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Activity of Vesicular Stomatitis Virus M Protein Mutants in Cell Rounding Is Correlated with the Ability to Inhibit Host Gene Expression and Is Not Correlated with Virus Assembly Function

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In addition to its role in virus assembly, the matrix (M) protein of vesicular stomatitis virus (VSV) is involved in virus-induced cell rounding and inhibition of host-directed gene expression. Previous experiments have shown that two M protein mutants genetically dissociate the ability of M protein to inhibit host-directed gene expression from its function in virus assembly: M protein from tsO82 virus is fully functional in virus assembly but defective in the inhibition of host-directed gene expression, while the MN1 deletion mutant, which lacks amino acids 4–21, inhibits host-directed gene expression but cannot function in virus assembly. Experiments presented here compared cell rounding induced by these two mutant M proteins to that of wt M protein. BHK cells were transfected with M protein mRNA transcribed *in vitro,* and the extent of cell rounding was evaluated at 24 hr posttransfection. The MN1 protein was nearly as effective as wt M protein in the induction of cell rounding, while tsO82 M protein expressed from transfected RNA was not able to induce cell rounding above that observed in negative controls without M protein, although it did cause BHK cells to have a less elongated shape. These results indicate that the ability of MN1 and tsO82 M proteins to induce cell rounding is not correlated with their virus assembly function. Instead the cell rounding activity of these mutants is correlated with their ability to inhibit host-directed gene expression. Previous data suggesting that these two cytopathic activities could be dissociated can be readily accounted for by quantitative differences in M protein expression required. Infection of either BHK cells or L cells with tsO82 virus induced cell rounding, although cell rounding was delayed relative to that following infection with wt VSV, suggesting that tsO82 M protein retains some cytopathic activity. The distribution of actin, vimentin, and tubulin in transfected cells was determined by fluorescence microscopy. In cells transfected with tsO82 M mRNA, these cytoskeletal elements were indistinguishable from those of negative control transfected cells. In cells rounded as a result of transfection with wt M or MN1 mRNA, actin-containing filaments were reorganized into a thick perinuclear ring but were not depolymerized. In contrast, tubulin and vimentin appeared to be diffusely distributed throughout the cytoplasm of rounded cells. These results support the idea that cell rounding induced by M protein results from the depolymerization of microtubules and/or intermediate filaments.

INTRODUCTION

Vesicular stomatitis virus (VSV) induces a variety of cytopathic effects in infected cells. Cells that are adherent to their substrate undergo pronounced morphological changes leading to cell rounding as a result of the sequential disassembly of the elements of the cytoskeleton (Simon *et al.,* 1990). Virus-induced cell rounding is attributable, at least in part, to the activity of the viral matrix (M) protein. M protein plays a major role in virus assembly by mediating envelopment of the nucleoprotein core (nucleocapsid) by the host plasma membrane during the budding process (reviewed by Lenard, 1996). Expression of M protein in the absence of other viral components leads to cell rounding (Blondel *et al.,* 1990; Ye *et al.,* 1994); and inhibition of M protein expression in virus-infected cells reverses cell rounding (Blondel *et al.,* 1990). Furthermore, purified M protein is able to inhibit assembly of microtubules from tubulin subunits in an *in vitro* assay (Melki *et al.,* 1994).

Another prominent cytopathic effect of VSV infection is the inhibition of host-directed gene expression. This inhibition occurs at multiple levels, including inhibition of both host-directed transcription and translation (Wagner and Huang, 1966; Wertz and Youngner, 1970). M protein also plays a major role in this aspect of viral cytopathology. Host-directed transcription is inhibited by M protein expression in the absence of other viral components (Black and Lyles, 1992; Black *et al.,* 1993; Paik *et al.,* 1995; Ferran and Lucas-Lenard, 1997). In several cases, defects in inhibition of host translation by VSV mutants have been correlated with mutational changes in M protein (Coulon *et al.,* 1990; Francoeur *et al.,* 1987; Stanners *et al.,* 1977), suggesting that M protein is involved in the inhibition of translation. However, expression of M protein in the absence of other viral components actually stimulates translation of cotransfected mRNAs (Black *et*

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al., 1994). Thus other viral components must be involved, perhaps together with M protein, in this aspect of VSV cytopathology.

The ability of M protein to inhibit host-directed gene expression is genetically separable from its role in virus assembly (Black *et al.,* 1993). This was shown using two M protein mutants with complementary phenotypes. The tsO82 mutant is temperature sensitive only in certain cell types such as chick embryo cells, but replicates as well as wt VSV in BHK cells at all temperatures. This virus is defective in its ability to inhibit host gene expression and has a single substitution in the 229 a.a. M protein of arg for met at position 51 (M51R mutation) (Coulon *et al.,* 1990). This mutation dramatically reduces the ability of M protein to inhibit host-directed gene expression at all temperatures without affecting the level of M protein synthesis or turnover or the ability of M protein to function in virus assembly (Black *et al.,* 1993; Kaptur *et al.,* 1995). The MN1 mutant, whose phenotype is the converse of that of tsO82, was created by deletion of the coding sequence for amino acids 4–21 of the M protein (Black *et al.,* 1993). This deletion abolished M protein function in virus assembly but had little effect on its inhibition of host-directed gene expression.

The goal of the experiments described here was to determine whether the ability of M protein to induce cell-rounding was genetically correlated with its function in virus assembly or its ability to inhibit host gene expression. Previous experiments have been inconclusive on this question. TsO82 virus has been reported to induce visible cytopathic effects in BHK cells but not in chick embryo cells (Coulon *et al.,* 1990). Group III (M gene) ts mutants of VSV, which are defective in virus assembly, inhibit host gene expression at the nonpermissive temperature, while in some cases cell-rounding is delayed (Blondel *et al.,* 1990; Weck and Wagner, 1979; Ye *et al.,* 1994), suggesting that cell-rounding is correlated with virus assembly and separate from the inhibition of host gene expression. In addition, deletion mutants of M protein that are defective in their membrane-binding ability, which is required for virus assembly, are also defective in their ability to induce cell-rounding (Ye *et al.,* 1994). The problem with the experiments with group III ts mutants and deletion mutants is that it is difficult to evaluate the extent of misfolding of M protein and the quantitative effects this misfolding might have on its various activities.

In the experiments described here, the ability of mutant M proteins to induce cell-rounding was evaluated using the two mutants that most clearly separate the function of M protein in virus assembly from its ability to inhibit host gene expression, namely the tsO82 and MN1 mutants. Quantitative approaches were used in which the amount of M protein expressed was varied in a systematic manner by transfecting different amounts of *in vitro*-transcribed M protein mRNA (Black *et al.,* 1994), so that differences in level of expression among mutant M proteins could be controlled. The results showed that the ability of mutant M proteins to induce cell rounding was correlated with their ability to inhibit host-directed gene expression and not with their virus assembly function.

MATERIALS AND METHODS

Cells and viruses

Wild-type VSV (Indiana serotype, Orsay strain) and the tsO82 mutant were grown in BHK cells as described except that tsO82 virus was grown at 31° (Lyles *et al.,* 1996). BHK cells and L cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum.

Plasmids and *in vitro* transcription of mRNA

The plasmids used for *in vitro* transcription of mRNA encoding wt, tsO82, and MN1 M proteins or the VSV nucleocapsid (N) protein together with a 3′ poly(A) sequence have been described (Black *et al.,* 1994). M mRNAs containing 5′ caps and 3′ poly(A) were transcribed in the presence of cap analog, ^{7}mG(5′)ppp(5′)G, from appropriately linearized plasmid DNA by bacteriophage SP6 RNA polymerase using reagents and procedures from a commercial kit (Message Machine, Ambion, Inc.).

Transfection of mRNAs encoding M and N proteins

BHK cells were transfected with different amounts of *in vitro*-transcribed mRNA encoding either wt or mutant M proteins using Lipofectin reagent (Gibco/BRL) as described (Black *et al.,* 1994) to determine their relative levels of expression from the transfected mRNA. At 5 hr posttransfection cells were labeled for 1 hr with [^{35}S]-methionine; the cells were solubilized, and labeled M proteins were analyzed by immunoprecipitation and gel electrophoresis as described except that the solubilization buffer lacked SDS and deoxycholate (Kaptur *et al.,* 1995). To determine the extent of rounding of transfected cells, BHK cells were cotransfected with N mRNA (1200 ng/10^5 cells) together with wt M, tsO82 M, or MN1 mRNA, or no M mRNA as a negative control. The total amount of RNA transfected was held constant at 2400 ng/10^5 cells using yeast total RNA. At 24 hr posttransfection, cells were either stained with crystal violet to examine total cells by phase contrast microscopy or were formalin-fixed, permeabilized with Triton X-100, and labeled with monoclonal antibody 10G4 against the N protein and a fluorescein-conjugated secondary antibody (Lyles *et al.,* 1988) to specifically examine transfected cells by fluorescence microscopy.

Fluorescent labeling of cytoskeletal elements

BHK cells were transfected and processed for immunofluorescence microscopy as described in the previous

section. The primary antibody incubation contained anti-N protein and the secondary incubation contained rhodamine-phalloidin (Molecular Probes, Eugene, OR) as well as fluorescein-conjugated anti-mouse IgG for double-label fluorescence analysis of actin-containing filaments (Wulf *et al.,* 1979) as well as N protein. In order to analyze the distribution of intermediate filaments, the primary antibody incubation included polyclonal goat anti-vimentin (Sigma Chemical Co., St. Louis, MO) as well as monoclonal anti-N protein and the secondary incubation included fluorescein-conjugated rabbit anti-mouse IgG and rhodamine-conjugated rabbit anti-goat IgG. Analysis of tubulin distribution was performed in single-label immunofluorescence experiments using mouse monoclonal anti-α tubulin (Amersham/USB, Arlington Heights, IL). N protein expression was analyzed in separate samples prepared in parallel.

RESULTS

Levels of expression of wt and mutant M proteins from transfected mRNA

The ability of tsO82 and MN1 mutant M proteins to induce cell rounding was compared to that of wt M protein. This was tested by transfecting *in vitro*-synthesized mRNA encoding M protein. This approach avoids the problem that M protein inhibits its own synthesis from DNA-based vectors that require host transcriptional activity (Black and Lyles, 1992). In addition, the amount of mRNA transfected can be adjusted to give equivalent levels of expression of mutant and wt M proteins. In the experiment shown in Fig. 1A, BHK cells were transfected with different amounts of *in vitro*-transcribed mRNA encoding either wt or mutant M proteins to determine their relative levels of expression from the transfected mRNA. At 5 hr posttransfection cells were labeled for 1 hr with [^{35}S]methionine; the cells were solubilized, and labeled M proteins were analyzed by immunoprecipitation and gel electrophoresis. When equivalent amounts of mRNA were used (1200 ng/10^5 cells), less M protein was produced by the tsO82 M mRNA (lane 4) than wt M or MN1 mRNA (lanes 1 and 5). This is due to the ability of wt M and MN1 proteins to stimulate translation of transfected mRNAs, including their own, while tsO82 M protein does not stimulate translation (Black *et al.,* 1994). The differences in levels of M protein produced were due to differences in rate of synthesis, not degradation, since the turnover of the mutant M proteins is the same as wt M protein (Black *et al.,* 1993). The level of expression of wt M protein when cells were transfected with 120 ng/10^5 cells (lane 3) was similar to that of the tsO82 M protein when 1200 ng/10^5 cells were used (lane 4). The MN1 protein appeared as a doublet on SDS–PAGE (lane 5). The reason for this has not been fully explored. However, when the radioactivity in the two bands was taken together, the total level of expression appeared similar to that of wt M protein.

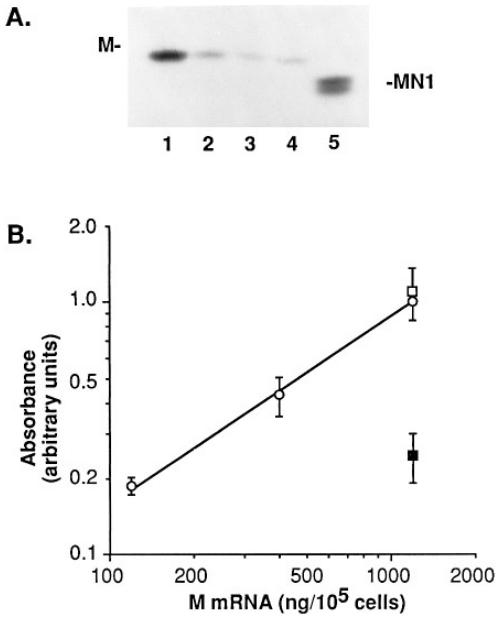


FIG. 1. Expression of wt and mutant M proteins from transfected mRNA. (A) BHK cells were transfected with mRNA encoding wt M protein (lanes 1–3), tsO82 M protein (lane 4), or MN1 protein (lane 5). The amount of M mRNA transfected (ng/10^5 cells) was 1200 ng (lanes 1, 4, 5), 400 ng (lane 2), or 120 ng (lane 3). At 5 hr posttransfection, cells were labeled for 1 hr with [^{35}S]methionine. The cells were solubilized, and the M proteins were analyzed by immunoprecipitation and gel electrophoresis, a fluorograph of which is shown. (B) Fluorographs similar to those in A were analyzed with a scanning densitometer, and the absorbance in each band was normalized to the value of wt M protein in lane 1, which was assigned an arbitrary value of 1.0. Data shown are means ± SEM for 3 independent experiments for wt M protein (open circles), tsO82 M protein (closed square), and MN1 protein (open square).

Figure 1B shows densitometry data obtained from three independent experiments similar to that in Fig. 1A. The integrated absorbance of each band was normalized to that of wt M protein from cells transfected with 1200 ng M mRNA/10^5 cells, which was assigned an arbitrary value of 1. The level of expression of tsO82 M protein (closed square) was 25% of that of wt M protein (open circle), while that of MN1 protein (open square) was equivalent to wt M protein. In order to achieve comparable levels of expression, cells had to be transfected with 10-fold less wt M mRNA (120 ng/10^5 cells) than tsO82 M mRNA.

Cell rounding induced by transfected M mRNAs

The ability of wt and mutant M proteins to induce cell-rounding was evaluated by microscopic examination of cells transfected with M mRNAs. Transfected cells were identified by immunofluorescent labeling of the VSV nucleocapsid (N) protein encoded by cotransfected N mRNA. Using N protein as a marker for transfected cells

instead of M protein avoided the problem that it is difficult to efficiently detect M protein by immunofluorescence when low amounts of mRNA are transfected. In the experiment shown in Fig. 2, BHK cells were cotransfected with N mRNA (1200 ng/10^5 cells) together with wt M, tsO82 M, or MN1 mRNA, or no M mRNA as a negative control. The amount of tsO82 M mRNA (1200 ng/10^5 cells) was 10-fold more than the amount of wt M or MN1 mRNA (120 ng/10^5 cells) to give approximately equal levels of M protein expression. The total amount of RNA transfected was held constant at 2400 ng/10^5 cells using yeast total RNA. At 24 hr posttransfection, cells were either stained with crystal violet to examine total cells by phase contrast microscopy or were processed for immunofluorescence labeling of N protein to specifically examine transfected cells.

Figure 2A shows phase contrast micrographs of cells transfected with wt M mRNA (panel 1) or yeast RNA as a negative control (panel 2). Many of the cells in panel 1 show the dramatic rounding typical of VSV-infected cells. In negative control cells transfected without M mRNA (panel 2), nearly all of the cells retain their polygonal morphology. The occasional rounded cells were usually mitotic. Immunofluorescent labeling of N protein in cells cotransfected with N mRNA is shown in Fig. 2B at higher magnification than in Fig. 2A. The punctate cytoplasmic labeling pattern of N protein seen in these micrographs is similar to that observed in previous studies of N protein expressed in the absence of other VSV gene products (e.g., Black *et al.,* 1993; Sprague *et al.,* 1983). Since labeling of N protein occurs throughout the cytoplasm, cells that are rounded as a result of transfection with wt M mRNA (Fig. 2B, panel 1) can be readily distinguished from those transfected with N mRNA alone, which retain their polygonal shape (panel 2). The punctate pattern of N protein labeling is more distinct in the control cells (panel 2) than in the cells transfected with M mRNA (panel 1). This is because more of the label is in one focal plane in the flatter cells than in those that are rounded. Cells transfected with tsO82 M mRNA (panel 3) remained flat with a polygonal morphology. However, there were fewer fully elongated cells than in the case of control cells that did not express M protein (panel 2) (described more fully in the next section). Cells transfected with MN1 mRNA (panel 4) were indistinguishable from those transfected with wt M mRNA (panel 1). These results indicate that the ability of the mutant M proteins to induce cell rounding is correlated with the ability to inhibit host-directed gene expression, as shown by the cell-rounding activity of the MN1 mutant, and is not correlated with their ability to function in virus assembly, as shown by the inability of the tsO82 M protein to induce cell rounding when expressed at levels equivalent to those of wt M or MN1 proteins.

The number of rounded cells was evaluated by microscopic examination to determine whether there were quantitative differences among the different M proteins in the induction of cell rounding. As seen in Fig. 2, rounded cells were usually easily distinguished from polygonal cells, but in ambiguous cases, cells were considered to be rounded if more than half of their circumference was round. The samples were evaluated in blinded assays to reduce as far as possible the subjectivity of the evaluation. Table 1 shows the results of three separate experiments similar to that in Fig. 2. There was no statistically significant difference between wt M protein versus MN1 protein in their ability to induce cell rounding (66 versus 61%). Likewise, there was no difference between the number of rounded cells in the presence of tsO82 M protein and that of the negative controls without M protein or N protein (approximately 20%). Although the differences are obscured in Table 1 by the variation between experiments, it was noted that in each experiment among cells transfected with wt M or MN1 mRNA, the percentage of fluorescent cells that were rounded was higher than the percentage of total cells that were rounded. This was expected, since the total cells would include some untransfected cells.

The number of rounded cells was determined as a function of the amount of M mRNA transfected to determine the potency of the different M proteins in the induction of cell rounding (Fig. 3). In these experiments, transfection with MN1 mRNA (open squares) resulted in slightly less cell rounding than transfection with wt M mRNA (open circles), particularly at lower mRNA concentrations. Interpolation along the lines drawn through the data indicates that the mRNA concentration (ng/10^5 cells) that resulted in rounding of 50% of the cells is approximately 10 ng for wt M mRNA and approximately 35 ng for MN1 mRNA. In no case was tsO82 M protein (closed squares) observed to induce cell rounding above the levels seen in the negative controls (horizontal lines).

Alterations in cell shape induced by tsO82 M protein

The cells expressing tsO82 M protein in Fig. 2B, panel 2, are polygonal, but none of them show the elongated morphology assumed by many normal BHK cells in Fig. 2A, panel 2, and at least one cell in Fig. 2B, panel 2. The number of fully elongated, labeled cells in randomly selected fluorescence micrographs was quantitated to determine whether this apparent difference in morphology was representative. To make this measurement, the circumference of the labeled cytoplasm was traced, and its length was measured by the longest straight line that could be placed entirely within the trace. The width of the trace was then measured by a perpendicular line drawn at half the length, and an "axial ratio" was calculated as the length divided by the width. The traces for the labeled cells in Fig. 2B, panels 2 and 3, are shown in Figs. 4A and 4B, respectively, along with their axial ratios. It is apparent from this limited sample that cells

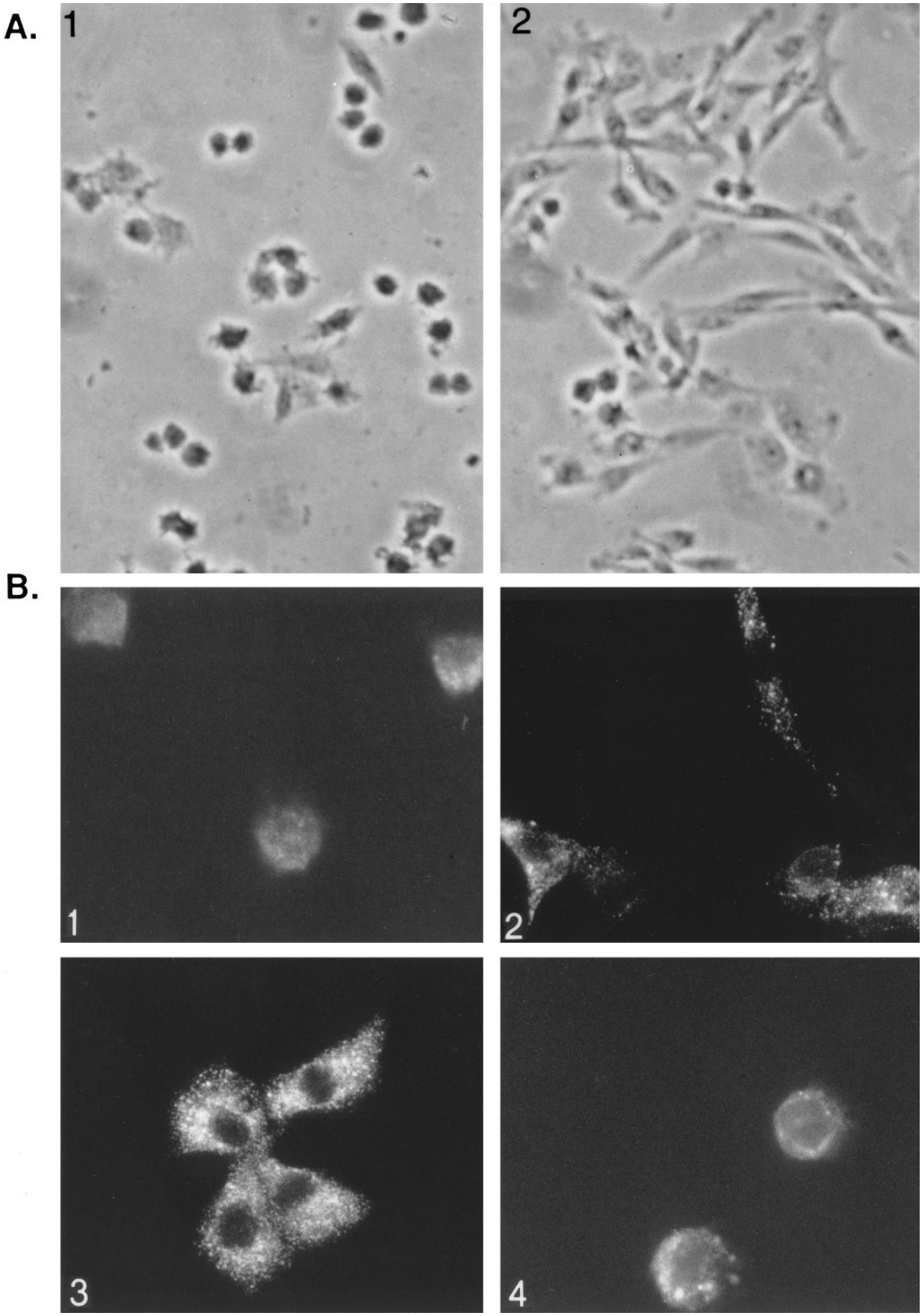


FIG. 2. Cell rounding induced by wt and mutant M proteins. BHK cells were cotransfected with N mRNA (1200 ng/10^5 cells) together with the indicated amount of M mRNA (ng/10^5 cells). At 24 hr posttransfection, cells were either stained with crystal violet for phase contrast microscopy (A) or processed for immunofluorescent labeling of N protein for fluorescence microscopy (B). (A) Panel 1, cells cotransfected with wt M mRNA (120 ng); panel 2, cells cotransfected with yeast RNA. (B) Panel 1, cells cotransfected with wt M mRNA (120 ng); panel 2, cells cotransfected with yeast RNA; panel 3, cells cotransfected with tsO82 M mRNA (1200 ng); panel 4, cells cotransfected with MN1 mRNA (120 ng).

TABLE 1

Efficiency of Cell Rounding by wt and Mutant M Proteins

M mRNA	Amount transfected (ng/10^5 cells)	Cotransfected RNA	Percentage of total cells rounded[a] (mean ± SEM; $N = 3$)	Percentage of fluorescent cells rounded[b] (mean ± SEM; $N = 3$)
wt	120	N	66 ± 5	69 ± 9
tsO82	1200	N	19 ± 1	24 ± 4
MN1	120	N	61 ± 9	66 ± 8
None	—	N	19 ± 5	20 ± 4
None	—	Yeast RNA	22 ± 4	—

[a] BHK cells cotransfected with the indicated RNAs for 24 hr were stained with crystal violet. The percentage of cells with a rounded morphology was determined by phase contrast microscopy. At least 400 cells were counted in each sample.

[b] Transfected cells were fixed, permeabilized, and labeled with an antibody against the VSV N protein together with a fluorescein-conjugated secondary antibody. The percentage of cells with a rounded morphology was determined by fluorescence microscopy. At least 200 cells were counted in each sample.

with axial ratios < 2 would not be considered elongated, those with axial ratios from 2 to 3 would be moderately elongated, and those with axial ratios > 3 would be considered fully elongated.

The distribution of axial ratios of approximately 90 labeled cells from three separate experiments similar to that in Fig. 2 is shown in Fig. 4C. In the case of control cells expressing the VSV N protein alone, 53% of cells had axial ratios ⩾ 3. In all three experiments, the distribution of axial ratios of cells expressing tsO82 M protein was markedly different, with the highest number of cells having ratios between 1 and 2. Only 27% of cells had axial ratios ⩾ 3. These data indicate that while tsO82 M protein expressed from transfected RNA was not able to induce cell rounding, it did cause BHK cells to have a less elongated shape. It is possible that the morphological change induced by M protein is a multistage process

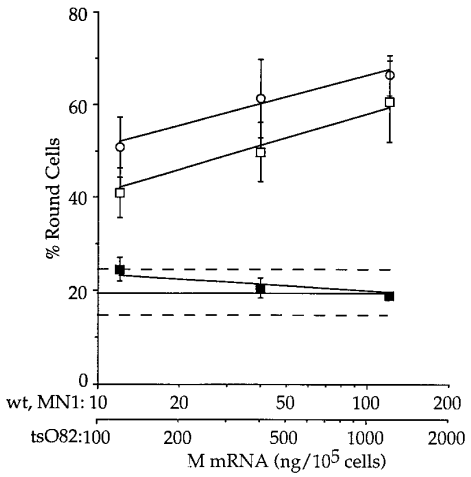


FIG. 3. Potency of wt and mutant M proteins in the induction of cell rounding. BHK cells were transfected with the indicated amounts of mRNA encoding wt M protein (open circles), tsO82 M protein (closed squares), or MN1 protein (open squares), or yeast RNA as a negative control. At 24 hr posttransfection, cells were stained with crystal violet and examined by phase contrast microscopy in a blinded assay to determine the percentage of cells that were round. Data shown are means ± SEM for three independent experiments. Solid and dashed horizontal lines indicate mean ± SEM for the negative controls.

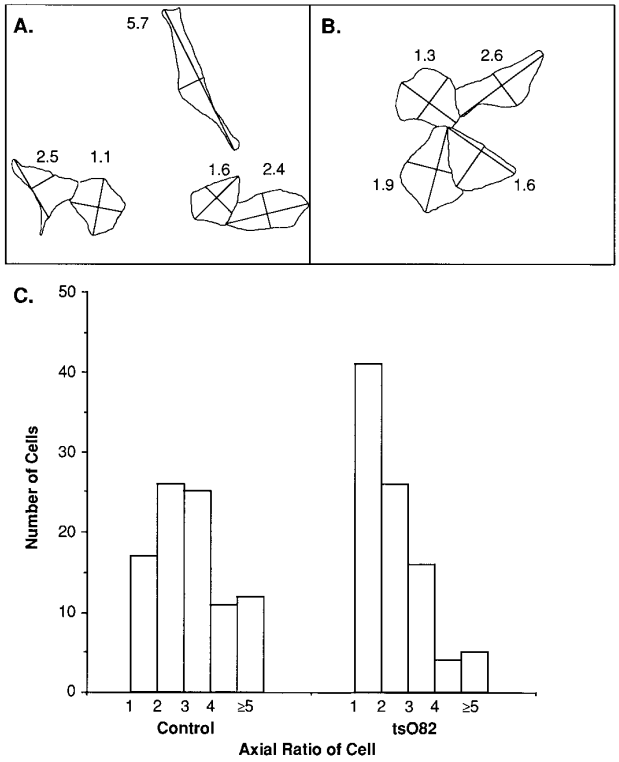


FIG. 4. Changes in cell shape induced by tsO82 M protein. The circumference of the labeled cytoplasm of the cells in Fig. 2B, panels 2 and 3, were traced and are shown in A (control cells) and B (cells transfected with tsO82 M mRNA), respectively. The longest straight line that could be placed entirely within the trace defines the length and a perpendicular line drawn at half the length defines the width of the cells. The "axial ratio" was calculated as the length divided by the width and is shown next to each trace. The distribution of axial ratios of approximately 90 labeled cells from three separate experiments similar to that in Fig. 2 is shown in C. Rounded cells were excluded from this analysis.

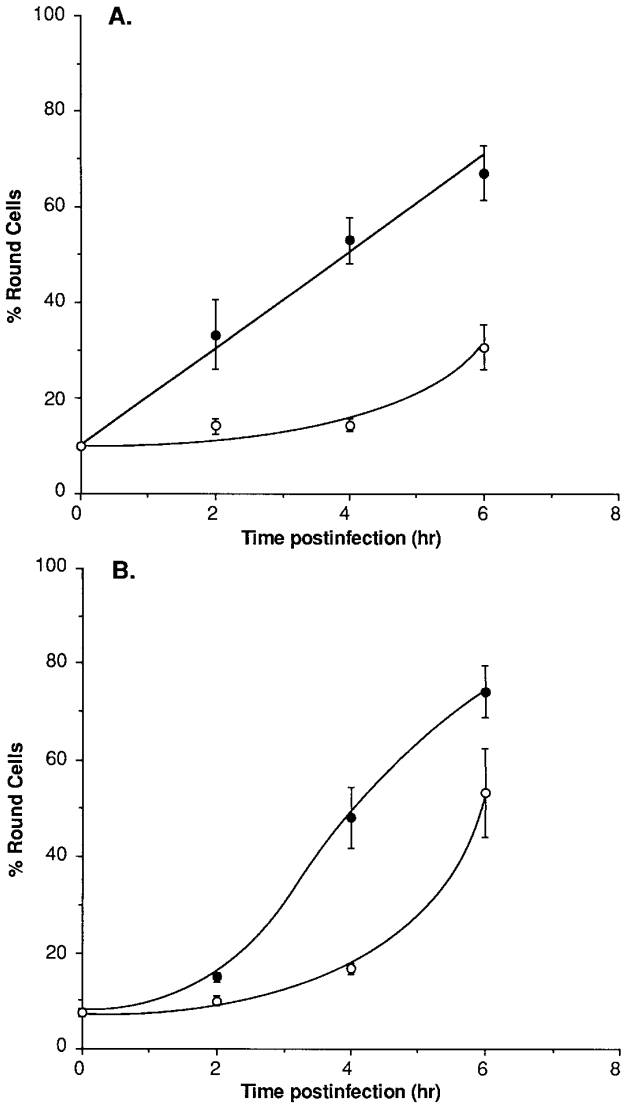


FIG. 5. Cell rounding induced by wt VSV and tsO82 virus. BHK cells (A) or L cells (B) were infected with either wt VSV (closed circles) or tsO82 virus (open circles) at an m.o.i. of 20. Cells were fixed and stained with crystal violet, and the extent of cell rounding was quantitated at 0, 2, 4, and 6 hr postinfection. Data shown are means ± SEM for three independent experiments.

and that the tsO82 M protein could induce some step in the change to occur.

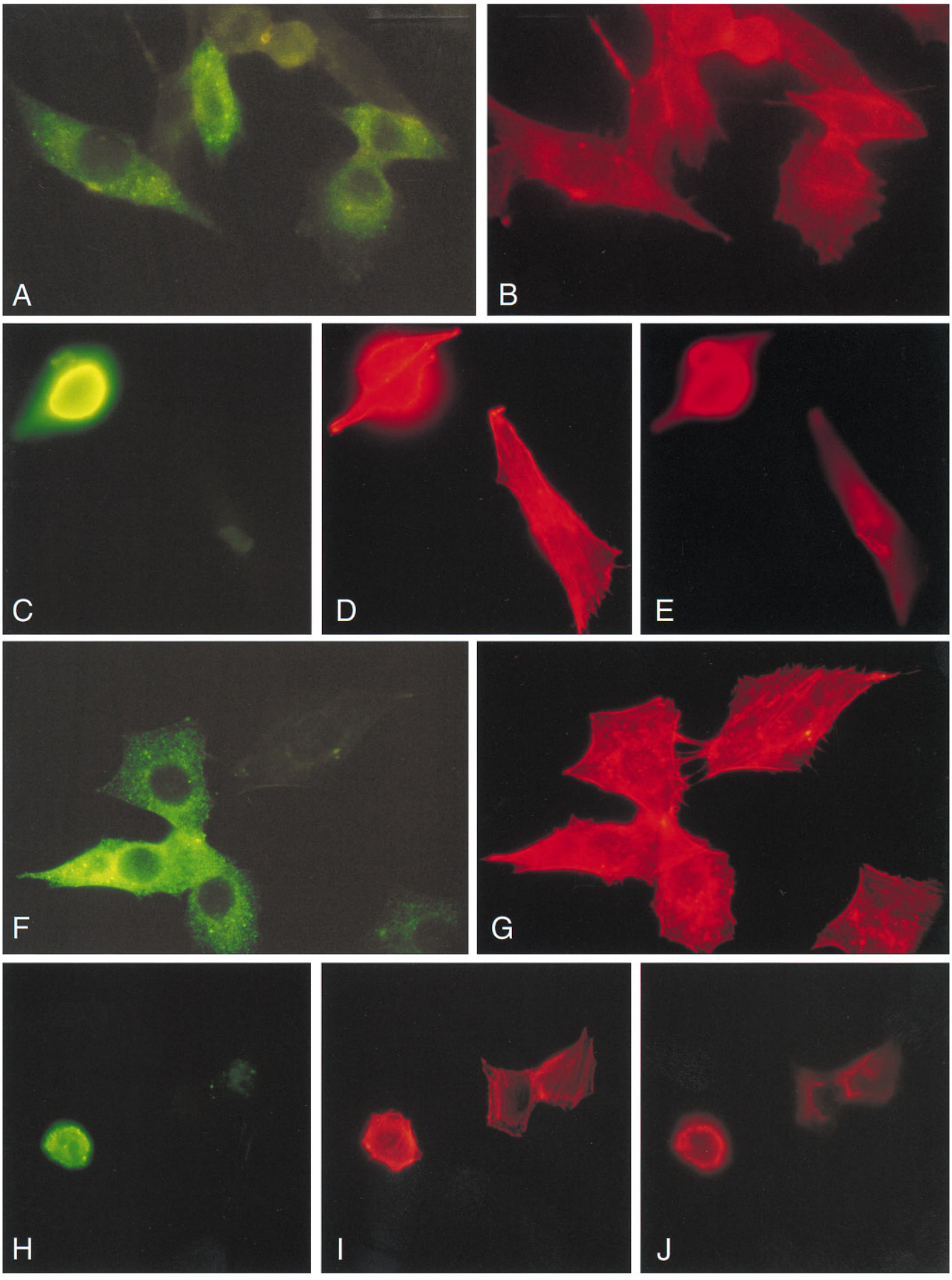
Cell rounding during infection with wt VSV and tsO82 virus

The data in Fig. 4 indicate that tsO82 M protein is not completely defective in its ability to alter cell shape, and suggest that cell rounding might be induced at higher levels of protein expression, such as those achieved during VSV infection. Indeed, tsO82 virus has been reported to induce visible cytopathic effects in some cell types, including BHK cells, but not in others (Coulon *et al.,* 1990). In the experiments shown in Fig. 5A, BHK cells were infected with either wt VSV or tsO82 virus at an m.o.i. of 20, and the extent of cell rounding was quantitated at 2, 4, and 6 hr postinfection to determine whether these two viruses differed in their cell-rounding ability. Similar experiments were conducted with mouse L cells, shown in Fig. 5B, to determine whether these observations would also apply to some other cell types in addition to BHK cells. In the case of BHK or L cells infected with wt VSV (Fig. 5, closed circles), the percentage of cells that were rounded increased steadily beginning about 2 hr postinfection. BHK cells infected with tsO82 virus (Fig. 5A, open circles) showed an approximately 4-hr delay in the onset of cell rounding, so that the percentage of round cells at 6 hr postinfection was approximately the same as at 2 hr postinfection with wt VSV. There was less of a delay in the onset of cell rounding in L cells infected with tsO82 virus (Fig. 5B, open circles), so that the percentage of round cells at 6 hr postinfection was approximately the same as at 4 hr postinfection with wt VSV. These data confirm the observations that tsO82 virus can induce cell rounding (Coulon *et al.,* 1990) and that cells differ in their sensitivity to the cell-rounding effects of tsO82 virus. However, cell rounding induced by this virus is delayed relative to that of wt VSV.

Distribution of cytoskeletal elements in cells transfected with M mRNA

The distribution of actin, vimentin, and tubulin filaments in BHK cells transfected with M mRNA was determined by fluorescence microscopy (Figs. 6, 7, and 8). In the experiment shown in Fig. 6, BHK cells were cotransfected with mRNA for N protein together with mRNA encoding wt, tsO82, or MN1 M proteins or yeast RNA as a negative control. At 24 hr posttransfection, cells expressing N protein were labeled with fluorescein-conjugated antibody to identify transfected cells, and cellular actin-containing filaments were labeled with rhodamine-phalloidin, which binds to polymerized, filamentous actin (F actin; Wulf *et al.,* 1979), but not to monomeric actin (G actin). In negative control cells transfected with N mRNA (Figs. 6A and 6B), the patterns of actin filament distribution were similar to those described by others (Simon *et al.,* 1990), and were not notably different in cells that expressed N protein compared to those that did not. In the focal plane of the base of the cell (Fig. 6B), parallel stress fibers with varying degrees of thickness were observed in most cells, as well as focal points from which several fibers radiated. The cell periphery was often outlined by cortical actin filaments.

Figures 6C–6E show different views of the same field containing a cell that was rounded as a result of transfection with wt M mRNA as well as an apparently normal cell that was not transfected as shown by the absence of fluorescein label. In the focal plane of the base of the cell (Fig. 6D), the untransfected cell contained the normal distribution of stress fibers, focal adhesions, and cortical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actin filaments, while the rounded cell contained only a brightly labeled, thick ring of actin filaments that outlined the base of the cell. This thick ring also conformed to the spherical shape of the rest of the cell, as shown in Fig. 6E, which is focussed on the middle of the cell body. Since rhodamine-phalloidin only labels filamentous actin, the fact that the round cell was intensely labeled implied that the actin filaments had not depolymerized, but instead had changed their organization. Most of the rounded cells expressing wt M protein displayed this distribution of actin filaments, and a few appeared to be in intermediate stages of filament reorganization.

Actin filaments in cells transfected with tsO82 M mRNA (Figs. 6F and 6G) showed the same types of distribution observed in the negative control cells. An occasional cell was observed that did not contain stress fibers, such as the one at the center bottom of Fig. 6G. However, this was also true in the negative control sample. There did not appear to be any correlation between the presence or absence of stress fibers and differences in "axial ratio" as defined in Fig. 4.

The organization of actin filaments in cells transfected with MN1 mRNA was indistinguishable from that of cells transfected with wt M mRNA. The predominant distribution in rounded cells was a thick ring of actin filaments present both at the base of the cell as well as throughout the cell body. Figures 6H–6J were chosen to illustrate the occasional cell that had not undergone complete reorganization of its actin filaments. Such cells were observed among cells transfected with both wt M as well as MN1 mRNA. The cell on the left in Figs. 6H–6J still contained a well-organized system of stress fibers and cortical actin filaments visible when focussed on the cell base (Fig. 6I), similar to that of the two small flat cells on the right side of the figure. However, the cell on the left was completely rounded (except at the base) and contained a thick ring of actin filaments around the cell body (Fig. 6J). The existence of such cells indicates that it was not necessary for a cell to undergo complete disruption of its system of stress fibers at the cell base in order to become round.

Figure 7 shows double-label immunofluorescence analysis of the distribution of N protein (Figs. 7A, 7C, 7E, and 7G) and vimentin (Figs. 7B, 7D, 7F, and 7H) in cells cotransfected with M and N mRNA. The distribution of vimentin-containing intermediate filaments in negative control cells transfected with N mRNA (Figs. 7A and 7B) consisted primarily of a very fine filamentous network that was concentrated around the nucleus and extended into the peripheral areas of the cytoplasm. This is seen most clearly in the flat cell to the right in Fig. 7B. The occasional round cells in the negative control are also seen in the middle of Fig. 7B. In such cases, bright staining throughout the cytoplasm was evident, but a filamentous organization could not be distinguished. Similarly in cells rounded as a result of expression of wt M protein (Figs. 7C and 7D), labeling of vimentin could be seen throughout the cytoplasm. However, since the antibody binds to soluble as well as filamentous vimentin, it could not be distinguished whether this reorganization of intermediate filaments resulted from depolymerization or from rearrangement of filaments. The organization of intermediate filaments in cells expressing tsO82 M protein (Figs. 7E and 7F) was similar to that of the negative control cells, while the distribution of vimentin in cells expressing MN1 protein (Figs. 7G and 7H) resembled that of cells expressing wt M protein.

Immunofluorescent labeling of tubulin in cells transfected with M mRNA is shown in Fig. 8. This was not a double-label experiment, but in parallel samples in which N protein was labeled, it was determined that approximately 70% of the cells were transfected. In negative control BHK cells transfected with N mRNA (Fig. 8A), there was a well-developed system of microtubules that radiated from a perinuclear microtubule organizing center that could be visualized in most cells by adjusting the focal plane. In cells rounded as a result of transfection of wt M mRNA (Fig. 8B), there was little or no organization of tubulin into microtubules, but instead the tubulin was diffusely distributed throughout the cytoplasm, suggesting that it was depolymerized. Although not evident in the cells in Fig. 8B, remnants of the microtubule organizing center, containing a few short microtubules, could occasionally be discerned in rounded cells. Cells transfected with tsO82 M mRNA (Fig. 8C) displayed a system of microtubules similar to those in the negative control cells. In the two cells shown in Fig. 8C, the perinuclear microtubule organizing centers are clearly visible.

FIG. 6. Distribution of actin-containing filaments in BHK cells transfected with mutant and wt M mRNAs. BHK cells were cotransfected with mRNA for N protein together with either yeast RNA as a negative control (A, B) or mRNA encoding wt (C, D, E), tsO82 (F, G), or MN1 (H, I, J) M proteins. At 24 hr posttransfection, cells expressing N protein were labeled with fluorescein-conjugated antibody to identify transfected cells, and cellular actin-containing filaments were labeled with rhodamine-phalloidin. In negative control cells transfected with N mRNA (A, B), the focal plane is at the base of the cell to show parallel stress fibers as well as focal points from which several fibers radiated. The cell periphery was often outlined by cortical actin filaments. The field in C, D, and E was chosen to illustrate a cell that was rounded as a result of transfection with wt M mRNA as well as an apparently normal cell that was not transfected as shown by the absence of fluorescein label. In the focal plane of the base of the cell (D), the rounded cell contained only a brightly labeled, thick ring of actin filaments that outlined the base of the cell. In the focal plane of the middle of the cell body (E), this thick ring can be seen to conform to the spherical shape of the rest of the cell. Cells transfected with tsO82 M mRNA (F, G) are shown in the focal plane of the base of the cells to illustrate the organization of stress fibers and focal adhesions. Most cells transfected with MN1 mRNA were similar to those in C, D, and E. The field in H, I, and J was chosen to illustrate the occasional cell that had not undergone complete reorganization of its actin filaments at the cell base (I) even though the cell body was completely rounded (J).

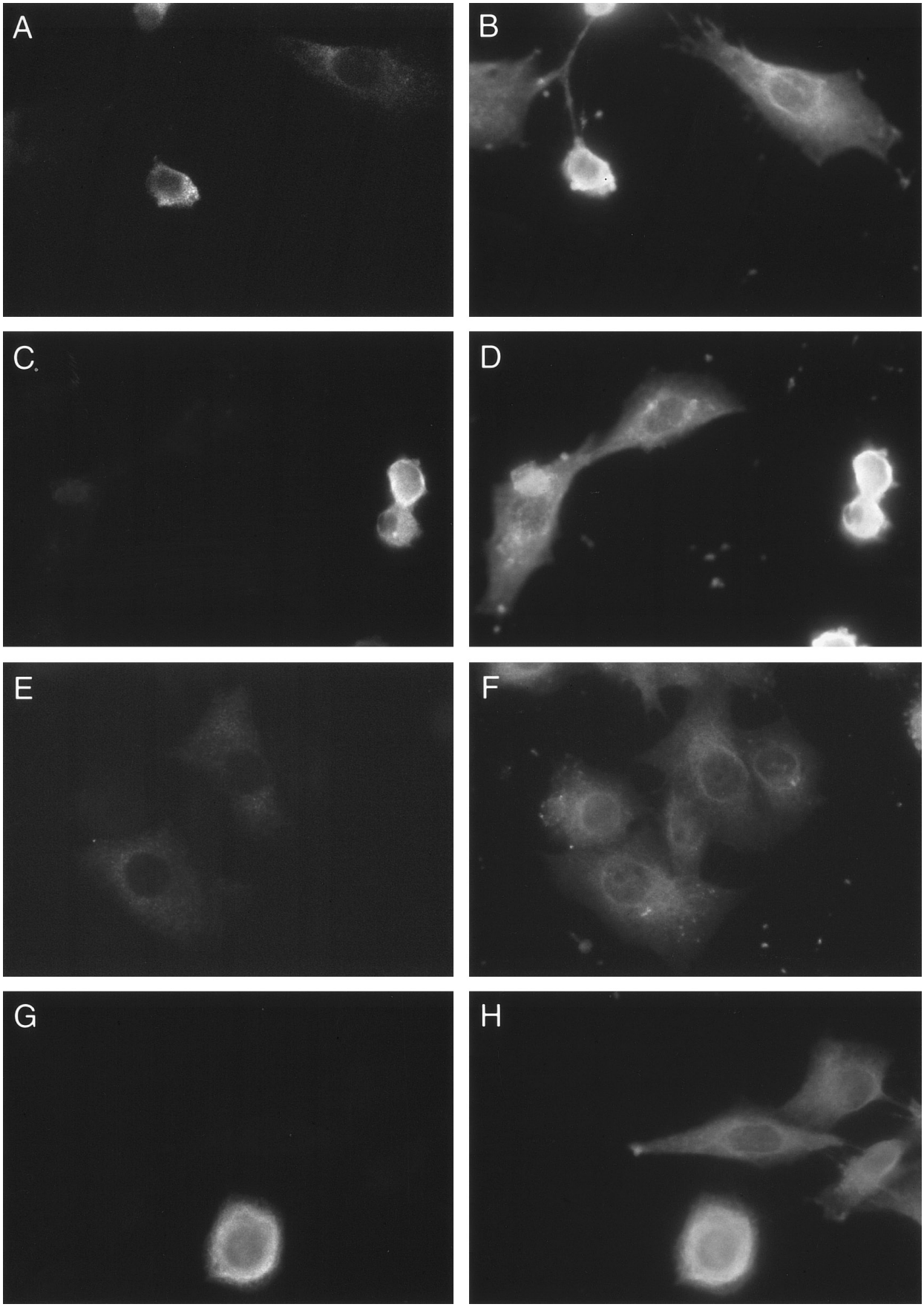

FIG. 7. Distribution of vimentin-containing intermediate filaments in BHK cells transfected with mutant and wt M mRNAs. BHK cells were cotransfected with mRNA for N protein together with either yeast RNA as a negative control (A, B) or mRNA encoding wt (C, D), tsO82 (E, F), or MN1 (G, H) M proteins. At 24 hr posttransfection, cells expressing N protein were labeled with fluorescein-conjugated antibody to identify transfected cells (A, C, E, G), and cellular vimentin-containing filaments were labeled with rhodamine-conjugated antibody (B, D, F, H).

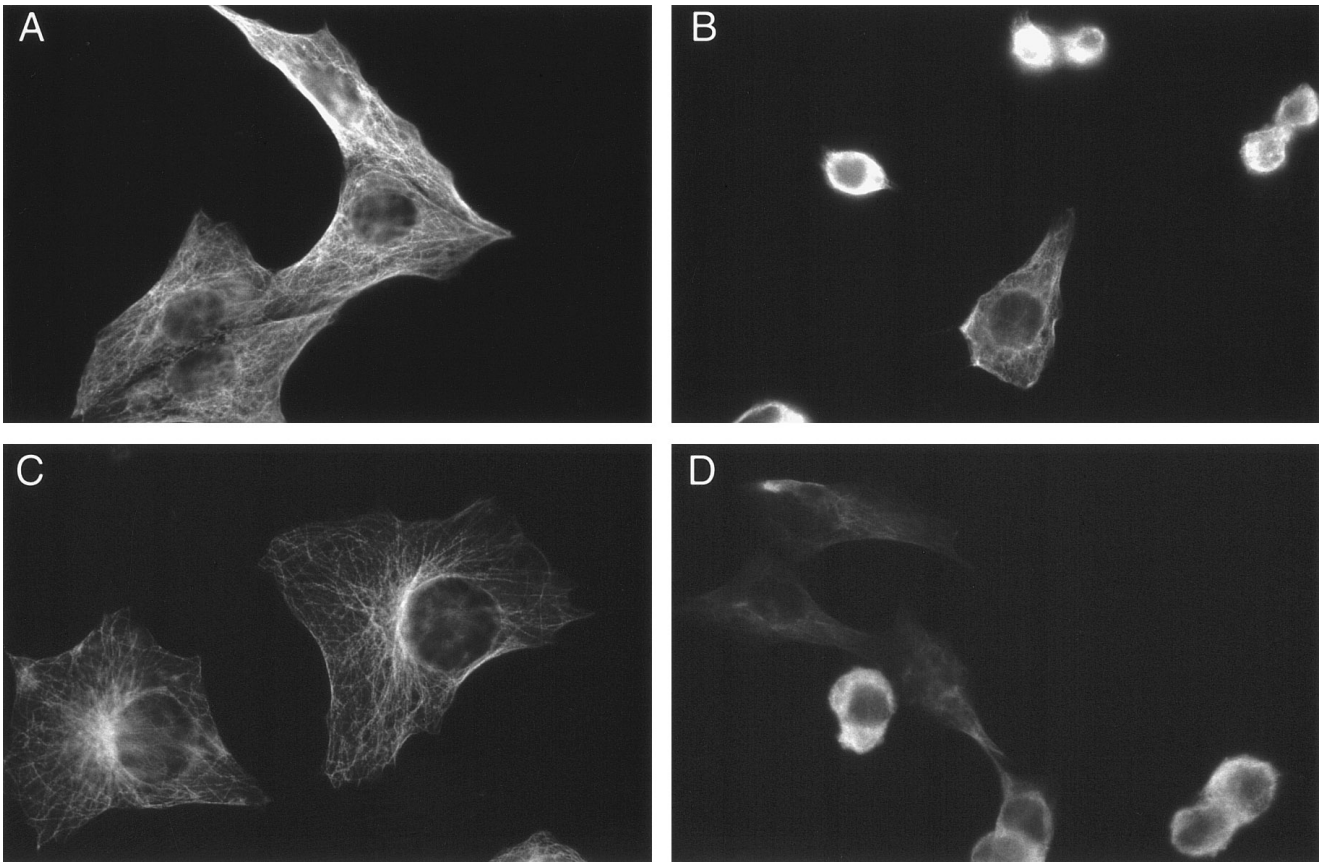

FIG. 8. Distribution of tubulin in BHK cells transfected with mutant and wt M mRNAs. BHK cells were cotransfected with mRNA for N protein together with either yeast RNA as a negative control (A) or mRNA encoding wt (B), tsO82 (C), or MN1 (D) M proteins. At 24 hr posttransfection, cells were labeled with antibody to cellular α-tubulin.

In large, polygonal cells such as those in Fig. 8C, the microtubules often appeared less densely organized than in the more elongated cells such as those in Fig. 8A. This was true regardless of whether such cells were transfected with tsO82 mRNA or with negative control RNA. Since such polygonal cells were more frequent among cells expressing tsO82 M protein, this distribution of microtubules was also more frequently observed than in the negative control cells. In cells rounded as a result of transfection with MN1 mRNA (Fig. 8D), tubulin was diffusely distributed throughout the cytoplasm in a manner that was indistinguishable from that of cells transfected with wt M mRNA.

CONCLUSIONS

The data in Figs. 2 and 3 and Table 1 clearly establish that the ability of M protein to induce cell rounding is genetically separate from its ability to function in virus assembly. The MN1 protein, which is nonfunctional in virus assembly, is nearly as effective as wt M protein in the induction of cell rounding. In contrast, tsO82 M protein was inefficient in the induction of cell rounding, despite its ability to function in virus assembly. The ability of tsO82 M protein to function in virus assembly at all temperatures has been amply documented. It is able to complement the growth of group III (M gene) ts mutants, which are defective in virus assembly, at the nonpermissive temperature (Black *et al.,* 1993; Flamand, 1970). It is also able to complement the assembly of infectious defective interfering particles in an assay in which no other M protein is present, because all of the VSV genes as well as the DI genome are derived from plasmid DNA (Kaptur *et al.,* 1995). Finally, tsO82 virus replicates as efficiently as wt VSV in BHK cells at all temperatures (Coulon *et al.,* 1990, and unpublished results).

Previous experiments have been inconclusive on the question of whether the tsO82 M protein can induce cell rounding. TsO82 virus has been reported to induce visible cytopathic effects in BHK cells but not in chick embryo cells (Coulon *et al.,* 1990). Similarly, another VSV mutant, the T1026R1 virus, containing the same M51R M protein mutation as tsO82 virus, has been reported to cause cell rounding in L cells (Ferran and Lucas-Lenard, 1997) but to be delayed in the induction of cell rounding in BHK cells (Simon *et al.,* 1990), suggesting that different cell types vary in their sensitivity to cell rounding induced by these viruses. This difference in cell-type sensitivity

was directly demonstrated by the data in Fig. 5. Cell rounding in BHK cells induced by tsO82 virus was delayed by approximately 4 hr compared to wt VSV, while the delay in L cells was only about 2 hr. The fact that tsO82 virus can induce cell rounding suggests that the tsO82 M protein retains some cytopathic activity that is apparent only at the high levels of M protein produced during virus infection but not at the lower levels produced following transfection with tsO82 M mRNA. The observation that transfection with tsO82 M mRNA induces subtle changes in cell shape (Fig. 4) would support this idea. Alternatively, other viral factors may be involved in cell rounding in addition to M protein.

In the experiments described here, the ability of mutant M proteins to induce cell rounding was genetically correlated with the ability to inhibit host-directed gene expression, since MN1 protein inhibits host-directed gene expression while tsO82 M protein does not (Black *et al.,* 1993). This conclusion would appear to be inconsistent with previous observations that the group III tsM mutants, which are defective in virus assembly, usually inhibit host gene expression at the nonpermissive temperature, while cell-rounding by some (though not all) of the group III mutants is delayed relative to a wt VSV infection (Blondel *et al.,* 1990; Weck and Wagner, 1979; Ye *et al.,* 1995). This apparent discrepancy could be explained by a difference in the amount of M protein required to induce the two different cytopathic effects. Under conditions similar to those used here, the amount of wt M mRNA required for 50% inhibition of expression of a target gene from cotransfected plasmid DNA is approximately 1 ng/10^5 cells (Lyles *et al.,* 1996), while somewhat higher levels of M mRNA were required to induce cell rounding (around 10 ng/10^5 cells). Thus a likely explanation for the results obtained with group III ts mutants is that at the nonpermissive temperature, enough functional M protein is made to inhibit host gene expression, but not enough to efficiently induce cell rounding. This would imply that even though the two cytopathic effects of M protein, cell rounding, and inhibition of host gene expression, are genetically related to each other, they differ in their quantitative requirements for M protein expression.

A likely explanation for the difference in quantitative requirements is that M protein has at least two cellular targets that are important for cytopathic effects, one that results in inhibition of gene expression and another that inhibits cytoskeletal function. If so, then the ability of M protein to interact with both targets must be prevented by the M51R mutation in the tsO82 M protein. It may be possible to identify other M gene mutations that affect these two targets differentially. This would indicate that these two cytopathic effects induced by M protein can be genetically dissociated from each other as they are dissociable from its function in virus assembly.

One candidate target of M protein action to inhibit cytoskeletal function would be the microtubule subunit tubulin. The idea that disruption of microtubules (and/or intermediate filaments) is the cause of cell rounding was originally suggested by the observation that actin-containing filaments were reorganized within approximately 1 hr postinfection with wt VSV, while microtubules and intermediate filaments were disrupted 3–4 hr postinfection, when cell rounding occurred (Simon *et al.,* 1990). The data presented here support this idea. In cells transfected with wt M or MN1 mRNA, actin-containing filaments were reorganized into a thick ring that conformed to the spherical shape of the cell body, but they were not depolymerized (Fig. 6). Also, occasional cells were observed that retained a well-organized system of stress fibers at the cell base, even though the cell body was completely rounded. In contrast, microtubules appeared to be almost completely depolymerized as shown by a diffuse distribution of tubulin throughout the cytoplasm of rounded cells (Fig. 8). Likewise, vimentin-containing intermediate filaments were retracted from the cell periphery in rounded cells and may have been depolymerized (Fig. 7). These results support the idea that cell rounding induced by M protein results from the depolymerization of microtubules and perhaps intermediate filaments.

M protein is able to inhibit assembly of microtubules *in vitro* (Melki *et al.,* 1994). However, a proteolytic fragment of M protein lacking the N-terminal 19 amino acids, similar to the MN1 protein, does not inhibit tubulin polymerization (Melki *et al.,* 1994), while MN1 protein is nearly as effective as wt M protein in inducing cell rounding. This apparent discrepancy could be due to the fact that MN1 protein retains the three N-terminal amino acids of M protein (Black *et al.,* 1993) or to potential posttranslational modifications of MN1 protein that are not present in the proteolytic fragment analyzed by Melki *et al.* (1994). Thus the question is unresolved as to whether the effect of M protein on microtubules *in vitro* is related to the mechanism of cell rounding *in vivo.*

An alternative explanation for the diverse cytopathic effects attributed to M protein is that they result from the interaction of M protein with a single cellular target rather than interacting directly with cytoskeletal elements or the host transcriptional apparatus. This interaction between M protein and its cellular target could induce a program of cytopathic effects of which cell rounding and transcription inhibition are two manifestations. An example of a single process leading to multiple endpoints would be activation of the pleiotropic effects of apoptosis. In the last several years many different viruses, including VSV (Koyama, 1995; Razvi and Welsh, 1995), have been observed to induce cellular effects that resemble apoptosis (reviewed by Razvi and Welsh, 1995). However, upon detailed examination there are a number of important differences between the effects of VSV M protein and the effects of the apoptotic pathway. In fact, VSV M gene mutants that are defective in the inhibition of host gene

expression, such as the T1026R1 virus, actually induce apoptosis more rapidly than wt VSV (Poliquin and Dunigan, 1996). This result suggests that one of the functions of the inhibition of host gene expression by M protein is to delay the onset of the apoptotic response to prevent premature death of the host cell. However, it is possible that a subset of the cellular pathways involved in apoptosis lead to cellular damage due to M protein. Such "atypical apoptosis" has been invoked recently to explain the effects of the adenovirus E3-11.6K protein that lead to cell lysis (Tollefson *et al.,* 1996). Future studies will test these possibilities.

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