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A Mutated Membrane Protein of Vesicular Stomatitis Virus Has an Abnormal Distribution within the Infected Cell and Causes Defective Budding

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Two temperature-sensitive (*ts*) mutants of the M protein of vesicular stomatitis virus (*ts*G31 and *ts*G33) are defective in viral assembly, but the exact nature of this defect is not known. When infected cells are switched from nonpermissive (40°C) to permissive (32°C) temperatures in the presence of cycloheximide, *ts*G33 virus release increased by 100-fold, whereas *ts*G31 release increased only by 10-fold. Thus, the *ts*G33 defect is more reversible than that of *ts*G31. Therefore, we investigated how the altered synthesis and cellular distribution of *ts*G33 M protein correlates with the viral assembly defect. At 32°C *ts*G33 M protein is stained diffusely in the cell cytoplasm and later at the budding sites. In contrast, at 40°C the mutant M protein formed unusual aggregates mostly located in the perinuclear regions of virus-infected cells and partially colocalized with G protein in this region. In temperature shift-down experiments, M can be disaggregated and used to some extent for nucleocapsid coiling and budding, which correlates with the virus titer increase. M aggregates also formed after shift-up from 32 to 40°C, indicating a complete dependence of M aggregation on the temperature. Biochemical analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting revealed that at 40°C M protein is detected exclusively in pellet fractions (nuclear and cytoskeleton components), whereas at 32°C M protein is mainly in the cytoplasmic soluble fractions. Furthermore, when the temperature is raised from 32 to 40°C, the distribution of M protein tends to shift from the soluble to the pellet and cytoskeletal fractions. Electron micrographs of immunoperoxidase-labeled M protein showed that at 40°C M aggregates are often associated with the outer nuclear membranes as well as with vesicular structures. No nucleocapsid coiling was observed in these cells, whereas coiling and budding were seen at 32°C in cells where M protein was partly associated with the plasma membrane. We suggest that the *ts*G33 M protein mutation may produce a reversible conformational alteration which causes M protein to aggregate at 40°C, therefore inhibiting the proper association of M protein with nucleocapsids and budding membranes.

It has been proposed that the M protein plays an essential role in the assembly of the rhabdovirus vesicular stomatitis virus (VSV). However, the exact mechanism by which M fulfills this function is not clearly understood. M protein is synthesized on free ribosomes and is detected in a diffuse pattern throughout the cells by immunofluorescence about 3 h after virus inoculation (reviewed in reference 9). Later in infection, M protein becomes more concentrated at the cell membrane where budding occurs. Within the virion, VSV M protein may be located immediately beneath the bilayer where this basic protein probably interacts with negatively charged lipids in the membrane (37). Using a deep-etch rotary replica technique to visualize the cytoplasmic face of the membrane of infected cells, we have found that the M protein can be localized on the surface of uncoiled but not coiled nucleocapsid (26). In that communication, we proposed that the interaction of M protein with the nucleocapsid triggered the coiling, which itself is necessary for viral assembly and budding. Earlier work also supports the role of M protein in coiling of the nucleocapsid inside the complete virion (24, 25).

How essential the M protein is to nucleocapsid coiling and virus budding can be further analyzed with temperature-sensitive (*ts*) mutants of the M protein like those of VSV *ts*G31 and *ts*G33 (10, 15, 16). The M gene of these mutants has been sequenced recently and shown to differ from the parental strain by only one amino acid (*ts*G31) or two amino acids (*ts*G33) (11). Previous studies with these mutants have shown that low amounts of M protein are synthesized at the nonpermissive temperature (40°C), that this M protein is unstable, and that virus release does not occur in these conditions (14–16).

We have used these mutants to analyze in more detail the defect in viral assembly at the nonpermissive temperature and the reversibility of this defect when the temperature is shifted down to 32°C. We find that M proteins of both mutants aggregate in perinuclear regions and are associated with the pellet fraction of infected cells maintained at nonpermissive temperature, whereas at the permissive temperature M protein is diffuse throughout the cells and associates only with the cytoplasmic soluble fraction. In addition, nucleocapsids stay uncoiled, and no budding occurs at the nonpermissive temperature. With one of the *ts* mutants (*ts*G33), M protein can be partly disaggregated after a 30-min shift down to the permissive temperature; this allows nucleocapsid coiling and budding to occur. Temperature shift-up experiments also induce M aggregation. We suggest that conformational changes of the mutant M protein at 40°C

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cause its abnormal distribution within the infected cells and inhibit budding.

MATERIALS AND METHODS

Virus and cell. Wild-type VSV (Mudd Summers strain, Indiana serotype) and the temperature-sensitive mutants *ts*G31 and *ts*G33 (Glasgow strain, Indiana serotype) were plaque purified in baby hamster kidney (BHK) cells grown in Eagle minimal essential medium supplemented with 10% fetal calf serum.

Cells were grown in 3.5- or 15-cm plastic tissue culture dishes or on glass cover slips. Stock viruses grown in BHK cells were diluted into Eagle medium and adsorbed to BHK cells at a multiplicity of infection of 10 to 100 at 37°C for 1 h. After unadsorbed virus was removed by washing with Eagle medium, the infected cells (in Eagle medium with 2% fetal calf serum) were kept at the permissive (32°C) or nonpermissive (40°C) temperature.

Titrations of the viruses were performed by the agar overlay plaque assay method. To enhance the accuracy of titrations, excess virus was neutralized after adsorption by rabbit anti-VSV antibodies (Microbiological Associates, Bethesda, Md.).

Antibodies and conjugates. Polyclonal rabbit antibody against M proteins of VSV was raised against electrophoretically purified M protein obtained from purified virus. The titer and specificity of this serum were determined by immunofluorescence. Mouse monoclonal antibodies against N and G proteins of VSV were a gift from Heinz Arnheiter (1, 2). Three mouse monoclonal antibodies that reacted with different epitopes of M protein were supplied by Robert Wagner (27, 29, 30). A monoclonal antibody that recognizes all classes of intermediate filaments was generously provided by Rebecca Pruss (31). Monoclonal anti-β-tubulin was purchased from Amersham Corp. (Arlington Heights, Ill.). Affinity purified goat anti-rabbit immunoglobulin G (IgG) antibody coupled to rhodamine, affinity-purified anti-mouse IgG conjugated to fluorescein, and both anti-rabbit IgG antibody and anti-mouse IgG antibody coupled with peroxidase were purchased from Cappel Worthington Biochemical (Malvern, Pa.).

Immunofluorescence. At various times after infection, infected or noninfected cells, grown on 13-mm glass cover slips, were fixed either in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 30 min, followed by 5 min of treatment with 0.05% Triton X-100 or in acid alcohol (5% acetic acid and 95% alcohol) for 15 min at −20°C. Antibodies directed against M, N, and G proteins of VSV, β-tubulin, or intermediate filament were added and then reacted with secondary antibody conjugates with the appropriate species specificity (see above). The cells were observed in a Zeiss photoscope III microscope equipped with epifluorescence or in a Zeiss ICM 405 inverted microscope equipped with epifluorescence and differential interference contrast optics. To detect potential colocalization of M and G proteins, M and N proteins, and M and intermediate filaments, double labeling was carried out on some specimens.

With a multiplicity of infection of 10, the intensity of immunofluorescence was not sufficient, and all cells could not be stained. Therefore, in most experiments cells were infected with a multiplicity of infection of 100.

Electron microscopy. For thin sections, cells were fixed in periodate lysine-paraformaldehyde fixative as described previously (22), and aldehyde groups were quenched with 50 mM NH_4Cl in phosphate-buffered saline (pH 7.4) for 30 min (20). Both M and N proteins were stained by the indirect immunoperoxidase method of Brown and Farquhar (3) with a rabbit polyclonal antibody and mouse monoclonal antibody, respectively. After cells were embedded in situ as described previously (17), they were checked for staining at the light microscopic level and drilled out of the epoxy layer, remounted, and thin sectioned parallel to the monolayer with a diamond knife. Grids were counterstained with uranyl acetate and lead citrate in some cases. For preparation of replicas of the cell surface, the procedure described by Odenwald et al. (26) was used.

All grids were examined with a Phillips 400T electron microscope operating with an accelerating voltage of 60 or 80 kV. Replicas were also examined at a ±6° tilt angle for stereo image and printed from photographically reversed negatives.

Pulse-labeling studies. The method used for the labeling of proteins produced by virus in BHK cells was based on that of Knipe et al. (15), with some modifications. Briefly, in time sequence experiments, cultures were labeled by adding [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) at 10 μCi/ml 2 h after adsorption. The cells were processed and fractionated every 1 h up to 8 h after adsorption. For temperature shift-down experiments, cells were labeled with the same amount of [^{35}S]methionine as above for 1 h at 40°C and rinsed with medium containing a normal level of unlabeled methionine. Cells were moved to 32°C for periods up to 2 h before fractionation. Fifteen minutes before shifting down, 100 μg of cycloheximide per ml was added into each dish to prevent protein synthesis after shifting down. For the temperature shift-up experiment, cells were labeled 2 h after adsorption and incubated for an additional 6 h at 32°C. The cells were then washed with nonradioactive normal prewarmed medium and maintained at 40°C for up to 3 h before fractionation.

Cell fractionation. Labeled cell extracts were separated by the method of Knipe et al. (15) and Lenk and Penman (19) into three subcellular fractions: soluble cytoplasmic, cytoskeletal, and nuclear fractions. Each fraction was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the soluble fractions were separated by using a 10 to 30% sucrose gradient in 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA. The radioactivity of each fraction was determined by scintillation spectrometry in 10 ml of Hydrofluor (National Diagnostics, N.J.). Subsequently, each fraction was also analyzed by SDS-PAGE.

SDS-PAGE of proteins and Western blot analysis. Labeled fractions from the cell extract were subjected to electrophoresis in a 10% polyacrylamide slab gel by the method of Laemmli (18). The proteins in the gels were transferred by electroblotting onto nitrocellulose sheets as described by Towbin et al. (35). The transferred proteins were incubated first with rabbit anti-M antibody and mouse monoclonal anti-intermediate filament antibody and then with a mixture of anti-rabbit and anti-mouse IgG conjugated with peroxidase.

RESULTS

Virus release at permissive (32°C) and nonpermissive (40°C) temperature and after temperature shift-down. At the permissive temperature, both mutants *ts*G31 and *ts*G33 induced cell rounding after 3 to 4 h, whereas discrete cell rounding was only observed after 8 h at the nonpermissive temperature. After 6 h at 32°C both mutant viruses yielded titers similar to those obtained with wild-type VSV, 10^8 PFU/ml. In con-

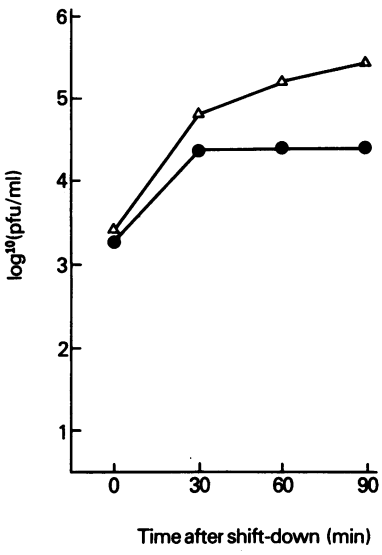

FIG. 1. Kinetics of virus release in cells infected with two VSV M mutants after shift-down to the permissive temperature. Cells infected with *ts*G31 (●) or *ts*G33 (△) were inoculated at 37°C, maintained for 6 h at 40°C, and then shifted back to 32°C (0 min) in the presence of 100 μg of cycloheximide per ml. Released virus was titered on BHK 21 cells at 32°C.

trast, at 40°C both *ts* mutants produced as few as 10^3 PFU/ml (Fig. 1), which is approximately 5 log units less virus than the wild type at this temperature. Virus budding was analyzed in thin sections of epoxy-embedded cells and in surface replicas of fast-frozen infected cells. In cells infected and maintained at 32°C, numerous bullet-shaped virions were seen budding at the plasma membrane and in intracytoplasmic vacuoles (Fig. 2A and B). In contrast, no complete virions were detected at the membrane of BHK cells maintained at 40°C (Fig. 2C and D). These results confirm that both *ts* mutants are defective in viral assembly at the nonpermissive temperature, even though a small number of viral particles can be detected by virus titration. These particles may be revertants of the *ts* mutant virus.

We also tested whether M protein presynthesized for 6 h at 40°C and then shifted to 32°C for up to 2 h could allow viral assembly and release. The synthesis of new M protein after shift-down was prevented by adding 100 μg of cycloheximide per ml 15 min before changing the temperature. With *ts*G33, the virus titer increased 100-fold (up to 10^5 PFU/ml) after 1 h of shift-down (Fig. 1). This indicates some reversibility of the defect in virus budding and renaturation of the M protein at 32°C. Such reversibility was less marked with *ts*G31-infected cells, which showed only a slight increase in virus release after shift-down (Fig. 1). Electron microscopy revealed a progressive increase in the number of viral buds on BHK cells infected with *ts*G33 after 30 min of temperature shift-down (Fig. 2E and F). Because the *ts*G33 mutation is more reversible at 32°C, this mutant virus was used in most of the following studies.

Intracellular localization of M protein at 32 and 40°C. We used immunocytochemical techniques on cells infected with the *ts* mutant to visualize the detailed localization of M protein in the cells and the relationship between M protein and other VSV proteins. Since M, N, and G proteins all appear essential to the process of viral assembly, we examined whether these three viral proteins were expressed at a specific time in virus-infected cells maintained at permissive and nonpermissive temperatures. We found that both N and G proteins were expressed in a location and at a time similar to that observed with the wild-type virus independent of the temperature (1, 2, 9, 28). Thus, N protein was first detected at 2 h postinoculation as small dots throughout the cytoplasm. These dots progressively increased in size and number until they formed large inclusions, sometimes apposed to the membrane at 6 h postinfection (Fig. 3A). The G protein was detected in the Golgi region at 2 to 3 h after infection (Fig. 3C) and at the cell membrane at 4 h postinoculation, as seen during wild-type infection.

In contrast to G and N proteins, M-protein distribution was very different at permissive and nonpermissive temperatures. At 32°C M protein was stained diffusely throughout the cytoplasm (Fig. 3B), whereas at 40°C M protein formed small dots mainly in the perinuclear regions in almost all cells (Fig. 3D). These dots increased in size with time and formed large aggregates later in the course of infection. Three different monoclonal anti-M antibodies, as well as a polyclonal anti-M serum, stained these aggregates.

To determine whether the M protein in these aggregates (formed at 40°C) was associated with any of the two other proteins involved in assembly, we performed double immunofluorescence staining for M and G as well as M and N on infected cells maintained at 40°C for 3 h. M and N were always found in different compartments of the cell, i.e., N-stained inclusions did not correspond to the M aggregates. The G protein, however, was partially colocalized with M aggregates in the perinuclear regions (Fig. 3C and D).

Redistribution of M protein in the cell after temperature shift-down or shift-up. When VSV *ts*G33-infected cells were maintained at 40°C and then shifted to 32°C, the M protein in the perinuclear aggregates decreased with time, whereas the amount of diffusely stained cytoplasmic M increased (Fig. 4A and B). However, aggregates did not disappear completely after 1 h. As described above (Fig. 1), the increase in virus budding and release correlated well with this disaggregation of the M protein.

Conversely, we examined whether M protein, diffusely stained throughout the infected cells after 5 h at 32°C, would aggregate within the cell when the temperature was shifted to 40°C. Approximately 30 min after shift-up, M protein started to form small aggregates mostly in the perinuclear regions (Fig. 4C), and these progressively increased in size with time (Fig. 4D). Simultaneously, cells which had become round at 32°C, adopted a polygonal shape at 40°C. We conclude that the distribution of the mutant M protein in the cell is entirely dependent on the temperature.

M-protein synthesis at 32 and 40°C. We then analyzed viral protein synthesis in *ts*G33-infected cells by SDS-PAGE. First, the soluble cytoplasmic fractions of infected cells were examined. Infected cells were pulse-labeled at 2 h with [^{35}S]methionine and examined every hour thereafter at both permissive and nonpermissive temperatures (Fig. 5). As expected, the labeling of individual viral proteins in each band of the soluble fractions progressively increased during the 2 h after the pulse-labeling and then remained stable. There were no significant differences in the intensity of labeling of viral proteins between *ts*G33 at 32°C and the wild-type virus (Fig. 5A and B). In contrast, in the *ts*G33-infected cells maintained at 40°C there was a marked reduction in the amount of M synthesized in the cytoplasm over 4 h, although the electrophoretic mobility of M protein appeared normal (Fig. 5B). Surprisingly, synthesis of other proteins was also decreased, especially L protein. N protein, however, was not affected significantly by the high temper-

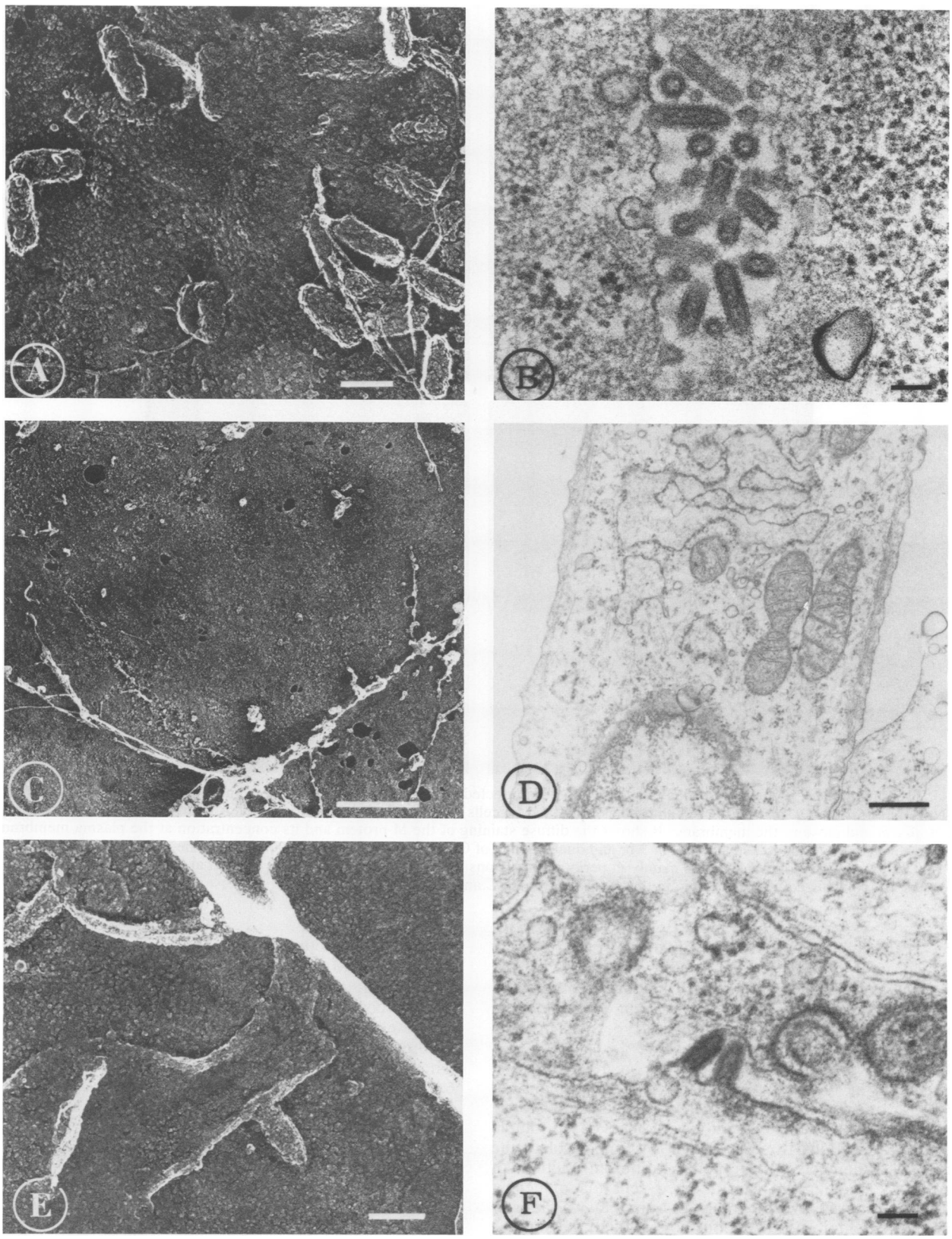

FIG. 2. Electron micrograph of BHK cells infected with VSV M mutant *ts*G33 at permissive and nonpermissive temperatures and after temperature shift-down. Platinum surface replicas are shown on the left, and thin sections are shown on the right. At the permissive temperature, numerous bullet-shaped virions were detected (A, B). At the nonpermissive temperature, however, no complete virion was detected at the surface of cells (C, D). In the shift-down experiment, a few budding viruses were observed 30 min after changing the temperature (E, F). Bars, 100 nm (A, B, E, F) and 500 nm (C, D).

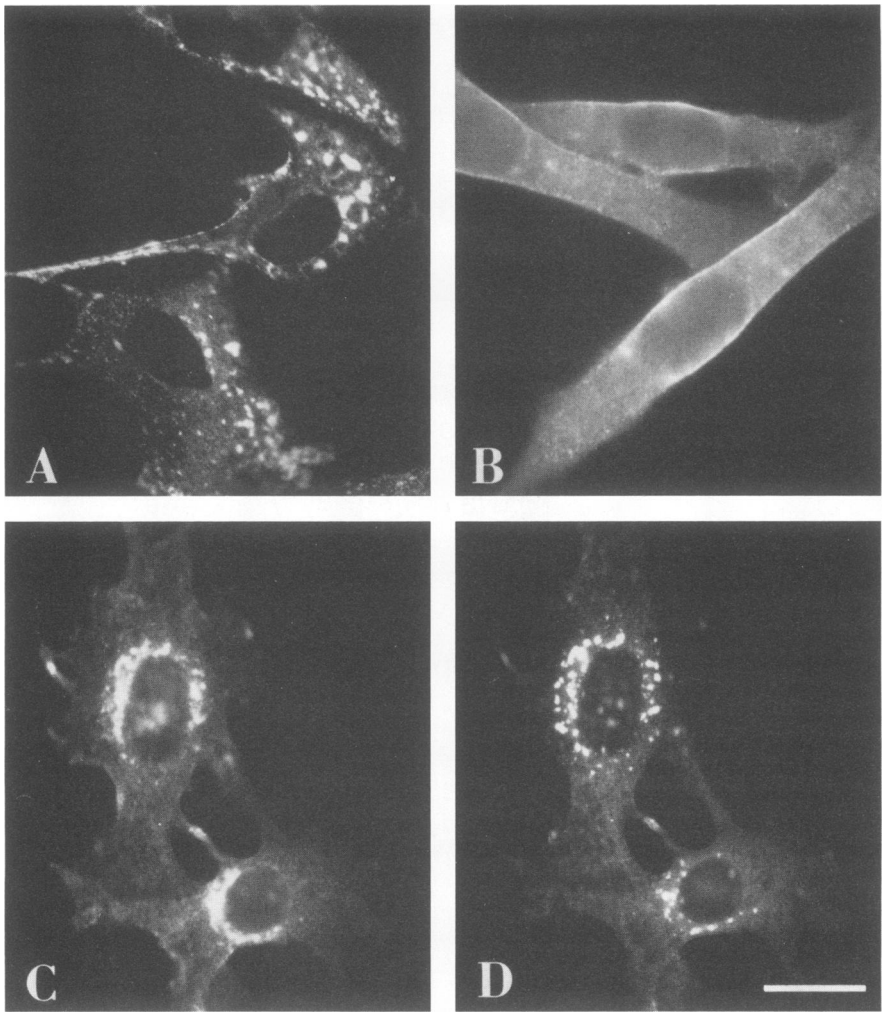

FIG. 3. Immunofluorescence microscopy of the BHK cells infected with *ts*G33 at the permissive and nonpermissive temperatures with anti-N (A), anti-M (B, D), and anti-G (C) antibodies. In A and B, cells were incubated at 32°C for 6 h. A shows N stained inclusions inside the cytoplasm and close to the membrane. B shows the diffuse staining of the M protein and its concentration at the plasma membrane. In C and D, the cells were incubated at 40°C for 3 h, and dual staining of G and M is shown in the same cells. The cells were incubated at 40°C for 3 h. The M protein shows aggregates mainly in perinuclear regions. The G protein seems to be located at the Golgi area (C). Parts of M aggregates are colocalized with G protein in these cells (compare C and D). Bar, 10 μm.

ature. To test whether the high temperature had a general effect on viral protein synthesis, cells infected with the wild-type virus were maintained at 40°C and compared with *ts*G33-infected cells. There was only a slight decrease in the M protein synthesis at 40°C with the wild-type virus in BHK cells (Fig. 5A).

Because M protein forms perinuclear aggregates in the infected cells at 40°C, we thought that M protein might not be able to migrate through the gel in its aggregated form. Therefore, we ran 10 to 30% sucrose gradients of the soluble cytoplasmic fractions to see whether we could isolate the aggregates. When gels were run on the different fractions of the sucrose gradient, M from infected cells maintained at 32°C was scattered over several fractions but was mostly found in the lighter ones (fractions 20 to 22). In the sucrose gradient fractions of virus-infected cells maintained at 40°C, M protein showed small peaks in heavier fractions (10 to 15), suggesting the presence of aggregates (data not shown).

Differences in subcellular localization of M protein at 32 and 40°C. Because weak label was found in the M protein band from virus-infected cells maintained at 40°C, we also searched for M protein aggregates in the insoluble fractions of the cell, which mainly consisted of cytoskeletal and nuclear components (Fig. 6A). These two fractions were separated as described previously (19). Significant amounts of M protein were detected at 40°C in both of these fractions, whereas almost none was seen in the soluble fraction (Fig. 6A; compare lanes 2 and 3 with lane 1 at 40°C). In contrast, at 32°C, the greatest amount of M was found in the soluble fraction as expected, whereas virtually none was detected in the nuclear fraction (Fig. 6A; 32°C). Some M protein was also associated with cytoskeletal fractions. As expected, bands corresponding to NS and N proteins were also found in the insoluble fractions.

Western blot analysis confirmed the presence of M protein in the fractions described above at 32 and 40°C (Fig. 6B). One cytoskeletal component (intermediate filaments) was stained to test the quality of the cytoskeleton preparation (Fig. 6B) and showed copurification with M protein. Since it has been reported that VSV mRNA and proteins are asso-

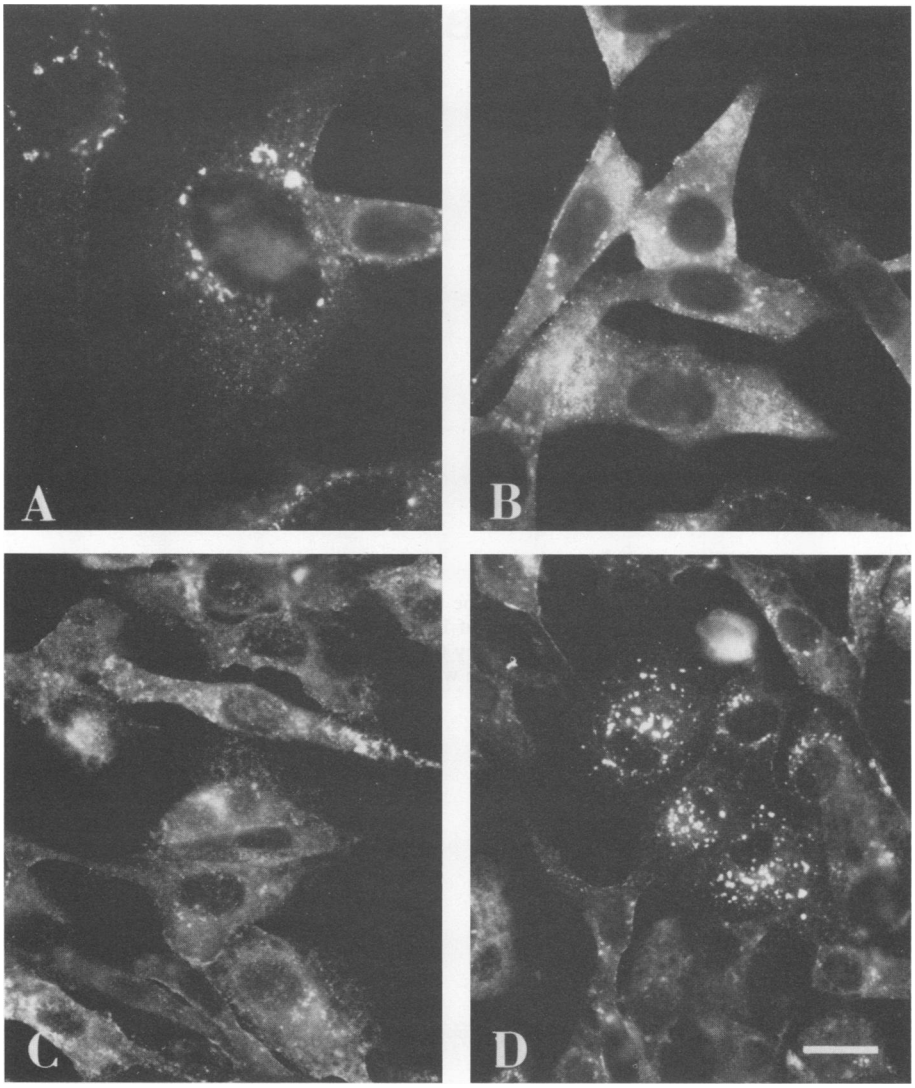

FIG. 4. Immunofluorescence microscopy of the M protein in cells infected with *ts*G33 in temperature shift-down (A, B) and shift-up (C, D) experiments. A shows the M aggregates of infected cells at 40°C for 6 h. One hour after shift-down to permissive temperature in the presence of cycloheximide, several cells showed diffuse M protein staining but still have M aggregates (B). In the shift-up experiment, the cells were incubated at 32°C 5 h after infection and moved to 40°C. Thirty minutes after shift-up, the M protein started to form aggregates (C), and aggregates increased in number and size after 1 h (D). Bar, 10 μm.

ciated with the cytoskeletal framework in HeLa cells (6), we performed double-labeling immunofluorescence tests on virus infected cells. M protein aggregates were closely associated with intermediate filaments around the nucleus in virus-infected cells (data not shown). However, no colocalization of M protein with microtubules was observed.

Analysis of soluble and insoluble fractions of infected cells was also performed after temperature shift-down and shift-up experiments. Temperature shift-down experiments revealed a slight increase in the amount of M protein in the soluble fractions with time (data not shown). Temperature shift-up experiments revealed that M protein synthesized at 32°C tends to move from the cytoplasmic to the nuclear fractions at 40°C (Fig. 6B).

Electron microscopic localization of M protein at 32 and 40°C. Although both immunofluorescence and cell fractionation data suggest that M protein is closely associated with the nucleus at 40°C, electron microscopic analysis was needed to elucidate further the M-protein localization. We used immunoperoxidase labeling of fixed and permeabilized cells to approach this question (Fig. 7). As expected, at 32°C M protein was scattered through the cytoplasm, where it is associated with free ribosomes. M protein also accumulated near the plasma membrane and on the cytoplasmic side of vacuoles, where some viral budding occurred (Fig. 7A and inset). In contrast, large clusters of electron opaque product (formed where anti-M antibody had bound) were seen in cells maintained at 40°C (Fig. 7B). These aggregates were seen in various compartments of the cytoplasm, but the largest ones were closely associated with the nuclear membrane. Inside these aggregates were small membrane vesicles to which M protein appeared attached (Fig. 7B, inset).

We also investigated whether the degree of coiling of the nucleocapsids would depend on the distribution of M protein in the cell. We therefore immunolabeled the virus-infected cells at permissive and nonpermissive temperatures by using

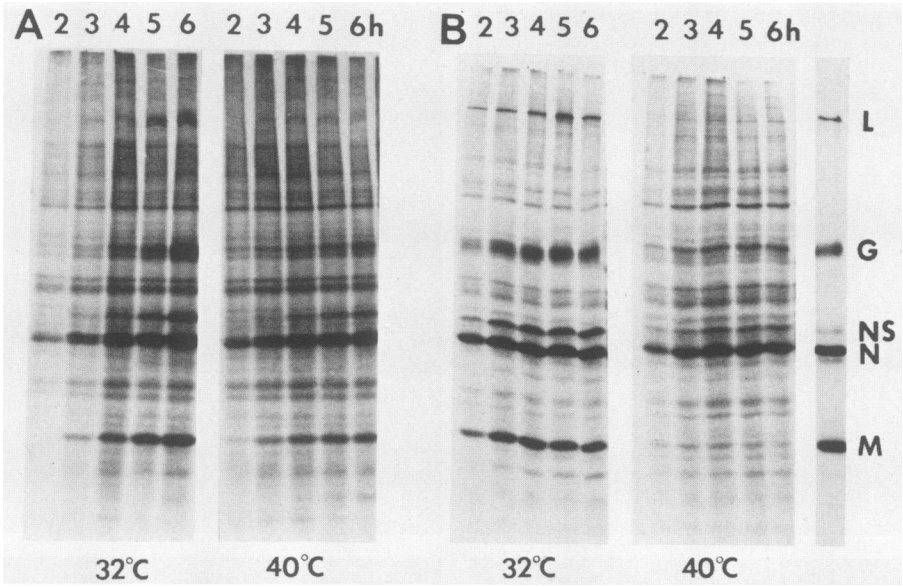

FIG. 5. Viral protein synthesis in cultures infected with wild-type VSV (A) or the *ts*G33 mutant (B) at permissive and nonpermissive temperatures. Cultures were infected at a multiplicity of infection of 100 at 32 and 40°C. [^{35}S]methionine (10 μCi/ml) was added 2 h after adsorption. At hourly intervals thereafter cells were lysed directly by the addition of 0.1 volume of 10% Nonidet P-40. In this experiment, only soluble fractions were analyzed by SDS-PAGE. Exposure time was 36 h.

an antibody to the nucleocapsid structural protein, N, and a peroxidase conjugate. After this labeling technique, the cytoplasmic nucleocapsids, which normally have low electron density, become highly electron dense. We found that when cells are maintained at 40°C, numerous nucleocapsids accumulated in various regions of the cytoplasm, but none of them are tightly coiled (Fig. 7D). In contrast, cells maintained at 32°C contain a number of coiled nucleocapsids inside growing viral buds and the nucleocapsids are labeled by the anti-N antibody, although it is hard to penetrate such a tight structure with antibodies (Fig. 7C). These coiled nucleocapsids were clearly connected to the uncoiled labeled nucleocapsids accumulating in the vicinity of the membrane. This suggests that normal transport and conformation of the M protein are a prerequisite for normal nucleocapsid coiling and budding.

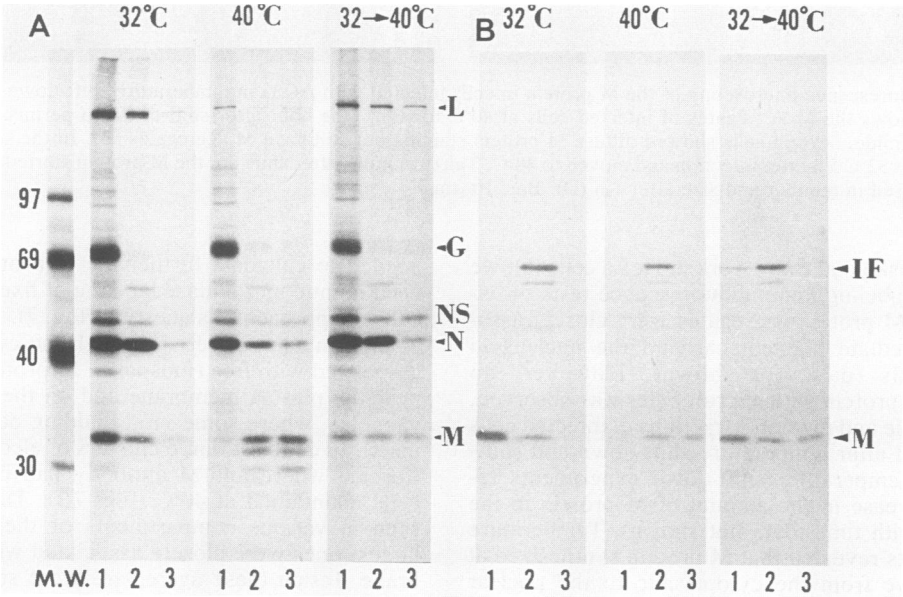

FIG. 6. Viral protein synthesis as analyzed by SDS-PAGE in three different kinds of cell fractions: cytoplasmic soluble (lanes 1), cytoskeletal (lanes 2), and nuclear (lanes 3) (A). Western blot analysis of similar fractions is shown in B. The cells infected with *ts*G33 were labeled 2 h after viral adsorption with [^{35}S]methionine (10 μCi/ml) at 32°C or 40°C or in a shift-up experiment and scraped off 6 h after adsorption. Western blot analysis was performed with rabbit anti-M polyclonal antibody and mouse anti-intermediate filament monoclonal antibody for the first antibody. Goat anti-rabbit IgG and goat anti-mouse IgG conjugated with peroxidase were used for the second antibody.

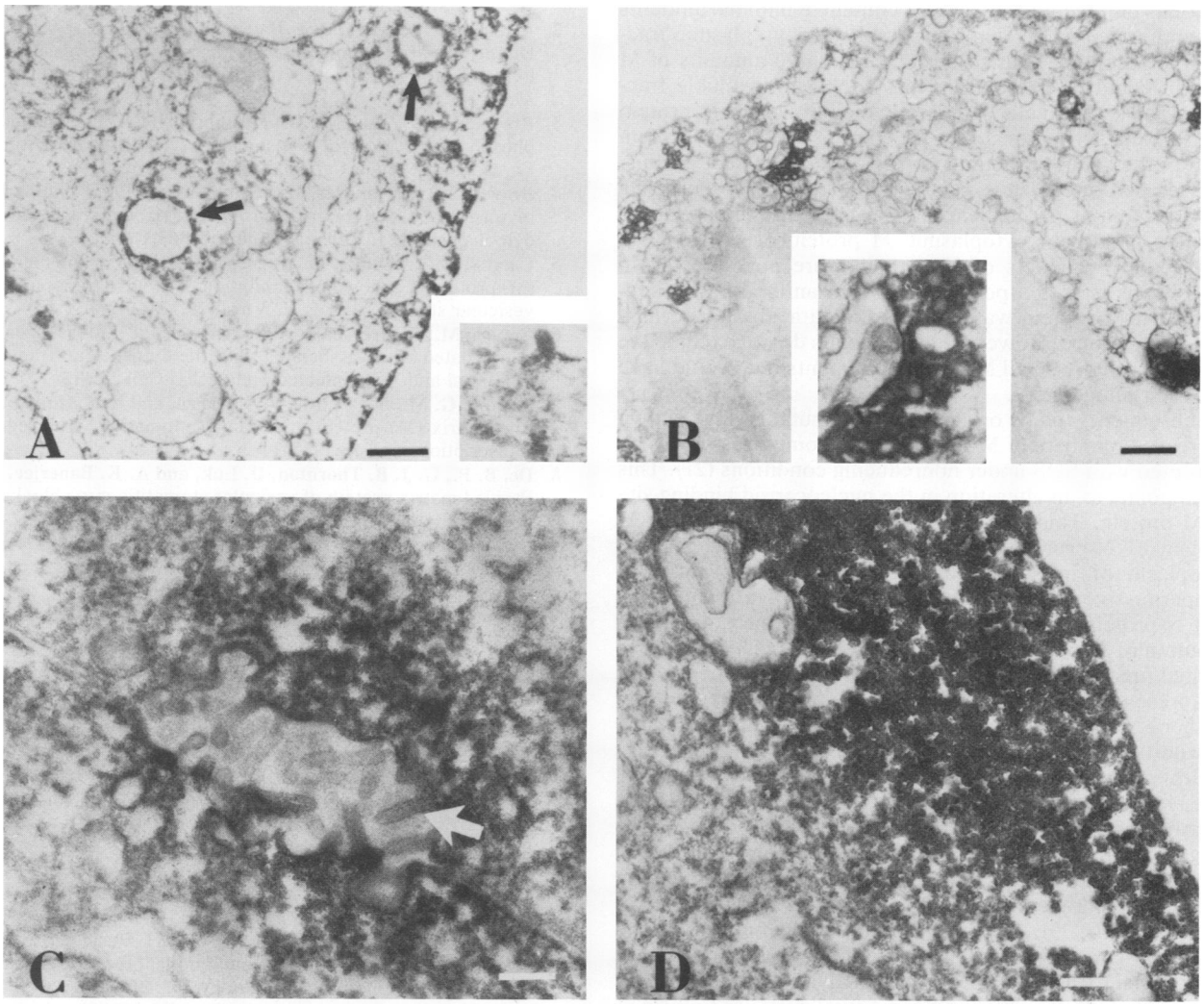

FIG. 7. Electron micrographs of cells infected with *ts*G33 and immunolabeled with rabbit anti-M protein (A, B) and mouse anti-N antibodies (C, D). The black electron-opaque reaction product corresponds to the binding sites of the antibodies. The cells were incubated for 6 h at 32°C (A, C) or 40°C (B, D). The M protein stain is scattered in the cytoplasm and concentrated near the plasma membrane and around vacuoles at 32°C (arrows) (A). M is also observed inside viral budding sites (A, insert). At 40°C, however, M forms large aggregates near the nucleus and also around vesicular structures in the cytoplasm (B). This is better seen at higher magnification (insert B). The aggregate in the lower right corner is clearly associated with the nuclear membrane. Coiled nucleocapsids inside growing viral buds are labeled by anti-N antibody at 32°C (C) (arrow). In contrast, at 40°C numerous uncoiled nucleocapsids accumulate in the cytoplasm. No budding or tight coils are detected at 40°C (D). Bars, 600 nm (A, B; insets magnified to 3×) and 200 nm (C, D).

DISCUSSION

In this study we show that the matrix or membrane-associated (M) protein of a *ts* mutant of VSV, *ts*G33, has a defective function resulting in the aggregation of M protein within the cell cytoplasm and a block in viral assembly at the nonpermissive temperature. The phenomenon is partially reversible after temperature shift-down and can be triggered by temperature shift-up. The M gene of *ts*G33 has two amino acid changes involving charged residues close to the carboxyl terminus (amino acids 204, 214) (11). It was proposed that the decreased binding to nucleocapsids may represent the primary effect of the mutation (11). The mutation may perturb the nucleocapsid binding site directly, or it may cause conformational changes in the protein or decrease accessibility of the binding site. Whatever the exact defect may be, it results in the loss of the ability of M protein to inhibit viral transcription (5, 7) and in the absence of nucleocapsid coiling. In addition, the mutant M protein appears unable to bind to proper membrane structures (smooth endoplasmic reticulum and plasma membrane of BHK cells) but instead preferentially associates with outer nuclear and small vesicular membranes. In the latter location, M protein is partially colocalized with G, an association which only occurred at assembly sites in permissive conditions. These unusual membrane associations of M protein fit well with previous observations indicating that the M protein of *ts* M mutants generally exhibits decreased binding to nucleocapsid and increased binding to membranes (21, 36).

Previous studies by Knipe and his colleagues (15, 16) have suggested that the M proteins of *ts* mutants (*ts*G31, *ts*G33) were degraded more rapidly at the nonpermissive temperature than wild-type proteins. This interpretation was based on the results of SDS-PAGE of the cytoplasmic soluble

fractions of infected cells, which contained minimal amounts of synthesized M protein. Our results on cytoplasmic fractions are identical, but to our surprise large amounts of M protein could be detected in the cytoplasm insoluble fractions. The size and electrophoretic mobility of the M protein from insoluble fractions were the same as those of M protein seen in the soluble fractions of the cells infected at permissive temperatures. Thus, it is possible that the sequestering of M protein at perinuclear sites account for the abnormal labeling pattern of cytoplasmic M protein of VSV *ts*G33. Degradation products of M protein were not detected in Western blots with a polyclonal anti-M antibody. This suggests that M protein was not degradated at the nonpermissive temperature. However, these results do not exclude the presence of very small M protein fragments that would have lost their antigenicity.

Subsequent studies on M protein *ts* mutants have shown decreased numbers of M-nucleocapsid complexes in virions disrupted with SDS under nonreducing conditions (21). This again points to an alteration in the nucleocapsid binding site of M protein. This is supported by the observation that M protein of N-protein *ts* mutants is found diffusely in the cytoplasm of infected cells at both the permissive and nonpermissive temperatures (data not shown), indicating that N-protein mutation does not influence the normal distribution of M protein in virus-infected cells. Therefore, the normal function of M protein does not appear to depend on the presence of other viral proteins. This property will be further examined in cells transfected with the cloned M gene of wild-type and *ts* mutants of VSV.

How can we now envision the problem of VSV assembly? The respective roles of G and M in promoting attachment of the nucleocapsid to the membrane and its coiling have been long debated (reviewed in references 9, 13, and 32). It has been shown that the sites of incorporation of G protein in the plasma membrane specify the sites of viral budding in polarized cells (33). Also, virus preferentially buds in regions with high concentrations of G protein in cells treated with cytochalasin B (4). A most recent analysis of the G protein mutant (*ts*O45), which allows some viral particles to form at 40°C, reveals that these spikeless particles contain a full complement of anchors corresponding to the carboxy-terminal end of G protein (23). This clearly favors the idea that an interaction between the G protein carboxy-terminal domain with the nucleocapsid is an essential event in viral assembly. Clustering of G-protein molecules at the plasma membrane may enhance nucleocapsid binding (26). The M protein, which normally diffuses freely throughout the cytoplasm, would then bind to the nucleocapsid, triggering its coiling and driving the budding process (23, 26). M protein, when added to purified nucleocapsids in vitro, can induce nucleocapsid coiling (8, 12). The fact that no nucleocapsid coiling is seen in cells synthesizing the mutant M protein of *ts*G33, which aggregates within the cytoplasm, supports our view on VSV assembly.

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