DIRECT ASSOCIATION OF MESSENGER RNA WITH MICROSOMAL MEMBRANES IN HUMAN DIPLOID FIBROBLASTS

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ABSTRACT

Messenger RNA (mRNA) of membrane-bound polysomes in a membrane fraction of WI-38 cells remains associated with the microsomal membranes even after ribosomes and their nascent polypeptide chains are removed by using puromycin in a high salt buffer or by disassembling the ribosomes in a medium of high ionic strength lacking magnesium. mRNA either was specifically labeled in the presence of actinomycin D, or it was recognized by virtue of its affinity for oligo-dT. Poly A segments in bound mRNAs have an electrophoretic mobility in acrylamide gels which is characteristic of cytoplasmic mRNAs and corresponds to 150–200 adenyl residues. Extensive RNase treatment did not lead to release of the poly A segments of membrane-associated mRNA molecules either from an intact membrane fraction or from a membrane fraction previously stripped of ribosomes. On the other hand, RNase treatment led to the release and digestion of the non-poly A segments of the mRNA molecules, indicating that the site of attachment of mRNA to the ER membranes is located near or at the 3′ end of the molecule which contains the poly A. A direct association of mRNAs and endoplasmic reticulum membranes is considered in a model to explain the assembly of bound polysomes and protein synthesis in a membrane-associated apparatus.

Eukaryotic cells specialized for the secretion of proteins contain a large fraction of their ribosomes bound to membranes of the endoplasmic reticulum (ER) (Palade, 1958). Secretory polypeptides synthesized by bound ribosomes are vectorially transferred to the lumen of ER cisternae as a first step in the process leading to their extracellular discharge (Redman and Sabatini, 1966; Sabatini and Blobel, 1970; Adelman et al., 1973). Other polypeptides destined to the content of storage granules or membrane-bound organelles such as peroxisomes (Higashi and Peters, 1963 *a*, *b;* Kashiwagi et al., 1971; but see Redman et al., 1972; Lazarow and de Duve, 1973), and lysosomes (de Duve and Wattiaux, 1966; Goldstone and Koenig, 1972, 1974), as well as polypeptides destined themselves to the ER membranes (Dallner, et al., 1966 *a*, *b;* Omura, 1973; Omura and Kuriyama, 1971) may also be synthesized in bound polysomes. Little is known, however, of the details of the process leading to the assembly of bound polysomes by which specific mRNAs are translated in association with ER membranes. An elucidation of this process is of interest since it should help in

understanding those mechanisms which insure that cellular proteins are conveyed to their correct destination, whether it be a subcellular structure, compartment, or the extracellular space.

It has been shown that membrane-bound ribosomes are attached to specific sites on ER membranes through the large ribosomal subunits (Sabatini et al., 1966) which contain the nascent polypeptide chains, and that the interaction of these chains with the membranes provides a link which maintains the ribosome-membrane association (Adelman et al., 1973). It has not yet been determined, however, if the association of the mRNA in bound polysomes with the membranes of the ER is exclusively mediated by the ribosomes and the nascent chains or if, in addition, the messenger itself is directly attached to the membranes. A direct association of mRNA and membranes may not be sufficient to insure the fate of products to be discharged in the ER lumen, which may also depend on amino acid sequences encoded by nucleotide sequences near the 5′ end (Sabatini et al., 1972; Milstein et al., 1972), but it may contribute to the spatial segregation of messengers to be translated on ribosomes bound to ER membranes.

To investigate the relationship between mRNA and the ER membranes we used a tissue culture system of normal human diploid fibroblasts which show a well-developed rough ER (Brandes et al., 1972) and actively secrete collagen into the medium (Houck et al., 1971). A membrane fraction containing rough microsomes was prepared from cells differentially labeled in their mRNA and ribosomes. The fate of the mRNA was followed after the in vitro disassembly of bound polysomes by artificial termination with puromycin or by unraveling the ribosomes through depletion of magnesium ions in a medium of high ionic strength (Borgese, 1972; Sabatini et al., 1972). It was found that the mRNA remained attached to the membranes after ribosome detachment and release of nascent chains, as would be expected if there is a direct association of the mRNA with the ER membranes. The poly A segment of the mRNA remained bound to the ribosome-stripped membranes even after extensive digestion with RNase, indicating that a site of attachment of the messenger to the membranes is at or near the 3′ end of the molecule. These observations lead us to formulate a model for the assembly of bound polysomes in which both ribosomes and mRNA make direct contact with ER membranes.

MATERIALS AND METHODS

Cell Cultures

Cultures of WI-38 fibroblasts (16–20 population doublings, obtained from Dr. L. Hayflick, Stanford University, Stanford, Calif.), were first subcultured (1:4) in T75 Falcon flasks (Falcon Plastics, Div. of B.-D. Laboratories, Los Angeles, Calif.) and grown according to Hayflick and Moorhead (1961). When confluent, these cultures were transferred to Falcon disposable roller bottles (690 cm²) and grown in a 5% CO_2, 95% air atmosphere with 120–150 ml of Eagle's minimal essential medium containing 10% fetal calf serum and supplemented with glutamine (20 μmol/ml), ascorbic acid (50 μg/ml), streptomycin (10 μg/ml), and penicillin (100 μg/ml). Tests for pleuropneumonia-like organisms (PPLO) (Grand Island Biological Co., Grand Island, N. Y.) were carried out routinely and only PPLO-free cultures were used for experiments. Roller bottles of cultures in passage levels corresponding to fewer than 28 population doublings were used for experiments.

RNA Labeling

For long-term labeling of ribosomal RNA (rRNA), 2 days after seeding the cells the medium in each bottle was replaced with fresh medium containing either 1 μCi of uniformly labeled [^{14}C]uridine (416 mCi/mmol) or [8-^{14}C]adenine (40–60 mCi/mmol). Cells were incubated with the labeled precursors for 72 h until confluent. For short-term labeling of mRNA in the presence of actinomycin D, the medium was changed and replaced with 50 ml of fresh medium containing 0.025–0.03 μg/ml actinomycin D. After 30 min, either 1 mCi of [5-^{3}H]uridine (sp act 20–30 Ci/mmol, New England Nuclear, Boston, Mass.) or 1 mCi of [2-^{3}H]adenosine (sp act 26 Ci/mmol, New England Nuclear) was added and incubation continued for 3 h until harvesting. For some experiments, cells were labeled with [^{3}H]adenosine or [^{3}H]uridine in the absence of actinomycin D.

Cell Fractionation and Preparation of the Postnuclear Supernate

Medium was removed from the roller bottles which were then rapidly cooled in an ice-water bath. The cell monolayers were washed twice with 50 ml of ice-cold Dulbecco's phosphate-buffered saline. Cells were harvested in the same buffer by scraping with a rubber policeman and recovered by centrifugation at 200 *g* for 5 min at 4°C. All further procedures were carried out at 4°C.

Washed cells from one roller bottle were resuspended in 2 ml of reticulocyte standard buffer (RSB) (1.5 mM $MgCl_2$, 10 mM KCl, 10 mM Tris-HCl buffer, pH 7.4) and allowed to swell for 10 min before being disrupted with 10 strokes of a tight-fitting Dounce glass homogenizer. Nuclei and cell debris were removed by sedimentation (800 *g* for 2 min) and the pellet was washed once in 1 ml of RSB. The combined supernates were centrifuged

once more (800 *g* for 2 min) to remove any residual nuclei and to obtain a final postnuclear supernate (PNS). The ionic composition of the postnuclear supernate was adjusted to high salt buffer (HSB) (5.0 mM $MgCl_2$, 500 mM KCl, 50 mM Tris-HCl, pH 7.4) before sucrose density gradient sedimentation in HSB to separate free and bound ribosomes or to obtain a membrane fraction. In all cases, the ionic composition in the sucrose gradients was the same as in the samples.

A membrane fraction (MF) was prepared from aliquots (0.5–1.0 ml) of the postnuclear supernates by centrifugation through a 15–30% sucrose density gradient for 30 min at 27,000 rpm in an SW41 Beckman rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The membrane fraction, deposited as a pellet, was resuspended in RSB (0.5–1.0 ml) with or without $MgCl_2$ by gentle homogenization in a glass-Teflon Potter-Elvehjem homogenizer and used for further studies. Electron microscopically, this membrane fraction was shown to contain rough and smooth microsomal vesicles, as well as plasma membrane and mitochondrial fragments.

Sucrose Density Gradient Analysis

For sedimentation analyses of the PNS or the MF, samples (0.5–1.0 ml) were layered onto 12.5-ml linear sucrose gradients, of the composition indicated in the figures, which were centrifuged in the Beckman SW41 rotor in the L3-50 or L5-50 centrifuges (Beckman Instruments, Inc.). Conditions of centrifugation are given in the figure legends.

After centrifugation the gradient solutions were withdrawn from the top of the tubes by using an Auto Densiflow probe (Buchler Instruments Div., Searle Analytic Inc., Fort Lee, N. J.) connected to a peristaltic pump, and passed through a UV analyzer (LKB Uvicord, II, type 830A, LKB Producter AB, Bromma-I, Sweden) equipped with a log converter attached to a linear recorder. The effluents were collected in about 30 fractions. For analytical experiments, 250 μg of bovine serum albumin was added to each fraction as a carrier before the addition of cold trichloroacetic acid (TCA) to a final concentration of 5%. Precipitates were collected on glass filter paper disks for measuring of radioactivity. When fractions were used for preparative procedures, 0.1-ml aliquots were spotted on Whatman 3 MM filter paper disks which were then processed according to Mans and Novelli (1961) for measurements of RNA radioactivity. Dried glass fiber or filter paper disks were counted in a Beckman model LS-200 scintillation spectrometer with a toluene-based 2,5-diphenyloxazole/1,4-bis[2-(5-phenyloxazolyl)]benzene (PPO/POPOP) scintillation fluid (Liquifluor, New England Nuclear).

Disassembly of Membrane-Bound Polysomes

The membrane fraction was resuspended in RSB and then the salt composition was adjusted to that of HSB. Puromycin was added to aliquots (0.5 ml) to a final concentration of 10^{-3} M. Samples were incubated at 37°C for 15 min and analyzed by sucrose density centrifugation at 20°C as previously described (Adelman et al., 1973). Alternatively, membrane-bound polysomes were disassembled and detached by treatment in a high salt buffer containing no Mg^{++} (Borgese, 1972; Sabatini et al., 1972). In this case, the membrane fraction was resuspended in RSB lacking Mg^{++}, and the salt concentration was adjusted to 0.5 M or 1.0 M KCl, and 50 mM Tris-HCl, pH 7.4. These samples (0.5 ml) either were analyzed directly in sucrose gradients (20–50% or 20–60% sucrose of the same salt composition), or stripped membranes were directly isolated by sedimentation through a 15–30% sucrose gradient of the same ionic composition.

RNA Extraction

Membrane fractions, resuspended in 2 ml of RSB containing 10 mM NaCl instead of KCl, were adjusted to the composition of NETS (0.2% sodium dodecyl sulfate ethylenediaminetetraacetic acid [SDS], 100 mM NaCl, 10 mM [EDTA], and 10 mM M Tris-HCl, pH 7.4) in a final volume of 5 ml. 5 ml of phenol saturated with NETS and 5 ml of chloroform containing 1% isoamyl alcohol were added and the mixture was shaken for 5 min at 37°C. The aqueous and phenol phases were separated by centrifugation (2,500 rpm for 5 min) at room temperature. The aqueous layer was removed and the phenol phase and interface were re-extracted with NETS. The RNA in the combined aqueous phases was precipitated with 2 vol of absolute alcohol, kept at −20°C overnight, resuspended in NETS, and reprecipitated with alcohol. In some cases the membrane fractions were dissolved in NETS and their RNA was analyzed directly by sedimentation in 15–30% sucrose gradients containing NETS.

RNA Analysis by Oligo-dT Cellulose Chromatography

RNA samples resuspended in binding buffer (0.4 M NaCl, 0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6) were applied to oligo-dT cellulose (Collaborative Research, Waltham, Mass.) columns. The columns were prepared with oligo-dT (20–40 mg) in 1 cm^3 hypodermic syringes and equilibrated with binding buffer. Each sample was passed three times through the same column to maximize binding. Columns were washed five times with 1-ml aliquots of binding buffer before elution of the bound RNA with four successive 1-ml aliquots of low ionic strength buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6). The radioactivity in aliquots of the column fractions was measured as described for sucrose gradient fractions.

Fractions containing bound or unbound RNA were precipitated with ethanol to recover the RNA. Bound fractions contained small amounts of RNA and therefore

received 100 μg of yeast RNA as a carrier before precipitation with ethanol.

Acrylamide Gel Electrophoresis of RNA

RNA samples were dissolved in a solution containing 0.2% SDS, 15% glycerol, and bromphenol blue at 1/20 saturation and analyzed by electrophoresis through either composite gels containing 2.6% acrylamide-0.13% bisacrylamide and 0.5% agarose by the buffer system of Peacock and Dingman (1968), or 3% acrylamide-0.15% bisacrylamide gels as described by Loening (1967) prepared in tubes 7.5 cm long and 6 mm in diameter. Gels (14 cm long, 6 mm in diameter) containing 10% acrylamide-0.5% bisacrylamide and the buffer system of Peacock and Dingman were used in the electrophoretic analysis of poly A. All gels and gel buffers contained 10% glycerol so that they could be frozen in a hexane-dry ice bath and sliced into 1-mm segments with a guillotine-type gel slicer (MRA Corp., Boston, Mass.). Gel slices were dissolved either in H_2O_2 and counted in 2-(4′-tert-butylphenyl)-5-(4″-biphenylyl)-1,3,4,-oxadiazone (butyl-PBD)-toluene-based scintillation fluid (Kreibich and Sabatini, 1974) or in Protosol and counted in a toluene-PPO scintillation fluid (Adesnik and Darnell, 1972).

Poly A Analysis

RNA samples dissolved in 0.1 M NaCl, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.4, were treated with pancreatic RNase (2.5 μg/ml) and T1 RNase (5 U/ml) and were incubated for 30 min at 37°C. The RNA was extracted with phenol and chloroform, as described, and precipitated with ethanol after addition of 100 μg of yeast RNA carrier. The poly A samples were then adsorbed and eluted from oligo dT cellulose, as described, before electrophoresis in 10% polyacrylamide gels. In some cases, RNase digests were analyzed directly by gel electrophoresis. The RNA radioactivity resistant to pancreatic and to T1 RNase under the digestion conditions just described was also tested for sensitivity to the same enzymes or to T2 RNase under conditions known to lead to digestion of poly A. These conditions were for the pancreatic and T1 digestion: 10 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, for 30 min at 37°C, and for T2 RNase: 2 U/ml of enzyme (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M ammonium acetate, pH 4.5, for 2 h at 37°C. RNA from membrane fractions was also tested for sensitivity to alkali (0.3 M KOH at 45°C for 2 h) and hot acid (5% TCA at 95°C for 15 min) hydrolysis conditions.

Analysis of Poly A Content by Hybridization with [^{3}H]Poly U

RNA extracted from intact or ribosome-stripped membrane fractions was hybridized to [^{3}H]polyuridylic acid ([^{3}H]polyU) (3.12 dpm, 80,000 cpm/μg, s_{20} = 6.5, from Miles Laboratories Inc., Miles Research Div., Elkhart, Ind.) under conditions described by Bishop et al. (1974). A 30-min incubation time of the RNA with the [^{3}H]polyU added in excess was required for maximum hybridization. Hybridized [^{3}H]polyU was measured after pancreatic RNase digestion at 0°C as described by Bishop et al. (1974).

RESULTS

a. Labeling of Membrane-Bound Ribosomes and Preparation of a Membrane Fraction

Confluent cultures of WI-38 cells harvested 3 h after a medium change contain approximately 50% of the cytoplasmic ribosomes bound to endoplasmic reticulum membranes (Fig. 1 *a*) (Tashiro et al., manuscript in preparation). These bound ribosomes can be separated from free polysomes, because they rapidly sediment to an isopycnic position ($\rho \cong 1.18$) in 20–65% sucrose gradients or can be recovered as a pellet after sedimenting through a 15–30% sucrose gradient (Rosbash and Penman, 1971). The ribosomes associated with the membrane fraction which is represented by the peak at fraction 20 in Fig. 1 *a* are released as polysomes and monomers when the PNS is treated with detergents to dissolve the membranes (Fig. 1 *b*).

It has previously been shown that a medium of high ionic strength, such as HSB, removes from ER membranes inactive ribosomes which may be bound to membrane sites at low ionic strength (Adelman et al., 1973; Tashiro et al., manuscript in preparation). Membrane fractions prepared from the PNS in media containing a high salt concentration are devoid of contaminating free polysomes and inactive ribosomes (Fig. 2 *a*, OD and ^{14}C profiles). The gradient profiles obtained from membrane fractions prepared in HSB and treated with detergent (Fig. 2 *b*) show that most of the ribosomes which remain associated with the membranes after treatment in a medium of high ionic strength are in polysomes.

b. mRNA in the Membrane Fraction

Using low doses of actinomycin D to suppress the labeling of rRNA, one can specifically label cytoplasmic mRNA (Perry and Kelley, 1970). Sodium dodecyl sulfate (SDS) sucrose gradient analysis of PNS showed that in WI-38 fibroblasts a concentration of actinomycin D between 0.02 and 0.035 μg/ml completely suppressed the labeling of rRNA and therefore could be satisfactorily

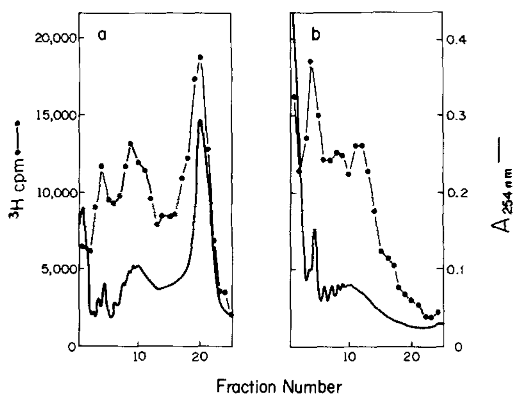

FIGURE 1 Distribution of free and membrane-bound ribosomes in postnuclear supernates from WI-38 cells. 100 μCi of [^{3}H]uridine (416 μCi/mmol) was added to a culture of WI-38 growing in a roller bottle. 72 h later, after the culture reached confluency, the medium was changed, and cells were harvested after 3 h. Aliquots (0.5 ml) of postnuclear supernates adjusted to the ionic composition of HSB were analyzed by sedimentation (1 h, 40,000 rpm, SW41) in 20–65% sucrose (S)-HSB gradients without (*a*) or with (*b*) previous addition of a detergent mixture (final concentration 0.5% DOC, 1% Triton X-100). Gradients were monitored for the optical density profile, fractions were collected, and the acid-precipitable radioactivity measured in each fraction.

used to prepare a membrane fraction specifically labeled in the mRNA. Fig. 2 *a* shows the isopycnic sedimentation analysis of a membrane fraction obtained from a culture labeled with [^{14}C]adenine for 3 days during growth and subsequently labeled with [^{3}H]adenosine for 3 h in the presence of 0.03 μg/ml actinomycin D. Oligo-dT cellulose chromatography of the RNA extracted from the membrane fraction showed that while the ^{3}H-labeled RNA was very efficiently (>80%) retained by the column, binding of the ^{14}C-labeled RNA to oligo-dT cellulose was minimal (<3%). It was therefore concluded that ^{3}H-labeled RNA contained poly A, which established its messenger-like character. When analyzed by electrophoresis in polyacrylamide gels (Fig. 3) or by sedimentation in sucrose gradients containing SDS (not shown), the ^{3}H-labeled RNA recovered from the oligo-dT columns was found to be of the size distribution expected of a mixture of mRNAs (Singer and Penman, 1973). Sedimentation analysis of detergent-treated membrane fractions (Fig. 2 *b*) showed that more than 50% of the [^{3}H]RNA radioactivity sedimented with bound polysomes while the rest of it corresponded to ^{3}H-labeled mRNA which remained in the top fractions of the gradient. It is possible that at least part of this nonpolysomal

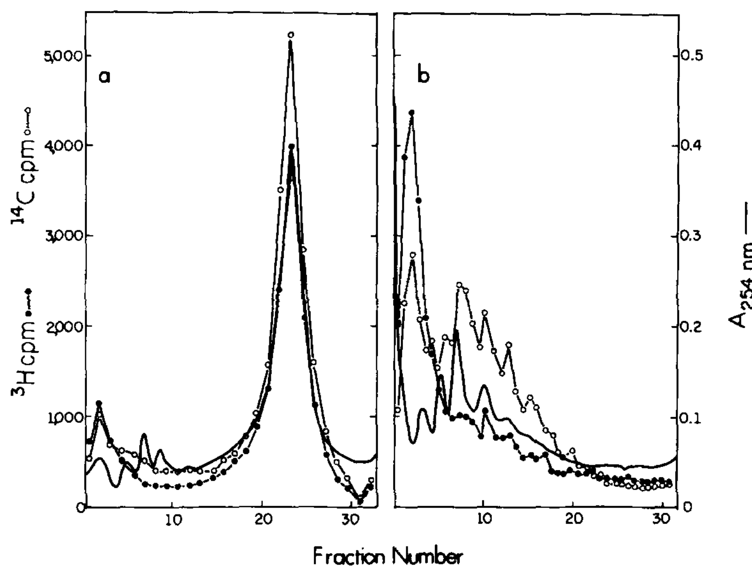

FIGURE 2 Distribution of labeled messenger and ribosomal RNAs in a membrane fraction and in bound polysomes. A culture was labeled with [^{14}C]adenine (1 μCi per roller bottle) added 72 h before confluency. After a medium change, actinomycin D was added to a final concentration of 0.03 μg/ml; 30 min later, [^{3}H]adenosine (1 mCi per roller bottle, sp act 26 Ci/mmol) was added, and the culture was harvested after 3 h. A membrane fraction adjusted to the salt composition of HSB was analyzed as in Fig. 1. (*a*) Control; (*b*) treated with detergent mixture, as in Fig. 1 *b*.

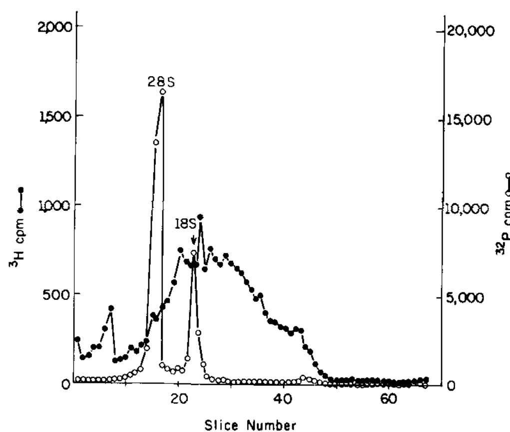

FIGURE 3 Size distribution of membrane-associated poly A containing ³H-labeled RNA. After a medium change, a confluent culture received actinomycin D (0.025 μg/ml), and 30 min later [³H]uridine (1 mCi per roller bottle, 26 Ci/mmol) was added. Cells were harvested 3 h later, a membrane fraction was prepared, and its RNA extracted. After addition of [³²P]rRNA marker derived from mouse cells, the fraction of labeled RNA retained by an oligo-dT cellulose column (80% of the total) was analyzed by electrophoresis in a composite polyacrylamide (2.6%)-agarose (0.5%) gel for 3 h at 3 mA per tube. Gels were sliced and the distribution of the ³²P and ³H radioactivity determined. O——O——O, ³²P radioactivity in RNA marker; ●——●——●, ³H radioactivity in mRNA.

RNA represents degradation of polysome-associated mRNA during detergent treatment.

c. The [³H]mRNA in the Membrane Fraction is Not Mitochondrial

A major difficulty in studying the membrane-bound protein synthesis apparatus of cultured cells stems from the fact that subcellular fractions derived from endoplasmic reticulum membranes cannot be prepared with high yields without major contamination by mitochondria and other cytoplasmic organelles (Penman et al., 1970; Rosbash and Penman, 1971). Hence, in most studies concerned with membrane-associated polysomes in tissue culture cells, a crude membrane fraction is used which may also contain mitochondrial ribosomes and mitochondrial mRNA (Zauderer et al., 1973; Hirsch and Penman, 1973). In order to assess what proportion of the ³H-labeled mRNA in the membrane fraction was mitochondrial, the relative effects of ethidium bromide and camptothecin in the presence of actinomycin D on the labeling of the membrane fraction RNA were measured.

It has been demonstrated (Perlman et al., 1973) in HeLa cells that the antibiotic, camptothecin, which abolishes the labeling of cytoplasmic RNA, has no effect on the labeling of mitochondrial RNA. Synthesis of mitochondrial RNA, however, can be selectively abolished by treatment with ethidium bromide (Zylber et al., 1969; Perlman et al., 1973). Since the labeling of RNA in the membrane fraction was nearly completely resistant to ethidium bromide and almost totally sensitive to camptothecin (Fig. 4), we concluded that the contribution of mitochondrial RNA to the labeling of mRNA from the membrane fraction was negligible. It will be shown in Section (*e*) that the poly A of the mRNA in the membrane fraction has the electrophoretic mobility characteristic of poly A in cytoplasmic mRNA, which is significantly lower than that of the poly A of mitochondrial mRNA (Perlman et al., 1973).

d. Fate of mRNA after Release of Ribosomes

Experiments with rat liver microsomes (Sabatini et al., 1966; Adelman et al., 1973) have shown that the attachment of ribosomes to ER membranes is mediated by bonds involving large ribosomal subunits and nascent polypeptide chains. While "inactive" ribosomes, containing no nascent polypeptides, are removed from the microsomal membranes in media of high salt containing Mg^{++} (HSB), active ribosomes can only be released from the membranes by HSB after discharge of the nascent polypeptides by treatment with puromycin (Adelman et al., 1973).

Using a membrane fraction derived from WI-38 cells differentially labeled in the rRNA with [¹⁴C]uridine (3 days) and in the mRNA with [³H]uridine (3 h in the presence of actinomycin D), we attempted to investigate the relationship of mRNA to the ER membranes by following its fate after disassembly of the bound polysomes. Fig. 5 shows the distribution of labeled ribosomes and mRNA in membrane fractions which were analyzed in sucrose density gradients containing HSB, before and after treatment with puromycin. A comparison of both patterns (Fig. 5 *a*, *b*) indicates that more than 70% of the [³H]mRNA remained associated with the membranes even after the puromycin treatment, despite the fact that this led to the removal of approximately 70% of the

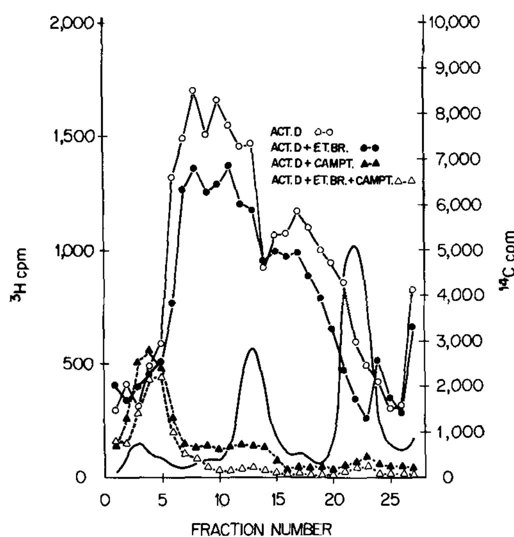

FIGURE 4 The effect of ethidium bromide and camptothecin on the labeling of membrane-associated mRNA. Four roller bottles were labeled for 72 h during growth with [^{14}C]adenine (1.5 μCi per bottle). At confluency, the medium was changed and actinomycin D (0.02 μg/ml) was added to each bottle. One bottle received only actinomycin, while others also received either ethidium bromide (1 μg/ml), camptothecin (20 μg/ml), or a combination of both drugs. 30 min after the addition of the drugs all bottles received [^{3}H]adenosine (1 mCi, 25 Ci/mmol), and the cells were harvested 3 h later. Membrane fractions were prepared, resuspended in 1 ml of NETS, and a portion (25% of each sample) was analyzed at 22°C in 15–30% sucrose-NETS gradients (17 h, 23,000 rpm, SW41). The ^{3}H radioactivity of each fraction was normalized, taking into account the relative amounts of ^{14}C-labeled ribosomal RNA in each gradient. For clarity, the ^{14}C radioactivity profile of the sample treated with actinomycin D alone is plotted as a continuous curve. ——O——O——, actinomycin D only; ●——●——●, actinomycin D and ethidium bromide; ▲——▲——▲, actinomycin D and camptothecin; △——△——△ actinomycin D, ethidium bromide, and camptothecin.

[^{14}C]rRNA from the membranes, which in the OD profiles are still visible mainly as a lower peak, due to light scattering by the membrane vesicles.

A more effective procedure for releasing ribosomes from the membranes employing a medium of high ionic strength lacking Mg^{++} which leads to the disassembly of polysomes into derivatized subunits (Borgese, 1972; Sabatini et al., 1972) was also used. Either membrane fractions were directly sedimented through sucrose gradients in high salt medium lacking magnesium (Fig. 6 *a*, *b*), or membrane fractions stripped in a high salt medium lacking magnesium were first recovered by sedimentation and subsequently analyzed in sucrose density gradients (Fig. 7 *a*, *b*). Both types of analysis showed that there was an extensive release (80–95%) of long term-labeled [^{14}C]RNA with almost quantitative retention of the mRNA which was pulse labeled with [^{3}H]uridine in the presence of actinomycin D.

Approximately 20–30% of the long term-^{14}C-labeled RNA which remained associated with the membranes after ribosome stripping was mitochondrial RNA which had the mobility of 12S and 23S in acrylamide gel electrophoresis (Fig. 8). The rest of the membrane-associated RNA was approximately equally distributed as rRNA with a

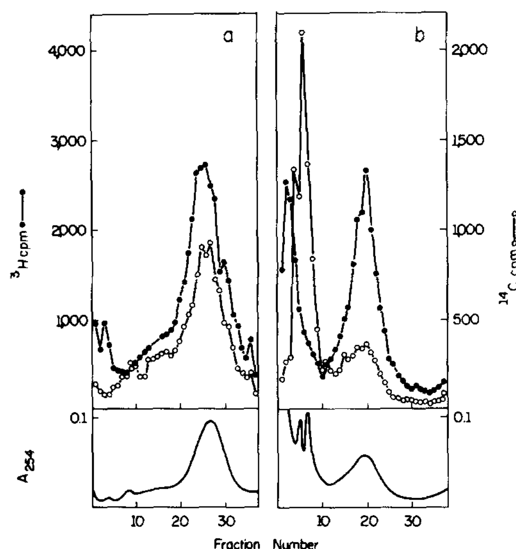

FIGURE 5 Release of ribosomal subunits by puromycin-KCl from a membrane fraction containing labeled messenger (^{3}H) and ribosomal (^{14}C)RNA. A double-labeled membrane fraction was prepared as described in Fig. 2, but with [^{14}C]uridine (1 μCi per roller bottle, sp act 55 mCi/mmol) and [^{3}H]uridine (1 mCi per roller bottle, sp act 26 Ci/mmol) for the long- and short-term labeling, respectively. The actinomycin concentration during the 3 h of labeling with [^{3}H]uridine was 0.03 μg/ml. The membrane fraction was resuspended in HSB and divided into two aliquots. One aliquot was treated by the puromycin-HSB procedure (see Materials and Methods) for ribosomal stripping (*b*), and the other used as control (*a*). Both samples were analyzed by sedimentation at 20°C (1 h, 40,000 rpm, SW41) in 20–50% sucrose gradients containing HSB. Optical density profiles were recorded and the radioactivity distributions were measured for each gradient.

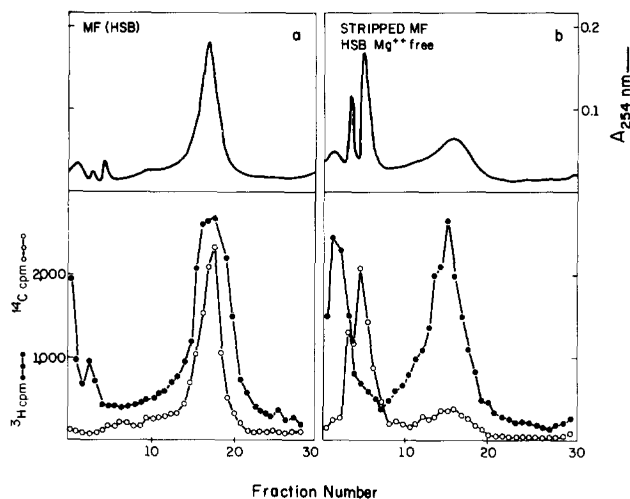

FIGURE 6 Release of ribosomal subunits and retention of mRNA in a membrane fraction treated with a medium of high ionic strength lacking Mg^{++}. A membrane fraction doubly labeled in ribosomal (^{14}C) and messenger (^{3}H)RNA was prepared as described in Fig. 2. One-half (*a*) was resuspended in HSB and analyzed at 4°C in a 20–60% sucrose gradient containing HSB. The other half (*b*) was resuspended in RSB containing no Mg^{++} and brought to 0.5 M KCl, 0.050 M Tris-HCl (no Mg^{++}), and analyzed in 20–60% sucrose gradients (1 h, 40,000 rpm, SW41) in HSB lacking Mg^{++}. Optical density and radioactivities are plotted separately for clarity.

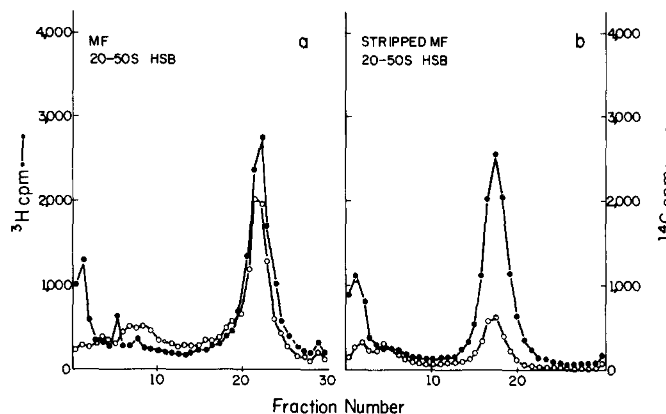

FIGURE 7 Retention of mRNA in membranes stripped of ribosomes in a medium of high ionic strength lacking Mg^{++}. A culture was labeled as indicated in the legend to Fig. 6. The harvested cells from one roller bottle were divided into two equal aliquots. From one, a control membrane fraction was prepared (*a*). Cells in the other aliquot were disrupted in RSB lacking magnesium (0.01 M Tris-HCl, pH 7.6, 0.01 M KCl), and a postnuclear supernate was prepared. The salt concentration in the supernate was adjusted to HSB lacking Mg^{++} (0.5 M KCl, 0.050 M Tris-HCl pH 7.6) to obtain a ribosome-stripped membrane fraction (*b*) by sedimentation through a 15–30% sucrose gradient of the same salt composition. Both the control and the stripped membrane fractions were resuspended in RSB and compensated to HSB for sedimentation analysis in a 20–50% sucrose HSB density gradient as in Fig. 6.

predominance of 28S and heterogeneous RNA (presumably, long term-labeled messenger).

A comparable demonstration of the retention of mRNA on the membranes after ribosome stripping was also obtained without the use of actinomycin D. In this case we monitored only poly A-containing labeled RNA, although it is known that a fraction of mRNA does not contain poly A

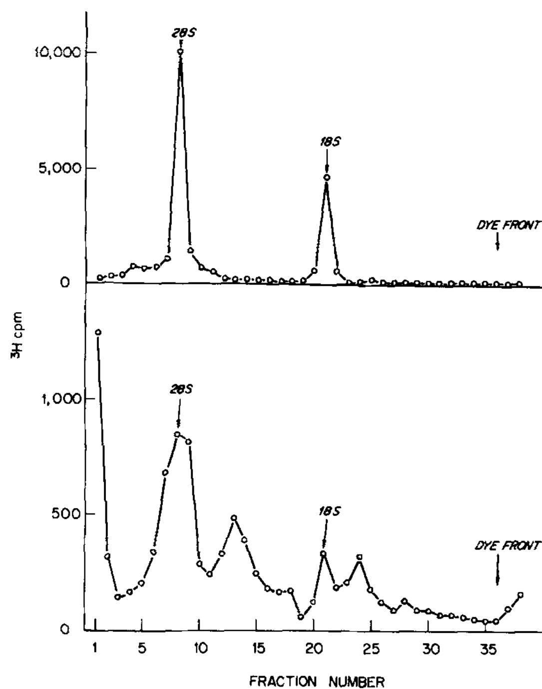

FIGURE 8 Quantitation of mitochondrial rRNA in a stripped membrane fraction by polyacrylamide gel electrophoresis. A roller culture was labeled with [^{3}H]uridine (100 μCi, 26 Ci/mmol) 72 h before confluence. After confluency, the medium was changed and 3 h later the cells were harvested. Intact and stripped membrane fractions were prepared as described in Fig. 7, each from half of the harvested cells. The RNA was extracted from each sample and amounts from an equivalent number of cells were analyzed by electrophoresis in 3% acrylamide gels by the Loening buffer system. Upper panel: RNA from intact membranes; lower panel: RNA from stripped membranes. Note the 10-fold difference in scale for the ordinate.

(Milcarek et al., 1974). A membrane fraction from cells labeled in both messenger and ribosomal RNAs with [^{3}H]adenosine for 3 h was prepared and treated for ribosome stripping in a high salt medium containing no Mg^{++}. The poly A-containing [^{3}H]mRNA fraction was purified from RNA extracts of intact and stripped membranes by oligo-dT cellulose chromatography and analyzed by electrophoresis in large pore polyacrylamide gels. Approximately 75% of the [^{3}H]poly A containing RNA in intact membranes remained associated with the membranes even after they were stripped of ribosomes. The electrophoretic profiles of the labeled poly A-containing RNA shown in Fig. 9 suggest that the lower recovery after stripping may, at least partially, be due to degradation of the mRNA.

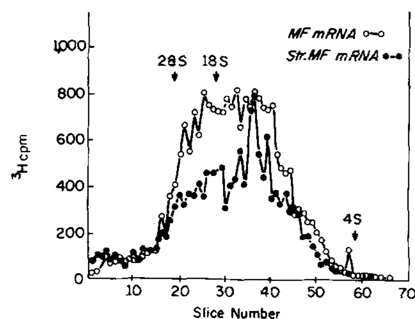

FIGURE 9 Retention of mRNA on membranes stripped of ribosomes from a culture labeled in the absence of actinomycin D. After medium change a confluent culture was labeled for 3 h with [^{3}H]adenosine (1 mCi per bottle, sp act 26 Ci/mmol). Control and ribosome-stripped fractions were prepared as indicated in the legend to Fig. 7. RNA was extracted from the membrane fractions and the poly A-containing molecules were isolated by chromatography on oligo-dT cellulose. These were analyzed by electrophoresis on polyacrylamide gels as indicated in the legend to Fig. 3.

The results of these experiments suggest that mRNA is attached directly to ER membranes independently of the large ribosomal subunits and the nascent polypeptide chains.

e. Poly A content of Membrane-Associated mRNA

By using [^{3}H]adenosine labeling and RNase treatment we measured directly the retention of labeled poly A on membranes stripped of ribosomes. First, it was demonstrated that if the mRNA in the membranes is labeled with [^{3}H]adenosine and stripping is effected by sedimentation through sucrose gradients containing high salt and no Mg^{++}, only the membrane band contains any radioactivity resistant to RNase (Fig. 10). The resistance to RNase of the [^{3}H]adenosine-labeled RNA which remained associated with the membranes was not due to the protection of non-poly A segments by association with the membranes since the resistance to RNase was measured in the presence of detergent.

A direct comparison of the RNase-resistant poly A segments contained in the mRNA of intact and stripped membranes was obtained by electrophoretic analysis (Fig. 11). Messenger RNA fractions were obtained by oligo-dT chromatography of RNA extracted from membrane fractions pre-

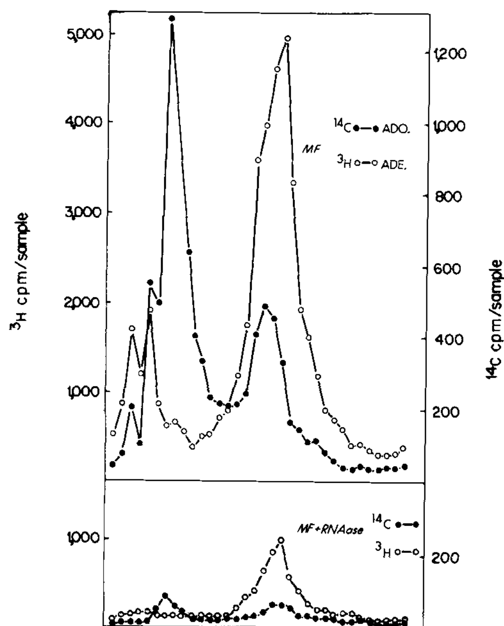

FIGURE 10 Quantitative retention of the RNase-resistant poly A segments of mRNAs on membranes stripped of ribosomes. Cultures doubly labeled in the rRNA ([^{14}C]adenine) and mRNA ([^{3}H]adenosine) as described in the legend to Fig. 2 were used to prepare a membrane fraction which was treated for ribosome stripping in a medium of high salt containing no Mg^{++} (1.0 M KCl, 0.010 M Tris-HCl, pH 7.6) and then fractionated in a 20–60% sucrose gradient of the same salt composition. Aliquots (100 μl each) of every fraction were diluted 10 times with water and treated with 0.5% sodium desoxycholate. One aliquot of each fraction was used to measure the ^{14}C and ^{3}H cold acid-precipitable radioactivity (top panel). The other aliquot received pancreatic ribonuclease (2.5 μg/ml) and Tl ribonuclease (5 U/ml) and was incubated for 30 min at 37°C before the measurement of acid-precipitable radioactivity (bottom panel).

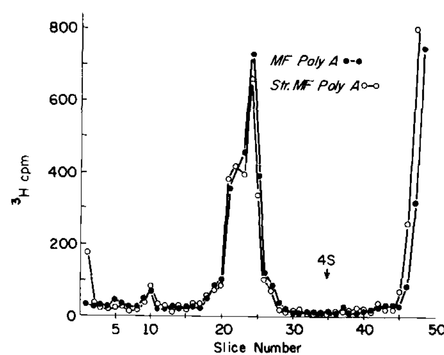

FIGURE 11 Poly A content of intact and ribosome-stripped membranes. The [^{3}H]adenosine-labeled poly A-containing mRNA from intact and stripped membranes which was characterized in Fig. 9 was digested with ribonuclease and the digest was directly analyzed by electrophoresis in 10% polyacrylamide gels as described in Materials and Methods. A parallel gel was run containing ^{32}P mouse cell soluble RNA, as a marker for 4S RNA.

pared from cells labeled for 3 h with [^{3}H]adenosine in the absence of actinomycin D and then digested with Tl and pancreatic RNase. The electrophoretic profiles clearly show that there was nearly a quantitative retention of [^{3}H]poly A of cytoplasmic mRNA (Adesnik and Darnell, 1972). It has been shown that poly A of mitochondrial mRNA has a considerably greater electrophoretic mobility (Hirsch and Penman, 1974).

The ribonuclease-resistant fraction of pulse-labeled, membrane-associated mRNA was shown to have the following characteristics of authentic poly A: (*a*) complete sensitivity to hydrolysis by alkali or hot TCA; (*b*) susceptibility (>90%) to attack by ribonuclease T2 or by pancreatic and T1 ribonuclease in solutions of low ionic strength.

f. Direct Measurement of Poly A Content in Intact and Ribosome-Stripped Membranes by Hybridization to [^{3}H]Poly U:

It should be noted that the preceding experiments do not provide a measure of the fraction of the total cellular mRNA which remains associated with the membranes after removal of the ribosomes, but rather of the newly synthesized mRNA which was labeled during a 3-h pulse in serum-stimulated confluent cultures. It is possible that the behavior of newly synthesized RNA is not representative of the bulk of the mRNA population. In fact, it has been proposed (Shiokawa and Pogo, 1974) that newly synthesized messenger emerges in the cytoplasm in association with ER membranes.

The total amount of poly A-containing mRNA in a cell fraction was assessed without previous labeling of the cultures by measuring the capacity of the extracted RNA to hybridize with radioactive poly U (Table I) by the procedure described by Bishop et al. (1974). These measurements indicated that 90% of the poly A content of a membrane fraction was present in membranes prepared in a high salt medium lacking magnesium despite the extensive release (85%) of ribosomes

which had occurred and was apparent from the loss of long term ^{14}C-labeled RNA.

g. A Site of Attachment of mRNA to the Membranes is at or near the Poly A Segment

Intact or ribosome-stripped membrane fractions containing [^{3}H]adenosine-labeled mRNA were treated with RNase under conditions which completely destroyed the rRNA. The membranes were recovered and the residual RNA was extracted and purified by oligo-dT cellulose chromatography. It was found (Fig. 12) that the poly A-containing RNA segments which remained with either type of membrane had an electrophoretic mobility similar to that of purified poly A prepared by RNase digestion and oligo-dT chromatography of RNA extracted from intact or stripped membranes. The results of these experiments suggest that poly A is at or close to the point of linkage of mRNA to the membranes and that mRNA in the membrane fraction is directly accessible to added RNase. The latter point was corroborated by the nearly complete susceptibility to RNase of the radioactivity in [^{3}H]uridine-labeled mRNA when intact membranes were digested in the presence or absence of detergent (not shown).

TABLE I
Poly A Content of RNA from Intact and Ribosome-Stripped Membrane Fractions Measured by Hybridization with [^{3}H]poly U

Source of RNA sample*	[^{3}H]poly U hybridized‡
	cpm
Intact membrane fraction	2,000
Stripped membrane fraction	1,800

* RNA was extracted from intact or stripped membrane fractions obtained, as described in the legend to Fig. 7, from a culture incubated with [^{14}C]adenine (1 μCi; 40 mCi/mmol) for 72 h before confluence to label the ribosomes. The stripping procedure removed from the membranes 85% of the ^{14}C-labeled RNA.

‡ Aliquots of each sample, corresponding to one-twelfth of the cells in a roller bottle, were hybridized with 8,000 cpm (100 ng) of [^{3}H]poly U as described in the text. RNase-resistant radioactivity was measured after cold acid precipitaion. Appropriate dual label counting procedures were used to correct for the small amount (< 2% of RNase-resistant ^{3}H radioactivity) of ribonuclease-resistant ^{14}C-labeled RNA.

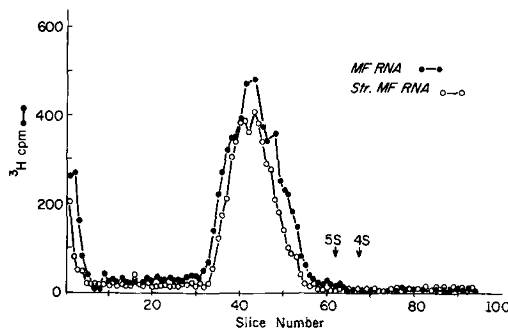

FIGURE 12 Poly A is not released when intact or ribosome-stripped membranes are treated with ribonuclease. Intact and ribosome-stripped membrane fractions containing [^{3}H]adenosine-labeled mRNA were prepared, as described in the legend to Fig. 7, from a culture labeled after a medium change for 3 h (1 mCi, 26 Ci/mmol) in the presence of 0.03 μg/ml actinomycin D. They were resuspended in 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA, and digested with a mixture of T1 (5 U/ml) and pancreatic (2.5 μg/ml) ribonucleases for 30 min at 37°C. After digestion the membranes were sedimented, the RNA was extracted, and the poly A-containing segments were purified by oligo-dT celulose chromatography and analyzed by electrophoresis in 10% polyacrylamide gels. A ^{32}P-labeled mouse soluble RNA marker was added to each sample before electrophoresis.

h. Exclusion of Artefactual Binding of mRNA and Poly A to Membranes

Free polysomes or bound polysomes (not shown) prepared by detergent treatment, containing labeled mRNA, were added to unlabeled membrane fractions before stripping. A negligible fraction of the added radioactivity was bound to the stripped membranes. Similarly, radioactive poly A did not bind to stripped membranes.

DISCUSSION

Our observations in following the fate of mRNA after disassembling membrane-bound polysomes in vitro indicate that most mRNA molecules present in membrane fractions previously washed in media of high ionic strength remain associated with endoplasmic reticulum membranes even after the ribosomes are detached and their associated nascent polypeptide chains are released. This was observed when disassembly of bound polysomes was effected either by incubation of a membrane fraction with puromycin in a high salt buffer, or by depletion of the Mg^{++} in the medium under

conditions which unravel and detach the ribosomes. The results of mixing experiments in which added messengers did not bind to microsomal membranes during the ribosome stripping procedure rendered unlikely the possibility that the mRNA was artefactually adsorbed to the membranes. It cannot be completely excluded, however, that binding occurred after disassembly and, because of the greater proximity of the messenger of bound polysomes to the membranes, added messengers could not compete as efficiently as local messengers for membrane sites with high messenger affinity.

The association of mRNA with the membranes apparently involves a segment of the messenger located near the 3′ end which contains the poly A stretch. This was inferred from the finding that ribonuclease digestion of intact or stripped membranes containing mRNA labeled with [^{3}H]adenosine destroyed the bulk of the labeled mRNA but left the poly A associated with the membranes. The poly A segment which, after extensive digestion with RNase, remained associated with the intact or stripped membranes, was of a similar size to that of the poly A segment which was recovered when extracted and purified mRNA was digested with RNase. The resolution of the polyacrylamide gel electrophoretic analysis, however, is not sufficient to exclude the possibility that the poly A segments left on membranes digested with RNase contain extra oligonucleotide sequences that may constitute recognition sites for the membranes or for proteins which serve to bind the mRNA to the ER membranes. Furthermore, it could not be ruled out that other specific sequences in the messenger not contiguous with the poly A which may have been protected by the membranes from the added RNase were at least partially degraded by residual RNase activity during extraction of the RNA from digested membranes. In fact, the small amount of [^{3}H]uridine pulse-labeled RNA which was not degraded by RNase treatment of intact or stripped membranes may represent such a sequence. In any case, it is clear that the poly A segment by itself cannot serve to determine the binding of the messenger to the membranes since mRNAs of both free and bound polysomes contain poly A segments of similar size. In this regard, it should be recalled that specific proteins are associated with poly A segments of messenger fractions from ascites tumor cells, rat liver, and cultured KB cells (Kwan and Brawerman, 1972; Blobel, 1973; Lindberg and Sundquist, 1974). It has yet to be determined if different specific proteins of this type are found in messengers of free and bound polysomes which may contribute to determine their subcellular distribution.

In fact, Milcarek and Penman (1974) have recently reported that after extensive RNase digestion of a HeLa cell membrane fraction, 57% of the poly A remained membrane associated, as a fragment with the same electrophoretic mobility as the poly A derived from purified mRNA.

Although our observations from using RNase indicate that within the mRNA molecule a binding site to the membranes occurs at or near the poly A segment, it cannot be excluded that there are also other binding sites for attachment to the membrane in regions of the messenger which were largely destroyed by the relatively rigorous RNase digestion conditions used in our experiments. It is possible that future experiments using milder or specific RNase treatments may reveal additional membrane binding sites in messengers of bound polysomes.

The conclusion that mRNA can be directly bound to ER membranes is further supported by the finding that when membranes prepared from confluent cultures labeled in the presence of actinomycin D were dissolved with detergents, not all the membrane-associated putative messenger RNA was recovered with the bound polysomes. A considerable fraction ($>40\%$) was consistently found in the top fraction of the gradients as expected of mRNA molecules which are membrane-bound but are not associated with ribosomes. Although at least part of this "non-ribosome-associated" mRNA may represent degradation of polysomal mRNA, the finding raises the possibility that even smooth portions of the endoplasmic reticulum, which at any given time do not contain bound ribosomes, may nevertheless contain some membrane-associated messenger RNA. The presence of "membrane-associated" RNA has been reported previously in rat liver microsomes (Petrović et al., 1965; Shapot and Pitot, 1966).

At variance with our observations, it has recently been reported (Harrison et al., 1974) that when bound polysomes in a membrane fraction derived from myeloma cells grown in culture are disassembled by a modification of our puromycin-KCl technique, mRNAs for the light and heavy chains of an immunoglobulin are quantitatively released. In these experiments, however, an equal

amount of labeled poly A-containing RNA remained associated with the membranes as was found in the released fraction. Although it was postulated that the RNA remaining in the membrane fraction (which had no messenger activity in a reticulocyte cell-free system) was of mitochondrial origin, this was not established experimentally. Furthermore, it should be noted that quantitative measurements of ^{32}P-labeled poly A-containing RNA by oligo-dT cellulose chromatography, which were used in the experiments with the myeloma cells to estimate the distribution of mRNA between released and membrane fractions, are very sensitive to nucleolytic cleavage. Thus, if the RNA retained in the membranes suffered, on the average, one more nucleolytic cleavage than the released RNA, its relative amount would have been underestimated by a factor or two. Similarly, any degradation of the immunoglobulin messenger RNA which was not released from the membranes would explain its being biologically inactive and the absence of characteristic oligonucleotide fragments in fingerprints from RNA species with the sedimentation values of intact light-chain mRNA. Further possible explanations for the apparent discrepancy between the results with fibroblasts and those with plasmacytoma cells may be related to the conditions of disassembly, which in the latter included a chelating agent, and to the tumoral-transformed state of the myeloma cell line.

One function of membrane-bound ribosomes is the vectorial transfer of nascent polypeptides across ER membranes. The operation of the mechanism effecting this transfer requires the fitting of a large ribosomal subunit into a specific receptor site which provides a passageway for the peptides across the membranes. Our results lead us to consider a model for protein synthesis in membrane-bound polysomes in which mRNA is directly bound to the membranes through a segment near the 3′ end (Fig. 13). Assembly of bound polysomes occurs by formation of initiation complexes between small subunits and the mRNA at the initiation site near the free 5′ end, which may be distant from the membranes. These complexes subsequently bind to large subunits which may be either free in the cytoplasm or already bound to membranes at the specific receptor sites. Those active ribosomes closest to the initiation site of the messenger contain growing polypeptide chains yet of insufficient length to anchor directly the large subunits at the membrane binding sites. Other ribosomes contain longer nascent peptides, the amino terminal segments of which have emerged from the large subunits and specifically function in anchoring the ribosome to the membrane receptor sites (Sabatini et al., 1972; Milstein et al., 1972). In cell extracts, only at low ionic strengths would those ribosomes which have translated relatively short stretches of mRNA be directly associated with the membranes, since at low ionic strength even inactive large subunits can be bound to the membrane binding sites (Borgese et al., 1974). At high ionic strengths, ribosomes with short chains may be released by mild RNase digestion which cleaves the mRNA.

It should be noted, however, that an independent binding of the mRNA to the membrane allows the possibility of assembling a different type of membrane-associated polysome than the one shown in Fig. 13, namely one containing ribosomes which do not necessarily ever come into contact with the underlying membrane. Membrane-bound polysomes which would contain only, or mainly, dangling or "loose" ribosomes of that type have been described (Rosbash and Penman, 1971; Lee et al., 1971) in cultured transformed cells in which numerous ribosomes are released from membrane-containing fractions by mild RNase treatment. According to our model of assembly, polysomes containing mostly loose ribosomes will be encountered in an unbalanced situation, if at any given time there are more ribosomes involved in the translation of membrane-associated messengers than there are available membrane sites for ribosome binding, even if the nascent polypeptide chains have expressed pertinent information for binding at their NH_2-terminal end. In this connection, it is interesting to note that few ribosomes of the loose type, i.e. easily released from the membranes by mild in vitro RNase treatment, are present in a membrane fraction of normal diploid WI-38 cells obtained by sedimentation through gradients containing high salt buffer. The demonstration, however, that in some cell types (Zauderer et al., 1973) loose polysomes, released as polysomes by washing in high salt media containing Mg^{++}, synthesize the same proteins as free polysomes, such as histones, together with the fact that at low ionic strength available sites in membranes can be occupied nonspecifically by free ribosomes or polysomes (Borgese et al., 1974) suggest that in most instances totally loose polysomes are likely to be artefactual. It should be noted that Milcarek and Penman (1974), using a

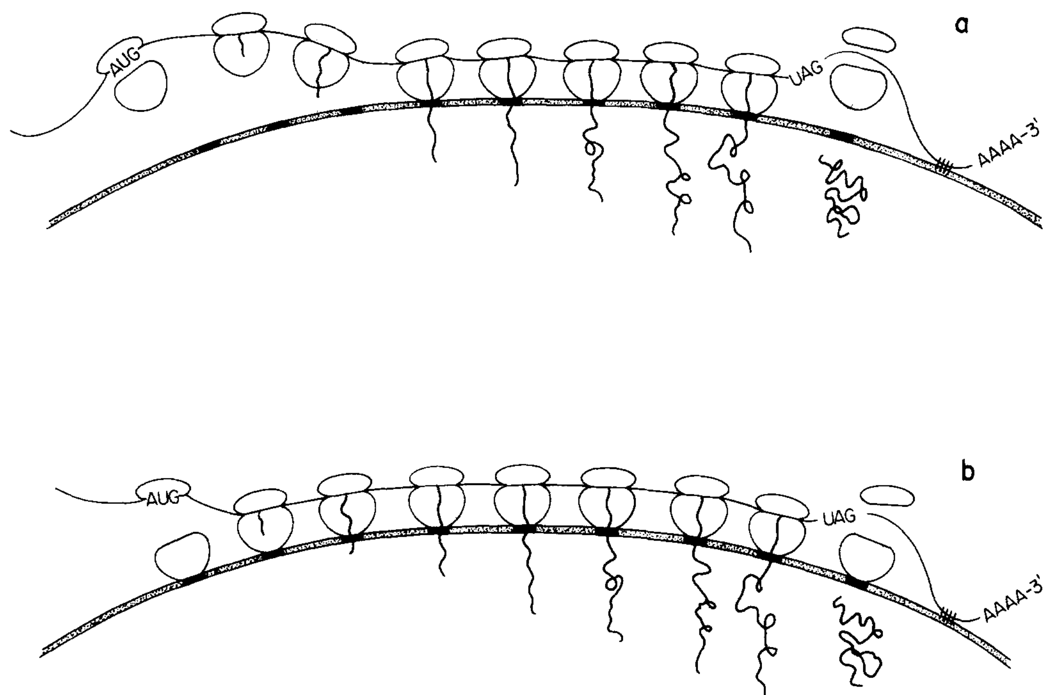

FIGURE 13 Scheme for assembly of bound polysomes with mRNA attached to the membrane through a segment near the 3′ end. Ribosome binding to the membranes has been shown to occur via the large subunits and to be stabilized by sufficiently long nascent polypeptide chains which emerge from the subunits and penetrate into the membrane (Sabatini et al., 1972). Ribosomes near the 5′ end, which contain short polypeptide chains, can be found in vitro either dangling (*a*) or directly contacting the membrane (*b*), depending on the ionic strength.

membrane fraction prepared in low salt media, have reported that 43% of the poly A could be released by RNase treatment followed by EDTA. As indicated in Fig. 13, under normal conditions and in the steady state of polysome assembly, only few ribosomes—namely those containing short polypeptide chains—will be found, at sufficiently high ionic strengths, dangling from the membranes bound solely through their associated messenger RNA.

Our model must also contain features which allow for the movement of messenger RNA with respect to the ribosomes which occurs during translation. Such movement may be facilitated by the fluidity of the membrane which may allow for extensive lateral displacement of binding sites for ribosomes and messenger (Ojakian et al., 1975). Alternatively, or in addition, the messenger may contain a long untranslated region between the termination codon and the membrane messenger binding site so that if the ribosomes and the messenger binding sites are fixed, the termination codon can still traverse all ribosomes within a polysome. In an extreme case the geometrical configuration of a polysome on a membrane could be fixed. In this case, the length of the untranslated segment between the termination codon and the anchoring point would determine the domain of the membrane to which a particular messenger RNA molecule is accessible for translation by bound ribosomes.

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