Proc. Nat. Acad. Sci. USA
Vol. 72, No. 1, pp. 274–278, January 1975

Translation and Identification of the mRNA Species Synthesized *In Vitro* by the Virion-Associated RNA Polymerase of Vesicular Stomatitis Virus

(wheat germ extracts/slab gel electrophoresis/peptide analysis/mRNA fractionation)

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Communicated by B. L. Horecker, October 29, 1974

ABSTRACT **Vesicular stomatitis virus messenger RNA has been transcribed *in vitro* from the viral genome by the virion-associated RNA polymerase in quantities suitable for translation. Wheat germ cell-free extracts programmed with the isolated *in vitro* 12–18S RNA fraction synthesize polypeptides similar to the viral N, NS, and M proteins, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and tryptic peptide mapping of the *in vitro* products and the viral marker polypeptides. In addition, the RNA synthesized *in vitro* also codes for a protein of molecular weight 63,000 which may be a nonglycosylated form of the viral glycoprotein G. The 12–18S RNA has been partially separated into individual messenger species and these have been identified by the proteins for which they code. There are four monocistronic messenger species in the *in vitro* 12–18S RNA and the coding capacity of three of these molecules agrees with the estimated molecular weight of the polypeptide assigned to it.**

Vesicular stomatitis virus (VSV) is a membrane-enveloped rhabdovirus (1) with a single-stranded RNA genome (2, 3) of molecular weight 3.6 to 4 $\times$ 10^6 (1, 3). The virion also contains five structural polypeptides (4–6); the glycoprotein G (7, 8) is located in the virion surface projections, the matrix protein M is found in the virion envelope and the nucleoprotein N, together with the genome RNA and two minor proteins, NS and L, constitutes the ribonucleoprotein core (9).

Polysomes of VSV-infected cells contain virus-specific messenger RNA (mRNA) which is complementary to the viral genome (10, 11). The mRNA can be resolved into two size classes by sucrose gradient sedimentation; a homogeneous species sedimenting at 28 S and heterogeneous RNAs sedimenting at 13–15 S (10). Further analysis of mRNA prepared from VSV-infected cells resolved the 13–15S component into at least three species (12–14) while the 28S RNA migrated as a homogeneous band (12). It was suggested, based on the molecular weights of these RNA species, that the 13–15S RNA could code for the G, N, NS, and M proteins (10) and that the 28S RNA could code for the L protein (6, 15). Indeed, subsequent work has shown that reticulocyte lysates programmed with 28S RNA isolated from VSV-infected cells can synthesize a protein which co-migrates with the viral L protein on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels (16). Similarly, it has also been shown that proteins which co-migrate with the viral N, NS, and M polypeptides can be synthesized in several different cell-free extracts programmed with 13–15S RNA, but it was not clear whether authentic G protein was made.

Abbreviations: VSV, vesicular stomatitis virus; $NaDodSO_4$, sodium dodecyl sulfate.

VSV has a virion-associated RNA polymerase which is capable of synthesizing, *in vitro*, RNA complementary to the viral genome (17, 18). Recent work in this laboratory (19) has shown that RNA species of two size classes, 31 S and 12–18 S, can be synthesized *in vitro* by the virion-associated RNA polymerase of VSV. The *in vitro* RNA products, similar in size to those found *in vivo* (10), are complementary to the genome and contain poly(A) segments (19–21), indicating that they could serve as messengers for the VSV proteins. In this report we show that the *in vitro* 12–18S RNA can be translated in cell-free extracts of wheat germ into proteins which seem identical to the viral polypeptides. In addition, we have partially separated these RNA species and concluded that the VSV 12–18S RNA synthesized *in vitro* consists of four monocistronic messengers which code for four viral polypeptides.

MATERIALS AND METHODS

Purification of Virus and Preparation of Lysates of Infected Cells. The VSV (Indiana serotype) used for synthesis of RNA was grown in baby hamster kidney (BHK) cells and purified as described previously (19). To prepare labeled virus, we infected 2.5 $\times$ 10^8 cells at a multiplicity of infection of 10 PFU per cell as described (22), except that the cells were diluted at 1.5 hr with medium containing one-fourth the usual level of amino acids. At 4 hr after infection, 1 mCi of L-[^{35}S]-methionine (specific activity 170 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added and virus was harvested at 20 hr after infection and purified as described (21). Cells (2 $\times$ 10^7) were infected as described above and [^{35}S]methionine (20 μCi/ml) was added at 4 hr after infection. Five minutes after the addition of the label, a 100-fold excess of unlabeled methionine was added and the cell lysate was prepared (23). A culture of mock-infected cells was treated identically. ^{35}S-Labeled proteins in cell lysates were mixed with sample buffer and analyzed directly on $NaDodSO_4$–polyacrylamide slab gels as described below.

In Vitro Synthesis of VSV RNA. VSV-specific RNA was synthesized *in vitro* from 1 mg of purified virus as previously described (19).

Preparation of Wheat Germ Extracts. Extracts of raw wheat germ (Niblack, Rochester, N.Y.) were prepared essentially as described (24), except that after swelling for 1 min in buffer, the tissue was ground vigorously for 30 sec in a chilled mortar and the homogenate was centrifuged at 23,000 $\times$ g for 10 min at 4°.

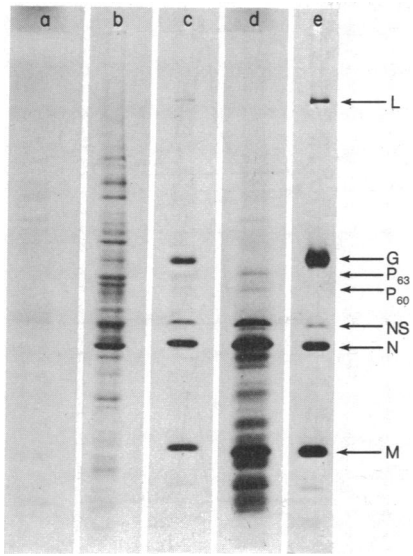

FIG. 1. Polypeptide products directed by VSV 12–18S RNA synthesized *in vitro*. Standard protein synthesis assays contained in a final volume of 25 μl: 0.85 $A_{260\ nm}$ units of preincubated wheat germ extract, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (Hepes, Calbiochem, San Diego, Calif.) (adjusted with KOH to pH 7.3), 2 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 40 μg/ml of creatine phosphokinase, 25 μM of the appropriate unlabeled amino acids, 90 mM KCl, and 3 mM magnesium acetate. L-[^{35}S]Methionine (specific activity 150–200 Ci/mmol, Amersham/Searle, Arlington Heights, Ill.) was used in amounts of 15–40 μCi/25 μl of assay. The amount of RNA added per 25 μl varied from 1 to 3 μg for either fractionated or unfractionated 12–18S VSV RNA preparations. Assays were incubated at 25° for 60 or 90 min. ^{35}S-Labeled polypeptides synthesized *in vitro* were analyzed by 10% polyacrylamide analytical slab gels containing 0.375 M Tris·glycine buffer (pH 8.7) and 0.1% $NaDodSO_4$ prepared according to Anderson *et al.* (25) following the methods of Laemmli (26), and electrophoresed at 125 V for 4 hr. Gels were stained and dried *in vacuo* (25). Autoradiograms were obtained by exposing Kodak BB54 medical x-ray film to the gel for 7 days. (a) No mRNA added; (b) proteins of mock, and (c) VSV-infected cell lysates; (d) products directed by VSV 12–18S RNA synthesized *in vitro;* (e) proteins of the purified virus.

RESULTS

Translation of In Vitro 12–18S VSV RNA in Cell-Free Extracts of Wheat Germ. VSV-specific RNA was synthesized *in vitro* by the RNA polymerase of purified virus and the 12–18S RNA was separated from the genome and 31S RNA by sucrose gradient sedimentation as previously described (19). The approximate yield of 12–18S RNA was 30 μg. Insufficient 31S RNA was synthesized during this reaction to allow translation of this species.

Pre-incubated extracts of wheat germ were chosen for translation experiments because of the very low level of endogenous protein synthesis (24) (see also Fig. 1a) and the absence of proteolytic cleavage in this system (27). Initially, 2–3 μg of the total 12–18S RNA was used to program the wheat germ extract. Under the conditions of protein synthesis described in Fig. 1, the incorporation of [^{35}S]methionine into polypeptide products continued at a linear rate for at least 1 hr. The proteins made in response to *in vitro* 12–18S RNA were analyzed by $NaDodSO_4$–polyacrylamide slab gel electrophoresis and

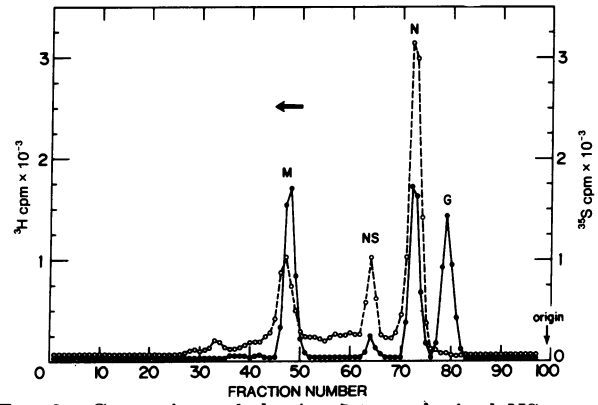

FIG. 2. Comparison of the *in vitro* synthesized NS protein with the authentic viral NS polypeptide. The polypeptide products directed by VSV *in vitro* 12–18S RNA in a wheat germ extract were dialyzed overnight against 0.01 M sodium phosphate buffer (pH 7.2) and $NaDodSO_4$ (0.1%), and 2-mercaptoethanol (0.1%), glycerol (10%) and amino-acid-labeled VSV [^{3}H]polypeptides were added. The sample was heated and subjected to electrophoresis in a 10% polyacrylamide gel in 0.1 M sodium phosphate (pH 7.2) and $NaDodSO_4$ (0.1%) at 22.5 V for 22 hr. The gel was cut into 1 mm slices and the radioactivities of the fractions were measured (23). Migration is from right to left. The ^{3}H radioactivity in each fraction was adjusted for ^{35}S spillover. ●——●, ^{3}H-labeled viral proteins; O– –O, ^{35}S-labeled products synthesized *in vitro*.

autoradiography. Fig. 1d shows that proteins were synthesized *in vitro* which co-migrate with the authentic M, N, and NS polypeptides present in VSV-infected BHK cells (Fig. 1c) and purified virions (Fig. 1e) but which are absent from mock-infected cells (Fig. 1b). Also synthesized were two proteins of molecular weights 63,000 and 60,000 (designated P_{63} and P_{60}, respectively) which are larger than the NS polypeptide but smaller than the glycoprotein G (Fig. 1d). Genome RNA (42 S), extracted from purified VSV, did not direct the synthesis of any detectable polypeptides under these conditions (data not shown).

Comparison of the NS Protein Synthesized In Vitro with the Authentic Viral Polypeptide. It has been previously observed that the viral NS protein migrates slower than (15) or faster than (5) the viral N protein, depending on the $NaDodSO_4$-polyacrylamide gel system used for its analysis. The reason for this anomalous migration is not understood but it provides a convenient way of testing the authenticity of the NS protein synthesized *in vitro*.

Figs. 1d and 1e show that both the *in vitro* synthesized and the viral NS polypeptide co-migrate with an apparent molecular weight of 52,000 (see Table 1) when they are analyzed by electrophoresis on the $NaDodSO_4$–polyacrylamide slab gel containing 0.375 M Tris·glycine buffer (pH 8.7). Moreover, when the protein products directed by the *in vitro* 12–18S RNA were mixed with amino-acid-labeled viral [^{3}H]polypeptides and analyzed by electrophoresis on a cylindrical 10% polyacrylamide gel in 0.1% $NaDodSO_4$ and 0.1 M sodium phosphate (pH 7.2) (Fig. 2), the NS proteins co-migrated with with an apparent molecular weight of only 40,000 (see Table 1). The fact that the two proteins co-migrate in both $NaDodSO_4$ gel systems further establishes the identity of the *in vitro* synthesized NS protein with the viral NS polypeptide. While the N proteins co-migrate in this gel system, it is apparent that

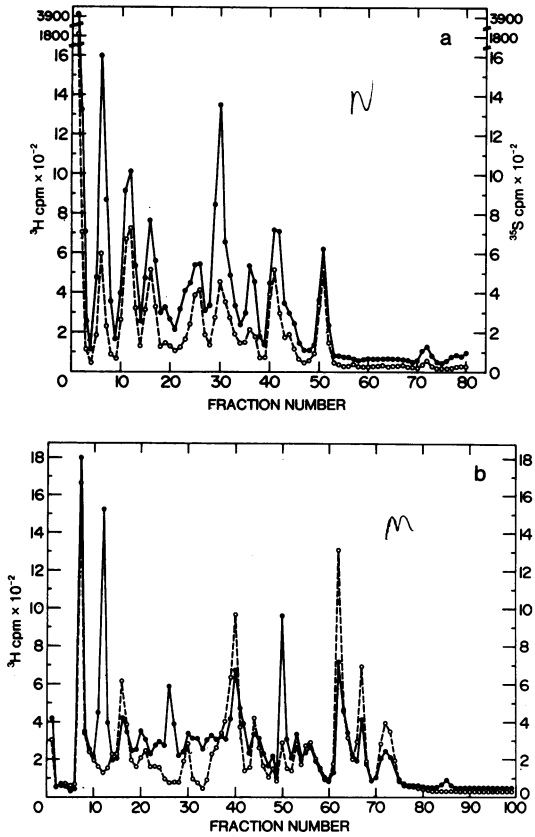

FIG. 3. Comparison of *in vitro* synthesized proteins and authentic viral polypeptides by trypsin digestion and ion exchange chromatography. Polypeptides were synthesized *in vitro* in a 50-μl wheat germ incubation mixture, mixed with [^{3}H]methionine-labeled purified virus, and separated by gel electrophoresis as in Fig. 1, and each protein was isolated from the gel (25). The purified proteins containing both ^{3}H and [^{35}S]methionine label were digested with L-1-tosylamido-2-phenethyl chloromethyl ketone (TPCK)-treated trypsin (1 mg/ml) in 1% ammonium bicarbonate at pH 7.8 for 16 hr at 37°. At 16 hr a further 500 μg of trypsin was added and after 20 hr the sample was diluted with three volumes of 0.025 M pyridine–acetate (pH 4.0) and chromatographed on a column (0.9 × 30 cm) of ion exchange resin (Sphenix resin XX907-10, Phoenix Precision Instrument Co., Philadelphia, Pa.) at 57°. The fractions were dried and their radioactivities were measured (29). (a) Chromatography of the tryptic peptides of (●——●) ^{35}S-labeled *in vitro* synthesized and (O– –O) ^{3}H-labeled viral N proteins. (b) As in (a), but for the M proteins.

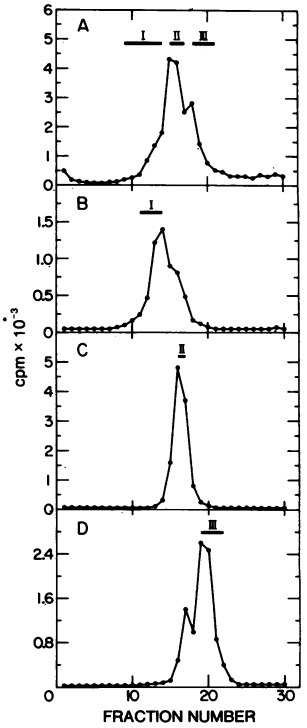

FIG. 4. Fractionation of *in vitro* 12–18S VSV RNA. VSV 12–18S RNA was synthesized as described in *Methods* and passed through an oligo(dT)-cellulose column (19). The RNA was recovered by precipitation with two volumes of ethanol in the presence of 0.2 M sodium chloride and centrifuged (A) on a 5–30% glycerol gradient in 20 mM Tris·HCl (pH 7.5), 0.1 M NaCl and 5 mM EDTA at 32,000 rpm in the Beckman SW41 rotor for 17 hr at 4°. Sedimentation is from right to left. The radioactivity in each fraction was determined by counting a small aliquot in Aquasol. The gradient fractions indicated were pooled and the RNA was recovered by ethanol precipitation. Each RNA sample was dissolved in water, adjusted to 6 M urea, heated at 80° for 30 sec, rapidly chilled, diluted 6-fold with water, and centrifuged on a separate gradient as described above. The RNA was recovered from the indicated fractions I, II, and III by precipitation (B–D). Prior to being used for translation, the RNA was washed with 95% ethanol and dried with N_2.

the M proteins are not coincident. We believe that this is due to the poor resolution of M, in this gel system, from the polypeptides migrating just ahead of it in Fig. 1d. Neither P_{63} nor P_{60} was detectable under these conditions because of the relatively poor resolution and sensitivity of this method of analysis compared with the techniques of slab gel electrophoresis and autoradiography used in Fig. 1.

Comparison of the In Vitro Synthesized and the Viral M and N Proteins by Trypsin Digestion and Ion Exchange Chromatography. In order to confirm the identities of the *in vitro* synthesized M and N proteins, we compared their tryptic peptides with those of the corresponding viral proteins. Wheat germ extracts were programmed with *in vitro* 12–18S RNA and [^{35}S]methionine-labeled proteins were synthesized during a 90-min incubation. The N and M proteins contained in the *in vitro* products and [^{3}H]methionine-labeled viral M and N polypeptides were separated as described. The ^{35}S- and ^{3}H-labeled N proteins and similarly, the corresponding M proteins, were combined, digested with trypsin and analyzed by ion exchange chromatography as described in Fig. 3. There was almost complete identity between the tryptic peptides of the viral and the *in vitro* synthesized N proteins (Fig. 3a). The tryptic peptides of the M proteins, although very similar (Fig. 3b), differ by two extra methionine-containing peptides (fractions 10–14 and fractions 25–28) present among the trypsin digestion products of the M protein synthesized *in vitro*. It may be that these represent peptides which normally would be cleaved from the M protein *in vivo*, but are present in the *in vitro* product because of the lack of proteolytic cleavage in this system (27). In any event, the data indicate that both the synthesis of the complementary product RNA by the virion-associated RNA polymerase and its subsequent translation in cell-free extracts of wheat germ occur with considerable biological accuracy.

Translation of Fractionated In Vitro 12–18S RNA. In vitro VSV RNA was prepared as described (19) and analyzed by velocity sedimentation (Fig. 4a). Under these conditions, the 12–18S RNA can be partially resolved into three size classes. The gradient fractions indicated were pooled and the RNA was recovered by precipitation. The RNA in each size class was further purified by urea treatment and sedimentation on a separate glycerol gradient (Fig. 4b–d) as described in the legend.

Approximately 2 μg of the urea-treated RNA fractions I, II, or III were used for translation in wheat germ extracts incubated for 60 min. Polyacrylamide gel electrophoresis of the translation products shows that the major proteins synthesized in response to fraction III RNA co-electrophorese with the M and NS proteins (Fig. 5c), while fraction II RNA codes predominantly for the N protein (Fig. 5b). Fraction I RNA is the only species which codes for two larger polypeptides, P_{63} and P_{60} (Fig. 5a) and in addition contains RNA which codes for the M, N and NS proteins. The array of minor proteins present among the *in vitro* products directed by RNA fractions II and III presumably arises through premature-termination of translation or in-phase internal initiation events.

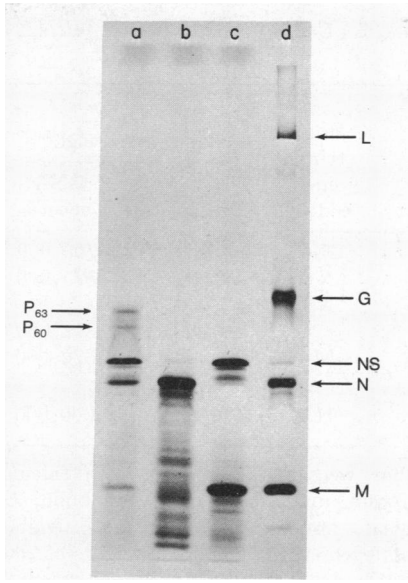

FIG. 5. Polypeptide products directed by fractionated VSV *in vitro* 12–18S RNAs. Polypeptide products were synthesized *in vitro* and analyzed as described in Fig. 1. Migration is from top to bottom. *In vitro* products directed by (a) fraction I RNA; (b) fraction II RNA; (c) fraction III RNA; and (d) as markers, the polypeptides of purified virus.

DISCUSSION

The messenger activity of the VSV 12–18S RNA species transcribed *in vitro* from the viral genome has been demonstrated by translation of the RNA into proteins, apparently identical to those of the virus, in cell-free extracts of wheat germ. From the results obtained and summarized in Table 1, it seems that there are four mRNA species present in the VSV *in vitro* 12–18S RNA. A viral polypeptide(s) can now be tentatively assigned to each VSV mRNA species (Fig. 5); fraction I RNA probably codes for P_{63}, fraction II RNA codes for N and fraction III RNA predominantly for M and NS. In general, the molecular weight of the polypeptide agrees with the coding potential of the mRNA assigned to it (see Table 1). There are, however, several points which require further clarification.

Although fraction I RNA directs the synthesis of P_{63} and P_{60}, which are completely absent from fractions II and III, it nevertheless contains RNA which codes for the N, NS, and M polypeptides (Fig. 5a). This may be due to the presence of residual aggregates of the smaller RNAs in this fraction, since the contamination of this region of the gradient by these mRNA species is greater if the RNA is not treated with urea prior to sedimentation (G. W. Both and S. A. Moyer, unpublished results). A similar result was observed with reovirus mRNA (G. W. Both, S. Lavi, and A. J. Shatkin, unpublished observations). In any event P_{63}, the largest polypeptide coded for by fraction I RNA, may be a nonglycosylated form of the viral glycoprotein G. This tentative identification comes from the following observations. The RNA isolated from membrane-bound polysomes of VSV-infected cells appears to be enriched for an RNA species which sediments predominantly in the same position as fraction I RNA synthesized *in vitro* (S. A. Moyer, M. J. Grubman, E. Ehrenfeld, and A. K. Banerjee, unpublished observations). Moreover, this *in vivo* RNA directs primarily the synthesis of a protein which comigrates with P_{63} synthesized in wheat germ extracts in response to the *in vitro* fraction I RNA and has tryptic peptides similar to those present in the viral glycoprotein G (unpublished observations). Since we know of no evidence that a protein can be glycosylated *in vitro* by extracts of wheat germ, we conclude that P_{63} may be a nonglycosylated G protein. Tryptic peptide analysis furthermore suggests that P_{60}, which is always present in the lesser amount, arises by premature termination during the synthesis of P_{63} (unpublished observations).

While the assignment of the N protein to the RNA in fraction II seems to be unambiguous (see Table 1 and Fig. 5b), the situation is not as clear for the products of the fraction III RNA. This RNA directs the synthesis of both the M and NS proteins (Fig. 5c) (with molecular weights of 29,000 and 52,000, respectively), but this result is totally unexpected, since the coding potential of fraction III RNA is only 32,700 (see Table 1). There are two possible explanations for this anomaly. One is that fraction III RNA is a dicistronic messenger with extensive secondary structure which might cause it to sediment with a low apparent molecular weight. Alternatively, this RNA might contain two monocistronic mRNA species of apparently equal molecular weight (see below), one of which codes for the M protein, the other for the NS polypeptide. If M and NS are translated from a single dicistronic mRNA, the ratio of NS to M protein synthesized *in vitro* in response to this RNA species should be constant. Clearly this is not the case, since fraction I RNA directs the synthesis of more NS protein relative to M than does fraction III RNA (Fig. 5a and c). This strongly suggests that the messages for the NS and M polypeptides are distinct monocistronic RNA species. However, while a monocistronic mRNA of molecular weight 3.72×10^5 is sufficient to code for the M protein (see Table 1), the NS protein is too large to be coded for by an RNA of this apparent size. It seems, though, that the sedimentation value of the NS protein mRNA is correct, since the RNA in fraction III appears to migrate with a molecular weight of 3.7×10^5 on denaturing gels (19). Moreover, the apparent molecular weight of the NS polypeptide varies from 52,000 to 40,000 (see Table 1), depending on the gel system

TABLE 1. *Coding potential of the mRNA species synthesized in vitro**

mRNA fractions	Proteins encoded	Molecular weight: Proteins	Molecular weight: RNA species	Coding potential
I	P_{63}	63,000	700,000	65,900
II	N	47,500	525,000	48,200
III	NS	52,000; 40,000	372,000	32,700
	M	29,000	372,000	32,700
31S RNA *in vitro*	(L)	195,000	2,090,000	206,000

* Molecular weights of proteins were calculated using as markers myosin, unreduced rabbit γ-globulin, *Escherichia coli* β-galactosidase, bovine serum albumin, ovalbumin, and α-chymotrypsinogen, with molecular weights of 200,000, 150,000, 130,000, 68,000, 45,000, and 25,000, respectively. The molecular weight of viral G protein was estimated to be 66,000 from these values. The molecular weights of *in vitro* mRNA species were taken from ref. 28. The molecular weights of NS proteins were taken from refs. 25 and 26 and estimated from the data in Fig. 2. The coding potentials of mRNA species were estimated subtracting an average of 140 adenylic acid residues per mRNA molecule to correct for poly(A) (22). The L protein has *not* been translated from 31S RNA synthesized *in vitro*. The data are included only to demonstrate that the 31S RNA could code for the viral L protein based on its estimated molecular weight.

used for its analysis, and the true molecular weight is unknown; it may be even lower and, therefore, in closer agreement with the coding potential of its mRNA species.

It has been previously shown that 28S RNA isolated from VSV-infected cells can code for a protein which co-migrates with the L protein of the virus (16). Indeed, the molecular weight of the L protein (see Table 1) indicates that it should be coded for by the 31S RNA transcribed from the viral genome *in vitro* (19). If this is shown to be the case, then the entire coding potential of the VSV genome complement would be accounted for by the five viral structural proteins L, G, NS, N, and M, and both the mRNA and proteins of the genome could be synthesized entirely in *in vitro* systems.

Note Added in Proof. We have recently compared the tryptic peptide map of the viral M protein and the M protein synthesized *in vitro* in response to purified mRNA from Peak III. The oligopeptide patterns obtained by ion-exchange chromatography were completely identical. The reason for the minor differences in the tryptic peptide patterns in Fig. 3b seems to have been due to protein impurities present in M protein arising from "early quitters" in response to total mRNA (see Fig. 1).

We thank Dennis Rhodes for skilled assistance and providing amino acid-labeled [^{3}H]VSV. We also wish to thank Dr. S. Lavi for helpful discussion and Drs. D. Summers and M. Grubman (Albert Einstein College of Medicine, New York) for [^{3}H]methionine-labeled VSV and for valuable advice and assistance with the ion exchange chromatography.

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