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Role of Matrix Protein in Cytopathogenesis of Vesicular Stomatitis Virus

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The matrix (M) protein of vesicular stomatitis virus (VSV) plays an important structural role in viral assembly, and it also has a regulatory role in viral transcription. We demonstrate here that the M protein has an additional function. It causes visible cytopathic effects (CPE), as evidenced by the typical rounding of polygonal cells after VSV infection. We have analyzed a temperature-sensitive mutant of the M protein of VSV (*ts*G33) which is defective in viral assembly and which fails to cause morphological changes of the cells after infection at the nonpermissive temperature (40°C). Interestingly, this defect in viral assembly as well as the CPE were reversible. Microinjection of antisense oligonucleotides which specifically inhibit M protein translation also inhibited the occurrence of CPE. Most importantly, when cells were transfected with a cDNA encoding the temperature-sensitive M protein of *ts*G33, no CPE was observed at the nonpermissive temperature. However, when these cells were shifted to the permissive temperature (32°C), they rounded up and detached from the dish. These results demonstrate that M protein in the absence of the other viral proteins causes rounding of the cells, probably through a disorganization of the cytoskeleton. The absence of CPE at the nonpermissive temperature is correlated with an abnormal dotted staining pattern of M in these cells, suggesting that the mutant M protein may self-aggregate or associate with membranes rather than interact with cytoskeletal elements.

All negative-strand RNA viruses possess a major structural protein, the matrix or M protein. The M protein of vesicular stomatitis virus (VSV) plays two important roles (for a review, see Wagner [41]). First, the protein plays a pivotal role in the assembly of the virus. The M protein is initially a soluble cytoplasmic protein which binds to cellular membranes during the budding process. Chemical cross-linking studies indicate that M protein is also in close proximity to other viral envelope components, in particular the viral glycoprotein G and the viral nucleocapsid (9, 20). Other studies show that the matrix protein interacts with nucleocapsids and promotes the condensation of nucleocapsids into tightly coiled structures both in vitro (28) and in vivo (29). The second function of M protein is directly linked to its ability to bind to nucleocapsids. This interaction inhibits and thereby controls viral transcription (5, 8). Besides these two well-characterized functional activities of M, we demonstrate here that M protein also plays a role in the cytopathogenesis of VSV.

Infection of cells with VSV results in a rapid inhibition of cellular macromolecular synthesis shortly after infection and the development of a cytopathic effect (CPE) which is manifested starting about 4 h after infection by the rounding of the cells (22, 23, 41). Subsequently, the cells detach from the plate and eventually lyse. One of the temperature-sensitive (*ts*) mutants of the M protein (*ts*G33) was of particular interest to us because it does not induce rounding of the cells at a nonpermissive temperature (40°C). Ono et al. (31) showed that this defect correlates with an abnormal distribution of M protein in these cells. M protein seems to form aggregates in these cells, and the assembly of new virus is inhibited (17). It should be pointed out that it is not known whether all M protein mutants which are deficient in assembly are also deficient in causing CPE.

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We have used two approaches to investigate the role of M protein in the CPE of VSV. First, we microinjected an antisense oligonucleotide complementary to M mRNA into cells in order to selectively inhibit only M protein translation during a VSV infection. Second, we cloned cDNA copies of the entire coding region for M protein of wild-type virus and of a *ts* mutant into a simian virus 40 (SV40) expression vector and compared the CPE in transfected cells at permissive and nonpermissive temperatures. The results presented here demonstrate that M protein alone is responsible for that part of the CPE which most likely affects the cytoskeleton and causes rounding of the cell.

MATERIALS AND METHODS

Virus and cells. Wild-type VSV (Mudd Summers strain, Indiana serotype) and the *ts* mutant *ts*G33 (Glasgow strain, Indiana serotype) were propagated, and titers were determined on monolayers of baby hamster kidney cells (BHK-21) grown in Eagle minimal medium supplemented with 10% fetal calf serum. Cos-1 cells (12) were used for DNA transfection experiments.

Preparation of VSV *ts*G33 mRNA. Confluent cultures of BHK-21 cells grown in 15-cm dishes were infected with 10 PFU of VSV *ts*G33 per cell. Six hours after infection, the cells were harvested by scraping them into phosphate-buffered saline and collecting them by low-speed centrifugation. RNA was extracted by the guanidinium thiocyanate method (21). Poly(A)$^+$ RNA was selected on an oligo(dT) cellulose (type 3; Collaborative Research, Lexington, Mass.). After ethanol precipitation, RNA was dissolved in water and stored at −70°C.

Oligonucleotide preparation. The oligonucleotides were synthesized with an Applied BioSystem 380 A DNA synthesizer. After deblocking (55°C; 8 to 12 h) and lyophilization, the oligonucleotides were suspended in deionized water, phenol extracted, chloroform extracted, and ethanol precip-

itated. The pellets were suspended in 10 mM Tris hydrochloride, pH 8–1 mM EDTA and kept at −20°C.

Cloning of cDNA and plasmid vectors. First, strands of DNA copies of VSV *ts*G33 mRNA were specifically synthesized by using a synthetic oligonucleotide primer A [AAGCGCGC(dT)$_{14}$ CAT] consisting of oligo(dT)$_{14}$ flanked at its 3′ end with three nucleotides complementary to the conserved nucleotides of all VSV mRNA preceding the poly(A) tail and flanked at its 5′ end with six nucleotides representing a *Bss*HII cloning site plus two additional A residues. The reverse transcription reactions were carried out in the presence of 1U of RNasin per ml as described previously (40). After 45 min at 42°C, the reaction was adjusted to 0.3 M NaOH and boiled for 5 min to hydrolyze the RNA. The solution was neutralized by adding HCl. The cDNA was phenol-chloroform (1:1) extracted, precipitated with ethanol, and dissolved in water.

The complementary plus-strand DNA was synthesized by the polymerase chain reaction (PCR) (36) by using a synthetic oligonucleotide primer B (AACTCGAGCCCAATC CATTCATCATGAGTTCC) which is identical in sequence to positions 27 to 50 at the 5′ end of the M mRNA preceded at its 5′ end by 6 nucleotides representing the *Xho*I cloning site and two additional A residues. The double-stranded DNA was amplified by PCR with both oligonucleotides A and B (conditions, 95°C for 1 min, 45°C for 2 min, 70°C for 3 min; 30 cycles) (see Fig 5). The amplified double-stranded cDNA was phenol-chloroform extracted, ethanol precipitated, digested by *Xho*I and *Bss*HII restriction enzymes, and subjected to 1.5% agarose gel electrophoresis. The approximately 820-base-pair (bp) fragment corresponding to the M mRNA cDNA of *ts*G33 was purified after electroblotting onto a NA-45 DEAE membrane (Schleicher & Schuell, Inc., Keene, N.H.). It was inserted into the unique and adjacent *Xho*I-*Bss*HII cloning sites of the SV40 expression vector pJC119 into which the *Bss*HII cloning site had been introduced earlier (24). *Escherichia coli* HB101 cells were transformed, and ampicillin-resistant colonies were picked.

The nucleotide sequences of two selected clones, ptsM16 and ptsM20, were determined by using Sequenase as directed by the manufacturer (U.S. Biochemical Corp., Cleveland, Ohio). The *Xho*I-*Bss*HII fragment containing the entire *ts*M gene was excised from ptsM16 and ptsM20 and subcloned into a modified pGEM4XB vector (10). These constructs were designated pGtsM16 and pGtsM20. Plasmids pKOM$_1$ and pKOM$_2$ had previously been isolated by K. Ono in our laboratory. pKOM$_1$ contains the cDNA copy of the wild-type M gene of the San Juan strain under the control of the SV40 late promoter. pKOM$_2$ contains the same wild-type M gene cDNA inserted into the pGEM4XB vector. The plasmid CDMM$_1$ contains the cDNA copy of the wild-type M gene under the control of a cytomegalovirus (CMV) promoter (1). The *Pst*I-*Hin*dIII fragment containing the entire wild-type M gene was excised from pKOM$_2$ and was subcloned into the corresponding site of the CDM8 vector (39).

The constructs pMΔ1 and pMΔ2 differ from pKOM$_1$ by a deletion of 140 bp at the 5′ terminus of the M gene and by a deletion of 267 bp at the 3′ terminus, respectively. These deletions were introduced by PCR amplification of the cloned San Juan wild-type M gene by using synthetic oligonucleotide primers AΔ1, BΔ1, AΔ2, and B. The primer AΔ1 (ATC GCG CGC ATA GGG ATA GAA AAG ACA GGA TAT TAG TTG T) is complementary to the 3′-end sequence of M mRNA and is flanked at its 5′ end with 6 nucleotides representing the *Bss*HII site plus 3 additional bases (ATC). The primer BΔ1 (ATC CTC GAG ATG GAG TAT GCT CCG AGC GCT CCA ATT GAC A) is identical in sequence to positions 138 to 168 of the M mRNA preceded at its 5′ end by 6 nucleotides representing the *Xho*I cloning site plus 3 additional residues (ATC). The primer AΔ2 (ATC GCG CGC TCT GAG CTC AAT CGT TCC CTT GTA AAG ACC T) is complementary to sequence 533 to 563 of the mRNA and is flanked at its 5′ end with the same 9 nucleotides described for AΔ1. The oligonucleotide B has been described above. Note that the 140-bp deletion of PMΔ1 includes a translational start codon so that the second in-frame AUG can be used for the translation of a truncated M protein which lacks the highly basic amino-terminal region of the wild-type protein (see Fig. 6).

Antibodies. Three mouse monoclonal antibodies that react with different epitopes of M protein (32) were kindly provided by R. R. Wagner, University of Virginia, Charlottesville. Polyclonal rabbit antibodies directed against a synthetic peptide corresponding to the amino terminus of the VSV polymerase L protein have been described earlier (38). Affinity-purified goat anti-rabbit immunoglobulin G (IgG) antibody and affinity-purified goat anti-mouse IgG conjugated to rhodamine or fluorescein were purchased from Cappel Worthington Biochemical (Malvern, Pa.). Biotinylated goat IgG was purchased from Vector Laboratories, Inc. (Burlingame, Calif.), and streptavidin-7-amino-4-methylcoumarin-3-acetic acid conjugate was purchased from Molecular Probes, Inc. (Eugene, Oreg.).

DNA transfection and immunofluorescent staining. Cos-1 cells were grown in 3.5-cm culture dishes until about 60 to 80% confluency. They were transfected with a mixture of 2.5 to 5 μg of supercoiled plasmid DNA and 5 μg of sonicated salmon sperm DNA by the calcium phosphate coprecipitation procedure (33). After transfection, cells were incubated for 1 or 2 days at 32, 37, or 40°C. Transfected cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 30 min at room temperature and permeabilized for 5 min with 0.05% Triton X-100 in phosphate-buffered saline. The viral M protein was stained with a murine monoclonal antibody and the corresponding goat anti-mouse IgG antibody conjugated to fluorescein isothiocyanate.

Microinjection, virus infection, and triple immunofluorescent staining. BHK-21 cells were grown in glass tissue culture dishes to about 50% confluency. The oligonucleotides (for the arrest of M protein translation, TCATGAATG GAT was used; for an unrelated control oligonucleotide, CTCATGATGAATG was used) were synthesized and prepared as described above. They were purified by electrophoresis on a 20% polyacrylamide gel containing 8 M urea. After elution from the gel, they were dissolved in 0.14 M KCl–2 mM piperazine *N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 7.6, and manually microinjected at a concentration of 1 mg/ml as described previously (14). After injection, cells were infected with wild-type VSV (Mudd Summers strain, Indiana serotype) at a multiplicity of infection of 10 PFU per cell for 30 min at 37°C. Six hours after infection, the cells were fixed and permeabilized as described above; they were then prepared for triple immunofluorescent staining according to procedures established by C. Godfraind, V. Friedrich, K. V. Holmes, and M. Dubois-Dalcq (J. Cell Biol., in press).

To identify injected cells, a biotinylated goat IgG (dialyzed against PIPES buffer) was injected with the oligonucleotide and labeled by exposing the cells to a streptavidin-7-amino-4-methylcoumarin-3-acetic acid conjugate. The viral M protein was stained with a murine monoclonal antibody and the corresponding goat anti-mouse IgG antibody conjugated to

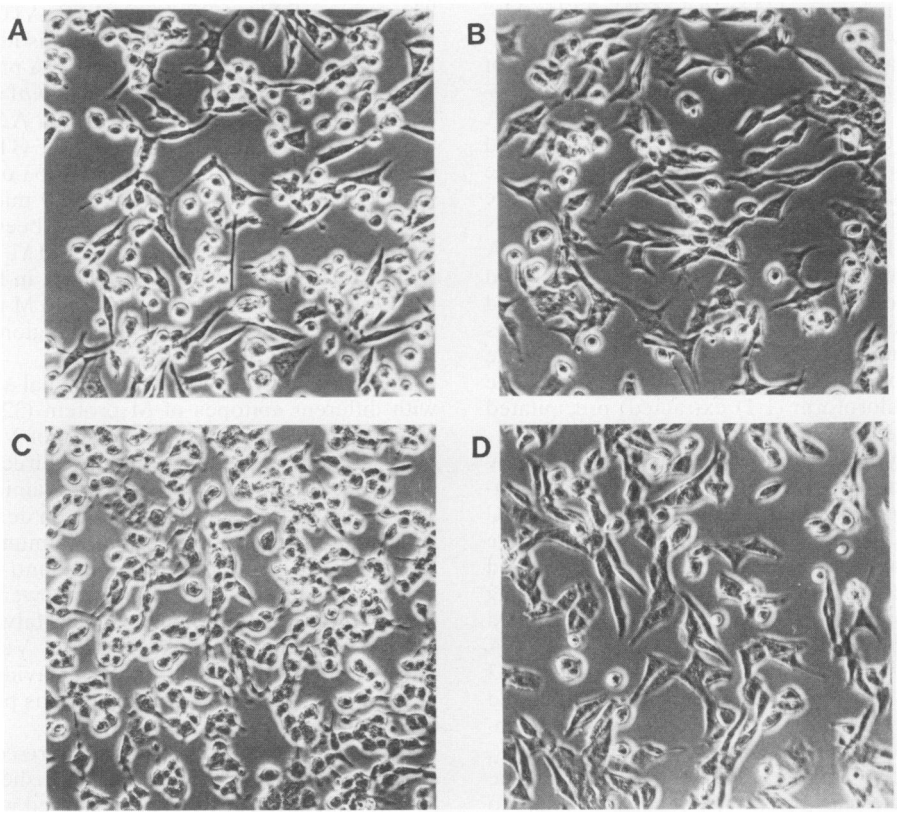

FIG. 1. BHK-21 cells infected with the VSV M protein mutant *ts*G33 after temperature-shift experiments. BHK-21 cells were infected with VSV *ts*G33 at a multiplicity of infection of 100 and incubated for 3 h at 32°C (A). The cells were also incubated for 3 h at 40°C (B) and then moved from 40 to 32°C for 3 h (C) and were subsequently shifted back to 40°C (D). The same experiment was also performed in the presence of 100 μg of cycloheximide per ml added 15 min before shift-down (C) or before shift-up (D); the same changes in the morphology of the cells during the course of the shift were seen both with and without the inhibitor.

fluorescein. The viral L protein was detected with a monospecific rabbit antibody and the corresponding goat anti-rabbit IgG antibody conjugated to rhodamine.

In vitro transcription and translation. pGem4XB (described elsewhere [10]) was used for in vitro transcription of the wild-type gene, the *ts*M gene, and the truncated M genes. Five micrograms of each plasmid was linearized by digestion at the unique *Bss*HII site. Capped runoff transcripts were synthesized by using the T7 microprobe kit (Promega Biotec, Madison, Wis.). RNA was translated in a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega Biotec) and labeled in the presence of [^{35}S]methionine (specific activity, >800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The proteins were analyzed on sodium dodecyl sulfate-polyacrylamide gels and visualized by fluorography according to standard procedures.

RESULTS

Reversibility of the CPE caused by *ts* M mutant. It has been previously mentioned that infection with the VSV M mutant *ts*G33 does not induce cell rounding of the cells when they are incubated at the nonpermissive temperature (40°C). In contrast, at the permissive temperature (32°C) the infected cells become round as early as 3 to 4 h after infection (31). We confirmed this result (Fig. 1A and B). When the infected cells were first maintained at 40°C for 3 to 4 h and were then shifted to 32°C for 3 h, the normally polygonal BHK cells became round (Fig. 1C). Moreover, the rounding of the cells could be reversed by shifting the infected cells again for 3 h to the nonpermissive temperature (Fig. 1D). Cells which had become round at 32°C again adopted a polygonal shape.

The proportions of round cells versus cells with a polygonal shape were quantified (Table 1). After the shift-up from 32 to 40°C, the percentage of round cells decreased from 74 to 30% within 3 h, whereas it increased from 20 to 84% within 3 h after shift-down to the permissive temperature. Furthermore, when the cells were moved back to the initial temperature, 89% of round cells were found again at the permissive temperature, compared with 36% at the nonpermissive temperature. We conclude that this morphological change (polygonal versus round) was entirely dependent on the temperature and that it was reversible.

In order to test whether M protein which was presynthesized for 3 h at 40°C could cause the rounding of the cell after the shift to 32°C, we prevented the synthesis of new M protein by adding 100 μg of cycloheximide per ml 15 min before the shift-down. Despite the inhibition of protein synthesis, we observed the same morphological changes as described previously without the drug (Fig. 1C and Table 1). This indicates a reversibility of the defect of the presynthesized M protein with respect to cell rounding. These results are in good agreement with the previous study that showed a reversibility of the defective function of M which resulted not only in the disaggregation of the M protein within the cell

TABLE 1. Effect of temperature on CPE caused by VSV *ts*G33[a]

Time [h] after Infection (0 3 6 9)	Number of Cells: Round	Number of Cells: Polygonal	Round Cells / Total Cells [%]
32°C	361	125	74
40°C	92	213	30
32°C 40°C	88	200	30
32°C 40°C 32°C	>200	24	>89
±cycloheximide; 40°C 32°C	>276	53	>84
40°C 32°C 40°C	113	205	36
Uninfected	12	152	7

[a] BHK-21 cells were infected as described in Fig. 1. The proportion of round versus polygonal cells was determined by counting the cells in different fields. Noninfected cells showed approximately 5 to 10% round cells. This background was subtracted from the number of round cells obtained with infected cells.

cytoplasm but also correlated with a partial restoration of viral assembly (31).

Selective inhibition of M protein expression and its effect on cell morphology during wild-type infection. In order to demonstrate whether M protein was involved in the rounding of the cells, we selectively inhibited the synthesis of M protein during a wild-type infection. This experiment was performed by microinjecting cells with a specific oligonucleotide (13 bases in length) complementary to the translation initiation site of M mRNA (18, 25). This oligonucleotide was injected into BHK-21 cells at a concentration of approximately 3×10^6 molecules per cell. The cells were then incubated for 1 h at 37°C and infected with wild-type VSV at a multiplicity of infection of 5 to 10 PFU per cell. The cells were fixed 6 h after infection, when the rounding of the cells was very pronounced. They were then processed for triple immunofluorescent staining that allowed us to (i) identify injected cells, (ii) screen for M protein expression, and (iii) identify infected cells by expression of the viral L protein, all within the same microscopic field. Six hours after infection, two injected cells (Fig. 2A) showed no M protein fluorescence (Fig. 2B) but bright L protein fluorescence (Fig. 2C), demonstrating that the expression of M protein was arrested in cells which were injected with the oligonucleotide. Injected cells which lacked M protein expression kept their initial morphology despite the wild-type infection, whereas cells which were not injected showed both M- and L-protein expression and were mostly round. M- and L-protein stain-

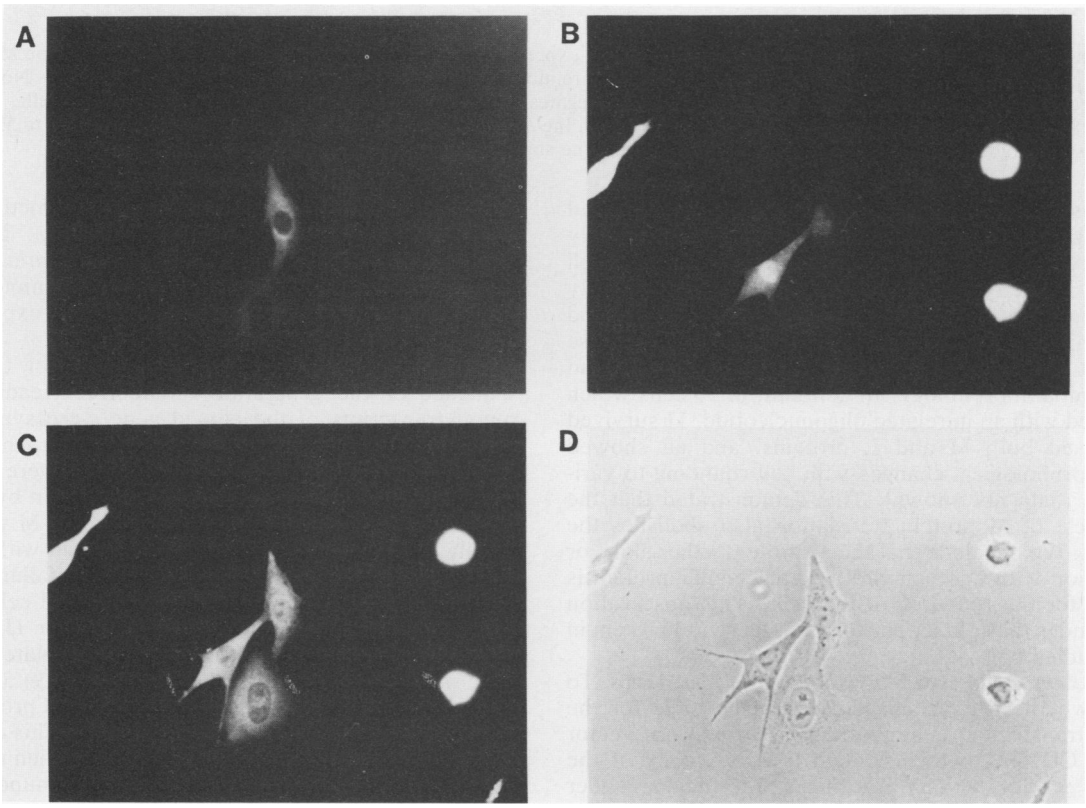

FIG. 2. Effect of microinjection of an antisense oligonucleotide to M protein mRNA on cell morphology. BHK-21 cells were microinjected, incubated for 1 h, and then infected with wild-type VSV at a multiplicity of infection of 10. After incubation for 6 h, the cells were fixed and prepared for triple immunofluorescent staining as described in Materials and Methods. They were stained with coumarin to identify injected cells (A) (two cells were microinjected in this field), with fluorescein to determine M protein expression (B), and with rhodamine to determine L protein expression (C). A phase-contrast micrograph of the same field is shown in panel D.

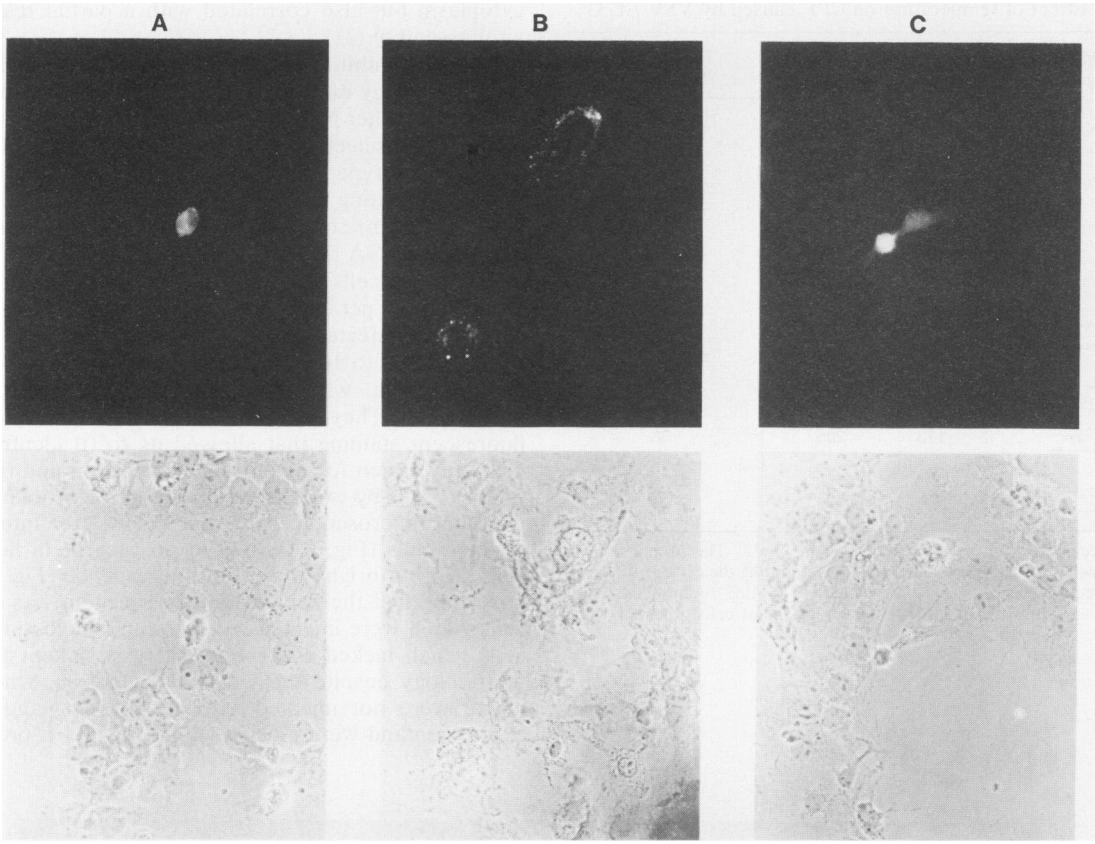

FIG. 3. Immunofluorescent staining of recombinant M protein expressed in Cos cells transfected with $pKOM_1$ (wild-type M protein) (A) or transfected with ptsM20 (*ts*G33 M protein) (B and C). Cells were incubated for 24 h at 37°C (A), 40°C (B), and 32°C (C). Note that at the nonpermissive temperature (40°C) the *ts* M protein formed aggregates located in the perinuclear regions of polygonal cells. Wild-type M protein was expressed in less than 0.1% of all cells, compared with the mutant M protein at 40°C, which was expressed in 1 to 5% of all cells. Phase-contrast micrographs of the same fields are shown below the stained cells.

ing as well as the cell rounding were typical for VSV-infected cells (Fig. 2D). The infection was, of course, not completely synchronized; therefore, a few infected cells, such as the cell close to the injected cells shown in Fig. 2, did not completely show the typical morphological changes. Overall, we found that of 45 injected cells, 26 survived and that in every case they did not express M protein but expressed L protein and kept their initial morphology. In contrast, of 36 cells which were injected with an unrelated oligonucleotide, 11 survived and expressed both M and L proteins, and all showed dramatic morphological changes with cell rounding to various extents (data not shown). This demonstrated that the specific arrest of M protein translation also abolishes the typical CPE. We conclude that the M protein either alone or in cooperation with another viral and/or cellular protein is responsible for the rounding of the cells. This observation clearly excludes the solitary role of any other viral protein in the CPE studied here.

Failure to detect wild-type M protein in transfected cells. To determine whether M protein alone is responsible for the CPE, we transfected cells with the expression vector $pKOM_1$ or $CDMM_1$, which contain a cDNA copy of the complete M gene of the VSV San Juan strain (Indiana) under the control of the late SV40 promoter or the CMV promoter, respectively. At different times after transfection (8, 24, and 48 h), the cells were fixed and stained for M protein expression. In most cases and when both vectors were used, no immunofluorescent staining for M protein was detected in Cos cells. Low levels of M protein were sometimes detectable in very few cells (<0.1%) (Fig. 3A). In dramatic contrast, the expression of the other four viral proteins of VSV under the control of the same SV40 promoter was very efficient with respect to the level of protein expression and the efficiency of transfection.

To rule out artifactual rearrangements of the M gene sequence or the generation of incorrect reading frames, runoff transcripts of the cloned gene were synthesized in vitro by T7 RNA polymerase by using the modified pGEM4XB vector (10). These transcripts were then translated in a cell extract. Analysis of the protein by polyacrylamide gel electrophoresis showed that the M protein was readily made in vitro and that it comigrated with M protein found in the virion (Fig. 4, lane 1). The failure to detect efficient M-protein expression in transfected cells has been observed by others using a similar vector (J. K. Rose, personal communication). The failure to isolate a recombinant vaccinia virus expressing M protein (19) also contributes to the idea that the inability to express M protein may be due to the toxicity of M protein to the cell. This cytotoxicity may simply cause the cell to round up and then detach from the dish so that it is not available for immunofluorescent staining.

Expression of *ts* M protein in transfected cells at the nonpermissive temperature. We postulated that transfected cells expressing M protein may lift off the dish and may be lost after the transfection because of cell rounding, possibly

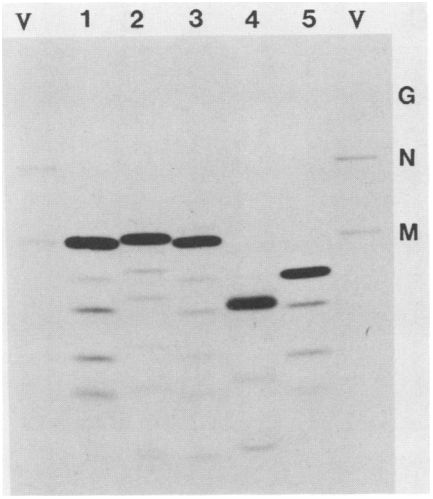

FIG. 4. Polyacrylamide gel electrophoresis of M proteins synthesized in vitro in rabbit reticulocyte lysates. Runoff transcripts from clones pKOM$_2$ (lane 1), pGtsM16 (lane 2), pGtsM20 (lane 3), pGMΔ2 (lane 4), and pGMΔ1 (lane 5) were translated in vitro, and the [^{35}S]methionine-labeled M proteins were electrophoresed on a 12.5% sodium dodecyl sulfate–polyacrylamide gel. Lanes V, [^{35}S]methionine-labeled viral proteins. G, Glycoprotein; N, nucleocapsid protein; M, matrix protein.

caused by M protein itself. To test this hypothesis, we cloned and sequenced the *ts* M gene of *ts*G33. Cos cells were transfected with the *ts*G33 M gene cloned into pJC119XB, and the cells were maintained at the nonpermissive (40°C) and permissive (32°C) temperatures. The cloning was carried out by PCR as outlined in Materials and Methods and in Fig. 5. Two clones (ptsM16 and ptsM20) were selected, and the sequences of both were determined by using Sequenase as described in Materials and Methods.

The ptsM20 sequence was identical to the corresponding consensus sequence reported by Morita et al. (26), but for ptsM16 one additional nucleotide at position 424 differed from the wild-type Glasgow strain sequence, resulting in a Gln-to-Arg amino acid change (Fig. 6A). This point mutation can be attributed either to the high degree of spontaneous mutability of the VSV genome (37) or to the error rate of the reverse transcriptase or Taq polymerase during the PCR reaction. These two *ts*M genes were subcloned into pGEM4XB. Transcripts made from these genes by T7 RNA polymerase were translated in vitro, and they produced a single protein (Fig. 4, lanes 2 and 3) which migrated closely to the wild-type M protein translated in vitro (Fig. 4, lane 1) as well as to authentic M protein from the virus particle (Fig. 4, lanes V). Cos cells were transfected with ptsM16 or ptsM20 and incubated for 20 and 40 h at permissive and nonpermissive temperatures, fixed, and stained for M protein. When the cells which were transfected with ptsM20 were maintained at 40°C, approximately 1% of them showed bright immunofluorescence in the form of small dots, mainly in the perinuclear region, as was also observed with *ts*G33 mutant-infected cells at the nonpermissive temperature (Fig. 3B). This demonstrated that the M protein appears to aggregate either with itself or with cellular components in the absence of the other viral proteins. In addition, these M-protein-expressing cells always appeared healthy, with a polygonal shape (Fig. 3B). In contrast, when the cells were incubated at 32°C, only very few round cells expressing M protein were detected (Fig. 3C). Surprisingly, after transfec-

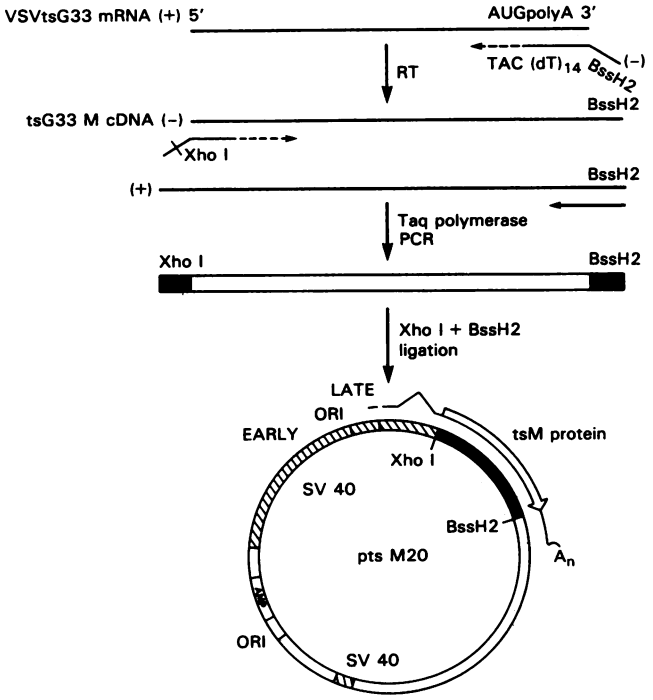

FIG. 5. Cloning strategy for a cDNA encoding the *ts* M protein. VSV *ts*G33 mRNAs were selected by oligo(dT) cellulose and reverse transcribed by using an oligo(dT) primer as described in Materials and Methods. The second-strand cDNA was initiated with an oligonucleotide complementary to the 3' terminal region of the full-length first-strand cDNA. The double-stranded cDNA was then amplified by PCR (36) and cloned into the cloning sites *Xho*I-*Bss*HII of the SV40 expression vector.

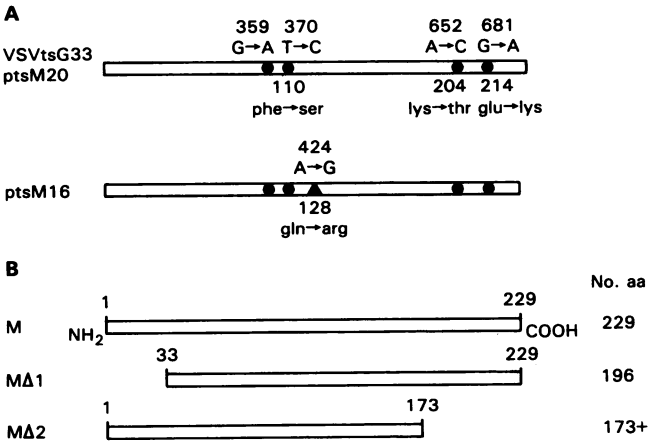

FIG. 6. Map of the M gene mutations. (A) M gene sequence comparison between two cDNA clones derived from *ts*G33 M (ptsM16 and ptsM20) and parental VSV Glasgow strain M gene (26). Nucleotides that differ from the corresponding wild-type sequence (●) and an additional mutation found only in clone ptsM16 (▲) are indicated. (B) Map of the N-terminal and C-terminal deletions of proteins MΔ1 and MΔ2, respectively. No. aa, Number of amino acids.

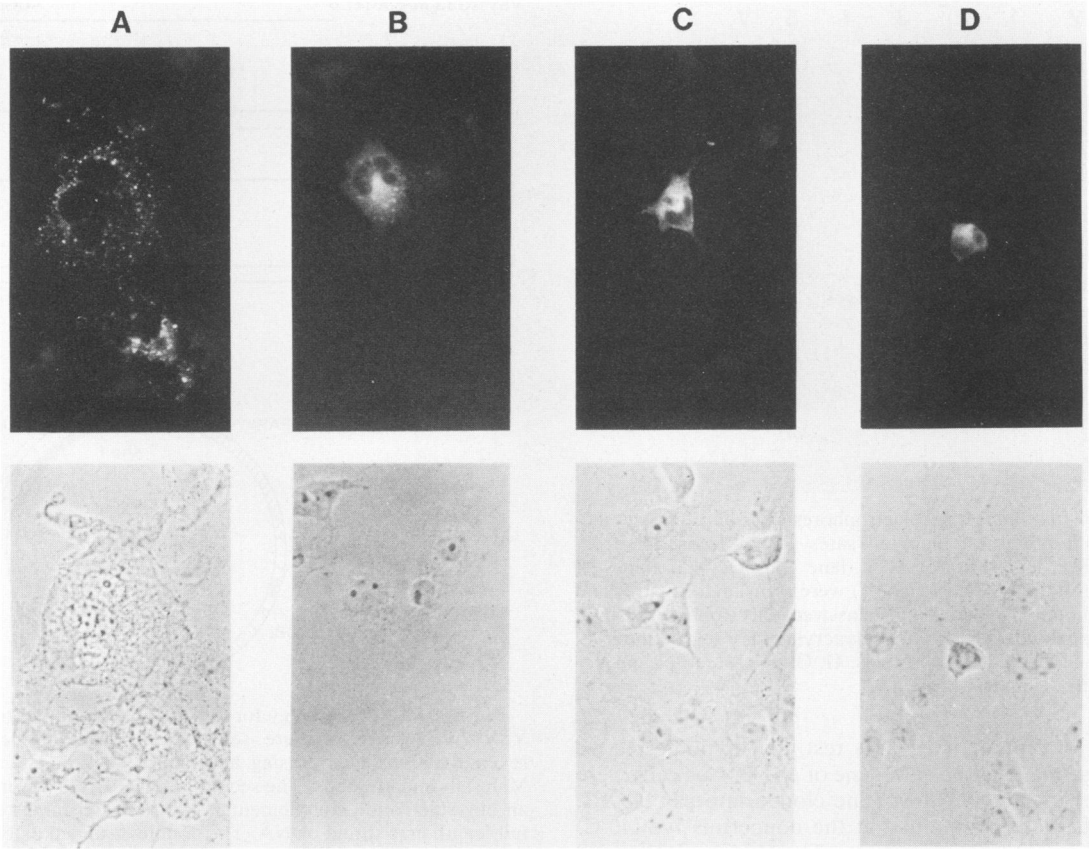

FIG. 7. Immunofluorescent staining of recombinant *ts* M protein expressed in Cos cells. Cos cells were transfected with ptsM20, which encodes the *ts* M protein of *ts*G33. The transfected cells were maintained for 20 h at 40°C (A) and then shifted down to 32°C for 3 (B), 6 (C), and 24 h (D). Note that the dotted perinuclear staining pattern of the *ts* M protein observed in polygonal cells at the nonpermissive temperature became gradually diffuse throughout the cytoplasm in round cells at the permissive temperature. Phase-contrast micrographs of the same fields are shown below the stained cells.

tion with ptsM16, not a single cell showed M protein staining at either temperature. We suspect that the absence of ptsM16 expression is the consequence of the additional point mutation present in its sequence. This mutation may have been present in the mutant virus stock, since its phenotype appears to be like that of wild-type virus (no visible expression of M protein). It is very likely that this clone was derived from a virus with a pseudoreversion.

These results are compatible with our above hypothesis that the expression of *ts* M protein at the permissive temperature induces the rounding of the cells, which consequently detach from the plate and are lost before staining. Together with its function during viral assembly and its control of viral transcription, this additional function of the M protein is also impaired at the nonpermissive temperature, and consequently no CPE is detected. At the nonpermissive temperature, the phenotype (the shape of the cells and the staining pattern of M) obtained after transfection with ptsM20 as well as after infection with the mutant virus *ts*G33 were very similar. In order to determine whether this phenotype is dependent on the temperature and is reversible, as is the case for virus-infected cells, Cos cells were transfected with ptsM20 and incubated for 20 h at 40°C. The temperature was then shifted down for different times. Like with *ts*G33-infected cells, the granular immunofluorescent staining which was observed at 40°C (Fig. 7A) disappeared with time and M protein was stained diffusely throughout the cell with only small remaining dots. This occurred rapidly within 3 h after the temperature change (Fig. 7B). After 6 h, the staining appeared to be entirely diffuse (Fig. 7C). After 24 h at 32°C, the transfected cells developed the typical CPE, and as expected, the number of positive cells was greatly reduced (Fig. 7D), whereas cells which were maintained at 40°C for 40 h kept their initial morphology and the dotted staining pattern for M protein. These results confirmed that the shape of the cells and the distribution of *ts*M protein in the cytoplasm are correlated and that they are both entirely dependent on the temperature. We conclude that M protein alone is sufficient to induce the morphological changes that lead to the characteristic visible CPE of a VSV infection.

Differential effect of the amino- and carboxy-terminal deletions on the cytopathic activity of M protein. In order to define the region of M protein that is responsible for the CPE, we constructed two clones, pMΔ1 and pMΔ2, which differed from the complete M gene clone $pKOM_1$ by deletions of 140 bp at the 5′ terminus and of 267 bp at the 3′ terminus, respectively, of the wild-type M protein (Fig. 6B).

The deletion mutant DNAs were constructed by using the PCR and specific synthetic oligonucleotide primers as described in Materials and Methods. The DNAs were cloned into pJC119XB for expression in Cos cells and into pGem4XB for in vitro transcription by T7 RNA polymerase

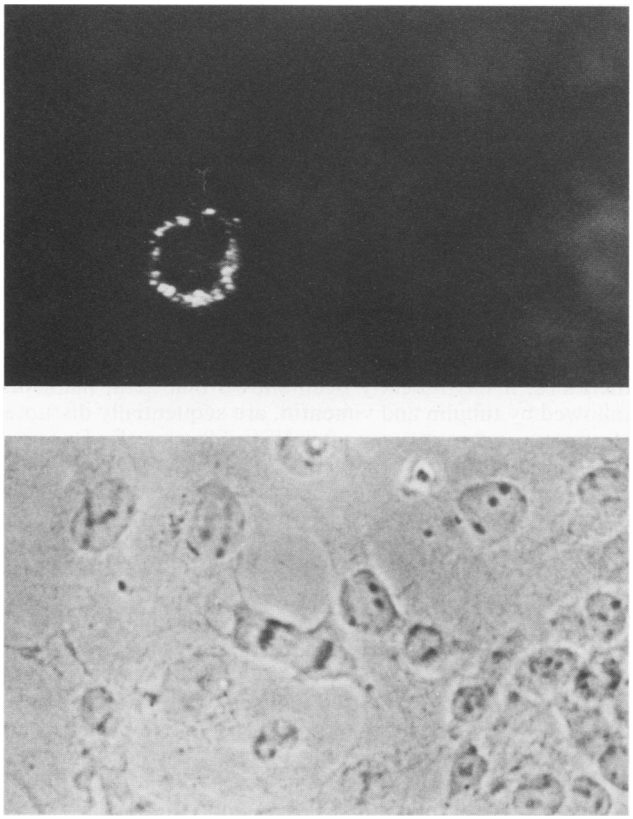

FIG. 8. Immunofluorescent staining of the carboxyl-terminal-deleted M protein expressed in Cos cells transfected with PMΔ2. After 24 h at 37°C, the truncated M protein was stained with a mixture of monoclonal antibodies MAb2 (specific for epitope 1), MAb3 (specific for epitope 2), and MAb 25 (specific for epitope 3) (32). Note the typical perinuclear dotted staining pattern. A phase-contrast micrograph of the same field is shown below the stained cells.

and translation. A schematic representation of the deleted proteins is shown in Fig. 6B.

Cos cells were transfected in parallel with both of these vectors. After 24 h of incubation at 37°C, the cells were stained for M expression with a mixture of three monoclonal antibodies (Mab 2 [directed to epitope 1], Mab 3 [directed to epitope 2], and Mab 25 [directed to epitope 3]). We anticipated that these would react with both truncated proteins because of the epitope mapping (32).

We observed the expression of a truncated M protein in cells transfected with pMΔ2, whereas we were unable to detect any cells expressing a truncated M protein with pMΔ1 (Fig. 8). However, both truncated M proteins, MΔ1 and MΔ2, could be translated in vitro and could be recognized by the mixture of the monoclonal antibodies during immunoprecipitations (not shown). Both proteins migrated on polyacrylamide gels as expected from their calculated molecular weights (Fig. 4, lanes 4 and 5, and Fig. 6B). The failure to detect M protein expression from pMΔ1 (as with the wild-type M protein gene) suggests that this truncated protein is able to cause CPE and that the amino-terminal region of the M protein may not be involved in this CPE.

It is interesting that the pattern of the truncated carboxyl-terminal M protein appeared to be granular and perinuclear (Fig. 8), as was also observed for *ts*M protein during infection with VSV *ts*G33 at the nonpermissive temperature. We can conclude from these data that the aggregate formation which results from interactions with cellular components or with M itself may not require the carboxyl-terminal region.

DISCUSSION

In this paper, we have described a novel third function of the VSV M protein. In addition to its structural role in virus maturation and its regulatory role in viral transcription, M protein alone causes a major portion of the visible CPE after infection, such as the rounding of cells.

We showed that the *ts* M protein of VSV *ts*G33 is defective at the nonpermissive temperature, resulting in the absence of these visible morphological changes of the infected cells. This defect was quickly reversed after a shift to the permissive temperature (as quickly as 2 h). The M gene of VSV *ts*G33 differs from the parental strain by three amino acid changes (amino acids 110, 204, and 214) (26). These mutations result in a decrease in the ability of M protein to inhibit viral transcription and in the lack of virus budding (41). Previous studies by K. Ono (31) have suggested that these mutations may produce a conformational change which causes M to aggregate at the nonpermissive temperature, thereby inhibiting the binding of the M protein to the nucleocapsid and consequently viral assembly. This defect is partially reversible after temperature shift-down and can be triggered by temperature shift-up (31). It is noteworthy that both CPEs (the inhibition of host macromolecular synthesis and the cellular rounding) are independent processes. This is predicted by the fact that cellular rounding is independent of protein synthesis and takes place in the presence of cycloheximide. At the nonpermissive temperature, VSV *ts*G33 used in this study inhibits the macromolecular synthesis of the host cell without causing a morphological change (22; D. Blondel and M. Schubert, unpublished data). Viral assembly, on the other hand, is reversibly inhibited, suggesting that there may be a link between cell rounding and viral budding. The only link known at this time, however, is that both processes require functional M protein.

The microinjection of antisense oligonucleotides demonstrated that the specific inhibition of M protein translation protects cells from morphological alteration caused by a wild-type VSV infection. These results did not exclude, however, that the M protein may interact with other viral proteins to cause cell rounding. The results of the transfection experiments allowed us to answer this question.

Initially, we were unable to detect M protein in Cos cells transfected with a cDNA copy of the wild-type M gene under the control of either a CMV promoter or the SV40 late promoter. These are both promoters which can readily be used to express the other VSV genes (15, 38, 41). The difficulty in expressing M protein has been previously found by others using the same vector (J. K. Rose, personal communication). The reason for the failure to isolate vaccinia virus recombinants expressing M protein may also be found in the toxicity of M protein itself (19). This is underlined by the fact that we observed efficient expression of a recombinant *ts* M protein at 40°C, whereas at 32°C we were unable or only very rarely able to detect cells positive for M protein. All these data lead us to propose that cells expressing M protein are specifically lost because of the CPE of the M protein itself. It appears that the defect of *ts* M protein results in a reduced CPE at nonpermissive temperature but not in a total loss of this effect. This could explain the observation of relatively low-level expression of M protein

in fewer cells (1 to 5%) compared with the other VSV proteins expressed in Cos cells.

The distribution of recombinant *ts* M protein in transfected cells is entirely dependent on the temperature, as it has been described during the infection with VSV *ts*G33 (31). This distribution correlates well with the morphology of the cells; the M protein appears in a dotted staining pattern in the perinuclear region of polygonal cells, but it is diffuse throughout the cytoplasm in rounded cells. These characteristics constitute part of the phenotype of the *ts*G33 M protein. This phenotype is reversible in both directions, as evidenced by shift-down experiments from 40 to 32°C as well as by shift-up experiments. The reversion occurs within 3 h after the temperature change. In summary, we conclude that the M protein is responsible for the rounding of the cells and that this function is independent of the presence or activity of any of the other viral proteins.

It is interesting that an additional mutation at nucleotide 424 of the *ts* M gene leading to a Gln-Arg substitution at amino acid 128 apparently restores the wild-type phenotype and seems to produce a pseudorevertant protein with respect to the CPE. At this time, we do not know the functionality of this mutant M protein in viral assembly. However, this is consistent with the data obtained by Morita et al. (26), who reported that 14 revertants derived from *ts* M protein mutants only differed from their *ts* parents by single amino acid changes. Therefore, with the M protein, many different point mutations can act individually in restoring the wild-type phenotype in a pseudoreversion, as measured for instance by the ability to inhibit in vitro transcription (26). Both from these data and from the complicated picture of overlapping epitopes defined by monoclonal antibodies (32), the M protein appears to be a tightly interacting structure whose functional domains are mostly determined by the conformation of the folded protein. However, less tertiary structure was suspected at the amino and carboxyl termini (32).

When we analyzed the effect of terminal deletions on the ability to induce morphological alterations, we observed that the mutant protein that lacks the amino-terminal region of M still induces cellular rounding, whereas the protein lacking the carboxyl-terminal segment does not. We conclude this because the amino-terminal-truncated protein was not detected in transfected cells, as was also the case with the wild-type protein. The carboxyl-terminal-deleted M protein, on the other hand, exerts the same phenotype as the *ts* mutant at the nonpermissive temperature (i.e., dotted perinuclear staining pattern in polygonal cells). The results with both deletion mutants suggest that the highly basic amino-terminal region of the M protein, which has been proposed to present a nucleocapsid-binding site (32, 34), is most likely not required for cell rounding. The complexity of the M protein conformation may not allow us to define a linear amino acid sequence which represents the domain involved in the rounding of cells. The dotted staining pattern is independent of G-protein expression, possibly suggesting an M-M or M-membrane interaction or both. Electron microscopic analysis of the distribution of the *ts* M protein at the nonpermissive temperature suggests an association of M with perinuclear vesicles (31). Our data indicate that the carboxyl-terminal region is not required for this interaction. For the CPE, however, the carboxyl terminus may be required.

The mechanism by which M protein causes cellular rounding has not been studied in this paper. We suspect that the rounding of the cells might be preceded by disruption of cytoskeletal elements. Morphological changes are often caused by viral infections, and these changes are often correlated with cytoskeletal alterations (13). The physical association of viral macromolecular synthesis with the cellular cytoskeletal framework has been suggested by Cervera et al. (7), who showed that VSV-specific polyribosomes were bound to the cytoskeletal framework. More recently it has been shown that microtubule-associated proteins and tubulin stimulate in vitro RNA synthesis by the VSV ribonucleocapsid-associated polymerase (16, 27). This raised the possibility that cytoskeletal proteins may be involved in M binding and may thereby remove the transcriptional block caused by M protein. Close associations between actin and the matrix proteins of Newcastle virus, Sendai virus, and influenza virus have been described elsewhere (4, 11). Furthermore, it has recently been shown that actin filaments, followed by tubulin and vimentin, are sequentially disrupted starting as early as 60 min after VSV infection (K. O. Simon et al., submitted for publication). We have preliminary evidence that the M protein may specifically disrupt microtubules (C. C. Widnell et al., manuscript in preparation). Further studies, however, will be required to define the precise mechanisms by which the M protein induces cellular rounding. It will be interesting to see whether it acts directly on the organization of the cytoskeleton or indirectly, possibly by affecting the permeability of the cells and thereby changing ion transport.

It has often been suggested that selective modifications of the viral matrix protein and/or viral envelope proteins may cause alterations of cytopathogenicity and host cell survival. This is a characteristic of most negative-strand RNA viruses during persistent infections. It may apply to Sendai virus in cell culture (35), to lymphocytic choriomeningitis virus (30) in vivo, and to measles virus during subacute sclerosing panencephalitis (2, 3, 6). It is striking that with the majority of subacute sclerosing panencephalitis isolates, the matrix protein gene was found to be specifically hypermutated compared with the other genes (6). This hypermutation may be necessary to maintain a persistent measles infection simply because it decreases the chances of pseudoreversion, which consequently may lead to a disorganization of the cytoskeleton and to a dissociation of the cells.

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