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A Novel Interferon-Inducible Domain: Structural and Functional Analysis of the Human Interferon Regulatory Factor 1 Gene Promoter

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We have cloned and functionally characterized the human interferon regulatory factor 1 (IRF-1) gene promoter. The promoter contains a CpG island, with several GC boxes, a CAAT box, but no TATA box. IRF-1 mRNA is strongly induced by gamma interferon (IFN-γ) but more weakly and transiently by IFN-α. There are several putative κB motifs and numerous AA(G/A)G(G/T)A and GAAANN motifs throughout the promoter. The IRF-1 promoter is not autoregulated by the IRF-1 gene product. IFN inducibility of the promoter was studied with 5′ deletion mutants linked to a heterologous reporter gene. Gel mobility shift assays were used to show IFN-inducible factor binding to the IRF-1 promoter. These studies showed that IFN inducibility is conferred by a novel imperfect inverted-repeat arrangement of two GAAANN motifs within a domain, 130 nucleotides upstream of transcription initiation. This inverted repeat binds a factor upon induction with IFN and can confer IFN inducibility on a heterologous promoter. Conversely, point mutations of the inverted repeat are not IFN inducible when linked to the same heterologous promoter.

Interferons (IFNs) activate signal transduction pathways leading to transcriptional activation of genes involved in antiproliferative, antiviral, immunomodulatory, and differentiating activities in mammalian cells. The type I IFN genes (IFN-α and -β) can be transcriptionally activated by viruses or double-stranded RNA [poly(I) · poly(C)]. The type II IFN gene (IFN-γ) is distinct from the type I IFNs in structure and has a distinct receptor (for reviews, see references 58 and 61). The transcriptional activation of the interferon genes and the IFN-inducible genes is mediated by binding of nuclear proteins to the transcriptional enhancers of these genes (27, 35).

The minimal sequence required for the transcriptional regulation of the IFN-β gene is limited to the IFN gene regulatory element (IRE). The IRE is composed of negative regulatory domains (NRDI) and two positive regulatory domains (PRDI and PRDII). Upon viral induction, the PRDII domain has been shown to bind NF-κB and a related protein, PRDII-BF1 (18, 32, 41, 60). PRDI is a 14-bp element that is distinct from PRDII (17, 28, 29, 63) and contains two copies of a hexamer motif, AAGTGA (21), which can function as a virus-inducible enhancer when multimerized (24, 38).

A number of PRDI-binding proteins have been identified. The IFN regulatory factor 1 (IRF-1) which, previously, was identified and cloned (23, 48), can bind to multimers of the PRDI hexamer AARKGA (where R = G or A and K = G or T) in vitro. IRF-1 partially activates endogenous IFN genes and cotransfected synthetic constructs containing IFN gene regulatory elements (19, 30), when overexpressed. Variations of the PRDI-like hexamer, GAAANN, are prevalent in transcriptional enhancers of IFN and IFN-induced genes (61). Direct repeat multimers of some types of GAAANN sequences are inducible by virus, by IRF-1, and/or by IFN (44, 49). IRF-1 is necessary, but not sufficient for maximal IFN-β production (20, 40). Taken together, these data have established that IRF-1 is a positive transcription factor involved in the activation of type I IFN genes. In addition, there is some evidence for the involvement of IRF-1 in the regulation of IFN-inducible genes (31, 53). The IFN-stimulated response element (ISRE) of IFN-inducible genes represents two direct repeats of the GAAANN motif and has been shown to bind the well-characterized factor ISGF2 (43, 51), also referred to as factors G and M (33). ISGF2 (or factor G) has been cloned and is identical to IRF-1 (51), although it is ISGF3 (or factor E) that is involved in the initial positive induction of IFN-inducible genes by IFN (11, 36, 43).

IRF-1 gene expression is induced by virus, double-stranded RNA, IFNs, and other cytokines or inducers of certain signal transduction pathways (1, 22, 30, 48, 51). Recently, the rat IRF-1 gene was identified by its induction in Nb2 T cells by prolactin (62). The activation and role of IRF-1 in signalling pathways may turn out to be more widespread than originally thought.

We have isolated the promoter for the human IRF-1 gene and characterized its regulation. The structure of the IRF-1 promoter is highly GC rich and contains a CpG island and many GC boxes. In addition, the promoter contains three putative NF-κB binding sites, numerous AARKGA hexamers, and GAAANN sequences that are not arranged as multimers. Transient expression and gel shift data suggest that IFN induction of the IRF-1 promoter is mediated by a factor(s) binding to a novel site consisting of an imperfect inverted-repeat arrangement of two GAAANN sequences.

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MATERIALS AND METHODS

Screening genomic library. Human placenta genomic DNA library (Clontech Laboratories, Inc.) was plated at 10^5 PFU per plate (150-mm plates), and duplicate filters (Schleicher & Schuell, Inc.) were hybridized overnight at 65°C with hybridization buffer containing 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 3× Denhardt's, 0.3% sodium dodecyl sulfate (SDS), 100 μg of denatured salmon sperm DNA per ml, and ^{32}P-IRF-1 cDNA labeled with a nick translation kit (Bethesda Research Laboratories). The filters were washed twice at 65°C with 0.1× SSPE and 0.1% SDS and then autoradiographed. Positive clones were subcloned into the pBS+ vector (Stratagene, Inc.) (54). The nucleotide sequence was determined by the dideoxy chain termination method with Sequenase version 2 (United States Biochemical Corp.) from denatured double-stranded templates (37, 55).

Cells. K562 cells (ATCC CCL 243) were grown in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. IFN-α (Intron A, Schering Corp.) and interferon-γ (a generous gift from Michael Shepard, Genentech Inc.) inductions were performed at 2,000 U/ml. Cycloheximide (CHX; Sigma) was used at 50 μg/ml and was added to the cells 15 min prior to induction with IFN, where applicable. Cell treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA; Sigma) was at 30 ng/ml for 15 min. All cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air.

RNA extraction and Northern (RNA) analysis. RNAs were extracted from cells, as previously described (8). Total cellular RNAs (15 μg per sample) were electrophoresed on 2.2 M formaldehyde–1% agarose gels. Following Northern transfer to Nytran membrane (Schleicher & Schuell), blots were hybridized with an IRF-1 cDNA probe ^{32}P labeled by nick translation. Blots were reprobed with a cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ATCC 57090) to ensure equal quantities of RNA per lane.

Primer extension analyses. A 40 nucleotide (nt) primer (5′-CGGCGTGGACTGGGCACGGCTCCGGGTGGCCTC GGTTCGG-3′) to the IRF-1 mRNA was 5′ end labeled with [γ-^{32}P]ATP (>6,000 Ci/mmol; NEN Research Products) by using T4 polynucleotide kinase (Boehringer Mannheim). The primer (0.1 pmol; specific activity, ~10^6 cpm/pmol) was hybridized to 80 μg of total RNAs and subjected to primer extension with 40 U of avian myeloblastosis virus reverse transcriptase (Life Sciences) as previously described (54). Extension products were separated on a 6.5% polyacrylamide sequencing gel in parallel with a ^{35}S-labeled sequencing reaction (Sequenase) by using the same primer on an IRF-1 cDNA subcloned into pUC18.

Sequence analysis. DNA sequence analyses were performed by using the University of Wisconsin sequence analysis software package (13). The human-mouse IRF-1 sequence comparisons were determined with the Bestfit program. CpG analysis was performed according to the criteria described by Gardiner-Garden and Frommer (25): CpG islands are GC-rich regions of at least 200 bp with a CpG content close to that expected from the base composition. The ratio of observed to expected (Obs/Exp) CpG was calculated as follows: Obs/Exp CpG = [number of CpG/(number of C × number of G)] × N, where N is the total number of nucleotides in the region being analyzed. The %G+C and Obs/Exp CpG were calculated for a sliding window of 100 bp across the IRF-1 promoter sequence. The 100-bp window was shifted in increments of 7 bp, giving a moving average across the DNA. CpG islands show an Obs/Exp CpG content of >0.6 in a region of >50% G+C.

Plasmid DNAs. The 1.3-kb IRF-1 *Sst*I promoter fragment was subcloned into the *Sst*I site of the cloning vector pBS+ (Stratagene). This fragment was then subcloned into the *Sst*I site of the promoterless luciferase reporter gene vector pXP2 (a gift from S. Nordeen [50]), generating the 5′Δ-1312-luc construct. The 5′Δ-410-luc construct was generated by exonuclease III digestion of the promoter in pBS+ (Erase-A-Base, Promega) followed by subcloning into pXP2. The 5′Δ-143-luc construct was generated by digesting the promoter in PBS+ with *Nhe*I and *Xba*I; the vector with deleted promoter was gel purified and religated, and the promoter fragment was excised with *Hin*dIII and *Sst*I and subcloned into pXP2. The IRF-1 cDNA, isolated from a human monocyte library (Clontech Laboratories, Inc.) was digested with *Pst*I and *Ava*II to remove some of the 5′ and 3′ untranslated regions. The fragment with the coding sequences was gel purified and subcloned into the eukaryotic expression vector pSVL (Pharmacia). The human IFN-β promoter (−274 to +15) was polymerase chain reaction (PCR) cloned from genomic DNA into pBluescript SK+ (Stratagene). The 5′ PCR primer included a *Hin*dIII restriction site, and the 3′ primer included an *Sst*I site, for cloning purposes. After sequence verification, the IFN-β promoter was subcloned into the promoterless luciferase reporter gene vector pXP2. A double-stranded oligonucleotide, spanning from −130 to −106 of the IRF-1 promoter, was synthesized with *Hin*dIII cohesive ends. Similarly, several point mutations of the same domain were synthesized for gel mobility shift assays and cloning purposes. One copy of the wild-type (wt) or point mutant oligonucleotides was subcloned immediately upstream of the herpes simplex virus type I thymidine kinase (TK) promoter, in the vector pT109luc (a gift from S. Nordeen [50]). pT109luc is a derivative of pXP2 and contains a functional herpes simplex virus TK promoter driving the luciferase reporter gene (50). The sequences of the subcloned oligonucleotides were confirmed by using a synthetic sequencing primer directed to the TK promoter.

Gel mobility shift assays. Nuclear and cytoplasmic protein extracts were prepared, from K562 cells, according to a slight modification of the method of Dignam et al. (14). Cells were washed in phosphate-buffered saline (PBS) and resuspended in hypotonic buffer (HB; 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9 at 4°C], 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 10 mM KCl) and allowed to swell on ice for 10 min (56). Cells were pelleted and resuspended in 2 packed-cell volumes of HB before homogenizing. After homogenization, 0.1 volume of sucrose restore buffer (1 part 10× HB, 9 parts 75% [wt/vol] sucrose) was added, and nuclei were pelleted at 16,000 × *g* for 30 s. Cytoplasmic proteins were retrieved as the supernatant from the nuclear pellet. Nuclear proteins were extracted with buffer C and processed by the method of Dignam et al. (14). Extracts were prepared in the presence of the protease inhibitors phenylmethylsulfonyl fluoride (0.25 mM) and aprotinin (5 μg/ml). Binding reactions were performed at 20°C and consisted of 20 μg of protein extract preincubated for 10 min with 4 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia) and 1 μg of pBR322 digested with *Msp*I. One hundred femtomoles of probe oligonucleotide, ^{32}P labeled by Klenow fill in of 3′ recessed ends, was added along with competitor DNAs, where applicable, and incubated for a further 20 min. Final salt concentrations of the 25-μl

binding reaction mixture were 12 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.12 mM EDTA, and 0.06 mM EGTA. Binding reactions were separated on 5% nondenaturing polyacrylamide gels (50 mM Tris [pH 7.9], 3.3 mM Na acetate, and 1 mM EDTA). Gels were dried and subjected to autoradiography.

Oligonucleotides used in this study were synthesized on a Cruachem PS-250 DNA synthesizer. The following oligonucleotides (and their complements) were used in these studies: −130/−97, AGCCTGATTTCCCCGAAATGACGGCA CGCAGCCG; −130/−106, AGCCTGATTTCCCCGAAAT GACGGC; −55/−31, AGGGCTGGGGAATCCCGCTAAG AGT; High-affinity NF-κB, AGCTTCAGAGGGGACTTTC CGAGAGGTCGA; IR1, GATTTCCCCGAAATG; IR2, GA CGGCACGCAGCCG; $(GAAATG)_4$, AGCTGAAATGGAA ATGGAAATGGAAATGAGCT; $(GAAATC)_4$, AGCTGAA ATCGAAATCGAAATCGAAATCAGCT; $(AAGTGA)_4$, GATCAAGTGAAAGTGAAAGTGAAAGTGAGATC; 6-16 ISRE, AGCTGTAGAGCTGGGAGAGAGGGGAAAATGA AACTGCAGAGTGCAGAGCT; 9-27 ISRE, CTAGATTTA CAAACAGCAGGAAATAGAAACTTAAGAGAAATAC ACACTGCA; CRE, CTCCTAGCCTGACGTCAGAGAG AGA; AP1, CTAGTGATGAGTCAGCCGGATG; Sp1, AG CTGGGGCGGGGCACGT; and IFN-β PRDI and II, GAGA AGTGAAAGTGGGAAATTCCT.

Transfections. DNAs were transfected into K562 cells by using radio frequency (RF) electric field poration (6, 7). Cells were washed in PBS and resuspended in poration buffer (15 mM KPO_4, 10 mM HEPES, 1 mM $MgCl_2$, and 200 mM sucrose [pH 7.4]). Each sample contained a total of 50 μg of DNA with 6×10^6 cells in a poration volume of 200 μl. Cells were porated in a 0.2-cm electroporation cuvette (Bio-Rad Laboratories) by pulsing with a DC-shifted RF wave pulse waveform. The RF pulses were 1 ms wide, oscillating at 40 kHz, with a field strength of 1.1 kV/cm. Pulses were applied in three trains with three pulses per train (the period was 1 s between pulses and 10 s between trains). Immediately following pulsing, 800 μl of complete tissue culture medium was added to the cuvette. After all samples were pulsed, cells were plated into 3.5-cm dishes containing 4 ml of medium with or without IFN.

Transfected samples contained 30 μg of IRF-1–promoter–luciferase reporter constructs (or equivalent molar amounts of each deletion) along with an internal control plasmid consisting of a β-galactosidase gene driven by the simian virus 40 (SV40) early promoter, pSVβ (a generous gift from Grant MacGregor [45]). The total amount of DNA was adjusted to 50 μg with pBS+ plasmid DNA. In each experiment, each transfection was performed in duplicate or, more usually, triplicate (as indicated in the figure legends), and standard errors were determined.

Transfected samples were grown in culture for up to 48 h before harvesting. Cells were lysed in a Triton X-100-based lysis buffer and analyzed for luciferase activity by using a luciferase assay kit (Promega). Light emissions were quantitated by using a model 1250 luminometer (LKB Wallace). Protein concentrations were determined by using the Bio-Rad protein assay kit with bovine serum albumin standards. Equal amounts of protein from each transfection sample were used for the reporter gene assays. β-Galactosidase activity was detected by a colorimetric reaction, using *o*-nitrophenyl-β-D-galactopyranoside as a substrate and reading the A_{420} (46). Both luciferase and β-galactosidase activities were determined in the linear range for each assay. Luciferase activities are represented as arbitrary light units and were corrected for different transfection efficiencies between samples by using the β-galactosidase activities.

Nucleotide sequence accession number. The sequence of the human IRF-1 promoter has been assigned GenBank no. L05078.

RESULTS

Isolation and sequence analysis of the genomic clone of the IRF-1 gene. A cDNA clone for IRF-1 was isolated from a monocyte cDNA library (53a) by use of a set of synthetic degenerate oligonucleotides homologous to the conserved exonic domains which code for the DNA-binding regions of the IRF-1 and IRF-2 proteins. A full-length IRF-1 cDNA was isolated and used to screen a human placental genomic library. Several IRF-1 positive genomic clones were isolated, and their restriction sites were mapped. The largest clone was ~9 kb in length. An *Sst*I digest yielded a promoter fragment which directly abuts the IRF-1 cDNA sequence.

The *Sst*I genomic fragment containing the IRF-1 promoter is 1.3 kb long, extending from −1312 to +7 (relative to transcription initiation at +1 [see next section]) (Figure 1A). The promoter and gene are GC rich from ~−450 to ~+250 nt. The %G+C and CpG content was analyzed in a sliding window of 100 bp across the region of interest. The window was moved across the promoter in 7-bp increments. To compute a moving average for %G+C, an Obs/Exp ratio for CpG was calculated (see Materials and Methods). This analysis shows that the IRF-1 promoter has a significant CpG island extending from ~−450 to a position downstream of transcription initiation, at ~+250 (Fig. 1B). As expected, this CpG island contains several GC boxes (GGGCGG) which are potential Sp1 binding sites (16, 26, 34). Interspersed with the GC boxes are AARKGA hexamers and GAAANN sequences which are commonly found in type I IFN gene promoters. There are no sequences similar to ISREs, such as are found in enhancers of IFN-inducible genes.

The promoter lacks a TATA box, but does contain a putative CAAT box at position −97 to −91. On either side of the CAAT box, there is a putative NF-κB motif or κB factor binding motif. The κB motif 3′ to the CAAT box (−49 to −40) conforms to the consensus GGGRNNYYCC (where R=A or G, Y=C or T, and N= any nucleotide [2]). The other potential κB motif (−117 to −126, opposite strand), 5′ to the CAAT box, differs from the consensus in two positions, but in the less conserved half-site of the motif. A comparison of the human IRF-1 promoter with that of the published mouse IRF-1 promoter (48) reveals extensive homology (83%) from around −230 to ~+60. The relative positions of the putative CAAT boxes, putative κB motifs, the major transcription initiation sites, and some GC boxes appear to be fairly well conserved between the human and mouse promoters (Fig. 2). It is apparent that the mouse IRF-1 promoter also contains a CpG island, as noted by Miyamoto et al. (48).

Analysis of the human IRF-1 mRNAs. Total RNAs were extracted from K562 cells following induction with either IFN-α or IFN-γ for various times. The RNAs were subject to Northern analysis and probed with an IRF-1 cDNA. IRF-1 mRNA was induced by both IFN-α and IFN-γ (Fig. 3A). The IFN-γ-induced message rose to higher levels and persisted longer than when IFN-α was used for induction. The IFN-α-induced IRF-1 mRNA was transient, reaching peak levels within 1 h and declining to near basal levels by 6 h (Fig. 3A). This suggests that the IRF-1 message is unsta-

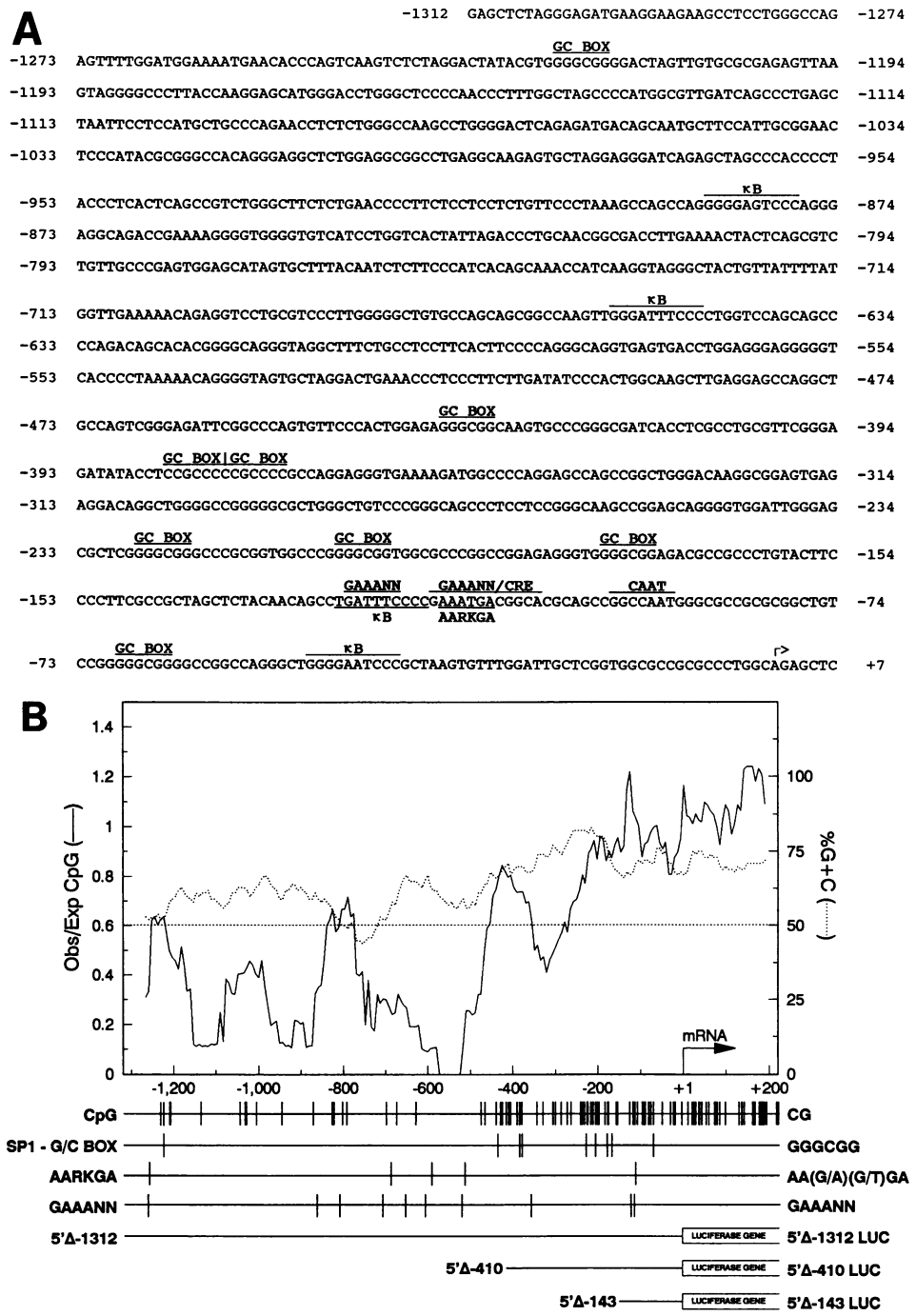

FIG. 1. Sequence and CpG analysis of the human IRF-1 promoter. (A) Nucleotide sequence of the human IRF-1 promoter. Locations of various potential regulatory motifs are marked. The GC boxes may be potential Sp1 binding sites. Two putative NF-κB binding sites are at nucleotide positions −126 (antisense strand) and −49 (sense strand). (B) CpG analysis of the human IRF-1 promoter. A CpG island is a stretch of DNA where the G+C composition is greater than 50% and the Obs/Exp ratio of CpG is greater than 0.6. The region of the promoter extending from ~−450 to +250 constitutes a CpG island. GC boxes are clustered within the CpG island. The locations of AARKGA hexamers and GAAANN motifs are shown along with the 5′Δ deletion mutants used in transfection experiments.

ble, as predicted by the presence of an AU motif (57) in the 3′-untranslated region. Northern analysis revealed two IRF-1 transcripts, one with the expected molecular weight and one close in size to the 28S rRNA. The higher-molecular-weight transcript may represent an unprocessed precursor that has a half-life similar to that of the mature IRF-1 mRNA (see Fig. 3A and B). Such a high-molecular-weight transcript was reported for the mouse IRF-1 mRNA (22).

Similar induction studies were performed in the presence of the protein synthesis inhibitor CHX. The purpose of this experiment was to determine if IFN induces de novo synthesis of an activating factor(s) for the inducible expression

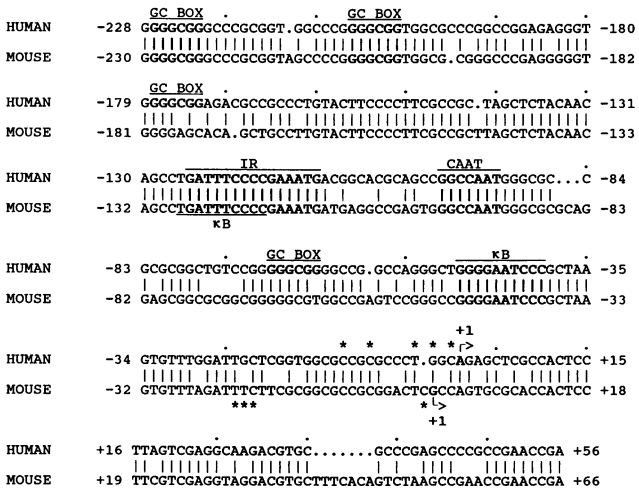

FIG. 2. Sequence homology between the human and the mouse IRF-1 promoters. The promoters are ~83% homologous from ~−230 to +60 (with respect to the major CAP site at +1). Several key sites are conserved between the two promoters: the CAAT box, the two putative κB motifs, two GC boxes, and an imperfect inverted repeat (IR). The mouse IRF-1 sequence was taken from Miyamoto et al. (48).

of the IRF-1 gene. Figure 3B shows that in the presence of CHX, both IFN-α and IFN-γ were able to induce IRF-1 gene transcription. The levels of IRF-1 message appear to be superinduced in the absence of protein synthesis. The apparent superinduction of the IRF-1 message is probably due to a decreased rate of mRNA degradation, rather than an increase in the rate of transcription. This phenomenon has been reported for other inducible genes and suggests the presence of a labile mRNA-degrading factor (9, 39). From nuclear factor binding studies (see below), it is unlikely that CHX, itself, is activating factors which could facilitate IRF-1 gene transcription. Nevertheless, it is clear from this experiment that the IRF-1 gene can be IFN induced in the absence of new protein synthesis, suggesting that IFN action is through posttranslational modification of a preexisting transcription factor(s).

Sequence analysis of the IRF-1 cDNA isolated from a monocyte library revealed an additional 37 nt 5′ to the published sequence for the human IRF-1 cDNA (47, 51). Primer extension analysis was used to identify the correct 5′ end(s) of IRF-1 mRNA from IFN-γ-induced K562 and HeLa cells. A primer was synthesized from +49 to +10 (5′ to 3′) of the published sequence for the human IRF-1 cDNA (47). The primer was used for both the primer extension assays and a sequencing reaction with the IRF-1 cDNA. The analysis revealed one major and several minor initiation sites (Fig. 3C). The same extension products were found with both K562 and HeLa RNAs. All minor initiation sites are further upstream from the major site. The major initiation site would generate a transcript that would include 3 nt in addition to the IRF-1 cDNA isolated from the monocyte library, suggesting that this cDNA is essentially full length. Taniguchi and colleagues found essentially the same transcription start site for the mouse IRF-1 mRNA, by using a nuclease protection assay (Fig. 2) (48). As different assays have revealed similar start sites, it is reasonable to assume that the major initiation site found in this study is valid and does not represent an RNA secondary structure artifact of the primer extension assay.

Functional analysis of the IRF-1 promoter. In order to study the IFN inducibility of the IRF-1 promoter, the 1.3-kb *Sst*I promoter fragment was subcloned into the promoterless luciferase reporter gene vector pXP2 (50). The full-length promoter construct was transfected into K562 cells and tested for inducibility with both IFN-α and -γ (Fig. 4A). The promoter was found to be active in the absence of IFN induction, which may be expected since the promoter contains nine GC boxes, some of which may be potential Sp1 binding sites. The promoter was highly inducible with both IFN-α and IFN-γ. The kinetics of induction differed between the type I and type II IFNs. IFN-α induced a transient severalfold increase in activity of the IRF-1 luciferase reporter gene (Fig. 4B). IFN-γ treatment, however, resulted in ~10- to 20-fold induction of luciferase activity that was sustained for 24 to 48 h.

Definition of the IFN-inducible transcriptional enhancer of IRF-1. In order to delimit an IFN-inducible element(s) in the IRF-1 promoter, deletion mutants of the 5′ region of the IRF-1 promoter were constructed. An exonuclease III deletion mutant was generated at position −410. The 5′ end of this mutant is at the 5′ boundary of the CpG island and excludes two κB motifs at −887 and −657, and three AARKGA hexamer motifs at positions −515, −594, and −690. A further deletion was made by using the *Nhe*I restriction site at position −143. This deletion removes all but one GC box and one AARKGA hexamer, AAATGA. Also included in the −143 promoter are a potential cyclic AMP response element (TGACGGCA), two GAAANN motifs, two potential κB motifs, and the CAAT box (see Fig. 1A and 6A). The 5′Δ-410 deletion shows similar induction kinetics as the full-length promoter (5′Δ-1312), with similar absolute levels of induction (Fig. 4C). The 5′Δ-143 deletion, on the other hand, shows a dramatic decrease in constitutive and induced activity (Fig. 4C). However, the relative fold-induction of the 5′Δ-143 promoter, with both IFN-α and IFN-γ, is almost identical to that of the 5′Δ-1312 and 5′Δ-410 promoters (Fig. 4D). The decreased activity of the 5′Δ-143 promoter could be explained by the deletion of all but one of the potential Sp1 binding sites. The Sp1 binding sites may serve to amplify the IFN-induced transcriptional response without actually contributing to the IFN inducibility.

The potential IFN-inducible elements in the 5′Δ-143 promoter may be the only domains responsible for IFN inducibility as the relative fold-inductions of all three promoter deletions are very similar. Deletion of most of the promoter does not change the overall fold-induction with either IFN-α or IFN-γ, suggesting that the same IFN-inducible element(s) may be shared by both types of IFN.

The IRF-1 promoter is not induced by IRF-1. To test the possibility of autoregulation, the 5′Δ410-luciferase construct was cotransfected with an IRF-1 expression vector. The IRF-1 expression vector was derived from our IRF-1 cDNA subcloned into an expression vector, pSVL, driven by an SV40 late promoter. From Fig. 5, it can be seen that the IRF-1 expression vector failed to influence the activity of the IRF-1 promoter. To ensure that the IRF-1 expression vector was functional, we used it in a cotransfection experiment with the IFN-β promoter. The human IFN-β promoter (from −274 to +15) was PCR cloned from genomic DNA and then, subcloned into the luciferase reporter gene vector, pXP2. Either the IRF-1 expression vector or the pSVL expression vector alone was cotransfected into K562 cells, with the IFN-β–luciferase construct (Fig. 5). The IFN-β construct showed a basal level of activity when cotransfected with the pSVL expression vector. However, when cotransfected

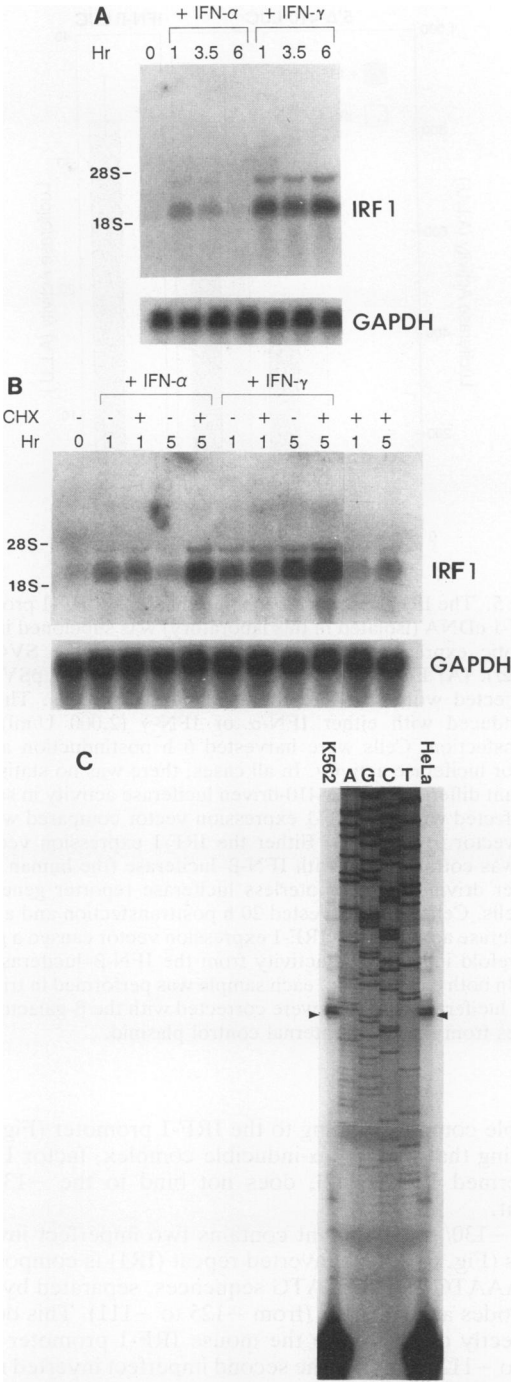

FIG. 3. Northern analysis and 5′ end analysis of IFN-induced IRF-1 mRNAs. Total RNAs (15 μg per lane) were separated on a 1% formaldehyde-agarose gel, transferred to Nytran membrane, and hybridized to a ^{32}P-labeled IRF-1 cDNA probe. The same blot was reprobed with a GAPDH probe to ensure that equal amounts of RNA were loaded in each lane. (A) Time-course of human IRF-1 mRNA induction with IFN-α and IFN-γ. K562 cells were treated with either IFN-α (1,000 U/ml) or IFN-γ (1,000 U/ml) for the indicated times. (B) IFN-induction of IRF-1 mRNA in the presence of the protein synthesis inhibitor CHX. K562 cells were treated with either IFN-α or IFN-γ (1,000 U/ml) for 1 and 5 h, with or without CHX (50 μg/ml; added 15 min prior to IFN induction). The location of the 28S and 18S rRNAs are indicated. There is a higher-molecular-weight IRF-1 transcript running close to the 28S rRNA. (C) Primer extension analysis of the 5′ ends of human IRF-1 mRNA. Eighty micrograms of total RNA from IFN-γ-induced (1,000 U/ml) K562 and HeLa cells was hybridized with a 40-nt 5′-end-labeled primer (see Materials and Methods). The same primer was used in a sequencing reaction with our IRF-1 cDNA subcloned into pUC18. The relative positions of the major (arrow) and minor (*) transcription start sites are shown. The same transcription initiation sites were detected for both K562 and HeLa cells. The initiations sites for uninduced cells were not detectable by this method (not shown).

with the IRF-1 expression vector, the IFN-β promoter activity was increased >4.5-fold. These data suggest that the presence of excess functional IRF-1 protein does not modulate the activity of the IRF-1 promoter, in transfection experiments.

Binding of factors to the potential IFN-inducible element of the 5′Δ-143 promoter. In order to determine where IFN-inducible transcription factors bound to the 5′Δ-143 promoter, we constructed double-stranded oligonucleotides from two regions. The first potential IFN-inducible domain is from positions −130 to −97 (Fig. 6A). This region contains an AARKGA motif (AAATGA) overlapping with a potential cyclic-AMP responsive element, CRE (TGACGGCA). In addition, the oligonucleotide contains two GAAANN sequences in an inverted repeat-like arrangement. These sites also coincide with a putative κB motif (GGGGAAAATCA), differing at two positions from the consensus (2). The GAAATG sequence also overlaps the AARKGA motif. The oligonucleotide was end labeled with ^{32}P and used in a gel mobility shift assay to determine the binding of putative IFN-inducible protein factors. Figure 6B shows a mobility shift assay in which the −130/−97 fragment was incubated with various extracts from K562 cells. After treating K562 cells with either IFN-α or IFN-γ, a new protein-DNA complex was formed (IRFi [Fig. 6B]). This complex was found in both nuclear and cytoplasmic extracts. The new complex was more strongly induced by treating K562 cells with IFN-γ than with IFN-α. The intensity of the complex correlates well with the level of induced IRF-1 mRNA with IFN-α versus IFN-γ (Fig. 3A). The kinetics of the induced complex also differs between inductions with each IFN. Both complexes were induced within 1 h. Yet, the IFN-α-induced complex had diminished considerably by 4 h postinduction, whereas the IFN-γ-induced complex was sustained.

Mobility shift competition experiments were performed with various unlabeled oligonucleotides to determine the nature of the IFN-induced complex. The −130/−97 fragment contains several sites of interest: a CRE, a AARKGA-hexamer, GAAATG, GAAATC, and a putative κB motif. The AARKGA hexamer and the GAAATG element are thought to efficiently bind factors only when tetramerized (21, 23, 24, 44, 49). Competition experiments were performed with $(AAGTGA)_4$, $(GAAATG)_4$, $(GAAATC)_4$, and a CRE (TGACGTCA). None of these elements competed with the −130/−97 probe for binding to the IFN-inducible complex (Fig. 6C and D). This suggests that none of the proteins which bind to the above sequences (the IRF-1, the TG protein, OTF-1, or CRE-binding proteins) are involved in the IFN-inducible complex formed with the IRF-1 transcription enhancer. Competitions with a high-affinity NF-κB binding site and the κB motif 3′ to the IRF-1 CAAT box failed to diminish the binding of IRFi (Fig. 7), suggesting that κB-like factors do not bind to the −130/−97 domain. ISREs from the 6-16 and 9-27 genes were also used in competition experiments. Neither of the ISREs (6-16, GGAAAATGAAACT, and 9-27, GGAAATAGAAACT) competed with the IFN-

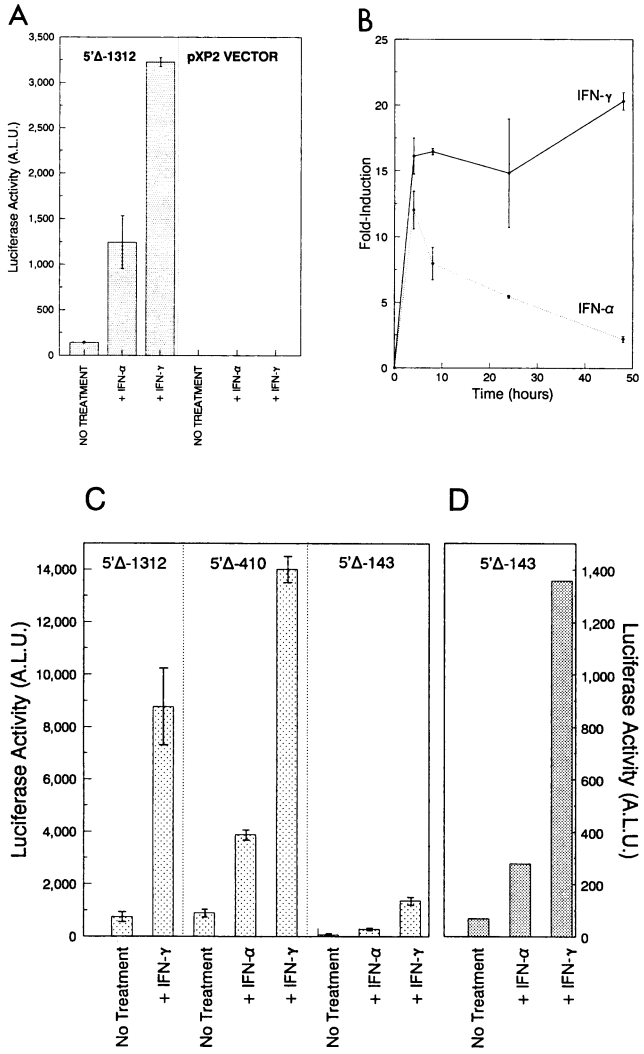

FIG. 4. IFN inducibility of the IRF-1 promoter and analysis of 5′Δ deletions linked to a luciferase reporter gene. (A) IFN induction of the full-length promoter. The full-length IRF-1 promoter was subcloned into a promoterless luciferase gene vector, pXP2, generating 5′Δ-1312-luciferase. The construct was transfected into K562 cells and treated with IFN-α (2,000 U/ml) or IFN-γ (2,000 U/ml). Cells were harvested 8 h postinduction and assayed for luciferase activity (arbitrary light units [A.L.U.]). Each transfection was performed in triplicate and corrected for transfection efficiency by cotransfecting with pSVβ (a β-galactosidase reporter gene driven by the SV40 early promoter). The promoterless vector, pXP2 showed negligible luciferase activity (all values below 5 A.L.U.), with or without IFN-α or IFN-γ induction. (B) Time course of the fold-induction of the IRF-1 promoter induced with IFN-α and IFN-γ. The 5′Δ-1312-luciferase construct was transfected into K562 cells and treated with either IFN-α or IFN-γ for 4.5, 8, 24, and 48 h. The IFN inducibility is represented as the fold-induction over constitutive levels of expression. The 48-h transfection was assayed in triplicate, all others in duplicate. (C and D) Functional analysis of 5′ deletions of the IRF-1 promoter. Two deletions of the IRF-1 promoter were subcloned into the promoterless luciferase reporter gene vector, generating 5′Δ-410-luciferase and 5′Δ-143-luciferase. The activities of these two deletions were compared to that the full-length promoter (5′Δ-1312-luciferase). The promoter constructs were transfected into K562 cells and treated with either IFN-α or IFN-γ (2,000 U/ml) for 15 h. The IFN inducibility of the 5′Δ-143-luciferase construct is represented on a magnified scale (D) to illustrate the similarity of fold-induction between the three promoter deletions.

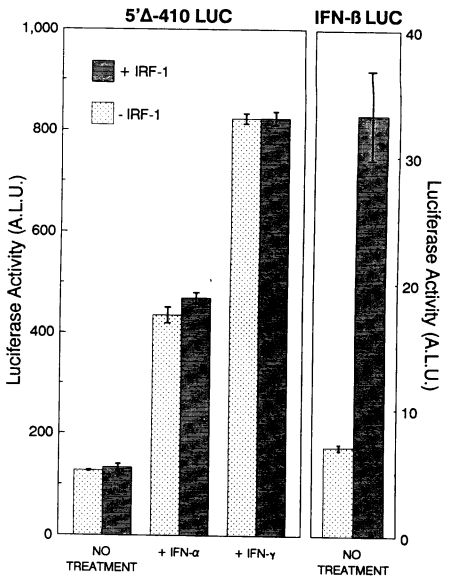

FIG. 5. The IRF-1 protein does not regulate the IRF-1 promoter. An IRF-1 cDNA (isolated in this laboratory) was subcloned into the eukaryotic expression vector, pSVL (containing the SV40 late promoter). (A) Either the IRF-1 expression vector or pSVL was cotransfected with 5′Δ-410-luciferase, into K562 cells. The cells were induced with either IFN-α or IFN-γ (2,000 U/ml) 16 h posttransfection. Cells were harvested 6 h postinduction and assayed for luciferase activity. In all cases, there was no statistically significant difference in 5′Δ-410-driven luciferase activity in samples cotransfected with the IRF-1 expression vector compared with the pSVL vector (n = 3). (B) Either the IRF-1 expression vector or pSVL was cotransfected with IFN-β–luciferase (the human IFN-β promoter driving the promoterless luciferase reporter gene), into K562 cells. Cells were harvested 20 h posttransfection and assayed for luciferase activity. The IRF-1 expression vector caused a greater than fivefold induction of activity from the IFN-β–luciferase construct. In both experiments, each sample was performed in triplicate and the luciferase activities were corrected with the β-galactosidase activities from the pSVβ internal control plasmid.

inducible complex binding to the IRF-1 promoter (Fig. 6D), indicating that the IFN-α-inducible complex, factor E (10), also termed ISGF3 (42), does not bind to the −130/−97 element.

The −130/−97 fragment contains two imperfect inverted repeats (Fig. 6A). One inverted repeat (IR1) is composed of the GAAATC and GAAATG sequences, separated by three nucleotides at the center (from −125 to −111). This domain is perfectly conserved in the mouse IRF-1 promoter (from −127 to −112 [Fig. 2]). The second imperfect inverted repeat (IR2) extends from −109 to −97 but is not conserved in the mouse promoter. Competitions were performed with IR1 and IR2 (Fig. 6E). IR1 efficiently inhibited the IFN-induced complex at 50-fold molar excess, whereas IR2 did not inhibit it at all. This suggests that the IFN-induced complex needs to bind to the GAAAT(C/G) elements in an inverted arrangement. Only two GAAANN sequences were required for binding. A search of the GenBank data base shows that IR1 is not found in any other IFN gene or IFN-inducible gene.

The second potential IFN-inducible domain of the 5′Δ-143 promoter is the κB motif located 3′ to the CAAT site. This motif conforms to the κB consensus and may bind NF-κB or another κB-like factor. Two double-stranded oligonucleotides were synthesized for use in mobility shift competition

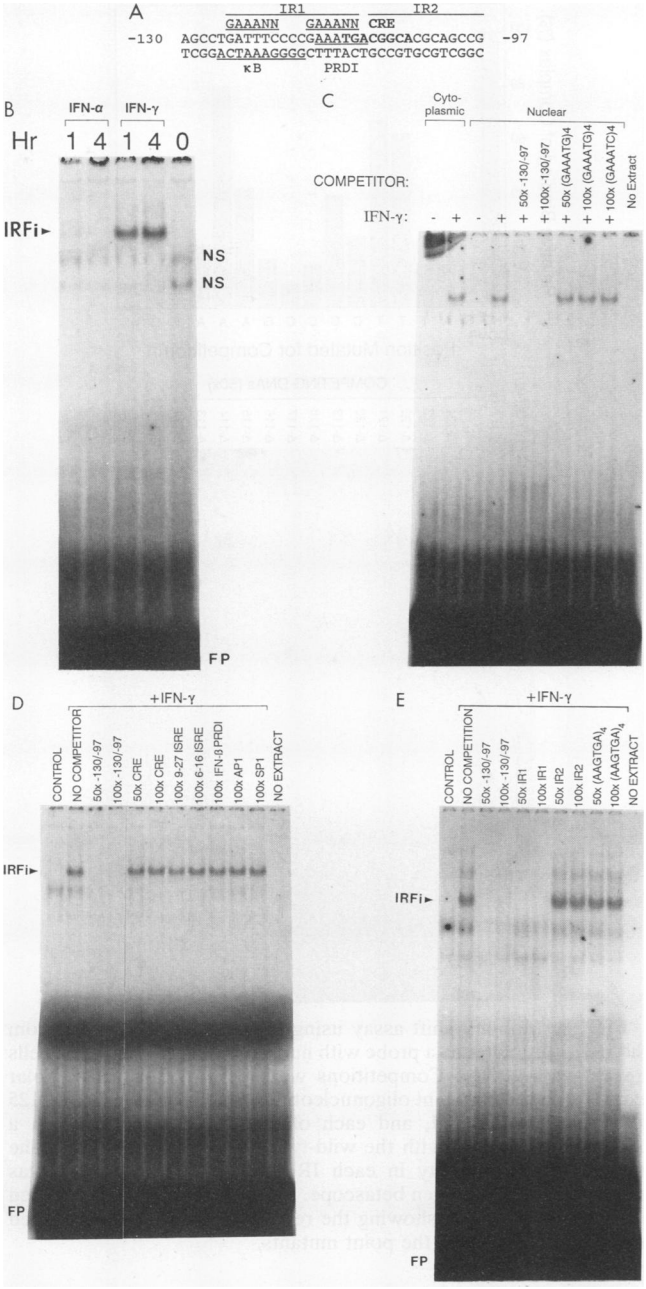

FIG. 6. IFN induced binding of a complex to the −130/−97 IRF-1 promoter fragment. Gel mobility shift assays were used to analyze the binding of IFN-inducible complexes to the putative IFN-inducible domain of the IRF-1 promoter (from −130 to −97). (A) Sequence of the double-stranded oligonucleotide used as a probe in the binding reactions. The domain extends from −130/−97 and includes a putative κB motif, an element with homology to a cyclic AMP response element (CRE), a PRDI-like AARGKA hexamer and two imperfect inverted repeats (IR1 and IR2), one of which contains two GAAANN motifs. (B) Cytoplasmic protein extracts were prepared from K562 cells treated with either IFN-α or IFN-γ (each at 2,000 U/ml) for 1 or 4 h. Binding reactions were performed with these extracts and a synthetic double-stranded oligonucleotide of the −130/−97 domain. An IFN-inducible complex (IRFi) is indicated. Other complexes were determined to be nonspecific (NS) and were found also in the control sample. FP, free (unbound) probe. C to E. Gel mobility shift competition experiments to determine to specificity of the IFN-induced complex (IRFi) binding to the −130/−97 promoter fragment. Nuclear and cytoplasmic extracts from K562 cells induced for 1 h with IFN-γ were used to determine which sequences were important for complex binding. Either 50- or 100-fold molar excesses of double-stranded oligonucleotides, over the probe, were used for competition in the binding reactions. (C) Binding reactions showing that the IFN-inducible complex, IRFi, is present in both nuclear and cytoplasmic extracts. Binding of IRFi is competed by unlabeled probe (−130/−97), but not by tetramerized GAAATG nor GAAATC motifs. (D) Further competition experiments showing that binding of IRFi is not competed by a CRE, ISREs (from the 6-16 and 9-27 genes), an IFN-β PRDI site, an AP1 motif, or an Sp1 binding site. (E) Competition experiments, using IFN-γ-induced nuclear extracts, showing that IRFi is inhibited by the imperfect inverted repeat (IR1), but not by the second imperfect inverted repeat (IR2) nor by a tetramerized AAGTGA hexamer. The more slowly migrating inducible complex may represent a doublet of the IRFi complex.

assays. One was a high-affinity NFκB binding site (63), the other was the κB motif found in a domain 3′ to the CAAT box of the IRF-1 promoter (−55 to −31). Neither of the κB motifs competed with the −130/−97 probe for binding of IRFi (Fig. 7A). In addition, radioactively labeled −55/−31 κB motif or the high affinity NF-κB motif failed to form an inducible complex with nuclear or cytoplasmic extracts from either IFN-α or IFN-γ treated K562 cells (data not shown).

We synthesized a different double-stranded oligonucleotide, from −130 to −106 of the IRF-1 promoter, to delimit the IFN-inducible inverted repeat (IR1). The −130/−106 domain was used as a probe in mobility shift assays with nuclear extracts from K562 cells treated with IFN-γ, with or without the presence of CHX (Fig. 7B). This experiment shows that IRFi can still bind to the −130/−106 probe in the absence of protein synthesis, upon induction with IFN-γ. Treatment of cells with CHX, alone, does not induce binding of IRFi nor a κB protein, to their respective binding sites, in K562 cells (Fig. 7B). These data indicate that the IRFi factor is constitutive within cells but requires posttranslational modification in order to bind to the IRF-1 promoter, upon induction with IFN. Furthermore, these data suggest that the apparent superinduction of IRF-1 mRNAs, in the presence of CHX (Fig. 3B), is not likely to be the result of transcription factors being activated by CHX. It is more probably that IRF-1 mRNAs are stabilized in the presence of CHX, because an mRNA degrading factor cannot be synthesized.

Treating K562 cells with the phorbol ester, TPA, induced binding of NF-κB to the high-affinity NF-κB binding motif, but did not induce binding of IRFi to the −130/−106 probe (Fig. 7B). Similarly, IFN-γ treatment induces binding of IRFi to the −130/−106 domain but does not induce factor binding to the NF-κB motif. These data suggest that IRFi is not a κB-like protein complex.

We conclude that the IRF-1 promoter binds a new protein complex upon IFN-γ induction. The same complex is apparent with IFN-α induction, but in considerably lower amounts and for a much shorter duration. The IFN-inducible complex binds at a unique inverted arrangement of two GAAANN motifs. This is the first report of strong binding of an inducible complex at just two GAAANN motifs in an inverted conformation, rather than as direct repeats. The binding of IRFi to IR1 is more strongly induced by IFN-γ than IFN-α and is dependent on posttranslational modification of a preexisting cellular factor(s).

Point mutation analysis of the IFN-inducible inverted repeat. To further characterize the binding of IRFi to the IR1

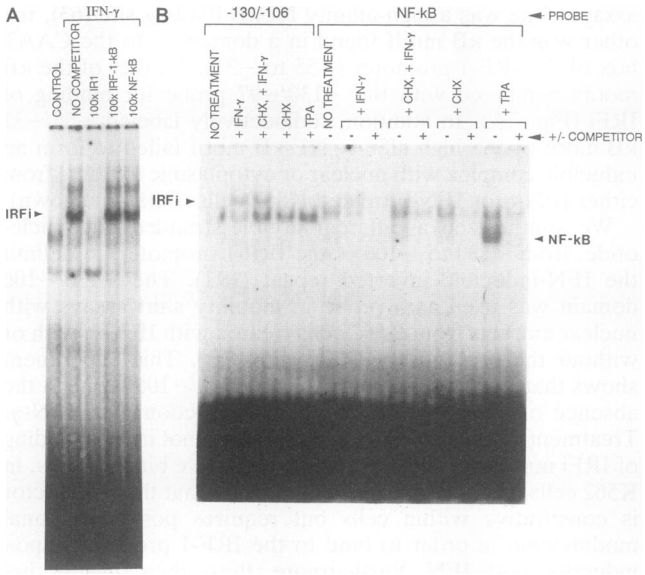

FIG. 7. (A) Mobility shift assays to determine the nature of complexes binding to the inducible domain of the IRF-1 promoter, using IFN-γ-induced nuclear extracts from K562 cells. The −130/−97 probe contains a motif with imperfect homology to the κB consensus. The IFN-inducible complex, IRFi, does not compete with a high-affinity NF-κB site or the κB motif found further downstream in the IRF-1 promoter (−55/−31 oligonucleotide, containing the 49/−40 κB motif). The only motif found to compete the IRFi complex is the IR1 sequence. (B) Mobility shift assays to show that the IR1 domain binds an IFN-inducible factor which is not a κB-binding protein. A domain from −130 to −106 was used as a probe (containing IR1, but not IR2). The binding of IRFi is not induced by CHX, nor is its induction by IFN prevented by the presence of CHX. In addition, a high-affinity NF-κB binding site was used as a probe with the same extracts. Binding of NF-κB was induced only with TPA treatment (30 ng/ml for 15 min). The competitor was the unlabeled NF-κB binding site, used at 100× molar excess over the probe. The positions of the IRFi and NF-κB complexes are indicated. The other complexes seen in these assays were determined to be nonspecific and are inhibited by most of the oligonucleotides used in the current study.

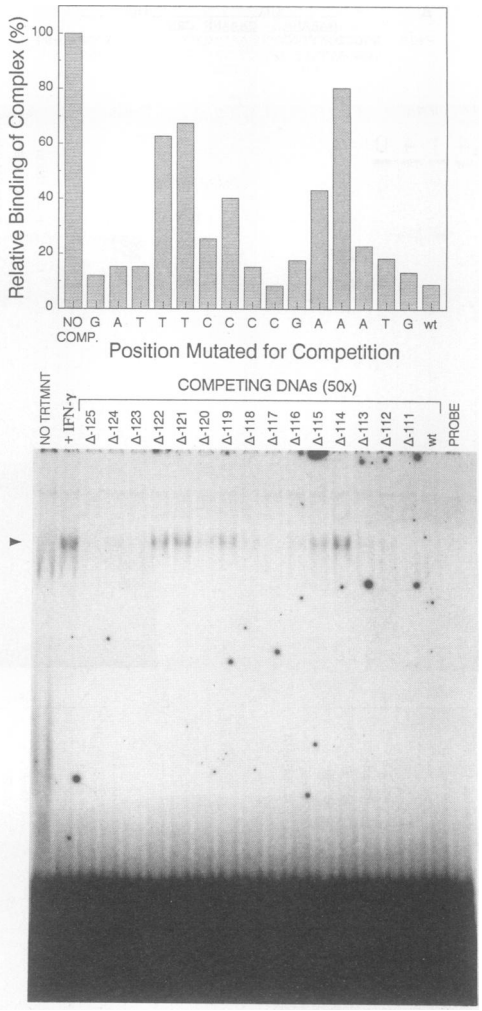

FIG. 8. Mobility shift assay using the −130/−106 domain from the IRF-1 promoter as a probe with nuclear extracts from K562 cells treated with IFN-γ. Competitions were performed at 50× molar excess with point mutant oligonucleotides. Each position from −125 to −111 was mutated, and each oligonucleotide was used in a competition reaction with the wild-type sequence as a probe. The amount of radioactivity in each IRFi complex (arrowhead) was measured with a Betagen betascope. The betascope data were used to generate the graph showing the relative amount of IRFi formed after competition with the point mutants.

domain, we synthesized a series of point mutations for use in competition experiments. Mobility shift assays were performed with nuclear extracts from IFN-γ induced K562 cells, using the −130/−106 domain as a probe. Each position within the inverted repeat region (IR1) of the −130/−106 domain was mutated (purine to pyrimidine and vice-versa). The point mutants were used as competitors for IRFi binding, in mobility shift binding reactions (Fig. 8). The point mutants that compete the least represent the most significant positions for binding of IRFi. The first two A positions of both of the GAAANN motifs appear to be most important for binding of IRFi (positions −114, −115, −121 and −122). In addition, one of the central C residues (at −119) also appears to be important for binding (Fig. 8). Mutation of any one of these residues significantly deminished the ability of that mutant to compete for binding of IRFi to the wild-type probe. Mutation of the NN positions of both GAAANN motifs had only a slight effect on the ability of that mutant to compete for IRFi binding, suggesting that there may well be a degree of degeneracy at these residues.

In conclusion, there is a symmetry in the positions of the residues that are important for binding of IRFi. The first two A residues of each of the inverted GAAANN motifs participate the most in IRFi binding, along with one of the central C nucleotides separating the two hexamer motifs.

The inverted repeat domain confers IFN inducibility upon a heterologous promoter. One copy of the IRFi binding site (from −130 to −106) was subcloned immediately upstream of a herpes simplex virus type I TK promoter driving a luciferase reporter gene (pT109luc [50]). The TK promoter construct, with or without the −130/−106 domain, was transfected into K562 cells (Fig. 9A). The TK promoter construct, alone, showed a basal level of activity, which did not change significantly upon treatment of cells with IFN-γ. However, the TK promoter construct containing an upstream −130/−106 domain showed a ~6-fold increase in activity over basal levels, upon IFN-γ induction.

In order to show that the binding of IRFi to the −130/−106

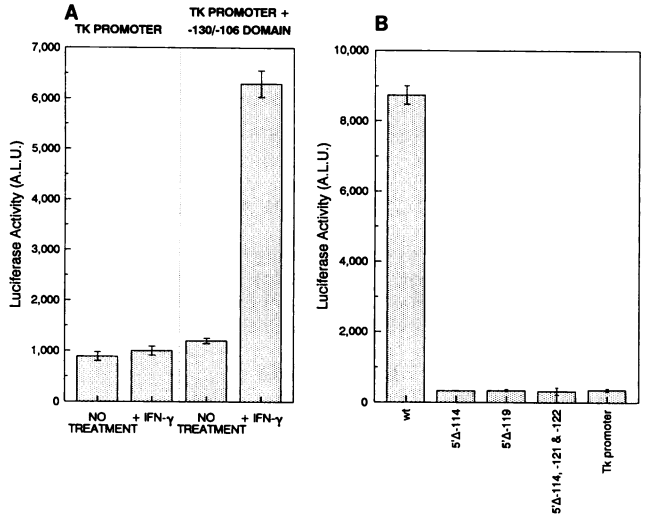

FIG. 9. Transfection experiment showing that the wt −130/−106 domain from the IRF-1 promoter is IFN-γ inducible. (A) The −130/−106 wt oligonucleotide was subcloned upstream of the herpes simplex virus type I TK promoter, driving a luciferase reporter gene. Both the TK and the TK with the −130/−106 domain constructs were transfected into K562 cells, with or without IFN-γ treatment. Cells were harvested 20 h postinduction and assayed for luciferase activity. Each transfection was performed in triplicate and corrected with an internal control reporter gene (β-galactosidase). (B) Three different mutants of the −130/−106 domain were subcloned upstream of the TK promoter driving the luciferase reporter gene. The mutants contained point mutations at position −114 (5′Δ-114), at −119 (5′Δ-119), or at the three positions −114, −121, and −122 (5′Δ-114, −121, and −122). The wt, mutant, and TK promoter (alone) constructs were transfected into K562 cells and treated with IFN-γ. Cells were assayed 24 h postinduction for luciferase activity. Each transfection was performed in duplicate and corrected with the β-galactosidase internal control plasmid.

domain, in vitro, correlates with conferring IFN-inducibility on the TK promoter, in vivo, it was necessary to show that point mutants of the IR1 domain do not confer a functional response to IFN-γ. Three different mutants of the −130/−106 domain were subcloned immediately upstream of the TK promoter in the above luciferase reporter gene vector (pT109luc). The wt, mutant, and TK promoter (without IR1 domains) were transfected into K562 cells and induced with IFN-γ (Fig. 9B). Only the wt IR1 domain conferred IFN-γ inducibility upon the TK promoter. All three of the mutated domains showed levels of luciferase comparable to that of the TK promoter alone, in the presence of IFN-γ.

Taken together, the data showed that the −130/−106 domain, containing IR1, acted as an IFN-inducible element, conferring IFN inducibility on a heterologous promoter. The IFN inducibility of the IR1 domain correlated with the binding of IRFi, in vitro, in gel mobility shift assays. The mutation of key nucleotides which deminished binding of IRFi, in vitro, abrogated the functional response to IFN, in vivo, in the context of the transfection experiments.

DISCUSSION

The IRF-1 promoter meets the proposed requirements for a CpG island (25) from ~−450 to >+250 nt, and thus, extends well beyond the transcription initiation site. It has been suggested that CpG islands may bind ubiquitous transcription factors (4). The general transcription factor, Sp1, binds to elements containing a GC box (GGGCGG) to facilitate transcription (34). There are eight GC boxes within the CpG island of the IRF-1 promoter which almost certainly contribute to the constitutive expression of this gene. The human IRF-1 promoter is shown here to be 83% homologous to the mouse promoter over a region extending from ~−230 to ~+60. Both the mouse and human promoters share the similar relative positions of CAAT boxes and initiation sites (Fig. 2, ignoring gaps inserted for clarity of alignment). The mouse promoter has two GC boxes in the conserved region, whereas the human promoter has four. The two putative κB motifs and the imperfect inverted repeat, IR1, also are conserved between the mouse and human promoters, whereas IR2 is not (see below).

We first identified the transcription initiation sites of the gene. The functional studies suggested that a region 143 nt upstream from the major transcription initiation site contains information necessary for the IFN-induced regulation of IRF-1 gene expression. The minimal promoter retained the same levels of fold-induction as the longer promoter constructs. The Sp1 sites of the promoter 5′ to the −143-nt fragment may serve to amplify the IFN-induced transcriptional activity.

There are no true IRF-1 factor binding sites in the IRF-1 promoter (such as a complete PRDI site or tandemly repeated AARKGA hexamers), suggesting that the gene is not autoregulatory. It is unlikely that these isolated hexamers could bind IRF-1, but we thought it necessary to test the possibility that novel flanking sequences may facilitate IRF-1 binding. The cotransfection experiments of the IRF-1 promoter with an IRF-1 expression vector (Fig. 5) demonstrate that our IRF-1 promoter construct is not autoregulatory. Interestingly, Taniguchi and colleagues recently presented evidence suggesting that the IRF-1 protein does have a positive autoregulatory action on IRF-1 mRNA accumulation (53). The study involved GM-637 cell lines stably transfected with sense or antisense IRF-1 expression vectors. The sense IRF-1-expressing cell line showed a more rapid and enhanced accumulation of endogenous IRF-1 mRNA upon either viral or poly(I) · poly(C) induction, than the control cell line. This result was interpreted as IRF-1 having a positive autoregulatory role in its own synthesis (53). However, this is an indirect assay of the effects of IRF-1 on its own promoter. It is possible that the excess of IRF-1 in the sense cell line may be activating other genes or pathways involved in IRF-1 gene expression. The data presented in the current study suggest that, at least for episomal IRF-1 promoter activity, the IRF-1 protein does not have an autoregulatory role.

We, then, conducted gel shift assays by using two potential IFN-inducible domains within the minimal IRF-1 promoter. These data suggested that IRF-1, the TG protein, OCT-1, nor κB-like factors are binding to the IFN-inducible domain 5′ to the CAAT box of the IRF-1 promoter. ISREs contain two direct repeats of the GAAANN motif and have been shown to be responsive to both IFN-α and IFN-γ (52). Our competition experiments with ISREs from the 6-16 and 9-27 genes also did not abolish binding of the IRFi complex, implying that complex E (10), or ISGF3 (42), does not bind to the −130/−97 fragment. A closer analysis of the −130/−97 fragment shows the presence of two palindrome-like domains, consisting of imperfect inverted repeats. One of the imperfect inverted repeats, IR1, includes the GAAATG and GAAATC motifs (*GATTTCCCCGAAATG*) and is conserved in the virus-inducible murine IRF-1 promoter (Fig. 2) (48). The IR1 domain also contains homology to the κB

consensus. The second imperfect inverted repeat, IR2, is composed of CGGCACGCAGCCG and is not conserved in the murine promoter. Competition experiments with these two inverted repeats shows that IR1, but not IR2 nor a high-affinity NF-κB motif, inhibits the IFN-inducible complex, IRFi (Fig. 6E and 7A). It appears that the hitherto undefined arrangement of two GAAANN motifs in an inverted fashion mediates the binding of a novel IFN-inducible factor to the IRF-1 promoter. To further characterize the IFN-inducible factor binding site, we performed binding competition assays with point mutants spanning the entire IR1 domain (Fig. 8). Mutation of either of the first two A residues of either GAAANN motif significantly reduced the ability of these mutants to compete with the wild-type −130/−106 domain for binding of IRFi. One of the central C residues also appears to play a significant role in factor binding. It is interesting to note the symmetry of the sites that are important for factor binding. It is the second and third residues of each inverted GAAANN motif that contribute the most to binding of IRFi. Although our experiments suggest a degree of degeneracy at the NN positions of the GAAANN hexamer, it is not yet clear if IRFi binding can still occur with complete degeneracy.

Other DNA elements, which strongly bind factors upon IFN-γ induction, have been described. These elements include the IFN-γ-activated site of the guanylate-binding protein gene (12), the interferon response sequence of the major histocompatibility complex MHC class I, *H-2K*^{2} gene (5) and other MHC class I genes (15), and the IFN-γ-responsive elements of various MHC class II genes (3, 59). However, there is little overall homology of these elements to the IR1 domain presented here.

The subcloning of the −130/−106 wt and mutant domains upstream of the TK promoter shows, definitively, that the wt IR1 region is capable of conferring IFN inducibility upon a heterologous promoter (Fig. 9A and B). There is a tight correlation between binding of IRFi to wt IR1 sequences, in vitro, and a functional response to IFN-γ, in vivo (at least in the context of transfection experiments). The mobility shift experiments, using CHX, strongly suggest that IRFi is a constitutive factor that is posttranslationally activated to bind to the inverted repeat, upon IFN induction. The IFN induction of the IRF-1 promoter does not appear to involve κB-like factors but may be conferred solely by binding of IRFi to the IR1 domain.

The data presented in this paper show that the human IRF-1 gene is constitutively expressed and is inducible with type I and type II IFNs. IFN induction of K562 cells results in the accumulation of IRF-1 mRNA, albeit with different kinetics between IFN-α and IFN-γ. The kinetics of mRNA accumulation correlates well with (i) the IFN-induced activity of the IRF-1 promoter on a heterologous reporter gene and (ii) the appearance of an IFN-inducible protein-DNA complex, IRFi. The IRF-1 gene does not appear to be autoregulatory in the presence or absence of IFN induction. However, IFN-induced regulation of the IRF-1 gene is mediated by factors binding to a novel IFN-inducible element. We are faced with the problem of how the IRFi factor(s) binding to the novel IFN-inducible element is, in turn, regulated.

It is possible that other inducers of IRF-1 expression may mediate their effects through the κB motifs of the IRF-1 promoter, as NF-κB is known to be activated by a number of agents which could, similarly, activate the IRF-1 gene (2).

We are currently purifying the IRFi factor to further elucidate its molecular properties and mode of action in IRF-1 gene expression as well as its potential involvement in regulating other IFN-inducible genes.

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