

In Vitro Synthesis of Proteins by Membrane-Bound Polyribosomes from Vesicular Stomatitis Virus-Infected HeLa Cells

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Membrane-bound polysomes from vesicular stomatitis virus (VSV)-infected HeLa cells synthesize predominantly three proteins in an *in vitro* protein synthesizing system. These three proteins have different molecular weights than the viral structural proteins, i.e., 115,000, 88,000, and 72,000. Addition of preincubated L or HeLa cell S10 or HeLa cell crude initiation factors stimulates amino acid incorporation and, furthermore, alters the pattern of proteins synthesized. Stimulated membrane-bound polysomes synthesize predominantly viral protein G and lesser amounts of N, NS, and M. *In vitro* synthesized proteins G and N are very similar to virion proteins G and N based on analysis of tryptic methionine-labeled peptides. Most methionine-labeled tryptic peptides of virion G protein contain no carbohydrate moieties, since about 90% of sugar-labeled peptides co-chromatograph with only about 10% of methionine-labeled peptides. Sucrose gradient analysis of the labeled RNA present in VSV-infected membrane-bound polysomes reveals a relative enrichment in a class of viral RNA sedimenting slightly faster than the total population of the 13 to 15S mRNA, as compared to a VSV-infected crude cytoplasmic extract. A number of proteins, other than the viral structural proteins, are synthesized in the cytoplasm of five lines of VSV-infected cells. One of these proteins has the same molecular weight as the major *in vitro* synthesized protein, P₈₈. *In vitro* synthesized protein P₈₈ does not appear to be a precursor of viral structural proteins G, N, or M based on pulse-chase experiments and tryptic peptide mapping. Nonstimulated membrane-bound polysomes from uninfected HeLa cells synthesize the same size distribution of proteins as nonstimulated VSV-infected membrane-bound polysomes.

We have previously shown that cytoplasmic extracts from vesicular stomatitis virus (VSV)-infected HeLa cells synthesize virus specific proteins when incubated in an *in vitro* protein synthesizing system (9). Furthermore, a crude fractionation of these extracts into cytoplasmic supernatant and cytoplasmic pellet resulted in a relative enrichment of specific protein-synthesizing capacities, suggesting a segregation of some VSV mRNAs.

In vivo studies by Wagner et al. (25, 26) with VSV-infected L cells have shown that, after a 1.5-h pulse with radioactive amino acids, viral proteins G and M (27) are associated with either a smooth membrane fraction enriched in plasma membranes or a fraction containing rough endoplasmic reticulum, whereas proteins N and NS (27) are initially found in the membrane-free cytoplasmic fraction. Cohen et al. (3) and David (6) further showed, using short

pulses with radioactive amino acids, that newly synthesized G and M proteins become rapidly associated with the host cell plasma membrane, whereas the N protein only becomes associated with the plasma membrane after a much longer period.

Since it is known that the G and M proteins are part of the lipoprotein envelope of VSV (2, 11, 24, 27), it would appear that the messages for these virion proteins are located on or near host cell membranes, and, after translation, these proteins are inserted directly into the cell plasma membrane where virus maturation occurs.

We have fractionated extracts from VSV-infected HeLa cells into membrane-bound polysomes using the Rosbash and Penman procedure (17), and have analyzed these extracts for their *in vitro* protein synthesizing activity and their mRNA content. We found that mem-

brane-bound polysomes, isolated from VSV-infected HeLa cells, only synthesize proteins of a higher molecular weight than four of the five viral structural proteins. Addition of preincubated extracts from uninfected HeLa cells (S10) to this system results in a varying degree of stimulation of incorporation of [³⁵S]methionine, but, most significantly, it results in the synthesis of large amounts of virion protein G and lesser amounts of N and M proteins. The activity that promotes the altered pattern of protein synthesis is present in the ribosomal salt wash of uninfected cells. Pulse-chase experiments and chromatography of tryptic peptides show no obvious precursor-product relationship between the high molecular weight proteins synthesized by the membrane polysomes in the absence of HeLa S10 and the viral structural proteins G, N, or M synthesized in the presence of HeLa S10. However, preliminary experiments indicate a possible host cell origin of the high molecular weight proteins.

Analysis of the RNA contained in the VSV-infected membrane-bound polysome fraction as compared to a crude cytoplasmic extract indicates a large increase in material sedimenting in the 13 to 15S region relative to the 26S region. Furthermore, the material in the 13 to 15S region is displaced towards the heavier side of this peak.

MATERIALS AND METHODS

Cell and virus cultures. Suspension cultures of HeLa, L, and murine Friend leukemia virus-induced leukemic cells (cell line number 745A) were grown in Eagle medium (Schwartz/Mann, Div. of Becton, Dickinson & Co., Orangeburg, N.Y.) plus 7% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) and 2 mM glutamine (Grand Island Biological Co.) at a concentration of about 4×10^5 to 6×10^6 cells per ml. Chinese hamster ovary and lung cells were grown in Blake medium supplemented with 5% fetal calf serum and 10% calf serum, and Eagle medium plus 7% fetal calf serum and 2 mM glutamine, respectively. Baby hamster kidney cells were grown in Eagle medium plus 5% fetal calf serum, 4 mM glutamine, nonessential amino acids, and vitamins.

VSV was of the Indiana serotype. Both unlabeled and ³H-labeled and ¹⁴C-labeled amino acid VSV were grown and purified as previously described (10, 15). Labeled virus was precipitated and solubilized as described previously for use as a marker on acrylamide gels (9).

Infection of HeLa cells. Cells, grown at 37 C and concentrated to 4×10^6 cells per ml, were infected with VSV at a multiplicity of 10 PFU per cell. Both actinomycin D (Merck & Co., Inc., Rahway, N.J.) and fetal calf serum were added during infection as previously described (9). [³H]adenosine (30 μ Ci/ml, 5 Ci/mmol) and [¹⁴C]adenosine (2.5 μ Ci/ml, 50 mCi/mmol) were added at 2.5 h postinfection. [³⁵S]meth-

ionine (100 Ci/mmol) was added as described in each experiment. All isotopes were from New England Nuclear, Boston, Mass. In all experiments the infection was stopped at 4.5 h postinfection unless otherwise stated.

Preparation of membrane-bound polysome extract. Cell extracts and fractions were prepared as previously described (9, 18). The cytoplasmic pellet was suspended in reticulocyte standard buffer (0.01 M NaCl or KCl, 0.0015 M MgCl₂, and 0.01 M Tris, pH 7.4) containing 50 μ g of heparin per ml, layered over a 36-ml, 15 to 30% (wt/wt) sucrose gradient in reticulocyte standard buffer, and centrifuged at 4 C in a Spinco SW27 rotor at 25,000 rpm for 30 min (17). The pellet was resuspended in reticulocyte standard buffer, containing 50 μ g of heparin per ml, glycerol was added to a final concentration of 10%, and the extracts were frozen at -70 C.

Preparation of S10 and crude initiation factors. Extracts from uninfected HeLa and L cells (S10) were prepared as previously described (9). Crude initiation factors were prepared from HeLa cells following essentially the procedure of Schreier and Staehelin (19).

Analysis of adenosine-labeled membrane-bound polysomes. The ³H-labeled adenosine pellet from the 15 to 30% sucrose gradient was resuspended in RSB plus heparin and mixed with a ¹⁴C-labeled adenosine crude cytoplasmic extract from VSV-infected HeLa cells. This was made 1% in sodium dodecyl sulfate (SDS), layered over a 36-ml, 15 to 30% (wt/wt) sucrose gradient in NETS (0.1 M NaCl, 0.01 M Tris [pH 7.4], 0.002 M EDTA, and 0.1% SDS), and was centrifuged in the SW27 rotor at 22,000 rpm for 18 h at 23 C.

Incorporation of amino acids in vitro. The contents of the cell-free protein synthesizing system was described previously (9). In addition, all complete protein synthesizing mixtures contained 165 μ g of stripped rabbit liver tRNA per ml.

Acrylamide gel electrophoresis and isolation of in vitro synthesized proteins. Samples from in vitro reaction mixtures were prepared for electrophoresis as described (9), and electrophoresis was performed on 18-cm 7.5% polyacrylamide-SDS gels, or on 7.5 to 30% polyacrylamide gradient slab gels with the Tris-glycine buffer system (1, 14, 20). Proteins were isolated from the 7.5% cylindrical gels by extrusion through a Savant autogel divider in 0.2% SDS, and were eluted in this buffer for 20 to 24 h at room temperature. Portions (50 μ liters each) were removed from each gel fraction, and the samples were assayed for radioactivity. The peak fractions were pooled, gel particles were removed by centrifugation, and trichloroacetic acid was added to a final concentration of 20%. Bovine gamma globulin (500 μ g) was added as carrier, and the samples were left at 4 C overnight. The protein was pelleted by centrifugation in a 65 rotor at 60,000 rpm for 1 h; the pellet was washed with acetone at 4 C and centrifuged in a Sorvall RC2-B at 12,000 rpm for 20 min. The acetone pellet was air dried and suspended in 1 ml of distilled water. A drop of phenol red was added as indicator, and 0.05 M ammonium bicarbonate was added dropwise until the pH was approximately 8. The isolation procedure

resulted in approximately a 50% recovery of radioactive proteins from the gel. A sample of each isolated protein was rerun on a 7.5% polyacrylamide-SDS gel to insure homogeneity.

Isolation of ^3H -labeled methionine virion proteins G, N, and M. ^3H -labeled methionine VSV was incubated with a final concentration of 10% Triton X100 for 45 min at 37 C to release G (12; J. J. McSharry, R. W. Compans, H. Lackland, and P. W. Choppin, *Abstr. Annu. Meet. Amer. Soc. Microbiol.*, 72nd, Philadelphia, Pa., Abstr. V178, p. 215, 1972; A. Scheid, L. A. Caligiuri, and P. W. Choppin, *Abstr. Annu. Meet. Amer. Soc. Microbiol.*, 72nd, Philadelphia, Pa., Abstr. V181, p. 215, 1972). After centrifugation at 43,000 rpm for 1 h in a 65 rotor, the supernatant, which contained only G protein, was dialyzed against 6 liters of distilled water. The pellet was solubilized in 0.1 M sodium phosphate buffer (pH 7.2), 1% SDS, and 0.1% 2-mercaptoethanol, and prepared for electrophoresis on 7.5% SDS-polyacrylamide gels. ^3H -labeled methionine N and M proteins were isolated from these gels as described above.

Trypsin digestion and ion-exchange chromatography of peptides. ^{35}S -labeled methionine *in vitro* synthesized protein and ^3H -labeled methionine virion protein were mixed and brought to a volume of about 1 ml, and 1 mg of trypsin (TPCK-treated; Worthington Biochemicals Corp., Freehold, N.J.) was added. Incubation was at 37 C for 5 h, and the pH of the reaction was monitored. Occasionally, prior to trypsin digestion, the samples were reduced and alkylated according to the procedure of Cooper et al. (4), and then were dialyzed overnight against 1,000 volumes of 0.01 M ammonium bicarbonate, pH 8.1. After trypsin digestion, the reaction mixture was centrifuged at 5,000 rpm for 10 min in a Sorvall RC2-B. The supernatant was removed and diluted with 3 volumes of 0.05 M pyridine acetate (pH 4.0) and stored at 4 C. The sample was applied to a 0.9×30 -cm column of ion-exchange resin (Spherix resin XX907-10, Phoenix Precision Instrument Co., Philadelphia, Pa.), which was maintained at 57 C. A four-chamber gradient of 200 ml of 0.05 M pyridine acetate (pH 4.0), 100 ml of 0.5 M pyridine acetate (pH 5.0), and 100 ml of 2.0 M pyridine acetate (pH 6.0) was run, and 3-ml fractions were collected every 4 min. Occasionally, an eight-chamber gradient containing twice the volume of the above buffers was used. The samples were dried in a chromatography oven to remove pyridine, dissolved in 1 ml of distilled water, and scintillation fluid was added. Radioactivity was measured in a Beckman scintillation spectrometer, and corrections were made for spill of ^{35}S into the ^3H channel.

High voltage paper electrophoresis. After trypsin digestion, the samples were lyophilized and dissolved in 100 μl of pyridine acetate buffer, pH 3.5, (1 ml of pyridine, 10 ml of glacial acetic acid, and distilled water to 300-ml total volume). The samples were spotted onto Whatman 3 MM paper, placed in a Gilson high voltage electrophoresis tank, and run at 3,000 V (200 mamp) for 1 to 1 $\frac{1}{4}$ h. After drying, the paper was cut into 1-cm strips and assayed for radioactivity.

RESULTS

Characterization of the proteins synthesized by membrane-bound polysomes. Membrane-bound polysomes were isolated from VSV-infected HeLa cells and were tested for *in vitro* protein synthesizing activity. The incorporation of [^{35}S]methionine into hot acid-precipitable material continued for about 40 min (Fig. 1). The addition of extracts from uninfected HeLa or L cells, preincubated in a complete protein synthesizing reaction mixture to eliminate their endogenous protein synthesizing activity, stimulated incorporation 2- to 5-fold depending upon the particular preparation of membrane-bound polysomes.

The products synthesized by the nonstimulated and stimulated systems were analyzed on 7.5% SDS-acrylamide gels. In the absence of preincubated L or HeLa S10, a number of proteins were synthesized; however, none of these proteins had the same electrophoretic mobility as VSV structural proteins (Fig. 2A). The molecular weights of these proteins were determined by extrapolation of their migration through the gels using the VSV structural proteins as markers (20). The largest protein

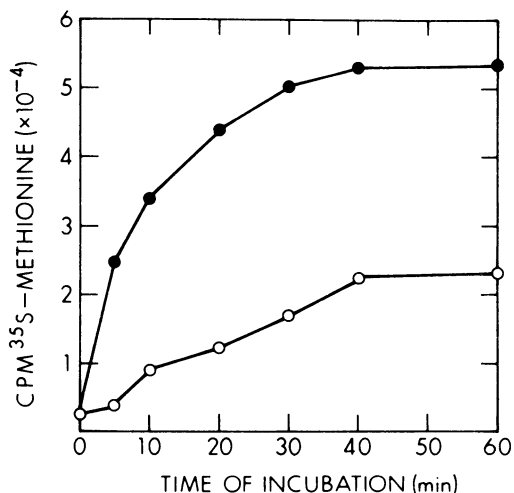


FIG. 1. Kinetics of incorporation of [^{35}S]methionine in an *in vitro* protein synthesizing system directed by membrane-bound polysomes from VSV-infected HeLa cells. A 0.150-ml sample of membrane-bound polysomes (9.5 OD_{260} U/ml) was added to a complete protein synthesizing system containing 20 μl of [^{35}S]methionine (850 $\mu\text{Ci}/\text{ml}$, 100 Ci/mmol). The total reaction volume was 0.3 ml. Incubation was for 60 min at 37 C. Forty- μl samples were removed at various times and assayed for hot acid-precipitable radioactivity. Symbols: \circ , complete system; \bullet , plus 80 μl HeLa S10 (63.5 OD_{260} U/ml).

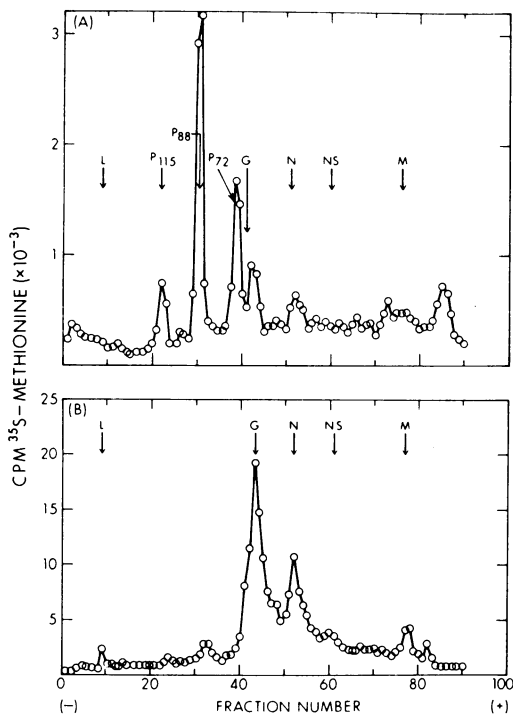


FIG. 2. Analysis by SDS-acrylamide gel electrophoresis of the products synthesized by VSV-infected membrane-bound polysomes in the absence or presence of preincubated S10 from uninfected L cells. A 0.15-ml sample of VSV-infected membrane-bound polysome (5.0 OD₂₆₀ U/ml) was incubated at 37 C for 60 min with a complete protein synthesizing system and 15 μ liters of [³⁵S]methionine (940 μ Ci/ml, 100 Ci/mmol). In one case, 60 μ liters of L cell S10 (26.8 OD₂₆₀ U/ml) was added. After 60 min, 10 μ liters of 1 mg of RNase per ml was added, and incubation was continued 5 min. The reaction was stopped by the addition of 0.3 ml of 10% trichloroacetic acid. The samples were then processed for electrophoresis on 7.5% SDS-acrylamide gels. (A), Membrane-bound polysome directed products; (B), L S10 stimulated products.

had a molecular weight of approximately 115,000 (P₁₁₅). The other major proteins had molecular weights of 88,000 (P₈₈) and 72,000 (P₇₂). The addition of preincubated L or HeLa S10 not only stimulated incorporation, but, more significantly, shifted the pattern of proteins synthesized. A large fraction of the material synthesized migrated with virion protein G, whereas lesser amounts migrated with virion proteins N, NS, and M (Fig. 2B). Furthermore, only relatively small amounts of the large molecular weight proteins were made. Similar results were obtained when a mixture of [³H]leucine, isoleucine, lysine, and tyrosine was used.

The addition of crude initiation factors from uninfected HeLa cells to membrane-bound polysomes from VSV-infected cells resulted in the same stimulation and pattern of proteins synthesized as the addition of preincubated L or HeLa S10.

To unequivocally establish the identity of in vitro synthesized proteins G and N with viral structural proteins G and N, we isolated the in vitro proteins from acrylamide gels and digested them with trypsin in the presence of the virion proteins isolated in a similar manner. The tryptic peptides were co-chromatographed on an ion-exchange column. The similarity of the methionine-containing peptides of in vitro and virion G and in vitro and virion N proteins is shown in Fig. 3 and 4. We have no definite explanation for the lack of 100% identity of the methionine-containing peptides.

Virion protein G is a glycoprotein. Since the extent of in vitro glycosylation of G protein was unknown, it might have been expected that the elution profiles of tryptic digests of in vitro synthesized G and virion G would show major differences corresponding to those methionine-labeled peptides which contained carbohydrate moieties. This did not occur, however (Fig. 3), and we therefore examined the tryptic peptides of virion G labeled with [³H]fucose and glucosamine and [³⁵S]methionine on an ion-exchange column (Fig. 5). We observed that about 90% of the radiolabeled sugar peptides are eluted from the column in the first few fractions, and only one methionine-labeled peptide also contained ³H sugar label. Thus, most methionine-labeled peptides contain no carbohydrate, and the identity of virion and in vitro G peptides is not masked by altered elution profiles of carbohydrate-containing fragments.

Since the peptide column did not resolve the sugar-containing peptides, we further analyzed tryptic methionine and fucose-labeled peptides on high voltage paper electrophoresis. There were few, if any, comigrating fucose and methionine-labeled peptides (Fig. 6). The resolution of peptides was much poorer on high voltage paper electrophoresis as compared with the ion-exchange column (Fig. 6A versus Fig. 3). High voltage paper electrophoresis resolved only about six methionine-labeled peptides, whereas at least twice the number of methionine-labeled peptides were resolved by the ion-exchange column. However, the resolution of tryptic glycopeptides was improved by high voltage paper electrophoresis, and there were at least six tryptic peptides from G protein which carry carbohydrate moieties in HeLa grown

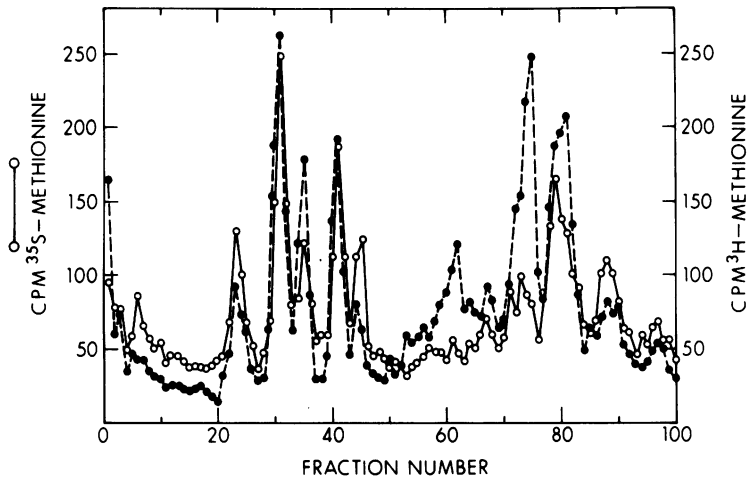


FIG. 3. Tryptic peptide analysis of ^{35}S -labeled methionine *in vitro* G protein and ^3H -labeled methionine virion G protein. 0.1 ml of ^{35}S -labeled methionine *in vitro* G protein (10 μl iters = 1503 counts/min) isolated from a 7.5% SDS-acrylamide gel as described was trypsin digested with 0.9 ml of ^3H -labeled methionine virion G protein (10 μl iters = 330 counts/min). The digested sample was chromatographed on an ion-exchange column containing Spherix resin XX907-10. Fractions were dried and counted as described.

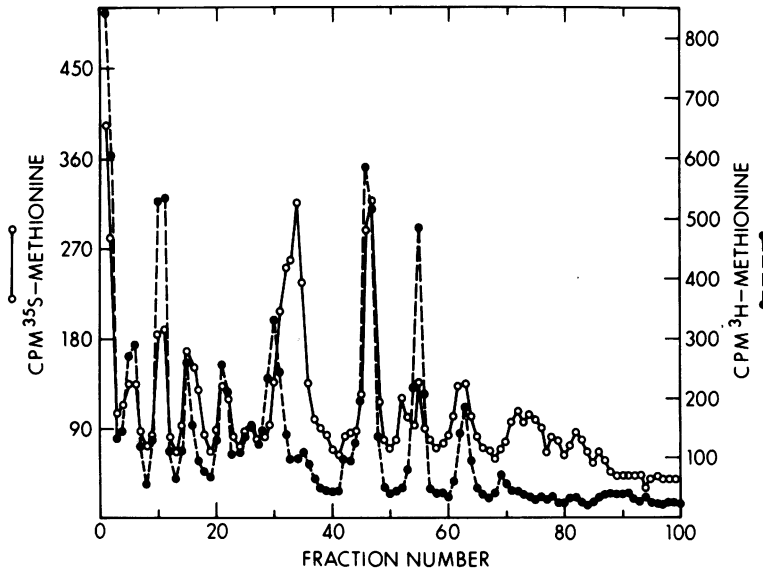


FIG. 4. Tryptic peptide analysis of ^{35}S -labeled methionine *in vitro* synthesized N protein and ^3H -labeled methionine virion N protein. Approximately 0.5 ml of ^{35}S -labeled methionine *in vitro* synthesized N protein (10 μl iters = 419 counts/min) was trypsin digested with 0.7 ml of ^3H -labeled methionine N protein (10 μl iters = 345 counts/min). The digested samples were applied to an ion-exchange column and fractions were collected and processed as described.

VSV (Fig. 6B). We are presently using alternative techniques to resolve the question of glycosylation of *in vitro* synthesized G.

Analysis of radiolabeled RNA in VSV-infected membrane-bound polysomes. The capacity of membrane polysomes to be stimulated to synthesize relatively large amounts of G protein suggested that it might be possible to detect an enrichment in a particular class of

VSV-specific mRNA. At 2.5 h postinfection, a culture of VSV-infected HeLa cells was divided and labeled with either [^3H] or [^{14}C]adenosine. Membrane-bound polysomes were prepared as usual from the ^3H -labeled adenosine culture, and mixed with a crude cytoplasmic extract prepared from the ^{14}C -labeled adenosine culture. This was made 1% in SDS, and the RNA species were analyzed by velocity sedimentation

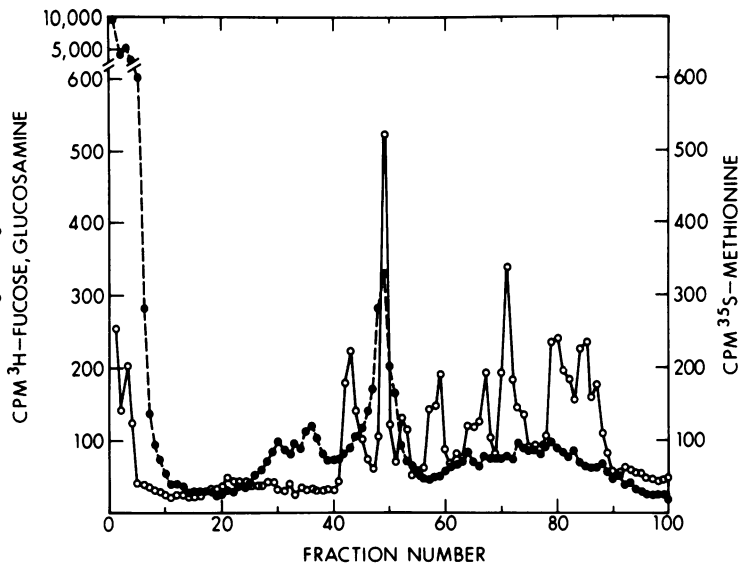


FIG. 5. Tryptic peptide analysis of ^3H -labeled fucose virion G protein and ^{35}S -labeled methionine G protein. 0.4 ml of ^3H -labeled fucose virion G protein (10 μl iters = 1060 counts/min) was trypsin digested with 0.2 ml of ^{35}S -labeled methionine virion G protein (10 μl iters = 1300 counts/min). The samples were applied to an ion-exchange column as described.

through sucrose gradients in the presence of SDS. The membrane-bound polysomes were enriched in the 13 to 15S class of mRNA relative to the 26S mRNA (Fig. 7). Furthermore, the material in the 13 to 15S region was skewed towards the heavier side of this peak.

Presence of proteins other than viral structural proteins in the cytoplasm of VSV-infected HeLa cells. Previously, *in vivo* studies of VSV-infected cells (15) have not revealed the presence of any of the large proteins synthesized *in vitro* by unstimulated membrane-bound polysomes. We sought to determine whether these proteins might be present transiently in infected cells. HeLa cells were infected with VSV at 32 and 37 C; the incorporation of [^{35}S]methionine at 32 C occurred at approximately 60 to 70% of the rate at 37 C, indicating a reduction in protein synthesis. In the experiments shown in Fig. 8, VSV-infected HeLa cells incubated at 32 and 37 C were pulsed for 5 min at 4 h postinfection with [^{35}S]methionine, and a portion of the culture was chased with cold methionine. Samples were removed during the chase and prepared for electrophoresis on polyacrylamide gradient slab gels. A number of newly-synthesized proteins larger than G could be seen in the cytoplasm of infected cells incubated at both 32 and 37 C. Also, a protein migrating electrophoretically with protein P_{88} , which is synthesized *in vitro* by the membrane-bound polysome fraction, could be clearly detected during the pulse and subsequent chase at

32 C. This protein also appeared to be present in infected cells incubated at 37 C, but was not present in as large amounts.

Possible precursor-product relationship of proteins P_{115} , P_{88} , and P_{72} and viral structural proteins. We considered that the large proteins synthesized by the membrane-bound polysomes in the absence of HeLa or L cell S10 or ribosomal salt wash, and also seen in infected cells, are precursors of one or more of the virion structural proteins. Factors present in HeLa S10 would thus be necessary to cleave these precursors *in vitro* into proper sized VSV proteins. Furthermore, incubation of infected cells at 32 C might cause a reduction in the rate of *in vivo* cleavage of these precursors.

To investigate the possible precursor-product relationship of the proteins synthesized *in vitro* in the nonstimulated and stimulated systems, we pulsed the nonstimulated membrane-bound polysome directed *in vitro* system for short periods of time with [^{35}S]methionine, and chased with a large excess of cold methionine and HeLa or L cell S10. In no instance were the large proteins converted into any of lower molecular weight (data not shown).

G protein labeled with [^3H]methionine was isolated from purified virions and digested with trypsin for comparison with ^{35}S -labeled methionine *in vitro* synthesized protein P_{88} . There is little similarity between the methionine tryptic peptides (Fig. 9). We then compared tryptic peptide digests of ^3H -labeled methionine virion

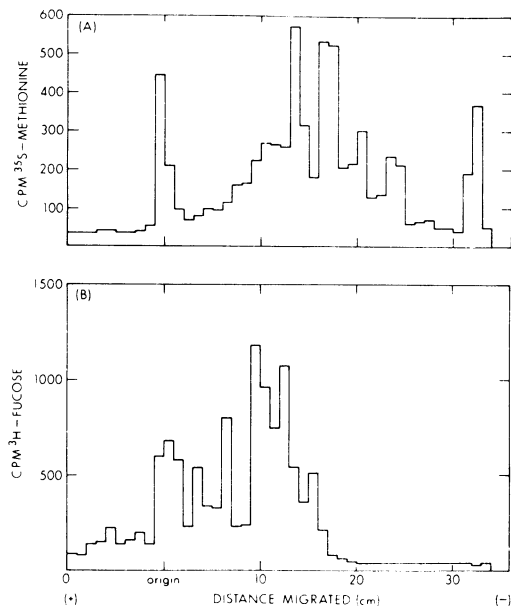


Fig. 6. High voltage paper electrophoresis of trypsin digested ³⁵S-labeled methionine virion G protein and ³H-labeled fucose virion G protein. 13,500 counts/min of ³⁵S-labeled methionine virion G protein and 47,500 counts/min of ³H-labeled fucose virion G protein prepared by Triton X100 treatment of VSV was trypsin digested, lyophilized, and chromatographed on Whatman 3MM paper. The paper was cut into 1-cm strips and counted. (A), ³⁵S-labeled methionine G peptides; (B), ³H-labeled fucose G peptides.

proteins N and M with ³⁵S-labeled methionine P₈₈. Figure 10 indicates less than a 25% matching of peptides. Tryptic peptide maps of [³⁵S]methionine P₁₁₅ versus [³H]methionine virion G and [³⁵S]methionine P₇₂ versus [³H]methionine virion G show no relationship of these in vitro synthesized proteins with protein G. Furthermore, comparison of tryptic digests of ³⁵S-labeled methionine P₁₁₅, P₈₈, and P₇₂ by high voltage paper electrophoresis indicated that these three proteins do not contain similar tryptic peptides (data not shown).

Possible host cell origin of proteins synthesized in vitro in the absence of S10. Since the large proteins synthesized in vitro by nonstimulated membrane polysomes do not appear to be precursors of viral proteins, we considered the possibility that they were of cellular origin. There were many labeled proteins present in the cytoplasm of VSV-infected HeLa cells other than the five viral structural proteins (Fig. 8). These proteins were still present in the cytoplasm after 4 h of infection and at the end of a 60-min chase period. Since VSV does not contain enough information to code for all of these proteins, it is conceivable that VSV infection

does not result in complete inhibition of host cell protein synthesis by 4 to 4.5 h postinfection.

Membrane polysomes from uninfected HeLa cells (non-actinomycin D treated) were isolated and tested for in vitro protein synthesizing activity. In the absence of HeLa S10, the predominant protein synthesized by the uninfected membrane polysomes had a molecular weight of about 90,000 (Fig. 11). A number of other proteins were also synthesized, including proteins of molecular weight 110,000 and 72,000. Incorporation of [³⁵S]methionine by S10-stimulated uninfected membrane polysomes was substantially increased, and there was a general stimulation of synthesis of proteins of many different molecular weights (data not shown).

Analysis of the cytoplasm of five VSV-infected cell lines. L, baby hamster kidney, and Chinese hamster ovary cells were infected with VSV, and the in vitro protein synthesizing activity of membrane-bound polysomes was examined. In the three VSV-infected cell lines, the predominant product synthesized in vitro by membrane-bound polysomes has a molecular weight of 86,000 to 90,000. The addition of preincubated HeLa S10 stimulates total [³⁵S]methionine incorporation, and again results in the synthesis of varying amounts of viral proteins G, N, and M.

The presence of a protein of about 88,000

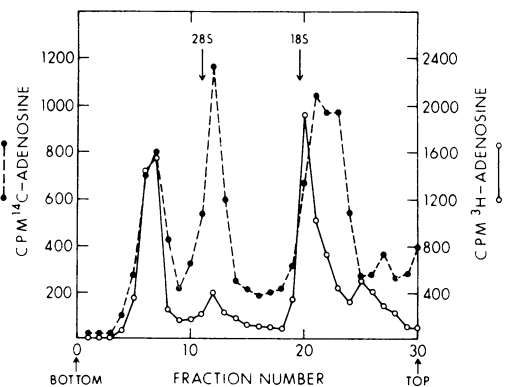


Fig. 7. Sucrose gradient analysis of RNA in VSV-infected membrane-bound polysomes. HeLa cells were infected with VSV and at 2.5 h postinfection the culture was divided. 250 μ Ci of [³H]adenosine (5 Ci/mmol) was added to one culture (8 ml), and 5 μ Ci of [¹⁴C]adenosine (50 mCi/mmol) was added to the other culture (2 ml). A crude cytoplasmic extract was prepared from the ¹⁴C-labeled adenosine culture, whereas membrane-bound polysomes were prepared from the ³H-labeled adenosine culture. The extracts were mixed, made 1% in SDS, layered onto a 36-ml 15 to 30% (wt/wt) sucrose gradient in NETS, and centrifuged in the SW 27 rotor at 22,000 rpm for 18 h at 23 C. Fractions were collected and assayed for trichloroacetic acid-precipitable radioactivity.

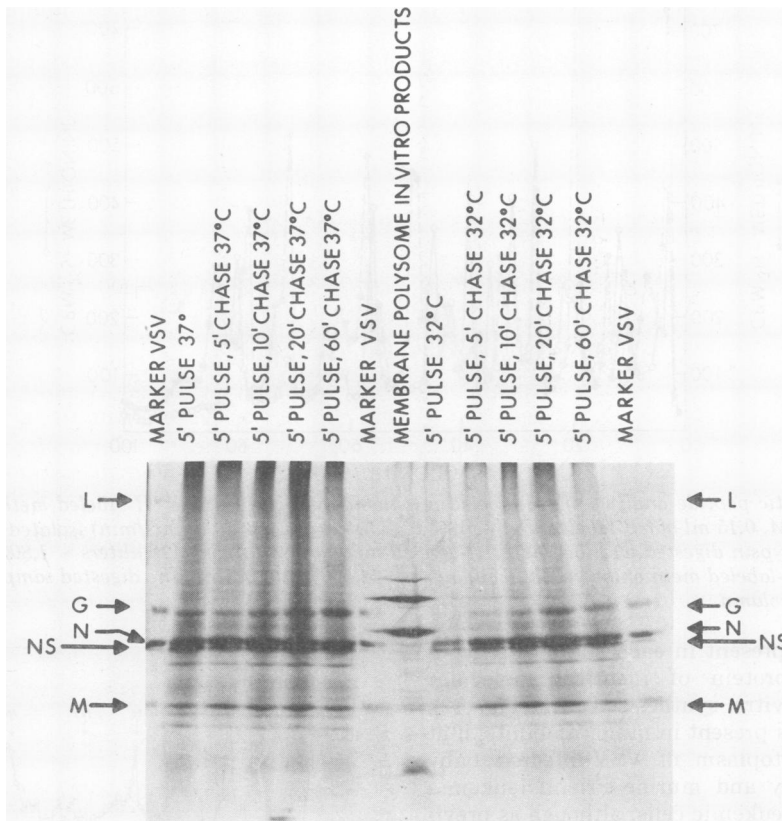


FIG. 8. Autoradiogram of a pulse-chase of VSV-infected HeLa cells. 500 ml of HeLa cells were concentrated 10-fold and infected with VSV. Actinomycin D and fetal calf serum were added. At 4 h postinfection, the cells were washed and suspended in Eagle medium missing methionine, and 500 μ Ci of [35 S]methionine (100 Ci/mmol) was added to each culture. One-half of the culture was incubated at 32 C and the other at 37 C. After a 5 min pulse, a 200-fold excess of cold methionine was added, and incubation was continued for 1 h. Samples were taken at the end of the pulse and at 5, 10, 20, and 60 min into the chase. 1% NP-40 was added to the samples. The nuclei were removed by centrifugation, and the cytoplasm was made 10% in trichloroacetic acid and processed for analysis by electrophoresis on 7.5 to 30% polyacrylamide gradient slab gels (1, 14). Migration is from top to bottom.

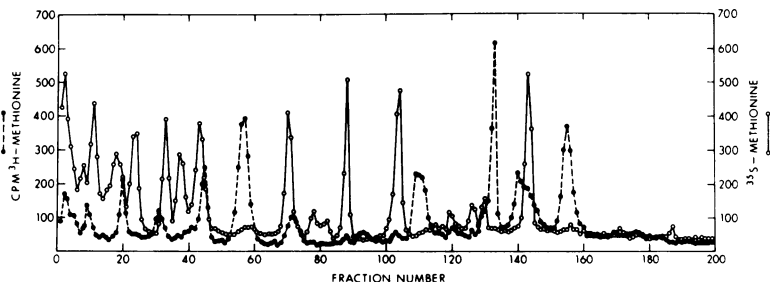


FIG. 9. Tryptic peptide analysis of 35 S-labeled methionine *in vitro* P_{88} and 3 H-labeled methionine virion G. 0.2 ml of 35 S-labeled methionine P_{88} (10 μ liters = 6336 counts/min) was trypsin digested with 0.5 ml. 3 H-labeled methionine virion G (10 μ liters = 1487 counts/min), and the sample was run on an ion-exchange column.

molecular weight was confirmed in the cytoplasm of various VSV-infected cells. HeLa, L, baby hamster kidney, Chinese hamster ovary, and murine Friend leukemia virus-induced leukemic cells were infected with VSV, radi-

labeled at 4 h postinfection for 30 min at 32 C with [35 S]methionine, and the labeled cytoplasmic proteins were analyzed on polyacrylamide gradient slab gels. In addition to the viral structural proteins, a number of other labeled

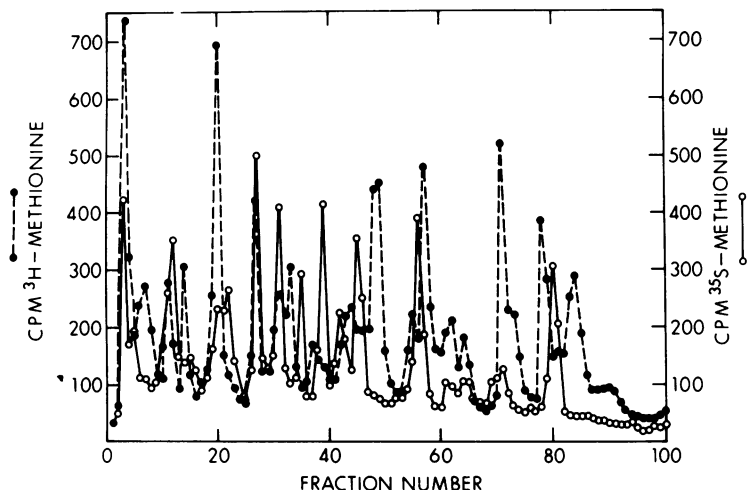


FIG. 10. Tryptic peptide analysis of ^{35}S -labeled methionine *in vitro* P_{88} and ^3H -labeled methionine virion proteins *N* and *M*. 0.15 ml of ^{35}S -labeled methionine P_{88} (10 μl iters = 6300 counts/min) isolated as previously described was trypsin digested with 0.4 ml of ^3H -labeled methionine virion *N* (10 μl iters = 1,800 counts/min) and 0.4 ml of ^3H -labeled methionine virion *N* (10 μl iters = 1,500 counts/min). The digested sample was run on an ion-exchange column.

proteins were present in each of the cell types including a protein of identical molecular weight to *in vitro* synthesized P_{88} (Fig. 12). However, P_{88} is present in minor amounts, if at all, in the cytoplasm of VSV-infected baby hamster kidney and murine Friend leukemia virus-induced leukemic cells; although as previously mentioned P_{88} is synthesized *in vitro* by membrane-bound polysomes from VSV-infected baby hamster kidney cells.

DISCUSSION

Membrane-bound polysomes from VSV-infected HeLa cells, in the absence of preincubated S10, synthesize predominantly three proteins in an *in vitro* protein synthesizing system, and these three proteins have different molecular weights than the viral structural proteins. The addition of HeLa or L cell S10 or HeLa cell crude initiation factors from the ribosomal salt wash stimulates amino acid incorporation 2- to 5-fold, and, furthermore, alters the pattern of proteins synthesized. Stimulated membrane-bound polysomes synthesize predominantly viral protein *G* and also *N*, *NS*, and *M*. The similarity of *in vitro* synthesized *G* and *N* with virion structural proteins *G* and *N* was substantiated by tryptic peptide mapping. However, we were not able to determine if *in vitro* synthesized *G* is glycosylated. Furthermore, the significant enrichment of a class of viral mRNA heavier than the total population of 13 to 15S mRNA in the membrane-bound polysome fraction suggests that this fraction may contain

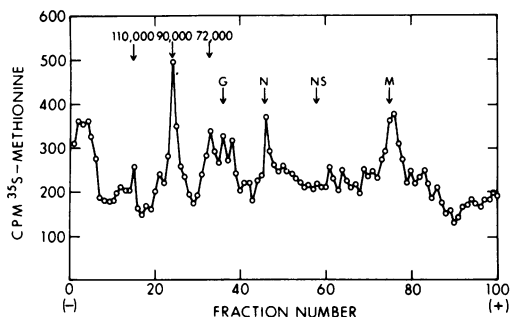


FIG. 11. Analysis by SDS-acrylamide gel electrophoresis of the products synthesized by uninfected HeLa membrane-bound polysomes. A 0.15-ml sample of uninfected HeLa membrane-bound polysomes (15.9 OD_{260} U/ml) was incubated at 37 C for 60 min with a complete protein synthesizing system and 20 μl iters of ^{35}S methionine (960 $\mu\text{Ci}/\text{ml}$, 100 Ci/mmol). After 60 min, RNase and trichloroacetic acid were added as described in Fig. 2, and the samples were processed for electrophoresis on 7.5% SDS-acrylamide gels.

unique species of mRNA, including the glycoprotein mRNA. The isolation of mRNA from this and other well-defined fractions for translation in messenger dependent cell-free systems may provide a way of identifying the individual mRNAs of the VSV structural proteins.

We found that there are a number of proteins synthesized at 4 h postinfection present in the cytoplasm of VSV-infected HeLa cells (and also the four other VSV-infected cell lines studied) other than the viral structural proteins. One of these nonstructural proteins corresponds in mo-

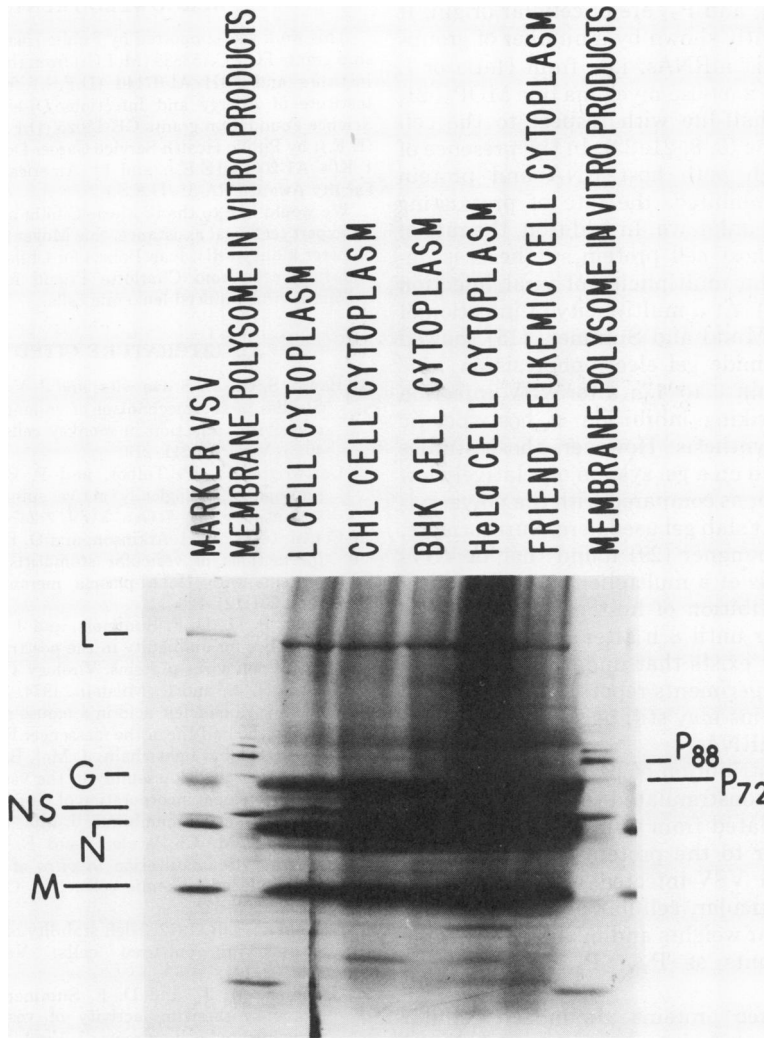


FIG. 12. Autoradiogram of the cytoplasm of VSV-infected HeLa, L, baby hamster kidney, Chinese hamster lung, and murine Friend leukemia virus-induced leukemic cells. The five cell lines were concentrated 10-fold and infected with VSV. Actinomycin D and fetal calf serum were added. At 4 h postinfection, the cells were washed and suspended in Eagle medium missing methionine and 100 μ Ci of [³⁵S]methionine (100 Ci/mmol) was added to each culture. The cultures were incubated at 32 C for 30 min. The infection was stopped by the addition of frozen Earle solution. 1% NP-40 was added to the samples. The cells were dounced, and the nuclei were removed by centrifugation. The cytoplasm was made 10% in trichloroacetic acid and processed for analysis by electrophoresis on 7.5 to 30% polyacrylamide gradient slab gels (1, 14). Migration is from top to bottom.

lecular weight to in vitro synthesized P₈₈. Since VSV does not contain enough information to code for all the additional nonstructural proteins present, it is conceivable that these proteins could be precursors of one or more of the viral structural proteins. We considered this possibility for the three major proteins synthesized in vitro by nonstimulated membrane polysomes. However, pulse-chase experiments and tryptic peptide mapping show no obvious relationship between proteins P₁₁₅, P₈₈, and P₇₂ and

virion proteins G, N, or M. Furthermore, addition of the protease inhibitor TPCK (13, 23) to VSV-infected HeLa cells does not cause the accumulation in the cytoplasm of large molecular weight polypeptides such as P₁₁₅, P₈₈, and P₇₂ (unpublished observation). The lack of peptide matching also rules out the possibility that synthesis of proteins P₁₁₅, P₈₈, and P₇₂ is the result of nonsense ribosomal read-through of one or more of the viral mRNAs.

We also considered the possibility that pro-

teins P_{115} , P_{88} , and P_{72} are of cellular origin. It has been recently shown by a number of groups that eukaryotic mRNAs, i.e., from HeLa or L cells and from a mouse myeloma line MOPC 21, have a long half-life with respect to the cell generation time (5, 8, 21, 22). In the presence of VSV, although both host RNA and protein synthesis is inhibited, the fate of preexisting host mRNA is unknown. In addition, the rate of inhibition of host cell protein synthesis is dependent on the multiplicity of viral infection (16, 28, 29, 30). At a multiplicity of infection of 10 PFU/cell, Mudd and Summers (15) showed by polyacrylamide gel electrophoresis on 7.5% gels that within 3 to 4 h after VSV infection there is a striking inhibition of host-specific polypeptide synthesis. However, those studies were performed on a gel system of relatively low resolving power as compared with the polyacrylamide gradient slab gel used here. Furthermore, Wertz and Youngner (29) found that in VSV-infected L cells at a multiplicity of infection of 10, a 90% inhibition of host protein synthesis does not occur until 8 h after infection. Thus, the possibility exists that under the conditions used in the experiments reported here, selected host cell proteins may still be synthesized from pre-existing mRNA.

The size distribution of proteins synthesized *in vitro* by nonstimulated membrane-bound polysomes isolated from uninfected HeLa cells is very similar to the proteins synthesized by nonstimulated VSV-infected membrane polysomes. In particular, cellular proteins with the same molecular weights and in almost the same relative amounts as P_{115} , P_{88} , and P_{72} are present.

If these three proteins are indeed cellular proteins, it is quite interesting that of all the cellular proteins synthesized in uninfected cells only a very limited number of cellular messages are able to functionally survive VSV infection.

Since proteins P_{115} , P_{88} , and P_{72} apparently are not precursors to virion proteins and are possibly host cell proteins, it is surprising that the synthesis of these proteins is not enhanced when incorporation of [35 S]methionine by VSV-infected membrane-bound polysomes is stimulated with HeLa S10. A reasonable explanation is that VSV-infected membrane-bound polysomes contain much more viral mRNA than host mRNA, and the host mRNA cannot successfully compete for the translational factors present in S10. However, we have no explanation for the preferential synthesis of proteins P_{115} , P_{88} , and P_{72} as compared to viral structural proteins G, N, and M in non-S10 stimulated extracts.

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