SUB-CELLULAR LOCALIZATION OF VESICULAR STOMATITIS VIRUS MESSENGER RNAs

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SUMMARY

Vesicular stomatitis virus (VSV) messenger RNAs (mRNAs) appear to be compartmentalized within the infected HeLa cells. Analysis by polyacrylamide gel electrophoresis in formamide of the RNA associated with the membrane bound polyribosomes from VSV-infected cytoplasmic extracts shows predominantly one size class of VSV mRNA, which is absent from the remaining cytoplasm. These results are consistent with the mRNA for the viral glycoprotein being exclusively associated with membrane bound polysomes since the latter have been shown to synthesize mainly the virion glycoprotein in an in vitro translation system.

INTRODUCTION

Infection of cells with vesicular stomatitis virus (VSV) results in the appearance in the infected cell cytoplasm of two size classes of RNA which are complementary to the viral genome and which serve as messenger RNAs (mRNAs) (1-5). The largest of the mRNA species sediments at 26S in sucrose gradients (1) and probably contains the nucleotide sequence which codes for the largest viral structural polypeptide, the L protein (6), which is required for the virion-associated RNA polymerase activity in vitro (7). The remainder of the mRNA species sediment heterogeneously with sedimentation coefficients ranging from 12 to 18S and presumably contains the sequences coding for the other four viral structural polypeptides (6); G (glycoprotein), M (membrane matrix protein), and N and NS (internal proteins associated with the RNA) (8).

Previous work by Wagner and his coworkers (9,10) has suggested that

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the synthesis of VSV proteins was compartmentalized in the infected cell cytoplasm, and in particular, that the G and M proteins are primarily associated with membrane fractions in the cell. The synthesis of VSV-specific proteins can be achieved in vitro with cell-free extracts prepared from infected cells (11,12). Moreoever, it appears from these results that the synthesis of the G protein, which ultimately appears as a "spike" on the outer surface of the virion, was restricted to the membrane bound class of polysomes (11,13). Sucrose gradient analysis of the virus-specific mRNA associated with the membrane bound polysomes suggested that this fraction was enriched for a class of mRNA sedimenting slightly faster than the total population of 12-18S cytoplasmic mRNA (13). In this paper we extend these observations and demonstrate that membrane bound polysomes of VSV-infected cells contain a unique size class of VSV mRNA, which is absent from the free cytoplasmic polysomes. This result, together with the data from the in vitro translation studies (12,13), is consistent with this size class of RNA being the mRNA for the G protein.

MATERIALS AND METHODS

Cells and virus. Suspension cultures of HeLa S3 cells were grown at a density of 4-8 x 10^5 cells/ml in minimum essential medium supplemented with 2 mM glutamine and 7% calf serum. Growth, purification and plaque assay of the Indiana serotype of VSV have been described (2).

Preparation of radioactive VSV RNA. HeLa cells were concentrated to a density of 4 x 10^6 cells/ml and infected with VSV at a multiplicity of 10 PFU/cell. Actinomycin D (gift of Merck, Sharp and Dohme) was added to 5 µg/ml at 1 hr post-infection, and ^3H uridine (20 Ci/mmol; 30 µCi/ml) (New England Nuclear) was added at 2.5 hr, and the culture was further incubated at 37° for 2 hr. ^{32}P labeling (30 µCi/ml of inorganic ^{32}P-phosphate) of VSV RNA was carried out from 2-6 hr post infection in phosphate-free minimum essential medium supplemented as above. Cells were harvested by centrifugation, washed with isotonic Earle's salts

solutions, and suspended at 3 x 10^7 cells/ml in 0.01 M Tris HCl, pH 7.4, 0.01 M NaCl, 0.0015 M $MgCl_2$ containing 50 µg/ml of heparin. After swelling for 10 min the cells were disrupted by Dounce homogenization, nuclei were removed by centrifugation, and the crude cytoplasmic extract was made 1% in sodium dodecyl sulfate (SDS). Samples were layered over linear 15-30% sucrose gradients in 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.02 M EDTA, 0.2% SDS and centrifuged for 18 hr at 22,000 rpm at 23°. Gradients were collected through a Gilford automatic recording spectrophotometer and small portions of each fraction were analyzed for radioactivity.

Isolation of membrane bound polysomes. The crude cytoplasmic extract was separated into a cytoplasmic pellet and supernatant by centrifugation at 20,000 x g for 30 min in the Sorvall SS-34 rotor. The cytoplasmic pellet was resuspended in the cell homogenization buffer and layered over a 36 ml 15-30% sucrose gradient prepared in the same buffer, and centrifuged at 4° in a Spinco SW 27 rotor at 25,000 rpm for 30 min (14). The pellet (membrane bound polysomes) was suspended in the cell homogenization buffer, treated with 1% SDS, and the RNA was sedimented through sucrose gradients as described above.

RESULTS AND DISCUSSION

HeLa S3 cells in suspension were infected with VSV (Indiana serotype) and the viral-specific RNA was labeled with ^3H uridine in the presence of actinomycin from 2.5-4.5 hr post-infection as described previously (12). Cytoplasmic extracts were prepared, treated with 1% SDS, and sedimented through SDS-sucrose gradients to purify the labeled RNAs as described in "Methods". Fractions with sedimentation coefficients from 12 to 18S were pooled, precipitated with ethanol and then further fractionated in a gradient at a higher speed. Fig. 1 shows that three distinct size classes of RNAs were obtained and designated by I, II and III with sedimentation coefficients of 17S, 14.5S and 12S, respectively. Similarly, three size classes of RNAs were discernable when the ^{32}P-labeled total

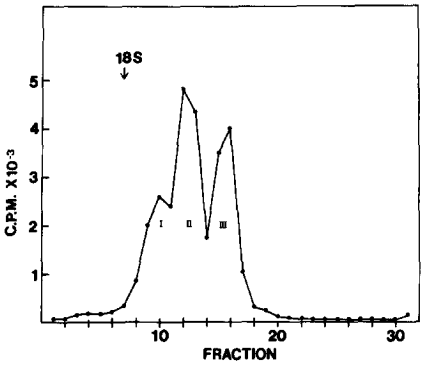

Fig. 1. Analysis of VSV-specific 12-18S mRNAs by velocity sedimentation. ^{3}H-uridine labeled VSV RNA was prepared as described in "Methods" and the 12-18S RNA fractions were pooled and precipitated with ethanol. A portion of the sample was dissolved in 0.5 ml of 0.01 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.001 M EDTA 0.5% SDS, and layered on a linear 15-30% sucrose gradient prepared in the same buffer. The sample was centrifuged for 17 hr at 33,000 rpm at 23° in an SW41 rotor. The fractions were collected from a hole pierced at the bottom of the tube, and acid-precipitable radioactivity in each fraction was determined. The position of 17S rRNA from BHK cells was determined in parallel gradients and is indicated by the arrow.

12-18S RNAs were separated in polyacrylamide gels in formamide (Fig. 2A). It was further shown that isolated fraction I RNA (Fig. 1) co-electrophoresed with the largest RNA species in the gel and fractions II and III RNAs with the other two RNA species in order of their sizes (data not shown). The patterns obtained under these conditions resemble that obtained by Schincariol and Howatson (4) on non-denaturing acrylamide gels. The heterogeneous migration rates of each size class is in marked contrast to the sharp bands obtained with HeLa cell ribosomal RNAs on these gels (data not shown), and is presumably the result of the widely variable lengths of poly(A) sequences attached to the VSV mRNA (15).

A portion of the VSV-infected cell cytoplasm described above was used for the isolation of membrane bound polysomes as described in "Methods" and the RNA species in the fractionated extract were analyzed by polyacrylamide gel electrophoresis in formamide. Fig. 2A shows that the membrane bound polysomes contain predominantly the largest of the 12-18S class of VSV mRNA (17S). The contamination of the predominant

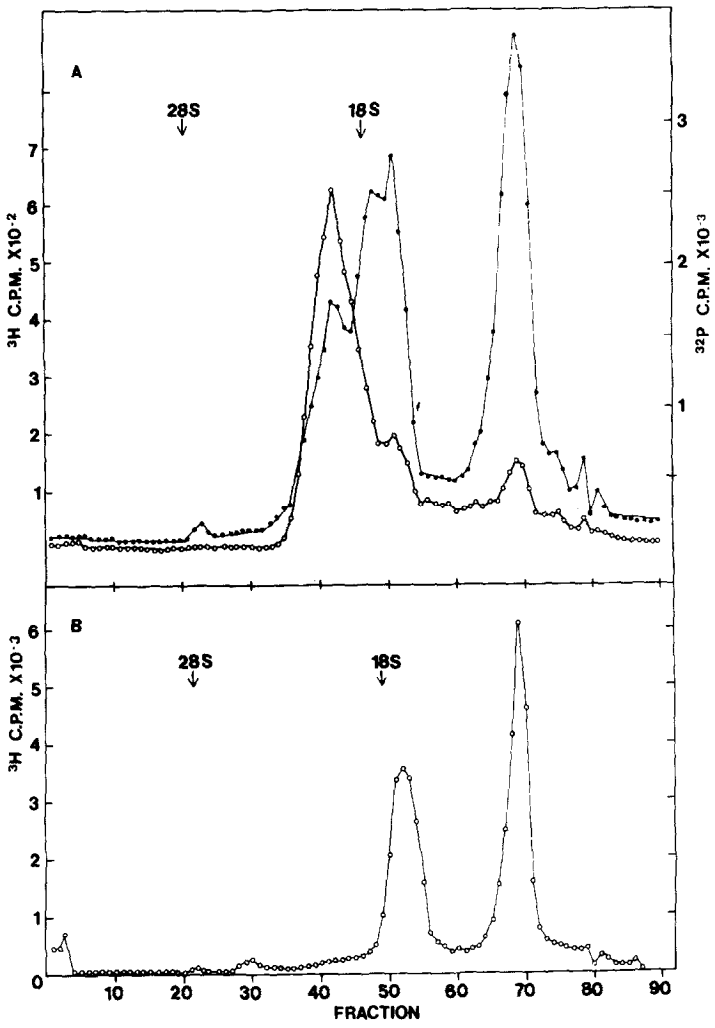

Fig. 2. Polyacrylamide gel electrophoresis in formamide of 12-18S VSV mRNAs isolated from membrane bound polysomes and from the cytoplasmic supernatant.
^{3}H-uridine-labeled VSV RNA was prepared as described in "Methods". (A) Membrane bound polysomes were prepared, treated with 1% SDS and sedimented through a sucrose gradient and the 12-18S RNA fractions were pooled and precipitated with ethanol. A portion was mixed with ^{32}P-labeled total cytoplasmic VSV 12-18S RNA and dissolved in 1 mM phosphate buffered formamide and 25% glycerol and heated at 70°C for 5 min, chilled and applied to gel containing 4.6% acrylamide and 0.69% bisacrylamide in 0.02 M phosphate buffered deionized formamide (19,20). Electrophoresis was performed at 80 V for 21 hr at 23°C with an aquous buffer of 0.04 M sodium phosphate (pH 6.8). The gels were crushed into 1 mM fractions and counted (20). Electrophoresis was toward the anode, represented to the right. (o—o), ^{3}H-labeled RNA from membrane bound polysomes; (•—•), ^{32}P-labeled cytoplasmic RNA). (B) The cytoplasmic supernatant from which the membrane bound polysomes had been removed was treated with 1% SDS and sedimented through a sucrose gradient and the 12-18S RNA fractions were pooled and analyzed by polyacrylamide gel electrophoresis in formamide.

component (Fig. 2A) with the two smaller mRNA classes is variable but always small. Fig. 2B shows that only the two smaller VSV RNA species (14.5S and 12S) are present in the remaining cytoplasm, and that the viral mRNA which is associated with the membrane bound polysomes is essentially absent. Apparently no pool of the 17S class of mRNA exists free in the cytoplasm, and its attachment to the membrane bound polysome fractions must occur quite rapidly after its synthesis. Since the membrane bound polysomes contain the capacity to synthesize significant amounts of G protein in vitro (11,12), these data suggest that this species of mRNA serves as the message for the G protein.

Previous studies have shown that VSV mRNA contains poly(A) sequences (15,16). RNA from the total infected cell cytoplasm and from the fractionated membrane bound polysomes and the remaining cytoplasm were analyzed for the presence of poly(A) sequences by passage through a column of oligo (dT)-cellulose. Table 1 shows that approximately 90% of the radioactivity in mRNA species derived from total cytoplasm or from either subcellular fraction binds to oligo(dT)-cellulose, indicating that the putative glycoprotein mRNA contains poly(A) sequences.

These results show that after subcellular fractionation of VSV-infected HeLa cells predominantly one species of viral mRNA (17S), which contains poly(A), is associated with the membrane bound polysomes and is absent from the remainder of the cytoplasm. This RNA is likely to be the mRNA coding for the G protein. In added support for this conclusion, in a wheat germ in vitro translation system, the RNA isolated from membrane bound polysomes of VSV-infected cells has been shown to yield predominantly a protein about the size of the VSV glycoprotein G (Both, Moyer and Banerjee, unpublished observations). It is believed that the 12-18S mRNA population includes at least four species, those coding for the G, N, NS and M proteins (6). We routinely see three size classes of RNAs analyzed either by formamide gels or by sucrose gradient centri-

Table 1. Polyadenylation of 12-18S VSV mRNA from sub-cellular fractions of infected HeLa cells.

DNA	CPM bound to oligo(dT)-cellulose	Total CPM	% Bound
Total cytoplasm	15,380	17,497	88%
Membrane bound polysomes	13,228	14,336	92%
Cytoplasmic supernatant	16,464	18,985	87%

^{3}H uridine labeled VSV-specific RNA from infected cell cytoplasm or from the subcellular fractions was obtained as described in "Methods" and was sedimented through sucrose gradients. The 12-18S material was pooled and analyzed by chromatography on an oligo(dT)-cellulose column (21).

fugation and it is likely that one of these size classes is comprised of two components.

Several investigators have suggested that both the M protein and the G protein are associated with subcellular membrane fractions, including the rough endoplasmic reticulum (9). Although it has been demonstrated that the M protein preferentially adsorbs to membrane fractions during cell homogenization and fractionation procedures at 0° (17), it nevertheless appears to be inserted into infected cell plasma membranes extremely rapidly (17,18). It was thus somewhat surprising to find only one species of mRNA associated with the membrane bound polysomes, that having been tentatively identified as the G protein mRNA. From our data it appears that the M protein, as well as the N and NS, is synthesized on free cytoplasmic polyribosomes, and that the G protein is the only polypeptide synthesized on membrane bound polysomes. The absence of the larger mRNA species in the cytoplasm from which membrane bound polysomes have been removed is very striking. It is not clear by what mechanism the mRNA species are selected for binding to free or membrane bound polysomes, but it seems that the process is extremely efficient and rapid. The requirement for post-translational glycosylation of the G

protein may account for the compartmentalization of its synthesis in the endoplasmic reticulum.

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