

## The Matrix Protein of Vesicular Stomatitis Virus Inhibits Nucleocytoplasmic Transport When It Is in the Nucleus and Associated with Nuclear Pore Complexes

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**The matrix (M) protein of vesicular stomatitis virus (VSV) is a potent inhibitor of bidirectional nuclear transport. Here we demonstrate that inhibition occurs when M protein is in the nucleus of *Xenopus laevis* oocytes and that M activity is readily reversed by a monoclonal antibody ( $\alpha$ M). We identify a region of M protein, amino acids 51 to 59, that is required both for inhibition of transport and for efficient recognition by  $\alpha$ M. When expressed in transfected HeLa cells, M protein colocalizes with nuclear pore complexes (NPCs) at the nuclear rim. Moreover, mutation of a single amino acid, methionine 51, eliminates both transport inhibition and targeting to NPCs. We propose that M protein inhibits bidirectional transport by interacting with a component of the NPC or an NPC-associated factor that participates in nucleocytoplasmic transport.**

Trafficking of large macromolecules (more than 50 kDa) between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs) via signal-dependent, carrier-mediated processes (reviewed in references 39 and 54). This transport is subject to control in response to a variety of stimuli such as progression through the cell cycle, exposure to stress, and infection by viruses (reviewed in reference 39). Thus, control of nucleocytoplasmic transport is an important element in the regulation of gene expression.

Much of the carrier-mediated movement through NPCs requires cargo-specific transport receptors called importins and exportins (or karyopherins), which are members of the importin  $\beta$  superfamily of proteins (16, 21, 45, 61). Transport receptors can bind their cargoes either directly or via specialized adapter proteins. For example, importin  $\beta$  mediates import of proteins containing basic nuclear localization sequences and small nuclear ribonucleoproteins (snRNPs) using the adapter proteins importin  $\alpha$  (1) and snurportin (28, 43), respectively. Importin  $\beta$  can also interact directly with import cargoes, such as cyclin B (40, 53) and certain ribosomal proteins (30). CRM1 (Exportin1) mediates the export of proteins containing leucine-rich nuclear export signals (NES) as well as unspliced viral mRNAs and pre-snRNAs that are bound to specific NES-containing adapter proteins (14, 17, 51, 57). Exportin-t binds directly to its RNA export cargo, tRNA (3, 33).

Directionality of nuclear transport appears to be governed largely by Ran, a small GTPase that is a central component of most known nucleocytoplasmic transport pathways (reviewed in references 9 and 41). Owing to the asymmetric localization of the Ran effector proteins RanGAP (the GTPase activating protein in the cytoplasm) and RCC1 (the guanine nucleotide exchange factor in the nucleus), a steep concentration gradient of RanGTP is presumed to exist across the nuclear envelope (29). This gradient plays a pivotal role in nucleocytoplasmic transport by triggering both assembly and disassembly of receptor-cargo complexes in the appropriate subcellular com-

partment (60). Thus, import complexes assemble in the cytoplasm in the absence of RanGTP and disassemble in the nucleus in the presence of RanGTP, whereas export complexes form upon binding to RanGTP in the nucleus and dissociate upon removal and hydrolysis of RanGTP in the cytoplasm. Consequently, collapse of the RanGTP-GDP gradient leads to a block of most nucleocytoplasmic transport (29).

Nucleocytoplasmic transport is subject to regulation during infection by many types of viruses. For example, the NS1 protein of influenza virus inhibits export of cellular poly(A)<sup>+</sup> mRNA (7). Expression of the Rev protein of human immunodeficiency virus type 1, which functions as an adapter for CRM1, allows export of incompletely spliced viral mRNAs (14, 17, 25, 38). The E1B oncoprotein of adenovirus type 5 promotes export of viral mRNAs and inhibits export of most cellular mRNA species (11). The matrix (M) protein of vesicular stomatitis virus (VSV) inhibits bidirectional nuclear transport of both RNAs and proteins (27).

Infection of cells by VSV, a negative-strand RNA virus that replicates in the cytoplasm, results in rapid shutoff of cellular gene expression (59) and snRNA processing (18). The M protein, a major structural component of the VSV virion, plays a central role both in the inhibition of host cell gene expression (5, 42) and in viral assembly (59). These two properties are genetically separable from each other (6, 8, 37) in that methionine 51 (Met-51) of the M protein is required for inhibition of host cell gene expression, but not for viral assembly, whereas amino acids 4 to 21 are needed for viral assembly but not for inhibition of host cell gene expression.

Previously, we showed that M protein synthesized in *Xenopus laevis* oocytes inhibits the import of snRNPs and karyophilic proteins, as well as the export of snRNAs and mRNAs, but not tRNAs (27). Here we use a monoclonal antibody that recognizes M protein ( $\alpha$ M) to investigate the mechanism of this inhibition of bidirectional nuclear transport. We demonstrate that inhibition of both export and import is readily reversed by this antibody and that M protein works from within the nucleus. Furthermore, we show that Met-51 and the adjacent residues 52 to 59 are necessary both for efficient recognition by  $\alpha$ M and for the inhibition of nuclear transport. Met-51 is also important for colocalization of M protein and NPCs,

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French press. The lysates were clarified by centrifugation, made 10% with respect to glycerol, and frozen at  $-70^{\circ}\text{C}$  until further purification.

For affinity purification of GST-M proteins, lysates (10 ml) were quick-thawed and loaded directly onto a 1-ml glutathione-Sepharose column (Sigma). Columns were washed with 10 ml of wash buffer (PBS, pH 7.4; 1 M NaCl; 1% Triton X-100) and eluted with 50 mM Tris-HCl (pH 8.0)–5 mM glutathione–150 mM KCl–0.01% Triton X-100. Then, 0.5-ml fractions were collected, and aliquots were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing GST-M protein were pooled, made 10% with respect to glycerol, and stored at  $4^{\circ}\text{C}$  or frozen at  $-70^{\circ}\text{C}$  until use.

**Analysis of RNA transport in *X. laevis* oocytes.** Stage VI oocytes were manually dissected from *X. laevis* ovaries and maintained in MBS-H medium at  $18^{\circ}\text{C}$  (24). Nuclei or cytoplasm were injected with 12 nl of  $\text{H}_2\text{O}$  containing  $\sim 5$  fmol each of  $^{32}\text{P}$ -labeled RNAs, along with blue dextran (44, 55). After incubation at  $18^{\circ}\text{C}$  for the indicated times, oocytes were manually dissected and analyzed as previously described (22, 35, 44).

For injection of mRNA encoding the wild-type VSV M protein, 25 nl ( $\sim 20$  fmol) was injected into the cytoplasm  $\sim 18$  h prior to the injection of import or export RNAs. For injections of mRNAs encoding M deletion proteins, 25 nl ( $\sim 200$  fmol) was injected. For injection of purified GST-M proteins ( $\sim 100$   $\mu\text{g}/\text{ml}$ ), 15 nl was injected into the nucleus or 25 nl was injected into the cytoplasm. For injection of GST-M(48–62) protein, the concentration of protein in the injection mix was 3.6 mg/ml. When GST-M protein and antibodies were coinjected, the concentration of GST-M protein in the injection mixes was  $\sim 25$   $\mu\text{g}/\text{ml}$ . For inhibition of protein synthesis, cycloheximide was added directly to the MBS-H medium at a final concentration of 200  $\mu\text{g}/\text{ml}$ .

**Analysis of protein synthesis in *X. laevis* oocytes.** Stage VI oocytes were injected into the cytoplasm with mRNAs for M proteins and incubated for 16 to 24 h in MBS-H containing 0.5 to 1.0  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine (Amersham) (24). The nuclear and cytoplasmic fractions from such oocytes were analyzed as previously described (27, 46).

**Antibodies.** The hybridoma cell line synthesizing the  $\alpha\text{M}$  monoclonal antibody (23H12) (36) was kindly provided by Douglas Lyles (Wake Forest University). Antibodies were purified from hybridoma supernatants on a protein G-Sepharose (Pharmacia) column using standard procedures (26). Antibodies were stored at  $-20^{\circ}\text{C}$  until use. For use in oocytes,  $\alpha\text{M}$  was concentrated to 27 mg/ml (Amicon), and 15 nl (nucleus) or 25 nl (cytoplasm) was injected. The nonimmune rabbit anti-mouse immunoglobulin G (IgG) antibody (Cappel) was concentrated to 22 mg/ml (Amicon) and either 15 nl (nucleus) or 25 nl (cytoplasm) was injected into oocytes. The monoclonal  $\alpha\text{GST}$  antibody (Pharmacia) was dialyzed against PBS at 0.2 mg/ml. For coinjection of  $\alpha\text{M}$  or  $\alpha\text{GST}$  antibody and GST-M protein, the concentration of antibody in the injection mixtures was  $\sim 0.18$  mg/ml.

**Western blotting and immunoprecipitations.** For Western blot analysis, purified GST-M proteins or oocyte extracts were fractionated by SDS-PAGE and the proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Membranes were probed with antibodies in TBS-T (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 0.25% Tween 20) containing 5% powdered milk (Carnation) and developed with LumiGLO (Kirkegaard and Perry).

For immunoprecipitations, 9  $\mu\text{g}$  of  $\alpha\text{M}$  was coupled to 20  $\mu\text{l}$  of protein A-Sepharose beads (Sigma) in TBS-T for 2 h at  $4^{\circ}\text{C}$ . Either 40 ng of recombinant M protein or 0.5 oocyte equivalent of [ $^{35}\text{S}$ ]methionine-labeled cytoplasmic extract, prepared from oocytes expressing M protein, was added to the  $\alpha\text{M}$  beads. After overnight rotation at  $4^{\circ}\text{C}$ , the supernatant was collected and the pellets were washed five times with 500  $\mu\text{l}$  of TBS-T. The supernatant and pellet fractions were visualized by SDS-PAGE, followed by autoradiography.

**Immunofluorescence.** To process cells for immunofluorescence, cells were either fixed with 2% paraformaldehyde for 15 min followed by permeabilization with 0.5% Triton X-100 or else extracted first with 0.5% Triton X-100 for 3 min, followed by paraformaldehyde fixation. GFP-M proteins were visualized using the  $\times 100$  objective of an Axioplan 2 fluorescence microscope (Zeiss). For double-labeling experiments, GFP-M protein expressing cells were extracted with Triton X-100, fixed with formaldehyde, and incubated with 5  $\mu\text{g}$  of mAb414 (kindly provided by Laura Davis, Brandeis University). The subsequent incubation was with Alexa 568-conjugated goat anti-mouse secondary antibody (Molecular Probes).

**Phage display.** Isolation of peptides that could be recognized by the  $\alpha\text{M}$  antibody (23H12) was done by phage display as described previously (49). The CW1 M13 phage library, a 12-mer random peptide library, was kindly provided by Brian Kay (University of Wisconsin—Madison). After three rounds of selection, the resulting phage were plaque purified. Binding activities of the cloned phage were confirmed by enzyme-linked immunosorbent assay, and the peptide sequences of isolated  $\alpha\text{M}$  binding phage were deduced from DNA sequence analysis.

## RESULTS

**$\alpha\text{M}$  antibody in either the nucleus or the cytoplasm reverses the inhibitory activity of M protein.** The M protein of VSV is a potent inhibitor of bidirectional nuclear transport (27). As we reported previously, export of RNAs (snRNA and mRNA

but not tRNA) from oocyte nuclei is selectively blocked upon the synthesis of M protein following cytoplasmic injection of in vitro-transcribed M protein mRNA (Fig. 1A, panel c). M protein also inhibits the import of karyophilic proteins (27; data not shown) and several RNAs, such as U1, U5, and U6 snRNAs and NL-15 RNA (Fig. 1C, panel d), which use different import pathways (22). U3 snoRNA, which normally is not exported (56) or imported (50), was included here as a control for accuracy of injection and dissection of the oocytes.

Here, we refer to the ability of M protein to block nucleocytoplasmic transport as M activity. To probe the mechanism of M activity, we utilized a mouse monoclonal IgG antibody, 23H12, which is specific for M protein ( $\alpha\text{M}$ ) (36). We first showed that  $\alpha\text{M}$  can neutralize M activity by introducing  $\alpha\text{M}$  into the cytoplasm of oocytes prior to the injection of M mRNA (Fig. 1A). The presence of this antibody in the cytoplasm eliminated the inhibitory activity of the newly synthesized M protein (panel d). As expected, RNA export in oocytes containing no M mRNA was unaffected by cytoplasmic (panel b) or nuclear  $\alpha\text{M}$  antibody (data not shown). The ability to neutralize M activity was specific to  $\alpha\text{M}$  since injection of nonspecific IgGs into the cytoplasm (panel e) was without effect.

We then asked if  $\alpha\text{M}$  could reverse M activity once the inhibition of transport had been established in oocytes expressing M protein (Fig. 1B and C). Inhibition of export of both mRNA and snRNA (Fig. 1B) was reversed regardless of whether  $\alpha\text{M}$  was injected into the cytoplasm (panel a) or the nucleus (panel b). RNA import (Fig. 1C) also was restored by  $\alpha\text{M}$ , independent of where the antibody was introduced (panels d to f); again, the antibody had little or no effect on RNA import in the absence of M protein (panels b and c). Likewise, protein import was restored by  $\alpha\text{M}$  that was injected into either the nucleus or the cytoplasm (data not shown). Thus,  $\alpha\text{M}$  in either the nucleus or the cytoplasm can reverse M activity, raising the possibility that M protein functions in the nucleus.

**$\alpha\text{M}$  antibody eliminates M activity by nuclear depletion or neutralization of M protein.** To determine how  $\alpha\text{M}$  neutralizes M activity, we monitored the intracellular distribution of M protein in the presence and absence of  $\alpha\text{M}$  (Fig. 2A). Because of its small size ( $\sim 28$  kDa), M protein might be able to enter and exit the nucleus either by diffusion or by carrier-mediated transport. In the absence of the antibody, M protein was present in both the nucleus and the cytoplasm (27) (Fig. 2A, lanes 1 and 4). Independent of the expression of M protein,  $\alpha\text{M}$  antibody remained in the cell compartment into which it was introduced (Fig. 2A, lanes 5 and 6; see also Fig. 2B, lanes 2 and 3). Injection of  $\alpha\text{M}$  into the cytoplasm prior to injection of M mRNA led to sequestration of M protein in the cytoplasm (Fig. 2A, lanes 3 and 6), showing that complexes of M protein plus  $\alpha\text{M}$  do not transit the NPCs (Fig. 2A, lane 6; Fig. 2B, lanes 2 and 3). Thus, neutralization of M activity by cytoplasmic  $\alpha\text{M}$  (Fig. 1A) is due either to the formation of antibody-M protein complexes in the cytoplasm or to the lack of M protein in the nucleus.

Because  $\alpha\text{M}$  also reversed M activity after transport had already been established (Fig. 1B and C), we analyzed the distribution of M protein in such oocytes (Fig. 2B). Injection of  $\alpha\text{M}$  into the cytoplasm resulted in the nuclear depletion of M protein (lanes 1 and 3), indicating that the nuclear M protein either exits to the cytoplasm or turns over. Injection of  $\alpha\text{M}$  into the nucleus led to increased levels of nuclear M protein but did not deplete the cytoplasmic pool (lanes 2 and 4), presumably because of continued synthesis of M protein. The fact that transport in these oocytes was normal indicates either that this

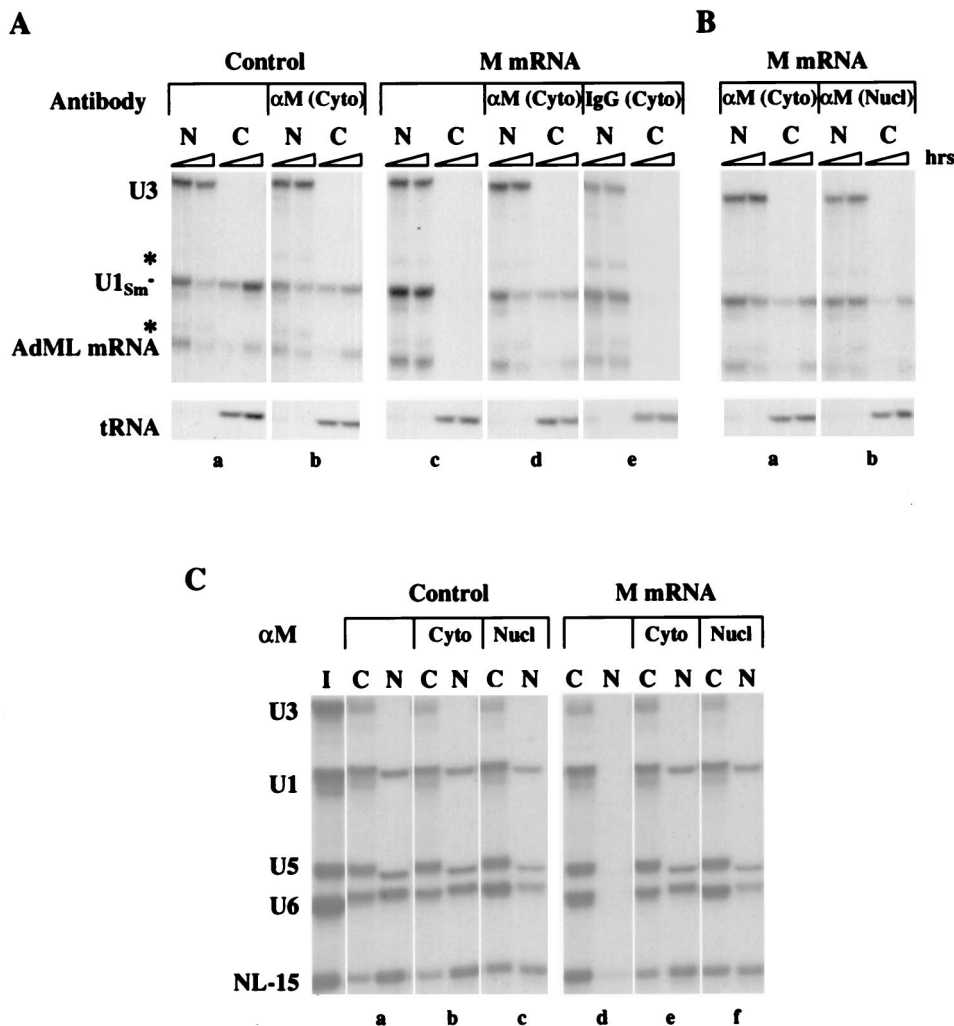


FIG. 1. Inhibition of bidirectional nuclear transport by M protein is reversed by  $\alpha$ M antibody. (A) Neutralization of M activity. A mixture of  $^{32}$ P-labeled U3 and U1<sub>sm</sub> snRNAs, AdML pre-mRNA, and tRNA was injected into the nuclei of oocytes that had been preinjected with M protein mRNA (M mRNA) and antibody in the cytoplasm.  $\alpha$ M or nonimmune rabbit anti-mouse IgG was injected 0.5 h prior to the injection of M protein mRNA, and oocytes were incubated for an additional 16 h prior to the injection of export RNAs. At 1 and 3 h after RNA injection, oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions; RNAs were isolated and 0.5 oocyte equivalents were resolved on 8% denaturing polyacrylamide gels and detected by autoradiography. AdML mRNA is generated by splicing of the injected pre-mRNA (not shown). Asterisks indicate degradation products arising from AdML pre-mRNA and U3 snoRNA. RNA export was monitored in control oocytes in the absence (a) and in the presence (b) of cytoplasmic  $\alpha$ M antibody and in oocytes expressing M protein (M oocytes) in the absence (c) and in the presence of cytoplasmic  $\alpha$ M antibody (d) or nonimmune rabbit anti-mouse IgG (e). (B) Reversal of inhibition of RNA export.  $\alpha$ M was injected into the nucleus (Nucl) or cytoplasm (Cyto) 16 h after the injection of M protein mRNA and 2 h prior to the injection of export RNAs. RNA export was monitored as in panel A in M oocytes in the presence of cytoplasmic (a) or nuclear (b)  $\alpha$ M antibody. (C) Reversal of inhibition of RNA import. A mixture of  $^{32}$ P-labeled U3, U1, U5, and U6 snRNAs and NL-15 RNA was injected into the cytoplasm of control and M oocytes in the absence or presence of  $\alpha$ M antibody.  $\alpha$ M was injected into the nucleus (Nucl) or cytoplasm (Cyto) 16 h after the injection of M protein mRNA and 2 h prior to injection of import RNAs. At 28 h after RNA injection, the nucleocytoplasmic distributions of RNAs were analyzed as in panel A. The RNAs in the injection mixture (I) are shown. Import was monitored in control and M oocytes in the absence (a and d) or in the presence of cytoplasmic (b and e) or nuclear (c and f)  $\alpha$ M antibody.

cytoplasmic M protein is in an inactive form or that the target of M protein resides in the nucleus. In any case, the elimination of M activity (Fig. 1) correlates with the lack of free M protein in the nucleus, due to binding of  $\alpha$ M to M protein in the nucleus, nuclear turnover, or sequestration of the protein in the cytoplasm. Therefore, we propose that M protein inhibits nuclear transport from within the nucleus.

**Inhibition of nuclear transport occurs from within the nucleus.** To introduce M protein directly into the nucleus (or the cytoplasm), we generated a fusion protein containing GST and M protein (GST-M protein). This recombinant protein had a molecular weight of about 56,000, thereby reducing its rate of passive diffusion through NPCs (54). Upon injection into ei-

ther the nucleus or the cytoplasm, GST-M protein was stable, and it gradually distributed between both compartments over a 20-h period (Fig. 3A). Injected recombinant GST-M protein inhibited both import of RNAs (Fig. 3B) and export of U1<sub>sm</sub> RNA (Fig. 3C, panels b and d) and AdML mRNA (Fig. 4b). Moreover, the inhibitory activity was independent of whether the protein was injected into the nucleus (Fig. 3B, panel b; Fig. 3C, panel b) or the cytoplasm (Fig. 3B, panel c; Fig. 3C, panel d).

As we observed with M protein synthesized in oocytes, the activity of cytoplasmically injected M protein was eliminated when the neutralizing antibody  $\alpha$ M was present in either the nucleus or the cytoplasm (Fig. 3C, panels e and f). Therefore,

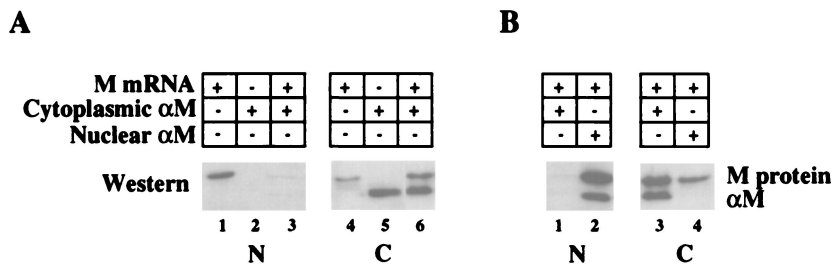


FIG. 2. Nucleocytoplasmic distribution of M protein in the absence or presence of αM. One oocyte equivalent of nuclear (N) or cytoplasmic (C) extract was analyzed by SDS-PAGE, followed by Western blotting with αM. (A) αM antibody was injected 0.5 h prior to the injection of M protein mRNA, and 21 h later extracts were prepared. The distribution of M protein was monitored in the absence (lanes 1 and 4) or presence (lanes 3 and 6) of cytoplasmic αM antibody. (B) αM antibody was injected 16 h after the injection of M protein mRNA, and 5 h later extracts were prepared. The distribution of M protein was monitored in the presence of cytoplasmic (lanes 1 and 3) or nuclear (lanes 2 and 4) αM antibody. In all cases, the antibody remained in the compartment into which it was injected, as shown by the location of the αM light chain (panel A, lanes 5 and 6; panel B, lanes 2 and 3).

we tested our model that M protein acts from within the nucleus by injecting GST-M protein into the nucleus and αM into the cytoplasm. When transport was monitored 1 h after injection of RNA substrates, GST-M protein inhibited RNA

export (panel c), demonstrating that nuclear M protein is sufficient for M activity. At later times, this GST-M activity was attenuated (data not shown), consistent with our previous finding that cytoplasmic αM eliminates M activity by sequestering

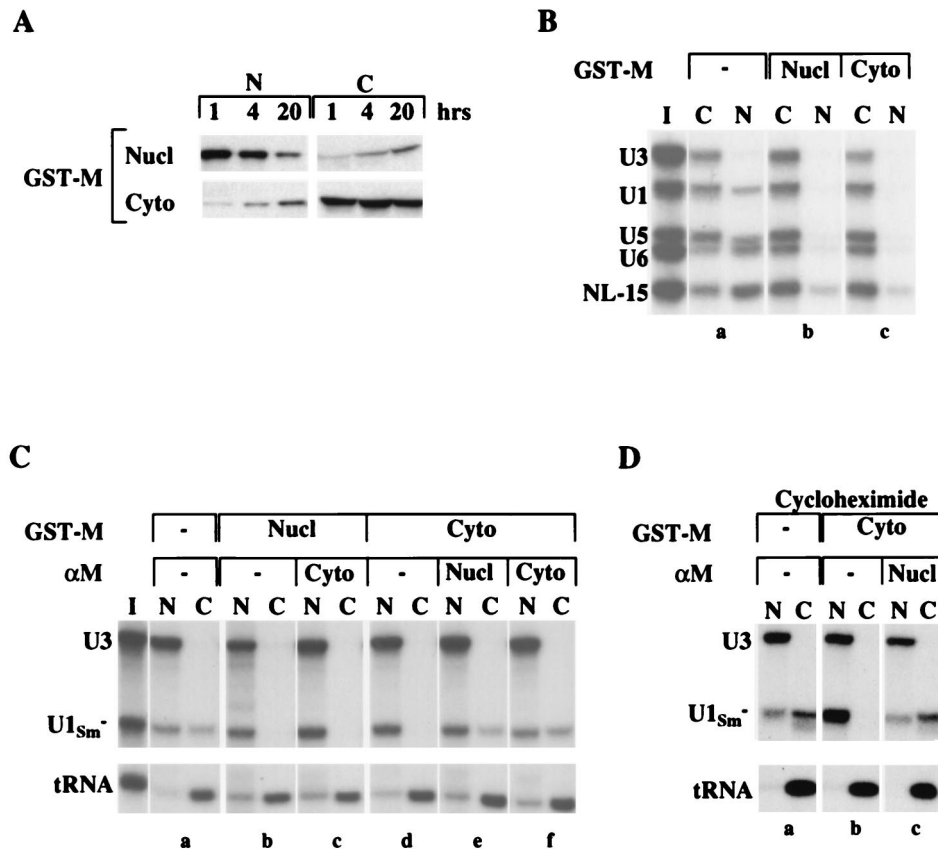


FIG. 3. Inhibition of transport by GST-M protein occurs from within the nucleus. (A) Stability and distribution of GST-M protein. The nucleocytoplasmic distribution of GST-M protein was monitored by Western blotting with αGST antibody. Extracts were prepared 1, 4, and 20 h after nuclear (top panels) or cytoplasmic (bottom panels) injection of GST-M protein; one oocyte equivalent of the nuclear (N) or cytoplasmic (C) extracts was analyzed. (B) Inhibitory activity of GST-M protein. Import of <sup>32</sup>P-labeled U1, U5, and U6 snRNAs and NL-15 RNA (import RNAs) was analyzed in control oocytes (a) and in oocytes preinjected with GST-M protein in the nucleus (b) or the cytoplasm (c). GST-M protein was injected 2 h prior to injection of import RNAs. Import was monitored 28 h after injection of the RNA mixture (I). (C) Nuclear function of GST-M protein. Export of <sup>32</sup>P-labeled U1<sub>sm</sub><sup>-</sup> snRNA and tRNA was analyzed in control oocytes (a) and in oocytes preinjected with GST-M protein (b and d) or with both GST-M protein and αM antibody (c, e, and f). αM was injected 1 h prior to the injection of GST-M protein, which was followed 1 h later by the injection of the export RNAs. Export was monitored 1 h after injection of the RNA mixture (I). (D) Reversal of transport inhibition in the absence of protein synthesis. Export of <sup>32</sup>P-labeled U1<sub>sm</sub><sup>-</sup> snRNA and tRNA was analyzed in the presence of cycloheximide in control oocytes (a) and in oocytes preinjected with GST-M protein (b) or with GST-M protein and nuclear αM antibody (c). GST-M protein was injected into the cytoplasm, and 1.5 h later cycloheximide (200 μg/ml) was added to the oocyte incubation medium. αM was injected 1 h after the addition of cycloheximide and 1 h prior to the injection of export RNAs. This amount of cycloheximide was sufficient to block protein synthesis as monitored by [<sup>35</sup>S]methionine labeling (data not shown).

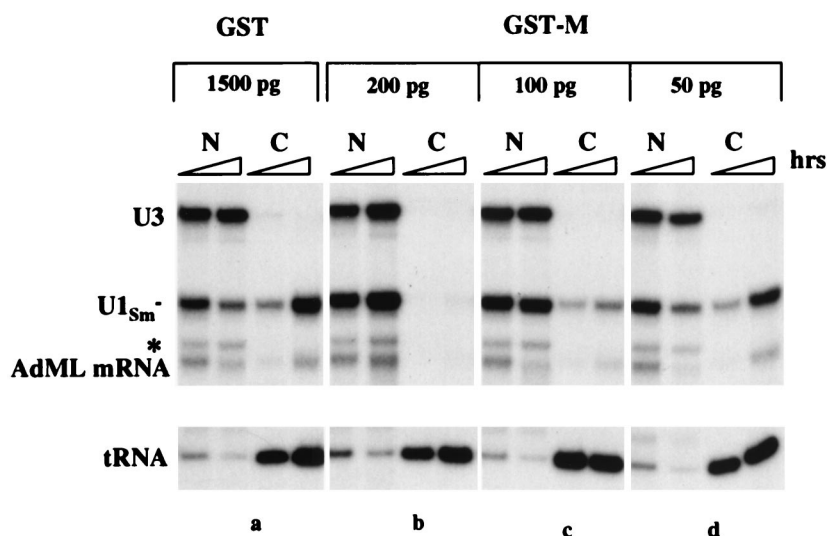


FIG. 4. Titration of the amount of nuclear GST-M protein required for M activity. Export of  $^{32}\text{P}$ -labeled  $\text{U1}_{\text{sm}}^-$  snRNA, AdML mRNA and tRNA was analyzed in oocytes preinjected 20 min earlier in the nucleus with 1,500 pg of recombinant GST protein (a) and with the indicated amounts (b to d) of GST-M protein. RNA export was monitored 1 and 3 h after injection of export substrates. The asterisk indicates a degradation product of U3 snoRNA.

M protein as it exits the nucleus (Fig. 1B and 2B). We conclude that GST-M protein functions from within the nucleus to inhibit nuclear transport.

**Nuclear M protein operates efficiently.** Only a low level of cytoplasmically injected GST-M protein appeared in the nucleus within 1 h (Fig. 3A), but this was sufficient for M activity (Fig. 3C, panel d). To determine the amount of nuclear GST-M protein required for inhibition of  $\text{U1}_{\text{sm}}^-$  RNA export, we varied the amount of protein injected. About  $2 \times 10^9$  molecules ( $\sim 200$  pg) per oocyte nucleus sufficed (Fig. 4b), but 100 pg did not (panel c). Because it is unlikely that all of the recombinant protein molecules were active, 200 pg of GST-M protein represents an upper limit of the amount required.

The rate at which nuclear GST-M protein inhibits export of RNA was estimated by coinjection of GST-M protein and RNA export substrates into the nucleus (data not shown). In this case,  $<10\%$  of the  $\text{U1}_{\text{sm}}^-$  RNA was exported within the first hour compared to 50% of the RNA in the absence of GST-M protein. Thus, M protein functions very quickly within the nucleus to inhibit transport.

**Nuclear  $\alpha\text{M}$  antibody reverses inhibition by shielding a region necessary for M activity.** Since reversal of transport inhibition by nuclear  $\alpha\text{M}$ , in principle, could be due to the synthesis of new protein(s) required for transport, we tested if  $\alpha\text{M}$  could restore transport in the absence of protein synthesis. Inhibition of RNA export by GST-M protein was reversed by nuclear  $\alpha\text{M}$ , even in the presence of cycloheximide (Fig. 3D), demonstrating that M activity does not involve the irreversible modification or destruction of a component that participates in nuclear transport. Also, the neutralization of GST-M protein by  $\alpha\text{M}$  was dependent on binding of the monoclonal antibody to its specific epitope, since a GST-specific monoclonal antibody ( $\alpha\text{GST}$ ) did not interfere with the activity of GST-M protein (data not shown). Thus,  $\alpha\text{M}$  may reverse transport inhibition by binding to a region of M protein necessary for M activity, thereby disrupting interactions between the protein and its target(s).

**Met-51 of M protein contributes to recognition by  $\alpha\text{M}$  antibody.** The region of M protein recognized by  $\alpha\text{M}$  was identified by Western blot analysis of mutant M proteins. Several

mRNAs encoding deletion mutants of M protein were generated, and the proteins were expressed in oocytes, as demonstrated by labeling with [ $^{35}\text{S}$ ]methionine (Fig. 5A, top panel). All of these mutant M proteins, except for the  $\Delta 47-75$  deletion protein, were recognized by  $\alpha\text{M}$  (bottom panel). In addition, an M13 phage library displaying random peptides 12 amino acids in length was screened for the ability to be recognized by  $\alpha\text{M}$ . A consensus sequence present in seven of the nine selected peptides, DPNQ, is present once in the immunizing antigen (M protein of the San Juan strain) (data not shown). This sequence is very similar to the amino acid sequence, DPHQ, found at the same position (amino acids 55 to 58) of the M protein (Orsay strain) used here (Fig. 5D). Thus, the  $\alpha\text{M}$  epitope is contained within amino acids 47 to 75 of M protein and includes residues 55 to 58.

A spontaneous temperature-sensitive mutant of VSV (*tsO82*) that is defective in blocking host cell gene expression has an altered M protein in which Met-51 is changed to arginine [M(51R) protein] (6, 8). Because Met-51 lies within the region of M protein containing the  $\alpha\text{M}$  epitope, we tested if this residue is important for recognition by  $\alpha\text{M}$ . GST-M(51R) mutant protein reacted poorly with  $\alpha\text{M}$  in Western blots (Fig. 5B, top panel, lane 2), a result consistent with the unpublished results of others analyzing *tsO82* mutant M protein synthesized in mammalian cells (D. Lyles, personal communication). Also, the native M(51R) protein was recognized inefficiently by  $\alpha\text{M}$  in an immunoprecipitation assay (Fig. 5C, compare lanes 2 and 3 with lanes 5 and 6).

Since the M51R mutation introduces a positively charged residue at position 51, we asked if a conservative substitution of Met-51 with the hydrophobic residues leucine or alanine could maintain  $\alpha\text{M}$  recognition. In Western blots, the GST-M(51L) but not the GST-M(51A) protein was recognized like wild-type GST-M protein (Fig. 5B, top panel, lanes 3 and 4). Thus, an amino acid with a long aliphatic side chain is required at position 51 of M protein for efficient binding to  $\alpha\text{M}$ .

**Met-51 is essential for M activity.** To test if the  $\alpha\text{M}$  epitope region is also required for inhibition of nuclear transport, we assayed the effect of the M51R mutation on M activity. GST-M(51R) protein injected into the nucleus was unable to inhibit

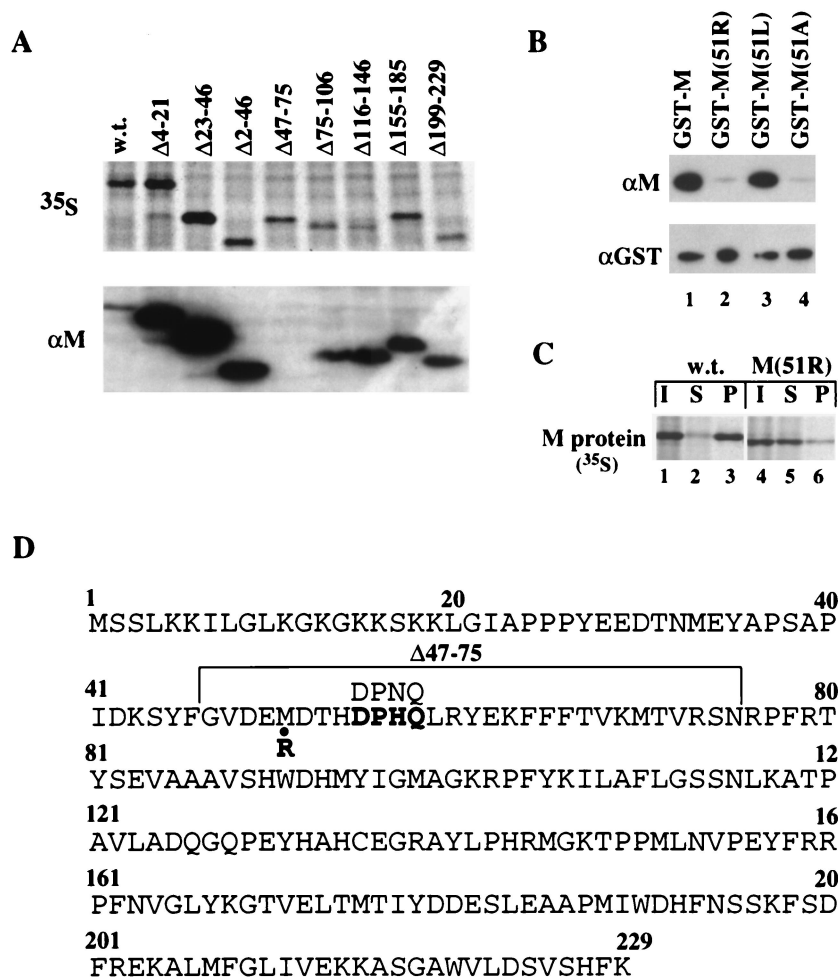


FIG. 5. Met-51 of M protein contributes to recognition by  $\alpha M$  antibody. (A) Mapping of the  $\alpha M$  epitope region. mRNAs encoding the wild type (w.t.) and the indicated deletion mutants of M protein were injected into the cytoplasm of oocytes, and the newly synthesized proteins were labeled by the addition of [ $^{35}S$ ]methionine to the incubation medium. After 18 h, whole-oocyte extracts were prepared and analyzed by SDS-PAGE, followed by autoradiography (top panel) or Western blotting with the  $\alpha M$  antibody (bottom panel). (B)  $\alpha M$  reactivity of wild-type and mutant M proteins. Western blot analysis was performed with 10 ng each of GST-M (lane 1), GST-M(51R) (lane 2), GST-M(51L) (lane 3), and GST-M(51A) (lane 4) proteins using  $\alpha M$  (top panel) or  $\alpha GST$  (bottom panel) antibody. (C) Inefficient recognition by  $\alpha M$  of native M(51R) protein. [ $^{35}S$ ]methionine-labeled M proteins were synthesized in oocytes as in panel A and 0.5 oocyte equivalents of cytoplasmic extracts (I) were immunoprecipitated with  $\alpha M$  antibody. Both the supernatant (S) and the pellet (P) fractions from immunoprecipitations of wild-type M (lanes 2 and 3) or M(51R) (lanes 5 and 6) proteins were analyzed by SDS-PAGE, followed by autoradiography. (D) Amino acid sequence of the VSV M protein of the Orsay strain. Amino acids 47 to 75 are bracketed. The DPHQ sequence (amino acids 55 to 58) is shown in boldface. The corresponding sequence (DPNQ) in the immunizing antigen, VSV M protein of the San Juan strain, is shown above the DPHQ sequence. The substitution of Met-51 to Arg-51, found in the M protein of the temperature-sensitive VSV mutant, *tsO82* (8), is indicated.

RNA export (Fig. 6A), showing that an arginine at position 51 of M protein eliminates M activity. Inhibition of transport was not observed even when the amount of injected mutant protein was 50 times that needed for inhibition by wild-type GST-M protein (cf. Fig. 4). Also, M(51R) protein was inactive for inhibition of protein import (data not shown). Western blot analysis, using  $\alpha GST$  antibody, demonstrated that the protein was stable in oocytes and that its distribution was similar to that of GST-M protein (Fig. 6B). Thus, the Met-51 to Arg-51 mutation abolishes the ability of M protein to inhibit both nuclear transport and gene expression (6, 13, 52), showing that the mechanisms of inhibition of these processes are closely linked.

We tested if the inactivity of GST-M(51R) protein was due to the introduction of a positive charge by assaying the activities of mutant M proteins having the conservative amino acid substitutions M51L and M51A. Neither of these proteins (which were stable in oocytes; Fig. 6B) was able to inhibit RNA

export (Fig. 6A, panels e and f) even when injected into the nucleus at 30 times the amount needed for inhibition by wild-type protein (data not shown). Thus, although leucine at position 51 allows for recognition by  $\alpha M$ , it does not suffice for M activity, showing that a methionine at position 51 is essential for the inhibition of nuclear transport.

**Amino acids 51 to 59 are necessary, but not sufficient, for inhibition of transport by M protein.** Secondary structure predictions by the PhD and PREDATOR algorithms (19, 20, 47) suggest that the  $\alpha M$  epitope is contained within a region of M protein (50-EMDTHDPHQ-58) that is likely to be in either a loop or a turn structure. This region probably is exposed on the surface of the protein because most of these amino acids are hydrophilic and  $\alpha M$  can bind and inactivate M protein in vivo. This model is in agreement with previous findings by others demonstrating that a major V8 protease cleavage site in M protein occurs at position 50 (31).

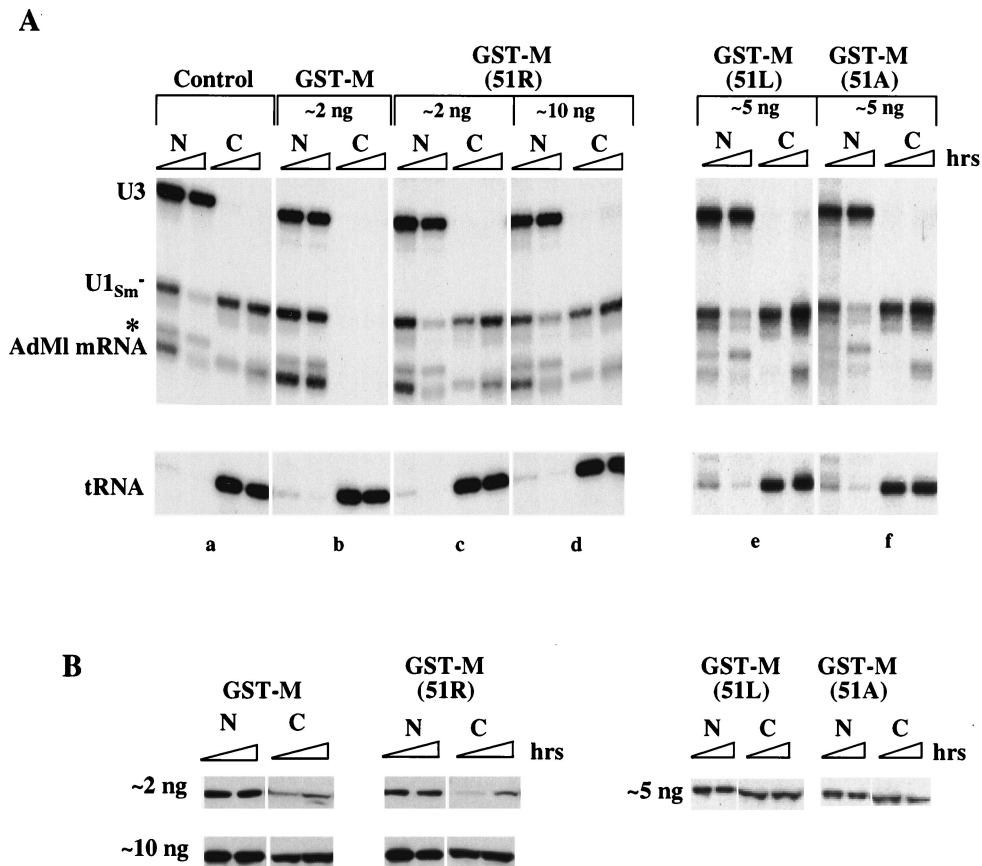


FIG. 6. Met-51 of M protein is essential for inhibition of nuclear export. (A) RNA export. Wild-type and mutant GST-M proteins were injected into the nucleus 1 h prior to the injection of RNA export substrates. Export of  $^{32}$ P-labeled U1<sub>Sm</sub>- snRNA, AdML mRNA, and tRNA was monitored 1 and 3 h after RNA injection. RNA export was analyzed in the absence (a) or in the presence of the indicated amounts of GST-M (b), GST-M(51R) (c and d), GST-M(51L) (e), or GST-M(51A) (f) proteins. (B) GST-M protein stability and distributions. The nucleocytoplasmic distributions of GST-M proteins were monitored 2 and 5 h after nuclear injection of the indicated amounts of GST-M, GST-M(51R), GST-M(51L), or GST-M(51A) proteins.

To determine if amino acids in the loop region surrounding Met-51 also are important for M activity, a series of templates was constructed encoding mutant GST-M proteins with triple alanine substitutions from positions 48 through 65 (three amino acids substituted in each mutant). As indicated, three of these proteins, mutated in amino acids 51 through 59, were not recognized by  $\alpha$ M (Fig. 7A), a result consistent with our  $\alpha$ M epitope mapping (Fig. 5). When tested for their abilities to inhibit transport, the same three mutant GST-M proteins were inactive, even though they were stable and distributed similarly to the GST-M protein in injected oocytes (Fig. 7A). Thus, in addition to Met-51, amino acids 52 to 59 are necessary for both  $\alpha$ M recognition and GST-M activity.

To test if the amino acids of this loop region were sufficient for M activity, we generated a fusion protein composed of GST and amino acids 48 to 62 of M protein. The chimeric protein was recognized by  $\alpha$ M in immunoprecipitations (Fig. 7B), showing that the  $\alpha$ M epitope region was presented in a context resembling that of the native M protein. Although a large nuclear pool of this protein remained for at least 18 h after injection into oocytes (data not shown), it had no effect on the export of mRNA or snRNAs (Fig. 7C). Thus, amino acids 51 to 59 are necessary, but not sufficient, for M activity.

**Met-51 is necessary for targeting M protein to the nuclear rim.** To determine where within the nucleus M protein might function, GFP-tagged wild-type and mutant [M and M(51R)]

proteins were expressed in transiently transfected HeLa cells. A comparable recombinant GFP-tagged M protein inhibited nuclear transport upon injection into oocytes (data not shown). The wild-type GFP-M protein localized to the nucleoplasm, the cytoplasm, and the nuclear rim (Fig. 8A, panel a). In contrast, the M(51R) mutant protein showed only nucleoplasmic and cytoplasmic localization; no nuclear rim association was readily observable (panel b), indicating that the intracellular distribution of the inactive M protein differs from that of wild-type M protein.

To visualize the nuclear-rim-associated fraction of GFP-M proteins, the transfected HeLa cells were treated with Triton X-100 prior to fixation with formaldehyde, thereby releasing soluble M protein (4). M protein associated with the nuclear rim was resistant to this treatment, whereas the nucleoplasmic and cytoplasmic fractions of GFP-M protein were extracted (Fig. 8B, panel a). As expected, in GFP-M(51R)-transfected cells, no nuclear-rim-associated protein was detectable (panel d). Thus, the elimination of M activity correlates with the loss of nuclear rim association, suggesting that targeting of M protein to the nuclear rim is essential for M activity.

We asked if a putative target for M protein might be contained within NPCs. To visualize the NPCs (Fig. 8B, panels b and e), we used monoclonal antibody mAb414, which recognizes FG repeat containing nucleoporins (10). As shown by the overlap of the fluorescence signals from GFP and mAb414



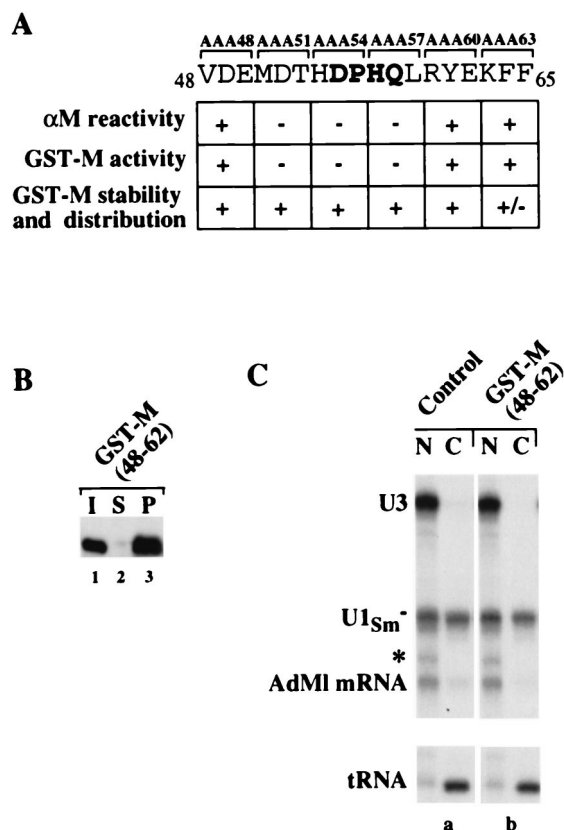


FIG. 7. Amino acids 51 to 59 of M protein are necessary, but not sufficient, for the inhibition of nuclear transport. (A) Activity and  $\alpha$ M reactivity of triple alanine scanning mutants of M protein. The  $\alpha$ M reactivities of the mutant M proteins were analyzed as in Fig. 5A and B, and the inhibitory activities, stabilities, and intracellular distributions were determined as in Fig. 6. +, Behavior indistinguishable from that of wild-type protein; -, behavior similar to that of GST-M(51R) mutant protein. (B)  $\alpha$ M reactivity of native GST-M(48-62) protein. GST-M(48-62) protein (I) was immunoprecipitated with  $\alpha$ M antibody and the supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE, followed by Western blotting with the  $\alpha$ GST antibody. (C) RNA export. Export of  $^{32}$ P-labeled U1<sub>sm</sub><sup>-</sup> snRNA, AdML mRNA, and tRNA was analyzed in the absence (a) or in the presence (b) of GST-M(48-62) protein. GST-M(48-62) protein was injected into the nucleus 1 h prior to the injection of export substrates. RNA export was monitored 1 h after RNA injection. The molar concentration of the injected GST-M(48-62) protein was  $\sim$ 75 times that of wild-type GST-M protein used in comparable experiments (e.g., Fig. 6A, panel b).

(panels c and f), GFP-M protein and NPCs colocalize at the nuclear rim (panel c). This suggests that wild-type, but not M(51R) mutant (panel f), M protein interacts with a nuclear component of NPCs. We propose that M protein acts as an inhibitor of bidirectional nuclear transport when associated with the intranuclear side of the NPC.

## DISCUSSION

We have demonstrated here that the M protein of VSV must be in the nucleus to inhibit bidirectional nuclear transport and that the target of M protein is likely to be a component of the NPC. Our results show that inhibition of transport is readily reversible, since a monoclonal antibody ( $\alpha$ M) can restore transport, even after inhibition has been established. Amino acids 51 to 59 of M protein are important both for the inhibition of nuclear transport and for recognition by  $\alpha$ M. Moreover, we have identified a single amino acid, Met-51, that is necessary for the association of M protein with the nuclear rim. We

propose that interaction of M protein with a nuclear component(s) of the NPC and/or an NPC-associated factor(s) is responsible for its inhibition of nuclear transport.

Our results show that M protein must be in the nucleus, and not complexed with  $\alpha$ M, in order to inhibit nucleocytoplasmic transport. Transport is restored either when the activity of M protein is neutralized by nuclear  $\alpha$ M or when M protein is sequestered and neutralized in the cytoplasm by  $\alpha$ M (Fig. 1 and 2). Moreover, GST-M protein introduced into the nucleus inhibits transport, even when neutralizing  $\alpha$ M antibody is present in the cytoplasm (Fig. 3C). Consistent with the need for a pool of nuclear GST-M protein, GST-M activity dissipates with time as the protein is depleted from the nucleus and sequestered by  $\alpha$ M in the cytoplasm. Thus, M protein exerts its inhibitory effect from within the nuclear compartment.

The interaction of M protein with a nuclear target(s) must be rapid, since inhibition of transport occurs soon after delivery of M protein to the nucleus. Also, this association must be dynamic, since  $\alpha$ M can restore transport even when protein synthesis is blocked (Fig. 3D), ruling out resynthesis of targets as a way to restore transport. Thus, M protein may modulate the activity of a nuclear transport factor through reversible modification (e.g., phosphorylation, ADP ribosylation, etc.). Alternatively, M protein could disrupt associations between factors that participate in nuclear transport by competitively binding to one of them.

The M(51R) mutant protein, originally identified in a spontaneous temperature-sensitive mutant of VSV (*tsO82*) (8), inactivates the ability of M protein to inhibit transport (Fig. 6A) and affects binding to  $\alpha$ M (Fig. 5B). Conservative substitution of Met-51 with either leucine or alanine also eliminates M activity (Fig. 6B), demonstrating that a methionine at this position is essential for the inhibitory effect of M protein. In the case of GST-M(51L) protein, its inactivity cannot be attributed to gross misfolding of the epitope region, since  $\alpha$ M recognition is not altered by this mutation (Fig. 5B). We propose that Met-51 of M protein is necessary for the interaction between M protein and its target(s) and that binding of  $\alpha$ M to the epitope region of M protein disrupts this association.

Met-51 lies within a region of M protein (amino acids 50 to 58) predicted to form an exposed loop or turn structure. Triple alanine scanning mutations of the adjacent amino acids in this presumptive loop region (amino acids 52 to 59) abolished both M activity and recognition by  $\alpha$ M (Fig. 7A). However, substitution of amino acids 53 to 59 of M protein with a sequence composed of both hydrophilic and hydrophobic amino acids, which are less likely than consecutive alanine residues to distort the structure of this region, did not eliminate M activity (unpublished data). This raises the possibility that amino acids 53 to 59 within the loop region do not play a direct role in the interaction of M protein and its target. We are currently testing essentially which amino acids in this region are involved in essential contacts between M protein and its nuclear target(s).

Our analyses of the intracellular localization of wild-type and mutant M proteins (Fig. 8) indicate that the nuclear components of the NPC and associated transport factors are likely targets for inactivation by M protein. In transiently transfected HeLa cells, GFP-M protein associated with the nuclear rim, and this localization was coincident with that of FG-repeat containing nucleoporins. In contrast, the nonfunctional mutant GFP-M(51R) protein did not colocalize with NPCs, leading to the model that M protein is associated with the NPC when it inhibits transport.

In support of this model, the pattern of inhibition by M protein resembles that observed when the activities of specific nucleoporins or transport factors are inhibited by antibodies or

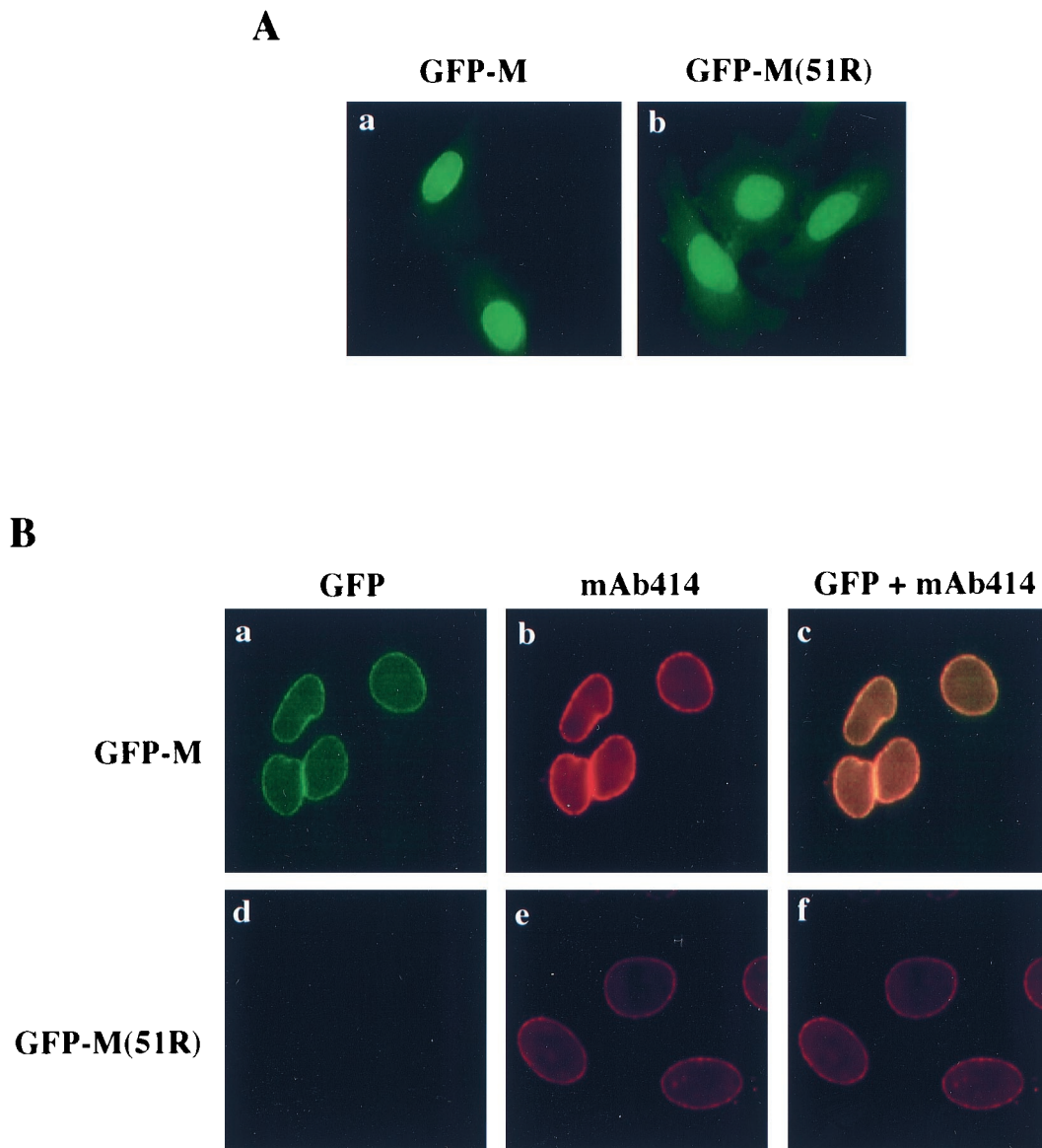


FIG. 8. Wild-type GFP-M protein, but not mutant GFP-M(51R) protein, colocalizes with NPCs. Plasmid DNAs encoding GFP-tagged wild-type and M(51R) mutant M proteins were transfected into HeLa cells, and 24 h later the cells were either fixed with formaldehyde (A) or extracted with 0.5% Triton X-100 prior to formaldehyde fixation and visualization by fluorescence microscopy (B). (A) Intracellular localization of GFP-M proteins in mammalian cells. The localization of GFP-M (a) and GFP-M(51R) (b) proteins in transiently transfected HeLa cells was monitored by direct fluorescence. (B) Colocalization of GFP-M protein and NPCs. The association of GFP-M protein with the nuclear rim was monitored in Triton-extracted cells that were expressing either GFP-M (a to c) or GFP-M(51R) (d to f) proteins. GFP-M (a) and GFP-M(51R) (d) proteins were detected by direct fluorescence, and NPCs (b and e) were visualized by immunostaining with mAb414 antibody and a rhodamine-labeled secondary antibody. Colocalization of NPCs and GFP-M (c) or GFP-M(51R) (f) proteins is indicated by the overlap (yellow) of the rhodamine (red) and the GFP (green) fluorescent signals.

dominant-negative mutant proteins. Antibodies to Nup98 or Nup153, two intranuclear components of the NPC, block the export of mRNA and snRNA, but not tRNA (46, 58). Likewise, the isolated nucleoporin binding domains of the transport factors, importin  $\beta$  and TAP, act as dominant-negative mutants to inhibit the export of mRNA and snRNA and, to a much lesser extent, tRNA (4, 32). The dominant-negative form of importin  $\beta$  is also an efficient inhibitor of protein import (32). Both the antibodies and dominant-negative mutants are proposed to inhibit NPC function by blocking docking sites for transporters and their respective cargoes. We propose that M protein functions in a similar manner to inhibit bidirectional nuclear transport. Consistent with this proposal, mAb414,

which inhibits the export of most classes of RNAs (23), does not block the export of ET202, an RNA that was selected solely for its ability to be exported in the presence of M protein (23). VSV replicates its genome in the cytoplasm and does not require nuclear factors for virus production (15); nonetheless, M protein can be detected in the nuclei of VSV-infected cells (36). In agreement with that observation, we have shown that M protein distributes to both the cytoplasm and the nucleus in *Xenopus* oocytes and in transfected HeLa cells (27) (Fig. 2 and 8) and that the protein is actively imported in an in vitro system (J. M. Petersen, unpublished data). The mutant M(51R) and M(51L) proteins also enter the nucleus both in vivo and in vitro (Fig. 8) (37; unpublished data), showing that Met-51 is

not required for nuclear uptake of M protein. It is unclear whether M protein exits the nucleus by diffusion or active transport.

In addition to inhibiting nucleocytoplasmic transport, wild-type M protein was previously reported to inhibit gene expression in cultured cells (5, 6, 13, 42) and transcription *in vitro* (2, 62). We have not observed an inhibition of transcription by M protein in *Xenopus* oocytes, but we recognize that oocyte nuclei may be unusual in that they contain large stockpiles of transcription and replication factors (24). In any case, the loss of inhibition of both gene expression and transport upon mutation of Met-51 indicates that a common mechanism is involved. We have proposed (27) that M protein may affect gene expression directly by blocking mRNA export. In addition, inhibition of transport might secondarily affect the import or function of factors that are essential for the assembly of active transcription complexes. However, M protein may use the same mechanism to inactivate different targets that affect either transport or transcription independently. Recent evidence suggests that inhibition of RNA polymerase II transcription by M protein alters the activity of the transcription factor TFIID (62).

Inhibition of transport by M protein, presumably via association with a nuclear component of NPCs, may recapitulate control systems that normally modulate nucleocytoplasmic transport in uninfected cells. For example, the target(s) of M protein could be a component of the NPC, the function of which could be altered during induction of a stress response, reduction of cell growth rate, or progression through the cell cycle (12, 34, 48). Thus, M protein might provide a potent tool for investigation of cellular mechanisms that regulate nuclear transport and transcription. Our current efforts are focused on the identification of the intranuclear components of the NPC that interact with M protein and on elucidation of how inhibition of transport and transcription are linked.

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