Reconstitution of Virus-mediated Expression of Interferon α Genes in Human Fibroblast Cells by Ectopic Interferon Regulatory Factor-7*

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Type I interferons constitute an important part of the innate immune response against viral infection. Unlike the expression of interferon (IFN) B gene, the expression of IFNA genes is restricted to the lymphoid cells. Both IFN regulatory factor 3 and 7 (IRF-3 and IRF-7) were suggested to play positive roles in these genes expression. However, their role in the differential expression of individual subtypes of human IFNA genes is unknown. Using various IFNA reporter constructs in transient transfection assay we found that overexpression of IRF-3 in virus infected 2FTGH cells selectively activated IFNA1 VRE, whereas IRF-7 was able to activate IFNA1, A2, and A4. The binding of recombinant IRF-7 and IRF-3 to these VREs correlated with their transcriptional activation. Nuclear proteins from infected and uninfected IRF-7 expressing 2FTGH cells formed multiple DNA-protein complexes with IFNA1 VRE, in which two unique DNA-protein complexes containing IRF-7 were detected. In 2FTGH cells, virus stimulated expression of IFNB gene but none of the IFNA genes. Reconstitution of IRF-7 synthesis in these cells resulted, upon virus infection, in the activation of seven endogenous IFNA genes in which IFNA1 predominated. These studies suggest that IRF-7 is a critical determinant for the induction of IFNA genes in infected cells.

The type I interferons (IFNs), IFN-α and IFN-β, are important effectors of innate and adaptive immunity. They are produced by infected cells as an early response to viral infection. Activated precursors of dendritic cells (CD4+CD11c+ cells) (1) and plasmacytoid monocytes (ILT3+/IL10− cells) (2) were identified as high producers of IFN-α in vivo. Interferons elicit antiviral effects as well as multiple biological responses such as activation of NK cells and macrophages, up-regulation of expression of MHC class I antigens (3), and protection of CD8+ cells from antigen-induced cell death (4). IFN-α is encoded by a family of 13 structurally related genes, whereas IFN-β is encoded by a single gene. Both IFNA and IFNB genes are localized on human chromosome 9p22 (5).

Induction of type I IFNs occurs at the level of gene transcription (6). Virus responsive elements (VRE) in the promoter region of IFNA and IFNB genes (7) alone can confer virus-mediated activation when inserted in front of a heterologous promoter. These regions show sequence motifs that are highly conserved in both IFNA and IFNB promoters. The VRE of IFNB gene has been well characterized, and it was shown that the activation of the IFNB gene in infected cells is a result of formation of the nucleoprotein complex (enhanceosome), consisting of NFκB, IRF-3, IRF-7, ATF-2/c-Jun, and high mobility group I(Y) (8−10). The enhanceosome has a very stable structure and can enhance multiple cycles of reinitiation of transcription (11).

The IFNA promoters are less well defined (12−18). The transcriptional regulation was shown to be a critical determinant for the relative levels of individual IFNA subtypes as well as for their cell type specific pattern of expression (6). In the murine (Mu) system, VRE of the highly inducible IFNA4 gene shows only six nucleotide differences from the uninducible IFNA6 gene (12, 13, 17). Differential expression of IFNA4 and IFNA11 genes in L929 cells was shown be due to A to G substitutions in the VRE of IFNA4 and to the presence of a negative regulatory sequence located upstream of VRE in IFNA11 promoter (16, 18). Binding sites for the family of IRFs are highly conserved within the VRE of IFNA and IFNB genes, which suggests that the members of IFR family play an important role in the regulated expression of these genes. Currently, nine human IRFs have been identified. All these proteins share homology in the amino-terminal region, consisting of a highly conserved DNA binding domain that is structurally related to Myb (19). Studies with knockout mice have shown that each of these factors exerts a distinct role in response to pathogens, cell growth regulation, or hematopoietic differentiation (20−24). Two members of the IRF family, IRF-3 and IRF-7, have been shown to play an essential role in the inducible expression of type I IFN genes. IRF-3 is constitutively expressed in a variety of cell lines and tissues and strongly synergizes with the virus-mediated transcriptional activation of IFNA and IFNB promoters (25−28). In infected cells, IRF-3 is phosphorylated at carboxyl-terminal serines and translocated to the nucleus where it binds to the transcription coactivator CBP/p300 (26−28). Over expression of oncoproteins such as adenovirus-encoded E1A (25), papilloma virus-encoded E6 (29), or HHV-8-encoded vIRF-1 (30) inhibit the IRF-3-mediated transactivation by competing with CBP/p300 or interacting with IRF-3.

Human IRF-7 gene is expressed in cells of lymphoid origin...
and both IFN and virus stimulate its expression (31). In mouse, however, expression of IRF-7 was also detected in embryonic fibroblasts (32, 33). Expression of IRF-7A or its spliced variant IRF-7H activates murine IFNA promoters in transient transfection assay and enhances virus-stimulated expression of these promoters (31).2 Viral infection facilitated transport of IRF-7 from cytoplasm to nucleus, and phosphorylation of carboxyl-terminal serine residues was found to be essential for virus-mediated activation (31–33). In infected cells, IRF-7 was shown to interact with IRF-3 to form the ternary complex named virus-activated factor, which binds weakly to the IFNB promoter (10).

The aim of this study was to analyze the role of IRF-3 and IRF-7 in the cell type-specific expression of individual human IFNA subtypes. By using a co-transfection assay, the expression of individual IFNA reporter genes was determined in the presence of overexpressed IRF-3 or IRF-7H. We have shown that although all the IFNA promoters (A1, A2, A4, and A14) were activated by IRF-7, only A1 was activated by IRF-3. We have further found that in human fibroblasts, in which viral infection led to the expression of the IFNB gene but not the IFNA genes, reconstitution of IRF-7 expression conferred the virus-mediated activation of seven endogenous IFNA genes. From these expression of IFNA1 was most predominant. These data indicate that the lack of IFNA gene expression in infected fibroblasts is due to the absence of IRF-7.

**EXPERIMENTAL PROCEDURES**

**Cells and Virus**—Bovine tracheal cells and 2FTGH cells, which were obtained from G. Stark (Cleveland Clinic Foundation, Cleveland, OH), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Sendai virus used in this study was purchased from Specific Pathogen Free Avian Supply (Preston, CT). Routinely, infection was conducted with 400 hemagglutinin unit/100-mm plate in front of the thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SA (34). The HuIFNB (pB-SAP) VRE, A1 mutants A1(NF-1) and A1(del21) were described previously (35). IRF-7H and IRF-3 expression plasmid were described previously (31, 36). The A1 mutant reporter plasmid, pA1(4PM)-SAP, was created by inserting the synthesized oligodeoxynucleotide corresponding to the mutation A1 VRE into thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SAP (34). The HuIFNB (pB-SAP) VRE, A1 mutants A1(NF-1) and A1(del21) were described previously (35). IRF-7H and IRF-3 expression plasmid were described previously (31, 36). The A1 mutant reporter plasmid, pA1(4PM)-SAP, was created by inserting the synthesized oligodeoxynucleotide corresponding to the mutated A1 VRE into thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SAP (34). The HuIFNB (pB-SAP) VRE, A1 mutants A1(NF-1) and A1(del21) were described previously (35). IRF-7H and IRF-3 expression plasmid were described previously (31, 36). The A1 mutant reporter plasmid, pA1(4PM)-SAP, was created by inserting the synthesized oligodeoxynucleotide corresponding to the mutated A1 VRE into thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SAP (34). The HuIFNB (pB-SAP) VRE, A1 mutants A1(NF-1) and A1(del21) were described previously (35). IRF-7H and IRF-3 expression plasmid were described previously (31, 36). The A1 mutant reporter plasmid, pA1(4PM)-SAP, was created by inserting the synthesized oligodeoxynucleotide corresponding to the mutated A1 VRE into thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SAP (34). The HuIFNB (pB-SAP) VRE, A1 mutants A1(NF-1) and A1(del21) were described previously (35). IRF-7H and IRF-3 expression plasmid were described previously (31, 36). The A1 mutant reporter plasmid, pA1(4PM)-SAP, was created by inserting the synthesized oligodeoxynucleotide corresponding to the mutated A1 VRE into thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SAP (34). The HuIFNB (pB-SAP) VRE, A1 mutants A1(NF-1) and A1(del21) were described previously (35). IRF-7H and IRF-3 expression plasmid were described previously (31, 36). The A1 mutant reporter plasmid, pA1(4PM)-SAP, was created by inserting the synthesized oligodeoxynucleotide corresponding to the mutated A1 VRE into thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SAP (34).

**Plasmids and Antibodies**—HuIFNA1, A2, A4, and A14 reporter genes designated as pA1-SAP, pA2-SAP, pA4-SAP, and pA14-SAP, were constructed by inserting the respective IFNA VRE (−110 to −51) in front of the thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SAP (34). The HuIFNB (pB-SAP) VRE, A1 mutants A1(NF-1) and A1(del21) were described previously (35). IRF-7H and IRF-3 expression plasmid were described previously (31, 36). The A1 mutant reporter plasmid, pA1(4PM)-SAP, was created by inserting the synthesized oligodeoxynucleotide corresponding to the mutated A1 VRE into thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SAP (34). The HuIFNB (pB-SAP) VRE, A1 mutants A1(NF-1) and A1(del21) were described previously (35). IRF-7H and IRF-3 expression plasmid were described previously (31, 36). The A1 mutant reporter plasmid, pA1(4PM)-SAP, was created by inserting the synthesized oligodeoxynucleotide corresponding to the mutated A1 VRE into thymidine kinase minimal promoter, which drives the expression of a reporter gene encoding the SAP (34).

**RESULTS**

**Distinct Activation of Human IFNA Promoters by IRF-3 and IRF-7**—The human IFNA gene family consists of 13 functional genes, which are clustered on the short arm of chromosome 9 (Fig. 1A) (5). All of these genes show a high degree of similarity in the nucleotide sequences, not only in the coding region, but also in the promoter region (Fig. 1B). The similarity can be also found between the human and mouse IFNA VRE. The AF-1 and virus-induced factor binding domains, identified as important elements in murine IFNA4 and A6 promoter (14, 17), are also preserved in the promoters of human IFNA genes. However, the levels of expression of individual IFNA subtypes in infected cells differ in a cell type-specific fashion.

The difference in the levels of expression of various IFNA subtypes is due to the difference in the transcription activation of these genes (6). Therefore, we have chosen four HuIFNA promoter regions of IFNA1, A2, A4, and A14 genes that were previously shown to be expressed at different levels in infected cells and examined the ability of IRF-3 and IRF-7 to activate the VRE of these promoters in transient transfection assays. The ability of these factors to activate IFNB promoter was also measured for comparison. 2FTGH cells express IRF-3 constitutively but do not express IRF-7, and virus infection in these cells stimulates the expression of IFNB but not IFNA genes. Transfection of pA1-, pA2-, pA4-, and pA14-SAP plasmids (containing IFNA1, A2, A4, and A14 promoters; Ref. 94) into 2FTGH cells did not result in expression of the SAP reporter gene in uninfected cells (Fig. 2A). Viral infection stimulated expression of the pB-SAP only. Co-transfection with IRF-3 led to an increase in the constitutive expression of pB-SAP (5-fold), which was further enhanced by viral infection (12-fold). However, the expression of pB-SAP in infected cells was similar in IRF-3 transfected or untransfected cells, possibly because the fusion proteins were purified on glutathione-Sepharose column as described previously (31). For EMSA, the binding reaction and analyses of the DNA-protein complexes were done as described previously (25, 37). The respective probes were labeled VRE fragments (−110 to −51) excised from pA-SAP by SnaI digestion. For the competition experiments, 100, 200, and 400 fmol of labeled VRE were added to GST-IRF-3, 20 min before addition of the probe. For DNase I footprinting, the IFNA probes used for the footprinting assay were generated by PCR and labeled with 32P by T4 kinase. The 3′ primer within the IFNA 5′-untranslated region (+35 to +55) was engineered to have XhoI cleavage site for the purpose of removing the linker between the antisense strand. For each binding reaction (final volume, 100 μl) probe of 1.2 × 106 cpm was incubated with 1 μg of GST fusion protein in a binding solution containing 10 mM Tris-Cl, pH 8, 5 mM MgCl2, 1 mM CaCl2, 2 mM dithiothreitol, 50 μg/ml bovine serum albumin, 2 μg of poly(dIdC), and 100 mM KCl. Following an incubation at 4 °C for 30 min, 7 × 105 units of DNase I was added to each binding reaction for 1–2 min at 25 °C, and the DNase activity was stopped by the addition of 350 μl of solution consisting of 325 μl of ethanol, 2.5 μl of tRNA (1 mg/ml), and 50 μl of 10 mM ammonium acetate. The DNase-digested products were recovered by centrifugation and resolved on a 6% denaturing acrylamide gel.

**Reverse Transcription PCR**—1 μg of total RNA isolated by Trizol method (Life Technologies, Inc.) was reverse transcribed to cDNA with oligo(dT) primers in a 30-μl reaction. From this mixture of cDNAs, IFNA, IRF-7, and β-actin cDNA were amplified by PCR using the following primer sets: (i) IFNA (consensus primers designed to recognize all human IFNA subtypes) sense 5′-GTACTGCGAGATCTCCTTTCTTCTCCTG-3′, antisense 5′-GGTTGCTATCGACAACTCTCCAGAGGACA-3′ (35); (ii) β-actin sense 5′-CAAATGACTGCTGGTGGCT-3′, antisense 5′-GATGGCACAGTGGTGGTGTA-3′; and (iii) IRF-7 sense 5′-TGCACAGTGCTCGGAG-3′, antisense 5′-TCAACTCCTCTGGCAGTCCACAGTCC-3′. The HuIFNA specific primers contain a Pst (sense primer) and a XbaI (antisense primer) restriction enzyme recognition sequence to facilitate the cloning of the PCR fragments. The amplified IFNA fragments were cloned into pBluescript KS II (-Stratagene) at the PstI/XbaI site. The clones were randomly chosen and sequenced.

**REFERENCES**

1. Gewert, and P. M. Pitha, unpublished data.
level of endogenous IRF-3 is already high in these cells. Transfection with IRF-3 resulted in a 2-fold increase in the pA1-SAP expression in infected cells but did not increase expression of pA2-, pA4-, or pA14-SAP. In contrast, transfection with IRF-7 led to an increase of both the constitutive and inducible expression of all the IFNA VRE tested. However, the relative expression of pA-SAP differed. Although IRF-7 increased the expression of pA1-SAP only 2-fold, viral infection increased IRF-7-mediated stimulation 6-fold, indicating a synergistic activation by IRF-7 and virus. The expression of pA2-SAP was increased 5-fold by IRF-7, and following viral infection the levels of SAP expression were increased by 16-fold. The pA4-SAP showed a similar response to IRF-7 activation but in the infected cells, its expression was slightly lower (12-fold) than that of pA2-SAP (16-fold). In contrast, the expression of pA14-SAP was only marginally enhanced (2-fold) by IRF-7 and was not further increased by viral infection. Overexpression of IRF-7 increased both constitutive and virus stimulated expression of pB-SAP; however, the stimulation by virus infection and IRF-7 overexpression were only additive. Altogether, these data show that in transient transfection assay in fibroblast cells, virus effectively induces only the IFNB VRE and none of the IFNA VRE tested. This correlates with the observed lack of expression of the endogenous IFNA genes in infected fibroblast cells.

To determine more precisely the sequences within VRE that respond to IRF-7 activation in infected cell, we examined the expression of IFNA1 VRE mutants inserted into the SAP reporter plasmid (Fig. 2C) (35). The pA1(del21)-SAP contains only sequences corresponding to PRDI and PRDIII in IFNB VRE, which were shown to bind IRF-3 (37). The four point mutations in IFNA1 VRE (pA1[4 PM]-SAP) completely abolished the transcription activity of the IFNA1 VRE, as well as its response to IRF-7. In contrast, the insertion of the NfκB binding site into IFNA1 VRE substantially enhanced the tran-
post-infection, and the presence of IFNA and IRF-7 mRNA was determined by immunoblotting with IRF-3 and IRF-7 antibodies, respectively. A, analysis of IFNA and IRF-7 mRNA. Total RNA was prepared from the infected transfected cells and parental cell line at 6 h post-infection, and the presence of IFNA and IRF-7 mRNA was determined by reverse transcription PCR as described under “Experimental Procedures.” IFNA cDNAs were amplified using primers corresponding to the conserved regions of all HuIFNA genes. The amplified cDNA fragments were analyzed on agarose gels.

The relative levels of IRF-7 and IFNA mRNAs in total RNA preparation isolated at 6 h post-infection from transfected cells were determined by reverse transcription PCR (Fig. 3B). The presence of IRF-7 mRNA and IFNA mRNA could be detected only in IRF-7 transfected cells but not in infected parental cells or cells transfected with IRF-3 expressing plasmid. The culture media from the infected cells were analyzed for the presence of HuIFNA-α by antiviral assay on bovine tracheal cells. These cells are sensitive only to IFN-α but not to IFN-β (39). It can be seen in Fig. 3B that the interferon activity (80 units/ml) was detected only in media from the IRF-7 transfected cells but not in media from any of the other infected cells. Because the transfection efficiency in 2FTGH cells is only about 25%, these levels of interferon represent an underestimation of the true values. However, neither IFNA mRNA nor biologically active IFN-α could be detected in cells expressing IRF-3. In immortalized mouse embryo fibroblast cells, the virus-induced activation of IFN-α4 and IFN-β stimulated the expression of IRF-7, which in turn induced the expression of non-IFNA4 genes (32, 33). In contrast, in human 2FTGH cells, IFN treatment neither induced the expression of IRF-7 nor primed virus-mediated induction of IFNA genes (31).

The presence of IFNA mRNA in IRF-7 transfected 2FTGH cells was detected by amplification with primers corresponding to the regions of IFNA genes that are conserved in all IFNA subtypes (Ref. 38; see “Experimental Procedures” for details). To determine which IFNA subtypes are expressed in IRF-7 transfected fibroblasts, we cloned the PCR-amplified cDNA fragment into the pBluescript KS II+ plasmid, and cDNA inserts isolated from 40 randomly selected clones were sequenced. DNA sequences obtained were compared with sequences of individual IFNA genes present in GenBankTM. Seven IFNA subtypes were expressed (Table I) and a significant difference in the relative frequency of the individual IFNA cDNA subtypes was found. IFNA1 and IFNA7 transcripts were most abundant (40 and 35%, respectively). IFNA4 gene was expressed at higher level (10%) than IFNA10 and IFNA17 (5%). IFNA2 and IFNA14 were only marginally expressed (2.5%).

In the transient expression assay in 2FTGH cells, IRF-7 activated IFNA2 VRE most efficiently. However, in the cells expressing ectopic IRF-7, the endogenous IFNA2 gene was induced only with low frequency. To determine whether the expression of individual IFNA subtypes shows the same profile in 2FTGH cells that constitutively expressed IRF-7, we generated permanently transfected 2FTGH cell lines (2FTGH/IRF-7). The lysates of transfected cells lines were analyzed by Western blot hybridization for the presence of IRF-7. Two of the cell lines, 2FTGH/IRF-7/7 and 9, that expressed high levels of IRF-7 (Fig. 4A) were further analyzed. Virus infection of both 2FTGH/IRF-7/7 and 9 cells induced high levels of biologically active IFN-α (1555 and 2048 units/ml, respectively). The individual IFNA genes expressed in these two cell lines and the frequency of their expression were determined as in transfected cells (Fig. 4B). The results showed that both transient and constitutive expression of IRF-7 in 2FTGH cell most efficiently induced IFNA1 gene. However, in both permanent cell lines constitutively expressing IRF-7, IFNA 17 was expressed more efficiently than in the transiently transfected cells (33 and 35%, respectively), whereas IFNA7 expressed at high levels in the transiently transfected cells was expressed less efficiently in the permanent lines (35 and 8%, respectively). Expression of IFNA2 was detected in neither of the two permanent cell lines; however, in 2FTGH/IRF-7/7 cells, IFNA8 that is located next to IFNA2 was expressed at low levels. Presently we cannot exclude the possibility that IFNA2 is expressed at low levels in these cells and therefore not detected in the set of clones analyzed. However, in both transiently and permanently IRF-7 expressing cell lines, with the exception of IFNA1, most of the genes expressed were present in the second IFNA cluster on chromosome 9.

**Table I**

| IFNA | Number of positives/40 clones |
|------|-----------------------------|
| A1   | 16                          |
| A2   | 1                           |
| A4   | 4                           |
| A7   | 14                          |
| A10  | 2                           |
| A14  | 1                           |
| A17  | 2                           |

**FIG. 3.** Expression of the endogenous IFNA genes in 2FTGH cells expressing ectopic IRF-7. A, analysis of the relative levels of IRF-3 and IRF-7 in transfected 2FTGH cells. Cells were collected 6 h post-infection, and the levels of IRF-3 and IRF-7 in cell lysates were determined by immunoblotting with IRF-3 and IRF-7 antibodies, respectively. B, analysis of IFNA and IRF-7 mRNA. Total RNA was prepared from the infected transfected cells and parental cell line at 6 h post-infection, and the presence of IFNA and IRF-7 mRNA was determined by reverse transcription PCR as described under “Experimental Procedures.” IFNA cDNAs were amplified using primers corresponding to the conserved regions of all HuIFNA genes. The amplified cDNA fragments were analyzed on agarose gels.
Infected with Sendai virus and the levels of biologically active IFN-
B before and 6 h after infection with Sendai virus were determined by Western blot hybridization with IRF-7 antibodies.

The levels of IRF-7 in total cell extracts from three of these lines (clones 3, 7, and 9) before and 6 h after infection with Sendai virus were determined by Western blot hybridization with IRF-7 antibodies. B, clones 7 and 9 were infected with Sendai virus and the levels of biologically active IFN-α were determined on bovine tracheal cells. RNA isolated from the infected cells was amplified by reverse transcription PCR, and the individual IFNA genes were identified as described under “Experimental Procedures.”

IFN-α subtypes. To determine whether this difference in activation reflects the binding affinity of these two factors to IFNA VREs, we analyzed (by EMSA) binding of the recombinant GST-IRF-3 (25) and GST-IRF-7 (31) to IFNA1, A2, A4, and A14 VRE. GST alone did not bind to any of these probes (Fig. 5A), and the presence of GST peptide did not affect the binding specificity of IRF-3 or IRF-7 (data not shown). GST-IRF-3 bound effectively to A1 probe resulting in the detection of a strong fast migrating band and a weak slower migrating band, probably representing IRF-3 dimer (40). GST-IRF3 binding to A2 and A4 probe resulted in a formation of only a weak fast moving band, indicating that binding of IRF-3 to these VREs is much weaker. However, no binding to the A14 VRE probe could be detected. In contrast, GST-IRF-7 bound effectively to both A1 and A2 VRE, and three protein-DNA complexes were detected. We assume that these may be derived from both binding of IRF-7 dimers as well as binding to the multiple IRF binding sites on the VRE probe (Fig. 1B). Binding of GST-IRF-7 to the A4 probe resulted in formation of only one slowly moving band, and no binding to A14 VRE could be detected. The binding specificity was confirmed by competition experiment. Binding of GST-IRF-3 to A1 VRE could be competed by unlabeled A1 VRE and PRDIII oligodeoxynucleotides (37) but not by A14 VRE or poly(dI-dC). Similar specificity was also confirmed for GST-IRF-7 binding (data not shown).

Because the VREs encompass 86 base pairs, we used the DNase protection assay to determine the region that binds the GST-IRF-3 and GST-IRF-7 in A1, A2, and A14 probes (Fig. 5B). The binding of GST-IRF-7 as well as binding of the GST-IRF-7 DNA-binding domain (amino acids 1–237) protected the region spanning nucleotides −103 to −67 in A1 VRE. The same region was protected by IRF-7 in A2 VRE, but no protection in this region was seen in A14 VRE. Binding of GST-IRF-3 to A1 VRE protected the identical region, but no protection by GST-IRF-3 was detected in A2 and A14 VRE. These data indicate that both GST-IRF-3 and GST-IRF-7 bind to the same region of A1 VRE. The lack of IRF-3 binding to IFNA VRE other than A1 correlates with its inability to activate the corresponding IFNA reporter gene.

It has been shown that viral infection facilitates transfer of GFP-IRF-3 and GFP-IRF-7 fusion proteins from cytoplasm to nucleus (29, 31). To determine whether endogenous IRF-3 and IRF-7 can be found not only in the cytoplasm but also in the nucleus of uninfected cells, we used Western blot hybridization to analyze for the presence of IRF-3 and IRF-7 in nuclear extracts of infected and uninfected 2FTGH/IRF-7 cells. It can be seen (Fig. 6A) that IRF-3 and IRF-7 could be detected in nuclei of both infected and uninfected cells. Although in the infected cells the majority of nuclear IRF-3 was phosphorylated, low levels of phosphorylated IRF-3 could be detected also in the uninfected cells as shown by slower mobility of the IRF-3 band. IRF-7 detected in the nuclear extracts of infected 2FTGH/IRF-7 cells also moved slower than in nuclear extracts from uninfected 2FTGH/IRF-7 cells, suggesting that IRF-7 may be phosphorylated following virus infection. Furthermore viral infection lead to an overall decrease in the relative levels of nuclear IRF-3 and IRF-7. In EMSA with IFNA1VRE probe, multiple bands representing the DNA-protein complexes were observed; however, there was no significant difference between binding profile of nuclear extracts from infected and uninfected cells (Fig. 6B). However, nuclear extracts from 2FTGH/IRF-7 cells show stronger binding and two new slowly moving bands, representing DNA-protein complexes I and II (Fig. 6B). The addition of IRF-3 antisemum abolished the formation of complex I, whereas the addition of IRF-7 antisemum resulted in the disappearance of both complex I and II. The preimmune serum did not affect the overall binding pattern. We therefore conclude that complex I contains both IRF-3 and IRF-7 and may represent binding of IRF-3/IRF-7 heterodimers, whereas the complex II contains only IRF-7 or its homodimers. We next examined the specificity of the binding by competition with IFNA1 and IFNA2 VRE. IFNA1 VRE that binds effectively both the recombinant IRF-3 and IRF-7 competed effectively formation of all the DNA-protein complexes including complex I and II. IFNA2 VRE competed effectively formation of complex I and II that contained IRF-7 but was much less effective competitor of the rest of the complexes, which we assume represent binding of the other cellular IRFs namely IRF-1 and IRF-2 to their multiple binding sites in IFNA1 VRE. These data clearly demonstrate that both IRF-3 and IRF-7 are present in the nuclear extracts of uninfected 2FTGH/IRF-7 cells. Additional experiments are in progress to determine whether phosphorylation can affect the DNA binding affinity of IRF-7.

**DISCUSSION**

We have demonstrated in this study that the virus-mediated expression of HuIFNA genes depends on the presence of the transcription factor IRF-7. In human fibroblast cells that do not express IRF-7 gene, virus infection stimulated the expression of the IFNB gene, whereas no expression of IFNA genes was detected. Only after the reconstitution of IRF-7 expression did virus infection stimulate transcription of the IFNA genes and the synthesis of biologically active IFN-α. The inducible expression of IFNA genes was observed both in cells transiently expressing IRF-7, as well as in permanent cell lines constitutively expressing IRF-7. The IRF-7 effect was not limited to 2FTGH cells because ectopic expression of IRF-7 in infected human foreskin fibroblast cells also resulted in transcription activation of IFNA genes (data not shown). During the course of this work two studies addressed the mechanism of IFNA gene
induction in murine system and came to a different conclusion from each other (32, 33). The results from these studies demonstrated that in fibroblasts derived from mice with homozygous deletion of STAT 1 gene, IFNA4 gene was found to be expressed as a primary response to viral infection (32), whereas all the other IFNA subtypes required synthesis of IFN induced protein identified as IRF-7 (32). In contrast, fibroblasts derived from mouse with homozygous deletion of IRF-8 (p48) were unable to induce any IFNA genes after viral infection but could do so after transfection with IRF-7 (33). These data are in agreement with our findings. However, there are two major differences between mouse embryo fibroblasts and human foreskin fibroblasts or the fibroblast cell line (2FTGH) used in this study. Firstly, mouse embryo fibroblasts express both IFNB and IFNA genes upon viral infection (32, 33), whereas human fibroblast express only the IFNB gene (41). Secondly, viral infection and IFN treatment induces expression of IRF-7 in murine but not in the human fibroblasts. Thus, the observed

**Fig. 5.** Binding of the recombinant GST-IRF-3 and GST-IRF-7 fusion proteins to the IFNA VREs. A, EMSA. An equal amount (1 μg) of purified GST, GST-IRF-3, or GST-IRF-7 was used in the binding reaction with individual IFNA VRE as indicated by the numbers 1, 2, 4, and 14. Each probe used in this EMSA was generated with the same amount of DNA and labeled to a similar specificity. The cold competitor DNA (200-fold excess) was introduced into the binding reaction 20 min prior to the addition of probe. PRDI/III was derived from the human IFNB promoter. B, analysis of the GST-IRF-3 and GST-IRF-7 binding by the DNase I protection assay. Each IFNA probe DNA was created by PCR using an universal 5′ primer and 3′ primer derived from the sequence located at the 5′-untranslated region and labeled at the sense strand. The IFNA1 and IFNA14 ladders were done by dideoxy sequencing using the same 5′ primer. The sequence of the footprint area is shown by the filled black box and the boundary is indicated by arrows. GST-IRF-7 (−DBD) containing only the carboxy-terminal portion (amino acids 300–514) of IRF-7 is used as a negative control.
differences could be related to the presence of low levels of IRF-7 in STAT-1 defective mouse fibroblasts (32). Interestingly, it has been shown that mouse fibroblasts containing homozygous deletion of IFNB gene were not able to induce expression of IFNA genes (42).

The contribution of IRF-3 and IRF-7 to the activation of the individual VRE of IFNA subtypes in infected cells was not equal. Thus, in a transient expression assay, IRF-3 enhanced virus activation of IFNB VRE and IFNA1 VRE but did not activate the other IFNA VREs tested. In this respect, the HuIFNA1 response resembled that of MuIFNA4 that was also activated by IRF-3 (31, 32) in a transient assay. All the IFNA VREs tested (A1, A2, A4, and A14) responded to IRF-3 in a transient transfection assay, but not to IRF-7 (31, 32). IRF-7-mediated activation and its binding to both IRF-3 and IRF-7. These results suggest the importance of GAAAG motif in activation of HuIFNA1 and MuIFNA4 promoters that was recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15).

The VRE regions of different IFNA subtypes including those tested show an extensive nucleotide homology in three GAAAG/C repeats. The G(C)/AAA(G/C) motif was recently identified as a consensus IRF-3 binding site,2 and IFNA1 activated by IRF-3 has all these repeats conserved. The IFNA4 VRE, which is not activated by IRF-3 and shows only a low response to IRF-7 has only one GAAAG domain conserved and binds neither recombinant IRF-3 or IRF-7. Point mutation in two GAAAG domains in IFNA1 VRE abolished its response to IRF-3 and IRF-7-mediated activation and its binding to both IRF-3 and IRF-7. These results suggest the importance of GAAAG domain in the VRE for the IFNA-3 and IRF-7-mediated activation. The role of the GAAAGC and GAAAGT motifs in activation of HuIFNA1 and MuIFNA4 promoters that was recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15). The crystal structure of the DNA binding domain of IRF-1 and IRF-2 revealed that GAAA motif is recognized previously (12–15).

In the transient transfection assay, transactivation of the individual subtypes of IFNA VREs generally correlated with the ability of IRF-3 and IRF-7 to bind to the respective VREs. However, the induction of the endogenous IFNA genes in 2FTGH/IRF-7 cells did not fully correlate with the results of the transient transfection assays. Most notably, while in transient transfection assay, IFNA2 VRE was activated most efficiently by IRF-7, and expression of endogenous IFNA2 gene was very low or undetectable in infected 2FTGH/IRF-7 cells. These data provide the first direct comparative analyses of the transcription activation of the integrated and unintegrated IFNA promoters and indicate that the levels of activation of the IFNA promoters in transient transfection assay do not always correlate with the expression levels of the endogenous gene.

The pattern of virus-stimulated IFNA gene expression in 2FTGH/IRF-7 cells differed from the pattern of IFNA genes induced in lymphoid cells that are natural producers of IFNA (45–48). Interestingly, in the 2FTGH/IRF-7 cells, most of the IFNA genes expressed were part of the distal IFNA gene cluster (Fig. 1A) (5). IFNB is localized at the far end of this cluster close to telomere (5). In virus-infected Namalwa cells, the IFNA genes expressed were localized both in the proximal and distal clusters (34), whereas in virus-stimulated PBMC, only genes localized close to the centromere were expressed (37). In virus-infected Namalwa cells, the IFNA genes expressed were localized both in the proximal and distal clusters (34), whereas in virus-stimulated PBMC, only genes localized close to the centromere were expressed (37). These data indicate that the overall transcription accessibility of the IFNA gene locus may depend on the cell type. The discordance between the levels of activation of individual IFNA promoters in transient transfection assays and the expression of the corresponding IFNA genes in infected cells suggests that factors whose role is to overcome the repressive effect of chromatin
may play an important role in the expression of the family of IFN genes in vivo. Several mechanisms may be in play. First, the factors that activate transcription were shown also to recruit chromatin modifiers such as acetyltransferases (e.g. CBP/p300 and P/CAF) to the promoters of genes they activate. The interaction of IRF-3 and NFκB with histone acetyltransferase p300 (10) was clearly demonstrated, and IRF-9 (ICSBP) was shown to bind histone acetyltransferase P/CAF (49). Thus, binding of the NFκB, IRF-3, and IRF-7 with these coactivators may rearrange the structure of the IFN locus. The importance of histone acetyltransferase in the activation of IFNB was recently suggested (50). Second, nucleosome response rapid phosphorylation of the nucleosome proteins, histone H3, and high mobility group 14 was found to be associated with growth factor-mediated stimulation of c-jun and c-fos genes (51). It was shown that the signaling pathway that activates transcription factors involved in the activation of these two genes also activates kinases responsible for phosphorylation of histone H3 and high mobility group 14 proteins. Because a virus-mediated activation of both IRF-3 and IRF-7 is associated with their phosphorylation, the possibility that the virus-mediated signaling also results in the phosphorylation of the nucleosome proteins warrants investigation. Thus further characterization of the critical factors that regulate expression levels of IFN genes in the infected cell in vivo as well as determination of the IFNA subtypes produced in these cells will be important for understanding of the role of IFN-α in the control of viral infection and autoimmunity.

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Reconstitution of Virus-mediated Expression of Interferon α Genes in Human Fibroblast Cells by Ectopic Interferon Regulatory Factor-7

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