

Identification of the Murine *Mx2* Gene: Interferon-Induced Expression of the *Mx2* Protein from the Feral Mouse Gene Confers Resistance to Vesicular Stomatitis Virus

HEE KYUNG JIN,¹ AYATO TAKADA,² YASUHIRO KON,¹ OTTO HALLER,³
AND TOMOMASA WATANABE^{1*}

Laboratory of Experimental Animal Science¹ and Laboratory of Microbiology,² Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan, and Department of Virology, Institute for Medical Microbiology and Hygiene, University of Freiburg, D-79104 Freiburg, Germany³

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The mouse genome contains two related interferon-regulated genes, *Mx1* and *Mx2*. Whereas *Mx1* codes for the nuclear 72-kDa protein that interferes with influenza virus replication after interferon treatment, the *Mx2* gene is nonfunctional in all laboratory mouse strains examined, since its open reading frame (ORF) is interrupted by an insertional mutation and a subsequent frameshift mutation. In the present study, we demonstrate that *Mx2* mRNA of cells from feral mouse strains NJL (*Mus musculus musculus*) and SPR (*Mus spretus*) differs from that of the laboratory mouse strains tested. The *Mx2* mRNA of the feral strains contains a single long ORF consisting of 656 amino acids. We further show that *Mx2* protein in the feral strains is expressed upon interferon treatment and localizes to the cytoplasm much like the rat *Mx2* protein, which inhibits vesicular stomatitis virus replication. Furthermore, transfected 3T3 cell lines of laboratory mouse origin expressing *Mx2* from feral strains acquire slight resistance to vesicular stomatitis virus.

Mammals have small families of interferon (IFN)-responsive genes that code for structurally related nuclear and cytoplasmic proteins collectively referred to as Mx proteins (2, 18). The *Mx1* and *Mx2* genes in the mouse show a high degree of sequence similarity (20) and they are both mapped to the same position on chromosome 16. The nuclear *Mx1* protein selectively blocks influenza virus multiplication (6). Cells from some laboratory strains synthesize *Mx2* mRNA, but their open reading frames (ORFs) are interrupted by an insertion, resulting in a translation frameshift. Alignment of the nonfunctional mouse and functional rat *Mx2* sequences has identified a supernumerary cytosine (C) residue at position 1366 of *Mx2* mRNA (20). As the *Mx2* gene in commonly used strains of laboratory mice is nonfunctional, the antiviral potential or other function of the *Mx2* protein is unknown. Swiss mouse 3T3 cells expressing the *Mx2* cDNA repaired by site-directed mutagenesis showed slight resistance to the vesicular stomatitis virus (VSV) multiplication (24). In this study, we found that the *Mx2* gene from feral strains was functional and could confer resistance to VSV infection.

MATERIALS AND METHODS

Mice. Two laboratory inbred strains, BALB/c and C57BL/6J, were purchased from Charles River Japan (Yokohama, Japan), and laboratory inbred strain SL/NiA was provided by H. Hiai, Kyoto University, Japan (1). The feral strains NJL (*Mus musculus musculus*) and SPR (*Mus spretus*), maintained as inbred lines in our laboratory, were originally captured in Denmark and Spain, respectively.

Cell culture. Fibroblast cultures were established from 16-day-gestation embryos of BALB/c, NJL, and SPR origin (5). The cells were seeded in 75-cm² tissue culture flasks (Falcon Labware; Becton Dickinson, Oxnard, Calif.) at a density of 5×10^7 cells/flask in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, Md.). The medium was changed after one day, and the cells were passaged every third day.

VSV strains. Recombinant VSV carrying the green fluorescent protein (GFP) gene instead of the G protein gene (VSVΔG⁺-G) was kindly provided by M. A. Whitt (University of Tennessee, Memphis, Tenn.). Infectivity of VSVΔG⁺-G on each 3T3 cell line was determined by counting the number of GFP-expressing cells in 10 to 20 microscopic fields (23).

Generation of full-length cDNA. 5' and 3' rapid amplification of cDNA ends (RACE) was used with reverse transcription (RT)-PCR to amplify mRNAs (3 μg) extracted from cultured macrophages taken from each mouse strain, using a Marathon cDNA amplification kit (Clontech, Palo Alto, Calif.) to generate double-stranded cDNA. The double-stranded cDNA was ligated with the Marathon cDNA adaptor and purified on a chromaspin-TE1000 column (Clontech) in a total volume of 100 μl. 5'- and 3'-RACE were performed by using the double-stranded cDNA as a template with the *Mx2* gene-specific primers GSP1 (5'-TTCTCGTCCACGATACTGCTTT-3') and GSP2 (5'-AAGTGAGGAGCTGCAGAAGTAC-3') and the adaptor primers AP1 (5'-CCATCTAATACGACTACTATAGGGC-3') and AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3'). Additional gene-specific primers (5'GSP [5'-TTCTGTGGAAGGATAGAGGATACC-3'], 5RA seqF [5'-AAGCCGGGAGGGGTGAGTATTG-3'], 5RA seqR [5'-TCCGCACCACATCCACGACCTT-3'], 3'GSP [5'-TCTGAGTGCTAGCATTATCAGAGC-3'], 3'NES [5'-CAGCAAGTAGATGGGTAGAGGA-3'], and 3'ESEQ [5'-ATCCATGGCTGAGATCTTCC-3']) were generated based on the sequences of progressively amplified 5'- and 3'-RACE products.

Sequencing and characterization of *Mx2* cDNAs. After RACE-PCR, amplified full-length cDNA fragments were cloned into pGEM-T Easy vector (Promega, Madison, Wis.). The nucleotide sequences were determined by using an ABI Prism dRhodamine Terminator cycle sequencing kit (Perkin-Elmer, Norwalk, Conn.) with an ABI Prism 310 genetic analyzer (Perkin-Elmer). All of the sequences were compared with that of the *Mx2* mRNA reported earlier for BALB/c mice (20), as analyzed by the GENETYX-MAC 7.2.0 program.

Construction of *Mx2* expression vector. After RACE-PCR, the *Mx2* cDNA fragments were cloned in the proper orientation into the *EcoRI* site of plasmid pCI-neo (Promega), which contains the human cytomegalovirus immediate-early enhancer/promoter region and the neomycin phosphotransferase gene.

Transfection of 3T3 cells with *Mx2* gene expression plasmid. 3T3 cells (BALB/c embryonic fibroblasts), purchased from Riken Cell Bank (Tsukuba, Japan), were grown in DMEM containing 10% (vol/vol) fetal bovine serum. After transfection with Lipofectin (Gibco-BRL), accomplished essentially as recommended by the manufacturer, clones were selected in medium containing 500 μg of G418 per ml (8, 16).

***Mx2* RT-PCR.** Total 3T3 cell RNA (3 μg) was reverse transcribed by using 200 U of SUPERScript II RT (Gibco-BRL) in a total volume of 20 μl. The oligonucleotide primers for the *Mx2* gene were 5'-AGTGAGGAGCTGCAGAAGTACG (bp 1158 to 1179) and 5'-ACTTGGTAGTCTGTGGAGGTT (bp 1609 to 1588) for the RT-PCR. As an internal control, β-actin mRNA was used in quantitative RT-PCR experiments as described previously (7). The reaction mixture for RT-PCR contained 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM

* Corresponding author. Mailing address: Laboratory of Experimental Animal Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan. Phone: 81-11-706-5106. Fax: 81-11-717-7569. E-mail: watanabe@vetmed.hokudai.ac.jp.

1 AACTCCGCTCCAAGCCGGGAGGGGTGAGTATTGCACAGGATTCTGTGGAAGGATAGAGGATACCTTCTAAGGCTTCTTTGTAGACACTGAGTACCAACTGAGAGAAG

111 AAATAAAAGATGGTTCTTAGCACAGAAGAAAACACAGGCGTTGATTCACTCAACTACCCAGTGGAGAGACGGGCTAGGAGAGAAAAGACCAGGAGTCTGTGAACAATCT
Met Val Leu SerThr Glu GluAsn Thr Gly Val Asp SerVal Asn Leu Pro Ser Gly Glu ThrGly Leu Gly Glu Lys Asp Gln Glu Ser Val AsnAsn Le

221 CTGCAGGAGATCAGTATGAGAGGTGCGGCCCTGCATTGACCTCATCGACTCCCTGCGGGCTCTGGTGTGGAGCAGGACCTGGCCCTGCCTGCCATCGCTGTCAATGGGG
u CysSer Gln Tyr Glu Glu Lys Val Arg ProCys Ile Asp Leu Ile Asp Ser LeuArg Ala Leu Gly Val Glu Gln AspLeu Ala Leu Pro Ala Ile Ala Val Ile Gly A

331 ACCAGAGTTACGGGAAGAGCTCTGTGCTGGAAGCACTGTCTGGAGTGGCCCTCCCGAGGCACTGGTATTGTACCAGATGCCCTCTGGTCTGAAATTGAGGAAGCTG
sp Gln Ser Ser Gly Lys SerSer Val Leu Glu Ala Leu Ser Gly Val Ala Leu ProArg Gly SerGly Ile Val ThrArg Cys ProLeu Val Leu Lys LeuArg Lys Leu

441 AATGAGGGAGAGGAGTGGAGAGGCAAAGTCTCCTATGATGACATCGAAGTGGAGCTCTCTGATCCTTCAGAGGTGGAAGAGGCCATCAACAAGGGTCAGAACTTCATTGC
Asn Glu Gly Glu Glu TrpArg Gly Lys Val SerTyrAsp Asp Ile Glu Val Glu Leu SerAsp Pro Ser Glu Val Glu Glu Ala Ile Asn Lys Gly Gln Asn Phe Ile Al

551 TGGGGTAGGTCTGGGGATCAGTATAAGCTCATTAGCCTGGATGTGAGCTCCCAATGTCCAGACCTGACTCTCATTGACCTGCCTGGAATTACCAGGGTGGCTGTAG
a Gly Val Gly Leu Gly Ile Ser AspLys Leu Ile Ser Leu AspVal Ser SerProAsn Val ProAsp LeuThr Leu Ile Asp Leu Pro Gly Ile ThrArg Val Ala Val G

661 GCAACCAGCCTGCAGACATAGAGCCAGATCAAGAGACTCAAGACATACATCCAAAACAGGAGACCATCAACCTGGTGGTGGTCCCGAGCAATGTGGACATTGCC
lyAsn Gln Pro AlaAsp Ile Gly Arg Gln Ile Lys Arg Leu Ile Lys ThrTyr Ile Gln Lys Gln Glu Thr Ile Asn Leu Val Val Val Pro SerAsn Val Asp Ile Ala

771 ACCACAGAGGCTCTGAGCATGGCTCAGGAGTGGACCTGAAGGGATAGGACCATAGGATCTTGACCAAGCCCGATCTGGTGGACAGAGGTAAGACAAGGCTGT
Thr Thr Glu Ala Leu Ser MetAla Gln Glu Val Asp Pro Glu GlyAspArg Thr Ile Gly Ile Leu ThrLys ProAsp Leu Val AspArg Gly Thr Glu AspLys Val Va

881 GGATGTGGTGGCAACCTGGTGTACCATCTGAAGAAGGGCTACATGATAGTCAAGTGCAGAGGCCAGCAGGACATCCAAGAGCAGCTGAGCCTGACTGAGGCTCTTCAGA
I Asp Val Val ArgAsn Leu Val Tyr His Leu Lys Lys GlyTyr Met Ile Val LysCysArg Gly Gln Gln Asp Ile Gln Glu Gln Leu SerLeu Thr Glu Ala Leu Gln A

991 ATGAGCAAATCTTCTCAAGGAACACCCCTCATTTCAGAGTTCTCTGGAGGATGGGAAGGCCACAGTGCCTGCCTGGCAGAGAGACTGACCCAGAGCTCATTTACAC
sn Glu Gln Ile Phe PheLys Glu His Pro His PheArg Val Leu Leu Glu Asp Gly Lys Ala Thr Val ProCys LeuAla GluArg Leu Thr Ala Glu Leu Ile Leu His

1101 ATCTGTAATCTCTTCTCTGCTAGAAAATCAAATAAAGAAAGTACCAGAGTCAAGTGGAGGCTGCAGAAGTACGGTATGGACATACCAGAAGATGACAGTGAAAA
Ile Cys LysSer Leu Pro Leu Leu GluAsn Gln Ile Lys Glu Ser His Gln Ser Ala Ser Glu Glu Leu Gln Lys Tyr Gly MetAsp Ile Pro GluAspAsp Ser Glu Ly

1211 AACGTTCTTCTGATTGAAAAATCAATGCTTTTAAACCAGGACATCACTGCCCTAGTACAAGGGGAAGAGAATGTGGCAGAGGGAGAATGTGCCCTATTCACCAGGCTCC
sThr Phe PheLeu Ile Glu Lys Ile Asn Ala Phe Asn GlnAsp Ile ThrAla Leu Val Gln Gly Glu Glu Asn Val Ala Glu Gly Glu CysArg Leu Phe ThrArg LeuA

1321 GAAAAGAGTTCTTGTCTGGAGTAAGGAGATCGAAAAGAATTCGAAAAGGTTATGCTGTTTTATATAATGAAGTCTGGGCATTTGAAAAGCAGTACCGTGGACGAGAA
rg Lys Glu Phe Leu Ser TrpSer Lys Glu Ile Glu LysAsn PheAla Lys Gly TyrAla Val Leu TyrAsn Glu Val Trp Ala Phe Glu Lys GlnTyrArg GlyArg Glu

1431 TTGCCAGGTTTGTGAATTACAAAACATTTGAGAACATTATAAGAAGACAATCAAACCCCTGGAAGAACCAGCTATAGAAATGCTGCACACGGTCACTGAAATTGTACG
Leu Pro Gly PheVal AsnTyr Lys Thr Phe GluAsn Ile Ile ArgArg Gln Ile LysThr Leu Glu Glu ProAla Ile Glu Met Leu His Thr Val Thr Glu Ile Val Ar

1541 AGCTGCCCTTACAAGTGTCTGAGAAAAATTTTCTGAGTTTTATAOCTCCACAGAACTACCAAGTCCAAACTGAAGACATCAGATTAGAACAAGAAAAGGAAGCAG
g Ala Ala Phe ThrSer Val Ser Glu LysAsn PheSer Glu Phe TyrAsnLeu His Arg Thr ThrLys Ser Lys Leu Glu Asp Ile Arg Leu Glu Gln Glu Lys Glu Ala G

1651 AGATGTCAATCCGACTCCACTTCAAGATGGAACAAATATCTACTGCCAGGACAGATTTACAGAGGACCTTGCAGAAGTCCAGAGAGGAGGAGCTGAGGAGGAGAAG
lu MetSer Ile Arg Leu His Phe LysMet Glu Gln Ile Ile Tyr CysGln Asp Gln Ile TyrArg Gly Ala Leu Gln Lys Val Arg Glu Glu Glu Ala Glu Glu Glu Lys

1761 AAGACAAAGCATGGCACTTCCAGTTCCTCTCAGTCCCAAGATTTACAGACCTCATOCATGGCTGAGATCTTCCAGCATCTGAATGCCTACCGCAAGAGGCTCACAAACCG
LysThr Lys His Gly Thr SerSer Ser Ser Gln Ser GlnAsp Leu Gln Thr Ser Ser MetAla Glu Ile Phe Gln His Leu AsnAla TyrArg Gln Glu Ala His Asn Ar

1871 CATCTCCAGCCAGTTCCTTGATCCTCAGTATTTTATCCTGAAAATGTTTGTGAGCGGCTGCAGAAGGGCATGCTCCAGCTCCTGCAGGACAAGGATTCTGCAGCT
g Ile Ser Ser His Val Pro Leu Ile Ile Gln Tyr Phe Ile Leu Lys Met PheAla Glu Arg Leu Gln Lys Gly MetLeu Gln Leu Leu GlnAsp LysAsp Ser CysSerT

1981 GGCTCTGAAGGAGCAGAGTGACACAAGCGAGAAGGAAGTTCTGAAGGAGAGGCTGCAAGGCTGGCCAGGCTCGGCGCGGCTAGCCAATTCCTGGTTAAGCT
rpLeu Leu Lys Glu Gln SerAsp ThrSer Glu LysArg Lys Phe Leu Lys Glu ArgLeu Ala Arg LeuAla Gln Ala ArgArgArg Leu Ala Lys PhePro Gly END

2091 GGCTCTGCTCCTTCCCTGTGTTTCTGGATTGTGATTACGGGACAGAAGGGCTCCTGCCTTCTGCTAGCTATACCACAGCCTTTATGCTCTGATAAGTGCTAGCACTC

2211 AGATTTGAAGGAGCTTTCTGCTCACTCTGAGATAGGAAGAGAAAGAGGCTCTCAAAGCTCAGCAAGTATAGGGTGGTGGAGGAGGCCACTTGTCTAATAAGACAACGGCTTC

2321 ACCTGAGTACTCTTCTGCCACCAATTTAGCACACTGCAGAAAATAAATATTTGATGTACAATGGGAGCTGCCTGTTACATTCCCTTAATAAACCACCTTTTCTTTA_(n)

FIG. 1. The nucleotide and amino acid sequences of *Mx2* mRNA in cells from strains NJL and SPR of feral mice.

EDTA, 1 mM dithiothreitol, 0.2 mM deoxynucleoside triphosphate (dNTP), 1 μ M each primer, and 200 U of SUPERScript II. PCR was performed in a DNA Thermal Cycler (Perkin-Elmer) with *Taq* polymerase in 1.5 mM $MgCl_2$, 0.2 μ M each primer, and 20 μ M each dNTP, as recommended by the supplier. The cycling profile was comprised of an initial denaturing step for 5 min at 94°C

followed by 35 cycles at 94°C for 1 min, 60°C for 2 min, 72°C for 1 to 3 min, and a final extension at 72°C for 5 min.

Northern blot analysis. Total RNA was isolated from embryonic fibroblast cells by using ISOGEN (Nippon Gene, Tokyo, Japan) as instructed by the manufacturer. RNA samples were electrophoresed on formaldehyde gels, trans-

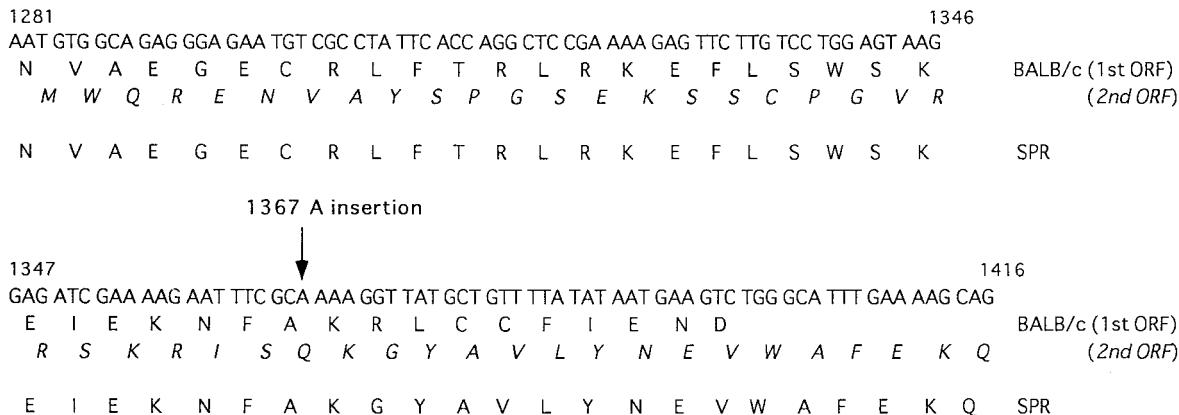


FIG. 2. Comparison of the nucleotide and amino acid sequences of *Mx2* proteins of BALB/c and wild-type strains NJL and SPR. The inserted A residue at position 1367 is found in *Mx2* mRNA of BALB/c where the two long ORFs overlap. The *Mx2* mRNA of the wild-type strains contained a single long ORF, as the A was excluded.

ferred, and hybridized as described previously (17). Northern blots were performed by hybridization with 2,161 bp of the *Mx2* cDNA (primers 5'GSP [5'-T TCTGTGGAAGGATAGAGGATAACC-3'] and 3'GSP [5'-TCTGAGTGCTAG CACTTATCAGAGC-3']) labelled with [³²P]dCTP.

Immunofluorescence staining. Cultured cells from peritoneal macrophages prepared as described previously (11) were treated for 18 h with 1,000 IU of mouse alpha/beta IFN (IFN-α/β) (Sigma Chemical Co., St. Louis, Mo.)/ml. Macrophages were then fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.3 for 30 min. The cells were permeabilized with 0.5% Triton X-100 in PBS, washed, and exposed to the monoclonal antibody 2C12, which cross-reacts with Mx proteins from many species (22, 25). The cells were then washed, exposed to tetramethyl rhodamine isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (Dako Co., Glostrup, Denmark), and mounted in 0.1 M Tris-HCl (pH 8.8) and glycerol. Finally, the cells were viewed and photographed with a BH2-RFCA microscope (Olympus Optical Co., Tokyo, Japan) equipped for epifluorescence.

Statistical analysis. Data were expressed as means ± standard errors of the means. Statistical significance was evaluated by using Fisher's protected least significant difference test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Characterization of cDNAs encoding the *Mx2* gene in the feral mouse strains. *Mx2* mRNAs of laboratory mouse strains BALB/c and CBA/J have ORFs interrupted by an insertional mutation (20). The *Mx2* cDNA sequences from two feral

strains, NJL and SPR, were compared with that from the BALB/c strain. It was indicated previously that the insertion of cytosine at position 1366 (C-1366) of BALB/c *Mx2* mRNA resulted in two overlapping ORFs encoding nonfunctional proteins. As shown in Fig. 1, C-1366 was also present in *Mx2* mRNA of two feral strains, but the adenine (A) at position 1367 was deleted. Therefore, the *Mx2* mRNA of feral strains is composed of a single long ORF, and the putative *Mx2* protein that is a product of this ORF consists of 656 amino acids (Fig. 2).

***Mx2* mRNA is highly expressed in IFN-α/β-activated wild-type embryonic cells.** To determine the expression pattern of *Mx2*, total RNAs derived from embryonic fibroblast cells of BALB/c, NJL, and SPR strains were isolated and analyzed by Northern blots. We treated the cells with 500 IU of mouse IFN-α/β per ml for 18 h and found that cells from both feral strains yielded higher concentrations of 2.1-kb mRNAs than the BALB/c cells when total RNA was hybridized with an *Mx2* probe (Fig. 3). No detectable 2.1-kb mRNA was found in BALB/c cells treated with IFN-α/β or in nontreated cells. NJL cells lacked a detectable level of 2.1-kb *Mx2* mRNA in the case of cells not treated with IFN-α/β, whereas SPR *Mx2* mRNA was detected even in such cells.

Cytoplasmic localization of *Mx2* expressed in two wild-type strains. The *Mx1*⁺ laboratory mouse strains A2G and SL/NiA are resistant to influenza virus, and Mx1 protein is localized in the nucleus (4, 6, 10). We have reported that the feral strains NJL and SPR also are positive for Mx1 protein (12). Rat *Mx2* cDNA is closely related to mouse *Mx2* cDNA and codes for a cytoplasmic protein that inhibits VSV but not influenza virus replication (14). We therefore explored whether the *Mx2* protein of the feral mouse strains synthesized in response to IFN is localized in the cytoplasm. Peritoneal macrophages were collected from each mouse strain and were treated for 18 h with 1,000 IU of IFN-α/β per ml. Proteins reacting with antibody 2C12, which cross-reacts with Mx1 and Mx2 proteins, accumulated both in the nuclei and in the cytoplasm of the macrophages from feral strains NJL and SPR (Fig. 4). Additionally, protein reacting with antibody 2C12 in the cytoplasm of the feral strains gave a punctate staining pattern. In contrast, no staining was observed in macrophages from BALB/c and C57BL/6 mice. These results suggested that the positive expression and localization of Mx2 protein in the feral strains were identical to those of the rat.

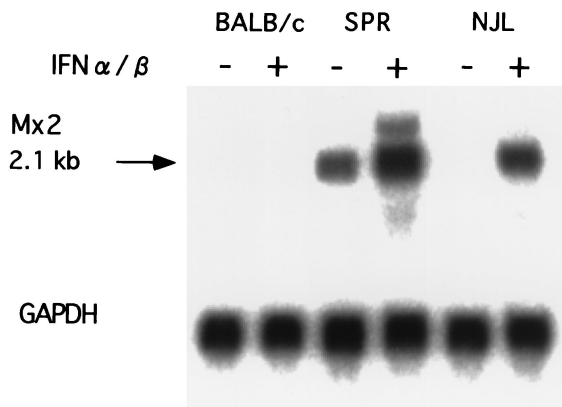


FIG. 3. Northern blot analysis of *Mx2* expression. Each lane contained 20 μg of total RNA isolated from embryonic cells. Embryonic cells were treated with 500 IU of IFN-α/β per ml for 18 h or were left untreated. The *Mx2* cDNA (top) was used as a probe and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (bottom) was used as a control for the probe.

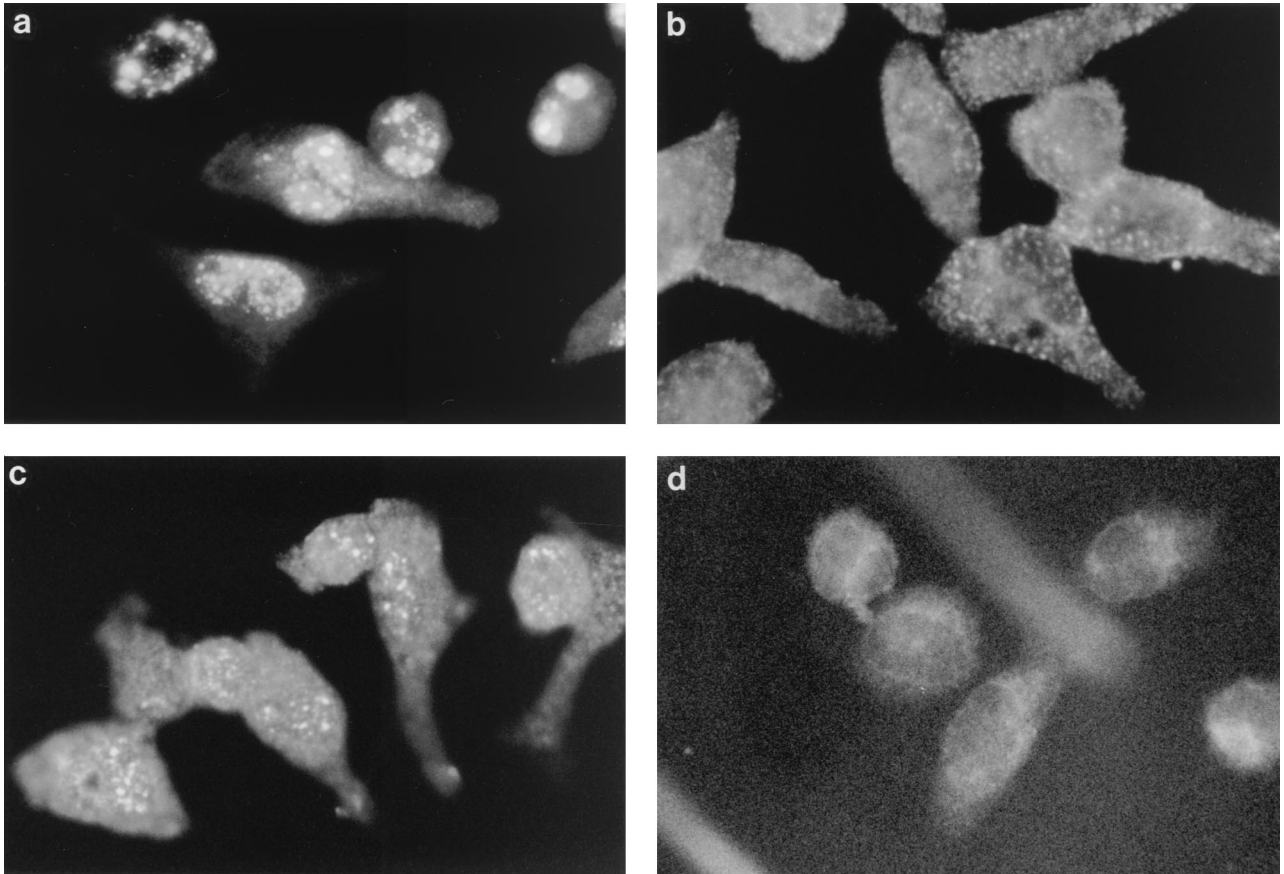


FIG. 4. Detection of Mx2 protein in IFN-treated peritoneal macrophages from feral mouse strains by immunofluorescence with the anti-Mx monoclonal antibody 2C12 as described previously (3). The cells shown are from the influenza virus-resistant strain SL/NiA (a), wild-type strain SPR (b), wild-type strain NJL (c), and susceptible strain C57BL/6 (d). All cells were treated with 500 IU of IFN- α/β per ml for 18 h.

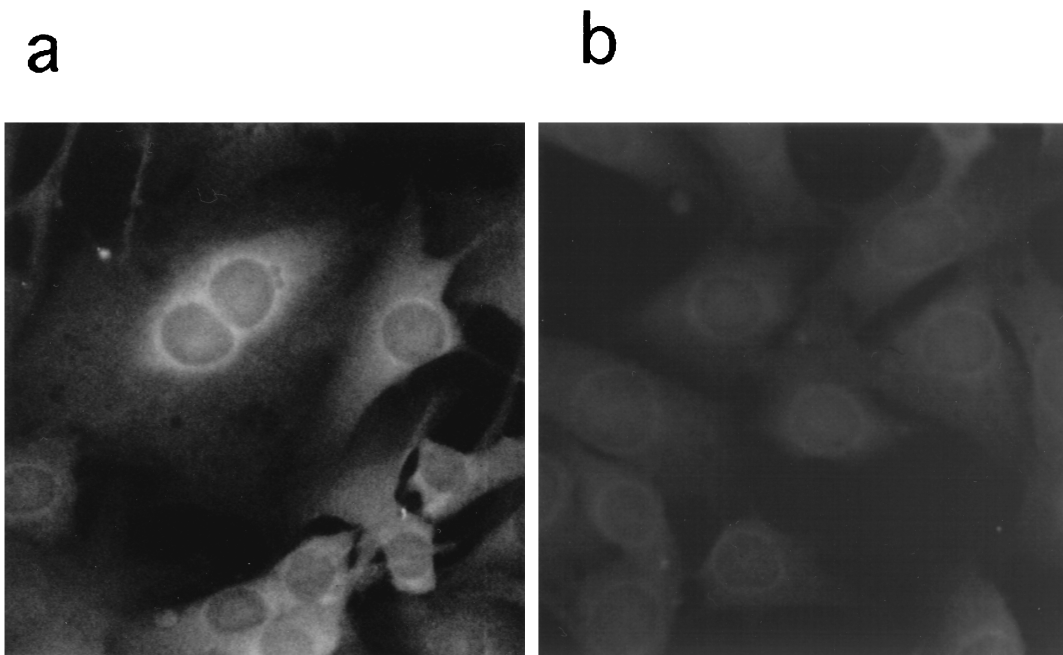


FIG. 5. Detection of Mx2 protein by immunofluorescence with the anti-Mx monoclonal antibody 2C12 in stably transfected 3T3 cells expressing the *Mx2* gene from feral strains NJL and SPR after IFN treatment. The transfected mouse 3T3 cells express the *Mx2* gene from feral strains (a) and control 3T3-pCI-neo (b). Both cell lines were treated with 500 IU of IFN- α/β per ml for 18 h.

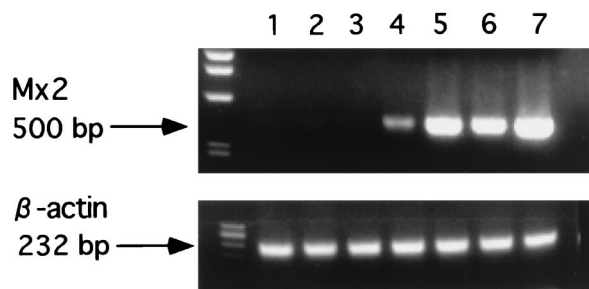


FIG. 6. Total RNA from transfected 3T3 cells was reverse transcribed and PCR amplified by using *Mx2* primers (top) and β -actin primers (bottom). Lanes: 1, 3T3 cells; 2 and 3, control 3T3-pCI-neo; 4 and 5, 3T3 cells expressing *Mx2* from SPR (S1 and S2); 6 and 7, 3T3 cells expressing *Mx2* from NJL (N2 and N4). An aliquot of each PCR product was electrophoresed on a 1.2% agarose gel and visualized with ethidium bromide.

Permanently transfected cell lines constitutively express *Mx2* proteins. The *Mx2* cDNA constructs with plasmid *Mx2*-pCI-neo were transfected into 3T3 cell lines. The 3T3 cells are derived from the BALB/c mouse strain lacking functional *Mx* genes and therefore are unable to synthesize endogenous *Mx* proteins (11, 19). Individual clones of stably transfected cells containing the *Mx2* cDNA constructed from feral strains NJL and SPR were tested for *Mx2* protein expression by immunofluorescence staining with specific antibody 2C12. In each clone, 80 to 90% of cells expressed *Mx2* protein (Fig. 5). The cytoplasm of the transfected 3T3 cells accumulating *Mx2* protein from feral strains gave a punctate staining pattern, whereas the cytoplasm of cells transfected with control pCI-neo showed no staining. Constitutive expression of these *Mx2* proteins did not significantly affect the growth rates of the transfected 3T3 cell lines.

The relative concentrations of *Mx2* mRNAs in transfected cells with the *Mx2* cDNA construct from feral strains (clones N2, N4, S1, and S2) were much higher than those in the control cells (Fig. 6).

VSV replication is inhibited in cells expressing *Mx2* protein from feral mouse strains. Since *Mx* proteins have been shown to exert an antiviral effect against influenza virus and VSV infection *in vitro* (6, 8, 21), the infectivities of VSV Δ G*-G were examined in each 3T3 cell clone expressing mouse *Mx2* protein from feral strains. Of the cell lines expressing the cytoplasmic *Mx2* protein of feral strains NJL and SPR, N2, N4, S1, and S2 showed resistance to viral infection, as they gave rise to significantly lower numbers of infected cells than those of control 3T3 cells (Fig. 7).

DISCUSSION

In all laboratory strains tested so far, the mouse *Mx2* gene is nonfunctional (20). No *Mx2* gene was detected in IFN- α / β -treated cells from strain A2G, which is one of the strains expressing the functional *Mx1* gene. Cells from BALB/c and other laboratory mouse strains tested synthesize low levels of *Mx2* mRNA under physiological conditions, but its ORF is interrupted by a nucleotide insertion with subsequent frame-shift (20).

Our sequence analysis showed that the putative coding region of the *Mx2* mRNA in the feral strains NJL and SPR differed from that of laboratory strains because of the absence of A at position 1367. Hence, the *Mx2* mRNA of these two feral strains contains a single long ORF and should be functional. It was indeed demonstrated by Northern blotting that IFN-treated embryonic cells from feral strains strongly expressed the *Mx2* gene. In contrast, expression of the BALB/c *Mx2* gene was not detected even after treatment with IFN- α / β . Immunostaining experiments indicated that *Mx2* protein was localized in the cytoplasm, and a punctate staining pattern was observed. Furthermore, by RT-PCR using an *Mx2*-specific primer, it was confirmed that transfected 3T3 cell lines from *Mx2* cDNA from feral strains expressed a high level of *Mx2* mRNA. Therefore, we demonstrated a functional *Mx2* gene in the mouse, although it remains to be determined how the *Mx2* protein interferes with virus infection.

Rat cells synthesize three related proteins, *Mx1*, *Mx2*, and

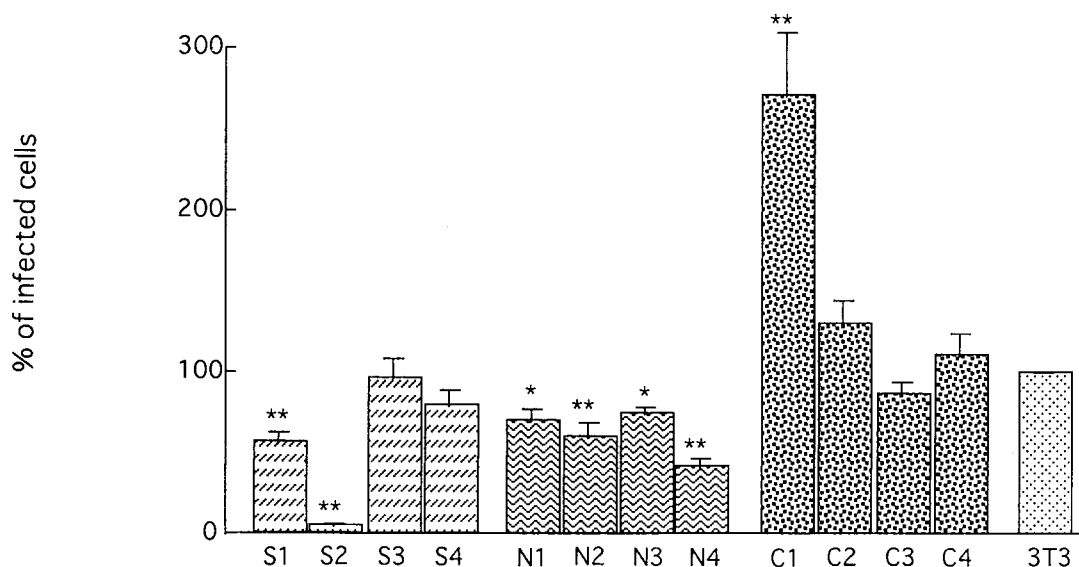


FIG. 7. The infectivity of VSV Δ G*-G in *Mx2* cDNA-transfected cell lines. S1 to S4, transfected 3T3 cells expressing *Mx2* of SPR; N1 to N4, transfected 3T3 cells expressing *Mx2* of NJL; C1 to C4, 3T3 cells transfected with pCI-neo. The infectivity on 3T3 cells is expressed as 100%. Shown are mean values \pm standard errors of the means ($n = 10$). Significance levels at $P < 0.05$ (*) and $P < 0.01$ (**) compared with 3T3 cells are indicated.

Mx3, upon IFN treatment (14). Rat Mx1 protein is predominantly localized in the nucleus and protects cells against influenza virus but only weakly against VSV. Rat Mx2 protein is cytoplasmic and protects against VSV. Rat Mx3 is also cytoplasmic, but it lacks activity. Though rat Mx2 differs from rat Mx3 in only eight amino acids (Leu-36, Ala-190, Lys-232, Ser-518, Ser-561, Lys-564, Arg-588, and His-630), mouse Mx2 matches rat Mx2 at four of these amino acids (Ala-190, Ser-518, Ser-561, and Arg-588) and rat Mx3 at two of them (Val-36 and Glu-232) (20). Johannes et al. (13) demonstrated direct evidence of the presence of anti-VSV determinants in the C terminus of rat Mx2, especially in the region between Arg-588 and His-630 (15, 24). However, the change of His in rat Mx2 protein to Gln in the mouse protein at position 630 might be a very drastic alteration, suggesting that this His residue plays a key role in rat Mx2 protein. On the other hand, the cells transfected with mouse *Mx2* cDNA repaired by the exclusion of a C residue at position 1366 showed a high degree of resistance to VSV, when the mouse 3-hydroxy-3-methylglutaryl coenzyme A reductase gene promoter was used (24). In the present study, the degree of resistance of *Mx2*-transfected cells was limited. This may be due to (i) the His-to-Gln change at position 630 or (ii) the difference of the promoter activity of expression vector and/or cell line used. It was recently found that VSV is less *Mx* sensitive than members of the *Bunyaviridae* family such as La Crosse virus, Hanta virus, and Rift Valley fever virus (9). It will be necessary to examine the antiviral potential of functional *Mx2*-expressing cell lines against bunyavirus infections.

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