Recruitment of Multiple Interferon Regulatory Factors and Histone Acetyltransferase to the Transcriptionally Active Interferon A Promoters*

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Type I interferon (IFN) plays a critical role in the innate immunity against viral infection. Expression of IFNA genes in infected cells is cell type-dependent and is regulated at the transcriptional level. The present study is focused on the molecular mechanism underlying the differential expression of human IFNA1 and A2 genes. Two nucleotides, at positions −98 and −91 of IFNA1 and A2 promoter, were pivotal to the differential expression. The DNA pull-down and chromatin preparation assays have shown that nuclear interferon regulatory factor (IRF)-3 and IRF-7 as well as IRF-1 bind to IFNA1 virus-responsive element (VRE). Interestingly, overexpression of IRF-7 increased the otherwise weak binding of both IRF-3 and IRF-7 to IFNA2 VRE. These data together with the results of two-step chromatin immunoprecipitation strongly suggest that the IRF-3 and IRF-7 bind to IFNA1 promoter as a dimer. Furthermore, binding of IRF-3 and IRF-7 to IFNA VRE is associated with the presence of acetylated histone H3, suggesting that histone acetyltransferase(s) is tethered together with virus-activated IRF-3 and IRF-7 to the IFNA1 promoter. In addition, the constitutively active IRF-3 (5D) and IRF-7 (2D) mutants activate the endogenous IFNA genes in uninfected cells; however, the expression profile of IFNA is not identical to that induced by viral infection.

Type I interferon (IFN) plays an essential role in innate immune response against viral infection. Virus-mediated activation of Type I IFN gene expression is regulated at the transcriptional levels. The minimal cis-acting elements that confer the response of Type I IFN genes to the virus-activated signaling are located within the 110 nucleotides 5' of the transcription initiation site (2, 3). The virus-responsive element (VRE) in the IFNA1 promoter region contains purine-rich GAAANN motifs that serve as a specific binding site for the interferon regulatory factor (IRF) family.

Nine cellular IRFs and three viral homologues have been identified (4–8). All of the cellular IRFs share a region of homology in the amino terminus encompassing a highly conserved DNA-binding domain characterized by five tryptophane repeats (4). Three of these repeats contact DNA recognizing the GAAA or AANNGAAA sequences (9–11). KSHV-encoded IRFs that contain an imperfect DNA-binding domain are not able to bind DNA with the same specificity as cellular IRFs.

Several IRFs were implicated in the regulation of Type I IFN gene expression in virus-infected cells. Among them, IRF-1 was first identified as an activator of IFNB gene, whereas IRF-2 antagonized the IRF-1-mediated activation and behaved as a suppressor. Despite the observation that the embryonic fibroblasts from IRF-1−/− mice express normal level of IFNA and B (12, 13), it was observed that IRF-1 is associated with the human IFNB promoter in vivo, thus suggesting that IRF-1 may contribute to the transcriptional regulation of human IFN B gene (14). Three IRFs (IRF-3, IRF-5, and IRF-7) were shown to be the direct transducers of virus-mediated signaling and to play a crucial role in the expression of Type I IFN genes (15–21). Although IRF-3 is constitutively expressed in all types of cell (22), constitutive expression of IRF-7 can be detected mostly in lymphoid cells. In most cell types, expression of IRF-7 can be stimulated by Type I IFN (16, 23). Expression of IRF-5 seems to be restricted to B cells and dendritic cells (24).

In the murine system, the induction of Type I IFN genes in virus-infected cells was proposed to proceed by two sequential phases. During the initial phase, which does not require protein synthesis, transcription of IFNB and IFNA4 genes is activated. The second phase, during which the rest of IFNA subtypes is induced, depends on IFN-mediated induction of IRF-7 expression (20, 26). Consistent with this observation are the recent results from infected mouse embryonic fibroblasts with homozygous deletion of IRF-3 gene that displayed significant reduction of Type I IFN expression, and an additional defect in IFN signaling pathway completely abolished the virus-mediated induction of Type I IFN genes (21). In human cells, however, overexpression of IRF-3 only stimulates expression of IFNB gene (28), and the expression of IRF-7 is essential for the induction of all IFNA genes. Cells, such as 2TGH fibroblasts, that do not express IRF-7 are impaired in virus-mediated induction of all IFNA genes, which can be rescued upon reconstitution of IRF-7 expression (17, 29). Furthermore, ribozyme-targeted reduction of IRF-3 levels also resulted in a dramatic decrease in expression of some IFNA subtypes, suggesting that IRF-3 is also indispensable for the induction of certain IFNA genes (18). Interestingly induction of IFNA genes can be also rescued in non-IRF-7-expressing cells by overexpression of IRF-5, but activation of this transcription factor is virus-specific and results in expression of different IFNA subtypes than found in infected cells expressing IRF-7 (24).
In infected cells both IRF-3 and IRF-7 are phosphorylated by an yet unidentified kinase and retained in the nucleus, where IRF-3 but not IRF-7 further interacts with the transcription co-activator, p300/CBP (30–33). Structure-function analysis of IRF-3 and IRF-7 proteins revealed that both of these IRFs contain the auto-inhibitory domain, which in transient transfection assay suppresses the transcription activity of these factors (34–36). Furthermore, the results of the study with IRF-7 dominant negative mutant suggested that IRF-3 and IRF-7 can form homodimers and heterodimers and that these interactions are critical for the stimulation of the transcriptional activity of endogenous IFNA genes (15, 35). The interaction between the members of IRF family was found to result in novel biological activities (38).

Although the promoters of all IFNA genes are highly homologous, differential expression of these genes as a function of cell type and inducing agents has been observed (39, 40). While IFNA2 was found to be a major subtype produced in B cell line, Namalwa (41), IFNA1 was the major subtype expressed in human fibroblast expressing ectopic IRF-7 (17). The aim of this study was to gain more insight into the molecular mechanism underlying the differential expression of human IFNA1 and IFNA2 genes. To this effect, we have identified the critical cis- and trans- regulatory elements regulating expression of the endogenous IFNA1 and IFNA2 genes in HeLa cells as well as in human 2TGH cells, in which the expression of IRF-7 was reconstituted by ectopic IRF-7 or its mutants. The results have indicated a critical role of IRF-3/IRF-7 heterodimers in the stimulation of transcription of these two IFNA genes and showed that the relative levels of IRF-7 in the cells affect the assembly of IRF-1, IRF-3, IRF-7, and acetyltransferase on the transcriptionally active promoters of IFNA1 and IFNA2 genes in infected cells.

MATERIALS AND METHODS

Cells, Virus, Plasmids, and Transfection—2TGH (kindly provided by Dr. G. Stark), 293T, and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For infection, Sendai virus purchased from Specific Pathogen Free Avian Supply (Preston, CT) at a concentration of 240 hemagglutinin units/60-mm plate or 15 hemagglutinin units/well of 24-well plate was used. Mutant IFNA SAP reporter genes were generated by inserting double-stranded oligomer corresponding to the IFN VRE (10^9 to 10^7) into plasmids and coupled with streptavidin magnetic beads (Dynal Inc.). Nuclear extract dialyzed against the binding buffer (10% glycerol, 12 mM HEPES, pH 7.9, 5 mM MgCl_2, 60 mM KCl, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonil fluoride) were then incubated with the DNA bound magnetic beads in the presence of salmon sperm DNA as nosppecific competitor for 4 h at 4 °C. After gentle washing of the beads, the bound proteins were resolved by SDS-polyacrylamide gel electrophoresis, and the presence of DNA-bound protein was identified by Western blot.

**DNA Pull-down Assay**—DNA pull-down assay was done as described recently (15). Briefly, biotinylated double-stranded oligomers corresponding to the IFNAVRE region (~110 to ~53 base pairs) were synthesized and coupled with streptavidin magnetic beads (Dynal Inc.). Nuclear extract dialyzed against the binding buffer (10% glycerol, 12 mM HEPES, pH 7.9, 5 mM MgCl_2, 60 mM KCl, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonil fluoride) were then incubated with the DNA bound magnetic beads in the presence of salmon sperm DNA as nosppecific competitor for 4 h at 4 °C. After gentle washing of the beads, the bound proteins were resolved by SDS-polyacrylamide gel electrophoresis, and the presence of DNA-bound protein was identified by Western blot.

**Chromatin Immunoprecipitation Assay**—The detailed assay procedure was described previously (24). Briefly, HeLa cells (5 × 10^6) were transfected with 1 μg of IFNA SAP reporter genes. At 16 h post-transfection, the cells were infected with Sendai virus for 5.5 h. To cross-link the proteins bound to DNA, the cells were treated with 11% formaldehyde (0.1 mM NaCl, 1 mM EDTA, 50 mM HEPES, pH 8.0) to a final concentration of 1% for 30 min at 37 °C. The in vivo cross-linking reaction was stopped by addition of glycine to final concentration of 0.125 M. The cell pellets were washed and resuspended in 400 μl of sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) on ice and sonicated for 10 s. These samples were then dialyzed 10-fold with 3.6 ml of dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) and precleared with protein A-agarose for 2 h. Following the preclearing, equal amounts of proteins (as determined by the Bio-Rad protein assay reagent) were immunoprecipitated with 1 μg of polyclonal anti-acetylated Histone 3 (Upstate Inc.), anti-IRF-7, or anti-NFκB p65 antibodies (Santa Cruz) or monoclonal anti-FLAG (M2, Stratagene Inc.) or IRF-3 antibody (PharMingen, Inc.) at 4 °C for 4 h. Immunocomplexes were extensively washed with the dilution buffer, resuspended in TE buffer (100μl), and treated with proteinase K (500 μg/ml) for 4 h. The cross-linked DNA- protein complexes were reverted by heating at 65 °C for 6 h, and the DNA was recovered by phenol/chloroform extraction. DNA purified by phenol/chloroform with ethanol in the presence of 2 M ammonium acetate was resuspended in 60 μl of water, and 15 μl were used as a template for PCR amplification with IFNA-SAP specific primers that could amplify both IFNA1 and IFNA2 VRE (sense, 5′-AAGTTGGTTAAC-GGCCAGGGT; antisense, 5′-ACAGTGCCCAGGGCAGAGTATTGCT). These primers detect selectively transfected plasmids and not the endogenous promoters. The PCR was performed with Vent DNA polymerase (New England BioLabs Inc.) with a PerkinElmer Life Sciences DNA thermal cycler at 94 °C, for 4 min for 1 cycle; 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min for 30 cycles; and 72 °C for 3 min for 1 cycle.

**RESULTS**

Presence of Two IRF-binding Sites Is Essential for the Expression of IFNA1 and IFNA2 Genes in Infected Cells—We have previously observed that in infected human fibroblast, 2TGH, ectopically expressing IRF-7, IFNA1 was the major induced subtype (17), although the expression of IFNA2 in these cells was very low. It was shown previously that recombinant IRF-7 but not IRF-3 binds efficiently to IFNA2 VRE (17, 41). This region contains two potential IRF-binding sites (Pharmingen, Inc.) for 4 h at 4 °C. Immunocomplexes were extensively washed with the dilution buffer, resuspended in TE buffer (100μl), and treated with proteinase K (500 μg/ml) for 4 h. The cross-linked DNA- protein complexes were reverted by heating at 65 °C for 6 h, and the DNA was recovered by phenol/chloroform extraction. DNA purified by phenol/chloroform with ethanol in the presence of 2 M ammonium acetate was resuspended in 60 μl of water, and 15 μl were used as a template for PCR amplification with IFNA-SAP specific primers that could amplify both IFNA1 and IFNA2 VRE (sense, 5′-AAGTTGGTTAAC-GGCCAGGGT; antisense, 5′-ACAGTGCCCAGGGCAGAGTATTGCT). These primers detect selectively transfected plasmids and not the endogenous promoters. The PCR was performed with Vent DNA polymerase (New England BioLabs Inc.) with a PerkinElmer Life Sciences DNA thermal cycler at 94 °C, for 4 min for 1 cycle; 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min for 30 cycles; and 72 °C for 3 min for 1 cycle.

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transcription of the reporter gene, SAP, was then tested by a transient transfection assay in HeLa cells. As shown in Fig. 1B, Sendai virus infection resulted in a 23-fold induction of IFNA1SAP, whereas the induction of IFNA2 SAP was only 3-fold. The IFNA1 mutant containing either three nucleotide changes (4PM) from IFNA1 to IFNA2, in the region corresponding to PRDI or a single nucleotide change in this region (M1) lost the virus-mediated inducibility. These results indicated that the −81 adenine is crucial for the inducibility of this VRE, whereas the cytosine and guanine at positions −86 (M2) and −79 (M4), respectively, were not critical. Interestingly, when a guanine at position −98 was deleted from IFNA1 VRE, thereby creating a perfect match to IFNA2 in the PRDIII corresponding region, the inducibility of the resulting mutant (M5) has decreased to a level similar to that of IFNA2 reporter. Also when the IRF-E in the PRDIII corresponding region was disrupted (M3), this mutant did not respond to virus infection. To further confirm that the −81 G and −81 A are the determinants of the differential expression of IFNA1 and IFNA2 genes, we generated IFNA2-VRE mutant (plasmid 2+1) in which we inserted guanine at position −98, replaced guanine with adenine at position −81, and found that the inducibility of this mutant was restored to about 60% of the activity of IFNA1 reporter. To determine whether the PRDIII-like regions are equally important for the induction, we replaced nucleotides either in position −98 or in position −81 of IFNA2 VRE (M6 and M7, respectively) and analyzed the activity of these mutants. The results have shown that the PRDIII-like element is more important for the virus-mediated induction of IFNA1 promoter, because the mutation in position −98 led to 12-fold induction, whereas the mutation in −81 led to only 7-fold induction. Taken together, these data indicated that two nucleotides within the two IRF-binding sites of IFNA1 VRE and IFNA2 VRE determine their response to viral infection. To determine whether spacing between these IRF-Es is important for the activation of these VREs, we have tested the expression of a mutant that contains a 5bp insertion between the two putative IRF-Es in the IFNA1 VRE and found that this mutant (5bp) is not inducible by virus. These results strongly suggest that the interaction between IRF proteins binding to these sites is important for the gene activation. Consistent with this notion, we found that mutation (M1 and M5) in either of the IRF-binding sites is sufficient to abolish the induction, indicating that these two IRF-binding sites cannot function independently.

Overexpression of IRF-7 Enhances Sendai Virus-mediated Activation of IFNA2 VRE—We have shown that previously the relative level of IRF-3 and IRF-7 modulated the expression profile of IFNA subtypes (18). To determine how the relative levels of IRF-3 and IRF-7 in the cells affect the expression of IFNA1 and IFNA2 genes, we examined the expression of the reporter plasmids containing the IFNA1, IFNA2 VRE, or their mutants in infected cells overexpressing either IRF-3 or IRF-7. The results of the transient transfection assay showed (Fig. 1B) that overexpression of IRF-3 resulted in a 2–3-fold increase of expression of IFNA1 reporter gene. There was little effect on the expression of all of the IFNA reporter plasmids tested, suggesting that the levels of IRF-3 in HeLa cells are not a rate-limiting factor for the induction of IFNA genes. Because IRF-3 was shown to form homodimers in vivo, we speculated that overexpression of IRF-3 may result in increased levels of IRF-3 homodimer in the cells, which by itself is unable to activate IFNA VRE. This result is consistent with our previous observation that overexpression of IRF-3 in the absence of...
IRF-7 failed to induce IFNA genes in infected cells (17). By contrast, overexpression of IRF-7 substantially enhanced the inducibility of IFNA VRE. Interestingly, expression of IFNA1 VRE mutants that completely lost virus inducibility (e.g., M1 and M5) was partially rescued by IRF-7. Furthermore, the expression of IFNA1 VRE with single nucleotide mutation in PRDI- or PRDIII-corresponding regions (M6 and M7) was about 2-fold more effective than the expression of wt IFNA2 VRE. These results indicate that increased levels of IRF-7, which may facilitate formation of IRF-7 homodimers and/or increase the relative levels of IRF-3/IRF-7 heterodimers, enhances the expression of otherwise silenced IFNA2 VRE in a transient transfection assay. These data also indicate that the presence of an AANGAAA motif in PRDI- or PRDIII-corresponding regions is involved in the IRF-7-mediated activation. It is also noteworthy that the only two mutants that failed to respond to the IRF-7 overexpression-mediated rescue are 5bp and M3. This result indicates that the spacing and integrity of these two IRF-Es are essential for the IRF-7-mediated activation of IFNA VRE.

**Activation of IFNA1 and IFNA2 VREs and Genes by Constitutively Active IRF-3 and IRF-7 Mutants**—It was previously shown that the constitutively active mutants of IRF-3 and IRF-7, IRF-3 (5D) and IRF-7 (2D), respectively, were able to activate IFNA and IFNB reporter genes in the absence of viral infection (34, 35), suggesting their virus-independent nuclear localization. We have therefore analyzed the ability of these mutants to activate expression of IFNA1 and IFNA2 VRE reporters and their mutants in uninfected cells. As shown in Fig. 2, overexpression of IRF-7 (2D) in uninfected HeLa cells resulted in 7.5-fold induction of IFNA1VRE reporter, whereas the induction of IFNA2 VRE reporter was slightly lower (5-fold) (Fig. 2). In contrast, IRF-3 (5D) activated very effectively the expression of IFNA1 VRE (25-fold enhancement), but it did not significantly activate the IFNA2 VRE. Similarly, the relative levels of expression of the IFNA1 and IFNA2 mutants in response to IRF-3 (5D) and IRF-7 (2D) were comparable with that seen in infected cells transfected with wt IRF-3 and IRF-7 (Fig. 1B). Thus, in the transient expression assay, the activation of IFNA promoter by virus and by the constitutively active mutants shows the same specificity.

**To determine the effect of IRF-3 (5D) and IRF-7 (2D) on the expression profile of the endogenous IFNA genes, we used 2TGH cells. We have shown previously that these cells do not express IRF-7 or IFNA upon viral infection, but reconstitution of IRF-7 expression rescue virus mediated induction of seven IFNA subtypes (17, 29). 2TGH cells were transfected with either the IRF-3 (5D) or IRF-7 (2D) expression plasmids. The induced IFNA RNAs were amplified by reverse transcription-PCR using a universal primer set designed to amplify all the subtypes of IFNA RNAs. The amplified cDNA was then isolated, cloned, and sequenced to identify the individual IFNA subtype gene (17). As shown in Table I, overexpression of both IRF3 (5D) and IRF-7 (2D) activated predominantly the expression of IFNA1 gene. The expression of other IFNA genes induced by the constitutively active mutants show several differences when compared with the expression pattern of IFNA subtypes expressed in infected IRF-7-expressing cells. Both the constitutively active mutants induced higher level of IFNA10 than viral infection (5-fold increase), and IRF-3 (5D) effectively induced the IFNA21 gene, which was not induced either by virus or IRF-7 (2D). In contrast IFNA7 was effectively induced both by viral infection and IRF-7 (2D) but to lesser extent by IRF-3 (5D). These results show that: 1) activation of IFNA genes by constitutively active IRF-3 and IRF-7 is not completely identical to the expression profile seen in infected cells and 2) IRF-3 (5D) is able to activate expression of IFNA genes in the absence of IRF-7, further indicating that activation by constitutively active IRF-3 (5D) is distinct from the virus activated wild type IRF-3. Although the mechanism underlying the difference remains to be investigated, it has been shown that IRF-3 (5D) exhibits stronger interaction with p300/CBP (42).**

**The Carboxyl-terminal IRF-3-interacting Domain of IRF-7 Is Required for Efficient Transcription Activation of Endogenous IFNA Genes**—Recently, the different functional domains of human IRF-7 were identified by using reporter-based transient transfection assay (35). IRF-7 was shown to contain an auto-inhibitory domain (amino acids 247–467; IRF-7A) that suppressed transactivation and DNA binding activity of unphosphorylated IRF-7. We have found that one of the domains (amino acids 418–473; IRF-7H) by which IRF-7 interacts with IRF-3 overlapped with the auto-inhibitory domain of human IRF-7 (15). We have therefore tested whether the IRF-7 (Δ247–467) mutant (denoted as IRF-7 -ID hereafter) that is lacking the IRF-3 interacting region can also activate expression of endogenous human IFNA genes in infected 2TGH cells. The activation of IFNA genes by IRF-7 and by the IRF-7 carboxyl-
Fig. 3. A, activation of endogenous IFNA genes in 2FTGH cells transfected with IRF-7 and its mutants. Total RNA of 2FTGH cells transfected with IRF-7, IRF-7/3 hybrid, or IRF-7 mutants was used for RT-PCR to examine the expression of endogenous IFNA, IFNB, β actin, genes, and transfected IRF-3 and IRF-7 plasmids. Two sets of primers (15, 17) were used to amplify either the 5′ (lanes 2 and 3) or 3′ (lanes 1, 5, and 6) portion of the IRF-7 cDNA and its mutants. Because the IRF-7 primer set cannot amplify the IRF-7/3 cDNA, IRF-3-specific primer set was used to detect the presence of IRF-7/3 fusion mRNA and endogenous IRF-3 mRNA (lane 4). For comparison, RT-PCR was also performed with mRNAs from uninfected, IRF-7 transfected 2FTGH cells using IRF-3-specific primers to determine the relative levels of endogenous IRF-3 mRNA (lane 5). B, co-precipitation of IRF-7-ID and IRF-3. Expression plasmids encoding IRF-7, IRF-7-ID, IRF-7 Leu–Pro and Gly–Glu (IRF-7 L-P, G-E), or IRF-7 (1–237) were co-transfected with IRF-3 expression plasmid in 293T cells. 16 h after transfection, the cells were either infected with Sendai virus or left uninfected for additional 6 h. Whole cell extracts (200 μg) were then immunoprecipitated (IP) with the indicated antibody, and the presence of IRF-7 or IRF-3 in the immunoprecipitates was detected by Western blotting (W). The levels of IRF-7, IRF-7-ID, IRF-7 Leu–Pro and Gly–Glu, and IRF-7 (1–237) present in the cell extracts used for immunoprecipitation are shown in the bottom panel. The arrows mark the location of IRF-7s (top panel) and IRF-3 (middle panel).

A terminal deletion mutant (amino acids 1–418), which is missing the virus-targeted phosphorylation sites, was examined for comparison. It can be seen in Fig. 3a that only the full-length IRF-7 but not IRF-7 -ID or IRF-7 (1–418) efficiently activated endogenous IFNA genes in infected cells (lanes 1–3). The analysis of the relative levels of IRF-7mRNAs and proteins (data not shown) in the transfected cells showed that IRF-7 and its mutants were expressed. These results indicated that both the IRF-3-interacting domain (within the auto-inhibitory domain) and the carboxyl-terminal portion (amino acids 418–514) of IRF-7 are required for the induction of endogenous IFNA genes in infected cells. Because IRF-7/3 fusion protein was shown to strongly activate IFNA reporter gene in transient transfection assay (19), we have also examined the ability of this fusion protein to activate the endogenous IFNA genes and found that IRF-7/3 (Fig. 3A) was able to activate the transcription of endogenous IFNA genes but to a lesser extent than the full-length IRF-7. Given the fact that overexpression of IRF-7/3 but not IRF-3 can activate endogenous IFNA genes in Sendai virus-infected 2FTGH cells, this observation suggests that the IRF-7 DNA-binding domain is an essential component in determining the activation of endogenous IFNA genes in 2FTGH cells.

Within the IRF-8 interaction domain, two amino acid residues, Leu331 and Gly351, were reported to be important for the interaction of IRF-8 with IRF-1 or IRF-2 (38). Because these two residues also are present in the IRF-3 association domain of IRF-7, we generated IRF-7 mutant, IRF-7 (Leu→Pro and Gly→Glu) with mutations in these two residues. However, this mutant activated endogenous IFNA genes to the same extent as the wild type IRF-7 (Fig. 3A, lane 6). These mutations also did not affect the protein-protein interaction between IRF-3 and IRF-7 (Fig. 3B). For comparison, the level of IFNB mRNA in these cells was also determined by the RT-PCR. In contrast to the expression of endogenous IFNA genes, IFNB gene was expressed in all infected cells including the IRF-7 (1–418) transfected cells. This result is consistent with the previous observation that virus-activated IFNB gene expression is not IRF-7-dependent. Interestingly, the levels of IFNB expression were lower in the IRF-7(1–418) transfected cells than that in the wt IRF-7 transfected cells; this inhibitory effect may result from a competition for the DNA binding between IRF-3 and IRF-7(1–418). It is noteworthy that in this transient transfection assay, only about 75% cells were transfected; therefore the effect of IRF-7(1–418) on the expression of IFNB gene may be underscored.

Using the glutathione S-transferase pull-down assay, we found that two regions (amino acids 1–237 and 418–473) of IRF-7 interact with IRF-3 (15). Using the co-immunoprecipitation assay, Lin et al. (35) demonstrated that the carboxyl-terminal part of IRF-7 is capable of interacting with IRF-3. To further investigate whether a decrease in the endogenous IFNA gene activation by the IRF-7-ID mutant resulted from a loss of IRF-3 binding, the FLAG epitope-tagged IRF-7 or IRF-7-ID mutant were co-transfected with IRF-3 expression plasmid to 293T cells and then infected with Sendai virus. The rational for selection of 293T cells was their high transfection efficiency (~90%), which facilitate the analysis. However, similar results were obtained in HeLa cells (data not shown). The interaction of IRF-3 and IRF-7-ID was examined in cell lysates by co-immunoprecipitation. As shown in Fig. 3B, both IRF-7 and IRF-7-ID-encoded proteins could be detected by immunoprecipitation with anti-IRF-3 antibodies, and IRF-3 could be detected in immunoprecipitates with anti-FLAG antibodies. This indicates that IRF-7-ID can still bind IRF-3 possibly by its amino-terminal region (15), whereas this mutant is unable to support an efficient activation of endogenous IFNA genes. Indeed, we found that IRF-7 (amino acids 1–237) can also interact with IRF-3 by co-immunoprecipitation. Taken together, these results indicated that the carboxyl-terminal region of IRF-7 is essential for the optimal activation of endogenous IFNA genes in the virus-infected cells.
Western blot are shown in the
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Cells
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whole cell extracts were about the same (data not shown). Cells, because the levels of IRF-1 in uninfected and infected
result of an overall increase of IRF-1 expression in infected
enhanced in infected cells, and this enhancement was not a
Interestingly, the nuclear localization of IRF-1 was greatly
is important for the DNA binding of both IRF-3 and IRF-7.
VREs bind IRF-7 very weakly (Fig. 2). We and others have shown that the recombinant
binds efficiently to IFNA1 VRE but not to IFNA2 VRE, whereas recombinant IRF-3 can bind to both of these VREs (17). We have therefore examined whether a similar difference in binding of IRF-3 and IRF-7 to these two VREs exists in infected cells. To analyze the DNA binding, we have used the DNA pull-down assay in which oligonucleotides corresponding to the IFNA VRE or its mutants were biotinylated and coupled to streptavidin-coated magnetic beads. The DNA-containing beads were then incubated with nuclear extracts from infected or uninfected HeLa cells, and the bound IRF-3 and IRF-7 were detected by Western blot (Fig. 4). The binding of IRF-1 was also analyzed, and the relative levels of these IRFs in nuclear extract used for the pull-down assay were determined by Western blot. As shown in Fig. 4, the relative levels of IRF-7 and IRF-1 in nuclear extract of uninfected cells were very low, and no nuclear IRF-3 was detected in these extracts. Accordingly, the binding of IRFs from these extracts to IFNA1 VRE was negligible. Virus infection resulted in nuclear accumulation of all three IRFs. The IRF-3 bound efficiently to the IFNA1, plasmid 2>1, and M1 VREs, whereas its binding to IFNA2 and 5bp was significantly lower, and no binding to M5 VRE was detected. The observation that IRF-3 bound to M1 VRE that has a single mutation in PRDII-like domain but not to M5VRE that contains a single mutation in PRDIII-like domain suggests that the PRDIII-corresponding IRF-E is preferentially targeted by IRF-3, which is in agreement with the binding pattern of recombinant IRF-3 shown previously (43). In contrast, about the same levels of IRF-7 and IRF-1 were bound to all the IFN VREs examined, except that 5bp VRE bound IRF-7 very weakly (Fig. 4). This suggests that IRF-7 binds IFNA VRE with lower selectivity than IRF-3 and that spacing between these two IRF-Es is important for the DNA binding of both IRF-3 and IRF-7. Interestingly, the nuclear localization of IRF-1 was greatly enhanced in infected cells, and this enhancement was not a result of an overall increase of IRF-1 expression in infected cells, because the levels of IRF-1 in uninfected and infected whole cell extracts were about the same (data not shown).

Analysis of IFNA Enhanceosome-like Complex in Infected Cells—It was shown that transcriptional activation of IFNB genes required the assembly of multiple transcription factors on IFNB VRE, forming a multi-component complex enhanceosome (44). To examine which factors assemble on IFNA1 and IFNA2 VRE in infected HeLa cells, we performed a chromatin immunoprecipitation assay. Because the endogenous IFNA1 and A2 gene promoters show a high degree of homology and are difficult to distinguish, we have analyzed the assembly of IRFs on the transfected IFNA1 and IFNA2 VRE reporter plasmids and their mutants. As a control for binding specificity, we have also analyzed the binding of a transcription factor, relA, unrelated to these VREs that does not contain the NFκB site. The HeLa cells were transfected either with IFNA1 or IFNA2 SAP plasmids or the 2>1 and 5bp mutants and either left uninfected or infected with Sendai Virus for 6 h. The proteins were then cross-linked to DNA, and the protein DNA complexes were precipitated with antibodies against IRF-1, IRF-3, IRF-7, acetylated histone H3, or relA (24, 33, 45). The DNA in the precipitates was then amplified by PCR with universal primers to the IFNA1 and IFNA2 VRE-containing reporter genes. To determine whether the PCR amplification of the immunoprecipitated cross-linked DNA was qualitative, we first defined the conditions of the PCR that give rise to a linear amplification. As shown in Fig. 5A, amplification of 5–25 μl of the cross-linked DNA solution resulted in linear amplification, and therefore 15 μl of this template was used in the following assays (Fig. 5B). As shown in Fig. 5B, the fragment containing the IFNA1 VRE was amplified from DNA-protein complexes immunoprecipitated by IRF-3 antibodies of infected cells, but no amplification was seen in uninfected cells. In contrast the amplification of IFNA2 VRE from these precipitates was very low. Interestingly the amplification of IFNA2 VRE in IRF-3 precipitates was much more effective from cells that overexpressed transfected IRF-7-expressing plasmid. 2>1 VRE was also amplified from the IRF-3 precipitates, but very little amplification of 5bp VRE from these precipitates could be detected. When precipitation was carried out with IRF-7 antibodies, again both IFNA1 VRE and 2>1VRE were amplified, but the amplification of 5bp VRE or IFNA2 VRE was as low as from uninfected cells. However, IFNA2 VRE could be amplified both from IRF-7 and IRF-3 precipitates of IRF-7-overexpressing cells. These results show that high levels of IRF-7 facilitate not only binding of IRF-7 to the IFNA2 VRE but also of IRF-3, thus suggesting that these two factors bind as heterodimers. Interestingly IFNA1, 2>1 and IFNA2 VREs could be amplified from cross-linked DNA of IRF-1 precipitates from infected cells, indicating that IRF-1 also is a component of the IFNA enhanceosome.

To determine whether IRF-7 homodimers or IRF-3/IRF-7 heterodimers are associated with the IFNA promoter in IRF-7-overexpressing cells, formaldehyde-treated extracts from infected HeLa cells and HeLa cells overexpressing IRF-7 were first precipitated with either anti-IRF-3 or anti-IRF-7 antibody. The supernatants from these precipitations were again precipitated with anti-IRF-7/IRF-3 antibodies. The IFNA1 VRE and IFNA2 VRE were amplified from both the first and second precipitates. As shown in Fig. 5C, the respective VRE could be amplified from the first precipitates but not from the second precipitates, indicating that all the IRF-3 and IRF-7 bound together to IFNA1 and IFNA2VRE. Thus, even in the IRF-7-overexpressing cells, IRF-7 binds the IFNA2 VRE as heterodimer with IRF-3, and no binding of IRF-7 dimer to IFNA VRE was detected.

Finally, it was determined previously that histone acetyltransferases are recruited to IFNB VRE in infected cells (14). To determine whether the histone acetyltransferase activity is also recruited to IFNA VRE in association with IRF binding, we precipitated the cross-linked DNA and proteins from infected
and uninfected cells with antibodies to acetylated histone H3 and amplified the respective VREs. As shown in Fig. 5B, effective amplification of IFNA1 VRE was detected in infected HeLa cells, whereas IFNA2 was effectively amplified only from infected, IRF-7 overexpressing cells. In the absence of IRF-3 and IRF-7 binding, the amplification of the respective VRE from histone H3 precipitates was much lower and about at the same level as in uninfected cells. Thus, the presence of acetylated histone H3 to IFNA VRE correlated well with the binding of IRF-3 and IRF-7 to these VRE, suggesting that activation of IFNA genes, like the IFNB gene, involves transcription complex containing acetyltransferase(s).

When antibodies reactive against acetylated histone H4 were used we observed enhancement in immunoprecipitation of the IFNA1 promoter region in response to viral infection, however, we also observed the basal level of histone H4 acetylation within the IFNA1 VRE in uninfected cells (data not shown). The observed difference in basal levels of H3 and H4 associated with the IFNA promoter could reflect not only the ability of the acetylated forms of H3 and H4 to associate with this promoter in uninfected cells but also the ability of the used anti-H3 and H4 acetylated antibodies to recognize different acetylated forms of H3 and H4.

**DISCUSSION**

Previous studies have shown that two transcription factors of the IRF family, IRF-3 and IRF-7, have critical roles in the induction of IFNA genes expression in infected human cells (15, 17, 18). Although the IFNA1 gene was expressed most abundantly in IRF-7-expressing human fibroblasts, the expression of IFNA2 gene was consistently very low. It was also shown that the expression profile of the other IFNA subtypes can be modulated by the relative levels of IRF-3 and IRF-7 (18). In this paper we have analyzed the elements in the IFNA1 and IFNA2 promoters that are responsible for this difference and shown that -98 G and -81 A are critical cis-acting determinants responsible for the distinct expression of IFNA1 and IFNA2 genes in infected cells. Two IRF-Es resembling PRDI and PRDIII of IFNB promoter can be identified in these IFNA VREs. Our data indicate that both of these IRF-Es and their close proximity are required for the inducible expression of these promoters, because mutation in either of these IRF-Es or their spatial separation by an insertion of 5bp abolished the virus inducibility of these promoters in infected cells. The expression of IFNA2 VRE, but not M3 could be partially rescued by overexpression of IRF-7, indicating that the presence of AANNGAAA motif in the PRDI- or PRDIII-like regions is involved in the IRF-7-mediated activation. Because the relative levels of constitutive and inducible IRF-7 are higher in lymphoid cells than in fibroblasts (16), this could partially explain why expression of IFNA2 gene is high in Namalwa cells and lymphocytes (39, 41).

It was shown previously that the recombinant DNA-binding domain of IRF-3 binds preferentially to PRDIII, whereas IRF-7 recognizes the PRDI-like domain (43). The differential affinity of recombinant IRF-3 and IRF-7 for the IFNA1 and IFNA2 VRE was also recognized. IRF-3 was able to bind only IFNA1 VRE. In contrast, IRF-7 could bind both IFNA1 and IFNA2 VREs (17, 19). Because both IRF-3 and IRF-7 are modified by phosphorylation at the carboxyl-terminal serine residues in infected cells (15, 20, 30–32), the binding of the unmodified recombinant IRF may not represent well the in vivo situation. The results of the DNA pull-down assay however showed that the binding of IRF-3 and IRF-7 from infected cells exhibited a similar specificity as binding of the recombinant proteins. These data together with the observation that IRF-3- and IRF-7-mediated activation of IFNB and histone H4 promoters oc-
curs in the absence of viral infection (25, 28) indicate that virus-mediated phosphorylation of these two IRFs is not an absolute requirement for their DNA binding capacity. The nuclear IRF-3 has shown strong binding affinity for IFNA1 VRE. Although introduction of a mutation in PRD3-corresponding IRF-3 did not have a significant effect, a single mutation in PRDII-like region abolished the IRF-3 binding. These results indicate that in the infected cells, the PRDII-corresponding site of the IFNA1 VRE is targeted by IRF-3. By contrast, the binding of IRF-7 to various IFNA VREs show little difference in DNA binding specificity. Interestingly IRF-1 also bound to all of the VRE tested.

The assembly of a multi-component transcription complex enhanceosome containing multiple transcription factors including IRFs was first demonstrated for IFNB promoter (44). Here we show by using a chromatin precipitation assay that IRF-1, IRF-3, and IRF-7 are recruited to the IFNA1 promoter in infected cells. Thus, although IRF-1 was found to be dispensable for the induction of murine IFNA and IFNB genes (14), the association of human IRF-1 with the IFNA promoters indicates that it may contribute directly to the transcriptional regulation of IFNA gene expression in infected cells. The IFNA1 enhanceosome also contains histone acetylase as demonstrated by the association of acetylated histone H3 with IFNA1 and IFNA2 promoters in infected cells. Previously we have demonstrated that IRF-3 associates with the carboxy-terminal half of CBP/p300 (19). However, it was also shown that IRF proteins recruit not only CBP/p300 but also PCAF and GCN5 to the interferon-stimulated response element (27). It remains to be determined which of the other histone acetylases are present in the IFNA enhanceosome. Under the same conditions as used for IFNA1 promoter, we have not detected binding of IRF-3 or IRF-7 to the IFNA2 promoter. However, overexpression of IRF-7 in infected cells increased binding not only of IRF-7 but also of IRF-3 to the IFNA2 promoter, indicating that these factors bind to this promoter as IRF-3/IRF-7 heterodimer. The presence of IRF-3/IRF-7 heterodimers in infected human cells was previously demonstrated (35). In contrast, it was suggested that in infected mouse cells, the IRF-7 homodimers were the major inducers of IFNA genes, and no association between IRF-3 and IRF-7 could be demonstrated in these cells (36). Using a sequential chromatin immunoprecipitation, we were not able to detect association of IRF-7 homodimers either with IFNA1 or IFNA2 promoters, indicating a possible difference between human and murine system in the regulated expression by IRF-3 and IRF-7. The binding of IRF-3 and IRF-7 complex to IFNB promoter as well as recruitment of IRF-1 and a transcription cofactor CBP/p300 to this promoter was also observed in infected human cells (14). Stimulation of the transcription activity of histone H4 promoter by IRF-1, IRF-3, and IRF-7 proteins was also recently demonstrated (25). The activity of this promoter was up-regulated by IRF-1, and further enhancement was observed in the presence of IRF-3 or IRF-7. Notably, this activation occurred in the absence of viral infection, indicating that the virus-mediated phosphorylation of IRF-3 and IRF-7 is not an absolute requirement for the function and DNA binding of these proteins. This finding correlates with our observation that overexpression of IRF-3 induces expression of IFNB gene in uninfected cells (28).

Functional analysis of the IRF-3 and IRF-7 proteins in the transfection assay identified the presence of both the transactivation domains and the auto-inhibitory domains in these two proteins (16, 34–36). However, none of the deletion mutants of IRF-7 that were able to activate IFNA and IFNB promoters in a transient expression assay (15) could effectively stimulate expression of the endogenous IFNA genes in infected cells. In contrast, Marie et al. (36) showed that murine IRF-7 mutant with a deletion of the auto-inhibitory domain is able to activate the endogenous murine IFNA6 gene independent of viral infection. It was shown previously that phosphorylation of IRF-3 on carboxy-terminal Ser385 and Ser386 is required for its retention in nucleus and interaction with CBP/p300 (32, 37). Replacement of the serine and threonine residues in the region between amino acids 395 and 407 by phosphomimetic Asp resulted in a constitutively active IRF-3, IRF-3 (5D) (30). Similarly, replacement of Ser377 and Ser379 of IRF-7 by Asp also led to a constitutively active form of IRF-7 (35). These constitutively active proteins accumulated in the nucleus and activated transcription activity of IFNA and IFNB promoters in a transient expression assay. We have shown that although the overexpression of both constitutively active IRF-3 (5D) or IRF-7 (2D) activates expression of endogenous IFNA genes in uninfected cells, two distinct differences were observed when compared with the induction in infected cells: 1) overexpression of IRF-3 (5D) was able to induced expression of endogenous IFNA genes in the absence of IRF-7, whereas IRF-3 wt alone is not sufficient to activate IFNA genes in infected cells and 2) the profile of expressed IFNA subtypes differed from that induced by viral infection. Although IFNA1 was still the major IFNA subtype expressed, IFNA10 was expressed more abundantly by constitutively active IRF-3 (5D) and IRF-7 (2D) than by virus. In addition, the IFNA21 gene was induced effectively by IRF-3 (5D) but not by IRF-7 (2D) or by viral infection. These results indicate that the stimulation by the constitutively active IRF-3 and IRF-7 is not identical to the virus-mediated activation. The difference in the mechanism of activation by virus activated wt IRF-3 and constitutively active IRF-3 (5D) is further supported by the observation that although overexpression of IRF-3 in infected HeLa cells did not enhanced virus-mediated induction of IFNA1 promoter, the activity of this promoter, in the uninfected HeLa cells, was greatly stimulated by IRF-3 (5D). Further experiments will have to clarify the molecular basis of the observed differences.

We have shown recently that another IRF family member, IRF-5, participates in the induction of IFNA genes and is able to replace function of IRF-7 and mediate the expression of IFNA genes in infected cells. However, this factor, in contrast to IRF-3 and IRF-7, shows virus specific activation and induces predominantly the expression of IFNA8 gene (24). Because the expression of this gene is restricted to only a few cell types, we have not considered its participation in the induction of IFNA genes in this study. One of the remaining questions is whether other transcription factors are part of the complex assembled on IFNAVRE and contribute to the differential expression of individual IFNA genes. Further studies will seek to determine the additional components of the IFNA enhanceosome and establish their role in the inducible expression of IFNA genes.

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IFNA Gene Activation Requires IRF Dimer and Acetyltransferase

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