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Organization of the Murine *Mx* Gene and Characterization of Its Interferon- and Virus-Inducible Promoter

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Specific resistance of Mx^+ mice to influenza virus is due to the interferon (IFN)-induced protein Mx. The *Mx* gene consists of 14 exons that are spread over at least 55 kilobase pairs of DNA. Surprisingly, the *Mx* gene promoter is induced as efficiently by Newcastle disease virus as it is by IFN. The 5′ boundary of the region required for maximal induction by both IFN and Newcastle disease virus is located about 140 base pairs upstream of the cap site. This region contains five elements of the type GAAANN, which occurs in all IFN- and virus-inducible promoters. The consensus sequence purine-GAAAN(N/−)GAAA(C/G)-pyrimidine is found in all IFN-inducible promoters.

Cells infected with a virus are stimulated to produce and secrete interferons (IFNs), which in turn induce a complex pattern of physiological changes, including establishment of an antiviral state in as yet uninfected cells. Cells treated with IFN synthesize some new proteins, and the levels of many other proteins are increased (60). Several cDNAs and genes corresponding to IFN-induced mRNAs have been isolated and characterized (for a review, see reference 44). In most cases it is not clear what role, if any, the induced proteins play in the antiviral state.

In this regard the IFN-induced Mx protein of the mouse, which confers selective resistance to influenza virus, is an exception (2, 3, 20, 21, 32, 54). Cells of influenza virus-resistant Mx^+ mice synthesize the nuclear 72-kilodalton Mx protein in response to treatment with type I IFN, while cells of influenza virus-susceptible Mx^- mice fail to do so (10, 23, 52, 54). A full-length cDNA encoding Mx protein was cloned and constitutively expressed in Mx^- cells. These cells resisted infection with influenza virus but were still susceptible to infection with vesicular stomatitis virus, a rhabdovirus, demonstrating that Mx protein is the only IFN-induced product required for resistance to influenza virus (53). Transfected nonmurine cells that constitutively express *Mx* cDNA are also resistant to influenza virus (41).

Little is known about the mechanism of gene activation by IFNs. The IFN receptors are only poorly characterized, and nothing is known about the mechanism of intracellular signal transmission. The structure of several IFN-responsive promoters such as metallothionein (27), $H\text{-}2K^b$ (28), $H\text{-}2D^d$ (29), $H\text{-}2L^d$ (56), HLA-A3 (55), HLA-DRα (8), an HLA class I gene (35), 202 (46), 56 K (58), 6-16 (42), IP-10 (33), ISG15 (43), ISG54 (31), and 2′,5′-oligo(A) synthetase (5) have been reported. A comparison of the regions upstream of the metallothionein and two HLA genes revealed a homologous sequence of about 30 base pairs (bp); it has been proposed (13, 56) that this so-called Friedman-Stark consensus sequence is involved in regulating transcription of IFN-responsive genes.

Here we show by analysis of cloned chromosomal DNA that the murine *Mx* gene consists of 14 exons that are distributed over at least 55 kilobase pairs (kbp) of DNA. A 5′-flanking DNA segment of about 450 nucleotides was found to contain the *Mx* promoter by fusing it to the rabbit β-globin transcription unit and showing that it mediates inducibility not only by IFN-α but also directly by virus. The 5′ boundary of the region required for maximal induction by either inducer is located about 140 bp upstream of the cap site, as determined by deletion analysis. Stepwise truncation showed that at least two distinct regions contribute to inducibility by both IFN and virus. Some sequence elements present in the Friedman-Stark consensus sequence were dispersed in the *Mx* gene promoter; however, there was no sequence similar to the consensus sequence as a whole.

MATERIALS AND METHODS

Primer elongation analysis of *Mx* mRNA. The 5′ ^{32}P-labeled oligonucleotide MxII (0.1 pmol; specific activity, 5×10^6 cpm/pmol; Fig. 1) was hybridized to 1 μg of poly(A)$^+$ RNA from BALB.A2G-*Mx* embryo fibroblasts that were either uninduced or induced for 3 h with 1,000 U of murine type I IFN (10^7 reference units per mg; LEE Biomolecular, San Diego, Calif.) per ml, as described previously (51). Primer elongation was with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (18). The products were separated on an 8% denaturing polyacrylamide gel (36) and sequenced individually (38).

Construction and screening of genomic mouse libraries. (i) Cosmid library. DNA of a BALB.A2G-*Mx* mouse was partially digested with *Mbo*I, and fragments of about 40 kb were cloned into the *Bam*HI site of cosmid pOPF1 (19). The resulting library was screened under stringent conditions with the nick-translated, 1.7-kb *Bam*HI fragment of pMx41 cDNA (53). One cosmid (8B) containing genomic *Mx* sequences was isolated. The second exon was identified by sequencing.

(ii) Lambda library. DNA of a BALB.A2G-*Mx* mouse was partially digested with *Mbo*I, *Bgl*II, and *Bam*HI. The purified reaction products were mixed, and 20-kb fragments were isolated from a sucrose gradient and cloned into the *Bam*HI site of lambda EMBL3 (14). To search for the first *Mx* exon, a 5′ ^{32}P-labeled, synthetic oligonucleotide consisting of the first 27 nucleotides of the longest sequenced *Mx* transcript (Fig. 1b) was used as a probe. Prehybridization and hybridization were done in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM sodium phosphate

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(pH 6.8)–5× Denhardt solution–0.1 mg of denatured, sonicated salmon sperm DNA per ml–0.2% sodium dodecyl sulfate at 37°C. The final wash was in 3.0 M tetramethylammonium chloride–50 mM Tris hydrochloride (pH 8.0)–2 mM EDTA–0.1% sodium dodecyl sulfate at 66°C (62). To search for the 3′ end of the *Mx* gene, the lambda library was screened with a nick-translated, 5.6-kb *Bam*HI fragment (devoid of repetitive sequences) from the 3′ end of cosmid 8B (Fig. 2b). Five positive clones (1-1, 6-2, 12-1, 12-2, 20-2) were isolated.

Mapping of lambda and cosmid clones. Restriction maps of the genomic clones were made (49), and the clones were characterized by Southern blotting (50) with the following *Mx* cDNA fragments: a 5′ probe (*Bam*HI-*Taq*I fragment; positions 1 to 278; numbering is as described previously [53]), a middle probe (*Pst*I-*Bam*HI fragment; positions 686 to 2314), and a 3′ probe (*Bam*HI-*Bam*HI; positions 2314 to 3218), or in the case of lambda MF4, the 5′ oligonucleotide described above.

Subcloning. The *Bam*HI fragments of cosmid 8B were subcloned into pSP65, and the *Hin*dIII fragments were subcloned into pSP64 (39). The *Hin*dIII, *Hin*dIII-*Sal*I, *Eco*RI, and *Eco*RI-*Sal*I fragments of lambda MF4 (the *Sal*I site was from the EMBL3 polylinker) were subcloned into pSP64. DNA from lambda clones 12-1 and 20-2 (which hybridized to the 3′ moiety of *Mx* cDNA) and the lambda clones 1-1, 6-2, and 12-2 (which hybridized to the 5′ moiety of the *Mx* cDNA) were combined in two pools and cut either separately with *Alu*I, *Hae*III, *Rsa*I, and *Mnl*I and mixed after cutting or with *Mbo*I only. In another approach, a sample from each pool was cleaved with DNase I (1). All fragments other than those generated by *Mbo*I were blunted with T4 DNA polymerase, and nicks were repaired with *Escherichia coli* ligase (1). DNA fragments of about 300 nucleotides were isolated from agarose gels and joined to Bluescript (Stratagene) that was cleaved with *Bam*HI and *Sma*I.

Sequencing. The 5′ and 3′ pools of the subcloned 300-nucleotide fragments were screened with 5′ and 3′ probes of the *Mx* cDNA, respectively, and both pools were screened with middle fragments of *Mx* cDNA. Single- and double-stranded DNAs were prepared from positive clones and sequenced by the Sanger method, using Sequenase (United States Biochemical Corp.); the protocol of the manufacturer was used.

Truncation analysis of the *Mx* promoter: constructions. For the construction of pSP65-rβgl, the *Pvu*II site at position −10 of the rabbit β-globin gene was converted into an *Xba*I site (59), and the *Xba*I-*Bam*HI fragment containing the upstream half of the β-globin gene was joined in a three-way ligation to the *Bam*HI-*Sal*I fragment of plasmid 20P (which contains the downstream half of the β-globin gene [30]) and to pSP65 (39) that was cleaved with *Xba*I and *Sal*I. For the construction of pSP64-Mxp(Eco-Pvu), the 2.3-kb fragment with the *Mx* promoter, extending from *Pvu*II (position +1; Fig. 3) to the *Eco*RI site (Fig. 2b) was cloned into pSP64 that was cleaved with *Eco*RI and *Sma*I. For *Mx* promoter truncation constructions, pSP64-Mxp(Eco-Pvu) was cleaved with *Bgl*II, and the ends were subjected to digestion with *Bal* 31 (36). After the addition of *Pst*I linkers and cleavage with *Pst*I and *Xba*I, fragments of about 40 to 450 nucleotides were isolated from agarose gels. These fragments were joined in a three-way ligation to the rabbit β-globin transcription unit excised from pSP65-rβgl with *Xba*I and *Hin*dIII (Fig. 4) and to the large *Hin*dIII-*Pst*I fragment (vector moiety) of pSP64-Mxp(Eco-Pvu). The *Mx* promoter deletions were characterized by sequencing (38). For the construction of pBlue-Mxp-rβglcDNA, the *Pst*I-*Xba*I fragment of the *Mx* promoter was isolated from pSP64-Mxp(Eco-Pvu). An *Xba*I linker (dCTC TAGAG) was inserted into the *Pvu*II site of the rabbit β-globin promoter-cDNA fusion plasmid RβGA4 (45), and the *Xba*I-*Bam*HI fragment (containing the β-globin cap site and the upstream moiety of the β-globin cDNA) was isolated. The *Bam*HI-*Eco*RI fragment of rabbit β-globin cDNA was isolated from plasmid pβG (11). These three fragments were cloned into Bluescript (minus) (Stratagene) that was cut with *Pst*I and *Eco*RI. The resulting plasmid contained the *Mx* promoter joined to the β-globin cDNA in the same way as it was joined to the β-globin gene in pSP64-Mxp(Eco-Pvu) and was used to prepare the probe for S1 nuclease mapping.

DNA transfection and induction. Murine L929 cells were transfected with 30 μg of test DNA and 10 μg of reference DNA by the DEAE-dextran method (17). The reference gene has been described by Ryals et al. (45) and is inducible by the Newcastle disease virus (NDV). Cells were induced after 48 h with 500 U of recombinant murine IFN-α2 per ml or with egg-grown, partially purified NDV (0.5 hemaglutinin U/ml; this amount has been found to be sufficient for the maximal induction of IFN under our experimental conditions [37]) in the presence of 320 U of polyclonal anti-murine IFN antibodies (kindly provided by I. Gresser) per ml. Less than 1 to 3 U of IFN activity per ml (as measured in a cytopathic assay on L cells) was measurable in the supernatants 8 h after induction by NDV. In other experiments protein synthesis was inhibited with 75 μg of cycloheximide per ml, which was added 1 h before induction and which was present until the cells were harvested.

RNA preparation and S1 nuclease mapping. Total RNA was prepared 8 h after induction or mock induction (4). To prepare the radioactive probe, the 5′ ^{32}P-labeled oligonucleotide 5′-GGATCCACGTGCAGC-3′ (which is complementary to β-globin cDNA between positions 352 and 367 in the second exon; specific radioactivity, 5×10^6 cpm/pmol) was hybridized to single-stranded pBlue-Mxp-rβglcDNA and elongated by using Klenow DNA polymerase (New England Biolabs) and unlabeled deoxynucleoside triphosphates. The product was cut at the *Cla*I site in the *Mx* promoter, and the 5′ ^{32}P-labeled, 549-nucleotide, single-stranded fragment was isolated from a denaturing polyacrylamide gel and purified (see Fig. 5a). Total RNA (usually 40 μg) was hybridized to the probe and subjected to S1 nuclease digestion (45) by using 800 U of S1 nuclease per ml. After polyacrylamide gel electrophoresis and autoradiography, bands were excised and the radioactivity was determined by Cerenkov counting. After background subtraction, the mean radioactivity ($R_{m,y}$) of the reference band for all noninduced ($y = ni$), virus-induced ($y = vi$), and IFN-induced samples ($y = ii$) of one experiment was calculated. Test signals were normalized by setting $T_y' = (T_y \times R_{m,y})/R_y$, where T_y' is the normalized test signal, T_y is the test signal, and R_y is the reference signal in the same lane as the test signal. The cell number (C) corresponding to the amount of RNA subjected to S1 nuclease analysis was calculated by assuming that one cell contains 20 pg of RNA (30). The number of strands per cell $N = (Ty' \times 3 \times 10^6)/Cb$, where b is radioactivity (in counts per minute) recovered after S1 nuclease mapping of 1 pg of pure β-globin RNA (equivalent to 3×10^6 strands) (45).

RESULTS

Analysis of 5′ ends of the major *Mx* transcripts. To define the start site(s) of *Mx* gene transcription, we primed the

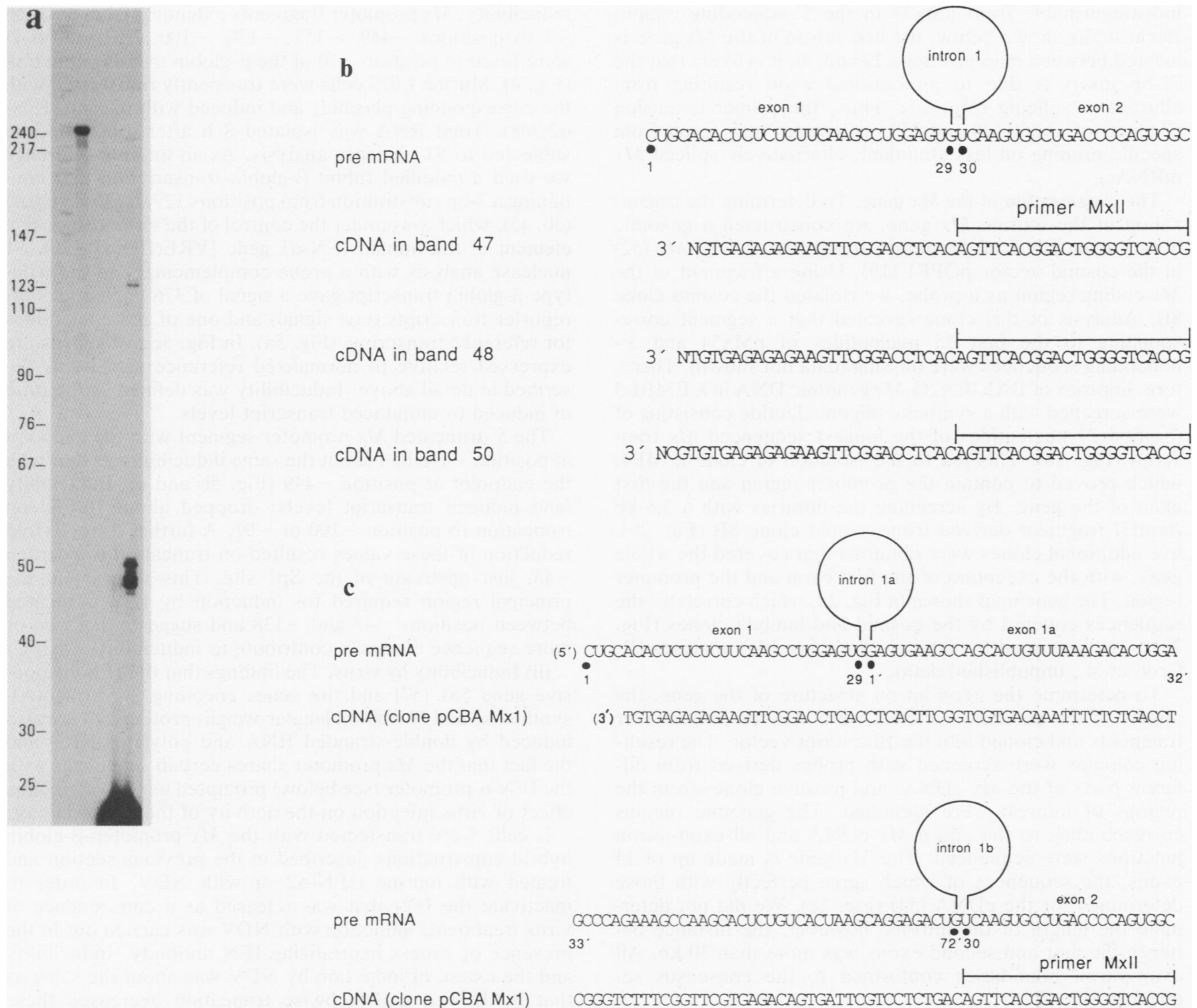


FIG. 1. Primer elongation analysis of *Mx* mRNA. (a) Poly(A)$^+$ RNA from BALB.A2G-*Mx* embryo cells treated for 3 h with 1,000 U of type I IFN per ml (+) or with nonsupplemented medium (−) was used as a template for elongation of the primer *MxII* (b and c). DNA size markers (M) were 5′ ^{32}P-labeled, *Hpa*II-digested pBR322, and a sequencing ladder of a 5′ ^{32}P-labeled 240-bp DNA fragment (obtained by pooling the A+G and C+T reactions). The major bands corresponded to products of 46 to 51 residues, and a minor band corresponded to a product of 123 nucleotides. (b) Products of 47, 48, and 50 residues were recovered from the gel and sequenced as described by Maxam and Gilbert (38). The cDNA sequences were compared with the pre-mRNA sequence deduced from the genomic *Mx* clones (see panel c). (c) The cDNA clone pCBAMx1 derived from IFN-induced cells of mouse strain CBA was isolated and sequenced. Comparison with the *Mx* pre-mRNA revealed the existence of an additional exon (exon 1a) of 72 nucleotides (1′ to 72′).

reverse transcription of *Mx* mRNA with an oligonucleotide (primer MxII) corresponding to positions 26 to 47 of the *Mx* cDNA insert of pMx34 (53) (positions 30 to 51 in Fig. 1b). This primer was efficiently extended in experiments with RNA from IFN-treated cells, but not with RNA from uninduced cells (Fig. 1a). The major cDNA products were between 46 and 51 nucleotides long. Signals corresponding to products of 47, 48, and 50 residues were about threefold stronger than those corresponding to products of 46, 49, and 51 residues, respectively. The sequence of the cDNAs in bands 47, 48, and 50 were identical, except for additional nucleotides at their 3′ ends (Fig. 1b). The common sequence, as represented in band 47, corresponded exactly to that found at the 5′ end of clone pMx34. The 3′ heterogeneity of the extension products could reflect a multiplicity of initiation sites of *Mx* mRNA, or could be artifactual because of multiple reverse transcription endpoints.

In addition to the reverse transcription products described above, weaker sets of signals corresponding to chain lengths of about 120 and 230 residues were detected. Each of these sets consisted of at least three bands that differed in length by only a few nucleotides (Fig. 1a). Because of their low abundance, the structures of the cognate cDNAs could not be determined by direct sequencing. However, in connection with other experiments, we isolated and sequenced *Mx* cDNA clones derived from IFN-induced cells of mouse strain CBA; these had a 72-bp insert between nucleotides 29 and 30 (designated 1′ to 72′; Fig. 1c) but were otherwise

indistinguishable from pMx34 in the 5′-noncoding region. Because, as shown below, the first intron of the *Mx* gene is located between map positions 29 and 30, it is likely that the 72-bp insert is due to an optional exon resulting from alternative splicing (Fig. 1c). Thus, the primer extension products of about 120 and 230 nucleotides may result from specific priming on less abundant, alternatively spliced *Mx* mRNAs.

The organization of the *Mx* gene. To determine the organization of the murine *Mx* gene, we constructed a genomic library from DNA of the mouse strain BALB.A2G-*Mx* (52) in the cosmid vector pOPF1 (19). Using a fragment of the *Mx*-coding region as a probe, we isolated the cosmid clone 8B. Analysis of this clone revealed that a segment corresponding to the first 25 nucleotides of pMx34 and 3′-noncoding sequences were missing (data not shown). Therefore, libraries of BALB.A2G-*Mx* genomic DNA in λ EMBL3 were screened with a synthetic oligonucleotide consisting of the first 27 nucleotides of the longest sequenced *Mx* transcript (Fig. 1b). This led to the isolation of clone λ MF4, which proved to contain the promoter region and the first exon of the gene. By screening the libraries with a 5.6-kb *Bam*HI fragment derived from cosmid clone 8B (Fig. 2b), five additional clones were obtained that covered the whole gene, with the exception of the first exon and the promoter region. The gene map shown in Fig. 2a, which correlates the sequences covered by the cosmid and lambda clones (Fig. 2b), was confirmed by genomic Southern blot analyses (R. Grob et al., unpublished data).

To determine the exon-intron structure of the gene, the inserts of the genomic λ clones were cleaved into smaller fragments and cloned into the Bluescript vector. The resulting colonies were screened with probes derived from different parts of the *Mx* cDNA, and positive clones from the regions of interest were identified. The genomic regions corresponding to the entire *Mx* cDNA and all exon-intron junctions were sequenced. The *Mx* gene is made up of 14 exons, the sequences of which agree perfectly with those determined for the cDNA (53) (Fig. 2c). We did not determine the length of the introns; however, the distance between the first and second exons was more than 30 kb. All exon-intron boundaries conformed to the consensus sequences (40, 47).

Structure of the 5′-flanking region of the *Mx* gene. The *Eco*RI and *Hin*dIII restriction map of clone λ MF4 is shown in Fig. 2b. Southern analysis demonstrated that the synthetic 27-nucleotide probe corresponding to positions 3 to 29 of the *Mx* mRNA shown in Fig. 1b hybridized to the 2.3-kb *Eco*RI-*Hin*dIII fragment of the insert of clone MF4. The nucleotide sequence of this region was determined (Fig. 2c and 3). The 3′ termini of the six primer elongation products shown in Fig. 1 are indicated by arrowheads. Because the cap site of *Mx* mRNA was not determined directly, we arbitrarily designated the most upstream putative initiation site as +1. The cloned chromosomal DNA fragment extended about 5.5 kb upstream of position 1. A TATA box at positions −27 to −20 and an SP1 binding site at positions −43 to −34 represented sequence elements common to many promoters. The 5′ splice region consensus sequence TG′GTGAGT marked the start of intron 1 at position 30.

Functional analysis of the *Mx* promoter. (i) Inducibility by IFN. Results of preliminary experiments showed that the 5′-noncoding segment of the *Mx* gene could be truncated from position −2300 to position −449 without reducing IFN-induced transcription or inducibility.

To localize the 5′ boundary of the region essential for inducibility, *Mx* promoter fragments extending from position −1 to positions −449, −151, −138, −100, −91, and −43 were fused to position −10 of the β-globin transcription unit (Fig. 4). Murine L929 cells were transiently transfected with the corresponding plasmids and induced with murine rIFN-α2 (48). Total RNA was isolated 8 h after induction and subjected to S1 nuclease analysis. As an internal reference we used a modified rabbit β-globin transcription unit containing a 7-bp substitution from positions 129 to 135 (IFC109) (30, 45), which was under the control of the virus-responsive element of the human IFN-α1 gene [VRE(IFNα)] (30). S1 nuclease analysis with a probe complementary to the wild-type β-globin transcript gave a signal of 376 nucleotides for reporter transcripts (test signal) and one of 218 nucleotides for reference transcripts (Fig. 5a). In Fig. 5c test signals are expressed relative to normalized reference signals, as described in detail above. Inducibility was defined as the ratio of induced to uninduced transcript levels.

The 5′-truncated *Mx* promoter segment with the endpoint at position −138 had about the same inducibility as that with the endpoint at position −449 (Fig. 5b and c). Inducibility (and induced transcript levels) dropped about 10-fold on truncation to position −100 or −91. A further 5- to 10-fold reduction of these values resulted on truncation to position −43, just upstream of the Sp1 site. This shows that the principal region required for induction by IFN is located between positions −43 and −138 and suggests that two or more sequence elements contribute to inducibility.

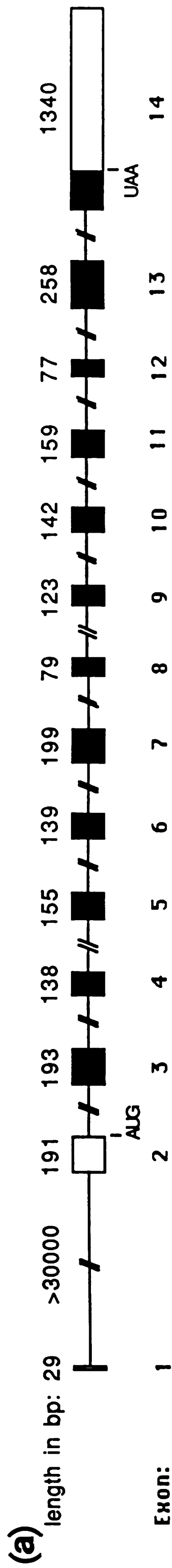
(ii) Inducibility by virus. The findings that the IFN-responsive gene 561 (57) and the genes encoding 2′-5′ oligo(A)-synthetase and 56,000-molecular-weight protein (58) are also induced by double-stranded RNA and poly(I)-poly(C) and the fact that the *Mx* promoter shares certain sequences with the IFN-α promoter (see below) prompted us to examine the effect of virus infection on the activity of the *Mx* promoter.

L cells were transfected with the *Mx* promoter–β-globin hybrid constructions described in the previous section and treated with murine rIFN-α2 or with NDV. In order to inactivate the IFN that was released as a consequence of virus treatment, induction with NDV was carried out in the presence of excess neutralizing IFN antibody. Inducibility and the extent of induction by NDV was about the same as that by IFN-α, and stepwise truncation decreased these parameters in parallel (Fig. 5c). Thus, at least at the level of resolution of this truncation analysis, the elements responsive to virus and IFN could not be resolved.

In one experiment, induction with NDV was carried out in the presence of 75 μg of cycloheximide per ml in order to prevent synthesis of IFN and to eliminate the possibility of intracellular induction by IFN. Cells that were induced with NDV in the presence of cycloheximide had 1,250 strands per cell; cells induced with IFN alone had 2,200 strands per cell compared with 20 strands per cell in the case of cells that were mock induced. Thus, induction by NDV does not come about via the action of IFN.

DISCUSSION

Structure of the murine *Mx* gene. Our analysis of the murine Mx^+ allele revealed that the *Mx* gene extends over 55 kb or more of the genomic sequence and contains 13 introns. The first intron was at least 30 kb long and separated a 5′-noncoding exon of only 29 bp from the second exon, which contained the remainder of the 5′-noncoding sequence and only the initiator triplet of the coding region. The *Mx*-coding region was interrupted by 12 more introns. The



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193
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155
139
199
79
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159
77
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UAA
Exon: 1
2
3
4
5
6
7
8
9
10
11
12
13
14
(b)
exon 1
intron 1
exon 2
exons 3-9
exon 10
exon 11
exons 12-13
exon 14
20-2
12-1
1-1
6-2
12-2
MF4
Cosmid 8B
probe 5.6 kb
10 kb

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LeuSerGlyValAlaLeuProArgGlySerG(66)
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cttttgtcaagttgatataaaactagcacagatgggaagaagccttgtgctgctggtggccagtgagagtggtgtttctcactgccctaggattgtgtgt
                              (210) lyValLeuThrLysProAspLeuValAspArgGlyAlaGluGlyLysValLeuAspValMetArgAsnLeu
gtcctctgatctcaatggctgatctacagGGGTCTTGACCAAGCCTGATCTGGTGGACAGAGGTGCTGAAGGCAAGGTCTTGGATGTGATGCGGAACCTG
ValTyrProLeuLysLysGlyTyrMetIleValLysCysArgGlyGlnGlnAspIleGlnGluGlnLeuSerLeuThrGluAlaPheGlnLysGluGlnV
GTGTATCCTCTGAAGAAGGGCTACATGATAGTCAAGTGCAGAGGTCAGCAGGACATCCAAGAGCAGCTGAGCCTGACTGAGGCTTTTCAGAAAGAGCAAG
alPhePheLysAspHisSerTyrPheSe (276)
TCTTCTTCAAGGATCACTCATACTTCAGgtgcctctgttatgctgtcccatgcagttaaaattgctggaattgctggtcaagaacaggcctgacattgtg
tgtgtgtgtgtgtttgtggggagagagagagagag..........................intron.7..............................
ctctggctatcctggaactaactctgtagaccaggctggcctcgaactcagaaatctgcctgcctctgcctcccatgtgctgggattaaaggtgtgcgcc
accactacccagcacaaacccacttctaagtactccatgagcaccaatcttaaggtatccctttcactgatagtacccaaactgtcccgatctcccccgc
                                                  (276) rIleLeuLeuGluAspGlyLysAlaThrValProCysLeuAlaG
agcaggctgcagacttgttttctggtttgataggaccctctcttctgttcccttagCATTCTTCTGGAAGATGGGAAGGCCACAGTGCCCTGCCTGGCAG
luArgLeuThrGluGluLeuThrSerHisIleCys (302)
AGAGACTGACTGAGGAGCTCACCTCCCACATCTGTgtaagcaggggtggctgtgtgttccacaggctaaactgggactgggactgtcctgggcacccaag
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..............................................intron.8............................................
                                                                                      (303) LysSe
tgcttgtgacacagccatgcttctgagtagccttggggcctccttcacattcccactccgtatttgcatgtctcactcatttctttccatttcagAAATC
rLeuProLeuLeuGluAspGlnIleAsnSerSerHisGlnSerAlaSerGluGluLeuGlnLysTyrGlyAlaAspIleProGluAspAspArgThrArg
ACTGCCACTATTGGAAGATCAAATAAATAGCAGTCATCAGAGTGCAAGCGAGGAGCTGCAGAAGTACGGTGCAGACATACCAGAAGATGACAGAACGAGG
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MetSerPheLeuValAsn (343)
ATGTCCTTTCTGGTGAATgtgagcatcagttgggacttctgtggaggacggctgaatcactatctcacagggatcaccataactgctaatcattcaaact
atttagtctgtgacagagccaacacgcagtctgtcactgtcaggaaagtggtaaaactgcaactcaattactgcaatgc.....................
.........intron.9..................gggagaggctgggatttcccaacacccaaaaccttttcttcccatgatgcaaccctcaaggtatg
                                                              (344)LysIleSerAlaPheAsnArgAsnIleMetAsnLeuIleGlnAlaGlnGluT
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hrValSerGluGlyAspSerArgLeuPheThrLysLeuArgAsnGluPheLeuAlaTrpAspAspHisIleGluGluTyrPheLysLysA (391)
CCGTATCAGAGGGAGACAGCCGGTTGTTTACCAAACTGCGAAATGAGTTTCTTGCTTGGGATGATCATATTGAGGAATATTTTAAAAAGgtgagtgtca
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                                                                      (391) spSerProGluValGlnSerLysMetLysGluPhe
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GluAsnGlnTyrArgGlyArgGluLeuProGlyPheValAspTyrLysAlaPheGluSerIleIleLysLysArgValLysAlaLeuGluGluSerAlaV
GAAAATCAGTATCGTGGCCGGGAGCTGCCAGGGTTTGTGGACTACAAGGCATTTGAGAGCATCATCAAAAAGCGAGTCAAAGCCCTGGAAGAGTCTGCTG
alAsnMetLeuArgArgValThrL (444)
TGAACATGCTGCGCAGGGTCACTAgtgagtagtgttctgagtcactggggaatcctacacaaggttactgaggaaccctgcacagggtcactaggcagta
gtgtgtagggttg...................................................intron.11.........................
                                                                                  (444) ysMetValGlnThrAlaP
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heValLysIleLeuSerAsnAspPheGlyAspPheLeuAsnLeuCysCysThrAlaLys (469)
TCGTAAAGATTTTATCAAATGATTTTGGTGATTTTTTAAACCTCTGCTGTACTGCTAAGgtaaatccaaatacatttatttttaagagaataataaata
agcatgaca................................intron.12...............................................
                                                                         (470) SerLysIleLysGluIleArgLeuA
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snGlnGluLysGluAlaGluAsnLeuIleArgLeuHisPheGlnMetGluGlnIleValTyrCysGlnAspGlnValTyrLysGluThrLeuLysThrIl
ACCAAGAAAAGGAAGCTGAAAACCTGATCCGACTTCACTTCCAGATGGAACAGATTGTCTACTGCCAGGACCAGGTTTACAAGGAAACCTTGAAGACGAT
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GluMetThrGlnHisLeuLysAlaTyrTyrGln (555)
GAGATGACCCAGCACCTGAAAGCCTACTACCAGgtaaatgcaggaatgctcccagcgtgctcccagcccattccctttcttcggggtgcctttt.......
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..........................................intron.13..........................................
                                                                                  (556) GluCy
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sArgArgAsnIleGlyArgGlnIleProLeuIleIleGlnTyrPheIleLeuLysThrPheGlyGluGluIleGluLysMetMetLeuGlnLeuLeuGln
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AspThrSerLysCysSerTrpPheLeuGluGluGlnSerAspThrArgGluLysLysLysPheLeuLysArgArgLeuLeuArgLeuAspGluAlaArgG
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lnLysLeuAlaLysPheSerAspE (631)
AGAAGCTTGCCAAATTCTCCGATTAACCAGGCTAGCTATGTGGCTTTCCTGGTCGCTGTGCAATGATCCTTTAGCTGCTAACCTTATGTCAGAATGTTGC
CTTTAGACTGTGGAAGCAAGCAAGCCCTTTGTAAACTCTGAGGAGATGAGGAGAGAGGAGCTATGGAGAGTCATTCTACTTCAGCAGGAAGGCATGACCC
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TAACAAGGATTCCACCTCTCTTTTAGCCCACCACCCATCAGAGGTAGTGGAAGAGAAAAGTTATTAGGCTATGGAGGAAGTGGACCTGTTCAGCAATAGT
TCTTTGGGGGCGAGCTCAGTCTTCCTTGGCAGCAGTTCAGTCCCATAACAAACACCAAGTATGACTCAGCAGCTGCAGACCAGTCCTCTAGGCAGGCAGA
CACCACATACAACCAGCAGCTATAGTCCAGTCCTTTCCACAGGCAGAAACTAAGAATGAATCAGCAGCTGAAGTTCAATCTTGATGAAAGCTTGCCATCT
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ATTCTTTCATGTGTTTGCTTCAGCAAGACATCATTTCACCTGTGTACCCCAGCAAAACATCATTTGATATAACTAACTTTCCAAGGAAACTGGAAGTTTC
CACTTTAGCGCTTCCAATAAAATTCTTGCTAAGTGAcacttgcatgattttcttttatttgggggaggggctgaaccccacaagaaggggaacaggtag
gggcagaggaaggtgcactgagtccacccttcctgataggggaggtagaacaaggagatagatcaagtgtttccaagggactacagggtagtcagtgtga
acagacaa
```

FIG. 2. Structure of the murine *Mx* gene. (a) Schematic overview of the intron-exon structure. Boxes represent exons. Interrupted lines indicate introns of unknown lengths. Coding sequences are black. (b) The restriction maps of the cosmid and lambda clones were established as described in the text; the wavy line indicates a region believed to be deleted from the cosmid, as judged by the absence of a *Hin*dIII restriction site present in the λ clone 12-2. Abbreviations: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI. A composite map is shown at the top; the dotted line indicates sequences of unknown length that have not yet been characterized. The cosmid-derived *Bam*HI fragment that was used as a probe to isolate the lambda clones is indicated at the bottom right. (c) Exons and exon-intron boundaries were identified by sequencing those segments of the λ inserts which hybridized to the cDNA probes described in the text. The amino acid numbering is that used previously for *Mx* cDNA (53). Capital letters indicate exon sequences. All sequences were determined on both strands, except for those indicated by a dotted line, which were established by repeated sequencing of only one strand.

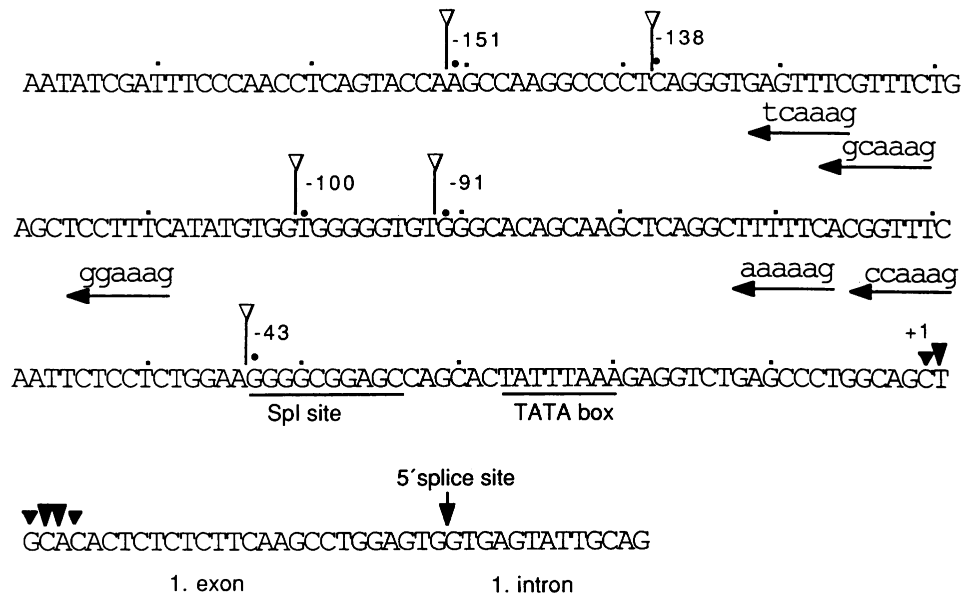


FIG. 3. Structure of the *Mx* promoter region. The Spl-binding site and the TATA box are underlined. The endpoints of the deletion mutants described in Fig. 4 are indicated. Arrowheads indicate the endpoints of the sequenced cDNAs of Fig. 1a and b. Position +1 of the nucleotide sequence is defined as the most upstream nucleotide determined by primer elongation (Fig. 1). Arrows indicate hexanucleotides of the type GAAANN which also occur in the human IFN-α1 promoter (Table 1).

TABLE 1. Homologies in the promoter region of IFN- and virus-induced genes

Gene	Sequence	
Friedman-Stark consensus	GAAACT[a]	TCTCCTCTC
	−120 −131	−55 −48
Mouse Mx (this report)	AGAAAC-GAAACT	TCTCCTCT
	−143 −131	−92 −87
Mouse 202 (46)[b]	GGAAATTGAAAGC	CTCCTC
	−99 −88	−113 −104
Human 2′,5′-(A) synthetase (5)[c]	GGAAAC-GAAACC	CTCCTCCCTT
	−152 −140	64 71
Human 6-16 (42)	GGAAAATGAAACT	TCTCCTCC
	−111 −99	
	GGAAAATGAAACT	
	−107 −119	−353 −347
Human 56,000-molecular weight protein (58)	GGAAAGTGAAACT	TCTCCTC
	−107 −95	
Human ISG15 (43)	GGAAACCGAAACT	
	−91 −103	
Human ISG54 (43)	GGAAAGTGAAACT	
	−221 −208	−181 −187
Human IP-10 (33)[d]	GGAAAGTGAAACC	CTCCTCT
	−128 −140	−193 −200
Human factor B (63)	GGAAACAGAAACT	CTCCTCTC
		−210 −215
		CTCCTC
Consensus	Purine-GAAAN(N/−)GAAA(C/G)-pyrimidine	
	−85 −73	
Human IFN-α1 (45)[e]	AGAAATGGAAAGT	
	−79 −67	−59 −54
Human IFN-β (15)[e]	GAGAAGTGAAAGT	TCCTCT

[a] Inverse complement.
[b] Numbering relative to the 5′ end of the longest cDNA clone.
[c] Numbering relative to the A of the initiation codon.
[d] Described as IFN-γ-inducible gene.
[e] Virus-inducible genes.

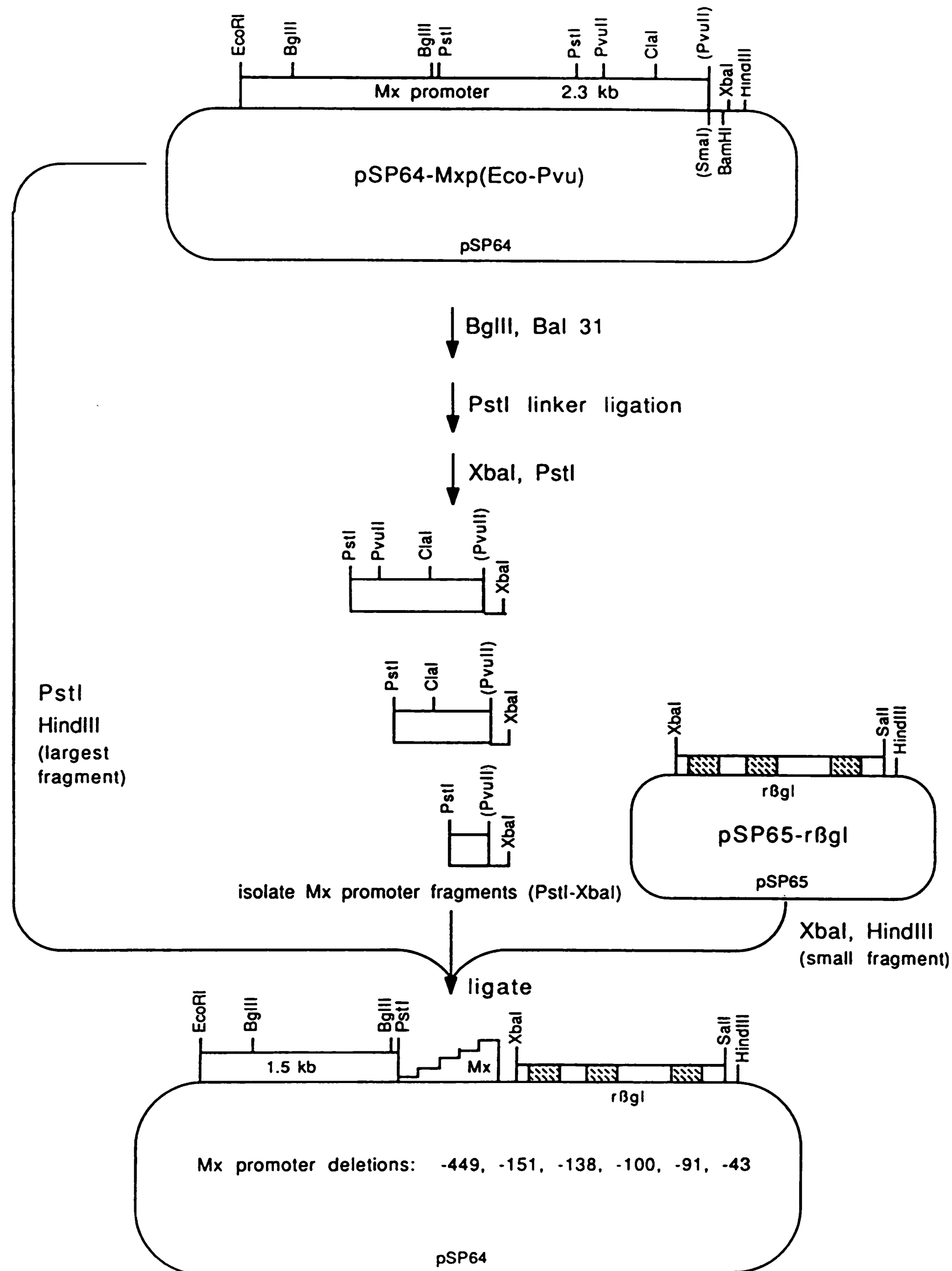
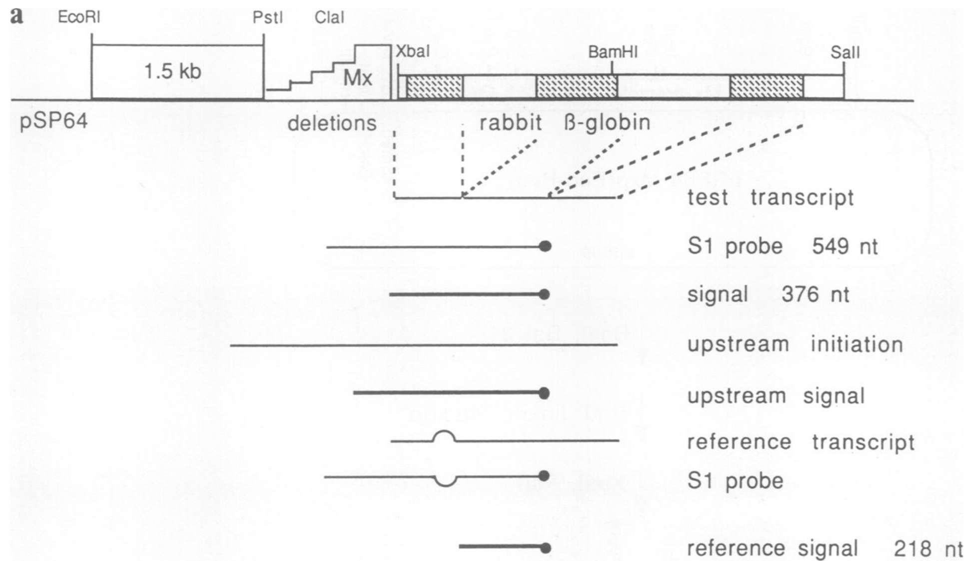


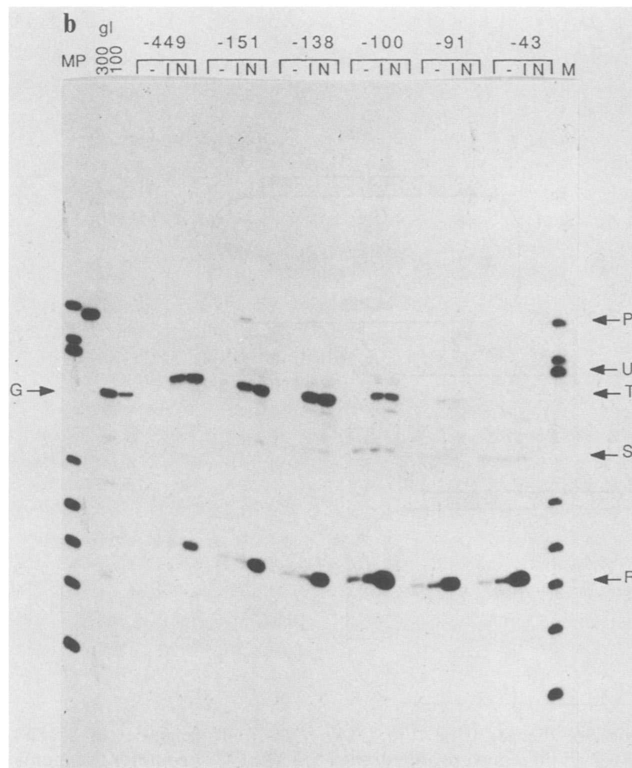
FIG. 4. The 5′ truncations of the *Mx* promoter. The 5′-noncoding region of the *Mx* gene (the 2.3-kb *Eco-Pvu*II fragment of λ MF4 subcloned in pSP64; see Fig. 2) was cleaved with *Bgl*II and treated with *Bal* 31. *Pst*I linkers were added; the *Pst*I-*Xba*I *Mx* promoter fragments in the range of about 40 to 450 bp were isolated and fused to the rabbit β-globin transcription unit (see text). About 1.5 kb of genomic mouse sequences of the *Mx* clone were retained upstream of the *Mx* promoter to diminish the effects of neighboring pBR sequences.

coding exons varied in length between 77 and 258 bp; the last exon, with 1,339 bp, encoded the 76 carboxy-terminal amino acids of the Mx protein, and in addition, it contained the whole 3′-untranslated sequence.

On Northern blots with RNA from IFN-treated Mx^+ cells, *Mx*-specific probes revealed a single band at about 3.5 kb. As shown by primer elongation, the more abundant *Mx* mRNAs appeared to result from transcription initiation within a cluster of six consecutive nucleotides. Some less-abundant *Mx* mRNAs had a 72-nucleotide insert between nucleotides 29 and 30, which marked the boundaries of the first intron. We presumed that the first intron contained a 72-bp optional exon, which is occasionally used in an alternative splicing event. Additional low-abundance *Mx* mRNAs may result from the rare use of other optional exons.

We recently identified a further IFN-inducible mouse gene, designated *Mx2*, which is closely related to but distinct from the *Mx* gene described in this paper (henceforth to be referred to as *Mx1*) (P. Staeheli and J. G. Sutcliffe, unpublished data). We have also established that there are at least two *Mx* genes in humans, both of which are induced by IFN (M. Aebi et al., unpublished data).





during induction or treated the cells with 75 μg of cycloheximide per ml; under these conditions IFN and NDV elicited about the same levels of *Mx* mRNA accumulation. However, when these treatments were omitted, induction by NDV (which entails induction by endogenous IFN) was far more efficient than induction by either component alone. Enoch et al. (12) have shown that in HeLa and 143 thymidine kinase-deficient cells, two signals are required for induction of the IFN-β gene, the first of which is elicited by IFN (and is attributed to a *trans*-acting factor produced in response to the latter) and the second of which is elicited by virus or double-stranded RNA. Tiwari et al. (57) have shown that in HeLa cells induction of the 56,000-molecular-weight gene (561 gene) also requires two signals, the first being provided by IFN-γ, platelet-derived growth factor, epithelial growth factor and the second being provided by IFN-α or double-stranded RNA. It is thus likely that in the experiments with mouse L cells we saw a similar cooperative effect of IFN and virus induction. In the case of the endogenous *Mx* gene of murine BALB.A2G-*Mx* embryo cells induction was equally efficient with NDV and IFN. Induction by a combination of NDV and IFN was stronger than that by either component alone. Perhaps the level of a factor required for virus inducibility is increased by IFN, in analogy to the situation described by Enoch et al. (12) and Tiwari et al. (57).

If not only NDV but also influenza virus induces *Mx* gene expression, the biological advantage of a dual response of the *Mx* gene to both IFN and virus would lie in the possibility of providing a rapid local *Mx* response in cells directly exposed to virus and a general, albeit slower, mobilization of *Mx* defense in regions of the organism not yet directly affected by the virus.

We also noted that the IFN-α promoter is slightly but distinctly stimulated by IFN alone (see the reference bands in Fig. 5B), suggesting that the IFN- and the virus-responsive sequences may be homologous and may show some cross-reactivity.

Inducibility of the *Mx* promoter by IFN and virus. *Mx* is one of the many genes whose expression is elicited or stimulated by type I IFN. It has been noted by others (57, 58) that the IFN-inducible 2′,5′ oligo(A)synthetase and the 56,000-molecular-weight genes are induced also by poly(I)-poly(C) or double-stranded RNA and that their promoters share certain sequences with the IFN-α promoter (58).

We determined that the 449-bp *Mx* promoter fragment and all of its truncated derivatives but the shortest one were activated as efficiently by NDV as by IFN. Because activation of the *Mx* promoter by NDV could be attributed to the IFN induced by the virus during the induction period, we either added an excess of IFN antibody to the medium

Structure of the *Mx* promoter. The promoter region between positions −138 and the cap site, when linked to the β-globin transcription unit, mediated a full response to IFN and virus. The cap site was not formally determined, but apparent initiation starts occurred with various degrees of efficiency at six positions, including A, G, C, and T residues.

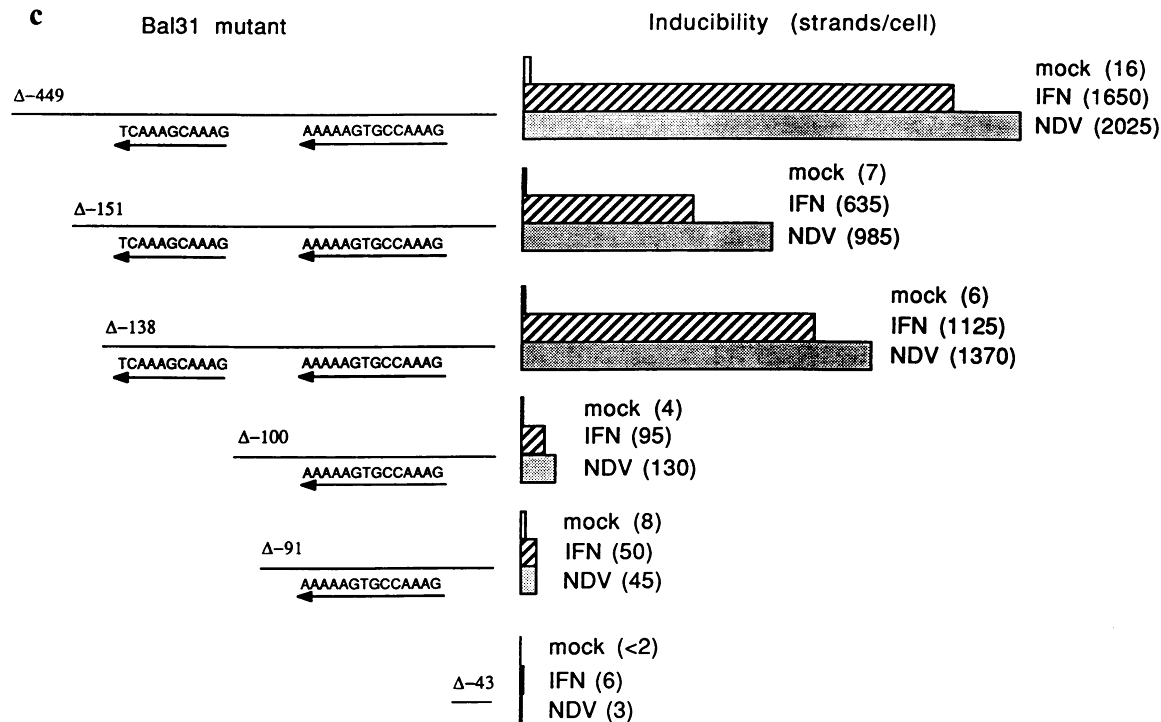


FIG. 5. Inducibility of *Mx* promoter 5′ deletion mutants by IFN and NDV. (a) The constructions used are those described in the legends to Fig. 3 and 4. The 5′ *Mx* promoter deletion fragments are indicated as steps in the diagram. About 1.5 kb of the genomic mouse sequence was located upstream of the *Mx* promoter fragment. Coding sequences of the rabbit β-globin gene are hatched. Filled circles indicate the 5′ ^{32}P label of the probe (thin lines) and signals (thick lines). Reference RNA and the S1 nuclease probe have a seven-nucleotide (nt) mismatch, which is indicated by a bulge. (b) Autoradiograph of S1 nuclease mapping. L929 cells were infected with IFN or NDV in the presence of excess IFN antibodies, as described in the text. Total RNA (40 μg) was mapped by S1 nuclease analysis as outlined above and described in detail in the text. The specific radioactivity of the probe was 4 × 10^6 cpm/pmol. Autoradiography was done for 2 days. Symbols and abbreviations: −, mock induced; I, murine IFN-α induced; N, NDV induced; gl, globin standards (in picograms); M, pBR327 digested with *Bsp*I and 5′ ^{32}P-labeled; P, undigested probe; U, readthrough β-globin transcripts; T, test signal; G, signal of globin standards; S, aberrantly spliced readthrough β-globin transcripts (9); R, signal from reference gene. (c) Response of truncated *Mx* promoter constructions to IFN or NDV. Bands were cut out from the S1 nuclease gel described above (b), and the radioactivity was determined. The number of strands per cell was calculated as described in the text. Open boxes represent mock-induced, hatched boxes represent IFN-induced, and dotted boxes represent NDV-induced values. The number of strands per cell is given in brackets. Arrows indicate hexanucleotides of the type GAAANN which also occurred in the human IFN-α1 promoter (Table 1).

A TATA box and a SP1-binding site were located between positions −43 and −20. The sequence CAAT at position −59 was not preceded by a C, and thus, it most likely does not constitute a CAAT box. A TATA box was found in most (but not all) controllable promoters and was mainly responsible for determining the initiation of transcription about 30 nucleotides downstream from it (6, 7). The particular sequence TATTTAAA is fairly uncommon but it occurs in all human, murine, and bovine IFN-α genes (61), as well as in some viral and plant genes (7). The Spl-binding site GGGGCGGAGC conformed with one of the three high-affinity variants of the consensus sequence of Kadonaga et al. (26). Spl-binding sites were found in many different promoters, both controlled and constitutive, and the cognate factor was ubiquitous. Spl-binding sites usually occurred in tandem arrays; nevertheless, a single site is sufficient to mediate transcriptional activation by the Spl factor (22, 25). Truncation of the *Mx* promoter at position −43 left the SP1-binding sequence intact; however, it abolished transcription almost completely. Perhaps the SP1-binding sites can act only as tandem arrays or in cooperation with other upstream sequences, such as the inducible elements or a CCAAT box.

Friedman and Stark (13) have suggested that a 30-nucleotide consensus sequence, TTCN[G/C]NACCTCNGCAG TTTCTC[C/T]TCT-CT, might be responsible for inducibility by IFN. This sequence was derived by comparison of the 5′-noncoding regions of four human genes that were weakly inducible by IFN-α, namely, three HLA genes and the metallothionein *MT2* gene. Sugita et al. (56) and Israel et al. (24) showed a weak, two- to threefold stimulation of a reporter gene by the Friedman-Stark consensus sequence. Whereas the murine *Mx* gene promoter showed no sequence that was clearly similar to the Friedman-Stark consensus sequence as a whole, elements found in the 3′ half of the latter were present, albeit widely separated: AGTTTC at positions 131 to −126 and TTCTCCTCT at positions −56 to −48. The sequence CTCCTC was also found in many other IFN-inducible promoters (Table 1). Results of our experiments indicate that a truncation to position −100 or −91, which removes the AGTTTC-containing DNA segment, decreased induced transcription and inducibility about 10-fold; however, the remaining segment still mediated 10- to 20-fold inducibility by both virus and IFN. A further truncation of the *Mx* promoter to position −43, removing the C/T-rich sequence and two elements of the type GAAANN (see below), abolished inducibility almost completely. We conclude that the Friedman-Stark consensus sequence may

well contain elements that mediate inducibility by IFN-α, but that it is not required in its entirety for this function.

It is remarkable that in the *Mx* promoter the region responsible for induction by IFN and virus contains five sequences of the type GAAANN, namely, GAAACT, GAAACG, GAAAGG, GAAAAA, and GAAACC, as inverse complements (Fig. 3). Elements of the type GAAANN occur in either orientation in all IFN- and virus-inducible genes (occasionally, as part of the Friedman-Stark consensus sequence), suggesting that they play a role in the response to both inducers, and that induction at the DNA level may involve related factors. We note that in all promoters that were inducible by type I IFN, two such elements were arranged in a structure of the type purine-GAAAN(N/−)GAAA(C/G)-pyrimidine or its inverse complement (Table 1); we have determined that an *Mx* promoter segment extending from positions −139 to −108 that makes up the inverse complement of AGAAACGAAACT is responsive to both virus and IFN (unpublished data). It is of interest that the IFN-γ-inducible promoter IP-10 contains a similar sequence, GGAAAGTGAAACC but shows only weak inducibility by IFN-β (33, 34).

Fujita et al. (16) and Kuhl et al. (30) have shown that tetrameric hexanucleotides of this type (or permutations thereof), for example, $(GAAAGT)_4$, can mediate inducibility by virus. We will show elsewhere that tetramers of GAAACT and of some other hexanucleotides can mediate induction by IFN as well as by virus (H. Hug, D. Kuhl, M. Chaturvedi, M. Aebi, and C. Weissmann, unpublished data).

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