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cDNA Structures and Regulation of Two Interferon-Induced Human Mx Proteins

MARKUS AEBI,[1] JÜRG FÄH,[2] NICOLE HURT,[2] CHARLES E. SAMUEL,[1]† DANIEL THOMIS,[1]† LUIGI BAZZIGHER,[2] JOVAN PAVLOVIC,[2] OTTO HALLER,[2] AND PETER STAEHELI[2]*

Institute for Molecular Biology I, University of Zurich, CH-8093 Zurich,[1] and Institute for Immunology & Virology, University of Zurich, CH-8028 Zurich,[2] Switzerland

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Human cells treated with interferon synthesize two proteins that exhibit high homology to murine Mx1 protein, which has previously been identified as the mediator of interferon-induced cellular resistance of mouse cells against influenza viruses. Using murine *Mx1* cDNA as a hybridization probe, we have isolated cDNA clones originating from two distinct human *Mx* genes, designated *MxA* and *MxB*. In human fibroblasts, expression of *MxA* and *MxB* is strongly induced by alpha interferon (IFN-α), IFN-β, Newcastle disease virus, and, to a much lesser extent, IFN-γ. MxA and MxB proteins have molecular masses of 76 and 73 kilodaltons, respectively, and their sequences are 63% identical. A comparison of human and mouse Mx proteins revealed that human MxA and mouse Mx2 are the most closely related proteins, showing 77% sequence identity. Near their amino termini, human and mouse Mx proteins contain a block of 53 identical amino acids and additional regions of very high sequence similarity. These conserved sequences are also present in a double-stranded RNA-inducible fish gene, which suggests that they may constitute a functionally important domain of Mx proteins. In contrast to mouse Mx1 protein, which accumulates in the nuclei of IFN-treated mouse cells, the two human Mx proteins both accumulate in the cytoplasm of IFN-treated cells.

Influenza viruses are important human pathogens (35), and one might therefore expect that humans possess a very efficient influenza virus defense system. During the course of viral infections, including influenza virus infections, interferons (IFNs) are synthesized which, in turn, induce an antiviral state in cells surrounding the initial site of virus replication. It is believed that IFNs thus help to limit virus spread and permit the immune system to destroy the invading virus without causing severe tissue damage. The beneficial role of IFNs in host defense against many viruses has been documented in animal model systems (see reference 7 for a review), suggesting that IFN may play a similar role in humans. From an experimental mouse model of influenza virus resistance, we have concluded that IFN exerts its protective effect through the activation of a cellular resistance gene, designated *Mx* (see reference 30 for a review). Exposure of mouse cells to IFN induces the synthesis of the 72-kilodalton (kDa) Mx1 protein, which, in turn, is capable of selectively blocking the multiplication of influenza viruses (1, 21, 31). The molecular mechanism principally responsible for the IFN-mediated inhibition of influenza virus multiplication is not yet resolved (16, 20, 25).

Evidence in favor of a similar influenza virus defense system in humans includes the observations that IFN can block influenza virus multiplication very efficiently in cultured human fibroblasts (10) and that IFN treatment causes the accumulation of an Mx-related protein in these cells (11, 29). This Mx-related human protein has an apparent molecular mass of about 78 kDa, and, unlike murine Mx1 protein, it accumulates in the cell cytoplasm rather than the nucleus. Although available data are compatible with the view that the Mx-related human protein plays a role in defense against influenza virus, no direct experimental evidence supporting this notion has yet been presented.

One approach to the elucidation of the physiological role of Mx-like proteins of humans is to molecularly clone the human *Mx* cDNAs and then to express them in transfected cells and to test such cells for newly acquired functions, in particular influenza virus resistance. In this paper we report the first step toward this goal, the isolation and characterization of two *Mx*-related human cDNAs. We show that the corresponding mRNAs originate from two distinct human *Mx* genes; the expression of both genes is stimulated by IFN-α, IFN-β, Newcastle disease virus (NDV), and, to a lesser extent, IFN-γ. The encoded proteins, designated human MxA and MxB, and Mx-like proteins of other species contain blocks of closely related sequences located near their amino termini. Indirect immunofluorescence analysis with specific antisera indicates that the human MxA and MxB proteins both accumulate in the cytoplasm of IFN-treated cells.

MATERIALS AND METHODS

IFNs. Recombinant human IFN-α_2 (10^8 U/mg) and recombinant IFN-γ (5×10^7 U/mg) were gifts from Biogen SA, Geneva, Switzerland. Highly purified, natural human IFN-β was purchased from Renschler, Laupheim, Federal Republic of Germany. Confluent cell monolayers were treated with the indicated concentrations of the different IFNs in culture medium containing 2% fetal calf serum. Where indicated, cycloheximide (CHX; final concentration, 75 μg/ml) was added to the culture medium 30 min before the IFN treatment was begun.

Cells. Human fetal lung (HFL) cells and the human glioblastoma cell line T98G (34) were grown in Dulbecco modified minimal essential medium containing 10% fetal calf serum. Confluent monolayer cultures were used for all experiments described here.

NDV. A working stock of NDV was grown in the allantoic

* Corresponding author.
† Present address: Department of Biological Sciences, Section of Biochemistry and Molecular Biology, University of California, Santa Barbara, CA 93106.

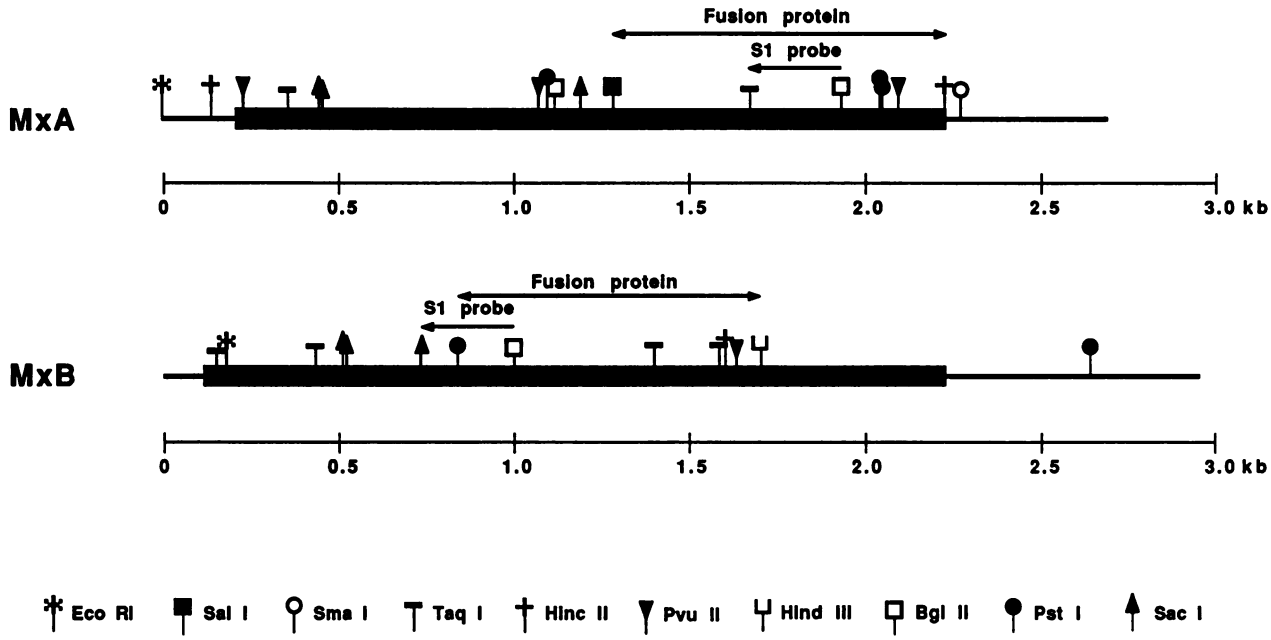

FIG. 1. Partial restriction maps of *MxA* and *MxB* cDNAs. Symbols: ■, relative positions of the protein-coding regions; ←, restriction fragments subcloned for subsequent use in S1 hybridization experiments; ↔, restriction fragments subcloned into β-galactosidase fusion protein constructs. Fusion proteins were produced in *E. coli* and used to immunize rabbits.

cavity of 10-day-old embryonated eggs. Viral infection was carried out as follows: confluent monolayers of HFL cells in a 90-mm dish were treated for 30 min at 37°C with 8 ml of serum-free medium containing CHX (75 μg/ml) before 80 μl of allantoic fluid containing NDV were added. The cell cultures were incubated at 37°C for another 3 h.

Isolation of RNA for cloning experiments and Northern (RNA) blot analysis. Cytoplasmic poly(A)$^+$ RNA was prepared as described previously (28). For some experiments, poly(A)$^+$ RNA was prepared from total cellular RNA isolated by the procedure described by Auffray and Rougeon (2).

Isolation of cDNA clones. Initially, a cDNA library was constructed from poly(A)$^+$ RNA of T98G cells treated with IFN-α_2. cDNA was prepared by the method of Maniatis et al. (17), using the hairpin-S1 method for the synthesis of the second strand. Size-selected cDNA was cloned into the *Eco*RI site of λgt10 (14). About 5,000 individual plaques were screened by using the nick-translated *Pst*I-*Bam*HI fragment of the murine *Mx* cDNA (positions 1181 to 2320 [31]) as a hybridization probe. Two cDNA clones were isolated that differed in their restriction maps but hybridized to the murine *Mx* cDNA probe. The two cDNA clones were designated *MxA* and *MxB*. (*MxA* corresponds to the previously designated *MxC* clone, and *MxB* corresponds to the previously designated *MxE* clone [M. Aebi, C. E. Samuel, H. Arnheiter, O. Haller, and C. Weissmann, J. Interferon Res. 7:719, 1987].) Additional cDNA libraries were produced in λgt11 (36) and λZap (Stratagene Inc., La Jolla, Calif.) from mRNAs of IFN-treated T98G cells and screened with probes specific for *MxA* (*MxA* cDNA positions 250 to 1077 in Fig. 4) or *MxB* (*MxB* cDNA positions 1690 to 3061 in Fig. 5). Several different, presumably full-length *MxA*-type cDNA clones were isolated, whereas no long *MxB*-type cDNA clone was obtained. Therefore, a new cDNA library was prepared in pHG327 (31) by the method of Okayama and Berg (22), with mRNA from HFL cells treated with IFN-α_2. One presumably full-length *MxB*-type cDNA clone was isolated from this library by using a fragment of *MxB* cDNA (positions 185 to 992 in Fig. 5) as the hybridization probe. The nucleotide sequences of representative cDNA clones were determined by the methods of Sanger (27) and Maxam and Gilbert (18).

S1 nuclease protection assays. (i) RNA isolation. Cell monolayers in 90-mm dishes were washed with ice-cold phosphate-buffered saline (PBS) before the RNA was extracted as described by Chomczynski and Sacchi (4), except that cell lysis was performed on the monolayers in culture dish by using 3 ml of their solution D.

(ii) Preparation of S1 probes. The *MxA*-specific S1 probe was prepared from plasmid pMxA-S1 containing the fragment of *MxA* cDNA indicated in Fig. 1. pMxA-S1 was constructed as follows. *MxA* cDNA was first cut with *Taq*I, and this site was blunted with Klenow polymerase (17). The DNA was next cut with *Bgl*II, and the 270-base-pair fragment was recovered from low-melting-temperature agarose. This DNA fragment was then cloned into pHG327 (31) cut with *Bgl*II and *Pvu*II. The *MxB*-specific S1 probe was prepared from plasmid pMxB-S1 containing the fragment of *MxB* cDNA indicated in Fig. 1. pMxB-S1 was constructed as follows. *MxB* cDNA was cut with *Sac*I and *Bgl*II, and the 275-base-pair fragment was recovered from low melting-temperature agarose. This DNA fragment was then cloned into pHG327 cut with *Bgl*II and *Sac*I. The β-actin S1 probe was prepared from plasmid pActin-S1, which was constructed as follows. pHF5 plasmid (8, 23) was cut with *Sau*3A and *Bam*HI, and the 200-base-pair fragment was recovered from low-melting-temperature agarose. This DNA fragment was then cloned into pHG327 cut with *Bgl*II and *Bam*HI, so that the *Sau*3A and the *Bgl*II sites became ligated and thus restored the *Bgl*II site. To prepare radiolabeled probes, pMxA-S1, pMxB-S1, and pActin-S1 were cut with *Bgl*II, treated with alkaline phosphatase, and labeled with ^{32}P at the 5′ end by using T4 polynucleotide kinase (17). The end-labeled DNAs were cut with *Eco*RI, and the 340-, 670- and 570-base-pair fragments, respectively, were recovered.

(iii) Hybridization, digestion with S1 nuclease, and analysis by gel electrophoresis. The assay was performed essentially as described by Berk and Sharp (3). A 15-μg sample of total cellular RNA was transferred to Eppendorf tubes, lyophilized, and dissolved in 20 μl of hybridization buffer, consisting of 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 1 mM EDTA, 400 mM NaCl, 80% formamide, and 0.01 pM of end-labeled S1 probe. A small drop of paraffin oil was added to minimize evaporation during subsequent steps. The samples were incubated for 15 min at 70°C to denature the probe, quickly transferred to a 46°C water bath, and incubated at this temperature overnight. To each tube was then added 250 μl of ice-cold S1 reaction mixture, consisting of 30 mM sodium acetate (pH 4.5), 250 mM NaCl, 1 mM zinc sulfate, 20 μg of carrier DNA per ml, and 200 U of S1 nuclease per ml, and the samples were incubated for 60 min at 37°C. The reaction mixtures were then extracted with phenol, 5 μg of tRNA was added to each sample, and the nucleic acids were precipitated with ethanol. The material was dissolved in 3 μl of 90% formamide gel loading buffer and electrophoresed through a 6% polyacrylamide–8 M urea sequencing gel (18). The gel was exposed to X-ray film at −70°C with an intensifying screen. The radioactivity associated with the gel slices corresponding to bands of interest was quantified by measurement of Cerenkov counts.

Production of antibodies. (i) Fusion protein constructs. A fragment of *MxA* cDNA containing all sequences downstream from the *Sal*I site was inserted into plasmid pUR291 (24) that was cut with *Sal*I and *Hin*dIII. Ligation at the *Sal*I site joined the β-galactosidase open reading frame (ORF) to that of MxA protein, yielding a fusion protein consisting of β-galactosidase and the fragment of MxA protein indicated in Fig. 1. The *Pst*I-*Hin*dIII fragment of *MxB* cDNA was cloned into pUR291 cut with *Pst*I and *Hin*dIII. Ligation at the *Pst*I site joined the β-galactosidase ORF to that of MxB protein. Since a fortuitous translational stop codon is located only a few codons downstream of the *Hin*dIII site, the resulting fusion protein consists of β-galactosidase and the fragment of MxB protein indicated in Fig. 1 and only a few additional amino acids.

(ii) Production of fusion proteins. Plasmids were transfected into *Escherichia coli* BMH 71-18 (24), and ampicillin-resistant clones were selected and grown in LB medium at 37°C to mid-log phase. Isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 1 mM) was added, and the cultures were incubated for another 4 h before they were harvested and lysed by boiling for 5 min in gel-loading buffer consisting of 125 mM Tris hydrochloride (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 5% 2-mercaptoethanol. The soluble material was loaded onto 8% polyacrylamide–SDS gels and electrophoresed. The gels were first soaked in ice-cold 250 mM KCl for 5 min and then in ice-cold water for 10 min. The fusion protein was then visible as white band. The gel material containing the fusion protein was cut out, finely minced by being forced through a 21-gauge needle, and diluted with PBS to yield a suspension of about 200 μg of protein per ml.

(iii) Immunizations. Every 2 weeks, female BALB/c mice received intraperitoneal injections of 200-μl portions of gel material containing MxA or MxB fusion protein. Hyperimmune sera were collected after four immunizations.

Western immunoblot analysis. Protein extracts were prepared by lysing T98G cells in gel-loading buffer containing bromophenol dye. The material was boiled for 3 min, and portions of 100 μg of protein were electrophoresed through 8% polyacrylamide–SDS gels. After electrophoresis, the proteins were transferred to nitrocellulose by standard procedures. The filters were incubated for 1 h in PBS containing 10% nonfat dry milk and then for 1 h in PBS containing 1% nonfat dry milk and 0.5% mouse hyperimmune serum. The filters were then washed in PBS and incubated for 1 h in PBS containing 1% nonfat dry milk and 0.2% peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (Nordic, Tilburg, The Netherlands). The filters were washed in PBS and stained with PBS containing 10% methanol, 300 μg of 4-chloro-1-naphthol per ml, and 0.01% H_2O_2.

Immunofluorescence analysis. The procedure for immunofluorescence analysis was described by Staeheli et al. (31). Mouse hyperimmune sera were diluted in 1:50 in PBS containing 5% normal goat serum.

RESULTS

Isolation of human *Mx*-related cDNAs. Southern blot analyses of genomic DNA from several human cell lines indicated that the previously cloned murine *Mx* cDNA (31) might be a suitable probe for isolating homologous human DNA sequences because low-stringency hybridization experiments demonstrated that several restriction fragments of human DNA contained *Mx*-related sequences (data not shown). We next tested whether the gene(s) containing these sequences was under IFN control. HFL cells and the glioblastoma cell line T98G were cultured in the presence or absence of 1,000 U of IFN-α_2 per ml for 18 h before RNA extraction. Northern blots from these RNAs were prepared and hybridized with the murine *Mx* cDNA probe under low-stringency conditions. RNAs of about 2.8 kilobases (kb) were detected in IFN-treated HFL and T98G cells; however, these RNAs were not detected in untreated control cells (data not shown).

To clone these human *Mx*-related mRNAs, we prepared a cDNA library from mRNAs of T98G cells treated with IFN-α_2 as described in Materials and Methods. The library was screened at low stringency of hybridization with a radiolabeled murine *Mx* cDNA fragment. Two distinct classes of human cDNA clones, here designated *MxA* and *MxB*, were isolated. Using fragments of *MxA* and *MxB* cDNAs as hybridization probes to rescreen different cDNA libraries prepared from mRNAs of T98G cells and of HFL cells, both treated with IFN-α_2, we eventually isolated several *MxA* and *MxB* clones containing long inserts (2.5 to 3.0 kb). Partial restriction maps of the longest of these cDNA clones are shown in Fig. 1. The maps of *MxA* and *MxB* cDNAs differed substantially from each other, suggesting that the corresponding mRNAs were derived from two distinct *Mx*-related genes, designated *MxA* and *MxB*.

***MxA* cDNA probes hybridize to an IFN-induced 2.8-kb mRNA, whereas *MxB* probes hybridize to a family of IFN-induced mRNAs.** To demonstrate that the cloned *Mx*-related human cDNAs indeed detected IFN-induced mRNAs, we studied the expression of the *MxA* and *MxB* genes in IFN-treated HFL cells. Poly(A)$^+$ RNAs isolated from monolayer cultures of HFL cells treated for 5 h either with 1,000 U of IFN-α_2, IFN-β, or IFN-γ per ml or with IFN-free medium were used to prepare a Northern blot, which was then hybridized sequentially to radiolabeled probes derived from cloned *MxA* and *MxB* cDNAs (Fig. 2).

At high stringency of hybridization, *MxA* probes hybridized to a single 2.8-kb mRNA from IFN-treated cells. This mRNA, designated *MxA* mRNA, was abundantly present in HFL cells treated with IFN-α or IFN-β, but was not

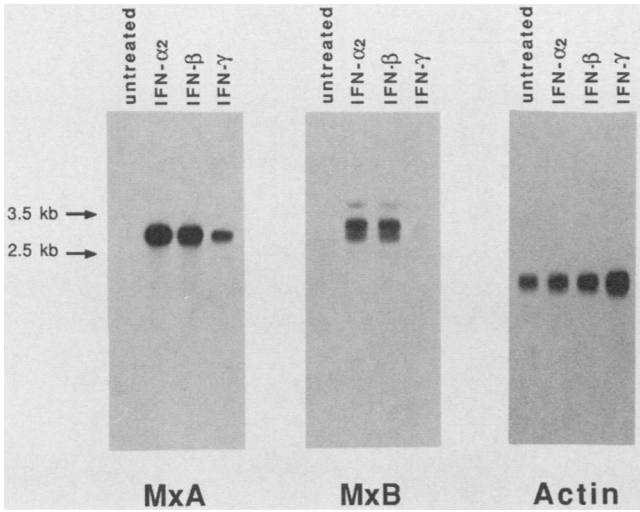

FIG. 2. IFN-induced *MxA* and *MxB* mRNAs in HFL cells. A Northern blot with poly(A)$^+$ RNA (about 1 μg) from HFL cells treated for 5 h with 1,000 U of IFN-α_2, IFN-β, or IFN-γ per ml was sequentially hybridized to *MxA* cDNA, *MxB* cDNA, and human β-actin cDNA. Arrows indicate the gel positions of mRNAs of known sizes, namely, murine *Mx1* mRNA (3.5 kb) and murine *Mx2* mRNA (2.5 kb).

detectable in untreated control cells. HFL cells treated with IFN-γ also contained *MxA* mRNA, although at a lower concentration. From the apparent size of *MxA* mRNA (2.8 kb), we concluded that our cloned 2.65-kb *MxA* cDNA (Fig. 1; also see Fig. 4) represents a near full-length copy of an *MxA* mRNA.

Radiolabeled *MxB* cDNA hybridized to a family of IFN-induced mRNAs from HFL cells. The three most abundant of these mRNAs, designated *MxB* mRNAs, were of 2.8, 3.1 kb, and 4 kb (Fig. 2). These *MxB* mRNAs were observed in HFL cells treated with IFN-α_2 or IFN-β, but were barely detectable in HFL cells treated with IFN-γ. In IFN-treated HFL cells, *MxB* RNAs were severalfold less abundant than *MxA* RNAs. (Note that the Northern blot probed with *MxB* cDNA in Fig. 2 was exposed about 5 times longer than the Northern blot probed with *MxA* cDNA.) We detected no *MxB* mRNAs in untreated HFL cells.

To define the relationships of the different *MxB* mRNA species, we sequentially reprobed the Northern blot with several small radiolabeled fragments of *MxB* cDNA. With all fragments except one, we observed efficient hybridization to all three *MxB* mRNA species. The exception was a probe derived from the 3′ untranslated region of *MxB* cDNA containing sequences downstream of the *Pst*I site located at map position 2.65 kb (Fig. 1). This probe hybridized to the 3.1- and 4-kb *MxB* mRNAs, but failed to hybridize to the 2.8-kb *MxB* mRNA (data not shown). Inspection of the *MxB* cDNA sequence (see Fig. 5) revealed the presence of two copies of the polyadenylation signal sequence AATAAA located about 25 and 300 nucleotides (nt), respectively, from the 3′ end of *MxB* cDNA. The simplest interpretation of these results, therefore, was that our cloned 2.96-kb *MxB* cDNA (Fig. 1; also see Fig. 5) represents a near-full-length copy of a 3.1-kb *MxB* mRNA. The 2.8-kb *MxB* mRNA most probably differs from the 3.1-kb *MxB* mRNA only by lacking about 300 nt of 3′ untranslated sequence owing to polyadenylation of pre-*MxB* mRNA at the first rather than the second polyadenylation site.

The precise structure of the rare 4-kb *MxB* RNA is not known. Since this RNA hybridized to all fragments of *MxB* cDNA tested, even at a very high stringency of hybridization, we believe that the 4-kb *MxB* transcript represents either unspliced, alternatively spliced, or differentially polyadenylated *MxB* RNA.

Kinetics of *MxA* and *MxB* expression in HFL cells treated with IFN-α_2. To quantify the *MxA* and *MxB* mRNA pools in IFN-treated HFL cells more precisely, we measured *Mx* mRNA concentrations by the S1 nuclease protection technique. Using the restriction fragments indicated in Fig. 1, we established assays as described in Materials and Methods that specifically detected either *MxA* mRNA or the *MxB* mRNAs. The high degree of specificity of these S1 assays was demonstrated with in vitro-synthesized *MxA* and *MxB* RNAs. Using these two RNAs, we could demonstrate that the S1 probes used were specific for the corresponding mRNAs; no cross-reactivity to the other RNA was observed (data not shown).

In the S1 assay used for *MxA* mRNA detection (Fig. 3a), the band at 341 nt represents undigested *MxA* probe, the band at 269 nt is the signal of mature *MxA* mRNA, whereas the band at 222 nt (asterisk in Fig. 3a) most probably indicates the presence of *MxA* precursor RNA (see legend to Fig. 3). In the S1 assay used for *MxB* mRNA detection (Fig. 3b), the band at 671 nt represents undigested *MxB* probe and the band at 274 nt is the signal of mature *MxA* mRNA. Because our S1 reaction mixtures further contained a human β-actin probe to provide an internal control to compare the concentrations of RNA in each reaction, our gels showed additional bands which are not of *Mx* origin. Undigested β-actin probe migrates at 570 nt, whereas β-actin mRNA yields a signal at 175 nt. Our β-actin probe also detected additional RNAs yielding signals of lower molecular masses, designated collectively as Actin RNAs in Fig. 3. These signals presumably result from imperfect hybridization of the β-actin probe to γ-actin mRNA.

We treated HFL monolayer cells for either 1.5, 3, 6, 12, or 24 h with 1,000 U of IFN-α_2 per ml before the RNA was extracted and assayed for the presence of *MxA* and *MxB* mRNAs (Fig. 3). Neither *MxA* nor *MxB* mRNAs were detectable in untreated control HFL cells, but they accumulated very rapidly after the beginning of IFN treatment and reached maximal concentrations within about 6 h. Thereafter, they decreased slowly. Counting the radioactivity in each signal and comparing these values with those obtained from hybridization of known concentrations of in vitro-synthesized *Mx* mRNAs, we calculated that HFL cells treated for 6 h with IFN-α_2 contained about 500 molecules of *MxA* mRNA per cell (Fig. 3a, lane 8) and about 50 molecules of *MxB* mRNA per cell (Fig. 3b, lane 8). Untreated control cells contained less than 1 *MxA* or *MxB* mRNA molecule per cell (Fig. 3, lanes 5 and 11), but after only 90 min of treatment with IFN-α_2, the levels of *Mx* mRNA pools were already about 25% of the maximum (Fig. 3, lanes 6). These results demonstrated that IFN-α_2 is a potent inducer of the human *Mx* genes.

From the Northern blotting experiment described above, we already knew that IFN-β is also capable of inducing human *Mx* genes very efficiently. The S1 experiment (Fig. 3) demonstrated that this rapid induction of *Mx* gene expression also occurred when cells blocked with the protein synthesis inhibitor CHX were treated with IFN. HFL cells were treated for 30 min with medium containing 75 μg of CHX per ml before IFN was added. The cells were then kept for 3 h in the medium with CHX and IFN before RNA was

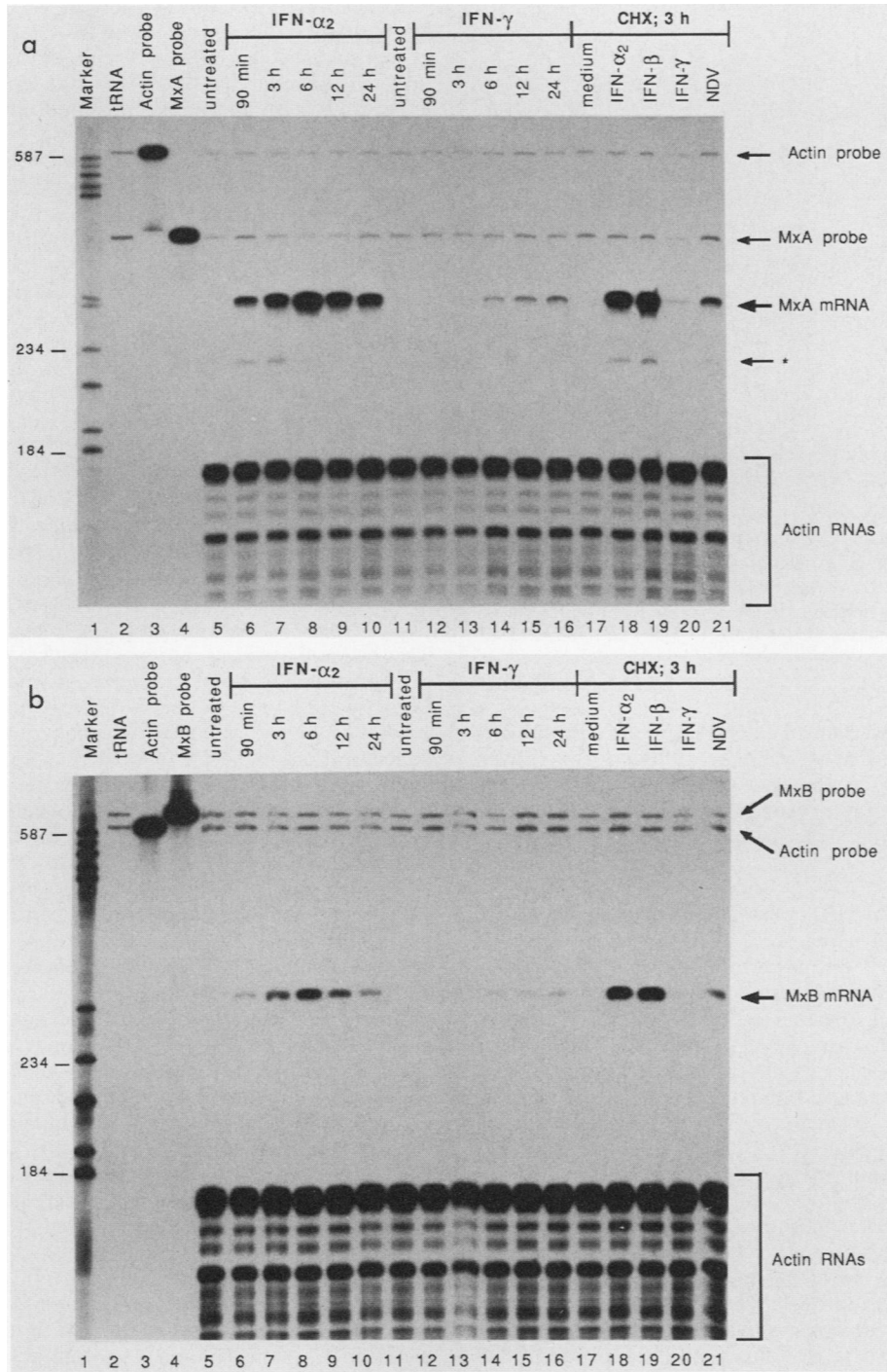

FIG. 3. *MxA* and *MxB* mRNA pools in HFL cells under various culture conditions. Monolayer cultures of HFL cells were treated for different times with different IFNs or with NDV in the presence or absence of CHX as indicated. Total RNAs were isolated, and 15 μg of each sample was tested for *MxA* (A) and *MxB* (B) mRNA content by S1 analysis. Expected bands are marked. The band at about 220 nt in panel A (asterisk) was not predicted. For two reasons it most probably indicates the presence of unspliced *MxA* RNAs: (i) this signal is observed at early times of induction only, and (ii) the signal maps to position 1719 of the *MxA* cDNA, which in the mouse *Mx1* gene marks the end of exon 12 (13). The heavy band at 180 nt is the β-actin signal, and the bands below probably are signals from cross-hybridizing γ-actin RNA. The size marker was pBR322, digested with *Hae*III and 5′ end labeled with ^{32}P.

extracted and analyzed. *MxA* and *MxB* mRNA pools were slightly higher in HFL cells treated with CHX and IFN than in cells treated with IFN alone (Fig. 3, compare lanes 7 and 18), demonstrating that activation of the *Mx* genes is not dependent on newly synthesized proteins.

Activation of *MxA* and *MxB* genes in HFL cells treated with IFN-γ. The Northern blot shown in Fig. 2 revealed that HFL cells treated with IFN-γ contained significant concentrations of both *MxA* and *MxB* mRNAs, but because hybridization of that blot with a β-actin probe (Fig. 2) demonstrated that the

lanes contained different amounts of RNA, it was difficult to determine the relative *Mx* mRNA levels. In a second experiment, we treated HFL monolayer cells with 1,000 U of IFN-γ per ml and determined *MxA* and *MxB* mRNA concentrations by S1 analysis at various times after induction. Again, we found *MxA* and *MxB* mRNAs in HFL cells treated with IFN-γ. In contrast to cells treated with IFN-α_2, the pools of *MxA* and *MxB* mRNAs constantly increased for at least 24 h after the onset of IFN-γ treatment (Fig. 3; compare lanes 6 to 10 with lanes 12 to 16). After 24 h of IFN treatment, we found about 30 *MxA* mRNA molecules per cell and about 2 to 3 *MxB* mRNA molecules per cell. Thus, in HFL cells treated with IFN-γ, maximal *MxA* and *MxB* mRNA levels were about 15-fold lower than in cells treated with IFN-α_2.

Cells in which protein synthesis was blocked with CHX still responded to IFN-γ by activation of *Mx* genes (Fig. 3, lanes 20), indicating that this activation is a bona fide effect of IFN-γ and is not likely to be an indirect effect of IFN-α, IFN-β, or other inducers that might be synthesized in IFN-γ-treated HFL cells.

Activation of *MxA* and *MxB* genes by virus. We tested whether infection of HFL cells with NDV caused an increase in the expression of the human *Mx* genes. To block the production of virus-induced IFN, we treated HFL cells with CHX before virus infection. At 3 h after infection, about 100 *MxA* and about 10 *MxB* mRNA molecules per cell were measured (Fig. 3, lanes 21). Thus, induction by virus of *MxA* and *MxB* gene expression does occur in HFL cells, but it is about fivefold less efficient than induction by IFN-α or IFN-β.

Nucleotide sequence of *MxA* cDNA and deduced amino acid sequence of MxA protein. The sequence of our longest cloned *MxA* cDNA is shown in Fig. 4. This cDNA contains 2,651 nt of heteropolymeric sequence followed by a poly(A) tail. A long ORF extends from an ATG initiation codon at position 211 to a TAA termination codon at position 2197. The ATG at the beginning of this ORF conforms to the consensus sequences for initiation of translation in vertebrates (15). The encoded protein, designated MxA protein, consists of 662 amino acids with a calculated molecular mass of 75,448 Da, in good agreement with its apparent molecular mass of about 76 kDa estimated by SDS-polyacrylamide gel electrophoresis (see below). MxA protein has an interesting amino acid composition: it contains 13% positively charged residues (5% arginine and 8% lysine) and 16% negatively charged residues (6% aspartic acid and 10% glutamic acid), reminiscent of murine Mx protein. A computer-assisted comparison of the predicted amino acid sequence of MxA protein with published sequences (NBRF-PIR data base, release 17) did not reveal any significant homologies.

Upstream of the beginning of the long ORF there are no additional ATG codons, but, rather, stop codons occur in all three reading frames, indicating that the cloned *MxA* cDNA contains the complete protein-coding sequence and most of the 5′ untranslated region of *MxA* mRNA. The 3′ untranslated region of *MxA* mRNA consists of about 450 nt and includes the polyadenylation signal sequence AATAAA.

Nucleotide sequence of *MxB* cDNA and deduced amino acid sequence of MxB protein. The sequence of our longest cloned *MxB* cDNA (Fig. 5) consists of 2,961 nt of heteropolymeric sequence followed by a poly(A) tail. A long ORF extends from positions 105 to 2249, encoding a putative protein of 715 amino acids with a calculated molecular mass of 81,994 Da. The nucleotide sequence surrounding the first ATG codon of the ORF does not conform to the consensus sequence for initiation of translation in vertebrates (15), suggesting that translation of *MxB* mRNA might initiate at an ATG codon that is located further downstream. Only the fifth ATG codon of the ORF at position 350 occurs in nucleotide surroundings consistent with a typical start site for translation. Initiation at this alternative position would yield a polypeptide of 633 amino acids, with a calculated molecular mass of 72,445 Da. Western blot analysis (see below) revealed that MxB protein present in IFN-treated T98G cells has an apparent molecular mass of about 73 kDa, rather than the expected 82 kDa if the 5′-proximal ATG codon were used to initiate translation. A computer-assisted comparison of the predicted amino acid sequence of MxB protein with published sequences (PIR data base, release 17) did not reveal any significant homologies.

The 3′ untranslated region of *MxB* mRNA consists of about 700 nt, including two copies of the polyadenylation signal sequence AATAAA. These copies are located 28 and about 300 nt from the poly(A) tail. For the reasons discussed above, we believe that the 2.8- and 3.1-kb *MxB* mRNAs observed in IFN-treated HFL cells result from alternative polyadenylation at these two sites.

Detection of MxA and MxB proteins with antibodies to β-galactosidase–Mx fusion proteins. We produced β-galactosidase–Mx fusion proteins containing the MxA or MxB protein fragment indicated in Fig. 1 (for details see Materials and Methods) and immunized BALB/c mice with the gel-purified fusion proteins. Hyperimmune sera from these mice were used to stain Western blots prepared from extracts of T98G cells treated with IFN-α_2 (Fig. 6). T98G rather than HFL cells were used for these experiments because large amounts of both *MxA* and *MxB* mRNAs were found in IFN-α_2-treated T98G cells (data not shown); we therefore expected that sufficient levels of the respective Mx proteins might be present within IFN-treated T98G cells to permit detection by Western analysis.

Sera from mice immunized with the MxA fusion protein detected an IFN-induced protein of about 76 kDa (Fig. 6A), roughly corresponding to the MxA protein size predicted from its cDNA sequence. Sera from mice immunized with the MxB fusion protein detected mainly an IFN-induced protein of about 73 kDa (Fig. 6B). A faint signal of a slightly larger protein was also detectable. To demonstrate that the observed signals resulted from specific detection of MxA and MxB proteins by the respective antisera, we stained a Western blot with a mixture of anti-MxA and anti-MxB sera. Two major IFN-induced proteins of similar but distinct migration properties were detectable (Fig. 6C), excluding the formal possibility that both sera detected the same IFN-induced Mx protein. We concluded that the antisera used exhibited a rather high degree of specificity for MxA and MxB proteins, respectively.

The major form of MxB protein synthesized by mouse 3T3 cells transfected with an expression plasmid containing the cloned *MxB* cDNA migrated on SDS-gels at 73 kDa (data not shown). This result suggested that the cloned *MxB* cDNA was derived from a representative *MxB* mRNA molecule and further supported the notion that the fifth ATG codon of the ORF might frequently serve as translation start site.

To determine the intracellular location of MxA and MxB proteins, we used indirect immunofluorescence to analyze T98G and HFL cells before and after treatment for 18 h with IFN-α_2. Antibodies to MxA protein and antibodies to MxB protein predominantly stained the cytoplasm of IFN-treated cells; no significant nuclear staining was observed (data not shown).

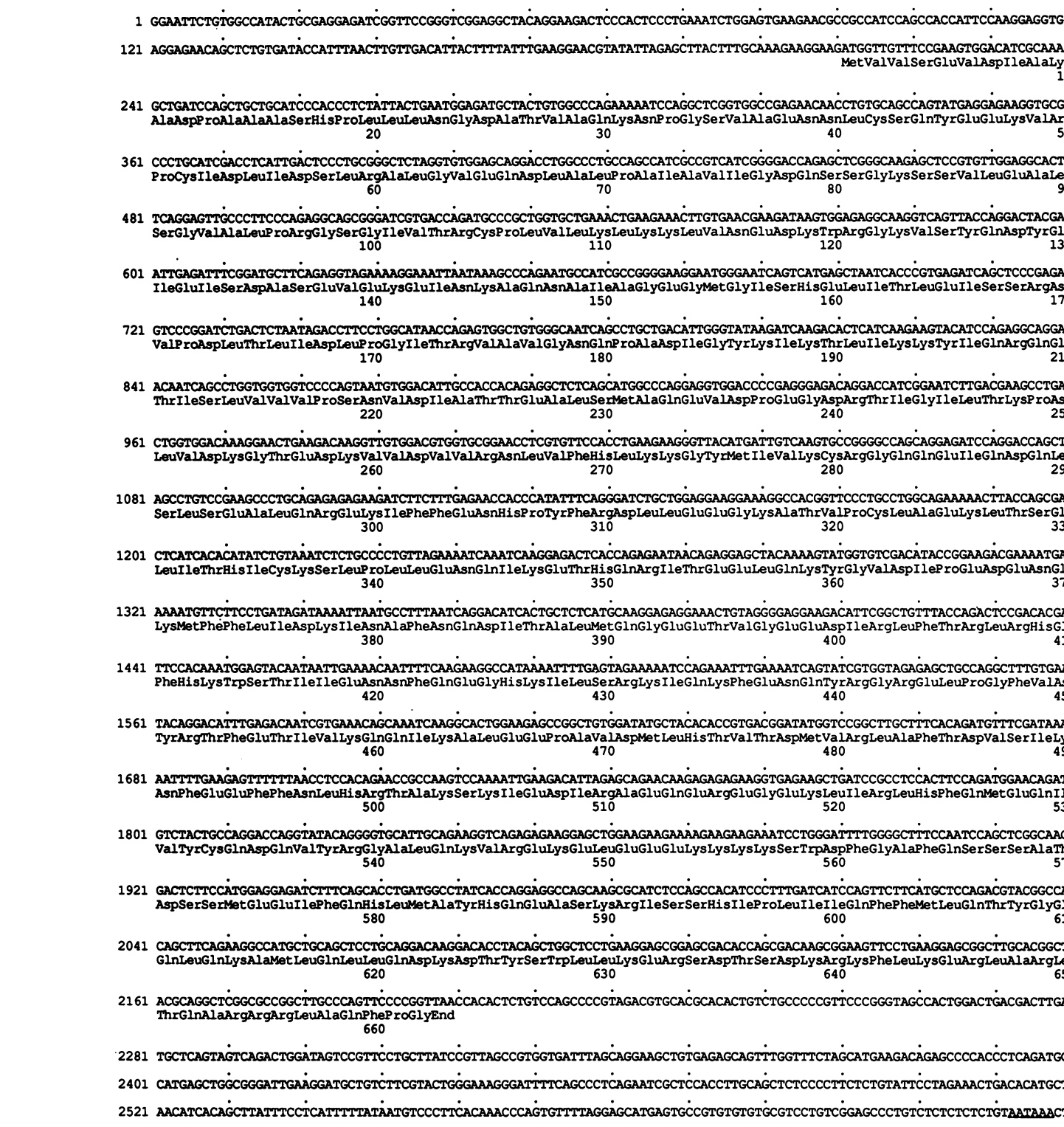

FIG. 4. Primary structure of *MxA* mRNA. The heteropolymeric nucleotide sequence of the longest *MxA* cDNA clone and the deduced amino acid sequence of MxA protein are shown. The polyadenylation signal sequence AATAAA is underlined.

Comparison of human, mouse, and fish Mx proteins. A comparison of all published vertebrate Mx protein sequences is shown in Fig. 7. The predicted sequences of the human MxA and MxB proteins, and those of hypothetical murine Mx2 and fish Mx-like proteins, were aligned for the best fit with mouse Mx protein (Mx1). The similarities among the five sequences are particularly striking in the regions that correspond to the amino-terminal moiety of

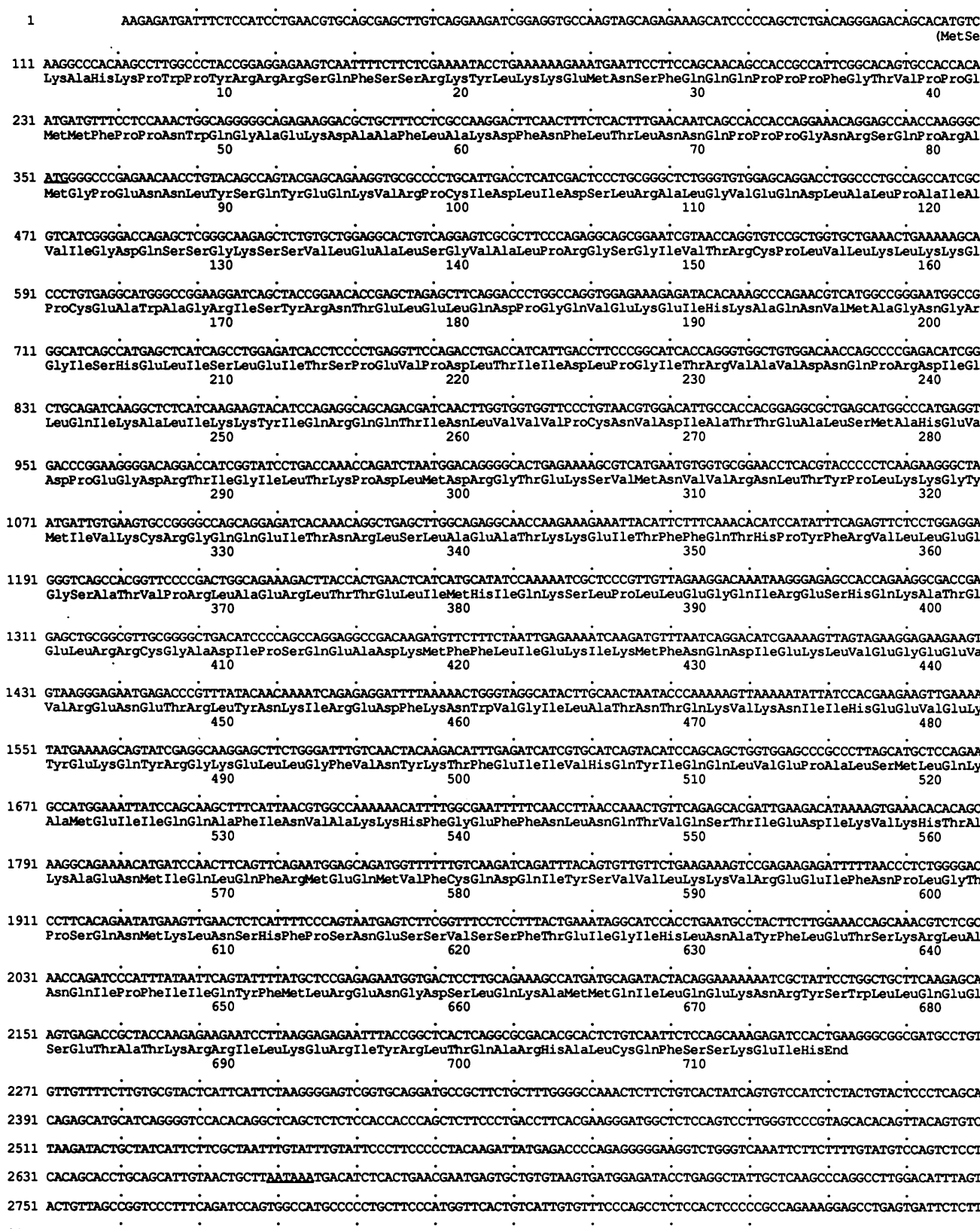

FIG. 5. Primary structure of *MxB* mRNA. The heteropolymeric nucleotide sequence of the longest *MxB* cDNA clone and the deduced amino acid sequence of MxB protein are shown. The two copies of the polyadenylation signal sequence AATAAA are underlined. The ATG codon proposed to serve as the translation start site is underlined, and the polypeptide sequence preceeding this start codon is shown in parentheses.

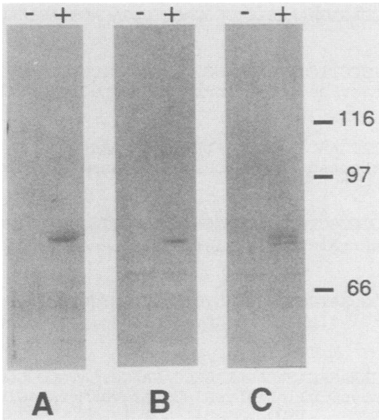

FIG. 6. Detection of MxA and MxB proteins on Western blots. T98G cells were grown for 18 h in IFN-free medium (−) or medium containing 1,000 U of IFN-α_2 per ml (+) before total cell lysates were prepared and 100-μg aliquots were loaded into individual lanes of an SDS–8% polyacrylamide gel. Mouse hyperimmune sera were used to stain the Western-blotted Mx proteins. (A) Serum to β-galactosidase-MxA fusion protein; (B) serum to β-galactosidase-MxB fusion protein; (C) 1:1 mixture of the two sera. Relative positions of protein size markers are indicated.

mouse Mx protein, encoded by *Mx1* exons 3 to 8 (13). In this region, the four mammalian Mx proteins contain a stretch of 53 identical amino acids. Conservation of the sequence of this particular stretch of amino acids is also remarkably high in the fish Mx-like protein (33), with 49 of these 53 amino acids being identical.

To learn more about the relationships of the two mouse and the two human Mx protein sequences, we estimated the degrees of sequence similarity by simply calculating the numbers of identical amino acids at corresponding positions, limiting the analysis to the 631 amino acids of mouse Mx1 protein (Fig. 7). By this analysis, the human MxA and mouse Mx2 protein are the most closely related, showing 77% sequence identity (Fig. 8), whereas the two human proteins are only 63% identical. The two mouse proteins are very similar at their N termini, but the overall identity of these two proteins is only 73%. Human MxB and mouse Mx1 protein are the least closely related, showing only 56% sequence identity.

DISCUSSION

Using murine *Mx1* cDNA as a hybridization probe, we have identified two classes of human *Mx*-related cDNAs. Sequence analysis revealed that the corresponding mRNAs must originate from two distinct human *Mx* genes, which we have designated *MxA* and *MxB*. A second *Mx* gene has recently been identified in the mouse system (32). In bovines, Mx-related proteins are most probably encoded by more than one gene (9), and there is evidence for three *Mx*-related genes in the rat (19). Thus, small *Mx* gene families are present in the genomes of all mammals studied to date.

Expression of the two human *Mx* genes is under tight control by IFN, like expression of the *Mx* genes of mice (28), rats (19), and cattle (9). In uninduced cells, *Mx* genes are not expressed at detectable rates; however, cells exposed to IFN-α, IFN-β, or NDV rapidly accumulate as many as a few hundred *Mx* transcripts per cell. IFN-γ is a less potent inducer of *Mx* genes in all systems that were studied (6, 19, 30).

MxA protein is most probably identical to the previously

```
                                                                                                       Exon 2 <--> Exon 3
mu Mx1                                                                                          MDSVNNLCRHYEEKVRPCIDLIDTLRALGVEQDLALPA   38
mu Mx2                                                         MVLSTEENTGVDSVNLPSGETGLGEKDQE......SQ.............S..............   65
hu MxA                                                MVVSEVDIAKADPAAASHPLLLNGDATVAQKNPGSVAE....SQ.............S..............   72
hu MxB  MSKAHKPWPYRRRSQFSSRKYLKKEMNSFQQQPPPFGTVPPQMMFPPNWQGAEKDAAFLAKDFNFLTLNNQPPPGNRSQPRA.GPE...YSQ..Q..........S..............  120
fish                                                                                       ITM.T.NQQ.............S..S...K......

                              Exon 3 <--> Exon 4                                Exon 4 <--> Exon 5
mu Mx1  IAVIGDQSSGKSSVLEALSGVALPRGSGIVTRCPLVLKLRKLKEGEEWRGKVSYDDIEVELSDPSEVEEAINKGQNFIAGVGLGISDKLISLDVSSPNVPDLTLIDLPGITRVAVGNQPA  158
mu Mx2  ..........................................N.............................................................................  185
hu MxA  .......................................K..VNEDK.......Q.Y.I.I..A...KE...A..A...E.M...HE..T.EI..RD.......................  192
hu MxB  .......................................K.-QPC.A.A.RI..RNT.L..Q..GQ..KE.H.A..VM..N.R...HE....EIT..E....I...........D...R  239
fish    ...................................E..MKRRQ.....Y..I..Q.C.K.IED.AD..KK.RDA.DEM....V....D....EIA..D............A....KG..E

 Exon 5 <--> Exon 6                         Exon 6 <--> Exon 7                                                      Exon 7 <-->
mu Mx1  DIGRQIKRLIKTYIQKQETINLVVVPSNVDIATTEALSMAQEVDPEGDRTIGVLTKPDLVDRGAEGKVLDVMRNLVYPLKKGYMIVKCRGQQDIQEQLSLTEAFQKEQVFFKDHSYFSIL  278
mu Mx2  ....................................................I.........T.D..V..V....H...........................L.N..I...E.PH.RV.  305
hu MxA  ...YK..T...K...R....S...............................I.......K.T.D..V..V...FH...............E..D....S..L.R.KI..EN.P..RD.  312
hu MxB  ...L...A...K...R.Q........C.............H...........I......M..T.KS.MN.V....T................E.TNR...A..TK..IT..QT.P..RV.  359
fish    N..D......QKF.KR....S.....C..........K.......D.E..L.I.......K.T.ET.V.IVH.E.I..........R...K..TDKV......IER.KA..N..VH.GT.

   Exon 8               Exon 8 <--> Exon 9                          Exon 9 <--> Exon 10                           Exon 10 <--> Exon 11
mu Mx1  LEDGKATVPCLAERLTEELTSHICKSLPLLEDQINSSHQSASEELQKYGADIPEDDRTRMSFLVNKISAFNRNIMNLIQAQETVSEGDSRLFTKLRNEFLAWDDHIEEYFKKDSPEVQSK  398
mu Mx2  ................A..I...........N..KE.............M......SEKTF..IE..N...QD.TA.V.GE.N.A..EC...R..K...S.SKE..KN.E.GYAVLYNE  425
hu MxA  ..E..........K..S..IT..........N..KET..RIT.......V.....ENEK.F..ID..N...QD.TA.M.GE...G.E.I...R..H..HK.STI..NN.QEGHKILSR.  432
hu MxB  ..E.S...R.......T..IM..Q.......G..RE...KAT...RRC.....SQEADK.F..IE..KM..QD.EK.VEGE.V.R.NET..YN.I.ED.KN.VGILATNTQ.VKNIIHEE  479
fish    YND.H....K...K..L..VH..E

                                                Exon 11 <--> Exon 12       Exon 12 <--> Exon 13
mu Mx1  MKEFENQYRGRELPGFVDYKAFESIIKKRVKALEESAVNMLRRVTKMVQTAFVKILSNDFGDFLNLCCTAKSKIKEIRLNQEKEAENLIRLHFQMEQIVYCQDQVYKETLKTIREKEAEK  518
mu Mx2  VWA..K...........N..T..N..RRQI.T...P.IE..HT..EI.RA..TSVSEKN.SE.Y..HR.T...LED...E......MS.....K....I.....I.RGA.QKV..E...E  545
hu MxA  IQK..............N.RT..T.V.QQI.....P..D..HT..D..RL..TDVSIKN.EE.F..HR......ED..AE..R.G.K...................RGA.QKV....L.E  552
hu MxB  VEKY.K....K..L...N..T..I.VHQYIQQ.V.P.LS..QKAMEII.Q..INVAKKH..E.F..NQ.VQ.T.ED.KVKHTAK...M.Q.Q.R...M.F....I.SVV..KV..EIFNP  599

                                  Exon 13 <--> Exon 14
mu Mx1  EKTKALINPATFQNNSQFPQKGLTTTEMTQHLKAYYQECRRNIGRQIPLIIQYFILKTFGEEIEKMMLQLLQDTSKCSWFLEEQSDTREKKKFLKRRLLRLDEARQKLAKFSD         631
mu Mx2  ..KTKHGTSSSS.SQDLQT---SSMA.IF...N..R..AHNR.SSHV..........M.A.RLQ.G.......KDS...L.K.....S..R....E..A..AQ..RR....PG         655
hu MxA  ..K.KSWDFGA..SS.ATD---SSME.IF...M..H..ASKR.SSH......F.M.Q.Y.QQLQ.A.......KDTY..L.K.R...SD.R....E..A..TQ..RR..Q.PG         665
hu MxB  LG.PSQNMKLNSHFP.NESSV-SSF..IGI..N..FL.TSKRLAN...F.....M.RENGDSLQ.A.M.I..EKNRY..L.Q...E.AT.RRI..E.IY..TQ..HA.CQ..SKEIH    716
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FIG. 7. Comparison of amino acid sequences of murine Mx protein (mu Mx1 [31]), hypothetical murine Mx2 protein (mu Mx2 [32]), human MxA protein (hu MxA), human MxB protein (hu MxB), and putative fish Mx protein (fish [33]). The sequences are aligned for the best fit relative to murine Mx1 protein (top sequence). Dots indicate the presence of identical amino acids at corresponding positions in murine Mx protein and in the Mx-related protein. The locations of the exon borders in the murine *Mx1* gene are indicated.

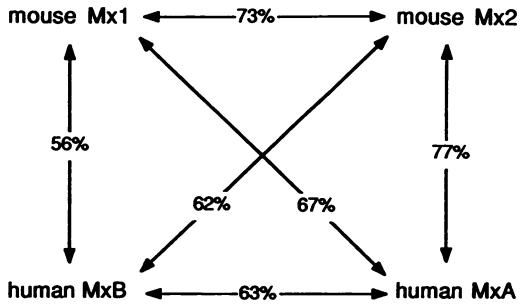

FIG. 8. Schematic presentation of relationships of mouse and human Mx proteins. The percentages of identical amino acids at corresponding positions are indicated. The calculations are based on the alignment shown in Fig. 7.

described IFN-induced human protein that is recognized by anti-mouse Mx protein monoclonal antibody 2C12 (29). Both proteins have molecular masses of about 76 kDa, and both are accumulated to very high levels in the cytoplasm of HFL cells and T98G cells treated with IFN-α_2. Furthermore, expression of *MxA* but not *MxB* cDNA in transfected mouse cells gives rise to a 2C12 monoclonal antibody-reactive protein (J. Pavlovic, unpublished results). Finally, the Mx protein purified from IFN-treated human foreskin fibroblasts by immunoaffinity chromatography with antibody 2C12 has the same amino-terminal sequence (G. Weitz, J. Bekisz, K. Zoon, and H. Arnheiter, J. Interferon Res., in press) as we predicted for MxA protein.

MxA protein is probably also identical to the IFN-induced p78 (IFI-78K) protein described by Horisberger and Hochkeppel (11). In addition to having similar molecular masses, the two proteins show the same intracellular location and are both induced by IFN-α and to a much lesser extent by IFN-γ (6). Furthermore, the restriction maps of *MxA* cDNA (Fig. 1) and *IFI-78K* cDNAs (12) are very similar, and the predicted N-terminal sequence of MxA protein is identical to that of p78 (M. Horisberger, personal communication). Horisberger et al. (12) have shown that the *IFI-78K* gene maps to human chromosome 21. We will show elsewhere that *MxA* and *MxB* both map to chromosome 21 (P. Huber and O. Haller, unpublished results).

MxB is probably a novel protein. In diploid fibroblasts (Fig. 3) or peripheral blood lymphocytes (data not shown) treated with IFN-α_2, the steady-state levels of *MxB* mRNA are 3 to 10 times lower than those of *MxA* mRNA. MxB protein concentrations in these cells are marginally high enough to permit detection by our anti-MxB sera. The cell line T98G is exceptional in that it responds to IFN-α_2 by synthesizing large amounts of both *MxA* and *MxB* mRNAs. In these cells, MxB protein is easily detectable by the Western blotting technique. In IFN-treated T98G cells as well as in mouse cells transfected with appropriate *MxB* cDNA constructs (J. Pavlovic, unpublished results), MxB protein accumulates in the cell cytoplasm. Thus, both human Mx proteins are confined largely to the cytoplasm. This is in marked contrast to the observations with rodent cells, in which Mx proteins are found in the nuclei of IFN-treated cells (5, 19). We have searched for additional human *Mx* genes that might encode a nuclear Mx protein, but have failed to find evidence for them; all cDNA clones with *Mx*-related sequences that we isolated proved to be derived from either *MxA* or *MxB* mRNAs (data not shown).

Western blot analysis revealed that MxB protein present in IFN-treated T98G cells has an apparent molecular mass of about 73 kDa, although the ORF of the cloned *MxB* mRNA predicts a 715-amino-acid protein with a calculated molecular mass of about 82 kDa. One possible explanation of this difference would be that MxB protein has aberrant migration properties on SDS-polyacrylamide gel electrophoresis. Another possibility would be that the 82-kDa translation product of *MxB* mRNA is a precursor of the mature 73-kDa MxB protein. We favor a third possibility, that translation of *MxB* mRNA does not initiate at the first ATG codon of the long ORF but rather at the fifth ATG codon, yielding a protein of about 72.5 kDa. Indeed, the −3/+4 flanking nucleotide context of the ATG codon at the beginning of the ORF is not optimal for translation initiation (15): CACATGT is considered to be a very poor initiation signal. By contrast, the fifth ATG codon of the ORF has a favorable −3/+4 flanking nucleotide context, GCAATGG. Initiation of translation at a downstream ATG codon is relatively rare in vertebrates, but has been demonstrated for a limited number of cellular and viral mRNAs (see references 15 and 26 for a review). Direct sequencing of the amino terminus of MxB protein is necessary to definitively establish its primary structure.

The Mx proteins of humans and mice have overall similarities of between 56 and 77%. A pairwise comparison reveals that the human MxA and the mouse Mx2 are most closely related proteins (Fig. 8). MxA protein shows a much lower degree of similarity to the human MxB and the mouse Mx1 proteins. This indicates that human MxA and mouse Mx2 proteins are encoded by homologous genes. However, MxB protein is probably not the true counterpart of mouse Mx1 protein, mainly for two reasons: MxB and mouse Mx1 proteins show a rather low degree of sequence similarity, and MxB is a cytoplasmic protein, whereas mouse Mx1 is a nuclear protein (5). It will be interesting to learn about the relationships of the two human Mx proteins to the two cytoplasmic rat proteins (19).

We have recently managed to establish several stable lines of transfected 3T3 cells that express high levels of either MxA or MxB protein in a constitutive manner. We will show elsewhere (J. Pavlovic, T. Zürcher, and P. Staeheli, submitted for publication) that transfected cell lines expressing human MxA protein have acquired a high degree of resistance to influenza virus, whereas cells expressing MxB protein do not show increased influenza virus resistance.

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