

Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus

(*Mx* gene/responsiveness to interferon/macrophages/two-dimensional gel electrophoresis/actinomycin D)

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ABSTRACT Mouse cells carrying the dominant resistance gene *Mx* develop a more efficient antiviral state toward influenza viruses in response to interferon than do *Mx*-negative cells. We have identified an *Mx* gene-associated product by labeling cultured peritoneal macrophages and embryonic cells with [³⁵S]methionine in the presence or absence of interferon. The radioactive proteins from unfractionated cytoplasmic extracts were separated electrophoretically in two dimensions and were revealed by fluorography. A protein with a *M_r* of 72,500 and an isoelectric point of 6.3 was induced by mouse interferon type I (a mixture of α and β interferons) in cells carrying the gene *Mx* but not in cells lacking *Mx*. The induction of this protein could be blocked by actinomycin D. The maximal rate of synthesis was reached in embryonic cells 4-5 hr after treatment with 10³ reference units of interferon per ml. When the allele *Mx* (present in the inbred mouse strain A2G) was repeatedly backcrossed on different genetic backgrounds (BALB/c, C57BL/6, A/J), a clear correlation between the inducibility by interferon of this protein and the presence of the allele *Mx* was observed. The results suggest that this protein induced by the interaction of interferon with *Mx* plays a role in the selective antiviral state against influenza viruses that is observed in interferon-treated *Mx*-bearing cells.

Exposure of cells to interferon (IFN) leads to an antiviral state in which the replication of most viruses is inhibited by various mechanisms and to different degrees. The development of antiviral resistance requires transcription and translation of cellular genes because it can be prevented by treatment of cells with actinomycin D and inhibitors of protein synthesis (1). IFN induces in cells a variety of responses including increased levels of certain enzymatic activities and the synthesis of a number of proteins, depending on cell type (2). However, the relative roles of these cellular proteins in antiviral resistance are still unclear. One possible approach to assigning specific proteins to particular antiviral states is to use cells that differ at alleles which govern the antiviral action of IFN towards specific viruses. For example, in mouse cells, a host gene *Mx* influences the efficacy of IFN in inhibiting influenza virus replication (for review, see ref. 3). Cells carrying the gene *Mx* can be protected by much smaller doses of IFN than are necessary to protect cells without *Mx* against infection with influenza viruses. This difference in response to IFN is observed with influenza viruses only. The degree of antiviral protection against other viruses tested so far is independent of *Mx* (4). We report here that IFN induces a unique protein in *Mx*-bearing cells but not in cells devoid of *Mx* and that the inducibility of this protein cosegregates with antiviral resistance governed by *Mx* in appropriate backcross mice.

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MATERIALS AND METHODS

Mice. A2G mice homozygous for the dominant resistance gene *Mx* (genotype *Mx/Mx*) were bred locally. The gene *Mx* confers to mice a high degree of resistance to infection with otherwise lethal doses of mouse-adapted influenza virus (5). BALB/c mice lacking *Mx* (genotype +/+) were obtained from F. L. Bomholtgård (Ry, Denmark). The gene *Mx* was introduced into the genetic background of BALB/c mice in the following way. (A2G × BALB/c)F₁ males (genotype *Mx/+*) were mated with BALB/c females. The resulting backcross generation was infected with lethal doses of influenza virus, and surviving *Mx*-bearing males were again backcrossed to BALB/c females. This procedure was repeated until the 11th backcross generation was reached. Similar backcrosses were produced with susceptible C57BL/6 and A/J mice. Cells obtained from backcross mice surviving infection were tested for IFN sensitivity as described (4) and were found to show increased antiviral activity selectively for influenza viruses (as compared to cells from susceptible BALB/c, C57BL/6, and A/J mice), thus confirming their *Mx* status (4).

Cells. Thioglycollate-induced peritoneal macrophages from the various mouse strains were harvested and cultured as described (4). Cells from 16-day-old mouse embryos were cultured and passaged as described (6).

IFN. Mouse IFN type I (containing both the α and β species) was induced by Newcastle disease virus in C-243 sarcoma cells and was partially purified to 10⁷ units of protein per mg as described (7). It was a gift from I. Gresser (Institut de Recherches Scientifiques sur le Cancer, Villejuif, France).

Labeling of Cells and Cytoplasmic Extracts. For long-term labeling (18 hr), 10 μ Ci (1 Ci = 3.7 × 10¹⁰ Bq) of [³⁵S]methionine (Amersham International) per ml was added at the same time as IFN to culture medium containing 2% fetal calf serum. For pulse labeling, cultures were washed with Hanks balanced salt solution supplemented with 2% fetal calf serum and incubated for 60 min with [³⁵S]methionine (12.5 μ Ci/ml) in the same buffer. The labeling was terminated by washing the culture with cold phosphate saline buffer, and the cultures were frozen at -80°C. Cells were then thawed and treated with 1.5 mM KCl/2.5 mM MgCl₂/5 mM Tris·HCl, pH 7.4/1% deoxycholate/1% Triton X-100 for 5 min at 0°C. The nuclei were centrifuged for 5 min at 2,300 × g; the supernatant was kept frozen at -80°C.

Analysis of ³⁵S-Labeled Proteins. The two-dimensional system combining nonequilibrium pH gradient electrophoresis (NEPHGE) with NaDodSO₄/polyacrylamide gel electrophoresis was used (8). An aliquot of cytoplasmic extract with ≈10⁵

Abbreviation: IFN, interferon.

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cpm was precipitated with 2 vol of ethanol. The proteins were solubilized in 50 μ l of "lysis buffer A" (9) and applied onto the acidic end of the nonequilibrium pH gradient electrophoresis gel, which contained 2% ampholytes, pH 3–10. The electrophoresis was run for 5 hr at 500 V. The separating gel for slab gel electrophoresis in the second dimension contained 12% acrylamide and 0.32% bisacrylamide. Radioactive proteins were visualized by fluorography on x-ray films (10). Microdensitometry was performed on the films with a Vitatron densitometer.

Isoelectric Point Determination. Isoelectric focusing of cellular proteins was performed for 8,500 V·hr on a pH gradient 5–8 as described (9). Three isoelectric-focusing gels were cut at 1-cm intervals, and each piece was equilibrated with 1 ml of degassed water for measurement of the pH gradient. One isoelectric-focusing gel with radioactive proteins was used for separation by polyacrylamide gel electrophoresis in the second dimension to locate the IFN-induced protein.

RESULTS

Proteins Synthesized in Control and IFN-Treated Macrophages Differing at the *Mx* Locus. Macrophages were obtained from the peritoneal cavity of A2G mice (homozygous for *Mx*) and of BALB/c mice (lacking *Mx*). They were then kept for 3 days in culture before treatment with 100 reference units of IFN per ml for 18 hr. Proteins synthesized during IFN treatment were labeled with [³⁵S]methionine. Cytoplasmic extracts were prepared, and the proteins were separated without further purification in the two-dimensional system as described. Extracts from cells not treated with IFN were prepared in the same way and served as controls. Fig. 1 shows the fluorographs

of a representative experiment. One major protein is present on gels of IFN-treated A2G macrophages (circle in Fig. 1E) but is absent in similarly treated BALB/c cells (circle in Fig. 1A). This protein is also missing on gels of A2G or BALB/c macrophages not treated with IFN (Fig. 1B and F). The induced protein has a M_r of \approx 72,500 and an isoelectric point of 6.3. The patterns of labeled proteins are otherwise quite similar on gels of IFN-treated and control cells, with only minor differences between the two mouse strains.

Because A2G and BALB/c inbred mice have many genetic differences besides *Mx*, it was mandatory to show that the IFN-induced protein was in fact associated with the gene *Mx*. Therefore, we introduced the allele *Mx* into different genetic backgrounds (BALB/c, C57BL/6, A/J) by using the breeding and selection scheme as outlined and established mouse strains nearly congenic at the *Mx* locus. Fig. 1C and D show protein gels of IFN-treated and control macrophages obtained from the 11th backcross generation of BALB/c mice carrying the gene *Mx* in heterozygous form. Again, IFN induced the M_r 72,500 protein in these cells (Fig. 1C), indicating that its appearance was due to gene(s) in or near the *Mx* locus and was not influenced by the BALB/c genetic background. Identical results were obtained with cells from backcrosses to C57BL/6 and A/J (results not shown). We could not find any other significant difference between *Mx*-positive and *Mx*-negative cells.

Induction of the *Mx*-Related Protein in Mouse Embryo Cells. IFN-induced selective resistance to orthomyxoviruses is not confined to macrophages but is expressed in various other cell types as well (e.g., embryo cells obtained from *Mx*-bearing animals). If the newly detected protein were part of the resistance mechanism, it would be induced also by IFN in nonphagocytic

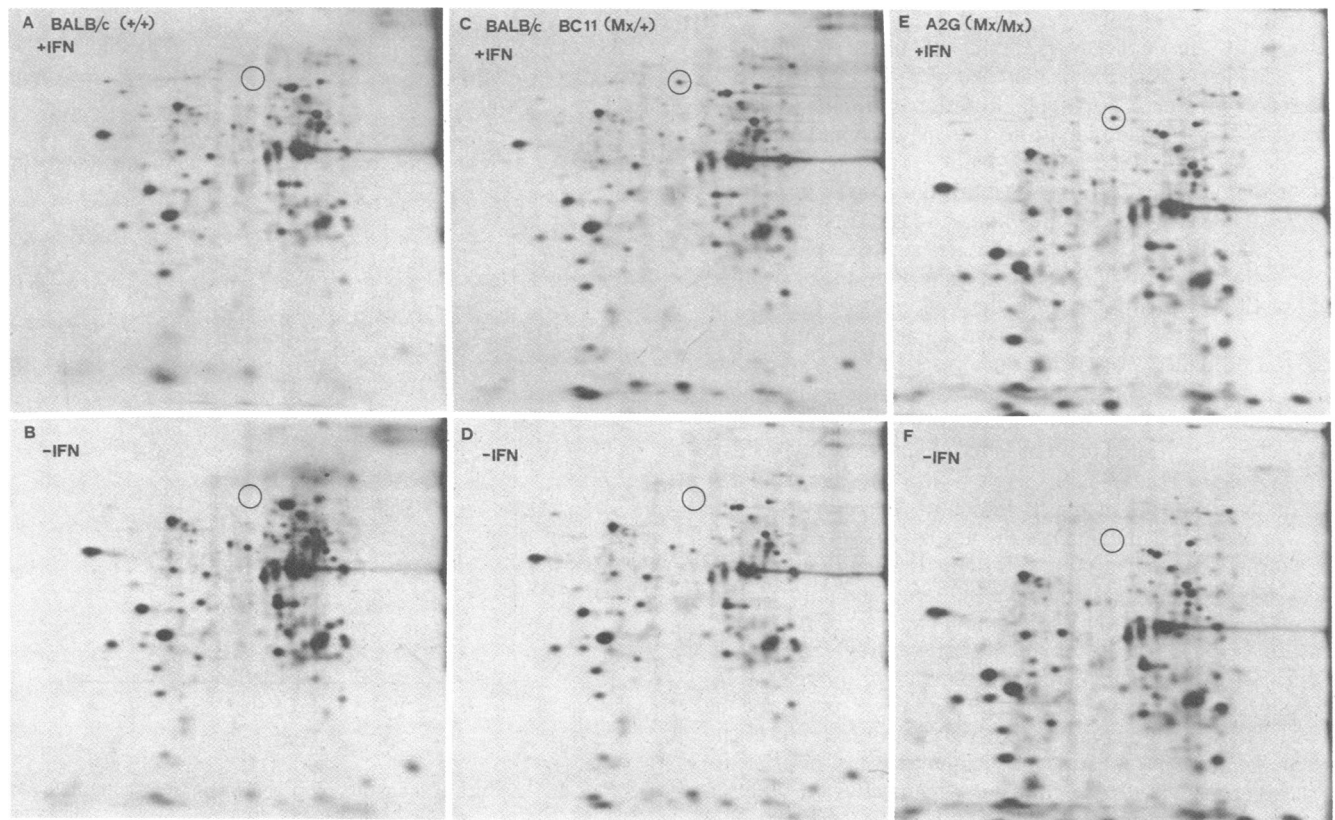


FIG. 1. Induction of the *Mx*-related protein in macrophages. Thioglycollate-induced peritoneal macrophages from BALB/c (+/+), BALB/c backcross 11th (*Mx*/+), and A2G (*Mx*/*Mx*) mice were cultured for 3 days. Control macrophages (B, D, and F) and macrophages treated with 100 reference units of IFN per ml (A, C, and E) were labeled for 18 hr with [³⁵S]methionine. The fluorographs represent the radioactive proteins of the unfractionated cytoplasmic extract separated in the two-dimensional system as described. The circles indicate the position of the IFN-induced M_r 72,500 protein. Exposure time was 4 days.

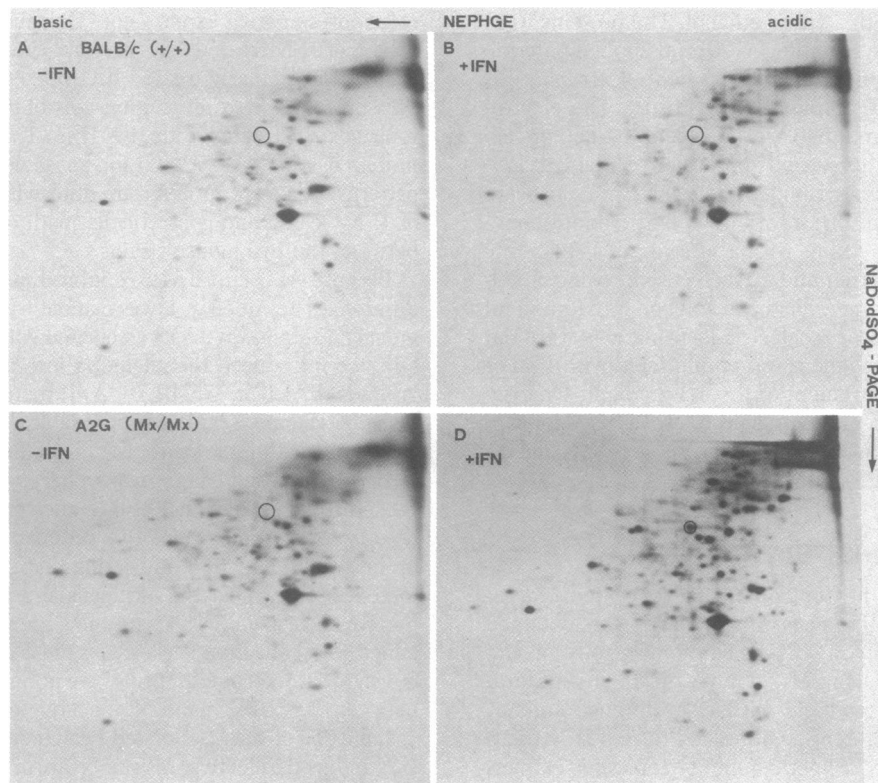


FIG. 2. Induction of the *Mx*-related protein by IFN in embryo cells from BALB/c (+/+) and from A2G (*Mx/Mx*) mice. Monolayers of control cells (A and C) and cells treated with 1,000 reference units of IFN per ml for 6 hr (B and D) were pulse-labeled with [³⁵S]methionine for 60 min. The fluorographs represent the radioactive proteins of the whole cytoplasmic extracts separated in the two-dimensional system. The circles indicate the position of the IFN-induced *M_r* 72,500 protein. Exposure time was 1 day.

cells. To test this, monolayer cultures of embryo cells differing at the *Mx* locus were established and were treated with 1,000 reference units of IFN per ml. In order to minimize the accumulation of [³⁵S]methionine into the many noninduced polypeptides synthesized in growing embryo cells, pulse-labeling rather than labeling during the entire duration of IFN treatment was performed. The induction of a major new protein by IFN in *Mx*-bearing cells but not in *Mx*-negative cells is demonstrated on the two-dimensional gels in Fig. 2. For comparison of the proteins induced in the two types of cells, equal parts of extracts from macrophages and embryo cells were mixed, and the proteins were coelectrophoresed in the two-dimensional system. The IFN-induced *M_r* 72,500 protein in this mixture migrated as a single spot, indicating a high degree of similarity of the *Mx*-related proteins induced in both cell types (data not shown).

Kinetics of Induction and Inhibition by Actinomycin D. The time-course of accumulation of the newly translated protein in *Mx*-positive embryo cells was determined. Monolayers of A2G embryo cells were treated with 1,000 reference units of IFN per ml and were labeled with [³⁵S]methionine for 60 min at various time intervals. Cell extracts were then prepared and analyzed. Fig. 3 shows a graphic representation of the relative intensities of the induced *M_r* 72,500 protein as measured by densitometric scanning (and expressed in arbitrary units relative to an internal standard). It is evident that synthesis of the induced protein occurred within a few hours after the addition of IFN. Peak values were obtained as early as 4 hr after IFN exposure and remained high thereafter throughout the observation period of 9 hr. Recent results have shown that maximal protection of *Mx*-bearing cells against influenza viruses is achieved within 4–6 hr after exposure to 1,000 reference units of IFN per ml (ref. 3; unpublished data). Thus, the induction

kinetics of the present protein would seem to parallel the development of the antiviral state towards influenza viruses in *Mx*-bearing cells.

If the appearance of the *Mx*-related protein were a consequence of IFN-induced transcription of cellular gene(s), in-

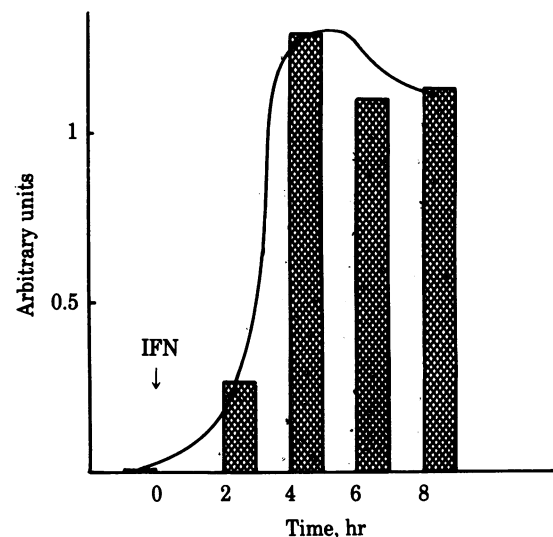


FIG. 3. Kinetics of induction by IFN of the protein unique to *Mx*-bearing cells. Embryo cells (third passage) from A2G (*Mx/Mx*) mice were grown to confluency, and 1,000 reference units of IFN per ml was added at time zero. Cells were pulse-labeled for 60 min with [³⁵S]methionine at times -1, 2, 4, 6, and 8 hr. Cytoplasmic extracts were prepared and analyzed by two-dimensional gel electrophoresis. The IFN-induced *M_r* 72,500 protein was scanned with a densitometer and quantitated in arbitrary units relative to an internal standard.

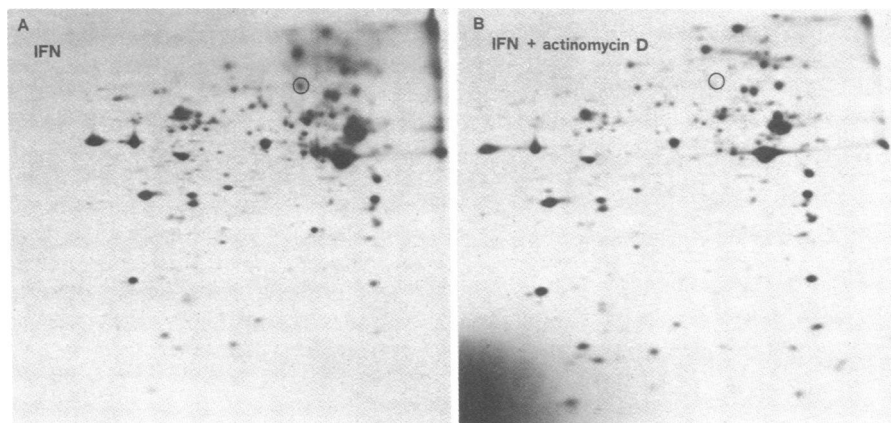


FIG. 4. Effect of actinomycin D on the synthesis of the IFN-induced M_r 72,500 protein in Mx -bearing cells. Embryo cells from A2G (Mx/Mx) mice were treated with IFN at 1,000 reference units/ml (A) or with a mixture of IFN at 1,000 reference units/ml and actinomycin D at 2 μ g/ml (B) for 9 hr in the presence of [35 S]methionine. Cytoplasmic extracts were prepared and analyzed by two-dimensional gel electrophoresis. The circles indicate the position of the IFN-induced M_r 72,500 protein. Exposure time was 2 days.

hibitors of RNA synthesis such as actinomycin D should block the synthesis of this polypeptide. Therefore, the polypeptides synthesized by embryonic cells in the presence of IFN and actinomycin D were compared to those synthesized in the presence of IFN only by two-dimensional gel analysis. Actinomycin D added at 2 μ g/ml simultaneously with IFN at 1,000 reference units/ml for 9 hr completely abolished synthesis of the M_r 72,500 protein (Fig. 4). Thus, the cellular control for the expression of this protein is at the transcriptional level.

DISCUSSION

The present results indicate that a M_r 72,500 protein with an isoelectric point of 6.3 is induced by mouse IFN type I in cells carrying the resistance gene Mx but not in cells lacking Mx . The induction of this protein by IFN in Mx -bearing cells was highly reproducible in independent experiments with various doses of IFN and different types of cells. Treatment of cells with actinomycin D blocked the induction of the protein, indicating a requirement for synthesis of new mRNA.

Our genetic analysis shows that the presence of the gene Mx (defined here functionally as increased responsiveness to the antiviral action of IFN toward influenza viruses) is necessary and sufficient for the induction of this protein by IFN. The gene Mx has been backcrossed to near congenicity on three different backgrounds, and the inducibility of the protein cosegregated with the resistance phenotype governed by Mx . This suggests that gene(s) in or near the Mx -locus are responsible for the appearance of this cellular protein, whereas differences of the genetic background would seem to be irrelevant. We do not know, however, whether Mx is the structural gene for the M_r 72,500 protein or a regulatory gene modulating its expression.

The evidence that the induced protein is part of the selective antiviral state towards orthomyxoviruses is by necessity indirect and rests on (i) the cosegregation of the protein with Mx in the backcross experiment; (ii) the inducibility of the protein in macrophages and embryo cells by doses of IFN known to elicit specific resistance in both these cell types (4, 6); (iii) the induction kinetics of the protein, which perfectly parallel the development of the antiviral state towards influenza viruses in Mx -bearing cells (ref. 3; unpublished results); and (iv) the ability of actinomycin D to prevent both the appearance of the protein and the establishment of an antiviral state (1). It remains to be determined whether the present protein possesses antiviral activity. It might by itself prevent virus growth or it might have to act in conjunction with the overall antiviral state induced by

IFN. If so, a most interesting feature of this protein would be its capacity to interfere selectively with biochemical events necessary for the growth of influenza viruses without touching the biochemical pathways of unrelated viruses.

Many reports have described new proteins synthesized in mouse cells after IFN treatment. However, this report differs by indicating a genetic correlation between the induction of a particular protein and the inhibition of particular viruses (namely, influenza viruses) by IFN. The proteins previously reported seem to be limited to some established cell lines (11–16); none of them seem to share the physico-chemical properties of the protein induced by IFN in Mx -bearing cells. This protein is not related to the (2'-5') (A)_n synthetase that has a M_r of 105,000 in mouse cells (17). The protein seems also unrelated to the protein kinase induced by IFN because it does not bind to poly(I)-poly(C) (unpublished data) and because there is no quantitative correlation between its induction and the level of kinase activity in macrophages (unpublished data). Therefore, it is unlikely that the protein we described here has been observed in other mouse cells. It is, however, quite possible that similar proteins may be found in cells from other species, where they may play a role in host defense against influenza viruses. Alternatively, the Mx system may have a wider biological significance than inhibition of influenza viruses, especially because mice are not known to be natural hosts for this virus. Analysis of the present protein and its structural gene should help to get more insight into the mechanism of IFN action.

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