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Activity of Rat Mx Proteins against a Rhabdovirus

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Upon stimulation with alpha/beta interferon, rat cells synthesize three Mx proteins. Sequence analysis of corresponding cDNAs reveals that these three proteins are derived from three distinct genes. One of the rat cDNAs is termed *Mx1* because it is most closely related to the mouse *Mx1* cDNA and because it codes for a nuclear protein that, like the mouse Mx1 protein, inhibits influenza virus growth. However, this protein differs from mouse Mx1 protein, in that it also inhibits vesicular stomatitis virus (VSV), a rhabdovirus. A second rat cDNA is more closely related to the mouse *Mx2* cDNA and directs the synthesis of a cytoplasmic protein that inhibits VSV but not influenza virus. The third rat cDNA codes for a cytoplasmic protein that differs from the second one in only eight positions and has no detectable activity against either virus. These results indicate that rat Mx proteins have antiviral specificities not anticipated from the analysis of the murine Mx1 protein.

Treatment of cells with interferon (IFN) usually results in the establishment of an antiviral state directed against many different viruses. Since different viruses use different replication strategies and are inhibited at different steps, it has been postulated that what is called the antiviral state is in fact composed of many distinct states, each brought about by distinct IFN-induced or IFN-modulated antiviral proteins (8). In fact, two such IFN-induced proteins have been implicated in inhibition of a distinct class of viruses. Cells constitutively expressing the 2′-5′oligoadenylate synthetase are nonpermissive for picornaviruses yet support the replication of other viruses (6). Cells expressing the murine Mx1 protein, a protein originally discovered in A2G mice (10), are nonpermissive for orthomyxoviruses yet are permissive for other viruses, including rhabdoviruses (17, 23). Of these two proteins, the Mx1 protein is of particular importance because it is the quintessential host factor controlling influenza virus pathogenicity in mice in vivo: *Mx1*-positive mice able to synthesize authentic Mx1 protein are resistant to influenza virus infection, whereas *Mx1*-negative mice with mutations in their *Mx1* gene are susceptible (for review, see reference 7). However, such *Mx1*-negative mice can be rendered resistant to lethal influenza infection by germ line transformation with an *Mx1* cDNA (4). The precise molecular mechanism for inhibition of influenza viruses is unknown, but it appears that an early step after entry and before accumulation of viral proteins is blocked (13, 16).

Mx genes are found in a variety of other organisms, including yeasts (19) and vertebrates ranging from fish to humans (for review, see reference 21). Some vertebrates are known to contain more than one *Mx* gene. Laboratory mice, for instance, have a second gene called *Mx2* with a potential coding capacity for an Mx1-related protein that represents, however, only the amino-terminal half of *Mx1* (24). Paradoxically, many of these *Mx*-containing species are not natural hosts for influenza viruses. It is conceivable that if their Mx proteins serve at all as antiviral proteins, they may have activities against other viruses.

We have shown earlier that laboratory rats have three IFN-inducible, Mx-related proteins (15). At least one of these three proteins must be responsible for IFN-induced protection against influenza viruses, since microinjection of an Mx-neutralizing antibody into IFN-treated rat cells specifically increases susceptibility to influenza virus (3). We now show that these proteins are derived from three different *Mx* genes. One of these proteins is most closely related to the mouse Mx1 protein and protects cells in transient expression assays against influenza viruses and, unlike mouse Mx1, also protects against vesicular stomatitis virus (VSV), a rhabdovirus. A second protein, more closely related to the mouse *Mx2* mRNA by sequence of its mRNA, is not at all an anti-influenza protein but protects against VSV. Surprisingly, the third protein, differing from the second one by only 8 residues, has no detectable activity against either one of these two viruses. Thus, it appears that Mx proteins of rats, though related to the Mx1 protein of mice, have different antiviral specificities, and the results suggest that Mx proteins of other vertebrate species may affect a wider range of viruses than hitherto presumed.

MATERIALS AND METHODS

Cells and viruses. DA rats were purchased from OLAC Ltd., Bicester, England, and bred at the Institute for Immunology and Virology, University of Zürich, Zürich, Switzerland. Rat embryo fibroblast cells were prepared from 16-day gestation embryos as described for mouse embryo fibroblasts (5). They were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and used between passages 3 and 10. Mouse 3T3 cells were maintained in Eagle minimum essential medium containing 10% fetal calf serum.

Stocks of influenza A/WSN virus were prepared in Madin Darby canine kidney cells. Stocks of VSV (Indiana serotype, Mudd-Summers strain) were prepared in baby hamster kidney cells.

IFN and IFN treatment. Partially purified rat IFN-α/β (specific activity, 2.3×10^6 reference units per mg of protein) was purchased from Lee Biomolecular (San Diego, Calif.) and added to monolayers of rat embryo cells at a final

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concentration of 500 U/ml. The cultures were incubated for 7 h before mRNA was isolated.

Isolation of mRNA and Northern (RNA) blot analysis. Rat embryo cells, either IFN-treated or untreated, were scraped off the plates and lysed with Nonidet P-40 (final concentration, 0.5%). Poly(A)$^+$ RNA was isolated by oligo(dT) chromatography, fractionated through 1% agarose–2.2 M formaldehyde gels, transferred to Genescreen membranes (New England Nuclear Corp.), and hybridized to ^{32}P-labeled probes (14). The probes used were the 2.3-kb *Bam*HI fragment (positions 1 to 2314) of the murine *Mx1* clone pMx34 (23), the 552-bp *Sac*I-*Eco*RI fragment (positions 2562 to 3114) of the rat *Mx1* clone pMx29, the 253-bp *Ssp*I-*Eco*RI fragment (positions 2275 to 2528) of the rat *Mx2* clone pMx36, and the 338-bp *Pvu*II-*Eco*RI fragment (positions 2105 to 2443) of the rat *Mx3* clone pMx223. The membranes were washed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1% sodium dodecyl sulfate at 55°C and autoradiographed.

DNA sequence analysis. Rat *Mx* cDNAs, inserted into the *Eco*RI site of pSP65 (Promega Biotec), were sequenced on both strands by the dideoxy chain termination technique (20) by using T7 sequenase and the double-stranded DNA sequencing procedure as described by the manufacturer (United States Biochemical Corp.). An SP6 promoter primer (20-mer, Promega Biotec) was used to determine the sequences of the termini. The remainder of the inserts was sequenced by using 20-mer oligonucleotide primers synthesized with a model 380 A DNA synthesizer (Applied Biosystems).

PCR amplification. Polymerase chain reactions (PCRs) were carried out in 100 μl of a solution containing the following components: 0.1 μg of plasmid DNA (pMx29, pMx36, or pMx223) or 1 μg of genomic rat DNA (Sprague-Dawley; Clontech Laboratories, Calif.); 1 μM each two oligonucleotide primers (see below); 200 μM each dATP, dGTP, dCTP, and dTTP; 50 mM KCl; 10 mM Tris hydrochloride (pH 8.3); 1.5 mM $MgCl_2$; 0.01% gelatin; and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus). Amplification was accomplished in 30 cycles of 1 min at 95°C, 2 min at 45°C, and 3 min at 70°C, followed by a 7-min extension time at 70°C. The primers used to amplify rat Mx1-specific sequences were 5′ CTCTGCAAACTCTGAGGAGA 3′ and 5′ TCAGGCTGGCTGGCGAGCTT 3′ (corresponding to positions 2182 to 2201 and 2680 to 2699 of pMx29). The primers used to amplify rat Mx2-specific sequences were 5′ CTAAC CACTACCCTTTATCCTATT 3′ and 5′ GCAATAGGC AGCTCCCAAACATCA 3′ (corresponding to positions 2248 to 2272 and 2489 to 2502 of pMx36). The primers used to amplify rat Mx3-specific sequences were 5′ CCAGAAA CTTCTCCCCATTGAAT 3′ and 5′ ATTGCCCACCTAAA-CATACAAAG 3′ (corresponding to positions 2144 to 2165 and 2401 to 2423 of pMx223).

In vitro transcription, in vitro translation, and immunoprecipitation. Approximately 5 μg of pSP65 plasmid DNA, containing the respective *Mx* cDNA, was cleaved at a unique *Sma*I site downstream of the insert. Capped runoff transcripts were synthesized with SP6 RNA polymerase (Promega Biotec) by the method of Krieg and Melton (12). These RNAs or total mRNA were translated in vitro in a rabbit reticulocyte lysate system from Promega Biotec. Triton X-100 was then added to a final concentration of 0.2%, and protein products were immunoprecipitated with the anti-mouse Mx1 protein antibody 2C12 as previously described (15). The samples were run on 8% sodium dodecyl sulfate-polyacrylamide gels.

Construction of expression plasmids. A simian virus 40 (SV40)-based expression vector was constructed as follows. The 450-bp *Eco*RI-*Bam*HI fragment of pMx34, containing the SV40 promoter (23), and the 1,606-bp *Bam*HI-*Xba*I fragment of the mouse β-globin gene (position 544 to 2150), containing part of the second exon, the second intron, and the third exon with the poly(A) addition site (11), were isolated. Both fragments and *Eco*RI-*Xba*I-cut pSP65 DNA were then ligated together. The resulting plasmid (pSS-1) contains a unique *Bam*HI site between the SV40 promoter and the 3′ half of the mouse β-globin gene.

To obtain plasmids expressing the three rat Mx proteins and herpesvirus thymidine kinase (htk), the following fragments were ligated to flush-ended *Bam*HI-cut pSS-1: (i) the *Mae*II fragment (positions 107 to 2827) of pMx29 to obtain pSVrMx1, (ii) the flush-ended *Dra*I-*Eco*RI fragment (positions 115 to 2528) of pMx36 to obtain pSVrMx2, (iii) the flush-ended complete *Eco*RI insert of pMx223 to obtain pSVrMx3, and (iv) the flush-ended 1,400-bp *Bgl*II fragment of htk cDNA to obtain pSVhtk. The mouse Mx1 expression plasmid pSV40Mx/βglobin has been described earlier (17).

Nuclear microinjection. Plasmid DNA was purified on an Elutip column (Schleicher and Schuell) and dialyzed against 0.002 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.2)–0.14 M KCl. The DNA was then injected into the nucleus of mouse 3T3 cells at a concentration of 0.4 mg/ml (17).

Double immunofluorescence staining of virus-infected cells. Microinjected mouse 3T3 cells were incubated for 16 to 44 h before they were infected with either influenza A/WSN virus or VSV as previously described (3). Seven hours after infection with influenza virus or 3 h after infection with VSV, the cells were fixed for 30 min with 2% formaldehyde in phosphate-buffered saline, pH 7.2, and permeabilized for 5 min with 0.05% Triton X-100 in phosphate-buffered saline.

Double indirect immunofluorescence staining for Mx proteins and viral proteins was done as follows. To detect Mx protein, cells were incubated with the monoclonal anti-mouse Mx1 antibody 2C12 (22), washed, and then incubated with affinity-purified goat $(Fab)_2$ anti-mouse immunoglobulin G (IgG) antibody conjugated with fluorescein isothiocyanate. To detect viral antigens, cells were incubated either with a rabbit anti-influenza A/WSN virus antiserum or with a rabbit anti-VSV antiserum, washed, and then incubated with affinity-purified goat $(Fab)_2$ anti-rabbit IgG antibody conjugated with tetramethylrhodamine isothiocyanate, and washed again.

Double immunofluorescence staining for htk and viral proteins was done as follows. To detect htk, cells were incubated with a rabbit anti-htk antiserum, washed, and then incubated with affinity-purified goat $(Fab)_2$ anti-rabbit IgG antibody conjugated with tetramethylrhodamine isothiocyanate. To detect viral antigens, cells were incubated either with a mouse anti-influenza A/PR8 virus antiserum or with a mouse monoclonal antibody (CIV/18) directed against the VSV G protein (2), washed, then incubated with affinity-purified goat $(Fab)_2$ anti-mouse IgG antibody conjugated with fluorescein isothiocyanate, and washed again. Preparations were observed with a Reichert-Jung Polyvar microscope equipped for incident light fluorescence microscopy.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this article can be accessed in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the following numbers: X52711 (rat *Mx1*), X52712 (rat *Mx2*), and X52713 (rat *Mx3*).

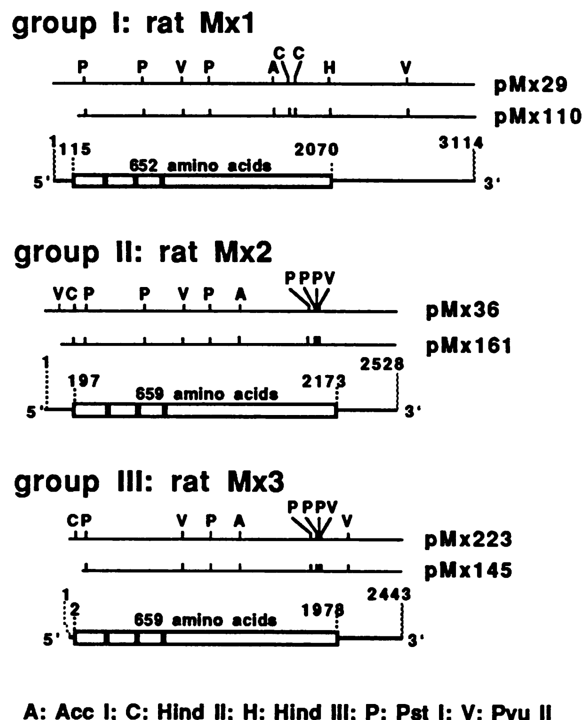

FIG. 1. Physical maps of rat *Mx* cDNA clones. The two largest cDNA clones of each group are shown. The letters depict restriction sites: A, *Acc*I; C, *Hin*dII; H, *Hin*dIII; P, *Pst*I; V, *Pvu*II. All clones are flanked by *Eco*RI sites contributed by linkers. The structures of the *Mx1*, *Mx2*, and *Mx3* mRNAs are shown, with the open reading frames indicated by the open boxes. The black areas depict the three consensus elements characteristic of GTP binding proteins. The numbers refer to nucleotide positions.

RESULTS

Three rat *Mx* cDNAs corresponding to three *Mx* genes, mRNAs, and proteins. We have previously identified a number of rat *Mx* cDNA clones which, on the basis of restriction maps, fall into three distinct groups, each represented by a similar number of clones (15). We now have completely sequenced on both strands the largest clone of each group and have deposited the sequences in the EMBL data library. The largest two cDNAs of each group are schematically depicted in Fig. 1. The largest clone from group I, pMx29, is composed of 3,114 nucleotides and contains an open reading frame with a putative initiation codon at nucleotides 115 to 117 and a coding capacity for a protein of 652 amino acids (calculated M_r = 74,422). The largest clone from group II, pMx36, is 2,528 nucleotides long and has an open reading frame with a putative initiation codon at nucleotides 197 to 199 and a coding capacity for a protein of 659 amino acids (calculated M_r = 75,026). The largest clone from group III, pMx223, comprises 2,443 nucleotides and contains an open reading frame with the first ATG at nucleotides 2 to 4 and a coding capacity for a protein of 659 amino acids (calculated M_r = 74,904).

Sequence comparisons (data not shown) revealed that the group I and group II cDNAs share 77% nucleotide identities in their putative coding regions and no significant similarity in their noncoding regions. The group III cDNA is identical to the group II cDNA, except for 16 positions in the coding region and 8 positions in the proximal 67 nucleotides of its 3′ noncoding region. However, the sequence completely diverges from that of the group II cDNA in the remainder of the 3′ noncoding region. Partial sequencing of two additional clones (pMx161 and pMx145, as indicated in Fig. 1) confirmed the differences. Comparisons with the published mouse *Mx1* and *Mx2* sequences (23, 24) revealed a high degree of conservation in both coding and noncoding regions between mouse *Mx1* and rat group I and mouse *Mx2* and rat group II cDNAs. As far as the coding and proximal 3′ noncoding regions are concerned, the rat group III cDNA is also more similar to the mouse *Mx2* cDNA than to the mouse *Mx1* cDNA. We designated the group I sequence as rat *Mx1*, the group II sequence as rat *Mx2*, and the group III sequence as rat *Mx3*.

The sequence comparisons suggest that the rat *Mx1* and *Mx2* cDNAs are derived from two different genes. Rat *Mx3* is most likely derived from a third gene, since the nucleotide differences between rat *Mx2* and *Mx3* are scattered over a large portion of the sequence. Whatever events have led to the generation of the three rat *Mx* genes, the evolutionary distance between *Mx1* and *Mx2* (or *Mx3*) appears to be larger than that between *Mx2* and *Mx3*. It was theoretically possible, though, that the inbred DA rat, from which the cDNA library had been prepared, was for unknown reasons heterozygous at the *Mx2* locus; consequently the *Mx2* and *Mx3* sequences might have been derived from allelic forms of the same gene. To rule this out, we obtained genomic DNA of a different inbred rat strain from a different source and examined whether or not this DNA contained sequences corresponding to all three *Mx* cDNAs. Mx1-, Mx2-, and Mx3-specific sequences were amplified by the PCR by using primer pairs derived from the unique regions at the 3′ termini of the three rat *Mx* cDNAs. The appearance of a 517-bp fragment indicates the presence of *Mx1* sequences, that of a 254-bp fragment indicates the presence of *Mx2* sequences, and that of a 279-bp fragment indicates the presence of *Mx3* sequences. The genomic DNA of this different inbred rat yields each of the expected fragments (Fig. 2, compare lanes 1 and 4 in panel A, lanes 2 and 4 in panel B, and lanes 3 and 4 in panel C). In addition, various inbred rats of different strains and sources (i.e., rats presumably homozygous at the *Mx2* locus) all synthesize the three easily distinguishable Mx proteins (see below). These observations make it highly unlikely that *Mx2* and *Mx3* are allelic forms of the same gene.

Northern blot analysis of rat poly(A)$^+$ RNA with a mouse *Mx1* cDNA probe (Fig. 3, lane 2) or with each of the full-length rat *Mx* cDNA probes (data not shown) shows one band at 3.2 and one at 2.5 kb. To identify which rat cDNA is derived from which mRNA, probes from the unique 3′ ends of the rat cDNAs were used. Each of these probes hybridizes specifically with its parental cDNA (data not shown). The *Mx1*-specific probe detects an INF-induced mRNA of 3.2 kb (Fig. 3, lane 4), and the *Mx2*- and *Mx3*-specific probes each detect an INF-induced mRNA of 2.5 kb (Fig. 3, lanes 6 and 8), indicating that the *Mx2* and *Mx3* mRNAs comigrate.

According to the designations of the cDNAs, the predicted proteins are termed rat Mx1, Mx2, and Mx3 proteins. Their sequences are shown in Fig. 4 along with the mouse *Mx1* sequence published earlier (23). The rat Mx1 protein sequence has 71% identity with that of rat Mx2 protein and 79% identity with that of mouse Mx1 protein. The predicted rat Mx2 protein sequence has 99% identity with that of rat Mx3 protein and shows differences in no more than 8 residues.

An interesting feature of the predicted amino acid sequences of the three rat Mx proteins is the presence in their

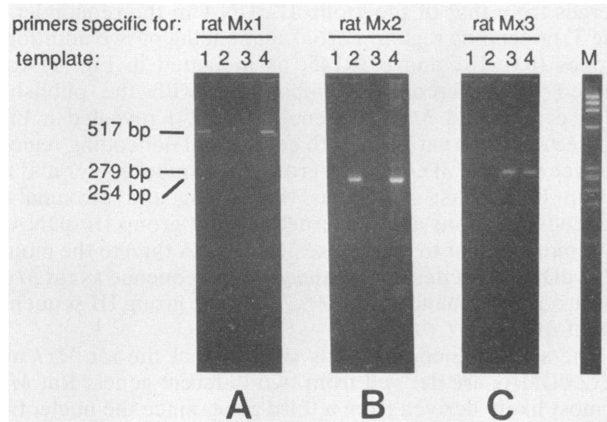

FIG. 2. PCR analysis of rat genomic DNA. DNA sequences were PCR amplified by means of oligonucleotide primer pairs specific for rat *Mx1* (panel A), rat *Mx2* (panel B), and rat *Mx3* (panel C). The templates used were pMx29 (lanes 1), pMx36 (lanes 2), pMx223 (lanes 3), or genomic DNA of a Sprague-Dawley rat (lanes 4). DNA samples were amplified for 30 cycles and then analyzed in a 1% agarose gel. The 517-bp fragment is diagnostic for rat Mx1, the 254-bp fragment is diagnostic for rat Mx2, and the 279-bp fragment is diagnostic for rat Mx3. Lane M, λ-*Hin*dIII and φX174-*Hae*III marker DNAs.

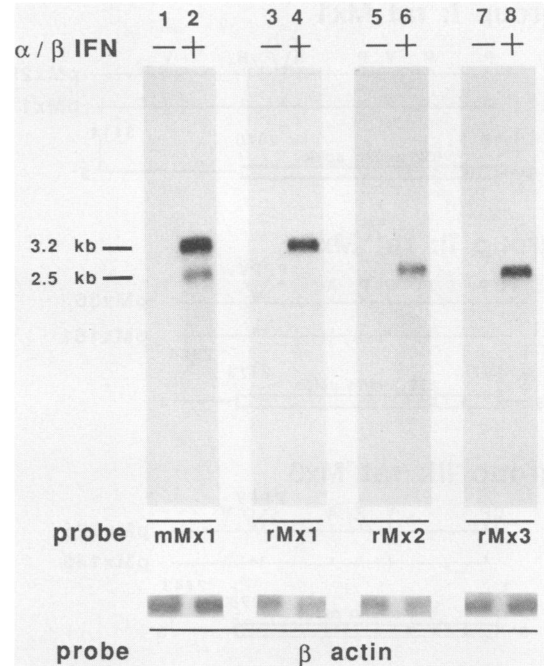

FIG. 3. Northern blot analysis of rat *Mx* transcripts. Poly(A)$^+$ RNA (0.5 μg per lane) was prepared from DA rat embryo cells that had been treated for 7 h with 500 U of rat IFN-α/β per ml (lanes +) or had been left untreated (lanes −). The RNA was fractionated by electrophoresis and transferred to a nylon membrane. The hybridization probes were the 2.3-kb *Bam*HI fragment of the mouse *Mx1* cDNA clone pMx34 that hybridizes with all rat *Mx* mRNAs (lanes 1 and 2) and restriction fragments specific for rat *Mx1* (lanes 3 and 4), rat *Mx2* (lanes 5 and 6), or rat *Mx3* (lanes 7 and 8). For details about the specific rat probes, see Materials and Methods. The hybridization signals obtained upon reprobing of the membranes with a chicken β-actin probe are shown at the bottom.

more conserved amino-terminal halves of three consensus elements characteristic of GTP-binding proteins (blocks in Fig. 1, stars in Fig. 4). These sequence elements are also conserved in all other published *Mx* sequences, that is, mouse, humans, fish and yeasts (9, 18, 19; for review, see reference 21). However, whether the respective proteins in fact bind GTP remains to be shown.

In vitro transcription/translation analysis of rat *Mx* cDNAs. To determine whether the cloned cDNAs are capable of directing the synthesis of the three authentic rat Mx proteins identified previously (15), RNA was transcribed in vitro from the largest clone of each group and then translated in vitro. The protein products were immunoprecipitated with the monoclonal antibody 2C12 which reacts with all three rat Mx proteins (15). The immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and compared with the three Mx proteins that are obtained by in vitro translation of poly(A)$^+$ RNA from IFN-treated rat cells and that are known to comigrate with the three authentic Mx proteins from cell lysates (15).

The results of the transcription and translation assays are shown in Fig. 5. The poly(A)$^+$ RNA-derived Mx proteins can be separated into a fast-, an intermediate-, and a slow-migrating protein (Fig. 5, lane 4). The *Mx1* clone pMx29 yields as a major product a protein that comigrates with the fast Mx protein (apparent M_r = 72,000; Fig. 5, lane 1), the *Mx2* clone pMx36 yields a protein that comigrates with the intermediate protein (apparent M_r = 75,000; Fig. 5, lane 2), and the *Mx3* clone pMx223 yields a protein that comigrates with the slow rat Mx protein (apparent M_r = 77,000; Fig. 5, lane 3). These results suggest that each of the three clones contains the full coding sequence of the respective proteins. However, the calculated M_rs of these three proteins are very similar (see above). Since we have no evidence for posttranslational modifications of these proteins (15), we assume that their anomalous electrophoretic mobility is due to particular features of their structure, as shown for other proteins.

Intracellular localization of rat Mx proteins. We have previously shown that the rat Mx protein with the fastest electrophoretic mobility accumulates in the nucleus of IFN-treated rat cells, while the two others are predominantly cytoplasmic (15). To determine the subcellular localizations of the proteins encoded by the individual cDNAs, SV40 early enhancer/promoter-based expression plasmids were prepared and needle injected into the nuclei of *Mx1*-negative mouse 3T3 cells unable to synthesize an Mx protein of their own. Eighteen hours after injection, the cells were fixed and stained for Mx protein by indirect immunofluorescence. The results are shown in Fig. 6. Cells injected with the Mx1 expression plasmid accumulate Mx1 protein in characteristic granules in the nuclei. Cells injected with either the Mx2 or Mx3 expression plasmids accumulate the corresponding proteins in the cytoplasm. However, Mx2 protein gives a granular staining, and Mx3 protein gives a diffuse staining. No Mx stain could be observed in cells that had not been injected, that had been injected with the vector containing no insert, or that had been injected with an unrelated insert (htk cDNA) (data not shown). Since cells stably transformed with these Mx plasmids synthesized recombinant Mx proteins with apparent molecular weights corresponding to those of their natural counterparts (data not shown), we assume that the Mx proteins expressed in the injected cells also correspond to their authentic counterparts.

Antiviral activities of rat Mx proteins. To assess the antiviral activities of the three rat Mx proteins, they could be expressed either transiently or stably in transformed cell

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m Mx1                                 .D..N...RH........  18
r Mx1        MKERTSACRHGTPQKHPDTSEESQAMESVDNLCSQYEEKVRPC  43
r Mx2 MVLSTEENRSVDLVNLPSV.LPDGEAGVGENNKD.LN.............  50
r Mx3 MVLSTEENRSVDLVNLPSV.LPDGEAGVGENNKD..N.............  50

m Mx1 .....T............................................  68
r Mx1 IDLIDSLRALGVEQDLALPAIAVIGDQSSGKSSVLEALSGVALPRGSGIV  93
r Mx2 .................................................. 100
r Mx3 .................................................. 100
                             *    **
m Mx1 .........RK..E..E.R...S.D.I.V.L.D..E..EA...G..F... 118
r Mx1 TRCPLVLKLKQLKQGEKWSGKVIYKDTEIEISHPSLVEREINKAQNLIAG 143
r Mx2 ..........K.N...E.K...T.D.I.V.L.D..E..EA..TG..H... 150
r Mx3 ..........K.N...E.K...T.D.I.V.L.D..E..EA..TG..H... 150

m Mx1 V..G..DK....D....N..................N.....GRQ..... 168
r Mx1 EGLKISSDLISLEVSSPHVPDLTLIDLPGITRVAVGDQPADIEHKIKRLI 193
r Mx2 V..G..DK....D.......................N.....GRQ..... 200
r Mx3 V..G..DK....D.......................N..S..GRQ..... 200
                          *  *
m Mx1 KT.........................S.......E......V....... 218
r Mx1 TEYIQKQETINLVVVPSNVDIATTEALKMAQEVDPQGDRTIGILTKPDLV 243
r Mx2 .N.........................S...K...D.............. 250
r Mx3 .N.........................S...E...D.............. 250
                                             * *
m Mx1 ...A.G..L..M....YP......................T..F...... 268
r Mx1 DRGTEDKVVDVVRNLVCHLKKGYMIVKCRGQQDIQEQLSLAEALQKEQVF 293
r Mx2 .................................................. 300
r Mx3 .................................................. 300

m Mx1 ..D.SY.SI.............E...E...........L..D...SS..S 318
r Mx1 FKEHPQFRVLLEDGKATVPCLAKRLTMELTSHICKSLPILENQINVNHQI 343
r Mx2 ........A.............E......I........L.....KES..S 350
r Mx3 ........A.............E......I........L.....KES..S 350

m Mx1 ................RT.M...V...SA..RN.MN.I....TV.EGD.. 368
r Mx1 ASEELQKYGADIPEDDSKRLSFLMNKINVFNKDILSLVQAQENISWEESR 393
r Mx2 T..............ENEKTL..IE...A..Q..TAI.EGE.IVREK.C. 400
r Mx3 T..............ENEKTL..IE...A..Q..TAI.EGE.IVREK.C. 400

m Mx1 ............D.H...Y...--D.P.VQ.K.KE..NQ........... 416
r Mx1 LFTKLRNEFLAWNDYIEEHFKKTLGSSEKHSQMEKFESHYRGRELPGFVD 443
r Mx2 ......K..FL.SEE..RN.Q.--..DALYKEVYT..MQ..........N 448
r Mx3 ......K..FL.SEE..RN.Q.--..DALYKEVYT..MQ..........N 448

m Mx1 .....S....R......S.V...R...K..QT..V.IL..D.......CC 466
r Mx1 YKAFENIIKKEVKALEEPALNMLHRVTTMVKNAFTKVSSNNFGDFLNLHS 493
r Mx2 ..T.....RRQI.T.....ME...K..EI.RA...T..EK..SE.F...R 498
r Mx3 ..T.....RRQI.T.....ME...K..EI.RA...T..EK..SE.F...R 498

m Mx1 ......KE..L.......N.........Q.......V..ET.KT...... 516
r Mx1 TAKSKIEDIRFNQEKEAEKLIRLHFQMEHIVYCQDQAYKKALQEIREKEA 543
r Mx2 .T...L....LE..T....S........Q.I.....I.R....KV..E.. 548
r Mx3 .T...L....LE..T....A........Q.I.....I.R....KV..E.. 548

m Mx1 ....TKALINPAT..N..QF.Q.G.......---...K......R..... 563
r Mx1 EKEKSTF----GAFQHNS--PRKELTTTEMT---QHLNAYYQECGRNIGR 584
r Mx2 .E.ERKH-----GKSRS.--QS.N.Q.SS.DEIF......R..AHNR.SS 591
r Mx3 .E.ERKH-----GKSRSA--QSPN.Q.SS.DEIF.....R..AHNC.SS 591

m Mx1 ........F..K...E.I..M...........S...E....T........ 613
r Mx1 QIPLIIQYSILQTFGQEMEKAMLQLLQDTSKCNWFLTEQSDSREKKKFLK 634
r Mx2 H.......F..KM.AEKLQ.G.......KDS.S.L.K.H..TS..RR... 641
r Mx3 H.......F..KM.AEKLQ.G.......KDS.S.L.K.K..TS..RR... 641

m Mx1 .........RQ......D 631
r Mx1 RRLLRLDEAQRKLAKFSN 652
r Mx2 E..A..AQ...R....PG 659
r Mx3 E..A..AQ...R....PG 659
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FIG. 4. Optimal alignment of the predicted amino acid sequences of rat (r) Mx1, Mx2, and Mx3 and mouse (m) Mx1 proteins. Dots indicate positions of amino acid identity with rat Mx1. Rat Mx2 and rat Mx3 proteins differ at the following eight positions: 36, 190, 232, 518, 561, 564, 588, and 630. The consensus elements characteristic of GTP-binding proteins are marked by stars.

lines. To avoid the risk of selecting transformed cell lines that are nonpermissive for virus growth for reasons other than Mx protein expression, we decided to assay the proteins' activities in transient expression experiments. As shown above, high levels of Mx protein expression can be achieved by microinjection of suitable plasmids. Therefore, 3T3 cells injected with any one of the three plasmids were infected with influenza A/WSN virus 18 h after injection. Seven hours thereafter, the cells were fixed and processed for double indirect immunofluorescence to visualize both Mx protein expression and accumulation of viral antigens. As in the absence of infection, rat Mx1 protein accumulated in the nuclei of these injected cells and both Mx2 and Mx3 proteins accumulated in the cytoplasm. Cells expressing rat Mx1 protein were always negative for influenza virus antigens. In contrast, the majority of cells that express either rat Mx2

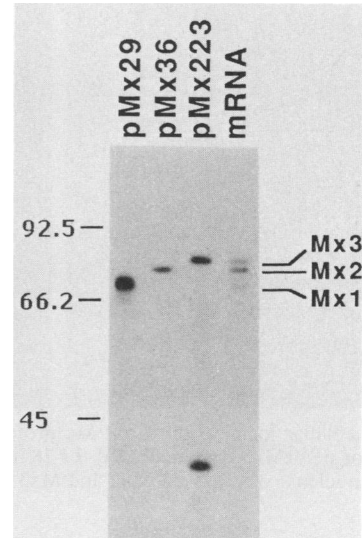

FIG. 5. In vitro translation of RNAs derived from rat *Mx* cDNA clones. pSP65 DNA containing the respective rat *Mx* cDNAs in its *Eco*RI site was linearized with *Sma*I. Capped runoff transcripts were synthesized with SP6 polymerase. These RNAs and poly(A)$^+$ RNA, isolated from IFN-treated rat embryo cells, were translated in vitro in a rabbit reticulocyte lysate. The [^{35}S]methionine-labeled products were immunoprecipitated with the anti-mouse Mx1 antibody 2C12, separated in an 8% sodium dodecyl sulfate-polyacrylamide gel, and visualized by autoradiography. RNAs were derived either from pMx29, pMx36, or pMx223; the lane marked "mRNA" shows the three Mx proteins obtained with poly(A)$^+$ RNA prepared from IFN-treated rat cells.

protein or rat Mx3 protein showed strong immunofluorescent staining for influenza virus antigens.

A quantitative analysis of the data is shown in Table 1, experiment 1. The percentage of influenza virus-positive cells is significantly reduced only after injection of the Mx1 expression plasmid. We noted that injection of the control htk plasmid slightly reduces the percentage of influenza virus proteins, compared with uninjected cells, as does injection of the Mx2 and Mx3 expression plasmids. This appears to be an unspecific effect of plasmid injection.

The effect of rat Mx proteins on VSV was tested much as described for influenza virus. Injected cells were exposed to virus, incubated for 3 h, and then fixed and stained for VSV proteins and Mx or htk proteins. The intracellular localization of Mx and htk proteins was not affected by infection with VSV (data not shown). A quantification of their anti-VSV activity is shown in Table 1, experiment 1. Thirty-six percent of cells expressing rat Mx1 and 2% of those expressing rat Mx2 stained positive for VSV. In contrast, 88% of the cells expressing Mx3 stained positive for VSV, comparable to 91% of control htk-expressing cells. Since 99% of uninjected cells were virus-positive, it appeared that as with influenza virus, plasmid injection had a slight and unspecific effect on VSV expression.

The activity of rat Mx1 and Mx2 against VSV was surprising, since the mouse Mx1 protein is not known to have activity against this virus (17, 23). To confirm these earlier results and to compare the mouse protein's activity directly with that of rat Mx1 protein, we tested these two proteins in a separate experiment. In this experiment (Table 1, experiment 2), 27% of rat Mx1 protein-expressing cells stained positive for VSV in contrast to 92% of those expressing mouse Mx1 protein. It is unlikely that rat Mx1 protein

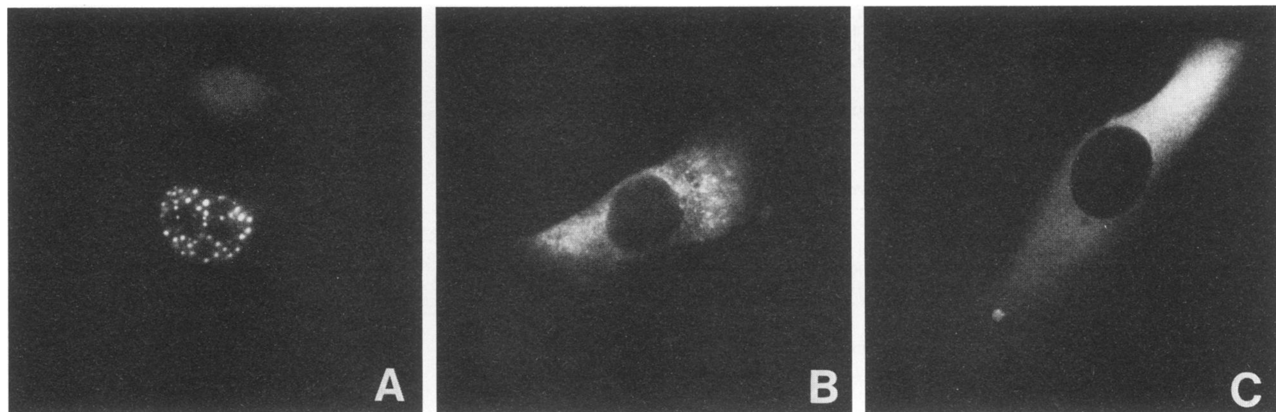

FIG. 6. Intracellular localization of rat Mx proteins. *Mx1*-negative mouse 3T3 cells were injected into their nuclei with pSVrMx1 (A), pSVrMx2 (B), or pSVrMx3 (C); incubated for 18 h; and then fixed and stained for Mx protein. Note that rat Mx1 protein accumulates in granules in the nucleus, whereas rat Mx2 and Mx3 proteins accumulate in the cytoplasm.

would inhibit viruses because it was generally toxic to cells, since we were able to generate cell lines which constitutively express rat Mx1 protein at levels comparable to those of injected cells. We conclude that both rat Mx1 and rat Mx2 but not rat Mx3 or mouse Mx1 proteins are able to inhibit VSV efficiently.

DISCUSSION

A characteristic feature of the nuclear Mx1 protein of mice is its specific anti-influenza virus activity. This feature has not been conserved in the three rat Mx proteins. Rat Mx1 protein, although closely related to mouse Mx1 protein, protects cells not only against influenza virus but also against VSV. Rat Mx2 and Mx3 proteins, both cytoplasmic and more distantly related to mouse Mx1 protein, fail to protect against influenza virus. However, rat Mx2 protein protects against VSV, whereas rat Mx3 protein, although differing in only 8 residues from the Mx2 protein, surprisingly is inactive against either test virus.

It is unclear what role Mx proteins play in protection of rats against viruses. Earlier experiments have shown that in rat fibroblasts, in which Mx proteins had been neutralized by microinjection of the monoclonal anti-Mx antibody 2C12, IFN can no longer establish an efficient antiviral state against influenza virus (3). Since this antibody reacts with all three rat Mx proteins, it was then concluded that at least one of them is required for arrest of influenza virus, but it was impossible to determine which one this was. The present data indicate that it is the Mx1 protein, and only the Mx1 protein, that is responsible for this anti-influenza activity. However, 2C12 antibody-injected, IFN-treated rat cells, although susceptible to influenza virus, are still protected against VSV (3). Therefore, it might at first sight be surprising to find that expression of Mx1 or Mx2 protein alone is sufficient to arrest VSV. It is possible that injection of antibody 2C12 results in incomplete neutralization of the anti-VSV activity of Mx1 or Mx2 proteins. However, it is more likely that IFN protects cells against VSV by two different mechanisms. One mechanism would be mediated by Mx1 or Mx2 protein or both and would be neutralized by antibody 2C12, and a second one would be independent of these two Mx proteins yet sufficient for protection. This latter hypothesis is supported by observations made of A2G mouse cells in which IFN establishes an efficient antiviral state against VSV. The Mx1 protein of these cells has no intrinsic activity against VSV, and the *Mx2* gene is not transcribed in them (24). Thus, for inhibition of VSV, rat Mx1 and Mx2 proteins may play but a synergistic role, along with other IFN-induced antiviral mechanisms.

Recently, human Mx proteins (1, 9) have also been found to display antiviral activities which differ from that of the murine Mx1 protein. When tested in mouse cells, the cytoplasmic human MxA protein has activity against influenza virus and VSV, whereas the cytoplasmic human MxB is inactive against any virus tested to date (18).

Our observations combined with those made with the human Mx proteins indicate that the subcellular localization of Mx proteins, as evidenced by immunofluorescence staining, does not determine their antiviral specificities in all cases. Influenza viruses whose replication and transcription are nuclear are inhibited by both the nuclear rat Mx1 and the cytoplasmic human MxA proteins, and VSV, whose replication takes place entirely in the cytoplasm, is also arrested by both nuclear (rat Mx1) and cytoplasmic (rat Mx2 and human MxA) proteins. This discrepancy could be explained in two ways. It is possible that Mx proteins operate against

TABLE 1. Antiviral activity of rat Mx proteins in mouse 3T3 cells[a]

Expt.	Injected plasmid	Intracellular localization	No. of virus-positive cells/no. of htk- or Mx protein-expressing cells (%)	
			Influenza A virus	VSV
1	htk	Cytoplasm	26/34 (76)	42/46 (91)
	Rat Mx1	Nucleus	0/26 (0)	10/28 (36)
	Rat Mx2	Cytoplasm	47/59 (80)	1/54 (2)
	Rat Mx3	Cytoplasm	32/45 (71)	36/41 (88)
2	Rat Mx1	Nucleus	ND	7/26 (27)
	Mouse Mx1	Nucleus	ND	24/26 (92)

[a] Plasmids were injected into the nuclei of *Mx1* negative mouse 3T3 cells. Sixteen hours after injection, cells were infected with either influenza virus or VSV, fixed, and double labeled for Mx proteins or htk and viral proteins. The intracellular localization of each of the Mx proteins or htk is the same in uninfected and influenza virus- or VSV-infected cells. Antiviral activity is given as percentage and absolute numbers of virus-positive cells per Mx protein- or htk-expressing cells. A high value indicates no or little antiviral activity and a low value indicates strong antiviral activity of a particular expressed protein. Of uninjected control cells, 90% were influenza virus positive and 99% were VSV positive. ND, Not done.

viruses indirectly, that is, by modifying other cellular proteins which then serve as modulators of viral replication in the relevant cellular compartment. Alternatively, they could interfere directly with one or several steps in the respective viral replication cycles, and some of them may do so at locations where they cannot be detected by the immunofluorescence assay. Thus, it is possible that a fraction of rat Mx1 protein inhibits VSV in the cytoplasm, even though rat Mx1 is undetectable in this cellular compartment. In either case, the virus specificity of an Mx protein is determined by its structure; for instance, the carboxyl-terminal region, in which the different Mx proteins show the least conservation, could be the determinant of specificity. It remains to be shown what role the more conserved amino-terminal portions (and within them the conserved sequence elements characteristic of GTP-binding proteins) play and at which precise step each of the Mx proteins interfere with the growth of the virus or the viruses they inhibit. Such analyses will reveal whether different viruses are inhibited at equivalent or distinct steps and why many viruses are not adversely affected by Mx proteins.

The murine Mx1 protein has been used successfully to confer influenza resistance to *Mx1*-negative, influenza virus-susceptible mice by germ line transformation (4). If, as anticipated from the present study, related Mx proteins indeed have a broader or different antiviral specificity in vivo, one could use them to obtain livestock resistant to other viruses. Animals may not be the only ones to benefit from such approaches. Recent advances in transformation of certain human cells such as hematopoietic cells may make it feasible in the future to employ recombinant proteins, including Mx proteins, in gene therapy of infections for which other antiviral therapies or standard vaccinations have proved difficult.

ADDENDUM IN PROOF

Obar et al. recently reported that rat dynamin, a microtubule-activated GTPase, has a high degree of similarity with mouse Mx1 (R. A. Obar, C. A. Collins, J. A. Hammarback, H. S. Shpetner, and R. B. Vallee, Nature (London) **347**:256–261, 1990). Sequence comparisons between rat dynamin and rat Mx proteins reveal similar degrees of similarity. These homologies are discussed in H. Arnheiter, and E. Meier, The New Biologist, vol. 2, no. 10, 1990.

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LITERATURE CITED

1. **Aebi, M., J. Fäh, N. Hurt, C. E. Samuel, D. Thomis, L. Bazzigher, J. Pavlovic, O. Haller, and P. Staeheli.** 1989. cDNA structures and regulation of two interferon-induced human Mx proteins. Mol. Cell. Biol. **9**:5062–5072.
2. **Arnheiter, H., M. Dubois-Dalcq, and R. A. Lazzarini.** 1984. Direct visualization of protein transport and processing in the living cell by microinjection of specific antibodies. Cell **39**:99–109.
3. **Arnheiter, H., and O. Haller.** 1988. Antiviral state against influenza virus neutralized by microinjection of antibodies to interferon-induced Mx proteins. EMBO J. **7**:1315–1320.
4. **Arnheiter, H., S. Skuntz, M. Noteborn, S. Chang, and E. Meier.** 1990. Transgenic mice with intracellular immunity to influenza virus. Cell **62**:51–61.
5. **Arnheiter, H., and P. Staeheli.** 1983. Expression of interferon dependent resistance to influenza virus in mouse embryo cells. Arch. Virol. **76**:127–137.
6. **Chebath, J., P. Benech, M. Revel, and M. Vigneron.** 1987. Constitutive expression of (2′-5′) oligo A synthetase confers resistance to picornavirus infection. Nature (London) **330**:587–588.
7. **Haller, O.** 1981. Inborn resistance of mice to orthomyxoviruses. Curr. Top. Microbiol. Immunol. **92**:25–52.
8. **Haller, O., H. Arnheiter, M. A. Horisberger, I. Gresser, and J. Lindenmann.** 1980. Interaction between interferon and host genes in antiviral defense. Ann. N.Y. Acad. Sci. **350**:558–565.
9. **Horisberger, M. A., G. K. McMaster, H. Zeller, M. G. Wathelet, J. Dellis, and J. Content.** 1990. Cloning and sequence analysis of cDNAs for interferon- and virus-induced human Mx proteins reveal that they contain putative guanine nucleotide-binding sites: functional study of the corresponding gene promoter. J. Virol. **64**:1171–1181.
10. **Horisberger, M. A., P. Staeheli, and O. Haller.** 1983. Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. Proc. Natl. Acad. Sci. USA **80**:1910–1914.
11. **Konkel, D. A., S. M. Tilghman, and P. Leder.** 1978. The sequence of the chromosomal mouse β-globin major gene: homologies in capping, splicing and poly(A) sites. Cell **15**:1125–1132.
12. **Krieg, P., and D. A. Melton.** 1984. In vitro RNA synthesis with SP6 polymerase. Methods Enzymol. **155**:397–415.
13. **Krug, R. M., M. Shaw, B. Broni, G. Shapiro, and O. Haller.** 1985. Inhibition of influenza viral mRNA synthesis in cells expressing the interferon-induced Mx gene product. J. Virol. **56**:201–206.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Meier, E., J. Fäh, M. S. Grob, R. End, P. Staeheli, and O. Haller.** 1988. A family of interferon-induced Mx-related mRNAs encodes cytoplasmic and nuclear proteins in rat cells. J. Virol. **62**:2386–2393.
16. **Meyer, T., and M. A. Horisberger.** 1984. Combined action of mouse α and β interferons in influenza virus-infected macrophages carrying the resistance gene Mx. J. Virol. **49**:709–716.
17. **Noteborn, M., H. Arnheiter, L. Richter-Mann, H. Browning, and C. Weissmann.** 1987. Transport of the murine Mx protein into the nucleus is dependent on a basic carboxy terminal sequence. J. Interferon Res. **7**:657–669.
18. **Pavlovic, J., T. Zürcher, O. Haller, and P. Staeheli.** 1990. Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein. J. Virol. **64**:3370–3375.
19. **Rothman, J. H., C. K. Raymond, T. Gilbert, P. J. O'Hara, and T. H. Stevens.** 1990. A putative GTP binding protein homologous to interferon-inducible Mx proteins performs an essential function in yeast protein sorting. Cell **61**:1063–1074.
20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
21. **Staeheli, P.** 1990. Interferon-induced proteins and the antiviral state. Adv. Virus Res. **38**:147–200.
22. **Staeheli, P., P. Dreiding, O. Haller, and J. Lindenmann.** 1985. Polyclonal and monoclonal antibodies to the interferon-inducible protein Mx of influenza virus-resistant mice. J. Biol. Chem. **260**:1821–1825.
23. **Staeheli, P., O. Haller, W. Boll, J. Lindenmann, and C. Weissmann.** 1986. Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. Cell **44**:147–158.
24. **Staeheli, P., and J. G. Sutcliffe.** 1988. Identification of a second interferon-regulated murine Mx gene. Mol. Cell. Biol. **8**:4524–4528.