Regulation of Type I Interferon Gene Expression by Interferon Regulatory Factor-3*

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The genes of the family of interferon (IFN) regulatory factors (IRF) encode DNA binding transcriptional factors that are involved in modulation of transcription of IFN and interferon-induced genes (ISG). The presence of IRF binding sites in the promoter region of IFNA and IFNB genes indicates that IRF factors recognizing these sites play an important role in the virus-mediated induction of these genes. We have described a novel human gene of this family, IRF-3, that is constitutively expressed in a variety of cell types. IRF-3 binds to the interferon-sensitive response element (ISRE) present in the ISG15 gene promoter and activates its transcriptional activity. In the present study, we examined whether IRF-3 can modulate transcriptional activity of IFNA and IFNB promoter regions. Our results demonstrate that IRF-3 can bind to the IRF-like binding sites present in the virus-inducible region of the IFNA4 promoter and to the PRDIII region of the IFNB promoter but cannot alone stimulate their transcriptional activity in the human cell line, 293. However, the fusion protein generated from the IRF-3 binding domain and the RelA(p65) activation domain effectively activates both IFNA4 and IFNB promoters. Cotransfection of IRF-3 and RelA(p65) expression plasmids activates the IFNB gene promoter but not the promoter of IFNA4 gene that does not contain the NF-kB binding site. Surprisingly, activation of the IFNA4 gene promoter by virus and IRF-1 in these cells was inhibited by IRF-3. These data indicate that in 293 cells IRF-3 does not stimulate expression of IFN genes but can cooperate with RelA(p65) to stimulate the IFNB promoter.

Viral infection leads to the transient expression of early inflammatory genes. The proteins encoded by these genes enhance recognition of the infected cells by the host immune system. A group of proteins, called interferons (IFNs),1 can directly inhibit viral replication. Type I IFNs are encoded by a family of closely related α genes and a single IFNB gene, which are all localized on chromosome 9 (1, 2). The sequences that regulate inducible transcription of these genes are localized within the 100-nucleotide 5’-end of the transcriptional start of IFNA and IFNB genes (3, 4). These regions contain a number of a short overlapping GAAAGT-rich sequences that serve as a binding sites for multiple transcriptional factors. Several elements that function as positive regulatory domains (PRD) in virus-infected cells were identified in the promoter region of the IFNB gene (5). It was further shown that the region designated as PRDIV contains an NF-kB binding site (6–8) to which the NF-kB heterodimers induced upon viral infection bind as well as the HMG protein (9). The PRDIV domain was shown to bind the ATF-2 factor and octamer binding protein (10), and the PRDI and PRDIII regions serve as high affinity sites for the binding of the interferon regulatory factors (IRF-1 and IRF-2) (6, 11, 12). The transcriptional activation of this promoter, in virus-infected cells, is a result of the interaction among these multiple transcriptional factors (13) where the virus-induced binding of p50p65 heterodimer plays a crucial role (14, 15). In contrast, the IFNA gene promoter region does not contain an NF-κB binding site. However, both the murine and human IFNA virus-inducible regions (VRE) contain multiple repeats to the GAAAGT and AAGTGA elements that could serve as binding sites for the IRF-1 and IRF-2 factors (16–18). In addition, the deletion and mutation analysis of the murine IFNA4 promoter region identified an essential element, named aF1 (19), which serves as a recognition site for DNA binding proteins p68 and p96 (20). In the human IFNA1 promoter region, another essential binding site interacting with the TG protein was identified (21). Recent studies have identified a distinct multisubunit complex in virus-induced cells (22).

The presence of IRF-like binding sites in the promoter region of the IFNA and IFNB genes indicated that the IRF factors recognizing these sites play an essential role in the induction of IFN genes. The original results of Harada et al. (11) suggested that the up-regulation of these genes is mediated by IRF-1, while the closely related IRF-2 suppresses the expression of these genes. However, the essential role of IRF-1 and IRF-2 in the regulation of IFNA and IFNB gene expression in infected cells was disputed by the finding that mice containing the homozygous deletion of IRF-1 or IRF-2, or fibroblast derived from these mice, were able to induce IFNA and IFNB gene expression upon infection with Newcastle Disease virus (NDV) to the same level as the wild-type mice or cells (23, 24). IRF-1 was shown to play an important role in the antiviral effect of IFNs (25). IRF-1 binds the ISRE element present in many IFN-inducible gene promoters and activates expression of some of these genes (26, 27). However, activation of ISG genes by IFNA and IFNB was shown to be mediated generally by the multiprotein ISGF3 complex (28). The binding of this complex to DNA is mediated by another member of the IRF family, p48,
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which, in IFN-treated cells, interacts with phosphorylated STAT1 and STAT2 transcriptional factors forming the heterodimer complex, ISGF3 (29–32). The homozygous deletion of p48 in mice abolishes the sensitivity of these mice to the antiviral effect of IFNs (25), and the infected macrophages from p48 mice show an impaired induction of IFNA and IFNB genes (33). However, induction of IFN genes in virus-infected p48 mice –/− spleenocytes is not affected.

Several other members of the IRF family have been identified. The ICSBP gene is expressed exclusively in the cells of the immune system (34, 35), and its expression can be enhanced by IFNy. ICSBP was shown to form a complex with IRF-1 and inhibit the transactivating potential of IRF-1 (36, 37). The homozygous deletion of ICSBP in mice leads to the alteration in the development of the cell of macrophage lineage (38). Another lymphoid cell-specific IRF, Pip/LSIRF, was identified (39, 40), which can interact with phosphorylated PU.1. It was shown that the Pip/PU.1 heterodimer can bind to the immunoglobulin light chain enhancer and function as a B cell-specific transcriptional activator. Expression of Pip/LSIRF can be induced by antigenic stimulation, but not by IFN, and it was recently shown that the Pip/LSIRF –/− mice failed to develop mature T and B cells (41). Another novel member of the IRF family, IRF-7, was recently identified by its ability to bind to an ISRE-like element in the promoter region of the Qp gene of EBV (42, 43). Furthermore, the genome of human herpesvirus 8 contains four open reading frames, which show homology to the cellular IRF family of genes (44). These data indicate that transcriptional factors of the IRF family may modulate not only the expression of cellular genes but viral genes as well.

We previously identified and described another member of the human IRF family, IRF-3 (45). The IRF-3 gene encodes a 55-kDa protein and is expressed constitutively in all tissues. Recombinant IRF-3 binds to the ISRE element of the IFN-induced gene, ISG15, and overexpression of IRF-3 activates transcription of this promoter in the transient expression assay. Viral infection or IFN treatment does not activate the expression of the IRF-3 gene.

In the present study, we address the question whether IRF-3 can modulate the expression of IFNA and IFNB genes. The IRF-1 site(s) plays an important role in the transcriptional activation of these genes; therefore, it is important to determine which member of the IRF family plays a role in the activation of IFNA and IFNB genes in virus-infected cells. Our results demonstrate that: 1) recombinant IRF-3 can bind to the IRF-like binding sites present in the virus-inducible region of the IFNA and IFNB promoters; 2) in 293 cells, overexpression of IRF-3 neither activates transcription of the IFNA or IFNB promoters in a transient expression assay nor induces expression of the endogenous IFN genes; 3) fusion of the IRF-3 DNA binding domain with the RelA(p65) transactivation domain generated a fusion protein that effectively activated both the IFNA and IFNB promoters; and 4) activation of the IFNA4 promoter region by IRF-1 in these cells is inhibited in the presence of IRF-3.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—293 cells were grown in Dulbecco’s modified Eagle’s medium. In the transfection assays, subconfluent 293 cells (2.5 × 106 cells/plate) were cotransfected with 2.5 μg of reporter plasmid chloramphenicol acetyltransferase (CAT) and the indicated amounts of the IRF or p65 expression plasmids, using the calcium phosphate coprecipitation method (46). When indicated, treatment with IFNα (500 units/ml) or infection with NDV (multiplicity of infection = 5) was done 24 h after transfection for 16 h. Protein extracts, prepared by the freeze-thaw method, and CAT assays were done as described previously (19). To compensate for the possible differences in transfection efficiency, each sample was cotransfected with β-galactosidase expressing plasmid (2.5 μg), and CAT activity was normalized to the constant levels of β-galactosidase (47). The total transfected DNA was kept constant in each experiment. Each transfection with the CAT reporter plasmids was repeated at least three times, and the data presented represents averages of these experiments.

**Plasmids**—The IRF-3 and the expression plasmids in which expression of IFN cDNA is under the regulation of the IE cytomegalovirus promoter region and GST-IRF-1 and GST-IRF-3 fusion plasmids were described previously (20, 45). The GST-IRF-3 plasmids consist of the 5′-DNA fragment that codes for the first 133 amino acids of IRF-3 cloned into pGEX2T (Pharmacia Biotech Inc.). The plasmids containing the various lengths of the IFNA4 promoter regions inserted 5′ of the CAT gene were described previously (17). The IRF-1/p65, IRF-2/p65, and IRF-3/p65 plasmids encode the amino-terminal portion of the indicated IRF protein (1–204 aa, IRF-1; 1–133 aa, IRF-3) fused to the carboxyl-terminal transactivating domain of RelA(p65) (397–550 aa) (48). The IFNB-CAT and TH-CAT plasmids were described previously (13).

**Expression of GST Fusion Proteins**—The GST-IRF-1 and IRF-3 fusion proteins were purified from bacterial lysates by affinity chromatography on a glutathione-agarose column (Sigma).

**Electrophoretic Mobility Shift Assays**—The indicated amounts of purified GST-IRF-3 (full-length or 133 aa) fusion proteins were preincubated for 10 min in a total volume of 20 μl of binding buffer (10 mm Tris-HCl, pH 7.5, 50 mm NaCl, 1 mm EDTA, 1 mm diethyrlthetol, 5% glycerol, 5% Nonidet P-40, 0.1 mg/ml bovine serum albumin, and 2 μg/ml poly(dI-dC)). The extracts were then incubated with the respective 32P-labeled oligonucleotide probe corresponding to the different regions of the IFNA4 promoter for 15 min at room temperature. Protein-DNA complexes were then resolved in a nondenaturing 5% polyacrylamide gel. The following IE probes were used in these experiments: WT, GAGTGAGTTAAAGAGTTGGGAAGAAG; 92, GAGTGAGATAAACAGTGAAATGGAAGAAG; 94, GAGTGAGTTAACAGTGAAATGGAAGAAG; 87, GAGTGAGTTAACAGTGAAATGGAAGAAG; 103, GAGTGAGTTAACAGTGAAATGGAAGAAG; 92, GAGTGAGTTAACAGTGAAATGGAAGAAG; 87, GAGTGAGTTAACAGTGAAATGGAAGAAG; 103, GAGTGAGTTAACAGTGAAATGGAAGAAG; 92, GAGTGAGTTAACAGTGAAATGGAAGAAG; 87, GAGTGAGTTAACAGTGAAATGGAAGAAG; 103, GAGTGAGTTAACAGTGAAATGGAAGAAG; 92, GAGTGAGTTAACAGTGAAATGGAAGAAG; 87, GAGTGAGTTAACAGTGAAATGGAAGAAG; 103, GAGTGAGTTAACAGTGAAATGGAAGAAG; 92, GAGTGAGTTAACAGTGAAATGGAAGAAG; 87, GAGTGAGTTAACAGTGAAATGGAAGAAG; 103, GAGTGAGTTAACAGTGAAATGGAAGAAG.

**RESULTS**

**Modulation of IFNA4 Promoter Activity by IRF-3, IRF-1, and Mutants of IRF-3**—The amino-terminal region of IRF-3 shows a high degree of homology to IRF-1 and IRF-2 (Fig. 1A). Au et al. (49) showed that IRF-1 is an effective activator of the IFNA4 promoter in transient cotransfection assays, whereas IRF-2 inhibits the IRF-1-mediated activation (14). We have analyzed whether IRF-3 plays any role in the regulation of the IFNA4 promoter. The IFNA4 promoter contains an IRF-1 binding sites in its inducible region (IE) (Fig. 1B) and additional IRF-1-like sites can be located in the upstream region of this promoter. Human 293 cells were cotransfected with murine IFNA4 (464)-CAT reporter plasmid and IRF-1 or IRF-3 (Fig. 2). Results of the transient transfection showed that IRF-1 is an effective transactivator as shown previously (20), whereas IRF-3 does not transactivate the IFNA promoter. Next, we wanted to determine whether IRF-3 modulates the IRF-1-mediated transactivation of this promoter. Cotransfection of IRF-1 and IRF-3 showed that IRF-3 inhibited IRF-1-mediated activation of IFNA4 promoter in a concentration-dependent manner. At the ratio of IRF-3 to IRF-1 of 2:1, the activation was reduced by 50%. The next question was if IRF-3-mediated inhibition of IFNA4 was due to competition for the same binding sites or due to protein-protein interaction as shown with ICSBP and IRF-1 (34). We, therefore, examined whether variants of IRF-3 with carboxyl-terminal deletions would also be inhibitory. Since proline-rich regions are often involved in protein-protein interaction, we also cotransfected IRF-1 with IRF-3 that had its proline-rich region deleted (Fig. 3). However, the proline-deleted form of IRF-3 could still inhibit IRF-1 activation of IFNA4 promoter, indicating that the proline-rich region of IRF-3 did not
play any role in the observed inhibition. Plasmids encoding the truncated forms of the IRF-3 protein (327 and 240 aa) were also potent inhibitors of IRF-1 activity. These data suggest that IRF-3 and IRF-1 compete for the same or overlapping binding site on the IFNA promoter and that the truncated proteins may bind more strongly than the full-length IRF-3.

Activation of IFNA4 Promoters by IRF-3, IRF-1, and RelA (p65) Chimeric Fusion Proteins—Since IRF-3 could not trans-activate the IFNA4-CAT promoter in 293 cells, we wanted to determine if this was a result of weak binding or the inability to transactivate this promoter in 293 cells. Therefore, a plasmid encoding a chimeric protein containing the amino-terminal binding domain of IRF-3 (1–133 aa) and the carboxyl-terminal transactivating domain of RelA(p65) (397–550 aa) was generated. 293 cells were cotransfected with IFNA4-CAT and the chimeric fusion protein IRF-3/p65 or with the previously described chimeric plasmid IRF-1/p65 used as a positive control (48). The levels of transactivation show that IRF-1/p65 and IRF-3/p65 chimeric fusion proteins were equally effective transactivators of the full-length IFNA4 promoter (Fig. 3), indicating that IRF-3 does bind but does not transactivate the IFNA4 promoter. In contrast to the IRF-3/p65 plasmids, cotransfection of IRF-3 and p65 plasmids in trans could not transactivate the IFNA4 promoter (data not shown). Since there is no obvious NF-kB binding site in IFNA promoters, the inability of p65 to cooperate with IRF-3 in trans further supports the idea that NF-kB binding does not play a role in the activation of the IFNA4 promoter. Transfection of the chimeric fusion plasmids with a series of IFNA promoter deletions resulted in a gradual reduction in reporter gene transactivation, suggesting the presence of multiple IRF-3 or IRF-1 fused to the carboxyl-terminal transactivation domain of p65. Standard deviations are indicated by the T-bars.

Virus Induction of IFNA4-CAT—We previously found that in L929 cells transfection of IRF-3 alone did not activate the IFNA promoter, but it enhanced NDV-induced activation (45). To determine whether IRF-3 also cooperates with NDV in 293 cells, the cells were cotransfected with 2.5 μg of the different IFNA4-CAT plasmids and 5 μg of the indicated expression plasmids, IRF-3, IRF-1, IRF-3/p65, and IRF-1/p65. Cultures were harvested 48 h after transfection and assayed for CAT activity. The data from IRF-3/p65, IRF-3(327), and IRF-3(244) are not shown, but they exhibited the same low level of activation as wild-type IRF-3. Standard deviations are shown by the T-bars.

**Fig. 1.** A, amino-terminal amino acid homology of IRF-1, -2, and -3. Conserved amino acids between the proteins are in **bold type**. B, schematic representation of IFNA and IFNB promoters.

**Fig. 2.** Modulation of the IFNA4 promoter by IRF-3, IRF-1, and mutants of IRF-3. 293 cells were cotransfected with 2.5 μg of IFNA4-CAT plasmid and 5 μg of the indicated expression plasmids IRF-3, IRF-3dPro, IRF-3(327), IRF-2(244), except IRF-1, for which 2.5 μg was used. Cultures were harvested 48 h after transfection and assayed for CAT activity. The data from IRF-3dPro, IRF-3(327), and IRF-3(244) are not shown, but they exhibited the same low level of activation as wild-type IRF-3. Standard deviations are shown by the T-bars.

**Fig. 3.** Modulation of the IFNA4 promoter and its deleted mutants by IRF-3, IRF-1, and their chimeric fusion proteins. 293 cells were cotransfected with 2.5 μg of the different IFNA4-CAT plasmids and 5 μg of the indicated expression plasmids, IRF-3, IRF-1, IRF-3/p65, and IRF-1/p65. Cultures were harvested 48 h after transfection and assayed for CAT activity. The chimeric fusion plasmids have the amino-terminal DNA binding domain of IRF-3 or IRF-1 fused to the carboxyl-terminal transactivation domain of p65. Standard deviations are indicated by the T-bars.

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Effect of IRF-3 on the Activation of the IFNB Promoter—The VRE of IFNB promoter contains two IRF binding sites (PRDI and PRDIII) and an NF-kB binding site (PRDII). To examine the effect of IRF-3 on the activity of IFNB promoter, we used the IFNB-CAT plasmid, which contains the IFNB promoter (~281 to +19) inserted in front of the CAT gene (Fig. 5A). In a transient transfection assay, overexpression of IRF-1 and IRF-3 did not transactivate the IFNB-CAT promoter in 293 cells, while a slight activation of this promoter was seen upon cotransfection with RelA(p65) expression plasmid. When IRF-1 and IRF-3 expressing plasmids were cotransfected together with the RelA(p65) expression plasmid (in trans), transactivation of the IFNB promoter was observed (13- and 8-fold, respectively), demonstrating that IRF-3 and IRF-1 can synergize with p65 and activate transcriptional activity (2-fold) of the IFNB promoter. The chimeric fusion protein IRF-1/p65 did not significantly transactivate the IFNB promoter. However, the IRF-3/p65 chimeric fusion protein was a strong transactivator (16-fold), suggesting that IRF-3/p65 is able to bypass the requirement for cooperation between NF-kB and IRF-3 binding for the activation of the IFNB promoter (14, 15). These results also suggest that IRF-3 can strongly interact with one or more PRD domains of the IFNB promoter.

To further characterize the IFNB transactivation mediated by IRF-3, we used the TH-CAT reporter, which contains a tetramer (AAGTGA) element that functions as a strong binding site for IRF-1 (Fig. 5B). As expected, both IRF-1 and IRF-3/p65 chimeric fusion proteins strongly transactivated reporter activity. However, transactivation of TH-CAT by IRF-3/p65 was only marginal, suggesting that IRF-3 does not bind effectively to this PRD-like element. These data suggested that IRF-3 binding to the IFNB promoter and IRF-3/p65 transactivation may be mediated by an IRF site present in the IFNB promoter that is distinct from the PRDI element.

Effect of IRF-3 on the Activation of the IFNA Promoter—Binding of recombinant IRF-3 to the PRD domains of the IFNB promoter was, therefore, analyzed by gel mobility shift analysis. The amino-terminal 133-aa DNA binding fragment of IRF-3, produced as a GST-fusion protein in Escherichia coli, bound with high affinity to PRDIII but only weakly to PRDI; no stable binding with PRDII was observed (Fig. 6). Interestingly, the IRF-3 protein bound efficiently to probes consisting of PRDI-II and PRDIII-I regions. At low concentrations of IRF-3, only a single DNA-protein complex was formed with the PRDIII-I probe; however, as the concentration of IRF-3 was increased, a second complex of slower mobility was formed, indicating a cooperative interaction of IRF-3 with other sites within the PRDIII-I element.

Binding of IRF-3 to the IFNA Promoter—Since IRF-3 displayed strong binding to the PRDIII region of the IFNB promoter, we searched for the sequence homologies to PRDIII (AGGAAAAGTG) region in the IFNA promoter. Two sequences similar to PRDIII were found. One sequence TGGAGTACG was located at positions −252 to −243, and the second, TGGAAAAAG, is located in the IE region of the promoter from position −94 to −85. This latter sequence overlaps with the IRF-1 binding site. The binding of GST-IRF-3 and truncated GST-IRF-3 (133 aa) to the −252 to −243 radiolabeled oligonucleotide probe was analyzed by EMSA. However, neither the full-size IRF-3 nor its amino-terminal fragment were able to bind this sequence (data not shown). We next analyzed the binding of the GST-IRF-3 proteins to IE probe (~109 to −75) and compared it with binding of GST-IRF-1 to this probe. As shown in Fig. 7A, 50 ng of GST-IRF-1 bound the radiolabeled IE probe, resulting in a formation of single complex. As the concentration of protein was increased from 50 to 100 ng, a second complex of slower mobility was formed. Full-length GST-IRF-3 bound very effectively to the IE probe with formation of multiple bands...
In this paper, we have analyzed the role of IRF-3 in the virus-mediated induction of the IFNA and IFNB genes. Our results show that overexpression of IRF-3 in 293 cells does not activate the transcription of IFNA4 or IFNB promoter regions in a transient expression assay while it can bind to the VRE of the IFNA and IFNB genes. Two IRF-1 binding sites, PRDII and PRDIII, were identified in the virus-inducible region of the IFNB promoter. Our binding studies demonstrated that the recombinant IRF-3, or its 3′-deleted mutant (133 aa), binds strongly to PRDIII but not to PRDI. When binding of IRF-3 to an oligonucleotide encompassing PRDIII-PRDI was analyzed, only a single protein-DNA complex was detected at low concentration; however, at higher IRF-3 protein concentrations, IRF-3 bound as two molecules either as a dimer or more likely to two distinct sites. This result indicates cooperativity of IRF-3 bind-

**FIG. 7.** Binding of IRF-1, IRF-3, and the amino-terminal region of IRF-3(133) to the IE element of the IFNA promoter. A, the indicated amount of GST-IRF-1 was incubated with radiolabeled IE probe and resolved by EMSA. B, the indicated amounts of GST-IRF-3 and GST-IRF-3 amino-terminal fragment (133) were incubated with radiolabeled IE probe and resolved by EMSA. C, 50 ng of GST-IRF-3(133) was preincubated with the 50× and 100× cold IE probe or 50× cold ISRE, PRDI (which is a tetramer of the PRDI site), and αF1 probes followed by incubation with radiolabeled IE and resolved by EMSA.

**FIG. 8.** Binding of GST-IRF-3(133) to the IE element and its mutants. 50 ng of GST-IRF-3(133) was incubated with the indicated radiolabeled probes. The numbers indicate the position of the mutation. The exposure of the autoradiogram of B is lighter than A.

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ing to high affinity (PRDIII) and low affinity (PRDI) binding sites.

Although IRF-1 contains an activation domain in its carboxy-terminal end, it cannot by itself activate transcription of the IFNB promoter in a transient transfection assay. In our previous study (45), we had not detected the presence of an activation domain in IRF-3. However, our recent results with the two-hybrid yeast assay as well as the results of others (50) indicate that IRF-3 has transactivation potential when expressed as a Gal4 fusion protein. Similarly to IRF-1, IRF-3 alone does not activate the IFNB promoter in 293 cells. In contrast, cotransfection of IRF-3 with a RelA(p65) expressing plasmid resulted in transcriptional activation of the IFNB promoter. These data indicate that the interaction between NF-kB (p50/p65) and IRF-3 is sufficient for a transcriptional activation of this promoter. Interestingly, the requirement for this interaction can be bypassed by the IRF-3/p65 and IRF-2/p65 (48) chimeric fusion proteins but not by the chimeric fusion protein IRF-1/p65. We assume that this difference indicates that IRF-3 binds more strongly to the IFNB promoter than IRF-1. It has been shown that virus-induced binding of p50/p65 heterodimer to the NF-kB site in the IFNB promoter plays a critical role in the activation of IFNB promoter in infected cells (14, 15). Mutations of the PRDI site in the IFNB promoter that abolish binding of IRF-1 were shown to decrease virus-mediated inducibility of this promoter (6, 51), indicating that nuclear factor binding to this element is essential for the transcriptional activation of this promoter. However, neither IRF-1 nor IRF-3 can act alone as a transcriptional activator of this promoter in transient cotransfection assays, indicating that the activation may require cooperation between different transcription factors (14, 15). The requirement of complex formation between Pip and PU.1 for activation of the immunoglobulin light chain gene was recently demonstrated (39). Our recent data show complex formation between IRF-3 and the p300/CBP proteins, which are basal components of the transcriptional complexes (52). This interaction is facilitated by virus-mediated phosphorylation of IRF-3. Since the interaction of RelA(p65) with the amino-terminal domain of p300/CBP was recently demonstrated (53), it is likely that the cooperation between IRF-3 and p65 may be facilitated by p300/CBP.

Promoters of the various IFNA genes differ from the majority of other cytokine gene promoters by the absence of a consensus NF-kB binding site. Nevertheless, these genes are induced in cells of lymphoid origin by viral infection in a cell type-specific manner (17, 54). Au et al. (49) has shown that, in a transient expression assay in L929 cells, overexpression of IRF-1 can induce both the murine IFNA promoters and synergize with the NDV-activated stimulation of the IFNA4 promoter. Furthermore, in these cells, overexpression of IRF-3 significantly enhanced virus-mediated activation of this promoter (45). In contrast, in 293 cells, IRF-3 does not activate the IFNA4 promoter region but inhibits the IRF-1-mediated activation. The IFNA4 promoter contains in its inducible element (1E) an IRF binding site (55); the right half of the 1E element of the IFNA4 promoter contains four adenosine residues (GAAAAG), thus resembling the half of the PRDIII site (GAAAAC) that strongly binds IRF-3. This is in contrast to the PRDI binding site that contains only three adenosine nucleotides (GAAA) and is a weak binding site for IRF-3 but a strong site for IRF-1. In this study, we have shown that IRF-3 binds effectively to IFNA4 1E and that mutations in positions 92 and 90 affect IRF-3 binding, while the replacement of the 3′ G by A in position 87 is without any effect. By mutation analysis, we have previously shown (49) that the IE site is important for the virus-inducible transcriptional activation of this promoter and that a single mutation at nucleotide 92 completely abolished virus-mediated induction of the IFNA4 promoter in 293 cells.

Two other IRF family members have been identified that inhibit IRF-1 mediated transactivation. IRF-2 shows the same DNA binding specificity as IRF-1 and inhibits IRF-1-stimulated transcription of both the IE and ISRE elements by competing for the binding site (48, 56). In contrast, ICSBP, which binds only weakly to the ISRE element and not to PRD, inhibits the IRF-1 transactivation by direct interaction with the 3′-end of the IRF-1 protein, thus interfering with its transcriptional activation capability (34, 37). Our data indicate that the observed IRF-3-mediated inhibition of IRF-1 in 293 cells occurs through competition for the binding site(s) since the 5′ part of IRF-3(133) protein, containing only the DNA binding domain, is also an effective competitor of IRF-1. Furthermore, we have been unable to demonstrate a direct interaction between immobilized GST-IRF-3 and IRF-1 (data not shown). These data indicate that several members of the IRF family may function as conditional repressors of IRF-1-targeted genes. Since IRF-1 is effectively induced both by virus and IFNs, the expression of IRF-1-regulated genes may reflect a balance between levels of IRF-1 and the negative regulators of the IRF family. However, experiments with mice in which various IRF genes were deleted indicate that the role of the transcriptional factors of the IRF family extends beyond the response to viral infection. Most of these factors were found to be critical for the proper development and/or function of immune lineage cells. In this respect, it is interesting to note that IRF-4/LSIRF/Pip can be induced only by antigenic stimulation of lymphocytes (41) and IRF-3 can be induced in peripheral blood mononuclear cell and macrophages by phytomagglutinin of phorbol 12-myristate 13-acetate, respectively.

In conclusion, our data demonstrate that IRF-3 modulates the expression of type I IFN genes in 293 cells. It cooperates with RelA(p65) to stimulate the transcripational activity of the IFNB gene promoter, while it inhibits IRF-1 and virus-mediated transactivation of the IFNA4 gene promoter. Studies done since this manuscript has been reviewed indicate that in mouse cells, containing homozygous deletions of IRF-1 and IRF-2 genes, and in embryo fibroblasts, high levels of overexpression of IRF-3 alone can stimulate transcriptional activity of IFNA4 and IFNB promoters and greatly enhance the virus-mediated induction of these promoters. Interestingly, our preliminary results also indicate that overexpression of E1A can inhibit the IRF-3-mediated transcriptional modulation of the IFNA4 and IFNB promoters. The fact that we failed to detect activation of IFNA4 and IFNB promoters by IRF-3 alone in 293 cells may be due to the expression of E1A in these cells. Although the exact role of IRF-3 in the virus-mediated pathway is presently unclear, the fact that IRF-3 is phosphorylated in infected cells but not in IFN-treated cells suggests that IRF-3 may be an important component of virus-induced signaling.

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