Translation of Individual Species of Vesicular Stomatitis Viral mRNA

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Vesicular stomatitis virus mRNAs from three of the four bands fractionated by polyacrylamide gel electrophoresis in 99% formamide have been eluted from gels and translated in the Krebs II ascites cell-free system. Band 2 mRNA (0.7 x 10^6 daltons) directed the synthesis of the protein moiety of the glycoprotein (G), and band 3 (0.55 x 10^6 daltons) coded for the nucleocapsid (N) protein. Band 4 mRNA (0.28 x 10^6 daltons) directed the synthesis of the NS and matrix (M) proteins. The authenticity of viral proteins synthesized in vitro was shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by analysis of [35S]methionine-labeled tryptic peptides. These results are consistent with the complexity analysis and coding capacities for the vesicular stomatitis virus mRNA species presented in the accompanying paper.

Vesicular stomatitis virus (VSV) mRNA is complementary (2, 11) to the single piece of RNA in the virion (1, 4) and is synthesized in VSV-infected cells from both the parental and progeny negative strands (3). Centrifugation of viral mRNA in sucrose gradients yields a homogeneous 28S species and a heterogeneous group of RNA species sedimenting at 13 to 15S (2, 11). Polyacrylamide gel electrophoresis of the 13 to 15S RNA resolves it into at least three species (D. Baltimore, T. Morrison, M. Stampfer, and H. Lodish, Negative Strand Virus Meet. Abstr., 1973, in press; 16).

Morrison et al. showed that translation of the 28S mRNA in reticulocyte extracts yields a protein comigrating on polyacrylamide gels with the L protein (9). Translation of the 13 to 15S RNA in reticulocyte and wheat germ extracts gave proteins comigrating with M, NS, N, and possibly G proteins (9). Further studies have shown that the G protein mRNA is found exclusively in the membrane fraction of infected cells. These same studies have been shown by analysis of [35S]methionine-labeled tryptic peptides that the proteins synthesized in the wheat germ extract are authentic viral proteins (T. Morrison and H. Lodish, manuscript in preparation).

Results in the previous paper (15) have shown the resolution of total poly(A)-containing VSV RNA into four species, termed bands 1 to 4. These RNAs were characterized both by size and nucleotide complexity. In this paper we show that the RNA from the three smaller bands (bands 2, 3, and 4) can be eluted from 99% formamide-polyacrylamide gels and translated in ascites cell-free extracts to yield authentic VSV proteins. This has allowed us to identify the messenger RNAs in the 13 to 15S group for the G, N, NS, and M proteins.

MATERIALS AND METHODS

Cells, virus, and purification of mRNA. Infections of Chinese hamster ovary cells with standard VSV B particles and labeling of VSV mRNA with 32P were as described (15).

Formamide-polyacrylamide gel electrophoresis of RNA. 32P-labeled VSV mRNA species were fractionated by preparative gel electrophoresis in 99% formamide as described (15). The cytoplasmic poly(A)-containing mRNA from approximately 2 x 10^8 VSV-infected cells was resolved on one preparative gel as described in the previous paper (15). The individual RNA species were eluted from the gel and purified by oligo(dT) cellulose chromatography. This RNA was precipitated by adding sodium acetate (pH 5.2) to 0.4 M and 2.5 volumes of ethanol, followed by centrifugation at 50,000 rpm for 2 h (10 C) in a Beckman SW50.1 rotor. For translation reactions, the pellet of RNA was washed twice with 0.1 M sodium acetate (pH 5.2) in 70% ethanol by adding this solution to the tube and centrifuging for 1 h at 50,000 rpm. The precipitate was then dried, resuspended in 100 μl of sterile, distilled water, and stored at -70 C.

Cell-free protein synthesis. Preparation of cell-free extracts from Krebs II mouse ascites cells and conditions for cell-free protein synthesis were as described previously (8), except that reactions were incubated at 37 C with 78 mM KCl for the first 15 min, and then the KCl concentration was increased to 100 mM.
138 mM for a further 105 min of incubation. This procedure allowed initiation at a KCl concentration optimal for initiation, but completion of protein chains at a higher KCl concentration, which allowed a higher rate of translocation. This technique has been shown to yield a larger percentage of the full-size poliovirus RNA translation product and less "early termination" products in reactions primed by poliovirus RNA in ascites extracts (L. Villa-Komaroff, D. Baltimore, and H. Lodish, manuscript in preparation).

Approximately 10 to 20 µg of total cytoplasmic poly(A)-containing mRNA were added to each reaction, whereas approximately 5 µg of fractionated VSV mRNA species (10 µl of the solution of purified mRNA) were added to each cell-free reaction. No decrease in efficiency of translation was observed in the polyacrylamide gel-purified mRNA species relative to equivalent amounts of mRNA which had not been subjected to electrophoresis in formamide.

**Polyacrylamide gel electrophoresis of proteins.** Proteins synthesized by cell-free reactions were analyzed on cylindrical 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 5 M urea as described (7). For analytical purposes, a 20-µl portion of a reaction mixture was used. For trypsic peptide analysis, a full 50-µl reaction mixture was fractionated on one gel.

Ten percent polyacrylamide gels as described by Weber and Osborn (22) were utilized for calibration of molecular weights of cytoplasmic and in vitro synthesized VSV proteins. These proteins were subjected to coelectrophoresis with five proteins used as standards: bovine serum albumin (68,000), ovalbumin (45,000), glyceraldehyde-phosphate dehydrogenase (35,000), chymotrypsinogen A (25,000), and sperm whale myoglobin (17,700). Each standard protein (5 µg) was run on gels with [35S]methionyl cytoplasmic VSV proteins or [35S]methionyl-labeled in vitro synthesized VSV proteins. The gels were stained, sliced, and dried, and the stained bands were identified by comparison with gels run in parallel containing individual standard proteins. Positions of the VSV proteins were determined by measurements on an autoradiogram of the dried gel.

**Labeling of cytoplasmic proteins.** VSV cytoplasmic proteins were labeled with [35S]methionine by harvesting cells at 4.0 h postinfection and resuspending them at 2 x 10⁶ cells/ml in Earle saline with 20 µCi of [35S]methionine per ml (200 Ci/mmol, New England Nuclear, Boston, Mass.). After 30 min of labeling, the cells were harvested and resuspended in 1 ml of reticulocyte standard buffer with 1% Nonidet P-40. Nuclei were removed by centrifugation at 800 x g for 5 min.

**Analysis of tryptic peptides.** Tryptic digests of [35S]methionyl-labeled proteins were prepared by digesting the protein-containing gel bands directly (T. Morrison and H. Lodish, manuscript in preparation). Excised, dried gel bands were placed in 1 ml of 1% ammonium bicarbonate containing 50 µg of trypsin and digested at 37°C for 24 h. The gel pieces were placed in 1 ml of fresh trypsin solution and further digested for 4 h. Tryptsinized peptides were eluted from the gel piece into the two supernatants and were lyophilized and washed twice. The resulting peptides were analyzed by high-voltage paper electrophoresis at pH 3.5, and radioactivity was determined in 1-cm strips by scintillation counting in a toluene-1,4-bis-(5-phenyloxazolyl)-benzene cocktail.

**RESULTS**

**Translation of cytoplasmic RNA.** Poly(A)-containing mRNA prepared from the cytoplasm of VSV-infected Chinese hamster ovary cells was added to cell-free extracts from Krebs II ascites cells, and the reaction products were analyzed by electrophoresis on 10% polyacrylamide gels. Proteins synthesized during the reactions were analyzed in parallel with [35S]methionine-labeled VSV proteins synthesized in infected cells. Figure 1 shows the microdensitometer tracings of the autoradiograms of the dried gels. Three of the proteins synthesized in the cell-free reaction comigrated with marker cytoplasmic proteins, M, NS, and N (Fig. 1a and b). The fourth major protein migrated slightly faster than the cytoplasmic G protein. In previous work, synthesis of G protein in vitro was only barely detectable (9). More recently, translation in wheat germ extracts of RNA isolated from the membrane fraction of VSV-infected cells has produced a protein which migrates on gels slightly faster than cytoplasmic G protein (T. Morrison and H. Lodish, manuscript in preparation). Since the VSV mRNA used here was isolated from cytoplasm which contains membranes, it appears that G protein is being synthesized by the ascites extracts. Further evidence will be presented later indicating the authenticity of the apparent G protein formed in vitro. The synthesis of L protein would not have been evident in these gels because the large amount of endogenous proteins at the top of these gels (see Fig. 1c) would have obscured it.

**Fractionation of VSV mRNA.** To determine the identity of the VSV mRNA coding for the individual VSV proteins, [32P]-labeled cytoplasmic VSV poly(A)-containing RNA was fractionated by preparative formamide-polyacrylamide gel electrophoresis into the species described in the previous paper (15), as shown in Fig. 2. Lane a of Fig. 2 shows that total poly(A)-containing VSV mRNA was resolved into four species, called bands 1 to 4. The RNA in bands 2, 3, and 4 were eluted from the gel and purified by oligo(dT) cellulose chromatography as described previously (15). Lanes b to d show analyses of a small amount of the RNA isolated from each of the four bands. The RNA in each sample migrates as a single band at the same

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**Figure 1**

**Figure 2**
position as it did prior to elution, indicating the high purity of each sample.

**Translation of fractionated VSV mRNA.**

The remainder of the RNA samples eluted from the gel bands were used to test for synthesis of VSV proteins in ascites extracts. After incubation, each reaction was analyzed on 10% polyacrylamide gels. Figure 3 shows the microdensitometer scans of the autoradiograms of the dried gels. Panel a shows a gel in which [\(^{35}\)S]methionine-labeled VSV cytoplasmic proteins were analyzed in parallel.

Panel b shows the reaction product of band 2 mRNA. The major product is a protein migrating slightly faster than the cytoplasmic G protein. To identify this product, the regions of equivalent preparative gels containing [\(^{35}\)S]methionine-labeled cytoplasmic G protein and the [\(^{35}\)S]methionine-labeled protein made in vitro analogous to region 1 of Fig. 3 were excised and treated with trypsin (see above). The resulting peptides were resolved by paper electrophoresis at pH 3.5 (Fig. 4A and B). The profiles were very similar. We conclude that VSV band 2 mRNA directs the synthesis in vitro of a protein which contains all the methionine-containing peptides and presumably all the sequences of the cytoplasmic G protein. The slower mobility of the cytoplasmic protein relative to the in vitro product may be due to the presence of sugar residues on the former (17). We have no evidence at the present concerning glycosylation of the apparent G protein made in the ascites cell-free system. The product of band 2 mRNA also contains two significant minor proteins which approximately comigrate with N and NS and thus could be synthesized from contaminating mRNA for N and NS proteins. This could be explained by a high efficiency of translation of a small amount of these mRNAs contaminating band 2 mRNA. We have not thus far recovered sufficient amounts of these proteins to test whether this is the case or whether these result from premature termination of G polypeptide chains.

Panel c of Fig. 3 shows the translation product of band 3 VSV mRNA. The predominant protein made comigrates with authentic VSV N protein. Panels C and D of Fig. 4 show the pattern of tryptic peptides of the cytoplasmic N protein and of the band 3 mRNA product comigrating with it (a gel band on another gel equivalent to region 2 in Fig. 3). The peptide patterns are similar, indicating that band 3 VSV mRNA directs the synthesis of authentic N protein. We have no explanation for the quantitative differences in the relative amounts of the peptides. Band 3 RNA also directs the synthesis of two proteins, regions 3 and 4 of Fig. 3, which migrate on gels faster than authentic N protein. Panel E of Fig. 4 shows that these products yield only some of the [\(^{35}\)S]methionine-labeled tryptic peptides found in authentic
N protein. These minor products thus result from either premature polypeptide chain termination or from initiations in correct phase in the interior of the mRNA. It can be concluded that, in terms of its translation product, band 3 is a pure mRNA species.

Panel c of Fig. 3 also shows that band 4 VSV mRNA directs the synthesis of two proteins which comigrate with the VSV proteins NS and M. Panels F and G in Fig. 4 compare the methionine-labeled tryptic peptides of cytoplasmic NS protein and the band 4 mRNA product comigrating with NS. The peptide patterns are similar with the exception that the peptide migrating at 11 cm is present in larger amounts in the in vitro product than in the cytoplasmic protein. However, the patterns are qualitatively similar, and we conclude that the protein being synthesized in vitro is NS protein.

Panels H and I in Fig. 4 show the similarity in tryptic peptides between the cytoplasmic M protein and the band 4 mRNA product comigrating with M (region 6 of Fig. 3), a result demonstrating that authentic M protein is also encoded by band 4 mRNA. It is clear from the tryptic peptide analysis that NS and M are

Fig. 2. Autoradiogram of wet 3.75% formamide-polyacrylamide slab gel in which VSV mRNA species were subjected to electrophoresis for 20 h at 80 V. (a) Total poly(A)-containing VSV mRNA (exposure time, 3 days); (b) purified band 2 VSV mRNA (exposure time, 3 days); (c) purified band 3 VSV mRNA (exposure time, 2 days); (d) purified band 4 VSV mRNA (exposure time, 2 days).
Panel J of Fig. 4 shows the tryptic peptides of the minor peak migrating slightly faster than M protein in the band 4 mRNA product (region 7 of Fig. 3). This product contains all the methionine-labeled tryptic peptides of full-sized M protein, yet migrates as a smaller protein. There is one extra peak at fraction 10, and this could conceivably have been derived from overlap with incomplete NS protein molecules. Presumably this smaller protein is an early termination product of an M polypeptide chain or the product of an internal initiation in correct phase.

**Determination of protein molecular weights.** Our finding that band 4 encodes VSV NS protein was unexpected, since the coding capacity of band 4 (molecular weight of 0.28 x 10^6; reference 15) should be a protein with a molecular weight of 31,000, but previous estimates of the size of NS protein ranged from 40,000 to 54,000 daltons (6, 11, 13). Hence, we found it desirable to redetermine the molecular weights of the cytoplasmic and in vitro synthesized VSV proteins by the procedure outlined in Materials and Methods.

The migration of the protein standards was obtained as shown in Fig. 5. From this, the molecular weights of the VSV proteins were determined. The cytoplasmic G protein was found to migrate at 66,000 daltons, as if it were larger than the in vitro synthesized G at 63,500. As stated previously, we believe this difference to be due to glycosylation of the cytoplasmic protein, and thus the molecular weight of the in vitro G protein should more closely approximate the molecular weight of the protein moiety of the glycoprotein. Cytoplasmic N and in vitro synthesized N comigrated on these gels and were determined to have a molecular weight of 52,500. The cytoplasmic and in vitro NS proteins comigrated with a molecular weight of 43,000, and the in vitro and cytoplasmic M proteins also comigrated with a molecular weight of 27,000. While it has previously been shown that the NS protein synthesized in reticulocyte and wheat germ extracts is larger than the cytoplasmic NS protein by 2,000 to 3,000 daltons (9), we found this not to be the case for the NS protein synthesized in ascites extracts.

**DISCUSSION**

In our earlier studies mRNA was isolated from the polyribosomes of VSV-infected cells which had been disrupted by nonionic detergents. When added to cell-free extracts of reticulocytes or ascites cells, this RNA directed the synthesis of the VSV L, M, N, and NS proteins, but little G protein was made (9). Subsequently, it has been shown that all G mRNA is
found in the membrane fraction of infected cells (T. Morrison and H. Lodish, manuscript in preparation). If infected cells are disrupted by Dounce homogenization, and if the nuclei are washed with a mixture of ionic and nonionic detergents (14), all the 13 to 15S VSV mRNA, including the G mRNA, are recovered in the pooled cytoplasmic extracts.

In the Krebs ascites cell-free system, this mRNA directs the synthesis primarily of the VSV proteins, even though much host mRNA is present. We do not know whether the VSV mRNA is present in much larger quantities than the host cell mRNA or whether the VSV mRNA is more efficiently translated than the host cell mRNA.

Elution of fractionated species of 13 to 15S VSV mRNA from 99% formamide-polyacrylamide gels has allowed us to identify the mRNA for each of the four smaller VSV proteins. Band
2 codes for the protein moiety of the glycoprotein (G), band 3 codes for the nucleocapsid (N) protein, and band 4 codes for 2 proteins, NS and the matrix (M) protein. Attempts to further resolve band 4 have not succeeded; it migrates as a single band in several aqueous gel systems and on electrophoresis at pH 3.5 on cellulose acetate strips (data not shown). Fingerprint analyses of band 4 RNA substantiated the notion that it is a mixture of two RNA species because its complexity was more than twice as great as the molecular weight determined by migration on polyacrylamide gels (Table 1; 15). Corroborating evidence for band 2 being the mRNA for the apparent G protein also exists; RNA isolated from membrane-bound polyribosomes is enriched both for the ability to stimulate the synthesis of the apparent G protein in vitro and for band 2 mRNA (T. Morrison and H. Lodish, manuscript in preparation).

Table 1 summarizes the information about the VSV RNA species and the proteins which they encode. The coding capacity of bands 2 and 3 is only slightly larger than the size of the corresponding proteins. There may be a small untranslated region on each of these messenger RNAs. The analysis for band 4 shows that the total coding capacity is slightly larger than the combined molecular weights of NS and M. Thus, one or both of these messengers could have a short untranslated region. However, a dilemma exists since a mRNA with a molecular weight of 280,000 (band 4) codes for a protein (NS) whose apparent molecular weight is 42,500. We have no data with which to explain this apparent contradiction. We feel that because band 4 migrates as a single band in gels containing 99% formamide or aqueous buffers with 6 M urea it is doubtful that the mRNA for NS is migrating anomalously. Also, band 4 mRNA migrates as a single 12S peak on sodium dodecyl sulfate-sucrose gradients (15). On the other hand, NS protein has been shown to migrate differently in different gel systems. Mudd and Summers (11) reported NS had a molecular weight of 40,000, and Obijeski et al. (13) showed NS to have a molecular weight of 42,000 on continuous Maizel gels. However, Stampfer and Baltimore (20), as well as Obijeski et al. (13), have found that on Laemmli discontinuous buffer gels NS migrated more slowly than N and thus would have a molecular weight greater than 50,000. It seems that NS migrates anomalously on sodium dodecyl sulfate polyacrylamide gels, so that the molecular weights obtained from such gels are questionable. NS protein is phosphorylated quickly after its synthesis in the cytoplasm of infected cells.

![Figure 5](image-url)

**Fig. 5. Calibration of molecular weights of cytoplasmic and in vitro synthesized VSV proteins on 10% polyacrylamide-sodium dodecyl sulfate gels. Positions of protein standards and labeled viral proteins were determined as described.**

<table>
<thead>
<tr>
<th>VSV mRNA</th>
<th>Mol wt from gel mobility*</th>
<th>Nucleotide sequence complexity (daltons)*</th>
<th>Complexity minus poly(A) (daltons)*</th>
<th>Coding capacity (daltons of protein)*</th>
<th>Protein encoded</th>
<th>Mol wt of encoded protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 1</td>
<td>≥ 1.65 × 10⁴</td>
<td>Not determined</td>
<td>0.73 × 10⁴</td>
<td>74,500</td>
<td>G</td>
<td>63,500</td>
</tr>
<tr>
<td>Band 2</td>
<td>0.7 × 10⁴</td>
<td>0.78 × 10⁴</td>
<td>0.53 × 10⁴</td>
<td>54,200</td>
<td>N</td>
<td>52,500</td>
</tr>
<tr>
<td>Band 3</td>
<td>0.55 × 10⁴</td>
<td>0.56 × 10⁴</td>
<td>0.72 × 10⁴-0.98 × 10⁴</td>
<td>74,000-100,000</td>
<td>NS</td>
<td>43,000</td>
</tr>
<tr>
<td>Band 4</td>
<td>0.28 × 10⁴</td>
<td>0.76 × 10⁴-1.0 × 10⁴</td>
<td></td>
<td></td>
<td>M</td>
<td>27,000</td>
</tr>
</tbody>
</table>

* Determined by 99% formamide-polyacrylamide gel electrophoresis as described (15).
* Determined using fingerprint analysis of ³²P-labeled mRNA species as described (15) and calculated assuming an average of 360 daltons per nucleotide (J. Rose, unpublished data).
* Calculated by subtracting the content of nucleotides in poly(A) from the nucleotide sequence complexity of the total molecule.
* Calculated from the complexity minus poly(A), assuming an average of 110 daltons per amino acid encoded.
(5, 10, 18), and this could affect the mobility of the protein. However, if this were the case, the NS protein synthesized in the cell-free extracts must then also be phosphorylated. We are currently investigating how phosphate groups affect the mobility of the NS protein.

We have not attempted to translate the band 1 RNA because of its low yield and large size. This band has been shown to be heterogeneous (15), but presumably contains the 28S mRNA species identified on sucrose gradients (2, 11, 21) and which has been shown to have poly(A) (19). This species has been shown to code for the L protein (9), and if band 1 contains this mRNA, this would complete the identification of the mRNAs for the known VSV proteins.

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