RETENTION OF mRNA ON THE ENDOPLASMIC

RETICULUM MEMBRANES AFTER IN VIVO DISASSEMBLY OF

POLYSOMES BY AN INHIBITOR OF INITIATION

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The mechanism which determines the selective translation of specific classes of mRNA in polysomes associated with endoplasmic reticulum (ER) membranes is yet to be elucidated. It is known, however, that ER membranes have specific sites for ribosome binding (cf. Sabatini et al., 1975) and that nascent polypeptide chains contained in bound large subunits associate tightly with the underlying membranes (Adelman et al., 1973). Our recent work (Lande et al., 1975) using membrane fractions of human diploid fibroblasts, and that of Milcarek and Penman (1974) with HeLa cells, suggest that there is also a direct association between the mRNA of bound polysomes and the ER membranes. We found that after in vitro disassembly and release of the subunits of bound polysomes mRNA nevertheless remained associated with the membranes. In this note, we demonstrate that the mRNA-membrane association found in human fibroblasts is maintained even after initiation of protein synthesis is inhibited in vivo by Verrucarin A. This drug causes ribosomal run-off and extensive polysome disaggregation in eukaryotic cells (Wei and Mc-Laughlin, 1974), blocking steps subsequent to the formation of 80S initiation complexes which contain both ribosomal subunits joined to mRNA.[1]

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[1] Martin, T. Manuscript in preparation.

MATERIALS AND METHODS

WI38 human diploid fibroblasts were obtained from Dr. L. Hayflick, Stanford University. Cultures (p.d. 20–30) in stationary phase were used in the experiments. Conditions for labeling and cell fractionation were given in a previous publication (Lande et al., 1975). Puromycin treatment of the membrane fraction was carried out as previously described (Lande et al., 1975). Additional experimental details are provided in the figure legends. A stock solution of Verrucarin A (courtesy of Sandoz Pharmaceuticals, East Hanover, N. J.) was prepared in dimethyl sulfoxide (DMSO) (25 mg/ml). Appropriate amounts were rapidly diluted in 1 ml of water at 37°C and added to the incubation medium.

RESULTS

The distribution of ribosomes and mRNA in the free and membrane-bound compartments of WI38 cells treated with Verrucarin A was determined. Cultures were incubated with [^{14}C]adenine for several days to label the ribosomal RNA (rRNA). Subsequently, mRNA was labeled specifically for 3 h with tritiated RNA precursors, by use of a low dose (Lande et al., 1975) of actinomycin D to suppress rRNA synthesis. This pulse-labeled RNA was identified as mRNA because of its size distribution in polyacrylamide gel electrophoresis and its efficient binding (>80%) to oligo-dT cellulose (Lande et al., 1975). Postnuclear supernates from controls and Verrucarin-treated cultures were prepared in a low salt-containing buffer (RSB) and analyzed in sucrose density gradients of the same ionic composition. The sedimentation conditions were chosen to separate ribosomes and mRNA associated with the membranes, which banded isopycnically in the bottom third of the gradients, from soluble cell constituents, free polysomes, and ribosomes, which sedimented in the upper two-thirds. A comparison of the sedimentation profiles in Fig. 1 a and b shows that treatment with Verrucarin A led to ribosomal run-off and disassembly of free polysomes as indicated by the conversion of polysomes into monomers; the mRNA from these polysomes cosedimented with ribosomal structures slightly larger than the free 80S monomers. Nevertheless, the association of ribosomes and mRNA with the membranes appeared to be unaffected by the Verrucarin A treatment when postnuclear supernates were analyzed in low salt-containing gradients (Fig. 1). The nature of the association between the ribosomes and the membranes, however, was altered. While in untreated cells, most ribosomes remained associated with the membranes in media
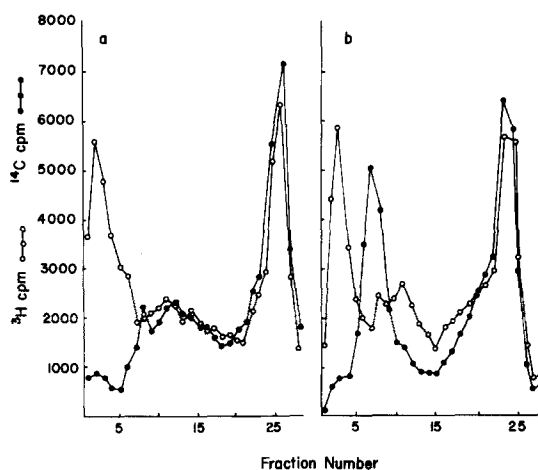

FIGURE 1 Distribution of labeled mRNA and ribosomes in postnuclear supernates of Verrucarin A-treated cells. WI-38 cells were grown in disposable roller bottles (690 cm²) and labeled during growth in their ribosomal RNA (rRNA) with 1 μCi/bottle of [8-^{14}C]adenine (40 mCi/mmol) for 72 h until confluence. mRNA was subsequently labeled with 1 mCi/bottle of [2-^{3}H]adenosine (26 Ci/mmol) for 3 h in the presence of a low dose of actinomycin D (0.03 μg/ml). Verrucarin A was added to a final concentration of 5 μg/ml 10 min before harvesting. Postnuclear supernates (PNS) were prepared in RSB (10 mM KCl, 10 mM Tris, pH 7.4, 1.5 mM MgCl₂) as described (Lande et al., 1975). Aliquots of the PNS (corresponding to material derived from one-fifth of the cells in a roller bottle) from control (a) and Verrucarin cultures (b) were analyzed in 15–55% sucrose density gradients containing RSB which were centrifuged for 60 min at 40,000 rpm, at 4°C in an SW41 Beckman rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Gradients were fractionated and the RNA was precipitated with cold 5% trichloroacetic acid (TCA) before collection on glass fiber filter papers for measuring of radioactivity in a scintillation counter. In all gradient profiles, the direction of sedimentation is from left to right.

of high ionic strength unless puromycin was used to release the nascent chains (Fig. 2a, b), in Verrucarin-treated cells most of the ribosomes were removed from the membranes simply by sedimentation in a high salt medium containing Mg^{++} (Fig. 2c). Incubation of the membrane fraction with puromycin, before sedimentation in high salt medium, did not lead to a significant release of additional ribosomes (Fig. 2d). In all cases, a very large fraction (>80%) of the ^{3}H-labeled mRNA remained associated with the membrane despite nearly complete removal of ribosomes in high salt
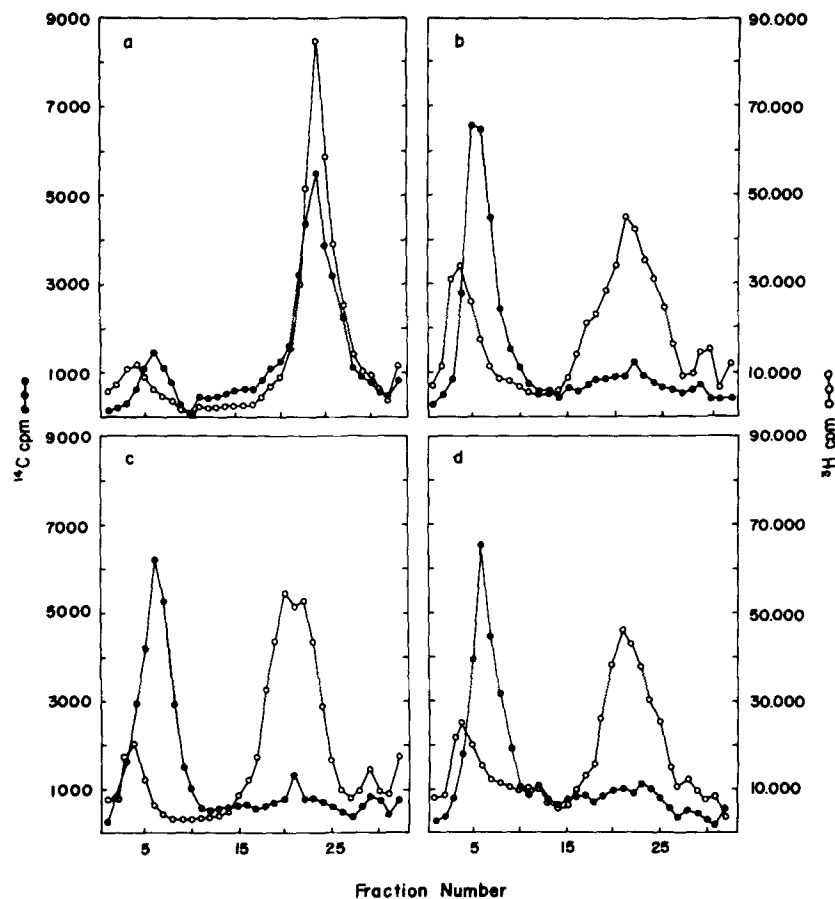
Figure 2 Release of ribosomes from membrane fractions of Verrucarin A-treated cells by incubation in a medium of high ionic strength. Cells were labeled as described in Fig. 1, and postnuclear supernatant fractions (PNS) were obtained from untreated and Verrucarin A-treated cultures. Membrane fractions (MF) were prepared from the PNS by sedimentation through 15–30% sucrose density gradients containing RSB (27,000 rpm, 30 min at 4°C, in an SW41 Beckman rotor). Six gradients were used to prepare membrane fractions from the PNS derived from three bottles. Membrane fraction pellets were gently resuspended in RSB and adjusted to the ionic concentration of HSB (500 mM KCl, 50 mM Tris, pH 7.4, 5 mM MgCl₂). Aliquots (containing material derived from cells in one-third of a bottle) were analyzed in 15–55% sucrose gradients containing HSB which were centrifuged for 60 min at 40,000 rpm, at 4°C in an SW41 Beckman rotor. (*a*) Membrane fraction from control cells; (*b*) puromycin-treated membrane fraction from control cells; (*c*) membrane fraction from cells treated with Verrucarin A; (*d*) membrane fraction from cells treated with Verrucarin A, incubated with puromycin before sedimentation. Radioactivity in fractions was determined as indicated in Fig. 1.

medium by either Verrucarin treatment in vivo or puromycin treatment in vitro, or both.

These experiments demonstrate that membrane fractions isolated in media of low salt concentration from Verrucarin-treated cells contain membrane-associated mRNA and inactive ribosomes, but only the latter can be extensively released from the membranes by treatment with a medium of high salt concentration. A role of inactive ribo-

somes in maintaining the mRNA on the membranes can therefore be excluded.

Residual [¹⁴C]RNA radioactivity found in membrane fractions stripped of ribosomes (Fig. 2 *b–d*) corresponds primarily to ribosomal RNA of contaminating mitochondria (Lande et al., 1975). Since mitochondria contain at most 5–10% of the [¹⁴C]RNA radioactivity (Lande et al., 1975), a small proportion of bound cytoplasmic

ribosomes must remain membrane-associated after Verrucarin and/or puromycin and high salt treatment. Some of these ribosomes may be trapped in inverted microsomal vesicles, which we have observed by electron microscopy; other residual ribosomes may be part of initiation complexes blocked by Verrucarin A which are known to be resistant to dissociation into subunits by high salt treatment.[1] Although there is no precedent for a high salt-resistant linkage between ribosomes and membranes in the absence of an associated nascent polypeptide chain, we nevertheless considered this possibility and examined the effect of treatment with EDTA on the retention of mRNA by the membranes of Verrucarin-treated cells. This treatment was carried out under conditions which should lead to the disassembly of initiation complexes as well as translating ribosomes. Membrane fractions prepared in a high salt buffer were treated with EDTA in a high salt medium containing no Mg^{++} and analyzed in sucrose density gradients of the same composition (Fig. 3). The retention of mRNA could not be accounted for by its involvement in initiation complexes since EDTA had only a small effect on reducing the amount of ribosomes and mRNA which associ-
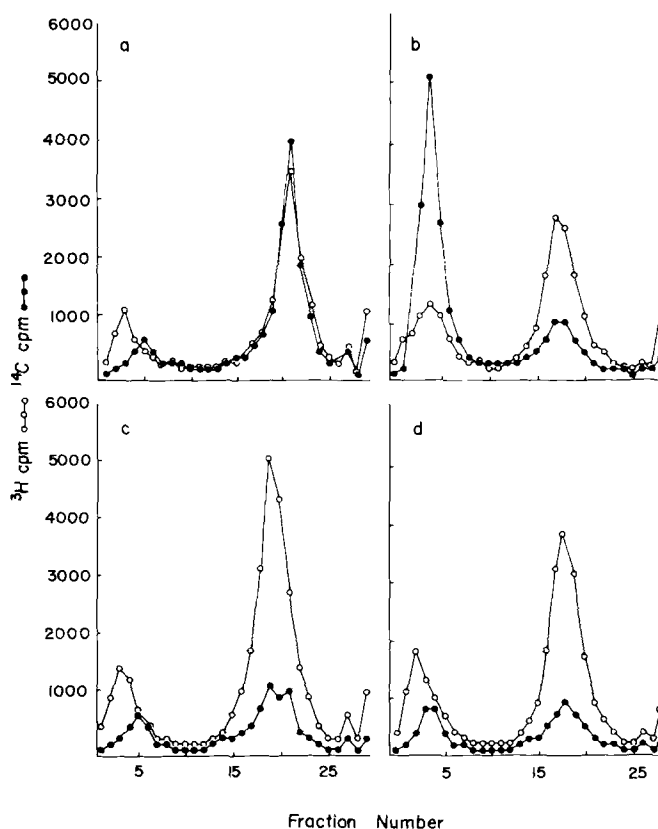

FIGURE 3 Retention of mRNA on membranes after EDTA treatment. Postnuclear supernates were obtained from control or Verrucarin A-treated cells as described in Fig. 1, and their ionic composition was adjusted to that of HSB. Membrane fractions were then obtained by sedimentation through 15–30% sucrose gradients containing HSB (27,000 rpm, 30 min at 4°C, SW41 Beckman rotor). After resuspension in HSB, aliquots (material derived from cells in one-third of a roller bottle) were kept as control or received 20 mM Na₂EDTA. The aliquots not treated with EDTA were analyzed in 15–55% sucrose density gradients containing HSB (panels a and c) while EDTA-treated aliquots were analyzed in gradients containing 500 mM KCl, 50 mM Tris, pH 7.4, and no Mg^{++} (panels b and d). Radioactivity in fractions was determined as described in Fig. 1. (a) Membrane fraction for control cells; (b) EDTA-treated membrane fraction from control cells; (c) membrane fraction from Verrucarin-treated cells; (d) EDTA-treated membrane fraction from cells incubated with Verrucarin.

ated with the membranes prepared from Verru-carin-treated cells in a medium of high salt concentration.

From these studies with cell fractionation procedures, it cannot be established whether the inactive ribosomes that were recovered with the membrane fraction were actually bound to the membranes "in vitro" or adsorbed onto unoccupied ribosome binding sites after cells were lysed in the low salt medium. However, electron microscope examination (Figs. 4 and 5) indicates that, intracellularly, numerous ribosomes remain bound to the ER membranes after Verrucarin A treatment. Similar observations concerning retention of bound ribosomes after polysome disaggregation have been reported in rat liver after inhibition of protein synthesis by treatment with ethionine (Baglio and Farber, 1965; Sarma et al., 1972).

DISCUSSION

These results support the conclusion, derived from experiments involving "in vitro" dissociation of membrane-bound polysomes, that in diploid human fibroblasts a large fraction of mRNA of bound polysomes is directly associated with the ER membranes (Lande et al., 1975). They also allow us to exclude the possibility that the binding of this mRNA to the membranes occurs only after polysome disassembly in vitro since, in the present case, polysome disassembly and ribosome run-off took place in the intact cells.

In myeloma cells, it has also been concluded from indirect evidence that there is an association between mRNA and the ER membranes. It was shown (Mechler and Vassalli, 1975) that small ribosomal subunits containing initiator tRNA remained associated with microsomal membranes— presumably through the mRNA—even after treatment of the cultures with pactamycin to inhibit initiation of protein synthesis. These observations, however, must be reconciled with the report of Harrison et al. (1974) that translatable light-chain mRNA is released from microsomes of cultured myeloma cells when bound polysomes are disassembled in vitro. In these experiments, despite the extensive release of light-chain mRNA, however, 50% of the poly A-containing RNA in the membrane fraction was not released. It was suggested that the latter fraction of poly A-containing RNA which could not be translated in a reticulocyte cell-free system was in mitochondria which contaminate the membrane fraction. It appears to us that this is unlikely since only a small fraction of the

ribosomal RNA in the myeloma membrane fraction appeared to be mitochondrial in its sedimentation characteristics (Harrison et al., 1974). We therefore suggest that at least part of the RNA retained in the membranes of myeloma cells after polysome disassembly may be bona fide membrane-bound microsomal mRNA, degraded during preparation and treatment of the membrane fraction and therefore relatively inactive for protein synthesis in vitro.

We have also examined the release of poly A-containing mRNA from rat liver rough microsomes and have found that, contrary to the situation in the tissue culture cells, release of ribosomes from these microsomes leads to an extensive concomitant release of poly A-containing mRNA (Kruppa and Sabatini, 1975). The possibility should therefore be considered that there is more than one class of mRNA associated with the ER membranes. One of these classes would correspond to the majority of mRNA in the human fibroblasts and HeLa cells which is labeled during short pulses and is directly associated with the membranes (Lande et al., 1975; Milcareck and Penman, 1974). This class of mRNA would be represented in myeloma cells by the poly A-containing fraction of RNA retained in the membranes after polysome disassembly. Other mRNAs such as light-chain mRNA (Harrison et al., 1974) and mRNA of rat liver microsomes (Kruppa and Sabatini, 1975) may not be directly associated with the membranes, or their association may be weak. It has been proposed that membrane-bound ribosomes are not only engaged in the synthesis of secretory proteins; in fact, in HeLa cells there is no significant protein secretion and yet ~15% of the cytoplasmic ribosomes are associated with ER membranes (Rosbach and Penman, 1971). It cannot yet be assessed what fraction of the pulse-labeled RNA in our experiments indeed codes for collagen, a secretory protein characteristic of fibroblast cultures. In fact, it is possible that mRNA coding for this protein represents no more than 20% of the total pulse-labeled mRNA, and therefore its fate during polysome disassembly cannot yet be determined. In addition to their involvement in the synthesis of secretory proteins, membrane-bound ribosomes are thought also to be involved in the synthesis of proteins destined for the ER cisternae which are later diverted to other membrane-bound organelles such as lysosomes and peroxisomes. They are also thought to manufacture proteins destined for
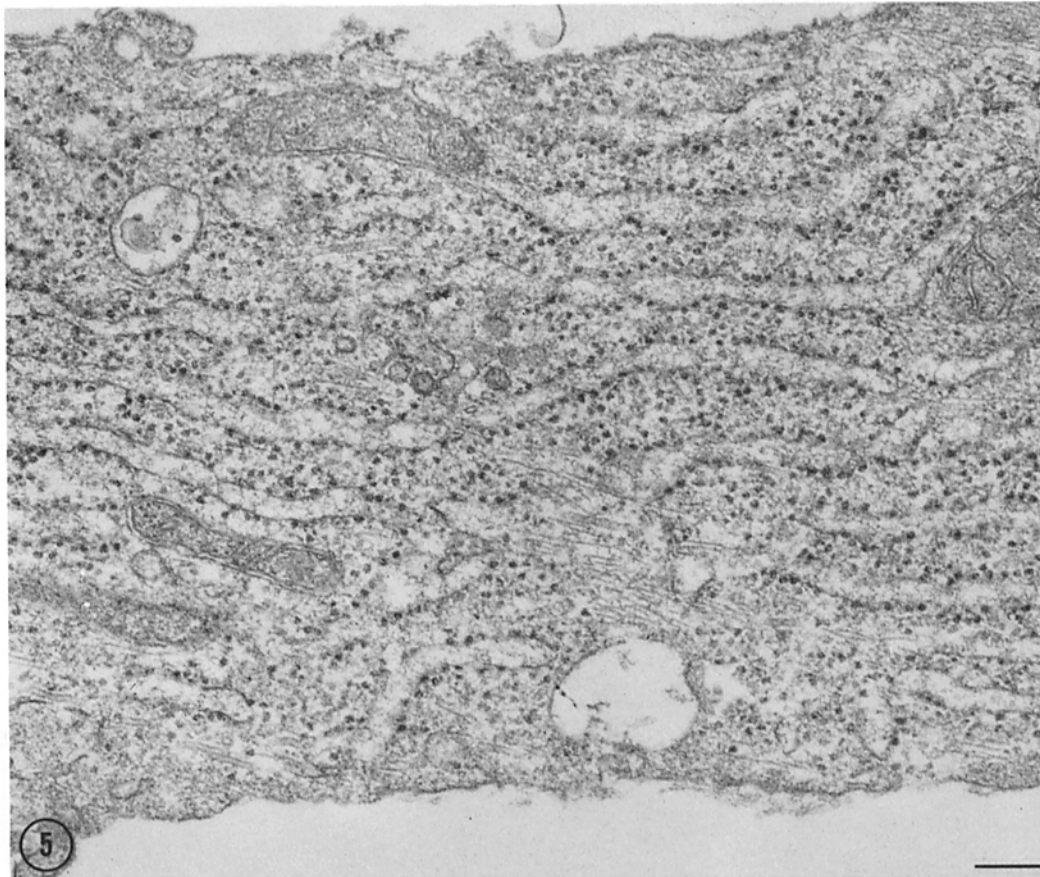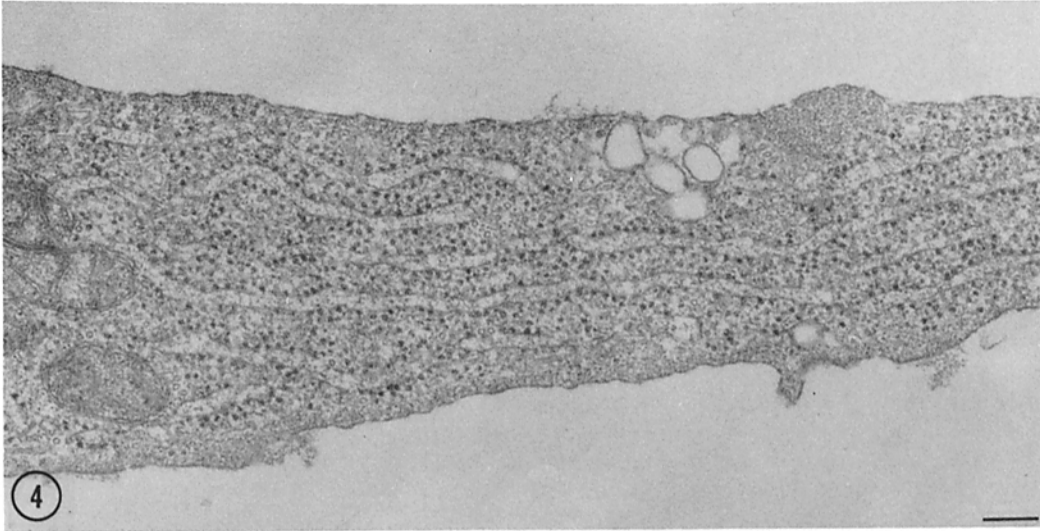
Figures 4 and 5 Retention of ribosomes bound to ER membranes after inhibition of initiation by Verrucarin. Electron micrographs show portions of the cytoplasm of WI38 fibroblasts after Verrucarin treatment for 10 min. Numerous ribosomes remain associated with membranes of the ER cisternae. No patterns of bound polysomes are visible. Bars correspond to 0.2 μm. WI38 human fibroblasts were grown to confluence in 60-mm petri dishes. After Verrucarin treatment (5 μg/ml) for 10 min the medium was removed and the cells were fixed in situ with 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 5 min at 37°C and for 15 min at room temperature. Cells were scraped off with a rubber policeman, sedimented, and fixed in glutaraldehyde for an additional hour at room temperature. Pellets were washed and stored in a cacodylate buffer overnight at 4°C and postfixed for 2 h in 2% osmium tetroxide at room temperature. After staining in 1% aqueous uranyl acetate, blocks were embedded in Epon and thin sections observed in a Philips 301 electron microscope. Fig. 4, × 33,000; Fig. 5, × 45,000.

the ER membranes themselves (e.g., Negishi et al., 1975). Thus, it is possible that while mRNAs for secretory proteins are not tightly bound to the membranes, other messengers which code for nonsecretory proteins and predominate in HeLa cells and fibroblasts have a direct association with the ER membrane on which they are retained after polysome disassembly in vivo and in vitro.

SUMMARY

Membrane-bound ribosomes and messenger RNA remained associated with the microsomal membranes of human fibroblasts after cultures were treated with Verrucarin A, an inhibitor of initiation which led to extensive run-off of ribosomes from polysomal structures. When a membrane fraction from Verrucarin-treated cells containing such inactive ribosomes and mRNA was suspended in a medium of high salt concentration, extensive release of ribosomal subunits occurred without the need for puromycin. The mRNA nevertheless remained associated with the membranes. These results add support to the conclusion that, in human fibroblasts, mRNA is bound directly to ER membranes, independently of the ribosomes and nascent polypeptide chains.

REFERENCES

Adelman, M. R., D. D. Sabatini, and G. Blobel. 1973. Nondestructive disassembly of rat liver rough microsomes into ribosomal and membranous components. *J. Cell Biol.* **56**:206.

Baglio, C. M., and E. Farber. 1965. Correspondence between ribosome aggregation patterns in rat liver homogenates and in electron micrographs following administration of ethionine. *J. Mol. Biol.* **12**:466.

Harrison, T. M., G. G. Brownlee, and C. Milstein. 1974. Preparation of light-chain mRNA from microsomes without the use of detergent. *Eur. J. Biochem.* **47**:621.

Kruppa, J., and D. D. Sabatini. 1975. Release of messenger RNA from rat liver rough microsomes upon disassembly of bound polysomes. *J. Cell Biol.* **67**(2, Pt. 2):453a. (Abstr.).

Lande, M. A., M. Adesnik, M. Sumida, Y. Tashiro, and D. D. Sabatini. 1975. Direct association of messenger RNA with microsomal membranes in human diploid fibroblasts. *J. Cell Biol.* **65**:513.

Long, B., J. Cardelli, and H. C. Pitot. 1975. Characteristics of fluoroorotate labelling in membrane-bound polysomes from rat liver. *Fed. Proc.* **34**:583.

Mechler, B., and P. Vassalli. 1975. Membrane-bound ribosomes of myeloma cells. III. The role of messenger RNA and nascent polypeptide chains in the binding of ribosomes to membranes. *J. Cell Biol.* **67**:25.

Milcarek, C., and S. Penman. 1974. Membrane-bound polyribosomes in HeLa cells: Association of polyadenylic acid with membranes. *J. Mol. Biol* **89**:327.

Negishi, M., T. Sawamura, T. Morimoto, and Y. Tashiro. 1975. Localization of nascent NADPH-cytochrome c reductase in rat liver microsomes. *Biochim. Biophys. Acta* **381**:215.

Rosbach, M., and S. Penman. 1971. Membrane-associated protein synthesis of mammalian cells. I. The two classes of membrane-associated ribosomes. *J. Mol. Biol.* **59**:227.

Sabatini, D. D., G. Ojakian, M. A. Lande, J. Lewis, W. Mok, M. Adesnik, and G. Kreibich. 1975. Structural and functional aspects of the protein synthesizing apparatus in the rough endoplasmic reticulum. *In* Advances in Experimental Medicine and Biology, Volume 62. Activation, Differences, and Modulation of the Genetic Program. R. Meints and E. Davies, editors. Plenum Press, New York. 151–180.

Sarma, D. S. R., I. M. Reid, E. Verney, and H. Sidransky. 1972. Studies on the nature of attachment of ribosomes to membranes in liver. I. Influence of ethionine, sparsomycin, CCl_4, and puromycin on membrane-bound polyribosomal disaggregation and on detachment of membrane-bound ribosomes from membranes. *Lab. Invest.* **27**:39.

Wei, C., and C. S. McLaughlin. 1974. Structure-function relationship in the 12,13 epoxitrichothecenes. *Biochem. Biophys. Res. Commun.* **57**:838.