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Translation of Vesicular Stomatitis Messenger RNA by Extracts from Mammalian and Plant Cells

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RNA was isolated from polyribosomes of vesicular stomatitis virus (VSV)-infected cells and tested for its ability to direct protein synthesis in extracts of animal and plant cells. In cell-free, non-preincubated extracts of rabbit reticulocytes, the 28*S* VSV RNA stimulated synthesis of a protein the size of the vesicular stomatitis virus L protein whereas the 13 to 15*S* RNA directed synthesis of the VSV M, N, NS, and possibly G proteins. In wheat germ extracts, 13 to 15*S* RNA also directed synthesis of the N, NS, M, and possibly G proteins. Analysis of extracts labeled with formyl [^{35}S]methionine showed that the 28*S* RNA directed the initiation of synthesis of one protein, whereas the 13 to 15*S* RNA directed initiation of at least four proteins. It is concluded that the 28*S* RNA encodes only the L protein, whereas the 13 to 15*S* RNA is a mixture of species, presumably monocistronic, which code for the four other known vesicular stomatitis virus proteins.

Vesicular stomatitis virus (VSV) is a negative-strand RNA virus: the virion contains a single molecule of RNA (2, 8) that is complementary to the viral mRNA (5). Viral mRNA is defined as that RNA found on polyribosomes after VSV infection (5). The mRNA is transcribed from both the parental and progeny negative strands (7). Centrifugation of viral mRNA in sucrose gradients resolves it into two size classes: a homogeneous RNA species sedimenting at 28*S* and a heterogeneous group of RNA species sedimenting between 13 and 15*S* (5). Electrophoresis of the mRNA on polyacrylamide gels further resolves the 13 to 15*S* species into at least three species, whereas the 28*S* RNA migrates as a homogeneous molecule (1, 19).

After VSV infection, host protein synthesis is rapidly inhibited (9) and virus-specific proteins are readily resolved by electrophoresis on polyacrylamide gels. Five major virus-specific proteins are made, all of which are found in the mature virion (3, 10, 17, 26). Which mRNA species codes for each protein is unknown but assignments have been made by comparing the coding capacity of each mRNA species with the size of the viral protein and assuming that each mRNA is monocistronic (M. Stampfer, Ph.D. thesis, Mass. Inst. Technology, 1972). Tentatively, the 13 to 15*S* mRNA species are thought to code for viral matrix (M) protein, the nucleocapsid (N) protein, the NS protein, and the glycoprotein (G), whereas the 28*S* mRNA species is thought to encode the viral large (L) protein (21).

In this paper we show that when RNA, isolated from the polyribosomes of VSV-infected cells, is added to cell-free extracts of several mammalian and plant cells, it directs the synthesis of viral-specific proteins. Furthermore, the 13 to 15*S* mRNA species direct the synthesis of the viral M, N, NS, and possibly G proteins, whereas the 28*S* mRNA directs the synthesis of a polypeptide the size of viral L protein. Finally, we present evidence that the viral mRNA is a collection of monocistronic messages and that there is very little post translational modification of the VSV proteins.

MATERIALS AND METHODS

Virus and cells. Stocks of pure standard B particles of the Indiana serotype of VSV were grown in Chinese hamster ovary (CHO) cells and purified as described previously (6, 22, 23, 25).

Purification of VSV mRNA. CHO cells growing at 37 C were infected with VSV at a multiplicity of 3 to 10 PFU/cell as described by Huang et al. (5), except that actinomycin D was used at a final concentration of 5 μg/ml. ^{3}H-uridine (70 Ci/mM, New England Nuclear Corp.; 5 μCi/ml) was added at 2 h postinfection. Infected cells were harvested at 4 h and disrupted in 1% NP-40 (Shell Oil Co.). Nuclei were removed by centrifugation and the cytoplasmic extract was layered on a 15 to 30% sucrose gradient made in 0.01 M NaCl, 0.01 M Tris (pH 7.4), 1.5 mM $MgCl_2$. The gradients were centrifuged at 95,000 × *g* for 2.5 h in a Beckman SW 27 rotor. Fractions were collected through a Gilford recording spectrophoto-

meter, and the polyribosome region of the gradient was pooled. Polyribosomes were precipitated with two volumes of ethanol and resuspended in buffer containing 0.5% sodium dodecyl sulfate, 0.01 M Tris, pH 7.4, 0.1 M NaCl, and 0.001 M EDTA. The solubilized RNA was layered on a 15 to 30% sucrose gradient in the same buffer and was centrifuged at 60,000 × *g* for 17 h in the Beckman SW 27 rotor in order to resolve the VSV 13 to 15*S* and the VSV 28*S* mRNA (23). The fractions containing the 28*S* mRNA were pooled as were the fractions containing the 13 to 15*S* RNA species. The RNA was twice precipitated with ethanol and finally dissolved in water.

Preparation of cell extracts for protein synthesis. Cell extracts derived from Krebs II mouse ascites cells, CHO cells, the rabbit reticulocytes were prepared as described by McDowell et al. (15) and Housman et al. (4). Cell extracts derived from wheat germ were prepared as described by Roberts and Paterson (18). All extracts except the reticulocyte extracts were preincubated.

Cell-free protein synthesis. Conditions for cell-free protein synthesis in CHO and ascites extracts are described by McDowell et al. (15). Conditions for reticulocyte cell-free synthesis have been described by Housman et al. (4) with the modification that reactions containing VSV 28*S* mRNA contained 100 mM KCl and 400 μg of rabbit tRNA per ml (General Biochemicals). Wheat germ cell-free reactions (50 μliters) contained per ml 20 μmol of Hepes, pH 7.6, 2 μmol of dithiothreitol, 1 μmol of ATP, 0.02 μmol of GTP, 8 μmol of creatine phosphate, 40 μg of creatine phosphokinase, 0.020 to 0.030 μmol of unlabeled amino acids, 90 μmol of KCl, 3 μmol of Mg acetate, 0.20 ml of S-30 and 250 μCi of [^{35}S]methionine (120 Ci/mM, New England Nuclear Corp.).

Polyacrylamide gel electrophoresis. Cell-free reactions were terminated by the addition of ribonuclease A (50 μg/ml). The products of the reaction were resolved on 5 or 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) and 8 M urea as described previously (14).

Hydroxylapatite chromatography. Radioactively labeled proteins [^{35}S] made in cell-free reactions and ^{3}H-labeled viral proteins made in infected cells were mixed and fractionated together by electrophoresis through polyacrylamide gels. The location of the ^{35}S-labeled products was determined by autoradiography of the dried gels. Each double-labeled [^{35}S/^{3}H] polypeptide was eluted from the gel in 0.5% SDS and chromatographed on hydroxylapatite columns as described by Moss and Rosenblum (16).

High voltage paper electrophoresis. For analysis of initiator tryptic peptides, formyl [^{35}S] methionyl $tRNA_{metf}$ was included in the cell-free reactions (4) and tryptic peptides containing [^{35}S]fmet were prepared and analyzed by paper ionophoresis at pH 3.5 as described previously (13).

Marker virion and cytoplasmic proteins. Radioactively labeled [^{3}H] or [^{14}C] leucine virus was purified (6) and viral proteins were solubilized in 1% SDS. Viral proteins present in cytoplasmic extracts of VSV-infected cells were prepared as described by Stampfer (M. Stampfer, Ph.D. thesis, Mass. Inst. of Technology, 1972).

RESULTS

The rabbit reticulocyte cell-free system was chosen for initial studies of translation of VSV mRNA because of previous success with this extract in studies of reovirus cell-free protein synthesis: of the five extracts tested, only nonpreincubated extracts of rabbit reticulocytes would direct synthesis of all eight known reovirus proteins, including the two largest proteins (λ_1 and λ_2) which have molecular weights of 155,000 and 140,000 (15).

Nonpreincubated reticulocyte extracts synthesize a variety of cell proteins in addition to globin (12). Addition of mRNA to such extracts leads to an inhibition of overall incorporation of amino acids, but the synthesis of virus-specific proteins can be detected as new radioactively labeled polypeptides evident after electrophoresis of the cell-free reaction products on polyacrylamide gels.

Translation of VSV 13 to 15S mRNA. VSV 13 to 15*S* RNA, prepared from virus-specific polyribosomes, was added to a cell-free system derived from rabbit reticulocytes. After incubation at 25 C for 90 min, the reaction products (labeled with [^{35}S]methionine) were analyzed by electrophoresis on 10% polyacrylamide gels. Autoradiographs of the dried gels are presented in Fig. 1. Marker proteins derived from [^{14}C]leucine-labeled virion and from [^{14}C]leucine-labeled viral proteins made in the cytoplasm of infected cells were analyzed in parallel.

Radioactively labeled proteins approximately the size of authentic viral M, N, and NS proteins were present among the products of the cell-free reaction directed by VSV 13 to 15*S* RNA but were absent from reactions directed only by endogenous reticulocyte mRNA (no added mRNA). However, a protein labeled in the reaction without added mRNA had the same mobility as viral G protein, so it is impossible to determine from this experiment whether the VSV 13 to 15*S* mRNA directs the synthesis of viral G protein.

In addition to the major virus-specific products of the cell-free reaction, there was an additional polypeptide migrating between the M and NS proteins. This polypeptide was also present in the cytoplasmic protein marker but not in the virion marker and it comigrated with a major protein present in uninfected CHO cells (data not shown). It was presumably encoded by residual host mRNA still associated with the polyribosomes after VSV infection.

Figure 2 portrays densitometer scans of autoradiographs of polyacrylamide gels similar to those shown in Fig. 1. As can be seen, the 13 to

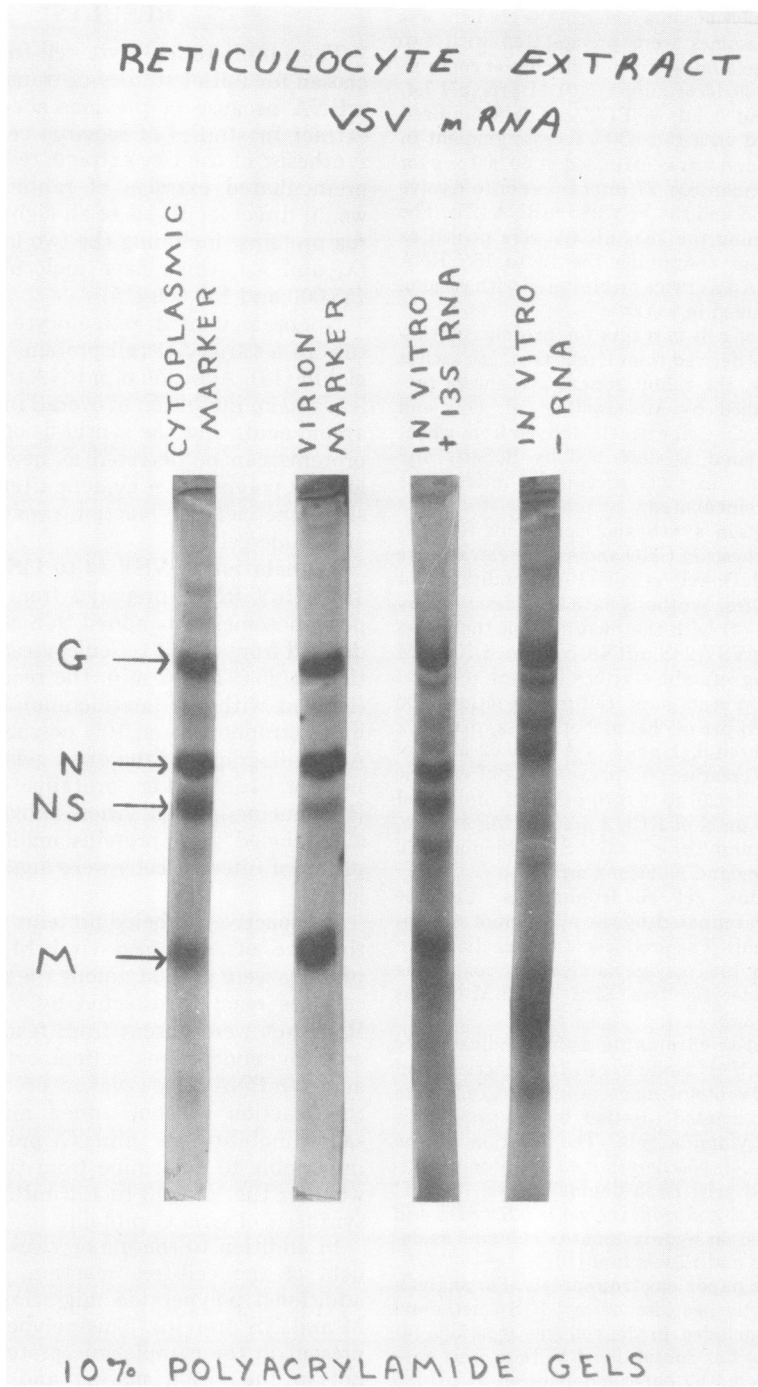

FIG. 1. *Acrylamide gel electrophoresis of 13 to 15S RNA-directed products. Reactions (50 μliters) contained extract from rabbit reticulocytes, VSV 13 to 15S RNA and [^{35}S]methionine. After incubation, the reaction was layered on 10% acrylamide gels (0.6 by 12 cm) which were run for 24 h at 4 mA/gel. The top of the gel is at the top of the figure. Marker proteins were labeled with [^{14}C]leucine. The exact concentration of VSV RNA present in our preparations is impossible to determine because of contaminating ribosomal RNA. The amount of RNA optimal for these extracts was determined by adding various amounts of the RNA preparation to reaction mixtures and separating the products of the reaction on polyacrylamide gels.*

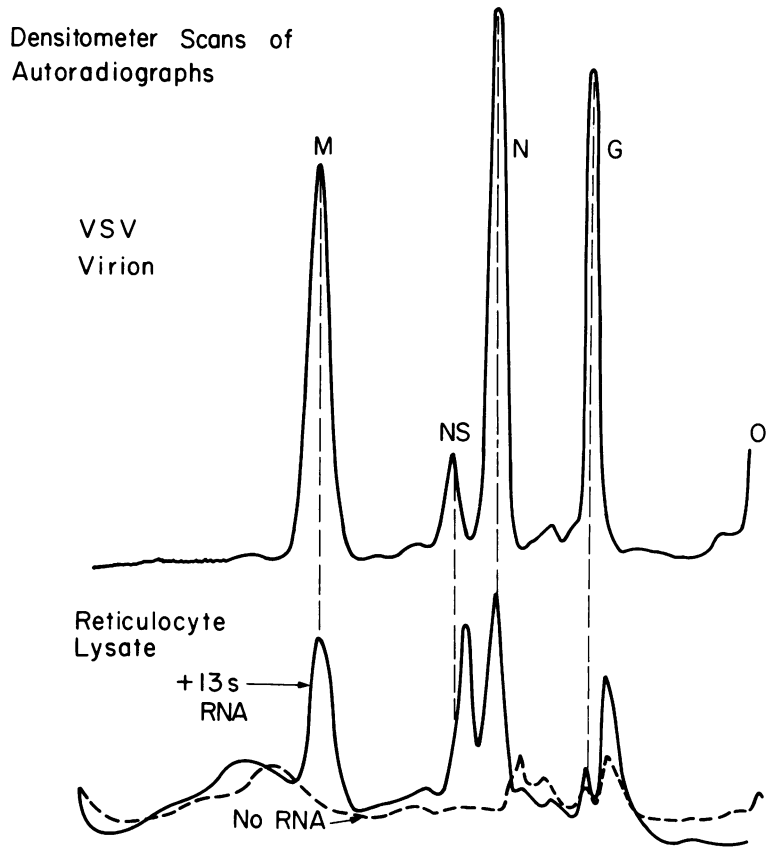

FIG. 2. *Densitometer scans of autoradiographs. See legend to Fig. 1.*

15*S* mRNA-directed reaction products contained polypeptides with electrophoretic mobilities identical to viral M and N proteins, but the authentic NS protein was smaller (by 2,000 to 3,000 molecular weight) than the corresponding product of the cell-free reaction.

To confirm that the products of the reticulocyte cell-free system indeed are authentic VSV proteins, we analyzed the proteins by chromatography on hydroxylapatite in buffers containing SDS. This procedure separates polypeptides on the basis of properties other than molecular weight (16). [^{35}S]labeled products directed by 13 to 15*S* RNA were mixed with [^{3}H]labeled proteins made in the cytoplasm of VSV-infected cells. The labeled proteins were resolved by electrophoresis through polyacrylamide gels and the location of the [^{35}S] labeled protein was determined by autoradiography. Each [^{35}S] and [^{3}H] labeled polypeptide was eluted in 0.5% SDS and analyzed by chromatography on a hydroxylapatite column with a sodium phosphate gradient. Figures 3 to 5 show that authentic viral [^{3}H] labeled M, NS, and N proteins cochromatographed with the corresponding protein made in the cell-free reaction. Hydroxylapatite column chromatography of the N protein, produced both in vivo and in cell-free protein synthesizing reactions, gave rise to two peaks. This phenomenon is currently under investigation. The shoulder present in the elution pattern of NS protein produced in the cell-free system is due to contaminating N protein which migrates very close to NS in the preparative polyacrylamide gel.

As noted above, reticulocyte cell-free reactions without added mRNA synthesize a protein which comigrates on polyacrylamide gels with VSV G protein. Cochromatography of this reticulocyte protein with authentic G protein showed that hydroxylapatite was able to partially separate the reticulocyte protein from G protein (Fig. 6). The products of the cell-free reaction directed by VSV 13 to 15*S* mRNA which migrate on acrylamide gels in the position of the authentic G protein cochromato-

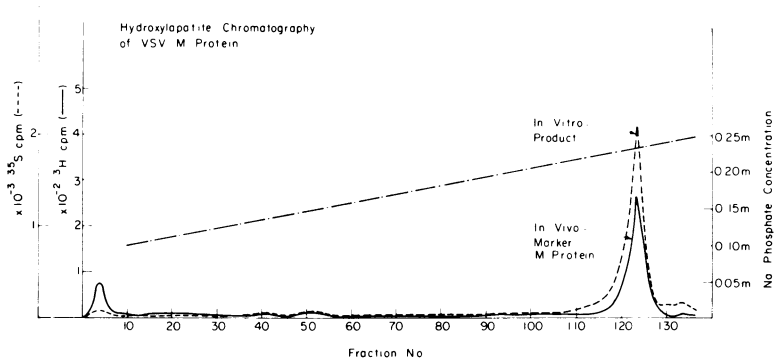

FIG. 3. *Hydroxylapatite chromatography of VSV M protein. Reactions (50 μliters) contained extract from reticulocytes, optimal amounts of VSV 13 to 15S RNA and [^{35}S]methionine. [^{3}H]leucine-labeled cytoplasmic viral proteins were mixed with the reaction products. Samples were run on 6% acrylamide gels for 36 h. The M protein was eluted and chromatographed on hydroxylapatite columns with a gradient of 0.05 to 0.25 M sodium phosphate, pH 6.4. Symbols: ——, marker protein; -----, cell-free reaction product.*

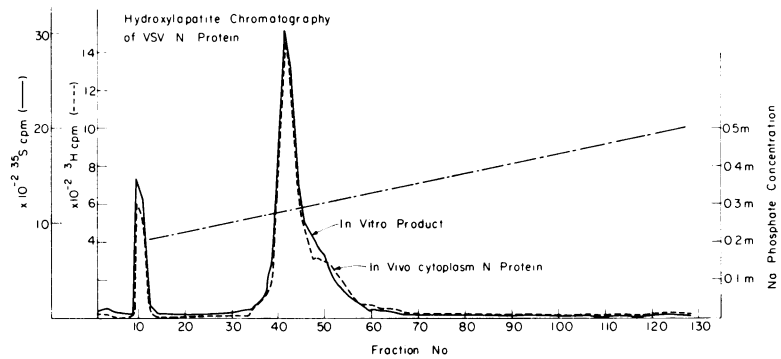

FIG. 4. *Hydroxylapatite column chromatography of VSV N protein. See legend to Fig. 3. The hydroxylapatite column was eluted in a sodium phosphate gradient of 0.2 to 0.5 M. Symbols:——, marker protein; -----, in vitro product.*

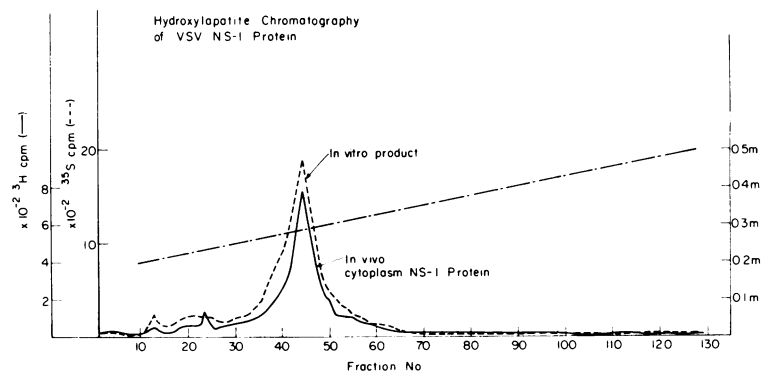

FIG. 5. *Hydroxylapatite column chromatography of VSV NS protein. See legend to Fig. 4. Symbols: ——, marker protein; -----, in vitro product.*

graphed exactly with authentic [^{3}H]-labeled G protein (Fig. 7). There was also a shoulder of [^{35}S] radioactivity which eluted after the G protein which could represent the reticulocyte background protein. This result suggests that 13 to 15*S* mRNA is able to direct synthesis of VSV G protein.

In order to investigate the synthesis of the viral G protein in cell-free reactions containing 13 to 15*S* mRNA, we used extracts from other types of cells. VSV 13 to 15*S* mRNA was added to extracts derived from Krebs II mouse ascites cells and from wheat germ. The [^{35}S]-labeled reaction products were resolved by electrophoresis on polyacrylamide gels. Figure 8 shows that with both extracts, 13 to 15*S* RNA-directed synthesis of material which comigrated with authentic VSV, M, NS, and N proteins. Ascites cell extracts did not synthesize any material comigrating with VSV G protein; however, a small amount of labeled protein migrating like G protein was evident in the wheat germ reaction. This is further evidence that 13 to 15*S* RNA codes for the viral G protein.

Translation of VSV 28S mRNA. The 28*S* VSV polyribosomal RNA directed synthesis of a polypeptide of very high molecular weight in cell-free extracts derived from rabbit reticulocytes (Fig. 9). Reactions containing 13 to 15*S* RNA or reactions without added mRNA formed no such polypeptide. This new, very large polypeptide comigrated with authentic VSV L protein (Fig. 9) derived from VSV virions.

Some polypeptides of the size of VSV N and

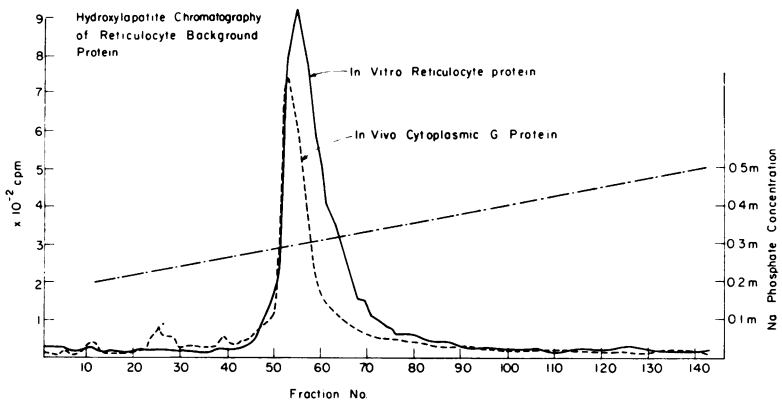

FIG. 6. *Hydroxylapatite column chromatography of the reticulocyte protein of 69,000 molecular weight. Reactions were processed as outlined in legend to Fig. 4 except that VSV mRNA was omitted from the cell-free reaction. Symbols: ——, VSV G protein; -----, reticulocyte protein.*

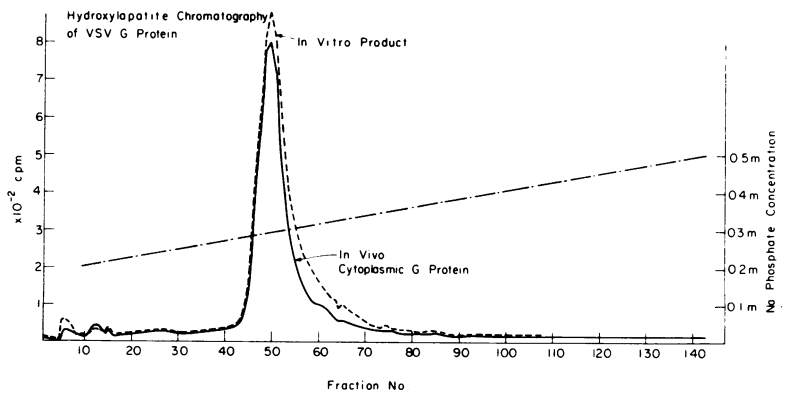

FIG. 7. *Hydroxylapatite column chromatography of G-sized polypeptide made in reticulocyte extracts directed by VSV 13 to 15S RNA. See legend to Fig. 4. Reactions of 150 μliters were used for preparation of this polypeptide. Symbols: ——, VSV G protein; -----, in vitro product.*

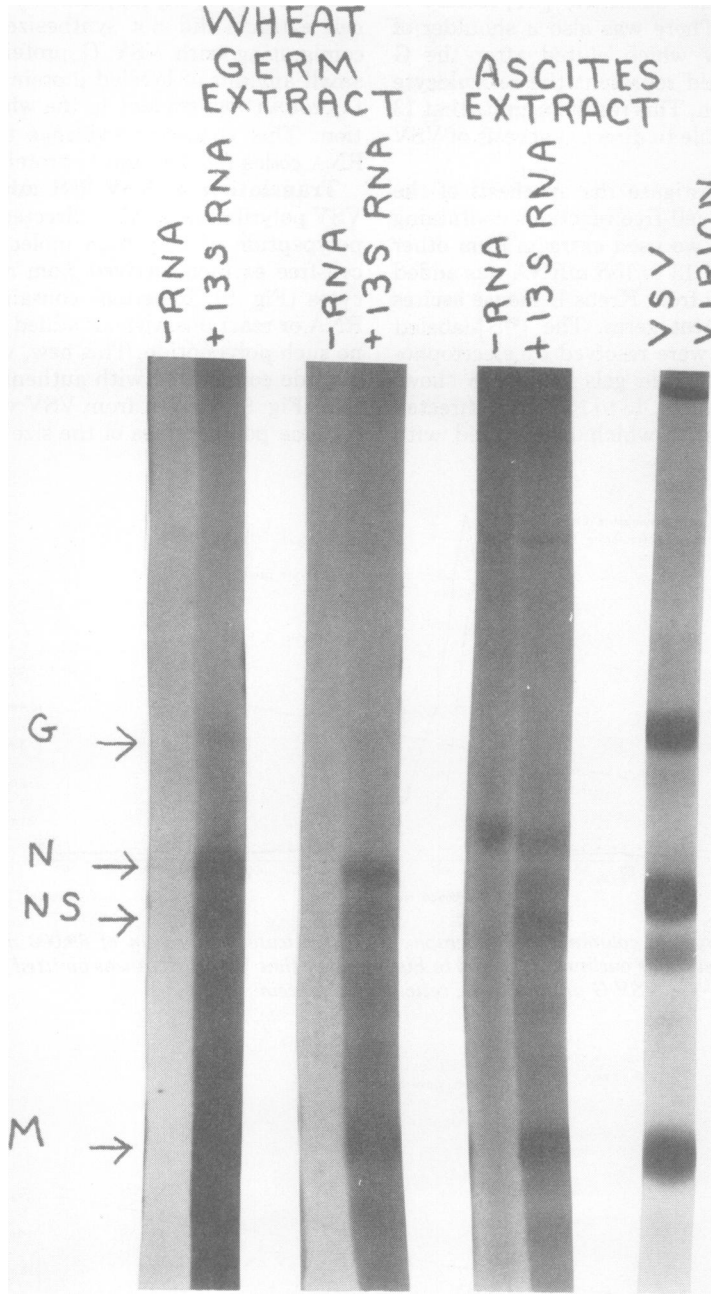

FIG. 8. *Polyacrylamide gel electrophoresis of VSV products made in ascites cell and wheat germ extracts. Reactions (50 μliters) contained optimal amounts of VSV RNA. Products were electrophoresed on 7.5% acrylamide gels (0.6 to 12 cm) for 24 h at 4 mA/gel. The top of the gel is at the top of the figure. The two sets of wheat germ gels are autoradiographs of the same gel which have been exposed for different times to X ray film.*

NS proteins were also formed under direction of the 28*S* RNA. However, the amounts of these polypeptides made varied with the VSV 28*S* mRNA preparation. Their synthesis is presumably due to the efficient translation of residual N and NS mRNA which contaminate all 28*S* mRNA preparations (1; M. Stampfer, thesis, Mass. Inst. of Technology; 1972).

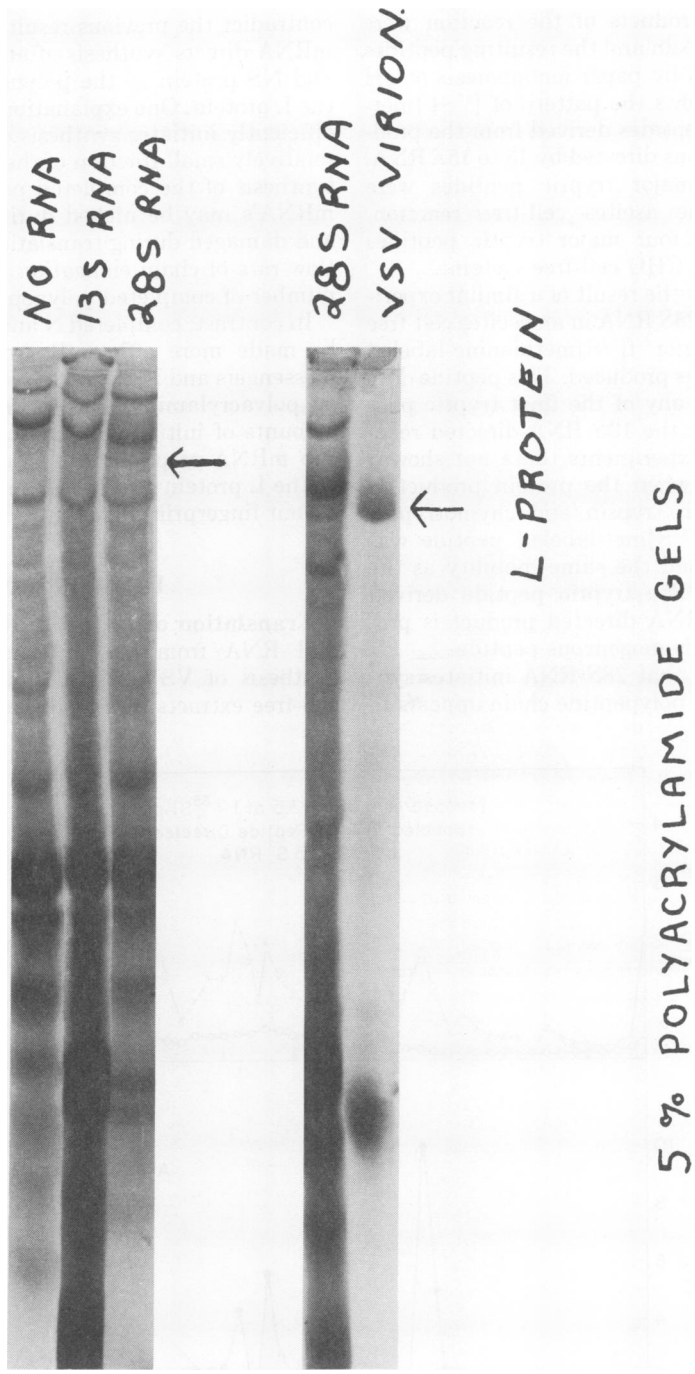

FIG. 9. *Polyacrylamide gel electrophoresis of 28S RNA-directed products. Reactions (50 μliters) contained optimal amounts of 13 to 15S RNA or VSV 28S RNA. The products were electrophoresed on 5% gels for 40 h. Marker proteins are from [^{14}C]leucine-labeled virion.*

Initiation tryptic peptides. In order to estimate the number of independent initiation sites of VSV mRNA, [^{35}S] formylmethionyl tRNA$_f^{met}$ (4) was added to reactions directed by VSV 13 to 15*S* RNA or VSV 28*S* mRNA. Extracts of ascites cells or CHO cells were used for these experiments because these extracts exhibit a relatively low amount of initiation of endoge-

nous proteins. Products of the reaction were digested with trypsin and the resulting peptides were fractionated by paper ionophoresis at pH 3.5. Figure 10 shows the pattern of [^{35}S] fmet-labeled tryptic peptides derived from the products of the reactions directed by 13 to 15*S* RNA. At least three major tryptic peptides were derived from the ascites cell-free reaction, whereas at least four major tryptic peptides were made in the CHO cell-free system.

Figure 11 shows the result of a similar experiment using VSV 28*S* RNA in an ascites cell-free system. One major f[^{35}S]methionine-labeled tryptic peptide was produced. This peptide does not migrate with any of the fmet tryptic peptides produced in the 13*S* RNA-directed reaction. In similar experiments (data not shown) we showed that when the protein product is treated with both trypsin and chymotrypsin again a single f[^{35}S]met-labeled peptide was obtained which had the same mobility as the tryptic peptide. The tryptic peptide derived from the 28*S* mRNA directed product is presumably a single homogeneous peptide.

The conclusion that 28*S* RNA initiates synthesis of only one polypeptide chain appears to contradict the previous result (Fig. 9) that 28*S* mRNA directs synthesis of at least as much N and NS protein as the polypeptide the size of the L protein. One explanation is that 28*S* RNA efficiently initiates synthesis of L protein but a relatively small fraction of these starts result in synthesis of the completed polypeptide. Large mRNA's may be nicked during their isolation and damaged during translation. In addition, a slow rate of chain elongation would reduce the number of completed polypeptide chains.

In contrast, completed N and NS protein may be made more efficiently from their smaller messengers and N and NS proteins are detected on polyacrylamide gels. However, the relative amounts of initiation of these proteins by the 28*S* mRNA preparation is much less than that of the L protein and are essentially undetected by our fingerprinting assay (Fig. 10).

DISCUSSION

Translation of 13 to 15S RNA. Polyribosomal RNA from VSV-infected cells directed synthesis of VSV M, N, and NS proteins in cell-free extracts. Evidence has been presented

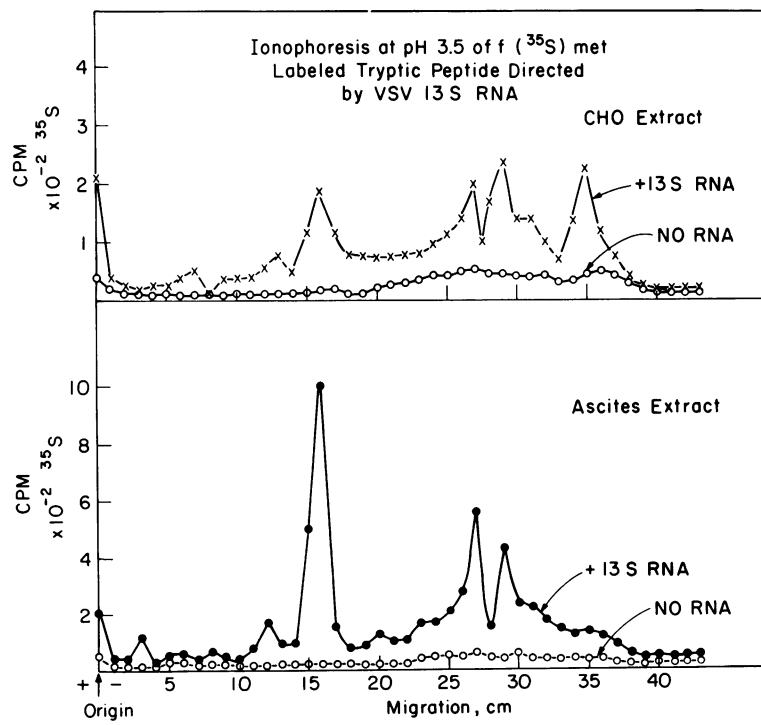

FIG. 10. [^{35}S] *formyl methionine labeled tryptic peptides from 13 to 15S RNA protein product. Ascites and CHO reactions (100 μliters) contained optimal amounts of VSV 13 to 15S RNA and* [^{35}S] *formyl methionyl tRNA. Reaction was incubated for 40 min at 30 C. The minor peptide migrating at position 12 is free methionine.* ——, *13 to 15S RNA;* -----, *no added RNA.*

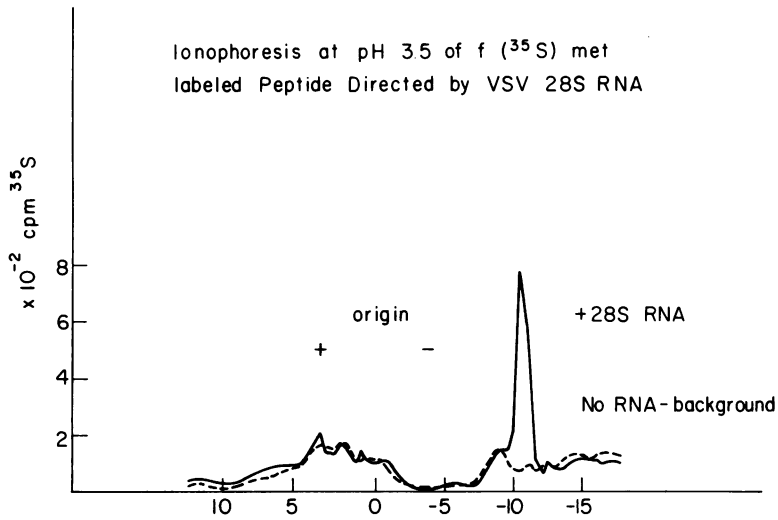

FIG. 11. [^{35}S] *formyl methionine tryptic peptide derived from VSV 28S RNA protein product. See legend to Fig. 10.*

which suggests that polysomal RNA from VSV-infected cells also directs VSV L and G protein synthesis. The heterogeneous group of VSV mRNA's which sediment at 13 to 15*S* appeared to encode the smallest viral proteins: N, NS, M, and possibly G. Identity of the proteins made in vitro with authentic VSV proteins was shown mainly by co-electrophoresis in SDS-polyacrylamide gels. In the case of proteins M, NS, N, and G synthesized by reticulocyte lysates, it was also shown that the polypeptides co-chromatograph on hydroxylapatite columns with the corresponding authentic VSV proteins.

The NS protein synthesized in the reticulocyte lysate appeared to be 1 to 2% larger than the authentic VSV NS protein. Presumably, this difference in molecular weight is due to synthesis in vitro of a precursor polypeptide which is usually modified intracellularly by removal of a few amino acids. It could be argued that the in vitro product is not related to NS protein but is some new polypeptide. The co-chromatography of the cell-free product with NS protein on hydroxylapatite, however, suggests that the two proteins are related. Further analysis of this question is being pursued.

As judged by the number of [^{35}S]formyl methionine initiation tryptic peptides derived from reaction products directed by the 13 to 15*S* RNA, these RNAs direct the initiation of synthesis of four peptides. Each of these [^{35}S]fmet peptides is presumably derived from one of the VSV proteins, M, NS, N, and G, although we have not yet determined which peptide corresponds to which protein. This finding adds support to the idea that each of these four proteins is synthesized by an independent initiation event, and that no two of these proteins are derived from a longer precursor protein. This point will be conclusively established if it can be shown directly that each of the completed proteins contains an N-terminal f[^{35}S] methionine residue.

Previous work on translation of poliovirus RNA (24), EMC RNA (20), and reovirus mRNA (15) in cell-free extracts from reticulocytes, ascites, and CHO cells has provided no evidence that these extracts are capable of post-translational proteolytic cleavage of large protein molecules. Hence, the fact that all of the VSV proteins made in these lysates (with the possible exception of NS) have virtually the same molecular weights as the authentic proteins is also consistent with the idea that they are not derived from much larger precursor proteins.

Translation of VSV 28S RNA. Work presented here showed that addition of the 28*S* mRNA to a cell-free protein synthesizing system from rabbit reticulocytes results in the synthesis of a polypeptide the size of the L protein. That the 28*S* RNA is a homogeneous species of messenger is also suggested by the fact that 28*S* RNA directs the synthesis of protein which contains one initiator tryptic peptide. These results are consistent with the work of Stampfer and Baltimore (21) which showed that the VSV L protein is a unique viral protein and not an aggregate of one of several VSV proteins.

VSV G protein. Relative to the VSV M, N,

and NS proteins, the VSV G protein is made in very small amounts in wheat germ and possibly in rabbit reticulocyte extracts. The reason for this result is unknown, but it is possible that either the mRNA which encodes viral G protein is present in small amounts in the VSV 13 to 15*S* RNA preparation, or the conditions for VSV G protein synthesis are not optimal. This question is now under investigation. We emphasize that we do not know whether or not the G protein made in any of these extracts is glycosylated.

Similar studies of D. Kingsbury with Sendai virus mRNA have also shown negligible synthesis of viral glycosylated proteins in cell-free systems (11). Grubman and Summers (3a) using extracts of VSV-infected cells have synthesized in cell-free reactions polypeptides corresponding in size to authentic VSV proteins.

ACKNOWLEDGMENTS

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