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Nuclear Localization of Mouse Mx1 Protein Is Necessary for Inhibition of Influenza Virus

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The interferon-induced Mx1 protein of mice confers selective resistance to influenza virus. It inhibits viral mRNA synthesis in the nucleus of influenza virus-infected cells. The related human MxA protein is localized in the cytoplasm and can inhibit influenza virus and vesicular stomatitis virus but not other viruses. MxA blocks a poorly defined cytoplasmic multiplication step of influenza virus that follows primary transcription of the viral genome. We previously showed that nuclear variants of MxA that carry an artificial nuclear translocation signal were also active against influenza virus. However, these variants blocked primary transcription of influenza virus. In the present study, we addressed the question of whether cytoplasmic forms of Mx1 were capable of mimicking the antiviral action of MxA by determining the antiviral activities of mutant mouse Mx1 protein. Cytoplasmic Mx1(E_{614}), which differs from wild-type Mx1 by a single amino acid substitution in its nuclear transport signal, failed to inhibit the multiplication of influenza virus and vesicular stomatitis virus. Relocation of Mx1(E_{614}) to the nucleus with the help of the simian virus 40 large T nuclear translocation signal attached to its amino terminus restored the influenza virus-inhibiting activity. Other changes in the carboxy-terminal region of Mx1 also abolished transport to the nucleus and simultaneously abolished antiviral activity. One of these variants, Mx1/A, gained activity against influenza virus upon relocation to the nucleus. These results demonstrate that unlike human MxA, the mouse Mx1 protein can function only in the nucleus. This finding has important implications regarding the mechanistic details of Mx protein action.

In cell culture and in animals, interferons stimulate the synthesis of at least 20 interferon-induced proteins, including the Mx proteins (21, 26, 27). Mx1 of mice is a nuclear 72-kDa protein (6, 11) that selectively inhibits the multiplication of influenza virus (7, 22, 29). It interferes with mRNA synthesis of influenza virus (14, 20), a process that takes place in the nucleus of infected cells (13). Human MxA protein is a cytoplasmic 76-kDa protein (1, 10, 28) that inhibits the multiplication of influenza virus and vesicular stomatitis virus (VSV) but has no inhibitory effect on other viruses (22). As expected from its cytoplasmic localization, human MxA has no inhibitory effect on influenza virus mRNA synthesis; rather, it inhibits a later viral multiplication step (20). The human MxA protein inhibits VSV by blocking its mRNA synthesis, which occurs in the cytoplasm of infected cells (30). The other Mx proteins analyzed to date are either inactive against all of the viruses that were tested (human MxB [22] and rat Mx3 [15]), not active against influenza virus but active against VSV (rat Mx2 [15] and mouse Mx2 [34]), or strongly active against influenza virus but only weakly active against VSV (rat Mx1 [15]).

The molecular mechanism of Mx protein action is not known. Mouse Mx1 (18) and human MxA (9) proteins were shown to possess GTPase activity, although it remains unclear whether this activity is necessary for antiviral activity. We recently showed that a mutant form of MxA with a Glu-to-Arg substitution at position 645 exhibits altered antiviral specificity; it has lost its activity against VSV, but it is still active against influenza virus (35), suggesting that a domain near the carboxy terminus of MxA is responsible for antiviral specificity. Depending on its intracellular localization, MxA can block different steps of the influenza virus multiplication cycle. We found that unlike the cytoplasmic wild-type MxA, which has no effect on influenza virus primary transcription (20), nuclear forms of MxA that were equipped with a foreign nuclear transport signal efficiently blocked primary transcription of the influenza virus genome (35).

Since MxA is able to block the multiplication of influenza virus in the cytoplasm as well as in the nucleus (35), we set out to determine whether the mouse Mx1 protein would also function in both cell compartments. An earlier attempt to define functional domains of Mx1 protein (19) revealed that the carboxy-terminal region harbors a sequence required for its nuclear translocation. We therefore generated Mx1 variants that lack a functional nuclear transport signal and determined their antiviral activities. We found that unlike wild-type MxA, the cytoplasmic variants of mouse Mx1 protein were all inefficient inhibitors of both influenza virus and VSV. We excluded the trivial possibility that mutations which inactivate the nuclear transport signal of Mx1 would simultaneously inactivate a functionally important domain: we relocated the mutant Mx1 proteins to the nucleus with a foreign nuclear transport signal and demonstrated that some of the relocated Mx1 proteins regained the ability to block influenza virus replication.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* MC1061 (3) was used for the construction and maintenance of plasmids; *E. coli* TG1 was used for the propagation of M13 phage vectors. For digestion of undermethylated DNA by *Cla*I and *Stu*I, the plasmids were grown in the Dcm^- Dam^- strain GM48.

Construction of pHMGMx1/A and pHMGMx1/2. Plasmids coding for the chimeric proteins Mx1/A and Mx1/2 were constructed by taking advantage of a *Pvu*II restriction site

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present at corresponding positions in the Mx1, MxA, and Mx2 cDNAs near the 3′ ends of their coding regions (Fig. 1). First, the Mx cDNAs were cloned into derivatives of cloning vector pSP64 (16), designated pSP64Δ191-*Pvu*II and pSP64Δ180-*Pvu*II, which both lack *Pvu*II sites. To construct pSP64Δ191-*Pvu*II, pSP64 was digested with *Sma*I and *Pvu*II, and the truncated vector was religated. To construct pSP64Δ180-*Pvu*II, pSP64 was digested with *Eco*RI and *Pvu*II, the 5′ protruding end of the *Eco*RI site was blunted with T4 DNA polymerase, and the truncated vector was religated. pSP64Mx1 was constructed by inserting the 2.2-kb *Bam*HI fragment of plasmid pHG327Mx (19) into the *Bam*HI site of pSP64Δ191-*Pvu*II. To generate pSP64Mx2, the 2.4-kb *Acc*I-*Bam*HI restriction fragment containing the complete coding region of mouse Mx2 cDNA (position 83 to the 3′ end [31]) was excised from plasmid pHG327Mx2, blunted with T4 DNA polymerase, and cloned into the *Hin*dII site of pSP64Δ191-*Pvu*II. pSP64MxA was obtained by subcloning the 2.2-kb *Sma*I restriction fragment of pBSMxA containing the complete MxA coding region (1) into the *Sma*I site of pSP64Δ180-*Pvu*II.

To construct pSP64Mx1/A and pSP64Mx1/2, samples of pSP64MxA, pSP64Mx1, and pSP64Mx2 were digested with *Pvu*II and *Sph*I. The 1.2-kb *Sph*I-*Pvu*II restriction fragment of pSP64Mx1 was then ligated to either the 2.5-kb *Pvu*II-*Sph*I fragment of plasmid pSP64MxA or the 2.7-kb *Pvu*II-*Sph*I fragment of plasmid pSP64Mx2. The resulting constructs, pSP64Mx1Δ/A and pSP64Mx1Δ/2, were next linearized with *Pvu*II and treated with alkaline phosphatase; the 1.0-kb *Pvu*II restriction fragment of pSP64Mx1 was then inserted, yielding plasmids pSP64Mx1/A and pSP64Mx1/2.

To construct expression vectors pHMGMx1/A and pHMGMx1/2, the chimeric cDNAs coding for Mx1/A and Mx1/2 were isolated as either a *Bam*HI-*Eco*RI fragment from pSP64Mx1/A or a *Bam*HI fragment from pSP64Mx1/2, blunted with T4 DNA polymerase, and ligated into the unique *Eco*RV site of the eukaryotic expression plasmid pCL642 (8), downstream of the constitutive promoter of the murine 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene.

Construction of pHMGMx1(E_{614}). The arginine residue (codon AGG) at position 614 of the Mx1 protein was replaced by glutamic acid (codon GAG) in the mutant protein Mx1(E_{614}). Site-directed mutagenesis was performed with the oligonucleotide 5′ CCAGCCTTAAAAGCCGCTC TTTCAGGAAC 3′, which is complementary to positions 2043 to 2071 of Mx1 cDNA (29) but differs from the wild-type sequence by the two underlined bases. The oligonucleotide was annealed to single-stranded M13mp18 phage DNA carrying the 250-bp *Hin*dII-*Hin*dIII Mx1 fragment corresponding to positions 1835 to 2086 of Mx1 cDNA (29). Synthesis of double-stranded DNA, its purification by agarose gel electrophoresis, and transfection of *E. coli* TG1 were performed as described previously (2). Phages carrying DNA with the mutation were identified by colony filter hybridization using the radiolabeled oligonucleotide as a probe. Successful mutagenesis was confirmed by DNA sequencing. The *Hin*dII-*Hin*dIII fragment of wild-type Mx1 cDNA was eventually replaced by the corresponding fragment carrying the newly introduced mutation. Subcloning of the modified Mx1 cDNA into the *Eco*RV restriction site of pCL642 (8) yielded the expression plasmid pHMGMx1(E_{614}).

Construction of pHMGMx1(−19). cDNA coding for a mutant Mx1 protein lacking the 19 carboxy-terminal amino acids (19) was cloned into the *Eco*RV restriction site of expression plasmid pCL642 (8), yielding the construct pHMGMx1(−19).

cDNAs encoding Mx proteins with SV40 large T nuclear translocation signal. The simian virus 40 (SV40) large T nuclear translocation signal was introduced at the amino terminus of the Mx1 protein as follows. First, a new *Cla*I restriction site, which destroyed the initiator ATG codon of Mx1, was generated by oligonucleotide-directed site-specific mutagenesis. The 30-mer oligonucleotide 5′ ATTATTCA CAGAATCGATCGTCTGGCTCTC 3′, which is complementary to positions 202 to 231 of Mx1 cDNA (29) except for the underlined base, was used for these manipulations. Next, a DNA fragment coding for the nuclear translocation signal of the SV40 large T antigen (12) was prepared by annealing two complementary 63-mer oligonucleotides, namely, the plus-strand oligonucleotide 5′ CGATACCATG GATAAAGAGTTCCTTGAGGCTCCTAAGAAGAAGAG GAAGGTGGAGTTCAGGAT 3′ and the minus-strand oligonucleotide 5′ CGATCCTGAACTCCACCTTCCTCTTCT TCTTAGGAGCCTCAAGGAACTCTTTATCCATGGTAT 3′. The ends of these oligonucleotides matched those of *Cla*I-restricted DNA. Annealing of the two oligonucleotides, T4 polynucleotide kinase treatment, and ligation into the newly created *Cla*I restriction site of Mx1 cDNA were performed by standard procedures (25). The correct orientation of the *Cla*I fragment in the resulting plasmid was verified by DNA sequencing. Plasmid pSP65TMx1 was eventually constructed by replacing the 420-bp *Eco*RI-*Stu*I fragment of the original Mx1 cDNA with the corresponding Mx1 cDNA fragment carrying the additional sequences.

To construct plasmids pSP65TMx1(E_{614}), pSP64TMx1 (−19), pSP64TMx1/A, and pSP64TMx1/2, the 0.8-kb *Sph*I-*Stu*I fragments of pSP65Mx1(E_{614}), pSP64Mx1(−19), pSP64 Mx1/A, and pSP64Mx1/2 were replaced with the corresponding fragment of pSP65TMx1. Construction of expression vectors pHMGTMx1, pHMGTMx1(E_{614}), pHMGTMx1 (−19), pHMGTMx1/A, and pHMGTMx1/2 was carried out as described above for the corresponding mutants lacking the SV40 large T nuclear translocation signal at the amino terminus.

Permanent transfection of Swiss 3T3 cells. Murine Swiss 3T3 cells were cultured in Dulbecco modified minimal essential medium (DMEM) containing 5 to 10% fetal calf serum (FCS). Transfected cells were selected and grown in the same medium supplemented with 500 μg of G418 (GIBCO) per ml. Transfection of plasmid DNA into Swiss 3T3 cells was performed by the standard calcium phosphate coprecipitation technique (32). Cells were split at a ratio of 1:6 the day before transfection. Five micrograms of pSV2neo plasmid DNA and 20 μg of the desired Mx cDNA constructs were used in each 90-mm-diameter culture dish. After overnight incubation at 37°C, the DNA precipitate was removed, and the cells were washed twice with phosphate-buffered saline (PBS) and incubated with new medium for another 24 h. The cells were then trypsinized and split at a ratio of 1:6 into medium containing 500 μg of G418 per ml. G418-resistant cell clones were examined for expression of Mx proteins by indirect immunofluorescence analysis as described previously (29). Positive clones were subcloned by limiting dilution, and stable cell clones expressing the different Mx protein variants were isolated.

Western immunoblot analysis. Sample preparation, electrophoresis, protein transfer to nitrocellulose membranes, and immunostaining were carried out as previously described (1) with a polyclonal rabbit antiserum against *E. coli*-produced murine Mx1 protein.

Virus stocks and infection procedures. Virus stocks of influenza virus A strain FPV-B and VSV serotype Indiana were prepared as described previously (22). For viral RNA analyses, confluent cell monolayers were infected for 30 min at room temperature with FPV-B at a multiplicity of 3 PFU per cell in medium containing 2% FCS and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.3. Unadsorbed virus was removed by two washings with PBS, and the cells were incubated in medium containing 2% FCS and 20 mM HEPES (pH 7.3) for 5 h at 37°C before RNA was prepared. For viral plaque assays, the cells were grown to about 80% confluency in 60-mm-diameter dishes. The monolayers were washed once with PBS and infected with about 100 PFU of influenza virus or VSV per dish in medium containing 2% FCS and 20 mM HEPES, pH 7.3. After 1 h at 37°C, unadsorbed virus was removed, prewarmed overlay agar (DMEM containing 2% FCS, 20 mM HEPES [pH 7.3], 0.4% Noble agar, and 0.002% DEAE-dextran) was added, and the dishes were incubated for 24 to 36 h at 37°C. To visualize viral plaques, the agar overlay was removed and the cells were stained with 1% crystal violet in 20% ethanol.

RNA isolation and Northern (RNA) blot analysis. Total cellular RNA was prepared by the acid guanidinium-phenol-chloroform procedure (4). RNA was subjected to electrophoresis through 1.2% agarose gels containing formaldehyde, blotted to nitrocellulose membranes, and hybridized to radiolabeled probes as described previously (1).

Hybridization probes. The probe for the PB1 gene of influenza virus A/Puerto Rico/8/34 was the *Hin*dIII insert of plasmid pAPR102 (33). The probe for the ubiquitously expressed cyclophilin gene was the *Bam*HI insert of plasmid pCD15:8-1 (5). DNA fragments were radiolabeled by nick translation with [α-^{32}P]dCTP.

RESULTS

Mutant forms of mouse Mx1 protein lacking a functional nuclear transport signal. We used different strategies to obtain cytoplasmic variants of mouse Mx1 protein (Fig. 1). It was previously shown that truncation of the carboxy terminus by 19 or more amino acids, which removes a stretch of basic residues (Fig. 2), results in cytoplasmic forms of Mx1 (19). As expected, the mutant protein Mx1(−19) was also excluded from the nucleus of permanently transfected Swiss 3T3 cells and instead accumulated in the cytoplasm (Fig. 3). In an attempt to inactivate the putative signal sequence that determines nuclear localization of Mx1 by less severe manipulations, we replaced the carboxy-terminal 35 residues of Mx1 with the corresponding sequences of the cytoplasmic mouse Mx2 and human MxA proteins. The resulting hybrid proteins, Mx1/2 and Mx1/A (Fig. 1), also accumulated in the cytoplasm of transfected Swiss 3T3 cells (Fig. 3). Finally, we changed the Arg residue at position 614 of Mx1 to Glu. This manipulation reduced the basic character of the arginine- and lysine-rich sequence near the carboxy terminus of Mx1, making it similar to the corresponding sequences of the cytoplasmic Mx2 and MxA proteins (Fig. 2). The resulting mutant protein, Mx1(E_{614}) (Fig. 1), indeed failed to migrate to the nucleus and accumulated in the cell cytoplasm (Fig. 3).

Nuclear transport of Mx1 was thus dependent on a motif located near the carboxy terminus, which includes the arginine residue at position 614. Although necessary for nuclear localization of Mx1, by itself this motif was not sufficient to translocate other proteins across the nuclear membrane. For example, a hybrid Mx protein consisting entirely of human MxA sequences, except for the carboxy-terminal 35 amino acids derived from Mx1, remained cytoplasmic. In contrast, a hybrid Mx protein consisting of the 289 amino-terminal residues of MxA and the 375 carboxy-terminal residues of Mx1 was transported to the cell nucleus (data not shown), indicating the presence of an additional nuclear transport signal sequence between residues 375 and 596 of Mx1. Taken together, these results suggested that Mx1 contains a bipartite or multipartite nuclear localization signal.

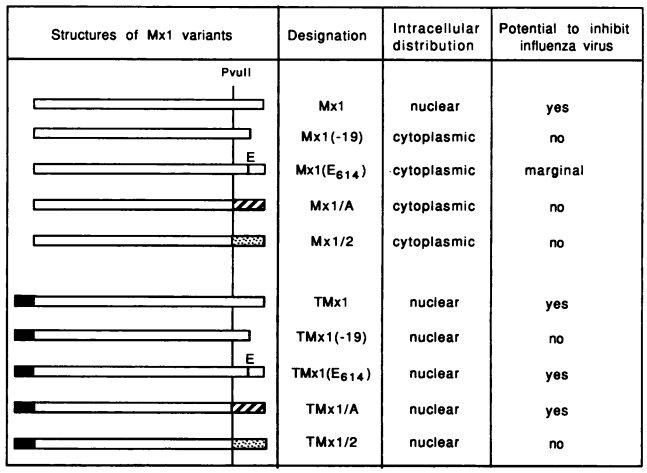

FIG. 1. Schematic representation of structures, subcellular distributions in transfected Swiss 3T3 cells, and antiviral potentials of wild-type and mutant forms of mouse Mx1 protein. Open bars indicate sequences derived from mouse Mx1, dotted bars indicate sequences derived from mouse Mx2, and striped bars indicate sequences derived from human MxA protein. Filled bars represent sequences corresponding to the nuclear translocation signal of the SV40 large T antigen. Mx1(−19) and TMx1(−19) lack the 19 carboxy-terminal amino acids of wild-type Mx1. The Arg codon at position 614 of Mx1 was converted to a Glu codon in the cDNAs coding for Mx1(E_{614}) and TMx1(E_{614}). Constructs encoding the hybrid proteins Mx1/2, Mx1/A, TMx1/2, and TMx1/A resulted from recombination of suitable cDNAs at a common *Pvu*II restriction site.

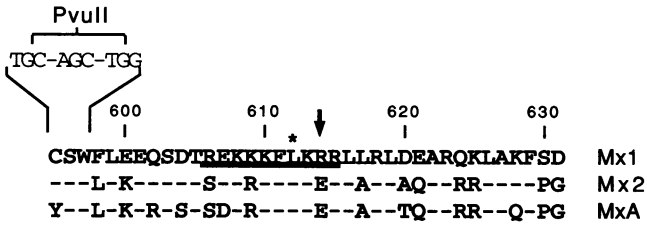

FIG. 2. Comparison of the carboxy-terminal amino acids of nuclear mouse Mx1 protein and the cytoplasmic mouse Mx2 and human MxA proteins. The cDNAs for these three proteins all contain a *Pvu*II restriction site at homologous positions, permitting convenient construction of hybrid proteins. A dash in the Mx2 or MxA sequence indicates identity to Mx1 at the corresponding position. The solid bar highlights a basic Mx1 sequence held responsible for the nuclear location of this protein (19). The arrow marks the Arg residue at position 614 of Mx1 that was mutated to Glu in the mutant proteins Mx1(E_{614}) and TMx1(E_{614}). The asterisk marks the Leu residue at position 612 of Mx1 that constitutes the new carboxy terminus of mutant proteins Mx1(−19) and TMx1(−19).

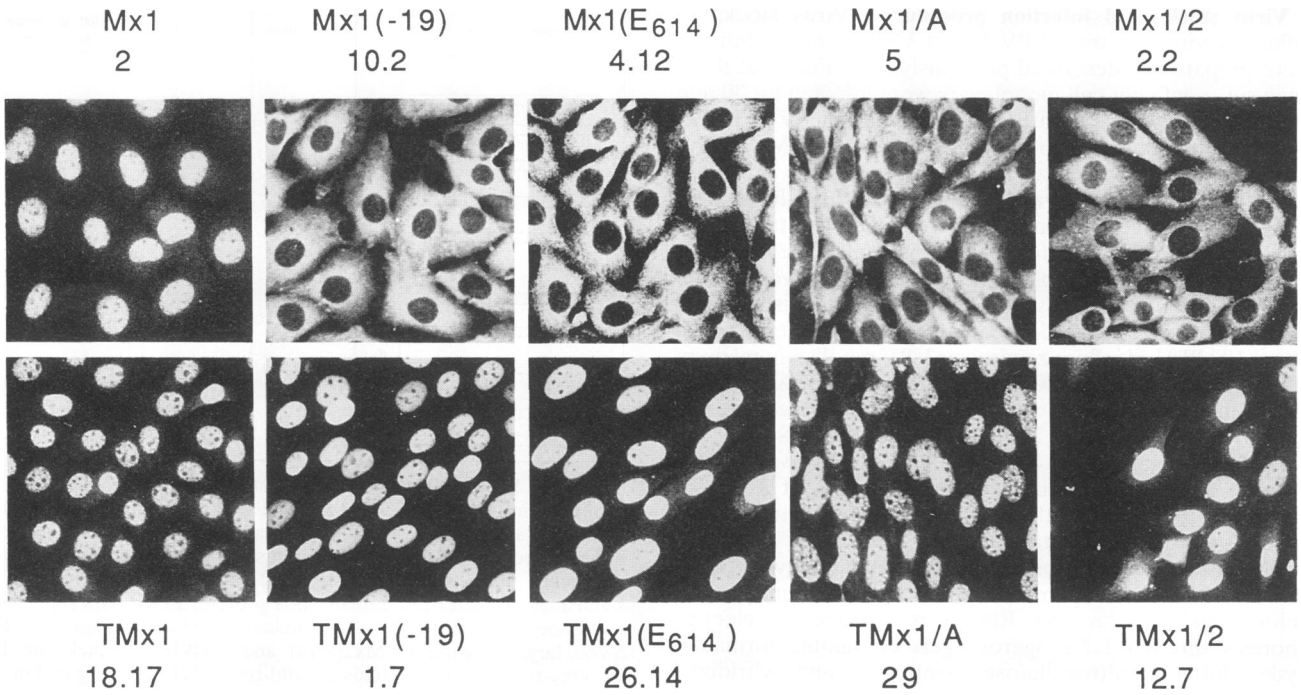

FIG. 3. Intracellular distribution of mutant forms of Mx1 proteins in permanently transfected Swiss 3T3 cells. The Mx1 variants expressed by the cell clones indicated were visualized by indirect immunofluorescence.

Mx1 variants lacking a functional nuclear transport signal are inefficient inhibitors of influenza virus and VSV. For each cDNA construct, we produced several independent permanent clonal cell lines that constitutively expressed the corresponding Mx1 variant proteins. Cell lines expressing amounts of the desired mutant Mx proteins equal to or larger than the amounts expressed by cell line 3T3mMx1 clone 2 were selected (Fig. 4A). Cell line 3T3mMx1 clone 2 expressed wild-type Mx1 protein at a level that resulted in a high degree of resistance to influenza virus (Fig. 5A). For the subsequent studies, we chose only cell clones that expressed the desired proteins at a fairly uniform level in close to 100% of the cell populations. As expected, the Mx1 variants produced by the different cell lines showed similar mobilities on denaturing protein gels, except for Mx1(−19), which migrated slightly faster because of its truncation at the carboxy terminus (Fig. 4A).

To determine the influenza virus-inhibiting activities of the cytoplasmic Mx1 variants, we infected cell cultures expressing these proteins at a multiplicity of 3 PFU of influenza virus per cell. At 5 h postinfection, we measured the relative influenza virus PB1 RNA levels in the infected cells by Northern blotting. Under these experimental conditions, high levels of PB1 RNA were found in infected control cells not expressing Mx proteins, whereas only low levels of PB1 RNA were detected in infected cells expressing wild-type Mx1 (Fig. 5A). The cell lines expressing cytoplasmic variants Mx1(−19), Mx1(E_{614}), Mx1/A, or Mx1/2 all contained high levels of PB1 RNA, indicating susceptibility to influenza virus (Fig. 5A).

We also tested whether influenza virus could form plaques on monolayers of Swiss 3T3 cells expressing the cytoplasmic Mx1 variants. In our laboratory, plaque reduction assays allow us to measure the antiviral activities of Mx proteins with very high sensitivity and sometimes reveal residual antiviral activity not detectable with other assays that require virus infections to be carried out at high multiplicities. Similar numbers of influenza virus plaques were observed in the cell line lacking Mx proteins and in the cell lines expressing either Mx1(−19), Mx1/2, or Mx1/A (Fig. 6). The plaque sizes were also similar. No influenza virus plaques were detectable in monolayers of control cells expressing wild-type Mx1. Interestingly, a reduced number of rather small plaques were observed when monolayers of cell clone 4.12, which expresses very high concentrations of Mx1 (E_{614}), were challenged with influenza virus (Fig. 6). This effect was not seen with cell clone 16.3.10 or others (data not shown) that express lower levels of Mx1(E_{614}) protein.

Next, we determined whether the cytoplasmic variants of mouse Mx1 protein could inhibit the multiplication of VSV. Viral plaque assays showed that Mx1(−19), Mx1/2, and Mx1/A had no significant activity against VSV (Fig. 7). Cell clone 4.12, which expresses Mx1(E_{614}) at a very high level, showed a reduced number of rather small VSV plaques (Fig. 7), whereas cell clone 16.3.10 and others that expressed lower levels of Mx1(E_{614}) remained fully susceptible to VSV (data not shown). Similarly, no protective effect was observed when VSV resistance was monitored by measuring VSV N mRNA accumulation (data not shown).

Some Mx1 variants gain influenza virus-inhibiting activity upon relocation to the nucleus. Is Mx1 indeed unable to function outside the cell nucleus, or did our modifications simultaneously destroy the nuclear transport signal and a functionally important domain of Mx1? In the first case, relocation to the cell nucleus with the help of a foreign nuclear transport signal should restore the antiviral activity of our Mx1 mutants. We therefore engineered the SV40 large T nuclear transport signal to the amino termini of the different Mx1 variants. The sequence added included the motif PKKKRKV, which can direct certain marker proteins

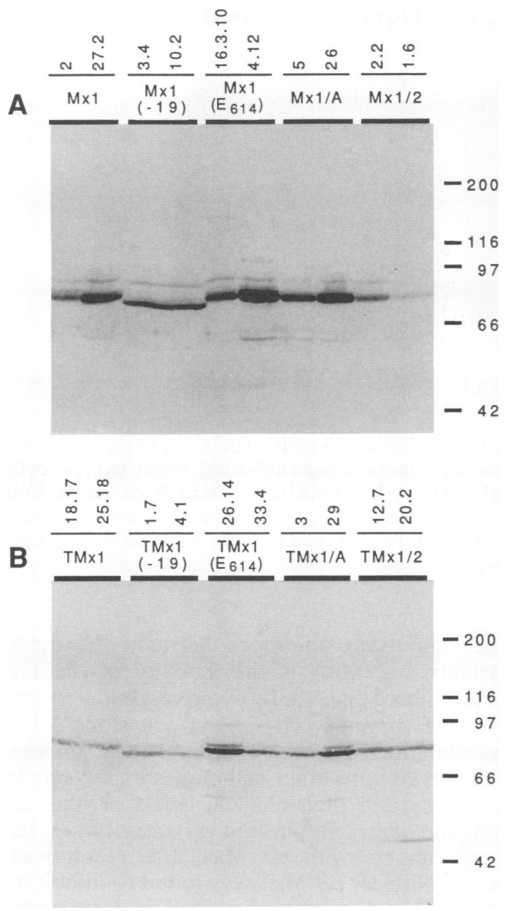

FIG. 4. Comparison of Mx protein expression levels in permanently transfected Swiss 3T3 cell lines. Mx1 concentrations in various clonal cell lines expressing wild-type Mx1 or the indicated Mx1 variants were measured by Western blotting. The numbering at the top of each gel refers to the cell lines used in this experiment. The bars and numbers at the sides indicate the relative gel positions of protein size markers (in kilodaltons). (A) Comparison of cell lines expressing wild-type Mx1 and derived cytoplasmic variants. (B) Comparison of cell lines expressing Mx proteins containing the SV40 large T nuclear transport signal.

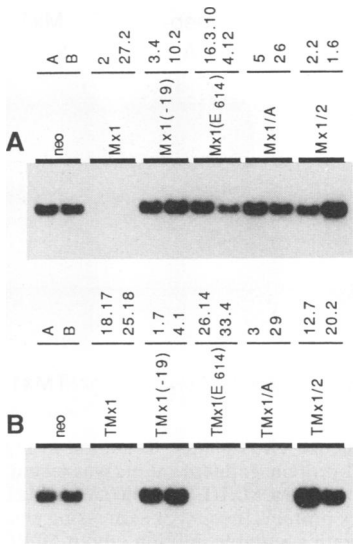

FIG. 5. Influenza virus RNA synthesis in permanently transfected Swiss 3T3 cell lines expressing cytoplasmic (A) or nuclear (B) variants of Mx1 protein. Two independent pools (lanes A and B) of cells transfected with the G418 resistance gene (*neo*) only served as virus-susceptible control cultures. The indicated cell lines were infected with influenza virus at a multiplicity of infection of 3 PFU per cell. At 5 h postinfection, the cells were lysed and total cellular RNA was prepared. Samples (10 μg) were then analyzed for their relative influenza virus PB1 RNA contents by Northern blotting. To show that similar concentrations of RNAs were loaded into each lane, the membranes were reprobed with radiolabeled 1B15 cDNA (5), which hybridizes to the transcripts of the ubiquitously expressed cyclophilin gene.

to the cell nucleus (12). These manipulations resulted in cDNA expression constructs encoding proteins TMx1, TMx1(−19), TMx1(E_{614}), TMx1/A, and TMx1/2, which differ from wild-type Mx1, Mx1(−19), Mx1(E_{614}), Mx1/A, and Mx1/2 by having 18 additional amino acids at the amino terminus (Fig. 1). Immunofluorescence analysis of Swiss 3T3 cells transfected with these expression plasmids revealed that Mx proteins carrying the SV40 sequence accumulated in the cell nucleus (Fig. 3). The nuclear staining patterns of wild-type Mx1, TMx1, TMx1(−19), TMx1(E_{614}), TMx1/A, and TMx1/2 were virtually identical, although the Mx1 variants with the SV40-derived nuclear transport signal relocated slightly faster to the nucleus after mitosis than wild-type Mx1 (data not shown).

As in the case of the cytoplasmic Mx1 variants, we again selected two or more independent stable clones of transfected Swiss 3T3 cells that constitutively expressed TMx1(−19), TMx1(E_{614}), TMx1/A, and TMx1/2 at levels equal to or higher than the levels of TMx1 expressed by our positive control cell lines (Fig. 4B). As expected, Mx proteins containing the 18 extra amino acids at the amino terminus migrated slightly more slowly on denaturing protein gels than their counterparts lacking the SV40 sequence (data not shown).

Wild-type Mx1 protein carrying the extra SV40 large T nuclear transport signal at the amino terminus retained its potential to block influenza virus replication (Fig. 5B and 6). However, the nuclear form of the truncated Mx1 protein, TMx1(−19), failed to inhibit the multiplication of influenza virus. Infected cells expressing this protein contained high levels of influenza virus PB1 RNA (Fig. 5B), and influenza virus formed plaques of normal size on monolayers of cells expressing Mx1(−19) (Fig. 6). In contrast, infected cells expressing either TMx1(E_{614}) or TMx1/A contained very low levels of influenza virus PB1 RNA (Fig. 5B) and did not allow plaque formation by influenza virus (Fig. 6). However, infected cells expressing TMx1/2 contained about as much influenza virus PB1 RNA as susceptible control cells lacking Mx proteins (Fig. 5B) and allowed influenza virus to form plaques (Fig. 6). Thus, two of the four Mx1 variants that we tested gained activity against influenza virus upon relocation to the cell nucleus.

These results indicated that sequences close to the carboxy terminus of Mx1 constitute a functionally important domain. The active protein TMx1/A and the inactive protein TMx1/2 differ by only five amino acids (Fig. 1 and 2). At four of these positions, the TMx1/2 sequence matches that of Mx1. The fifth amino acid is a threonine in TMx1/A, an alanine in TMx1/2, and an aspartic acid in Mx1. We therefore tested whether replacing the alanine at position 620 of TMx1/2 with a threonine would yield a protein with activity

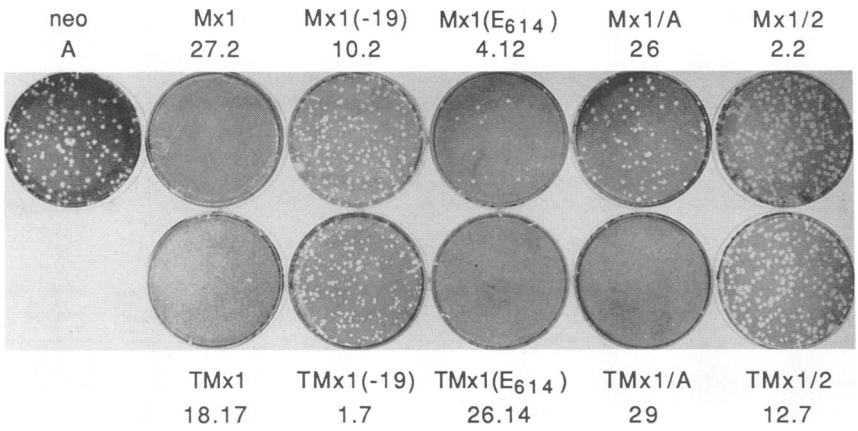

FIG. 6. Influenza virus plaque formation in monolayers of permanently transfected Swiss 3T3 cells expressing cytoplasmic or nuclear variants of Mx1 protein. Influenza virus was tested for its ability to form plaques on the clonal cell lines indicated, which express cytoplasmic Mx1 variant Mx1(−19), Mx1(E_{614}), Mx1/A, or Mx1/2 or nuclear Mx1 variant TMx1, TMx1(−19), TMx1(E_{614}), Mx1/A, or Mx1/2. Control cell lines lacking Mx proteins (neo A) or expressing wild-type Mx1 (Mx1 27.2) were included. Confluent cell monolayers in 60-mm-diameter dishes were infected with a suitable dilution (about 50 PFU per dish) of influenza virus A strain FPV-B, and plaques were allowed to develop for 40 h under soft agar.

against influenza virus. However, cell lines expressing TMx1/2 with this mutation remained susceptible to influenza virus (data not shown).

DISCUSSION

Our results demonstrate that the mouse Mx1 protein can function as an inhibitor of influenza virus replication only when present in the nucleus of Swiss 3T3 cells. Cytoplasmic forms of Mx1 with a nonfunctional nuclear transport signal were all inactive. The possibility that all mutations which inactivated the nuclear transport signal simultaneously inactivated a functionally important domain of Mx1 was excluded by relocating the Mx1 variants to the nucleus with a foreign nuclear transport signal and by showing that they could block influenza virus in the nucleus. Mx1 thus differs markedly from human MxA protein, which can inhibit the multiplication of influenza virus in both cell compartments (35).

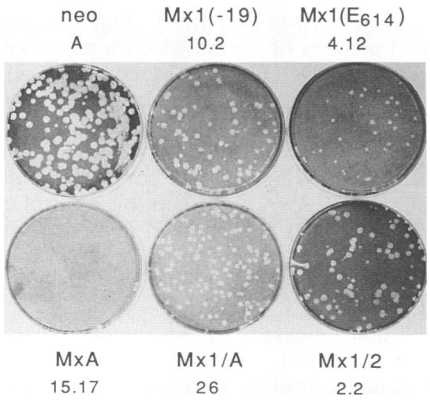

FIG. 7. VSV plaque formation in monolayers of permanently transfected Swiss 3T3 cells expressing cytoplasmic variants of Mx1 protein. Clonal cell lines expressing the indicated Mx1 variants, wild-type MxA (positive control), or no Mx proteins (neo) were infected with a suitable dilution of VSV stock virus (about 50 PFU), and viral plaques were allowed to develop for 40 h under soft agar.

Another important difference between the cytoplasmic Mx1 variants and MxA is the inability of the former to efficiently inhibit VSV. Only overexpression to extremely high levels of variant Mx1(E_{614}) in cell clone 4.12 caused marginal inhibition of VSV. No such residual antiviral activity was observed with other cell clones expressing levels of Mx1(E_{614}) closer to physiological levels or with cell lines expressing the other cytoplasmic variants of Mx1. In light of recent experiments with rat Mx1, this result was rather unexpected. Nuclear rat Mx1 was found to inhibit influenza virus and VSV when overexpressed in transiently transfected mouse cells (15). Since VSV replicates in the cytoplasm of infected cells, this inhibitory effect was thought to be due to traces of rat Mx1 residing outside the cell nucleus, suggesting that cytoplasmic Mx1 is a potent inhibitor of VSV. Our experiments thus seem to indicate that this is not true for the mouse Mx1 protein. However, since we used mutant forms of Mx1, we cannot rule out the possibility that Arg-614 is required for activity of Mx1 against VSV. This is rather unlikely because human MxA, rat Mx2, and mouse Mx2, which are all efficient inhibitors of VSV (15, 22, 34), have a Glu residue at the corresponding position.

Comparing Mx1 variants with and without anti-influenza virus activity after relocation to the cell nucleus revealed that the carboxy terminus of Mx1 contains a functionally important domain with complex sequence requirements. Changing Arg-614 to Glu abolished nuclear transport of Mx1 but did not abolish antiviral function. In contrast, truncation of Mx1 by 19 amino acids abolished both nuclear transport and antiviral function. Interestingly, the chimeric protein Mx1/A, which differs from wild-type Mx1 at 16 of the 35 carboxy-terminal positions, was able to inhibit influenza virus in the nucleus, whereas the chimeric protein Mx1/2, which differs at only 12 positions, was not. This correlated with the anti-influenza virus activities of human MxA and mouse Mx2, respectively; wild-type MxA is active against influenza virus (22), whereas Mx2 is not (34).

All of our conclusions regarding the antiviral activity of mouse Mx1 protein are based on experiments with permanent cell lines which expressed the different Mx variants at a level similar to that observed in mouse fibroblasts treated for 15 h with a saturating concentration of alpha/beta interferon

(22). We believe that this system allows us to measure the antiviral activities of the different Mx variants with reasonable accuracy. This goal is achieved less readily with transient expression systems, which tend to yield nonphysiologically high levels of Mx protein pools in the transfected cells (15, 19). Controlling Mx protein expression is particularly important for evaluating the biological significance of residual antiviral activity associated with certain Mx variants. For example, variant Mx1(E_{614}), which was marginally active when expressed at a very high level, clearly failed to confer virus resistance when expressed at physiological levels, and we therefore classified it as an inactive Mx1 variant. Similarly, the marginal activity of rat Mx1 protein against VSV (15), which was measured in a transient transfection assay, could not be verified with permanently transfected cells expressing rat Mx1 protein at approximately physiological levels (23). A shortcoming of the permanent expression system is that stable expression of certain Mx variants was very difficult or even impossible (32, 34). Furthermore, it was not possible to firmly quantify the antiviral activity of the different Mx1 variants, because the clonal lines often contained a low number of cells that expressed significantly reduced Mx1 levels (22). These cells showed decreased virus resistance and distorted dose-response curves.

It was previously found that transport of Mx1 to the nucleus depended on a basic carboxy-terminal sequence. The sequence REKKKFLKRR between positions 606 and 615 of Mx1 was suggested to represent the functional transport signal (19). Our results confirmed that this sequence is required for nuclear transport in mouse Swiss 3T3 cells. As expected, introducing Glu in place of Arg at position 614 yielded a cytoplasmic Mx1 variant. The corresponding sequences SEKRKFLKER of mouse Mx2 and SDKRK FLKER of MxA, which are both less basic than the wild-type Mx1 sequence, failed to mediate nuclear localization of Mx1, stressing the crucial role of Arg-614. We found that the REKKKFLKRR sequence by itself was not sufficient to mediate nuclear localization of the ordinarily cytoplasmic MxA protein. An additional motif located between positions 375 and 596 of Mx1 was required, indicating that Mx1 contains a bipartite or multipartite nuclear transport signal, such as nucleoplasmin (24) or PB2 of influenza virus (17). Alternatively, MxA might contain a strong cytoplasmic retention signal, which is overcome by the nuclear transport signal of SV40 large T (35) but not by that of Mx1.

Earlier work revealed that wild-type Mx1 was also transported to the nucleus in a variety of cell lines originating from other species, including humans, monkeys, and chickens (7, 19). Nuclear transport of Mx1 was rather inefficient in the human lung fibroblast cell line A549 and in canine MDCK cells. Nonetheless, A549 and MDCK cells expressing Mx1 predominantly in the cytoplasm were resistant to influenza virus (19). Under these experimental conditions, Mx1 presumably accumulated in the nucleus to a level that was sufficient for influenza virus resistance. Alternatively, Mx1 may have functioned in the cytoplasm of these cells, which would indicate that they contained a cytoplasmic factor that rendered inactive Mx1 protein active. In fact, our finding that Mx1 function is restricted to the nucleus of Swiss 3T3 cells whereas MxA works in both cell compartments (35) could also be explained by assuming that some accessory factors modulate Mx activity. The putative activating factor of Mx1 might be a nuclear protein, whereas the activating factor of MxA might be present in both compartments. Interaction of Mx and an accessory factor(s) might stimulate the Mx-associated GTPase activity.

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