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Identification of the Vesicular Stomatitis Virus Large Protein as a Unique Viral Protein

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Previous studies have noted the existence of a 190,000-dalton vesicular stomatitis virus (VSV) protein called the large (L) protein. To determine whether this protein is a nonspecific aggregate, a precursor to the other VSV proteins, or a unique viral protein, its synthesis relative to the other VSV proteins was studied under conditions of inhibition of initiation of protein synthesis. Also, its tryptic peptides were compared to those of the other VSV proteins. In both cases the results were consistent with the identification of the large protein as a unique viral protein.

Polyacrylamide gel electrophoresis has resolved five proteins which are present in virions of vesicular stomatitis virus (VSV) and are synthesized in VSV-infected cells (2, 10, 14, 23). These proteins have been designated the M, NS, N, G, and L proteins with estimated molecular weights of 29,000, 45,500, 50,000, 69,000, and 190,000, respectively (21). The three major VSV structural proteins are the M (matrix), N (nucleocapsid), and G (glycoprotein). The function of the NS and L (large) proteins is presently unknown, but one or both of them have been implicated as components of the virion-associated transcriptase (4).

Although there is general agreement that the M, NS, N, and G proteins are distinct polypeptides, it has been suggested that the L protein might be an aggregate of the other viral proteins (14, 23). The L protein is found only in small amounts in both the cytoplasm and the virion, but this one protein would require about 50% of the coding capacity of the VSV genome to specify its structure. Attempts to dissociate the L protein in 8 M urea have not been successful (22). To determine whether the L protein is an aggregate, a precursor of the other VSV proteins, or a unique viral protein, two experimental approaches have been employed in the present study. (i) VSV protein synthesis was studied in the presence of low concentrations of pactamycin, a drug which inhibits initiation of protein synthesis but permits continued translation once initiation has occurred. Under these circumstances, proteins translated from large mRNA molecules should continue to be synthesized after the time that translation directed by the smaller mRNA has ceased. Therefore, if the L protein is translated from a larger mRNA,

addition of pactamycin should cause an increase in the amount of L protein synthesized relative to the synthesis of all of the other VSV proteins, but there should be no increase if the L protein is an aggregate. (ii) The tryptic peptides of the L protein were compared to those of all the other VSV proteins. If the L protein is an aggregate or a precursor of all the other VSV proteins, then its peptide profiles should be identical to that of the other VSV proteins. If the L protein is an aggregate of only one or two of the other VSV proteins, then it should contain a smaller number of peptides, all of which should correspond to peptides generated by the other VSV proteins. If the L protein is unique, its peptide profile should be dissimilar to that of the other VSV proteins.

MATERIALS AND METHODS

Infection of cells. The growth of VSV in Chinese hamster ovary (CHO) cells has been described previously (5, 17, 18).

Incorporation of radioactive precursor into virus-specific protein. Cells were harvested from the growth medium and suspended at 2×10^6/ml in either Earle saline (3) or minimal essential medium minus lysine. The appropriate amino acids were then added for the indicated times. Samples were removed and assayed for radioactivity as previously described (20).

Polyacrylamide gel electrophoresis. Cytoplasmic extracts were prepared by disrupting cells in Nonidet P-40 and removing the nuclei by centrifugation. The extracts were treated as previously described (20) and subjected to the Maizel and Laemmli modification of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11, 13) for approximately 3.5 h at 100 V.

Column chromatography of tryptic peptides. Radioactive VSV proteins were eluted from polyacrylamide gels by shaking overnight with 0.1% sodium dodecyl sulfate, reduced by reaction with 12 mg of dithiothreitol for 60 min at 37 C, and then alkylated by incubation with 40 mg of iodoacetamide for 1 h at 4 C. The protein was precipitated with 15% trichloroacetic acid, washed with 5% trichloroacetic acid, and lyophilized. The precipitate was then dissolved in 1 ml of 0.1 M ammonium bicarbonate, pH 8.0, lyophilized, and suspended in 1 ml of ammonium bicarbonate and 50 μg of trypsin. After 12 to 16 h at 37 C, an additional 50 μg of trypsin was added, and the solution was incubated for a further 4-h period. The digest was then lyophilized, dissolved in 1 ml of 0.2 M pyridine-acetate buffer, pH 3.1, lyophilized, and suspended in the pyridine-acetate buffer. The sample was applied to a 55- by 0.9-cm column of Technicon type P chromobeads ion exchange resin maintained at 50 C and eluted as previously described (8). Fractions of about 1.5 ml were added to 20-ml cocktail D (1) and counted in a Beckman LS-250 scintillation counter.

Materials. ^{3}H-L-leucine at 39 Ci/mmol, ^{14}C-L-leucine at 261 mCi/mmol, ^{3}H-L-lysine at 26.8 Ci/mmol or 2 Ci/mmol, and ^{14}C-L-lysine at 312 mCi/mmol were purchased from New England Nuclear Corp., Boston, Mass. Pactamycin was a kind gift of the Upjohn Company; actinomycin D was a kind gift of Merck, Sharpe, and Dohme.

RESULTS

Polyacrylamide gel electrophoresis of VSV cytoplasmic and virion proteins. A mixture of a cytoplasmic extract of VSV-infected CHO cells labeled with ^{3}H-lysine with a preparation of virion proteins labeled with ^{14}C-leucine was analyzed by electrophoresis on 5%, 7.2%, and 10% polyacrylamide gels (Fig. 1). In 5% gels, the large L protein was clearly resolved, whereas the smaller VSV proteins migrated together with the dye marker. Electrophoresis through 7.2% gels resolved the L, G, NS, and N proteins, but the M protein still migrated with the front. In previous reports (10, 13, 21) the NS protein was found to migrate slightly faster than the N protein; we have seen no such protein but have seen a peak of the expected size migrating more slowly than the N protein. This peak, migrating slower than the N protein, is apparently the same as the one seen in other studies because it represents a much larger proportion of cytoplasmic protein than it does of virion protein. Its migration relative to the N protein is apparently a function of the conditions of gel electrophoresis. When we used the electrophoresis system employed in other studies, we could confirm that the NS protein migrated

more rapidly than the N protein, but, in the Laemmli-Maizel gel system, which we have used in the present studies, it migrated more slowly than the N protein. In agreement with previous reports, the G protein isolated from the virion migrated more slowly than the cytoplasmic G protein (10). The 10% gels resolved the M protein from the dye front but gave poor separation of the NS and N proteins.

Effect of pactamycin on VSV-specific protein synthesis. At 5×10^{-7} M, pactamycin will selectively inhibit initiation of protein synthesis in a reticulocyte cell-free system, but it will also inhibit chain elongation when it is present at higher concentrations (12). Since initiation of protein synthesis cannot be directly assayed in intact VSV-infected CHO cells, the ability of various concentrations of pactamycin to produce a delayed but complete stoppage of VSV-specific protein synthesis was assayed. Figure 2 shows that a pactamycin concentration between 2×10^{-7} M and 5×10^{-7} M was sufficient to produce total inhibition of protein synthesis within 12 to 16 min after addition of the drug. This value is similar to that found in poliovirus-infected HeLa cells, where 10^{-7} M pactamycin was the lowest concentration of the drug which ultimately produced complete inhibition of protein synthesis (20).

To determine if pactamycin could increase the amount of L protein relative to the other viral proteins, cells infected for 3.25 h were exposed to 4×10^{-7} M pactamycin, and ^{3}H-lysine was added after 0, 3, or 6 min. All cells were harvested 14 min after addition of pactamycin. Control cells which had not received pactamycin were also exposed to ^{3}H-lysine for 14 min. Cytoplasmic extracts were analyzed on 7.2% polyacrylamide gels (Fig. 3). Although 7.2% gels do not resolve the M protein from the gel front, this concentration was chosen because it gave better resolution of the L protein relative to the background level of radioactivity in that region of the gel.

The total radioactivity present in the L, G, NS, and N protein peaks was determined for each gel, and the percentage of this radioactivity represented by the L protein was calculated. In the control cells (Fig. 3a), the L proteins contained 2.8% of the total radioactivity; in cells labeled for 14 min after addition of pactamycin, 7.5% (Fig. 3b); in cells labeled 3 to 14 min after addition of pactamycin, 12.5% (Fig. 3c); and in cells labeled 6 to 14 min after addition of pactamycin, 9.3% (Fig. 3d). The percentage of the total radioactivity present in the G, NS, or N pro-
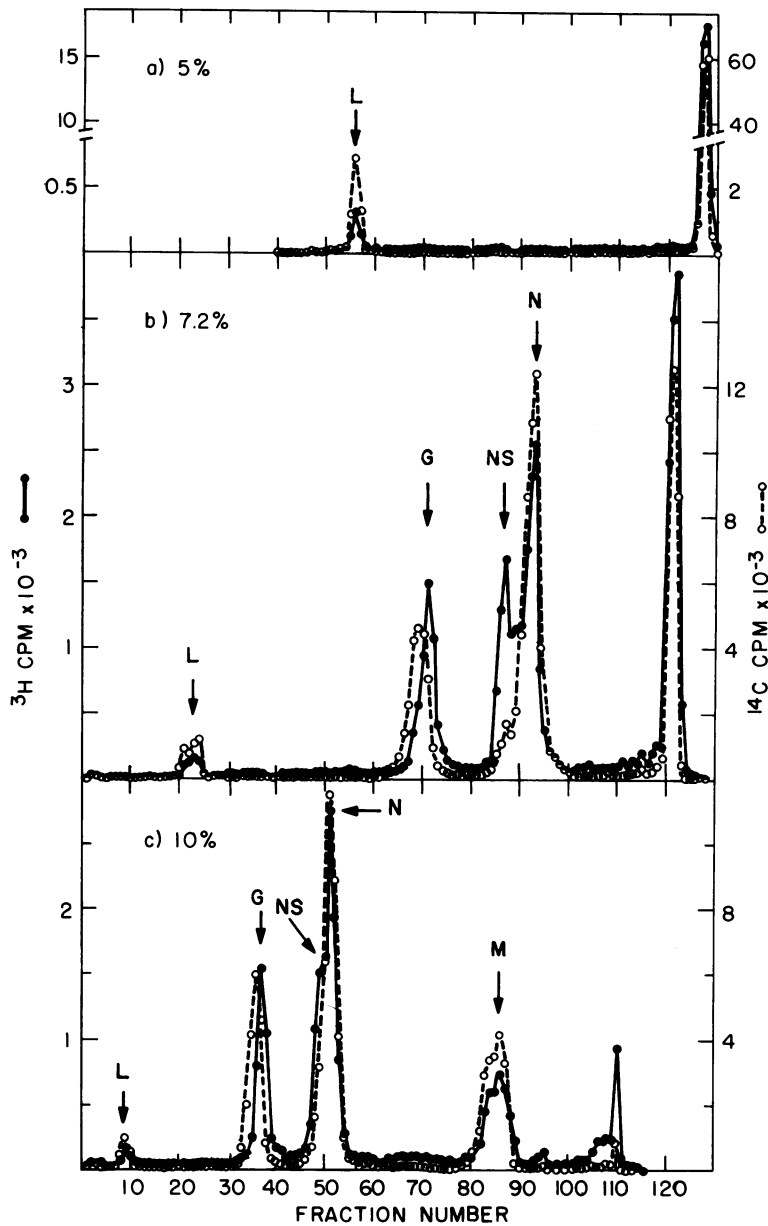
Fig. 1. *Polyacrylamide gel electrophoresis of VSV cytoplasmic and virion proteins.* ^{14}C-virion proteins were obtained by infecting CHO cells at an input multiplicity of 10 plaque-forming units per cell and labeling cells from 1.86 to 4 h postinfection with 5 μCi of ^{14}C-L-leucine per ml in leucine-free minimal essential medium. The released viral particles were harvested at 9 h postinfection, and the purified virus was disrupted in 1% sodium dodecyl sulfate. ^{3}H-cytoplasmic proteins were obtained by infecting cells at an input multiplicity of 10 plaque-forming units per cell and labeling the cells for 14 min at 3.25 h postinfection with 150 μCi of ^{3}H-lysine per ml at 2 Ci/mmol. Electrophoresis was as described in Materials and Methods. The fronts were in fractions 127 to 128 for the 5% gel, fractions 119 to 121 for the 7.2% gel, and fractions 109 to 110 for the 10% gel.

teins remained approximately constant in the various samples. Similar experiments have shown that the percentage of total radioactivity present in the M protein does not increase after addition of pactamycin. Therefore, in the presence of pactamycin, the amount of L protein synthesized relative to all of the other viral proteins can be increased at least fourfold.
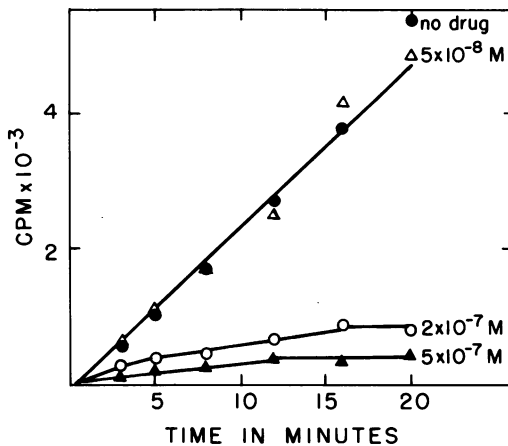
FIG. 2. *Time course of incorporation of leucine in VSV-infected CHO cells at different concentrations of pactamycin. CHO cells were infected with VSV at an input multiplicity of 10 plaque-forming units per cell. At 3 h postinfection, cells were harvested and suspended in Earle saline at 2 × 10⁶ cells/ml. The cells were separated into four equal portions, and pactamycin was added at concentrations of 5 × 10⁷ M, 2 × 10⁻⁷ M, 5 × 10⁻⁸ M, or no drug was added to each. ³H-L-leucine was added to a final concentration of 10 μCi/ml simultaneously with the pactamycin. Samples were taken at the indicated times and assayed for acid-insoluble radioactivity as described (19).*

Comparison of the tryptic peptides of the L protein and the other VSV proteins. Cytoplasmic extracts of VSV-infected cells labeled 2.75 to 4 h postinfection with ³H-lysine or ¹⁴C-lysine were subjected to electrophoresis on 5, 7.2, and 10% polyacrylamide gels. The relative proportions of the five VSV proteins were similar to those shown in Fig. 1 except for a decrease in the relative amount of the M protein, possibly due to the longer labeling period. The approximate molar ratios of the G, NS, N, and M proteins were calculated to be 1:1:2:1 in the preparation. As also seen in Fig. 1, there were no detectable cellular protein peaks.

Those fractions from a 5% gel containing the L protein and those containing all of the other VSV proteins were prepared for analysis of their tryptic peptides. Figure 4 shows a comparison of the tryptic peptides of ¹⁴C-lysine-labeled L protein and ³H-lysine-labeled M, N, NS, and G proteins. A similar result was obtained when the radioactive labels were reversed. Approximately 23 tryptic peptides were generated by the L protein and about 30 by the other VSV proteins. The two profiles were grossly dissimilar, although a few L protein peptides did chromatograph with peptides present in the other VSV proteins.

DISCUSSION

The experiments reported here argue that the L protein is indeed a unique VSV-specified protein. Since most of the L protein tryptic peptides differ from those of the other VSV proteins, it cannot be a precursor or an aggregate of all of the other VSV proteins. At least 23 tryptic peptides are generated by the L protein so it is unlikely to be an aggregate of just one of the smaller VSV proteins because they generate together a total of about 30 tryptic peptides. The fourfold enhancement of L protein synthesis by pactamycin relative to that of each of the other VSV proteins also argues that the L protein is not an aggregate of the smaller VSV proteins but is translated from a larger mRNA. We cannot rule out, however, that there exist some overlaps between the RNA sequences translated from the L protein mRNA and the mRNA species for the other VSV proteins since 5 to 6 of their tryptic peptides were not resolved.

The VSV proteins are translated from mRNA species which have a base sequence complementary to that of the virion RNA (5). These mRNA species are transcribed by a virion-associated, RNA-dependent RNA polymerase (1, 6) and consist of at least three species which sediment together at about 13 to 15S but separate on polyacrylamide gels and one species which sediments at 28S (5). The molecular weights of the 13 to 15S RNA species are consistent with their acting as monocistronic mRNAs for the synthesis of the M, NS, N, and G proteins (16; Stampfer et al., manuscript in preparation). The estimated molecular weight of the 28s mRNA species (2.6 × 10⁶, unpublished observations) is more than sufficient to code for a protein of 190,000 daltons. Therefore, although it may seem unusual for a virus to use 50% of its coding capacity for the synthesis of one large protein, the existence of the L protein is entirely consistent with the known properties of VSV mRNAs. In addition, the L, G, N, NS, and M proteins have a combined molecular weight of approximately 380,000, which is within the coding capacity of the VSV genome which is approximately 3.6 × 10⁶ to 4.0 × 10⁶ daltons (6, 7, 14, 15, 16).

The 28S RNA represents about 30 to 40% of the virus-specific RNA synthesized in VSV-infected CHO cells (18), whereas the L protein represents only about 2 to 3% of VSV-specific protein. It has also been observed that the 28S mRNA is present on small polyribosomes and must therefore have a low concentration of
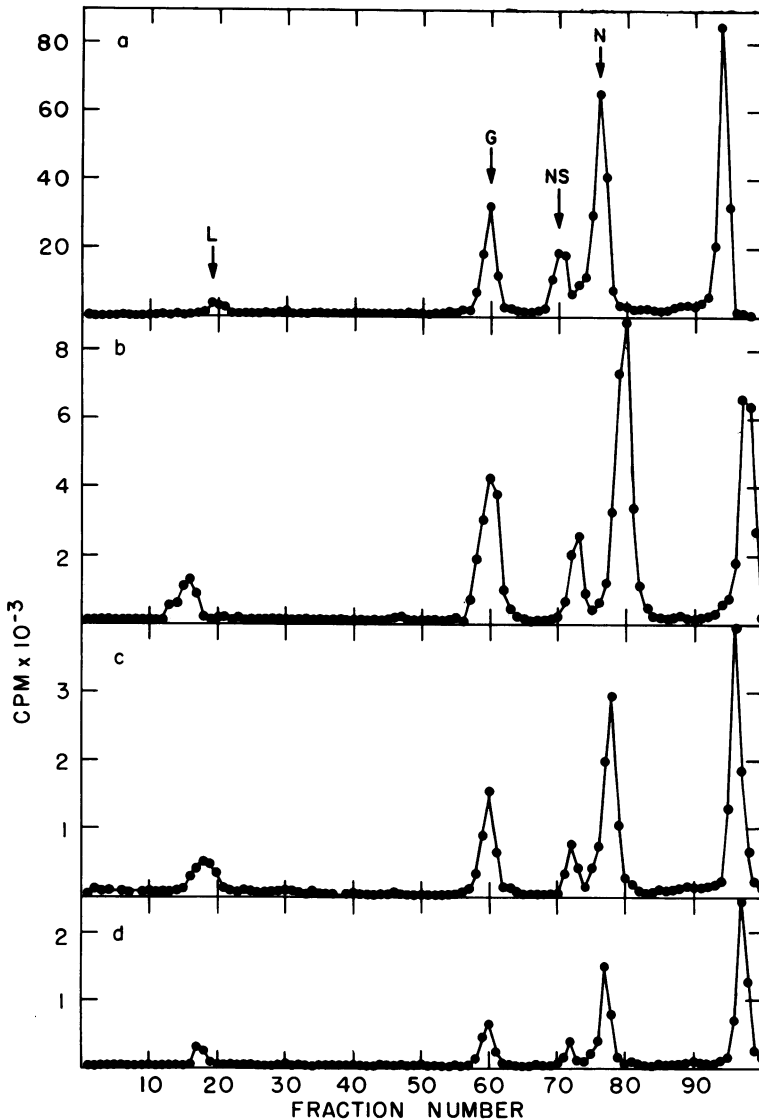
Fig. 3. *Polyacrylamide gel electrophoresis of VSV cytoplasmic proteins synthesized by CHO cells after addition of pactamycin. CHO cells were infected with VSV at an input multiplicity of 10 plaque-forming units per cell. At 3.25 h postinfection, cells were harvested and suspended in Earle saline at 2 × 10⁶ cells/ml.* The cells were separated into four equal portions, and pactamycin at a concentration of $4 \times 10^7 M$ was added to three of the portions. ³H-L-lysine at 2 Ci/mmol at a final concentration of 150 μCi/ml was added to each portion for the indicated times. The cells were harvested, and the cytoplasmic extracts were subjected to electrophoresis on 7.2% polyacrylamide gels. a, No drug added, cells labeled for 14 min; b, cells labeled 0 to 14 min after addition of pactamycin; c, Cells labeled 3 to 14 min after addition of pactamycin; d, Cells labeled 6 to 14 min after addition of pactamycin.*

ribosomes per length of RNA (5). Poliovirus mRNA, which is comparable in size to the VSV 28S mRNA, is present on much larges polyribosomes (19). Since there is a large amount of 28S mRNA present in infected CHO cells, yet only small amounts of L protein are synthesized on small polyribosomes, there must be differential translation of the various viral messenger RNAs, presumably determined by the frequency of initiation of translation on each mRNA.
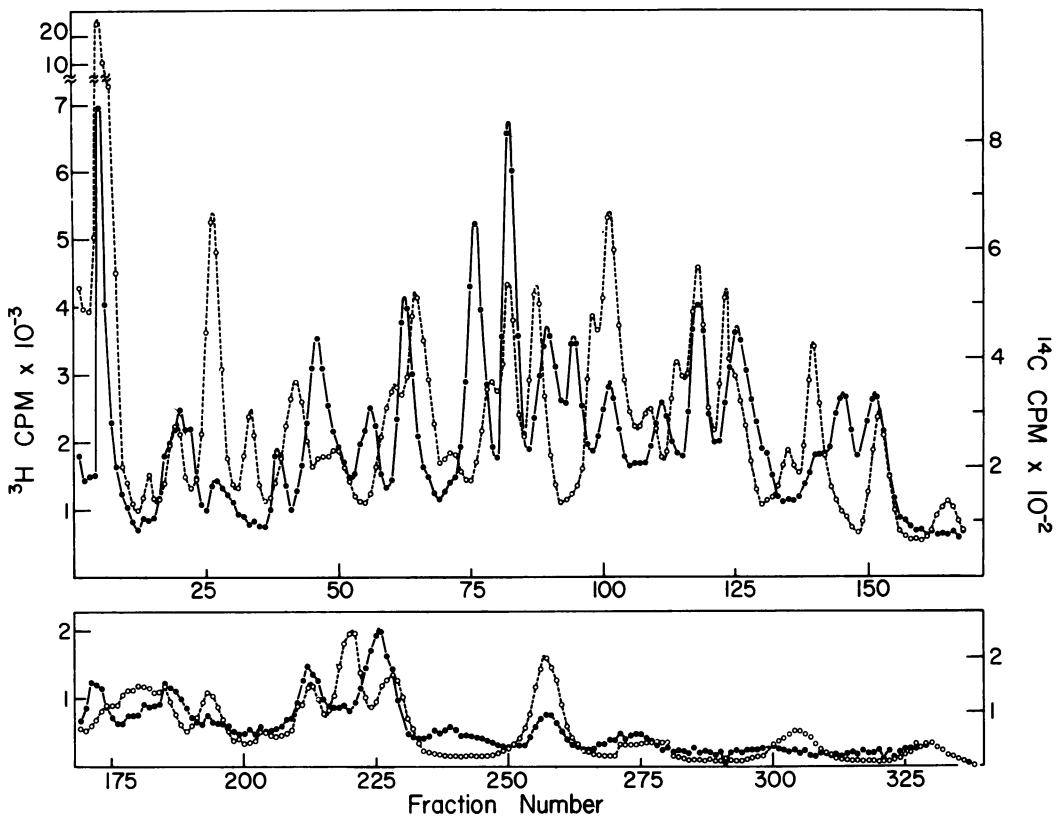
FIG. 4. *Tryptic peptides of ¹⁴C-L-lysine-labeled VSV L protein compared to the tryptic peptides of ³H-L-lysine-labeled other VSV proteins. CHO cells were infected with VSV at an input multiplicity of 10 plaque-forming units per cell. At 2.75 h postinfection, cells were harvested and suspended in minimal essential medium minus lysine. ³H-lysine at 26.8 Ci/mmol at a final concentration of 100 μCi/ml or ¹⁴C-lysine at 10 μCi/ml was added, and the cells were harvested at 4 h postinfection. The cytoplasmic extracts were subjected to electrophoresis on 5% polyacrylamide gels, sliced into 1-mm fractions, and the proteins were eluted by shaking overnight in 0.1% sodium dodecyl sulfate. The fractions containing the L protein and those containing the other VSV proteins were pooled and prepared for analysis of their tryptic peptides as described. The two panels represent the fractions from a single column analysis. Symbols: ●, ¹⁴C; ○, ³H.*

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