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Transcriptional Activation of the Mouse *Mx* Gene by Type I Interferon†

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Mouse cells of the Mx^+ genotype accumulate Mx mRNA in response to type I interferon (IFN). Nuclear runoff experiments show that IFN stringently regulates *Mx* gene expression at the level of transcription. Mx mRNA synthesis peaks about 3 h after IFN treatment, and within 5 h, Mx mRNA concentration rises from undetectable levels to about 0.1% of polyadenylated RNA.

Mouse strains carrying different alleles at the influenza virus resistance locus *Mx* (12) differ from one another in relative susceptibility to infection with influenza viruses (6). The allele Mx^+ confers resistance to influenza viruses but not to unrelated viruses (6, 11, 12). The *Mx* locus has been assigned to mouse chromosome 16 (19). Allele Mx^+ encodes Mx protein, a 75-kilodalton nuclear protein whose complete sequence has been deduced from a cDNA clone (3, 9, 16, 17). Transfection of influenza virus-susceptible (Mx^-) 3T3 mouse cells with the Mx cDNA results in expression of Mx protein and confers influenza virus resistance on these cells (17). In Mx^+ animals and Mx^+ tissue culture cells, type I (α and β) interferon (IFN) mediates influenza virus resistance (7, 8). Synthesis of the Mx protein is detectable in Mx^+ cells after treatment with type I IFN but not in untreated control cells or in cells treated with type II (γ) IFN (9, 16, 18). Mx mRNA, which migrates as a single band on Northern blots, is readily detectable in polyadenylated [poly(A)$^+$] RNA preparations from type I IFN-treated Mx^+ cells but is not found in RNA preparations from untreated control cells (17). Thus far, it has been unclear whether Mx mRNA accumulation in IFN-treated cells is a consequence of increased transcription of the *Mx* gene or of increased stability of otherwise rapidly degraded Mx mRNA. In this study we used an Mx cDNA clone as a probe to analyze the nature of induction by type I IFN of the mouse gene *Mx*. We show that IFN regulates the synthesis of Mx protein by dramatically increasing the transcription rate of the *Mx* gene, after which Mx mRNA rapidly accumulates. Despite the continuous presence of IFN in the culture medium, its stimulatory effect on *Mx* gene transcription is short-lived and is no longer detectable 8 h after the onset of IFN treatment. Transcriptional regulation can fully account for the induction of *Mx* gene expression by IFN.

Induction of Mx mRNA synthesis by both alpha and beta IFNs. Mouse embryo cells were prepared from BALB.A2G-*Mx* (Mx^+) mice (18). Cells were cultured in Dulbecco modified minimal essential medium containing 10% fetal calf serum and passaged at a dilution of 1:3 onto fresh plates every 3 to 4 days. In the first set of experiments, 4-day-old confluent cell monolayers from passages 5 to 10 were treated for 5 h at 37°C with culture medium containing various concentrations of natural type I IFN. Cytoplasmic poly(A)$^+$ RNA was isolated from each culture and analyzed by the Northern blotting technique as follows. Cells were scraped off the plates into ice-cold phosphate-buffered saline, collected by low-speed centrifugation, suspended in 1 ml of buffer (10 mM Tris hydrochloride at pH 8.5, 0.14 M NaCl, 1.5 mM $MgCl_2$) per 10^7 cells, and lysed with Nonidet P-40 (final concentration, 0.25%) for 5 min at 0°C. The nuclei were spun down (10 min; 1,000 × *g*) and processed for nuclear runoff assays as described below. The supernatant was recovered, sodium dodecyl sulfate (SDS; final concentration, 0.2%) and EDTA (final concentration, 10 mM) were added, and this mixture was extracted with phenol-chloroform. RNA in the aqueous phase was precipitated with ethanol, and poly(A)$^+$-enriched RNA was prepared by oligo(dT)-cellulose column chromatography. Poly(A)$^+$ RNA samples (2 μg) were fractionated by electrophoresis on 1.2% agarose–1 M formaldehyde gels and were transferred to nitrocellulose. The prehybridization and hybridization of RNA blots were done as described previously (13), except that 56% formamide and a 37°C incubation temperature were chosen. All blots were washed at 68°C in 0.2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.5% SDS. As a Northern hybridization probe for Mx mRNA we used the 2.3-kilobase *Bam*HI fragment of clone pMx34 (17), which contains the entire Mx protein coding region. To show that similar amounts of poly(A)$^+$ RNA were loaded into each lane, the blots were also probed with radiolabeled 1B15 cDNA (13), which hybridizes to the transcripts of a ubiquitously expressed gene. Mx mRNA was not detectable in control cells treated with IFN-free medium. Cells treated with 250 or 2,500 reference units of IFN per ml contained high concentrations of Mx mRNA, which appeared on Northern blots as a single 3.5-kilobase species (Fig. 1A). The abundance of Mx mRNA in these fully induced cells was about 0.1%, as estimated from the abundance of Mx in a cDNA library prepared from this RNA and from the relative intensities of the Northern signals obtained with Mx and 1B15. The abundance of 1B15 mRNA was about 1% (13). Mx mRNA concentration in cells treated with 25 reference units per ml was about 10-fold lower than in fully induced cells. Since our Northern conditions should allow the detection of transcripts occurring at frequencies higher than about 0.001%, Mx mRNA concentration in Mx^+ cells rose at least 100-fold during the first 5 h of type I IFN treatment. The 1B15 mRNA concentration was neither detectably increased nor decreased in IFN-treated cells. The type I IFN routinely used was partially purified (10^7 reference units per mg) and

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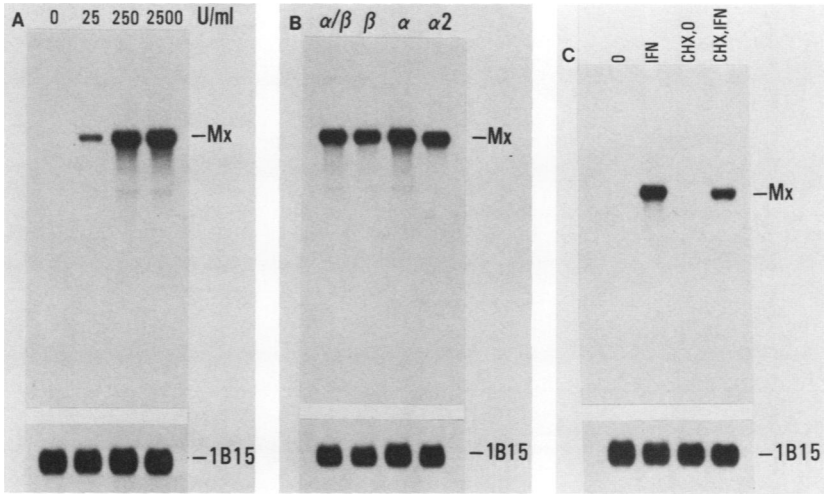

FIG. 1. Mx mRNA induction under several physiological conditions. BALB.A2G-*Mx* (*Mx*$^+$) embryo cells were incubated for 5 h at 37°C (A) with the indicated concentrations of partially purified type I IFN, (B) in medium containing 1,000 reference units of partially purified type I IFN per ml consisting of approximately 90% IFN-β and 10% IFN-α (α/β), highly purified IFN-β (β), partially purified IFN-α not containing detectable amounts of IFN-β (α), or partially purified *E. coli*-produced recombinant IFN-α_2 (α2). (C) Four parallel cultures were subjected to different regimens of IFN and CHX treatments. One culture received fresh medium without supplements (0), the second culture was treated for 3 h with 1,000 reference units of type I IFN (IFN) per ml, the third culture was treated for 4 h with 50 μg of CHX per ml (CHX,0), and the fourth culture was incubated with 50 μg of CHX per ml for 1 h before the addition of 1,000 reference units of type I IFN per ml and further incubation for 3 h (CHX,IFN). Hybridization was for 18 h at 5×10^6 cpm of either the ^{32}P-labeled nick-translated 2.3-kilobase *Bam*HI restriction fragment of the Mx cDNA clone pMx34 (17) or the insert of clone 1B15 (13) labeled by nick translation per ml. The specific activities of the probes were about 10^9 cpm/μg.

consisted of a mixture of about 90% IFN-β and about 10% IFN-α. We therefore treated parallel cultures of *Mx*$^+$ embryo cells with 1,000 reference units per ml of this mixture of IFN-α and IFN-β, highly purified IFN-β (1.8×10^8 reference units per mg), partially purified IFN-α (2×10^6 reference units per mg) not containing detectable amounts of IFN-β (these IFNs were purchased from Lee Biomolecular, San Diego, Calif.), or *Escherichia coli*-produced (10^7 reference units per mg) recombinant mouse IFN-α_2 (14). Clearly (Fig. 1B), all these IFNs were potent inducers of *Mx* gene transcription, indicating that triggering the type I IFN cell surface receptor by any of these IFNs may induce the same cascade of events that results in Mx mRNA accumulation.

Induction of Mx mRNA synthesis in cells with blocked protein synthesis. To distinguish between a primary IFN response, which would not require protein synthesis, and a secondary response, for which protein synthesis would be necessary, we performed experiments in which treatments with cycloheximide (CHX) and IFN were combined. We treated the cells with CHX at a concentration of 50 μg/ml, which, within 1 h after the onset of treatment, inhibited protein synthesis at least 95% (data not shown). We observed significant cell losses when CHX was present in culture media for longer than 5 h. Cells kept in CHX-containing medium synthesized almost as much Mx mRNA during the 3 h of IFN treatment as parallel control cultures treated with the same amount of IFN but not with CHX (Fig. 1C). CHX by itself did not induce Mx mRNA synthesis. Thus, synthesis of new proteins is not required for efficient transcriptional activation of the *Mx* gene.

Transcriptional regulation of Mx mRNA synthesis. Stimulated transcription of the *Mx* gene or, alternatively, posttranscriptional control events could account for the observed accumulation of Mx mRNA in IFN-treated *Mx*$^+$ cells. To distinguish between these possibilities, we performed a series of in vitro transcription experiments with nuclei isolated from IFN-treated and untreated control cells. Isolated nuclei are able to complete the synthesis of in vivo-initiated mRNA chains when incubated in appropriate buffers containing all metabolic precursors. Since under these conditions de novo initiation of RNA polymerase II complexes does not occur, transcription in isolated nuclei is believed to be an accurate reflection of the RNA synthesis activity of a given cell population at a given time (1, 2). For the results shown in Fig. 2, groups of three 150-mm-diameter dishes of confluent cell monolayers were incubated for variable time periods in medium containing 1,000 reference units of IFN per ml. After cell lysis, cytoplasmic poly(A)$^+$ RNAs were prepared from all cultures as described above, and the nuclei were immediately processed for transcription experiments as follows. Nuclei were washed in 10 mM Tris hydrochloride (pH 7.8)–150 mM KCl–5 mM $MgCl_2$–1 mM $MnCl_2$–5 mM dithiothreitol–10% glycerol and suspended in the same buffer containing 1 mM each of ATP, CTP, and UTP (nuclear runoff buffer) at approximately 10^8 nuclei per ml. Nuclear runoff buffer (minus $MnCl_2$) (38 μl; 4×) and 12 μl of 12.5 mM $MnCl_2$ were added to 100 μl (1.0 mCi) of aqueous [α-^{32}P]GTP (800 Ci/mmol; New England Nuclear Corp., Boston, Mass.), and 25 μl was added to each sample (75 μl) of suspended nuclei. After 15 min at 25°C, the nuclei were spun down, the supernatant was discarded, and the nuclei were lysed in 300 μl of 10 mM Tris hydrochloride (pH 8)–1 mM EDTA–1% SDS–50 μg of yeast tRNA per ml. Proteinase K (final concentration, 0.4 mg/ml) was added to the lysates, and the samples were incubated for 5 min at 37°C. After two extractions with phenol-chloroform-isoamyl alcohol, the nucleic acids in the aqueous phase were precipitated with ethanol. Precipitates were dissolved in 300 μl of 40 mM Tris hydrochloride (pH 8)–5 mM $MgCl_2$–0.1 mM $CaCl_2$–0.1 mM EDTA–1 mM dithiothreitol, and the DNA was digested for 10 min at 37°C with 20 U of RNase-free DNase I (Promega Biotec, Madison, Wis.). After two ex-

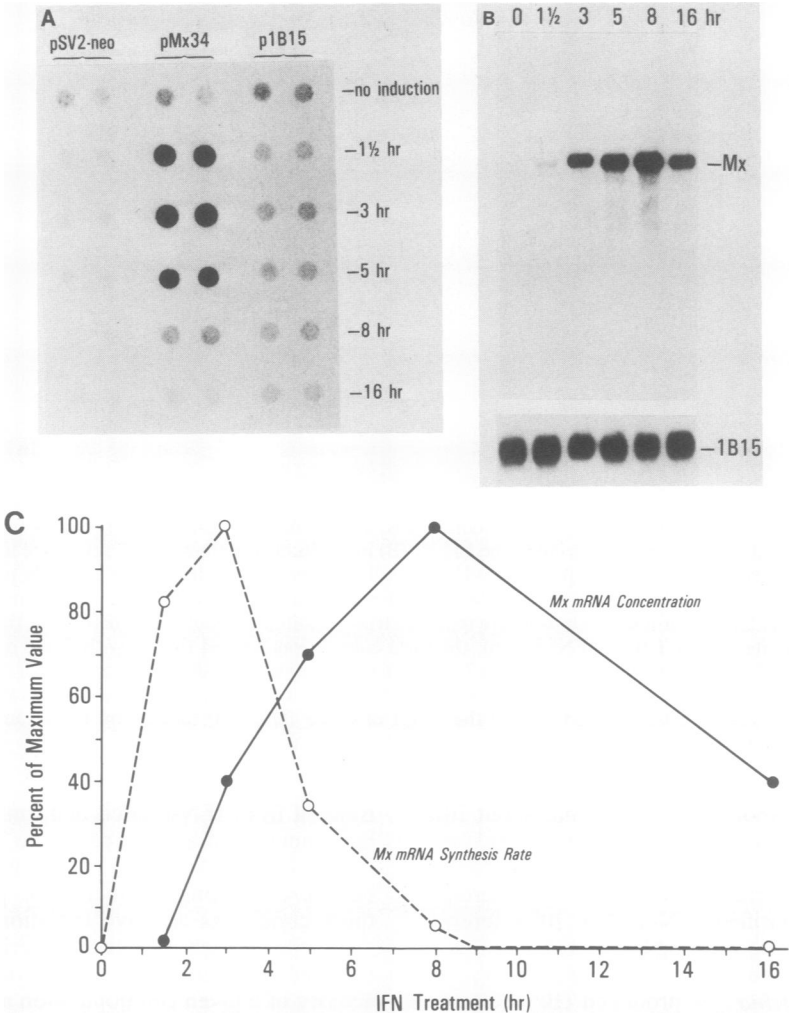

FIG. 2. Synthesis rates and concentrations of Mx mRNA at different times after IFN induction. (A) Isolated nuclei were used to synthesize radiolabeled runoff RNA in vitro, and the DNAs were hybridized to filter-immobilized DNA. (B) A Northern blot with the poly(A)$^+$ RNAs was assayed for Mx mRNA and 1B15 mRNA with appropriate nick-translated probes. (C) Graphical presentation of the data obtained from scanning the X-ray films shown in panels a and b. Differences between samples in background hybridization and RNA concentration were taken into account as described in the text.

tractions with phenol-chloroform, the RNA in the aqueous phase was precipitated with ethanol. Runoff RNAs were hybridized in duplicates to nitrocellulose filters with either immobilized Mx cDNA, 1B15 cDNA, or pSV2-neo DNA. Linearized plasmid DNAs were bound to nitrocellulose filter circles (10 μg/5-mm-diameter circle). Sets of filters containing no more than one of each type were prehybridized for 8 h and hybridized for 40 h as described elsewhere (5), except that hybridization was in the absence of dextran sulfate. The filters were washed for 10 min at room temperature in 1× SSC and 0.1% SDS and then twice for 45 min at 67°C in 0.1× SSC and 0.1% SDS. The filters were exposed to Kodak XRP-1 film at −70°C with an intensifying screen.

IFN treatment had no significant effect on the rate of overall RNA synthesis (data not shown). To determine the effect of IFN on the transcription rate of the *Mx* gene, we hybridized the different radiolabeled RNAs to a large excess of Mx cDNA immobilized on nitrocellulose filters (Fig. 2A). To detect possible differences between individual RNA preparations in hybridization efficiencies, i.e., RNA concentrations and content of impurities that would increase the filter backgrounds, we also hybridized runoff RNAs to nitrocellulose filters containing either cDNA derived from the ubiquitously expressed mRNA 1B15 or pSV2-neo DNA (15), which does not hybridize to mouse RNA. We found that *Mx* gene transcription was dramatically stimulated by IFN treatment. In untreated control cells, the rate of *Mx* gene transcription was very low. In fact, with the nuclear runoff assay, we could not demonstrate any transcription from the *Mx* gene in untreated cells. (In the results shown in Fig. 2A, the RNA prepared from noninduced cells showed unusually high background hybridization to all three types of filters. In other experiments, all background signals were low, including those with RNA from noninduced cells.) Ninety minutes after the onset of IFN treatment, the *Mx* gene transcription rate was at least 50-fold increased. The transcription rate was highest at about 3 h postinduction. Of a total of 10^7 cpm of runoff RNA from fully induced cells, around 500 cpm on the average hybridized to the filter with Mx DNA, about 50 cpm hybridized to the filter with 1B15 DNA, and about 15 cpm hybridized to the background filter. Since the Mx probe used was about three times longer than

the 1B15 probe, we conclude that 3 h after the onset of treatment of quiescent Mx^+ embryo cells with IFN, the Mx promoter was about fivefold more active than the 1B15 promoter. The rate of *Mx* gene transcription dropped sharply after 3 h postinduction. By 8 h it was about 20-fold lower than at 3 h, and by 16 h after induction, *Mx* gene transcription was apparently shut off completely, having returned to its preinduction level despite the continuous presence of IFN in the culture medium. The 1B15 transcription rate was not affected by IFN.

To determine the relative Mx mRNA pools in the different cultures at the time of the harvest, we probed Northern blots of the poly(A)$^+$ RNAs with nick-translated Mx probe and with 1B15 probe to verify equal amounts of RNA in each lane (Fig. 2B). Mx mRNA was undetectable in the control culture not treated with IFN. Ninety minutes after the onset of IFN treatment, Mx mRNA became detectable. The Mx mRNA pool was about half maximal after 3 h. The highest cytoplasmic Mx mRNA concentration was found in the culture which had been treated with IFN for 8 h. Mx mRNA concentration was about twofold lower in the culture treated with IFN for 16 h. These results are in good agreement with earlier observations that in vivo pulse labeling of Mx protein is most efficient in cells pretreated with IFN for 4 to 8 h (9). Fig. 2C shows the relative *Mx* gene transcription rates and the relative Mx mRNA concentrations as a function of time after IFN treatment. After scanning the X-ray films, we normalized all Mx scores for differences in background and in RNA concentration by subtracting the corresponding pSV2-neo scores and by expressing the Mx scores as a fraction of the corresponding 1B15 scores. The highest values resulting from these calculations for *Mx* gene transcription rate and Mx mRNA concentration, respectively, were then defined as 100%. Mx mRNA continued to accumulate as long as the *Mx* gene transcription rate was substantial, as one would predict if the concentration of Mx mRNA were mainly controlled by its rate of synthesis. Between hours 8 and 16 of IFN treatment, the rate of de novo Mx mRNA synthesis was very low. During this period, Mx mRNA concentration decreased to about 40% of the value at 8 h. Thus, the IFN-mediated control of *Mx* gene expression occurred mainly, if not exclusively, at the level of gene transcription.

Conclusions. The IFN-regulated mouse gene *202* (4) and the human genes encoding the mRNAs pIF-1 and pIF-2 (10) were found to be controlled at the transcriptional level, very much like the *Mx* gene. In contrast, expression of the human gene *1-8* seems to be controlled by IFN-mediated transcriptional and posttranscriptional regulation events (5). It was observed that the cytoplasmic concentration of 1-8 mRNA continued to increase between hours 8 and 24 of IFN treatment, although the transcription rate of the *1-8* gene was very low during this whole period. We were unable to demonstrate similar regulatory events in the Mx system. As in the case with the *1-8* gene, *Mx* gene transcription rate was very low between 8 and 16 h after the onset of IFN treatment, but in contrast to the 1-8 situation, Mx mRNA concentration decreased about twofold during this time. From this result we conclude that the bulk of the Mx mRNA production in IFN-treated cells is a consequence of an increased rate of its synthesis and that mRNA stabilization does not significantly contribute to increased Mx mRNA concentrations. We evaluated the remote possibility that the *Mx* gene might be controlled at the posttranscriptional level by a specific ribonuclease which, in untreated cells, would selectively destroy Mx mRNA shortly after its synthesis and whose activity would be inhibited by IFN. We would expect such a hypothetical nuclease to cut specifically at defined sites. Resulting fragments might never leave the nuclei and would thus escape detection by Northern analysis of cytoplasmic RNA. However, we would expect to find these fragments in the nuclei, preferentially in the nuclei of cells not treated with IFN. In contrast to these expectations, nuclear runoff RNA from untreated cells did not hybridize to filters with Mx DNA (Fig. 2A). Furthermore, in transfected cells, Mx cDNA expression under the control of the simian virus 40 early promoter was not dependent on IFN (17). These results argue against the possibility of posttranscriptional control by a specific nuclease.

The effect of IFN on *Mx* gene expression was transient, despite the continuous presence of IFN in the culture medium. Although we have not measured the IFN concentrations in the culture media at the time of the cell harvest, it is conceivable that a substantial fraction of the added IFN retained its biological activity for at least 8 h. Since we routinely used 1,000 reference units of IFN per ml, which is at least twice the IFN concentration required for full-scale induction, we believe that the short-lived effect of IFN on *Mx* gene transcription cannot be sufficiently explained by IFN depletion. Transient effects of IFN on the transcription rates of other inducible genes have been reported (5, 10). In these cases it was postulated that IFN receptor down-regulation may have caused the transient effects. It is interesting to note that not all IFN-inducible genes behave in the same fashion. Increased transcriptional activity of mouse gene *202* in IFN-treated Ehrlich ascites tumor cells was observed for at least 24 h (4), suggesting either that IFN receptor down-regulation does not occur in those cells or, alternatively, that IFN acts via more than one secondary messenger and that these intracellular factors may differ significantly in their half-lives. A remote possibility is that the products of some IFN-induced genes actively shut down the expression of certain IFN-activated genes.

Its strength and its stringent regulation by type I IFN make the Mx promoter a potentially valuable regulatory element to control the expression of cloned DNAs in transfected cells. Production of certain proteins by genetic engineering techniques may largely depend on the effectiveness of the control element, which should not only effectively suppress transcription of the cloned gene, but also allow for its efficient induction late in the production process. One would also favor a control element which responds to a well-defined inducer to be added to the culture medium. The control element of choice might be the Mx promoter. By supplementation of the culture medium with IFN, transcription from the Mx promoter in quiescent Mx^+ cells can be increased from a nondetectable level to a very high rate such that over a relatively short period (a few hours) a substantial pool (0.1%) of Mx mRNA accumulates. During this short period, the *Mx* gene is possibly one of the most active polymerase II transcription units in these cells. It was found, for example, to be about fivefold more active in the nuclear runoff experiments than the ubiquitously expressed gene *1B15*, which gives rise to about 1% of the stable mRNA. All of our experiments were performed with confluent monolayers of diploid cells. It will be interesting to learn whether the isolated *Mx* promoter performs as well in recipient cells after DNA-mediated gene transfer as it does in its natural environment on chromosome 16.

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