Regulation of Viral Transcription by the Matrix Protein of Vesicular Stomatitis Virus Probed by Monoclonal Antibodies and Temperature-Sensitive Mutants

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The ability of the matrix (M) protein of wild-type vesicular stomatitis virus (VSV) to regulate viral transcription was studied with monoclonal antibodies and temperature-sensitive (*ts*) mutants in complementation group III, the M proteins of which are restricted in transcription inhibition. The marked inhibition of transcription by VSV ribonucleoprotein (RNP) cores complexed with M protein (RNP/M) was reversed by antibody to epitope 1. Antibodies to epitopes 2 and 3 not only failed to reverse the transcription-inhibitory activity of isolated M protein but actually increased M-protein inhibition of transcription in a reconstituted system. Monoclonal antibodies to epitopes 2 and 3 strongly bound to M proteins from all wild-type and *ts*-mutant virions, but monoclonal antibody to epitope 1 completely failed to bind to the M protein of *ts*O23(III) even though it reacted strongly with M proteins of mutants *ts*G31(III) and *ts*G33(III). The M protein of a *ts*O23 revertant (R11) completely recovered its capacity to inhibit transcription and to bind monoclonal antibody to epitope 1, whereas the M proteins of three other revertants remained restricted in their capacity to inhibit transcription and to bind monoclonal antibody to epitope 1. These studies indicate that exposure of epitope 1 on the surface of M protein is essential for inhibiting transcription by VSV RNP cores.

Vesicular stomatitis virus (VSV), the prototype rhabdovirus, is membrane enclosed and contains a ribonucleoprotein (RNP) core (24). Two of the five virally coded VSV proteins are membrane associated: the externally oriented transmembrane glycoprotein, which serves for cell attachment and as the type-specific antigen (15, 21), and the peripheral matrix (M) protein which lines the inner surface of the lipid bilayer in close association with RNP core (16, 28). The three other VSV proteins (N, L, and NS) constitute the RNP core and collectively serve as an RNA-dependent RNA polymerase that sequentially transcribes five messenger RNAs from the negative-strand RNA template (1, 2, 10).

The M protein of VSV plays a regulatory role in virus-directed RNA synthesis in infected cells (7) and serves as an inhibitor in vitro of viral transcription (5, 8, 9, 18, 26). Temperature-sensitive (*ts*) mutants in complementation group III of VSV-Indiana have lesions in the M-protein gene (19); those derived from the Orsay wild-type (*wt*) strain, *ts*O23(III) and *ts*O89(III), contain M proteins completely devoid of transcription-inhibitory activity, and those derived from the Glasgow *wt* strain, *ts*G31(III) and *ts*G33(III), exhibit limited ability to inhibit transcription (5, 26). The M protein of *wt* VSV-Indiana is rich in lysines and arginines (20) and is positively charged (pI of ~ 9.1; 5), which apparently contributes to binding of M protein to phospholipids with negatively charged headgroups (27). Relatively little is known about the interaction of M protein with RNP cores, but this is presumably also electrostatic in nature since inhibition of transcription by M protein is reversed by polyglutamic acid (4) or by high-ionic-strength buffers (26). This RNP-M protein interaction was shown to be weaker for the four group III *ts* mutants (5, 26).

In this study, we report the effects of monoclonal antibodies (mAbs) on the capacity of *wt* M protein to regulate transcription of VSV RNP cores. Also reported are the different capacities of various mAbs reacting with separate epitopes to bind to a mutant M protein incapable of inhibiting transcription but not to other mutant M proteins or revertant M proteins. By these combined immunologic and genetic techniques, we hope to be able eventually to identify the region of the M protein that specifies its transcriptional regulatory activity.

MATERIALS AND METHODS

Virus and cells. The *wt* San Juan strain of VSV (Indiana serotype) was obtained originally from the U.S. Agriculture Research Center, Beltsville, Md. The *ts* group III mutant of VSV designated as *ts*O23(III) and the *wt* from which it was derived (Orsay strain) were kindly provided by A. Flamand, Faculté des Sciences, Université de Paris-Sud, Orsay, France. The mutant *ts*G31(III) and the corresponding *wt* Glasgow VSV strain were kindly provided by C. R. Pringle, Institute of Virology, Glasgow, Scotland, and Pringle's mutant *ts*G33(III) was a gift from Harvey Lodish, Massachusetts Institute of Technology, Cambridge, Mass. Baby hamster kidney (BHK-21) cells were grown to 95% confluency at 37°C in Dulbecco modified Eagle medium supplemented with 10% tryptose-phosphate broth, 10% calf serum, and antibiotics as described previously (3). Plaque-purified VSV was used to infect cell monolayers at a multiplicity of 0.1 PFU per cell. Bullet-shaped virions were harvested at 21 h postinfection and were purified by differential, rate zonal, and equilibrium centrifugation (3). Purified virions were stored in phosphate-buffered saline (pH 7.4) at a concentration of 2 to 4 mg/ml at -70°C until further use.

Preparation of RNP cores. Enzymatically active RNP cores from VS virions were prepared in two different forms as follows. To isolate RNP cores with M protein attached

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(RNP/M), VSV (1 mg/ml) was dialyzed overnight against 10 mM Tris hydrochloride (pH 7.5) to remove salt from the viral suspension and then disrupted with 30 mM octylglucopyranoside (Calbiochem-Behring, La Jolla, Calif.) in 10 mM Tris hydrochloride (pH 7.5). The suspension was allowed to stand at room temperature for 1 h, and the RNP cores were pelleted by centrifuging in an SW50.1 rotor at $189,000 \times g$ for 2 h through a 50% (vol/vol) glycerol pad. The supernatant fluid containing G protein and the lipids was discarded, and the pellet, containing the RNP/M cores, was suspended in 10 mM Tris hydrochloride (pH 8.0).

To isolate RNP cores containing only trace amounts of M protein (RNP), VSV (0.5 mg/ml) was disrupted in 10 mM Tris hydrochloride (pH 8.0)–1% Triton X-100–0.25 M NaCl, and the solution was allowed to stand at room temperature for 30 min. RNP cores were then pelleted by centrifuging in an SW50.1 rotor at $189,000 \times g$ for 2 h through a 50% (vol/vol) glycerol pad. The supernatant fluid containing the lipids, G protein, and almost all the M protein was discarded or used for isolating pure M protein. The pellet, containing the RNP cores, was then suspended in 10 mM Tris (pH 8.0).

Preparation of VSV M protein. VSV M protein was isolated from purified virions by exposing them to 1% Triton X-100 and 0.25 M NaCl in 10 mM Tris hydrochloride buffer (pH 8.0). After removing the RNP by centrifugation at $150,000 \times g$, the M protein was purified by column chromatography on Whatman P11 phosphocellulose as described previously (25).

Preparation of mAbs to M protein of VSV. As reported previously (17), mAbs to M protein of VSV were prepared by fusion of SP2/0 myeloma and spleen cells obtained from BALB/c mice hyperimmunized with purified M protein by techniques described in detail elsewhere (23). Clones producing the antibodies to M protein were selected by solid-phase enzyme-linked immunosorbent assay tests and further subcloned twice by serial dilution of cell suspensions before single cell clones were selected for each final hybridoma. Large amounts of each mAb were obtained by injecting 10^7 cloned hybridoma cells intraperitoneally into BALB/c mice which had been primed 4 weeks before with an intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane). Purified immunoglobulins were obtained from the ascites fluid by protein A-Sepharose column chromatography as described previously (12, 23). The separate antigenic determinants (epitopes) of the M protein were identified by competitive binding of each unlabeled and ^{125}I -labeled mAb to M protein coated on a plastic surface, as described previously (23).

Western blot analysis. VSV (1 μg) was subjected to 12.5% polyacrylamide-sodium dodecyl sulfate (SDS) slab gel electrophoresis (5), and the proteins were transferred by electroblotting onto nitrocellulose sheets (0.1 μM ; Schleicher & Schuell, Inc., Keene, N.H.) as described by Towbin et al. (22). The nitrocellulose sheet was then incubated with buffered bovine serum albumin (3% bovine serum albumin, 0.9% NaCl, 10 mM Tris hydrochloride [pH 7.4]) for 1 h at 37°C and then exposed for 2 h, first to mAbs (10 $\mu\text{g}/\text{ml}$) and then to ^{125}I -labeled *Staphylococcus* protein A (Amersham Corp., Arlington Heights, Ill.) (specific activity, 33 mCi/mg; 0.1 $\mu\text{Ci}/\text{ml}$) in 50 mM Tris (pH 7.4)–10 mM NaCl–5 mM EDTA–0.25% gelatin–0.05% Nonidet P-40. After extensive washing with buffer containing 50 mM Tris (pH 7.4), 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.5% Triton, and 0.1% SDS, nitrocellulose sheets were air-dried and exposed for autoradiography to Kodak X-Omat film at -70°C for several hours with an intensifying screen.

Transcription assays. Transcription assays of RNP cores were performed as described elsewhere (14). Briefly, RNP or RNP/M cores were suspended in transcription buffer containing 7.5 mM MgCl_2 , 10 mM Tris hydrochloride (pH 8.0), 1 mM dithiothreitol (DTT), 1 mM each of ATP, GTP, CTP, and 0.1 mM UTP containing 10 to 100 μCi of [α - ^{32}P]UTP (Amersham) (400 Ci/mmol), and various concentrations of NaCl as indicated for each experiment. Transcription mixtures were incubated for 2 h at 31°C, and the reactions were stopped by adding 1/10 volume of 10 $\times$ SET (1 $\times$ SET is 0.15 M NaCl–5 mM EDTA–50 mM Tris [pH 8.0]). Carrier RNA (50 μg) was then added to the reaction mixture, and the total RNA was precipitated with equal volumes of 10% trichloroacetic acid (TCA). The mixture was kept on ice for 20 min, and the acid-insoluble RNA was collected by filtration through glass-fiber filters (GSA; Whatman, Inc., Clifton, N.J.). The radioactive content in the filter paper was measured by counting the dried filter by using scintillation spectrometry.

For detection of transcription products by gel electrophoresis, the transcription reaction was terminated by the addition of a 1/10 volume of 10 $\times$ SET, and the mixture was extracted with an equal volume of water-saturated phenol. The resulting aqueous phase was extracted from one to three times with CHCl_3 -isoamyl alcohol (24:1 [vol/vol]) adjusted to 0.3 M with sodium acetate, and the RNAs were precipitated at -80°C for 2 h after the addition of three volumes of 95% ethanol. The precipitate was collected by centrifugation at $10,000 \times g$ for 10 min and suspended in 1 $\times$ SET. Transcription products were separated by electrophoresis at 1,600 V on 8 M urea–20% polyacrylamide gels in buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA, and 5 mM boric acid. The products were detected by autoradiograms.

RESULTS

Production of mAbs and specificity of binding to M protein. Hybridomas that secrete mAb to M protein were prepared by standard techniques of fusing SP2/0 myeloma cells with spleen cells of BALB/c mice hyperimmunized with purified VSV M protein, as previously described for G-protein mAbs (23). The characteristics of these M-protein mAbs are described in detail in a separate communication (17). In brief, we obtained 29 separate hybridoma clones, of which 11 secreted mAbs of the immunoglobulin M (IgM) isotype and could not be conveniently purified or used in certain experiments. By competitive binding studies between each pair of mAbs, it was possible to delineate four antigenic determinants (epitopes) on the M protein, two of which (epitopes 2 and 3) exhibited considerable overlap. Epitope 1 was unique and provided high-titered mAbs of the IgG2b isotype, whereas epitope 4 had only a single mAb of the IgM isotype and was discarded. In the experiments presented here we used only three mAbs representing three separate epitopes: mAb2 (epitope 1), mAb3 (epitope 2), and mAb25 (epitope 3).

Figure 1 illustrates Western blots of the three epitope-specific mAbs for VS virion proteins separated by electrophoresis on a 12.5% polyacrylamide-SDS gel and electroblotted onto nitrocellulose. As noted, all three antibodies were highly specific for M protein, with only minimal cross-reaction of mAb3 (epitope 2) and mAb25 (epitope 3) with N protein, far less than 1% of their binding to M protein.

Reactivity with mAbs of RNP/M. M protein appears to have strong affinity for the VSV RNP core. The RNP/M complex is evidently electrostatic in nature since the presence of salt during detergent disruption of the virion enve-

lope also results in dissociation of M protein from the RNP core (26). We prepared RNP/M simply by treating virions with 30 mM β -octylglucopyranoside in the absence of salt; this resulted in cores largely devoid of G protein but with almost a full complement of M protein. Exposure of VS virions to 1% Triton X-100 containing 0.25 M NaCl resulted in RNP cores largely devoid of M protein but retaining full transcription activity and essentially all of proteins N, L, and NS (data not shown). However, trace amounts of M and G proteins (<0.1%) were still present in association with RNP cores exposed to 0.35 M NaCl, and higher salt concentrations result in dissociation of the polymerase from RNP cores (10).

The capacity of mAbs to react with RNP/M cores was determined by an enzyme-linked immunosorbent assay. In these experiments RNP/M, RNP essentially devoid of M, or M protein alone were coated in wells of microtiter plates and reacted with each of the mAbs. It was found that RNP/M cores reacted strongly and as efficiently with all three mAbs as did isolated M protein, suggesting that all three antigenic sites of RNP/M were accessible to each mAb (data not shown). RNP cores prepared in 0.25 M NaCl also reacted slightly with the mAbs, indicating residual M protein could not be completely removed by high salt. Similar data were obtained by binding of 125 I-labeled mAb2, mAb3, and mAb25 to RNP/M, M protein alone, and RNP alone.

It was of interest to determine, in light of the subsequent studies on the effect of mAbs on VSV transcription, whether the mAbs affected the stability of RNP/M protein complexes. To this end, RNP/M cores isolated from virions labeled with 3 H-labeled amino acids were exposed for 2 h to mAb2 (epitope 1) or mAb3 (epitope 2) in a transcription buffer containing 10 mM Tris (pH 8.0), 0.08 M NaCl, 7 mM MgCl_2 , and 1 mM DTT. These mixtures of RNP/M and mAbs were then subjected to rate zonal centrifugation in a 0 to 66% sucrose gradient for 1 h at $125,000 \times g$ in an SW50.1 rotor.

Figure 2 compares the sedimentation profiles of 3 H-

FIG. 1. Western blot analysis of the selective binding to VSV proteins of the mAbs that react with three separate antigenic determinants (epitopes) of the VSV-Indiana M protein. VS virions (1 μ g) were subjected to electrophoresis on a 12.5% polyacrylamide slab gel. The viral proteins were then transferred by electroblotting to nitrocellulose sheets and then reacted with mAbs followed by 125 I-labeled *Staphylococcus* protein A for autoradiography as described in Materials and Methods. Lane 1, Unblotted VSV-Indiana proteins stained with Coomassie brilliant blue; lanes 2 to 4, VSV-Indiana proteins blotted into nitrocellulose and reacted with mAb2 to epitope 1, with mAb3 to epitope 2, and with mAb25 to epitope 3, respectively.

FIG. 2. Velocity gradient centrifugation of VSV-Indiana RNP/M cores before or after exposure to mAbs directed to epitope 1 or epitope 2 of the M protein. RNP/M cores labeled with 3 H-labeled amino acids were prepared by solubilization of VSV (1 mg/ml) in 10 mM Tris hydrochloride (pH 7.4) containing 30 mM β -octylglucopyranoside in the absence of salt to prepare RNP cores with almost a full complement of adherent M protein (RNP/M), as described in Materials and Methods. Aliquots of these RNP/M cores were then incubated at 31°C for 2 h in a buffer containing 10 mM Tris hydrochloride (pH 8.0), 0.08 M NaCl, 7.5 mM MgCl_2 , and 1 mM DTT either in the absence of or containing 100 μ g each of mAb2 directed to epitope 1 or mAb3 directed to epitope 2. The samples were then loaded on a 0 to 66% discontinuous sucrose gradient and centrifuged in an SW50.1 rotor at $125,000 \times g$ for 1 h. Gradient fractions of 0.2 ml each were collected, and their 3 H radioactivity was counted by scintillation spectroscopy. Symbols: $\circ$, RNP/M alone; Δ , RNP/M plus mAb2 (epitope 1); $\bullet$, RNP/M plus mAb3 (epitope 2).

labeled RNP/M complexes alone and in the presence of mAb2 or mAb3. As noted, RNP/M in the absence of antibody sedimented as a sharp peak at a density of 1.30 mg/ml. Interaction of RNP/M with mAb2 or mAb3 resulted in two peaks each, one containing nucleocapsids that sedimented only slightly slower than the control RNP/M, but another population of nucleocapsids reacting with either mAb sedimented much slower. When the content of M protein associated with the rapidly sedimenting peaks was compared by polyacrylamide gel electrophoresis with that in the slowly sedimenting peaks, it was found that 60% of the M protein was removed from the slowly sedimenting RNP/M complex exposed to mAb2 or mAb3 based on ratios of N to M protein (data not shown). Similar results were obtained in a separate experiment in which RNP/M cores were exposed to mAb25 directed to epitope 3 (data not shown). It appears, therefore, that reaction of RNP/M cores with mAbs to three different epitopes removes about 60% of the M protein from about half of the RNP/M cores. The decreased sedimentation rate of RNP/M exposed to mAbs is probably related to greater frictional resistance of uncoiled RNP cores from which M protein has been removed compared with more rapidly sedimenting RNP/M cores that are tightly coiled by the bound M protein (16).

Transcription of the RNP/M complex in the presence or absence of mAbs. It was of interest to determine whether reaction with the three mAbs, which cause partial dissociation of M protein, also affects *in vitro* transcription of

FIG. 3. Differential effects on in vitro transcription by RNP/M cores of mAbs to each of three epitopes of VSV-Indiana M protein under the following conditions. (A) Various salt concentrations and no antibody or constant 140 µg of mAb2 (epitope 1) or mAb3 (epitope 2) per ml; (B) a constant 0.08 M NaCl but increasing concentrations of mAb2 (epitope 1), mAb3 (epitope 2), or mAb25 (epitope 3); and (C) autoradiographic [³²P]UMP-labeled RNA transcripts synthesized by RNP/M cores in the absence of antibody (lane 1) or in the presence of mAb2 to epitope 1 (lane 2), mAb3 to epitope 2 (lane 3), or mAb25 to epitope 3 (lane 4). VSV (1 mg/ml) was solubilized by 30 mM β-octylpyranoside in 10 mM Tris hydrochloride (pH 7.5) in the absence of salt to prepare RNP cores with a full complement of M protein (RNP/M) as described in Materials and Methods. Transcription reaction mixtures (50 µl) in duplicate contained 180 µg of RNP/M in 10 mM Tris hydrochloride (pH 8.0)–7.5 mM MgCl₂–1 mM DTT–1 mM each of ATP, GTP, and CTP–0.1 mM [α-³²P]UTP (10 µCi). Amounts of NaCl and mAbs varied in the different reactions as indicated in each experiment. Reaction mixtures were incubated for 2 h at 31°C after which incorporation of [³²P]UMP into TCA precipitates was measured by scintillation spectroscopy. For analysis of transcripts shown in panel C, RNA synthesized for 2 h was phenol extracted, ethanol precipitated, and analyzed by electrophoresis on 20% polyacrylamide gels as described in Materials and Methods. 1, Leader RNA; 11, 12, 13, 14, oligonucleotide transcripts composed of 11, 12, 13, or 14 nucleotides (see reference 18).

RNP/M. To this end, RNP/M cores were prepared by disrupting VS virions with octylglucoside in the absence of salt. Transcription was carried out in a standard reaction mixture containing 0.08 M NaCl, all four nucleoside triphosphates, and [α-³²P]UTP (5) in the presence or absence of the individual mAbs.

Figure 3 shows that marked inhibition of transcription by M protein in RNP/M cores is considerably reversed by increasing concentrations of NaCl or by mAb2 directed to epitope 1. In the absence of mAb, transcription of RNP/M cores increased progressively at NaCl concentrations increasing from 0.02 to 0.12 M. This effect of salt on RNP/M transcription was greatly augmented in the presence of mAb2 (160 µg/ml) but not by an equal amount of mAb3 (Fig. 3A). In fact, mAb3 partially counteracted the reversal of inhibition of RNP/M by NaCl. Partial reversal of transcription inhibition by 0.12 M NaCl is presumably caused by dissociation of M protein from the RNP/M cores. Stimulation of transcription by mAb2 was noted at every salt concentration (Fig. 3A). Figure 3B demonstrates linear enhancement of RNP/M transcription when increasing concentrations of mAb2 (epitope 1) were present in a regular transcription mixture (0.08 M NaCl) containing RNP/M cores markedly inhibited in transcription. In sharp contrast, mAb3 (epitope 2) and mAb25 (epitope 3) had no effect on inhibited RNP/M transcription at any antibody concentration.

Figure 3C depicts the RNA species synthesized by RNP/M cores transcribed in the absence or presence of the three mAbs as analyzed by electrophoresis on a 20% polyacrylamide slab gel. The primary RNA species synthesized by RNP/M cores in the absence of antibody were leader

RNA and other low-molecular-weight RNA species as described by Pinney and Emerson (18). The presence of mAb2 (epitope 1) dramatically increased RNA synthesis by RNP/M, particularly of high-molecular-weight species representing VSV messengers. The presence of mAb3 and mAb25 did result in some increased synthesis of leader RNA, small RNAs, and mRNAs, but this was quite limited compared with the dramatic effect of mAb2. These results clearly demonstrate the striking capacity of mAb2 (directed to epitope 1) to reverse inhibition of transcription by *wt* VSV M protein compared with minor if any effect by mAbs to two other epitopes.

Effects of mAbs on the transcriptional regulatory activity of M protein reconstituted with RNP cores. In addition to demonstrating that one of three mAbs stimulates transcription of endogenous RNP/M complexes, it was important to determine whether mAbs can affect the transcriptional inhibitory activity of isolated M protein reconstituted with RNP cores essentially free of M protein. In these experiments, RNP cores were prepared from virions exposed to 1% Triton X-100 in the presence of 0.25 M NaCl to remove almost all the endogenous M protein but retaining maximal levels of in vitro transcriptional activity. The M protein was isolated from supernatants fluids after centrifugation of purified virions that had been treated with Triton X-100 and 0.25 M NaCl and then purified by phosphocellulose chromatography (25). Isolated M protein (>98% pure) was incubated for 30 min at room temperature in the regular transcription buffer containing 0.08 M NaCl with or without individual mAbs and then added to RNP cores; the level of transcription was measured by incorporation of [³²P]UMP into TCA-precipitable material.

FIG. 4. Differential effects of mAbs on in vitro transcription by RNP cores reconstituted with M protein alone (A) or with 160 μ g of M protein per ml preincubated with increasing concentrations of mAb2 (epitope 1), mAb3 (epitope 2), or mAb25 (epitope 3) (B). Panel C portrays autoradiographs of [³²P]UMP-labeled transcription products made by RNP cores alone (lane 1), in the presence of 160 μ g of M protein per ml (lane 2), or M protein (160 μ g/ml) preincubated with 140 μ g/ml each of mAb2 to epitope 1 (lane 3), mAb3 to epitope 2 (lane 4), or mAb25 to epitope 3 (lane 5). VSV-Indiana virions (0.5 mg/ml) were solubilized in a buffer of 10 mM Tris hydrochloride (pH 8.0) containing 1% Triton X-100 and 0.25 M NaCl to prepare transcribable RNP cores essentially free of M protein as described in Materials and Methods. Separate suspensions of purified M protein were prepared by phosphocellulose chromatography. Each transcription reaction mixture (50 μ l) in duplicate contained naked RNP cores (180 μ g/ml) in 10 mM Tris hydrochloride (pH 8.0)–0.08 M NaCl–7.5 mM MgCl₂–1 mM DTT–1 mM each of ATP, GTP, and CTP–0.1 mM of [α -³²P]UTP (10 μ Ci). Each transcription reaction was carried out in the absence of M protein, in the presence of M protein alone, or in the presence of M protein (160 μ g/ml) which had been previously incubated at 31°C for 30 min with mAb2, mAb3, or mAb25 reactive with epitopes 1, 2, or 3, respectively. Transcription reaction mixtures were incubated for 2 h at 31°C to determine incorporation of [³²P]UMP into TCA-precipitable RNA as measured by scintillation spectroscopy. For the RNA transcripts shown in panel C, the RNA synthesized for 2 h was phenol extracted, precipitated by ethanol, and analyzed by electrophoresis and autoradiography of 20% polyacrylamide gels as described in Materials and Methods. 1, Leader RNA; 11, 12, 13, 14, oligonucleotide transcripts composed of 11, 12, 13, or 14 nucleotides (see reference 18).

Figure 4 shows transcription reactions and transcription products of RNP cores reconstituted with isolated M protein before or after incubation with mAbs. Increasing concentrations of M protein alone resulted in progressive reduction in RNP transcription to a level ~40% that of M-protein-free RNP (Fig. 4A); transcription inhibition is somewhat greater (~90%) in RNP/M cores isolated in the absence of salt from virions endogenously complexed with M protein (Fig. 3). Such an inhibitory effect of M protein has been attributed to condensation of the RNP core after interaction with M protein (9, 16). Figure 4B shows that the three different mAbs had two contrasting effects on the transcriptional inhibitory activity of M protein reconstituted with RNP cores. Preincubation of M protein with increasing concentrations of mAb2 (epitope 1) resulted in the progressive loss of the capacity of M protein to inhibit transcription; however, this increased level of transcription never reached that of RNP cores devoid of M protein. In marked contrast, incubation of RNP cores with M protein exposed to increasing concentrations of mAb3 (epitope 2) and mAb25 (epitope 3) resulted in the completely opposite effect of considerably enhancing the transcriptional inhibitory activity of M protein to the 90% level or greater observed in the endogenous RNP/M complex. It is important to note that none of the mAbs directly affected transcription of naked RNP cores in the absence of M protein (data not shown). It should also be noted that variation occurred in the capacity of mAb2 to reverse the transcriptional inhibitory activity of M protein, apparently depending on the degree of residual M protein

present on the RNP cores (data not shown); enhancement of transcriptional inhibitory activity was invariable for M protein incubated with mAb3 and mAb25.

Figure 4C illustrates on 20% polyacrylamide gels the RNA species synthesized by RNP cores alone or RNP cores reconstituted with M protein before or after preincubated with the three mAbs. As noted, M protein caused moderate reduction in synthesis of large RNA transcripts but not the leader or other small RNA species. This inhibition of transcription by M protein was completely reversed by preincubation with mAb2 (epitope 1). Quite striking is the degree to which mAb3 (epitope 2) and mAb25 (epitope 3) enhanced the transcriptional inhibitory activity of M protein, resulting in marked diminution in amount of all transcripts except, perhaps, the leader and other small RNA species. These data confirm the striking difference in the action of mAb to epitope 1 compared with those that recognize other epitopes of M protein.

Reactivity of mAbs to M proteins isolated from group III *ts* mutants. The capacity of mAb2 to reverse the inhibitory effect of M protein on VSV transcription led us to investigate whether our mAbs that bind to *wt* VSV M protein would also bind to M proteins of group III *ts* mutants, which are restricted in their capacity to inhibit VSV transcription (5, 26). The group III *ts* mutants available for these studies were *ts*G31 and *ts*G33, derived from the Glasgow strain of VSV-Indiana (19), and *ts*O23, derived from the Orsay strain (13). Since the Glasgow and Orsay *wt* strains differ somewhat by oligonucleotide fingerprints (6) and by nucleotide sequences

FIG. 5. Comparison by Western blot analysis of the capacity of mAb2 (directed to epitope 1) to bind to M proteins of VSV strain Orsay *wt* (lane 1), *tsO23* (lane 2), Glasgow strain *wt* (lane 3), *tsG31* (lane 4), and *tsG33* (lane 5), and the capacity of mAb3 (directed to epitope 2) to bind to VSV M proteins of Orsay strain *wt* (lane 6), *tsO23* (lane 7), Glasgow strain *wt* (lane 8), *tsG31* (lane 9), and *tsG33* (lane 10). The virion proteins of each cloned VSV *wt* strain and *ts* mutants grown at 31°C were subjected to electrophoresis on 12.5% polyacrylamide gels. The virion proteins were then transferred by electroblotting to nitrocellulose sheets for reaction with mAb2 (epitope 1) and mAb3 (epitope 2); bound IgG was detected by reaction with ¹²⁵I-labeled *Staphylococcus* protein A and autoradiography as described in Materials and Methods. Only the M protein of VSV exhibited significant binding of each mAb.

(J. Lenard, personal communication) from the San Juan strain of VSV-Indiana, the M protein of which was used to prepare our mAbs, it was also necessary to test each of the three *wt* strains. It is also important to note that the M proteins of *tsO23* are completely devoid of transcriptional inhibitory activity compared with the M proteins of *tsG31* and *tsG33*, which have more limited transcriptional inhibitory activity (5, 26).

In these studies *wt* and *ts* mutant virions were grown in BHK-21 cells at the permissive temperature (31°C), and their proteins were separated by electrophoresis on 12.5% polyacrylamide-SDS gels. The viral proteins were then transferred to a nitrocellulose sheet to test their reactivity with mAbs by Western blot analysis, using ¹²⁵I-labeled *Staphylococcus* protein A to locate the bound immunoglobulin.

Figure 5 shows equivalent binding of mAb2 (epitope 1) and mAb3 (epitope 2) to M proteins of *tsG31* and *tsG33*, as well as to the M proteins of the Glasgow and Orsay wild types. In striking contrast, the M protein from *tsO23* failed to bind any detectable mAb2 immunoglobulin despite intact capacity to bind mAb3. M proteins of all *wt* and *ts* mutants, including *tsO23*, avidly bound mAb25 to epitope 3 (data not shown). These experiments were confirmed by repeated Western blots and enzyme-linked immunosorbent assay techniques.

These results demonstrate that epitope 1 is not present on the surface of the *tsO23* M protein, which is also incapable of inhibiting VSV transcription.

Reactivity with mAbs and transcription inhibition by M proteins of *tsO23*(III) revertants. It seemed essential to compare the M proteins of *tsO23* and its revertants for their capacity to bind mAbs and to inhibit VSV transcription. If these two phenotypic expressions of *tsO23* are related, one

might expect simultaneous reversion of these two properties. To address this question, we proceeded to isolate revertants of *tsO23* by cloning viruses from individual plaques of *tsO23* plated on L-cell monolayers incubated at 39°C. Orsay *wt*, *tsO23*, and four revertants (R11, R12, R13, and R14) were titrated by plaque assay on L-cell monolayers incubated at 31°C (permissive) and 39°C (restrictive). Cloned stocks of *wt* and all four revertant viruses all had titers of $\sim 1.0 \times 10^9$ PFU/ml at 31°C and titers of 8.0×10^8 to 1.8×10^9 PFU/ml at 39°C; the mutant *tsO23* had titers of 1.5×10^9 PFU/ml at 31°C and 5.0×10^3 PFU/ml at 39°C (data not shown).

The M proteins from virions of *wt*, *tsO23*, and all four revertants were tested by Western blot analysis for their reactivity with mAb2 (epitope 1) and mAb3 (epitope 2). Figure 6 shows the autoradiographs of ¹²⁵I-labeled protein A binding on nitrocellulose to monoclonal IgG reacting to M proteins of *wt*, *tsO23*, or revertant virions. As noted above, *tsO23* M protein showed no detectable binding of mAb2 compared with its marked affinity for *wt* M protein. Of the four revertants, only R11 reverted to the *wt* phenotype and showed strong binding of mAb2 compared with R12, R13, and R14, the M proteins of which bound no detectable mAb2. By comparison, mAb3 bound equally well to M proteins from the *wt*, *tsO23*, and all four revertants (Fig. 6), as did the epitope 3 antibody mAb25 (data not shown). The fact that M protein in only one of four *tsO23* revertants reacquires reactivity with mAb2 suggests that reversion need not expose epitope 1.

These data provided another possible means for correlating exposure of epitope 1 to mAb2 with the capacity of M protein to inhibit VSV transcription. Therefore, inhibition of transcription at a high concentration (0.8 mg/ml) of VSV (5)

FIG. 6. Comparison by Western blot analysis of the capacity of mAb2 (directed to epitope 1) to bind to M proteins of VSV Orsay strain *wt* (lane 1), *tsO23* (lane 2), revertant R12 (lane 3), revertant R13 (lane 4), revertant R11 (lane 5), and revertant R14 (lane 6), and the capacity of mAb3 (directed to epitope 2) to bind to M proteins of VSV Orsay *wt* (lane 7), *tsO23* (lane 8), revertant R12 (lane 9), revertant R13 (lane 10), revertant R11 (lane 11), and revertant R14 (lane 12). The virion proteins of each cloned VSV Orsay strain *wt*, *tsO23*, and the four revertants of *tsO23* were subjected to electrophoresis on 12.5% polyacrylamide gels, followed by transfer by electroblotting to nitrocellulose sheets for reaction with mAb2 (epitope 1) and mAb3 (epitope 2). IgG bound to M protein was detected by reactivity with ¹²⁵I-labeled *Staphylococcus* protein A followed by autoradiography as described in Materials and Methods. None of the VSV proteins other than M protein was significantly labeled.

was compared for Orsay *wt*, *tsO23*, and revertants R11 and R13. Orsay *wt* M protein inhibits transcription by ~90% (mean of six determinations), compared with no inhibition at all by M protein of *tsO23*, which also fails to bind mAb2 (Table 1). The M protein of the R11 revertant of *tsO23* reverted almost completely to the *wt* phenotype based on 84.6% inhibition of transcription and marked binding by mAb2. On the other hand, the M protein of R13, which showed no reversion in its capacity to bind mAb2, recovered only 60% of its capacity to inhibit VSV transcription.

These experiments suggest that when epitope 1 reappeared on the surface of the M protein of the *tsO23* R11 revertant, the capacity of its M protein to inhibit VSV transcription is restored to about the *wt* level. However, the R13 revertant appears to provide an example of a secondary mutation in *tsO23* that restores infectivity at the restrictive temperature and partially restores transcriptional inhibitory activity of the M protein without the reappearance of epitope 1. Notwithstanding, these data indicate that the domain of M protein recognized by epitope 1 mAb is at least partially responsible for regulating VSV transcription.

Comparative effects of mAbs on transcription by *tsG33*(III) and Glasgow VSV *wt*. In what was originally designed as a negative control experiment, we set out to test the effects of mAbs on transcription inhibition by the partially restricted M protein of *tsG33*(III) compared with the Glasgow *wt* from which this mutant was derived. RNP/M were isolated by exposure to octylglucoside in the absence of salt from *wt* and *tsG33* virions both grown at permissive temperature (31°C). The effects on transcription of 160 µg each of mAb2 (epitope 1) and mAb3 (epitope 2) per ml were tested under conditions of increasing salt concentrations similar to those shown in Fig. 3A. Figure 7 compares the transcription reactions of RNP/M cores from *wt* (Fig. 7A) and *tsG33* (Fig. 7B) in the absence or presence of mAb2 or mAb3 under the stimulating conditions of concentrations of NaCl increasing from 0.02 to 0.1 M. As noted, increasing salt concentrations enhanced the transcriptional activity of both *wt* and *tsG33* RNP/M cores, but this effect, even in the absence of antibody, was significantly greater for *tsG33*. As noted previously, mAb2 augmented the transcriptional activity of *wt* RNP/M cores, whereas mAb3 did not. However, the most striking effects of both mAbs were marked augmentation of transcription by

FIG. 7. Comparative effects of mAbs on transcriptional activity at different salt concentrations of RNP/M cores isolated from VSV virions of Glasgow *wt* (A) or its mutant *tsG33*(III) (B). Cloned virions (1 mg/ml) of the VSV-Glasgow *wt* and its mutant *tsG33*(III), each grown at 31°C, were solubilized in a buffer of 10 mM Tris hydrochloride (pH 7.5) containing 30 mM β -octylglucopyranoside in the absence of salt to prepare RNP/M as described in Materials and Methods. Transcription reactions were carried out in mixtures containing RNP/M cores (180 µg/ml) in 10 mM Tris hydrochloride (pH 8.0)–7.5 mM $MgCl_2$ –1 mM DTT–1 mM each of ATP, GTP, and CTP–0.1 mM [α - ^{32}P]UTP (10 µCi). Each transcription reaction contained various concentrations of NaCl (as indicated) and 160 µg of mAb2 (directed to epitope 1) (▲) or mAb3 (directed to epitope 2) (●) per ml or no antibody (○). Reaction mixtures were incubated at 31°C for 2 h, and the incorporation of [^{32}P]UMP into TCA-precipitable material was measured by scintillation spectroscopy.

tsG33 RNP/M cores to levels comparable to those of RNP cores in the absence of M protein. The addition of mAb2 to the reaction mixture stimulated the transcriptional activity of both *wt* and *tsG33* RNP/M cores at all salt concentrations. Quite remarkably, mAb3 directed to epitope 2, which had no effect on transcription by *wt* RNP/M cores, greatly enhanced the transcriptional activity of *tsG33* RNP/M cores.

The results of these experiments with *tsG33* do not lend themselves to simple interpretations. Clearly, the M proteins of VSV *wt*, *tsO23*, and *tsG33* differ considerably in their capacity to inhibit VSV transcription and their reactivity with non-cross-reacting mAbs. The M protein of *tsG33* has been reported by Wilson and Lenard (26) to exhibit weaker interaction with RNP cores than does the *wt* M protein. This may explain the anomalous effect of epitope 2 mAb3 which, on reacting with *tsG33* M protein in the RNP/M complex, may dissociate the M protein from RNP or may alter its conformation, thus resulting in complete reversal of its capacity to inhibit transcription. The mAb reactive with epitope 1 may cause the same effects in binding or conformation of M proteins, but in this case affecting *wt* as well as mutant *tsG33* M protein.

DISCUSSION

The basic objectives of the experiments reported here were to study the topological and conformational properties of the VSV M protein that are responsible for regulating transcription by RNP cores. Two probes were used in these preliminary studies. mAbs to *wt* M protein and M proteins

TABLE 1. Comparative transcription inhibition and binding by three distinct mAbs to the M proteins isolated from *wt*, mutant, and revertant Orsay strain viruses

Virus	% Transcription inhibition ^a	Epitope binding ^b		
		mAb2	mAb3	mAb25
<i>wt</i> Orsay	90.5 ± 6.8 ^c	+	+	+
<i>tsO23</i>	0	0	+	+
Revertant 11	84.6 ± 11.1	+	+	+
Revertant 13	60.6 ± 15.5	0	+	+

^a Transcription reaction mixtures contained virions (0.8 mg/ml), 10 mM Tris (pH 8.0), 0.2% Triton X-100, 7.5 mM $MgCl_2$, 0.14 M NaCl, 1 mM DTT, 1 mM each of ATP, CTP, and GTP, and 0.1 mM of [α - ^{32}P]UTP (10 µCi). Reaction mixtures were incubated at 31°C for 2 h, and the incorporation of [^{32}P]UMP into TCA-insoluble material was measured. The percent inhibition was calculated by considering the transcription level for *tsO23* virions of 55×10^4 cpm as completely uninhibited.

^b +, Strong binding by Western blot analysis of mAbs to each of three M-protein epitopes; 0, no detectable binding of mAb (see Fig. 5 and 6 for examples of each).

^c Standard deviation from six separate experiments with three sets of virus stocks.

from *ts* mutants partially or completely restricted in transcription regulation. mAbs to three distinct antigenic determinants affected *in vitro* transcription of *wt* RNP/M cores in widely divergent ways. Antibody directed to one determinant (epitope 1) greatly stimulated transcription by the inhibited *wt* RNP/M core complex. mAbs to epitopes 2 and 3 had the directly opposite effect of considerably enhancing the inhibitory activity of M protein, possibly by steric hindrance of the transcriptase as it ostensibly moves along the template (11). Epitope 1 was not present in the M protein of *tsO23* but was in a revertant in which transcription inhibition was almost completely restored but not in another revertant in which transcription inhibition was only partially restored. Other mutants from the Glasgow *wt* strain, *tsG31* and *tsG33*, contained M proteins with functional epitope 1 that retained partial ability to inhibit RNP core transcription. This partial transcriptional inhibitory activity of the M protein of *tsG33* was completely reversed by mAbs directed to both epitopes 1 and 2. It seems quite likely from these data that mutations in the VSV M-protein gene occurring at different regions of the cistron result in phenotypic differences based on conformational changes in M protein that alter its binding affinity to RNP cores. The different properties of *tsO23* revertants strongly suggest secondary mutations at various sites which partially or completely restore conformational changes in the mutant M protein affecting RNP binding or transcription inhibition or both.

The capacity of mAbs to epitopes 2 and 3 to bind to RNP/M cores indicates that these antigenic determinants of the *wt* M protein are not hidden by complexing with RNP. Equally efficient binding to *wt* RNP/M cores by mAb2 indicates that epitope 1 is also quite accessible to antibody despite the fact that this epitope, absent in *tsO23*, is apparently the critical M-protein domain responsible for transcription inhibition. These data tend to obscure, rather than clarify, the mechanism by which mAb2 reverses transcription inhibition by *wt* M protein. It is conceivable that mAb2 binds to a domain of the M protein close, but not identical, to the RNP-binding and polymerase-inhibitory site of the *wt* M protein. This postulate is supported by evidence that preincubation of M protein with mAb2 was able to reverse only to some extent the transcriptional inhibitory activity and, perhaps, the RNP-binding affinity of *wt* M protein. Of course, the major evidence for juxtaposition or identity of the RNP-binding and transcription-inhibitory sites of *wt* M protein is the complete absence of both transcription inhibition and mAb2 binding in the M protein *tsO23* and parallel restoration of these functions in a *tsO23* revertant.

Definitive identification of the M-protein site responsible for transcription inhibition of VSV RNP must await detailed biochemical studies which may be difficult to perform if secondary structure of the protein is the dominant feature. Fortunately, the M protein has no disulfide bonds because it contains only a single cysteine residue (20). The fact that monoclonal antibodies bind quite efficiently to SDS-denatured M protein suggests a significant role for primary amino acid sequences rather than specific conformational attitudes for certain functions. We have some preliminary evidence, based on chemically and enzymatically cleaved peptides, that transcriptional inhibitory activity and epitope 1 are both located in the amino-terminal third of the *wt* M protein (J. R. Ogden et al., research in progress). The amino-terminal region of the M protein is rich in hydrophilic amino acids and particularly lysines which may contribute to the basic nature (pI of ~9.1) of the M protein (5). These data have led Rose and Gallione (20) to postulate that the amino

terminus of the VSV-Indiana M protein is responsible for its binding to the negatively charged RNP cores. mAbs recognizing these or adjacent domains could provide useful probes for studying interaction with RNP cores of *wt* and mutant M proteins and relevant peptides. We are also undertaking preparation of cDNA clones and expression vectors of *wt*, mutant, and revertant M-protein genes to locate critical M-protein sites that determine mAb binding, RNP binding, and transcription inhibition, always mindful of the potential and critical role played by secondary and tertiary structure in determining the various functions of M proteins. Such studies will hopefully lead to a better understanding of the critical role played by M proteins of various viruses in the assembly of virions and regulation of transcription.

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LITERATURE CITED

1. Abraham, G., and A. K. Banerjee. 1976. Sequential transcription of the genes of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* 73:1504-1508.
2. Ball, L. A., and C. H. White. 1976. Order of transcription of genes of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* 73:442-446.
3. Barenholz, Y., N. F. Moore, and R. R. Wagner. 1976. Enveloped viruses as model membrane systems: microviscosity of vesicular stomatitis virus and host cell membranes. *Biochemistry* 15:3563-3570.
4. Carroll, A. R., and R. R. Wagner. 1978. Reversal by certain polyanions of an endogenous inhibitor of the vesicular stomatitis virus-associated transcriptase. *J. Biol. Chem.* 253:3361-3363.
5. Carroll, A. R., and R. R. Wagner. 1979. Role of the membrane (M) protein in endogenous inhibition of *in vitro* transcription by vesicular stomatitis virus. *J. Virol.* 29:134-142.
6. Clewley, J. P., D. H. L. Bishop, C.-Y. Kang, J. Coffin, W. E. Schnitzlein, M. E. Reichmann, and R. E. Shope. 1977. Oligonucleotide fingerprints of RNA species obtained from rhabdoviruses belonging to the vesicular stomatitis virus subgroup. *J. Virol.* 23:152-166.
7. Clinton, G. M., S. P. Little, F. S. Hagen, and A. S. Huang. 1978. The matrix (M) protein of vesicular stomatitis virus regulates transcription. *Cell* 15:1455-1462.
8. Combard, A., and C. Printz-Ané. 1979. Inhibition of vesicular stomatitis virus transcriptase complex by the virion envelope M protein. *Biochem. Biophys. Res. Commun.* 88:117-123.
9. De, B. P., G. B. Thornton, D. Luk, and A. K. Banerjee. 1982. Purified matrix protein of vesicular stomatitis virus blocks viral transcription *in vitro*. *Proc. Natl. Acad. Sci. USA* 79:7137-7141.
10. Emerson, S. U. 1976. Vesicular stomatitis virus: structure and function of virion components. *Curr. Top. Microbiol. Immunol.* 73:1-34.
11. Emerson, S. U. 1982. Reconstitution studies detect a single polymerase entry site on the vesicular stomatitis virus genome. *Cell* 31:635-642.
12. Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. *Immunochimistry* 15:429-436.
13. Flamand, A. 1970. Etude génétique du virus de la stomatite vésiculaire: classement de mutants thermosensible spontanés en groupes de complémentation. *J. Gen. Virol.* 8:187-195.
14. Grinnell, B. W., and R. R. Wagner. 1984. Nucleotide sequence and secondary structure of VSV leader RNA and homologous DNA involved in inhibition of DNA-dependent transcription. *Cell* 36:533-543.
15. Kelley, J. R., S. U. Emerson, and R. R. Wagner. 1972. The glycoprotein of vesicular stomatitis virus is the antigen that give

- rise to and reacts with neutralizing antibody. *J. Virol.* **10**:1231–1235.
16. Newcomb, W. W., G. J. Tobin, J. J. McGowan, and J. C. Brown. 1982. In vitro reassembly of vesicular stomatitis virus skeletons. *J. Virol.* **41**:1055–1062.
 17. Pal, R., B. W. Grinnell, R. M. Snyder, J. R. Wiener, W. A. Volk, and R. R. Wagner. 1985. Monoclonal antibodies to the M protein of vesicular stomatitis virus (Indiana serotype) and to a cDNA M gene expression product. *J. Virol.* **55**:298–306.
 18. Pinney, D. F., and S. U. Emerson. 1982. In vitro synthesis of triphosphate-initiated *N*-gene mRNA oligonucleotides is regulated by the matrix protein of vesicular stomatitis virus. *J. Virol.* **42**:897–904.
 19. Pringle, C. R. 1977. Genetics of rhabdoviruses, p. 239–289. In "H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 9. Plenum Press, New York.
 20. Rose, J. K., and C. J. Gallione. 1981. Nucleotide sequences of the mRNA's encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. *J. Virol.* **39**:519–528.
 21. Schloemer, R. H., and R. R. Wagner. 1975. Association of vesicular stomatitis virus glycoprotein with virion membrane: characterization of the lipophilic tail fragment. *J. Virol.* **16**:237–249.
 22. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
 23. Volk, W. A., R. M. Snyder, D. C. Benjamin, and R. R. Wagner. 1982. Monoclonal antibodies to the glycoprotein of vesicular stomatitis virus: comparative neutralizing activity. *J. Virol.* **42**:220–227.
 24. Wagner, R. R. 1975. Reproduction of rhabdoviruses, p. 1–93. H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 4. Plenum Press, New York.
 25. Wiener, J. R., R. Pal, Y. Barenholz, and R. R. Wagner. 1983. Influence of the peripheral matrix protein of vesicular stomatitis virus on the membrane dynamics of mixed phospholipid vesicles: fluorescence studies. *Biochemistry* **22**:2162–2170.
 26. Wilson, T., and J. Lenard. 1981. Interaction of wild-type and mutant M protein of vesicular stomatitis virus with nucleocapsids *in vitro*. *Biochemistry* **20**:1349–1354.
 27. Zakowski, J. J., W. A. Petri, and R. R. Wagner. 1981. Role of the matrix protein in assembling the membrane of vesicular stomatitis virus: reconstitution of matrix protein with negatively-charged phospholipid vesicles. *Biochemistry* **20**:3902–3907.
 28. Zakowski, J. J., and R. R. Wagner. 1980. Localization of membrane-associated proteins in vesicular stomatitis virus by use of hydrophobic membrane probes and cross-linking reagents. *J. Virol.* **36**:93–102.