BINDING OF VIRAL GLYCOPROTEIN mRNA TO ENDOPLASMIC RETICULUM MEMBRANES IS DISRUPTED BY PUROMYCIN

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ABSTRACT

Previous studies showed that the glycoprotein (G) of vesicular stomatitis virus is synthesized in association with the endoplasmic reticulum (ER) membrane and that all G mRNA co-fractionates with ER membrane. Here, we show that treatment of infected cells with puromycin results in dissociation of G mRNA, and presumably the associated ribosomes, from the ER membrane. Even if extracts from treated cells are kept at low ionic strength (0.01 M KCl), over 80% of G mRNA is found unattached to membranes. There is no evidence for direct interaction of G mRNA with membranes; rather, the linkage apparently is mediated by the nascent G polypeptide.

All mammalian cells contain polyribosomes which are bound to the endoplasmic reticulum (ER) membrane. Bound polysomes synthesize secretory proteins and certain integral membrane glycoproteins (2, 7, 17, 18, 21); these proteins must interact with the ER membrane, either by being transferred across the membrane or by being imbedded in it.

It is not yet clear what determines the selective translation of specific classes of mRNA in polysomes associated with the ER. Three types of experiment suggest that the specificity for this attachment resides in the amino-terminal sequence of the nascent polypeptide (2, 3, 4, 16). First, the amino-terminal sequence of a number of secretory proteins is very hydrophobic and exhibits considerable sequence homology (6). Second, this amino-terminal "leader" sequence appears to be cleaved from the protein concomitantly with its transfer into the cisternae of the ER (2, 3). Blobel and Dobberstein (2, 3) and Blobel and Sabatini (4) suggest that, subsequent to this initial recognition by the amino-terminus, the binding of a polysome to the membrane is a composite of two interactions—a salt-labile linkage of the 60S ribosome subunit to the membrane and a direct interaction of the nascent polypeptide with a proteinaceous channel in the membrane. Third, analysis of the subcellular localization and translation of Sindbis virus 26S mRNA, an RNA which encodes two membrane glycoproteins and one soluble capsid protein, has suggested that the nascent glycoproteins are of major importance in directing the binding of 26S mRNA to the ER membrane (21). Since the same ribosomes and mRNA synthesize both soluble and membrane proteins, it is unlikely that either of these contributes to the specificity of the recognition by membranes (21).

In contrast, recent work utilizing membrane fractions of HeLa cells or cultured human diploid fibroblasts suggests that there is a direct interaction between the mRNA of bound polysomes and the ER membrane (14, 15). The key evi-

dence supporting this interaction is that labeled mRNA remains associated with membranes after puromycin-induced in vitro disassembly of polysomes and release of the ribosome subunits. Also, the mRNA-membrane interaction was maintained even after initiation of polypeptide synthesis was inhibited by Verrucarin (1).

To probe further into the mRNA-membrane interaction, we have been studying the mRNA which encodes the vesicular stomatitis virus (VSV) glycoprotein (G). The great majority of the G mRNA is bound to the ER membrane (7, 8, 17). Immediately after its synthesis, G is a transmembrane protein; only about 30 amino acid residues are susceptible to proteolytic digestion of ER vesicles[1]. G appears on the cell surface 30 min after its synthesis; during the intervening period it is invariably bound to intracellular membranous elements (10, 11).

In this paper, we show that treatment of intact cells with puromycin results in dissociation of G mRNA, and presumably the associated ribosomes, from the ER membrane. At least in this system, there is no evidence for direct interaction of mRNA with membranes.

MATERIALS AND METHODS

Labeling and Fractionation of Infected Cells

Modifications of earlier procedures (20, 21) were used. One liter of Chinese hamster ovary (CHO) cells in spinner culture at 4×10^5 cells/ml were concentrated by centrifugation, washed twice with 50 ml of PO_4-free medium, and then resuspended in 100 ml of PO_4-free medium. Actinomycin D (final concentration 5 μg/ml) and VSV (multiplicity of infection, 10) were then added, and incubation at 37°C was continued. 1 h after addition of virus, 10 mCi of carrier-free $^{32}PO_4$ (New England Nuclear, Boston, Mass.) was added, and incubation was continued for 3 h.

15 min before harvesting, puromycin (5×10^{-4} M) was added to half of the culture. Cultures were chilled on ice. All subsequent steps were done at 4°C. Cells were recovered by centrifugation, washed once with phosphate-buffered saline, once with buffer A (0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid [Hepes], pH 7.5, 0.01 M KCl, 0.0015 M $Mg[OAc]_2$), and resuspended in buffer A at a concentration of 2.5×10^7 cells/ml. After swelling on ice for 10 min, the cells were disrupted with 40 strokes of a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). Nuclei were removed by centrifugation for 4 min at 4,000 *g*; controls established that <10% of ^{32}P-labeled RNA was in the nuclear pellet and that it had a gel profile similar to that of total cellular VSV RNA. Of the postnuclear supernate, 2.5 ml was layered on a gradient, made up in the ionic composition of buffer A, containing 5 ml of 55% (wt/wt) sucrose and a 30-ml linear 15–40% sucrose gradient. Centrifugation was carried out at 26,000 rpm for 4 h at 4°C in the SW27 rotor of the Beckman ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Fractions of 1.5 ml were collected by pumping from the bottom into tubes containing 0.15 ml of 10% sodium dodecyl sulfate (SDS) and 12 μl of diethylpyrocarbonate (DEPC).

In studies in which the membranes were to be banded to equilibrium, the above-described gradients were collected into tubes without SDS or DEPC. The membrane fractions were homogenized 10 times with a tight-fitting Dounce homogenizer; sufficient 62% sucrose solution was added to yield a final concentration of 59%. Gradients contained 5 ml of 62% sucrose, 9 ml of sample, and 8 ml of 55%, 8 ml of 40%, and 8 ml of 30% sucrose. Centrifugation was carried out for 16 h and 26,000 rpm at 4°C in the Beckman SW27 rotor. Fractions of 1.5 ml were collected into tubes containing SDS and DEPC.

Quantitation of RNA and Phospholipids

The following procedure was devised to quantitate radioactivity in RNA and in phospholipid in the gradient fractions. Aliquots of 0.1 ml were diluted with 0.2 ml of water. To these was added 1.2 ml of a solution of chloroform-methanol (2:1); the suspension was mixed vigorously for 1 min, then subjected to centrifugation for 5 min at 500 *g*. From the top (aqueous) layer, an aliquot of 200 μl was removed; to this was added 100 μg of carrier RNA and 1 ml of a 10% solution of trichloroacetic acid. The RNA precipitate was collected on fiberglass filters, washed, dried, and counted in a scintillation counter.

The lower (organic) phase was removed quantitatively and transferred to a glass scintillation vial. 10 mg of Triton X-100 (as carrier) was added, and the material was dried at 95°C. The material was dissolved in 0.2 ml of $CHCl_3$; 10 ml of Aquasol (New England Nuclear) was added, and the mixture was counted in a liquid scintillation counter.

Isolation of RNA

To the pooled gradient fractions were added 3 vol of water, 0.4 vol of buffer B (1 M NaCl, 0.1 M Tris, 0.01 M EDTA, pH 7.5), and 2 vol each of phenol and chloroform. After vigorous mixing for 2 min, the phases were separated by centrifugation at 7,000 *g* for 10 min. The organic phase was back-extracted with an

[1] Katz, F., and H. F. Lodish. Manuscript in preparation.

equal volume of one-tenth concentrated buffer B. To the pooled aqueous layers were added sodium acetate (pH 4.7) to 0.4 M, and then 2 vol of ethanol. After sitting at −20°C for 1 h, the RNA precipitate was recovered by centrifugation. It was dissolved in 0.4 M sodium acetate (pH 4.7) and reprecipitated with ethanol. Finally, the RNA pellet was dried at room temperature and dissolved in 0.1 ml of water. Recovery of acid-precipitable radioactivity from each of the various gradient fractions was between 55 and 80%, and in different experiments no gradient fractions yielded consistently abnormal recoveries.

Gel Electrophoresis

Except as noted, 5-μl samples of RNA were denatured and subjected to electrophoresis on a 3.75% polyacrylamide slab gel containing 98% formamide as described previously (19). The wet gel was subjected to autoradiography with Kodak No-Screen X-ray film. A Joyce-Loebl microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, Eng.) was used to scan the autoradiographs, using a maximum pen deflection of 1.1 OD units. The height of the peaks was proportional to the radioactivity, provided the OD was below 1.0 OD units.

RESULTS

The distribution of VSV G mRNA in the free and membrane-bounded compartments of infected CHO cells was determined. Cells were labeled with [^{32}P]PO_4 from 1–4 h postinfection. Postnuclear supernates from control cultures and from cells treated with puromycin were prepared in a buffer containing a low concentration of salt (0.01 M KCl). They were then analyzed in a sucrose density gradient of the same ionic composition; the gradient contained a cushion of 55% (wt/wt) sucrose overlaid with a linear 15–40% sucrose (wt/wt) gradient. The centrifugation conditions were chosen to separate membranes, which banded isopycnically at the 40–50% sucrose interface, from free polysomes and ribosomes which sedimented in the top half of the gradient. Analysis of ^{32}P-radioactivity in each gradient fraction which was extractable into a solution of chloroform:methanol (2:1) provided a measure of the distribution of phospholipids; as expected, the great majority of the labeled phospholipids were found at the 40–55% interface, coincident with the visible band of membranes (Fig. 1, panel II).

In the control cells, ~35% of the acid-precipitable RNA co-sedimented with the membrane fraction (Fig. 1, panel I). Polyacrylamide gel electrophoresis resolves VSV mRNA into four

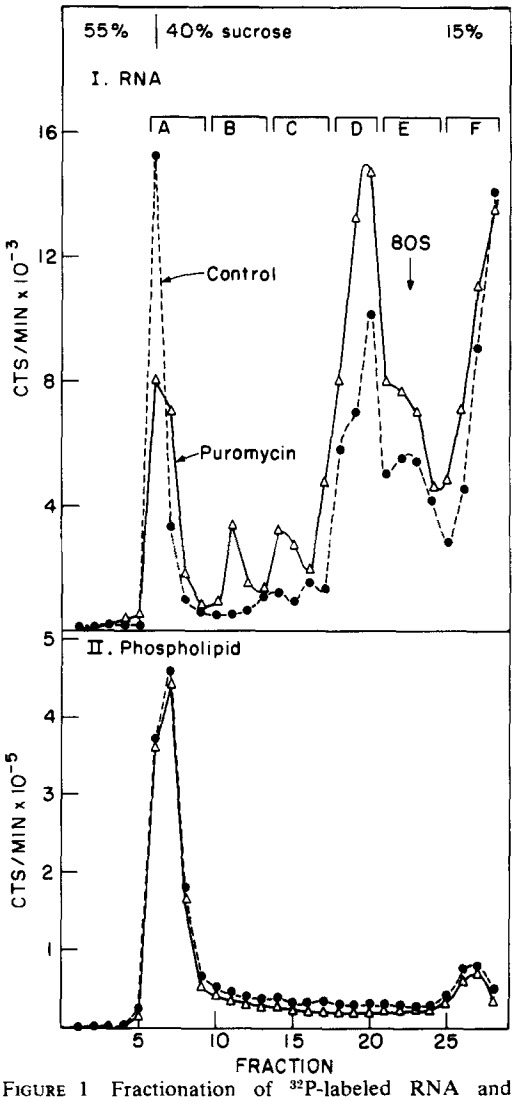

FIGURE 1 Fractionation of ^{32}P-labeled RNA and phospholipid in VSV-infected cells. A postnuclear supernate prepared from ^{32}P-infected cells treated (triangles) or not (circles) with puromycin was fractionated on a sucrose gradient as detailed in Materials and Methods. Aliquots of each fraction were extracted with a mixture of chloroform and methanol. Shown in the top panel (I) is the distribution of radioactivity which was not extracted into the organic phase but which was precipitable with trichloroacetic acid. The bottom panel (II) shows the distribution of radioactivity which was extracted into the organic solvent. Sedimentation is from right to left. Brackets indicate fractions which were pooled for isolation of RNA. CTS, counts.

classes (c.f. Fig. 2). Band 2 (mol wt = 7.4×10^5) is the mRNA for VSV G protein. Band 1 is a mixture of 42S virion RNA and 26S mRNA for L

protein. Band 3 (mol wt=5.5 × 10^5) encodes the N protein, and band 4 (mol wt=2.8 × 10^5) is a mixture of mRNAs for the M and NS proteins (12, 19). Infection of cells with VSV in the presence of actinomycin D results in complete inhibition of synthesis of ribosomal and messenger RNAs. However, generally 20–25% of ^{32}P-radioactivity in RNA is incorporated into 4S RNA, probably representing terminal addition into transfer RNA; this material has migrated off the gels depicted in Figs. 2–4. Thus, not all of the acid-precipitable radioactivity in Fig. 1 is VSV-specific. Fig. 2 does show that over 90% of the G mRNA (band 2) is associated with the membrane fraction. Over 80% of the ^{32}P-radioactivity in bands 3 and 4, by contrast, sediments with free polyribosomes, in agreement with our earlier results (17). Note that while fraction F at the top of the initial gradient (Fig. 1) contains 14% of the acid-precipitable RNA radioactivity, none co-migrates with authentic VSV mRNA (Figs. 2 F and 4 F). Analysis of this fraction by sedimentation through a sucrose gradient established that all of the radioactivity is in transfer RNA (data not shown).

To demonstrate the tight interaction of band 2 RNA with membranes, samples of fractions 6, 7, and 8 from a gradient similar to that of Fig. 1 were made 59% (wt/wt) in sucrose. They were overlaid with a discontinuous gradient of 55, 40, and 30% sucrose, and centrifuged to equilibrium. Floating to the 40–55% interface were 90% of the phospholipids and 65% of the ^{32}P-VSV RNA. Fig. 3 shows that the VSV-specific RNA in these purified membranes migrated as band 2 (G mRNA) and band 1; possibly, band 1 RNA radioactivity is encapsidated 42S virion RNA.

In control cells, G mRNA is greatly enriched in the membrane fraction. Treatment of cells with puromycin 15 min before harvesting results in selective release of G mRNA from membranes (Figs. 1 and 4). Only 20% of G mRNA remains associated with membranes; 80% sediments as free polysomes (fractions B–D) or ribosomes (panel E). In both puromycin-treated and control cells, ~20% of the VSV mRNA bands 3 and 4 – encoding the soluble proteins N, NS, and M – co-sediments with the membrane fraction. Presumably, this is due to nonspecific adsorption since

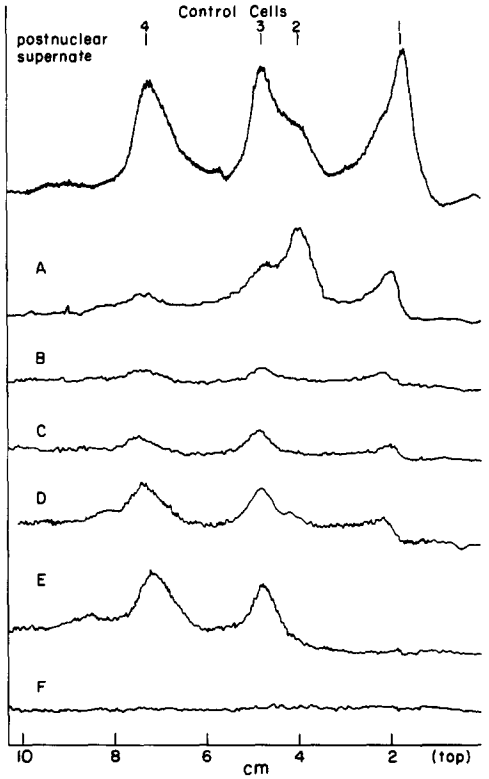

FIGURE 2 Polyacrylamide gel analysis of ^{32}P-labeled RNA isolated from subcellular fractions from control VSV-infected cells. RNA was extracted from the postnuclear supernate or from pooled gradient fractions of the experiment depicted in Fig. 1, and $^1/_{20}$ of each preparation was analyzed. Shown are scans of the autoradiographs of the polyacrylamide gel which were exposed for 15 h.

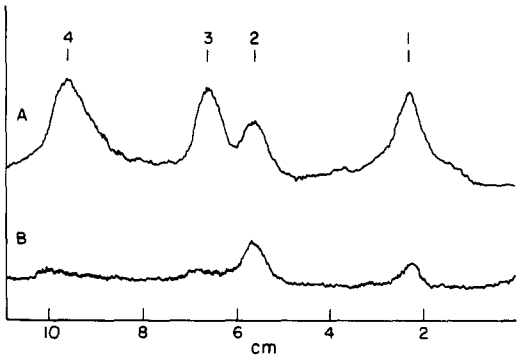

FIGURE 3 Polyacrylamide gel analysis of ^{32}P-labeled RNA isolated from membranes of VSV-infected cells which were purified by equilibrium gradient centrifugation. Panel A is a gel analysis of total labeled RNA isolated from the postnuclear supernate; Panel B is that of RNA isolated from membranes which were centrifuged to equilibrium as described in Materials and Methods. The gel was exposed for 15 h.

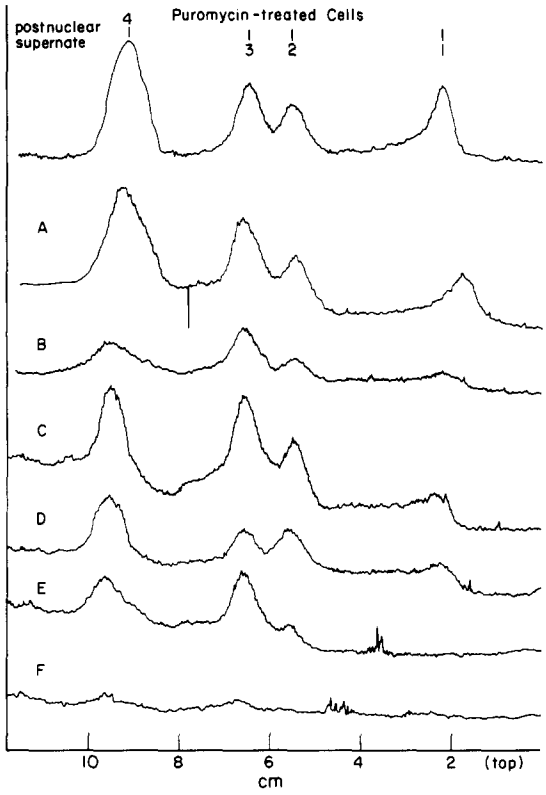

FIGURE 4 Polyacrylamide gel analysis of ^{32}P-labeled RNA isolated from subcellular fractions from puromycin-treated VSV-infected cells. RNA was extracted from the postnuclear supernate or from pooled gradient fractions of the experiment depicted in Fig. 1. As detailed in Materials and Methods, $^1/_{20}$ of each preparation of RNA was analyzed, except for fraction *A*, for which $^1/_{10}$ of the preparation was used. Hence, to compare *A* with the other fractions, areas under the peaks in *A* should be divided by two. This experiment was done in parallel with that of Fig. 2, but the gels of samples *A*-*F* were exposed for 40 h, $2^1/_2$ times longer than in Fig. 2.

mRNAs for bands 3 and 4 are selectively lost during equilibrium centrifugation of the membranes (Fig. 3). The 20% of the G mRNA remaining in the membrane fraction of puromycin-treated cells might also be due to nonspecific adsorption.

Release of G mRNA from membranes was not due merely to the inhibition of protein synthesis since treatment of cells with 1 mg/ml of emetine, an inhibitor of polypeptide chain elongation, before fractionation, resulted in the same amount of all VSV mRNA species bound to membranes as in control cultures (data not shown).

DISCUSSION

The principal conclusion from this study is that attachment of ribosome-G mRNA complexes to membranes is mediated primarily by the nascent G chain. It has been known for some time that G mRNA is attached to the ER membrane, suggesting a close coupling of G biosynthesis to insertion into the membrane (8, 17). The finding that puromycin causes release of G mRNA now establishes this central point.

Puromycin, a drug which causes premature termination of the growing peptide chain, releases G mRNA from the membrane fraction, presumably the result of dissociation of ribosomes from the ER membrane. It is of interest that this puromycin-induced dissociation does not require solutions of high ionic strength (Figs. 2 and 4), nor does a high-salt buffer (0.5 M KCl, 0.005 M Magnesium acetate, 0.05 M Hepes, pH 7.5) cause removal of G mRNA from membranes of infected cells which had not been inhibited with puromycin (data not shown). Treatment of extracts from puromycin-treated cells with this high-salt buffer had no effect on the distribution of RNA radioactivity in the initial gradient analysis, nor did it affect the resultant gel profiles of RNA; again, ~20% of all VSV mRNAs remain bound to membranes, presumably in a nonspecific manner.

Treatment with puromycin in a buffer of high ionic strength (100–500 mM KCl) is required to dissociate from the ER of pancreas, myeloma, and liver, most of the ribosomes and also mRNA active in directing synthesis of secretory proteins (4, 5, 9, 13). A similar result was obtained with the Sindbis 26S mRNA, which directs the synthesis of two virus glycoproteins and one soluble protein: removal of ribosomes and of 26S mRNA from ER membranes required treatment of cells with puromycin and also addition of high-salt buffer to the isolated membranes (21). Presumably, ionic linkages between the ribosomes and/or nascent chain and the ER membrane, supposedly those disrupted by the high-salt solutions, are of less importance for binding of VSV G mRNA than for binding of Sindbis 26S RNA or mRNAs encoding secretory proteins.

All of the studies of identified polypeptides mentioned above demonstrate a direct interaction between the nascent chain and the membrane and imply the absence of a direct mRNA-membrane interaction. How can one reconcile these findings with work in cultured mammalian

cells which suggests a direct linkage of mRNA to ER membranes (1, 14, 15)? It is not clear whether in these studies all of the labeled RNA bound to membranes is indeed active messenger, nor is it known what proteins might be encoded by these species. It is also likely that treatment of isolated membranes with puromycin might be incomplete. If a membrane-bound mRNA is being translated by, say, 10 ribosomes, dissociation of all of these ribosomes with puromycin would be necessary to remove completely mRNA from the membranes. Since in these studies 10% or more of the ribosomes remain bound to membranes after puromycin treatment (14, 15), this might be sufficient to retain most of the mRNAs. Indeed, in our hands, release of labeled VSV G mRNA or Sindbis 26S mRNA from membranes by treatment, in vitro, with puromycin (with or without high-salt) has always been incomplete and frequently nonexistent. Had we not treated the whole cells with puromycin before fractionation, our results would have suggested a direct interaction of mRNA and membranes.

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