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Assembly of Vesicular Stomatitis Virus: Distribution of the Glycoprotein on the Surface of Infected Cells

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This study demonstrates that the glycoprotein of vesicular stomatitis virus clusters in the plasma membrane of infected Chinese hamster lung cells during morphogenesis and suggests that viral nucleocapsids are required for this clustering. A mutant virus (*ts* E-1) which is temperature sensitive for the synthesis of viral nucleocapsids but not viral membrane proteins was used. The surface distribution of the viral glycoprotein in cells infected by this virus was determined by a specific indirect immunoferritin stain. Early in infection at permissive temperatures, the glycoprotein was randomly distributed on membrane ghosts. Later, clusters of ferritin the size and shape of virus particles were seen. In contrast, ghosts prepared from virus-infected cells maintained at a restrictive temperature always had a random distribution of viral glycoprotein.

Integral membrane proteins span the membranes with which they are associated and may participate in significant molecular interactions on both sides of the membrane. In many cases, these proteins are freely diffusible in the plane of the membrane (37). An increasing number of membrane proteins, however, are found to be distributed over the surface of the cell in a nonrandom fashion. For example, Ash et al. (1) have shown that several antibody-patched integral membrane proteins are arranged in linear arrays on the surface of cultured fibroblasts, specific receptor molecules are frequently localized in defined regions of the membrane, and viral glycoproteins of lipid-containing viruses are localized in regions of the membrane active in virus assembly (8). The molecular interactions which lead to the nonrandom distribution of integral proteins are currently under active study in a number of laboratories. Evidence is accumulating which indicates that the linear arrays of membrane proteins visible on the surface of fibroblasts result from interactions of domains of these proteins present on the cytoplasmic surface with elements of the cytoskeleton (1, 14, 22). In the case of the best-studied receptor system, that of the low-density lipoproteins, the molecules appear to be inserted into the membrane at random sites and then to migrate laterally in the plane of the membrane until they reach a "coated pit." A domain of the receptor presumed to be on the cytoplasmic side of the membrane forms an association with clathrin, resulting in fixation of the receptor into the coated-pit structure (15). A similar model has been proposed for distribution of viral glycoproteins during viral assembly. In particular, Compans and Kemp (8) have suggested that the viral glycoproteins are in a freely mobile state immediately after insertion into the plasma membrane and become clustered only after the interaction of cytoplasmic domains of the glycoproteins with internal viral components. In this report, we provide evidence for the validity of this model for one virus system, vesicular stomatitis virus (VSV).

VSV is a relatively simple, enveloped RNA-containing virus. The interior of the virus particle is a ribonucleoprotein structure consisting of a single strand of RNA and three protein species, L, NS, and N (5). Two of these proteins (L and NS) comprise the virion-associated transcriptase (11), whereas the third (N) is required for the viral RNA to serve as a template for transcription in vitro (38). A layer of matrix protein (M) is thought to surround the viral nucleocapsid (10). A host-derived lipid bilayer surrounds the nucleocapsid-matrix protein structure (6). Embedded in the bilayer are copies of a virus-coded glycoprotein (G) (36). A small number of specific host glycoproteins are also found in the viral membrane (16, 27).

Final assembly of VSV from its components usually occurs at the plasma membrane of infected cells (39). Pulse-chase experiments suggest that viral proteins take three independent paths to the site of assembly (20, 21). Viral nucleocapsids are preassembled in the cytosol

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(2, 9, 20). Matrix protein is synthesized on nonmembrane-bound polysomes but becomes associated with membranes rapidly after synthesis (2, 9, 20). The glycoprotein is synthesized on membrane-bound ribosomes (3, 29) and migrates through internal membranous structures to the plasma membrane (35). The nucleocapsids and matrix proteins then associate with the plasma membrane and exit the cell, picking up the G-containing lipid bilayer in the process (20).

Electron microscopic analysis of budding intermediates which occur during the assembly of VSV indicates that the viral glycoprotein is clustered in areas of the plasma membrane active in viral assembly (39). It is not currently known which viral functions are required for this clustering. We have approached this problem by characterizing a temperature-sensitive mutant (*ts* E-1) of VSV which does not assemble nucleocapsids (25) but which does synthesize all viral proteins. We then determined the two-dimensional distribution of G in cells permissively and restrictively infected with this mutant by staining plasma membrane ghosts with an indirect immunoferritin stain. These studies indicate that the viral nucleocapsid is required for clustering of G in the membrane. In the absence of nucleocapsids, the G protein appears to be randomly distributed in the plane of the membrane. Thus, our studies provide direct evidence in support of the random insertion and clustering model for virus maturation and strongly suggest that the clustering process is driven by the interaction of the membrane proteins with the nucleocapsid.

MATERIALS AND METHODS

Cells and virus. Baby hamster kidney cells (clone 21, subculture 13) and Chinese hamster lung (CHL) cells were maintained in suspension and monolayer culture as previously described (32). Stocks of VSV, New Jersey serotype (wild type and temperature-sensitive mutants) were obtained by infecting flasks (150 cm^2) of baby hamster kidney cells at a multiplicity of infection (MOI) of 0.01 to 0.001 with virus which had been plaque purified four times.

Immunological reagents. Large quantities of VSV, New Jersey serotype, were produced in baby hamster kidney cells, and the G protein was purified from this material as previously described (32). Rabbits were injected intradermally with an emulsion containing 1 mg of VSV G protein in Freund complete adjuvant. Five weeks later, 1 mg of G protein was injected intravenously. The animals were bled 7 days later, and the serum was obtained after clotting at 4°C overnight. The immunoglobulin G (IgG) fraction of this serum was purified by standard methods.

Goat anti-rabbit IgG serum was obtained from Antibodies Inc. The IgG fraction of this serum was purified by the method of Fanger et al. (13).

Goat anti-rabbit IgG-ferritin conjugates were made by the glutaraldehyde method of Kishida et al. (19). The ferritin used in this technique was E.M. grade horse spleen ferritin (Polysciences, Inc.) which was further purified by recrystallization (four times). The conjugates formed consisted mainly of one molecule of IgG linked to one molecule of ferritin as judged by agarose A-5M chromatography and the ferritin-to-IgG ratio.

All immunological reagents were preabsorbed against glutaraldehyde-fixed CHL cells before use.

Preparation of anti-G [^{125}I]IgG and binding to infected cells. The IgG fraction of anti-G serum was iodinated with Na^{125}I, using lactoperoxidase-glucose oxidase containing Enzymobeads (BioRad Laboratories) as described by the manufacturer. The final specific activity of the IgG was appproximately 6×10^6 cpm/μg. CHL cells (10^5) which had been fixed in 1% glutaraldehyde for 30 min at 4°C and quenched by incubation in 100 mM $(NH_4)_2CO_3$ (pH 8.0) for 5 min at 4°C were incubated with 10^6 cpm of anti-G [^{125}I]IgG and various concentrations of unlabeled anti-G IgG in phosphate-buffered saline (PBS) containing 1 mg of bovine serum albumin (BSA) per ml (PBS-BSA) for 30 min at 37°C. The cells were collected by centrifugation and washed two times with PBS-BSA, and the amount of [^{125}I]IgG bound to the cells was determined by counting in a Searle gamma counter.

Preparation of VSV-infected CHL cell membrane ghosts. Suspension cultures of CHL cells were infected by the method of Knipe et al. (20). At various times postinfection, ghosts were prepared by a modification of the technique of Nicolson and Singer (31). Infected cells (2×10^6) were washed with PBS two times by centrifugation at the temperature at which the cells were growing. The pelleted cells were resuspended in 50 μl of PBS and immediately mixed with 50 μl of ice-cold 0.1% glutaraldehyde (EM grade; Polysciences) in PBS. Incubation was allowed to proceed for 10 s, after which time the entire mixture was dropped onto a surface of deionized, distilled water. The material remaining on the water surface was picked up onto a Parlodion-coated, carbon-strengthened grid and fixed for 5 min at 4°C in 1% glutaraldehyde in PBS. Unreacted glutaraldehyde was quenched by floating the grid onto five changes of 100 mM $(NH_4)_2CO_3$ (pH 8.0) at 4°C for 1 min per change.

Staining of membrane ghosts. Grids with membrane ghosts on the surface were touched to a solution of 5% BSA in PBS and immediately placed onto a solution of 0.5 mg of rabbit IgG per ml in PBS-BSA which was held in a Microtest tissue culture plate (Falcon Plastics). After incubation for 10 min at 4°C, unbound IgG was washed off by sequentially transferring the grid onto five changes of PBS-BSA at 4°C for 1 min per change. The grid was incubated on a solution of ferritin goat anti-rabbit IgG conjugate in PBS-BSA for 10 min at 4°C. Unbound conjugate was washed off the grid by floating it on five changes of PBS-BSA. The grid was washed for 10 s on a surface of deionized distilled water. Excess water was removed by placing a piece of Whatman no. 1 filter paper at the edge of the grid. The grid was allowed to air dry. Specimens were viewed in a Philips EM 301 electron microscope.

[^{35}S]methionine labeling of infected cells. Infected cells were collected at various times postinfection by centrifugation, washed once with PBS, and suspended in 0.1 ml of methionine-free suspension medium containing 15 mM TES [*N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [pH

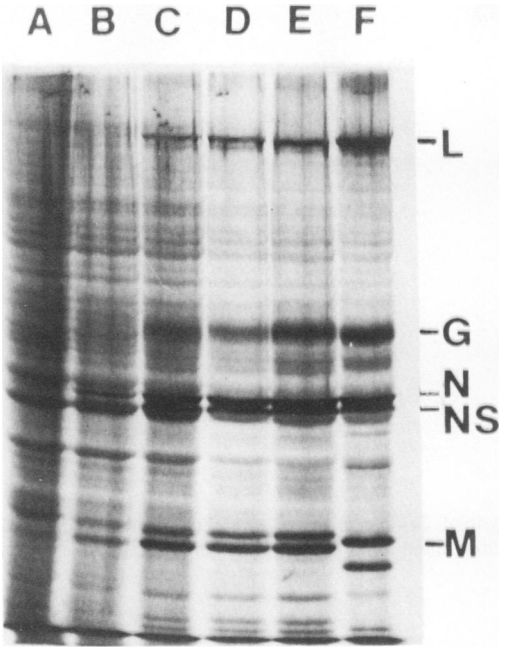

FIG. 1. Synthesis of viral proteins at restrictive temperature. Cells were infected at an MOI of (A) 10, (B) 20, (C) 50, (D) 100, or (E) 150 with *ts* E-1 or (F) at an MOI of 10 with wild-type VSV and maintained at 39°C. Incorporation of [^{35}S]methionine into viral proteins was analyzed by PAGE on a 10% gel.

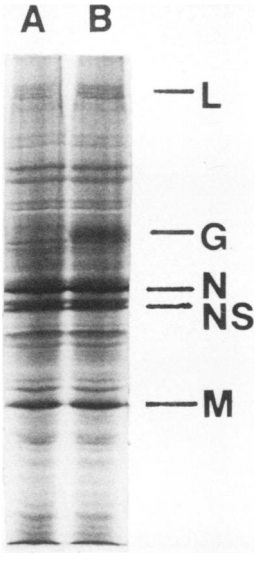

FIG. 2. Susceptibility of G in *ts* E-1-infected CHL cells to chymotrypsin. Cells were infected with *ts* E-1, maintained under restrictive conditions, and labeled with [^{35}S]methionine from 3 to 4 h postinfection. Incorporated label was chased for 1 h by the addition of 1 mM nonradioactive methionine. The cells were then washed with PBS and treated with chymotrypsin (lane A) or chymotrypsin plus phenylmethylsulfonyl fluoride (lane B), and the labeled proteins were analyzed by PAGE.

7.2]), 2% dialyzed fetal calf serum, and 10 μCi of methionine (850 Ci/mmol). Thirty minutes later, the cells were collected by centrifugation and lysed in sodium dodecyl sulfate (SDS) buffer (1% SDS–50 mM Tris-chloride [pH 6.8]–10% glycerol), and the proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (23). The dried gels were autoradiographed at −80°C with Kodak No-Screen X-ray film.

Chymotrypsin treatment of [^{35}S]methionine-labeled cells. Infected cells were labeled from 3 to 4 h postinfection with [^{35}S]methionine as described above. At 4 h postinfection, incorporation of label was stopped by bringing the medium to 1 mM methionine. At 5 h postinfection, the cells were washed three times with PBS and incubated for 10 min at 37°C with 100 μg of chymotrypsin in PBS or 100 μg of chymotrypsin plus 2 mM phenylmethylsulfonyl fluoride (prepared fresh from a 1 M stock in 95% ethanol) in PBS. The culture containing only chymotrypsin was brought to 2 mM phenylmethylsulfonyl fluoride, and both cultures were incubated for 5 min at 37°C and 30 min at 4°C. The cells were washed two times with ice-cold PBS, lysed, and analyzed by PAGE.

RESULTS

Characterization of *ts* E-1. The *ts* E-1 mutant of VSV, New Jersey serotype, has previously

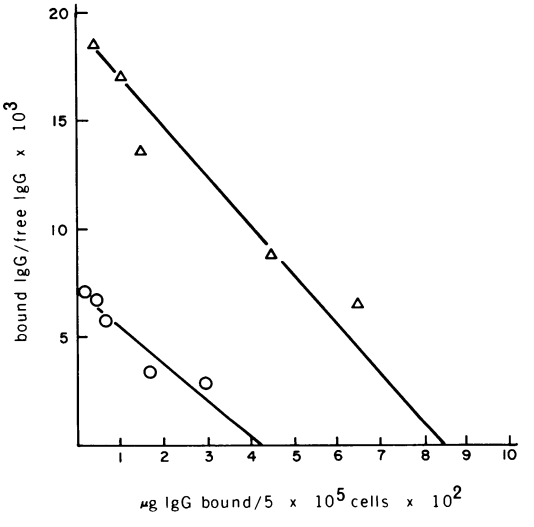

FIG. 3. Binding of anti-G [^{125}I]IgG to permissively and restrictively infected cells. Glutaraldehyde-fixed CHL cells which had been infected with *ts* E-1 and maintained at 31°C (△) or 39°C (○) were incubated with various concentrations of anti-G [^{125}I]IgG for 30 min at 37°C and washed extensively, and the amount of bound anti-G [^{125}I]IgG was determined.

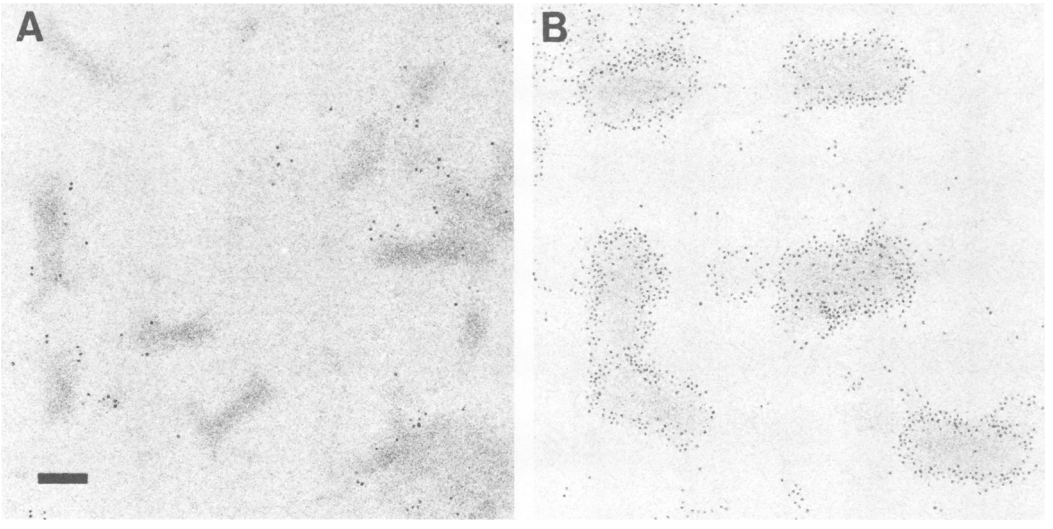

FIG. 4. Immunoferritin staining of purified virions. Purified virions absorbed to electron microscope grids were stained with ferritin-goat anti-rabbit IgG conjugate after incubation with normal rabbit IgG (A) or rabbit anti-G IgG (B).

been shown to be defective in replication of viral nucleocapsids (25). We confirmed this finding; infected cells maintained at permissive temperatures incorporated [^{3}H]uridine into material which had S values similar to those of purified virions and nucleocapsids, whereas cells maintained at restrictive temperatures showed little incorporation into either fraction.

If the sole defect in the growth cycle of the *ts* E-1 mutant is in replication of viral RNA, then cells infected at a restrictive temperature should support primary transcription from input nucleocapsids. In this case, an MOI-dependent synthesis of viral proteins should be detectable. To test this hypothesis, the level of incorporation of [^{35}S]methionine into virus-specific proteins was determined at various MOIs by SDS-PAGE. Below an MOI of 50 (Fig. 1, lane C), little virus-specific protein synthesis could be detected. At an MOI of 150 (Fig. 1, lane E), the level of virus-specific protein synthesis was similar to that in wild-type infected cells (Fig. 1, lane F) maintained at 39°C.

It is apparent (Fig. 1) that the electrophoretic mobility of M protein synthesized in cells infected with *ts* E-1 is different from that of M protein synthesized in cells infected with the wild-type virus used in this experiment. Analysis of non-temperature-sensitive revertants of *ts* E-1 by PAGE and tryptic peptide mapping (28; C. A. Maack, Ph.D. thesis, University of California at Berkeley, Berkeley, Calif., 1978) has demonstrated that the temperature-sensitive phenotype is associated with alterations in the NS protein and not in the M protein.

Virus glycoprotein synthesized at a restrictive temperature in *ts* E-1-infected cells can be shown to migrate to the plasma membrane of the cell by several criteria. Radiolabeled glycoprotein becomes accessible to externally added chymotrypsin after a 1-h chase (Fig. 2). Externally added anti-G will bind to *ts* E-1-infected cells maintained at a restrictive temperature. A quantitative binding study indicates that about half as much anti-G will bind to restrictively infected *ts* E-1 cells as to permissively infected cells at 4 h postinfection (Fig. 3).

Staining procedure. An indirect immunoferritin staining procedure was used to visualize the glycoprotein in virus preparation samples. After fixation for 10 min at 4°C with 1% glutaraldehyde, the samples were incubated with the IgG fraction of either rabbit anti-G serum or normal rabbit serum. Samples were extensively washed and then treated with a conjugate consisting of ferritin covalently linked to the IgG fraction of goat anti-rabbit IgG serum. The extent and pattern of conjugate binding were determined by electron microscopy. Purified virions prepared in this way and treated with normal rabbit IgG had little ferritin associated with slightly electron-opaque virus particles (Fig. 4A). Purified virions treated with anti-G IgG before conjugate treatment (Fig. 4B) had large amounts of ferritin associated with virus particles. A similar procedure was used to visualize VSV G protein in plasma membrane ghosts. As in the case of virus particles, ghosts treated with normal rabbit IgG showed very low levels of conjugate binding (see below and Fig. 6B).

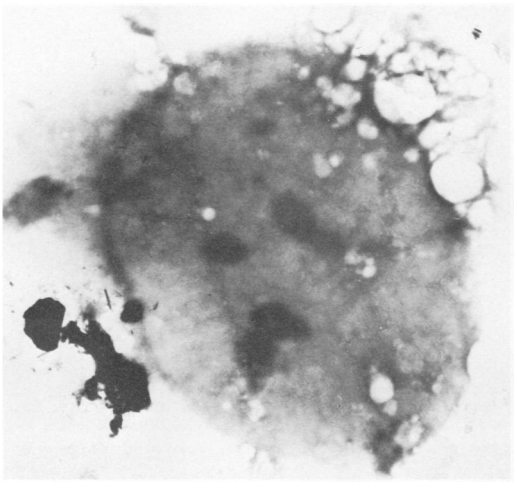

FIG. 5. Electron micrograph of CHL cell plasma membrane ghosts. Ghosts of CHL cells were prepared by lysis at an air-water interface after partial fixation with 0.05% glutaraldehyde. Magnification, ×4,800.

Analysis of ghosts of cells infected with *ts* E-1 and maintained at permissive or restrictive temperatures. To determine the two-dimensional distribution of G in the plasma membrane of infected cells, we used a modification of the hypotonic lysis technique of Nicolson and Singer (31) to prepare plasma membrane ghosts. Visualization of these preparations at a low magnification (Fig. 5) demonstrates that this technique results in ghosts with large areas of well-preserved plasma membrane. Individual ferritin molecules can easily be seen on the relatively electron-transparent ghosts (Fig. 6B through F).

At various times postinfection, permissively infected cells were lysed and stained as described above. Figure 6 shows representative micrographs of cells prepared from 3 to 6 h postinfection. At 3 h, ghosts had large amounts of conjugate bound to them (Fig. 6B). The pattern of binding was fairly dispersed, with only a few small clusters of conjugate seen. By 4 h postinfection, extensive clusters of conjugate could be seen (Fig. 6C). Many of these clusters were of the same size and shape as virus particles. Directly adjacent to these areas, no clustering of ferritin could be seen (Fig. 6D). At 5 h postinfection, large areas of membrane had clusters associated with them (Fig. 6E). At 6 h postinfection, the membranes were still densely stained with ferritin, but few virus-like clusters could be seen (Fig. 6F).

Representative micrographs of ghosts prepared from 3 to 6 h postinfection from restrictively (39°C) infected cells are shown in Fig. 7. Before 3 h postinfection, little binding of conjugate could be seen (data not shown). By 4 h postinfection (Fig. 7B), the level of conjugate staining was similar to that of permissively infected cells at 3 to 4 h postinfection (Fig. 6B through D). At no time were any large clusters of ferritin seen on ghosts prepared from restrictively infected cells. The lack of clustering of G is not merely a result of incubation at 39°C, since ghosts prepared from cells infected with wild-type VSV and maintained at 39°C have clusters of ferritin on their surfaces (Fig. 8).

DISCUSSION

The molecular interactions occurring during assembly of VSV are ill defined. It is clear from a number of previous studies that clustering of G occurs in the plasma membrane of infected cells; most host glycoproteins are excluded from released virions (26), and clusters of G can be seen in areas of the cell membrane active in virus assembly (39). The functions (viral or host) required for this clustering are not known. It has been previously suggested that viral M protein interacting with cytoplasmic domains of G molecules and specific cellular glycoproteins (8, 17, 26) is responsible for clustering. This is consistent with the facts that (i) M binds to the plasma membrane of infected cells (30), (ii) M self-aggregates under certain conditions (4), and (iii) M is a late function, presumably involved in the assembly of virions (2). Alternatively, it has been suggested that viral nucleocapsids may be involved in clustering of G, perhaps in association with M (26).

To study clustering of G in the plasma membrane during morphogenesis, we used indirect immunoferritin staining of plasma membrane ghosts. This method of sample preparation was used to analyze large areas of plasma membrane. We feel that this approach allows for a less ambiguous determination of the two-dimensional distribution of G in the membrane than would the use of a technique which yields a one-dimensional view of the plasma membrane (e.g., thin sectioning).

The work in this paper confirms that the VSV G protein does in fact cluster during morphogenesis. This conclusion is based on the fact that clusters of ferritin are seen associated with the plasma membranes of infected cells. For the conclusion that viral nucleocapsids are required for clustering to be valid, *ts* E-1 must have no lesion other than that responsible for the failure to form nucleocapsids. Available evidence suggests that this is true. The *ts* E-1 mutant has been shown to have a lesion in the NS gene (12, 24, 28). NS is present in virions in small amounts (5) and probably does not have a role in assembly of virus particles. *ts* E1 complements mutants in all other groups of VSV, New Jersey serotype, and reverts at reasonable frequency,

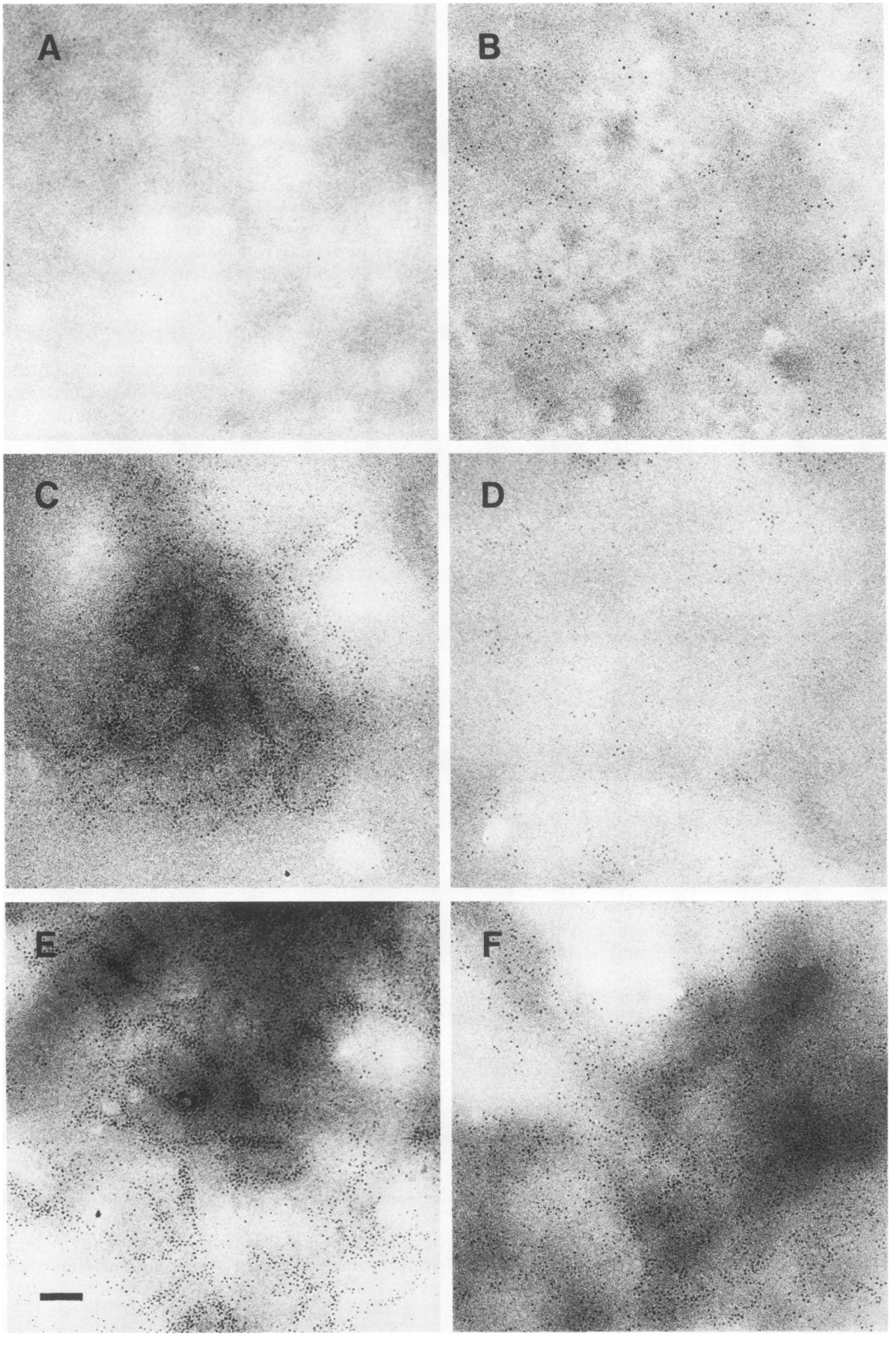

FIG. 6. Staining of permissively infected (*ts* E-1) CHL cell membrane ghosts. Cells infected with 150 PFU of *ts* E-1 per cell were maintained at 31°C. At various times postinfection, the cells were lysed, fixed, and stained as described in the text. (A) Normal rabbit IgG control; (B) ghosts of cell lysed at 3 h postinfection; (C) ghosts of cell lysed at 4 h postinfection, showing clustered array of ferritin conjugate; (D) same cell as that shown in (C); (E) ghosts of cell lysed at 5 h postinfection; (F) ghosts of cell lysed at 6 h postinfection. Bar = 100 nm.

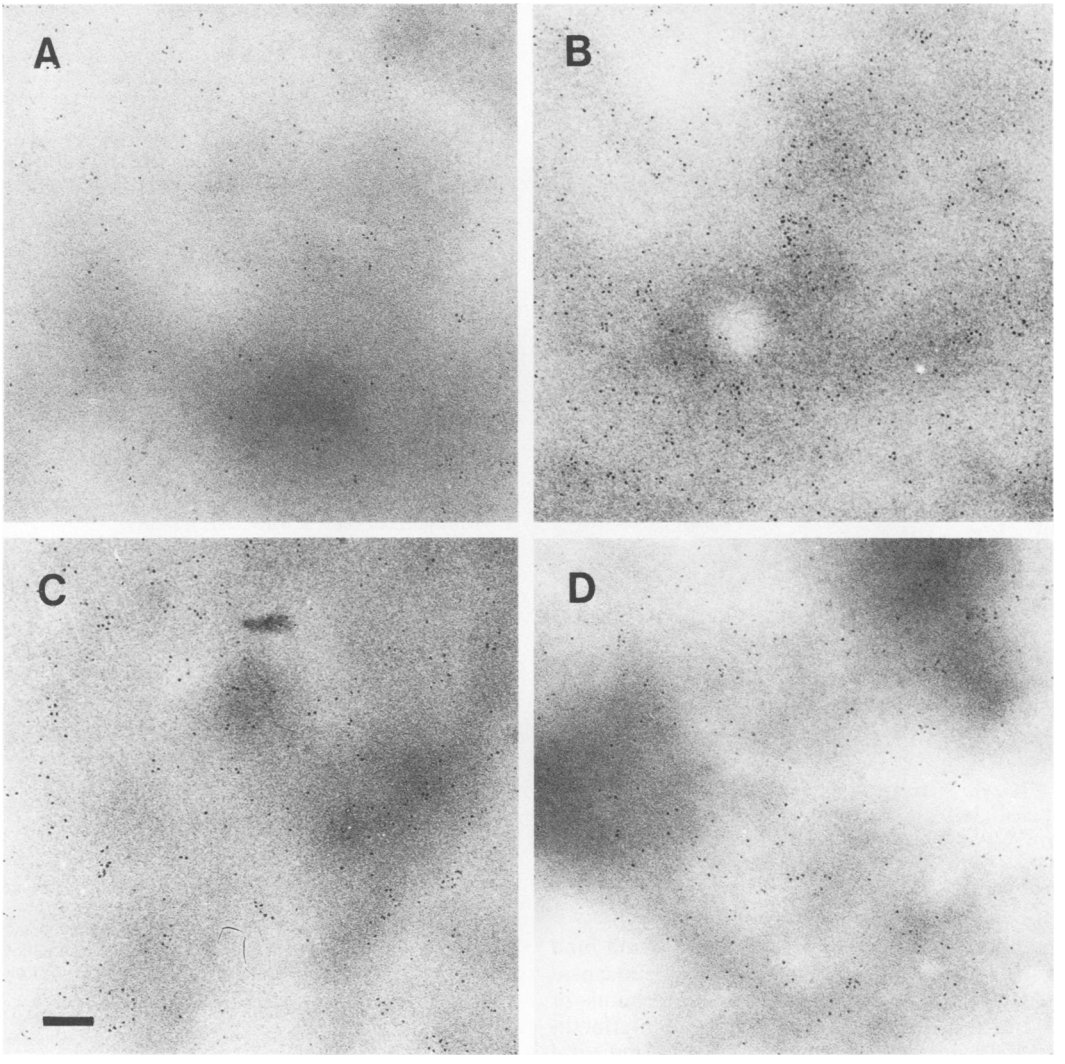

FIG. 7. Staining of infected (39°C) CHL cell membrane ghosts. Cells infected with 150 PFU of *ts* E-1 per cell were maintained at 39°C. At various times postinfection, the cells were lysed, fixed, and stained as described in the text. (A) Ghosts of cell lysed at 3 h postinfection; (B) ghosts of cell lysed at 4 h postinfection; (C) ghosts of cell lysed at 5 h postinfection; (D) ghosts of cell lysed at 6 h postinfection; Bar = 100 nm.

suggesting that there is only a single mutation responsible for the temperature-sensitive phenotype. Non-temperature-sensitive revertants of *ts* E-1 consistently have NS proteins which show altered electrophoretic mobility and peptide map patterns (28). None of the other proteins of these revertants had altered electrophoretic mobility. In addition, the peptide map pattern of the M protein was identical, regardless of whether the protein was isolated from cells infected with *ts* E-1 or non-temperature-sensitive revertants of *ts* E-1 (Maack, Ph.D. thesis). Since the average amount of G present on the cell surface at a restrictive temperature is only one-half of that present at a permissive temperature, we cannot rule out the possibility that there is a concentration-dependent clustering of G. We do not believe that this is true, since individual cells with a greater-than-average level of G on their surfaces (as determined by electron microscopy) still do not cluster G at a restrictive temperature.

The results presented in this paper are consistent with either of two models for assembly. One model suggests that M binds to the carboxy-terminus of G (34) at the plasma membrane but does not cluster G. Viral nucleocapsids would recognize these G-M pairs, bind to them, and induce clustering by the multivalent nature of

FIG. 8. Staining of infected (39°C) CHL cell membraned ghosts. Cells infected with 150 PFU of wild-type VSV per cell were maintained at 39°C for 4 h. Cell ghosts were lysed, fixed, and stained as described in the text.

the nucleocapsids. Alternatively, M could bind first to nucleocapsids, and this M-nucleocapsid structure could bind to the carboxy terminus of G. In either model, M functions as an intermediate with dual recognition sites for G and viral nucleocapsids but does not itself induce G clustering. Such interactions are consistent with the known binding properties of M. M binds to membranes (30) and may directly associate with the cytoplasmic portion of G. Dissociation of virions in Tx-100 and low salt leaves M stably associated with viral nucleocapsids (18). This interaction is probably physiologically relevant, since M inhibits viral transcription in vitro (4, 40) and in vivo (7).

We believe that these models are consistent with results previously published by Reidler et al. (33), who have shown that mutations in the M gene lead to an increased fraction of G being mobile at a restrictive temperature as compared with the fraction which is mobile at a permissive temperature. Our results suggest that this phenomenon is due to the inability of nucleocapsids to cluster G without the presence of functional M protein.

ACKNOWLEDGMENTS

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