JOURNAL OF VIROLOGY, Mar. 1994, p. 1532–1543
0022-538X/94/$04.00+0

Vol. 68, No. 3

Transcription Inhibition and Other Properties of Matrix Proteins Expressed by M Genes Cloned from Measles Viruses and Diseased Human Brain Tissue

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Received 13 August 1993/Accepted 22 November 1993

Ribonucleoprotein (RNP) cores extracted from virions of wild-type (Edmonston strain) measles virus (MV) or obtained from MV-infected cells (cRNP) were shown to be capable of transcribing RNA in vitro but at relatively low efficiency. The tightly bound matrix (M) protein could be effectively removed from virion RNP (vRNP) and from cRNP by exposure to buffers of high ionic strength (0.5 to 1.0 M KCl) but only at pH 8.0 or higher. The vRNP and cRNP cores complexed with M protein exhibited markedly reduced transcriptional activity at increasing concentrations, whereas vRNP and cRNP cores free of M protein exhibited linear and substantially higher transcriptional activity; these data suggest that M protein is the endogenous inhibitor of MV RNP transcription. M-gene cDNA clones derived from three strains of wild-type (*wt*) MV and 10 clones from mRNAs isolated from the brain tissue of patients who had died from subacute sclerosing panencephalitis (SSPE) and from measles inclusion body encephalitis (MIBE) were recloned in the pTM-1 expression vector driven by the bacteriophage T7 RNA polymerase expressed by a coinfecting vaccinia virus recombinant. All 10 mutant SSPE and MIBE clones expressed in vitro and in vivo M proteins that reacted with monospecific anti-M polyclonal antibody and migrated on polyacrylamide gels to positions identical to or only slightly different from those of the M proteins expressed by *wt* MV clones. When reconstituted with cRNP cores, the three expressed *wt* M proteins and 6 of the 10 mutant-expressed M proteins showed equivalent capacity to down-regulate MV transcription. Three of the M proteins from SSPE clones and one from the MIBE clone showed little or no capacity to down-regulate transcription when reconstituted with cRNP cores. The only plausible explanations for loss of transcription inhibition activity by the four SSPE/MIBE M proteins were exceedingly high degrees of hypermutations leading to U→C transitions and cloning-corrected mutations in the initiator codon (ATG→ACG) of the four M genes. However, only the hypermutated M protein expressed by the MIBE cDNA clone exhibited virtually no capacity to bind cRNP cores in a reconstitution assay. These experiments provide some preliminary data to support the hypothesis that MV encephalitis may result from certain selective mutations in the M gene.

Measles virus (MV) is an enveloped, negative-strand, nonsegmented RNA virus of the genus *Morbillivirus* and the family *Paramyxoviridae*. The internal ribonucleoprotein (RNP) core of the virion contains an RNA-dependent RNA polymerase that transcribes at least seven mRNAs from the genome in the gene order 3′-N-P(C)-M-H-F-L-5′. Six of the seven mRNAs are translated into structural proteins of the virion (8, 23, 44, 45). The RNP core consists of the genomic RNA template encapsidated by the major N protein as well as the large (L) protein and the phosphoprotein (P), which collectively comprise the RNA polymerase. The MV virion envelope contains two integral glycoproteins designated H for hemagglutinin and F for fusion protein; also associated with the envelope and the RNP core is the matrix (M) protein, which has 335 amino acids (10), is not glycosylated, and is synthesized in the cell cytoplasm by a pathway different from that of the H and F proteins (28, 29, 39, 44). Nonstructural proteins found in MV-infected cells are designated C and V and are translated from mRNAs transcribed from the P gene in a reading frame different from that of the P mRNA (11, 17).

Matrix proteins are major components of the envelope and RNP core of all negative-strand viruses and down-regulate transcription from RNP cores. Quite extensive studies have been done on the effect of M proteins on transcription of the rhabdovirus vesicular stomatitis virus (VSV) (14) and influenza orthomyxovirus (57). Paramyxoviruses have a very similar polymerase (49) and very likely a similar strategy of transcription initiating at the 3′ end of the genome coding for a leader sequence (15, 32). Quite reliable systems have been developed for in vitro transcription of paramyxoviruses and their messenger products (43) and for the effects thereon of cellular components (34, 37, 38). However, the role of the M protein in down-regulating transcription of paramyxoviruses has been less well studied than the effect of equivalent M proteins in regulating transcription of rhabdoviruses (40) or of influenza virus (54).

Infection with MV is confined to humans and usually results in a self-limiting childhood disease, but defective forms of the virus can persist for years in host tissue, resulting in fatal degenerative diseases of the central nervous system known as subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE) (7, 39, 50). The virus survives intracellularly in the brain, inaccessible to humoral and cellular immune systems of the host; the ordinarily defective virus can sometimes be rescued by cocultivation with susceptible cells (13, 51). MVs isolated from the brains of patients dying of

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SSPE ordinarily have undergone a high degree of mutation and are generally defective (16). These genetically altered SSPE viruses frequently retain the coding potential for synthesizing the envelope-associated proteins H, F, and M (1, 3, 6, 7, 13, 18–20, 22, 48, 55). Mutations have been found particularly in the M gene of patients dying with SSPE and in one case with MIBE; some of these M-gene mutations show unidirectional uridine (U)-to-cytodine (C) transitions, called biased (A/I) hypermutations (5, 19, 20, 53). As a likely explanation for these hypermutations, the activity of double-stranded RNA unwindase has been reported to be very high in cells of central nervous system origin (9, 42). A study of M genes cloned from the same brain tissue of a patient who died of SSPE showed wide variation, ranging from essentially wild-type MV sequences to hypermutations in the M gene (5). Only recently have studies been initiated on functional differences among M proteins coded by genes with varying degrees of mutation. Hirano et al. (31) reported that the M protein of an SSPE virus is functionally different from that of its progenitor wild-type strain in being localized entirely to the cytoplasm of infected cells and by being incapable of binding to MV nucleocapsids.

The principal aim of this study was to compare the functional capacity of wild-type MV M proteins to inhibit RNP transcription with that of SSPE and MIBE M proteins. The validity of these experiments required devising a system not previously achieved for obtaining transcribable RNP cores free of endogenous M protein and to prepare pure M proteins. The experimental system demanded reduction in transcription activity when M protein-free RNP cores were reconstituted with wild-type M protein. The results demonstrate that M proteins of MV and of minimally mutated SSPE clones consistently inhibit transcription by RNP cores, whereas transcription inhibition activity is frequently lost by hypermutated M proteins expressed by SSPE and MIBE clones.

MATERIALS AND METHODS

Viruses and cells. As described elsewhere (12, 32), MV (Edmonston strain) was grown in monolayer cultures of Vero cells (both obtained from Raymond Marusyk and Linda Chui, University of Alberta). To produce large amounts of MV, monolayers of Vero cells in 850-cm^2 plastic roller bottles were infected with plaque-purified MV at a multiplicity of infection (MOI) of 0.05 PFU per cell. After incubation at 37°C for 3 to 4 days in minimal essential medium (Gibco/BRL) containing 5% fetal bovine serum (FBS), 0.2% bovine serum albumin, and antibiotics, the culture supernatants were harvested and clarified. The released virions were pelleted through a 25% glycerol pad in HNE buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 1 mM EDTA, 0.1 M NaCl) for 4 h at 100,000 × *g*. The pelleted virions were resuspended in HNE, layered on a linear 15 to 60% sucrose gradient in HNE, and centrifuged for 16 to 18 h at 100,000 × *g*. The visible virion band was collected, dialyzed and resuspended in reticulocyte standard buffer (RSB; 10 mM HEPES [pH 7.4], 10 mM NaCl, 1 mM dithiothreitol [DTT], 5% glycerol), and stored at −70°C for future use. The vaccinia virus recombinant vTF1-6,2 (obtained from Bernard Moss) expressing the bacteriophage T7 RNA polymerase (25) was grown in CV-1 monkey kidney cells in the presence of Dulbecco modified Eagle medium (DMEM) supplemented with FBS.

Preparation of RNP complexes from virions and infected cells. A modification of the procedures described by Peluso and Moyer (41) and Ray and Fujinami (43) was used to prepare RNP cores from infected cells (cRNP) and from purified virions (vRNP), using the optimal pH, salt, and detergent concentrations. Confluent Vero or CV-1 cell monolayers were infected with measles virus at an MOI of 2 PFU per cell and grown for 48 h at 32.5°C. The monolayer was washed, and the cells were scraped into phosphate-buffered saline (PBS). Following low-speed centrifugation, the pellet was treated with lysolecithin (250 μg/ml; Sigma) for 1 min, repelleted, and suspended in lysis buffer (0.1 M HEPES [adjusted to pH 8.0 with KOH], 50 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate, 1 mM DTT, 2 μg of actinomycin D per ml, 1 mM spermidine, 40 U of creatine kinase per ml, 50 mM creatine phosphate). The cells were lysed by pipetting up and down at least 15 to 20 times and by brief sonication at the end. The nuclei and the cell debris were removed by centrifugation at 1,000 × *g* for 5 min. The KCl concentration was adjusted either to low salt (no addition) or to high salt (with 0.5 M KCl) and made 2% with respect to Triton N-101. The RNP cores from the infected cell lysates (1 ml) were pelleted through a 40% glycerol cushion (2.5 ml) containing 20 mM HEPES (pH 8.0) and 1 mM DTT by centrifugation at 100,000 × *g* for 2 h at 4°C. The RNP cores were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) for their protein content and suspended in RSB (pH 8.0) for storage at −70°C. The same procedure was used for preparing RNP cores from measles virions disrupted in detergent buffers containing 2% Triton N-101 and adjusted either to low salt (no addition) or to high salt (with 1.0 M KCl). The vRNP cores were also pelleted through a 40% glycerol cushion, analyzed by SDS-PAGE for protein content, and stored at −70°C in RSB. All samples tested were prepared in Laemmli buffer containing SDS, and the electrophoretic analyses described were carried out in 15% polyacrylamide gels containing 0.1% SDS.

General strategy for construction of recombinant plasmids. The vaccinia virus-based transient expression vector pTM-1, generously supplied by Bernard Moss (National Institutes of Health), was used for cloning and expressing in vitro and in vivo the wild-type (*wt*) and mutant M genes used in this research. This vector is a pUC-derived plasmid with strong promoter and terminator sequences for bacteriophage T7 RNA polymerase as well as multiple cloning sites and a cap-independent sequence derived from the encephalomyocarditis virus genome, all flanked by the thymidine kinase gene (36). Expression is driven by the phage T7 polymerase supplied in vivo by coinfection with the vaccinia virus recombinant vTF1-6,2 (25). For cloning of the MV (Edmonston) M gene in pTM-1, we used plasmid pSP64, containing the full-length M (FLM+) gene as well as upstream 5′-terminal sequences of the P gene and the M-gene leader sequence (nucleotides 1 to 32). An *Nco*I site was introduced at the M-gene ATG site (nucleotide 33) by PCR. This PCR-modified full-length *wt* M gene was cloned into the *Nco*I-*Pst*I sites of pTM-1 to generate the construct designated pTM-EDM, containing the T7 polymerase-expressible M gene of Edmonston MV.

The vector pTM-1 and the *wt* M-gene construct pTM-EDM were used for cloning 2 other *wt* M genes of MV (designated JM and CM) and 10 mutant M genes cloned from the brains of patients who died from SSPE or MIBE. These *wt* and mutant M genes cloned in the expression vector pTM-1 are described in greater detail in Results. A unique *Bgl*II site present in the M gene at nucleotides 39 to 40 was used to clone the 2 *wt* (JM and CM) and 6 of 10 mutant genes into pTM-EDM. A *Bgl*II site was created by PCR for mutant M-gene clones AMS-M9 and BDS-M7, using *Bgl*II and reverse primers. These *wt* and mutant M genes were isolated by digesting their plasmids and PCR products with *Xba*I, blunt ended with Klenow polymerase, and then digested with *Bgl*II. The *Bgl*II-*Stu*I fragment

was removed from pTM-EDM and replaced with *Bgl*II and *Xba*I blunt-ended M-gene fragments to generate the *wt* expression vectors pTM-JM and pTM-CM and the mutant vectors pTM-A, pTM-B1, pTM-M6, pTM-M15, pTM-M7, pTM-M9, pTM-K, and pTM-PP2 (described in Results). The M genes from mutant C-MIBE and SSPE-P4-M2 were amplified by PCR directly, using oligonucleotide primers with an *Nco*I site and T7 polymerase or reverse primers. The amplified mutant M genes were restricted with *Nco*I and *Bam*HI and cloned into pTM-1 to generate clones pTM-P4 and pTM-MIB.

In vitro transcription and translation of cloned M genes. Protein products of *wt* and mutant M genes cloned in pTM-1 were synthesized by the coupled transcription-translation system TnT (Promega), using the phage T7 polymerase for transcription and the reticulocyte lysate system for translation. Linearized plasmid DNA templates of each construct (1 to 2 μg) were used in the reactions containing [^{35}S]methionine (specific activity, 1,100 Ci/mmol; ICN). Transcription-translation products were analyzed by SDS-PAGE and fluorographed by using Resolution (EM Corp.). The remaining samples were immunoprecipitated with a monoclonal antibody (ascites fluid) against MV M protein (gift from Aimo Salmi, University of Turku, Turku, Finland), and the immune complexes were analyzed by PAGE.

In vivo expression of M protein in plasmid-transfected cells. M-gene recombinant plasmid DNA purified by isopycnic banding in CsCl was used to transfect cells by the procedures of Felgner and Ringold (24) and Rose et al. (46), with certain modifications. Monolayer cultures of CV-1 cells grown to confluency in 60-mm-diameter dishes in DMEM or Opti-MEM (Gibco/BRL) were first infected at an MOI of 10 to 15 PFU per cell with the vaccinia virus recombinant vTF1-6,2 expressing the phage T7 polymerase. After a 30-min adsorption at 37°C, the monolayers were transfected with the pTM-M gene recombinants in 1.0 ml of Opti-MEM mixed with 30 to 50 μl of Lipofectin reagent (Gibco/BRL), premixed for 15 to 20 min at room temperature to promote binding of DNA to liposomes. For optimal transfection, the DNA-liposome mixture was incubated with the cell monolayer for 3 to 5 h at 37°C with periodic swirling in a CO_2 incubator. After transfection, 2 ml of Opti-MEM containing 10% FBS and antibiotics was added to the monolayer without removal of the transfecting mixture and incubated for 36 to 48 h before harvesting of the transfected cells. After removal of the cells from the monolayer by scraping and washing twice in ice-cold PBS, the transfected cells were suspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.2 M NaCl, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml) for 20 to 30 min in an ice water bath. After brief sonication, the cell lysates were clarified by microcentrifugation, denatured by suspension in Laemmli buffer, and subjected to electrophoresis. The proteins were then electrophoretically transferred onto nitrocellulose membranes (0.2-μm pore size; Schleicher & Schuell). The membranes were reacted with either a polyclonal anti-MV serum (a gift of Aimo Salmi, University of Turku, Turku, Finland) or a polyclonal anti-M serum (a gift of Timothy Wong, University of Washington, Seattle) and later with ^{125}I-labeled *Staphylococcus aureus* protein A (specific activity, 33 mCi/mg; Amersham). The blots were washed extensively and autoradiographed by using Kodak X-Omat RP5 film.

Bulk production and purification of *wt* and mutant M proteins by electroelution from gels. Transcription inhibition studies require large quantities of reasonably pure M proteins. Many attempts to purify moderately large quantities of virion or cellular M proteins by ion-exchange or affinity chromatography were unsuccessful, particularly when attempted with cytoplasm of plasmid-transfected cells. Therefore, we resorted to the technique of electroelution of M proteins separated on polyacrylamide gels. Confluent monolayers of CV-1 cells were grown in 150-cm^2 Corning tissue culture flasks. After infection of each flask with 1.5 ml of vTF1-6,2 for 30 min at 37°C, the inoculum was replaced with a transfecting mixture of 60 to 70 μg of M-gene recombinant plasmid DNA in 2 ml of Opti-MEM mixed with 2 ml of Opti-MEM containing 100 to 120 μg of Lipofectin. After incubation of cells with the plasmid-Lipofectin mixture for 3 to 5 h at 37°C, 20 ml of Opti-MEM containing 10% FBS and antibiotics was added to each flask. After incubation for 48 h at 37°C, the transfected cells were harvested and lysed as described above, and the expressed M protein was purified by electroelution. The clarified lysates were subjected to electrophoresis on 15% polyacrylamide gels in which 0.1 mM sodium thioglycolate had been added to the cathode reservoir to prevent destruction of tryptophan, histidine, and methionine side chains of the M proteins. Following electrophoresis, the gels were stained with 0.5% Coomassie blue in acetic acid-isopropanol-water (1:3:6) for 30 min at room temperature and destained at 4°C for 2 to 3 h with acetic acid-methanol-water (50:165:785). The M-protein bands that were identified by coelectrophoretic migration of immunoblotted M proteins were excised, and electroelution was carried out in an electroelution cell (ECU-040; CBS Scientific, Del Mar, Calif.) as described by Hunkapiller et al. (33), using an ammonium bicarbonate-SDS buffer system. Following dialysis, the protein samples were precipitated after extraction with chloroform-methanol. The dried samples were resuspended in RSB and stored at −70°C to be used for transcription studies.

Measles virion-RNP transcription in vitro. In vitro transcription assays were carried out according to published procedures (32, 43), with minor modifications. A typical reaction mixture (50 μl) consisted of 0.1 M HEPES (pH 8.0, adjusted with KOH), 0.1 M KCl, 4.5 mM magnesium acetate, 1 mM DTT, 1 mM spermidine, 2 μg of actinomycin D per ml, 20 U of RNasin (Boehringer Mannheim), 5% glycerol, 1 mM each ATP, CTP, and GTP, 10 μM UTP, and 10 to 15 μCi of [α-^{32}P]UTP (specific activity, >400 Ci/mmol; Amersham), with various amounts of virion or cellular RNPs prepared as described above. The reaction mixtures were incubated for 4 h at 31°C, and the incorporation of [α-^{32}P]UTP in an acid-insoluble form was measured by trichloroacetic acid (TCA) precipitation. The reactions were stopped by the addition of 20 μg of yeast tRNA and 1 ml of 10% cold TCA containing 67 mM sodium pyrophosphate and incubated on ice for 30 min. The acid-insoluble material was collected on glass fiber filters (GF/A; Gelman Sciences) and washed with 50 ml of 5% ice-cold TCA. The filters were dried, and the incorporation of [^{32}P]UMP was monitored by liquid scintillation counting. Transcription reaction mixtures with purified measles virions contained 0.1% Triton X-100.

The reconstituted in vitro transcription reaction mixtures contained RNP complexes either from purified virions (vRNP) or from MV-infected Vero cell extracts (cRNP). To measure the transcription-inhibitory activity of *wt* and mutant M proteins, we prepared in vitro transcription reaction mixtures containing cRNPs (500 to 700 μg of protein per ml) depleted of M protein by treatment with high salt (1.0 M KCl) and detergent (2% Triton N-101) into which various amounts of vector-expressed electroeluted M proteins were added. Transcription inhibition assay buffer was prepared without KCl, but the KCl concentration was adjusted to 0.1 M (final) when the assay was initiated by adding purified M protein prepared in RSB containing 0.2 M KCl. Following incubation for 4 h at

31°C, the incorporation of [^{32}P]UMP was monitored as described above. All transcription reactions were carried out in duplicate and were repeated at least twice for all M-protein samples tested. The transcription inhibition effect of *wt* (Edmonston strain) M protein was also tested with vRNP cores as the template.

RESULTS

Preparation of MV transcription-active RNP cores and the effect of endogenous M protein. It has been shown that high-salt buffers can remove M proteins from RNP cores of VSV and influenza virus, resulting in enhanced transcription; moreover, reconstitution of these RNP cores with purified homologous M proteins results in down-regulation of transcription (14, 40, 54, 57). In the case of MV and other paramyxoviruses, far less conclusive data have been obtained for the transcription inhibition activity of their M proteins, to a great extent because the RNP-M protein complex is not readily dissociable in high-salt solutions. After several futile attempts to remove significant amounts of M protein from vRNP cores exposed to high-ionic-strength solutions, we resorted to the technique used by Zhirnov (56), who found that alkaline pH as well as high salt concentration of the solution was important for dissociation of M protein from the cores of Sendai paramyxovirus.

Purified measles virions or gradient-fractionated RNP cores from MV-infected cells were suspended in Tris-morpholineethanesulfonic acid (MES) buffer (adjusted to pH 5.5, 6.0, 7.0, 8.0, 8.5, or 9.0) and treated with increasing concentrations of KCl (0.1 to 1.0 M) in 2% Triton N-101 (which gave results superior to those obtained with use of Triton X-100). The disrupted virions and cellular RNPs were fractionated and collected by centrifugation through a glycerol cushion (see Materials and Methods). vRNP and cRNP cores were tested for polymerase activity by incorporation of [^{32}P]UMP and examined for protein content by SDS-PAGE and electroelution onto nitrocellulose membranes for reactivity by Western blotting (immunoblotting) with a polyclonal antibody to the entire measles virion. Residual M protein on RNP cores was determined by integrated laser densitometry and compared with residual N protein (M/N ratio). Treatment of measles virions with 2% Triton N-101 at low salt concentrations (0.1 to 0.15 M) at pH 5.5 and 6.5 efficiently removed glycoproteins H and F but very little M protein; at pH 7.0, only 50% of M protein was dissociated from virions even at 1.0 M KCl (data not shown). Only at pH 8.0 and a KCl concentration of 1.0 M was 80 to 90% M protein removed from RNP cores.

Figure 1 shows the protein profiles by SDS-PAGE and Western blotting of vRNP and cRNP cores after dissociation with Triton N-101 buffer at pH 8.0 at concentrations of KCl ranging from 0.01 to 1.0 M, as well as the transcriptional activity of vRNP and cRNP at various protein concentrations before and after removal of M protein. When virions were exposed to increasing concentrations of KCl, about 40% of the M protein was removed from vRNP cores at 0.2 M KCl; virtually complete dissociation of M protein from vRNPs was reached at KCl concentrations of 0.75 to 1.0 M (Fig. 1A). At KCl concentrations of 0.2 to 0.75 M, virtually none of the N protein was dissociated from vRNP, but about 15% of N was removed at 1.0 M KCl. Even at concentration of KCl up to 1.0 M, very little P protein and L protein was dissociated from vRNP cores, which retained virtually complete transcriptional activity after exposure to 1.0 M KCl. Similar results were obtained upon dissociation of M protein from vRNP cores at KCl concentrations of 0.1 to 1.0 M when vRNP cores were probed with a monospecific anti-M polyclonal antibody (data not shown). Higher alkalinity of the Triton N-101 buffer at pH 8.5 and 9.0 was somewhat more efficient in removing M protein from vRNP cores, but at these pH levels, 30 to 40% of N protein was dissociated at 0.75 to 1.0 M KCl (data not shown). Therefore, all future studies on dissociation of M protein from RNP cores were performed in Triton N-101 buffers at pH 8.0.

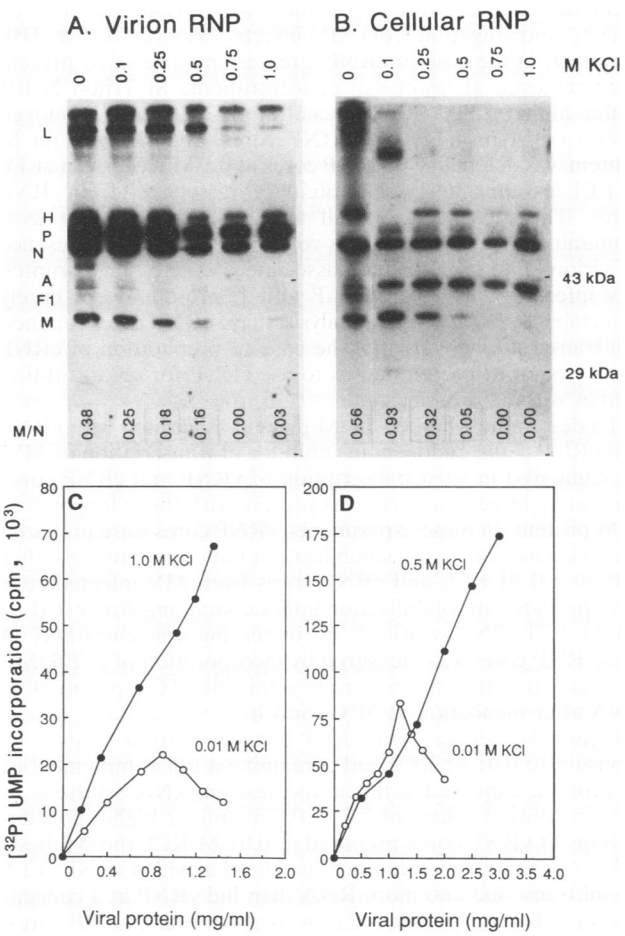

FIG. 1. Protein composition analyzed by SDS-PAGE and Western blotting (A and B) and transcriptional efficiency (C and D) of vRNPs and cRNPs prepared at different salt concentrations. As described in Materials and Methods, RNPs were extracted from measles virions (vRNP) or from MV-infected cells (cRNP) with Triton N-101 in a Tris-MES buffer at pH 8.0 containing KCl at concentrations of 0 to 1.0 M. For protein analysis, the vRNPs (A) and cRNPs (B) were collected by pelleting through a 40% glycerol pad and dissolved in Laemmli sample buffer, and the proteins were subjected to electrophoresis on 15% polyacrylamide–SDS gels. After transfer by electroblotting onto nitrocellulose sheets, the fractionated proteins were exposed to polyclonal antibodies directed to all MV proteins and then to ^{125}I-labeled staphylococcal protein A. After autoradiography, the M/N protein ratio was determined by integrated laser densitometry. Positions of molecular weight markers and locations of measles virion proteins L, H, P, N, F1, M, and A (cellular actin) are illustrated. The band on top of the L protein is the aggregate that did not enter the gel. Absence of salt is indicated as a molar concentration of 0. Transcription by vRNPs prepared in 0.01 M versus 1.0 M KCl (C) and cRNPs prepared at 0.01 M versus 0.5 M (D) was carried out in a regular transcription reaction mixture (see Materials and Methods) at increasing protein concentrations of vRNP and cRNP. Transcription was measured by incorporation of [^{32}P]UMP in TCA-precipitable RNA.

Similar studies were performed with cRNP isolated by density centrifugation from MV-infected Vero cells (Fig. 1B). However, in the case of cRNP, greater amounts of M protein were removed at lower salt concentrations. In Triton N-101 buffer at pH 8.0, a KCl concentration of 0.25 M removed ~90% of M protein from cRNP cores, and virtually no M protein was detectable on RNP cores at 0.5 M KCl. Even at 1.0 M KCl, less than 20% of N protein was dissociated from cRNP cores. The presence of small amounts of H protein as a contaminant of cRNP cores is very likely due to the presence of virions and/or H-protein-associated vesicles in disrupted MV-infected cells. Although P and L proteins were barely detectable by Western blot analysis, these cRNP cores retained full transcriptional activity. The ease of preparation of cRNP cores free of M protein led us to use cRNP for reconstitution studies with M protein.

To determine whether the M protein associated with vRNP and cRNP is the endogenous inhibitor of transcription of MV, we compared in vitro transcription of vRNP and cRNP cores containing large amounts of M protein with those largely free of M protein. In these experiments, vRNP cores were prepared from virions exposed to solubilization buffer containing either 0.01 or 1.0 M KCl, and cRNP cores from MV-infected cells were prepared in solubilization buffer containing either 0.01 or 0.5 M KCl. RNA synthesis by increasing concentrations of these RNP cores was measured by incorporation of [^{32}P]UMP in a standard 50 μl of transcription mix into TCA-precipitable RNA after incubation at 31°C for 4 h.

Figure 1C shows that vRNP prepared from virions by exposure to 0.01 M KCl, and containing a full complement of M protein, exhibited a linear increase in RNA synthesis at protein concentrations of 0.2 to 0.8 mg/ml; at higher concentrations of vRNP cores prepared in 0.01 M KCl, the synthesis of RNA decreased continuously to a level at which vRNP at 1.5 mg/ml transcribed no more RNA than did vRNP at a concentration of 0.4 mg/ml (Fig. 1C). In sharp contrast, vRNP cores, in which >95% M protein was removed by 1.0 M KCl, exhibited a steeper linear increase in RNA synthesis up to at least an RNP protein concentration of 1.4 mg/ml (Fig. 1C). cRNP treated with 0.01 M KCl showed a similar increase and decline in RNA synthesis with increasing concentrations of cRNP (Fig. 1D). The cRNP cores prepared in the presence of 0.5 M KCl also exhibited a linear increase in capacity to synthesize RNA with increasing concentrations of cRNP proteins until at least a concentration of 3.25 mg/ml. It seems likely, as shown in Fig. 1A and B, that the M protein removable from RNP cores by high-ionic-strength buffer is the endogenous inhibitor of transcription by vRNP and cRNP cores of MV. These effects on inhibition of transcription by M protein at increasing virion concentration are due to greater reassociation of endogenous M protein with RNP cores, as described for VSV by Carroll and Wagner (14).

To determine whether MV subgenomic mRNAs were synthesized under the in vitro transcription assays described here, slot blot analyses (43) were performed by hybridization of transcribed ^{32}P-labeled mRNAs, using plasmids containing cDNAs of five MV genes (N, P, M, H, and F). The results indicated that all MV mRNAs for these structural proteins were synthesized under the assay conditions described here (data not shown). We did not measure L-gene transcripts because of unavailability of an L cDNA probe.

Construction of *wt* and mutant M-gene expression vectors. To study the transcription inhibition activity of M proteins from *wt* MV and SSPE measles-like virus, it was necessary to prepare pure M proteins for reconstitution with transcriptionally active RNPs free of endogenous M proteins. Numerous attempts to recover and purify M proteins from measles virions by chromatographic methods were unsuccessful. Therefore, we resorted to the procedure for cloning *wt* and mutant M genes in an expression vector and then purify the M proteins after expression. By this technique, we could also produce mutant M proteins from M genes cloned from brains of patients who had died of SSPE and MIBE and test their capacity to down-regulate MV RNP transcription.

All *wt* and mutant M-gene clones were recloned in the vaccinia virus-based expression vector pTM-1 as described in Materials and Methods. The full-length *wt* M gene of MV (Edmonston) cloned in pSP64 (FLM+) was a gift from William Bellini of the Centers for Disease Control and Prevention, Atlanta, Ga. (10). The two other *wt* M-gene clones, designated CM and JM, were cloned from isolates of MV (3). The SSPE-A M-gene clone came from patient A, a 9-year-old child who died after only a 3-month course of SSPE (6). The SSPE-B1, BDS-M6, BK-M15, AMS-M9, and BDS-M7 M-gene clones were obtained from patient B, a 10-year-old child diagnosed as suffering from SSPE for approximately 6 months before death; B1 is the original clone from the brain of patient B (20), whereas M6, M15, M9, and M7 are clones isolated from the left frontal and left and right occipital lobes and the cerebellum of the same patient (5). SSPE-K is the M-gene clone from a 14-year-old child who died after an 18-month illness with SSPE (4). SSPE-PP2-M2 and SSPE-P4-M2 are two additional SSPE clones (2). C-MIBE is a clone from a 3-year-old child who died of MIBE 22 months after the diagnosis of leukemia, 4 months after clinical measles, and 2 months after symptoms of neurological disease (20). All patients exhibited hyperimmune responses with high antibody titers in blood and cerebrospinal fluid to all MV antigens except the M proteins. Neuropathological findings consisted of perivascular mononuclear cell infiltrations, Cowdry type A inclusion bodies in neurons and oligodendrocytes, and reactive fibrillar astrocytes in the grey and white matter of the entire brain. Tissue processing, RNA extractions, cloning, and sequencing are described in earlier publications (6, 47). The vector used for cloning M-gene cDNAs was pBluescript II SK(+) in all cases except SSPE-A, SSPE-B1, and C-MIBE, for which pBluescript KS(+) was used.

Figure 2 provides a summary of all *wt* and mutant M genes cloned in pTM-1; also illustrated is the general structure of the protein expressed by each vector, the source of the cDNA cloned, and the reference describing the original cDNA clone. Also noted are the hypermutations in SSPE and MIBE M-gene clones expressed as the number of U→C transitions. The stick model of expressed proteins illustrates certain alterations in N-terminal amino acids and read-through beyond the termination codon at amino acid 335. The *wt* Edmonston (vaccine) MV M gene (pTM-EDM) has a long untranslated 3′ region (~400 nucleotides) and codes for a protein of 335 amino acids. The noncoding region is missing in clone pTM-M15. Translation initiation site ATG is at position +33 in the M gene (10). The expression vector pTM-1 has an *Nco*I restriction site (ATGG) where the insert needs to be cloned for efficient expression. Introduction of an *Nco*I site at the translation initiation site of all M-gene clones involved an A-to-G change (ATGA to ATGG at position +36) produced by PCR amplification. This resulted in change of an amino acid residue from threonine to alanine next to the initiator methionine. Original M-gene cDNA clones of mutants AMS-M9, BDS-M7, SSPE-P4, and C-MIB had their ATG altered to ACG because of a hypermutation event (U→C transition), thus altering the normal initiation site of the M protein (9, 42). Cloning these mutant genes into the *Nco*I site of pTM-1 has thus restored the

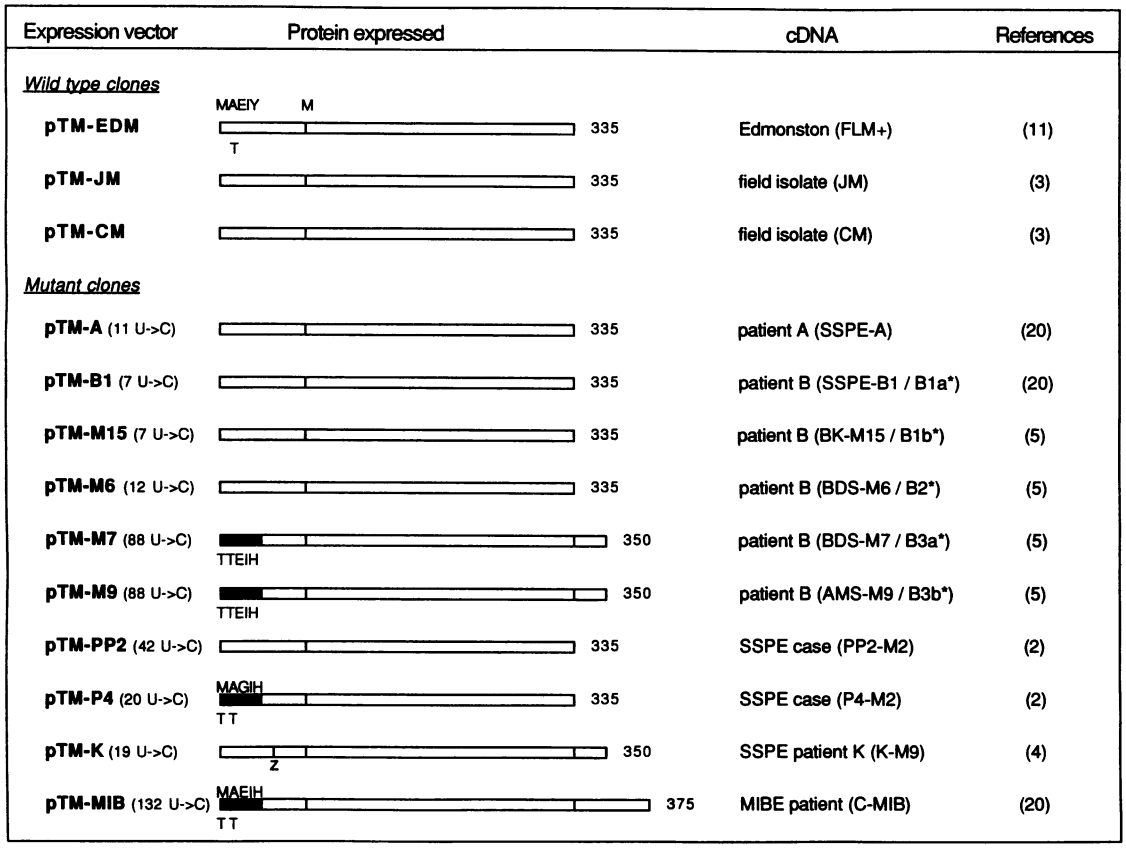

FIG. 2. Summary of MV M-gene constructs. Three wild-type and ten mutant (SSPE and MIBE) M genes were cloned in the pTM-1 expression vector driven by the phage T7 RNA polymerase. The plasmids are named for the original cDNA isolates, which are also shown along with the references which provide more detailed information. Also presented are stick models for the M proteins expressed by each clone showing amino acid totals. The black boxes (proteins expressed by pTM-M7, pTM-M9, pTM-P4, and pTM-MIB) indicate the regions modified by PCR during the process of introducing an *Nco*I site in the plasmid; these four M genes were originally isolated with a mutation which altered the initiation codon to ACG, which was converted to ATG by PCR. The sequence of the first five amino acids (MAEIY) shown for *wt* pTM-EDM holds for all proteins except those expressed by pTM-P4 and pTM-MIB (shown above the stick models). The original amino acid sequences of proteins expressed by pTM-M7, pTM-M9, pTM-P4, and pTM-MIB are shown at the bottom of each stick model before conversion by PCR. Also noted in the stick model for pTM-EDM is conversion of alanine to threonine by introducing the *Nco*I site (ATGG), which applies to all 13 constructs. The z in pTM-K represents the premature termination site at amino acid residue 22. An ATG in-frame codon at residue 51 is designated M (for methionine) as an alternative initiation site in all constructs. U→C transitions at nucleotide +1036 ablated the termination codons of pTM-M7, pTM-M9, pTM-K, and pTM-MIB, adding 15 amino acids to three of these proteins and 40 to the pTM-MIB product. The designation of cDNA with an asterisk (B1a, B1b, B2, B3a, and B3b) in clones from patient B represents the various clonal subgroups of B to which each individual clone belongs (5).

original ATG in clones pTM-M9, pTM-M7, pTM-P4, and pTM-MIB. However, in clone pTM-K, although the initiation site is normal, a change of nucleotide G to A at position +67 introduces an early terminator site at amino acid residue 11. The next in-frame ATG site is at position +183 (amino acid residue 51) in all M genes including pTM-K. Additional U-to-C mutations have extended the translation termination sites beyond the normal position at nucleotide +1038 (amino acid residue 335), introducing an additional 15 amino acids to the deduced amino acid sequence of M proteins of clones pTM-M7, pTM-M9, and pTM-K and an additional 40 amino acids to the M-protein C-terminal sequence of pTM-MIB.

Expression of M-gene clones in vitro and in vivo. It was important to determine whether the various measles, SSPE, and MIBE M genes could be expressed when cloned in the pTM-1 vector. In particular, would the hypermutated M genes be capable of being transcribed and translated into M proteins recognized by antibodies directed to *wt* M protein? In addition, could these M-gene clones be expressed efficiently enough to produce and purify *wt* and mutant M proteins in amounts sufficient to test their capacity to down-regulate transcription of MV RNP? To address these questions, all of the M-gene constructs were tested for their capacity to express M proteins in vitro and in vivo.

All M-gene constructs were transcribed and translated in vitro by using the TnT system (Promega), and the ^{35}S-labeled products were immunoprecipitated with anti-M monoclonal antibody, collected on protein A-Sepharose beads, and then analyzed by SDS-PAGE (data not shown). All 13 M-gene clones expressed in vitro M proteins precipitable by a single monoclonal antibody. Quite remarkable is the efficient expression of antigenically intact M proteins by pTM-M7, pTM-M9, and pTM-MIB, which have 88, 88, and 132 U→C mutations, respectively (Fig. 2). Quite evident were slight differences in migration of these in vitro-expressed M proteins subjected to

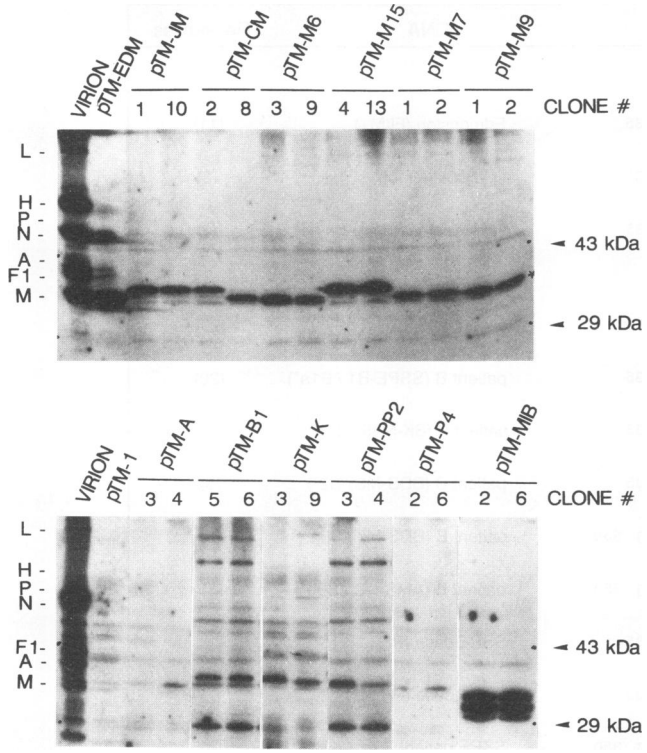

FIG. 3. Western blot identification of electrophoretically separated M proteins synthesized in CV-1 cells transfected with *wt* and mutant M-gene cDNA plasmids. As detailed in Materials and Methods, after infection of cell monolayers with T7 polymerase-expressing vTF1-6,2 at an MOI of 10 PFU per cell, cells were transfected with 10 μg of plasmid DNA by use of Lipofectin. After incubation for 36 to 48 h at 37°C, transfected cell lysates were subjected to electrophoresis on 15% polyacrylamide–SDS gels, and the separated proteins were electroblotted onto nitrocellulose sheets for reactivity with polyclonal antibodies directed to all MV structural proteins. Expressed M proteins and marker measles virion proteins L, H, P, N, F1, M, and A (actin) were identified by MV antibody by coupling with ^{125}I-labeled staphylococcal protein A and autoradiography. The molecular masses of two protein markers are also shown. The numbers under each designated M-gene plasmid refer to the two separate clones tested for in vivo expression of each M gene. The vector alone, pTM-1, was tested as a negative control for transfection expression. Note differences in migration for certain mutant M-gene products and the three rapidly migrating M-protein bands expressed by pTM-MIB.

PAGE; only the M protein expressed by pTM-MIB had moderately increased mobility, and the migration of the M protein expressed by pTM-P4 was somewhat retarded (data not shown).

The same 13 M-gene constructs (3 *wt* MV and 10 cloned from SSPE- and MIBE-infected brains) were tested for in vivo expression of M proteins. Figure 3 illustrates the Western blots for the M-gene products of duplicate clones expressed in CV-1 cells. Each clone expressed respectable amounts of M protein except pTM-A and pTM-P4, as judged by reduced reactivity with the polyclonal anti-MV antibody. There was some variation in SDS-PAGE migration of the mutant-expressed M protein compared with virion M protein, which migrated as a 37-kDa protein, as did the *wt* (Edmonston strain) M protein expressed by pTM-EDM. There were only slight differences in migration by SDS-PAGE of the M proteins expressed by the other two *wt* clones and by the mutant SSPE clones despite major hypermutations and altered termination codons and restored initiation codons. The only marked difference in migration was noted for the M-gene product(s) of mutant clone pTM-MIB, which appeared as three distinct bands, all of which migrated faster (~36, 35, and 34 kDa) than the *wt* 37-kDa protein (Fig. 3). All three of these pTM-MIB-expressed proteins were readily recognized by an anti-M polyclonal antibody. The mutated ATG initiator codon of this pTM-MIB clone was also restored by the cloning procedure, and 40 additional amino acids were potentially added by reading through the hypermutated termination codon to a termination signal at nucleotide +1158. The remarkable feature of these studies on expression of quite dissimilar M-gene clones is that every one of the hypermutated SSPE and MIBE clones expressed an M protein recognizable by anti-M polyclonal and monoclonal antibodies. Also remarkably, the M-gene products migrated by SDS-PAGE exactly like or only slightly different from *wt* M protein of MV.

Transcription inhibition by *wt* and mutant M proteins. Partially purified M proteins expressed by MV, SSPE, and MIBE clones were next tested for their capacity to down-regulate transcriptions by MV cRNP. As described in Materials and Methods, M proteins were produced in bulk by transfecting monolayers of CV-1 cells in large flasks previously infected with vTF1-6,2 to generate phage T7 polymerase. Pooled cytoplasmic extracts for each clone were fractionated by SDS-PAGE, and the M protein was electroeluted from carefully excised gel fragments. Although these extraction procedures do not ensure renaturation, the *wt* M proteins retain antigenicity and their capacity to down-regulate transcription. Transcription inhibition by increasing concentrations of each M protein was tested in 50-μl reaction mixtures containing, per ml, 300 to 400 μg of cRNP cores isolated from MV-infected CV-1 cells and freed of endogenous M protein by exposure to 0.5 M KCl at pH 8.0. In one experiment, vRNP cores, from which 80 to 90% of endogenous M protein had been removed by 1.0 M KCl, were also tested for down-regulation of RNA synthesis by *wt* M protein. Transcription reactions, containing various amounts of each expressed M protein, were carried out for 4 h at 31°C, and the amount of RNA synthesized was determined by incorporation of [^{32}P]UMP in TCA-precipitable RNA.

Figure 4 compares the transcription inhibition activities of increasing concentrations of *wt* and mutant vector-expressed M proteins added to fully transcribable cRNP cores. The *wt* Edmonston strain of M protein expressed by pTM-EDM efficiently inhibited transcription by cRNP cores free of endogenous M protein, reducing RNA synthesis by 40% at *wt* M-protein concentrations of 2 μg/ml and progressing to 68% inhibition of transcription at an *wt* M-protein concentration of 12 μg/ml (Fig. 4A). The same Edmonston *wt* M protein showed a considerably reduced capacity to down-regulate transcription of virion-derived vRNP cores, requiring 12 μg/ml to reduce transcription of the vRNP template by only 35% (Fig. 4A). It seems logical to assume that this reduced transcription inhibition activity by *wt* M protein reconstituted with vRNP cores is due to failure to remove 10 to 20% of endogenous M protein (Fig. 1A). Two other *wt* M proteins cloned from other MV strains and expressed by pTM-JM and pTM-CM also down-regulated transcription by cRNP cores but somewhat less effectively (Fig. 4B). The reason for this discrepancy among *wt* M proteins from different MV strains is unclear but could conceivably be due to reconstitution with heterogeneous cRNP cores derived from the Edmonston strain of MV. For these reasons, different ordinates and abscissas were used to display the data in these experiments, which of

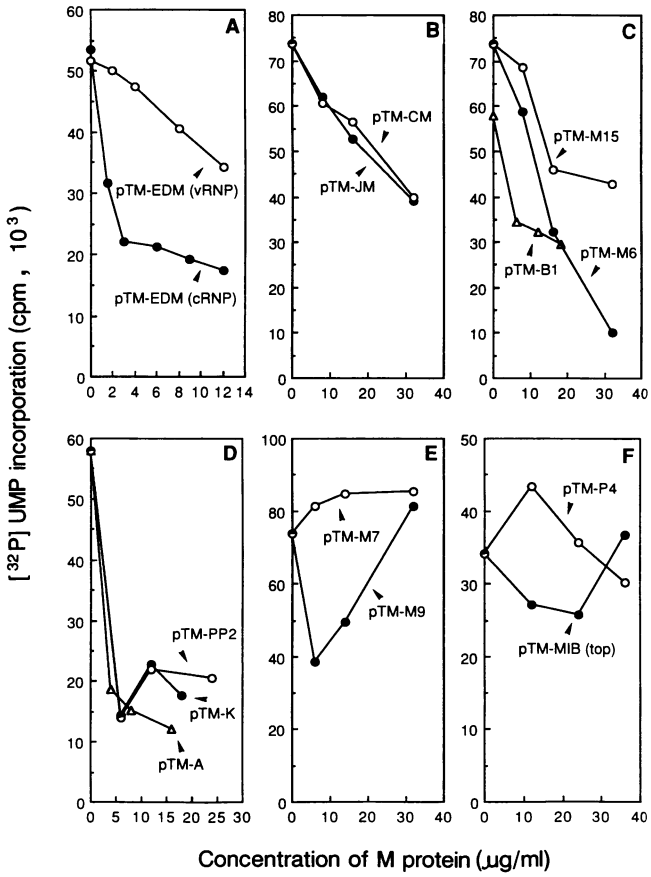

FIG. 4. Comparative transcription inhibition activity of plasmid-expressed *wt* and mutant M proteins reconstituted with RNP cores. Bulk quantities of M protein were produced in CV-1 cells transfected with 3 *wt* M-gene clones or 10 M genes cloned from SSPE- or MIBE-infected brains; *wt* and mutant M proteins were purified by electroelution after SDS-PAGE separation (see Materials and Methods). Increasing concentrations of each vector-expressed, purified M protein (or buffer alone) were added to 50 μl of transcription reaction mixtures containing 300 to 400 μg of cRNP cores extracted from Edmonston MV-infected CV-1 cells and rendered free of endogenous M protein (similar vRNP cores were tested in one experiment). Transcription was allowed to proceed for 4 h at 31°C before measurement of RNA synthesis by incorporation of [^{32}P]UMP into TCA-precipitable material. The data are plotted as 4-h transcription of RNPs in the absence of M protein or compared with increasing concentrations of each vector-expressed M protein. Panel A shows the effect of *wt* M protein expressed by pTM-EDM on transcriptional activity of vRNP prepared from measles virions compared with cRNP prepared from MV-infected CV-1 cells. Panel B compares the inhibition of cRNP transcription by M proteins expressed by two other *wt* M-gene clones. Panels C and D show some variable capacity to inhibit transcription by cRNP reconstituted with M proteins expressed by six separate M genes cloned from brains of patients who had died from SSPE. Panels E and F portray no significant effect (or only erratic effects) on cRNP transcription of M proteins cloned from the brains of patients with SSPE or MIBE.

necessity could not all be done simultaneously; portraying the data as percent inhibition could be misleading. Notwithstanding, these experiments clearly show for the first time that exogenous M protein inhibits transcription by MV RNP cores.

Among the 10 M genes cloned from brains of patients with SSPE and MIBE, six of the expressed M proteins inhibited MV cRNP transcription to various degrees (Fig. 4C and D), and four of the expressed mutant M proteins showed little or no capacity to down-regulate cRNP transcription (Fig. 4E and F). There was some variation in the degree of transcription inhibition displayed by M proteins expressed by SSPE clones. Transcription inhibition equivalent to or greater than that of *wt* M protein expressed by pTM-EDM (Fig. 4A) was exhibited by M proteins expressed by clone pTM-A from SSPE patient A (80% inhibition; Fig. 4D), clone pTM-K (76% inhibition; Fig. 4D), clone pTM-M6 (80% inhibition; Fig. 4C), and clone pTM-PP2 (75% inhibition; Fig. 4D). Somewhat less effectiveness in cRNP transcription inhibition was exhibited by M proteins cloned from SSPE patient B; the M proteins expressed by pTM-B1 inhibited transcription by 45%, and that expressed by pTM-M15 inhibited transcription by 43 and 10% at M-protein concentrations of 30 and 5 μg/ml, respectively (Fig. 4C). All of these six SSPE clones that expressed transcription-inhibitory M protein have intact ATG initiator codons at nucleotide +33; all six also have U→C transitions varying from 7 for pTM-B1 and pTM-M15 to 19 for pTM-K and 42 for pTM-PP2 (Fig. 2). The only individual differences among these six SSPE clones that could possibly be considered significant were the absence of a noncoding region in pTM-M15 and the presence of an early terminator codon in pTM-K (5, 21).

The M proteins expressed by 4 of the 10 M genes cloned from brains of SSPE and MIBE patients exhibited little or no capacity to down-regulate transcription (Fig. 4E and F). As noted, there was certain variation in cRNP transcription at various concentrations of M protein; however, in no case was cRNP transcription inhibited by concentrations of >30 μg/ml of M proteins expressed by pTM-M7 and pTM-M9 (Fig. 4E) or pTM-P4 and pTM-MIB (Fig. 4F). The genetic basis for the loss of transcription inhibition activity of the M proteins expressed by these M-gene clones compared with the M proteins of the six SSPE clones that do down-regulate transcription (Fig. 4C and D) is not clear. In general, the four M proteins that do not inhibit cRNP transcription are from M genes with hypermutations: U→C transitions of 88 for pTM-M7 and pTM-M9 and 132 for pTM-MIB (leading to 57 and 69 amino acid substitutions, respectively) but only 20 for pTM-P4 (Fig. 2). Of some interest is the striking variability in the transcription inhibition activity of M proteins expressed by M genes cloned from different regions of the brain of patient B; clones pTM-M7 and pTM-M9 each have 88 U→C transitions and lost their capacity to inhibit cRNP transcription, whereas pTM-B1, pTM-M15, and pTM-M16 have 7, 7, and 12 U→C transitions, respectively, and retained transcription inhibition activity. All of the M genes of pTM-M7, pTM-M9, pTM-P4, and pTM-MIB originally had mutated initiation codons (ATG→ACG), but these were restored to ATG during the cloning procedure. Clone pTM-MIB, from a patient with MIBE, had undergone the most drastic hypermutation, with 70% (132) U→C transitions and 40 additional amino acids added beyond the termination codon (at nucleotide +1138). Although the M protein(s) expressed by pTM-MIB was recognized by the anti-M polyclonal antibody, one would expect that this mutant M protein might lose its transcription inhibition activity.

Comparative affinities of *wt* and mutant M proteins binding to RNP cores. In view of the variation among M proteins in their capacity to down-regulate RNP transcription, it was of interest to compare their capacity for physical interaction with the RNP core. In previous studies, Hirano et al. (31) reported considerable variation in binding to RNP cores of M proteins isolated from different *wt* strains of MV and certain neurovirulent strains. In our experiments, we tested the RNP-binding

affinities of *wt* and mutant M proteins expressed from M genes cloned in pTM-1 vectors, using the TnT coupled transcription-translation system; this system provided M proteins heavily labeled with [^{35}S]methionine which were prepared from three *wt* MV clones and certain SSPE and MIBE clones.

Initially, we used the technique of Hirano et al. (31) to study binding of M proteins to RNP cores (data not shown). The ^{35}S-M protein expressed by an M-gene clone of the MV Edmonston vaccine strain (pTM-EDM) was used as the arbitrary baseline of 100% binding to Edmonston strain cRNP cores. By comparison, the cRNP affinities of heterologous M proteins varied from 40 to 42% binding for the two other *wt* clones pTM-CM and pTM-JM to 44 to 76% for mutant M proteins expressed by cDNA M genes cloned from the brains of patients A and B, both of whom died from SSPE (data not shown). The clonally expressed M proteins that were incapable of down-regulating transcription (pTM-M7 and pTM-M9) were capable of binding to cRNP cores as well as did the M proteins expressed by clones that did inhibit transcription (pTM-A, pTM-B1, and pTM-M6). In sharp contrast, the M protein expressed by the cDNA cloned from the patient with MIBE (pTM-MIB) not only failed to inhibit cRNP transcription (Fig. 4F) but exhibited little or no capacity to bind cRNP above the baseline controls (data not shown).

In an effort to confirm these data on binding of *wt* and mutant M proteins to cRNP cores, a second series of experiments was performed by isopycnic centrifugation in order to minimize background radioactivity noise. Figure 5 compares the isopycnic gradient fractionation of ^{35}S-M protein alone, ^{3}H-labeled RNP cores before and after interaction with ^{35}S-M proteins synthesized by plasmids expressing the M gene cloned from Edmonston MV (pTM-EDM), from the brain of SSPE patient B (pTM-M6 and pTM-M7), and from a patient with MIBE (pTM-MIB). Quite clearly, ^{3}H-cRNP alone bands at a much higher buoyant density than does *wt* ^{35}S-M protein in the absence of cRNP (Fig. 5A and B). In contrast, ~85% of *wt* ^{35}S-M protein bands at the same density as the ^{3}H-RNP with which it had been incubated (Fig. 5C), clearly indicative of RNP-M binding. Despite the fact that the M protein expressed by pTM-M7 has lost its capacity to down-regulate MV RNP transcription compared with the pTM-M6-expressed M protein, which markedly inhibits transcription (Fig. 4), both of these SSPE M proteins bind to cRNP cores (Fig. 5D and E) at an efficiency not significantly different from that of pTM-EDM M protein (Fig. 5C). In sharp contrast, the ^{35}S-M protein expressed by the pTM-MIB clone shows a markedly reduced capacity to bind ^{3}H-RNP cores (Fig. 5F). The M protein expressed by pTM-MIB has also lost most of its capacity to down-regulate cRNP transcription and has by far the greatest number of mutations among the SSPE M-protein clones.

Except for the M protein expressed by the cDNA cloned from the brain of the MIBE patient, these data reveal no direct correlation between the physical reassociation of mutant M proteins with RNP cores and their capacity to down-regulate transcription. It would seem likely that the functions of RNP binding and down-regulation of transcription are located at separate domains of the M protein.

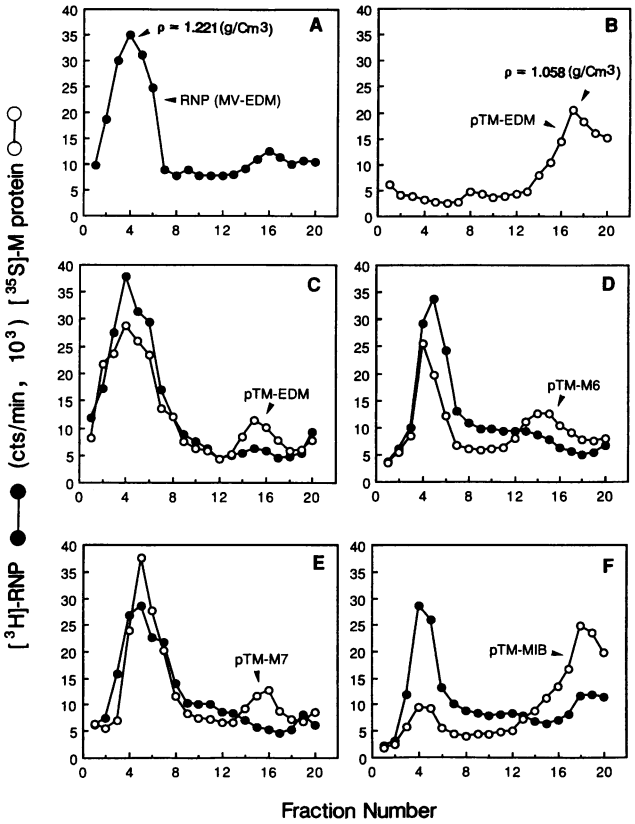

FIG. 5. Isopycnic gradient profiles demonstrating binding affinity to ^{3}H-labeled cRNP cores of *wt* and mutant ^{35}S-M proteins. (A) ^{3}H-cRNP in the absence of M protein; (B) ^{35}S-M protein in the absence of cRNP; (C) pTM-EDM *wt* ^{35}S-M protein; (D) pTM-M6 SSPE ^{35}S-M protein; (E) pTM-M7 SSPE ^{35}S-M protein; (F) pTM-MIB MIBE ^{35}S-M protein. cRNP cores prepared from CV-1 cells infected with MV (Edmonston) in the presence of [^{3}H]uridine were rendered free of endogenous M protein and incubated at 4°C in binding buffer (pH 7.5) for 2 h with M proteins synthesized in vitro by M gene-recombinant pTM1 plasmids in a TnT coupled transcription-translation system rendered free of background ^{35}S radioactivity by overnight dialysis. Incubated mixtures of equivalent amounts of ^{3}H-cRNP and ^{35}S-M proteins, or cRNP and *wt* ^{35}S-M protein alone, were layered on 15 to 75% linear sucrose gradients and subjected to equilibrium centrifugation at 200,000 × *g* for 22 h at 4°C. Twenty fractions collected from each gradient were counted by double-labeling scintillation spectrometry corrected for ^{3}H and ^{35}S spill. Peaks of ^{35}S radioactivity were verified by immunoprecipitation with an anti-M antibody or coprecipitation with cRNP by an anti-N antibody. Buoyant density was determined by refractometry.

DISCUSSION

These studies clearly show that, as is the case for VSV and influenza virus (14, 57), removal of M protein from vRNP or cRNP cores resulted in greatly enhanced transcriptional activity of MV, strongly suggesting that the M protein is the endogenous inhibitor of RNP transcription in vitro. Support for this conclusion came from experiments in which partially purified *wt* M protein was found to down-regulate transcription when reconstituted with RNP cores free of endogenous M protein. Better results were obtained, and most of the subsequent experiments were performed, by using cRNP cores derived from MV-infected CV-1 cells, as was the experience of other investigators who used cell-derived RNP cores to study MV transcription (43, 49) and polymerase enzyme reconstitution of RNP transcription (37, 38). One possible complication of using cRNP cores as templates to study in vitro transcription is the possibility that host cell components may influence transcription activity. However, we found that cytoplasmic extracts of uninfected CV-1 cells had no detectable influence on the rate of transcription by cRNP (data not reported), nor did HeLa cell extracts alter transcription by MV RNPs in a

previous study (43). However, Moyer et al. (37, 38) have reported that cytoskeleton elements may augment transcription by both paramyxoviruses and rhabdoviruses. Actin, which is present in virions of MV and other paramyxoviruses, could also play a role in RNP transcription, as indicated by evidence for interaction of M protein and actin (26, 35). The technique that we used to purify M protein, by electroelution from fragments of polyacrylamide gels (27, 33), would seem to preclude association of cytoskeletal elements as a factor in transcription inhibition by M protein.

Comparing the M proteins of measles, SSPE, and MIBE viruses required cloning in a competent expression vector. Bacterial, baculovirus, and various mammalian cloning systems were previously tried unsuccessfully (data not shown). Of great advantage was the vaccinia virus-based system using the cap-independent vector pTM-1 driven by the phage T7 polymerase (36), which is also ideal for the in vitro TnT coupled transcription-translation system and for abundant transient expression in CV-1 cells. Expression in vitro and in vivo of 3 MV *wt* M genes and 10 mutant M genes (cloned from brains of SSPE and MIBE patients) resulted in ample production of M proteins from each clone. Despite numerous mutations in many of the SSPE and MIBE M genes, the 13 M proteins all reacted with monoclonal and specific polyclonal anti-M antibodies, and only minor variations in migration were apparent on PAGE. The only major exception was the in vivo-expressed product of the MIBE M gene (pTM-MIB), which migrated by SDS-PAGE as three separate bands, each at a molecular weight lower than that of *wt* or the nine other mutant M proteins. Each of the 3 *wt* M proteins and 6 of the 10 mutant M proteins were capable of down-regulating transcription by cRNP cores with only moderate differences in efficiency of transcription inhibition. All of these six M-gene SSPE clones had mutations leading to U→C transitions, but these mutations were generally limited in number except for clone pTM-PP2, which had 42 U→C transitions. Three SSPE M-gene clones and the one MIBE M-gene clone gave rise to M proteins which possessed little or no capacity to down-regulate cRNP transcription; two of these SSPE M genes had 88 U→C transitions, the MIBE M gene had 132 U→C transitions (70% uridines were mutated), and one SSPE M gene (pTM-P4) had only 20 U→C transitions. The only other potentially significant difference in the M-gene mutants that expressed M proteins incapable of down-regulating transcription is a mutation in the initiator codon at nucleotide +33 resulting in conversion of ATG to ACG, which was corrected back to ATG during the cloning procedure. With the exception of the hypermutated M protein expressed by pTM-MIB, there was no correlation between binding of M proteins to RNP cores and their failure to down-regulate transcription.

Since isolation of infectious viruses from patients suffering from SSPE or MIBE is rather difficult, many recent studies have resorted to cloning the genomic RNA or mRNA present in brains of patients who have died of these diseases. Numerous studies have indicated great abundance of intracellular RNP cores in brains of SSPE and MIBE patients because defective maturation of measles virions occurs, presumably as a result of extensive mutations in the genes coding for H and F proteins, as well as the M-protein gene (1, 5–7, 16, 19, 20, 22, 55). These mutations are linked to unidirectional U→C transitions, called biased hypermutations, that have been found to affect the M gene in particular and other genes of MV (16, 19, 20, 52). These biased hypermutations have also been attributed to the activity of double-stranded RNA unwindase present in many cells (9, 13) and particularly of high activity in cells of the central nervous system (42).

Recent studies by Baczko et al. (5) compare the genetic variability of M genes cloned from different regions of the brain of patient B, who died of SSPE, as well as variant pTM-B1 originally cloned from the brain of the same SSPE patient (20). Some of these M genes cloned from patient B had nucleotide sequences with few mutations, not very different from those of M genes from *wt* MV B1b (pTM-15) and B2 (pTM-M6). Several other M genes cloned from patient B exhibited marked hypermutations, primarily U→C transitions. However, no M protein could be detected in the brain of SSPE patient B; moreover, the RNA extracted from the brain of SSPE patient B could not be translated in vitro (5, 22). Many of these cDNA M genes cloned from SSPE patient B were examined in the studies reported here, but in each case, these M genes were cloned for in-frame reading sequences and mutated initiator codons (ACG) were corrected to ATG for MV B3a (pTM-M7) and virus B3b (pTM-M9).

A major obstacle in studying the cause of SSPE and MIBE is difficulty in isolating the progenitor virus, which makes it a problem to devise a functional assay for SSPE/MIBE virus proteins, particularly the defective M, H, and F proteins. Cloning techniques for SSPE/MIBE brain material, especially by PCR, have provided significant data on the genetic variability between the genes of SSPE/MIBE viruses and wild-type MV; what has been lacking are functional assays for the proteins coded by the mutant genes of SSPE and MIBE viruses. Recently, Hirano et al. (30, 31) reported functional differences between *wt* and SSPE M proteins, based on inability of SSPE M protein to bind in vitro to virion RNP cores compared with competency of binding M protein from the *wt* Niagata strain of MV. Their studies demonstrated the reduced capacity of mutant M proteins to interact with heterotypic RNP cores, suggesting strict conformational requirements for M-protein function. Our studies show various degrees of binding of different *wt* MV and SSPE M proteins with a heterologous RNP core from the Edmonston vaccine strain of MV. Such observations indicate that RNP-binding domains are not completely altered in SSPE M proteins and that the binding domains are presumably different from those involved in transcriptional regulation. The hypermutated M protein cloned from the MIBE-infected brain appears to represent an extreme case in which virtually complete absence of RNP binding prevents its functional activity.

Further studies are needed to analyze the stoichiometry of binding in both homologous and heterologous systems and to determine the stages at which M protein exerts its effect in attenuation of transcription. The experiments reported here illustrate another functional assay for comparing *wt* and mutant M proteins of MV. The striking abundance of RNP cores in brain cells of patients with SSPE could possibly result from unrestricted transcription and replication of RNP cores as a result of certain mutated M proteins defective in transcription inhibition (or binding to RNP cores). Far more research is required to test these hypotheses.

ACKNOWLEDGMENTS

We express our deep appreciation to William Bellini, Centers for Disease Control and Prevention, Atlanta, Ga.; Aimo Salmi, University of Turku, Turku, Finland; Ray Marusyk and Linda Chui, University of Alberta, Edmonston, Alberta, Canada; and Tim Wong, University of Washington, Seattle, for generously providing cDNA clones and antibody directed to M protein. Druen Robinson and Oneida Mason provided excellent technical assistance.

This research was supported by grant R37 AI-11112 from the National Institute of Allergy and Infectious Diseases and by the Deutsche Forschungsgemeinschaft and the Alexander von Humboldt Siftung.

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