harvested from the peritoneal cavity 5–7 days after inoculation, were washed several times in warm Krebs bicarbonate buffer. For the starvation treatment, the cells were incubated with gentle agitation at 37°C for 30 min in the same buffer, at a cell density of 1×10^7/ml. Recovery was initiated by addition of glucose, bovine serum, and a complete mixture of amino acids.

The cells were harvested after rapid cooling at 0°C, and washed once with ice-cold 1% NaCl. For lysis, the cells were swollen by washing twice with ice-cold 10 mM Tris-HCl (pH 7.6), 10 mM KCl, and 1 mM $MgCl_2$, then gently suspended in 2.5 vol of 50 mM Tris (pH 7.6), 130 mM KCl, 6.5 mM $MgCl_2$, 6.5 mM β-mercaptoethanol, 0.13% Triton X-100, and 13% sucrose. The resulting lysate was centrifuged at 500 *g* for 5 min. The pellet was used as nuclear fraction and the supernatant as cytoplasmic fraction.

Nucleic Acid Determinations

The samples were precipitated with ice-cold 0.2 N $HClO_4$ and the pellets were washed with 5 ml of the same solution. They were next suspended in 4 ml of 0.1 N KOH and kept at 100°C for 10 min to hydrolyze the RNA. After chilling in ice, 1 ml of 2.5 N $HClO_4$ was added to precipitate the DNA and proteins. The suspensions were centrifuged, the supernatants were used for RNA determinations, and the pellets were treated with 0.5 N $HClO_4$ at 70°C for 20 min to solubilize the DNA. After chilling, the suspensions were centrifuged and the supernatants were used for DNA determinations. The amounts of DNA and RNA were obtained by optical density measurements at 260 mμ. The average nucleotide molar extinction values at 260 mμ used in this study were 9200 for DNA and 11,900 for RNA.

Electron Microscopy

The preparation of the specimens, as well as the examination of the electron micrographs, were kindly performed by Dr. Samuel Dales (The Public Health Research Institute of The City of New York, Inc.). The nuclear pellets were washed by suspension in 10 ml of ice-cold 10% sucrose, 50 mM Tris (pH 7.6), and 3 mM $CaCl_2$, and by recentrifugation. The cell samples, washed in 1% NaCl, were used as such. The pellets were prepared for thin sectioning and electron microscopy as described by Dales et al. (5). Sections 700–1000 A in thickness were mounted on naked grids and examined in a Philips EM 300 electron microscope fitted with an anticontamination device. The images were recorded on Kodak electron image plates at instrumental magnifications of 5000–40,000.

RESULTS

Changes in Ribosome Distribution in S-180 Cells Subjected to Starvation and Recovery

Addition of nutrients to cells incubated in Krebs bicarbonate solution (see Experimental section) leads to rapid conversion of the ribosomal monomers accumulated during the starvation treatment to polysomes (Fig. 1). The disappearance of polysomes during starvation appears to be due to ribosome runoff coupled with a block in the initiation process, as indicated by the fact that the resulting ribosomal monomers are free of mRNA and peptidyl-transfer tRNA (6). The mRNA released from the polysomes appears to be fully functional, as evidenced by its rapid re-incorporation into polysomes after the addition of nutrients (6).

The formation of polysomes in starved cells supplemented with nutrients is accompanied by the attachment of a portion of the ribosomes to structures sedimenting at 500 *g*, as indicated by determinations of RNA distribution in subcellular fractions (Tables I and II). The procedure used for cell lysis leaves the great bulk of the RNA distributed between the nuclear fraction (500 *g* pellet) and the 17,000 *g* supernatant fraction (Table I). The ribosomal material in the nuclear fraction appears to be firmly bound, since washing hardly reduces the RNA content of this fraction. The amount of RNA in the nuclear fraction of starved cells tended to vary. Values as low as 12% of the total cellular RNA were obtained in some experiments. The value of 25% shown in Table II, however, was more typical. The shift of ribosomes from cytoplasm to nuclear fraction was most rapid within the first 10 min, but it continued for about 1 hr (Table II).

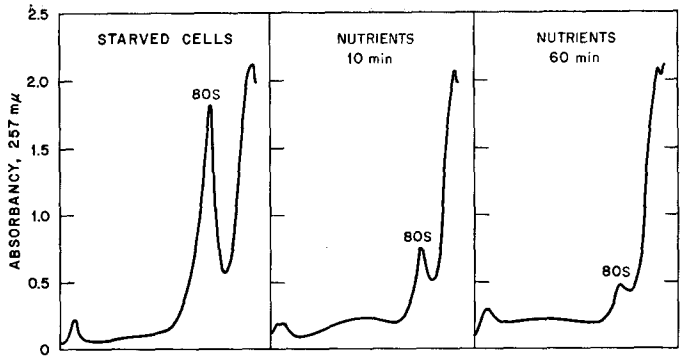

FIGURE 1 Polysome formation in starved S-180 cells after administration of nutrients. Cells were subjected to starvation and recovery treatments as described in the Experimental section. 0.15 ml samples of cytoplasmic fractions (containing 40 μg RNA) were layered over 4.5 ml of linear 10–30% sucrose gradients in 50 mM Tris (pH 7.6), 50 mM KCl, and 1 mM $MgCl_2$, with a 0.5 ml cushion of 2 M sucrose at the bottom of the centrifuge tubes. Centrifugation was done at 45,000 rpm for 25 min in the SW 50 Spinco rotor. Contents of tubes were then pumped from the bottom through a UV detector and the absorbancy at 257 mμ was recorded.

TABLE I

Distribution of RNA in Subcellular Fractions of Recovered Cells

Cell subjected to recovery treatment for 30 min. Subcellular fractions obtained by successive centrifugations of cell lysate at 500 *g* for 5 min and 17,000 *g* for 10 min; 500 *g* pellet was washed by resuspending in lysing medium (see Experimental section) and by recentrifuging at 500 *g* for 5 min.

Fraction	RNA content	
	μg	% of total
500 *g* pellet, crude	314	40
500 *g* pellet, washed	294	37
17,000 *g* pellet	7.4	1
17,000 *g* supernatant	465	59

TABLE II

Changes in RNA Content of Nuclear Fraction During Polysome Formation

Cell suspension preincubated in complete medium for 30 min, then washed and subjected to procedure for starvation and recovery. Nuclear fraction represents 500 *g* pellet washed with 10% sucrose, 50 mM Tris (pH 7.6), and 3 mM $CaCl_2$. Values expressed as micrograms nucleic acid per sample.

State of cells	Nuclear fraction			Total RNA in cell*	% of total RNA in nuclear fraction
	RNA‡	DNA*	RNA/DNA		
Starved	170	522	0.33	670	25
Recovering,					
10 min	214	520	0.41	669	32
60 min	242	484	0.50	620	39
120 min	282	545	0.52	698	40

* Variations in DNA and in total RNA can be attributed to sampling errors, since no consistent pattern of variations was observed in different experiments.

‡ Microscopic examination of cell lysates showed the virtual absence of intact cells. Thus, the changes in RNA content of nuclear fractions cannot be attributed to contamination by intact cells.

Distribution of Ribosomes in Cytological Preparations of Whole Cells

Examination of the recovering cells by electron microscopy showed the expected change from a random distribution of ribosomes in the cytoplasm of starved cells (Fig. 2) to the clustering typical for polysomal aggregates in the cells exposed to nutrients for 10 min (Fig. 4). Most of the ribosomes are free in the starved cells, but frequent association with the endoplasmic reticulum is evident in the recovering cells. After 60 min of recovery, the appearance of the cells is about the same as in the 10 min sample, although there appears to be a greater proportion of membrane-bound polysomes (Fig. 6). The characteristic arrangement of ribosomes in rosette-like patterns is evident where the cisternae of the endoplasmic

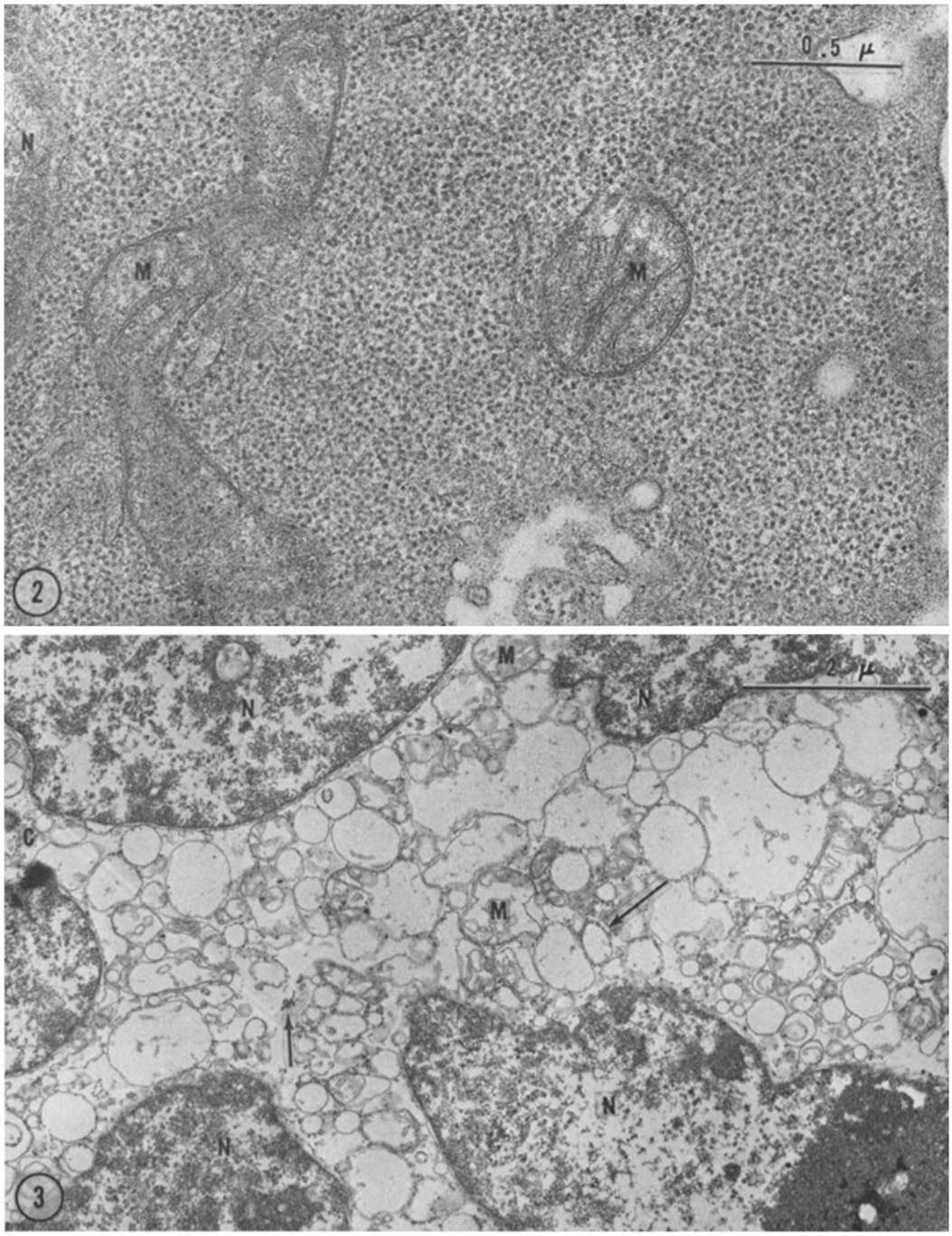

FIGURE 2 Portion of an intact cell from a suspension subjected to starvation treatment. The cytoplasm contains mitochondria, elements of the endoplasmic reticulum, and ribosomes. The "free" ribosomes appear to be randomly distributed throughout the cytoplasmic matrix. *N*, nucleus; *M*, mitochondria. × 44,500.

FIGURE 3 Low power micrograph of section through a washed 500 *g* pellet from lysate of starved cells, showing nuclei (*N*) and remaining cytoplasmic components (*C*) of several cells. Numerous swollen mitochondria (*M*) and vesicles of the endoplasmic reticulum remain associated with nuclei, but few ribosomes remain (arrows). × 14,000.

reticulum were sectioned tangentially. These patterns have been described by Palade (9).

Distribution of Ribosomes in Preparations from Nuclear Fractions

The significance of ribosome attachment to structures in the nuclear fraction became apparent when the latter was examined by electron microscopy. The method of cell lysis used in this study leaves the nuclei enclosed by a swollen endoplasmic reticulum network. Numerous ribosome clusters are associated with this network in preparations from recovering cells (Figs. 5 and 7), while relatively few ribosomes are present in preprations derived from starved cells (Fig. 3). Fig. 7, with its higher magnification, reveals some details of the polysome-membrane association. Many of the clusters appear to overlie and hang away from the membranes.

Nature of Attachment of Ribosomes to Endoplasmic Reticulum

The conversion of ribosomes previously "free" in starved cells to polysomes attached to membranes could be the result of interaction of the ribosomal monomers with membrane-associated mRNA. In this case the attachment should be through the intermediary of the mRNA molecule. Direct attachment of ribosomes to membranes through the large subunit, as described by Sabatini et al. (11), could also take place. Ribosomes bound in the latter fashion are not released by RNase treatment (4), and EDTA treatment leads to preferential release of the small ribosomal subunit (11). As seen in Fig. 8, RNase treatment of the nuclear fracton of recovered cells releases ribosomal monomers, and EDTA leads to release of the two ribosomal subunits in apparently equal proportion. Sedimentation profiles of material released from the equivalent fraction from starved cells showed considerably less ribosomal material, but nearly equal amounts of the slowly sedimenting UV-absorbing material. The latter probably represents nonribosomal RNA, since the amounts released were not dependent on the ribosome content of the nuclear fraction. The same appears to be true of the acid-soluble material produced by the RNase treatment, since the absolute amounts produced from the two types of nuclear fractions were the same (see Table III). Moreover, the conditions for RNase digestion used here do not lead to ribosome destruction (unpublished experiments).

The total amounts of RNA released from the nuclear fractions of starved and recovered cells are listed in Table III. The differences between the amounts released from the two types of nuclear fractions account for only a portion of the ribosomes shifted to the nuclear fraction during recovery (60% in the case of the EDTA treatment and 46% in the case of the RNase treatment).

TABLE III

Release of Ribosomal Material from Nuclear Fraction of Recovering Cells

Samples of washed nuclear fractions (1.2 mg DNA per sample), suspended in 10% sucrose, 50 mM Tris (pH 7.6), and 3 mM $CaCl_2$, were treated as described in Fig. 8, or left untreated. All samples were then centrifuged at 600 *g* for 10 min, and nucleic acid analyses were done on both pellets and supernatant fluids. Values are expressed as ratios of RNA to DNA in untreated nuclear fractions.

	Treatments of nuclear fraction						
	Wash		EDTA		RNase		
	Released	Residue	Released	Residue	Released	Residue	Acid soluble‡
Starved	0.016	0.271	0.077	0.212	0.055	0.144	0.089
Recovering, 60 min	0.020	0.437	0.180	0.280	0.132	0.234	0.092
Material shifted or released*		0.166	0.103		0.077		

* Values express, in the case of "Wash," the amount of ribosomal material shifted to structures in the nuclear fraction during recovery, and in other cases, the portions of this material released by the RNase and EDTA treatments.

‡ Represents material hydrolyzed to acid-soluble components by enzyme treatment, as determined by difference between amounts of RNA originally in nuclear fraction and amounts remaining after treatment.

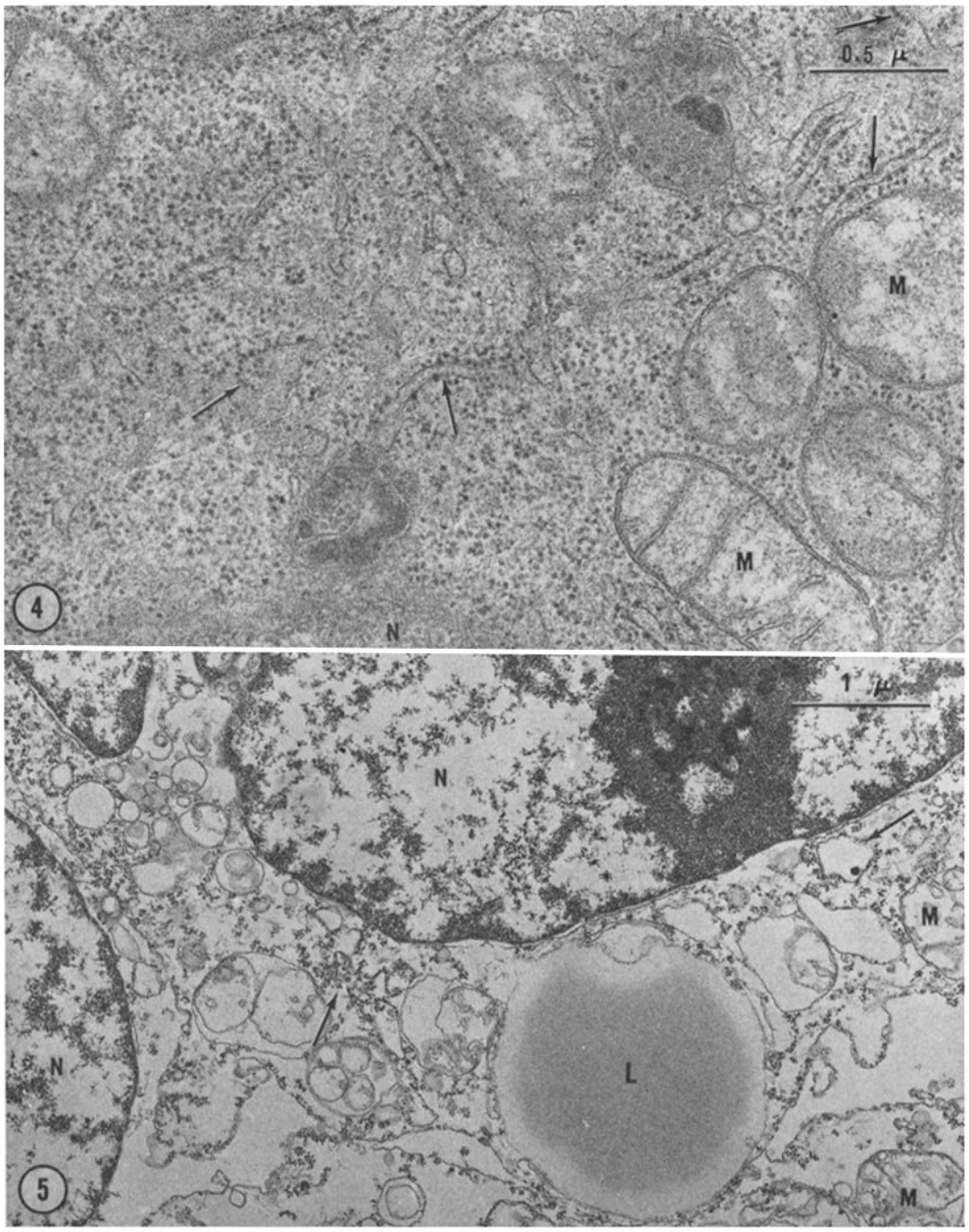

FIGURE 4 Appearance of intact cell 10 min after addition of nutrients to suspension of starved cells. Many ribosomes, some in rosette configuration, are associated with endoplasmic reticulum cisternae (arrows). Clustering of ribosomes in the cytoplasmic matrix is also apparent. *N*, nucleus; *M*, mitochondria. × 45,000.

FIGURE 5 Section through a washed 500 *g* pellet of lysed cells from 10 min recovered culture. Numerous clusters of ribosomes apparently connected to membranous cell components are clearly evident (arrows). *N*, nucleus; *M*, mitochondria; *L*, lipid-filled vacuole. × 20,000.

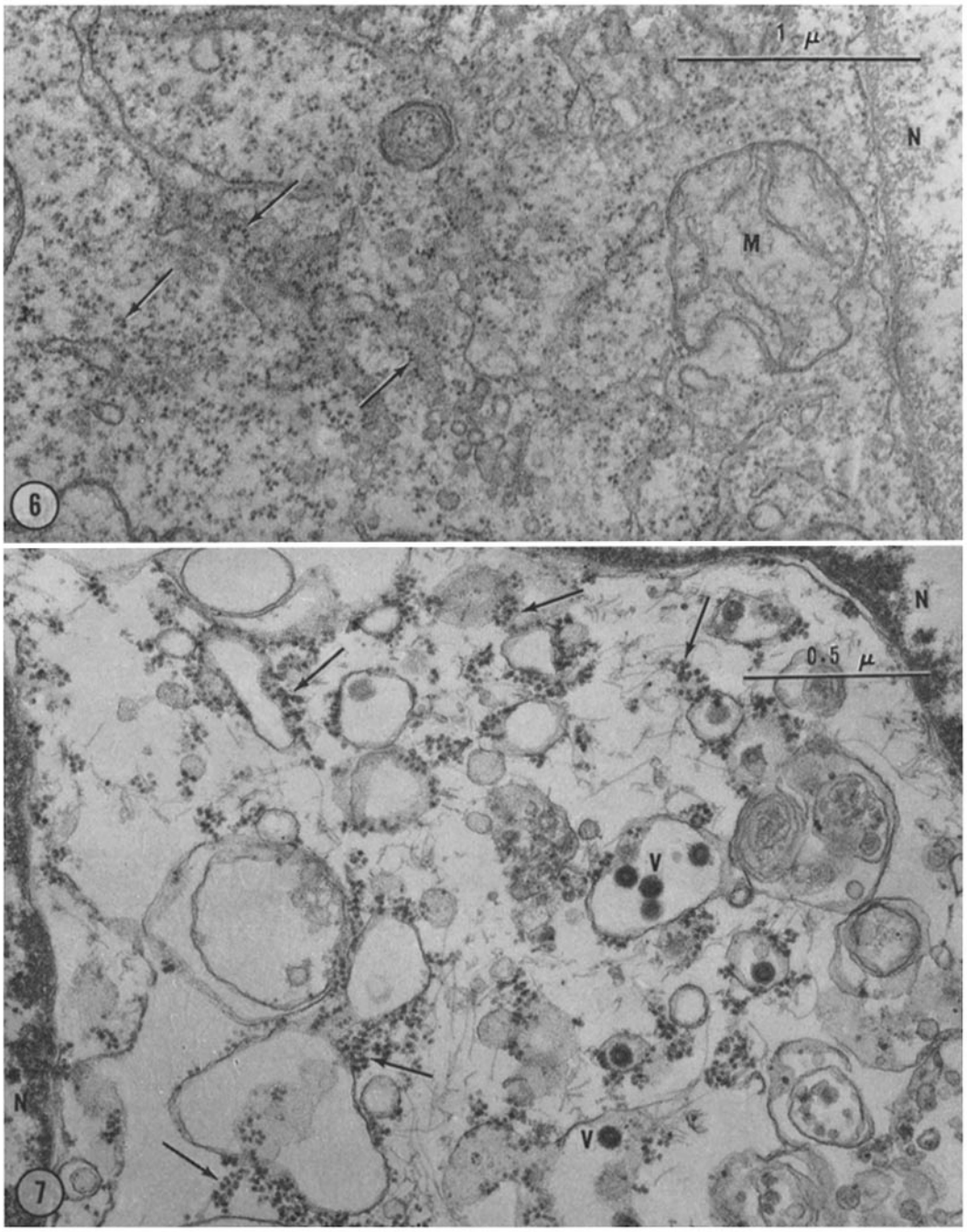

FIGURE 6 Appearance of intact cell 60 min after addition of nutrients to starved cell suspension. Clusters of ribosomes in the matrix and associated with the cisternae are evident (arrows). Where the membranes of tangentially sectioned cisternae are displayed *en face*, the rosette pattern of ribosomes is observed (upper arrow). *N*, nucleus; *M*, mitochondria. × 35,000.

FIGURE 7 Section through a washed 500 *g* pellet of lysed cells from 60 min recovered culture. Clusters of membrane-related ribosomes (arrows) are readily apparent. In this sample, several A type virus-like particles (*V*) occur, possessing the morphology described for the leukemia-sarcoma murine agents (3). *N*, nucleus. × 55,000.

Treatment of the nuclear fraction of recovered cells with EDTA concentrations ranging from 10 to 30 mM yielded similar amounts of ribosomal subunits. The release of monomers by treatment with RNase was very inefficient at 0°C, and increased wtih the temperature of incubation. It is not known whether the temperature of 10°C selected for the experiments described in Fig. 8 and Table III represents the optimum, since higher temperatures were not tried. Incubation of the nuclear fraction at 10°C without enzyme did not lead to any release of ribosomes.

Attempts to separate the membranous structures from the nuclei, in order to permit further characterization of the membrane-bound polysomes, proved unsuccessful. The structures in the nuclear fraction resisted vigorous mechanical shear, even in the presence of 2 M sucrose. Treatment with 1% Triton X-100 released very little, if any, ribosomal material. 0.2% sodium deoxycholate caused little release, and higher concentrations led to lysis of the nuclei.

DISCUSSION

This report describes the rapid attachment of ribosomes to membranes of the endoplasmic reticulum during polysome formation in mouse sarcoma cells recovering from starvation. The membrane-bound ribosomes are seen in the electron micrographs as clusters, an indication that they are attached in the form of polysomes. The appearance of the ribosomal particles in sections of whole cells, as well as in the nuclear fractions, indicates that two different types of ribosome binding may occur in the recovering cells. Ribosomes aligned in rows along the cisternae of the endoplasmic reticulum, as well as the rosette-like patterns, are clearly evident. These patterns are typical for ribosomes bound to membranes through the intermediary of the large ribosomal subunits, as described in microsomal fractions of guinea pig liver and of HeLa cells (1, 11). A second type of binding is suggested by the appearance of many of the ribosome clusters, which seem to hang away from the membranes (Figs. 5 and 7). The occurrence of two types of binding is also suggested by the effect of treatment with RNase, which causes the release of about half of the ribosomes as monomers. Ribosomes attached directly to the membranes have been shown not to be released by treatment with RNase (1, 4). The sensitivity to RNase in the present case indicated that the material is more susceptible to the enzyme than are the ribosomes. Polysomes attached through the intermediary of mRNA could account for the RNase effect as well as for the appearance in the electron micrographs.

The effect of EDTA, which dissociates ribosomes into subunits, is more difficult to understand in terms of the two types of binding postulated above. This treatment should release the small subunits preferentially from the ribosomes bound directly to the membranes (11) and both subunits equally from the ribosomes not attached in this manner. Zone centrifugation of the released particles (Fig. 8) should have shown an

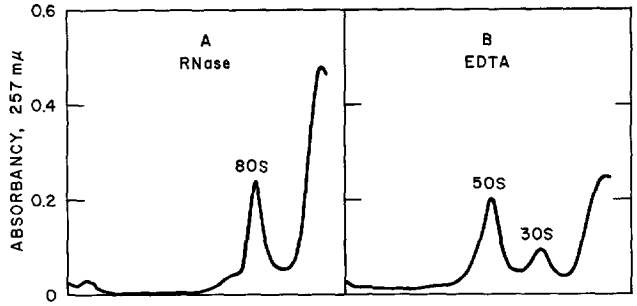

FIGURE 8 Zone centrifugation patterns of material released by RNase and EDTA from washed nuclear fraction of cells subjected to recovery treatment for 30 min. 500 *g* pellet of cell lysate was washed once in 10% sucrose, 50 mM Tris (pH 7.6), and 3 mM $CaCl_2$, and resuspended in the same solution at a concentration equivalent to the one in the original lysate. Suspensions were incubated with 10 μg/ml of pancreatic RNase at 10°C for 20 min (A); and with 20 mM EDTA in the cold (B). After treatments, suspensions were centrifuged at 600 *g* for 10 min, and 0.2 ml samples of supernatants (containing 0.5 A_{260} mμ units for A and 0.4 units for B) were centrifuged through 5-ml linear 10–30% sucrose gradients in 50 mM Tris (pH 7.6), 50 mM KCl and 1 mM $MgCl_2$ at 45,000 rpm in the SW Spinco rotor for 30 min (A) and 90 min (B).

excess amount of the small subunits. It is possible that a portion of the released small subunits became degraded. Also, the amount of ribosomal material not released by EDTA (Table III) seems excessive. This suggests that a substantial portion of the ribosomes is in such a configuration that it resists dissociation into subunits. The occurrence of "stuck" monomers is also indicated in the study of Sabatini et al. (11).

The present results suggest that S-180 cells may contain mRNA associated with membranes and that this RNA may function as a template for protein synthesis in this state. The concept of mRNA association with membranes is not a novel one (12), but convincing evidence has been lacking due to the difficulty in characterizing the mRNA. Membrane-associated polysome structures similar to those described here have been found in poliovirus-infected HeLa cells (5) and in rat liver (2). Uninfected cells also appear to possess membrane-bound polysomes that can be released by RNase (Rosbach and Penman, personal communication). Additional evidence, however, will be required before this type of polysome structure can be established as a normal feature of animal cells.

The release of ribosomes from membrane-bound mRNA during starvation and their reattachment during recovery could be readily explained in terms of interruption and resumption of polypeptide chain initiation (6). The relation between attachment to membranes and initiation of protein synthesis, however, may not be a direct one, since ribosome attachment seems to proceed more slowly than polysome formation (compare Fig. 1 and Table II). Ribosomes bound directly to the membranes also appear to be released and reattached during the starvation-recovery treatment. The mechanism of attachment is now known in this latter case. It would appear from the present results that the ribosomes must be engaged in protein synthesis in order to remain bound to the membranes.

The authors are very grateful to Dr. Samuel Dales for the preparation and interpretation of the electron micrographs, as well as for useful discussions. They also wish to thank Mrs. Mary Ann Krutz for her very competent technical assistance.

This work was supported by a United States Public Health Service Research Career Development Award (GM-K3-3295) from the National Institute of General Medical Sciences to Dr. Brawerman and a research grant from the United States Public Health Service (GM-11527).

Received for publication 24 August 1970, and in revised form 11 January 1971.

REFERENCES

1. ATTARDI, B., B. CRAVIOTO, and G. ATTARDI. 1969. *J. Mol. Biol.* **44**:47.
2. BENEDETTI, E. L., W. S. BONT, and H. BLOEMENDAL. 1966. *Lab. Invest.* **15**:196.
3. BERNHARD, W., and M. GUÉRIN. 1959. *C. R. Acad. Sci. Ser. D.* **248**:160.
4. BLOBEL, G., and V. R. POTTER. 1967. *J. Mol. Biol.* **26**:293.
5. DALES, S., H. J. EGGERS, I. TAMM, and G. E. PALADE. 1965. *Virology.* **26**:379.
6. LEE, S. Y., V. KRSMANOVIC, and G. BRAWERMAN. 1971. *Biochemistry.* **10**:895.
7. PALADE, G. E. 1956. *J. Biophys. Biochem. Cytol.* **2** (4, Suppl.):85.
8. PALADE, G. E. 1958. *In* Microsomal Particles and Protein Synthesis. R. B. Roberts, editor. Pergamon Press, New York. 36.
9. PALADE, G. E. 1964. *Proc. Nat. Acad. Sci. U. S. A.* **52**:613.
10. Porter, K. R. 1961. *In* The Cell. J. Brachet and A. Mirsky, editors. Academic Press, Inc., New York. **2**:621.
11. SABATINI, D. D., Y. TASHIRO, and G. E. PALADE. 1966. *J. Mol. Biol.* **19**:503.
12. SHAPOT, V., and H. C. PITOT. 1966. *Biochim. Biophys. Acta.* **119**:37.