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Structure and Regulation of the Human Interferon Regulatory Factor 1 (IRF-1) and IRF-2 Genes: Implications for a Gene Network in the Interferon System

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Interferon regulatory factor 1 (IRF-1) and IRF-2 are structurally similar DNA-binding factors which were originally identified as regulators of the type I interferon (IFN) system; the former functions as a transcriptional activator, and the latter represses IRF-1 function by competing for the same *cis* elements. More recent studies have revealed new roles of the two factors in the regulation of cell growth; IRF-1 and IRF-2 manifest antioncogenic and oncogenic activities, respectively. In this study, we determined the structures and chromosomal locations of the human IRF-1 and IRF-2 genes and further characterized the promoters of the respective genes. Comparison of exon-intron organization of the two genes revealed a common evolutionary structure, notably within the exons encoding the N-terminal portions of the two factors. We confirmed the chromosomal mapping of the human IRF-1 gene to 5q31.1 and newly assigned the IRF-2 gene to 4q35.1, using fluorescence in situ hybridization. The 5′ regulatory regions of both genes contain highly GC-rich sequences and consensus binding sequences for several known transcription factors, including NF-κB. Interestingly, one IRF binding site was found within the IRF-2 promoter, and expression of the IRF-2 gene was affected by both transient and stable IRF-1 expression. In addition, one potential IFN-γ-activated sequence was found within the IRF-1 promoter. Thus, these results may shed light on the complex gene network involved in regulation of the IFN system.

Interferons (IFNs) are a family of multifunctional cytokines which were originally identified as antiviral proteins. IFNs elicit antiviral activities by inducing the expression of various genes, referred to as IFN-inducible genes (7, 44, 51, 55). In addition, IFNs are well known as regulators of cell growth and differentiation (6, 7, 33, 38, 49, 55). Previously, we identified two novel DNA-binding factors, IFN regulatory factor 1 (IRF-1) and IRF-2, as regulators of the type I IFN (IFN-α and -β) genes (17, 19, 37). The two factors are structurally related, particularly in the N-terminal regions, which confer specificity for DNA binding, and they both bind to the same sequence within the promoters of IFN-α and IFN-β genes (19, 50). DNA sequences recognized by IRFs have been also found in the regulatory regions, termed IFN-stimulated regulatory elements, in a number of IFN-inducible genes (50). cDNA transfection studies have shown that IRF-1 functions as a transcriptional activator and IRF-2 represses IRF-1 action (13, 21, 40). In addition, the genes for IRF-1 and IRF-2 are both virus and IFN inducible (19). In IFN-stimulated cells, IRF-2 gene induction occurs only after induction of IRF-1 (19). In IFN-treated or virus-infected cells, the IRF-2 protein is more stable than the IRF-1 protein (half-lives of 8 h and 30 min, respectively) (57). Thus, in growing cells, IRF-2 is more abundant than IRF-1, but after stimulation by IFN or viruses, the amount of IRF-1 increases relative to the amount of IRF-2 (57). The IRF-1 gene is shown to be also induced by other cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, leukemia inhibitory factor (LIF), and prolactin (1, 16, 39, 61). Previously, the 5′ flanking region of the mouse IRF-1 gene (spanning up to −299 from the cap site) was shown to contain a virus-inducible DNA element by using a transient expression system (37). More recently, promoter elements affecting human IRF-1 gene expression have been reported (47). In the context of cell growth regulation, it has been recently demonstrated that IRF-1 and IRF-2 have antioncogenic and oncogenic potentials, respectively, in NIH 3T3 cells (20). Moreover, the human IRF-1 gene is frequently deleted in patients with leukemia or myelodysplastic syndromes (59). These observations indicate that restrained cell growth depends on a balance between these two mutually antagonistic transcription factors and that subtle changes in the IRF-1/IRF-2 ratio perturb cell growth control (20). Taken together, these finding suggest that a complex gene network is involved in regulation of the IFN system and cell growth and that the interplay between IRF-1 and IRF-2 genes may be critical for the proper balance of the IRF-1/IRF-2 ratio.

To gain more insight on the functional relationship between IRF-1 and IRF-2, the structures and the chromosomal locations of human IRF-1 and IRF-2 genes were determined. In addition, the promoters of the respective genes were characterized. We demonstrate that the IRF-1 and IRF-2 genes consist of 10 exons and 9 exons, respectively. Comparison of the exon-intron organization reveals a common evolutionary origin corresponding to the region from exons 2 to 4 which contains the N-terminal portion of the proteins. Furthermore, the human IRF-1 and IRF-2 genes were mapped to 5q31.1 and 4q35.1, respectively, by applying

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fluorescence in situ hybridization (FISH). We have examined the 5′ regulatory regions of both genes and have found highly GC-rich sequences and consensus binding sequences for several known transcription factors. Interestingly, both promoters contain the binding sequence of NF-κB, which has also been shown to play a role in expression of the IFN-β gene (14, 22, 32, 56). Interestingly, one IRF binding site is found within the IRF-2 but not the IRF-1 promoter. Furthermore, expression of the IRF-2 gene is affected by both transient and stable IRF-1 expression. We will discuss the significance of our findings in the context of the gene network for the regulation of the IFN system and cell growth.

MATERIALS AND METHODS

Isolation of human IRF-2 genomic clones. A human genomic library which has also been used in our previous studies (45) was generously provided by R. Lawn (Genentech, Inc., South San Francisco, Calif.) (31). Additional clones for the IRF-2 gene were isolated from a cosmid library generously provided by T. Tokino (Osaka University). For the isolation of specific clones corresponding to the human IRF-2 cDNA, in situ hybridization was performed with pHIRF4S-51 (25) as a probe. The probes were labeled by a multiprime DNA labeling reaction (Amersham).

DNA sequence analysis. The nucleotide sequences were determined by a dideoxy method (Sequenase; United States Biochemical, Inc.), using synthetic primers complementary to human IRF-1 and IRF-2 cDNAs and the Maxam-Gilbert procedure (36).

Chromosomal localization by FISH. A genomic clone (COS1; 30-kb insert) for IRF-2 was used as a DNA probe. A direct mapping system based on FISH combined with replicated prometaphase R bands (48) was used. This system allows the detection of fluorescent signals on R-banded prometaphases. The procedure for FISH with a biotin-avidin system has been previously described (48). A suppression hybridization procedure with total human DNA as previously described was used to eliminate repetitive sequences in these two clones (23). A 30-fold excess amount of total human placenta DNA was added for both clones. The concentration of the probe DNA was 300 ng/20 μl of hybridization mixture per slide. Routine procedures for hybridization, rinsing, and detection were followed. Ektachrome film (Kodak ASA 100) was used for microphotography (filter combinations, Nikon B-2A and B-2E).

Construction of plasmids. The DNA fragment containing positions −492 to +177 relative to the major cap site of the IRF-1 gene was recloned into Bluescript KS M13+ (Stratagene) to construct pBSIRF1B. The IRF-1 promoter-thymidine kinase gene (*tk*) fusion plasmids were constructed by replacing the region of the chloramphenicol acetyltransferase gene (*cat*) in the IRF-1 promoter-*cat* fusion plasmids (*Hin*dIII-*Bam*HI fragment) with herpes simplex virus-*tk* in pRSVtk (46). The IRF-1 promoter-*cat* fusion plasmids were constructed as follows. Plasmid p-492IRF1cat was constructed by ligating a *Bam*HI-*Hin*dIII fragment from pBSIRF1B and a *Bgl*II-*Hin*dIII backbone fragment from pA10cat2 (41). Plasmid p-333IRF1cat was constructed by ligating a *Bst*XI-*Hin*dIII fragment from pBSIRF1B, whose *Bst*XI site became blunt by T4 DNA polymerase, and a *Sal*I-*Hin*dIII backbone fragment from pA10cat2, whose *Sal*I site was rendered flush by fill-in. Plasmids p-216IRF1cat, p-143IRF1cat, and p+8IRF1cat were constructed similarly to p-333IRF1cat by ligating *Sac*II-*Hin*dIII, *Nhe*I-*Hin*dIII, and *Sac*I-*Hin*dIII fragments, respectively from pBSIRF1B and a *Sal*I-*Hin*dIII backbone fragment from pA10cat2. Plasmid p-3500IRF1cat was constructed by using the following three pieces of DNA: (i) a 3-kb *Eco*RI-*Hin*dIII fragment containing positions −3500 to −492, whose *Eco*RI site was rendered flush by fill-in; (ii) a 501-bp *Hin*dIII-*Sac*I fragment containing positions −492 to +8; and (iii) a 5.2-kb *Sal*I-*Sac*I fragment from p-492IRF1cat, whose *Sal*I site was rendered flush by fill-in. The IRF-2 promoter-*cat* fusion plasmid, p-414IRF2cat, was constructed by using the following two pieces of DNA: (i) an *Ava*II-*Taq*I fragment containing positions −414 to +19 relative to the major cap site of the IRF-2 gene, whose sites became blunt by T4 DNA polymerase; and (ii) *Hin*dIII-digested pSV00cat (2), whose sites were rendered flush by fill-in.

Gel shift assays. For the detection of NF-κB, L929t (14), cells were harvested 9 h after Newcastle disease virus (NDV) infection and nuclear extracts were prepared by the method of Dignam et al. (8). For factor binding, a reaction mixture (10 μl) containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EGTA, 4% Ficoll, 1 μg of herring sperm DNA, 2 μg of poly(dI-dC) · poly(dI-dC), ^{32}P-labeled probe DNA (4 fmol; 8,000 cpm/fmol), and 10 μg of nuclear extract, in the presence or absence of 4 pmol of unlabeled competitor DNA, was incubated for 15 min at 0°C. The sequence of probe DNA containing the NF-κB motif of the IRF-2 promoter is

5′-CGGGGGGTGGGGGGATTTCCAGCCGCGGCTCTTCGCAGTT-3′
3′-GCCCCCCACCCCCCTAAAGGTCGGCGCCGAGAAGCGTCAA-5′

The sequence of competitor DNA containing the NF-κB motif of IRF-1 promoter is

5′-GGGCCGGCCAGGGCTGGGGAATCCCGCTAAGTGTTTGGAT-3′
3′-CCCGGCCGGTCCCGACCCCTTAGGGCGATTCACAAACCTA-5′

The sequences of competitor DNAs containing the NF-κB motifs of the IFN-β and *H-2K*^{b} promoters, A1 and A4, respectively, are given in reference (14).

For detection of IRF, in vitro-translated IRF-1 and IRF-2 proteins were prepared as described previously (50). For factor binding, a gel shift assay was performed as previously described (21). Oligomer C13, which has one IRF binding motif (18, 50), was used as the probe DNA. The sequence of competitor DNA containing the IRF motif of the IRF-2 promoter is

5′-TCGACGCCGCGACCTGGGGAAGCGAAAATGAAATTGACTTTTCCG-3′
3′-GCGGCGCTGGACCCCTTCGCTTTTACTTTAACTGAAAAGGCCTAG-5′

DNA transfection and CAT assay. DNA transfection was performed by the calcium phosphate method (15). To obtain stable transformants, 5 × 10^5 L929t cells were transfected with 0.3 μg of pSTneoB (29) and 15 μg of pUCHIRF1B or each IRF-1–*tk* chimeric plasmid. G418-resistant cells were selected in ES medium (Nissui) containing 1 mg of G418 per ml. Usually, approximately 100 colonies were obtained in each transfection, and they were cultivated as a mixture. This procedure has been used to average out the possible fluctuations observed with individual colonies (15). DNA transfection into P19 cells and the CAT assay were performed as previously described (21).

RNA isolation and S1 mapping analysis. Cells were harvested 9 h after NDV infection and 1 h after IFN-β (Toray, Tokyo, Japan) treatment. Isolation of cytoplasmic RNA and S1 mapping analysis were performed as previously described (18). The human IRF-1 probe, which spans from nucleotide

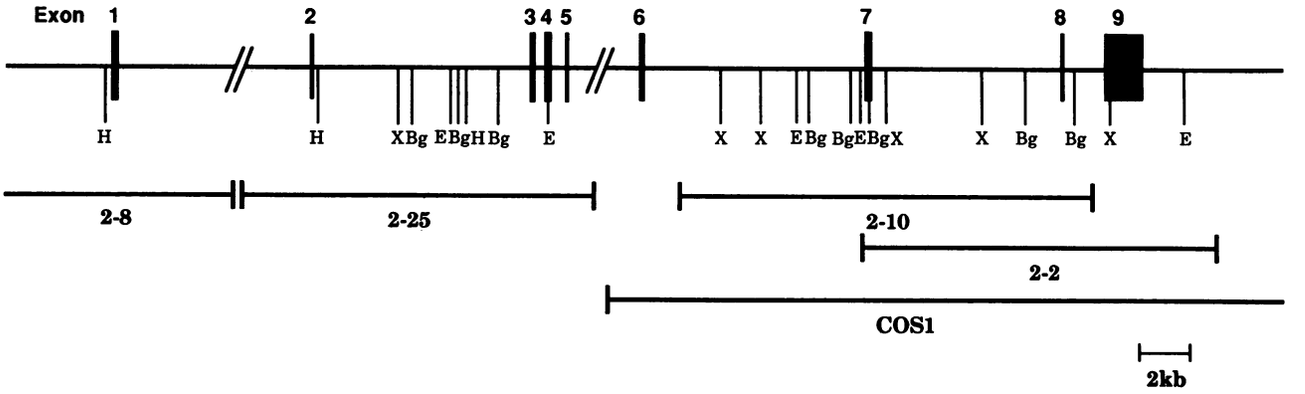

FIG. 1. Organization of the human IRF-2 gene. A restriction map of the human IRF-2 gene constructed from the overlapping bacteriophage clones and a cosmid clone is depicted beneath the map. Positions of the exons are shown. H, *Hin*dIII; E, *Eco*RI; X, *Xho*I; Bg, *Bgl*II.

residues −46 to +97, and the mouse IRF-1 probe were prepared as described previously (21). The RNA copy numbers were estimated as described previously (18).

RNA blotting analysis. The same filter previously used (40) was rehybridized with the ^{32}P-labeled DNA probe. To prepare the IRF-2 probe, a 1.4-kb *Xba*I fragment from pHIRF4S-51 (25) was labeled by a multiprime DNA labeling reaction (Amersham).

Nucleotide sequence accession numbers. The sequences of the human IRF-1 and IRF-2 promoters have been assigned EMBL accession number X53095 and EMBL/GenBank accession number D14082, respectively.

RESULTS

Isolation and characterization of the human IRF-1 and IRF-2 genes. To isolate chromosomal DNA fragments encompassing the gene for human IRF-1, a gene library in which the human DNA partially cleaved by *Hae*III-*Alu*I and cloned into λ Charon 4A arms (31, 45) was screened by the in situ procedure, using the pHIRF31 (35) cDNA insert as a probe. As previously shown (59, 60), clone 1-B carrying a 19-kb insert contained DNA sequences for both 5′- and 3′-terminal regions of the IRF-1 cDNA. The IRF-1 gene consists of 10 exons, in which the initiator ATG and the terminator TAG sequences are encoded by the second and last exons, respectively (5) (see also Fig. 2). Comparison of the nucleotide sequences of the exons with that of the pHIRF31 cDNA which was isolated from a human T-cell line-derived library (35) revealed several mismatches, resulting in three amino acid changes, Leu-34 to Phe, Glu-35 to Gln, and Ile-220 to Thr, as also reported by others (39, 58).

Similar analysis was performed on phage clones isolated from the same human genomic library, using the human IRF-2 cDNA pHIRF4S-51 (25) as a probe. By screening 1.2 × 10^6 plaques, five positive clones were identified. Restriction enzyme maps of the five inserts and locations of the exons within the inserts are shown in Fig. 1. Clone 2-8 contains the DNA sequences of the 5′-terminal region of the IRF-2 cDNA and extends beyond the 3′ terminus of exon 1. Clone 2-25 contains sequences from the 5′-terminal region of exon 2 to the 3′-terminal region of exon 5, whereas clone 2-2 contains the 5′-terminal region of exon 7 and the 3′-terminal region of the IRF-2 cDNA. Since these clones did not contain the entire IRF-2 gene, a cosmid library, in which total human DNA was partially cleaved by *Sau*3AI and cloned into pWE15, was screened, using probes derived from the 1.2- and 3.6-kb *Eco*RI fragments of clones 2-2 and 2-25, respectively (Fig. 1). One cosmid clone, COS1, which contained the 5′-terminal region of exon 6 and the 3′-terminal region of the cDNA was isolated. Nucleotide sequence and restriction enzyme analysis of these clones revealed that the IRF-2 gene consists of nine exons, in which the initiator ATG and the terminator TAA sequences are encoded by the second and last exons, respectively. The nucleotide sequences of all of the exons were in complete agreement with that of the pHIRF4S-51 cDNA (data not shown). All sequences at exon-intron boundaries conform to the canonical sequences (data not shown).

Comparison of the exon-intron organization of the IRF-1 and IRF-2 genes (Fig. 2) (5) reveals extensive sequence identities in the regions of exon 2 through exon 4 corresponding to the DNA binding domain located in the N-terminal portion of the respective proteins (19). Outside this region, the exons show little if any sequence identities. These results suggest that both genes diverged from a common ancestor by gene duplication and that selective pressure was exerted during evolution on the exons encoding the DNA binding domains of the two genes.

Chromosomal assignment of the IRF-1 and IRF-2 genes. To determine the chromosomal locations of the IRF-1 and IRF-2 genes, FISH analyses were performed. We examined 100 (pro)metaphase plates showing typical R bands by using both pUCHIRF1B (a derivative of pUC19 carrying a 19-kb insert of clone 1-B) and COS1 clones for IRF-1 and IRF-2, respectively. The IRF-1 gene is assigned to band 5q31.1, further confirming previous reports (data not shown) (26, 42, 59). In the case of COS1 (i.e., the IRF-2 gene), 97% of the plates exhibited symmetrical double spots on both homologs localized to band q35.1 of the long arm of chromosome 4. No signals were observed on other chromosomes. Thus, the IRF-2 gene is mapped to band 4q35.1 (Fig. 3).

The IRF-1 and IRF-2 promoters and factor binding sites. Previous studies have demonstrated that both the IRF-1 and IRF-2 genes are induced by NDV and IFN-β (19). Furthermore, the IRF-1 gene was found to be induced by other stimuli such as TNF-α, IL-1, IL-6, LIF, prolactin, and second-messenger pathways in human or mouse cell lines (1, 16, 39, 61). To gain insight on the regulatory mechanism of the IRF genes, we next analyzed the sequences of the IRF-1 and IRF-2 promoter regions. As shown in Fig. 4, typical TATA box-like sequences were not identifiable in the up-

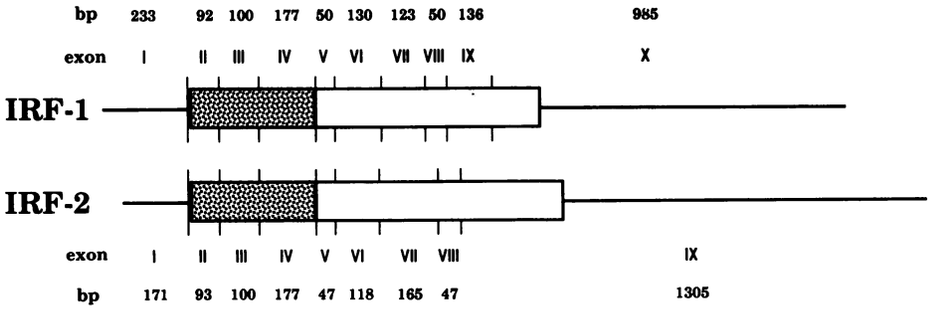

FIG. 2. Exon-intron organization of the human IRF-1 and IRF-2 genes. The size of each exon (in nucleotides) is given. The protein-coding regions are boxed. Dotted regions in exons indicate extensive sequence identities in the region between the IRF-1 and IRF-2 genes.

stream region of either gene. One major and one minor cap site were identified in both genes by S1 mapping analysis, using RNAs from FL fibroblast or Jurkat T-cell lines, respectively, induced by NDV or tetradecanoyl phorbol acetate plus phytohemagglutinin (data not shown; see Fig. 4). As shown in Fig. 4, the 5′ flanking regions of both genes contain highly GC-rich sequences. Furthermore, several putative binding sites for known transcription factors can be detected; they include GC boxes (Sp1 binding sites) (11) and CAAT boxes. In addition, one potential IFN-γ-activated sequence (27) was found within the IRF-1 promoter (−122 to −112) (Fig. 4).

It has been reported that NF-κB is involved in virus- or double-stranded RNA-induced IFN-β gene regulation (14, 22, 32, 56). Moreover, it has been reported that the IRF-1 promoter contains one NF-κB site (27, 43, 47). Interestingly, the IRF-2 promoter also possesses a potential NF-κB binding sequence (−33 to −24) (Fig. 4B). To test whether the sequence indeed binds NF-κB, gel shift assays were performed with an IRF-2 promoter fragment containing the potential NF-κB binding site as a probe. In the nuclear extract of mouse L929 cells infected with NDV, a virus-inducible complex was detected (Fig. 5A, lane 1). This complex was abolished by the DNA fragments containing the NF-κB sites within the promoters of human IFN-β (−66 to −57) (15), human IRF-1 (−47 to −38), human IRF-2 (−33 to −24), and mouse major histocompatibility complex class I (−171 to −162) (4) genes (Fig. 5A, lanes 2 to 5). Hence, these results suggest the involvement of NF-κB (or an NF-κB-like factor) in the induction of not only the IFN-β gene but also the IRF-1 and IRF-2 genes in virus-infected cells.

It has been shown that in IFN-stimulated cells, IRF-2 gene induction occurs only after induction of IRF-1 (19), suggesting the possible involvement of IRF-1 in IRF-2 gene induction. From this point of view, it is interesting that one potential IRF site was found within the IRF-2 promoter (−247 to −258; Fig. 4B) (50). To examine IRF binding to this sequence, IRF proteins were synthesized in vitro and subjected to a gel shift assay using a synthetic C13 oligomer DNA which contains one high-affinity IRF binding site as the probe (18, 50) (see Materials and Methods). As shown in Fig. 5B, the resulting DNA–IRF-1 and DNA–IRF-2 complexes were specifically abolished by the fragment containing the IRF site of the IRF-2 promoter. The affinities of both proteins to this IRF site are about one-third to one-fifth of that of the C13 oligomer (Fig. 5B, lanes 1 to 5 and 9 to 13). These results suggest that expression of the IRF-2 gene may be regulated by IRFs (see below).

Functional analysis of IRF-1 and IRF-2 gene promoters. To characterize further the *cis* elements controlling IRF-1 gene expression, we first transfected pUCHIRF1B (a derivative of pUC19 carrying a 19-kb insert of clone 1-B) with the neomycin resistance gene into mouse L929 cells. Approximately 100 G418-resistant colonies were pooled, and the extracted DNA was subjected to Southern blot analysis. The transfected gene was estimated to be present in about seven copies (data not shown). Cells were then either mock induced or induced by NDV and IFN-β, and the human and endogenous mouse IRF-1 mRNA levels were determined by

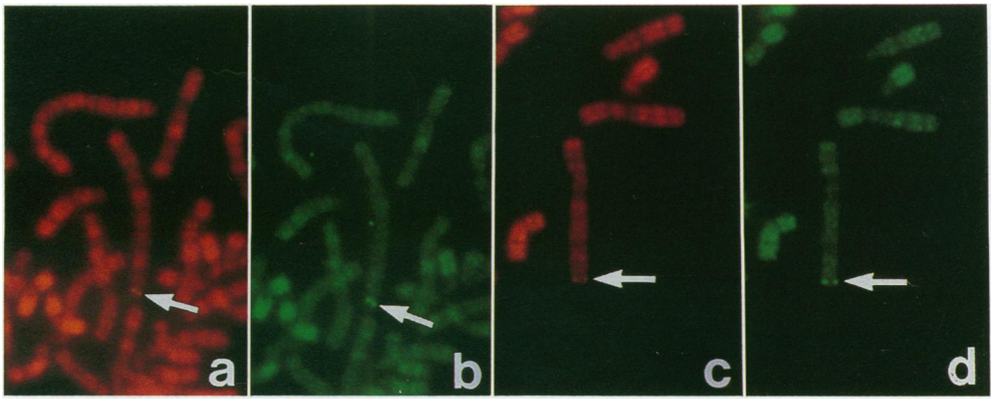

FIG. 3. Chromosomal localization of the human IRF-2 gene. Shown are partial R-banded (pro)metaphase plates hybridized with the biotinylated human IRF-2 gene (Nikon filter combinations B-2A [a and c] and B-2E [b and d]). Arrows indicate signals on 4q35.1.

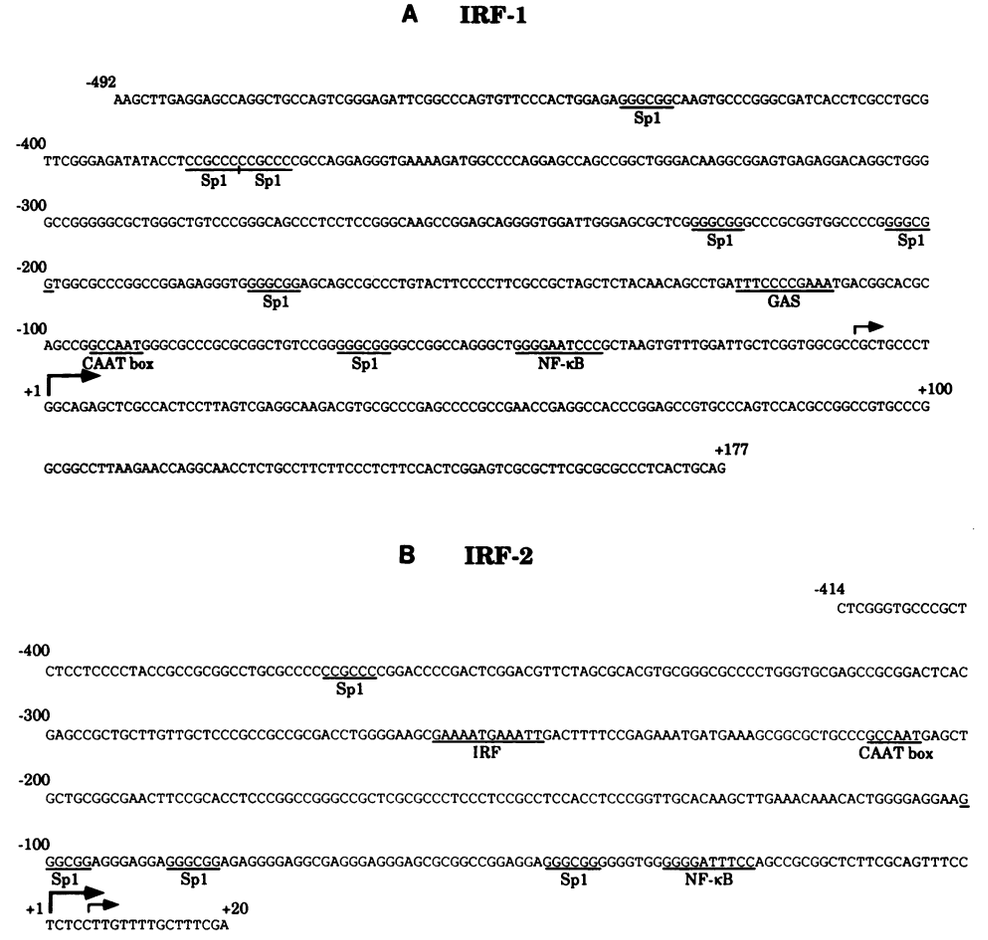

FIG. 4. Sequences of genomic fragments containing human IRF-1 and IRF-2 promoters. (A) Nucleotide sequence of the human IRF-1 promoter. The thick and thin arrows indicate the major and minor cap sites, respectively, determined by S1 mapping analysis. These sites are similar but not identical in position to the cap sites described by Sims et al. (47). This difference may be due to the use of different methods (i.e., S1 mapping in this study and primer extension in the study of Sims et al. [47]). The putative recognition sites for known transcriptional factors are indicated. GAS, IFN-γ-activated sequence. (B) Nucleotide sequence of the human IRF-2 promoter. The thick and thin arrows indicate the major and minor cap sites, respectively, determined by S1 mapping analysis. The putative recognition sites for known transcriptional factors are indicated.

S1 analysis. The RNA expression levels of human and mouse IRF-1 were about 1.9 and 6.1 copies per cell, respectively, in the mock-induced L929 transformants. In addition, the mRNA levels increased following treatment of the cells with NDV or IFN-β (77.0 or 21.7 copies per cell for human IRF-1 and 188.3 or 78.2 copies per cell for mouse IRF-1) (Fig. 6A). The mRNA levels of the transfected human IRF-1 gene were lower than those of endogenous mouse IRF-1 gene. However, the relative fold inductions with both NDV and IFN-β are similar among those genes. These results show that the transfected gene responds to the extracellular stimuli in a manner analogous to the endogenous gene.

We next focused on the promoter region for further analyses. To dissect the function of the promoter region of IRF-1, DNA fragments containing various 5′ deletion mutants were abutted to the herpes simplex virus type 1 *tk* gene as depicted in Fig. 6B and were each cotransfected with the neomycin resistance gene into mouse L929 cells. Approximately 100 G418-resistant colonies were pooled, and Southern blot analysis was performed. For each pool, the transfected gene was estimated to be present in about six copies on average (data not shown). Cells were then either mock induced or induced by NDV or IFN-β, and the *tk* mRNA levels were determined by S1 analysis. As shown in Fig. 6C and D, the RNA expression levels were low but detectable in the mock-induced L929 transformants carrying p-3500IRF1TK, p-492IRF1TK, p-333IRF1TK, p-216IRF1TK, or p-143IRF1TK (about 1.1, 0.3, 0.4, 0.4, or 0.3 copies per cell, respectively) but not in the transformants carrying p+8IRF1TK, indicating the presence of a weak, constitutive promoter activity. The mRNA levels increased following treatment of the cells with NDV or IFN-β in the transformants transfected with p-3500IRF1TK (48.1 and 8.2 copies), p-492IRF1TK (5.3 and 1.4 copies), p-333IRF1TK (10.2 and 2.0 copies), and p-216IRF1TK (6.9 and 1.6 copies) but not with p-143IRF1TK and p+8IRF1TK (Fig. 6C and D). Interestingly, the the fold induction of inducible promoter activity decreased as the 5′ deletion extended from −3500 to −492 and −216 to −143. The results indicate the presence of virus- or IFN-inducible elements which are distributed within the upstream region between −3500 to −492 and −216 to −143. Essentially the same DNA sequences were also found to

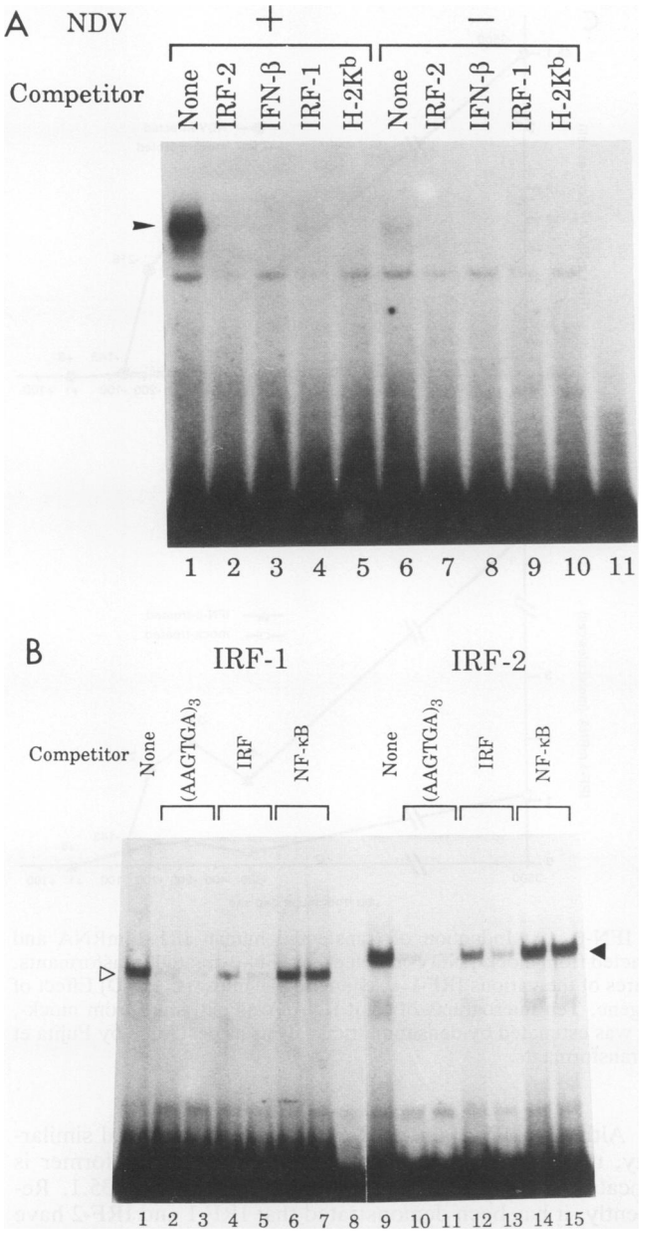

FIG. 5. (A) Identification of the factor binding to the NF-κB motif in the human IRF-1 and IRF-2 promoters. Nuclear extracts from mock- or NDV-induced L929 cells were subjected to a gel shift assay using a ^{32}P-labeled oligomers containing the NF-κB motif from the IRF-2 promoter (see Materials and Methods). Lanes: 1 and 6, no competitor DNA; 2 to 5, 7 to 10- and 1,000-fold molar excesses of unlabeled oligomers containing the NF-κB motif from the IRF-2, IFN-β, IRF-1, and *H-2K^b* promoters, respectively, included in the reaction mixture; 11, no extract. The arrowhead indicates the position of the factor-DNA complex. (B) Identification of IRF factors binding to the IRF motif in the human IRF-2 promoter. In vitro-translated IRF-1 (lanes 1 to 7) and IRF-2 (lanes 9 to 15) proteins were subjected to a gel shift assay using a ^{32}P-labeled oligomer containing the IRF motif from the IRF-2 promoter (see Materials and Methods). The oligomers used as competitors were as follows: lanes 2, 3, 10, and 11, oligomer C13 (see Materials and Methods); lanes 4, 5, 12, and 13, the oligomer containing the IRF motif from the IRF-2 promoter; lanes 6, 7, 14, and 15, the oligomer containing the NF-κB motif from the IFN-β promoter. In lanes 2, 4, 6, 10, 12, and 14, a 333-fold molar excess of unlabeled oligomer was included in the reaction mixture; in lanes 3, 5, 7, 11, 13, and 15, a 1,000-fold molar excess of unlabeled oligomer was included in the reaction mixture. Lanes 1 and 9 contained no competitor DNA; lane 8 contained no extract. The open and closed arrowheads indicate positions of the IRF-1–DNA and IRF-2–DNA complexes, respectively.

respond to TNF-α in L929 cells (24) and mitogen stimulation in a human T-cell line, Jurkat, by using a transient expression system (data not shown). Taken together, the results indicate that the cloned 5′ flanking region of the IRF-1 gene contains promoter sequences inducible by various stimuli.

For analyses of the IRF-2 gene promoter, we used an approach previously used for analysis of the mouse IRF-1 promoter. We constructed a reporter gene (p-414IRF2cat) which contains a bacterial *cat* gene linked to the *Ava*I-*Taq*I (−414 to +20) IRF-2 promoter region. In a transient transfection assay, the gene showed low-level but constitutive CAT activity and was activated in NDV-infected L929 cells (data not shown), indicating that the cloned 5′ flanking region of the IRF-2 gene also contains virus-inducible promoter sequences. To test whether the IRF-2 promoter can be activated by IRF-1, the P19 embryonal carcinoma cell line, which is devoid of detectable IRF activity (21), was cotransfected with the aforementioned reporter gene and the IRF expression plasmids pAct-1 and pAct-2 (21). As shown in Fig. 7A, the IRF-2 promoter was strongly activated by IRF-1 expression, and this activation was strongly repressed by coexpression of IRF-2. On the other hand, the IRF-1 promoter was not affected by either IRF-1 or IRF-2 expression (Fig. 7A, columns 1 to 4).

We next compared the levels of IRF-2 mRNA in a human fibroblast line, GM637, expressing IRF-1 mRNA in either the sense or antisense orientation (40). Interestingly, the accumulation of the inducible IRF-2 mRNA upon stimulation with NDV was more rapid and enhanced in the line expressing sense IRF-1 mRNA (S1) compared with cells transfected with the control plasmid (C1). On the other hand, the levels of IRF-2 mRNA were significantly lower in the line expressing antisense IRF-1 mRNA (AS11) than in the C1 line (Fig. 7B and C). Collectively, these results suggest that the activator IRF-1 induces the expression of its own repressor IRF-2.

DISCUSSION

Regulated expression of cytokine genes is an essential part of the control of many biological processes such as viral infection, inflammation, immunity, and hematopoiesis. IFNs are best known as cytokines involved in the host defense against viral infection. In addition, they have been found to be crucial regulators of cell growth and differentiation (6, 7, 33, 38, 49, 55). Although the molecular mechanisms of how cytokines are produced and how the cytokine-induced cellular response is regulated still remain largely unknown, recent studies have revealed the nature of many transcription factors affecting such events (reviewed in references 28 and 51). In the regulation of the type I IFN system, several transcription factors have been identified (10, 12, 19, 30, 34, 37, 52, 54), and it has been shown that many of these factors become induced and/or activated by viruses and other IFN inducers. Among these factors, we have been focusing on the IRF factors which bind to the two IRF-E regions within the IFN-β promoter as well as to the IFN-stimulated regulatory elements found in many of the IFN-inducible genes (19, 21, 50). It has been shown previously that both IRF-1

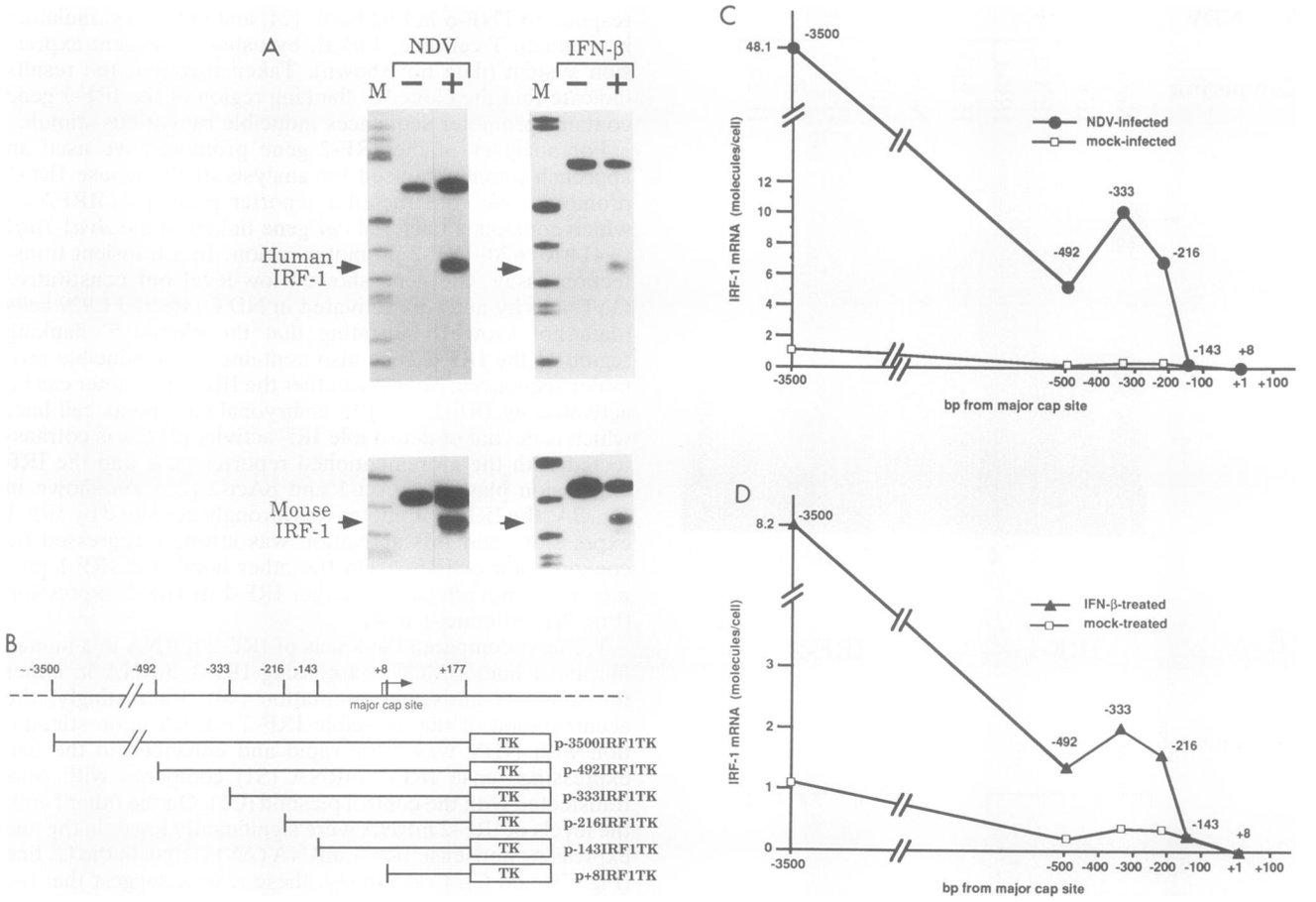

FIG. 6. Expression of the human IRF-1 gene induced by NDV or IFN-β. (A) Induction of transfected human IRF-1 mRNA and endogenous mouse IRF-1 mRNA. Ten micrograms of total RNAs was extracted from mock-, NDV-infected-, or IFN-β-treated transformants. Lane M, ^{32}P-labeled *Hae*III-digested pBR322 DNA fragments. (B) Structures of the various IRF-1–*tk* chimeric plasmids. (C and D) Effect of 5′ deletion on NDV- or IFN-β-induced expression of the human IRF-1 gene. Ten micrograms of total RNAs was extracted from mock-, NDV-infected, or IFN-β-treated transformants. The mRNA copy number was estimated by densitometric analysis as described by Fujita et al. (18). (C) NDV-infected L929 transformants. (D) IFN-β-treated L929 transformants.

and IRF-2 genes are induced by virus and IFNs (19). To gain further insight on the regulation of the IRF-1 and IRF-2 genes, we cloned and analyzed the human IRF-1 and IRF-2 genes.

The IRF-1 and IRF-2 genes are split into 10 and 9 exons, respectively. Comparison of the exon-intron organization of the two genes revealed conservation of the organization and structure of exons from exons 2 to 6 for both genes (Fig. 2). It has been shown previously that the DNA binding domains are located within the N-terminal regions of IRF-1 and IRF-2 (19, 53) and that these domains (consisting of 113 amino acids) are encoded by exons 2 to 4 (Fig. 2). In addition, there is structural conservation of exons 5 and 6. Although the functional roles of these regions for IRF-1 and IRF-2 are unknown, they may play a common role (e.g., interaction with other factors). Recently, the cDNAs encoding ICSBP and ISGF3γ, the DNA-binding factors also involved in IFN signaling, have been cloned (9, 54). These factors also have weak but significant homologies with IRFs only within the N-terminal DNA binding domain, but clearly the genes for these factors are more distantly related to the two IRF genes (9, 54). Furthermore, the exon-intron organization of the recently cloned genomic murine ICSBP gene (27) shows that it has no obvious close evolutionary relationship to the IRFs.

Although IRF-1 and IRF-2 genes show structural similarity, they differ in chromosomal localization; the former is located at 5q31.1, and the latter is located at 4q35.1. Recently, it has been demonstrated that IRF-1 and IRF-2 have antioncogenic and oncogenic potentials, respectively (20). Moreover, the human IRF-1 gene is frequently deleted in patients with leukemia or preleukemic myelodysplastic syndromes (59). On the other hand, the IRF-2 gene, which maps to a different chromosome (Fig. 3), is not affected in these patients (59). Such an imbalance of the genes for mutually antagonistic factors may result in the perturbation of restrained cell proliferation (20).

It is interesting that the promoters of both IRF genes possess NF-κB-binding elements. The role of NF-κB (or an NF-κB-like factor) in the expression of a wide range of signal-responsive genes has been well documented (3). In fact, it has been reported that NF-κB is also involved in virus- or double-stranded RNA-induced IFN-β gene regulation (14, 22, 32, 56). Although treatment with cycloheximide induces NF-κB, the IFN-β gene cannot be induced, indicating that induction of NF-κB alone is not sufficient for IFN-β gene induction (57). In the case of IRF-1 gene expression, a point mutation within the NF-κB binding site of the IRF-1 gene promoter which abolishes factor binding drastically

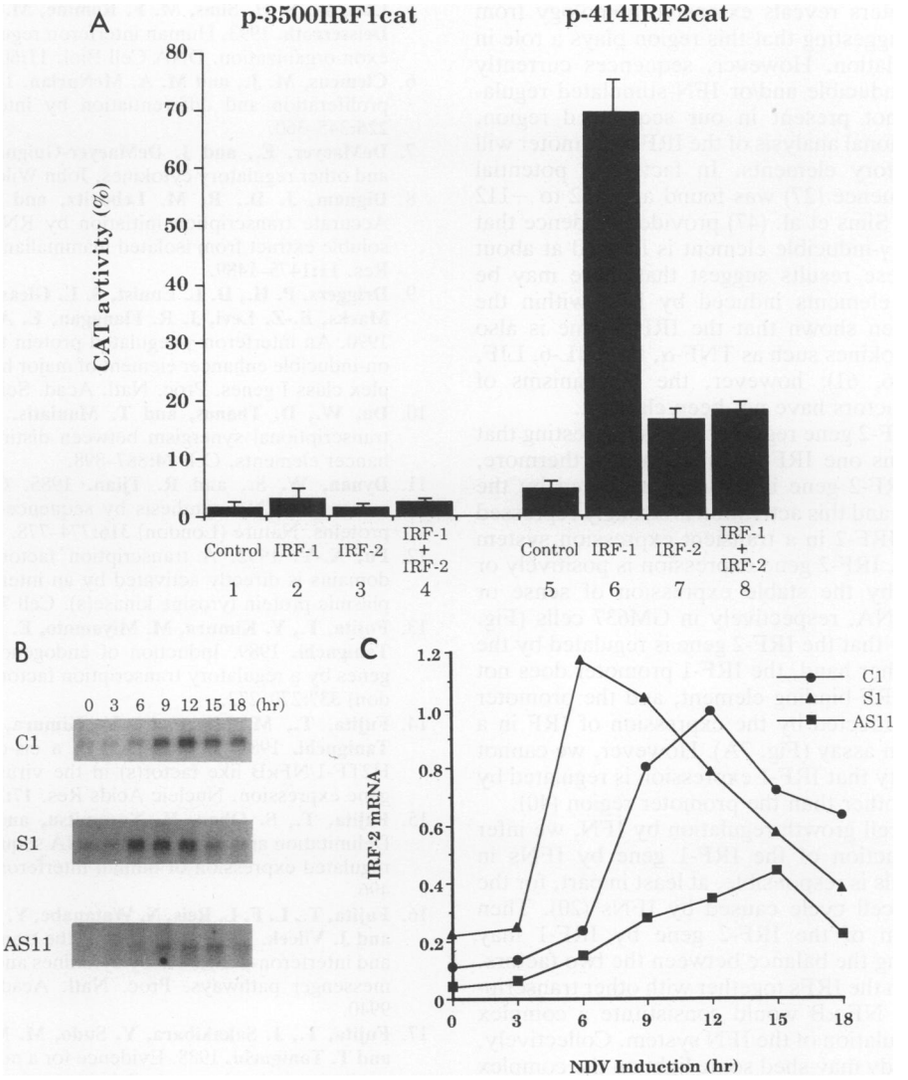

FIG. 7. (A) Levels of CAT activities in the transfected P19 cells. P19 cells were transfected with 5 μg of the *cat* reporter gene and 5 μg of the effector genes. The transfected effector genes were as follows: control, 5 μg of pAct-C; IRF-1, 2.5 μg of pAct-C and 2.5 μg of pAct-1; IRF-2, 2.5 μg of pAct-C and 2.5 μg of pAct-2; IRF-1 plus IRF-2, 2.5 μg of pAct-1 and 2.5 μg of pAct-2. The transfection was duplicated, and the assay was repeated at least three times; results were essentially reproducible. (B and C) Induction of IRF-2 mRNA by NDV. (B) Cultures of C1, S1, and AS11 cells were induced by NDV, and 5 μg of total RNA isolated from the cells at the indicated times was subjected to Northern (RNA) blot analysis. (C) From the results shown in panel B, the IRF-2 mRNA levels were quantitated by densitometric analysis. The peak expression level of C1 cells was assigned a value of 1.0.

reduced the virus inducibility of p-216IRF1cat mRNA in L929 cells (24). Thus, NF-κB (or an NF-κB-like factor) may play a role in the induction of IRF-1 and IRF-2 and synergize with NF-κB in the regulation of IFN-β gene expression in virus-infected cells. On the other hand, in view of the finding that TNF-α but not IFN-β induces NF-κB activity in various cell lines, including L929 (57), it is likely that the two cytokines utilize distinct signaling mechanisms, at least in part, in inducing the IRF-1 gene.

To gain further insight on the regulation of the IRF-1 gene, we first established the transformants carrying pUCHIRF1B into mouse L929 cells. The results in Fig. 6A demonstrate that the transfected gene behaves similarly to the endogenous IRF-1 gene in the responses to NDV and IFN-β and also indicate that some elements important for gene induction may be located within the cloned human IRF-1 gene. Then we constructed several deletion mutants of the 5′ region of the gene and established the transformants carrying these constructs. The 5′ flanking region of the gene contains promoter sequences, which give rise to a low-level, constitutive expression of the gene in uninduced L929 cells. In fact, such a constitutive expression has been demonstrated in various types of mouse primary cells (37). Furthermore, these sequences are activated by NDV and IFN-β. Expression studies of the 5′ deletion mutants did not permit us to dissect clearly the distinct sequence elements functioning in gene induction by various stimuli. Our findings that p-3500IRF1TK is more active than other constructs and that the inducible promoter activity decreased as the 5′ deletion extended from −3500 to −492 and −216 to −143 suggest that the gene contains multiple regulatory elements within these regions. In fact, sequence comparison between human and

mouse IRF-1 promoters reveals extensive homology from −228 to +38 (37), suggesting that this region plays a role in virus or IFN stimulation. However, sequences currently known to be virus-inducible and/or IFN-stimulated regulatory elements are not present in our sequenced region, suggesting that additional analysis of the IRF-1 promoter will reveal novel regulatory elements. In fact, one potential IFN-γ-activated sequence (27) was found at −122 to −112 (Fig. 4A). Recently, Sims et al. (47) provided evidence that an IFN-α- and IFN-γ-inducible element is located at about −130. Together, these results suggest that there may be multiple regulatory elements induced by IFN within the promoter. It has been shown that the IRF-1 gene is also induced by other cytokines such as TNF-α, IL-1, IL-6, LIF, and prolactin (1, 16, 61); however, the mechanisms of induction by these factors have not been clarified.

With respect to IRF-2 gene regulation, it is interesting that the promoter contains one IRF binding site. Furthermore, expression of the IRF-2 gene is strongly activated by the expression of IRF-1, and this activation is strongly repressed by coexpression of IRF-2 in a transient expression system (Fig. 7A). Moreover, IRF-2 gene expression is positively or negatively affected by the stable expression of sense or antisense IRF-1 mRNA, respectively in GM637 cells (Fig. 7B). Thus, it is likely that the IRF-2 gene is regulated by the two IRFs. On the other hand, the IRF-1 promoter does not contain a potential IRF-binding element, and the promoter up to −3500 is not affected by the expression of IRF in a similar cotransfection assay (Fig. 7A). However, we cannot exclude the possibility that IRF-1 expression is regulated by the IRFs in regions other than the promoter region (40).

In the context of cell growth regulation by IFN, we infer that the abrupt induction of the IRF-1 gene by IFNs in normally growing cells is responsible, at least in part, for the perturbation of the cell cycle caused by IFNs (20). Then subsequent induction of the IRF-2 gene by IRF-1 may contribute to restoring the balance between the two factors. This balance between the IRFs together with other transcription factors such as NF-κB would consistitute a complex gene network for regulation of the IFN system. Collectively, the results of this study may shed some light on the complex network of the genes involved in regulation of the IFN system.

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