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Expression of the M Gene of Vesicular Stomatitis Virus Cloned in Various Vaccinia Virus Vectors

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Initial attempts to clone the matrix (M) gene of vesicular stomatitis virus (VSV) in a vaccinia virus expression vector failed, apparently because the expressed M protein, and particularly a carboxy-terminus-distal two-thirds fragment, was lethal for the virus recombinant. Therefore, a transient eucaryotic expression system was used in which a cDNA clone of the VSV M protein mRNA was inserted into a region of plasmid pTF7 flanked by the promoter and terminator sequences for the T7 bacteriophage RNA polymerase. When CV-1 cells infected with recombinant vaccinia virus vTF1-6,2 expressing the T7 RNA polymerase were transfected with pTF7-M3, the cells produced considerable amounts of M protein reactive by Western blot (immunoblot) analysis with monoclonal antibodies directed to VSV M protein. Evidence for biological activity of the plasmid-expressed wild-type M protein was provided by marker rescue of the M gene temperature-sensitive mutant *ts*O23(III) at the restrictive temperature. Somewhat higher levels of M protein expression were obtained in CV-1 cells coinfected with a vaccinia virus-M gene recombinant under control of the T7 polymerase promoter along with T7 polymerase-expressing vaccinia virus vTF1-6,2.

Vesicular stomatitis virus (VSV), the prototype of the family *Rhabdoviridae*, contains a single-stranded negative-sense RNA genome which encodes five mRNAs that are translated into five proteins (25). The N, NS, and L proteins are associated with the RNA genome to form the ribonucleoprotein (RNP) core, whereas the G protein is an integral membrane protein that constitutes the spikes protruding from the surface of the virion envelope (3, 23, 25, 27). The matrix (M) protein, on the other hand, appears to serve the dual purpose of adhering to the inner surface of the virion membrane and to the maturing RNP core in the process of virion maturation and budding (15, 27). The M protein also plays a role in regulation of viral transcription (4, 6, 8, 19, 26).

Our laboratory has recently undertaken structural studies of the M protein in an attempt to locate its functional domains. By the use of epitope-specific monoclonal antibodies (MAbs) and proteolytic cleavage, we have tentatively located the membrane-binding site of the M protein within its carboxy-terminus-distal (trypsin-resistant) region (16). In sharp contrast, the RNP-binding and transcription inhibition region of the M protein (as well as epitope 1) is apparently located in the amino-terminal end, somewhere between amino acids 19 and 43 (16–18). Confirmatory evidence for this location of transcription inhibition and epitope 1 sites has been obtained by the use of synthetic oligopeptides homologous to this region (J. B. Shipley, R. Pal, and R. R. Wagner, manuscript in preparation).

In an effort to confirm and extend these biochemical and immunological studies, we have been cloning wild-type and mutant M genes in procaryotic and eucaryotic expression vectors. By these techniques we hope to be able to identify critical M protein sequences that determine sites for binding of MAbs as well as membrane-binding, RNP-binding, and transcription inhibition sites. We report here our failure in the use of one recombinant DNA system with a vaccinia virus vector and our success with a highly efficient transient expression system with another vaccinia virus vector. This latter system also permits experiments for marker rescue of a conditionally defective phenotype.

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MATERIALS AND METHODS

Cells and viruses. VSV (Indiana serotype, San Juan strain) and the complementation group III mutant *ts*O23 were grown in BHK-21 cells and isolated and purified as described previously (1). Vaccinia virus (strain WR), recombinant vaccinia virus containing the T7 RNA polymerase gene (v-TF1-6,2), and human TK^- 143 cells have been described by Fuerst et al. (10) and were kindly provided by Bernard Moss of the National Institutes of Health. Human TK^- 143 cells were grown in Eagle minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and 25 μg of 5-bromodeoxyuridine (BUdR) per ml. CV-1 monkey kidney cells were grown in Dulbecco modified Eagle medium containing 10% FBS. HeLa cells were grown in Eagle MEM supplemented with 5% horse serum.

Plasmids. The vaccinia virus coexpression vectors pSC11 (5) and pTF7IHB-1 (10) were also provided by Bernard Moss. Plasmid pM309 containing the VSV Indiana M gene was a generous gift of John K. Rose. Recombinant plasmids were constructed and used to transform *Escherichia coli* by standard methods described by Maniatis et al. (14). Plasmids were prepared by the alkaline sodium dodecyl sulfate (SDS) method as described by Birnboim and Doly (2) and purified by CsCl-ethidium bromide equilibrium density gradient centrifugation. Plasmids were routinely checked by agarose gel electrophoresis to ensure that the majority of the DNA was in the supercoiled configuration. DNA fragments were isolated from agarose gels by electroblotting onto NA-45 DEAE membranes (Schleicher and Schuell).

Isolation of recombinant virus. CV-1 cells were infected with wild-type vaccinia virus at a multiplicity of infection (MOI) of 0.05 PFU/cell and transfected with calcium phosphate-precipitated plasmids; 20 to 40 μg of plasmid recombinant DNA was added in 1 ml of HEPES-buffered saline and precipitated by addition of $CaCl_2$ to a final concentration

of 125 mM. Recombinant viruses formed by homologous recombination of the foreign gene into the TK^- locus were selected by plaque assay on TK^- 143 cell monolayers in the presence of BUdR (25 μg/ml). TK^- recombinant virus plaques were distinguished from spontaneous TK^- mutant virus by DNA-RNA dot blot hybridization instead of DNA-DNA dot blot hybridization. After two consecutive plaque purifications, recombinant virus was amplified by infecting TK^- 143 cell monolayers in the presence of BUdR, and then large stocks were made in HeLa cells without selective medium.

Transient expression and Western blot (immunoblot) analysis. CV-1 cells were grown to 80% confluence in 35-mm plates (≈3 × 10^5 cells) and infected at an MOI of 30 with purified recombinant vaccinia virus vTF1-6,2 expressing T7 RNA polymerase, which is identical to the vTF7-3 recombinant vaccinia virus described by Fuerst et al. (10). The virus was allowed to adsorb for 1 h at 37°C with occasional rocking of the plate. The inoculum was then removed, and each plate was washed once with 2 ml of serum-free medium. We then added simultaneously 0.4 ml of calcium phosphate-precipitated DNA (10 to 30 μg of recombinant plasmid) and 3 ml of MEM with 5% FBS. Cells incubated at 37°C were harvested at 24 h after infection, lysed, and subjected to 12.5% polyacrylamide–SDS slab gel electrophoresis as described previously (4). The proteins were transferred by electroblotting onto nitrocellulose sheets (0.1 μM; Schleicher & Schuell, Inc., Keene, N.H.) as described by Towbin et al. (24). The nitrocellulose sheets were then reacted with MAbs and ^{125}I-labeled *Staphylococcus* protein A as previously described in detail (17, 18).

Marker rescue. CV-1 cells were grown to 80% confluence in 35-mm plates (≈ 3 × 10^5 cells) and infected with VSV temperature-sensitive mutant *ts*O23 at an MOI of 1 PFU/cell. The virus was allowed to adsorb for 30 min at room temperature. The inoculum was then removed, and 2 ml of MEM containing 5% FBS was added to each plate. The infected cultures were incubated at 39°C, and 2.5 h after infection, the cells were infected with vTF1-6,2 at a multiplicity of 30 PFU/cell at 39°C for 1 h. Then, 0.4 ml of calcium phosphate-precipitated DNA (15 μg of recombinant plasmid) and 3 ml of MEM containing 5% FBS were added to each plate. All the plates were then incubated at 39°C for 14 h. The supernatant fluids were harvested, and virus was titrated by plaque assay on L-cell monolayers at 31 and 39°C.

RESULTS

Attempts to construct a vaccinia virus-M gene recombinant expression vector. The objective of these studies was to insert the entire coding region of the VSV M protein into the vaccinia virus genome in order to synthesize authentic M protein in quantities sufficient for biological and biochemical studies. To ensure efficient expression, homopolymeric GC tails of pM309 that had been added during the original cDNA cloning by Rose and Gallione (22) were trimmed off by treatment with Bal31 nuclease and blunt-ended by Klenow fragment DNA polymerase before cloning into the unique *Sma*I site of the plasmid coexpression vector pSC11 (5, 7). A considerable number of pSC11-M recombinants were obtained with the M gene insert in either orientation (Fig. 1A). The features of this construct include: (i) the M gene cDNA is under the control of the early-late vaccinia virus promoter P7.5; (ii) the vector contains the *E. coli* β-galactosidase gene under the control of the late vaccinia virus promoter P11; and (iii) both chimeric genes are flanked by vaccinia virus thymidine kinase (TK) sequences. Upon transfection of vaccinia virus-infected cells, the pSC11-M plasmids should undergo homologous recombination in the *tk* locus of the vaccinia virus genome, yielding infectious recombinant vaccinia virus in medium containing BUdR. Recombinant vaccinia virus plaques are identified by a blue color developing on the addition of an agar overlay containing the 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) indicator.

In these transfection experiments with pSC11-M plasmids, vaccinia virus recombinants yielding blue plaques were readily isolated consistently, but only when the M gene inserted in the *tk* region of the vaccinia virus genome was in the wrong orientation. Unexpectedly and rather surprisingly, numerous plaque isolates of pSC11-M vaccinia virus recombinants were tested but none ever contained the entire M gene inserted in the correct orientation for transcription (data not shown). However, pSC11-vaccinia virus recombinants with the M gene in the correct orientation were readily obtained when the coding region of the M gene cDNA downstream from nucleotide 258 was removed by *Bgl*II restriction and by filling in the recessed end with Klenow fragment DNA polymerase and religating the vector. This procedure adds four nucleotides and the UAA stop codon adjacent to the *Bgl*II site, providing a reading frame that terminates translation at amino acid 74 of the M protein. In another experiment, the amino-terminal portion of the M gene in pSC11-M was removed with *Hae*II, which cuts the M gene DNA at nucleotide 153, leaving a fragment coding for amino acids 51 to 219. No plaques were formed by vaccinia virus-pSC11 recombinants in the correct orientation, even though they were ostensibly capable of expressing the carboxyl-distal end of this truncated M gene, whereas vaccinia virus recombinants with truncated carboxyl-terminal M genes in the wrong orientation produced numerous plaques (data not shown). These experiments indicate that expression of the M gene, and particularly the carboxyl-distal region, is lethal for recombinant vaccinia virus. In marked contrast, Mackett et al. (13) successfully cloned and expressed the entire VSV G gene in a similar vaccinia virus vector.

Construction of a transient expression vector with the T7 phage polymerase promoter and terminator. In an attempt to overcome the lethality problem encountered with the pSC11-vaccinia virus vector, we cloned the M gene in the novel plasmid pTF7 used for transient expression in the system recently developed by Fuerst et al. (10). The basic strategy in this system is to infect cultured cells with a recombinant vaccinia virus that expresses the T7 bacteriophage RNA polymerase, followed by transfection with a plasmid containing the gene of interest flanked by the T7 polymerase promoter and terminator sequences. As shown in Fig. 1B, the plasmid pTF7IHB-1 used in these studies contained the hepatitis B surface antigen (HBsAg) gene, which was excised with *Bam*HI and filled in with the DNA polymerase Klenow fragment. The M gene DNA was then inserted into the unique *Bam*HI site to form a plasmid designated pTF7-M3. For M protein expression, this plasmid with the M gene in the correct reading frame was used to transfect cells that had previously been infected with vaccinia virus vTF1-6,2 expressing the T7 phage RNA polymerase.

It also seemed feasible for obtaining higher M protein expression levels to construct a recombinant vaccinia-pTF7-M3 virus. As originally described by Fuerst et al. (9), this construct would place the M gene integrated in the vaccinia virus genome under control of the T7 polymerase promoter, expressible only when cells are coinfected with the vaccinia

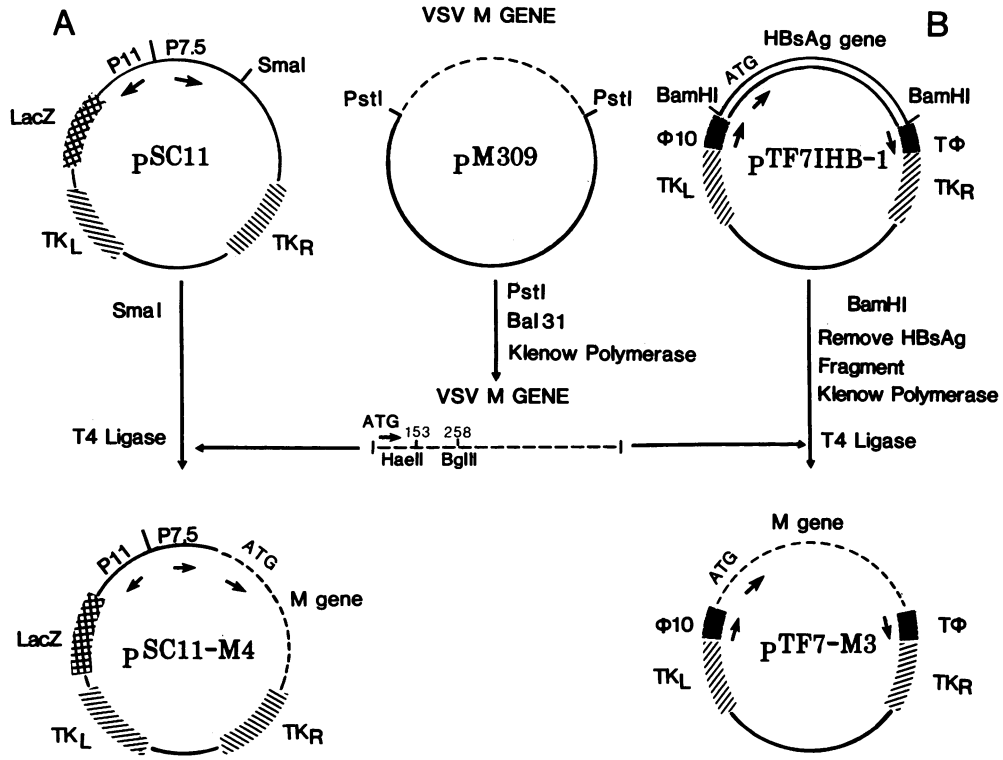

FIG. 1. Construction of VSV M gene recombinant plasmids. (A) cDNA copy of the VSV M protein gene was cleaved from pM309 with *Pst*I and inserted into the unique *Sma*I site of pSC11 to form pSC11-M4, in which the M cDNA is under the control of the early-late vaccinia virus promoter P7.5 and the *E. coli* β-galactosidase gene is under the control of the late vaccinia virus promoter P11. Both chimeric genes are flanked by the left (TK_L) and right (TK_R) vaccinia virus *tk* gene sequences. (B) The same cDNA copy of the VSV-M protein gene was blunt-end ligated to pTF7 that had been cleaved with *Bam*HI to remove the HBsAg gene and treated with Klenow polymerase. The resulting plasmid, pTF7-M3, has the M cDNA juxtaposed to the bacteriophage T7 polymerase promoter (ϕ10) and terminator (Tϕ) and flanked by the left (TK_L) and right (TK_R) sequences of the vaccinia virus *tk* gene. Both vectors were subsequently used to direct the insertion, by homologous recombination, of the M cDNA construct into the *tk* locus of the vaccinia virus genome. The DNA segments are not drawn to scale.

virus recombinant expressing the T7 phage RNA polymerase. To select the vaccinia virus-pTF7-M3 recombinant, cells infected with wild-type vaccinia virus (strain WR) were transfected with pTF7-M3; after incubation for 48 h, virus progeny were assayed for their ability to form plaques on TK^- human 143 cells cultivated in the presence of BUdR. Recombinants were identified and distinguished from spontaneous TK^- mutants by dot blot hybridization with ^{32}P-labeled VSV M protein mRNA. Autoradiographs showed that 20 of 49 TK^- virus plaques were positive for VSV M gene DNA (data not shown). After two consecutive plaque purifications, recombinant vaccinia virus stocks containing pTF7-M3 inserts were collected, grown to high virus titer, and designated vM3. Plasmid pTF7-M3 and vM3 were both tested by coinfection with the vaccinia virus T7 polymerase recombinant vTF1-6,2 for expression of the VSV M gene.

Transient expression of M gene. We next tested the ability of the transfected pTF7-M3 plasmid to express M protein in CV-1 cells infected with vaccinia virus vTF1-6,2, which expresses the T7 phage RNA polymerase. It was also of interest to determine whether the pSC11-M4 plasmid under the control of the P7.5 vaccinia virus promoter could transiently express the M gene in cells infected with the WR strain of wild-type vaccinia virus. This latter experiment should identify or rule out the vaccinia virus P7.5 promoter as the lethal factor in vaccinia virus recombinants with integrated pSC11-M4.

CV-1 cells infected with wild-type vaccinia virus or T7 polymerase recombinant vaccinia virus vTF1-6,2 were transfected by calcium phosphate precipitation with the M gene-containing plasmid pSC11-M4 or pTF7-M3 under control of the vaccinia virus P7.5 or T7 polymerase promoter, respectively. Cells harvested at 24 h after infection or transfection were extracted in SDS and subjected to electrophoresis on 12.5% polyacrylamide–SDS gels. The proteins were then blotted onto nitrocellulose sheets for detection of M protein by its ability to bind immunoglobulin G purified from M-specific MAbs, followed by ^{125}I-labeled protein A and autoradiography, as described in Materials and Methods.

Figure 2 shows the Western blot autoradiograms for transient expression of M proteins in wild-type- or vTF1-6,2-infected CV-1 cells transfected with pSC11-M4 or pTF7-M3, respectively. As noted, a mixture of three MAbs readily detected M protein in CV-1 cells transfected with either pSC11-M4 or pTF7-M3, provided that the cells were also infected with wild-type vaccinia virus or vTF1-6,2. In each case the electrophoretic mobility of expressed M protein was indistinguishable from that of authentic M protein extracted from VSV virions. The degree of expression was proportional to the amount of each transfecting plasmid over a range of 10 to 30 μg. Quite clearly, the pTF7-M3 plasmid with the T7 polymerase promoter and the vaccinia virus helper vTF1-6,2 T7 polymerase were far superior to the transient expression of the pSC11-M4 plasmid with the

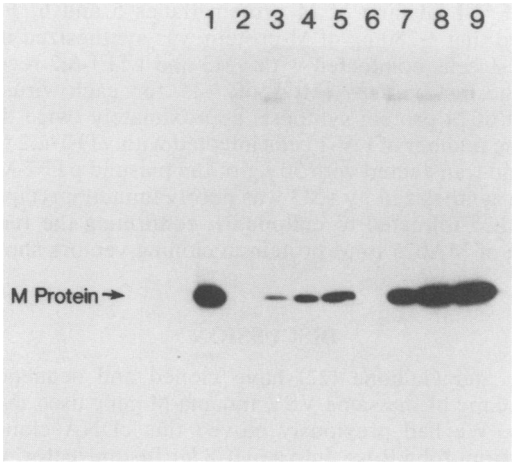

FIG. 2. Western blot analysis of transiently expressed M protein gene. CV-1 cells were infected with either wild-type vaccinia virus (strain WR) or vTF1-6,2 recombinant vaccinia virus expressing T7 RNA polymerase at an MOI of 30 PFU/cell for 1 h at 37°C. The virus inoculum was replaced with the indicated amount of calcium phosphate-precipitated pSC11-M4 or pTF7-M3 DNA added with 3 ml of MEM containing 5% FBS. Cells were collected at 24 h and disrupted in SDS, and the cell extracts were analyzed by 12.5% polyacrylamide–SDS gel electrophoresis. The proteins were then blotted onto a nitrocellulose sheet and detected by a mixture of anti-M protein MAbs to epitopes 1, 2, and 3, followed by ^{125}I-protein A. Lane 1, Purified VSV virion M protein (150 ng); lane 2, extract from uninfected cells transfected with 30 μg of pSC11-M4 DNA; lanes 3 to 5, extracts from wild-type vaccinia virus-infected cells transfected with 10, 20, or 30 μg of pSC11-M4 DNA, respectively; lane 6, extract from uninfected cells transfected with 30 μg of pTF7-M3 DNA; lanes 7 to 9, extracts from vTF1-6,2-infected cells transfected with 10, 20, or 30 μg of pTF7-M3 DNA, respectively.

vaccinia virus promoter and polymerase; laser densitometry scanning revealed a sixfold greater synthesis of M protein in vTF1-6,2-infected cells transfected with pTF7-M3 (data not shown). Parenthetically, transient expression of the M gene by the plasmid pSC11-M4 indicates that the vaccinia virus promoter P7.5 is not responsible for the lethality of pSC11-M4 integrated into the *tk* gene of vaccinia virus in the correct reading frame.

To determine whether the pTF7-M3 plasmid expresses an M protein as competent antigenically as native virion M protein, individual MAbs directed to three separate epitopes (16–18) were compared for their ability to bind to M protein expressed by pTF7-M3 in CV-1 cells infected with vaccinia virus vTF1-6,2. M protein transiently expressed in CV-1 cells by pTF7-M3 efficiently bound MAb2 (directed to epitope 1) and MAb3 (directed to epitope 2) (Fig. 3). However, the same pTF7-M3 plasmid-expressed M protein was considerably less efficient in binding MAb25 (directed to epitope 3) than was the native virion M protein (compare lanes 1 and 4 in Fig. 3). Similar results were obtained by Western blotting of M protein expressed in vTF1-6,2-infected cells transfected with pTF7 plasmids expressing the M genes of the Orsay wild-type strain or its mutant *ts*O23 (data not shown). We can only conclude that, for reasons unknown, epitope 3 of VSV M protein expressed from plasmid pTF7 is less accessible to its MAb than are two other epitopes.

Rescue of mutant *ts*O23 by pTF7-M3 plasmid-expressed wild-type M protein. Marker rescue experiments were performed to determine whether the M protein transiently expressed by pTF7-M3 is sufficiently authentic to complement a conditionally lethal M gene mutation. The question addressed was whether the epitope 3-deficient M protein expressed by pTF7-M3 is competent to serve its virus maturation function at the restrictive temperature. For this purpose, monolayer cultures of CV-1 cells were infected with complementation group III mutant *ts*O23 and incubated at the nonpermissive temperature (39°C) for 2.5 h prior to infection with T7 phage polymerase vaccinia virus recombinant vTF1-6,2 (MOI, ≈30); half of the cultures were transfected with the M gene plasmid pTF7-M3, and half served as controls. These doubly infected and transfected cells were incubated at 39°C for 14 h, at which time the supernatant fluid was assayed for plaque-forming VSV by plating on L-cell monolayers incubated at either 31 or 39°C.

Table 1 compares the yield of VSV *ts*O23 released from vTF1-6,2 vaccinia virus-infected cells transfected or not tranfected with plasmid pTF7-M3. As noted, control cells not transfected with pTF7-M3 produced significant amounts of VSV progeny, a finding attributable to the high reversion frequency and probable leakiness of *ts*O23 and other complementation group III mutants (20). In cells transfected with pTF7-M3 and infected with *ts*O23 at an MOI of 1 PFU/cell, the yield of *ts*O23 plated at 31°C was 200 times greater than that from *ts*O23-infected cells not transfected with pTF7-M3. As expected, this rescued virus was genotypically still temperature sensitive because the plaque titers at 39°C, due to revertants, were the same for *ts*O23 grown in the presence or absence of pTF7-M3. In a futile attempt to enhance mutant rescue by pTF7-M3, cells were infected with *ts*O23 at an MOI of 10 without increasing the MOI of vTF1-6,2 or transfecting dose of pTF7-M3. This higher MOI of *ts*O23 resulted in a lesser increase in titer of only 10-fold (Table 1), a finding which is difficult to explain but could be due to

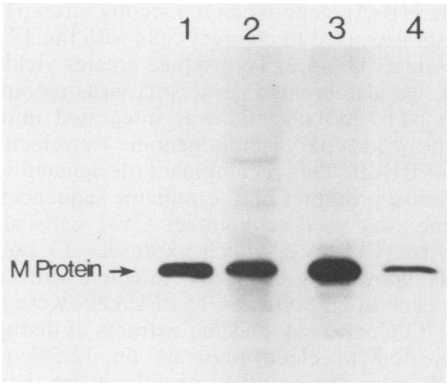

FIG. 3. Western blot analysis of three epitope-specific MAbs to M protein expressed in the vaccinia virus-T7 polymerase transient expression system. CV-1 cells were infected with vTF1-6,2 (recombinant vaccinia virus expressing T7 RNA polymerase) at an MOI of 30 PFU/cell for 1 h of adsorption at 37°C. The virus inoculum was then replaced with 15 μg of calcium phosphate-precipitated pTF7-M3 DNA added with 3 ml of MEM containing 5% FBS. Cells were collected at 24 h, and the cell extracts were subjected to 12.5% polyacrylamide–SDS gel electrophoresis. The proteins were transferred to nitrocellulose sheet and tested for their ability to bind various MAbs detected by binding of ^{125}I-labeled protein A. Lane 1, Purified VSV virion M protein (150 ng) tested with MAb25 to epitope 3; lane 2, extract from vTF1-6,2-infected cells transfected with 15 μg of pTF7-M3 DNA and tested with MAb2 to epitope 1; lane 3, extract from vTF1-6,2-infected cells transfected with 15 μg of pTF7-M3 DNA and tested with MAb3 to epitope 2; lane 4, extract from vTF1-6,2-infected cells transfected with 15 μg of pTF7-M3 DNA and tested with MAb25 to epitope 3.

TABLE 1. Rescue of *ts*O23 M protein mutant by plasmid pTF7-M3 transfected into CV-1 cells infected with the T7 polymerase-expressing vaccinia virus recombinant vTF1-6,2[a]

MOI of *ts*O23	Plasmid pTF7-M3 DNA transfected (μg)	Virus yield (PFU/ml)	
		31°C	39°C
1	None	2.8×10^3	6.2×10^2
	15	5.5×10^5	7.5×10^2
10	None	1.1×10^3	5×10^1
	15	1.3×10^4	$<10^1$

[a] CV-1 cells were infected with *ts*O23 virus at an MOI of 1 or 10 PFU/cell at room temperature for 30 min. Then, the virus inoculum was replaced with 2 ml of MEM containing 5% FBS and incubated at 39°C for 2 h. At 2.5 h after infection, the cells were infected with vTF1-6,2 (recombinant vaccinia virus expressing T7 RNA polymerase) and transfected with 0 or 15 μg of the pTF7-M3 plasmid DNA. The cells were incubated further at 39°C for 14 h. Supernatant culture fluids were then collected, and *ts*O23 virus was titrated by plaque assay on L-cell monolayers at 31 and 39°C.

limited expression of wild-type M protein by pTF7-M3 in the presence of excessive competing *ts*O23-restricted M protein.

Similar results in three repeated experiments indicate that pTF7-M3 driven by the vTF1-6,2 polymerase can synthesize wild-type M protein capable of rescuing the M protein-defective mutant *ts*O23 to an even more significant extent (200-fold). The variability in results of repeated marker rescue experiments was no greater than that shown in Table 1.

Expression of M gene by vaccinia virus-pTF7-M3 recombinant under control of the T7 polymerase promoter. One would expect greater expression of a gene integrated in the vaccinia virus genome than in a transfecting plasmid because the virus can enter virtually all the cells, whereas the plasmid cannot. In developing such a system, Fuerst et al. (9; personal communication) reported higher levels of expression by the HBsAg gene when a vaccinia virus-pTF7IHB-1 recombinant was used to coinfect cells with the T7 polymerase-expressing vTF1-6,2. To produce greater yields of VSV M protein, we also created a vaccinia virus recombinant in which the pTF7-M3 plasmid was integrated into the TK locus of the wild-type vaccinia genome by selection in the presence of BUdR. This recombinant (designated vM3) with T7 polymerase promoter and terminator sequences flanking the M gene was used to coinfect CV-1 cells along with vaccinia virus vTF1-6,2, which expresses T7 polymerase. CV-1 cells coinfected or singly infected with vM3 and vTF1-6,2, each at an MOI of ~15 PFU/cell, were incubated for 24 h at 37°C, and cytoplasmic extracts of disrupted cells were subjected to electrophoresis on 12.5% polyacrylamide–SDS gels. The resolved proteins were then electroblotted onto nitrocellulose sheets and exposed to a mixture of two MAbs (MAb2 and MAb3) directed to epitopes 1 and 2, respectively, of VSV M protein.

No M protein was produced in mock-infected CV-1 cells or CV-1 cells infected with vTF1-6,2 alone (Fig. 4, lanes 2 and 3), but a very small amount of MAb-binding M protein was present in cells infected only with vM3 (lane 4) and in cells infected with wild-type vaccinia virus WR and transfected with plasmid pTF7-M3 (lane 7). In contrast, CV-1 cells coinfected with vM3 and vTF1-6,2 produced very large amounts of M protein, as demonstrated either by Western blotting (lane 5) or by immunoprecipitation of cytoplasm with MAb2 plus MAb3 (lane 8), whereas CV-1 cells infected with vTF1-6,2 and transfected with 30 μg of pTF7-M3 yielded somewhat lesser amounts of immunoblotting M protein (lane 6). Laser densitometry scanning of MAb binding and ^{125}I labeling of M protein (lanes 5 and 6, Fig. 4) revealed that ~280 ng of M protein was synthesized in 3×10^5 CV-1 cells coinfected with vM3 and vTF1-6,2 recombinant vaccinia viruses (MOI of ≈15 for each virus), an amount of M protein synthesis approximately twice that in the same number of CV-1 cells infected with vTF1-6,2 (MOI, ≈30) and transfected with 30 μg of the plasmid pTF7-M3. M protein synthesized by vM3 was poorly immunoprecipitated by MAb25 (directed to epitope 3), confirming the reduced binding of MAb25 to M protein in cloning vectors shown in Fig. 3.

DISCUSSION

Rose and Gallione (22) have cloned and sequenced a cDNA copy of the same VSV Indiana M gene used in these studies. We had previously moved this cDNA clone, received from John Rose, into a pUC8 *lac* fusion vector, where it was readily expressed in *E. coli* as an authentic M protein that reacted by Western blotting with all three epitope-specific MAbs (17). We had been informed by John Rose (personal communication) that he had difficulty in expressing the VSV M gene in the same eucaryotic simian virus 40-based vector which readily expressed the VSV G gene

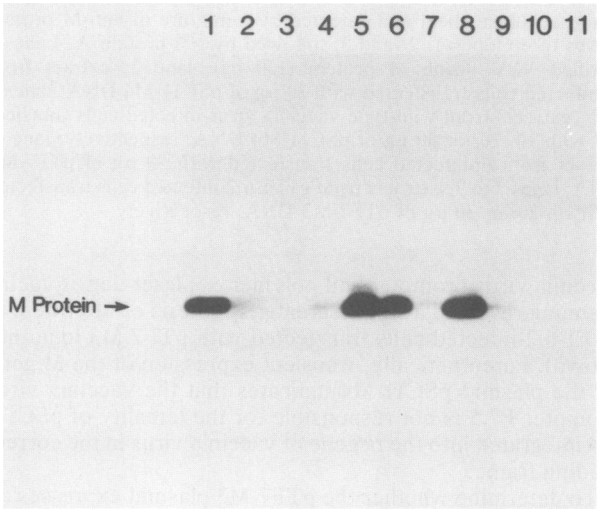

FIG. 4. Western blot analyses of the VSV M protein synthesized by recombinant vaccinia virus. CV-1 cells were coinfected with vTF1-6,2 (recombinant vaccinia virus expressing T7 RNA polymerase) and vM3 (recombinant vaccinia virus containing the VSV M gene) at an MOI of 15 PFU/cell for each virus. At 24 h after infection, cell lysates were prepared, separated by 12.5% polyacrylamide–SDS gel electrophoresis, and transferred to nitrocellulose; M protein was detected by immunoblotting with a mixture of anti-M protein MAbs to epitopes 1 and 2. Lane 1, Purified VSV virion M protein (150 ng); lane 2, extract from mock-infected cells; lane 3, extract from cells infected with vTF1-6,2 alone; lane 4, extract from cells infected with vM3 alone; lane 5, extract from cells coinfected with vTF1-6,2 and vM3; lane 6, extract from vTF1-6,2 (30 PFU/cell)-infected cells transfected with 30 μg of pTF7-M3 DNA; lane 7, extract from wild-type vaccinia virus (30 PFU/cell)-infected cells transfected with 30 μg of pTF7-M3 DNA; lane 8, extract from vTF1-6,2- and vM3-coinfected cells immunoprecipitated by a mixture of MAb2 and MAb3 to epitopes 1 and 2; lane 9, extract from vM3-infected cells immunoprecipitated by a mixture of MAb2 and MAb3; lane 10, extract from vTF1-6,2-infected cells immunoprecipitated by a mixture of MAb2 and MAb3; lane 11, extract from mock-infected cells immunoprecipitated by a mixture of MAb2 and MAb3.

(21). Therefore, it is perhaps not surprising that we encountered the same difficulty in obtaining cDNA M gene recombinants of vaccinia virus capable of expressing intact M protein. There is no obvious reason why integration of the M gene in the correct orientation for expression of M protein should be lethal for vaccinia virus recombinants, whereas M gene cDNA integration in the reverse, nonexpressible orientation resulted in viable TK^- vaccinia virus plaques. The only other evidence we were able to obtain for vaccinia virus lethality by M gene expression was the finding that integration of truncated M gene cDNA, coding for the first 74 amino acids, resulted in viable TK^- vaccinia virus recombinants, but correct integration of the distal two-thirds segment of M gene cDNA also resulted in a nonviable vaccinia virus recombinant. It is possible, of course, that the membrane-binding affinity of the VSV M protein, particularly at the carboxyl-terminal region (16), is responsible for its interference with vaccinia virus maturation. Therefore, we were forced to abandon our use of the pSC11 plasmid as a means for integrating M gene cDNA into a stable coexpression vaccinia virus vector.

It is perhaps of some interest that M gene cDNA integrated in the pSC11 plasmid transfected into wild-type vaccinia virus-infected cells can transiently express moderate quantities of antigenically competent M protein, whereas the integrated M gene cDNA does not and is lethal. One possible clue may reside in the fact that M protein binds to VSV RNP cores and, in so doing, inhibits VSV transcription (4, 6, 8, 19, 26). Conceivably, the M protein synthesized by the vaccinia virus-pSC11-M4 recombinant binds to vaccinia virus DNA, inhibiting a vital transcriptional function resulting in death of the virus.

Of some interest was the finding that the polymerase expressed by wild-type vaccinia virus could use the vaccinia virus P7.5 promoter in the transfected plasmid pSC11-M4, resulting in quite significant synthesis of VSV M protein. However, the vTF1-6,2 T7 polymerase was at least six times more efficient in transcribing M protein mRNA from the T7 promoter of transfected plasmid pTF7-M3. It seems clear that the unique system devised by Fuerst et al. (10) of vaccinia virus T7 polymerase and cotransfected pTF7 plasmids with the T7 polymerase promoter and terminator provides a useful system for expressing foreign genes in eucaryotic cells. It seems remarkable that the M gene driven only by the ϕ10 T7 polymerase promoter can be expressed, albeit minimally, in the complete absence of the T7 RNA polymerase (Fig. 4, lanes 4 and 7). Another somewhat unexpected and unexplained finding in our studies was weak Western blot binding by epitope 3-specific MAb25 to M protein expressed by recombinant plasmids or vaccinia virus in CV-1 cells. Previous studies (17) and ongoing research in our laboratory (unpublished data) reveal expression in *E. coli* by transfecting recombinant plasmids pUC8 and pKK223 of M protein that binds all epitope-specific MAbs as efficiently as does native M protein from VSV virions. It seems likely that recombinant-plasmid-expressed M protein in CV-1 cells undergoes some conformational alteration that results in partial loss of epitope 3 but not of epitopes 1 and 2.

It has been known for some time that wild-type VSV M protein promotes maturation and budding of the virus, properties defective in complementation group III *ts* mutants infecting cells at nonpermissive temperatures (11, 12, 20). The marker rescue experiments presented here with *ts*O23(III) reveal that pTF7-M3-expressed M protein can complement the mutant M protein at the restrictive temperature, increasing some 200-fold the production of VSV *ts*O23(III). These marker rescue studies indicate that the recombinant vaccinia virus-T7 polymerase clones express a biologically competent M protein. Therefore, this technique should provide the means for producing recombinant M gene constructs with deletions or site-directed mutations that will serve as useful probes for exploring the biological and antigenic properties of matrix proteins and other proteins. It is also likely that the new coinfection procedure devised by Fuerst et al. (9) with hybrid vaccinia virus-T7 RNA polymerase systems will result in expression of foreign genes in eucaryotic cells sufficient for detailed chemical and physical studies of genetically altered M proteins.

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