

STUDIES ON THE MECHANISM FOR ENTRY OF VESICULAR STOMATITIS VIRUS GLYCOPROTEIN G mRNA INTO MEMBRANE-BOUND POLYRIBOSOME COMPLEXES

MARVIN J. GRUBMAN, JOEL A. WEINSTEIN, and DAVID A. SHAFRITZ

From the Departments of Medicine and Cell Biology and the Liver Research Center, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT

Glycoprotein mRNA (G mRNA) of vesicular stomatitis virus is synthesized in the cytosol fraction of infected HeLa cells. Shortly after synthesis, this mRNA associates with 40S ribosomal subunits and subsequently forms 80S monosomes in the cytosol fraction. The bulk of labeled G mRNA is then found in polysomes associated with the membrane, without first appearing in the subunit or monomer pool of the membrane-bound fraction. Inhibition of the initiation of protein synthesis by pactamycin or muconomycin A blocks entry of newly synthesized G mRNA into membrane-bound polysomes. Under these circumstances, labeled G mRNA accumulates in the cytosol. Inhibition of the elongation of protein synthesis by cycloheximide, however, allows entry of 60% of newly synthesized G mRNA into membrane-bound polysomes. Furthermore, prelabeled G mRNA associated with membrane-bound polysomes is released from the membrane fraction *in vivo* by pactamycin or muconomycin A and *in vitro* by 1 mM puromycin – 0.5 M KCl. This release is not due to nonspecific effects of the drugs. These results demonstrate that association of G mRNA with membrane-bound polysomes is dependent upon polysome formation and initiation of protein synthesis. Therefore, direct association of the 3' end of G mRNA with the membrane does not appear to be the initial event in the formation of membrane-bound polysomes.

In eukaryotic cells, polyribosomes are found either free in the cytoplasm or in association with membranes comprising the rough endoplasmic reticulum (RER) (35). The percentage of polyribosomes in each class is dependent upon the cell type, and cells synthesizing large amounts of secretory proteins contain a high proportion of membrane-bound polysomes (35). Furthermore, it has been demonstrated that proteins secreted from the cell are synthesized primarily on membrane-bound polysomes, whereas protein serving intracellular functions are synthesized primarily on free polysomes (12, 17, 37). Dallner et al. (10,

11) and Lodish and Small (25) have postulated that membrane-bound polysomes also synthesize proteins which became structural units of various membrane fractions.

From many studies, it appears that mRNAs for specific cellular functions are segregated in eukaryotic cells; however, the mechanism for this segregation process has not been fully elucidated (12, 17, 37). Certain investigators have reported a direct association of mRNA with membranes via the 3' poly (A) and/or adjacent regions of the messenger and have suggested that specificity for mRNA-membrane interaction may reside in the

primary sequence of mRNA (8, 24, 26, 29, 30). Blobel and co-workers (3, 5, 6) have obtained evidence for an alternative mechanism in which the specificity for segregation of mRNA in membrane-bound polysomes is conferred by a common N-terminal hydrophobic nascent polypeptide chain for all secretory proteins. This region, termed the "signal sequence", is cleaved before completion of protein synthesis and does not appear in the final secreted product.

In the present study, we have examined interaction of a specific eukaryotic mRNA with membranes, utilizing vesicular stomatitis virus (VSV)-infected HeLa cells. VSV-infected cells contain two size classes of viral specific mRNAs of sedimentation values 26S and 12-18S (19, 33). The 12-18S size class can be subdivided further into 17S, 14.5S and 12S species (15, 40, 41). The 17S viral specific mRNA codes for glycoprotein G (7, 15, 22, 32) and is found on membrane-bound polysomes. The 14.5S and 12S VSV mRNAs code for nucleocapsid (N), membrane matrix (M) and NS proteins and are found predominantly on free polysomes (7, 15, 22, 32). We have attempted to follow the fate of G mRNA from the time of its synthesis to its association with polysomes in the membrane. We have found that glycoprotein mRNA (G mRNA) is synthesized in the cytosol and then rapidly associates with membrane-bound polysomes. Pactamycin and muconomycin A, inhibitors of initiation of protein synthesis, almost totally block association of G mRNA with the membrane fraction. In contrast, cycloheximide, an inhibitor of elongation, allows association of G mRNA with membrane-bound polysomes to a level ~60% that found in control infected cells. Furthermore, puromycin -0.5 M KCl specifically releases labeled VSV mRNA from membranes of infected cells, indicating that the nascent chain serves as an anchor for attachment of polysomes containing VSV mRNA. These results are consistent with an interpretation that events early in protein synthesis, related to formation of the N-terminal peptide, are required before glycoprotein G mRNA enters into a stable complex with membrane structures.

MATERIALS AND METHODS

Suspension cultures of HeLa cells were grown at 37°C to a concentration of $4-8 \times 10^6$ cells/ml in Eagle's medium (MEM) supplemented with 2 mM glutamine and 7% fetal calf serum. Vesicular stomatitis virus (Indiana serotype) was grown as previously described (16). Actinomycin D was a gift of Merck Chemical Div., Merck & Co.

Inc., Rahway, N.J., and muconomycin A was kindly provided by Dr. Susan Horwitz of the Department of Pharmacology, Albert Einstein College of Medicine, New York. Isotopes were obtained from New England Nuclear Corp., Boston, Mass. All glassware, buffers and sucrose solutions were autoclaved.

Infection of HeLa Cells

Log-phase HeLa cells were concentrated to a density of 4×10^6 cells/ml and infected with VSV at a multiplicity of infection (MOI) of 10 PFU/cell. 1 h postinfection, actinomycin D was added to 5 μ g/ml and fetal calf serum was added 1.5 h postinfection. [³H]uridine (30-40 Ci/mmol, 30 μ Ci/ml) was added 2.5 h postinfection or as stated in the appropriate figure legend. Inhibitors of protein synthesis were added as indicated. Labeling of infected cells was stopped by pouring the cells over frozen, crushed Earle's solution.

Preparation of Cytoplasmic Extracts

Cells were harvested by centrifugation, washed with Earle's solution and resuspended in RSB (0.01 M NaCl, 0.01 M Tris-HCl, pH 7.4, and 0.0015 M MgCl₂) containing 100 μ g/ml heparin. The cells were left at 0°C for 10-15 min and then lysed with a glass Dounce homogenizer. Cell lysis was monitored by light microscopy. Nuclei were removed by centrifugation at 1,600 rpm for 3 min and the crude cytoplasmic extract was separated into a cytoplasmic pellet and a postmembrane supernatant fraction by centrifugation at 13,000 rpm for 30 min. The cytoplasmic pellet was resuspended in isotonic buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.0015 M MgCl₂) containing 100 μ g/ml heparin and layered over replicate 35-ml 15-30% (wt/wt) linear sucrose gradients in isotonic buffer and 100 μ g/ml heparin. Centrifugation was carried out at 4°C in a Beckman #SW27 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) at 25,000 rpm for 30 min. The pellet, containing membrane-bound polysomes, was resuspended in isotonic buffer containing the nonionic detergent NP-40 and used directly for separation of polysomes and ribosomal subunits or adjusted to a final sodium dodecyl sulfate (SDS) concentration of 1% for analysis of RNA. The postmembrane supernatant fraction was also separated into polysomes and ribosomal subunits after addition of the NP-40 or was analyzed directly for VSV mRNA components.

Sucrose Gradient Centrifugation of the Membrane Pellet or Postmembrane Supernatant Fraction

Membrane pellets or postmembrane supernatant fractions were layered over replicate 35-ml 7-52% (wt/wt) linear sucrose gradients in isotonic buffer, containing 100 μ g/ml heparin, and were centrifuged in the Beckman #SW27 rotor at 4°C for 16 h at 16,000 rpm. Gradients were collected through an Altex automatic recording

spectrophotometer, model #152 (Altex Scientific Inc., Berkeley, Calif.). Small portions of each fraction were analyzed for radioactive RNA by TCA precipitation and liquid scintillation spectroscopy as described previously (16). In order to determine whether [³H]uridine-labeled RNA in the 40S region was associated with 40S ribosomal subunits or was present as mRNPs, this region of the gradient was rerun on a second 35-ml 15–30% (wt/wt) linear sucrose gradient in isotonic buffer containing 100 μg/ml heparin. Centrifugation in the Beckman #SW27 rotor was carried out at 4°C for 15 h at 25,000 rpm. Gradients were collected as described above, and small portions of each fraction were analyzed for radioactive RNA. Appropriate fractions from each gradient were pooled and precipitated with 2.5 vol of absolute ethanol for subsequent analysis of VSV mRNA components.

Sucrose Gradient Analysis of VSV mRNA

Fractions in ethanol were precipitated by centrifugation at 12,000 rpm for 30 min, resuspended in NETS buffer (0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.002 M EDTA and 0.1% SDS), layered on 11.6-ml 15–30% (wt/wt) sucrose gradients in NETS buffer, and centrifuged in a Beckman #SW41 rotor at 23°C for 17 h at 33,000 rpm. Gradients were monitored as indicated above.

RESULTS

Synthesis and Subcellular Localization of VSV G mRNA

To analyze subcellular sites for synthesis and accumulation of VSV mRNAs, HeLa cells were infected with VSV and at appropriate time points postinfection aliquots of cells were labeled with [³H]uridine for 5, 20, or 120 min. In each case, the time at which the cells were harvested was 4.5 h postinfection. After the various labeling periods, membrane-bound and non-membrane-bound fractions were prepared and the distribution of [³H]uridine-labeled 12–18S viral mRNA species determined (Fig. 1). After a 5-min pulse, labeled 17S mRNA is present in both the nonmembrane and membrane-bound fractions, although the proportion of labeled 17S mRNA in total 12–18S mRNA is higher in the membrane-bound fraction (Fig. 1 A and D). After a 20-min pulse, the relative proportions of labeled 14.5S and 12S VSV mRNAs increase in the nonmembrane fraction (Fig. 1 B), whereas the proportion of labeled 17S mRNA increases in the membrane-bound fraction (Fig. 1 E). The profile for 12–18S VSV mRNAs in the membrane-bound and nonmembrane fractions changes only slightly between 20 min and 120

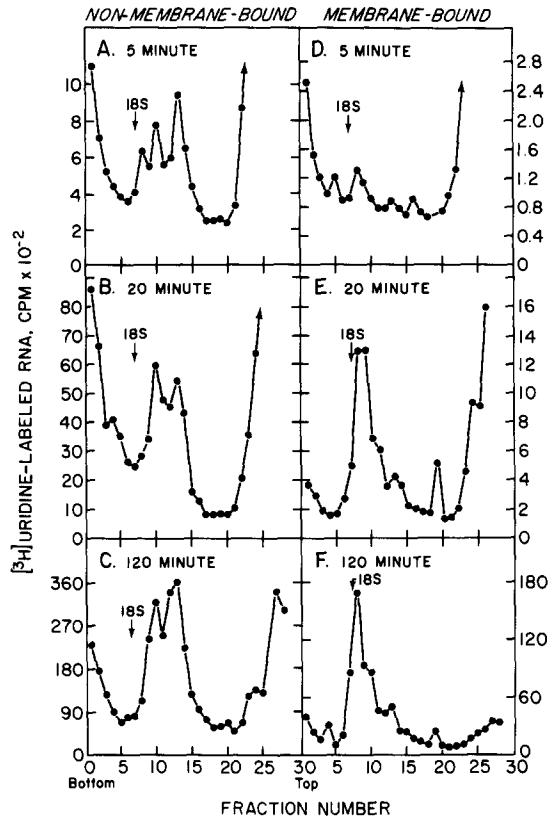


FIGURE 1 Sucrose gradient analysis of 12–18S VSV mRNA in infected HeLa cells after 5, 20 and 120 min of [³H]uridine labeling. HeLa cells were infected with VSV, and, at various times postinfection, 0.75×10^8 infected cells were labeled with 0.75 mCi [³H]uridine (39.3 Ci/mmol). Equivalent numbers of infected cells were labeled with [³H]uridine for 5 min (4 h 25 min postinfection), 20 min (4 h 10 min postinfection) or 120 min (2 h 30 min postinfection). All cells were harvested at 4.5 h postinfection. Labeling was stopped by pouring cells over frozen, crushed Earle's solution, and a crude cytoplasmic extract was prepared. Non-membrane and membrane fractions, prepared as described in Materials and Methods, were adjusted to 1% in SDS and layered over a 11.6-ml linear 15–30% (wt/wt) sucrose gradient in NETS buffer. Centrifugation was carried out in the Beckman SW41 rotor at 33,000 rpm for 17 h at 23°C. Fractions were collected from the bottom of each gradient and were assayed for trichloroacetic acid-precipitable radioactivity as previously reported (16).

min. After a 120-min pulse, 17S mRNA comprises >75% of labeled 12–18S VSV mRNA in the membrane-bound fraction, whereas it represents only a small shoulder in the 17S region in the non-membrane fraction (Fig. 1 C and F). These results demonstrate that all the VSV 12–18S

mRNA species, including 17S G mRNA, are synthesized in the nonmembrane fraction and that G mRNA rapidly associates with the membrane-bound fraction.

Experiments were performed to follow the movement of labeled 17S VSV mRNA from the nonmembrane to the membrane-bound fraction of infected cells after short pulses with [³H]uridine (Fig. 2). In infected cells labeled for 5 min, most of the radioactivity associated with the non-membrane-bound fraction is found in 40S subunits and monosomes, with higher activity in the 40S region (Fig. 2 A). After a 15-min pulse, labeled RNA in 40S subunits and monomers of the nonmembrane fraction is increased and some radioactivity has entered the polysome region (Fig. 2 B). Labeling of polysomes, however, is partially obscured by 120S nucleocapsids, and some larger polysomes in the nonmembrane fraction may be lost during

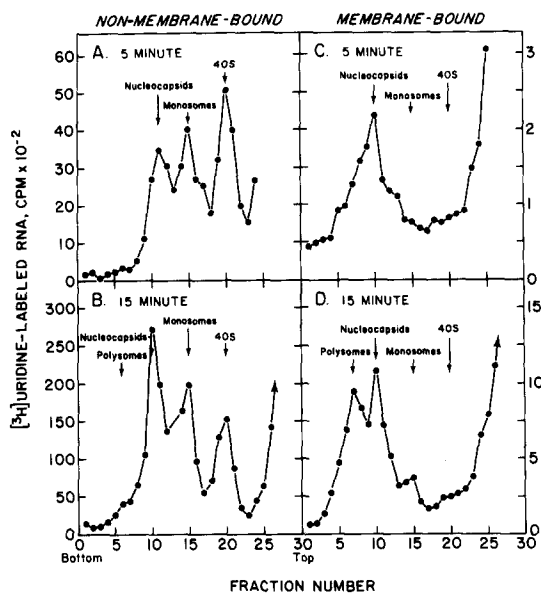


FIGURE 2 Labeling of VSV mRNA in polysomes, ribosomes, and ribosomal subunits in the membrane and non-membrane-bound fractions of infected HeLa cells. 1×10^9 HeLa cells were infected with VSV and at 4.5 h postinfection equal numbers of cells were labeled with 3 mCi of [³H]uridine (37.6 Ci/mmol) for 5 or 15 min. Labeling was stopped by the addition of frozen, crushed Earle's solution. Membrane and nonmembrane fractions were prepared, and polyribosomes were separated and analyzed for [³H]uridine-labeled RNA by centrifugation through a 7–52% (wt/wt) linear sucrose gradient as described in Materials and Methods. Absorbance at 254 nm and the TCA-precipitable radioactivity were determined in each fraction as noted in Fig. 1.

preparation of subcellular components. In the membrane-bound fraction of cells pulsed for 5 min (Fig. 2 C), little radioactivity is found in 40S subunits or monosomes, but some radioactivity is already present in polysomes. Essentially, the same results were obtained after a 2.5-min pulse, although the level of radioactive labeling was lower. After a 15-min pulse, 40S subunits and monomers still show only a low level of labeling; however, membrane-bound polysomes are highly labeled (Fig. 2 D). Various regions of the gradient shown in Fig. 2 A (i.e. polysomes, monosomes, 40S subunits and supernatant mRNPs) were separated and analyzed for 12–18S VSV mRNA species by SDS-sucrose gradient centrifugation. As shown in Fig. 3, labeled 17S mRNA can be identified in material isolated from the 40S subunit and monomer regions but not in material isolated from the mRNP or polysome region. Since the experiments in Figs. 2 and 3 were performed under conditions of continuous labeling, the results indicate that 17S G mRNA associates initially with nonmembrane ribosomes and is then transferred to membrane-bound polysomes without first cycling through membrane-bound subunits or monosomes.

To be certain that labeled 12–18S RNA in this system represents VSV mRNA, a number of experiments were performed. Polysomes were isolated from VSV-infected cells, treated with 5 μ g/ml actinomycin D, and labeled with [³H]uridine for 1 h. RNA was isolated from these polysomes by phenol extraction, and the 12–18S fraction was separated by sucrose gradient centrifugation. 85% of labeled RNA in the 12–18S region adsorbed to oligo (dT)-cellulose at 0.5 M NaCl–10 mM Tris HCl, pH 7.4 and was eluted with 10 mM Tris HCl, pH 7.4 (16). This fraction was hybridized to 42S RNA isolated from intact virus and, as shown in Fig. 4, there was complete protection from T₁ and pancreatic RNase degradation as the level of 42S virion RNA input was increased. These results show that all of the labeled RNA in the 12–18S region represents sequences complementary to 42S virion RNA, and that the bulk of this material is polyadenylated, i.e. represents VSV mRNA.

Effect of Inhibitors of Protein Synthesis on Subcellular Localization of G mRNA

In order to determine whether protein synthesis is necessary for attachment of G mRNA to the membrane-bound fraction, the effect of certain

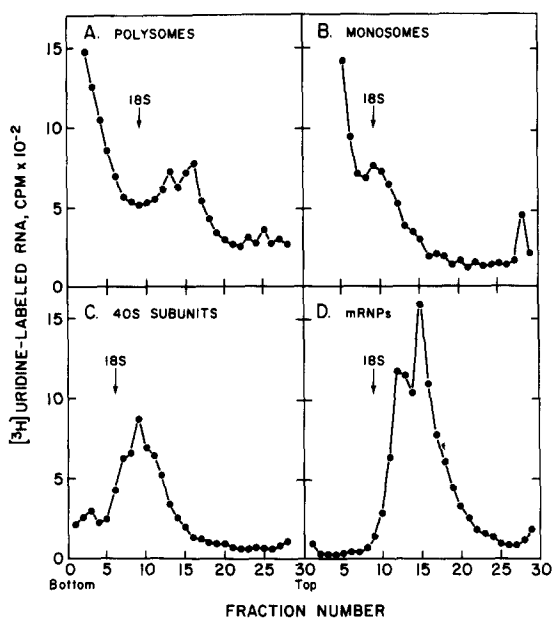


FIGURE 3 Ribosomal localization of [³H]uridine-labeled VSV G mRNA in the non-membrane fraction after a 5-min pulse. 3.3×10^8 HeLa cells were infected with VSV, and at 4 h 45 min postinfection the cells were labeled for 5 min with 3 mCi [³H]uridine (37.6 Ci/mmol). Cells were poured over frozen, crushed Earle's solution, and a nonmembrane fraction was prepared. The nonmembrane fraction was layered over a 35-ml linear 7–52% (wt/wt) sucrose gradient in isotonic buffer containing 100 μ g/ml heparin, and centrifuged in the Beckman SW 27 rotor at 16,000 rpm for 16 h at 4°C. Fractions were collected and aliquots assayed for trichloroacetic acid-precipitable radioactivity. The polysome, monosome and 40S regions of the gradient were pooled separately, and polysomes and monosomes were precipitated by addition of 2 vol of absolute ethanol. To separate 40S-mRNA initiation complexes from supernatant mRNPs, the 40S region from a gradient comparable to that shown in Fig. 2 A or B (fractions 19–22) was pooled and pelleted, resuspended in isotonic buffer containing 100 μ g/ml heparin, and centrifuged through a 35-ml linear 15–30% (wt/wt) sucrose gradient in isotonic buffer containing 100 μ g/ml heparin. Under the conditions of the second gradient, 40S subunits sediment more rapidly than supernatant mRNPs, and these components can be separated clearly (16). Aliquots from each gradient fraction were assayed for trichloroacetic acid-precipitable radioactivity, and appropriate fractions were pooled and precipitated by addition of 2 vol of absolute ethanol. Aliquots of each pooled sample were centrifuged in the Sorvall RC-5 at 10,000 rpm for 30 min at 4°C, resuspended in NETS buffer, layered over an 11.6-ml linear 15–30% (wt/wt) sucrose gradient and centrifuged in the Beckman SW41 rotor at 33,000 rpm for 17 h at 23°C. Fractions were collected and assayed for

protein synthesis inhibitors was studied. At a concentration of 1×10^{-6} M, pactamycin inhibits protein synthesis in VSV-infected cells by ~95% (Fig. 5 A). As reported previously by Perlman and Huang (35), pactamycin at this concentration stimulates viral mRNA synthesis (Fig. 5 B). At 4 h postinfection, VSV-infected cells were preincubated at 37°C for 10 min with 1×10^{-6} M pactamycin, and cells were labeled with [³H]uridine. Portions of drug-treated and control infected cells were removed after various time periods, and membrane-bound and nonmembrane fractions were prepared and analyzed for labeled RNA (Fig. 6). In control cells, the amount of labeled viral RNA increases with time in both the membrane-bound and nonmembrane fractions. However, in drug-treated cells, the rate of increase of viral RNA is reduced in the membrane-bound fraction and is increased in the nonmembrane fraction (Fig. 6 A and B).

To determine whether the relative decrease in membrane-bound viral RNA in pactamycin-treated cells resulted from a block in the association of viral mRNA (specifically G mRNA) with membranes, [³H]uridine-labeled viral RNA from drug-treated and control cells was analyzed by sucrose gradient centrifugation (Fig. 7). Addition of pactamycin reduced the level of all three 12–18S VSV mRNA components in the membrane-bound fraction, the most prominent effect being that on the 17S component (Fig. 7 A). At the same time, the proportion of 17S mRNA in the nonmembrane fraction increased, so that it now represented a defined peak (Fig. 7 B). The levels of [³H]uridine-labeled 17S G mRNA in the membrane-bound fraction of equivalent numbers of cells, in the presence or absence of various protein synthesis inhibitors, are listed in Table I. Pactamycin reduces G mRNA in the membrane-bound fraction to 14% of control and muconomycin A to 7–8% of control. Cycloheximide, at 100 μ g/ml, has a lesser influence on the amount of labeled G mRNA entering the membrane-bound fraction, reducing this value to 57% of control (Table I). At this concentration, cycloheximide inhibited protein synthesis in VSV-infected HeLa cells by 97%.

trichloroacetic acid-precipitable radioactivity. [¹⁴C]uridine-labeled HeLa 18S RNA was used as a marker in a parallel gradient. A: 12–18S VSV mRNA in polysome region. B: 12–18S VSV mRNA in monosome region. C: 12–18S VSV mRNA in 40S subunit region. D: 12–18S VSV mRNA in supernatant mRNP region.

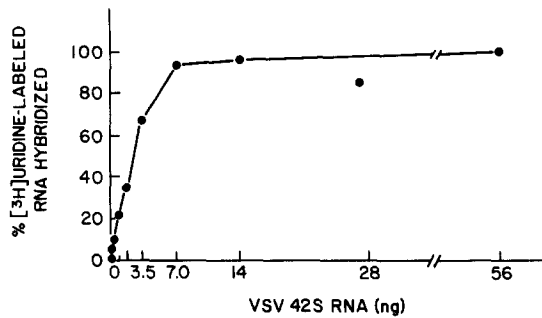


FIGURE 4 Hybridization of [³H]uridine-labeled 12-18S RNA from VSV-infected HeLa cells to VSV 42S virion RNA. HeLa cells were infected with VSV for 2.5 h and labeled with [³H]uridine (30-40 Ci/mmol, 60 μCi/ml) for 4 h as noted in Materials and Methods. [³H]uridine-labeled 12-18S, poly A⁺ RNA was prepared from total cytoplasm by phenol extraction, ethanol precipitation, sucrose gradient centrifugation, and oligo (dT)-cellulose chromatography as described previously (16). Unlabeled VSV 42S RNA was prepared from intact virus by SDS-phenol extraction, ethanol precipitation and sucrose gradient centrifugation to isolate the 42S component. Hybridization assays were performed in a 5-μl volume in sealed capillary tubes containing 200 mM NaPO₄ buffer, pH 6.8-0.5% SDS, 437 cpm 12-18S VSV mRNA, and increasing amounts of VSV 42S RNA. The tubes were heated at 100°C for 5 min and then incubated at 65°C for 15 h. The tubes were placed on ice, broken, and the samples diluted into 1 ml of 300 mM NaCl-30 mM Na citrate. 10 μg of pancreatic RNase plus 4 U of T₁ RNase in 1 ml of 300 mM NaCl-30 mM Na citrate were added and incubation was performed at 37°C for 30 min. The samples were then placed on ice for 10 min, adjusted to 10% TCA, and left on ice for 10 min. The samples were filtered through nitrocellulose filters, washed 3 times with 4 ml of 5% TCA, and counted by liquid scintillation spectroscopy. Blanks of 38 cpm, representing machine background plus cpm resistant to nuclease digestion with labeled RNA samples incubated in the absence of 42S virion RNA, were subtracted from each point. 56 ng of VSV 42S RNA protected [³H]uridine-labeled poly A⁺ RNA from uninfected HeLa cells to only 18 cpm above the blank.

Experiments were then performed to show that G mRNA was accumulating in the nonmembrane fraction of pactamycin-treated cells. The [³H]uridine-labeled 17S component from the nonmembrane fraction of VSV-infected and pactamycin-treated cells was isolated and applied to an oligo (dT)-cellulose column. Approximately 85% of this material was adsorbed to oligo (dT)-cellulose, indicating that it contained a poly (A) sequence. Bound material was eluted from oligo (dT)-cellulose at low ionic strength (16) and ana-

lyzed further by sucrose gradient centrifugation. Fig. 8 shows that this mRNA sedimented at 17S, the normal position for glycoprotein G mRNA. A 17S component was also identified in the nonmembrane fraction of drug-treated cells by sucrose gradient centrifugation in the presence of 85% formamide.

Unlabeled, poly A⁺ RNA from the membrane-bound and non-membrane-bound RNA fraction of control and pactamycin-treated VSV-infected cells was isolated and translated in a wheat germ mRNA-dependent cell-free system. Both et al. (7) have demonstrated previously in this system that 17S VSV mRNA codes for a 63,000-dalton polypeptide which is glycoprotein G protein by tryptic peptide analysis (presumably minus the carbohydrate region, since in vivo synthesized G protein has a mol wt of 67,000 daltons). As shown by the autoradiogram of the slab gel electrophoresis of our cell-free reaction product (Fig. 9, upper portion), a polypeptide of 63,000 daltons is produced when RNA from VSV-infected cells is added to the wheat germ system. The relative proportion of the 63,000-dalton polypeptide (G_{63K}) is highest and represents a major component with control

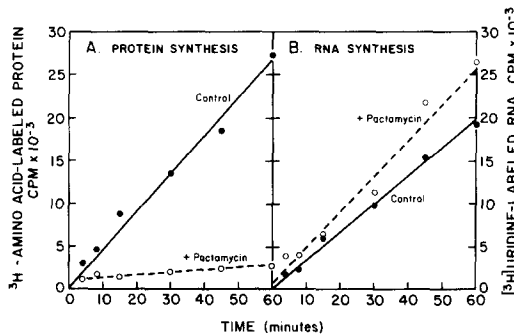


FIGURE 5 Effect of pactamycin on protein synthesis and RNA synthesis in VSV-infected HeLa cells. 1.5×10^6 HeLa cells were infected with VSV, and at 5 h postinfection the cells were divided into 4 equal portions. Two control fractions were labeled with either 150 μCi of [³H]-amino acids or 150 μCi of [³H]uridine. The other two fractions were labeled with 150 μCi of either [³H]-amino acids or [³H]uridine in the presence of 1×10^{-6} M pactamycin. Incubation was carried out at 37°C for 60 min. Duplicate 100-μl aliquots were removed at 4, 8, 15, 30, 45 and 60 min and transferred to 1 ml of cold Earle's solution. Labeled cells were collected by centrifugation and resuspended in 1 ml of distilled H₂O. 1 ml of 10% TCA was added, and the precipitated material was collected on a Whatman GF/C glass fiber filter. Radioactivity was determined as described previously (16). A: Protein synthesis, B: RNA synthesis.

membrane-bound RNA (slot F) and is lowest with control non-membrane-bound RNA (slot D). After addition of pactamycin to VSV-infected

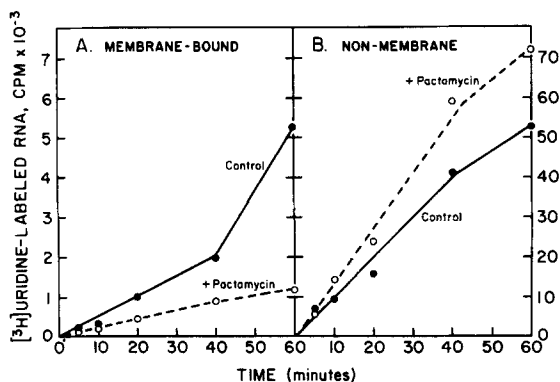


FIGURE 6 Effect of pactamycin on RNA synthesis in non-membrane and membrane fractions of VSV-infected HeLa cells. 8×10^8 HeLa cells were infected with VSV. 1×10^8 cells were removed at 4 h, 4 h 20 min, 4 h 40 min, 4 h 50 min, or 4 h 55 min postinfection. At each time interval, half the cells were treated with 1×10^{-6} M pactamycin for 10 min at 37°C , while the other half served as control. Cells were labeled with $400 \mu\text{Ci}$ of $[^3\text{H}]$ uridine (37 Ci/mmol) per 10^8 cells. In each case, labeling was stopped at 5 h 10 min postinfection by addition of frozen, crushed Earle's solution. Cells were lysed in RSB containing $100 \mu\text{g/ml}$ heparin, and non-membrane and membrane fractions were prepared as described. Aliquots from each fraction were assayed for TCA-precipitable radioactivity. *A*: RNA synthesis in the membrane fraction. *B*: RNA synthesis in the non-membrane fraction.

cells, the proportion of $G_{63\text{K}}$ synthesized in the wheat germ system is decreased under direction of membrane-bound RNA (slot C) and is increased under direction of non-membrane-bound RNA (slot E). Fig. 9 (lower portion) shows the densitometry scans of the cell-free product in the wheat germ system under the direction of VSV-mRNA from control and pactamycin-treated cells. In these tracings, the profile of the cell-free product is normalized to a constant peak height for N protein, the major VSV-directed cell-free product. With RNA from control VSV-infected cells, the relative amount of G synthesized under the direction of membrane-bound RNA (determined as the area under the 63-k dalton region of the tracing in scan f) is approximately 9-fold greater than that obtained with non-membrane-bound RNA (scan d). This is consistent with the distribution profiles for labeled "17S" VSV-mRNA in the membrane-bound versus the non-membrane-bound RNA fraction after a 2-h pulse. The amount of $G_{63\text{K}}$ synthesized under the direction of membrane-bound RNA is reduced approximately 50% after treatment of cells with pactamycin (scan f vs. scan c), whereas synthesis of $G_{63\text{K}}$ under the direction of non-membrane-bound RNA is increased 400% after treatment of cells with pactamycin (scan d vs. scan e). However, the relative amount of $G_{63\text{K}}$ synthesized by the sum of membrane-bound plus non-membrane-bound RNA from control versus pactamycin-treated cells remains relatively constant (scans f plus d vs. scans c plus e). This is

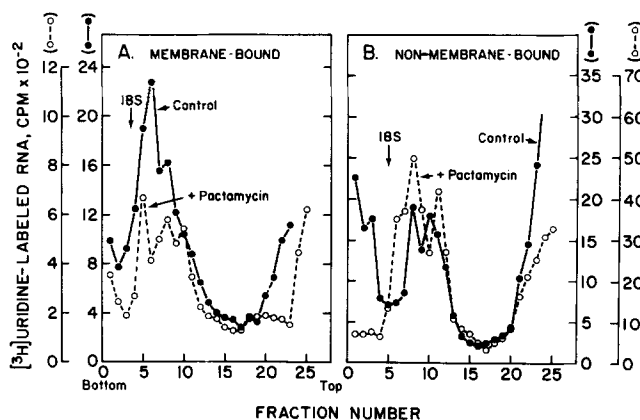


FIGURE 7 Influence of pactamycin on distribution of VSV mRNA in membrane and non-membrane-bound fractions of infected HeLa cells. Membrane and nonmembrane fractions from VSV-infected cells, labeled with $[^3\text{H}]$ uridine for 60 min in the presence or absence of 1×10^{-6} M pactamycin, were resuspended in buffer containing 1% SDS. Samples were layered over a 15–30% (wt/wt) sucrose gradient in NETS buffer and centrifuged in the Beckman SW 41 rotor at 33,000 rpm for 17 h at 23°C . Fractions were assayed for TCA-precipitable radioactivity. *A*: Membrane-bound fraction. *B*: Non-membrane-bound fraction.

TABLE I
³H]Uridine-Labeled G mRNA in Membrane-Bound Fraction after Treatment of VSV-Infected HeLa Cells with Antibiotic Inhibitors of Protein Synthesis

Membrane sample	³ H]Uridine-labeled 17S G mRNA	
	cpm	% of control
Control	84,200	—
+10 ⁻⁶ M pactamycin	11,500	13.6
+10 ⁻⁶ M muconomycin A	6,040	7.2
+100 μg/ml cycloheximide	47,600	57

At 4 h postinfection, equal numbers of VSV-infected HeLa cells were preincubated for 10 min in the presence of an antibiotic and subsequently labeled for 60 min with ³H]uridine. A control sample was labeled for 60 min with ³H]uridine. Membrane-bound fractions were prepared, resuspended in NETS buffer, and layered on 15–30% sucrose gradients in NETS buffer. The samples were centrifuged in the Beckman SW41 rotor at 33,000 rpm for 17 h at 23°C. Fractions were assayed for TCA-insoluble radioactivity, and the amount of 17S G mRNA in each sample was determined by estimating the area under the 17S region in each gradient.

consistent with earlier findings demonstrating a shift in labeled G mRNA from the membrane-bound to the non-membrane-bound fraction after addition of pactamycin to VSV-infected HeLa cells.

In order to show that the decrease of 12–18S mRNA in membrane-bound polysomes of cells incubated with pactamycin was not the result of non-specific release of mRNA, VSV-infected HeLa cells were labeled for 60 min with ³H]uridine and the membrane-bound polysome fraction was prepared. This fraction was resuspended in buffer, incubated at 37°C for 30 min with various agents, and membranes were re-collected through a sucrose cushion by a modification of the method of Cardelli et al. (8). Exposure of the membrane-bound fraction to pactamycin, cycloheximide, or 0.5 M KCl resulted in release of little ³H]uridine-labeled VSV RNA (Table II). However, agents which are known to release mRNA from polysomes, namely, puromycin – 0.5 M KCl and EDTA, reduced ³H]uridine-labeled RNA in the membrane-bound fraction by 85%.

Fate of Prelabeled mRNA in Membrane-Bound Fraction

The results presented in Tables I and II suggested that pactamycin and muconomycin A

blocked entry of G mRNA into the membrane-bound fraction, but that cycloheximide was considerably less effective in this function. We performed experiments to follow the fate of pre-labeled G mRNA already present in the membrane-bound fraction. VSV-infected HeLa cells were labeled with ³H]uridine for 30 min; pactamycin, muconomycin A or cycloheximide was added and incubation continued for an additional 30 min. Membrane fractions were prepared, counted for radioactivity, and 12–18S VSV mRNA was analyzed by sucrose gradient centrifugation. Fig. 10 A shows that total labeled VSV RNA in the membrane-bound fraction is reduced after treatment of cells with pactamycin or muconomycin A but that it continues to increase after treatment with cycloheximide. In the non-membrane-bound fraction (Fig. 10 B), the amount of labeled VSV RNA increases, as expected. Table III demonstrates that 70% and 90% of pre-labeled G mRNA are removed from the membrane-bound fraction after addition of pactamycin and muconomycin A, respectively. Since G mRNA continues to enter the membrane-bound fraction after addition of

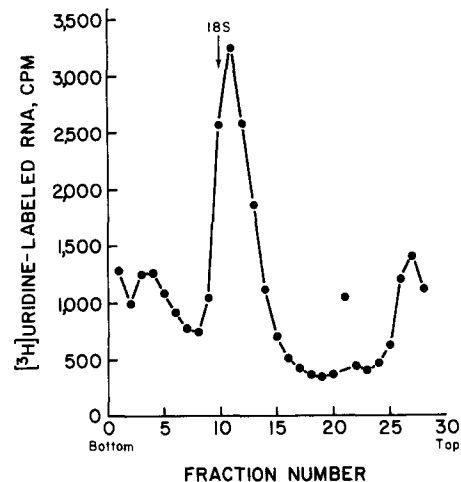


FIGURE 8 Sucrose gradient analysis of G mRNA synthesized in the presence of pactamycin. ³H]uridine-labeled 17S mRNA was isolated from the nonmembrane fraction of pactamycin-treated cells and applied to an oligo (dT)-cellulose column in 0.5 M NaCl, 0.01 M Tris, pH 7.4, 0.5% SDS buffer. Polyadenylated mRNA was eluted from the column with 0.01 M Tris, pH 7.4 and precipitated with absolute ethanol. Labeled RNA was pelleted, resuspended in NETS buffer and centrifuged on a 15–30% sucrose gradient in the Beckman SW 41 rotor as described in the legend to Fig. 7. Fractions were collected and radioactivity was determined as described.

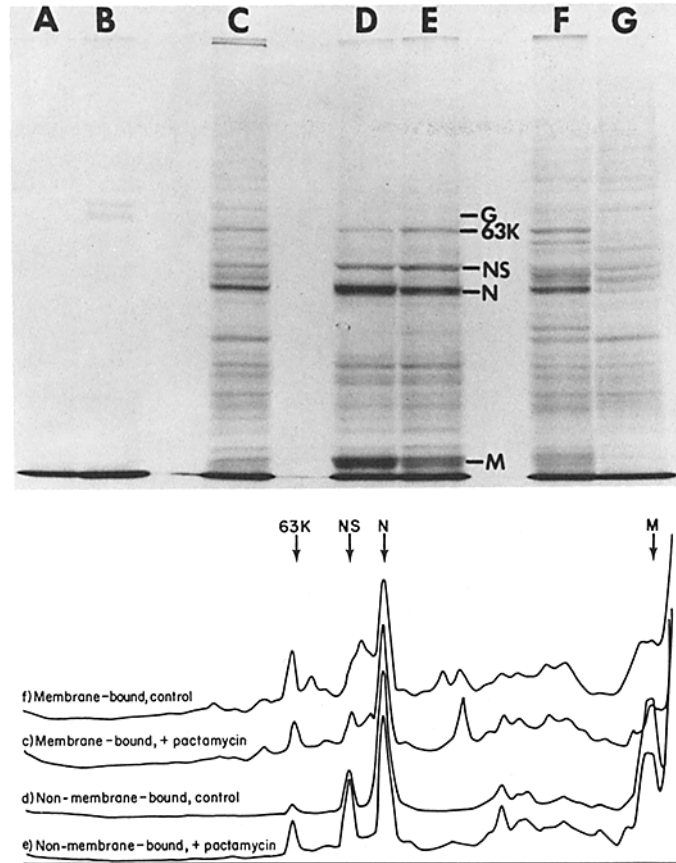


FIGURE 9 Autoradiogram and densitometry scans of SDS slab gel electrophoresis of [³⁵S]methionine-labeled cell-free reaction products synthesized under the direction of membrane-bound and non-membrane-bound RNA fractions isolated from VSV-infected HeLa cells. 2×10^9 HeLa cells, infected with VSV for 2.5 h, were divided into two equal aliquots, and 1×10^{-6} M pactamycin was added to one aliquot. Incubation of cells was continued for 4 h, and RNA was isolated by SDS-phenol extraction and ethanol precipitation of total membrane-bound and non-membrane-bound fractions. Poly A⁺ RNA was prepared from these RNA fractions as described in Fig. 8 and translated in a messenger-dependent wheat germ cell-free system prepared according to the method of Marcu and Dudock (27). Incubations in a total volume of 25 μ l were performed at 23°C for 2.5 h and contained 20 mM HEPES, pH 7.4, 2.5 mM Mg(Ac)₂, 60 mM KAc plus 40 mM KCl, 2 mM dithiothreitol, 1 mM ATP, 20 μ M GTP, 8 mM creatine phosphate, 16 μ g/ml creatine phosphokinase, 40 μ M phenylmethyl sulfonyl fluoride (a protease inhibitor), 8 μ l wheat germ extract, 27 μ M 19 unlabeled L-amino acids minus methionine, 6 μ Ci L-[³⁵S]methionine (sp act 315 Ci/mmol), and various RNA fractions including poly A⁺ RNA from uninfected cells and poly A⁺ RNA from rabbit reticulocytes as controls. Incorporation of [³⁵S]methionine into protein was measured as polypeptide insoluble in 5% TCA after heating to 90°C for 20 min. SDS-slab gel electrophoresis was performed with approximately 200,000 cpm of each cell-free reaction product as previously described, using intact virus for authentic VSV protein markers (16). Upper portion: Autoradiogram of SDS-slab gel electrophoresis. *A*: No RNA added, 72,566 cpm of cell-free product (the maximum we could apply to the gel) was used in this case. *B*: Poly A⁺ rabbit reticulocyte RNA (under the conditions used, α - and β -globin polypeptide chains migrated with dye front). *C*: Poly A⁺ RNA from membrane-bound fraction of pactamycin-treated cells, *D*: Poly A⁺ RNA from control non-membrane-bound fraction. *E*: Poly A⁺ RNA from non-membrane-bound fraction of pactamycin-treated cells. *F*: Poly A⁺ RNA from control membrane-bound fraction. *G*: Poly A⁺ RNA from uninfected HeLa cells. Lower portion: Densitometry scans of slots *C*, *D*, *E* and *F* from the autoradiogram of the SDS-slab gel electrophoresis shown above. In these tracings, the sensitivity of the scanner was adjusted so that the peak height of VSV N protein (the major VSV-directed cell-free product) was 60% of full scale. Since the absolute amount of mRNA added to the system in each experiment is unknown, this normalization was required to compare the amount of glycoprotein G synthesized under the direction of the four separate RNA fractions; (*f*) membrane-bound RNA, control VSV-infected cells; (*c*) membrane-bound RNA, pactamycin-treated, VSV-infected cells; (*d*) non-membrane-bound RNA control VSV-infected cells; (*e*) non-membrane-bound RNA, pactamycin-treated, VSV-infected cells. The position of authentic VSV protein markers NS, N and M and the 63-k dalton band from the autoradiogram are indicated.

TABLE II
*In-vitro Release of [³H]Uridine-Labeled VSV-RNA
 from the Membrane-Bound Fraction of Infected
 HeLa Cells*

Fractions	cpm	% release
A Control	5,338	0
B +0.5 M KCl	4,959	9
C +0.5 M KCl-1 mM puromycin	875	84
D +30 mM EDTA	796	85
E +10 ⁻⁶ M pactamycin	4,580	14
F +100 μg/ml cycloheximide	4,550	15

At 3.5 h postinfection, 1.5×10^8 VSV-infected HeLa cells were labeled for 60 min with 3 mCi [³H]uridine. A membrane-bound fraction was prepared, resuspended in buffer (20 mM Tris HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 100 μg/ml heparin), and divided into six equal portions. Each of these fractions was incubated at 37°C for 30 min under varying conditions: (A) buffer; (B) buffer containing 0.5 M KCl; (C) buffer containing 0.5 M KCl and 1 mM puromycin; (D) buffer containing 30 mM EDTA; (E) buffer containing 10⁻⁶ M pactamycin; and (F) buffer containing 100 μg/ml cycloheximide. After incubation, the various membrane-bound fractions were layered over a 5-ml 30% (wt/wt) sucrose cushion in buffer (samples A and D-F) or in 0.5 M KCl buffer (samples B and C) and centrifuged in the Beckman SW 41 rotor at 30,000 rpm for 30 min at 4°C. The pellets were resuspended in 250 μl of buffer containing heparin. 2 ml of 10% TCA was added, and the precipitated radioactivity was determined as in previous tables and figures.

cycloheximide, similar analysis could not be performed with this inhibitor.

Ribosomal Localization of G mRNA in Drug Treated Cells

We were interested in determining the ribosomal localization of G mRNA in drug-treated cells. VSV-infected HeLa cells were preincubated with either muconomycin A or cycloheximide and subsequently labeled for 60 min with [³H]uridine. Cells were lysed and polysomes resolved on sucrose gradients. Fig. 11 A shows that, in control cells, VSV mRNA is present in 40S subunits, 80S monomers and polysomes of various sizes. In the presence of 1×10^{-6} M muconomycin A (Fig. 11 B), labeled mRNA is present predominantly in monomers, with a very small amount of label in 40S subunits. With cycloheximide, however, residual dimers, trimers and tetramers are present

and are labeled with VSV-mRNA (Fig. 11 C). The ribosomal fraction of VSV-infected cells treated with cycloheximide also contains twice as much [¹⁴C]leucine-labeled nascent polypeptide chain as that obtained from cells treated with muconomycin A (data not shown).

The 40S subunit and 80S monomer regions from the nonmembrane fraction of muconomycin A-treated cells were isolated after a 60-min pulse with [³H]uridine (Fig. 12). Both the 40S subunit and 80S monomer fractions contained all three species of 12-18S mRNA.

DISCUSSION

The results presented in this paper suggest that formation of membrane-bound polysomes in VSV-infected HeLa cells occurs after protein synthesis is initiated and that the nascent polypeptide chain is required to maintain integrity of mRNA-membrane interaction. Our results are based on analysis of a specific viral mRNA, glycoprotein G mRNA. This mRNA is found almost exclusively in the membrane-bound fraction, and its translation product, G protein, is transferred across the plasma membrane to become part of the outer envelope of the mature virus (18). We have followed the fate of newly synthesized G mRNA by labeling VSV-infected cells with [³H]uridine in the presence or absence of inhibitors of protein synthesis. Pulse-labeling experiments indicate that 17S G mRNA is synthesized in the cytosol fraction. This is consistent with the finding that a soluble VSV-transcription complex sediments at 140S in VSV-infected Chinese hamster ovary cells (33). Newly synthesized G mRNA rapidly associates with 40S ribosomal subunits and subsequently with 80S monomers in the nonmembrane fraction. We have not been able to detect G mRNA in polysomes of the non-membrane-bound fraction.

Labeled VSV G mRNA is present in the membrane-bound fraction of infected HeLa cells within 5 min after a pulse with [³H]uridine. However, even after this very short labeling period, radioactive VSV mRNA is found in small polysomes of the membrane-bound fraction, whereas little radioactivity is present in 40S subunits or monomers. Within 20 min after pulse labeling of these cells, G mRNA comprises more than 75% of the 12-18S mRNA in the membrane-bound fraction, and this mRNA is present predominantly in polysomes. The amount of G mRNA in the polysome region of the membrane-bound fraction is also

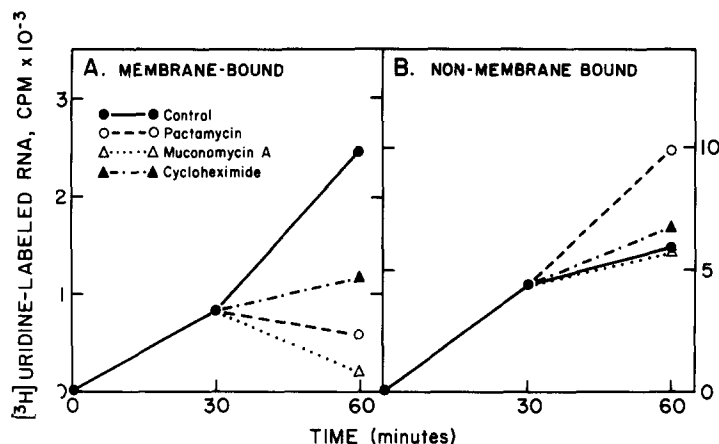


FIGURE 10 Redistributiion of [³H]-VSV RNA in subcellular polysome fractions of HeLa cells after addition of protein synthesis inhibitors. HeLa cells were infected with VSV, and, at 3 h 40 min postinfection, equal portions of cells were labeled with [³H]uridine. After 30 min, one portion was analyzed for [³H]uridine-labeled RNA in the membrane-bound and non-membrane-bound fractions. No drug (control), 1×10^{-6} M pactamycin, 1×10^{-6} M muconomycin A, or 100 μ g/ml cycloheximide was added to the remaining portions of cells and incubation was continued for an additional 30 min. The membrane-bound and non-membrane-bound fractions from these cells were isolated, and [³H]uridine-labeled VSV RNA was determined by trichloroacetic acid precipitation. *A*: Membrane-bound fraction. *B*: Non-membrane-bound fraction.

TABLE III
Release of Prelabeled [³H]-Uridine G mRNA from the Membrane-Bound Fraction after Treatment of HeLa Cells with Antibiotic Inhibitors of Protein Synthesis

Membrane sample	17S G mRNA	
	cpm	% release
Control (30')	37,600	—
Control (30') + 10^{-6} M pactamycin (30')	11,212	70
Control (30') + 10^{-6} M muconomycin A (30')	3,800	90

At 3 h 40 min postinfection, VSV-infected HeLa cells were divided into equal portions, and each was labeled for 30 min with [³H]uridine. The membrane-bound fraction was prepared from one sample, while the other samples were incubated for an additional 30 min in the presence of 1×10^{-6} M pactamycin or 1×10^{-6} M muconomycin A. Membranes were prepared from each of these samples, and 12-18S mRNA was analyzed by sucrose gradient centrifugation. The amount of G mRNA in the membrane-bound fraction of drug-treated cells was compared to the amount of G mRNA present in 30-min labeled, untreated cells. Based on the data in Table I, a correction was made for entry of [³H]uridine-labeled G mRNA into the membrane-bound fraction during the second 30-min period of incubation with pactamycin or muconomycin A.

greater than that found in the 40S subunit or monomer pool after a 5-min or 15-min pulse. Since we have been unable to detect a progression of labeled 17S G mRNA from subunits to polysomes within the membrane-bound fraction, it appears that G mRNA is present in polysomes, or at least in oligosomes, at the time of its association with membranes.

We reasoned that, if association of G mRNA with the membrane-bound fraction requires prior mRNA-ribosome interaction and synthesis of a nascent polypeptide chain, then inhibition of protein synthesis at the appropriate step would prevent formation of an mRNA-ribosome-membrane complex. To examine this question, we used a number of inhibitors of protein synthesis to determine the requirements for entry of VSV G mRNA into the membrane-bound fraction. Pactamycin and muconomycin A (also known as verrucaric acid) are inhibitors of initiation of protein synthesis, the former allowing formation of an mRNA-40S subunit complex (9, 20, 21), and the latter blocking initiation after formation of an mRNA-80S monomer complex (2, 26). The very low level of protein synthesis observed in the presence of these drugs at 1×10^{-6} M (less than 5% of control) probably represents individual cells or polysomes

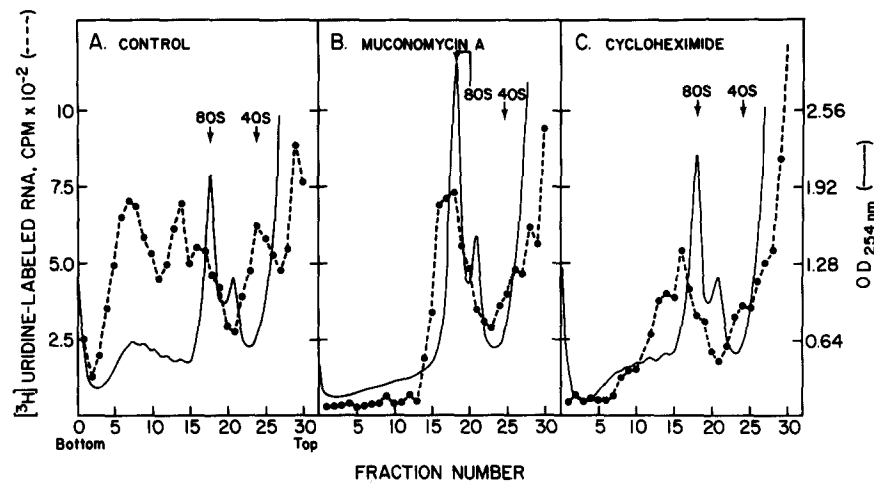


FIGURE 11 Influence of muconomycin A and cycloheximide on polysome size and VSV mRNA distribution. 1×10^8 HeLa cells were infected with VSV, and at 4 h postinfection the cells were divided into 3 equal portions. 1×10^{-6} M muconomycin A or 100 μ g/ml cycloheximide was added to separate portions, and one portion was left as control. After 10 min in the presence or absence of muconomycin A or cycloheximide, 0.8 mCi [3 H]uridine (37 Ci/mmol) was added to control and drug-treated cells and incubation was continued for an additional 60 min. Total polysome fractions were prepared, layered on 7–52% (wt/wt) sucrose gradients, and centrifuged in the SW27 rotor as described. Gradient fractions were monitored for optical density at 254 nm, and TCA-precipitable radioactivity was determined in each fraction. A: Control cells. B: Muconomycin A-treated cells. C: Cycloheximide-treated cells.

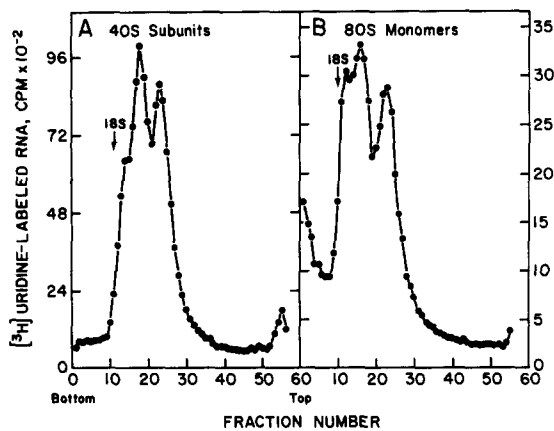


FIGURE 12 Sucrose gradient analysis of VSV 12–18S mRNA species present in 80S monomers and 40S subunits of non-membrane-bound fraction of muconomycin A-treated VSV-infected HeLa cells. The 80S and 40S regions from the non-membrane fraction of muconomycin A-treated VSV-infected cells were isolated and precipitated with ethanol. The samples were resuspended in NETS buffer, layered on a 15–30% sucrose gradient, and centrifuged in the Beckman SW41 rotor as described in the legend to Fig. 7. Fractions were collected and radioactivity was determined.

which escaped inhibition. Utilizing these antibiotics, we find that formation of polysomes is inhibited and that entry of G mRNA into the membrane fraction is suppressed up to 80–90%. Under these circumstances, labeled 17S mRNA accumulates in the non-membrane fraction.

Rosbash (39) has previously studied the influence of cycloheximide on association of mRNA with membranes in uninfected HeLa cells. Cycloheximide inhibits elongation of polypeptide chains, rather than initiation of protein synthesis, and allows formation of small polysomes (9, 13). In Rosbash's studies, there was only a 30–35% reduction of labeled HeLa cell mRNA in the membrane-bound polysome fraction after addition of cycloheximide, although protein synthesis was inhibited by 98%. In addition, labeled HeLa cell mRNA was found predominantly in monosomes and disomes, although some material was present in trisomes (39). Using the VSV-infected HeLa cell system, we have confirmed the observations of Rosbash for a specific mRNA, G mRNA. Thus, we find that cycloheximide reduces the level of [3 H]uridine-labeled G mRNA in the membrane-bound fraction to 57% of control (Table I).

Our results demonstrate further that pactamycin and muconomycin A, which inhibit the initiation of protein synthesis but do not prevent mRNA-40S subunit association (pactamycin) or mRNA-80S monomer formation (muconomycin A), have a significantly greater effect on G mRNA association with membranes than cycloheximide. This is consistent with an interpretation that synthesis of the nascent chain is a prerequisite for association of G mRNA with the membrane fraction. Furthermore, from *in vitro* experiments with membranes prelabeled with VSV mRNA and incubated in the presence of 1 mM puromycin -0.5 M KCl, we find no evidence for direct association of labeled mRNA or mRNA-40S subunits with membranes.

Since pactamycin and muconomycin A inhibited interaction of G mRNA with membranes but did not cause dissociation of preformed mRNA-membrane complexes, we were able to perform "pulse-chase" experiments to follow the fate of G mRNA which had already entered the membrane-bound fraction. If G mRNA were directly associated with membranes, then addition of inhibitors of protein synthesis to preformed complexes should have little effect. However, we found that 70-90% of prelabeled G mRNA is released from the membrane-bound fraction after addition of pactamycin or muconomycin A. This suggested that G mRNA-membrane interaction depends upon a linkage which is sensitive to protein synthesis inhibition. Release of labeled mRNA from the membrane-bound fraction *in vitro* by puromycin -0.5 M KCl is consistent with this interpretation.

The results reported here support a model for segregation of membrane-bound polysomes recently proposed by Blobel and co-workers (3, 5, 6). According to this model, termed the "signal hypothesis", formation of polyribosomes and translation of mRNA for all proteins begins in the cytosol compartment of the cell. Those mRNAs whose translation products are to be transferred across a membrane first synthesize a hydrophobic N-terminal peptide, which serves as the signal for recognition by the membrane. Polysomes containing this hydrophobic peptide or "signal sequence" attach to the membrane, polypeptide synthesis continues, and vectorial transfer of protein across the membrane occurs (3, 5, 6). Therefore, it is the N-terminal sequence of the growing polypeptide chain, rather than the primary sequence of

mRNA, which serves as the signal for recognition by the membrane.

We would like to emphasize that our present results have been obtained with one specific labeled RNA, glycoprotein G mRNA. Studies by other investigators reporting a direct association of poly (A) containing mRNA with membranes are based primarily on *in vitro* treatment of isolated membranes with various agents known to remove ribosomes from membranes (8, 24, 30). Under such conditions, a varying portion of labeled mRNA may remain with membranes, depending upon the cell type, labeling conditions and treatment utilized (8, 24, 30). Certain studies have suggested that mRNA-protein complexes (mRNPs) may be involved in mRNA-membrane interaction (8, 24, 28-30), and mRNP complexes involving the 3'-poly (A) region of mRNA have been described (4, 23, 42). Recently, Adesnik et al. (2) reported a direct association of mRNA with the membrane fraction in human fibroblasts after *in vivo* inhibition of initiation of protein synthesis (2). However, these and other studies (8, 24, 30) have examined total or a mixed population of membrane mRNAs rather than an mRNA for a specific protein. Since membranes may contain mRNAs for secreted proteins, membrane constituent proteins and possibly certain intracellular proteins (10-12, 17, 25, 38), specific mRNAs in different systems may have varying affinities or mechanisms for interaction with ribosomes and membranes, and further studies will be needed to examine this question.

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