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Translation and Identification of the Viral mRNA Species Isolated from Subcellular Fractions of Vesicular Stomatitis Virus-Infected Cells

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The cytoplasm of vesicular stomatitis virus (VSV)-infected BHK cells has been separated into a fraction containing the membrane-bound polysomes and the remaining supernatant fraction. Total poly(A)-containing RNA was isolated from each fraction and purified. A 17*S* class of VSV mRNA was found associated almost exclusively with the membrane-bound polysomes, whereas 14.5*S* and 12*S* RNAs were found mostly in the postmembrane cytoplasmic supernatant. Poly(A)-containing VSV RNA synthesized in vitro by purified virus was resolved into the same size classes. The individual RNA fractions isolated from VSV-infected cells or synthesized in vitro were translated in cell-free extracts of wheat germ, and their polypeptide products were compared by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. The corresponding in vivo and in vitro RNA fractions qualitatively direct the synthesis of the same viral polypeptides and therefore appear to contain the same mRNA species. By tryptic peptide analysis of their translation products, the in vivo VSV mRNA species have been identified. The 17*S* RNA, which is compartmentalized on membrane-bound polysomes, codes for a protein of molecular weight 63,000 (P_{63}) which is most probably a nonglycosylated form of the viral glycoprotein, G. Of the viral RNA species present in the remaining cytoplasmic supernatant, the 14.5*S* RNA codes almost exclusively for the N protein, whereas the 12S RNA codes predominantly for both the NS and M proteins of the virion.

Vesicular stomatitis virus (VSV) is a rhabdovirus (7) which contains a single-stranded RNA genome (~4 × 10^6 daltons) of negative polarity (8, 16). In infected cells, VSV synthesized mRNA species of size classes 13 to 15*S* and 28*S* (7, 14, 16, 17, 20). In addition, similar RNA species, with sedimentation coefficients of 12 to 18*S* and 31*S*, are transcribed in vitro by the virion-associated RNA polymerase in purified virus (11). Recently, we demonstrated that in cell-free extracts of wheat germ 12 to 18*S* RNA synthesized in vitro can function as message for four of the five VSV structural polypeptides (3). In addition, we partially fractionated the in vitro 12 to 18*S* RNA into three size classes with sedimentation coefficients of 17*S*, 14.5*S*, and 12*S* and identified the mRNA(s) in each class by the protein(s) for which it codes.

Previous reports suggest that the VSV mRNAs and proteins may be compartmentalized within the infected cell (18, 19). Experiments utilizing polypeptide chain completion in vitro by membrane-bound polysomes from infected cell lysates suggested that they contain primarily the mRNA for the viral glycoprotein G (4, 5). Direct analysis of the RNA species of the membrane-bound polysomes indicated that this fraction was greatly enriched for a single 17*S* viral mRNA species which was entirely absent from the remaining cytoplasmic supernatant (5, 6). Our work with the in vitro synthesized VSV messages (3) indicated that translation might be a useful tool for the identification of the mRNAs in subcellular fractions of VSV-infected cells. In this report we show that the smaller in vivo VSV mRNAs, sedimenting from 12 to 18*S*, also contain three classes of RNA which are compartmentalized in the cell and code for four VSV proteins.

MATERIALS AND METHODS

Purification of the virus. The procedures for the growth and purification of VSV (Indiana serotype) in BHK cells and the preparation of [^{35}S]methionine-labeled virus have been previously described (3, 12).

Purification of VSV mRNA species synthesized in vivo and in vitro. BHK cells (4 × 10^6 cells/ml) were infected with VSV at a multiplicity of infection

of 10 PFU/cell (2) and labeled with [^{3}H]uridine (15 μCi/ml; specific activity 27.7 Ci/mmol; New England Nuclear, Boston, Mass.) from 2.5 to 5 h after infection. A cell extract was prepared after Dounce homogenization and separated into a fraction containing membrane-bound polysomes and a cytoplasmic supernatant fraction from which the membrane-bound polysomes were removed (5, 15). The RNA from each fraction was recovered following phenol-chloroform extraction by precipitation with ethanol and purified by binding to and elution from an oligo(dT)-cellulose column (2). The total poly(A)-containing membrane-bound polysomal RNA and, similarly, the cytoplasmic supernatant RNA was heated in the presence of urea (3), and the components were separated by sedimentation of the samples on 5 to 30% glycerol gradients containing 20 mM Tris-hydrochloride (pH 7.5), 100 mM NaCl, and 5 mM EDTA in the Beckman SW41 rotor at 32,500 rpm for 17 h at 4 C. The major RNA peak from the membrane-bound polysomes and the two RNA peaks from cytoplasmic supernatant were repurified by urea treatment and sedimentation for 19 h on glycerol gradients as described above.

VSV-specific RNA was synthesized in vitro from 1 mg of purified virus (11) and the 17*S*, 14.5*S*, and 12*S* RNA species were fractionated by gradient centrifugation as previously described (3). Prior to being used for translation, RNA was washed with 95% ethanol and dried with N_2.

Preparation of wheat germ extracts and protein synthesis assays. The preparation of wheat germ extracts and the conditions used for protein synthesis have been described (3).

Polyacrylamide gel electrophoresis and analysis of tryptic peptides. The conditions for analysis of viral and in vitro synthesized proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis have been described (3). The conditions for the purification of proteins, digestion with trypsin, and analysis of the peptides by ion exchange chromatography were as follows. In vitro synthesized proteins and [^{3}H]methionine-labeled VSV polypeptides were separated by electrophoresis on a 10% polyacrylamide-SDS slab gel as described (3). The gel was stained, the regions containing the desired protein were cut out, and the polypeptide was subjected to electrophoresis from the combined gel fragments (1). After the addition of 500 μg of bovine serum albumin as carrier, the protein was recovered by precipitation with 10 volumes of acetone-hydrochloride (40:1, vol/vol, 1 N HCl). The double-labeled protein pellet was washed twice with ethanol-ether (1:1) and dried with N_2, and a sample of the preparation was reanalyzed on a gel to ensure its homogeneity. The pellet containing both ^{3}H and [^{35}S]methionine-labeled protein was dissolved in 1.0 ml of 1% ammonium bicarbonate containing phenol red as indicator and adjusted to pH 7.8 with ammonium hydroxide. Trypsin (1 mg; TPCK treated; Worthington Biochemical Corp., Freehold, N.J.) was added to the sample, which was incubated at 37 C at pH 7.8 for 16 h. The sample was centrifuged at 2,000 rpm in the Sorvall RC-3 to remove the slight insoluble residue, and the supernatant was diluted with 3 volumes of 0.025 M pyridine-acetate (pH 4.0). The sample was applied to a column (0.9 by 30 cm) of ion exchange resin (Sphenix resin XX907-10, Phoenix Precision Instrument Co., Philadelphia, Pa.) which was equilibrated with 0.025 M pyridine-acetate (pH 4.0) and maintained at 57 C. Peptides adsorbed to the column were eluted with a gradient of 0.025 M (pH 4.0)-2.0 M (pH 5.8) pyridine acetate buffer contained in a four-chamber gradient (2 by 100 ml of 0.025 M pyridine acetate [pH 4.0], 100 ml of 0.5 M pyridine-acetate [pH 5.0], and 100 ml of 2.0 M puridine-acetate [pH 5.8]). Fractions were collected at a rate of 3 ml every 4 min. For the analysis of the peptides of P_{63} and P_{60} (see below), 0.05 M buffer was used. The samples were dried in an oven to remove pyridine, dissolved in 1 ml of water, and counted in 10 ml of Aquasol. Radioactivity was measured in a Beckman scintillation spectrometer, and corrections were made for the spill of ^{35}S into the ^{3}H channel.

RESULTS

Fractionation and translation of in vivo and in vitro 12 to 18S VSV mRNAs. A cytoplasmic extract of VSV-infected BHK cells labeled with [^{3}H]uridine was prepared as described above. The extract was separated into a membrane-bound polysome fraction and the remaining cytoplasmic supernatant fraction by the procedure of Grubman et al. (5). Total RNA was recovered from each fraction after phenol extraction, and the poly(A)-containing RNA was purified by oligo(dT)-cellulose chromatography (2). Analysis of the RNA by velocity sedimentation showed that the post-membrane cytoplasmic supernatant of VSV-infected BHK cells contained predominantly the 14.5*S* (fraction II) and 12*S* (fraction III) classes of RNA, but very little 17*S* (fraction I) RNA, whereas the membrane-bound polysomal RNA was greatly enriched for the latter class (data not shown), a result similar to that previously found in VSV-infected HeLa cells (5, 6). Each class of RNA was treated with urea and purified (to a homogeneous peak) by velocity sedimentation as described above and in the legend to Fig. 1. Similarly, the corresponding classes of RNA were purified from the products synthesized in vitro by purified virus (3. 11).

We have previously demonstrated that each class of VSV mRNA synthesized in vitro can be translated in cell-free extracts of wheat germ into apparently authentic viral polypeptides (3). Therefore, to identify the in vivo VSV mRNA species and to study their distribution in the infected cell, the polypeptides directed by these RNA fractions and the corresponding classes of RNA synthesized in vitro were compared by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 2). Neither the total poly(A)-containing RNA prepared from

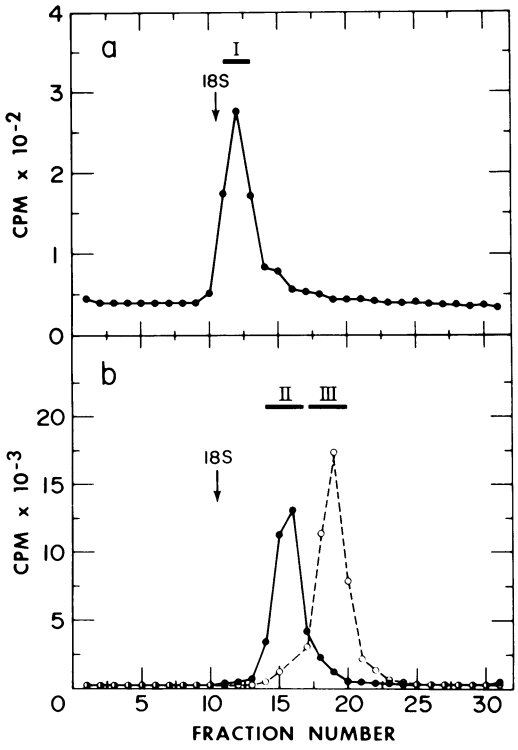

FIG. 1. *Purification of in vivo VSV mRNA species. The total poly(A)-containing membrane-bound polysomal RNA and, similarly, the cytoplasmic RNA fractions were prepared and centrifuged on glycerol gradients as described. The radioactivity in each fraction was determined by counting a portion in Aquasol (New England Nuclear, Boston, Mass.). Sedimentation was from right to left. The RNA was recovered from the indicated fractions I, II, and III by ethanol precipitation. (a) Membrane-bound polysomal RNA; (b) cytoplasmic supernatant RNA species. The RNA species in (b) were purified on individual gradients, and the data was plotted on the same graph. The position of 18S RNA from BHK cells was determined on a parallel gradient and is indicated by the arrow.*

the cytoplasmic extract of mock-infected cells (Fig. 2c) nor the wheat germ extract alone (Fig. 2b) directed the synthesis of any polypeptides which comigrated with the viral polypeptide markers. However, it is apparent that both the in vivo and the in vitro fraction I, II, and III VSV mRNAs code for similar polypeptides in wheat germ extracts, indicating that each in vivo mRNA fraction contains the same mRNA species as its counterpart synthesized in vitro. It seems that only the fraction I RNAs code for the proteins of molecular weights 63,000 (P_{63}) and 60,000 (P_{60}) (reference 3, Fig. 2d and e), whereas the fraction II RNAs direct the synthesis predominantly of a protein comigrating with the viral N polypeptide (Fig. 2f and g). Fraction III RNAs code almost exclusively for proteins comigrating with the viral NS and M polypeptides (Fig. 2h and i) and, therefore, appear to contain two mRNA species (3).

The other two bands present in Fig. 2a may correspond to those seen in Fig. 2c coded for by cellular RNA from mock-infected cells. Alternatively, they may be "early quitters" arising from premature termination during the synthesis of the NS protein, since these bands are also present in small amounts after translation of fraction III RNA synthesized in vitro (Fig. 2i). In any event, it was more apparent when electrophoresis was carried out for longer times that neither band comigrated with any viral marker polypeptides (data not shown).

Comparison by tryptic peptide analysis of authentic viral proteins with the polypeptides coded for by the in vivo VSV mRNA fractions. The authenticity of the N, NS, and M polypeptides coded for by the in vitro synthesized VSV mRNA fractions has already been demonstrated (3). However, in order that a more positive identification of the species present in the in vivo mRNA fractions can be made, we have compared the tryptic peptides of the translation products with those of authentic viral proteins.

It was suggested that P_{63}, the primary product coded for by the in vitro fraction I RNA, represents the nonglycosylated form of the viral glycoprotein G (3). We have compared P_{63} synthesized by the in vivo fraction I RNA with the viral G protein by ion exchange chromatography of their tryptic peptides. Figure 3a shows that there is considerable homology between the methionine-containing tryptic peptides of the two proteins, indicating that P_{63}, the largest protein coded for by the fraction I RNAs, may be the virus-specified polypeptide backbone of the viral glycoprotein G. In addition, it seems that P_{60}, which is always synthesized in vitro in a lesser amount than P_{63}, is also closely related to G, since it also has many methionine-containing peptides in common with G (Fig. 3b).

When the methionine-containing tryptic peptides of the N protein synthesized in vitro in response to the in vivo fraction II RNA were compared with those of the viral N protein, there was almost complete homology (Fig. 4a). A similar result was obtained when the viral M protein and the M polypeptide coded for by in vivo fraction III RNA were compared in the same way (Fig. 4b). In addition, Fig. 5a shows that the NS protein also coded for in vitro by the fraction III RNA isolated from fractionated

VSV-infected cells contains tryptic peptides common to the viral NS protein. There is, however, one major methionine-containing peptide (fractions 26 to 30) in the in vitro synthesized NS protein which has no counterpart in the viral polypeptide. Nevertheless, the in vitro synthesized NS protein comigrates with the viral NS polypeptide in two different SDS-polyacrylamide gel systems (Fig. 2h and j and Fig. 5b), where the NS proteins migrate with apparent molecular weights of 52,000 and 40,000, respectively (3). This suggests that despite the extra peptide, the NS protein synthesized in vitro is closely related to the viral NS polypeptide.

These results are consistent with (i) the

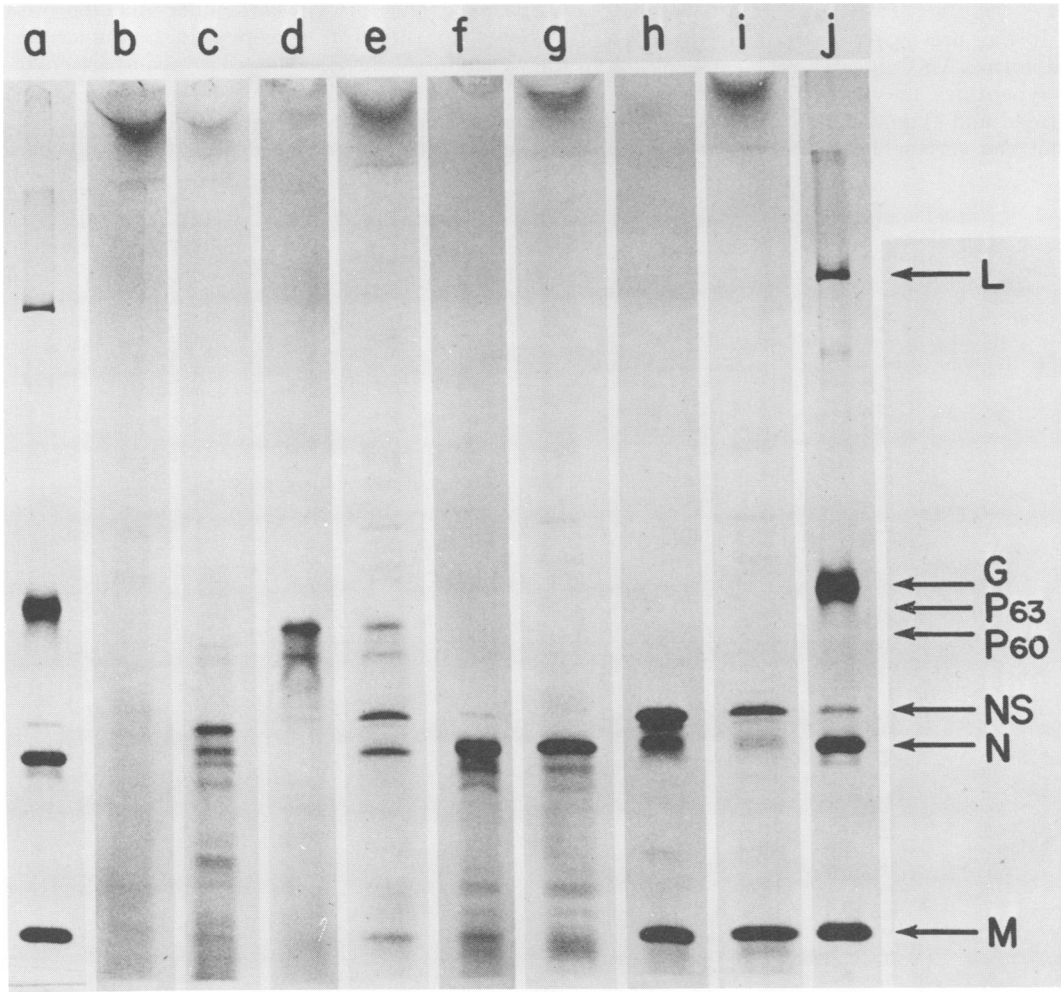

FIG. 2. *Comparison of polypeptides synthesized by wheat germ extracts in response to VSV RNA synthesized in vivo or in vitro. VSV mRNA species were extracted from fractionated infected cell extracts, and the purified classes I, II, and III were isolated as described in the legend to Fig. 1. The VSV 12 to 18S RNA synthesized in vitro from purified virus was fractionated and purified in a similar manner (3). Total poly(A)-containing RNA was prepared from a cytoplasmic extract of mock-infected cells by the procedures described. Each class of in vitro or in vivo RNA was used to direct the synthesis of virus-specific polypeptides in cell-free extracts of wheat germ during a 90-min incubation period using the conditions previously described (3). The preparation of the samples and the procedures for electrophoresis and autoradiography have been described (3). Migration is from top to bottom. (a) Proteins of the virion; proteins synthesized in wheat germ extracts (b) in the absence of RNA and (c) in response to RNA from mock-infected cells; fraction I RNA (d) from membrane-bound polysomes; (e) synthesized in vitro; fraction II RNA (f) from infected cell cytoplasmic supernatant; (g) synthesized in vitro; fraction III RNA (h) from infected cell cytoplasmic supernatants; (i) synthesized in vitro; and (j) marker proteins of purified virus.*

synthesis of the polypeptide backbone of the viral glycoprotein G, coded for by the fraction I RNAs, (ii) the synthesis of authentic N protein coded for by the fraction II RNAs, and (iii) the synthesis of authentic NS and M proteins coded for by the fraction III RNAs. The results also indicate that there are two mRNAs in fraction III RNA as discussed previously (3).

DISCUSSION

It was previously shown that the RNA isolated from VSV-infected cells can code for viral polypeptides in vitro (10), and we have confirmed and extended this observation. It seems that the corresponding classes of mRNA prepared from VSV-infected cells or synthesized in vitro from purified virus qualitatively direct the synthesis of the same viral proteins, as judged by their comigration on SDS-polyacrylamide slab gels.

We have demonstrated that the in vivo fraction I (17S) RNA, which is compartmentalized on membrane-bound polysomes, directs the synthesis of P_{63} (molecular weight of 63,000). This protein is smaller than but related to the viral glycoprotein G (molecular weight of 66,000) as judged by comparison of their tryptic peptides. Under the conditions of ion exchange chromatography of the tryptic peptides used in these experiments, only one

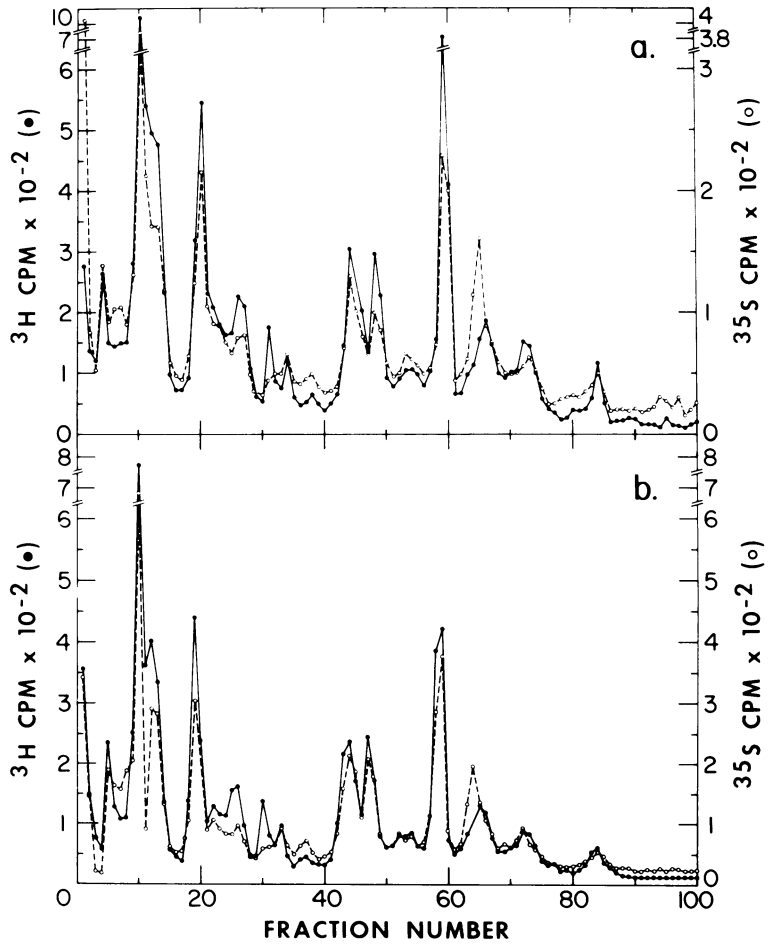

FIG. 3. *Comparison of in vitro synthesized P_{63}, P_{60}, and authentic viral glycoprotein G by trypsin digestion and ion exchange chromatography. [^{35}S]methionine-labeled P_{63} (a) and P_{60} (b) synthesized in a wheat germ extract in response to in vivo fraction I RNA were purified, digested, and chromatographed with [^{3}H]methionine-labeled peptides of the viral glycoprotein G as described.*

carbohydrate-containing methionyl-peptide of the G protein is retained on the column (5), and glycosylation of the viral G protein interferes little in its comparison with P_{63} synthesized in vitro. We do not know whether P_{63} contains any carbohydrate moieties, but we know of no evidence that a protein can be glycosylated in vitro by extracts of wheat germ. Presumably, P_{63} represents the nonglycosylated form of the viral glycoprotein G. P_{60} (molecular weight of 60,000), which is related to P_{63} and G according to tryptic peptide analysis (Fig. 3b), is synthesized in a lesser amount than P_{63} and presumably arises by premature termination of translation during the synthesis of this protein. Alternatively, it may arise through in-phase internal initiation events on the 17S mRNA.

It is apparent (Fig. 2e) that the in vitro fraction I RNA is contaminated by other RNA species, probably due to aggregation of the species in the smaller RNA fractions, such that they now sediment at 17S (3). However, it can be seen from the translation products directed by the in vivo fraction I RNA (Fig. 2d) that this RNA is much less contaminated by the messenger species from the other fractions. Presum-

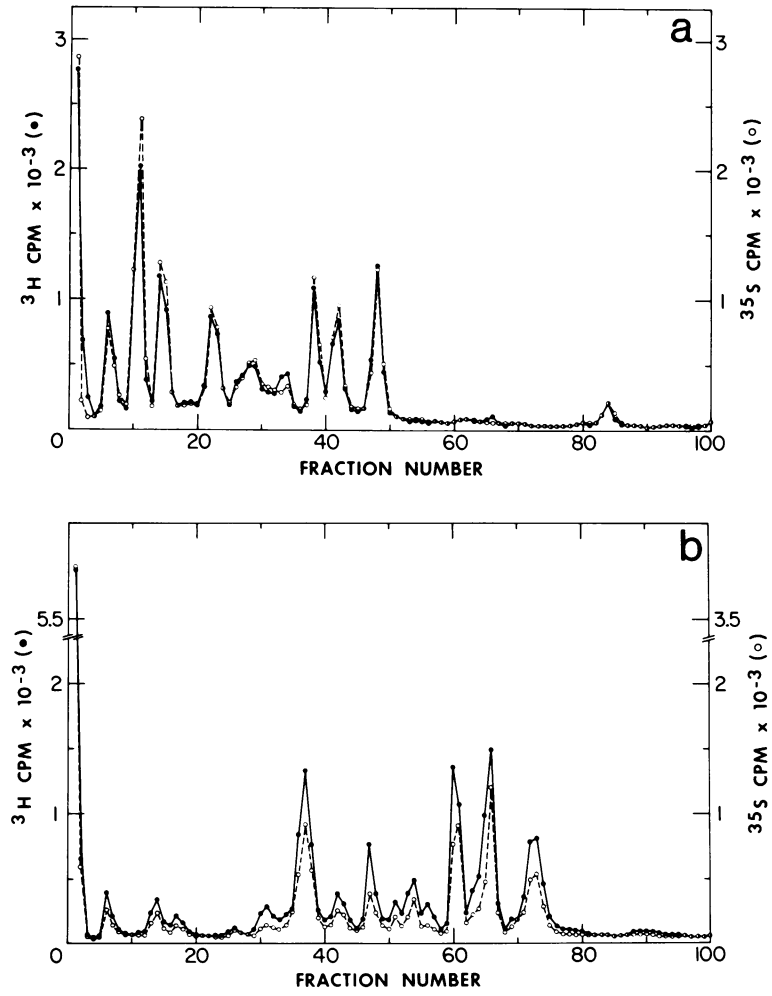

FIG. 4. *Comparison of the tryptic peptides of the in vitro synthesized N and M proteins with the corresponding viral polypeptides.* [^{35}S]*methionine-labeled N protein (a) and M protein (b) synthesized in wheat germ extracts in response to in vivo fractions II and fractions III RNA, respectively, were purified, digested, and chromatographed with* [^{3}H]*methionine-labeled peptides of the viral glycoprotein G as described.*

ably, this is a result of the compartmentalization of in vivo fraction I RNA in the membrane-bound polysomes.

Tryptic peptide analysis of the in vitro synthesized N and M proteins coded for by the in vivo fraction II and III RNAs indicates that they contain peptides identical to those found in the corresponding authentic viral proteins. However, in our earlier work (3) we found that the M protein, coded for in wheat germ extracts by the in vitro synthesized fraction III RNA, contained two methionyl-peptides not present in the viral M protein. The most likely explanation of this result is that our in vitro synthesized M protein band possibly contained other protein(s), since it was isolated from the products directed by total VSV mRNA synthesized in vitro rather than the purified fraction III RNA. In addition, although the in vitro synthesized NS protein coded for by the in vivo fraction III RNA comigrates with the viral NS protein in two SDS-polyacrylamide gel systems (Fig. 2h and j and Fig. 5b), it contains one additional major methionyl-peptide which has no counterpart in the viral protein. However, NS is a phosphoprotein (13), and this may represent an unphosphorylated peptide whose phosphorylated virion counterpart is not resolved on the column under these conditions. This assumption further implies that phosphorylation of the NS protein does not affect its migration in the SDS-polyacrylamide gel system used in these studies.

In any event, it is now apparent that species of viral mRNA, similar to those synthesized in vitro by purified VSV, can be isolated from partially fractionated extracts of VSV-infected cells. Moreover, the in vitro and in vivo mRNA species can be partially resolved into the same three size classes with sedimentation coeffi-

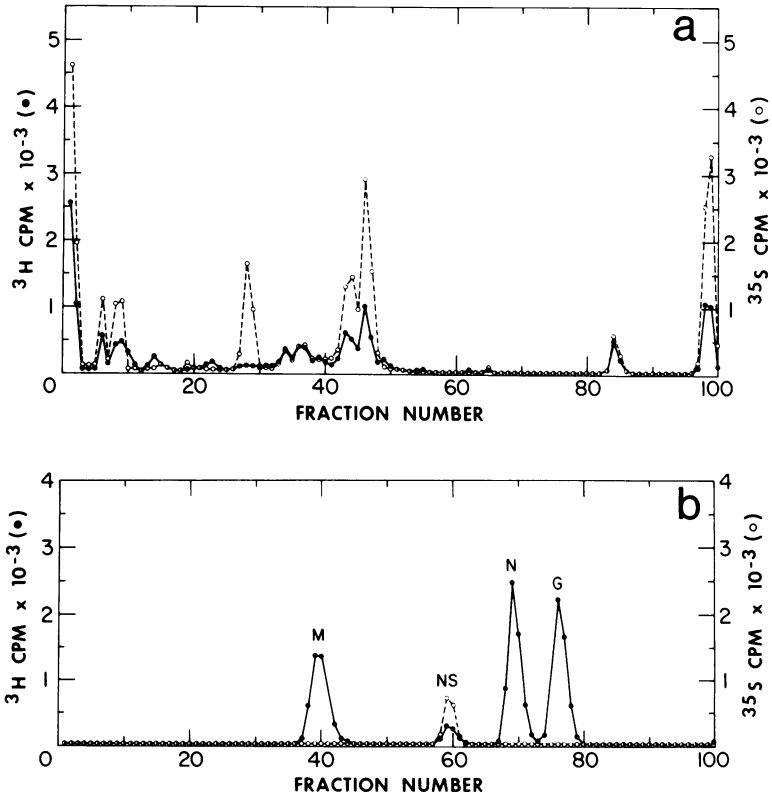

FIG. 5. *Comparison of the in vitro synthesized NS protein with the viral NS polypeptide. (a) [^{35}S]methionine-labeled NS protein synthesized in wheat germ extracts in response to in vivo fraction III RNA was purified, digested, and chromatographed with [^{3}H]methionine-labeled peptides of the viral NS polypeptide as described. (b) A sample of the purified ^{35}S-labeled NS protein was mixed with [^{3}H]amino acid-labeled VSV, heated, and subjected to electrophoresis at 22.5 V for 22 h on a 10% polyacrylamide gel containing 0.01 M sodium phosphate buffer (pH 7.2) and 0.1% SDS, fractionated, and counted (3).*

cients of 17S, 14.5S, and 12S, and these qualitatively direct the synthesis of polypeptides which comigrate on an SDS-polyacrylamide gel. In addition, tryptic peptide analysis allows a positive identification of the in vivo VSV mRNA species. Thus, fraction I (17S) RNA, which is compartmentalized on the membrane-bound polysomes, codes for P_{63} which is most probably a nonglycosylated form of the viral glycoprotein G. In vivo fraction II (14.5S) RNA codes almost exclusively for the N protein and together with fraction III (12S) RNA, which codes for the NS and M polypeptides, is found predominantly in the cytoplasmic supernatant of the cell.

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