Journal of Cellular Biochemistry 60:83–94 (1996)

ARTICLES

Host Genotype Controls the Ability of the ISGF3 Complex to Activate Transcription of IFN-Inducible Genes

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Abstract C57BL/6 mice are unable to express the Ifi 202 type genes upon injection in vivo of multiple dsRNA, poly rI:rC, or IFN-treatment in vitro. For this purpose the 5′ terminal flanking region (called the b segment of 804 bp) was linked to a heterologous reporter gene chloramphenicol acetyl transferase (CAT) and transfected into NIH3T3 cells or BLK cells derived from the C57BL/6 strain. IFN-α induced strong CAT activity in NIH3T3 but not in BLK cells. This lack of transcription activation was not due to a defect in STAT factor activity, since IFN-α treatment in the presence of IFN-γ priming induced translocation of the ISGF3 into the nucleus, and binding to the ISRE (IFN-Stimulated Response Element) of the 202 gene even in C57BL/6 derived cells. Surprisingly when three tandem copies of the 202 ISRE (42 bp) were linked to a heterologous promoter (c-fos promoter) driving the reporter CAT gene, activation was also observed in C57BL/6 cells upon IFN-treatment. Finally, another IFN-inducible gene, namely the Mx, was activated in C57BL/6 mice. Thus, the primary defect of the C57BL/6 strain leading to an impaired Ifi 202 type gene response to IFN appears to be an inability of the ISGF3 complex to activate the endogenous promoter. Altogether these results suggest that unidentified nuclear factors related to the host genotype control the ability of the STAT factors to activate transcription upon IFN-treatment.

Key words: IFNs, ISGF3 complex, host genotype

Interferons (IFNs) are a family of cytokines whose pleiotropic effects on cells range from inhibition of viral replication and cell growth to regulation of cell functions and modulation of the immune response [De Mayer and De Mayer-Guignard, 1988]. Studies in mice show that the animal genotype has a clear-cut effect on the various biological actions of IFNs, which depends on the transcriptional activation of a group of structural genes, whose protein products appear to mediate different biochemical pathways and hence various biological activities [Revel and Chebath, 1986].

Several IFN-induced genes have now been cloned. Comparison of their 5′-flanking region sequences has revealed highly homologous sequences defined as IFN-stimulated response elements (ISREs) [Darnell et al., 1994]. ISREs located upstream or downstream from reporter genes activate transcription in an IFN dependent manner. Biochemical and genetic experimental approaches exploring the induction of transcription by both IFN-α and IFN-γ have permitted a general understanding of the signal transduction pathway that directly connects events at the cell surface to gene activation [Kerr and Stark, 1991; Shuai et al., 1993; Ziemiecki et al., 1994].

The current model envisions that ligand binding activates protein tyrosine kinases of the Janus family, which consists of at least five members, Jak1-4 and Tyk2, resulting in phosphorylation of latent cytoplasmic proteins termed STATs (signal transducers and activators of transcription) [Darnell et al., 1994; Ihle et al., 1994; Zhong et al., 1994a,b].

The primary positive regulator of IFN-α activity, ISGF3, consists of a 91/84 kDa protein and a 113 kDa protein, termed p91 or Stat1α, p84 or Stat1β, and p113 or Stat2, respectively [Kessler et al., 1990; Fu et al., 1992]. The sequences of the 91 and 84 kDa proteins are identical, except that the 38 COOH-terminal amino acids of the 91 kDa protein are absent in the 84 kDa protein

Received January 17, 1995; accepted April 26, 1995.

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[Schindler et al., 1992a; Improta et al., 1994]. The DNA binding component of the ISGF3 complex is a 48 kDa protein, p48 [Veals et al., 1992]. Its sequence analysis has assigned it to a family of DNA binding proteins, that includes IRF1, IRF2, and ICSBP [Pine et al., 1990]. Upon activation, ISGF3 promptly moves into the nucleus where it binds to the ISREs and switches on transcription of inducible genes [Levy et al., 1989; Schindler et al., 1992b]. IFN-resistant mutant cell lines and complementation of their defects have proved a powerful tool in clarifying the signal transduction cascade from ligand binding to ISGF3 binding to the ISRE [John et al., 1991]. The subsequent events leading to activation of the basal transcription machinery, however, are still obscure.

We have used the enhancer-promoter of the IFN-activatable Ifi 202 gene to investigate IFN-induced gene activation. The 202 gene is part of a cluster of at least six IFN-activatable genes, initially designated Ifi 200, located on murine chromosome 1. This family also comprises Ifi 201 and 204, which cross-hybridize both to each other and to the 202 gene [Opdenakker et al., 1989]. The 5′ promoter region of the 201, 202, and 204 genes (known as the b segment) is 800 bp in length and displays a remarkable similarity in sequence (over 82%) extending through both the untranslated exon 1 and intron 1. This segment does not contain any canonical TATA element, and its ISRE (designated GA box), homologous to the ISRE of other stimulatable genes, is located in the first exon at approximately +40 bp downstream from the major transcription start sites [Samanta et al., 1986; Gribaudo et al., 1987]. An inducible GA box-binding activity, homologous to the ISGF3, is present in nuclear extracts of IFN-α treated cells in electrophoretically mobility shift assays [Gariglio et al., 1994].

Previous studies have demonstrated that both injection in vivo of an IFN-inducer, the synthetic dsRNA poly rI:rC, and IFN-α treatment in vitro augment the expression of the Ifi 202 gene in various strains of mice with the sole exception of the C57BL/6 strain, suggesting that host genotype somehow controls gene induction by IFNs [Gariglio et al., 1992].

This report further shows that even though IFN-α does not stimulate the expression of the Ifi 202 gene in C57BL/6 mouse strain (1) the ISGF3 complex is normally assembled and bound to the 202 ISRE, and (2) in transient transfection assays, the 202 ISRE driving the endogenous promoter is not activated in C57BL/6-derived cell lines, whereas it is fully active when linked to a heterologous promoter (c-fos). Evidence is thus available for the first time to support the view that cell factors determined by host genotype control the ability of the ISGF3 complex to activate the transcription of IFN-inducible genes. This finding is also open to exploitation in the establishment of a suitable model for the characterization of these still unidentified regulatory genes.

MATERIALS AND METHODS

Mice

Pathogen-free, 6- to 8-week-old DBA/2 and C57BL/6 female mice were purchased from Charles River Laboratories (Calco, Italy). All animals were maintained in our animal house only for the duration of the experiments.

Cells

NIH-3T3 cells (from American Tissue Culture Collection [ATCC], Rockville, MD) were cultured in Dulbecco minimal essential medium (DMEM) supplemented with 10% donor calf serum (Serolab). BLK SV HD.2 A.5R.1 A.3R.1 cells (transformed mouse fibroblast, from ATCC) (BLK), were cultured in DMEM supplemented with 10% fetal calf serum (Serolab). L12R4 and EL-4 T cell lymphomas were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Only exponentially growing cells were used.

Oligonucleotide Probes

Double-stranded oligonucleotides corresponding to wild and mutated 202 gene ISRE were synthesized on an Applied Biosystem (Foster City, CA) 308A DNA synthesizer: 202 ISRE (GA box), 5′-TCCAAAGCCAGGGAAATTGAAAG-CTATGAACGAAACTGGGAG-3′ annealed to 5′-CTCCCAGTTTCGTTCATAGCTTTCAA-TTTCCCTGGCTTTGGA-3′ [Ito et al., 1989]; mutated GA box, 5′-TCCAAAGCCAGGCACA-TTGACAGCTATGAACGAAACTGGGAG-3′ annealed to 5′-CTCCCAGTTTCGTTCATAGC-TGTCAATGTGCCTGGCTTTGGA-3′ (mutated bases are underlined). They were labeled at the 5′ end with γ-^{32}P-ATP by T4 polynucleotide kinase, annealed and purified by polyacrylamide gel electrophoresis.

Interferons

Recombinant hybrid human IFN-α A/D (spec. act.: 4×10^8 U/mg of protein), used as source of murine IFN-α, and recombinant murine IFN-γ (spec. act.: 4×10^7 U/mg of protein) were kindly provided by Drs. Michael Brunda, Hoffmann-La Roche, Nutley, NJ, and Gianni Garotta, Hoffmann-La Roche, Basel, respectively.

Plasmids

Plasmid J21 (kindly provided by P. Matthias, Basel, Switzerland) was cut with HindIII and treated with Klenow DNA polymerase to generate blunt ends. Three tandem copies of the 202 gene ISRE (GA box) were inserted into this site in order to link this GA trimer to the minimal fos promoter and the CAT reporter gene (see Fig. 7). Plasmid pBLCAT6b was obtained by ligation of the 804-nucleotide-long PstI-HindIII fragment (segment b) from the 202 gene to pBLCAT6 (kindly provided by Dr. G. Schutz, Heidelberg, Germany) linearized with PstI and HindIII (Fig. 1) [Boshart et al., 1992].

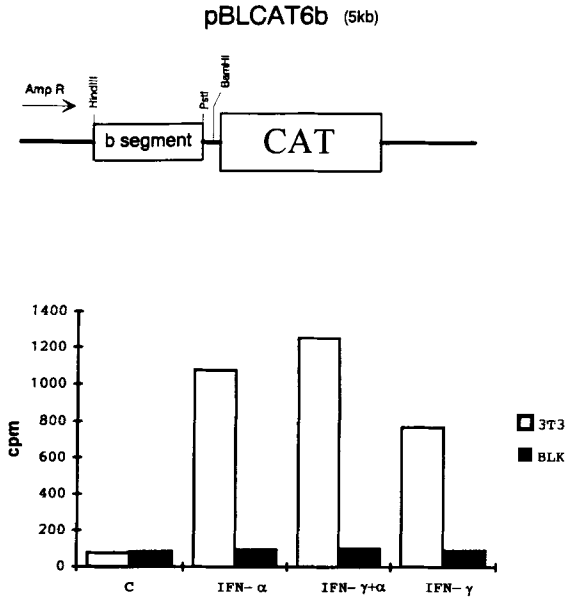

Fig. 1. Transactivation of the 202 gene promoter by IFN in NIH3T3, but not in BLK cells. Plasmid pBLCAT6b containing the b segment from the 202 gene was transiently transfected into both NIH3T3 and BLK cell lines by calcium phosphate method. Thirty-six hours after transfection, cells were left untreated or treated with IFN-α, IFN-γ for 16 h, or IFN-γ followed by IFN-α (priming) for further 8 h (see Materials and Methods), collected, and assayed for CAT activity. Each transfection was independently repeated at least three times and one representative is reported.

Nuclear Extracts

Nuclear extracts were prepared according to Dignam et al. with some modifications [Dignam et al., 1983]. The cells were treated in three conditions: 2 h with 1,000 U/ml of IFN-α; 16 h with 1,000 U/ml of IFN-γ, followed by 1 h with 1,000 U/ml of IFN-α; 16 h with 1,000 U/ml of IFN-γ. Controls received no IFN treatment. Cells were harvested, washed twice in cold PBS, and lysed by homogenization in hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT) with the addition of 0.2 mM PMSF, 1 μg/ml pepstatin, 0.1 mM benzamidine, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. The homogenate was checked microscopically for cell lysis and centrifuged for 10 min at 600*g* to pellet the nuclei. Nuclear proteins were extracted from the nuclear pellet with 20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitors. Nuclear extracts were clarified at 25,000*g* for 30 min, and stored in liquid nitrogen. Protein concentration was determined by the Bio-Rad (Richmond, CA) assay.

Electrophoretic Mobility Shift Assay (EMSA)

Protein-DNA binding reactions were carried out in a final volume of 20 μl of solution containing 15 μg of nuclear extracts, 300 ng of sonicated calf thymus DNA, and 1 ng of 5′ end labeled probe in the presence of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT. Extracts were incubated for 20 min at room temperature with 1 μg of anti-91N (Affiniti, Nottingham, U.K.), then the probe was added and incubated for 20 min, and the reaction mixtures were loaded onto 6% non-denaturing, polyacrylamide gel. Specific competitor DNA was added just before the radiolabeled probe. Gels were run for 3–4 h in 0.5 × TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA), dried, and autoradiographed on X-ray film.

RNA Analysis

Total cellular RNA was prepared from either tissue culture or mouse organs by guanidinium thiocyanate/cesium chloride centrifugation method, as previously described [MacDonald et al., 1987]. Twenty microgram RNA samples were transferred to Zeta-Probe membranes (Bio-Rad) and hybridized to the following cDNA probes: (1) a murine 202 cDNA probe (1.1-kbp EcoRI fragment from pC51 clone) [Samanta et al.,

1986]; (2) a murine Mx-1 cDNA probe (2,167 bp BamHI-HindIII fragment from pSP64-Mx1 clone) [Noteborn et al., 1987]. Hybridization to (^{32}P)dCTP-labeled cDNA probes was performed at 65°C in 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% SDS. The washes were all performed at 65°C for 20 min, twice in 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 5% SDS, and twice in the same buffer, but with 1% SDS. Filters were then exposed at −80°C to Hyperfilm MP films (Amersham, Arlington Heights, IL) with intensifying screens for autoradiography.

DNA Transfections and CAT Assays

DNA transfection was performed with the calcium phosphate method [Wigler et al., 1979]. The day before transfection the cells were plated in growth medium at a density of 0.5×10^6 cells/60 mm diameter dish. This was replaced by fresh growth medium 3 h before transfection. Supercoiled plasmid DNA (4 μg/plate of each plasmid) was transfected by $CaPO_4$ precipitation. The precipitates were left on the cells overnight. At 16 h before harvesting, the cells were treated with 1,000 U/ml of IFN-α. Cell extracts were prepared 48 h post-transfection for CAT activity assays by incubating 30 μg of protein for 2 h at 37°C with (14 l)chloramphenicol (50–60 mCi/mmol; Amersham) [Gorman et al., 1982]. The assay of chloramphenicol acetyltransferase activity was performed according to standard methods [Sambrook et al., 1989].

Southwestern Blotting

This was performed by protocols described previously with some modifications [Hübscher, 1987]. NIH 3T3 or BLK cells were left untreated or treated for 16 h with 1,000 U/ml of IFN-γ, followed by 1 h with 1,000 U/ml of IFN-α. Nuclear extracts (100 μg) were denatured in SDS-sample buffer, heated at 37°C for 10 min, and size-fractionated by analytical 8.5% SDS-PAGE. Proteins were electrotransferred onto nitrocellulose filters (Amersham), in 25 mM Tris-190 mM glycine, 1 mM EDTA, and 0.01% SDS. Proteins bound on the filter were renatured by incubation at 4°C for 24 h in 100 ml of 10 mM Hepes, ph 7.9, 50 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 5% milk-powder (Carnation non-fat). For DNA-binding the filter was transferred into a bath containing 8 ml binding buffer (same composition as above, except only 0.25% milk-powder is used) with 6 μg denatured sonicated calf thymus DNA and 1×10^6 cpm/ml ^{32}P labelled GA box, and was gently shaken at 4°C for 12–15 h. Filters were washed twice in 50 ml binding buffer each (without DNA) for 15 min, air-dried, and exposed to X-ray film.

Immunofluorescence

Cells were plated on chamber slides at low density (approximately 10^4 cells per cm^2), grown for 2 to 3 days, and treated as described by Loh et al. [1994]. The samples were blocked with 5% FCS in TBS-T. The primary monoclonal antiserum was diluted in 1% FCS in TBS-T at 1:40 for 91N (anti-91 kDa protein) and incubated at 37°C for 1 h. Anti-mouse immunoglobulin G conjugated to a fluorescein isothiocyanate (Sigma Immunochemicals, St. Louis, MO) secondary reagent was diluted at 1:40 in 1% FCS in TBS-T, and incubated at 37°C for 1 h. The cells were washed in TBS-T, mounted in Mowiol 20%, and examined with the ×50 objective.

RESULTS

Functional Analysis of the 202 Promoter in C57BL/6 Derived Cell Lines

We have previously reported that the 202 gene induction by IFN treatment is significantly reduced in C57BL/6 derived cells [Gariglio et al., 1992]. To determine whether this reduction is related to a defect in the transacting factors involved in transcriptional activation of the 202 gene, transient transfections were therefore performed in both positive and negative cell lines. Plasmid pBLCAT6b, containing the 804 bp (b segment) regulatory region of the 202 gene linked to the bacterial CAT-coding region, was transfected into NIH3T3 and in C57BL/6-derived BLK cells by $CaPO_4$ coprecipitation. Thirty-six hours after transfection, the cultures were treated with IFN-α, IFN-γ, or IFN-γ followed by IFN-α (priming). Extracts were prepared and assayed for CAT activity. Both IFN-α and IFN-γ led to a strong induction of CAT activity in the NIH3T3 cells (14-fold and 10-fold, respectively), that further increased when the two IFNs were combined in the priming protocol (Fig. 1). By contrast, stimulation of CAT activity in extracts from BLK cells was almost undetectable regardless the IFN-treatment (Fig. 1). Altogether these results indicate that the b segment is not activatable by IFNs in the BLK cells.

IFN-α-Induced Formation of ISGF3 Is Intact in C57BL/6 Derived Cell Lines Displaying Decreased 202 Activation

The synthetic 42-mer oligonucleotide containing the ISRE of the 202 gene, called GA box, produced in EMSA a strongly retarded band when mixed with nuclear extracts of IFN-treated L1210 cells [Gariglio et al., 1994]. This band, which is only induced by type I IFN, corresponds to the binding of the mouse transcription factor ISGF3 to the ISRE [Gariglio et al., 1994]. To see whether this lack of induction was related to a defect in the STAT factors, the GA box was mixed with nuclear extracts from NIH3T3 or BLK cells treated with IFN-α and IFN-γ separately, or pretreated with IFN-γ and subsequently stimulated with IFN-α; the latter treatment has been reported to induce optimal levels of ISGF3 activity. The presence of the ISGF3 complex was assessed by EMSA. Little specific DNA binding activity was present in extracts from NIH3T3 after IFN-α treatment (Fig. 2, lane 3) but abundant activity was detected after the superinduction protocol (lane 7). Extracts from control cells or IFN-γ treated cells had no ISGF3 binding activity (lanes 1 and 5). Treatment with IFN-γ indeed does not by itself induce ISGF3 formation but prepares the cells to better respond to IFN-α [Darnell et al., 1994]. The specificity of the activated DNA binding complex was confirmed by demonstrating that unlabeled GA box inhibited the formation of the band (lane 9). By contrast a 200-fold excess of an unlabeled oligo mutated in the ISGF3 binding site only slightly competed in the case of NIH3T3 extracts (Fig. 2, lane 10) and did not compete in the case of BLK extracts (Fig. 3, lane 10). Footprinting analysis has indeed demonstrated that the binding site of ISGF3 within the 42 bp 202 ISRE is limited to the repeated GAAA motifs; ISGF3 binding was completely abolished by mutations in these motifs (data not shown). Moreover, anti-91N antibodies shifted the band corresponding to ISGF3 (Fig. 2, lane 8), suggesting

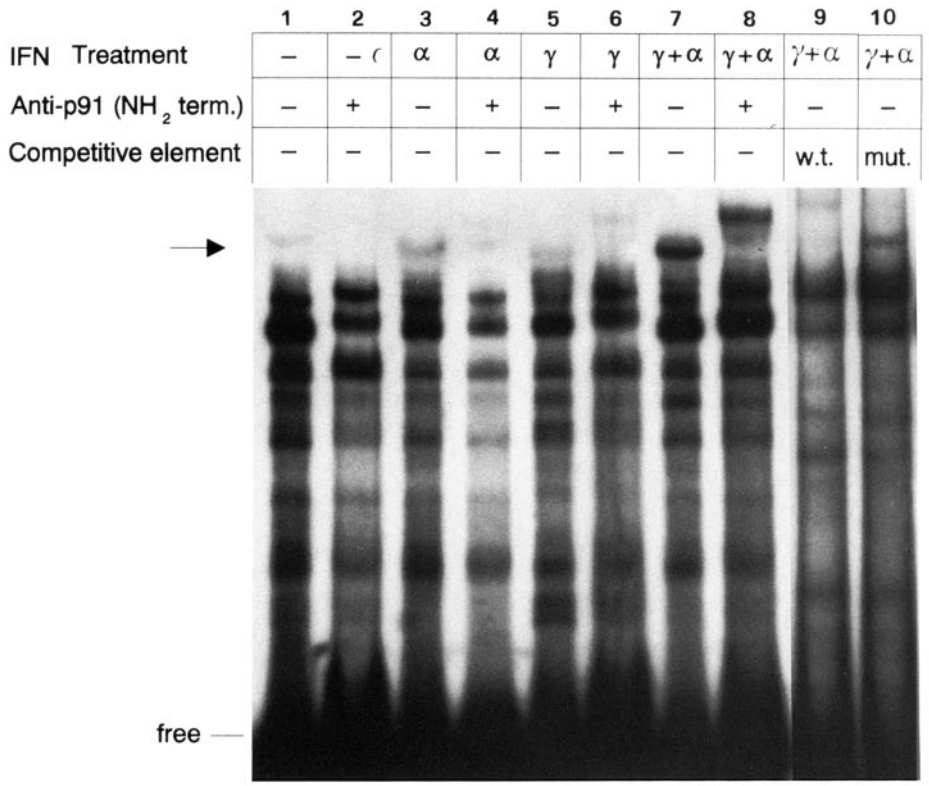

Fig. 2. Time course for IFN-α induced ISGF3 formation with NIH3T3 nuclear extracts. EMSA. The probe used is the 42 bp GA box from the 5′ regulatory region of the 202 gene. Nuclear extracts were made with cells uninduced (*lanes 1 and 2*), induced with IFN-α for 2 h (*lanes 3* and *4*), induced with IFN-γ for 16 h (*lanes 5 and 6*), induced with IFN-γ for 16 h and then with IFN-α for 1 h (*lanes 7, 8, 9, and 10*). Lanes 1 to 8 contain the probe mixed with nuclear extracts. Lane 9, in addition to the probe and nuclear extracts, contains a 50-fold excess of specific DNA competitor (the unlabeled GA box), while lane 10 contains the mutated GA box. Antibodies to p91 were added for lanes 2, 4, 6, and 8, respectively.

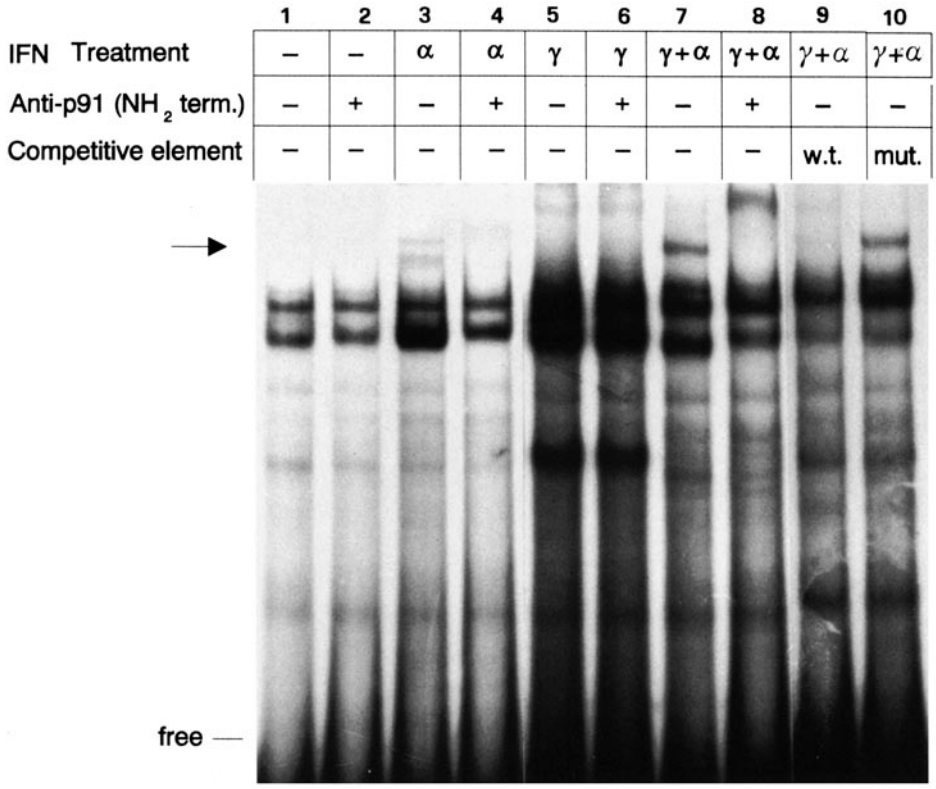

Fig. 3. Time course for IFN-α induced ISGF3 formation with BLK nuclear extracts. EMSA. The probe used is the 42 bp GA box from the 5′ regulatory region of the 202 gene. Nuclear extracts were made with cells uninduced (*lanes 1 and 2*), induced with IFN-α for 2 h (*lanes 3* and *4*), induced with IFN-γ for 16 h (*lanes 5 and 6*), induced with IFN-γ for 16 h and then with IFN-α for 1 h (*lanes 7, 8, 9, and 10*). Lanes 1 to 8 contain the probe mixed with nuclear extracts. Lane 9, in addition to the probe and nuclear extracts, contains a 50-fold excess of specific DNA competitor (the unlabeled GA box), while lane 10 contains the mutated GA box. Antibodies to p91 were added for lanes 2, 4, 6, and 8, respectively.

that at least the p91 component or Stat1 is present in this IFN-inducible complex. When nuclear extracts from IFN-treated BLK cells were examined, a retarded complex similar to the ISGF3 activity was detected particularly with the super-induction protocol (Fig. 3, lane 7). The identity with the canonical ISGF3 is strongly supported by the findings that the BLK complex is super-shifted by anti-p91N antibodies and is not displaced by the unlabeled oligo mutated in the ISGF3 binding site (Fig. 3, lanes 8 and 10).

We also checked ISGF3 binding in other C57BL/6-derived cell lines. As shown in Figure 4, two lymphoid cell lines, namely L12R4 and EL-4, displayed upon IFN-treatment a retarded complex corresponding to the ISGF3 activity. Two hours of IFN-treatment was chosen as the optimum for generating the appearance of the ISGF3 complex in the light of previous kinetic studies [Gariglio et al., 1994]. In this case IFN-α alone was sufficient to induce detectable amounts of the ISGF3 complex.

Despite its presence, ISGF3 failed to activate the 202 promoter upon IFN treatment in C57BL/6 derived cell lines.

ISGF3 p48 Subunit-Binding to the 202 ISRE Is Not Defective in C57BL/6 Derived Cell Lines

The ISGF3 complex contacts DNA through the p48 subunit [Veals et al., 1992]. The possibility of an altered contact between GA box and p48 in BLK cells was investigated by "Southwestern" analysis of nuclear extracts from NIH3T3 or BLK cells untreated or treated with IFN, separated onto an SDS-PAGE, and then transferred to a nitrocellulose filter. After allowing the proteins to renature, the filter was incubated with ^{32}P-labelled GA box. Autoradiography of the filter revealed that nuclear extracts from both NIH3T3 and BLK cells treated with IFN-α contained a strong band migrating in the 48 kDa area (Fig. 5, lanes 2 and 4), whereas those from untreated cells contained only a faint band in this area (lanes 1 and 3), whose presence

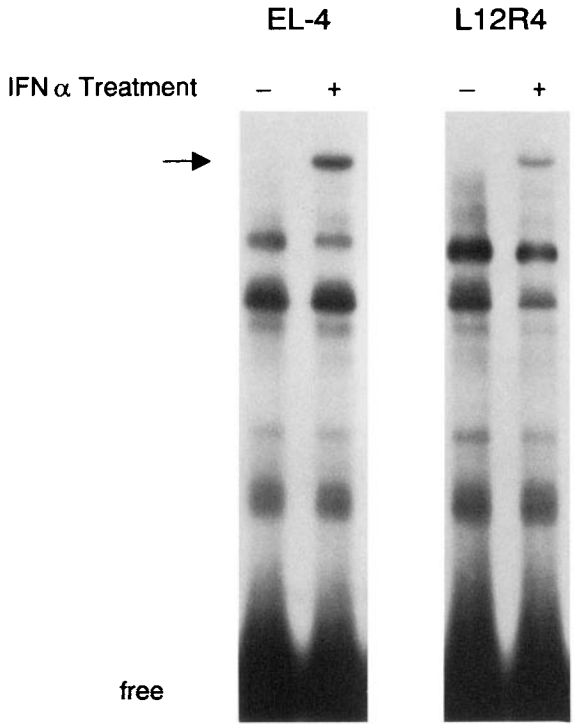

Fig. 4. ISGF3 binding is also induced in lymphoid cell lines derived from C57BL/6 mouse strain. EMSA. The end-labeled GA probe was incubated with nuclear extracts generated from both L12R4 and EL-4 cells untreated or treated with IFN-α for 2 h. The retarded complex corresponding to ISGF3 is indicated by the arrow.

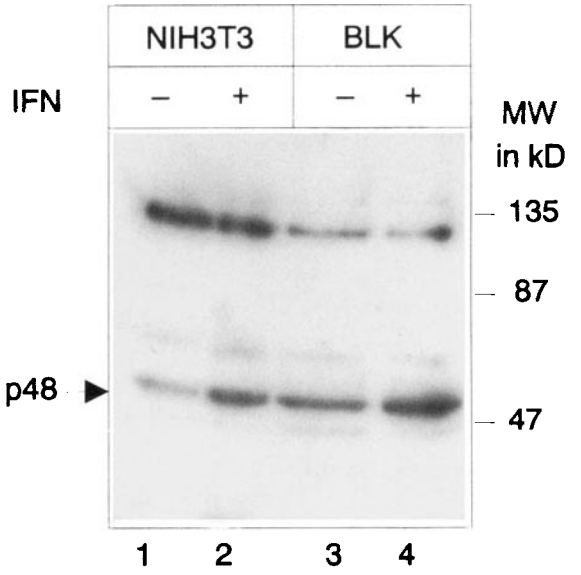

Fig. 5. "Southwestern" blot analysis with nuclear proteins from NIH3T3 and BLK cells. NIH3T3 or BLK cells were left untreated or treated for 16 h with 1,000 U/ml of IFN-γ followed by 1 h with 1,000 U/ml of IFN-α. Nuclear extracts (100 μg per lane) from both cell lines were size-separated in a denaturing protein gel, blotted to a nitrocellulose filter, renatured, and incubated with radiolabeled GA probe. The signal seen at 48 kD is specific for the GA box. The signal at position of 120 kD is due to non-specific binding, as we have ascertained by incubating a control filter with a different probe which also binds to this high mol. wt. protein, but not to the region in which the p48 migrates (data not shown). Molecular sizes are indicated at right (in kilodaltons).

could have been due to cytoplasm contamination during the extraction. As expected IFN treatment significantly increased the intensity of the migrating band corresponding to the p48 subunit.

We conclude that ISGF3 binding to the 202 ISRE through the p48 subunit is comparable in both inducible NIH3T3 and uninducible BLK cells.

Nuclear Translocation of p91 Is Not Defective in C57BL/6 Derived Cell Lines

It has previously been shown that p91 is activated for nuclear translocation in response to either IFN-α or IFN-γ [for a review, see Darnell et al., 1994]. Immunofluorescence staining was performed to examine its intracellular distribution in both BLK and NIH3T3 cell lines before and after IFN treatment. The 91 kDa protein was stained prominently in the cytoplasm of the untreated cells (Fig. 6), but after the superinduction protocol (IFN-γ for 16 h plus IFN-α for 1 h) it was rapidly localized in the nuclei of both cell lines. This translocation was almost undetectable after IFN-α only (data not shown), very likely because the immunofluorescence technique was not sufficiently sensitive to small amounts of translocated p91.

These data clearly demonstrate that, even though p91 is normally translocated to the nucleus upon IFN treatment, 202 gene transcription activation is not revealed in C57BL/6 derived cells. Other components downstream from the ISGF3 complex are therefore required for 202 gene expression.

ISGF3 Complex Activates the 202 ISRE When Linked to a Heterologous Promoter in C57BL/6 Cells

At this stage, it was important to show that STAT factors in BLK cells, and more in general in the C57BL/6 inbred strain, were not functionally defective per se. Plasmid J21D4 (Fig. 7, upper panel), containing three tandem copies of the 202 ISRE linked to the minimal fos promoter and the CAT gene, was therefore transfected in both inducible (NIH3T3) and uninduc-

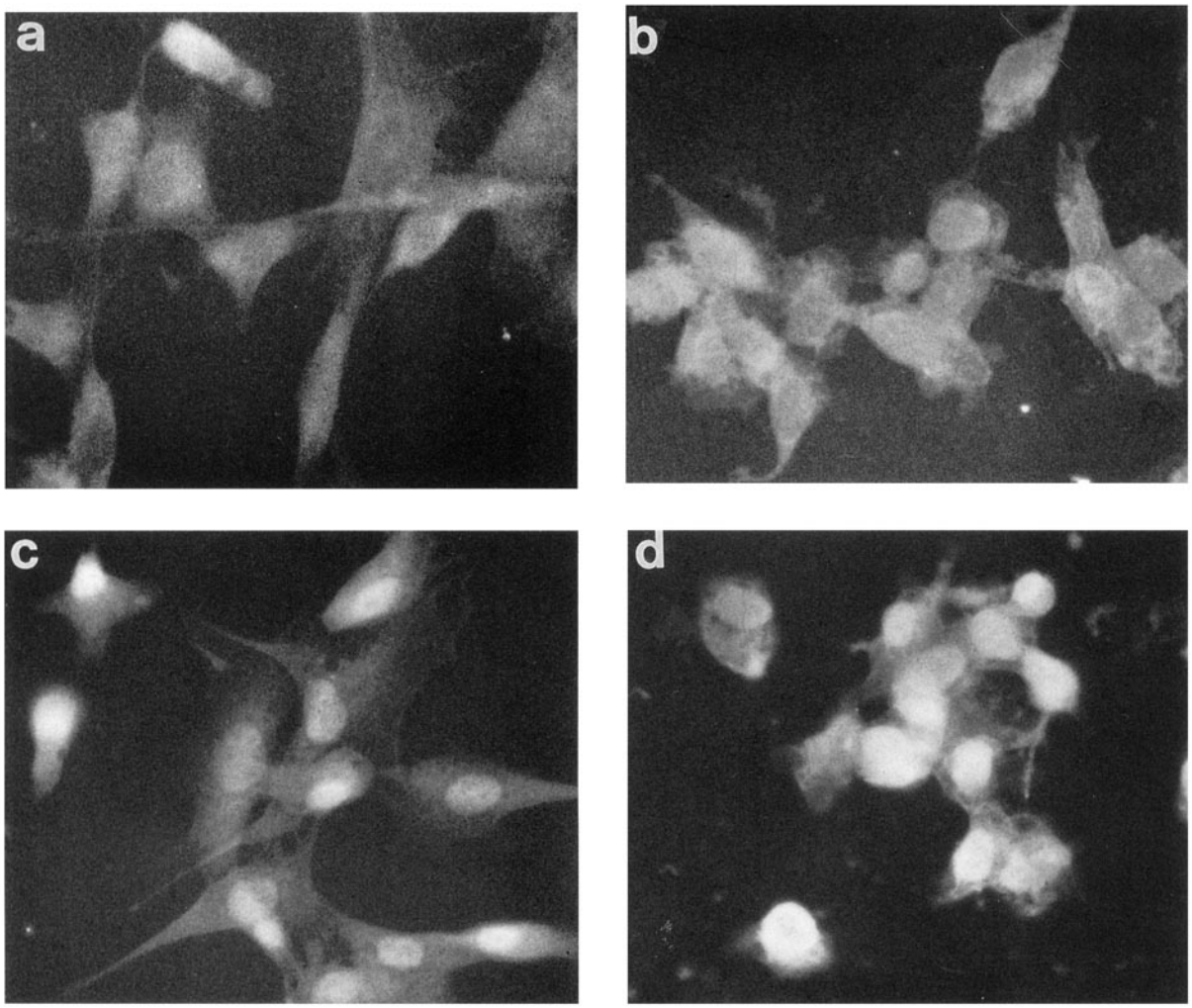

Fig. 6. Translocation of the 91kDa protein to the nucleus after treatment of both NIH3T3 and BLK with IFNs. NIH3T3 cells and BLK cells were left untreated (**a** and **b**, respectively) or treated with IFN-γ for 16 h followed by 30 min IFN-α treatment (**c** and **d**, respectively), and then examined by immunofluorescence with anti-p91N antibodies.

ible (BLK) cell lines. As shown in Figure 7, CAT activity was increased 10-fold and 9.6-fold in NIH 3T3 and BLK cells, respectively, following IFN-α treatment. As expected IFN-γ did not activate CAT activity in both cell lines. These results are in accordance with the findings that ISGF3 binding to the 202 ISRE is detectable in the C57BL/6 cell lines after IFN treatment.

Lack of 202 But Not Mx mRNA Induction by IFN in C57BL/6 Mice

The finding that 202 ISRE is stimulatable by IFN-α when linked to a heterologous promoter (c-fos promoter) suggested that this defect is limited to the 202 system. To confirm that this defect reflected the in vivo situation, total RNA was isolated from spleen cells of DBA/2 or C57BL/6 mice pretreated with dsRNA. The RNA samples were then analyzed by Northern blotting using both 202 and Mx cDNAs as probes. In agreement with the in vitro results previously reported [Gariglio et al., 1992], the 202 mRNA was not induced by dsRNA in C57BL/6 mouse strain (Fig. 8, lane 4), whereas it was strongly increased in the DBA/2 mouse strain. By contrast, treatment with poly rI:rC increased the level of Mx mRNA more than 10-fold (Fig. 8, lanes 2 and 4) in both DBA/2 and C57BL/6 mice.

DISCUSSION

Previous in vivo studies have shown that the Ifi 202-type genes are induced by IFN-α in the DBA/2, BALB/c and C3H/HeJ mouse strains, but not in inbred C57BL/6 mice, and that non-

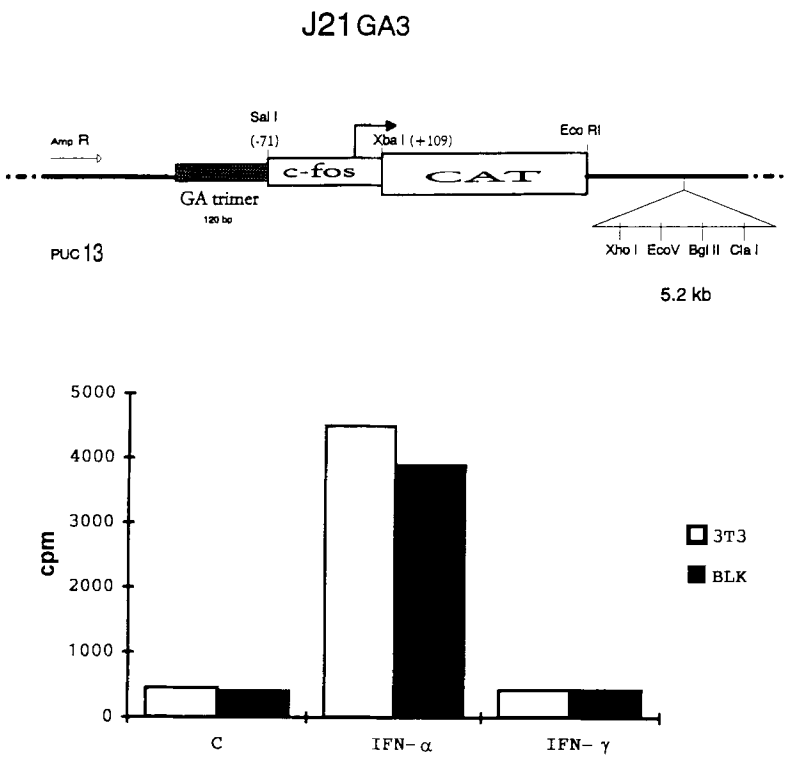

Fig. 7. Interferon inducibility of the 202 ISRE linked to a heterologous promoter in both NIH3T3 and BLK cell lines. Three tandem copies of the GA box were fused to the HindIII site of the plasmid J21, a CAT reporter construct linked to the minimal fos promoter. The resulting plasmid J21GA3 was transfected in NIH3T3 and BLK cell lines by calcium phosphate method. Thirty-six hours after transfection cells were treated with IFN-α or IFN-γ for further 16 h, or left untreated, collected, and assayed for CAT activity. Each transfection was independently repeated at least three times and one representative is reported.

induction was limited to this cluster of genes, since the 2′–5′$(A)_n$ synthetase, another IFN-activatable gene, was normally expressed [Gariglio et al., 1992].

This study demonstrates that the lack of 202 inducibility by IFN in the C57BL/6 strain is not caused by a defect in the STAT factor family, but by non-cooperation between STAT proteins and unidentified transacting factors related to the strain's genetic background. This statement is based on the following observations. Treatment of C57BL/6 derived cell lines with IFN-α indeed induces a retarded complex similar if not identical to the canonical ISGF3. This complex is superinduced by IFN-γ priming and contains both p48 and p84/91, as shown by the Southwestern blot and the supershift with anti-p91 antibodies, respectively. Following IFN-treatment, the complex translocates into the nucleus, as observed in fluorescence studies, where it binds to the GA motif, as demonstrated by the lack of competition with an oligo mutated in the GA frame. Thereafter, binding to the ISRE induces the expression of other genes, such as the Mx. Lastly, that the C57BL/6 strain contains functional STAT factors can also be deduced from the finding that the 202 ISRE, when linked to a heterologous promoter (minimal c-fos promoter), is also activated in BLK cells. The exact mechanisms whereby the 202 promoter is activated by IFNs depending on the host genotype remain to be elucidated.

The Ifi 200 cluster was mapped to murine chromosome 1, between the erythroid α-spectrin and the amyloid P-component loci [Choubey et al., 1989]. Upon treatment of cells with IFN, the Ifi 202 gene encodes a protein of 52 kDa that is phosphorylated on serine and threonine residues and accumulates in the cytoplasm. After prolonged IFN-treatment, it mainly localizes in the nucleus, where it binds dsDNA [Choubey and Lengyel, 1993]. Another well-characterized member of this family, namely the 204 gene, encodes for a protein of 72 kDa that upon IFN-treatment translocates into the nucleolus and nucleoplasm [Choubey and Lengyel, 1992]. In the human genome, a cluster of related genes localized to the human chromosome 1q21-22,

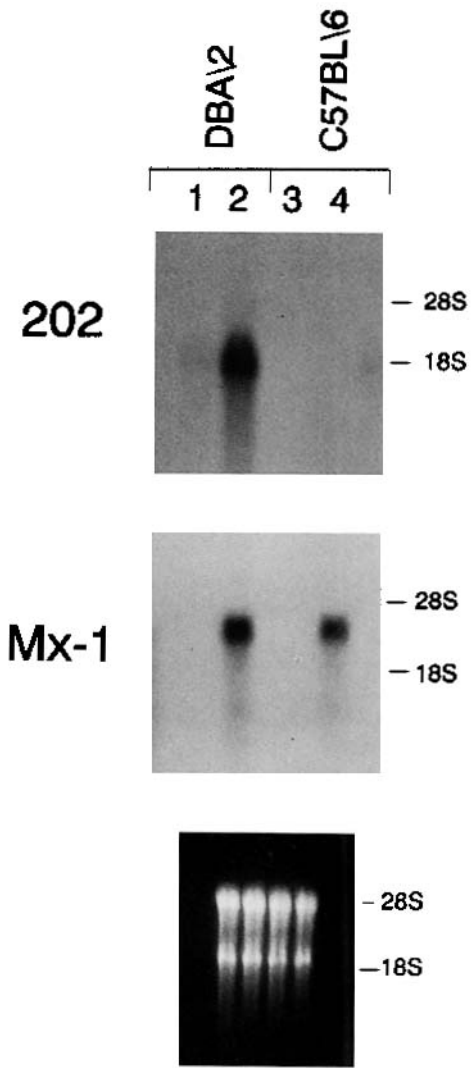

Fig. 8. Northern blot analysis of total RNA from DBA/2 and C57BL/6 mice untreated or treated with poly rI:rC. Total RNA was prepared from the spleens of DBA/2 or C57BL/6 mice untreated or treated for 18 h with poly rI:rC (200 μg i.p.). Total RNA was extracted and subjected to gel electrophoresis (10 μg per lane). After transfer to nitrocellulose, the blot was hybridized with the mouse 202 cDNA probe (**upper panel**). The same blot was then stripped and rehybridized with a cDNA probe of the murine Mx-1 cDNA (**lower panel**). *Lanes 1, 3:* no treatment; *lanes 2, 4:* IFN-α treatment. Ethidium bromide-stained RNA separated on the agarose gel used on the Northern blots, representing the analysis of equivalent amounts of intact total RNA isolated from the different mice. The positions of the rRNAs are indicated.

homologous to the mouse Ifi 200 cluster, has been recently identified [Briggs et al., 1992; Trapani et al., 1992; Tannenbaum et al., 1993]. One of them encodes a protein, designated as human myeloid cell nuclear differentiation antigen (MNDA), that specifically occurs in monocytes and granulocytes, as well as in more differentiated leukemia cells [Burrus et al., 1992; Briggs et al., 1994].

The cascade of events between IFN-binding and generation of ISRE-ISGF3 complex has been partly illustrated through the construction of mutant cell lines defective in the IFN response. Many independent mutants in which IFN-α was unable to induce gene expression have been isolated and classified in complementation groups determined by the type of complementing protein. To date groups U2, U3, and U6 have corroborated the importance of each component of the ISGF3 complex (p48 kDa, p91, and p113) in response to IFN-α and, as far as p91 is concerned, to IFN-γ [Velazquez et al., 1992; Müller et al., 1993]. In addition, the U1 and U4 mutants have emphasized the importance of the Jak family of protein tyrosine kinases in response to both IFN-α and IFN-γ [Watling et al., 1993]. Similar conclusions have been drawn by other investigators using mutant cell lines obtained by different selection strategies.

The difference in Ifi 200 gene induction in the C57BL/6 as opposed to the DBA/2 mouse strain represents a step further to understand the chain of events that lead to gene activation by IFNs. Several technical approaches have shown that STAT proteins do bind to the ISRE and activate gene transcription when the ISRE drives a heterologous promoter (c-fos) in C57BL/6 cell lines. Two conclusions can be drawn from our results: binding of ISGF3 complex to the ISRE is necessary, but not sufficient to activate the basal transcription machinery in response to IFNs and other unidentified elements are required to complete the transcription process. These elements are controlled by the host genotype and must interplay with the ISGF3 complex to induce gene transcription. The C57BL/6 inbred strain and the Ifi 200 gene cluster could be exploited to characterize these host-genotype dependent elements.

A few years ago, DeMayer and De Mayer-Guignard [1988] postulated the existence of regulatory genes influencing the IFN action, considered bona fide the result of structural gene activation. Our research has shown that structural genes, such as the Ifi 202-type genes, are differentially induced in relation to the genetic background, and hence provides the first evidence of how the host genotype, through the activity of undefined regulatory genes, may generate variability in the response to IFN action, and in resistance to infections. The immune

response to infection, in fact, appears to be controlled both by genes linked to the major histocompatibility complex (MHC-linked genes) and NOT-MHC-linked genes. In this regard, it is worthy of note that a locus, designated Lsh/Ity/Bcg gene, governing the early response to infection by Leishmania, Salmonella, and Mycobacteria, when IFNs appears to play an important biological role, has been mapped in chromosome 1 close to the Ifi 200 cluster [for a review, see De Mayer and De Mayer-Guignard, 1988]. Since the C57BL/6 strain displays a different pattern of resistance to such infection, one can speculate that some indirect relationship between this pattern of resistance and differential induction of Ifi 202-type genes could exist.

ACKNOWLEDGMENTS

This study was supported by grants from the Italian National Research Council (C.N.R.) (P.F. A.C.R.O., and F.A.T.M.A.) and from the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.). M.G. was supported by a fellowship from "Comitato Regionale Piemontese Gigi Ghirotti."

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