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Vesicular Stomatitis Virus-Infected Cells Fuse when the Intracellular Pool of Functional M Protein is Reduced in the Presence of G Protein

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Five highly cytolytic strains of both Indiana and New Jersey serotypes of vesicular stomatitis virus were shown to induce cell fusion in BHK-21 and R(B77) cells. Inhibition of protein synthesis after the eclipse period of viral replication is a prerequisite for vesicular stomatitis virus-induced cell fusion. Pulse-chase experiments showed that inhibition of protein synthesis would lead to a drastic reduction in the intracellular pool of M protein as compared with other proteins. A temperature-sensitive mutant defective in M protein function (G31) was the only mutant of the five complementation groups to spontaneously induce polykaryocytes at the nonpermissive temperature. Previously, G protein has been shown to play a role in vesicular stomatitis virus-induced cell fusion. These results suggest that the combination of the presence of G protein on the virus-infected cell surface and the absence of functional M protein or a reduced level of intracellular M protein promotes cell fusion. On the basis of this study, we propose that vesicular stomatitis virus infection can induce cell fusion when the functional M protein pool declines to a critical level while G protein remains on the cell surface.

The cytopathic effects induced by vesicular stomatitis virus (VSV) occur within a few hours after infection. These include inhibition of cellular macromolecular synthesis as well as morphological alterations in the host cells (1, 2). The usual result of an in vitro VSV infection is cell rounding, followed by rapid cell destruction (1, 18). In contrast, there have been a number of reports which illustrate that VSV and some of its temperature-sensitive mutants can spontaneously induce the formation of polykaryocytes in specific hosts (4, 8, 10, 19, 25). Takehara reported that VSV (IND) induces marked polykaryocyte formation in two strains of BHK-21 cells but shows the typical cytolytic effect in LLC MK2, L, and HeLa cells (25). VSV (NJ) also induces a high level of polykarycytosis in mouse L and C-243-3 cells and a lower level of polykaryocytosis in BHK-21 cells (19). Temperature-sensitive mutants of VSV (IND), ts052 (II), and tsG31 (III) have been shown to induce cell fusion in rat XC cells (4, 8). The tsG31 (III) mutant also induces polykaryocytes in mouse Ehrlich ascitic tumor cells and murine neuroblastoma cell lines N-18 and N-2A (8, 10). In all these instances it is apparent that the correct combination of virus and host is needed for virus-induced cell fusion. Although it has been suggested that G protein plays a role in VSV-induced cell fusion (7, 8, 10, 22), the mechanism of cell fusion is not understood.

We have observed that cytolytic strains of VSV induce polykaryocytes when viral protein synthesis is inhibited shortly after infection. In this report, five different strains of VSV are shown to induce cell fusion in two cell lines: BHK-21 and R(B77). The requirements for VSV-induced cell fusion are characterized, and an explanation is proposed.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney cell clone 21 (BHK-21) was obtained from the American Type Culture Collection. Cultures of the B77 strain of avian sarcoma virus-transformed rat cells [R(B77)] were originally obtained from Howard Temin, McArdle Laboratory, University of Wisconsin, Madison. R(B77) cells and BHK-21 cells were grown as monolayer cultures in Dulbecco modified minimal Eagle medium (DMEM), supplemented with 5% heat-treated fetal bovine serum. Media and serum were purchased from the Grand Island Biological Co. The VSV strains used in this study have been described previously (6).

Growth of cells in amino acid-deficient media. The standard medium contained Earle balanced salt solution (50 ml), sodium bicarbonate (2.54 g), minimal essential medium vitamins (20 ml), L-glutamine (5 ml), fetal bovine serum (25 ml), and distilled water (355 ml). Normal amino acid concentration was obtained with the addition of 20 ml of a minimal essential medium-amino acid mixture. To prepare amino acid-deficient media, 4, 2, 1, or 0.67 ml of a minimal essential medium-amino acids mixture was added to the standard medium to obtain 1/5, 1/10, 1/20, or 1/30 the normal amino acid concentration, respectively. The amount of distilled water was adjusted to bring the volume to 500 ml.

Twelve hours before infection, media in the cultures were removed and replaced with media containing the appropriate concentration of amino acids. The cells were infected with virus at a multiplicity of infection (MOI) of 1. After adsorption, the cultures were incubated in fresh media containing the appropriate amino acid concentration. Observations were made every 4 h. In other experiments, actinomycin D (Merck Sharp & Dohme) was added to one set of duplicate plates at a concentration of 1 μg/ml 12 h before infection.

Growth of VSV-infected cells in the presence of protein synthesis inhibitors. Puromycin and cycloheximide were purchased from Aldrich Chemical Co. In these experiments, DMEM containing one of the inhibitors at concentrations of 0, 1, 10, 50, 100 μg/ml was added 15 h before infection. Just before infection, the cells were washed once with phosphate-buffered saline to remove any inhibitor not incorporated into

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the cells. The cells then were infected with VSV at an MOI of 1. Fresh DMEM free of inhibitors was added to the cultures after the adsorption period. Photographs were taken at 12 and 24 h.

Growth of VSV-infected cells treated with cycloheximide before or after infection. The medium from confluent cultures of BHK-21 cell was replaced with DMEM containing 100 μg of cycloheximide per ml at 1 or 2 h before infection or at 1, 2, 3, 4, or 5 h after infection. Observations were made every 4 h after infection. In this experiment, the infected cells were incubated in the continued presence of cycloheximide.

Pulse-chase of virus-specific proteins. Cells were pretreated with 2 μg of actinomycin D per ml in DMEM for 18 h and then infected with VSV at an MOI of 1. After adsorption, the infected cultures were overlaid with methionine-free Eagle modified minimal essential media (Flow Laboratories, Inc.) and incubated for 1 h. The viral proteins were labeled for 3 h in the presence of 3.5 μCi of [^{35}S]methionine (Amersham Corp.) per ml. Then the cells were washed, and the label was chased with medium containing an excess of unlabeled methionine. One set of infected cultures were harvested immediately and designated as time equals 0 h. At 0, 1, 2, and 3 h of the chase, proteins in the infected cultures and extracellular virion-associated proteins were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Analysis of viral proteins by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis of the viral proteins was carried out in a 15% acrylamide–0.09% bisacrylamide separating gel with a 3% acrylamide–0.09% bisacrylamide stacking gel. The discontinuous buffer system has been previously described (14). After electrophoresis, the proteins were transferred onto nitrocellulose paper by electroblotting (3) and exposed to X-Omat AR X-ray film (Kodak) at −70°C.

RESULTS

Cytopathic effects caused by VSV infection in various cell lines. VSV infections of most cell lines produce a rapid cytolytic response, which includes rounding and degeneration of the infected cells. The cytopathic effects caused by VSV are, to some extent, a reflection of the infected host. For example, L cells, BHK-21 cells, and R(B77) cells show the typical cytolytic effect when infected with VSV (Fig. 1). In contrast, RAT-1 cells form polykaryocytes as a result of VSV infection, and chicken embryo fibroblasts form vacuolar ghosts (Fig. 1). The R(B77) cells are avian sarcoma virus-transformed Rat-1 cells, yet the R(B77) and Rat-1 cell lines differ in their response to VSV infection. It was for this reason that R(B77) cells were selected as one of the cell lines for this study. BHK-21 cells were used for this study because VSV infection leads to rapid rounding and degeneration.

Effect of amino acid deprivation on VSV-induced cytopathic effects. Preliminary observations suggested that inhibition of protein synthesis by deprivation of amino acids induced polykaryocytes (data not shown). We examined the possibility that inhibition of protein synthesis altered the cytopathic effect causd by VSV. BHK-21 and R(B77) cells were grown, before and after infection, in normal medium or medium containing 1/5, 1/10, 1/20, or 1/30 the normal amino acid concentrations. VSV (IND-HR) infection in cells grown at the normal concentration of amino acids showed typical rounding and degeneration (Fig. 2). However, cells grown in 1/5 to 1/30 the normal amino acid concentrations formed polykaryocytes as a result of VSV infection (Fig. 2). The size and number of polykaryocytes formed varied according to the concentration of amino acids. At 1/5 the normal amino acids concentration, 30 to 40% of the monolayer was involved in polykaryocytes (data not shown). At 1/10 and 1/20 the normal concentration, 50 to 70% of the cells fused (Fig. 2), and at 1/30 the normal concentration, 80 to 90% of the cells were involved in polykaryocytosis (data not shown).

We have repeated this study with another serotype and different strains of VSV to show that cell fusion is not a unique cytopathic effect of VSV (IND-HR). We chose four other prototypes of VSV (New Jersey [Concan], Indiana [HR, New Mexico, and Glasgow]) to repeat the amino acid deprivation experiment. All strains tested induced cell fusion when either BHK-21 or R(B77) cells were grown before and after infection in medium containing 1/20 the normal amino acid concentration (data not shown). Although there was variation in the amount of cell fusion observed, all strains induced cell fusion. This indicates that VSV-induced cell fusion is not strain dependent.

To determine whether defective interfering particles in the stock cultures are responsible for cell fusion, we repeated the experiment with low-MOI progeny of VSV (IND-HR) (i.e., virus containing no detectable level of defective interfering particles). The progeny strain induced cell fusion in cells grown in amino acid-deficient media at the same level as that induced by the parent (data not shown). This suggests that defective interfering particles were not involved in VSV-induced cell fusion.

We examined the effect of inhibition of host protein synthesis on VSV-induced cell fusion by actinomycin D treatment of infected cells during amino acid deprivation. Actinomycin D did not affect the formation of VSV-induced polykaryocytes when the infected cells were grown in medium containing 1/20 the normal amino acid concentration (i.e., polykaryocytosis occurred). VSV-induced polykaryocytes did not form when cells were pretreated with actinomycin D in the presence of the normal concentration of amino acids. It is important to note that neither BHK-21 nor R(B77) cells spontaneously fused when they were grown in the presence of amino acid-deficient media and were not infected.

These results indicate that VSV-infected BHK-21 and R(B77) cells form polykaryocytes as a result of an inadequate concentration of amino acids. It also is apparent (Fig. 2) that the two cell lines differed in their response to amino acid deprivation. The VSV-induced polykaryocytes in R(B77) cells were smaller and fewer than those in BHK-21 cells.

Induction of polykaryocytes by VSV as a result of inhibition of protein synthesis. To determine whether inhibition of protein synthesis would promote VSV-induced polykaryocytosis, we treated cells with either cycloheximide or puromycin. When BHK-21 cells were treated with as little as 1 μg of either cycloheximide (Fig. 3 and Table 1) or puromycin (Table 1) per ml, VSV infection induced polykaryocytosis. R(B77) cells treated with either inhibitor also fused, but a higher concentration of inhibitor was required for VSV to fuse these cells. It is important to note that pretreatment with either puromycin or cycloheximide did not induce fusion of uninfected cells, which suggests that newly synthesized viral specific proteins are necessary. To determine the level of protein synthesis at each concentration of inhibitor, duplicate cultures were labeled with a mixture of ^{3}H-amino acids at the time of infection, and the amount of label incorporated at 12 h after infection was determined. A comparison of the level of protein synthesis

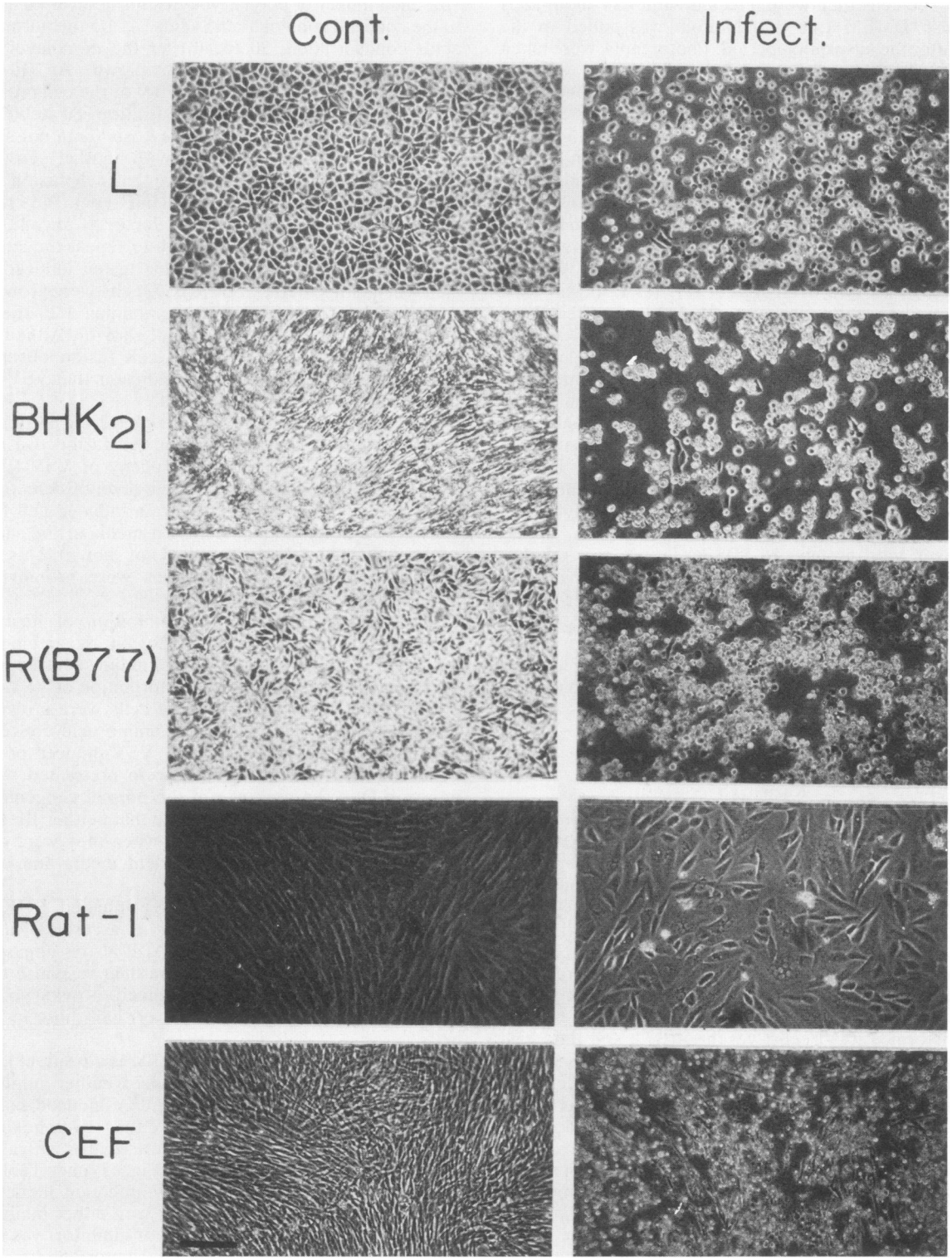

FIG. 1. Cytopathic effects caused by VSV in various cell lines. Cells were infected at an MOI of 1. The bar represents 0.5 μm. Note the polykaryocyte formation in VSV-infected Rat-1 cells and the lack of polykaryocyte formation in infected BHK-21 and R(B77) cells.

in BHK-21 and R(B77) cells shows that as much as 90% of protein synthesis was inhibited by 100 μg of cycloheximide or puromycin per ml (Table 1). However, R(B77) and BHK-21 cells responded differently to the two inhibitors, in that a greater inhibition of protein synthesis was observed in BHK-21 cells than in R(B77) cells. The level of inhibition of protein synthesis at which VSV induced polykaryocytosis also varied. In BHK-21 cells, VSV induced cell fusion when protein synthesis was inhibited by as little as 38% (Table 1; 1 μg of puromycin per ml). In contrast, VSV-induced

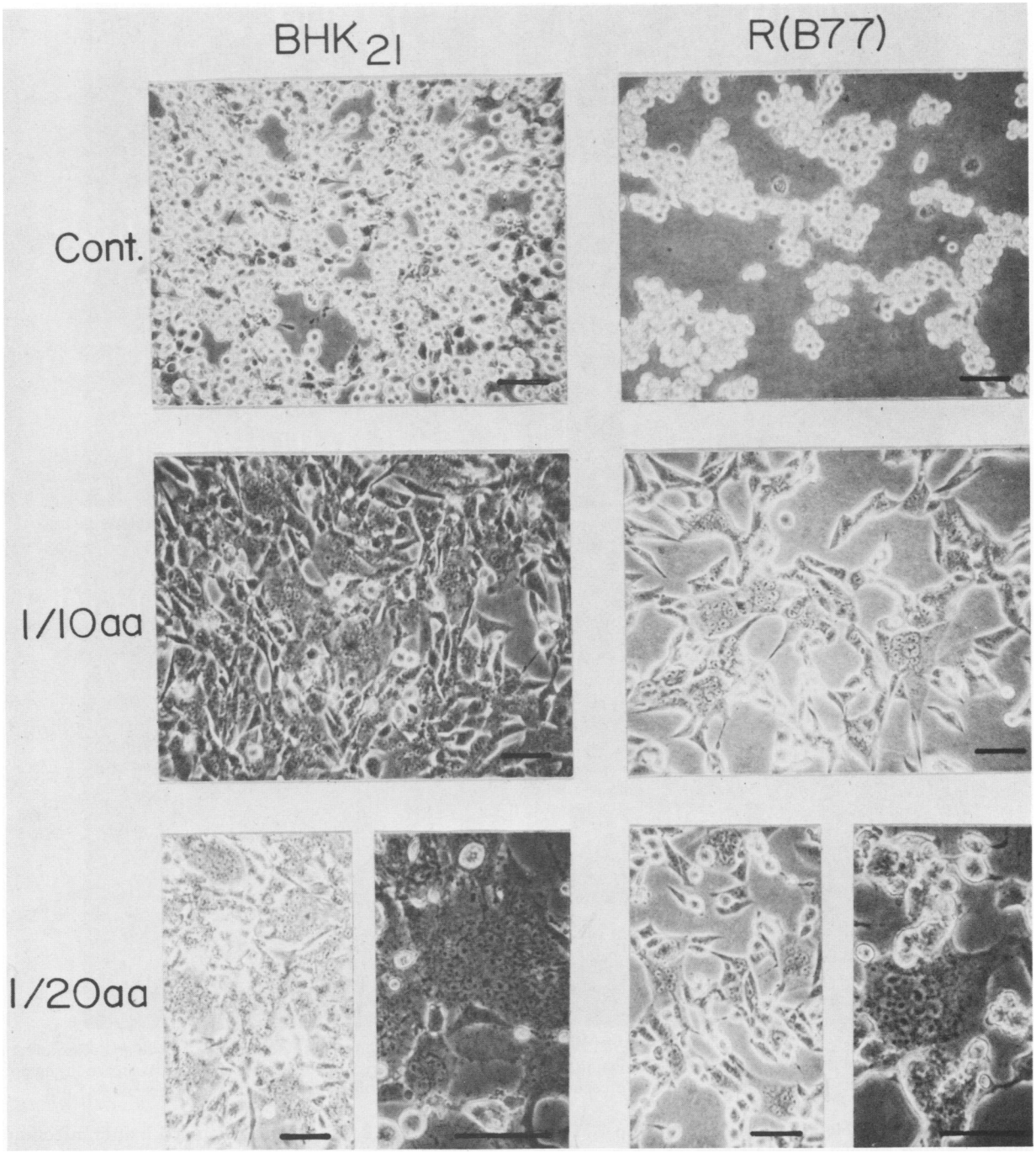

FIG. 2. Cytopathic effects caused by VSV when infected host cells are grown in normal DMEM or DMEM containing 1/10 or 1/20 the normal amino acid concentration. The bar represents 0.5 μm. Second micrograph in the panels containing micrographs of cells grown in 1/20 amino acid concentration are higher magnifications showing large polykaryocytes.

polykaryocytosis occurred in R(B77) cells only when protein synthesis was inhibited by greater than 60% (Table 1; 50 μg of cycloheximide per ml, 100 μg of puromycin per ml). An interesting similarity between the two cell lines is that VSV infection resulted in the formation of polykaryocytes when protein synthesis was inhibited by 90% (Table 1), indicating that a high level of protein synthesis was not required for fusion. Moreover, as the concentration of inhibitor increased, the size and number of polykaryocytes increased (Fig. 3). These results indicate that VSV-induced polykaryocytosis is dependent on the level of protein synthesis and that the host cell plays a minor role in fusion.

Effect of total inhibition of protein synthesis before or after infection. Although inhibition of viral protein synthesis has been shown to be an important prerequisite for the induction of cell fusion by VSV infection (Fig. 3 and Table 1), we wanted to determine whether the inhibition of the host protein synthesis must occur before, during, or after adsorp-

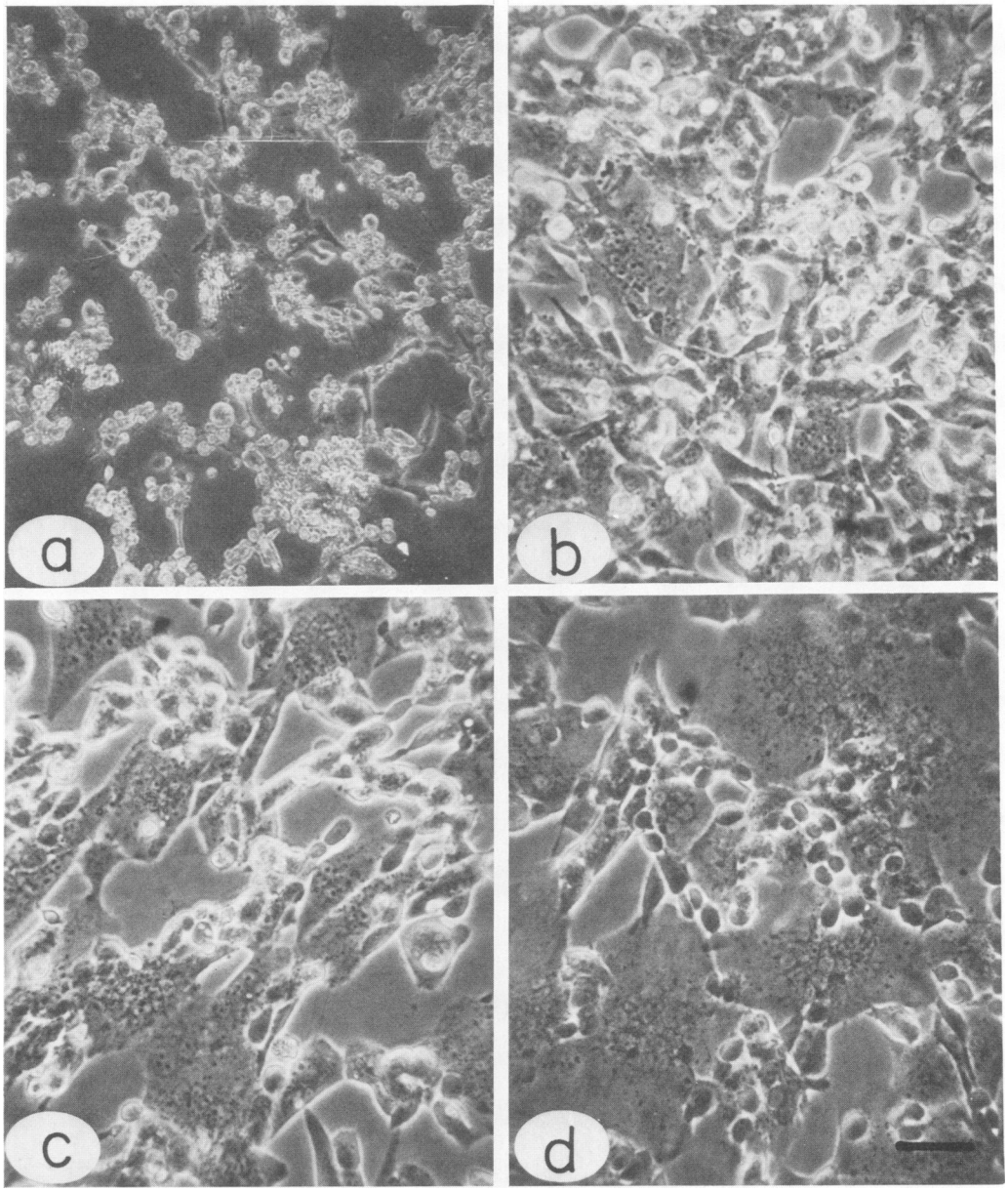

FIG. 3. Cytopathic effects caused by VSV when BHK-21 cells are grown in the presence of cycloheximide before and after infection. a, Cells grown in the absence of cycloheximide; b, cells grown in the presence of 1 μg of cycloheximide per ml; c, cells grown in the presence of 10 μg of cycloheximide per ml; d, cells grown in the presence of 100 μg of cycloheximide per ml. The bar represents 0.5 μm.

tion. To explore this problem, cells were grown in DMEM containing 1/20 the normal concentration of amino acids. After infection, medium was replaced with DMEM containing a normal concentration of amino acids. As a comparison, cells also were grown in DMEM with normal levels of amino acids before infection; this medium was replaced with DMEM containing 1/20 the normal concentration of amino acids after infection. VSV-induced cell fusion occurred only in the cells that were starved during the infection. This result suggests that protein synthesis inhibition after the initiation of infection was the important prerequisite for VSV-induced cell fusion. To determine the crucial time period, induction of polykaryocytes after VSV infection was examined when 100 μg of cycloheximide per ml was added at various intervals before and after infection (Fig. 4). Only minor cytopathic effects were observed in cells treated with cycloheximide 1 or 2 h before infection or 1 h after infection (Fig. 4). When cycloheximide was added at 2 to 5 h after infection, the majority of the monolayers were involved in polykaryocytes (Fig. 4). This result suggests that early protein synthesis is required for the infection to progress. It also indicates that when protein synthesis is inhibited early in infection, the cells will be predisposed to form polykaryocytes.

Intracellular synthesis and extracellular appearance of virus-specific proteins. It is clear from the data presented above that inhibition of protein synthesis early in infection is a requirement for VSV-induced cell fusion. To determine whether this requirement could be traced to particular proteins, we studied the rates of synthesis and transport of the viral-specific proteins in a pulse-chase experiment. VSV-infected BHK-21 cells were grown at 37°C for 1 h and labeled with [^{35}S]methionine for 3 h, and the radioactivity

TABLE 1. Inhibition of protein synthesis and its effect on cytopathogenic changes of VSV-infected cells

Cell lines	Inhibitor	% of ^{3}H-amino acid incorporation of untreated cultures[a] (cytopathic effect) at the following concn of inhibitor (μg/ml)				
		0	1	10	50	100
BHK-21	Puromycin	100 (R + D)	62 (PK)	ND	ND	10 (PK)
R(B77)	Puromycin	100 (R + D)	71 (R + D)	ND	ND	10 (PK)
BHK-21	Cycloheximide	100 (R + D)	38 (PK)	18 (PK)	12 (PK)	9 (PK)
R(B77)	Cycloheximide	100 (R + D)	71 (R + D)	40 (R + D)	25 (PK)	18 (PK)

[a] Calculated by dividing total counts found in the treated sample by total counts found in the untreated samples.
[b] R + D, Rounding and degeneration; PK, polykaryocyte formation; ND, not determined.

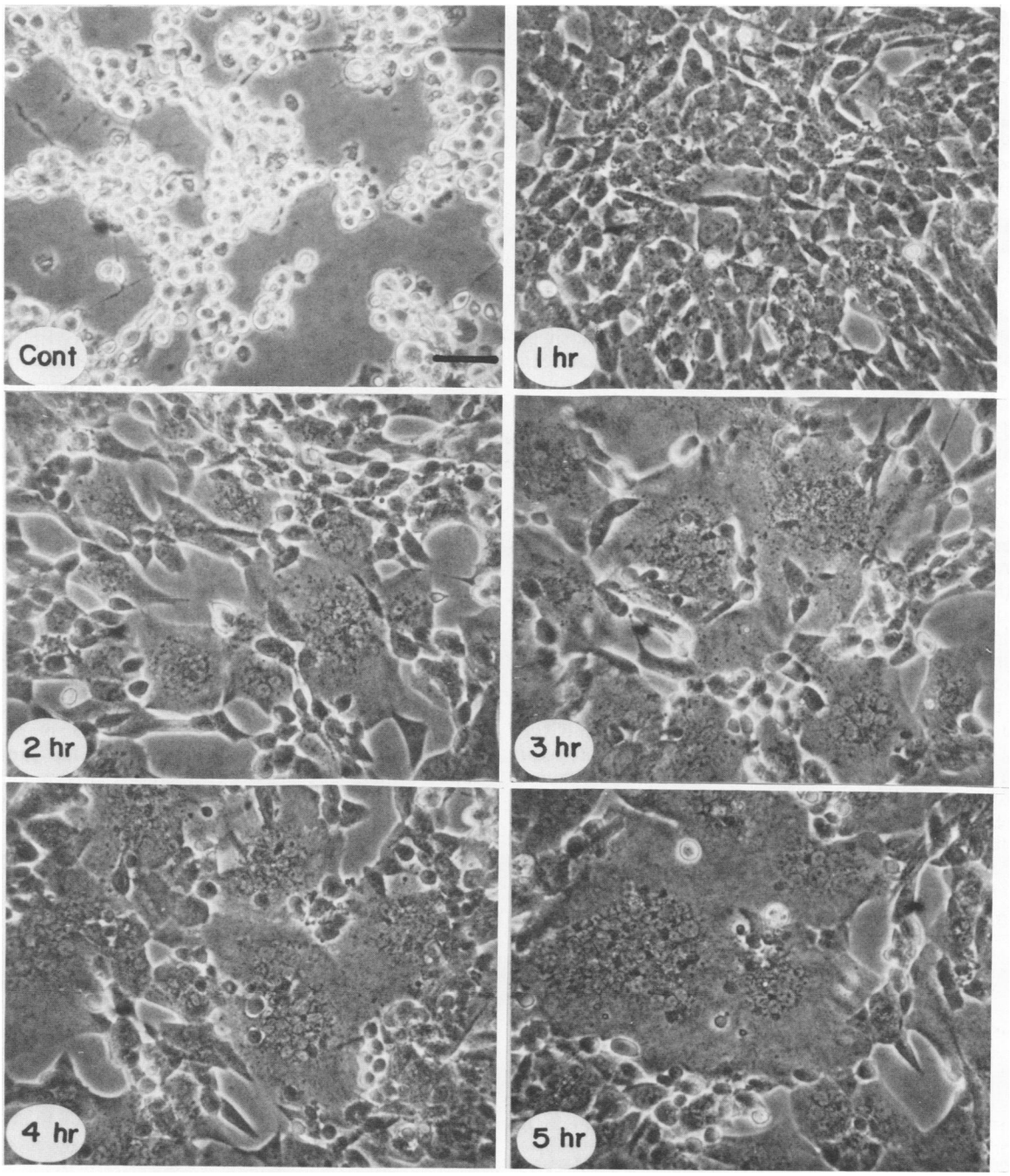

FIG. 4. VSV-mediated cytopathic effect when cycloheximide is added at various times before and after infection. All micrographs were taken at 12 h after infection. The control culture (Cont) did not receive cycloheximide. The cytopathic effect of VSV on BHK-21 cells that received cycloheximide at 1 and 2 h before infection is identical to that shown for the culture that received cycloheximide at 1 h after infection (1 hr). Note the sequential increase in the size of the polykaryocytes in the cultures that received cycloheximide at 2 h after infection (2 hr), 3 h after infection (3 hr), 4 h after infection (4 hr), and 5 h after infection (5 hr).

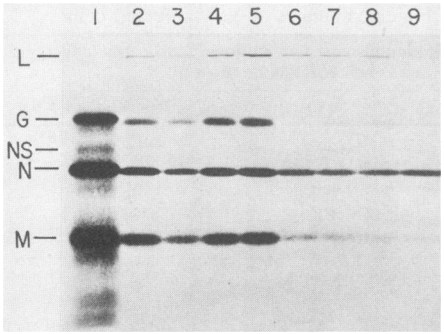

FIG. 5. SDS-polyacrylamide gel electrophoresis of pulse-labeled virion-associated and intracellular proteins of VSV (IND-HR). Cells were pretreated for 18 h with actinomycin D and infected at an MOI of 1. At 1 h after the adsorption, the cells were labeled with [^{35}S]methionine for 3 h and washed, and the radioactivity was chased with nonradioactive methionine. Lanes: 1, standard VSV (IND-HR) proteins; 2, virion-associated proteins immediately after the pulse; 3, virion-associated proteins 1 h after the pulse; 4, virion-associated proteins 2 h after the pulse; 5, virion-associated proteins 3 h after the pulse; 6, intracellular proteins immediately after the pulse; 7, intracellular proteins 1 h after the pulse; 8, intracellular proteins 2 h after the pulse; 9, intracellular proteins 3 h after the pulse.

was chased with cold methionine for 3 h. Samples were taken after 0, 1, 2, and 3 h of chase. The intracellular polypeptides at time equals 0, 1, 2, and 3 h of chase are seen in Fig. 5 (lanes 6 to 9). To quantitate the amount of protein represented by each band, the autoradiograph of the gel was scanned with a Beckman DU-8B spectrophotometer by the gel-scanning system. Knowing the amount of each protein present in the cell at each time period after the pulse, the percentage of the total viral proteins for each protein was calculated (Table 2). After the pulse, N protein was the most prominent protein, accounting for 71% of the total intracellular viral protein (Fig. 5, lane 6, and Table 2). During the chase, the amount of intracellular N protein remained relatively constant, but its percentage of the total increased to 87%. The percentage of G and NS proteins remained constant during the chase period at ca. 3 and 1%, respectively. The percentages of the total protein dropped for two proteins (L and M proteins) during the chase, accounting for the increase in the percentage of N protein. However, all of the drop in total percentage of L protein was accounted for between 2 and 3 h of the chase. In contrast, the percentage of M protein dropped continually during the chase period. Moreover, the percentage drop of M protein was much greater than that of L protein. M protein accounted for 19% of the total immediately after the chase and dropped to 8% of the total after 3 h of chase (Table 2).These results suggest that over the chase period, M protein disappears more rapidly from the infected cells than do the other proteins.

The virion-associated proteins at times of 0, 1, 2, and 3 h of chase are shown in Fig. 5 (lanes 2 to 5). Immediately after the pulse, all the structural proteins were present (Fig. 5, lane 2); however, M protein accounted for the highest percentage (45%) of the total virion-associated proteins (Table 2). During the case, the percentage of M protein dropped slightly from 45 to 39% (Table 2). This indicates that the relative amount of M protein remained approximately the same during the chase period, whereas the amounts of the other proteins increased. The combination of a rapid disappearance of M protein intracellularly and the rapid appearance of M protein in the virion-associated proteins suggest that it is the protein most rapidly transported from the cell into virions, as shown previously (12). Consequently, inhibition of protein synthesis early in infection would most severely affect the intracellular pool of M protein as compared with the other protein pools. This leads to the suggestion that reduction of the intracellular pool of M protein may be a requirement for VSV-induced polykaryocytosis.

Formation of polykaryocytes by temperature-sensitive mutants of VSV at the nonpermissive temperature. To examine the putative role of M protein in VSV-induced cell fusion, temperature-sensitive mutants from each complementation group were tested for their ability to produce polykaryocytes. We observed that at the nonpermissive temperature only tsG31 (III) induced cell fusion and that even at the permissive temperature tsG31 (III) induced a low level of polykaryocyte formation (Table 3). Mutant tsG31 (III) previously was shown to form polykaryocytes in a number of other cell lines (4, 7, 9). It also was shown that the temperature-sensitive mutation in tsG31 (III) is in the M protein gene (11, 13, 15). We analyzed intracellular and virion-associated proteins of tsG31 (III) produced at permissive (31°C) and nonpermissive (40°C) temperatures. N, NS, G, and M proteins from tsG31 (III) had slightly different electrophoretic mobilities as compared with the proteins from the wild-type VSV (IND-HR) strain (Fig. 6). G and N proteins of tsG31 (III) moved faster, NS protein moved slightly slower, and M protein moved much slower than their counterpart VSV (IND-HR) proteins (cf. Fig. 6, lanes 1 and 6). It is obvious that less intracellular viral-specific proteins are made at the nonpermissive temperature (Fig. 6, lane 3) than at the permissive temperature (Fig. 6, lane 4). It is of interest

TABLE 2. Percentage of the viral-specific proteins[a]

Viral proteins	Virion-associated proteins at following h of chase:				Intracellular viral-specific proteins at following h of chase:			
	0	1	2	3	0	1	2	3
L	1.5	1.4	2.3	4.2	6.2	7.2	8.4	0.2
G	11.6	11.1	18.0	18.2	3.1	5.1	7.7	3.3
NS	0.4	0.4	0.5	0.9	0.7	4.0	2.6	1.1
N	41.1	46.3	40.4	37.2	71.3	65.0	69.5	86.8
M	45.4	40.8	38.8	39.5	18.7	18.7	11.8	8.6

[a] The autoradiograph from Fig. 5 was scanned with the gel-scanning system of a Beckman DU-8B spectrophotometer. The area of each viral-specific peak was calculated, and this was used to determine the percentage of total viral-specific protein in each peak.

TABLE 3. Cytopathic effects caused in BHK-21 cells by temperature-sensitive mutants of VSV at permissive and nonpermissive temperatures

VSV mutants	Complementation group	Cytopathic effect at:			
		31°C (permissive)		40°C (nonpermissive)	
		12 h PI	24 h PI	12 h PI	24 h PI
G114	I	50% R	R + D	NC	NC
G23	II	50% R	R + D	NC	NC
G31	III	50% R	5% PK	30% PK	70% PK
G44	IV	50% R	R + D	NC	NC
O45	V	80% R	R + D	NC	NC
IND-HR	WT[b]	70% R	R + D	80% R	R + D

[a] PI, postinfection; R + D, rounding and degeneration; NC, no cytopathic effects; PK, polykaryocyte formation.
[b] WT, Wild type.

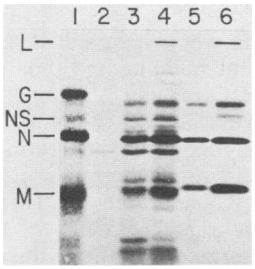

FIG. 6. Intracellular and virion-associated proteins of tsG31 (III). Cells were pretreated with actinomycin D and infected at an MOI of 1. Then the infected cultures were incubated at 31°C for 3 h. After this incubation time, the cultures were split into two groups; one was incubated at 40°C and the others were incubated at 31°C. The cells were incubated for 1 h after the split. The infected cells then were labeled with 3.5 μCi of [^{35}S]methionine (Amersham Corp.) per ml and incubated for 4 h at 31 or 40°C. At this time, the cultures were harvested and fractionated into viral or intracellular components, and the proteins were subjected to SDS-polyacrylamide gel electrophoresis. Lanes: 1, VSV (IND-HR) standard proteins; 2, mock-infected BHK-21 cells; 3, intracellular tsG31 (III)-specific proteins generated at nonpermissive temperature; 4, intracellular tsG31 (III)-specific proteins generated at permissive temperature; 5, virion-associated proteins of tsG31 (III) produced at nonpermissive temperature; 6, virion-associated proteins of tsG31 (III) produced at permissive temperature.

that the percentages of each of the proteins remained constant between the permissive and nonpermissive temperatures, except for the L protein which dropped from 5 to 1% of the total viral protein (Table 4). In contrast, in the virion-associated proteins (Fig. 6, lanes 5 and 6) M protein accounted for 40% of the total viral protein at the permissive temperature but dropped to 33% of the total at the nonpermissive temperature (Table 4). These results suggest that synthesis of all the viral-specific proteins of tsG31 (III) is inhibited at the nonpermissive temperature. More importantly, the results indicate that transport of M protein from the cell to the virions was specifically affected at the nonpermissive temperature. The implication of these results is that when nonfunctional M protein is present intracellularly and normal concentrations of the other viral proteins are produced in infected cells, the cells may be predisposed toward cell fusion.

DISCUSSION

In this report, we have shown that highly cytolytic strains of VSV infection induced polykaryocytes when the pattern of viral protein synthesis was modified after infection. This observation is different from the VSV-induced polykarycytosis reported previously (4, 8, 10, 19, 25) in a number of ways. Nevertheless, it seems unlikely that there would be more than one mechanism of VSV-induced cell fusion.

It has been observed that VSV-induced cell fusion is strictly dependent on the strain of VSV and the host involved (4, 8). However, we show that when protein synthesis is modified, cell fusion is not dependent on the strain of VSV used, suggesting that VSV has the ability to fuse cells when certain conditions are met. Our results also suggest that the host cell plays a role in virus-induced cell fusion, since there are differences between R(B77) and BHK-21 cells in their tendency to form polykaryocytes (Fig. 3 and Table 1). Previous studies of VSV-mediated cell fusion also show that host-associated factors are involved, with susceptibility of the host membrane to fusion being the most important feature (20, 21). We would like to emphasize the fact that differences of VSV-induced cell fusion between BHK-21 cells and R(B77) cells, reported in this study, are minor compared with the all-or-none response reported previously (4, 8, 19).

We have performed a series of experiments to determine when, and by how much, inhibition of protein synthesis is necessary as a requirement for VSV-induced cell fusion. Since we found that inhibition of protein synthesis by 75% is sufficient for both BHK-21 and R(B77) cells to fuse as a result of VSV infection (Fig. 3 and Table 1) and since a reduction of host protein synthesis alone, by actinomycin D pretreatment, did not result in VSV-induced cell fusion, we conclude that host proteins do not play a major role in the fusion potential of these cell lines.

We have shown that total inhibition of protein synthesis before or during the eclipse period of viral replication prevented polykaryocyte formation (Fig. 4). This finding is consistent with data published by Nishiyama et al. (19), who found that cycloheximide completely suppressed cell fusion when the inhibitor was added at 1 h postinfection but that fusion was not suppressed when the drug was added at 3 h postinfection. It also has been shown in this paper and by others (4, 10) that when cycloheximide is added immediately after infection and maintained during infection, viral replication will be blocked. We and others (4, 10, 19, 25) have shown clearly that inhibition of viral-specific protein synthesis after the eclipse period of virus replication promotes cell fusion (Fig. 3 and 4).

There have been a number of reports on the pH dependence of VSV-induced cell fusion (7, 22, 27). It was found that VSV-induced cell fusion had a sharp pH threshold at ca. 6 (27). We believe that there was little pH dependence on the VSV-induced cell fusion we are observing because our experiments were conducted in media of neutral pH. In these types of experiments, however, one cannot completely rule out a pH dependence because amino acid starvation or the addition of a inhibitor for protein synthesis may have a subtle effect on the pH of the medium or the cell surface.

Glycoproteins of a number of enveloped viruses have been implicated as the fusion proteins, specifically F protein of Sendai (5, 9, 22–24) and B_2 glycoprotein of herpes simplex virus (17). There is accumulating evidence that glycoprotein G of VSV plays a role in cell fusion (7, 8, 10, 22). Hughes et al. found that when glycosylation was inhibited by 10 mM D-glycosamine, the process of cell fusion also was blocked (10). This block of VSV-induced polykaryocytosis occurred even when the glucosamine was added at a concentration that inhibited neither host nor viral protein synthesis. Monospecific antibody directed towards G protein of VSV

TABLE 4. Percentage of the viral-specific proteins of tsG3 (III) grown at permissive temperatures

Viral proteins	Intracellular viral-specific proteins at following incubation temp:		Virion-associated proteins at following incubation temp:	
	40°C	31°C	40°C	31°C
L	0.3	4.8	0.4	6.3
G	13.2	12.5	12.7	11.5
NS	12.0	8.1	1.2	3.6
N	46.4	47.3	52.1	38.4
M	28.1	27.3	33.6	40.2

[a] The autoradiograph from Fig. 6 was scanned with the gel-scanning system of a Beckman DU-8B spectrophotometer. Percentages of viral-specific proteins were calculated as described in footnote *a* of Table 2.

also has been shown to inhibit VSV-induced cell fusion (8, 10). Antibodies directed against M and N proteins do not block polykaryocyte formation as a result of VSV infection (8). It is important to note that anti-M antibody may promote cell fusion; however, this possibility has not yet been tested. Recently, it has been shown that expression of cloned cDNA of VSV G protein in the absence of the other viral proteins will induce cells to fuse when the pH of the culture medium is reduced (7, 22). The conclusion to be reached is that G protein is required for cell fusion to take place.

In a typical VSV infection, G protein is abundantly expressed on the surface of infected cells, yet cell fusion does not occur. This may well be due to a rapid destruction of cells by insertion of M and G proteins into the membrane and the sequential budding of the virus from the cell. This suggests that cell surface G protein in the presence of M protein or other viral proteins will not promote fusion. Our observations support this hypothesis. We have found that viral protein synthesis must be inhibited after the eclipse period for cell fusion to take place (Fig. 3 and 4 and Table 1), at which time G protein has already been synthesized. Thus, a reduction in the amount of another protein must be required for VSV to cause polykaryocytosis. The most compelling evidence for the involvement of another protein is the work done with the temperature-sensitive mutant tsG31 (III). This mutant has a temperature-sensitive defect in M protein (13, 15). When grown at the nonpermissive temperature, tsG31 (III) does not produce infectious virus (11), produces a smaller amount of M protein (16) having an aberrent mobility as compared with that of wild type (Fig. 6), and induces cell fusion in two neuroblastoma cell lines (10), mouse EAT and rat XC cells (8), and BHK-21 and R(B77) cells (Table 3). These characteristics, coupled with the observation that no mutant from the four other complementation groups of VSV-induced cell fusion, leads us to conclude that when a nonfunctional M protein is produced, normal maturation cannot occur and the cell may be ripe for fusion mediated by G protein.

It seems that when nonfunctional M protein is present in infected cells, then G protein may promote cell fusion, as shown by the work done on tsG31 (III). But can reduced levels of M protein have an effect on the cytopathic effect of VSV? There are significant differences among the rates of transport of the five VSV proteins into virions (12, 26) (Fig. 5). VSV M protein disappears most rapidly from the cells and is rapidly incorporated into virions (Fig. 5) (12, 13). If protein synthesis, a requirement for VSV-induced cell fusion, is inhibited early in the infection, the intracellular pool of M protein would be the most drastically reduced. However, G protein is not as rapidly transported from the cell into virions. Thus, if protein synthesis is inhibited early in infection, M protein would be lost from the cells, but G protein would slowly accumulate in the cell surface. This line of reasoning leads to the hypothesis that when protein synthesis is inhibited early in infection, the ratio of G protein to M protein would be disturbed and G protein would be able to mediate cell fusion.

On the basis of this study, two models for VSV-induced cell fusion can be put forward. First, VSV infection could lead to cell fusion when the initial molar ratio of G protein to M protein is disturbed intracellularly. Specifically, when G protein accumulates on the membrane in the absence of functional M proteins, the infected cells will be ripe for cell fusion. An alternative model is that the total amount of cell surface G protein or the amount of time required for the cells to remain in contact with a G protein on the cell surface might determine whether the cells will fuse. In this model, any effects M protein would have might be indirect since M protein is required for budding and thus would reduce the level of cell surface G protein. Although both models are possible, the evidence presented favors the first. Specifically, in tsG31 (III)-infected cells, the ratio of M protein to G protein is the same at both the permissive and nonpermissive temperatures (Fig. 6 and Table 2). Therefore, lack of a functional M protein in infected cells and the presence of G protein on the cell surface lead to cell fusion.

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