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Reovirus μ2 Protein Determines Strain-Specific Differences in the Rate of Viral Inclusion Formation in L929 Cells

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Reovirus infection induces the formation of large cytoplasmic inclusions that serve as the major site of viral assembly. Reovirus strains type 3 Dearing (T3D) and type 1 Lang (T1L) differ in the rate of inclusion formation in L929 cells. The median time of inclusion formation is 18 h in cells infected with T3D and 39 h in cells infected with T1L. Using reassortant viruses that contain combinations of gene segments derived from T1L and T3D, we found that the M1 gene, which encodes the μ2 protein, is the primary determinant of the rate of inclusion formation. The S3 gene, which encodes the nonstructural protein σNS, plays a secondary role in this process. The subcellular location of the μ2 protein was determined by confocal laser scanning microscopy using dual-fluorescence labeling of μ2 and the outer-capsid protein μ1/μ1C. In virus-infected cells, μ2 protein colocalized with other viral proteins in inclusions and was also distributed diffusely in the cytoplasm and nucleus. Expression of recombinant T1L and T3D μ2 proteins resulted in the formation of protein complexes resembling inclusions in both the cytoplasm and the nucleus with kinetics that reflected the strain of origin. The median time of μ2 protein complex formation was 22 h in cells transfected with the T3D M1 gene and 43 h in cells transfected with the T1L M1 gene. These findings suggest that the μ2 protein influences the rate of inclusion formation and contributes to inclusion morphogenesis. The requirement of μ2 protein in inclusion formation was tested by determining the subcellular localization of μ2 in cells infected with temperature-sensitive (ts) mutants that are defective in viral assembly. In contrast to infection with wild-type virus, μ2 did not colocalize with μ1/μ1C protein in subcellular structures that formed in cells infected at nonpermissive temperature with ts mutants tsH11.2, tsC447, and tsG453 with mutations in the M1, S2, and S4 genes, respectively. These results suggest that despite the role of the μ2 protein in controlling the rate of inclusion formation, this process is a concerted function of several reovirus proteins.

INTRODUCTION

In association with the production of nucleic acid and protein, viral infection often involves the reorganization of cellular architecture to generate sites that facilitate viral assembly. All members of the *Reoviridae* induce cytoplasmic inclusions (Francki and Boccardo, 1983) that contain fibrillar constituents. In the case of orthoreoviruses, these filamentous structures have been identified as intermediate filament proteins (Sharpe *et al.,* 1982). Insect reoviruses (cypoviruses) encode a polyhedrin protein that forms the matrix of inclusions (Payne and Mertens, 1983). Viral proteins that mediate inclusion formation have not been identified for vertebrate reoviruses.

Mammalian reoviruses have a segmented genome consisting of 10 double-stranded (ds) RNA gene segments enclosed within a double-shelled capsid. Each segment encodes a single protein with the exception of S1, which is dicistronic (reviewed by Nibert *et al.,* 1996). During viral entry into cells, the outer capsid is removed to produce transcriptionally active single-shelled, viral core particles. The resulting primary transcripts are translated to produce viral proteins that assemble with genomic sets of 10 mRNAs to produce replicase particles that synthesize minus-sense RNAs resulting in the formation of progeny dsRNA. Viral proteins and RNA are directed to inclusions that first appear by phase-contrast microscopy as dense granules scattered in the cytoplasm that coalesce around the nucleus later in infection (Sharpe *et al.,* 1982; Gomatos *et al.,* 1962). Reovirus inclusions are large cytoplasmic structures that are detectable by a variety of light and electron microscopic techniques (Zarbl and Millward, 1983). These inclusions are thought to be the primary sites of viral assembly since they contain paracrystalline arrays of virions late in infection (Gomatos *et al.,* 1962; Spendlove *et al.,* 1963; Dales *et al.,* 1965; Fields *et al.,* 1971).

Mechanisms by which viral proteins and RNA are directed to inclusions are not known. The involvement of host cytoskeletal proteins in reovirus inclusion formation and replication has previously been investigated. Reovi-

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rus infection of CV-1 cells results in disruption of the vimentin, intermediate filament network followed by its reorganization into a fibrous network within viral inclusions (Sharpe *et al.,* 1982). Microtubule and microfilament organization is not affected. However, virions and viral proteins appear to be attached to microtubules (Babiss *et al.,* 1979), which are thought to be involved in viral transport rather than morphogenesis. Colchicine treatment of reovirus-infected cells prevents development of large perinuclear inclusions without reducing viral yield (Babiss *et al.,* 1979; Spendlove *et al.,* 1964).

One of the three medium-sized gene segments, M1, encodes the μ2 protein, which is a minor component of the viral core. The μ2 protein is present in about 18 copies per virion (Coombs, 1998a) and is located in the viral core along with the viral polymerase at the base of the icosahedral vertices (Dryden *et al.,* 1998). Studies using reassortant viruses derived from reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) have demonstrated that the μ2-encoding M1 gene segregates with strain-specific differences in several biological properties including replication in myocardial cells (Matoba *et al.,* 1991), bovine aortic endothelial cells (Matoba *et al.,* 1993), and Madin–Darby canine kidney cells (Rodgers *et al.,* 1997). The M1 gene also segregates with strain-specific differences in induction of myocarditis (Sherry and Fields, 1989; Sherry and Blum, 1994; Sherry *et al.,* 1998) and hepatic injury (Haller *et al.,* 1995) in mice.

In addition to its role in reovirus growth in cell culture and pathogenesis in mice, the μ2 protein is involved in RNA synthesis. The M1 gene segment segregates with strain-specific differences in the temperature optimum of transcription, the extent of transcription (in conjunction with the L1 gene segment) (Yin *et al.,* 1996), and NTPase activity (Noble and Nibert, 1997). The M1 gene temperature-sensitive (ts) mutant tsH11.2 is defective in dsRNA synthesis, which suggests that the μ2 protein is required for genome replication (Coombs, 1996). Recombinant μ2 protein binds both single-stranded (ss) and dsRNA (Brentano *et al.,* 1998). The M1 genes of T1L and T3D contain a single open reading frame of 736 amino acids that corresponds to the μ2 protein. The deduced amino acid sequences of T1L and T3D μ2 proteins are very conserved, differing at only 10 amino acid positions (Wiener *et al.,* 1989; Zou and Brown, 1992, 1996b). These 10 amino acid differences must account for the differences in T1L and T3D attributable to the μ2 protein.

We conducted experiments to identify the genetic basis for inclusion formation in reovirus. Following the observation that T1L and T3D differ in the rate of inclusion formation we used reassortant viruses to identify viral genes that control this phenotype. We identified the M1 gene encoding the μ2 protein as the primary determinant in the rate of inclusion formation with a secondary role for the S3 gene encoding the nonstructural protein σNS. The intracellular localization of μ2 protein was monitored by fluorescence microscopy in cells infected with wild-type (wt) and ts mutants as well as M1 gene transfected cells. The results suggest that the μ2 protein plays a crucial role in the formation of reovirus inclusions, perhaps by influencing the rate of biochemical events or by forming a structural lattice for inclusion formation.

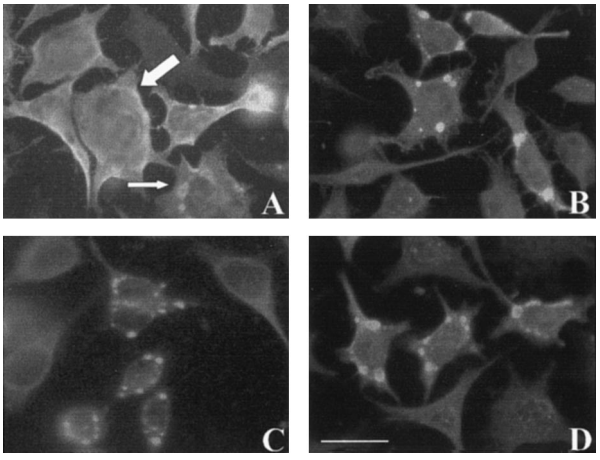

FIG. 1. Subcellular localization of reovirus proteins in cells infected with reovirus strains T1L or T3D. L929 cells were infected with T1L and labeled with rabbit anti-T1L antiserum at 24 (A) and 48 (C) h p.i. or with T3D and labeled with rabbit anti-T3D antiserum at 24 (B) and 48 (D) h p.i. Infections were done at an m.o.i. of 10 PFU per cell. The secondary antibody used was Cy3-conjugated donkey anti-rabbit antiserum. Images were obtained using an epifluorescence microscope. An antigen-positive cell without inclusions is indicated with a heavy arrow and a cell with inclusions is indicated with a thin arrow in (A). Bar represents 10 μm.

RESULTS

Reovirus exhibits strain-specific differences in the rate of inclusion formation in L929 cells

To determine whether T1L and T3D differ in kinetics of inclusion formation in L929 cells, we used indirect immunofluorescence to assess the relative proportion of antigen-positive cells that formed cytoplasmic inclusions. Cells were infected with either T1L or T3D at a multiplicity of infection (m.o.i.) of 10 plaque-forming units (PFU) per cell. The cells were then fixed in acetone at varying times after infection and stained with strain-specific rabbit antisera followed by donkey anti-rabbit antiserum conjugated to Cy3. Viral inclusions were seen as local concentrated areas of staining within the cytoplasm relative to generalized cytoplasmic staining (Fig. 1A, thin and heavy arrows, respectively). Cells showing intermediate staining patterns were scored as inclusion-negative. Immunofluorescence staining showed that viral antigen was diffusely distributed in the cytoplasm in the majority of T1L-infected cells at 12 and 24 h postinfection (p.i.) but was seen to localize into inclusions by 48 h p.i. (Figs. 1A and 1C and Table 1). In contrast,

TABLE 1

Rate of Inclusion Formation in T1L- or T3D-Infected L929 Cells

Time postinfection (hours)	Percentage inclusions[a]	
	T1L (*n*)[b]	T3D (*n*)
15	4 ± 2 (328)	46 ± 2 (35)
20	7 ± 8 (79)	67 ± 15 (37)
24	11 ± 9 (219)	66 ± 4 (84)
48	63 ± 10 (251)	nd

[a] Average of two to three experiments.
[b] *n* is the number of antigen-positive cells counted; nd, not done.

inclusion formation in T3D-infected cells was observed at much earlier time points, with a majority of cells showing inclusions by 20 h p.i. (Figs. 1B and 1D and Table 1). T1L and T3D differed strikingly in the rate of inclusion formation with median times of 39 and 18 h, respectively. These results demonstrate that reovirus strains T1L and T3D differ in the rate of inclusion formation in L929 cells.

Strain-specific differences in the rate of reovirus inclusion formation are determined primarily by the M1 gene

To identify viral genes that segregate with differences in the kinetics of inclusion formation exhibited by T1L and T3D, we tested 15 T1L × T3D reassortant viruses for the rate of inclusion formation in L929 cells by immunofluorescence staining at 24 h p.i. (Table 2). This time point was chosen because it demonstrated a maximum difference in the extent of inclusion formation by T1L- and T3D-infected L929 cells (Table 1). Those reassortant viruses that produced the greatest percentage of infected cells with inclusions at 24 h p.i. possessed a T3D M1 gene, while those that produced the least contained a T1L M1 gene. Reassortant viruses containing a T3D M1 gene and a T1L S3 gene produced inclusions with faster kinetics than the T3D parent. The rate of inclusion formation of reassortant viruses containing a T1L M1 gene was, however, not influenced by the allelic nature of the S3 gene. The association of the M1 gene with the rate of inclusion formation was highly statistically significant (*t* test, $P < 0.0001$; Mann–Whitney (MW) test, $P < 0.001$). The remaining genes were not significantly associated with the rate of inclusion formation in these experiments (*t* test and MW test, $P > 0.05$). We used parametric stepwise linear regression analysis to determine the individual contribution of genes to the rate of inclusion formation. This analysis showed that 89.8% ($P = 0.027$) of the variability in inclusion formation is accounted for by the 10 reovirus genes with 86% contributed by the M1 gene ($P < 0.001$), 7% by the S3 gene ($P = 0.169$), and 4% by the S1 gene ($P = 0.649$). There was no detectable contribution by the other viral genes to this phenotypic difference. These results indicate that M1 is the only viral gene that independently contributes to the rate of reovirus inclusion formation. When only those reassortant viruses containing a T3D M1 gene ($n = 10$) were analyzed, the S3 gene was the only gene significantly associated with the rate of inclusion formation (*t* test, $P = 0.004$; MW test, $P = 0.014$). These results indicate that the rate of viral inclusion formation is

TABLE 2

Inclusion Formation in L929 Cells Exhibited by T1L × T3D Reassortant Viruses

Virus strain	Parental origin of gene segments										Percentage inclusion formation (*n*)[a]	Rank
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4		
EB129	3	3	3	**3**	3	1	3	1	**1**	3	96.2 (157)	1
G16	1	1	1	**3**	1	1	1	3	**1**	1	88.4 (69)	2
EB123	3	3	1	**3**	3	3	3	3	**1**	3	79.4 (160)	3
EB146	1	1	1	**3**	1	1	1	1	**1**	3	76.3 (97)	4
EB88	3	3	3	**3**	1	3	3	3	3	3	65.6 (162)	5
T3D	3	3	3	**3**	3	3	3	3	3	3	65.5 (84)	6
EB96	1	3	1	**3**	1	1	1	1	3	1	64.8 (88)	7
EB28	3	3	1	**3**	3	3	3	1	3	3	64.1 (167)	8
EB86	1	3	3	**3**	3	1	3	3	3	1	46.0 (174)	9
EB31	1	1	1	**3**	1	1	1	3	3	1	38.2 (110)	10
H60	3	3	1	1	3	3	3	3	3	1	29.6 (81)	11
H15	1	3	3	1	3	3	3	3	3	1	27.0 (89)	12
EB39	1	3	3	1	3	3	3	3	3	3	19.3 (88)	13
EB47	1	3	1	1	1	1	1	1	1	1	14.5 (124)	14
T1L	1	1	1	1	1	1	1	1	1	1	11.0 (219)	15
H17	3	3	1	1	3	3	1	3	3	1	7.6 (92)	16
EB73.1	1	3	1	1	3	3	3	3	3	3	6.5 (124)	17

[a] *n* is the total number of antigen-positive cells counted by immunofluorescence staining with rabbit polyclonal anti-T1L and -T3D antisera.

due primarily to the M1 gene, which encodes the μ2 protein, and is modulated by the S3 gene, which encodes the nonstructural protein σNS.

Colocalization of μ2 and other reovirus proteins in cytoplasmic inclusions

The observation that the M1 gene is the primary determinant of strain-specific differences in the rate of viral inclusion formation led us to examine the subcellular localization of μ2 protein with respect to other reovirus proteins during infection of L929 cells. A monoclonal antibody (8H6) specific for μ1/μ1C (Virgin *et al.,* 1991), a reovirus outer-capsid protein, was used to identify reovirus inclusion bodies, and rabbit anti-T1L-μ2 polyclonal antiserum was used to detect μ2 protein. The polyclonal rabbit anti-T1L-μ2 antiserum demonstrated equivalent reactivity with both T1L and T3D μ2 proteins, which are 98.6% identical in amino acid sequence, by both immunoprecipitation (Zou and Brown, 1996b) and immunoblot (data not shown). To determine the subcellular distribution of μ2 relative to other reovirus proteins during infection, double-immunofluorescence labeling of cells infected with either T1L or T3D was performed using confocal laser scanning microscopy. T3D μ2 protein was primarily concentrated in inclusions, whereas T1L μ2 protein had a more diffuse distribution (Figs. 2E and 2F). However, in cells infected with either strain, μ2 and μ1/μ1C proteins colocalized in cytoplasmic inclusions (Figs. 2E, 2F, 2H, 2I, 2K, and 2L). In addition, μ2 protein localized to the nucleus in both T3D- and T1L-infected cells (Figs. 2E and 2F). The presence of the μ2 protein in the nucleus was confirmed by costaining with a dsDNA-specific dye, ToPro3 (Figs. 2K and 2L). Occasionally, μ2 protein was observed to accumulate in small cytoplasmic foci that were not associated with μ1/μ1C staining (Fig. 2L). These results indicate that μ2 localizes to sites of viral assembly and is also distributed in the nucleus.

μ2 protein localization in cells infected with reovirus temperature-sensitive mutants

We used temperature-sensitive mutants that are defective in viral assembly to further examine the role of μ2 protein in inclusion formation. The temperature-sensitive mutant tsH11.2 does not replicate at nonpermissive temperature (39.5°C) due to a conditionally defective μ2 protein (Coombs, 1996). Progeny virions are not observed in tsH11.2-infected cells due to a defect in viral assembly and genome replication. Infection of L929 cells with tsH11.2 at a permissive temperature (33°C) resulted in normal inclusions with colocalization of μ2 and μ1/μ1C proteins (data not shown). Infection of L929 cells with tsH11.2 at a nonpermissive temperature resulted in the accumulation of μ1/μ1C protein in subcellular structures that did not contain μ2 protein and were generally smaller than inclusions observed in cells infected with wild-type virus (Figs. 3G and 3J). Instead, μ2 protein had a diffuse cytoplasmic and nuclear localization (Figs. 3D and 3J). These results demonstrate that subcellular structures that resemble inclusions by phase-contrast microscopy can form without μ2 protein as in wt infection. To determine whether viral inclusion formation was affected by defects in other viral genes, two other ts mutants, tsC447 and tsG453, with mutations in the σ2-encoding S2 and σ3-encoding S4 genes, respectively, were used to infect L929 cells. Infection with either ts mutant resulted in the accumulation of μ1/μ1C protein in subcellular structures that did not stain for μ2 protein (Figs. 3H, 3I, 3K, and 3L). In contrast to tsH11.2, infection with tsC447 and tsG453 resulted in the accumulation of μ2 in small protein complexes seen as punctate cytoplasmic staining adjacent to subcellular structures containing μ1/μ1C protein, in addition to having diffuse nuclear staining (Figs. 3E, 3F, 3K, and 3L). Occasionally, μ2 protein was also seen to accumulate in discrete structures in the nucleus (Fig. 3F).

Recombinant T1L and T3D μ2 proteins form nuclear and cytoplasmic complexes at different rates

Genetic analysis of the rate of reovirus inclusion formation indicated that this property is linked to the μ2-encoding M1 gene. Accumulation of μ2 in protein complexes independent of μ1/μ1C protein also was observed in cells infected with either wt viruses or ts mutants (Figs. 2L, 3K, and 3L). These observations suggested that μ2 would be capable of forming protein complexes in cells independent of other viral proteins. To examine this possibility, recombinant μ2 protein was expressed in L929 cells by transfection with either T1L or T3D M1 gene-containing plasmids under control of a bacteriophage T7 RNA polymerase promoter. Immunoblot analysis of transfected cells showed high levels of μ2 protein expression 24 h posttransfection for both the T1L and the T3D M1 gene constructs (Fig. 4). In contrast, expression of T1L μ2 was more robust than T3D μ2, in cells infected with these virus strains (Fig. 4). M1-gene transfected cells were stained for μ2 protein at 24 and 48 h after transfection. The μ2 protein of both T1L and T3D formed complexes in the absence of the other reovirus proteins (Figs. 5C, 5D, 5E, and 5F). The rate of complex formation varied in a strain-specific manner. The median time of complex formation by T3D μ2 protein was 22 h posttransfection compared to 43 h posttransfection for cells expressing T1L μ2 protein (Table 3; Figs. 5B, 5C, 5D, and 5E). Differences in the rate of complex formation exhibited by T1L and T3D μ2 proteins paralleled that of inclusion formation in reovirus-infected cells. Recombinant μ2 protein formed complexes in both the nucleus and the cytoplasm (Fig. 5D). The presence of nuclear μ2 complexes was confirmed using confocal laser scanning microscopy and dual staining for μ2 and

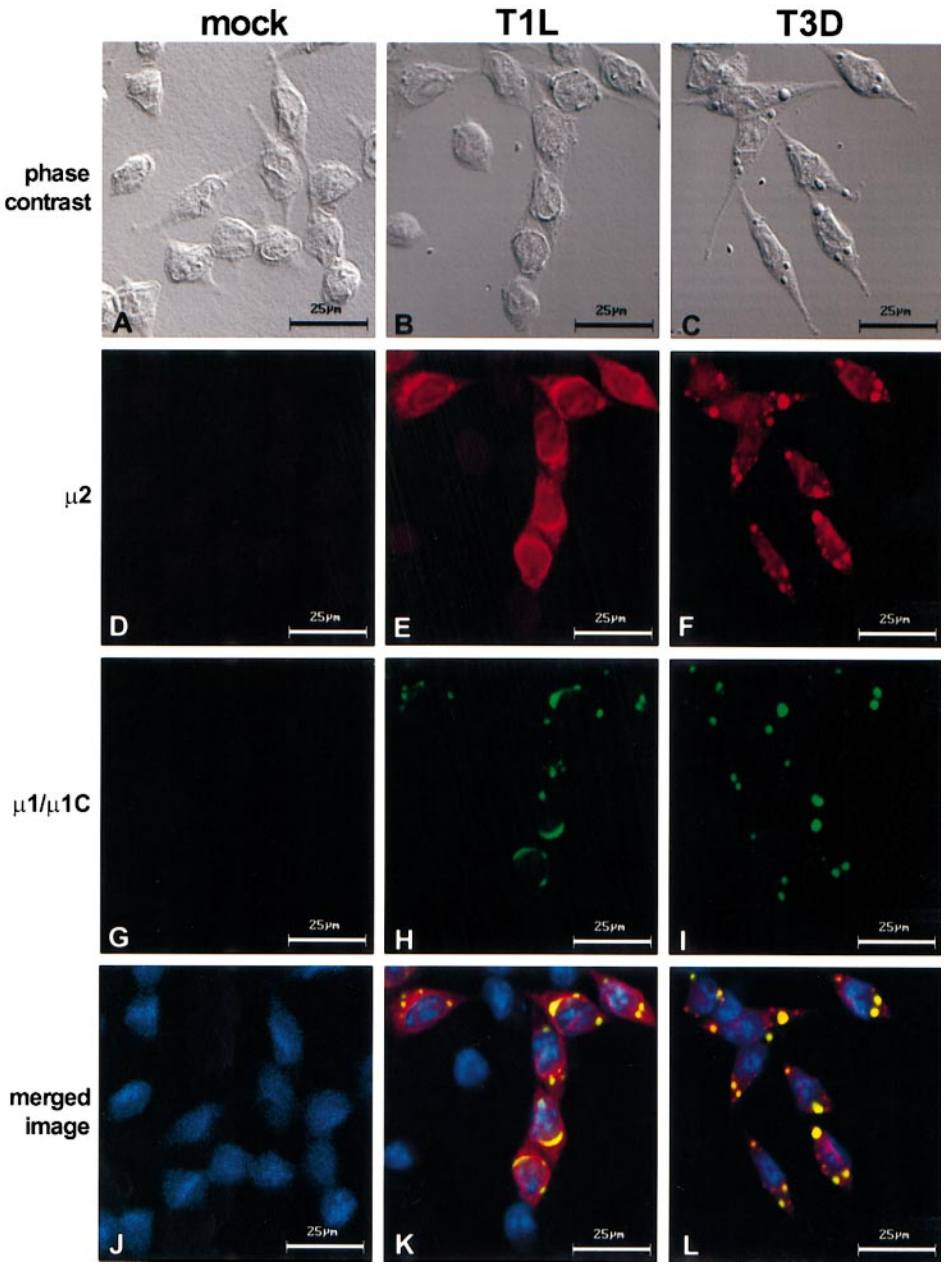

FIG. 2. Subcellular localization of reovirus $\mu 2$ and $\mu 1/\mu 1C$ proteins in cells infected with reovirus strains T1L or T3D. L929 cells were either mock-infected (A, D, G, J) or infected with T1L (B, E, H, K) or T3D (C, F, I, L) at an m.o.i. of 10 PFU per cell. Following adsorption, cells were incubated at 37°C for 18 h. Cells were stained for $\mu 2$ using a $\mu 2$-specific polyclonal antiserum (D–F) and for $\mu 1/\mu 1C$ using $\mu 1/\mu 1C$-specific mAb 8H6 (G–I) as primary antibodies followed by goat anti-mouse Alexa488 and goat anti-rabbit Alexa546 as secondary antibodies. The $\mu 2$ protein is colored red, and the $\mu 1/\mu 1C$ protein is colored green. ToPro3, a dsDNA-specific dye, was incubated with the secondary antibodies and is colored blue (J–L). Images were obtained using a confocal laser scanning microscope. A phase-contrast image of each field is shown in A–C. In the merged images, colocalization of $\mu 2$ and $\mu 1/\mu 1C$ is indicated by the yellow color, and colocalization of $\mu 2$ and ToPro3 is indicated by the pink color.

the nucleus (data not shown). Expression of recombinant tsH11.2 $\mu 2$ protein resulted in temperature-sensitive complex formation at permissive relative to nonpermissive temperature at 48 h posttransfection (17 ± 9% vs 7 ± 7% of antigen-positive cells; *t* test, $P = 0.047$). However, the number of cells containing $\mu 2$ complexes after transfection with tsH11.2 $\mu 2$ at both permissive and nonpermissive temperatures was significantly less than that after transfection with wt T1L $\mu 2$ protein (17 ± 9% and 7 ± 7% vs 56 ± 8% for T1L $\mu 2$; *t* test, $P < 0.00001$). The expression level of recombinant tsH11.2 $\mu 2$ protein was comparable to that of wt T1L (data not shown). This finding suggests that the M1 mutation in tsH11.2 impairs the capacity of tsH11.2 $\mu 2$ to form protein complexes when expressed on its own.

To further assess the localization of recombinant $\mu 2$

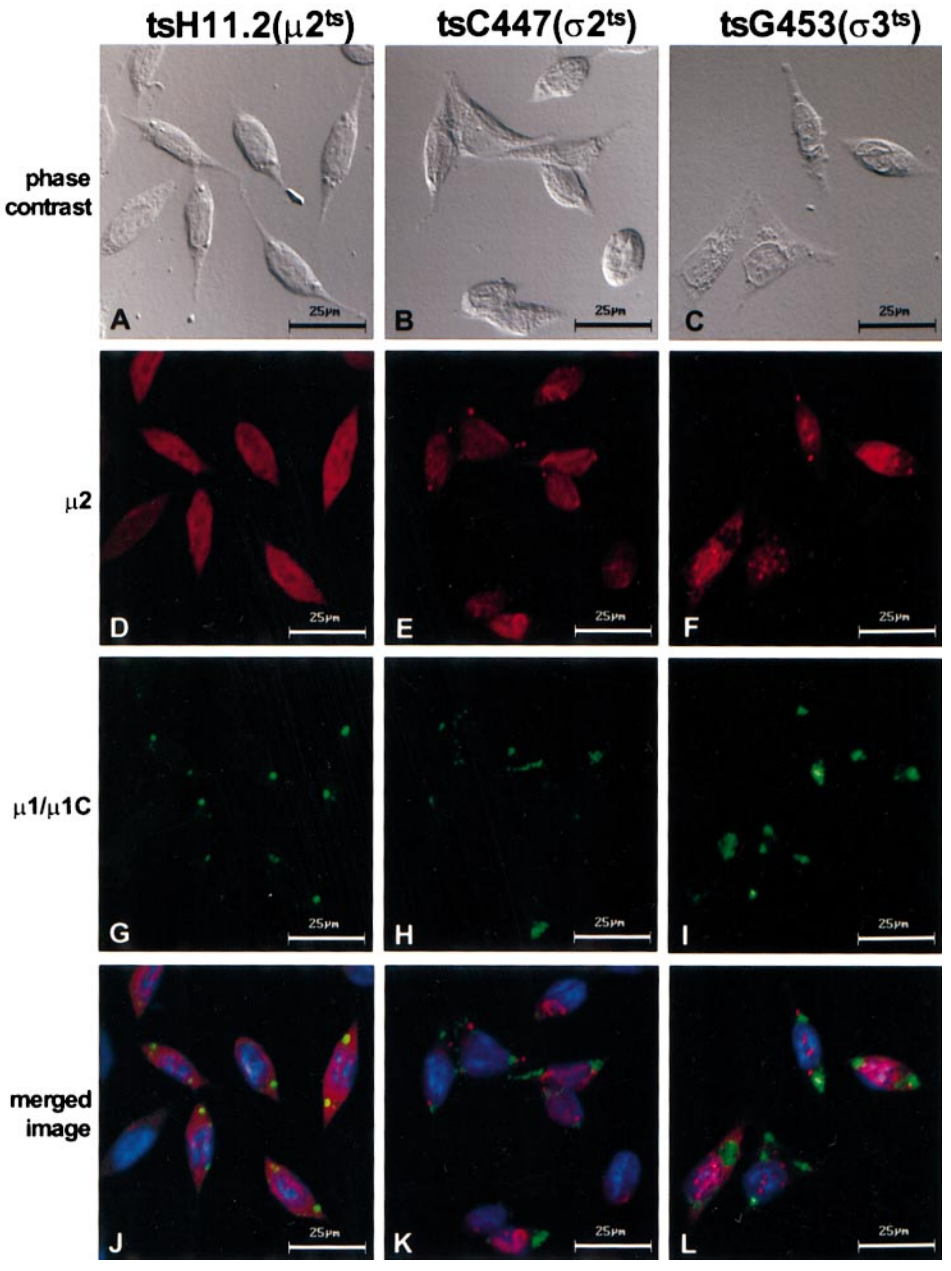

FIG. 3. Subcellular localization of reovirus μ2 and μ1/μ1C proteins in cells infected with ts mutant reoviruses tsH11.2, tsC447, and tsG453. L929 cells were infected with either tsH11.2 (A, D, G, J), tsC447 (B, E, H, K), or tsG453 (C, F, I, L) at an m.o.i. of 10 PFU per cell. Following adsorption, cells were incubated at 39.5°C for either 24 h (tsH11.2 and tsC447) or 36 h (tsG453). Cells were stained for μ2 using a μ2-specific polyclonal antiserum (D–F) and for μ1/μ1C using μ1/μ1C-specific mAb 8H6 (G–I) as primary antibodies followed by goat anti-mouse Alexa488 and goat anti-rabbit Alexa546 as secondary antibodies. The μ2 protein is colored red, and the μ1/μ1C protein is colored green. ToPro3, a dsDNA-specific dye, was incubated with the secondary antibodies and is colored blue (J–L). Images were obtained using a confocal laser scanning microscope. A phase-contrast image of each field is shown in A–C. In the merged images, colocalization of μ2 and μ1/μ1C is indicated by the yellow color, and colocalization of μ2 and ToPro3 is indicated by the pink color.

protein, transfected cells were fractionated into soluble (cytoplasmic) and pellet (nuclear) fractions by detergent extraction and sedimentation. The resulting samples were analyzed by immunoblot using a T1L μ2-specific antiserum. Both T1L and T3D μ2 proteins were found in the soluble and pellet fractions (Fig. 6). Quantification by phosphorimaging showed that 61% of the T3D μ2 protein compared to 49% of the T1L μ2 protein had accumulated in the pellet by 24 h posttransfection. This observation is consistent with the nuclear localization of T3D and T1L μ2 proteins as seen by immunofluorescence. The integrity of the fractionation procedure was verified by staining the same samples for tubulin, a cytoplasmic protein, which was present only in the soluble fraction (Fig. 6). These data support the nuclear localization of μ2 protein observed in reovirus-infected cells.

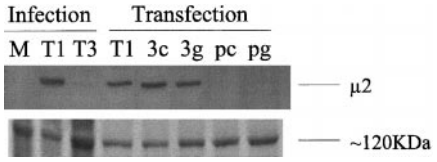

FIG. 4. Immunoblot analysis of $\mu 2$ protein expression in infected and transfected L929 cells. Cell lysates were collected 24 h p.i. or posttransfection, and the blot was probed with anti-T1L-$\mu 2$ antiserum. Lane annotation from left to right: Infection: M, mock-infected; T1, T1L-infected; T3, T3D-infected. Transfection: T1, pCMV-M1CN T1-7; 3c, pCMV-M1CN T3-13; 3g, pGEM-M1 T3-18; pc, pcDNA3 vector control; and pg, pGEM7 vector control transfected. (Bottom) The same samples stained with Coomassie blue showing a protein of approximately 120 kDa, as a loading control.

TABLE 3

Rate of $\mu 2$ Complex Formation in L929 Cells Transfected with the T1L or T3D M1 cDNA

Time posttransfection (hours)	Percentage cells with protein complex formation[a]	
	T1L M1 cDNA (n)[b]	T3D M1 cDNA (n)
24	15 ± 10 (132)	61 ± 11 (88)
48	56 ± 8 (115)	98 ± 5 (26)

[a] Average of two to four experiments (at least one with pGEM and pcDNA3).

[b] n is the number of antigen-positive cells counted by immunofluorescence staining with rabbit polyclonal anti-T1L $\mu 2$ antiserum.

DISCUSSION

In this study, we show that reovirus strains T1L and T3D differed in the rate of inclusion formation in L929 cells. This finding is consistent with early studies of the rate of inclusion formation by T1L (Rhim *et al.,* 1962) and T3D (Gomatos *et al.,* 1962). Genetic analysis using T1L × T3D reassortant viruses showed that the M1 gene is the primary determinant of strain-specific differences in the kinetics of inclusion formation. Since T1L synthesizes several-fold more $\mu 2$ protein and other reovirus proteins than T3D (Fig. 4; Mbisa and Brown, unpublished data; Schmechel *et al.,* 1997; Matoba *et al.,* 1991) and yet forms inclusions at a slower rate, it is likely that an intrinsic difference in T1L and T3D $\mu 2$ proteins influences the rate of inclusion formation in reovirus-infected cells.

The S3 gene modulated the rate of inclusion formation in conjunction with the M1 gene such that pairing of a T1L S3 gene with a T3D M1 gene in T1L × T3D reassortant viruses resulted in a faster rate of inclusion formation than for the parental strain T3D. This finding indicates that the T3D S3 gene attenuates the rate of inclusion formation by the cognate T3D M1 gene. The strain of origin of the S3 gene did not affect the kinetics of inclusion formation when paired with the T1L M1 gene, indicating that the T1L $\mu 2$ protein differs from T3D $\mu 2$ in its interactions with σNS. It is possible that the σNS protein modulates the rate of inclusion formation by direct interactions with $\mu 2$.

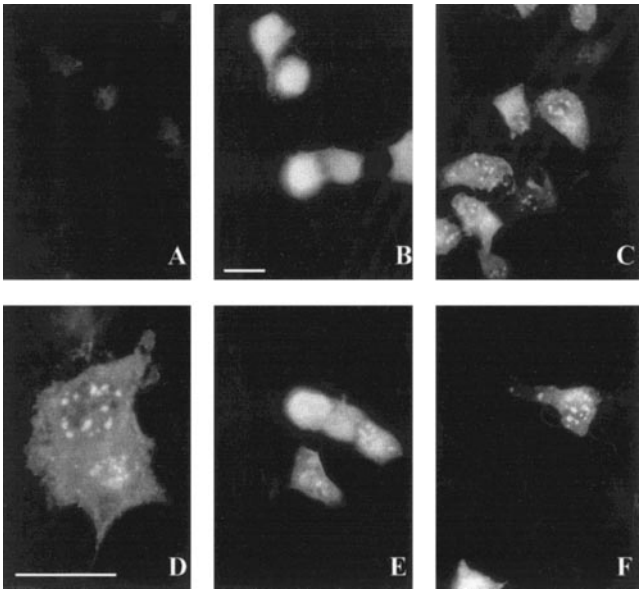

FIG. 5. Localization of recombinant $\mu 2$ protein in transfected cells. L929 cells were transfected with control vector (pcDNA3) (A), pCMV-M1CN clone T1-7 expressing the T1L M1 gene (B, E), or transfected with pCMV-M1CN clone T3-13 expressing the T3D M1 gene (C, F). Following incubation for either 24 h (A, B, C) or 48 h (E, F), cells were stained for $\mu 2$ using $\mu 2$-specific rabbit polyclonal antiserum followed by FITC-conjugated goat anti-rabbit antiserum. (D) Higher magnification of cells transfected with pGEM-M1 clone T3-18 expressing the T3D M1 gene, stained for $\mu 2$ at 24 h posttransfection showing nuclear and cytoplasmic complexes of $\mu 2$. The bars represent 10 μm.

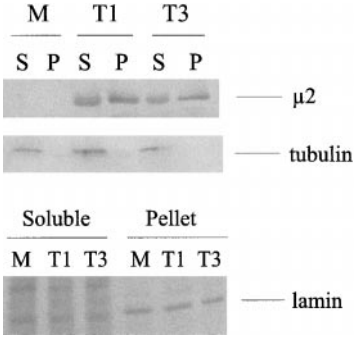

FIG. 6. Immunoblot analysis of recombinant $\mu 2$ protein in the soluble (cytoplasmic) and pellet (nuclear) fractions of cells transfected with either the T1L or the T3D M1 gene. Cell lysates were collected and fractionated at 24 h posttransfection, and the blot was probed with polyclonal rabbit anti-T1L-$\mu 2$ antiserum and detected by phosphorimaging. The middle panel shows an immunoblot of the same samples stained for tubulin, a cytoplasmic protein that is present only in the soluble fraction. (Bottom) The same samples stained with Coomassie blue showing a protein in the pellet fractions of 67 kDa corresponding to lamin and unidentified proteins in the soluble fractions as loading controls. M, mock transfected; S, soluble fraction; P, pellet fraction; T1, T1L M1 gene transfected; T3, T3D M1 gene transfected.

In addition to controlling the rate of inclusion formation, the μ2 protein might play a direct physical role in the inclusion formation process. This hypothesis is supported by expression of recombinant μ2 protein in L929 cells, which resulted in the formation of protein complexes that resemble inclusions. Although these μ2 complexes were not associated with other viral proteins or the host protein vimentin (Mbisa and Brown, unpublished data), they were formed with kinetics that reflect the parental origin of the M1 gene. However, μ2 protein is probably not the only reovirus protein involved in inclusion formation. Infection of L929 cells with mutants tsH11.2, tsC447, and tsG453 resulted in the formation of subcellular structures that resembled reovirus inclusions but did not contain μ2 protein. In addition, at least one other reovirus protein, μ1/μ1C, has been shown to form protein complexes when expressed alone (Yue and Shatkin, 1996). The σ1 protein also has been shown to have a uniform cytoplasmic distribution or to accumulate in punctate foci in different studies using different cell lines for transfection (Banerjea *et al.,* 1988; Belli and Samuel, 1991). Inclusion formation may thus be a concerted function of several reovirus proteins with μ2 protein controlling the formation rate. Alternatively, μ2 could coordinate the formation of a subset of protein complexes that serve as a prerequisite for inclusion formation.

In addition to localizing to cytoplasmic inclusions, μ2 localizes to the nucleus in cells infected with wt reovirus, in cells infected with mutant viruses tsH11.2, tsC447, and tsG453, and in M1-gene-transfected cells. The localization of the protein to the nucleus was unanticipated, considering that reovirus replication is thought to be entirely cytoplasmic. It is possible that μ2 protein localizes to the nucleus to induce cellular events involved in reovirus replication, including inclusion formation. Both recombinant T1L and T3D μ2 proteins accumulate in the nucleus to similar levels in transfected cells, although T3D μ2 protein produced complexes more rapidly than T1L μ2 protein. This observation may be relevant to the inclusion formation process since the activity parallels the rate of inclusion formation during infection. The μ2 protein is not the only reovirus protein that localizes to the nucleus. The σ3 protein accumulates in the nucleus of both infected and transfected cells (Yue and Shatkin, 1996; Schmechel *et al.,* 1997), and σ1s, a nonstructural protein encoded by the S1 gene, localizes to the nucleolus (Belli and Samuel, 1991; Rodgers *et al.,* 1998).

Sequence analysis of the μ2 protein reveals a prototypical nuclear localization signal motif at positions 261 to 264 (KRLR) that fits a consensus sequence, K R/K X R/K (Garcia-Bustos *et al.,* 1991; Mukaigawa and Nayak, 1991), required for the nuclear localization of several proteins including the PB1 and PB2 subunits of the influenza virus RNA polymerase (Nath and Nayak, 1990; Mukaigawa and Nayak, 1991). Nuclear localization signals have also been shown to consist of a single stretch of basic amino acids (Chelsky *et al.,* 1989; Garcia-Bustos *et al.,* 1991) and one such region is present in the μ2 protein from position 100 to 114 (RRLRKRLMLKKDLRK). Experiments are in progress to identify sequences in the M1 gene required for nuclear localization and complex formation of the μ2 protein.

The μ2 protein did not accumulate in protein complexes in cells infected at nonpermissive temperature with tsH11.2, and although complexes were observed in cells expressing high levels of recombinant tsH11.2 μ2 protein they were formed less efficiently, even at permissive temperatures, than wt T1L μ2 protein. These findings suggest that the mutations in the tsH11.2 μ2 protein affect the accumulation of μ2 in complexes. The tsH11.2 M1 gene has two mutations resulting in 2 amino acid substitutions that are 11 residues apart, $M^{399} \rightarrow T$ and $P^{414} \rightarrow H$ (Coombs, 1996). Constitutive expression of wt μ2 protein in L929 cells complements the M1 defect of tsH11.2 at nonpermissive temperatures (Zou and Brown, 1996a). Expression of μ2 proteins containing either single mutation is capable of partially complementing the tsH11.2 defect, which indicates that both mutations contribute to the ts phenotype (Zou, Brown, and Coombs unpublished results; Coombs, 1998b). The $P^{414} \rightarrow H$ mutation resides in a region with partial homology to the A motif of known ATPases (Noble and Nibert, 1997). This observation raises the possibility that μ2 protein mediates the rate of inclusion formation directly by an ATP- or other nucleotide-dependent event or indirectly through an ATP-dependent interaction with host regulatory proteins. Consistent with this idea, the M1 gene, in addition to the L3 gene, influences the NTPase activity of reovirus cores (Noble and Nibert, 1997).

Reovirus assembly is poorly understood, but the earliest ssRNA-containing structures termed "assortment" complexes are composed of σNS, μNS, σ3, μ1/μ1C, λ1, and λ2 (Antczak and Joklik, 1992). Progeny, core-like, subviral particles also acquire three other proteins that are not found in assortment complexes; μ2, λ3, and σ2. This results in the formation of replicase particles containing all the viral structural proteins as well as μNS (Zweerink *et al.,* 1976). These particles are transcriptionally active progeny subviral particles and synthesize dsRNA. It is possible that ssRNA-containing complexes associate with another complex containing μ2, λ3, and σ2 and that the μ2 protein controls the formation of this complex at the site of inclusion formation. Since the M1 gene determines the rate of inclusion information, the formation of this complex could be the rate-limiting step. The secondary role of the S3 gene in kinetics of inclusion formation suggests that σNS influences the rate of formation of the ssRNA-containing complex. Alternatively, σNS might directly interact with μ2 to form replicase particles. Such an association could be modulated by the different M1 and S3 genes to affect the rate of inclusion formation. This model predicts that the μ2 protein does

not associate with μ1/μ1C-containing complexes in either tsC447- or tsG453-infected cells that express mutant σ2 and σ3 proteins, respectively. The absence of μ2 in μ1/μ1C-containing complexes in cells infected with these ts mutants would result from failure of the μ2 protein to recognize the aberrant structures produced during these infections due to mutations in σ2 and σ3. Our future experiments will address the interactions of individual viral proteins in wt and ts reovirus infections to clarify the sequence of events leading to inclusion formation. Nonetheless it is interesting that the only reovirus ts mutants that are completely defective in virion assembly—where capsid structures do not form—are tsH (M1 gene) and tsE (S3 gene) (reviewed by Coombs, 1998b), which supports the hypothesis that virion assembly and inclusion formation are functionally linked and that μ2 and σNS play crucial roles in both of these processes.

Host tissue-dependent effects of reovirus infection that segregate with the M1 gene may be explained in part by the differential capacity of the μ2 protein to influence the rate of inclusion formation. This μ2-mediated property might in turn be dependent on the capacity of the μ2 protein to interact with host factors and form inclusions, which are indicative of successful viral assembly. Identification of host constituents associated with viral inclusions in reovirus-infected cells should provide insight into mechanisms of reovirus genome replication and assortment. Knowledge of the inclusion formation process, including identification of the protein–protein and protein–RNA interactions required for inclusion formation, will contribute to an understanding of viral assembly and identify new therapeutic targets for dsRNA-containing viruses.

MATERIALS AND METHODS

Cells and viruses

Mouse fibroblast L929 cells were propagated in adherent culture in MEM (Gibco BRL, Burlington, Ontario, Canada) supplemented to contain 5% fetal calf serum (Biocell Laboratories, Carson, CA). Reovirus strains T1L and T3D were laboratory stocks initially obtained from Dr. B. N. Fields. The ts mutants tsC447, tsG453, and tsH11.2 were provided by Dr. K. Coombs (University of Manitoba). The T1L × T3D reassortant viruses were produced previously (Brown *et al.,* 1983). The recombinant vaccinia virus vTF7.3 that expresses the T7 RNA polymerase was a kind gift from Dr. K. Dimock (University of Ottawa) and was grown in CV-1 cells. Reoviruses were propagated and titrated by plaque assay in L929 cells as described previously (Zou and Brown, 1992).

Antibodies

Antisera to T1L and T3D μ2 proteins were produced in rabbits immunized with Trp-E–μ2 fusion protein expressed in *Escherichia coli* (Zou and Brown, 1996a). Antisera to reovirus strains T1L and T3D were generated by immunizing rabbits with purified reovirus. Prior to use in indirect immunocytochemistry the antisera were adsorbed to acetone-fixed L929 cell extract and used at a dilution of 1 in 500. Monoclonal antibody 8H6, specific for μ1/μ1C protein, was used at a concentration of 10 μg/ml to detect reovirus inclusions in dual-labeling experiments. The primary antibody used to detect cytoskeletal proteins was murine monoclonal antibody against β-tubulin (Clone DM1B, Amersham, Baie d'Urfe, Quebec, Canada; at 1:1000).

Secondary antibodies used were fluorescein-conjugated goat anti-rabbit IgG (Sigma Chemical Co., Oakville, Ontario, Canada) and Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Goat anti-mouse Alexa488 and goat anti-rabbit Alexa546 (Molecular Probes, Inc., Eugene, OR) were used in dual-labeling experiments at a dilution of 1:1000. ToPro3, a dsDNA-specific dye (Molecular Probes), was used to detect the nucleus at a dilution of 1:1000. Protein A conjugated to alkaline phosphatase and anti-mouse IgG conjugated to alkaline phosphatase were obtained from Sigma Chemical Co.

Indirect immunostaining

L929 cells seeded on 22 × 22 mm glass coverslips (Corning Inc., Big Flats, NY) in 6-well plates were infected with reovirus strains T1L or T3D at a m.o.i. of 10 PFU per cell. Prior to being stained, the cells were fixed in prechilled acetone for 5 min. After being rinsed in PBS (3× 5 min), 100 μl of an appropriate dilution of antisera was applied and incubated in a humidified chamber at room temperature for 30–90 min. The coverslips were then rinsed in PBS (3× 5 min) and treated with the appropriate dilution of the secondary conjugated antibody. After another 30- to 90-min incubation period at room temperature the coverslips were rinsed in PBS (3× 5 min) and once in ddH_2O and mounted on glass slides in Gel/Mount (Biomeda Corp). All antibody dilutions were done in PBS/3% BSA. Mock infections were done and analyzed for every experiment as a negative control. The samples were visualized using a Zeiss microscope equipped with epifluorescence and a 63X or 100X, 1.40 NA PlanApo objective. The images were collected using Image One Metamorph software (Universal Imaging Corp., West Chester, PA) and a Hamamatsu chilled charge-coupled digital camera (Model C5985, Hamamatsu Corp., Bridgewater, NJ).

For double-immunolabeling the cells were fixed for 2 min in a 1:1 (v/v) mixture of methanol and acetone. Cells were kept in methanol at −20°C until stained. Cells were then washed twice in PBS and incubated for 15 min in PBS containing 5% BSA (Sigma, St. Louis, MO). Nonspecific binding of antibody to cells was blocked by incuba-

tion for 10 min in PBS containing 1% BSA, 1% Triton X-100 (Bio-Rad Laboratories, Hercules, CA), and 2% normal goat serum (Vector Laboratories, Inc., Burlingame, CA). All washes and antibody dilutions were done in the same solution. Cells were then incubated with primary and secondary antibodies and ToPro3 as described above. Cells were washed twice for 15 min with PBS/1% BSA, 1% Triton X-100 and then twice for 10 min with PBS. Coverslips were washed with deionized water and then mounted on glass slides using ProLong Antifade (Molecular Probes). Cells were visualized using a Zeiss confocal fluorescence microscope (Carl Zeiss, New York, NY), and the images were processed and colored using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

Transfection

L929 cells seeded in 6-well plates containing 22 × 22 mm glass coverslips (Corning) were transfected with DNA using lipofectin reagent when they were at 80–85% confluence using the protocol from Gibco BRL. Five micrograms of DNA and 15 μl of lipofectin were used for each transfection reaction. M1 DNA constructs pCMV-M1CN (clones T1-7 and T3-13) and pGEM-M1 (clones T1-7 and T3-18) both under control of the bacteriophage T7 promoter were used (Zou and Brown, 1996b). After a 5-h incubation period the cells were infected with the recombinant vaccinia vTF7.3 at an m.o.i. of 0.5 to 1. The cells were then incubated for 24 or 48 h at 37°C before analysis.

Immunoblotting

Infected or transfected cell monolayers were lysed in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue, and 5% 2-mercaptoethanol). The proteins were separated by SDS–PAGE and transblotted onto Immobilon P membrane (Millipore, Mississauga, Ontario, Canada) at 25 V overnight at 4°C. The dried membrane was blocked with 5% skim milk in PBS for 1 h at RT. This was followed by the addition of primary antibody in fresh milk and incubation for 2 h at 4°C. The membrane was then washed three times in PBS and once in TBS to remove phosphate and incubated in 5% milk in TBS containing 1 μg/ml PAAP. Finally the membrane was washed 4× in TBS before reaction with chromogenic substrate, nitro blue tetrazolium (33 μl/ml) plus 5-bromo-4-chloro-3-indolyl phosphate (3.3 μl/ml) (Sigma), in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris–HCl, pH 9.5) or Attophos reagent (JBL Scientific, San Luis Obispo, CA) for phosphorimaging using a Storm 860 PhosphoImager equipped with ImageQuant software (Molecular Dynamics). For quantification purposes known amounts of TrpE–μ2 fusion protein were run as reference and only μ2 protein readings falling within the linear range of the reference were considered significant.

Cell fractionation

Adherent transfected cells were washed with cold PBS, lysed in lysis buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 4 mM vanadyl riboside complex, 0.5% (v/v) Triton X-100, and 1.2 mM PMSF), and scraped into a microfuge tube (He *et al.,* 1990). To collect the cytoplasmic and nuclear fractions, a modification of the procedure described by He *et al.* (1990) was used. Briefly, the samples were centrifuged (600 *g* for 3 min), the supernatant was decanted (cytoplasmic fraction), and the pellet was then rinsed with RSB buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, and 1 mM PMSF) and resuspended in 1× sample buffer (nuclear fraction). The samples were then resolved by SDS–PAGE and electroblotted to a PVDF membrane before immunoblotting as described above.

Statistical analysis

The association of reovirus gene segments with the rate of inclusion formation was determined by using *t* test (parametric) and MW test (nonparametric) statistical techniques. The contributions of individual genes were analyzed further by parametric stepwise linear regression and additional linear regression analysis was performed for all combinations of genes found to be statistically significant. Statistical analyses were performed by using the Minitab (release 8) statistical software package (Addison-Wesley, Reading, MA).

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