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Vesicular Stomatitis Virus M Protein in the Nuclei of Infected Cells

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The M protein of vesicular stomatitis virus (VSV) was localized in the nuclei and cytoplasm of VSV-infected cells by subcellular fractionation and immunofluorescence microscopy. Nuclei isolated from VSV-infected Friend erythroleukemia cells were fractionated into a nuclear membrane and a nucleoplasm fraction by DNase digestion and differential centrifugation. G protein was present in the membrane fraction, and M protein was present in the nucleoplasm fraction. Immunofluorescence detection of M protein in the nucleus required that fixed cells be permeabilized with higher concentrations of detergent than were required for detection of M protein in the cytoplasm of VSV-infected BHK cells.

The M protein of vesicular stomatitis virus (VSV) is a major structural component of the virion and plays a central role in virus assembly. Newly synthesized M protein is initially found in the cytosol as a soluble precursor to M protein associated with the cytoplasmic surface of the plasma membrane during virus assembly (1, 7, 16, 22). Chemical cross-linking studies indicate that the virion M protein interacts both with viral envelope components and with the nucleocapsid (9, 20, 29). In vitro reconstitution experiments show that the M protein condenses the nucleocapsid into a tightly coiled structure (23) and inhibits the in vitro RNA polymerase activity of the viral nucleocapsid (5, 28). In addition, studies with temperature-sensitive M protein mutants suggest a role for M protein in regulating viral transcription in vivo (6). Thus, the well-characterized functional activities of M protein occur in the cytoplasm of infected cells.

However, in addition to that present in the cytoplasm, M protein is detected in a highly purified nuclear fraction from VSV-infected cells (26). In the experiments described here, M protein was also observed in the nucleus of infected cells by immunofluorescence microscopy of unfractionated cells. In contrast, Ono et al. (24) have presented both immunofluorescence and subcellular fractionation data indicating that M protein is absent from infected-cell nuclei. The experiments described here show that the discrepancy in these results is due to the use of different detergent concentrations in permeabilization of cells for immunofluorescence microscopy and in isolation of nuclei. The weight of the evidence indicates that the VSV M protein is present in the nucleus of infected cells. The idea that differential permeabilization of cells could account for the difference in immunofluorescence microscopy results was obtained from a recent study showing that the M protein of Newcastle disease virus is localized primarily in the nucleus of infected cells (25).

The distribution of VSV proteins within the nuclear fraction isolated from infected cells was examined to determine whether M protein was associated with the nuclear envelope or with internal nuclear constituents (nucleoplasm). Friend erythroleukemia cells (clone 745a, cell line GM-86) obtained from the Human Genetics Mutant Cell Repository, Camden, N.J., were used in subcellular fractionation experiments. From these cells, nuclei can be readily isolated essentially intact and free of cytoplasmic contamination, as shown by electron microscopy (19) and by the fact that they are largely devoid of cytoplasmic marker enzymes (19) and cytoplasmic RNAs such as the mRNAs for the VSV N, NS, and M proteins (26). Erythroleukemia cells were infected with VSV (Indiana serotype) at a multiplicity of 10 PFU per cell as described elsewhere (26). At 4 h postinfection, cells were incubated for 10 min in methionine-deficient Dulbecco modified Eagle medium and then labeled for 10 min with [^{35}S]methionine (25 μCi/ml, >600 Ci/mmol) in methionine-deficient Dulbecco modified Eagle medium containing 2.5% dialyzed fetal calf serum. Nuclei were isolated essentially as described elsewhere (19). Briefly, cells were disrupted by Dounce homogenization in 10 mM Tris–10 mM NaCl–1.5 mM $MgCl_2$ (reticulocyte standard buffer). The homogenate was layered over a two-step gradient of 45 and 61% (wt/wt) sucrose in reticulocyte standard buffer including 1 mg of bovine serum albumin per ml and was centrifuged at low speed. Purified nuclei were collected from the interface.

Nuclear membranes and a nucleoplasmic fraction were isolated by a modification of the procedure of Kay and Johnston (15). Isolated nuclei were sedimented and suspended to 5×10^7/ml in 0.1 mM $MgCl_2$. DNase I (Sigma Chemical Co., St. Louis, Mo.) was added (5 μg/ml), and then 4 volumes of buffer A (10% sucrose in 10 mM Tris, 0.1 mM $MgCl_2$, and 6 mM 2-mercaptoethanol, pH 8.5) were added. Digestion was for 15 min at room temperature with constant agitation. An equal volume of ice-cold water was added, and membranes were sedimented by centrifugation at 38,000 × *g* for 15 min at 4°C. The membranes were suspended in buffer A (pH 7.5), redigested with 5 μg of DNase per ml for 20 min at room temperature, and sedimented again. The nucleoplasm fraction consisted of the pooled supernatants from the two centrifugations following digestion with DNase. These supernatants contain most of the chromatin in the form of small, DNase-digested fragments and most of the nucleoplasmic proteins (15). The nuclear membranes obtained had a 4.7-fold increase in specific activity (counts per million per mg of protein) over intact nuclei as determined by [^{14}C]ethanolamine labeling of phospholipids. The nuclear membrane fraction also showed enrichment of membranes upon examination by electron microscopy, although some fragments of chromatin remained attached to the inner nuclear membrane. [^{35}S]methionine-labeled nuclei, nuclear membranes, and nucleoplasm were analyzed by sodium dodecyl sulfate-gel electrophoresis on 10% polyacrylamide

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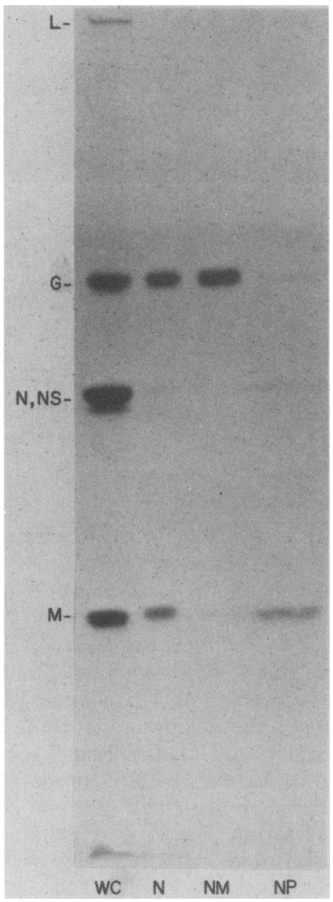

FIG. 1. Localization of VSV proteins in the nuclear fraction of infected cells. VSV-infected Friend erythroleukemia cells were labeled for 10 min with [^{35}S]methionine, and nuclear membranes were isolated from purified nuclei as described in the text. The protein composition of each fraction was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A fluorograph of the dried gel is shown. Lanes: WC, proteins extracted from unfractionated whole cells; N, purified intact nuclei; NM, purified nuclear membranes; and NP, nucleoplasm. The identities of the VSV proteins are indicated on the left. N and NS comigrate in this electrophoresis system.

gels (17) followed by fluorography (3). The results are shown in Fig. 1.

As shown previously (26), nuclei isolated from Friend erythroleukemia cells (Fig. 1, lane N) contain a substantial fraction of newly synthesized G and M proteins but are largely devoid of the VSV nucleocapsid proteins N, NS, and L. The G protein was present in the nuclear membrane (Fig. 1, lane NM) but not in the nucleoplasm fraction (Fig. 1, lane NP). This was expected, since G protein is synthesized on ribosomes bound to the outer nuclear membrane coordinately with synthesis on ribosomes bound to the endoplasmic reticulum (26). In erythroleukemia cells which contain large nuclei and relatively little endoplasmic reticulum, nearly half of the total newly synthesized viral glycoproteins can be found in the nuclear membrane (19, 26). The M protein initially associated with purified nuclei (Fig. 1, lane N) was not present in the nuclear membrane fraction but instead was found in the nucleoplasm (Fig. 1, lane NP). In the subcellular fractionation experiments described by Ono et al. (24), neither M protein nor G protein was detectable in their nuclear preparation. We have determined that the discrepancies between our results and those of Ono et al. can be accounted for by the absence or presence of detergents (Triton X-100 and deoxycholate) in the nuclear isolation. Our nuclei were purified in the absence of detergent because our experiments demonstrated that Triton X-100 solubilized the G protein and that deoxycholate solubilized both the G and M proteins from nuclei isolated from erythroleukemia cells, yielding results similar to those of Ono et al. (Table 1).

TABLE 1. Detergent solubilization of VSV proteins in nuclei isolated from infected cells[a]

Detergent	% in supernatant	
	M protein	G protein
Expt 1		
None	25	23
Triton X-100 (0.5%)	20	77
Expt 2		
None	15	5.0
Sodium deoxycholate (0.2%)	92	96

[a] VSV-infected Friend erythroleukemia cells were labeled for 10 min with [^{35}S]methionine at 4 h postinfection, and nuclei were isolated as described in the text. Nuclei were suspended to approximately 2×10^7/ml in reticulocyte standard buffer, and either detergents were added to the indicated final concentration or no detergent was added to control for the small amount of nuclear disruption that occurs upon repeated centrifugation. Samples were sedimented at 1,600 × *g* for 30 min. The supernatant and pellet were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography, and the labeled G and M proteins in each fraction were quantitated by densitometry. Data shown are from a representative of two experiments with each detergent.

The presence of M protein in the nucleus of VSV-infected cells was also demonstrated by immunofluorescence microscopy of fixed and permeabilized cells. However, the ability to detect M protein in the nucleus depended on the conditions used to permeabilize the cells, as recently described for the Newcastle disease virus M protein (25). Monoclonal antibodies against the VSV M protein used for immunolocalization were produced as described elsewhere (18). Two different antibodies (23H12 and 24H6), which have been determined to be serotype specific for the M protein of VSV Indiana by enzyme-linked immunosorbent assay and by immunoprecipitation of labeled infected cell extracts (unpublished results), were used. Antibodies were purified from ascites fluid by chromatography on protein A-Sepharose and labeled with fluorescein isothiocyanate as described elsewhere (26). BHK cells were seeded onto 12-mm glass cover slips (10^5 cells per sample) and incubated overnight in Dulbecco modified Eagle medium plus 10% fetal calf serum. Cells were infected with VSV at a multiplicity of 10 to 30 PFU per cell. At 4 h postinfection, the cells were fixed in 4% paraformaldehyde in 10 mM sodium phosphate–0.9% NaCl, pH 7.4 (PBS), at 4°C for 1 h and then washed three times in PBS containing 0.1 M glycine (blocking buffer). Cells were permeabilized by incubation for 10 min at room temperature with 0.05, 0.2, or 1% Triton X-100 in PBS and then washed in blocking buffer. Labeling with fluorescein-conjugated antibodies was for 1 h at 4°C in blocking buffer with 5% fetal calf serum. Cover slips were mounted in Aqua-mount (Lerner Laboratories, New Haven, Conn.) and photographed with an Olympus fluorescence microscope. The data were shown in Fig. 2.

When fixed cells were permeabilized with 1 or 0.2% Triton X-100 (Fig. 2a and b), immunofluorescence labeling of the VSV M protein was observed to be diffusely distributed

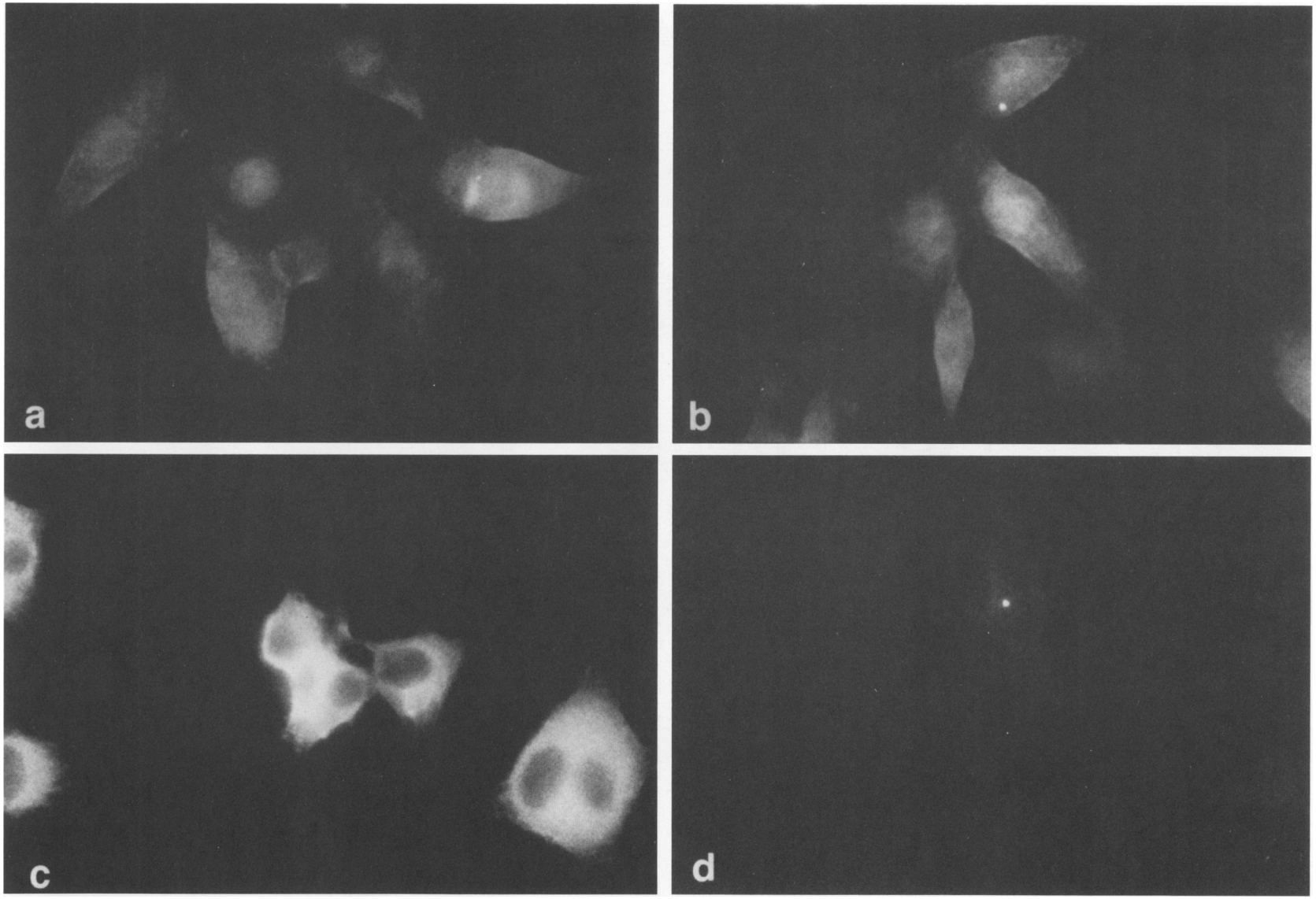

FIG. 2. Immunofluorescence localization of M protein in VSV-infected BHK cells. Cells were infected with either VSV Indiana (a–c) or VSV New Jersey (d). At 4 h postinfection, cells were fixed and permeabilized with Triton X-100 at a concentration of 1% (a and d), 0.2% (b), or 0.05% (c). Cells were labeled with fluorescein-conjugated monoclonal antibody 24H6 specific for the M protein of VSV Indiana.

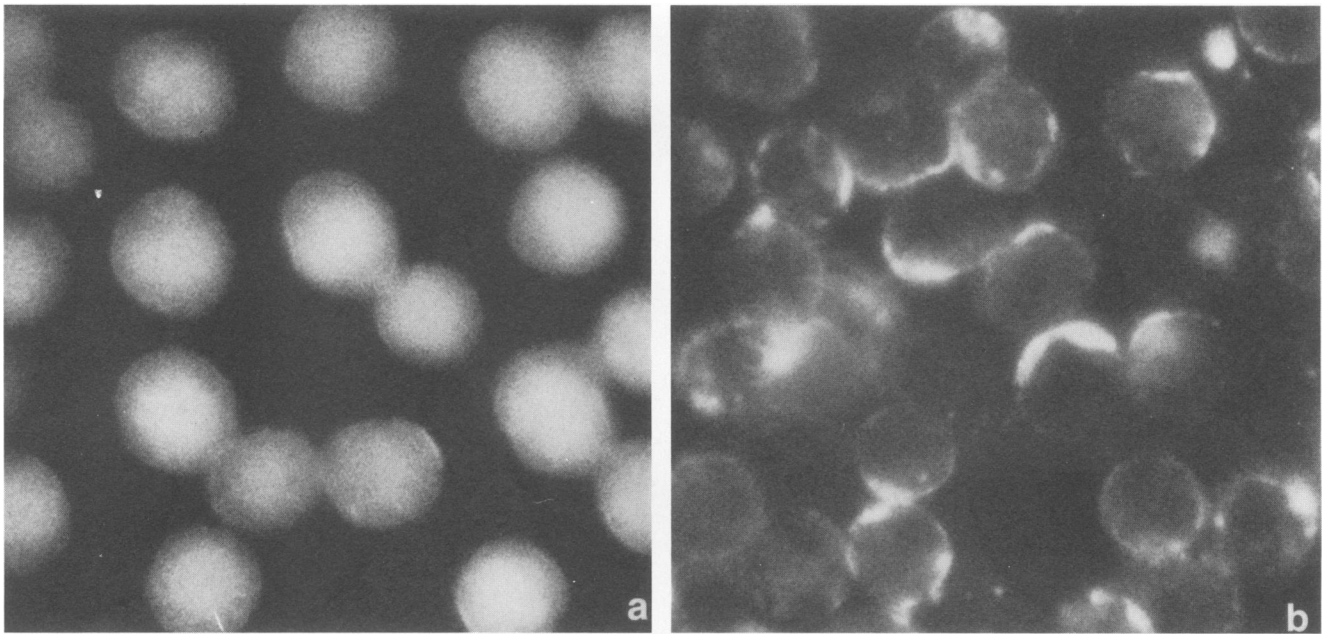

FIG. 3. Immunofluorescence of VSV-infected Friend erythroleukemia cells with antibodies to the M and N proteins. VSV-infected cells were fixed with Formalin and permeabilized with Triton X-100 at 4 h postinfection. Cells were labeled with fluorescent monoclonal antibodies to the VSV Indiana M protein (a) or N protein (b).

throughout the cytoplasm and the nucleus of infected cells. In some cells, the nucleus is clearly visible above the level of fluorescence seen in the cytoplasm. This is probably because of the greater thickness of the cell in the area around the nucleus compared with the thinness of the peripheral cytoplasm. In slightly rounded cells, the nucleus is not so clearly discernible because the nucleus and cytoplasm appear to be stained to approximately equal intensities. As reported previously (24), when cells were permeabilized with 0.05% Triton X-100, labeling of M protein was observed in the cytoplasm, but labeling was not observed in the nucleus (Fig. 2c). As a negative control, cells infected with the New Jersey serotype of VSV showed only background fluorescence (Fig. 2d). This control rules out the possibility that the labeling of the nucleus was due to cross-reactivity of the M protein antibodies with a host component induced by VSV infection. Results virtually identical to those in Fig. 2a, b, and d, including labeling of the nucleus in fixed cells permeabilized with Triton X-100, have been obtained under a variety of experimental conditions (data not shown). Two different monoclonal antibodies have been used, making it unlikely that the M protein distribution observed is due to a unique epitope accessibility. No difference in the distribution of M protein was observed as a function of time from 2 to 10 h postinfection. Cells have been labeled with Texas red-conjugated antibodies or by indirect rather than direct immunofluorescence techniques. Fixation in Formalin, paraformaldehyde, and glutaraldehyde all gave virtually identical results. The only other fixation procedure we have tried that gave results similar to those shown in Fig. 2c, in which labeling of the nucleus was not detected, was the use of 5% acetic acid in ethanol as described by Ono et al. (24).

The intracellular distribution of the M protein and nucleocapsid (N) protein in Friend erythroleukemia cells is shown in Fig. 3. At 4 h postinfection, cells were fixed in 3% formaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. Parallel samples were labeled with fluorescein-conjugated antibody 23H12 specific for M protein or with Texas red-conjugated antibody 10G4 specific for N protein. The staining pattern observed for M protein in erythroleukemia cells (Fig. 3a) was diffuse and evenly distributed throughout the cell cytoplasm and nucleus. In contrast, the staining observed for the N protein (Fig. 3b) was concentrated in focal areas of the cytoplasm and was not observed in the nucleus. The same results were obtained by immunofluorescence labeling of N protein in VSV-infected BHK cells (data not shown). These results show that detection of M protein in the nucleus by immunofluorescence microscopy of fixed and permeabilized cells is not unique to BHK cells and is not the result of detergent-induced redistribution of VSV antigens, since N protein is observed only in the cytoplasm.

The results presented here, together with our previously published results (26), demonstrate that the envelope proteins of VSV are found in the nucleus of infected cells when both subcellular fractionation and immunolabeling techniques are used. The G protein is associated with the nuclear envelope, whereas the M protein is associated with internal nuclear structures. In our subcellular fractionation experiments, we have used erythroleukemia cells, which contain large nuclei that are readily isolated with a very low level of cytoplasmic contamination. We avoided nuclear isolation procedures that used detergents to remove residual cytoplasmic elements, since such procedures solubilize the nuclear membrane and can also solubilize proteins from within the nucleus. For example, deoxycholate has been shown to solubilize the structural proteins of the nuclear lamina (13). Similarly, isolation of nuclei in the presence of detergents failed to demonstrate the nuclear localization of the influenza virus NS_2 protein (4), which was later shown by immunofluorescence microscopy of fixed and permeabilized cells (12). The ability to detect the VSV M protein in the nucleus by immunofluorescence microscopy depends on the conditions used for permeabilization, as shown recently for the M

protein of Newcastle disease virus (25). The ability to detect viral antigens in the nucleus is a function of differential accessibility of the nucleus to antibodies rather than a difference in antigen distribution (25). The conditions required for entry of antibodies into the nuclei of fixed cells probably vary somewhat according to cell type, as briefly mentioned previously (12), since permeabilization of the nuclei of Vero cells (25) requires lower concentrations of Triton X-100 than those used here to permeabilize BHK cell nuclei.

The mechanism by which a portion of the M protein is localized to the nucleus could be passive partitioning from the soluble cytoplasmic pool. Such behavior has been observed when molecules that are not normal intracellular components are introduced into cells. However, such partitioning is rare among normal cytoplasmic proteins (8). Alternatively, M protein may contain targeting signals that result in nuclear localization, as recently described for several nuclear proteins. For example, the highly basic amino-terminal domain of the M protein (27) may resemble the sequence responsible for localization of the simian virus 40 T antigen in the nucleus, which contains five contiguous basic amino acids (14).

Finally, the role VSV proteins in the nucleus may play during virus infection must be considered. They are clearly not essential for virus production, since VSV replication can occur in enucleated cells with only minimal reduction in virus yield (11). G protein in the nuclear envelope is found to be present in both the inner and outer nuclear membranes (2; L. Puddington and D. S. Lyles, unpublished results). As mentioned above, the G protein in the nuclear envelope is a quantitatively important source of newly synthesized G protein in some cell types. Likewise, a substantial fraction of the newly synthesized M protein is found in the nucleus. It seems likely that this pool of viral proteins may be more than just a reservoir of proteins for virus assembly. For example, these proteins may play a role in the inhibition of nuclear function that is part of the cytopathology of VSV infection. Recent attention has focused on the role of the VSV leader RNA (21) and possibly the N protein (10) in the inhibition of host RNA synthesis. However, the role of M protein or G protein or both in viral alteration of other nuclear functions should also be evaluated.

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