Transcription Inhibition Site on the M Protein of Vesicular Stomatitis Virus Located by Marker Rescue of Mutant *tsO23*(III) with M-Gene Expression Vectors

YAN LI, LIZHONG LUO, AND ROBERT R. WAGNER*

Department of Microbiology and Cancer Center, University of Virginia School of Medicine,
Charlottesville, Virginia 22908

Received 19 December 1988/Accepted 20 February 1989

The matrix (M) protein of vesicular stomatitis virus serves as an endogenous inhibitor of viral transcription, a function missing or deficient in M proteins of temperature-sensitive (*ts*) mutants assigned to complementation group III. Previous studies with mutant *tsO23*(III) and vaccinia virus M-gene expression vectors revealed that the temperature-sensitive phenotype is due to a mutation leading to substitution of phenylalanine for leucine at amino acid III, whereas loss of the major antigenic determinant (epitope 1) of the mutant M protein results from the substitution of glutamic acid for the wild-type amino acid glycine at position 21 (Y. Li, L. Luo, R. M. Snyder, and R. R. Wagner, J. Virol. 62:3729-3737, 1988). We demonstrate here that transcription inhibition activity is restored to rescued *tsO23* virus only when the rescuing vaccinia virus recombinant expresses M protein with glycine and not glutamic acid at amino acid 21. These experiments indicate the importance of the conformational integrity of the amino-terminal domain in determining the capacity of the vesicular stomatitis virus M protein to down regulate endogenous transcription.

The vesicular stomatitis virus (VSV) matrix (M) protein plays an essential role in virion assembly (9, 11, 12) and a negative role in regulating endogenous viral transcription (1, 2, 13). The extremely basic, lysine-rich N-terminal region of the M protein seems a likely candidate site for interaction with genomic ribonucleoprotein (RNP) cores in down regulating RNA synthesis. Studies with an epitope-specific monoclonal antibody (MAb) and proteolytically cleaved M protein fragments (7, 8), as well as homologous synthetic oligopeptides (10), indicate that the transcription inhibition activity of the wild-type (*wt*) VSV M protein is located within the first 31 N-terminal amino acids. Widely used in these studies are complementation group III temperature-sensitive (*ts*) mutants, the M proteins of which have greatly reduced or no capacity to inhibit VSV transcription (1, 2, 13); RNA sequencing by primer cDNA extension of the M genes of these mutants revealed base changes leading to amino acid substitutions widely distributed along the M protein (6). One of these mutants, *tsO23*, was of particular interest to us because its M protein completely lost a major antigenic determinant (epitope 1) which is the site for binding of MAb that reverses inhibition of VSV transcription (7, 8, 10). Unfortunately, *tsO23* has three widely separated mutations leading to amino acid substitutions: Gly→Glu at position 21, Leu→Phe at position 111, and His→Tyr at position 227 (see Fig. 1).

To identify more precisely the regions of the VSV M protein that serve as determinants for binding epitope 1-specific MAb, for conversion to the temperature-sensitive phenotype, and for mediating transcription inhibition, we have cloned M-gene cDNAs of *wt* VSV-Indiana (Orsay strain) and its mutant *tsO23* in procaryotic and eucaryotic expression vectors; we have also introduced by primer extension site-specific mutations in these clones and constructed *wt*-mutant chimeras (4, 5). Expression in CV-1 cells of *wt* or mutant M genes cloned in plasmids under control of

the T7 polymerase promoter and driven by T7 polymerase expressed by a vaccinia virus recombinant enabled us to determine by Western blot (immunoblot) analysis that epitope 1 is specified by glycine and not by glutamic acid, alanine, or proline at position 21 of M protein (5). Marker rescue experiments, in which transfecting plasmids expressing M-gene constructs were tested for their capacity to complement *tsO23* in cells infected at restrictive temperature, demonstrated that the temperature sensitivity phenotype of mutant *tsO23* is attributable to a phenylalanine substituted for a leucine at position 111 in *wt* VSV (5). We were unable, in these earlier studies, to determine which mutation in *tsO23* M protein is responsible for loss of its capacity to inhibit viral transcription, because the available vectors expressed insufficient amounts of mutant or *wt* M proteins to test in a reconstituted in vitro VSV transcription system. Also, a recurring problem is insolubility of the isolated M protein, particularly *ts* mutant M proteins, which makes assays for their capacity to inhibit RNA synthesis in a reconstituted system quite difficult.

The capacity of certain vector-expressed M proteins to rescue *tsO23* in cells coinfecting at restrictive temperature provided a possible means of producing VSV virions containing genetically altered M protein. The only constraint in this experiment is that leucine must be present at amino acid 111 of the vector-expressed M protein to make *tsO23* rescuable. This procedure allowed us to test the hypothesis that transcription inhibition by virion-associated M protein requires the presence of glycine, and not glutamic acid, at position 21.

To address the questions of which amino acids substituted at the three mutational sites in *tsO23* M protein inactivate its capacity to inhibit VSV transcription and, conversely, which of the three corresponding amino acids in *wt* VSV M protein provides its transcription inhibition activity, we used the procedure of Fuerst et al. (3) to construct four vaccinia virus recombinants which express *wt* or mutant M proteins in cells coinfecting with the T7 polymerase-expressing vaccinia virus

* Corresponding author.

FIG. 1. Constructs of *wt* and *tsO23* M genes in pTF7 plasmids flanked by left and right segments of the thymidine kinase gene (TK_L and TK_R) as well as the T7 phage promoter (Φ10) and terminator (TΦ) used for creation of vaccinia virus recombinants vOM79(*wt*), vOM79(Glu21), vMts23, and vMts23(R2). Construction of M-gene plasmids with site-directed mutants has been previously described (4). Recombinants were then prepared by infecting CV-1 cells with *wt* vaccinia virus (WR strain) at a multiplicity of 0.05 PFU per cell and then transfecting them with calcium phosphate-precipitated plasmid DNA: pTF7-OM79 (*wt*), pTF7-OM79(Glu 21), pTF7-*tsM23* (*tsO23*) and pTF7-*tsM23*(R2) (see reference 5 for details). Vaccinia virus virions harvested 48 h postinfection were screened for TK⁻ recombinants by plaque assay on TK⁻ human 143 cells in the presence of 5-bromodeoxyuridine (25 µg/ml). The presence of M genes in vaccinia virus recombinants was determined by dot blot hybridization with ³²P-labeled M-gene cDNA.

vector vTF1-6,2. We used vaccinia virus recombinants with integrated plasmid pTF-OM79, pTF-OM79(Glu21), pTF7-*tsM23*, or pTF7-*tsM23*(R2) rather than transfecting cells with the plasmids alone, because earlier studies have revealed yields of expressed M protein 4 to 15 times greater in cells coinfecting with vaccinia virus M-gene recombinants (5). Each of these recombinants was created by integration into the vaccinia virus thymidine kinase locus of the respective plasmids with *wt* or mutant M genes flanked by the T7 polymerase promoter and terminator and then by transfection of vaccinia virus-infected CV-1 cells and selection of 5-bromodeoxyuridine-resistant recombinants (5). Figure 1 portrays the vaccinia virus M-gene recombinants, designated vOM79(*wt*), vOM79(Glu21), vMts23, and vMts23(R2), and shows the location on expressed M proteins of the amino acids at positions 21, 111, and 227.

Mutant *tsO23* virus was produced by marker rescue in cells infected at restrictive temperature (39°C) and coinfecting with vaccinia virus recombinants expressing Leu-111 M proteins as previously described (4, 5) except that production was on a much larger scale. BHK-21 cells in roller bottles (3 × 10⁸ cells per bottle) were infected with *tsO23* at a multiplicity of 1 PFU per cell at 31°C for 30 min to allow adsorption. The virus inoculum was then replaced with Dulbecco modified Eagle minimum essential medium with 5% fetal bovine serum and incubated at 39°C for 2 h. At 2.5 h postinfection, the cells were coinfecting with the vaccinia virus vTF1-6,2 expressing the T7 bacteriophage RNA polymerase as well as the vaccinia virus recombinants containing each of the four M-gene constructs shown in Fig. 1, each at a multiplicity of 15 PFU per cell. After further incubation of the triply infected cells for 14 h, the rescued *tsO23* virus released into the surrounding medium was collected, titrated by plaque assay at 31 and 39°C, and purified by rate zonal and equilibrium centrifugation as previously described (8).

Because of the marked leakiness of *tsO23*, it was essential to determine the relative amounts of *wt* M protein compared with the amount of mutant M protein still present in *tsO23* rescued by coinfection at restrictive temperature with vOM79 (*wt*). To this end, vOM79-rescued *tsO23* was subjected to electrophoresis on 12.5% polyacrylamide-sodium dodecyl sulfate slab gels and electroeluted onto nitrocellulose paper for Western blot analysis with MABs detected by ¹²⁵I-labeled staphylococcal protein A. The Glu-21 mutant M protein can be readily detected by its lower mobility and its reactivity with epitope 2-specific MAB 3 and failure to recognize epitope 1-specific MAB 2, whereas the more rapidly migrating Gly-21 *wt* M protein readily binds to epitope 1-specific MAB 2. Laser density scanning revealed that *tsO23* rescued at restrictive temperature by vOM79 contained 19.2 µg of mutant M protein per ml and only 1.3 µg of *wt* M protein per ml in the rescued virions (data not shown). Therefore, these experiments were performed with rescued *tsO23* virions in which only ~6.7% of the M protein was of *wt* phenotype.

The three purified *tsO23* preparations rescued by coinfection with vaccinia virus M-gene recombinants and a fourth *tsO23* preparation unrescued by a coinfecting vaccinia virus Phe-111 M-gene recombinant were tested for their capacity to transcribe VSV mRNA in vitro. The transcription inhibition activity of the endogenous mutant M proteins was determined by the method of Carroll and Wagner (1), in which VSV RNA synthesis in vitro is measured by [³²P] UMP incorporation at 31°C for 90 min in a complete transcription mixture containing virus at protein concentrations varying from 0.1 to 1.0 mg/ml. This procedure measures the capacity of increasing concentrations of virion-associated M protein to inhibit the transcription activity of increasing concentrations of RNP cores. If the M protein being tested is devoid of transcription inhibition activity, RNA synthesis will increase by an amount commensurate with the increased concentrations of RNP template and its polymerase.

Figure 2 compares in vitro transcription of rescued *tsO23* virions containing RNP-associated M proteins expressed by the four vaccinia virus-M-gene constructs shown in Fig. 1. As previously described (1), no transcription inhibition activity was exhibited by mutant M-protein present in leaky *tsO23* virions released from cells coinfecting with the vaccinia virus recombinant vMts23 expressing Phe-111 M protein not capable of rescuing *tsO23* (5). A similar pattern of increasing levels of RNA synthesis at increasing virus concentrations was exhibited by *tsO23* rescued by coinfection with revertant vMts23(R2) expressing M protein with Phe-111→Leu amino acid substitution. In both of these reactions, RNA synthesis increased almost linearly with virus concentrations increasing from 0.1 to 1.0 mg/ml. In sharp contrast, the transcriptase activity of *tsO23* virus rescued by the *wt* M protein expressed by coinfecting vOM79 was drastically inhibited despite increasing concentrations of RNP template and polymerase. However, when *tsO23* was rescued by vOM79(Glu21), which expresses the same *wt* M protein except for a Gly-21→Glu substitution, the transcription inhibition activity of the rescuing M protein was completely reversed, as shown by a linear increase in RNA synthesis (Fig. 2).

These data clearly show that loss of transcription inhibition activity by the *tsO23* M protein is due to the substitution of glutamic acid for glycine at position 21. Moreover, the same Gly-21→Glu substitution was previously shown to result in a mutant M protein devoid of epitope 1, the antigenic determinant for binding of the MAB that reverses

FIG. 2. Transcription in vitro at increasing concentrations of *tsO23* virus rescued by M proteins expressed by coinfecting vaccinia virus recombinants vOM79 (*wt*) (▲), vOM79(Glu21) (*), vMts23 (○), and vMts23(R2) (■). Transcription assays of the rescued *tsO23* viruses was performed at the indicated increasing concentrations of viral protein (1). The *tsO23* virus containing different M proteins was suspended in transcription buffer containing 50 mM Tris hydrochloride (pH 8.0); 5 mM MgCl₂; 4 mM dithiothreitol; 100 mM NaCl; 0.025% Triton X-100; 1 mM each of ATP, GTP, and CTP; and 0.1 mM UTP containing 20 μCi of [³²P]UTP (Amersham Corp.; 400 Ci/mmol). Duplicate 0.1-ml reaction mixtures were incubated at 31°C for 90 min. The transcription reaction was terminated by the addition of 0.2 ml of 67 mM sodium PP_i containing 200 μg of yeast cell RNA. Total RNA was precipitated with an equal volume of 10% trichloroacetic acid, and radioactivity was counted by scintillation spectrometry after filtration through GF/A filters (Whatman, Inc.).

transcription inhibition by *wt* M protein (5, 10). These and earlier results lead to the postulate that glycine at position 21 determines the three-dimensional structures at the N-terminal region of *wt* M protein that imparts its transcription inhibition activity as well as a major antigenic determinant (5, 10). This Gly-21→Glu substitution is not present in M proteins of other group III *ts* mutants sequenced by Morita et al. (6), who also selected numerous spontaneous revertants with second-site mutations scattered throughout the M gene but none at the N terminus. Many of these revertants had M proteins in which the capacity to inhibit VSV transcription was restored to various degrees. In conflict with our results, Morita et al. (6) also reported that a *tsO23* revertant (*tsO23r1*) with a Phe-111→Leu "amino acid reversion was sufficient for recovery of *wt* polymerase inhibitory activity" (data on transcription were not provided). In our experiments, in which we produced the exact same Phe-111→Leu substitution in the *tsO23* M protein expressed by

vMtsO23(R2), the revertant *tsO23* Leu-111 M protein exhibited no capacity to inhibit VSV transcription. The third mutation in *tsO23*, leading to a His-227→Tyr substitution, had no effect on transcription inhibition. The conclusion that a glycine at position 21 of the VSV M protein is essential for down regulation of VSV transcription seems inescapable.

This research was supported by Public Health Service grant R37 AI-11112 from the National Institute of Allergy and Infectious Diseases and by grant MV-9 from the American Cancer Society.

We are grateful to Joshua Shipley for very helpful suggestions and to Bernard Moss for supplying the original vaccinia virus vectors.

LITERATURE CITED

1. 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.
2. 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.
3. Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**:8122-8126.
4. Li, Y., L. Luo, R. M. Snyder, and R. R. Wagner. 1988. Expression of the M gene of vesicular stomatitis virus cloned in various vaccinia virus vectors. *J. Virol.* **62**:776-782.
5. Li, Y., L. Luo, R. M. Snyder, and R. R. Wagner. 1988. Site-specific mutations in vectors that express antigenic and temperature-sensitive phenotypes of the M gene of vesicular stomatitis virus. *J. Virol.* **62**:3729-3737.
6. Morita, K., R. Vanderoef, and J. Lenard. 1987. Phenotypic revertants of temperature-sensitive M protein mutants of vesicular stomatitis virus: sequence analysis and functional characterization. *J. Virol.* **61**:256-263.
7. Ogden, J. R., R. Pal, and R. R. Wagner. 1986. Mapping regions of the matrix protein of vesicular stomatitis virus which bind to ribonucleocapsids, liposomes, and monoclonal antibodies. *J. Virol.* **58**:860-868.
8. Pal, R., B. W. Grinnell, R. M. Snyder, and R. R. Wagner. 1985. Regulation of viral transcription by the matrix protein of vesicular stomatitis virus probed by monoclonal antibodies and temperature-sensitive mutants. *J. Virol.* **56**:386-394.
9. Schnitzer, T. J., and H. F. Lodish. 1979. Noninfectious vesicular stomatitis virus particles deficient in the viral nucleocapsid. *J. Virol.* **29**:443-447.
10. Shipley, J. B., R. Pal, and R. R. Wagner. 1988. Antigenicity, function, and conformation of synthetic oligopeptides corresponding to amino-terminal sequences of wild-type and mutant matrix proteins of vesicular stomatitis virus. *J. Virol.* **62**:2569-2577.
11. Wagner, R. R. 1987. Rhabdovirus biology and infection: an overview, p. 9-74. In R. R. Wagner (ed.), *The rhabdoviruses*. Plenum Publishing Corp., New York.
12. Weiss, R. A., and R. L. R. Bennett. 1980. Assembly of membrane glycoprotein studied by phenotypic mixing between mutants of vesicular stomatitis virus and retrovirus. *Virology* **100**:252-274.
13. 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.