Increased Levels of Interferon Regulatory Element-binding Activities in Nuclei of High Interferon-producing If-1<sup>h</sup> Mice<sup>1</sup>

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Abstract
The transient induction of type I interferon (IFN) genes following a viral infection involves transcriptional derepression and activation, mediated by positive and negative factors which bind to upstream cis-acting elements. We have transfected the human IFN-β gene into primary splenocytes from mice regulated by the If<sup>1</sup> locus and have shown that the exogenous gene is regulated by this locus in a manner similar to that of endogenous IFN genes. Using nuclear extracts from splenocytes of C57BL/6 (If-i<sup>h</sup>) and BALB/c (If-i<sup>h</sup>) mice in gel retardation assays, we found that levels of DNA-binding activities for the interferon regulatory element and its subelements were constitutive in nuclear extracts of spleen cells. Levels of DNA binding to the interferon regulatory element were higher in extracts from the nuclei of If-i<sup>h</sup> mice and thus correlate with the higher levels of human IFN-β mRNA detected in these transfected cells and the transcription of the endogenous IFN genes. Higher levels of DNA-binding/transcription factors found in nuclei from spleen cells of If-i<sup>h</sup> mice may be involved in the expression of the If-1 phenotype.

Introduction
The IFNs<sup>2</sup> constitute a family of cytokines which exert varied and potent biological effects on animals and cultured cells. This group of proteins is subdivided into the type I or viral IFNs (IFN-α and IFN-β) and the type II immune IFNs (IFN-γ) (for reviews, see Ref. 1). Type I IFNs are not produced under normal physiological conditions, but an exposure to inducers (typically a virus or a double stranded RNA) leads to transcriptional activation and a transient peak of expression of IFN mRNA and protein. The cis-acting elements involved in induction of the IFN genes have been studied extensively by several groups (2–6).

Mutational analysis of the HuIFN-β promoter has identified three positive regulatory domains and two negative regulatory domains. Two distinct PRDs (I and II: −77 to −64 and −66 to −55, respectively) within the IRE (−77 to −37 relative to the mRNA start site) were required for induction (3, 4). PRDII, which was very similar to PRDII, was shown to synergize with PRDI and PRDII to increase virus inducibility (7). On the other hand, Fujita et al. (5, 6) have identified a more extensive region in the IFN-β promoter (−117 to −39 from the cap site) that contains a repetitive hexanucleotide motif which, when multi-merized, mediates virus-induced activation of transcription.

At present, four different PRDI-binding factors or hexamer-binding factors have been identified: PRDI-BF1 and PRDII-BF1 (8), IRF-1 (9), and IRF-2 (10). A number of factors have also been shown to bind to PRDII: PRDII-BF (8) and NF-κB (11–13). No DNA-binding activities were detected in the negative regulatory domain. Although all of these factors bind specifically to PRDI or PRDII, their relative contribution to the regulation of the IFN-β gene is not clear.

The complexity of the regulation of the IFN-β promoter provides the possibility for numerous levels of regulation. It is also probable that the actual induction mechanism may differ in vivo as opposed to in vitro and in different cell types. Among other parameters, we must note that in inbred mice, the levels of IFN expressed following induction are modulated by a number of factors (14–16), including the genotype. A series of genetic loci, known as the If loci, are involved in this regulation. If-1 controls the levels of both IFN-α and IFN-β expression in mice or in mouse splenocytes in response to induction by NDV. This locus exists as two alleles, and mice (or their spleen cells) carrying the If-1<sup>h</sup> allele (e.g., the C57BL/6 strain) produce IFN levels which are 10- to 15-fold higher than those carrying the If-1<sup>h</sup> allele (e.g., BALB/c) following infection with NDV (15, 16). We have shown previously that this effect is due to higher levels of transcription of the IFN genes in induced splenocytes of If-1<sup>h</sup> mice as compared to those of If-1<sup>l</sup> mice (17). The If-1 phenotype is specific to lymphoid cells and is not expressed, for example, in fibroblasts (16, 17).

Our results show that 280 bp of the HuIFN-β promoter contain sufficient information for induction and If-1 regulation in primary splenocytes. Factors binding to the regulatory region of the HuIFN-β gene are present constitutively in spleen cell nuclei, indicating that IFN induction in these cells occurs by a different mechanism than that described for L929 cells. Finally, levels of factors binding to the IRE in gel retardation assays correlate to the levels of transcription of the endogenous IFN genes in these cells.

Received 9/3/91.

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3 The abbreviations used are: IFN, interferon; Hu, human; PRD, positive regulatory domain; IRE, IFN-β regulatory element; HEX, hexamer; NDV, Newcastle disease virus; Mu-, murine; kD, kilodalton(s); kb, kilobase(s); bp, base pair(s); MEM, minimal essential medium; PCV, packed cell volume(s); NP-40, Nonident P-40; BrdUrd, 5-bromodeoxyuridine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; poly(A)<sub>3</sub>, polyadenylated; poly[d(T)<sub>12</sub>]; poly(dI:dC); poly(deoxyinosinic-deoxycytidylic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Vol. 3, 93–100, February 1992 Cell Growth & Differentiation 93
Table 1 Interferon titers* in culture media of spleen cell suspensions electroporated with the HuIFN-β gene*

<table>
<thead>
<tr>
<th>Mouse IFN</th>
<th>Human IFN</th>
</tr>
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<tbody>
<tr>
<td>Mock induced</td>
<td>Induced</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>&lt;6</td>
<td>60</td>
</tr>
<tr>
<td>&lt;6</td>
<td>960</td>
</tr>
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</table>

* IFN titers were determined 6 h postinduction, and all titers are in laboratory units.

Five × 10^6 cells were electroporated with 100 μg of pGEMHuIFN-β gene and 20 μg of pSVkneoΔ.

The human IFN-β gene is the 1.8-kb EcoRI fragment including 282 bp of 5' upstream sequence.

Results

Regulation of Expression of the HuIFN-β Gene by the IF-1 Locus in Transfected Murine Spleen Cells. Several groups have reported that expression of the HuIFN-β gene was regulated during induction in a manner similar to that of the endogenous MuIFN-β gene when transfected into murine cells (18-20).

We transfected the HuIFN-β gene (1.8-kb EcoRI fragment) into primary splenocytes from C57BL/6 (If-i') and BALB/c mice (If-i). The culture supernatants of the transfected cells were titrated (Table 1), and polyadenylated mRNAs were prepared and subjected to RNase mapping (Fig. 1). The levels of HuIFN-β mRNA and HuIFN product of the transfected gene followed the same pattern as that of the endogenous MuIFN. In splenocytes of If-i mice, we detected 10-fold more HuIFN activity and 5-10-fold more HuIFN-β mRNA after induction than with splenocytes of If-i mice. The length of the protected fragment confirms that transcription was initiated at the normal capping site. This result indicates that the exogenous gene is appropriately regulated by If-i. Transfection efficiencies were controlled by cotransfecting plasmid pSVkneoΔ, which constitutively expresses the neo gene, and determining the levels of neo-specific mRNA (data not shown).

The high homology demonstrated between the HuIFN-β and MuIFN-β promoters, especially within the IRE (Fig. 2; Ref. 21), suggests similar mechanisms of regulation of induction for the two genes.

Nuclear Factor Binding to the IRE. We carried out gel retardation experiments in order to examine nuclear proteins binding to different IFN-β regulatory elements in splenocytes and fibroblasts (L929 cells).

Nuclear extracts from mock-induced L929 cells yielded one major band (complex D) with this fragment (Fig. 3A, Lane 2). The intensity of complexes B, C, and D increased during induction, and complex A became detectable (Fig. 3A, Lane 3). The pattern of retarded bands for spleen cell nuclear extracts was similar to that observed with induced L929 cells (Fig. 3; compare A and B) with minor differences in the migration of complexes A and B. No significant difference was observed when extracts from mock-induced and induced splenocytes were compared. We obtained the same result with another inducer (polynosinic-polycytidylic acid) and after different times of induction (1, 2, and 5 h after induction) (data not shown).

It is also clear from the results presented in Fig. 3 that, in splenocyte nuclear extracts, the levels of DNA-binding activities specific to the IRE are higher (3- to 4-fold difference) in extracts from If-i' strains (C57BL/6 and DBA) than in those from If-i (BALB/c and C3H) strains, indicating that the level of binding to the IRE probe correlated with the If-i' genotype (Table 2; Fig. 3C). This difference was also observed when we used a larger probe (-202 to -39 from the cap site) in gel retardation assays (Fig. 4).

Efficiency of Complex Formation for the IRE with Nuclear Extracts from High and Low Producers. The observed higher binding activities for the IRE in the nuclear extracts from splenocytes of C57BL/6 mice could be due to higher levels of the nuclear protein(s) which bind to this sequence or to higher efficiency of complex formation (more stable binding, changes in cooperativity, or other causes). In order to compare the total efficiency of the binding complexes for the IRE in different extracts, we carried out a competition experiment with different amounts of C57BL/6 and BALB/c splenocyte nuclear extracts adjusted to give equivalent levels of binding activity. The formation of complexes was competed by adding increasing amounts of the unlabeled IRE. The results are presented in Fig. 5. To decrease the binding activities for the IRE to 50%, it was necessary to add 50-100-fold molar excess of competitor DNA to the C57BL/6 nuclear extract as compared to a 5- to 10-fold molar excess for BALB/c nuclear extract. This result suggests that the complexes obtained using C57BL/6 splenocyte nuclear extract were formed with higher efficiency than those from BALB/c splenocyte nuclear extract, rather than merely being due to higher levels of the given DNA-binding proteins.

Nuclear Factor Binding to HEX (PRDI-like) and PRDII. Several groups have reported a number of cellular factors...
which specifically bind to PRDI [or a PRDI-like sequence created by multimerization of a hexamer (AAGTGA)] and to PRDII (8–13). We used the oligonucleotides HEX and PRDII as probes to determine whether the observed difference in binding activities with the IRE probe between nuclear extracts of IF-1\(^\text{a}\) and IF-1\(^\text{I}\) splenocytes could be due to PRDI-binding factor(s), to PRDII-binding factor(s), or to the interaction between them.

We detected three complexes (P1, P2, and P3) in the mock-induced L929 cell nuclear extracts (Fig. 6A, Lane 2) with the PRDII probe. Levels of complexes P1 and P2 were increased following induction (Fig. 6A, Lane 3). Both complexes (P1 and P2) were detected in splenocyte nuclear extracts (Fig. 6A, Lanes 4 to 7). Again, no difference was observed between mock-induced and induced splenocyte nuclear extracts (Fig. 6A; compare Lane 4 with Lane 5, and Lane 6 with Lane 7). However, the binding activities in C57BL/6 nuclear extract and BALB/c nuclear extract were equivalent. All of the complexes observed with the PRDII probe (P1, P2, and P3) were competed by the unlabeled probe itself (data not shown). However, in C57BL/6 splenocyte nuclear extracts, only the P2 complex was competed by different oligonucleotides, including NF-\(\kappa\)B binding sites (IgKappa enhancer, H-2K\(^b\) promoter, and Mut-1). No competition was observed with Mut-2, which is not recognized by NF-\(\kappa\)B (Fig. 6A, Lanes 7 to 11). We observed the same competition using BALB/c splenocyte nuclear extracts and L929 cell nuclear extracts (data not shown). Moreover, treatment of uninduced L929 cell cytosol by the detergents deoxycholate and NP-40 resulted in the production of a complex with a mobility similar to that of the P2 complex (Fig. 6A, Lanes 12 and 13). These results indicate that only the P2 complex was indistinguishable from the NF-\(\kappa\)B factors previously described (11–13).

With the HEX probe, we observed two major complexes (H2 and H3) in the mock-induced L929 cell nuclear extracts (Fig. 6B, Lane 2). We detected complex H1 in the induced L929 cell nuclear extracts (Fig. 6B, Lane 3), and the intensity of complexes H2 and H3 was increased 3- to 4-fold (Fig. 6B, Lane 3). In splenocyte nuclear extracts, the same complexes were detected (H1, H2, and H3), and no changes occurred during induction (Fig. 6B, Lanes 4 to 7). Again, the levels of HEX-binding activities were similar between extracts from nuclei of IF-1\(^\text{a}\) and IF-1\(^\text{I}\) splenocytes.

The full length IRF-1 protein was prepared by in vitro translation of mRNA encoded by the coding sequence of an IRF-1 complementary DNA (22) and used in gel
Table 2. Interferon titers* in sera* from mice inoculated i.v. with NDV (in vivo) and in culture media from spleen cell suspensions induced with NDV (in vitro)

<table>
<thead>
<tr>
<th>Mouse strains</th>
<th>Serum</th>
<th>Spleen cell suspensions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mock induced</td>
<td>Induced</td>
</tr>
<tr>
<td></td>
<td>Mock induced</td>
<td>Induced</td>
</tr>
<tr>
<td>BALB/c</td>
<td>&lt;6</td>
<td>960</td>
</tr>
<tr>
<td>C3H</td>
<td>&lt;6</td>
<td>768</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>&lt;6</td>
<td>9,600</td>
</tr>
<tr>
<td>DBA</td>
<td>&lt;6</td>
<td>13,400</td>
</tr>
</tbody>
</table>

Each number is the average of five mice of each strain.
Serum and supernatant of spleen cell suspensions were collected after 8 h of induction.

Discussion

In this report, we have compared IFN induction in splenocytes of inbred mice to that observed in cultured murine fibroblasts. The lymphoid system in general and the spleen in particular are the main producers of IFN in cases of systemic exposure to virus or other inducers (1). One particular characteristic of lymphoid cells in general (and of spleen cells in particular), as opposed to other tissues, was already known: they express the Il-1 regulatory locus, which controls the levels of transcription of the IFN genes in splenocytes following induction with NDV (17) and which has been studied extensively from the point of view of classical genetics (for a review, see Ref. 16).

When the HULFN-β gene was transfected into splenocytes from C57BL/6 (II-1β) and BALB/c (II-1α) mice, it was inducible, and the relative levels of induction were similar to those observed for the endogenous genes (Table 1; Fig. 1). This finding clearly indicates that the portion of the gene used in these transfections carries sufficient information for both induction and Il-1 regulation of the gene. The kinetics of induction were found to be much more rapid than in L929 cells, as we have already reported (17).

As a next step, we analyzed the nature and levels of activities of factors binding to various elements of the HULFN-β promoter in nuclear extracts from splenocytes and L929 cells. Two major conclusions could be made from these experiments. First of all, although nuclear extracts from induced L929 cells contained highly increased levels of factors binding to the IRE, PRDII, and PRDI as compared to extracts of nuclei of uninduced cells, in spleen cell extracts, induction had no effect on the levels of these complexes. Neither the pattern nor the levels of DNA binding to these elements changed in spleen cell extracts following induction. At the same time, the pattern of gel retardation observed in extracts from uninduced and induced spleen cells was very similar to
Fig. 5. Competition of the complexes formed with the IRE probe. The $^{32}$P-labeled oligonucleotide IRE was incubated with 6 μg of BALB/c nuclear extract (open columns) or 2 μg of C57BL/6 nuclear extract plus 4 μg of bovine serum albumin (filled columns) and 2.5 μg of poly[dI:dC]. The amounts of unlabeled oligonucleotide IRE indicated were added to the reaction after 15 min, and a further incubation of 15 min was carried out. The binding activities were quantitated by densitometric analysis of the autoradiogram, and the binding activities observed in the absence of competitor DNA were taken as 100%.

that of induced L929 cells. Thus, the factors necessary for IFN induction are present constitutively in spleen cell nuclei. This indicates that the mechanism of induction in these cells is different from that observed in L929 cells and does not involve increased levels of the known activators. Constitutive levels of DNA-binding activities could be due to a priming phenomenon related to spontaneous IFN production. However, we were unable to detect any IFN activity in serum or spleen cell culture fluid prior to induction.

The second observation from the gel retardation experiments is that nuclear extracts from splenocytes of IF-1<sup>+</sup> mice consistently contain higher levels of complex formation with both the IRE and the DraI-Aval fragment, which contains all of the regulatory motifs described to date. The difference was on the order of 3- to 4-fold, which correlates well with the difference in the levels of HuIFN-β mRNA found in transfected cells (Fig. 1) and with the levels of transcription of the MuIFN genes in spleen cells from corresponding mouse strains [3- to 5-fold difference between IF-1<sup>+</sup> and IF-1<sup>-</sup> splenocytes, as we have reported previously (17)].

Competition curves with unlabeled IRE as competitors (Fig. 5) and the observation that the levels of factors binding to the HEX and PRDII probes are equivalent in nuclei from both C57BL/6 and BALB/c splenocytes (Fig. 6, A and B) indicate that the different levels of binding activity for the IRE are due to overall efficiency of complex formation and not due to physically different levels of DNA-binding proteins involved. This difference in the efficiency of complex formation could be explained by an increased cooperativity between such factors or by participation in the complex of other proteins than those
which can be detected as binding directly to the subregions of the IRE.

Several groups have reported that the inducible PRDII-binding factor is indistinguishable from transcription factor NF-xB (11–13) and suggest that this factor is strongly implicated in IFN expression. NF-xB (=50 kD) is present constitutively (22–25) in B-lymphocytes (which constitute a major component of the splenocyte population). In other cells, NF-xB is sequestered in the cytoplasm by I-xB (23–25) and is translocated into the nucleus after induction by a variety of factors (4β-phorbol 12-myristate 13α-acetate, lipopolysaccharide, double stranded RNA, virus) (11–13, 26, 27). The 60 kD protein (P2 complex), which is inducible in L929 cell nuclear extracts and is constitutive in splenocyte nuclear extracts, is consistent with the 58 kD protein observed by Xanthoudakis and Hiscott (27) in different cell lines (HeLa, Jurkat, and U937) induced with different agents (12-O-tetradecanoylphorbol-13-acetate, polyinosinic-polycytidylic acid, and virus) and with the 50 to 55 kD polypeptides characterized by affinity chromatography of the NF-xB transcription factor complex (24–26).

Cotransfection experiments with complementary DNA clones encoding IRF-1 and IRF-2 proteins suggest that the control of IFN expression may be mediated through competitive interactions of IRF-1 and IRF-2 (10). Expression of both IRF-1 and IRF-2 genes is virus inducible (10, 28). In UV cross-linking analysis of proteins binding to the HEX (PRDII-like) probe, the 50 kD band (inducible in L929 cell nuclear extracts but constitutive in splenocyte nuclear extracts) corresponds to the H3 complex and comigrates with the IRF-1 protein (Fig. 7B, Lanes 9 and 10). This is consistent with the purified 56 kD IRF-1 characterized by affinity chromatography (29). The 72 and 85 kD proteins may represent IRF-2 and other proteins not yet fully characterized.

Thus, no differences in the protein patterns of the complexes binding to IFN promoter elements in If-1 or If-10 nuclei were found. The only difference observed between spleen cells and L929 cells is the presence of an additional protein of 85 kD with the PRDII probe in the latter.

In conclusion, we have observed that in contrast to the situation in L929 fibroblasts, in splenocyte nuclear extracts, no significant changes were seen in the DNA-binding activities specific to the IRE or its subregions after induction. We have observed that higher levels of DNA-binding activities specific for regions involved in induction of the HulFN-β gene correlate with higher levels of HulFN-β mRNA in transfected splenocytes and the levels of transcription of IFN genes in If-10 splenocytes (17). The difference in activity seems to be due to other reasons than concentrations of the DNA-binding proteins involved. We are continuing our studies on the nature of these factors and their interactions with their binding sequences and among themselves in order to obtain further insight into the action of the Il-1 regulatory locus and into the mechanism of IFN induction in spleen cells and in general.

Materials and Methods

Animals, Cells, and Plasmids. Male mice of the C57BL/ 6, BALB/c, C3H, and DBA lines, age 6 to 8 weeks, were obtained from the Jackson Laboratory (Bar Harbor, ME) or Charles River Canada, Inc.

L929 cells and T98G cells were cultured as described previously (17, 30). Splenocyte suspensions were prepared by the dissection of spleens onto a Millipore filter support (AP 32; 75-mm diameter); support filters were discarded, and the suspension was centrifuged and washed twice in Eagle’s MEM without fetal bovine serum. pHulFN-β was prepared by cloning the 1.8-kb genomic EcoRI fragment of the IFN-β gene (19) into the poly linker of pGEM1 (Promega Biotec). pGEM MulFN-β, was pre-
pared by cloning the PstI insert of pMβ3 (31) into the PstI site of the polylinker of pGEM1.

**Induction and Titration of IFN.** For induction of IFN, cells were exposed to NDV (approximately 50 plaque-forming units/cell) for 2 h. At the end of this time, virus was replaced with Eagle's MEM.

IFN titrations were carried out by the method of cytopathic effect reduction. L929 cells infected with vesicular stomatitis virus were used for murine IFN titration, and T98G cells infected with encephalomyocarditis virus were used for human IFN titration as previously described (17).

**DNA Transfection.** Transfections were performed by electroporation using a Bio-Rad electroporator. One hundred μg of pGEMHuIFN-β with 20 μg of pSVKneoβ were cotransfected into 5 × 10^7 cells at 1000 V with a capacitance at 25 μF. Cells were induced with NDV after 36 h of incubation at 37°C with 5% CO2.

**Isolation and Analysis of RNA.** Total cellular RNA was isolated as previously described (32). The poly(A)+ fraction was purified by oligo(dT)-cellulose chromatography (Type 7; Pharmacia).

RNAse protection analysis was performed essentially as previously described (2). Poly(A)+ RNA (2 μg) were hybridized with complementary radiolabeled probes for 18 h at 45°C. RNA-RNA hybrids were digested with RNase A and T1 for 1 h at 30°C. Resistant hybrids were analyzed on 5% sequencing gels.

The 32P-labeled cRNA probe complementary to the MuIFN-β mRNA and the 5’ of the HuIFN-β mRNA were synthesized using pGEMMuIFN-β and pGEMS’HuIFN-β linearized with HindIII and transcribed with T7 RNA polymerase. The pGEMS’HuIFN-β contains 282 bp of 5’ upstream sequence. Multiple exposures of the dried gel were taken and were quantitated by densitometry (GS 300 densitometer; Hoefer Scientific Instruments).

**Preparation of Nuclear Extracts.** Nuclear extracts were prepared in the same way for L929 cells and splenocytes, using a modification of the method of Dignam et al. (33). The modification was found to be necessary as isolated splenocytes were too fragile for the standard procedure. L929 cells were removed by scraping from Petri dishes, whereas single cell suspensions from spleens were prepared as described above.

The splenocyte suspensions from 20 spleens/preparation were treated for 10 min with 0.17 mM NH4Cl to lyse erythrocytes and washed twice with Eagle’s MEM. After 2 h in a CO2 incubator (37°C), the cells were resuspended in 2 PCV of 25 mM Tris-HCl (pH 7.5), 25 mM KCl, 7.5 mM MgCl2, 0.2 mM phenylmethylsulfonyl fluoride, 30% sucrose, and 0.002% NP-40 and incubated for 15 min on ice. At this point, an additional 5 PCV of the same buffer (without NP-40) were added, and the cells were pelleted in the cold. The nuclear pellet was resuspended in 0.1 PCV of the original volume of low salt buffer [25% glycerol, 200 mM NaCl, 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotinin, and 5 μg/ml of leupeptin]. An equal volume of high salt buffer (same composition but with 400 mM NaCl) was added slowly with gentle mixing. Cells were left on ice for 30 min, during which time they were gently resuspended every 10 min. At the end of this time, nuclei were centrifuged at 18,000 × g for 15 min, and the supernatant fraction was dialyzed overnight against 1,000 ml for 20% glycerol, 20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 20 units/ml aprotinin, and 0.5 μg/ml of leupeptin. After dialysis, extracts were clarified for 20 min at 18,000 × g and then aliquoted and stored in liquid nitrogen. Extracts from L929 cells prepared in this way gave identical results to those obtained with extracts made by the standard Dignam protocol (33). Cytosolic extracts were prepared as previously described (12).

Protein determinations were performed using the Bio-Rad protein assay kit (Bio-Rad, Mississauga, Ontario, Canada).

**Gel Retardation Assays and UV Cross-Linking Analysis.** DNA fragment and synthetic oligonucleotides (synthesized with the Milligen BioSearch Cyclone DNA synthesizer) spanning the HuIFN-β regulatory element (see Fig. 2) and hexamer ([AAGTGA]6) were labeled using [γ-32P]ATP (ICN, 3888 Ci/mmole) and polynucleotide kinase (Pharmacia). The IgKappa oligonucleotide and the H-2Kb oligonucleotide were: 5’-AGGGGACTTTC3’ and 5’-GTGGGATTCC3’, and the Mut-1 and Mut-2 oligonucleotides were: 5’-ATGGGACTTTC3’ and 5’-TGGGATTC3’, respectively.

BrdUrd-incorporated oligonucleotides (B) were generated by substituting selected thymidine residues in the nucleotide sequence (shown below) with BrdUrd CED phosphoramidite (Pharmacia).

**PrDII: 5’-GGGAAABCCGGAAABCC3’**

**Hex: 5’-AAGBGAAGBGAAGBGAAGBGA3’**

**PrDIIa: 5’-CCCTBTAAGcccTTBTAaGGG3’**

The gel retardation assay was performed as previously described (20) with the following modifications. Reactions contained: nuclear extract (2.5 to 5 μg), 2.5 μg of poly[dI:dC] as nonspecific competitor, 25 mM HEPES (pH 7.9), 1 mM DTT, 40 mM NaCl, 1 mM EDTA, and 5% glycerol (for a total volume of 25 μl). After 20 min at 25°C, the probe (5,000 to 10,000 cpm) was added, and the reaction was incubated for another 20 min at 25°C. At this point, 3 μl of BrdUrd III (34) sample buffer were added, and the samples were electrophoresed in 5% polyacrylamide gels containing 50 mM Tris (pH 7.5), 380 mM glycine, and 2 mM EDTA. The gels were run at 150 V for 1.5 to 2.5 h, after which they were dried and exposed to autoradiographic film at −70°C. The relative intensities of the protein-DNA complexes from various exposures of the dried gel were measured using the GS 300 densitometer (Hoefer Scientific Instruments).

The UV cross-linking analyses were carried out as previously described (27). Essentially, the protein-DNA complexes were resolved in gel retardation assays, and complexes were cross-linked in situ by exposure to UV light (302 nm) for 1 h at 4°C. The region corresponding to the shifted complexes was localized by autoradiography, excised, and soaked in 30 ml SDS-sample buffer (1% SDS, 2% v/v 2-mercaptoethanol, 20% glycerol, 10 μg/ml bromophenyl blue, and 62 mM Tris-HCl (pH 6.8)) for 5 min at 68°C. The cross-linked proteins were analyzed on a 9% SDS-polyacrylamide gel.

**In Vitro Transcription and Translation of IFR-1.** A XbaI fragment from pCMIRS (22) containing the entire IRF-1 coding sequence was subcloned into pGEM. To produce
the full length IRF-1 protein, pGEMIRF-1 (5 µg) was completely digested with BamHI and transcribed with SP6 polymerase. Run-off RNA transcripts (2 µg) were translated in the rabbit reticulocyte lysate (Promega) in the presence of 135S)methionine, according to the manufacturer's instructions.

Acknowledgments

We thank A. Haggarty for useful discussions, R. Duclos for photography, M. Vosguian for secretarial work, and D. Forget and A. Steyaert for technical assistance.

References