Formation in Vitro of Membrane-Bound Polysomes Containing Parathyroid Hormone Messenger Ribonucleic Acid†

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ABSTRACT: We have utilized the binding of radioactive parathyroid hormone messenger ribonucleic acid (mRNA) to dog pancreatic microsomal membranes as an assay for the formation of membrane-bound ribosomes in vitro. Parathyroid hormone mRNA was labeled by RNA ligase catalyzed addition of cytidine 3′,5′-[5′-^{32}P]diphosphate to the 3′ terminus. Radioactive parathyroid hormone mRNA was incubated in a reticulocyte cell-free protein-synthesizing system that was supplemented with dog pancreatic microsomal membranes. As determined by discontinuous sucrose gradient centrifugation, 25% of the total mRNA bound to the membranes in the reaction. Inhibition of protein synthesis by puromycin, addition of membranes after termination of the cell-free reaction, or disruption of the membranes after the reaction by deoxycholate resulted in less than 10% of the mRNA in the membrane region of the gradient. The binding of the mRNA to the membranes was resistant to incubation in 290 mM KCl, and only 25% of the mRNA was released by incubation in 540 mM KCl. This suggests that the nascent polypeptide chain is in part stabilizing the mRNA–ribosome membrane complex; this conclusion is strengthened by the observation that limited proteolysis caused the release of mRNA from the complex in high ionic strength buffer but not in low ionic strength buffer. Preformed mRNA–membrane complexes were disrupted within 5 min by addition of puromycin to the reaction, suggesting that direct interaction between the mRNA and membrane is not required to stabilize functional membrane-bound ribosomes. These experiments demonstrate that the binding of radioactive mRNA to microsomal membranes in vitro is a useful assay for analyzing the formation and nature of membrane-bound polysomes.

It is widely accepted that proteins that are destined to be secreted from a cell are synthesized on membrane-bound polyribosomes (Palade, 1975). Recent studies have suggested that functional membrane-bound ribosomes can be formed in vitro (Blobel & Dobberstein, 1975). Membrane-dependent cleavage of presequences from secretory proteins (Lingappa et al., 1977; Shields & Blobel, 1977; Boime et al., 1977; Dorner & Kemper, 1978; Strauss et al., 1978) and glycosylation of polypeptide chains (Rothman & Lodish, 1977; Katz et al., 1977; Lingappa et al., 1978; Bielinska & Boime, 1978) have been demonstrated. In addition, the newly synthesized secretory proteins in many of these studies have been shown to be resistant to proteolysis, indicating that they have been sequestered with the lumen of the microsomal membranes. Each of these processes represents a relatively late event in the formation and functioning of the membrane–ribosome complex. It would be useful to have an assay for an earlier step in the process, the binding of the ribosome to the membrane, so that the factors involved in the formation of membrane-bound ribosomes could be more precisely analyzed. The binding of isolated ribosomes to microsomal membranes in vitro has been used in many studies as an assay for the formation of membrane-bound ribosomes [reviewed by Sabatini & Kreibich (1976)]. In these studies a completely functional membrane–ribosome complex is not formed because the nascent chains of these molecules are not vectorially transported into the vesicle lumen (Borgese et al., 1974). The role of the nascent polypeptide chain, for example, in the formation of membrane-bound polysomes can not be assessed in these systems. On the other hand, the cell-free systems in which functional membrane-bound polysomes are formed generally are unfractionated and the ribosomes themselves are not conveniently labeled with radioactivity. To circumvent this problem, we have developed a method which utilizes the association of radioactive parathyroid hormone (PTH)[1] mRNA with microsomal membranes as an assay of the formation of functional membrane-bound ribosomes.

The translational product of PTH mRNA in the wheat germ and reticulocyte cell-free systems is pre-proparathyroid hormone (pre-ProPTH) (Kemper et al., 1974; Dorner & Kemper 1978), which is presumably rapidly cleaved in vivo to produce proparathyroid hormone (ProPTH) (Habener et al., 1976). If these two cell-free systems are supplemented with dog pancreatic microsomal membranes, accurate conversion of pre-ProPTH to ProPTH can be demonstrated and the ProPTH is sequestered within the microsomal membranes (Dorner & Kemper, 1978). RNA preparations may be obtained from parathyroid tissues which contain ~50% PTH mRNA (Stolarsky & Kemper, 1978). About 60–70% of the translational products of these preparations of RNA are PTH-containing peptides. In the present paper, we have incorporated cytidine 3′,5′-[5′-^{32}P]diphosphate ([^{32}P]pCp) onto the 3′ terminus of PTH mRNA and have measured the binding of the radioactive mRNA to membranes in the reticulocyte lysate cell-free system supplemented with dog pancreatic microsomal membranes.

Experimental Procedures

mRNA Isolation. Globin mRNA was isolated by sequential isolation of globin messenger ribonucleoprotein and mRNA by sucrose gradient centrifugation (Labrie, 1969). PTH mRNA was isolated and partially purified to ~50% purity, as described previously (Stolarsky & Kemper, 1978). The fraction of mRNA used was poly(A)-containing RNA fractionated on a sucrose gradient containing 70% formamide. For

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[1] Abbreviations used: PTH, parathyroid hormone; pre-ProPTH, pre-proparathyroid hormone; ProPTH, proparathyroid hormone; [^{32}P]pCp, cytidine 3′,5′-[5′-^{32}P]diphosphate.

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convenience, this RNA fraction is referred to as PTH mRNA.

Incorporation of [^{32}P]pCp into PTH mRNA. The synthesis of [^{32}P]pCp was as described by England et al. (1979), with some modifications (D. M. Hinton, personal communication). In a final volume of 25 μL, 1–3 μM [γ-^{32}P]ATP (1000–3000 Ci/mmol), 1 unit of T4 polynucleotide kinase, 0.96 mM 2′- or 3′-CMP, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM dithiothreitol were incubated for 70 min at 37 °C. The reaction was stopped by addition of 100 μL of ethanol, and [^{32}P]pCp was purified by descending chromatography on Whatman 3 MM paper with propanol–concentrated NH_3–water (55:10:35) as solvent.

[^{32}P]pCp was incorporated into PTH mRNA, as described by England et al. (1979), with modifications. The reaction mixture, in a final volume of 9 μL, contained 0.4 mM mRNA, 0.4 mM [^{32}P]pCp (1000–3000 Ci/mmol), 8 mM ATP, 10 mM $MgCl_2$, 3.6 mM dithiothreitol, 9% dimethyl sulfoxide, and 12 μM T4 RNA ligase, obtained from R. Gumport, University of Illinois, Urbana, IL, and isolated as described by McCoy et al. (1979) after one pass through an Affi-Gel Blue column. The reaction mixture was incubated for 16 h at 4 °C. Intact ^{32}P-labeled mRNA was recovered by centrifugation in a 5–20% sucrose gradient in 0.1 M NaCl, 0.5% sodium dodecyl sulfate, 0.001 M EDTA, and 0.01 M Tris (pH 7.4) at 50000 rpm in a Beckman SW50.1 rotor for 4 h at 20 °C. RNA migrated as a single major peak of radioactivity.

Cell-Free Protein Synthesis. Reticulocyte lysate was prepared as described by McDowell et al. (1972) and treated with micrococcal nuclease as described by Pelham & Jackson (1976). Reaction conditions were described previously (Dorner & Kemper, 1978), except that 30 μM amino acids and 20 μg/mL *Escherichia coli* RNA were added to the reaction mixtures. For mRNA binding studies, incubation was for 30 min at 23 °C.

Dog pancreatic membranes were isolated according to the methods of Blobel & Dobberstein (1975), with modifications previously described (Dorner & Kemper, 1978).

Analysis of Initiation Complex Formation. Formation of initiation complexes was analyzed under standard conditions for protein synthesis in wheat germ cell-free systems (Dorner & Kemper, 1978). The reactions were preincubated for 20 min at 23 °C before addition of mRNA and 1 mM anisomycin. After an incubation of 15 min at 23 °C, the reaction was stopped by chilling on ice, 50 μL of 0.01 M Tris–acetate (pH 7.5), 0.1 M KCl, and 0.005 M $MgCl_2$ was added, and the sample was layered on a 15–30% sucrose gradient in a 0.8-mL centrifuge tube for the Beckman SW50.1 rotor. The sample was centrifuged for 60 min at 50000 rpm, 2-drop fractions were collected, and radioactivity was assayed by scintillation counting in Aquasol (Beckman) containing 10% H_2O. In control experiments, 2 mM 7-methylguanosine 5′-phosphate was added instead of anisomycin to prevent formation of initiation complexes.

Analysis of mRNA–Membrane Complexes. Membranes were separated from the reaction mixture by a modification of procedures described by Mechler & Vassalli (1975a). Twenty microliters of a reticulocyte lysate reaction mixture was adjusted to 2.1 M sucrose by the addition of 110 μL of 2.5 M sucrose. In a 0.8-mL centrifuge tube, the sample was layered over 100 μL of 2.5 M sucrose, and 250 μL of 2.05 M sucrose and 170 μL of 1.3 M sucrose were layered over the sample. All sucrose solutions contained 50 mM triethanolamine (pH 7.4, 20 °C), 50 mM KCl, and 5 mM $MgCl_2$. The sample was centrifuged at 31000 rpm in a Beckman SW50.1 rotor for 4.5 h at 4 °C. Membranes floated to the interface between the 2.05 M sucrose and 1.3 M sucrose. Two-drop fractions were collected, and radioactivity was assayed by scintillation counting in 3 mL of Aquasol (New England Nuclear) containing 10% water.

RNA was analyzed by electrophoresis on 3% acrylamide gels, as described previously (Stolarsky & Kemper, 1978). RNA associated with membranes was prepared for electrophoresis by addition of 2 volumes of ethanol to precipitate the RNA. The pellet was dissolved in 0.01 M Tris (pH 7.4), 0.1 M NaCl, 0.001 M EDTA, and 0.5% sodium dodecyl sulfate and extracted with phenol–chloroform–isoamyl alcohol.

Treatment of Membrane-Bound mRNA with High Concentrations of KCl and with Proteolytic Enzymes. In experiments to determine the effect of high ionic strength on the binding of the mRNA to the membranes, cell-free reactions were stopped by cooling on ice. Fifteen-microliter aliquots of the reaction mixture were adjusted to 90, 290, and 540 mM KCl by the addition of either no KCl, 4 μL of 1 M KCl, or 3 μL of 3 M KCl, respectively, and to a final volume of 20 μL with water. The reactions were then incubated at 0 °C for 60 min before analysis of mRNA binding to membranes by sucrose gradient centrifugation.

In other experiments, reaction mixtures were treated with chymotrypsin and trypsin before incubation in high or low concentrations of KCl. In these experiments, 35-μL aliquots were adjusted to a final volume of 40 μL, containing 1.0 μg/mL each of trypsin and chymotrypsin. After incubation at 0 °C for 40 min, aliquots of the sample were adjusted to 78 or 528 mM KCl and incubated for an additional 50 min at 0 °C.

Analysis of Polysomes. For analysis of the incorporation of PTH mRNA into polysomes, 20 μL of a reaction mixture was diluted with 100 μL of 0.01 M Tris (pH 7.2), 0.01 M $MgCl_2$, and 0.1 M KCl and 7 μL of 10% Nonidet P40. The samples were layered on 10–30% sucrose gradients and centrifuged at 4 °C for 80 min at 40000 rpm in a Beckman SW41 rotor. Radioactivity in ~1-mL fractions was assayed by scintillation counting in Aquasol (Beckman).

Results

Characterization of ^{32}P-Labeled PTH mRNA. PTH and mRNA was a poor acceptor of [^{32}P]pCp compared to other RNA molecules studied by England et al. (1979). Under our conditions ~10% of the 3′ termini theoretically available actually accepted a pCp residue. About 3×10^5 cpm of radioative mRNA was isolated after sucrose gradient centrifugation per μg of RNA added to the ligase reaction.

The RNA preparation used in these experiments had been estimated previously by gel electrophoretic (Stolarsky & Kemper, 1978) and excess RNA hybridization techniques to be ~50% pure (D. Gordon and B. Kemper, unpublished experiments). An analysis of the ^{32}P-labeled PTH mRNA by electrophoresis on 3% acrylamide gels containing 98% formamide revealed that 68% of the total radioactivity on the gel migrated as a single peak with a mobility expected for PTH mRNA (Figure 1). The higher percentage of radioactivity than expected in PTH mRNA might be explained if phosphorylated 3′ termini, which are nonaccepting termini for RNA ligase, are present on degraded RNA molecules in the sample. Since the preparation of RNA used was only 50% pure and ~10% of the RNA molecules accepted a radioactive pCp residue, it is possible that PTH mRNA was not radioactively labeled. However, the radioactive profile of the [^{32}P]-pCp-labeled RNA after gel electrophoresis was similar to the profile of ^{125}I-labeled RNA (Stolarsky & Kemper, 1978) with one major peak, suggesting that the major species present,

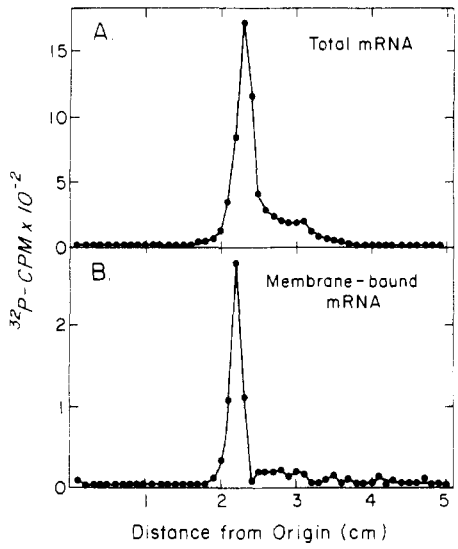

FIGURE 1: Analysis of [^{32}P]pCp-labeled PTH mRNA by acrylamide gel electrophoresis. RNA was analyzed on 3% acrylamide gels containing 98% formamide as described under Experimental Procedures. Total mRNA is the initial starting PTH mRNA. Membrane-bound mRNA is RNA recovered from the membrane region of sucrose gradients and reisolated by extraction with phenol–chloroform–isoamyl alcohol.

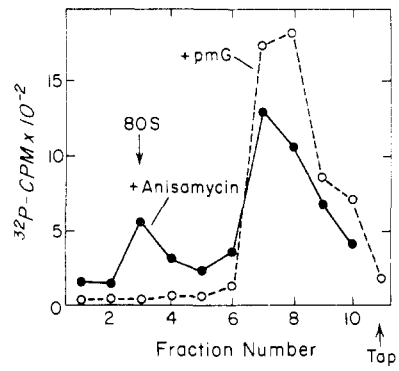

FIGURE 2: Formation of an initiation complex by [^{32}P]pCp-labeled PTH mRNA. Radioactive PTH mRNA was incubated in wheat germ cell-free protein-synthesizing systems in the presence of anisomycin or 7-methylguanosine 5′-phosphate (pmG). The approximate positions of 80S ribosomes were determined on a separate gradient.

PTH mRNA, was labeled. We thus refer to the radioactive mRNA as PTH mRNA in this paper. Since radioactivity was incorporated into PTH mRNA only at the 3′ terminus, we analyzed the nature of the radioactive RNA that could be recovered from a membrane fraction after incubation in a reticulocyte cell-free system. As shown in Figure 1, the radioactive profile of the recovered RNA was very similar to the initial input RNA with 70% of the radioactivity on the gel present in a single peak. The binding of the radioactivity to the membrane was, therefore, not due to the binding of 3′-terminal fragments of the mRNA to the membranes.

For assessment of the translational activity of the ^{32}P-labeled mRNA, the accumulation of radioactivity into initiation complexes was studied in a wheat germ cell-free system in the presence of an inhibitor of elongation, anisomycin. Approximately 25% of the total radioactivity was present in a peak which sedimented at around 80 S (Figure 2). This peak was absent in reactions containing an inhibitor of initiation, 7-methylguanosine 5′-phosphate. This percentage of apparently active mRNA is about the same as that found in polysomes and that bound to membranes in the reticulocyte cell-free system.

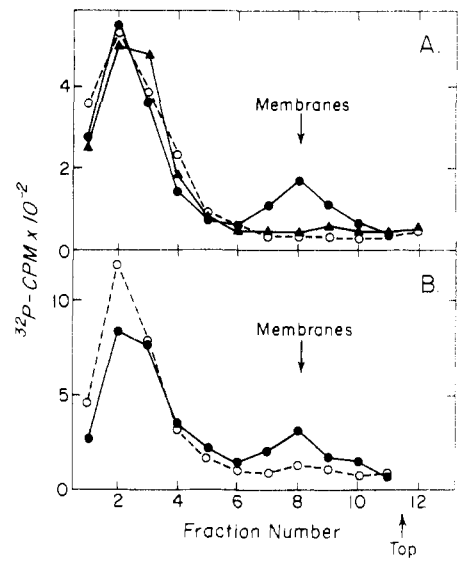

FIGURE 3: Binding of PTH mRNA to microsomal membranes. ^{32}P-Labeled PTH mRNA was incubated in a reticulocyte lysate cell-free system supplemented with dog pancreatic microsomal membranes. The reaction mixture was adjusted to 2.05 M sucrose and layered near the bottom of a discontinuous gradient as described under Experimental Procedures. After centrifugation, the membranes and associated radioactive mRNA floated to the upper portion of the gradient as indicated. Panel A: (●) complete reaction; (○) reaction mixture treated with 0.5% deoxycholate before layering on the gradient; (▲) microsomal membranes added after the termination of the cell-free incubation. Panel B: (●) complete reaction; (○) 0.5 mM puromycin present during the cell-free reaction.

Characterization of the Binding of PTH mRNA to Microsomal Membranes. Binding of radioactive mRNA to microsomal membranes in the reticulocyte cell-free system was assayed by discontinuous sucrose gradient centrifugation, as shown in Figure 3. After an incubation of 25 min at 23 °C, ~25% of the radioactivity floated with the membranes to the upper portion of the gradient. As shown in Figure 3A, if the membranes were not present during the reaction but were added just before the sucrose gradient analysis or if the structure of the membranes was destroyed by treatment with 0.5% deoxycholate before centrifugation, no peak of radioactivity was present in the region of the membranes and only about 6 to 7% of the radioactivity was present in this region of the gradient. If protein synthesis was inhibited by the addition of puromycin from the beginning of the reaction at a concentration of 0.5 mM, the peak of radioactivity was essentially eliminated and less than 10% of the radioactivity was present in the membrane region of the gradient. The dependence of binding on (1) the presence of the membranes in the reaction during translation, (2) the structural integrity of the membranes, and (3) protein synthesis indicates that the observed binding of the mRNA to the membranes results from the formation of membrane-bound polysomes.

If the binding of PTH mRNA to the microsomal membranes is the result of membrane-bound polysome formation in vitro, then the mRNA of a protein that is not synthesized on membrane-bound polysomes such as globin mRNA should not bind to the membranes under the same conditions. Incorporation of pCp into globin mRNA was 2 to 3 times as efficient as incorporation into PTH mRNA and ~30% of the radioactive globin mRNA entered initiation complexes in the wheat germ cell-free system. In Figure 4, an analysis of the binding of globin mRNA to microsomal membranes is shown. About 11% of the total radioactivity was present in the region of the gradient containing membranes, and no peak of radio-

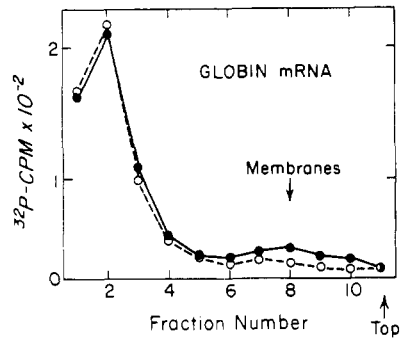

FIGURE 4: Lack of binding of globin mRNA to microsomal membranes. ^{32}P-Labeled globin mRNA was incubated in a reticulocyte cell-free system supplemented with microsomal membranes and analyzed as described in the legend to Figure 3. (●) Complete reaction; (○) microsomal membranes added after termination of the cell-free reaction.

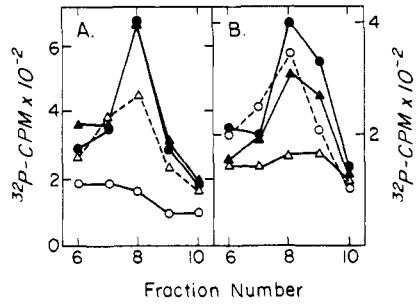

FIGURE 5: Effect of treatment with high concentrations of KCl and limited proteolysis on binding of PTH mRNA to microsomal membranes. ^{32}P-Labeled PTH mRNA was incubated in reticulocyte cell-free systems and analyzed by sucrose gradient centrifugation as described in Figure 3. Only the region of the gradient containing the membranes is shown. Panel A: complete reactions were incubated for an additional 60 min at 0 °C before layering on the sucrose gradient in 90 (●), 290 (▲), or 540 mM KCl (△). As a control, puromycin was added during the cell-free synthesis (○). Panel B: complete reactions were incubated in 78 mM KCl without proteolytic treatment (●) or with proteolytic treatment (○), or complete reactions were incubated in 528 mM KCl without proteolytic treatment (▲) or with proteolytic treatment (△).

activity was observed in this region. Some of the "nonspecific" binding of globin mRNA appeared to be time or temperature dependent since slightly less radioactivity was present in the region of the gradient containing membranes when the membranes were added after the termination of the cell-free incubation.

Effect of High Concentrations of KCl on the Binding of PTH mRNA to Membranes. Membrane-bound ribosomes formed in vivo are resistant to dissociation by high concentrations of KCl (Adelman et al., 1973; Harrison et al., 1974a). In Figure 5A, the effect of incubation of reactions in 90, 290, and 540 mM KCl before analysis by sucrose gradient centrifugation is shown. No decrease in binding was observed when the reactions were treated with 290 mM KCl, and ~20% of the specific binding was released in the presence of 540 mM KCl. In four experiments, the decrease in specific binding after treatment with 540 mM KCl ranged from 20 to 34% with an average release of 26%. The decrease in binding was not the result of disruption of nonspecific binding of mRNA to the membranes since no decrease in radioactivity in the membrane region of gradients was observed for puromycin-treated reactions incubated with 540 mM KCl.

The resistance of the radioactive mRNA–membrane complex to dissociation by high concentrations of KCl suggested that the nascent polypeptide chain was also involved in the binding. In an effort to examine this possibility, we subjected

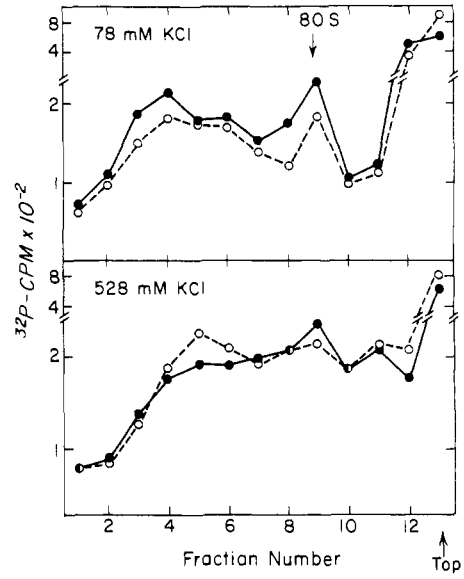

FIGURE 6: Effect of treatment with high concentrations of KCl and limited proteolysis on polysomes containing PTH mRNA. Aliquots of reaction mixtures analyzed in panel B of Figure 5 were treated with 0.5% Nonidet P40, and the polysomes were analyzed by sucrose gradient centrifugation as described under Experimental Procedures. Upper panel: complete reactions were incubated in 78 mM KCl without proteolytic treatment (●) or with proteolytic treatment (○). Lower panel: complete reactions were incubated in 528 mM KCl without proteolytic treatment (●) or with proteolytic treatment (○).

the mRNA–membrane complexes to limited proteolysis. Sabatini & Blobel (1970) reported that a treatment of rough microsomes with 50–100 μg/mL trypsin and chymotrypsin at 0 °C for 5–24 h did not disrupt polysomal complexes and that the membranes remain as vesicles. Under these conditions, however, ribosomes were released from membranes. In our experiments, more limited proteolytic treatment with 1 μg/mL trypsin and chymotrypsin for 40 min at 0 °C did not result in the release of PTH mRNA from the membranes in 78 mM KCl (Figure 5B). After proteolysis and treatment of the reaction with 528 mM KCl, the peak of radioactivity associated with the membranes is eliminated. In other experiments, soybean trypsin inhibitor was added after proteolysis treatment with trypsin alone to minimize additional proteolysis during the following incubation with 78 or 528 mM KCl and results similar to those shown in Figure 5B were obtained.

It is possible that the observed release of mRNA from the membranes after treatment with proteolytic enzymes and 528 mM KCl was the result of release of mRNA from the ribosome on the membrane rather than as a consequence of release of the ribosome from the membrane. To examine this possibility, we analyzed the polysome profile of detergent-treated reactions by sucrose gradient centrifugation, as shown in Figure 6. Proteolysis treatment did not affect the fraction of PTH mRNA sedimenting in the polysomal region of the gradient after incubation of the reactions with either 78 or 528 mM KCl, indicating that the mRNA was not dissociated from the ribosome but that the ribosomes were released from the membrane in 528 mM KCl. These results suggest that the nascent chain is involved in the binding of the ribosomes to the membrane, but the possibility that the release is due to destabilizing effects of proteolysis on the membrane or ribosome cannot be ruled out.

Effect of Puromycin on Binding of PTH mRNA to Membranes. Although puromycin, present from the beginning of the reaction, inhibits protein synthesis and clearly prevents binding of PTH mRNA to the membranes, we wanted to

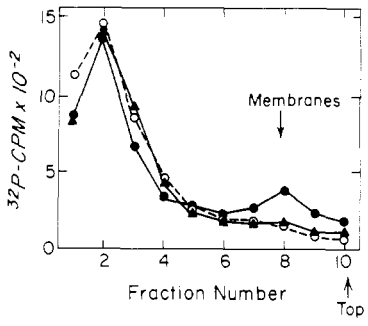

FIGURE 7: Effect of puromycin on preformed mRNA–membrane complexes. ^{32}P-Labeled PTH mRNA was incubated in a reticulocyte cell-free system and analyzed as described in Figure 3. (●) Standard reaction incubated for 25 min; (▲) standard reaction incubated for 25 min and for an additional 5 min in the presence of 0.5 mM puromycin; (○) reaction containing 0.5 mM puromycin during the entire 25-min reaction.

examine the effects of addition of puromycin to the reaction after the mRNA–membrane complex had formed. The decrease of the binding of mRNA to membranes after the addition of puromycin to a reaction in which PTH mRNA–membrane complexes had formed is illustrated in Figure 7. The addition of puromycin for 5 min caused a complete loss of specific binding of mRNA from the membranes, and the radioctive profile of PTH mRNA was the same as that for a reaction that contained puromycin throughout the incubation. Since puromycin causes runoff of the polysome under conditions of protein synthesis (Lawford, 1969), mRNA is presumably released from the ribosome under these conditions. We cannot conclude, therefore, that the ribosome is released from the membrane by puromycin at these relatively low concentrations of KCl. The results suggest, however, that there is no direct interaction between the membrane and the mRNA in the PTH mRNA–membrane complexes since puromycin would not be expected to affect interactions of RNA with membranes.

Discussion

These studies demonstrate the incorporation of radioactive PTH mRNA into membrane-bound polysomes, in vitro. The binding of PTH mRNA to microsomal membranes does not occur if protein synthesis is blocked, and globin mRNA, which is not expected to enter membrane-bound polysomes, binds only slightly to the membranes. The presence of PTH mRNA in the membrane region of the gradients is eliminated if the membranes are disrupted with detergents or if membranes are added after the protein synthetic incubation. The main disadvantages of the assay at present are the relatively low ratio of specific binding to nonspecific binding and the fact that binding of the mRNA to the membranes can be inhibited by blocking interaction of the mRNA with ribosome as well as blocking interaction of the mRNA–ribosome complex with the membrane. The latter problem can be eliminated by demonstrating that the released mRNA is still associated with polysomes. This assay was useful for examining some of the properties of the membrane-bound polysomes formed in vitro and should be useful in examining factors involved in the formation of membrane-bound polysomes.

It is generally accepted that in membrane-bound polysomes the ribosome interacts with the microsomal membranes through KCl-labile electrostatic bonds and through an interaction of the nascent chains that is stable to high concentrations of KCl (Adelman et al., 1973; Harrison et al., 1974a). About 20–30% of the PTH mRNA is released from the membranes by incubation with 540 mM KCl, which is similar to the percentage of ribosomes released from microsomal membranes isolated from cells. Ribosomes released from microsomal membranes by high ionic strength alone have been postulated to represent either free polymers nonspecifically bound to membranes at low ionic strength, inactive ribosomes not synthesizing protein, or ribosomes at the 5′ terminus of the mRNA, which do not have a nascent chain sufficiently long to anchor the ribosome to the membrane and which are released by RNase cleavage of mRNA (Sabatini & Kreibich, 1976). In the present studies specific mRNA–membrane complexes represent only active membrane-bound ribosomes, and the mRNA recovered from the membrane fraction is intact, which indicates that the second and third of the proposed possibilities do not explain the release of 20–30% of the PTH mRNA in vitro. Since ribosomes bind to microsomal membranes in vitro without interaction of the nascent chain being involved (Sabatini & Kreibich, 1976), the released mRNA–ribosomes may represent ribosomes which contain very short nascent chains and which bind by this mechanism. It is also possible that small polysomes are formed in the less efficient in vitro system and that the released mRNA–ribosome complexes represent ribosomes in which the nascent chain is long enough to initiate the binding of the ribosome to the membrane but not to anchor the ribosome to the membrane at high ionic strength (Adelman et al., 1973).

Adelman et al. (1973) and Harrison et al. (1974a) demonstrated the importance of the nascent chain in binding the ribosome to the membrane by the requirement for puromycin treatment to release nearly all the ribosomes from the membrane at high ionic strength. We were unable to do this experiment because puromycin results in runoff of the polysomes and, thus, release of the radioactive mRNA from the ribosome. The evidence that treatment with 540 mM KCl releases only a small percentage of the mRNA from the membranes indicates that the nascent chain is involved in binding of the ribosome to the membrane, as in polysomes formed in vivo. Mild proteolytic treatment causes release of the mRNA from the membrane after treatment with 528 mM KCl but not with 78 mM KCl. Since mRNA remains attached to the membranes at low ionic strength, the proteolytic treatment has presumably not greatly affected the direct interaction of the ribosomal subunit with the membrane in contrast to that observed previously (Sabatini & Blobel, 1970) with more extensive proteolytic treatment which released the ribosomes from the membrane. The release of ribosomes at 528 mM KCl after proteolysis thus probably results from a change in the interaction of the nascent polypeptide chain with the membrane. The release could result from cleavage of the nascent chain by trypsin at the junction of the ribosome and membrane. The alternate possibility that proteolysis alters the membrane to cause release seems less likely since the membrane-associated proteins that anchor to the nascent chain might be expected to be well within the membrane and protected from the proteolytic enzymes. On the other hand, Walter et al. (1979) have demonstrated that limited proteolytic digestion inhibits the activity of the membranes in translocating the nascent polypeptide chain across the membrane. Although the actual mechanism of this proteolytic inhibition has not been determined, it is possible that a membrane protein(s) that interacts with the nascent polypeptide chain is inactivated by proteolytic cleavage. If so, the release of mRNA from the membranes in our experiments might be explained by proteolytic cleavage of the same protein. An analysis of the ability of PTH mRNA to bind to trypsin-treated membranes will be required to distinguish between these two alternatives.

Evidence that mRNA interacts directly with the membrane to stabilize the membrane-bound polysomes has been obtained primarily in studies with cultured cells (Milcarek & Penman, 1974; Lande et al., 1975; Mechler & Vassalli, 1975b). In contrast, in bacteria (Smith et al., 1978), viral-infected cells (Lodish & Froshauer, 1977; Grubman et al., 1977), myeloma cells (Harrison et al., 1974b), and liver (Kruppa & Sabatini, 1977) evidence has been obtained that the mRNA does not bind directly to the membrane. In the present study, addition of puromycin to incubations containing mRNA bound to membranes results in a rapid release of the mRNA from the membrane. This result supports the previous studies suggesting that an interaction of the mRNA with the membrane is not necessary to form functional polysomes. Furthermore, in the earlier studies, the release of mammalian mRNA from microsomal membranes in vivo was attained by treatment either with puromycin in the presence of high ionic strength buffer or with EDTA (Grubman et al., 1977; Harrison et al., 1974b; Kruppa & Sabatini, 1977). Possible direct interactions of the mRNA with the membranes could have been disrupted by the unusual ionic conditions used in these experiments. In our studies, the release of PTH mRNA from membranes by puromycin occurs at low ionic strength and, thus for this system at least, the possibility of such interactions that are sensitive to ionic conditions can be eliminated.

The properties of the heterologous membrane-bound ribosomes formed in vitro are remarkably similar to those of homologous ribosomes. In both cases the mRNA–ribosome complex resists dissociation from the membrane at high ionic strength, and in both the primary stabilizing interactions involve direct binding of the ribosome and nascent chain to the membrane. It had been clearly demonstrated that posttranslational processing and sequestration of secretory proteins could be accomplished by heterologous components (Blobel & Dobberstein, 1975). In the present paper, the probable binding of cow nascent parathyroid polypeptides to receptors in dog pancreatic micorsomal membranes is similar to the binding in homologous membrane-bound polysomes formed in vivo. These experiments thus further illustrate the universality in different tissues and species of the molecular mechanisms involved in the biosynthesis of proteins on rough microsomes.

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